Mechanistic Studies of Conjugated Fatty Acid Biosynthesis

By

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Abstract

The mechanism of conjugated fatty acid biosynthesis as it occurs in plants has been examined by incubating deuterium-labelled linoleates with a number of yeast strains expressing the requisite FAD2 type enzymes: $\Delta^{8,10}$ -desaturase (*Calendula officinalis*); $\Delta^{11,13}$ -desaturase (*Aleurites fordii* Hemsl.) and $\Delta^{10,9OH}$ -desaturase (*Dimorphotheca sinuata*).

The stereochemistry of calendic acid (octadec-8*E*,10*E*,12*Z*-trienoic acid) formation was probed by monitoring the fate of deuterium from (*R*)- and (*S*)-8- d_1 -octadec-9*Z*,12*Z*-dienoates and (*R*)- and (*S*)-11- d_1 -octadec-9*Z*,12*Z*-dienoates (linoleates). The latter substrates were synthesized by a chemoenzymatic route that involved the use of a yeast Δ^{12} mutant to introduce the 12*Z* double bond. Calendate is formed from linoleate by *syn*-removal of the 8H_R and 11H_S hydrogens as expected.

Using similar methodology, it was shown that α -eleostearic acid (octadec-9*Z*,11*E*,13*E*-trienoic acid) formation was formed from linoleate by *syn*-removal of the 8H_S and 11H_R hydrogens. This result required the postulation of a revised substrate conformation. By comparing the deuterium kinetic isotope effects at C-11 (large) and C-14 (small), the site of initial hydrogen abstraction was determined to be at C-11.

Dimorphecolate ((*R*)-9-hydroxyoctadec-10*E*,12*E*-octadecadienoic acid) is biosynthesized from oleate by tandem 12*E*-desaturation /allylic oxidation. It was demonstrated that the latter step is initiated by isotopically sensitive hydrogen abstraction at C11, followed by hydroxyl rebound to C-9 of the allylic radical intermediate.

All biotransformations could be described by an active site model that is common to all FAD2 type homologues operating on linoleate substrates.

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List of Abbreviations

(<i>R</i>)-AMA	(<i>R</i>)-(9-Anthranyl)methoxy acetic acid
(<i>R</i>)-MPAA	(<i>R</i>)-Methoxyphenylacetic acid
(<i>R</i>)-TFAE	(<i>R</i>)-(-)-1-(9-Anthranyl)-2,2,2-trifluoroethanol
(<i>S</i>)-AMA	(<i>S</i>)-(9-Anthranyl)methoxy acetic acid
(<i>S</i>)-(+)-MPAA	(<i>S</i>)-(+)- α -Methoxyphenylacetic acid
(<i>S</i>)-TFAE	(<i>S</i>)-(+)-1-(9-Anthranyl)-2,2,2-trifluoroethanol
ACP	Acyl carrier protein
Amp	Ampicillin
9-BBN	9-Borabicyclo [3.3.1]nonane
bp	Boiling point
BSTFA	<i>N,O</i> -Bis(trimethylsilyl)trifluoroacetamide
CIP	Cahn-Ingold-Prelog
CLA	Conjugated linoleic acid
CLnA	Conjugated linolenic acid
CoA	Coenzyme A
CS2	Clavamate synthase 2
CSA	Chiral solvating agent
CSM-URA	Complete supplement mixture - uracil
DAST	Diethylaminosulfur trifluoride
DEPT	Distortionless enhancement by polarization transfer
DGPC	Deoxyguanidinoproclavaminic acid
DHP	3,4-Dihydro-2 <i>H</i> -pyran

List of Abbreviations *continued*

DMAP	4-(Dimethylamino)pyridine
DMF	N,N-Dimethylformamide
DMS	Dimethyl sulphide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
<i>dr</i>	Diastereomeric ratio
EDA	Ethylenediamine
EDC HCl	<i>N</i> -(3-Dimethylaminopropyl)- <i>N</i> '-ethylcarbodiimide hydrochloride
<i>ee</i>	Enantiomeric excess
EI	Electrospray ionization
ER	Endoplasmic reticulum
EtMgBr	Ethylmagnesium bromide
EtOAc	Ethyl acetate
EtOH	Ethanol
<i>Fac2</i>	Fatty acid conjugase 2
FAD	Fatty acid Δ^9 -Desaturase
FAD2	Fatty acid Δ^{12} -Desaturase
FAD3	Fatty acid Δ^{15} -Desaturase
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry

List of Abbreviations *continued*

HMPA	Hexamethylphosphoramide
HPLC	High-performance liquid chromatography
IR	Infrared
α -KG	α -Ketoglutarate
KHMDS	Postassium bis(trimethylsilyl)amide
KIE	Kinetic isotope effect
LA	Linoleic acid
LB	Lysogeny broth
<i>m</i> -CPBA	<i>meta</i> -Chloroperbenzoic acid
MeCN	Acetonitrile
MeLi	Methyl lithium
MeOH	Methanol
MMA	Methyl mandelate
MMO	Methane monooxygenase
mp	Melting point
MS	Mass spectrometry
MTPA	α -Methoxy- α -(trifluoromethyl)phenylacetic acid
MTPA-Cl	α -Methoxy- α -(trifluoromethyl)phenylacetyl chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
<i>n</i> -BuLi	<i>n</i> -Butyllithium
NMR	Nuclear magnetic resonance

List of Abbreviations *continued*

PC	Phosphatidylcholine
PDC	Pyridinium dichromate
PCR	Polymerase chain reaction
PL	Phospholipid
PPTS	Pyridinium <i>p</i> -toluenesulfonate
<i>p</i> -TsOH	<i>p</i> -Toluenesulfonic acid
Py	Pyridine
RuCl ₃ . <i>n</i> H ₂ O	Ruthenium(III) chloride hydrate
SCD	Stearoyl CoA Δ^9 -Desaturase
SD	Synthetic dextrose
SIM	Selected ion monitoring
S _N 2	Bimolecular nucleophilic substitution
SOMO	Singly occupied molecular orbital
TAG	Triacylglycerol
TBS	<i>tert</i> -Butyl dimethylsilyl
THF	Tetrahydrofuran
THP	Tetrahydropyran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSCHN ₂	Trimethylsilyldiazomethane
TsCl	<i>p</i> -Toluenesulfonyl chloride
YPD	Yeast peptone dextrose

Chapter 1: Introduction

1.1 Conjugated Fatty Acids

1.1.1 Lipids and Fatty acids

Lipids are essential hydrocarbon-based components of every living organism where they serve as a source of energy and as hydrophobic building blocks of membranes. In the latter role, lipids protect the inner constituents of the cell from the outer environment and selectively allow foreign substances to pass through (cellular transport). Other biological functions performed by lipids include antifeedant activity, regulation of membrane protein activities and chemical signalling.¹⁻⁴ Lipids can be subdivided into two major categories - firstly, fatty acids and their derivatives; and secondly, substances related biosynthetically or functionally to these compounds, such as

Figure 1.1. Biosynthesis of fatty acids in plants and its subsequent modifications by employing several different enzymes to produce complex lipids. The Figure was adapted from Gunstone *et al.*⁵

polyketides and terpenoids (Figure 1.1). Fatty acids are the long chain carboxylic acid containing compounds that commonly exist in biological systems as esters of triglycerides and phospholipids.^{6,7} In addition to the carboxyl terminus, fatty acids can contain various other functionalities including monoolefinic, polyolefinic (methylene interrupted and/or conjugated), acetylenic, hydroxyl, hydroperoxy, epoxy, keto and cyclopropenyl groups.^{3,6-8} The fatty acyl chain lengths can vary but fatty acids with 16 and 18 carbons are most common.^{2,7,8}

1.1.2. Fatty acid biomodification

The topic of this thesis is focused on the mechanism of a desaturase-mediated, post assembly fatty acid biomodification known as conjugated fatty acid formation (Panel C, Figure 1.2). To place this biochemical reaction in its proper context, the general subject of biomodified fatty acids will be reviewed.¹ These compounds arise by transformation of the common saturated (e.g. stearate), monounsaturated (e.g. oleate) and polyunsaturated (e.g. linoleate) fatty acids as shown in Figure 1.2. A common theme that unites many of the biosynthetic pathways is that they are mediated by desaturase or desaturase-like enzymes.^{1,9} The various functional groups generated during these reactions include allene,³ cyclopropene and cyclopropane,⁹ epoxide,¹⁰ alkyne,^{1,10} polyacetylene,⁹ allylic alcohol,¹¹ homoallylic alcohol,¹² conjugated triene and fused polycyclobutane.³

Most of these unusual fatty acids are thought to confer antifeedant properties that are used by the corresponding species to defend against various predators in that eco-habitat.^{1,13} Introduction of the allenic moiety in the fatty acyl chain is one of the rarest example of fatty acid modifications that have been observed to present in several

Figure 1.2. Examples of some naturally occurring exotic fatty acids and their derivatives.

subtropical plants (Panel A, Figure 1.2).¹⁴ The seed oil of *Leonotis neptaeifolia* (Lion's ear) has been observed to contain (*R*) 5,6-monoallenic octadecadienoic acid or laballenic acid.¹⁵ It is thought that the allene is biosynthesized by stepwise dehydrogenation at the 6, 7 and 5,6 positions.

The production of ricinoleic acid (Panel B, Figure 1.2) in the seeds of castor plant (*Ricinus communis* L.) has had a high impact in the oleochemical industry as a replacement of petrochemicals in some important processes and as an engine lubricant (Castrol ^{TM}),⁹ 90 % of the total fatty acids in the seed oil of the castor plant is (*R*)-12-hydroxy-(9*Z*)-octadecenoic acid or ricinoleic acid and this fact contributes to its bioactivity as a purgative. It has been observed that the plant biosynthesizes this fatty acid from oleic acid using an enzyme that is very closely related to the desaturase responsible for double bond introduction in linoleate biosynthesis.^{1,9,16} The very high toxicity of ricin in the seed coating of this plant complicates its cultivation in large quantity for industrial production.¹⁷ Cloning of the 12-hydroxylase gene into more benign plant hosts has been a major biotechnological objective.

Certain tropical plants have also been observed to accumulate a remarkable fatty acid containing highly strained cyclopropene ring in its seeds oil known as sterculic acid (Panel B, Figure 1.2).¹ Following the first structural elucidation of this fatty acid from the seed oil of *Sterculia foetida* in 1952 by Nunn, a search for natural products containing cyclopropenyl rings and their biological functions began.¹⁸ Today it is known that a number of tropical plant species belonging to the families of *Sterculiaceae*, *Bombacaceae*, *Tiliaceae* and *Malvaceae* possess sterculic acid in their seeds.¹⁹ Among all the seeds of *Sterculia foetida* contains the highest amount of this fatty acid as 78 % of the

total fatty acid content whereas the cotton seed (*Gossypium hirsutum*) oil contains far less amount (~ about 1 %). Sterculic acid shows strong inhibition of mammalian stearoyl-CoA Δ^9 -desaturase leading to various disorders, which might explain the reason for the biogenesis of this unusual fat as it part of their antifeedant defence strategy.²⁰⁻²³ It is thought that sterculate is generated from a cyclopropyl intermediate *via* desaturation at C-9 and C-10.⁹

Vernolic acid is another example of industrially important exotic phyto fatty acid that is derived from a plant source (Panel C, Figure 1.2). Epoxide containing fatty acids are industrially important exotic substances that are commonly used in manufacturing adhesives, varnishes, paints as well as industrial coatings and resins.⁹ The presence of an epoxide ring at the allylic position of a double bond makes it less viscous, easily oxidizable and therefore makes any oil-based paints more durable. That is why it is mostly used as an environment friendly non-volatile solvent for many oil-based paints.⁹ The seeds of *Vernonia galamensis*,²⁴ *Euphorbia lagascae*,²⁵ *Crepis palaestina*¹⁰ are the major source of vernolic acid. It is generated by epoxidation of linoleic acid involving a variant desaturase, however the details of the mechanistic pathway are still unknown.

Probably the most remarkable of all fatty acid biomodification features the abstraction of vinylic hydrogens (=C-H) to generate alkyne (Panel C, Figure 1.2) - the biotransformation process responsible for production of crepeynic acid in *Crepis alpina*.²⁶⁻²⁸ A similar kind of acetylenase activity has been observed in the moss *Ceratodon purpureus* with the biofunctional Δ^6 -desaturase/acetylenase.²⁹ These reactions are particularly noteworthy in the sense that it shows the commitment of an enzyme towards a particular mechanistic pathway in preference to the energetically more

favourable epoxidation or allylic oxidation leading to conjugated fatty acids.^{1,26} Cloning of the cDNA and functional analysis of the acetylenase responsible for the biosynthesis of crepenynic acid from linoleic acid shows high resemblance with parent FAD2 enzyme and another variant FAD2 enzyme (for epoxide formation) found in the genus *Crepis* itself.^{9,10,26} It has been predicted that the formation of crepenynic acid from linoleic acid is the first step towards the biosynthesis of relatively more widespread, large family of polyacetylenes such as falcarindiol (Panel C, Figure 1.2)^{10,27,28,30,31} The most common source of polyacetylene compounds are the fungi, algae, marine sponges as well as higher plants and these unusually conjugated natural products have been observed to exhibit a broader range of biological activities including anti-inflammatory, antitumor, antifungal, antimicrobial, insecticidal and many others.^{30,32}

Perhaps the most exotic fatty acids are the ladderanes (Panel D, Figure 1.2), first reported by L. A. van Niftrik and M. S. M. Jetten *et al.*³³ These lipids are isolated from two anaerobic ammonia-oxidizing bacteria where they create an exceptionally dense, tight protective barrier in the intracytoplasmic compartment to prevent any diffusion of toxic intermediates such as hydrazine during oxidation of ammonia to dinitrogen intermediate.³³ Multiple cyclobutyl rings (as many as five) are fused linearly in *cis* ring junction fashion such that it looks like a ladder and possibly biosynthesized by anaerobic cyclization of an unsaturated fatty acid.³ Such kind of ladderane molecules function as a promising building blocks for optoelectronics.³³

Isolation, characterization and engineering of the enzymes responsible for the biogenesis of these important lipids in different species is an important task for the biochemist, biotechnologist and bioorganic chemist.³ In this way, new drugs against

various metabolic and terminal diseases may be discovered and the value of green oleochemical feedstocks and renewable energy source (hydrocarbon biofuels (Panel A, Figure 1.2)) may be enhanced.

1.1.3 Conjugated Fatty Acids

Conjugated fatty acids are the naturally occurring compounds that have drawn much attention because of their useful applications as nutraceuticals (putative anticancer, antidiabetic, antiobesity properties) as well as feedstocks for industrial uses (paints, inks, varnishes, lubricants, dyes, plastics, resins).³⁴ Conjugated fatty acids and their derivatives are present in many organisms including algae, plants, insects and mammals. Chain lengths of the conjugated fatty acids can vary from 12 to 22 carbons, but a chain length of 18 carbons is the most common. Variation in position and geometries of the conjugated double bonds as well as the presence of additional functional groups leads to much diversification of the structures of conjugated fatty acids as shown in Figure 1.3.

Three different types of mechanisms have been reported in the literature to date for the biosynthesis of conjugated fatty acids.³⁴ Firstly, desaturation (1,2-dehydrogenation) of olefinic fatty acids at the allylic position to give conjugated dienes (Figure 1.3A).³⁵ Secondly, an anaerobic isomerisation mechanism that has been used by the marine algae to biosynthesize conjugated trienes and tetraenes (Figure 1.3C).^{36,37} It has also been indicated that CLA (conjugated linoleic acid) such as 9Z,11E-isomer of linoleic acid may be biosynthesized in ruminants *via* isomerisation of linoleic acid (Figure 1.3A).³⁸ Thirdly, a mechanistic variation of 1,2-dehydrogenation (such as 1,4-dehydrogenation) applied to *cis*-olefinic substrates is featured in the formation of insect pheromones³⁹⁻⁴¹ (Figure 1.3B) and conjugated fatty acid accumulation in the seed oils of

selected higher plants^{34,42,43} (Figure 1.3D). The latter process is catalyzed by desaturase variants.

A Animals

B Insects

C Algae

D Plants

Figure 1.3. Representative structures of naturally occurring conjugated fatty acid derivatives from animals, insects, algae and plants.

1.1.4 Conjugated Fatty Acids in Animals

In animals, conjugated fatty acids predominantly exist as conjugated linoleic acids (CLA). In 1935, Booth *et al.* reported the presence of conjugated fatty acids in food products derived from ruminant animals.⁴⁴ Subsequently, Parodi *et al.* confirmed the structure of this conjugated linoleic as 18:2-9Z,11E *i.e.* most commonly now known as rumenic acid.⁴⁵ Milk, meat and other dairy products from ruminants are a rich source of CLA isomers that are thought to have many beneficial properties and are used as healthy food supplements.⁴⁶⁻⁵³ There are 28 different isomers that have been isolated so far from the rumen CLA but among them the two isomers, 18:2-9Z,11E (about 1 % of the total milk fat and about 80 to 90 % of the total CLA in milk) and 18:2-10E,12Z are present to a major extent.^{45,46,50,54,55}

It has been demonstrated that the CLA in ruminants are biosynthesized from dietary forages containing unsaturated fats (linoleic or α -linolenic acid) in two pathways where 18:2-9Z,11E is formed as an intermediate or by-product.^{35,51,55,56} In pathway one, CLA is produced as an intermediate during the ruminal biohydrogenation of dietary linoleic or α -linolenic acid to stearic acid. Investigations have revealed that biohydrogenation which is a multiple step biochemical process is actually mediated by several different species of rumen bacteria.^{56,55} In pathway two, CLA is biosynthesized in the adipose tissue of mammary glands of the animal from 18:1-11E fatty acid that is formed during biohydrogenation (Figure 1.4).

In 1987 N. K. Grimm and M. W. Pariza *et al.* first reported the anticarcinogenic properties of conjugated linoleic acid from fried ground beef extract.^{57,58} Following their discovery many other research groups reported numerous physiological activities by

Figure 1.4. Biogenesis of CLA in the milk and meat fat of ruminants.

this class of compounds. Included in this feature - inhibition of tumorigenesis, modulation of the immune response, antiinflammatory effect, potent antiatherosclerotic effect as well as play major role in regulating the membrane lipid level in animals body (antiobesity) as evident by reduction in body fat and increase in lean body mass.^{11,46-50,53,54,59-66} Conjugated fatty acids have also shown promise for serum lipid lowering effect in mammals, increases the C-reactive protein concentration and lipid peroxidation and to decrease the insulin insensitivity *in vivo* i.e. has antidiabetic effect.^{48,52,53} In

particular, 18:2-9*E*,11*E* has also been shown to inhibit platelet aggregation^{67,68} and exhibit antiproliferative effect.⁶⁹

Recent studies indicate that average daily requirement of CLA in human body is <500 mg/day.⁷⁰ However, ruminant milk and meat fat contains maximum up to 1 % (w:w) CLA which is not enough (as food supplement for animals) to consider this as health-promoting food supplement.⁵⁹ Oils that contains high proportions of essential fatty acids such as safflower oil, canola oil, vegetable oil are treated with alkali to generate CLA and supply the health food market.

Research on the physiological effects of conjugated linolenic acid (CLnA) has not progressed as much as work on the benefits of CLA. Only recently, it was reported that this family of conjugated fatty acids also exhibits significant cytotoxic effect on tumor cells, shows anticarcinogenic effect and play critical role in lipid metabolism of animals.^{34,46,64-66} In fact, both *in vivo* and *in vitro* studies established that conjugated linolenic acids (CLnA) have much stronger antitumour activity in comparison to CLA.⁵⁹⁻⁶¹ These findings have increased interest in CLnA.

1.1.5 Conjugated Fatty Acids in Insects

Female processionary moths of several lepidopteran species have been observed to use a blend of modified lipids including conjugated π systems known as sex pheromones to attract the mate.^{41,71,72} Pheromone blends are usually composed of several long chain lipophilic unsaturated fatty acetates, alcohols or aldehydes where the number as well as the position and stereochemistry of the C-C double bonds can vary along the backbone to constitute a species-specific semiochemical language.⁷²⁻⁷⁵ Experimental investigations have shown that the desaturation of fatty acyl-CoA esters involving

membrane-bound acyl-CoA desaturases is the most important reaction that shows high commitment for the production of olefinic pheromone blends.^{14,74,76} Unlike desaturases found in animal and fungi, insect desaturases show enormous enzymatic plasticity and are capable of producing a wide range of unsaturated fatty acyl-CoA esters of varying chain lengths, double bond stereochemistry as well as position.^{39,41,71,75,77}

Gemma Fabrias *et al.* has reported that the female moth pheromone glands contain several C14 acetates including (9*Z*), (11*E*) and (11*Z*)-11-tetradecenyl as well as (10*E*,12*E*) and (9*Z*,11*E*)-tetradecadienyl acetates.^{39,71,76,78} Using radiolabeled precursors, (it was) demonstrated that (9*Z*,11*E*)-tetradecadienoate is biosynthesized from (11*E*)-tetradecenoate using a Δ^9 -desaturase, whereas the (10*E*,12*E*)-tetradecadienoate is derived from (11*Z*)-tetradecenoate catalyzed by a Δ^{11} -desaturase enzyme (Figure 1.5).^{39,40,71,79}

Figure 1.5. The initial involvement of various membrane-bound desaturases and the bifunctional activity of Δ^{11} -desaturase in generating conjugated fatty acid derivatives in the pheromone blend of female processionary moth, *Spodoptera littoralis*.^{39,40,41}

The pheromone blend of *Bombyx mori* was reported to contain a similar conjugated fatty acid derivative, (10*E*,12*Z*)-hexadecadienoate that was believed to be generated from (11*Z*)-hexadecenoic acid employing a Δ^{11} -desaturase enzyme that itself catalyzes the formation of (11*Z*)-hexadecenoic acid from palmitic acid.⁸⁰

Conjugated eneynes are found in another female processionary moth, *Thaumetopoea pityocampa*.^{41,75,81} It has been observed that there exists a remarkable multifunctional desaturase enzyme which is responsible for exhibiting Δ^{11} -desaturase, Δ^{11} -acetylenase and Δ^{13} -desaturase activities equally (Figure 1.6).

Figure 1.6. Catalytic diversity of a multifunctional Δ^{11} -desaturase that is responsible for the biosynthesis of sex pheromone in processionary moth, *Thaumetopoea pityocampa*. The Figure was adapted from Gemma Fabrias.⁷⁵

1.1.6 Conjugated Fatty Acids in Plants

It is estimated that the seeds of various plants contain thousands of structurally distinct fatty acid compounds in a species-specific distribution pattern.^{8,82,83} Conjugated fatty acids constitute an important subset of the so-called “unusual” fatty acids. Higher

plants biosynthesize these compounds in developing seed tissues where they are stored primarily in the form of triacylglycerols (TAG) for future use as a source of energy and for structural purposes.⁸³⁻⁸⁵ An early biogenetic scheme that relates the various conjugated fatty acids to linoleic acid precursor by oxidative pathways is shown in Figure 1.7.

The existence of α -eleostearic acid was first reported by Cloez in 1875⁸⁶ and subsequently the chemical structure of this conjugated fatty acid was elucidated by Böeseken and Ravenswaay in 1925.⁸⁷ It was not until 1957 that L. Crombie and A. G. Jacklin first established the position and configuration of the conjugated trienes in α - and β -eleostearic acid as well as punicic acid *via* total synthesis and analyzing the products based on their physical and spectroscopic properties.⁸⁸ The α -eleostearic and punicic acid were synthesized using several stereoselective reactions, whereas β -eleostearate (all *trans* isomer) was obtained from α -eleostearate following the treatment with I_2 in presence of UV-light. Typically the structures of conjugated fatty acids can be determined by a combination of M.P., UV absorbance, molar extinction coefficients, IR data, intensity characteristics of IR bands, quantitative hydrogenation, GC-EI/MS, ^1H and ^{13}C NMR, respectively.⁵⁹

18:2-10*E*,12*E* CLA is the only non-oxygenated conjugated dienoic fatty acid that has been isolated from a plant source (10 % of the seed oil of *Chilopsis linearis*). Screening of the seed oil derived from *Dimorphotheca* species (*Dimorphotheca aurantiaca*, *Dimorphotheca sinuata*) revealed that more than 60 % of the total fatty acid content in the oil contains dimorphecolic acid - a unique hydroxylated dienoic acid. Examples of conjugated trienoic fatty acids include α -eleostearic acid (Tung oil),

Figure 1.7. Proposed biogenesis of the conjugated fatty acids in plants beginning with H-abstraction at C-11 of linoleic acid. The Figure was adapted from Crombie *et al.*⁴³

calendic acid, α -parinaric acid, jacaric acid, catalpic acid and punicic acid; these can be found in over nine different plant families.^{46,62,64,89} α -Eleostearic acid is the principal fatty acid in the seed oil of tung tree (*Aleurites fordii* Hemsl.) and contains ca. 80 % of the total fatty acid content.^{34,90} Other sources of α -eleostearic acid include bitter melon (*Momordica charantia*) where the seeds accounts for ~ 65 % of this fatty acid, but the flesh contains much less.^{89,91,92} The seed oil of marigold plant (*Calendula officinalis*) has been reported to contain about 51-53 % of calendic acid.^{16,63,93} α -Parinaric acid is the major fatty acid constituent in the seed oil of *Parinarium laurinum* and *Impatiens balsamina* plant species where it present as 46 % and 29 % of the total fatty acid content in the oil, respectively.^{82,94,95}

Oils enriched with conjugated fatty acids have many useful commercial applications such as drying agents in the formulations of inks, paints, plastics, nylon, dyes, resins and varnishes.^{11,34} Conjugated fatty acids are highly unstable and easily prone to oxidation.^{62,92,96,97} As a result, coating materials that contain oil from conjugated fatty acids dry more quickly compared to oil containing methylene interrupted unsaturations. The resultant coating gives more resistance to the extreme weather and adds value to the drying qualities.⁹⁷

Among the medicinal properties of this family of compounds are associated with the anticancer, anti-inflammatory, antiatherosclerotic, antidiabetes, antiobesity (reduce body fat accumulation) properties.^{46,60-64} α -Eleostearic acid has shown stronger anticancer activity by inhibiting the growth of tumour cells and inducing the apoptosis of colon cancer cells.^{60,61,98} This fatty acid has also been shown to alter lipid metabolism by lowering the serum lipid level.⁶⁵ Calendic acid has antiinflammatory properties that is used to treat burns, cuts, rashes, any skin wounds⁹⁹ and causes the reduction of body fat content in mice.^{63,48} Dimorphecolic acid is an aromatase and acetylcholine esterase inhibitor and is used to treat familial Mediterranean fever, a hereditary inflammatory disorder.¹⁰⁰ α -Parinaric acid is commercially used as fluorescent binding tag that selectively binds to the peroxisome proliferator activated receptors (PPARs), and target proteins for drugs against cancer, diabetes, atherosclerosis.¹⁰¹ It is also used as an fluorescent tag to measure lipid peroxidation in cardiomyocytes during ischemia and reperfusion and has also shown promising cytotoxic activity against malignant glioma cell lines.^{102,103}

1.2 Fatty acid desaturases

1.2.1 Historical Overview

As illustrated in the previous section, conjugated fatty acids in plants are generated from unsaturated fatty acyl precursors (Figure 1.7) *via* an O₂-dependent dehydrogenation pathway that is catalyzed by a superfamily of enzymes known as fatty acid desaturases (FAD).^{1,2,104} To differentiate this highly exceptional aerobic dehydrogenation of unactivated methylene groups from the anaerobic removal of hydrogens at an activated position, the term “desaturation” was proposed.^{1,2,105} Fatty acid desaturases are non-heme diiron proteins that introduce double bonds in a manner that cannot be reproduced by synthetic organic catalysts.^{1,4,106} The enzymes are highly regioselective, stereoselective as well as chemoselective and are universally present in every aerobic living organisms examined to-date including bacteria, algae, fungi, plants, insects, protozoa and mammals. Desaturases are unanimously denoted as Δ^n , where “n” signifies the position of the double bond going to be introduced into the fatty acid chain with respect to the carboxyl carbon. For example, a desaturase enzyme that

Figure 1.8. A prototypical O₂-dependent desaturase-mediated biotransformation reaction (X can be CoA, PL or ACP).

introduces a double bond at the C-9,10 position of a 18C fatty acyl chain is referred to as Δ^9 -desaturase (Figure 1.8).

Some desaturases also use the terminal methyl group as a reference point and introduce the double bond relative to that position. This group of desaturases are designated as “ ω -n”. As an example, a desaturase enzyme that introduces a double bond three carbons away from the methyl terminus, *i.e.* at C-15 carbon of a 18 carbon fatty acyl chain is designated as a ω -3 desaturase.

The intriguing biosynthetic process was discovered more than 50 years before when Bloch and Bloomfield first revealed that a membranous fraction of yeast, *Saccharomyces cerevisiae* bioassay can desaturate palmitoyl-CoA (and by implication stearoyl-CoA) to generate oleoyl-CoA in the presence of O₂ and NADPH (Figure 1.8).^{1,107,108} The cryptoregiochemistry (site of initial H-abstraction) and stereospecificity of this biotransformation was also subsequently examined by this group by using radiolabelled isotopes; it was demonstrated that the Δ^9 desaturase from *Corynebacterium diphtheriae* is initiating the oxidation by selectively removing the *pro R* hydrogen at C-9 and then the *pro R* hydrogen at the C-10 position of substrate.^{2,109} This implies that desaturation is a *syn* dehydrogenation process. It is now known that the essential biochemistry of this reaction involves four-electron reduction of molecular oxygen to two molecules of water where two electrons come from NAD(P)H *via* an electron transfer chain involving a NAD(P)H reductase and one or more electron transfer protein such as ferredoxin or cytochrome b5; and other two electrons comes from the fatty acid substrate.^{1-4,110} The electron donors (NADH, NADPH) and the carrier protein (cytochrome b5 or ferredoxin) can vary with the type of desaturase operating.¹

1.2.2 Structural Biology of Desaturases

Significant progress have been made in the last few decades in understanding the structural and molecular biology associated with this remarkable class of enzymes. This activity has led to the identification of many more desaturases with unique regioselectivities.¹¹⁰⁻¹¹² Amino acid sequence comparison for a large set of desaturases were examined by Shanklin and Fox and as a result of their effort, it was apparent that desaturases could be divided into two major structurally unrelated categories: a) a smaller group of soluble desaturases that is found only in plants and utilizes specifically the fatty acid substrates as acyl carrier protein (ACP) thioester derivatives¹¹³; b) a relatively large set of widely distributed integral membrane-bound desaturases that act on CoA- or phospholipid (PL)-linked fatty acid substrates.^{1,112,114,115} These two classes of desaturases have also been shown to differ significantly with respect to their reaction kinetics.¹¹¹

1.2.3 Soluble Plant Desaturases

An example of a typical soluble plant desaturation reaction is the production of oleoyl-ACP from stearoyl-ACP catalyzed by a soluble plant stearoyl Δ^9 -desaturase in the castor bean (*Ricinus communis* L.) (Figure 1.8).² Plant seed oil is the major source of oleic acid where it is stored as triacylglyceride derivative. This observation has led to the investigation of several plant tissues such as safflower seeds, heder helix (English ivy), soybean cotyledons, spinach and avocado. Considerable attempts have been made in the last two decades to biochemically characterize the soluble plant desaturases which eventually lead to the identification and determination of the crystal structures of several soluble plant desaturases.¹¹⁶⁻¹²³ The most significant breakthrough in desaturase research took place in 1991, when Shanklin and Somerville as well as Thompson *et al.*

individually succeeded in isolating and purifying the soluble plant stearyl-ACP Δ^9 -desaturase from castor seed¹²⁴ and safflower embryos¹²⁵ respectively, and overexpressed this protein in *E. coli*.^{117,118} Lindqvist and Shanklin also resolved the three-dimensional crystal structure associated with castor stearyl-ACP Δ^9 -desaturase. The crystallographic model revealed the presence of a recombinant homodimeric desaturase (molecular wt ~ 70 kDa) where each of the monomeric unit contain a diiron cluster in the vicinity of a hydrophobic binding cavity (Figure 1.9a).^{118,124,125} In agreement with spectroscopic data,¹¹⁸ it was demonstrated that the active site of this enzyme features a non-heme diiron core that is

Figure 1.9. Three-dimensional crystal structure of soluble plant stearyl-ACP Δ^9 -desaturase. **a)** View of the homodimer along the α -axis. The two subunits are coloured green and blue, respectively and two iron units are indicated as red spheres; **b)** Stereoview of the diiron centre with ligands. Images taken from Lindqvist *et al.*¹¹⁸

coordinated with four carboxylate μ -oxo bridged and non-bridged glutamate ligands and two histidine units of the amino acid side chain (Figure 1.9b).^{118,126} A water molecule was also found in the second coordination sphere from the iron atoms. This catalytic core was found to be surprisingly similar to that of well characterized methane monooxygenase (MMO) enzyme that is responsible for the hydroxylation of methane to yield methanol.¹²⁶

Analysis of the structure by Lindqvist *et al.* suggested a deep hydrophobic binding cavity at the active site that is presumed to accommodate the substrate fatty acid and extends from the surface to the interior of the enzyme.¹¹⁸ The topology of this hydrophobic binding channel is boomerang-shaped and forces the substrate fatty acid to adopt a *quasi-gauche* conformation at the C9-C10 position (Figure 1.10).^{1,2,118} This arrangement allows the C-9 and C-10 *pro R* hydrogens to come very close to the catalytic

Figure 1.10. Computer model of a soluble plant Δ^9 -desaturase docked with a stearyl-ACP substrate in the hydrophobic binding pocket showing the topology of the active site. Image taken from Buist.²

centre that would facilitate the regioselective *syn* dehydrogenation process and generate thermodynamically unfavorable Z-double bond in the product oleoyl-ACP.^{116,118} The

model was supported by the stereochemical investigation of this enzyme using stereospecifically monodeuterated fatty acid substrates (see Section 1.3.1).¹²⁷ Using thia-substituted analogues it was demonstrated that the catalytic non-heme diiron core is not actually present at the symmetrical position between C-9 and C-10 carbons, but slightly towards the C-10 carbon (see Section 1.3.2).¹¹⁰

It is worthwhile mentioning here that other soluble plant desaturase enzymes that show different regioselectivities and chain length specificities exhibit strong similarities to that of the castor stearyl-ACP Δ^9 -desaturase in terms of their overall amino acid sequence and diiron binding mode.¹⁰⁴ Crystal structures of a soluble Δ^4 -desaturase from *Hedera helix* was recently documented; sequence comparison showed that this regioselective desaturase enzyme shows 74 % homology in terms of its overall amino acid sequence with castor Δ^9 -desaturase.¹¹⁷ The primary determinant of regioselectivity turned out to be the interaction between the particular amino acid (ASP280 in castor Δ^9 -desaturase and the corresponding LYS275 in ivy Δ^4 -desaturase) located at the entrance of the binding cavity and the acyl carrier protein, ACP (phospho-serine 38 in castor Δ^9 -desaturase and phosphate of the pantetheine in ivy Δ^4 -desaturase).¹²⁸ Most of the mechanistic, structural and bioinorganic development in this area of desaturase research has been carried out using the castor stearyl-ACP Δ^9 -desaturase as a model.^{3,4}

1.2.4 Membrane-Bound Desaturases

As mentioned previously, dehydrogenation reaction at C-9 and C-10 of a stearyl substrate has also been found to be catalyzed by a set of membrane-bound Δ^9 -desaturases.² An example of such biotransformation has already been given in the historical overview section 1.2.1: Bloch first demonstrated that in yeast, Δ^9 -desaturation

of stearyl-CoA generates oleoyl-CoA. In contrast to the situation described above for soluble desaturases, there has not been much progress in isolation, purification and characterization of the membrane-bound desaturases.^{1,2} This is mostly because of the fact that the membrane-bound desaturases are highly unstable outside of the native hydrophobic environment.¹ Purifying membrane-bound desaturases to generate sufficient quantity for structural and spectroscopic determination has proven to be difficult.¹ Strittmatter's group first reported the isolation and purification of an integral membrane-bound Δ^9 -desaturase along with its electron transport chain consisting of an NADH (nicotinamide adenine dinucleotide) reductase and cytochrome-b5 components by fractionation of rat liver microsomes.¹²⁸ This group has also reported the presence of a non-heme iron functionality instead of a heme group at the active site of rat liver microsomal stearyl-CoA Δ^9 -desaturase.¹²⁹ Subsequent comprehensive examination by Shanklin and Fox of the amino acid sequences of a larger set of membrane-bound desaturases from mammals, fungi, insects, higher plants and cyanobacteria revealed that there exists eight essential histidine residues and these are present in three highly conserved HX_(2,4)H boxes.¹¹⁴ This hypothesis was confirmed using site-directed mutagenesis experiments. As described above, the enzymatic properties of desaturation *i.e.* the requirement of O₂ and NADPH and a catalytic iron centre matches that of hydroxylation by mixed function monooxygenases.¹³⁰ Therefore, it has long been believed that the active site features of both types of enzymes should be similar. Thus, membrane-bound alkane hydroxylases from *Pseudomonas oleovorans* and related organisms also feature the presence of such three highly conserved histidine boxes. In addition, the existence of a non-heme diiron core at the active site was established for the

P. oleovorans enzyme by Mössbauer spectroscopy.¹³¹ It is presumed that the essential histidine residues of membrane-bound desaturases/hydroxylases are coordinating with the non-heme diiron center at the catalytic site.^{1,2} A fused cytochrome b5 component *i.e.* responsible for transporting electrons from NADH reductase to the catalytic diiron center was also documented with a number of membrane-bound desaturases.¹³²

At present, only a generalized two-dimensional topological model is available (Figure 1.11) that was developed by hydropathy analyses of the overall amino acid sequence of the rat liver stearyl-CoA Δ^9 -desaturase with a homologous yeast Δ^9 -desaturase.^{133,134} The model demonstrates that the enzyme is actually anchored in the endoplasmic reticulum (ER) membrane where it forms two pairs of closely-spaced membrane-spanning domains and majority of the enzymatic part is suspended in the cytosolic side of the endoplasmic reticulum (ER) membrane.¹ However, due to the lack of proper structural information, how the interaction between the substrate fatty acid and amino acid side chains at the active site of these classes of enzymes facilitates such selective dehydrogenation still remains highly illusive.

Figure 1.11. Proposed topological model of the membrane-bound Δ^9 -desaturase (FAD2) showing the presence of two closely spaced membrane-spanning domains in the ER membrane and three essential histidine regions in the cytosolic part are coordinating with the non-heme diiron center at the active site. Image adapted from Meyer *et al.*¹³⁵

1.2.5 FAD2 Family of Desaturases

A very important subset of membrane-bound fatty acid desaturases is the family of phytoenzymes that are responsible for the biosynthesis of the essential fatty acid, linoleic acid.¹³⁶ One of the two pathways by which linoleic acid is biosynthesized in plants involves an extraplastidial oleate Δ^{12} -desaturase enzyme (FAD2).^{2,137} In addition, a number of closely related species-specific FAD2 variants generate a number of exotic fatty acids as discussed previously in this thesis (Figure 1.12). In 1996, Pat Covello's group was the first to successfully express FAD2 in *Saccharomyces cerevisiae* - a convenient yeast strain.¹³⁸ This early triumph greatly facilitated subsequent mechanistic studies of FAD2 and related enzymes using *in vivo* yeast expression systems instead of plant tissues.

Figure 1.12. Biosynthesis of several exotic oxidized fatty acid compounds in plants catalyzed by a family of FAD2 variants. The Figure was adapted from Buist.¹

1.3 Mechanistic investigations of desaturases

The currently accepted mechanistic model for fatty acid desaturation is depicted in Figure 1.13.¹ This model is equally acceptable for both soluble and membrane-bound desaturases since both of the enzymatic systems utilizes a non-heme diiron catalytic center, molecular O₂ and NADH as a source of electrons.^{1,2} Experimental evidence from different sources suggested that the removal of hydrogens from two vicinal carbons takes place in a non-synchronous (step-wise) manner.^{1,26,127,139} It has also been demonstrated that during the course of dehydrogenation a *syn* removal of hydrogens takes place by abstracting the *pro R* (or topological equivalent) hydrogens from the vicinal carbons.^{1,40,109,140} A highly constrained active site forces the substrate to bind in a

Figure 1.13. The currently accepted mechanistic model for fatty acid desaturation. The Figure was adapted from Buist.²

quasi-gauche/near-eclipsed conformation.^{1,2} The desaturation is initiated with the abstraction of a hydrogen atom (*pro R*) using a compound Q-type high valent iron-oxo species¹⁴¹ leading to the formation of a carbon centered radical intermediate.¹ This step is believed to be kinetically the slowest step of the reaction as it involves the breaking of a very strong, unactivated secondary C-H bond.¹⁴² This radical intermediate is highly unstable and collapses immediately *via* the elimination of second hydrogen from the adjacent carbon and generating a *cis* C-C double bond along with iron-bound water.^{1,110} According to the current scenario, removal of the second hydrogen could occur either directly or *via* a one electron oxidation/deprotonation sequence.^{1,110}

Support for this model (Figure 1.13) was obtained from many membrane-bound desaturases that produce the corresponding regiospecifically hydroxylated alcohol as low-level error product.¹⁶ It is thought that generation of hydroxylated product could only be possible if instead of a second hydrogen removal, in fact a competing “hydroxyl rebound” step is followed.² Early evidence by Bloch suggested that the hydroxylated intermediates are not forming as an intermediate during the course of olefin production.¹⁴³ It can be assumed that the relative positioning of the FeOH species with respect to the radical intermediate as well as the orientation of the second C-H bond compared to the half-filled orbital of carbon-centered radical (*syn*-periplanar for double bond formation) could determine the reaction outcome.^{1,110}

In summary, for each desaturase or desaturase variant, three fundamental questions associated with this generic mechanistic model need to be answered:²

1. The stereochemical issue - *i.e.* which hydrogens are removed during the course of desaturation? This is related to the enantioselectivity of desaturation at each prochiral centers.

2. The cryptoregiochemistry or site of initial oxidation - *i.e.* assuming there is a preference, then which hydrogen is getting abstracted first by this highly oxidizing iron-oxo species?
3. The chemoselectivity of desaturation - *i.e.* what factor(s) controls the fate of putative radical intermediate outcome such that the formation of hydroxylated product is prevented?

Answers to the first two questions give critical information on the position of the oxidant with respect to the substrate and help design possible computational experiments to tackle the third. Mechanistic investigations of desaturase-mediated reactions are very much dependent on the use of sterically non-demanding probes that can function as an alternative to the substrate.¹ For example, the use of methyl substituted fatty acid analogues as alternative substrates by James *et al.* where a methyl group was present adjacent to the site of initial oxidation resulted in unproductive substrate binding.¹⁴⁴ This experimental finding put some limitation over the types of substrate analogues that can be used as mechanistic probes for the active site. In general the substrates bearing isotopic labels such as deuterium ($^2\text{H}_1$), tritium ($^3\text{H}_1$), ^{18}O and isosteric substitution of hydrogen with fluorine, or methylene unit(s) with sulfur or oxygen are used as possible mechanistic probes.^{1,2} Fatty acid analogues must be enzymatically activated as ACP, CoA or phospholipid ester derivatives which place further restriction on their use.¹ Critical analysis of all these issues are explained in the following sections.

1.3.1 Stereochemistry

There are two stereochemical issues associated with the course of desaturation. These are firstly, the enantioselective way of hydrogen removal from each methylene

carbons by the reactive diamond-shaped diiron oxo species and secondly, the relative stereochemistry of C-H bond breaking at the two adjacent carbons to produce either *cis* or *trans*-double bonds.

Traditionally the stereochemistry of hydrogen abstraction is analyzed by using isotopically labelled fatty acid substrates (Figure 1.14). Deuterium or tritium is used as the isotopic label for this purpose.¹

Figure 1.14. Use of chiral monodeuterated substrates as isotopic mechanistic probe to determine the enantioselectivity of Δ^9 -desaturation.

The approach was first recognized as a powerful tool back in the 1960's when Bloch revealed the stereochemical course of oleate formation in yeast using four stereospecifically monotritiated stearic acids and monitored the loss of isotope labelling with respect to ^{14}C -labeled substrate.¹⁰⁹ These isotopically labelled stearates were synthesized from naturally occurring hydroxystearic acids of known absolute configuration.^{109,140,145} Since then, this general approach continues to be fruitfully applied in investigating fatty acid desaturation. Nowadays, the chiral monodeuterated fatty acids are prepared from alcohols that are produced in high enantiomeric excess using well established synthetic methodology.¹⁴⁶ In some cases, especially *in vivo* experiments where chances of isotopic dilution due to endogenous product formation is high, the substrates also contain extra remote deuterium labels to facilitate mass spectrometric

analysis.^{40,81} The desaturated product of the incubations *in vitro* or *in vivo* are analyzed by gas chromatography coupled to mass spectrometry to monitor the loss or retention of deuterium labelling (Figure 1.14). In an alternative way, the chiral monodeuterated substrates can also be generated *in situ* from [2-¹³C, 2-²H₃]-acetate *via* fatty acid synthase and the loss of isotope labels can be traced at even-numbered carbons using ¹³C NMR.^{147,148}

The second stereochemical issue of desaturation *i.e.* whether the conversion is operating in *syn* or *anti* fashion can be most easily estimated using *threo*- and *erythro*-dideuterated substrates, which can be synthesized from the corresponding olefinic precursor using stereospecific reduction (see Figure 1.15).¹ The resultant desaturated product is analyzed by gas chromatography-mass spectrometry to determine whether the biosynthetic process yielded mainly monodeuterated or non-deuterated/dideuterated product.

Figure 1.15. Determination of the relative stereochemistry of C-H bond breaking *via* the racemic *erythro* and *threo*-dideuterated fatty acids as probes which confirmed the *syn*-removal of hydrogens from the adjacent methylene carbons.

A novel approach to ascertain the stereochemistry of hydrogen removal has been developed by Buist *et al.* that exclude the necessity of synthesizing chiral monodeuterated substrates. This approach features the use of fatty acid thia analogues acting as an oxo trap to yield chiral sulfoxides in high enantiomeric excess (Figure 1.16).^{149,150}

Figure 1.16. Application of thia-fatty acid analogue to elucidate the stereochemistry of desaturation. **A)** Stereochemical relationship between the fatty acid desaturation and sulfoxidation; **B)** Determination of the absolute stereochemistry at the sulfoxide center using a chiral solvating agent (CSA) showing the effect of complexation on the chemical shifts of neighbouring atoms in NMR.

The absolute stereochemistry of the sulfoxide center is ascertained through the use of Pirkle-type chiral NMR solvating agents (Figure 1.16B).^{150,151} These form a collision complex by forming a H-bond between the reagent's hydroxyl or carboxyl hydrogen and sulfoxide oxygen.^{149,152} In addition to the hydrogen bond, to account for the specific shielding effects a stabilizing interaction between the lone pair of the sulfoxide

and the methine of the CSA has also been proposed. Such complexation induces shift non-equivalence in selected NMR signals of the sulfoxide. Use of larger aromatic groups than phenyl, such as in (*S*)-AMA, (*R*)-TFAE or (*R*)-Naproxen increase the magnitude of non-equivalence on the selected NMR signals which leads to greater separation of the particular peaks in terms of chemical shift (δ).^{153,154} The results obtained to date from these oxo transfer reactions have matched exactly with the known enantioselectivity of hydrogen removal as determined *via* isotopically-labelled fatty acid substrates (Figure 1.16A).¹⁵¹

More recently, the sensitivity of this chiral sulfoxide procedure in determining the enantioselectivity of desaturation was further enhanced through the use of ω -fluoro-tagged thiasubstrates in combination with well-characterized chiral solvating agents (CSA) (Figure 1.17).^{153,155,156} The procedure was first developed by Buist and coworkers who showed that the presence of a fluorine atom at the remote ω -position can be used to differentiate between the two enantiomeric sulfoxides by ^{19}F NMR /CSA methodology.¹⁵⁵ Surprisingly, the sense of the non-equivalency generated due to the complexation of the ω -fluoro-tagged chiral sulfoxides with the CSA is opposite to that observed with ^1H NMR as was determined using synthetic standards.^{153,155,156} This technique can be applied to resolve the stereochemistry of chiral sulfoxides at the trace level (nanomole scale). The high natural abundance of ^{19}F isotope, inherently high sensitivity and wide chemical shift range has made the ^{19}F -NMR based methodology highly successful.

To date, employing all these mechanistic tools, a *syn* dehydrogenation pattern has been observed with all desaturases, including soluble and membrane-bound, that again

support the proposed mechanistic mode (Figure 1.13).¹ Also for all desaturases, it has been shown that both hydrogens are removed with *pro R* (or equivalent) selectivity.

Figure 1.17. A) Castor stearyl Δ^9 -desaturase-mediated chiral sulfoxidation of 18-fluoro-10-thiooctadecanoyl ACP; B) Pirkle-binding complexation model for the interaction of (*R*)-AMA with the two enantiomers of 18-fluoro-10-thiooctadecan-1-ol S-oxide; C) its effect on ^1H -decoupled ^{19}F NMR spectroscopy - i) Enzymatically produced 18-fluoro-10-thiooctadecan-1-ol S-oxide (*Biosyn-1*), ii) (*Biosyn-1*) + (*R*)-AMA, iii) (*Biosyn-1*) spiked with racemic 18-fluoro-10-sulfoxy-1-octadecanol in presence of (*R*)-AMA.¹⁵³

1.3.2 Cryptoregiochemistry

Cryptoregiochemistry¹⁵⁷ is the most general term used to indicate the “site of initial oxidation” during the course of desaturation. As the desaturation reaction involves the abstraction of two hydrogens, the reaction could potentially start from either carbon generating a carbon centered radical that would ultimately yield the same double bond following the second removal of hydrogen. According to the literature, there are three ways to verify the cryptoregiochemistry of hydrogen abstraction. These are - i) the KIE measurement of each C-H bond breaking step, ii) use of thia-analogues and evaluating the relative amount of sulfoxide formation and iii) isolation of the hydroxyl product that is formed by a low-level “error” pathway during dehydrogenation.

The most widely used and straightforward methodology of determining the cryptoregiochemistry of double bond formation is *via* the KIE (kinetic isotope effect) measurement.^{1,111,157} The approach is based on the principle that the abstraction of the first hydrogen by breaking the unactivated, very strong C-H bond should be energetically more difficult as compared to the second hydrogen removal. As a result, the initial C-H bond breaking step should be more sensitive towards isotopic substitution than the later one. Therefore, if one can replace that particular C-H bond by C-D then there should be a kinetic isotope effect associated with its cleavage as breaking the C-D bond is energetically more difficult compare to a C-H bond. However, there should be essentially no KIE associated with the second C-H bond cleavage step as it is not kinetically important (fast step). The primary deuterium KIE is only observed at the rate determining step.¹⁵⁷ By measuring the primary deuterium KIE on each C-H bond breaking step one can easily estimate the site of initial oxidative attack by the active diiron oxo species. To

measure the primary deuterium KIE a competitive approach has been used, where typically a $\sim 1:1$ mixture of regiospecifically dideuterated to non-deuterated fatty acids is used as substrates and incubated with a convenient desaturating system. The biosynthetic conversion is quenched at low substrate conversion ($<10\%$) and analyzed by using GC-MS.^{1,111,158} The primary deuterium KIE is then computed by comparing the d_0/d_1 ratio of the olefinic product with the d_0/d_2 ratio of the substrate.¹⁵⁷ Racemic monodeuterated substrates can also be used for this purpose and will essentially give the same result.¹⁵⁹ However, it should be remembered that the anticipated primary deuterium KIE value on each C-H bond cleavage step have already some embedded secondary deuterium KIE in it, but this perturbation is expected to be small ($<10\%$).¹⁶⁰ Differential kinetic isotope

Figure 1.18. Use of intermolecular primary deuterium kinetic isotope effect measurement to determine the cryptoregiochemistry of oleate formation by a membrane-bound Δ^9 -desaturase. The site of initial oxidation is observed to be at C-9 as demonstrated by large KIE at C-9 and KIE of ~ 1 at C-10.

effects on each C-H bond cleavage for all membrane-bound desaturases studied to date (one large and one close to unity) clearly support the step-wise nature of the desaturation reaction as originally postulated by Bloch.^{107,108}

However, the KIE protocol could not be used to determine the cryptoregiochemistry of dehydrogenation for soluble plant desaturases where it was found that C-H bond breaking steps are not kinetically important steps of the reaction.^{1,161} Instead, other enzymatic events such as the substrate binding or product release step were found to be kinetically more important.¹ Both competitive and non-competitive estimation of the intermolecular primary deuterium KIE on C-H bond cleavage at C-9 and C-10 by the soluble plant Δ^9 -desaturase support this comment (KIE estimated to be ~ 1 in both cases).^{161,162} A more broadly applicable method of determining the site of initial oxidative attack has been developed by Buist and coworkers.^{1,151,163} Systematic replacement of the substrate methylene groups by the isosteric sulphur atom can potentially induce regioselective oxo transfer from the diiron oxo species leading to the formation of sulfoxy-fatty acid (Figure 1.19).^{1,2}

Figure 1.19. Schematic diagram preferential 9-sulfoxide formation by membranous Δ^9 -desaturase.

The hypothesis is that the relative amount of sulfoxide production formed from each positional thia-analogue *via* oxo transfer should indicate the positioning of the putative diiron species relative of the substrate.^{1,151} This approach was initially validated for a membrane-bound yeast Δ^9 -desaturase as can be seen by comparing the results shown in Figure 1.18 and 1.19. In contrast, cryptoregiochemical investigation of a soluble plant Δ^9 -desaturase revealed that 10-thiastearoyl ACP was efficiently converted into the corresponding sulfoxide, whereas the 9-thia analogue resulted in only a lower amount of (10 %) sulfoxide conversion.²

The sensitivity of this thia approach can be further enhanced through the incorporation of a fluorine tag at the remote ω -position (Figure 1.20).^{164,165} Relative changes in the position of sulfoxidation from C-9 to C-10 can greatly affect the chemical shift of fluorine even though this atom is many bonds away from the position of the oxidative event.¹⁶⁶ The oxidized ω -fluoro-tagged-sulfoxy fatty acid products isolated

Figure 1.20. Determination of cryptoregiochemistry of castor stearyl soluble-ACP Δ^9 desaturase involved formation oleoyl-ACP by using ω -fluoro-tagged thia-fatty acid analogues as mechanistic probe.

from the cultures and subsequently analyzed by using ^1H -decoupled ^{19}F NMR spectroscopy where an efficient measurement can be done even at the nanomole

scale.^{165,167} The cryptoregiochemical result obtained from this fluoro-tagged thia analysis corroborate well with what have been observed using non-labelled the thia-analogues.¹⁶⁶

Valuable information about the cryptoregiochemistry can also be obtained through the use of another mechanistic probe, namely by identifying the hydroxyl byproduct that is generated during the course of double bond formation as a low-level error product.¹⁶ As depicted in Figure 1.13, in a complementary pathway the putative radical intermediate can collapse to give alcohol.^{1,4} In such circumstance, the position and stereochemistry of the hydroxyl byproduct should match with the site of initial oxidation as well as the enantioselectivity of initial hydrogen abstraction.¹ The yeast membrane-bound Δ^9 -desaturase from several sources produces ca. 1 % of 9-hydroxystearate as byproduct during dehydrogenation which clearly shows the site of initial hydrogen abstraction is at C-9 (Figure 1.21).¹⁶ Support for this observation has already obtained from the KIE methodology and sulfoxidation strategy.¹⁶⁸ Hydroxylated by-products are usually detected in GC-MS as silylated ether derivatives that dramatically increases the peak intensity.²

Figure 1.21. Regioselective desaturase-mediated hydroxyl byproduct formation.

1.3.3 Highly Conserved Active Site Structural Motif

By comparing all of the stereo and cryptoregiochemical results presented in the literature for the desaturase-mediated oxidation of fatty acids, it is apparent that for both soluble and membrane-bound desaturases there exists a highly conserved active site structural motif that determines the outcome of those desaturations.^{1,2,4} The active site architecture of soluble plant desaturases is different from that of the membrane-bound enzymes and shows different substrate specificity: for example, the soluble Δ^9 -desaturase strongly prefers fatty acids with chain length C18, whereas the membrane-bound Δ^9 -desaturase can act on the fatty acids with variable chain lengths from C15 to C19.¹⁶⁹ As demonstrated in Figure 1.22, for the soluble plant desaturase, the reaction starts at the carbon that is further from the carboxyl end or closest to the methyl terminus, whereas for the membrane-bound desaturases (1,2-dehydrogenation mode) the site of initial oxidation

Membrane-Bound Desaturases:

Soluble Desaturases:

Figure 1.22. Highly conserved active-site structural motif of membrane-bound as well as soluble plant desaturases.

is always at the carbon closer to the carboxyl end.^{1,161} However, the overall enantioselectivity of hydrogen removal is the same for both desaturating systems *i.e.* the

pro R hydrogens are abstracted from the prochiral methylene groups during the course of desaturation.

1.3.4 Chemoselectivity: Dehydrogenation/hydroxylation Connection

As alluded to before, many membrane-bound desaturases as well as other members of the family of iron-bound proteins, such as non-heme monoiron (MMO) and heme iron (Cytochrome P450) have been documented to produce either dehydrogenated or hydroxylated products.^{1,16,112,170} However, the critical issue that still remains to be understood is the nature of the switch that controls the reaction outcome for those systems. Due to the scarcity of the proper crystallographic information regarding the desaturase-mediated enzyme/substrate complex, proper explanation is difficult.^{1,2} It is thought that there are at least three different factors that may influence the dehydrogenation vs. oxygenation channels. These are the electronic, steric and metal ion-based effects that may be operating either singly or in combination to determine the chemoselectivity of desaturases.¹

The generic mechanistic model of desaturation (Figure 1.13, 1.22) was developed based on the assumption that both dehydrogenation and hydroxylation reactions proceeds *via* a common radical intermediate step. As an explanation of the electronic factor, redox potential of this putative radical intermediate plays an important role to decide whether the reaction should direct towards the dehydrogenation or hydroxylation pathway.¹ While the ‘hydroxyl rebound’ would lead to the formation of the oxygenated product, the double bond formation can proceed either by direct removal of a second hydrogen or *via* a rapid one electron oxidation to give a carbocation followed by deprotonation sequence. Thus it can be said that the carbocation formation would facilitate the dehydrogenation

sequence, whereas the radical intermediate would favor the ‘hydroxyl rebound’ step.¹ As a result, if somehow this radical intermediate is oxidized to a carbocationic intermediate then that will eventually yield the olefinic product.¹⁵⁰ Support for such a hypothesis has already been obtained from an experimental model where it was observed that the sMMO enzyme, which is typically a hydroxylating protein, can also produce the dehydrogenated product using ethylbenzene as substrate (π -cation stabilization at the benzylic position).¹ On the other hand, castor stearyl-ACP Δ^9 -desaturase, which is known to catalyze only the formation of oleoyl product (>99.99 % detection limit), produces the 10-hydroxylated fatty acids upon the introduction of a fluorine atom into the substrate namely as (*R*)- and (*S*)-9-fluorostearate (Figure 1.23).¹²⁷ It has been suggested that the strong inductive electron withdrawing effect of highly electronegative fluorine disfavours the formation of a carbocation at the β -position and allows the radical step to compete, yielding fluorohydrins along with the olefinic products.^{1,127}

Figure 1.23. Comparison of (*R*)- and (*S*)-9-fluorostearate oxidation by a soluble plant Δ^9 -desaturase leading to novel hydroxylated and olefinic products.¹²⁷

There are two different issues associated with the steric explanation of the chemoselectivity of the desaturase-mediated reaction outcome. These are - firstly, the proper alignment of the β C-H bond with respect to the singly occupied molecular orbital (SOMO) of the radical intermediate and secondly, the relative positioning of the iron-hydroxyl species compared to the two reactive carbon centers (Figure 1.24).^{1,110} Slight changes in the active site architecture of the enzyme could affect these issues that would lead to the alteration of the hydroxylation/dehydrogenation ratio. Briefly, the β C-H bond must be placed precisely in *syn*-periplanar arrangement with respect to the half-filled orbital to promote the double bond formation.¹ In addition, the iron-hydroxyl species

Figure 1.24. Positioning of the iron-hydroxyl species relative to the two carbons of the incipient double bond. Active site scenarios leading to **A** desaturation and **B** hydroxylation.¹

should also reside close to the second hydrogen so that a favorable removal takes place (Figure 1.24). Violation of any one of these two conditions that favor dehydrogenation would lead to the hydroxylation.

Hepatic cytochrome P450 family of enzymes are known to catalyze primarily hydroxylation reactions, as in the example found by Baillie *et al.* in the hydroxylation/dehydrogenation of the epileptic drug, valproic acid (Figure 1.25).^{1,171}

Figure 1.25. C-4 initiated bimodal oxidation of valproic acid.¹

This can be rationalized since cytochrome P450 families of enzymes are known to act on a wide variety of substrates, which means that the active site morphology for these class of enzymes are highly flexible to accommodate various substrates. In contrast, the binding cavities of desaturases are highly conserved for a particular fatty acid chain length and its conformation. Therefore, the alignment required to get dehydrogenation can't be sufficiently controlled by P450 enzymes and that's why they rarely exhibit desaturase activity.¹ Moreover, this concept of steric factors is further supported through the result obtained from a recent site-directed mutagenesis experiment on a bifunctional membrane-bound 12-hydroxylase/12-desaturase enzyme.¹⁷² The result demonstrated that a relatively small change of the hydrophobic residues in the substrate-binding cavity

significantly influenced the relative ratio of hydroxylated vs. dehydrogenated product formation. Both enzymes proceed *via* a C-12 carbon-centered free radical.

Figure 1.26. Relationship between 12-hydroxylation / 12-dehydrogenation.

In consideration of the metal ion-based effect, the ligand environment of the iron center and the relative energies associated with the coordination of the iron center with the water product (desaturation) vs. no ligand (hydroxylation) may act to drive the selection of reaction pathway.^{1,170} A case in point is a non-heme monoiron-based enzyme, clavamate synthase 2 that selectively catalyzes the hydroxylation of deoxyguanidinoproclavaminic acid (DGPC) during the course of clavulanic acid biosynthesis, but also generates desaturated product (with DPC) depending upon the nature of a pendant amino substituent present at the γ -position relative to the site of initial oxidation (Figure 1.27).¹⁷⁰ It has been postulated that the switch between the hydroxylation and desaturation reaction is determined by the relative stability of the coordinated iron-water complex, which in turn is affected by how the different substrates bind in the active site pocket. The greater the stability of the coordinated iron-water complex, the more the reaction favours the dehydrogenation sequence over hydroxylation (Figure 1.27).¹⁷⁰

Figure 1.27. The role of associated ligand environment at the reaction binding site in controlling the switch between the hydroxylation vs. desaturation channel during the biosynthetic pathway of clavulanic acid.¹⁷⁰

1.4 Mechanistic Investigations of 1,4-Desaturation

FADs responsible for the introduction of the conjugated double bonds by 1,4-dehydrogenation reactions have been investigated in several higher plants and insects.^{34,39,40,43,63,71} Examples of such fatty acid modifications are already illustrated in the previous sections 1.1.5 & 1.1.6. In the early literature, the enzymes responsible for 1,4-dehydrogenation process were termed as “conjugases”. This misleading name is better replaced with “1,4-desaturase” or “Δ^{n,m} desaturase” where “n” and “m” represent the carbon number of the incipient diene system. Sequence analysis of the enzymes responsible for 1,2- and 1,4-dehydrogenation reveals a close relationship between them (Figure 1.28).^{1,34,63}

Figure 1.28. CLUSTAL comparison between the amino acid sequences of FAD2 variants responsible for biosynthesis of conjugated fatty acids in plants.

The detailed descriptions of their mechanism of action both in insects and plants are given below.

1.4.1 Insects

A description of the conjugated fatty acids found in insect pheromone blends and their species-specific uses as chemical signals in different moth species has already been described in section 1.1.5. *In vivo* mechanistic work by Gemma Fabrias and coworkers showed that (10*E*,12*E*)-dodecadienoic acid is produced from (*Z*)-11-tetradecenoic acid by the action of a bifunctional Δ^{11} -desaturase (Figure 1.5).^{39,40} The compound generated by Δ^{11} -desaturation of tetradecanoic acid is further used as a substrate by the same Δ^{11} -desaturase to introduce the conjugation at C-10,C-12 position (1,4-dehydrogenation) *via* the modification of the existing double bond at C-11. Stereochemical analysis of this

bifunctional enzyme by employing stereospecifically monodeuterated fatty acids revealed that the 1,4-dehydrogenation is taking place *via* the *syn* removal of two hydrogens from the non-adjacent positions C-10 and C-13 (Figure 1.29)^{40,71}; the enantioselectivity of this process is identical to that of Δ^{11} -desaturation of tetradecanoic acid.^{1,74}

Figure 1.29. Topological model for the stereochemical investigation of the biosynthesis of (10*E*,12*E*)-fatty acetate as a component of sex pheromone blend in moth *Spodoptera littoralis* using stereospecifically monodeuterated fatty acid analogues.

Incubating ~ 1:1 mixtures of non-deuterated to regiospecifically dideuterated FA precursors (C-10 and C-13) and analysis of the resultant primary deuterium kinetic isotope effects showed that the 1,4-dehydrogenation is initiated by rate determining removal of hydrogen from C-10.³⁹ Thus a large KIE was observed with the C-10 dideuterated species (10.2 ± 1.6) whereas the C13-H cleavage was found to be insensitive to isotope substitution (1.2 ± 0.2).

1.4.2 Plants

It has been speculated that fatty acids bound to phosphatidylcholine (PC) serve as precursors for the biosynthesis of these conjugated fatty acids from a linoleoyl precursor in the corresponding developing plant seeds.^{42,62,63,92} Isolation and sequence comparison of the genes responsible for the biosynthesis of unusual fatty acids revealed that a family

stereospecifically deuterated linoleates were synthesized by Crombie and Holloway in order to investigate the stereochemistry of this process but insufficient incorporation of labelled precursors in the incubation experiments utilizing plant tissues prevented completion of these experiments.

Cryptoregiochemical analysis of this $\Delta^{8,10}$ -desaturase enzyme by Buist *et al.* using KIE methodology established that formation of calendate was initiated at C-11 (Figure 1.31).¹⁵⁸ Incubating the appropriately labelled linoleates with a mutant yeast strain expressing the *Fac2* – *Calendula officinalis* gene, it was observed that there is a large KIE associated with C11-H abstraction (5.7 ± 1.0) whereas the removal of hydrogen from C-8 position was found to be insensitive to deuterium substitution (1.0 ± 0.1).¹⁵⁸ This result clearly demonstrates the step-wise nature of the 1,4-dehydrogenation reaction and supports the hypothesis that FAD2 variants initiate the reaction from a position that is closest to C-12 - the site of initial oxidation for the parent FAD2 enzyme that introduces a double bond at 12,13 (Figure 1.26).^{1,2}

Figure 1.31. Determination of the cryptoregiochemistry of calendic acid biosynthesis using the KIE measurements.

1.5 Goals of the Thesis

Examples of several FAD2-like desaturating systems in various higher plants have been described in the previous sections that are capable of biosynthesizing a diverse array of conjugated fatty acids such as calendic, α -eleostearic and dimorphecolic acid.^{11,34,62,63,158}

Figure 1.32. Involvement of several variant FAD2 enzymes to generate calendic, α -eleostearic and dimorphecolic acid.

However, only one detailed mechanistic study has been carried out on this class of desaturases, namely the determination by our laboratory of the site of initial oxidation for calendate formation.¹⁵⁸ The enantioselectivity of this 1,4-desaturation has not been reported yet. In addition, the stereochemistry as well as the cryptoregiochemistry of α -eleostearic acid formation has not been studied.^{1,34} Detailed functional analysis of the enzymes from *Dimorphotheca sinuata* showed that there exist two FAD2-like enzymes that work in a tandem fashion to generate the (*R*)-9-hydroxy-(10*E*,12*E*)-octadecadienoic acid from oleic acid (Figure 1.32).¹¹ However, the detailed mechanism of hydroxylation

is still not well understood. As part of ongoing research into the structure-function relationships for this class of FAD2-like enzymes, a closer examination of the conjugated fatty acids biosynthesis is clearly necessary. The availability of a convenient yeast^{11,34,158} expression system for these enzymes offers us a unique opportunity to carry out the mechanistic investigations *in vivo*.

In summary, the objective of the research described in this thesis was to:

- 1) Elucidate the stereochemistry of calendic acid biosynthesis using the FAD2 variant found in the seeds of *Calendula officinalis*.
- 2) Investigate the stereo- and cryptoregiochemistry of α -eleostearic acid biosynthesis using the FAD2 variant of developing tung tree, *Aleurites fordii* Hemsl. seeds.
- 3) Determine the stereochemistry and KIEs associated with the site of initial oxidation in dimorphecolic acid formation using the FAD2 variants operating in the seeds of *Dimorphotheca sinuata*.

Chapter 2: Stereochemistry of Calendic acid Biosynthesis in *Calendula officinalis*

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2.1 Experimental Approach

Previous investigations in our laboratory have shown that calendic acid biosynthesis is initiated *via* initial abstraction of hydrogen from the C-11 position of the fatty acid chain.¹⁵⁸ It was proposed that only a slight shift in the position of the highly reactive diiron oxidant (Figure 2.1) with respect to the substrate is enough to switch the mechanism from the parent desaturation (FAD2) to 1,4-dehydrogenation (FAD2 variant).

As shown below, the C-8 hydrogens and C-11 hydrogens of the fatty acid chain are positioned to allow *syn* dehydrogenation with the same facial selectivity as the *syn* removal of hydrogens from C-12 and C-13 positions during linoleic acid formation (FAD2). The correctness of this mechanistic model could be tested through the determination of the enantioselectivity of polyunsaturated fatty acid formation using stereospecifically deuterated fatty acids as mechanistic probes.

Figure 2.1. Mechanistic model showing the relationship between oxidant position in Δ^{12} -desaturation and the (1,4)-desaturase reaction leading to the formation of the conjugated 8,10-diene system of calendate.¹⁵⁸ The double bonds are quasi-coplanar.

A convenient *Saccharomyces cerevisiae* expression system for gene *Fac2* from *Calendula officinalis* that encoded the (1,4)-desaturase enzyme was available.^{93,158} The host yeast culture system does not endogenously produce the substrate linoleic acid which made the use of other mechanistic probes such as thia-substituted analogues or unnaturally chain-shortened substrates unnecessary. The use of thia-substituted fatty acid analogues as oxo traps to determine the position of the oxidant (Section 1.3.1) was not a viable option as the required vinyl thioether substrates are difficult to synthesize and are unstable.

Our approach required the synthesis of four stereospecifically monodeuterated methyl linoleates with chirotopic center in one pair at C-8 and in other at C-11 (Figure 2.2) *i.e.* methyl (8*R*)-[8-²H₁]-octadeca-9*Z*,12*Z*-dienoate **1a**, methyl (8*S*)-[8-²H₁]-octadeca-9*Z*,12*Z*-dienoate **1b**, methyl (11*S*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate **2a** and methyl

(11*R*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate **2b**. It was anticipated that incubation of each isomer with the mutant yeast cultures containing the $\Delta^{8,10}$ -desaturase enzyme (FAD2 variant) would generate enough calendate product to allow tracking the fate of deuterium label by gas chromatography - mass spectrometry (GC-MS).

Figure 2.2. The target chiral monodeuterated linoleates required to investigate the stereochemistry of calendate formation.

2.2 Results and Discussion

2.2.1 Synthesis of methyl (8*R*)- and (8*S*)-[8-²H₁]-octadeca-9*Z*,12*Z*-dienoate, **1a** & **1b**

2.2.1.1 Overview

There are principally two strategies involved in the synthesis of stereospecifically deuterium labeled linoleates - firstly, by preparing a suitably functionalized, long chain, chiral secondary alcohol followed by subsequent S_N2-type displacement of the corresponding tosylate or mesylate using deuteride (D⁻)^{43,127}; secondly, *via* the synthesis of a shorter chain chiral monodeuterated alcohol followed by subsequent S_N2-type displacement of the corresponding tosylate or mesylate using a terminal acetylide of the correct chain length.^{40,75,116,127,173-179} Alkyne functionality of the synthetic intermediates can be stereoselectively reduced to give the requisite *cis*-olefins.^{158,173,180}

Figure 2.3. Scheme for the synthesis of chiral (8*R*)- and (8*S*)-[8-²H₁]-linoleates.

In this thesis, the -CHD chiral centre was introduced into the C18 fatty acid framework by using the approach described in the second method (Figure 2.3). The synthesis of the title compounds (~ 4 % overall yield) was adapted from that previously reported by Crombie and Heavers¹⁷³ and features the preparation of both enantiomers of ethyl 8-d₁-8-hydroxyoctanoate **6a**, **b** followed by stepwise elongation using acetylide alkylation reactions and subsequent Lindlar reduction. Trial reactions were carried out using non-deuterated reagents.

2.2.1.2 Preparation of (8*S*)- and (8*R*)-Ethyl [8-²H₁]-8-Hydroxyoctanoate, **6a** & **b**

Chiral monodeuterated primary alcohols can be synthesized from the corresponding monodeuterated aldehyde which in turn is readily accessible from the respective dideuterated alcohol *via* various oxidation mechanisms.¹⁷³ As shown in Figure 2.3, diethyl suberate was converted into ethyl 8-hydroxy-[8,8-²H₂]octanoate **4** in 35% yield using half-equivalent of lithium aluminium deuteride in THF.^{174,179,181,182} Flash chromatography was required to remove the diol by-product and starting material. The dideuterated monoethyl ester was then oxidized to the corresponding monodeuterated aldehyde, ethyl 8-oxo-[8-²H₁] octanoate **5** through the use of the Swern oxidation method (93% yield).¹⁸³ This approach represented a substantial improvement (33 % vs. 6 %) over an initial attempt to follow the method of Crombie²⁰¹ as illustrated in Figure 2.4:

Figure 2.4. Scheme for Crombie's method for the synthesis of monodeuterated aldehyde.

Enantioselective reduction of monodeuterated aldehyde **5** was effected using Alpine-Boranes® as the chiral reducing agent¹⁸⁴⁻¹⁸⁶ (Figure 2.3 and 2.5) rather than the Baker's yeast methodology employed by Crombie (the latter involves somewhat cumbersome product isolation procedures). Analyses of the mass spectra (MS) of the TMS derivatives¹⁸⁷ of each chiral asymmetric reduced alcohols indicated that the deuterated intermediates consisted entirely of monodeuterated species (within experimental error). The use of chiral boranes was first pioneered by Midland and coworkers and is still widely used for the asymmetric reduction of aliphatic, aromatic as well as α,β -unsaturated aldehydes to the corresponding chiral primary 1-deuterio alcohols in a simple, efficient manner with high enantiomeric excess.^{174,184,185,188} It has been observed that the use of (+)-(*R*)-Alpine-Borane® results in the synthesis of (*S*)-[1-²H₁]-alcohol from

deuterio aldehyde (Figure 2.5), whereas the (*R*)-[1-²H₁]-alcohol could be obtained by using (-)-(*S*)-Alpine-Borane® from the same aldehyde.

Figure 2.5. Preparation of (+)-(*R*)-Alpine-Borane® and its use in chiral enantioselective reduction of deuterio aldehyde to synthesize the (*S*)-[1-²H₁]-alcohol.

The reactions are usually carried out in an inert atmosphere as B(III) is highly sensitive to oxidation in presence of O₂. The reaction can be successfully performed under exceptionally mild conditions even at room temperature and has been observed that the use of slight excess of Alpine-Borane® reagents (1.2 equivalent) instead of one equivalent leads to an increase in enantiomeric excess.^{185,188} The chiral (+)-(*R*)-Alpine-Borane® reagent is derived from (+)-(α)-pinene and 9-BBN, while the chiral (-)-(*S*)-Alpine-Borane® reagent is made from the reaction between (-)-(α)-pinene and 9-BBN and both of them are commercially available as a solution in THF. The origin of the enantioselective reduction is explained by using the model proposed by Midland *et al.* as described in Figure 2.6.^{185,188} From the model, it is apparent that during reduction, the aldehyde must approach the chiral borane in such a manner so that the steric interaction between the aldehyde R group and the pinane methyl group is minimized.

Figure 2.6. Explanation of the enantiomeric preference Alpine-Borane® reagents in the reduction of aldehydes.

2.2.1.3 Stereochemical Analysis of (8*R*)- and (8*S*)-Ethyl [8-²H₁]-8-Hydroxyoctanoate

The absolute configuration and enantiomeric purity of the derived chiral deuterio alcohols were determined using the Mosher's α -methoxy- α -(trifluoromethyl)phenylacetic esterification (MTPA) derivatization method and comparing the results with previously reported observations.^{173,189} Upon derivatization of primary alcohols with chiral reagents (MTPA-chloride) the enantiotopic hydrogens of the CH₂OH group become diastereotopic and ¹H NMR-nonequivalent due to the presence of another chiral centre nearby. Thus the amount of minor enantiomer in the samples of chiral ethyl 8-d₁-8-hydroxyoctanoate could be estimated by integration of the peak areas for the ¹H signals appearing at diastereotopic positions.

(8*S*)-[X₁ = D, X₂ = H]; δ 4.335 (*t*, J = 6.6 Hz, 1H), (α -D isotope shift $\sim$ - 0.019ppm)
 (8*R*)-[X₁ = H, X₂ = D]; δ 4.284 (*t*, J = 6.6 Hz, 1H), (α -D isotope shift $\sim$ - 0.027ppm)
 [X₁ = H, X₂ = H]; δ 4.354 (*H_R*); 4.311 (*H_S*)

Figure 2.7. Determination of the absolute configuration and enantiomeric purity of the chiral ethyl 8-d₁-8-hydroxy octanoates using Mosher's esterification method.

It was observed that the ¹H signal of the CHD group for each Mosher ester was a broadened triplet due to a combination of small ²*J* HD coupling and large ³*J* HH vicinal coupling (Figure 2.7). In contrast, the Mosher ester of the corresponding non-labeled alcohol features an AB quartet of triplets due to magnetic non-equivalence of the diastereotopic geminal protons. As is well documented in the literature,¹⁷³ the ¹H signals at C-8 of the (8*S*)-alcohol are deshielded relative to the corresponding peaks of the (8*R*)-alcohol. The enantiomeric excess of the chiral alcohols was estimated to be $\sim$ 96 %.

2.2.1.4 Conversion of Chiral Ethyl [8-²H₁]-8-Hydroxyoctanoates to Corresponding Chiral [8-²H₁]-Linoleates

As shown in Figure 2.3, the chiral alcoholic esters **6a** and **6b** were hydrolyzed to the corresponding acids in order to avoid the possibility of ester attack by acetylide in the subsequent steps. The tosylates of the hydroxy acids **7a** and **7b**^{173,179,182,184} were displaced by acetylide ion to give the compounds **9a** and **9b**^{39,173}. The nucleophilic displacement of the tosylate by the acetylide ion is believed to proceed through configurational inversion as demonstrated in an unambiguous manner by Crombie.¹⁷³ Initially the terminal alkynes **9a** and **9b** were coupled directly with 1-(*p*-tolylsulfonyloxy)-oct-2-yne **12** in presence of ethyl magnesium bromide and copper(I) bromide-dimethyl sulfide complex as catalyst as suggested by Crombie²⁰¹, but yields were very low. As an alternative^{175-178,190} the coupling reaction was performed with the terminal alkyne as methyl ester **10a** and **10b**¹⁹¹ instead of the free acids in the presence of K₂CO₃ as a base, copper (I) iodide as a catalyst and sodium iodide (NaI) as an additive in DMF at room temperature. The C18 skipped 1,4-diynes **11a** and **11b** were obtained in good yields; semi-hydrogenation of these compounds using Lindlar catalyst in hexane afforded the crude target compounds, methyl (8*R*)- and (8*S*)-[8-²H₁] octadeca-9*Z*,12*Z*-dienoates (**1a** and **1b**) as light yellowish clear oils that were purified first by flash chromatography (1:19 Et₂O-hexane) for spectral characterization and followed by HPLC when incubated with the *in vivo* yeast culture.^{158,173} The analytical data of the title compounds were in accord with their assigned structures and similar to that previously reported in the literature.¹⁷³ The title compounds were obtained in high purity as determined by the appearance of a large major peak in GC for both of them (Figure 2.8 and 2.9) whose retention time also

matches with that of the non-labeled linoleate. Minor peaks could be attributed to the unreacted short-chain methyl 8-d₁-9-decenoate, its saturated ester, some of the semi-partially reduced C18 eneyne and *cis*, *trans*-isomer of the expected product.

Figure 2.8. GC-MS chromatogram of the HPLC purified (8*R*)-[8-²H₁]-linoleate.

Figure 2.9. GC-MS chromatogram of the HPLC purified (8*S*)-[8-²H₁]-linoleate.

The mass spectra (MS) of each enantiomer (e.g. Figure 2.10, methyl (8*R*)-[8-²H₁]-linoleate) are virtually indistinguishable from each other and featured the following

characteristic fragment ions: m/z 295 (molecular ion, $[M]^+$), 280 ($[M - CH_3]^+$), 264 ($[M - OCH_3]^+$) and ($[M - 74]^+$). These are consistent with their assigned structures and corresponds well with the MS of the non-deuterated linoleate¹⁹²⁻¹⁹⁴ (Figure 2.11) as well as with that of the previously reported results.¹⁷³ From the analyses of the MS of each enantiomers it is apparent that the title compounds are obtained mainly as monodeuterated species which is consistent with the isotopic purity of the intermediates and their spectroscopic data.

Abundance

Figure 2.10. Mass spectrum of methyl (8*R*)-[8-²H₁]-linoleate.

Figure 2.11. Mass spectrum of methyl linoleate.

Since scrambling of allylic deuterium label can occur during catalytic hydrogenation, the location of the deuterium label was confirmed by comparing the ^1H and ^{13}C NMR spectra of both enantiomers (**1a** and **1b**) with that of non-labelled methyl linoleate.¹⁷³ Thus, while the ^1H multiplet due to the bisallylic hydrogens at C-11 was similar in appearance and integration for the d_1 - and non-labelled compound (Figure 2.12 and 2.13), the overlapping multiplets at 2.05 ppm due to protons at C8 and C14 integrated for 3 and 4 protons, respectively.

Figure 2.12. ^1H NMR spectrum of methyl (8*R*)-[8- $^2\text{H}_1$]-linoleate. * due to unknown impurity. † Along with the product methyl group, small amount of solvent peak also present.

Figure 2.13. ^1H NMR spectrum of methyl linoleate.

The location of deuteration was easily pinpointed in the ^{13}C NMR spectrum of **1a** and **1b**. The signal assigned¹⁷³ to C-8 at δ 27.20 ppm in the ^1H -decoupled ^{13}C NMR spectrum of the non-labeled compound was replaced by a triplet ($^2J_{\text{CD}} = 19.2$ Hz) at δ 26.84 ppm (α -deuterium isotope shift of - 0.362 ppm) for (8*R*)-[8- $^2\text{H}_1$]-linoleate **1a** (Figure 2.14). As further detailed in Appendix A, β -isotope shifts of - 0.093 and - 0.051 ppm, respectively were also observed for signals assigned to C-7, C-9. The same scenario was observed with the (8*S*)-isomer **1b** (data not shown).

Figure 2.14. ^{13}C NMR spectrum of methyl (8*R*)-[8- $^2\text{H}_1$]-linoleate. The ^{13}C signals due to C9 (129.99 ppm) and C10 (128.06 ppm) are reduced in intensity due to the presence of deuterium at C8. * Peaks are due to the presence of unreacted methyl (8*R*)-[8- $^2\text{H}_1$]-dec-9-enoate. † Unknown impurities.

Figure 2.15. ^{13}C NMR spectrum of methyl linoleate.

The presence of deuterium label in **1a** and **1b** was also detected by IR spectroscopy with the appearance of an intense absorption peak at 2145 cm^{-1} corresponding to a C-D bond stretch¹⁷³ (Figure 2.16).

Figure 2.16. IR spectrum of methyl (8*R*)-[8-²H₁]-linoleate.

2.2.2 Chemoenzymatic Synthesis of Methyl (11*R*)- and (11*S*)-[11-²H₁]-Octadeca-9*Z*,12*Z*-Dienoate

2.2.2.1 Overview

Skipped dienes that are stereospecifically deuterated at the bisallylic position have traditionally been synthesized by enzymatic desaturation of the appropriate chiral precursors.^{40,195} A literature procedure¹⁷³ for a purely synthetic route to the title compounds was initially attempted, as described in the next section, but the products were subsequently discovered to be racemic. The successful route (Figure 2.17), is described here:

Figure 2.17. Scheme for the chemoenzymatic synthesis of chiral [11-²H₁]-linoleates via Δ¹²-desaturation of chiral [11-²H₁]-oleate precursors, methyl (11*S*)-[11-²H₁]-oleate 3a and methyl (11*R*)-[11-²H₁]-oleate 3b.

2.2.2.2 Synthesis of Methyl (11*S*)- and (11*R*)-[11-²H₁]-Octadeca-9*Z*-enoate, 3a & 3b

Reduction of ethyl octanoate to the corresponding dideuterated alcohol **13**^{174,179} using LiAlD₄, followed by Swern oxidation furnished [1-²H₁]-octanal **14**¹⁸³. Enantioselective reduction of the 1-deuteriooctanal employing chiral Alpine-Borane® reagents afforded the required chiral monodeuterio octanols **15a** and **15b** as colorless clear oils.^{174,185} As the expected absolute configuration and high enantiomeric purity (ee ~ 90 %) of closely related homologous alcohols ((1*R*)- and (1*S*)-[1-²H₁]-pentanol) produced in a similar manner had already been confirmed (Section 3.2.3.1), it was assumed that the course of Alpine-Borane® reduction had proceeded as described in Figure 2.18. The chiral deuterio alcohols were then tosylated^{174,179,184} for the purpose of nucleophilic substitution by lithium acetylide to generate (3*S*)- and (3*R*)-[3-²H₁]-1-decyne.^{39,173} The nucleophilic substitution of the tosylate by acetylide is believed to proceed through inversion of configuration as noted above. The acetylide of each chiral deuterio 1-decyne was coupled¹⁹⁶ with the THP ether derivative of 8-iodo-1-octanol to yield the C18 intermediate **18a** and **18b**. A standard deprotection^{174,196}/oxidation^{180,196}/methylation¹⁹¹/Lindlar hydrogenation^{158,173} sequence generated the required chiral methyl [11-²H₁]-oleates after purification by flash chromatography (1:19 Et₂O-hexane). The extent to which Lindlar semi-hydrogenation produced *cis*-olefin without positional scrambling and the isotopic content of the d₁-oleates was checked through the use of GC-MS, IR, ¹H and ¹³C NMR spectroscopy. The appearance of a major peak in the gas chromatograms of both enantiomers (Figure 2.18 and 2.19) showed that compounds of high chemical purity were obtained through the use of our synthetic strategy.

Figure 2.18. GC-MS of methyl (11*S*)-[11-²H₁]-oleate **3a**.

Figure 2.19. GC-MS of methyl (11*R*)-[11-²H₁]-oleate **3b**.

The preponderance of monodeuterated species in the target compounds were further confirmed through the mass spectral analysis (e.g. Figure 2.20) of the corresponding GC peak and showed molecular ion 297 that is consistent with the molecular formula $C_{19}H_{35}DO_2$. The mass spectral fragmentation pattern was similar (1 mass unit increase) to that observed with the non-labeled analogue^{192-194,197} (Figure 2.21). The following fragment ions were observed: 297 ($[M]^+$), 266 ($[M - OCH_3]^+$), 265 ($[M - CH_3OH]^+$) and a characteristic peak at 223 ($[M - 74]^+$) and are in accord with our expectations.

Abundance

Figure 2.20. Mass spectrum of methyl (11*S*)-[11-²H₁]-oleate **3a**.

Figure 2.21. Mass spectrum of methyl oleate.

The chemical shifts (δ) and splitting patterns (J) of the signals in the ^1H NMR spectra of methyl (11*S*)-[11- $^2\text{H}_1$]-oleate **3a** (e.g. Figure 2.22) and its enantiomer (data not shown) were in accord with their assigned structures and similar to that observed for the non-deuterio oleate (Figure 2.23). The presence of one deuterium at the allylic position of C-11 was indicated by the reduction of the integral of the multiplet at δ 2.01 ppm from 4H to 3H relative to that of the unlabelled compound. However, the effect of deuteration on the neighboring and germinal proton's splitting pattern as well as the associated isotopic shifts couldn't be calculated as in most of the cases multiplets were observed due to the overlap of more than one proton signal.

Figure 2.22. ¹H NMR spectrum of methyl (11*S*)-[11-²H]-oleate **3a**.

Figure 2.23. ¹H NMR spectrum of methyl oleate.

The ^{13}C NMR spectrum of methyl (11*S*)-[11- $^2\text{H}_1$]-oleate (Figure 2.24) and its enantiomer (data not shown) featured a triplet ($^2J_{\text{CD}} = 19.3$ Hz) at 26.87 ppm that is upfield shifted (α -deuterium isotope shift) by 0.352 ppm relative to the singlet that appears at δ 27.22 ppm assigned to the C-11 carbon in the ^{13}C NMR of non-deuterated oleate.^{192-194,197} In addition, typical β -deuterium isotope shifts at C-10 (- 0.04 ppm) and C12 (- 0.08 ppm) and γ -deuterium isotope shifts at C9 (0.03 ppm) and C11 (- 0.02) were noted (Appendix A3).

Figure 2.24. ^{13}C NMR spectrum of methyl (11*S*)-[11- $^2\text{H}_1$]-oleate **3a**. The ^{13}C signal due to C11 (26.85 ppm) splits into a triplet and is significantly reduced in intensity because of coupling with the deuterium atom. Also, the ^{13}C signal due to C9 (129.77 ppm) and C10 (129.95 ppm) is reduced in intensity due to the presence of deuterium at C11.

The observed chemical shifts for the allylic carbons, C-8 and C-11 at 27.18 ppm and 26.87 ppm respectively are in accord with the assigned *Z*-configuration of the double

bond in **3a** and **3b**. The corresponding chemical shifts of *E*-configured olefins occur further downfield at 32.64 ppm and 32.66 ppm as documented by Gunstone *et al.* for the corresponding free acid.^{192-194,197} Absence of such peak in the ^{13}C NMR of methyl (11*S*)-[11- $^2\text{H}_1$]-oleate confirmed that the *Z*-configured isomer was obtained upon reduction.

Figure 2.25. ^{13}C NMR spectrum of methyl oleate.

Finally, the presence of a diagnostic C-D stretch was observed in the IR spectra of deuterated samples (e.g. Figure 2.26) at 2149 cm^{-1} .

Figure 2.26. IR spectrum of methyl (11S)-[11-²H₁]-oleate 3a.

Figure 2.27. IR spectrum of methyl oleate. OH Stretch at 3400 cm⁻¹ peak due to traces of H₂O.

2.2.2.3 Enzymatic Conversion of Methyl (11*S*)- and (11*R*)-[11-²H₁]-Octadeca-9*Z*-enoate to Corresponding Linoleates, 2a and 2b

Saponified samples (150 mg) of methyl (11*S*)- and (11*R*)-[11-²H₁] octadeca-9*Z*-enoate dissolved in Tergitol (type NP-40) were incubated at 225 rpm for 6 days at 20°C with a *S. cerevisiae* mutant strain pSAS050/DTY10-a2 containing a functional Δ^{12} -desaturase from *Caenorhabditis elegans*.²⁶ In earlier experiments, this particular Δ^{12} -desaturating system has been demonstrated to utilize the exogenous oleic acids as substrate to produce linoleates in relatively good yields.²⁶ This yeast strain is also incapable of endogenous fatty acid biosynthesis through a FAS mutation and therefore the chances of dilution of the deuterium labeled linoleic acid product is prevented. The fatty acid mixture containing biosynthetic methyl [11-²H₁]-linoleates were fractionated by reverse-phase HPLC and the title compounds methyl (11*S*)- and (11*R*)-[11-²H₁]-linoleates were obtained in ~ 6 % overall yield. GC-MS analysis of this material (Figure 2.28 and 2.29) revealed that a small amount of tetradecanoate was present as impurity. However, the presence of tetradecanoate didn't interfere in our targeted biosynthetic experiment as the $\Delta^{8,10}$ -desaturase enzyme doesn't use this particular fatty acid as substrate. The selected ion monitoring chromatograms of the above GC profiles indicated the high deuterium content in the biosynthetic samples and demonstrated that both enantiomers were obtained nearly exclusively in monodeuterio form.

Figure 2.28. GC-MS of biosynthetic methyl (11*S*)-[11-²H₁]-linoleate **2a**.

Figure 2.29. GC-MS of biosynthetic methyl (11*R*)-[11-²H₁]-linoleate **2b**.

The mass spectra of the corresponding GC peak of both enantiomers were found to be essentially identical with each other (Figure 2.30 and 2.31). Appearance of the molecular ion signal at m/z 295 ($[M]^+$) in both isomers are in accord with the molecular formula

Abundance

Figure 2.30. Mass spectrum of methyl (11*S*)-[11-²H₁]-linoleate **3a**.

Abundance

Figure 2.31. Mass spectrum of methyl (11*R*)-[11-²H₁]-linoleate **3b**.

$C_{19}H_{33}DO_2$ and clearly demonstrated that the chiral deuterio linoleates consisted mainly as monodeuterated species. Measurement of the deuterium content in the biosynthetic samples **2a** and **2b** revealed the same isotopic purity as observed with the monodeuterio oleates **3a** and **3b**.

2.2.2.4 Attempted Synthesis of Chiral Methyl [11- $^2H_1$]-Octadeca-9Z,12Z-Dienoates

A synthesis of chiral methyl [11- $^2H_1$]-octadeca-9Z,12Z-dienoates **2a** and **2b** has been reported by Crombie and Heavers,¹⁷³ and a modified version was attempted (Figure 2.32):

Figure 2.32. Scheme for the attempted synthesis of chiral methyl [11- $^2H_1$]-linoleates.

The synthesis started with the preparation of one of the key intermediates [1-²H₁]-2-octyn-1-al by deprotonation of 1-heptyne with *n*-BuLi, followed by addition of the carbanion to a solution of N,N-dimethylformamide-*d*₁ to give **24**.¹⁹⁸ This replaced the less efficient Crombie method featuring heptyne acylation with ethyl chloroformate in the presence of Grignard reagent (EtMgBr),¹⁷³ reduction of the ester with lithium aluminum deuteride and MnO₂-mediated oxidation (Figure 2.33).

Figure 2.33. Scheme showing the synthesis of [1-²H₁]-2-octyn-1-al by Crombie *et al.*¹⁷³

The deuterio propargyl aldehyde was then stereoselectively reduced to the corresponding chiral monodeuterated alcohols **25a** and **25b** using Alpine-Borane® reagents as described above.^{174,185,188} Stereochemical analysis of these products was carried out using Mosher's MTPA esterification method as described previously in Section 2.2.1.3.¹⁸⁹ As shown in Figure 2.34, the residual C-1 proton resonance in the ¹H NMR spectrum of the MTPA ester is broadened due to coupling with geminal deuterium and long range coupled to the methylene group at C-4 (⁵*J*_{HH} = ~ 1.9 Hz). It is clear from this analysis that each enantiomer is obtained in high enantiomeric excess and with the expected absolute configuration based on literature precedent.¹⁷³

Figure 2.34. Stereochemical analysis of chiral- d_1 -alkyn-1-ols using Mosher's esterification method.

Several attempts to couple the tosylate of the chiral monodeuterated alkynols with the Grignard reagent derived from $\text{H}-\text{C}\equiv\text{C}-(\text{CH}_2)_7-\text{CO}_2\text{H}$ in the presence of Copper(I) bromide dimethyl sulfide complex, as described by Crombie, were found to be very low yielding processes.¹⁷³ Variations in the amounts of Grignard reagent, tosylate, CuBr-DMS complex or temperature did not lead to significant improvement. Finally, it was decided that the synthesis would be completed^{175-178,190} (Figure 2.32) to generate what was in all likelihood racemic 11- d_1 -linoleate due to use of sodium iodide catalysis in the coupling

step. This material could be used to evaluate the primary deuterium kinetic isotopic effect at C-11 in a manner that was complementary to the methodology employed in a previous work (See Section 1.4.2).¹⁵⁸ Samples of (1*R*)- and (1*S*)-[1-²H₁]-2-octyn-1-ol-derived methyl [11-²H₁]-linoleates were obtained as described in Figure 2.32. The proposed structures, chemical and isotopic purity of these compounds were confirmed by comparing the GC-MS (Figure 2.35), ¹H and ¹³C NMR (Figure 2.36 AB), and IR (Figure 2.37) spectra with that of non-labeled linoleate¹⁹²⁻¹⁹⁴ as well as with other isotopically substituted linoleates in this series. Representative spectra are given below:

Figure 2.35. Mass spectrum of (1*R*)-[1-²H₁]-2-octyn-1-ol-derived methyl [11-²H₁]-linoleate.

Figure 2.36 A: ^1H NMR and B: ^{13}C NMR of methyl $[11\text{-}^2\text{H}_1]$ -linoleate.

Figure 2.37. IR spectrum of (1*R*)-[1-²H₁]-2-octyn-1-ol-derived methyl [11-²H₁]-linoleate. The C-D stretch due to C₁₁HD at 2158 cm⁻¹ is blue shifted (13 cm⁻¹) relative to that of methyl (8*R*)-[8-²H₁]-linoleate.

2.2.3 Incubation Experiments Using Chiral Methyl [8-²H₁]- and [11-²H₁]-Linoleates

2.2.3.1 Trial Incubation of d₀-Linoleate

Prior to the incubation of the synthetic chiral monodeuterio linoleic acids, the efficiency of calendate formation from d₀-linoleate using cultures of a *S. cerevisiae* mutant expressing the *Calendula officinalis* Δ^{8,10}-desaturase (*Fac2*) gene was probed in a preliminary trial experiment. Thus ca. 0.5 mL of sterilized linoleic acid solution in 10 % Tergitol (10 mg/mL) was incubated with a 50 mL growing shake culture (200 rpm) of the pYJ/DTY10a2 strain of *S. cerevisiae* at 20 °C for 3 days and another 3 days at 15 °C.

These conditions had previously been shown to optimize substrate conversion rates.¹⁵⁸ A corresponding control experiment using *S. cerevisiae* (*Fac2*- plasmid) was also run. The cellular fatty acid fractions were isolated from the centrifuged yeast cells *via* a standard hydrolysis (10 % w/v KOH/MeOH at 80 °C for 2 h) /methylation (CH₂N₂) procedure known not to decompose conjugated fatty ester¹⁵⁸ and analyzed by GC-MS (Figure 2.38). Comparison of the fatty acid methyl ester (FAME) profile from *S. cerevisiae* (*Fac2*-plasmid) (data not shown) and the pYJ/DTY10a2 strain of *S. cerevisiae* (Figure 2.38) revealed that only in the latter culture, was exogenously added linoleic acid converted into the product calendic acid (0.6 % of the total cellular fatty acids). This result corresponds well with that observed previously.¹⁵⁸ The product was identified on the basis of retention times using a reference standard mixture that distinguishes between the triene isomers.¹⁵⁸ The mass spectrum of the GC peak assigned to methyl calendate (Figure 2.39) revealed the presence of the molecular ion at m/z 292 ($[M]^+$) and the other fragment ions at m/z 261 ($[M - OCH_3]^+$), 163 ($[Me(CH_2)_4CH=CH-CH=CH-CH=CH-CH_2]^+$), 149 ($[Me(CH_2)_4CH=CH-CH=CH-CH=CH]^+$), which are in accordance with the assigned structure and similar to that reported before.^{63,158} Silylation¹⁸⁷ of a portion of the FAMES did not reveal hydroxylated product formation as a low-level “error” compound during 1,4-desaturation. A similar examination of the methylated CH₂Cl₂ extract of the culture medium did not uncover any hydroxylated fatty acids.

Figure 2.38. The cellular fatty acid profile of *S. cerevisiae* grown in a medium supplemented with linoleic acid.

Figure 2.39. Mass spectrum of biosynthetic methyl calendate derived from methyl linoleate.

2.2.3.2 Incubation of Chiral [8-²H₁]- and [11-²H₁]-Linoleates

To determine the enantioselectivity of the $\Delta^{8,10}$ -desaturase enzyme, the saponified methyl esters of chiral [8-²H₁]-linoleates **1a** and **1b** and chiral [11-²H₁]-linoleates **2a** and **2b** (5 mg each) were administered separately, in triplicate, to growing cultures of pYJ/DTY10a2 strain of *S. cerevisiae* (50 mL) using the conditions identical to that of the trial experiment. The cellular fatty acid fractions were subsequently isolated by hydrolysis/methylation and analyzed by GC-MS to determine the fate of the deuterium label in each case. The d_1/d_0 ratio of the cellular linoleic acid fractions and the d_1/d_0 ratio of the administered compounds were similar (data not shown). This result was not unexpected since *S. cerevisiae* is not able to biosynthesize linoleic acid.

Mass spectral analysis of the calendate product derived from (8*R*)-[8-²H₁]-linoleic acid indicated that the most abundant molecular ion appeared at m/z 292 (Figure 2.40); this represents the overall loss of deuterium during the enzymatic reaction.

Figure 2.40. Representative mass spectrum of biosynthetic methyl calendate derived from methyl (8*R*)-[8-²H₁]-linoleate **1a**.

In contrast, the mass spectrum of the calendate product derived from (8*S*)-[8-²H₁]-linoleic acid featured the most abundant molecular ion at *m/z* 293 (Figure 2.41); this represents the overall retention of deuterium from the *pro R* position during the enzymatic reaction.

Figure 2.41. Representative mass spectrum of biosynthetic methyl calendate derived from methyl (8*S*)-[8-²H₁]-linoleate **1b**.

A similar pattern of deuterium incorporation was observed for the calendate derived from (11*S*)-[11-²H₁]-linoleic acid **2a** (loss of label) and (11*R*)-[11-²H₁]-linoleic acid **2b** (retention of label) as indicated by the mass spectra shown in Figure 2.42 and Figure 2.43, respectively.

Figure 2.42. Representative mass spectrum of biosynthetic methyl calendate derived from methyl (11*S*)-[11-²H₁]-linoleate **2a**.

Figure 2.43. Representative mass spectrum of biosynthetic methyl calendate derived from methyl (11*R*)-[11-²H₁]-linoleate **2b**.

Figure 2.44 Observed results for calendate formation from stereospecifically deuterated linoleates. Note CIP priority rules dictate that the deuterium atoms that are removed from the C-8 and C-11 positions are labeled D_R and D_S respectively.

This result tells us that during calendate biosynthesis, the C-8 and C-11 hydrogens that are removed are topologically equivalent to the hydrogens removed during formation of linoleic acid by the parent Δ^{12} -desaturase (FAD2)^{1,112,140} as shown by comparing Figure 2.44 with Figure 2.1. This interpretation assumes that the conformations of substrate in each case are similar. Interestingly, this result also correlates well with the observed enantioselectivity for an insect $\Delta^{10,12}$ -desaturase that converts a *cis*-11 ene to the corresponding (*E,E*)-10,12-conjugated diene (Chapter 1, Figure 1.29).⁴⁰ This suggests that the overall contours of the binding site for all these enzymes are similar.

The quantitation of deuterium loss vs. retention was accomplished by analyzing molecular ion clusters of the biosynthetic calendate products (Figures 2.40 - 2.43) and carrying out the appropriate corrections of ion intensities at m/z 293 due to $M+1$ contribution (ca. 21.5 %) from d_0 product (m/z 292). The $M+1$ correction factors were determined experimentally using d_0 calendate and compared favourably with the theoretical values. The results of this analysis are given in Table 2.1. It is assumed that

the enantiomeric purity of the chiral starting materials cannot be better than 90 % ee (see above) and is probably somewhat less due to the lack of perfect stereospecificity in the acetylenation step. This can account for the presence of 5 - 10 % of d_0 product that is obtained in cases where deuterium is retained overall (Experiment # 2, 4).

Table 2.1. Isotopic content of stereospecifically labeled monodeuterated linoleates and $\Delta^{8,10}$ -desaturated products (calendate).

Experiment #	Compound	Substrates ^a		Products ^a	
		% d_0	% d_1	% d_0	% d_1
1	(8 <i>R</i>)-[8- ² H ₁]-LA	1.2	98.8	73.2	26.8
2	(8 <i>S</i>)-[8- ² H ₁]-LA	1.1	98.9	9.6	90.4
3	(11 <i>S</i>)-[11- ² H ₁]-LA	0.8	99.2	61.4	38.6
4	(11 <i>R</i>)-[11- ² H ₁]-LA	2.5	97.5	5.1	94.9

^a Each type of chiral incubation was carried out three times and the % isotopic content of each species is given as an average value of three independent GC-MS runs. The average standard deviation is ± 0.4 for each species expressed as a percentage.

For the cases where deuterium is mainly lost (Exp. # 1, 3), the amount of d_1 -product is comparatively much higher (27 - 40 %). For the (11*S*)- d_1 -substrate, the apparent reduced stereoselectivity can be accounted for in terms of the known large primary deuterium kinetic isotope effect associated with C11-H₅ bond cleavage.¹⁵⁸ Consequently, the (11*R*)- d_1 -contaminant in this sample would react substantially faster than the (11*S*)- d_1 -substrate and lead to enhanced % d_1 product content at low conversion rates. Since it is known that C-8 bond rupture proceeds without a substantial KIE, the

appearance of substantial amounts of d_1 -product in the (8*R*)-[8- $^2H_1$] incubation is puzzling but may be due to an anomalously low substrate % ee.

2.2.3.3 Incubation of Racemic [11- $^2H_1$]-Linoleates

An independent assessment of the intermolecular primary deuterium kinetic isotope effect for C11-H_S bond cleavage became possible in serendipitous fashion due to the availability of racemic methyl [11- $^2H_1$]-linoleate (See above). Thus (1*R*)-[1- $^2H_1$]-2-octyn-1-ol-derived- and (1*S*)-[1- $^2H_1$]-2-octyn-1-ol-derived methyl [11- $^2H_1$]-linoleate samples (5 mg each) were incubated, in duplicate, as a solution in 10 % Tergitol (10 mg/mL) with a growing culture of pYJ/DTY10a2 strain of *S. cerevisiae* (50 ml) expressing the *Fac2* gene under the growth conditions as previously described. The yeast cultures were grown up to the normal extent following which the cells were harvested by centrifugation and hydrolyzed by KOH in methanol at 80 °C for 2 h to recover the cellular fatty acid fraction that was subsequently methylated for the GC-MS analysis. Mass spectral analyses of the GC peak assigned to product calendate derived from all samples of 11- d_1 -linoleate were essentially the same within experimental error: 78.2 % $\pm$ 1.0 d_1 and 21.8 % $\pm$ 1.0 d_0 (Table 2.2). This indicated that the samples were stereochemically identical *i.e.* racemic.

Table 2.2. Intermolecular isotopic discrimination by the *Calendula officinalis* $\Delta^{8,10}$ -desaturase in the 1,4-dehydrogenation of racemic methyl [11- $^2\text{H}_1$]-linoleate.

Position of Isotope in d_1 -Species	Trial	Cellular Linoleic acid Substrate ^{a,b,c}			Cellular Calendic acid Product ^{a,c}		KIE
		d_0	d_1 -(R)	d_1 -(S)	d_0	d_1	
C-11	1	2.7	48.65	48.65	22.5	77.5	4.3
	2	2.2	48.9	48.9	21.1	78.9	4.5
	Average						4.4 ± 0.1^d

^a The isotopic content of both substrate and product were evaluated by considering the (M+1) and (M-1) contributions from d_0 - and d_1 -species, respectively. ^b The isotopic ratio of the cellular linoleic acid fractions are similar to that measured prior to the incubation. ^c The deuterium content (%) of each species is given as an average value based on two GC-MS runs. ^d The average KIE (two incubations) $\pm$ standard deviation. The average standard deviation is ± 0.4 for each species expressed as a percentage.

The product deuterium KIE for C-H cleavage at C-11 can be calculated using the following ratio:¹⁹⁹

$$\frac{k_H}{k_D} = \frac{P_{d_1}/P_H}{S_{d_{1R}}/S_{d_{1S}}}$$

Where, P_{d_1}/P_H is the ratio of monodeuterated to nondeuterated calendate and $S_{d_{1R}}/S_{d_{1S}}$

is the ratio of monodeuterated (11R)- to (11S)- d_1 -linoleate. Since the racemic sample

contains a small amount of d_0 -substrate (S_{d_0}) that contributes to P_H , a modified KIE expression is used:

$$\frac{k_H}{k_D} = \frac{[\% P_{d_1} + \{(\% P_{d_1} \times \% S_{d_0}) / \% S_{d_{1R}}\}] / [\% P_{d_0} - \{(\% P_{d_1} \times \% S_{d_0}) / \% S_{d_{1R}}\}]}{[(\% S_{d_{1R}} + \% S_{d_0}) / \% S_{d_{1S}}]}$$

The analytical result obtained in this experiment is displayed in Table 2.2, which is showing the involvement of a large primary deuterium KIE (4.4 ± 0.1) for the C-H bond cleavage step at C-11. This value is substantially lower than the previously reported result ($\text{KIE} = 5.7 \pm 1.0$) for the same biotransformation reaction,¹⁵⁸ where a ~ 1:1 mixture of nondeuterated to regiospecifically dideuterated linoleates was used. This discrepancy can be explained by considering the potential impact of normal α -secondary kinetic isotope effects¹⁶⁰ in the two modes of calculating KIEs (Figure 2.45).

Figure 2.45. Comparison of the relative rates of C-H vs. C-D bond breaking in the two modes of calculating KIE at C-11.

The α -secondary deuterium isotope effect reduces the rate of d_1 -product formation from d_{1S} substrate relative to the rate of d_0 -product formation from d_0 -substrate, which in turn **reduces** the numerator of the ratio k'_H/k'_D relative to k_H/k_D . On the other hand, the α -secondary deuterium isotope effect reduces the rate of d_1 -product formation from d_2 -substrate relative to d_0 -product formation from d_{1R} substrate, which in turn **increases** the denominator of the ratio k'_H/k'_D relative to k_H/k_D . The net effect is that the KIE calculated using the racemic 11- d_1 samples is $\sim 77\%$ or 100 ($1/1.15 \times 1/1.15$) of the KIE calculated using $\sim 1:1$ mixture of nondeuterated to regiospecifically dideuterated linoleates. Values of α -secondary isotope effects of 1.15 for iron oxo-mediated hydrogen abstraction reactions are within the expected range.^{160,199}

2.3 Conclusion

The enantioselectivity of the 1,4-desaturase (FAD2 variant) involved in formation of conjugated fatty acids in plants has been investigated for the first time. This completes a study that was begun by Crombie and coworkers⁴³ some 26 years ago. Our investigation established that the formation of calendic acid in *Calendula officinalis* (pot marigold) involves the initial, isotopically sensitive removal of a *pro S* hydrogen from C-11 of linoleate and subsequent cleavage of the *pro R* C8-H bond *i.e.* two hydrogens are removed in *syn* fashion from two neighbouring allylic positions of the fatty acid chain to biosynthesize the conjugated trienoic acid (Figure 2.46).

Figure 2.46. Mechanistic model for 1,4-dehydrogenation reaction of linoleate to biosynthesize calendate as it occurs in *Calendula officinalis*. The double bonds are quasi-coplanar.

The observed stereochemistry and cryptoregiochemistry of hydrogen removal in calendate biosynthesis correlates well with that observed for the parent FAD2 enzyme involved in formation of linoleic acid ($\text{CH}_3-(\text{CH}_2)_5-\text{C}_{13}=\text{C}_{12}-\text{CH}_2-\text{C}_{10}=\text{C}_9-(\text{CH}_2)_7-\text{CO}_2\text{H}$) from oleate and validates the proposed mechanistic model (Figure 2.1). The results of this investigation strengthens the proposal that there exists a highly conserved, active-site structural motif for the family of membrane-bound FAD2-like enzymes, where the oxidation is initiated by approaching the olefinic plane at or near the C-12 position of substrate from the top (C1 terminus at right hand side of structure ($\text{CH}_3-(\text{CH}_2)_5-\text{C}_{13}=\text{C}_{12}-\text{CH}_2-\text{C}_{10}=\text{C}_9-(\text{CH}_2)_7-\text{CO}_2\text{H}$)).

Chapter 3: Mechanistic Studies of α -Eleostearate Formation in *Aleurites fordii* Hemsl.

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3.1 Experimental Approach

To further explore the biosynthesis of conjugated fatty acids involving an apparent 1,4-desaturation reaction, the formation of α -eleostearic acid (a calendic acid regioisomer) in the seeds of *Aleurites fordii* Hemsl. commonly known as Tung tree³⁴ was targeted. The mechanism by which this important fatty acid is biosynthesized in the developing seeds of the tung tree has not been explicitly investigated before. Phylogenetic analysis demonstrated that the enzyme tung FADX can be grouped with parent FAD2 and hydroxylases which is consistent with its desaturase activity.³⁴ Expression and functional analysis of this FAD2 homologue in yeast *S. cerevisiae* in the presence of the exogenously added linoleic acid showed that the externally supplied linoleic acid is transformed to α -eleostearic acid.³⁴ One may assume that the formation of α -eleostearic acid features the removal of the hydrogens from two non-adjacent

methylene groups (C-11 and C-14) *via* an apparent 1,4-dehydrogenation mechanism (hereafter will be called $\Delta^{11,13}$ -desaturase, Figure 3.1).^{34,200}

Figure 3.1. A proposed mechanism for the 1,4-desaturase-mediated formation of α -eleostearate featuring initial H-abstraction at C-11. The accepted mechanism for parent FAD2 formation is included for the purposes of comparison. The double bonds are quasi-coplanar.

We decided to probe the cryptoregiochemistry (site of initial oxidation) of $\Delta^{11,13}$ -desaturation by measuring the deuterium kinetic isotope effect (KIE) on the two C-H cleavage steps during oxidation - an approach that has already been validated on related systems. As described in Section 1.3.2, the approach is based on the premise that only the cleavage of the first C-H bond is likely to be the rate-determining step of the enzyme catalyzed reaction, then substitution of that particular hydrogen with deuterium should in principle generate a primary deuterium KIE (Figure 1.18). On the basis of the similarity between FAD2 and its variant, we propose that α -eleostearate formation is initiated at C-

11, the carbon closest to C-12 (the site of initial oxidation for linoleate formation). The enzymatic isotope effects in our investigation will be determined *via* intermolecular competitive experiments as described in Section 1.3.2, where a ca. equimolar (1:1) mixture of non-deuterated and regiospecifically dideuterated substrate compete in the same incubation.¹⁹⁹ The d_1/d_0 ratio of the olefinic product is then compared with the d_2/d_0 ratio of the substrate. Our approach required the synthesis of regiospecifically dideuterated methyl [11,11-²H₂]- and [14,14-²H₂]-linoleates (**31** and **32**) (Figure 3.2) and a *S. cerevisiae* transformant that functionally expresses the $\Delta^{11,13}$ -desaturase enzyme from *Alurites fordii* Hemsl. The latter was constructed through a collaborative effort with the Covello laboratory (NRC- PBI, Saskatoon).

The proposed mechanism for α -eleostearate formation as shown in Figure 3.1 predicts that the substrate adopts a conformation that allows the hydrogens at C-11 and C-14 of the fatty acid chain to be removed with the same facial selectivity as observed with the *syn* removal of hydrogens from C-12 and C-13 positions during linoleic acid formation (FAD2). The correctness of this proposed topology could be tested through the determination of the enantioselectivity of α -eleostearate formation using stereospecifically labeled deuterated fatty acids as mechanistic probes.

Our approach required the use of four stereospecifically monodeuterated linoleates with the chirotopic center in one pair at C-11 and in the other pair at C-14 (Figure 3.2) *i.e.* methyl (11*S*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate **2a**, methyl (11*R*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate **2b**, methyl (14*S*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate **33a** and methyl (14*R*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate **33b**. The former pair of

enantiomers were available from the caldate experiments (See Chapter 2). The latter compounds were synthesized as described in Section 3.2.3.

Figure 3.2. The dideuterated and chiral monodeuterated linoleates required to investigate the cryptoregiochemistry and stereochemistry α -eleostearate formation.

3.2 Results and Discussion

3.2.1 Construction of Yeast Mutant Containing $\Delta^{11,13}$ -Desaturase

3.2.1.1 Amplification of the template DNA, Cloning and Expression in *E. coli*

The plasmid pRTL2 myc-FADX containing the FADX gene that encodes the $\Delta^{11,13}$ -desaturase enzyme from *Aleurites fordii* Hemsl. responsible for the biosynthesis of α -eleostearate from linoleate was originally obtained from John M. Dyer (US Department of Agriculture-Agricultural Research Services Southern Regional Research Center, New Orleans, Louisiana).³⁴ The plasmid vector was then amplified using the PCR reaction, where a FDX-forward primer (5'-gaagcttgctagaATGGGAGCTGGTGGCCGAATGTCT-3', $T_m = 76.1^\circ\text{C}$) and FDX-reverse primer (5'-aaggatccACTCCATATCTCGTAACAA-GGTCAAACCTC-3', $T_m = 69.4^\circ\text{C}$) were used as described by Dyer *et al.*³⁴ (the FDX-reverse primer contain the BamHI site as indicated in the paper) along with the 2x Phusion® high-fidelity PCR master mix (New England BioLabs). During the PCR

reaction, the cycle was repeated 30 times and the primers were annealed at 72 °C with the single-stranded DNA template. Following the completion of the cycle, TAQ polymerase was added and the mixture was heated to 72 °C for 10 minutes to ensure “A overhang” on the PCR products that were then analyzed by agarose gel electrophoresis and visualized by ethidium bromide (Figure 3.3).

Figure 3.3. Agarose gel electrophoresis of the PCR amplified FADX genes. Expected size of the entire FADX gene was 1.2 kb. The two amplified FADX gene was compared with the reference ladder and showed that the amplification resulted in the production of correct amino acid.

The PCR products were then TA cloned into pYES2.1 vector by using the pYES2.1 TOPO® TA Expression Kit from Invitrogen and the recombinant vector was transformed into chemically competent *E. coli* cells. This vector provides for galactose-inducible expression mediated by the GAL1 promoter. The mixture was then plated on LB media plates under ampicillin selection and individual colonies were picked from the well-spaced bacterial colonies to grow in liquid culture media on LB_{amp} (100 µg/mL) at 200 rpm in 37 °C. The newly constructed plasmid DNAs were purified by Promega

Wizard® Plus Midipreps DNA Purification kit and quantitated by spectrophotometry. The purified plasmid DNA solutions were submitted for sequencing and the sequence data was analyzed by Lasergene SeqMan software and shown to be identical with the reported FADX sequence³⁴. The purified plasmid DNA (see Chapter 5) was subsequently inserted into *S. cerevisiae* using the *S. c.* EasyComp transformation kit (Invitrogen).

3.2.1.2 Preparation of *S. cerevisiae* mutant expressing $\Delta^{11,13}$ -desaturase enzyme from *Aleurites fordii* Hemsl.

For transformation, INVSc-1 strain of *S. cerevisiae* was used and selected on SDglucose-uracil selection media plates. Briefly, the freshly prepared cultures of the INVSc-1 strain of *S. cerevisiae* were grown to mid-log phase on YPD medium and the harvested cells were resuspended in highly concentrated solution of lithium acetate (solution II) in order to make the cells competent for transfection using the method described by *S. c.* EasyComp™ transformation kit (Invitrogen). The competent cells were then cooled to - 80 °C since better transformation efficiencies have been observed with thawed cells compared to freshly prepared cells. After equilibrating to room temperature, the frozen cells were mixed with the vector DNA and finally diluted with the appropriate transformation solution. The cell/DNA mixture was subsequently mixed thoroughly and incubated at 30 °C to induce the uptake of DNA. The transformed yeast cells were streaked on SDglucose-uracil selection media plates containing 2 % agar and allowed to grow at 30 °C for 4 days to yield sufficient discrete colonies. Individual colonies were selected and cultured in selection/induction media, S-Galactose-uracil to prepare the starter culture for incubation experiments with labeled substrates.

3.2.2 Cryptoregiochemistry of $\Delta^{11,13}$ -Desaturation

3.2.2.1 Synthesis of Methyl [11,11- $^2\text{H}_2$]-Linoleate

Methyl [11,11- $^2\text{H}_2$]-linoleate **31** was synthesized in 54 % overall yield starting with 1-heptyne as starting material using the synthetic strategy as depicted in Figure 3.4. A key intermediate [1,1- $^2\text{H}_2$]-2-octyn-1-ol **30** was prepared with high deuterium content as previously discussed in Chapter 2 (Figure 2.33).

Figure 3.4. Synthesis of methyl [11,11- $^2\text{H}_2$]-linoleate.

This intermediate was elaborated to give the title compound as previously described for the synthesis of racemic 11-d₁-linoleate (Figure 2.32). Methyl [11,11-²H₂]-linoleate was obtained in high purity following flash column chromatography on silica gel as determined by GC-MS (data not shown). Analysis of the MS revealed that the deuterated substrate consisted mainly of dideuterated species as evidenced by the appearance of the molecular ion peak at m/z 296 (Figure 3.5) and is in accord with the molecular formula C₁₉H₃₂D₂O₂.

Abundance

Figure 3.5. Mass spectrum of methyl [11,11-²H₂]-linoleate 31.

The location of the CD₂ group was confirmed by the absence of C11 ¹H resonances (Figure 3.6) that appear at δ 2.77 ppm in the ¹H spectrum of the non-labeled material (Figure 2.13). The splitting pattern of the multiplet at 5.30-5.45 ppm due to the olefinic protons is also altered.

Figure 3.6. ^1H NMR spectrum of methyl [11,11- $^2\text{H}_2$]-linoleate. The arrow indicates the positioning of the signals due to the bis-allylic protons at C-11 that are missing here due to dideuteration at this location.

In addition, the dideuterium labeling at C-11 position causes the ^{13}C resonance to split into a pentet due to coupling with the two deuteria ($2nI + 1$ rule, $I = 1$ and $n = 2$) and isotope shifted upwards by 0.83 ppm (α -shift). The β and γ -isotope shifts are documented in Appendix A2.

Figure 3.7. ^{13}C NMR spectrum of methyl $[11,11\text{-}^2\text{H}_2]$ -linoleate. The arrows are showing the pentet. The ^{13}C signals due to C9 (130.07 ppm), C10 (127.93 ppm), C12 (127.79 ppm) and C13 (130.24 ppm) are all reduced in intensity due to the presence of deuterium at C11. The effect is largest for C10 and 12.

Additional evidence for the presence of -CD_2 unit at the bis-allylic position into the fatty acid chain was obtained *via* the appearance of two absorption bands at 2200 and 2099 cm^{-1} in the IR spectrum of the title compound (Figure 3.8). These two absorption bands correspond to the symmetric as well as asymmetric stretching frequencies of the two associated C-D bonds and is absent in the analogous non-deuterated linoleate.

Figure 3.8. IR spectrum of methyl [11,11- $^2\text{H}_2$]-linoleate. Asymmetric and symmetric C-D stretches due to C_{14}DD appear at 2099 and 2200 cm^{-1} . Peak at $\sim 2360\text{ cm}^{-1}$ is part of a band that may be due to the CO_2 stretch.

3.2.2.2 Synthesis of Methyl [14,14- $^2\text{H}_2$]-linoleate

Methyl [14,14- $^2\text{H}_2$]-linoleate **32** was synthesized in 37 % overall yield from the commercially available methyl valerate as starting material (Figure 3.9). The route mimics that outlined for the synthesis of the corresponding chiral 14- d_1 -linoleates (Figure 3.18) as discussed in the later section. The GC-MS, IR, ^1H and ^{13}C NMR data obtained on the purified title compound confirmed the proposed structure, location of deuterium labeling and the high d_2 -content. The relevant spectra are listed below.

Figure 3.9. Synthesis of methyl [14,14-²H₂]-linoleate.

Figure 3.10. Mass spectrum of methyl [14,14- $^2\text{H}_2$]-linoleate.

Figure 3.11. ^1H NMR spectrum of methyl [14,14- $^2\text{H}_2$]-linoleate. Doublet of triplets due to C_{14}HH is absent and leaves C_8HH at 2.05 ppm integrating for 2H. * diethyl ether. † unknown impurities.

Figure 3.12. ^{13}C NMR spectrum of methyl [14,14- $^2\text{H}_2$]-Linoleate. The arrows are showing the pentet which is due to C14D₂ is isotope shifted to 26.47ppm (α - shift of - 0.719 ppm) relative to the corresponding signal in non-labelled linoleate. The ^{13}C signals due to C12 (127.94 ppm) and C13 (130.11 ppm) are reduced in intensity due to the presence of deuterium at C14.

Figure 3.13. IR spectrum of methyl [14,14- $^2\text{H}_2$]-linoleate. Asymmetric and symmetric C-D stretches due to C_{14}DD appear at 2099 and 2186 cm^{-1} .

3.2.2.3 Incubation of Substrates: Trial Incubation of d_0 -Linoleate

Before running the intermolecular competition between the d_0 -linoleate and stereospecifically dideuterated linoleates, the efficiency of formation of the α -eleostearate product from d_0 -linoleate was examined in a redundant experiment using the newly constructed yeast mutant expressing the $\Delta^{11,13}$ -desaturase enzyme (FADX gene) from *Aleurites fordii* Hemsl. In order to probe this, ca. 0.5 mL of sterilized linoleic acid solution in 10 % Tergitol (10 mg/mL) was inoculated with a 50 mL growing culture of the pRTL2 myc-FADX strain of *S. cerevisiae* and allowed to shake at 20 °C for 3 days followed by another 3 days at 15 °C at 200 rpm. This incubation condition is similar to that used for the calendate experiments. A corresponding control experiment using only wild type *S. cerevisiae* without having the mutant expression (FADX - plasmid) was also

run. The cellular fatty acid fractions were subsequently isolated from the centrifuged *S. cerevisiae* cells via a standard hydrolysis (10 % w/v KOH/MeOH at 80 °C for 2 h) / methylation (CH_2N_2) procedure known not to decompose the conjugated fatty ester¹⁵⁸ and analyzed by GC-MS (Figure 3.14). Comparison of the fatty acid methyl ester (FAME) profile of the control experiment (FADX - plasmid) (data not shown) with the culture containing the pRTL2 myc-FADX strain of *S. cerevisiae* (Figure 3.14) revealed that only in the latter experiment was the exogenously supplied linoleic acid converted into the product α -eleostearic acid to an extent of 2 % of the total cellular fatty acids. The host yeast culture system was also shown to be unable to biosynthesize the substrate linoleic acid endogenously (data not shown here), as is necessary for the type of mechanistic investigations we were using for. The product was identified based on the comparison of the retention times of a reference standard mixture that distinguishes between different triene isomers.¹⁵⁸ Comparison of the retention time of methyl α -eleostearate (at 16.25 min) with methyl calendate (at 16.43 min) revealed that the former is eluting somewhat faster than the latter under comparable conditions. Analysis of the mass spectrum of the GC peak assigned to methyl α -eleostearate (Figure 3.15) revealed that the presence of the molecular ion at m/z 292 ($[\text{M}]^+$) and the other fragment ions at m/z 261 ($[\text{M} - \text{OCH}_3]^+$), 163 ($[\text{Me}(\text{CH}_2)_4\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2]^+$), 150 ($[\text{Me}(\text{CH}_2)_3\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2]^+$) which are in accord with the proposed structure and similar to that reported previously.³⁴ No oxygenated intermediate could be detected by silylation of the FAME as low-level “error” compound formation during 1,4-desaturation. A similar examination of the methylated CH_2Cl_2 extract of the culture medium did not reveal any hydroxylated fatty acids.

Figure 3.14. The cellular fatty acid profile of *S. cerevisiae* grown in a medium supplemented with linoleic acid.

Figure 3.15. Mass spectrum of the biosynthetic methyl α-eleostearate derived from methyl linoleate.

3.2.2.4 Intermolecular Kinetic Isotope Effect (KIE) Studies

To determine the intermolecular primary deuterium kinetic isotope effects on each C-H cleavage steps during α -eleostearic acid formation by $\Delta^{11,13}$ -desaturase enzyme from linoleic acid, a mixture consisting of approximately equivalent amount (1:1) of saponified methyl esters of non-labeled to stereospecifically labeled [11,11- $^2\text{H}_2$] or [14,14- $^2\text{H}_2$] linoleates was incubated as a solution in 10 % Tergitol (~ 5 mg each) to the growing cultures of pRTL2 myc-FADX strain of *S. cerevisiae* (50 mL). A small portion of the fatty acid mixture was withdrawn from the Tergitol solution in order to determine the isotopic deuterium content (d_0/d_2 ratio) in the incubated mixtures by GC-MS. For each type of mixture, the incubation was carried out four times and the inoculated cultures were allowed to grow to the normal extent under the conditions identical to that of the trial experiment. The cellular fatty acid fractions were isolated from the harvested cells by the hydrolysis/methylation technique and the deuterium content of the recovered linoleic (d_0/d_2) acid as well as the eleostearic acid products (d_0/d_1) was assessed by GC-MS. Comparison of the d_2/d_0 ratio of the cellular linoleate fraction with that measured in the administered mixture revealed that the ratios were essentially identical in both incubations, indicated that no dilution of the deuterium label occurred during the feeding experiment (data not shown). Maintenance of this ratio before and after the incubation is essential for this type of KIE study and the result was according to the expectation, since the host yeast system has been documented in the trial experiment not able to biosynthesize the d_0 -substrate.

Mass spectral analysis of the GC peak assigned to α -eleostearate fraction (Figure 3.16 and 3.17) demonstrated that in both incubations, the product consisted entirely as a

Figure 3.16. Representative mass spectrum of biosynthetic α -eleostearate derived from 1:1 mixture of d_0 -linoleate and 11- d_2 -linoleate.

Figure 3.17. Representative mass spectrum of biosynthetic α -eleostearate derived from 1:1 mixture of d_0 -linoleate and 14- d_2 -linoleate.

mixture of d_0/d_1 which clearly indicating the loss of one deuterium from d_2 -substrate as expected.

Since, the overall % conversion was less than 10 % in both incubations as clearly evident from the gas chromatograms of the FAMES, the product kinetic isotope effects (k_H/k_D) could be calculated. The following equation is used:¹⁹⁹

$$\frac{k_H}{k_D} = \frac{P_H/P_D}{S_H^0/S_D^0}$$

Where, P_H/P_D is the ratio of the nondeuterated to monodeuterated methyl α -eleostearate

and S_H^0/S_D^0 is the ratio of the nondeuterated to regiospecifically dideuterated [11,11- $^2H_2$]-

or [14,14- $^2H_2$]-linoleate. Thus, the product kinetic isotope effects resolved to:

$$\frac{k_H}{k_D} = \frac{[\% d_0 \text{ Product}]/[\% d_1 \text{ Product}]}{[\% d_0 \text{ Substrate}]/[\% d_2 \text{ Substrate}]}$$

The analytical data obtained from our competitive experiment is exhibited in Table 3.1. Using the formulation described above, the presence of a large primary deuterium isotope effect (4.9 ± 0.4) for the carbon-hydrogen bond cleavage at C-11 was calculated, whereas the carbon-hydrogen bond cleavage at C-14 was found to be insensitive to isotopic substitution ($KIE = 1.12 \pm 0.02$). It should be remembered that the magnitude of the 'intrinsic KIE' on C-11 C-H bond cleavage may well be much higher than the determined value, since the estimated value was derived *via* an intermolecular

competition that is subjected to partial masking by other enzymatic steps such as, substrate binding or product release.^{1,201} It is also true that the observed isotope effects calculated in this manner typically incorporates a small amount (~ 10 %) of a normal α -secondary KIE associated with conversion of an sp^3 centre to an sp^2 carbon.¹⁶⁰

Table 3.1. Intermolecular isotopic discrimination by the *Aleurites fordii* Hemsl. $\Delta^{11,13}$ -desaturase mediated 1,4-desaturation of methyl [11,11- $^2H_2$]-linoleate **31** and methyl [14,14- $^2H_2$]-linoleate **32**.

Position of Isotope in d_2 -Species	Trial	Cellular Linoleic acid Substrate ^{a,b,c}		Cellular Eleostearic acid Product ^{a,c}		KIE
		d_0	d_2	d_0	d_1	
C-11	1	49.7	50.3	83.8	16.2	5.2
	2	48.6	51.4	82.0	18.0	4.8
	3	49.3	50.7	83.6	16.4	5.2
	4	51.1	48.9	81.8	18.2	4.3
	Average					4.9 ± 0.4^d
C-14	1	54.0	46.0	56.3	43.7	1.10
	2	52.8	47.2	55.6	44.4	1.12
	3	53.4	46.6	56.9	43.1	1.15
	4	52.3	47.7	54.6	45.4	1.10
	Average					1.12 ± 0.02^e

^a The isotopic content of both substrate and product mixtures were evaluated by considering the (M+1), (M+2) as well as (M-1), (M-2) contributions from d_0 -, d_1 - and d_2 -species. d_0 , d_1 and d_2 refer to the nondeuterated, monodeuterated and dideuterated species, respectively. ^b The isotopic composition of the cellular linoleic acid fractions in both incubations were similar to that measured prior to the incubation. ^c The deuterium content (%) of each species is given as an average value based on four GC-MS runs. The average standard deviation for these measurements is ± 0.4 expressed as a percentage. ^d The average KIE at C-11 (four incubations) $\pm$ standard deviation. ^e The average KIE at C-14 (four incubations) $\pm$ standard deviation.

As also described in Chapter 2 (calendate biosynthesis), an independent evaluation of the deuterium isotope effect at C-11 during the biosynthesis of α -eleostearate became possible due to the availability of racemic methyl [11-²H₁]-linoleate. Thus, two independently synthesized samples of methyl [11-²H₁]-linoleates (~ 5 mg), one derived from (1*R*)-[1-²H₁]-2-octyn-1-ol **25a** and the other from (1*S*)-[1-²H₁]-2-octyn-1-ol **25b** were incubated separately with the growing *S. cerevisiae* strain expressing $\Delta^{11,13}$ -desaturase enzyme from *A. fordii* Hemsl. After saponification with a growing culture of pRTL2 myc-FADX strain of *S. cerevisiae* (50 mL) under the optimal incubation conditions as previously described to let the cultures grow to its normal extent. The fatty acid methyl esters (FAMES) from the harvested yeast cells were isolated as described before and analyzed by GC-MS. Mass spectral analysis of the GC peak assigned to the product α -eleostearate derived from both [11-²H₁]-linoleates revealed that the deuterium content was essentially identical (within experimental error): 76.8 ± 0.4 d₁- and 23.2 ± 0.4 d₀-eleostearate. This results confirmed that both of the [11-²H₁]-linoleates were stereochemically alike *i.e.* racemic. This permitted calculation of the product KIE for C-H cleavage at C-11 in the manner discussed in Chapter 2.

$$\frac{k_H}{k_D} = \frac{[\% P_{d_1} + \{(\% P_{d_1} \times \% S_{d_0}) / \% S_{d_{1R}}\}] / [\% P_{d_0} - \{(\% P_{d_1} \times \% S_{d_0}) / \% S_{d_{1R}}\}]}{[(\% S_{d_{1R}} + \% S_{d_0}) / \% S_{d_{1S}}]}$$

Table 3.2. Intermolecular isotopic discrimination by the *Aleurites fordii* Hemsl. $\Delta^{11,13}$ -desaturase in the 1,4-dehydrogenation of racemic methyl [11- $^2\text{H}_1$]-linoleate.

Position of Isotope in d_1 -Species	Trial ^a	Cellular Linoleic acid Substrate ^{b,c,d}			Cellular Eleostearic acid Product ^{b,d}		KIE
		d_0	d_1 -(R)	d_1 -(S)	d_0	d_1	
	1a				23.5	76.5	4.0
C-11	2a	2.7	48.65	48.65	23.5	76.5	4.0
	3a				23.3	76.7	4.0
	Average						4.0 ± 0.1^e
	1b				23.4	76.6	3.8
C-11	2b	2.2	48.9	48.9	22.4	77.6	4.1
	3b				23.3	76.7	3.9
	Average						3.9 ± 0.2^e

^a Trial 1a-3a were accomplished by using the (1R)-[1- $^2\text{H}_1$]-2-octyn-1-ol-derived [11- $^2\text{H}_1$]-linoleate and trial 1b-3b were accomplished by using the (1S)-[1- $^2\text{H}_1$]-2-octyn-1-ol-derived [11- $^2\text{H}_1$]-linoleate. ^b The isotopic content of both substrates and products were evaluated by considering the (M+1) and (M-1) contributions from d_0 - and d_1 -species, respectively. d_0 and d_1 refers to non-deuterated and monodeuterated species, respectively. ^c The isotopic ratio of the cellular linoleate fractions were similar to that measured prior to the incubation. ^d The deuterium content (%) of each species is given as an average value based on three GC-MS runs. The average standard deviation for these measurements is ± 0.4 expressed as a percentage. The standard deviation is considered to be similar to the standard error for each individual KIE value. ^e The average KIE at C-11 (three incubations) $\pm$ standard deviation.

The results clearly point to the existence of a large primary deuterium kinetic isotope effect (4.0 ± 0.2) for the carbon-hydrogen bond cleavage at C-11 during the formation of α -eleostearic acid from linoleic acid. Once again, the calculated value was found to be substantially lower ($\sim 81\%$) compared to that obtained using equal amount (1:1) of non-deuterated to 11- d_2 -linoleate (4.9 ± 0.4) as reported above for the same biotransformation reaction. As described in the calendate Section 2.2.3.3, here also this

result can be explained by considering the potential impact of α -secondary isotope effects on both ways of calculating KIE.¹⁶⁰ It is interesting to note that the consequence of including the α -secondary isotope effects on the primary deuterium KIEs appears to be similar, 81 % vs. 77 % (calendate KIE) in both cases, as might be expected for reactions following a similar mechanism by the same family of enzymes.

In conclusion, the data presented above (*i.e.* one large KIE at C-11 and one small KIE at C-14) clearly demonstrates the stepwise nature of the 1,4-desaturation reaction to form α -eleostearate. Based on our experimental finding, it can now be described that the α -eleostearate formation is initiated by a relatively slow and hence isotopically sensitive hydrogen abstraction at C-11 and completed by a second facile and kinetically unimportant hydrogen removal at the C-14 position. The observed result was similar to that reported for the biosynthesis of calendic acid (Chapter 2), which has also been observed to initiate the oxidation at C-11 of the linoleyl chain.¹⁵⁸ It is significant that in both cases the cryptic site of initial oxidation is close to C-12, the site of initial oxidation for parent FAD2^{1,112} enzyme.

The stepwise removal of hydrogens from two non-adjacent carbons during the conjugated double bond formation also matches closely with the pattern observed in insects during the biosynthesis of (*E,E*)-10,12-tetradecadienoic acid from (*Z*)-11-tetradecenoic acid as a component in moth *Spodoptera littoralis* sex pheromone (Figure 1.29).³⁹ The site of initial oxidation for this insect 1,4-desaturase enzyme was revealed to be at C-10³⁹ which is close to C-11, the cryptic site of initial oxidation for its parent Δ^{11} -desaturase¹³⁹ (Figure 1.5). This observation signifies that like tung $\Delta^{11,13}$ -desaturase, this

insect $\Delta^{10,12}$ -desaturase enzyme also maintains a close relationship with its parent Δ^{11} -desaturase in terms of cryptoregiochemistry^{39,139} of fatty acid oxidation.

3.2.3 Stereochemistry of $\Delta^{11,13}$ -Desaturation

3.2.3.1 Synthesis of Chiral Methyl [14-²H₁]-Octadeca-9Z,12Z-Dienoates

The syntheses of methyl (14*S*)- and (14*R*)-[14-²H₁]-octadeca-9*Z*,12*Z*-dienoates **33a** and **33b** were performed in ~ 2 % overall yield, starting with commercially available *N,N*-dimethylformamide-*d*₁ (99 atom % D) (Figure 3.18). The intermediate [1-²H₁]-pentanal **41**¹⁹⁸ was reduced in asymmetric fashion to the enantiomeric monodeuterio 1-pentanol **42a** and **42b** using well established Alpine-Borane® method described previously in this thesis (Figure 2.5).^{174,185} The absolute configuration as well as the enantiomeric purity of these chirally reduced alcohols was assessed by ¹H NMR after derivatization using (*S*)-MTPA chloride.^{173,189}

Figure 3.18. Synthesis of chiral methyl $[14\text{-}^2\text{H}_1]\text{-linoleates}$.

Integration of the diastereotopic ^1H resonances assigned to the C-1 CHD group of the MTPA ester of (1*S*)-[1- $^2\text{H}_1$]-pentanol (major) vs. (1*R*)-[1- $^2\text{H}_1$]-pentanol (minor) shown in the left panel of Figure 3.19 yielded an estimated ee of 90 %. The presence of impurities in the (1*R*)-[1- $^2\text{H}_1$]-pentanol MTPA ester (right panel of Figure 3.19) prevented a similar analysis for this compound. As expected¹⁸⁹, the C-1 CHD resonance of the (+)-(*R*)-MTPA ester of (1*S*)-[1- $^2\text{H}_1$]-pentanol appears down field relative to the corresponding signal of the (1*R*)-alcohol.

Figure 3.19. ^1H resonances at C-1 of MTPA ester of (1*S*)-, (1*R*)-[1- $^2\text{H}_1$]-pentanol (triplet) and d_0 -pentanol (AB quartet). * are due to the impurities. α -Deuteration causes a small upfield shift (~ 0.018 , 0.019 ppm) of the signal due to the geminal proton.

Analyses of the mass spectra of these chiral deuterio alcohols as TMS derivative¹⁸⁷ revealed that each enantiomer was obtained mainly as monodeuterated species. The tosylates^{174,179,184} of chiral deuterio 1-pentanol were reacted with the THP-derivative of propargyl alcohol in the presence of methyllithium in HMPA to give the THP protected [4-²H₁]-2-octyn-1-ols,¹⁷⁴ **44a** and **44b**. Displacement of the tosylate by acetylide anions is believed to take place *via* inversion of configuration and proof for such a claim has been provided as discussed in the next Section 3.2.3.2. Following the deprotection of the THP ether derivatives, the resultant chiral deuterio 2-octyn-1-ols **45a** and **45b**^{174,196} were tosylated and then coupled^{175-178,190} with methyl 9-decynoate in presence of mild base K₂CO₃, copper iodide and sodium iodide in dry DMF. The skipped 1,4-diynoates **47a** and **47b** generated in this manner were semi-hydrogenated by Lindlar catalyst in hexane to generate the title compounds **33a** and **33b** as faint-yellowish clear oils after flash chromatography.^{158,173}

The proposed structures, chemical and isotopic purity of these compounds were confirmed by comparing the GC-MS (Figure 3.20), ¹H NMR (Figure 3.21), ¹³C NMR (Figure 3.22) and IR (Figure 3.23) spectra with that of non-labeled linoleate as well as with other isotopically substituted linoleates in this series. Representative spectra are shown below:

Figure 3.20. Mass spectrum of methyl (14S)-[14- $^2\text{H}_1$]-linoleate **33a**. Molecular ion at m/z 295.

Figure 3.21. ^1H NMR spectrum of methyl (14S)-[14- $^2\text{H}_1$]-linoleate **33a**. The overlapping resonances due to C_{14}HD and C_8HH at 2.06 ppm is reduced in intensity relative to that of unlabeled linoleate and integrated to 3H as expected. * are due to the presence of minor amount of impurities.

Figure 3.22. ^{13}C NMR spectrum of methyl (14*S*)-[14- $^2\text{H}_1$]-linoleate 33a. The triplet ($J_{\text{CD}} = 76.2$ Hz) due to C_{14}HD at 26.84 ppm is isotope shifted (-0.359 ppm) relative to that of unlabeled linoleate. The ^{13}C signals due to C12 (127.94 ppm) and C13 (130.17 ppm) are reduced in intensity due to the presence of deuterium at C14. * are due to the presence of minor amount of impurities.

Figure 3.23. IR spectrum of methyl (14*S*)-[14-²H₁]-linoleate **33a**. The C-D stretch due to C₁₄HD appears at 2150 cm⁻¹.

3.2.3.2 Stereochemical analysis of Acetylide Anion Attack on Chiral CHD Tosylates

During the syntheses of chiral monodeuterio fatty acids, it is assumed that the nucleophilic displacement of chiral tosylates by acetylide ion takes place predominantly in S_N2 fashion with inversion of configuration. In this section, an attempt to investigate the extent of configurational inversion is described (Figure 3.24). The chiral monodeuterio [4-²H₁]-2-octyn-1-ols **45a** and **45b** prepared during the synthesis of chiral [14-²H₁]-linoleates were semi-hydrogenated *via* Lindlar catalyst to produce the *cis*-[4-²H₁]-linoleates were semi-hydrogenated *via* Lindlar catalyst to produce the *cis*-[4-²H₁]-2-octen-1-ols **48a** and **48b**, respectively.^{202,203} The allylic alcohols generated in this fashion were further processed for oxidative cleavage of the olefinic double bond to synthesize the corresponding chiral [2-²H₁]-hexanoic acids **49a** and **49b** under extremely

mild conditions *via* the use of periodic acid ($\text{IO}(\text{OH})_5$) and the ruthenium(III) chloride hydrate as catalyst.^{204,205} Subsequently, the resultant chiral deuterio hexanoic acids were esterified with the Parker reagent, (+)-(*S*)-methyl mandelate and these esters analyzed by ^1H NMR²⁰⁶ (Figure 3.25).

Figure 3.24. Stereochemical analysis of tosylate displacement by alkyne anion.

While a major unknown impurity in the samples of the chiral d_1 -hexanoates prevented accurate assessment of the % ee of these compounds, it is clear that tosylate displacement did proceed with inversion as the major pathway. As illustrated in Figure 3.25, it is apparent that in the MMA ester of (2*R*)-[2- $^2\text{H}_1$]-hexanoic acid, the H_B resonance of -CHD moiety is shifted upfield due to phenyl shielding relative to the corresponding ^1H resonance in the MMA ester of (2*S*)-[2- $^2\text{H}_1$]-hexanoic acid as expected.

Figure 3.25. ¹H resonances at C-2 of (*S*)-O methyl mandelate (MM) ester of (2*S*)-, (2*R*)-[2-²H₁]-hexanoic acid (triplet of triplets) and d₀-hexanoic acid (AB quartet). α-Deuteration causes a small upfield shift (~ 0.017, 0.015 ppm) of the signal due to the geminal proton. * signals of the major unknown impurity.

3.2.3.3 Incubation of Chiral d₁-Linoleates

The enantioselectivity of hydrogen removal by Δ^{11,13}-desaturase during α-eleostearate formation was investigated in analogous fashion to that described for Δ^{8,10}-desaturation in Chapter 2 (Section 2.2.3.2). Chiral [11-²H₁]-linoleates **2a** and **2b** and chiral [14-²H₁]-linoleates **33a** and **33b** (~ 5 mg of each saponified substrate) were administered independently, in triplicate and quadruplicate respectively, to growing cultures of pRTL2 myc-FADX strain of *S. cerevisiae* (50 ml). The deuterium content of

methyl α -eleostearate obtained in each experiment was ascertained by GC-MS analysis of the isolated fatty acid methyl ester (FAME) fraction of the collected yeast cells.

Mass spectral analysis of the α -eleostearate product derived from (11*R*)-[11- $^2\text{H}_1$]-linoleic acid revealed the appearance of the most abundant molecular ion at m/z 292 (Figure 3.26), this represents the overall loss of deuterium from the fatty acid during enzymatic oxidation.

Abundance

Figure 3.26. Representative mass spectrum of biosynthetic methyl α -eleostearate derived from methyl (11*R*)-[11- $^2\text{H}_1$]-linoleate **2b**.

In contrast to the outcome displayed above, the mass spectrum of the α -eleostearate derived from (11*S*)-[11- $^2\text{H}_1$]-linoleic acid demonstrated the appearance of the most abundant molecular ion at m/z 293 (Figure 3.27), this represents the overall

retention of deuterium in the fatty acid product during enzymatic oxidation when it is positioned at the *pro R* position of the d_1 -substrate.

Abundance

Figure 3.27. Representative mass spectrum of biosynthetic methyl α -eleostearate derived from methyl (11*S*)-[11- $^2H_1$]-linoleate **2a**.

A similar pattern of deuterium incorporation was observed for the α -eleostearate derived from (14*R*)-[14- $^2H_1$]-linoleic acid (loss of label) and (14*S*)-[14- $^2H_1$]-linoleic acid (retention of label) as featured by the mass spectra shown in Figure 3.28 and Figure 3.29, respectively.

Abundance

Figure 3.28. Representative mass spectrum of biosynthetic methyl α -eleostearate derived from methyl (14*R*)-[14- $^2\text{H}_1$]-linoleate **33b**.

Abundance

Figure 3.29. Representative mass spectrum of biosynthetic methyl α -eleostearate derived from methyl (14*S*)-[14- $^2\text{H}_1$]-linoleate **33b**.

The quantitative aspects of these results are displayed in Table 3.3. It is interesting to note that the elevated level of d₁-product derived from (11*R*)-[11-²H₁]-LA **2b** was anticipated due to large primary deuterium KIE (4.0) operating at C-11. This causes the (11*S*)-d₁ contaminant in this substrate to lose the 11*R* hydrogen much faster than the corresponding (*R*)-enantiomer loses deuterium from this position.

Table 3.3. Isotopic content of stereospecifically labeled cellular monodeuterated linoleates and Δ^{11,13}-desaturated products (α-eleostearate) .

Compounds Name	Substrates		Products	
	% d ₀	% d ₁	% d ₀	% d ₁
(11 <i>R</i>)-[11- ² H ₁]-LA ^a	2.5	97.5	74.9	25.1
(11 <i>S</i>)-[11- ² H ₁]-LA ^a	0.8	99.2	8.2	91.8
(14 <i>R</i>)-[14- ² H ₁]-LA ^b	1.2	98.8	94.3	5.7
(14 <i>S</i>)-[14- ² H ₁]-LA ^b	1.3	98.7	10.7	89.3

^a Incubations of chiral 11-d₁-substrates were carried out three times and the % isotopic content of each species is given as an average value ± standard deviation of three independent GC-MS runs. ^b Incubations of chiral 14-d₁-substrates were carried out four times and the % isotopic content of each species is given as an average value ± standard deviation of four independent GC-MS runs. The average standard deviation for each species is ± 0.4 expressed as a percentage.

These results show that during the course of α-eleostearic acid biosynthesis, linoleic acid loses *pro R* hydrogens at both C-11 and C-14 positions. Our observation is in accord with the *syn* dehydrogenation pattern at the two non-adjacent positions, but in

relation to our predicted model (Figure 3.1 and 3.30) the hydrogens are removed topologically from the opposite side of the olefinic plane compared to that removed during the course of linoleic acid formation by the parent Δ^{12} -desaturase (FAD2)^{2,140}. The observed enantioselectivity also does not correlate with the enantioselectivity identified during calendic acid biosynthesis in *Calendula officinalis* by $\Delta^{8,10}$ -desaturase (Chapter 2, Figure 2.46) and for an insect $\Delta^{10,12}$ -desaturase that converts (*Z*)-11-tetradecenoate to the corresponding (*E,E*)-10,12-tetradecadienoate⁴⁰ (Chapter 1.4.1, Figure 1.29).

Figure 3.30. Predicted outcomes for α -eleostearate formation from stereospecifically labeled linoleates.

It is difficult to imagine how the diiron peroxo species can abstract hydrogens from below the plane of the olefin, as the associated interactions with its surrounding ligands as well as the amino acid sequence near the active site would have to change dramatically. Looking at the many different fatty acid products that can be generated by the FAD2 family^{1,2} and the enormous enzymatic plasticity of tung FAD2 variant³⁴, it is most likely that the enzyme active site cavity can be altered to accommodate different substrate conformations relative to a fixed diiron position. Considering all these factors, we have proposed a revised topological model as shown in Figure 3.31 for the

explanation of the observed stereochemical outcome in our experiment. In this revised topological model, it is assumed that the substrate can present the *pro R* hydrogens at C-11 and C-14 positions of the fatty acid substrate to the diiron peroxo species through rotation at the C-10,11 and C-14,15 C-C bonds. In this conformation, the cryptoregiochemistry of the $\Delta^{11,13}$ -desaturase enzyme still remains same at C-11. It is understood that the double bonds are quasicoplanar.

Figure 3.31. The revised topological model and observed results for eleostearate formation from stereospecifically deuterated linoleates. Note CIP priority rules dictate that the deuterium atoms that are removed from the C-11 and C-14 positions are labeled as both D_R and those deuterium atoms retained in the products are labeled as D_S .

This is not the only case where the enantioselectivity of a desaturase is opposite to expectations. This type of instance was also reported in the literature previously namely during Δ^{13} -desaturation of (*Z*)-11-hexadecenoate and 11-hexadecynoate to biosynthesize (*Z,Z*)-11,13-hexadecadienotate and (*Z*)-hexadeca-11-yne-13-eneoate respectively in the moth *Thaumetopoea pityocampa* (Section 1.1.5, Figure 1.6).^{41,75} Using stereospecifically labeled deuterated palmitoyl precursors, it was shown that the Δ^{13} -desaturase removes two *pro S* hydrogens from C-13 and C-14 positions of (*Z*)-11-hexadecenoate but the *pro R*

hydrogens from the 11-alkyne analogue.⁷⁵ A topological model for this substrate-dependent reversal in enantioselectivity was proposed (Figure 3.32).

Figure 3.32. Schematic diagram showing the possible conformational arrangement of two different substrates at the binding cavity of Δ^{13} -desaturase in *T. pityocampa*. **A)** Good fit for 11-hexenoate substrate; **B1)** Good fit for 11-hexynoate substrate; **B2)** Bad fit for 11-hexynoate substrate.

3.3 Conclusions

The cryptoregiochemistry and enantioselectivity of another important plant 1,4-desaturase enzyme belonging to the family of FAD2 homologue has been determined. The site of initial oxidation (cryptoregiochemistry) for the tung $\Delta^{11,13}$ -desaturase-mediated formation of α -eleostearic acid has been determined to be at C-11, the carbon closest to C-12 which is the site of initial oxidative attack for the parent FAD2 enzyme. Our cryptoregiochemical result correlates well with prediction and to that observed with parent FAD2 and other FAD2 homologues catalyzed formations of various other fatty acids as discussed in Section 1.2.5.

However, the observed enantioselectivity of hydrogen removal doesn't correlate with prediction as well as with the observed enantioselectivity in plant $\Delta^{8,10}$ -desaturase and insect $\Delta^{10,12}$ -desaturase during the formation of calendic acid and (*E,E*)-10,12-

tetradecadienoic acid, respectively. A revised topological model is proposed to explain the reversal in enantioselectivity (Figure 3.33). According to this revised model, the biosynthesis of α -eleostearic acid in *Aleurites fordii* Hemsl. (tung) involves the initial, isotopically sensitive removal of a *pro R* hydrogen from C-11 of linoleate and subsequent cleavage of the *pro R* C14-hydrogen bond *i.e.* two hydrogens are removed in *syn* fashion from two neighboring allylic positions of the fatty acid chain to generate the conjugated trienoic acid (Figure 3.33).

Figure 3.33. Mechanistic model for 1,4-dehydrogenation reaction of linoleate to biosynthesize α -eleostearate as it occurs in *Aleurites fordii* Hemsl. The double bonds are quasi-coplanar.

Chapter 4: Stereochemistry of Dimorphecolic acid Biosynthesis in *Dimorphotheca sinuata* and associated KIE Evaluation

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4.1 Experimental Approach

In the context of understanding the structure-function relationships of the family of FAD2 homologues that leads to the formation of unusual conjugated fatty acids in plants, the formation of dimorphecolic acid in the seeds of *Dimorphotheca sinuata* was investigated here. Determination of the mechanism of dimorphecolic acid formation is clearly necessary, as the normally least preferable hydroxyl rebound step dominates over the generally observed second hydrogen removal step.^{1,2} Molecular biological studies revealed that two FAD2-like enzymes (DsFAD2-1 and DsFAD2-2) work in tandem fashion to produce dimorphecolic acid from oleic acid *via* the 12-*trans* isomer of linoleate as shown in Figure 4.1.¹¹

Figure 4.1. The biosynthetic pathway of dimorphecolic acid formation from oleic acid in the seeds of *Dimorphotheca sinuata*.

Based on the close taxonomical relationship¹¹ between *Dimorphotheca sinuata* and *Calendula officinalis* and observed high amino acid sequence identity between DsFAD2-2 and $\Delta^{8,10}$ -desaturase enzyme, we propose a mechanistic model for the former enzyme that has been shown in Figure 4.2. The substrate 9*Z*,12*E*-18:2 adopts a conformational arrangement that is similar to that suggested during calendate formation¹⁵⁸ (see Chapter 2, Figure 2.1) and the reaction starts at C-11 with the removal of a hydrogen atom.

Figure 4.2. Proposed mechanistic model for Δ^9 -hydroxylation leading to the formation of 9-hydroxy-(*E,E*)-10,12-octadecadienoic from *trans*- Δ^{12} -linoleic acid and showing how iron-OH is not able to reach the C8H to form calendate.

The resultant allylic radical is trapped by iron-bound hydroxyl in a regioselective manner and generates the C-9 allylic alcohol that is (*S*)-configured.¹¹

In this spatial arrangement, the removal of hydrogen at C-11 and hydroxyl rebound at C-9 occurs with the same facial selectivity as the *syn* removal of hydrogens from C-11 and C-8 positions of linoleic acid that was reported during calendate formation (see Chapter 2). The correctness of this proposed topology could be tested through the determination of the enantioselectivity of dimorphecolic acid formation using stereospecifically labeled deuterated fatty acids as mechanistic probes. The availability of a convenient *S. cerevisiae* system for the co-expression of the genes that encoding the enzymes DsFAD2-1 and DsFAD2-2 from *Dimorphotheca sinuata* allowed the determination to be done *in vivo*.¹¹

Our approach required the use of two stereospecifically monodeuterated oleates, methyl (11*S*)-[11-²H₁]-octadec-9*Z*-enoate **3a** and methyl (11*R*)-[11-²H₁]-octadeca-9*Z*-enoate **3b** that were available from the calendate experiment (see Chapter 2, Section 2.2.2). The availability of methyl [11,11-²H₂]-oleate **51** in Patrick S. Covello's laboratory (NRC - PBI, Saskatoon) prompted us to carry out a measurement of the primary deuterium KIE for C-11 H abstraction.

Figure 4.3. The chiral monodeuterated and dideuterated oleate required to investigate the mechanism of dimorphecolate formation.

4.2 Incubation Experiments Using Dideuterated Substrates: KIE Studies

4.2.1 Trial Incubation of d₀-Oleate

The efficiency of formation of dimorphecolic acid from d₀-oleate using the cultures of *S. cerevisiae* strain that functionally co-expressing both DsFAD2-1 and DsFAD2-2 genes from *Dimorphotheca sinuata* was probed in a preliminary trial experiment. Accordingly, ca. 0.5 mL of sterilized oleic acid solution in 10 % Tergitol (10 mg/mL) was administered to a 50 mL growing culture of pESC-HIS/YPH499 strain of *S. cerevisiae* and allowed to grow at 28 °C for 7 days at 310 rpm. These optimized incubation conditions have been demonstrated to give the best substrate conversion rate and were used throughout the mechanistic studies. A corresponding control experiment under the same incubation conditions, but without the addition of exogenous oleic acid substrate was also run. The cellular fatty acid fractions were isolated from the harvested yeast cells *via* a standard hydrolysis/methylation protocol as described earlier (see Chapter 2 and 3) and analyzed by GC-MS. For the purpose of analysis the methylated fatty acid extract was silylated using BSTFA/pyridine¹⁸⁷ to obtain the TMS derivative of methyl dimorphecolate (Figure 4.4).¹¹ Silylation of alcohols is known to dramatically enhance their detectability by GC.

The product was identified on the basis of retention time (13.60 min for d₀-molecular ion cluster) of the TMS derivative of methyl dimorphecolate and its comparison with the retention time of a reference standard.¹¹

Figure 4.4. The cellular fatty acid profile of *S. cerevisiae* grown in a medium supplemented with oleic acid.

The mass spectrum of the GC peak assigned to silylated derivative of methyl dimorphecolate product (Figure 4.5) revealed the presence of the molecular ion at m/z 382 ($[M]^+$) and a diagnostic base peak at m/z 225 ($[Me(CH_2)_4CH=CH-CH=CH-CH-O-Si(CH_3)_3]^+$) due to the fragmentation between the C-8 and C-9 atoms. The fragmentation pattern is in accord with the assigned structure and similar to that reported before.¹¹

The dehydrogenated product (8*E*,10*E*,12*E*-octadeca-trienoic acid) or the *cis*- Δ^{12} -isomer of dimorphecolic acid could not be detected as trace “error” compounds in the FAME of the total cellular fatty acids. This observation shows the commitment of DsFAD2-1 and DsFAD2-2 to the production of specific fatty acids. The result matches well with that reported earlier by Cahoon and Kinney.¹¹ A similar examination of the methylated CH_2Cl_2 extract of the culture medium did not identify any “error” products.

Figure 4.5. Mass spectrum of TMS derivative of the methyl ester of dimorphecolic acid.

Comparison of the fatty acid methyl ester (FAME) profile of the control experiment (no oleate in medium) with the culture expressing the pESC-HIS/YPH499 strain of *S. cerevisiae* (Figure 4.4) revealed that in both incubations the product dimorphecolic acid (as trimethylsilyl derivative) has been produced which is consistent with the endogenous formation of the substrate oleic acid. The amount of dimorphecolic acid formed *via* the tandem fatty acid oxidation sequence was ~0.5 % of the total cellular fatty acids. The result corresponds well with transformation levels reported previously.¹¹

4.2.2 Intermolecular Kinetic Isotope Effect (KIE) Studies

The primary deuterium KIE for C11-H cleavage was evaluated in an intermolecular competitive experiment where ca. equimolar (1:1) mixture of 18-d₃-oleate and 11-d₂-oleate was used.¹⁹⁹ The ω-tagged 18-d₃-oleate was used as the “11-d₀ substrate” since the contribution from endogenous oleate pool could not be controlled. As

a control, we also performed a set of incubation experiments using a mixture of 18-d₃-oleate and [9,10-²H₂]-oleate; in this case a KIE of ~ 1 was expected since the latter isotopomer does not contain deuterium label at C-11. All labeled compounds were available in the Covello laboratory (NRC - PBI, Saskatoon) and had been synthesized by Pat Tulloch (NRC - Saskatoon) some four decades ago. Sterilized mixtures consisting of saponified methyl esters of [18,18,18-²H₃]-oleate in combination with [11,11-²H₂]- or [9,10-²H₂]-oleates were administered separately as a solution in 10 % Tergitol (~ 5 mg each) to growing cultures of pESC-HIS/YPH499 strain of *S. cerevisiae* that co-expresses both DsFAD2-1 and DsFAD2-2. For each type of mixture the reaction was run in triplicate. The cellular fatty acid fractions of the harvested cells were isolated by hydrolysis/methylation procedure and treated with BSTFA/pyridine to obtain the TMS derivative of methyl dimorphecolate that was then analyzed by GC-MS. The d₃/d₂ ratio of the recovered oleate fractions was found to be similar to the d₃/d₂ ratio of the administered mixtures. Maintenance of this ratio during the incubation is essential for competitive product KIE measurements as explained earlier (Section 1.3.2).¹⁹⁹ We can reasonably assume that the rate of *trans*-olefin at C12 catalyzed by DsFAD2-1 is not affected by deuterium at C11 or C9,10.

Mass spectral analysis of the TMS derivative of the dimorphecolate fraction revealed that when 18-d₃-oleate and 11-d₂-oleate was used as a substrate, the product (Figure 4.6) consisted of a mixture of d₁ and d₃-species, in addition to d₀ content from endogenous oleate. This indicated the loss of one deuterium from 11-d₂ substrate as expected. On the other hand, incubation of 18-d₃-oleate and 9,10-d₂-oleate as a substrate resulted in the formation of the product (Figure 4.7) that contained a mixture of d₀, d₂ and

Figure 4.6. Representative mass spectrum of TMS derivative of the methyl ester of dimorphecolic acid derived from 1:1 mixture of 18-d₃-oleate and 11-d₂-oleate. * The peak at m/z 383 is enhanced due to presence of d₁ species in addition to the d₀ M+1 contribution.

Figure 4.7. Representative mass spectrum of TMS derivative of the methyl ester of dimorphecolic acid derived from 1:1 mixture of 18-d₃-oleate and 9,10-d₂-oleate.

d_3 -species, which indicated the retention of both of the deuterium in the product from 9,10- d_2 -substrate as expected.

The primary deuterium product kinetic isotope effect (k_H/k_D) for C-H bond cleavage at C-11 was calculated using the following ratio:¹⁹⁹

$$\frac{k_H}{k_D} = \frac{P_H/P_D}{S_H^0/S_D^0}$$

Where, P_H/P_D is the ratio of ([18,18,18- d_3]- and d_0 -dimorphecolate) to [11- d_1]-dimorphecolate and S_H^0/S_D^0 is the ratio of ([18,18,18- d_3]- and d_0 -oleate) to [11,11- d_2]-oleate. Thus, the above ratio simplifies to:

$$\frac{k_H}{k_D} = \frac{[\% P_{d_0} + \% P_{d_3}]/[\% P_{d_1}]}{[\% S_{d_0} + \% S_{d_3}]/[\% S_{d_2}]}$$

Table 4.1. Competitive isotopic discrimination in the *Dimorphotheca sinuata* DsFAD2-1 and DsFAD2-2 desaturase-mediated formation of dimorphecolic acid from [11,11-²H₂]-oleic acid or [9,10-²H₂]-oleic acid vs. [18,18,18-²H₃]-oleic acid.

Position of Isotope in d ₂ -Species	Trial	Oleate Substrate ^{a,b,c}			TMS Derivative of Dimorphecolate Product ^{a,c}			KIE
		d ₀	d ₂	d ₃	d ₀	d ₁	d ₃	
C-11	t ₀	0.0	43.7	56.3				
	1	26.2	31.2	42.6	43.0	6.0	51.0	7.1
	2	26.5	31.8	41.7	42.3	5.1	52.6	8.7
	3	25.8	31.4	42.8	42.2	5.6	52.2	7.7
	Average							7.8 ± 0.8 ^d
C-9,10					d ₀	d ₂	d ₃	
	t ₀	0.0	28.3	71.7				
	1	30.8	17.0	52.2	35.0	18.3	46.7	0.91
	2	31.7	16.3	52.0	35.0	18.8	46.2	0.84
	3	31.8	16.1	52.1	35.8	18.5	45.7	0.85
	Average							0.87 ± 0.04 ^e

^a The isotopic content of both substrate and product mixtures were evaluated by considering the (M+1), (M+2) as well as (M-1), (M-2) contributions from the d₀-, d₁-, d₂- and d₃-species. d₀, d₁, d₂ and d₃ refer to the non-deuterated, monodeuterated, dideuterated and trideuterated species, respectively. ^b The isotopic composition of the cellular oleic acid fractions in both incubations were similar to that measured prior to the incubation. ^c The deuterium content (%) of each species is given as an average value based on three GC-MS runs. The average standard deviation for these measurements is ± 0.4 expressed as a percentage. ^d The average KIE at C-11 (three incubations) ± standard deviation. ^e The average secondary KIE at C-9,10 (three incubations) ± standard deviation.

The analytical results obtained from these competitive experiments are displayed in Table 4.1. The existence of a large primary deuterium kinetic isotope effect (KIE = 7.8 ± 0.8) for the C-H bond cleavage at C-11 was calculated. In contrast, the secondary kinetic isotope effect due to deuterium at C-9 and C-10 of the fatty acid chain (KIE =

0.87 ± 0.04) was found to be close to unity as expected. The observation of a very large primary deuterium isotope effect on C-11 bond rupture is in accord with what has been observed with other members in the family of FAD2 homologues and indeed all membrane-bound desaturases studied to date (see Section 1.2.5).^{1,26,112,158} In general, the magnitude of the observed kinetic isotope effects are difficult to interpret precisely since partial masking of the intrinsic values by other enzymatic steps such as, substrate binding or product release may be occurring.^{1,201} Primary deuterium KIE that are larger than the theoretical maximum (6.8 at 28 °C)²⁰⁷ such as we have observed here have been observed before in desaturase-mediated reactions^{26,79} and may indicate a tunneling phenomenon¹⁹⁵. In any case, these data can now be used to help interpret the results of the stereochemical investigations as reported in the next section.

4.3 Incubation of Chiral d₁-Oleates: Stereochemical Studies

To determine the enantioselectivity of dimorphecolate formation from oleate in the cascade reaction involving two FAD2 variants, the sterilized solution of saponified methyl esters of chiral [11-²H₁]-oleates **3a** and **3b** (5 mg each) were administered separately to growing cultures of pESC-HIS/YPH499 strain of *S. cerevisiae* (50 mL) under conditions identical to that of the trial experiment. A small portion of the chiral fatty acid solution in Tergitol was also withdrawn prior to the incubation to evaluate the isotopic deuterium content (d_1/d_0) of the administered compounds by GC-MS. The cellular fatty acid fraction of the harvested yeast cells were collected by hydrolysis/methylation procedure similar to the trial experiment and treated with

BSTFA/pyridine¹⁸⁷ in order to produce the trimethylsilyl derivative of methyl dimorphecolate.

Mass spectral analysis of the TMS derivative of the dimorphecolate product derived from (11*S*)-[11-²H₁]-oleic acid **3a** revealed that the most abundant ion in the molecular and base peak cluster appeared at *m/z* 382 and 225, respectively (Figure 4.8); this represents the overall loss of deuterium. In contrast, the mass spectrum of the TMS derivative of the dimorphecolate product derived from (11*R*)-[11-²H₁]-oleic acid **3b** indicated that the ions at *m/z* 383 and 226, respectively (Figure 4.9), were greatly enhanced; this represents the overall retention of deuterium labeling during the enzymatic reaction. The substantial amount of *d*₀ product in this mass spectrum can be traced to the substantial amount of endogenous *d*₀ oleate that is produced in this yeast mutant. The quantitation of deuterium loss vs. retention at C-11 was accomplished by analyzing the base peak of the TMS derivative of methyl dimorphecolate which is the diagnostic peak for the trimethylsilylated derivative (Figure 4.8 and 4.9) and carrying out the appropriate correction of ion intensity at *m/z* 226 due to *M*+1 contribution (ca. 19.3 %) from its *d*₀-counterpart (*m/z* 225). The *M*+1 correction factor was determined experimentally using *d*₀-dimorphecolate and compared to the theoretical values. The deuterium content of oleate, the 9*Z*,12*E*-octadecadienoate intermediate and dimorphecolate product is displayed in Table 4.2.

Figure 4.8. Representative mass spectrum of TMS derivative of the methyl ester of dimorphecolic acid derived from methyl (11*S*)-[11-²H₁]-oleate **3a**.

Figure 4.9. Representative mass spectrum of TMS derivative of the methyl ester of dimorphecolic acid derived from methyl (11*R*)-[11-²H₁]-oleate **3b**.

Table 4.2. Isotopic content of stereospecifically labeled monodeuterated oleates and DsFAD2-1 and DsFAD2-2 involved hydroxylated products (dimorphecolate).

Compound	Cellular Oleate ^a		Dimorphecolate Product ^a	
	% d ₀ ^b	% d ₁	% d ₀	% d ₁
(11 <i>S</i>)-[11- ² H ₁]-18:1	39.1	60.9	84.4 (88) ^c	15.6 (12) ^c
(11 <i>R</i>)-[11- ² H ₁]-18:1	39.4	60.6	48.3 (43) ^c	51.7 (57) ^c

^a Incubation of each enantiomer was carried out three times and the % isotopic content of each species is given as an average value of three independent GC-MS runs. The average standard deviation for these measurements is ± 0.4 expressed as a percentage. ^b d₀ Content is due to endogenous oleate production. ^c Predicted values are based on 80 % ee of the chiral substrates and KIE of 6 at C-11.

The deuterium content of the dimorphecolate product can be rationalized by considering that:

- 1) 40 % of the oleate substrate is non-deuterated due to endogenous oleate production.
- 2) The chiral d₁-substrates may contain as much as 10 % of the minor enantiomer. This is based on 90 % ee of the chiral alcohol intermediate (Figure 3.19) used in the synthesis and a stereospecificity of ~ 90 % in the inversion reaction using acetylide anion (Figure 3.24).
- 3) C-11-D cleavage is subject to a large KIE isotope effect as demonstrated in the previous section.

The predicted values for % d₀ and % d₁ of dimorphecolate product derived from the two chiral 11-d₁-oleates were calculated as follows:

For the case when deuterium is lost from (11*S*) substrate with large KIE

$$\% \text{ d}_0 \text{ Product} = (39.1 + 7.0) / \text{Total} \times 100 = 46.1/52.2 \times 100 = 88 \%$$

$$\% \text{ d}_1 \text{ Product} = 100 - 88 = 12 \%$$

For the case when deuterium is retained from (11*R*) substrate

$$\% \text{ d}_0 \text{ Product} = (39.4 + 0.8) / \text{Total} \times 100 = 40.2/94.7 \times 100 = 43 \%$$

$$\% \text{ d}_1 \text{ Product} = 100 - 43 = 57 \%$$

Inspection of Table 4.2 revealed that the observed results are in reasonable agreement with the predicted deuterium content in both cases, given the uncertainty in the % ee of the starting materials.

This result signifies that during the course of dimorphecolic acid biosynthesis, the hydroxylase enzyme removes the *pro S* hydrogen from the C-11 position of *trans*- Δ^{12} -linoleic acid and delivers the hydroxyl group topologically on the same side of the olefinic plane at C-9 position. Our observed topology of hydrogen removal at C-11 clearly corresponds to the established topology of hydrogen abstraction by the parent FAD2 enzyme during the formation of linoleic acid (Figure 2.1).^{1,2,140} The observed

stereochemistry also matches the reported enantioselectivity of calendate formation by the same family of FAD2 enzyme, $\Delta^{8,10}$ -desaturase which was also found to initiate the bioconjugation by removing the *pro S* hydrogen at C-11 position of linoleic acid (see Chapter 2). Overall, the result obtained in this experiment further enhance the proposed active site architectural motif for the family of FAD2 homologues that the overall contours of the binding site for all these enzymes are similar.

Figure 4.10. Observed results for dimorphecolate formation from stereospecifically deuterated *trans*- Δ^{12} -linoleate. Note CIP priority rules dictate that the deuterium atom that is removed from C-11 position is labeled D_S .

Interestingly, this experimental finding also coincides well with the overall topology of another hydroxylated fatty acid formation, namely ricinoleic acid by a different FAD2 variant in the seeds of *Ricinus communis L.*, where the desaturase enzyme act as Δ^{12} -hydroxylase and produces the alcohol in a stereoselective manner (Figure 4.11 and 1.26).^{1,9,16} The regiochemistry of hydroxyl delivery in dimorphecolate formation may well be dictated by which iron oxygen bond of the diiron dioxo species is cleaved during the initial hydrogen abstraction step.

Figure 4.11. Comparison of relative stereochemistry of hydroxylation by FAD2 variants in the biosynthesis of ricinoleate and dimorphecolate.

In the current context of understanding the biosynthesis of unusual hydroxylated fatty acid in plants, it is necessary to mention the serendipitous discovery by Shanklin and coworkers of a new triple mutant T117R/G188L/D280K of soluble plant castor stearyl-ACP Δ^9 -desaturase that shows activity analogous to dimorphecolate formation.¹¹⁶ It was observed that the mutant desaturase converts oleyl-ACP substrate into a major 9-hydroxy-(10*E*)-octadecenoic acid.

The mutant desaturase initiates the oxidation at C-11 of oleyl-ACP and generates an allylic intermediate that contains a radical at C-9 which abstracts the hydroxyl group from diiron catalytic center in highly regiospecific fashion (Figure 4.12). O¹⁸ labeling experiment demonstrated that the hydroxyl oxygen is well derived from molecular oxygen *via* the hydroxyl rebound to the radical intermediate at C-9. The mutant desaturase enzyme was also identified to use *trans*-isomer of oleoyl-ACP as substrate and transform it into two major products, 9-hydroxy-(10*Z*)-octadecenoic acid and (9*E*,11*Z*)-

CLA isomer. It was proposed that the fate of the reaction outcome from various substrates is primarily governed by the curvature of the substrate binding cavity, which is boomerang shaped with a bend adjacent to the active site (Figure 4.12).

Figure 4.12. Proposed substrate binding modes and reaction outcomes for various substrates with mutant soluble desaturase. The mechanism of (Z)-9-18:A (panel A) and (E)-10-18:1-9-OH (panel B) formation are shown in the context of the bend in the desaturase substrate channel (shown in gray) adjacent to the active site oxidant. Postulated substrate binding modes of the nonnatural elaidyl substrate (panel C) that leads to the formation of (Z)-10-18:1-9-OH and (E)-9-(Z)-11-18:2 are shown. The Figure was adapted from Shanklin *et al.*¹¹⁶

A precise positioning of the substrate in this binding curvature is necessary to allow exclusive formation of specific hydroxylated product, as it occurs with oleyl-ACP, which fits nicely in the substrate binding cavity (Figure 4.12B) and allows the hydrogen abstraction to proceed at C-11 followed by stereospecific hydroxylation at C-9 leading to the production of 9-hydroxy-(10E)-octadecenoic acid. Conversely, in case of *trans*-oleyl isomer because of its inability to completely insert into the substrate binding cavity, the

corresponding reaction results in the formation of 9-hydroxy-(10*Z*)-octadecenoic acid as the major and 11-hydroxy-(9*E*)-isomer as minor product (Figure 4.12C). It would be of interest to determine more precisely how the DsFAD2-2 hydroxylase favours formation of hydroxylation over dehydrogenation.

4.4 Conclusions

The enantioselectivity of another important unusual plant fatty acid biotransformation as it occurs in *Dimorphotheca sinuata* has been determined in this chapter. Our investigation established that the formation of dimorphecolic acid involved the initial, isotopic sensitive removal of *pro S* hydrogen from C-11 of *trans*- Δ^{12} -linleate and generates an allylic radical intermediate that removes the oxygen atom from the catalytic diiron center to hydroxylate in *syn* fashion at the *pro S* position (Figure 4.13). A large primary deuterium KIE was observed for the initial hydrogen abstraction.

Figure 4.13. Mechanistic model for DsFAD2-2 hydroxylase involved biotransformation of *trans*- Δ^{12} -linoleate to dimorphecolate as it occurs in the seeds of *Dimorphotheca sinuata*.

The observed stereochemistry and site of initial oxidation in dimorphecolate formation correlates well with that observed for the parent FAD2 enzyme involved desaturation of oleate to generate linoleate ($\text{CH}_3\text{-(CH}_2)_5\text{-C}_{13}=\text{C}_{12}\text{-CH}_2\text{-C}_{10}=\text{C}_9\text{-(CH}_2)_7\text{-CO}_2\text{H}$) and validates the proposed mechanistic model (Figure 4.13). Since no dehydrogenated product could be detected, this militates against the possibility of the presence of a minor pathway involving further dehydrogenation at C9-C8 position followed by hydration at C-9. The results obtained from this experiment further strengthen the proposal that there exists a highly conserved, active site structural motif for the family of membrane-bound FAD2 homologues.

Chapter 5: Summary and Future Directions

5.1 Summary

All of the research objectives that were formulated at the outset of this thesis work have been achieved. The results of mechanistic studies of various conjugated fatty acid biosyntheses are as follows:

- 1) The enantioselectivity of *Calendula officinalis* $\Delta^{8,10}$ -desaturase (FAD2 variant) - mediated formation of calendic acid from linoleic acid has been determined for the first time using deuterium-labelled mechanistic probes. It was observed that the $\Delta^{8,10}$ -desaturase enzyme initiates the oxidation by removing the *pro S* hydrogen from the C-11 position of linoleic acid and completes the sequence by removing another hydrogen from the non-adjacent *pro R* position at C-8 in *syn* fashion. The primary deuterium KIE at C-11 was also assessed using racemic methyl [11- $^2\text{H}_1$]-linoleate and the calculated value (4.4 ± 0.1) was found to be in agreement with that previously reported¹⁵⁸ when secondary isotope effects are taken into account.
- 2) We have also investigated the cryptoregiochemistry (site of initial oxidation) and enantioselectivity of *Aleurites fordii* Hemsl. $\Delta^{11,13}$ -desaturase involved in the formation of α -eleostearic acid from linoleic acid. The site of initial oxidation for this enzyme has been determined to be at C-11 using KIE methodology; this result is similar to that observed during calendic acid biosynthesis and matches with our prediction. The deuterium kinetic isotope effect at C-11 was also evaluated by using racemic methyl [11- $^2\text{H}_1$]-linoleate and the anticipated result (4.0 ± 0.1) is in accord with that estimated using

approximately 1:1 (w/w) mixture of non-deuterated to regiospecifically dideuterated linoleates. However, using chiral monodeuterated fatty acids as mechanistic probes it was revealed that the enantioselectivity of α -eleostearic acid formation doesn't correlate with our prediction. Thus, to explain the stereochemical outcome, a revised substrate conformation was proposed where $\Delta^{11,13}$ -desaturase enzyme initiates the oxidation by removing the *pro R* hydrogen from the C-11 position of linoleic acid and completes the sequence following the removal of non-adjacent *pro R* hydrogen from the C-14 position in a *syn* dehydrogenation fashion.

- 3) The enantioselectivity of dimorphecolic acid formation from the 12*E*-isomer of linoleic acid in *Dimorphotheca sinuata* was also investigated. Using chiral [11-²H₁]-oleates as precursors, it was demonstrated that the oxidation was initiated by isotopically sensitive removal of *pro S* hydrogen from C-11 position of the *trans*- Δ^{12} -linoleate substrate to generate an allylic radical that captures iron-bound hydroxyl at C-9 in an enantioselective manner from the same side of the olefinic plane.

Overall, the results obtained from these mechanistic studies clearly establish the fact that there exists a highly conserved, active site architectural motif for the family of membranous FAD2 homologues (Figure 4.14), where the oxidation is always initiated by approaching the olefinic plane at or near the C-12 position of the substrate from the top (C1 terminus at the right hand side of the structure (CH₃-(CH₂)₅-C₁₃=C₁₂-CH₂-C₁₀=C₉-(CH₂)₇-CO₂H)):

Figure 5.1. Highly conserved active site model of the membrane-bound family of FAD2 homologues showing that the biooxidation is normally initiated via the removal of topologically top hydrogen from the plane of the olefin at the carbon closest to C-12, the site of initial oxidation for parent FAD2 enzyme.

5.2 Future Directions

As a part of ongoing research towards the understanding of the structure-function relationships of the family of FAD2-like enzymes, our KIE methodology and chiral deuterium labeled mechanistic probes can be used to further investigate the formation of other unusual fatty acids, such as α -parinaric acid (1,4-desaturation) and vernolic acid (epoxidation). In doing so, we hope to correlate the various regio- and enantioselectivities observed for this important set of catalysts with the geometric relationship between oxidant and substrate.

As already discussed in Chapter 3, since the $\Delta^{11,13}$ -desaturase enzyme from *Aleurites fordii* Hems³⁴ exhibits multifunctional enzymatic plasticity by inserting either a *trans* double bond at C12,13 of an oleate substrate or performing another 1,4-desaturation of α -linolenic acid to produce α -parinaric acid with the same ease. Therefore, it would be

interesting to compare the cryptoregiochemistry and stereochemistry of these transformations with that observed for α -eleostearic acid biosynthesis. In this manner, the contours of the active site can be further defined.

Experiments could also be performed in the future to determine the origin of oxygen atom in dimorphecolic acid. Also the validity of the proposed active site model for the castor stearyl-ACP Δ^9 -desaturase derived triple mutant T117R/G188L/D280K¹¹⁶ could be further tested by determining the stereochemistry of hydrogen abstraction and hydroxyl capture.

Finally, the interpretation of the results obtained from these mechanistic studies would be greatly aided by characterizing the membranous FAD2-like enzymes *via* X-ray crystallography. Analysis of enzyme-substrate complexes will improve our understanding about how the active-site architecture controls the reaction outcome. From a broader point of view, understanding structure-function relationships will assist plant biotechnologists to develop novel transgenic plants and produce lipid-based bio-products with desired properties.

Chapter 6: Part I Experimental

6.1 Materials and Methods

^1H (300 MHz), ^{13}C NMR (75 MHz), and DEPT NMR spectra were obtained on a Bruker Avance 300 spectrometer. Analytes were dissolved in CDCl_3 , that was dried by passing the solvent through a short-column packed with neutral alumina (Activated aluminum oxide, Brockmann I standard grade, 150 mesh, 58 Å). Typical analyte concentration was 10 mg/mL for ^1H NMR and 20 mg/mL for ^{13}C NMR and DEPT. Chemical shifts for ^1H and ^{13}C NMR spectra are reported in ppm (δ) relative to TMS (0.00 ppm) and standardized to a chemical shift value of 7.2650 ppm (^1H) and 77.0275 ppm (^{13}C) for residual CHCl_3 (^1H) and CDCl_3 (^{13}C). Abbreviations used to assign multiplicity of peaks are as follows: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *m* = multiplet and *b* = broad. *J*-values are reported in Hertz (Hz). The isotope shifts on the ^{13}C NMR resonances of deuterated compounds are reported in Appendix A and B.

GC-MS data of the synthetic compounds were obtained courtesy of the University of Ottawa's mass spectrometry facility (Dr. Clem Kazakoff) using a Kratos Concept ^1H mass spectrometer at 70 eV interfaced with a J & W 30 m x 0.21 mm, DB-5 capillary column. The temperature program started at 120 °C and increased at 10 °C/min until 320 °C and held for 2 minutes.

GC-MS (EI, 70 eV) of the biological extracts were obtained at Plant Biotechnology Institute, National Research Council, Saskatoon using an Agilent 5973 Mass Selective Detector coupled to an Agilent 6890 gas chromatograph equipped with J & W Scientific 30 m x 0.253 mm DB-23 capillary column (with a film thickness of 0.25 μm), where the oven temperature was programmed from 160 °C to 240 °C with an

increment rate of 4 °C/min and then isothermal at 240 °C for 15 minutes with a flow rate of 1.0 mL/min. Helium flow was set at 30 mL/min. Analyses were carried out under a selected ion monitoring (SIM) mode with a narrow EI⁺ scan range of 280-305 m/z used at a rate of 1500 mass units / second that resulted in ~ 250 scans/GC peak. The integrated intensities of the individual ions in the pertinent ion cluster were recorded using Agilent MSD Chemstation software and corrected for natural isotopic abundances. The entire GC peak in the integration was carefully included to prevent errors due to fractionation of isotopic species during chromatography.

Purification of the biosynthesized (11*R*)- and (11*S*)-[11-²H₁]-linoleates were carried out on a Hewlett-Packard 1100 series HPLC system (Hewlett-Packard, Pal Alto, CA) equipped with an auto-sampler, solvent degasser, quaternary pump, column heater, and diode array detector (monitored at 205 and 254 nm) which were all controlled by HP Chemstation software. The samples were separated on a Phenomenex Gemini-5µm C18 110A 250mm x 10 mm column, eluting at a rate of 3 mL/min isocratically for 5 minutes with acetonitrile followed by 0-30 % acetone gradient in 20 minutes and subsequent flushed with 100 % acetone to rinse and then re-equilibrate. The spectral data obtained for these compounds were in accord with that reported in the literature. The purity of the deuterated fatty acid substrates were estimated using GC-MS by means of an Agilent 5973 Mass Selective Detector coupled to an Agilent 6890 gas chromatograph equipped with J & W Scientific 30 m x 0.253 mm DB-23 capillary column (with a film thickness of 0.25 µm) and controlled by Chemstation software and found that all of them were > 95-99 % chemically pure.

IR spectra were recorded on a Varian 1000 Scimitar series FT-IR spectrometer and the vibrational frequencies of the chemical bonds were reported in cm^{-1} (ν).

Purification of synthetic compounds by flash chromatography was performed using Merck silica gel (60 A), grade 9385 (230-400 mesh) with an air pressure of ca. 5 psi. Thin layer chromatography was carried out using glass-backed plates coated with Merck silica gel (60 A). Visualization of TLC spots was achieved by a combination of UV (aromatic compounds), I_2 vapour (unsaturated compounds) and water spray (universal detection of all compounds) as appropriate.

All reagents obtained commercially were acquired from Sigma-Aldrich and used without purification unless indicated. Freshly purified *p*-toluenesulfonyl chloride was used whenever required: reagent grade *p*-toluenesulfonyl chloride (10 g) was dissolved in minimum amount of chloroform (25 mL) and the solution was diluted with five volumes of spectrograde hexane (125 mL), swirled. The solid precipitate, that formed was removed by gravity filtration. The solvent was evaporated by rotary evaporation and the compound was dried over vacuum. Pyridine was dried over NaOH pellets. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were freshly distilled from Na-benzophenone ketyl. Toluene was freshly distilled from NaH. Dichloromethane was distilled from CaH_2 . For use in moisture-sensitive reactions, Spectrograde™ acetone was dried over activated molecular sieves (grade 4A, 1.6 mm pellets) and under N_2 for 1 h. All air and moisture-sensitive reactions were performed under ultra pure N_2 grade 5.0. When required, the reaction mixtures were cooled down to 0 to - 4 °C, - 10 to -12 °C, - 48 to - 50 °C and - 68 to - 72 °C using ice-water, ice-NaCl salt, acetone-dry ice and isopropanol-dry ice slurry, respectively. Moisture sensitive and reactive reagents were

added via glass or plastic syringes and 21 gauge needles. Organic extracts were typically worked up by drying over anhydrous Na_2SO_4 in a cone filter paper followed by subsequent gravity filtration. The solvents were evaporated in vacuo on a Büchi RE 111 Rotavapor and then dried to constant weight under N_2 .

All of the non-deuterated compounds prepared in this thesis have been synthesized previously and in each case, the analytical data was compared with the literature values.

Non-deuterated fatty acids (> 99 %) were obtained from Nu-Chek Prep, Inc. (Elysian, MN). $[11,11\text{-}^2\text{H}_2]$ -oleic acid, $[9,10\text{-}^2\text{H}_2]$ -oleic acid and $[18,18,18\text{-}^2\text{H}_3]$ -oleic acid were synthesized previously by A. P. Tulloch (NRC-Saskatoon).

6.2 Synthesis of Deuterium-Labeled Fatty Acids

6.2.1 Chiral $[8\text{-}^2\text{H}_1]$ -Linoleates Synthesis

6.2.1.1 Ethyl 8-hydroxyoctanoate

To a stirred solution of diethyl suberate (8.2 mL, 35 mmol) in anhydrous THF (60 mL) was added dropwise a solution of 2.0 M lithium aluminium hydride in THF (9.3 mL, 18.5 mmol) at 0 to -4 °C. After stirring the reaction mixture for 2.5 h at 0 °C, EtOAc (10 mL) was added to quench excess reducing agent and the solvent was removed in vacuo. The residue was suspended in CH_2Cl_2 (70 mL) and poured into an ice-water mixture (75 mL). The aqueous layer was acidified with 2.0 M HCl to pH 1 and the organic layer was separated. The aqueous layer was re-extracted with CH_2Cl_2 (3 x 50 mL) and the organic

layers combined. The combined organic phase was worked up in the normal manner to afford a mixture of the desired compound as well as the corresponding diol and starting material. After flash chromatography of the product mixture using EtOAc:hexane (1:4) as eluent, the pure title compound was obtained as a clear colorless liquid (1.35 g, 21 %). bp 85-87 °C at 0.1 mm Hg; R_f 0.48 (EtOAc/hexane, 9:1); IR (film): ν 3361 (O-H), 2933, 2858 (C-H), 1735 (C=O), 1465, 1374, 1248, 1184, 1096, 1036, 860 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.11 (*q*, $J = 7.3$ Hz, 2 H, OCH_2Me), 3.63 (*t*, $J = 6.0$ Hz, 2 H, CH_2OH), 2.28 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Et}$), 1.62 (*tt*, $J = 7.5, 6.0$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Et}$), 1.56 (*tt*, $J = 7.0, 6.0$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{OH}$), 1.42 (*s*, 1 H, OH), 1.39-1.29 (*m*, 6 H, C-4, C-5, C-6), 1.25 (*t*, $J = 7.3$ Hz, 3 H, CH_3); ^{13}C NMR (100 MHz, CDCl_3) δ_{C} 173.89 (C-1), 34.32 (C-2), 24.86 (C-3), 29.02 (C-4), 29.05 (C-5), 25.53 (C-6), 32.67 (C-7), 62.91 (C-8), 60.19 ($-\text{OCH}_2\text{Me}$), 14.24 (CH_3); EI-MS: m/z 158 ($[\text{M}-32]^+$), 143 ($[\text{M}-45]^+$), 124, 115, 101, 96, 88, 83, 73, 70, 60, 55, 41; TMS Derivative EI-MS: m/z 259 ($[\text{M} - 1]^+$), 245 ($[\text{M} - 15]^+$), 215 ($[\text{M} - 45]^+$), 199 ($[\text{M} - 61]^+$), 173 ($[\text{M} - 87]^+$), 157, 129, 115, 103, 88, 75, 73, 55, 43.

6.2.1.2 Ethyl 8-hydroxy-[8,8- $^2\text{H}_2$]-octanoate

The title compound was synthesized from diethyl suberate (44 mL, 188 mmol) using lithium aluminium deuteride (1.0 M in THF, 99.5 mL, 99.4 mmol) as reducing agent by following the same experimental procedure as used in the synthesis of ethyl 8-hydroxyoctanoate. The pure compound was obtained as a clear colorless liquid (13.9 g, 35 %). bp 85-87 °C at 0.1 mm Hg; R_f 0.29 (EtOAc:hexane, 1:1); and the spectral data of

the compound was similar to that reported above for the non-deuterated parent compound as well as with that listed in the literature¹⁷³. IR (film): ν 3387 (O-H), 2981, 2930, 2858 (C-H), 2193, 2087 (C-D), 1739 (C=O), 1465, 1373, 1257, 1184, 1108, 1034, 967, 859 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.10 (*q*, $J = 7.1$ Hz, 2 H, OCH_2Me), 2.27 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Me}$), 1.70 (*s*, 1 H, OH), 1.60 (*tt*, $J = 7.5, 7.2$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$), 1.53 (*t*, $J = 7.3$ Hz, 2 H, $\text{CH}_2\text{CD}_2\text{OH}$), 1.39-1.27 (*m*, 6 H, C-4, C-5, C-6), 1.23 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C NMR (100 MHz, CDCl_3) δ_{C} 173.88 (C-1), 34.31 (C-2), 24.85 (C-3), 29.02 (C-4), 29.05 (δ -shift - 0.003, C-5), 25.48 (γ -shift - 0.051, C-6), 32.45 (β -shift - 0.217, C-7), 62.15(*p*, $J_{\text{CD}} = 21.6$ Hz, α -shift - 0.755, C-8), 60.18 ($-\text{OCH}_2\text{Me}$), 14.23 (CH_3); EI-MS: m/z 173 ($[\text{M}-17]^+$), 158 ($[\text{M}-32]^+$), 145 ($[\text{M}-45]^+$), 126, 99, 88, 83, 70, 60, 5, 40; TMS Derivative EI-MS: m/z 247 ($[\text{M}-15]^+$), 217 ($[\text{M}-45]^+$), 201 ($[\text{M}-61]^+$), 173 ($[\text{M}-89]^+$), 159, 131, 117, 103, 83, 75, 73, 55, 43.

6.2.1.3 Ethyl 8-oxo-[8- $^2\text{H}_1$] octanoate

A stirred solution of oxalyl chloride (6.2 mL, 71.0 mmol) in dry CH_2Cl_2 (90 mL) was cooled to -65°C (external temperature) and anhydrous DMSO (10.1 mL, 142.0 mmol) was added dropwise for 20 min. A strongly exothermic reaction took place that turned the reaction mixture viscous after the completion of DMSO addition. The reaction mixture was stirred for an additional 5-10 minutes prior to the addition of ethyl 8-hydroxy-[8,8- $^2\text{H}_2$]-octanoate (6.75 g, 35.5 mmol) in dry CH_2Cl_2 (30 mL), in portions, at -

55 to - 60 °C. Immediately after the addition of the alcohol, the reaction mixture turned cloudy white in color and was diluted with dry CH₂Cl₂ (20 mL) to facilitate stirring. Stirring was continued for another 20 min before warming the reaction mixture to - 12 °C (external temperature). After stirring for a further 20 min, triethylamine (29.6 mL, 213.1 mmol) was added and the resultant yellowish reaction mixture was allowed to warm to room temperature and stirred for additional 30 min before the reaction was terminated. The reaction was worked up by the addition of distilled water (150 mL) and the organic layer was separated. The aqueous layer was re-extracted with CH₂Cl₂ (3 x 120 mL) and the combined organic layers were treated in the normal manner to yield a residue that was diluted further with Et₂O (180 mL) and washed successively with 2.0 M HCl (3 x 80 mL), H₂O (2 x 70 mL) and brine (3 x 70 mL). Filtration of the organic phase through anhydrous Na₂SO₄ followed by the removal of the solvent in vacuo gave the title compound as a yellowish liquid (6.16 g, 93 %). bp 62-63 °C at 0.05 mm Hg; *R_f* 0.34 (EtOAc:hexane, 1:3); IR (film): ν 2983, 2936, 2860 (C-H), 2070 (C-D), 1735 (C=O, CO₂Et), 1713 (C=O, CDO), 1465, 1373, 1252, 1184, 1096, 1033, 875 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 4.11 (*q*, *J* = 7.3 Hz, 2 H, OCH₂Me), 2.41 (*t*, *J* = 7.4 Hz, 2 H, CH₂CDO), 2.28 (*t*, *J* = 7.4 Hz, 2 H, CH₂CO₂Et), 1.72-1.54 (*m*, 4 H, CH₂CH₂CDO, CH₂CH₂CO₂Et), 1.34 (Overlapping *tt*, *J* = 7.4, 3.0 Hz, 4 H, C-4, C-5), 1.24 (*t*, *J* = 7.3 Hz, 3 H, CH₃); ¹³C NMR (100 MHz, CDCl₃) δ _C 173.67 (C-1), 34.19 (C-2), 24.68 (C-3), 28.80 (C-4), 28.75 (C-5), 21.79 (C-6), 43.59 (*t*, ²*J*_{CD} = 3.6 Hz, C-7), 202.36 (*t*, *J*_{CD} = 26.1 Hz, C-8), 60.19 (-OCH₂Me), 14.23 (CH₃); EI-MS: *m/z* 188 ([M]⁺), 168 ([M-19]⁺), 159 ([M-28]⁺), 143 ([M-44]⁺), 142 ([M-45]⁺), 130, 123, 116, 115, 102, 96, 88, 74, 69, 60, 55, 45, 41.

6.2.1.4 Ethyl (8*S*)-8-hydroxy-[8-²H₁] octanoate

A 3-neck round-bottom flask equipped with a reflux condenser, stir bar, glass stopper and rubber septa was charged with ethyl 8-oxo-[8-²H₁]-octanoate (6.21 g, 33.2 mmol) and anhydrous THF (90 mL). To the stirred solution was added (+)-(R)-Alpine-Borane® (0.5 M in THF, 119.6 mL, 59.8 mmol) in portions at room temperature. 30 minutes after the addition, the rubber septum was quickly replaced with a glass stopper and the light yellow reaction mixture was refluxed overnight to ensure completion of the reaction. Excess Alpine-Borane® reagent was quenched by the addition of acetaldehyde (5 mL) and the mixture was stirred for additional 30 min. After replacing the glass stopper was replaced with a one-piece distillation apparatus, THF and the α -pinene byproduct was removed under vacuum (at 15 mm Hg and 0.02 mm Hg, respectively). Particularly, to remove α -pinene, slight warming (40-45 °C) of the reaction flask was required. The residue was taken up in anhydrous Et₂O (150 mL) whilst maintaining an inert atmosphere. The solution was cooled to 0 °C and ethanolamine (3.6 mL, 59.8 mmol) was added dropwise to produce a thick white precipitate that was removed by filtration through celite (grade 521). The precipitate was rinsed with additional portions of ether (2 x 50 mL) and the combined organic phase was treated in the normal manner to yield the crude compound that was purified by column chromatography on silica gel using a gradient eluent system of EtOAc:hexane (5 %, 10 %, 15 %, 20 % and pure EtOAc, respectively). The title compound was obtained as a faint yellowish clear oil (4.71 g, 75

%). bp 85-87 °C at 0.1 mm Hg; R_f 0.26 (EtOAc:hexane, 1:1); IR (Neat): ν 3416 (O-H), 2981, 2932, 2858 (C-H), 2145 (C-D), 1737 (C=O), 1465, 1374, 1254, 1183, 1104, 1034, 941 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.11 (*q*, $J = 7.1$ Hz, 2 H, OCH_2Me), 3.59 (*tt*, $J = 6.5, 1.4$ Hz, 1 H, CHDOH), 2.27 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Et}$), 1.55 (*s*, 1 H, OH), 1.61 (*tt*, $J = 7.5, 7.3$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Et}$), 1.54 (*td*, $J = 6.6, 6.5$ Hz, 2 H, CH_2CHDOH), 1.40-1.28 (*m*, 6 H, C-4, C-5, C-6), 1.24 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C NMR (100 MHz, CDCl_3) δ_{C} 173.86 (C-1), 34.30 (C-2), 24.84 (C-3), 29.01 (C-4), 29.04 (δ -shift - 0.008, C-5), 25.50 (γ -shift - 0.028, C-6), 32.55 (β -shift - 0.118, C-7), 62.50 (*t*, $J_{\text{CD}} = 21.7$ Hz, α -shift - 0.403, C-8), 60.17 (- OCH_2Me), 14.22 (CH_3); TMS Derivative EI-MS: m/z 260 ($[\text{M}-1]^+$), 246 ($[\text{M}-15]^+$), 216 ($[\text{M}-45]^+$), 200 ($[\text{M}-61]^+$), 173 ($[\text{M}-88]^+$), 158, 141, 130, 116, 102, 88, 75, 73, 55, 43.

6.2.1.5 8-Hydroxyoctanoic acid

A 2.0 M solution of KOH (30 mL, 60 mmol) in 1:1 EtOH- H_2O [prepared by dissolving 5.6 g of KOH in 44 mL of 1:1 EtOH- H_2O] was added to a stirred solution of ethyl 8-hydroxyoctanoate (1.32 g, 7.0 mmol) in absolute EtOH (20 mL) at room temperature. After stirring at room temperature for 10 min, the reaction mixture was refluxed for 50 minutes at 80 °C after which the EtOH was removed in vacuo. The residue was dissolved in $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$ (40:20 mL) and the aqueous layer was acidified to pH 2 using 2.0 M HCl. The organic layer was separated and the aqueous layer washed with CH_2Cl_2 (3 x 25 mL). The combined organic layers were worked up in the usual way

to furnish the desired compound as a white amorphous solid (702 mg, 62 %). mp 59-61 °C; R_f 0.32 (EtOAc:hexane, 19:1); IR (KBr): ν 3248 (O-H), 3500-2600br (CO₂H), 2937, 2858 (C-H), 1709 (C=O), 1467, 1296, 1095, 1054, 907 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 6.48-4.38 (*br s*, 2 H, OH, CO₂H), 3.65 (*t*, J = 6.0 Hz, 2 H, CH₂OH), 2.35 (*t*, J = 7.5 Hz, 2 H, CH₂CO₂H), 1.65 (*tt*, J = 7.5, 7.5 Hz, 2 H, CH₂CH₂CO₂H), 1.57 (*tt*, J = 7.5, 6.0 Hz, 2 H, CH₂CH₂OH), 1.36 (*s-m*, 6 H, C-4, C-5, C-6); ¹³C NMR (75 MHz, CDCl₃) δ_C 179.21 (C-1), 33.92 (C-2), 24.59 (C-3), 28.98 (C-4), 28.98 (C-5), 25.50 (C-6), 32.60 (C-7), 62.96 (C-8); bis-TMS Derivative EI-MS: m/z 303 ([M-1]⁺), 289 ([M-15]⁺), 273 ([M-31]⁺), 245, 217, 199, 185, 173, 157, 147, 129, 117, 107, 97, 83, 75, 55, 45.

6.2.1.6 (8*S*)-8-Hydroxy-[8-²H₁]-octanoic acid

The title compound was prepared from ethyl (8*S*)-8-hydroxy-[8-²H₁] octanoate (4.49 g, 23.7 mmol) and KOH (2.0 M in 1:1 EtOH-H₂O, 102.2 mL, 0.2 mol) as reagent using the same experimental protocol that used for the synthesis of 8-hydroxyoctanoic acid. The anticipated compound was obtained as a white amorphous solid (3.27 g, 86 %). mp 60-61 °C; R_f 0.73 (MeOH:CH₂Cl₂, 1:19); IR (KBr): ν 3393 (O-H), 3600-2600br (CO₂H), 3019, 2934, 2859 (C-H), 2156 (C-D), 1710 (C=O), 1465, 1412, 1216, 1105, 1064, 937 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 8.87 (*br s*, 2 H, OH, CO₂H), 3.61 (*t*, J = 6.4 Hz, 1 H, CHDOH), 2.37 (*t*, J = 7.5 Hz, 2 H, CH₂CO₂H), 1.62 (*tt*, J = 7.5, 7.2 Hz, 2 H, CH₂CH₂CO₂H), 1.54 (*td*, J = 6.4, 5.8 Hz, 2 H, CH₂CHDOH), 1.39-1.29 (*m*, 6 H, C-4, C-5, C-6); ¹³C NMR (75 MHz, CDCl₃) δ_C 179.14 (C-1), 33.98 (C-2), 24.59 (C-3), 28.95 (C-

4), 28.95 (δ -shift - 0.033, C-5), 25.44 (γ -shift - 0.059, C-6), 32.39 (β -shift - 0.215, C-7), 62.48 (*t*, J_{CD} = 20.3 Hz, α -shift - 0.485, C-8); TMS Derivative EI-MS: m/z 304 ($[M-1]^+$), 290 ($[M-15]^+$), 274 ($[M-31]^+$), 246, 217, 200, 186, 174, 158, 147, 129, 117, 108, 98, 84, 75, 55, 45.

6.2.1.7 8-(*p*-Tolylsulfonyloxy)-octanoic acid

To a stirred solution of 8-hydroxyoctanoic acid (408 mg, 2.6 mmol) in anhydrous pyridine (4 mL) was added dropwise, *p*-toluenesulfonyl chloride (778 mg, 4.1 mmol) in anhydrous pyridine (2 mL) at 0 °C. After stirring at 0 °C - 4 °C for 5 h, the reaction mixture was poured into H₂O (15 mL). The aqueous layer was extracted with Et₂O (4 x 35 mL) and the combined organic layer was washed successively with 1.0 M HCl (3 x 30 mL), water (40 mL) and brine (40 mL). The organic layer was treated in the normal manner to yield the title compound as a colorless oil (655 mg, 82 %). R_f 0.60 (EtOAc:hexane, 3:2); IR (film): ν 3500-2600br (O-H), 3029, 2936, 2861 (C-H), 1709 (C=O), 1599 (C=C, Ar), 1465, 1359, 1189, 1177, 1098, 914, 815 cm⁻¹; (¹H NMR (300 MHz, CDCl₃) δ_H 10.58 (*br s*, 1 H, CO₂H), 4.02 (*t*, J = 6.0 Hz, 2 H, CH₂OTs), 2.33 (*t*, J = 7.5 Hz, 2 H, CH₂CO₂H), 1.62 (Overlapping *tt*, J = 7.5, 6.0 Hz, 4 H, CH₂CH₂OTs, CH₂CH₂CO₂H), 1.40-1.21 (*m*, 6 H, C-4, C-5, C-6), 2.46 (*s*, 3 H, CH₃Ar), 7.79 (*d*, J = 9.0 Hz, 2 H, C_o-Ph), 7.35 (*d*, J = 9.0 Hz, 2 H, C_m - Ph); ¹³C NMR (75 MHz, CDCl₃) δ_C 178.72 (C-1), 33.71 (C-2), 24.48 (C-3), 28.75 (C-4), 28.77 (C-5), 25.16 (C-6), 28.55 (C-7), 70.54 (C-8), 144.69 (C_{ipso} - Ph), 127.91 (C_o - Ph), 129.84 (C_m - Ph), 133.20 (C_p -

Ph), 21.66 (CH₃ - Ph); EI-MS: m/z 296 (- H₂O, [M-18]⁺), 281, 232, 207, 173, 155, 125, 107, 91, 73, 65, 41.

6.2.1.8 (8*S*)-8-(*p*-Tolylsulfonyloxy)-[8-²H₁]-octanoic acid

To a mixture of (8*S*)-8-hydroxy-[8-²H₁]-octanoic acid (4.25 g, 26.4 mmol) and *p*-toluenesulfonyl chloride (9.06 g, 47.5 mmol) in dry CH₂Cl₂ (55 mL) was added anhydrous pyridine (5.4 mL, 66 mmol) at room temperature and the reaction was stirred for 24 h. The mixture was poured into a saturated solution of NH₄Cl (60 mL) and the organic layer was separated after dilution with CH₂Cl₂ (35 mL). The organic layer was then washed with saturated NH₄Cl (3 x 60 mL), saturated NaCl (2 x 60 mL) and dried over anhydrous Na₂SO₄. The residue obtained after the removal of the solvent was diluted again with CH₂Cl₂ (50 mL) and washed with H₂O (3 x 50 mL), brine (50 mL) to decompose and remove remaining excess of *p*-toluenesulfonyl chloride. The title compound was obtained as a pale yellowish oil (6.53 g, 78 %). R_f 0.11 (EtOAc:hexane, 3:2); IR (film): ν 3500-2700br (O-H), 3028, 2935, 2862 (C-H), 2194 (C-D), 1709 (C=O), 1596 (C=C, Ar), 1455, 1367, 1189, 1176, 1098, 941, 814 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 7.79 (*d*, J = 7.5 Hz, 2 H, C₂ - Ph), 7.35 (*d*, J = 7.5 Hz, 2 H, C₄ - Ph), 4.00 (*t*, J = 6.3 Hz, 1 H, CHDOTs), 2.45 (*s*, 3 H, CH₃Ar), 2.33 (*t*, J = 7.5 Hz, 2 H, CH₂CO₂H), 1.62 (*m*, 4 H, CH₂CHDOTs, CH₂CH₂CO₂H), 1.39-1.22 (*m*, 6 H, C-4, C-5, C-6); ¹³C NMR (75 MHz, CDCl₃) δ_C 177.53 (C-1), 34.10 (C-2), 24.71 (C-3), 28.61 (C-4), 28.85 (δ -

shift 0.081, C-5), 25.11 (γ -shift - 0.045, C-6), 28.58 (β -shift 0.029, C-7), 70.25 (t , J_{CD} = 22.8 Hz, α -shift - 0.283, C-8), 144.65 (*Cipso* - Ph), 127.86 (C_Q - Ph), 129.81 (C_m - Ph), 133.16 (C_P - Ph), 21.62 (CH_3 - Ph) ; EI-MS: m/z 297 (- H_2O , $[M-18]^+$), 281, 233, 207, 173, 155, 126, 107, 91, 73, 65, 41.

6.2.1.9 Dec-9-ynoic acid

A solution of 8-(*p*-tolylsulfonyloxy)-octanoic acid (398 mg, 1.3 mmol) in anhydrous DMSO (4 mL) was added dropwise to a stirred suspension of lithium acetylide-ethylenediamine complex (466 mg, 5.1 mmol) in DMSO (8 mL) at room temperature and the reaction mixture was stirred at room temperature for 3 h. Excess reagent was quenched by pouring the mixture on to a chilled ice-1.0 M HCl mixture (40 mL) and the acidity was adjusted to pH 2 using 1.0 M HCl. The aqueous layer was extracted with diethyl ether (3 x 30 mL) and the combined organic phase was worked up in the usual way generated the crude compound as an oil (202 mg, 95%) and used further without purification. mp 4-6 °C; R_f 0.35 (MeOH: CH_2Cl_2 , 1:19); IR (film): ν 3302 (terminal $HC\equiv C$), 3400-2600br (O-H), 3035, 2935, 2859 (C-H), 2118 ($C\equiv C$), 1710 (C=O), 1463, 1431, 1413, 1287, 1250, 938 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 11.16 (*br s*, 1 H, CO_2H), 2.35 (*t*, J = 7.5 Hz, 2 H, CH_2CO_2H), 2.17 (*td*, J = 6.9, 2.6 Hz, 2 H, $CH_2C\equiv C$), 1.93 (*t*, J = 2.6 Hz, 1 H, $HC\equiv C$), 1.63 (*tt*, J = 7.5, 7.2 Hz, 2 H, $CH_2CH_2CO_2H$), 1.52 (*tt*, J = 7.1, 6.9 Hz, 2 H, $CH_2CH_2C\equiv C$), 1.46-1.24 (*m*, 6 H, C-4,

C-5, C-6); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 180.44 (C-1), 34.05 (C-2), 24.57 (C-3), 28.88 (C-4), 28.49 (C-5), 28.36 (C-6), 28.70 (C-7), 18.34 (C-8), 84.60 (C-9), 68.17 (C-10); EI-MS: m/z 149 ($[\text{M}-19]^+$), 135 ($[\text{M}-33]^+$), 126 ($[\text{M}-42]^+$), 121 ($[\text{M}-47]^+$), 108 ($[\text{M}-60]^+$), 93 ($[\text{M}-75]^+$), 81, 67, 60, 55, 41.

6.2.1.10 (8*R*)-[8- $^2\text{H}_1$]-Dec-9-ynoic acid

Same experimental procedure has been used for the synthesis of the title compound from (8*S*)-8-(*p*-Tolylsulfonyloxy)-[8- $^2\text{H}_1$]-octanoic acid (4.476 g, 14.203 mmol) and lithium acetylide-ethylenediamine complex (5.231 g, 56.812 mmol) as followed to synthesize dec-9-ynoic acid. The desired compound was furnished as a pale yellowish clear liquid that solidifies at 0 °C (1.87 g, 78 %). mp 4-6 °C; R_f 0.34 (MeOH: CH_2Cl_2 , 1:19); IR (film): ν 3308 (terminal $\text{HC}\equiv\text{C}$), 3500-2700br (O-H), 2933, 2860 (C-H), 2157 (C-D), 2117 ($\text{C}\equiv\text{C}$), 1710 (C=O), 1551, 1463, 1412, 1310, 1224, 758 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 8.29 (*br s*, 1 H, CO_2H), 2.33 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{H}$), 2.15 (*dt*, $J = 7.6, 2.6$ Hz, 1 H, $\text{CHDC}\equiv\text{C}$), 1.93 (*d*, $J = 2.6$ Hz, 1 H, $\text{HC}\equiv\text{C}$), 1.63 (*tt*, $J = 7.5, 7.2$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.51 (*td*, $J = 7.6, 6.3$ Hz, 2 H, CH_2CHD), 1.45-1.24 (*m*, 6 H, C-4, C-5, C-6); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 179.39 (C-1), 34.00 (C-2), 24.61 (C-3), 28.88 (C-4), 28.45 (δ -shift - 0.039, C-5), 28.24 (γ -shift - 0.115, C-6), 28.69 (β -shift - 0.005, C-7), 18.04 (*t*, $J_{\text{CD}} = 19.9$ Hz, α -shift - 0.305, C-8), 84.58 (β -shift - 0.012, C-9), 68.17 (γ -shift 0.003, C-10); EI-MS: m/z 169 ($[\text{M}]^+$), 150 ($[\text{M}$ -

19]⁺), 136 ([M-33]⁺), 126 ([M-42]⁺), 122 ([M-47]⁺), 109 ([M-60]⁺), 94 ([M-75]⁺), 82, 68, 60, 55, 44, 41.

6.2.1.11 Methyl dec-9-ynoate

Dec-9-ynoic acid (1.44 g, 5.1 mmol) was dissolved in 3:2 toluene/methanol (18 mL) and treated with (trimethylsilyl) diazomethane (2.0 M in hexanes, 2.6 mL, 5.1 mmol). When the evolution of gas from the reaction mixture was estimated to be complete, an additional few drops of reagent was added until a light yellow color persisted. Excess reagent was destroyed by the addition of a few drops of AcOH. The solvent was removed in vacuo and the crude methyl ester was purified by flash chromatography using Et₂O:hexane (1:49) as eluent to afford the title compound as a colorless oil (1.33 g, 88 %). *R_f* 0.38 (EtOAc:hexane, 1:9); IR (film): ν 3298 (terminal HC $\equiv$ C), 2935, 2859 (C-H), 2117 (C $\equiv$ C), 1741 (C=O), 1461, 1437, 1247, 1198, 1173, 1098, 1016 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 3.65 (*s*, 3 H, OCH₃), 2.29 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.16 (*td*, *J* = 7.0, 2.6 Hz, 2 H, CH₂C $\equiv$ C), 1.92 (*t*, *J* = 2.6 Hz, 1 H, HC $\equiv$ CCH₂), 1.61 (*tt*, *J* = 7.5, 7.3 Hz, 2 H, CH₂CH₂CO₂Me), 1.51 (*tt*, *J* = 7.2, 7.0 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.45-1.23 (*m*, 6 H, C-4, C-5, C-6); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 174.30 (C-1), 34.07 (C-2), 24.88 (C-3), 28.99 (C-4), 28.53 (C-5), 28.38 (C-6), 28.73 (C-7), 18.37 (C-8), 84.66 (C-9), 68.14 (C-10), 51.48 (-OMe); EI-MS: *m/z* 181 ([M-1]⁺), 167 ([M-15]⁺), 151 ([M-31]⁺), 143 ([M-39]⁺), 135 ([M-47]⁺), 127, 122, 108, 93, 87, 81, 74, 67, 55, 41.

6.2.1.12 Methyl (8*R*)-[8-²H₁]-dec-9-ynoate

The title compound was synthesized from (8*R*)-[8-²H₁]-dec-9-ynoic acid (1.77 g, 23.7 mmol) using the same experimental protocol used for the preparation of methyl dec-9-ynoate. The title compound was obtained as a faint yellowish oil (863 mg, 45 %). *R_f* 0.28 (Pure CH₂Cl₂); IR (film): ν 3296 (terminal HC $\equiv$ C), 2934, 2859 (C-H), 2145 (C-D), 2117 (C $\equiv$ C), 1740 (C=O), 1462, 1437, 1252, 1200, 1173, 1104, 1016, 847 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 3.66 (*s*, 3 H, OCH₃), 2.30 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.15 (*m*, 1 H, CHDC $\equiv$ C), 1.92 (*d*, *J* = 2.6 Hz, 1 H, HC $\equiv$ C), 1.62 (*tt*, *J* = 7.5, 7.4 Hz, 2 H, CH₂CH₂CO₂Me), 1.51 (*td*, *J* = 7.0, 6.8 Hz, 2 H, CH₂CHD), 1.45-1.26 (*m*, 6 H, C-4, C-5, C-6); ¹³C NMR (75 MHz, CDCl₃) δ _C 174.22 (C-1), 34.03 (C-2), 24.86 (C-3), 28.97 (C-4), 28.48 (δ -shift - 0.053, C-5), 28.27 (γ -shift - 0.118, C-6), 28.71 (β -shift - 0.020, C-7), 18.05 (*t*, *J*_{CD} = 20.0 Hz, α -shift - 0.314, C-8), 84.59 (β -shift - 0.062, C-9), 68.14 (γ -shift - 0.005, C-10), 51.43 (-OMe); EI-MS (rel. intensity): *m/z* 182 ([M-1]⁺), 168 (1, [M-15]⁺), 152 (4, [M-31]⁺), 143 (2, [M-40]⁺), 136 (6, [M-47]⁺), 128 (2), 123 (12), 109 (55), 94 (38), 87 (31), 82 (52), 74 (100), 68 (41), 55 (82), 41 (73).

6.2.1.13 1-(*p*-Tolylsulfonyloxy)-oct-2-yne¹⁷³

To a stirred solution of 2-octyn-1-ol (500 mg, 4.0 mmol) and freshly prepared *p*-toluenesulfonyl chloride (832 mg, 4.4 mmol) in anhydrous Et₂O (12 mL) was added powdered KOH (1.92 g, 34.1 mmol) at - 50 °C. The reaction mixture was stirred at 0 °C for 2 h and then poured into H₂O (35 mL). The organic layer was separated and the aqueous layer was extracted with Et₂O (3 x 25 mL) and the combined organic phase washed successively with water (30 mL), brine (2 x 30 mL) and filtered through anhydrous Na₂SO₄. The solvent was evaporated in vacuo and concentrated to afford the crude compound as a pale yellowish oil (959 mg, 86 %). *R*_f 0.21 (EtOAc:hexane, 1:4); The spectral data of the compound was similar to that reported in the literature.⁶ IR (film): ν 2957, 2933, 2862 (C-H), 2310, 2238 (C $\equiv$ C), 1599 (C=C, Ar), 1456, 1369, 1190, 1177, 1097, 942, 838, 815 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 7.81 (*d*, *J* = 8.2 Hz, 2 H, C_Q - Ph), 7.34 (*d*, *J* = 8.2 Hz, 2 H, C_m - Ph), 4.70 (*t*, *J* = 2.2 Hz, 2 H, CH₂OTs), 2.44 (*s*, 3 H, CH₃Ar), 2.06 (*tt*, *J* = 7.0, 2.2 Hz, 2 H, CH₂C $\equiv$ C), 1.38 (*tt*, *J* = 7.0, 6.9 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.32-1.22 (*m*, 4 H, 2 x CH₂), 0.88 (*t*, *J* = 6.8 Hz, 3 H, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ _C 58.80 (C-1), 71.76 (C-2), 90.61 (C-3), 18.60 (C-4), 27.73 (C-5), 30.89 (C-6), 22.09 (C-7), 13.88 (C-8), 144.80 (C_{ipso} - Ph), 128.09 (C_Q - Ph), 129.70 (C_m - Ph), 133.43 (C_p - Ph), 21.61 (CH₃ - Ph); EI-MS: *m/z* 239 ([M-41]⁺), 225, 201, 173, 155 ([M-125]⁺), 139 ([M-141]⁺), 125, 117, 107, 91, 79, 65, 55, 41.

6.2.1.14 Methyl octadeca-9,12-diynoate

The following components were dissolved in anhydrous DMF (10 mL) at -25°C : methyl dec-9-ynoate (500 mg, 2.7 mmol); K_2CO_3 (378 mg, 2.7 mmol); CuI (520 mg, 2.7 mmol); and NaI (410 mg, 2.7 mmol). After stirring the reaction mixture for 15 minutes at -25°C , a solution of 1-(*p*-tolylsulfonyloxy) oct-2-yne (766 mg, 2.7 mmol) in anhydrous DMF (10 mL) was added and stirred was continued for another 20 minutes at -20°C and a further 24 h at room temperature. The reaction was terminated by pouring the mixture on to a saturated solution of aqueous NH_4Cl (50 mL) and the aqueous layer was extracted with Et_2O (4 x 30 mL). The combined organic layers were worked up in the normal manner to afford a residue that was diluted again with hexanes (30 mL) and washed with H_2O (3 x 25 mL), brine (3 x 25 mL) and finally dried over anhydrous Na_2SO_4 . The solvent was removed in vacuo and the crude compound was purified by flash chromatography using Et_2O :hexane (1:19) as eluent to give the title compound as a pale yellowish oil (580 mg, 73 %). R_f 0.31 (EtOAc :hexane, 1:9); IR (film): ν 2933, 2859 (C-H), 2210 ($\text{C}\equiv\text{C}$), 1738 (C=O), 1459, 1437, 1248, 1200, 1173, 1017, 737 cm^{-1} ; ^1H NMR

(300 MHz, CDCl_3) δ_{H} 3.66 (*s*, 3 H, OCH_3), 3.11 (*tt*, $J = 2.3, 2.3$ Hz, 2 H, C-11), 2.30 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Me}$), 2.15 (Overlapping *tt*, $J = 7.2, 2.3$ Hz, 4 H, C-8, C-14), 1.62 (*tt*, $J = 7.5, 7.2$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$), 1.55-1.42 (*m*, 4 H, C-7, C-15), 1.43-1.23 (*m*, 10 H, 5 $\times$ CH_2), 0.89 (*t*, $J = 7.0$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 174.28 (C-1), 34.07 (C-2), 24.90 (C-3), 28.76 (C-4), 28.65 (C-5), 28.65 (C-6), 29.01 (C-7), 18.71 (C-8), 80.37 (C-9), 74.58 (C-10), 9.72 (C-11), 74.44 (C-12), 80.53 (C-13), 18.71 (C-14), 28.47 (C-15), 31.08 (C-16), 22.22 (C-17), 13.99 (C-18), 51.45 (-OMe); EI-MS: (rel. intensity) m/z 259 (1, $[\text{M}-31]^+$), 201 (2, $[\text{M}-89]^+$), 189 (1, $[\text{M}-101]^+$), 173 (3), 159 (5), 148 (13), 133 (33), 119 (58), 105 (90), 91 (100), 79 (45), 67 (38), 55 (39), 41 (42).

6.2.1.15 Methyl (8*R*)-[8- $^2\text{H}_1$] octadeca-9,12-diynoate

The following components were dissolved in anhydrous DMF (10 mL) at -25°C : methyl (8*R*)-[8- $^2\text{H}_1$] dec-9-ynoate (500 mg, 2.7 mmol); K_2CO_3 (378 mg, 2.7 mmol); CuI (520 mg, 2.7 mmol); and NaI (410 mg, 2.7 mmol). After stirring the reaction mixture for 15 minutes at -25°C , a solution of 1-(*p*-tolylsulfonyloxy)-oct-2-yne (766 mg, 2.7 mmol)

Lindlar catalyst (16 mg) was suspended in a solution of methyl octadeca-9,12-dienoate (50 mg, 0.2 mmol) in hexane (8 mL) containing 0.00624 % quinoline (0.4 μ L). The reaction flask was attached to a hydrogenation apparatus and the system was evacuated and flushed with H₂ three times. The hydrogenation apparatus was then filled with H₂ and the reaction was stirred at 1 atmosphere pressure for 6 h. The catalyst was removed by filtration and washed with hexane (20 mL). The solvent was removed *in vacuo* to afford the crude compound that was purified by flash chromatography using Et₂O:hexane (1:19) as eluent and the final product was resulted as faint yellowish clear oil (49 mg, 96 %). *R_f* 0.44 (EtOAc:hexane, 1:9); and the spectral data of this compound was similar to that reported in the literature for methyl linoleate.¹⁹²⁻¹⁹⁴ IR (film): ν 2932, 2859 (C-H), 2233, 2211, 1739 (C=O), 1596 (C=C), 1459, 1437, 1248, 1198, 1173, 1018, 727 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 5.45-5.27 (*m*, 4 H, 4 x HC=C), 3.67 (*s*, 3 H, OCH₃), 2.77 (*t*, *J* = 5.8 Hz, 2 H, C-11), 2.30 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.05 (*m*, 4 H, 2 x CH₂C=C), 1.62 (*tt*, *J* = 7.5, 7.1 Hz, 2 H, CH₂CH₂CO₂Me), 1.41-1.22 (*m*, 14 H, 7 x CH₂), 0.89 (*t*, *J* = 6.8 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 174.32 (C-1), 34.11 (C-2), 24.96 (C-3), 29.11 (C-4), 29.17 (C-5), 29.13 (C-6), 29.60 (C-7), 27.20 (C-8), 130.06 (C-9), 128.06 (C-10), 25.64 (C-11), 127.92 (C-12), 130.23 (C-13), 27.22 (C-14), 29.36 (C-15), 31.54 (C-16), 22.59 (C-17), 14.08 (C-18), 51.45 (-OMe); EI-MS: *m/z* 294 ([M]⁺), 263 ([M-31]⁺), 220 ([M-74]⁺), 178, 164, 150, 123, 109, 95, 81, 67, 55.

6.2.1.17 Methyl (8*R*)-[8-²H₁]-octadeca-9*Z*,12*Z*-dienoate

Lindlar catalyst (50 mg) was suspended in a solution of methyl (8*R*)-[8-²H₁] octadeca-9,12-dienoate (178 mg, 0.6 mmol) in hexane (16 mL) containing 0.00624 % quinoline (0.9 μL). The reaction flask was attached to a hydrogenation apparatus and the system was evacuated and flushed with H₂ three times. The hydrogenation apparatus was then filled with H₂ and the reaction was stirred at 1 atmosphere pressure for 6 h. The catalyst was removed by filtration and washed with hexane (30 mL). The solvent was removed *in vacuo* to afford the crude compound that was purified by flash chromatography using Et₂O:hexane (1:19) as eluent and the final product was obtained as a faint yellowish clear oil (180 mg, 99.8 %). *R_f* 0.47 (EtOAc:hexane, 1:9); IR (film): ν 3010, 2955, 2928, 2857 (C-H), 2146 (C-D), 1743 (C=O), 1463, 1436, 1363, 1272, 1171, 1073, 728 cm^{-1} ; ¹H NMR (300 MHz, CDCl₃) δ_{H} 5.44-5.28 (*m*, 4 H, 4 x HC=C), 3.67 (*s*, 3 H, OCH₃), 2.77 (*t*, *J* = 6.0, Hz, 2 H, C-11), 2.30 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.05 (*m*, 3 H, C-8, C-14), 1.62 (*m*, 2 H, CH₂CH₂CO₂Me), 1.42-1.23 (*m*, 14 H, 7 x CH₂), 0.89 (*t*, *J* = 6.8 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_{C} 174.29 (C-1), 34.11 (C-2),

24.96 (C-3), 29.08 (C-4), 29.17 (δ -shift 0.006, C-5), 29.13 (γ -shift 0.002, C-6), 29.51 (β -shift - 0.093, C-7), 26.84 (*t*, $J_{\text{CD}} = 19.2$ Hz, α -shift - 0.362, C-8), 130.01 (β -shift - 0.051, C-9), 128.08 (γ -shift 0.019, C-10), 25.64 (δ -shift 0.003, C-11), 127.93 (C-12), 130.22 (C-13), 27.22 (C-14), 29.36 (C-15), 31.54 (C-16), 22.58 (C-17), 14.07 (C-18), 51.43 (-OMe); EI-MS: m/z 295 ($[\text{M}]^+$), 280 ($[\text{M}-15]^+$), 264 ($[\text{M}-31]^+$), 235, 221 ($[\text{M}-74]^+$), 207, 192, 179, 164, 150, 136, 124, 110, 96, 81, 67, 55, 41.

6.2.1.18 Methyl (8*S*)-[8- $^2\text{H}_1$]-octadeca-9*Z*,12*Z*-dienoate

The title compound was synthesized in 3.8 % overall yield from diethyl suberate using the same procedures as described for the synthesis of the corresponding (*R*)-enantiomer. The chiral starting material was prepared by the reduction of ethyl 8-oxo-[8- $^2\text{H}_1$]-octanoate using a 0.5 M solution of (-)-(*S*)-Alpine-Borane in THF. R_f 0.47 (EtOAc:hexane, 1:9); The analytical data for the title compound was essentially identical to that obtained for the corresponding (*R*)-enantiomer and similar to that reported above with the non-deuterio parent compound (see Section 6.2.1.16). IR (film): ν 3010, 2927, 2856 (C-H), 2147 (C-D), 1743 (C=O), 1640 (C=C), 1462, 1436, 1363, 1251, 1199, 1172, 1016, 911, 727 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 5.44-5.27 (*m*, 4 H, 4 x HC=C), 3.66 (*s*, 3 H, OCH₃), 2.77 (*t*, $J = 6.0$ Hz, 2 H, C-11), 2.30 (*t*, $J = 7.5$ Hz, 2 H, CH₂CO₂Me), 2.04 (*m*, 3 H, C-8, C-14), 1.62 (*m*, 2 H, CH₂CH₂CO₂Me), 1.41-1.23 (*m*, 14 H, 7 x CH₂), 0.89 (*t*, $J = 6.8$ Hz, 3 H, CH₃); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 174.28 (C-1), 34.10 (C-2), 24.95 (C-3), 29.08 (C-4), 29.17 (δ -shift 0.002, C-5), 29.13 (γ -shift - 0.004, C-6), 29.50

(β -shift - 0.098, C-7), 26.83 (t , J_{CD} = 19.2 Hz, α -shift - 0.368, C-8), 130.00 (β -shift - 0.060, C-9), 128.07 (γ -shift 0.012, C-10), 25.63 (δ -shift 0.004, C-11), 127.92 (C-12), 130.21 (C-13), 27.21 (C-14), 29.36 (C-15), 31.54 (C-16), 22.58 (C-17), 14.07 (C-18), 51.42 (-OMe); EI-MS: m/z 295 ($[M]^+$), 264 ($[M-31]^+$), 235, 221 ($[M-74]^+$), 207, 192, 179, 164, 151, 136, 123, 110, 96, 81, 67, 55, 41.

6.2.2 Attempted synthesis of Chiral $[11-^2H_1]$ -Linoleates

6.2.2.1 Ethyl oct-2-ynoate

A solution of 1-heptyne (24.5 mL, 187.2 mmol) in anhydrous THF (80 mL) was added dropwise during 1 h to a stirred solution of ethylmagnesium bromide (1.0 M in THF, 224.6 mL, 224.6 mmol) in anhydrous THF at 0 to - 5 °C. After the addition, the reaction mixture slowly warmed up to room temperature for 15 min and subsequently refluxed for 1 h during which the preformed precipitate dissolved into the yellowish solution. Afterward, the reaction mixture was cooled to room temperature, then to 0 °C and to that was slowly admitted a solution of ethyl chloroformate (19.6 mL, 205.9 mmol) in anhydrous THF (40 mL) during 1 h. The resultant yellowish reaction mixture was then refluxed for another 1.5 h during which the solution became deep-brownish in color. The reaction terminated by the addition of a saturated solution of aqueous NH_4Cl to the ice-cold reaction mixture. The organic layer was separated in a separatory funnel and the aqueous layer was re-extracted with Et_2O (3 x 150 mL). The collected organic layers

were combined and worked up in a regular fashion to afford the crude compound that was purified by short-path distillation under vacuum. The purified material was isolated as a colorless oil (25.8 g, 82 %). bp 62-64 °C at 0.1 mm Hg; R_f 0.46 (EtOAc:hexane, 1:4); IR (film): ν 2960, 2936, 2873, 2864 (C-H), 2314, 2234 (C $\equiv$ C), 1713 (C=O), 1466, 1367, 1252, 1076, 863, 753 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.21 (*q*, J = 7.2 Hz, 2 H, OCH_2Me), 2.31 (*t*, J = 7.1 Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.58 (*tt*, J = 7.2, 7.1 Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.43-1.25 (*m*, 4 H, 2 x CH_2), 1.30 (*t*, J = 7.2 Hz, 2 H, OCH_2CH_3), 0.89 (*t*, J = 7.0 Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 153.86 (C-1), 73.13 (C-2), 89.43 (C-3), 18.60 (C-4), 27.21 (C-5), 30.95 (C-6), 22.06 (C-7), 14.01 (C-8), 61.71 ($-\text{OCH}_2\text{Me}$), 13.82 ($-\text{OCH}_2\text{CH}_3$); EI-MS: m/z 169 ($[\text{M}+1]^+$), 153 ($[\text{M}-15]^+$), 139 ($[\text{M}-29]^+$), 123 ($[\text{Me}(\text{CH}_2)_4\text{C}\equiv\text{CCO}]^+$, $[\text{M}-45]^+$), 111, 95, 79, 67, 55, 41.

6.2.2.2 Oct-2-yn-1-ol

To a stirred solution of ethyl oct-2-ynoate (6.94 g, 41.3 mmol) in anhydrous THF (50 mL) was slowly added a solution of lithium aluminium hydride in anhydrous THF (2.0 M, 7.4 mL, 14.8 mmol) in portions at 0 °C. After stirring the reaction mixture for 2.5 h at 0 °C (during which the color of the solution changed to light yellow), excess ethyl acetate (4 mL) was added to quench any excess of reducing agent. THF was removed in vacuo and the residue was suspended in ether (50 mL) at 0 °C to which cold water (40 mL) was admitted in small portions. The pH of the solution was adjusted to 1 using 2.0

M HCl and the organic layer was separated using liquid-liquid extraction method. The aqueous layer was re-extracted with ether (3 x 40 mL) and the collected organic layers were combined. The combined organic layers were treated in the normal manner to give the crude compound that was purified by flash chromatography using EtOAc:hexane (1:9) as eluent and the title compound was obtained as a colorless liquid (3.28 g, 63 %). bp 48-50 °C at 0.1 mm Hg; R_f 0.22 (EtOAc:hexane, 1:4); IR (film): ν 3331 (O-H), 2958, 2933, 2861 (C-H), 2289, 2226 (C $\equiv$ C), 1459, 1379, 1229, 1138, 1013, 725 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 4.24 (*t*, J = 2.2 Hz, 2 H, CH₂OH), 2.20 (*tt*, J = 7.2, 2.2 Hz, 2 H, CH₂C $\equiv$ C), 1.97-1.81 (*br s*, 1 H, OH), 1.50 (*tt*, J = 7.2, 7.1 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.42-1.24 (*m*, 4 H, 2 x CH₂), 0.89 (*t*, J = 7.1 Hz, 3 H, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ_C 51.29 (C-1), 78.28 (C-2), 86.50 (C-3), 18.66 (C-4), 28.27 (C-5), 31.01 (C-6), 22.16 (C-7), 13.90 (C-8); EI-MS: m/z 125 ([M-1]⁺), 111 ([M-15]⁺), 95 ([M-31]⁺), 83 ([M-43]⁺), 79, 70, 55, 41.

6.2.2.3 [1,1-²H₂]-oct-2-yn-1-ol

The title compound was prepared using the same experimental protocol as described in 6.2.2.2, from ethyl oct-2-ynoate (23.34 g, 138.9 mmol) and lithium aluminium deuteride (1.0 M in anhydrous THF, 50 mL, 50 mmol) as reagent. The estimated compound was resulted as a colorless liquid (16.18 g, 91 %). bp 48-50 °C at 0.1 mm Hg; R_f 0.22 (EtOAc:hexane, 1:4); IR (film): ν 3335 (O-H), 2958, 2933, 2861 (C-H),

2247, 2206 ($\text{C}\equiv\text{C}$), 2102 (C-D), 1460, 1379, 1331, 1173, 1080, 1043, 965, 830 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 2.20 (*t*, $J = 7.1$ Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.72 (*s*, 1 H, OH), 1.51 (*tt*, $J = 7.2, 7.1$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.43-1.24 (*m*, 4 H, 2 x CH_2), 0.89 (*t*, $J = 7.0$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 50.84 (*p*, $J_{\text{CD}} = 22.5$ Hz, α -shift - 0.343, C-1), 78.21 (β -shift - 0.059, C-2), 86.59 (γ -shift 0.166, C-3), 18.68 (δ -shift 0.039, C-4), 28.29 (C-5), 31.03 (C-6), 22.19 (C-7), 13.94 (C-8); TMS Derivative EI-MS: m/z 185 ($[\text{M}-15]^+$), 167 ($[\text{M}-33]^+$), 153 ($[\text{M}-47]^+$), 143 ($[\text{M}-57]^+$), 129, 117, 109, 96, 83, 75, 59, 41.

6.2.2.4 2-Octynal

To a three-neck round-bottom flask equipped with a magnetic stir bar was charged with oxalyl chloride (0.7 mL, 8 mmol) and subsequently diluted with dry CH_2Cl_2 (12 mL). After cooling the solution to -66°C (external temperature), anhydrous DMSO (1.1 mL, 15.9 mmol) was added to it dropwise leading to a strong exothermic reaction. The solution turned more viscous after the addition of DMSO and the reaction mixture was stirred for an additional 5 min at -66°C . To the reaction mixture was added a solution of oct-2-yn-1-ol (500 mg, 4.0 mmol) in dry CH_2Cl_2 (10 mL) in portions for 10 min and the reaction mixture became cloudy, off-white in color. The reaction mixture was then warmed up to -12°C (external) and stirred for additional 30 min before triethylamine (3.3 mL, 23.8 mmol) was added dropwise. The resultant yellowish reaction mixture was stirred for an additional 10 min before being warmed to room temperature and stirred for

another 20 min. The reaction was terminated by pouring the mixture into water (50 mL) and the organic layer was separated on separatory funnel. The aqueous layer was extracted with dichloromethane (3 x 25 mL) and the collected organic layers were treated with 1.0 M HCl (40 mL), H₂O (40 mL) and brine (50 mL). Filtration through anhydrous Na₂SO₄ followed by removal of the solvent in rota-evaporator gave the crude compound as a yellowish oil (24.6 mg, 5 %). bp 105-110 °C at 15 mm Hg; *R*_f 0.44 (EtOAc:hexane, 1:9); IR (film): ν 2959, 2935, 2863, 2739 (C-H), 2281, 2201 (C $\equiv$ C), 1671 (C=O), 1467, 1388, 1138, 817 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 9.18 (*t*, *J* = 0.3 Hz, 1 H, CHO), 2.41 (*td*, *J* = 7.2, 0.3 Hz, 2 H, CH₂C $\equiv$ C), 1.61 (*tt*, *J* = 7.2, 7.1 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.46-1.23 (*m*, 4 H, 2 x CH₂), 0.91 (*t*, *J* = 7.1 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 177.20 (C-1), 81.61 (C-2), 99.33 (C-3), 19.00 (C-4), 27.15 (C-5), 30.87 (C-6), 22.00 (C-7), 13.76 (C-8); EI-MS (rel. intensity): *m/z* 123 (16, [M-1]⁺), 109 (44, [M-15]⁺), 95 (100, [Me(CH₂)₄C $\equiv$ C]⁺, [M-29]⁺), 91 (24), 81 (73, [M-43]⁺), 79 (34), 67 (84), 55 (46), 51 (22), 41 (74), 29 (44).

6.2.2.5 [1-²H₁]-Oct-2-ynal

The same experimental procedure was followed to oxidize [1,1-²H₂]-oct-2-yn-1-ol (3.05 mg, 23.8 mmol) to the corresponding monodeutrio propargylic aldehyde as described in 6.2.2.4. The reaction resulted in the formation of the expected product as a pale yellowish oil with very poor yield (149 mg, 5 %). Therefore, the Swern oxidation

procedure could not be used to synthesize a large quantity of propargylic aldehyde from the corresponding alcohol. bp 105-108 °C at 15 mm Hg; R_f 0.44 (EtOAc:hexane, 1:9); IR (film): ν 2959, 2934, 2863 (C-H), 2322, 2216 (C $\equiv$ C), 2120 (C-D), 1649 (C=O), 1467, 1328, 1160, 796, 728 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 2.38 (t, J = 7.2 Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.58 (tt, J = 7.2, 7.1 Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.44-1.24 (m, 4 H, 2 x CH_2), 0.88 (t, J = 7.1 Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 176.98 (t, J_{CD} = 29.3 Hz, α -shift - 0.218, C-1), 81.66 (t, $^2J_{\text{CD}}$ = 4.9 Hz, β -shift 0.049, C-2), 99.30 (γ -shift - 0.031, C-3), 19.08 (δ -shift 0.082, C-4), 27.22 (C-5), 30.95 (C-6), 22.08 (C-7), 13.90 (C-8); EI-MS: m/z 124 ($[\text{M}-1]^+$), 123 ($[\text{Me}(\text{CH}_2)_4\text{C}\equiv\text{CCO}]^+$, $[\text{M}-2]^+$), 110 ($[\text{M}-15]^+$), 95 ($[\text{Me}(\text{CH}_2)_4\text{C}\equiv\text{C}]^+$, $[\text{M}-30]^+$), 93, 82 ($[\text{M}-43]^+$), 79, 68, 55, 51, 41.

6.2.2.6 Oct-2-ynal¹⁷³

Oct-2-yn-1-ol (785 mg, 6.2 mmol) and activated MnO_2 (5.89 g, 67.7 mmol) were mixed together in dry CH_2Cl_2 (40 mL) and stirred for 48 h at room temperature. The reaction mixture was filtered through celite on Buchner funnel and the solvent was removed in vacuo to afford the crude compound that was purified by flash chromatography on silica gel using CH_2Cl_2 :hexane (1:19) as eluent. The title compound was obtained as a colorless liquid (270 mg, 35 %). bp 105-110 °C at 15 mm Hg; R_f 0.35 (EtOAc:hexane, 1:19); IR (film): ν 2959, 2935, 2863, 2739 (C-H), 2281, 2201 (C $\equiv$ C), 1671 (C=O), 1467, 1388, 1138, 817 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 9.14 (t, J = 0.3

Hz, 1 H, CHO), 2.38 (*td*, $J = 7.1, 0.3$ Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.57 (*tt*, $J = 7.2, 7.1$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.43-1.22 (*m*, 4 H, 2 x CH_2), 0.88 (*t*, $J = 7.0$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 177.30 (C-1), 81.70 (C-2), 99.43 (C-3), 19.11 (C-4), 27.24 (C-5), 30.96 (C-6), 22.09 (C-7), 13.87 (C-8).

6.2.2.7 [1- $^2\text{H}_1$]-Oct-2-ynal

The title compound was obtained in a similar manner to that described in 6.2.2.6, from [1,1- $^2\text{H}_2$]-oct-2-yn-1-ol (9.43 g, 73.7 mmol) as a colorless oil (3.22 g, 35 %). bp 105-108 °C at 15 mm Hg; R_f 0.44 (EtOAc:hexane, 1:9); IR (film): ν 2958, 2933, 2862 (C-H), 2321, 2216 ($\text{C}\equiv\text{C}$), 2117 (C-D), 1650 (C=O), 1467, 1329, 1160, 1070, 796, 728 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 2.41 (*t*, $J = 7.1$ Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.61 (*tt*, $J = 7.2, 7.1$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.47-1.25 (*m*, 4 H, 2 x CH_2), 0.91 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 177.02 (*t*, $J_{\text{CD}} = 29.3$ Hz, α -shift - 0.177, C-1), 81.67 (*t*, $^2J_{\text{CD}} = 4.9$ Hz, β -shift 0.063, C-2), 99.38 (γ -shift 0.046, C-3), 19.10 (δ -shift 0.107, C-4), 27.24 (C-5), 30.96 (C-6), 22.09 (C-7), 13.86 (C-8); EI-MS: m/z 124 ($[\text{M}-1]^+$), 123 ($[\text{Me}(\text{CH}_2)_4\text{C}\equiv\text{CCO}]^+$, $[\text{M}-2]^+$), 110 ($[\text{M}-15]^+$), 95 ($[\text{Me}(\text{CH}_2)_4\text{C}\equiv\text{C}]^+$, $[\text{M}-30]^+$), 93, 82 ($[\text{M}-43]^+$), 79, 68, 55, 51, 41.

6.2.2.8 2-Nonynal

n-Butyllithium in hexanes (2.5 M in hexanes, 4 mL, 10 mmol) was added dropwise to a stirred solution of 1-octyne (1.12 g, 10 mmol) in anhydrous Et₂O (10 mL) at - 74 °C and allowed to stir for 30 minutes. After slowly admitting [1-²H₁] N,N-dimethylformamide (1.2 mL, 15 mmol) to the reaction mixture, the reaction was warmed up to room temperature and stirred for an additional 30 minutes. Ice-water (15 ml) was added to the reaction mixture and acidified using 3.0 M HCl to pH 3, followed by neutralization with saturated aqueous NaHCO₃ to pH 7. The organic layer was separated and the aqueous phase was extracted repeatedly with ether (4 x 10 ml). Combined organic phases were treated with water (25 mL), brine (2 x 25 mL) and finally dried over anhydrous Na₂SO₄. The solvent was removed initially by short-path distillation at normal atmospheric pressure and then over to the N₂ flash until constant weight was recorded. The anticipated compound was afforded as a yellowish oil (1.25 g, 90 % yield). *R*_f 0.42 (EtOAc:hexanes, 1:19); IR (film): ν 2932, 2860 (C-H), 2281, 2201 (C≡C), 1672 (C=O), 1467, 1387, 1138, 824, 791, 726 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 9.17 (*s*, 1H, CHO), 2.40 (*t*, *J* = 7.1 Hz, 2H, CH₂C≡C), 1.59 (*m*, 2H, CH₂CH₂C≡C), 1.22-1.45 (*m*, 6H, 3 x CH₂), 0.88 (*t*, *J* = 6.8 Hz, 3H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C: 177.21 (C-1), 81.63 (C-2), 99.34 (C-3), 19.06 (C-4), 27.45 (C-5), 28.44 (C-6), 31.13 (C-7), 22.40 (C-8), 13.94 (C-9); EI-MS (rel. intensity): *m/z* 137 (2, [M-1]⁺), 123 (7), 109 (28), 95 (24), 81 (45), 68 (48), 67 (48), 55 (36), 43 (100), 41 (74).

6.2.2.9 [1-²H₁]-Oct-2-ynal

The title compound was synthesized using the same experimental procedure as described in 6.2.2.8, from 1-heptyne (4.15 g, 43.1 mmol) and [1-²H₁] N,N-dimethylformamide (5.0 mL, 64.6 mmol) as a colorless clear oil (4.87 g, 93 %). bp 105-108 °C at 15 mm Hg; *R_f* 0.44 (EtOAc:hexane, 1:9); IR (film): ν 2959, 2934, 2873 (C-H), 2321, 2216 (C $\equiv$ C), 2119 (C-D), 1649 (C=O), 1467, 1328, 1160, 796, 729 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_{H} 2.40 (*t*, *J* = 7.1 Hz, 2 H, CH₂C $\equiv$ C), 1.60 (*tt*, *J* = 7.2, 7.1 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.46-1.25 (*m*, 4 H, 2 x CH₂), 0.90 (*t*, *J* = 7.1 Hz, 3 H, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ_{C} 176.96 (*t*, *J*_{CD} = 29.3 Hz, α -shift - 0.233, C-1), 81.63 (*t*, ²*J*_{CD} = 4.9 Hz, β -shift - 0.024, C-2), 99.30 (γ -shift - 0.032, C-3), 19.06 (δ -shift 0.062, C-4), 27.21 (C-5), 30.93 (C-6), 22.06 (C-7), 13.91 (C-8); EI-MS: *m/z* 124 ([M-1]⁺), 123 ([Me(CH₂)₄C $\equiv$ CCO]⁺, [M-2]⁺), 110 ([M-15]⁺), 95 ([Me(CH₂)₄C $\equiv$ C]⁺, [M-30]⁺), 93, 82 ([M-43]⁺), 79, 68, 55, 51, 41.

6.2.2.10 Oct-2-yn-1-ol

A three-neck round-bottom flask equipped with a reflux condenser, one-piece distillation apparatus, stir bar, and rubber septa was flushed with N₂ and charged with

Oct-2-ynal (504 mg, 4.1 mmol). The reactant was diluted with anhydrous THF (17 mL) and to the stirred solution was added a solution of (+)-(*R*)-Alpine-Borane® in THF (0.5 M, 9 mL, 4.5 mmol) in portions at room temperature. The reaction mixture turned light yellow in color after the completion of addition and was allowed to stir overnight at room temperature. Excess

Borane® reagent was quenched by the addition of excess acetaldehyde (1 mL) and the mixture was stirred for additional 1 h to ensure completion of reaction. The rubber septum was quickly replaced with a glass stopper and the solvent (THF) as well as the byproduct α -pinene was distilled under vacuum (at 15 mm and 0.02 mm Hg, respectively). To remove α -pinene, slight warming (40-45 °C) of the reaction flask was required. The residue was diluted with anhydrous Et₂O (20 mL) whilst maintaining an inert atmosphere and the solution was subsequently cooled to 0 °C. Ethanolamine (0.3 mL, 4.5 mmol) was slowly admitted to the stirred solution leading to the formation of a white precipitate that was removed by filtration through celite on Buchner funnel. The precipitate washed with additional ether (2 x 20 mL) and the collected liquids were combined. The combined organic layer was treated in the usual way to give the crude compound that was purified by Kugelrohr distillation under vacuum and the desired alcohol was obtained as a colorless liquid (369 mg, 72 %). bp 52-55 °C at 0.1 mm Hg; *R*_f 0.23 (EtOAc:hexane, 1:3); IR (film): ν 3339 (O-H), 2957, 2933, 2861 (C-H), 2289, 2226 (C $\equiv$ C), 1459, 1432, 1359, 1137, 1095, 1014, 725 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 4.23 (*t*, *J* = 2.2 Hz, 2 H, CH₂OH), 2.19 (*tt*, *J* = 7.2, 2.2 Hz, 2 H, CH₂C $\equiv$ C), 1.94 (*s*, 1 H, OH), 1.50 (*tt*, *J* = 7.2, 7.1 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.42-1.23 (*m*, 4 H, 2 x CH₂), 0.88 (*t*, *J* = 6.9 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 51.19 (C-1), 78.27 (C-2), 86.42

(C-3), 18.64 (C-4), 28.26 (C-5), 30.99 (C-6), 22.14 (C-7), 13.88 (C-8); TMS Derivative
EI-MS: m/z 197 ($[M-1]^+$), 183 ($[M-15]^+$), 165 ($[M-33]^+$), 153 ($[M-45]^+$), 142 ($[M-56]^+$),
127, 115, 109, 96, 83, 75, 59, 41.

6.2.2.11 (1*R*)-[1-²H₁]-Oct-2-yn-1-ol

The title compound was prepared in a similar manner to that described in
6.2.2.10, from [1-²H₁]-Oct-2-ynal (4.6 g, 36.8 mmol) as a colorless liquid (3.32 g, 71 %).
bp 51-55 °C at 0.1 mm Hg; R_f 0.27 (EtOAc:hexane, 1:3); IR (film): ν 3346 (O-H), 2957,
2932, 2872, 2861 (C-H), 2294, 2230 ($C\equiv C$), 2180, 2134 (C-D), 1467, 1316, 1142, 1031,
946, 825 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_H 4.23 (*tt*, $J = 2.3, 2.1$ Hz, 1 H, CHDOH),
2.20 (*td*, $J = 7.1, 2.1$ Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.75 (*br s*, 1 H, OH), 1.51 (*tt*, $J = 7.2, 7.1$ Hz,
2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.43-1.25 (*m*, 4 H, 2 x CH_2), 0.89 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C
NMR (75.5 MHz, CDCl_3) δ_C 51.16 (*t*, $J_{\text{CD}} = 22.6$ Hz, α -shift - 0.026, C-1), 78.23 (β -shift
- 0.032, C-2), 86.64 (γ -shift 0.219, C-3), 18.70 (δ -shift 0.063, C-4), 28.30 (C-5), 31.04
(C-6), 22.20 (C-7), 13.96 (C-8); EI-MS: m/z 126 ($[M-1]^+$), 112 ($[M-15]^+$), 98 ($[M-29]^+$),
95, 84, 80, 71, 67, 55, 53, 41; TMS Derivative EI-MS: m/z 198 ($[M-1]^+$), 184 ($[M-15]^+$),
166 ($[M-33]^+$), 153 ($[M-46]^+$), 143 ($[M-56]^+$), 128, 116, 109, 96, 83, 75, 59, 41.

6.2.2.12 (1*R*)-1-(*p*-tolylsulfonyloxy)-[1-²H₁] oct-2-yne¹⁷³

The anticipated compound was obtained in a similar manner to that described in **6.2.1.15**, from (1*R*)-[1-²H₁]-Oct-2-yn-1-ol (1.35 g, 10.6 mmol) as a pale yellowish oil (2.67 g, 89 %). *R_f* 0.41 (EtOAc:hexane, 1:4); IR (film): ν 2957, 2933, 2861 (C-H), 2312, 2241 (C $\equiv$ C), 1598 (C=C, Ar), 1458, 1369, 1190, 1178, 1097, 938, 815, 738 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_{H} 7.80 (*d*, *J* = 8.2 Hz, 2 H, C_o - Ph), 7.33 (*d*, *J* = 8.2 Hz, 2 H, C_m - Ph), 4.68 (*tt*, *J* = 2.2, 2.1 Hz, 1 H, CHDOTs), 2.44 (*s*, 3 H, CH₃Ar), 2.05 (*td*, *J* = 7.0, 2.2 Hz, 2 H, CH₂C $\equiv$ C), 1.37 (*tt*, *J* = 7.0, 6.9 Hz, 2 H, CH₂CH₂C $\equiv$ C), 1.31-1.19 (*m*, 4 H, 2 x CH₂), 0.87 (*t*, *J* = 6.8 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_{C} 58.57 (*t*, *J_{CD}* = 23.7 Hz, α -shift - 0.230, C-1), 71.72 (β -shift - 0.042, C-2), 90.60 (γ -shift - 0.014, C-3), 18.60 (δ -shift 0.002, C-4), 27.74 (C-5), 30.90 (C-6), 22.10 (C-7), 13.90 (C-8), 144.80 (C_{ipso} - Ph), 128.09 (C_o - Ph), 129.70 (C_m - Ph), 133.43 (C_p - Ph), 21.62 (CH₃ - Ph); EI-MS: *m/z* 281 (1, [M]⁺), 240 (1, [M-41]⁺), 226 (1), 202 (3), 173 (5, [M-108]⁺), 155 (34, [M-126]⁺), 139 (30, [M-142]⁺), 126 (5), 118 (6), 107 (10), 91 (100), 80 (16), 77 (19), 65 (50), 53 (22), 41 (35).

6.2.2.13 Octadeca-9,12-diynoic acid¹⁷³

Ethylmagnesium bromide in anhydrous THF (1.0 M, 1.8 mL, 1.8 mmol) was added dropwise to a stirred solution of dec-9-ynoic acid (142 mg, 0.8 mmol) in dry THF (3 mL) at 0 °C. Following the addition, the reaction mixture was heated up to 49 °C and stirred for 30 minutes. The reaction mixture was then cooled to room temperature and CuBr.DMS (22 mg) complex was added to it in portions during 15 minutes. The reaction mixture was cooled to 0 °C and to that was added a solution of 1-(*p*-tolylfonyloxy)-Oct-2-yne (260 mg, 0.9 mmol) in dry THF (5 mL). After stirring at 0 °C for 1 h, the reaction was warmed to room temperature and allowed to stir another 3 h. The reaction was quenched by the addition of saturated aqueous solution of NH₄Cl (15 mL) and the suspension was diluted with Et₂O (10 mL). The organic layer was separated by liquid-liquid extraction and the aqueous layer extracted with additional portions of ether (3 x 12 mL). The collected organic layers were combined and treated in the normal manner to afford the crude compound as yellowish semi-solid. The crude compound was purified by column chromatography on silica gel using EtOAc:hexane (1:9 in presence of 0.1 % AcOH) to give the purified anticipated compound as a light yellowish oil that crystallized

at 4 °C (79 mg, 34 %). mp - 16°C; R_f 0.33 (MeOH:CH₂Cl₂, 1:19); IR (film): ν 3500-2700 (O-H), 3035, 2934, 2859 (C-H), 2214, 2117 (C $\equiv$ C), 1711 (C=O), 1460, 1413, 1287, 1247, 937 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 3.12 (*tt*, J = 2.3, 2.3 Hz, 2 H, C-11), 2.35 (*t*, J = 7.3 Hz, 2 H, CH₂CO₂H), 2.28-2.10 (*m*, 4 H, C-8, C-14), 1.64 (*tt*, J = 7.3, 7.0 Hz, 2 H, CH₂CH₂CO₂H), 1.58-1.44 (*m*, 4 H, C-7, C-15), 1.42-1.23 (*m*, 10 H, 5 x CH₂), 0.89 (*t*, J = 7.1 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 180.15 (C-1), 34.02 (C-2), 24.60 (C-3), 28.77 (C-4), 28.65 (C-5), 28.65 (C-6), 28.94 (C-7), 18.72 (C-8), 80.36 (C-9), 74.61 (C-10), 9.73 (C-11), 74.45 (C-12), 80.55 (C-13), 18.72 (C-14), 28.48 (C-15), 31.09 (C-16), 22.23 (C-17), 14.00 (C-18).

6.2.2.14 (11*S*)-[11-²H₁]-octadeca-9,12-diynoic acid

The title compound was obtained in a similar manner to that described in 6.2.2.14, from (1*R*)-1-(*p*-tolylsulfonyloxy)-[1-²H₁]-oct-2-yne (1.11 g, 4.0 mmol) and dec-9-ynoic acid (600 mg, 3.6 mmol) as a faint yellowish oil that solidifies at 4 °C (89 mg, 9 %). mp - 16 °C; R_f 0.17 (EtOAc:hexane, 1:4 in presence of 0.2 % AcOH); IR (film): ν 3600-2600

(O-H), 2933, 2859 (C-H), 2235, 2116 ($\text{C}\equiv\text{C}$), 1710 (C=O), 1458, 1431, 1295, 1162, 929 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 3.10 (*tt*, $J = 2.1, 2.1$ Hz, 1 H, C-11), 2.35 (*t*, $J = 7.4$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{H}$), 2.26-2.15 (*m*, 4 H, C-8, C-14), 1.64 (Overlapping *tt*, $J = 7.4, 6.5$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.58-1.46 (*m*, 4 H, C-7, C-15), 1.45-1.24 (*m*, 10 H, 5 x CH_2), 0.88 (*t*, $J = 6.9$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 179.62 (C-1), 33.99 (C-2), 24.61 (C-3), 28.72 (C-4), 28.64 (C-5), 28.64 (C-6), 28.91 (C-7), 18.70 (δ -shift - 0.018, C-8), 80.36 (γ -shift 0.003, C-9), 74.57 (β -shift - 0.044, C-10), 9.51 (*t*, $J_{\text{CD}} = 20.5$ Hz, α -shift - 0.214, C-11), 74.40 (β -shift - 0.045, C-12), 80.54 (γ -shift - 0.003, C-13), 18.70 (δ -shift - 0.018, C-14), 28.47 (C-15), 31.08 (C-16), 22.22 (C-17), 13.95 (C-18).

6.2.2.15 Methyl octadeca-9,12-diynoate

To a stirred solution of octadeca-9,12-diynoic acid (299 mg, 1.1 mmol) in 3:2 toluene-methanol (10 mL) was added commercially available (trimethylsilyl) diazomethane (2.0 M in hexanes, 0.6 mL, 1.1 mmol) dropwise until evolution of the gas from the reaction mixture was estimated to be complete. Few more drops of the reagent were added further to the reaction mixture until a persistent yellow color appeared and the reaction was stirred for another 5 min. Excess diazomethane was quenched by the addition of few drops of acetic acid. The solvent was removed in vacuo and the crude

compound was purified by flash chromatography using Et₂O:hexane (1:49) as eluent to afford the pure compound as a pale yellowish oil (243 mg, 77 %). *R*_f 0.25 (EtOAc:hexane, 1:32); IR (film): ν 2933, 2859 (C-H), 2212 (C $\equiv$ C), 1740 (C=O), 1460, 1437, 1247, 1198, 1172, 1019, 728 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 3.66 (*s*, 3 H, OCH₃), 3.11 (*tt*, *J* = 2.4, 2.4 Hz, 2 H, C-11), 2.30 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.14 (Overlapping *tt*, *J* = 7.1, 2.3 Hz, 4 H, C-8, C-14), 1.62 (*tt*, *J* = 7.5, 7.2 Hz, 2 H, CH₂CH₂CO₂Me), 1.486 (*m*, 4 H, C-7, C-15), 1.43-1.22 (*m*, 10 H, 5 x CH₂), 0.89 (*t*, *J* = 7.0 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 174.29 (C-1), 34.07 (C-2), 24.89 (C-3), 28.76 (C-4), 28.65 (C-5), 28.65 (C-6), 29.01 (C-7), 18.70 (C-8, C-14), 80.37 (C-9), 74.57 (C-10), 9.72 (C-11), 74.43 (C-12), 80.53 (C-13), 28.46 (C-15), 31.08 (C-16), 22.22 (C-17), 13.99 (C-18), 51.48 (C-19); EI-MS: (rel. intensity) *m/z* 259 (1, [M-31]⁺), 189 (1, [M-101]⁺), 175 (2), 159 (5), 147 (12), 133 (35), 119 (59), 105 (90), 91 (100), 79 (44), 67 (35), 55 (42), 41 (44).

6.2.2.16 Methyl (11*S*)-[11-²H₁] octadeca-9,12-diynoate

The title compound was synthesized using the same experimental procedure to that described in 6.2.2.16, from (11*S*)-[11-²H₁]-octadeca-9,12-diynoic acid (110 mg, 0.4

mmol) as a pale-yellowish oil (92 mg, 79 %). R_f 0.19 (EtOAc:hexane, 1:19); IR (film): ν 2933, 2859 (C-H), 2152 (C-D), 1740 (C=O), 1460, 1436, 1267, 1198, 1173, 1016, 738 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 3.66 (s, 3 H, OCH_3), 3.10 (tt, $J = 2.3, 2.3$ Hz, 1 H, C-11), 2.30 (t, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Me}$), 2.14 (Overlapping td, $J = 7.1, 2.3$ Hz, 4 H, C-8, C-14), 1.62 (tt, $J = 7.5, 7.3$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$), 1.56-1.42 (m, 4 H, C-7, C-15), 1.42-1.23 (m, 10 H, 5 x CH_2), 0.89 (t, $J = 7.0$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 174.25 (C-1), 34.05 (C-2), 24.88 (C-3), 28.75 (C-4), 28.64 (C-5), 28.64 (C-6), 29.00 (C-7), 18.69 (δ -shift - 0.020, C-8, C-14), 80.35 (γ -shift - 0.020, C-9), 74.53 (β -shift - 0.042, C-10), 9.49 (t, $J_{\text{CD}} = 20.6$ Hz, α -shift - 0.231, C-11), 74.40 (β -shift - 0.041, C-12), 80.51 (γ -shift - 0.020, C-13), 28.46 (C-15), 31.07 (C-16), 22.21 (C-17), 13.98 (C-18), 51.43 (-OMe); EI-MS: m/z 260 ($[\text{M}-31]^+$), 248 ($[\text{M}-43]^+$), 235, 218, 202, 188, 174, 160, 146, 134, 120, 106, 92, 79, 59, 41.

6.2.2.17 Methyl (11*S*)-[11- $^2\text{H}_1$] octadeca-9*Z*,12*Z*-dienoate¹⁷³

The title compound was obtained in a similar manner to that described in 6.2.1.20, from methyl (11*S*)-[11- $^2\text{H}_1$]-octadeca-9,12-diynoate (202 mg, 0.7 mmol) as a pale-yellowish clear oil (195 mg, 95 %). R_f 0.44 (EtOAc:hexane, 1:9); The spectral data was

similar to that reported above with the non-deuterio parent compound (see Section 6.2.1.16). IR (film): ν 3009, 2928, 2856 (C-H), 2163 (C-D), 1743 (C=O), 1463, 1436, 1363, 1246, 1197, 1172, 1016, 853 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 5.45-5.28 (*m*, 4 H, 4 x HC=C), 3.67 (*s*, 3 H, OCH_3), 2.75 (*br t*, $J = 6.8$ Hz, 1 H, C-11), 2.30 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Me}$), 2.05 (*m*, 4 H, C-8, C-14), 1.62 (*tt*, $J = 7.5, 7.1$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$), 1.42-1.21 (*m*, 14 H, 7 x CH_2), 0.89 (*t*, $J = 6.7$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 174.32 (C-1), 34.11 (C-2), 24.95 (C-3), 29.11 (C-4), 29.17 (C-5), 29.13 (C-6), 29.60 (C-7), 27.20 (δ -shift 0.001, C-8), 130.07 (γ -shift 0.010, C-9), 127.998 (β -shift - 0.060, C-10), 25.32 (*t*, $J_{\text{CD}} = 19.3$ Hz, α -shift - 0.316, C-11), 127.86 (β -shift - 0.060, C-12), 130.24 (γ -shift 0.009, C-13), 27.22 (δ -shift 0.002, C-14), 29.37 (C-15), 31.54 (C-16), 22.59 (C-17), 14.08 (C-18), 51.45 (-OMe); EI-MS: m/z 295 ($[\text{M}]^+$), 280 ($[\text{M}-15]^+$), 264 ($[\text{M}-31]^+$), 235, 221 ($[\text{M}-74]^+$), 207, 192, 179, 165, 150, 136, 124, 110, 96, 82, 68, 55, 41.

6.2.2.18 Methyl (11*R*)-[11- $^2\text{H}_1$] octadeca-9*Z*,12*Z*-dienoate

The title compound was synthesized in 4 % overall yield from 1-heptyne using the same procedures as described for the synthesis of the corresponding (*S*)-enantiomer. The chiral starting material was prepared by the reduction of [1- $^2\text{H}_1$] 2-octynal using a solution of (+)-(*R*)-Alpine-Borane®. R_f 0.44 (EtOAc:hexane, 1:9); The analytical data for the title compound was essentially identical to that obtained for the corresponding (*S*)-enantiomer and similar to that reported above for the non-deuterio parent compound (see

Section 6.2.1.16. IR (film): ν 3009, 2928, 2856 (C-H), 2158 (C-D), 1743 (C=O), 1463, 1436, 1363, 1246, 1197, 1172, 1016, 852 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 5.45-5.27 (*m*, 4 H, 4 x HC=C), 3.66 (*s*, 3 H, OCH_3), 2.74 (*br t*, $J = 6.6$ Hz, 1 H, C-11), 2.30 (*t*, $J = 7.5$ Hz, 2 H, $\text{CH}_2\text{CO}_2\text{Me}$), 2.04 (*m*, 4 H, C-8, C-14), 1.70-1.54 (*tt*, $J = 7.5, 6.9$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$), 1.41-1.20 (*m*, 14 H, 7 x CH_2), 0.89 (*t*, $J = 6.6$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 174.27 (C-1), 34.08 (C-2), 24.93 (C-3), 29.09 (C-4), 29.15 (C-5), 29.11 (C-6), 29.58 (C-7), 27.18 (δ -shift - 0.024, C-8), 130.03 (γ -shift - 0.029, C-9), 127.97 (β -shift - 0.086, C-10), 25.30 (*t*, $J_{\text{CD}} = 19.3$ Hz, α -shift - 0.339, C-11), 127.83 (β -shift - 0.086, C-12), 130.20 (γ -shift - 0.030, C-13), 27.19 (δ -shift - 0.023, C-14), 29.34 (C-15), 31.52 (C-16), 22.57 (C-17), 14.05 (C-18), 51.41 (-OMe); EI-MS: m/z 295 ($[\text{M}]^+$), 280 ($[\text{M}-15]^+$), 264 ($[\text{M}-31]^+$), 235, 221 ($[\text{M}-74]^+$), 207, 192, 179, 165, 150, 136, 124, 110, 96, 82, 68, 55, 41.

6.2.3 Chiral $[11\text{-}^2\text{H}_1]$ -Linoleates (via Δ^{12} -Desaturation of Chiral $[11\text{-}^2\text{H}_1]$ -Oleates

6.2.3.1 2-(8-Bromooctyl-1-oxy)tetrahydropyran¹⁹⁶

3,4-Dihydro-2H-pyran (2.7 mL, 29.9 mmol) and pyridinium *p*-toluenesulfonate (0.9 g, 15 mol %) was added to a stirred solution of 8-bromooctan-1-ol (5.0 g, 23.9 mmol) in anhydrous CH_2Cl_2 (116 mL) at 0 °C. After stirring the reaction mixture for another 30 minutes at 0 °C, it was slowly warmed up to room temperature and allowed to stir for another 16 h. During stirring, the reaction mixture turned into light brownish in

color. The reaction mixture was poured into a solution of saturated NaHCO_3 (120 mL) and extracted with CH_2Cl_2 (40 mL). The aqueous layer was re-extracted using CH_2Cl_2 (3 x 60 mL) and the combined organic layers were worked up in the normal manner to give a crude THP ether that was purified by flash chromatography using EtOAc:hexane (1:13) as eluent. A pale yellow oil was obtained (7.0 g, 99.8 %). R_f 0.31 (EtOAc:hexane, 1:9); IR (film): ν 2935, 2856 (C-H), 1465, 1454, 1440, 1352, 1200, 1135, 1120, 1078, 1034, 987, 869 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.57 (*t*, J = 3.6 Hz, 1 H, OCHO), 3.92-3.81 (*m*, 1 H, CHO), 3.78-3.67 (*m*, 1 H, CHO), 3.56-3.44 (*m*, 1 H, CHO), 3.44-3.32 (*m*, 1 H, CHO), 3.40 (*t*, J = 6.9 Hz, 2 H, CH_2Br), 1.85 (overlapping *td* and *tt* J = 7.1, 7.1 Hz, 4 H, C-7, C-2'), 1.65-1.46 (*m*, 6 H, 3 x CH_2), 1.46-1.26 (*m*, 8 H, 4 x CH_2); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 62.32 (C-1), 29.67 (C-2), 26.11 (C-3), 28.67 (C-4), 28.08 (C-5), 29.23 (C-6), 32.77 (C-7), 33.92 (C-8), 98.83 (C-1'), 30.76 (C-2'), 19.68 (C-3'), 25.49 (C-4'), 67.56 (C-5').

6.2.3.2 2-(8-Iodo-octyl-1-oxy)tetrahydropyran¹⁹⁶

To a stirred solution of 2-(8-bromooctyl-1-oxy)tetrahydropyran (7.06 g, 24.1 mmol) in dry acetone (250 mL) was added NaI (7.21 g, 48.1 mmol) and the reaction mixture was refluxed for 20 h. A white precipitate, that formed was removed by filtration and the solvent was evaporated. The residue was diluted with CH_2Cl_2 (140 mL), transferred to a separatory funnel and extracted with water (160 mL). The aqueous layer was re-extracted with CH_2Cl_2 (2 x 140 mL) and the organic layers were combined. The

combined organic layers were worked up as usual to afford a crude sample, that was purified by flash chromatography using ethyl acetate-hexane (1:13) as eluent to furnish pure 2-(8-iodooctyl-1-oxy)tetrahydropyran as a pale yellow non-viscous liquid (7.26 g, 89 %). R_f 0.33 (EtOAc:hexane, 1:9); IR (film): ν 2932, 2855 (C-H), 1464, 1453, 1440, 1352, 1200, 1135, 1120, 1078, 1034, 984, 869 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.57 (*t*, $J = 3.5$ Hz, 1 H, OCHO), 3.92-3.81 (*m*, 1 H, CHO), 3.78-3.67 (*m*, 1 H, CHO), 3.56-3.44 (*m*, 1 H, CHO), 3.44-3.32 (*m*, 1 H, CHO), 3.18 (*t*, $J = 7.0$ Hz, 2 H, CH_2I), 1.81 (Overlapping *td* and *tt* = 7.1, 7.1 Hz, 4 H, 2 x CH_2 , C-7, C-2'), 1.66-1.46 (*m*, 6 H, 3 x CH_2), 1.45-1.25 (*m*, 8 H, 4 x CH_2): ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 62.35 (C-1), 30.43 (C-2), 26.14 (C-3), 28.46 (C-4), 29.69 (C-5), 29.23 (C-6), 33.52 (C-7), 7.21 (C-8), 98.85 (C-1'), 30.79 (C-2'), 19.71 (C-3'), 25.51 (C-4'), 67.59 (C-5'); EI-MS: m/z 339 ($[\text{M}-1]^+$), 267 ($[\text{M}-73]^+$), 239 (- OTHP, $[\text{M}-101]^+$), 212, 197, 183, 169, 155, 127, 101, 85 ($[\text{OTHP}]^+$), 69, 55, 41.

6.2.3.3 1-[1,1- $^2\text{H}_2$]-Octanol¹⁷⁹

A 1.0 M solution of lithium aluminium deuteride in anhydrous THF (50 ml, 50 mmol) was added dropwise to a stirred solution of ethyl octanoate (7.83 g, 45.5 mmol) in anhydrous diethyl ether (50 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for another 3 h. Ethyl acetate (4 mL) was added to quench any excess of reducing agent, THF was removed by rotary evaporation. The solids were suspended in ether (200 mL) at 0 °C to which cold water (120 mL) was added in small

portions. The pH of the solution was adjusted to 1 using 2M HCl and the organic layer was separated. The aqueous layer was extracted with ether (2 x 90 mL) and the combined organic layers were treated in the usual manner to obtain the title compound as a colorless oil (5.95 g, 99 %). bp 194-197 °C; R_f 0.23 (EtOAc:hexane, 1:3); The spectral data of this material was similar to that reported in the literature for the non-deuterated parent compound². IR (film): ν 3331 (O-H), 2957, 2926, 2856 (C-H), 2197, 2093 (C-D), 1466, 1379, 1297, 1134, 1093, 968, 723 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 1.54 (*br t*, $J = 6.9$ Hz, 2 H, $\text{CH}_2\text{CD}_2\text{OH}$), 1.45 (*s*, 1 H, OH), 1.38-1.21 (*m*, 10 H, 5 x CH_2), 0.87 (*t*, $J = 6.8$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 62.26 (*p*, $J_{\text{CD}} = 21.5$ Hz, α -shift - 0.370, C-1), 32.58 (β -shift - 0.063, C-2), 25.70 (γ -shift - 0.028, C-3), 29.40 (δ -shift 0.027, C-4), 29.27 (C-5), 31.81 (C-6), 22.64 (C-7), 14.06 (C-8); TMS Derivative EI-MS: m/z 204 ($[\text{M}]^+$), 189 ($[\text{Me}(\text{CH}_2)_6\text{CD}_2\text{OSiMe}_2]^+$, $[\text{M}-15]^+$), 131 ($[\text{Me}(\text{CH}_2)_6\text{CD}_2\text{O}]^+$, $[\text{M}-73]^+$), 117, 105, 103, 91, 75, 70, 55, 43, 41.

6.2.3.4 1-[1- $^2\text{H}_1$] Octanal¹⁸³

A three-neck 500 ml round-bottom flask, equipped with a magnetic stir bar was purged with N_2 , prior to the addition of oxalyl chloride (11.1 mL, 127.2 mmol). After diluting the reagent with dry CH_2Cl_2 (90 mL), the solution was cooled to -72 °C (external temperature) and anhydrous DMSO (18.1 mL, 254.3 mmol) was added in portions. The reaction was strongly exothermic and turned viscous after the DMSO addition was complete. The reaction mixture was stirred for additional 5-10 minutes after which was

added a solution of 1-[1,1-²H₂]-octanol (8.41 g, 63.6 mmol) in dry CH₂Cl₂ (40 mL) dropwise for 30 minutes at - 65 to - 70 °C. Soon after the addition of the alcohol the reaction mixture become cloudy and was stirred for additional 15 minutes before being warmed to - 12 °C (external temperature) and stirred for another 3 h. NEt₃ (53.1 mL, 381.5 mmol) was added and the resultant yellowish reaction mixture was stirred for an additional 40 minutes before being allowed to warm to room temperature and stirred for another 1.5 h. The reaction was worked up by the addition of distilled water (300 mL) and the mixture was transferred to a separatory funnel. The organic layer was diluted with another 40 mL of CH₂Cl₂ before being collected. The aqueous layer was extracted several times with dichloromethane (3 x 120 mL) and the combined organic layers treated in the normal manner to give the crude compound (7.89 g, 96 %). This material was purified by column chromatography on silica gel using ethyl acetate-hexane (1:19) as eluting solvent to provide the title compound as a pale yellow oil (4.52 g, 55 %). bp 170-172 °C; *R*_f 0.44 (EtOAc:hexane, 1:19); The spectral data of the product was similar to that reported in the literature for the non-deuterated parent compound.³ IR (film): ν 2956, 2926, 2856 (C-H), 2184, 2090 (C-D), 1731 (C=O), 1466, 1378, 1211, 1159, 1071, 967, 723 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 2.41 (*t*, *J* = 7.4 Hz, 2 H, CH₂CDO), 1.62 (*tt*, *J* = 7.4, 7.3 Hz, 2 H, CH₂CH₂CDO), 1.39-1.20 (*m*, 8 H, 4 x CH₂), 0.87 (*t*, *J* = 6.8 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 202.53 (*t*, *J*_{CD} = 26.1 Hz, α-shift - 0.357, C-1), 43.72 (*t*, ²*J*_{CD} = 3.6 Hz, β-shift - 0.102, C-2), 22.05 (γ-shift 0.038, C-3), 29.11 (δ-shift 0.053, C-4), 29.00 (C-5), 31.61 (C-6), 22.56 (C-7), 14.01 (C-8); EI-MS: *m/z* 111 ([Me(CH₂)₆C]⁺, [M-18]⁺), 101 ([M-28]⁺), 96 ([M-33]⁺), 84, 82, 69, 58, 55, 45, 41, 39, 30.

6.2.3.5 (1*R*)-[1-²H₁]-Octanol^{185,208}

A 3-neck round-bottom flask equipped with a reflux condenser, a one-piece distillation apparatus, stir bar, and rubber septa was flushed with N₂. The flask was then charged with monodeuterio-aldehyde (6.53 g, 50.6 mmol) that was then diluted with anhydrous THF (120 mL). A 0.5 M solution of (-)-(S)-Alpine-Borane® in THF (152 mL, 75.8 mmol) was added slowly to the stirred solution at room temperature and the reaction mixture became light yellow in color. The reaction mixture was stirred at room temperature for another 40 h. Excess Alpine-Borane® reagent was quenched by the addition of excess acetaldehyde (5 mL) and the mixture was stirred for additional 1 h to ensure completion of the reaction. The rubber septum was quickly replaced with a glass stopper and the solvent (THF) as well as the byproduct α-pinene was distilled under vacuum (at 15 mm and 0.02 mm Hg, respectively). To remove α-pinene, slight warming (40-45 °C) of the reaction flask was required. The residue was taken up in anhydrous Et₂O (150 mL) whilst maintaining an inert atmosphere. The solution was cooled to 0 °C and ethanolamine (4.6 mL, 75.8 mmol) was added dropwise to produce a white precipitate that was removed by filtration through celite 521. The precipitate rinsed with additional portions of ether (2 x 25 mL). The combined organic phase was treated in the normal manner to yield the crude compound that was then purified by column chromatography on silica gel using a gradient eluent system of EtOAc:hexane (4 %, 6 %, 8 % and 10 %). The title compound was obtained as a clear colorless liquid (6.37 g, 96

%), bp 194-197 °C; R_f 0.28 (EtOAc:hexane, 1:3); and the spectral data of the compound was similar to that reported above for the dideuterated compound (see Section 6.2.3.3) as well as with that of the non-deuterated parent compound reported in the literature³. IR (film): ν 3345 (O-H), 2926, 2858 (C-H), 2169 (C-D), 1468, 1452, 1411, 1326, 1300, 1166, 1040, 1003 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 3.62 (*tt*, J = 6.6, 1.4 Hz, 1 H, CHDOH), 1.55 (Overlapping *td*, J = 6.8, 6.6 Hz, 2 H, CH_2CHDOH), 1.43 (*s*, 1 H, OH), 1.40-1.23 (*m*, 10 H, 5 x CH_2), 0.88 (*t*, J = 6.7 Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 62.68 (*t*, J_{CD} = 21.7 Hz, α -shift 0.046, C-1), 32.71 (β -shift 0.063, C-2), 25.73 (γ -shift 0.004, C-3), 29.41 (δ -shift 0.030, C-4), 29.28 (C-5), 31.82 (C-6), 22.66 (C-7), 14.09 (C-8); TMS Derivative EI-MS: m/z 203 ($[\text{M}]^+$), 188 ($[\text{Me}(\text{CH}_2)_6\text{CHDOSiMe}_2]^+$, $[\text{M}-15]^+$), 130 ($[\text{Me}(\text{CH}_2)_6\text{CHDO}]^+$, $[\text{M}-73]^+$), 116, 104, 102, 90, 75, 70, 55, 43, 41.

6.2.3.6 (1*R*)-1-(*p*-Tolylsulfonyloxy)-[1- $^2\text{H}_1$]-Octane¹⁷⁹

To a solution of (1*R*)-[1- $^2\text{H}_1$]-octanol (3.57 g, 27.2 mmol) and *p*-toluenesulfonyl chloride (5.71 g, 30.0 mmol) in dry CH_2Cl_2 (55 mL) was added dropwise anhydrous pyridine (3.1 mL, 38.1 mmol) at room temperature. The reaction was stirred for 24 h and then poured into a saturated solution of NH_4Cl (50 mL). The organic layer was diluted with CH_2Cl_2 (25 mL) that was separated and washed successively with saturated NH_4Cl (3 x 50 mL) and saturated NaCl (2 x 50 mL). Filtration through anhydrous Na_2SO_4 followed by removal of solvent in vacuo afforded the crude compound that was purified by flash chromatography using ethyl acetate-hexane (1:9) as eluent to yield the pure

tosylate (6.26 g, 81 %). R_f 0.44 (EtOAc:hexane, 1:4); and the spectral data of the compound was similar to that reported in the literature for the non-deuterated parent compound. IR (film): ν 2928, 2858 (C-H), 1596 (C=C, Ar), 1455, 1366, 1190, 1176, 1098, 943, 814 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.79 (d , $J = 8.2$ Hz, 2 H, $\text{C}_{\text{O}} - \text{Ph}$), 7.34 (d , $J = 8.2$ Hz, 2 H, $\text{C}_{\text{M}} - \text{Ph}$), 4.00 (t , $J = 6.5$ Hz, 1 H, CHDOTs), 2.45 (s , 3 H, CH_3Ar), 1.62 (td , $J = 6.7, 6.5$ Hz, 2 H, CH_2CHDOTs), 1.35-1.14 (m , 10 H, 5 x CH_2), 0.87 (t , $J = 6.9$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 70.39 (t , $J_{\text{CD}} = 22.6$ Hz, C-1), 28.68 (C-2), 25.28 (C-3), 29.03 (C-4), 28.86 (C-5), 31.68 (C-6), 22.58 (C-7), 14.06 (C-8), 144.61 ($\text{C}_{\text{ipso}} - \text{Ph}$), 129.79 ($\text{C}_{\text{O}} - \text{Ph}$), 127.87 ($\text{C}_{\text{M}} - \text{Ph}$), 133.24 ($\text{C}_{\text{P}} - \text{Ph}$), 21.62 ($\text{CH}_3 - \text{Ph}$); EI-MS: m/z 173, 155, 139, 113, 107, 91, 84, 70, 65, 56, 41.

6.2.3.7 (3*S*)-[3- $^2\text{H}_1$]-Dec-1-yne¹⁷³

A solution of (1*R*)-1-(*p*-tolylsulfonyloxy)-[1- $^2\text{H}_1$]-octane (6.17 g, 21.6 mmol) in anhydrous DMSO (20 mL) was added dropwise to a stirred suspension of lithium acetylide-ethylenediamine complex (2.59 g, 28.1 mmol) in DMSO (20 mL) at room temperature. The reaction mixture was stirred for 3 h. The reaction was worked up by pouring into an ice-water mixture (100 mL) and pH of the solution was adjusted to 1 using 1.0 M HCl. The aqueous layer was extracted with diethyl ether (3 x 40 mL) and the combined organic phase was worked up in the usual manner to yield the crude compound. This material was purified by flash chromatography using Et_2O -pentane (1:32) as eluent to obtain the pure title compound as a light yellowish oil (1.17 g, 39 %).

bp 174-178 °C; R_f 0.83 (Pure Et₂O); and the spectral data of the title compound was similar to that obtained using the authentic non-deuterated parent compound. IR (film): ν 3314 (HC $\equiv$ C), 2956, 2925, 2855 (C-H), 2164 (C-D), 2120 (C $\equiv$ C), 1465, 1378, 1299, 1060, 967, 724 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 2.17 (*m*, 1 H, CHDC $\equiv$ C), 1.93 (*d*, J = 2.6 Hz, 1 H, HC $\equiv$ C), 1.51 (*td*, J = 7.0, 7.0 Hz, 2 H, CH₂CHD), 1.46-1.35 (*m*, 2 H, CH₂, C-9), 1.28 (*m*, 8 H, 4 x CH₂, C-5, C-6, C-7, C-8), 0.89 (*t*, J = 6.7 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 68.03 (γ -shift 0.005, C-1), 84.77 (β -shift 0.003, C-2), 18.13 (*t*, J_{CD} = 20.0 Hz, α -shift - 0.283, C-3), 28.43 (β -shift - 0.091, C-4), 28.76 (γ -shift - 0.031, C-5), 29.10 (δ -shift 0.001, C-6), 29.19 (C-7), 31.85 (C-8), 22.67 (C-9), 14.09 (C-10); EI-MS: m/z 124 ([CH₂(CH₂)₅CHDC $\equiv$ CH]⁺, [M-15]⁺), 110 ([CH₂(CH₂)₄CHDC $\equiv$ CH]⁺, [M-29]⁺), 96 ([CH₂(CH₂)₃CHDC $\equiv$ CH]⁺, [M-43]⁺), 82 ([CH₂(CH₂)₂CHDC $\equiv$ CH]⁺, [M-57]⁺), 68 ([CH₂CH₂CHDC $\equiv$ CH]⁺, [M-71]⁺), 55, 41.

6.2.3.8 2-((11*S*)-[11-²H₁]-Octadeca-9-ynyl-1-oxy)tetrahydropyran¹⁹⁶

n-BuLi (2.5 M in hexanes; 4.2 mL, 10.4 mmol) was added dropwise to a stirred solution of (3*S*)-[3-²H₁]-decyne (1.29 g, 9.3 mmol) in dry THF (17 mL) at 0 °C. After 20

min, HMPA (17 mL) was added to the reaction mixture and the solution changed from orange to wine red in color. After stirring the reaction mixture for another 15 min, a solution of 2-(8-iodooctyl-1-oxy)tetrahydropyran (3.78 g, 11.1 mmol) in anhydrous THF (17 mL) was added, and the solution turned yellow-orange in color. The reaction mixture was kept at 0 °C for 1.5 h (during which the solution become much darker in color) and then allowed to stir at rt for another 48 h. THF was removed in vacuo, the residue was diluted with Et₂O (40 mL) and the solution was poured into an ice-water mixture (140 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (3 x 90 mL). The combined organic layers were worked up in the normal manner to afford the crude compound that was purified by column chromatography on silica gel using a gradient eluent system (pentane, 4 % Et₂O-pentane, 4 % EtOAc-hexane, 8 % EtOAc-hexane, 12 % EtOAc-hexane and 16 % EtOAc-hexane). The title compound was obtained as a pale yellowish liquid (1.88 g, 58 %). *R*_f 0.30 (EtOAc: hexane, 1:11); and the spectral data of the compound was similar to that reported in the literature for the non-deuterated parent compound.¹ IR (film): ν 2930, 2856, 2804 (C-H), 2145 (C-D), 1465, 1456, 1352, 1201, 1136, 1121, 1078, 1034, 990, 906, 870 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 4.57 (*t*, *J* = 3.6 Hz, 1 H, OCHO), 3.92-3.81 (*m*, 1 H, CHO), 3.78-3.68 (*m*, 1 H, CHO), 3.56-3.45 (*m*, 1 H, CHO), 3.44-3.32 (*m*, 1 H, CHO), 2.13 (Overlapping *t*, *J* = 6.3 Hz, 3 H, C-8, C-11), 1.93-1.19 (*m*, 30 H, 15 x CH₂), 0.88 (*t*, *J* = 6.7 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 67.67 (C-1), 30.80 (C-2), 29.15 (C-3), 29.40 (C-4), 29.08 (C-5), 28.85 (C-6), 29.15 (C-7), 18.76 (C-8), 80.19 (C-9), 80.28 (C-10), 18.47 (*t*, *J*_{CD} = 19.6 Hz, C-11), 29.15 (C-12), 28.82 (C-13), 29.12 (C-14), 29.24 (C-15), 31.86 (C-16), 22.67 (C-17), 14.11 (C-18), 98.84 (C-1'), 29.75 (C-2'), 19.72 (C-3'), 25.52 (C-4'), 62.33

(C-5'); EI-MS: m/z 351 ($[M]^+$), 308 ($[M-43]^+$), 278 ($[M-73]^+$), 266 (-OTHP, $[M-85]^+$), 252 ($[CHDC \equiv C(CH_2)_8OTHP]^+$), 237 ($[C \equiv C(CH_2)_8OTHP]^+$), 224, 195, 153, 136, 124, 110, 101, 96, 85, 67, 55, 41.

6.2.3.9 (11*S*)-[11-²H₁]-Octadec-9-yn-1-ol¹⁹⁶

To a stirred solution of 2-((11*S*)-[11-²H₁]-octadeca-9-ynyl-1-oxy)tetrahydropyran (1.78 g, 5.1 mmol) in methanol (40 mL) was added *p*-toluenesulfonic acid, monohydrate (97 mg, 10 mol %, 0.5 mmol) and allowed to stir for overnight at room temperature. Reaction mixture was poured into a saturated solution of NaHCO₃ (15 mL) and methanol was removed in vacuo. The residue was diluted with Et₂O (30 mL) and the organic layer was separated. The aqueous phase was re-extracted with Et₂O (3 x 20 mL) and the collected organic layers were washed with brine (3 x 25 mL) until the washings were found neutral with litmus paper. The solvent was removed in vacuo after drying over anhydrous Na₂SO₄ and concentrated to furnish the crude compound as a crystalline white solid (1.34 g, estimated yield 99 %). The crude compound was used for further reaction without purification. R_f 0.25 (EtOAc:hexane, 1:3); and the spectral data of the compound

was similar to that reported in the literature for the non-deuterated parent compound.¹ IR (film): ν 3342 (O-H), 2928, 2856 (C-H), 2145 (C-D), 1465, 1378, 1057, 723 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 3.64 (*t*, J = 6.5 Hz, 2 H, CH_2OH), 2.13 (Overlapping *t*, J = 6.3 Hz, 3 H, C-8, C-11), 1.56 (*tt*, J = 6.8, 6.5 Hz, 2 H, $\text{CH}_2\text{CH}_2\text{OH}$), 1.45 (Overlapping *tt*, J = 6.6, 6.3 Hz, 4 H, C-7, C-12), 1.41-1.22 (*m*, 18 H, 9 x CH_2), 0.88 (*t*, J = 6.7 Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 63.05 (C-1), 32.78 (C-2), 25.70 (C-3), 29.33 (C-4), 29.07 (C-5), 28.85 (C-6), 29.14 (C-7), 18.744 (C-8), 80.17 (C-9), 80.28 (C-10), 18.46 (*t*, J_{CD} = 19.8 Hz, C-11), 29.14 (C-12), 28.78 (C-13), 29.14 (C-14), 29.23 (C-15), 31.85 (C-16), 22.67 (C-17), 14.11 (C-18); EI-MS: m/z 339 ($[\text{M}-1]^+$), 324 ($[\text{M}-16]^+$), 296 ($[\text{M}-44]^+$), 268, 248, 225, 183, 169, 153, 135, 121, 103, 96, 82, 75, 68, 55, 41.

6.2.3.10 (11*S*)-[11- $^2\text{H}_1$]-Octadec-9-ynoic acid¹⁹⁶

Freshly prepared Jones reagent (4.1 mL) [prepared by mixing CrO_3 (16 g), H_2O (10 mL) and 3.0 M H_2SO_4 (60 mL)] was slowly admitted to a stirred solution of (11*S*)-[11- $^2\text{H}_1$]-octadeca-9-yn-1-ol (1.37 g, 5.1 mmol) in acetone (110 mL). During addition, temperature of the reaction mixture was maintained below 25°C (using an ice-water bath) and the reaction was stirred for 2 h at rt. Excess oxidant was destroyed by passing

SO₂ through the reaction mixture to yield an emerald green color and the precipitate was dissolved in H₂O (50 mL). The aqueous layer was extracted with Et₂O (3 x 40 mL) and the combined organic layers were washed with water (50 mL) and saturated NaCl (made slightly acidic to pH 5 by adding few drops of 2.0 M HCl) until the aqueous layer was colorless. The solvent was removed in vacuo and the residue was further diluted with hexane and worked up in the usual manner to afford the title compound as an amorphous white solid (1.426 g, 99 %). *R_f* 0.28 (MeOH:CH₂Cl₂, 1:19); and the spectral data of the compound was similar to that reported in the literature for the non-deuterated parent compound.¹ IR (KBr): ν 3500-2800br (O-H), 3020, 2930, 2858 (C-H), 1711 (C=O), 1463, 1413, 1286, 1216, 1125, 1074, 914, 758 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 11.18 (*br s*, 1 H, CO₂H), 2.36 (*td*, *J* = 7.5, 1.9 Hz, 2 H, CH₂CO₂H), 2.14 (Overlapping *t*, *J* = 6.3 Hz, 3 H, C-8, C-11), 1.65 (*tt*, *J* = 7.5, 6.3 Hz, 2 H, CH₂CH₂CO₂H), 1.53-1.21 (*m*, 20 H, 10 x CH₂), 0.88 (*t*, *J* = 7.0 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 179.79 (C-1), 33.98 (C-2), 24.64 (C-3), 29.07 (C-4), 28.97 (C-5), 28.87 (C-6), 29.07 (C-7), 18.73 (C-8), 80.08 (C-9), 80.36 (C-10), 18.47 (*t*, *J*_{CD} = 19.9 Hz, C-11), 28.62 (C-12), 28.78 (C-13), 29.15 (C-14), 29.24 (C-15), 31.86 (C-16), 22.68 (C-17), 14.12 (C-18); EI-MS: *m/z* 284 (+ 2H, [M+3]⁺), 267 (- OH⁺, [M-14]⁺), 253 ([M-28]⁺), 241, 224, 210, 197, 183, 174, 167 ([C $\equiv$ C(CH₂)₇CO₂H]⁺), 153, 147, 137, 123, 110, 96, 82, 68, 60, 55, 41.

6.2.3.11 Methyl (11*S*)-[11-²H₁]-Octadec-9-ynoate

The deuterated fatty acid (1.44 g, 5.1 mmol) was dissolved in 3:2 toluene/methanol (18 mL) and treated with (trimethylsilyl) diazomethane (2.0 M in hexanes, 2.6 mL, 5.1 mmol) as described above for the methylation of decynoic acid. The crude product was purified by flash chromatography using EtOAc:hexane (1:49) as eluent and the title compound was obtained as colorless oil (1.33 g, 88 %). R_f 0.37 (Et₂O:hexane, 1:3); and the spectral data of the compound was similar to that reported in the literature for the non-deuterated parent compound.¹⁹⁷ IR (film): ν 2929, 2856 (C-H), 2145 (C-D), 1743 (C=O), 1463, 1436, 1362, 1247, 1197, 1171, 1098, 1017, 725 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 3.66 (*s*, 3 H, OCH₃), 2.30 (*t*, J = 7.5 Hz, 2 H, CH₂CO₂Me), 2.13 (Overlapping *t*, J = 6.2 Hz, 3 H, C-8, C-11), 1.62 (*tt*, J = 7.5, 7.3 Hz, 2 H, CH₂CH₂CO₂Me), 1.46 (*m*, 4 H, C-7, C-12), 1.41-1.19 (*m*, 16 H, 8 x CH₂), 0.88 (*t*, J = 6.7 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 174.28 (C-1), 34.08 (C-2), 24.92 (C-3), 29.08 (C-4), 29.06 (C-5), 28.86 (C-6), 29.08 (C-7), 18.73 (C-8), 80.09 (C-9), 80.31 (C-10), 18.47 (*t*, J_{CD} = 19.7 Hz, C-11), 28.65 (C-12), 28.80 (C-13), 29.15 (C-14), 29.24 (C-15), 31.86 (C-16), 22.68 (C-17), 14.11 (C-18), 51.44 (-OMe); EI-MS: m/z 295 ([M]⁺),

264 ($[M-31]^+$), 246 ($[M-49]^+$), 221 ($[M-74]^+$), 211, 197, 179, 165, 153, 137, 123, 110, 96, 82, 69, 55, 41.

6.2.3.12 Methyl (11*S*)-[11- $^2H_1$]-Octadeca-9*Z*-enoate

Methyl (11*S*)-[11- $^2H_1$]-octadeca-9-ynoate (100 mg, 0.3 mmol) in hexane (10 mL) was hydrogenated essentially as described above using Lindlar catalyst (17 mg) and 1.5 h reaction time. The final product was obtained as clear colorless oil (100 mg, 99 %). R_f 0.53 (EtOAc:hexane, 1:9); and the spectral data of the monodeuterated compound was similar to the non-deuterated parent compound reported in the literature.^{192-194,197} IR (film): ν 3004, 2926, 2855 (C-H), 2149 (C-D), 1744 (C=O), 1464, 1436, 1362, 1246, 1196, 1171, 1016, 724 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 5.42-5.28 (*m*, 2 H, 2 x HC=C), 3.67 (*s*, 3 H, OCH_3), 2.30 (*t*, $J = 7.5$ Hz, 2 H, CH_2CO_2Me), 2.01 (Overlapping *tt* and *td*, $J = 6.0, 6.0$ Hz, 3 H, C-8, C-11), 1.62 (*tt*, $J = 7.5, 7.3$ Hz, 2 H, $CH_2CH_2CO_2Me$), 1.39-1.21 (*m*, 20 H, 10 x CH_2), 0.88 (*t*, $J = 6.7$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 174.32 (C-1), 34.12 (C-2), 24.97 (C-3), 29.11 (C-4), 29.17 (C-5), 29.15 (C-6), 29.70 (C-7), 27.18 (δ -shift 0.011, C-8), 129.78 (γ -shift 0.027, C-9), 129.97 (β -shift - 0.037, C-10), 26.87 (*t*, $J_{CD} = 19.3$ Hz, α -shift - 0.352, C-11), 29.70 (β -shift - 0.078, C-12),

29.32 (γ -shift - 0.022, C-13), 29.55 (δ -shift 0.007, C-14), 29.34 (C-15), 31.93 (C-16), 22.70 (C-17), 14.13 (C-18), 51.44 (-OMe); EI-MS: m/z 297 ($[M]^+$), 266 ($[M-31]^+$), 265 (-MeOH $^+$, $[M-32]^+$), 236 ($[M-61]^+$), 223 ($[M-74]^+$), 208, 181 ($[M-116]^+$), 167, 152, 139, 123, 110, 98, 83, 74, 69, 55, 41.

6.2.3.13 Methyl (11*R*)-[11- $^2\text{H}_1$]-Octadeca-9*Z*-enoate

The title compound was synthesized in 7.3 % overall yield from ethyl octanoate using the same procedures as described for the synthesis of the corresponding (*S*)-enantiomer. The chiral starting material was prepared by the reduction of 1-[1- $^2\text{H}_1$]-octanal using (+)-(*R*)-Alpine-Borane. R_f 0.53 (EtOAc:hexane, 1:9); The analytical data for the title compound was essentially identical to that obtained for the corresponding (*S*)-enantiomer and similar to that reported in the literature for the non-deuterio compound.^{192-194,197} IR (film): ν 3004, 2926, 2855 (C-H), 2147 (C-D), 1744 (C=O), 1465, 1436, 1248, 1197, 1171, 1018, 725 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 5.41-5.27 (*m*, 2 H, 2 x HC=C), 3.67 (*s*, 3 H, OCH₃), 2.31 (*t*, J = 7.6 Hz, 2 H, CH₂CO₂Me), 2.01 (Overlapping *tt* and *td*, J = 6.0, 6.0 Hz, 3 H, C-8, C-11), 1.62 (*tt*, J = 7.6, 7.3 Hz, 2 H, CH₂CH₂CO₂Me), 1.39-1.21 (*m*, 20 H, 10 x CH₂), 0.88 (*t*, J = 6.7 Hz, 3 H, CH₃); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 174.34 (C-1), 34.12 (C-2), 24.97 (C-3), 29.11 (C-4), 29.17 (C-5), 29.14 (C-6), 29.70 (C-7), 27.18 (δ -shift 0.007, C-8), 129.78 (γ -shift 0.025, C-9), 129.97 (β -shift - 0.039, C-10), 26.87 (*t*, J_{CD} = 19.3 Hz, α -shift - 0.356, C-11), 29.70 (β -shift - 0.081, C-12), 29.31 (γ -shift - 0.024, C-13), 29.55 (δ -shift 0.006, C-14), 29.34 (C-

15), 31.92 (C-16), 22.70 (C-17), 14.13 (C-18), 51.46 (-OMe); EI-MS: m/z 297 ($[M]^+$), 266 ($[M-31]^+$), 265 (- MeOH $^+$, $[M-32]^+$), 236 ($[M-61]^+$), 223 ($[M-74]^+$), 208, 181 ($[M-116]^+$), 167, 152, 139, 123, 110, 98, 84, 74, 69, 55, 41.

6.2.4 Chiral [14- $^2H_1$]-Linoleate Synthesis

6.2.4.1 1-Pentanal

A 2.5 M solution of *n*-butyllithium in hexane (54.5 mL, 135.6 mmol) was added dropwise over a period of 50-55 minutes to a stirred solution of N,N-dimethylformamide (10 mL, 129.2 mmol) in a 5:1 mixture of anhydrous Et₂O-THF (120 mL) at 0 °C. The reaction was exothermic and the reaction mixture turned viscous and become off-white in color after the addition was complete. The reaction mixture was warmed up to rt and allowed to stir for 3 h. The solvent was removed in vacuo and the residue was diluted with ether (120 mL) that was poured into chilled H₂O (110 mL). After acidifying the aqueous layer with 3.0 M HCl, the organic layer was separated and the aqueous layer was extracted with Et₂O (3 x 80 mL). The combined organic layers were washed successively with water (120 mL), saturated NaHCO₃ (120 mL), brine (2 x 120 mL) and finally dried over anhydrous Na₂SO₄. The solvent was initially removed by short-path fractional distillation at atmospheric pressure and when the solvent was judged to evaporated; the title compound was distilled using diethyl suberate as a high boiling chase. The title compounds was obtained as a pure colorless liquid (6.90 g, 62 %). bp 100-102 °C; *R*_f 0.48

(EtOAc:hexane, 1:3); IR (film): ν 2960, 2935, 2875, 2821 (C-H), 2720 (C-H, CHO), 1727 (C=O), 1467, 1412, 1391, 1147, 1120, 1001, 888 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 9.75 (*t*, $J = 1.9$ Hz, 1 H, CHO), 2.41 (*td*, $J = 7.4, 1.9$ Hz, 2 H, CH_2CHO), 1.60 (*tt*, $J = 7.4, 7.4$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CHO}$), 1.35 (*qt*, $J = 7.4, 7.3$ Hz, 2 H, CH_2CH_3), 0.91 (*t*, $J = 7.3$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 202.88 (C-1), 43.59 (C-2), 24.13 (C-3), 22.25 (C-4), 13.74 (C-5).

6.2.4.2 1-[1- $^2\text{H}_1$]-Pentanal²⁰⁹

The title compound was synthesized from [1- $^2\text{H}_1$] N,N-dimethylformamide (10.6 mL, 135.0 mmol) using the same experimental procedure to that described above during the preparation of 1-pentanal. The title compound was obtained as a pure colorless liquid (7.19 g, 61%). bp 99-104 $^{\circ}\text{C}$; R_{f} 0.48 (EtOAc:hexane, 1:3); and the spectral data of the monodeuterated compound was similar to that of the non-deuterated parent compound reported above. IR (film): ν 2964, 2875 (C-H), 2191, 2071 (C-D), 1715 (C=O), 1467, 1406, 1380, 1094, 954 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 2.41 (*t*, $J = 7.3$ Hz, 2 H, CH_2CDO), 1.60 (*tt*, $J = 7.5, 7.3$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{CDO}$), 1.34 (*qt*, $J = 7.5, 7.3$ Hz, 2 H, CH_2Me), 0.91 (*t*, $J = 7.3$ Hz, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 202.63 (*t*, $J_{\text{CD}} = 25.9$ Hz, α -shift - 0.251, C-1), 43.43 (*t*, $^2J_{\text{CD}} = 3.6$ Hz, β -shift - 0.156, C-2), 24.10 (γ -shift - 0.023, C-3), 22.27 (δ -shift 0.016, C-4), 13.77 (C-5); EI-MS: m/z 85 ($[\text{M}-2]^+$), 70 ($[\text{M}-17]^+$), 57 ($[\text{M}-30]^+$), 41, 28.

6.2.4.3 1-Pentanol

The spectral data and physical characteristics of the compound was recorded using the authentic sample to establish the formation of the synthetic deuterated materials as well as to calculate the isotopic shifts. bp 138-140 °C; R_f 0.23 (EtOAc:hexane, 1:3); IR (film): ν 3338 (O-H), 2958, 2932, 2873 (C-H), 1467, 1379, 1116, 1076, 1056, 1006, 889, 729 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 3.59 (*t*, $J = 6.5$ Hz, 2 H, CH_2OH), 2.19-2.06 (*br s*, 1 H, OH), 1.54 (*tt*, $J = 6.8, 6.5$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{OH}$), 1.39-1.25 (*m*, 4 H, 2 x CH_2), 0.88 (*t*, $J = 6.8$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 62.81 (C-1), 32.38 (C-2), 27.88 (C-3), 22.44 (C-4), 13.97 (C-5); EI-MS: m/z 87 ($[\text{M}-1]^+$), 70 ($[\text{M}-18]^+$), 55 ($[\text{M}-33]^+$), 42, 31, 29, 27.

6.2.4.4 (1*S*)-[1- $^2\text{H}_1$]-pentanol

The title compound was synthesized from monodeuterated [1- $^2\text{H}_1$]-pentanal (5.36 g, 61.5 mmol) and (+)-(R)-Alpine-Borane® (185 mL, 92.3 mmol) using the same experimental protocol as that described above for the synthesis of (1*R*)-[1- $^2\text{H}_1$]-octanol. The crude compound was purified by flash chromatography using Et_2O :pentane (1:9) as eluent and the pure monodeuterated alcohol was isolated as colorless oil (1.35 g, 25 %). bp 138-140 °C; R_f 0.18 (EtOAc:hexane, 1:3); and the spectral data of the monodeuterated

compound was similar to that reported above for the non-deuterated parent compound. IR (film): ν 3339 (O-H), 2959, 2931, 2874 (C-H), 2153 (C-D), 1467, 1379, 1132, 1067, 1015, 940, 736 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 3.59 (*tt*, $J = 6.8, 1.5$ Hz, 1 H, CHDOH), 2.47 (*s*, 1 H, OH), 1.53 (*tt*, $J = 6.9, 6.8$ Hz, 2 H, CH_2CHD), 1.38-1.24 (*m*, 4 H, 2 x CH_2), 0.87 (*t*, $J = 6.9$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 62.56 (*t*, $J_{\text{CD}} = 21.6$ Hz, α -shift - 0.252, C-1), 32.32 (β -shift - 0.069, C-2), 27.87 (γ -shift - 0.017, C-3), 22.46 (δ -shift 0.018, C-4), 14.00 (C-5); EI-MS: m/z 88 ($[\text{M}-1]^+$), 71 ($[\text{M}-18]^+$), 56 ($[\text{M}-33]^+$), 42, 32, 29, 27; TMS Derivative EI-MS: m/z 161 ($[\text{M}]^+$), 146 ($[\text{M}-15]^+$), 133 ($[\text{M}-28]^+$), 116 ($[\text{M}-45]^+$), 104, 90, 75, 59, 45.

6.2.4.5 1-(*p*-Tolylsulfonyloxy)-pentane¹⁷³

To a stirred solution of 1-pentanol (1.5 g, 17.0 mmol) and *p*-toluenesulfonyl chloride (4.05 g, 21.3 mmol) in anhydrous Et_2O (50 mL) was added powdered KOH (8.2 g, 146.2 mmol) at - 50 °C. The reaction mixture was stirred at 0 °C for 2 h, poured into H_2O (50 mL) and worked up as described above for the preparation of 1-(*p*-tolylsulfonyloxy)-oct-2-yne to afford a colorless clear liquid (3.62 g, 88%). R_f 0.41 (EtOAc:hexane, 1:4); IR (film): ν 2959, 2933, 2873 (C-H), 1599 (C=C, Ar), 1467, 1360, 1177, 1098, 961, 913, 815, 762 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.78 (*d*, $J = 8.2$ Hz, 2 H, C_ortho - Ph), 7.34 (*d*, $J = 8.2$ Hz, 2 H, C_meta - Ph), 4.01 (*t*, $J = 6.5$ Hz, 2 H, CH_2OTs), 2.44 (*s*, 3 H, CH_3Ar), 1.63 (*tt*, $J = 6.9, 6.5$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{OTs}$), 1.36-1.18 (*m*, 4 H, 2 x CH_2), 0.84 (*t*, $J = 7.0$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 70.68 (C-1), 28.47

(C-2), 27.40 (C-3), 21.98 (C-4), 13.78 (C-5), 144.61 (*Cipso* - Ph), 129.77 (*C_o* - Ph), 127.83 (*C_m* - Ph), 133.19 (*C_p* - Ph), 21.58 (CH₃ - Ph); EI-MS: m/z 242 ($[M]^+$), 185, 173, 155, 139, 107, 91, 77, 65, 55, 41.

6.2.4.6 (1*S*)-1-(*p*-Tolylsulfonyloxy)-[1-²H₁]-pentane¹⁷³

The anticipated compound was synthesized in a similar manner to that described in 6.2.4.5, from (1*S*)-[1-²H₁]-pentanol (1.35 g, 10.6 mmol) as a colorless clear oil (2.67 g, 89%). R_f 0.41 (EtOAc:hexane, 1:4); and the spectral data of the compound was similar to the non-deuterated parent compound reported above. IR (film): ν 2959, 2933, 2873 (C-H), 2193 (C-D), 1599 (C=C, Ar), 1458, 1364, 1189, 1178, 1098, 943, 816, 755 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 7.78 (*d*, J = 8.2 Hz, 2 H, *C_o* - Ph), 7.34 (*d*, J = 8.2 Hz, 2 H, *C_m* - Ph), 3.99 (*tt*, J = 6.5, 1.2 Hz, 1 H, CHDOTs), 2.44 (*s*, 3 H, CH₃Ar), 1.62 (*td*, J = 6.9, 6.5 Hz, 2 H, CH₂CHDOTs), 1.36-1.18 (*m*, 4 H, 2 x CH₂), 0.84 (*t*, J = 7.0 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 70.37 (*t*, J_{CD} = 22.8 Hz, α -shift - 0.309, C-1), 28.37 (β -shift - 0.10, C-2), 27.38 (γ -shift - 0.023, C-3), 21.99 (δ -shift 0.005, C-4), 13.77 (C-5), 144.61 (*Cipso* - Ph), 127.83 (*C_o* - Ph), 129.77 (*C_m* - Ph), 133.22 (*C_p* - Ph), 21.58 (CH₃ - Ph); EI-MS: m/z 243 ($[M]^+$), 186, 173, 155, 139, 107, 91, 77, 71, 65, 41.

6.2.4.7 2-((4*R*)-[4-²H₁]-octa-2-ynyl-1-oxy)tetrahydropyran¹⁷⁴

Methyl lithium (1.6 M in Et₂O, 7.3 mL, 11.7 mmol) was added dropwise to a stirred solution of the tetrahydro-2-(2-propynyloxy)-2*H*-pyran (1.64 g, 11.7 mmol) in dry HMPA (10 mL) at 0 °C. After stirring the reaction mixture for 45 minutes at 0 °C during which the reaction mixture turned from yellow to deep purple, a solution of (1*S*)-1-(*p*-tolylsulfonyloxy)-[1-²H₁]-pentane (2.35 g, 9.6 mmol) in dry HMPA (5 mL) was added dropwise over a period of 15 minutes. During stirring, the solution color changed from green to brown. The reaction mixture was stirred at 0 °C for 2.5 h, at 40 °C for 24 h and at 45 °C for another 24 h. The reaction was quenched by pouring the mixture into an ice-water mixture (30 mL), stirred for 15 minutes and additional H₂O (100 mL) was added to it. The suspension was transferred to a separatory funnel and the organic layer was diluted with 1:3 mixture of Et₂O-pentane (40 mL). The organic layer was separated and the aqueous phase was extracted with additional 1:3 mixture of Et₂O-pentane (3 x 40 mL). The collected organic layers were combined and worked up in the usual manner to result the crude compound that was purified by flash chromatography using Et₂O:hexane (1:9) as eluent. The pure compound was obtained as a light yellowish oil (1.83 g, 89 %). *R*_f 0.21 (EtOAc:hexane, 1:19); and the spectral data of the compound was similar to that reported in the literature for the non-deuterated parent compound.¹⁰ IR (film): ν 2943, 2872 (C-H), 2237 (C≡C), 2118 (C-D), 1455, 1443, 1357, 1202, 1121, 1030, 903, 871,

816 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.81 (*td*, $J = 4.5, 3.2$ Hz, 1 H, OCHO), 4.34-4.16 (*m*, overlapping *ddd*, 2 H, CH_2OTHP), 3.84 (*m*, 1 H, CHO (5')), 3.67-3.47 (*m*, 1 H, CHO (5')), 2.19 (*t*, $J = 7.0, 2.3$ Hz, 1 H, $\text{CHDC} \equiv \text{C}$), 1.94-1.45 (*m*, 8 H, 4 x CH_2 , C-5, C-2', C-3', C-4'), 1.42-1.24 (*m*, 4 H, 2 x CH_2 , C-6, C-7), 0.89 (*t*, $J = 6.7$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 54.64 (δ -shift - 0.003, C-1), 79.76 (γ -shift - 0.002, C-2), 88.97 (β -shift 2.212, C-3), 18.50 (*t*, $J_{\text{CD}} = 20.0$ Hz, α -shift - 0.295, C-4), 28.20 (β -shift - 0.101, C-5), 31.02 (γ -shift - 0.032, C-6), 22.18 (δ -shift - 0.004, C-7), 13.96 (C-8), 97.55 (C-1'), 30.30 (C-2'), 19.14 (C-3'), 25.39 (C-4'), 61.99 (C-5'); EI-MS: m/z 210 ($[\text{M}-1]^+$), 182 ($[\text{M}-29]^+$), 168 ($[\text{M}-43]^+$), 154 ($[\text{M}-57]^+$), 140 ($[\text{M}-71]^+$), 127, 111, 101, 94, 85, 80, 68, 55, 41.

6.2.4.8 (4*R*)-[4- $^2\text{H}_1$]-2-Octyn-1-ol¹⁷⁴

To a stirred solution of 2-((4*R*)-[4- $^2\text{H}_1$]-octa-2-ynyl-1-oxy)tetrahydropyran (1.71 g, 8.1 mmol) in methanol (40 mL) was added *p*-toluenesulfonic acid, monohydrate (28 mg). The resulting reaction mixture turned cloudy, but became clear after 20 minutes of stirring. After stirring the reaction for 1 h at 0 °C, it was warmed up to room temperature and allowed to stir another 3 h. Saturated aqueous solution of NaHCO_3 (9 mL) was added to the reaction mixture and methanol was removed in vacuo. The resulting residue was extracted with Et_2O (3 x 25 mL) and the collected organic layers were combined. Combined organic layers were worked up with brine (3 x 25 mL) until the washings were found neutral to pH paper. After filtering the ether layer through anhydrous Na_2SO_4 , the

solvent was removed in vacuo and concentrated to afford the crude compound (646 mg, 63 %). The crude compound was purified by flash chromatography using EtOAc:hexane (1:19) as eluent and pure compound was obtained as a light yellowish clear liquid (267 mg, 26 %). bp 50-54 °C at 0.1 mm Hg; R_f 0.23 (EtOAc:hexane, 1:3); the spectral data of the compound was similar to that reported above for the non-deuterated parent compound (see Section 6.2.2.2) and with the closely related compound in the literature¹⁰. IR (film): ν 3340 (O-H), 2958, 2932, 2862 (C-H) 2275, 2237 (C $\equiv$ C), 2147 (C-D), 1469, 1348, 1161, 1114, 1010, 722 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 4.25 (*dd*, J = 5.8, 2.2 Hz, 2 H, CH₂OH), 2.19 (*tt*, J = 7.0, 2.2 Hz, 1 H, CHDC $\equiv$ C), 1.64 (*br t*, J = 5.8 Hz, 1 H, OH), 1.50 (*td*, J = 7.0, 6.9 Hz, 2 H, CH₂CHD), 1.43-1.24 (*m*, 4 H, 2 x CH₂), 0.90 (*t*, J = 7.0 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 51.42 (δ -shift 0.229, C-1), 78.28 (γ -shift 0.013, C-2), 86.63 (β -shift 0.209, C-3), 18.41 (*t*, J_{CD} = 20.0 Hz, α -shift - 0.230, C-4), 28.20 (β -shift - 0.056, C-5), 31.01 (γ -shift 0.021, C-6), 22.20 (δ -shift 0.055, C-7), 13.95 (C-8); TMS Derivative EI-MS (rel. intensity): m/z 198 (1, [M-1]⁺), 184 (26, [M-15]⁺), 166 (2, [M-33]⁺), 154 (100, [M-45]⁺), 143 (6, [M-56]⁺), 128 (14), 115 (6), 110 (6), 97 (16), 83 (5), 75 (52), 59 (9), 41 (13).

6.2.4.9 (4*R*)-1-(*p*-Tolylsulfonyloxy)-[4-²H₁]-oct-2-yne¹⁷³

The title compound was synthesized from (4*R*)-[4-²H₁]-2-octyn-1-ol (213 mg, 1.7 mmol) and *p*-toluenesulfonyl chloride (400 mg, 2.1 mmol) using the same experimental procedure that used for the preparation of 1-(*p*-tolylsulfonyloxy)-oct-2-yne. The desired

compound was obtained as a pale yellowish oil (356 mg, 76 %). R_f 0.41 (EtOAc:hexane, 1:4); and the spectral data of the monodeuterated compound was similar to that reported with the non-deuterated parent compound (see Section 6.2.1.13).⁶ IR (film): ν 2957, 2932, 2863 (C-H), 2244 (C $\equiv$ C), 2153 (C-D), 1599 (C=C, Ar), 1457, 1370, 1190, 1177, 1097, 940, 815 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.82 (*d*, J = 8.2 Hz, 2 H, C $_{\text{O}}$ - Ph), 7.34 (*d*, J = 8.2 Hz, 2 H, C $_{\text{M}}$ - Ph), 4.70 (*d*, J = 2.3 Hz, 2 H, CH_2OTs), 2.45 (*s*, 3 H, CH_3Ar), 2.05 (*tt*, J = 7.0, 2.3 Hz, 1 H, $\text{CHDC}\equiv\text{C}$), 1.38 (*td*, J = 7.0, 6.6 Hz, 2 H, CH_2CHD), 1.33-1.21 (*m*, 4 H, 2 x CH_2), 0.88 (*t*, J = 6.7 Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 58.82 (δ -shift 0.019, C-1), 71.80 (γ -shift 0.041, C-2), 90.63 (β -shift 0.019, C-3), 18.34 (*t*, J_{CD} = 20.0 Hz, α -shift - 0.253, C-4), 27.67 (β -shift - 0.066, C-5), 30.90 (γ -shift 0.003, C-6), 22.12 (δ -shift 0.030, C-7), 13.91 (C-8), 144.82 (*Cipso* - Ph), 128.12 (C $_{\text{O}}$ - Ph), 129.72 (C $_{\text{M}}$ - Ph), 133.48 (C $_{\text{P}}$ - Ph), 21.64 (CH_3 - Ph); EI-MS (rel. intensity): m/z 281 (1, $[\text{M}]^+$), 239 (1, $[\text{M}-42]^+$), 226 (1), 202 (1), 172 (3, $[\text{M}-109]^+$), 155 (30, $[\text{M}-126]^+$), 139 (27, $[\text{M}-142]^+$), 126 (4), 117 (5), 107 (10), 91 (100), 80 (17), 77 (19), 65 (54), 53 (27), 41 (40).

6.2.4.10 Methyl (14*R*)-[14-²H₁]-octadeca-9,12-diynoate¹⁷⁶

The following components were mixed in anhydrous DMF (6 mL) at - 25 °C in the following order: methyl 9-decynoate (113 mg, 0.6 mmol); K₂CO₃ (86 mg, 0.6 mmol); CuI (118 mg, 0.6 mmol); and NaI (93 mg, 0.6 mmol). After stirring the reaction mixture for 15 minutes at - 25 °C, a solution of (4*R*)-1-(*p*-tolylsulfonyloxy)-[4-²H₁]-oct-2-yne (168 mg, 0.6 mmol) in anhydrous DMF (6 mL) was added and stirred for another 20 minutes at - 20 °C. The reaction mixture was slowly warmed up to room temperature and kept stirring for next 24 h. The reaction was quenched by pouring the mixture into a saturated solution of aqueous NH₄Cl (40 mL) and the aqueous layer was extracted with Et₂O (4 x 25 mL). The collected ether layers were combined and worked up in the normal manner to afford a residue that was diluted with hexane (30 mL) and washed further with H₂O (3 x 20 mL), brine (3 x 25 mL). After drying the organic phase over anhydrous Na₂SO₄, the solvent was removed in vacuo to give the crude compound that was purified by flash chromatography using Et₂O:hexane (1:49) as eluent. The title compound was isolated as a faint yellowish clear oil (117 mg, 65 %). *R*_f 0.43 (Et₂O:hexane, 1:3); and the spectral data of the monodeuterated compound was similar to that reported above in Section 6.2.1.14 for the non-deuterated parent compound. IR (film): ν 2933, 2859 (C-H), 2211 (C≡C), 1740 (C=O), 1459, 1437, 1248, 1198, 1172, 1021, 882, 727 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_H 3.66 (*s*, 3 H, OCH₃), 3.11 (*td*, *J* = 3.5, 2.3 Hz, 2 H, C-11), 2.29 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.14 (Overlapping *tt*, *J* = 7.1, 2.4 Hz, 3 H, C-8, C-14), 1.61 (*tt*, *J* = 7.5, 6.9 Hz, 2 H, CH₂CH₂CO₂Me), 1.55-1.42 (*m*, 4 H, C-7, C-15), 1.42-1.21 (*m*, 10 H, 5 x CH₂), 0.88 (*t*, *J* = 7.1 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 174.23 (C-1), 34.03 (C-2), 24.86 (C-3), 28.73 (C-4), 28.62 (C-5), 28.62 (C-6), 28.98 (C-7), 18.67 (C-8), 80.32 (C-9), 74.55 (C-10), 9.68 (δ-shift -0.045, C-11), 74.53 (γ-shift -

0.015, C-12), 80.46 (β -shift -0.073, C-13), 18.38 (t , $J_{CD} = 19.9$ Hz, α -shift -0.326, C-14), 28.34 (β -shift -0.125, C-15), 31.02 (γ -shift -0.061, C-16), 22.19 (δ -shift -0.031, C-17), 13.95 (C-18), 51.41 (-OMe); EI-MS: m/z 291 ($[M]^+$), 260 ($[M-31]^+$), 248 ($[M-43]^+$), 235, 216, 202, 188, 174, 160, 146, 134, 120, 106, 92, 79, 67, 59, 55, 41.

6.2.4.11 Methyl (14*R*)-[14- $^2H_1$]-octadeca-9*Z*,12*Z*-dienoate¹⁷³

The experimental procedure used for the preparation of the title compound from methyl (14*R*)-[14- $^2H_1$]-octadeca-9,12-diynoate (83 mg, 0.3 mmol) was identical to that described above for the preparation of (8*R*)-[8- $^2H_1$] isotopomer (see Section 6.2.1.17). The title compound was isolated as a faint yellowish clear liquid (84 mg, 99.8 %). R_f 0.44 (EtOAc:hexane, 1:9); and the spectral data of the monodeuterated compound was similar to that obtained with the non-deuterated parent compound (see Section 6.2.1.16). IR (film): ν 3010, 2954, 2927, 2856 (C-H), 2150 (C-D), 1743 (C=O), 1461, 1436, 1247, 1197, 1172, 1018, 725 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 5.45-5.27 (m , 4 H, 4 x HC=C), 3.67 (s , 3 H, OCH₃), 2.77 (t , $J = 6.00$ Hz, 2 H, C-11), 2.30 (t , $J = 7.5$ Hz, 2 H, CH₂CO₂Me), 2.04 (m , 3 H, C-8, C-14), 1.63 (m , 2 H, CH₂CH₂CO₂Me), 1.42-1.21 (m , 14 H, 7 x CH₂), 0.89 (t , $J = 6.8$ Hz, 3 H, CH₃); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 174.30 (C-

1), 34.11 (C-2), 24.95 (C-3), 29.11 (C-4), 29.16 (C-5), 29.13 (C-6), 29.60 (C-7), 27.19 (C-8), 130.05 (C-9), 128.06 (C-10), 25.64 (δ -shift - 0.001, C-11), 127.94 (γ -shift 0.016, C-12), 130.17 (β -shift - 0.053, C-13), 26.85 (*t*, J_{CD} = 19.2 Hz, α -shift - 0.363, C-14), 29.27 (β -shift - 0.096, C-15), 31.51 (γ -shift - 0.031, C-16), 22.59 (δ -shift 0.001, C-17), 14.07 (C-18), 51.43 (-OMe); EI-MS: m/z 295 ($[M]^+$), 264 ($[M-31]^+$), 235, 221 ($[M-74]^+$), 207, 192, 179, 164, 150, 136, 121, 110, 96, 81, 67, 55, 41.

6.2.4.12 Methyl (14*S*)-[14- $^2H_1$] octadeca-9*Z*,12*Z*-dienoate¹⁷³

The title compound was synthesized in 1.5 % overall yield from [1- $^2H_1$] N,N-dimethylformamide using the same procedures as described for the synthesis of the corresponding (*R*)-enantiomer. The chiral starting material was prepared by the reduction of [1- $^2H_1$] pentanal using (+)-(*S*)-Alpine-Borane®. R_f 0.44 (EtOAc:hexane, 1:9); The analytical data for the title compound was essentially identical to that obtained for the corresponding (*R*)-enantiomer and similar to that reported above with the non-deuterated parent compound (see Section 6.2.1.16). IR (film): ν 3010, 2927, 2856 (C-H), 2155 (C-D), 1743 (C=O), 1460, 1436, 1247, 1197, 1172, 1019, 726 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 5.44-5.26 (*m*, 4 H, 4 x HC=C), 3.67 (*s*, 3 H, OCH₃), 2.77 (*t*, J = 5.9 Hz, 2 H, C-11), 2.31 (*t*, J = 7.6 Hz, 2 H, CH₂CO₂Me), 2.04 (*m*, 3 H, C-8, C-14), 1.61 (*m*, 2 H, CH₂CH₂CO₂Me), 1.41-1.19 (*m*, 14 H, 7 x CH₂), 0.89 (*t*, J = 6.7 Hz, 3 H, CH₃); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 174.31 (C-1), 34.11 (C-2), 24.96 (C-3), 29.11 (C-4), 29.17 (C-5), 29.13 (C-6), 29.60 (C-7), 27.20 (C-8), 130.05 (C-9), 128.06 (C-10), 25.64 (δ -shift 0.001,

C-11), 127.94 (γ -shift 0.018, C-12), 130.17 (β -shift - 0.051, C-13), 26.86 (t , J_{CD} = 19.2 Hz, α -shift - 0.359, C-14), 29.27 (β -shift - 0.092, C-15), 31.51 (γ -shift - 0.028, C-16), 22.59 (δ -shift 0.005, C-17), 14.07 (C-18), 51.43 (-OMe); EI-MS: m/z 295 ($[M]^+$), 264 ($[M-31]^+$), 235, 221 ($[M-74]^+$), 207, 192, 179, 164, 150, 136, 121, 110, 96, 81, 67, 55, 41.

6.2.5 Assessment of Enantiomeric Purity of the Chiral Synthetic Intermediates

6.2.5.1 (4*R*)-[4-²H₁]-2-octen-1-ol

Lindlar catalyst (18 mg) was added to a solution of (4*R*)-[4-²H₁]-2-octyn-1-ol (50 mg, 0.4 mmol) in Spectrograde hexane (10 mL) containing quinoline (180 μ L, 2 mol % with respect to solvent). Hydrogenation was allowed to proceed as described above for 1 h at atmospheric pressure and the reaction mixture worked up in the normal manner to yield the crude alcohol product as a brownish liquid (37 mg, 71 %). R_f 0.25 (EtOAc:hexane, 1:3); and the spectral data of the compound was similar to that reported in the literature for the non-deuterated parent compound. ¹H NMR (300 MHz, CDCl₃) δ_H 5.69-5.49 (m , 2 H, 2 x HC=C), 4.19 (d , J = 5.8 Hz, 2 H, CH₂OH), 2.05 (tdt , J = 7.0, 1.9 Hz, 1 H, CHDC=C), 1.42-1.19 (m , 6 H, 3 x CH₂), 0.89 (t , J = 6.8 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 58.77 (δ -shift 0.275, C-1), 128.34 (γ -shift 0.027, C-2), 133.17 (β -shift 0.072, C-3), 27.03 (t , J_{CD} = 19.2 Hz, α -shift - 0.327, C-4), 29.18 (β -shift -

0.074, C-5), 31.37 (γ -shift - 0.013, C-6), 22.50 (δ -shift 0.019, C-7), 14.01 (C-8); EI-MS: m/z 201 ($[M-1]^+$), 186 ($[M-16]^+$), 172 ($[M-30]^+$), 157 ($[M-45]^+$), 144, 129, 114, 101, 82, 75, 73, 68, 59, 55, 41.

6.2.5.2 (2*R*)-[2-²H₁]-Hexanoic acid^{204,210}

To a stirred solution of (4*R*)-[4-²H₁]-2-octen-1-ol (29 mg, 0.2 mmol) in 2:2:3 CH₃CN (1.2 mL), CCl₄ (1.2 mL) and H₂O (1.8 mL) was added periodic acid (266 mg, 1.2 mmol) and the reaction was allowed to proceed at room temperature for 3 min. RuCl₃ · *n*H₂O (3 mg, 0.01 mmol) was added to the reaction mixture and stirring at room temperature was continued for another 5 h during which the solution turned from brown to blackish in color. The product was isolated by extraction with ether (3 x 10 mL) and the combined organic extracts were dried over Na₂SO₄. The solvent was evaporated in vacuo to give the crude compound (26 mg, estimated 99 %) that was used in the next step without purification. *R_f* 0.80 (MeOH:CH₂Cl₂, 1:19); The spectral data of the compound was similar to that of the non-deuterated parent compound reported in the literature. ¹H NMR (300 MHz, CDCl₃) δ_H 9.03 (*br s*, 1 H, CO₂H), 2.33 (*tt*, *J* = 7.6, 2.2 Hz, 1 H, CHDCO₂H), 1.64 (*td*, *J* = 7.2, 6.8 Hz, 2 H, CH₂CHD), 1.40-1.20 (*m*, 4 H, 2 x CH₂), 0.90 (*t*, *J* = 6.9 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 180.14 (β -shift -0.576, C-1), 33.74 (*t*, *J_{CD}* = 19.6 Hz, α -shift -0.371, C-2), 24.31 (β -shift -0.053, C-3), 31.18 (γ -shift -

0.029, C-4), 22.29 (δ -shift -0.006, C-5), 13.87 (C-6); TMS Derivative EI-MS: m/z 188 ($[M-1]^+$), 176 ($[M-13]^+$), 147 ($[M-42]^+$), 100 ($[M-89]^+$), 73 ($[M-116]^+$), 59, 45, 28.

6.2.5.3 (*S*)-2-Methoxy-2-oxo-1-phenylethyl (2*R*)-[2-²H₁]-hexanoate²⁰⁶

(+)-(*S*)-Methyl 2-hydroxy-2-phenylethanoate (31 mg, 0.2 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (36 mg, 0.2 mmol) were added to a stirred solution of (2*R*)-[2-²H₁]-hexanoic acid (22 mg, 0.2 mmol) and 4-(dimethylamino)pyridine (5 mg) in CH₂Cl₂ (5 mL) at -10 °C. The resultant mixture was stirred at -10 °C for 1 h and at room temperature for 3 h. The suspension was diluted with CH₂Cl₂ and washed with water (3 x 15 mL), brine (2 x 15 mL) and filtered through anhydrous Na₂SO₄. The solvent was removed under reduced pressure and the crude compound was purified by flash chromatography using ethyl acetate:hexane (1:9) as eluent to furnish the pure compound as a light yellowish oil (16 mg, overall yield from (4*R*)-[4-²H₁]-2-octyn-1-ol: 20 %). *R*_f 0.40 (EtOAc:hexane, 1:4); The spectral data of the compound was similar to that reported before with the analogous material.⁹ ¹H NMR (300 MHz, CDCl₃) δ _H 7.48 (*dd*, *J* = 6.6, 3.3 Hz, 1 H, C_p - Ph), 7.43-7.35 (*m*, 4 H, C_m - Ph, C_o - Ph), 5.940 (*s*, 1 H, CHPhCO₂Me), 3.73 (*s*, 3 H, OCH₃), 2.472 (2*S*, *tt*, *J* = 7.6, 2.2 Hz, 1 H, CHDCO₂R', 26.002 %), 2.415 (2*R*, *tt*, *J* = 7.6, 2.2 Hz, 1 H, CHDCO₂R', 73.998 %), 1.691 (*td*, *J* = 7.6, 7.1 Hz, 2 H, CH₂CHD), 1.42-1.19 (*m*, 4 H, 2 x CH₂), 0.89 (*t*, *J* = 7.0 Hz, 3 H, CH₃).

6.2.5.4 (*S*)-2-Methoxy-2-oxo-1-phenylethyl (2*S*)-[2-²H₁] hexanoate²⁰⁶

The title compound was synthesized in 14 % overall yield from (4*R*)-[4-²H₁]-2-octyn-1-ol using the same procedures as described for the synthesis of the corresponding (*S,R*)-diastereomer. The analytical data for the title compound is given here: *R_f* 0.40 (EtOAc:hexane, 1:4); The spectral data of the compound was similar to that reported before with the analogous material.²⁰⁶ ¹H NMR (300 MHz, CDCl₃) δ_H 7.48 (*dd*, *J* = 6.6, 3.3 Hz, 1 H, *C_p* - Ph), 7.43-7.35 (*m*, 4 H, *C_o* - Ph, *C_m* - Ph), 5.941 (*s*, 1 H, CHPhCO₂Me), 3.73 (*s*, 3 H, OCH₃), 2.470 (2*S*, *tt*, *J* = 7.4, 2.2 Hz, 1 H, CHDCO₂R', 86.401 %), 2.415 (2*R*, *tt*, *J* = 7.4, 2.2 Hz, 1 H, CHDCO₂R', 13.599 %), 1.691 (*td*, *J* = 7.4, 7.2 Hz, 2 H, CH₂CHD), 1.42-1.21 (*m*, 4 H, 2 x CH₂), 0.90 (*t*, *J* = 7.0 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_C 174.06 (C-1), 33.60 (*t*, *J_{CD}* = 19.7 Hz, C-2), 24.36 (C-3), 31.11 (C-4), 22.24 (C-5), 13.85 (C-6), 74.21 (CHCO₂Me), 169.38 (CO₂Me), 52.53 (OCH₃), 138.35 (*C_{ipso}* - Ph), 129.16 (*C_o* - Ph), 128.75 (*C_m* - Ph), 127.57 (*C_p* - Ph).

6.2.5.5 (*R*)-[1(*S*)-²H₁]-pentyl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate¹⁸⁹

To an oven-dried 5 mL reaction vial were added dry pyridine (440 μL), (+)-(*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (50 mg, 0.2 mmol), carbon tetrachloride (430 μL) and the substrate 1-(1*S*)-[1-²H₁]-pentanol (13 mg, 0.1 mmol), accordingly. After

stirring the reaction mixture for 30 min at rt the reaction was judged to be complete when crystalline pyridine hydrochloride ceased to precipitate. At this point, excess 3-dimethylamino-1-propylamine (50 μ L) was added and stirring was continued for additional 10 min. The reaction mixture was diluted with ether (10 mL) and washed successively with chilled 2.0 M HCl (8 mL), chilled saturated Na_2CO_3 (8 mL) and brine (8 mL). The solvent was removed in vacuo after filtration through anhydrous Na_2SO_4 and the filtrate was diluted with CCl_4 and evaporated three times to remove last traces of ether. The product was obtained as a light yellowish clear oil (40 mg, 92 %). R_f 0.57 (EtOAc:hexane, 1:4); ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.54 (*dd*, $J = 6.5, 2.6$ Hz, 2 H, C_Q - Ph), 7.46-7.38 (*m*, 3 H, 2 x C_m - Ph, C_p - Ph), 4.322 (*tt*, $J = 6.6, 1.4$ Hz, 1 H, CHDOMTPA, 96.622 %), 4.287 (*tt*, $J = 6.7, 1.3$ Hz, 1 H, CHDOMTPA, 3.378 %), 3.57 (*q*, $J = 1.2$ Hz, 3 H, OCH_3), 1.697 (*td*, $J = 7.0, 6.6$ Hz, 2 H, $\text{CH}_2\text{CHDOMTPA}$), 1.39-1.24 (*m*, 4 H, 2 x CH_2 , C-3, C-4), 0.89 (*t*, $J = 6.8$ Hz, 3 H, CH_3).

6.2.5.6 (*R*)-[1(*R*)- $^2\text{H}_1$]-pentyl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate¹⁸⁹

The title compound was synthesized in 89 % overall yield from 1-(1*S*)-[1- $^2\text{H}_1$]-pentanol using the same procedure as described for the synthesis of the corresponding (*R,S*)-diastereomer. The analytical data for the title compound is given here: R_f 0.57 (EtOAc:hexane, 1:4); ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.54 (*dd*, $J = 6.6, 2.5$ Hz, 2 H, C_Q - Ph), 7.46-7.38 (*m*, 3 H, 2 x C_m - Ph, C_p - Ph), 4.322 (*tt*, $J = 6.7, 1.4$ Hz, 1 H, CHDOMTPA, 3.378 %), 4.287 (*tt*, $J = 6.7, 1.3$ Hz, 1 H, CHDOMTPA, 96.622 %), 3.57

(*q*, $J = 1.1$ Hz, 3 H, OCH₃), 1.697 (*td*, $J = 7.0, 6.7$ Hz, 2 H, CH₂CHDOMTPA), 1.39-1.24 (*m*, 4 H, 2 X CH₂, C-3, C-4), 0.89 (*t*, $J = 6.8$ Hz, 3 H, CH₃).

6.2.5.7 (*R*)-[1(*R*)-²H₁]-Oct-2-yne 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate¹⁸⁹

To a small round-bottom flask the following reagents were added according to the order: anhydrous pyridine (0.4 mL, 5.3 mmol), (+)-(*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (50 mg, 0.2 mmol), carbon tetrachloride (0.4 mL, 4.4 mmol) and the substrate alcohol, (*1R*)-[1-²H₁] oct-2-yn-1-ol (18 mg, 0.1 mmol). The reaction was then allowed to stir for 30 minutes at room temperature (completion of the reaction was evidenced by no more formation of crystalline pyridine hydrochloride) subsequently was added excess 1,4-diaminobutane (0.04 μL, 0.4 mmol) and stirred for additional 5 minutes. The reaction mixture was diluted with diethyl ether (10 mL) before being washed with chilled 2.0 M HCl (8 mL), chilled saturated aqueous Na₂CO₃ (8 mL), saturated NaCl (8 mL) and finally dried over anhydrous Na₂SO₄. The solvent was removed in vacuo and the content was diluted with CCl₄ and evaporated three times to remove the last traces of ether. The product was obtained as a colorless liquid (47 mg, 96

%). The ^1H NMR spectrum was recorded with the crude sample and compared with reference standard to calculate the enantiomeric excess. ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.59-7.47 (*m*, 2 H, C_Q - Ph), 7.44-7.35 (*m*, 3 H, 2 x C_M - Ph, C_P - Ph), 4.927 (1*R*, *t*, J = 1.7 Hz, 1 H, CHDOMTPA, 95.548 %), 4.810 (1*S*, *t*, J = 1.8 Hz, 1 H, CHDOMTPA, 4.452 %), 3.59 (*q*, J = 1.2 Hz, 3 H, OCH_3), 2.204 (*tt*, J = 7.1, 2.13 Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.58-1.49 (*m*, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.38-1.28 (*m*, 4 H, 2 x CH_2), 0.89 (*t*, J = 7.2 Hz, 3 H, CH_3).

6.2.5.8 (*R*)-[1(*S*)- $^2\text{H}_1$]-Oct-2-yne 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate¹⁸⁹

The title compound was synthesized in 95 % overall yield from (1*S*)-[1- $^2\text{H}_1$]-2-octyn-1-ol using the same procedure as described for the synthesis of the corresponding (*R,R*)-diastereomer. The analytical data for the title compound is given here: ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.57-7.47 (*m*, 2 H, C_Q - Ph), 7.44-7.36 (*m*, 3 H, 2 x C_M - Ph, C_P - Ph), 4.925 (1*R*, *t*, J = 1.8 Hz, 1 H, CHDOMTPA, 3.108 %), 4.808 (1*S*, *t*, J = 1.9 Hz, 1 H, CHDOMTPA, 96.892 %), 3.59 (*q*, J = 1.2 Hz, 3 H, OCH_3), 2.205 (*tt*, J = 7.1, 1.9 Hz, 2

H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.60-1.52 (*m*, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.40-1.27 (*m*, 4 H, 2 x CH_2), 0.89 (*t*, $J = 6.0$ Hz, 3 H, CH_3).

6.2.6 Methyl [11,11- $^2\text{H}_2$]-Linoleate Synthesis

6.2.6.1 [1,1- $^2\text{H}_2$]-1-(*p*-Tolylsulfonyloxy)-oct-2-yne¹⁷³

The estimated compound was synthesized from [1,1- $^2\text{H}_2$]-2-octyn-1-ol (2.5 g, 19.5 mmol) using the similar procedure as described in 6.2.1.13, as a clear colorless liquid (5.4 g, 98 %). R_f 0.46 (EtOAc:hexane, 1:4); and the spectral data of this material was similar to that reported above for the non-deuterated material (see Section 6.2.1.13). IR (film): ν 2957, 2933, 2862 (C-H), 2255 ($\text{C}\equiv\text{C}$), 2154 (C-D), 1598 (C=C, Ar), 1458, 1367, 1190, 1177, 1098, 954, 846 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.81 (*d*, $J = 8.2$ Hz, 2 H, C_o - Ph), 7.34 (*d*, $J = 8.2$ Hz, 2 H, C_m - Ph), 2.45 (*s*, 3 H, CH_3Ar), 2.06 (*t*, $J = 7.0$ Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.38 (*tt*, $J = 7.0, 6.9$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.33-1.18 (*m*, 4 H, 2 x CH_2), 0.88 (*t*, $J = 6.7$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 58.33 (*p*, $J_{\text{CD}} = 21.7$ Hz, α -shift - 0.475, C-1), 71.68 (β -shift - 0.085, C-2), 90.58 (γ -shift - 0.029, C-3), 18.59 (δ -shift - 0.003, C-4), 27.74 (C-5), 30.90 (C-6), 22.09 (C-7), 13.90 (C-8), 144.80 (*Cipso* - Ph), 128.09 (C_o - Ph), 129.70 (C_m - Ph), 133.44 (C_p - Ph), 21.63 (CH_3 - Ph); EI-MS: m/z 241 ($[\text{M}-41]^+$), 227 ($[\text{M}-55]^+$), 203, 173 ($[\text{M}-109]^+$), 155 ($[\text{M}-127]^+$), 139 ($[\text{M}-143]^+$), 119, 107, 91, 77, 65, 54, 41.

6.2.6.2 Methyl [11,11-²H₂]-octadeca-9,12-diynoate

The title compound was obtained in a similar manner to that described in 6.2.1.14, from [1,1-²H₂]-1-(*p*-tolylsulfonyloxy)-oct-2-yne (2.64 g, 9.3 mmol) and methyl 9-decynoate (1.7 g, 9.3 mmol) as a colorless clear oil (2.04 g, 74 %). *R_f* 0.21 (EtOAc:hexane, 1:19); and the spectral data of the compound was similar to that reported above for the non-deuterated parent compound (see Section 6.2.1.14). IR (film): ν 2933, 2859 (C-H), 1741 (C=O), 1461, 1436, 1247, 1198, 1172, 1098, 1016, 726 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ _H 3.66 (*s*, 3 H, OCH₃), 2.29 (*t*, *J* = 7.5 Hz, 2 H, CH₂CO₂Me), 2.14 (Overlapping *t*, *J* = 7.0 Hz, 4 H, C-8, C-14), 1.61 (*tt*, *J* = 7.5, 7.2 Hz, 2 H, CH₂CH₂CO₂Me), 1.48 (*m*, 4 H, C-7, C-15), 1.42-1.23 (*m*, 10 H, 5 x CH₂), 0.88 (*t*, *J* = 7.0 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ _C 174.24 (C-1), 34.04 (C-2), 24.88 (C-3), 28.75 (C-4), 28.63 (C-5), 28.63 (C-6), 29.00 (C-7), 18.68 (δ -shift - 0.029, C-8), 80.35 (γ -shift - 0.023, C-9), 74.51 (β -shift - 0.069, C-10), 9.28 (*p*, *J*_{CD} = 20.0 Hz, α -shift - 0.445, C-11), 74.37 (β -shift - 0.067, C-12), 80.51 (γ -shift - 0.023, C-13), 18.68 (δ -shift - 0.029, C-14), 28.45 (C-15), 31.06 (C-16), 22.20 (C-17), 13.97 (C-18), 51.42 (-OMe); EI-MS:

m/z 292 ($[M]^+$), 277 ($[M-15]^+$), 261 ($[M-31]^+$), 205, 189, 177, 161, 149, 135, 121, 107, 93, 79, 74, 67, 59, 43.

6.2.6.3 Methyl [11,11- $^2H_2$]-octadeca-9Z,12Z-dienoate¹⁷³

The title compound was synthesized using the similar procedure that described in 6.2.1.16, from methyl [11,11- $^2H_2$]-octadeca-9,12-diynoate (250 mg, 0.9 mmol) as a colorless oil (253 mg, 100 %). R_f 0.45 (EtOAc:hexane, 1:9); and the spectral data of the dideuterated material was similar to that reported above for the non-deuterated parent compound (see Section 6.2.1.16). IR (film): ν 3009, 2929, 2856 (C-H), 2200, 2099 (C-D), 1742 (C=O), 1461, 1437, 1248, 1198, 1172, 1015, 726 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 5.46-5.28 (*m*, 4 H, 4 x HC=C), 3.66 (*s*, 3 H, OCH_3), 2.30 (*t*, $J = 7.5$ Hz, 2 H, CH_2CO_2Me), 2.05 (Overlapping *td*, $J = 7.0, 6.8$ Hz, 4 H, C-8, C-14), 1.62 (*tt*, $J = 7.5, 7.1$ Hz, 2 H, $CH_2CH_2CO_2Me$), 1.42-1.20 (*m*, 14 H, 7 x CH_2), 0.89 (*t*, $J = 6.8$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 174.33 (C-1), 34.10 (C-2), 24.94 (C-3), 29.10 (C-4), 29.16 (C-5), 29.12 (C-6), 29.60 (C-7), 27.20 (δ -shift - 0.005, C-8), 130.07 (γ -shift 0.014, C-9), 127.93 (β -shift - 0.124, C-10); 24.81 (*p*, $J_{CD} = 9.8$ Hz, α -shift - 0.829, C-11), 127.80 (β -shift - 0.124, C-12), 130.24 (γ -shift 0.013, C-13), 27.21 (δ -shift - 0.009, C-14), 29.36 (C-

15), 31.53 (C-16), 22.58 (C-17), 14.07 (C-18), 51.45 (-OMe); EI-MS: m/z 296 ($[M]^+$), 281 ($[M-15]^+$), 265 ($[M-31]^+$), 236, 222 ($[M-74]^+$), 207, 193, 180, 165, 150, 137, 125, 111, 97, 83, 69, 55, 41.

6.2.7 Methyl [14,14- $^2H_2$]-Linoleate Synthesis

6.2.7.1 [1,1- $^2H_2$]-pentanol

The title compound was synthesized by following the similar procedure that described in 6.2.3.3, from methyl valerate (6.0 g, 51.7 mmol) using lithium aluminium deuteride (1.0 M in anhydrous THF, 57 ml, 56.8 mmol) as reagent and the estimated compound was obtained as a clear colorless liquid (4.25 g, 92 %). bp 138-140 °C; R_f 0.18 (EtOAc:hexane, 1:3); and the spectral data of the compound was similar to that reported above for the non-deuterated compound (see Section 6.2.4.3). IR (film): ν 3336 (O-H), 2959, 2930, 2862 (C-H), 2194, 2093 (C-D), 1467, 1379, 1161, 1130, 1083, 970 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.96 (*s*, 1 H, OH), 1.53 (*br t*, $J = 6.4$ Hz, 2 H, CH_2CD_2), 1.39-1.26 (*m*, 4 H, 2 x CH_2), 0.89 (*t*, $J = 6.8$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ_C 62.14 (*p*, $J_{CD} = 21.5$ Hz, α -shift - 0.669, C-1), 32.20 (β -shift - 0.187, C-2), 27.85 (γ -shift - 0.035, C-3), 22.46 (δ -shift 0.013, C-4), 13.99 (C-5); TMS Derivative EI-MS: m/z 162 ($[M]^+$), 147 ($[M-15]^+$), 131 ($[M-31]^+$), 117 ($[M-45]^+$), 105, 91, 75, 59, 45.

6.2.7.2 1-Bromopentane²¹¹

To a stirred solution of HBr (48 % in H₂O, 7.5 g, 44.5 mmol) and concentrated H₂SO₄ (2.2 mL) was added 1-pentanol (3.08 g, 35 mmol) followed by conc. H₂SO₄ (1.0 mL) in several portions and allowed the reaction mixture to reflux for 3 h. Direct short-path distillation of the contents in vacuo (15 mm Hg) afforded the title compound as a colorless clear liquid (4.56 g, 86 %). bp 28-30 °C at 15 mm Hg; *R_f* 0.59 (EtOAc:hexane, 1:19); IR (film): ν 2960, 2933, 2873, 2861 (C-H), 1466, 1296, 1250, 1208, 1036, 920, 729 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ_{H} 3.41 (*t*, *J* = 6.9 Hz, 2 H, CH₂Br), 1.87 (*tt*, *J* = 7.1, 6.9 Hz, 2 H, CH₂CH₂Br), 1.48-1.28 (*m*, 4 H, 2 x CH₂), 0.92 (*t*, *J* = 7.1 Hz, 3 H, CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ_{C} 34.01 (C-1), 32.56 (C-2), 30.34 (C-3), 21.89 (C-4), 13.91 (C-5); EI-MS: *m/z* 151 (Br⁸¹, [M-1]⁺), 149 (Br⁷⁹, [M-1]⁺), 121 ([M-30]⁺), 119 ([M-30]⁺), 109 ([M-42]⁺), 107 ([M-42]⁺), 81, 79, 69, 55, 53, 41.

6.2.7.3 1-[1,1-²H₂]-Bromopentane

The title compound was synthesized in a similar manner to that described in 6.2.7.2, from [1,1-²H₂]-pentanol (4.23 g, 47.0 mmol) as colorless liquid (5.88 g, 82 %). bp 40-44 °C at 15 mm Hg; *R_f* 0.59 (EtOAc:hexane, 1:19); and the spectral data of this material was similar to that reported above for the non-deuterated parent compound and

the analogous compound reported in the literature.¹⁴ IR (film): ν 2959, 2933, 2873, 2861 (C-H), 2158 (C-D), 1466, 1380, 1217, 1121, 992, 932, 731 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 1.85 (*br t*, $J = 7.1$ Hz, 2 H, $\text{CH}_2\text{CD}_2\text{Br}$), 1.48-1.28 (*m*, 4 H, 2 x CH_2), 0.91 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 33.46 (*p*, $J_{\text{CD}} = 23.0$ Hz, α -shift - 0.543, C-1), 32.32 (β -shift - 0.243, C-2), 30.27 (γ -shift - 0.068, C-3), 21.89 (δ -shift - 0.008, C-4), 13.89 (C-5); EI-MS: m/z 153 (Br^{81} , $[\text{M}-1]^+$), 151 (Br^{79} , $[\text{M}-1]^+$), 121 ($[\text{M}-32]^+$), 119 ($[\text{M}-32]^+$), 109 ($[\text{M}-44]^+$), 107 ($[\text{M}-44]^+$), 81, 79, 71, 55, 51, 42.

6.2.7.4 2-Octyn-1-ol²¹²

n-Butyllithium (2.5 M in hexane, 21 mL, 52.3 mmol) was added dropwise to a stirred solution of propargyl alcohol (1.4 mL, 23.8 mmol) in anhydrous THF-HMPA (24:19.5 mL) at - 25 to 30 °C. After stirring the reaction mixture for 30 min at 0 °C, a solution of 1-bromopentane (4.0 g, 26.2 mmol) in anhydrous HMPA (20 mL) was added to it in portions over 25-30 min, keeping the temperature below 0 °C. The reaction mixture was warmed up to room temperature and stirred overnight. The reaction was terminated by pouring the mixture to chilled water (120 mL) and the aqueous layer was extracted several times with ether (4 x 60 mL). The combined organic layers were washed with H_2O (80 mL), saturated NaCl (2 x 80 mL) and dried over anhydrous Na_2SO_4 . The solvent was removed in vacuo to afford the crude compound as a yellowish oil (2.68 g, 61 %). bp 48-50 °C at 0.1 mm Hg; R_f 0.25 (EtOAc:hexane, 1:3); IR (film): ν 3351 (O-H), 2957, 2932, 2860 (C-H), 2285, 2224 ($\text{C}\equiv\text{C}$), 1460, 1299, 1198, 1136,

1029, 987 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.21 (*t*, $J = 2.2$ Hz, 2 H, CH_2OH), 2.36 (*s*, 1 H, OH), 2.17 (*tt*, $J = 7.2, 2.2$ Hz, 2 H, $\text{CH}_2\text{C}\equiv\text{C}$), 1.48 (*tt*, $J = 7.2, 7.1$ Hz, 2 H, $\text{CH}_2\text{CH}_2\text{C}\equiv\text{C}$), 1.40-1.22 (*m*, 4 H, 2 x CH_2), 0.87 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 51.13 (C-1), 78.30 (C-2), 86.34 (C-3), 18.63 (C-4), 28.26 (C-5), 30.98 (C-6), 22.13 (C-7), 13.85 (C-8); TMS Derivative EI-MS: m/z 197 ($[\text{M}-1]^+$), 183 ($[\text{M}-15]^+$), 165 ($[\text{M}-33]^+$), 153 ($[\text{M}-45]^+$), 142 ($[\text{M}-56]^+$), 127, 115, 109, 96, 83, 75, 59, 41.

6.2.7.5 [4,4- $^2\text{H}_2$]-Oct-2-yn-1-ol²¹²

The anticipated compound was obtained in a similar manner as described in 6.2.7.4, from 1-[1,1- $^2\text{H}_2$]-bromopentane (4.0 g, 26.2 mmol) and propargyl alcohol (1.33 g, 23.8 mmol). The crude compound was purified by flash chromatography using EtOAc-hexane (1:9) as eluent to give the pure compound as a clear colorless oil (1.86 g, 61 %). bp 50-53 $^{\circ}\text{C}$ at 0.1 mm Hg; R_f 0.33 (EtOAc:hexane, 1:3); and the spectral data of the title compound was found similar to that reported above for the non-deuterated counterpart. IR (film): ν 3336 (O-H), 2931, 2862 (C-H), 2245, 2201 ($\text{C}\equiv\text{C}$), 2100 (C-D), 1459, 1379, 1234, 1184, 1040, 999 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 4.24 (*d*, $J = 5.8$ Hz, 2 H, CH_2OH), 1.78 (*t*, $J = 5.8$ Hz, 1 H, OH), 1.49 (*t*, $J = 6.7$ Hz, 2 H, CH_2CD_2), 1.42-1.22 (*m*, 4 H, 2 x CH_2), 0.89 (*t*, $J = 7.1$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 51.36 (δ -shift 0.175, C-1), 78.31 (γ -shift 0.040, C-2), 86.57 (β -shift 0.147, C-3), 18.11 (*p*, $J_{\text{CD}} = 19.9$ Hz, α -shift - 0.528, C-4), 28.10 (β -shift - 0.159, C-5), 30.98 (γ -shift - 0.013, C-6),

22.19 (δ -shift 0.046, C-7), 13.93 (C-8); EI-MS: m/z 127 ($[M-1]^+$), 113 ($[M-15]^+$), 97 ($[M-31]^+$), 85 ($[M-43]^+$), 80, 72, 67, 57, 41; TMS Derivative EI-MS: m/z 199 ($[M-1]^+$), 185 ($[M-15]^+$), 167 ($[M-33]^+$), 155 ($[M-45]^+$), 144 ($[M-56]^+$), 129, 115, 109, 98, 83, 75, 59, 41.

6.2.7.6 [4,4- $^2H_2$]-1-(*p*-Tolylsulfonyloxy)-oct-2-yne¹⁷³

The title compound was synthesized from [4,4- $^2H_2$]-2-octyn-1-ol (1.5 g, 11.7 mmol) using the same experimental procedure that used in 6.2.1.13, and the purified compound was isolated as a pale yellowish oil (3.23 g, 98 %). R_f 0.41 (EtOAc:hexane, 1:4); and the spectral data of the di-deuterio material was similar to that reported above for the non-deuterated compound. IR (film): ν 2958, 2932, 2863 (C-H), 2251 ($\text{C}\equiv\text{C}$), 2107 (C-D), 1599 (C=C, Ar), 1456, 1370, 1190, 1177, 1097, 941, 838, 815 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ_{H} 7.82 (*d*, $J = 8.2$ Hz, 2 H, C_2 - Ph), 7.34 (*d*, $J = 8.2$ Hz, 2 H, C_m - Ph), 4.70 (*s*, 2 H, CH_2OTs), 2.45 (*s*, 3 H, CH_3Ar), 1.37 (*br t*, $J = 6.2$ Hz, 2 H, CH_2CD_2), 1.33-1.19 (*m*, 4 H, 2 x CH_2), 0.88 (*t*, $J = 6.8$ Hz, 3 H, CH_3); ^{13}C NMR (75.5 MHz, CDCl_3) δ_{C} 58.81 (δ -shift 0.005, C-1), 71.81 (γ -shift 0.052, C-2), 90.60 (β -shift - 0.013, C-3), 18.05 (*p*, $J_{\text{CD}} = 20.0$ Hz, α -shift - 0.548, C-4), 27.56 (β -shift - 0.171, C-5), 30.86 (γ -shift - 0.033, C-6), 22.11 (δ -shift 0.02, C-7), 13.90 (C-8), 144.81 (C_{ipso} - Ph), 128.12 (C_2 - Ph), 129.71 (C_m - Ph), 133.4775 (C_p - Ph), 21.63 (CH_3 - Ph); EI-MS (rel. intensity): m/z 282 (1, $[M]^+$), 239 (1, $[M-43]^+$), 227 (1), 203 (1), 173 (4, $[M-109]^+$), 155

(38, $[M-127]^+$), 139 (56, $[M-143]^+$), 127 (6), 117 (8), 107 (10), 91 (100), 81 (11), 77 (11), 65 (40), 55 (19), 41 (24).

6.2.7.7 Methyl [14,14- $^2H_2$]-octadeca-9,12-diynoate

The title compound was synthesized by following the similar route that described in 6.2.1.14, from [4,4- $^2H_2$]-1-(*p*-tolylsulfonyloxy)-oct-2-yne (3.0 g, 10.6 mmol) and methyl 9-decynoate (1.94 g, 10.6 mmol) as a light yellowish clear oil (2.58 g, 83 %). R_f 0.21 (Et₂O:hexane, 1:19); and the spectral data of the title compound was similar to that reported above for the non-deuterated compound (see Section 6.2.1.14). IR (film): ν 2932, 2859 (C-H), 2204 (C $\equiv$ C), 2185, 2098 (C-D), 1740 (C=O), 1462, 1436, 1363, 1315, 1248, 1198, 1172, 1018 cm^{-1} ; ^1H NMR (300 MHz, CDCl₃) δ_H 3.66 (s, 3 H, OCH₃), 3.11 (t, J = 2.2 Hz, 2 H, C-11), 2.29 (t, J = 7.5 Hz, 2 H, CH₂CO₂Me), 2.14 (tt, J = 7.0, 2.2 Hz, 2 H, C-8), 1.63 (tt, J = 7.5, 7.3 Hz, 2 H, CH₂CH₂CO₂Me), 1.472 (m, 4 H, C-7, C-15), 1.42-1.21 (m, 10 H, 5 x CH₂), 0.88 (t, J = 7.0 Hz, 3 H, CH₃); ^{13}C NMR (75.5 MHz, CDCl₃) δ_C 174.27 (C-1), 34.06 (C-2), 24.90 (C-3), 28.76 (C-4), 28.65 (C-5), 28.65 (C-6), 29.01 (C-7), 18.70 (C-8), 80.35 (C-9), 74.59 (C-10), 9.71 (δ -shift - 0.019, C-11), 74.47

(γ -shift 0.030, C-12), 80.47 (β -shift - 0.062, C-13), 18.12 (p , J_{CD} = 20.0 Hz, α -shift - 0.588, C-14), 28.27 (β -shift - 0.195, C-15), 31.03 (γ -shift - 0.056, C-16), 22.22 (δ -shift 0.002, C-17), 13.97 (C-18), 51.43 (-OMe); EI-MS: m/z 291 ($[M-1]^+$), 281 ($[M-11]^+$), 262 ($[M-30]^+$), 248 ($[M-44]^+$), 241, 221, 205, 191, 175, 161, 147, 135, 121, 107, 93, 79, 67, 59, 41.

6.2.7.8 Methyl [14,14- $^2H_2$]-octadeca-9Z,12Z-dienoate¹⁷³

The title compound was obtained in a similar manner to that described in 3.2.1.16, from methyl [14,14- $^2H_2$]-octadeca-9,12-diynoate (57 mg, 0.2 mmol) as a pale yellowish clear oil (59 mg, 99 %). R_f 0.45 (EtOAc/hexane, 1:9); and the spectral data of the dideuterated compound was similar to that reported above for the monodeuterated (see Section 6.2.4.11 & 6.2.4.12) as well as non-deuterated parent compound (see Section 6.2.1.16). IR (film): ν 3009, 2959, 2927, 2856 (C-H), 2186, 2099 (C-D), 1744 (C=O), 1459, 1436, 1261, 1171, 1101, 1019, 802 cm^{-1} ; ^1H NMR (300 MHz, $CDCl_3$) δ_H 5.44-5.27 (m , 4 H, 4 x HC=C), 3.66 (s , 3 H, OCH₃), 2.77 (t , J = 5.6 Hz, 2 H, C-11), 2.30 (t , J = 7.5 Hz, 2 H, CH₂CO₂Me), 2.05 (td , J = 6.4, 6.4 Hz, 2 H, C-8), 1.62 (tt , J = 7.5, 7.0 Hz, 2 H, CH₂CH₂CO₂Me), 1.40-1.22 (m , 14 H, 7 x CH₂), 0.89 (t , J = 6.8 Hz, 3 H, CH₃); ^{13}C NMR

(75.5 MHz, CDCl₃) δ_c 174.30 (C-1), 34.12 (C-2), 24.96 (C-3), 29.11 (C-4), 29.17 (C-5), 29.13 (C-6), 29.60 (C-7), 27.20 (C-8), 130.05 (C-9), 128.08 (C-10), 25.65 (δ -shift 0.014, C-11), 127.97 (γ -shift 0.051, C-12), 130.14 (β -shift - 0.090, C-13), 26.50 (p , J_{CD} = 18.6 Hz, α -shift - 0.719, C-14), 29.17 (β -shift - 0.195, C-15), 31.49 (γ -shift - 0.053, C-16), 22.59 (δ -shift 0.006, C-17), 14.06 (C-18), 51.42 (-OMe); EI-MS: m/z 296 ([M]⁺), 281 ([M-15]⁺), 265 ([M-31]⁺), 236, 222 ([M-74]⁺), 207, 193, 180, 164, 150, 136, 121, 111, 97, 81, 74, 67, 55, 41.

Chapter 6: Part II Biological Experiments

6.3 General

All components of the biological media were purchased from Difco with the exception of D-glucose, D-galactose, Tergitol (type NP-40), diazald, BSTFA, pyridine, potassium hydroxide and the spectrograde organic solvents which were obtained from Sigma-Aldrich Canada LTD. (Oakville, Ontario, Canada). The CSM-URA or “dropout powder” was obtained from MP Biomedicals, LLC. All buffers and salts were purchased from Merck (Kirkland, Quebec, Canada). Distilled deionized water (ddH₂O) was used in the preparation of all media and obtained using a Millipore Milli Q water system (CD0F01205) equipped with a Super C Carbon cartridge, two ion exchange cartridges, an Organex Q cartridge and a Milli Pak Filter. All glassware was sterilized in an Amsco Eagle series (model 2021) gravity autoclave at 120 °C for 30 minutes. All biological transfers were performed in sterile laminar-flow fume hood under a Bunsen burner flame. All cultures were grown in either a New Brunswick Scientific G25 incubator shaker, or a

LabLine Instruments Environ-Shaker model #3597. Centrifugations were carried out either on a Sorvall RC 5C Plus floor centrifuge, Beckmann GS 6 tabletop centrifuge or an Eppendorf 5414D bench top centrifuge at ca. 4 °C (PBI, NRC, Saskatoon).

The following *S. cerevisiae* strains, used in this thesis were available in the laboratory of Dr. Patrick S. Covello (PBI, NRC-CNRC, Saskatoon): *S. cerevisiae* pSAS050/DTY10-a2 (*MAT α* , *fas2 Δ ::LEU2*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11*, *his3-15*, pSAS050) for enzymatic conversion of chiral 11-d₁-oleic acid to chiral 11-d₁-linoleic acid; *S. cerevisiae* pYJ/DTY10-a2 (*MAT α* , *fas2 Δ ::LEU2*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11*, *his3-15*) containing the plasmid pYJ comprised of the vector pYES2 and a *Fac2* gene (expressing $\Delta^{8,10}$ -fatty acid desaturase) from *Calendula officinalis* for the conversion of linoleate to calendate; yeast strain pESC-HIS/YPH499 derived from the strain YPH499 using the vector pESC-HIS (Stratagene) *i.e.* co-expressing the two cDNAs of DsFAD2-1 and DsFAD2-2 fatty acid desaturases for the conversion of oleate to dimorphecolate via the formation of *trans*- Δ^{12} -linoleate.

For the construction of the tung 1,4-desaturase (FADX)-containing yeast strain, 2x Phusion® high-fidelity PCR master mix for PCR reaction was ordered from New England BioLabs, Inc. pYES2.1 TOPO® TA Expression Kit from Invitrogen was used to TA clone the PCR product into pYES2.1 vector and transformed into chemically competent *E. coli* cells. The plasmid DNAs were purified by Promega Wizard® Plus Midipreps DNA Purification system. The yeast strain INVSc-1 was obtained from Invitrogen, Carlsbad, California. The purified plasmid DNA was transformed into *S. cerevisiae* using a *S. cerevisiae* EasyComp transformation kit from Invitrogen.

6.4 General Procedures

6.4.1 Saponification of Fatty Acid Substrates

Fatty acid methyl ester was weighed in Teflon sealed, 12 mL culture tube and 3 mL of 10 % KOH in MeOH and 10 % ddH₂O was added. The fatty ester was dispersed in the solution by vortexing the tube and flushed under N₂ for about 4 minutes to remove any trace of O₂. The tube was sealed tightly and allowed to heat at 80 °C for 2 hrs. It was removed from the heating block and cooled on ice for about 2 minutes. The alkaline fatty acid solution was then diluted with 2 mL of deionized water and acidified with acetic acid (400 µL). Two mL of hexane was added and the crude fatty acid was extracted by vortexing the culture tube vigorously followed by centrifugation at 2000 rpm for 1 minute. The aqueous layer was re-extracted with hexanes (2 x 2 mL) and the collected organic layers were combined and subsequently dried over anhydrous Na₂SO₄. After removing the solvent under a stream of N₂ at 25 °C, the neat free fatty acid sample was weighed. Yields were typically > 95 %.

6.4.2 Incubation Experiments

An equal volume of ethanol was added to the neat free fatty acid sample in a 12 mL glass culture tube that was sealed and heated at 80 °C for 10 minutes to sterilize. A sterile solution of 10 % Tergitol in ddH₂O (filter sterilized) was added to the tube to make a final concentration of 5-10 mg/mL of the fatty acid. The sample was vortexed, the tube was sealed and heated again at 80 °C for 5 minutes at which time the solution was clear.

A typical incubation experiment was carried out as follows: 0.5 mL of the above fatty acid-Tergitol solution containing the desired fatty acid was transferred to a sterile 250 mL Erlenmeyer flask containing 50 mL of the growth media (SDgalactose - uracil)

and 1 mL of the starter yeast culture. The Erlenmeyer flask was plugged with cotton wool, covered with tinfoil and placed in a shaking incubator. The incubation period, temperature and shake speed varied with each organism and is specified in the appropriate Results and Discussion sections. Cultures typically became turbid at the end of the incubation period. We have observed that the addition of exogenous fatty acid did not affect the growth of the yeast strain when compared with the control culture.

6.4.3 Extraction of the Cellular Fatty Acids

Fatty acids were isolated from the harvested yeast cells by using standard hydrolysis-methylation protocol. The yeast cultures (50 mL) of each incubation experiments were removed from the shaker and transferred to Falcon™ tubes (50 mL). The yeast cells were pelleted by centrifugation at 3000 x g for 5 minutes and the supernatants were carefully decanted from each centrifuge tubes. The yeast pellets were first washed with 10 mL of 10 % Tergitol solution and the mixtures were transferred to 12 mL culture tubes. The solutions were centrifuged at 3000 x g for 5 minutes, the supernatants were carefully discarded and the pellets were washed another two times with deionized water (10 mL). The harvested cells were first saponified, non-saponifiable lipids were removed and then the partially purified free fatty acids were methylated. To accomplish this, the yeast pellets (~ 1 g) were diluted with 2 mL of 10% KOH in MeOH in each tube and flushed with N₂ to remove any trace of O₂. The culture tubes were then quickly sealed and vortexed to disperse the material. The mixtures were saponified by heating the solution at 80 °C for 2 hr which leads to the lyses of the cells and saponification of any esters. The culture tubes were cooled down to room temperature and 2 mL of dH₂O was added to each tube. The aqueous layer was extracted with

hexanes (2 x 2 mL) by vortexing the mixtures to remove any nonsaponifiable lipids. In this particular step, centrifugation at 3000 x g for 2 minutes helped to get a clear phase separation. The aqueous layer was neutralized with 50 % acetic acid, diluted with 2 mL of deionized water and extracted with hexanes (3 x 2 mL). The organic layers were combined and the solvent was removed under a stream of N₂ to yield the fatty acid residue as free long-chain carboxylic acid for GC-MS analysis.

6.4.4 Derivatization of Fatty Acids for GC-MS Analysis

Fatty acids were derivatized to fatty acid methyl ester (FAME) for GC-MS analysis. An ice-cold solution of diazomethane was used for that purpose and was prepared by the following procedure: to a diazomethane generator were added 2.5 g of KOH in 4 mL of water and 5 mL of EtOH and the mixture was heated up to 65 °C using a water bath. A solution of 2.5 g of Diazald™ in 30 mL Et₂O was slowly admitted to the mixture using a dropping funnel and the freshly prepared diazomethane solution was collected by condensation from a dry-ice cold finger to an ice-cold RBF as a bright yellow liquid. Once the Diazald™ addition was completed and the distillation had ceased, 1 mL ether was added until a clear distillate appeared.

Freshly prepared ice-cold diazomethane solution was then slowly added to the crude fatty acid solutions until the yellow color persisted. The solvent and excess reagent was removed under a stream of N₂ and the residual fatty acid methyl esters (FAME) were finally taken in 100 µL of hexane for GC-MS analysis. Use of diazomethane to methylate the fatty acid samples containing sensitive functionalities was found to be most suitable over other methylation techniques such as Fischer esterification, as it esterifies under extremely mild condition. When analyzing fatty acid methyl ester samples containing

dimorphecolate, the FAMES were silylated using BSTFA and pyridine.¹⁸⁷ For silylation, 0.2-5.0 mg of the FAMES, 0.2-0.4 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 0.1-0.2 mL of pyridine were combined in a 1.0 mL reacti-vial^{TM}; the mixture was shaken at room temperature for 30 seconds and subsequently heated at 60 °C for 10 minutes to ensure completion of the reaction.

6.5 Microbial synthesis of methyl (11*R*)- and (11*S*)-[11-²H₁]-octadeca-9*Z*,12*Z*-dienoate by Δ^{12} -desaturation of corresponding methyl octadeca-9*Z*-enoates

The yeast strain pSAS050/DTY10-a2 (*MATa*, *fas2* Δ ::*LEU2*, *can1*-100, *ura3*-1, *ade2*-1, *his3*-11, *his3*-15, pSAS050) used for this experiment is defective in FAS (Fatty Acid Synthase) to prevent endogenous oleic from diluting the deuterium labeled 18:2 product and functionally expresses a Δ^{12} -desaturase gene. Methyl (11*R*)-[11-²H₁] oleate (163 mg) was saponified with 2 mL of 10 % KOH in methanol and 10 % deionized water in a sealed 12 mL glass culture tube at 80 °C for 2 hours. The mixture was cooled to room temperature, acidified with 2 mL of 6M HCl and extracted with 3 x 2 mL of hexane. The combined hexane layer was dried over anhydrous Na₂SO₄ and the solvent was removed under a stream of N₂ to recover 150 mg of free fatty acid. 100 μ L of ethanol was added and the tube was heated to 80 °C for 20 minutes to sterilize, then 10 mL of sterile 10 % Tergitol was added to make a 15 mg/mL solution. The solution was warmed until dissolved/emulsified to give a clear solution. 10 flasks each containing 150 mL of SDgalactose-uracil media was inoculated with 1 mL of stationary phase starter culture of the pSAS050/DTY10-a2 yeast strain. 1 mL of oleic acid/Tergitol mixture was then

added. The cultures were capped with a foam plug, covered with tinfoil and shaken at 225 rpm for 6 days at 20 °C. The cultures were spun down by centrifugation; the pellets were washed with 10 mL of water and pelleted into a glass culture tube. The pellets were suspended in 2 mL of 10 % methanolic-KOH, sealed to vortex and heated at 80 °C for 2 hours to saponify the harvested cells. The mixture was cooled to room temperature, 2 mL of deionized water added and acidified with 2 mL of 6M HCl before extraction with 3 x 2 mL of hexane. The combined hexane extracts were dried under nitrogen and esterified with 2 mL of 1 % H₂SO₄ in dry methanol for 1 h at 60 °C. The mixture was cooled; 2 mL of water was added and the fatty acid methyl esters were extracted with 3 x 2 mL of hexane. The organic layers from all 10 cultures were combined, the solvent was evaporated under nitrogen and the crude FAME (147 mg in total) was dissolved in 300 µL of 50 % acetone:acetonitrile mixture. The purification was carried out on a Hewlett-Packard 1100 series HPLC system equipped with an auto-sampler, solvent degasser, quaternary pump, column heater, and diode array detector (monitored at 205 and 254 nm) controlled by HP Chemstation software. The crude sample was divided 6 x 50 µL aliquots and each aliquot injected on a Gemini-5 µm C18 110A 250 mm x 10 mm column, eluting at a rate of 3 mL/min isocratically for 5 minutes with acetonitrile, followed by a 0-30 % acetone gradient for 20 minutes and finally with 100 % acetone to rinse and re-equilibrate. Methyl (11*R*)-[11-²H₁]-linoleate was collected between 12 and 13 minutes and the unreacted labeled oleate was recovered between 14 and 16 minutes. After removing the solvent (acetone:acetonitrile), pure methyl (11*R*)-[11-²H₁]-linoleate (10 mg, 7 % of the total crude FAME) was obtained. The exact same procedure was followed to convert methyl (11*S*)-[11-²H₁]-oleate into methyl (11*S*)-[11-²H₁]-linoleate. In

this case, 8 mg of pure methyl (11*S*)-[11-²H₁]-linoleate was recovered after purification ~ 7 % of the total crude FAME.

6.6 Construction of the Tung 1,4-Desaturase (FADX)-containing Yeast Mutant

6.6.1 Amplification of the Template Plasmid DNA using PCR Reaction

The DNA plasmid pRTL2 myc-FADX, containing the tung (*Aleurites fordii* Hemsl.) seed FADX gene, that encodes the $\Delta^{11,13}$ -fatty acid conjugase/desaturase enzyme was a generous gift from J. M. Dyer (US Department of Agriculture-Agricultural Research Services Southern Regional Research Center, New Orleans, Louisiana) and amplified using the PCR reaction using a FDX-forward primer (5'-gaagcttgcttagaATGGGAGCTGGTGGCCGAATGTCT-3') and a FDX-reverse primer (5'-aaggatccACTCCATATCTCGTAACAAGGTCAAACCTC-3').

50 μ L of PCR mixture was set up on ice using the following components, which were added in the following order:

Component	50 μ L reaction
Sterile water	22 μ L (to make the mixture up to 50 μ l)
2x Phusion ® high-fidelity master mix	25 μ L
FADX forward primer, T _m = 76.1 °C	1 μ L (50 pmoles)
FADX reverse primer, T _m = 69.4 °C	1 μ L (50 pmoles)
pRTL2-myc-FADX plasmid as template DNA	1 μ L (0.5 ng)

2x Phusion® high-fidelity PCR master mix is a convenient 2x mix containing Phusion high-fidelity DNA polymerase (0.04 U/ μ L), nucleotides (400 μ L of each dNTP) and optimized 2x Phusion HF reaction buffer including MgCl₂. The PCR reaction was then started on a thermal cycler after mixing the contents of the tube and amplified by using the following cycling parameters:

Cycle step	2-Step protocol		Cycles
	Temperature	Time	
Step 1. Initial denaturation	98 °C	30 sec	1
Step 2. Denaturation	98 °C	10 sec	30
Step 3. Annealing	72 °C	30 sec	
Step 4. Extension	72 °C	30 sec	
Step 5. Final extension	72 °C	10 min	1
	4 °C	hold	

1 μ L of Taq polymerase (thermostable DNA polymerase) was added to the mixture at step 5 and heated up to 72 °C for 10 minutes to ensure A-overhang on PCR products. The Phusion DNA polymerase has the ability to stabilize primer-template hybridization. The annealing temperature used is the lowest primer T_m + 3 °C = 72 °C for 30 seconds. The final extension of 10 minutes at 72 °C after the last cycle was included to ensure that all PCR products are full length and 3' adenylated. After the completion of amplification, 10 μ L of PCR product was removed from the reaction and analyzed by agarose gel electrophoresis. DNA was visualized by ethidium bromide staining. A single, discrete band was obtained.

6.6.2 TOPO® Cloning of PCR Products and Expression in *E. coli*

TOPO® cloning technology provides an opportunity to construct the required PCR product, recombined into the TOPO vector, and then transform the recombinant vector into *E. coli* easily. pYES2.1 TOPO TA Expression Kit was used to TA clone the PCR product into pYES2.1 vector and transform TOP10 chemically competent one shot® *E. coli* cells.

6.6.2.1a LB Medium

10 g Tryptone (1 %), 5 g yeast extract (0.5 %) and 10 g NaCl (1 %) were dissolved into 950 mL of deionized water in a 2 L sterile bottle and the pH of the solution was adjusted to 7.0 using NaOH. After adjusting the final volume to 1 L, the solution was autoclaved on liquid cycle for 20 minutes at 120 °C and 15 psi. The solution was cooled and ampicillin was added as antibiotic (100 µg/mL) and stored at room temperature (LB_{amp}).

6.6.2.1b LB agar Plates

10 g Tryptone (1 %), 5 g yeast extract (0.5 %), 10 g NaCl (1 %) and 15 g Bacto agar (1.5 %) were dissolved into 935 mL of deionized water in a 2 L sterile bottle and the pH of the solution was adjusted to 7.0 using NaOH. After adjusting the final volume to 1 L, the solution was autoclaved on liquid cycle for 20 minutes at 120 °C and 15 psi. The solution was cool down to 55 °C before 100 mg of ampicillin was added as antibiotic (100 µg/mL), stirred well and poured into 10 cm petri plates (~ 30 ml / plate). Plates were allowed to harden, inverted and stored at 4 °C in the dark for future use.

6.6.2.1c SOC Medium

2 % Tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose added together to make a final volume of 6 mL.

6.6.2.2 Bacterial Transformation

Before starting the cloning reaction, SOC media was warmed to room temperature, the (LB_{amp}) agar plate was placed in the incubator to warm up to 37 °C for 30 minutes, a water bath was equilibrated to 42 °C for the chemical transformation, the vial of TOP10 chemically competent one shot® *E. coli* cells (50 µL each) was thawed on ice.

6.6.2.2.1 Preparation of TOPO® Cloning Reaction

For each transformation, 2 µL of sterile ddH₂O was added to a mixture of 2 µL of fresh PCR product and 1 µL of salt solution to make a final volume of 5 µL and the TOPO Cloning reaction was started by gently mixing 1 µL of TOPO® vector pYES2.1 with the combination. The reaction was then incubated for 5 minutes at room temperature then placed on ice and proceeds immediately for One Shot® chemical transformation.

6.6.2.2.2 Chemical Transformation

2 µL of the TOPO Cloning reaction was pipetted directly into the vial containing the TOP10 One Shot® chemically competent *E. coli* cells (50 µL) and mixed by gently tapping the vial. The reaction mixtures were incubated on ice for 30 minutes, removed from the ice bath and given a heat-shock treatment for 30 seconds at 42 °C without shaking the vial and then placed immediately back on ice. Room temperature SOC media (250 µL) was added to the tube. After sealing the vial, the reaction was shaken at 200 rpm for 1 hour at 37 °C, removed from the incubator and 5µL was spread equally on two pre-

warmed (LB_{amp}) plates. The plates were inverted and incubated for overnight at 37 °C. From one of the two plates, 10 well-spaced bacterial colonies were picked and incubated overnight in 15 mL of liquid culture (LB_{amp})(100 µg/mL) in 50 mL sterile Falcon™ tubes at 200 rpm at 37 °C. The plasmids were purified and submitted to DNA Services at PBI for sequencing.

6.6.2.2.3 Long-term storage

A single colony was picked up from the plate(s) that contains the correct clone and was inoculated into 2 mL of LB media under ampicillin selection (100 µg/mL). After growing the culture(s) to saturation ($OD_{600} = 1-2$), 0.85 mL of the culture was pipetted to a cryovial containing 0.15 mL of sterile glycerol and stored at - 80 °C.

6.6.3 Plasmid DNA Purification

Small scale purification of the plasmid DNA known as “minipreps” is commonly used in molecular biology procedures. The plasmid DNA was purified by using Promega Wizard® *Plus* Midipreps DNA Purification kit. The following procedures were used in the purification:

6.6.3.1 Preparation of Bacterial Lysate

The overnight grown cultures of *E. Coli* (15 mL) in 50 mL conical tubes were pelleted by centrifugation at 10,000 x g for 10 minutes at 4 °C. The supernatants were carefully decanted and discarded and the centrifuge tubes were blotted upside down with a paper towel to remove excess liquid. The pellets were suspended in Cell Resuspension Solution (3 mL each) and the cell lumps broken up using an applicator stick. Cell Lysis Solution (3 mL) was added to each Falcon™ tube and left 3-5 minutes for efficient lysis of the bacterial cells. The tubes were capped and inverted 4 times for proper mixing

which made the cell suspension completely clear. Subsequently, Neutralization Solution (3 mL) was added to the tubes and the solutions were mixed again by inverting the capped tubes 4 times. The lysed cells were centrifuged at 14,000 x g for 15 minutes at 4 °C to produce a tight pellet and the supernatant liquid containing the plasmid DNAs were carefully decanted off from the top of the white precipitates to the new 50 mL Falcon™ tubes.

6.6.3.2 Plasmid DNA Purification

Wizard® Midipreps DNA Purification resin was thoroughly shaken and 10 mL resin solution was added to the plasmid DNA solutions. The resin/DNA mixtures were swirled before being transferred to the Midicolumns that were attached to the vacuum manifold ports. The vacuum was applied and when all liquids from the reservoir have been judged to pass through the column the vacuum was released. A total of 30 mL of column wash solution was passed through the column and the resin was allowed to dry under vacuum suction for 30 seconds. The Midicolumns were removed from the vacuum manifold, separated from the reservoirs and placed in 1.5 mL microcentrifuge tubes that were then centrifuged at 10,000 x g for 2 minutes (to remove any residual Column Wash Solution). The Midicolumns were transferred to new microcentrifuge tubes (1.5 mL) and to each was added 100 µL of preheated (80 °C) nuclease-free water. After 1 min, the plasmid DNA was isolated by centrifugation at 10,000 x g for 20 seconds. The Midicolumns were removed from the microcentrifuge tubes and discarded. The supernatant was centrifuged for additional 5 minutes at 10,000 x g to separate the resin fines and the supernatant containing the plasmid DNA was carefully pipetted to new microcentrifuge tubes. The solutions containing the purified plasmid DNAs in water were

finally stored at - 20 °C. Purified plasmid was quantitated by spectrophotometer and submitted for sequencing to DNA services at PBI. The sequence data was analyzed using Lasergene SeqMan software to verify that the sequence was correctly translated.

```
tttggctagaATGGGAGCTGGTGGCCGAATGTCTGTTGCCCCAACAAACAGCAAGTGCAGAAAAAGGAAAGCAGAAG
CGTGAAACGAGTCCCACACACAAAGCCTCCATTCACTCTTGGCCAATTAAAGCAAGCCATACCGTCCCATTGCTTTAA
ACGCTCCCTTCTTCGCTCCTTCTTATGTTGTTTATGACCTTTCTTAAAGTTTTATTTCTACTCCATTGCCACCACCTA
CTTCCATCTCCTCCCTTCTCCGATAACCTACATCGCTTGGCCTGTCTACTGGGCTTTCCAAGGCTGCATTCTCACTAGT
GTTTGGGTGCTCGGCCATGAATGTGGTCACCATGCTTTTAGCGAATATAATTGGCTTGACGACACTATTGGCCTAATT
CTCCACTCTTCACTTCTAGTTCATACTTTTCATTTAAAATTAGCCATCGTCGCCATCACTCTAACATTGCATCACTTGA
ACGCGACGAGGTGTTGTCCCTAGGCTCAAGTCTGCAATTCATGGTATTCCAAGTACCTCAACAACCCACCAGGCC
GAGCTTTAACCCCTGTAGCTACACTCTTCATTGGCTGGCCTTTATATTTAGCCTTCAATGTTTCGGGCGCGATACTATGA
TCGCTTTGCCTGCCATTATGATCCTTATAGTCTATATATTCTGATAGAGAAAGGCTTCAGATTTACATTTCTGATGCT
ATGATTTTCGTTGCAGCTTATGTGTTGTATAAGATTGCCATGGCAAAAGGGCTAGCATGGCTGGTATGTATCTATGG
GGTACCATTGCTTATTGTTAATGCTCTTGTGTGACTATCACATCCTTGACGACACCCACGTTGCATTGCCACATTAT
GACTCCTCAGAATGGGATTGGCTTCGGGGAGGTTTAGCAACAGTAGATAGAGATTATGGAGTGTTCAATAAGATT
TCCATAATGCTACAGATACTCATGTAATACACCATCTATTCTCTTCAATGCCTCATTATCATGGAGTGGAAGCTACTAG
AGCAATCAAGCGATATTGGGTGACTATTATCTGTTTGATGATACTCCTATTCATGTGGCACTGTGGAGGGAGGCAA
AGGAGTGTCTGTTTGTGAGCCAGATGAGGGAGATAACAACAATGGTGTCTTCTGGTATAGTAATAAGTTTTGAggat
cc
```

Figure 6.1. Sequence of the FADX from tung (*Aleurites fordii* Hemsl.) inserted in the pYES2.1 plasmid. The capital letters indicates open reading frame (ORF) and the lower case is untranslated.

```
MGAGGRMSVAPNNSKCEKESRSVKRVPHTKPPFTLGQLKQAIPSHCFKRSLRSFSYVYDLSLSFIFYSIATT
YFHLLPSPITYIAWPVYWAFQGCILTSVWVLGHECGHHAFSEYNWLDDTIGLILHSSLLVPYFSFKISHRRHHSN
IASLERDEVFVPRKLSAIPWYSKYLNPPGRALTLVATLFIGWPLYLAFNVSGRYYDRFACHYDPYSPYSDRERL
QIYISDAMIFVAAAYVLYKIAMAKGLAWLVCIYGVPLLVNALVVTITSLQHTHVALPHYDSSEWDWLRGGLATV
DRDYGVFNKIFHNATDTHVIHHLFSSMPHYHGVEATRAIKPILGDYFLFDDTPIHVALWREAKECLFVEPDEG
DNNNGVFWYSNKF
```

Note: amino acid 122 D (Asp) translated from “gat” (FADX) and from “gac” (FADX19).

Figure 6.2. Translated gene sequence of tung FADX from *Aleurites fordii* Hemsl.

6.6.4 Transformation of *S. cerevisiae*

The FADX plasmid DNA was then transformed into the INVSc-1 strain of *S. cerevisiae* using the method described in the manual for *S. cerevisiae* EasyComp

transformation kit (Invitrogen) and selected on SDglucose-uracil selection media plates. The following protocol was used in the preparation of competent *S. cerevisiae* cells that were transformed with the purified plasmid DNA.

6.6.4.1a Stock Solution

200 g of dextrose (D-glucose) was dissolved in 800 mL of ddH₂O to make a final volume of 1 L. The solution was filtered into a sterilized 2 L bottle and stored at 4 °C.

6.6.4.1b YPD Solution

10 g of yeast extract (1 %), 20 g of bacto-peptone (2 %) and 20 g of dextrose (2 %) [D-glucose] were dissolved in 950 mL of ddH₂O to make a final volume of 1 L. The solution was filtered into a sterile 2 L bottle. The YPD medium was stored at room temperature for future use.

6.6.4.1c YPD Agar Medium

10 g of yeast extract (1 %), 20 g of bacto-peptone (2 %) were dissolved in 900 mL of ddH₂O and 20 g of bacto-agar (2 %) was added to it. The solution was filtered into a sterile 2 L bottle. 100 mL of stock solution (2 % D-glucose) was added to the bottle, swirled well. The solution was then poured into sterile petri plates (~ 30 ml/plate) to make the YPD plates and stored in the dark at 4 °C for future use.

6.6.4.2 Preparation of Competent Yeast Cells

The INVSc-1 strain of *S. cerevisiae* was streaked on a YPD (Yeast Extract Peptone Dextrose) plate and incubated at 28-30 °C for 2 days. Solution I (wash solution) and II (Lithium cation solution for making cells competent) were removed from the *S. cerevisiae* EasyComp Transformation Kit and allowed to equilibrate to room temperature. 10 mL of YPD solution was transferred into a 50 mL sterile Falcon™ tube

and to that was added the INVSc-1 strain of *S. cerevisiae* using a platinum loop that was flamed before use. The yeast culture was allowed to grow overnight at 28 °C in a shaking incubator at 250 rpm to an OD₆₀₀ of between 3.0 and 5.0. The cells from the overnight grown culture were diluted to an OD₆₀₀ of 0.2 to 0.4 in a total volume of 10 mL of YPD. The diluted YPD culture was again allowed to grow in a shaking incubator at 28-30 °C for 4-5 hrs at 250 rpm until the OD₆₀₀ reached a value of 0.6 to 1.0. The cells were then pelleted by centrifugation at 500 x g for 5 minutes at room temperature and the supernatant was discarded. The cell pellet was resuspended in 10 mL of Solution I (wash solution) and after 2 minutes, pelleted by centrifugation at 500 x g for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended for the final time in 1 mL of Solution II (Lithium cation solution for making cells competent). The competent cells were aliquoted into several 1.5 mL sterile screw-capped microcentrifuge tubes, each containing 50 µL of solution and placed in a Styrofoam box (to avoid damage to the cell wall, it is important that the cells freeze slowly) and stored at - 80 °C for future use (higher transformation efficiencies have been observed with the frozen cells compared to freshly prepared cells).

6.6.4.3 Transformation of the Competent Cells

The Solution III (transformation solution) from the *S. cerevisiae* EasyComp Transformation Kit was equilibrated to room temperature. Five selective SD-ura agar plates were equilibrated to room temperature. One 1.5 mL microcentrifuge tube containing 50 µL of competent cells was defrosted at room temperature and to that was added 4 µL of purified FADX plasmid DNA solution (vector DNA) followed by 500 µL of room temperature solution III. The cell/DNA mixture was mixed thoroughly by

vigorously vortexing the tube and subsequently incubated in a water bath for 1 hr at 30 °C. During heating in the water bath, the transformation reaction was mixed by vortexing the tube vigorously every 15 minutes. The microcentrifuge tube was removed from the water bath and by using a sterile spreader the transformed yeast was streaked on 2 SD-uracil selection media plates each containing 10 and 100 µL of cell/DNA mixture. The plates were then incubated at 30 °C for 4 days during which each transformation yielded sufficient number of discrete colonies (each transformation yielded at least 100 colonies).

6.6.4.4 Starter Culture

A starter culture was prepared from a single colony of INVSc-1 strain of *S. cerevisiae* that was grown on a plate of selection media (SD-uracil + 2 % agar)²¹³ and functionally expressing the fatty acid $\Delta^{11,13}$ -desaturase/conjugase enzyme of *Aleurites fordii* Hemsl.. A platinum loop was flamed and the colony was transferred from the plate to 2 mL liquid selection/induction media (SDgalactose-ura) and grown to stationary phase for 2 days at 28 °C in a rotary shaking incubator set at 150 rpm. The starter culture was kept at 5 °C for up to 3 weeks. The selection media (solid media) for the petri plates was prepared from 20 g of glucose (20 %), 6.8 g of Difco bacto-Peptone (0.7 %) [Yeast nitrogen base without amino acids], 1.5 g of CSM-URA or “dropout powder” (1.5 g, 0.2 %) [Complete supplemental mixture without uracil, the essential base and amino acid mixture for the yeast strain to grow] and 20 g of Difco bacto-agar (2 %) were dissolved in 951 mL of ddH₂O to make a 1 L solution, filtered in a 2 L Erlenmeyer flask that was plugged with cotton wool, covered with tinfoil and autoclaved on liquid cycle for 20 minutes to sterilize. The solution was cool down to 55 °C and poured into several 10 cm petri plates which were stored at 4°C in the dark for future use. The selection/induction

media was prepared as follows: 20 g of galactose (2 %) [99 % (D)-(+)-galactose], Difco bacto-Peptone (6.8 g, 0.7 %) [Yeast nitrogen base without amino acids] and CSM-URA or “dropout powder” (1.5 g, 0.2 %) [Complete supplemental mixture without uracil, the essential base and amino acid mixture for the yeast strain to grow] were dissolved in 971 mL of ddH₂O to make a 1 L solution, filtered in a 2 L Erlenmeyer flask that was capped with cotton wool, covered with tinfoil and autoclaved to sterilize.

Appendix A1.

^{13}C NMR data for methyl [8- $^2\text{H}_1$]-octadec-9Z,12Z-dienoate and corresponding synthetic intermediates: Deuterium Isotope Shifts (ppm)

Compound	C11	C10	C9	C8	C7	C6	C5
Et 8-OH-octanoate				62.91	32.67	25.53	29.05
Et 8- d_1 -8OH-octanoate				62.50 (α : - 0.40)	32.55 (β : - 0.12)	25.50 (γ : - 0.03)	29.04
Et 8- d_2 -8OH-octanoate				62.15 (α : - 0.76)	32.45 (β : - 0.22)	25.48 (γ : - 0.05)	29.05
Me dec-9-ynoate		68.14	84.66	18.37	28.73	28.38	28.53
Me 8- d_1 -dec-9-ynoate		68.18 (γ : + 0.04)	84.63 (β : - 0.03)	18.09 (α : - 0.28)	28.75 (β : + 0.02)	28.31 (γ : - 0.07)	28.52
Me octadec-9,12-dienoate	9.72	74.58	80.37	18.71	29.01	28.65	28.65
Me 8- d_1 -octadec-9,12-dienoate	9.71	74.60 (γ : + 0.02)	80.34 (β : - 0.03)	18.40 (α : - 0.31)	28.02 (β : + 0.01)	28.56 (γ : - 0.09)	28.63
Me octadec-9Z,12Z-dienoate	25.64	128.06	130.06	27.20	29.60	29.13	29.17
Me 8- d_1 -octadec-9Z,12Z-dienoate	28.64	128.08 (γ : + 0.02)	130.01 (β : - 0.05)	26.84 (α : - 0.36)	29.51 (β : - 0.09)	29.08 (γ : - 0.03)	29.17

Appendix A2.

^{13}C NMR data for methyl [11- $^2\text{H}_1$]- and [11- $^2\text{H}_2$]-octadec-9Z,12Z-dienoate and corresponding synthetic intermediates: Deuterium Isotope Shifts (ppm)

Compound	C14 (C4)	C13 (C3)	C12 (C2)	C11 (C1)	C10	C9	C8
2-octyn-1-ol	19.00	99.33	81.61	177.20			
1-d ₁ -2-octyn-1-ol	19.08	99.30 (γ : - 0.03)	81.66 (β : + 0.05)	176.98 (α : - 0.22)			
2-octyn-1-ol	18.66	86.50	78.28	51.29			
1-d ₁ -2-octyn-1-ol	18.65	86.49 (γ : - 0.01)	78.24 (β : - 0.04)	51.01 (α : - 0.28)			
1,1-d ₂ -2-octyn-1-ol	18.68	86.59 (γ : + 0.09)	78.21 (β : - 0.07)	50.84 (α : - 0.35)			
Me octadec-9,12-dienoate	18.71	80.53	74.44	9.72	74.58	80.37	18.71
Me 11-d ₁ -octadec-9,12-dienoate	18.69	80.51 (γ : - 0.02)	74.40 (β : - 0.04)	9.49 (α : - 0.23)	74.53 (β : - 0.05)	80.35 (γ : - 0.02)	18.69
Me 11-d ₂ -octadec-9,12-dienoate	18.68	80.51 (γ : - 0.02)	74.37 (β : - 0.07)	9.28 (α : - 0.44)	74.51 (β : - 0.07)	80.35 (γ : - 0.02)	18.68
Me octadec-9Z,12Z-dienoate	27.22	130.23	127.92	25.64	128.06	130.06	27.20
Me 11-d ₁ -octadec-9Z,12Z-dienoate	27.22	130.24 (γ : + 0.01)	127.86 (β : - 0.06)	25.32 (α : - 0.32)	128.00 (β : - 0.06)	130.07 (γ : + 0.01)	27.20
Me 11-d ₂ -octadec-9Z,12Z-dienoate	27.21	130.24 (γ : + 0.01)	127.80 (β : - 0.12)	24.81 (α : - 0.83)	127.93 (β : - 0.13)	130.07 (γ : + 0.01)	27.20

Appendix A3.

^{13}C NMR data for methyl $[11\text{-}^2\text{H}_1]$ -octadec-9Z-enoate and corresponding synthetic intermediates: Deuterium Isotope Shifts (ppm)

Compound	C14 (C4)	C13 (C3)	C12 (C2)	C11 (C1)	C10	C9	C8
octan-1-ol	29.33	25.70	32.78	63.07			
1-d ₁ -octan-1-ol	29.34	25.66 (γ : - 0.04)	32.64 (β : - 0.14)	62.61 (α : - 0.46)			
1,1-d ₂ -octan-1-ol	29.35	25.65 (γ : - 0.05)	32.53 (β : - 0.25)	62.26 (α : - 0.81)			
1-decyne	29.10	28.79	28.52	18.41	84.76	68.02	
3-d ₁ -1-decyne	29.10	28.76 (γ : - 0.03)	28.43 (β : - 0.09)	18.13 (α : - 0.28)	84.77 (β : + 0.01)	68.03 (γ : + 0.01)	
Me octadec-9Z-enoate	29.54	29.34	29.78	27.22	130.01	129.76	27.17
Me 11-d ₁ -octadec-9Z-enoate	29.55	29.31 (γ : - 0.03)	28.70 (β : - 0.08)	26.87 (α : - 0.35)	129.97 (β : - 0.04)	129.78 (γ : + 0.02)	27.18

Appendix A4.

^{13}C NMR data for methyl $[14\text{-}^2\text{H}_1]\text{-}$ and $[14\text{-}^2\text{H}_2]\text{-}$ octadec-9Z,12Z-dienoate and corresponding synthetic intermediates: Deuterium Isotope Shifts (ppm)

Compound	C17 (C7)	C16 (C6)	C15 (C5)	C14 (C4)	C13 (C3)	C12 (C2)	C11 (C1)
2-octyn-1-ol	22.14	30.99	28.26	18.64	86.42	78.27	51.19
4-d ₁ -2-octyn-1-ol	22.14	30.95 (γ : -0.04)	28.15 (β : -0.11)	18.35 (α : -0.29)	86.55 (β : +0.13)	78.23 (γ : -0.04)	51.33
4,4-d ₂ -2-octyn-1-ol	22.14	30.93 (γ : -0.06)	28.05 (β : -0.21)	18.11 (α : -0.53)	86.52 (β : +0.10)	78.26 (γ : -0.01)	51.31
Me octadec-9,12-dienoate	22.22	31.08	28.47	18.71	80.53	74.44	9.72
Me 14-d ₁ -octadec-9,12-dienoate	22.23	31.06 (γ : -0.02)	28.38 (β : -0.09)	18.42 (α : -0.29)	80.50 (β : -0.03)	74.47 (γ : +0.03)	9.72
Me 14,14-d ₂ -octadec-9,12-dienoate	22.22	31.03 (γ : -0.05)	28.27 (β : -0.20)	18.12 (α : -0.59)	80.47 (β : -0.06)	74.47 (γ : +0.03)	9.71
Me octadec-9Z,12Z-dienoate	22.59	31.54	29.36	27.22	130.23	127.92	25.64
Me 14-d ₁ -octadec-9Z,12Z-dienoate	22.59	31.51 (γ : -0.03)	29.27 (β : -0.09)	26.86 (α : -0.36)	130.17 (β : -0.06)	127.94 (γ : +0.02)	25.64
Me 14,14-d ₂ -octadec-9Z,12Z-dienoate	22.59	31.49 (γ : -0.05)	29.17 (β : -0.19)	26.50 (α : -0.72)	130.13 (β : -0.10)	127.96 (γ : +0.04)	25.65

References:

1. Buist, P. H. *Nat. Prod. Rep.* **2004**, *21*, 249.
2. Buist, P. H. *In Comprehensive Natural Products II Chemistry and Biology*; Mander, L.; Lui, H.-W., Eds.; Elsevier: Oxford, **2010**, *1*, pp. 5-33.
3. Buist, P. H. *Nat. Prod. Rep.* **2007**, *24*, 1110.
4. Buist, P. H. *Wiley Encyclopedia of Chemical Biology*; Begley, T. P., Ed.; John Wiley & Sons: Hoboken, **2009**, *2*, pp. 1-7.
5. Gunstone, F. D.; Harwood, J. L.; Dijkstra, A. J. *Lipid Handbook*, 3rd Ed.; Taylor CRC Press: Boca Raton, **2007**, pp. 37-141.
6. Weete, J. D. *Lipid Biochemistry of Fungi and Other Organisms*, New York: Plenum Press, **1980**.
7. Gurr, M. I.; Harwood, J. L. *Lipid Biochemistry: An Introduction*, 4th ed., Chapman and Hall: London, **1991**.
8. Millar, A. A.; Smith, M. A.; Kunst, L. *Trends Plants Sci.* **2000**, *5*, 95.
9. Jaworski, J.; Cahoon, E. B. *Curr. Opin. Plant Biol.* **2003**, *6*, 178.
10. Lee, M.; Lenman, M.; Banas, A.; Bafor, M.; Singh, S. et al *Science*, **1998**, *280*, 915.
11. Cahoon, E. B.; Kinney, A. J. *J. Biol. Chem.* **2004**, *279*, 12495.
12. Van de Loo, F. J.; Broun, P.; Turner, S.; Summerville, C. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 6743.
13. Van de Loo, F. J.; Fox, B. G.; Somerville, C. *Lipid Metabolism in Plants*; Moore, T. S. Jr., Ed., CRC Press: Boca Raton, **1993**, pp. 91-126.
14. Hoffmann-Roder, A.; Krause, N. *Angew. Chem. Int. Ed.* **2004**, *43*, 1196.
15. Bagby, M. O.; Jr. Smith, C. R.; Wolff, I. A. *J. Org. Chem.* **1965**, *30*, 4227.
16. Broadwater, J. A.; Whittle, E.; Shanklin, J. *J. Biol. Chem.* **2002**, *277*, 15613.
17. Lu, C.; Wallis, J. G.; Browse, J. *BMC Plant Biol.* **2007**, *7*, 42.
18. Nunn, J. R. *J. Chem. Soc.* **1952**, 313.

19. Yano, I.; Morris, L. J.; Nichols, B. W.; James, A. T. *Lipids*, **1972**, *7*, 35.
20. Gomez, F. E.; Bauman, D. E.; Ntambi, J. M.; Fox, B. G. *Biochim. Biophys. Res. Commun.* **2003**, *300*, 316.
21. Jeffcoat, R.; Pollard, M. R. *Lipids*, **1977**, *12*, 480.
22. Johnson, A. R.; Pearson, J. A.; Shenstone, F. S.; Fogert, A. C. *Nature*, **1967**, *214*, 1244.
23. Fogert, A.C.; Johnson, A. R.; Pearson, J. A. *Lipids*, **1972**, *7*, 335.
24. Billault, I.; Duan, J. R.; Guiet, S.; Robins, R. J. *J. Biol. Chem.* **2005**, *280*, 17645.
25. Cahoon, E. B.; Ripp, K. G.; Hall, S. E.; McGonigle, B. *Plant Physiol.* **2002**, *128*, 615.
26. Reed, D. W.; Polichuk, D. R.; Buist, P. H.; Ambrose, S. J. et al *J. Am. Chem. Soc.* **2003**, *125*, 10635.
27. Minto, R. E.; Blacklock, B. J. *Prog. Lipid Res.* **2008**, *47*, 233.
28. Cahoon, E. B.; Schnurr, J. A.; Huffman, E. A.; Minto, R. E. *Plant J.* **2003**, *34*, 671.
29. Sperling, P.; Lee, M.; Thomas, G.; Zahringer, U.; Stymne, S.; Heinz, E. *Eur. J. Biochem.* **2000**, *267*, 3801.
30. Bohlmann, F.; Burkhardt, T.; Zdero, C. *Naturally Occurring Acetylenes*; Academic Press: London, **1973**.
31. Christensen, L. P.; Lam, J. *Phytochemistry*, **1991**, *30*, 2453.
32. Shi Shun, A. L. K.; Tykwinski, R. R. *Angew. Chem. Int. Ed.* **2006**, *45*, 1034.
33. Damste, J. S. S.; Strous, M.; Rijpstra, W. I.; Hopmans, E. C.; Geenevasen, J. A.; Van Duin, A. C.; Van Niftrik, L. A.; Jetten, M. S. *Nature*, **2002**, *419*, 708.
34. Dyer, J. M.; Chapital, D. C.; Kuan, J. et al *Plant Physiol.* **2002**, *130*, 2027.
35. Corl, B. A.; Baumgard, L. H.; Dwyer, D. A.; Griinari, J. M.; Phillips, B. S.; Bauman, D. E. *J. Nutr. Biochem.* **2001**, *12*, 622.
36. Zheng, W.; Wise, M.; Wyrick, A. Metz, J.; Yuan, L.; Gerwick, W. *Arch. Biochem. Biophys.* **2002**, *401*, 11.

37. Wise, M. L.; Hamberg, M.; Gerwick, W. H. *Biochemistry*, **1994**, *33*, 15223.
38. Griinari, J. M.; Bauman, D. E. *Advances in Conjugated Linoleic Acid Research*; AOCS Press, Champaign, IL, pp. 180-200.
39. Rodriguez, S.; Camps, F.; Fabrias, G. *J. Org. Chem.* **2001**, *66*, 8052.
40. Rodriguez, S.; Clapes, P.; Camps, F.; Fabrias, G. *J. Org. Chem.* **2002**, *67*, 2228.
41. Serra, M.; Pina, B.; Abad, J. L.; Camps, F.; Fabrias, G. *Proc. Natl. Acad. Sci. USA*, **2007**, *104*, 16444.
42. Liu, L.; Hammond, E. G.; Nikolau, B. J. *Plant Physiol.* **1997**, *113*, 1343.
43. Crombie, L.; Holloway, S. L. *J. Chem. Soc. Perkin Trans. 1* **1985**, 2425.
44. Booth, R. G.; Kon, S. K.; Dann, W. J.; Moore, T. *Biochem. J.* **1935**, *29*, 133.
45. Parodi, P. W. *J. Dairy Sci.* **1977**, *60*, 1550.
46. Yuan, G.; Sun, H.; Sinclair, A. J.; Li, D. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 537.
47. Park, Y.; Pariza, M. W. *Food Res. Int.* **2007**, *40*, 311.
48. Chardingy, J. M.; Hasselwander, O.; Genty, M.; Kraemer, M.; Ptock, A.; Sebedio, J. L. *Lipids*, **2003**, *38*(9), 895.
49. Liavonchanka, A.; Hornung, E.; Feussner, I.; Rudolph, M. G. *Proc. Natl. Acad. Sci. USA*, **2006**, *103*(8), 2576-2581.
50. Park, Y. *J. Food Comp. Anal.* **2009**, *225*, S4.
51. Corl, B. A.; Barbano, D. M.; Bauman, D. E.; Ip, C. *J. Nutr. Biochem.* **2003**, *133*, 2893.
52. Belury, M. A.; *Nutr. Rev.* **1995**, *53*, 83.
53. Park, Y.; Albright, K. J.; Storkson, J. M.; Liu, W.; Pariza, M. W. *J. Food Sci.* **2007**, *72*, S612.
54. Kelly, N. S.; Hubbard, N. E.; Erickson, K. L. *J. Nutr.* **2007**, *137*, 2599.
55. Bauman, D. E.; Baumgard, L. H.; Corl, B. A.; Griinari, J. M. *Proc. Am. Soc. Animal Sci.* **1999**, 1.

56. Kemp, P.; Lander, D. J. *J. Gen. Microbiol.* **1984**, *130*, 527; Kepler, C. R.; Hirons, K. P. McNeill, J. J.; Tove, S. B. *J. Biol. Chem.* **1966**, *241*, 1350.
57. Ha, Y. L.; Grimm, N. K.; Pariza, M. W. *Carcinogenesis*, **1987**, *8*, 1881.
58. Pariza, M. W.; Hargraves, W. A. *Carcinogenesis*, **1985**, *6*, 591.
59. Tsuzuki, T.; Tokuyama, Y.; Igarashi, M. et al *J. Nutr.* **2004**, *134*, 2634.
60. Igarashi, M.; Miyazawa, T. *Cancer Lett.* **2000**, *148*, 173.
61. Tsuzuki, T.; Tokuyama, Y.; Igarashi, M.; Miyazawa, T. *Carcinogenesis*, **2004**, *25*, 1417.
62. Cahoon, E. B.; Dietrich, C. R.; Meyer, K. Damude, H. G.; Dyer, J. M.; Kinney, A. *J. Phytochemistry*, **2006**, *67*, 1166.
63. Cahoon, E. B.; Ripp, K. G.; Hall, S. E.; Kinney, A. J. *J. Biol. Chem.* **2001**, *276*, 2637.
64. Hennessy, A. A.; Ross, R. P.; Devery, R. *Lipids*, **2011**, *46*, 105.
65. Koba, K.; Akahoshi, A.; Yamasaki, M. Tanaka, K. et al *Lipids*, **2002**, *37*, 343.
66. Yamasaki, M.; Kitagawa, T.; Koyanagi, N. et al *Nutrition*, **2006**, *22*, 54.
67. Al-Madaney, M. M.; Kramer, J. K.; Deng, Z.; Vanderhoek, J. Y. *Biochim. Biophys. Acta*, **2003**, *1635*, 75.
68. Li, G.; Barnes, D.; Butz, D.; Bjorling, D.; Cook, M. E. *J. Lipid Res.* **2005**, *46*, 2134.
69. Lai, C.; Yin, J.; Li, D.; Zhao, L.; Chen, X. *Archives Animal Nutr.* **2005**, *59*, 41.
70. Ritzenthaler, K. L.; McGuire, M. K. et al *J. Nutr.* **2001**, *131*, 1548.
71. Serra, M.; Pina, B.; Bujons, J.; Camps, F.; Fabrias, G. *Insect Biochem. Mol. Biol.* **2006**, *36*, 634.
72. Oldham, N. J.; Boland, W. *Naturwissenschaften*, **1996**, *83*, 248.
73. Howard, R. W.; Blomquist, G. J. *Ann. Rev. Entomol.* **2005**, *50*, 371.
74. Tillman, J. A.; Seybold, S. J.; Jurenka, R. A.; Blomquist, G. J. *Insect Biochem. Mol. Biol.* **1999**, *29*, 481.

75. Abad, J. L.; Camps, F.; Fabrias, G. *J. Am. Chem. Soc.* **2007**, *129*, 15007.
76. Rodriguez, S.; Hao, G.; Liu, W.; Pina, B.; Rooney, A. P. Camps, F.; Roelofs, W. L.; Fabrias, G. *Insect Biochem. Mol. Biol.* **2004**, *34*, 1315.
77. Serra, M.; Gauthier, L. T.; Fabrias, G.; Buist, P. H. *Insect Biochem. Mol. Biol.* **2006**, *36*, 822.
78. Villorbina, G.; Roura, L.; Camps, F.; Joglar, J.; Fabrias, G. *J. Org. Chem.* **2003**, *68*, 2820.
79. Abad, J. L.; Camps, F.; Fabrias, G. *Angew. Chem. Int. Ed.* **2000**, *39*, 3279.
80. Moto, K.; Suzuki, M. G.; Hull, J. J.; Kurata, R. et al *Proc. Natl. Acad. Sci. USA*, **2004**, *101*, 8631.
81. Abad, J. L.; Villorbina, G.; Fabrias, G.; Camps, F. *J. Org. Chem.* **2004**, *69*, 7108.
82. Smith, C. R. Jr. *In Progress in the Chemistry of Fats and Other Lipids*; Holman, R. T. Ed.; Pergammon: London, **1970**, *11*, 137.
83. Napier, J. A. *Annu. Rev. Plant Biol.* **2007**, *58*, 295.
84. Cahoon, E. B.; Shockey, J. M.; Dietrich, C. R.; Gidda, S. K.; Mullen, R. T.; Dyer, J. M. *Curr. Opin. Plant Biol.* **2007**, *10*, 236.
85. Broun, P.; Gettner, S.; Somerville, C. *Annu. Rev. Nutr.* **1999**, *19*, 197.
86. Cloez, *Compt. Rend.*, **1875**, *81*, 469; **1876**, *82*, 501; **1876**, *83*, 943.
87. Boeseken and Ravenswaay, *Rec. Trav. Chim.*, **1925**, *44*, 241.
88. Crombie, L.; Jacklin, A. G. *J. Chem. Soc.* **1957**, 1632.
89. Yang, P.; Li, X.; Shipp, M.; Shockey, J. M.; Cahoon, E. B. *BMC Plant Biol.* **2010**, *10*, 250.
90. Hopkins, C. Y.; Chisholm, M. J. *J. Am. Oil. Chem. Soc.* **1968**, *45*, 176.
91. Chisholm, M. J.; Hopkins, C. Y. *Can. J. Biochem.* **1967**, *45*, 1081.
92. Cahoon, E. B.; Carlson, T. J.; Ripp, K. G. et al, *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 12935.
93. Qiu, X.; Reed, D. W.; Hong, H.; Mackenzie, S. L.; Covello, P. S. *Plant Physiol.* **2001**, *125*, 847.

94. Gunstone, F. D.; Subbarao, R. *Chem. Phys. Lipids*, **1967**, *1*, 349.
95. Bagby, M. O.; Smith, C. R. Jr.; Wolff, I. A. *Lipids*, **1966**, *1*, 263.
96. Formo, M. W. In *Bailey's Industrial Oil and Fat Products*; Swern, D. Ed.; John Wiley & sons: New York, **1979**, pp. 708-709.
97. Sonntag, N. O. V. In *Bailey's Industrial Oil and Fat Products*; Swern, D. Ed.; John Wiley & sons: New York, **1979**, pp. 289-477.
98. Yasui, Y.; Hosokawa, M.; Kohno, H.; Tanaka, T.; Miyashita, K. *Anticancer Res.* **2006**, *26*, 1855.
99. Brown, D. J.; Dattner, A. M. *Archives of dermatology*, **1998**, *134*, 1401.
100. Ley, S. V.; Meek, G. J. *Chem. Soc. Perkin Trans. 1*, **1997**, 1125.
101. Palmer, C. N. A.; Wolf, C. R. *FEBS Letters*, **1998**, *431*, 476.
102. Steenbergen, R. H. G.; Drummen, G. P. C.; Kamp, J. A. F.; Post, J. A. *Biochim. Biophys. Acta*, **1997**, *1330*, 127.
103. