Biosynthesis of C_{11} hydrocarbons in the brown alga

Ectocarpus siliculosus

Dissertation

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Gutachter:

1. Prof. Dr. Wilhelm Boland	Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena
2. Prof. Dr. Reiner Beckert	Institute für Organische Chemie und Makromolekulare Chemie, Friedrich Schiller Universität, Jena
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List of abbreviations

CLS	closed loop stripping
DCC	dicyclohexylcarbodiimide
DiHODA	dihydroxyoctadecadienoic acid
DMAP	4-Dimethylaminopyridine
DMSO	dimethylsulfoxide
GC/MS	gas chromatography/mass spectrometry
HETE	hydroxyeicosatetraenoic acid
HKR	hydrolytic kinetic resolution
HPETE	hydroperoxyeicosatetraenoic acid
HPL	hydropeoxyde lyase
HPOT	hydroperoxyoctadecatetraenoic
IBX	1-Hydroxy-1,2-benziodoxol-3-(1H)-one 1-Oxide
LDS	linoleate diol synthase
LOX	lipoxygenase
PFBHA	pentafluorobenzyl hydroxylamine
PGG_2	prostaglandin G_2
PGHS	prostaglandin H synthase
PUFA	polyunsaturated fatty acid
SPME	solid phase microextraction
THF	tetrahydrofurane

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1. Introduction

1.1 Chemical ecology of brown algal pheromones

The class of brown algae (Phaeophyceae) consists of around 2000 species living on marine coasts worldwide. They evolved multicellularity independently from other eukaryotic organisms like green and red algae, fungi and animals, and they are regarded as an alternative model of development (*1*). Brown algae reproduce both sexually and asexually, with motile gametes and zoospores having two flagella, and because of this morphological feature they are classified in the phylum Heterokontophyta. In the sexual phase of their life cycle, meio-spores generate haploid filaments called gametophytes. These, in turn, release either male or female gametes, which fuse and produce a zygote from which arise a new sporophyte, the diploid form of the organism (*2*). Settling of sporophytes is fundamental in the population dynamics of brown algae (*3*, *4*). Many brown algae, such as Laminaria and Ectocarpus species, release gametes in water, where tidal current and turbulence favour their dispersal. Nevertheless settling of new sporophytes does occur. From this perspective it is not surprising that cells of opposite sex use chemical cues to come in contact (*5*). As these substances mediate communication between individuals of the same species, they are classified as pheromones, according to the definition of Karlson and Lüscher (*6*).

Fig. 1 Gametophyte of the brown alga *Ectocarpus siliculosus*. Vegetative filament (left) and mature filament with two sexual bodies containing gametes (right).

The function of pheromones has been thoroughly studied in the small filamentous brown alga *Ectocarpus siliculosus* (Fig. 1). Settled female gametes emit a substance that affects flagellar movement of male cells, and directs them towards the pheromone source (Fig. 2) (*7*). One of the male cells eventually fuses with a female and a zygote is formed. This kind

of substances have a pheromone function in most brown algae, although in some cases, like some Dictyoperis species, they are emitted also by vegetative thalli without a connection to reproductive role (*8*).

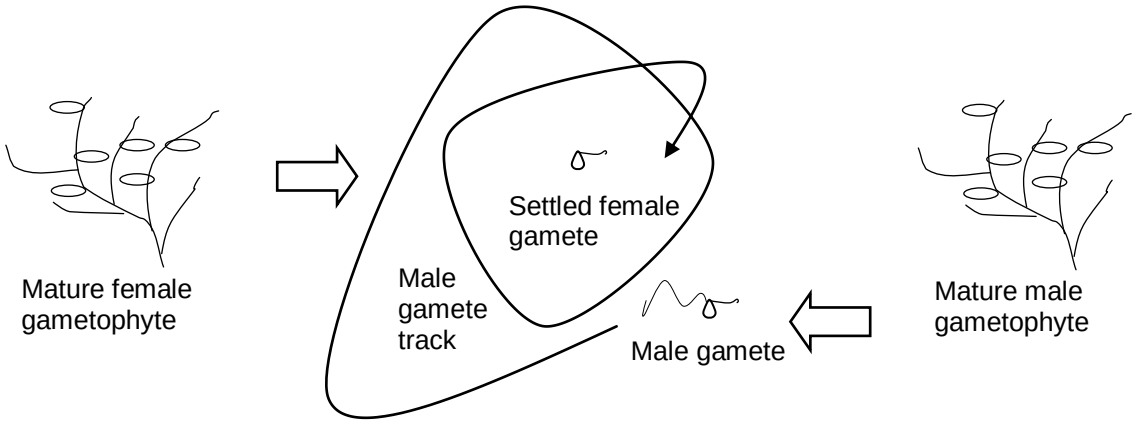


Fig. 2 Pheromone function in gametes of *E. siliculosus*, adapted from (*7*)

The diversity of pheromones is relatively limited compared to the number of brown algal species and actually the same molecule is active in more than one species. Similar structural elements are: a C_{11} hydrocarbon skeleton (or C_8 hydrocarbon in the case of Fucus species), that can be linear or cyclic; multiple unsaturations; and the lack of methyl branches as well as polar groups, except for the oxirane ring of lamoxirene (**1**) (Fig. 3). Depending on species, released pheromones are either optically pure or enantiomeric mixtures. In the first case, concerning for example multifidene (**2**), the natural enantiomer has higher attractivity, showing that male cells can discriminate chiral pheromones (*9*). In the mixtures, like for example hormosirene (**3**), the enantiomeric ratio depends on the species or even on the geographical region, although the biological function of this variability has not been fully explained (*10*). A C_{11}-hydrocarbon structurally similar to hormosirene, namely pre-ectocarpene (**4**), plays an important role in male gamete attraction. A concerted reaction, namely a [3,3] sigmatropic rearrangement, transforms the *cis*-disubstituted three membered ring into the unsaturated seven membered ring (6*S*)-but-(1*Z*)-enyl-1,4-cycloheptadiene (**5**) (Fig. 4). The pheromone function was initially assigned to this substance, that was isolated for the first time from female gametes of *E. siliculosus* and named ectocarpene (*11*). In fact, after the low-temperature synthesis of the *cis*-bisalkenylcyclopropane **4**, it was shown that this substance is several orders of magnitude more active than the corresponding cycloheptadiene **5**, therefore it was named pre-ectocarpene, and it is now considered as the genuine pheromone of *E. siliculosus* (*12*). As this rearrangement is spontaneous at natural temperatures (ca. 16 °C), it is regarded as an effective mechanism of pheromone deactivation. Sigmatropic reactions occur without

intermediates, thus the enantiomeric purity of the product reflects that of the precursor. The products of the rearrangement of pre-ectocarpene (**4**) and of the more saturated analogue (**6**), which are respectively ectocarpene (**5**) and dictyotene (**8**), have high optical purity (*13* , *14*).

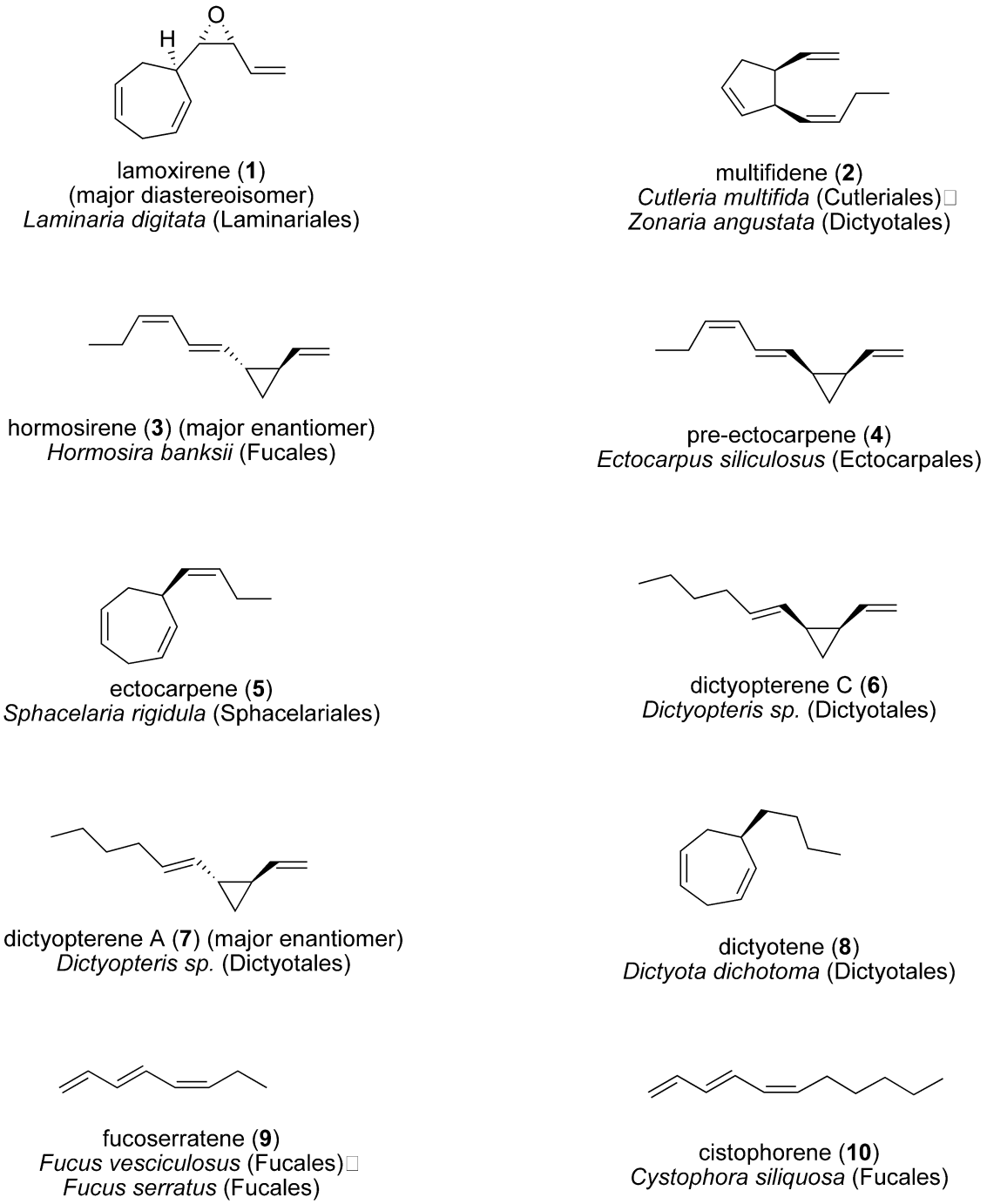


Fig. 3 Structure of C_{11} hydrocarbons of different brown algal species.

The *trans*-bisalkenylcyclopropanes **3** and **7** are often emitted as enantiomeric mixtures, whereas *cis*-bisalkenylcyclopropanes **4** and **6** are, so far as known, optically pure, suggesting that the enzyme systems responsible for the biosynthesis of these two classes of pheromones are different.

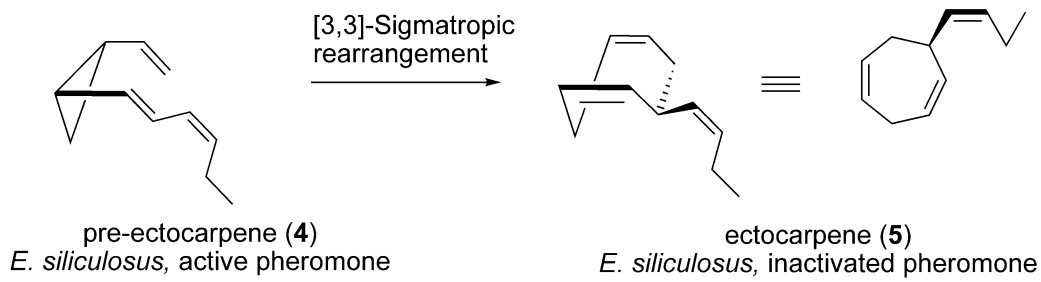


Fig. 4 Spontaneous deactivation pheromone of *E. siliculosus* pre-ectocarpene.

1.2. Biosynthesis of C_{11} hydrocarbons in *E. siliculosus* and other organisms

As sexual cells of brown algae are difficult to obtain in suitable amounts for biosynthetic studies, the hypotheses on the natural precursors of C_{11} hydrocarbons have been tested also in other organisms, easier to cultivate. These additional experimental models allowed repeated incubations with isotopically labelled precursors. In the case of brown algae, incubation of female gametes of *E. siliculosus* and *Sphacelaria rigidula* with deuterated analogues of arachidonic acid resulted in labelled C_{11}-hydrocarbons. This indicates that C_{20}-polyunsaturated fatty acids (C_{20}-PUFAs) are the genuine pheromone precursors, the ω3-aliphatic terminus being incorporated in the 1-butenyl moiety of ectocarpene, as illustrated in Fig. 5 (*15*).

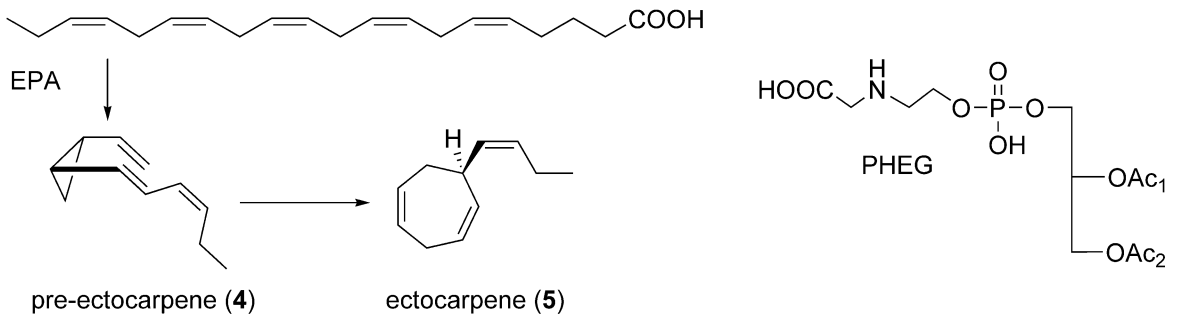


Fig. 5 Biosynthesis of pre-ectocarpene (**4**) in the Brown alga *E. siliculosus* and proposed lipid precursor phosphatidyl-*O*-[*N*-(hydroxyethyl)-glycine] (PHEG). EPA: eicosapentaenoic acid; AA: arachidonic acid; Ac_1 and/or Ac_2 = EPA and AA.

Studies on gamete lipids additionally support this hypothesis, showing a different fatty acid composition in the phospholipids of female and male sexual cells (*16*). In particular phosphatidylethanolamine (PE) and phosphatidyl-*O*-[*N*-(hydroxyethyl)-glycine] (PHEG) contain more eicosapentaenoic acid in female than in male cells (*17*). It is believed that these lipids may be the storage molecules for the pheromone precursor (Fig. 5).

The hypothesis of C_{20} PUFAs precursors had been initially tested on the terrestrial plant *Senecio isatidaeus,* which, among other substances, produces the same volatile hydrocarbons of *E. siliculosus* (*18*). However labelling experiments showed that plants do not use long chain PUFAs, which they do not possess, but the shorter all-(*Z*)-3,6,9-dodecatrienoic acid, suggesting that β-oxidation of α-linolenic acid is involved (Fig. 6a) (*19*). In this case it was also possible to envisage a mechanistic model of the enzymatic reaction, based on incubation with fatty acids isotopically labeled in specific positions (*20*). The mechanism involves hydrogen abstraction from a bis-allylic methylene group, in analogy with the widespread enzyme class of lipoxygenases (Fig. 6b). A spatial arrangement of the C_{12} fatty acid precursor was suggested to explain the formation of enantiopure volatile **5** (*20*).

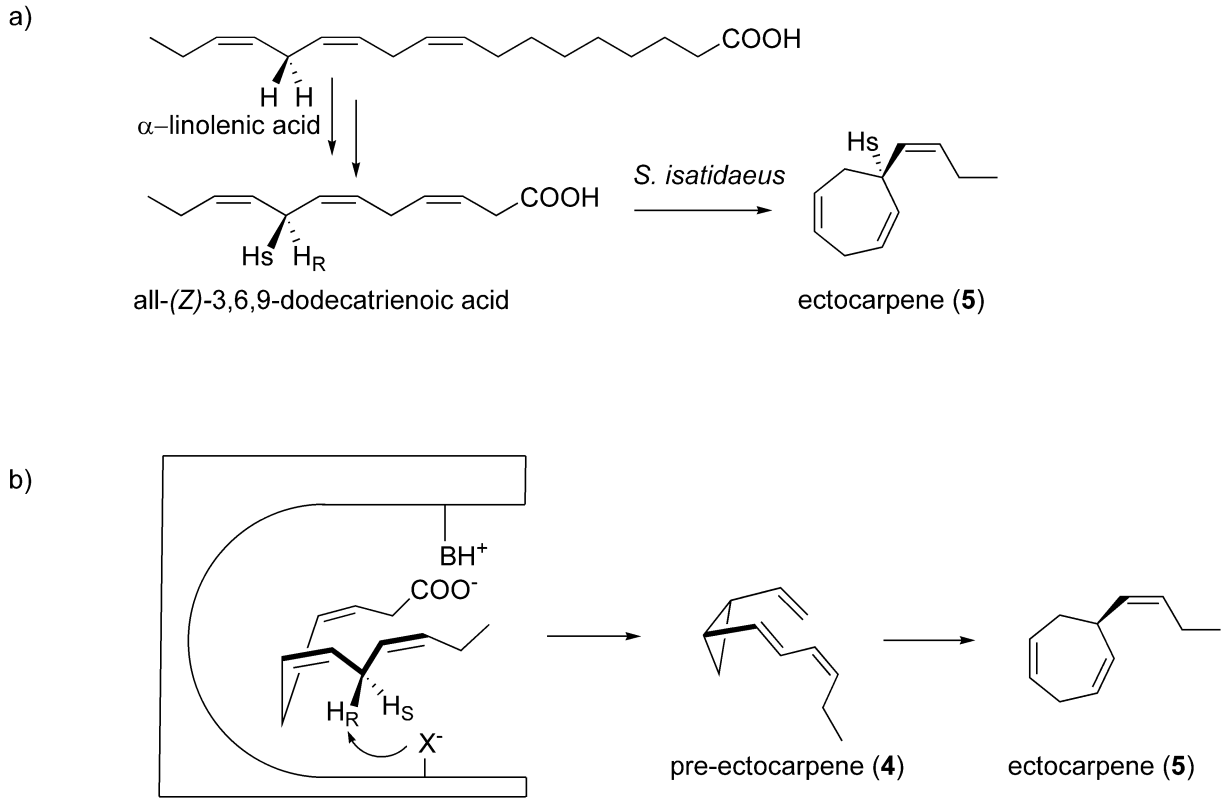


Fig. 6 Biosynthesis of C_{11}-hydrocarbons in the plant *S. isatidaeus.* (a) Fatty acid precursors. (b) Mechanistic model for the formation of the *cis*-bisalkenylcyclopropane **4**.

Additional information about the intermediates and the stereochemical course of the biosynthetic pathway involving C_{20} PUFAs is available thanks to studies with diatoms. Some of these unicellular organisms, which are evolutionary related to brown algae, produce C_{11} hydrocarbons (*21*). Moreover, in the diatoms studied, the oxygen dependent, simultaneous production of hydrocarbons and ω-oxoacids has been shown (*22*, *23*). Upon cell wounding, for instance, the diatom *Gomphonema parvulum* transforms arachidonic acid (AA) into the hydrocarbon dictyopterene A (**7**) and in the polar compound (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid (**29**) (*24*) (Fig. 7a).

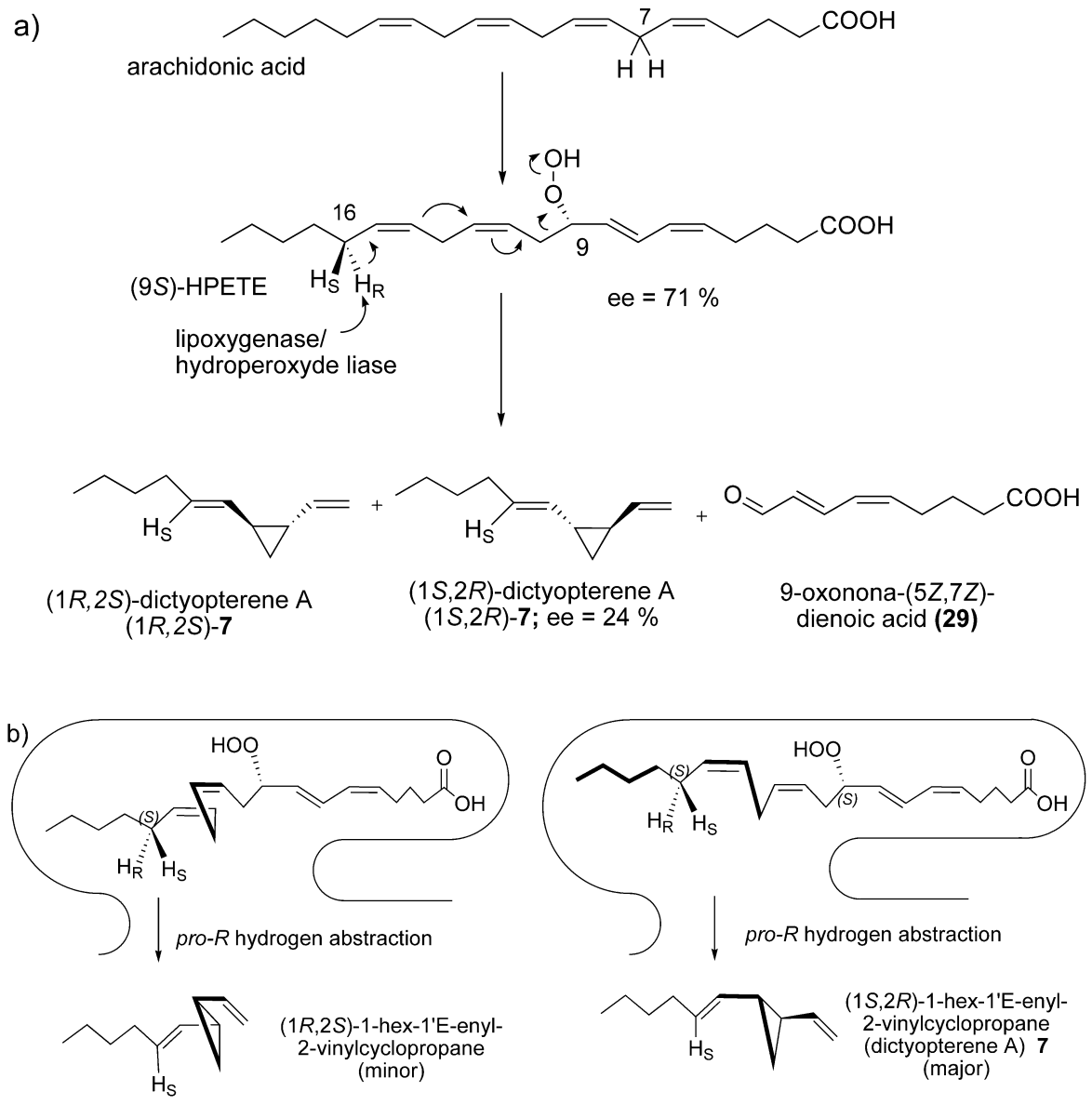


Fig. 7 Biosynthesis of *trans*-bisalkenylcyclopropanes in diatom *G. parvulum*: a) mechanism involving lipoxygenation of the C_{20} fatty acid precursor; b) proposed spatial arrangement of the substrated in the binding site of the enzyme.

Similarly eicosapentaenoic acid (EPA) is transformed into hormosirene and the same ω-oxoacid. Moreover, the C_{20} hydroperoxy fatty acid (9*S*)-hydroperoxy eicosatetraenoic acid ((9*S*)-HPETE) is the first intermediate (Fig. 7a). This has been observed by trapping the corresponding hydroxy fatty acid (9*S*)-HETE and subsequent HPLC analysis after incubation with arachidonic acid. The formation of these substances indicates an enzymatic activity capable of both oxygenation and cleavage of the fatty acid, which has therefore been called lipoxygenase/hydroperoxyde lyase activity (LOX/Lyase) (*25*). In analogy with the mechanism known for C_{11} hydrocarbon biosynthesis in plants, hydrogen abstraction takes place, although, in this case, twice. The first hydrogen abstraction, occurring in position C-7 of arachidonic acid, leads to the intermediate (9*S*)-hydroperoxyeicosatetraenoic acid (9*S*)-HPETE. The second occurs in position C-16 of the hydroperoxide intermediate, and results in the formation of the hydrocarbon dictyopterene A (**7**). As mentioned before (see 1.1), dictyopterene A (**7**) and its unsaturated analogue hormosirene (**3**) are released as defined enantiomeric mixtures in nature. Therefore the enzyme stereospecificity of hydrocarbon formation was investigated in *G. parvulum*. The fatty acid substrate undergoes hydrogen abstraction and oxygen insertion on a pentadiene segment, a typical feature of lipoxygenases (*26*). The resulting hydroperoxyde produced by the diatom has low enantiomeric excess (ee 71 %); this is due partially to the autooxydation process and partially to the uncomplete stereochemical control by the enzyme. The second hydrogen abstraction, which takes place on the intermediate (9*S*)-HPETE, occurs preferentially at the pro-*R* hydrogen, but the resulting hydrocarbon dictyopterene A has low optical purity (ee 24%). Based on these data it was suggested that the stereochemistry of *trans*-bisalkenylcyclopropanes depends on the enantiomeric excess of the hydroperoxy fatty acid (9*S*)-HPETE as well as the conformation in the substrate binding site of the enzyme, which would exert a weak control on the spatial arrangement of the substrate (Fig. 7b) (*25*). Thus, the formation *cis*-bisalkenylclopropanes like pre-ectocarpene (**4**) and dictyopterene C (**6**), could depend on a different folding of the polyunsaturated fatty acid, as these hydrocarbons have high optical purity (see 1.1).

Another open issue regarding the biosynthesis of pre-ectocarpene in *E. siliculosus* is the direct transformation of complex lipids. Oxidation and cleavage of the fatty acid backbone could also occur directly on a phospholipid. In fact the products of the cleavage of free fatty acids, like the ω-oxoacids released by diatoms, could not be detected in the brown alga. During autooxidation of the lipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (OxPAPC), for example, the carboxylic terminus of the unsaturated fatty

acid is not hydrolysed but remains as glutaryl- or 5-oxovaleryl-ester in the complex lipid (*27*). Specific oxygenation of complex lipids has been shown in the activity of soybean lipoxygenase on dilinoleyl phosphatidylcholine (*28*), and glycolipids esterified with dihydroxy fatty acids are present in the red alga *Gracilaria chilensis* (*29*).

1.3 Selected enzymes catalysing hydrogen abstraction from polyunsaturated fatty acids.

The knowledge about the mechanism of formation of C_{11} hydrocarbons is based on labelling experiments and on the identification of the intermediates. Enzymes catalysing the formation of these volatile substances have never been isolated, so far as known. Nevertheless, the mechanistic hypotheses on the formation of C_{11} hydrocarbons available in literature could benefit from the study of isolated enzymes involved in the metabolism of polyunsaturated fatty acids in other organisms. Some information about well studied enzymes is given hereafter. The enzyme class of lipoxygenases (LOX) is widespread in plants and animals, where these enzymes play a key role, respectively, in the production of oxylipins and leukotrienes. They catalyse the stereospecific abstraction of bis-allylic hydrogen from a polyunsaturated fatty acid followed by antarafacial oxygen insertion, that is at the opposite face of the plan defined by the 1,4-pentadiene (Fig. 8a). Kinetic isotope effect provided evidence that the abstraction of the hydrogen atom is the first step of the process (*30*).

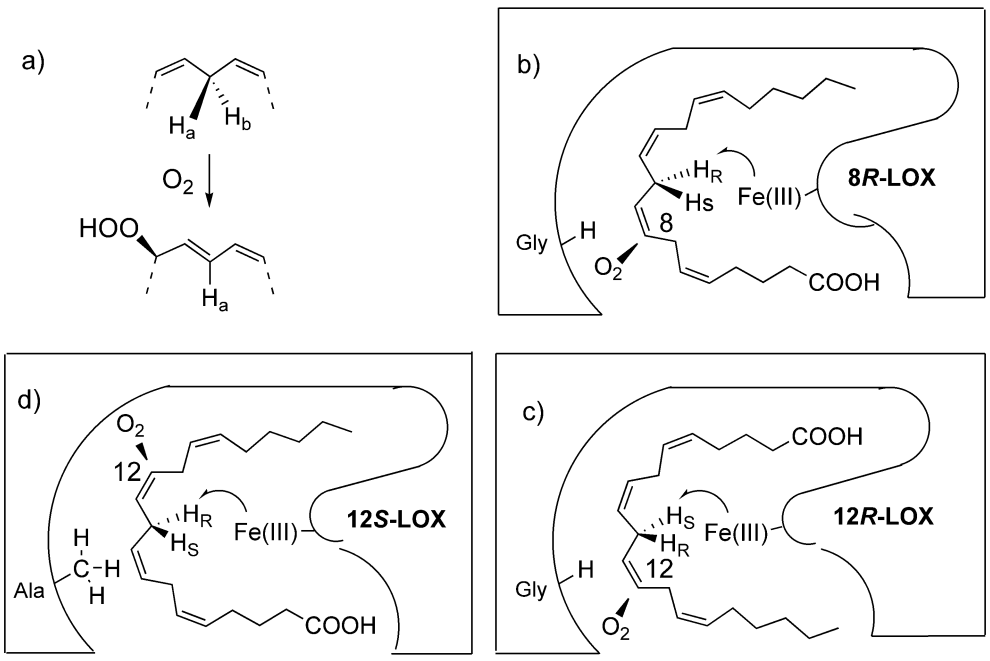


Fig. 8. Stereospecificity of lipoxygenase activity (LOX). a) Antarafacial relation between oxygen insertion and hydrogen abstraction. b,c,d) Model for LOX regio- and stereospecificity based on recombinant wild-type and mutant proteins of mammals, plants and one coral, adapted from (*31*).

The catalytic domain contains a non-heme iron atom coordinated to three hystidine residues and the carboxyl group of a C-terminal isoleucine (*32*). These enzymes are stereospecific, producing either (*S*)- or (*R*)-hydroperoxy fatty acids. Nevertheless, some exceptions have been reported, like in the case of maize lipoxygenase-1, which produces mixtures of stereo- and regioisomers, probably because the oxygen binding cavity is disconnected from the substrate binding cavity (*33*). In lipoxygenases the regio- and stereospecificity is determined by the orientation of the fatty acid substrate in the enzyme active site. With respect to the abstracted hydrogen, oxygen insertion occurs always with antarafacial geometry. However, the position of the oxygenation depends in first instance on the orientation of the fatty acid in the hydrophobic pocket (Fig. 8b and 8c). In second instance, given a certain orientation of the fatty acid, it depends on the size of a specific aminoacidic residue of the enzyme. If a glycine is substituted by a more bulky alanine, the enzymatic activity has different stereo- and regiospecificity, switching from 8*R*-LOX to 12*S*-LOX (Fig. 8b and 8d) (*31*). Another important enzyme class in lipid metabolism is that of hydroperoxyde lyases (HPL), which catalyse the cleavage of a hydroperoxy fatty acid deriving from the lipoxygenase activity into an aldehyde and a ω-oxoacid. Although the catalytic mechanism of HPL is not completely understood, it is known that these enzymes contain one iron atom coordinated to a heme prosthetic group and that they belong to the family of cytochrome P450. Moreover, there is evidence that the aldehyde and the oxoacid are foremed *via* an epoxyallylic radical intermediate and hemiacetals, followed by isomerase activity (*34*) (Fig. 9).

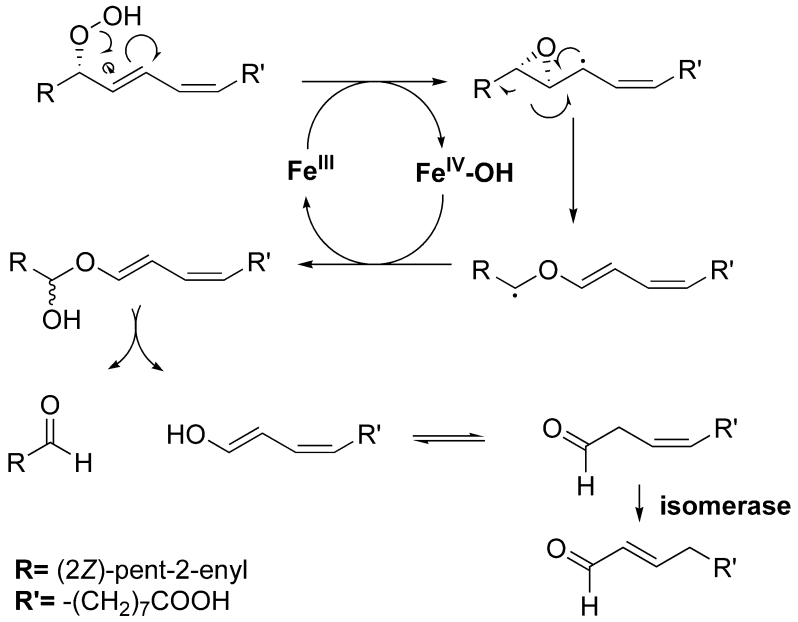


Fig. 9. Mechanism of hydroperoxide lyase based on recombinant enzymes of melon, alfalfa (*Medicago stativa)* and guava (*Psidium guajava*) (*34*).

Stereospecific hydrogen abstraction on polyunsaturated fatty acids has been observed also in other enzymes belonging to the suprafamily of prostaglandine H synthases (PGHS) or cyclooxygenases. In mammalian PGHS, *pro-S* hydrogen abstraction in position C-13 of arachidonic acid leads to the formation of prostaglandine PGG_2 after insertion of two molecules of oxygen (*35*) (Fig. 10a). The active domain contains a heme-coordinated iron atom and a conserved residue of tyrosine which participates to the reaction as tyrosyl radical. Recently, enzymes with high sequence homology to PGHS have been discovered in plants and fungi.

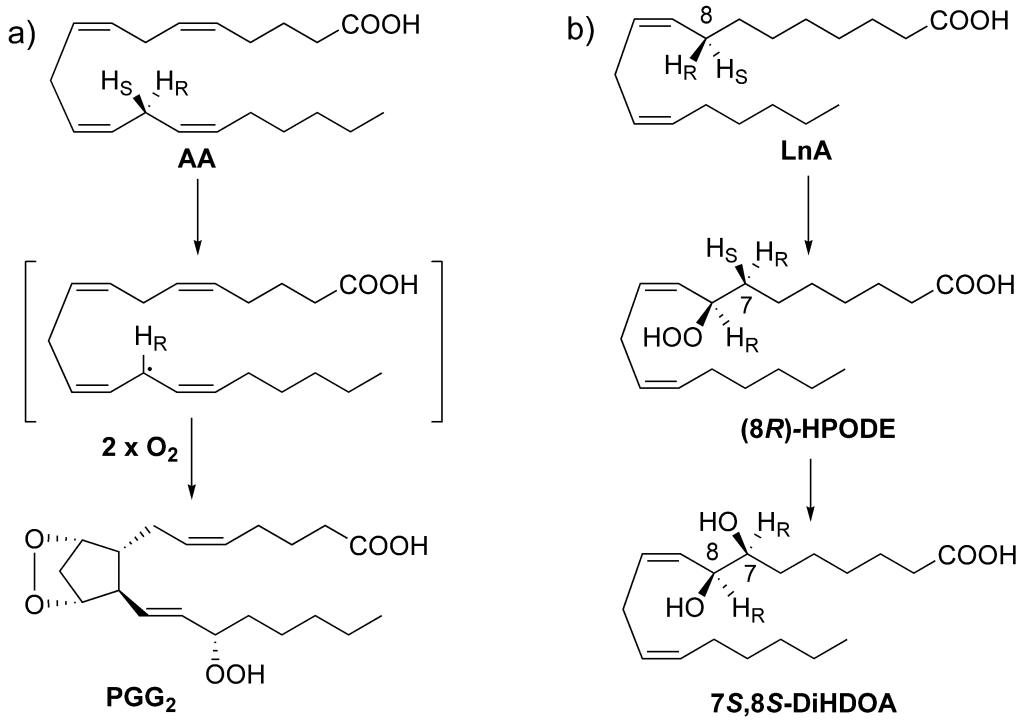


Fig. 10 Hydrogen abstraction in a) human prostaglandin H synthase and b) linoleyl diol synthase from the fungus *Gaeumannomyces graminis*.

Among these, linoleate diol synthase (LDS) catalysed the formation of (7*S*,8*S*)-dihydroxyoctadecadienois acid [(7*S*,8*S*)-DiHODE]. This enzyme abstracts the *pro-S* hydrogen in C-8 position of linoleic acid followed by antarafacial insertion of molecular oxygen at C-8 to generate (8*R*)-hydroperoxy octadecadienoic acid [(8*R*)-HPODE]. The latter is then isomerised to (7*S*,8*S*)-DiHODE by elimination of the *pro-S* hydrogen at C-7 and intramolecular oxygen insertion from the hydroperoxyde group (*36*) (Fig. 10b).

Besides these well-characterized enzyme classes there are reports of proteins capable of particular transformations of polyunsaturated fatty acids. For example in the coral *Plexaura homomalla*, an organism known for its high content of prostaglandin esters, it was discovered an enzyme that catalyses the transformation of arachidonic acid into an allene oxide, a putative precursor of prostanoids. This enzyme has two distinct catalytic

domains. The first contains a non–heme iron center and catalyses the formation of (8*R*)-hydroperoxyeicosatetraenoic acid [(8*R*)-HPETE]. The second is a catalase-related domain containing an iron atom coordinated to a heme group and is responsible for the production of an allene oxide, a potential precursor of coral prostanoids (*37*). This type of transformation also occurs in Plants, where an allene oxide derived from linolenic acid is a key intermediate for the formation of the five-membered carbon ring of jasmonic acid; the plant pathway, however, involves distinct enzymes (*38*). More recently it has been reported that the maize lipoxygenase *ZmLOX6* encodes for a protein which has lost lipoxygenase activity and accepts 13-hydroperoxyoctadecatetraenoic acid (13-HOTE) as substrate. Among other products, the enzyme converts this oxygenated fatty acid into (9*Z*,11*E*)-13-oxotrideca-(9,11)-dienoic acid and a putative C_5 alkane (*39*).

1.4 Synthesis of deuterium labelled fatty acids

1.4.1 Deuterium labelling

Isotopically labelled fatty acids are extremely useful in the study of biochemical pathways and in research concerning enzymatic mechanisms (*40-42*). Monitoring the transformation products by mass spectrometry gives information whether or not the isotope label is involved in the enzymatic reaction, and requires minimal amounts of substance.

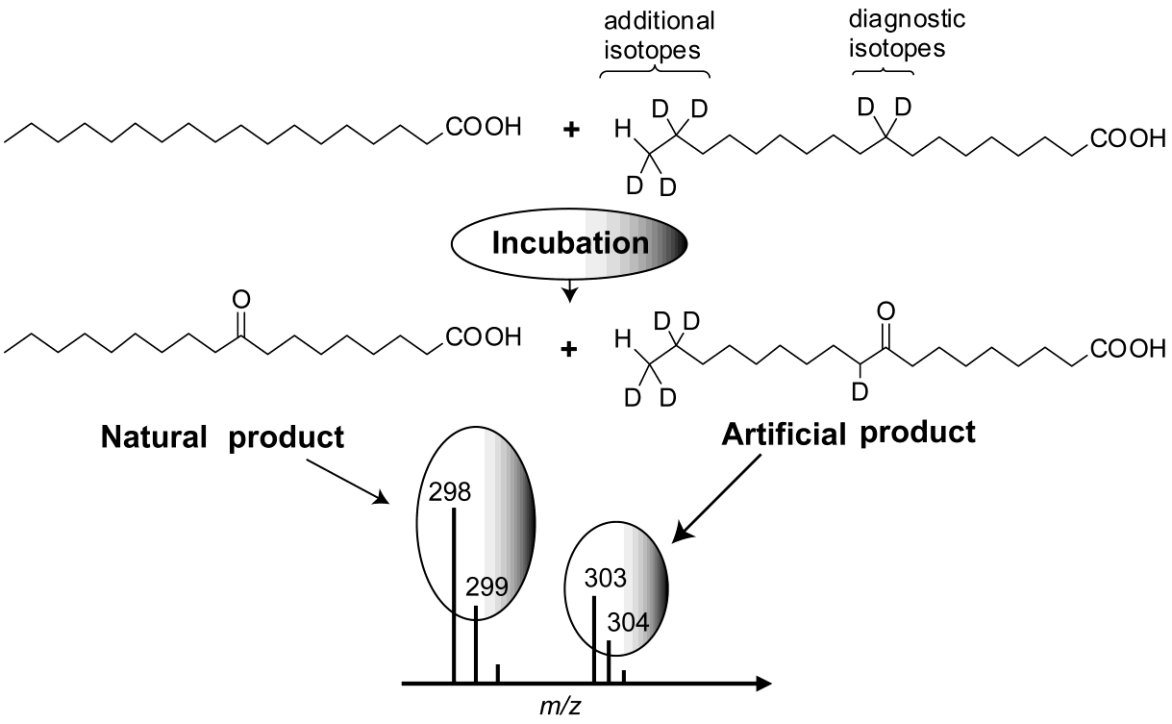


Fig. 11 The use of deuterium loading to discriminate natural and artificial products in biosynthetic experiments, adapted from (*43*)

In order to separate the contribution to mass spectra of natural products and metabolites of externally added precursors it is advisable to label the probe with isotopes that will not take part in the reaction - so called deuterium loading (Fig. 11) (*43*). It is crucial that the labelling procedures are very accurate, because errors in isotope position or degree of incorporation could falsify the interpretation of the results. A convenient way for deuterium loading is to introduce triple or double bonds, and to saturate them with deuterium. Heterogeneous catalysis is not recommended, because the unsaturated bonds of the molecules, adsorbed on the solid substrate, can undergo isomerisation and partial deuteration, resulting in deuterium scattering (*43*). This problem does not occur in homogeneous catalysis, because the 1:1 ratio of molecules of gas and unsaturated substrate at the reaction site is determined by the coordination complex formed with the transition metal of the catalyst (*44*). Stereochemistry plays an important role in fatty acid metabolism, as tested in the 5*S*-lipoxygenation of arachidonic acid in rat leukemic cells with a deuterated substrate (*40*).

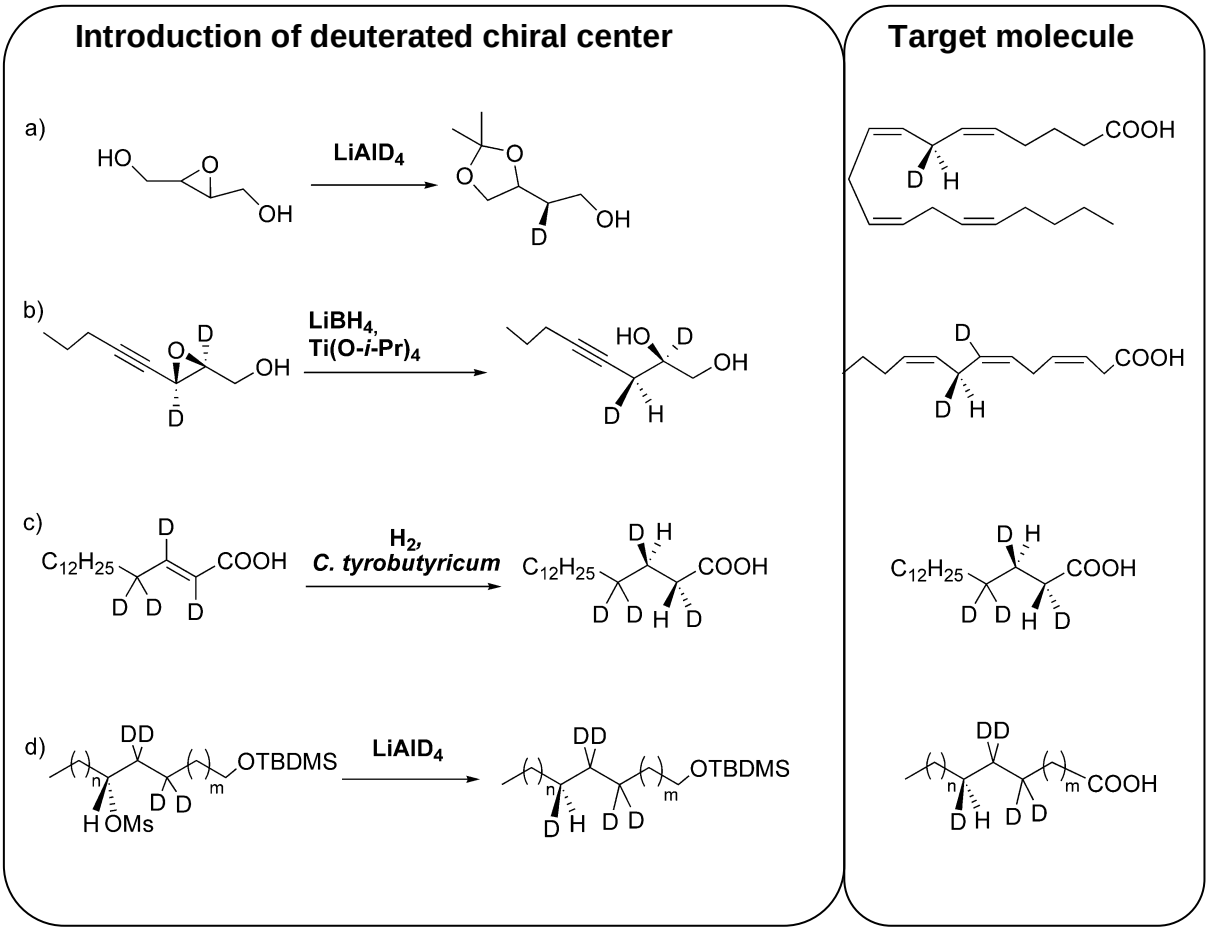


Fig. 12 Different synthetic approaches to stereospecifically labelled fatty acids: a) opening of a C_2-symmetric epoxide by $LiAlD_4$; b) opening of a deuterated epoxide by $LiBH_4$ and Ti tetra-*iso*-propoxide; c) enzymatic reduction of a deuterated α,β-unsaturated acid; d) nucleophylic substitution of a chiral alkyl mesylate by $LiAlD_4$.

Therefore the need for stereospecifically labelled polyunsaturated fatty acids has favoured the development of different synthetic strategies for these substances. A concept of Corey is based on the opening of a diol epoxide with C_2 symmetry with lithium aluminium deuteride (Fig. 12a). In the synthesis of (7*R*)-[7-$^2H_1$]-arachidonic acid, for example, the vicinal diol obtained with this method was protected as an acetal and the remaining alcohol group was transformed, *via* several steps, into a phosphonium salt. The fatty acid was obtained by Wittig olefination (*40*).

A different approach using Sharpless stereospecific epoxidation allows, however, higher enantiomeric excess, up to 95 % (Fig. 12b) (*45*). In the synthesis of stereospecifically labelled tridecatrienoic acid (*20*), a deuterium labelled epoxide was prepared with this reaction. After that, stereospecific ring opening with lithium borohydride and titanium tetra-*iso*-propoxide gave a stereospecifically deuterated functionalised diol. The latter was then hydrogenated and oxidised to obtain a carbonyl precursor for Wittig olefination. The sequence of Sharpless epoxidation and stereoselective ring opening has been applied also to the stereospecific synthesis of both enantiomers of [15,16-$^2H_2$]-arachidonic acid (*46*). In this case the stereospecifically deuterated carbonyl precursor for the aliphatic terminus of the acid was transformed in a C_6-phosphorane and used for the olefination of the C_{14} oxoester methyl 14-oxo-(all-*E*-5,8,11)-tetradecatrienoate. An efficient strategy involving a biocatalytic reduction has been carried out with broken cells of *Clostridium tyrobutyrricum* in presence of hydrogen and a cofactor. This organism has an enzyme system consisting of a hydrogenase and an enoate reductase which is able to transfer two hydrogen atoms to the double bond of an α,β-unsaturated acid with enantiomeric excess much higher than 95 % (Fig. 12c) (*47*). The product can be reduced to aldehyde and olefinated in order to synthesize the unsaturated fatty acid. Unfortunately the production of these cells, which requires industrial fermentation and strong safety measures, has been discontinued, preventing the use of this biocatalytic reduction. Stereospecific labelling with deuterium can also be achieved by mesylation of an optically pure secondary alcohol followed by substitution with lithium aluminium deuteride. In the case of chiral deuterated palmitic acid, for example, at first a racemic secondary alcohol was resolved by lipase-catalysed kinetic resolution. Next mesylation and reaction with lithium aluminium deuteride on the complete fatty acid scaffold afforded the labelled product, that was used in the study of desaturase stereochemistry (*48*) (Fig. 12d).

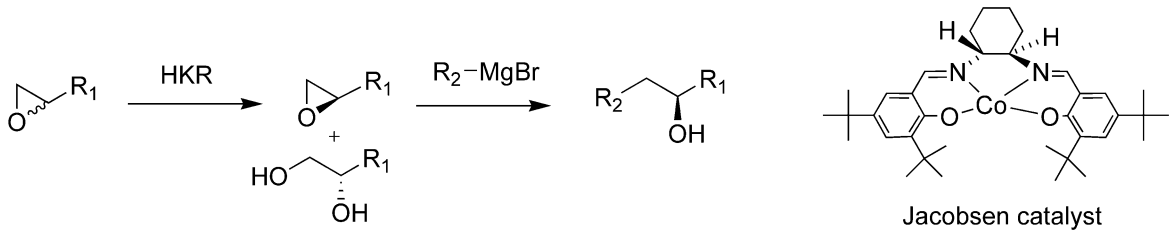


Fig. 11 Synthesis of enantiopure secondary alcohols by means of hydrolytic kinetic resolution (HKR) and Grignard alkylation.

This approach is particularly interesting because since the development of the hydrolytic kinetic resolution of epoxides, a straight forward access to optically pure secondary alcohols has become available. The resolution is based on the reaction of water and racemic substrate in presence of a cobalt-containing chiral catalyst and provides large scale amounts of functionalised epoxides with enantiomeric excess higher than 99 % (*49*). Regiospecific opening of the optically pure epoxide by a Grignard reaction catalysed by Cu (I) affords the secondary alcohol, a versatile chiral functionalised intermediate, in only two steps and large scale amount, as illustrated in Fig. 13 (*50*).

1.4.2 Polyunsaturated fatty acids by Wittig olefination

The first syntheses of polyunsaturated fatty acids, which were lengthy and produced mixtures of isomers, were based on a sequential acetylenic homologation followed by a partial reduction of the polyyne system (*51*). A versatile alternative to obtain *Z*-polyolefines is the Wittig reaction in Lithium-salt-free conditions. In order to generate the ylide, bulky bases sodium or potassium hexamethyldisilylamide are used because of their low nucleophilic and highly basic character. Combination of these conditions with low temperatures results in a *Z*-stereoselectivity up to 98 % (*52*). The olefination can be used to introduce a stereocenter in the aliphatic terminus of the target polyunsaturated fatty acid; this requires a chiral precursor containing either a carbonyl or an ylide. One example is the synthesis of both enantiomers of [15,16-$^{2}H_2$]-arachidonic acid mentioned before (see 1.4.1.) (*46*). The fatty acid was obtained *via* olefination of 14-oxo-(all-*E*-5,8,11)-tetraenoate with stereospecifically deuterated hexylidenetriphenyl phosphorane (Fig. 14a). The polyunsaturated ω-oxoester was prepared in five steps from arachidonic acid starting with the regioselective epoxidation of its Δ14 double bond (*53*). In a complementary approach, four additional steps afforded a C_{14} triphenylphosphonium acid, that was used in the preparation of (16*R*)-hydroperoxy arachidonic acid (*54*). In this case the precursor for the aliphatic terminus of the fatty acid was the chiral C_6 aldehyde (2*R*)-2-benzoyloxy-

hexanal. More recently a route to methylene skipped polyunsaturated fatty acid based on double-Wittig olefination was developed (*55*) (Fig. 14b). The use of an unsaturated bis-phosphonium salt allows sequential coupling with two different carbonyls and the formation of a triene in a one-pot reaction. Although the products deriving from the symmetric coupling, that is an olefin and a diester, are also formed, they can be easily separated from the product of non-symmetric coupling by column chromatography, because of their different polarity. The extent of the desired coupling can be improved with a modified procedure, called reductive olefination. In such a procedure the precursor for the aliphatic terminus of the fatty acid is an ester rather than an aldehyde, which is reduced *in situ* by diisobuthylaluminium hydride. Controlled thermal decomposition of the aluminium alkoxide complex slowly releases the carbonyl which reacts with the more reactive bis-ylide, rather than with the monoylide intermediate.

