Perspectives in Flavor and Fragrance Research

Perspectives in Flavor and Fragrance Research. Edited by Philip Kraft and Karl A. D. Swift

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Philip Kraft, Karl A. D. Swift (Eds.)

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Preface

What began as an annual one-day event in the late 1980's, the '*Flavor & Fragrance*' conference of the *Royal Society of Chemistry* and the *Society of Chemical Industry* has grown into the multi-day international meeting it is today. Since the '*International Congresses of Flavors, Fragrances and Essential Oils*' were discontinued after the Istanbul *ICEOFF* in 1995, the '*Flavor & Fragrance*' conference of the *RSC* and *SCI* constitutes the only international forum for both flavor and fragrance chemists. Both flavor and fragrance chemistry continue to be rapidly growing domains fueled by the constant demand for innovation and the ever-changing trends in the F&F industry. Previous meetings held in 1997 and 2001 had their respective presentations published in proceedings books. The '*F&F 2004*' meeting held 12th – 14th May at the UMIST Conference Centre, Manchester, is the subject of this proceedings book, which continues the series.

This proceedings book contains 18 of the 24 presentations, the majority of which has also appeared in a special issue of *Chemistry & Biodiversity* dedicated to this conference. Four chapters are devoted to natural-product chemistry, two relate to the biochemistry of olfaction and malodor production, three to foods and flavors, and nine cover the many aspects of fragrance chemistry. The book is directed primarily towards anyone who works in an R&D environment within the flavor and fragrance industry and to academic researchers interested in this field. As most of the chapters have a review-like character, it may, however, also be used as an advanced textbook to complement an introductory work such as '*Chemistry and Technology of Flavors and Fragrances*', Ed. D. Rowe, Blackwell Publishing, Oxford, 2005. But our primary aim is to promote this exciting area of science and keep it visible to a wider scientific community.

The logo of the conference, also shown on the cover of this book, features *Ambrocenide*®, a new powerful ambery odorant that emerged from classical cedrene chemistry. It should symbolize the endless possibilities in the search for new aroma chemicals, even in the '*well-explored*' areas of terpene-derived materials or ambery odorants, and the strong momentum of this industry in general. We plan to highlight a different new odorant in the logo of the conference every three years, each time from a different company. In September 2007, the next flavor and fragrance conference will take place at the Imperial College in London, England. This event promises to be an exciting occasion and will have an equally impressive line up of prominent academics and industrialists. Please check the following website for future plans as they evolve: www.confsec.co.uk.

The editors would like to express their sincere thanks to all the contributors who painstakingly transformed their respective presentations into the manuscripts found in this book. Our thanks also go to all the speakers and delegates at the 2004 conference, the conference secretariat, and to the *RSC* and *SCI* for their continued financial

backing of the event. Finally, we would like to express our gratitude to Dr. *M. Volkan Kısakürek* and Dr. *Richard J. Smith* of the *Verlag Helvetica Chimica Acta* for their help in preparing this book, and for their enthusiasm for the project overall.

February 2005 *Philip Kraft*
Karl A. D. Swift

Contents

Molecular and Cellular Basis of Human Olfaction

by **Hanns Hatt**

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The human olfactory systems recognize and discriminate a large number of different odorant molecules. The detection of chemically distinct odorants begins with the binding of an odorant ligand to a specific receptor protein in the ciliary membrane of olfactory neurons. To address the problem of olfactory perception at a molecular level, we have cloned, functionally expressed, and characterized some of the human olfactory receptors from chromosome 17. Our results show that a receptor protein is capable of recognizing the particular chemical substructure of an odor molecule and, therefore, is able to respond only to odorants that have a defined molecular structure. These findings represent the beginning of the molecular understanding of odorant recognition in humans. In the future, this knowledge could be used for the design of synthetic ideal receptors for specific odors (biosensors), or the perfect odor molecule for a given receptor.

Introduction. – Even if we have lost faith in our noses, we are still strongly influenced by smells, even if only subconsciously. Smells can evoke memories and emotions, influence our mood, and are important for our enjoyment when eating. We often use the word *taste* erroneously, when we actually mean *smell*; for we can only taste something if it is salty, sour, sweet or bitter. With closed eyes and nose, we can maximally discriminate between a sweet banana and a sour cucumber. All the delicate nuances of an epicurean cuisine or of a noble glass of wine are, in the final analysis, savored through our sense of smell. In addition, before the spirit and beauty of a person can fascinate us, our nose must become infatuated.

So, how do we register a smell? That our knowledge of the molecular background is still too scant is due to the complexity of the world of smell. More than 10 000 odors can be distinguished, even in extremely low concentrations. The sense of smell is, thus, exceptionally specific and sensitive, and might best be compared in terms of complexity with the immune system.

What happens in our nose, when we smell the odor of a rose? A basic question asked by *Shakespeare*. A flower or any odorous subject has to release molecules according to their vapor pressure into the air. During inhalation, they can reach our nasal cavity. As long as we breathe, we smell.

The olfactory sense organ is a mucus membrane made up of an epithelium and a subepithelial lamina propria of connective tissue, blood vessels, and glands. In vertebrates, the olfactory epithelium consists of three mature cell types: bipolar primary sensory olfactory neurons, supporting cells, and two types of basal cells (adult stem cells), which generate olfactory-receptor neurons and supporting cells throughout our whole life. The turnover of the *ca.* 30 million olfactory neurons is less than one month. A proximal pole of the cell body of sensory neurons narrows into an axon that joins with other axons to form small nerve bundles that then project to the olfactory

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bulb. There, they terminate within glomeruli by forming synapses on mitral cells. At the apical pole of the cell body is a narrow dendrite that usually ends in an extension, the dendritic knob at the epithelial surface. Projecting from the dendritic knob are several cilia, in human *ca.* 20, overlying the surface of the olfactory epithelium in a matrix of mucus. Their cell membranes contain all the molecular components necessary to convert the chemical odor stimulus into an electrical signal in the cell by way of a cascade-like biochemical amplification mechanism. Meanwhile, it is generally accepted that the interaction of odor molecules with the receptor protein leads to the activation of the so-called G_{olf}-protein as mediator to activate the enzyme adenylate cyclase, which produces large amounts of cyclic adenosine monophosphate (cAMP) as second messenger (*Fig. 1*). The cAMP molecules now act directly within the cell membrane to change the structure (conformation) of a channel protein into its open state. The so-called CNG (cyclic-nucleotide-gated) channel is permeable unspecifically for cations (Na^+, Ca^{2+}), which can flow from the nasal mucosa into the cell [1][2]. As a result, the negative membrane potential (*ca.* -70 mV at rest) is shifted to more-positive values. Above a certain threshold (-50 mV), this analog sensor potential is converted into a digital action-potential frequency, which is conducted along the axon of the olfactory cell into the brain. All the molecular components involved in the transduction of a chemical stimulus in an electric cell signal have been known for approximately ten years [3–6].

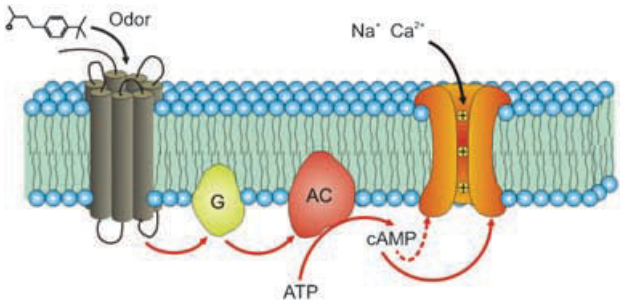

Fig. 1. *Schematic representation of the cAMP-mediated transduction pathway operating in the sensory cilia of olfactory-receptor neurons*

In 1990, a breakthrough was achieved in olfactory research. *Randy Reed* and co-workers [7] discovered the first of three different subunits of the CNG channel. The biophysical and pharmacological properties of this olfactory channel have been examined in detail in our and in other laboratories during the last couple of years. Interestingly, it was only recently that we could show that subunit composition and modulation can be adapted to different functional roles, *e.g.*, adaptation [8].

In 1991, *Linda Buck* and *Richard Axel* [9] discovered a large multigene family expressed exclusively in rat-olfactory-receptor neurons. The members show a heptahelical transmembrane structure, as expected for G-protein-coupled receptors. The *ca.* 320 amino acids are highly homologous and Southern blots of genomic libraries suggested that the gene family consists of at least 1000 members. It is the largest gene family in rat, but also in our genome. Out of the 1000, each olfactory sensory cell expresses only one type of olfactory-receptor protein, which implies a sophisticated

mechanism of olfactory gene choice [10]. The repertoire of human olfactory-receptor genes contains a large fraction of pseudo genes. Recent studies indicate that *ca.* 70% of all olfactory-receptor genes in human may be pseudo genes, suggesting that olfaction became less important during the course of evolution. In comparison with rodents or some of the primates, we have lost two thirds of our olfactory-receptors [11]. The members of the olfactory-receptor gene family are distributed on nearly every human chromosome, except 20 and Y [12]. Altogether, 347 putative full-length olfactory-receptor genes have been identified in the human genome, the rest are pseudo genes. Many of the human olfactory genes appear in genomic clusters with 10 or more members. We concentrated our work on a cluster from chromosome 17, which contains 18 olfactory receptor-coding regions, six of which are pseudo genes. As shown in the *Table*, we cloned the remaining twelve human olfactory-receptors out of human genomic DNA *via* the polymerase chain reaction (PCR).

Table. *Cluster of Olfactory-Receptor Genes of Human Chromosome 17*

Code	Symbol	Loc-Name	Trivial name	Coord	Pseudo
H38g886	OR1D3P	OR17-23		34731	Yes
H38g096	OR1E3P	OR17-210		36192	Yes
H38g894	OR1A2	OR17-6		36192	No
H38g893	OR1A1	OR17-7		36192	No
H38g896	OR1P1P	OR17-208		36192	Yes
H38g809	OR1D2	OR17-4		36192	No
H38g099	OR1G1	OR17-130		36192	No
		OR17-209			
H38g888	OR1D4	OR17-30		36192	No
H38g153	OR1R1P	OR17-1		3.00	Yes
H38g180	OR1E1	OR17-2	HGMP071	37014	No
		OR17-32			
H38g183	OR3A3	OR17-137		37075	No
		OR17-16			
		OR17-201			
H38g877	OR3A1	OR17-40	OLFRA03 hg138	37075	No
H38g884	OR3A2	OR17-228		37075	No

Results and Discussion. – First, we concentrated our interest on the olfactory receptor 17-4 (hOR17-4). For many years, nobody could verify that these genes, discovered by *Linda Buck*, really code for olfactory receptors, because it was not possible to get a functional expression and characterization of any of the receptors. Just like many others, we tried to solve this problem, and were successful with the idea that an inefficient translocation of expressed receptors to the plasma membrane may be responsible for all failed attempts to achieve a functional expression. As a result, we constructed a vector (pS myc-plasmid) that contains a CMV-promoter and a membrane-import sequence we found when cloning the human serotonin-gated receptor channel (5 HT3) out of human intestine material [13], followed by a human myc epitop and the cDNA of the olfactory receptor 17-4. Using normal amplification and transfection methods (calcium precipitation and *Semliki Forest* virus infection), it

can be shown by localizing the myc sequence with antibodies that the receptor protein is expressed in HEK-293 cells and transported to the plasma membrane.

To test the functionality of the protein, one has to find a parameter for demonstrating a cell response. Calcium-imaging measurements on isolated human olfactory cells demonstrated that, after odor applications, the neurons show an increase in the intracellular calcium (*Fig. 2*). Similar experiments with transfected HEK cells, upon application of a complex mixture of odorants, gave a clear response of all infected HEK-293 cells to the odorant mixture, using the *Semliki Forest* virus-infection system [14]. From evaluation of the ratio obtained from integrated fluorescence (f 340/f 380), measured over time for different cells, it could be seen that, shortly after odor application, the calcium concentration increases transiently.

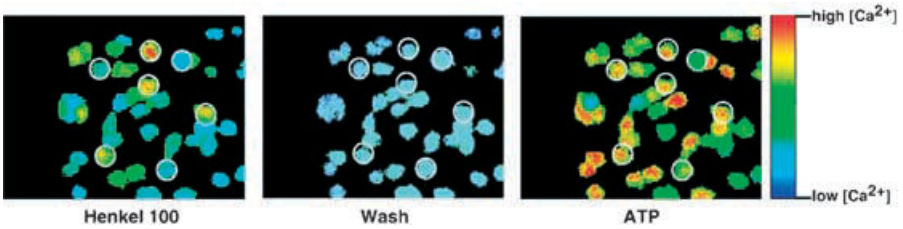

Fig. 2. *The olfactory-receptor hOR17-4 can be recombinantly expressed in human embryonic kidney cells* (HEK). In a randomly selected field of view, the complex odorant mixture *Henkel 100* induced transient calcium signals in transfected HEK-293 cells (left trace). ATP served as a control of HEK cell excitability (right trace). Calcium changes in individual cells are indicated in pseudo colors. Both *Henkel 100* and ATP were applied for 5 s each.

To identify the specific ligand of the receptor hOR17-4, a mixture of 100 structurally different odorous chemicals were tested. The chemicals chosen, frequently used in perfumes, were prepared for us by the *Henkel* company [15]. Fortunately, this mixture did stimulate the cells transfected with the receptor hOR17-4. By splitting into subgroups and testing the stimulatory effect of each group, among the 100 odorants initially tested, only a single compound, *Cyclamal*® (=2-methyl-3-[4-(1-methylethyl)-phenyl]propanal; see *Fig. 4*), elevated responses in the transfected cells (*Fig. 3*).

Next, the molecular receptive field of the receptor was determined. The effectiveness of *Cyclamal*® could be modified by changing the size, shape, functional group, and chemical properties of the molecule. First, the i-Pr group on the aromatic ring was changed. Second**,** we looked at the importance of the chain length and branching of the propanal chain, and third, the aldehyde (CHO) functional group was modified. Complete removal of the *para*-substituent from the benzene ring, which results in 3-PPA, led to a strong decrease in the effectiveness, whereas a *t*-Bu group (*Lilial*®) increased the effectiveness. Interestingly, additionally removing the 2-Me group next to the CHO function led to the most-effective substance identified, *i.e.*, *Bourgeonal*®, having an odor like lily of the valley. Next, the influence of the length of the main alkyl chain was tested. The molecule with the shortest chain, benzaldehyde, was found to be completely inactive. Increasing the number of C-atoms demonstrated that the most-effective substance had four C-atoms. The compound with five C-atoms

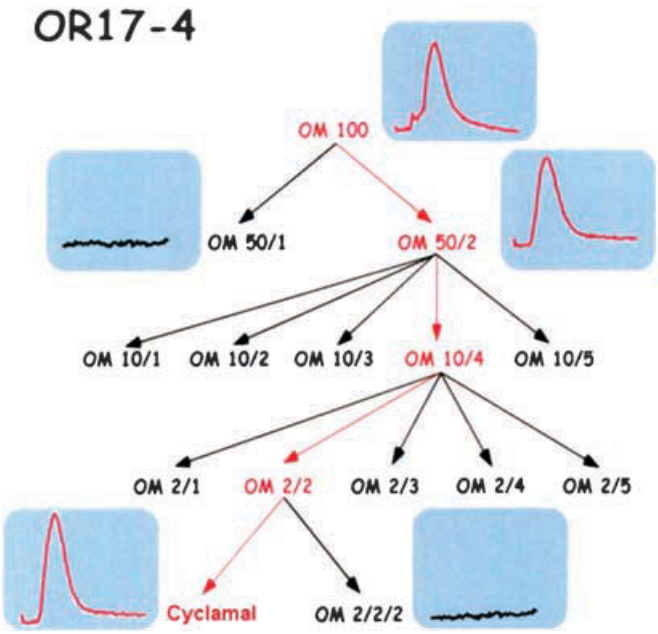

Fig. 3. *To identify the effective component in the odorant mixture* Henkel 100, *the mixture was subdivided into smaller fractions and then tested for activity.* Only application of *Cyclamal*® induced a transient increase in calcium concentration.

(5-phenylvaleraldehyde) decreased the effectiveness dramatically. Finally, the functional group was modified. Oxidation of the aldehyde (CHO) group to an acid (COOH) or reduction to an alcohol (CH_2OH) abolished the molecules' effectiveness completely (*Fig. 4*).

Our results demonstrated the absolute requirement of the formyl (CHO) group for activation, and suggested that the receptor recognizes a particular feature of different ligands, in analogy to a *pharmacophore* in medicinal chemistry. For the first time, we could show another analogy to pharmacology, the existence and the effectiveness of antagonists (*Fig. 5*). It was speculated for many years that it should be possible to construct antagonists for olfactory receptors in a similar way as in the case of the medically used blockers of adrenergic or dopaminergic receptors. Interestingly, under the many substances tested, undecanal ($Me(CH_2)_9CHO$) showed a clear competitive antagonistic effect.

These results, from both agonists and antagonists, provide information as to the structure and geometry of the partner of this interaction *i.e.*, the receptive area (binding pocket) of the receptor protein. To answer the question of whether the binding pocket is situated in the extracellular loop – as suggested by many groups – or in the transmembrane region, we studied other olfactory receptors occurring in the gene cluster of chromosome 17, *e.g.*, hOR17-40 and hOR17-44. The amino acid sequences of the receptors differ mostly in the residues located in the transmembrane regions. Functional expression of hOR17-40 showed [16][17] that this receptor is highly

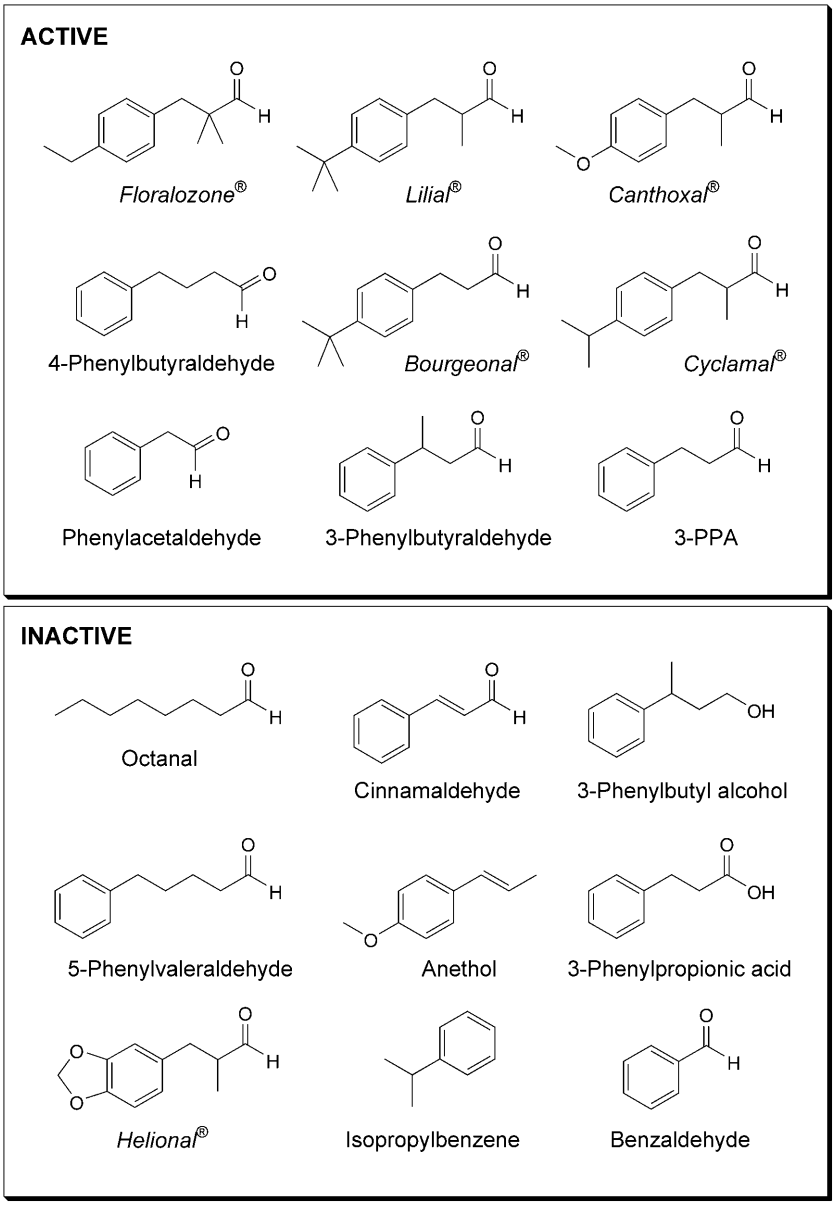

Fig. 4. *Molecular receptive field of the hOR17-4 olfactory-receptor protein. Cyclamal®* and several structurally related molecules were tested for activity at the heterologously expressed protein.

sensitive to *Helional®* (= 3-(1,3-benzodioxol-5-yl)-2-methylpropanal; *Fig. 6*), and the expression of hOR17-44 demonstrated that aliphatic aldehydes (C_{11}) are the best agonists. None of the ligands tested, even in the millimolar concentration range, activated either, the hOR17-40 or the hOR17-44 receptors.

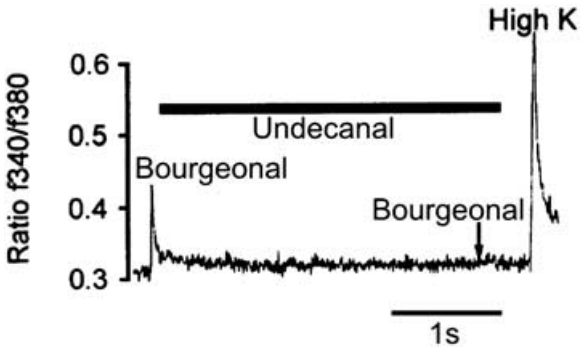

Fig. 5. *Undecanal* (A11) *competitively inhibits the activation of the hOR17-4 receptor by* Bourgeonal®*, as determined by fluorescence spectroscopy*

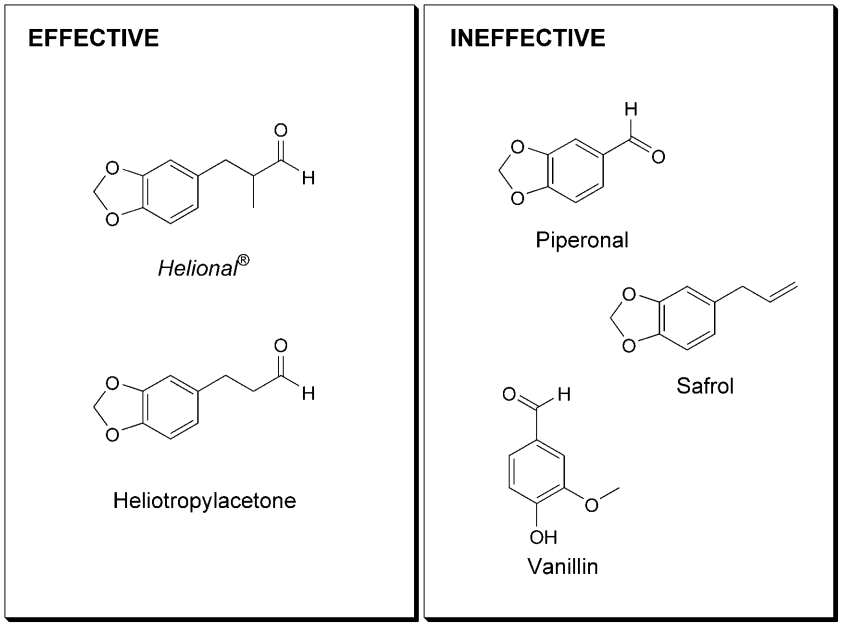

Fig. 6. *Effective* vs. *ineffective agonists towards hOR17-40*

The above data strongly indicate that the amino acids responsible for the interaction with the odorant ligand are located in the transmembrane region, similar to retinal in vertebrate rhodopsin. Such data also allows us to construct a very preliminary computer-generated three-dimensional working model of an olfactory-receptor, based on the vertebrate rhodopsin template for the receptor hOR17-40. The model predicts the binding pocket to be *ca.* 10 Å away from the extracellular surface formed by the transmembrane domains TM3-6, and that some amino acids, *e.g.*, Asp^{69}, interact with the CHO functional group of the substrate (*Fig.* 7).

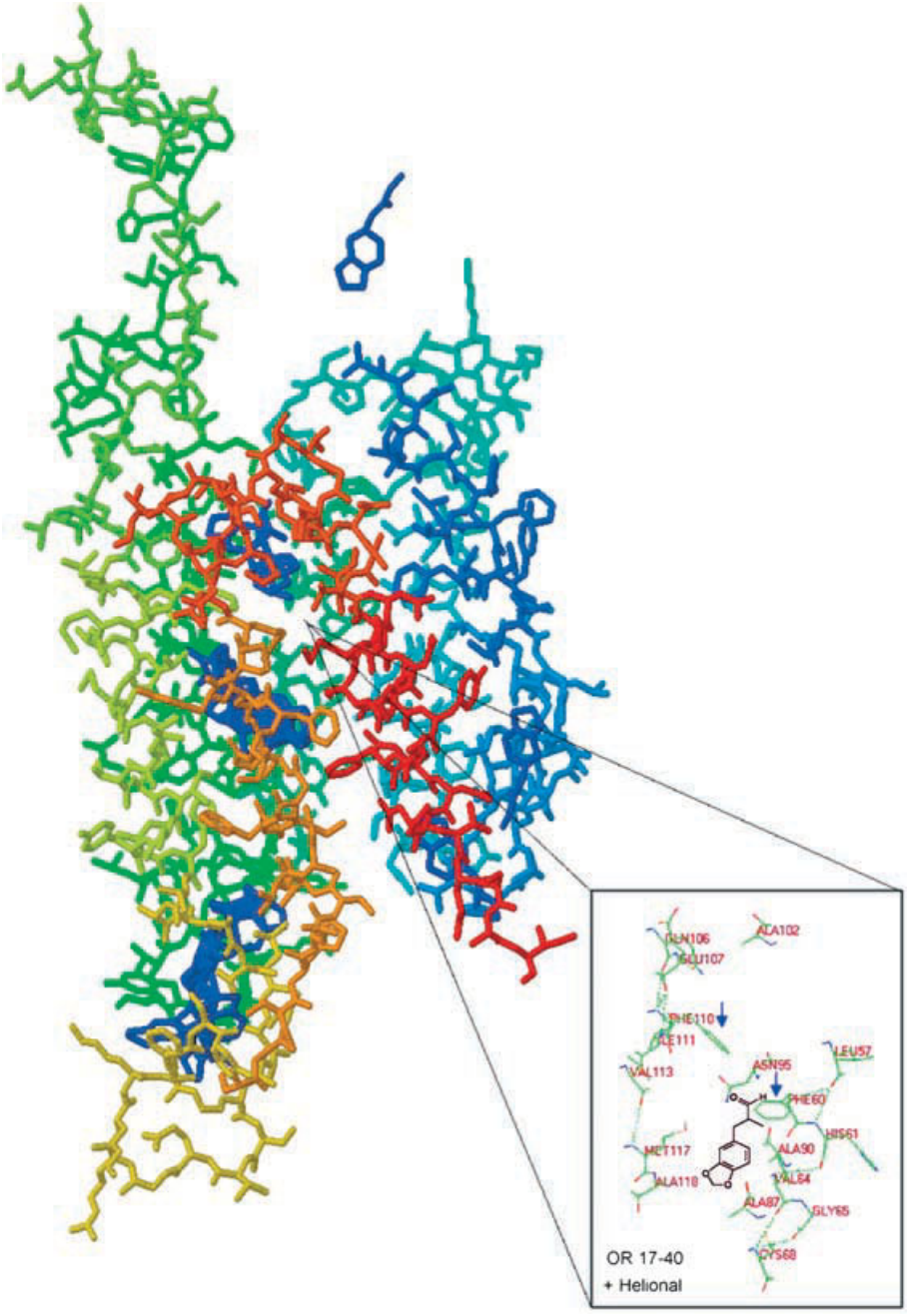

Fig. 7. *Molecular modeling of the human olfactory-receptor hOR17-40.* A possible olfactory binding pocket for *Helional*® is shown in the box.

Such molecular modeling may help us in the future to generalize our findings and to construct synthetic ideal receptors for specific odors, or the perfect odor molecule for a given receptor. The knowledge of the binding requirements for olfactory-receptors at the atomic level is also important for the design of biosensors for defense, perfumes, or food-industry applications.

Recently, we have shown that olfactory-receptors also exist and play an important functional role outside the olfactory epithelium: in human sperm cells. The latter possess olfactory-receptor proteins, as well as all the other members of the second-messenger cascade, the G-protein, the AC, and the CNG channel [18]. Oversimplifying, one could say *that a sperm cell is nothing more than an olfactory neuron with a tail*. Nearly ten years ago, we showed that in human sperm a CNG channel exists whith a high degree of homology to the olfactory and cone channels. Now, we were able also to demonstrate the presence of members of the olfactory-receptor-protein family. Upon analyzing human testis material *via* PCR to search for receptors from chromosome 17, the receptors hOR17-2 and hOR17-4 were detected. Using calcium imaging, it was shown that sperm can 'smell' *Bourgeonal*® (and *Cyclamal*®) in a concentration-dependent manner [15]. The threshold for the calcium increase was *ca.* 10^{-6} M for *Bourgeonal*® (*Fig. 8*).

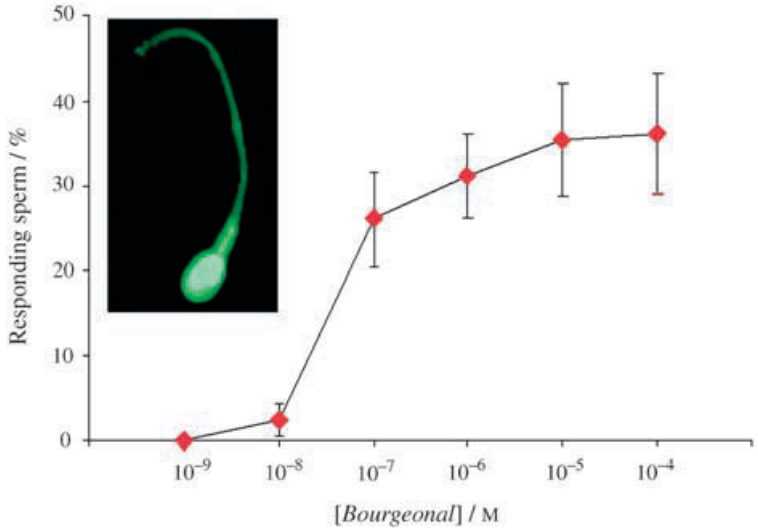

Fig. 8. *The percentage of sperm cells showing calcium signals in response to* Bourgeonal® *is dose-dependent* (mean populations of responsive sperm). The inset shows the fluorescent imaging of a fura-2-loaded spermatocoon.

Next, the other effective agonists from the recombinant-expressed hOR17-4 receptor were tested in sperm. They produced exactly the same profile of active and inactive substances. This result shows that sperm expresses functionally the receptor hOR17-4 in the membrane. To get information about the functional role of this receptor 'behavior', experiments on human sperm cells were undertaken in cooperation with the group of *Richard Zimmer* (University of California). We found that the

sperm showed a concentration-dependent positive chemotactic behavior to *Bourgeonal*® and doubled their speed in the presence of the odor (*Fig. 9*).

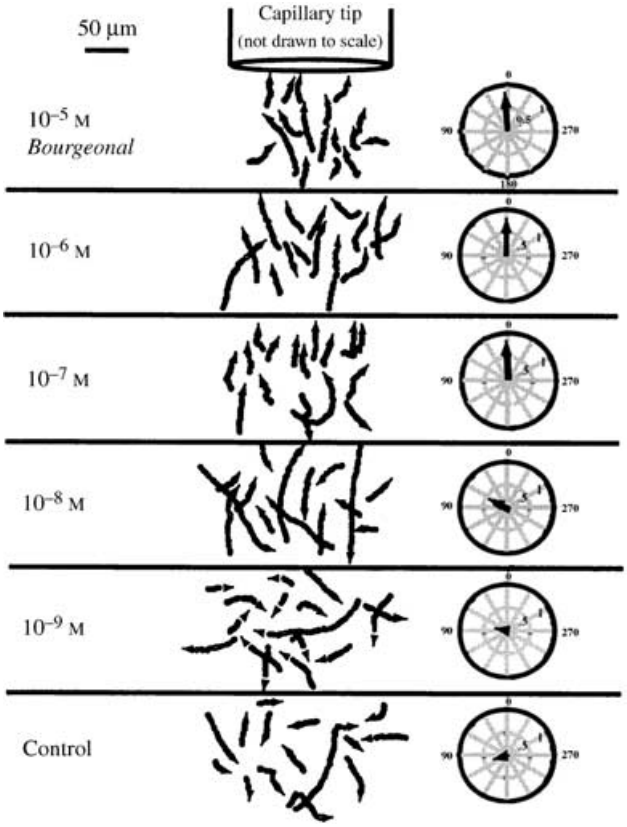

Fig. 9. *Swimming behavior of human sperm imaged near a micro-capillary tip from which* Bourgeonal® *was released.* Concentrations of 10^{-7} M *Bourgeonal*® elicited a positive chemotaxic sperm orientation in the ascending gradient.

Interestingly, when the antagonist undecanal was applied together with the agonist, the effects of *Bourgeonal*® on sperm navigation and swim speed were strongly inhibited. This data suggests that hOR17-4 signaling potentially governs chemical communication between sperm and egg. Thus, the hOR17-4 signaling system could potentially be used to manipulate fertilization with important consequences for contraception and procreation [19].

How can the molecular information obtained at the level of sensory neurons be followed to successive levels of synaptic processing in the olfactory bulb? In these processing steps, the determinant structures of the odor molecules are first mapped into glomeruli, which are the smallest functional units in the olfactory bulb. The synaptic circuits in the glomeruli contain mechanisms for fine tuning, for comparing the responses, and also for plasticity and learning. Each olfactory neuron expresses only one type of the 350 receptor genes present in the human genome. In other words, each

olfactory neuron is relatively specific for a group of certain odorants (according to the molecular receptive field), *e.g.*, '*Helional*-type' or '*Bourgeonal*-type' neurons. However, structurally related olfactory receptors recognize overlapping sets of odorants with distinct affinities and specificities. These data support the existence of a receptor code, in which the identities of different odorants are specified by distinct combinations of odorant receptors that possess unique molecular receptive fields. The olfactory neurons expressing a given receptor can be distributed across a large region of the sensory epithelium, as shown by *in situ* hybridization data. The expression pattern is genetically determined. New fascinating neuroanatomical and immunohistochemical data [20–22] have shown that neurons, expressing a specific receptor, project to only two topographically fixed locations among the 2000 glomeruli in the olfactory bulb of rats. It means, a developing olfactory neuron makes two precise choices from a vast repertoire: first, it selects one receptor from a thousand possibilities in rat (350 in human), and second, it finds one target glomerulum out of a thousand (*Fig. 10*). The basic olfactory map is probably established by a developmental hard-wired strategy.

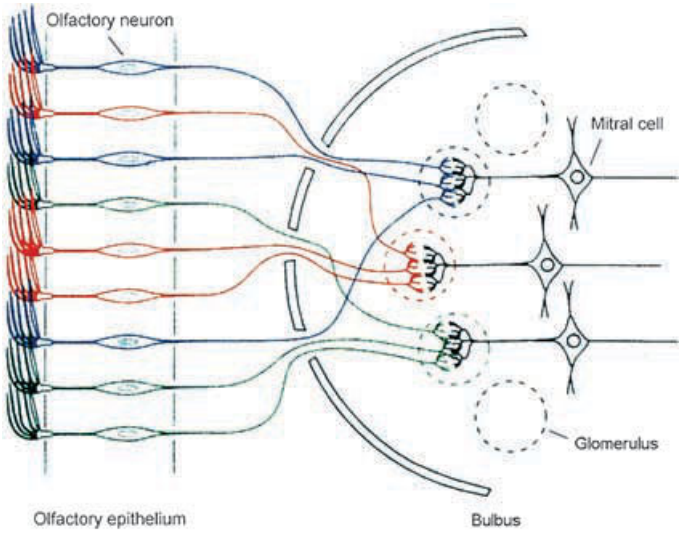

Fig. 10. *Olfactory sensory neurons expressing the same olfactory-receptor protein project to a specific glomerulus in the olfactory bulb*

The convergence of signals from thousands of neurons expressing the same olfactory-receptor protein onto a few glomeruli may optimize sensitivity to low concentrations of odorants by allowing the integration of weak signals from many olfactory epithelium neurons. The invariant pattern of inputs might have a different advantage, ensuring that the neural representation ('code') for an odorant remains constant over time, even though olfactory epithelium neurons are short-lived cells that are continuously replaced [23].

It was also shown that the olfactory-receptor protein is also present in the axonal structures of the olfactory cells. These results raise the interesting possibility that olfactory proteins themselves help to organize the map in the olfactory bulb [24][25].

This data provides direct support for a model in which a certain combination of activated glomeruli encodes the odor quality in the olfactory bulb. The spectra of different odors can overlap.

Conclusions. – Many natural odors such as flower scents and perfumes consist of hundreds of individual chemical components. How can we discriminate a scented rose from an orange? When we inhale such a complex mixture, out of the *ca.* 350 different types of olfactory sense cells, the only ones to be activated are those bearing receptors for one of the chemicals the scent contains. Bearing in mind that all the sensory cells have the same receptor proteins, wherever they may be in the nose, all send their neural processes to one and the same spherical group of cells (glomerulus) in the olfactory bulb, thus producing a constant activation.

For instance, all of the axonal 50000 processes from the 'vanillin-sense *cells*' terminate in the 'vanilla glomerulus', which, at the threshold vanillin concentration, gets activated selectively. When someone smells a mixture of several chemical components, correspondingly more olfactory-receptor types are activated, and, hence, so are the associated glomeruli. The result is a reproducible, but complex, pattern of glomerular activation, from which it is possible to infer by reverse logic which odor mixture has been smelled. The rose-scent activation pattern is clearly distinct from the orange-scent pattern (*Fig. 11*). When individual chemical components are present in both odor mixtures, the patterns in activated glomeruli can overlap.

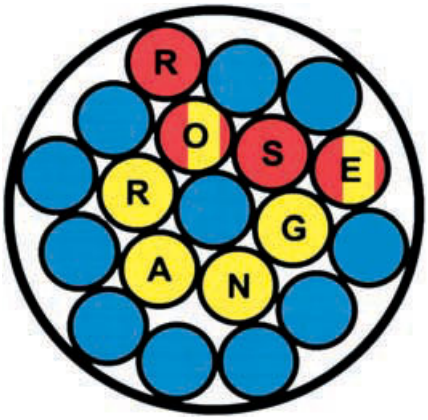

Fig. 11. *Schematic activation model of the glomeruli after stimulation with the scent of roses or oranges*

In psychology, this representation by a particular shape could be described with the terms '*odor gestalt*' or '*gestalt recognition*'. Once we have 'learned' an odor, we can recognize it again [26], even though some of the information it normally contains may be missing. The severely reduced rose or orange scents that are artificially produced take advantage of this fact.

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Vanishing Flora – Lost Chemistry: The Scents of Endangered Plants around the World

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As part of our broad and ongoing evaluation of the olfactory components of fragrant plants and flowers during the past 25 years, we have encountered an astounding number of interestingly scented, but endangered plant species. In appreciation of nature's marvels in these species, we are compiling a report on their scent compositions and complementary information in an upcoming book '*Vanishing Flora – Lost Chemistry*'. In this paper, a few examples of endangered plant species and their scent components are presented as a brief introduction to the concept of the book project.

Introduction. – Plants deliver food, fiber, construction material, colors, fragrances, flavors, fuel, medicine, and, through their beauty, inspiration to mankind. Furthermore, plant life is absolutely essential to the survival of all living things. Botanists believe that, due to actions of humankind, five plant species now disappear from nature each day, most without ever having been recognized, much less classified or analyzed. What has happened to the human spirit that so much of the basic stuff of life is so threatened today?

Dugald Stermer shows in his beautiful and unique book '*The Vanishing Flora*' [1], with lovely and scrupulously accurate illustrations, a selection of 74 of these species and attempts in his strongly worded introduction to make people aware that plants are also threatened – that, globally, at least 10% of all species are in imminent danger of extinction.

In my ongoing search for new scent molecules and scent concepts in nature, I have encountered over the past years a respectable number of interestingly scented endangered species. In our appreciation of nature's marvels and in the hope that we can sensitize people, to the plight of these wondrous plants, we decided to compile their scent compositions as well as complementary information in a book '*Vanishing Flora – Lost Chemistry*'. The purpose of this paper is to give a brief introduction to the concept of this project.

Background and Methods. – Until the middle of the 19th century, natural extracts of scented flowers or other plant parts, and, to a certain extent, animal secretions, were the only raw materials used in the creation of fragrances. No wonder that chemists working in the fragrance industry have been investigating these natural products extensively since the dawn of modern organic chemistry. As a result of this (continuing) research work, the perfumer now has at his disposal not only the 500 or so regularly used natural products for the preparation of his creations but at least double this number of synthetic fragrance compounds that have originated in one way or an other from natural products.

Perspectives in Flavor and Fragrance Research. Edited by Philip Kraft and Karl A. D. Swift

In spite of all these synthetic and natural products available from the shelf, the huge range of fascinating natural scents that surround us is still a great source of stimulation and is yet far from exhausted. For a long time, however, many of these scents could not be analytically investigated because they could not be captured in sufficient amounts and/or in adequate quality.

By the 1970s, methods of instrumental analysis, particularly capillary gas chromatography and mass spectrometry, had reached such a high level of sensitivity, thanks to modern electronics that also the analytical investigation of microsamples could be envisaged. This motivated us to look for methods to trap the scents in the quality emitted by the living flower/plant and as perceived by the human nose. Destructive isolation methods, such as micro-extraction/distillation, that influence the original scent did not come under consideration. However, a close-to-nature trapping technique to enable, for the first time, the analytical investigation of rare and endangered species was deemed the method of choice. Such a method was soon developed to trap the emanated scent on a small amount of suitable adsorbent porous polymer, such as *Porapak* or *Tenax*, or charcoal, followed by solvent extraction.

In the mid 1970s, we at *Givaudan*, thus, implemented a long-term research program with the aim to investigate, and, in promising cases, subsequently synthetically reconstitute these original and attractive scents that are not available as commercial essential oils or related products. The method has since proved to be effective, and has been specially adapted over the past ten years to field experiments conducted under extreme conditions such as those existing in rain forests.

To collect the flower scent of, *e.g.*, *Pachira insignis*, a fascinating Bombacaceae native to the neotropics, a single flower is inserted into a glass vessel of adapted size and shape without damaging the flower (*Fig. 1*).

The scented air surrounding the flower is then drawn through the adsorption trap by means of a battery-operated pump over a period of 30 min to 2 h (30 ml/min), depending on the intensity of the scent. The adsorption trap containing 2–5 mg of adsorbent, in this case *Porapak Super Q*, is placed as closely as possible to the scent source within the glass vessel. While air and moisture pass unhindered these micro-traps, the scent is adsorbed and accumulates to amounts of 10–200 μg during the collection time. For flowers or plant parts with very complex shapes, it is more practical to simply isolate the scent source from the environment to the extent possible with a suitably shaped object, *e.g.*, a glass funnel. The adsorption trap is then centered as near as possible to the position where the scent release is judged to be maximum. Afterwards, the adsorbed scent is eluted with an adequate amount of a suitable solvent, usually 20–60 μl of hexane/acetone 10 : 1, directly into a micro-ampoule, which is then sealed and kept cool until the return to the laboratory. Finally, the samples thus obtained are investigated by a combination of capillary gas chromatography and mass spectrometry, and complementary methods.

By applying these methods over the past 25 years, we have investigated more than 1800 flower, plant, fruit, wood, and herb scents from a selection of *ca.* 9000 species of scented plants olfactorily evaluated during this time. Publications, *e.g.*, on the scent of orchids [2][3] and of cacti [4], and, more generally, on new or uncommon volatile compounds among the most diverse floral scents [5][6], and on scents found in rain forests [7] give partial overviews of these investigations.

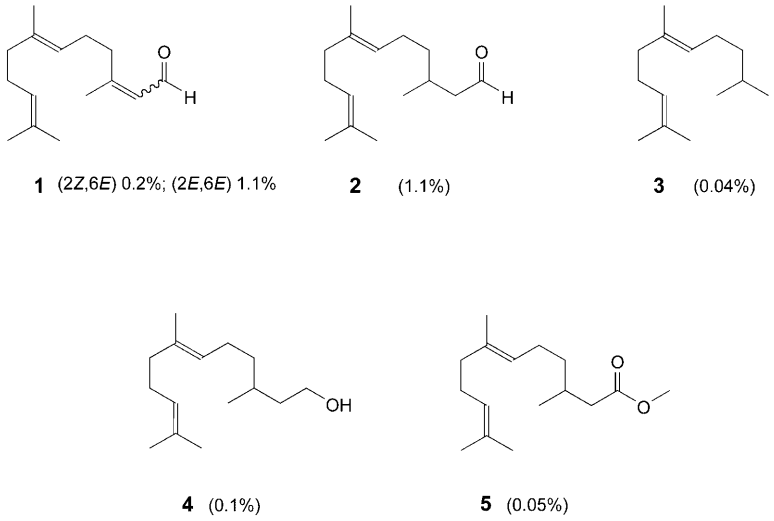
Fig. 1. *Trapping scent samples in the field* (*Pachira insignis*)

As illustrated in another publication [8], our approach to trapping, investigating, and reconstructing natural scents is designed, however, to study not only well-defined flower, herb, fruit, and wood scents, but it can also be applied to the investigation of entire olfactory 'scenarios', perceived in a certain environment as, *e.g.*, in a 'Maquis' biotope at the Ligurian coast [8]. Papers published by *Mookherjee et al.* [9], *Joulain* [10], *Brunke et al.* [11], *Nakamura* [12], *Surburg et al.* [13], and others show that other groups in the fragrance industry probably began to address the topic of scent trapping at more or less the same time. Related and complementary methods have been reviewed by *Bicchi* and *Joulain* [14], *Kaiser* [15], *Dobson* [16], and *Knudsen et al.* [17].

Examples Intended for '*Vanishing Flora – Lost Chemistry*'. – Rothmannia annae, *A Species from a Fragile Eden*. We begin our introductory survey of endangered scented plant species with the most endangered of the 40 *Rothmannia* species that occur in tropical and southern Africa, Asia, and the Seychelles: *Rothmannia annae* (WRIGHT) KEAY (syn. *Gardenia annae*), the so-called '*Wright*'s Gardenia' [18] (*Fig. 2*). 100 Years ago, this species was found on several islands but is now restricted to the privately owned Aride island where 900 trees survive. The *Nature Protection Trust of Seychelles* (*NPTS*), a member of the *World Conservation Union* (*IUCN*), is working to keep this last natural population alive and has a project in cooperation with *Royal Botanic Gardens*, Kew, to re-establish the species on other islands known to have hosted it once. The attractive flowers emit a rich aromatic, white-floral scent, reminiscent of aspects of orange flowers, carnation, and tuberose, which is at its peak during dusk. Of special importance to this scent are methyl benzoate, 2-phenylacetaldehyde, methyl salicylate, 2-phenylethyl alcohol, eugenol, indole, and (*E*)-2,3-dihydrofarnesal (**2**; *cf.* analytical data in the *Appendix*). We found compound **2** (*Fig. 3*) originally some years ago as a new olfactorily important compound in the scent of lemon flowers (*Citrus limon*) [8] and have introduced it in the meantime, as a new fragrance compound. It is easily accessible from farnesal (**1**) by catalytic hydrogenation. In *Rothmannia annae*, **2** occurs also together with the derivatives **3**–**5**, which, as we have observed during the recent years, are frequently associated with **2** in natural scents.

Fig. 2. Rothmannia annae (WRIGHT) KLAY, *a highly endangered species endemic to the Seychelles*

The Star of Madagascar and Other Fascinating 'Moth Orchids'. I have always been struck by the richness of white-flowering, night-active, moth-pollinated species in the orchid flora of Africa [3], including Madagascar as a highly important area. This unique



1 (2Z,6*E*) 0.2%; (2*E*,6*E*) 1.1% **2** (1.1%) **3** (0.04%)

4 (0.1%) **5** (0.05%)

Fig. 3. *(*E*)-2,3-Dihydrofarnesal* (**2**) *and derivatives in the floral scent of* Rothmannia annae (WRIGHT) KLAY (for entire analytical composition, see *Appendix*)

island, characterized by extremely high endemism, is home to arguably the most famous orchid, a symbol of co-evolution and adaptation between a flower and its pollinator, *Angraecum sesquipedale* THOU., also known as the 'Comet Orchid' or as the 'Star-of-Madagascar'. In 1862, *Charles Darwin* prophesied to botanists and biologists the identification of an insect, whose proboscis would be long enough to reach the nectar at the bottom of the 25- to 35-cm long spur. 40 Years later, the corresponding pollinator, the hawkmoth *Xanthopan morgani*, was discovered in the orchid's natural habitat, and, an other 90 years later, Prof. *Wasserthal* was the first to photograph this incredible encounter [19] (*Fig. 4*). *Angraecum sesquipedale*, which we studied in regard to the scent composition already some years ago [2][3], is among the most threatened species in the wild. Thanks to the declaration of the majority of the Masoala Peninsula in northeast Madagascar as a national park in 1997, this unique orchid as well as many other endangered plants have been given an opportunity to survive. Among them are also some representatives of the orchid genus *Aerangis*, which includes *ca.* 50 species occurring on the African mainland and Madagascar. We can only hope that also the enchanting *Aerangis confusa* J. STEWART (*Fig. 5*) will have a future in its natural habitat in Kenya. Fully scentless during day, its flowers, which exhibit all the characteristics for moth pollination, emit after sunset an attractive so-called white-floral scent characterized by notes reminiscent of tuberose and gardenia. For the latter aspects, the *cis*-4-methyldecano-5-lactone (**7**) [2][3] (*Table*) is mainly responsible. We have identified **7** only twice from the 1800 scented plant species, investigated during the past 25 years, in the *Aerangis* species under discussion and in *A. kirkii* (ROLFE) SCHLTR. We named it, therefore, Aerangis lactone. In both floral scents, the Aerangis lactone (**7**) is accompanied by the structurally simple methyl ester **6**, which we have, so far, found also in only these two *Aerangis* species. In the meantime, the absolute configuration of

Fig. 4. Angraecum sesquipedale Thou. *with its pollinator* Xanthopan morgani

Aerangis lactone (**7**) has been established as (4*S*,5*S*) [20] and that of the methyl ester **6** (*S*) [21].

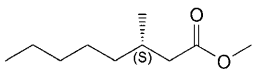
Endangered Treasures of Neotropics. The last examples we present to illustrate the concept of '*Vanishing Flora – Lost Chemistry*' bring us to habitats on the American continent. Probably the most strongly scented flowers can be found within certain neotropical orchid genera that are visited and pollinated exclusively by male euglossine bees (*Fig. 6*). This orchid flower – euglossine bee relationship is especially developed in the genera *Coryanthes*, *Gongora*, *Stanhopea*, and *Catasetum*. In contrast to the situation for 'bee flowers', the pollinators of these species do not associate the strong floral scent with food in form of nectar, since, in fact, these flowers do not produce any. Instead, the bees visit the orchids to collect the fragrance for use afterwards in their own reproduction biology [22][23]. This orchid – euglossine bee relationship is often highly specific, meaning that the scent and shape of a given flower is often designed to attract only one or perhaps a few of the 180 species of euglossine bees. To achieve this high degree of selectivity, the flowers are not only optimally adapted in their morphology,

Fig. 5. Aerangis confusa J. STEWART

but also their scents often contain very uncommon constituents. Thus, the rare *Coryanthes mastersiana* LEHM. from Colombia is totally dominated by the 2-(methylamino)benzaldehyde (**8**) [3][23] (*Fig. 6*), a highly typical scent constituent for the genus *Coryanthes*, while the scent of the even rarer second Colombian species *Coryanthes vieirae* GERLACH contains 80% ipsdienol (**12**), accompanied by the derivatives **13**–**15** [3][23].

Interestingly, many species of the section *Coryanthes* within the genus *Coryanthes* contain the (3*E*,5*Z*)-undeca-1,3,5-triene (**18**; *Scheme*), sometimes amounting to over 50%, and always accompanied by only small amounts of the corresponding (*E*,*E*)-isomer. Interestingly, in all these scent samples, **18** was accompanied by an O-containing compound of molecular weight of 194 exhibiting a much longer retention time on GC. Based on its mass fragmentation, a dodecadieno-4- or -5-lactone could be assumed. The scent sample trapped from *Coryanthes elegantium* LINDEN & RCHB.f., a rare epiphyte from the hot and humid rain forests of western Ecuador and Colombia, contained around 50% of this compound and was sufficiently concentrated for us to be

Table. cis-*(4S,5S)-4-Methyldecano-5-lactone* (**7**), *the So-Called Aerangis Lactone, and Other Constituents of* Aerangis confusa J. STEWART *and* A. kirkii (ROLFE) SCHLTR.



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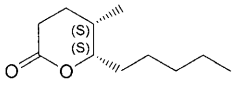


7

Compound	*A. confusa* Area-%	*A. kirkii* Area-%
Methyl 3-methyloctanoate (**6**)	3.2	1.9
4-Methylanisole	–	2.8
Linalool	2.2	3.3
Germacrene D	0.8	33.0
Benzyl acetate	31.2	17.3
(*E*,*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	28.0	–
Phenylethyl acetate	1.1	4.7
Methyl cinnamate	5.7	–
p-Cresol	0.7	1.0
cis-4-Methyldecano-5-lactone (**7**) (Aerangis lactone)	2.7	26.2

able to isolate 10 μg by preparative capillary gas chromatography and to elucidate its structure, (*Z*)-dodeca-2,6-dieno-5-lactone (**16**) by NMR [6].

It is clear that **16** is the biological precursor of **18**, which is present in high isomeric purity. A literature search revealed that **16** was described already in 1977 by *Priestap et al.* [24] as a constituent of the rhizomes of *Aristolochia argentina*, and that it was synthesized four years later by *Fehr et al.* [25], together with a series of other 6-substituted 5,6-dihydropyran-2(2*H*)-ones by the approach outlined in the *Scheme*. In the flower scent of the very rare *Coryanthes panamensis* GERLACH (*Fig. 7*), we most recently found, besides the dodecadienolactone **16** (9.5%; *Scheme*) and **18** (8.9%), also the corresponding degradation product of linolenic acid, the (*Z*,*Z*)-dodeca-2,6,9-trieno-5-lactone (**17**; 0.1%) accompanied by the (*E*,*Z*,*Z*)-undeca-1,3,5,7-tetraene (**19**; 0.3%). In both species, these interesting compounds are accompanied by minor amounts of the unsaturated lactones **20**–**23**. Looking back over three decades of research on natural scents, I can hardly find a better example to illustrate the exquisite chemistry offered to us by nature in these highly endangered plant species.

Turbinicarpus pseudomacrochele *ssp.* krainzianus, *the Mouldiest Scent on Earth.* One of the most unusual floral scents encountered during our broad olfactory evaluation of fragrant flowers, the mouldy, musty-earthy odor typical of geosmin was found in a series of diurnal cacti species [4][26]. The compound responsible for this peculiar odor was most often dehydrogeosmin (**24**) (*Fig. 8*), which was a new natural product in the 1990s, in a few cases occurring together with geosmin (**25**). Among the 1800 fragrant plant species investigated to date in our laboratory, we have found geosmin (**25**) in the flower scents of only three species of Cactaceae and in that of *Dorstenia turneraefolia* FISCH & MAY., a Moraceae native to the Brazilian Amazonas, while dehydrogeosmin (**24**) has now already been identified in 55 cacti species [4][27], but in no other plant family. The extreme is represented by the flower scent of *Turbinicarpus pseudomacrochele* ssp. *krainzianus* (G. FRANK), BACKEB. (*Fig. 9*), in

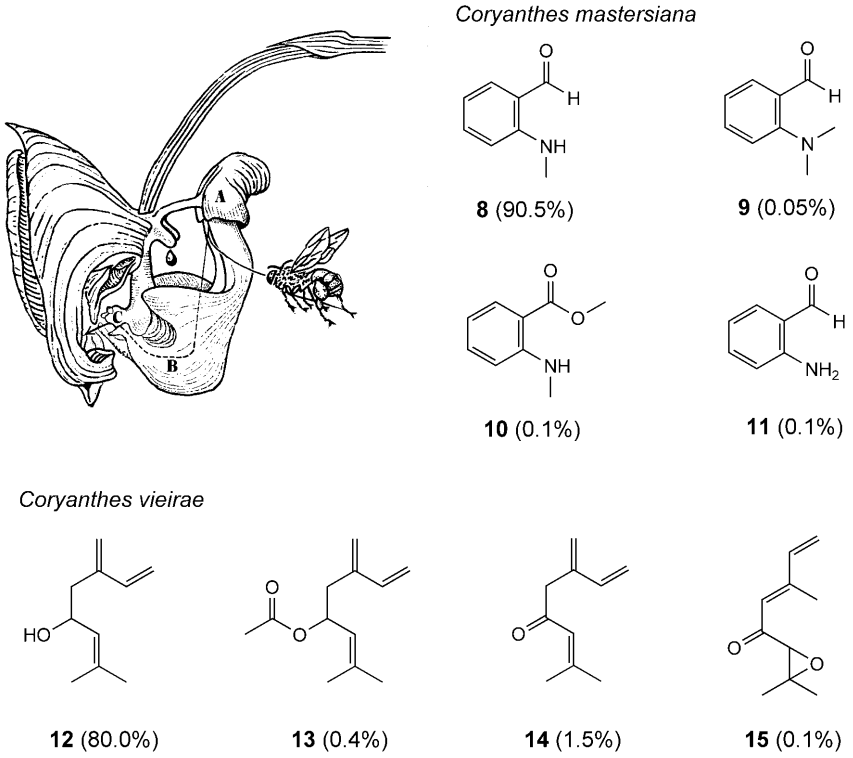
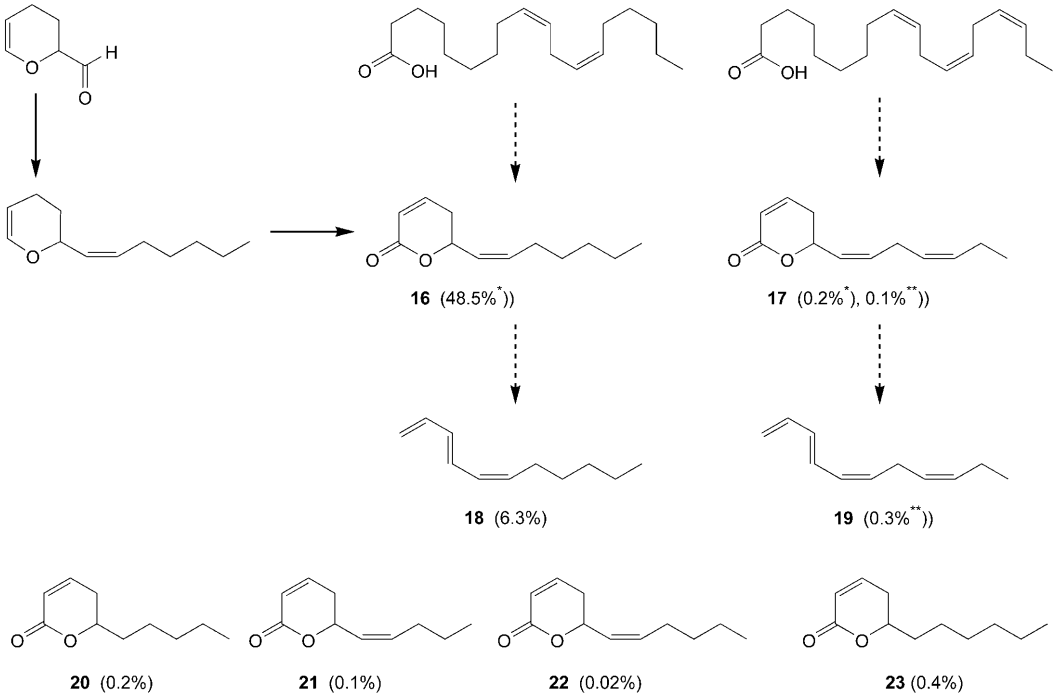


Fig. 6. *Characteristic scent constituents of* Coryanthes mastersiana LEHM. *and* Coryanthes vieirae GERLACH *and a representation of the* Coryanthes *flower together with its pollinator, an euglossine bee.* The attracted respective euglossine bee starts to collect the fragrance in region **A** of the flower, where most of it is exuded. Because of the exposed site the bees often sip on the waxy surface and fall into the fluid-filled bucket **B** formed by the complex lip. Their only escape route is through the narrow tunnel between the bucket and the top of the column **C**, where the pollinia ultimately attach themselves to the pollinator.

which **24** occurs to 78%, accompanied by traces of **25**. This very rare and highly endangered species (CITES, Appendix 1) is native to the Mexican states of Hidalgo and Queretaro. *Boland*, *König*, and co-workers [28] could show that the product emitted by flowers of *Rebutia marsoneri* and *Dolichothele sphaerica* BRITTON & ROSE is optically pure (+)-(4*S*,4a*S*,8a*S*)-**24**, which, thus, has the same absolute configuration as the microbial metabolite (−)-**25**. It is certainly most striking that the flower scent of such representatives of the Cactaceae family, growing mostly under extremely dry and hot conditions, are olfactorily dominated by a compound of extreme musty-earthy character, which – for the human nose – is always associated with moist/damp places. It would not be too surprising if this new natural product were of significant importance to the pollination biology of these Cactaceae. A comprehensive study of dehydrogeosmin-producing species by *Schlumpberger* [27] furnished many new facts to this interesting group of Cactaceae; the biological significance of these compounds, however, remains unclear.

Concluding Remarks. – The five species of highly endangered flowering plants chosen to illustrate our project '*Vanishing Flora – Lost Chemistry*' revealed in their

Scheme *(3*E,*5*Z*)-Undeca-1,3,5-triene* (**18**) *and Its Precursor* **16** *as Highly Characteristic Scent Constituents of the Section* Coryanthes *within the Genus* Coryanthes



*) Percentages refer to the trapped scent of *Coryanthes elegantium*. **) Percentages refer to the trapped scent of *Coryanthes panamensis*.

Fig. 7. Coryanthes panamensis GERLACH, *a rare and endangered species offering beautiful scent chemistry*

scents structurally and olfactorily interesting new constituents that were not previously known in the literature. Furthermore, some of these elucidated 'missing pieces' helped us, in the context of their precursors and derivatives, to understand the respective biogenetic pathways. Finally, it can be assumed that all of these constituents are involved in systems of chemical communication that are vital to the survival of the respective species. Who can imagine what chemical treasures we have already lost with the number of species that have disappeared during recent decades. Current estimates classify 13% of the global flora as threatened with extinction. However, a recent publication [29] makes clear that this figure is already considered to be a serious underestimate, because it does not include a reliable tally of species at risk in the tropical latitudes where most of the world's plants grow.

'*When the last individual of a race of living things breathes no more, another heaven and another earth must pass before such a one can be again*'.

William Beebe (1877 – 1962)

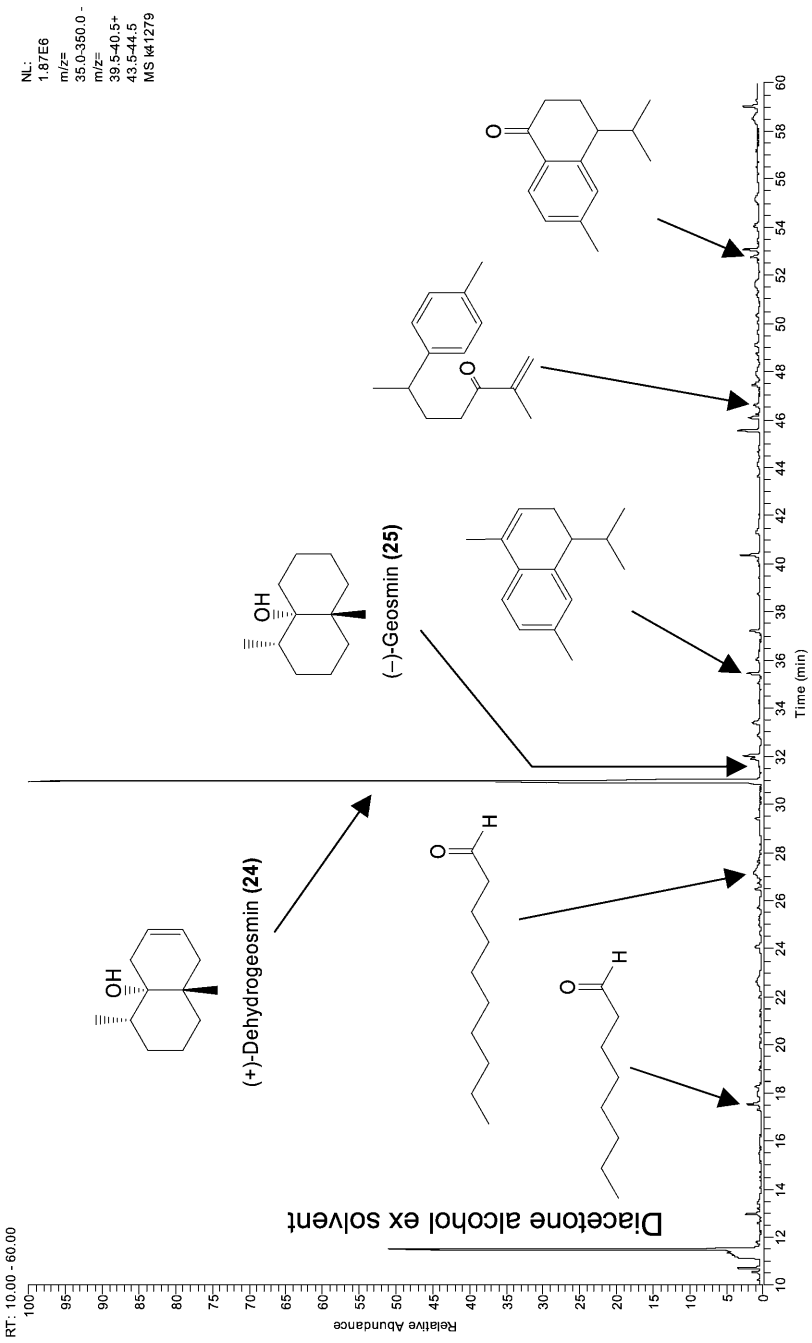


Fig. 8. *GC/MS of the trapped scent of* Turbinicarpus pseudomacrochele *ssp.* krainzianus (G. Frank) Backeb., *revealing (+)-dehydrogeosmin* (**24**) *as the dominating scent constituent*

Fig. 9. Turbinicarpus pseudomacrochele *ssp.* krainzianus (G. FRANK) BACKEB.

Appendix: Analytical Compositions of Flower Scents Discussed in More Detail. – For experimental details regarding trapping and investigating of flower/plant scents, see, *e.g.*, [4][7].

***Rothmannia annae* (WRIGHT) KEAY (**trapped on *Porapak Super Q* at *Botanical Garden of Munich–Nymphenburg*, Germany, 09.50–14.10, November 3, 2000): methyl 2-methylbutyrate 0.20%, methyl valerate 0.05%, α-pinene 0.10%, β-pinene 0.10%, sabinene 0.05%, myrcene 0.05%, methyl tiglate 0.02%, limonene 0.20%, eucalyptol 0.20%, (*E*)-hex-2-enal 0.10%, (*Z*)-ocimene 0.05%, (*E*)-ocimene 13.30%, hexyl acetate 0.04%, 6-methylhept-5-en-2-one 0.08%, anisole 0.02%, hexan-1-ol 0.20%, (*Z*)-3,4-epoxy-3,7-dimethylocta-1,6-diene 0.07%, benzyl methyl ether 0.05%, nonanal 0.06%, (*E*)-3,4-epoxy-3,7-dimethylocta-1,6-diene 0.10%, (*E*)-2,6,10-trimethylundeca-2,6-diene 0.04%, 4-methylanisole 0.03%, 2-methoxy-3-isopropylpyrazine 0.001%, oct-1-en-3-ol 0.02%, citronellal 0.05%, (*E*)-2,3-epoxy-2,6-dimethylocta-5,7-diene 0.20%, decanal 0.04%, benzaldehyde 3.00%, linalool, 0.60%, octan-1-ol 0.07%, methyl benzoate 48.00%, 2-phenylacetaldehyde 1.40%, ethyl benzoate 0.20%, (*E*)-β-farnesene 0.10%, 2-phenylacetaldehyde *O*-methyl oxime ((*E*)/(*Z*) *ca.* 1:1) 0.20%, (*E*,*E*)-α-farnesene 0.10%, methyl salicylate 4.10%, citronellol 0.07%, 2-phenylethyl formate 0.04%, 2-phenylethyl acetate 0.10%, (*E*)-geranylacetone 0.03%, benzyl alcohol 0.40%, 2-phenylethyl alcohol 15.90%, 2-phenylacetonitrile 0.10%, (*E*)-2,3-dihydrofarnesal 1.10%, cinnamaldehyde 0.01%, 3-phenylpropan-1-ol 0.03%, (*E*)-nerolidol 0.30%, methyl (*E*)-cinnamate 0.03%, methyl 2-methoxybenzoate 0.03%, methyl (*E*)-3,7,11-trimethyldodeca-6,10-dienoate 0.05%, *p*-cresol 0.50%, 1-nitro-2-phenylethane 0.05%, (*E*)-cinnamyl acetate 0.07%, eugenol 0.02%, (*Z*,*E*)-farnesal 0.20%, methyl anthranilate 0.05%, 3-hydroxy-4-phenylbutan-2-one 0.03%, (*E*,*E*)-farnesal 1.10%, (*E*)-2,3-dihydrofarnesol 0.10%, (*E*)-cinnamic alcohol 0.06%, 2-phenylacetaldehyde oxime ((*E*)+(*Z*)) 0.40%, 1*H*-indole 1.60%, vanilline 0.01%, benzyl benzoate 0.40%, 2-phenylethyl benzoate 1.90%, 2-phenylethyl salicylate 0.10%.

***Angraecum sesquipedale* THOU.** (trapped on *Porapak Super Q* at Point Tampolo, Masoala Peninsula, Madagascar, 21.15–04.30, November 21, 2001): isovaleronitril 0.20%, limonene 0.10%, eucalyptol 0.10%, (*Z*)-ocimene 0.05%, (*E*)-ocimene 0.50%, 1-nitro-3-methylbutane 0.20%, (*Z*)-hex-3-en-1-ol 0.05%, nonanal 0.10%, isovaleraldoxime ((*E*)+(*Z*)) 26.00%, benzaldehyde 0.80%, methyl benzoate 1.20%, phenylacetaldehyde 4.50%, benzyl acetate 0.30%, neral 0.10%, *trans*-linalool oxide (pyranoid) 0.20%, geranial 0.10%, geranyl acetate 0.05%, methyl salicylate 0.08%, *cis*-linalool oxide (pyranoid) 0.20%, 2-phenylethyl acetate 0.10%, benzyl alcohol 25.00%, 2-phenylethyl alcohol 7.50%, 2-phenylacetonitrile 0.40%, β-ionone 0.30%, β-ionone epoxide 0.03%, anisaldehyde 2.20%, *p*-cresol 2.10%, 1-nitro-2-phenylethane 0.30%, 6,10,14-trimethylpentadecan-2-one 0.80%, methyl anthranilate 0.05%, eugenol 0.10%, anisyl alcohol 0.30%, (*E*)-cinnamic alcohol 0.10%, 2-phenylacetaldehyde oxime ((*E*)+(*Z*)) 2.20%, 1*H*-indole 15.00%, (*E*)-4-methoxycinnamaldehyde 0.10%, (*Z*)-4-methoxycinnamic alcohol 0.10%, benzyl benzoate 0.50%, (*E*)-*p*-methoxycinnamic alcohol 1.00%, benzyl salicylate 0.02%.

***Aerangis confusa* J. STEWART** (trapped on charcoal at *Botanical Garden of Zurich*, Switzerland, 18.00–20.00, October 28, 1990): α-pinene 0.30%, butyl acetate 0.05%, myrcene 0.01%, limonene 1.50%, eucalyptol

0.30%, (*E*)-ocimene 0.02%, nonanal 0.10%, methyl 3-methyloctanoate 3.20%, menthone 0.02%, decanal 0.05%, benzaldehyde 1.80%, linalool 2.20%, bornyl acetate 0.02%, methyl benzoate 4.50%, menthol 0.02%, benzyl formate 0.50%, germacrene D 0.80%, benzyl acetate 31.20%, methyl phenylacetate 0.10%, (*Z*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene 0.20%, (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene 28.00%, 2-phenylethyl acetate 1.10%, benzyl alcohol 0.30%, 2-phenylethyl alcohol 0.10%, methyl (*E*)-2,6,10-trimethylundeca-5,9-dienoate 1.30%, (*E*)-nerolidol 0.20%, methyl (*E*)-cinnamate 5.20%, *p*-cresol 0.70%, (*E*)-cinnamyl acetate 0.80%, methyl (*E*,*E*)-3,7,11-trimethyldodeca-2,6,10-trienoate 0.70%, *cis*-4-methyldecano-5-lactone 2.70%, methyl (*E*,*E*)-4,8,12-trimethyltrideca-3,7,11-trienoate 6.20%, 1*H*-indole 0.10%, benzyl benzoate 1.20%, benzyl salicylate 0.20%.

***Coryanthes panamensis* Gerlach** (trapped on *Porapak Super Q* at *Botanical Garden of Munich – Nymphenburg*, Germany, 08.50 – 13.15, March 24, 2003): α-pinene 4.70%, β-pinene 2.90%, sabinene 3.80%, δ-3-carene 0.30%, myrcene 0.30%, limonene 1.50%, dodecane 0.80%, eucalyptol 0.60%, styrene 5.50%, hexyl acetate 2.50%, (*E*)-4,8-dimethylnona-1,3,7-triene 0.30%, (*Z*)-hex-3-en-1-yl acetate 0.20%, hexan-1-ol 1.90%, (*Z*)-hex-3-en-1-ol 0.10%, (*E*,*Z*)-undeca-1,3,5-triene 8.90%, (*E*,*E*)-undeca-1,3,5-triene 0.80%, *trans*-linalool oxide (furanoid) 0.05%, (*E*,*Z*,*Z*)-undeca-1,3,5,7-tetraene 0.30%, octyl acetate 0.10%, decanal 0.20%, pentadecane 0.30%, linalool 0.05%, *trans*-α-bergamotene 1.40%, terpinen-4-ol 0.20%, hexadecane 0.30%, methyl benzoate 0.10%, β-bisabolene 1.00%, 1,2-dimethoxybenzene 0.70%, germacrene D 0.20%, 1,4-dimethoxybenzene 0.60%, germacrene A 20.70%, (*E*,*Z*)-deca-2,4-dien-1-ol 0.10%, methyl (*Z*)-cinnamate 0.10%, (*E*)-cinnamyl acetate 5.10%, 6-pentyl-α-pyrone 0.20%, massoia lactone 0.04%, elemol 0.50%, methyl (*Z*)-4-methoxycinnamate 2.20%, dodeca-2-eno-5-lactone 0.20%, (*Z*)-dodeca-2,6-dieno-5-lactone 9.50%, (*Z*,*Z*)-dodeca-2,6,9-trieno-5-lactone 0.30%, methyl (*E*)-4-methoxycinnamate 2.80%, (*E*)-4-methoxycinnamyl acetate 2.70%.

***Turbinicarpus pseudomacrochele* ssp. *krainzianus* (G. Frank) Backeb.** (trapped on *Porapak Super Q* at the *Collection of Succulent Plants of the City of Zurich*, Switzerland, 13.40 – 15.20, October 9, 2002): nonanal 1.00%, decanal 1.40%, benzaldehyde 0.50%, dehydrogeosmin 69.00%, geosmin 0.02%, calamenene 1.50%, (*E*)-geranylacetone 0.80%, α-calacorene 1.00%, γ-calacorene 0.80%, 2-methyl-6-(*p*-tolyl)hept-1-en-2-one 0.80%, cadaline 0.50%, 3,4-dihydro-4-isopropyl-6-methyl-2*H*-naphthalen-1-one 1.00%.

I am grateful to Dr. *G. Gerlach*, *Botanical Garden Munich – Nymphenburg*, for samples of the trapped scents of *Rothmannia annae* and *Coryanthes panamensis*, and for the corresponding photographs shown in *Figs. 2* and *7*, to Mr. *W. Philipp*, custodian of the orchid collection at Zurich University for samples of the trapped scent of *Aerangis confusa*, to Dr. *Thomas Bolliger* and Dr. *Urs Eggli*, *Collection of Succulent Plants of the City of Zurich*, for samples of the trapped scent of *Turbinicarpus pseudomacrochele*, to Prof. Dr. *L. T. Wasserthal*, University of Erlangen – Nürnberg, Germany, for permission to reproduce *Fig. 4*, to my colleagues at *Givaudan Fragrance Research* for discussions and assistance, in particular to Dr. *J. Schmid* for many discussions on the mass spectra of new components, to Mr. *H. Gfeller* for all the GC/MS measurements, to Mr. *E. Senn* and Mr. *H. J. Vögeli* for their skillful assistance throughout the investigations summarized in this paper, and, last but not least, to Mrs. *Aurelia Kreis* for all the organizational and secretarial work.

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From the Linden Flower to Linden Honey – Volatile Constituents of Linden Nectar, the Extract of Bee-Stomach and Ripe Honey

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Honey is produced by honeybees (*Apis mellifera*), which collect nectar from flowers, digest it in their bodies, and deposit it in honeycombs, where it develops into ripe honey. We studied the evolution of the volatile constituents from the nectar of linden blossoms (*Tilia cordata*) to honey *via* the 'intermediate' honeybee.

The sampling of the contents of the honey stomach or honey sack of the bee is unique. Extracts were prepared from nectar, from the liquid of the honey stomach, and from ripe honey. The chemistry is extremely complex, and compounds spanning from monoterpenes (hydrocarbons, ethers, aldehydes, acids, and bifunctional derivatives), isoprenoids, aromatic compounds (phenylpropanoids, phenols), and products degraded from fatty acids to alkaloids, were identified. Some compounds definitely stem from the plants, whereas other interesting constituents can be attributed to animal origin. Two derivatives of decanoic acid, 9-oxodec-2-enoic acid (**12**) and 9-hydroxydec-2-enoic acid, identified in the honey are known to be constituents of the so-called '*Queen's pheromone*'. Two metabolites of these acids were identified in the extract of the honey stomach: 8-oxononanal (**10**), a new natural product, and 8-oxononanol (**11**). There structures were confirmed by synthesis.

Nectar and honey stomach contain many aldehydes, which, due to the highly oxidative atmosphere in the honeycomb, are found as corresponding acids in the honey. Two acids were newly identified as 4-isopropenylcyclohexa-1,3-diene-1-carboxylic acid (**14**) and 4-(1-hydroxy-1-methylethyl)-cyclohexa-1,3-diene-1-carboxylic acid (**15**).

Introduction. – Ever since prehistoric times, honey has played an important role in human nutrition, principally as a flavorsome sweetener, but also for its medicinal properties, which include alleviating insomnia, lowering blood pressure and nervousness, and preventing arteriosclerosis. Honey is produced by the honeybees (*Apis mellifera*), which collect nectar, digest it, and stock it in honeycombs, where it is ripened. The nectar, a sticky liquid, is produced in the blossom by nectary glands and collected in cup-forming sepals (*Fig. 1*).

The nectar gathered is stocked in the honey stomach, which can contain up to 60 µl of liquid (*Fig. 2*). Enzymes in the saliva, produced in the cervical gland, degrade sucrose into glucose and fructose and cleave glycosides of the nectar into sugar moieties and volatile aglycones. Only a portion of the nectar is transferred to the intestines through a special valve and digested to produce energy for long-distance flights, or stored for hibernation.

On returning to the hive, the content of the stomach is regurgitated into the waxy honeycomb, either as feed for the bees carrying out their duties in the hive or to ripen into honey. In this investigation, we tried to follow the transformation of the volatiles from the nectar to the ripened honey. The volatile compounds of the nectar, and then of the liquid contained in the honey stomach of the bee, having collected nectar from

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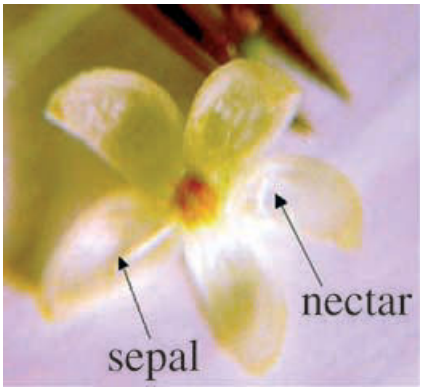


Fig. 1. *Sepals of a linden flower with a droplet of nectar*

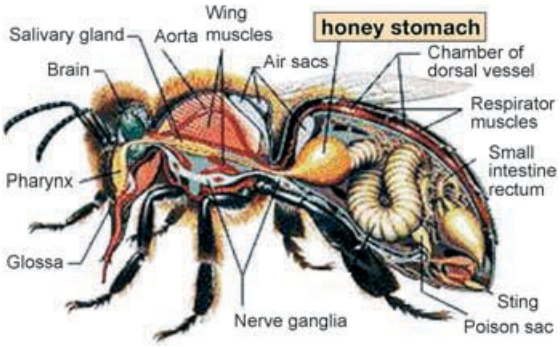


Fig. 2. *Anatomy of the bee* [1]

linden flowers (*Tilia cordata*), were studied. The only trees in bloom in the surrounding neighborhood of the honeycombs were linden trees, and it was, thus, assumed that the nectar preferentially gathered was from linden flowers, and that the honey sample was also monofloral. The nectar of 50 flowers was collected, extracted, and analyzed, as well as the contents of the stomach of 25 bees caught at the entrance of the hive on their way back from nectar gathering. Gentle pressure with two fingers applied on their backs ejected the liquid into a glass capillary tube (*Fig. 3*).

There are only a few studies in which the chemical constituents of the flower have been correlated with those of the corresponding honey or nectar: the volatiles of the flower, the nectar, and the honey of leatherwood, an endemic plant of Tasmania [2], and constituents of the nectar of *Citrus* flowers, concentrating, however, on the content of caffeine [3]. Most recently, a comparison of the components of extracts of entire *Citrus* flowers and of *Citrus* honey showed similar monoterpenoids as identified in our study [4]. The analyses of linden honey and the fresh linden flowers led to the identification of a unique monoterpenoid ether called '*linden ether*' (=2,4,5,7a-tetrahydro-3,6-dimethylbenzofuran; **1**) [5]. To the best of our knowledge, no

Fig. 3. *Manual sampling of the contents of the honey stomach of a bee*

investigation has described the isolation and analysis of linden nectar and of the liquid extracted from bee stomach.

The present paper will highlight some observations that seem relevant for the processing of nectar to honey. It is, however, out of the scope of this report to give the exhaustive results of the investigation, since *nearly 500 different compounds* have been identified!

Results and Discussion. – The extracts prepared from linden nectar, linden nectar recovered after being digested in the honeybee stomach, and of the ripe linden honey are extremely complex. An impressive number of unknown compounds show mass-spectral patterns that are characteristic for mono- and bifunctional monoterpenoids. Due to the minute quantities of samples of the first two types of extracts, fractionation and isolation of compounds for further analytical measurement could only be performed with the third sample, *i.e.*, the finished honey. Most results were established only by GC/MS injection and interpretation of the mass spectra, by comparison with reference substances, and by confirmation of their retention times.

Nectar. The nectar extract (see *Fig. 4*) is composed of all the essential groups of natural products: compounds of fatty acid degradation (nonanal, decanal, tetradec-1-ene), phenylpropanoids (3-(4-methoxyphenyl)propan-1-ol, 3-(4-methoxyphenyl)propanal, 3-(4-methoxyphenyl)prop-2-enal), isoprenoids (vomifolione, vomifoliol, and 3,5,5-trimethyl-4-(3-oxobutyl)cyclohex-2-en-1-one), alkaloids (caffeine, theophylline, a trace of nicotine), and a complex mixture of monoterpenes, among them the above mentioned linden ether (**1**), 1,8-cineole, diols **2** and **3**, and unknown compounds having molecular weights (MW) of 148, 150, 152, 166, 168, and 170, respectively (see *Fig. 5*). As a consequence of the results described later for the ripe honey, the following hypothetical structures might be proposed for two of the honey compounds: *p*-mentha-1,3,8-trien-7-al (**4**) and 8-hydroxy-*p*-mentha-1,3-dien-7-al (**5**). Their mass spectra are depicted in *Fig. 6*,*a* and *6*,*b*, respectively.

Honey Stomach. In the honey stomach (see *Fig. 7*), the aliphatic compounds, the isoprenoids, and the alkaloids all remain unchanged. The presumption that active

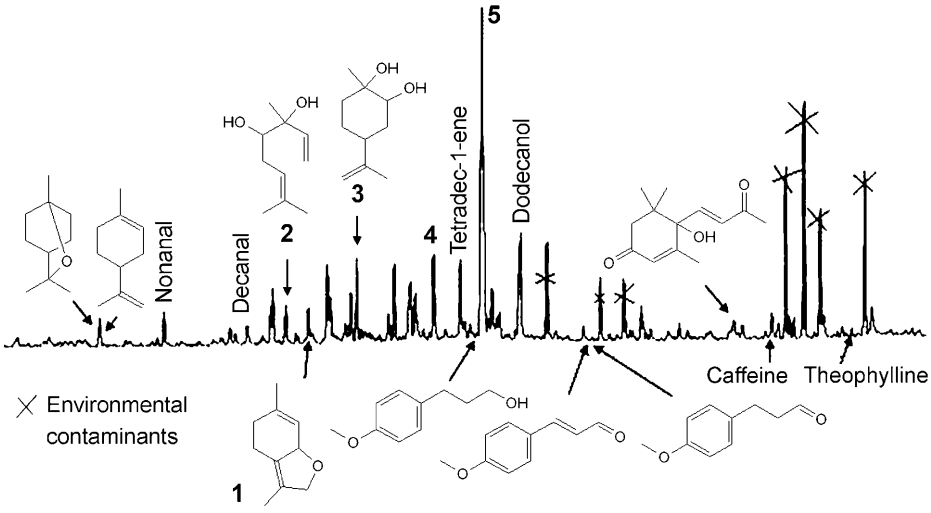


Fig. 4. *Gas-chromatographic* (GC) *profile of linden-flower nectar extract*

glycosidases are present in the saliva was confirmed by the appearance of new monoterpenic alcohols in the extract of the liquid isolated from the bee stomach: the three linalool derivatives 3,7-dimethylocta-1,5-dien-3,7-diol (**6**), 3,7-dimethylocta-1,6-dien-3,5-diol (**7**), and 2,6-dimethyl-6-hydroxyocta-2,7-dienal (**8**), also identified in *Citrus* honey [4]. The latter aldehyde, giving rise to the isomers of lilac aldehyde [4] (compounds identified in linden honey as well), has already been identified in lavandin oil; it is formed during the '*noble rot*' of grape must by *Botrytis cinerea* [6]. The most-abundant compound (MW 164) is highly unsaturated and, most probably, corresponds to the unknown aromatic compound 4-(1-hydroxy-1-methylethyl)benzaldehyde (**9**; see *Fig. 6,c*).

Two new *aliphatic* compounds, with mass fragments at *m*/*z* 43 and 58, respectively, typical for methyl ketones, and with molecular weights of 156 and 158, appeared as trace components. Their hypothetical structures, 8-oxononanal (**10**) and 9-hydroxynonan-2-one (**11**) were confirmed by synthesis (see *Exper. Part*); and their resemblance with the '*Queen's substance*' (= 9-oxodec-2-enoic acid; **12**) was evident. This semiochemical is the main constituent of the '*Queen's pheromone*', a well-equilibrated cocktail of fatty acids and aromatic compounds [7], produced in the mandibular gland of the queen and having the function of regulating the social behavior of the bee colony.

α-Oxidation of the '*Queen's substance*' (**12**), or of the saturated analog **13**, another constituent of the pheromone, to 2-hydroperoxy-9-oxodecanoic acid, followed by decarboxylation, leads to the keto aldehyde **10** [8] (see *Scheme 1*). These two compounds are the only metabolites of animal origin absorbed into the plant liquid extracted from the bee stomach. The alcohol **11** is known from milk [9], *Pimenta racemosa* [10], krill products [11], and, most interestingly, from the sternal gland of the elephant shrew [12]. The aldehyde **10** has been described in a synthetic context only [13], and is, therefore, a new natural product.

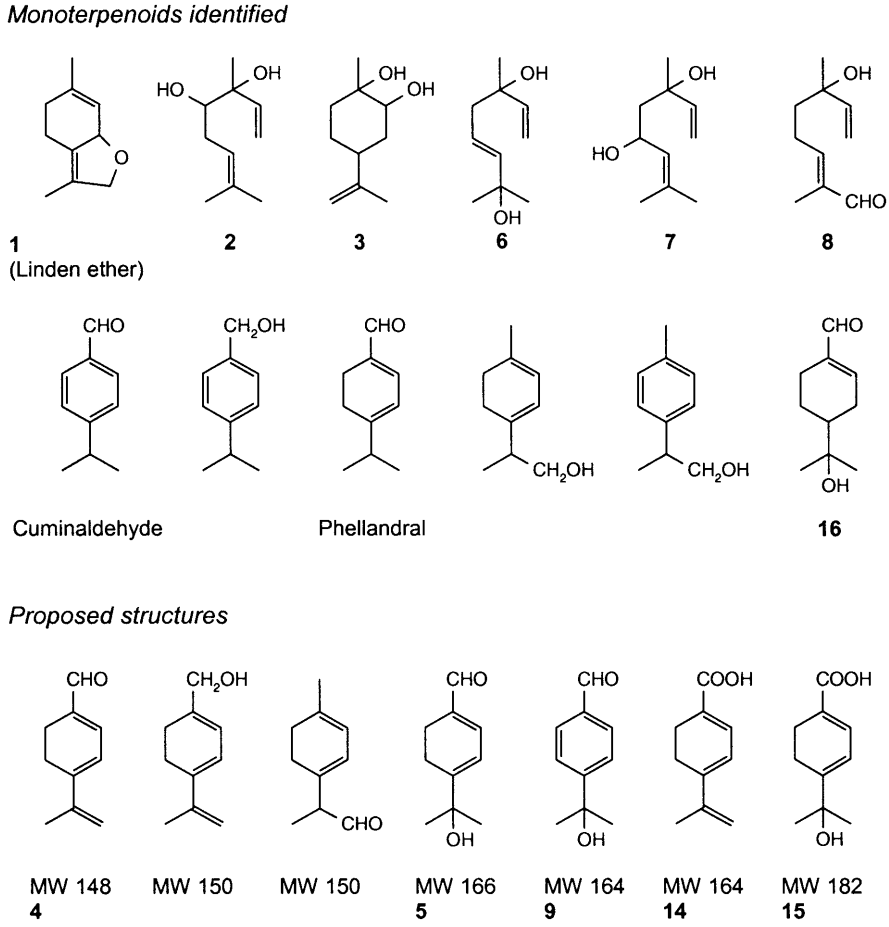


Fig. 5. *Identified and proposed strucutres of monoterpenoids*

Ripe Honey. After one month, the mature honey is recovered from the waxy honeycomb by centrifugation. The preparation of a sugar-free extract by solid-phase extraction (SPE) on an *OASIS®-HBL* cartridge (rather than solvent extraction with CH_2Cl_2 with the formation of nasty emulsions), provided a product with excellent organoleptic properties truly representing the starting material. The monoterpenoid diols **3** and **7**, vomifolione, caffeine, and theophylline remain intact under the highly oxidative atmosphere of the honeycomb (35° and abundant air (O_2) provided by 'ventilation' performed by the bees moving their wings at the entrance of the hive), whereas other compounds do undergo oxidation. Benzoic acid and phenylacetic acid, very characteristic of the honey-like smell, together with two new monoterpenic acids, 4-isopropenylcyclohexa-1,3-diene-1-carboxylic acid (**14**) and 4-(1-hydroxy-1-methyl-ethyl)cyclohexa-1,3-diene-1-carboxylic acid (**15**) are abundant components (see *Fig. 8*). The isolation, identification, and spectral data of **14** and **15** will be discussed in a separate paper. The structures of these compounds, established by NMR

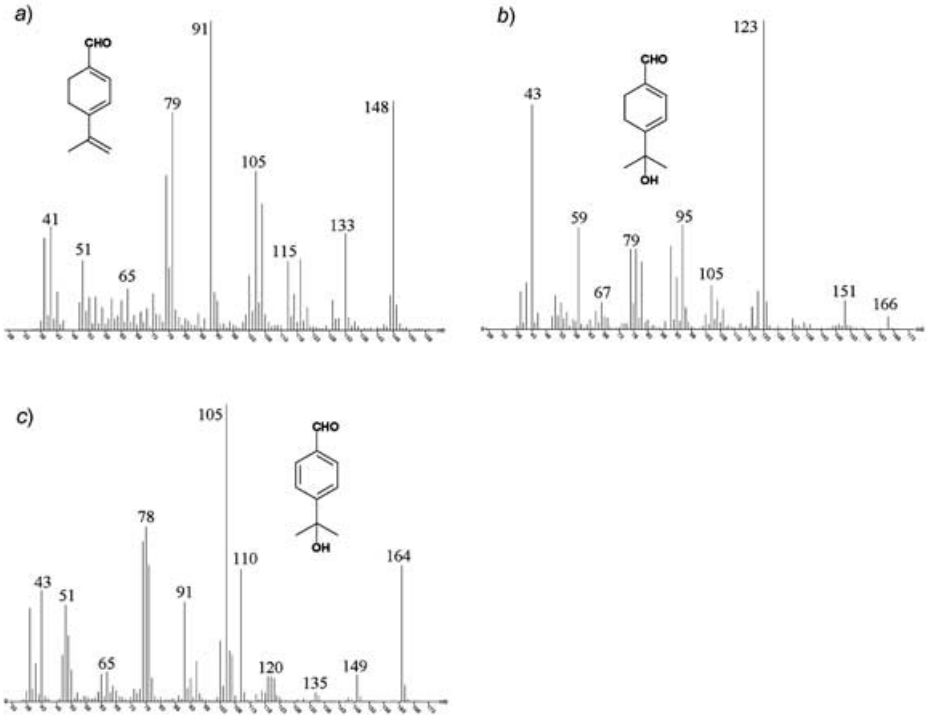
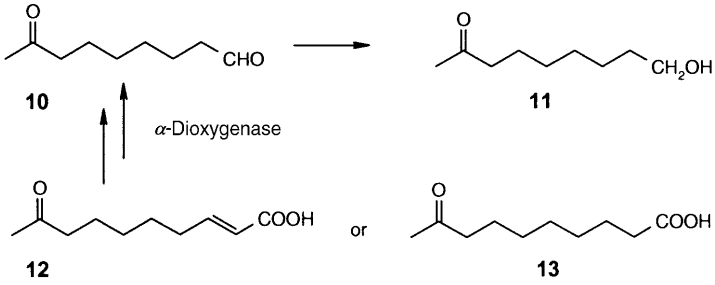


Fig. 6. *Mass spectra and anticipated chemical structures of the* (unknown) *compounds* **4** (*a*), **5** (*b*), and **9** (*c*)

Scheme 1. *Transformation of Compounds Related to* 'Queen's substance' (see text)



experiments, support the hypothetical structures of aldehydes **4** and **5** proposed in nectar. Compound **5** was still present in the ripe honey, eluting just after 8-hydroxy-*p*-menth-1-en-7-al (**16**), a known compound [14]. The content of linden ether (**1**) increased, and among the trace compounds, a series of further *p*-menthofuranoids, including dill ether and menthofuran, and rose oxide, are compounds with strong odor impacts (*Fig. 9*) [5][15].

Methyl syringate (**17**; see *Fig. 8*) is most probably absorbed into the lipophilic honey from propolis, a resinous material collected by the worker-bees from tree barks and used, mixed with beeswax, to construct and strengthen the combs. Within the methyl

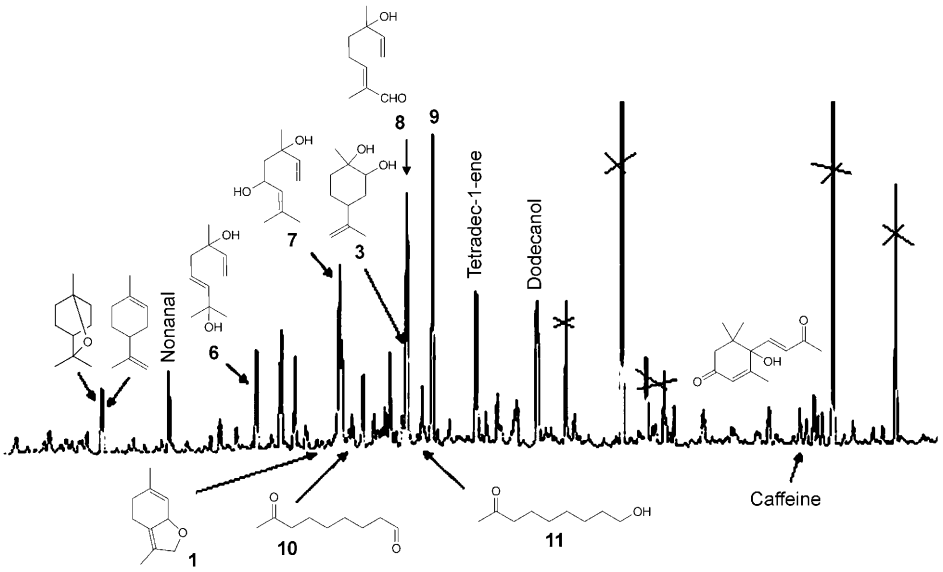


Fig. 7. *Gas-chromatographic profile of the extract of the honey stomach*

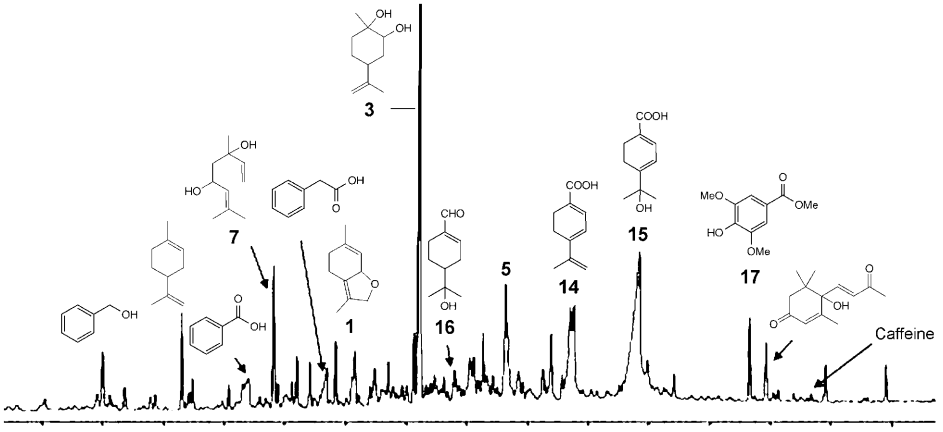


Fig. 8. *Gas-chromatographic profile of linden honey* (*OASIS*® extraction)

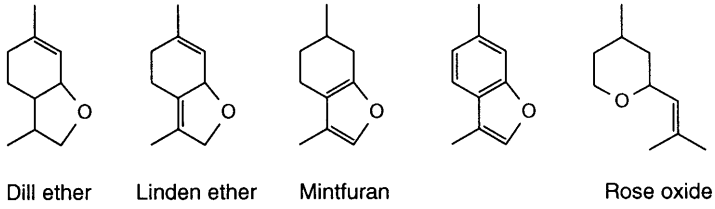


Fig. 9. *Structures of 'menthofurans' identified in linden honey*

ester fractions, among 115 acids, the '*Queen's substance*' (**12**) and 9-hydroxydec-2-enoic acid [16] (another compound of the '*Queen's pheromone*') were identified. This is, to our knowledge, the first report of these pheromones in finished honey. Many saturated (C_6 to C_{14}) and unsaturated (dec-2-enedioic and dodec-2-enedioic) diacids, having their origin in royal jelly [17][18] – a secretion of the pharyngal gland, and used as protein-rich feed for the larvae of the queen – are present together with a panoply of aromatic acids [19] and a trace amount of abscisic acid. The alkaloids caffeine and theophylline have been postulated as markers in *Citrus* honeys [3], where their concentration is significantly higher; and caffeine, together with theobromine, was identified in tea flowers [20].

Conclusions. – Our observations are based on the interpretation of restricted samples, quasi '*snapshots*' of a complex sequence of events. This report is a subjective selection of results that seem to delineate the evolution of the volatile constituents from the flower nectar to honey. Many compound structures could not be fully elucidated, and some hypothetical compounds were proposed. However, compounds **10** and **11**, related to the '*Queen's substance*', were confirmed. The monoterpenoid acids **14** and **15** as well as their glycosidic precursor, which could be isolated from various honeys, will be the subject of a detailed subsequent publication.

We are indebted to Dr. *F. Brühlmann* and Dr. *B. Maurer* (*Firmenich SA*) for stimulating discussions about natural-products chemistry.

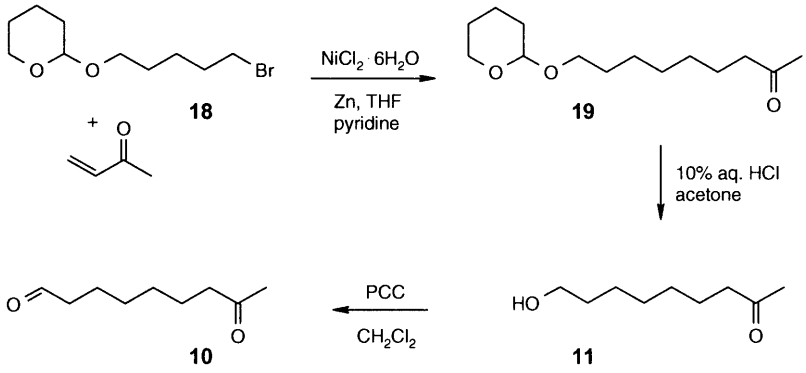
Experimental Part

General. GC/MS Experiments were performed on a *6890* gas chromatograph (*Agilent*) coupled to a quadrupole *5971* mass spectrometer (*Agilent*) equipped with an apolar column (*SPB-1* capillary column, 30 m × 0.25 mm, film thickness 1 μm; temp. program: 5 min at 60°, then 60° – 250° at 5°/min; injector temp. 250°, transfer-line temp. 250°; carrier gas: He), or on a *6890* gas chromatograph (*Agilent*) coupled to a quadrupole *5972* mass spectrometer (*Agilent*) equipped with a polar column (*Supelcowax®*; 30 m, film thickness 0.25 μm; temp. program: 5 min at 50°, 50 – 240° at 5°/min; carrier gas: He); EI mass spectra were generated at 70 eV at a scan range from *m/z* 27 – 350. Linear retention indices (*RI*) were determined after injection of a series of n-alkanes (C_5 – C_{28}) under identical GC conditions. ^{1}H- and ^{13}C-NMR Spectra were measured on a *Bruker AMX-360* instrument in $CDCl_3$; δ values in ppm rel. to Me_4Si as internal standard. Solid-phase-extraction cartridges: *OASIS®-HLB* (*Waters*), 20 ml, filled with 1 g of polymer. Linden flowers were picked in a linden trees path in Cormondrèche (Neuchâtel, Switzerland) on a dry, sunny day (June 2002) and immediately transferred to the laboratory for workup. Linden honey: ripe honey produced from the same trees with the same hives in 2001 was obtained ('*Les Miels Suisses*', Mr. *Boris Bachofen*). 9-Oxo-dec-2-enoic acid was purchased from *Maybridge Chemical*, Cornwall; 3,4-dihydro-2*H*-pyran from *Acros Organics*, Geel; 5-bromopentan-1-ol from *TCI Organic Chemicals*, Oregon; and Zn powder (< 45 μm) from *Merck*, Darmstadt. 9-Hydroxynonan-2-one (**11**) was prepared according to [21] and [22] (following *Scheme 2*).

Isolation and Extraction of the Nectar. – Approximately 50 flowers were dissected with tweezers to access the droplets of nectar situated in the concave sepals. These droplets were aspirated into a glass capillary (i.d. 0.7 mm); rich flowers gave up to 2 cm of liquid, which was blown into H_2O (25 ml) with Ar gas. The capillaries were rinsed with H_2O. The aq. layer was extracted with CH_2Cl_2 (5 ml), and the org. extract was dried and concentrated.

Isolation and Extraction of the Liquid from the Honey Stomach. 25 Worker-bees were caught at the entrance of the hive, and pressure was applied on their backs with two fingers, which pushed the contents of the honey-stomach back to the mouth where it could be aspirated with a small glass capillary (*Fig. 3*). The bees were then released without being harmed. The pieces of capillary tubes containing the extracted liquid were covered with deionized H_2O (25 ml), rinsed with H_2O, and covered with CH_2Cl_2 (10 ml). The aq. layer was extracted

Scheme 2. *Synthesis of Compounds* **10** *and* **11**



with CH_2Cl_2 (3 × 20 ml), and the combined org. extracts were dried ($MgSO_4$) and concentrated by distilling off the solvent (*Vigreux* column).

Preparation of the Extract of Ripe Linden Honey. – An *OASIS®-HLB* cartridge was put on top of a vial with a rubber adapter and branched to a water pump. It was conditioned with MeOH (10 ml), and equilibrated with deionized H_2O (10 ml), applying a slight vacuum. A soln. of linden honey (50 g) in H_2O (400 ml) was loaded, and the cartridge was rinsed with H_2O (100 ml) and then with 5% aq. MeOH (10 ml). The compounds were eluted with Et_2O (20 ml), and the solvent was dried ($MgSO_4$) and removed by distillation (*Vigreux* column).

Preparation of the Methyl Ester Fraction of the Honey Extract. The extract (780 mg), prepared as described above, was dissolved in Et_2O (150 ml) and treated with sat. aq. $NaHCO_3$ soln. (150 ml). After neutralization with 10% aq. HCl soln. (70 ml), drying, and concentration under reduced pressure, the fraction (300 mg) containing org. acids was treated with an ethereal soln. of diazomethane (CH_2N_2), until the yellow color persisted. Removal of the solvent afforded a mixture of methyl esters (240 mg), which were separated by column chromatography (CC; SiO_2; gradient of ether/pentane, twelve fractions).

*2-[(5-Bromopentyl)oxy]tetrahydro-2*H*-pyran* (**18**; *Scheme 2*). Conc. aq. HCl (0.1 ml) was added at r.t. to 3,4-dihydro-2*H*-pyran (1.76 g, 21.0 mmol). Then 5-bromopentan-1-ol (3.32 g, 20 mmol) was added dropwise with cooling in a water bath. After 4 h of stirring at r.t., conc. aq. HCl (0.2 ml) was added, and stirring was continued for 3 h. Then, Et_2O was added, and the org. phase was separated, washed with aq. Na_2CO_3, dried ($MgSO_4$), and concentrated (rotary evaporator). The resulting residue (4.94 g) was distilled to provide **18** (1.25 g, 25%). B.p. 131–136° (12 mm Hg). ^{1}H-NMR: 1.44–1.95 (*m*, 12 H); 3.35–3.90 (*m*, 6 H); 4.58 (*t*, 1 H). ^{13}C-NMR: 98.9 (*d*); 67.2 (*t*); 62.4 (*t*); 33.7 (*t*); 32.6 (*t*); 30.8 (*t*); 28.9 (*t*); 25.5 (*t*); 25.0 (*t*); 19.7 (*t*). EI-MS: 251 (4, M^+), 249 (5), 151 (10), 149 (11), 85 (100), 69 (47), 56 (23), 41 (54), 29 (18).

9-Hydroxynonan-2-one (**11**). Zn Powder (740 mg, 11.4 mmol) was introduced into a flame-dried flask under Ar. THF (8.0 ml), $NiCl_2 \cdot 6\,H_2O$ (120 mg, 0.505 mmol), pyridine (550 mg, 6.95 mmol), and but-3-en-2-one (660 mg, 9.42 mmol) were introduced. The mixture was heated to 60° for 30 min. Compound **18** (1.25 g, 4.98 mmol) was added, and the mixture was heated for 72 h at 80°. After cooling, Et_2O and THF were added, the soln. was filtered, and the filtrate was concentrated under reduced pressure. Acetone (100 ml) and 10% aq. HCl (200 ml) were added to the resulting crude mixture, which contained 9-(tetrahydro-2*H*-pyran-2-yloxy)nonan-2-one (**19**; 66%). The mixture was stirred for 30 min. at r.t., and saturated with NaCl, and extracted with Et_2O (3 ×). The org. layer was dried ($MgSO_4$), the solvent was removed *in vacuo*, and the resulting residue (580 mg) was purified by CC (SiO_2; pentane/ether 3 : 7) to provide **19** (190 mg, 15% from **18**) and the desired keto alcohol **11** (170 mg; 22% from **18**).

Data of **19**: ^{13}C-NMR: 209.3 (*s*); 98.9 (*d*); 67.6 (*t*); 62.4 (*t*); 43.8 (*t*); 30.8 (*t*); 29.9 (*q*); 29.7 (*t*); 29.2 (*t*); 29.1 (*t*); 26.1 (*t*); 25.5 (*t*); 23.8 (*t*); 19.7 (*t*). EI-MS: 242 (0, M^+), 157 (3), 141 (4), 123 (30), 85 (100), 67 (10), 55 (28), 43 (95), 29 (15).

Data of **11**: GC/MS Retention indices (RIs): 2223 (polar), 1337 (apolar). ^{1}H-NMR: 1.25–1.40 (*m*, 6 H); 1.52–1.62 (*m*, 4 H); 2.00 (br. *s*, 1 H); 2.14 (*s*, 3 H); 2.42 (*t*, 2 H); 3.62 (*t*, 2 H). ^{13}C-NMR: 209.6 (*s*); 62.8 (*t*); 43.7 (*t*); 32.7 (*t*); 29.9 (*q*); 29.2 (*t*); 29.1 (*t*); 25.6 (*t*); 23.7 (*t*). EI-MS: 140 (1, M^+), 125 (2), 111 (2), 101 (3), 97 (5), 82 (20), 71 (17), 58 (75), 55 (40), 43 (100), 31 (17).

8-Oxononanal (**10**; *Scheme 2*). A soln. of **11** (1.06 g, 6.70 mmol) in CH_2Cl_2 (10 ml) was added dropwise at r.t. to a stirred soln. of pyridinium chlorochromate (PCC; 2.18 g, 10.1 mmol) in CH_2Cl_2 (10 ml). The mixture was stirred for 90 min. Then, Et_2O was added, the mixture was filtered through a pad of SiO_2, and the filtrate was concentrated *in vacuo*. The resulting residue (0.98 g) was purified by CC (SiO_2; pentane/ether 1:1) to afford **10** (550 mg; 53%). RI: 1967 (polar), 1266 (apolar). ^{1}H-NMR: 1.28–1.40 (*m*, 4 H); 1.55–1.68 (*m*, 4 H); 2.13 (*s*, 3 H); 2.42 (*m*, 4 H); 9.76 (*t*, 1 H). ^{13}C-NMR: 209.0 (*s*); 202.7 (*d*); 43.8 (*t*); 43.6 (*t*); 29.9 (*q*); 28.9 (*t*); 28.8 (*t*); 23.5 (*t*); 21.8 (*t*). EI-MS: 156 (0, M^+), 138 (6), 113 (3), 95 (10), 81 (4), 71 (13), 58 (52), 43 (100), 29 (12).

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Pyrazines and Pyridines from Black Pepper Oil (*Piper nigrum* L.) and Haitian Vetiver Oil (*Vetiveria zizanioides* (L.) NASH)

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An investigation of nitrogen containing compounds in the basic fraction of black pepper oil and vetiver oil was carried out by capillary GC, GC/MS, and HPLC. A range of compounds were identified in both oils; 20 previously unreported pyrazines and pyridines were observed in black pepper, whilst only four of the 23 pyrazines and pyridines detected in vetiver are described to occur in this oil in the literature.

Introduction. – Black pepper and vetiver oils are important natural ingredients in the flavor and fragrance industry. Each has a characteristic odor that, in the case of black pepper has '*roast*' notes and in the case of vetiver has '*earthy*' notes, is often associated with nitrogen containing compounds. Analysis of other essential oils has shown the importance of nitrogenous compounds [1]. Whilst the volatile compounds of black pepper have been studied in the literature and over 135 compounds have been identified [2–6], its volatile nitrogenous materials have not yet been investigated in detail. A series of acyl amides have been reported in *Muntok* pepper by *Kollmannsberger et al.* [5], and three pyrazine compounds (2,5-dimethyl-3-methoxypyrazine, 2-isopropyl-3-methoxypyrazine and 2,3-diethyl-5-methylpyrazine) were found previously in a study identifying the potent odorants of black and white pepper [4], although these compounds were not the primary focus of the work.

Black pepper oil is extracted from the fruit of *Piper nigrum* of which several varieties exist. The fruits are picked by hand and normally dried in the sun, then further dried in ovens to remove most of the moisture. There may then be further light roasting before the peppercorns are finally extracted. The black pepper oil used in this study was supplied by *Sensient Ltd.* as 'Pepper Black – C1522' produced by supercritical fluid extraction.

The most comprehensive studies of vetiver are by *Weyerstahl et al.* [7–9], who concentrated on the neutral and polar fractions of Haitian essential oil, identifying in excess of 175 components. However, few references to any pyrazines or pyridines of vetiver oil are to be found in the literature [1].

The vetiver oil used in this study was supplied by the *Haiti Essential Oil Company* as '*Vetiver Haiti Pure*'. The oil originated from the south western peninsula of Haiti, around Les Cayes. The root of the vetiver plant is picked by hand, the bulked roots steam distilled, and the vetiver oil collected after condensation of the steam. The oil is then centrifuged to remove any remaining water.

Results and Discussion. – This analysis of black pepper has revealed 20 pyrazines and pyridines that the authors believe have not previously been reported as

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components of black pepper oil (*Figs. 1* and *2*). Volatile nitrogenous compounds that have been previously reported include piperidine [5][10], 1-acetylpiperidine [5] and 1-formylpiperidine [5][6]. Whether the reported nitrogen compounds are present in their native form in the plant as the product of biochemical pathways, or whether they are the products of chemical reactions such as the *Maillard* reaction on amino acids during the various post-harvest processes cannot be determined by this investigation, however, it is likely that there is a contribution from both processes.

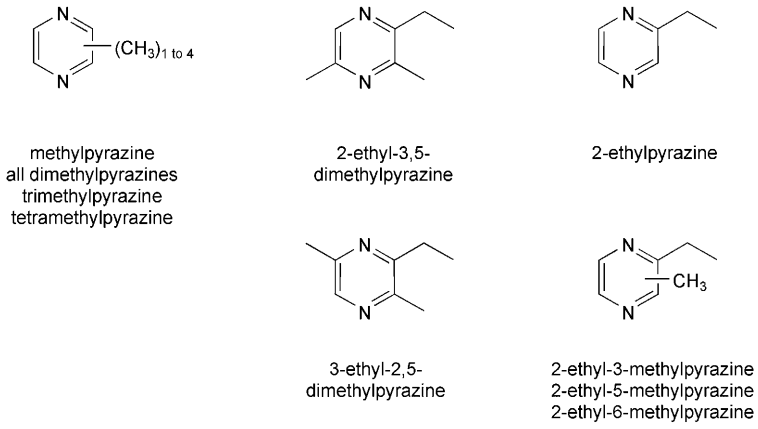

Fig. 1. *Pyrazines from black pepper*

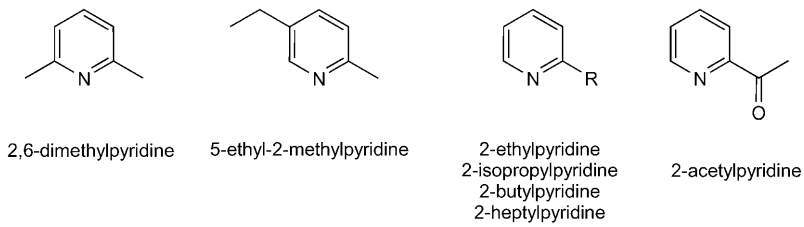

Fig. 2. *Pyridines from black pepper*

The most abundant pyrazine identified in black pepper is 2,3,5,6-tetramethylpyrazine estimated at 7 ppm in the oil (*Table 1*). The odor character determined by GC-O and odor threshold values [11 – 13] reveal that the compounds contributing most to the characteristic odor are 3-ethyl-2,5-dimethylpyrazine (*earthy, roasty*), 2-ethyl-3-methylpyrazine (*roasted, aromatic*) and 2-ethyl-3,5-dimethylpyrazine (*roasted, green vegetable, coffee* and *cocoa* note).

In this study, a total of 23 pyrazines and pyridines have been identified in vetiver oil, four of which are known previously to occur in vetiver (2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-6-methylpyrazine and 2-pentylpyridine) [1] (*Figs. 3* and *4*). The compounds identified as contributing to the odor of vetiver oil are 2-isopropyl-3-methoxypyrazine, which has a green earthy note and is estimated to be present in the oil at about 500 ppb, well above its published detection threshold of 1 ppb [13], and 2-ethyl-

Table 1. *Pyrazines and Pyridines Identified in Black Pepper Oil*

Compound	RRI	Identification	Estimated conc. [ppm]	Odor description
pyridine	752	[a])	0.23	sour, putrid, fishy (conc.), warm burnt, smoky (dil.) [11]
2-methylpyrazine	815	[a])	0.66	nutty, roasted [14]
2,6-dimethylpyridine	888	[a])	0.15	green, astringent, earthy [11]
2-ethylpyridine	906	[a])	0.15	green [14]
2,5-dimethylpyrazine	908	[a])	0.97	aromatic, roasted, cocoa, coffee, nut nuances [15]
2,6-dimethylpyrazine	910	[a])	0.52	chocolate, roasted nuts, fried potato odor[b])
2-ethylpyrazine	910	[a])	0.15	musty, nutty, peanut, woody [15]
2,3-dimethylpyrazine	916	[a])	1.70	green, nutty, potato, cocoa, coffee, caramel, meaty [14]
2-isopropylpyridine	957	[c])	0.15	narcotic, terpeney, green roots [15]
2-ethyl-6-methylpyrazine	998	[a])	0.30	roasted hazelnut [14]
2-ethyl-5-methylpyrazine	1004	[a])	0.15	nutty, roasted, somewhat grassy[b])
2,3,5-trimethylpyrazine	1005	[a])	1.40	nutty, roasted [14]
2-ethyl-3-methylpyrazine	1007	[a])	0.52	potato, burnt nutty, roasted, cereal, earthy[b])
5-ethyl-2-methylpyridine	1024	[d])	0.73	amine-like, nauseating [15]
2-acetylpyridine	1037	[a])	0.84	popcorn, bready, tobacco [11]
3-ethyl-2,5-dimethyl-pyrazine	1082	[d])	0.49	cocoa, chocolate, nutty, burnt almond[b])
2-ethyl-3,5-dimethyl-pyrazine	1090	[d])	1.50	roasted, green vegetable, coffee and cocoa note [15]
2,3,5,6-tetramethylpyrazine	1091	[a])	7.0	aromatic, smoky, dry herbal, weak, nutty, musty [15]
2-butylpyridine	1099	[c])	0.39	green, herbal, fruity[b])
diethyl-methylpyrazine[e])	1163	[d])	0.39	earthy, roasty[b])
2-heptylpyridine	1407	[d])	0.40	green, floral, geranium, earthy[b])

[a]) Identification by comparison with RRI and MS data from a reference sample held in these laboratories. [b]) Odor assessment by in-house panel. [c]) Identification by comparison with RRI and MS data from synthesized sample. [d]) Identification by comparison with MS data from external libraries. [e]) Correct isomeric form not identified.

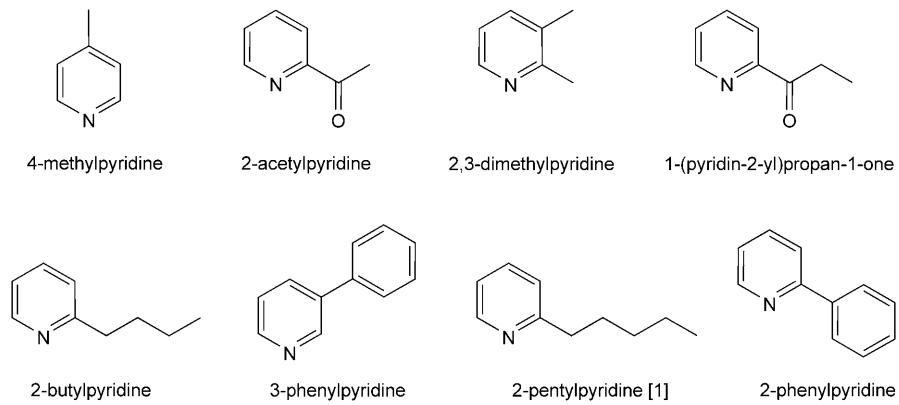

Fig. 3. *Pyridines from vetiver oil*

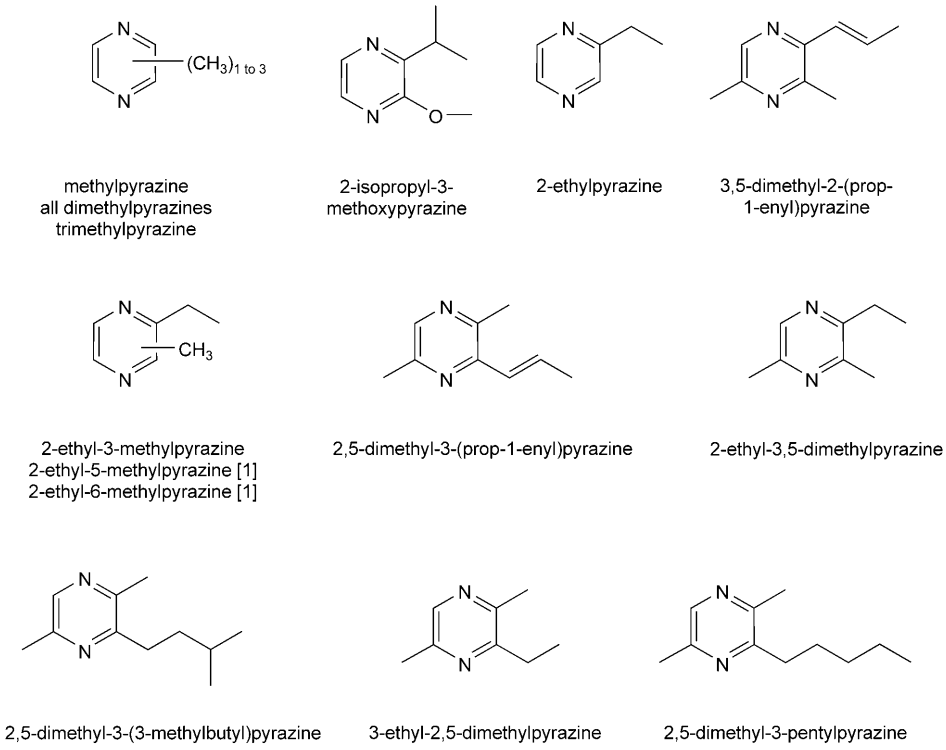

Fig. 4. *Pyrazines from vetiver oil*

3,5-dimethylpyrazine, which has been estimated at 300 ppb also above its known threshold value [12] (*Table 2*).

Experimental Part

Extraction. The oils were washed successively with 10% (*v*/*v*) aq. H_2SO_4 (5×100 ml) and then H_2O (50 ml). The washings were combined and back washed with pentane (5×20-ml portions) to remove any remaining org. compounds. The aq. layer was treated with aq. $NaHCO_3$ until it reached pH 8, and the basic materials were removed by washing with AcOEt (5×20 ml). The org. extract was dried (anh. $MgSO_4$), filtered and concentrated to yield a dark brown residue. GC-FID/NPD analysis of the basic fraction revealed the presence of a number of nitrogen containing compounds.

Fractionation. The basic extract from black pepper was fractionated by prep. HPLC, while the basic extract of vetiver was fractionated by flash column chromatography.

Prep. HPLC. Both normal and reverse-phase conditions were used since some loss of components due to irreversible adsorption of certain compounds was observed in normal phase. In both instances, a *Laserchrom SDS 9404* HPLC pump and *Rheodyne* 100-µl fixed volume injection loop were used. Data acquisition and processing were carried out with *HP ChemStation* software (Rev. A.06.01 [403]).

Reverse-Phase Conditions. Whatman ODS-3 – Partisil 5 column (10 cm × 9 mm × 5 µm), isocratic elution, mobile phase MeCN/H_2O (50 : 50), flow rate 1.8 ml min^{-1}. Detection was performed by UV/VIS diode array (*Hewlett-Packard 1040*).

Collected fractions were treated with dil. HCl (to pH 1), concentrated by rotary evaporation and reconstituted in H_2O. Treatment with aq. $NaHCO_3$ (to pH 9) was followed by partition into AcOEt, and drying (anh. $MgSO_4$).

Table 2. *Pyrazines and Pyridines Identified in Haitian Vetiver Oil*

Compound	RRI	Identification	Estimated conc. [ppm]
2-methylpyrazine	815	[a])	0.3
4-methylpyridine	856	[a])	1.3
2,5-dimethylpyrazine	908	[a])	0.3
2,6-dimethylpyrazine	910	[a])	0.3
2-ethylpyrazine	910	[a])	0.3
2,3-dimethylpyridine	912	[a])	0.3
2-ethyl-6-methylpyrazine	998	[a])	0.6
2-ethyl-5-methylpyrazine	1004	[a])	5.4
2,3,5-trimethylpyrazine	1005	[a])	trace[b])
2-ethyl-3-methylpyrazine	1007	[a])	trace[b])
2-acetylpyridine	1037	[a])	0.3
3-ethyl-2,5-dimethylpyrazine	1082	[a])	0.9
2-ethyl-3,5-dimethylpyrazine	1090	[a])	0.3
2-isopropyl-3-methoxypyrazine	1098	[a])	0.6
2-butylpyridine	1099	[a])	0.3
1-(pyridin-2-yl)propan-1-one	1140	[c])	0.3
2-pentylpyridine	1200	[a])	0.6
2,5-dimethyl-3-(prop-1-enyl)pyrazine	1232	[a])	trace[b])
3,5-dimethyl-2-(prop-1-enyl)pyrazine	1235	[a])	0.6
2,5-dimethyl-3-(3-methylbutyl)pyrazine	1318	[c])	0.6
2,5-dimethyl-3-pentylpyrazine	1370	[c])	0.3
2-phenylpyridine	1467	[a])	0.3
3-phenylpyridine	1471	[a])	0.3

[a]) Identification by comparison with RRI and MS data from a reference sample held in these laboratories. [b]) Trace level less than 0.1 ppm. [c]) Identification by comparison with MS data from external libraries.

Normal-Phase Conditions. Dynamax silica column (25 cm × 10 mm × 5 μm), mobile phase of 20% *t*-BuOMe and 0.5% MeOH in hexane, isocratic elution, flow rate 1 ml min^{-1}. Detection was performed with a UV/VIS diode array detector (*Hewlett-Packard 1040*) and refractive-index detector (*Laserchrom RI 2000*).

Flash Column Chromatography. A *Flash 40S KP-Sil* silica-cartridge column (*Biotage, Dynax Corp.*) was used (32–63 μm, 40 g, 4.0 × 7.0 ml), with a *t*-BuOMe/hexane (50:50; *v/v*) mobile phase. Visualization of the components was achieved first by irradiation with a UV/VIS lamp, set at wavelength 365 nm, then with molybdophosphoric acid in EtOH (ca. 20%) applied as a spray. After recombining fractions of similar composition, the solvent was removed by rotary evaporation and the samples reconstituted in hexane (*ca.* 0.5 ml) for further analysis.

Component Identification. GC-FID/NPD analysis was carried out with a *Hewlett-Packard 6890* gas chromatograph, fitted with a *HP-5* cap. column (25 m × 0.2 mm × 0.33 μm), split injection (50:1) with H_2 carrier gas (1.2 ml min^{-1}). A column splitter leads to separate FID and NPD detectors. The oven was programmed from 50° to 280° (held for 6 min) at 10° min^{-1}. The injector and detector temp. were held constant at 250° and 300°, resp. Data were acquired and processed with *HP ChemStation* software (Rev. A.06.01 [403]).

Gas Chromatography/Mass Spectrometry. GC/MS was performed on a *Varian 3400* gas chromatograph coupled to a *Finnigan MAT ITS40* ion-trap mass spectrometer, operating at electron impact of 70 eV, source temp. 220°. An *Ultra 2* capillary column (50 m × 0.2 mm × 0.33 μm) with He carrier gas at 1.6 ml min^{-1}, split injection (50:1) was used. The oven was programmed from 50° to 270° at 2° min^{-1} with a constant injector temp. of 250°. Data acquisition and processing were handled by *DataMaster* software (version II). Individual components were identified by comparison to fragmentation patterns and relative retention indices with existing literature [16–18] and authentic reference samples, some of which were synthesized in this laboratory. Relative retention indices were calculated against a series of *n*-alkanes. Compounds were quantified by calibration with external standards where possible. The concentration of nitrogenous compounds in the original oil was estimated by extrapolation of the quantities determined in the fractions.

Odor Assessment. GC-O was performed using an *Hewlett-Packard 5890 Series II* instrument with a *HP-5* column (25 m × 0.32 mm × 0.52 μm), which splits to a FID and a custom built sniffing port held at 200° with humidified N_2 at 20 ml min^{-1}. The carrier gas was He at a flow rate of 1.2 ml min^{-1}, and the oven was programmed from 50° to 280° at 3° min^{-1}. The injector and detector temp. were 250° and 280°, resp. Odor descriptions for pure materials were provided by a trained in-house panel, literature sources [11][12][14][15], and compared to those obtained by GC-O.

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14-Methylpentadecano-15-lactone (Muscolide): A New Macrocyclic Lactone from the Oil of *Angelica archangelica* L.

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The chemical composition of seed and root oils from *Angelica archangelica* L. was investigated. Analyses were performed by GC/MS and GC using two columns of different polarities (polyethylene glycol (*DB-Wax*) and 5% phenyl/95% polydimethylsiloxane (*HP-5*)), for the separation of several co-eluting components. A total of 58 compounds were identified, accounting for 96.3% (seed) and 93.5% (root) of the oils, respectively. A high content of β-phellandrene (74.7%) was found in *Angelica* seed oil. Root oil contained a larger amount of macrocyclic lactones (1.3%) in comparison to the seed oil (0.4%). Different harvest dates produced only slight changes in the root-oil composition. In root oil harvested in summer, the β-phellandrene content increased by *ca.* 36%, but no significant changes in the relative compositions of other components were observed. Fresh root oils were collected in five fractions (constant time intervals) during steam distillation (see *Table*). The highest-boiling fraction contained 9.3% of macrocyclic lactones such as tridecano-13-lactone (5.0%), 12-methyltridecano-13-lactone (0.4%), tetradecano-14-lactone (0.1%), pentadecano-15-lactone (3.5%), 14-methylpentadecano-15-lactone (**1**; trace), hexadecano-16-lactone (trace), and heptadecano-17-lactone (0.2%). This is the first report of the occurrence of 14-methylpentadecano-15-lactone (muscolide; **1**) in a natural product.

Introduction. – Oils from *Angelica archangelica* L. are important ingredients in flavor formulations used in the alcoholic-beverage industry, and in lower dosages also in the preparation of fine fragrances. The seed oil is a light yellow liquid with a *fresh*, *sweet*, and *peppery* odor. The root oil is a pale yellow to deep amber liquid with a *green*, *herbaceous*, *peppery*, *musk-like* odor and a *bittersweet* taste [1]. The most-valuable root oils, obtained in the last high-boiling fractions collected during steam distillation, are comprised of unique musk-like compounds [2].

Several reviews have described the chemical composition of *Angelica* seed and root oils [3–5]. Macrocyclic lactones in *Angelica* oils have been described as essential odor components responsible for the *musky* note [6]. Pentadecano-15-lactone was the first natural musk identified in *Angelica* root oil [7]. Subsequently, *Taskinen* and *Nykanen* [8] analyzed in detail the chemical composition of *Angelica* root oil. Tridecano-13-lactone (0.4%), pentadecano-15-lactone (0.4%), and heptadecano-17-lactone (trace) were the main macrocyclic lactones identified, together with an unknown compound found in less than 0.1% content. The new component was further identified as 12-methyltridecano-13-lactone based on ^{1}H-NMR, MS, and IR data [9]. *Schultz* and *Kraft* [10] obtained a fraction rich in macrolides by fractional distillation and silica-gel column chromatography of a commercial sample of *Angelica* root oil. GC/MS Analysis of this fraction allowed identifying the following macrocyclic lactones: tridecano-13-

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lactone, 12-methyltridecano-13-lactone, tetradecano-14-lactone, pentadecano-15-lactone, hexadecano-16-lactone, and heptadecano-17-lactone. The enantiomer composition of 12-methyltridecano-13-lactone was established as $(R)/(S) = 72:28$, and the major enantiomer was found to have a clean musk-like odor, with a sandalwood tonality.

A comparison between the *Angelica*-root extracts obtained with liquid CO_2 *vs.* liquid CO_2/EtOH/H_2O indicated that the plain CO_2 extract was richer in macrocyclic lactones, tridecano-13-lactone (0.4%), 12-methyltridecano-13-lactone (1.1%), and pentadecano-15-lactone (7.7%) [11].

Chalchat and *Garry* [12] examined the effect of drying and distilling procedures with different root parts on the chemical composition of *Angelica* root oil. Increased drying time appreciably lowered the yield of oil from whole roots. Whereas the yield of oil was strongly influenced by the nature of the roots, the composition remained fairly stable. Extractions performed with the plant out of (or in) H_2O afforded oils with similar compositions. High oil recovery was obtained by steam distillation [4][12].

Ojala et al. [13] studied the composition of *Angelica* oil obtained from roots harvested in Norway, Finland, and Ireland. It was found that the chemical composition depended only slightly on the geographical origin of the plant, but showed wide variations according to the year of harvest. Also, *Letchamo et al.* showed that the oil yield is influenced by light intensity and growing medium [4][14].

The aim of the present work was to determine in detail the chemical composition of *Angelica archangelica* oils, focusing on macrocyclic lactones, and to describe qualitative and quantitative differences related to processing procedures, plant part, and harvesting date.

Results and Discussion. – A total of 58 components were identified on the basis of their mass spectral (MS) characteristics, GC/MS retention indices (RI), and co-elution with standards. The percentage of each component within the essential oils, determined on an *HP-5* GC/MS column, is presented in the *Table*. The contents of limonene and β-phellandrene, which were separated on a *DB-Wax* column, are also included in this *Table*.

A high content of β-phellandrene (74.7%) was found in *Angelica archangelica* seed oil, and together with other monoterpenes, they comprised 94.1% of the total oil (*Fig. 1*). Small amounts of macrocyclic lactones were found in the seed oil: tridecano-13-lactone (0.3%), pentadecano-15-lactone (0.2%), and heptadecano-17-lactone (trace). The seed oil typically is higher in β-phellandrene (35–61%) and lower in the macrocyclic components than the root oil. The composition is in agreement with previous publications, especially regarding other monoterpenes and sesquiterpenes identified [4][5].

Root oil contained 1.3% of macrocyclic lactones, such as tridecano-13-lactone (0.6%), 12-methyltridecano-13-lactone (0.1%), pentadecano-15-lactone (0.5%), and heptadecano-17-lactone (0.1%). High amounts of β-phellandrene (26.6%), α-phellandrene (19.1%), and α-pinene (15.7%) were also found. Among the sesquiterpenes, α-copaene (0.9%) and α-humulene (1.1%) were the most representative.

A comparison of the quantitative composition of root oils extracted in different seasons, spring *vs.* summer, showed that different harvest dates result in slight changes in the composition of the oil. The β-phellandrene content of *Angelica* root oil harvested

Table. *Constituents from the Seed and Root Oils of* Angelica archangelica L. *Fractions* (*Fr.*) *1–5* were collected at constant time intervals during distillation and analyzed by GC/MS (see *Exper. Part*).

Entry	Compound	RI[a])	RI	Area [%]						
		HP-5	*Wax*	*Fr. 1*	*Fr. 2*	*Fr. 3*	*Fr. 4*	*Fr. 5*	Root	Seed
1	Hexanal	801	1078	≤ 0.05	trace	trace	trace	trace	trace	
2	Heptanal	902	1181	trace	0.06	0.06	trace	trace	trace	
3	α-Thujene	930	1022	0.74	0.46	0.34	0.26	0.22	0.40	0.18
4	α-Pinene	938	1015	14.35	13.71	12.64	10.85	9.15	15.70	6.58
5	Camphene	953	1057	0.36	0.33	0.30	0.25	0.20	0.44	0.26
6	Thuja-2,4(10)-diene	958	1117	0.10	0.22	0.28	0.29	0.19	0.10	
7	Sabinene	977	1113	2.70	2.52	2.20	1.66	1.25	0.68	0.37
8	β-Pinene	980	1096	0.86	0.80	0.72	0.59	0.47	1.07	0.59
9	Myrcene	992	1164	2.89	2.64	2.35	1.90	1.56	2.81	2.91
10	δ-2-Carene	1003	1125	0.16	0.21	0.23	0.19	0.18	0.12	
11	α-Phellandrene	1006	1158	14.54	16.40	15.78	11.80	11.08	19.11	3.65
12	δ-3-Carene	1012	1142	7.01	6.64	6.00	4.86	3.96	5.71	0.20
13	α-Terpinene	1020	1172	0.11	0.14	0.16	0.13	0.12	0.11	trace
14	*p*-Cymene	1027	1261	11.00	9.09	7.61	5.87	3.49	5.03	0.62
15	Limonene	1032	1189	24.90/ 6.52^{wax}	23.28/ 6.16^{wax}	20.72/ 5.55^{wax}	15.95/ 4.36^{wax}	12.50/ 3.39^{wax}	31.59/ 5.91^{wax}	77.20/ 2.67^{wax}
16	β-Phellandrene	1033	1195	18.97^{wax}	17.82^{wax}	16.01^{wax}	12.57^{wax}	10.09^{wax}	26.61^{wax}	74.66^{wax}
17	(*Z*)-β-Ocimene	1041	1234	0.87	0.84	0.76	0.59	0.50	0.88	0.16
18	(*E*)-β-Ocimene	1051	1250	2.22	2.15	1.94	1.46	1.29	2.28	0.32
19	γ-Terpinene	1062	1238	0.39	0.38	0.36	0.27	0.25	0.26	trace
20	*cis*-Sabinene hydrate	1070	1467	0.08	0.09	0.06	trace	trace		
21	Isoterpinolene	1088	1270	0.36	0.38	0.33	0.26	0.21	0.31	trace
22	Terpinolene	1090	1274	0.54	0.59	0.59	0.50	0.44	0.51	0.09
23	*trans*-Sabinene hydrate	1099	1549	0.12	0.15	0.19	0.24	0.21	0.06	
24	Linalool	1100	1552							0.27
25	*cis*-*p*-Menth-2-en-1-ol	1124	1560	0.16	0.24	0.33	0.42	0.26	0.10	
26	*trans*-*p*-Menth-2-en-1-ol	1142	1625	0.11	0.16	0.22	0.27	0.18	0.07	
27	*cis*-Verbenol	1144	1663	0.07	0.12	0.17	0.19	0.12	trace	
28	*trans*-Verbenol	1147	1673	0.39	0.54	0.66	0.67	0.41	0.16	
29	Camphor	1148	1490							trace
30	Borneol	1168	1692	0.10	0.18	0.33	0.50	0.42	trace	0.13
31	Terpinen-4-ol	1179	1594	0.32	0.32	0.37	0.42	0.24	0.09	0.16
32	Cryptone	1188	1645	0.32	0.25	0.30	0.46	0.35	0.16	0.12
33	*p*-Cymen-8-ol	1186	1844							–
34	α-Terpineol	1192	1692	trace	trace	trace	0.10	0.10	trace	trace
35	*cis*-Piperitol	1196	1674	trace	trace	trace	0.11	0.24	trace	
36	*trans*-Piperitol	1208	1742	0.06	0.08	0.11	0.17	0.13	trace	
37	Linalyl acetate	1259	1555							0.19
38	Bornyl acetate	1288	1567	0.32	0.24	0.23	0.26	0.16	0.14	0.06
39	α-Copaene	1377	1475	2.20	2.92	3.61	4.16	4.09	0.91	0.24
40	β-Bourbonene	1386	1500							trace
41	β-Caryophyllene	1420	1576	0.31	0.34	0.42	0.53	0.50	0.20	0.08
42	β-Copaene	1430	1686	0.34	0.49	0.67	0.87	0.85	0.18	0.06
43	α-Humulene	1454	1646	1.28	1.98	3.04	4.29	4.43	1.13	0.63
44	(*E*)-β-Farnesene	1459	1662							trace
45	γ-Muurolene	1478	1671	0.50	1.00	1.63	2.20	2.46	0.64	0.45
46	α-Muurolene	1500	1707	0.49	0.78	1.28	1.92	2.07	0.37	0.07
47	β-Bisabolene	1509	1715	0.24	0.40	0.68	1.12	1.28	0.20	
48	δ-Cadinene	1525	1742	0.22	0.38	0.69	1.23	1.46	0.25	trace
49	Germacrene B	1557	1802	0.10	0.20	0.38	0.78	1.09	0.35	0.28

Table (cont.)

Entry	Compound	RI[a])	RI	Area [%]						
		HP-5	*Wax*	*Fr. 1*	*Fr. 2*	*Fr. 3*	*Fr. 4*	*Fr. 5*	Root	Seed
50	Caryophyllene oxide	1583	1953							trace
51	Humulene epoxide II	1608	2008	trace	0.08	0.14	0.44	0.80	0.12	
52	Tridecano-13-lactone	1627	2030	0.15	0.30	0.69	2.42	5.04	0.65	0.32
53	12-Methyltridecano-13-lactone	1681	2056		trace	0.06	0.21	0.45	0.06	
54	Tetradecano-14-lactone	1727	2132			trace	trace	0.08		
55	Pentadecano-15-lactone	1828	2233	0.08	0.17	0.41	1.52	3.51	0.53	0.15
56	14-Methylpentadecano-15-lactone	1877	2253					trace		
57	Hexadecano-16-lactone	1928	2334					trace		
58	Heptadecano-17-lactone	2028	2433			trace	0.10	0.24	0.06	trace
	Total			**92.06**	**92.25**	**90.04**	**83.28**	**78.23**	**93.54**	**96.34**

[a]) Retention Index.

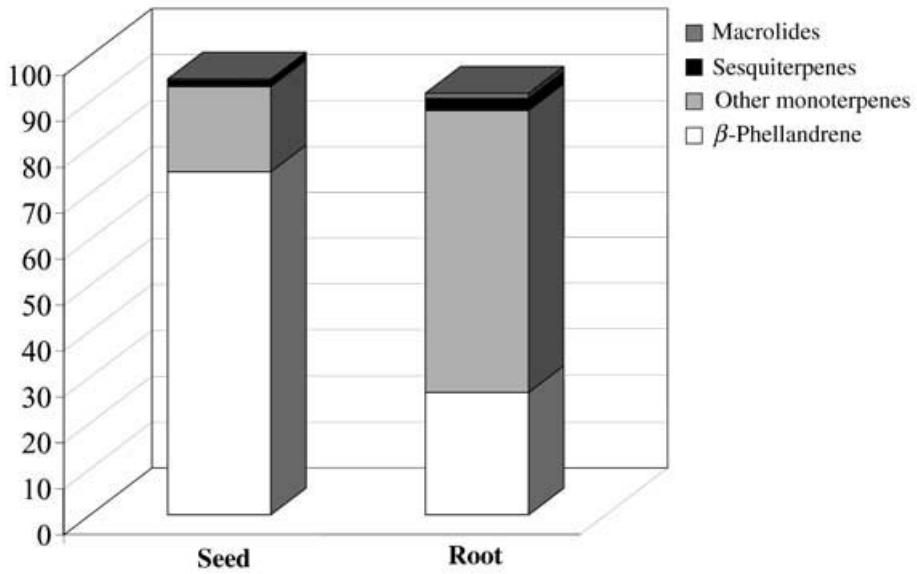


Fig. 1. *Quantitative composition* (%) *of seed and root oils of* Angelica archangelica L.

in summer increased approximately by 36%. However, no significant changes in the percentage of other components were observed (*Fig. 2*).

A batch of fresh *Angelica* roots were steam distilled, and the oil fractions were collected in equal time intervals throughout the distillation. It was possible to obtain fractions containing high- and low-boiling components (*Fig. 3*). As shown in the *Table*, the content of macrolides in the highest-boiling fraction increased up to sevenfold in comparison to the standard oil.

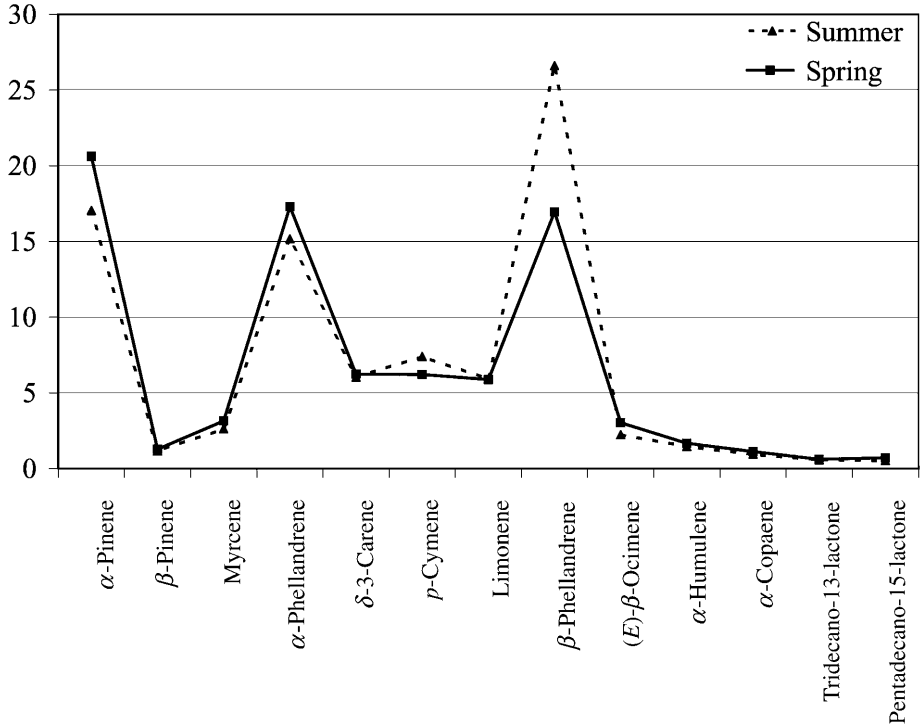


Fig. 2. *Quantitative composition* (%) *of root oil of* Angelica archangelica *harvested during summer* vs. *spring*

The highest-boiling fraction contained 9.3% of macrocyclic lactones, which were identified as tridecano-13-lactone (5.0%), 12-methyltridecano-13-lactone (0.4%), tetradecano-14-lactone (0.1%), pentadecano-15-lactone (3.5%), hexadecano-16-lactone (trace), heptadecano-17-lactone (0.2%), and a trace component which was identified as 14-methylpentadecano-15-lactone (=15-methyloxacyclohexadecan-2-one; **1**), known as *muscolide*.

Its molecular formula, $C_{16}H_{30}O_2$, evident from the mass spectrum, was very similar to those of known macrolides, displaying a molecular ion at *m/z* 254, a base peak at *m/z* 55, and typical $[M-18]^+$, $[M-28]^+$, $[M-36]^+$, and $[M-60]^+$ fragments. Some anomalous peaks were present, however, the most abundant in the high-mass region were at *m/z* 199 (7%) and *m/z* 181 (16%). A small peak (1%) for the $[M-15]^+$ ion was also observed.

The formation of the ions *m/z* 199 and 181, analogues of which are not present in the spectrum of unbranched macrolides such as 16-hexadecanolide, can be rationalized by *McLafferty* rearrangement of H–C(14) followed by cleavage of the bond β to the newly formed C=C bond, which leads to a fragment ion of *m/z* 199. Loss of H_2O would then give the *m/z* 181 ion.

Unequivocal identification of muscolide (**1**) in *Angelica* root oil was achieved by co-injection with an authentic (racemic) sample synthesized in our laboratory, and by comparison of their mass spectra. The *Baeyer–Villiger* oxidation of commercially

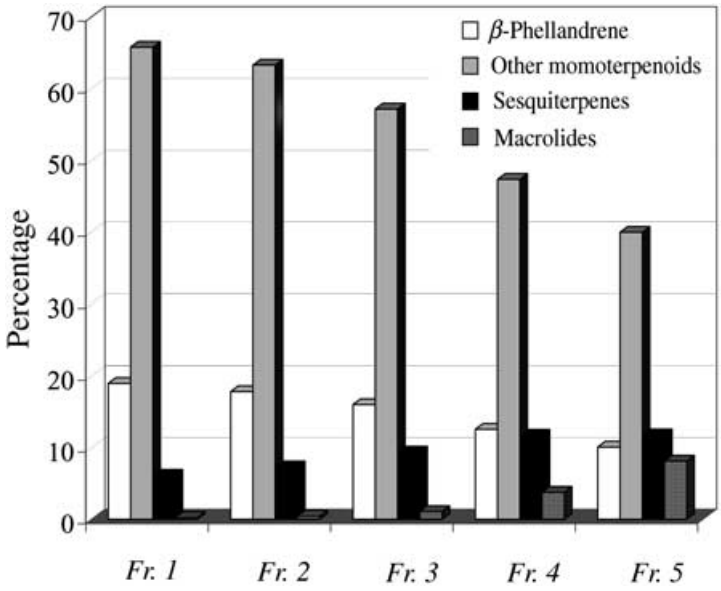
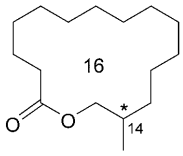


Fig. 3. *Quantitative composition* (%) *of steam-distilled root-oil fractions of* Angelica archangelica L.



Muscolide (**1**)

available muscone to muscolide was performed in one step according to the procedure of *Wiberg* and *Snoonian* [15]. The reaction was extremely slow, and only 12% of muscone was converted to **1** within 18 h.

This is the first report of the occurrence of muscolide (**1**) in a natural product. Muscolide was first synthesized by *Ruzicka* in 1928, also starting from muscone. Recently, its enantiomers were prepared *via* a ring-enlargement sequence, starting from enantiomerically pure chiral synthons available by enzymatic methods. The odor of (*R*)-muscolide has been described as weaker than that of pentadecano-15-lactone, but '*distinct musky*, *erogenous*, *animalic*, *resembling that of natural musk tincture*'; (*S*)-muscolide is of similar intensity than its antipode, but possesses a '*very pleasant musk note with a far more distinctive erogenous-animalic character*' [16].

Conclusions. – The chemical composition of seed and root oils of *Angelica archangelica* L. was studied in detail, resulting in the first identification of 14-methylpentadecano-15-lactone (muscolide; **1**) in a natural product. Extractions of root oil performed during spring and summer gave similar qualitative compositions. The β-phellandrene content of the *Angelica* root oil harvested in summer increased by

approximately 36%, but no significant changes in the percentage of other components were found. The overall yield of essential oil was, however, higher in the roots harvested in summer.

Experimental Part

General. Muscone was obtained from *Givaudan* Schweiz AG, CH-8600 Dübendorf. Sodium sulfite, sodium bicarbonate and *m*-chloroperbenzoic acid (MCPBA) were purchased from *Sigma-Aldrich* Chemie GmbH, D-89555 Steinheim, Germany. Reactions were conducted in oven-dried glassware under N_2 atmosphere. Mass spectra were obtained on an *Agilent 5973* MSD mass spectrometer, coupled directly to an *Agilent 6890* gas chromatograph fitted with an *HP-5MS* column (5% phenyl/95% polydimethylsiloxane; 0.25 mm (i.d.) × 30 m, 0.25 micron film thickness, fused silica capillary). EI-MS: 70 eV; in *m/z* with rel. peak intensities in % of the base peak (100%). Complementary analyses were performed on a *DB-Wax* column (polyethylene glycol; 0.32 mm (i.d.) × 30 m, 0.25 micron film thickness; fused silica capillary). The GC/MSD apparatus was operated under the following conditions: *HP-5MS* column: injector temp. 250°; transfer line 280°; oven temp. 60–260° at 3°C/min; final hold time depending on sample; solvent delay 2 min. *DB-Wax* column: injector temp. 250°; transfer line 240°, oven temp. 40° for 5 min, with 3°C/min to 220°; final hold time depending on sample; solvent delay 3 min. Carrier gas: He (1 ml/min); injection volume 1 µl (1% soln. in CH_2Cl_2), split 1:20. Ion source 230°, 70 eV; MS quadrupole 150°. Quantification was performed with an *Agilent 6890* gas chromatograph equipped with a flame-ionization detector under the same conditions described above, detector temp. 280° and 250°, resp. The concentrations of each component were calculated as percentage by internal normalization, assuming identical mass-response factor for all compounds. Plants were grown in Cultus Lake, British Columbia, under a certified organic production system, and the harvest was after two years of cultivation.

Isolation of Essential Oils. Fresh roots (10 kg) were sliced in pieces of *ca.* 2.5 cm, and steam distilled for 1 h using a commercial distillation unit. Five fractions were collected during equal time periods throughout the distillation process. Pale yellow to pale yellow-greenish oils were recovered, and a more-intense *musky* odor was present in the last fraction. The plant material used was harvested in fall 2002. Two single distillations (1 h) of fresh roots were also performed during spring and summer, yielding 0.12 and 0.18% (*v/wt*) of pale yellow oils, resp.

Seeds and sliced roots collected in fall 2002 were dried in a commercial electric dryer at 40° with ventilation for 3 days. Dried seeds (710 g) and roots (10 kg) were comminuted using a hammer mill, and steam distilled for 3 h (seeds) or 1 h (roots). The yields of oil were 1.13 and 0.17% (*v/wt*), resp. Seed oil was a light, pale-yellow oil with a *strong peppery* odor. The oils were dried (Na_2SO_4) and stored in a refrigerator prior to analysis.

Identification of Components. The oils were analyzed by GC/MS and GC using two different capillary columns (*HP-5* and *DB-Wax*). The identification of single components was performed by comparison of GC retention indices (RI) on both polar and nonpolar columns, mass spectra, and co-injection with authentic standards [17]. A standard soln. of n-alkanes (C_7–C_{26}) was used to obtain the RI values.

14-Methylpentadecano-15-lactone (=15-Methyloxacyclohexadecan-2-one; Muscolide; **1**). To a stirred mixture of MCPBA (0.70 g, 4.1 mmol) and $NaHCO_3$ (1.3 g, 16 mmol) in CH_2Cl_2 (10 ml) was added muscone (0.55 g, 2.3 mmol) dissolved in CH_2Cl_2 (2 ml). Stirring was continued for 18 h, and the mixture was then washed with 10% Na_2SO_3 soln. (5 ml) and sat. $NaHCO_3$ soln. (5 ml). The org. layer was dried (Na_2SO_4) and concentrated *in vacuo* [15]. The resulting residue was purified by column chromatography (SiO_2; hexane/Et_2O 5:1), and muscolide (**1**) was identified by GC/MS. EI-MS: 254 (4, M^+), 239 (1, $[M-CH_3]^+$), 236 (30, $[M-H_2O]^+$), 226 (4, $[M-CO]^+$), 199 (7, $C_{12}H_{23}O_2^+$; *McLafferty*), 181 (16, $[C_{12}H_{23}O_2-H_2O]^+$, 163 (9, $C_{12}H_{19}^+$), 152 (14, $C_{11}H_{20}^+$), 139 (14, $C_{10}H_{19}^+$), 125 (19, $C_9H_{17}^+$), 111 (33, $C_8H_{15}^+$), 97 (60, $C_7H_{13}^+$), 83 (64, $C_6H_{11}^+$), 69 (88, $C_5H_9^+$), 55 (100, $C_4H_7^+$), 41 (64, $C_3H_5^+$).

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Odor and (Bio)diversity: Single Enantiomers of Chiral Fragrant Substances

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Our sense of smell is enantioselective. This review reports interesting examples of single stereoisomers of natural and synthetic odorants, prepared *via* bioorganic routes, that support this statement. This article is the summary of a talk given at the *Flavours & Fragrances 2004* conference in Manchester at the MCC/UMIST, 12–14 May, 2004.

Introduction. – The study of biodiversity in nature is both fascinating and challenging. When dealing with the diverse chemicals used by living beings to communicate, the so-called *semiochemicals*, surprising findings can be made. For instance, female Asian elephants release in their urine the same pheromone, (*Z*)-dodec-7-enyl acetate[1]) [1], to signal that they are ready to mate, as does the turnip looper or the cabbage looper. On the other hand, there are insects that use very specific pheromones, *i.e.*, single enantiomers of chemically rather complex chiral molecules. The (*R*)-enantiomer of japonilure is the active pheromone of the gypsy moth (*Lymantria dispar*), but is strongly inhibited by its antipode [3]. (+)-*exo*-Brevicomin shows activity as aggregation pheromone of the western pine beetle (*Dendroctonus brevicomis*), while the (−)-enantiomer is completely inactive [4].

Unlike other flowers that lure pollinators with nectar, the Australian orchid *Chiloglottis trapeziformis* attracts its pollinator, the thynnine wasp *Neozeleboria cryptoides*, by producing the pheromone that the insect uses to attract the opposite sex [5], a strategy known as *sexual deception*. There are also examples of sexually deceptive European orchids that employ mixtures of alkanes and alkenes in different proportions to function as attractants [6], but what is surprising is that the Australian orchid uses only one single compound, namely 2-ethyl-5-propylcyclohexane-1,3-dione, which is effective with only one specific type of pollinator.

Mankind has been trying for more than a century to reproduce by synthesis the complex world of odors and perfumes provided by nature. Fragrance chemists try to diversify odor response by synthesizing new, but structurally often very similar, derivatives of known odorous compounds. An alternative is to seek different or purer odor sensations in the stereoisomers of known chiral odorants. Nature, indeed,

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[1]) For other interesting examples of organisms from different parts of the natural world that use structurally similar communication substances, see [2].

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produces only one enantiomer of menthol, the active one showing the best organoleptic properties and the most-powerful cooling effect. In contrast, carvone is present in nature in both enantiomeric forms: (+)-carvone smells *caraway-like*, while the (−)-enantiomer has a *typical spearmint* odor, and both occur in the respective essential oils.

When a molecular-recognition process is involved, as it does happen in olfaction, *diversity* originates from both constitutional and configurational isomers. The enantioselectivity of odor perception is rather obvious on the basis of the following considerations. The macromolecules responsible for life are enantiomerically pure; proteins being made up from L-amino acids, nucleic acids being based on D-sugars. The physiological processes controlled by these macromolecules should, therefore, proceed stereoselectively. When chiral, exogenous compounds are introduced into the body, they can be discriminated by their different interactions with enantiomerically pure targets, such as receptors and enzymes. Fragrances are also such exogenous compounds that are introduced into our body to produce a certain effect, in this case odor sensation.

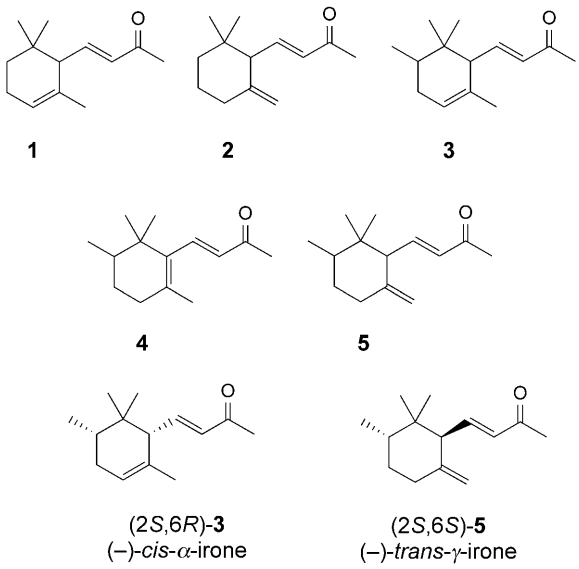
We have been studying odor-related phenomena for the last years [7] by preparing all the possible single stereoisomers of selected natural and synthetic chiral fragrances. When possible, we established also the absolute configurations to extend our knowledge on the relationship between odor and molecular structure. In this paper, we describe our approach to enantiomerically pure chiral fragrances *via* bio-organic syntheses. For each stereoisomer, we will highlight the odor description given by professional perfumers, and show that the olfactory discrimination of enantiomers takes place in a specific way and to a specific extent for each chiral fragrance.

Results and Discussion. – Our approach is to take advantage of biocatalyzed processes to obtain enantiomerically pure or enriched products. As organic chemists, we are fascinated by nature's exquisite enzymatic chemistry, which tends to be highly selective, efficient, mild, and environmentally benign.

Two types of enzymatic transformations are possible: kinetic resolution with isolated enzymes, and stereoselective reactions with microorganisms. Several enzymes are commercially available and can be used like traditional heterogeneous chemical catalysts since they do not need cofactors. They can be employed under mild reaction conditions in common organic solvents, under atmospheric pressure, and at room temperature. They do not require specific equipment, are generally safe for the environment, and can be recycled without loss of activity.

Lipases are the most-widely employed biocatalysts. They are generally used to catalyze the transesterification of alcohols or the hydrolysis of esters. The technique of kinetic resolution of racemates by lipase catalysis requires the identification of a suitable alcohol/ester substrate.

Ionones and Irones. As the ionones **1** and **2** and the irones **3**–**5** are carbonyl compounds, the allylic alcohols obtained by $NaBH_4$ reduction of the racemic compounds are the simplest substrates for kinetic resolution [8–12]. The reduction of the carbonyl group introduces an additional stereocenter and, thus, increases the number of stereoisomers to be separated, but the fractional crystallization of derivatives of α- and γ-ionols, and the manipulation of the epoxy derivatives of α-irols offered the chance to separate the corresponding diastereoisomers. These



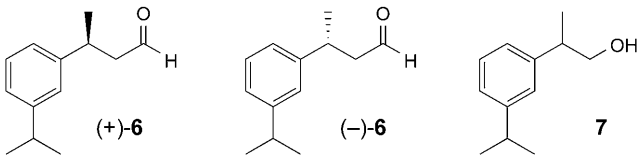
latter derivatives were then obtained in enantiomerically pure form by treatment with lipase.

Both (+)-(*R*)- and (−)-(*S*)-α-ionone (**1**, 98 and 97% ee[2]), resp.) were characterized by a *floral-woody note with an additional honey aspect*. The two samples showed similar odor thresholds within experimental error: 3.2 and 2.7 ng/l air, for (*R*)- *vs.* (*S*)-**1**, respectively [13]. A different situation was found for the two enantiomers of γ-ionone (**2**). (−)-(*R*)-γ-Ionone (99% ee) was found to be *weak green, fruity, pineapple-like with metallic aspects and only slightly woody, ionone-type nuances*, *i.e.*, quite different from the typical ionone odor, but with a rather weak odor threshold of 11 ng/l. (+)-(*S*)-γ-Ionone was described as *very pleasant, floral, green, woody, with a very natural violet tonality*. With an odor threshold of 0.07 ng/l air, it was the most-powerful and -pleasant isomer.

We also had in our hands all ten pure stereoisomers of irones. Only (−)-*cis*-α-irone ((2*S*,6*R*)-**3**) and (−)-*trans*-α-irone ((2*S*,6*S*)-**5**) were found to possess the delicate and characteristic scent of *orris butter*, though the other irone samples were also described to smell pleasant of *violet and woody notes*.

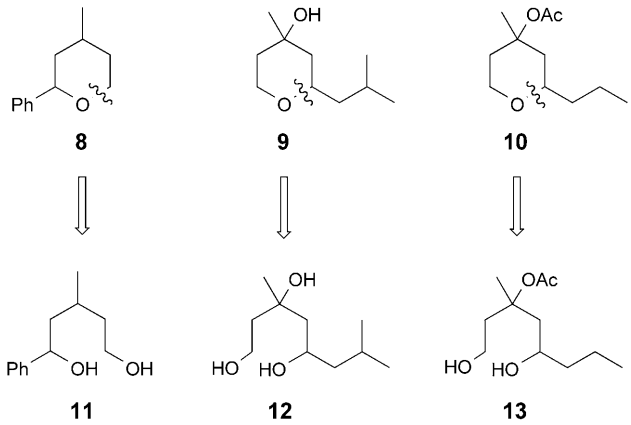
Florhydral®. The key intermediate in our syntheses of *Florhydral*® was the racemic primary alcohol **7**, which was submitted to porcine pancreatic lipase (PPL) mediated acetylation [14]. Pure (+)-**6** (> 99% ee) was found to smell *floral, watery, green, yet with an acidic touch, even in the dry down note*. In comparison with racemic **6**, this enantiomer was *more green, a bit less watery, and more powerful* (odor threshold: 0.035 ng/l air). Its antipode (−)-**6** (> 99% ee) has *a typical Florhydral smell, floral, fresh, green, muguet-like, but more marine, and more plastic* (odor threshold: 0.88 ng/l air). The most-relevant difference between the two enantiomers lies in their odor

[2]) Enantiomeric and diastereoisomeric excesses are referred to as 'ee' and 'de', resp.



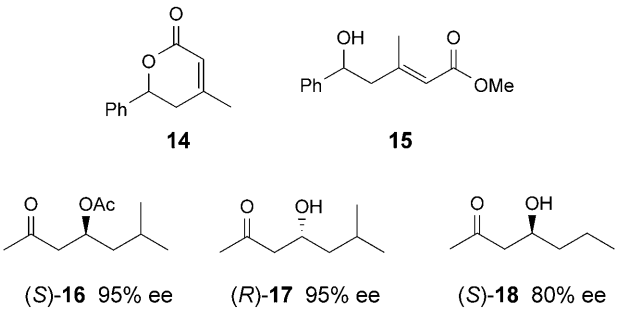
strengths rather than in their odor characters, the odor threshold of (−)-**6** being 25 times higher than that of its antipode.

Floral Tetrahydropyran Odorants. The general strategy for the preparation of the single stereoisomers of *Doremox*® (**8**) [15], *Florol*® (**9**) [16], and *Clarycet*® (**10**) [16] was the kinetic resolution of an open-chain precursor. The obvious retrosynthetic disconnection of these tetrahydropyran derivatives is at one of the C−O bonds, providing the corresponding 1,5-diols **11**, **12**, and **13**, respectively. The two racemic diastereoisomers of **11** were obtained separately by reduction of the hydroxy ester **14** and the unsaturated lactone **15**. When the diacetates of diols **11** were exposed to lipase PS, the primary ester function was hydrolyzed both regio- and enantioselectively.



Compounds **12** and **13** were obtained as 1:1 mixtures of enantiomerically enriched diastereoisomers by *Grignard* reaction of allyl magnesium chloride with (*S*)-**16** (95% ee), (*R*)-**17** (91% ee), and (*S*)-**18** (80% ee), followed by ozonolysis and reductive workup with $NaBH_4$. The precursors (*S*)-**16** and (*R*)-**17** were prepared by lipase PS-mediated acetylation of the racemic hydroxy ketone **17**. Transesterification of hydroxy ketone **18** with lipase from *Candida cylindraceae* (CCL) afforded the acetate of (*S*)-**18** in 60% ee, which was increased to 80% ee by subsequent CCL-mediated saponification. The antipode (*R*)-**18** could not be isolated as an unreacted alcohol of the CCL-mediated acetylation of racemic **18**, since the enantiomeric excess was poor.

Separation of the diastereoisomers was performed by column chromatography after ring closure, which was effected by treatment with *p*-toluenesulfonyl chloride in pyridine, with retention of configuration. The fourth pair of tetrahydropyran derivatives was obtained by ring closure under inversion of configuration.



14 **15**

(*S*)-**16** 95% ee (*R*)-**17** 95% ee (*S*)-**18** 80% ee

Olfactory evaluation of the *Doremox*® samples gave the following results: *cis*-(2*R*,4*S*)-**8** (92% ee, >99% de[2]): *rose oxide, diphenyl oxide, metallic, slightly plastic*; *cis*-(2*S*,4*R*)-**8** (80% ee, >99% de): *rose oxide, powerful, nice*; *trans*-(2*S*,4*S*)-**8** (72% ee, 70% de): *weak, rosy, plastic, citronellol, slightly rose oxide*; and *trans*-(2*R*,4*R*)-**8** (50% ee, 77% de): *rosy, rose oxide, metallic, off note*. Thus, *cis*-(2*S*,4*R*)-**8** was found to be the nicest and the most-powerful isomer of this series.

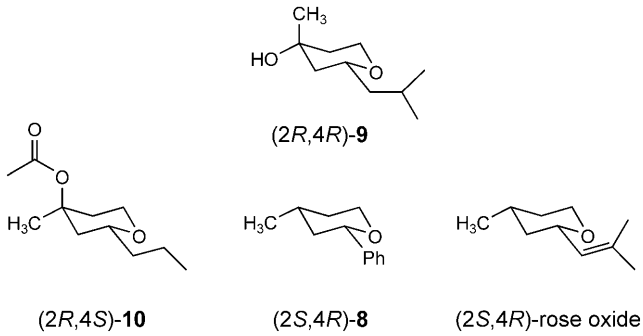
A gradual variation of odor threshold was noticed in the four *Florol*® isomers, from 1.21 ng/l for (2*R*,4*R*)-**9** to over 600 ng/l for (2*S*,4*R*)-**9**. The following characteristics were found: (2*R*,4*R*)-**9**: Most-pronounced and -intense stereoisomer (odor threshold: 1.21 ng/l air), *fresh, soft, sweet, and natural floral odor reminiscent of muguet with some rose oxide side note, and earthy nuances*; (2*S*,4*S*)-**9**: second-most-intense stereoisomer, but already much weaker (odor threshold: 26 ng/l air), *similar fresh-floral note as its antipode, but less sweet and also more linalool-like, more herbaceous, and more earthy in tonality*; (2*R*,4*S*)-**9**: second-weakest stereoisomers (odor threshold: 520 ng/l air); *relatively weak, mainly fruity, grape-like, but also reminiscent of linalyl acetate and clary sage oil, with some nuances of dry herbs*; and (2*S*,4*R*)-**9**: odorless on GC-olfactometry (odor threshold: >600 ng/l air), weakest of the stereoisomers, *very weak in odor, mainly linalool- and coumarine-like, with some citric and hesperidic nuances.*

So, the two *Floral* enantiomers of the diastereoisomer bearing the OH group and the isobutyl chain in equatorial positions are decidedly the most intense, and are responsible of the odor of commercial *Florol*®.

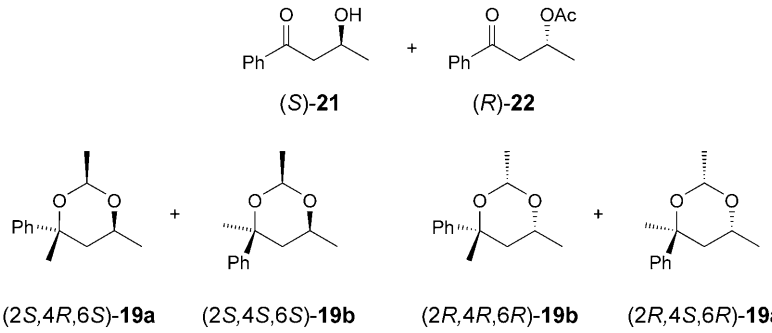
The odor evaluations of the *Clarycet*® stereoisomers were as follows: (2*S*,4*S*)-**10**: *green, fresh, earthy, fruity (sage), on dry down odorless*; (2*S*,4*R*)-**10**: *floral, agrestic, fruity, touch acetic-green-tobacco, on dry down slightly fruity, but very weak*; (2*R*,4*R*)-**10**: *pine, pine oil, terpenic, woody, on dry down dusty-dirty*; and (2*R*,4*S*)-**10**: *fruity, rosy, reminiscent of rose ketones, touch earthy, dry, sweet, woody, and sage-like, on dry down floral and sage-like.*

So, for *Clarycet*®, only (2*R*,4*S*)-**10** emanates a nice fruity-floral odor devoid of green, terpenic notes, which distinguishes it from the other three stereoisomers. (2*R*,4*S*)-**10** is related to the best isomer of (2*S*,4*R*)-rose oxide and to the best isomer of *Doremox*® (2*S*,4*R*)-**8**. All three have the Me group at C(4) and the substituent at C(2) in a *cis*-diequatorial arrangement.

Floropal® (**19**) and *Magnolan*® (**20**) are structurally related, dioxygenated commercial odorants. In 1999, a patent [17] disclosed that the olfactory properties of



the two racemic diastereoisomers **19a** and **19b** differ significantly. Acetal **19a** was described as *strong, herbal-fresh, green, and typical grapefruity*, while **19b** was found to be *very weak, chemical solvent-like*, and to have a detracting influence upon the sensory properties of the commercial mixture.

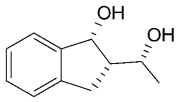


To investigate the influence of the absolute configuration on the odor of *Floropal*®, we exploited lipase-mediated esterification of hydroxy ketone **21** to obtain acetate (*R*)-**22** (97% ee) and unreacted alcohol (*S*)-**21** (93% ee) [18]. Addition of MeMgBr, and reaction with acetaldehyde afforded two mixtures, one consisting of enantiomerically pure (2*R*,4*R*,6*R*)-**19b** (66%) and (2*R*,4*S*,6*R*)-**19a** (33%), the other of enantiomerically pure (2*S*,4*R*,6*S*)-**19a** (33%) and (2*S*,4*S*,6*S*)-**19b** (66%). These two mixtures of diastereoisomers **19a** and **19b** were enriched in the most-valuable diastereoisomer by treatment with $BF_3 \cdot Et_2O$ according to reference [17]. The following samples were obtained: (+)-(2*R*,4*S*,6*R*)-**19a** (80% ee, 76% de) from (2*R*,4*R*,6*R*)-**19b** (97% ee, 33% de); (−)-(2*S*,4*R*,6*S*)-**19a** (68% ee, 73% de) from (2*S*,4*S*,6*S*)-**19b** (93% ee, 33% de).

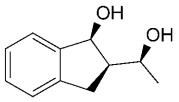
The samples of the *Floropal*® isomers were submitted for olfactory evaluation. The 1:2 mixture (2*S*,4*R*,6*S*)-**19a**/(2*S*,4*S*,6*S*)-**19b** (33% de) was more powerful than commercial *Floropal*®, but also reminiscent of *Corps Pamplemousse*® (=4,7,7-trimethyl-6-thiabicyclo[3.2.1]octane), especially regarding its sulfury aspects; it is similar to the 1:2 mixture (2*R*,4*S*,6*R*)-**19a**/(2*R*,4*R*,6*R*)-**19b**, but not so pungent. The 1:2 mixture (2*R*,4*S*,6*R*)-**19a**/(2*R*,4*R*,6*R*)-**19b** (33% de) shows a more sulfury-sweaty character, almost a bit of H_2S, and is more technical in smell than the corresponding mixture of

enantiomers, yet still reminiscent of *Floropal*®; it smells *green, floral, musty, strong, pungent, and not very nice*. The single isomers are characterized as follows: (+)-**19a**: *little bit acidic, floral, gardenia, a little bit of Vertacetal*®; (−)-**19a**: *Vertacetal*®*-like, more like styrolyl acetate, with a rhubarb note*. So, (2*S*,4*R*,6*S*)-(−)-**19a** is the most interesting of the enantiomers of the valuable diastereoisomers **19a**. Its presence confers to the 1 : 2 mixture (2*S*,4*R*,6*S*)-**19a**/(2*S*,4*S*,6*S*)-**19b** a nice character, despite (2*S*,4*S*,6*S*)-**19b** being the major component.

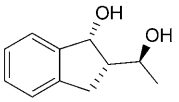
The commercial perfumery raw material *Magnolan*® is a mixture of only two racemic diastereoisomers, **20a** and **20b**. We optimized a biocatalytic approach to the single enantiomers by lipase-mediated acetylation of the racemic diols **23a** and **23b** [18]. Different regio- and enantioselectivities were observed with two different enzymes, lipase PS and *Candida rugosa* lipase (CRL).



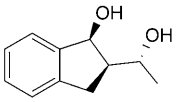
(1*R*,2*S*,1′*R*)-**23a**



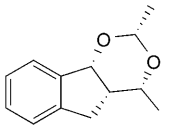
(1*S*,2*R*,1′*S*)-**23a**



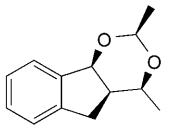
(1*R*,2*S*,1′*S*)-**23b**



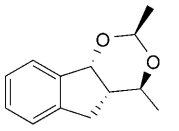
(1*S*,2*R*,1′*R*)-**23b**



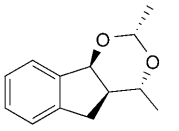
(+)-**20a**



(−)-**20a**



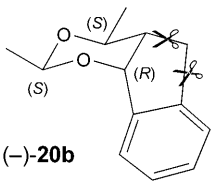
(−)-**20b**



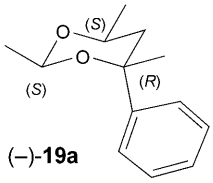
(+)-**20b**

The above approach offered the chance to prepare (1*R*,2*S*,1′*R*)-**23a** (93% ee), (1*S*,2*R*,1′*S*)-**23a** (87.5% ee), (1*R*,2*S*,1′*S*)-**23b** (93% ee), and (1*S*,2*R*,1′*R*)-**23b** (93% ee), which were converted into the *Magnolan*® stereoisomers (+)- and (−)-**20a** and (−)- and (+)-**20b**, respectively, by reaction with acetaldehyde in CH_2Cl_2 in the presence of pyridinium *p*-toluenesulfonate. The samples of the *Magnolan*® stereoisomers, evaluated by professional perfumers, were characterized as follows: (−)-**20a**: *wet, earthy, greasy, and technical*; (+)-**20a**: *reminiscent of caryophyllene, after 24 h very weak, with a touch of a woody character*; (+)-**20b**: *weak, acidic, floral, rosy, sweet, and warm*; and (−)-**20b**: *rosy, floral in the direction of geranium and magnolia, reminiscent of citronellyl acetate, citric, and fruity with a slight green nuance* (the most-interesting enantiomer).

So, the two racemic diastereoisomers **20a** and **20b**, constituents of commercial *Magnolan*®, show a similar difference in their olfactory properties as reported for the diastereoisomers **19a** and **19b** of *Floropal*®. In the most-appreciated isomer **20b**, the phenyl ring at C(4a) is situated in the axial position, resembling the axial phenyl group of **19a**. Since (−)-**20b** is the most-interesting enantiomer of the diastereoisomers **20b**, the configuration of the three stereogenic centers is the same as in (−)-**19a**, the most-appreciated enantiomer of racemic **19a**.



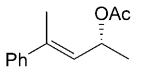
(–)-**20b**



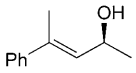
(–)-**19a**

Rhubafuran®. For the biocatalyzed preparation of all enantiomers of *Rhubafuran*® (**24**), the allylic alcohol **25** was devised as a suitable key intermediate [16]. It was subjected to acetylation by lipase PS to afford the acetate (*R*,*E*)-**26** (> 99% ee) and the alcohol (*S*,*E*)-**25** (> 99% ee), which were converted into the desired stereoisomers of **24** *via* the diastereoisomeric diols (2*S*,4*RS*)- and (2*R*,4*RS*)-**27**, which were separated by column chromatography. The following odor descriptions were obtained for *Rhubafuran*® stereoisomers: (2*R*,4*R*)-**24**: *nuts, acidic, animalic, and slightly rhubarb*; (2*S*,4*R*)-**24**: *citric, rhubarb, slightly green, and slightly animalic*; (2*S*,4*S*)-**24**: *grapefruit-like, bitter, cassis, slightly reminiscent of Oxane® and of dimethyloctenone* (dry down (24 h): *bitter, grapefruit, oxane*; and (2*R*,4*S*)-**24**: *the most-pleasant isomer, floral, linalool-like, rhubarb and citrus, green, and slightly reminiscent of eucalyptus.*

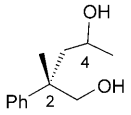
So, (2*R*,4*S*)-**24**, followed by its enantiomer, are the most-typical odorants of *Rhubafuran*®. As in the case of *Florol*®, *Floropal*®, and *Magnolan*®, the odor vectors of the commercial fragrant substance are again two enantiomers of the same diastereoisomer, the best one with Me groups *cis* to one another. This *Rhubafuran*® isomer can be correlated with *Floropal*® by exchanging the O–CH–Me unit for a CH_2 group. Then, the best diastereoisomers of both odorants possess the same relative configuration.



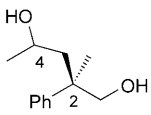
(*R*,*E*)-**26**



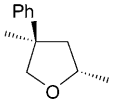
(*S*,*E*)-**25**



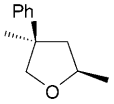
(2*S*,4*RS*)-**27**



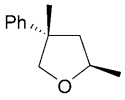
(2*R*,4*RS*)-**27**



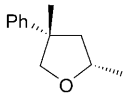
(2*S*,4*R*)-**24**



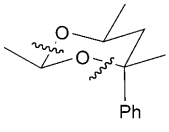
(2*R*,4*R*)-**24**



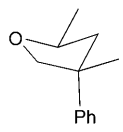
(2*R*,4*S*)-**24**



(2*S*,4*S*)-**24**



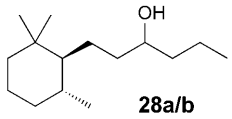
Floropal®
(rel. configuration)



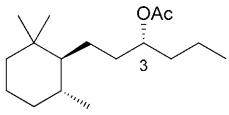
Rhubafuran®
(rel. configuration)

Timberol®. The commercial perfumery raw material *Timberol*® (**28**) is a mixture of four isomers of 1-(2,2,6-trimethylcyclohexyl)-hexan-3-ol. Shortly after its introduction to the market, it was, however, established that the main *woody animalic note* of *Timberol*® is due to the *anti*-diastereoisomers **28a/b** [19]. The 1:1 mixture of the two racemic *anti*-diastereoisomers **28a** and **28b** was, therefore, industrially manufactured by *Firmenich*, and is in captive use under the name *Norlimbanol*®.

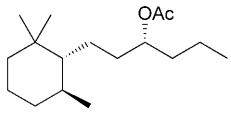
The single enantiomers of **28a** and **28b** were also synthesized at *Firmenich* [20], starting from (1*R*,6*S*)- and (1*S*,6*R*)-dihydrocyclocitral and (*R*)- and (*S*)-2-propyloxirane. (+)-(1′*R*,3*S*,6′*S*)-**28a** was described as the best of the series, *powerful and long lasting, with a very nice woody-ambery note.* (−)-(1′*S*,3*S*,6′*R*)-**28b** possesses an odor resembling that of (+)-(1*R*,3*S*,6′*S*)-**28a**, but less powerful and decidedly inferior. The (3*S*)-isomers were *devoid of the animalic character and very weak* [20][21]. A few years ago, we subjected the mixture of the four stereoisomers of **28a/b** to enzymic acetylation [22]. The (3*R*)-acetate **29a** was obtained first after 24 h of reaction, then, after 7 d the (3*R*)-configured acetate **29b** was recovered. Hydrolysis of (3*R*)-**29a** and (3*R*)-**29b** provided (3*R*)-**28a** and (3*R*)-**28b**, respectively. Inversion of the configuration by acetate displacement afforded the corresponding odoriferous (3*S*)-isomers.



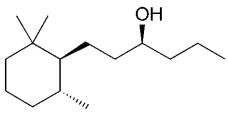
28a/b



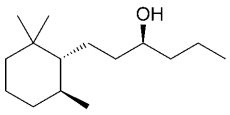
(3*R*)-**29a**
>99% ee, 82% de



(3*R*)-**29b**
>99% ee, 95% de



(3S)-**28b**

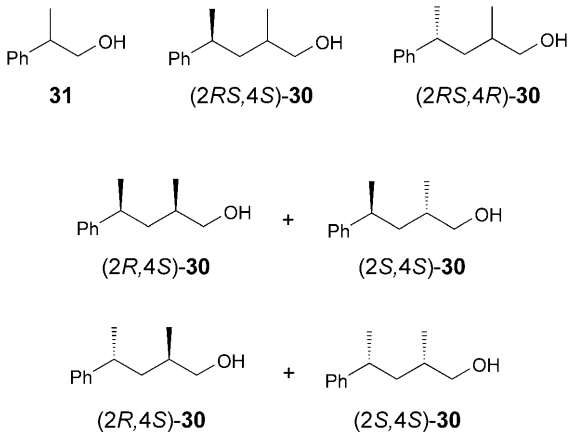


(3S)-**28a**

Pamplefleur®. This odorant, 2-methyl-4-phenylpentanol (**30**), represents a rather unexpected case in our investigation of the olfactory properties of stereoisomers. Racemic alcohol **31** was submitted to PPL-catalyzed transesterification, and separated (*R*)- and (*S*)-**31** were converted to (2*RS*,4*S*)-**30** and (2*RS*,4*R*)-**30**, respectively [23]. Separation of the diastereoisomers was accomplished by PPL-mediated acetylation. The following interesting odor evaluations were obtained for the samples of *Pamplefleur*® isomers: (2*R*,4*S*)-**30**: *natural, fruity odor in the direction of grapefruit and rhubarb, close to gardenol ('methyl phenyl carbinyl acetate') and 2,5-dimethyloct-2-en-6-one, slightly metallic*; (2*S*,4*S*)-**30**: *floral-fruity odor in the direction of grapefruit*

and linalool, with earthy, woody, and bitter nuances, also reminiscent of 2,5-dimethyloct-2-en-6-one and of some aspects of vetiver oil; (2*S*,4*R*)-**30**: *fruity-citric odor, with some harsh, animalic, and slightly woody nuances, also a bit rubbery*; and (2*R*,4*R*)-**30**: *floral-fruity odor in the direction of rhubarb with a touch of grapefruit, also reminiscent of gardenol*.

In the *IFF* catalogue[3]), *Pamplefleur*® is described as *citrus, grapefruit, floral, and vetiver*. Thus, from the odor descriptions given above, it is clear that the different stereoisomers contribute to different facets of the commercial odorant, resulting in a unique blend. However, they all share a *hesperidic grapefruit tonality*. With respect to this grapefruit note, (2*R*,4*S*)-**30** is the most typical, but *Pamplefleur*® is a complex blend, and all stereoisomers play a definite role.



Conclusions. – The use of kinetic resolution mediated by lipases is a convenient, flexible, and widely applicable method for the preparation of the stereoisomers of chiral fragrant substances in enantiomerically enriched or enantiomerically pure form. This method requires only the identification of a suitable alcohol or acetate as substrate for the biocatalyzed transesterification or hydrolysis. This key intermediate may be an allylic alcohol (as for ionones, irones, or *Rhubafuran*®), or a primary alcohol/acetate (as for *Florhydral*®, *Doremox*®, or *Pamplefleur*®), a hydroxy ketone (as for *Florol*®, *Clarycet*®, or *Floropal*®), a diol (as for *Magnolan*®), or a secondary alcohol (as for *Timberol*®). After resolution, simple and straightforward chemistry can usually be employed to convert the intermediates into the final odorants in good yields.

The enzyme-mediated approach allowed us to rapidly obtain samples of high enantiomeric purity for olfactory evaluations. The odor profiles of these single stereoisomers reveal that no predictions can be made on the odor discrimination of chiral fragrances. There are enantiomers with the same odor character and the same odor threshold (*e.g.*, α-ionones), enantiomers with the same odor character but different odor thresholds (*e.g.*, *Florhydral*®), or enantiomers with different odor character and thresholds (*e.g.*, γ-ionones). When there is more than one stereogenic C-atom, it happens that the odor vectors are the enantiomers of the *same* diastereoisom-

[3]) Catalogue of *International Flavors & Fragances, Inc.*, U.S.A.

ers (in the case of *Floropal*®, *Magnolan*®, *Rhubafuran*®, and *Florol*®) or of *different* diastereoisomers (in the case of *Timberol*®). It may also happen that each stereoisomer contributes to a particular aspect of the racemic mixture (*Pamplefleur*®), so that no isomer prevails, each being essential for the overall odor sensation.

The authors are indebted to Dr. *Philip Kraft* and Mr. *Jean-Jacques Rouge*, *Givaudan Schweiz AG*, Fragrance Research, Dübendorf, Switzerland, for the odor descriptions. The authors would also like to thank Professor Dr. *Wilhelm Pickenhagen* and Dr. *Johannes Panten*, *Symrise GmbH Co. & KG*, Holzminden, Germany, for the odor evaluation of the *Floropal*® samples. *COFIN-Murst* is acknowledged for financial support.

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Scent through the Looking Glass

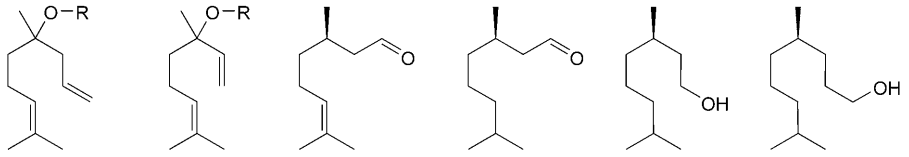
by **Charles S. Sell**

Quest International, Ashford, Kent, TN24 0LT, England

In 1896, *Tiemann* first raised the question of whether or not enantiomers might exhibit different odors. The classic work of *von Braun* should have settled the issue in 1927 but the debate continued for decades, often rather acrimoniously. Various factors such as purity of samples and subjectivity of odors provided the confusion necessary to perpetuate disagreement. Nowadays, we have enough good-quality data to know that sometimes, absolute configuration affects odor perception and sometimes it does not. We are, therefore, left with some interesting academic and commercial questions to be addressed.

Introduction. – In '*Alice through the Looking Glass*', the young heroine steps through a looking glass into a mirror image world. On finding a glass of milk there, she muses that '*perhaps looking glass milk isn't good to drink*'. The author of the book, *Lewis Carroll*, was in reality, *Charles Dodgson*, a reader in mathematics at Oxford University. The book was written in 1878, 18 years after *Pasteur* resolved tartaric acid, and so it is tempting to speculate that *Dodgson* had been involved in Common Room discussions on the subject of chirality, and that these had prompted the remark of *Alice's*, or perhaps even the whole idea of the book.

The first recorded question about odor differences between enantiomers came another 18 years later when *Tiemann* and *Schmidt* suggested the possibility in 1896 [1]. They had synthesised homolinalool (**1**) and its acetate (**2**), and noticed that they had odors which were similar to but weaker than those of optically active linalool (**3**) and linalyl acetate (**4**), respectively, which they had isolated from natural sources. They suggested that this might be because optically active materials experience stronger interactions with olfactory receptors than do racemates. Nowadays, this difference would be attributed to the fact that the analogues they had produced were different molecules, for example, they had higher molecular weights than the natural counterparts and the functional groups had changed from allylic to homoallylic. Nonetheless,



1 R = H
2 R = Ac

3 R = H
4 R = Ac

5 **6** **7** **8**

Perspectives in Flavor and Fragrance Research. Edited by Philip Kraft and Karl A. D. Swift

Tiemann and *Schmidt* had made a significant contribution by opening up a line of investigation which could then be taken up by other workers.

Among those to rise to the challenge laid down by *Tiemann* and *Schmidt* was *von Braun*. He and his various co-workers carried out some first-class experiments in the 1920s into the question of the effect of chirality on odor, and produced sound evidence to support the view that odor differences between enantiomers did exist.

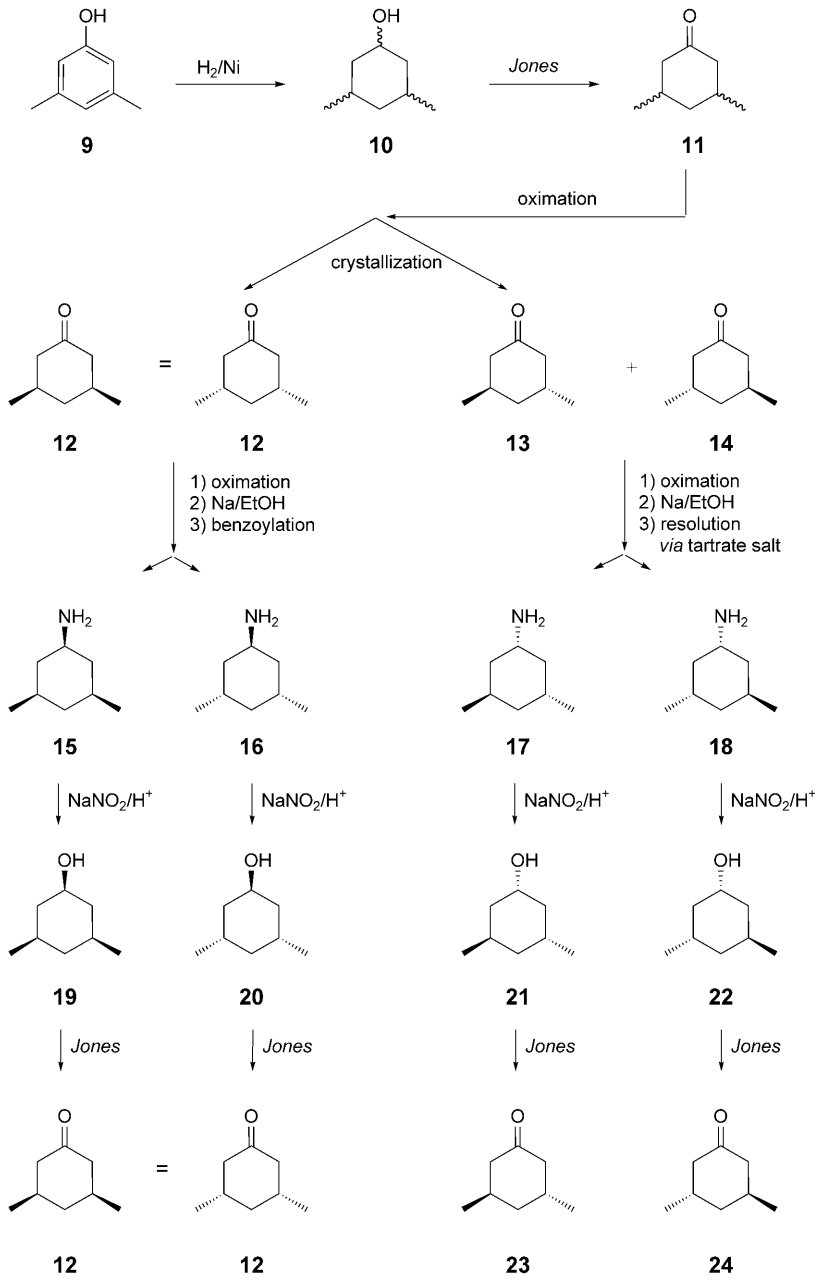
In some of his earlier work, *von Braun* used chiral starting materials obtained from natural sources. For example, from (+)-(*R*)-citronellal (**5**) he prepared the saturated aldehyde **6**, the saturated alcohol **7** and the homologated alcohol **8**, and compared their odors with those of the corresponding racemates, which he had prepared by total synthesis. There was no significant difference in the character of the single enantiomers relative to the racemates but he did observe differences in intensity. (+)-(*R*)-Tetrahydrogeraniol (**7**) was stronger than the corresponding racemate, whereas the other two single enantiomers were weaker than the corresponding racemates [2].

However, *von Braun* was well aware of the issue of organoleptic quality [3]. He realised that, at that time, it was well nigh impossible to guarantee that subtle differences in odor between samples were not due to traces of impurities. This was particularly so when starting from different feedstocks and especially those of natural origin. He, therefore, designed a synthesis of all stereoisomers of 3,5-dimethylcyclohexanone starting from a common achiral precursor, *m*-xylenol, *i.e.*, 3,5-dimethylphenol (**9**) [3] [4] (*Scheme 1*).

In this carefully thought out experiment, *von Braun* and co-workers started by hydrogenating 3,5-dimethylphenol (**9**), and then oxidised the resultant cyclohexanol **10** to the ketone **11**. Oximation of ketone **11** allowed them to separate the *meso*-isomer **12** from the pair of enantiomers **13** and **14**. Oximation of the racemate, followed by reduction to the amine, and fractional crystallisation of the tartrate salt gave the pure individual enantiomeric amines **17** and **18**. These could then be converted, *via* the enantiomerically pure alcohols **21** and **22**, to the isomerically pure, optically active ketones **23** and **24**. As a witness to *von Braun*'s academic rigor, he also converted the *meso*-isomer **12** to the pair of diastereosiomeric amines **15** and **16**, and hence to the diastereoisomeric alcohols **19** and **20**. He was able to demonstrate that, in this case, a single ketone **12** gave a pair of products, whereas the same conversion applied to each of the optically active ketones **13** and **14** gave a single product. Upon oxidation, both **19** and **20** provided the same ketone **12**. This confirmed that **12** was indeed the *meso*-isomer, and **13** and **14** the pair of optically active antipodes.

By starting from a single achiral precursor, *von Braun* had eliminated any issues regarding purity differences in feedstock and, by using identical chemistry, he had reduced to a minimum any chance of different impurities creeping in during the synthetic sequence. The only likely complication would be that of differences resulting during the resolution, as *Posvic* was to discover later in a different case, *vide infra*. This was dealt with by rigorous purification at each intermediate stage. Thus, *von Braun* had prepared samples of three chemically and enantiomerically pure materials, which differed from each other only in their relative configuration. The *meso*-isomer **12** was found to have a camphoraceous odor, similar to that of thujone, whereas one of the optically active isomers, the dextrorotatory one, possessed a fruity, ester-like odor, whilst its enantiomer was minty, resembling isopulegone in character.

Scheme 1



Von Braun's result is of considerable significance in the search for understanding the mechanism by which we perceive odor. As *Naves* [5] pointed out, the observation of odor differences between enantiomers is only possible if the receptors in the nose are

chiral. Interestingly, the opposite logic has also been employed in reaching the conclusion that our sense of smell is chiral. For instance, *Friedman* and *Miller* [6] argued that, since most life processes are chiral, then odor perception must also be chiral and so enantiomers might be expected to display different odor properties. The approach adopted by *von Braun* and *Naves* is more in keeping with the scientific method.

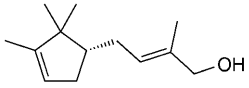
Confusion. – Despite the fact that *von Braun*'s elegant experiment had demonstrated unequivocally that enantiomers can differ from each other in their odor quality, many subsequent generations of chemists continued to argue about the possibility of finding enantiomers with different odors and confusion reigned for many decades. For example, in 1963, *Wright* published the following assertion: '*The suggestion that the first stage in the process of smelling involves an enzymic process is rejected because enzymes are stereospecific and optical isomers have the same or nearly the same odors. The initiation of smelling must be physical rather than chemical. Enzymes can be involved only in the secondary process.*' [7].

In the early part of the 20th century, pairs of pure single enantiomers were not easy to obtain, and, consequently, research into the relationship between odor and chirality was limited. At the same time, many theories concerning the mechanism of olfaction existed, none of which was entirely consistent with all of the observed facts but none of which could be rigorously disproved. Many workers believed that whether or not enantiomers had different odors potentially provided data in favour of or against various contending theories. For example, such differences would be more difficult, though not impossible [8] to accommodate within the vibrational theory of odor. The debate was highly charged emotionally and, for instance, *Friedman* and *Miller* [6] comment that the arguments against their stereochemical thesis included invective as well as more rational objections. This emotional element tended to cloud evaluation of facts, and it took a cool mind to retain objectivity. One such example was *Posvic* [9] who, having demonstrated (*vide infra*) that one particular pair of enantiomers possessed identical odors, reminded readers of his paper that this did not constitute a proof of the vibrational theory.

There are many other factors leading to confusion and conflicting results in the literature. Some are probably simply the result of typographical errors (*e.g.*, the inversion of carvone odor descriptors in one report [10]). Others are at a basic level of understanding. For example, there are cases of workers (mostly biologists but, sadly, sometimes chemists as well) dealing with diastereoisomers but referring to them as enantiomers or to their detection as examples of chiral perception. In some cases, one wonders if the authors are sufficiently aware of the definitions of the descriptors *d* and *l*, D and L, (*R*) and (*S*), or (+) and (−), and of the differences between these.

Directly conflicting results are all too common. For example, of the three papers describing the odors of the enantiomers of 2-methyl-4-(2,2,3-trimethylcyclopent-3-en-1-ylidene)but-2-en-1ol, two agree that the (+)-(*R*)-isomer **26** (*vide infra*) possess a strong sandalwood odor, and that the (−)-(*S*)-antipode **25** is either weak [11], or odorless [12]. However, the third paper [13] claims that the (−)-(*S*)-enantiomer **25** possesses a strong sandalwood odor, whereas the (+)-(*R*)-isomer **26** is sweet and floral in character. The rather different values of the specific rotations reported in the third

paper, might suggest an explanation in this case. The literature itself, or rather careless use of it, can contribute to confusion. For example, *Spreitzer et al.* [14] give an example of how an incorrect observation becomes a myth through citation by later authors who do not check either the facts or the original publication for themselves.



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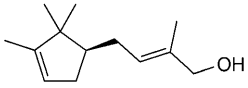

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By far the greatest source of confusion is the fact that odor is a very difficult property to measure. Most chemists do not understand sensory science, and conversely, most sensory scientists do not understand chemistry. Until the formation of interdisciplinary bodies such as the *Association for Chemoreception Sciences* (*AChemS*) and the *European Chemoreception Research Organisation* (*ECRO*), the two schools did not even share a common language. There are many publications in the literature concerning the perception of odorous molecules but only few where both the chemistry and the sensory science are strong. Significantly, the majority of these authors, such as *Haring*, *Ohloff*, *Polak*, *Theimer*, and *Yamamoto*, are from the *fragrance and flavor* industry.

This is hardly surprising as *fragrance and flavor* companies must invest in both chemistry and sensory science in order to operate effectively. Since the tools are there, they can be used for more academic research. However, since the tools are provided in a commercial environment, commercial justification for their use will always be a factor in deciding which experiments will be done. Rigorous sensory evaluation is expensive, and so will only be carried out in cases where there is a good chance of a worthwhile return on the investment. In other cases, pragmatic short-cuts will be taken. In the academic environment, where the tools are much less likely to be available and/or understood, pragmatic short-cuts again rule the day. Consequently, papers containing sound experimental data are few and far between.

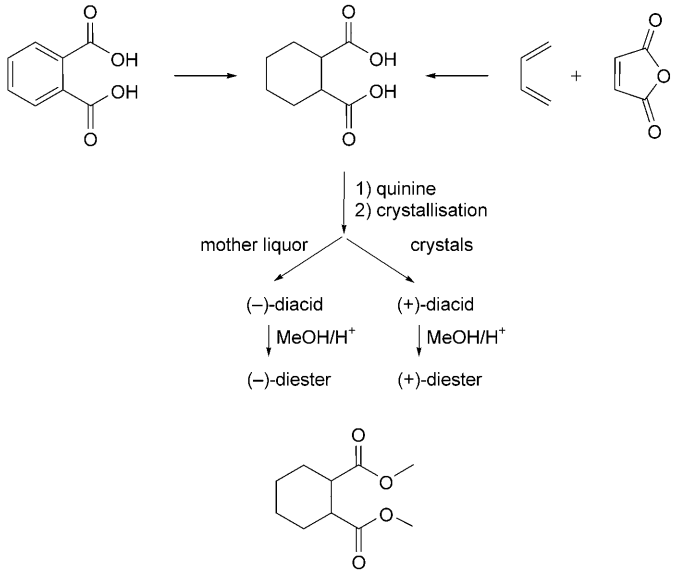
Problems in Measurement of the Odors of Enantiomers. – Any attempt to make sense of the growing volume of data on the chirality of odor, or to relate it to the mechanism of olfaction is fraught with practical and intellectual pitfalls. Sadly, despite all of the advances in chemical analysis and sensory science, many workers today still repeat the mistakes of the past, and some modern data are no more-reliable than those published before the development of modern techniques. It is, therefore, prudent to consider these before embarking on a discussion of the literature on chiral odorants. The key difficulties arise from organoleptic purity, enantiomeric purity, the subjectivity of odor, and the problems in measuring odor.

Organoleptic Purity. – One of the topics of debate in the 1940s was on the question as to whether citronellol from various oils such as *Eucalyptus citriodora* was the same as rhodinol from rose (it was), or if they were structural or optical isomers. In a paper on the subject, *Naves* [15] wrote: '*The origin of many of the errors and failures of workers attempting to elucidate this problem is to be found in the difficulty of recognising*

chemically well-defined preparations and in appreciating the isomerising effect of the reagents employed in the isolation of products and in their structural analysis. Lack of objectivity of chemists inclined to jump to conclusions has done the rest'. Since then, both gas chromatography and high performance liquid chromatography have made enormous advances and one might expect that issues of purity would be a thing of the past. However, this is not the case and many authors have continued to publish results which indicate either ignorance of or disregard for the purity issues, which rigorous workers such as *von Braun* and *Naves* stated so clearly.

Dimethyl *trans*-cyclohexane-1,2-dicarboxylate (**27**) provides us with a nice example of issues arising from the chemical purity of samples. The individual enantiomers had been prepared by *Werner* and *Conrad* [16], and they reported that the dextrorotatory diester was almost odorless whereas the laevorotatory enantiomer possessed a strong odor. Over fifty years later, *Posvic* [9] re-investigated this work, and made an interesting discovery. *Werner* and *Conrad* had prepared the isomers from phthalic acid as shown in *Scheme 2*. *Posvic* decided to use a different route also shown in *Scheme 2*, starting from butadiene and maleic anhydride but using the resolution procedure described by *Werner* and *Conrad*. He confirmed that the odors were as had been described, the (−)-isomer having a strong peppermint smell whereas the (+)-isomer had only a weak, spearmint like odor. However, on examination of the IR spectra of the two, *Posvic* noticed a small absorption at 14 μm, which was present in the case of the (−)-isomer but not the (+). *Posvic* demonstrated that this peak was due to traces of methyl benzoate in the *l*-isomer. This originated from the quinine used in the resolution. The quinine contained small traces of benzoic acid which remained in the

Scheme 2



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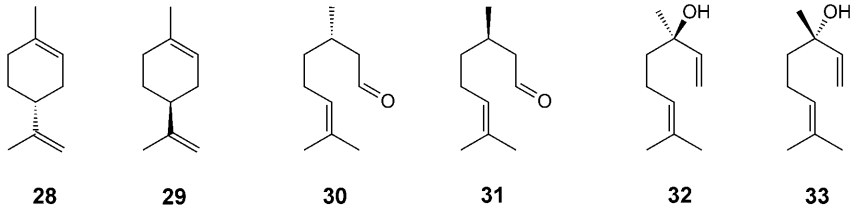
mother liquors during the resolution but separated out with the (−)-isomer when these liquors were acidified. On rigorous purification of the (−)-isomer, *Posvic* showed that it had exactly the same odor character and strength as its antipode.

Limonene is often cited as an example of a material possessing enantiomers with different odors. (+)-Limonene (**28**) is always described as orange, and it is always obtained from orange oil. *Boelens et al.* [17] suggest that the orange odor of **28** is actually due to the presence of minute traces of highly odorous aldehydes, which are the odor impact compounds of orange oil. The present author's own experience supports this view as, in our hands, the more rigorously limonene is purified (not an easy task as it seems to have enormous affinity for aldehydes), the less odor it displays. The description of (−)-limonene (**29**) as being lemon seems to derive from one publication [6] where this odor is attributed to it. However, these authors claim that they obtained their (−)-limonene **29** from lemons, a very odd claim since lemons, like all citrus fruits, produce the (+)-enantiomer **28** of limonene in high enantiomeric purity.



28 **29** **30** **31** **32** **33**

34 **35** **36** **37**

Enantiomeric Purity. – Materials from natural sources are not always enantiomerically pure. *Werkhoff et al.* [18] give several good examples of variations in a number of cases and clearly demonstrate that natural isolates must not be assumed to be all of the same chirality or enantiopure. For example, *Table 1* shows the balance which they have found between (−)-(*S*)-citronellal (**30**) and (+)-(*R*)-citronellal (**31**) in four different essential oils.

Table 1. *Optical Properties of Citronellal from Different Natural Sources*

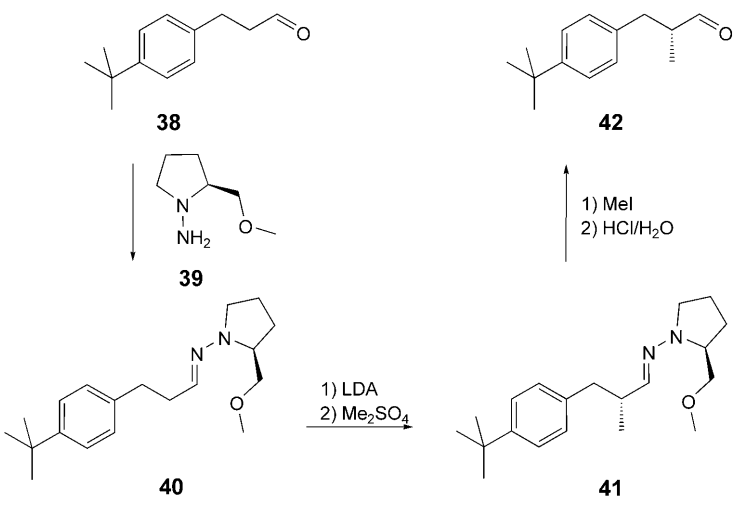
Oil	(−)-(*S*) [%]	(+)-(*R*) [%]
E. citriodora	46.0	54.0
Lemongrass	46.8	53.2
Citronella (Ceylon)	13.5	86.5
Litsea cubeba	62.7	37.3

Despite this, many workers (particularly biologists with little chemical training) use natural isolates without checking organoleptic and enantiomeric purity, and publish results based on these dubious samples. For example, in one study on brain electrical activity in response to smelling chiral odorants, the samples of linalool used for the experiments were clearly not pure. [19] The (−)-(*R*)-linalool (**32**) was extracted from lavender and had an $[\alpha]_D$ of −15.1 whereas the (+)-(*S*)-linalool (**33**) had an $[\alpha]_D$ of +17.4. Even with synthetic materials, authors often report odor differences between enantiomers when it is clear (from $[\alpha]_D$ or % ee values) that their materials are not enantiomerically pure. Such results must, therefore, be treated with caution. In one paper [20], the authors even claim that the rate of reaction of an (+)-(*S*)-isomer was 30% faster than that of the (−)-(*R*)-enantiomer, under the same conditions employing achiral reagents. This should have told them that something was amiss with their materials.

One very good example of the effects of enantiomeric purity below 100% is given by *Weber* and *Mosandl* [21]. Having separated and evaluated the enantiomers of 3-(methylsulfanyl)butanal using chiral GC-olfactometry, they point out that the (*R*)-enantiomer **34** has a threshold so low that 20 ng of the odorless (*S*)-enantiomer **35** with an enantiomeric purity of 99.9% ee, smelt from an achiral GC column, would still smell of potatoes because of the 0.02 ng of the (*R*)-enantiomer present. Similarly, in a paper on *Vulcanolide*®, *Fehr* and *Chaptal-Gradoz* [22] point out that the weak odor of the (+)-(*R*,*R*)-isomer **36** could be due to traces of the extremely potent (−)-(*S*,*S*)-enantiomer **37**.

The effect of the synthetic route on the enantiomeric purity is demonstrated by the example of *Lilial*® (**44**). *Enders* and *Dyker* [23] synthesised both enantiomers using their (*S*)-1-amino-2-(methoxymethyl)pyrrolidine (SAMP)/(*R*)-1-amino-2-(methoxymethyl)pyrrolidine (RAMP) hydrazone methodology as shown in *Scheme 3*. Treatment of *Bourgeonal*® (**38**) with SAMP (**39**) gave the hydrazone **40**. The hydrazone **40**

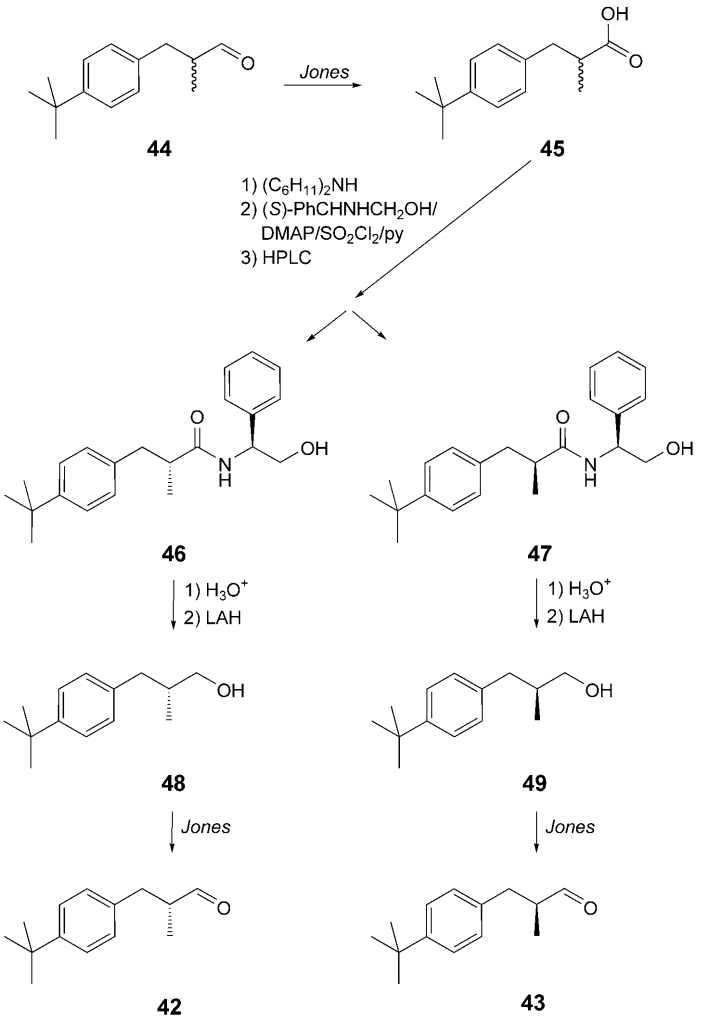
Scheme 3



was deprotonated using LDA, and the anion alkylated with Me_2SO_4. This alkylation was stereospecific and afforded **41** with 93–95% diastereoselectivity. *N*-Alkylation and subsequent hydrolysis of **41** provided (−)-(*R*)-*Lilial*® (**42**) with 93–96% ee. The (+)-(*S*)-isomer **43** was prepared in the same way using the RAMP hydrazone. Contrary to an earlier report by *Sotoguchi et al.* [24], *Enders* and *Dyker* found that both enantiomers smelt but with the (−)-(*R*)-enantiomer being much stronger.

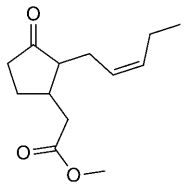
Several years later, *Mosandl* and co-workers [25], synthesised both enantiomers of *Lilial*® **42** and **43** by a different route (*Scheme 4*). They started from racemic *Lilial*® (**44**) and oxidised it to the corresponding acid **45**. Treatment of the dicyclohexylammonium salt with (*S*)-2-amino-2-phenylethan-1-ol in the presence of 4-(dimethylami-

Scheme 4



no)pyridine (DMAP), SO_2Cl_2 and pyridine, gave the diastereoisomeric amides **46** and **47**, which were separated by HPLC. Hydrolysis of the diastereoisomer **46** with H_2SO_4 in aqueous THF, followed by reduction with LAH, provided the (*R*)-configured alcohol **48**, which was oxidised to (−)-(*R*)-*Lilial*® (**42**) with *Jones*' reagent. Similarly, (+)-(*S*)-*Lilial*® (**43**) was obtained from the (*S*,*S*)-amide **47** *via* the (*S*)-configured alcohol **49**. They checked the enantiomeric purity of their materials by chiral GC against a racemic standard and carried out their odor evaluations from the outlet port of a GC-olfactometer fitted with a chiral stationary phase. In this way, they were assured that any observed odors were indeed derived from a single enantiomer. Their findings were in agreement with those of *Sotoguchi et al.* [24], in that they found the (−)-(*R*)-enantiomer **42** to possess a powerful lily of the valley odor, whereas the (+)-(*S*)-isomer **43** is odorless. The odor detected in the latter by *Enders* and *Dyker*, must, therefore, have been due to the small amount of the active enantiomer which it contained. In general, any researcher who finds two enantiomers to have similar odor character but different intensity would be well advised to check the enantiomeric purity of the weaker isomer, taking into account the odor threshold of the stronger.

The example of *Lilial*® also raises the interesting question of enantiomers in which the stereogenic centre is epimerisable. *Mosandl* and co-workers were able to verify that the final stages of their synthesis had not caused racemisation despite the fact that each conversion involved the use of acid and/or base, an important point which should not be overlooked. Since there are many examples of enantiomeric compounds with epimerisable stereogenic centres possessing different odors, the mechanisms for detection of odorants and their removal from the olfactory system must be faster than *in vivo* racemisation. Other examples include methyl jasmonate (**50**) [26] and α-damascone (**51**) [27–29]. The majority of commercial fragrance applications involve media which are not at neutral pH [30], and so racemisation does occur with loss of optical activity of materials in this category thus limiting the commercial utility of homochiral versions.



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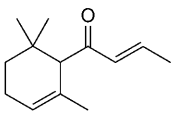

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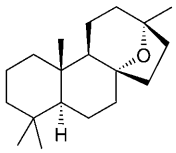

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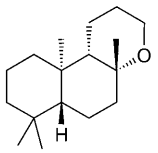

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Subjectivity of Odor. – Odor is subjective on three levels: semantic, hedonic, and intrinsic [31]. Semantic and hedonic effects can be eliminated in properly designed experiments, but the intrinsic differences between individual humans can only be measured and results expressed either as pertaining to an individual or in statistical terms for a group of individuals.

A publication, which demonstrates variation between individuals very effectively, is one by *Ohloff et al.* [32] on the subject of the odor of selected analogues of *Jeger*'s ketal (**52**) and their enantiomers. When 27 panelists were asked to assign the ether **53** to an odor category, the results were as shown in *Table 2*. The variation in the results is not a

Table 2. *Odor Classification of Compound* **53**

Odor category	Women	Men
Minty/camphor	6	8
Fruity	2	4
Balsamic	0	3
Musky/woody	2	2

consequence of their not understanding the language as all were accustomed to dealing with and describing odorous materials. There were genuine differences in perception of **53** by the subjects involved. As in the case of **53**, a sexual bias in perception was evident for most of the compounds studied. Perception of both odor character and intensity varied from one subject to another. Some materials were found to be more polarising than others, in that they tended to scored either '*strong*' or '*odorless*' ratings rather than '*detectable*'. Rapid fatigue, *i.e.*, loss of ability to smell on continued exposure, proved a serious issue when carrying out these experiments. *Ohloff* commented on people giving '*wrong*' answers for odor character, *e.g.*, '*isopropanol-like*' for a material which most would describe as amber. However, in that sense, there is no such thing as a wrong answer. People report what they perceive and that is what they perceive, even if they classify or perceive differently from others. Furthermore, in this instance, to many people, there is a connection between the odors of isopropanol and *Jeger*'s ketal, and so isopropanol is not such a '*wrong*' answer. The only '*wrong*' answers are when people cannot repeatedly pick out the different sample from a triangle test[1]), indicating that they cannot distinguish between two test materials.

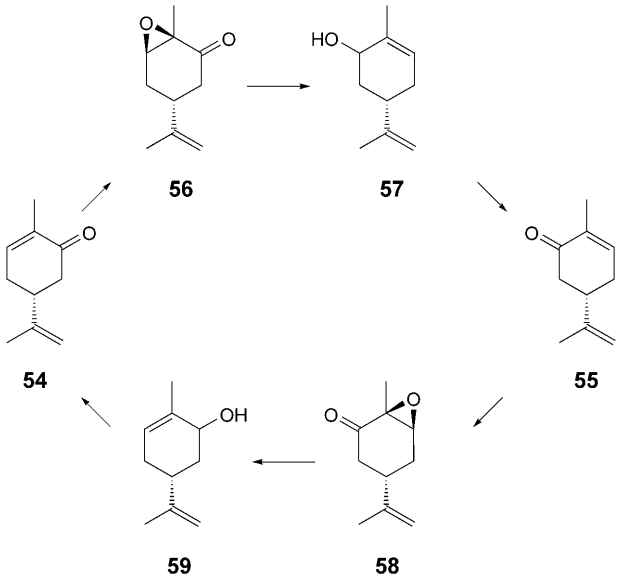
Blank samples were included in the test, and these sometimes attracted positive scores with ratings being given for both character and intensity. *Ohloff* postulated that this effect was due to strong samples being absorbed in the nose and then are desorbed when smelling a blank. An alternative explanation might be found in the *Slossen* experiment [33]. In this classical experiment of sensory science, a liquid was poured onto cotton wool at the front of the theatre at the beginning of a lecture and the audience asked to indicate when they could smell it. Those in the front row responded quickly and eventually *ca.* 70% of audience believed they could smell it. The liquid was distilled water. Thus expectations can influence perception, and great care must be taken with data which is not gathered from a properly organised statistical experiment using double blind samples, in which neither the subject nor the person administering the test knows the identity of the samples. In another example, *Laska* and *Teubner* [34] investigated the odor of ten pairs of enantiomers, and found that the ability to distinguish between the isomers in each pair was time stable for each individual subject but that there were large differences between individuals.

The issue of organoleptic purity was clearly settled in the case of carvone by *Friedman* and *Miller* [6] who not only synthesised both enantiomers independently, but also converted the (−)-isomer **54** to the (+)-isomer **55** and *vice versa* as shown in

[1]) In a triangle test, the subject is given three samples, two of which are identical, and is asked to identify the odd one out. In a more stringent form of the test, there are five samples, two of one material and three of a second. The subject must then correctly identify two out of five.

Scheme 5. They thus demonstrated that the spearmint scent of the (−)-isomer **54** and the dill-caraway character of its antipode **55** were intrinsic properties of the two compounds and not due to impurities. The interconversion was possible by virtue of the fact that the unsaturated ketone function of carvone can be manipulated without affecting the configuration of the stereogenic centre. Thus, allylic transposition has the effect of inverting the configuration as shown in *Scheme 5*. Treatment of (−)-carvone (**54**) with alkaline peroxide gave the epoxide **56**, which could be reduced to (+)-carveol (**57**), and this in turn was oxidised to (+)-carvone (**55**). Starting from (+)-carvone (**55**), the same sequence of reactions led through **58** and **59** to (−)-carvone (**54**).

Scheme 5



However, in the same year as *Friedman* and *Miller*'s publication, *Leitereg et al.* [35] published an interesting and well executed piece of sensory work on the enantiomers of carvone **54** and **55**. They showed that not all subjects could distinguish between the enantiomers. Surprisingly, a greater percentage of subjects described (−)-carvone (**54**) as caraway if the sample had been obtained from spearmint oil than if the sample was synthetic in origin. The results of this part of their work are shown in *Table 3*. So, although *Friedman* and *Miller* showed conclusively that there is a difference in the average of odor perception between the enantiomers, *Leitereg et al.* remind us that the interpretation of the difference, and even the ability to perceive it, is subject to variation between individuals.

Variability is not limited to humans. *Apfelbach* and co-workers [36] showed that the ability of rats to detect the enantiomers of carvone varied by a factor of 10^4 for the (+)-isomer **55** and 5×10^4 for the (−)-isomer **54**. Therefore, individual variation cannot be merely a consequence of some higher level process in humans. For comparison, the range in humans has been shown to be of the order of 10^3 [37–39].

Table 3. *Odor Classification of the Carvone Enantiomers* **54** *and* **55**.

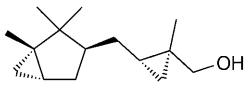
Sample	% of subjects who identified it as	
	caraway	spearmint
(+)-Carvone from caraway	71	29
(+)-Carvone, synthetic	80	20
(−)-Carvone from spearmint	19	91
(−)-Carvone, synthetic	29	71

Odor Measurement. – Odor measurement is much more difficult than is imagined by those who do not do it properly. To obtain good-quality results, the subjective nature of olfaction requires the use of large panels and the results should be reported in statistical terms. Very often, authors report figures (*e.g.*, for thresholds) but do not say how many subjects were involved in the measurement, what ranges of values were observed and what were the reproducibility and statistical reliability of the results. This is particularly misleading if the results are reported to high precision since the subject to subject variation probably covers orders of magnitude. Panel testing requires reasonably large quantities of test material. Modern synthetic chemists tend to work on very small scale and use chiral GC-olfactometry to obtain rigorously enantiomerically pure samples. This precludes the use of large panels and so, in most cases, there is a pragmatic trade-off between chemistry and sensory science.

There are no fixed reference points for odor character, and all descriptions are associative. There are no agreed universal standards and apparent agreement can be misleading. Therefore, comparison of results from different laboratories always carries an element of risk. Odor classification is arbitrary and tends to rely on factors such as botanical origin rather than anything to do with receptors [40].

If we think of odor character as existing in *n*-dimensional space, then sometimes there are surprising examples of apparently very different odors coming close together in that space. For example, in the author's experience, muguet (lily of the valley) and sandalwood seem to be closely related in terms of chemical structure in that a small structural modification will flip a material from one camp to the other. Thus, it is not so surprising when *Bajgrowicz et al.* [41] report that the enantiomer **60** of the strong sandalwood material **61** possesses a muguet odor.

60



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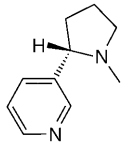

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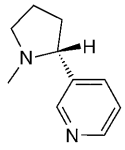


67

Measurement of odor intensity is equally fraught, and there is no objective scale. When comparing different objects or phenomena, humans will adjust the scale to suit the overall context. For example, one person asked to give a percentage difference between an elephant and a butterfly may well come up with a similar value to another who is asked to compare a red admiral with a cabbage white. In the same way, when

asked to compare the strength of two odors, the scale will be adapted to the difference between them.

A good example of this issue is provided by the work of *Haring et al.* [42] on the enantiomers of nootkatone **62** and **63**, and α-vetivone **64** and **65**. *Fig. 1* shows the odor profiles of the enantiomers of both of these isomeric sesquiterpenes. In each case, the isomers are distinguishable by smell, but the differences are more significant in the case of nootkatone.

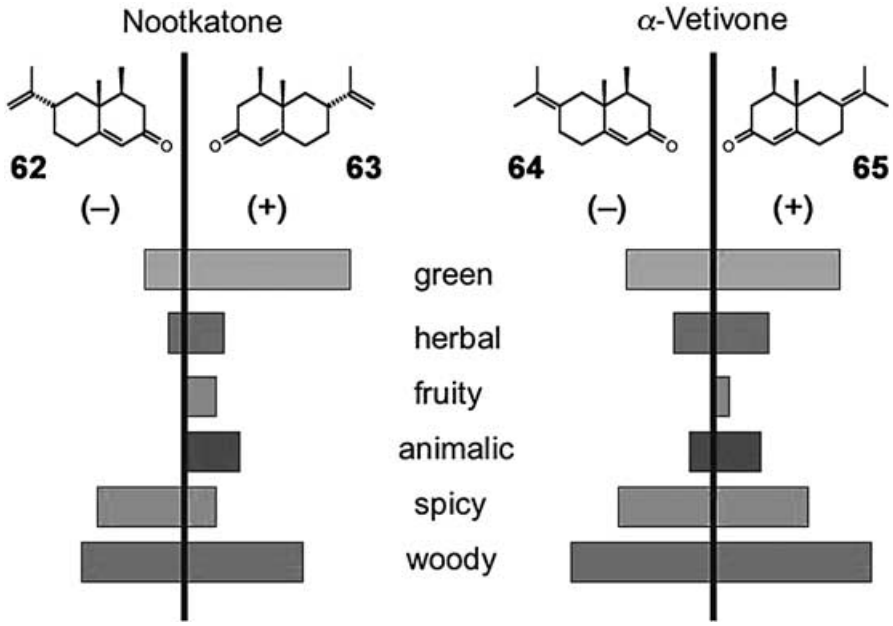


Fig. 1. *Odor profiles of the enantiomers of Nootkatone* (**62** and **63**) *and* α*-vetivone* (**64** and **65**)

Intensity is often measured in terms of thresholds, but this is erroneous as the two are not the same. Because compound A has a lower threshold of detection than compound B, this does not necessarily mean that A will be stronger than B when both are presented at the same super-threshold concentration, as is clearly demonstrated using *Stevens*' Law plots [43]. Many authors, even '*experts*', confuse intensity with threshold, and it is always worthwhile checking the experimental section before interpreting reported results. An illustration of the difference is found in the work of *Hummel et al.* [44] on the enantiomers of nicotine. The detection thresholds of both the naturally occurring (−)-(*S*)-isomer **66** and its enantiomer **67** are identical, but the latter is perceived as more intense. Interestingly, non-smokers experience a greater difference between them than do smokers. The *Stevens*' Law plot of the nicotine enantiomers must be something like that shown in *Fig. 2*. In other work, it is suggested that the ability to discriminate between the odor of the nicotine enantiomers lies in the trigeminal rather than the olfactory system [45].

Intensity and threshold are always determined in terms of concentration. Different workers use different frames of reference for concentration. Direct determination of

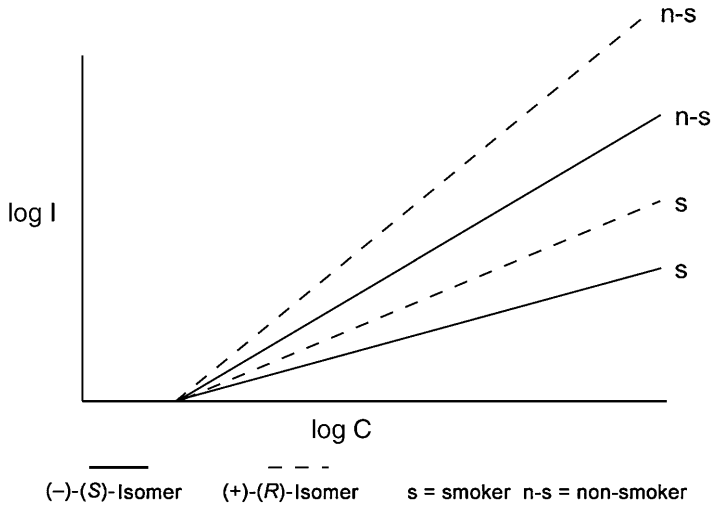
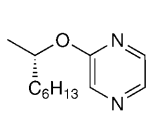


Fig. 2. *The* Stevens' *Law plot of the nicotine enantiomers*

the actual concentration of a molecule in inhaled air is difficult, and so it is common practice to refer to the concentration relative to the solvent in which the material to be tested is dissolved. Sometimes the quoted concentration is that in the solvent and sometimes this is corrected, using *Raoult*'s Law, to give an estimate of the concentration in the headspace (air above the solution under study). Sometimes activity coefficients are included in the calculation and sometimes not. In smelling from GC instruments, the concentration or the actual weight eluted is quoted. However, such figures are calculated rather than measured and make many assumptions about material loss, air flows *etc.* in the instrument. Once again, it is important to look carefully at the experimental details before attempting to compare results between different laboratories.

Odor signals are not necessarily additive in a linear way and this applies also to the odors of enantiomers. For example *Masuda* and *Mihara* [46] found that the measured odor thresholds of (+)-(*S*)-2-[(1-methylheptyl)oxy]pyrazine (**68**), (−)-(*R*)-2-[(1-methylheptyl)oxy]pyrazine) (**69**) and racemic 2-[(1-methylheptyl)oxy]pyrazine were 30, 90, and 90 ppb, respectively. Similarly for (+)-2-(menthyloxy)pyrazine (**70**), (−)-2-(menthyl)oxypyrazine (**71**), and racemic 2-(menthyloxy)pyrazine the values were 10, 2, and 3 ppb, respectively. There are examples in the literature where the threshold of one enantiomer is estimated from those of its antipode and the racemate. Any such values must be treated with caution. Similarly, *Polak et al.* has demonstrated that the odor properties of one enantiomer cannot be predicted from those of its antipode [39][47].

The subjectivity of odor perception leads us to the use of statistical measurement in order to give a picture of the population as a whole rather than one individual. However, even when an experiment is executed in an exemplary manner, as was *Polak*'s study [47] on the enantiomers of geosmin, there is still a danger in taking the headline results and using them, for example, to build a QSAR model. *Polak* found that the mean odor threshold of (−)-geosmin (**72**, the enantiomer found in nature) was



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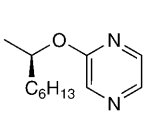

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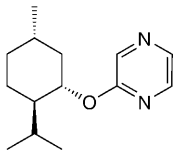

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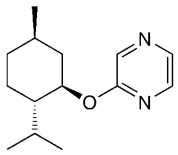

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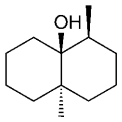

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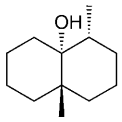


73

0.0095 ppb in H_2O, whereas the (+)-enantiomer (**73**) had a threshold of 0.078 ppb in H_2O. The averages indicate that the natural isomer can be detected at a concentration of less than one tenth that of its antipode. However, some people could detect the unnatural isomer at concentrations which were one thirtieth that of their limit for detection of the natural one. This brings into question the significance of any model derived from the average figures.

Recent Developments. – Of the many advances which have been made over the last three decades there are four which have played a particular role in significantly increasing our understanding of the odor perception of enantiomeric compounds. The development of chiral chromatography provided a huge fillip in that the enantiomeric purity of materials can now be determined relatively easily and coupling of smelling ports to GC instruments fitted with chiral columns enables single enantiomers to be smelt unambiguously. Chiral chromatography has been reviewed by *Mosandl* [48], *Mosandl et al.* [49], *Werkhoff et al.* [50] as well as *Schurig* and *Nowotny* [51]. Nowadays, NMR spectroscopy provides a less expensive alternative to X-ray crystallography for the determination of absolute configuration, and developments in chiral catalysis and dynamic resolution have enabled larger amounts of enantiomerically pure materials to be prepared. The contribution of *Knowles*, *Noyori*, and *Sharpless* to chiral catalysis was recognised by their being awarded the *Nobel Prize* in 2002, and their methods are used every day to produce materials for the fragrance industry.

In the light of these three developments, it is not surprising that there is now an increasing flow of papers into the literature describing the resolution or enantioselective synthesis of odorous molecules complete with odor descriptions of single enantiomers or diastereoisomers. One of the factors which added emotional heat to the debate in the past was the subjectivity of odor. In 1973, *Polak* postulated that the mechanism of odor perception is combinatorial in nature [52]. His theory was vindicated in 1999 when *Buck* and co-workers [53] demonstrated that olfactory receptor proteins are broadly tuned so that each receptor type responds to a range of odorant molecules, and each odorant molecule interacts with a range of receptors. Thus, each odorant generates a signal '*fingerprint*' and the perception of '*odor*' is the result of pattern recognition and interpretation. The genome contains codes for *ca.* 1,000

olfactory receptors [54] although each human uses only *ca.* 350 of them, and there is variation between individuals as to which 350 of the 1,000 are active genes and which are ψ-genes [55]. Thus, the subjectivity of olfaction starts at the most basic level in that each of us detects odors with a unique combination of receptors. Experience and other inter-individual differences result in an increase in subjectivity higher up in the neurotransmission pathway.

In reality, the situation is even more complex. The perceived odor of a material is the sum of the inputs, not just from the array of 350–400 olfactory receptor types in use, but also from other inputs. About 70% of odorants also stimulate the trigeminal nerves which innervate the nasal cavity. In 1971, *Theimer* and *McDaniel* [56][57] suggested that the trigeminal nerve made a contribution to the totality of odor perception. This hypothesis has since been confirmed by later workers. For example, as mentioned earlier, *Hummel et al.* [44] showed that the trigeminal nerve is involved in the perception of nicotine (**66**/**67**), and *Kobal* and co-workers [45] postulate that the ability to discriminate between the enantiomers of nicotine depends on the trigeminal rather than the olfactory sense.

Recent work by *Apfelbach* and co-workers [58] has thrown some light onto the issues with the perception of carvone which were discussed above. Lectins are proteins that recognise and bind to saccharides. The olfactory receptors are glycosylated on the face outside the cell and are thus subject to binding and hence blocking by specific lectins. *Apfelbach* and co-workers showed that application of the lectin concanavalin-A to the olfactory mucosa, inhibited perception of (+)-carvone (**55**) but not of (−)-carvone (**54**). On the other hand, another lectin, wheatgerm agglutinin (WGA), inhibited perception of (−)-carvone (**54**) but not of (+)-carvone (**55**) [59]. The conclusion is, therefore, that each of the enantiomers is detected by a different population of receptors from the other. There is an old flavorists' trick of adding a small amount of nonan-1-ol to (−)-carvone (**54**) to give it an odor more like that of the less readily available (+)-carvone (**55**). It would now seem that this effect might be due to the combined receptor firing patterns resulting from exposure to a mixture of nonan-1-ol and (−)-carvone (**54**) fortuitously resembling the pattern resulting from (+)-carvone (**55**).

Evaluating Published Data. – In view of all the above practical difficulties involved in determining odor properties of enantiomers, it is not surprising that there is a great deal of data of dubious quality in the literature. Assessing the quality of literature data is not straightforward and is, by necessity, often a rather subjective exercise in itself.

There are various approaches to obtaining enantiomerically pure samples for evaluation. Some of these are listed below in order of increasing rigor. This scale can be used as a rough guide to assessing the likelihood of materials being adequately pure:

– Isolate each enantiomer from natural sources.
– Resolve enantiomers.
– Synthesise each enantiomer from enantiomerically pure natural starting materials.
– Synthesise each enantiomer from different achiral starting materials.
– Synthesise both enantiomers from the same achiral precursor.
– Synthesise both enantiomers from the same homochiral precursor.
– Interconvert the two enantiomers.

For examples of good practice, the reader is referred to such work as that of *Ohloff* and co-workers [60], or *Theimer* and *McDaniel* [56]. For odor evaluation, similarly, there are various techniques some of which are listed below, also in order of increasing rigor:

– Single subject assessment of bulk samples.
– Single subject assessment of samples from a chiral GC column.
– Full sensory panel assessment using samples whose purity has been established by chiral GC *etc.*

Clearly, the more-rigorous assessments are very expensive and consequently relatively rare. To evaluate the quality of published results, the researcher must make a pragmatic judgment based on the rigor of the experimental approaches employed as far as both the chemistry and sensory science are concerned.

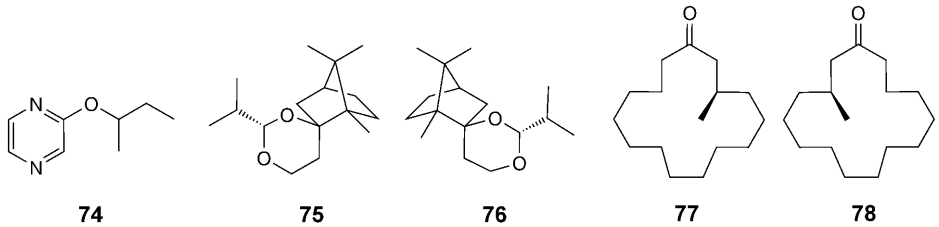
Variety of Effects of Chirality on Odor. – There are a number of reviews [17] [61 – 68] on the subject of odor differences between enantiomers, and there would be no point in attempting to summarise them in this present work.

To search for patterns relating absolute configuration to structural features, the author has scanned both the literature and *Quest*'s proprietary data, bearing in mind the above criteria regarding the purity of samples and the rigor of evaluation. The simple conclusion is that there is nothing to contradict *Polak*'s [39][47] assertion that differences in odor between enantiomers are unpredictable.

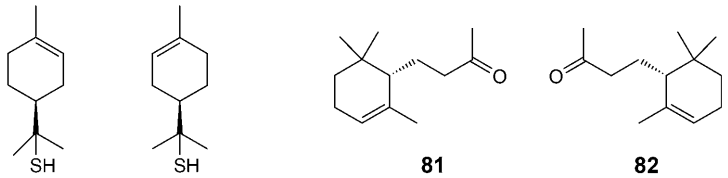
There follow some general observations about the effect of structure on chirality, but it must be stressed that these are based on chemical instinct and '*rule of thumb*' and, in view of the limited data set available, have not been subjected to proper statistical analysis. Rigid molecules are slightly more likely to have a large intensity difference than are flexible ones. On the other hand, flexible molecules are more likely to have a character difference than rigid ones. Overall, it would appear that a difference in threshold or intensity is more likely than a difference in character. This third observation is more puzzling in the light of what we know about the combinatorial nature of olfaction than are the previous two. Relative difference in size of substituents around a stereogenic centre does not seem to correlate with intensity difference but does seem to affect character difference; the greater the size difference, the more likely it is that there will be a significant character difference. *Rossitol*® [69], *Galaxolide*® [70] and wine lactone [71] provide examples where the absolute configuration is more important in determining the odor than is *cis*/*trans*-geometry around the ring systems of the molecules.

There does not appear to be any correlation between odor difference and either the absolute configuration, *i.e.*, (*R*)- or (*S*)-configuration, or the direction or magnitude of the specific rotation, $[\alpha]_D$. From the work of *Spreitzer et al.* [72] on vetiver fragments, it is clear that the size of odor difference between enantiomers is not related to the size of the CD curve, therefore indicating that receptors do not see chiral odorants in the same way that polarised light sees them. *Masuda* and *Mihara* [46] investigated a series of chiral 2-alkoxypyrazines and found that there were no odor character differences between enantiomers but that differences in odor intensity increased with alkyl chain length. It would be interesting to know whether this phenomenon is due to recognition or transport effects or even a combination of the two.

Sound examples of all possible combinations of effects of chirality on odor intensity and character can be found in the literature, and a few examples will suffice to establish this fact. In some cases, the optical activity has no effect whatsoever on the odor. For example, *Masuda* and *Mihara* [46] showed that both (+)- and (−)-enantiomers of the 2-(1-methylpropoxy)pyrazine (**74**) have exactly the same odor character, and detection threshold (100 ppb) and these are exactly the same as those of the racemate.



74 **75** **76** **77** **78**



79 **80** **81** **82**

Bajgrowicz and *Frank* [73] found that the enantiomers **75** and **76** had identical odor thresholds (40 ng/l air) but different odor characters. Whereas **74** had pineapple aspects combined with wood, **75** had rose in addition to wood. Conversely, *Pickenhagen* [74] found that the enantiomers of muscone both had the same musky odor, but that the threshold (61 ppb) of (−)-muscone (**76**) was lower than that (233 ppb) of (+)-muscone (**78**). Similarly, the enantiomers of thioterpineol both display a grapefruit character but the threshold (2.1×10^{-5} ppb) of the (−)-enantiomer **79** is four times lower than that of its antipode **80** [75]. The enantiomers of dihydro-α-ionone differ in both character and intensity. The (−)-enantiomer **81** has an odor of orris with a threshold of 100 ng/l air, whereas the (+)-enantiomer **82** has a violet character and a lower threshold of 31 ng/l air [76]. As mentioned above, *Mosandl* and co-workers [25] showed that in the case of *Lilial*® one enantiomer is totally odorless.

In view of the combinatorial mechanism of olfaction, this last observation is the most puzzling. With carvone, we see each enantiomer firing a range of receptor types, some different and some in common, thus generating two different patterns – which is what one would expect. What is unexpected is that, if a material can reach the olfactory epithelium and generate a signal pattern, its enantiomer should be odorless. The sense of smell has evolved to be capable of detecting as wide a range of volatile materials as possible, so why should this be? One possible simple explanation is that there is a single specific receptor which the odorous enantiomer fires, but which the odorless one does not. However, how likely is this in view of the breadth of tuning of receptors and the enormous diversity within the family? Could it be that the brain just ignores, *i.e.*, fatigues or adapts to, the signals generated by the wrong enantiomer?

Commercial Considerations. – There are some sound commercial reasons for preparing enantiomerically pure or enantiomerically enriched fragrance ingredients. If there are odor quality differences between the enantiomers, then enantiomerically pure grades will provide unique odor properties, distinct from those of the racemate. If the difference is in intensity, then the use of the stronger isomer will provide a higher impact. This will enable the use of a lower dose, and hence a lower environmental load. However, unless there is a suitable starting material from nature, the enantiomerically pure material will always be more expensive than the corresponding racemate. It might also be that adverse environmental effects of a stereoselective synthesis could outweigh the environmental advantage of an enantiomerically pure or enantiomerically enriched product. Thus, the use of pure enantiomers in fragrance will always depend on the outcome of a cost–benefit analysis, and it is likely that, for the foreseeable future, racemates and enantiomerically pure materials will exist side by side in a competitive market place.

Conclusions. – One important theme to come out of this study is the need for scientific rigor in the design and execution of experiments and in interpreting the results derived from them. Of course, rigor in data interpretation applies equally to one's own results as well as those obtained from the literature. Use of inadequate or dubious data will inevitably lead to inadequate or dubious conclusions being drawn. It is also important not to disregard the existing literature, as some excellent work was carried out in the past despite the constraints on practical techniques under which earlier generations of scientists worked. It is also necessary to maintain as broad and interdisciplinary an outlook as possible. For example, no matter how good the chemistry is, if one tries to link molecular structure to biological data, one must understand the significance of the biological data.

The confirmation of the combinatorial mechanism of olfaction has increased our understanding of odor very significantly. However, the complexity, which is a necessary part of such a mechanism, presents us with many fascinating questions and challenges. The existence of small character differences between most pairs of enantiomers is not at all surprising in view of their being recognised by a range of broadly tuned receptors. On the other hand, the fact that there are odorless enantiomers of chiral odorants raises some interesting questions. Flexibility of molecules is always a problem for modellers when attempting to understand the interaction between a small molecule and a protein. In the vast majority of cases, there will be a variety of conformations of the small molecule, which could be present under physiological conditions, and binding energy could increase this number. When faced with a receptor array, the problem increases dramatically as each receptor might interact with a different conformation of the ligand. The receptor tuning is broad yet clearly can be subject to even subtle stereochemical effects. Furthermore, each of us uses a unique set of receptors. The total picture is even more complex, since signal attenuation and/or amplification occurs at various points higher in the chain of neuroprocessing events, which convert the raw signals into the phenomenon, which we call odor.

Complete understanding of the entire process and, therefore, the ability to predict exactly how any given molecule will smell, is still a very long way off. The structure–activity models, which we build and use, are purely statistical in nature.

Experienced chemists develop an instinctive model, which we have found will outperform any SAR model adding weight to the aphorism that '*it is better to be approximately right than precisely wrong*'. Furthermore, experienced chemists will also be able to build commercial factors, such as production feasibility and cost, into their molecular design. Early last century, *Ernest Beaux*, creator of '*Chanel N°5*' (*Chanel*, 1921), said that '*One has to rely on chemists to find new aroma chemicals creating new, original notes. In perfumery, the future lies primarily in the hands of chemists*'. In the light of the above discussion, I would conclude that his assertion is as true today as it was then.

I would like to thank my colleagues *Roger Duprey* and *Angus MacMaster* for their work on evaluation of the odor of chiral odorants, and *Richard Butcher* and *Martin Till* for their work in helping me to build a database of published and proprietary descriptions of the odors of pairs of enantiomers. I would also like to thank the organisers, Dr. *Karl Swift* and Dr. *Philip Kraft*, for inviting me to present this work.

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Silylating Reagents: A Powerful Tool for the Construction of Isosteric Analogs of Highly Branched Odorants

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We have discovered that α-[dimethyl(thexyl)silyl]acetaldehyde (= [dimethyl(1,1,2-trimethylpropyl)silyl]-acetaldehyde; **31**) has a strong, woody odor. Structural analysis has shown resemblance to known odorants with similar organoleptic properties. On the basis of structure–odor relationships, new and more-powerful woody and ambery sila odorants were prepared. Further derivatization led to a set of compounds with very interesting organoleptic properties. Selected chiral compounds were also prepared stereoselectively. The influence of the absolute configuration on the olfactory properties was in agreement with theoretical assumptions. We also designed other groups of organosilicon odorants. The compounds discovered can be obtained in a few simple steps from commercially available reagents, and may find application in the fragrance and flavor industry. Their structures provide interesting data for further research on structure–odor relationships.

Introduction. – Silicon (Si), an element of group IV, and carbon (C) have similar properties and reactivities [1]. However, there are also distinct differences, which can be summarized as follows: *1*) nucleophilic substitution at Si is much easier than at C; *2*) double bonds to Si are weak (hence, elimination does not affect substitution); *3*) single bonds from Si to oxygen (O), chlorine (Cl), and fluorine (F) are very strong; and *4*) Si stabilizes a positive charge in the β-position and a negative one in the α-position. These properties and other features [1] make Si derivatives widely applicable in organic synthesis, especially as protecting and directing groups in asymmetric synthesis [2][3] (for reviews see [1][4]). Moreover, organosilicon compounds are generally nontoxic [1][5] and have found broad application in industry and daily life. The most popular application is the production of a wide variety of linear (**1**), branched (**2** and **3**), as well as cyclic (**4**) polylsiloxanes (*Fig. 1*).

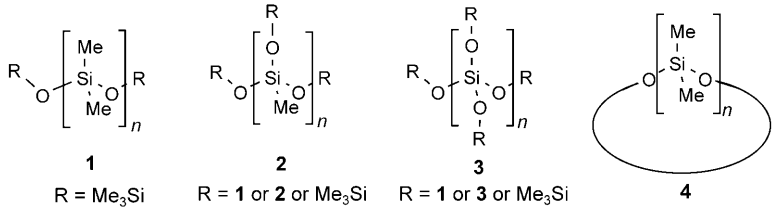

Fig. 1. *Simplest linear, branched, and cyclic polysiloxanes*

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Polysiloxanes are used as thermally and chemically resistant oils, plastics, and glues. They find application in cosmetics, and as odor- and tasteless food additives such as antifoaming agents in juice production. Other well-known applications are in contact lenses and a wide variety of human organ prostheses. Low-molecular-weight linear polydimethylsiloxanes, known under the trade name *Symethicone*®, are used in medicine for the treatment of heartburn and flatulence even in infants [5]. In the last decades, so called Si switches have been extensively investigated. For instance, the Si analog **5** of *Hexahydrodifenidol*® appeared to be a very strong antimuscarinic drug [6] (*Fig. 2*). It is commercially available and used for the classification of muscarinic receptors. Recently, the Si-derived phthalocyanine **6** (*PC-4*®) has been tested in photodynamic cancer therapy (*Fig. 2*) [7].

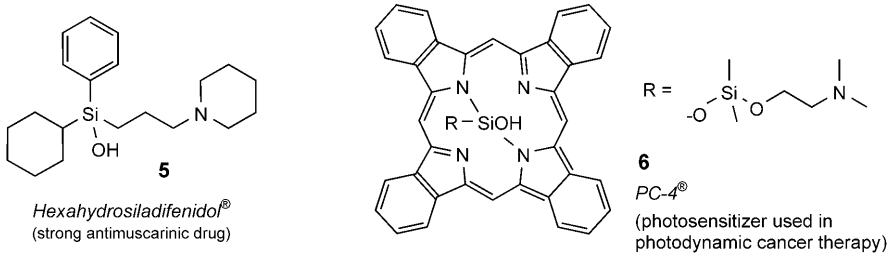

Fig. 2. *Examples of 'sila drugs'*

However, there are no organosilicon odorants on the market at present. Previous data on Si odorants have been reported mainly by the group of *Wannagat* and others [8–11]. They investigated the *floral-smelling* compounds **7**–**13**, musks such as **14**, and a few terpene analogs such as **15**–**17** (*Fig. 3*), and found that most of these analogs have very similar olfactory properties both in quality and intensity. The best example is, perhaps, dimethyl(phenylmethyl)silanol (**9**), for which was reported that 60 panelists out of 100 were not able to distinguish it from the parent C analog.

Recently, *Tacke et al.* prepared the Si analog **18** of *Majantol*® (*Fig. 4*) and found it *less intensive and not as characteristic as Majantol*® (**19**) [10]. They also prepared both enantiomers of **20**, a hybrid structure of *Majantol*® (**19**) and linalool (**21**) or its Si switch **22** [11]. While the *leavo* forms of compounds **20** and **22** are similar, but weak, the *dextrorotary* silanol (+)-**22** appeared to be totally different, without any floral tonality. It was described as *strong, mushroom-like, resembling octen-3-ol* (**23**).

Wannagat and co-workers obtained during the synthesis of the Si analog of ionone the acetylenic derivative **24** with an e*xtremely strong and diffusive violet odor accompanied by woody notes* [9]. Recently, a Chinese group also systematically investigated the odor of derivatives of 3- and 4-(Me_3Si)-substituted cyclohexanols [12], which are *mainly woody* in smell. For instance, the alcohol **25** possesses a *very persistent, radiant sweet-woody smell* [13]. These results encouraged us to investigate the field of sila odorants in a more-systematic way.

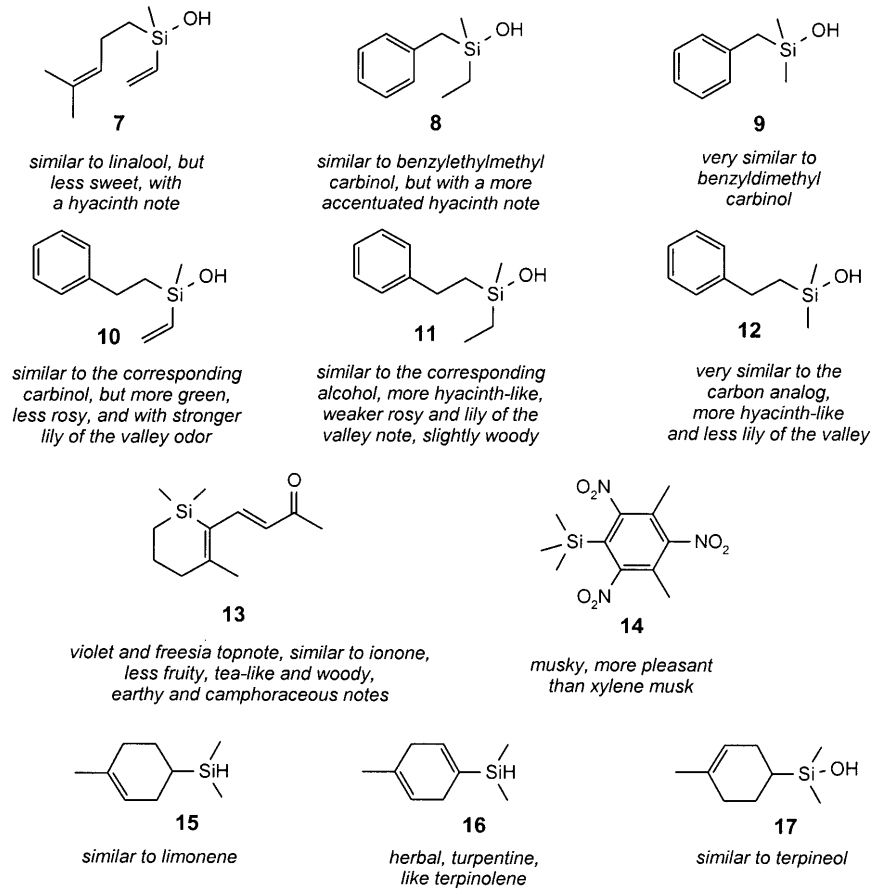

Fig. 3. *Sila odorants with organoleptic properties similar to those of their parent carbon counterparts*

Results. – While working on the asymmetric synthesis of amino acid derivatives of type **26**, using the SAMP-hydrazones[1]) **27**–**29**, we found that, upon purification by silica-gel chromatography, one of the starting materials, the silylated hydrazone **28**, furnished traces of *strong, woody-floral smelling* products, recalling us of *Koavone*® (**30**). We, therefore, presumed that the active principle of the obtained mixture might be the structurally related [dimethyl(thexyl)silyl]acetaldehyde (**31**)[2]), formed in small amounts by acidic hydrolysis of **28**. To verify this, hydrazone **28** was ozonolyzed, and the desired aldehyde **31** was isolated in 80% yield (*Scheme 1*).

Indeed, **31** turned out to be a strong odorant, described by perfumers as *sweet woody, floral and somewhat cardboard-like.* As this compound is quite flexible, different conformations could partially mimic the structure of known woody, ambery

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[1]) SAMP = (*S*)-1-Amino-2-(methoxymethyl)pyrrolidine.

[2]) Thexyl = 1,1,2-trimethylpropyl.

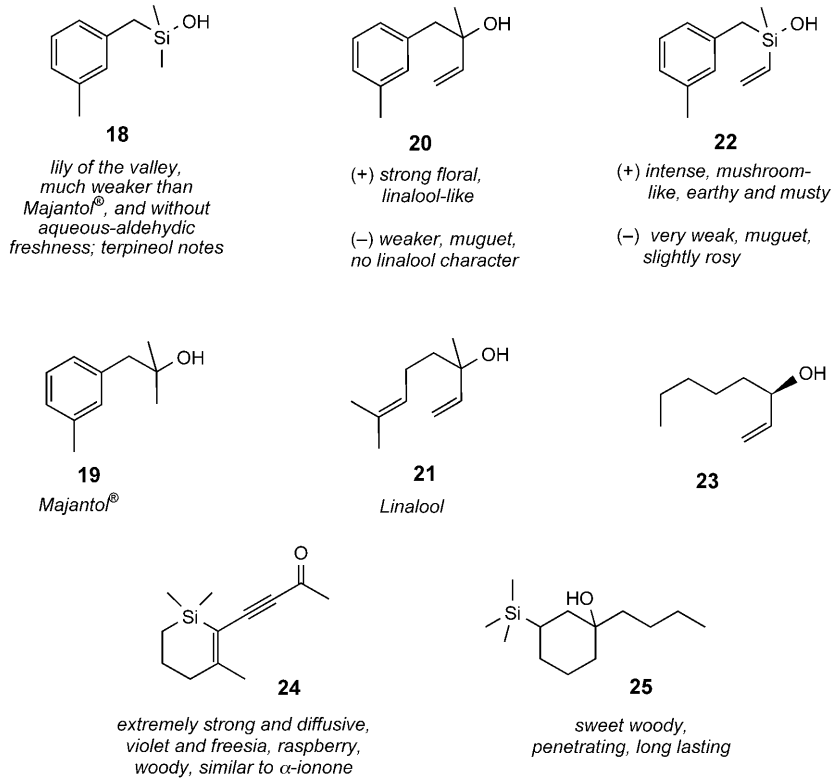
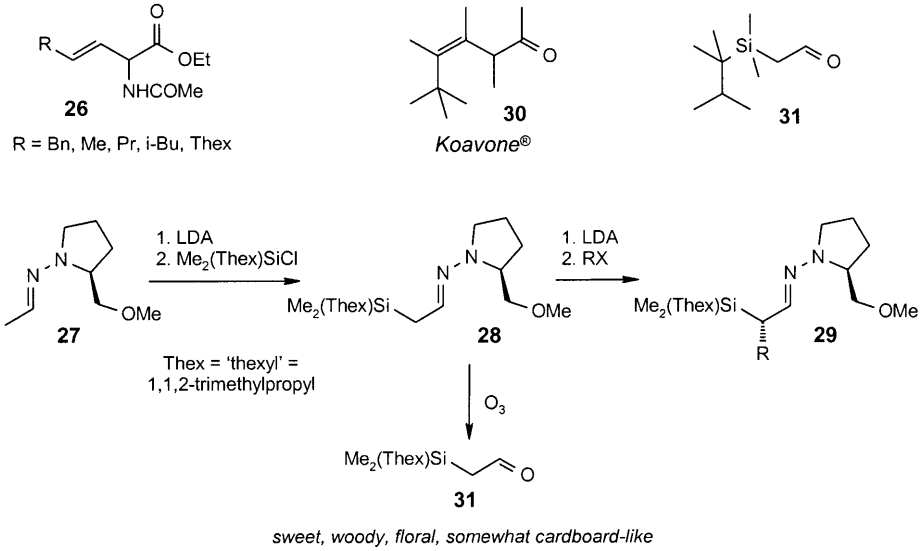


Fig. 4. *Differences between C and Si odorants.* Compounds **24** and **25** are outstanding organosilicon odorants.

Scheme 1. *Discovery of Novel Si-Derived Odorants and Synthesis of* **31**



and floral odorants such as **30** and **32**–**36** (*Fig. 5*). These compounds possess additional substituents in α-position to the C=O group and are often ketones. Therefore, we decided to prepare a few homologs of the parent structure **31** to study their olfactory properties. In a few simple steps shown in *Scheme 2*, we prepared the seven new compounds **48**–**54**, starting from the *N*,*N*-dimethylhydrazones **37**–**40**, *via* the hydrazones **41**–**44** and **45**–**47**.

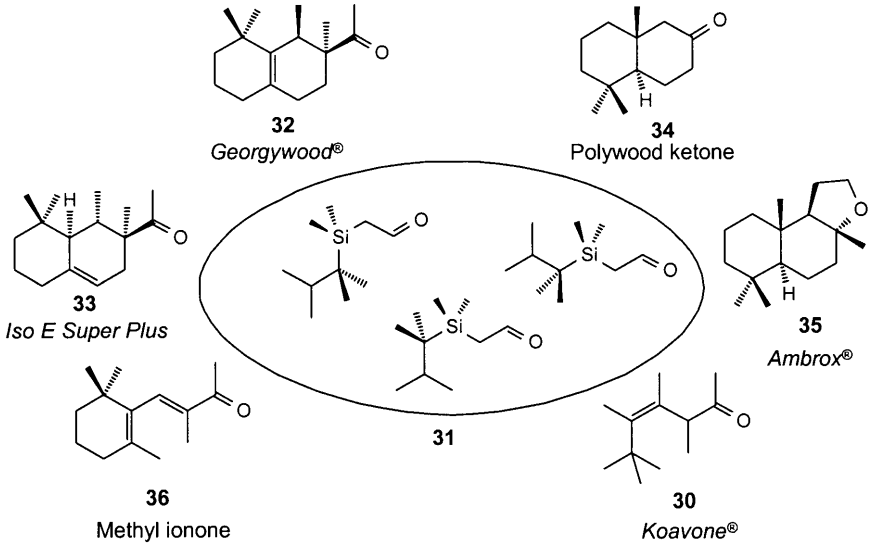
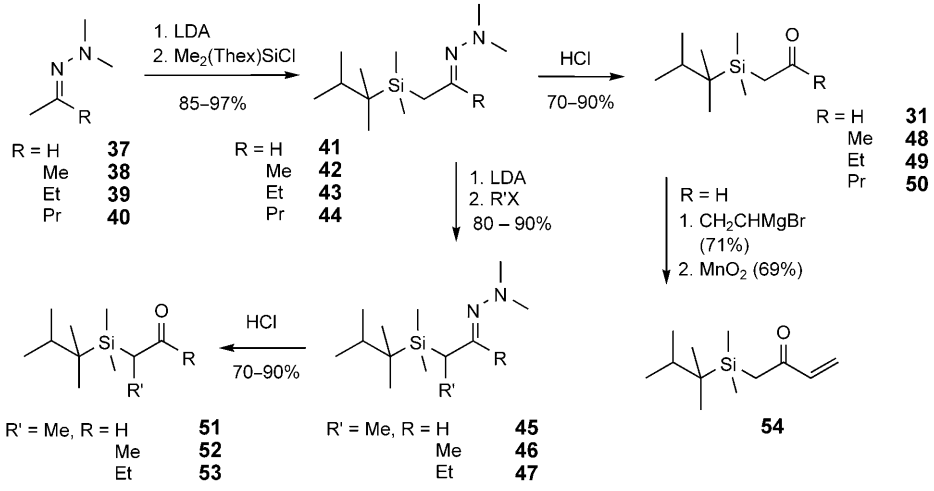


Fig. 5. *Resemblance between the silylated acetaldehyde* (**31**) *and selected woody, ambery, and floral odorants*

Scheme 2. *Synthesis of α-Silylated Aldehydes and Ketones*



Interestingly, the dimethyl(thexyl)silylpropanal **51** appeared to be much stronger than the parent aldehyde **31**, with an additional ambery note, and was highly appreciated by the evaluating perfumer. Its odor threshold was determined to be *ca.* 10 ng/l air. The conformation of **51** overlaps well with the essential structural elements of the amber odorants **34** and **35** (*Fig. 6*).

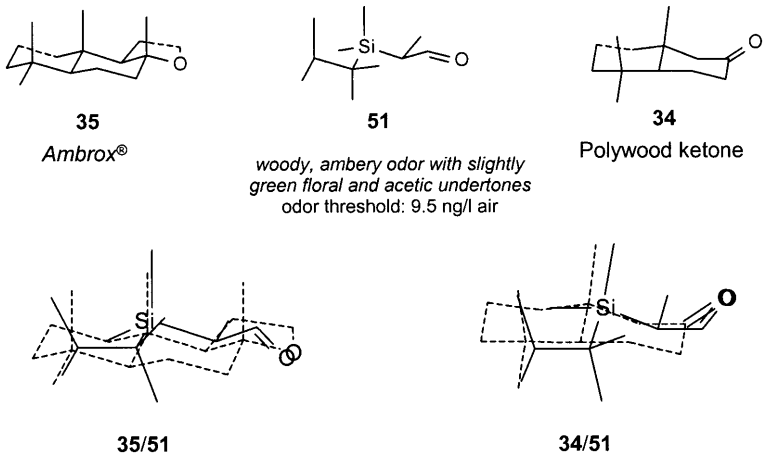


Fig. 6. *Resemblance of the sila odorant* **51** *to compounds with ambery odors*

For 3-[dimethyl(thexyl)silyl] butanone (**52**), a four times lower odor threshold of 2.5 ng/l air was measured. This *woody-fruity* smelling compound can be considered a very simple *seco*-structure of the powerful woody odorants **33** and *Georgywood*® (**32**). The conformation of **52**, shown in *Fig. 7*, overlaps well with essential structural elements of these woody odorants. Both enantiomers of **52** were prepared *via* SAMP/RAMP-hydrazone methodology (*Scheme 3*) [3]. The enantiomer (*S*)-**52** was obtained with $>98\%$ ee (enantiomeric excess) in three steps by silylation of (*S*)-**55**, methylation of the resulting silyl derivative **56**, and subsequent ozonolysis of **57**. The antipode (*R*)-**52** was obtained analogously in $>95\%$ ee, starting from (*R*)-**55**. (*S*)-**52** was found to be *much stronger, fruity, floral, ambery, and woody*, while its antipode (*R*)-**52** was described as being *slightly woody, technical, acidic, and food-like*, which confirms our modeling studies (*Fig. 7*).

The properties of the silyl ketones **48**–**50**, **53**, and **54** allow the conclusion that elongation of the side chain lowers the intensity of the odor and reduces the woody notes. Interestingly, the introduction of a C=C bond in compound **54** leads to additional *green* notes (*Fig. 8*).

With these woody-smelling aldehydes and ketones in hand, we thought of also mimicking ambery-smelling alcohols like α-ambrinol **58** or nordrimanol **59** (*Fig. 9*). Therefore, we reduced the silylated compounds **15**, **48**, and **49** with $NaBH_4$ and obtained the alcohols **60**–**62** in almost quantitative yields. The silyl alcohols **60** and **62** appeared to be strong ambery odorants. Surprisingly, compound **61**, expected to be the strongest in this series, lacks any amber tonality (*Fig. 9*).

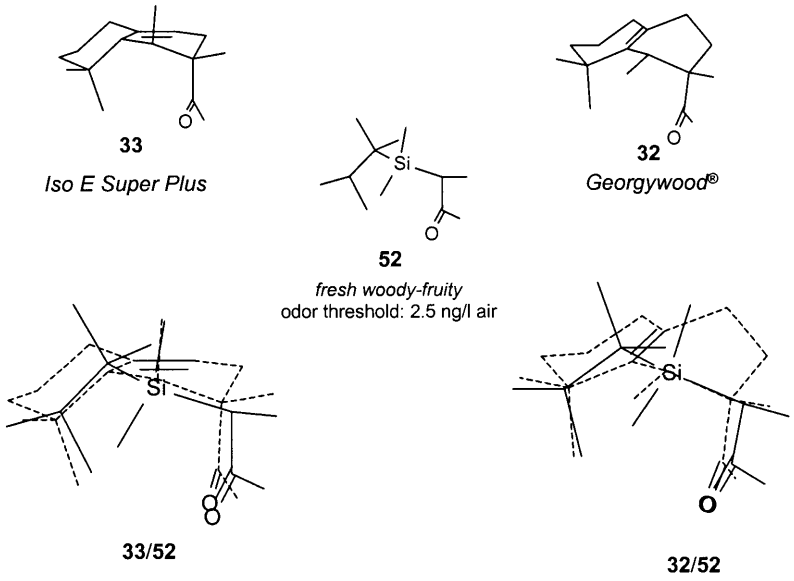
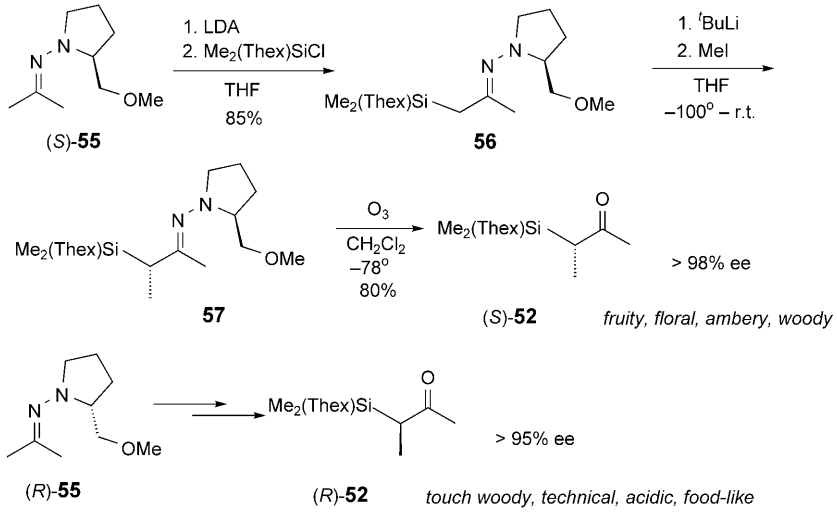
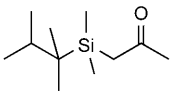


Fig. 7. *Resemblance of the sila odorant* **52** *to known woody odorants*

Scheme 3. *Asymmetric Synthesis of 3-[Dimethyl(thexyl)silyl]butanone*[2]) (**52**)

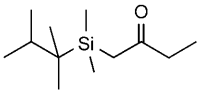


As the discovery of this new class of woody-ambery odorants was serendipitous, we wanted to test whether new odorants could be designed rationally on the basis of known structure–activity relationships. Sandalwood odorants are highly branched compounds, and their structural requirements have been thoroughly investigated (for reviews, see, *e.g.*, [14]). The most-appealing model to us was the superstructure **63**



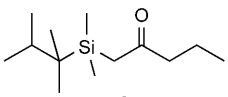
48

earthy, woody, floral, slightly minty and green, somewhat reminiscent of patchouli oil, but more earthy



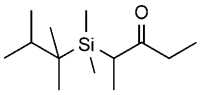
49

green, acidic odor with a smokey cedarwood note



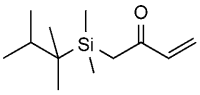
50

fruity, citrus and some facetes of methyl ionone



53

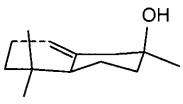
acidic buttery-fatty, floral, spicy, reminiscent of p-tertbutylcyclohexylacetate



54

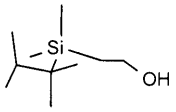
pleasant, green-watery and somewhat fruity note reminiscent of Undecavertol® and violet leaves oil, a woody-cardboard character

Fig. 8. *Organoleptic properties of silyl ketones* **48**–**50**, **53**, *and* **54**

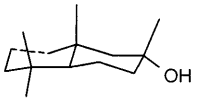


58

α-Ambrinol

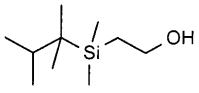


60



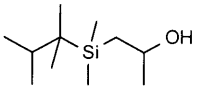
59

Nordrimanol



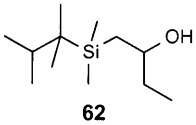
60

nice, relatively powerful woody-ambery odor, with earthy-camphoraceous nuances and a creamy side



61

fruity in the direction of red fruits (raspberry), but rather weak



62

woody-ambery, rich, floral, muguet, creamy

Fig. 9. *Ambery alcohols and their Si analogs*

devised by *Chapuis* and co-workers at *Firmenich* (*Fig. 10*) [15]. By simple removal of bonds, the model allows one to construct most *sandalwood-like smelling* compounds, *e.g.*, **64**–**66**. In addition, it rationalizes why opposite enantiomers of the same racemic compound **65** reveal similar organoleptic properties. We do not know whether this model was known to *Givaudan* researchers when *Bajgrowicz et al.* [16] discovered *Javanol*® (**66**), but it predicts this most-powerful sandalwood odorant to be active.

We wondered whether a C-atom in essential position of this model could be replaced with Si (*Fig. 10*). To test the resulting isosteric model **67**, we decided to prepare the simple allyl silane **68** (*Fig. 10*). However, we were aware that it would probably not be a strong sandalwood odorant, because it lacks electron-rich features like a C=C bond, an O-atom, or a cyclopropyl ring in the position indicated by an

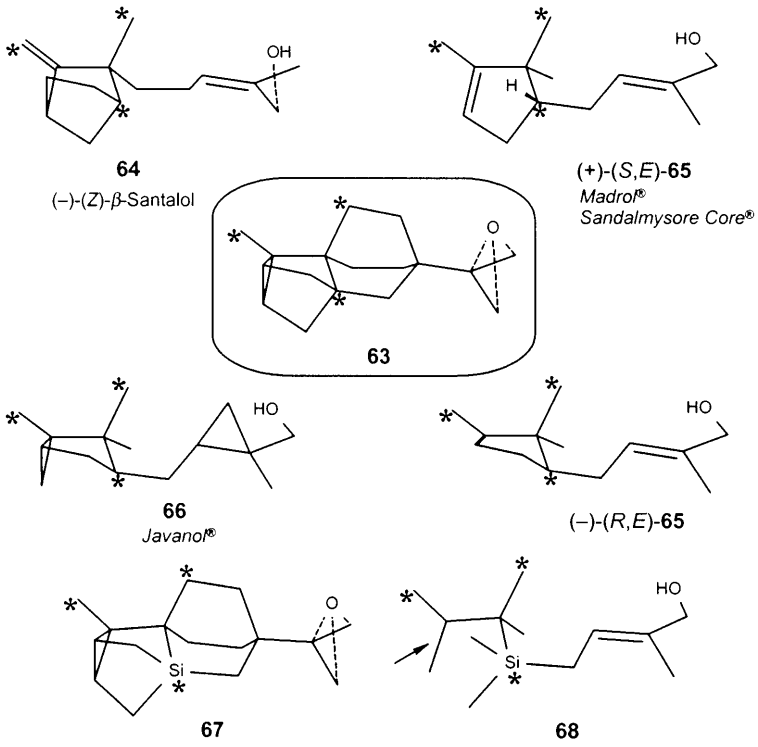
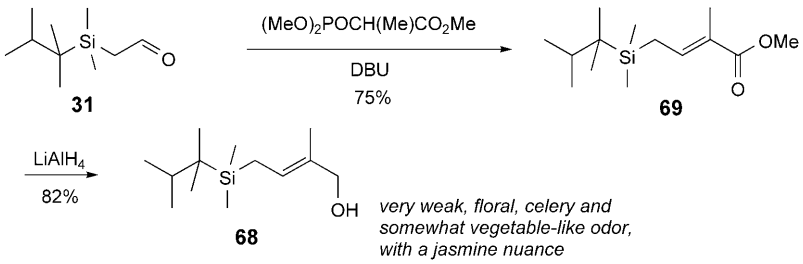


Fig. 10. *Model of sandalwood odorants by* Chapuis *and co-workers* [15] *and derived sila analogs*

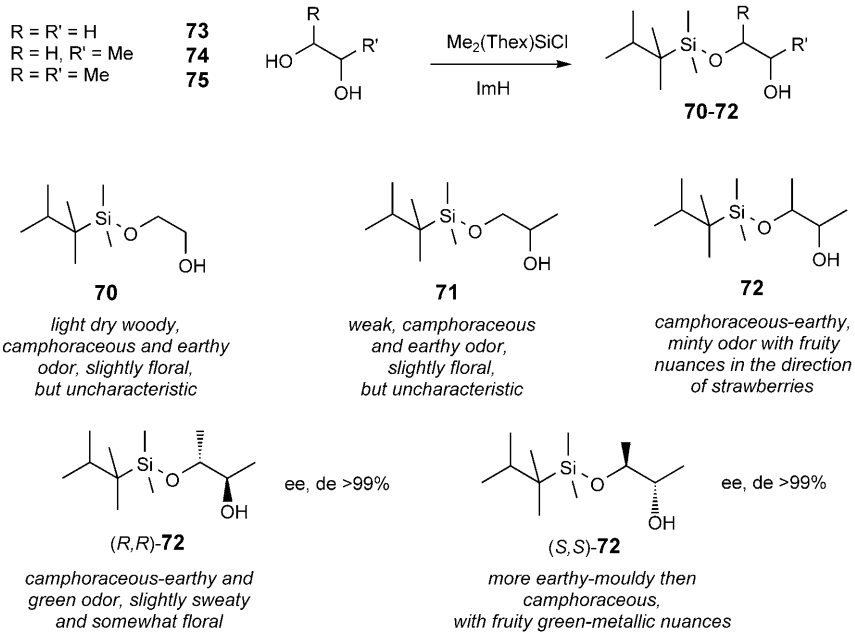
arrow, which have been shown to intensify the sandalwood-odor impression. The designed alcohol **68** was prepared in two simple steps from our parent aldehyde **31**, *via Horner* olefination and subsequent reduction of the formed ester **69** (*Scheme 4*). However, **68** was not sandalwood-like in smell, but was described by perfumers as *very weak, floral, celery, and somewhat vegetable-like with a jasmine touch.*

Scheme 4. *Synthesis of the Silylated (*E*)-But-2-enol* **68**



We were also curious about the organoleptic properties of compounds **70**–**72** possessing a shorter side chain and one C-atom replaced by an O-atom, which quite often has no significant influence on the odor quality. These compounds were prepared

Scheme 5. *Synthesis and Odors of α-Silyloxy Alcohols* **70**–**72**

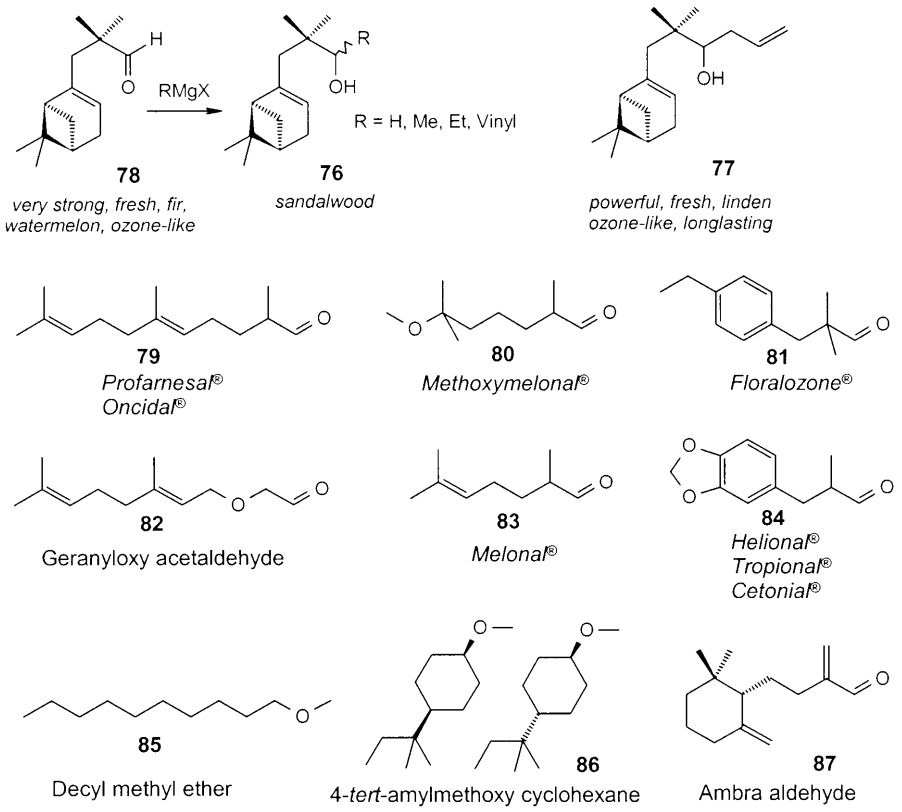


by silylation of the respective diols **73**–**75** (*Scheme 5*); however, again, the products lack the desired sandalwood tonality.

Another interesting group of fragrant substances are those with *ozone-like* odors, although these are not well documented in the chemical literature. During work related to sandalwood chemicals with the pinane skeleton **76** (*Scheme 6*) [17], *Doszczak* and *Góra* prepared the homoallylic alcohol **77**, which emanates a *powerful ozone-like smell with linden blossom facets*. One intermediate used in these syntheses, mirtenyl isobutyraldehyde (**78**) has a *very strong fresh fir, watermelon smell with ozone-like notes*, which prompted us to investigate structure–activity relationships for this group of compounds.

Ozone-like smelling substances such as **79**–**87** represent a structurally diverse group of compounds (*Scheme 6*). Most of them are aldehydes, even straight-chain aldehydes. At first glance, they differ significantly, and, due to their conformational flexibility, they are quite hard to compare. An obvious question is why these relatively large compounds smell like the very small ozone (O_3) molecule. Eventually, O_3 does not interact with odorant receptors directly, but causes oxidation (ozonolysis) of lipids in the mucus layer or the nasal cavity. Traces of the corresponding aldehydes formed may then trigger the respective receptors. This could explain why some other oxidants give similar impressions. The family of ozone-like odorants is structurally and organoleptically closely related to marine and watery (*i.e.*, melon and cucumber-like) odorants such as **88**–**93** (*Fig. 11*). Many, but not all of them, can be aligned on the almost planar *Calone*® (**94**), which is reminiscent of the typical sea-breeze odor, or on compounds **95**–**98**, products of algae contribution to sea-shore impression [18][19].

Scheme 6. *Diversity of Ozone-like Odorants and Synthesis of* **76**



However, most of the known ozone-like smelling compounds can be superimposed on the rigid, powerful, green-ozonic smelling *Maceal*® (**99**), for instance compounds **78**–**87** and **100**–**103** (*Fig. 12*).

So far, the limited data on the absolute configuration of the most-powerful enantiomers did not allow constructing a stereochemically defined model. However, the absolute configuration of the sea-breeze-like smelling, so called *ambra aldehyde* (**87**) is known [20], and, recently, we have prepared both enantiomers of *Helional*® (**84**) [21] (*Scheme 7*; for a related asymmetric synthesis of the perfumery raw material *Lilial*®, see [22]). Alkylation of the hydrazone (*S*)-**104** with piperonyl bromide (**105**), subsequent cleavage of the resulting hydrazone **106** to the nitrile **107**, followed by DIBAL-reduction, furnished (*S*)-*Helional*® ((*S*)-**84**) with 90% ee. The same sequence of reactions, but starting from (*R*)-**104**, provided (*R*)-*Helional*® ((*R*)-**84**) with similar enantiomeric excess. It was shown that only (*S*)-**84** possesses the characteristic *ozone-like* note, while both enantiomers share *floral, aldehydic aspects.*

On the basis of these data, and encouraged by the practical model of *Chapuis* and co-workers [15], we propose **108** as a model for ozone-like odorants (*Scheme 8*). This is a simplified superposition of chosen conformations of ozone-like-smelling compounds.

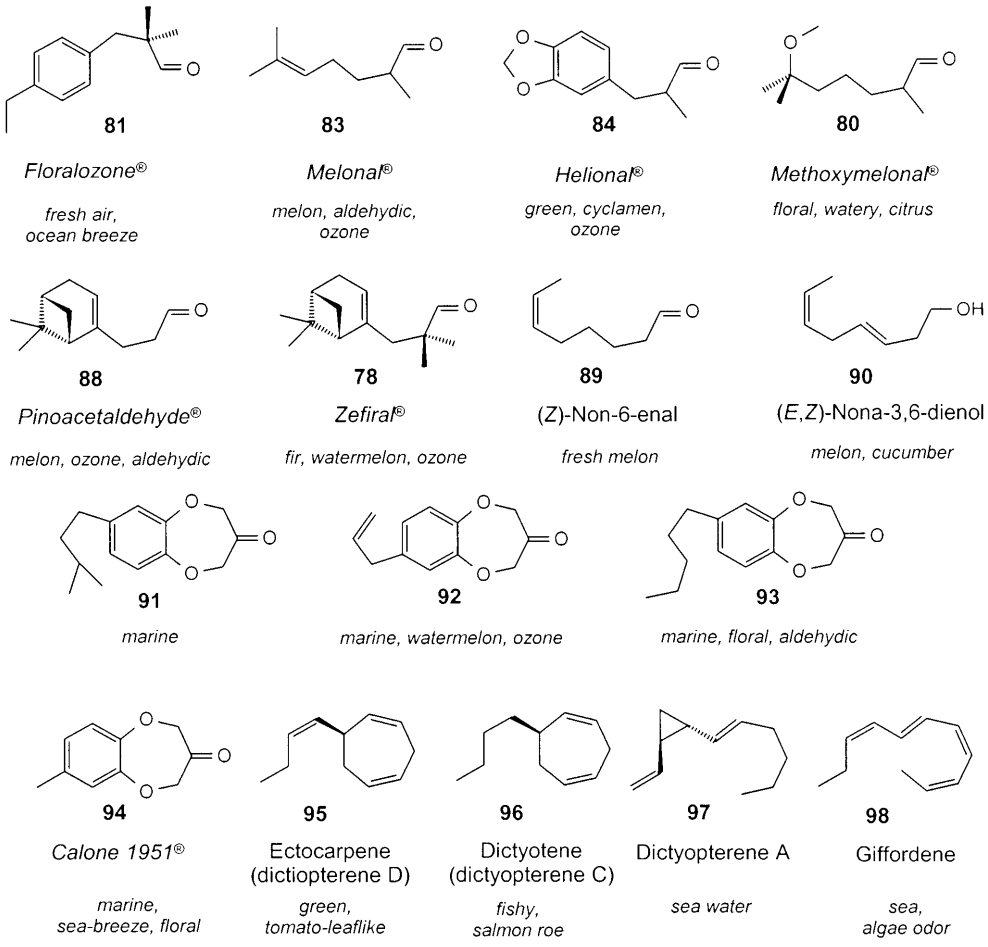


Fig. 11. *Common structural features of watery, marine, and ozone-like odorants*

It allows deriving the structures of numerous known ozone-like smelling compounds by removal or occasional addition of bonds. Like for the sandalwood model, we were also curious whether a bridgehead C-atom in **108** could be replaced with a bulky Si-atom bearing additional Me groups. To access the isosteric model **109**, we decided to prepare the simple structure **110**. Oxidation of the monosilylated glycol **70** with pyridinium chlorochromate (PCC) furnished the expected [dimethyl(thexyl)silyl]oxy acetaldehyde **110**, which was described by the evaluating perfumer as a *strong, fresh minty, green-fruity smelling* substance, however, *without ozone-like character*. At present, we are working on the evaluation of the predictive abilities of the presented models.

Conclusions. – We have discovered a new class of woody and ambery sila odorants, which can be obtained in a few simple steps from commercially available reagents, and demonstrated potential application of organosilicon compounds in fragrance chemistry.

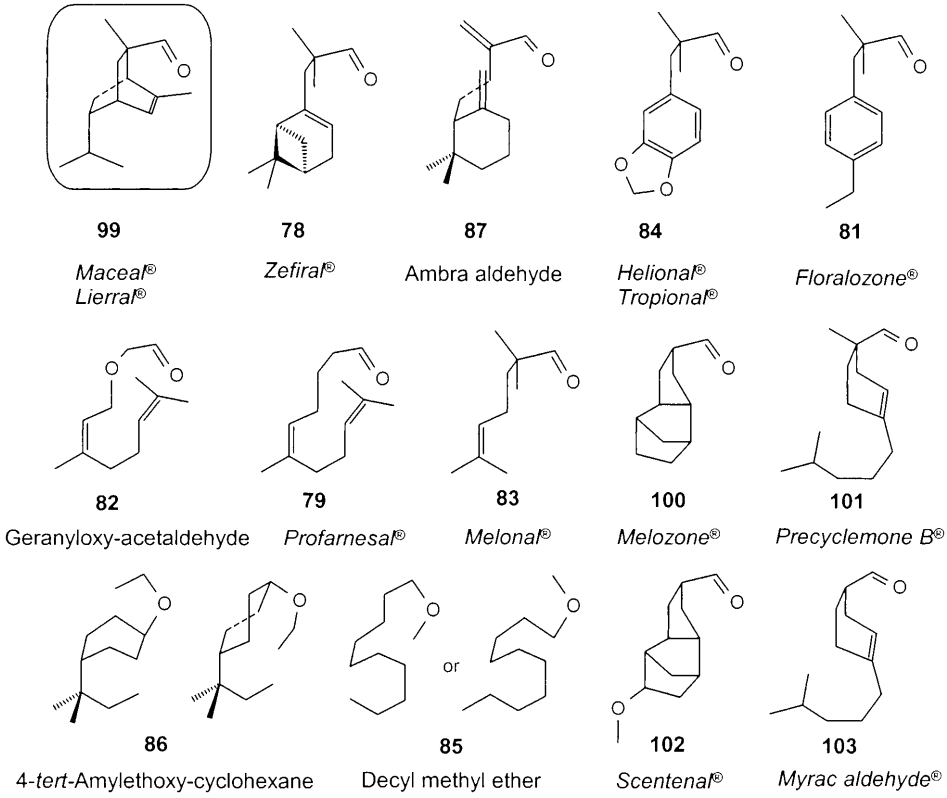
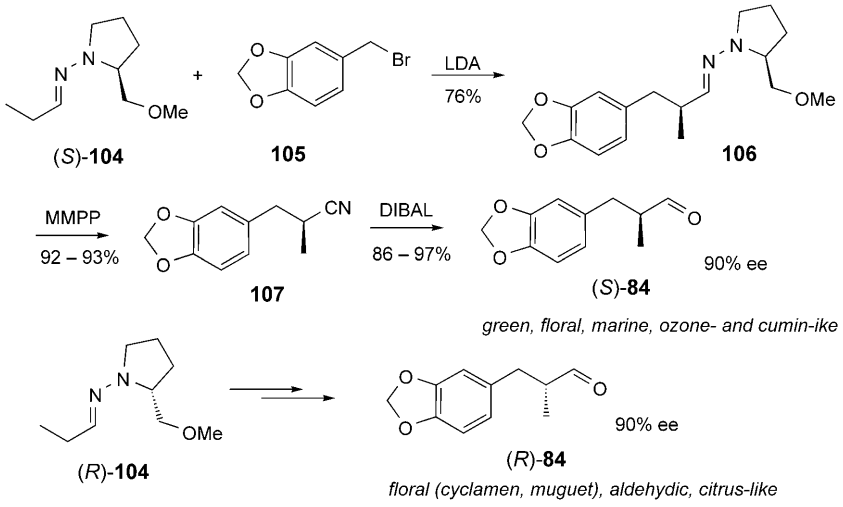
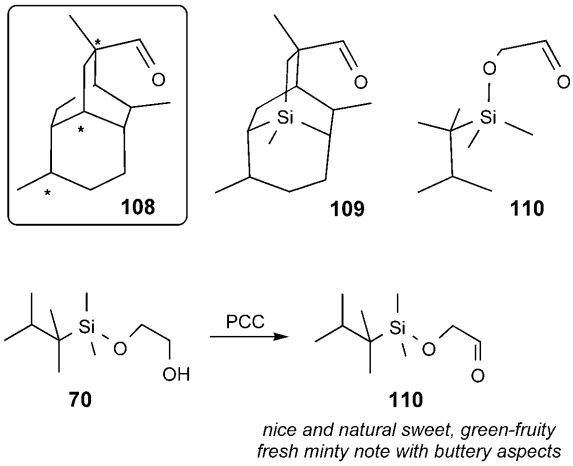


Fig. 12. *Structural similarities of odorants having ozone-like notes*

Scheme 7. *Asymmetric Synthesis of* Helional® (**84**) [20]



Scheme 8. *Model for Ozone-Like Odorants and Their Si Analogs, and Synthesis of* **110** *from* **70**



We have also designed a simplified general model for ozone-like odorants, combining common structural features of different ozone-like smelling compounds. Together with a similar model for sandalwood odorants [15], this was employed in the design of new sila-odorants. Though the desired ozone- and sandalwood notes were not yet attained with the designed sila odorants, our work provides interesting data for research on structure–odor relationships, and may encourage the fragrance industry to undertake more-systematic research on organosilicon odorants.

This work was supported by the *Fonds der Chemischen Industrie* and a *Roman Herzog fellowship* of the *Hertie* and *Alexander von Humboldt* foundations to *L. D.* We thank *Degussa AG*, *BASF AG*, *Bayer AG*, and *Wacker Chemie* for the donation of chemicals, and would like to express our gratitude to Dr. *Philip Kraft*, senior perfumer *Jean-Jacques Rouge*, and *Givaudan Schweiz AG* for the olfactory evaluation of samples, measurement of odor-threshold values, and helpful discussions.

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New Woody and Ambery Notes from Cedarwood and Turpentine Oil[1])

by **Johannes Panten***, **Heinz-Jürgen Bertram**, and **Horst Surburg**

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The development of a new product in the chemical industry is still driven by needs like technical properties, price/performance ratio, biodegradability, or product safety. However, in terms of improving more and more on ecological criteria, summarized under such catchphrases as *sustainable development* or *green chemistry*, another important aspect is to use renewable resources as starting materials. This is not significantly new in fragrance chemistry, and there are a lot of raw materials in the perfume oils that are derived from molecules of renewable resources. Two commonly used materials are: *longifolene* (from turpentine oil) and *cedrene* (from cedarwood oil). These compounds are very suitable for the synthesis of *woody* and *ambery* notes, and even though it seemed that all possibilities were exhausted, it is actually still feasible to discover new molecules with excellent olfactory properties such as *Ambrocenide*® (**50a**), which is available in three steps from α-cedrene. Some of these molecules will be treated in this review, both with respect to synthesis as well as structural and sensory aspects.

Introduction. – In the chemical industry, many companies have introduced rules and programs under the generic term *responsible care*. All efforts towards *green chemistry* can be seen as an important step in this direction. One of the mental fathers of *green chemistry*, *Paul Anastas*, has defined this term by means of twelve principles [1]. This review focuses on one of these principles and demonstrates how fragrance chemists, involved in the discovery of new odorants could contribute to this subject.

The principle we are referring to is: '*Use renewable resources as raw materials wherever it is possible*'. This is not significantly new in the domain of fragrance chemistry. Perfumery on the whole has its origin in the utilization of natural resources such as essential oils and natural extracts. But, after a triumphant progress in the area of synthetic fragrant substances, natural ingredients are nowadays used only to a relatively small extent, *e.g.*, in fine-fragrance perfumery. However, several natural raw materials consist mainly of hydrocarbons that are not suitable as smelling substances *per se*. These hydrocarbons often possess complex carbon skeletons, which are difficult to synthesize, but offer excellent possibilities for functionalization with oxygen-containing goups – transformations that often lead to interesting new fragrant materials. Some examples of naturally occurring raw materials that are especially useful in the synthesis of *woody* and *ambery* materials are depicted in *Fig. 1*.

Sclareol (**1**) is recovered from clary sage oil (*Salvia sclarea*) by solvent extraction [2]. The same technique is used for the isolation of manool (**2**) from the coniferous tree *Dacrydium biforme* [3]. Although both compounds are already functionalized, they represent the starting materials for two of the most-important ambery notes

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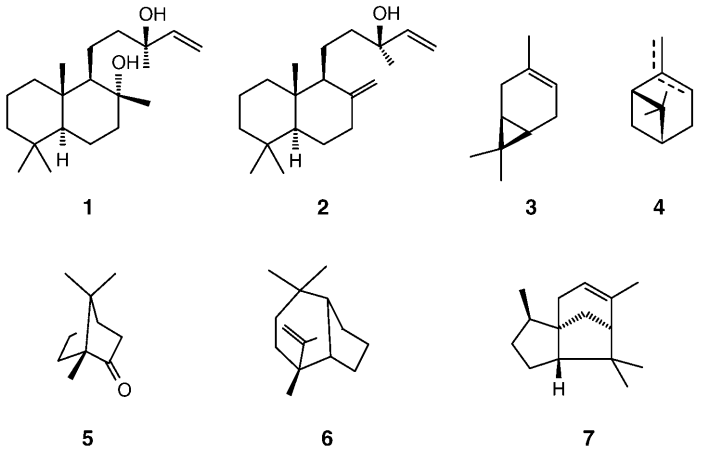


Fig. 1. *Naturally occuring raw materials for the synthesis of fragrances with woody and ambery notes*

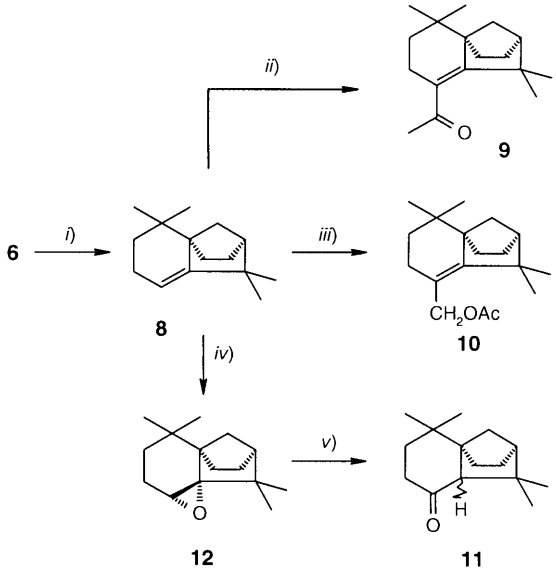
(ambroxide (**52**) and amberketal (**47**); see below). Further examples include the cyclopropyl-monoterpene carene (**3**), a major ingredient of turpentine oil (*Pinus silvestris*; content: 40%), and, from the same essential oil, α- and β-pinene (**4**). These compounds are produced by steam distillation of the plant material, followed by fractional distillation of the resulting oil. Camphor (**5**) is produced from camphor oil (*Ho oil*, content: *ca.* 50%) by crystallization. As the two most-promising starting materials in the search for new woody and ambery odorants, we chose longifolene (**6**), which is obtained from turpentine oil (*Pinus longifolia*; content: 5–10%) [4], and α-cedrene (**7**), which is obtained from cedarwood oil (*Juniperus chinensis*; content: 20–46%) [5]. Woody and ambery notes play a decisive role in modern perfumery. They form the foundation of a lot of perfumes, and it is difficult to imagine a perfume without any woody or ambery notes.

Fragrance Raw Materials from Longifolene. – The synthesis of nearly all fragrant substances derived from longifolene (**6**) commences with an acid-catalyzed rearrangement of **6** to isolongifolene (**8**), *Brønsted* and *Lewis* acids working equally well (*Scheme 1*).

A simple derivatization of the first-generation product **8** is, *e.g.*, the acetylation to acetylisolongifolene (**9**; *Capinone*®), which is described as *woody* (*Scheme 1*). Chemists from *Bush, Boake & Allen* investigated the *Prins* reaction of **8** with formaldehyde already in the 1970s [6], and commercialized **10**, which possesses *ambery, cedarwood-* and *vetiver-like aspects*, as *Amborylacetate*®. Vetiver oil is also a natural product of the woody family, though it is not obtained from wood, but from the roots of the grass *Vetiveria zizanoides.*

The most-important smelling substance from isolongifolene (**8**) up to the mid 1990s was isolongifolanone (**11**), which is synthesized by oxidation with a peracid, providing epoxide **12** (*Scheme 1*), which was commercialized under the name of *Folenox*®. The latter can easily be converted to **11** (*Piconia*®) in the presence of a *Lewis* acid, is being produced in hundreds of tons annually, and possesses a *pleasant warm woody smell* [7].

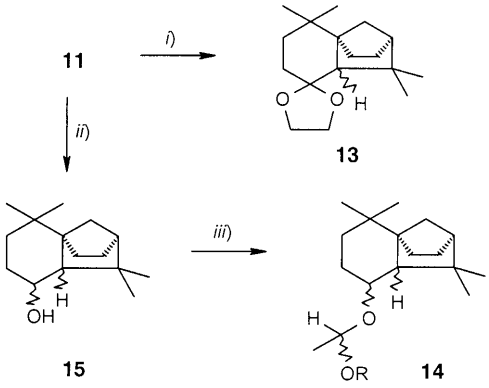
Scheme 1. *First-Generation Products from Longifolene* (**6**; see also *Scheme* 4)



i) H^+ or *Lewis* acid. *ii*) Ac_2O, *Lewis* acid. *iii*) CH_2O, H^+. *iv*) Peracid. *v*) *Lewis* acid.

Isolongifolanone (**11**) was the starting point of our research activities in the early 1990s. To improve the performance, we systematically synthesized acetals of **11**, and, at last, discovered *Ysamber K*® (**13**), the ethyleneglycol acetal of (**11**) (*Scheme 2*) [8].

Scheme 2. *Acetals Derived from Isolongifolanone* (**11**)



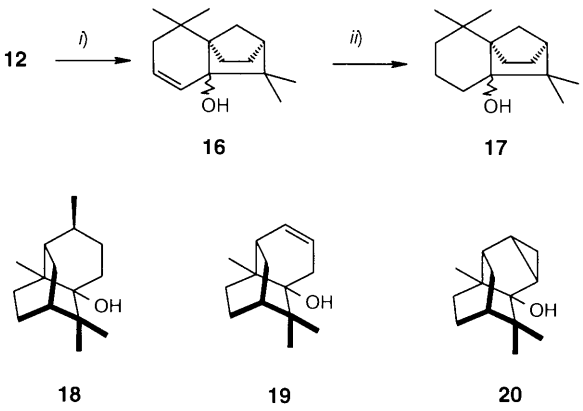
i) $HO(CH_2)_2OH$, H^+. *ii*) $NaBH_4$, NaOH. *iii*) $MeHC(OR)_2$, H^+ (R=Me, Et).

Ysamber K® (**13**) has a powerful woody smell, and combines harmoniously *ambery* and *woody* elements. The importance of the acetal structure is discussed later, but the success of *Ysamber K*® (**13**) inspired us to look for other acetals, and we decided to investigate noncyclic acetals. Therefore, a number of linear formaldehyde- and

acetaldehyde-based acetals **14** were synthesized (*Scheme 2*) [9], which are easily available on $NaBH_4$ reduction of **11** to **15**, followed by transacetalization. The new substances emanated an *interesting woody smell with herbaceous side notes.*

Some years ago, a sudden shortage of patchouli oil, a highly popular natural woody ingredient in perfumery, produced from the dried leaves of *Pogostemon cablin*, drove our attention to isolongifolenol (**16**). It is obtained by reaction of the epoxide **12** with *t*-BuOK (*Scheme 3*) [10].

Scheme 3. *Synthesis of Isolongifolenol* (**16**) *and Its Derivative* **17**. Compounds **18**–**20** are the so-called *patchouli alcohols.*



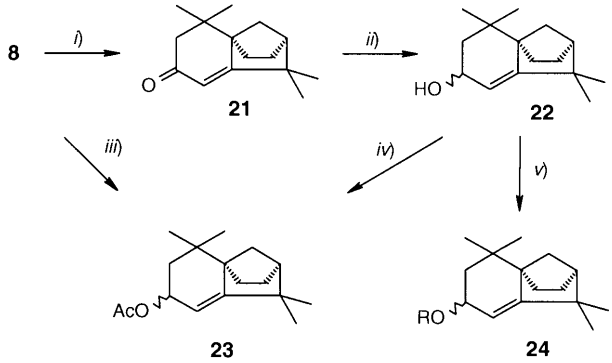
i) *t*-BuOK. *ii*) H_2, catalyst.

The unsaturated tertiary alcohol **16** possesses a *woody smell combined with strong earthy, patchouli-type aspects.* Hydrogenation of the C=C bond afforded the saturated alcohol **17** (*Scheme 3*), which is not so distinctly earthy as **16**. Indeed, both **16** and **17** show similarities to the structures of the alcohols occuring in patchouli oil, which are patchoulol (**18**) [11], *nor*-patchoulenol (**19**) [12], and *nor*-patchoulol (**20**) [13]. In combination with other *patchouli-like* smelling materials, isolongifolenol (**16**) has shown promising results in attempts for the reconstitution of this highly desired oil in perfumery.

The synthesis of another important commercial longifolene-derived first-generation product started with allylic oxidation of isolongifolene (**8**). Subsequent $NaBH_4$ reduction of the resulting ketone **21**, followed by esterification of **22**, yielded isolongifolyl acetate (**23**), (*Scheme 4*). The latter possesses a *woody smell with vetiver-like nuances*, and is reported to combine well with vetiveryl acetate, a material synthesized from vetiver oil. Researchers from *Bush, Boake & Allen* published a one-step synthesis of this compound in the early 1970s, employing AcOH, CuCl, and di(*tert*-butyl) peroxide [14].

We used the unsaturated isolongifolenol (**22**) to synthesize ethers like **24**, because the ether function is another frequently occurring structural element in woody and ambery materials (*cf.* ambroxide (**52**) below). And, indeed, simple substitution of the ester group with an ether function shifted the scent to an ambery direction and, what really surprised us, with musky side notes [15].

Scheme 4. *First-Generation Products from Isolongifolene* (**8**)



i) AcOH, Cr^{6+}. *ii*) $NaBH_4$. *iii*) O_2, CuCl, $(t\text{-BuO})_2$. *iv*) Ac_2O. *v*) Me_2SO_4, NaOH.

As described above, most products derived from longifolene (**6**) are synthesized *via* isolongifolene (**8**). However, other isomers are also useful for the creation of ambery molecules, as described in reference [16], and according to this publication, the *allo*-isolongifolene ketone **25** does also exhibit an *amber-like* odor (*Fig. 2*).

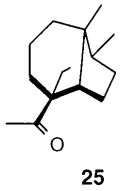
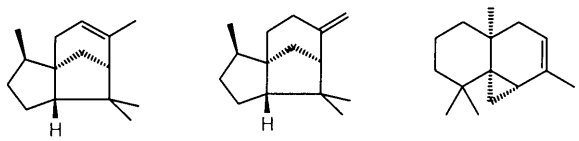


Fig. 2. *Structure of* allo-*isolongifolene ketone, an amber-like odorant*

Fragrance Raw Materials from *α*-Cedrene. – α-Cedrene (**7**) is a major constituent of the sesquiterpene fraction of cedarwood oil. Two other ingredients are β-cedrene (**26**) and thujopsene (**27**) (*Fig. 3*). By far, the commercially most-important cedrene-derived perfumery materials are produced by acylation of the sesquiterpene fraction of cedarwood oil, and are sold under the names of *Lignofix*®, *Vertofix*®, *MCK*®, and *methyl cedryl ketone*, to name just the most-important ones. The industrial syntheses basically make use of acetic anhydride (Ac_2O) and polyphosphoric acid (H_3PO_4) as acetylating agents. Numerous investigations have been carried out to identify the compounds responsible for the *attractive warm and woody smell*. Today, it is generally accepted that the acetylated thujopsene isomer **29** is the principal odorant of the commercial mixture, the formation of which is detailed in *Scheme 5*, according to *Kitchens* and co-workers [17].

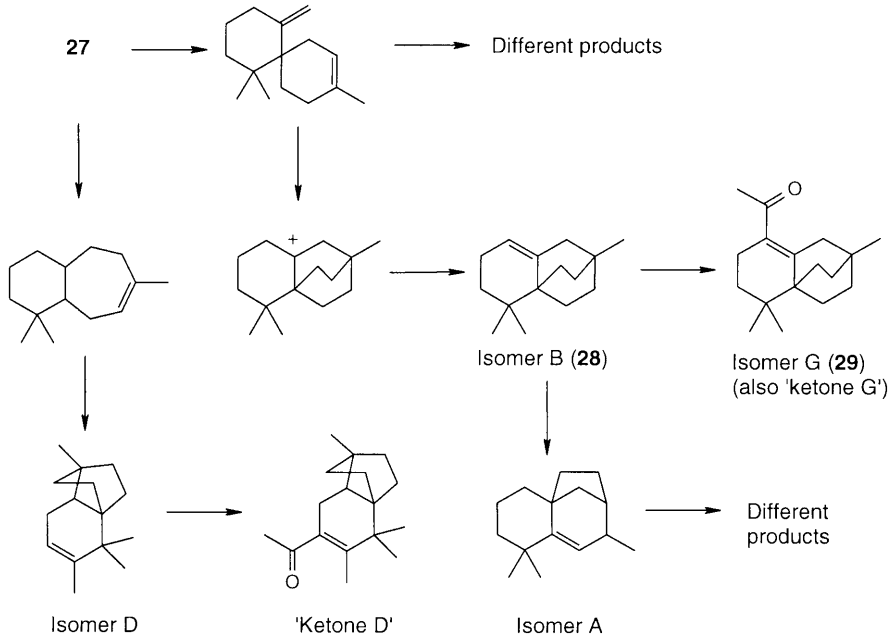
A series of *Wagner–Meerwein* rearrangements results in the unsaturated tricyclic compound **28**, which was coined *isomer B*. Acetylation of **28** then affords *isomer G* (**29**) in a highly unselective reaction, accompanied by numerous other compounds. The acetylation products of α-cedrene (**7**) and β-cedrene (**26**) were also identified in the reaction mixture, but do not have any significant impact on the overall smell of the



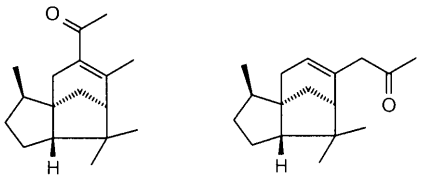
7 **26** **27**

Fig. 3. *Main constituents of the sesquiterpene fraction of cedarwood oil*

Scheme 5. *Acetylation Products of Thujopsene* (**27**)



mixture, as verified by olfactory evaluation of the pure substances. The acetylated α-cedrene **30** possesses a *warm woody smell* [18], while the acetylated β-cedrene **31** is very weak in smell (*Fig. 14*) [19].

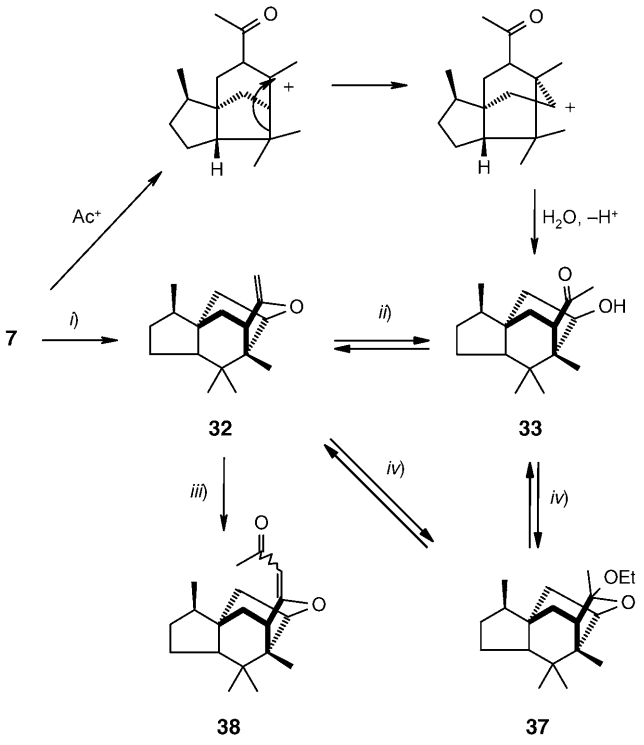


30 **31**

Fig. 4. *Acetylation products of α- and β-cedrene*

Much effort was put into getting a more-definite picture of the acylation products of the sesquiterpene fraction of cedarwood oil. In 1973, *Sell* and co-workers [20] reported on the acetylation of α-cedrene (**7**) in the presence of *Lewis* acids; they isolated and identified a compound of the new and unexpected structure **32**, an enol ether with a tetracyclic skeleton (*Scheme 6*). Upon hydration, the ring was opened, and hydroxy ketone **33** was obtained, which is *nearly odorless*, while **32** has a *weak, cedarwood-like odor.* In the initial step, the C=C bond of **7** is protonated, and the resulting *tertiary* carbocation undergoes a *Wagner–Meerwein* rearrangement prior to nucleophilic attack by a H_2O molecule to afford **33** (*Scheme 6*). This unusual structure has not yet been found in nature, but the cyclic form **32** shows some structural similarity to the unnatural khusimone-like compound **34**, which was reported by *Büchi* [21] in 1978 (*Fig. 5*).

Scheme 6. *Acetylation Products of α-Cedrene* (**7**), *and Mechanism for the Formation of the Unexpected Product* **33**



i) Ac_2O, $TiCl_4$. *ii*) H_2O. *iii*) Ac_2O, $SnCl_4$. *iv*) EtOH.

Khusimone (**35**) and β-vetivone (**36**) are responsible for the typical scent of vetiver oil [22]. When we synthesized the enol ether **32** to study its odor, we noticed that it emanates a *surprisingly strong and woody odor* when dissolved in EtOH. This was due to the acetal **37** (*Scheme 6*), which, indeed, exhibits a *powerful woody scent* [23]. The

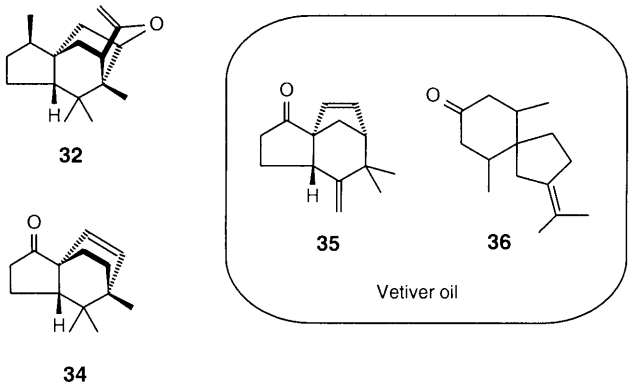


Fig. 5. *Khusimone* (**35**) *and β-vetivone* (**36**), *constituents of vetiver oil, and structural relationship between the khusimone-like compounds* **32** *and* **34**

usage of **37** in perfume formulations is, however, limited owing to its instability to heat and H_2O. Yet, in the course of our investigations on the acylation of α-cedrene (**7**), we isolated some other compounds with interesting structures such as the vinylogous ester **38**, which is formed by acylation of **32** (*Scheme 6*), but possesses only a weak odor [24].

As in the case of isolongifolene, other commercial cedrene-derived first-generation products include the epoxide **39**, the *tertiary* alcohol **40**, the acetate **41**, and the very popular methyl ether **42**, which was commercialized as *Cedramber*® (*Fig. 6*). Their industrial syntheses are commonly known and, thus, not detailed here.

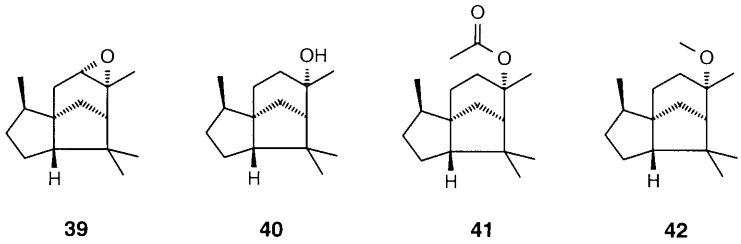


Fig. 6. *Structures of commercial cedrene-derived products*

Woody and ambery molecules have especially challenged fragrance chemists to investigate their structure–activity relationship (SAR). The main reason is probably that these molecules show great structural diversity and possess relatively similar scents. Until the beginning of the 1990s, the two most-important theories in this domain were *Ohloff*'s *triaxial rule* [25] and *Vlad*'s *ambergris triangle* [26]. The *triaxial rule* describes the relative position of three Me groups in a *trans*-decalin system. These should be in an *axial* position if the molecule smells ambery. The *ambergris triangle* specifies a range of distances of an O-atom to two adjacent H-atoms for an amber odorant.

In the mid 1980s, acetals became popular structural elements in the synthesis of woody and ambery odorants. Some examples, delineated in *Fig. 7*, include *Spirambrene*® (**43**), which is synthesized from carene (**3**) [27], *Karanal*® (**44**) [28], *Okoumal*®

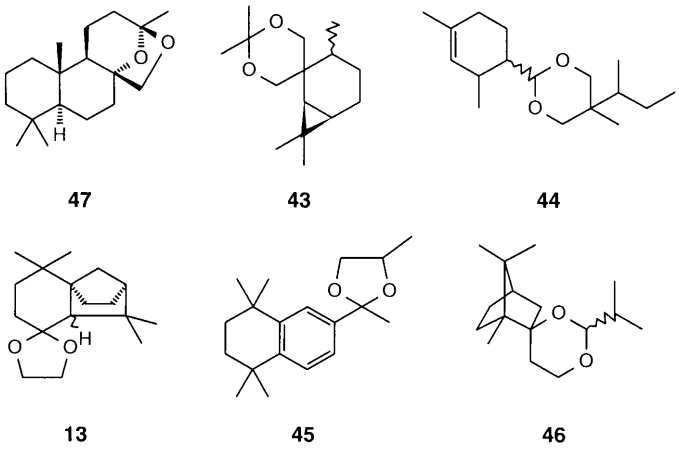
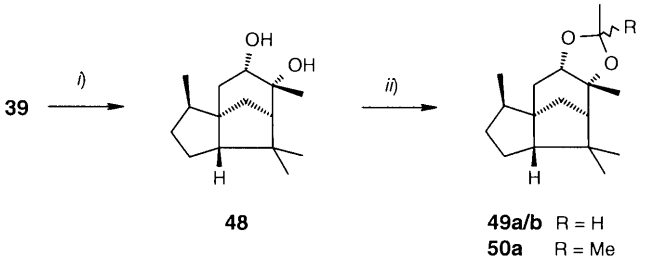


Fig. 7. *Structures of woody- and ambery-smelling, acetal-based molecules*

(**45**) [29], *Ysamber K*® (**13**), and *Belambre*® (**46**), which is synthesized from camphor (**5**) [30]. Known for a long time is, in addition, amberketal (**47**) [31], which is also synthesized from a renewable source, namely manool (**2**).

With this in mind, we decided to synthesize acetals possessing an α-cedrene (**7**) skeleton. Starting material was the epoxide **39**, and, after ring opening of **39** to the diol **48**, we first obtained the acetaldehyde acetal **49a/b**, which, indeed, possesses a *woody, ambery smell*, with an odor threshold of 50 ppb in H_2O (*Scheme 7*). The odor threshold of a new substance is a very interesting and important figure in addition to the sensory properties. The real breakthrough was, however, the synthesis of the corresponding acetone acetal, which led to the new captive *Ambrocenide*® (**50a**) [32]. The olfactory properties of **50a** differ not so significantly from that of **49a/b**, but the odor threshold, 0.06 ppb in H_2O, is extremely low. The molecular weight of **50a** (278 g/mol) is very high compared to other fragrant chemicals, and very rarely do so large molecules possess such a low odor threshold, making **50a** very substantive in applications. Careful structural analysis confirmed that the diol moiety of **50a** is *syn*-configured, as shown in the calculated structure [33] depicted in *Fig. 8*.

Scheme 7. *Synthesis of Acetals with α-Cedrene Skeleton*



i) NaOH. *ii*) $RCH(OMe)_2$ (R=H, Me).

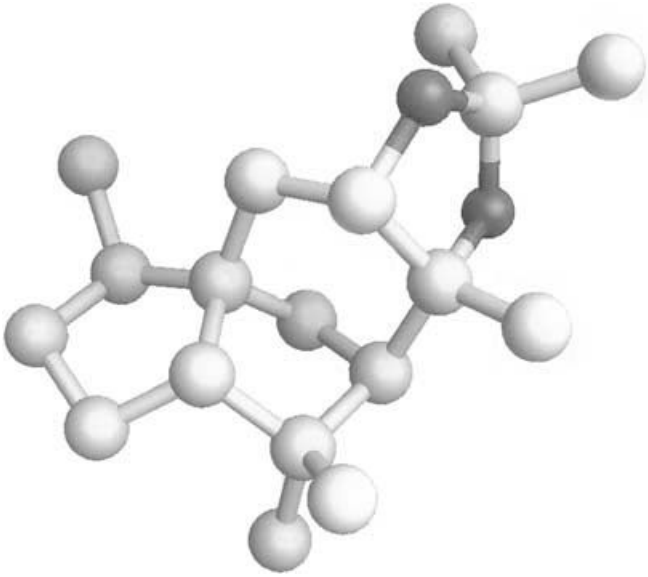


Fig. 8. *'Ball-and-stick' representation of* Ambrocenide® (**50a**). Molecular structures and relative energies were calculated by means of the MAB all-atom force field without additional constraints, as implemented in MOLOC. Due to the rigid system of fused rings, only a few conformers had to be taken into account. Interatomic distances *d* are given in Å for the conformer of the lowest internal energy.

To further improve the odor threshold and, thus, the strength of *Ambrocenide*® (**50a**), we systematically varied its structure (*Fig. 9*). But neither the stereoisomers nor the *nor*-analogs did possess lower thresholds than the parent compound **50a**. In an additional attempt, we wanted to employ molecular-modeling techniques. Recently, three new theories were published on the structure–odor correlation of ambery

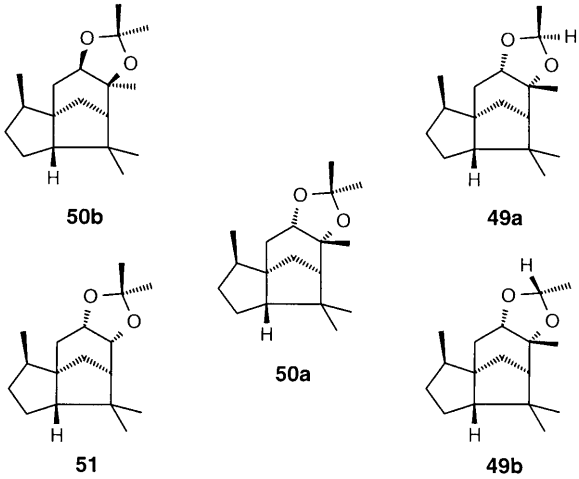


Fig. 9. *Structural variations of* Ambrocenide® (**50a**)

odorants: *1*) *Gorbachov* and *Rossiter* [34] defined an '*active amber-fragment, which consists of one oxygen and three carbon atoms which show certain atom partial charges in characteristic distances to each other*'; *2*) *Bajgrowicz* and *Frank* [35] introduced '*a hypothesis generated from a special software, which is composed of one oriented hydrogen-bond-acceptor (HBA) function and four hydrophobic function*'; and *3*) *Buchbauer* and co-workers [36] followed '*a combinatorial QSAR-approach, which considers all possible independant models and different descriptor collection*'.

In our own SAR calculations [33], we followed the general approach of *Gorbachov* and *Rossiter* [34]. We first determined the distances of the designated atoms in the *Ambroxide*® (**52**) molecule (*Fig. 10*), and then calculated the distances of the respective atoms in the hypothetical, THF-fused molecules **53** and **54** (*Fig. 11*). The interatomic distances of **53** and **54** were compared to those of **52**. Thereby, the data for **54** corresponded better with those of *Ambroxide*® (**52**) (*Table*).

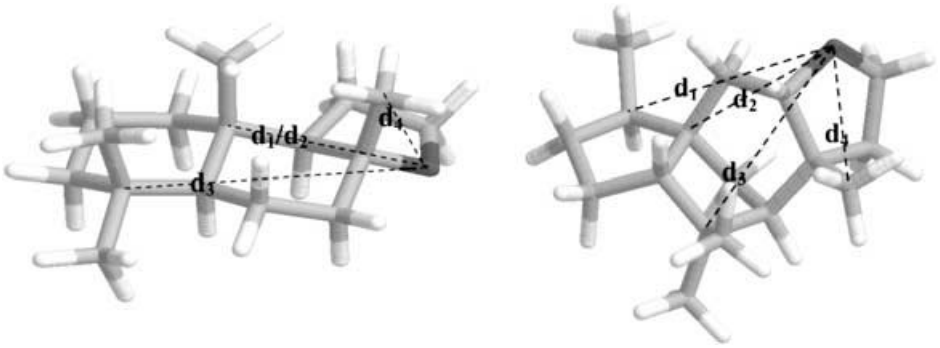
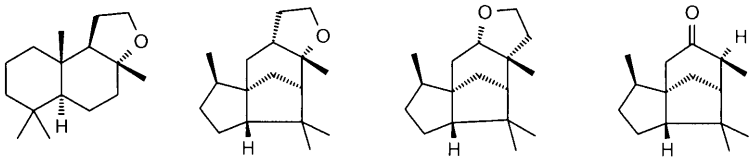


Fig. 10. *Calculated structures and selected interatomic distances of* **52** (left) *and* **53** (right). For experimental details, see the *Table* and the legend to *Fig. 8*.



52 **53** **54** **55**

Fig. 11. *Structures of* Ambroxide® (**52**) *and of the two new, analogous compounds* **53** *and* **54**, *which were* (incorrectly) *anticipated to be ambery odorants* (see text). Compound **54** was synthesized from cedranone (**55**).

Table. *Comparison of Selected Interatomic Distances* (see *Fig. 10*) *of Compounds* **52**–**54**

Distance [Å]	*Ambroxide*® (**52**)	Analog **53**	Analog **54**
d_1	3.72 (0.10)	5.03 (0.10)	4.91 (0.10)
d_2	3.72 (0.10)	3.76 (0.05)	3.56 (0.05)
d_3	5.73 (0.10)	4.56 (0.10)	3.89 (0.10)
d_4	2.41 (0.05)	3.04 (0.05)	2.37 (0.05)

Compound **54** was readily synthesized from commercially available cedranone (**55**), [37]. However, contrary to our expectations, **54** showed neither the ambery odor characteristics and strength of *Ambrocenide*® (**50a**) nor of *Ambroxide*® (**52**), being not only much weaker, but with *dominating cedar and woody notes*. This finding shows that odor predictions on the basis of SAR theories still seem to be difficult.

In summary, we have shown that longifolene (**6**) and α-cedrene (**7**) are still attractive starting materials for the synthesis of new woody and ambery fragrant substances. *Ambrocenide*® (**50a**) seems to be the best currently available molecule from these two resources. Predictions with existing SAR theories on the ambery odor of a given compound seem, however, to be difficult.

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A Novel Approach to Prezizaane Sesquiterpenes

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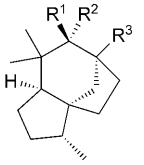
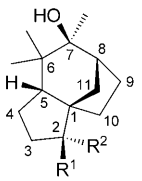
Prezizaane sesquiterpenes are an olfactorily interesting class of tricyclic natural products, which occur in some precious perfumery raw materials. These compounds are biosynthetically derived from farnesyl pyrophosphate *via* cyclization, but some questions regarding the stereoselectivity of this process have not yet been answered. We discuss a novel and concise access to the tricyclic framework of these sesquiterpenes, as exemplified by the synthesis of (±)-5-*epi*-sesquithuriferone (5-*epi*-**4**).

Introduction. – In the family of woody, ambery, and balsamic odorants, sesquiterpenoids play an important role in the fragrance industry [1]. Irrespective of a few exceptions, sesquiterpenes are not used in pure form, but as essential oils, which usually are complex mixtures. In most cases, minor constituents contribute olfactorily to the complexity, strength, volume, and substantivity, hence to the beauty of these natural scents. Several of the most-precious essential oils in perfumery contain a number of minor sesquiterpenic constituents that share the same tricyclo[6.2.1.0^{1,5}]undecane[1]) skeleton, the so-called *prezizaanes*. The dextrorotatory prezizaene was first isolated from the vetiver species *Vetiveria zizanioides* [2]. Later, (−)-prezizanol (**1**) and related compounds were found in *Eremophila georgei* DIELS, a kind of sandalwood [3]. (−)-Jinkohol (**2**) was isolated from agarwood (*Aquilaria malaccensis* BENTH.) [4] in 1981; its structure was corrected only recently [5]. During the last decade, more than 15 epimeric and enantiomeric prezizaanes were isolated from various natural sources [6], among them (+)-prezizaan-7-ol (**3**) from Haitian vetiver oil [5], the most appreciated vetiver oil in perfumery, and (+)-sesquithuriferone (**4**) as well as (−)-sesquithuriferol (**5**) from *Juniperus thurifera* L. [7].





1 (–)-Prezizanol R^1 = OH, R^2 = Me, R^3 = H
2 (–)-Jinkohol R^1 = H, R^2 = Me
3 (+)-Prezizaan-7-ol R^1 = Me, R^2 = H
4 (+)-Sesquithuriferone R^1 = R^2 = O, R^3 = Me
5 (–)-Sesquithuriferol R^1 = H, R^2 = OH, R^3 = Me

[1]) Octahydro-1*H*-3a,6-methanoazulene (IUPAC name).

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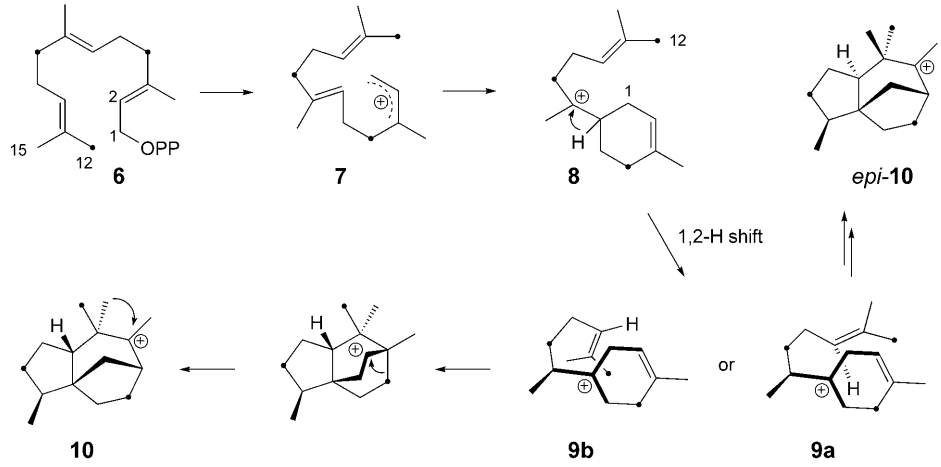
A characteristic feature of prezizaane derivatives is the *syn* relationship between H–C(5) and the CH_2 bridge, while the relative configurations at C(2) and C(7) vary, depending on the natural source. The underlying reason for this is the biosynthetic cyclization of farnesyldiphosphate (**6**) (*Scheme 1*) [8][9]. As shown by *Andersen* and *Falcone* [2], and *Akhila et al.* [10], the starting conformation of enzyme-bound **6** is judged to be a critical determinant for the configuration of the final cyclization product. Loss of the pyrophosphate (OPP) group generates the allyl cation **7** (or a biosynthetic equivalent), which cyclizes to the transient compound **8**. This cation undergoes a 1,2-H shift to **9a**, which was assumed to attack the distal C=C bond from the *si*-face. Support for this hypothesis came from the *in vivo* conversion of labeled **6** (dotted positions in *Scheme 1*) to prezizanol, followed by degradation. From these results, it was deduced that the labeled Me(12) group in **6** becomes the *endo*-Me group in **3** [10]. However, cyclization of the intermediate **9a** would rather lead to the formation of *epi*-**10**, but natural products with this relative configuration have not yet been identified. Therefore, it appears to be more likely that the cationic center in **9b** approaches the *re*-face of the C=C bond being in an s-*cis* conformation. This mode results in the proper configuration at C(5) of the natural product **3**, but the labeled Me group should be *exo* in cation **10**, which is the direct precursor of **3**.

Scheme 1. *Biosynthesis of Prezizaane Sesquiterpenes*

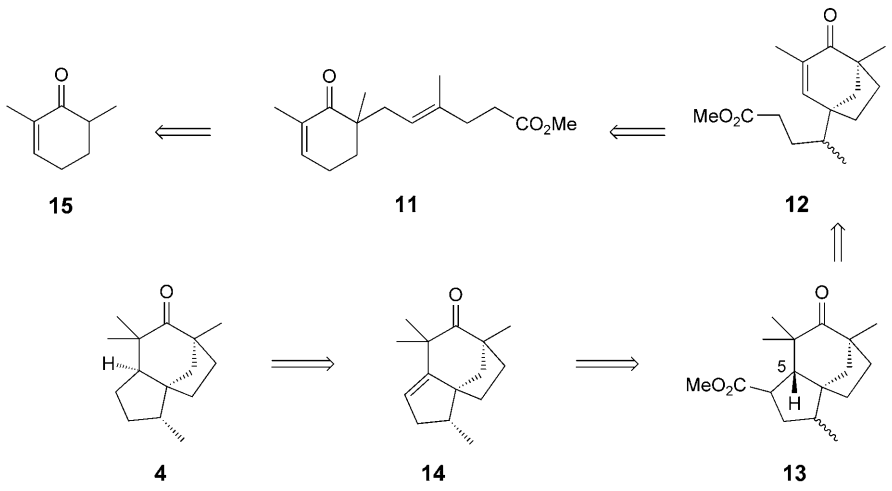


The first total syntheses of (–)-**1**, starting from (+)-pulegone, were reported by *Coates* and co-workers, and *Mori* and co-workers [11]. *Subba Rao* and co-workers [12] described different routes to racemic sesquiterpenes of *Eremophila georgei* and to prezizanol and jinkohol II. An important step in these syntheses was a *Lewis* acid induced rearrangement of a MeO-substituted bicyclo[2.2.2]octenol derivative into a bicyclo[3.2.1] system.

Results and Discussion. – We now report a new access to the tricyclic framework of prezizaane sesquiterpenes. The route is outlined in *Scheme 2* for the synthesis of 5-*epi*-sesquithuriferone (5-*epi*-**4**). The key step in our synthetic plan was the $EtAlCl_2$-

promoted cyclization of keto ester **11** by a series of hydride and alkyl shifts [13]. It should then be possible to cyclize the resulting compound **12** to the tricyclic keto ester **13** by an intramolecular ester-enolate addition to the enone unit, followed by *in situ* alkylation with MeI. This addition of the enolate was expected to proceed from the sterically less demanding *exo*-face of **12**, which would lead to the C(5)-epimer. However, after separation of the diastereoisomers, oxidative decarboxylation to **14** would destroy this stereogenic center, and subsequent hydrogenation to **4** was expected to be also directed by the *exo*-face.

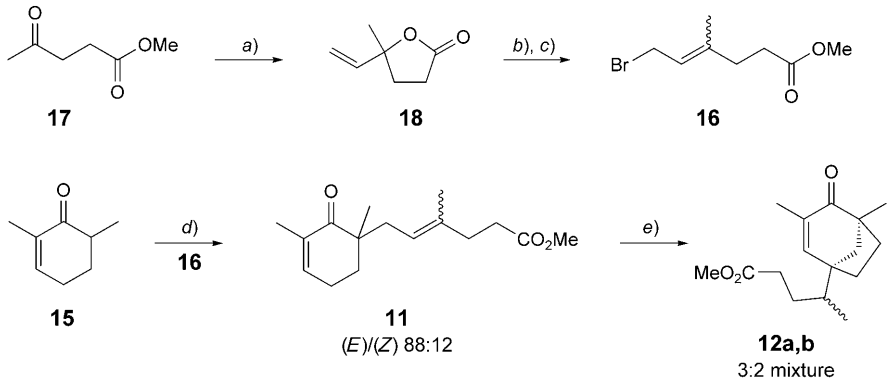
Scheme 2. *Retrosynthetic Approach*



The keto ester **11** was easily accessible by alkylation of 2,6-dimethylcyclohex-2-enone **15** with bromide **16**, which was prepared from methyl laevulinate (**17**) according to *Scheme 3*. Lavender lactone (**18**) was saponified with KOH in MeOH, esterified with MeI, and converted into **16** with PBr_3. Mild conditions for these transformations were necessary, as any acid or base treatment of the intermediate hydroxy acid or hydroxy ester resulted in rapid re-lactonization to **18**. The cationic cyclization of **11** proceeded at 70° with 3 equiv. of $EtAlCl_2$ to yield **12** as a 3 : 2 mixture of diastereoisomers [13], which were separated at a later stage (*vide infra*).

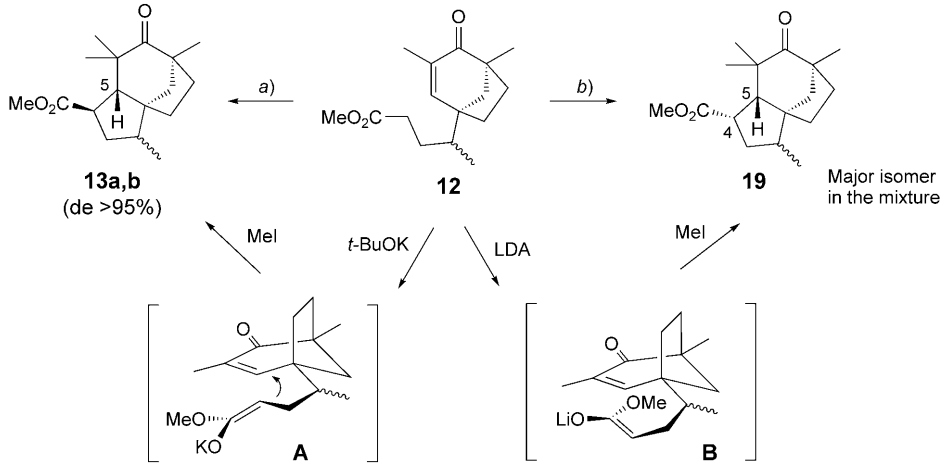
Good selectivity in the formation of tricyclic **13** was obtained by treatment of **12** with *t*-BuOK in hot THF (*Scheme 4*). Presumably, the intermediate, (*E*)-configured enolate **A**, being in an s-*trans* conformation, adds to the *exo*-face of the bicyclic enone unit. The keto enolate generated this way is then alkylated with MeI to **13**, with a diastereoisomeric excess (de) of $>95\%$ with respect to the newly formed stereogenic centers. In contrast, kinetically controlled ester-enolate formation should result in (*Z*)-enolate **B** [14], which approaches the enone group in an s-*cis* conformation. This results in a predominantly *anti* relationship at positions 4 and 5 in compound **19**.

Scheme 3. *Synthesis of the Keto Ester* **12a**,**b**



a) $CH_2=CHMgBr$, THF/benzene; 54%. *b*) KOH, MeOH, then DMF, CH_3I; 86%. *c*) PBr_3, pyridine; 72%. *d*) LDA, THF, **16**, $-78° \rightarrow$ r.t.; 76%. *e*) $EtAlCl_2$ (3 equiv.), toluene, 80°, 8 h; 87%.

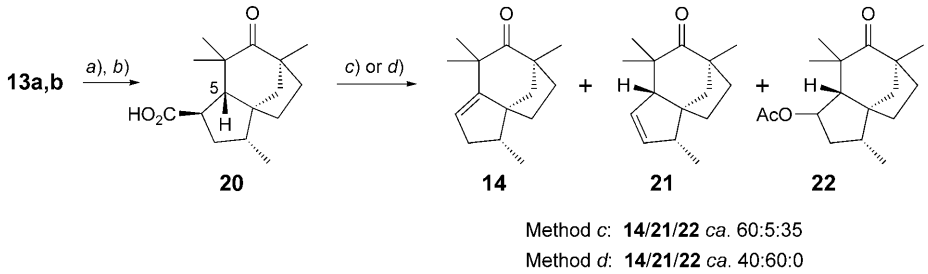
Scheme 4. *Formation of the Tricyclic Keto Ester* **13a**,**b**



a) *t*-BuOK, THF, 60°, 2 min, then MeI, 10 min; 66%. *b*) LDA, THF, $-78°$, 1 h, then MeI, $-78° \rightarrow$ r.t.; 58%.

Saponification of the diastereoisomer mixture **13a**,**b**, followed by fractional crystallization, led to **20** in 98% de (*Scheme 5*). According to our synthetic plan (*Scheme 2*), it should now be possible to invert the configuration at position 5 by an oxidative decarboxylation/hydrogenation sequence. $Pb(OAc)_4$-Promoted decarboxylation appeared to be an appropriate tool for the first part of this transformation, as competitive alkane formation *via* H transfer to the initially formed secondary alkyl radical usually occurs only in the case of 'primary' acids [15]. Treatment of **20** with $Pb(OAc)_4$ alone led to the desired olefin **14**, together with a small amount of isomer **21** and acetate **22**. The latter was formed by oxidative substitution, which can frequently be suppressed by adding a catalytic amount of $Cu(OAc)_2$. In fact, in the presence of

Scheme 5. *Oxidative Decarboxylation of* **20**



a) KOH, H_2O/MeOH, 80°, 8 h. *b*) Fractional crystallization from AcOEt. *c*) $Pb(OAc)_4$ (2 equiv.), benzene, reflux, 8 h; 58%. *d*) $Pb(OAc)_4$ (2 equiv.)/cat. $Cu(OAc)_2$, pyridine, benzene, 80°; 78%.

$Cu(OAc)_2$, compound **22** was not observed; the amount of isomer **21** was, however, significantly increased. Fortunately, compounds **14**, **21**, and **22** could be separated by flash chromatography. Surprisingly, the hydrogenation of **14** produced *epi*-sesquithuriferone (5-*epi*-**4**) exclusively.

In an attempt to reduce the C=C bond from the opposite face, alcohol **23** was treated with Et_3SiH in the presence of trifluoro acetic acid (TFA) [16]: an initially generated cation **C** should be reduced at the *exo*-face, giving rise to compound **24** with the correct configuration. However, the migration of both the methano- and ethano bridges resulted in a mixture of **25** and **26** *via* the intermediate cations **D** and **E**, respectively (*Scheme 6*).

In summary, a novel and flexible route for the synthesis of the tricyclic framework of prezizaane sesquiterpenes has been developed. Further studies on this process and on the synthesis of other prezizaanes are in progress.

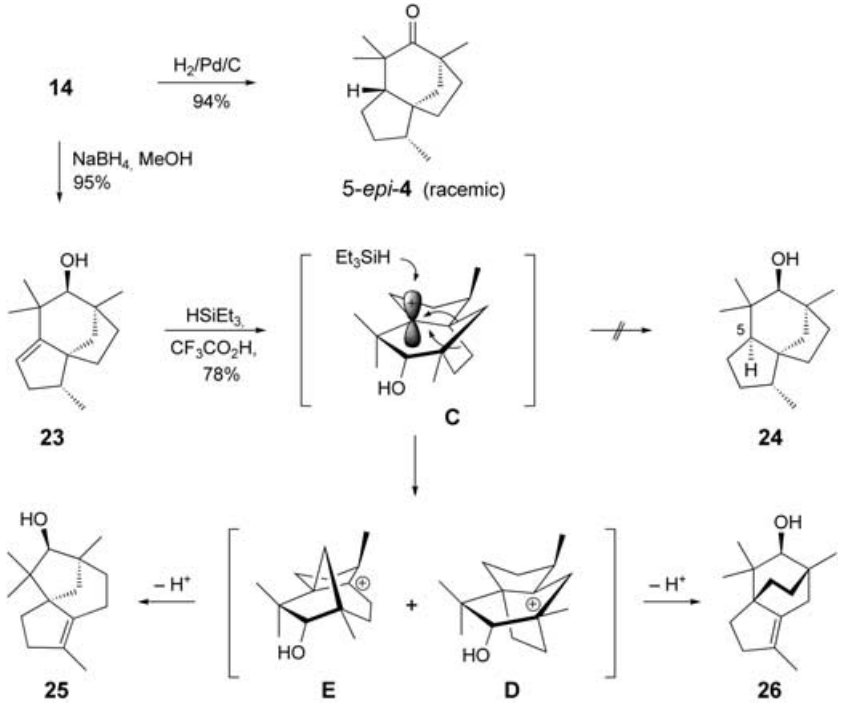
We are grateful to Dr. *Peter Gygax*, Dr. *Georg Fráter*, and Dr. *Markus Gautschi* for fruitful discussions, and to Dr. *Markus Gautschi* also for proofreading. Thanks are also due to Dr. *Joachim Schmid* and *Hans Gfeller* for MS data, and to *Ronnie Heusser* for additional experimental work.

Experimental Part

General. Unless otherwise stated, all reactions were performed under N_2. Reagents and solvents: *Fluka* (*puriss.* or *purum*), used without further purification. Flash chromatography (FC): *Merck Kieselgel 60*, particle size 40–63 μm. Thin-layer chromatography (TLC): *Merck Kieselgel 60*, particle size 5–20 μm, layer thickness 250 μm on glass, 5 cm × 10 cm, visualization reagent: phosphomolybdic acid (PMA) spray soln. for TLC, *Merck* 1.00480.0100. IR Spectra: *VECTOR 22/Harrick SplitPea ATR* spectrometer; in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: *Bruker AVANCE DPX-400* or *Bruker Avance 500* spectrometers; δ in ppm rel. to $SiMe_4$, J in Hz. GC/MS: *Finnigan MAT-95* instrument and *HP Chemstation 6890 GC/5973 Mass Sensitive Detector*; rel. int. in % of the base peak. Microanalyses were obtained from *Ilse Beetz*, *Mikroanalytisches Laboratorium*, 96301 Kronach, Germany.

*5-Ethenyl-4,5-dihydro-5-methylfuran-2(3*H*)-one* (*Lavender lactone*; **18**). A soln. of vinylmagnesium bromide, prepared from Mg (75 g, 3.1 mol) and vinyl bromide (321 g, 3.0 mol) in THF (750 ml) was diluted with benzene (720 ml) and added to a soln. of *methyl laevulinate* (**17**) in benzene (1260 ml) during 3 h at 10°. The mixture was stirred for an additional 30 min, poured on a mixture of ice (500 g), H_2O (2 l) and conc. HCl (1 l), and extracted with *t*-BuOME. The combined org. phases were washed with H_2O, sat. aq. $NaHCO_3$ soln., and brine, dried ($MgSO_4$), and concentrated *in vacuo* to yield a brown oil (362 g), which was distilled over a *Vigreux* column at 54–58°/0.03 mbar to give **18** as a colorless oil (182.2 g, 54%). ^1H-NMR (400 MHz, $CDCl_3$): 5.91 (*dd*, J = 17.2, 10.8, 1 H); 5.27 (*d*, J = 17.2, 1 H); 5.15 (*d*, J = 10.8, 1 H); 2.58–2.54 (*m*, 2 H); 2.25–2.06 (*m*, 2 H); 1.51 (*s*, 3 H).

Scheme 6. *Hydrogenation, Reduction, and Rearrangement of* **14**



Methyl 6-Bromo-4-methylhex-4-enoate (**16**). To a soln. of **18** (48 g, 0.38 mol) in MeOH (0.5 l) was added KOH (23.4 g, 0.41 mol). The temp. rose to 40°, and the mixture was stirred for 2 h and then concentrated *in vacuo*. To the waxy residue were added DMF (0.5 l) and MeI (64.9 g, 0.46 mol), and this mixture was stirred overnight, then poured on ice, and extracted with *t*-BuOMe (3 ×). The combined org. phases were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo* at 25° to yield a yellow oil (52 g, 86%). To a portion of this oil (45 g, 0.285 mmol) was added Et_2O (0.5 l) and pyridine (10 ml), and the mixture was cooled to 5°. PBr_3 (61.6 g, 0.23 mol) was added during 2 min. The suspension was stirred for 10 min, poured on ice, and extracted with pentane. The combined org. phases were washed with H_2O and brine, dried ($MgSO_4$), and concentrated *in vacuo*. The brown residue was rapidly distilled bulb-to-bulb to yield a colorless oil (38.6 g, 72%), which turned brown on standing. ^{1}H-NMR (400 MHz, $CDCl_3$): 5.58–5.53 (*m*, H–C(5)); 4.99 (*d*, *J* = 8.6, CH_2(6)); 3.68 (*s*, MeO); 2.47–2.37 (*m*, CH_2(2), CH_2(3)); 1.74 (*s*, Me). ^{13}C-NMR ((*E*)-isomer; 100 MHz, $CDCl_3$): 173.2 (*s*, C(1)); 141.4 (*s*, C(4)); 121.3 (*d*, C(5)); 51.6 (*q*, MeO); 34.3 (*t*); 32.2 (*t*); 28.9 (*t*); 15.8 (*q*, *Me*–C(4)).

Methyl 6-(1,3-Dimethyl-2-oxocyclohex-3-en-1-yl)-4-methylhex-4-enoate (**11**). To a soln. of LDA – prepared from BuLi (1.6M in hexane; 96 ml, 154 mmol) and diisopropylamine (15.55 g, 154 mmol) in THF (100 ml) – was added **15** (17.36 g, 140 mmol) at –78°, and the soln. was stirred for 1 h. Then **16** (28.3 g, 150 mmol) was added, and the mixture was allowed to warm to 20° over 7 h. The mixture was poured on H_2O and extracted with *t*-BuOMe (3 ×). The combined org. phases were washed with H_2O and brine, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by FC (SiO_2; hexane/AcOEt 80:20) to yield **11** (26.2 g, 76%) as a mixture of (*E*)- and (*Z*)-isomers in a ratio of 88:12. IR (neat): 2924*m*, 1737*s*, 1666*s*, 1435*m*, 1357*m*, 1158*s*, 1029*m*. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.65–6.61 (*m*, 1 H); 5.16–5.10 (*m*, 1 H); 3.67, 3.66 (2*s*, 3 H); 2.45–2.14 (*m*, 8 H); 1.94–1.87 (*m*, 1 H); 1.77–1.68 (*m*, 4 H); 1.61 (br. *s*, 3 H); 1.06, 1.04 (2*s*, 3 H). ^{13}C-NMR ((*E*)-Isomer; 100 MHz, $CDCl_3$): 204.0 (*s*); 173.7 (*s*); 143.5 (*d*); 135.9 (*s*); 134.0 (*s*); 120.7 (*d*); 51.4 (*q*); 45.0 (*s*); 34.9 (*t*); 34.8 (*t*); 33.3 (*t*); 33.0 (*t*); 22.8 (*t*); 21.8 (*q*); 14.4 (*q*); 16.1 (*q*). GC/MS (EI): 264 (3, M^+), 233 (4), 141 (8), 124 (100), 109 (50), 81 (39), 67 (19), 55 (20), 41 (21). Anal. calc. for $C_{16}H_{24}O_3$: C 72.69, H 9.15; found: C 72.64, H 9.23.

Methyl 4-(3,5-Dimethyl-4-oxobicyclo[3.2.1]oct-2-en-1-yl)pentanoate (**12a**,**b**). To a soln. of **11** (22.00 g, 83.3 mmol) in toluene (130 ml) was added a soln. of $EtAlCl_2$ in toluene (3 equiv. 139 ml, 1.8M, 250 mmol) at 0°. The mixture was stirred for 8 h at r.t., and then for an additional 8 h at 80°. The resulting soln. was cooled and carefully poured on ice. The org. phase was separated, and the aq. phase was extracted with *t*-BuOMe (2 × 100 ml). The combined org. phases were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by FC (SiO_2; hexane/AcOEt 90:10) to yield 19.04 g (86.5%) of **12** as a colorless oil (mixture of two isomers in a ratio of 3:2). IR (neat): 2958*m*, 2866*w*, 1737*s*, 1671*s*, 1437*m*, 1364*m*, 1171*s*, 1034*m*. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.85–6.84 (*m*, 1 H); 3.70, 3.68 (2*s*, 3 H); 2.52–2.40 (*m*, 1 H); 2.36–2.25 (*m*, 1 H); 1.97–1.34 (*m*, 9 H); 1.75 (br. *s*, 3 H); 1.24 (*s*, 3 H); 1.01, 0.93 (2*d*, J = 6.8, 6.3, 3 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 204.9 (*s*); 174.0 (*s*); 151.7; 151.0 (*d*); 133.5; 133.3 (*s*); 52.4; 52.3; 52.2; 51.9 (2*s*); 51.5 (*q*); 50.9; 50.5 (*t*); 39.8; 39.7 (*d*); 35.1; 34.9 (*t*); 33.2; 33.0 (*t*); 32.5; 32.4 (*t*); 28.5; 27.9 (*t*); 20.7 (*q*); 15.6 (*q*); 15.4; 14.7 (*q*). GC/MS (EI): 264 (14, M^+), 236 (39), 177 (15), 162 (31), 149 (100), 121 (47), 110 (52), 91 (39), 79 (37), 55 (30), 41 (37). Anal. calc. for $C_{16}H_{24}O_3$: C 72.69, H 9.15; found: C 72.68, H 8.99.

*Methyl (1*R**,2*S**,4*R**,5*S**,8*S**)-* (**13a**) *and Methyl (1*R**,2*R**,4*R**,5*S**,8*S**)-2,6,6,8-Tetramethyl-7-oxotricyclo[6.2.1.0^{1,5}]undecane-4-carboxylate* (**13b**). A soln. of **12a**,**b** (6.00 g, 22.7 mmol) in THF (10 ml) was added to a suspension of *t*-BuOK (2.67 g, 23.9 mmol) in THF (40 ml) at 60°. The mixture was kept for 5 min at this temp., and was then cooled to r.t. MeI (3.87 g, 27.2 mmol) was added, and the mixture was stirred for 1 h. The resulting white suspension was poured on H_2O and extracted with *t*-BuOMe. The org. phase was washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by FC (SiO_2; hexane/*t*-BuOMe 90:10) to yield 4.18 g (66%) of a colorless oil (**13a**/**13b** 1.8:1). Anal. calc. for $C_{17}H_{26}O_3$: C 73.34, H 9.41; found: C 73.38, H 9.44.

Data of **13a**. ^{1}H-NMR (500 MHz, $CDCl_3$): 3.70 (*s*, MeO); 2.77 (*ddd*, $J_{4,5}$ = 11.0, $J_{4,3a}$ = 7.3, $J_{4,3b}$ = 10.4, H–C(4)); 2.30 (*d*, $J_{5,4}$ = 11.0, H–C(5)); 2.15 (*ddd*, $J_{3a,3b}$ = 12.3, $J_{3a,4}$ = 7.3, $J_{3a,2}$ = 6.3, H_a–C(3)); 1.97–1.91 (*m*, H–C(2)); 1.86–1.73 (*m*, H_a–C(10), H_a–C(9)); 1.73 (*dd*, $J_{11s,11a}$ = 12,3, J = 1.9, H_s–C(11)); 1.67–1.48 (*m*, H_b–C(9), H_b–C(10), H_b–C(3)); 1.47 (*dd*, $J_{11a,11s}$ = 12.3, $J_{11a,5}$ = 1.3, H_a–C(11)); 1.16 (*s*, Me–C(8)); 1.12 (2*s*, 2 Me–C(6)); 1.01 (*d*, J = 6.9, Me–C(2)). ^{13}C-NMR (126 MHz, $CDCl_3$): 219.1 (*s*, C(7)); 176.9 (*s*, CO_2Me); 58.0 (*d*, C(5)); 53.2 (*s*, C(1)); 51.7 (*q*, CO_2*Me*); 50.5 (*s*, C(8)); 45.4 (*d*, C(4)); 44.2 (*s*, C(6)); 44.2 (*t*, C(11)); 40.1 (*d*, C(2)); 38.7 (*t*, C(3)); 34.0 (*t*, C(10)); 33.8 (*t*, C(9)); 29.9, 25.5 (2*q*, 2*Me*–C(6)); 21.4 (*q*, *Me*–C(8)); 16.2 (*q*, *Me*–C(2)).

Data of **13b**. ^{1}H-NMR (500 MHz, $CDCl_3$): 3.69 (*s*, MeO); 2.85 (*ddd*, $J_{4,3b}$ = 10.8, $J_{4,3a}$ = 4.7, $J_{4,5}$ = 9.3, H–C(4)); 2.36 (*dd*, $J_{5,4}$ = 9.3, $J_{5,11a}$ = 1.8, H–C(5)); 2.11–2.03 (*m*, H–C(2)); 1.99 (*ddd*, $J_{3a,3b}$ = 12.9, $J_{3a,2}$ = 8.5, Hz, $J_{3a,4}$ = 4.7, H_a–C(3)); 1.97–1.90 (*m*, H_a–C(10)); 1.87–1.76 (*m*, H_a–C(9), H_a–C(10)); 1.62–1.56 (*m*, 2 M, H_b–C(3), H_b–C(9)); 1.51 (*d*, $J_{11s,11a}$ = 12.2, H_s–C(11)); 1.37 (*dd*, $J_{11a,11s}$ = 12.2, $J_{11a,5}$ = 1.9, H_a–C(11)); 1.21 (*s*, Me_a–C(6)); 1.17 (*s*, Me–C(8)); 1.07 (*s*, Me_b–C(6)); 0.92 (*d*, J = 6.6, Me–C(2)). ^{13}C-NMR (126 MHz, $CDCl_3$): 219.6 (*s*, C(7)); 177.5 (*s*, CO_2Me); 60.1 (*d*, C(5)); 54.5 (*s*, C(1)); 52.0 (*s*, C(8)); 51.8 (*q*, 4-CO_2*Me*); 43.9 (*s*, C(6)); 43.8 (*d*, C(4)); 38.3 (*d*, C(2)); 37.7 (*t*, C(11)); 37.0 (*t*, C(3)); 35.4 (*t*, C(9)); 33.9 (*t*, C(10)); 31.6 (*q*, Me_a–C(6)); 25.0 (*q*, Me_b–C(6)); 21.7 (*q*, *Me*–C(8)); 13.9 (*q*, *Me*–C(2)).

*(1*R**,2*S**,4*R**,5*S**,8*S**)-2,6,6,8-Tetramethyl-7-oxotricyclo[6.2.1.0^{1,5}]undecane-4-carboxylic Acid* (**20**). A soln. of **13a**,**b** (4.00 g, 14.39 mmol) and KOH (8.0 g, 0.14mol) in H_2O/MeOH 2:1 (50 ml) was heated to 70° for 8 h. The mixture was acidified (pH 1) and extracted with *t*-BuOMe. The org. phase was washed with H_2O and brine, dried ($MgSO_4$), and concentrated *in vacuo* to yield 4.0 g of a semicrystalline crude, which was recrystallized from AcOEt/hexane to give **20** (68% de). Further crystallization from AcOEt yielded **20** (1.08 g) in 98% de. M.p. 171–174°. IR (neat): 3046 (br.), 2955*m*, 2872*m*, 1691*s*, 1426*m*, 1212*m*. ^{1}H-NMR (400 MHz, $CDCl_3$): 2.87 (*ddd*, $J_{4,3b}$ = 10.9, $J_{4,3a}$ = 4.1, $J_{4,5}$ = 9.1, H–C(4)); 2.36 (*dd*, $J_{5,4}$ = 9.1, $J_{5,11a}$ = 1.8, H–C(5)); 2.15–2.05 (*m*, 2 H); 1.99–1.01 (*m*, 1 H); 1.89–1.80 (*m*, 1 H); 1.70–1.55 (*m*, 4 H); 1.52 (*d*, $J_{11s,11a}$ = 12.1, H_s–C(11)); 1.37 (*dd*, $J_{11a,11s}$ = 12.1, $J_{11a,5}$ = 1.8, H_a–C(11)); 1.24 (*s*, Me_a–C(6)); 1.18 (*s*, Me–C(8)); 1.12 (*s*, Me_b–C(6)); 0.93 (*d*, J = 6.3, Me–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 219.8 (*s*, C(7)); 183.2 (*s*, CO_2Me); 60.1 (*d*, C(5)); 54.7 (*s*, C(1)); 52.1 (*s*, C(8)); 44.1 (*s*, C(6)); 43.9 (*d*, C(4)); 38.5 (*d*, C(2)); 37.7 (*t*, C(11)); 37.3 (*t*, C(3)); 35.6 (*t*, C(9)); 34.1 (*t*, C(10)); 31.7 (*q*, Me_a–C(6)); 25.3 (*q*, Me_b–C(6)); 21.8 (*q*, *Me*–C(8)); 13.8 (*q*, Me–C(2)). MS (EI): 264 (16, M^+), 236 (17), 191 (4), 121 (100), 108 (19), 93 (11), 81 (16), 55 (10), 43 (12), 41 (17). Anal. calc. for $C_{16}H_{24}O_3$: C 72.69, H 9.15; found: C 72.72, H 9.11.

*(1*R**,2*R**,8*S**)-2,6,6,8-Tetramethyltricyclo[6.2.1.0^{1,5}]undec-4-en-7-one* (**14**) *and (1*R**,2*R**,5*S**,8*S**)-2,6,6,8-Tetramethyltricyclo[6.2.1.0^{1,5}]undec-3-en-7-one* (**21**). A suspension of **20** (2.00 g, 7.58 mmol), $Pb(OAc)_4$ (6.71 g, 15.2 mmol), $Cu(OAc)_2$ (100 mg), and pyridine (10 ml) in benzene (100 ml) was heated to 80° for 7 h. The mixture was cooled, poured on ice-cold 2N aq. HCl soln., and extracted with *t*-BuOMe. The org. layer was washed with H_2O and brine, dried ($MgSO_4$), and concentrated. The residue was purified by FC (SiO_2; hexane/AcOEt 95:5) to give a mixture of **14** and **21** (1.28 g, 78%) in a ratio of 4:6.

Data of **14**: IR (neat): 2958*m*, 2927*m*, 2869*w*, 1705*s*, 1449*m*, 1376*m*, 1025*m*. ^{1}H-NMR (500 MHz, C_6D_6): 5.31 (*t*, *J* = 2.4, H−C(4)); 2.65 (*ddd*, *J* = 16.1, 7.6, 2.0, H_a−C(3)); 2.23 (*d*,*d*,*q*, *J* = 7.1, 7.1, 3.6, H−C(2)); 1.90–1.75 (*m*, H_a−C(3), CH_2(10), H_a−C(9)); 1.70–1.62 (*m*, H_b−C(9)); 1.60 (*s*, H−C(11)); 1.32 (*s*, Me_a−C(6)); 1.24 (*s*, Me_b−C(6)); 1.19 (*s*, Me−C(8)); 0.94 (*d*, *J* = 7.1, H−C(8)). ^{13}C-NMR (126 MHz, C_6D_6): 218.7 (*s*, C(7)); 155.3 (*s*, C(5)); 119.1 (*d*, C(4)); 59.6 (*s*, C(1)); 53.0 (*s*, C(8)); 45.5 (*s*, C(6)); 41.9 (*t*, C(11)); 39.7 (*t*, C(3)); 39.3 (*d*, C(2)); 37.5 (*t*, C(10)); 34.6 (*t*, C(9)); 31.0 (*q*, *Me*$_a$−C(6)); 29.3 (*q*, Me_b−C(6)); 21.8 (*q*, *Me*−C(8)); 17.5 (*q*, *Me*−C(2)). GC/MS (EI): 218 (29, M^+), 189 (30), 175 (47), 161 (28), 147 (100), 133 (31), 119 (47), 105 (61), 91 (50), 77 (29), 41 (27).

Data of **21**: IR (neat): 2956*m*, 2868*m*, 1703*s*, 1455*m*, 1023*m*. ^{1}H-NMR (500 MHZ, C_6D_6): 5.48 (*ddd*, $J_{4,3}$ = 5.7, $J_{4,5}$ = 2.6, $J_{4,2}$ = 1.7, H−C(4)); 5.44 (*dt*, $J_{3,4}$ = 5.7, *J* = 2.2, H−C(3)); 2.57–2.52 (*m*, H−C(2)); 2.38–2.35 (*m*, H−C(5)); 1.70 (*d*, $J_{11s,11a}$ = 12.3, H_s−C(11)); 1.65–1.54 (*m*, H_a−C(9), H_a−C(10)); 1.36–1.24 (*m*, H_b−C(9), H_b−C(10)); 1.24 (*s*, Me−C(8)); 1.19 (*s*, Me_a−C(6)); 0.99 (*d*, $J_{11a,11s}$ = 12.3, H_a−C(11)); 0.98 (*s*, Me_b−C(6)); 0.81 (*d*, *J* = 7.2, Me−C(2)). ^{13}C-NMR (126 MHz, C_6D_6): 218.5 (*s*, C(7)); 136.1 (*d*, C(4)); 129.2 (*d*, C(3)); 62.4 (*d*, C(5)); 54.8 (*s*, C(1)); 50.8 (*s*, C(8)); 45.3 (*d*, C(2)); 43.5 (*s*, C(6)); 38.0 (*t*, C(11)); 37.5 (*t*, C(10)); 35.4 (*t*, C(9)); 29.7 (*q*, *Me*$_a$−C(6)); 26.0 (*q*, *Me*$_b$−C(6)); 22.5 (*q*, *Me*−C(8)); 14.7 (*q*, *Me*−C(2)). GC/MS (EI): 218 (8, M^+), 189 (6), 175 (16), 147 (100), 119 (23), 105 (35), 91 (37), 77 (22), 55 (11), 41 (19). Anal. calc. for $C_{15}H_{22}O$: C 82.51, H 10.16; found: C 82.57, H 10.14.

*(1*R*,2*R*,5*S*,8*S*)-2,6,6,8-Tetramethyltricyclo[6.2.1.0^{1,5}]undecan-7-one (5*-epi-*Sesquithuriferone*; 5*-epi-***4**). A soln. of **14** (100 mg, 0.46 mmol) in MeOH (20 ml) was hydrogenated over Pd/C (10%) during 24 h. The mixture was filtered, and the residue was distilled bulb-to-bulb to yield 5*-epi-***4** (95 mg, 94%) as a colorless oil. IR (neat): 2953*m*, 2868*m*, 1699*s*, 1455*m*, 1376*m*, 1024*m*. ^{1}H-NMR (500 MHz, C_6D_6): 1.76–1.70 (*m*, H_a−C(9)); 1.70–1.60 (*m*, H_a−C(2), H−C(3), H_a−C(10)); 1.54 (*ddd*, *J* = 10.3, 8.4, 1.9, H−C(5)); 1.47–1.39 (*m*, H_a−C(4)); 1.37–1.21 (*m*, H_b−C(4), H_b−C(9), H_b−C(10), H_s−C(11)); 1.24 (*s*, Me−C(8)); 1.13 (*s*, Me_a−C(6)); 1.05 (*s*, Me_b−C(6)); 1.03 (*dd*, *J* = 12.3, *J* = 1.9, H_a−C(11)); 1.01–0.97 (*m*, H_b−C(3)); 0.76 (*d*, *J* = 6.5, Me−C(2)). ^{13}C-NMR (126 MHz, C_6D_6): 218.0 (*s*, C(7)); 57.8 (*d*, C(5)); 54.0 (*s*, C(1)); 52.0 (*s*, C(8)); 44.1 (*s*, C(6)); 39.3 (*d*, C(2)); 38.1 (*t*, C(11)); 35.3 (*t*, C(9)); 34.3 (*t*, C(11)); 32.4 (*q*, Me_b−C(6)); 31.4 (*t*, C(3)); 26.2 (*t*, C(4)); 25.3 (*q*, Me_a−C(6)); 22.4 (*q*, *Me*−C(8)); 14.7 (*q*, *Me*−C(2)). GC/MS (EI): 220 (20, M^+), 192 (30), 149 (10), 135 (14), 121 (100), 108 (64), 93 (29), 81 (40), 67 (13), 55 (20), 41 (43). HR-MS: 220.1827 (M^+, $C_{15}H_{24}O$; calc. 220.1827.

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'Brain Aided' Musk Design

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'Musks are my favorite perfumery raw materials. There are countless possibilities to incorporate them into a fragrance. They are like cotton in the textiles industry: You can make cheap, vulgar dresses from it, but also very elegant well cut ones…'
Olivia Giacobetti in an interview of *Bolero* [1]

This brief review, including new experimental results, is the summary of a talk at the RSC/SCI conference *flavours & fragrances 2004* in Manchester, United Kingdom, 12–14 May, 2004. Musk odorants have been a classical domain for computer aided structure–odor relationship (SOR) studies, but, contrary to sandalwood or amber odorants, they belong to structurally very different substance classes, *e.g.*, macrocycles, aromatic polycycles, and nitro arenes. Most SOR computer models are restricted to one class, excluding structural diversity to increase predictability. But even within a musk family, structural similarities are often due to a common synthetic access, and do not reflect binding requirements for the musk receptor. Beyond that, the importance of structural key features can be missed, which is discussed on the example of the (4*S*)-Me group of *Galaxolide*®. By synthesis and olfactory evaluation of *Galaxolide*®-like shaped macrobicycles as model compounds for conformationally constrained (12*R*)-12-methyltridecano-13-lactone, it was investigated how likely there is more than one musk receptor. Finally, the new family of so-called linear musks is discussed, especially with respect to the conformational importance of the gem-2′,2′-dimethyl moiety in *Helvetolide*® and the additional 2′-carbonyl group of Romandolide – structural features that strongly diminish the musk odor of macrocycles. On the example of 2-methyl-2-[(E)-1,2,4-trimethylpent-2-enyloxy]propyl esters, the 'brain-aided' design and conformational analysis of musk odorants is illustrated. The overview concludes with the synthesis, odor evaluation, and conformational discussion of the new musk odorant 2-(3,3-dimethylcyclohexyl)propanoic acid ethoxycarbonylmethyl ester.

Introduction. – Musks are certainly indispensable to impart sensuality to a perfume, and perhaps they are even the most versatile odorants used in perfumery. They can make a unisex fragrance more vibrant, sheer, and crisp in the top note, or they can turn the dry-down note of a floriental perfume into a lush and luscious experience. They can be elegant, eclectic, eccentric, emotional, exciting, evocative, exotic, extreme, ecstatic, extravagant, energizing, or exalting in a perfume; but always in an erogenous way. Of course, this plenitude of attributes is not covered by one substance alone: there are numerous odorants that share a common musk character, whilst they differ in their tonality and their facets [2], but, so far, almost all of them are derivatives of three common lead motives being quite different in their chemical structure (*Scheme 1*):

1) Macrocyles, such as the natural lead (−)-(*R*)-muscone (**1**), or such as pentadecano-15-lactone (**2**; *Exaltolide*®, *Thibetolide*®),
2) nitro arenes, such as Musk ketone (**3**),
3) polycyclic benzene derivatives, such as *Phantolide*® (**7**) or *Galaxolide*® (**8**).

At first glance, there is little structural similarity between these three musk families, which raises the question: are there three musk receptors? Considering the fact that

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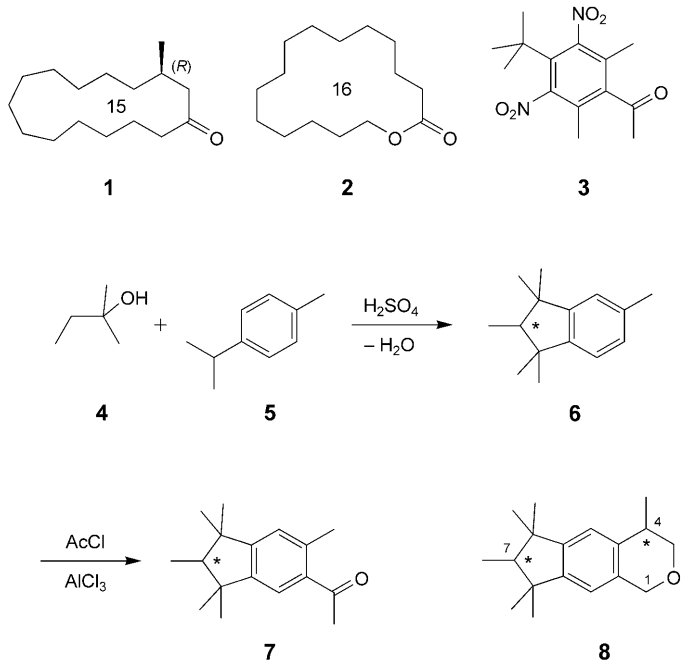
only 347 olfactory receptors are functionally expressed in humans, it seems a lot to dedicate 1% of these for musk odorants. Yet, most computer-aided models [3][4] for the structure–odor relationship (SOR) of musks are restricted to one class only as if there was one receptor for each family. This certainly increased predictability of these SOR models, but it also makes it more difficult to use them for the discovery of new generations of musk odorants. However, if one looks at the structures in *Scheme 1* more closely, one recognizes common spatial arrangements even in the two-dimensional structural formulae: there is a similarity of the outer periphery of (−)-(*R*)-muscone (**1**) and *Galaxolide*® (**8**), including the distance of the Me group to the functional group, the so-called olfactophore, which is assumed to orient the molecule on the receptor site. Yet, transannular strain would prevent **1** from adopting a conformation as drawn in *Scheme 1*, and pentadecano-15-lactone (**2**) does not even possess a Me group. So it is probably (much) more complicated than it appears at first sight.

Scheme 1. *The Different Motives of Musk Odorants, and the Synthesis of* Phantolide® (**7**)



***Galaxolide*® Isomers.** – A data set of 362 compounds comprising musk odorants of all three structural classes, as well as of their inactive analogs mainly from the literature up to the late 1980s, was the basis of a computer model by *Bersuker et al.* [5], which claimed two independent molecular features responsible for the musk odor of a compound:

1) A functional group, *e.g.*, a C=O, NO_2, or CN group, flanked in a distance of 6.7 Å by two hydrophobic moieties, which are 2.5 Å apart from each other, *e.g.*, a gem-dimethyl group.

2) Two hydrophobic moieties situated in a distance of 5.5 Å from each other.

Bersuker et al. [5] reported nearly 96% of the compounds to be correctly predicted, but *Kansy et al.* [6], who evaluated the model, found only 54% of the data set predicted correctly. Based on the same data set, *Kansy et al.* [6] could, however, derive a different model, which had a prediction rate of 65%. This computer-generated model consists of three hydrophobic binding spheres 5.5, 6.7, and 8.0 Å apart from a H-bond acceptor, *e.g.*, a C=O, NO_2, or CN group. The three hydrophobic moieties are situated 3.6 and 4.0 Å apart from each other, and bind to three adjacent Me groups of the 1,1,2,3,3-pentamethylindane moiety of *Galaxolide*® (**8**; *Fig. 1*, *top*). For pentadecano-15-lactone (**2**; *Fig 1*, *bottom*), $CH_2(4)/CH_2(5)$, $CH_2(7)$, and $CH_2(11)/CH_2(12)$ take up the space of these three hydrophobic spheres. But as the macrocycle **2** is conformationally flexible, and consequently adapts to a variety of different binding geometries, more rigid polycycles like **7** and **8** are determinant in the computer-aided generation of the olfactophore model. As the 2,3-dihydro-1,1,2,3,3-pentamethyl-1*H*-indene moiety occurs in a plenitude of musk odorants, it seems most characteristic for the pattern-recognition algorithms.

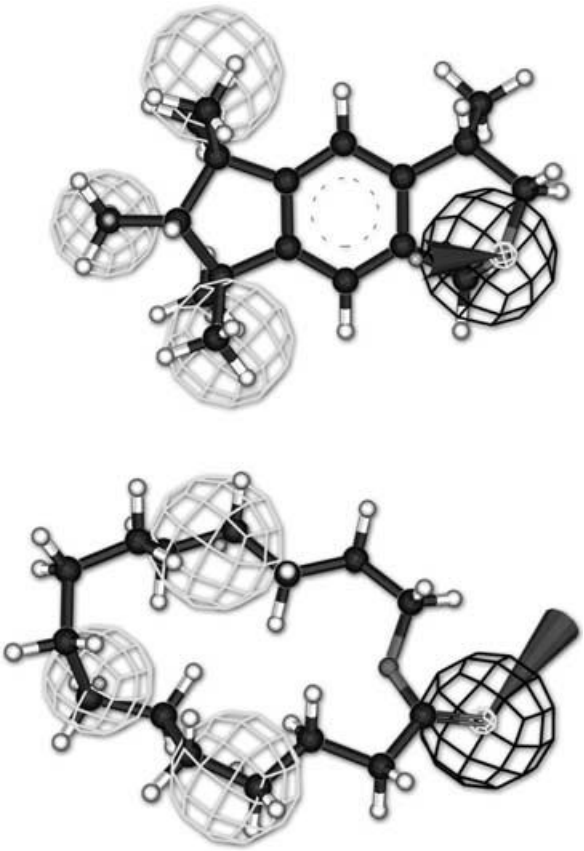


Fig. 1. *The olfactophore model of* Kansy et al. *with* Galaxolide® (**8**; *top*) *and pentadecano-15-lactone* (**2**; *bottom*) *bound to the calculated features*

The reason for the wide occurrence of this structural motive is, of course, the easy access to these polycyclic musks by chemical synthesis. *Phantolide*® (**7**), for instance, is prepared by acid-catalyzed *Friedel–Crafts* alkylation of *p*-cymene (**5**) with 1,1-dimethylpropan-1-ol (**4**), which provides 1,1,2,3,3,5-hexamethylindane (**6**) as central intermediate. This is then transformed to the industrial end product **7** by *Friedel–Crafts* acylation with AcCl in the presence of $AlCl_3$ (*Scheme 1*). Polymethylated indane systems are easy to synthesize, and thus they occur frequently – but that does not mean that this structural element is necessary for a musky odor.

The olfactophore model of *Kansy et al.* implies that the 4-Me group of *Galaxolide*® (**8**, see *Scheme 1* for atom numbering), and especially the configuration at C(4), is of no importance for the musk odor, while the configuration at C(7) should have a significant effect on the odor characteristics of **8**. This prediction turned out to be wrong when *Fráter et al.* [7] prepared and characterized the four stereoisomers of the isochromane musk **8**. This was carried out by *Friedel–Crafts* alkylation of 1,1,2,3,3-pentamethylindane with (*R*)- and (*S*)-methyloxirane, respectively, and separation of the resulting diastereoisomers *via* tricarbonyl chromium complexes.

The odor description and threshold data of the four stereoisomers are compiled in *Fig. 2*, and, contrary to what would be expected according to the computer model of *Kansy et al.* [6], the configuration at C(4) turned out to be crucial. The (−)-(4*S*)-**8** isomers possess strong, typical musk odors of 0.63 and 1.0 ng/l air thresholds, compared to weak, uncharacteristic notes and thresholds of 130 and 440 ng/l air for the (+)-(4*R*)-**8** isomers, respectively [7][8]. On the other hand, the configuration at C(7) is of less importance to both odor character and intensity, even though, in the computer model, it should be situated in a crucial hydrophobic binding site. It thus appears that the structural fragments $Me_2C(6)$, Me−C(7), and $Me_2C(8)$ just roughly outline the extent of a bulky hydrophobic site, while Me−C(4) is key for the musk odor. In analogy to the terminology of *Beets* [9], we thus call the (*S*)-configured Me−C(4) a *profile* group, the spatial distance of which to the functional group is of utmost importance. The (most polar) functional group is thought to orient the molecule on the binding site of the receptor, and was coined *osmophore* by *Rupe* and *von Majewski* [10] as well as *Ruzicka* [11]. Their criterion for an *osmophore* was its interchangeability with another functional group of similar polarity without loss of the principal odor characteristics of a molecule – so, in modern terms, a test for the function as a H-bond donor or acceptor group. The term *osmophore* is sometimes confused with the complete set of structural features responsible for a given odor, for which we use the term *olfactophore* according to *Ham* and *Jurs* [12], and *Kansy et al.* [6]. Transferred to the old but vivid *lock-and-key* concept of *Fischer* [13], we thus obtain the picture (*Fig. 3*) of the *osmophore* inserting the odorant into the receptor binding site, in which mainly the *profile group* codes the information, and bulky substituents provide a *firm grip* by filling out a larger hydrophobic volume. As depicted in *Fig. 3*, this terminology is not only applicable to musks like **8**, but also to sandalwood odorants like *Javanol*® [14] or to ambra odorants like *Ambrocenide*® [15]. We will use this simple *brain-aided* concept rather than a *computer-aided* model, which overrates common elements due to similar steps in the synthesis of musk odorants, and we will use it to look into the question, whether it is likely that there are one or more musk receptors.

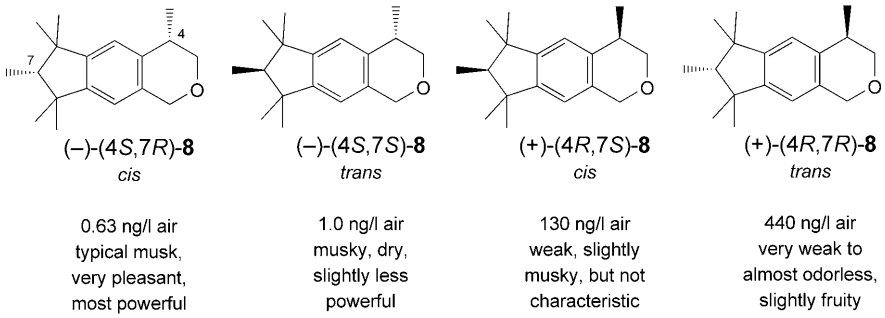


Fig. 2. *Olfactory characterization of the four stereoisomers of* Galaxolide® (**8**)

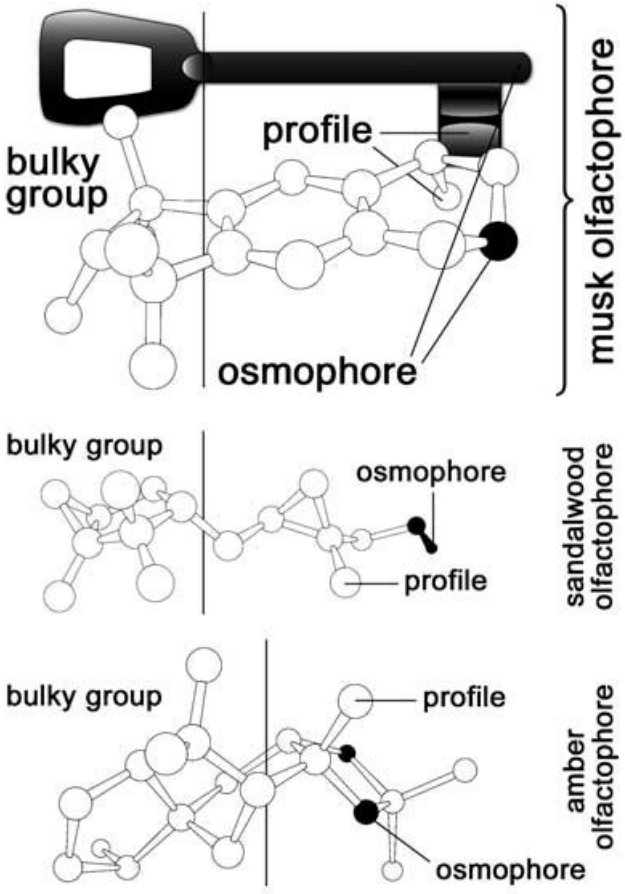


Fig. 3. *The molecular parameters of odorants illustrated with the old but vivid* lock-and-key *analogy*

Constructing Macrobicycles. – Considering the current state of our biological and biochemical understanding of olfaction, the question as to whether a common musk receptor exists is actually impossible to answer in a definite way. But we may gain some further insight from the chemical perspective if we construct hybrids of polycyclic musks such as *Galaxolide*® (**8**) and macrocycles. Thereby, we can test if the conformation of macrocyclic musks on the receptor site might resemble the outer perimeter of polycyclic benzene derivatives – what actually the computer-aided models would suggest, since their algorithms used the rigid polycyclic structures as templates.

12-Methyltridecano-13-lactone (**9**) is an example of a macrocyclic musk, in which the Me substitution is of crucial importance, as it is in *Galaxolide*® (**8**). Whereas **9** smells musky, the parent 14-membered macrolide tridecano-13-lactone does not. The enantiomers of **9**, which both occur in *Angelica* root oil in the (*R*)/(*S*) ratio of 72:28 [16], differ significantly in their odor character (*Fig. 4*): while the (+)-(12*R*)-isomer of **9** possesses a strong clean musk odor with sandalwood-like and fruity aspects, the enantiomer (−)-(12*S*)-**9** emanates an animalic musk odor with camphoraceous aspects [17][18]. 12-Methyltridecano-13-lactone (**9**) is also ideally suited for the construction of hybrid structures with *Galaxolide*® (**8**) because of its low molecular weight, which allows the introduction of a bridge without exceeding the mass boundaries of macrocyclic musks (*ca.* 286 u [19]). To constrain (+)-(12*R*)-**9** in a *Galaxolide*®-type shape, the bridge has to be situated symmetrical to the $-COO-CH_2-CMeH-$ side to match both the osmophore and the profile group. In addition, five- and six-membered ring systems should be avoided in order to keep the molecule as flat as possible (*Fig. 4*). Therefore, we planned to introduce a CH_2 bridge between C(3) and C(8) or C(9) of (+)-(12*R*)-**9**, leading to bicyclo[7.5.1]- and bicyclo[8.4.1]-macrolides. Furthermore, to investigate the importance of the absolute configuration of C(12), bearing the Me group, on the odor of these macrobicycles, we were also aiming at the demethyl analogs of these bicyclomacrolides [20].

The retrosynthetic plan, according to which the syntheses were carried out, is summarized in *Scheme 2* on the example of (+)-(1*R*,6*R*,9*R*)-6-methyl-4-oxabicyclo[7.5.1]pentadecan-3-one (**10**), which turned out to be the most musky target compound synthesized. Introducing an auxiliary C=O function adjacent to the CH_2 bridge and in 1,6-relation to the carbonyloxy group in **10** gives the oxo lactone **11** as a suitable intermediate, which should be easily accessible by oxidative cleavage of the hexahydrochromene system of **12**, either by ozonolysis or by the catalytic RuO_4 oxidation of *Sharpless*. Dissecting the dihydropyranyl ring to a hydroxy ketone by *retro* enol ether formation then offers the possibility of introducing the stereogenic Me-substituted center *via* **13** by alkylation of (1*R**,7*S**)-bicyclo[5.3.1]undecan-9-one with a protected hydroxy halide, which can be prepared from the corresponding hydroxy esters by protection of the OH group as silyl ether, reduction of the ester moiety with DIBAH, and halogenation of the resulting OH group. The diaxially linked (1*R**,7*S**)-bicyclo[5.3.1]undecan-9-one, on the other hand, is available by heterogeneous hydrogenation of the bicyclic enone **14**. Introducing an auxiliary EtOCO function in the 10-position of bicyclo[5.3.1]undec-7-en-9-one (**14**) reveals **15** as *Michael* addition product of ethyl acetoacetate to cyclooct-2-enone with subsequent intramolecular aldol condensation [21]. According to the same synthetic scheme, bicyclo[8.4.1]macrolides

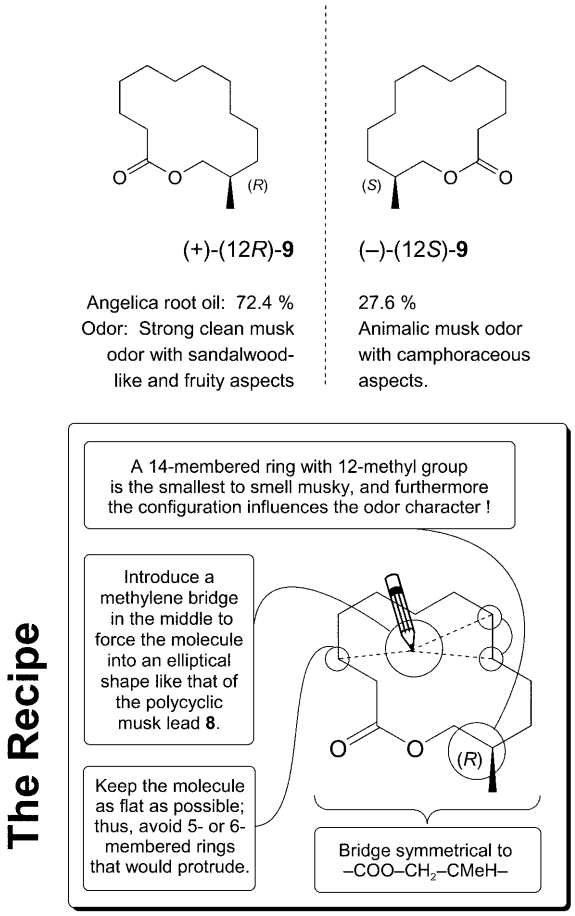
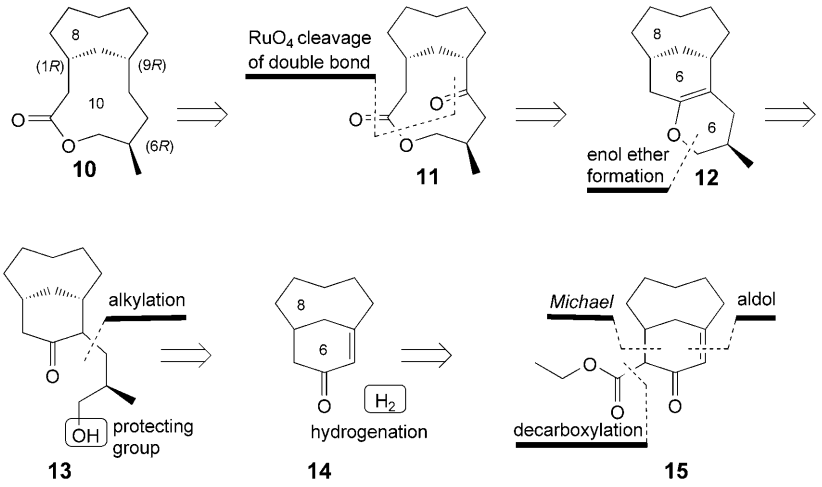


Fig. 4. *The naturally occurring 12-methyltridecano-13-lactone, and how to conformationally constrain it by introduction of a CH_2 bridge*

can also be synthesized, starting from cyclohept-2-enone and employing 4-halobutoxy silanes in the alkylation step.

Besides (+)-(1*R*,6*R*,9*R*)-6-methyl-4-oxabicyclo[7.5.1]pentadecan-3-one (**10**), (+)-(1*S*,6*R*,9*S*)-6-methyl-4-oxabicyclo[7.5.1]pentadecan-3-one (**16**), (1*R**,10*S**)-4-oxabicyclo[8.4.1]pentadecan-3-one (**17**), (1*R**,9*R**)-4-oxabicyclo[7.5.1]pentadecan-3-one (**18**), and (+)-(1*S*,6*R*,10*R*)-6-methyl-4-oxabicyclo[8.4.1]pentadecan-3-one (**19**) were prepared following this synthetic route [20]. They are depicted with their X-ray crystal structure or with the structure calculated (PM3) from the crystal structure of the corresponding oxo lactones in *Fig. 5* in the order of their muskiness: **10** > **16** ≫ **17** > **18** ≫ **19**. So at first sight it seems that there is just one receptor for all classes of musk odorants, and it also seems correct to choose *Galaxolide*® (**8**) as the template for the olfactophore model of musk odorants, since **10** indeed superimposes best on the crystal structure of (−)-(4*S*,7*R*)-**8** [20], while **19**, which possesses a woody-ambery odor devoid

Scheme 2. *Retrosynthetic Analysis of (+)-(1*R,*6*R,*9*R*)-6-Methyl-4-oxabicyclo[7.5.1]pentadecan-3-one* (**10**), *the Most Musky Macrobicycle Synthesized*



of any musk tonality, does worst. But then in terms of intensity, the picture looks quite different: **19** > **10** > **16** ≫ **17** > **18**. Furthermore, **10** is much weaker than both racemic 12-methyltridecano-13-lactone (**9**) and racemic *Galaxolide*® (**8**), while actually it should be (much) stronger because it would correspond to the active conformation of (+)-(12*R*)-**9** on the receptor. How does this all make sense? We can only speculate, but while the shape similarity of **8** and **10** is apparent and is an argument for a common receptor, the higher intensity of **8** may account for an aromatic ring binding site on the receptor, to which benzenoid musks could dock, while their macrocyclic counterparts such as **9** could only address hydrophobic binding sites, and thus need a higher flexibility and a larger perimeter to gain the same affinity on the receptor.

Linear Musk Origami. – We have just seen that it is better to use macrocyclic rather than polycyclic musks as templates in the design of new musk structures if these were to be non-aromatic. Such new musk structures are the so-called linear musks, which had harbingers in ethyl citronellyl oxalate (**20**) and *Rosamusk* (**21**); two odorants that emanate floral, rosy, geranium-like odors, but that also possess discernible musk notes, however, by far not characteristic enough to be considered and used as musks. In 1975, *von Fraunberg* and *Hoffmann* of *BASF* [22] discovered in *Cyclomusk* (**22**) the first representative of a new family of musk odorants, which are neither macrocyclic nor benzenoid, and became known as linear musks. *Cyclomusk* (**22**) possesses a fruity, strawberry-type musk odor. Samples of it were shown to the market, but **22** had at that time no chance against polycyclic musks such as *Galaxolide*® (**8**), and, therefore, never made it to full production stage. Then in 1990, *Giersch* and *Schulte-Elte* of *Firmenich* [23] discovered *Helvetolide*® (**23**), which became the first representative of the family to make it to production scale. It exhibits a musky-floral, fruity, pear-like musk odor, and was, for instance, used at 3.8% in '*Flower*' (*Kenzo*, 2000), at 6.1% in '*Miracle*' (*Lancôme*, 2000), and even at 8.8% in '*Emporio White Her*' (*Armani*, 2001) [2].

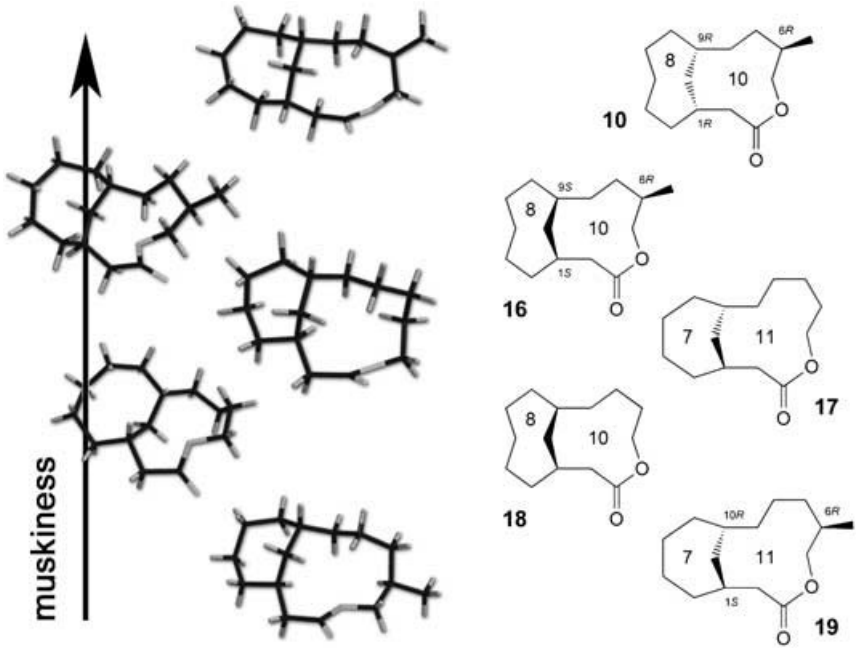


Fig. 5. *Comparison of the muskiness of the macrobicyclic lactones synthesized.* The 3D structures were derived by X-ray crystallography of either the target compounds or the corresponding oxo lactones.

Williams of *Firmenich* [24] then made in 1998 the surprising discovery that the gem-dimethyl ether motive of **23** can be replaced by an ester moiety without loss of the musk odor, in fact even with a slight gain in intensity. The resulting musk odorant **24** was introduced into perfumery as '*Romandolide*', and found for instance use in '*Absolu*' (*Rochas*, 2002) and '*Murmure*' (*Van Cleef & Arples*, 2002) [2].

The structures of *Helvetolide*® (**23**) and *Romandolide* (**24**) are remarkable for the gem-dimethyl group and the additional C=O group, respectively. An additional C=O group, except when in 1,5- or 1,6-position, causes macrocyclic ketones and lactones to become weak to odorless [2][25], and a gem-dimethyl substitution in macrocycles generally decreases the muskiness dramatically [2][25]. So, these linear musks must have different conformational preferences than macrocycles, and apparently those structural elements have a decisive influence. As is depicted in *Fig. 6*, the donor–acceptor interaction of the bonding σ-orbital of Me–C(2′) with the antibonding σ*-orbital of C(1′)–O forces O–C(1′)–C(2′)–O in *Helvetolide*® (**23**) into a *synclinal* conformation, while the steric interaction of Me–C(1″) and $Me_2C(2')$ forces C(2′)–O–C(1″)–C(1‴) into an *anticlinal* conformation. In effect, the molecule is bent twice, and adopts a horseshoe-shaped conformation, which makes up three-quarters of the perimeter of a macrocyclic musk. In the diester *Romandolide* (**24**), the '*eclipsed*' C(1′)=O function performs the role of the bulky gem-dimethyl group of **23** in terms of steric interaction with Me–C(1″). The electrostatic interaction of the carbonyloxy O-atom with the partially positive carbonyloxy C-atom C(1) induces the

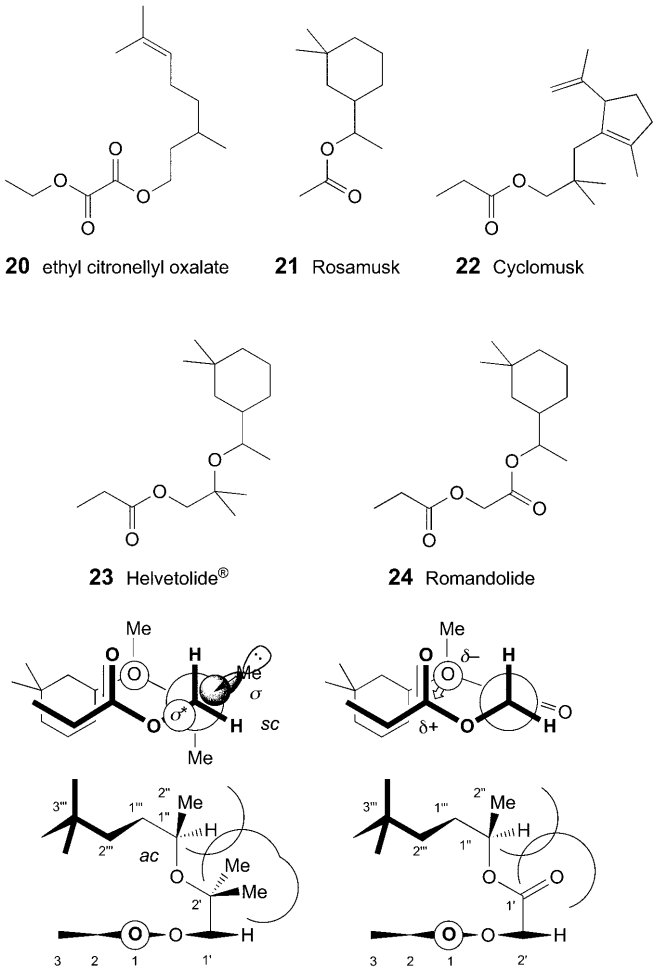


Fig. 6. *Overview of linear musk structures and conformational analyses of* Helvetolide® (**23**) *and* Romandolide (**24**)

synclinal conformation of O–C(2′)–C(1′)–O to lead to a similarly shaped minimum-energy conformer for *Romandolide* (**24**).

These conformational considerations gave us the idea of introducing an additional cycloalkyl moiety in *Helvetolide*® (**23**) [26] right opposite to the dimethylcyclohexyl ring. Indeed, the cyclopropanoate **25** (*Fig. 7*) turned out to be a very powerful musk, more substantive and also 4–5 times more intense than **23**. Even the cyclopentanoate **26** still smells musky with a similar threshold as *Helvetolide*® (**23**), and, with a molecular weight of 325 u for $C_{20}H_{36}O_3$, it also holds the record as the heaviest musk odorant known to date, perhaps even the heaviest of all families if *Schiff* bases are not considered actual odorants. Next, we wanted to investigate the influence of a C=C bond in the dimethylcyclohexyl ring on the musk odor. Placing this C=C bond at the

ring stereocenter adjacent or opposite to the gem-dimethyl group not only avoids the formation of diastereoisomers, but, most importantly, offers the possibility to use this C=C bond later on as a rigid structural element in the construction of new musk odorants without branched alicyclic moieties. Such '*all-aliphatic*' musks should possess higher vapor pressures, and better diffusivity.

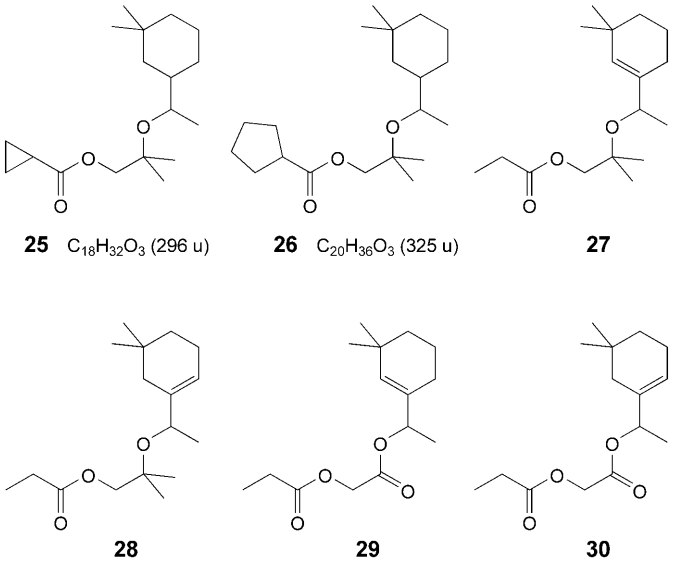


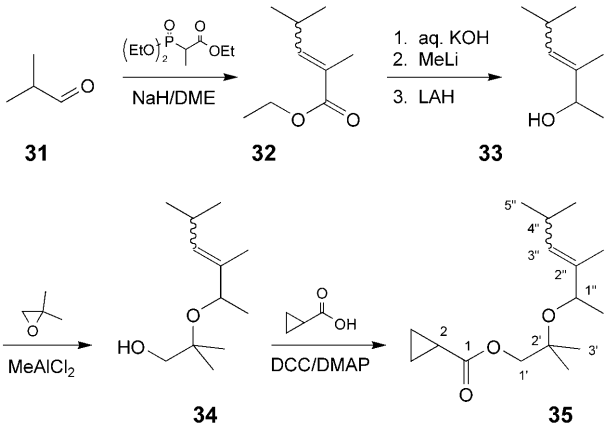
Fig. 7. *Exploration of the structural features of linear musks.* The cyclopentanecarboxylate **26** constitutes the odorant with the highest molecular weight known.

Starting from artemone (=1-(3,3-dimethylcyclohex-1-enyl)ethanone) and 1-(5,5-dimethylcyclohex-1-enyl)ethanone, available by *Rupe* rearrangement of ethynylcyclohexanol, we prepared the unsaturated analogs **27**, **28**, **29**, and **30** of *Helvetolide*® (**23**) and *Romandolide* (**24**) [27]. We found that the introduction of the C=C bond indeed had a major impact on the intensity and character of the compounds. The *Helvetolide*® analog **27** with the C=C bond adjacent to the gem-dimethyl group turned out to be the most-intense and most-typical musk odorant of the series, being *ca.* 5–6 times more powerful than *Helvetolide*® (**23**). It is followed by **29**, which is about as strong as *Romandolide* (**24**). Next in the order of intensity and muskiness are **28** and **30**, with the latter being already *ca.* 10 times weaker than **23** [27]. In conclusion, the 3,3-dimethylcyclohexyl derivatives possess the more distinct and more intense musk odors.

This finding keyed the design of structurally new musk odorants, the synthesis of a typical representative of which is detailed in *Scheme 3*. The target odorant **35** was devised conceptually by cutting C(4‴) and C(5‴) out of **27**, and with the superior olfactory properties of **25** in mind, by exchanging the propanoate for a cyclopropanecarboxylate group. The synthesis of **35** was carried out by *Wittig–Horner–Emmons* reaction of 2-methylpropanal (**31**) with triethyl 2-phosphonopropanoate, saponification of the resulting ester **32** with KOH, reaction of the formed acid with MeLi and $LiAlH_4$ (LAH) reduction to the corresponding pentenol **33**. Etherification of

33 with dimethyloxirane and subsequent *Steglich* esterification of the hydroxy ether **34** with cyclopropanecarboxylic acid completed the synthesis of **35**, which indeed emanated a powerful, sweet musk odor with slightly fruity nuances.

Scheme 3. *Synthesis of 2-Methyl-2-[(1,2,4-trimethylpent-2-enyl)oxy]propyl Cyclopropanecarboxylate* (**35**)



Does **35** show similar conformational preferences as **23** and **24**, which could account for its pronounced musk character? *Fig. 8* details selected lowest-energy conformers of **35**, all of which are horseshoe-shaped. In the global-minimum-energy conformer, the O–C(1′)–C(2′)–O unit is *synclinal* like in *Helvetolide*® (**23**) and *Romandolide* (**24**) shown in *Fig. 6*. The torsion angle of C(2′)–O–C(1″)–C(2″) in **35** is with almost exactly 90° for **35** smaller than the corresponding one in **23** (130°), but the molecular shapes of **23** and **35** superimpose very well. The same is true for the next conformer of **35**, which is 0.42 kcal/mol higher in energy than the global minimum. The torsion angle for C(2′)–O–C(1″)–C(2″) is again almost exactly 90°, the O–C(1′)–C(2′)–O moiety is *synclinal* though with a 17° greater dihedral angle. Then, 1.54 kcal/mol above the global minimum follows a conformer, in which $Me_2C(2')$ constitutes a *gauche*-corner, and the cyclopropanecarboxylate edge is consequently two atoms longer. The next two conformers, 1.83 kcal/mol and 1.85 kcal/mol, above the global energy minimum, however, correspond very well with the first two lowest-energy conformers, and, at 1.88 kcal/mol, we even find a conformer that equals the *Helvetolide*® (**23**) conformer in *Fig. 6*. So the only outlier is the conformer at 1.54 kcal/mol with the corner atom at $Me_2C(2')$ and a torsion angle of 80° for C(1′)–C(2′)–O–C(1″). How could we investigate if such a conformer is responsible for or at least contributes to the distinct musk note of **35**?

The easiest way to ensure that the corner atom is not in α-position to the gem-dimethyl-substituted quaternary C-atom is to introduce an (*E*)-configured C=C bond in between the C=O osmophore and the gem-dimethyl-substituted C-atom (*Scheme 4*). The resulting α,β-unsaturated ketone **36**, which, for synthetic ease, bears an Et rather than a cyclopropyl substituent, can still be s-*cis*- or s-*trans*-configured, but it cannot adopt the model conformation of *Fig. 6*. The synthesis of **36** started from compound **34**, the intermediate hydroxy ether in the synthesis of **35**. Oxidation with

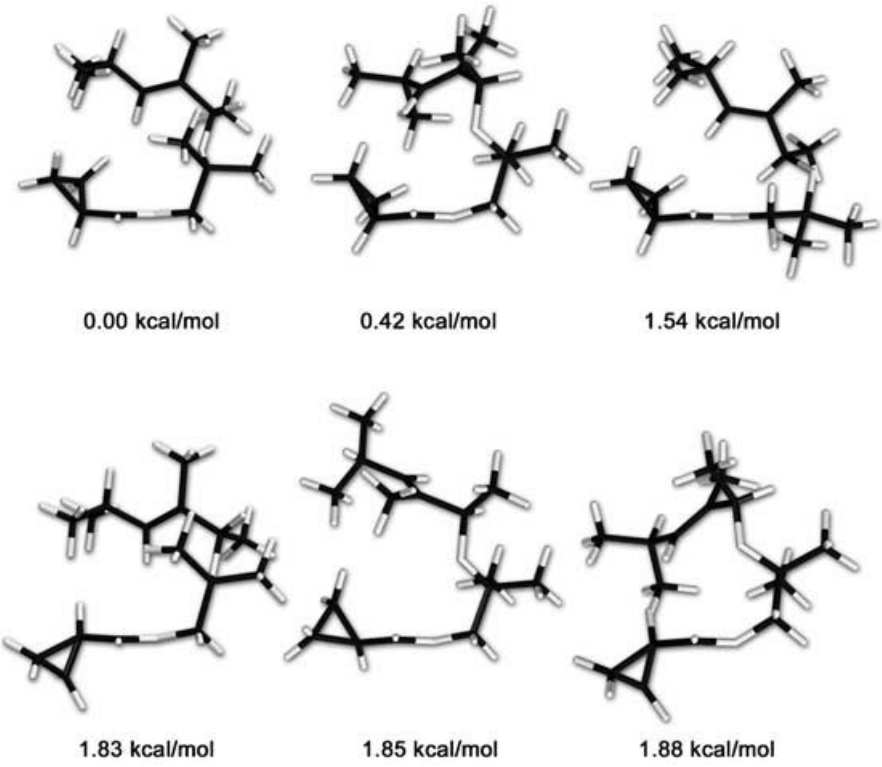


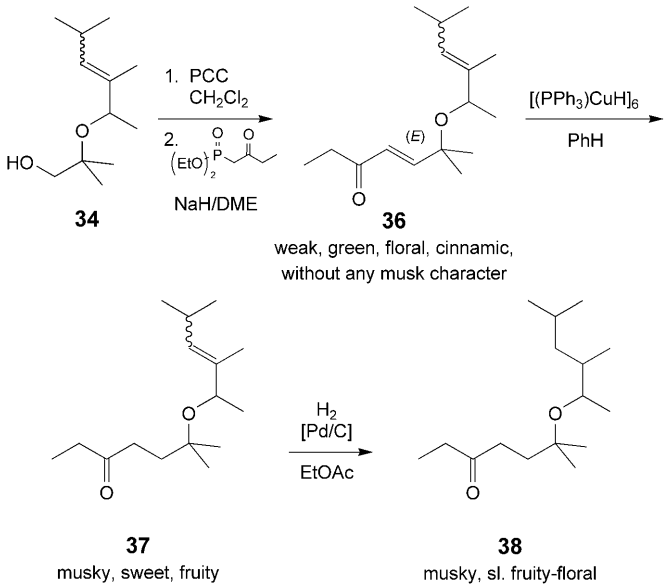
Fig. 8. *Selected lowest-energy conformers* (PM3) *of the musk odorant* **35**

pyridinium chlorochromate (PCC) on *Celite*® furnished the corresponding aldehyde, which was transformed to **36** by *Wittig–Horner–Emmons* reaction with diethyl (2-oxobutyl)phosphonate [27]. The synthesized α,β-unsaturated ketone **36** indeed did not smell musky at all, but emanated a relatively weak green, floral, cinnamic odor.

If our considerations were correct, we should be able to *switch on* the musk odor by selective hydrogenation of the α,β-unsaturated C=C bond of **36** as conformers with corner atoms in α-position to $Me_2C(6)$ should prevail in **37**. This was realized by employing the copper(I) hydride cluster $[(Ph_3P)CuH]_6$ in deoxygenated benzene for the synthesis of **37**. After flash chromatography, the heptanone **37** was isolated in 81% yield, and, in fact, possessed a musky, sweet, and fruity odor of even lower threshold than **23** or **24**. Of course, we were also eager to investigate the fully saturated 6-(alkoxy)-6-methylheptan-3-one **38**, and so we hydrogenated **37** in the presence of Pd on activated carbon. Even though the 3,5-dimethylhex-2-yl tail makes **38** conformationally more flexible than **37**, **38** also possesses a typical musk odor of slightly fruity-floral connotation, and it is only insignificantly weaker than **37** in terms of its threshold.

As the final example of our *adventures in 'brain aided' musk design*, the synthesis, olfactory evaluation and conformational analysis of the ethoxycarbonylmethyl 2-(3,3-dimethylcyclohexyl)propanoate (**42**) is presented here. The idea behind this target

Scheme 4. *Synthesis of All-Aliphatic Ketones*

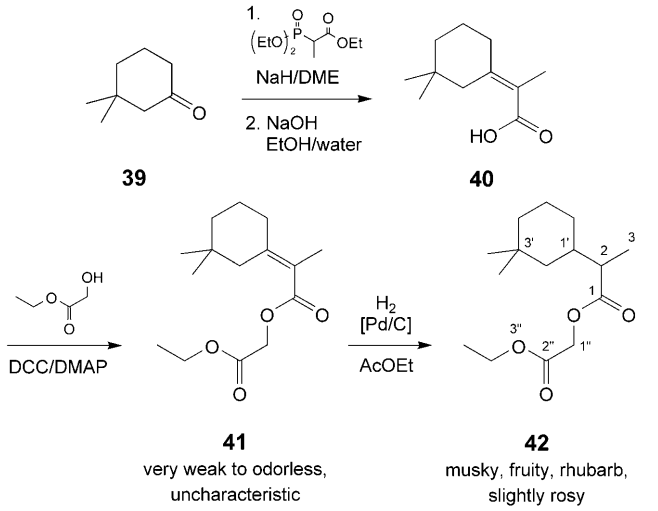


structure was to investigate the *non-osmophoric* ester function of *Romandolide* (**24**) by inverting it. Since there will be less electrostatic interaction between the two ester functions in **42** than in **24**, we cannot expect the same conformation as depicted in *Fig. 6* for **24**; however, the alkoxy C–O bonds of both ester groups have considerable double-bond character, and steric repulsion in the (*E*)-conformation, as well as the n–σ* donor–acceptor interaction should strongly favor a (*Z*)-configuration of the two ester functions. Thus, the $CH_2(1''')$ C-atom between the two (*Z*)-configured ester functions is expected to be situated in a corner position with C(O)–C(1‴)–O–C(O) in *synclinal* conformation. Thus, due to the resulting shape similarity with **23** and **24**, we would expect **42** to smell musky as well.

We synthesized **42** by *Wittig–Horner–Emmons* reaction of 3,3-dimethylcyclohexanone (**39**) with triethyl 2-phosphonopropionate, saponification of the formed α,β-unsaturated ester, *Steglich* esterification of the corresponding acid **40** with ethyl glycolate and subsequent hydrogenation of the unsaturated intermediate **41** in the presence of Pd on activated carbon (*Scheme 5*). As anticipated, the '*inverted*' diester **42** had a typical musk character with fruity nuances in the direction of rhubarb and some rosy facets. Both diastereoisomers of **42** smell relatively similar in tonality, but compared to *Romandolide* (**24**) they are both weaker, in terms of threshold by a factor of *ca.* 4 and 12, respectively. The unsaturated intermediate **41** is, however, only uncharacteristic in smell, and very weak to almost odorless.

The lowest-energy conformers of the linear musk **42** are compiled in *Fig. 9*. For the global-minimum conformer, we find a *synclinal* torsion angle of 85° for C(O)–C(1‴)–O–C(O) and an *anticlinal* torsion angle of 115° for O–C(1)–C(2)–C(1′), leading again to a horseshoe-shaped molecular structure. The

Scheme 5. *Synthesis of the Linear Musk* **42** *with an Inverted Diester Motive*



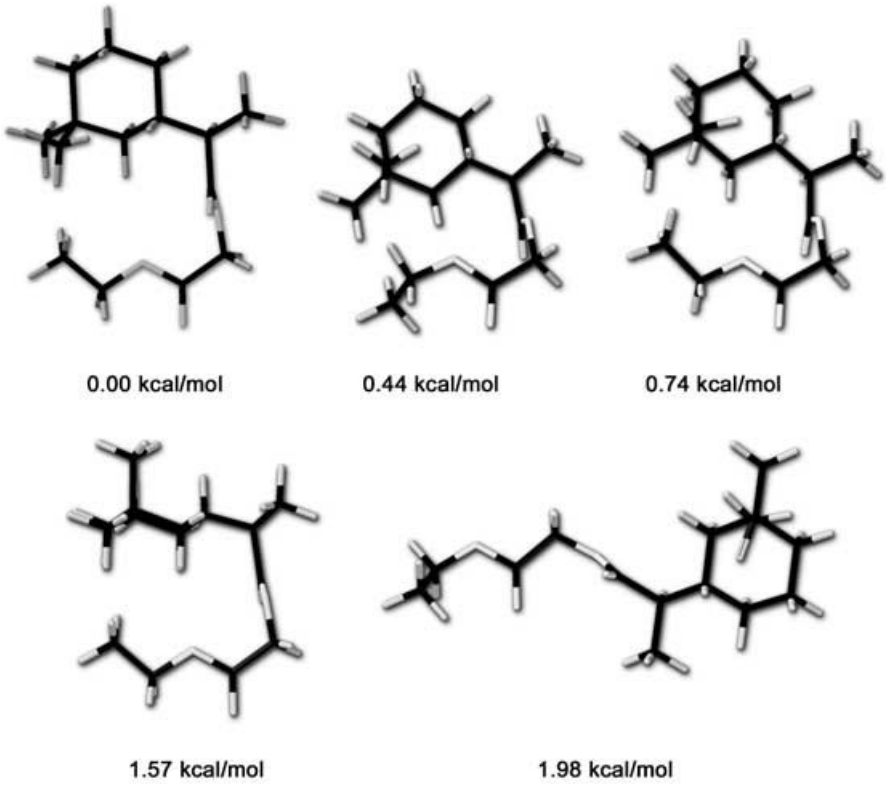


Fig. 9. *Selected lowest-energy conformers* (PM3) *of the linear musk* **42**

two next higher conformers at 0.44 and at 0.74 kcal/mol resemble the global energy minimum quite closely, and also the one 1.57 kcal/mol above the global minimum is still horseshoe-shaped, though the dihedral angles deviate somewhat from those of the lower-energy conformers with, for instance, 90° for O–C(1)–C(2)–C(1′). At 1.98 kcal/mol, we find, however, a conformer that is not horseshoe-shaped, but drawn out long. Based on our previous reflections, we would not expect such conformers to contribute to a musky odor sensation, and thus the occurrence of such conformers may account for the weaker intensity of **42** as compared to *Romandolide* (**24**). In the intermediate **41**, the α,β-unsaturated ester moiety favors in both (*Z*)- and (*E*)-configuration of the C=C bond, and in both s-*cis*- and s-*trans*-configuration of the carbonyl group, conformers that resemble the local minimum of **42** at 1.98 kcal/mol. This would explain why compound **41** does not smell musky, and why it is almost completely odorless.

In summary, we hope to have demonstrated that the critical challenge of computational models can provide new insights into structure–odor relationships, and how simple and inspiring concepts derived from such investigations can lead to the creative '*brain aided*' design of structurally new odorants.

Experimental Part

General. Unless otherwise stated, all reactions were performed under N_2. Reagents and solvents: *Fluka* (*puriss.* or *purum*), used without further purification. Flash chromatography (FC): *Merck Kieselgel 60*, particle size 40–63 μm. TLC: *Merck Kieselgel 60*, particle size 5–20 μm, layer thickness 250 μm on glass, 5 cm × 10 cm, visualization reagent: PMA spray solution for TLC, *Merck* 1.00480.0100. IR: *VECTOR 22/Harrick SplitPea ATR* (attenuated total reflection) spectrometer, Si, $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR: *Bruker AVANCE DPX-400* spectrometer, δ in ppm rel. to Me_4Si, *J* in Hz. MS: *Finnigan MAT 95* instrument and *HP Chemstation 6890 GC/5973 Mass Sensitive Detector* GC/MS station, rel. int. in % of the base peak.

Ethoxycarbonylmethyl 2-(3,3-Dimethylcyclohexyl)propanoate (**42**). A soln. of triethyl 2-phosphonopropionate (79.8 g, 335 mmol) in 1,2-dimethoxyethane (DME; 70 ml) was added dropwise within 30 min to a stirred suspension of 95% NaH (7.54 g, 300 mmol) in 1,2-dimethoxyethane (350 ml). The mixture was heated to reflux, and 3,3-dimethylcyclohexanone (**39**; 63.0 g, 500 mmol) was added during 5 min. After refluxing for 15 h, the mixture was poured onto crushed ice (600 g), acidified to pH 5 by addition of AcOH (*ca.* 18 ml, 315 mmol), and extracted with Et_2O (2 × 1 l). The combined org. extracts were washed with H_2O and sat. aq. NaCl, dried ($MgSO_4$), and concentrated on the rotary evaporator to provide the crude α,β-unsaturated ester (89.2 g). Distillation *in vacuo* afforded at 89–90°/3 mbar ethyl 2-(3,3-dimethylcyclohexylidene)propanoate (61.3 g, 97%). The ethyl 2-(3,3-dimethylcyclohexylidene)propanoate (30.0 g, 143 mmol) was dissolved in EtOH/H_2O 1:1 (300 ml), and NaOH (28.6 g, 715 mmol) was added with stirring. After heating to reflux for 5 h, EtOH was distilled off, and the mixture was diluted with H_2O (500 ml) prior to extraction. The ethereal washings were discarded, the aq. soln. was acidified with conc. H_3PO_4 and extracted with Et_2O (3 × 700 ml). The combined org. extracts were dried ($MgSO_4$), and the solvent was evaporated on a rotary evaporator to furnish 2-(3,3-dimethylcyclohexylidene)propanoic acid (**40**; 24.6 g, 95%) sufficiently pure for further transformations. At 0°, this crude **40** (3.00 g, 16.5 mmol) was dissolved in CH_2Cl_2 (40 ml) and treated with ethyl glycolate (1.71 g, 16.4 mmol). Then, 4-(dimethylamino)pyridine (DMAP; 2.01 g, 16.5 mmol) was added at 0°, and, after stirring at this temp. for 5 min, a soln. of 1,3-dicyclohexylcarbodiimide (DCC; 3.74 g, 18.1 mmol) in CH_2Cl_2 (20 ml) was added dropwise. The cooling bath was removed, and stirring was continued at r.t. for 15 h before separating the precipitates by vacuum filtration. The precipitate was washed with CH_2Cl_2 (100 ml), and the combined filtrates were concentrated on the rotary evaporator. The crude product (7.55 g) was purified by FC (silica gel; pentane/Et_2O, 19:1) to provide **41** (3.96 g, 90%) as a colorless liquid of very weak odor. Pd 10% on activated carbon (0.10 g, 0.094 mmol) was added to a stirred soln. of **41** (1.00 g, 3.73 mmol) in AcOEt (10 ml). After two cycles of evacuating the reaction flask and flushing with N_2, the flask was evacuated again, and flushed with H_2. After stirring in an H_2 atmosphere for 1 d, the reaction flask was again evacuated twice and flushed with N_2. The

catalyst was separated by vacuum filtration over a pad of *Celite*® and washed with AcOEt (100 ml) to provide the crude material (1.02 g), which was purified by bulb-to-bulb distillation to furnish at 75–85°/0.04 mbar odoriferous **42** (0.96 g, 96%). IR (neat): 1744*s*, 1764*m* (C=OO); 1144*s* (C–O). ^{1}H-NMR ($CDCl_3$): 0.90 (*s*, $Me_2C(3')$); 0.91–1.60 (*m*, $CH_2(2')$, $CH_2(4')$, $CH_2(5')$, $CH_2(6')$); 1.15/1.16 (*d*, *J* = 7.5, Me(3)); 1.28 (*t*, *J* = 7.0, Me(5″)); 1.76 (m_c, H–C(1′)); 2.29/2.30 (*quint.*, *J* = 7.5, H–C(2)); 4.22 (*q*, *J* = 7.0, $CH_2(4'')$); 4.60 (*s*, $CH_2(1'')$). ^{13}C-NMR ($CDCl_3$): 13.7/13.8/13.9/14.0 (*q*, C(3, 5″)); 22.0/22.1 (*t*, C(5′)); 24.4/24.5 (*q*, Me_{ax}–C(3′)); 29.0/30.8 (*t*, C(6′)); 30.6/30.7 (*s*, C(3′)); 33.3/33.4 (*q*, Me_{eq}–C(3′)); 36.1/36.2 (*d*, C(1′)); 38.8/38.9 (*t*, C(4′)); 42.4/43.9 (*t*, C(2′)); 45.1/45.2 (*d*, C(2)); 60.3/60.3/61.0/61.1 (*t*, C(1″,4″)); 167.7/167.8 (*s*, C(2″)); 175.6/175.7 (*s*, C(1)). MS (70 eV): 270 (1, M^+), 255 (1, $[M - Me]^+$), 225 (8, $[M - EtO]^+$), 185 (5, $C_{11}H_{21}O_2^+$), 167 (11, $[C_{11}H_{21}O_2 - H_2O]^+$), 160 (100, $C_7H_{12}O_4^+$; *McLafferty* rearr.), 114 (78, $[C_7H_{12}O_4 - EtOH]^+$), 95 (54, $C_7H_{11}^+$), 69 (48, $C_5H_9^+$), 56 (59, $C_4H_8^+$). Odor description: Musky, fruity, rhubarb, slightly rosy. Odor thresholds: 2.6 and 8.0 ng/l air for the two diastereoisomers, resp.

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New Alicyclic Musks: The Fourth Generation of Musk Odorants

by **Marcus Eh**

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Musk odorants are one of the most important classes of fragrances in perfumery because they impart sensuality to perfume-oil compositions. Among the three well-known classes of musks, a new and very exciting generation of musk odorants, the so-called *alicyclic musks*, was discovered recently, of which *Helvetolide*® (**2**) and *Romandolide*® (**3**) are the most popular representatives so far. To find new, structurally related alicyclic musks, we have synthesized a library of 114 unique alicyclic molecules with modified cyclohexyl moieties. The olfactory properties of all compounds were evaluated to identify the structural requirements to be met for a musk odorant.

Introduction. – Musk can be considered the king of fragrances. In centuries past, musk was a true luxury, an exquisite scent, a noble fragrance that only the truly wealthy could afford. Perfume manufacturers used to pay the equivalent of more than 50 Euros per gram of musk, approximately five times the price of gold. Today, the typical scent of musk, described as *warm, sensual, animalic*, and *natural* [1] can be found in shampoos, soaps, perfumes, and laundry detergents.

In general, three main structural classes of musk odorants are known: nitro musks, polycyclic musks (PCMs), and macrocyclic musks (MCMs) [2], all of which possess excellent sensory properties. Today, nitro musks and PCMs are discussed very controversially, because massive production volumes and nonbiodegradability have led to bio-accumulation [3]. In contrast, the MCMs, which exhibit an even higher tenacity and substantivity than nitro musks and PCMs, are biologically degradable. However, some MCMs are still comparatively expensive, especially those that occur naturally, *i.e.*, *Muscone*®, *Exaltone*®, and *Civetone*. Hence, the discovery of musk odorants that are biodegradable and economical to manufacture is still of great interest. This led to the development of a new generation of musk fragrances, the so-called *alicyclic musks* (ACMs).

The history of ACMs began in 1975 with the discovery of the trisubstituted cyclopentene derivative *Cyclomusk*® (**1**) by *Hoffmann* and *von Fraunberg* at *BASF* (*Fig. 1*) [4]. *Cyclomusk*® (**1**) possesses a *fruity, strawberry-like musk odor*. Some 15 years later, *Helvetolide*® (**2**), a further representative of this exceptional family of musk odorants, was discovered by *Giersch* and *Schulte-Elte* at *Firmenich* [5]. It emanates a *fruity and pear-like musk odor*. In 2000, *Williams* of *Firmenich* [6] found that the $OCMe_2$ ether moiety of **2** can be replaced by an ester function without losing the musk note. The resulting musk odorant **3**, which was introduced into perfumery as *Romandolide*®, was claimed to be *less fruity and more ambrette-like in smell than Helvetolide*® (**2**). Both odorants can be found in several perfume oils in different applications.

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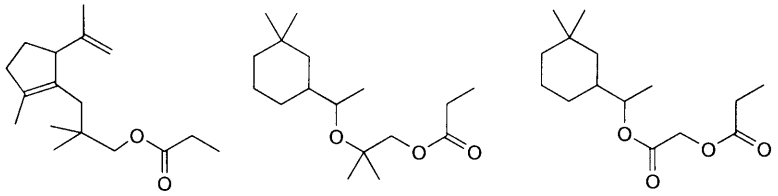


Fig. 1. *Renowned representatives of the new generation of acyclic musk odorants*

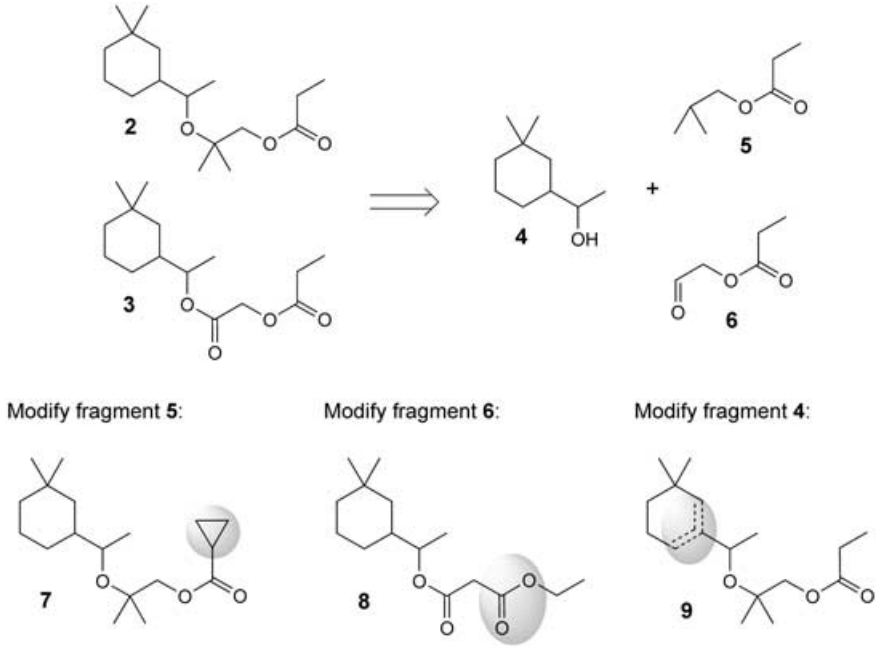
The aforementioned ACMs do not belong structurally or sensorily to the three known generations of musk odorants. The perfumery materials **2** and **3** can be disconnected from 1-(3,3-dimethylcyclohexyl)ethanol (**4**) and the propanoate side chains **5** and **6**, respectively (*Scheme 1*). Recent strategies for the discovery of new ACMs were as follows: one fragment, either the cyclohexyl moiety or the propionate side chain, was kept unchanged, while the other was modified. Following this strategy, *Kraft* and *Cadalbert* of *Givaudan* [7] modified the side chains of **2** and **3** in a way that they obtained cycloalkane carboxylates, of which the cyclopropyl compounds turned out best. Interestingly, only the cyclopropane carboxylate **7**, analog of **2**, possesses a *strong and intense musk odor*, while the corresponding analog of **3** was *weak and uncharacteristic*. In a second approach, *Bledsoe et al.* of *IFF* [8], also leaving the cyclohexyl moiety intact, synthesized carbonates, oxalates, and malonates from **4**. The ethyl malonate **8** was described as the molecule with the strongest musk odor. Last but not least, *Kraft* and *Eichenberger* of *Givaudan* [9] introduced a C=C bond in the cyclohexyl moiety of **9**, with the side chains unaltered. All synthesized molecules of this type possess musk odors with different connotations.

In our own studies, we synthesized alicyclic musk odorants in which we left the side chains **5** and **6** intact, but modified the cyclohexyl moiety. Our concept was to vary the ring size and the substitution pattern, and, in some cases, to introduce also a C=C bond in the ring. This approach promised that many different cycloalkanols could be prepared and subsequently esterified or etherified with fragments **5** and **6**, respectively.

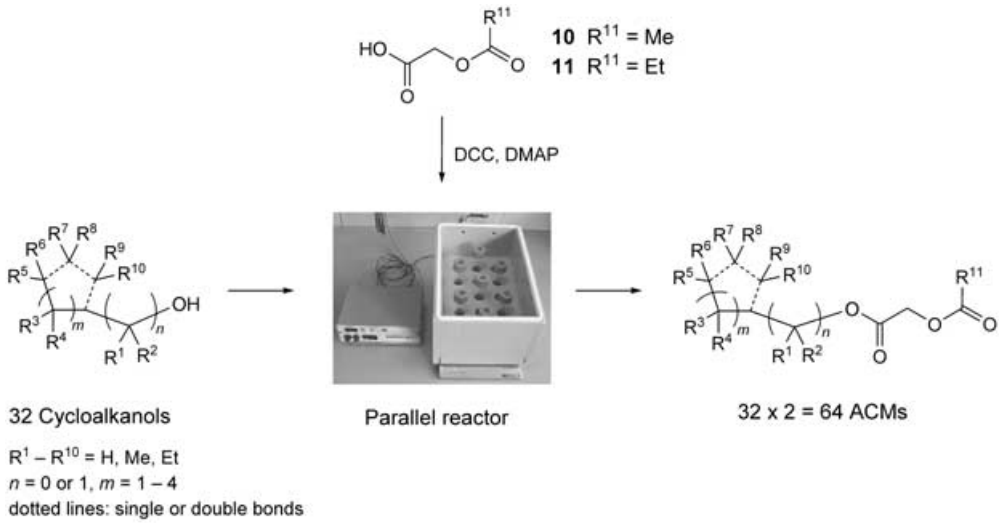
Results and Discussion. – We focused on a solution-phase parallel synthesis of ACM libraries, since it allowed the preparation of the library compounds in sufficient quantities by means of existing methodologies. The side-chain compounds **10** and **11** can be easily obtained by reaction of glycolic acid with acetyl or propanoyl chloride (*Scheme 2*) [10]. After the reaction was complete, the excess acid chloride was removed under reduced pressure, and the residue was distilled *in vacuo* to afford **10** and **11** in good yields. Esterification with a set of 32 different cycloalkanols under *Steglich* conditions [11] in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 was performed in the parallel reactor (*Scheme 2*). The classical workup procedure was replaced by simple parallel filtration of the reaction mixtures over silica gel/magnesium sulfate. After parallel evaporation of the solvent, the residues were purified by flash chromatography.

In the parallel reactor, we were able to carry out 15 reactions simultaneously, with a maximum reaction volume of 25 ml. The reactions could be performed under inert

Scheme 1. *Strategy for the Discovery of New Acyclic Musks*



Scheme 2. *Synthesis of* Romandolide® (**3**) *Analogs*



atmosphere or *in vacuo*, and the temperature was adjustable from $-60°$ to $+150°$ by means of a cryostat. With 32 different cycloalkanols and two side-chain compounds, we synthesized in five runs 64 crude alicyclic esters of the *Romandolide*® (**3**)-type. The ACM library was subsequently submitted to sensory evaluation by a panel of professional perfumers. However, all compounds with cyclopentenyl-, cycloheptyl-, or cyclooctyl-ring moieties were found to possess no musk odor. Interestingly, the ten molecules of the library that emanated musk odors exhibited exclusively a cyclohexyl moiety, with at least one Me group in the ring (*Fig. 2*).

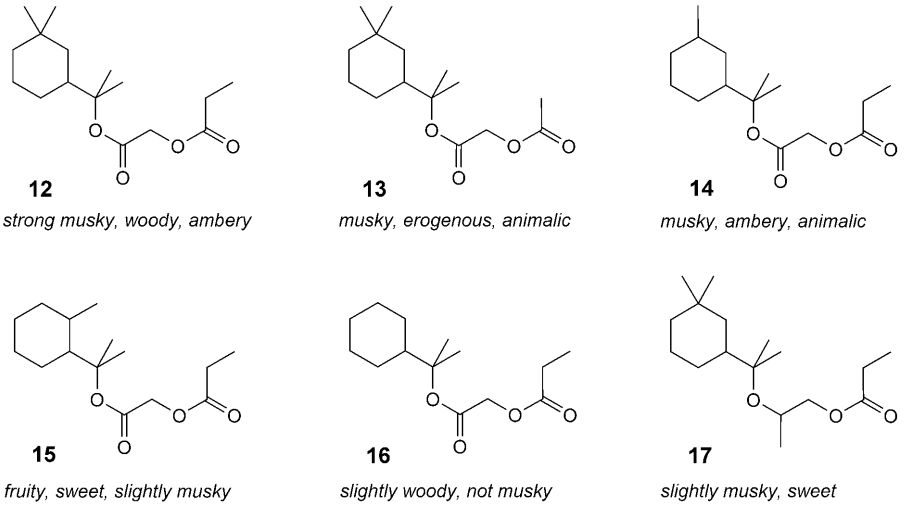


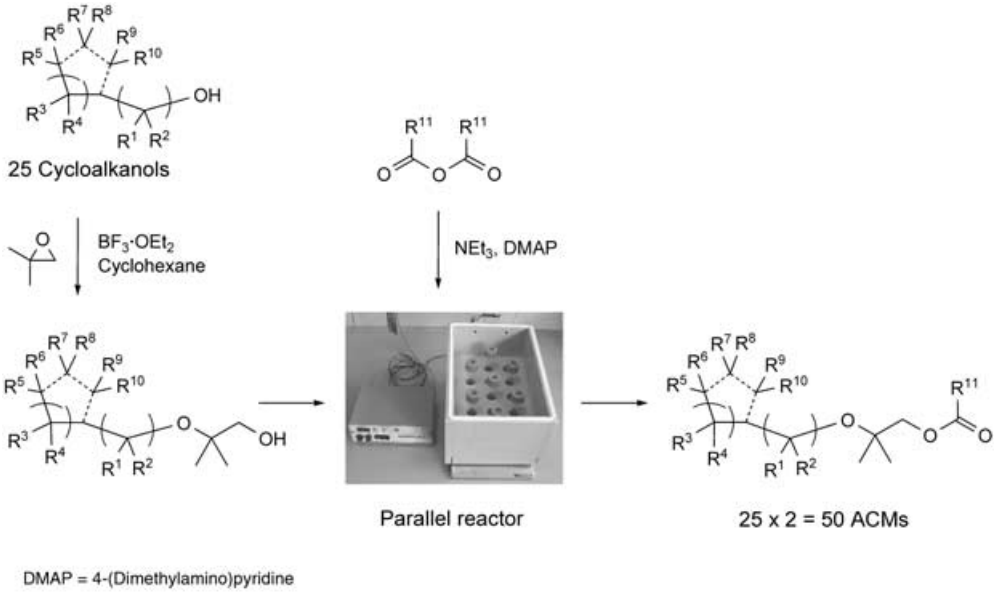
Fig. 2. *Odor descriptions of the* Romandolide® *analogs* **12**–**17**

Compound **12** was the most-intense musk odorant of this series; its musk odor was accompanied by *woody and ambery connotations*. Exchanging the propanoate group of **12** for an acetate group led to a decrease in the musk intensity of **13**, and the tonalities were *less ambery and more erogenous*. When we removed one of the two Me groups of the cyclohexyl ring, the musk intensity of the resulting molecule **14** was much weaker than that of **12**, but an *ambery aspect* was still clearly present. Shifting the Me group from C(3) to C(2) of the ring led to compound **15**, which had a *weak musk odor paired with some fruity and sweet tonalities*. The complete removal of both Me groups from the cyclohexyl ring of **12** provided the propanoate **16**, which no longer possessed a musk odor, but had only *slightly woody* aspects. Finally, we replaced the ester moiety of **12** by an α,α-disubstituted ether fragment, in analogy to the conversion **3** → **2**. Unfortunately, the etherification of 2-(3,3-dimethylcyclohexyl)propan-2-ol with isobutylene oxide (=2,2-dimethyloxirane) did not work. Therefore, we etherified 2-(3,3-dimethylcyclohexyl)propan-2-ol with propylene oxide, and the primary alcohol obtained was subsequently esterified with propanoic anhydride to provide the corresponding propanoate **17**. In **17**, the ester moiety of **12** is formally replaced by a methyl ether fragment. The odor of **17** was described as *slightly musky with sweet tonalities*.

In a second approach, we selected a different set of 25 cycloalkanols, most of which were, however, identical to the ones described above. These cycloalkanols were

regioselectively etherified with isobutylene oxide in the presence of $BF_3 \cdot OEt_2$ (10 mol-%) [12]. These reactions were carried out with a large excess of the cycloalkanols, which could be regenerated once the etherifications were complete. In the next step, we esterified the resulting 25 primary alcohols in the parallel reactor (*Scheme 3*) with two different acid anhydrides in the presence of Et_3N and DMAP [13].

Scheme 3. *Synthesis of* Helvetolide® (**2**) *Analogs*



With these 25 different primary cycloalkanols and two anhydrides, we obtained a set of 50 alicyclic esters of the *Helvetolide*® (**2**)-type, which were synthesized in four runs in the parallel reactor. After purification, the ACM library was submitted again to sensory evaluation by a panel of professional perfumers. In general, the evaluation results were comparable to the findings of our olfactory studies of the *Romandolide*® (**3**) analogs. When a compound of the ACM library contained a cyclopentenyl, cycloheptyl, or cyclooctyl ring, we observed the absence of any musk odor. In contrast, eleven molecules, exhibiting exclusively a cyclohexyl moiety, with or without Me substitution in the ring, emanated musk odors. To our very surprise, musk odorant **18**, without Me substitution in the ring, possessed an *erogenous musk odor with floral aspects* (*Fig. 3*). The additional single Me groups in **19** and **20** did not diminish the musk odor, but, instead, resulted in *pleasant musk notes*, in the case of **19**, combined with *floral tonalities*, and, in the case of **20**, *reminiscent of muscone*. Interestingly, the shift of a Me group to the 4-position in **21** led to a *total loss of musk odor*, and we observed instead only *a weak floral and fruity odor*. Finally, in analogy to the approach described above, we replaced the branched ether fragment of the best musk odorants **18**–**20** by an ester moiety. This replacement led to a total loss of musk odor in the cases of **22** and **23**, whereas the propanoate **24** possessed a *more-erogenous musk odor* in comparison to the strong musk odorant **19**.

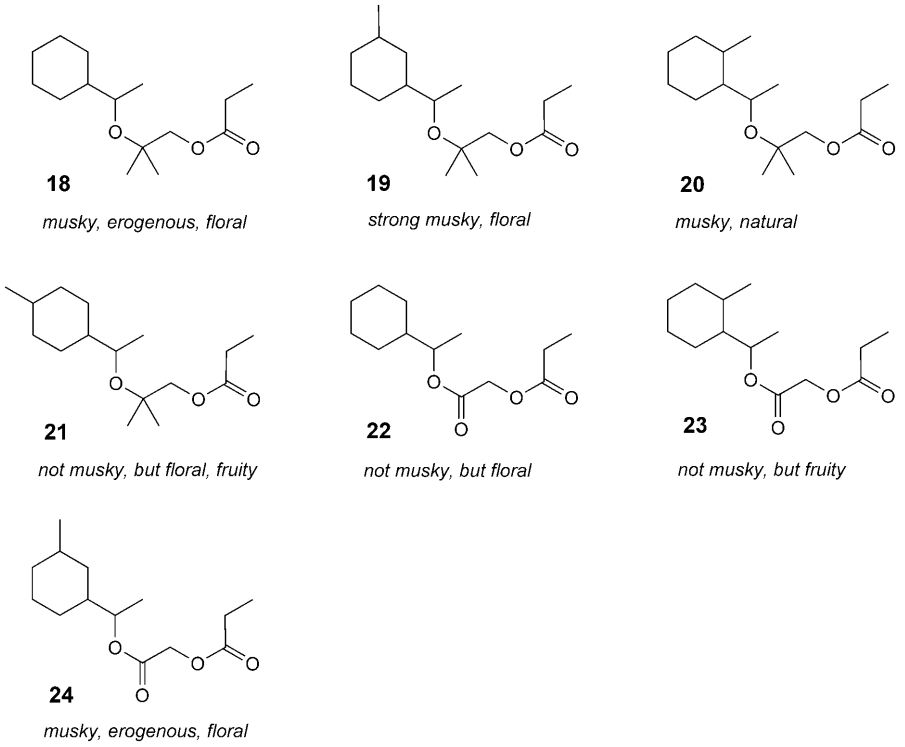


Fig. 3. *Odor descriptions of the* Helvetolide® *analogs* **18**–**24**

Conclusions. – We have established a solution-phase parallel synthesis of a large number of new alicyclic musk odorants. The advantage of this approach is that it allows the rapid preparation of various analogs for the study of structure–odor relationships. The structural requirements for alicyclic musk odorants identified in this study are, in the case of *Romandolide*® (**3**) analogs: *1*) a cyclohexyl ring with at least one Me group in position 3; and *2*) an additional Me group at the bridging C-atom between the cyclohexyl ring and the ether O-atom.

In the case of *Helvetolide*® (**2**) analogs with musk odors, we have identified the following structural requirements: *1*) a cyclohexyl ring is of crucial importance, but the substitution pattern can be more flexible in comparison with analogs of **3**; *2*) either no or one Me group at position C(2) or C(3) of the cyclohexyl ring, but not at C(4), which leads to total loss of musk odor.

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Experimental Part

General. Reagents and solvents were purchased from *Sigma-Aldrich* (Deisenhofen, Germany) or *Acros Organics* (Schwerte, Germany) and used without purification, *Herbac*® (=1-(3,3-dimethylcyclohexyl)ethanone) was purchased from *IFF*, and isobutylene oxide was supplied by *BASF*. Parallel reactor: the reactor was developed by *Bayer AG* in cooperation with *Symrise*. Flash chromatography (FC): *Biotage Flash 40*, equipment with disposable prepacked columns filled with *Merck* silica gel *60* (particle size 40–63 μm), pressure 0.8–1.0 bar. Workup: *Chromabond*® vacuum manifold for simultaneous preparation of up to 12 samples from *Macherey-Nagel*, and *Chromabond*® solid-phase-extraction columns filled with *Merck* silica gel *60* (1.0 g) and $MgSO_4$ (1.0 g). Thin-layer chromatography (TLC): *Merck* silica gel *60* F_{254} (layer thickness 200 μm, on aluminum plates; 5 cm × 10 cm); UV detection and visualization by dipping into a solution of '*Mo-stain*' (25 g phosphomolybdic acid, 10 g $Ce(SO_4)_2 \cdot H_2O$, 60 ml conc. H_2SO_4, 940 ml H_2O), or vanillin (1 g of vanillin, 10 ml conc. H_2SO_4, 20 ml AcOH, 170 ml MeOH). NMR: *Varian* Unity Inova and *Varian* Mercury^{Plus}, operating at 400.42 (^{1}H) and 100.69 (^{13}C) MHz in $CDCl_3$; δ in ppm rel. to Me_4Si, *s* in Hz. GC/MS: *Hewlett Packard MSD-5972-A* (EI: 70 eV).

General Procedure (*GP 1*) *for the Parallel Synthesis of* Romandolide® (**3**) *Derivatives*. Under N_2, a soln. of DCC (3.4 g, 16.5 mmol) in CH_2Cl_2 (5 ml) was added at 0° to a soln. of *(acetoxy)acetic acid* (**10**; 1.96 g, 16.5 mmol) or *carboxymethyl propanoate* (**11**; 2.18 g, 16.5 mmol), DMAP (122 mg, 1.0 mmol), and the cycloalkylalkanol (15 mmol) in CH_2Cl_2 (10 ml). The cooling bath was removed, and the mixture was stirred for 16 h at r.t. prior to parallel vacuum filtration over $SiO_2/MgSO_4$ 1:1. The filtrate was concentrated under reduced pressure, and the resulting residue was purified by FC.

2-[1-(3,3-Dimethylcylohexyl)-1-methylethoxy]-2-oxoethyl Propanoate (**12**). A soln. of 1-(3,3-dimethylcyclohexyl)ethanone (50.0 g, 324 mmol) in Et_2O (200 ml) was added dropwise with stirring under N_2 atmosphere to MeMgCl (3M in THF, 130 ml, 390 mmol), and the mixture was then heated to reflux. After 3 h, the mixture was allowed to cool to r.t., then poured into cold sat. aq. NH_4Cl soln. (250 ml). The layers were separated, and the aq. phase was extracted with Et_2O (3 × 250 ml). The combined org. extracts were washed with sat. aq. $NaHCO_3$ soln. (250 ml) and brine (250 ml), dried (Na_2SO_4), and concentrated in a rotary evaporator. The resulting 2-(3,3-dimethylcyclohexyl)propan-2-ol (52.7 g, 95%) was used in the next step without further purification. Following *GP 1*, *Steglich* esterification with **11**, purification by FC (SiO_2; cyclohexane/AcOEt 10:1; R_f = 0.23), and bulb-to-bulb distillation (221°/1.3 mbar) afforded **12** (3.10 g, 73%). Odor: *strong musky, woody, ambery*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80–1.40 (*m*, 2 CH_2); 0.88 (*s*, Me); 0.92 (*s*, Me); 1.18 (*t*, *J* = 7.6, Me); 1.42 (*d*, *J* = 0.6, Me); 1.50–1.76 (*m*, 2 CH_2); 2.03 (*tt*, *J* = 12.4, 3.1, CH); 2.44 (*q*, *J* = 7.6, C(O)CH_2); 4.46 (*d*, *J* = 15.8, 1 H of C(O)CH_2–O); 4.55 (*d*, *J* = 15.8, 1 H of C(O)CH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 8.9 (Me); 22.2 (CH_2); 23.3 (2 Me); 24.5 (Me); 27.0 (CH_2); 27.1 (C(O)*C*H_2); 30.7 ((CH_2)$_2$*C*Me_2); 33.6 (Me); 39.0 (CH_2); 40.0 (CH_2); 42.0 (CH); 61.0 (C(O)*C*H_2–O); 87.4 (($Me)_2$*C*–O); 166.7 (O–*C*(O)CH_2); 173.5 (O–*C*(O)CH_2).

1-(3,3-Dimethylcyclohexyl)-1-methylethyl (Acetoxy)acetate (**13**). Following the procedure for the synthesis of **12**, *Steglich* esterification of 2-(3,3-dimethylcyclohexyl)propan-2-ol with **10**, purification by FC (SiO_2; cyclohexane/AcOEt 10:1; R_f = 0.25), and bulb-to-bulb distillation (192°/0.35 mbar) afforded **13** (2.82 g, 70%). Odor: *musky, erogenous, woody*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80–1.08 (*m*, 3 H of CH_2); 0.88 (*s*, Me); 0.92 (*s*, Me); 1.30–1.45 (*m*, 3 H of CH_2); 1.42 (*s*, Me); 1.43 (*s*, Me); 1.50–1.72 (*m*, CH_2); 2.03 (*tt*, *J* = 12.5, 3.1, CH); 2.15 (*s*, C(O)Me); 4.47 (*d*, *J* = 15.7, 1 H of C(O)CH_2–O); 4.52 (*d*, *J* = 15.7, 1 H of C(O)CH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 20.5 (Me); 22.2 (CH_2); 23.3 (Me); 23.4 (Me); 24.6 (Me); 27.0 (CH_2); 30.7 ((CH_2)$_2$*C*($Me)_2$); 33.7 (C(O)Me); 39.0 (CH_2); 40.1 (CH_2); 42.1 (CH); 61.1 (C(O)*C*H_2–O); 87.6 (Me_2*C*–O); 166.8 (O–*C*(O)CH_2); 170.3 (O–*C*(O)CH_2).

2-[1-Methyl-1-(3-methylcyclohexyl)ethoxy]-2-oxoethyl Propanoate (**14**). In the presence of 5% Ru on activated carbon (15.0 g, 7.4 mmol, 0.35 mol-%), 1-(3-methylphenyl)ethanone (300 g, 2.23 mol) was hydrogenated at a H_2 pressure of 18 bar during 8 h at 90°. The catalyst was removed by vacuum filtration over a pad of *Celite*, and the filtrate was concentrated in a rotary evaporator. The residue was distilled (45–49°/1.1 mbar) to provide the intermediate 1-(3-methylcyclohexyl)ethanol (279 g, 88%) as a colorless oil. To a soln. of this intermediate (11.1 g, 77.8 mmol), tungstic acid (35.5 mg, 0.14 mmol), *Aliquat® 336* (54.9 mg, 0.14 mmol), and H_2O (1.85 g, 102 mmol) in toluene (20 ml) was carefully added 30% aq. H_2O_2 (9.38 g, 156.0 mmol) at 90°. After 3 h, the mixture was allowed to cool to r.t., before Et_2O (100 ml) was added. The layers were separated, and the org. one was washed with sat. aq. $Na_2S_2O_3$ soln. (3 × 50 ml). The org. extract was dried (Na_2SO_4), filtered, and concentrated in a rotary evaporator. The resulting 1-(3-methylcyclohexyl)ethanone (10.8 g, 99%) was used in the next step without further purification. A soln. of the ethanone (10.5 g, 74.8 mmol) in Et_2O (75 ml) was added dropwise with stirring under N_2 atmosphere to MeMgCl (3M in THF, 31.0 ml, 93.0 mmol), and the mixture was

then heated to reflux. After 3 h, the mixture was allowed to cool to r.t., then poured into cold sat. aq. NH_4Cl soln. (50 ml). The layers were separated, and the aq. phase was extracted with Et_2O (3×100 ml). The combined org. extracts were washed with sat. aq. $NaHCO_3$ soln. (100 ml) and brine (100 ml), dried (Na_2SO_4), and concentrated in a rotary evaporator. The resulting 2-(3-methylcyclohexyl)propan-2-ol (10.8 g, 93%) was used in the next step without further purification. Following *GP 1*, *Steglich* esterification with **11**, purification by FC (SiO_2; cyclohexane/AcOEt, 10:1; $R_f = 0.22$), and bulb-to-bulb distillation (205°/0.8 mbar) afforded **14** (3.4 g, 84%). Odor: *musky, ambery.* ^{1}H-NMR (400 MHz, $CDCl_3$): 0.68 (*q*, $J = 12.1$, 1 H of CH_2); 0.80 (*ddd*, $J = 13.1$, 11.8, 3.8, 1 H of CH_2); 0.89 (*d*, $J = 6.5$, Me); 0.94–0.99 (*m*, 1 H of CH_2); 1.18 (*t*, $J = 7.6$, Me); 1.25 (*dt*, $J = 13.1$, 3.6, 1 H of CH_2); 1.29–1.44 (*m*, CH); 1.42 (*s*, Me); 1.43 (*s*, Me); 1.63–1.73 (*m*, 3 H of CH_2); 1.77 (*dquint.*, $J = 13.1$, 3.2, 1 H of CH_2); 1.89 (*tt*, $J = 12.1$, 3.1, CH); 2.43 (*q*, $J = 7.6$, C(O)CH_2); 4.51 (*s*, C(O)CH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 8.9 (Me); 23.0 (Me); 23.3 (Me); 23.4 (Me); 26.1 (CH_2); 26.7 (CH_2); 27.2 (C(O)*C*H_2); 32.7 ((CH_2)$_2$*C*H(Me)); 35.1 (CH_2); 35.9 (CH_2); 46.4 (CH); 61.0 (C(O)*C*H_2–O); 87.4 (Me_2C–O); 166.9 (O–*C*(O)CH_2); 173.7 (O–*C*(O)CH_2).

2-[1-Methyl-1-(2-methylcyclohexyl)ethoxy]-2-oxoethyl Propanoate (**15**). This compound was prepared according to the same procedures as described for **14**, with the exception that 1-(2-methylphenyl)ethanone was used in the initial reduction. Purification by FC (SiO_2; cyclohexane/AcOEt, 10:1; R_f 0.22) and bulb-to-bulb distillation (193°/0.9 mbar) afforded **15** (2.9 g, 72%). Odor: *musky, ambery.* ^{1}H-NMR (400 MHz, $CDCl_3$): 0.95 (*d*, $J = 7.2$, Me); 1.18 (*t*, $J = 7.6$, Me); 1.15–1.30 (*m*, 1 H of CH_2); 1.36–1.44 (*m*, 5 H of CH_2); 1.50 (*s*, Me); 1.51 (*s*, Me); 1.60–1.70 (*m*, CH_2); 1.70–1.82 (*m*, CH and 1 H of CH_2); 2.11–2.18 (*m*, CH); 2.44 (*q*, $J = 7.6$, C(O)CH_2); 4.48 (*s*, C(O)CH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 8.7 (Me); 14.0 (Me); 20.2 (CH_2); 21.2 (CH_2); 24.3 (Me); 24.4 (Me); 27.0 (CH_2); 27.2 (C(O)*C*H_2); 28.6 (CH); 35.3 (CH_2); 49.8 (CH); 60.9 (C(O)*C*H_2–O); 87.1 (Me_2C–O); 166.7 (O–*C*(O)CH_2); 173.5 (O–*C*(O)CH_2).

2-(1-Cyclohexyl-1-methylethoxy)-2-oxoethyl Propanoate (**16**). A soln. of cyclohexyl methyl ketone (142 g, 1.13 mol) in Et_2O (500 ml) was added dropwise with stirring under N_2 to MeMgCl (3M in THF, 470 ml, 1.41 mol), and the mixture was then heated to reflux. After 1 h, the mixture was allowed to cool to r.t., before poured into cold sat. aq. NH_4Cl soln. (300 ml). The layers were separated, and the aq. phase was extracted with Et_2O (2×300 ml). The combined org. extracts were washed with sat. aq. $NaHCO_3$ soln. (250 ml) and brine (250 ml), dried (Na_2SO_4), and concentrated in a rotary evaporator. The resulting 2-cyclohexylpropan-2-ol (151 g, 94%) was used in the next step without further purification. Following *GP 1*, *Steglich* esterification with **11**, purification by FC (SiO_2; cyclohexane/AcOEt, 10:1; R_f 0.19), and bulb-to-bulb distillation (184°/0.9 mbar) afforded **16** (3.1 g, 81%). Odor: *woody, slightly fruity.* ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.35 (*m*, 5 H of CH_2); 1.18 (*t*, $J = 7.5$, Me); 1.43 (*s*, 2 Me); 1.60–1.76 (*m*, 5 H of CH_2,); 1.89 (*tt*, $J = 11.2$, 3.1, CH); 2.44 (*q*, $J = 7.5$, C(O)CH_2); 4.51 (*s*, C(O)CH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 8.9 (Me); 23.3 (2 Me); 26.5 (3 CH_2); 27.2 (C(O)*C*H_2); 27.3 (2 CH_2); 46.5 (CH); 60.9 (C(O)*C*H_2–O); 87.5 (Me_2C–O); 166.7 (O–*C*(O)CH_2); 173.5 (O–*C*(O)CH_2).

2-(1-Cyclohexylethoxy)-2-oxoethyl Propanoate (**22**). In the presence of 5% Ru on activated carbon (3.6 g, 1.8 mmol, 0.18 mol-%), acetophenone (120 g, 1.00 mol) was hydrogenated at a H_2 pressure of 18 bar for 6 h at 85°. The catalyst was removed by vacuum filtration over a pad of *Celite*, and the filtrate was concentrated in a rotary evaporator. The resulting residue was distilled (49–52°/1.2 mbar) to provide 1-cyclohexylethanol (117 g, 91%) as colorless oil. Following *GP 1*, *Steglich* esterification with **11**, purification by FC (SiO_2; cyclohexane/AcOEt, 10:1; R_f 0.18), and bulb-to-bulb distillation (167°/0.7 mbar) afforded **22** (3.1 g, 86%). Odor: *slightly fruity and floral.* ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.10 (*m*, CH_2); 1.10–1.30 (*m*, 3 H of CH_2); 1.19 (*t*, $J = 7.5$, Me); 1.20 (*d*, $J = 6.4$, Me); 1.46 (*ttd*, $J = 11.6$, 6.4, 3.1, CH); 1.62–1.70 (*m*, CH_2); 1.71–1.79 (*m*, 3 H of CH_2); 2.45 (*q*, $J = 7.5$, C(O)CH_2); 4.59 (*s*, C(O)CH_2–O); 4.82 (*quint.*, $J = 6.4$, MeC*H*–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.0 (Me); 16.9 (Me); 25.9 (CH_2); 26.0 (CH_2); 26.3 (CH_2); 27.1 (C(O)*C*H_2); 28.3 (CH_2); 28.4 (CH_2); 42.5 (CH); 60.8 (C(O)*C*H_2–O); 76.1 (Me*C*H–O); 167.6 (O–*C*(O)CH_2); 173.7 (O–*C*(O)CH_2).

2-[1-(3-Methylcyclohexyl)ethoxy]-2-oxoethyl Propanoate (**24**). Following *GP 1*, *Steglich* esterification of 1-(3-methylcyclohexyl)ethanol (for synthesis, see the preparation of **14**) with **11**, purification by FC (SiO_2; cyclohexane/AcOEt, 10:1; $R_f = 0.20$), and bulb-to-bulb distillation (205°/0.5 mbar) afforded **24** (2.9 g, 76%). Odor: *musky, erogenous, fruity.* ^{1}H-NMR (400 MHz, $CDCl_3$): 0.58–0.72 (*m*, 1 H of CH_2); 0.74–0.98 (*m*, CH_2); 0.89 (*d*, $J = 6.6$, Me); 1.18 (*t*, $J = 7.6$, Me); 1.19 (*d*, $J = 6.4$, Me); 1.19–1.40 (*m*, CH_2); 1.49 (*ttd*, $J = 11.9$, 6.4, 3.2, CH); 1.57–1.82 (*m*, 4 H of CH_2 and CH); 2.44 (*q*, $J = 7.6$, C(O)CH_2); 4.59 (*s*, C(O)CH_2–O); 4.81 (*quint.*, $J = 6.4$, Me*C*H–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.0 (Me); 16.9 (Me); 22.9 (Me); 25.8 (CH_2); 27.2 (C(O)*C*H_2); 27.8 (CH_2); 32.4 (CH); 35.0 (CH_2); 37.0 (CH_2); 42.4 (CH); 60.8 (C(O)*C*H_2–O); 76.1 (Me*C*H–O); 167.6 (O–*C*(O)CH_2); 173.7 (O–*C*(O)CH_2).

2-[1-(2-Methylcyclohexyl)ethoxy]-2-oxoethyl Propanoate **23**. Following *GP 1*, *Steglich* esterification of 1-(2-methylcyclohexyl)ethanol (for synthesis, see preparation of **15**) with **11**, purification by FC (SiO_2; cyclohexane/AcOEt, 10:1; R_f 0.21), and bulb-to-bulb distillation (197°/0.4 mbar) afforded **23** (2.7 g, 71%). Odor: *slightly fruity*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.86 (*d*, *J* = 6.3, Me); 1.14 (*t*, *J* = 7.6, Me); 1.17 (*d*, *J* = 6.3, Me); 0.95–1.30 (*m*, 2 CH_2); 1.30–1.80 (*m*, 6 H of CH_2 and CH); 2.40 (*q*, *J* = 7.6, C(O)CH_2); 4.54 (*s*, C(O)CH_2–O); 4.78 (*quint.*, *J* = 6.3, Me*C*H–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 8.9 (Me); 17.0 (Me); 18.5 (Me); 22.9 (CH_2); 25.6 (CH_2); 27.1 (C(O)*C*H_2); 27.2 (CH_2); 33.1 (CH_2); 33.2 (CH); 45.5 (CH); 60.8 (C(O)*C*H_2–O); 74.1 (Me*C*H–O); 167.6 (O–*C*(O)CH_2); 173.7 (O–*C*(O)CH_2).

General Procedure (*GP 2*) *for the Parallel Synthesis of* Helvetolide® (**2**) *Derivatives*. To a soln. of the cycloalkanol (275 mmol) and $BF_3 \cdot OEt_2$ (0.1 ml) was added dropwise with stirring under N_2 atmosphere isobutylene oxide (25 mmol). After 3 h of stirring, an additional portion of $BF_3 \cdot OEt_2$ (0.1 ml) was added, and the mixture was stirred for 16 h. The mixture was then adjusted to pH 8–9, and the excess cycloalkylalkanol was removed by vacuum distillation. The resulting residue was dissolved in Et_2O (100 ml) and washed with brine (2 × 50 ml). The org. extract was dried (Na_2SO_4), filtered, and concentrated in a rotary evaporator. The resulting primary alcohol was used in the next step without further purification. Under N_2 atmosphere, 2.0 equiv. of acetic anhydride (for R^{11} = Me) or propanoic anhydride (for R^{11} = Et), Et_3N (2.0 equiv.), and DMAP (0.05 equiv.) were added to a stirred soln. of the crude cycloalkanol in the parallel reactor. The reaction mixtures were stirred for 2 h at r.t., before Et_2O (1 ml/mmol cycloalkylalkanol) and 2M aq. HCl soln. (0.5 ml/mmol cycloalkylalkanol) were added. The org. layers were separated and subjected to parallel vacuum filtration over $SiO_2/MgSO_4$, 1:1. The filtrates were concentrated under reduced pressure, and the resulting residues were purified by FC.

2-(1-Cyclohexylethoxy)-2-methylpropyl Propanoate (**18**). Following *GP 2*, etherification of 1-cyclohexylethanol (for synthesis, see preparation of **22**) with isobutylene oxide, followed by esterification of the resulting 2-(1-cyclohexylethoxy)-2-methylpropan-1-ol with propanoic anhydride, purification by FC (SiO_2; cyclohexane/AcOEt, 20:1; R_f 0.21), and bulb-to-bulb distillation (137°/0.5 mbar) afforded **18** (2.8 g, 44% over 2 steps). Odor: *musky, erogenous, floral*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80–1.35 (*m*, 3 CH_2); 1.06 (*d*, *J* = 6.1, Me); 1.16 (*t*, *J* = 7.5, Me); 1.18 (*s*, 2 Me); 1.55–1.85 (*m*, 5 H of CH and CH_2); 2.37 (*q*, *J* = 7.5, C(O)CH_2); 3.40 (*quint.*, *J* = 6.1, CH); 3.95 (*s*, Me_2CCH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.1 (Me); 19.7 (Me); 23.8 (Me); 24.0 (Me); 26.5 (CH_2); 26.6 (CH_2); 26.7 (CH_2); 27.7 (C(O)*C*H_2); 28.5 (CH_2); 29.5 (CH_2); 44.9 (CH); 70.3 (C(O)O–*C*H_2); 71.6 (Me*C*H–O); 73.7 (Me_2C–O); 174.3 (O–*C*(O)CH_2).

2-Methyl-2-[1-(3-methylcyclohexyl)ethoxy]propyl Propanonate (**19**). Following *GP 2*, etherification of 1-(3-methylcyclohexyl)ethanol (for synthesis, see preparation of **14**) with isobutylene oxide, followed by esterification of the resulting 2-methyl-2-[1-(3-methylcyclohexyl)ethoxy]propan-1-ol with propanoic anhydride, purification by FC (SiO_2; cyclohexane/AcOEt, 20:1; R_f 0.23), and bulb-to-bulb distillation (153°/0.75 mbar) afforded **19** (3.3 g, 49% over 2 steps). Odor: *musky, floral, fruity*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.55 (*q*, *J* = 12.1, 1 H of CH_2); 0.72–0.86 (*m*, 1 H of CH_2); 0.88 (*d*, *J* = 6.6, Me); 1.07 (*d*, *J* = 6.1, Me); 1.16 (*t*, *J* = 7.6, Me); 1.18 (*s*, 2 Me); 1.20–1.40 (*m*, 2 CH_2); 1.60–1.82 (*m*, 4 H of CH and CH_2); 2.37 (*q*, *J* = 7.6, C(O)CH_2); 3.40 (*quint.*, *J* = 6.1, CH); 3.94 (s, Me_2CCH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.1 (Me); 19.7 (Me); 23.0 (Me); 23.8 (Me); 24.1 (Me); 26.3 (CH_2); 27.7 (C(O)*C*H_2); 29.0 (CH_2); 32.7 (CH); 35.4 (CH_2); 37.1 (CH_2); 44.9 (CH); 70.3 (C(O)O–*C*H_2); 71.6 (Me*C*H–O); 73.8 (Me_2C–O); 174.2 (O–*C*(O)CH_2).

2-Methyl-2-[1-(2-methylcyclohexyl)ethoxy]propyl Propanoate (**20**). Following *GP 2*, etherification of 1-(2-methylcyclohexyl)ethanol (for synthesis, see preparation of **15**) with isobutylene oxide, followed by esterification of the resulting 2-methyl-2-[1-(2-methylcyclohexyl)ethoxy]propan-1-ol with propanoic anhydride, purification by FC (SiO_2; cyclohexane/AcOEt, 20:1; R_f 0.21), and bulb-to-bulb distillation (166°/0.90 mbar) afforded **20** (2.9 g, 43% over 2 steps). Odor: *musky, reminiscent of muscone*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.87 (*d*, *J* = 7.1, Me); 0.95–1.05 (*m*, 1 H of CH_2); 1.09 (*d*, *J* = 6.1, Me); 1.16 (*t*, *J* = 7.6, Me); 1.19 (*s*, 2 Me); 1.25–1.57 (*m*, 6 H); 1.67–1.75 (*m*, 1 H of CH_2); 1.84–1.96 (*m*, CH); 2.14–2.22 (*m*, CH); 2.36 (*q*, *J* = 7.6, C(O)CH_2); 3.48 (*dq*, *J* = 7.0, 6.1, CH); 3.96 (*s*, Me_2CCH_2–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.1 (Me); 12.9 (Me); 20.4 (CH_2); 21.1 (Me); 23.7 (CH_2); 23.9 (Me); 24.5 (Me); 26.6 (CH_2); 27.7 (C(O)*C*H_2); 28.7 (CH); 34.0 (CH_2); 47.4 (CH); 70.4 (Me*C*H–O); 70.9 (C(O)O–*C*H_2); 73.6 (Me_2C–O); 174.3 (O–*C*(O)CH_2).

2-Methyl-2-[1-(4-methylcyclohexyl)ethoxy]propyl Propanoate (**21**). Following the hydrogenation protocol described for the synthesis of **22**, 1-(4-methylphenyl)ethanone was hydrogenated to provide 1-(4-methylcyclohexyl)ethanol. The latter was etherified with isobutylene oxide, and the resulting 2-methyl-2-[1-(4-methylcyclohexyl)ethoxy]propan-1-ol was esterified with propanoic anhydride following *GP 2*. Purification by FC (SiO_2; cyclohexane/AcOEt, 20:1; R_f 0.21) and bulb-to-bulb distillation (159°/0.56 mbar) afforded **21** (3.4 g, 50% over 2 steps). Odor: *slightly fruity and floral*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.92 (*d*, *J* = 7.1, Me); 1.08 (*d*, *J* =

6.1, Me); 1.16 (*t*, *J* = 7.6, Me); 1.19 (*s*, 2 Me); 1.26 – 1.53 (*m*, 4 CH_2); 1.66 – 1.74 (*m*, CH); 1.75 – 1.84 (*m*, CH); 2.36 (*q*, *J* = 7.6, C(O)CH_2); 3.49 (*quint.*, *J* = 6.1, CH); 3.95 (*s*, Me_2CCH_2-O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.1 (Me); 18.6 (Me); 20.2 (Me); 23.7 (CH_2); 23.8 (Me); 23.9 (CH_2); 24.2 (Me); 27.6 (C(O)CH_2); 28.4 (CH); 31.5 (CH_2); 31.6 (CH_2); 44.1 (CH); 70.5 (Me_2CCH_2-O); 70.6 (Me*C*H–O); 73.8 (Me_2C-O); 174.3 (O–*C*(O)CH_2).

2-[1-(3,3-Dimethylcyclohexyl)-1-methylethoxy]propyl Propanoate (**17**). Following *GP 2*, etherification of 2-(3,3-dimethylcyclohexyl)propan-2-ol (for synthesis, see preparation of **12**) with propylene oxide, followed by esterification of the resulting 2-[1-(3,3-dimethylcyclohexyl)-1-methylethoxy]propan-1-ol with propanoic anhydride, purification by FC (SiO_2; cyclohexane/AcOEt, 20 : 1; R_f 0.21), and bulb-to-bulb distillation (169°/0.61 mbar) afforded **17** (2.8 g, 39% over 2 steps). Odor: *slightly musky, sweet*. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.77 – 0.85 (*m*, CH_2); 0.88 (*s*, Me); 0.90 (*s*, Me); 0.99 – 1.09 (*m*, 1 H of CH_2); 1.04 (*s*, Me); 1.06 (*s*, Me); 1.11 (*d*, *J* = 5.7, Me); 1.16 (*t*, *J* = 7.6, Me); 1.30 – 1.46 (*m*, 3 H of CH_2); 1.54 – 1.63 (*m*, CH_2); 1.67 – 1.75 (*m*, CH); 2.36 (*q*, *J* = 7.6, C(O)CH_2); 3.81 – 3.88 (*m*, Me$CHCH_2-O$); 3.95 – 4.02 (*m*, Me*C*H–O). ^{13}C-NMR (100 MHz, $CDCl_3$): 9.1 (Me); 20.0 (Me); 22.5 (Me); 22.6 (CH_2); 23.1 (Me); 23.5 (Me); 24.8 (Me); 27.3 (CH_2); 27.7 (C(O)CH_2); 30.9 ((CH_2)$_2$*C*Me_2); 39.4 (CH_2); 40.4 (CH_2);42.9 (CH); 64.3 (Me*C*H–O); 68.7 (C(O)O–*C*H_2); 76.8 (Me_2C-O); 171.0 (O–*C*(O)CH_2).

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New Macrocyclic Musk Compounds

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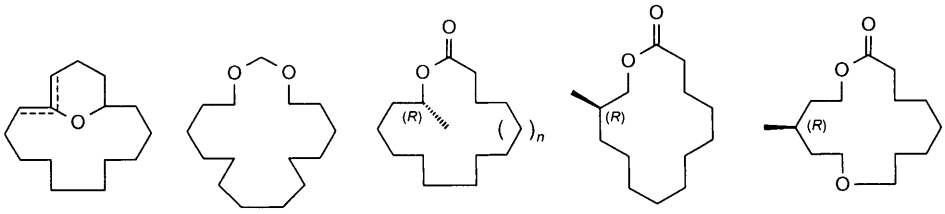
The syntheses and olfactory evaluations of eight new macrocyclic musks with a 1,6-dioxa structure (**1a–d** and **1′a–d**) as well as of twelve optically active 3-methyl macrolides (**5a–c** and **5′a–c**) are reported. These macrocycles were synthesized *via* intramolecular metathesis mediated by the *Grubbs* catalyst. Despite the absence of a C=O function, the 1,6-dioxa compounds, both unsaturated (**1**) and saturated (**1′**), possess musky odors similar to those of macrocyclic ketones and lactones. Especially 16-membered rings were found to display an intense and pleasant musk character. However, in the case of optically active 3-methyl macrolides (**5**, **5′**), only the (*R*)-configured 15- and 16-membered rings had intense and pleasant musk notes.

Introduction. – For some time now, safety and environmental issues have become very important in the perfume industry, and these apply obviously also for the development of new musk odorants, which is still an essential topic of fragrance chemistry. Macrocyclic musks are attractive molecular targets because they have superior odor characteristics and are environmentally friendly. Only few macrocyclic musks with an ether function were originally known, *e.g.*, the bicyclic pyran **A** [1] and the acetal **B** [2]. In the substance class of optically active methyl-substituted macrocycles, (−)-(*R*)-muscone, the pheromone of the male musk deer, is a prominent example for an optically active macrocyclic ketone. But also optically active *ω*-methyl macrolides of type **C** [3] and (*ω* − 1)-methyl macrolides **D** [4] occur in nature. The methyl macrolides **C** and **D** have been identified in galbanum resin and *Angelica* root oil, respectively. Interestingly, the principal enantiomers of these methyl macrocycles are (*R*)-configured. For the (*ω* − 2)-methyl oxamacrolide **E** [5], which does not occur in nature, it was even found that only the (*R*)-enantiomer smells.



Results and Discussion. – In the course of our investigations on new musk odorants with high chemical stabilities, we first focused on macrocycles bearing ether functions. We synthesized and evaluated the odor characteristics of a series of new macrocyclic

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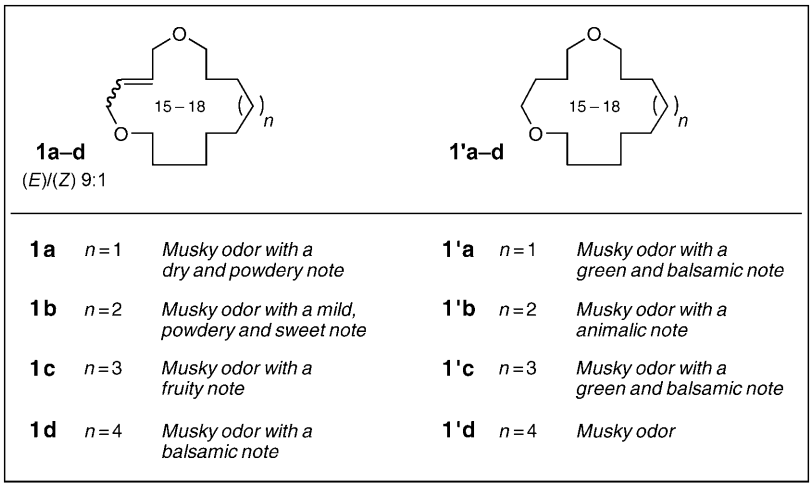
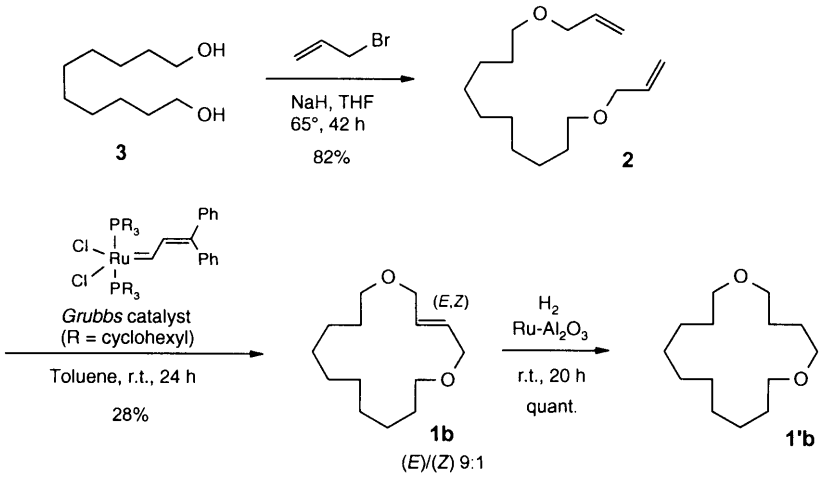
musks **1a–d** of 1,6-dioxa structure (*Fig. 1*) [6]. These compounds, with 15- to 18-membered rings, were synthesized by intramolecular metathesis mediated by the *Grubbs* catalyst (*Scheme 1*) [7]. Substrates for the ring-closing metathesis were diallyl ethers, *e.g.* **2**, which is easily available by etherification of the 1,ω-diol **3** with allyl bromide (*Scheme 1*). In addition to the unsaturated 1,6-dioxacycloalk-3-enes, the saturated analogs **1′a–d** were also prepared by hydrogenation, and investigated by experienced perfumers for their olfactory properties.



1a	*n* = 1	*Musky odor with a dry and powdery note*	**1'a**	*n* = 1	*Musky odor with a green and balsamic note*
1b	*n* = 2	*Musky odor with a mild, powdery and sweet note*	**1'b**	*n* = 2	*Musky odor with a animalic note*
1c	*n* = 3	*Musky odor with a fruity note*	**1'c**	*n* = 3	*Musky odor with a green and balsamic note*
1d	*n* = 4	*Musky odor with a balsamic note*	**1'd**	*n* = 4	*Musky odor*

Fig. 1. *Olfactory characterization of 15- to 18-membered saturated* (**1a–d**) *and unsaturated* (**1′a–d**) *1,6-dioxacycloalkanes*

Scheme 1. *Synthesis of 1,6-Dioxacyclohexadec-3-ene* (**1b**) *and Its Saturated Analog* **1′b**



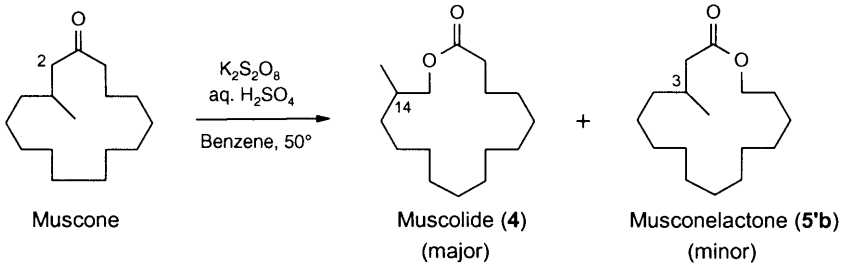
Despite the absence of a C=O function, both the unsaturated and saturated 1,6-dioxa macrocycles possess musky odors, like macrocyclic ketones and lactones; in

particular, the 16-membered rings emanate intense and pleasant musk notes. While the unsaturated compounds have a more-*powdery musk* character, the saturated analogs possess *animalic and balsamic* side notes (*Fig. 1*).

Besides the ring size, the geometry of the C=C bond has a distinct influence on the odor profile, as discovered in the case of **1b**. (*E*)-1,6-Dioxacyclohexadec-3-ene ((*E*)-**1b**) constitutes the most valuable musk odorant of the series; it emanates a much liked, powdery, sweet musk odor.

Since (−)-(*R*)-muscone was considered by our perfumers to be one of the best musk odorants, we used it as a model structure in our investigations on new musk odorants. In the ω-methyl macrolides **C**, naturally occurring in galbanum resin, the $CH_2(2)$ group of (−)-(*R*)-muscone was replaced by an O-atom (*Scheme 2*). The resulting ($\omega-1$)-methyl-substituted macrolide, muscolide (**4**), had already been obtained in 1928 as the major product of the *Baeyer–Villiger* oxidation of racemic muscone [8]; almost 70 years later, both enantiomers of **4** were synthesized, and their odor characteristics were evaluated [9].

Scheme 2. Baeyer–Villiger *Oxidation of Racemic Muscone*



On the basis of these structural relationships, we were interested in the minor product of the above *Baeyer–Villiger* oxidation, '3-methyl-15-pentadecanolide' (**5′b**)[1]), for which we propose the name *musconelactone*. We also synthesized and evaluated the odor characteristics of the unsaturated (**5a–c**) and saturated (**5′a–c**) homologs and their optical isomers (*Fig. 2*) [10]. These compounds with 15- to 17-membered rings were synthesized according to *Scheme 3* from enantiomerically pure β-butyrolactone (=4-methyloxetan-2-one; **6**) [11] by reaction with ω-unsaturated alkyl *Grignard* reagents (such as **7**), esterification of the products with ω-unsaturated alcohols (such as **9**), and intramolecular ring-closing metathesis employing *Grubbs* catalyst. The sequence is outlined in *Scheme 3* for the synthesis of musconelactone (**5′b**). Opening of **6** with the Grignard reagent in the presence of catalytic amounts of CuCl proceeded under almost complete retention of configuration [12], as monitored by chiral HPLC (*Fig. 3*). The (*R*)-isomers of the 15- and 16-membered 3-methyl macrolides possessed the most-intense and preferred musk characters of the series, while the (*S*)-isomers, in general, had only weak and flat musk odors (*Fig. 2*).

Upon comparing the minimum-energy conformers of (−)-(*R*)-muscone, (+)-(*R*)-muscolide (**4**), and (−)-(*R*)-musconelactone (**5′b**), generated with the CAChe software

[1]) Systematic name: 4-methylpentadecano-15-lactone or 4-methyloxacyclohexadecan-2-one.

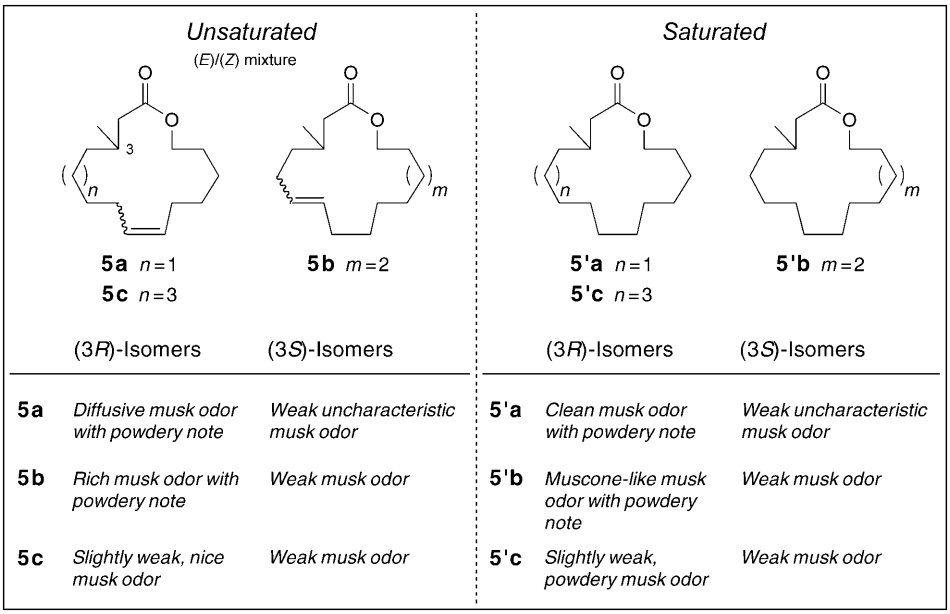
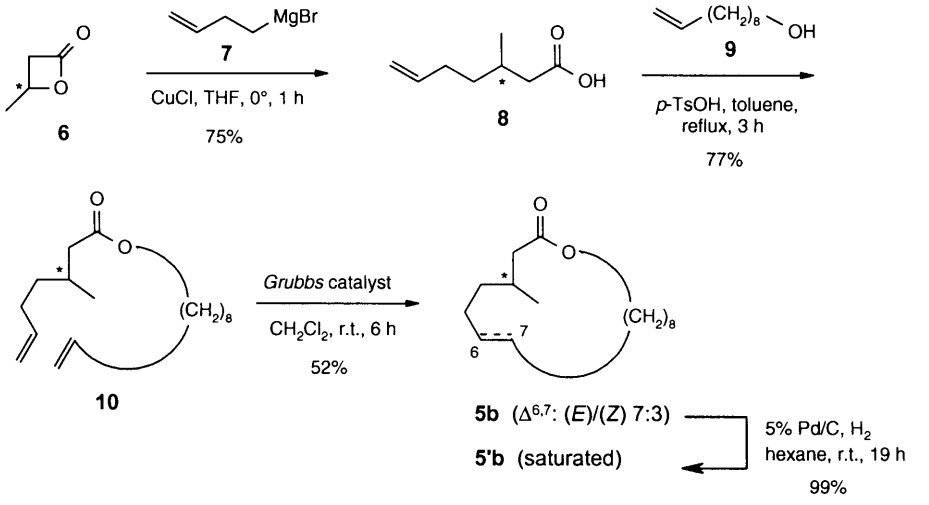


Fig. 2. *Olfactory characterization of the enantiomers of 15- to 17-membered unsaturated as well as saturated 3-methyl macrolides of type* **5** *and* **5′**, *resp.*

Scheme 3. *Stereoselective Synthesis of Musconelactone* (**5′b**)



package [13], the C=O and Me groups of (−)-(*R*)-muscone and (−)-(*R*)-**5′b** were found to overlap well, while they did not in the case of (*R*)-**4** and (*R*)-muscone (*Fig. 4*).

We gratefully acknowledge the support of Dr. *T. Yamasaki* and Dr. *T. Hagiwara*, and the olfactory evaluations by the perfumers in the Hiratsuka laboratory.

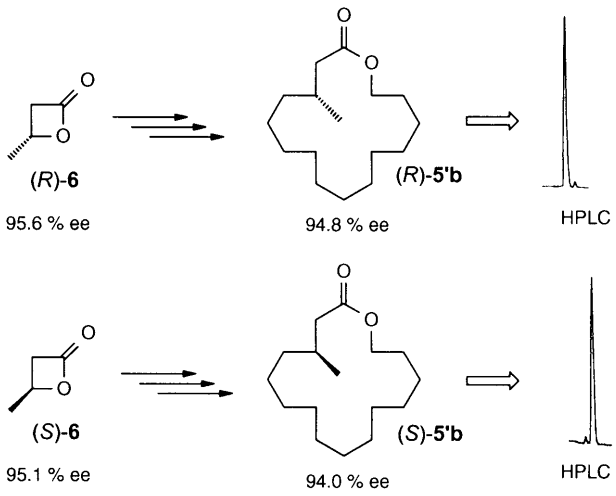


Fig. 3. *Retention of configuration in the syntheses of (*S*)- and (*R*)-***5′b***, as monitored by HPLC (Chiralcel OD-H*, hexane, 0.5 ml/min)

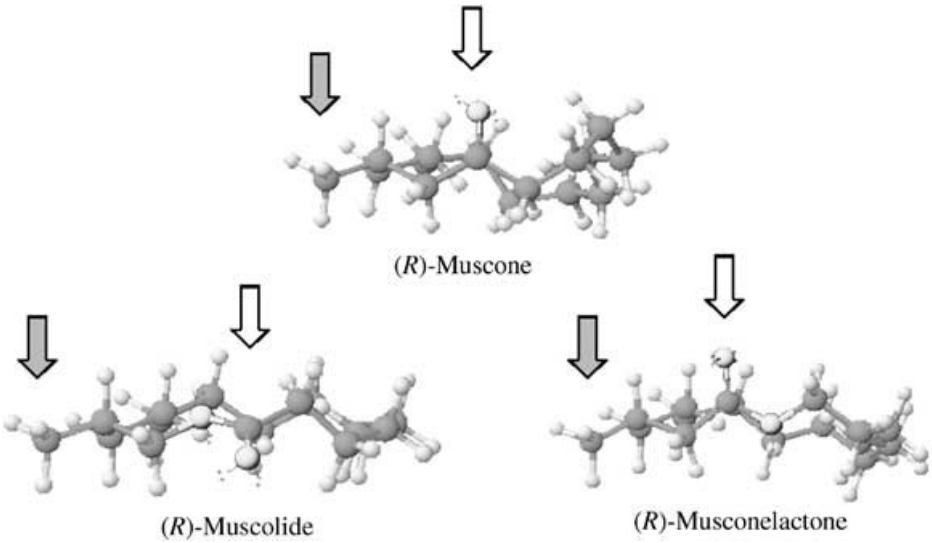


Fig. 4. *Comparison of the minimum-energy conformers of (−)-(*R*)-muscone, (+)-(*R*)-muscolide* (**4**), *and (−)-(*R*)-musconelactone* (**5′b**). Calculations were performed with CAChe software [13].

Experimental Part

General. All reagents and solvents were commercially available and used without further purification. HPLC: *Hitatchi L-6200* and *L-4000*. GC: *Hewlett-Packard HP-6890*. Optical rotation: *Nihon Bunkou DIP-360*. IR Spectra: *Nihon Bunkou IR-800*; in cm^{-1}. ^{1}H-NMR Spectra: *Bruker AMX-400*; δ in ppm, J in Hz. GC/MS Spectra: *Hitachi M-80B*; in m/z.

1,6-Dioxacyclohexadec-3-ene (**1b**). A soln. of *decane-1,10-diol* (**3**; 100 g, 574 mmol), 60% NaOH (50.5 g, 1.26 mol), and allyl bromide (153 g, 1.26 mol) in THF (300 ml) was stirred at 65° for 42 h. After the mixture had

cooled to r.t., it was poured into H_2O (1 l). The org. layer was separated and washed with H_2O (1 l) and brine (1 l), and concentrated *in vacuo*. The resulting crude material was distilled to provide pure *1,5-bis(prop-2-enyloxy)pentane* (**2**; 120 g, 82%). Under N_2 atmosphere, *Grubbs* catalyst [7] (822 mg, 10.0 mmol) was added to a soln. of **2** (12.0 g, 50.0 mmol) in toluene (2.4 l), the mixture was stirred at r.t. for 24 h, and the solvent was removed *in vacuo*. The crude product was purified by column chromatography (CC; SiO_2) to furnish pure **1b** (3.00 g, 28%; (*E*)/(*Z*) 9 : 1). The double-bond isomers were separated by HPLC (*Inertsil ODS-2*, 4.6 mm i.d. × 250 mm; 80% aq. MeOH). ^{1}H-NMR ($CDCl_3$): 5.78 (*m*, 2 H); 3.98 (*d*, *J* = 6.8, 4 H); 3.47 (*t*, *J* = 5.9, 4 H); 1.62 (*m*, 4 H); 1.45 – 1.27 (*m*, 12 H). ^{1}H-NMR ($CDCl_3$; (*Z*)-Isomer): 5.77 (*m*, 2 H); 4.05 (*d*, *J* = 5.7, 4 H); 3.50 (*t*, *J* = 6.3, 4 H); 1.60 (*m*, 4 H); 1.43 – 1.28 (*m*, 12 H).

1,6-Dioxacyclohexadecane (**1′b**). A soln. of **1b** (3.00 g, 13.3 mmol) and 5% Ru/Al_2O_3 (300 mg, 0.15 mmol) in EtOH (20 ml) was stirred at r.t. under H_2 atmosphere for 20 h. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo*. The resulting crude material was distilled to provide pure **1′b** (2.80 g, 93%). ^{1}H-NMR ($CDCl_3$): 3.45 (*m*, 8 H); 1.67 (*m*, 4 H); 1.58 (*m*, 4 H); 1.47 – 1.30 (*m*, 12 H).

1,6-Dioxacyclopentadec-3-ene (**1a**). Prepared in analogy to **1b**, but with nonane-1,9-diol. ^{1}H-NMR ($CDCl_3$): 5.81 (*m*, 2 H); 3.99 (*d*, *J* = 4.1, 4 H); 3.49 (*t*, *J* = 5.9, 4 H); 1.58 (*m*, 4 H); 1.48 – 1.31 (*m*, 10 H).

1,6-Dioxacyclopentadecane (**1′a**). Prepared in analogy to **1′b**, but from **1a**. ^{1}H-NMR ($CDCl_3$): 3.48 (*m*, 8 H); 1.67 (*m*, 4 H); 1.55 (*m*, 4 H); 1.49 – 1.36 (*m*, 10 H).

1,6-Dioxacycloheptadec-3-ene (**1c**). Prepared in analogy to **1b**, but from undecane-1,11-diol. ^{1}H-NMR ($CDCl_3$): 5.80 (*m*, 2 H); 3.99 (*d*, *J* = 4.0, 4 H); 3.48 (*t*, *J* = 6.1, 4 H); 1.60 (*m*, 4 H); 1.42 (*m,* 4 H); 1.38 – 1.30 (*m*, 10 H).

1,6-Dioxacycloheptadecane (**1′c**). Prepared in analogy to **1′b**, but from **1c**. ^{1}H-NMR ($CDCl_3$): 3.46 (*m*, 8 H); 1.64 (*m*, 4 H); 1.55 (*m*, 4 H); 1,42 (*m*, 4 H); 1.47 – 1.30 (*m*, 10 H).

1,6-Dioxacyclooctadec-3-ene (**1d**). Prepared in analogy to **1b**, but from dodecane-1,12-diol. ^{1}H-NMR ($CDCl_3$): 5.79 (*m*, 2 H); 3.98 (*d*, *J* = 4.0, 4 H); 3.47 (*t*, *J* = 6.0, 4 H); 1.58 (*m*, 4 H); 1.47 – 1.27 (*m*, 16 H).

1,6-Dioxacyclooctadecane (**1′d**). Prepared in analogy to **1′b**, but from **1d**. ^{1}H-NMR ($CDCl_3$): 3.43 (*m*, 8 H); 1.66 (*m*, 4 H); 1.55 (*m*, 4 H); 1.43 (*m*, 4 H); 1.33 (*m*, 12 H).

*(4*R*)-4-Methyloxacyclohexadec-7-en-2-one* ((*R*)-**5b**). Under N_2 atmosphere, a soln. of but-3-enyl magnesium bromide in THF (128 ml, 1.16 M, 148 mmol) was added dropwise at 0° within 15 min to a soln. of CuCl (0.61 g, 6.16 mmol) in THF (350 ml). At the same temp., a soln. of (*R*)-*β*-butyrolactone ((*R*)-**6**, 95% ee; 10.6 g, 0.123 mol) in THF (120 ml) was added dropwise during 70 min to the stirred mixture, and stirring was continued for 1 h at this temp. After acidification with 2N aq. HCl soln. (100 ml), H_2O (100 ml) was added, and the org. layer was separated and washed with H_2O (1 l) and brine (1 l). The solvent was removed *in vacuo*, and the crude product was purified by CC (SiO_2) to provide *(3*R*)-3-methylhept-6-enoic acid* ((*R*)-**8**; 13.4 g, 75%). The latter, *dec-9-en-1-ol* (9; 22.0 g, 141 mmol), and *p*-TsOH · H_2O (670 mg, 3.52 mmol) in anh. toluene (30 ml) were azeotropically distilled for 3 h, with removal of separated H_2O. After the mixture had cooled to r.t., H_2O (50 ml) was added, and the org. layer was separated and washed with sat. aq. $NaHCO_3$ soln. (50 ml) and brine (50 ml). After evaporation of the solvent *in vacuo*, the crude product was purified by CC (SiO_2) to afford 91.5% (GC) pure *dec-9-enyl-(3*R*)-3-methylhept-6-enoate* ((*R*)-**10**; 16.2 g, 77%). At r.t., a solution of the latter (13.5 g, 48.1 mmol) was added dropwise within 5 h to a stirred soln. of the *Grubbs* catalyst (2.00 g, 2.41 mmol) in CH_2Cl_2 (2 l), and stirring was continued for 1 h. Then, the org. layer was separated and washed with 10% aq. NaOH soln. (1 l), H_2O (3 × 1 l), and brine (1 l). After concentration of the org. phase *in vacuo*, the resulting residue was purified by CC (SiO_2) to provide (*R*)-**5b** (5.70 g, 52%, (*E*)/(*Z*) 7 : 3). $[\alpha]_D^{24} = -6.06$ (*c* = 1.04, MeOH). IR (neat): 1735, 970, 710. ^{1}H-NMR ($CDCl_3$): 5.32 (*m*, 2 H); 4.19 (*m*, 1 H); 4.04 (*m*, 1 H); 2.31 (*m*, 1 H); 2.16 – 1.94 (*m*, 6 H); 1.65 (*m*, 2 H); 1.49 – 1.17 (*m*, 12 H); 0.90 (*d*, 3 H). MS: 252 (M^+), 234, 210, 192, 177, 163, 150, 137, 124, 109, 95, 81, 67, 54, 41, 28, 14.

*(4*R*)-4-Methyloxacyclohexadecan-2-one* ((*R*)-**5′b**). A mixture of (*R*)-**5b** (1.80 g, 7.13 mmol) and 5% Pd/C (200 mg, 0.094 mmol) in hexane (4.0 ml) was stirred at r.t. for 19 h under H_2 atmosphere. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo*. The crude product was distilled to afford 94.3% (GC) pure (*R*)-**5′b** (1.86 g, 98.7%; 94.3 % ee). $[\alpha]_D^{24} = +4.51$ (*c* = 1.02, MeOH). IR (neat): 1740. ^{1}H-NMR ($CDCl_3$): 4.20 (*m*, 1 H); 4.05 (*m*, 1 H); 1.61 (*m*, 2 H); 1.47 – 1.14 (*m*, 20 H); 0.96 (*d*, 3 H). MS: 254 (M^+), 236, 211, 194, 179, 166, 152, 138, 124, 110, 96, 82, 69, 55, 41, 27, 12.

*(4*S*)-4-Methyloxacyclohexadec-7-en-2-one* ((*S*)-**5b**). Prepared from (*S*)-*β*-butyrolactone (95.1% *ee*), in analogy to (*R*)-**5b**: 92.6% (GC) pure (*S*)-**5b** ((*E*)/(*Z*) 8 : 2). $[\alpha]_D^{24} = +4.80$ (*c* = 1.00, MeOH).

*(4*S*)-4-Methyloxacyclohexadecan-2-one* ((*S*)-**5′b**). Prepared from (*S*)-**5b** in analogy to (*R*)-**5b**: 91.3% (GC) pure (*S*)-**5′b** (94.9% ee). $[\alpha]_D^{23} = -1.22$ (*c* = 0.98, MeOH).

*(4*R*)-4-Methyloxacyloheptadec-11-en-2-one* ((*R*)-**5c**). Prepared from oct-7-enyl magnesium bromide and hept-6-enol in analogy to **5b**: 99.2% (GC) pure (*R*)-**5c** ((*E*)/(*Z*) 8:2). $[\alpha]_D^{24} = +3.50$ ($c = 1.00$, MeOH). IR (neat): 1730, 965, 715. ^{1}H-NMR ($CDCl_3$): 5.37–5.24 (*m*, 2 H); 4.20–4.09 (*m*, 1 H); 4.07–3.95 (*m*, 1 H); 2.30–2.22 (*m*, 1 H); 2.20–2.11 (*m*, 1 H); 2.10–1.99 (*m*, 4 H); 1.95 (*m*, 1 H); 1.68–1.59 (*m*, 2 H); 1.47–1.04 (*m*, 14 H); 0.93 (*d*, 3 H). MS: 266 (M^+), 248, 238, 224, 206, 191, 178, 163, 149, 138, 123, 109, 95, 82, 67, 55, 41, 29, 18.

*(4*R*)-4-Methyloxacycloheptadecan-2-one* ((*R*)-**5′c**). Prepared from (*R*)-**5c** in analogy to (*R*)-**5′b**: 99.2% (GC) pure (*R*)-**5′c**. $[\alpha]_D^{24} = +1.71$ ($c = 1.00$, MeOH). IR (neat): 1735. ^{1}H-NMR ($CDCl_3$): 4.21–4.13 (*m*, 1 H); 4.09–4.02 (*m*, 1 H); 2.23 (*d*, 1 H); 2.19 (*m*, 1 H); 2.04–1.93 (*m*, 1 H); 1.68–1.59 (*m*, 2 H); 1.45–1.13 (*m*, 22 H); 0.95 (*d*, 3 H). MS: 266 (M^+), 250, 235, 225, 217, 208, 193, 180, 166, 152, 138, 124, 110, 96, 82, 69, 55, 41, 29, 18.

*(4*R*)-4-Methyloxacyclopentadec-9-en-2-one* ((*R*)-**5a**). Prepared from hex-5-enyl magnesium bromide and hept-6-en in analogy to (*R*)-**5b**: 98.2% (GC) pure (*R*)-**5a** ((*E*)/(*Z*) 9:1). $[\alpha]_D^{24} = -18.8$ ($c = 1.01$, MeOH). IR (neat): 1740, 970, 715. ^{1}H-NMR ($CDCl_3$): 5.44 (*m*, 2 H); 4.18–4.06 (*m*, 2 H); 2.33–2.25 (*m*, 1 H); 2.14–2.07 (*m*, 1 H); 2.06–1.93 (*m*, 5 H); 1.58 (*m*, 2 H); 1.47–1.15 (*m*, 10 H); 0.96 (*d*, 3 H). MS: 238 (M^+), 220, 210, 195, 187, 178, 163, 149, 136, 122, 109, 95, 82, 67, 55, 41, 29, 18.

*(4*R*)-4-Methyloxacyclopentadecan-2-one* ((*R*)-**5′a**). Prepared from (*R*)-**5a** in analogy to (*R*)-**5b**: 98.2% (GC) pure (*R*)-**5′a**. $[\alpha]_D^{24} = +1.49$ ($c = 1.01$, MeOH). IR (neat): 1740. ^{1}H-NMR ($CDCl_3$): 4.19 (*m*, 1 H); 4.05 (*m*, 1 H); 2.28 (*m*, 1 H); 2.17 (*m*, 1 H); 2.01 (*m*, 1 H); 1.72–1.57 (*m*, 2 H); 1.49–1.18 (*m*, 18 H); 0.98 (*d*, 3 H). MS: 240 (M^+), 222, 207, 197, 189, 180, 165, 152, 138, 124, 110, 96, 82, 69, 55, 41, 29, 18.

*(4*S*)-4-Methyloxacyclopentadec-9-en-2-one* ((*S*)-**5a**). Prepared from (*S*)-β-butyrolactone (95.1% *ee*) in analogy to (*R*)-**5a**: 97.1% (GC) pure (*S*)-**5a** ((*E*)/(*Z*) 9:1). $[\alpha]_D^{24} = +17.7$ ($c = 1.00$, MeOH).

*(4*S*)-4-Methyloxacyclopentadecan-2-one* ((*S*)-**5′a**). Prepared from (*S*)-**5a** in analogy to (*R*)-**5′a**: 96.5% (GC) pure (*S*)-**5′a**. $[\alpha]_D^{25} = -3.56$ ($c = 1.01$, MeOH).

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The Search for New Fragrance Ingredients for Functional Perfumery

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Functional perfumery is an integral part of the fragrance business. It demands that the ingredients chosen for compounding withstand the aggressive nature of some of the bases used for soaps, detergents, softeners, bleach, and personal-care products. The synthetic efforts in this area reported in this short personal account, presented in a talk at the *RSC/SCI* conference *Flavours & Fragrances 2004* (Manchester), have resulted in the discovery of the two new proprietary molecules *Fleuranil*® (**5**/**6**) and *Khusinil*® (**7**), which fulfill the criteria of functional perfumery. The structure – odor relationships of several analogs of *Fleuranil*® and *Khusinil*® prepared in the course of these investigations are also presented.

Introduction. – This article provides a brief overview of our ongoing endeavors at *International Flavors & Fragrances* (*IFF*) in the relentless pursuit of new molecules with unique sensory properties. Carbon – carbon bond-formation is at the heart of organic synthesis [1]. Diverse approaches are being pursued to synthesize novel structures for exploratory purposes. In previous publications [2][3], we have reported the utility of, *e.g.*, *Diels – Alder*, *Mannich*, and ene reactions to prepare new lead compounds, which has led to the discovery of three new proprietary fragrance molecules: *Cassiffix*® (**1**/**2**), a long-lasting cassis note, *Prismantol*® (**3**), a woody-spicy ingredient, and *Prismylate*® (**4**), a woody-ambery, vetiver-like odorant (*Fig. 1*). In the light of regulatory and labeling issues [4] facing the fragrance and flavor industry, there is an urgent need to discover new molecules that enhance the performance in functional-perfumery applications. As a consequence, we have discovered and commercialized two new fragrance ingredients that address these requirements: *Fleuranil*® (**5**/**6**), *a powerful, green-anisic and ozone-like, sweet floral note* [5], and *Khusinil*® (**7**), *a strong, fresh, long-lasting nootkatone-type (bergamot, grapefruit), vetiver-woody odorant* [6], which both will hopefully advance the art of perfumery [7].

Background. – Functional perfumery is an integral part of the fragrance-compounding business, and demands that the fragrance ingredients used have good application properties in physical, chemical, and sensory terms [8]. In addition, the ingredients chosen for the perfume must perform well, and must be stable in the bases and alcohols of perfumes, cosmetics, toiletries, deodorants, soaps, detergents, softeners, bleach, LADD-perborate, and personal care products. Aldehydes are widely used as perfumery ingredients, but they suffer from several disadvantages (*Fig. 2*). Because of the lack of stability of aldehydes, the corresponding nitriles have often been used [9], and this replacement of functional groups has been in vogue for functional perfumery

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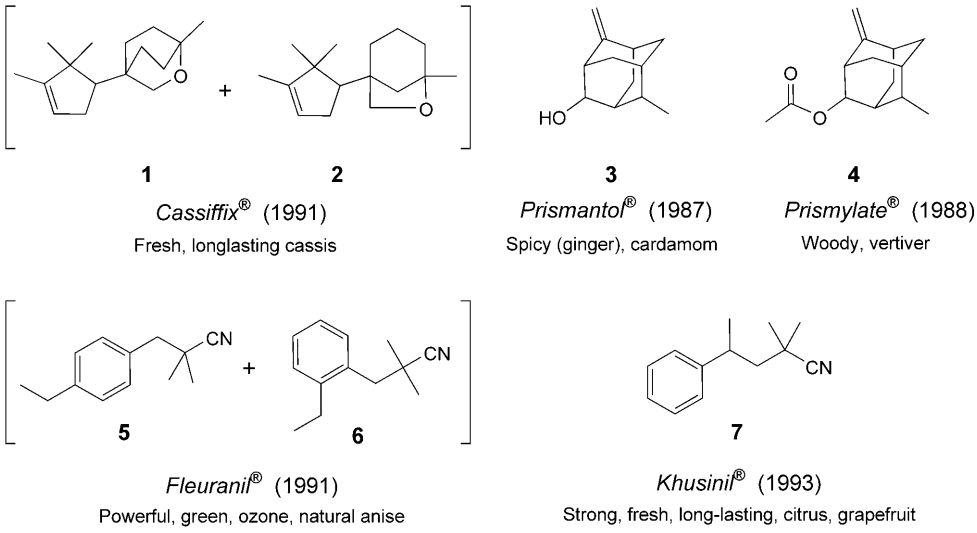


Fig. 1. *New proprietary fragrance ingredients of* IFF

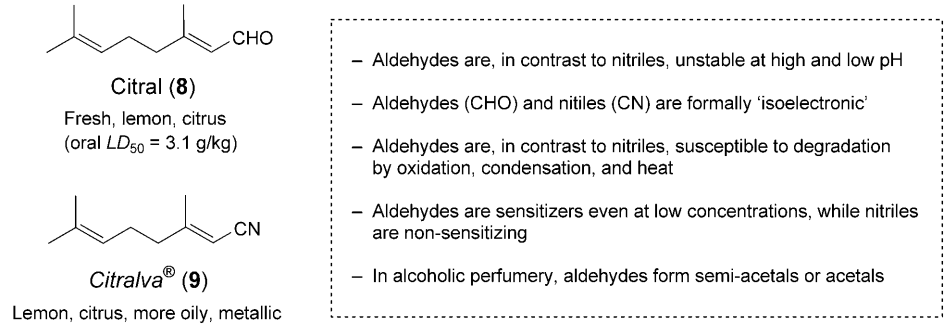


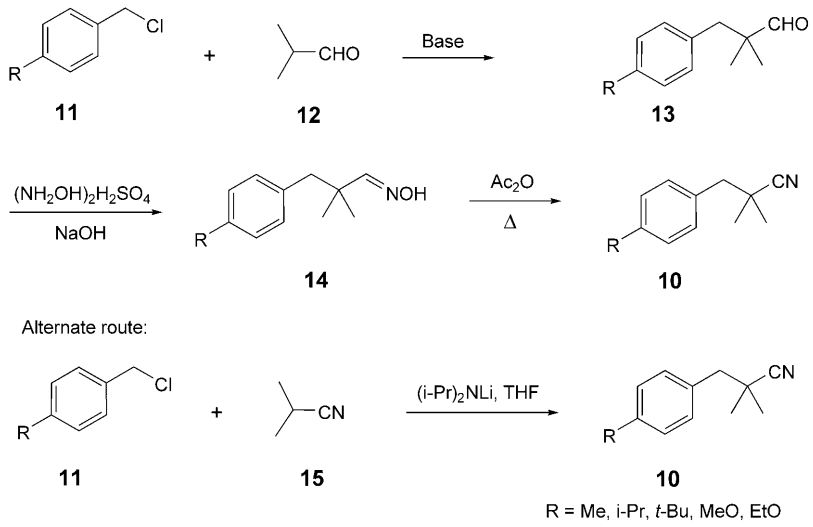
Fig. 2. *Strucutres and properties of citral* (**8**) *and* Citralva® (**9**), *and general comparison of odor and performance of volatile aldehydes* vs. *nitriles*

for well over 50 years now. In *Fig. 2*, a comparison of the aldehyde citral (**8**) with the corresponding nitrile, *Citralva*® (**9**), is made under diverse aspects.

Synthesis of Novel Aromatic Nitriles. – As stated above, several years ago, we began an exploratory program aimed at the re-evaluation of the odor of aromatic nitriles prepared from well-known aldehydes such as *Floralozone*®, *Cyclemax*®, *Lilial*®, or *Cyclamal*®. This research investigation turned into a detailed structure–odor-relationship study. Several new molecules with varying alkyl substituents on the aromatic ring were prepared for olfactory evaluation, and diverse synthetic routes were employed to prepare these analogs (*Schemes 1–6*). A brief description of how these novel structures were prepared is given in the following for some of these exploratory chemicals.

Various *p*-alkyl-substituted *Fleuranil*® (**5**/**6**) analogs of type **10** were synthesized by reacting an appropriately substituted benzyl chloride **11** with the carbanion of isobutyraldehyde **12**, generated under basic conditions, to provide the aldehydes **13** (*Scheme 1*). The desired nitriles **10** were prepared by converting the aldehydes **13** into the corresponding oximes **14** with hydroxylammonium sulfate, and heating the resulting oximes with acetic anhydride at reflux temperature. Alternatively, selected *Fleuranil*® analogs **10** were prepared in one step by reacting an appropriately substituted benzyl chloride **11** with the carbanion of isobutyronitrile (**15**) generated from lithium diisopropylamide (LDA) in tetrahydrofuran (THF).

Scheme 1

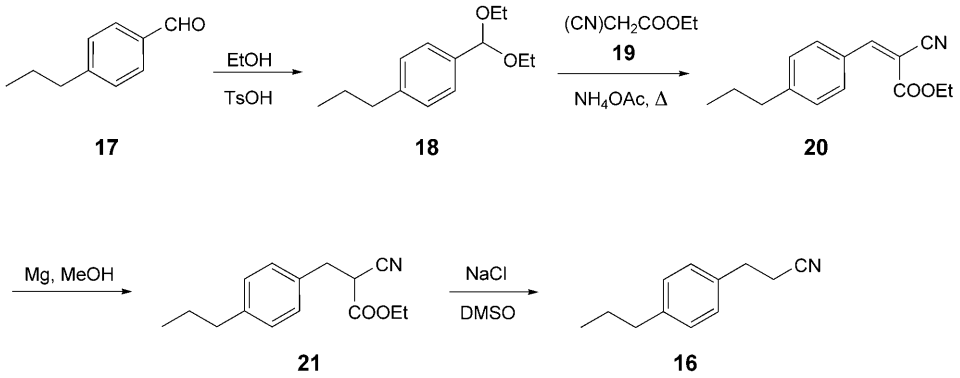


A multistep synthesis of 3-(4-propylphenyl)propanenitrile (**16**) was developed from *p*-propylbenzaldehyde (**17**), which was converted to its diethyl acetal **18**. The latter was condensed with ethyl cyanoacetate (**19**), using NH_4OAc as a base. The intermediate unsaturated ester **20** was hydrogenated to **21** with Mg in MeOH, and subsequent decarboxylation with NaCl in DMSO afforded the *Fleuranil* analog **16** (*Scheme 2*).

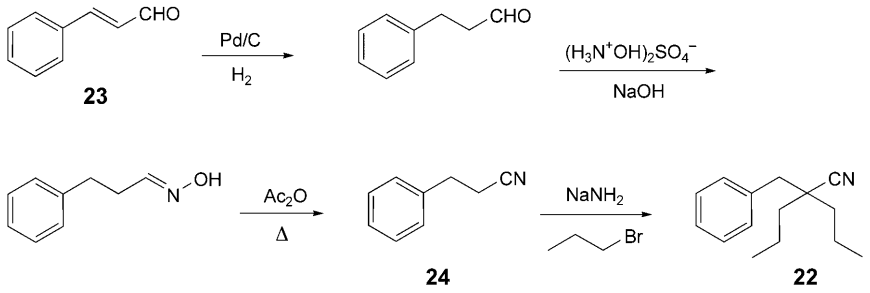
2-Benzyl-2-propylpentanenitrile (**22**) was synthesized from cinnamic aldehyde (**23**; *Scheme 3*), which was first hydrogenated over Pd on activated carbon, and then converted to 3-phenylpropanenitrile (**24**) *via* its oxime, using the standard methodology described before. The nitrile **24** was then doubly alkylated with NaH and propyl bromide to furnish **22**.

3-(4-Ethylphenyl)-3-methylbutanenitrile (**25**) was prepared by conjugate addition of 4-ethylphenyl magnesium bromide (**26**) to the unsaturated ester **27** in the presence of a catalytic amount of elemental Cu (*Scheme 4*). Saponification of the resulting product **28** with 10% aqueous NaOH solution at 40°, and subsequent *in situ* decarboxylation of the intermediary free acid at 200° furnished the target molecule **25** in good yield.

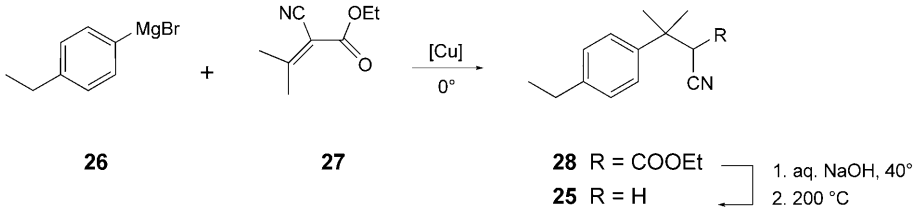
Scheme 2



Scheme 3



Scheme 4



The olfactory comparison of the *Fleuranil*® analogs **31**–**44**, and of the muguet aldehydes **45**–**47**, prepared according to the synthetic routes detailed above, is given in *Fig. 3*. Among the new molecules **31**–**44**, none was found to possess the interesting odor characteristics typical for *Fleuranil*® (**5**/**6**). Unfortunately, most nitriles synthesized had only weak odor profiles.

Since many alkoxy substituted aromatic aldehydes are widely used in perfumery, *e.g.*, *Canthoxal*® and *Helional*® (**48**), we envisaged that it would be interesting from an

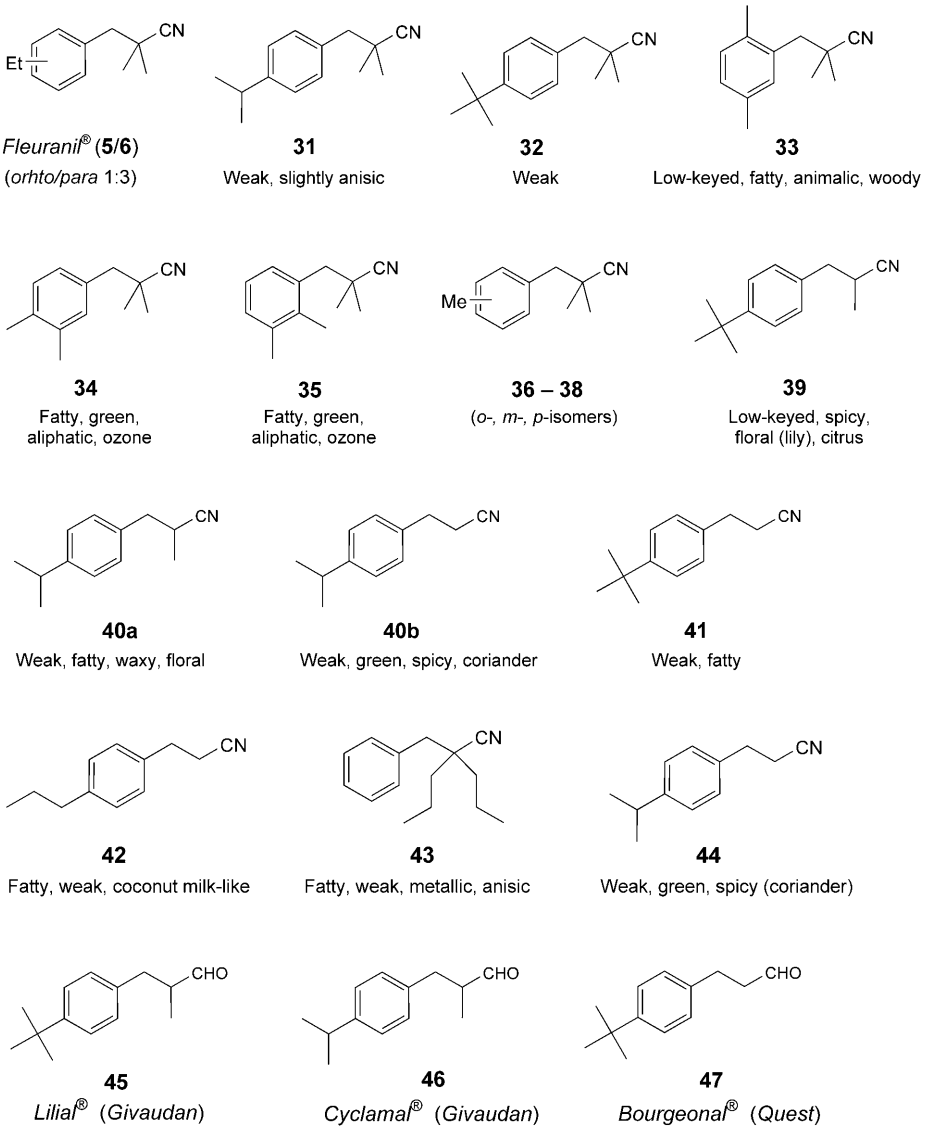


Fig. 3. *Odor comparison of* Fleuranil® *anologs of* IFF. For comparison, the muguet aldehydes **45**–**47** of other companies are also depicted.

olfactory perspective to prepare also alkoxy nitriles [10][11] and to compare their odors with those of the corresponding alkyl nitriles. Again, these derivatives were synthesized by means of the standard methodology elaborated for *Fleuranil®* analogs (see *Schemes 1* and *2*). The structures and odor characteristics of the synthesized alkoxy nitriles **49**–**54** are presented in *Fig. 4*.

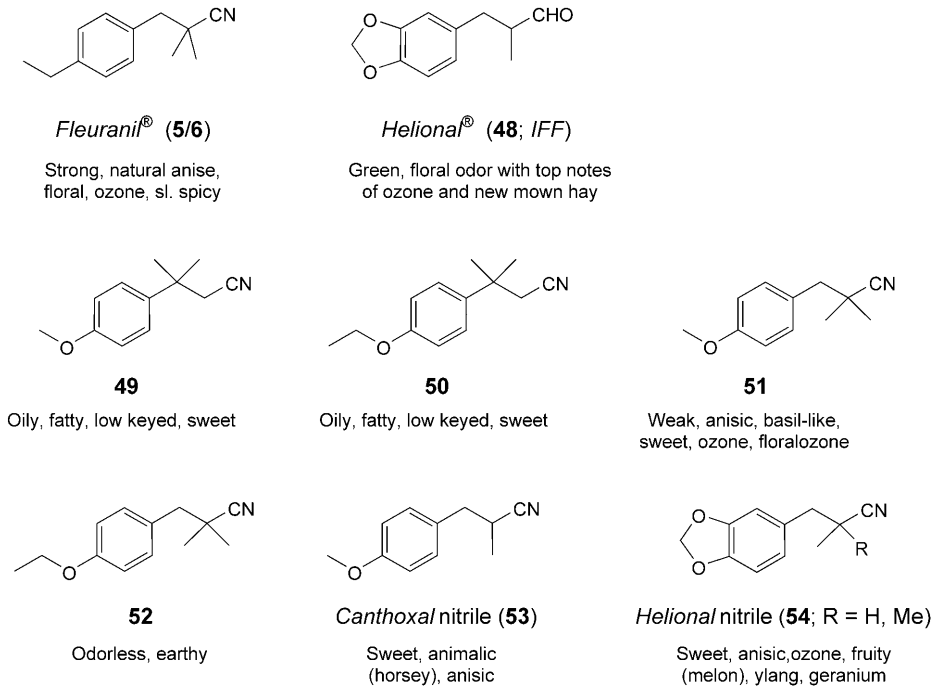


Fig. 4. *Odors of* Fleuranil®*-related alkoxy analogs*

Discovery of *Khusinil*®. – The discovery of *Khusinil*® (**7**; *Fig. 1*) came about as an outgrowth of this structure–odor relationship study [5] carried out during the exploratory work on analogs of *Fleuranil*® (**5**/**6**). Again, several dimethyl-substituted aryl nitriles were prepared for olfactory comparison, starting from readily available styrenes of type **55** (*Scheme 5*). The nucleophilic addition of a carbanion generated from isobutyronitrile (**15**) to, *e.g.*, styrene proper (**55a**) or the derivative **55b** at 120° gave **56** and *Khusinil*® (**7**), respectively, in good yields. Demethyl Khusinil (**57**) was prepared in two steps from 2-phenylpropanal (**58**) by reaction with propanal (**59**) in the presence of a base. On catalytic hydrogenation of the resulting 2-methyl-4-phenylpent-2-enal (**60**), the saturated aldehyde **61** was obtained in high yield. The latter was converted to the target nitrile **57** *via* its oxime, as described above.

An odor comparison of the *Khusinil*® analogs **63**–**66** with both *Khusinil*® (**7**) and *Pamplefleur*® (**67**) is made in *Fig. 5*. Compounds **63** and **64** have essentially the same odor profiles (*vetiver, grapefruit*) as *Khusinil*® (**7**), but have a *more-metallic odor tonality*, and are weaker as well.

Since there are several valuable fragrance ingredients that contain a pyridine ring, *e.g.*, **68**–**71** (*Fig. 6*), we felt that it would be interesting to prepare also some pyridyl analogs of *Khusinil*® [12] for structure–odor correlation. The two analogs **72** and **73** were prepared from readily available 2- and 4-vinylpyridine (*Scheme 6*), following the same route as developed for *Khusinil*® (*cf. Scheme 5*).

Scheme 5

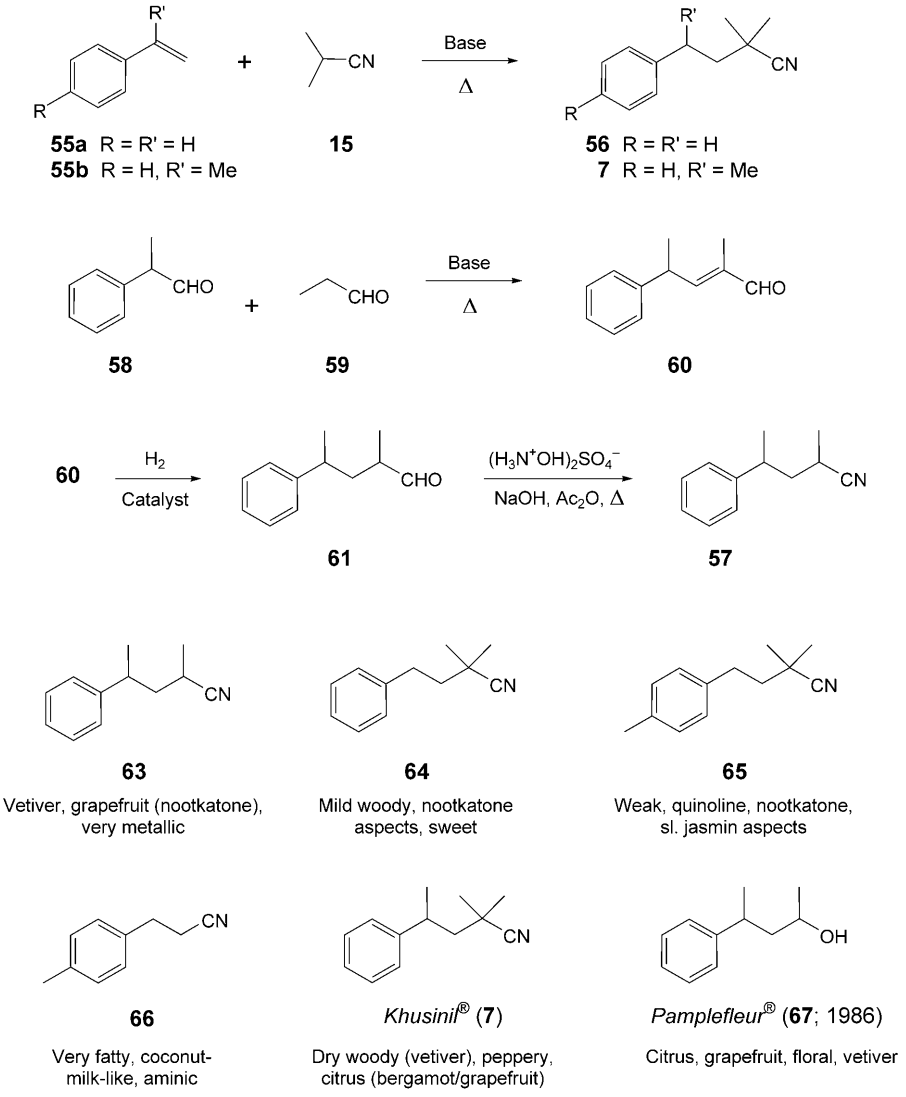


Fig. 5. *Odor relationships of* Khusinil® *analogs*

Finally, we would like to illustrate the importance of nitriles in functional perfumery with a list of nitrile ingredients, including compounds **5**–**7** and **74**–**78** (*Fig. 7*)[1]),

[1]) *Citralva*® and *Citronalva*® were first made at *IFF* in 1949. All trademarks noted in this article are assigned to *International Flavor & Fragrances, Inc.*, unless noted to the contrary. *Lilial*® (**45**) and *Cyclamal*® (**46**) are registered trademarks of *Givaudan SA*, while *Bourgeonal*® is a registered trademark of *Quest International*.

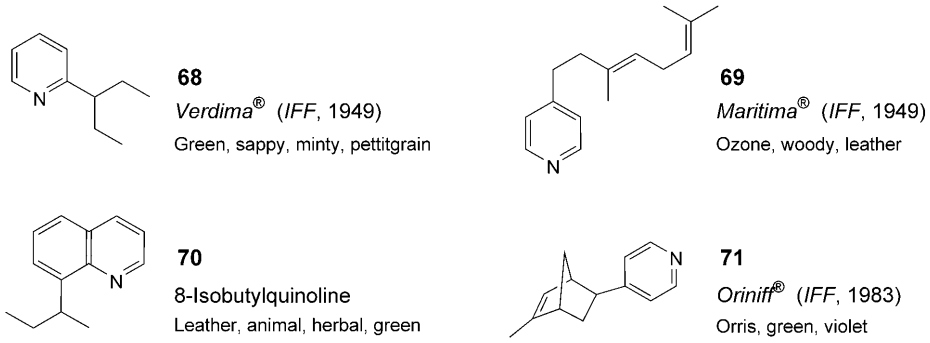


Fig. 6. *Structures and odor characteristics of the known pyridines* **68**–**71** *used in perfumery*

Scheme 6

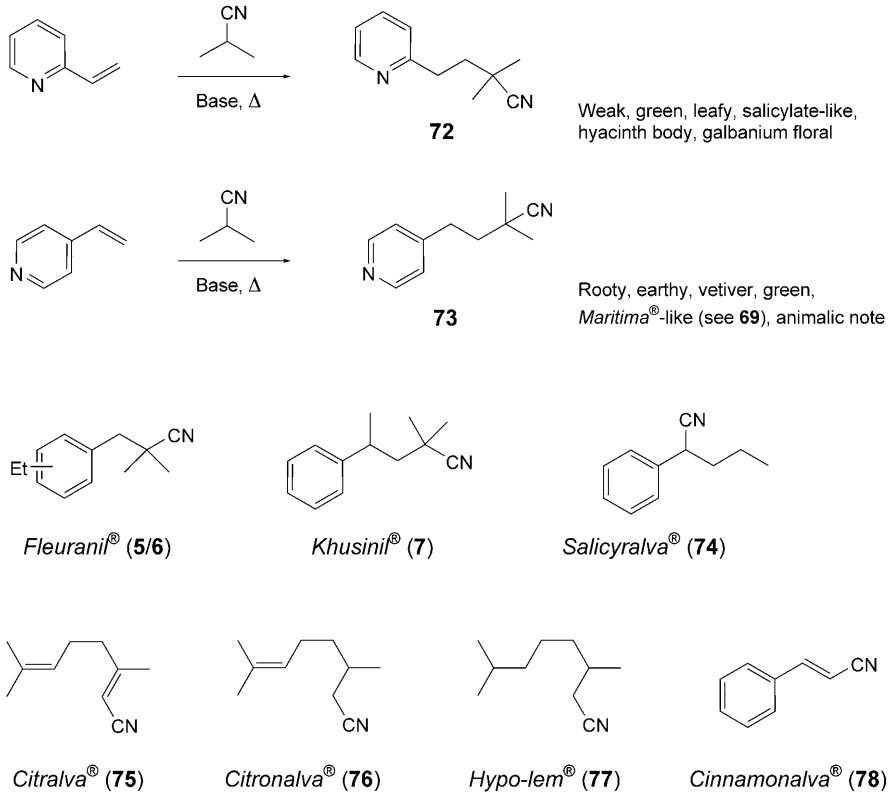


Fig. 7. *Important nitrile-based ingredients developed at* IFF *for functional perfumery*

developed over the years at *IFF*. These ingredients have enhanced the creativity of perfumers engaged in functional perfumery around the world.

Conclusions. – This account has provided just a glimpse of our research efforts on the example of the discovery of *Fleuranil*® (**5**/**6**) and *Khusinil*® (**7**). It is, indeed, refreshing to note that a perfumer's need for new molecules never wanes in spite of *ca.* 3000 fragrance ingredients to choose from. Hence, we would like to assure perfumers world-wide that they can bank on the ingenuity of synthetic organic chemists to provide them with new and unique fragrance ingredients. This would not only enhance their creativity, but also address toxicological as well as environmental concerns. An organic chemist's quest in the pursuit of new fragrance molecules will continue unabated towards that aim.

The author wishes to acknowledge his colleagues at *IFF*, whose names are cited in the following references, for their dedicated synthetic work, and several renowned perfumers for their expert fragrance evaluations of the new chemicals disclosed in this article.

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Binding Studies and Computer-Aided Modelling of Macromolecule/Odorant Interactions

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Odorant-to-biopolymer (proteins and polysaccharides) binding properties influence the partition coefficients of odorant/matrix mixtures and depend on the molecular structure of the guest and the host. While air/solvent partition coefficients of odorants influence the flavor intensity of compounds in the headspace above the solvent, more complex mechanisms are proposed for the binding of a molecule to a food matrix. Odorant air/solvent partition coefficients are dependant on both the physico-chemical properties of odorant and solvent. Binding affinities for flavor compounds on various biopolymers can be estimated by calculation of physico-chemical descriptors for the odorants. Binding affinities of γ- and δ-lactones (C_7–C_{11}) to bovine serum albumin (BSA) and β-lactoglobulin (BLG) were investigated by ultracentrifugation and equilibrium-dialysis techniques. Quantitative structure–activity relationships (QSAR) of lactone binding on proteins (BLG and BSA) were performed by the measurement of lipophilicity and H-bond strength. Large differences in observed protein-binding properties for the various compounds clearly demonstrated that structure–activity relationship was significantly influenced by the lipophilicity of the odorant. If the structure of the receptor molecule is known, computational ligand–macromolecule docking experiments can be used to predict binding affinities for unknown compounds with the receptor molecule. A BLG–lactone binding position, not previously reported in the literature, has been identified and confirmed by competitive binding studies. A model has been developed to estimate the free energy of binding of odorants to biopolymers. Estimated free energies of binding of lactones with BLG from computational methods were in very good agreement with the experimentally obtained results.

Introduction. – Odorants can interact with food ingredients such as proteins and carbohydrates. This interaction can result in a chemical reaction between the odorant and the macromolecule, leading to a change in the flavor and a loss of the aroma effectiveness. In particular, reactive odorant functional groups such as an aldehyde can react with the amine functionality of, *e.g.*, the amino acid lysine in a protein resulting in the formation of an imine. An odorant can also interact with the macromolecule in a non-covalent way, such as by means of H-bond formation, *Van der Waals*, electrostatic, π-π, ionic, and dipole–dipole interactions. These types of interaction influence the stability of a loosely associated complex. The strength of a ligand–protein complex can be experimentally determined and expressed as either an association constant (K_a [mol^{-1}]) or dissociation constant (K_d [mol]) [1]. The following relationship exists between the binding energy (ΔG) and the dissociation constant (K_d) or association constant (K_a) (*Eqn.* 1)

$$\Delta G = \Delta H - T\Delta S = RT \ln K_d = -RT \ln K_a \quad (1)$$

The magnitude of the interaction strength is, therefore, dependent on the enthalpy (ΔH) and the entropy ($T\Delta S$) of the interaction. The entropy of a system increases by

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the association of a ligand to a macromolecule with displacement of H_2O molecules at the binding site of the macromolecule. This process parallels an entropy increase of the system and a decrease of the degrees of freedom of the ligand. The entropy increase of a system delivers an essential contribution to the binding affinity of a protein – ligand complex. This statement reveals a relationship between the lipophilicity of a ligand and the entropy contribution of the interaction. The more H_2O molecules that are released from the protein environment by the ligand, the greater is the contribution to the affinity. The number of the released H_2O molecules is roughly proportional to the size of the hydrophobic part of the ligand on the binding position on the protein.

Franzen and *Kinsella* [2] used headspace analysis to carry out the first investigations on the binding properties of odorants on a protein. They investigated the influence of the addition of protein (α-lactalbumin, bovine serum albumin, yeast protein isolate, and soy beans protein isolate) to odorants solved in H_2O. Headspace concentrations of the odorants hexanal, heptanal, octanal, hexan-2-one, heptan-2-one, and octan-2-one were measured. It was found that the headspace concentrations of the compounds above H_2O/protein solutions decrease in comparison to a similar soln. without protein addition. The magnitude of the decrease in the headspace concentrations varied depending on protein and odorant. However, a correlation between the partition coefficients of the odorants (protein soln./headspace) and the binding constants of the investigated proteins was not found.

Damodaran and *Kinsella* [3] carried out binding experiments of heptan-2-one and nonan-2-one to bovine serum albumin (BSA). From the differences in the association constants for heptan-2-one ($270\ mol^{-1}$) and nonan-2-one ($1800\ mol^{-1}$), the authors concluded that the binding strength is dependant on the lipophilicity of the odorant. The calculated free binding enthalpy was -4.4 kcal/mol for nonan-2-one and -3.3 kcal/mol for heptan-2-one. On the assumption that the free energy of binding (ΔG) increased in a linear fashion from heptan-2-one to nonan-2-one, one CH_2 group rises the binding strength by -0.55 kcal/mol. Binding affinities of heptan-2-one, octan-2-one, and nonan-2-one on β-lactoglobulin (BLG) were investigated by *O'Neill* and *Kinsella* [4]. The association constants of $150\ mol^{-1}$ (heptan-2-one), $480\ mol^{-1}$ (octan-2-one), and $2440\ mol^{-1}$ (nonan-2-one) indicate an increase of the binding affinity with an increase in the lipophilicity of the odorant. This is in accordance to the data reported for BSA [3]. In the case of BLG, one CH_2 group increased the binding strength by -0.7 kcal/mol (heptan-2-one to octan-2-one) and by -1.0 kcal/mol (octan-2-one to nonan-2-one).

Further work relating to structure – property relationships of odor-active substances and their binding behavior on casein were carried out by *Landy et al.* [5] with the fruity smelling ethyl esters of acetic, butanoic, and hexanoic acid. The authors found an increase in the binding affinity corresponded with a rise in the molecular weight of the ethyl ester.

Sostmann et al. [6] determined the binding behavior on BLG for selected lactones and esters. They likewise ascertained a dependence of binding affinity to the number of C-atoms in the odorants. Increasing the lipophilicity of an odor-active component increased the association constant of the odorant to BLG. *Pelletier et al.* [7] confirmed the investigations of *Sostmann et al.* [6] on a huge number of methyl, ethyl, propyl, butyl, and hexyl esters of the $C_2 - C_7$ fatty acids. The binding constant (K_a) increased by lengthening of the alkyl chain by one CH_2 group.

Dufour and *Haertle* [8] carried out binding experiments with α- and β-ionone as well as geraniol, and (*R*)- and (*S*)-limonene on native and modified BLG. By means of fluorescence spectroscopy, the authors determined a dissociation constant of 6×10^{-7} for the protein – ligand complex of β-ionone and BLG; however, they found no binding for α-ionone, geraniol, and (*R*)- and (*S*)-limonene. In view of the structural similarities of α- and β-ionone they proposed a relationship between the three-dimensional structure of a compound and its binding affinity. Comparison of the three-dimensional structures of α- and β-ionone indicates that β-ionone has, in contrast to α-ionone, a planar structure. Based on these findings, it is suggested that the key – lock principle applies and, therefore, a precise three-dimensional structure must be assumed to achieve a strong protein – ligand interaction. This assumption was supported by a comparison of these results with data on the three-dimensional structure and binding behavior of retinol to BLG [9]. A comparison of the dissociation constants of β-ionone (6×10^{-7} mol) with those of retinol (2.0×10^{-8} mol) [8] revealed high binding affinities for both compounds on BLG. It was showed that retinol also has a planar structure in homology to the odorant β-ionone. On the basis of these results, it was concluded that the three-dimensional structure defines the affinity of a ligand to a macromolecule. *Papiz et al.* [10] determined the three-dimensional structure of BLG by X-ray structure analysis and modelled a protein – ligand complex with retinol. They suggested that retinol binds in the β-barrel, a central deep hydrophobic pocket of the protein, which is built from the eight antiparallel β-strands of BLG, labeled A – H.

Further research on whey proteins by *Jasinski* and *Kilara* [11] used equilibrium dialysis to determine the binding constants of nonan-2-one and nonanal to whey protein concentrate, BSA, BLG, and α-lactalbumin. The binding constants for nonan-2-one and nonanal varied from 1.0×10^6 mol^{-1} – 2.0×10^6 mol^{-1} ($n = 61$ and $n = 68$). With a binding constant of 1.41×10^4 mol^{-1} ($n = 15$), the binding strength of nonan-2-one to BSA was much higher than to BLG (1.21×10^2 mol^{-1} and $n = 13.6$). The lowest binding affinity of nonan-2-one was found to the protein α-lactalbumin (1.05×10^2 mol^{-1} and $n = 33.3$). Based on these results, they suggested whey protein concentrate as a suitable flavor carrier for foodstuffs.

Tromelin and *Guichard* [12] carried out quantitative structure – activity relationship (3D-QSAR) studies using the *Catalyst* software to explain the nature of interactions between flavor compounds and BLG. A set of 35 odorants, for which dissociation constants had been previously determined by affinity chromatography, were chosen. On the basis of these results, it appears that aroma binding to BLG is caused by both hydrophobic interactions and H-bonding. *Guth et al.* [13] investigated the binding properties of a series of γ- and δ-lactones to BLG and BSA. By means of molecular modelling experiments and neural networks, with Kohonen Maps [14][15], it could be shown that hydrophobicity and the three-dimensional structure of odorants are important factors for the binding properties. Binding energies of selected lactones were calculated in the central deep hydrophobic pocket of BLG using the software package *Sculpt* [16]. Relative binding-energy differences for the investigated lactones correlated with the experimentally determined differences in association constants.

Lübke et al. [17] studied the binding position of different small ligands to BLG. They investigated the interaction of BLG with small ligands in aqueous solutions (pH 2.0 and 7.5) by means of *Fourier*-transform infrared (FT-IR) spectroscopy.

Comparison of the IR spectra from BLG with and without ligands allowed conclusions relating to binding position in accordance to changes in the conformation of BLG. This technique allowed observation of changes in the conformation of BLG in the presence of relatively low amounts of retinol, tetradecanoic acid, and γ-decalactone. Based on the literature results stating that fatty acids bind in the central hydrophobic pocket of BLG [18], it was concluded that the above-mentioned substances have the same binding position. Different behavior was found for the binding of β-ionone to BLG and this was confirmed by the 2D-NMR study of *Lübke et al.* [19]. The 2D-NMR results suggested binding of γ-decalactone (pH 2.0) in the central hydrophobic pocket of BLG and, for β-ionone, an external binding site in a groove on the outer surface of BLG that is built up by the helix and the external part of the β-barrel.

By means of X-ray-diffraction studies, *Monaco et al.* [20] suggested the groove on the surface of BLG as the retinol-binding site. This is in contrast to the X-ray-diffraction studies of *Kontopidis et al.* [21] and the modelling studies of *Papiz et al.* [10] who found retinol in the central pocket of BLG.

Sostmann and *Guichard* [22] investigated the binding constants of various odorants by affinity chromatography (investigations carried out at pH 3). According to the results of the competition experiments, they concluded that the binding sites of γ- and δ-octalactone correspond to that of β-ionone in the central pocket of BLG. For the other investigated compounds (*e.g.*, nonan-2-one, octan-2-one, and α-ionone) a non-specific hydrophobic interaction with BLG was presumed.

Methods for the determination of the binding constants (association constant, K_a; dissociation constant, K_d; capacity, R; number of the binding sites, n) can be found in two reviews by *Sebille* [23], and *Hage* and *Tweed* [24]. According to the literature, different methods for the determination of the binding parameters are suggested, depending on the matrix and ligand to be investigated. The standard method is equilibrium dialysis, which is used in particular for the determination of binding constants of pharmacologically active substances. Further methods for the determination of binding constants are: dynamic dialysis, ultrafiltration, fluorescence spectroscopy, affinity chromatography, and liquid chromatography techniques [25].

Comparison of the experimentally determined binding data of retinol to BLG by means of equilibrium dialysis ($K_d = 6.62 \times 10^{-5}$ mol) [26] and fluorescence spectroscopy ($K_d = 2.0 \times 10^{-8}$ mol) [9] shows that the different techniques resulted in different binding constants. The fluorescence method is based on a change in the chemical environment of a tryptophane residue in the protein in the presence of a ligand. The measured fluorescence intensity or the binding affinity derived from it depends, therefore, on the presence of a tryptophane residue in a protein, and on the distance of the ligand binding position to the tryptophane residue. A detailed discussion of the differences in binding constants obtained by fluorometry and equilibrium dialysis was reported by *Muresan et al.* [27] who compared the bindings of several small ligands to BLG by fluorometry and equilibrium dialysis techniques [27].

The results published in the literature, some of which have been briefly discussed here make it clear, that the molecular basis of the BLG interaction is not well understood. The binding sites of odorants are only partly or insufficiently described, and, for various ligands, the proposed binding positions are not in agreement between authors. It should also be noted that the majority of this research did not include

discussions relating to the pH-dependent conformation changes of the central hydrophobic pocket of BLG [28][29].

The aim of the present study was to evaluate the benefits of molecular-modelling methods for the determination of the binding properties of flavor compounds to BLG. For this, we selected a series of γ- and δ-lactones (C_7-C_{11}) and measured the association constants (K_a) by ultracentrifugation and equilibrium dialysis techniques. Furthermore, physico-chemical properties (partition coefficients: air/H_2O, octanol/H_2O, and cyclohexane/H_2O) of selected lactones were determined, and the relationship between binding affinities and physico-chemical properties was investigated. It was envisaged that molecular-modelling methods should indicate important binding sites of lactones to BLG and allow the prediction of binding affinities of unknown compounds.

Results and Discussion. – *Determination of the Binding Constants of Odorants on BLG and BSA*. For the evaluation of the binding constants of odorants to proteins, a method was developed that was suitable for the determination of the protein-bound odorant and free odorant concentration. Due to the high volatility and instability of odorants, a method was chosen that permitted a rapid realization of the experiments. As a method of choice, an ultrafiltration technique, the so-called ultracentrifugation method, was used [13]. The data obtained by ultracentrifugation was compared with the values yielded by equilibrium dialysis. The results of the binding studies are summarized in *Table 1* for the protein BSA and in *Table 2* for the protein BLG. Literature data of lactone binding constants on BLG are also included in *Table 2*.

The ultracentrifugation method showed odorant losses of *ca.* 20% for γ-nonalactone and 36% for γ-undecalactone due to the high volatility of the substances and/or by adsorptive effects to the filtration unit. The recovery of protein-bound odorant was 95–100%. With respect to the binding constants obtained, it was assumed that the odorant losses during the filtration process have minimal effect on the equilibrium and no influence on the accuracy of the calculated association constants. This is because in this study the odorant concentrations were determined in the eluate (free odorant fraction) and in the residue fraction (free and bound odorant fraction). Calculation of the protein-bound odorant concentration was achieved from the difference between both fractions. The binding isotherms of the odorants were investigated by use of non-linear regression (Origin 6.0) techniques for a one-site- (ν_1) and a two-site-binding model (ν_2) (*Eqn. 2*).

$$\nu_1 = \frac{n_1 \times F}{K_{d1} + F}, \nu_2 = \frac{n_1 \times F}{K_{d1} + F} + \frac{n_2 \times F}{K_{d2} + F}, \; K_a = 1/K_d \qquad (2)$$

(ν_i: odorant bound [M]/protein [M]; *F:* free odorant concentration [M], n_i*:* number of binding sites)

Depending on temp., pH value, ionic strength of the protein soln., and protein concentration, BLG resides in a monomeric-dimeric equilibrium [30]. According to *Aymard et al.* [30] and the conditions used in the present study (0.08M phosphate buffer soln., temperature 20°, protein concentration 8 mg BLG/ml, pH 6.5–7.0), BLG exists in a dimeric form.

Table 1. *Binding Constants* (association constant K_a, number of binding sites n, free energy of binding ΔG) *of* γ- *and* δ-*Lactones to Bovine Serum Albumin (BSA)*

Compound[a])	K_a [mol^{-1}][b])	n	Global affinity	ΔG [kcal/mol]	
			$n \times K_a$	$(n \times K_a)$	(K_a)[c])
γ-Heptalactone					
0 – 100 mg/l	5263 ± 1052	0.1 ± 0.0	526	– 3.71	–
100 – 1000 mg/l	230 ± 20	1.3 ± 0.1	299	– 3.37	
γ-Octalactone					
0 – 100 mg/l	950 ± 100	1.0 ± 0.1	950	– 4.05	– 4.05
100 – 1000 mg/l	277 ± 50	2.7 ± 0.3	748	– 3.91	
γ-Nonalactone					
0 – 100 mg/l	2080 ± 300[d])	1.8 ± 0.3	3744	– 4.86	– 4.52
100 – 1000 mg/l	606 ± 300	5.6 ± 1.8	3394	– 4.81	
γ-Decalactone					
0 – 100 mg/l	6290 ± 700[d])	1.8 ± 0.3	11322	– 5.52	– 5.17
γ-Undecalactone					
0 – 50 mg/l	16700 ± 1700[d])	2.0 ± 0.2	33400	– 6.16	– 5.75
δ-Heptalactone					
0 – 100 mg/l	650 ± 300	0.3 ± 0.1	195	– 3.12	–
δ-Nonalactone					
0 – 100 mg/l	813 ± 300	1.2 ± 0.4	976	– 4.07	– 3.96
100 – 2000 mg/l	30 ± 6	25.0 ± 3.0	750	– 3.91	
δ-Decalactone					
0 – 100 mg/l	2000 ± 500	1.4 ± 0.4	2800	– 4.69	– 4.49
100 – 1000 mg/l	83 ± 10	31.0 ± 1.0	2573	– 4.64	
δ-Undecalactone					
0 – 50 mg/l	6250 ± 2000	0.9 ± 0.3	5625	– 5.11	– 5.17

[a]) Concentration range of odorant for the binding experiments. [b]) Binding constants were determined by ultracentrifugation technique (BSA: 4.46×10^{-4} M, phosphate buffer: pH 7.0, 0.08M, KH_2PO_4/Na_2HPO_4). The binding constants (K_a and n) were calculated from the binding isotherms with the software package Origin 6.0 for a one-site- (ν_1) and a two-site-binding model (ν_2): $\nu_1 = n \times F/(K_D + F)$ and $\nu_2 = [n_1 \times F/(K_{d1} + F)] + [n_2 \times F/(K_{d2} + F)]$, $K_a = 1/K_d$; ν: odorant bound (M)/protein (M), F: free odorant concentration (M), n_i: number of binding sites). [c]) Free energy of binding for the binding site with the highest affinity to BSA. [d]) The two-site-binding model (ν_2) showed that $K_{a1} = K_{a2}$ ($n_1 = 1$ and $n_2 = 1$).

Within the series of the γ- and δ-lactones the highest association constants (K_a) to BSA (*Table 1*) were found for γ-undecalactone (1.6×10^4 mol^{-1}, $n = 2$) and δ-undecalactone (6.25×10^3 mol^{-1}, $n = 0.9$). For the association constants of γ- and δ-undecalactone to BLG (*Table 2*), values of 5.88×10^3 mol^{-1} ($n = 1.1$) and 1.64×10^3 mol^{-1} ($n = 1$) were obtained, respectively. According to these results, the binding affinities of the corresponding lactones to BSA were much higher than those for BLG. The evaluation of the binding isotherms of γ-decalactone and γ-undecalactone to BSA by statistical methods (Origin 6.0) indicated two possible binding models. One model has two distinguished binding sites for the lactone on BSA with similar binding affinities (*Table 1*); the second model has one binding site which can accommodate two molecules of lactone. The two-site-binding model will only be valid if both binding sites are independent of each other, *i.e.*, a ligand binding on one site causes no change in the

Table 2. *Binding Constants* (association constant K_a, number of binding sites n, free energy of binding ΔG) *of* γ- *and* δ-*Lactones to* β-*Lactoglobulin (BLG)*

Compound	Method[a])	pH	K_a (M^{-1})	n	ΔG (kcal/mol)
γ-Heptalactone	UCF	7.0	< 50	–	< 2.3
γ-Octalactone	UCF	7.0	77 ± 35	3.0 ± 1.0	-2.57
	AC	3.0	450[b])	–	–
γ-Nonalactone	UCF	7.0	215 ± 40	1.9 ± 0.2	-3.18
γ-Decalactone	UCF	7.0	910 ± 200	0.9 ± 0.3	-4.03
	MED	7.0	1020 ± 200	1.0 ± 0.3	-4.09
	MED	6.5	2050 ± 100	1.1 ± 0.1	-4.51
	AC	3.0	3230[b])	–	–
γ-Undecalactone	UCF	7.0	5880 ± 150	1.1 ± 0.3	-5.13
	AC	3.0	9924[b])	–	–
δ-Heptalactone	UCF	7.0	< 50	–	< 2.3
δ-Octalactone	UCF	7.0	< 50	–	< 2.3
	AC	3.0	231[b])	–	–
δ-Nonalactone	UCF	7.0	145 ± 10	1.0 ± 0.1	-2.94
δ-Decalactone	UCF	7.0	625 ± 120	1.3 ± 0.1	-3.81
δ-Undecalactone	UCF	7.0	1640 ± 370	1.0 ± 0.2	-4.38

[a]) UCF: ultracentrifugation (BLG: 4.45×10^{-3} M, phosphate buffer: pH 7.0, 0.08M, KH_2PO_4/ Na_2HPO_4); MED: micro equilibrium dialysis (BLG: 4.45×10^{-4} M, phosphate buffer: pH 7.0, 0.08M, KH_2PO_4/Na_2HPO_4); AC: affinity chromatography with immobilized BLG ($1.69 - 5.20 \times 10^{-3}$ M) on the HPLC column [27]. [b]) Values are taken from [27]. The reported values are global affinity constants ($n \times K_a$).

binding affinity of the other site (non-cooperative binding). By critical evaluation of the binding curves, the number of binding sites of a protein can be determined. In relation to this, one needs to be aware of the publication of *Klotz* and *Hunston* [31] that describes the shortcomings of the evaluation according to the *Scatchard* plot [32]. Accordingly, the analysis was performed from plots of the logarithm of the free concentration of the odorant (log *F*, *x*-axis) against the bound odorant concentration [M]/protein [M] (*y*-axis) [13]. In contrast to γ- and δ-heptalactone, for γ- and δ-undecalactone, γ- and δ-decalactone, and γ- and δ-nonalactone, the saturation of the binding sites could not be achieved due to poor H_2O solubility (*Table 3*). The calculation of the number of binding sites was achieved by extrapolation of the curve using the Origin 6.0 software with non-linear curve adaptation. The addition of an organic solvent to raise the solubility of the lactones was not desirable, because this can lead to changes in the binding behavior.

Investigations into the enantioselectivity of the lactone binding to BLG and BSA showed that the (*R*)- and (*S*)-enantiomers of the corresponding lactones bound with identical affinity. By means of chiral gas chromatography (*Fig. 1*) the enantiomeric ratio was evaluated after equilibration of the racemic lactones ((*R*)/(*S*) 1:1) with BLG and BSA. After extraction of the lactones (free and protein-bound fractions) with solvent and separation by chiral gas chromatography (*Fig. 1*), the enantiomeric ratio of the fractions did not change after binding of the lactones ((*R*)/(*S*) 1:1) to the proteins.

The influence of pH on the binding of lactones to BLG is shown in *Table 2* for selected γ- and δ-lactones. The association constants of lactones increases when the pH value decreases from 7.0 to 3.0. In the homologous series of the γ- and δ-lactones, the binding affinities to BSA as well as to BLG increased with the increasing number of C-

Table 3. *Partition Coefficients* ($\log P$)[a]) *and Solubility Parameters* (S)[b]) *of γ- and δ-Lactones in Different Solvent Systems*

Compound	$\log P$		$\Delta\log P$[c])	S_{H_2O}[b]) [M]	$S_{\text{phosphate buffer}}$[b]) [M]
	octanol/H_2O[a])	cyclohexane/H_2O[a])			
γ-Heptalactone	0.65	− 0.10	0.75	3.2×10^{-1}	2.1×10^{-1}
γ-Octalactone	1.22	0.56	0.66	5.9×10^{-2}	3.9×10^{-2}
γ-Nonalactone	1.95	1.22	0.73	1.5×10^{-2}	1.2×10^{-2}
γ-Decalactone	2.72	1.87	0.85	5.0×10^{-3}	2.8×10^{-3}
γ-Undecalactone	3.30	2.37	0.93	1.0×10^{-3}	5.9×10^{-4}
δ-Heptalactone	0.27	− 0.51	0.78	n.d.	n.d.
δ-Octalactone	0.97	0.13	0.84	8.1×10^{-2}	5.0×10^{-2}
δ-Nonalactone	1.54	0.76	0.78	5.6×10^{-2}	3.6×10^{-2}
δ-Decalactone	2.34	1.47	0.87	1.4×10^{-2}	8.5×10^{-3}
δ-Undecalactone	2.93	2.10	0.83	3.8×10^{-3}	2.8×10^{-3}

[a]) Experimental determined partition coefficient (P) of lactones, calculated as $\log P$ values: octanol/H_2O ($\log P_{\text{octanol/H}_2O}$) and cyclohexane/$H_2O$ ($\log P_{\text{cyclohexane/H}_2O}$). [b]) Solubilities of lactones in pure H_2O (S_{H_2O}) und phosphate buffer ($S_{\text{phospate buffer}}$) at pH 7.0 ($KH_2PO_4$ 0.066 M, Na_2HPO_4 0.083 M); n.d.: not determined. [c]) $\Delta\log P = \log P_{\text{octanol/H}_2O} - \log P_{\text{cyclohexane/H}_2O}$.

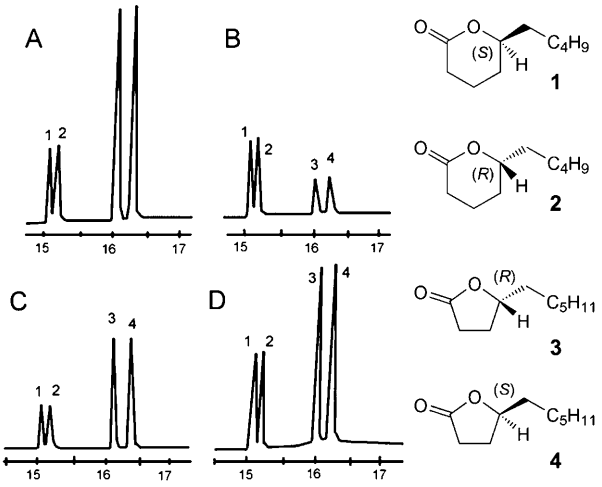

Fig. 1. *Investigation of the enantioselectivity of the lactone binding to BLG und BSA by chiral gas chromatography* (2,3-Diacetyl-6-[(*tert*-butyl)dimethylsilyl]-β-cyclodextrine): *(*S*)-δ-decalactone* (**1**), *(*R*)-δ-decalactone* (**2**), *(*R*)-γ-decalactone* (**3**), *and (*S*)-γ-decalactone* (**4**). *A*) BSA-Bound fraction of lactone, *B*) free fraction (protein BSA), *C*) free fraction (protein BLG), and *D*) BLG bound fraction of lactone.

atoms of the lactones. For example, the association constant of γ-undecalacton to BLG is 27-fold higher compared to γ-nonalactone (*Table 2*). The results in *Table 2* indicate that BLG binds around 1 mol of γ- and δ-decalactone, and γ- and δ-undecalactone per mol of dimeric BLG (M_r *ca.* 36000). The computed binding energy ($\Delta G = -RT \ln K_a$) to BLG for γ-undecalactone was − 5.13, for γ-decalactone − 4.51, for δ-undecalactone

– 4.38, and for δ-decalactone – 3.81 (*Table 2*). In the case of the above-mentioned two γ-lactones, the addition of one CH_2 group increased the binding strength to BLG by – 0.62 kcal/mol and for the two δ-lactones by – 0.57 kcal/mol (*Table 2*). For the binding to BSA, the two γ-lactones (C_{10} and C_{11}) differ by – 0.58 kcal/mol, and the two δ-lactones (C_{10} and C_{11}) by – 0.68 kcal/mol (*Table 1*). Depending on the odorant concentration range applied to the binding studies with BSA, a different number of binding sites (n) could be observed. The data summarized in *Table 1* indicates that one or two high-affinity binding sites, and a large number of binding sites with lower affinity exist.

Determination of the Physico-chemical Properties of Selected Lactones: Partition Coefficients in Octanol/H_2O and Cyclohexane/H_2O, Solubility in H_2O, Solubility in Phosphate Buffer. The log P values were determined in the systems octanol/H_2O and cyclohexane/H_2O, and are summarized in *Table 3*. The partition coefficients of the octanol/H_2O system ($\log P_{\text{octanol/H}_2\text{O}}$) for the investigated lactones ranged from 0.27 (δ-heptalactone) to 3.30 (γ-undecalactone), and the $\log P_{\text{cyclohexane/H}_2\text{O}}$ values from – 0.51 (δ-heptalactone) to 2.37 (γ-undecalactone). An elongation of the alkyl side chain of the γ- and δ-lactones by one CH_2 group leads to an increase of the $\log P_{\text{octanol/H}_2\text{O}}$ values by around 0.66 log P units and for the $\log P_{\text{cyclohexane/H}_2\text{O}}$ values by around 0.63 log P units. From the data in *Table 3*, it becomes clear that γ-lactones are more hydrophobic than δ-lactones when considering an identical number of C-atoms in the molecule. From the data, it can be deduced that a CH_2 group in the lactone ring performs a weaker contribution to the lipophilicity than a CH_2 group in the alkyl side chain of the lactone. The H_2O-accessible surfaces of the corresponding lactones are responsible for this effect, which is smaller if the CH_2 group is located in the ring in contrast to a CH_2 group in the alkyl side chain of the lactone. The data relating to the solubility of the compounds in H_2O and phosphate buffer (*Table 3*) confirms the assumption that γ-lactones have a higher log P value, and a lower solubility in H_2O and phosphate buffer when compared to δ-lactones with an identical number of C-atoms.

The difference between the log P values in octanol/H_2O and in the cyclohexane/H_2O system is according to *Seiler* [33] a measure of the H-bond strength of a compound. Substances with large differences ($\log P_{\text{octanol/H}_2\text{O}} - \log P_{\text{cyclohexane/H}_2\text{O}}$) can form stronger H-bonds than those with small differences. Also, a ligand of a protein must strip its hydrate shell, before the binding to the protein can occur. The strength of the H-bond rebuilt to the protein can then give a positive contribution to the enthalpic (ΔH) part of the reaction. When considering the fact that the hydrate shell must first be stripped, followed by the rebuilding of the H-bonds to the protein, it is postulated that there is an optimum $\Delta\log P$ value for a given protein–ligand interaction. These representations make clear the significance of the $\Delta\log P$ value for a protein–ligand interaction. However, the observed differences of the $\Delta\log P$ values of the investigated lactones ($\Delta\log P$: 0.66–0.93) are too small to explain the differences found for protein–ligand binding affinities. Rather, it is to be assumed that the enthalpic contribution for a possible H-bond of the lactones formed to BLG or BSA is very similar.

Within a class of compounds, the increase of the binding affinity is depending on the lipophilicity: if the lipophilicity increases, *e.g.*, from γ-nonalactone ($\log P_{\text{octanol/H}_2\text{O}} = 1.95$) to γ-undecalactone ($\log P_{\text{octanol/H}_2\text{O}} = 3.30$), the free binding energy to BSA

increases to -1.23 kcal/mol (*Table 1*). For both lactones, which differ by two CH_2 groups, a $\Delta\log P$ value of 1.35 (*Table 3*) was found. Therefore, a ΔG value of -1.0 kcal/mol corresponds to 1.1 $\log P$ units. If one compares these results with the study of *Damodaran* and *Kinsella* [3] for the binding differences of nonan-2-one and heptan-2-one ($\Delta G = -1.1$ kcal/mol), a $\Delta\log P_{\text{octanol/H}_2\text{O}}$ of 1.21 units for both compounds can be expected. The actual, experimentally ascertained $\Delta\log P_{\text{octanol/H}_2\text{O}}$ value for both ketones extracted from the literature [34] was 1.16. Thus, the actual and calculated values are in good agreement and confirm, therefore, the relationship between binding affinity and $\log P$ value.

A correlation of the $\log P_{\text{octanol/H}_2\text{O}}$ values and the binding affinities of the investigated lactones on BLG (*Fig. 2*) and on BSA (*Fig. 3*) confirm the recognized relationship between lipophilicity and the binding affinity of a compound. From the linear regression analyses (*Figs. 2* and *3*), the relationships can be expressed in form of two regression equations. These allow the estimation of binding energies of γ- and δ-lactones on BLG and BSA. As the three-dimensional structure of the compound remains disregarded, the binding energy of other classes of compounds cannot be described with the two equations.

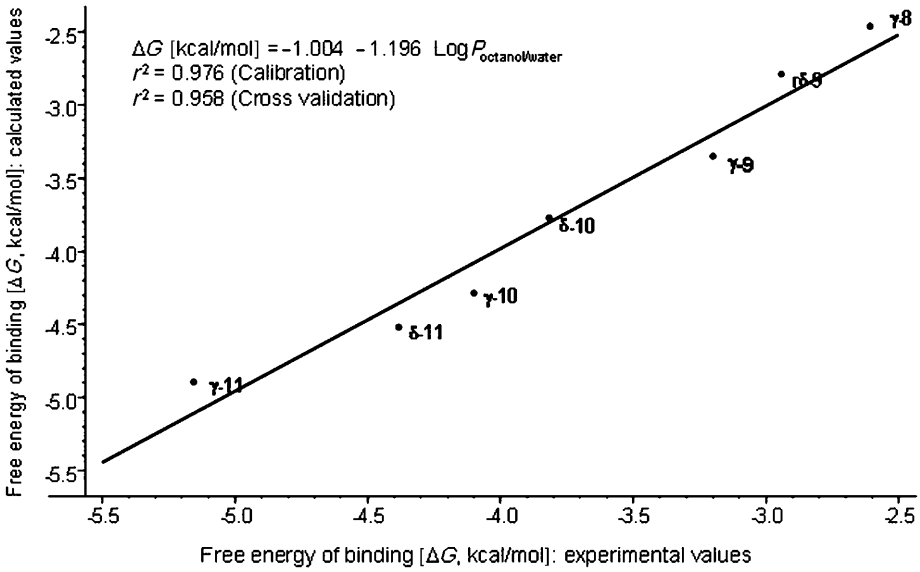

Fig. 2. *Correlation of log* $P_{octanol/H_2O}$ *values and free energy of binding of lactones to BLG by means of linear regression analysis.* γ-8: γ-Octalactone, γ-9: γ-nonalactone, γ-10: γ-decalactone, γ-11: γ-undecalactone, δ-9: δ-nonalactone, δ-10: δ-decalactone, δ-11: δ-undecalactone.

Molecular-Modelling studies of BLG–Lactone Binding. For this molecular-modelling investigation, the crystal structure of BLG was taken from the *Brookhaven Protein Databank* (http://www.rcsb.org). To allow comparison of the experimentally obtained binding data (BLG mixture of variants A and B) with the molecular-modelling studies, the crystal structure of BLG containing the mixture of the genetic variants A and B (1beb) was taken and modelled [35]. The two genetic variants differ

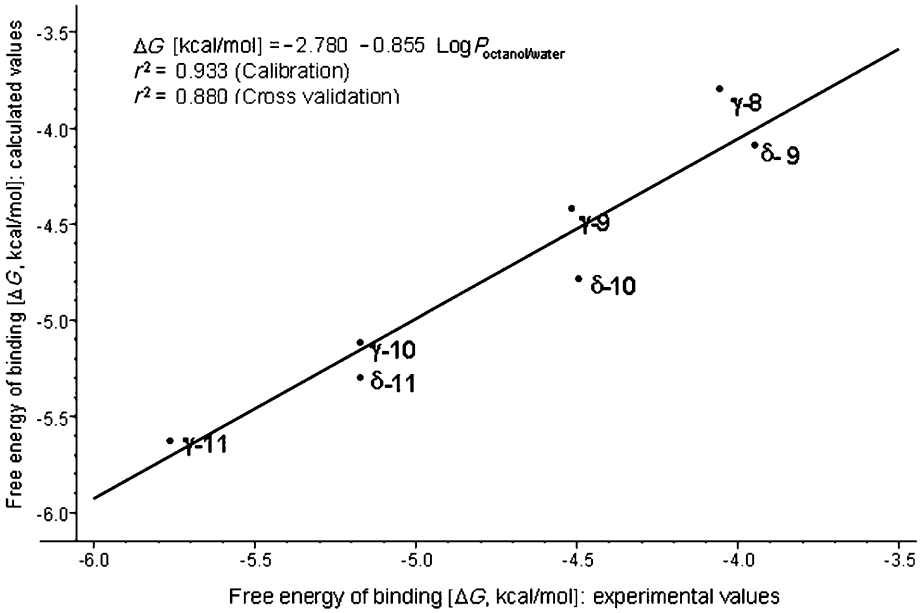

Fig. 3. *Correlation of log* $P_{octanol/H_2O}$ *values and free energy of binding of lactones to BSA by means of linear regression analysis.* γ-8: γ-Octalactone, γ-9: γ-nonalactone, γ-10: γ-decalactone, γ-11: γ-undecalactone, δ-9: δ-nonalactone, δ-10: δ-decalactone, δ-11: δ-undecalactone.

in the amino acid positions 64 and 118. These positions consist of asparagine (Asp) and valine (Val) in variant A, and of glycine (Gly) and Alanin (Ala) in variant B. The conformation differences ascertained by means of X-ray analyses of the genetic variants A and B are very small [36]. The structure of the mixed genetic variant A and B of BLG is displayed in *Fig. 4*. The contact positions of the BLG dimer (variant A and B) are the AB loops, and the two β-sheets I of variants A and B. On the contact points *Brownlow et al.* [35] found twelve H-bond interactions between the following amino acids: His 146 (variant B) → Ser 150 (variant A), His 146 (variant A) → Ser 150 (variant B), Asp 33 (variant B) → Arg 40 (variant A), Asp 33 (variant A) → Arg 40 (variant B), Arg 148 (variant A) → Arg 148 (variant B), Asp 33 (variant B) → Ala 34 (variant A), and Asp 33 (variant A) → Ala 34 (variant B). For BLG, a pH-dependent conformation change of the central deep binding pocket is described [28]. *Qin et al.* [28] investigated the structural changes of BLG (variant A) at three different pH values (6.2, 7.1, and 8.2) by means of X-ray analysis. The authors observed, as a function of pH value, conformation changes of the loop EF (amino acids 85–90 in *Fig. 5*). The structures published in the protein database, were examined in the present study by means of molecular modelling. At a pH value of 6.2, the loop EF (*Fig. 4*) lies over the binding pocket, so that the pocket of BLG is no longer accessible (*Fig. 5,A*). With rising pH (7.1 and 8.2) a movement of the loop EF takes place, so that access to the central binding pocket is possible (*Fig. 5,B* and *C*). These described observations can have a significant influence on the protein–ligand interaction. In the present study, we have found increasing binding affinities of, *e.g.*, γ-decalactone with decreasing pH

values (*Table 2*). From these results, one can expect a binding position of γ-decalactone to BLG, which is different from that of the central hydrophobic pocket. In contrast, 2D-NMR studies at pH 2.0 [19] and molecular-modelling investigations [13] suggested binding of γ-decalactone in the central hydrophobic pocket of BLG.

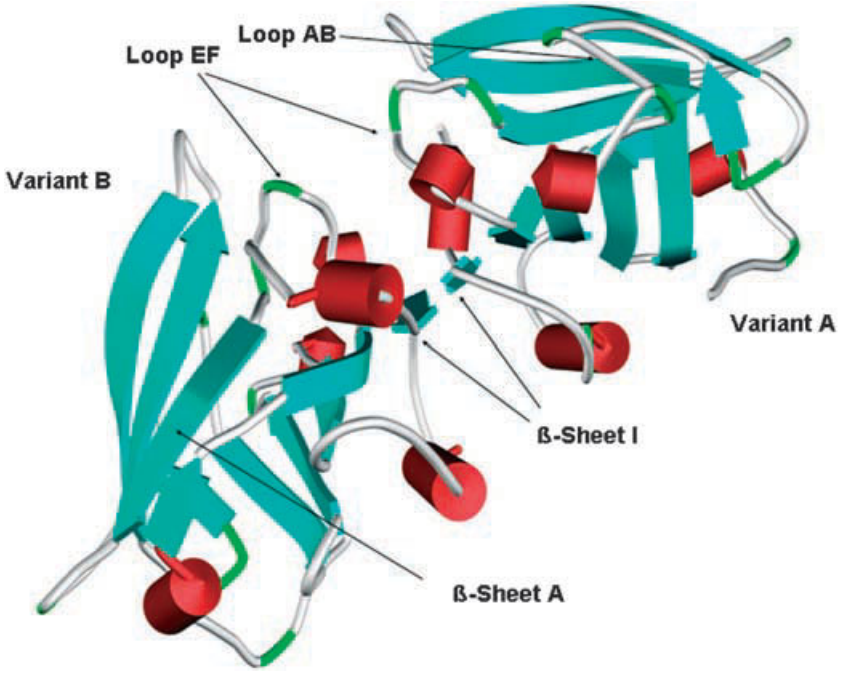

Fig. 4. *Dimeric structure of BLG (Brookhaven Protein Data Bank:* 1beb; X-ray analysis: mixture of variant A and B, resoln. 1.8 Å, pH 6.5) [35]

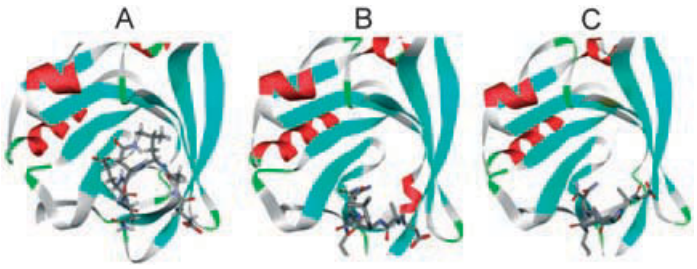

Fig. 5. *Detailed view of the central hydrophobic binding pocket of BLG at different pH values* (*Brookhaven Protein Data Bank:* 3blg (*A*), 1bs y (*B*), und 2blg (*C*); X-ray analyses: variant A, resolution 2.56 Å, *A:* pH 6.2, *B:* pH 7.1, *C:* pH 8.2; amino acids 85 – 90 of BLG displayed as sticks [28].

To clarify the different binding behavior of the investigated lactones on BLG, the ligand – protein complexes were examined by means of molecular modelling. The approach and software programs used are shown in *Fig. 6*. The energetically favorable three-dimensional structures of lactones were generated using the molecular-mechan-

ics force field MM+ (Hyperchem 5.0). The conformation analyses were carried out by means of Monte Carlo and molecular-dynamics simulations. The energetically most stable lactones were used as source structures for the docking studies.

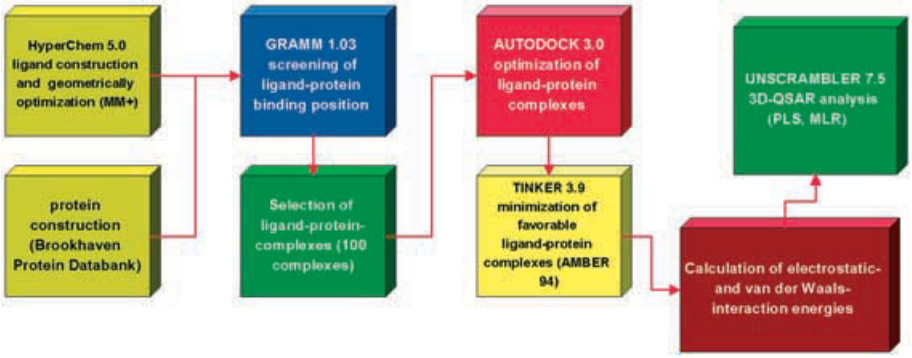

Fig. 6. *Schematic approach for the investigation of binding positions and free binding energies of lactones to BLG*

For the detection of possible binding positions of the lactones on BLG, the software package GRAMM (*Fig. 6*) was used as the first screening process. *GRAMM* (Global Range Molecular Matching) is a program that was used in the literature for protein–protein interaction simulations [37][38]. GRAMM carries out a six-dimensional search describing translation and rotation degrees of freedom for both ligand and macromolecule. The possible binding positions (100 BLG–lactone complexes) preserved by means of GRAMM on BLG are displayed, *e.g.*, for δ-decalactone in *Fig. 7*. Subsequently, aprox. 10 of the 100 lactone complexes on BLG were extracted according to their locations on the protein and further processed by the software package Autodock [39], which was compiled on Linux. Autodock was used for the optimization of the binding positions and calculation of the free binding energy (ΔG) for the protein ligand complex. Autodock calculates the free energy upon binding according to *Eqn. 3*.

$$\Delta G = \Delta G_{vdw} + \Delta G_{hbond} + \Delta G_{elec} + \Delta G_{conf} + \Delta G_{tor} + \Delta G_{sol} \quad (3)$$

The first four terms of the *Eqn.* take into consideration the dispersion/repulsion (ΔG_{vdw}), H-bonding (ΔG_{hbond}), electrostatic (ΔG_{elec}), and ligand conformation (ΔG_{conf}) binding energies. ΔG_{vdw} was calculated according to the *Lennard–Jones* 12-6 potential, ΔG_{hbond} is a directional 12–10 H-bonding term, ΔG_{elec} the Coulombic electrostatic potential, and ΔG_{conf} the energy change of ligand upon binding to the receptor molecule. ΔG_{tor} is the contribution of the translation and rotation energy (more positively, unfavorable contribution) to free binding energy. The term of ΔG_{tor} is proportional to the number of sp^3 bonds in the ligand. ΔG_{sol} models desolvation upon binding, which contributes negatively (favorable contribution) to the binding energy by a rise in entropy. The calculation of ΔG_{sol} is based on the determination of the molecular surfaces. By means of Autodock, the ten most-stable complexes are charted for δ-decalactone on BLG in *Fig. 8*. The most-stable protein–ligand complex showed a free energy of binding of -7.4 kcal/mol (complex 1 in *Fig. 8*). For γ-decalactone, the

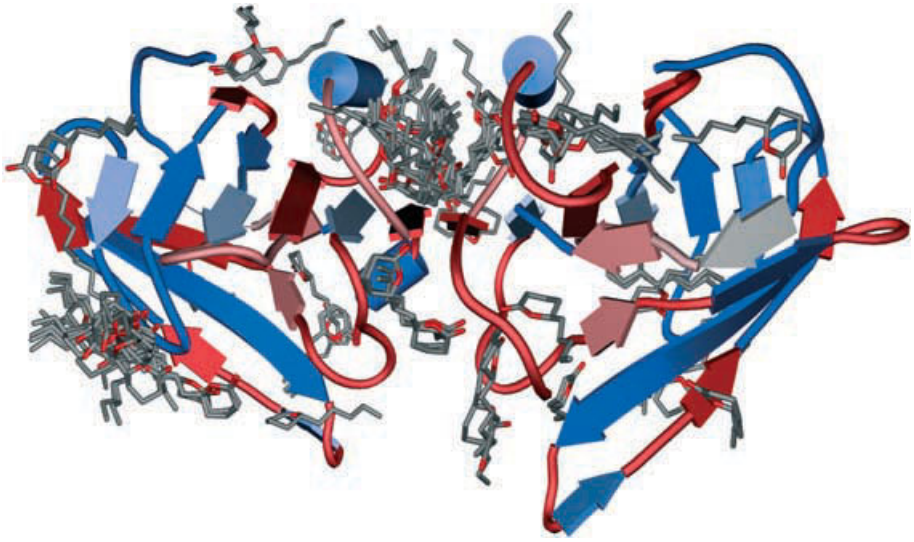

Fig. 7. *Presentation of the favored binding positions of (R)-δ-decalactone on BLG* (100 complexes) *by means of software package GRAMM.* Docking parameter: fixed protein structure, lactone minimized (HyperChem 5.0, MM +) and flexible (translation and rotation, no torsion changes allowed). Docking mode: generic; grid step size: 1.7 Å; repulsion: 30; increment for the rotation degree: 10°; cumulative projection; potential range type: *Van der Waals* radius (high resoln. docking).

most favorable binding position ($\Delta G = 7.2$ kcal/mol), suggested by Autodock, has the same location as found for δ-decalactone on BLG (complex 1 in *Fig. 8*). The free energy of binding found for selected lactones by Autodock was − 7.8 kcal/mol for δ-undecalactone and − 6.6 kcal/mol for γ-nonalactone. The discovered binding position of lactones to BLG corresponds to none of the literature reported binding sites. According to the literature, three main hydrophobic binding positions have been discussed for proteins belonging to the lipocalin protein superfamily [40]. A δ-decalactone – BLG complex on the external binding site in a groove on the outer surface of BLG (proposed as a retinol-binding site [40]), which is built up by the α-helix and external parts of the β-barrel, yielded a free energy of binding of − 5.1 kcal/mol (complex 7 in *Fig. 8*). The free energy of binding in the deep β-barrel of BLG (proposed as a palmitic acid- and retinol-binding site [21][41]) amounts to − 5.3 kcal/mol (complex 4 in *Fig. 8*). Under the conditions applied for the current investigation (pH 6.5 – 7.0), BLG exists in a dimeric form [30], and a pocket forms between both monomers as further ligand coordination takes place. The binding energy ascertained by means of Autodock for this protein – ligand complex was − 6.1 kcal/mol (complex 3 in *Fig. 8*). As the binding affinities of, *e.g.*, γ-decalactone (*Table 2*) increase with decreasing pH value, and a dissociation of dimeric BLG to monomers is observed under these conditions [30], one can assume that this binding position is unlikely. The same is true for the palmitic acid BLG binding site, because the binding pocket of the β-barrel is closed by the loop EF at low pH values (*Fig. 5,A*). The investigations described here give further clues for the fact that the suggested BLG – lactone binding site (complex 1 in *Fig. 8*) is appropriate.

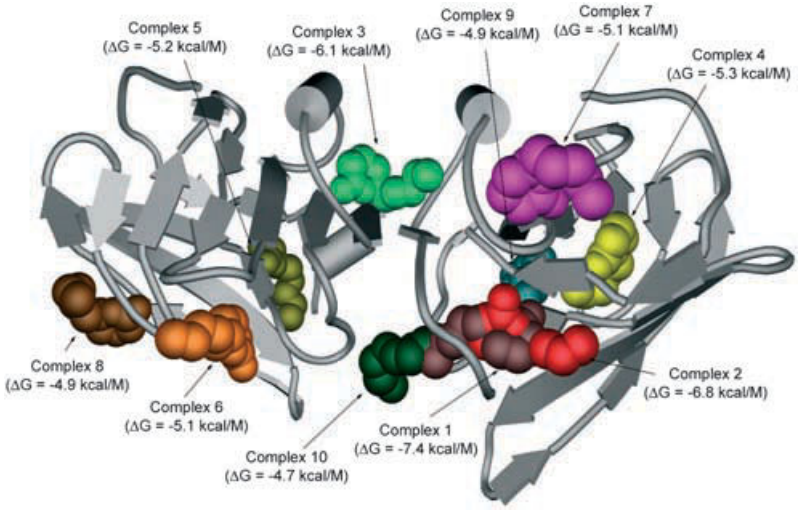

Fig. 8. *Binding position of* (R)*-δ-decalactone on BLG obtained by GRAMM* (*Fig. 7*) *and refined by Autodock.* Docking parameter: fixed protein structure; ligand flexible (translation-, rotation, and torsion changes are allowed); Lamarckian Genetic Algorithm (LGA); affinity maps calculated for the atoms C and O, as well as for the electrostatic potential: grid points $60 \times 60 \times 60$ around the ligand center, point size 0.375 Å.

For further confirmation of the binding position suggested here for the lactones, competitive binding studies were carried out. Ligands with known binding positions on BLG were equilibrated together with γ-decalactone. Methyl palmitate and retinol were used as competition. The results of the binding studies are summarized in *Table 4*. The data in *Table 4* shows that, in the presence of retinol and methyl palmitate, only slight inhibition of binding of γ-decalactone can be observed. In comparison, looking at the published binding affinity of retinol and palmitic acid on BLG (equilibrium dialysis: retinol: 1.5×10^4 mol^{-1}; palmitic acid: 5.2×10^5 mol^{-1}) [20], γ-decalactone has a much lower binding affinity: by a factor of 10 and 500 compared to retinol and palmitic acid, respectively. An entire inhibition of the binding of γ-decalactone would be expected on the postulated 'retinol- and palmitic acid-binding sites' if γ-decalactone binds to these positions.

Interesting to note is, that *Qin et al.* [28] suggested the exact binding position described here for lactones on BLG, because of the gel formation property of BLG. The following amino acids are assumed to be responsible for the oligomerization of BLG: the 'key' on one monomer is Lys 8 and the 'lock' on the second monomer is Tyr 20, Ser 21, Val 41, Tyr 42, Val 43, Leu 156, Glu 157, and Glu 158. Nearly identical amino acids are found for the lactone binding pocket as displayed in *Fig. 9*. The results obtained by *Qin et al.* [28] are in harmony with the binding position postulated here for the lactones on BLG. Further confirmation of the postulated lactone binding site (complex 1 in *Fig. 8*) was obtained from the preceding binding studies by means of equilibrium dialysis and ultracentrifugation techniques that showed that one mol of lactone is bound per mol of BLG dimer (*Table 2*). The molecular-modelling studies also indicate only one possible binding position on BLG dimer, on the A unit (*Fig. 8)*. The

Table 4. *Effect of Methyl Palmitate and Retinol on BLG Binding of γ-Decalactone*

Additive	Concentration additive [mM]	pH	Concentration γ-decalactone [mM]	Bound fraction [%]
None (control)	–	7.0	0.10	29
Methyl palmitate	0.22	7.0	0.10	24
Retinol	0.21	7.0	0.10	25
None (control)	–	6.5	0.12	27
Methyl palmitate	0.22	6.5	0.12	28
Retinol	0.21	6.5	0.12	24
None (control)	–	7.0	0.87	23
Methyl palmitate	0.22	7.0	0.87	20
Retinol	0.21	7.0	0.95	16
None (control)	–	7.0	1.30	18
Methyl palmitate	0.44	7.0	1.30	17
None (control)	–	6.5	0.92	21
Methyl palmitate	0.44	6.5	0.92	16

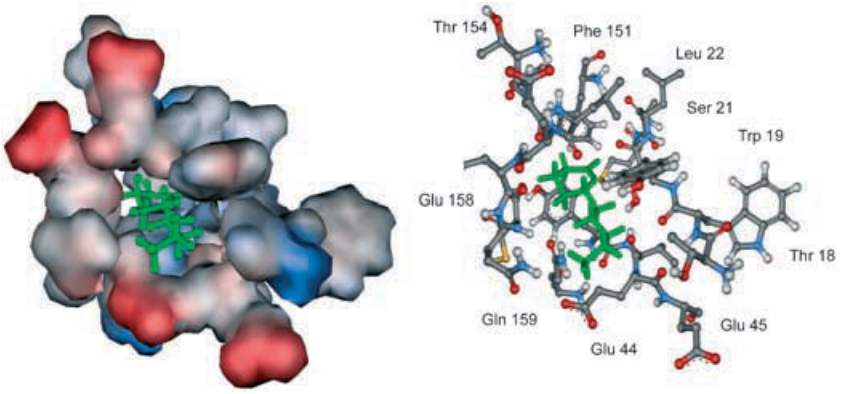

Fig. 9. *Details of the binding position of γ- and δ-lactones on BLG* (*Van der Waals* surface with mapped molecular electrostatic potential). Amino acids are labelled with a maximum distance of 6 Å to the lactone. Two H-bonds are found from Ser 21 to (*R*)-δ-decalactone.

corresponding place on the B unit is not accessible; this binding position is presumably blocked by another BLG monomer as suggested by *Qin et al.* [28].

If one compares the binding energy of the most-stable complex of δ-decalactone on BLG (– 7.4 kcal/mol, complex 1 in *Fig. 8*) with the experimentally obtained value of – 3.8 kcal/mol (*Table 2*), a free energy of binding lower by *ca.* – 3.6 kcal/mol is predicted with Autodock. For this reason a thermodynamic cycle was developed, similar to that which is integrated into Autodock, and which is able to predict more precisely the free energy of binding of lactones to BLG (*Eqn. 4*).

$$\Delta G = \Delta G_{vdw} + \Delta G_{elec} + \Delta G_{conf} + \Delta G_{tor} + \Delta G_{\log P} \quad (4)$$

For the investigations into the intermolecular interaction energies of BLG and lactones (ΔG_{vdw} and ΔG_{elec}), the software TINKER with the force-field parameters of AMBER (pam94) was used. The intramolecular-energy difference of ligand conformer before and after binding to BLG (ΔG_{conf}) was calculated using TINKER for the isolated lactone. ΔG_{tor} is proportional to the number of sp^3 bonds in the ligand and has the same meaning as in Autodock. The relationship between the $\log P_{octanol/H_2O}$ value and the binding affinity of a lactone to BLG was recognized in this study (*Fig. 2*), and, as a result, the $\Delta G_{\log P}$ value was included in *Eqn. 4*. The $\Delta G_{\log P}$ value is responsible for desolvation effects of the lactone upon binding to BLG.

Due to no force-field torsion parameters for lactone and ester groups being available in TINKER, the parameters had to be developed. The most stable ligand–protein complexes for γ- and δ-decalactone obtained by Autodock (complex 1 in *Fig. 8*) were further examined in TINKER by means of molecular-dynamics (MD) and molecular-mechanics (MM) simulations. The remaining lactones listed in *Table 5* are generated by the addition or removal of CH_2 groups of the lactone, followed by MD and MM simulations. The minimized BLG complexes of δ-decalactone and γ-decalactone are displayed in *Fig. 10*.

The interaction energies (ΔG_{vdw} and ΔG_{elec}; obtained by using TINKER) of the investigated lactone BLG complexes are listed in *Table 5*. Strong *Van der Waals* interactions were found with increasing number of C-atoms in the lactone. A correlation study of the free energy of binding with the variables mentioned in *Eqn. 4* and calculated in *Table 5* was performed using Unscrambler 7.5. The result is shown in *Fig. 11*. Regression analysis was carried out by means of partial least square (PLS) analysis. The obtained regression coefficient (r^2) of 0.985 (calibration) and of 0.960 (cross-validation) shows the model to be good. Noteworthy is also the correct prediction of the (*R*)- and (*S*)-lactone enantiomers by molecular modelling which both have similar binding energies on BLG.

Conclusions. – Large differences in the binding properties on proteins observed for the various lactones clearly demonstrated that the binding affinity was significantly influenced by the lipophilicity of the odorant. Possible binding positions of odorants on BLG were screened by the software packages GRAMM and Autodock. In the present study, a BLG–lactone binding position, not reported in the literature until now, could be identified and confirmed by competitive binding studies. Autodock is a powerful tool for detection of favorable binding positions on macromolecules. There is some evidence from experimental and molecular-modelling studies that the reported 'lactone-binding site' on BLG is likely, but this has yet to be further confirmed by X-ray-diffraction analysis.

A model was developed to estimate the free energy of binding (ΔG) of odorants to biopolymers by calculating the following descriptors: *Van der Waals* and electrostatic intermolecular energies of lactone–BLG complex, torsional degrees of freedom of lactone, internal energy of ligand, and $\log P_{octanol/H_2O}$ of ligand. The estimated free energies of binding (ΔG) of lactones with BLG obtained from the molecular-modelling experiment were in good agreement with the results of the experimentally determined binding affinities.

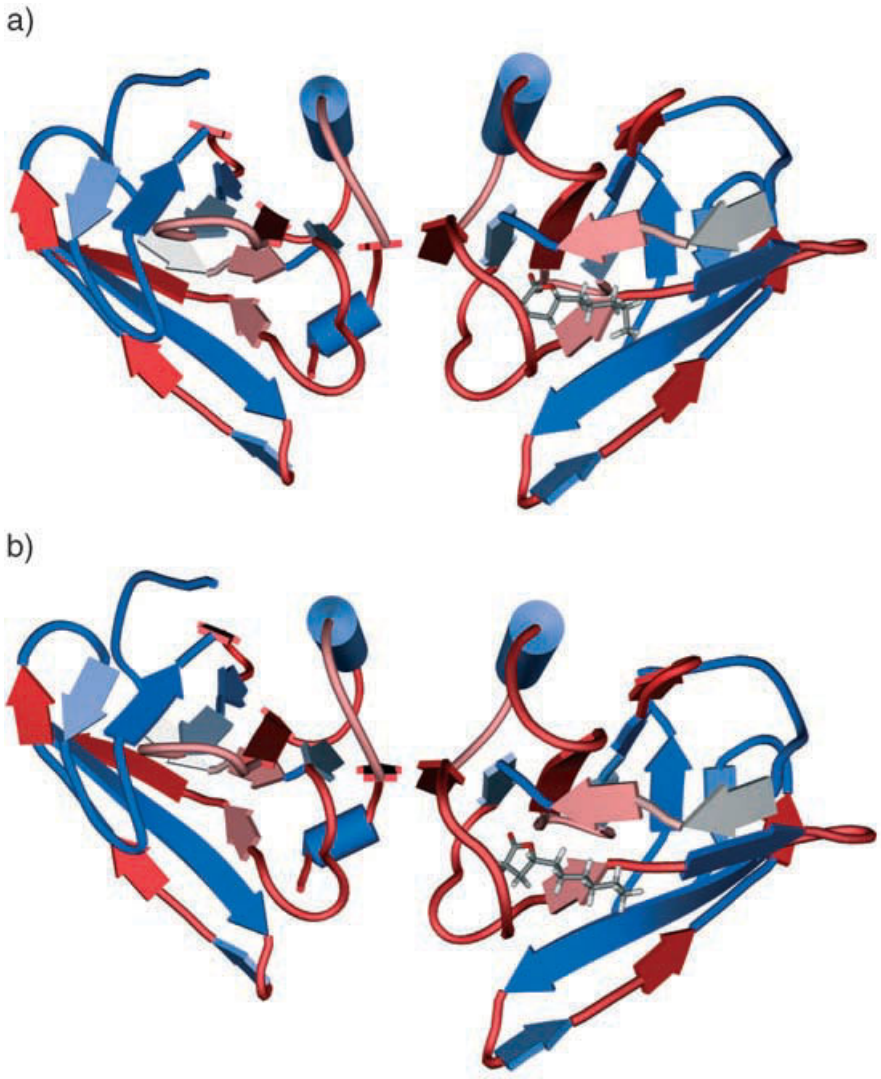

Fig. 10. *Binding positions of* (R)-δ- (*a*) *and* (R)-γ-*decalactone* (*b*) *on BLG dimer after refinement of the Autodock complexes with TINKER.* TINKER MD Calculation parameter: Dynamic module, force field AMBER, MD simulation 10 ps, time step 1 fs, NPT ensemble ($T = 300$ K, 1 atm), velocity verlet algorithm, windows of 1 ps. Ten structures obtained by MD simulation were further investigated by the TINKER minimize module: force field AMBER 94, L-BFGS, RMSD: 0.01 kcal/mol. Conditions for all calculations: flexible ligand and amino acids (Thr 18, Trp 19, Tyr 20, Ser 21, Leu 22, Met 24, Tyr 42, Val 43, Glu 44, Gln 59, Phe 151, Gln 155, Leu 156, Glu 157, Gln 159, and Cys 160) in the binding pocket.

Table 5. *Energetic Contributions to the Predicted Free Energy of Binding* (ΔG_{pred}) *for Selected Lactones Bound to BLG Dimer*

Compound	ΔG_{vdw} [a] [kcal/mol]	ΔG_{elec} [b] [kcal/mol]	ΔG_{conf} [c] [kcal/mol]	ΔG_{tor} [d] (n)	$\Delta G_{log\,P}$ [e] [kcal/mol]	ΔG_{pred} [kcal/mol]	ΔG_{exp} [f] [kcal/mol]
(*R*)-γ-Undecalactone	−36.68	−18.76	2.20	6	−4.50	−5.0	−5.1
(*S*)-γ-Undecalactone	−37.19	−18.62	2.33	6	−4.50	−5.1	−5.1
(*R*)-γ-Decalactone	−34.86	−18.99	2.56	5	−3.71	−4.1	−4.1
(*R*)-γ-Nonalactone	−32.53	−19.39	2.38	4	−2.66	−3.2	−3.2
(*R*)-γ-Heptalactone	−29.30	−19.47	1.70	2	−0.88	−1.4	< −2.3
(*R*)-δ-Undecalactone	−35.93	−18.91	2.57	5	−3.99	−4.6	−4.4
(*S*)-δ-Undecalactone	−34.65	−21.36	4.12	5	−3.99	−4.4	−4.4
(*R*)-δ-Decalactone	−34.98	−18.15	2.40	4	−3.19	−3.8	−3.8

[a]) Intermolecular *Van der Waals* interaction energy of ligand in the protein complex (TINKER). [b]) Intermolecular electrostatic interaction energy of ligand in the protein complex (TINKER). Lactone charges calculated with Gaussian 98 (basis set: HF/6–31G*, Prop = fitcharge, Opt) from the electrostatic potential on the *Van der Waals* surface of lactone. [c]) Intramolecular energy difference of ligand conformer before and after binding to BLG (TINKER): ΔG_{conf} [kcal/mol] = intramolecular energy($ligand_{min}$) − intramolecular energy($ligand_{complex}$); intramolecular *Van der Waals* 1–4 scaling factor: 2.0; intramolecular charge 1–4 scaling factor: 1.2. [d]) ΔG_{tor}: number (n) of sp^3 bonds in the ligand (not $C-CH_3$ and ring atoms). [e]) Experimental by determined log $P_{octanol/H_2O}$ value: $\Delta G_{log\,P}$ [kcal/mol] = $-RT\ \ln K$ (T = 298.15 K, K: partition coefficient octanol/H_2O, *Table 3*). [f]) Experimentally determined free energy of binding (ΔG_{exp}) according to *Table 2*.

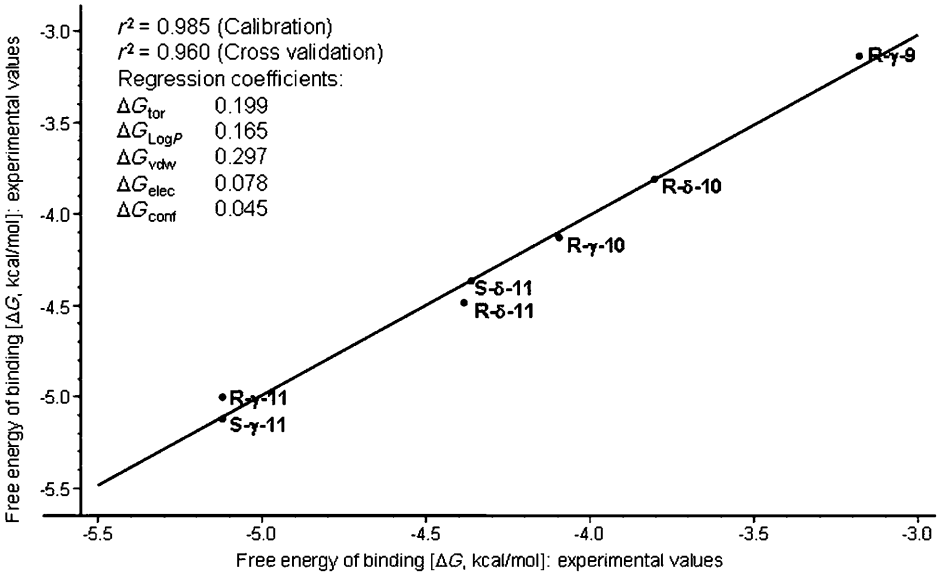

Fig. 11. *Correlation of molecular-modelling binding studies and experimentally determined binding affinities of lactones on BLG by means of partial least squares (PLS) analysis*

Financial support of the *Deutsche Forschungsgemeinschaft* (DFG, project 515/1-1) and scientific exchange and discussions in the framework of COST Action 921 are gratefully acknowledged.

Experimental Part

General. Commercial available β-lactoglobulin (BLG) from cow milk (mixture of genetic variants A and B, L-3908) and bovine serum albumin (A-7511, lipid-free) were obtained from *Sigma-Aldrich* (D-Steinheim). The powders were dispersed in phosphate buffer soln. (80 mM), and the pH was adjusted to pH 7.0 and 6.5, resp., with conc. phosphoric acid. γ-Heptalactone, γ-octalactone, γ- and δ-nonalactone, γ- and δ-decalactone, and γ- and δ-undecalactone were purchased from *Sigma-Aldrich* (D-Steinheim), and δ-heptalactone and δ-octalactone from *Roth* (D-Karlsruhe). Aroma solns. were prepared daily in phosphate buffer soln. (80 mM) with the pH adjusted to pH 7.0 and 6.5, resp., with conc. phosphoric acid. Chiral lactones ((*R*)-γ-nonalactone, (*R*)-δ-decalactone, (*S*)-δ-decalactone, and (*S*)-γ-undecalactone) were obtained from *Fluka*. The molecular structure of BLG was taken from the *Brookhaven Protein Data Bank*.

Lactone–Protein Binding: Ultracentrifugation. Binding experiments were carried out at constant concentrations of bovine serum albumin (BSA) and β-lactoglobulin (BLG, 0.45 mM) and at variable concentrations of various γ- and δ-lactones (3.0 mM – 0.2 μM) in a phosphate buffer soln. (80 mM, pH 7.0 and 6.5) by ultracentrifugation (*Centricon 10*; cut-off 10000, *Millipore*, D-Eschborn). The protein/odorant mixture (2 ml) was incubated for 30 min at r.t. and then centrifuged (3400 rpm) for 5 min. The obtained filtrate (*ca.* 0.2 ml) was discarded and the protein/odorant buffer soln. centrifuged (4700 U/min, $g = 2270$) for a further 20 min. The obtained filtrate (*ca.* 0.8 ml) and the residue (*ca.* 1.0 ml) were used for the determination of the odorant concentration in the protein fraction (free and bound odorant) and in the filtrate (free-odorant fraction) by cap. gas chromatography/mass spectrometry (HR-GC/MS). Quantification experiments of the lactones were performed as follows: an internal lactone standard soln. was added to the protein/odorant soln. (0.5 ml) that contained the bound and free odorant, as well as to the filtrate (0.5 ml), which contained the free odorant. For γ-lactones, the corresponding δ-lactones and for δ-lactones the corresponding γ-lactones were used as an internal standard. After addition of the internal standard, the fractions were stirred for 15 min and then extracted with pentane (2×2 ml). The combined extracts were dried (Na_2SO_4) and then concentrated by microdistillation [42]. HR-GC/MS analyses were performed by means of a *HP 5890* gas chromatograph connected to a *HP 5971* mass spectrometer (*Agilent*, D-Waldbronn) operating in the EI mode. HR-GC separation of odorants was performed on a DB-FFAP capillary (*J&W Scientific, Fisons*, D-Mainz). The samples were applied by the on-column injection technique at 35°. After 2 min, the temp. of the oven was raised at 40°/min to 60° and held for 1 min isothermally, then raised at 8°/min to the final temp. of 240°. For quantification of γ-lactones and δ-lactones by MS, the molecular ions at m/z 85 and 99 were monitored and used for calculations of odorant concentrations. The following mass-spectrometer correction factors for γ-lactones and δ-lactones, resp., were used for calculation of analyt concentration: γ-heptalactone (0.33), γ-octalactone (0.32), γ-nonalactone (0.44), γ-decalactone (0.44), γ-undecalactone (0.48), δ-heptalactone (2.96), δ-octalactone (2.19), δ-nonalactone (2.28), δ-decalactone (2.26), and δ-undecalactone (2.12). The odorant bound to protein was calculated according to *Eqn. 5*.

$$\begin{aligned}\text{odorant bound [μg/sample]} = &(\text{odorant residue [μg/ml]} \times \text{volume residue [ml]}) \\ &- (\text{odorant filtrate [μg/ml]} \times \text{volume residue [ml]})\end{aligned} \quad (5)$$

Lactone–Protein Binding: Equilibrium Dialysis. As a standard method for the determination of the binding parameters of lactones to BSA and BLG, the equilibrium dialysis technique was applied [4]. A dialysis cell (*Thomaphor*, acrylic glass) consisting of four separated micro chambers (total volume of one chamber 1 ml) was used. The cell chambers were separated by a membrane (regenerated cellulose, cut-off 10000). The proteins BSA and BLG (0.45 mM), resp., were solved in phosphate buffer soln. (80 mM, 0.5 ml) and added to one side of the microdialysis cell. The other compartment of the cell contained the odorant (0.24–3.0 mM) solved in buffer soln. (80 mM, 0.5 ml). The sample was incubated for 24 h, and then a defined aliqote (200 μl) of the soln. of each compartment was removed. The quantification experiments were carried out as described previously for the ultracentrifugation method.

Depending on the solubility of the analyt for each binding isotherm, 5 to 10 different odorant concentrations were applied at a constant protein concentration. Binding constants (association constant K_a and number of binding sites n) were calculated from the binding isotherms by non-linear regression (Origin 6.0,

Microcal Software, Northampton, USA) for a one-site and two-site binding model (see *Results and Discussion* for details).

Partition Coefficient (*log* P). Partition coefficients (log *P* values) of odorants were determined by the shaking-flask method [34] in solvent mixtures consisting of octanol/H_2O ($\log P_{octanol/H_2O}$) and cyclohexane/H_2O ($\log P_{cyclohexane/H_2O}$). To a mixture of H_2O (4 ml) and org. solvent (3.9 ml), a soln. of the odorant in cyclohexane and octanol (0.1 ml, 5–10 μg), resp., was added and stirred for 30 min. After centrifugation (5 min, 3400 rpm), the org. layer was separated from the H_2O layer, and an internal standard (corresponding to γ-lactone and δ-lactone, resp.) was added to both fractions. Quantification experiments of the odorant in the H_2O and org. fractions were performed by HR-GC/MS analysis as described previously for protein-binding experiments.

Computational Methods. The *PDB* data files were processed using WebLab. Viewer software (*Accelrys Inc.*, San Diego, CA). The following molecular-modelling methods were applied to obtain energy-minimized molecular structures of the odorants and protein–odorant complexes: for the generation of energy-minimized lactone structures the software package Hyperchem 5.0 (*Hypercube*, Ganesville, Florida, USA) was used. Conformations of lactones were generated by Monte Carlo (MC) and molecular-dynamics (MD) simulations, and minimized by molecular-mechanics force field (MM+). The protein structures (1beb, 3blg, 1bsy, and 2blg) were downloaded *via* the Internet from the *Brookhaven Protein Data Bank*.

The missing force-field parameters of the lactones were developed in AMBER (pam94, Department of Pharmaceutical Chemistry, University of California, San Francisco, USA) [43] according to the instructions of the official Amber homepage. For the calculation of the electrostatically derived charges and the dihedral parameters of the lactones, the software Gaussian 98 (*Gaussian, Inc.*, Carnegie, USA) was applied. Increments of the dihedral angles for the well-chosen structure elements (AcOEt and propyl propanoate) were computed by means of Gaussian 98 (conditions: HF/6-31G*, opt = modredundant, scan, 20–30° increments).

The relative energy differences [kcal/mol] of the dihedral angles of the corresponding fragments were plotted, and the obtained potential curves were transferred by means of TableCurve 2D (software version 4.0, *SPSS Science*, D-Erkrath) in *Fourier* series: F_n, $n = 1-9$, X-variable (dihedral angle), Y-variable (relative-energy difference in kcal/mol), $F_1 = \mathrm{DTOR(X)}$, $F_2 = \mathrm{DTOR(A2)}$, $F_3 = (\mathrm{A0}) \cdot (1 + \mathrm{COS}[(F_1) \cdot \mathrm{A1} - F_2])$, $F_4 = (\mathrm{A3}) \cdot (1 + \mathrm{COS}[(F_5) \cdot \mathrm{A4} - F_6])$, $F_5 = \mathrm{DTOR(X)}$, $F_6 = \mathrm{DTOR(A5)}$, $F_7 = (\mathrm{A6}) \cdot (1 + \mathrm{COS}[(F_8) \cdot \mathrm{A7} - F_9])$, $F_8 = \mathrm{DTOR(X)}$, $F_9 = \mathrm{DTOR(A8)}$, fit: $Y = F_3 + F_4 + F_7$.

The following parameters were added to the AMBER (pam94) force field (atom labels according to AMBER): bond **C-OS** ($K_\theta = 450$ kcal mol^{-1} Å^{-2}, $r_\theta = 1.33$ Å); angles **O2-C-OS** ($K_\theta = 80$ kcal mol^{-1} rad^{-2}, $\theta_\theta = 119.7°$), **OS-C-CT** ($K_\theta = 80$ kcal mol^{-1} rad^{-2}, $\theta_\theta = 118.1°$), **C-OS-CT** ($K_\theta = 80$ kcal mol^{-1} rad^{-2}, $\theta_\theta = 124.3°$), and **OS-CT-HC** ($K_\theta = 50$ kcal mol^{-1} rad^{-2}, $\theta_\theta = 106.8°$); dihedral angles **CT-C-OS-CT** (IDIVF = 1, PK = 2.34 kcal mol^{-1} rad^{-1}, phase = 180, periodicity = 2, IDIVF = 1, PK = 0.86 kcal mol^{-1} rad^{-1}, phase = 0, periodicity = 1), **OS-C-CT-HC** (IDIVF = 1, PK = 0.043 kcal mol^{-1} rad^{-1}, phase = 0, periodicity = 3), **OS-C-CT-CT** (IDIVF = 1, PK = 0.031 kcal mol^{-1} rad^{-1}, phase = 0, periodicity = 3, IDIVF = 1, PK = 0.079 kcal mol^{-1} rad^{-1}, phase = 180, periodicity = 2), **C-OS-CT-CT** (IDIVF = 1, PK = 0.121 kcal mol^{-1} rad^{-1}, phase = 0, periodicity = 1), **C-OS-CT-HC** (IDIVF = 1, PK = 0.12 kcal mol^{-1} rad^{-1}, phase = 0, periodicity = 3), **O2-C-OS-CT** (IDIVF = 1, PK = 2.34 kcal mol^{-1} rad^{-1}, phase = 180, periodicity = 2, IDIVF = 1, PK = 0.89 kcal mol^{-1} rad^{-1}, phase = 180, periodicity = 1); improper torsions **CT-O2-C-OS** (PK = 10.5 kcal mol^{-1} rad^{-1}, phase = 180, periodicity = 2), **HC-CT-CT-OS** (PK = 10.5 kcal mol^{-1} rad^{-1}, phase = 180, periodicity = 3).

As the first screening process for the detection of possible binding positions of the lactones on BLG, the software package GRAMM (*Bioinformatics Lab.*, Department of Applied Mathematics and Statistics, SUNY at Stony Brook., NY) [37][38] was used. GRAMM carries out a six-dimensional search describing translation and rotation degrees of freedom of both ligand and macromolecule. Docking parameter: fixed protein structure, flexible ligand (translation and rotation). Docking mode: generic; grid step size: 1.7 Å; repulsion: 30; increment for the rotation degree: 10^0; cumulative projection; potential range type: *Van der Waals* radius (high resoln. docking).

Automated docking was performed with Autodock 3.0 [39] (*The Scripps Research Institute*, MB-5 Department of Molecular Biology, La Jolla, CA, USA). Atomic and electrostatic interaction energy grids (module Autogrid, 60 × 60 × 60 cubic box, point size 0.375 Å) were calculated around the ligand positions, which were obtained by GRAMM. Rotatable bonds in the ligand were generated with the AUTOTORS module. The following docking parameters were used: fixed protein structure, ligand flexible (translation, rotation, and torsion changes are allowed), Lamarckian Genetic Algorithm (LGA), 10 runs, affinity maps calculated for C and O as well as for the electrostatic potential.

Parameters (ΔG_{vdw}, ΔG_{elec}, and ΔG_{conf}) for the empirical binding-free-energy function (see *Eqn. 4*) were determined by TINKER (Department of Biochemistry and Molecular Biophysics, Washington University

School of Medicine, Saint Louis, Missouri, USA). The most-stable odorant–protein complex from Autodock was used for further investigations in TINKER. The intermolecular interaction energies (ΔG_{vdw} and ΔG_{elec}) of BLG–lactone complex were evaluated by the force-field parameters of AMBER (pam94) and the developed parameters for lactones. The following docking parameters were used in TINKER modules Dynamic and Minimize: MD simulation 10 ps, time step 1 fs, NPT ensemble ($T = 300$K, 1 atm), velocity verlet algorithm, windows of 1 ps. The ten structures obtained by MD simulation were further investigated by the TINKER Minimize module and the interaction energies (ΔG_{vdw} and ΔG_{elec}) were calculated: force field AMBER (pam94), L-BFGS, RMSD: 0.01 kcal/mol. Conditions for all calculations: flexible ligand and amino acids (Thr 18, Trp 19, Tyr 20, Ser 21, Leu 22, Met 24, Tyr 42, Val 43, Glu 44, Gln 59, Phe 151, Gln 155, Leu 156, Glu 157, Gln 159, and Cys 160) at the binding pocket.

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Studies on the Volatile Compounds of Roasted Spotted Shrimp

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The aroma of spotted shrimp (*Sergia lucence* HANSEN) was analyzed upon roasting to determine the components that constitute the characteristic roasted shrimp flavor. Our analyses resulted in the identification of *ca.* 200 volatiles, including high-impact sulfur and nitrogen compounds. In addition, we synthesized all possible stereoisomers of the pyrrolidine derivatives **1** and **4**, and of the imine derivatives **16** and **18**–**20**, which are very characteristic for the aroma. The odor evaluation of these chemicals revealed distinct differences, each possessing different aroma characteristics.

Introduction. – The spotted shrimp (*Sergia lucence* HANSEN), a small shrimp with a length of *ca.* 5 cm, is mainly fished in Japan during 2 months in spring and autumn. It possesses many pale-pink dots, and constitutes a valuable nutriment (DHA, EPA, taurine, *etc.*). Besides, upon roasting, a strong and pleasant odor is generated, which makes it very tasty. Thus, the spotted shrimp is widely used in the traditional Japanese cuisine, especially in meals such as *kaki-age*, deep-fried slices of assorted seafood in tempura dough, and *okonomi-yaki*, a sort of Japanese pizza pancake. In the following, we summarize our analytical and synthetic work concerning the constituents that contribute to the aroma of roasted spotted shrimp. The compounds identified are of potential use as flavoring ingredients.

Results and Discussion. – We used sun-dried spotted shrimps caught in Suruga bay of Japan. The shrimps (100 g) were placed in a flask and roasted at 160° on a hot plate, while the surrounding air, the so-called 'headspace', was pumped through a filter at a rate of 125 ml/min. In this case, we used *Tenax TA resin* (1 g), and sampled the aroma for a period of 30 min. Then, the aroma was desorbed from the resin with Et_2O, and the ethereal extract was dried and concentrated. This operation was repeated ten times. As a result, 35 mg of aroma concentrate was obtained from 1 kg of spotted shrimps, amounting to a yield of 35 ppm. This aroma concentrate was then analyzed by aroma-extract dilution analysis (AEDA), gas chromatography (GC), and mass spectrometry (MS) or GC-MS.

The gas chromatogram of the aroma concentrate is shown in the *Figure*. Approximately 200 compounds were identified by GC and GC-MS analysis, among which 13 were sulfur-containing and 80 nitrogen-containing. We also indentified by GC-MS 29 high-impact compounds with flavor dilution (FD) factors >3 (*Table 1*).

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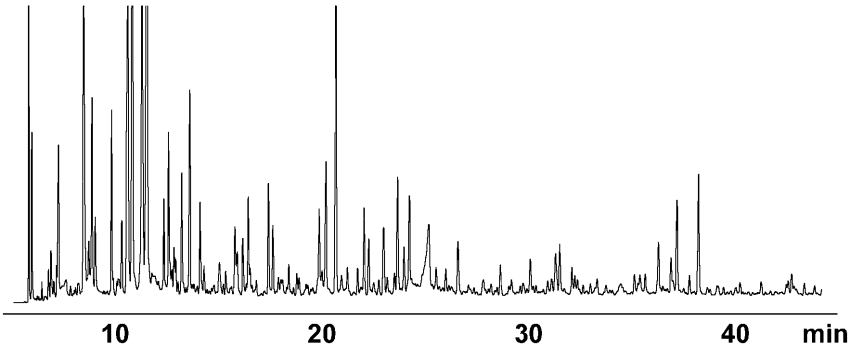


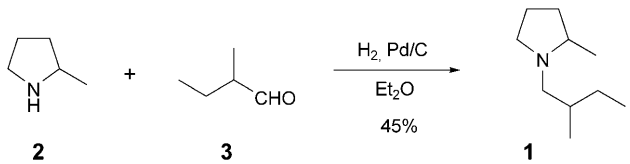
Figure. *Gas chromatogram of the aroma concentrate obtained from roasted spotted shrimp*. The chromatogram shows *ca*. 200 distinct volatiles of which the most-prominent ones have been identified (see *Table 1*).

The six compounds with the highest FD factors (*Entries 2*, *8*, *9*, *11*, *16*, resp.) are methanethiol, 1-pyrroline, *N*-(2-methylbutyl)pyrrolidine, *N*-(3-methylbutyl)pyrrolidine, isopropyl methyl disulfide and 3-methylpyridine [1].

Regarding *Entry 12* in *Table 1*, no chemical structure could be assigned at this stage. It was the major constituent of the aroma extract in terms of peak area, and possessed a pleasant roasted-seafood odor on GC-olfactometry. We, therefore, wanted to determine the structure of this compound. After careful re-investigation of the GC/MS data, we proposed the tentative structure 2-methyl-*N*-(2-methylbutyl)pyrrolidine (**1**). To confirm this structural proposal, we synthesized the racemate of **1**.

Condensation of 2-methylpyrrolidine (**2**) with 2-methylbutanal (**3**) in the presence of palladium on carbon under H_2 atmosphere provided **1** as a mixture of diastereoisomers (*Scheme 1*). Although the mass spectra of the synthetic and natural samples were quite similar, the GC retention indices (RI) were not completely identical. The odor of synthetic **1** was natural seafood-like, pleasant, and roasty. As we were also interested in the relationship between configuration and odor, we planned to synthesize all four stereoisomers of **1**. In addition, both enantiomers of *N*-(2-methylbutyl)pyrrolidine (**4**), with an even higher FD factor, also had to be synthesized and evaluated (*Scheme 2*).

Scheme 1



As shown in a retrosynthetic analysis (*Scheme 2*), we planned to derive all the stereoisomers of both **1** and **4** from the amides **5** as central intermediates. The latter could be dissected into pyrrolidines **6** and 2-methylbutyric acid (**7**) by cleavage of the amide bond. Both enantiomers of 2-methylpyrrolidine (**6a**) could be derived from proline (**8**). Concerning 2-methylbutyric acid (**7**), the (*S*)-isomer is commercially

Table 1. *Most-Dominant* (FD > 3) *Odorants of Roasted Spotted Shrimp* as *Characterized by Gas Chromatography*

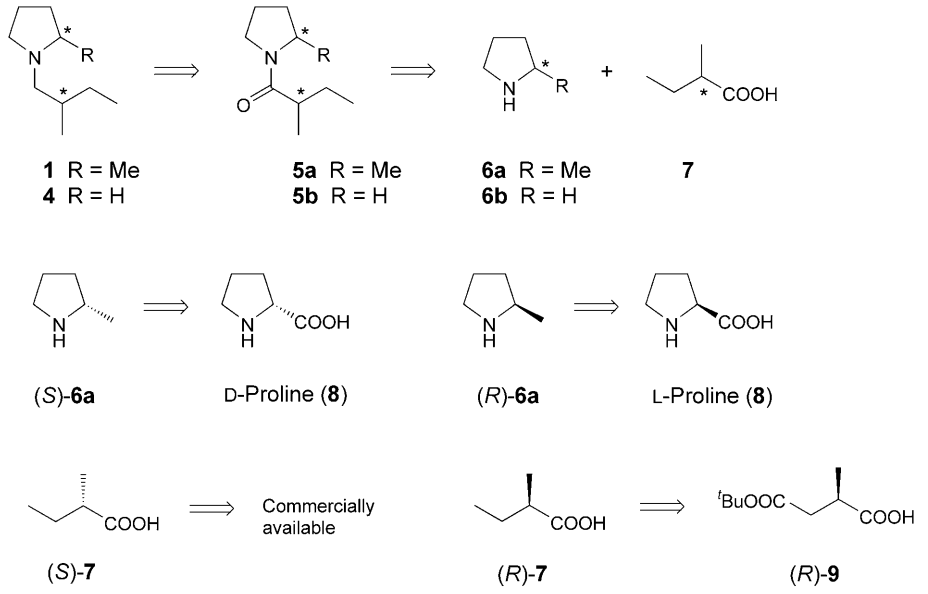
Entry	Compound	Odor Description[a])	Peak area [%]	FD Factor	RI[b])
1	Hydrogen sulfide	Sulfur	trace	3	480
2	Methanethiol	Sulfur, meaty	0.9	2187	702
3	2-Methyl-1-propanethiol	Roast	0.1	3	901
4	2-Methylbutanal	Malty	0.4	27	922
5	3-Methylbutanal	Malty	0.4	27	923
6	*N*-(2-Methylpropyl)pyrrolidine	Roast	0.1	3	975
7	2-Methyl-1-butanethiol	Tropical fruit-like	0.1	243	1004
8	1-Pyrroline	Shrimp meat-like	3.3	2187	1021
9	*N*-(2-Methylbutyl)pyrrolidine	Roasted seafood-like	0.6	2187	1093
10	Dimethyl disulfide	Sulfur	0.6	243	1095
11	*N*-(3-Methylbutyl)pyrrolidine	Roasted seafood-like	0.2	2187	1116
12	Unknown compound[c])	Dried seafood-like	4.5	9	1128
13	2-Methyl-*N*-(3-methylbutylidene)-butanamine	Internal organs of shrimp, green	0.1	243	1137
14	3-Methyl-*N*-(2-methylbutylidene)-butanamine	Seafood, roast	4.3	3	1155
15	3-Methyl-*N*-(3-methylbutylidene)-butanamine	Seafood, green	7.2	3	1164
16	Isopropyl methyl disulfide	Shrimp meat-like, roast	0.1	2187	1192
17	Pyridine	Scallop-like	1.3	81	1200
18	2-Methylpyridine	Fishy	0.2	3	1240
19	Methyl 2-methylpropyl disulfide	Cooked shrimp-like	0.1	243	1280
20	3-Methylpyridine	Fishy, green	0.6	2187	1305
21	2,5-Dimethylpyrazine	Roast, nutty	0.9	3	1338
22	2-Isopropyl-5-methylhex-2-enal	Dried seafood-like	0.2	3	1373
23	Nonan-2-one	Green	0.9	3	1398
24	Dimethyl trisulfide	Fermented	1.5	81	1406
25	2-Ethyl-3-methylpyrazine	Roast, nutty	0.1	27	1410
26	2,3,5-Trimethylpyrazine	Roast, nutty	2.8	27	1418
27	2-Ethyl-3,5-dimethylpyrazine	Roast, nutty	0.4	729	1474
28	3,5-Diethyl-2-methylpyrazine	Dried seafood-like	0.1	27	1501
29	2,5-Dimethyl-3-(3-methylbutyl)-pyrazine	Dried seafood-like	0.3	9	1666

[a]) Odor description assigned during AEDA. [b]) Retention index on *TC-WAX* [c]) Later identified as **16** (see text and *Scheme 7*).

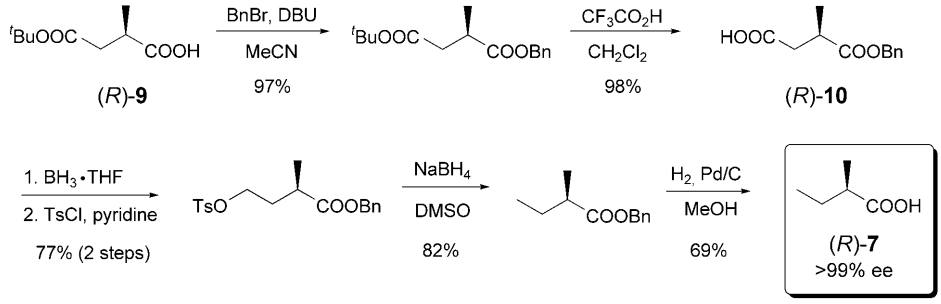
available, while we planned to derive the (*R*)-isomer from the (*R*)-2-methylsuccinate (*R*)-**9** as chiral building block. The synthesis of (*R*)-**7** from (*R*)-**9** is outlined in *Scheme 3*. Exchange of the protective group of **9** provided half-ester **10** [2]. Selective reduction of the carboxygroup, tosylation, reductive cleavage [3], and deprotection then furnished (*R*)-2-methylbutyric acid ((*R*)-**7**) in 41% overall yield from **9**.

The synthesis of the stereoisomers of **4** is summarized in *Scheme 4*. Treatment of (*R*)-**7** with pivaloyl chloride afforded the mixed anhydride **11** [4], which was reacted with pyrrolidine to provide the amide **12**. Finally, reduction of the amide group with $LiAlH_4$ furnished (*R*)-**4**. In the same manner, (*S*)-**4** was prepared from commercially available (*S*)-2-methylbutyric acid ((*S*)-**7**).

Scheme 2



Scheme 3



The synthesis of all the stereoisomers of **1** was achieved according to *Schemes 5* and *6*. *N*-CBz-L-Prolinol (**13**)[1]) was prepared from L-proline (**8**) in three steps according to known procedures [5–7]. Tosylation of **13** and reductive cleavage furnished CBz-protected (*R*)-2-methylpyrrolidine ((*R*)-**14**). In the same manner, (*S*)-**14** was prepared from D-proline.

In the presence of the mixed anhydride **11** (*Scheme 6*), deprotection of the CBz group of **14** with palladium on carbon in H_2 atmosphere gave amide **15** in one pot [4][8]. Finally, reduction of the amide group with $LiAlH_4$ afforded (2*R*,2′*R*)-**1** and (2*R*,2′*S*)-**1**, respectively. The other diastereosiomers, (2*S*,2′*R*)-**1** and (2*S*,2′*S*)-**1**, were prepared on the same route from (*S*)-**14**.

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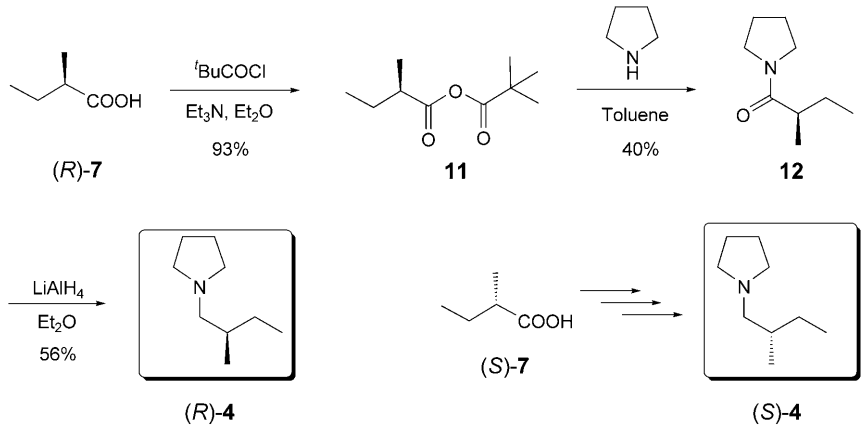
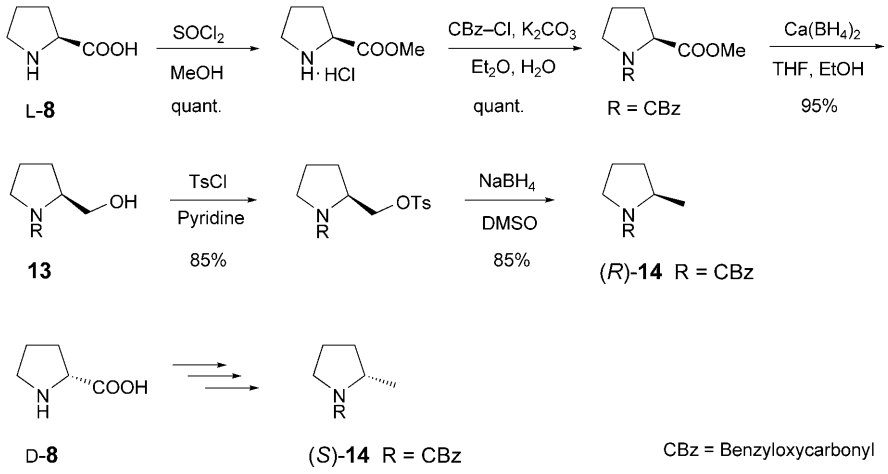
[1]) CBz = (Benzyloxy)carbonyl.

Scheme 4



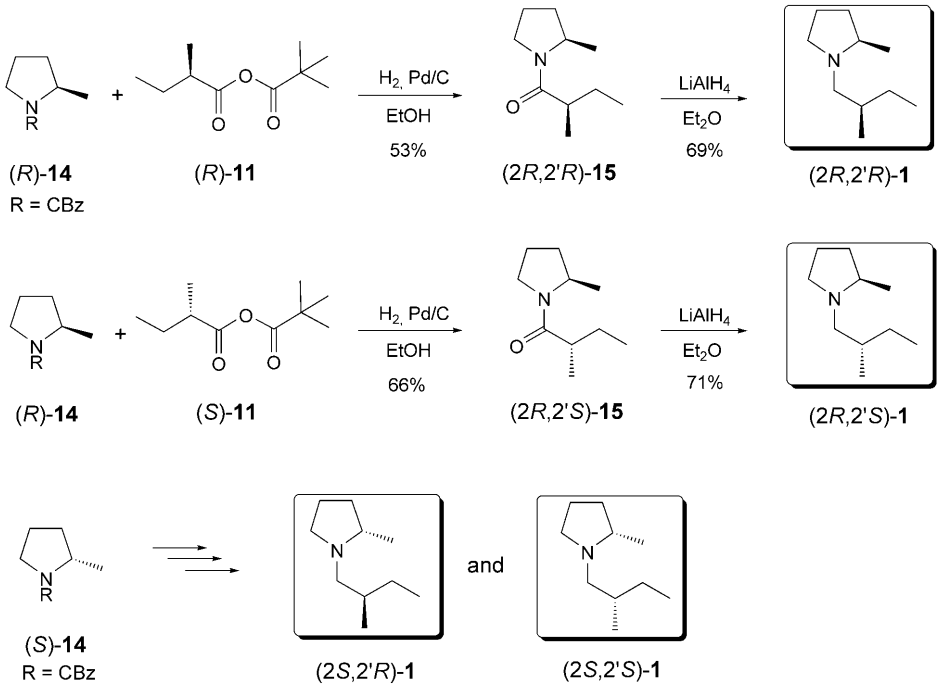
Scheme 5



The results of the odor evaluation of **1** and **4** are shown in *Table 2*. All stereoisomers of **1** possess typical odor characteristics of seafood, but each isomer has a different nuance. That the (2*R*)-isomers of **1** emanate roasty odors, while the (2*S*)-isomers possess mouldy notes. We also observed that the (2′*S*)-isomers of **1** are weaker in odor than the (2′*R*)-isomers. On the other hand, in the case of **4**, we found that the (*R*)-isomer has a roasted-seafood note, while the (*S*)-isomer has only a seafood-like note [9][10].

At this point, we re-investigated the GC-MS data of the unknown compound (*Entry 12* in *Table 1*), and proposed a new structure: 2-methyl-*N*-(2-methylbutylidene)butan-1-amine (**16**). To confirm this proposal, we synthesized a diastereoisomeric mixture of **16** by condensation of **3** with neat 2-methylbutanamine (**17**) (*Scheme 7*).

Scheme 6



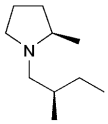
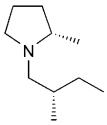
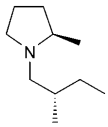
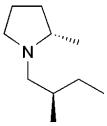
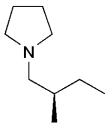
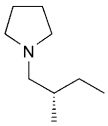
The spectral data of synthetic **16** were abosoutely identical with those of the unknown compound. Thus, the unknown compound in *Table 1* was identified as **16**.

We were interested in the relationship between the configuration and the odor of the stereoisomers of **16**. Therefore, we synthesized all four stereoisomers and evaluated their odor characteristics. In addition, all stereoisomers of **18**–**20**, analogs of the parent imine **16**, were synthesized, and their odors were evaluated, too.

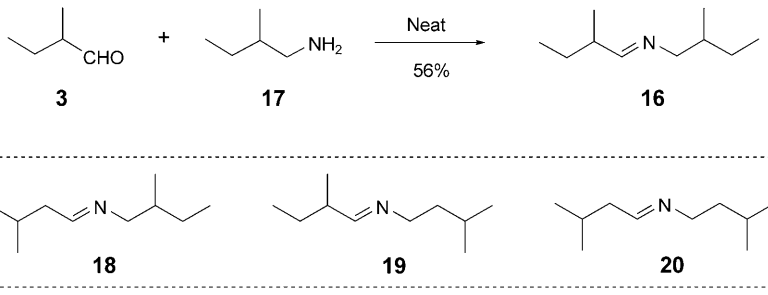
The syntheses of optically active 2-methylbutanal (**3**) and 2-methylbutanamine (**17**) as precursors of the above imine derivatives are presented in *Schemes 8* and *9*. Reduction of (*R*)-**7** with $LiAlH_4$ gave (*R*)-2-methylbutanol (**21**), which was converted to (*R*)-**17** in three steps by tosylation, azidation, and reduction. Compound (*R*)-**21** was then converted to (*R*)-**3** by standard TEMPO oxidation [11]. (*S*)-2-Methylbutanal ((*S*)-**3**) and (*S*)-2-methylbutanamine ((*S*)-**17**) were prepared in the same manner from commercially available (*S*)-**21**. All stereoisomers of the imines **16** and **18**–**20** were synthesized by reaction of the corresponding aldehydes and amines in neat (*Scheme 9*).

The results of odor evaluation of the four stereoisomers of **16** are shown in *Table 3*. All isomers have seafood odors, each with different nuances. The (2*S*)-isomers smell metallic, while the (2*R*)-isomers have phenolic nuances. Moreover, all the stereoisomers share a fruity tonality. A sensory evaluation complemented the olfactory evaluation of **16** (*Table 3*). The odor thresholds were determined in H_2O by triangular tests with 24 trained panelists using odorless H_2O as blank. Slight differences in the

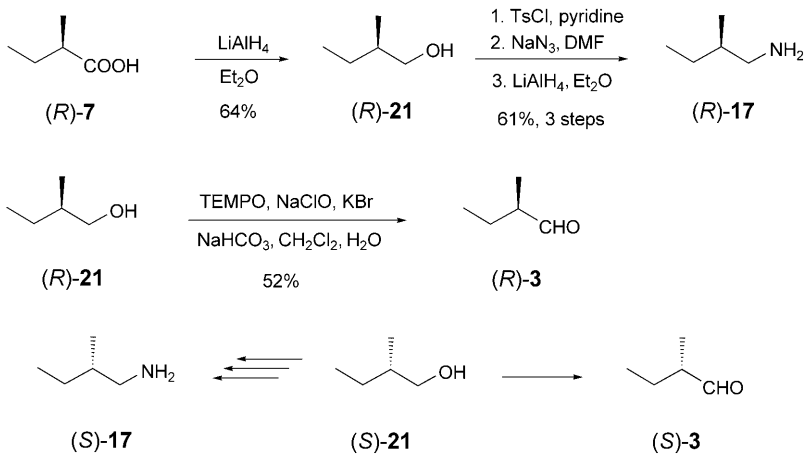
Table 2. *Odor Evaluations of Compounds* **1** *and* **4**

Structure	Number	Description
	(2*R*,2′*R*)-**1**	Irritating roasty note (metallic)
	(2*S*,2′*S*)-**1**	Chocolate-like note (mould-like, weak)
	(2*R*,2′*S*)-**1**	Green note (weak, roast)
	(2*S*,2′*R*)-**1**	Mouldy note (earthy, chocolate-like)
	(*R*)-**4**	Roasted-seafood note (strong, metallic)
	(*S*)-**4**	Seafood-like note (strong, mild)

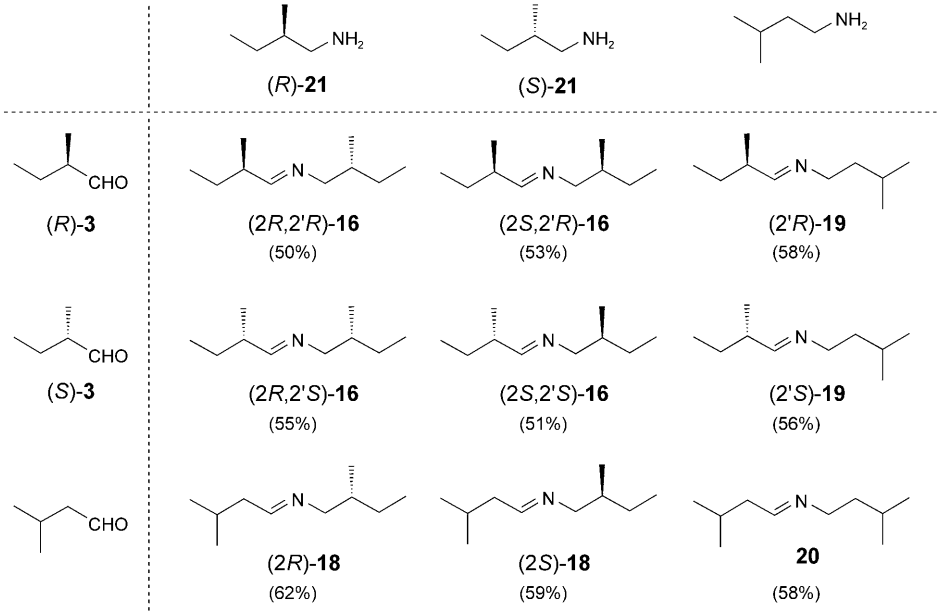
Scheme 7



Scheme 8



Scheme 9. *Starting Materials for the Syntheses of the Target Odorants* **16** *and* **18**–**20**. Yields are given in parentheses.



thresholds of the enantiomers, and distinct differences in those of the diastereoisomers were observed. Thereby, (2*S*,2′*S*)-**16** had the lowest threshold of all four isomers.

The results of the olfactory evaluation of compounds **18**–**20** are shown in *Table 4*. All stereoisomers have, again, seafood odors, each isomer differing in specific nuances.

Table 3. *Odor Evaluations and Thresholds* (in H_2O) *of the Four Stereoisomers of Compound* **16**

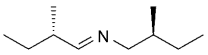
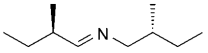
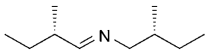
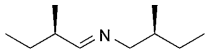
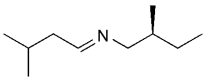
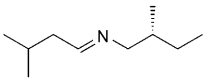
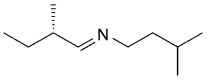
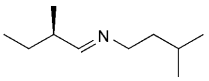
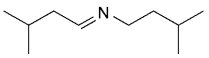
Structure	Number	Description	Odor threshold
	(2*S*,2′*S*)-**16**	Green note (fruity, metallic, mild, estery)	2.6 ppb
	(2*R*,2′*R*)-**16**	Medicine-like note (fruity, phenolic)	6.1 ppb
	(2*R*,2′*S*)-**16**	Fruity note (phenolic, estery)	46 ppb
	(2*S*,2′*R*)-**16**	Fruity note (metallic, amine-like, sweet)	91 ppb

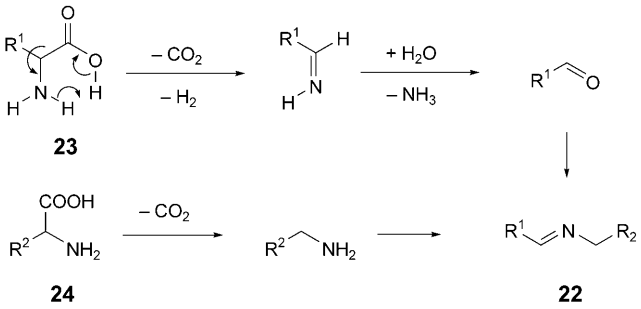
Table 4. *Odor Evaluations of Compounds* **18**–**20**

Structure	Number	Description
	(2*S*)-**18**	Amine-like note (sweet, cocoa-like)
	(2*R*)-**18**	Fruity note (heavy, esteric, cereal-like)
	(2′*S*)-**19**	Light note (strong, fruity)
	(2′*R*)-**19**	Heavy note (strong, sweet)
	**20**	Aldehydic, roasted-seafood note

Moreover, we discovered that all of the imine derivatives cannot be used only as seafood flavors, but also as fruit flavors, *e.g.*, in mango aromas [12].

Yaylayan and co-workers [13] recently reported the mechanism for the formation of the imine derivatives **22** from the amino acids **23** and **24** (*Scheme 10*). We considered that the imines **16** and **18**–**20** are also generated according to this pathway, *i.e.*, from leucine and isoleucine, naturally occurring in spotted shrimp. We, therefore, predicted the stereocenters of all these imines to be (*S*)-configured. In order to verify or falsify this hypothesis, separation of racemic imines by GC analysis on various chiral stationary phases is currently in progress.

Scheme 10. *Proposed Mechanism* [13] *for the Generation of Imines of Type* **22** *from an Amine* (**23**) *and an α-Amino Acid* (**24**)



In conclusion, we have discovered the mayor compounds responsible for the characteristic aroma of roasted spotted shrimp. We have also presented the stereoselective syntheses of these pyrrolidine and imine derivatives, and found each isomer to differ in its specific aroma characteristics, while having in common a general aspect of seafood. For experimental details, see references [9] and [12].

We are most grateful to Dr. *Hidenori Watanabe* and Dr. *Ken Ishigami* (The University of Tokyo) for their continuing encouragement. Our thanks are also due to Dr. *Akihiro Sakimae* (*Mitsubishi Rayon Co., Ltd.*) for his generous gift of (*R*)-**9**. This study was carried out with the help of our fellow workers of *T. Hasegawa Co., Ltd.*, which is highly appreciated.

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Fun with Furans

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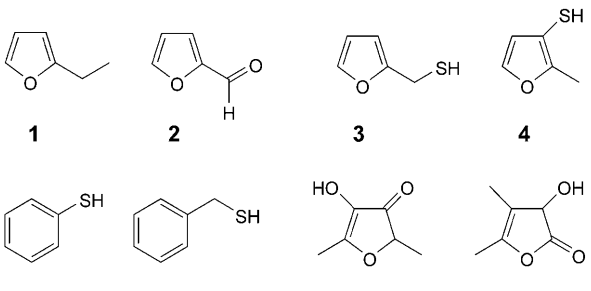
We're Gonna Have Fu(ra)n, Fu(ra)n, Fu(ra)n
'Till Daddy Takes The T-Bird Away
The Beach Boys (1966), '*Fu(ra)n, Fu(ra)n, Fu(ra)n*' (Almost)

The aromatic furan ring is found in a wide range of aroma chemicals, especially those produced by the *Maillard* reaction. They include both low-odor materials such as furfural and high-impact materials such as 2-methylfuran-3-thiol. Among the most important are the hydroxyfuranones, especially 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF, **7**), which is a material of unusual reactivity. This contrasts with the inert character of its isomer sotolone. Furans are also found as trace components where they can either contribute a desirable character or an *off-note* depending on context.

Introduction. – Of all the functionalities, heterocycles, and chemical groupings found among aroma chemicals, furans are often neglected. The reason for this is simply that, whereas concepts such as a *pyrazine note* or *sulfur aroma* are often referred to, there is no corresponding *furan note* or character. This may even lead to furans being considered simply to be expensive, more awkward, and troublesome versions of benzenoids. As with many generalizations, there is a grain of truth in this; the π-excessive furan ring makes it much more prone to electrophilic attack than analogous benzene derivatives, and the directing effect of the heteroatom makes the introduction of substituents at any position other 2 or 5 a challenge to the art and science of the organic chemist. In addition, simple, relatively unfunctionalized furans such as 2-ethylfuran (**1**) and furfural (**2**) have high odor thresholds and not easily recognized odors. The assumption, therefore, is that, in functionalized furans, one smells the functional group, and, again, at first sight, the low odor thresholds of the furan-thiols **3** and **4** seem to confirm this.



1 **2** **3** **4**

5 **6** **7** **8**

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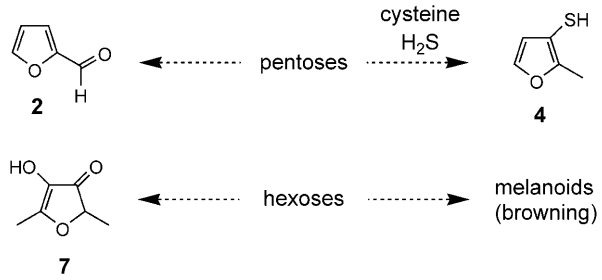
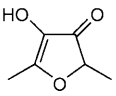
However, that this is an over-simplification can be gleaned from the character of **3** and **4**; while benzenethiol (thiophenol; **5**) is a trace component in beef aroma, it lacks the character of **4**, which is the single most important component of roast beef (and other meats) and benzenemethanethiol (**6**) is absent from foods such as coffee where furan-2-methanethiol (**3**) dominates. Finally, there are the hydroxyfuranones such as **7** and **8**, which are powerful and characteristic materials and for these, there are no benzenoid analogues.

A second reason for emphasizing the importance of furans is simply their ubiquitousness; especially, their formation in the *Maillard* reaction. Pentoses give rise to furfural and associated derivatives, and, in the presence of sulfur sources such as cysteine, highly odorous materials such as **4**. Hexoses give **7** and the melanoids responsible for browning (*Scheme 1*).

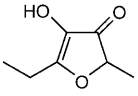
Scheme 1



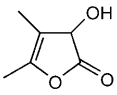
Hydroxyfuranones. – *4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one* (HDMF, **7**). The most important of these, with some of their common and trade names, are shown below.



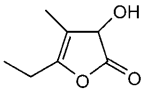
4-hydroxy-2,5-dimethylfuran-3[2*H*]-one (**7**)
Furaneol®
Fraision®
Strawberry furanone
Pineapple ketone



Homofuranol®
Ethylfraision®

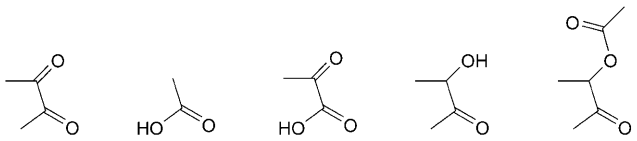


3-hydroxy-4,5-dimetylfuran-2[3*H*]-one (**8**)
sotolone
fenugreek lactone
caramel furanone



ethyl sotolone
ethyl fenugreek lactone
maple furanone

Of these, by far the most important is **7**, the previously mentioned 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF), which has a *sweet, candyfloss/cotton-candy-type aroma* and is familiar to anyone working in the flavor industry. It was first identified nearly 40 years ago in both pineapple and roast beef, and since then has been found to be a major contributor to the flavor of coffee, wine, and a variety of fruits. In strawberry it has been found to increase with ripeness [1], making it almost a feeding pheromone for humans! This was well illustrated in a study of strawberry volatiles. A reconstitution using the top twelve aroma-active volatiles was developed, and the influence of each of the volatiles was investigated by omitting each in turn. HDMF (**7**) was found to be the most critical component, as, in its absence, the formulation lacked *ripeness*, and the *green character* of (*Z*)-hex-3-enal dominated the flavor [2]. In meat and savory flavors, it is often used with sulfur-containing molecules such as 2-methylfuran-3-thiol (MFT, **4**) and, in this instance, it appears to act as a flavor enhancer rather than contributing sweetness. This range of applications makes it one of the most widely used materials in the flavor industry. However, it remains one of the more difficult materials to work with due to its high reactivity. The pure material, which is available in both natural and synthetic forms, is a colorless or pale yellow crystalline material, the natural form often being a little more highly colored. On standing, this free-flowing crystalline solid becomes sticky, the color increases and eventually the material becomes a gummy mass, most closely resembling set honey. Because of this physical change, HDMF (**7**) is often described as deliquescent, *i.e.*, absorbing moisture to the level of forming a solution, as is seen with simple inorganics such as $CaCl_2$ and NaOH. However, the situation here is much more complex. In addition to the visible changes, the odor of the material also alters, developing a *sharp and more powerful aroma* that is readily recognized as butane-2,3-dione (**9**) and acetic acid (**10**). GC shows some of the changes taking place (*Fig. 1*).



9 **10** **11** **12** **13**

The top GC trace in *Fig. 1* shows the purity of the synthetic material; a high loading was used to demonstrate this, and hence the peak is rather broad and has split into several peaks. In contrast, the older material shows a range of peaks, including butane-2,3-dione (**9**), which is partly merged with the solvent (acetone) peak. The aged sample, having become liquefied, could be analyzed by GC/MS without solvent (*Fig. 2*).

Two of the main contributors to the *aged* odor, **9** and **10**, co-eluted as a large peak. Pyruvic acid (**11**) also contributes a *burnt, sharp caramel aroma*. Other small molecules include acetoin (**12**) and its acetate **13**. The molecule **14** gives a clue to what might be happening. This molecule is a lower oxidation state equivalent to intermediate **15** (*Schemes 2* and *3*), which is cyclized to HDMF (**7**) as the last stage in its synthesis.

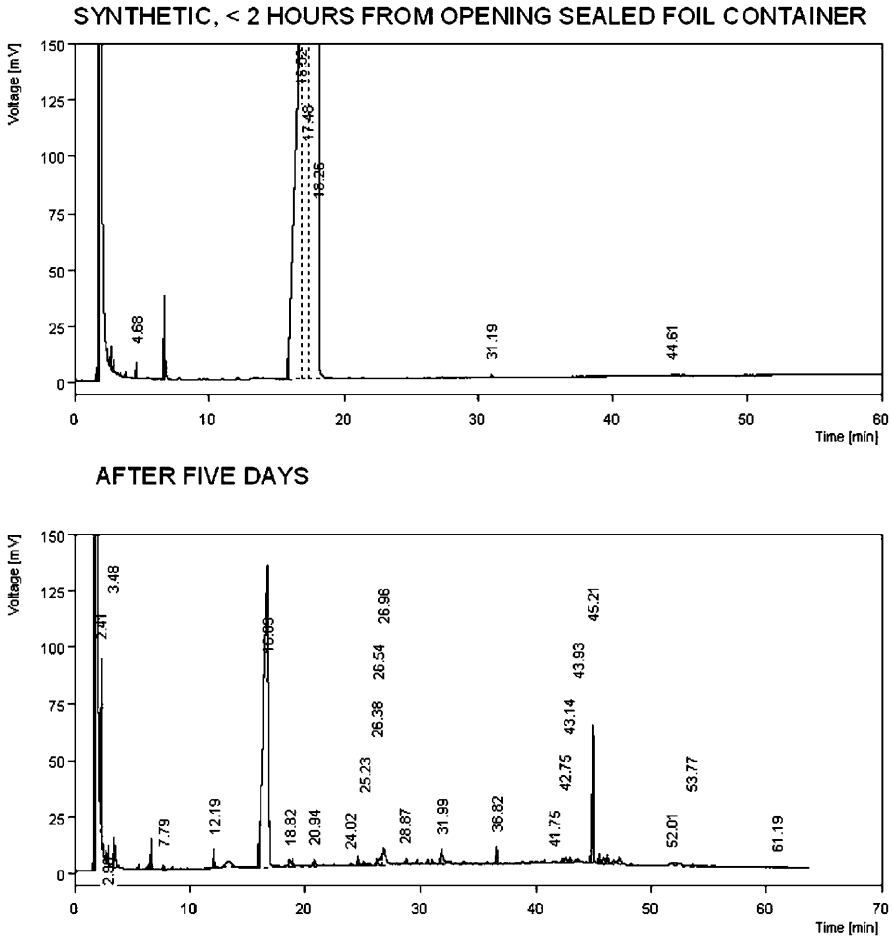
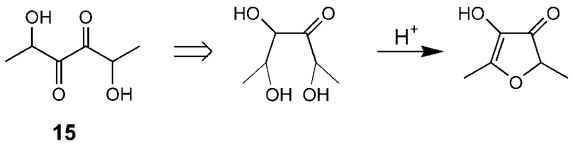


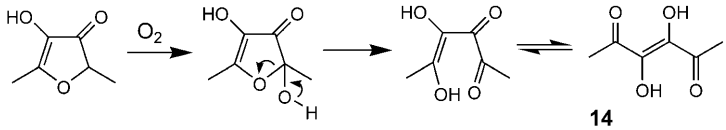
Fig. 1. *GC Comparison of fresh synthetic HDMF* (**7**) *with a sample that has been opened for five days*

Scheme 2



15

Scheme 3



14

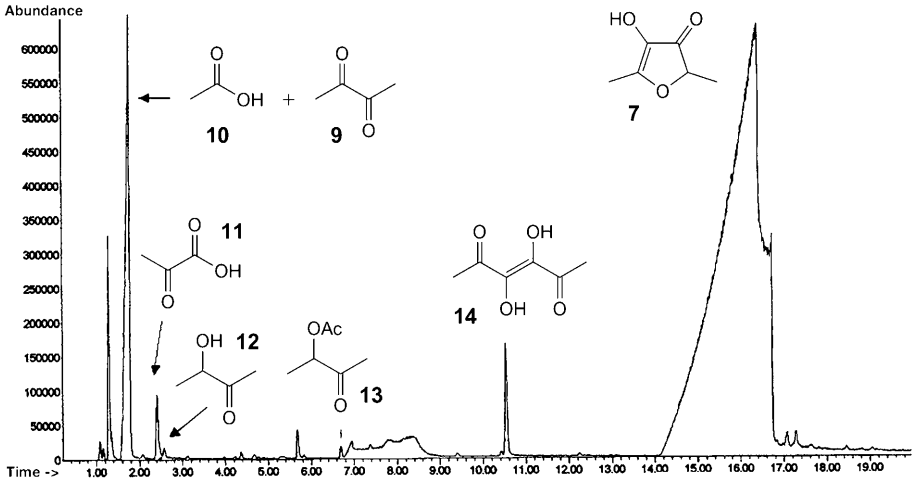
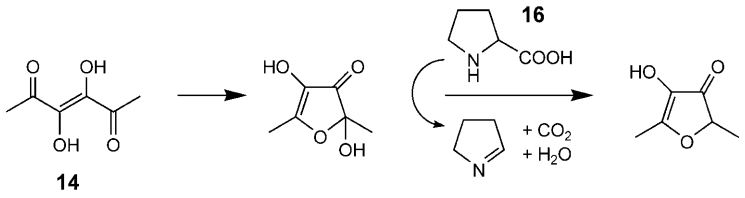


Fig. 2. *GC of aged sample of HDMF* (**7**) *showing the identity of some of the other components*

In essence, the breakdown is a combination of oxidation and hydrolysis (*Scheme 3*) and the apparent deliquescence is due to formation of liquid breakdown products, and possibly also the lowering of the melting point of HDMF (**7**) as the purity falls. We may also note that, in a way, this is the reverse of the proposed pathway for its formation in the *Maillard* reaction [3], the final step of which is the cyclization of a 3,4-dihydroxyhex-3-ene-2,5-dione (**14**) followed by reduction by means of the *Strecker* reaction with proline (**16**; *Scheme 4*). Is this an example of a material *uncooking* itself?

Scheme 4



In turn, this is only part of the story. When we amplify the GC trace of the *aged* HDMF (**7**; *Fig. 3*), we see that the base line is *Himalayan*, the mountainous *humps and bumps* characteristic of material containing a lot of involatiles that are breaking down in the injector or on the column.

We can see this in a more scientific manner by use of an internal standard. 2,3,4,5-Tetrahydro-2-methylfuran-3-one (*Coffee Furanone*; **17**) used at a 1:1 ratio with *fresh* and *aged* HDMF (**7**) analyzed at 48:52 and 63:32, respectively, and 5-methylfurfural (**18**) gave 54:46 and 69:29. This shows that *ca.* 30% of the HDMF (**7**) had disappeared.

Sotolone (= *3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one*; **8**). Compared to its isomer **7**, **8** is much less widely used. Found in some of the same places as HDMF (**7**), *e.g.*, coffee and roast beef, its most important occurrence is in the herb fenugreek, where it is the

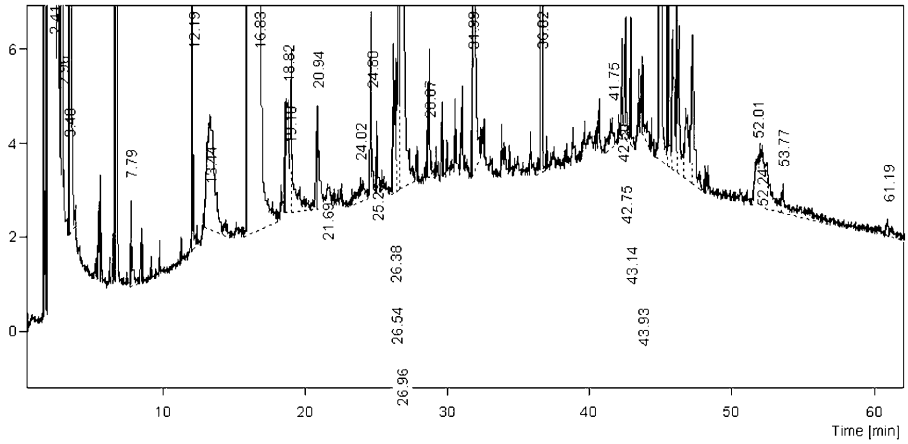
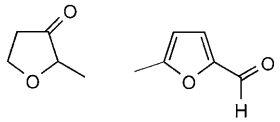


Fig. 3. *GC trace of HDMF* (**7**) *showing amplified baseline*

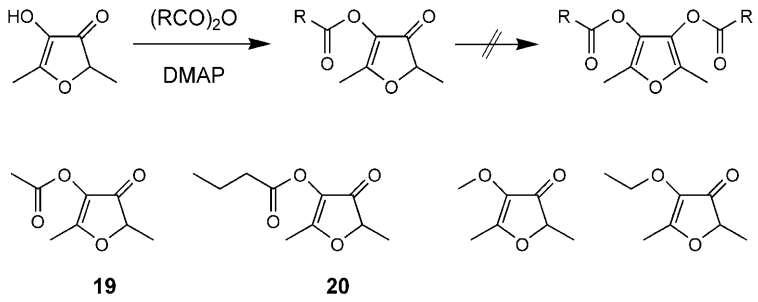
key odorant. The pure material has an overwhelming *burnt*, *caramel*, *fenugreek aroma* that persists in the environment, and especially on the skin, for days. In contrast to HDMF (**7**), sotolone (**8**) is annoyingly stable! A study on the influence of human saliva on number of aroma chemicals showed that, in contrast to aldehydes, sotolone was unaffected [4]. This fact comes as no surprise to those of us who have spilled this material onto their hands, and hence know that it is unaffected by hot water, soap, bleach, and prolonged scrubbing! The power and persistence of this material may, however, be turned to its advantage. Progressive and adventurous perfumers are encouraged to make use of the materials *intensely diffusive and fenugreek* tonality in fragrances [5].



17 **18**

HDMF Esters and Ethers. In general, these have a similar *sweet aroma* to their respective parent compound and are less familiar to flavorists and chemists. The acetate **19** is the most important, a pale yellow liquid with a *sweet candy aroma*; the butyrate **20** was first identified in mango [6] and is now *FEMA GRAS*. The esters are formed by reaction of HDMF (**7**) with acyl anhydrides. This apparently trivial reaction also indicates that the 'standard' way of denoting HDMF (**7**), as a mono-enol, is consistent with its behavior, as only the monoester is formed under normal conditions (*Scheme 5*).

Scheme 5

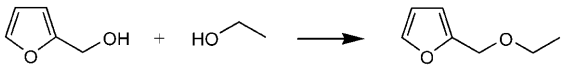


No Fu(ra)n, my babe, No Fu(ra)n
No Fu(ra)n, my babe, No Fu(ra)n
No Fu(ra)n to hang around, feeling that same old way
No Fu(ra)n to hang around, freak out, for another day.
Iggy & The Stooges (1970), '*No Fu(ra)n*' (nearly)

Furans as Off-Notes. – The widespread nature of furans almost inevitably means that they will also appear where they are not wanted; an aroma chemical in one context adds desirable character, whereas in another it is an unwanted *off-note*. The two main ways of generating furan *off-notes* are oxidation and the *Maillard* reaction. Oxidation due to simple exposure to the air can generate excessive quantities of HDMF (**7**) in orange juice and sotolone (**8**) in white wine [7]. Inappropriate heating can lead to the *Maillard* reaction that can generate the very powerful sulfur-containing furans, such as 2-methylfuran-3-thiol (**4**), which has been identified as an *off-note* in orange juice [8]. Compound **4** can also form by thermal degradation of thiamine (**23**).

Ethyl furfuryl ether (**21**) has been identified as an *aging* compound in beer [9]. Formed simply by the reaction of EtOH with furan-2-methanol (**22**; *Scheme 6*), its level rises with time, making it a useful marker for the age of beer. Given sufficient time it can exceed its taste threshold and become an *off-note*, lending a *solvent off-note* to the beer.

Scheme 6

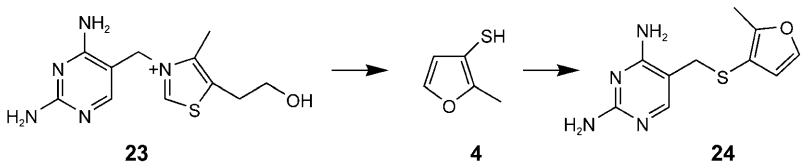


22 **21**

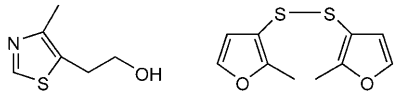
The line between desirable character and an *off-note* can sometimes be a fine one. Thiamine (**23**) degradation in food leads to the formation of 2-methylfuran-3-thiol (**4**), which can react with another thiamine molecule to generate the pyrimidine **24** (*Scheme 7*) [10]. Of course, one part of thiamine is 4-methylthiazole-5-ethanol (sulfurol; **25**), a low-odor molecule whose *meaty*, *dairy* or *beany* character is determined by trace components and, in this context, it would be unfair to refer to

these traces as off-notes. Furan disulfides are strong candidates for these modifiers, especially since they have a similar boiling point to that of sulfurol (**25**) and hence will codistil; **25** and bis(2-methylfuran-3-yl) disulfide (**26**) both have boiling points of around 280°.

Scheme 7



23 **4** **24**



25 **26**

Conclusions. – Furans have been identified in a very wide range of foodstuffs, in both desirable and undesirable contexts. The range of organoleptic properties, in terms both of character and odor threshold, is correspondingly wide. Furans are probably the most widespread heterocycle in the flavor industry. Indeed, the range of characters and uses has perhaps led to furans not being considered a group in their own right – to use an old English expression, '*we cannot see the wood for the trees*'.

My thanks to *Martin Gill*, *Peter Cannon*, and *Peter Whipps* at *De Monchy Aromatics*, UK, and to Dr. *Nikitas Ragoussis* and the Mass Spectrometry Department at *Vioryl S. A.*, Greece.

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Identification of New Odoriferous Compounds in Human Axillary Sweat

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3-Hydroxy-3-methylhexanoic acid (**1**) and the 3-sulfanylalkan-1-ols **2** – **5** were identified to contribute to the odor of human axillary sweat. Quantitative analyses of axillary sweat extracts from 50 healthy men showed an unambiguous correlation between the detected levels of **1** and the intensity of the axillary odor. Chiral-GC analyses revealed **1** to be a 72 : 28 mixture of the (*S*)/(*R*)-isomers. Optically pure (*S*)-**1** (> 97% ee) emanated a strong *spicy* note, which recalled typical axillary odors. 3-Methyl-3-sulfanylhexan-1-ol (**2**), the enantiomeric ratio of which equaled that of **1**, was present in greater quantity than any of the other 3-sulfanylalkanols. Optically pure (*S*)-**2** (> 97% ee) had a strong meaty, fruity note, also reminiscent of axillary odor. The compounds identified, in particular (*S*)-**1** and (*S*)-**2**, contribute significantly to the olfactory impression of human axillary odor.

Introduction. – The human body generates a variety of different odors. Scalp, hair, mouth, axillae, foot, and even the general skin surface all possess characteristic odors that are formed by septic action due to bacterial degradation. Of all the human scents, axillary odor is probably the most powerful and impressive. The axillary odor has been examined and discussed from an analytical, biological, and behavioral-physiology point of view. In particular, there is a wealth of information suggesting that it may contain chemical signals that affect the menstrual cycle [1] or may be involved in mate selection depending on a major-histocompatibility-complex (MHC) allele [2]. There are also several reports on actual axillary odorants [3], of which (*E*)-3-methylhex-2-enoic acid (3M2 H; see below) is considered one of the most important. 3M2 H was first reported by *Zen* and co-workers in 1991 [3d], who analyzed axillary odors collected from Americans, and, since then, researchers have mainly focused on this unsaturated acid. However, our own experiments have established that 3M2 H is not solely responsible for axillary odors. After a thorough analysis of the chemical composition of axillary odor, we discovered two new constituents, which also contribute significantly to the overall odor. One of them possesses a *spicy* note, while the other has a *sulfury* odor.

Results and Discussion. – 1. *Analysis of the Spicy Constituent*. A total of 50 healthy Japanese male volunteers were asked to collect their underarm odors by wearing clean white T-shirts that had fully defatted cotton inserts stitched into both of the armpit positions. Each insert was evaluated: 20% of the volunteers showed typical axillary odors, and 80% had a *sour, acidic* odor. The inserts were extracted with diethyl ether, and the ethereal extracts were treated with base. As this made the strong distinctive spicy odor disappear, the characteristic axillary odor was apparently caused by acidic

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constituents. Thus, acidic extracts of axillary odors collected from subjects having strong axillary odors were prepared and analyzed by GC-olfactometry and GC/MS. The total-ion-chromatography (TIC) plot of a typical extract is depicted in *Fig. 1*.

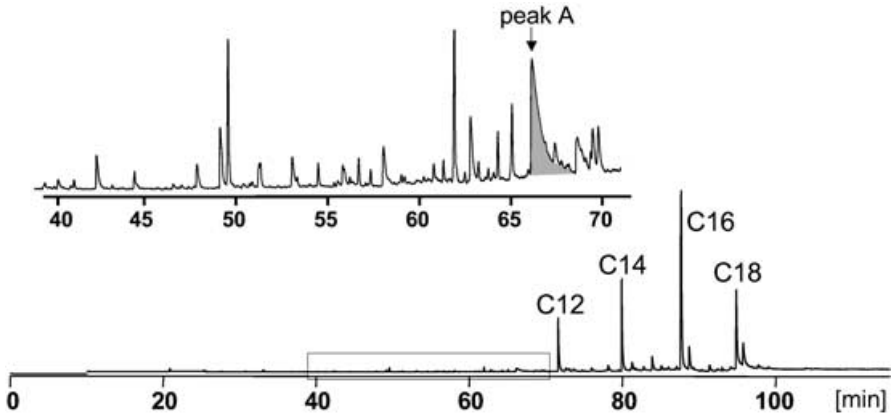


Fig. 1. *Total-ion chromatogram of the acidic fraction of axillary odor from an intensely smelling male test person.* The boxed part in the lower chromatogram has been enlarged. Compounds giving rise to peak *A* had a spicy note.

Aliphatic acids with 12 – 14 C-atoms constituted the bulk of the extract. The marked area in the TIC chromatogram of *Fig. 1* indicates when substances belonging to axillary odors eluted. Quantitative olfactory evaluation established peak *A* to correspond to the compound with the strongest spicy note, resembling typical axillary sweat. Comparison with the chromatogram obtained from an individual with *sour*, *acidic* underarm odor indicated saturated aliphatic acids, such as hexanoic and octanoic acid, and γ-lactones to be present in both. Peak *A* was, however, found only in the extracts from subjects with *spicy* axillary odor (*Fig. 2*). Peak *A* was therefore, specific to subjects with strong axillary odor, and we concluded that it was also responsible for the specific spicy note.

The next task was to elucidate the chemical structure of the constituent(s) giving rise to peak *A*. The order of elution of the TIC peaks from a nonpolar column indicated the compound to comprise seven or eight C-atoms. The mass spectrum with peaks at *m*/*z* 85 and 103 suggested loss of H_2O, and, thus, the presence of an OH function. The vapor-phase IR spectrum established the molecule to contain a C=O function as well. We observed a sharp absorption band at 1750 cm^{-1}, which was assigned to a C=O stretching fequency, and another band at 3550 cm^{-1}, which indicated a COOH group (*Fig. 3*). Based on this structural information, we proposed the compound to be 3-hydroxy-3-methylhexanoic acid (**1**), and this was verified by direct comparison with an authentic sample. Synthetic **1**, indeed, exhibited a spicy note that reminded us of the characteristic spicy axillary odor [4].

Fig. 4,*a*, illustrates the relationship between the observed strength of the spicy axillary odor and the detected amount of acid **1**. The bar chart clearly shows a correlation between the amount of **1** and the intensity of the axillary odor observed. The component was not detectable, though, in individuals with *sour, acidic* axillary odor. Acid **1** is, therefore, a useful indicator of whether or not a subject is prone to

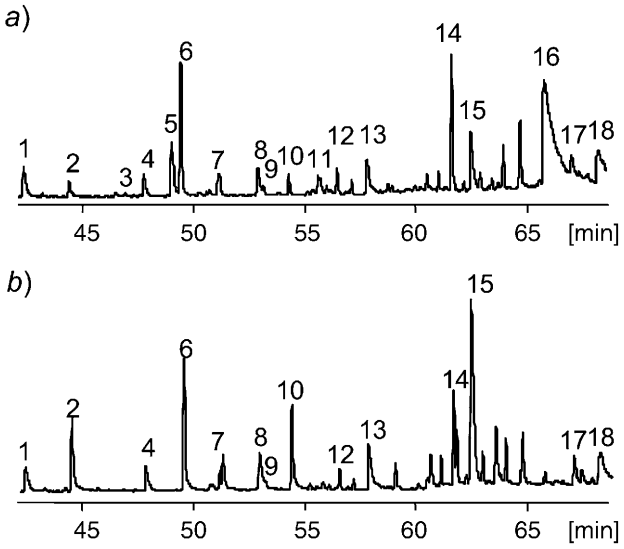
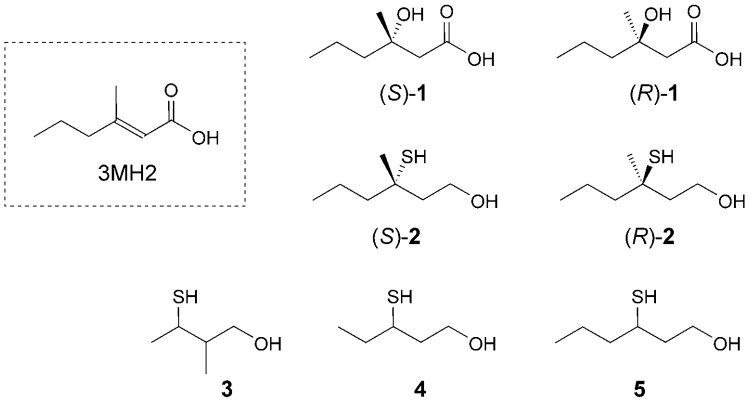


Fig. 2. *Total-ion chromatograms of test persons with 'typical'* (*a*) vs. *'sour, acidic'* (*b*) *axillary odors.* The following compounds were identified: hexanoic acid (*1*), undecanol (*2*), (*Z*)-3-methylhex-2-enoic acid (*3*), 2-ethylhexanoic acid (*4*), (*E*)-3-methylhex-2-enoic acid (*5*), dodecanol (*6*), γ-nonalactone (*7*), octanoic acid (*8*), *p*-cresol (*9*), tridecanol (*10*), oct-7-enoic acid (*11*), γ-decalactone (*12*), nonanoic acid (*13*), γ-undecalactone (*14*), decanoic acid (*15*), peak *A* (*16*; see *Fig. 1*), undecanoic acid (*17*), and benzoic acid (*18*).



strong axillary odor. It is also relevant for assessing the strength of axillary odors, since, typically, precise olfactory evaluations of underarm odors are difficult to perform due to odor adaptation.

Compound **1** has a stereogenic center at C(3), and, since enantiomers often possess different odor characteristics and intensities, we determined the enantiomeric ratio. Indeed, isolated **1** was not racemic, but occurred in a ratio of (*R*)/(*S*) 28 : 72, almost independent of the individual evaluated (*Fig. 5*). Both enantiomers of **1** were synthesized and characterized by olfactory evaluation. The dextrorotatory (*S*)-

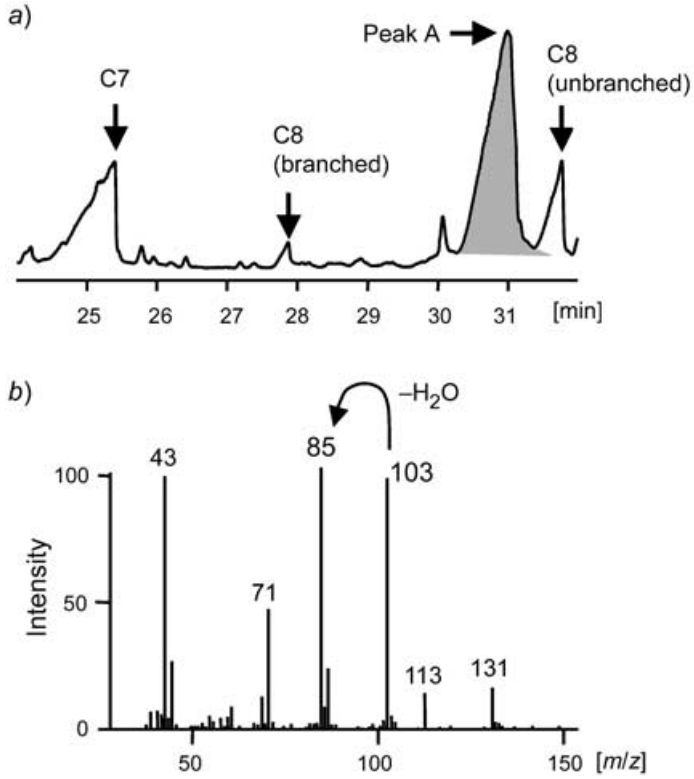


Fig. 3. *Expanded total-ion chromatogram* (*a*) *and mass spectrum of peak* A (*b*)

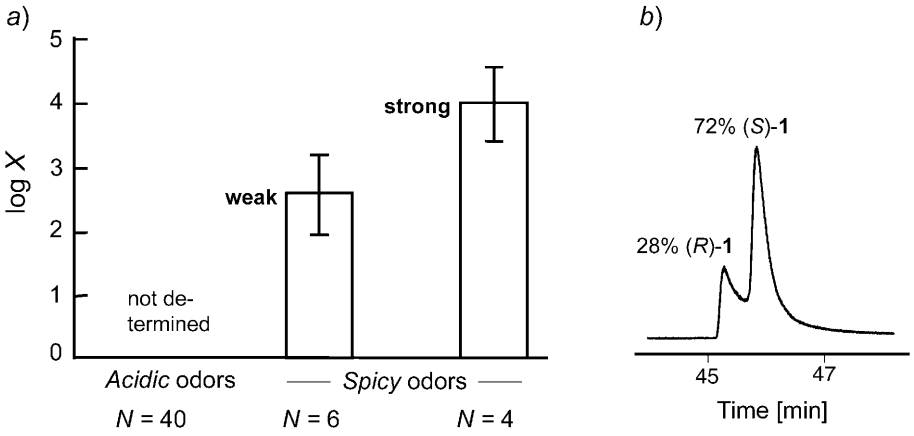


Fig. 4. a) *Quantitative analysis of compound* **1** *in different odor samples.* The (logarithmic) ordinate refers to X = amount of **1** (in ng) per cotton insert used for sweat collection (see text and *Exper. Part*); N refers to the number of male test persons investigated. Only spicy notes were examined. b) *Ion-chromatographic determination of the enantiomeric distribution of* **1** (*m*/*z* 103; see *Exper. Part*) *isolated from the spicy sweat of male volunteers.* The odor thresholds for the (*R*)- and (*S*)-isomers of **1** were found to be 10 and 0.1 ppm, resp.

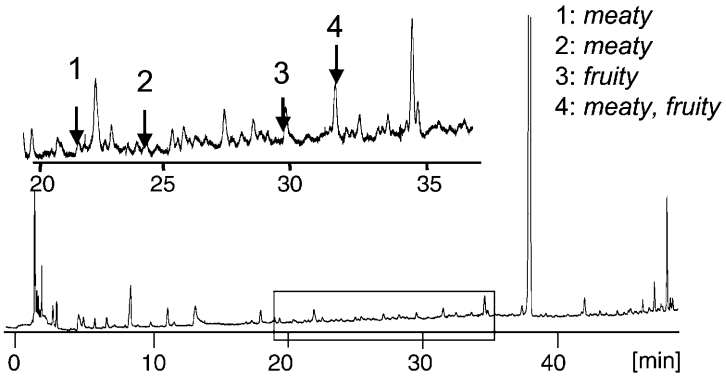


Fig. 5. *GC-Olfactometry trace of sulfury axillary sweat*

configured compound (*S*)-**1** emanates a *strong*, *spicy odor*, while its antipode (*R*)-**1** has a rather *weak animalic odor* (*Fig. 4*,*b*) [5].

2. *Analysis of the Sulfury Constituent.* The evaluation of the sulfury odor was easily possible by directly smelling the armpit of a subject. The chemical analysis of this odor, however, turned out to be extremely difficult due to the limited amount of material available. The application of SBSE (stir-bar sorptive extraction) methodology allowed us, nevertheless, to study the compounds involved. The odorants were absorbed by placing the sorptive stir bars directly into axillary sweat, which was collected from subjects with axillary odors who had taken a sauna at 40^0 (80% humidity, 30 min). As in the case of the spicy odorant, the analysis of the sulfury odor was performed by GC-olfactometry and GC/MS.

Fig. 5 shows a typical chromatogram obtained by GC-olfactometry. The outlined area marks the part of the chromatogram where sulfury odors were detected, four strong-smelling peaks being indicated with arrows. By GC/MS analysis peak 4 was identified as 3-methyl-3-sulfanylhexan-1-ol (**2**) [6][7]. During our investigations, we observed that the sulfury odor of the samples was more pronounced under anaerobic incubation. By analysis of incubated sweat samples, we were able to identify the remaining three sulfury-smelling compounds as homologous 3-sulfanylalkan-1-ols [8], *i.e.*, 2-methyl-3-sulfanylbutan-1-ol (**3**), 3-sulfanylpentan-1-ol (**4**), and 3-sulfanylhexan-1-ol (**5**; *Fig. 6*).

It is worth noting that sulfury-smelling odorants were detected neither in fresh nor incubated sweat that had been collected from individuals exhibiting a *sour*, *acidic* sweat odor. Like the analogous hydroxy acid **1**, the 3-methyl-3-sulfanylhexan-1-ol (**2**) possesses a stereogenic center at C(3), and in connection with the observed enantiomeric excess of **1**, we were also interested in the enantiomeric composition of isolated **2**. By GC analysis on a chiral column, it was demonstrated that the (*S*)/(*R*) ratio of **2** was the same as in the case of **1** (*Fig. 7*). Thereby, synthetic (−)-(*S*)-**2** (> 97% ee) possesses a *strong meaty*, *fruity note* with the characteristic *sulfury odor* searched for, whereas (+)-(*R*)-**2** (> 97% ee) has a *green*, *fruity note*.

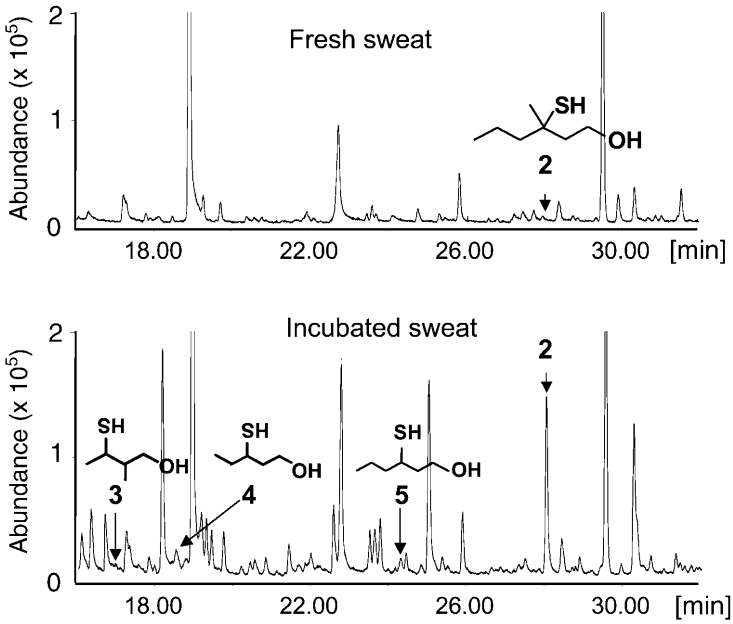


Fig. 6. *Chromatographic comparison between fresh and incubated human sweat* (see *Exper. Part*)

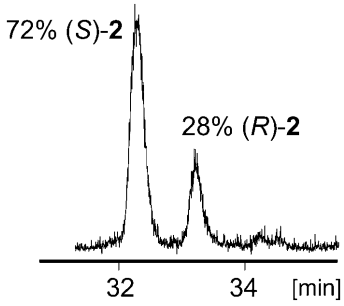


Fig. 7. *Chiral ion chromatogram of natural* **2** (*m*/*z* 114; see *Exper. Part*). The (*R*)/(*S*) ratio of 28 : 72 was found to be conserved for both **1** and **2**, inicating a biogenetic relationship. The odor thresholds for (*R*)- and (*S*)-**2** were determined as 10 ppb each.

Conclusions. – We have identified (+)-(*S*)-3-hydroxy-3-methylhexanoic acid ((*S*)-**1**) and (−)-(*S*)-3-methyl-3-sulfanylhexan-1-ol ((*S*)-**2**) as two new volatile compounds that make an important contribution to the overall olfactory impression of human axillary sweat. Both **1** and **2** share the same C skeleton and occur in the same enantiomeric ratios, which suggests a biochemical relationship. The formation of human axillary odor has been the subject of several reports [9][10]. In particular, the 2003 article by *Natsch et al.* [8] deals with malodor precursors, in which the odorants are conjugated with the N-terminal glutamine residue of *Aporipo protein D*. Similar precursors may also exist for **1** and **2**.

The majority of methods applied in deodorant products are based on excessive sterilization of the axillary region, which can cause irritation and may even turn out to be detrimental to the immune system. Although it is controversial, we think that axillary odor is not just malodor. It probably does have a function, maybe pheromonal or emotional effects [11], and only became undesirable in the modern age. With the discovery of the strong, offensive odorants **1** and **2**, it might now be possible to develop less-aggressive methods for a more-targeted deodoration that respects the symbiotic relationship between humans and microorganisms, without affecting a healthy skin condition.

Experimental Part

General. – All reagents and solvents were purchased from *Tokyo Kasei Kogyo Co.*, *Aldrich*, *and Wako Chemicals*. GC/MS, GC/IRD, and GC-Olfactometry analyses were performed on *Agilent 6890N*, *Agilent HP5973*, and *Agilent HP5965B* systems, resp. The TDS (thermal-desorption system) and CIS (cooled injection system) instruments were manufactured by *Gerstel GmbH*, Germany. HPLC was performed with a *Shimazu* pump, *LC-10ATVP*, detector *SPD-10AVP*, and autosampler *SIL-10ADVP*. Optical rotations were measured on *Atago Polax-D* (10 cm, 1.0 cm^3, Na lamp, 25°; in EtOH, $c = 42.4$). SBSE (Stir-bar sorptive extraction) was performed with equipment from *Gerstel GmbH*, Germany.

Odor-Threshold Measurements. The odor-threshold values were determined by triangle tests. A defined amount of each compound was dissolved in mineral oil (*Nakarai tesque*). In every dilution step, three glass beakers were used, each containing 5 ml of liquid. One of the beakers contained the soln. with the compound. The samples were presented in order of increasing concentrations. Five panelists were used to evaluate each threshold value, the results of which were averaged.

Preparation and Analysis of Acidic Extracts from Axillary Sweat. The odor was collected with clean white T-shirts into which were stitched fully defatted oval-shaped cotton inserts (15 cm × 18 cm) at both armpit positions. The test persons, 50 healthy male volunteers (age 25–42), had to wear the T-shirt for 24 h without using deodorants, deodorant soaps, or cologne in the axillary region. Each insert was then soaked with Et_2O (2 × 50 ml) and squeezed, and the combined ethereal solns. were extracted with aq. 2M NaOH soln (2 × 50 ml). The combined aq. extracts were adjusted to pH 2 with aq. 2M HCl soln., and then extracted with Et_2O (2 × 50 ml). The combined org. layers were concentrated under reduced pressure, and subjected to GC/MS analysis (70 eV): *DB-WAX* (60 m × 0.25 mm i.d. fused silica, 0.25 μm, *J &W Scientific*; He flow of 1 ml/min). The acidic extract was diluted with 100 μl of Et_2O, and 2 μl of the diluted sample was injected splitless at 250° (temp. program: 40° at 6°/min to 70°, then at 2°/min to 240°, then 240° for 40 min). Chiral GC/MS analysis (70 eV) of *3-hydroxy-3-methylhexanoic acid* (**1**: *Chiraldex D-DM* (30 m × 0.25 mm i.d. fused silica, 0.25 μm, *Advanced Separation Technologies, Inc.*, He flow 2.2 ml/min). The acidic extract was diluted with 100 μl of Et_2O, and 1 μl of the diluted sample was injected splitless at 250° (temp. program: 1 min at 40°, at 6°/min to 60°, at 2°/min to 120°, then 120° for 60 min).

Analysis of the Sulfury Note of Axillary Sweat. Fresh axillary sweat was collected from individuals with strong axillary odor who had taken a sauna at 40^0 (80% humidity, 30 min). A tube (1.5 cm i.d.) was placed directly onto the axillae whilst massaging the area with the edge of the tube and scooping. This procedure did not discriminate between apocrine and eccrine secretions. For incubation, the tube containing the collected sweat sample was placed in the sealed box containing *AnaeroPack®* (*Mitsubishi Gas Chemical Company, Inc.*) under an atmosphere of less than 1% O_2 and *ca.* 21% CO_2, and stored at 30° for 48 h. An SBSE stir bar (*Twister®, Gerstel GmbH*) was allowed to stir in both the fresh and the incubated sweat samples, resp., for 5 min prior to wiping it dry and placing it into a glass tube (6 mm i.d.) of the TDS system. The desorbed volatile compounds (desorbtion temperature 250°, purge flow 50 ml/min during 3min, CIS at −150°, with 12°/min to 250°) were automatically injected in solvent-vent mode into the GC/MS (70 eV) apparatus equipped with *DB-1* (60 m × 0.25 mm i.d. fused silica, 0.25 μm, *J & W Scientific*, He flow of 1 ml/min; temp. program: 40° at 6°/min to 70°, at 2°/min to 300°, then 300° for 40 min). Chiral GC/MS analysis (70 eV) of 3-methyl-3-sulfanylhexan-1-ol (**2**): *Chiraldex D-DM* (30 m × 0.25 mm i.d. fused silica, 0.25 μm, *Advanced Separation Technologies, Inc.*, He flow of 2.2 ml/min). The sample was desorbed at 250° (purge flow 50 ml/min during 3 min, CIS at −150° at 12°/min to

250°) and injected in the solvent-vent mode into the GC/MS apparatus (temp. program: 1 min at 40°, at 6°/min to 60°, at 2°/min to 120°, then 120° for 60 min).

3-Hydroxy-3-methylhexanoic Acid (**1**). Under N_2, Zn powder (13.1 g, 200 mmol) was suspended in anh. THF (50 ml) and pentan-2-one (21.3 ml, 200 mmol) was added with stirring. Benzyl bromoacetate (31.60 ml, 200 mmol) was added dropwise, and stirring was continued at r.t. for 8 h. The reaction was quenched with sat. aq. $NaHCO_3$ soln. (20 ml). The org. layer was separated, dried (Na_2SO_4), filtered, and evaporated to provide crude, *racemic* benzyl 3-hydroxy-3-methylhexanoate (41.1 g, 174 mmol), which was separated into the respective optically pure compounds by means of HPLC (*Chiralcel AD*; hexane/MeOH, 100:3, 1.0 ml/min, 25°; detection at 220 nm).

To a stirred soln. of benzyl 3-hydroxy-3-methylhexanoate (98% ee; 1.40 g, 6 mmol) in EtOH (1.40 g) was added aq. 1M NaOH soln. (5.94 g, 149 mmol). The mixture was stirred for 2 h, washed with CH_2Cl_2 (5 × 20 ml) to remove $PhCh_2OH$, and adjusted to pH 2 with aq. 2M HCl soln. The resulting soln. was extracted with CH_2Cl_2 (3 × 20 ml), dried (Na_2SO_4), filtered, and evaporated to yield (*S*)-**1** (810 mg, 91%) or (*R*)-**1** (760 mg, 85%). $[\alpha]_D^{25} = +1.6$ ((*S*)-**1**), $[\alpha]_D^{25} = -1.6$ ((*R*)-**1**). EI-MS (70 eV): 146 (M^+), 131 (12), 113 (14), 103 (88), 87 (39), 85 (97), 71 (41), 69 (14), 43 (100).

Identification of 3-Sulfanylalkan-1-ols. Identification of these compounds was carried out by comparison with synthetic reference samples, which were prepared according to the following procedure.

3-Methyl-3-sulfanylhexan-1-ol (**2**). Racemic 3-(benzylsulfanyl)-3-methylhexan-1-ol was prepared according to [6]. The crude racemate was separated by chiral HPLC (*Chiralcel OJ-H*, *Daicel Chemical Industries*; hexane/i-PrOH, 9:1, flow 0.5 ml/min, 40°; detection at 254 nm). To a soln. of enantiomerically pure 3-(benzylsulfanyl)-3-methylhexan-1-ol (1.10 g, 4.62 mmol) in Et_2O (12.0 ml) was added at −78° liq. NH_3 (15.0 ml). Na (*ca.* 230 mg, 47.9 mmol) was added, until the mixture remained blue for more than 20 min. The mixture was allowed to warm to r.t. overnight, and EtOH was added until the blue color disappeared. The mixture was acidified with aq. 1M HCl soln., and extracted with Et_2O (3 × 2 ml). The combined org. layers were dried (Na_2SO_4) and concentrated under reduced pressure. Distillation at 130°/40 mbar provided (*S*)-**2** (564 mg, 82%) or (*R*)-**2** (533 mg, 78%), respectively, depending on the starting material [6] employed. EI-MS: 148 (1, M^+), 114 (12), 97 (25), 71 (37), 55 (100), 41 (68).

2-Methyl-3-sulfanylbutan-1-ol (**3**). Tiglic acid methyl ester (2.36 g, 20 mmol), $PhCH_2SH$ (4.96 g, 21 mmol), and piperidine (10 ml) were mixed together and heated to reflux for 48 h. The mixture was then allowed to cool to r.t., and the excess piperidine was removed at reduced pressure. To a suspension of $LiAlH_4$ (0.37 g, 10 mmol) in Et_2O (5 ml) was added slowly with stirring the above residue (2 g), keeping the temp. below 10°. Stirring was continued for 1 h, and then sat. aq. NH_4Cl soln. (20 ml) was added slowly. The mixture was extracted with Et_2O, and the combined org. extracts were dried (Na_2SO_4). More anh. Et_2O (30.0 ml) was added, and the soln. was cooled to −78°. Liq. NH_3 (30.0 ml) was added, followed by Na (*ca.* 660 mg), until the mixture remained blue for more than 20 min. The mixture was then allowed to warm to r.t. overnight, and EtOH was added until the blue color disappeared. The mixture was acidified with 1M aq. HCl soln. and extracted with Et_2O (3 × 20 ml). The combined org. extracts were dried (Na_2SO4), concentrated under reduced pressure, and then distilled at 60°/0.2 mbar to afford **5** (800 mg, 78%). EI-MS: 120 (15, M^+), 102 (4), 86 (84), 71 (57), 61 (80), 60 (100), 55 (65), 45 (62), 43 (30), 42 (17).

3-Sulfanylpentan-1-ol (**4**). *Pent-2-enoic acid* (5.00 g, 50 mmol) and propanol (7.50 g, 125 mmol) were mixed in pyridine (15 ml), and dicyclohexylcarbodiimide (15 g, 72.8 mmol) was added. The mixture was stirred for 3 h, and then filtered. CH_2Cl_2 (10 ml) was added to the filtrate, and the org. layer was washed with 1M aq. HCl soln., dried (Na_2SO_4), and evaporated. The residue (2 g), $PhCH_2SH$ (1.50 g, 12 mmol), and piperidine (3.00 ml) were mixed together and heated to reflux for 48 h. The mixture was allowed to cool to r.t., and the excess piperidine removed by distillation under reduced pressure. The resulting residue (2 g) was added slowly with stirring to a suspension of $LiAlH_4$ (0.40 g, 10 mmol) in Et_2O (10 ml), keeping the temp. below 10°. Stirring was continued for 1 h, and then sat. aq. NH_4Cl soln. (20 ml) was added slowly. The mixture was extracted with Et_2O, and the combined org. layers were dried (Na_2SO_4). To the org. soln. was added anh. Et_2O (30 ml). At −78°, liq. NH_3 (30 ml) and then Na (*ca.* 0.45 g) were added, until the mixture remained blue for more than 20 min. The mixture was allowed to warm up to r.t. overnight, and EtOH was added, until the blue color disappeared. The mixture was acidified, washed with 1M aq. HCl soln., and extracted with Et_2O (3 × 20 ml). The combined org. layers were dried (Na_2SO_4), concentrated under vacuum, and distilled at 80°/1.5 mbar to give **4** (850 mg, 59%). EI-MS: 120 (15, M^+), 102(3), 86 (85), 69 (64), 61 (47), 57 (100).

3-Sulfanylhexan-1-ol (**5**). Hex-2-enoic acid (5.00 g, 43 mmol) and PrOH (7.50 g, 125 mmol) were mixed in pyridine (15.0 ml), and dicyclohexylcarbodiimide (15.0 g, 72.8 mmol) was added. After 3 h of stirring at r.t., the mixture was filtered, and the filtrate was diluted with CH_2Cl_2 (10 ml). The org. soln. was washed with 1M aq. HCl

soln., and the solvent was evaporated under reduced pressure. The resulting residue (2 g) and $PhCH_2SH$ (1.50 g, 12 mmol) were mixed in piperidine (3.0 ml) and heated to reflux for 48 h. After the mixture had cooled to r.t., the excess piperidine was removed by distillation at reduced pressure. The residue (2 g) was added slowly with stirring to a suspension of $LiAlH_4$ (400 mg, 10 mmol) in Et_2O (10 ml) keeping the temp. below 10°. Stirring was continued for 1 h, and then sat. aq. NH_4Cl soln. (20 ml) was added slowly. The mixture was extracted with Et_2O, and the combined org. layers were dried (Na_2SO_4), and then diluted with anh. Et_2O (30 ml). This soln. was cooled to −78°, and liq. NH_3 (30 ml) was added. Na (*ca.* 450 mg, 19.6 mmol) was added, until the mixture remained blue for more than 20 min. The mixture was allowed to warm to r.t. overnight, and EtOH was added, until the blue color disappeared. The mixture was acidified with 1M aq. HCl soln. and extracted with Et_2O (3 × 20 ml). The combined org. extracts were dried (Na_2SO_4), concentrated under reduced pressure, and then distilled at 90°/2.6 mbar to provide **5** (940 mg, 58%). EI-MS: 134 (10, M^+), 116 (0.4), 100 (46), 82(26), 67 (32), 61 (37), 57 (56), 55 (100).

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