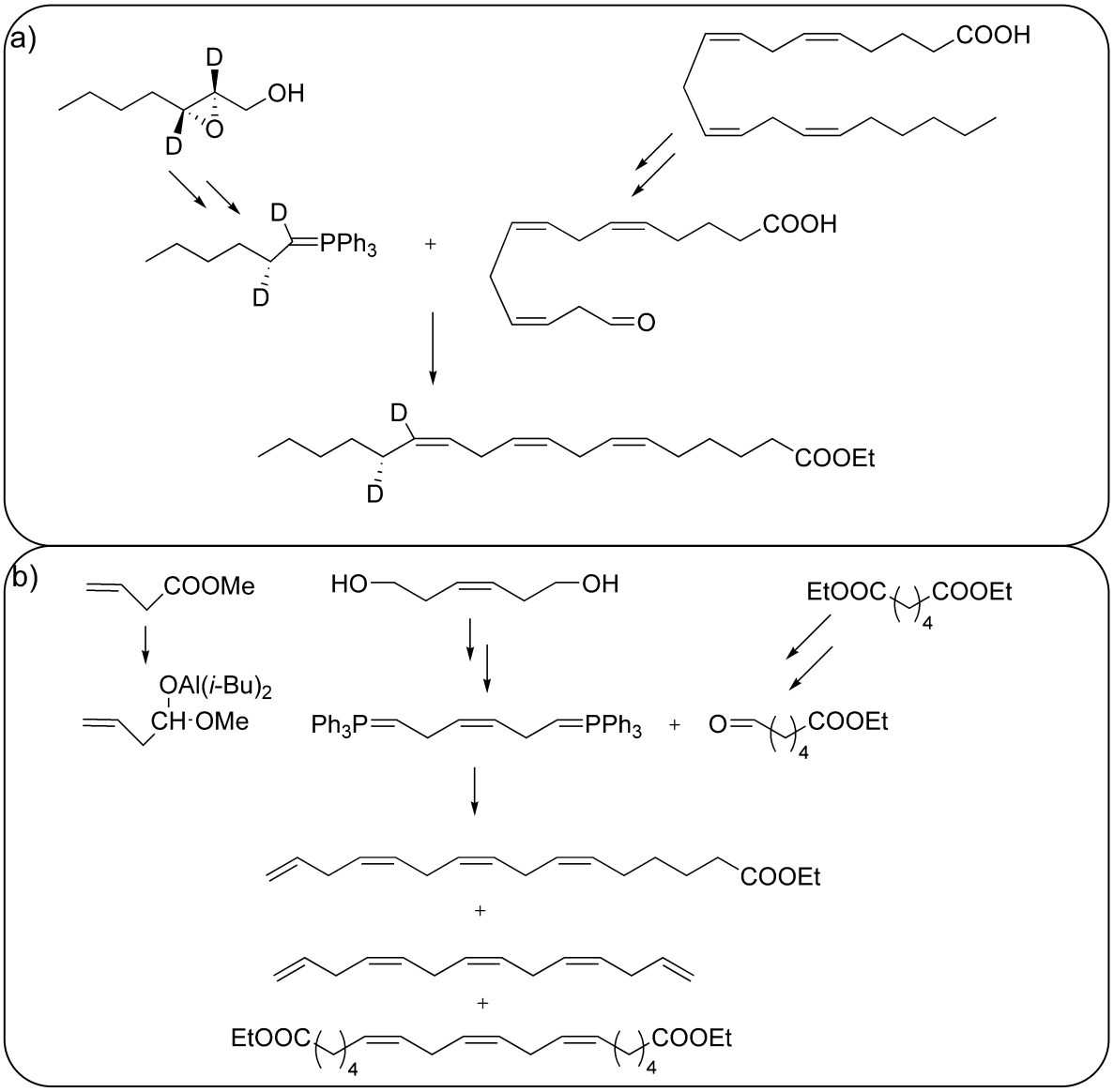


Fig. 14. Two examples of a) Wittig olefination and b) double-Wittig olefination.

In general, although the yield of a double Wittig olefination is lower than that of the normal Wittig approach, the carbonyl precursors do not need to have multiple double bonds; that facilitates their preparation. For the same reason the overall synthesis usually requires less purification steps. For example the Wittig salt bis-(1,6-triphenylphosphonium)-(*Z*)-hex-3-enyl halide can be prepared in only two steps from 1,6-(*Z*)-hex-3-endiol and is available in multi-gram scale (*55*). The double-Wittig approach was applied to the preparation of 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid, a polyunsaturated fatty acid found in diatoms (*56*) (Fig. 14b). In this case the precursors of both the carboxylic and aliphatic termini of the hexadecatetraenoic acid are easy to access.

1.5. Analysis of volatiles and oxylipins with GC/MS

1.5.1 Analysis of volatiles and chiral chromatography

Direct solvent extraction of C_{11} hydrocarbons in pentane is possible, but results in partial loss of the most volatile components during solvent evaporation. For this reason it is better to trap the volatile organic compounds present in the gas phase by adsorption on a solid material, with procedures similar to that used for the analysis of airborne pollutants. The closed loop stripping method consists in pumping the air present over the cell suspension (headspace) through a solid adsorbent with large specific surface, usually charcoal (*57*). As the air is circulated for several hours through a closed circuit, volatile components progressively adsorbe on the solid substrate. The system can be miniaturised, so that elution with a minimal amount of solvent, in the order of microliters, provides a solution of volatiles suitable for analysis with GC/MS (Fig. 14) (*58*). This system provides a time average sampling of the whole emission period, usually 12 or 24 hours, so that trapping is ensured also in case that no volatile emission should take place in the first hours. Solvent extracts can be stored and used for different kind of analyses as well as for development of the analytical metod, for instance chiral gas chromatography, and also for chemical degradation and hydrogenation. The latter two, for example, have been useful in the determination of absolute configuration of ectocarpene. An alternative method, called solid-phase microextraction (SPME), allows direct insertion of the sampling device in the gas chromatograph, resulting in low detection limits and shorter extraction times (*59*). This technology has found several applications in environmental, food, and medicine related analysis (*60, 61*). In this case the trapping material is a polymer similar to the inner coating of capillary columns for gas chromatography, deposited in a 10-100 µm thick layer on a glass fibre support.

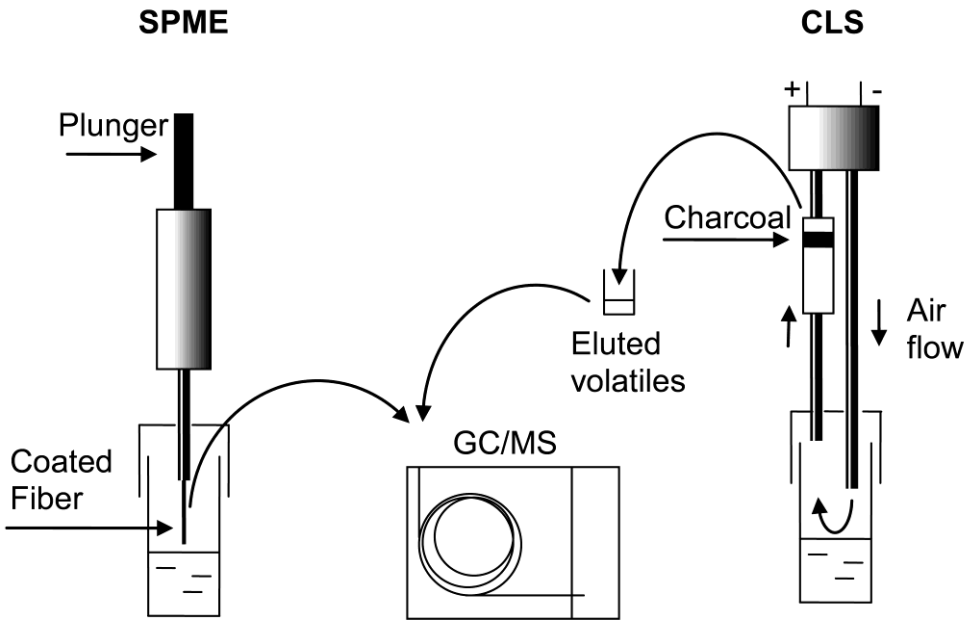


Fig. 15 Trapping headspace volatiles with solid phase microextraction (SPME) and closed loop stripping.

The fibre is incorporated into a syringe-like holder, consisting of a plunger and a protective needle. As illustrated in Fig. 15, the sampling material is exposed to the sample by pushing the plunger out of the needle, so that extraction time can be controlled accurately. By thermal desorption in the hot GC inlet the collected substances are cleared from the fibre and transported to the analytical column by the carrier gas. Although this method does not allow storage of the collected samples, it is useful for the analysis of small amount of volatiles, and has the advantage that the sampling time is in the order of minutes. SPME has been used, for example, in the chiral analysis of the pheromones of *Laminaria digitata* (*62*) and of deuterium labelled hydrocarbons in the diatom *G. parvulum* (*25*).

The optical purity of ectocarpene is known from optical rotation measurements, whereas its absolute configuration has been determined by selective hydrogenation followed by ozonolysis and comparison with butylsuccinic acid (*13*, *63*). However, optical rotation measurements cannot provide an accurate value of the enantiomeric purity. A powerful method of chiral analysis, instead, consists in gas chromatography on a chiral stationary phase. This technique is currently the method of choice for the analysis of enantiomers of volatile natural products, as it requires a minimal amount of substance and provides accurate information even on mixtures with high enantiomeric excess.

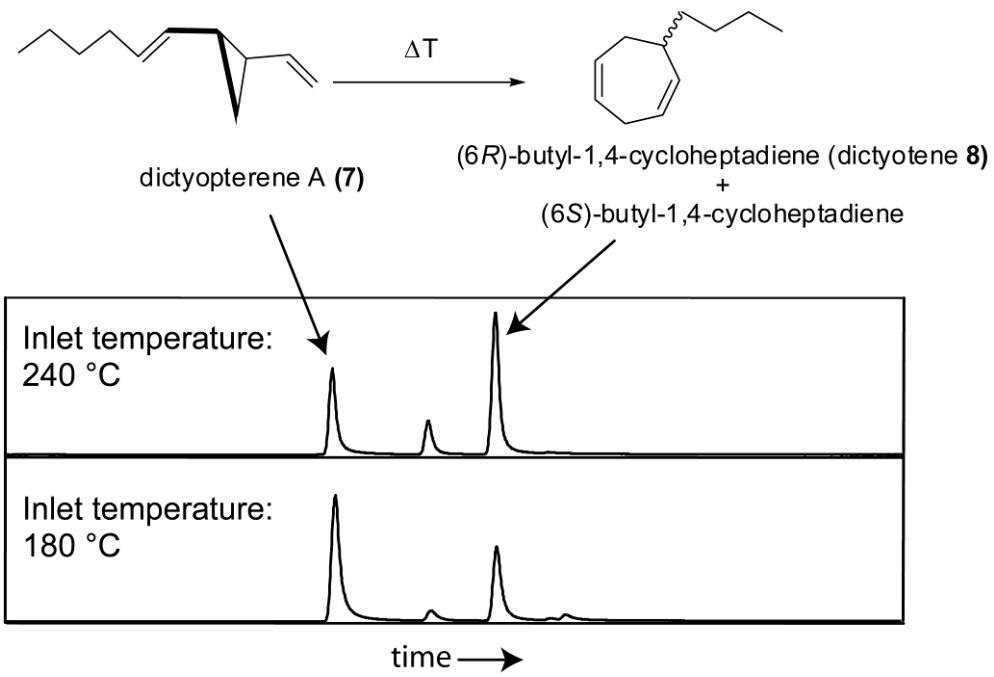


Fig. 16 Thermal rearrangement of substituted *trans*-1,2-divinylcyclopropane to substituted cycloheptadiene and assignment of GC peaks by injection at different inlet temperatures.

The stationary phase is based on modified cyclodextrins, differently functionalised at their hydroxyl groups. The substance class to which the chiral column performs better depends on the size of the polysaccharide ring as well as on the position and nature of the functional groups (*64*). In the case of C_{11} hydrocarbons, for instance, good results are possible with β- and γ-cyclodextrins with different functionalisation. Among these substances, analyses of structures containing cyclopropane, cyclopentane and cyclohexane rings result in baseline separations (*65*). On the contrary, substituted unsaturated cycloheptadienes are difficult to separate and a good result is reported only with two stationary phases: with a 25 meter column coated with (2,6-di-*O*-methyl-3-*O*-pentyl)–β-cyclodextrine diluted with polysiloxane (*66*) and with a 50 m column of pure (6-*O*-methyl-2,3-*O*-pentyl)-γ-cyclodextrine (*67*). Regarding the analysis of *trans*-1,2-bis-alkenylcyclopropanes it is important to mention that a temperature promoted rearrangement can take place. In this case the reaction is not a sigmatropic rearrangement like for the *cis*-isomers, but occurs through a diradical intermediate. Therefore the optical purity of the precursor is lost in the resulting cycloheptadiene (*68*). Usually this transformation takes place in the hot GC inlet. In fact the relative area of the three membered ring and the seven membered ring structures resulting from the rearrangement can be measured in the chromatogram (Fig. 16). However due to the short time spent by the sample in the GC inlet, it is possible to set the temperature at such a value that no significant rearrangement occurs. On the contrary, if a

high temperature is chosen, almost complete rearrangement is achieved, providing a mixture of enantiomers of the cycloheptadiene substance. These are useful for method development in chiral chromatography and for the identification of cyclopropane pheromones.

1.5.2 Analysis of oxylipins

Aldehydes from natural matrices have received attention in analytical chemistry because of their relevance as markers of cell injury and as off-flavours of food (*69*). In chemical ecology aldehydes are generally investigated because they occur as degradation products of plant oxylipins. These compounds, which derive from the oxygenation of polyunsaturated fatty acids, mediate physiological response of plants to stress and are markers of the activation of plant induced defenses (*38*). Additionally a role of aldehyde compounds in diatom chemical defense has been reported in marine environment (*70*). As a consequence analytical methods applied to plant tissues and marine water samples (*71, 72*) have been developed. As the carbonyl function reacts easily with substances containing amino groups present in the biological sample, like the lysine residues of proteins, their extraction is facilitated by previous *in situ* derivatisation with trapping reagents. This prevents further reaction of the aldehydes and provides useful properties for extraction or detection, like, for example, hydrophobicity and UV absorption.

The derivatising agent pentafluorobenzyl hydroxylamine (PFBHA) has the required properties: it reacts readily with aldehydes even in aqueous environment (*73*) and it increases the hydrophobicity of the molecule. Moreover, the *O*-(2,3,4,5,6)-pentafluorobenzyl oximes (PFB-oximes) can be selectively detected by negative ion mass spectrometry due to the electronegative fluorinated moiety. Instead, with the classical method of electron impact mass spectrometry of positive-ions (EI-MS), they provide a charachteristic fragment at m/z 181 as illustrated in Fig. 17. This feature is used to extract the signal of aldehydes or ketones from the chromatogram (extracted ion chromatogram). Due to the formation of *E/Z* isomers of the oxime derivatives, chromatograms often show twin peaks with nearly the same mass spectra, which simplify the identification of aldehyes and ketones in complex mixtures. An additional advantage in the analysis of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes is the formation of a pyridinium ion with a diagnostic peak in the MS spectrum (*69*) (Fig. 17).

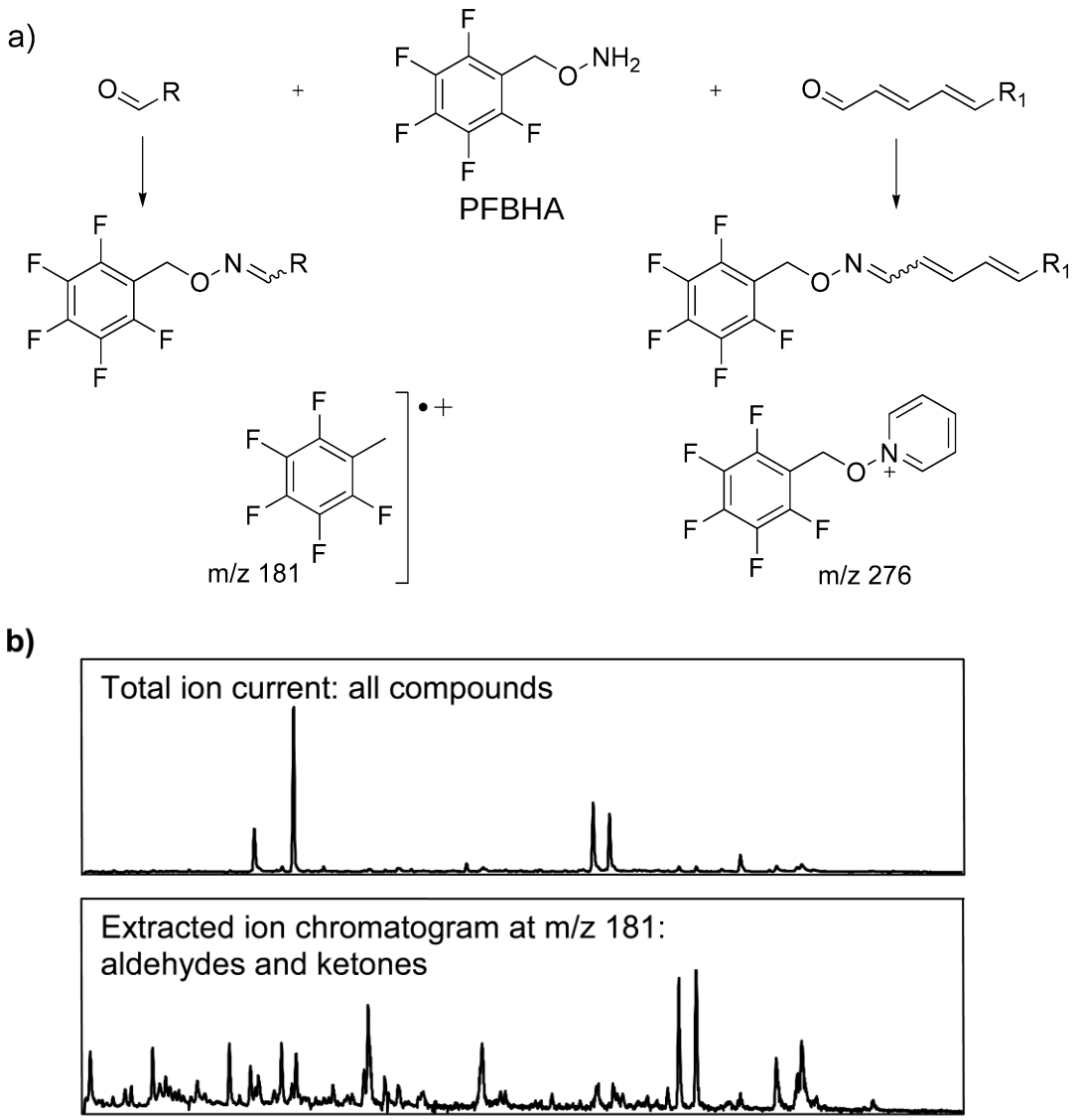


Fig. 17 Derivatisation of carbonyl compounds with PFBHA. a) Characteristic ions of PFBHA derivatives of aldehydes in general (m/z 181) and of α,β,γ,δ-unsaturated aldehydes (m/z 276). b) Extracted ion chromatogram of the signal of carbonyl compounds at m/z 181.

In the analysis of oxoacids, due to the presence of a carboxylic function, an additional derivatisation step is added in order to increase the volatility of the compounds for GC analysis. This can be obtained either by silylation or by methylation of the carboxyl group. In the case of biosynthetic studies, it is necessary to use deuterium labelling in order to establish the precursor role of a certain oxylipin metabolite. Incorporation of the labelled precursor into the oxylipin product *in vivo* can be established by mass spectrometry and provides evidence for the postulated pathway. This approach was used in the identification of 9-oxonona-(5Z,7*E*)-dienoic and 13-oxotrideca-(5*Z*,8*Z*,11*E*)-trienoic acid respectively in *G. parvulum* and *A. formosa* (*23, 24*). The compounds were identified with synthetic references and by comparison with the labelled analogues after incubation with labelled precursors.

2. Aim of the project

Certain brown algal species are known to emit pheromones as defined enantiomeric mixtures (*10*), while others release enantiomerically pure compounds (*9*). Most of these substances are unsaturated hydrocarbons that have eleven carbon atoms deriving from the aliphatic terminus of polyunsaturated fatty acids. Stereochemical studies in organisms used as models of C_{11} hydrocarbon biosynthesis in brown algae showed that the abstraction of (bis)allylic hydrogen from the fatty acid precursors is a key step of the process (*20, 25*), and biosynthetic studies have established that fatty acid oxygenation takes place in diatoms (*22, 24*). The aim of this project is to study the stereochemical course of pheromone biosynthesis and the occurrence of fatty acid oxygenation in a brown alga that produces pheromones with high optical purity. *E. siliculosus* is a suitable physological model for brown algae (*1*) because it is known to produce C_{11} hydrocarbon ectocarpene with high optical purity (*13*).

The model biosynthetic pathway established in the diatom *G. parvulum* showed that eicosapentaenoic acid is transformed into a highly reactive hydroperoxy intermediate by a 9-lipoxygenase; this intermediate is subsequently cleaved by a hydroperoxyde lyase to give hormosirene (**3**) and the 9-oxoacid **29** (*24*). Stereochemical studies with labelled arachidonic acid clarified that the hydrogen abstraction from the C-16 position of the C_{20} polyunsaturated fatty acid occurs with high *pro-R* stereoselectivity (*25*), although the released *trans*-bisalkenylcyclopropane **7** has low enantiomeric excess. In the plant *Senecio isatidaeus,* in contrast, *pro-R* abstraction of the bisallylic hydrogen in C-8 position of dodeca-3,6,9-decatrienoic acid results in the enantiopure hydrocarbon ectocarpene (**5**) (*20*). This substituted 1,4-cycloheptadiene is likely to originate by the Cope rearrangement of a termolabile pre-ectocarpene (**4**), as was established for the same substance released by *E. siliculosus* (*67*). It is believed that the same general mechanism found in the diatom combined with a different conformation of the fatty acid in the active site of the enzyme explains the biosynthesis of enantiopure *cis*-bisalkenylcyclopropanes in algae (*67*).

Given these findings, the same stereospecificity of *pro-R* hydrogen abstraction from the precursor is also expected in the model brown alga *E. siliculosus*, where ectocarpene (**5**) shows high optical purity. The enantiomeric excess of substituted 1,4-cycloheptadienes **5** and **8** will be measured with chiral gas chromatography, in order to confirm the available data obtained with less powerful optical rotation analyses (*13*). In addition, the stereochemical course will be studied by incubating living female gametes with the

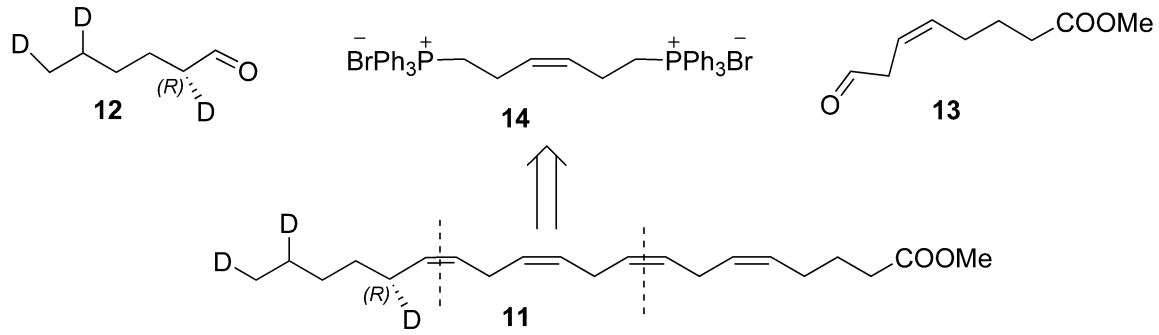
stereospecifically deuterated precursor (16*R*)-[16,19,20-$^{2}H_{3}$]-arachidonic acid (16*R*)-**28**), and subsequent use of mass spectrometry to analyse the labelled metabolites. The labelled substrate will be prepared *via* a double Wittig approach. The enantiomerically pure, deuterated (2*R*)-[2,5,6,]-hexanal (**13**) will be used as precursor of the aliphatic terminus of the fatty acid. After incubating gametes with the labelled fatty acid precursor (16*R*)-**28**, analysis of the labelled hydrocarbons produced by gamete suspensions should establish whether the diagnostic label is abstracted.

The lipoxygenase/hydroperoxyde lyase activity has been established only for diatoms (*24*, *74*). In order to provide evidence for fatty acid oxygenation in brown algae, an additional incubation experiment will be conducted. Algal gametes will be incubated with octadeuterated arachidonic acid, labelled at each double bond. The analysis of deuterated metabolites will be carried out in both the headspace and the homogenate of gamete suspensions, and in addition to hydrocarbons, also carbonyls will be searched for. Derivatisation with pentafluorobenzyl hydroxylamine is expected to trap these reactive substances, which should be identified by comparison with diatom metabolites.

3 Results and discussion

3.1 Synthesis of (16*R*)-16,19,20-[$^2H_3$]-arachidonic acid.

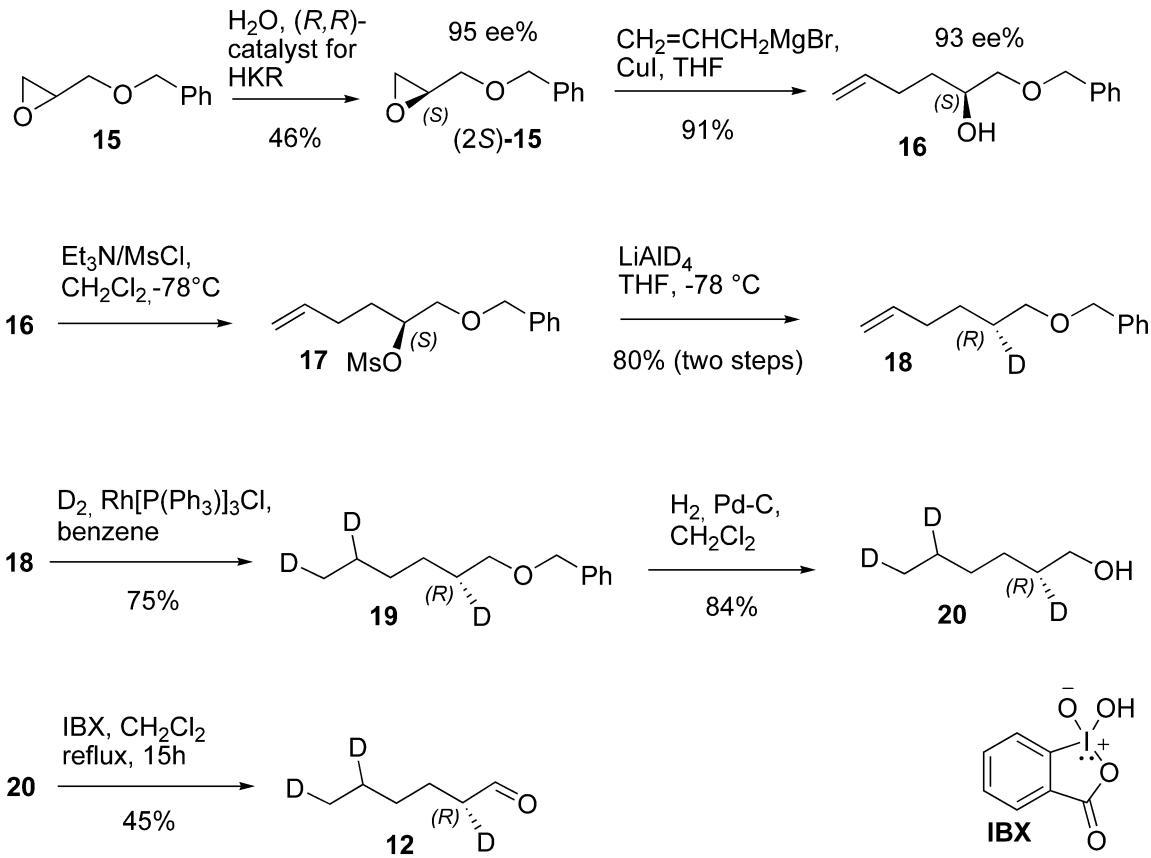
The tetraenoic acid scaffold was obtained by means of a double-Wittig olefination, which allows a straightforward route to labelled polyunsaturated fatty acids with only a few purification steps (see 1.4.2). Due to this approach, the synthesis of (16*R*)-[16, 19, 20-$^2H_3$]-arachidonic acid methyl ester **11**, could be divided in the preparation of two carbonyl precursors: (2*R*)-[2,5,6-$^2H_3$]-hexanal (**12**)**,** corresponding to the aliphatic terminus of the final fatty acid, and (5*Z*)-8-oxoocta-5-enoic acid methyl ester **13**, incorporated in the carboxylic terminus (Scheme 1). The *bis*-Wittig salt (3*Z*)-hex-3-ene-1,6-bis(triphenylphosphonium bromide) **11** was already available, and the general procedure for its preparation is described in literature (*55*). The synthesis of the carbonyl precursors and the olefination are discussed in the following paragraphs.



Scheme 1. Retrosynthetic analysis of (16*R*)-[16, 19, 20-$^2H_3$]-arachidonic acid methyl ester **11**.

3.1.1 Synthesis of (2*R*)-[2,5,6-$^2H_3$]-hexanal (12)

Asymmetric synthesis and deuterium labelling were applied only to the synthesis of the carbonyl precursor of the aliphatic terminus of the fatty acid. Hydrolytic kinetic resolution of 2-(benzyloxymethyl)oxirane **15** provided (2*S*)-**15** in large scale. The optical purity obtained, which was measured by chiral gas chromatography, was determined as ee = 95.3 ± 0.2 %. Regiospecific opening of the oxirane ring by allylic Grignard reagent in presence of Cu(I) gave (2*S*)-1-(benzyloxy)hex-5-en-2-ol **16** (Scheme 2). The enantiomeric excess of this mono-protected diol was determined by GC after derivatisation with 1-phenylethylisocyanate, a procedure developed for hydroxy fatty acids (*75, 76*). After derivatisation with optically pure and racemic 1-phenylethylisocyanate, the diastereoisomers formed could be separated by gas chromatography, as illustrated in Fig. 18.



Scheme 2. Synthesis of (2*R*)-[2,5,6-$^{2}H_3$]-hexanal (**12**) and structure of the oxidant IBX.

The sample derivatised with enantiopure reagent afforded a value of ee = 92.5 ± 0.2 %. This accounts for a minimal racemisation, which could not be explained, as the stereocenter at C-2 is not involved in the reaction. Nevertheless the optical purity was sufficient for the application to the incubation experiments and comparable with that of alternative methods (see 1.4.1). In the next step stereospecific labelling was achieved by mesylation of the free hydroxyl function of the diol **16** and nucleophylic substitution by lithium aluminium deuteride on the methylsulfonate intermediate **17**. This reactive compound was used without purification in order to avoid decomposition. The S_N2 mechanism for the displacement of alkyl tosylates by lithium aluminium hydride has been confirmed (*77*) and, accordingly, labelling should proceed with complete inversion of configuration at the stereocenter, as outlined in scheme 2. The displacement of secondary mesylates by lithium aluminium deuteride is a well established approach for the synthesis of deuterium labelled saturated fatty acids (*48*, *78*).

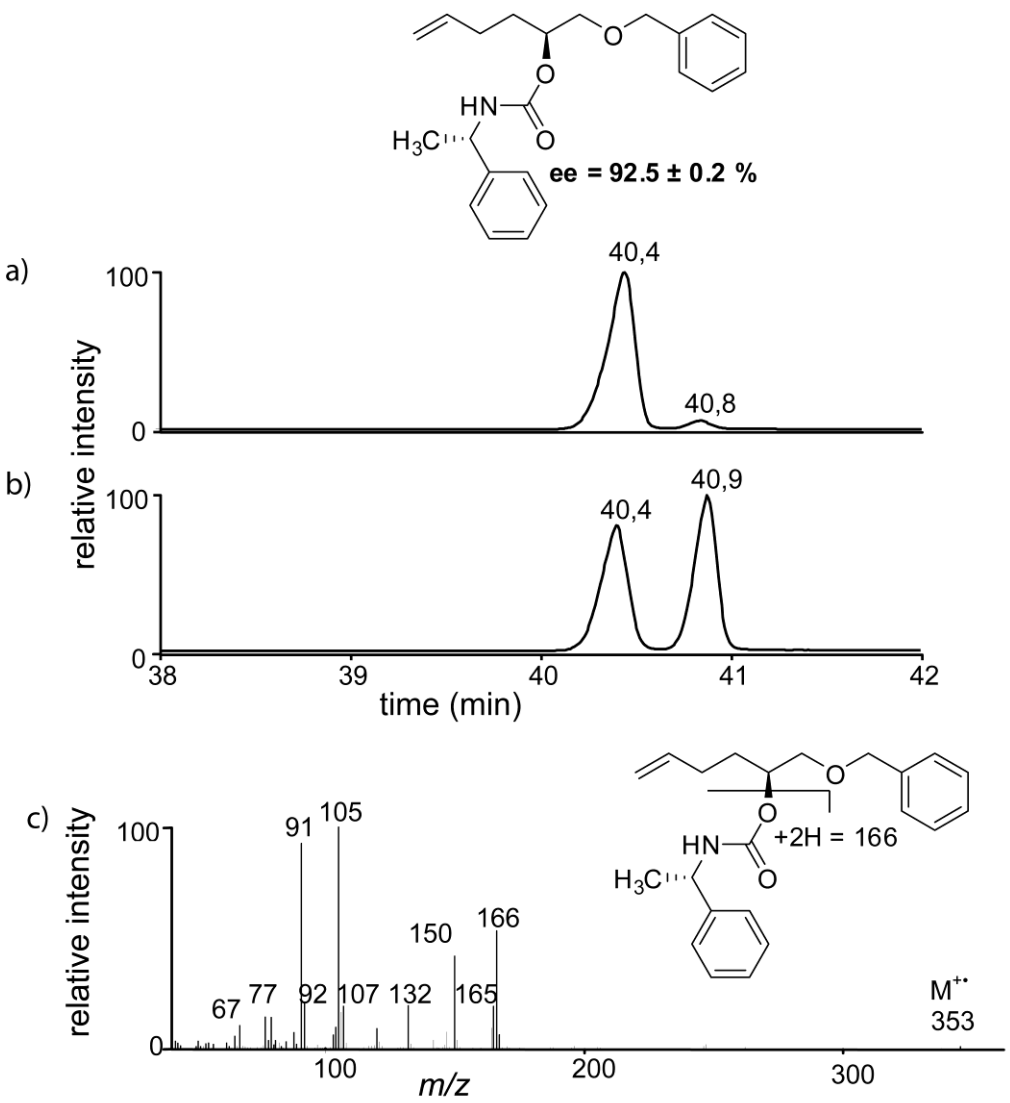
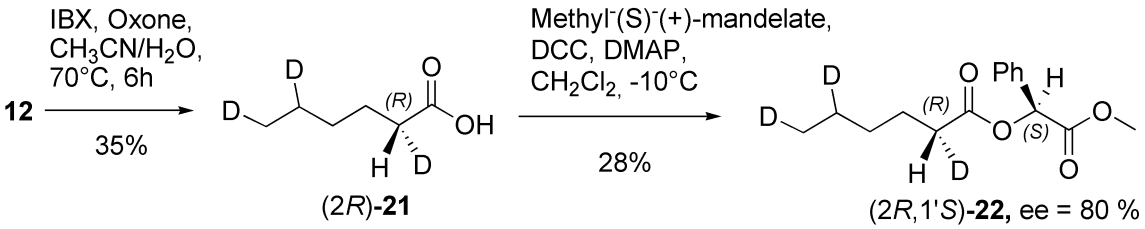


Fig. 18 Determination of the enantiomeric excess of (2*S*)-1-(benzyloxy)hex-5-en-2-ol (**16**). GC/MS of the carbamate formed after derivatisation of **16** a) with enantiopure (*S*)-1-phenylethylisocyanate and b) with racemic 1-phenylethylisocyanate. c) Representative mass spectrum of the chromatographic peaks.

After preparation of the stereospecifically labelled hex-5-enylbenzylether **18**, two additional deuterium atoms were required for the incubation experiments, in order to separate the labelled hydrocarbons from the background of natural products. These isotopic labels were introduced by homogeneous catalysis, which allows accurate deuteration of the double bond without deuterium scattering and isomerisation, which often occur at least partially with heterogeneous catalysts (see 1.4.1). Once the double bond was saturated, the benzyl ether **19** was cleaved by hydrogenolysis using palladium on carbon, which readily afforded (2*R*)-[2,5,6-$^{2}H_3$]-hexan-1-ol **20**. The subsequent oxidation step utilized 1-hydroxy-1,2-benziodoxol-3-(1H)-one 1-Oxide (IBX) in poorly dissolving solvents (*79*). The original protocol was adapted to the use of dichloromethane, a solvent that, due to its low boiling point, allows satisfying recovery of the volatile hexanal **12**.



Scheme 3. - Oxidation of aldehyde **12** and derivatisation for the determination of the enantiomeric excess by NMR.

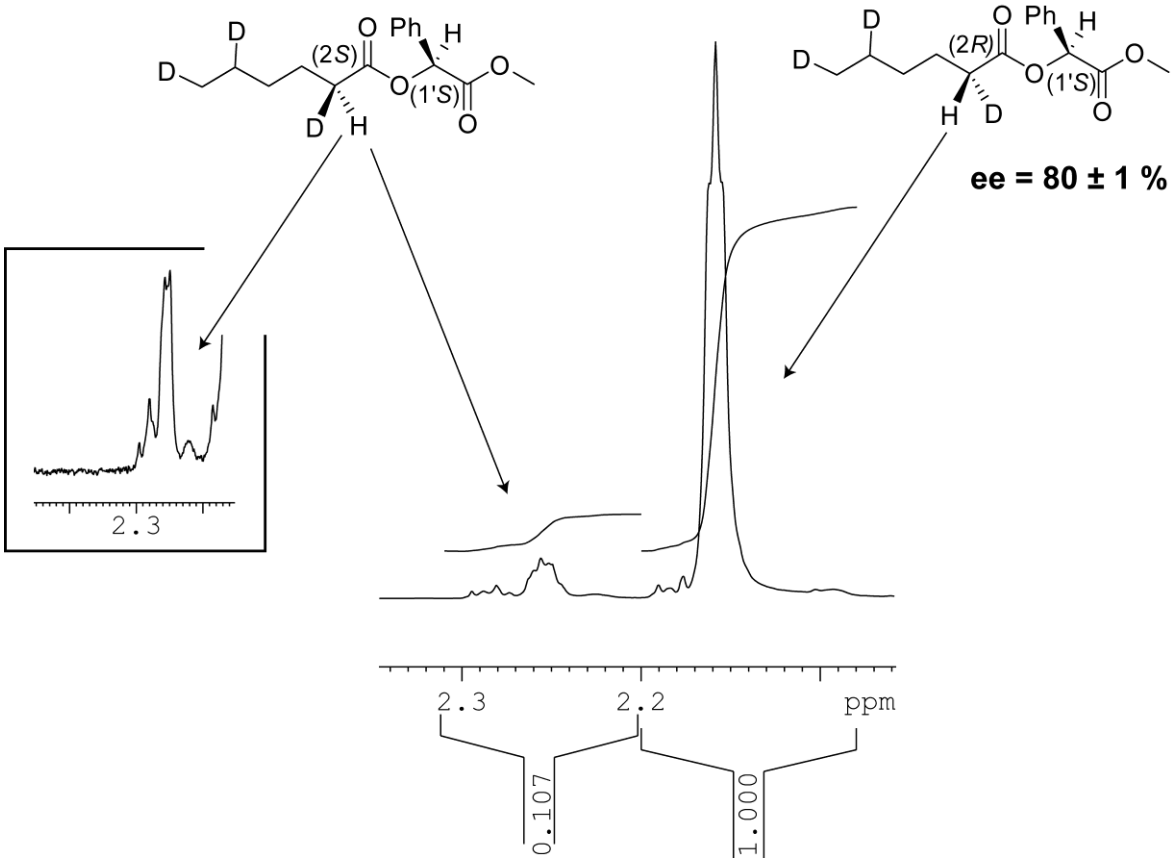


Fig. 19. ^{1}H NMR of the mandelate diester **22** for the determination of the enantiomeric excess. The protons in β to the carbonyl of the hexanoyl moiety were selectively decoupled. The error was estimated from the limit of detection of a diluted solution (spectum in the small frame).

Due to the lower temperature, the reaction was much slower than in the reference procedure and had to be run for several hours. Unexpectedly, after 24 hours a considerable amount of hexanoic acid **21** was observed with ^{1}H NMR.

As it was intended to measure the enantiomeric excess of **12**, this aliquot of the alcohol **20** was treated to complete the oxidation to carboxylic acid. After derivatisation with optically

pure (1*S*)-methyl mandelate, it was possible to separate the signals of the diastereotopic protons in α-position of the (2*R*,1'*S*) and (2*S*,1'*S*) diesters. The optical purity of the aldehyde **12** could not be determined by a chromatographic method. Indeed, since the enantiomers differing only for the substitution of one enantiotopic hydrogen with deuterium have similar chromatographic properties, they cannot be separated by this technique. Therefore in such cases the method of choice is ^{1}H NMR of the corresponding carboxylic acid derivatised with a chiral reagent. To complete the oxidation, a protocol involving the same oxidant IBX under different conditions was used. The hypervalent iodine reagent was used in catalytic amounts in the presence of the peroxysulfate Oxone® as a co-oxidant; a mixture of water and acetonitrile served as solvent (*80*). Esterification with methyl-(*S*)-mandelate was achieved with dicyclohexylcarbodiimide as condensing agent along with a catalytic amount of 4-dimethylaminopyridine in dichloromethane at low temperature (scheme 3). The prolonged reaction time in aqueous solution during oxidation of labelled hexanal **12** led to partial racemisation due to keto-enol tautomerism and the enantiomeric excess of **21** was considerably lower than that of **12**. As illustrated in Fig. 19, the two diastereoisomers display a chemical shift of ca. 0.1 ppm for the diastereotopic α-protons, provided that the β-protons are selectively decoupled (*81*). Therefore, by integration of the corresponding signals, it is possible to calculate the mole fractions and the diastereoisomer excess of **22**, hence the enantiomeric excess of labelled hexanoic acid **21**. The integrals of the enantiotopic α-protons at 2.31-2.20 ppm and 2.20-2.08 ppm were, respectively, $I_S = 0.11$, $I_R = 1.00$, thus the ratio of the enantiomers was (*R*): (*S*) = er = 90:10, and the enantiomeric excess was ee = 80 %. In order to estimate the error Δer, the uncertainty Δ*x* of the mole fraction *x* was estimated. Due to the fact that diastereoisomers have the same molecular weight, the mole fractions can be calculated either from the moles *n* or from the mass *m* (equations 1-3).

(1) $er = x_R \bullet 100$ (2) $ee = (x_R - x_S) \bullet 100$

(3) $$x_S = \frac{n_S}{n_R + n_S} = \frac{m_S}{m_R + m_S}$$

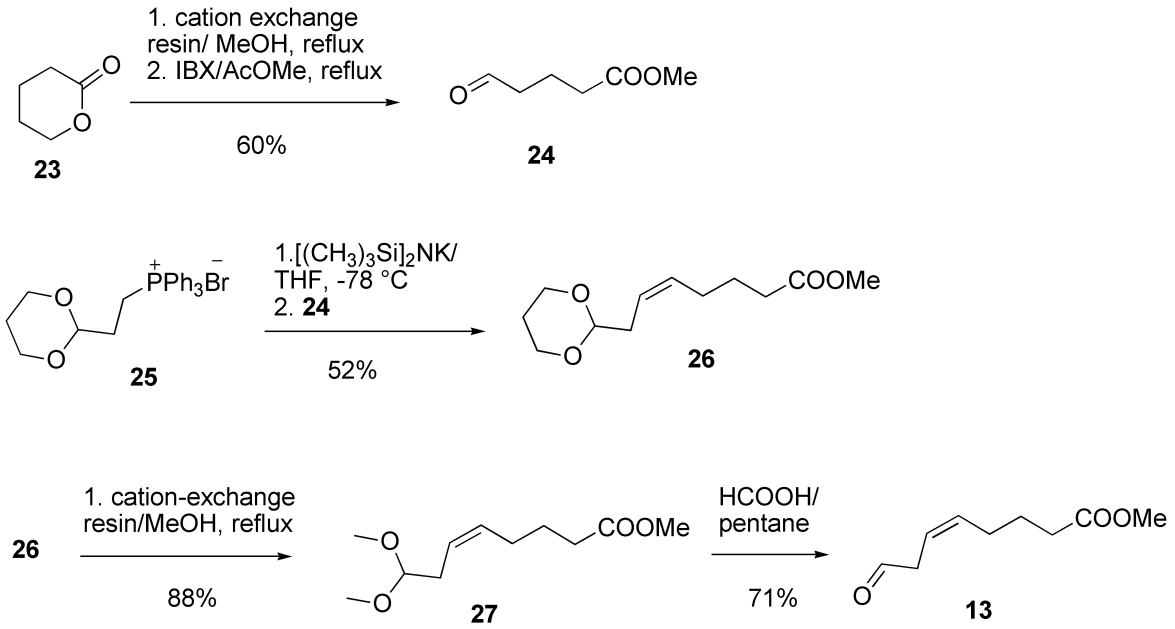
(4) $$\Delta x_S = \frac{m_{Sd}}{m_c}$$ (5) $$\Delta x_R = \frac{m_{Sd}}{m_c}$$ (6) $$m_{Sd} = \frac{m_c \bullet x_S}{d}$$

Uncertainty Δx was assumed equal to the minimal mass of substance m_{Sd} giving a signal distinguishable from the noise present in a diluted solution, divided by the total amount of substance m_c present in the concentrated solution used for ee determination (equation 4 and 5). Therefore the concentrated solution, containing a quantity of product m_c = 5 mg, was diluted d = 25 times and analysed. A signal to noise ratio S/N= 90 for the (*S*)-α-proton was set as suitable. The quantity m_{Sd} was calculated multiplying the amount m_c present in the concentrated solution for the mole fraction x_S and dividing by the dilution d (equation 6). Applying the relations described, the minimal detectable amount is m_{Sd} = 0.02 mg and the errors of the mole fractions are $\Delta x_S = \Delta x_R = 0.004$. Hence the uncertainty of the ratio is Δer = 0.4% and that of the enantiomeric excess is Δee = 0.8 %, which means that the enantiomeric excess of hexanoic acid **21** was measured with good accuracy. However, the method has systematic uncertainty, because the aqueous conditions necessary for the oxidation of (2*R*)-[2,5,6-$^2H_3$]-hexanal (**12**) to hexanoic acid **21** may have caused partial racemisation. The optical purity of the hexanal **12** is expected to be close to that of the secondary alcohol **16**, since the displacement of the mesylate by the lithium aluminium deuteride occurs with inversion of configuration at the stereocenter and the oxidation in water free conditions prevents racemisation by keto-enol tautomerism. In literature the enantiomeric purity of chirally deuterated fatty acids obtained from secondary mesylates and tosylates is usually obtained from that of the secondary alcohol precursor (*48, 78, 82*). For the oxidation of the remaining aliquot of labelled hexanol **20**, IBX was prepared on the same day in order to minimise water content, which could favour keto-enol tautomerism of the labelled hexanal and racemisation in α-position. Moreover the reaction was monitored at shorter time intervals and overoxidation was excluded in the first 15 hours. The isolated labelled hexanal **12** was used immediately for double-Wittig olefination.

3.1.2 Synthesis of (5*Z*)-8-oxooct-5-enoic acid methyl ester (13)

The precursor for the carboxylic terminus of the labelled arachidonic acid is the β,γ-unsaturated oxoester **13**. This building block has been used extensively in syntheses of arachidonic by Wittig olefination (*83*). In analogy to literature procedures the compound was obtained by C_3 homologation of 5-oxopentanoate (*83, 84*), but using reagents that can be removed by filtration like the oxidant IBX and cation-exchange resin, as outlined in scheme 4. First, commercially available δ-valerolactone **23** was hydrolysed with strong cation-exchange resin and oxidised with IBX, instead of Cr (VI)-based oxidant PCC, in one-pot procedure. Due to the low solubility of this reagent in most solvents, except

dimethylsulfoxide, the product can be recovered after simple filtration of the residual oxidant; chromatographic purification was not required. The resulting 5-oxopentanoic methyl ester **24** was olefinated with the ylide of 2-(1,3-dioxan-2-yl)-ethyltriphosphonium salt (**25**) (*85*). This C_3 homologating reagent afforded methyl 7-dioxanylhept-(5Z)-enoate (**26**), where a carbonyl function was protected as a cyclic acetal. Removal of the protecting group was achieved in two steps. Transacetalisation in methanol in presence of strong cation-exchange resin afforded methyl 8,8-dimethoxyoct-(5*Z*)-enoate ester (**27**). Then, acidic hydrolysis in a two phase system of pentane and formic acid led to (*Z*)-methyl 8-oxooct-5-enoate **13** (*86*). Using cation-exchange resin as acidic catalyst and formic acid, which is immiscible with pentane, allowed minimising isomerisation of the β,γ–unsaturated carbonyl so that the precursor **13** for the olefination was obtained in suitable yield and purity.

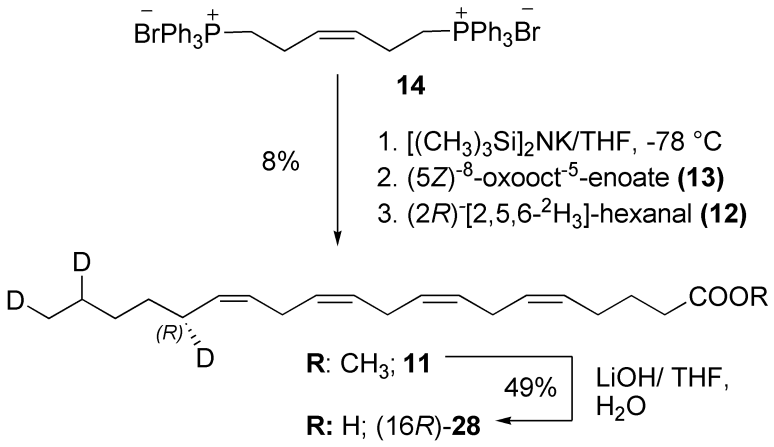


Scheme 4. Synthesys of the oxoester methyl 8-oxooct-(5*Z*)-enoate (**13**)

3.1.3 Double Wittig olefination

After preparation of the labelled carbonyl precursor **12** and of the unsaturated oxoester **13**, olefination with the bis(ylide) derived from (3*Z*)-hex-3-ene-1,6-bis(triphenylphosphonium bromide) **14** afforded labelled methyl arachidonate **11** (Scheme 5). As reported before (see 1.4.2), besides reducing the amount of synthetic steps, double Wittig olefination gives products of both symmetric and asymmetric coupling. Symmetric coupling reduces yield, on the other hand the resulting olefins have different polarities from the desired product

and they can be separated by chromatography. The labelled methyl ester was purified with normal phase HPLC affording a yield of 8 %. In order to minimise auto-oxidation of the free polyunsaturated fatty acid *(87),* the ester was hydrolysed in small batches with lithium hydroxide in aqueous THF. Due to the co-eluting impurities by 2,6-di-*tert*-butyl-4-methylphenol, further purification with reversed phase HPLC was necessary. The labelled arachidonic acid (16*R*)-**28** was obtained with an overall yield of 1 %. Wittig reactions require basic conditions for the formation of the ylide. Therefore an obvious concern in the case of carbonyl precursors with a stereocenter in α-position is keto-enol tautomerism and subsequent racemisation. Nevertheless, this undesired process was excluded in several analogous cases (*88*). Therefore the enantiomeric excess of (16*R*)-**28** should be the same as that of the chiral hexanal **12**. As discussed before (see 3.1.1), the optical purity of **12** could not be determined directly, but only estimated either from that of the unlabelled diol precursor **16** by chromatography, or from that of the oxidation product hexanoic acid **21** by NMR.



Scheme 5. Synthesis of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid [(16*R*)-**28**].

The enantiomeric excess of the fatty acid (16*R*)-**28** can be estimated between that of this aldehyde and that of the labelled hexanoic acid **21,** taking the last one as a lower limit, that is 92.5 ± 0.2 % ≥ ee > 80 ± 1 %. Another aspect of the characterization of the labelled arachidonic acid is the degree and position of deuterium incorporation. Therefore methyl ester **11** was analysed by high resolution mass spectrometry and GC/MS coupled with sector field mass analyser. Selected ion trace, corrected for the contribution of the ^{13}C natural isotopic abundance, provided a sharp isotopic pattern with a ratio d_2:d_3:d_4=1:98:1, the sum of the other isotopologues from d_0 to d_5 being below 1%. Despite of the low

yield, the product had good optical purity as well as high isomeric and isotopic purity, which are the major requirements for the experiments with algal gametes.

3.2 Analysis of volatiles and oxlipins

3.2.1 Determination of the enantiomeric excess of dictyotene in *E. siliculosus*

C_{11} Hydrocarbons of *E. siliculosus* were analysed with chiral gas chromatography. In order to establish the separation conditions, both enantiomers of 1,4-cycloheptadienes **5** and **8** were obtained by thermal rearrangement of *trans*-bisalkenylcyclopropanes **3** and **6**, which were produced by cultures of the diatom *G. parvulum* incubated with eicosapentaenoic or arachidonic acid (see 1.4.1). The process occurs within minutes, therefore volatile extraction with SPME was preferred to the CLS method because of the short sampling time. The same technique was used for the collection of algal volatiles which were extracted for one hour after complete gamete settling in three aliquots of cell suspension. The separation of ectocarpene (**5**) from its (6*R*)-enantiomer on chiral GC/MS proved to be challenging, and although different stationary phases and flow conditions were applied (*66*), the results were not sufficient for determination of the enantiomeric excess. The following analyses focused on the enantiomeric purity of the less unsaturated 1,4-cycloheptadiene **8**, which is present as a minor component in the volatile bouquet of *E. siliculosus*, and derives from arachidonic acid instead of eicosapentaenoic acid. As brown algae metabolise both fatty acids, it is assumed that the formation of the two hydrocarbons **5** and **8** occurs with the same stereoselectivity, as it was observed, for example, in the formation of *trans*-bisalkenylcyclopropanes **3** and **7** from the same precursors in *G. parvulum* (*24*).

The most satisfactory result was achieved with the stationary phase Hydrodex-β-6TBDM (Fig. 20). The peaks of dictyotene were baseline separated, and the peak area integration gave the enantiomeric excess ee = 95.8 ± 0.5 %. This is in accordance with the high optical purity reported for ectocarpene and dictyotene based on optical rotation measurements (*13, 14*), and provides an accurate value for the enantiomeric excess of dictyotene in *E. siliculosus.* Terefore the biosynthetic pathway in *E. siliculosus* proceeds highly stereospecific. The optical purity of the precursor of dictyotene, *cis*-(1*R*,2*R*)-1-hex-1'*E*-enyl-2-vinylcyclopropane (**6**)**,** must have the same enantiomeric excess, due to the stereospecificity of sigmatropic reactions.

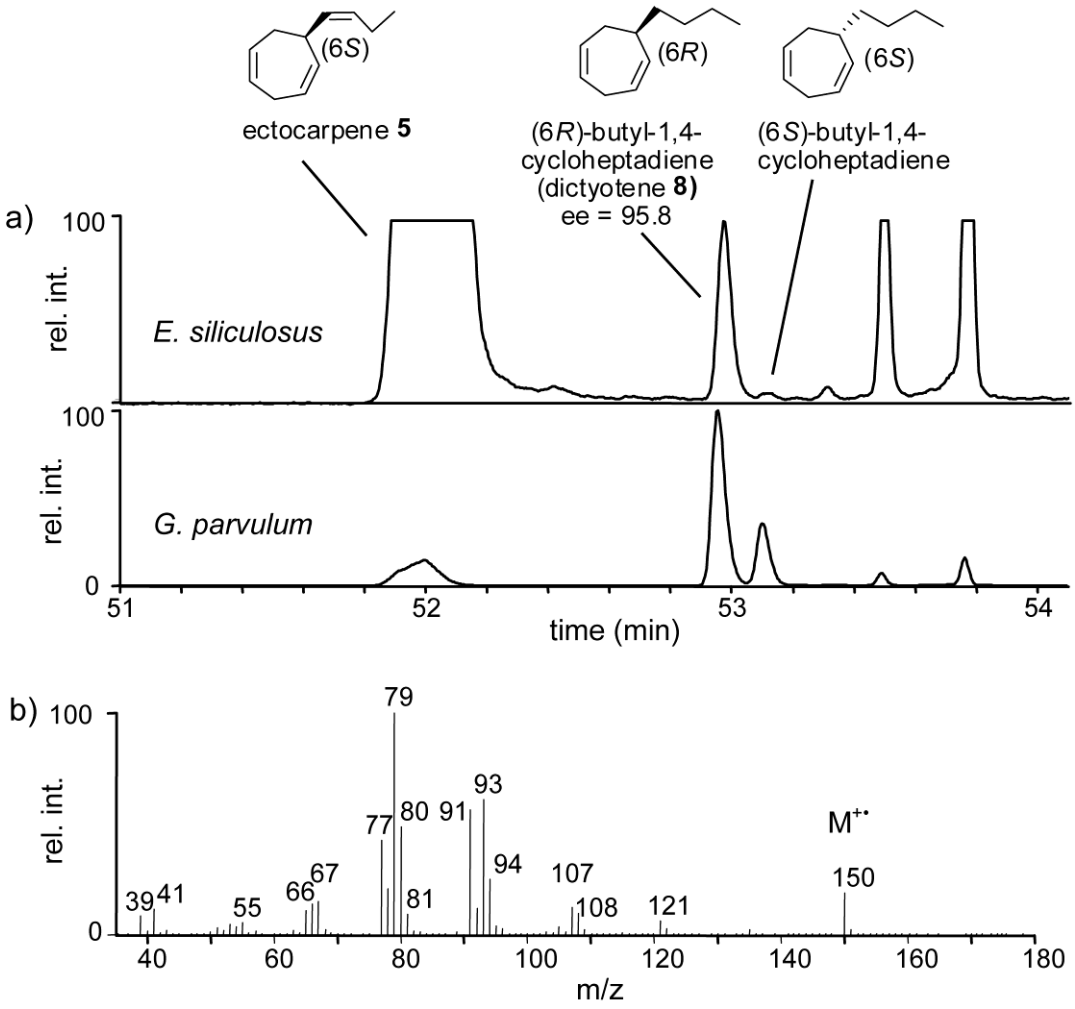


Fig. 20 Chiral chromatography of C_{11} hydrocarbons, ion trace m/z 150. a) Volatiles from a gamete suspension of *E. siliculosus* and homogenate of the diatom *G. parvulum.* b) Mass spectrum of dictyotene.

3.2.2 Stereochemical course of the enzymatic activity at C-16 of arachidonic acid

In order to investigate how the stereochemistry of C_{11}-hydrocarbons is established, gametes of *E. siliculosus* were incubated with labelled arachidonic acid (16*R*)-**28**. This substrate carries one diagnostic deuterium label at the C-16 position with an enantiomeric ratio 96 % ≥ er > 90 % (see 3.1.4), as well as two additional deuterium atoms at the end of the aliphatic terminus, in order to distinguish labelled metabolites from natural occurring volatiles (see 1.4.1). A small amount of concentrated solution of fatty acid (16*R*)-**28** in

dimethylsulfoxide (0.5% of the medium) was administered to suspensions of algal gametes, which assured that the solvent was not toxic for living cells (*15*).

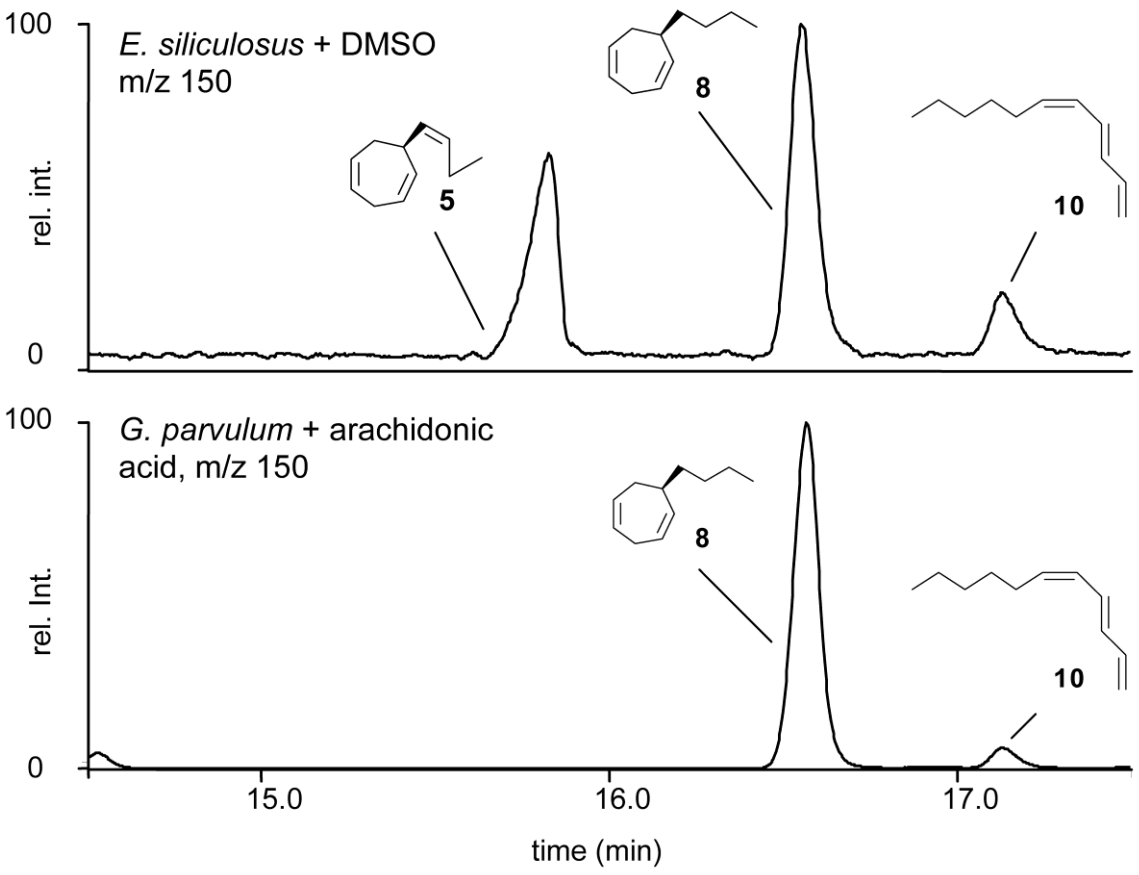


Fig. 21 Natural C_{11} hydrocarbons of *E. siliculosus* and of *G. parvulum*, ion trace at m/z 150.

The labelled substrate (16*R*)-**28** was present in excess, as it was shown by analysis of total fatty acids (*89*) in gametes at the end of a test incubation experiment. C_{11} hydrocarbons were identified by comparison with *G. parvulum* homogenates and injection at different inlet temperature (See 1.5.1). The typical volatile pattern of the extracted ion chromatogram at m/z 150, corresponding to the molecular mass of dictyotene **8**, is represented in Fig. 21. Ectocarpene **5** ($M^{+\bullet}$ + 2 peak), which is the main volatile component of the bouquet, does not interfere with the analyses of the other components. These are metabolites of arachidonic acid, and consist of the predominant dictyotene (**8**)**,** which elutes after ectocarpene (**5**), and the minor linear hydrocarbon cystophorene (**10**), which elutes at last, with relative ratio **8**:**10** = 84:16 in *E. siliculosus.*

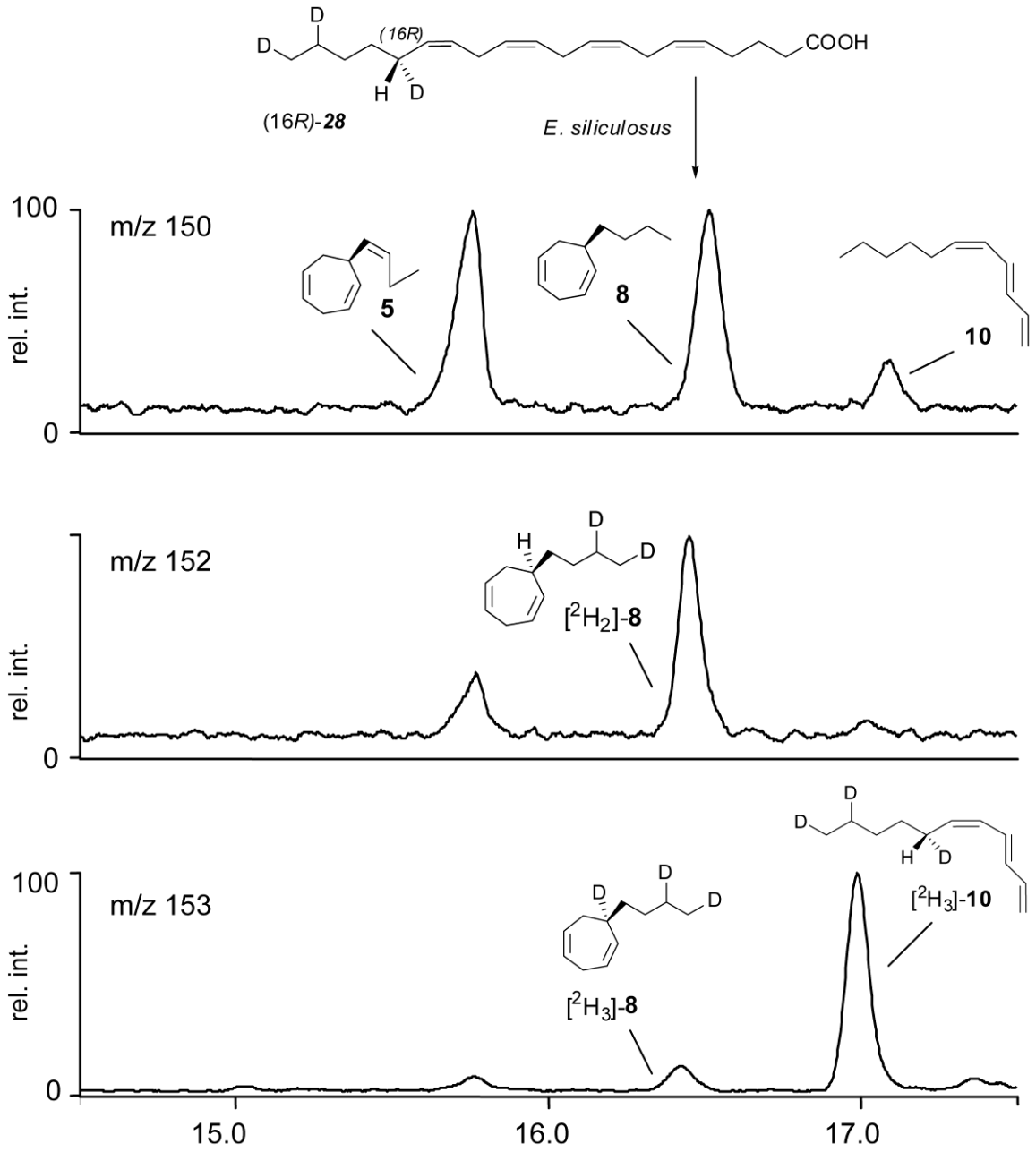


Fig. 22 Natural and labelled C_{11} hydrocarbons produced by *E.siliculosus* after incubation with (16*R*)-**28**, ion traces at m/z 150, 152, 153.

Upon incubation with the specifically deuterated (16*R*)-**28** labelled volatiles are formed as shown in the extracted ion chromatograms at m/z 152 and 153 (Fig. 22). Deuteration influences the retention time of the hydrocarbons, which is slightly lower than that of the natural analogues. With focus on the labelled dictyotene, [$^2H_2$]-**8** is the major one, as expected, whereas [$^2H_3$]-**8** is the minor. Surprisingly, the major labelled volatile is the linear hydrocarbon [$^2H_3$]-**10**, as it is evident from the ion trace at m/z 153. The relative distribution of the products is now [$^2H_2$]-**8 :** [$^2H_3$]-**8 :** [$^2H_3$]-**10** = 18:9:73 , which means that of the product pattern has been reversed.

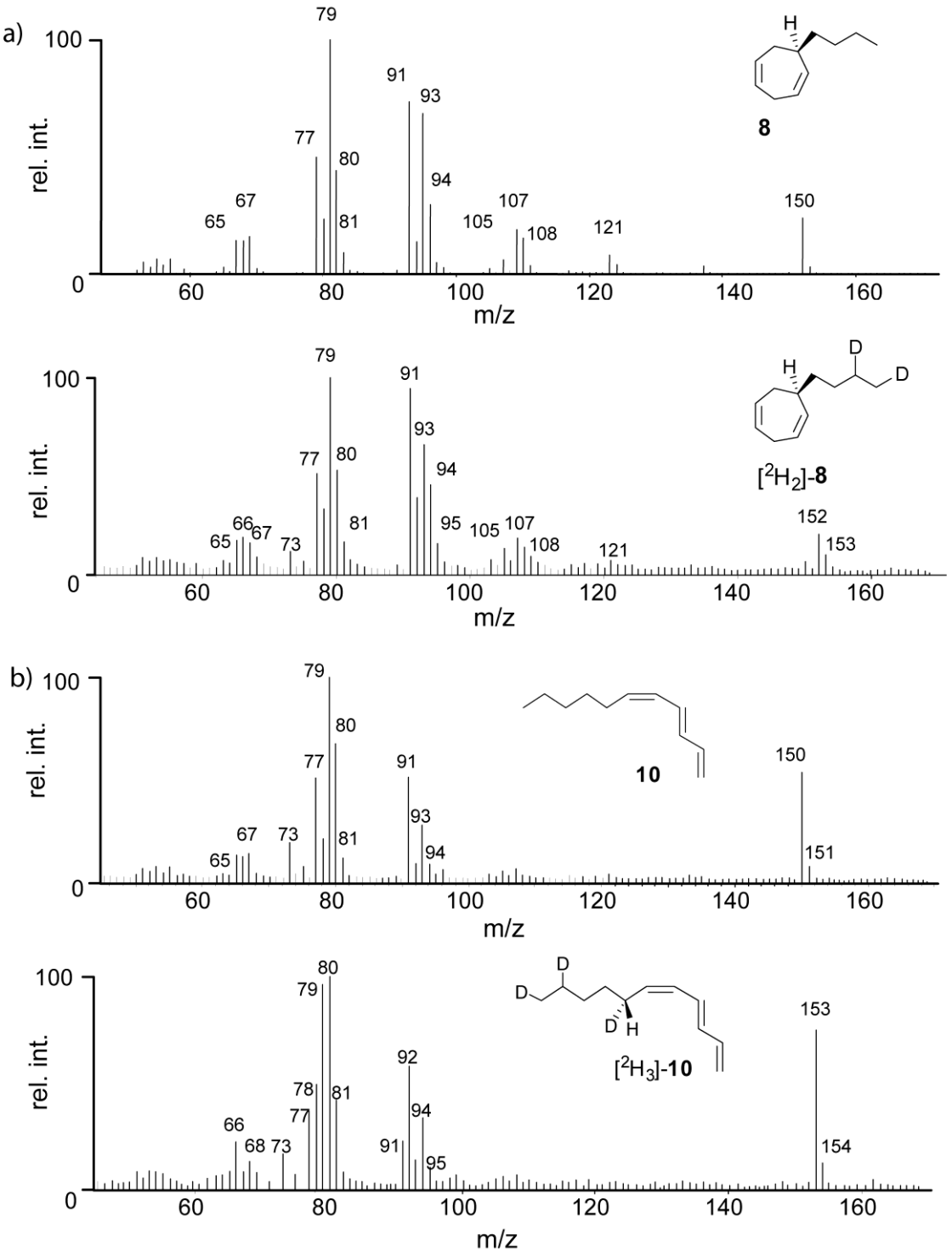


Fig. 23 Mass spectra of a) deuterated and natural dictyotene (**8**); b) deuterated and natural cystophorene (**10**).

The mass spectra of the volatiles reported in Fig. 23 show the mass shift of the molecular ion of two and three units.

The presence of the major dictyotene [$^2H_2$]-**8** implies the loss of the deuterium label from the (16*R*)-**28** precursor, and indicates that an enzymatic activity of algal gametes selectively abstracts the C16-*pro-R* atom of arachidonic acid during the formation of *cis*-bisalkenylcyclopropane **6**. However, substitution of the C-16-*pro-R* hydrogen with deuterium limits the rate of the transformation, and favours two alternative reaction pathways (Fig. 24). The conversion of the minor fatty acid precursor (16*S*)-**28** produces trideuterated dictyotene [$^2H_3$]-**8**. The second process is the attack at C-13 rather than at C-16 of (16*R*)-**28**, which results in the formation of the linear trideuterated hydrocarbon [$^2H_3$]-**10**. A shift from C-16 to C-13 regiospecificity after incubation with (16*R*)-[15,16-$^2H_2$]-arachidonic acid is known for the model study on the diatom *G. parvulum* (*25*). In that case a kinetic isotope effect (KIE) of k_{HD} = 4.5 was determined.

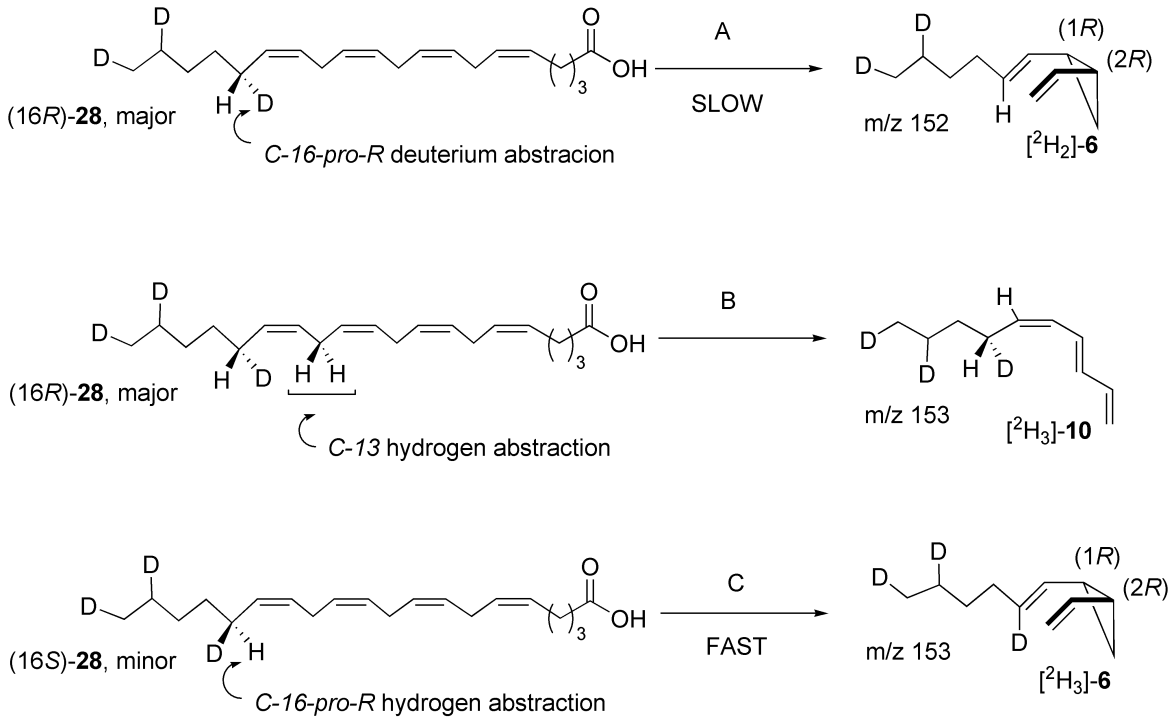


Fig. 24 Reaction pathways for the transformation of arachidonic acid (16*R*)-**28** in C_{11} hydrocarbons in *E. siliculosus.* A) C-16-*pro-R* abstraction from (16*R*)-**28**; B) C-13 abstraction from (16*R*)-**28**; C) C-16-*pro-R* abstraction from (16*S*)-**28**.

The cleavage of C-H bond in allylic position is particularly sensitive to isotopic substitution of hydrogen and the KIE often has extremely high values due the

quantumechanical process of hydrogen tunneling (*90*). A striking example is the lipoxygenation of racemic 11-(*R,S*)-[^{2}H]-linolenic acid by soybean lipoxygenase 1. The substrate labelled in the C-11-*pro-S* position is oxidized so slowly that the enzyme first converts (11*R*)-[^{2}H]-linolenic acid, so that the (11*S*)-[^{2}H]-enantiomer can be recovered from the enzymatic assay in high purity (*91*).

In the case of the conversion of arachidonic acid by *E. siliculosus* the substitution of the C-16-*pro-R* hydrogen by a deuterium atom causes even a change in the relative distribution of products. Due to the simultaneous reaction at C-13 and C-16 of (16*R*)-**28**, the KIE on C-16-*pro-R* hydrogen abstraction can be only estimated. The area of the trideuterated dictyotene [$^2H_3$]-**8** is obtained from of the peak at m/z 153 (S_{153}) after subtracting the contribution of the M+1 peak of [$^2H_2$]-**8** due to the natural isotopic abundance of ^{13}C (12 %), according to the equation:

(7) $S([^2H_3]\text{-}\mathbf{8}) = S_{153} - (S_{152} \bullet 0.121)$

The relative distribution of the labelled dictyotene calculated from three replicates is [$^2H_2$]-**8** :[$^2H_3$]-**8** = (73:27) ± 4 %. Comparing the ratio [$^2H_3$]-**8**/[$^2H_2$]-**8** with the ratio of the volatile precursors of each volatile (16*S*)-**28**/(16*R*)-**28** gives an estimation of the KIE relative to C-16-*pro-R* hydrogen abstraction:

(8) $$k_{HD} = \frac{[^2H_3]\text{-}\mathbf{8} \cdot ([^2H_2]\text{-}\mathbf{8})^{-1}}{(16S)\text{-}\mathbf{28} \cdot ((16R)\text{-}\mathbf{28})^{-1}}$$

which has different values according to the enantiomeric ratio of the precursor, as shown in Table 1.

precursors (16*S*)-**28**/(16*R*)-**28** (%)	products [$^2H_3$]-**8** /[$^2H_2$]-**8** (%)	KIE
3.8/96.2	27/73	9.4 ± 2
10/90	27/73	3.3 ± 0.8

Tab. 1 Estimated kinetic isotope effect for the *pro-R* hydrogen abstraction from position C-16 of arachidonic acid in the formation of dictyotene (**8**). Two results are calculated from different values of the enantiomeric ratio of the fatty acid (16*R*)-**28**.

In order to confirm that also the biosynthesis of the labelled hydrocarbons [$^2H_2$]-**8** and [$^2H_3$]-**8** is stereoselective, the incubation with (16*R*)-**28** was repeated and the volatiles were

analysed with chiral GC/MS. In contrast to the analysis of the natural product **8**, a slower temperature program was used, which results in broader peaks. In contrast to GC, chiral GC does not separate the linear hydrocarbon [$^2H_3$]-**10** from the cycloheptadienes, as shown in Fig. 25. The *cis*-bisalkenylcyclopropanes [$^2H_2$]-**6** and [$^2H_3$]-**6** produced have high enantiomeric purity, which means that the transformation of labelled fatty acid (16*R*)-**28** is stereoselective.

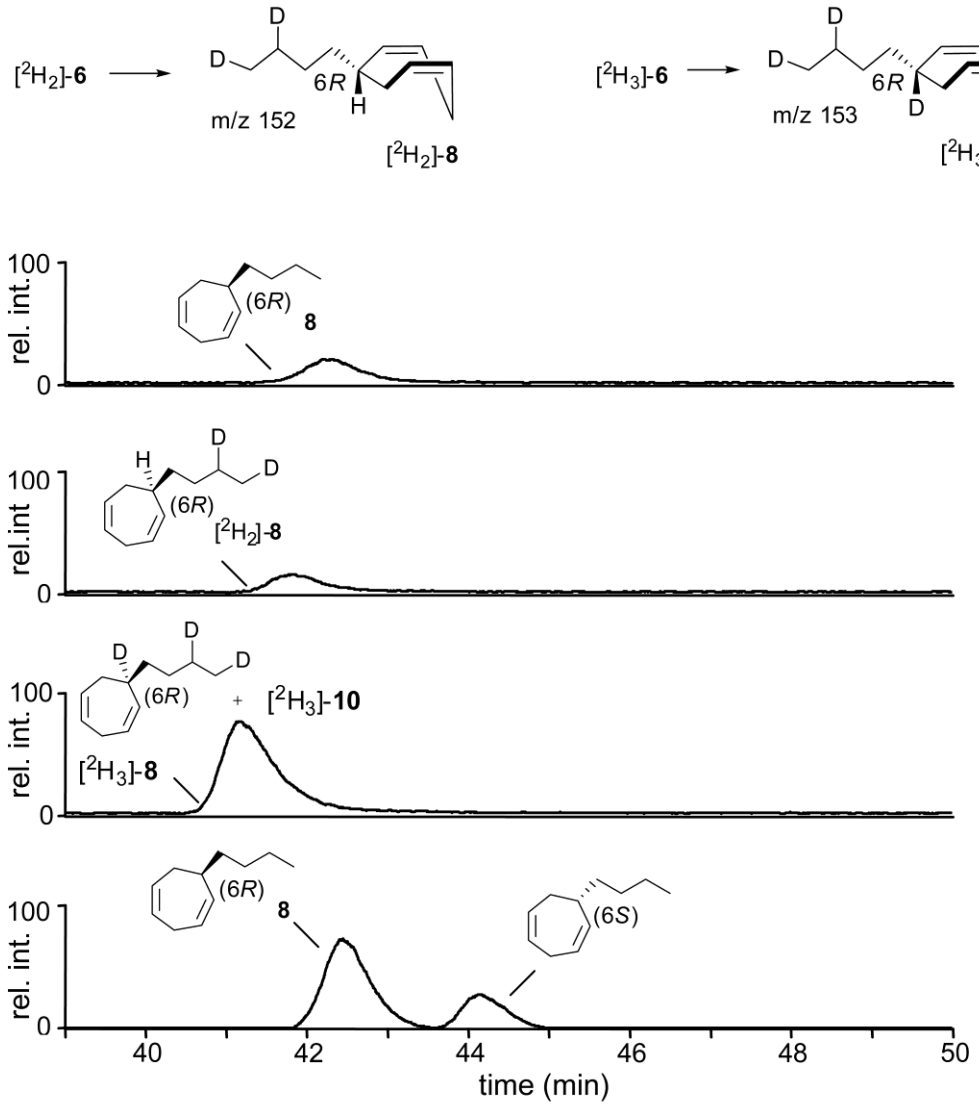


Fig. 25 Analysis of the labelled volatiles of *E. siliculosus* with chiral chromatography.

The selective abstraction of the C-16-*pro-R* hydrogen could be one of the necessary mechanistic requisites for the formation of enantiomerically pure pheromones. On the other hand a chiral putative hydroperoxy fatty acid intermediate could also play a role in the control of the stereochemical course. For example, the diatom *G. parvulum* produces enantiomeric mixtures of *trans*-bisalkenylcyclopropane **7** with abstraction of the C16-H_R

hydrogen. In this organism the C_{20} fatty acid is functionalised as (9*S*)-hydroperoxytetraeicosaenoic acid (9*S*-HETE) before cleavage, which is likely to happen also in the biosynthesis of the brown alga.

The high enantiomeric purity of **6** in *E. siliculosus* supports the hypothesis that the biosynthesis is catalysed by an enzyme enforcing the substrate in a U-shaped conformation in the active site, as illustrated in Fig. 26. The folding of the fatty acid backbone would force Δ11 and Δ14 double bonds and the methylene group of C-13 into a geometry that would favour the formation of the (1*R*,2*R*)-bisalkenylcyclopropane **6**. Formation of the (1*S*,2*S*) enantiomer, in contrast, would require an unfavourable transition state and the yield would be low. Such a U-shaped arrangement in the enzyme binding site was proposed also for the biosynthesis of enantiopure ectocarpene (**5**) from (*Z*)-dodeca-3,6,9-trienoic acid by the plant *S. isatidaeus* (*20*). On the other hand, an extended conformation of the substrate was postulated for the biosynthesis of *trans*-cyclopropanes **3** and **7** in the diatom *G. parvulum* (*25*) (See 1.2).

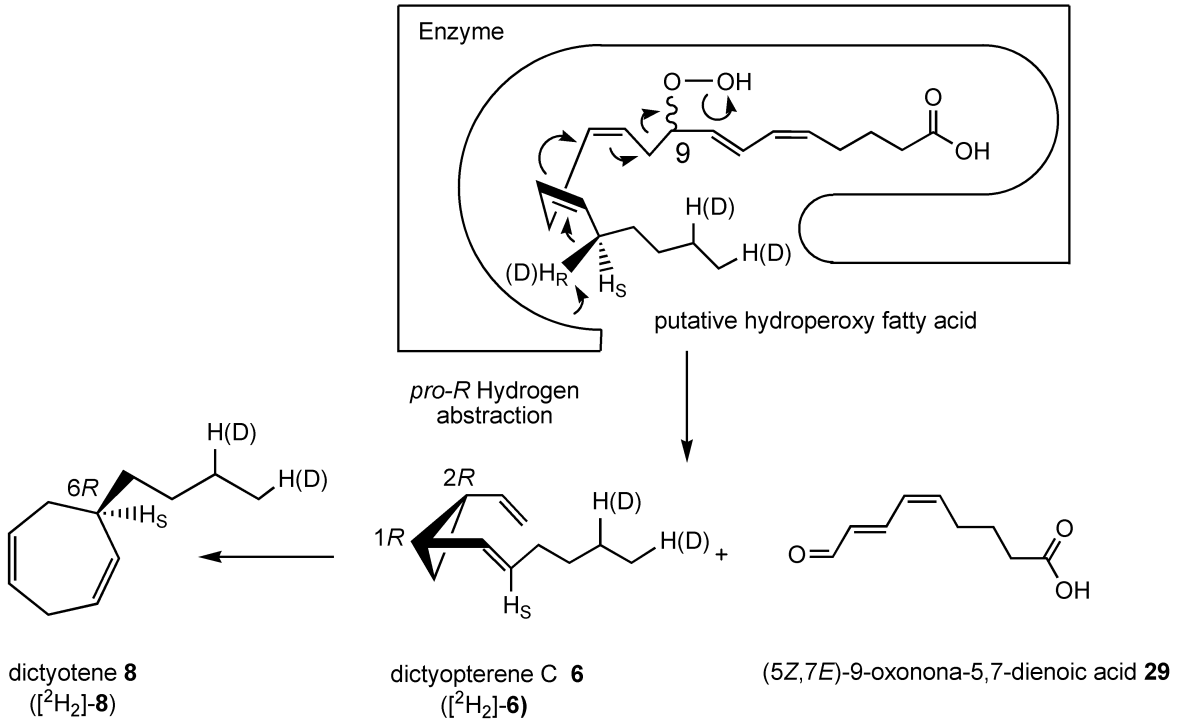


Fig. 26 Proposed U-shaped conformation of the C_{20} precursor in *E. siliculosus* (labelling products in brackets).

3.2.3 Identification of labelled (5*Z*,7*E*)-9-oxo-5,7-dienoic acid in gamete suspensions of *E. siliculosus.*

Female gametes of *E. siliculosus* were incubated with arachidonic acid carrying eight vinylic deuterium atoms (d_8-AA). After transformation of the fatty acid, the isotopic labels were retained both in the volatile C_{11}-hydrocarbon and in the C_9-oxoacid (Fig. 27). The formation of tetradeuterated dictyotene in the headspace, monitored by SPME and subsequent GC/MS, indicated that incorporation of the externally added precursor had occurred. At this time point the (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid was trapped *in situ* with aqueous pentafluorobenzyl hydroxylamine (PFBHA). After mild acidification and liquid extraction, the organic extract was treated with diazomethane.

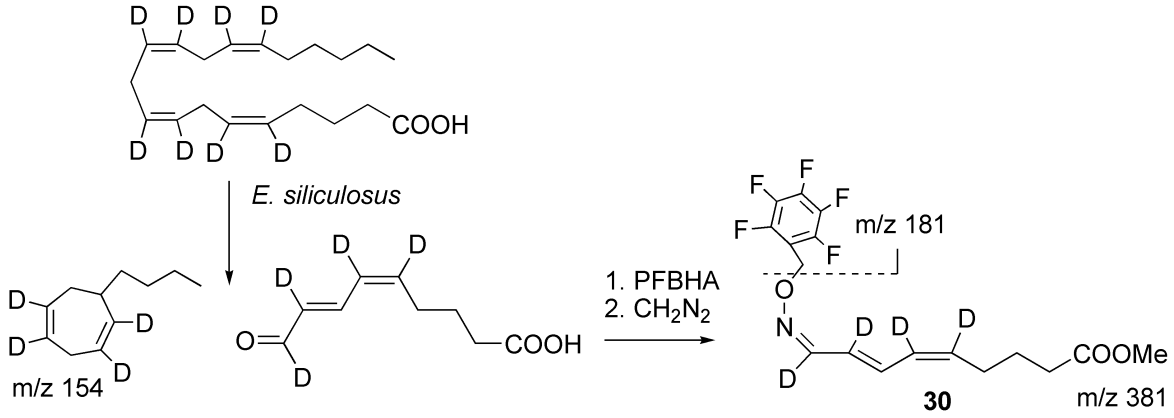


Fig. 27 Incorporation of d_8-arachidonic acid into labelled dictyotene and (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid by gamete suspensions of *E. siliculosus* and cell homogenates of *G. parvulum*.

Reference compounds for the analysis were produced with homogenates of the diatom *G. parvulum* incubated and treated similarly. As described before, this organism is known to synthesise simultaneously C_{11} hydrocarbons and 9-oxoacid (see 1.2). Due to the formation of *O*-(2,3,4,5,6)-pentafluorobenzyl oximes (PFB oximes) during derivatisation, it was possible to run two types of measurements. The first one was positive-ion mass spectrometry after electron-impact ionisation (EI), which showed the charachteristic fragmentation of the PFB oxime at m/z 181 and that of the pentafluorobenzyloxy piridynium ion typical of α,β,γ,δ-unsaturated aldehydes (see 1.5.2).

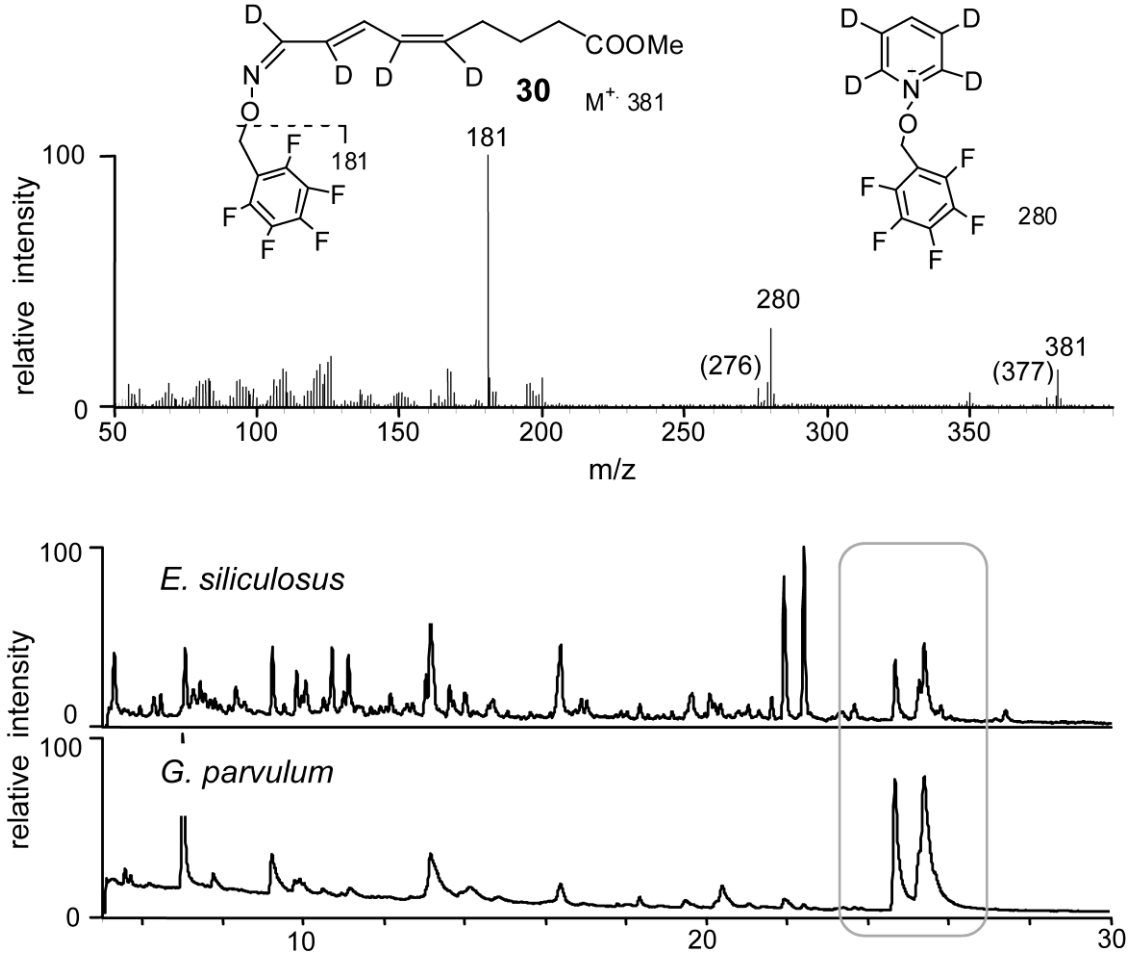


Fig. 28 GC/MS of the polar fraction after derivatisation showing the PFB oxime of 5,6,8,9-[$^2H_4$]-9-oxo-5Z,7E-dienoic acid methyl esther (**30**). EI-Positive mode with ZB-5 column (trace at m/z 181). Fragments of the non labelled product are in brackets.

This ion gave the signal at m/z 276 in the case of the PFB oxime of the natural 9-oxoester and that at m/z 280 for the tertradeuterated analogue **30** deriving from d_8-arachidonic acid (Fig. 28). The second type of analysis was negative-ion mass spectrometry after chemical ionisation (CI-NEG), which is particularly suitable for fluorinated compounds and which provided chromatograms free of background (Fig. 29). A different column could be used, and monitoring the $[M\text{-}HF]^{\bullet -}$ ion provided additional evidence for the presence of the PFB-oxime derivative of the 9-oxoacid (**29**).

Labelling of both 9-oxoacid and C_{11} hydrocarbon confirms the hypothesis that molecular oxygen is inserted in the fatty acid precursor during biosynthesis of C_{11} hydrocarbons in *E. siliculosus.* The role of oxygen in the biosynthesis could be similar to diatoms, where the formation of hydrocarbons is suppressed in oxygen-free homogenates (*22*).

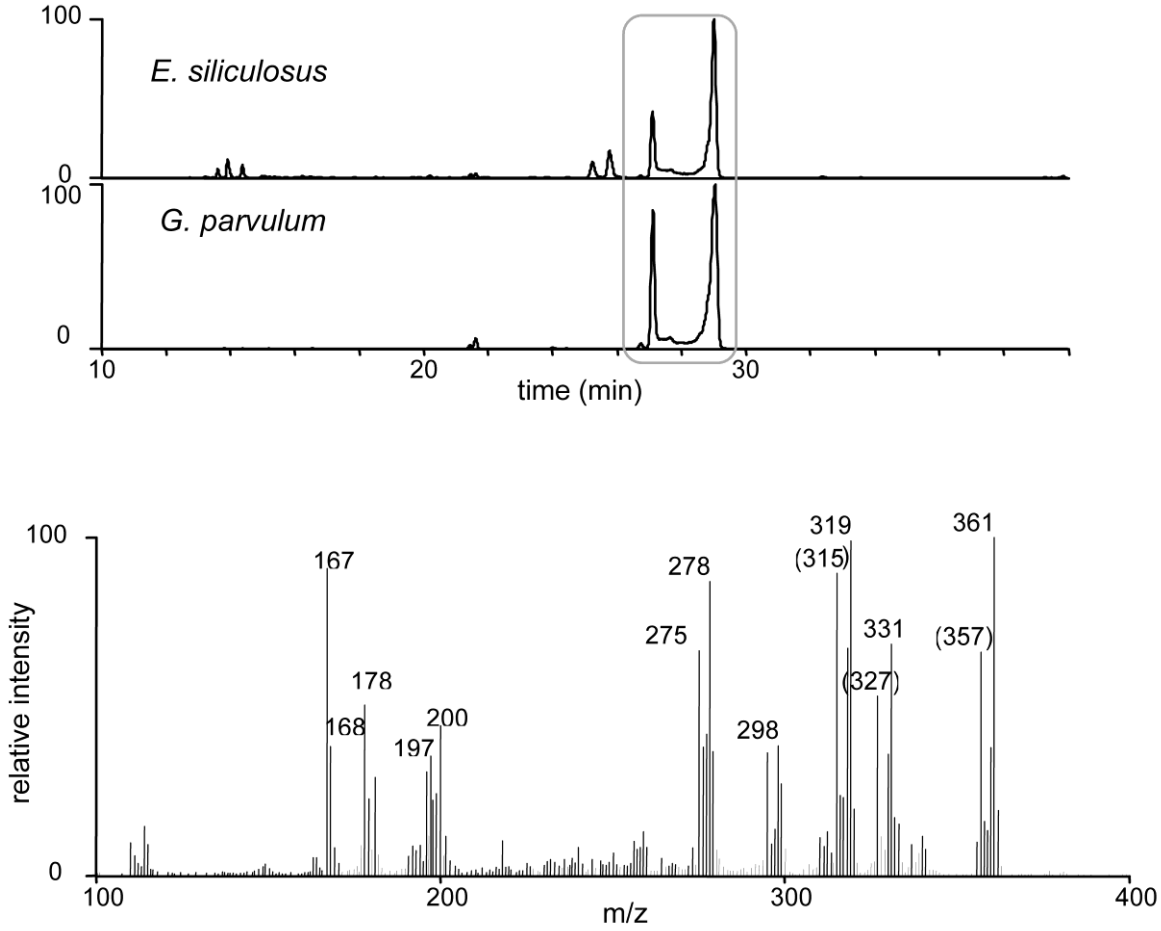


Fig. 29 GC/MS of f the polar fraction after derivatisation showing the PFB oxime of 5,6,8,9-[$^2H_4$]-9-oxo-5Z,7E-dienoic acid methyl ester (**30**). Analysis in CI-negative mode with column RTX-200 (trace at m/z 361 corresponding to the fragment $[M\text{-}HF]^-$). Fragments of the non labelled product are in brackets.

In the diatom *G. parvulum* (9*S*)-HPETE, a hydroperoxy fatty acid precursor of (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid (**29**), is formed (*25*). Although it is likely that (9)-HPETE is produced also by the brown alga, the identification of this intermediate was not successful. Pheromone formation in algal gametes requires a long time compared to the fast conversion of PUFAs by diatom cell homogenates; this could explain why trapping a reactive intermediate was not possible. The identification of (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid as free acid provides an argument against the oxygenation of esterified arachidonic acid on a complex lipid and supports the cleavage by a phospholipase before oxygenation (see 1.2.1). Interestingly, a biological role has been proposed for (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid (**29**). In feeding experiments with the amphipod *Amphitoe longimana*, which feeds on algae belonging to Dictyopteris species, it could be shown that this compound strongly deters this crustacean from feeding. Therefore it was suggested that its water solubility and its reactive unsaturated aldehyde function could play a role in chemical defense (*92*). The biosynthesis of this deterrent compound does actually occur in *E. siliculosus.*

4. Conclusions

This thesis investigated the mechanism of the formation of dictyotene (**8**) in the brown alga *E. siliculosus.* In this organism the C_{11} hydrocarbon dictyotene (**8**) is generated with high optical purity (*13*) from the aliphatic terminus of arachidonic acid (*15*). It was hypothesized that a novel lipoxygenase/hydroperoxyde lyase catalyses this transformation through the cleavage of a hydroperoxy fatty acid into a C_{11} hydrocarbon and a polar fragment (*24*). In the present study the stereochemical course of the reaction was investigated using stereospecifically labelled arachidonic acid. Furthermore, the polar C_9 cleavage product of a putative hydroperoxyde lyase was identified.

The sterospecificity of the biosynthesis was evaluated by measuring the enantiomeric excess of dictyotene (**8**) with high accuracy. In accordance with the optical rotation (*13*), the enantiomeric excess of dictyotene (**8**) is very high (ee = 95.8 ± 0.5 %) confirming that the biosynthesis in *E. siliculosus* is highly stereospecific. In order to study the stereochemical course of the formation of the dictyotene (**8**), a biosynthetic precursor stereospecifically labelled on its aliphatic terminus, (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid [(16*R*)-**28**)], was prepared. The synthesis of (16*R*)-**28** was achieved *via* double Wittig olefination, coupling the bis-(ylide) of (3*Z*)-hex-3-enyl-1,6-(bistriphenylphosphonium bromide) (**14**) with the two short-chain aldehydes (2*R*)-[2,5,6-$^2H_3$]-hexanal (**12**) and methyl 8-oxooct-5-enoate (**13**). For the precursor of the aliphatic terminus of the fatty acid (2*R*)-[2,5,6-$^2H_3$]-hexanal (**12**), asymmetric synthesis and deuterium labelling were required.

The synthesised (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid [(16*R*)-**28**] has extremely high isotopic purity and good enantiomeric excess. Thus the synthetic strategy is a competive alternative to established protocols for stereospecifically labelled analogues of unsaturated fatty acids (*40, 45*).

Analysis of volatiles of algal gametes after incubation with (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid [(16*R*)-**28**] by GC/MS revealed [$^2H_2$]-dictyotene ([$^2H_2$]-**8**) as the major labelled cycloheptadiene. This confirms that the enzyme attacks the *pro-R* deuterium atom at C-16 of arachidonic acid (16*R*)-**28**.

Moreover, due to the strong kinetic isotope effect (KIE) at position C-16 of (16*R*)-**28**, the enzyme alternatively attacks a hydrogen atom at C-13 instead of the *pro-R* deuterium atom at C- 16, resulting in the formation of the linear C_{11} hydrocarbon [$^2H_3$]-cystophorene ([$^2H_3$]-**10**).

The opposite enantiomer of the fatty acid (16*S*)-**28**, which is present in minor amounts in the precursor, leads to [$^2H_3$]-dictyotene ([$^2H_3$]-**8**) through the abstraction of the hydrogen atom at C-16. This allowed to estimate the KIE as 9.4 > k_{HD} > 3.3, which, however, does not consider the attack to the hydrogen at C-13 of (16*R*)-**28**.

In order to further characterize the mechanism of formation of Dictyotene (**8**) from arachidonic acid, the putative product (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid (**29**) of the oxidative cleavage of the fatty acid precursor, was searched for. The ω-oxoacid **29** could be identified so far only in cell homogenates of the diatom *G. parvulum* (*24*). Up to now its reactivity hampered its detection in brown algae. In the present study, using *in situ* derivatisation after incubation of gametes with [$^2H_8$]-arachidonic acid, the labelled oxoacid [$^2H_4$]-**29** was trapped and identified by GC/MS as pentafluorobenzyl oxime derivative. Thus (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid (**29**) is produced by both *G. parvulum* and gametes of *E. siliculosus* and it was identified for the first time in a brown alga.

In conclusion, the experiments confirm the *pro-R* hydrogen abstraction at C-16 of arachidonic acid by the lipoxygenase/hydroperoxyde lyase of *E. siliculosus*, which produces both dictyotene (**8**) and the (5*Z*,7*E*)-9-oxonona-5,7-dienoic acid (**29**) as predicted earlier (*24*) in analogy with the enzymatic mechanism in the diatom *G. parvulum*. In contrast to the diatom, however, the enzyme of the brown alga is highly stereospecific. This feature could be due to an U-shaped conformation of the hydroperoxy fatty acid intermediate enforced by the enzyme in the active site.

Zusammenfassung

Dieses Projekt untersuchte die mechanistischen Aspekte der Bildung von Dictyoten (**8**) in der Braunalge *E. siliculosus*. Dieser Organismus produziert Dictyoten (**8**) in hoher optischer Reinheit (*13*) aus dem aliphatische Ende der Arachidonsäure (*15*). Es wurde postuliert, dass eine Lipoxygenase/Hydroperoxid-Lyase diese Reaktion durch die Spaltung einer Hydroperoxy-Fettsäure in einen C_{11}-Kohlenwasserstoff und ein polares Fragment katalysiert (*24*). In dieser Studie wurde der stereochemische Verlauf der Reaktion durch eine spezifisch markierte Arachidonsäure untersucht. Außerdem wurde das Spaltungsprodukt der mutmaßlichen Hydroperoxid-Lyase identifiziert.

Die Stereospezifizität der Biosynthese wurde durch die Bestimmung des Enantiomerenüberschusses des Dictyotens (**8**) mittels chiraler Gaschromatographie evaluiert. Das Ergebnis stimmt mit polarimetrischen Messungen (*13*) überein und beweist, dass die Biosynthese von Dictyoten (**8**) in *E. siliculosus* über alle Schritte hoch stereospezifisch verläuft. Um den stereochemischen Mechanismus des Prozesses zu untersuchen, wurde ein Analogon der Arachidonsäure synthetisiert, nämlich (16*R*)-[16,19,20-$^2H_3$]-Arachidonsäure [(16*R*)-**28**)], die an ihrem aliphatischen Ende spezifisch deuteriert wurde. Die Synthese von (16*R*)-**28** wurde *via* bis-Wittigalkenylierung, mit dem Ylid von (3*Z*)-Hex-3-enyl-1,6-(bistriphenyl-phosphonium bromide) (**14**) mit (2*R*)-[2,5,6-$^2H_3$]-Hexanal (**12**) und Methyl 8-oxooct-5-enoat (**13**) durchgeführt. Die dafür notwendige Vorstufe (2*R*)-[2,5,6-$^2H_3$]-Hexanal (**12**) wurde durch asymmetrische Synthese und weiteren Deuterierungsschritten erreicht. Die (16*R*)-[16,19,20-$^2H_3$]-Arachidonsäure [(16*R*)-**28**)], wurde mit sehr hocher Isotopenreinheit und guten Enantiomerenüberschuss ereicht. Daher ist diese synthetische Strategie konkurrenzfähig mit den bekannten Protokollen zur Synthese markierter ungesättigter Fettsäuren (*40, 45*).

Die Analyse der Duftstoffe aus den Algengameten nach Inkubation mit (16*R*)-[16,19,20-$^2H_3$]-Arachidonsäure [(16*R*)-**28**] zeigte, dass [$^2H_2$]-Dictyoten ([$^2H_2$]-**8**) das häufigste markierte Cycloheptadien ist. Zudem abstrahiert das Enzym das *pro-R* Deuteriumatom von C-16 der Arachidonsäure (16*R*)-**28**. Alternativ abstrahiert das Enzym aufgrund eines starken kinetischen Isotopeneffekts (KIE) ein Wasserstoffatom in Position C-13 statt des *pro-R*-Deuteriumatoms an C-16. Daher entsteht der lineare C_{11} Kohlenwasserstoff [$^2H_3$]-Cystophorene ([$^2H_3$]-**10**). Das entgegengesetzte Enantiomer der Fettsäure (16*S*)-**28**, das in

geringerem Anteil in der biosynthetischen Vorstufe enthalten ist, führt durch die Abstraktion des Wasserstoffatoms an C-16 zu [$^2H_3$]-Dictyoten ([$^2H_3$]-**8**). Das erlaubt eine Abschätzung des KIE auf 9.4 > k_{HD} > 3.3, die aber die Abstraktion an C-13 von (16*R*)-**28** nicht umfasst.

Um den Mechanismus der Bildung des Dictyotens (**8**) aus der A3rachidonsäure weiter zu charakterisieren, wurde das vermutete oxygenierte Abbauprodukt (5*Z*,7*E*)-9-oxonona-5,7-diensäure (**29**) gesucht. Die ω-Oxosäure (**29**) konnte im Zellhomogenat der Diatomee *G. parvulum* identifiziert werden (*24*). Ihre Reaktivität verhindert die Detektion in Braunalgen. Mittels *in situ* Derivatisierung wurde nach der Inkubation von Algengameten mit [$^2H_8$]-Arachidonsäure die markierte ω-Oxosäure [$^2H_4$]-**29** als Pentafluorbenzyl-Oxim mit GC/MS detektiert. Daher produzieren sowohl die Diatomee *G. parvulum* als auch die Braunalge *E. siliculosus* (5*Z*,7*E*)-9-oxonona-5,7-diensäure (**29**); diese wurde zum ersten Mal in einer Braunalge identifiziert.

Die Experimente bestätigen die *pro-R* Abstraktion des Wasserstoffes von C-16 der Arachidonsäure durch eine Lipoxygenase/Hydroperoxid-Lyase aus *E. siliculosus*. Das Enzym stellt sowohl Dictyoten (**8**) als auch (5*Z*,7*E*)-9-Oxonona-5,7-diensäure (**29**) her, wie zuvor in Analogie zur Diatomee *G. parvulum* geschlossen wurde (*24*). Im Gegensatz zur Diatomee ist das Enzym der Braunalge hochstereospezifisch. Diese Eigenschaft bekräftigt die Hypothese einer U-förmigen Konformation der Hydroperoxy-Fettsäure in der Bindungstelle des Enzyms.

5.1 Materials and methods

Solvents were distilled prior to use. NMR system: Bruker Avance DRX 500 spectrometer, $CDCl_3$ as solvent. Chemical shifts of ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) are given in ppm relative to the solvent peak respectively at 7.26 and 77.0 ppm. NMR for compounds **21** and **28:** Brucker Avance II 550 MHz TCI500S2 C-H/N-D05Z; d_6-benzene as solvent, solvent peak at 7.15 ppm and 120.8 ppm. GC/MS used for synthetic purposes: Finnigan Magnum GC colum DB-5 30 m x 0.25 mm, ion trap, EI, 70 eV. GC/MS for analytical purposes: Thermo Finnigan Trace quadrupole, EI, 70 eV, Thermo GC, He as carrier gas; this instrument was used for mass spectrometry charachterisation of all compounds. The following columns were used: Zebron ZB-5 (15 m x 0.25 mm) by Phenomenex; *heptakis*-(2, 3-di-*O*-methyl-6-*O*-*t*-butyldimethyl-silyl)-β-cyclodextrin (25 m x 0.25mm), commercial name Hydrodex-β-6TBDM; *heptakis*-(2, 6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (25 m x 0.25 mm), commercial name Hydrodex-β-3P, by Macherey-Nagel. For the analysis of oxylipins in negative ion mode the GC/MS system Finnigan Mat GCQ (CI, CH_4), equipped with RTX-200 column (0.25mm x 30m) was used. HR-MS and sector field GC/MS, used additionally for methyl arachidonate **11**: Micromass MasSpec. IR: Perkin-Elmer Series 1600 FTIR Spectrophotometer; all samples as film between NaCl disks. HPLC: Gilson HPLC pump 420 equipped with the following columns: LiChrocart column RP-C18 250 x 10 mm 5µm; Nucleosil column 50-7 250 x 10 mm and Nucleosil column 50-5 250x4 mm; by Macherey-Nagel. HPLC grade solvents were purchased from VWR. Column chromatography: Silica gel, Si 60 (0.200-0.063 mm) from Merck, and florisil® from Sigma. Thin layer chromatography: aluminium sheets coated with silica gel 60 F254 by Merck. All chemicals were purchased from Fluka, except potassium *bis*-(trimethylsilyl)amide solution from Alfa aesar; δ-valerolactone and allylmagnesium bromide from Aldrich; and Wilkinson catalyst from STREM Chemicals. (*Z*)-hex-3-enyl-1,6-bis-(triphenylphosphonium bromide) was kindly provided by Dr. Chuanghai Xia. Deuterated solvents for NMR were purchased from Deutero GmbH. Sonication of cell suspension was carried out with sonotrode Bandelin Sonoplus set to 50 % intensity. SPME: 100 µm polydimethylsiloxane, bonded phase, by SUPELCO, Belafonte, U.S.A.

5.2 Synthesis of (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid

(2*S*)-Glycidyl benzyl ether [(2*S*)-15]

The catalyst (*R*,*R*)-(−)-N,N′-*bis*-(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexanediamino cobalt (II) (91 mg, 0.15 mmol, 0.005 eq.) was dissolved in a mixture of (±)-glycidyl benzyl ether (5 g, 30 mmol), acetic acid (34 µl, 0.6 mmol, 0.02 eq.) and 0.5 mL of tetrahydrofurane in a 10 mL round bottomed flask. After cooling the solution on ice, water (297 µl, 16.5 mmol, 0.55 eq.) was added, the mixture was allowed to come to room temperature and stirred for 23 h. After TLC control the epoxide was distilled with a glass oven apparatus at high vacuum (Oven temp. 150 °C, 0.15 mbar) into a cooled receiving flask (0 °C). Diol impurities were removed by eluting the liquid through a pad of Florisil® (PE/EA 1/1) and a clear liquid was obtained (2.27 g, 46 % yield). The enantiomeric excess was determined as average of three measurements by chiral GC/MS on Hydrodex-β-3P with the following program: 100 °C for 5 min, then to 160 °C at 1 °C/min and to 200 °C at 30 °C/min; RT(*S*) = 31.23; RT(*R*) = 31.82 min; ee = 95.3 ± 0.2 %. Reported error is one standard deviation.

^{1}H NMR: δ 7.40-7.27 (m, 5H, aromatics); 4.65-4.55 (m, 2H, C(1′)); 3.79-3.75 (m, 1H, C(1)); 3.47-3.42 (m, 1H, C(1)); 3.22-3.17 (m, 1H, C(2)); 2.81 (dd, *J*= 5 Hz; 4 Hz; 1H, C(3) *cis* to H-C(2)); 2.63 (dd, *J*=5 Hz; 3 Hz; 1H, C(3) *trans* to H-C(2)). ^{13}C NMR: δ 137.9 C(2′); 128.4 C(4′); 127.8 (C(3′), C(5′)); 73.3 (C(1′)); 70.8 (C(1)); 50.9 (C(2)); 44.3 (C(3)). MS (%): m/z 164 ($M^{+\cdot}$, 4); 107 (45); 105 (30); 91 (100); 79 (31); 65 (14). IR (cm^{-1}): ν(C-H) aromatic, 3031. $\nu_{as}(CH_2)$ of cyclopropane, 2999. $\nu_{as}(CH_2)$, 2922; $\nu_s(CH_2)$ 2863. 2000-1700. ν(C=C) aromatic, 1604. $\delta(CH_2)$, 1453.

(2*S*)-1-(Benzyloxy)hex-5-en-2-ol (16)

All the glassware was dried with a heating gun under argon flow. In a 100 mL flask with a cock containing copper (I) iodide (200 mg, 1 mmol, 0.07 eq.) were transferred 25 ml of dry THF, and the solution was cooled at -40 °C. Then a 1 M solution of ethyl magnesium bromide in THF was added (24 ml, 24 mmol) and the suspension stirred for 15 min. Benzyl-(*S*)-glycidyl ether (2.25 g, 13.6 mmol) dissolved in THF (2 ml) was added with a syringe and the mixture was stirred for 3 h. After GC control, the unreacted

allylmagnesium bromide was hydrolysed with 2N HCl. The aqueous phase was extracted with ether (3x 20 ml) and the collected organic phases were washed with saturated solution of ammonium chloride (3 x 50 ml) to complex the metal, then with brine. The upper phase was dried with sodium sulphate and the residue was filtered on a silica frit (PE/E 1/1). After removal of the solvent a transparent liquid was obtained (2.55 g; 91 % yield). Derivatisation for the determination of ee: an aliquot of 1µl of the diol **16** was transferred in GC microvial and treated with 4 µl of *R*-(+)-1-phenylethyl isocyanate. An equal aliquot was treated with 2 µl of the same reagent and 2 µl of its opposite enantiomer. Both samples were heated at 60 °C for two hours, then 30 µl of methanol were added and heating for 15 min followed. After solvent evaporation and dilution in dichloromethane, GC/MS was performed three times on ZB-5 with the following program: from 40 °C to 185 °C at 40 °C/min, then 185 °C for 35 min, then to 280 at 10 °C/min. RT(*S*) = 40.44 min; RT(*R*)= 40.83 min; ee = 92.5 ± 0.2 %. Reported error is one standard deviation

^{1}H NMR: δ 7.37-7.28 (5H, aromatics); 5.90-5.79 (m, 1H, C(5)); 5.1-4.95 (m, 2H, C(6)); 4.56 (s, 2H; C(1´)); 3.9-3.8 (m, 1H, C(2)); 3.52 (dd, *J*=10 Hz, 3 Hz ; 1H, C(1)); 3.35 (dd, *J*= 10 Hz, 8 Hz; 1H, C(1)); 2.29-2.19 (m, 3H, C(4), (-OH)); 1.63-1.48 (m, 2H, C(3)). ^{13}C NMR: δ 138.2 (C(5)); 137.9 (C(2´), 128.4 (C(4´)), 127.8 (C(5´)), 127.7 (C(3´)); 114.9 (C(6)), 74.5 (C(1´)); 73.3 (C(1)); 69.8 (C(2)); 32.2 (C(3)); 29.7 (C(4)). MS (%): m/z 206 ($M^{+\cdot}$; 1); 175 (2); 146 (2); 121 (5); 107 (24); 92 (66); 91 (100); 85 (21); 67 (21); 65 (11). IR (cm^{-1}): ν(O-H), 3500-3200. ν(C-H) vinylic, 3066. ν_{as}(C-H) aromatic, 3030. $\nu_{as}(CH_2)$, 2919. $\nu_s(CH_2)$, 2860. 2000-1700. ν(C=C) vinylic, 1640. ν(C=C) aromatic, 1604. $\delta(CH_2)$, 1454.

1-(2*R*)-[2-^{2}H]-Hex-5-enyloxy)methyl)benzene (18)

A solution of mesyl chloride (2.52 g, 22.0 mmol) in 50 mL of dichloromethane was added dropwise to a solution of triethylamine (3.70 g, 36.6 mmol) and (*S*)-1-(benzyloxy)hex-5-en-2-ol (**16**) (2.52 g, 12.2 mmol) in 2.5 mL of the same solvent in a 100 ml flask cooled at -78 °C. The mixture was warmed to 0 °C and stirred for 30 min. After GC/MS control the excess base was neutralized with 2N HCl, the solution was washed with brine and extracted with ether (3 x 50 mL). The organic phase was dried with sodium sulphate and evaporated. The (2*S*)-1-(benzyloxy)hex-5-en-2-yl methanesulfonate (**17**) (3.61 g, 12.2 mmol) was used directly without purification to avoid any risk of decomposition. It was

dissolved in 3 mL of sodium-dried diethyl ether in a 50 ml flask with cock and the solution was cooled to -78 °C under argon (some precipitate formed). Lithium aluminium deuteride (1.54 g, 36.6 mmol) was added, after 30 min the temperature was slowly raised and the suspension was stirred overnight at 20 °C. The excess of lithium aluminium deuteride was carefully hydrolysed with water/diethyl ether 1/10 and the inorganic precipitate was dissolved with 1M sulphuric acid. After extraction of the mixture with ether (3 x 10 ml), the organic phase was washed with brine, dried and evaporated to give a transparent liquid (1.87 g, yield 80%).

^{1}H NMR: δ 7.38-7.28 (m, 5H; aromatics); 5.86-5.76 (m, 1H, C(5)); 5.12 (m, 1H, C(6)); 4.95 (m, 1H, C(6)); 4.51 (s, 2H, C(1´)); 3.48 (d, *J*=8Hz; 2H, C(1)); 2.12-2.04 (m, 2H, C(4)); 1.62 (m, 1H, C(2)); 1.49 (m, 2H, C(3)). ^{13}C NMR: δ 138.8 (C(5)); 138.7(C(2´)); (128.4, (C(4´)); (127.6 (C(5´)); 127.5 (C(3´); 114.5, (C(6)); 72.9 (C(1´)); 70.2 (C(1)); 33.6 (C(4)); 28.9 (t, ^{CD}J= 19 Hz, C(2)); 25.4, (C (3)). MS (%): m/z 191 ($M^{+\cdot}$, 1); 162 (2), 107 (16), 91 (100), 82 (11), 65 (8). IR (cm^{-1}): ν(C-H) vinylic, 3070. ν(C-H) aromatic, 3031. $\nu_{as}(CH_2)$ methylenic, 2925. $\nu_{s}(CH_2)$, 2856. ν(C-D), 2156. 2000-1700. ν(C=C) vinylic, 1640. ν(C=C) aromatic 1605. $\delta(CH_2)$, 1452.

1-(2*R*)-[2,5,6-$^{2}H_3$]-Hexyloxy)methyl)benzene (19)

The solvent was degassed by freezing in liquid nitrogen, evacuating at high vacuum and thawing (freeze-thaw method) several times until no more gas developed. Then 40 mL were transferred with a syringe in a dry 100 mL flask with a septum, the solvent was cooled at 0 °C and the headspace purged with argon. Wilkinson catalyst chloro-*tris*-(triphenylphosphine) rhodium (I), (445 mg, 0.48 mmol, 0.05 eq.) and the substrate **18** (1.84 g, 9.6 mmol) were added to the solvent. A deuterium balloon connected with a Pasteur pipette to assure tightness was applied by piercing a rubber septum, then the mixture was purged with deuterium for one minute, warmed to 18 °C and stirred. After 45 min an intense dark red colour developed and after 8 hours the gas balloon was removed (GC/MS control). The solution was concentrated to a few ml, then the catalyst was precipitated with hexane and filtrated over celite. The slightly orange residue was filtered through a silica pad (hexane) and concentrated to give a clear liquid (1.41 mg, yield 75 %).

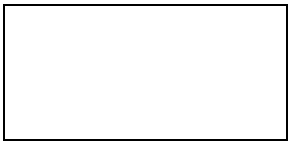
^{1}H NMR: δ 7.37-7.27 (m, 5H, aromatics); 4.51 (s, 2H, C(1´)); 3.47 (d, *J*=6.6 Hz, 2H, C(1)); 1.65-1.55 (m, 1H, C(2)); 1.40-1.34 (m, 2H, C(3)); 1.32-1.25 (m, 3H, (C(4), C(5)); 0.90-0.80 (m, 2H, C(6)). ^{13}C NMR: δ 138.7 (C(2´)); 128.3 (C(4´)); 127.6 (C(5´)); 127.4 (C(3´)); 72.8 (C(1´); 70.5 (C(1)); 31.5 (C(4)); 29.4 (^{CD}J=19 Hz; C(2)); 25.7 (C(3)); 22.1 (^{CD}J=19 Hz; C(5)); 13.6 (^{CD}J=19 Hz; C(6)). MS (%): m/z 195 ($M^{+\cdot}$, 1); 108 (10); 92 (69); 91 (100); 65 (7). IR (cm^{-1}): ν(C-H) aromatic, 3032. $\nu_{as}(CH_2)$, 2924. $\nu_{s}(CH_2)$, 2855. ν(C-D), 2165. 2000-1700. ν(C=C) aromatic, 1602. $\delta(CH_2)$, 1454.

(2*R*)-[2,5,6-$^{2}H_3$]-Hexan-1-ol (20)

A 50 mL flask equipped with cock and a septum previously dried by heating under argon flux, was loaded with 135 mg of palladium on charcoal (Pd-C 5%), heated again and cooled under argon, in order to remove adsorbed oxygen and decrease the risk of combustion in the presence of hydrogen. A hydrogen balloon connected with a Pasteur pipette was applied through a septum and the flask was flushed with hydrogen for 10 min, then 30 ml of dichloromethane and the benzyl ether **19** (1.35 g, 6.9 mmol) were added. After 3 hours the precursor was no longer detected by GC/MS, and the suspension was filtered on celite. Solvent and toluene were removed at 60 °C and 55 mbar in a rotary evaporator equipped with a Vigreux column. The transparent liquid solidified at room temperature (611 mg, 84%).

^{1}H NMR (for peak assignment see correlation spectra HSQC and HMBC): δ 3.63 (d, *J*=6.7 Hz, 2H, C(1)); 1.58-1.50 (m, 2H, C(2), (-OH)); 1.38-1.30 (m, 2H, C(3)); 1.30-1.20 (m, 3H, C(4), C(5)); 0.9-0.8 (m, 2H, C(6)). ^{13}C NMR: δ 63.0 (C(1)); 32.3 (t, ^{CD}J=19 Hz; C(2)); 31.5 (C(4)); 25.3 (C(3)); 22.1 (t, ^{CD}J=19 Hz C(5)); 13.6 (t, ^{CD}J=19 Hz C(6)). MS of TMS derivative (%): m/z 162 ($M^{+\bullet}$-15, 100); 116 (8); 103 (15); 89 (19), 75 (26); 73 ($^{+\bullet}SiC_3H_9$, 35). IR (neat): ν(O-H), 3500-3000. $\nu_{as}(CH_2)$, 2921. $\nu_{s}(CH_2)$, 2854. ν(C-D), 2166. $\delta_{as}(CH_2)$, 1456. ν(C-O), 1043.

(2*R*)-[2,5,6-$^{2}H_3$]-Hexanoic acid (21)



Deuterated hexanol **20** (310 mg, 3.0 mmol) was dissolved in 15 ml of dichloromethane in a 50 mL flask with cock and 1-hydroxy-1,2-benziodoxol-3-(1H)-one (IBX) (2.52 g, 9.0 mmol) was added. A reflux condenser closed by an argon balloon was applied and the suspension was stirred at 43 °C. Reaction controls were taken with the following procedure: 0.5 ml of suspension was cooled at -20 °C, filtered through glass wool, and the solvent was carefully evaporated to allow NMR analysis. After 24 hours the spectrum showed a considerable amount of hexanoic acid. After precitation of the oxidant at -20°C, filtration on a florisil frit and solvent evaporation, around 150 mg of substance were obtained. The product was dissolved in 50 ml of acetonitrile/water 2/1. Then IBX (250 mg, 0.9 mmol, 0.3 eq.) and Oxone (2.76 g, 4.5 mmol) were added to the solution, which was refluxed at 70°C for 6 h. The suspension was cooled on ice, the precipitate was filtrated and washed with 10 ml of water and 10 ml of dichloromethane, the collected filtrate was then extracted with dichloromethane (3 x 20 ml). After separation, the lower phase was dried, filtrated and the solvent was evaporated. In order to remove some impurities the white residue was dissolved in 5 ml of diluted $NaHCO_3$ (20 g/l) and the aqueous solution was washed with ether (2 x 2 ml). The lower phase was collected and acidified to pH 2.5 with diluted H_2SO_4. Extraction with dichloromethane (5 x 2 mL), filtration of the pooled lower phases through sodium sulphate and evaporation yielded an oil (125 mg, 35%).

^{1}H NMR: δ 2.36-2.29 (m, 1H, C(2)); 1.67-1.57 (m, 2H, C(3)); 1.35-1.25 (m, 3H, C(4), C(5)); 0.90-0.80 (2H, C(6)). ^{13}C NMR: δ 179.1 (C(1)); 33.6 (t, ^{CD}J= 19 Hz; C(2)); 31.1 (C(4)); 24.3 C(3); 21.8 (t, ^{CD}J= 19 Hz; C(5)); 13.4 (t, ^{CD}J= 19 Hz; C(6)). MS of methyl ester after diazomethane (%): m/z 133 ($M^{+\cdot}$, 19), 120 (3); 102 ($M^{+\cdot}$-OCH_3, 100); 75 (McL, 23). IR (cm^{-1}): (O-H), 3300-2500. $\nu_{as}(CH_2)$, 2928. $\nu_s(CH_2)$, 2854. ν(C-D), 2170. ν_s(C=O) of carboxylic acid dimer, 1709.

(2*R*)-[2,5,6-$^2H_3$]-Hexanoyl-(*S*)-methylmandelate diester (22)

(*S*)-methylmandelate (28 mg, 0.17 mmol), dicyclohexylcarbodiimide (35 mg, 0.17 mmol) and 4-dimethylaminopyridine (2 mg, 0.02 mmol) were added to a solution of labelled hexanoic acid **21** (20 mg, 0.17 mmol) in dichloromethane. The solution was stirred for 3 hours at -10 °C. After filtration of the precipitated urea, solvent was removed and the product was purified by direct phase HPLC. Column: Nucleosil 50-5, 250x4 mm; eluent: hexane (A) / ethyl acetate (B); gradient 1 % B for 6 min, then to 3 % B in 4 min; then 90 % B for 5 min; flow: 0.6 ml/min; UV detection at 254 nm. The fraction eluting at RT 6.30-7.00 min was collected and the solvent evaporated (13 mg, 28 %).

^{1}H NMR (in d_6-benzene): δ 7.48-7.44 (m, 2H, C(3´´)); 7.10-7.00 (m, 3H, aromatics (C(2´´), C(4´´)); 6.10 (s, 1H, C(1')); 3.18 (s, 3H, C(3')); 2.30-2.20 (m, 0.35H; C(2), (2*S*,1'*S*)-**22**); 2.20-2.05 (m, 1.15, C(2), (2*R*,1'*S*)-**22**); 1.60-1.50 (m, 2H, C(3)); 1.18-1.05 (m, 3H, C(4), C(5)); 0.80-.070 (m, 2H, C(6)). ^{13}C NMR: δ 172.8 (C(1)), 169.4 (C(2')), 134.8 (C(1´´)); 129.3 (C(3´´)); 128.9 (C(2´´), C(4´´)); 74.8 (C(1')); 51.9 (C(3')); 33.7 (J^{CD}= 19 Hz, C(2)); 31.2 (C(4)); 24.7 (C(3)); 22.1 (J^{CD}= 19 Hz; C(5)); 13.6 (J^{CD}= 19 Hz, C(6)). MS (%): m/z 267 ($M^{+\cdot}$, 0.4); 235 (4); 208 (6); 167 (5); 166 (6); 105 (12); 102 ($C_6H_8D_3O^{+\cdot}$, 100); 74 (24). IR (cm^{-1}): ν(C-H), aromatics, 3035. $\nu_{as}(CH_3)$, 2952. $\nu_{as}(CH_2)$, 2930. $\nu_s(CH_2)$ 2856. ν(C-D) 2169. ν(C=O), 1743. $\delta_{as}(CH_3)$, 1437. $\delta_s CH_3$, 1352. (C-H), 735.

^{1}H NMR of (22) with band selective homonuclear decoupling

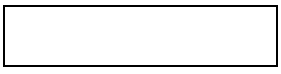
Mandelate diester **22** (5 mg) was dissolved in d_6-benzene. A pulse program (zghd) was used to decouple the signal at 1.60-1.50 ppm of the protons in (C(3))-position, and acquisition was carried out with N= 128 scans. The same experiment was carried out with 0.2 mg of product, and the signal to noise ratio at 2.25 ppm was calculated as S/N = 80. ^{1}H NMR: 7.50-7.45 (m, 2H, (C3´´)); 7.10-7.00 (m, 3H, (C(2´´),(C(4´´)); 6.10 (s, 1H, C(1')); 3.18 (s, 3H, C(3')); 2.31-2.20 (m, 0.11H; C(2), (2*S*,1'*S*)-[2,5,6-$^2H_3$]-hexanoyl mandelate diester); 2.20-2.08 (m, 1.00H; C(2), (2*R*,1'*S*)-[2,5,6-$^2H_3$]-hexanoyl mandelate diester); 1.15-1.05 and 0.80-0.70 (m, 5H, C(4,5,6)). Enantiomeric ratio = 90 ± 0.4 %. Enantiomeric excess ee = 80 ± 0.8 %. Reported error is calculated as ratio of the limit of detection of 0.2 mg of product and the total amount of substance measured (5 mg) (see 3.1.1).

(2*R*)-[2,5,6-$^{2}H_3$]-Hexanal (12)

The second aliquot of the labelled hexanol **20** (290 mg, 2.8 mmol) was dissolved in 15 ml of dichloromethane in a 50 mL flask with cock. After IBX (2.36 g, 8.4 mmol) was added, a reflux condenser closed by an argon balloon was applied and the suspension was stirred at 43 °C. Reaction controls were taken with the following procedure: 0.5 ml of suspension was cooled to -20 °C to induce precipitation, filtered on glass wool, and the solvent was carefully evaporated prior to NMR analysis. Reaction was completed within 15 hours. The whole suspension was kept at -20°C and the precipitate was filtered over a pad of pre-conditioned florisil, which was then rinsed with one volume of dichloromethane at low pressure. Evaporation at 450 mbar and 45 °C in a rotary evaporator equipped with a Vigreux column afforded a transparent liquid (186 mg) containing a small amount of dichloromethane. This was determined by ^{1}H NMR from the ratio of the integrals at δ = 5.29 ppm (-CH_2, int.= 0.89) and δ = 2.42-2.35 ppm (-CDH, int.= 0.80) as 36 mol % (31 % w/w). The product was used immediately for olefination (186 mg, 69 % w/w, 45 %).

^{1}H NMR: δ 9.76 (d, *J* = 1.8 Hz, 2H, C(1)); 2.42-2.35 (m, 1H, C(2)); 1.65-1.69 (m, 2H (C(3)); 1.33-1.22 (m, 3H, C(4), C(5)); 0.90-0.82 (m, 3H). ^{13}C NMR: δ 203.1 (C(1)); 43.5 (t, ^{CD}J= 19 Hz; C(2)); 31.1 (C(4)); 21.9 (^{CD}J=19 Hz, C(5)); 21.7 (C(3)); 13.4 (^{CD}J=19 Hz, C(6)). MS (%): m/z 103 ($M^{+\bullet}$, 1)); 85 (21), 84 (9), 74 (35), 59 (33); 58 (93); 46 (20); 45($[C_3H_5D_2]^{+\bullet}$, 100).

Methyl 5-oxopentanoate (24)



The cation exchange resin DOWEX EX50-WX8 (200 mg) was added to a solution of technical grade δ-valerolactone (4 g, 40 mmol) in 50 ml of Methanol and stirred under reflux for 3 hours in a 100 ml flask equipped with a “circulus” stirrer (GC/MS control). After addition of the same volume of ethyl acetate and evaporation of methanol, IBX (14.0 g, 50 mmol) was added and the mixture was refluxed for 3 more hours (GC/MS control). After cooling on ice, filtration and evaporation of the solvent, the residue was distilled in a glass oven (Kugelrohr) at 130 °C and 20 mbar, and a clear liquid was obtained (3.14 g; 60 %). The filtrated oxidant powder was collected to be recycled.

^{1}H NMR: δ 9.75 (t, 1.4Hz, 1H, C(5)); 3.67 (s, 3H, (C1')); 2.52 (td, J_1=7 Hz, J_2= 1.3 Hz, 2H, C(4)), 2.36 (t, J= 7 Hz, 2H, (C(2)), 1.93 (quint. J= 7 Hz, 2H, (C(3)). ^{13}C NMR: δ 201.4 (C(5)); 173.3 (C(1)); 51.6 (C(1´)); 42.9 (C(4)); 32.9 (C(2)); 17.3 (C(3)). MS (%): m/z 102 ($M^{+\cdot}$-18, 19); 99 (47), 98 (45), 74 (100), 59 (40). IR (neat) : $\nu_{as}(CH_3)$, 2955. aldehydic ν(C-H), 2836, 2730. ν(C=O), 1736. $\delta_{as}CH_3$, 1438. $\delta_s CH_3$, 1370. ν[C-C(=O)-O], 1167.

Methyl 7-(1,3,-dioxan-2-yl)-hept-(5*Z*)-en-oate (26)

Wittig salt 2-(1,3-dioxan-2-yl)-ethyltriphenylphosphonium bromide (2.51 g, 5.5 mmol) was loaded in a 100 mL round bottom flask with cock and dried by gentle heating at high vacuum. After closing with a septum and addition of 40 ml of dry THF, the mixture was cooled to -78 °C under argon atmosphere and a solution of potassium bis(trimethylsilyl)amide (0.91 M, 6.1 ml. 5.5 mmol) was added over 5 min. After heating to room temperature and recooling the orange solution to -78 °C, a solution of the oxoester **24** (650 mg, 5 mmol) in THF was added in 10 min. After 2 h GC/MS control showed no residual oxoester, the solution was diluted with petrol ether and the base was neutralised with 2 N HCl until the solution reached pH 3. The organic phase was separated and dried over sodium sulfate. After evaporation of the solvent and dissolution in THF/PE 1/10, white crystals of triphenylphpsphine oxide were separated by filtration. After removing the solvent, the residue was purified on a silica pad (PE/E 1/1) gave a clear liquid (595 mg, yield 52%).

^{1}H NMR (for peak assignment see H,H-COSY): δ 5.51-5.46 (m, 2H, C(5), C(6)); 4.52 (t, J= 5 Hz, 1H, C(8)); 4.12-4.08 (m, 2H, C(1'')); 3.78-3.73 (m, 2H, C(3'')); 3.66 (s, 3H, C(1´)); 2.37-2.33 (m, 2H, C(7)); 2.31 (t, J=7.5 Hz, 2H, C(2)); 2.13-2.02 (m, 3H, C(4), C(2'')), 1.69 (quint. J= 7.8 Hz, 2H, C(3)); 1.34-1.31 (m, 1H, C(2'')). ^{13}C NMR: δ 174.0 (C(1)); 131.2 (C(5)); 124.5 (C(6)); 101.8 (C(8)), 67.0 (C(1'') and C(2'')); 51.5 (C(1´)); 33.5 (C(7)); 33.4 (C(2)); 26.8 (C(4); 25.7(C(2'')); 24.7 (C(3)). MS (%): m/z 228 ($M^{+\cdot}$, 7); 197 (4); 152 (6); 87 (100); 59 (18). IR (cm^{-1}): ν(C-H) olefinic, 3018. $\nu_{as}(CH_3)$, 2955. $\nu_s(CH_2)$, 2851. ν(C=O) of ester, 1738. ν(*cis*-C=C), 1657. $\delta_{as}(CH_3)$, 1435. $\delta_s(CH_3)$, 1378.

Methyl 8,8-dimethoxyoct-(5*Z*)-enoate (27)

Methyl 7-(1,3,-dioxan-2-yl)-hept-5-en-oate (**26**) (590 mg, 2.6 mmol) was dissolved in 100 ml of methanol in a 250 ml flask equipped with a "circulus" stirrer and 400 mg of strong cation-exchange resin. A refrigerant was applied and the suspension was refluxed for 5 hours (GC/MS control). After filtration of the resin 2 ml of saturated solution of NaCl were added in order to provide an additional solvent for the 1,3-propandiol formed, then 50 mL of ethyl acetate were added and methanol was evaporated. The upper phase was dried and concentrated to give a clear liquid containing the product **27** (495 mg, 88 %).

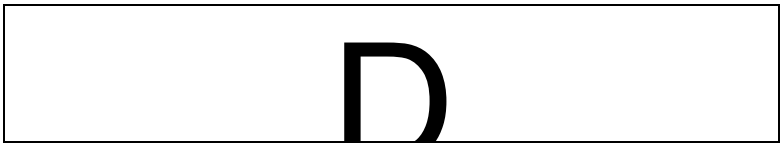
^{1}H NMR: δ 5.50-5.38 (m, 2H, C(5), C(6)); 4.36 (t, *J*= 5.5 Hz, 1H, C(8)); 3.66 (s, 3H, C(1´)); 3.32 (s, 6H, C(1''), C(1'''')); 2.38-2.34 (m, 2H, C(7)), 2.32 (t, *J*= 7.5 Hz, 2H, C(2)); 2.12-2.06 (m, 2H, C(4)), 1.70 (quint. *J*= 7.4 Hz, 2H, C(3)). ^{13}C NMR (APT): ^{13}C NMR (APT): δ 174.0 ↓ (C(1)); 131.1 ↑ (C(5); 124.6 ↑ (C(6); 104.2 ↑ (C(8); 53.0 ↑ (C(1''), C(1'''')); 51.4 ↑ (C(1´); 33.4 ↓ (C(7); 31.0 ↓ (C(2); 26.7 ↓ (C(4); 24.7 ↓ (C(3). MS (%): m/z 185 ($M^{+\cdot}$-31, 9); 153 (15); 75 (100). IR (neat): $\nu_{as}(CH_3)$, 2952. ν_s(C=O), 1739. ν(*cis*-C=C), 1657. $\delta_{as}(CH_3)$, 1438. $\delta_s(CH_3)$, 1364.

Methyl 8-oxooct-(5*Z*)-enoate (13)

Dimethyl acetal **27** (490 mg, 2.3 mmol) was dissolved in 70 mL of pentane in a 100 mL flask, then 2 ml of formic acid were added and the mixture was stirred vigorously at 20 °C. After 1,5 h (GC/MS control) the upper phase was separated and the lower phase was carefully extracted with pentane (3x50 ml, 5 min of equilibration). The collected upper phase was washed with brine (4 x 10 ml) until the washing solution reached pH 6, then it was dried and evaporated at 35 °C. The light yellow oil obtained contained the product (82 mol %: ^{1}H NMR, 9.66 ppm, t, 1.8 Hz, 0.81H) as well as impurities of the α,β-unsaturated isomer (10 mol % : ^{1}H NMR at 9.49 ppm, d, 7.8 Hz, 0.08H) and of the acetal **26** (10 mol % : ^{1}H NMR, 4.51 ppm, *t*, 5.2 Hz, 0.08H). Since the impurities do not interfere with the next reaction and the product is prone to isomerisation already under mild acidic conditions, it was used directly for the olefination. (360 mg, 77 % w/w purity, yield 71 %).

^{1}H NMR: δ 9.66 (t, *J*=1.8 Hz, 1H, C(8)); 5.70-5.62 (m, 1H, C(6)); 5.62-5.54 (m, 1H, C(5)); 3.56 (s, 3H, C(1')); 3.20-3.17 (m, 2H, C(7)); 2.31 (t, *J*=7.3 Hz, 2H, C(2)); 2.08 (m, 2H, C(4)); 1.70 (quint.; *J*=7.3 Hz, 2H C(3)). ^{13}C NMR (APT): δ 199.4 ↓, (C(8)); 173.8 ↑, (C(1)); 133.9 ↓ (C(5)); 119.3 ↓, (C(6)); 51.6 ↓, (C(1´)); 42.5 ↑, (C(7)); 33.2 ↑, (C(2)); 26.9 ↑, (C(4)); 24.4 ↑, (C(3)). MS (%): m/z 170 ($M^{+\bullet}$, 1); 152 (5); 139 (41); 110 (72); 74 (McL; 100); 67 (75). GC/MS: Program: T = 45 °C for 10 min, then to 240 °C at 10 °C/min; RT(*Z*) = 9.29 min; RT(*E*) = 9.43 min; *Z*:*E* = 95:5. IR (neat): ν(C-H) olefinic, 3019. $\nu_{as}(CH_3)$, 2953. $\nu_{as}(CH_2)$, 2852. ν(C-H), 2730. ν(C=O), 1737. ν(*cis*-C=C), 1639. $\delta_{as}(CH_3)$, 1437. $\delta_{s}(CH_3)$, 1378.

(16*R*)-[16,19,20-$^{2}H_3$]-Methyl arachidonate (11)

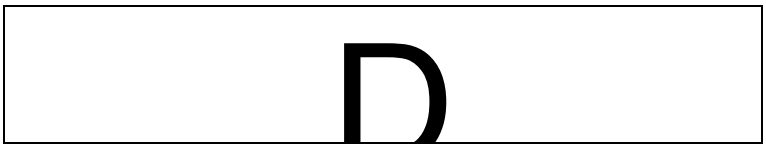


An oven-dried 50 ml flask with a cock loaded with (*Z*)-hex-3-enyl-1,6-bis-(triphenylphosphonium bromide) **14** (927 mg, 1.2 mol) was dried with high vacuum and gentle heating, cooled under argon atmosphere and closed with a rubber septum. 20 ml of dry THF were added and the suspension was cooled to -78 °C under argon atmosphere. After that, potassium bis(trimethylsilyl)amide (0.91 M, 2.6 ml. 2.4 mmol) was added over a period of 5 min and the mixture was allowed to warm to 0 °C. Orange colour was observed, the solution was stirred for 30 min and cooled again. The oxoester **13** (265 mg, 77 % w/w, 1.2 mmol) was dissolved into 2 mL of THF and added with a syringe pump over 40 minutes. Then the mixture was warmed to 0 °C in two hours, in which GC/MS reaction controls were taken to observe a decrease of the oxoester. After that, the suspension was cooled again to -78 °C and a solution of the aldehyde **12** (180 mg, 69 % w/w, 1.2 mmol) in 1 mL of THF was added. The mixture was let warm to room temperature and stirred for 30 min. After addition of 2N HCl the suspension was extracted with ether and the pooled organic phases were washed with brine until pH 6, dried with sodium sulphate in an ice bath and evaporated at 5 °C and 50 mbar. The substance was purified on a silica column (PE/E = 20/1), equipped with a cooling jacket and aluminium foil to protect the product from light and heat. Separation of the desired deuterated arachidonic acid methyl ester (R_f 0.32) and impurities of triphenylphosphine oxide and BHT (R_f 0.36) was incomplete. The mixed fractions were dried and further purified by semi-preparative direct HPLC with the following conditions: Hexane (A) / THF (B) 2 % B

to 10 % B in 20 min, then 90 B % for 6 min; 3 ml/min, UV detection 210 nm, affording the pure product as a clear liquid (31 mg, 8 %). The deuteration pattern is calculated from the molecular ion m/z 321 of the ester **13** and the molecular ion m/z 320 and 322 respectively of the [$^2H_2$]- and [$^2H_4$]- analogues. Extracted ion chromatograms were obtained by GC/MS with sector field analyser and the peak areas were corrected for the area due to the natural isotopic abundance. The resulting area values at m/z 320, 321, 322 were attributed, respectively, to d_2, d_3 and d_4-methyl arachidonate, resulting in $d_2 : d_3 : d_4 = 1 : 98 : 1$; the sum of d_0, d_1, d_5 was less than 1 %.

^{1}H NMR: δ 5.45-5.30 (m, 8H, olefinic protons, C(5/6/8/9/11/12/14/15)); 3.67 (s, 3H, C(1´)); 2.90-2.75 (broad, 6H, C(7/10/13)); 2.35 (t, *J*=7.6 Hz, 2H, C(2)); 2.14-2.08 (m, 2H, C(4)); 2.07-2.01 (m, 1H, C(16)); 1.71 (quintet, *J*=7.6 Hz, C(3)); 1.4-1.2 (broad, 5H, C(17/18/19)); 0.9-0.8 (broad, 2H, C(20)). ^{13}C NMR: δ 174.03 (C(1)); 130.45 (C(15)); 128.92 (C(5); 128.87 (C(6)); 128.58 (C(12)); 128.20(C(8)); 128.16 (C(14)); 127.86 (C(11)); 127.55 (C(9)); 51.45 (C(1´)); 33.44 (C(2)); 31.35 (C(18)); 29.20 (C(17)); 26.86 (t, ^{CD}J=19 Hz, C(16)); 26.55 (C(4)); 25.63; 25.62; 25.60 (C(7); (C(10)); (C(13)); 24.78 (C(3)); 22.08 (t, ^{CD}J=19 Hz, C(19)); 13.63 (t, ^{CD}J=19 Hz, C(20)). MS with sector field (%): 321 ($M^{+\bullet}$, 5); 180 (27); 153 (35); 120 (22); 119 (28); 106 (52); 106 (52); 105 (42); 93 (56); 91 (69); 80 (85); 79 (100). IR (cm^{-1}): ν(C-H) olefinic, 3012. $\nu_{as}(CH_2)$, 2923. $\nu_s(CH_2)$, 2854. ν(C-D), 2168. ν(C=O), 1741. ν(*cis*-C=C), 1656. $\delta_{as}(CH_3)$, 1438. $\delta_s(CH_3)$, 1365. ν(C-(C=O)-O-C), 1156. HR-MS: calculated for $C_{21}H_{31}D_3O_2$: 321.274711; found: 321.275711; deviation: -3.1 ppm. Deuteration pattern after GC/MS with sector field: d_2:d_3:d_4=1:98:1

(16*R*)-[16,19,20-$^2H_3$]-Arachidonic acid [(16*R*)-28]



Hydrolysis of the methyl ester was carried out in small batches short before incubation experiments with algal gametes, in order to avoid risk of auto-oxidation. For the same purpose solvents and solutions were flushed with argon before use. A water solution of lithium hydroxide (1 mol/l; 1 ml) was added to a solution of the ester **13** (13 mg; 0.04 mmol) dissolved in 3 ml of THF in a 5 ml conic flask at 0 °C, it was added in 30 min with a syringe pump. After applying a water bath at 20 °C the mixture was stirred for 11 hours (TLC control, Pentane/E/HAc = 85/14/1, R_f = 0.15). After that, it was poured into a 5 mL flask containing 1 ml of 2N HCl and 1 ml of diethyl ether stirred in an ice bath. Extraction

with diethyl ether (5 x 1 ml), drying of the organic layer (neutral pH) and evaporation at 18 °C afforded the raw product. The residue was purified by reversed phase HPLC with the following conditions. Eluent A: H_2O/CH_3CN 95/5 + 0.05 % HCOOH; eluent B: CH_3CN + 0.05 % HCOOH; flow: 3.6 ml/min; UV detection 210 nm. Instrument method: 85 % B for 3 min; then from 85 % to 100 % B in 15 min; hold 3 min, to 85 % B in 2 min. The pooled collected fractions were concentrated at 100 mbar and 25 °C, the residue was extracted with pentane (4x1 ml) and dried on a small pad of sodium sulphate. Evaporation under a stream of argon gave a clear oil (6 mg, 49 %).

^{1}H NMR (in d_6-benzene; for peak assignment see correlation spectra HSQC and HMBC): δ 5.50-5.35 olefinic (m, 7H, C (6/8/9/11/12/14/15)); 5.30-5.25 olefinic (m, 1H, C(5)); 2.90-2.84 (m, 4H, C(7), C(13)); 2.80-2.75 (m, 2H, C(10)); 2.03 (t, *J*=7.5 Hz, 3H, C(2) superimposed to C(16)); 1.95-1.89 (m, 2H, C(4)); 1.52 (quint., *J*=7.5 Hz, 2H, C(3)); 1.35-1.30 and 1.25-1.21 (m, 5H, C(17/18/19)); 0.88-0.83 (m, 2H, C(20)). ^{13}C NMR (chemical shift from correlation spectra): δ 174.8 (C(1)); 130.3 and 128.0 (C(6/8/9/11/12/14/15)); 128.9 (C(5)); 128.7 (C); 32.2 (C(2)); 31.6 (C(18)); 29.6 (C(17)); 26.9 (C(16)); 26.4 (C(4)); 25.7 (C(7) and C(13)); 25.6 (C(10)); 24.5 (C(3)); 22.2 (C(19)); 13.9, C(20). MS of TMS derivative (%): m/z 379 ($M^{+\cdot}$, 2); 364 ($M^{+\cdot}$-15, 4); 153 (51); 117 (100).

1-Hydroxy-1,2-benziodoxol-3(1*H*)-one 1-Oxide (IBX)

2-Iodobenzoic acid (12.5 g, 50 mmol) was added to a solution of oxone (46 g, 75 mmol) in 250 ml of water and the mixture was warmed to 70°C. After stirring for 3 h, the suspension was cooled to 4 °C, precipitated crystals were filtered and then rinsed with water and acetone in order to remove the inorganic salt in excess and unreacted iodobenzoic acid. The residual solvent was removed under vacuum filtration. White crystals were obtained (10.7 g, 76 %). The product was characterized by NMR and was free from impurities (7.70 (1H, t) iodosobenzoic acid, not detected; 7.47 (1H, t) iodobenzoic acid, not detected).

CAUTION: IBX has been reported to be explosive upon heavy impact and heating over 200 °C. (*93*)

5.3 Algae cultivation and incubation experiments

5.3.1 Cultivation of *E. siliculosus* and gamete release

Axenic cultures of female gametophytes of *E. siliculosus* (New Zealand strain) on agarised medium were kindly provided by Prof. D.G. Müller. Fragments of thalli were transferred in artificial sea water medium (ASM, as described in (*94*)) and kept in a climate chamber at 13°C, with a period of Light/Darkness 14:10 hours and light intensity around 10-15 $\mu E/m^2 \bullet s$. The growing filaments were transferred into new medium every 5 days using sterile Pasteur pipettes under a sterile bench. During the first 30 days of cultivation, Petri dishes of 6 cm diameter sealed with parafilm and containing around 20 ml of medium were used, later glass vessels with plastic cap with around 100 ml of medium. Formation of gametangia was monitored with a microscope and was complete around 90 days from the inoculation in liquid medium. For gamete release the cultures were kept in darkness at 4 °C overnight. After transferring filaments in empty Petri dishes under illumination, and removing the residual cold water with Pasteur pipette, to every culture was added a small amount (around 0.5 ml) of medium at 16 °C. Released gametes were collected with a Pasteur pipette and pooled into a graduated cylinder. Part of the suspension was used for cell counting with a hemocytometer (typical value 1×10^7 cells/ml), the rest was divided in aliquots for incubation with labelled fatty acids or collection of the released volatiles.

5.3.2 Cultivation of *G. parvulum*

Stock cultures on agar slant were purchased from the Culture Collection of Algae (SAG) at the University of Göttingen (Heterokontophyta – Bacillariophyceae – Gomphonema). A small amount of cells was transferred into modified WC medium (*95*) with double amount of Na_2SiO_3 solution and cultivated at 16 °C with a period of Light/Darkness 14:10 hours and light intensity around 10 to 15 $\mu E/m^2 \bullet s$. Every seven days half of the suspension was transferred into the same amount of new medium on a sterile bench. During the first two weeks the volume of the liquid cultures in 6 cm diameter Petri dishes sealed with parafilm was around 20 ml; after the second week it was around 100 ml in glass vessels. After around 30 days aliquots of the suspensions were used for incubation with fatty acids.

5.3.3 Determination of the enantiomeric excess of dictyotene

Cells of *G. parvulum* were harvested by filtering 5 ml of culture on 1 cm diameter glass fibre filter (Whatmann) and suspended into 2 ml of phosphate buffer (0.1 M KH_2PO_4, pH

7) in a 4 mL screw-cap vial with a septum (*24*). A solution of 6 µg of fatty acid in 2 µl of DMSO was added, and, after agitation, the suspension was sonicated at high power for 30 seconds with a 0.5 second pulse in an ice bath. The vial was closed quickly and after 60 minutes at room temperature SPME was carried out for 30 seconds. Female gametes of *E. siliculosus* were collected as described (se 5.3.1) and three aliquots of 2 mL were transferred in 4 mL vials (8.3 x 10^6 cell/ml). After 6 h volatiles were collected by SPME for 60 minutes and analysed with chiral GC/MS. Chromatographic conditions: Column Hydrodex-β-6-TBDMS, 0.25 mm x 25 m. Program: 50 min at 60°C then to 200 °C at 20°C/min. Inlet temperature 220 °C. Retention time: dictyotene 52.96 min; (6*S*)-6-butyl-1,4-cycloheptadiene 53.10 min. The enantiomeric excess is ee = 95.8 ± 0.5 %.

5.3.4 Incubation with (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid [(16*R*)-28]

2 ml aliquots of female gametes *E. siliculosus* were transferred into 4 mL screw-cap vials and incubated with 30 µg of labelled arachidonic acid (16*R*)-**28** dissolved in 1 µl of DMSO (final concentration in gamete suspension 15 µg/mL; 50 µM). After gentle agitation, the suspension was left for 5 h in a growth chamber at 16 °C to allow complete cell settling, and SPME was carried out for 60 minutes starting from the fifth hour. Analysis of total fatty acids during a test incubation: gametes were incubated with (16*R*)-**28** as described before. After the analysis of labelled volatiles, the gamete suspension was centrifuged, the pellet was transferred into a sealed hydrolysis vial, and homogenated in 0.5 mL of methanol. Then 0.5 mL of a mixture of acetyl chloride/methanol 1:9 and 0.5 ml of hexane were added together with 2 µg of d_{27} miristic acid as an internal standard. Heating at 100 °C for 10 minutes caused formation of single phase. After cooling on ice, phase separation was enhanced by addition of 1 ml of water, the upper phase was collected, dried with sodium sulphate and the solvent was evaporated. After dissolution in 40 µl of hexane, GC/MS analysis and external calibration using methyl arachidonate, the amount of arachidonic acid (16*R*)-**28** in the cell suspension was determined as ca. 22 µg. Column ZB-5, program 40 °C for 5 min, then to 100 °C at 3 °C/min, then to 280 °C at 30 °C/min, inlet 240 °C. Chiral GC/MS: Column Hydrodex-β-TBDM; program 70 °C for 50 min then to 200 at 20 °C/min, inlet 220 °C.

5.3.5 Incubation with [5,6,8,9,11,12,14,15-$^2H_8$]-arachidonic acid and analysis of oxylipins

Diatoms cells were harvested as described (see 5.3.3) and incubated with a solution containing 6µg/µl of [5,6,8,9,11,12,14,15-$^2H_8$]-arachidonic acid. After SPME, a water

solution of PFBHA (0.75 ml; 25 mmol/l) containing BHT (10 µg/mL) was added to the homogenate and mixed. After 30 min it was carefully acidified to pH 3 with diluted sulfuric acid and extracted three times with 0.5 ml of dichloromethane containing BHT (10 µg/mL) in two plastic Eppendorf tubes. Phase separation was achieved by centrifugation, then the combined lower phases were centrifuged again, transferred in a new plastic tube, dried with sodium sulphate, transferred in GC vial and evaporated. After treatment with diazomethane the residue was dissolved in dichloromethane, transferred in a microvial, and dissolved in 40 µl of the same solvent. For the incubation of *E. siliculosus*, 2 ml aliquots of gamete suspension were transferred into 4 mL screw-cap vials and incubated with 30 µg of [5,6,8,9,11,12,14,15-$^{2}H_{8}$]-arachidonic acid dissolved in 1 µl of DMSO (final concentration 15 µg/mL; 50 µM). After gentle agitation, the suspension was let for 5 h in a growth chamber at 16 °C to allow complete cell settling, and SPME was carried out for 60 minutes starting from the fifth hour. After volatile sampling, 0.75 mL of a water solution of PFBHA (25 mM) containing also BHT (10µg/mL, from 1000x stock solution in methanol) and the suspension was sonicated in an ice bath by high power sonication for 30 seconds with a 0.5 second pulse. Then the homogenate was acidified, extracted and derivatised as described for *G. parvulum* and dissolved in a microvial with 40 ml of dichloromethane. Temperature Program for EI-Positive ion: 90 °C, hold 4 min, then to 250 °C at 5 °C/min and to 300 °C at 20 °C/min, hold 2 min Flow 1.5 ml/min. Temperature program for CI-Negative ion: 90 °C, then to 180 °C at 10 °C/min, then to 210 °C at 1 °C/min, then to 290 °C at 40 °C/min, hold 2 min. Velocity 40 cm/sec.

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7. Curriculum Vitae

Name and Surname	**Fabio Rui**
Address	Oberlauengasse 4 - 07743 Jena/Germany
Telephone	+491631541998 Email: fabio.rui@gmx.de

Education

12/2003	**Master in Applied Biocatalysis and Industrial Microbiology** /University of Ferrara (Italy). "Optimisation of extraction and quantification methods for caffeic acid derivatives from cell cultures of *Echinacea angustifolia"*. Grades 24/30.
12/2001	**Degree in Chemistry**/University of Padua (Italy). "Synthesis of peptides corresponding to the fifth trans-membrane segment of the mt_1 receptor and interaction studies with melatonin". Grades 105/110.
07/1994	**Grammar school Certificate** "Liceo scientifico" A.Cornaro/Padova (Italy). Grades 50/60.

Research Experience

01/2005 - Present	**PhD student/**Max Plank Society/Jena (Germany) Topic: Biosynthesis of C_{11} hydrocarbons in the brown alga *E. siliculosus.*
01/2004 - 12/2004	**Chemical analyst/**IRB s.r.l./Vicenza (Italy) HPLC analysis of plant secondary metabolites, development and validation of HPLC methods for active pharmaceutical ingredients extracted from plants.
09/2000- 12/2001	**Undergraduate student/**Natonal Research Council/Padova (Italy) Topic: synthesis of peptides reproducing one fragment of the mt_1 melatonin receptor and study of their interaction with melatonin.

Conferences attended

April 1-4, 2007, York (UK) - 3rd European Symposium on Plant Lipids - Poster presentation "Eicosanoid derived pheromones in brown algae" by F. Rui and W. Boland.

July 22-26, 2007, Jena (Germany) - 23rd Annual Meeting of the International Society of Chemical Ecology - Poster presentation "Eicosanoid derived pheromones in brown algae" by F. Rui and W. Boland.

March 30 – 2 April, 2008, Lutherstadt Wittemberg (Germany) - Meeting of the phycological section of the German Botanical Society.

June 4-8, 2008, Oban (UK) - Ectocarpus 2008 - Oral presentation "Mechanistic aspects of pheromone formation in *Ectocarpus siliculosus*".

Supervision of undergraduate students

Practical training in the frame of the course Organic Chemistry V – Universiy of Jena, May-September 2007.

Language skills

Italian	Full Knowledge
English	Fluent - Wide Knowledge (Cambridge FCE)
German	Fluent – Intermediate (Zertifikat Deutsch - Level B1)

Other activities

Cycling, Photography, Percussions.

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegeben Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, 6 Februar 2009

Fabio Rui

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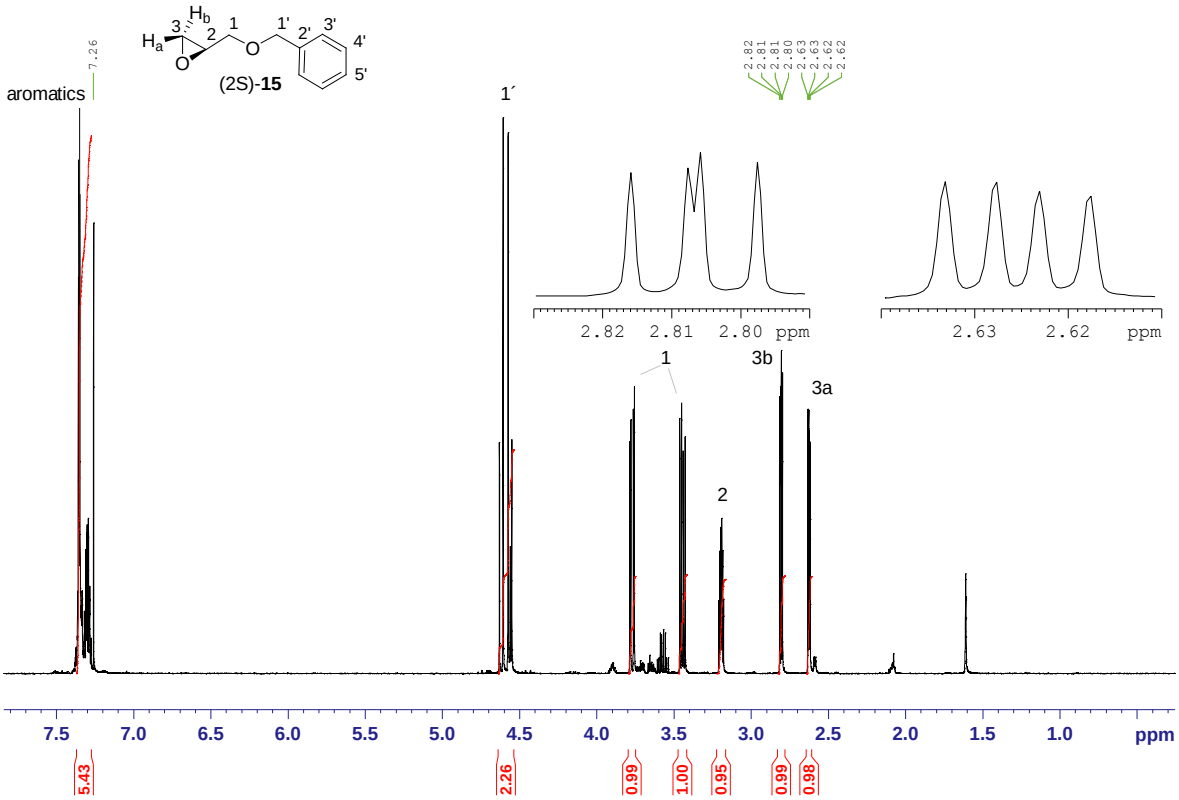
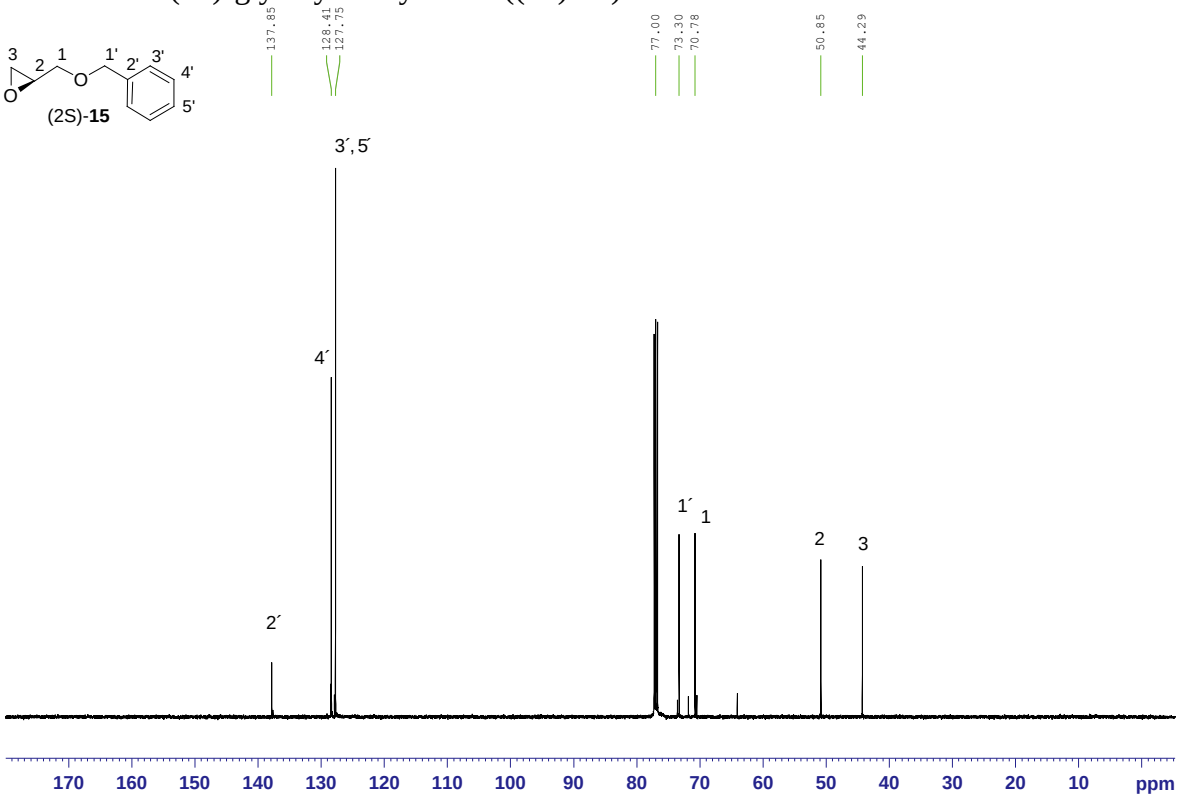
10. Attachment

10.1 NMR spectra

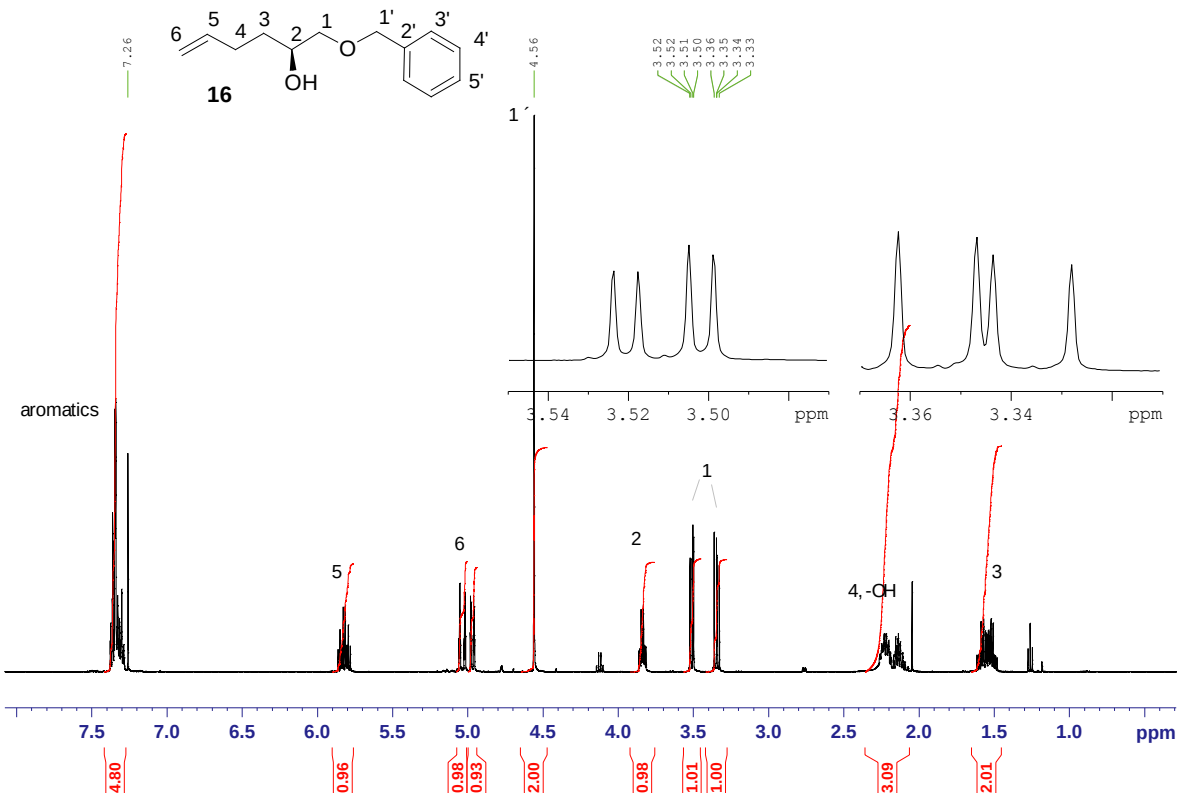
^{1}H NMR of (2*S*)-glycidylbenzyl ether ((2*S*)-**15**)



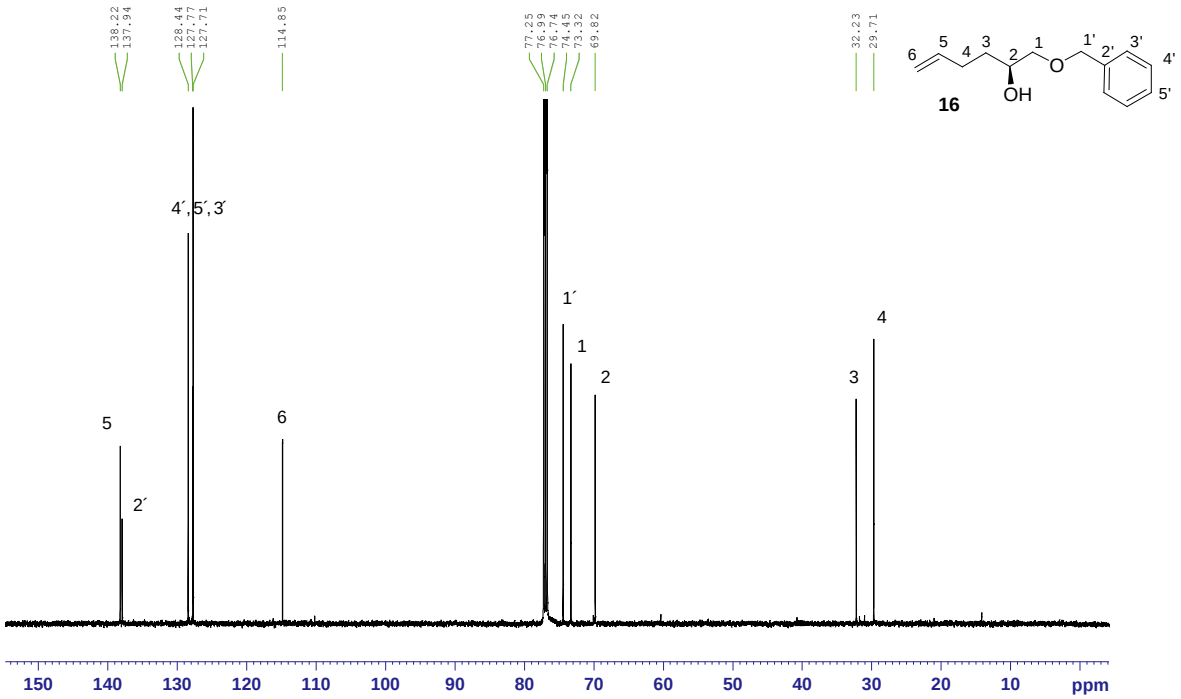
^{13}C NMR of (2*S*)-glycidylbenzyl ether ((2*S*)-**15**)



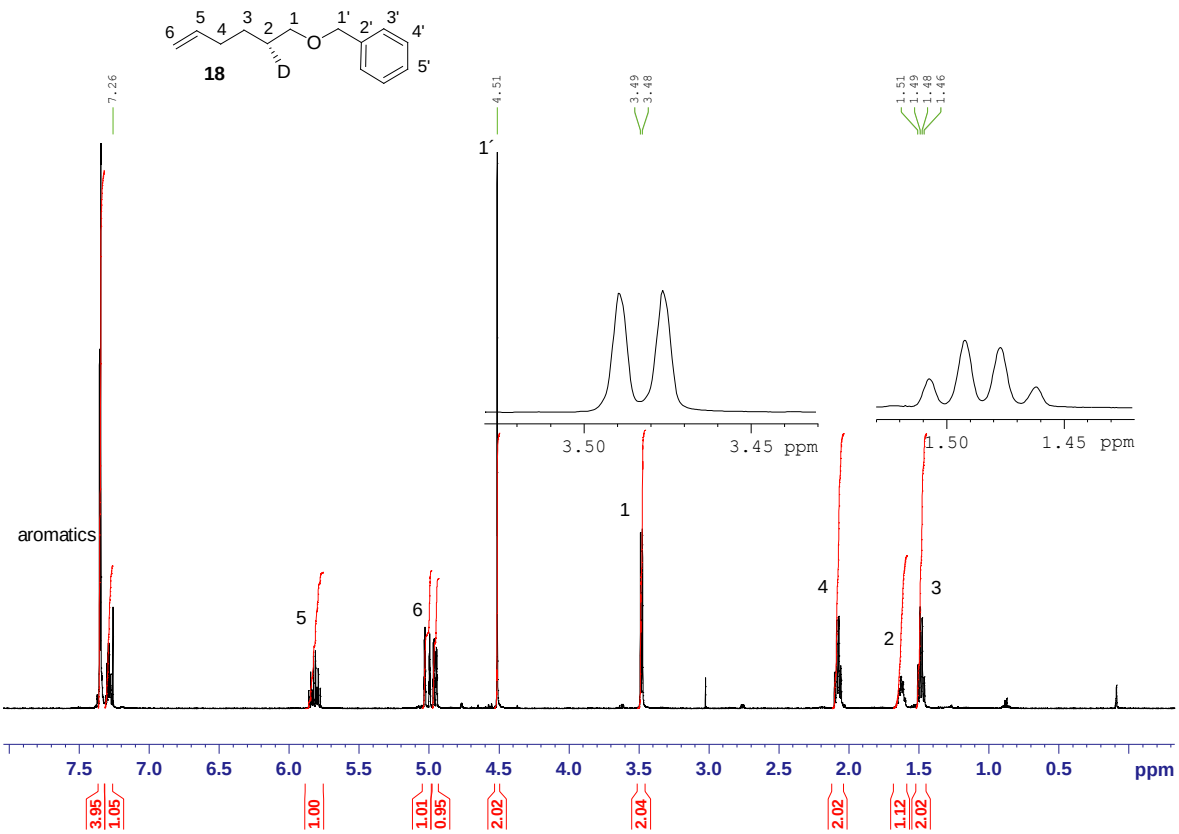
^{1}H NMR of (2*S*)-1-(benzyloxy)hex-5-en-2-ol (**16**)



^{13}C NMR of (2*S*)-1-(benzyloxy)hex-5-en-2-ol (**16**)



^{1}H NMR of 1-((2*R*)-[2-^{2}H]-hex-5-enyloxy)methyl)benzene (**18**)



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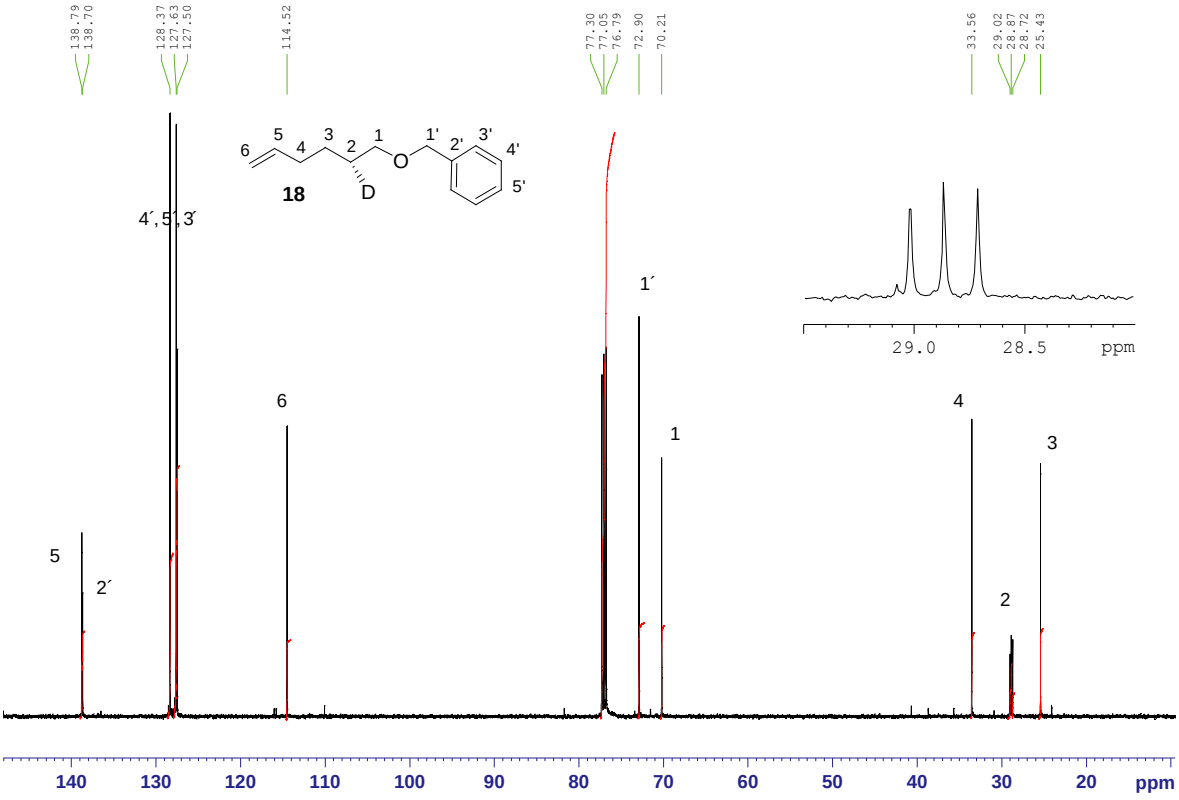
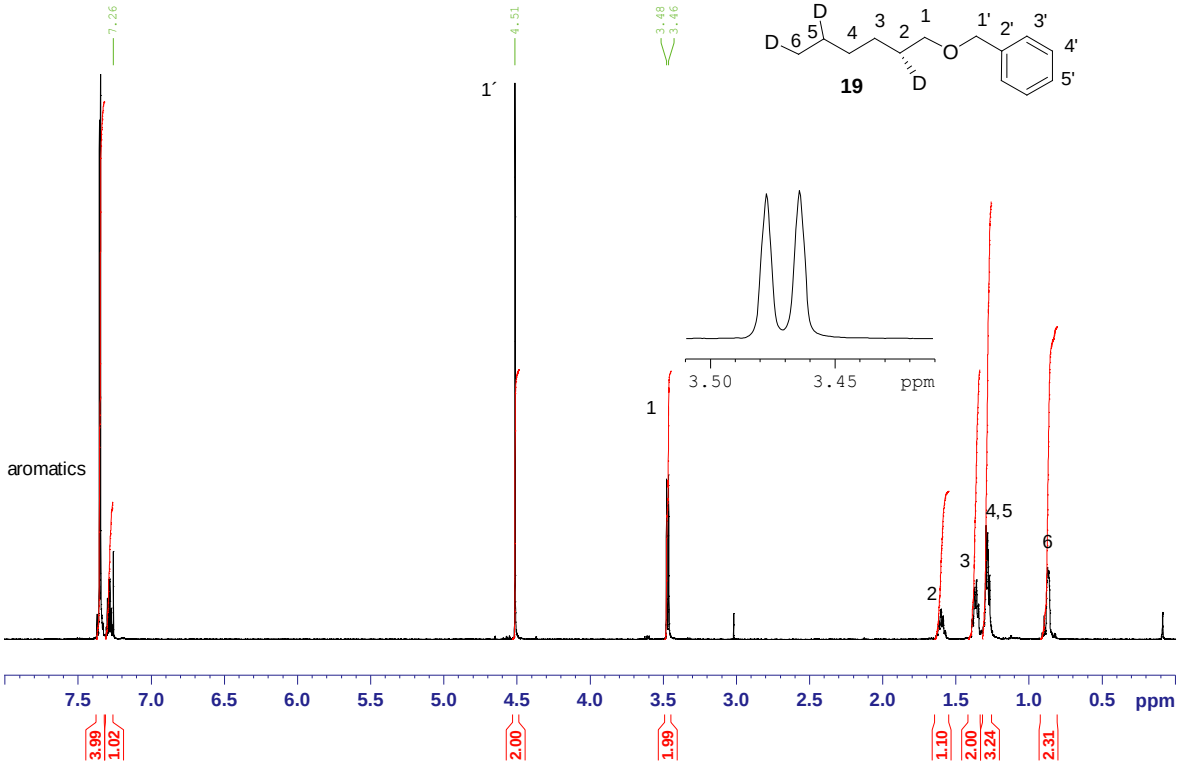
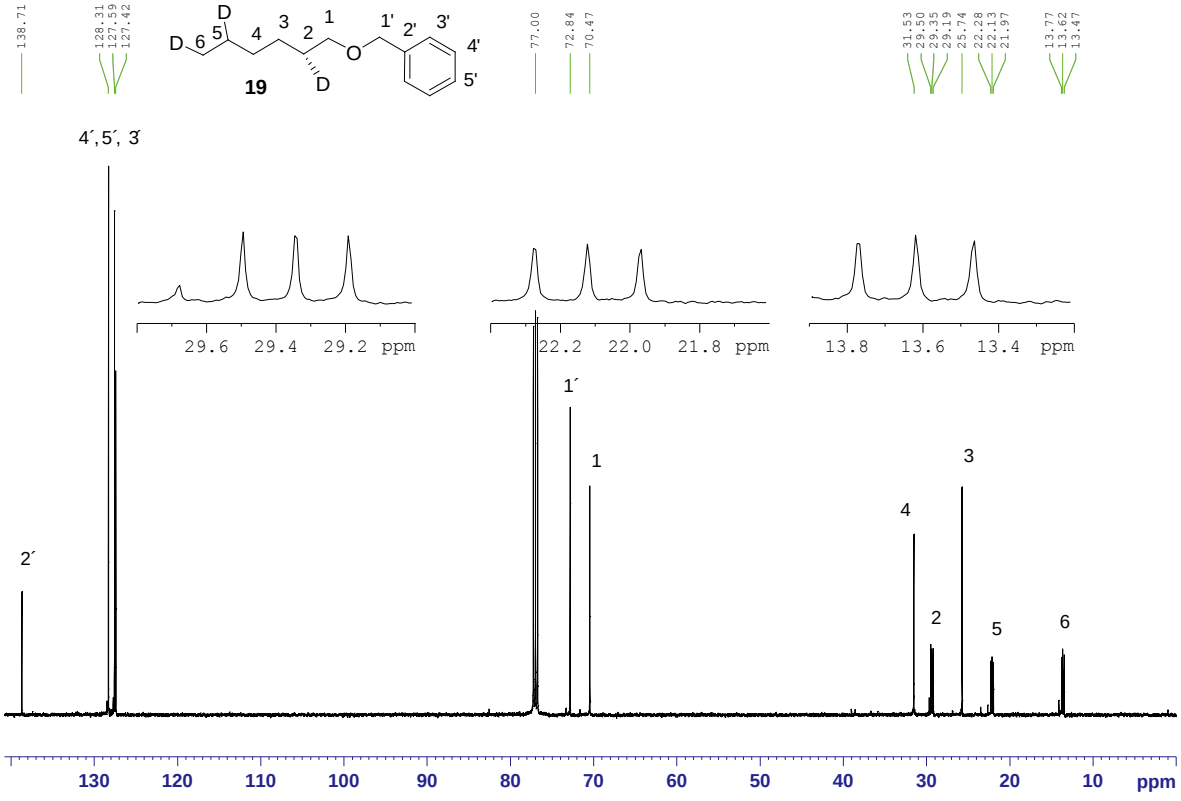


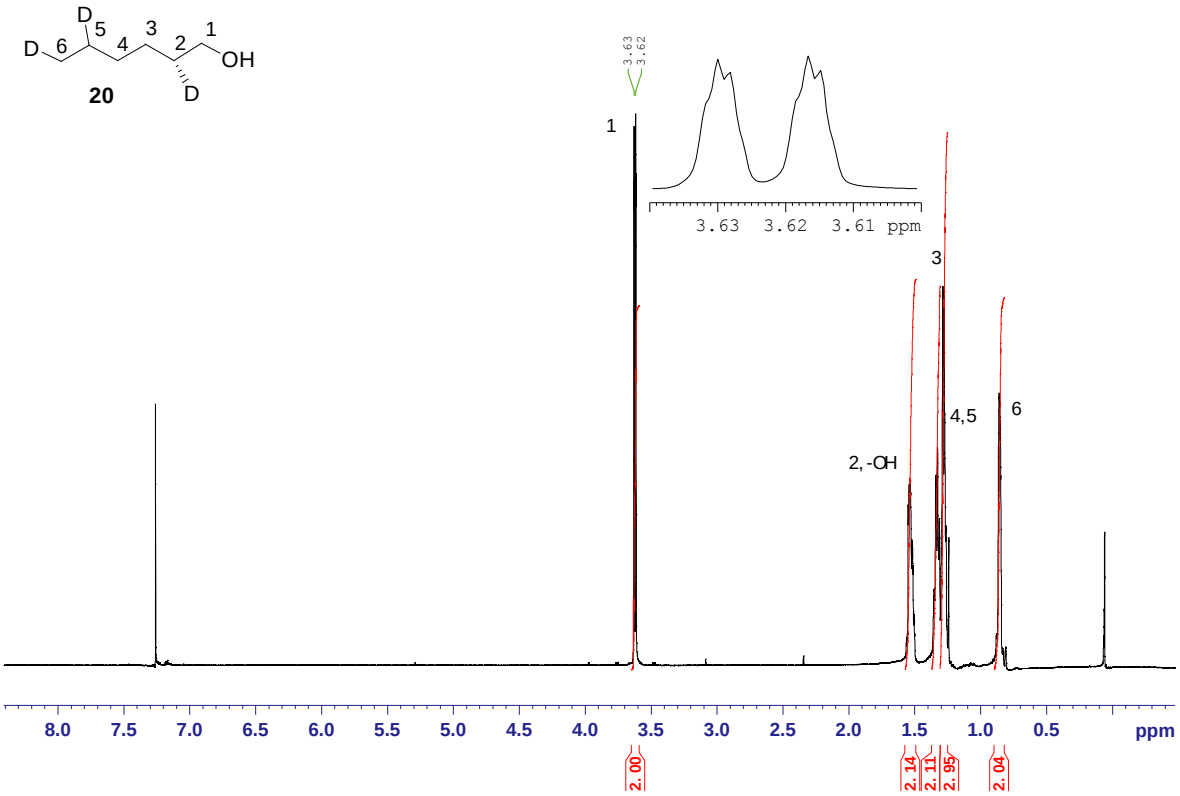
^{1}H NMR of 1-((2*R*)-[2,5,6-$^{2}H_3$]-hexyloxy)methyl)benzene (**19**)



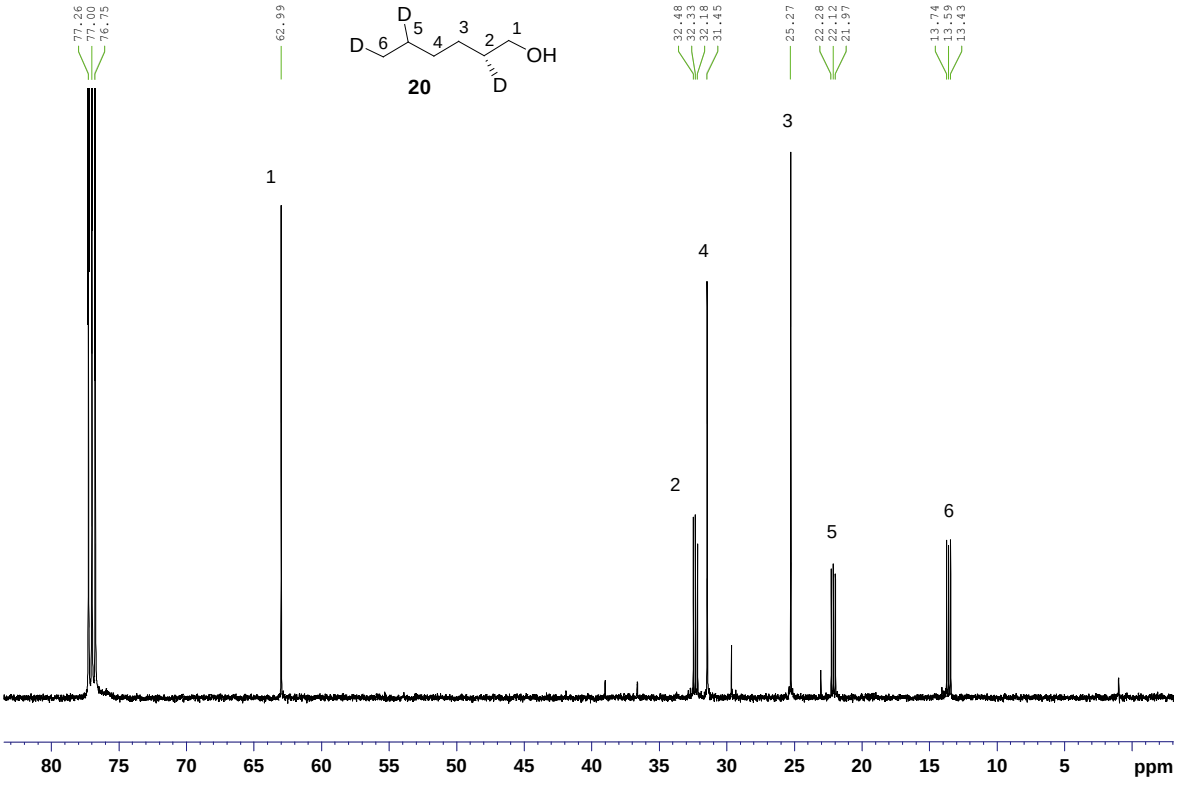
^{13}C NMR of 1-((2*R*)-[2,5,6-$^{2}H_3$]-hexyloxy)methyl)benzene (**19**)



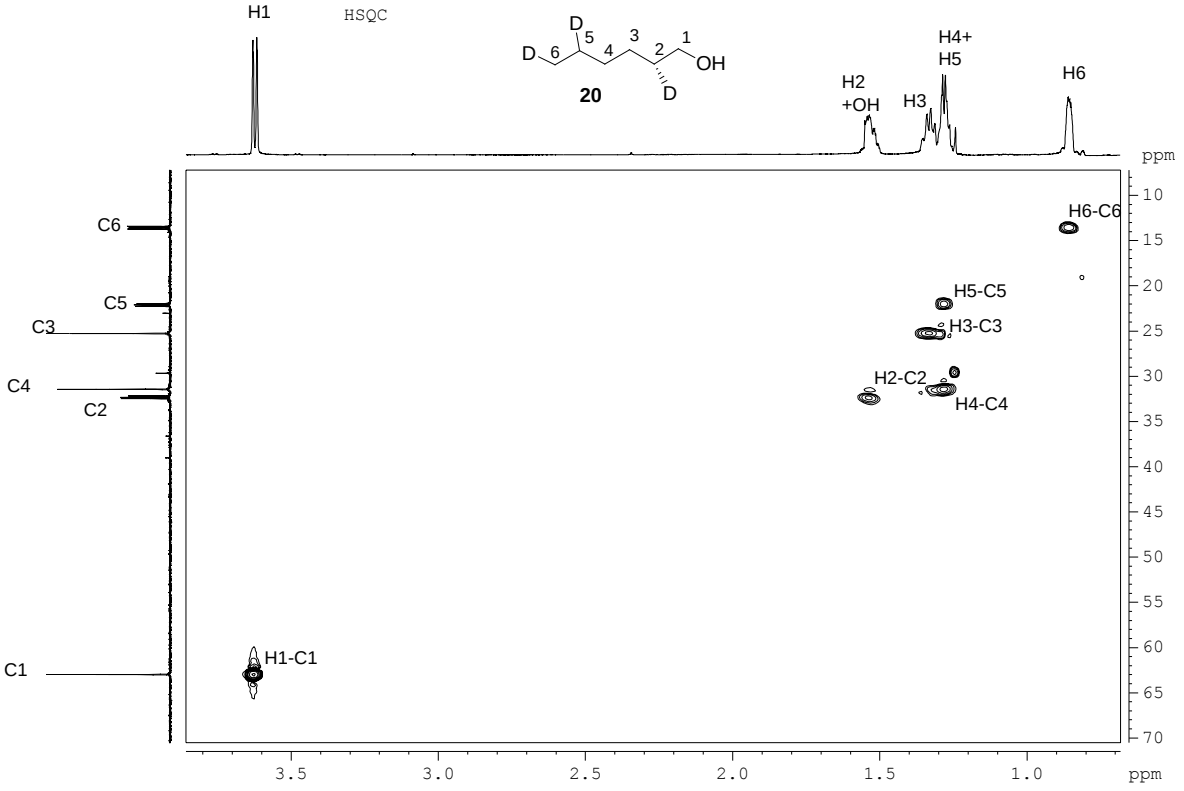
^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexan-1-ol (**20**)



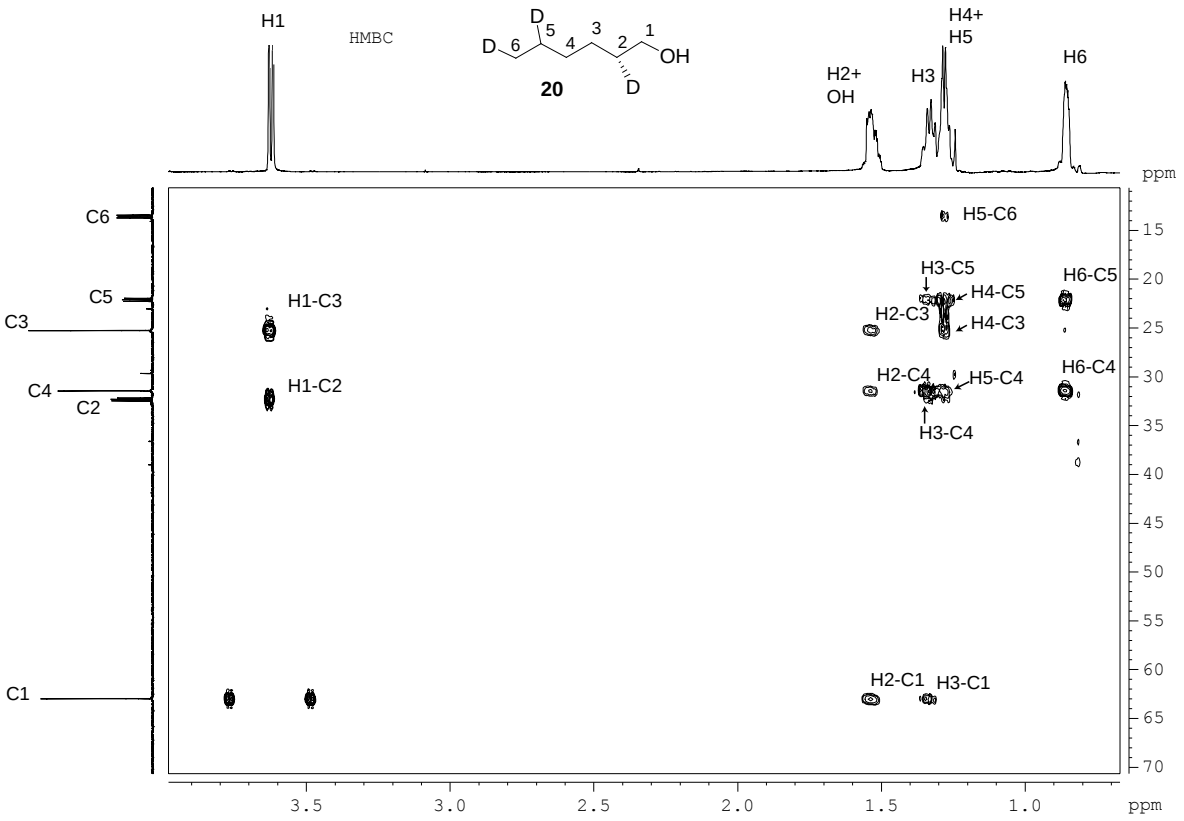
^{13}C NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexan-1-ol (**20**)



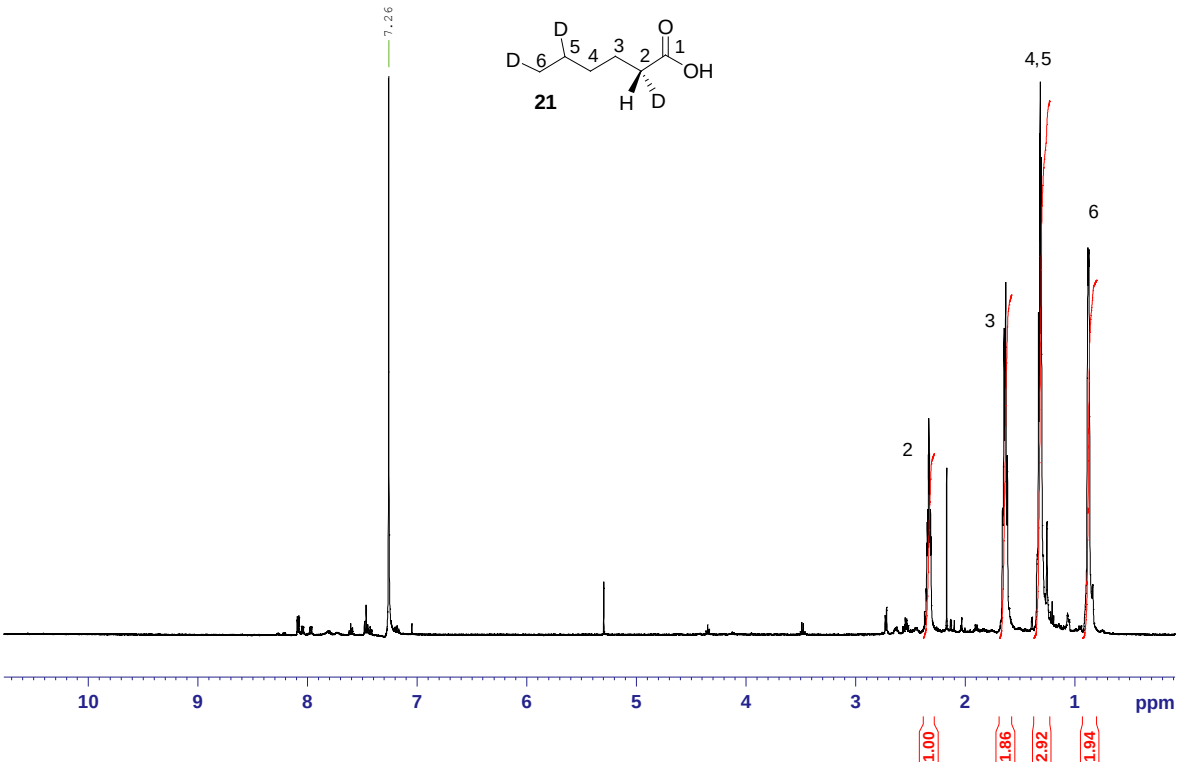
HSQC NMR of (2*R*)-[2,5,6-$^2H_3$]-hexan-1-ol (**20**)



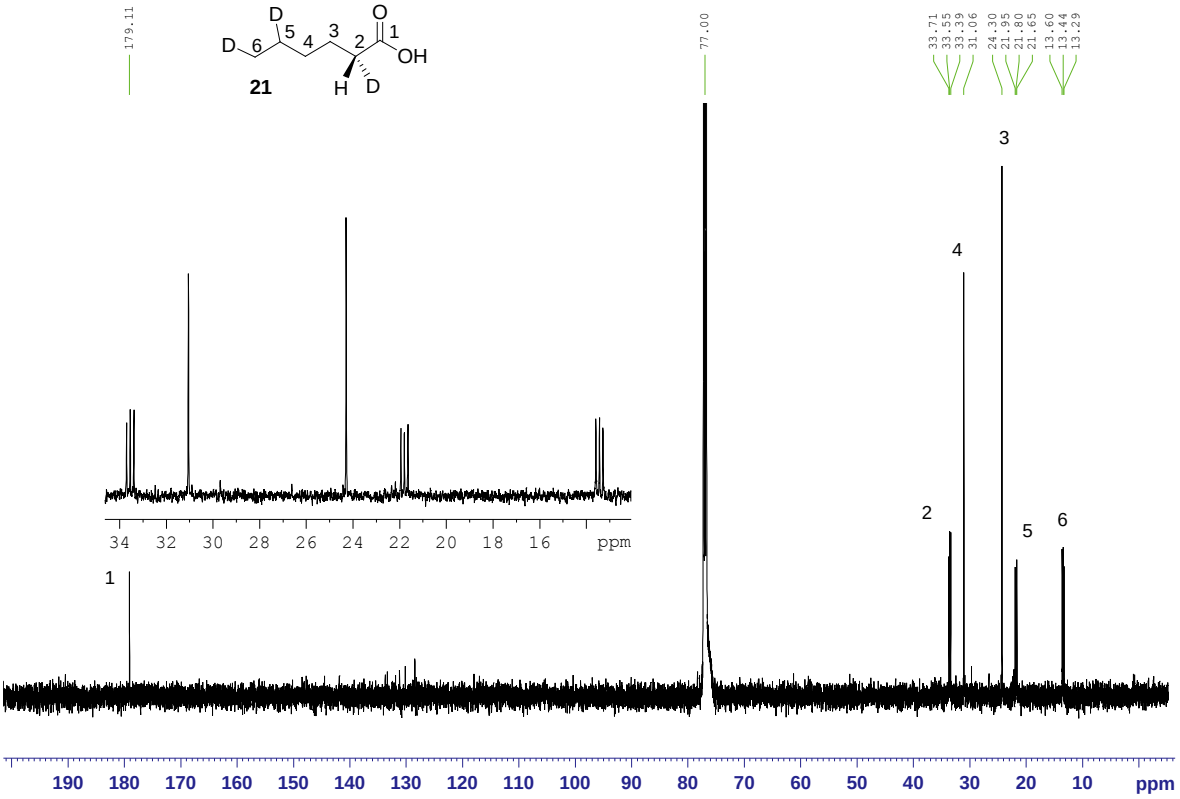
HMBC NMR of (2*R*)-[2,5,6-$^2H_3$]-hexan-1-ol (**20**)



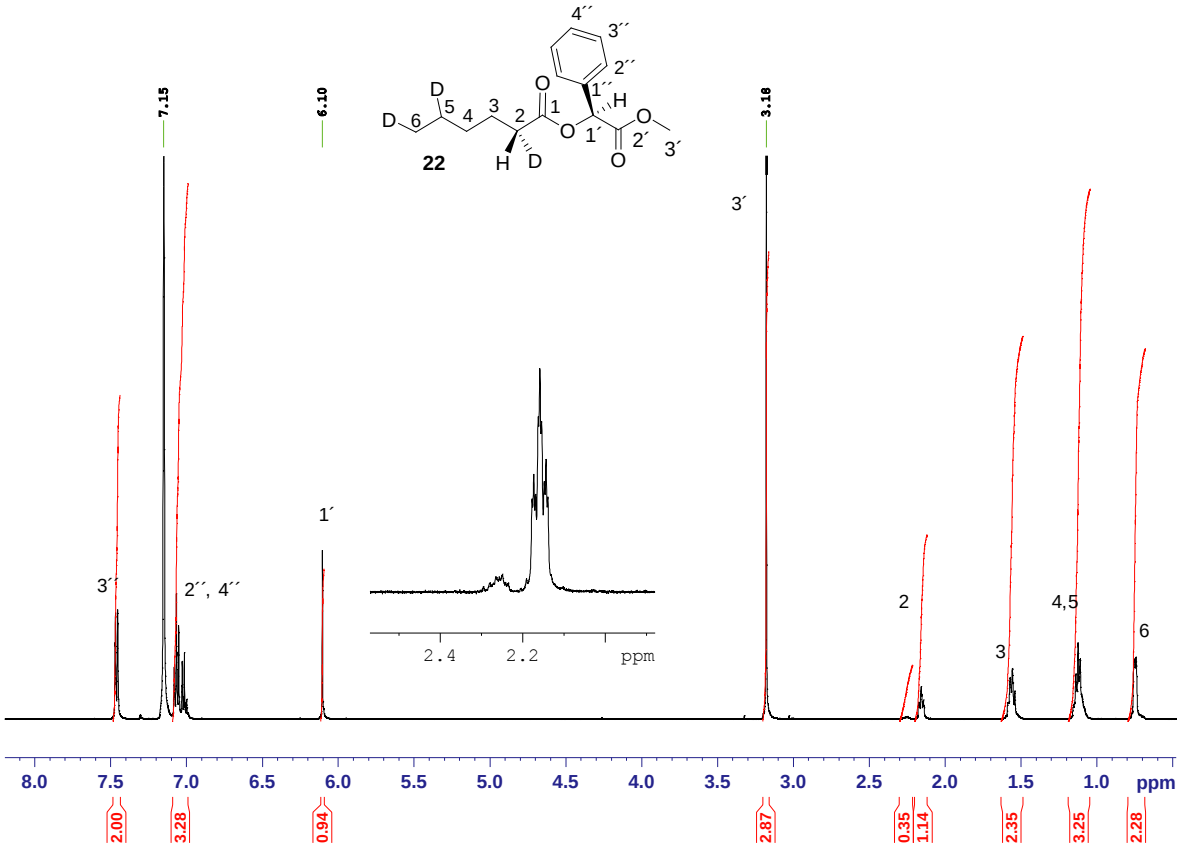
^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanoic acid (**21**)



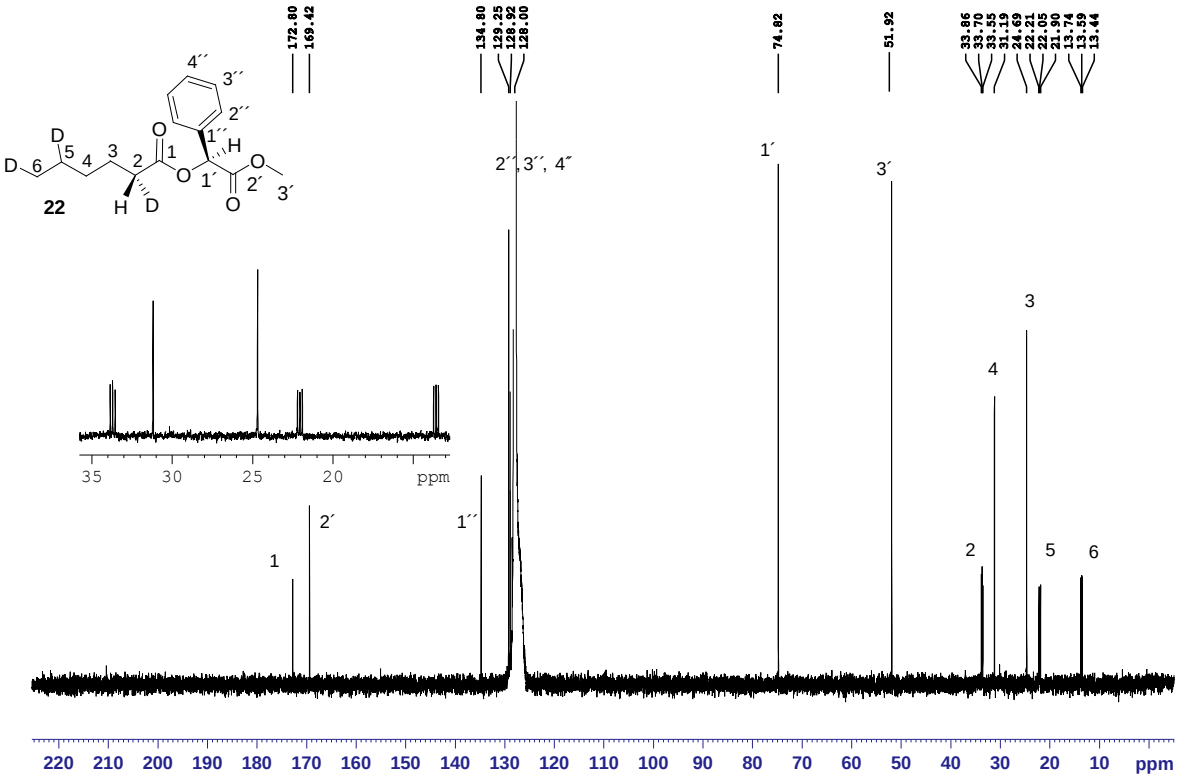
^{13}C NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanoic acid (**21**)



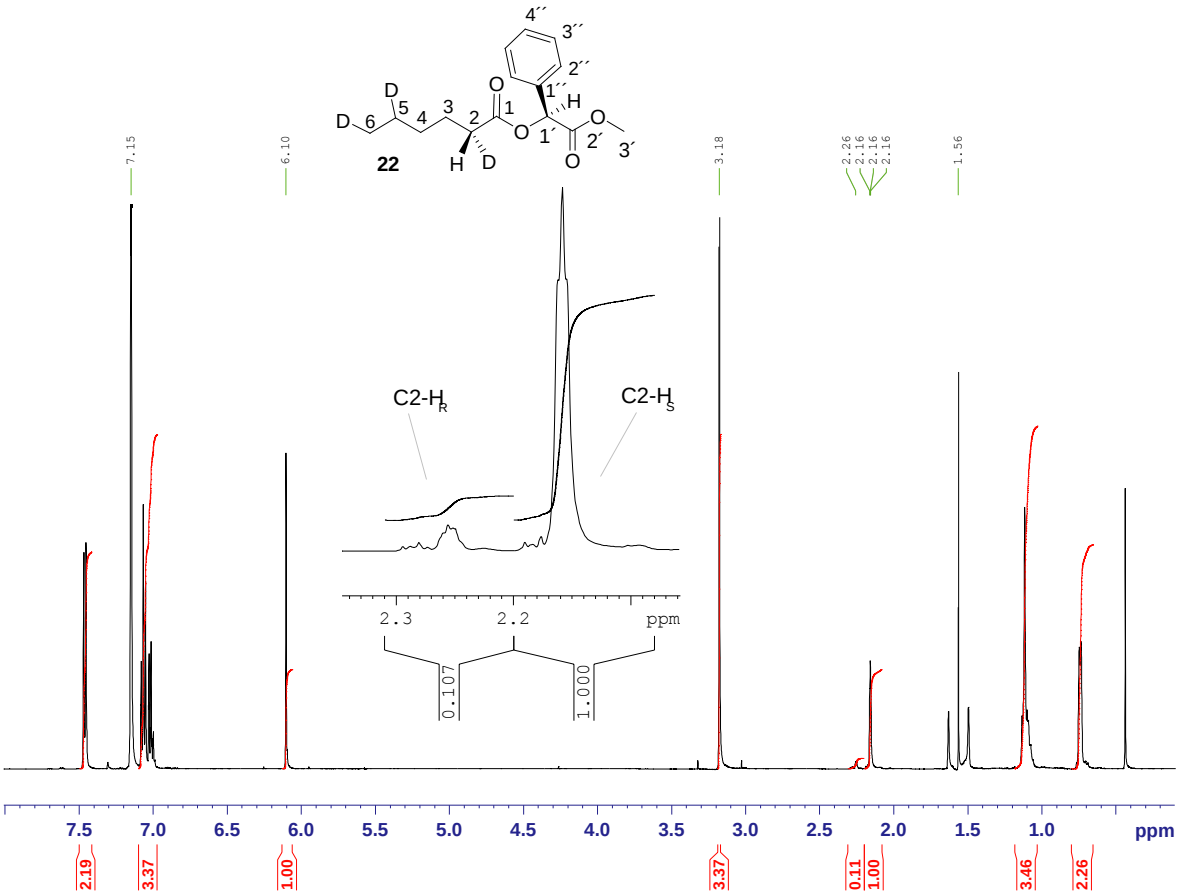
^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanoyl-(*S*)-methylmandelate diester (**22**)



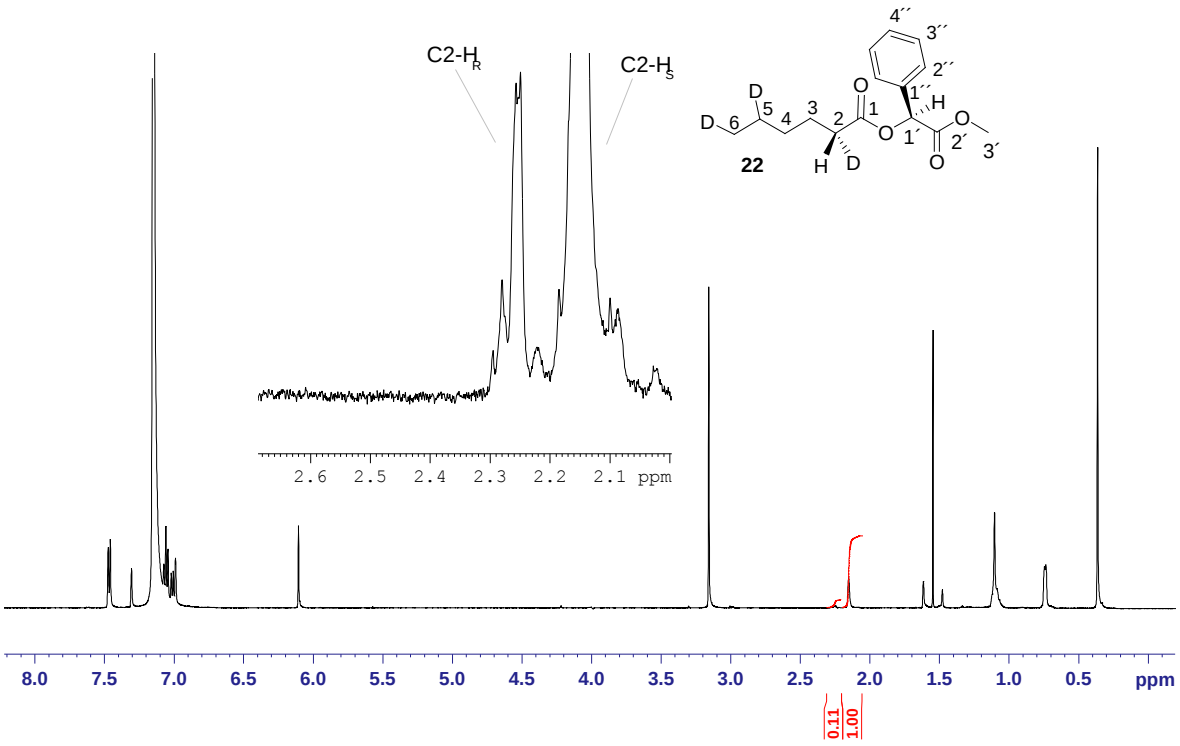
^{13}C NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanoyl-(*S*)-methylmandelate diester (**22**)



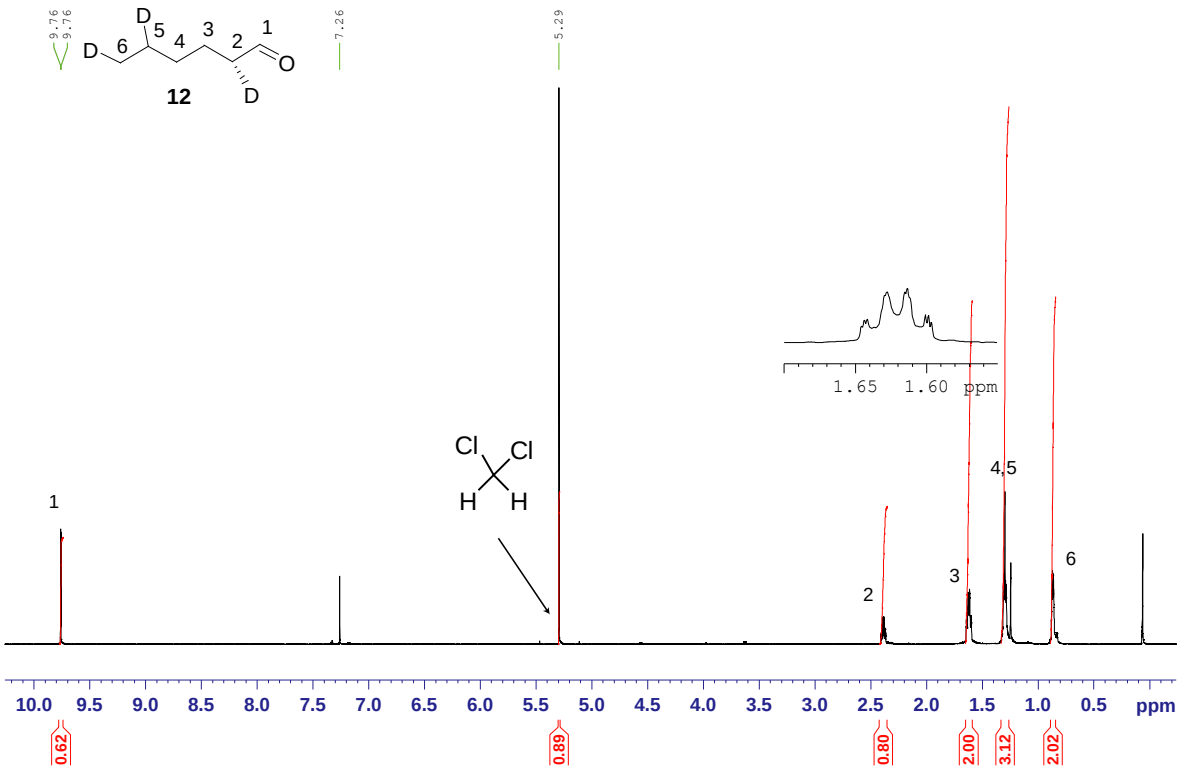
Decoupled ^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanoyl-(*S*)-methylmandelate diester (**22**), stock solution



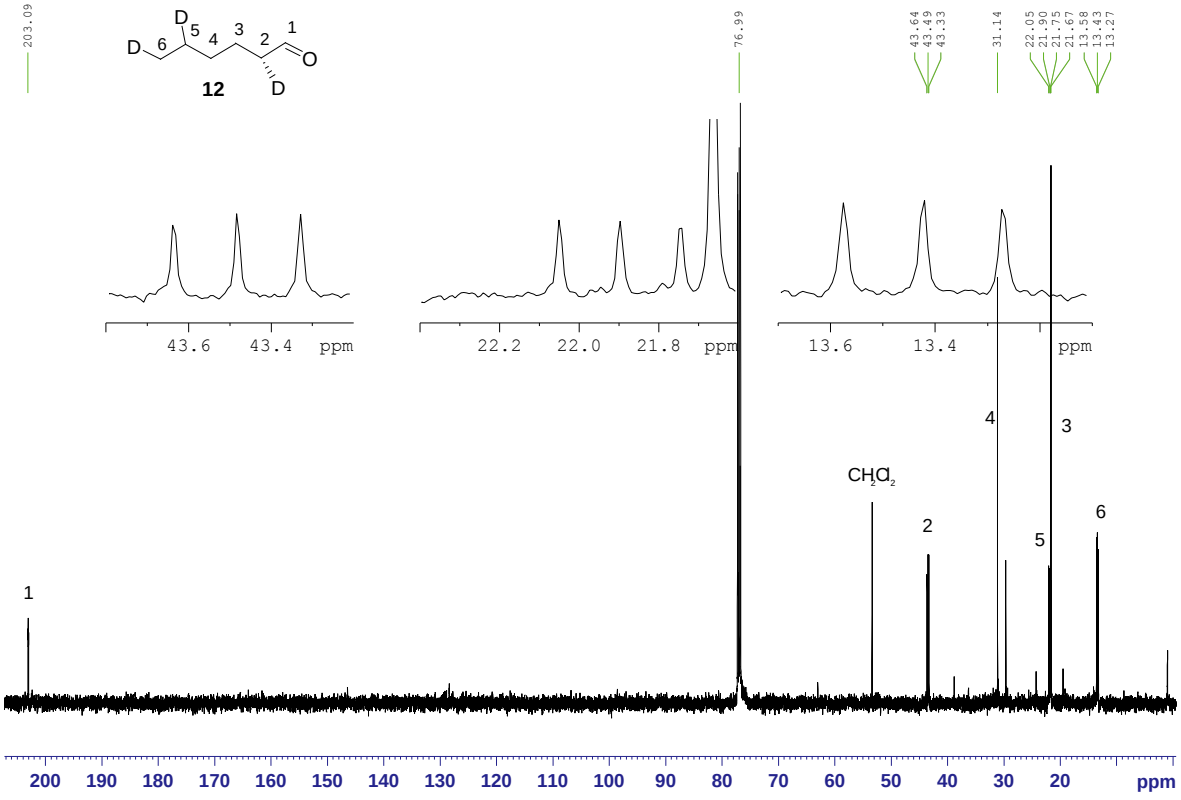
Decoupled ^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanoyl-(*S*)-methylmandelate diester (**22**), diluted solution



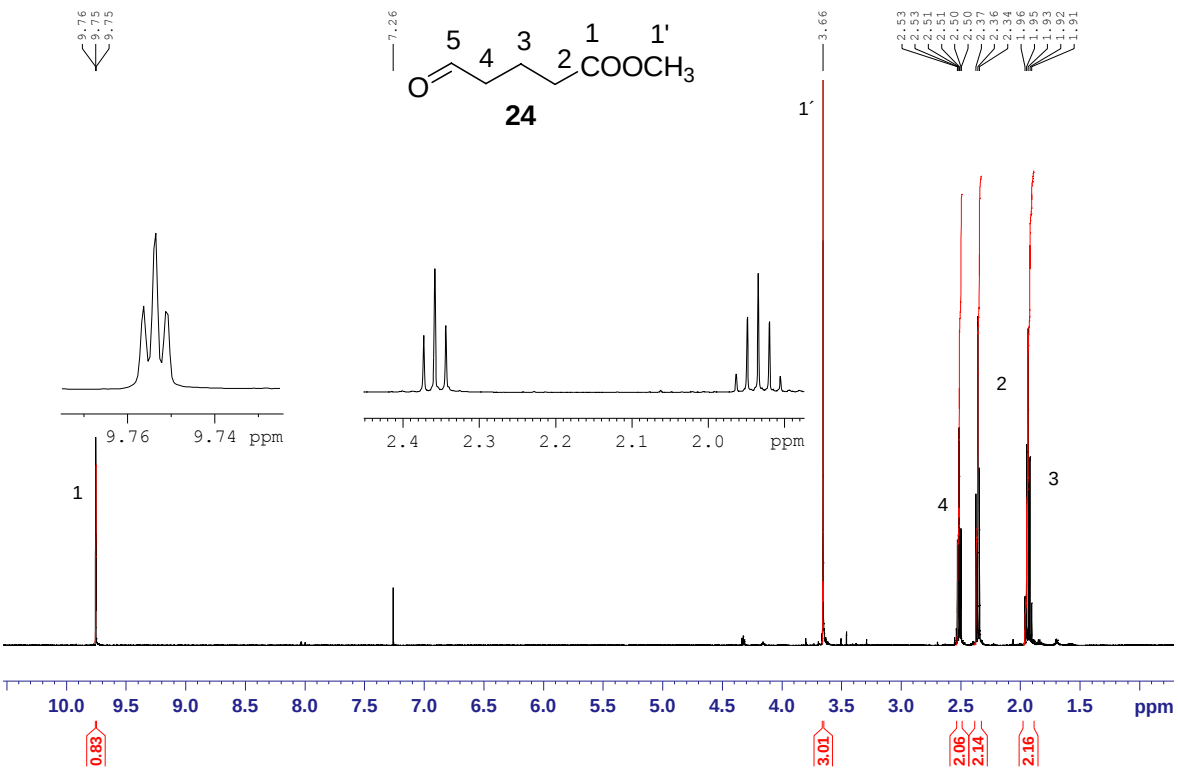
^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanal (**12**)



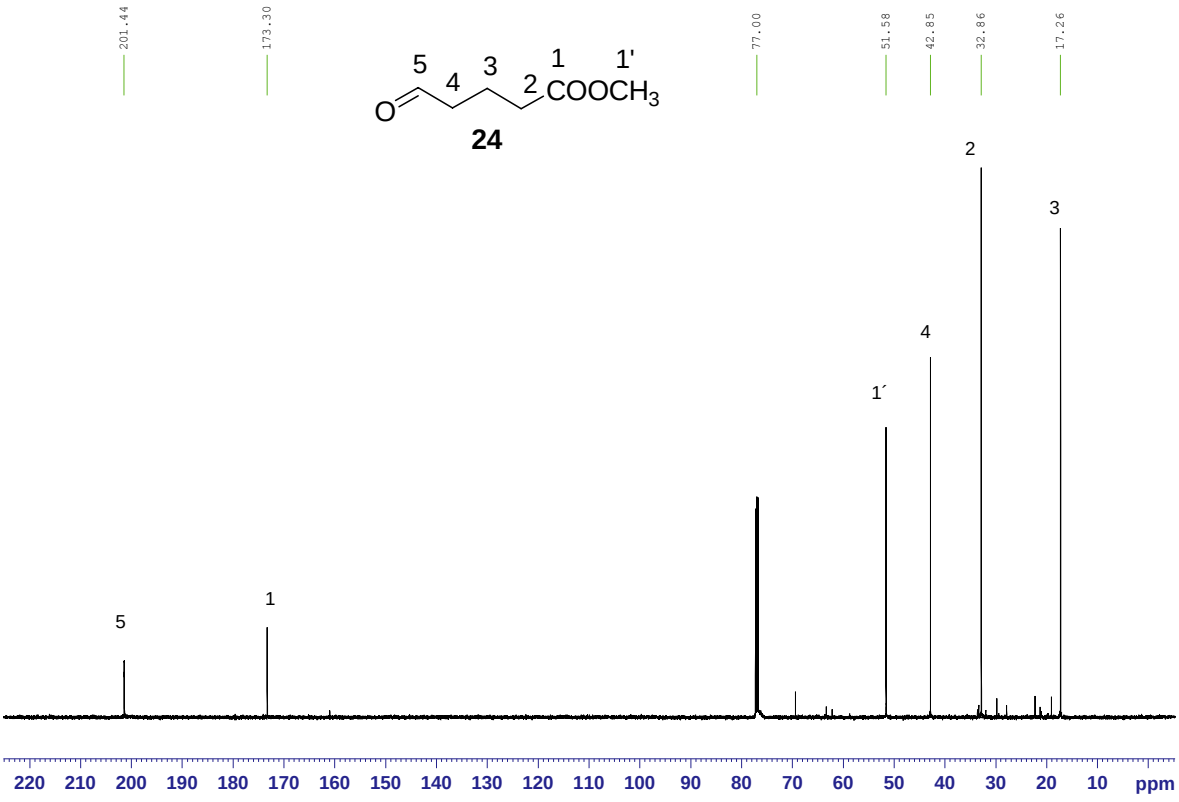
^{13}C NMR of 2(*R*)-[2,5,6-$^{2}H_3$]-hexanal (**12**)



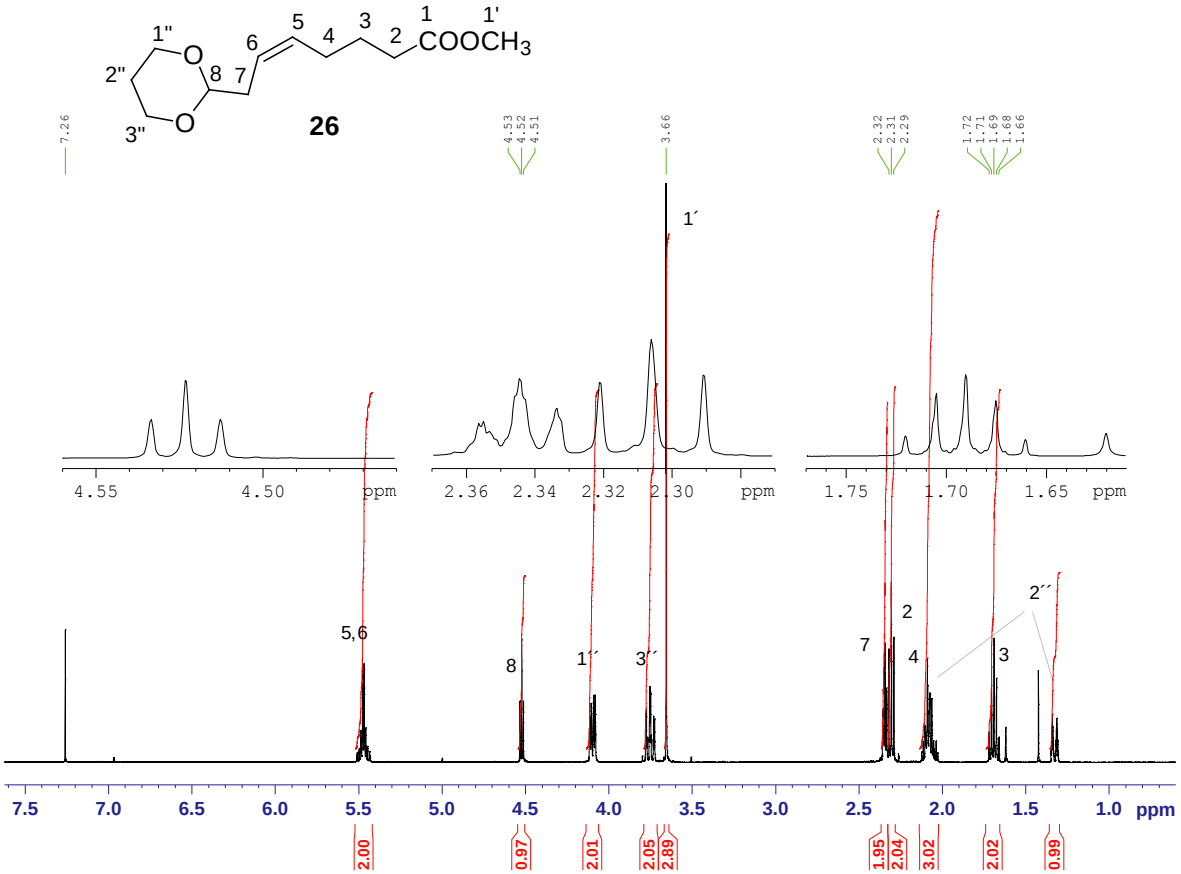
^{1}H NMR of methyl 5-oxopentanoate (**24**)



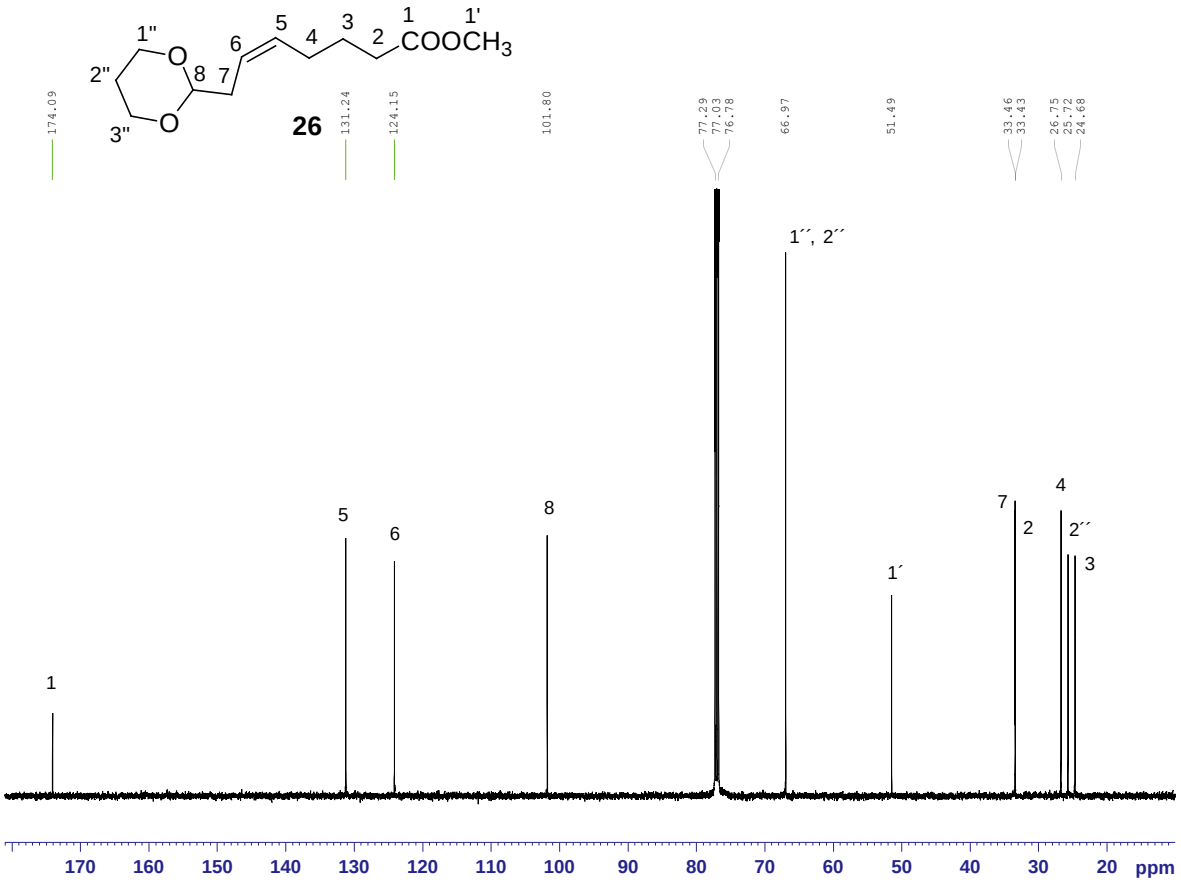
^{13}C NMR of methyl 5-oxopentanoate (**24**)



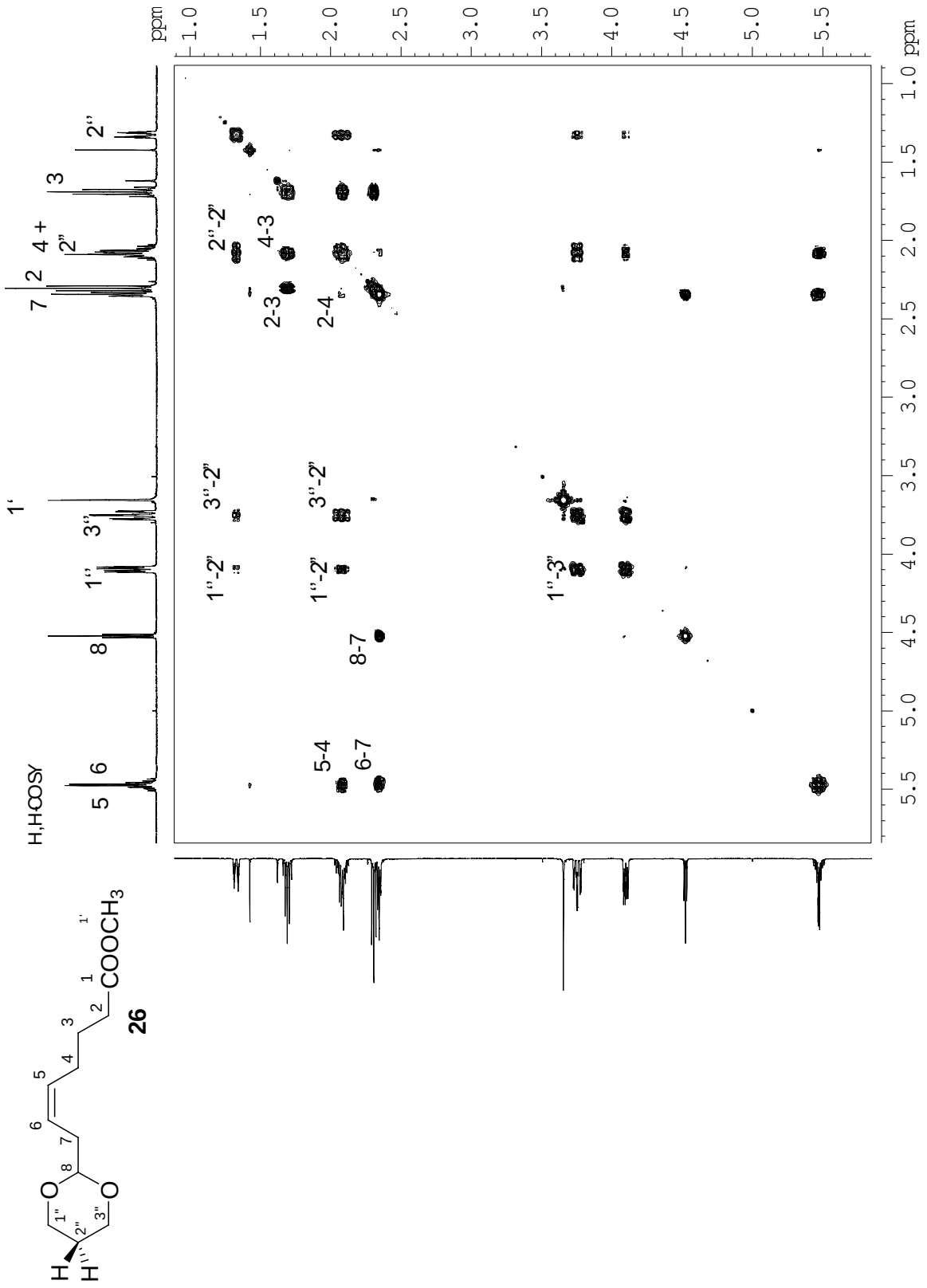
^{1}H NMR of methyl 7-(1,3,-dioxan-2-yl)-hept-5-en-oate (**26**)



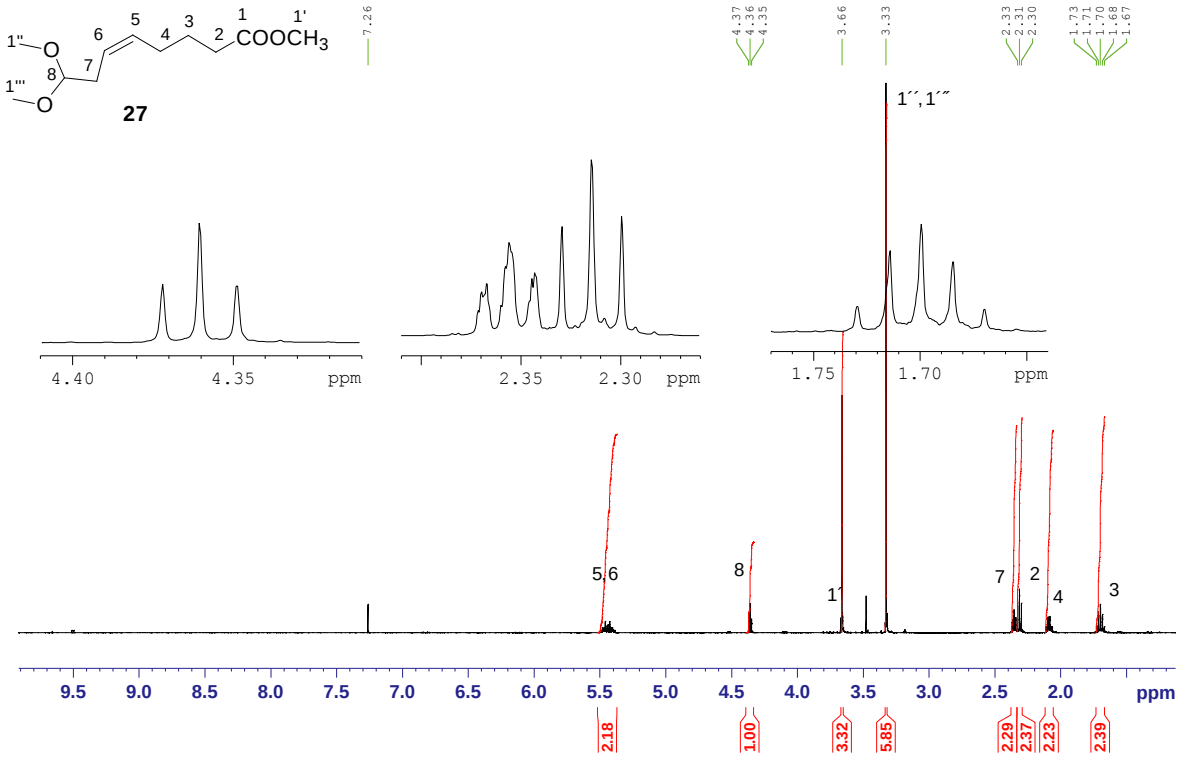
^{13}C NMR of methyl 7-(1,3,-dioxan-2-yl)-hept-5-en-oate (**26**)



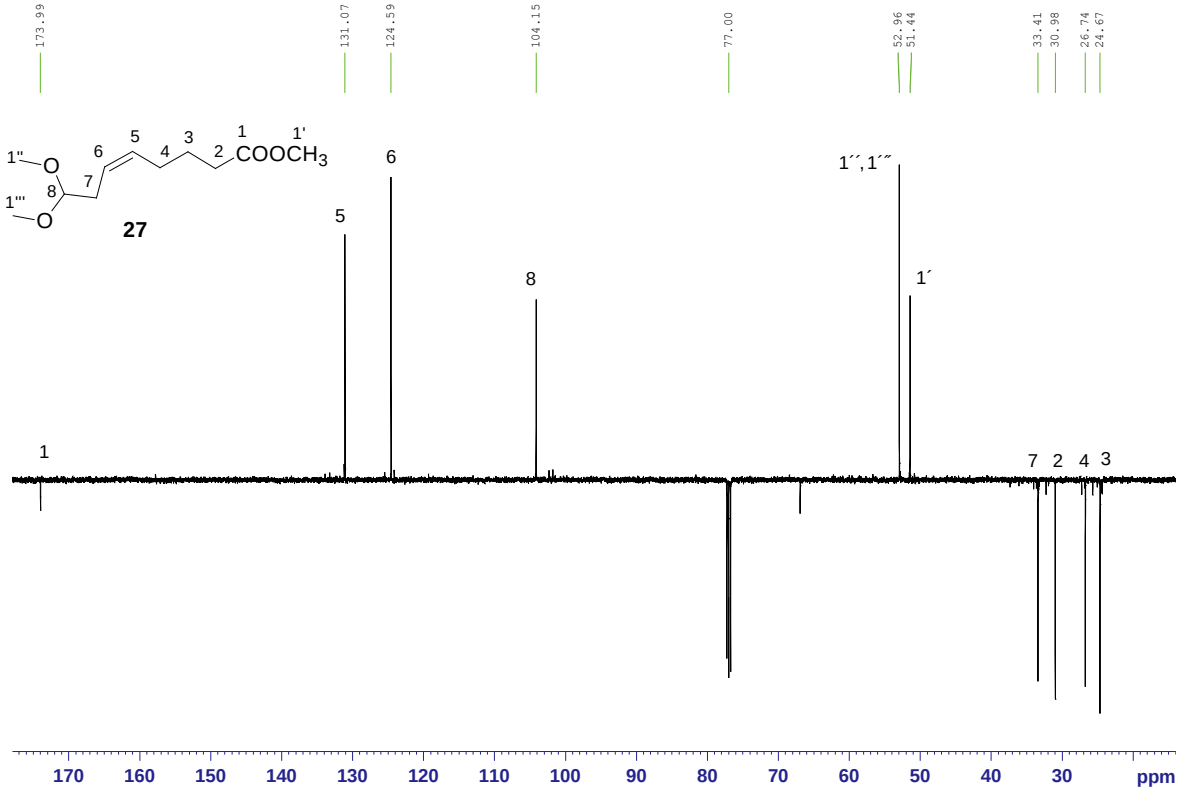
H,H-COSY-NMR of methyl 7-(1,3,-dioxan-2-yl)-hept-5-en-oate (**26**)



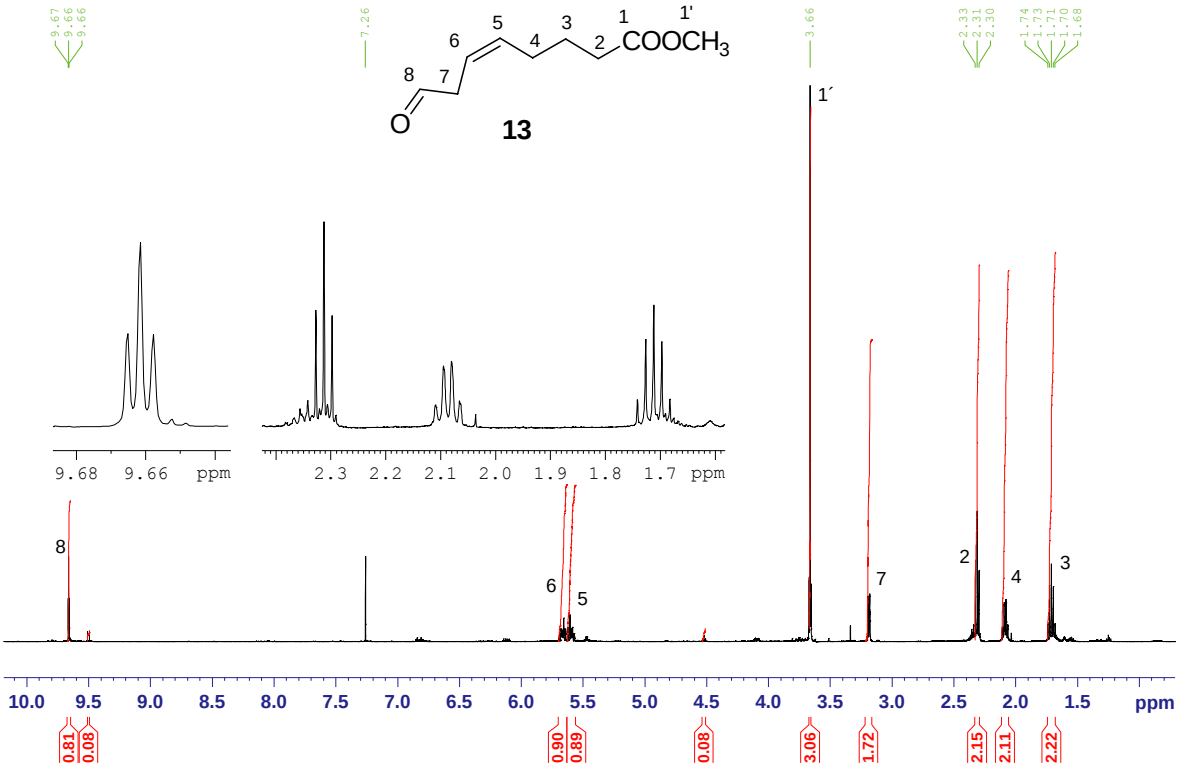
^{1}H NMR of (*Z*)-methyl 8,8-dimetoxyoct-5-enoate (**27**)



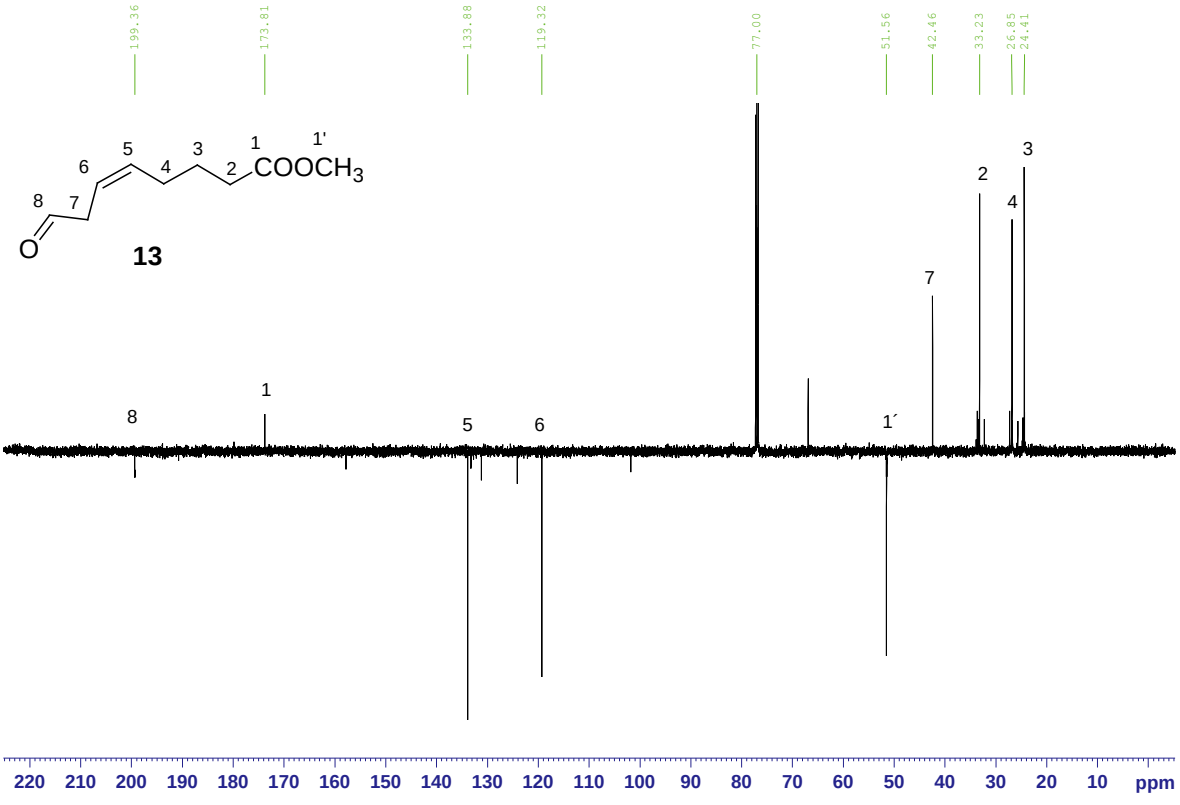
^{13}C NMR of (*Z*)-methyl 8,8-dimetoxyoct-5-enoate (**27**)



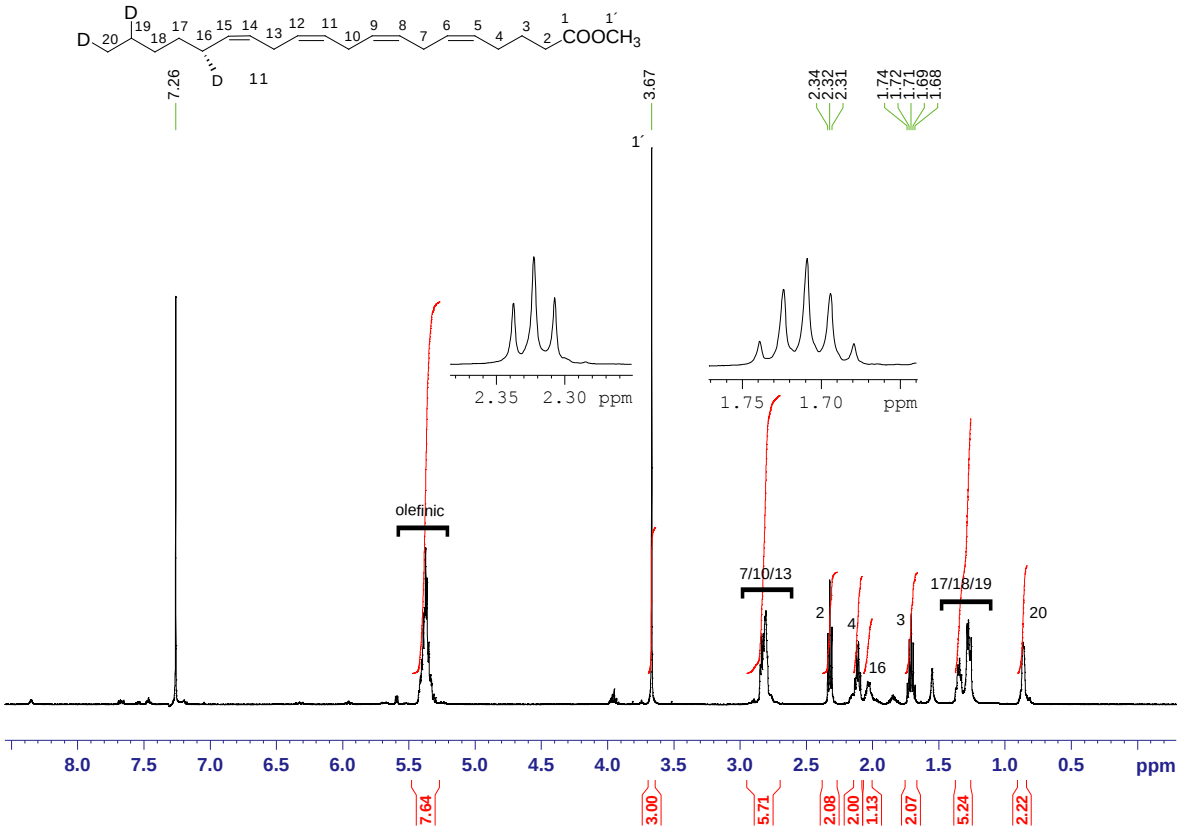
^{1}H NMR of methyl 8-oxooct-(5*Z*)-enoate (**13**)



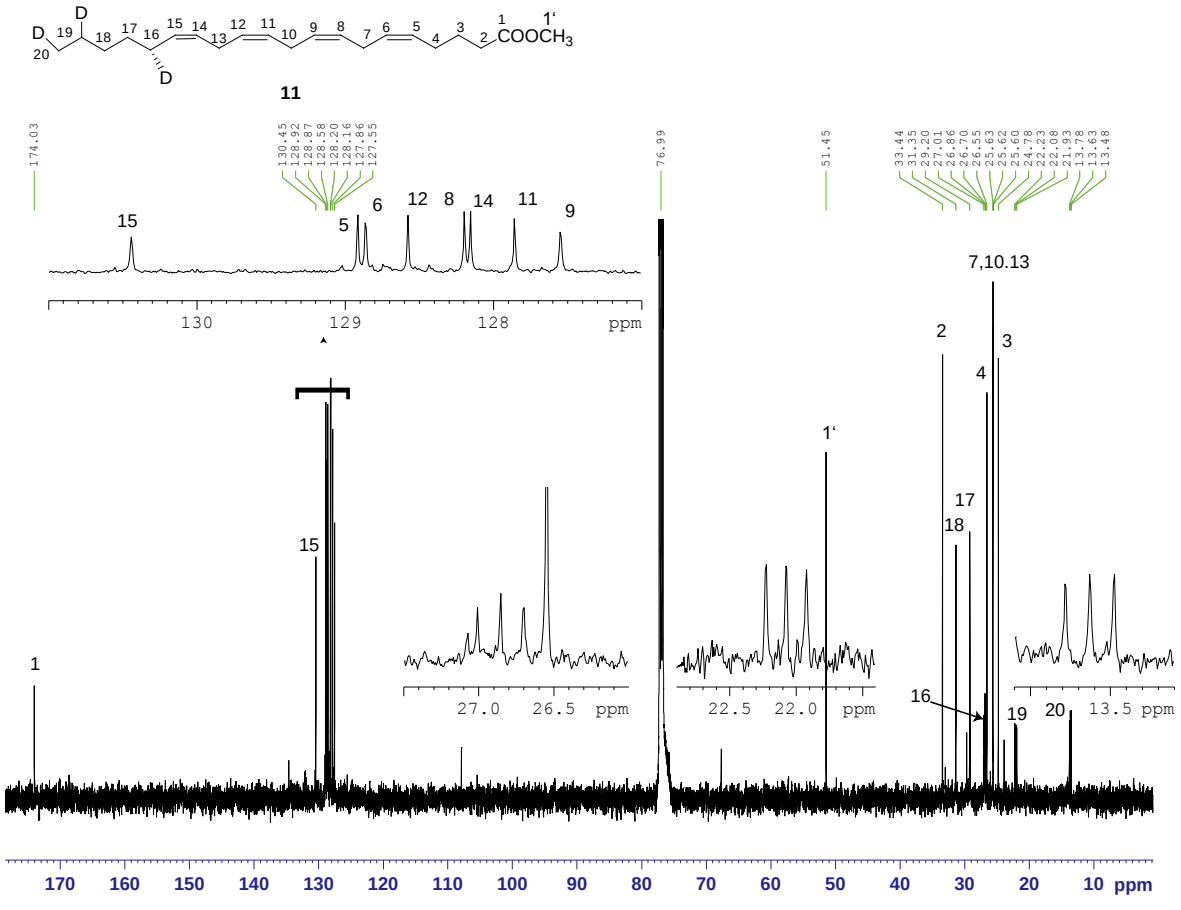
^{13}C NMR of methyl 8-oxooct-(5*Z*)-enoate (**13**)



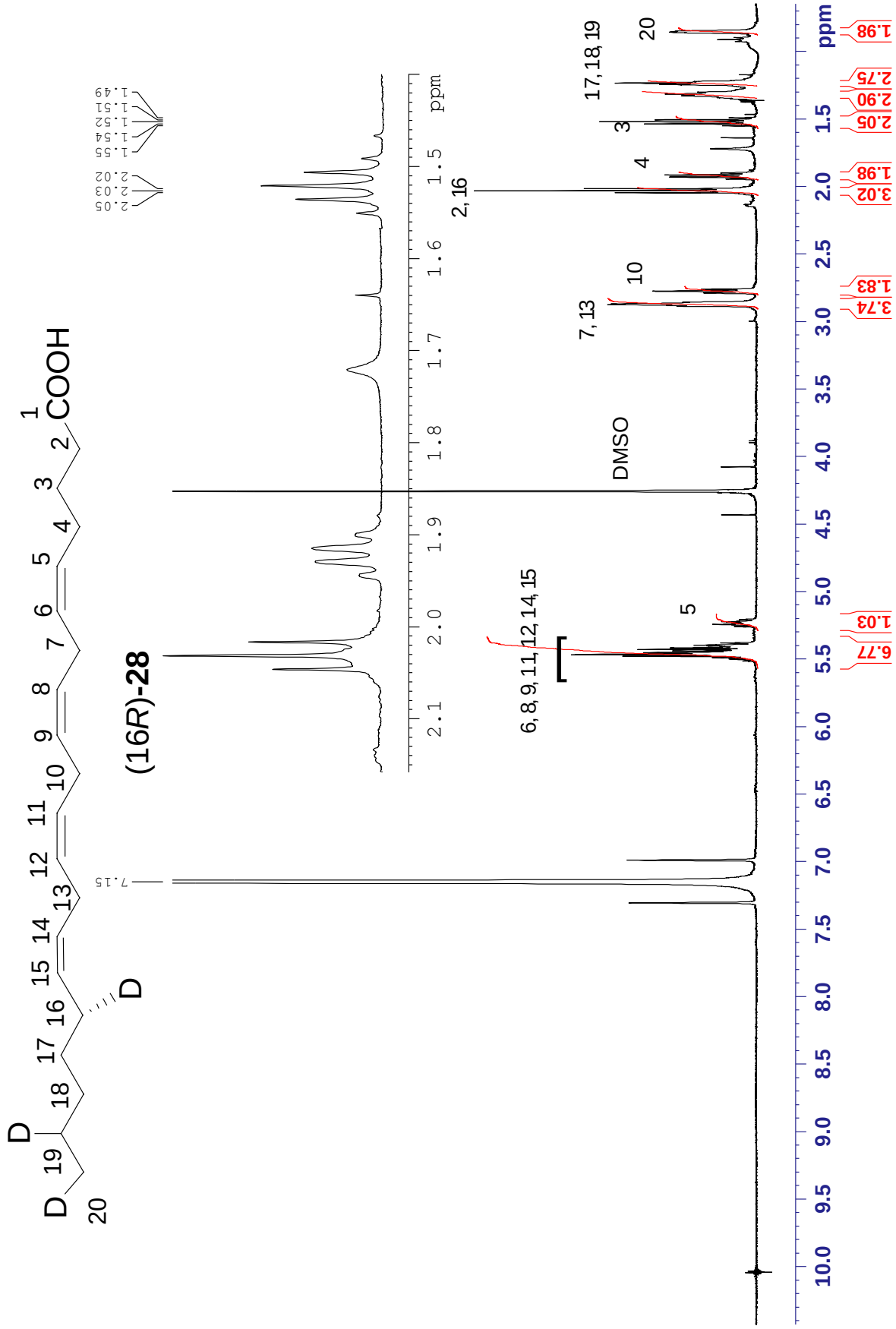
^{1}H NMR of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**11**)



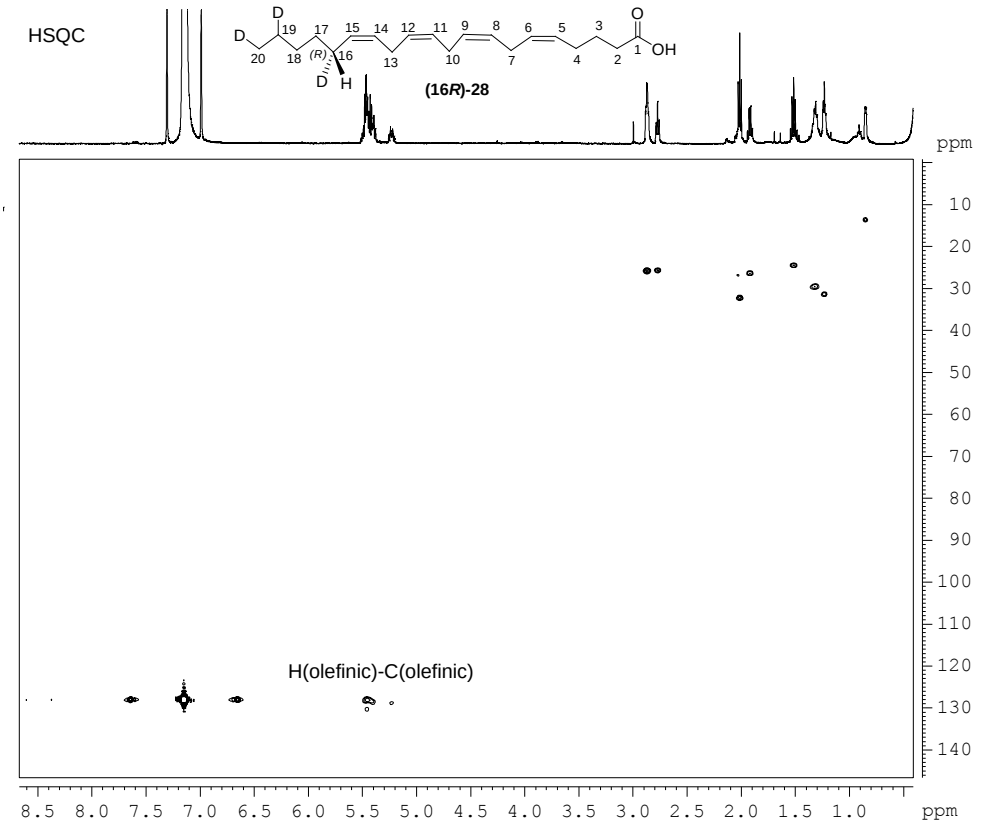
^{13}C NMR of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**11**)



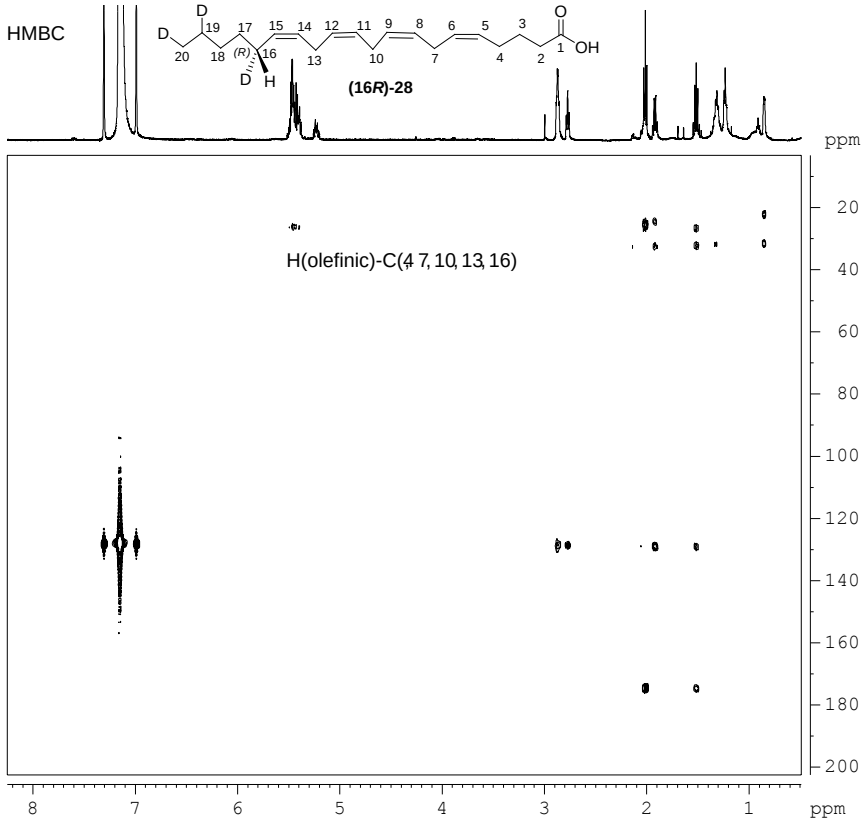
^{1}H NMR of (16*R*)-[16,19,20-^{2}H$_3$]-arachidonic acid [(16*R*)-**28**]



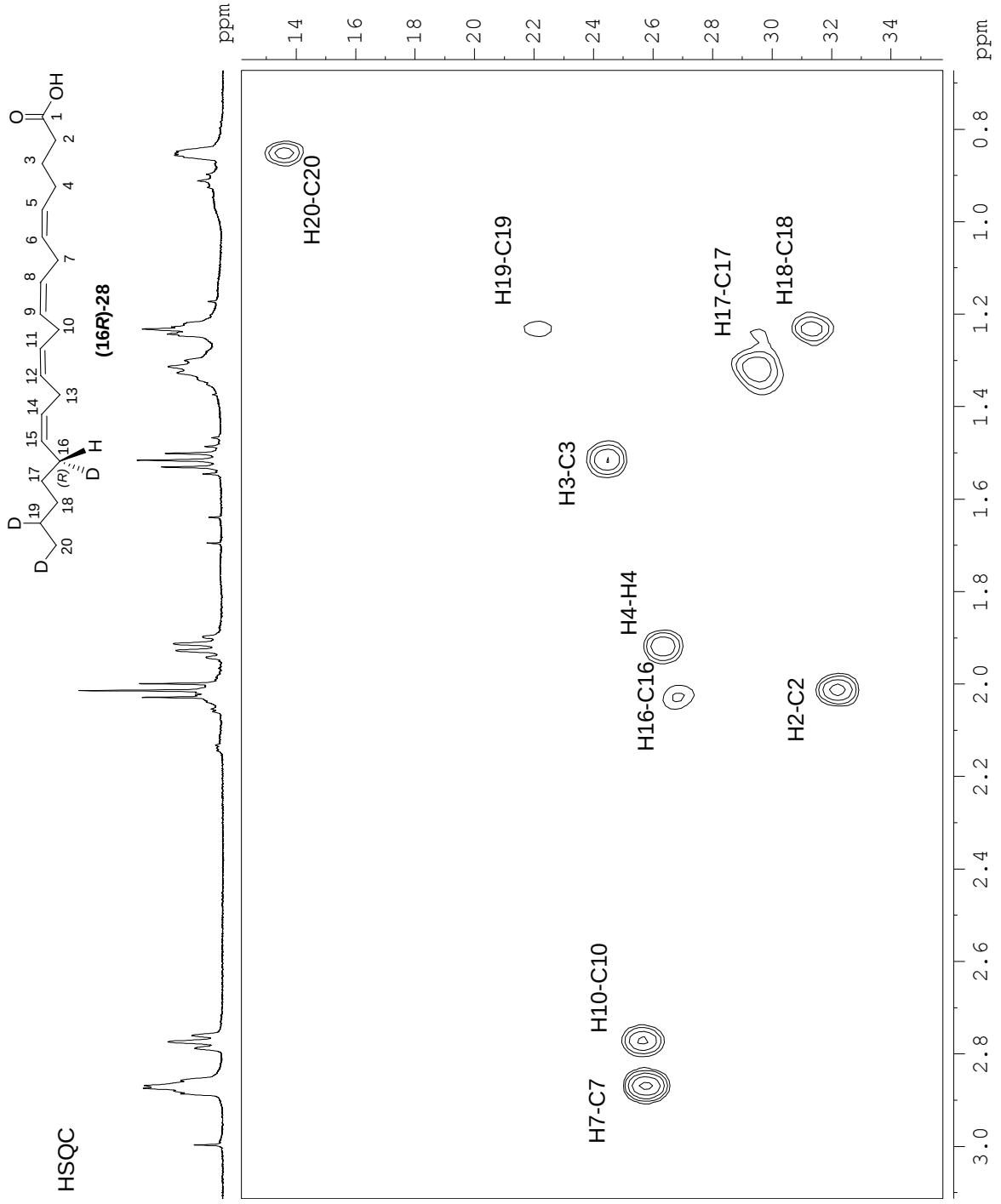
HSQC NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid [(16*R*)-**28**]



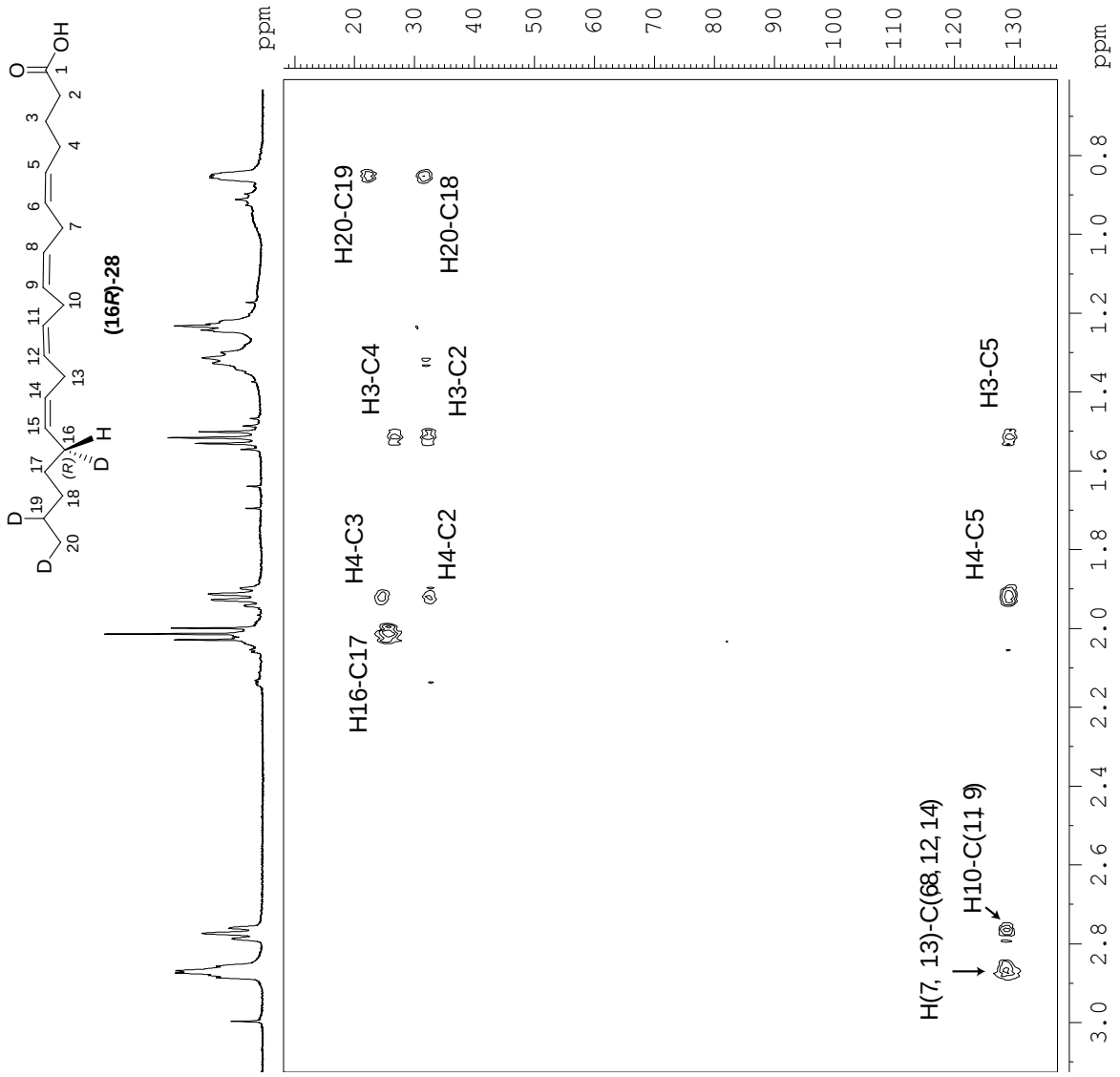
HMBC NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid [(16*R*)-**28**]



Enlarged view of HSQC NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid [(16*R*)-**28**]

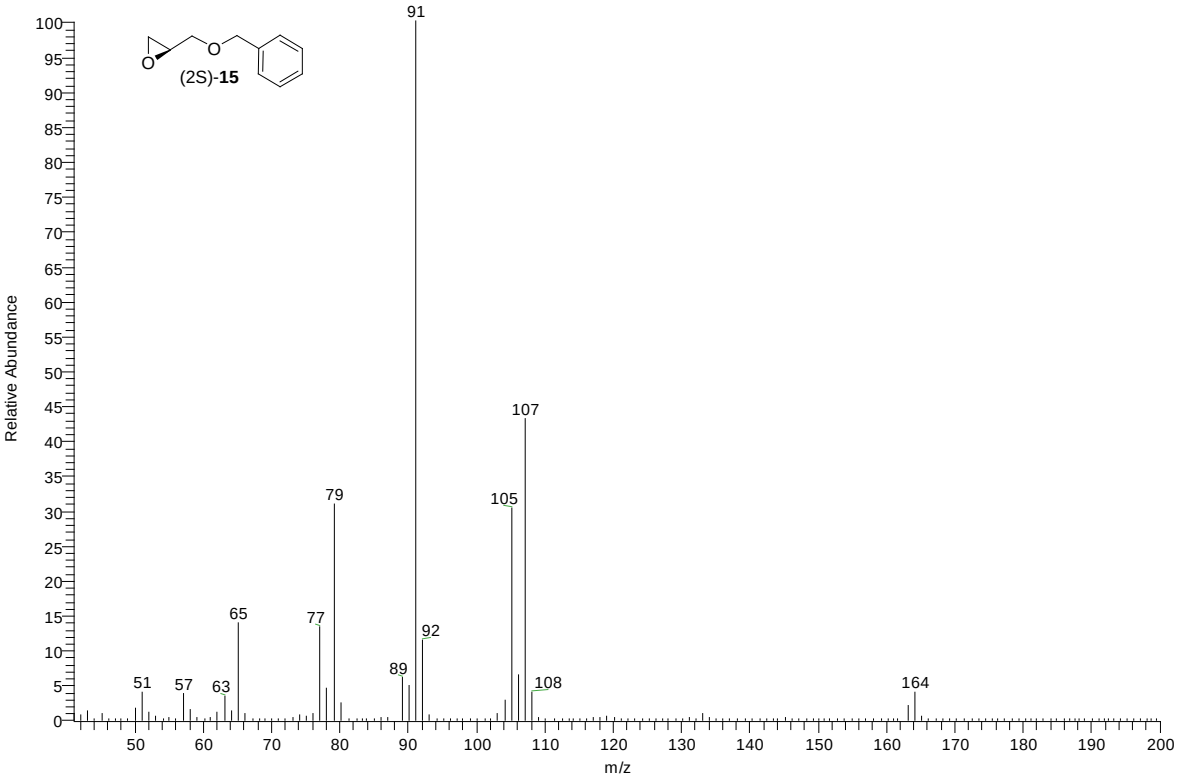


Enlarged view of HMBC NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid [(16R)-**28**]

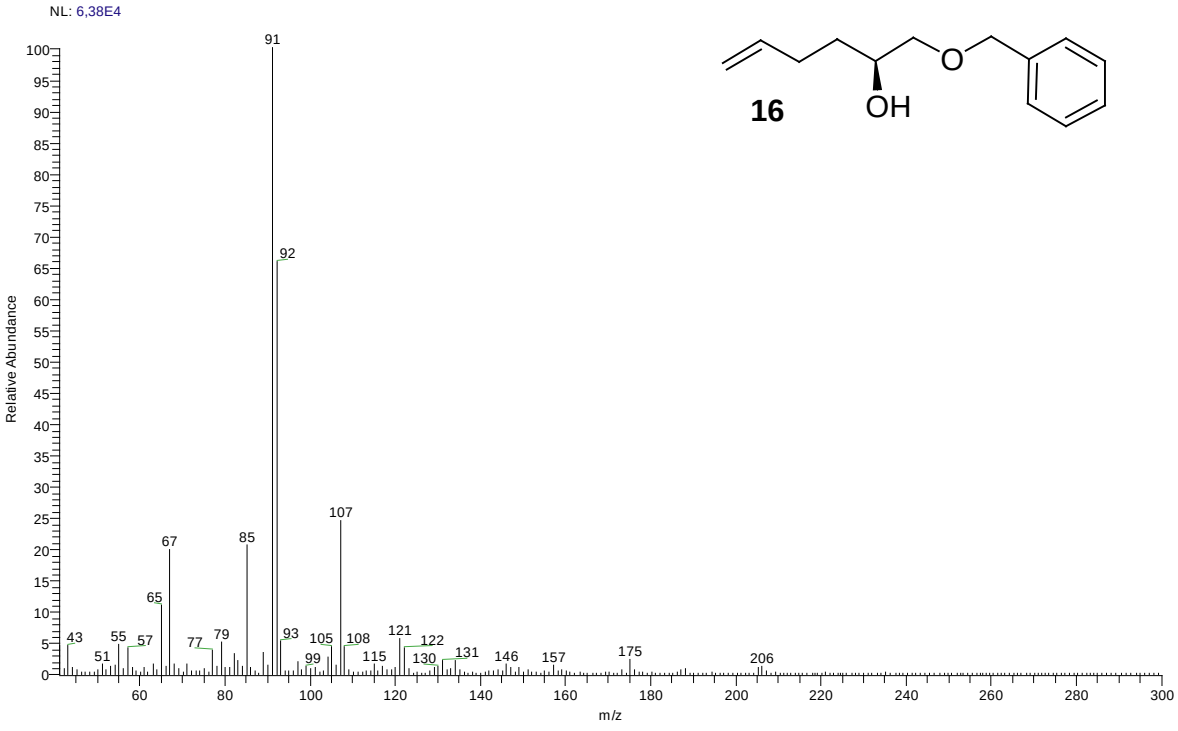


10.2 MS and GC/MS spectra

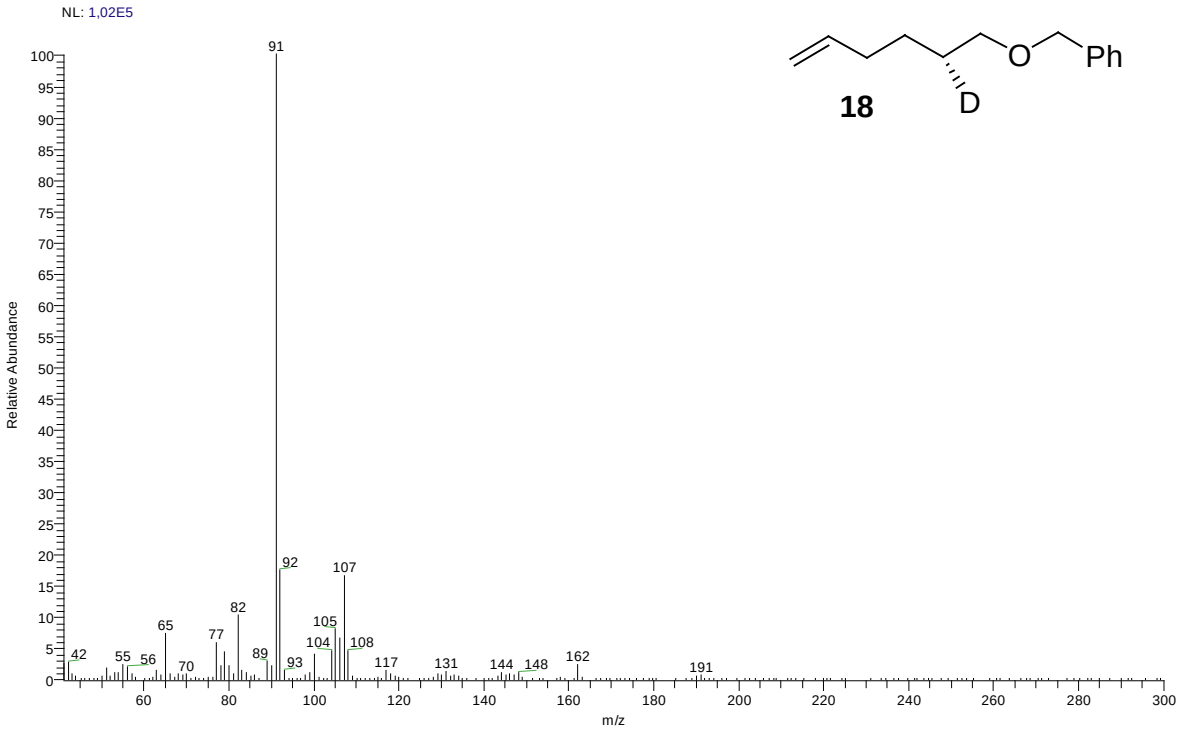
(2*S*)-glycidylbenzyl ether ((2*S*)-**15**)



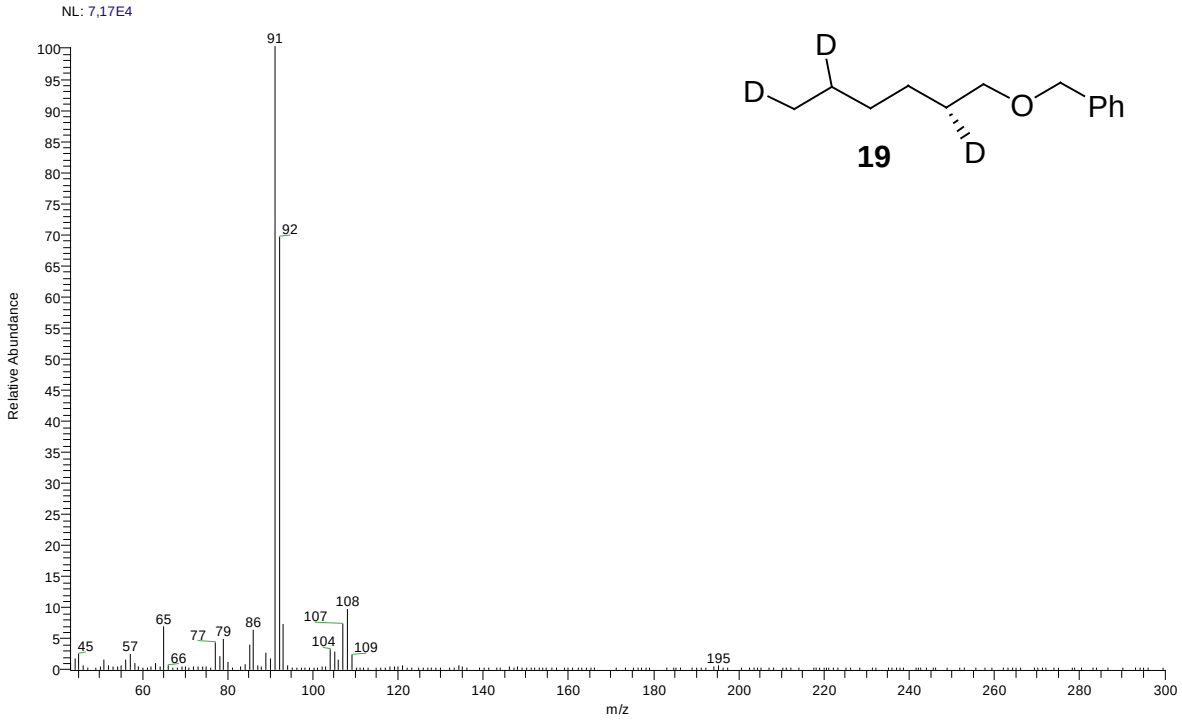
(2*S*)-1-(benzyloxy)hex-5-en-2-ol (**16**)



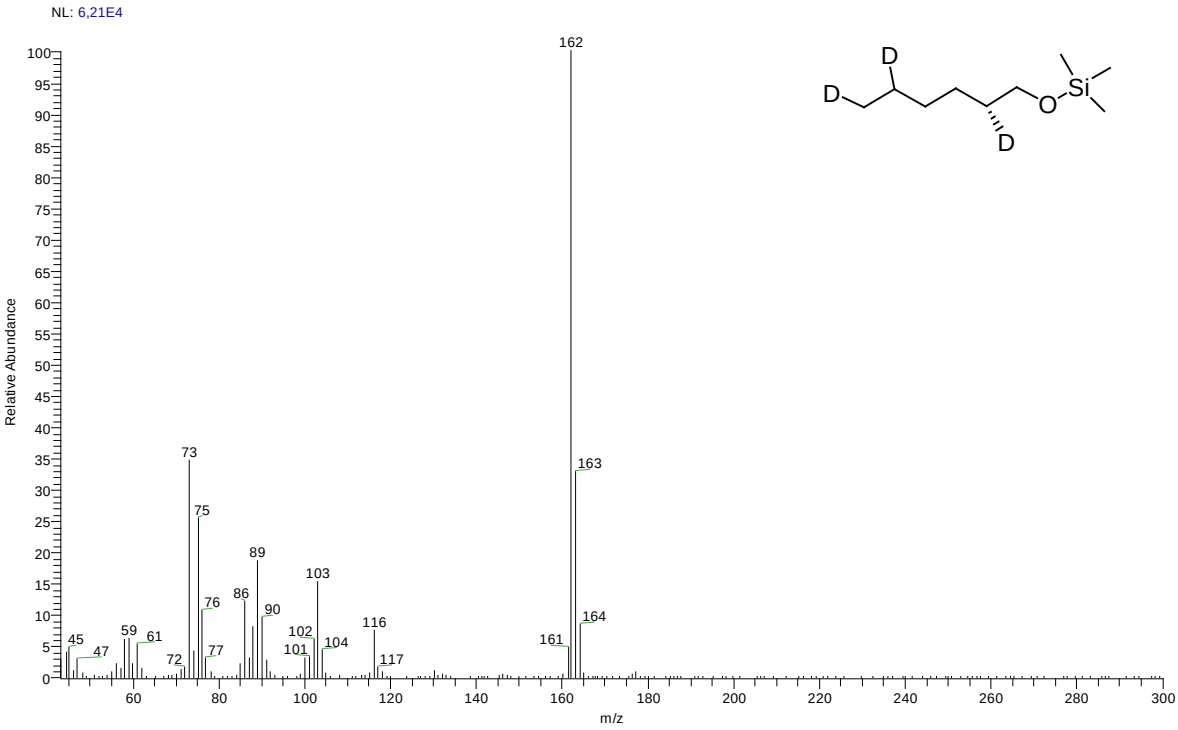
1-(2*R*)-[2-^{2}H]-hex-5-enyloxy)methyl)benzene (**18**)



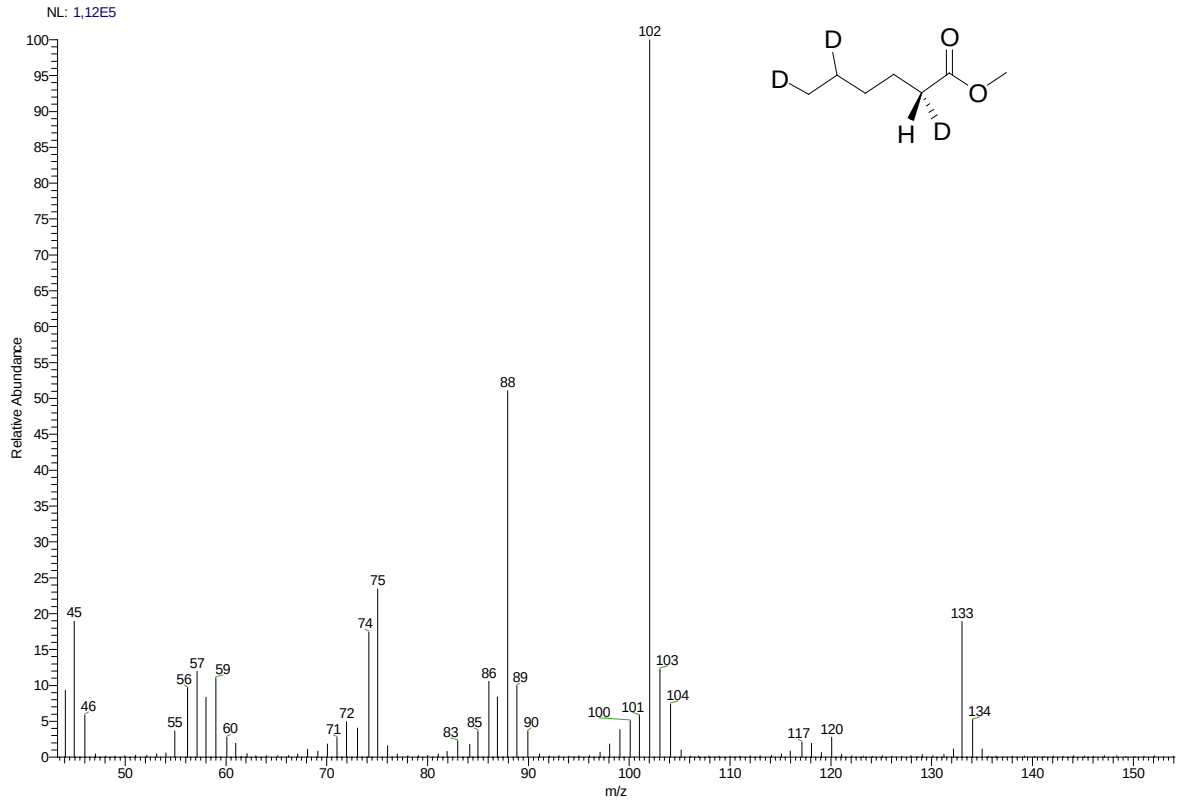
1-(2*R*)-[2,5,6-^{2}H$_{3}$]-hexyloxy)methyl)benzene (**19**)



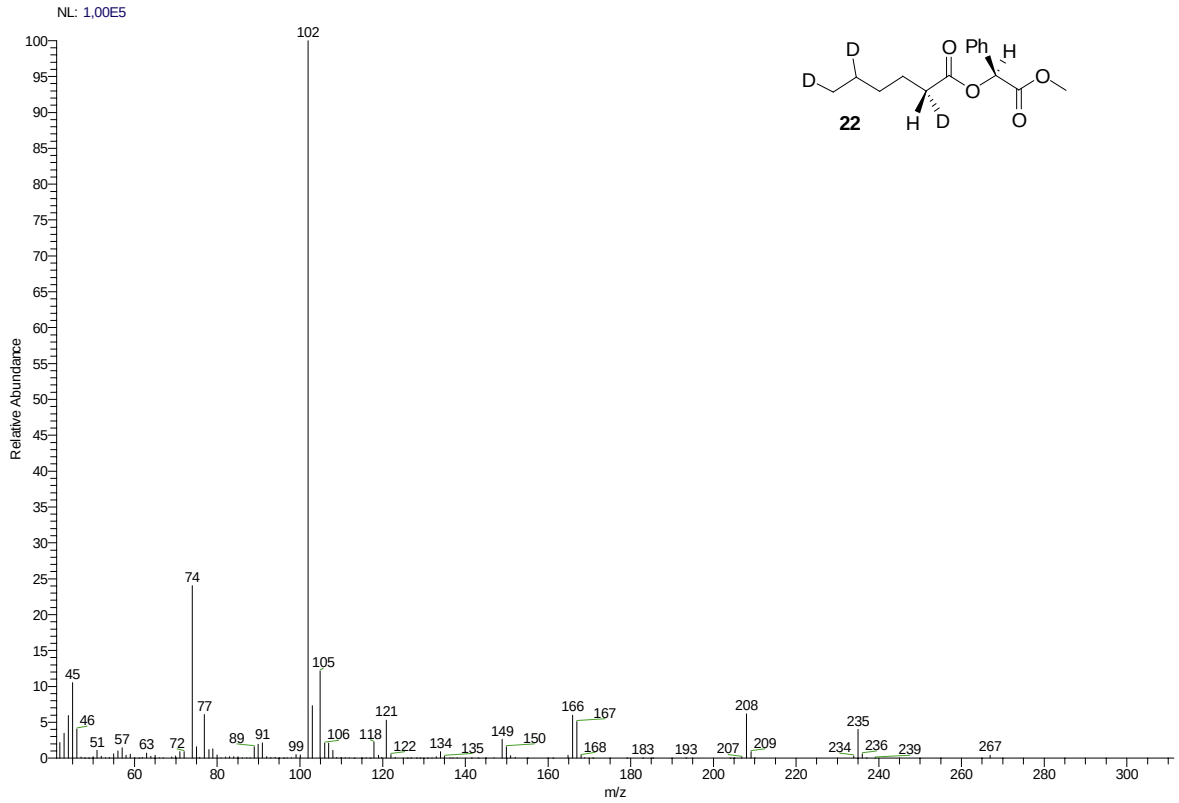
Trimethylsilyl-(2*R*)-[2,5,6-$^{2}H_{3}$]-hexan-1-ol



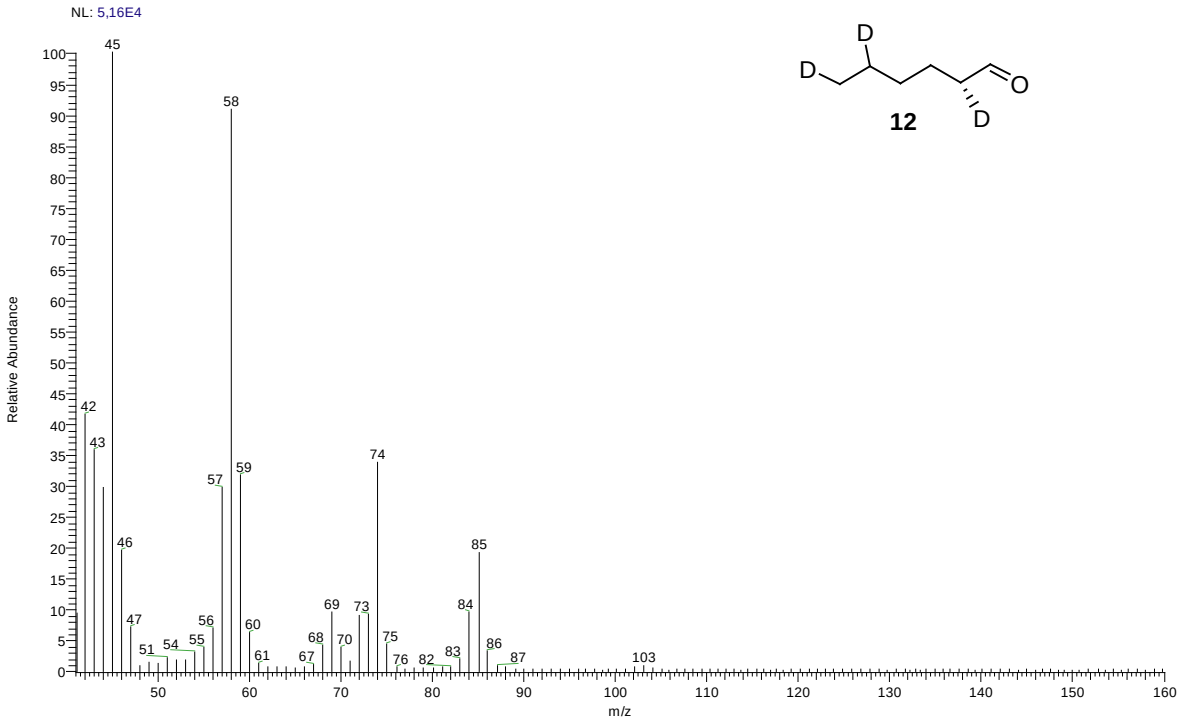
(2*R*)-[2,5,6-^{2}H]-Hexanoic acid methyl ester



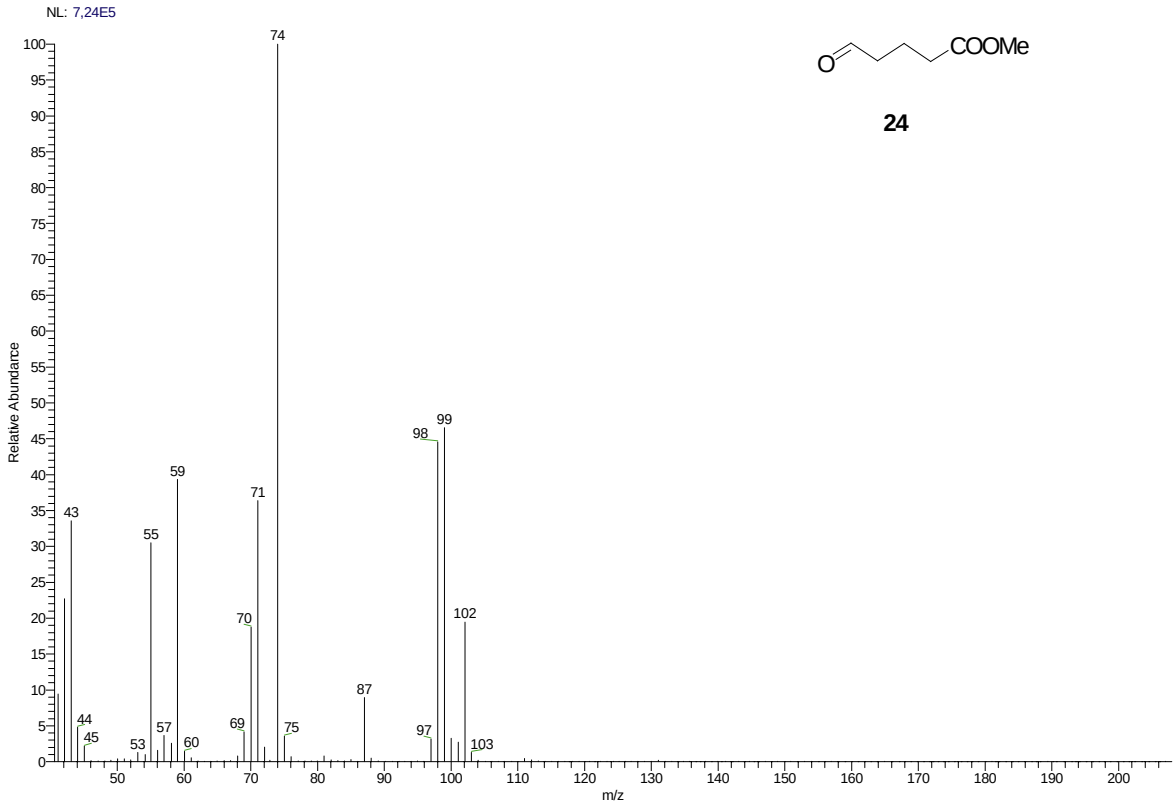
(2*R*)-[2,5,6-$^{2}H_3$]-Hexanoyl-(*S*)-methylmandelate diester (**22**)



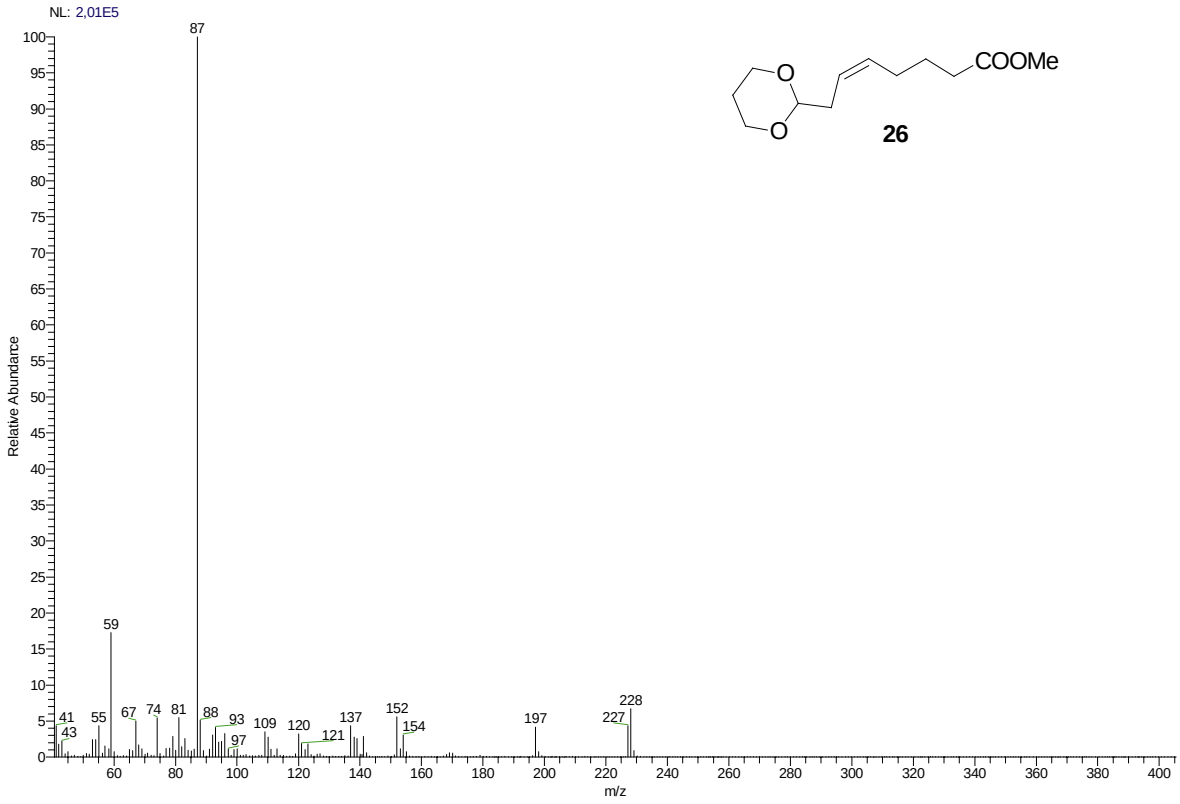
(2*R*)-[2,5,6-$^{2}H_3$]-Hexanal (**12**)



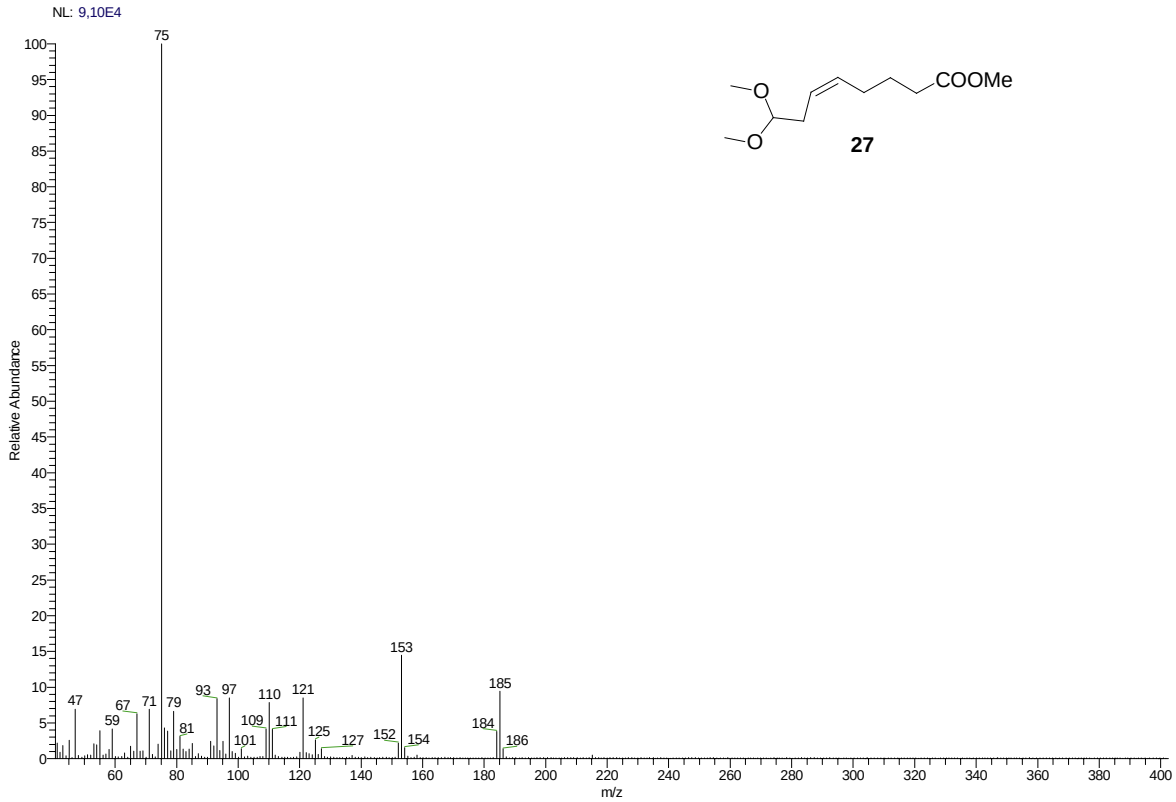
Methyl 5-oxopentanoate (**24**)



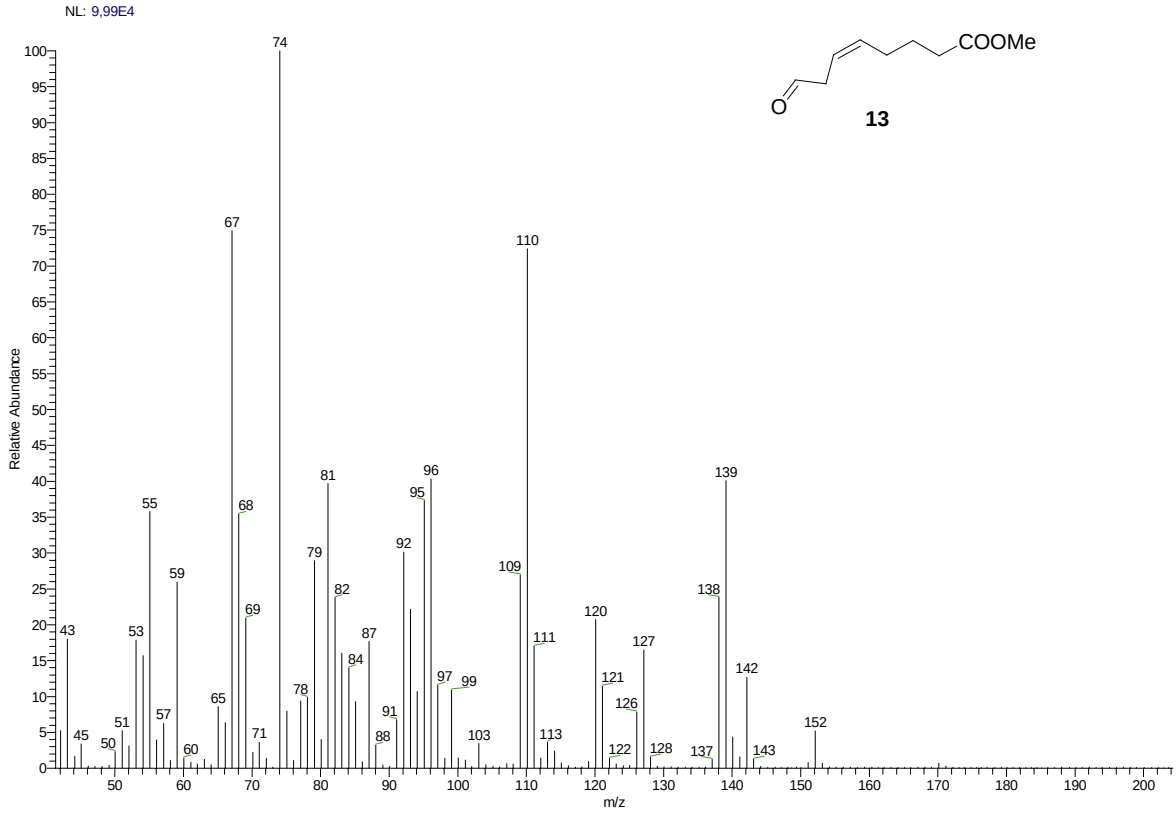
Methyl 7-(1,3,-dioxan-2-yl)-hept-(5*Z*)-en-oate (**26**)



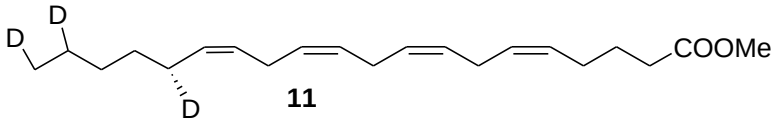
Methyl 8,8-dimethoxyoct-(5*Z*)-enoate (**27**)



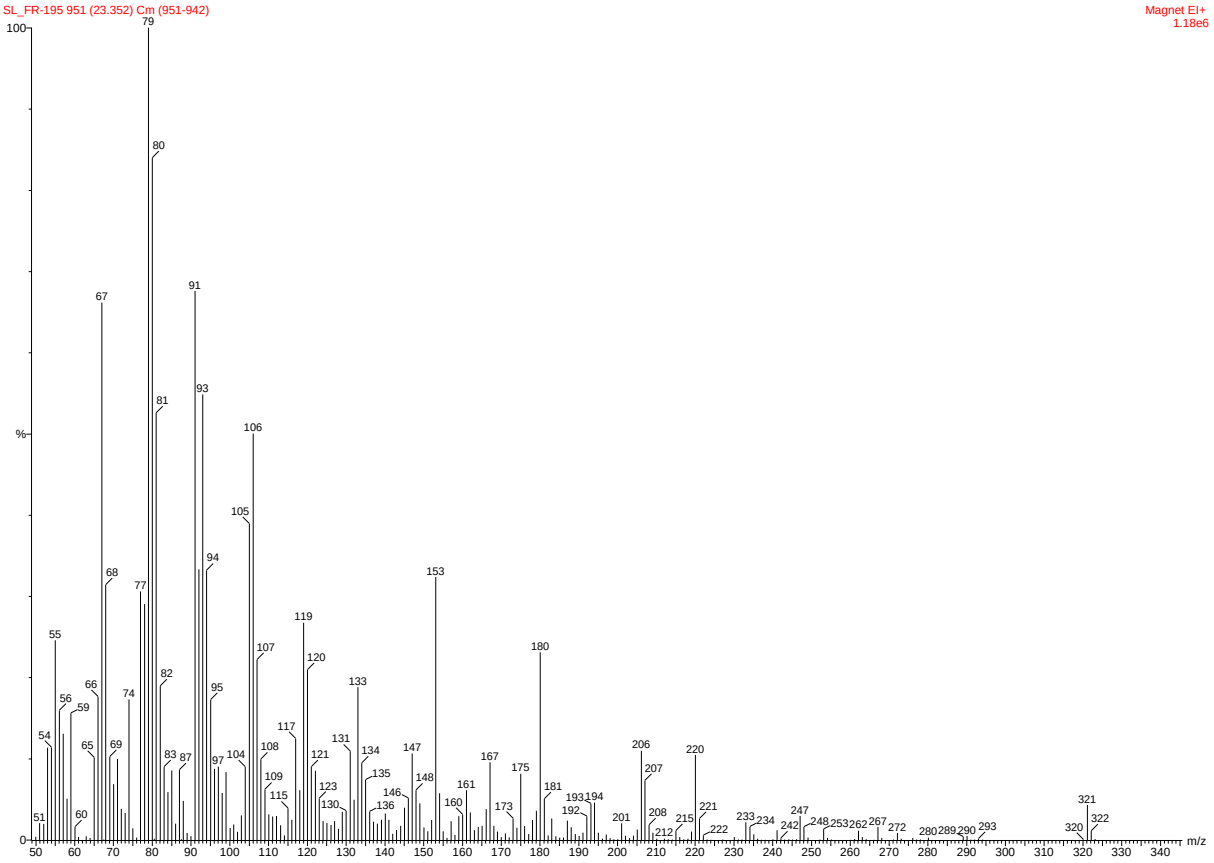
Methyl 8-oxooct-(5*Z*)-enoate (**13**)

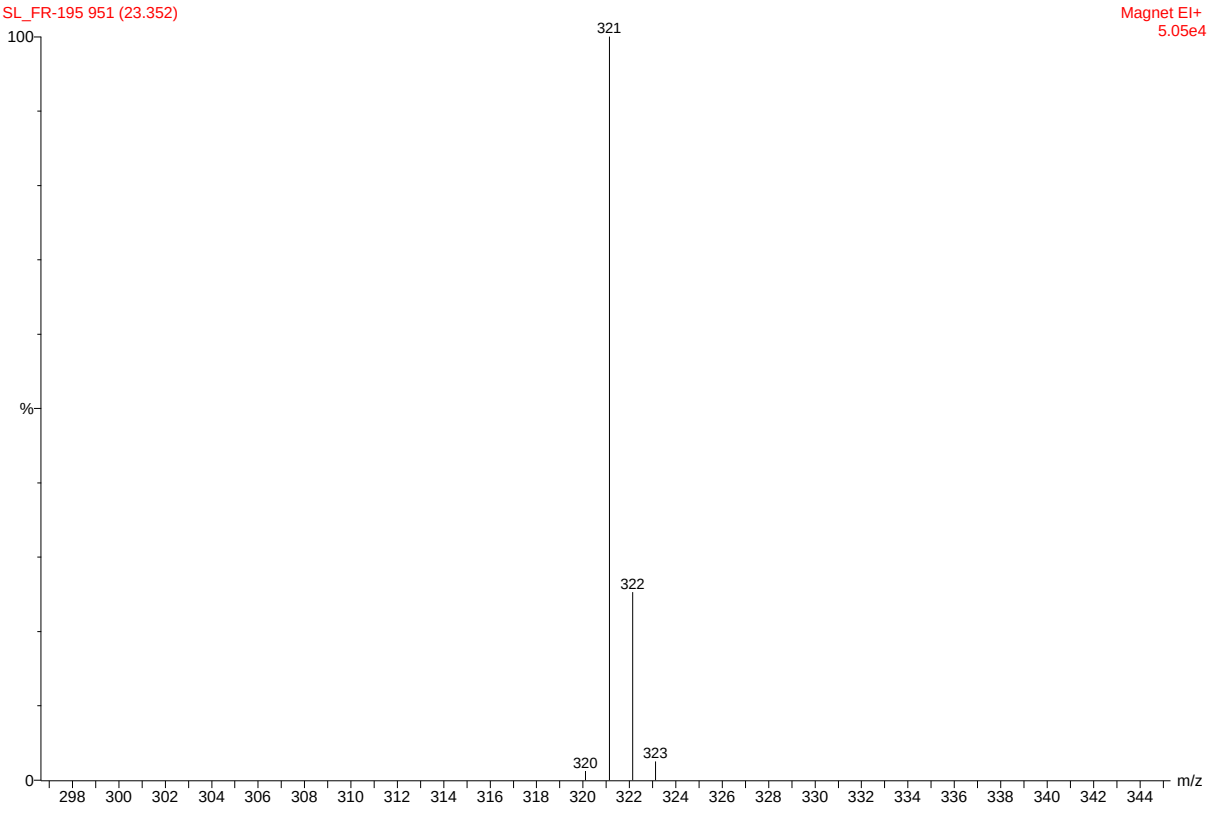


Sector field MS of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**11**)

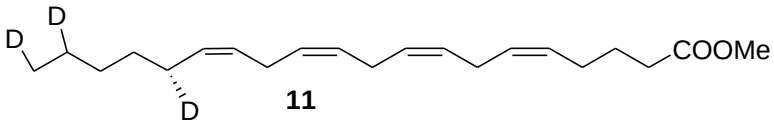


11



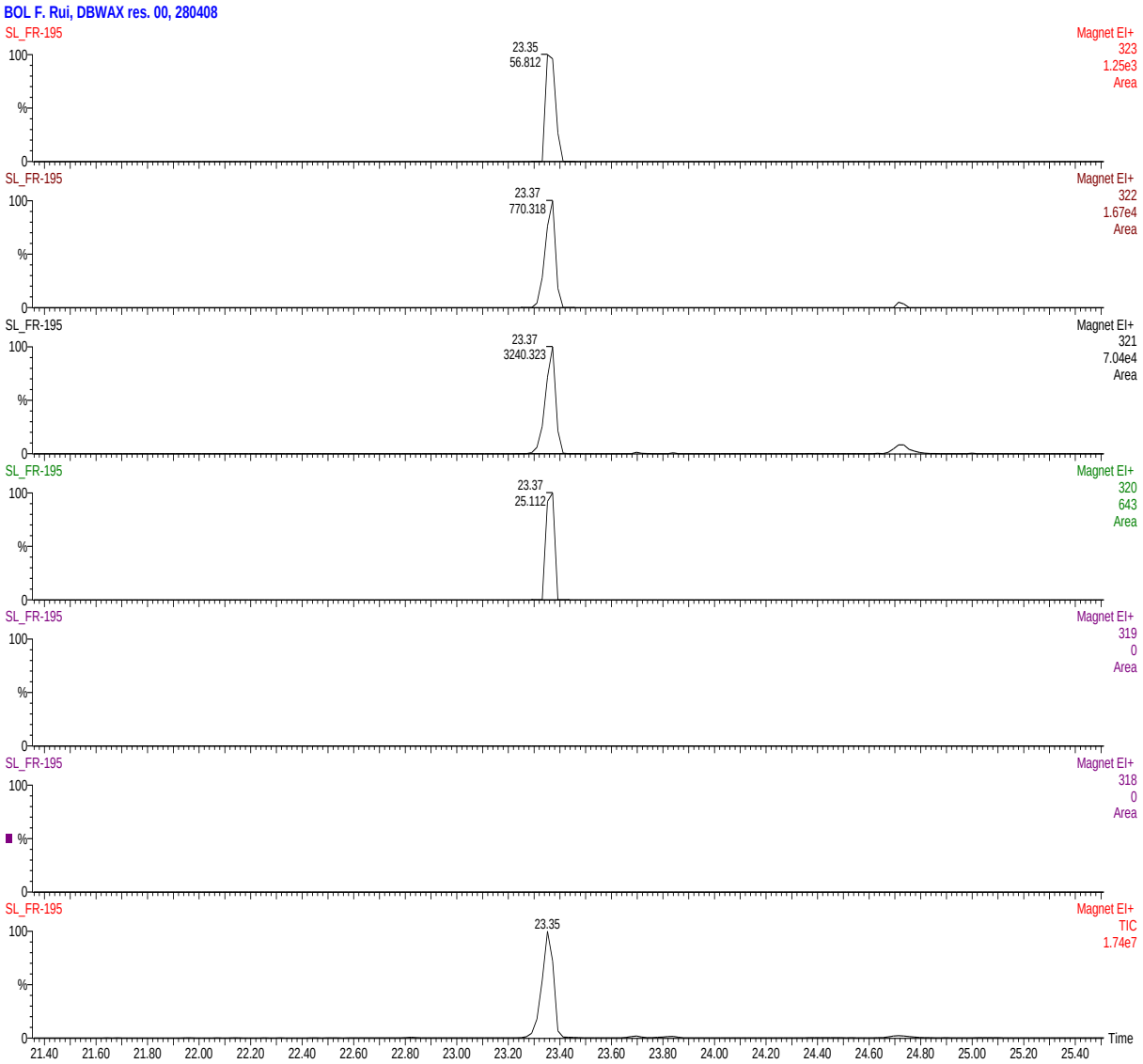


GC/MS analysis with sector field MS of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**11**), extracted ion chromatograms

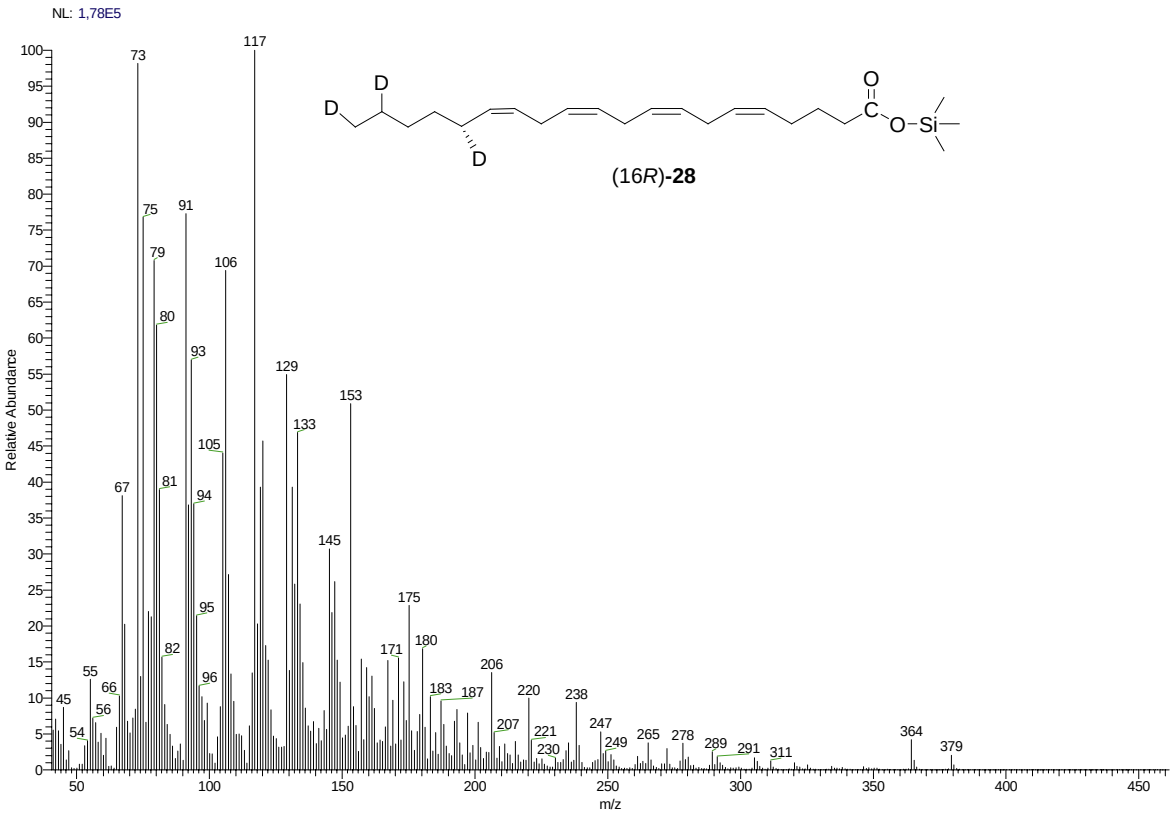


11

Ion trace (m/z)	deuterium content	RT	Area	Area corrected for the contribution of ^{13}C	%
323	d_5	23.372	57	<5	<0.2
322	d_4	23.372	770	22	0.7
321	d_3	23.372	3240	3233	98.5
320	d_2	23.372	25	25	0.8
319	d_1	-	not detected	-	<0.2
318	d_0	-	not detected	-	<0.2

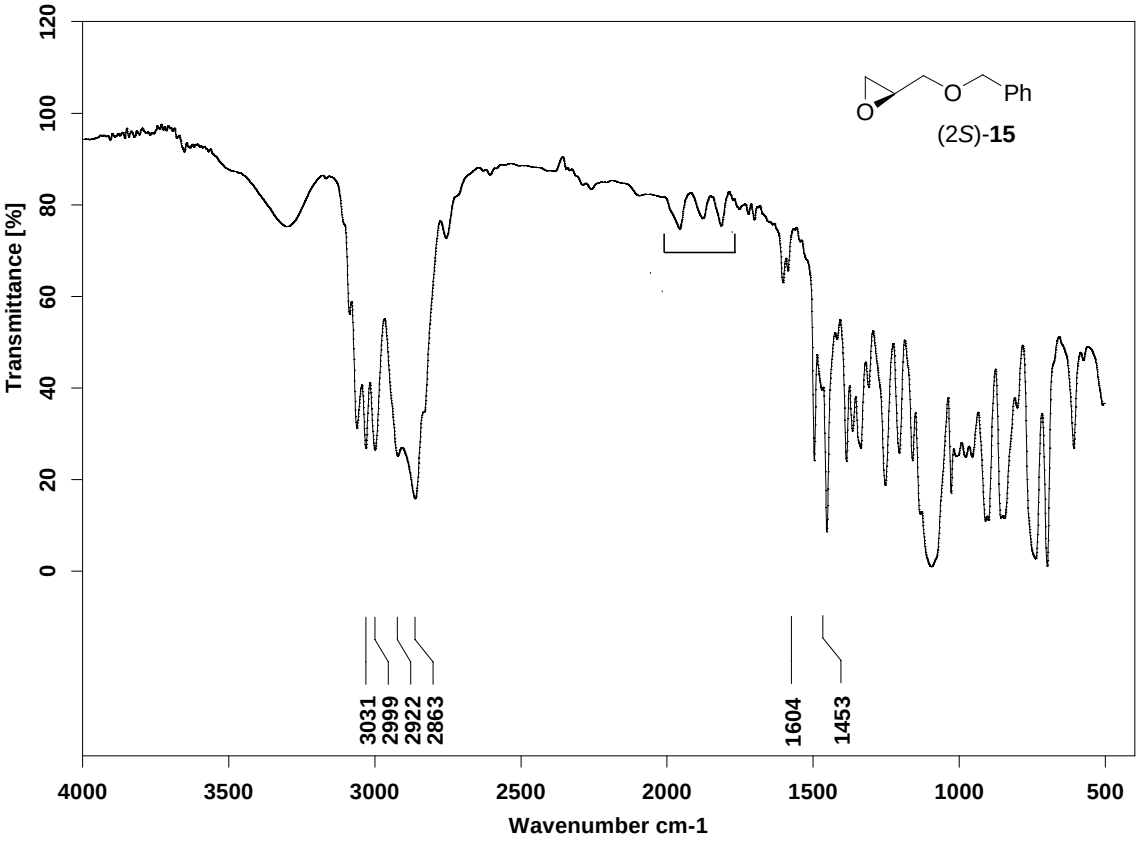


(16*R*)-[16,19,20-$^{2}H_{3}$]-Arachidonic acid trimethylsilyl ester

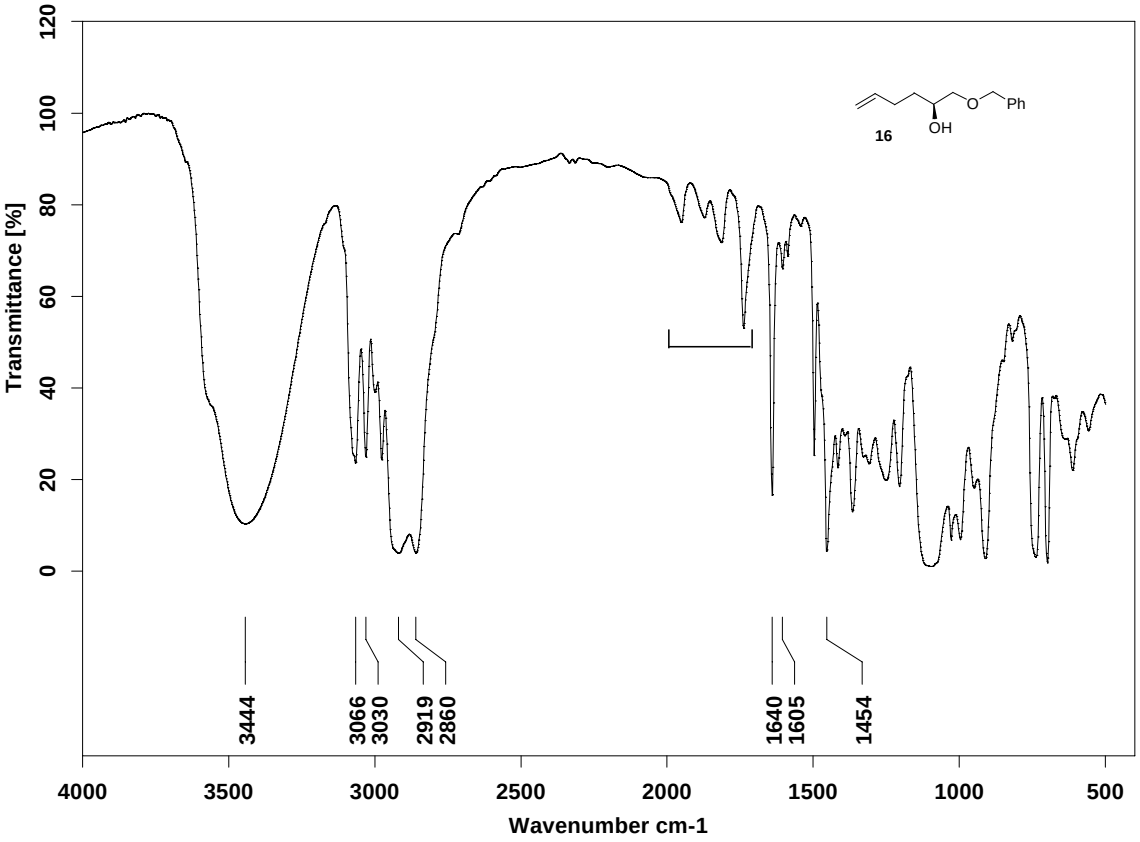


10.3 IR spectra

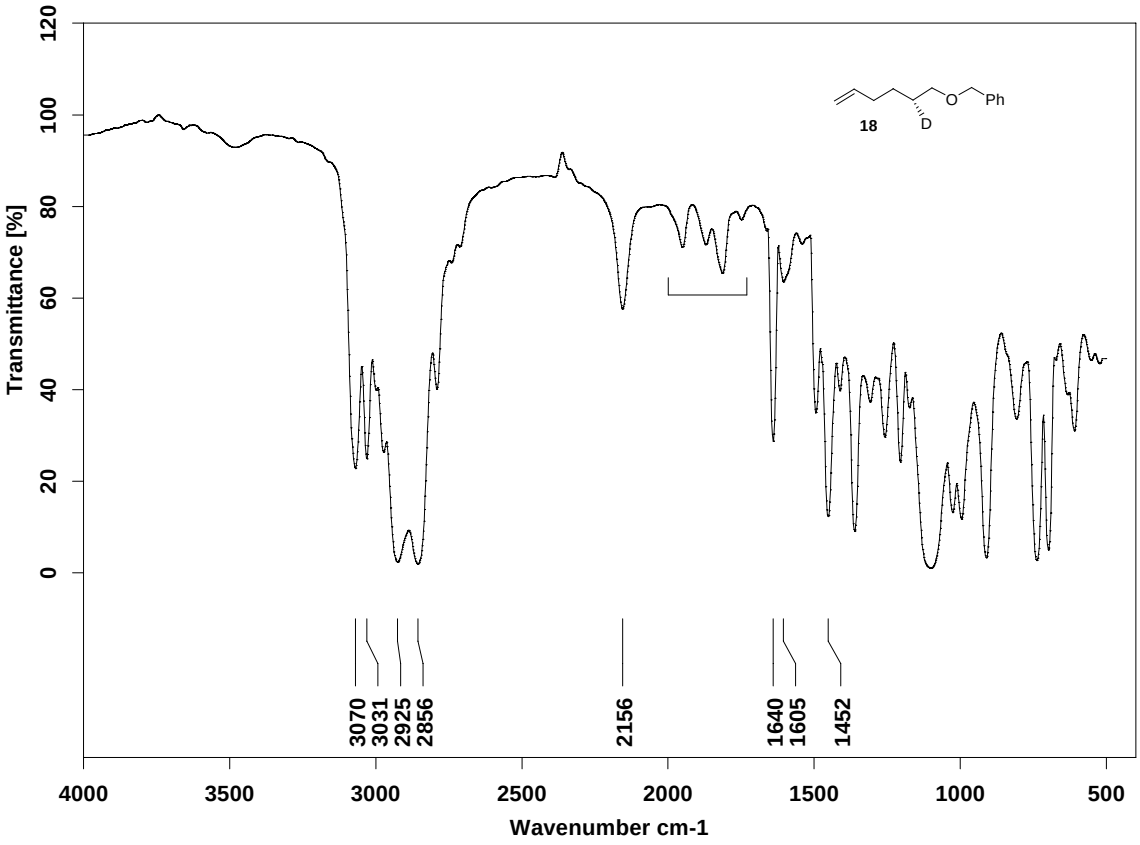
(2*S*)-glycidylbenzyl ether ((2*S*)-**15**)



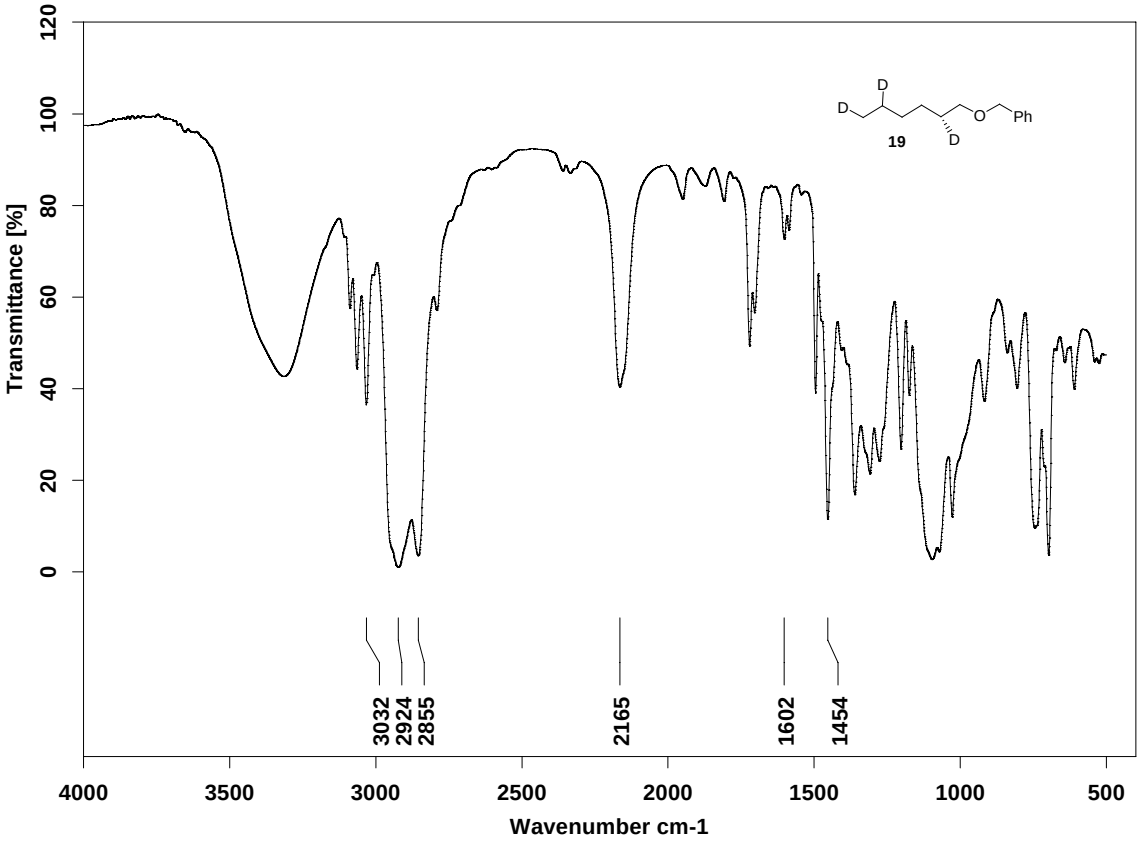
(2*S*)-1-(benzyloxy)hex-5-en-2-ol (**16**)



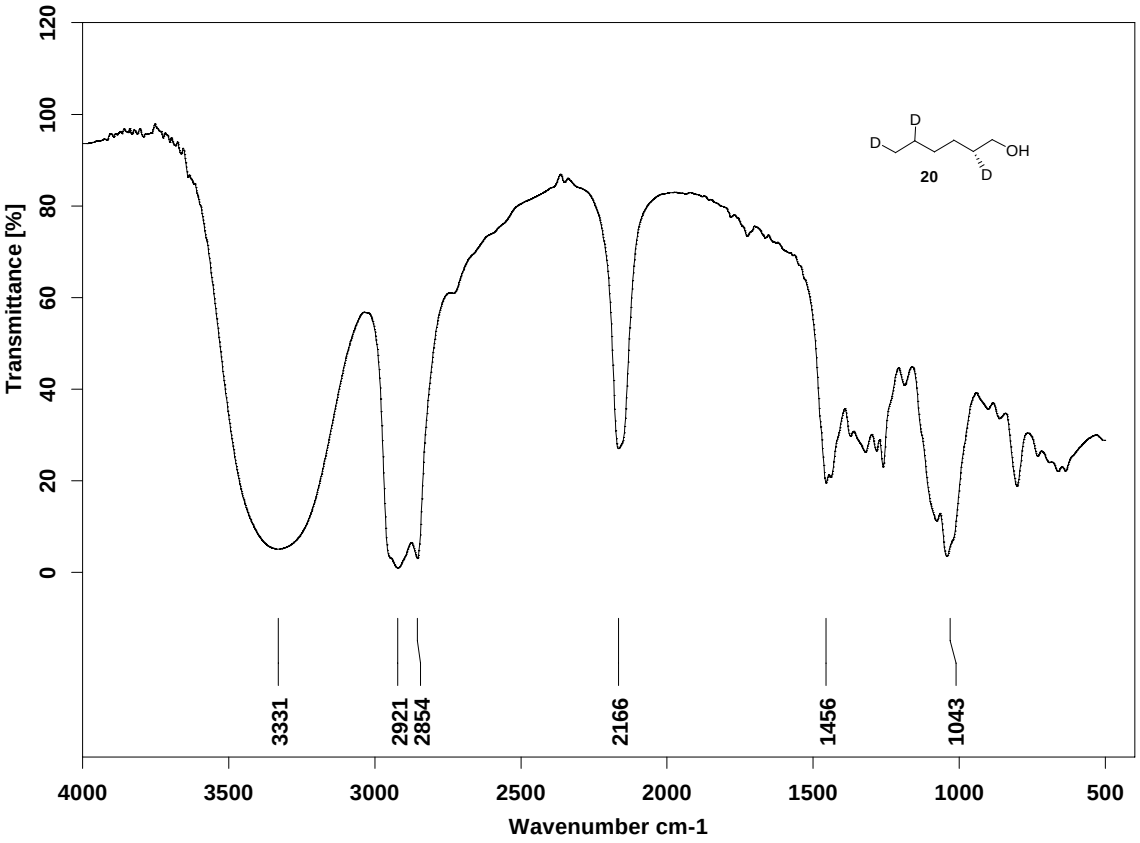
1-(2*R*)-[2-^{2}H]-hex-5-enyloxy)methyl)benzene (**18**)



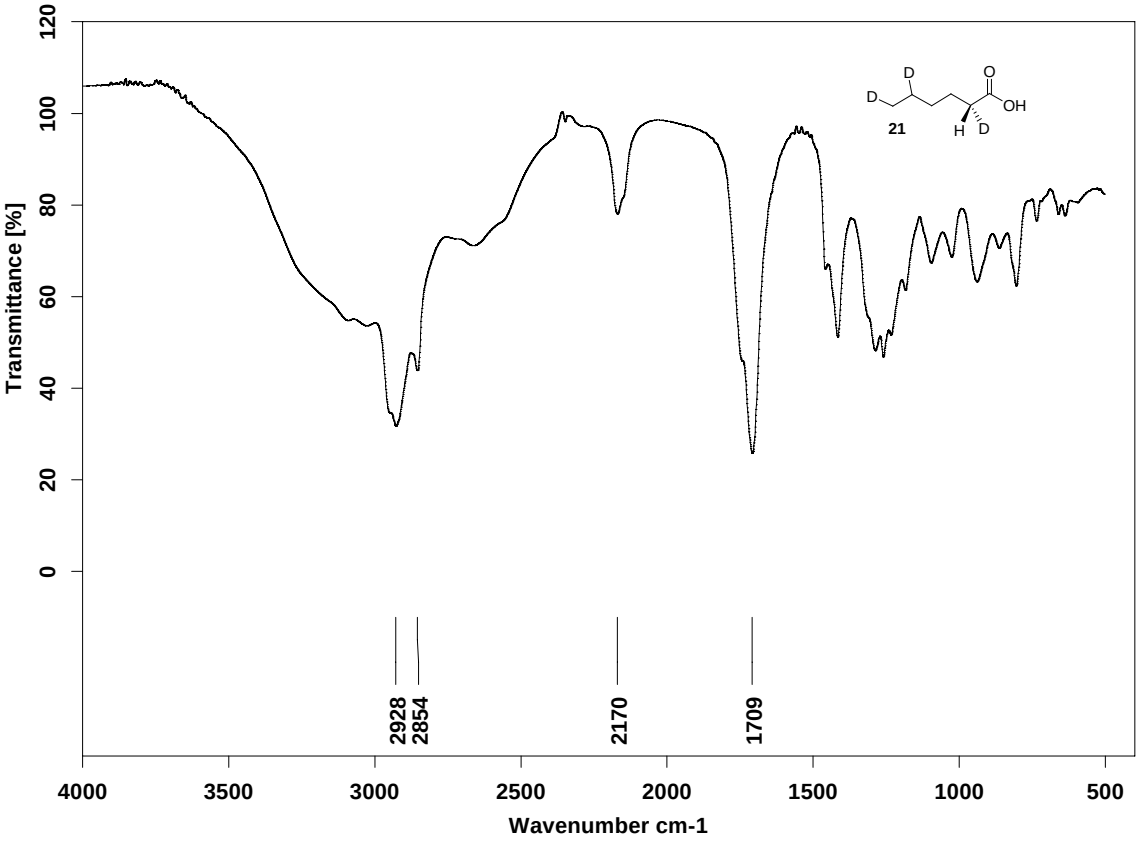
1-(2*R*)-[2,5,6-^{2}H$_3$]-hexyloxy)methyl)benzene (**19**)



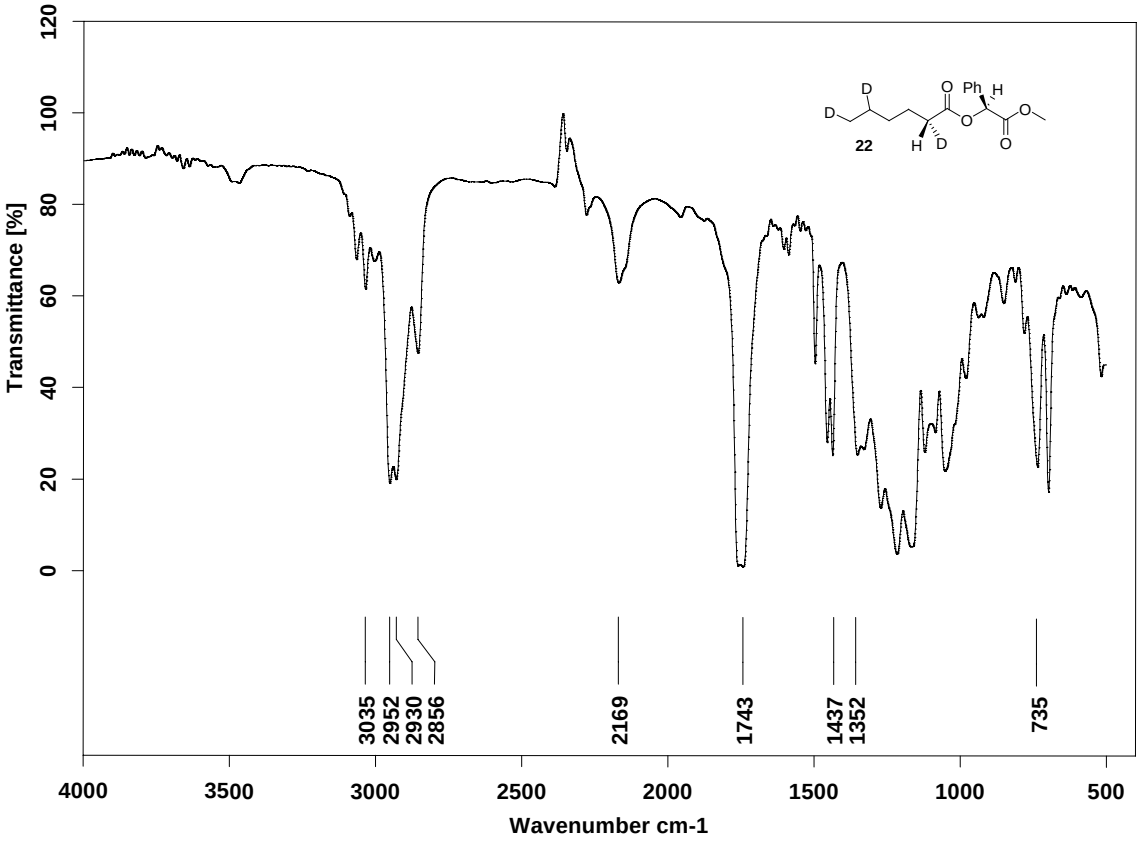
(2*R*)-[2,5,6-$^{2}H_3$]-hexan-1-ol (**20**)



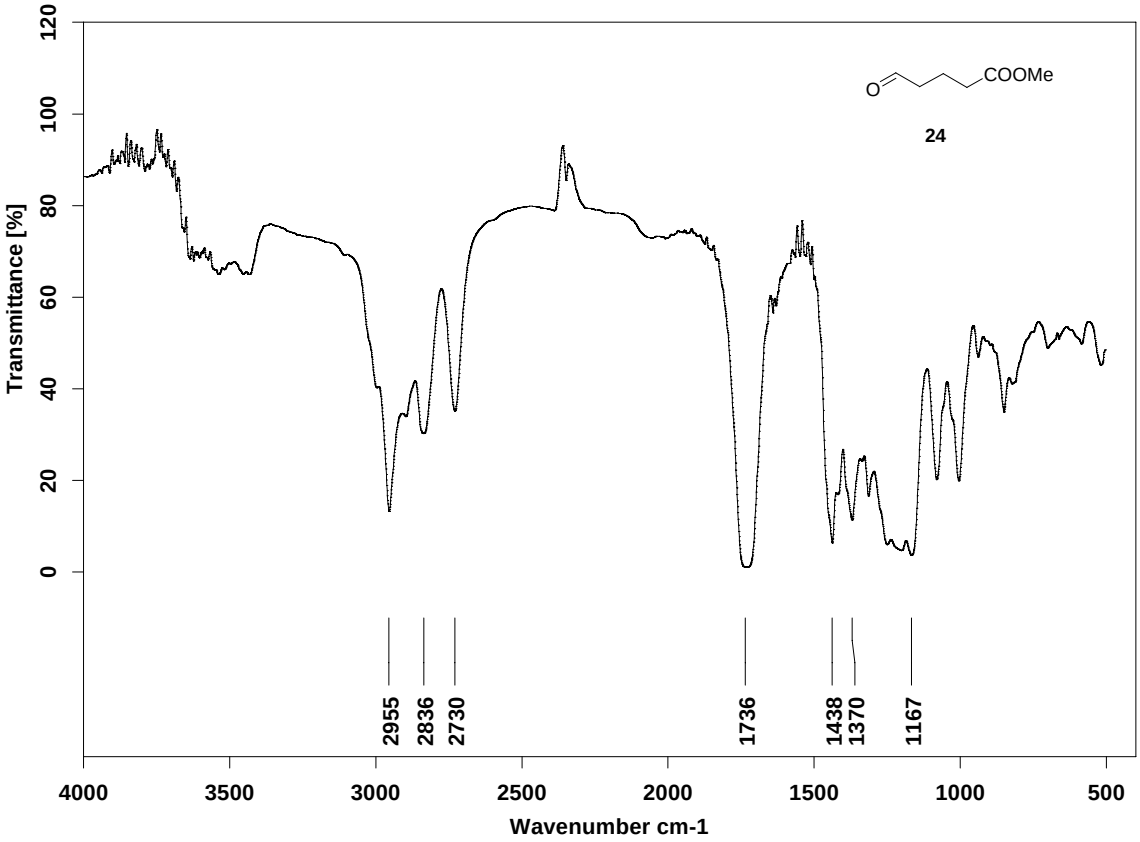
(2*R*)-[2,5,6-$^{2}H_3$]-Hexanoic acid (**21**)



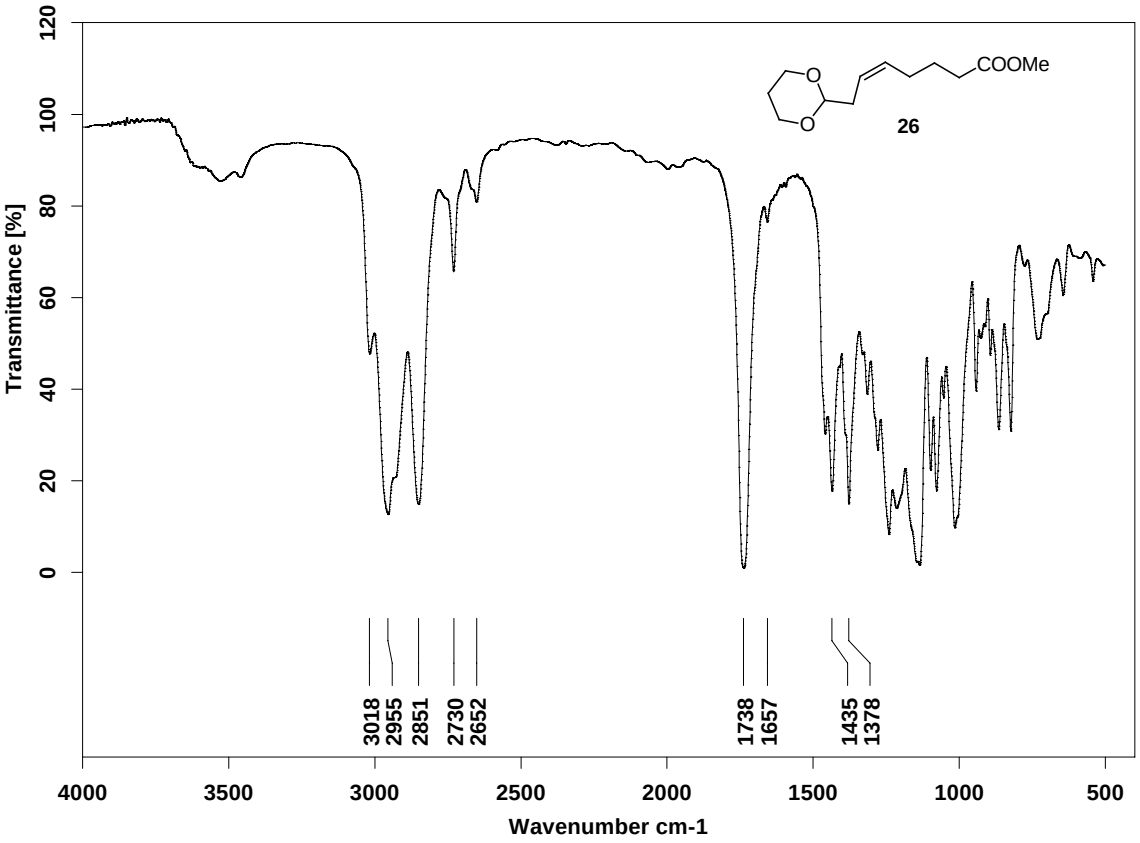
(2*R*)-[2,5,6-$^{2}H_3$]-Hexanoyl-(*S*)-methylmandelate diester (**22**)



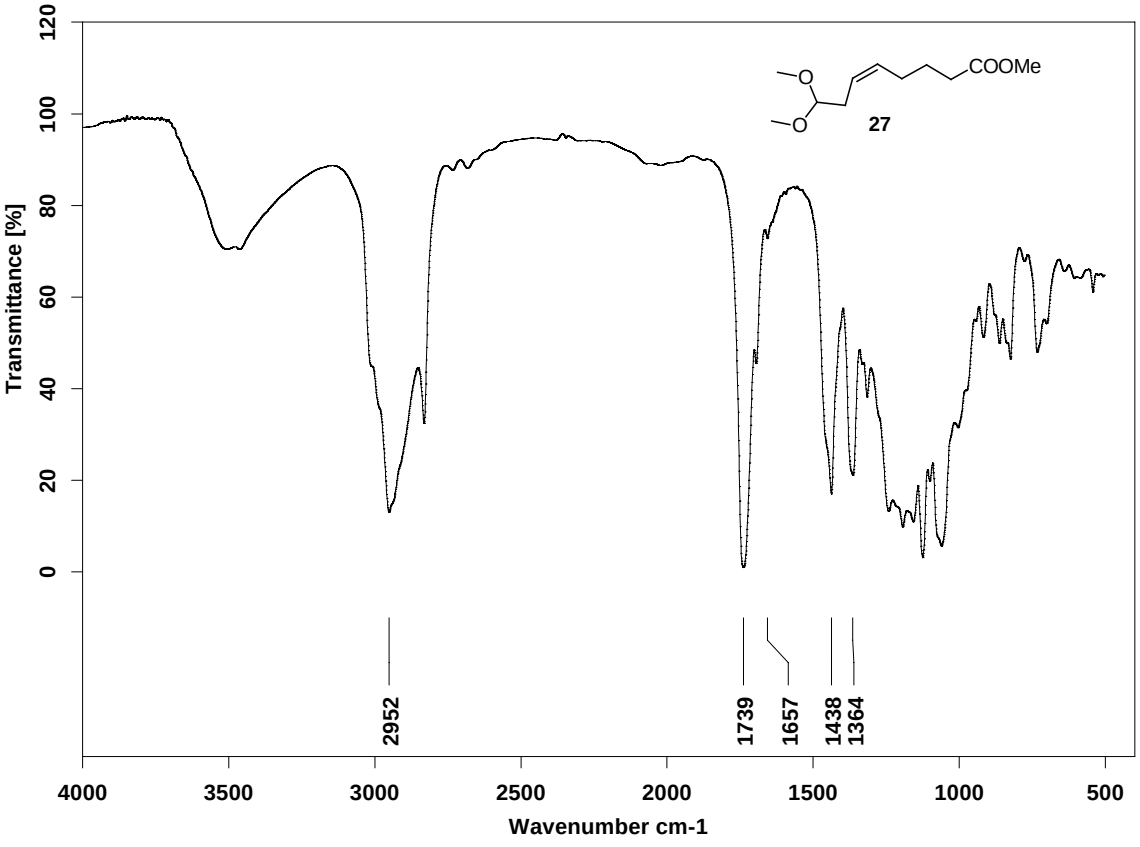
Methyl 5-oxopentanoate (**24**)

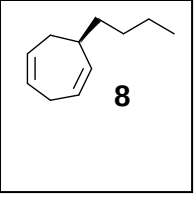


Methyl 7-(1,3,-dioxan-2-yl)-hept-(5*Z*)-en-oate (**26**)

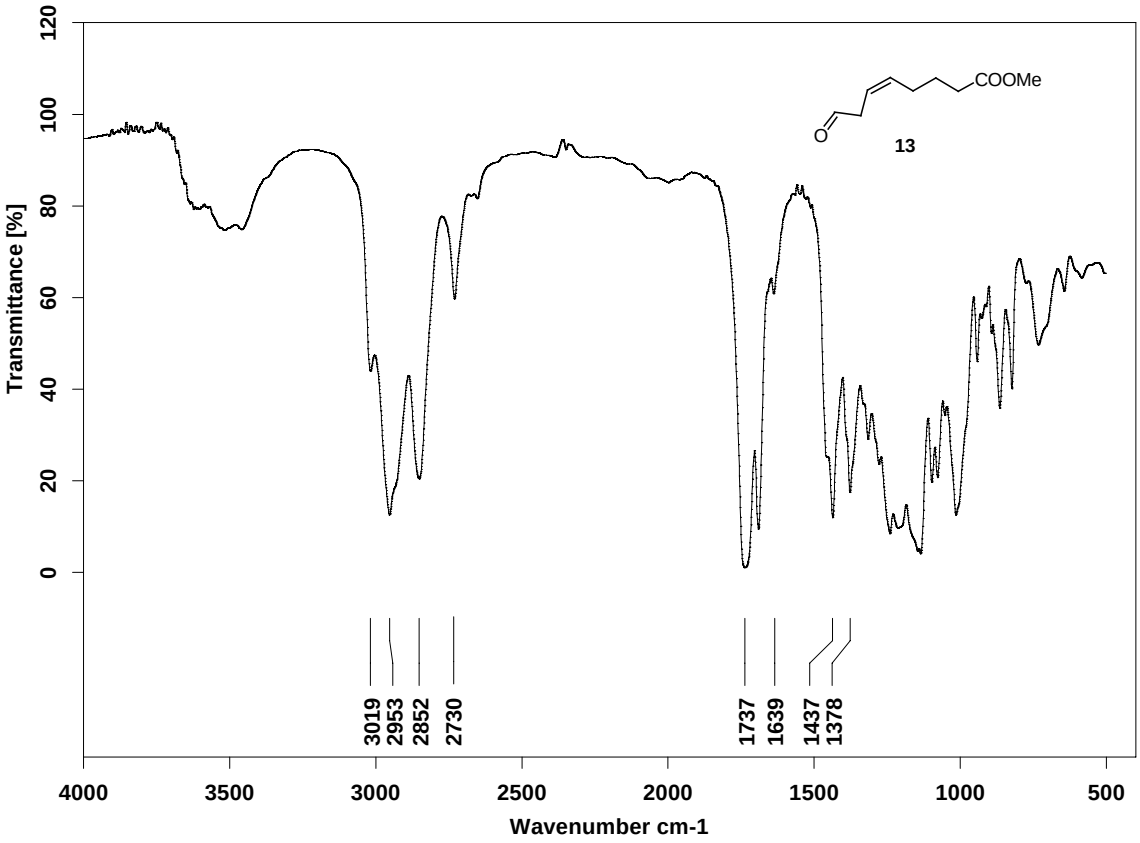


Methyl 8,8-dimethoxyoct-(5*Z*)-enoate (**27**)





Methyl 8-oxooct-(5*Z*)-enoate (**13**)



(16*R*)-[16,19,20-$^{2}H_{3}$]-methyl arachidonate (**11**)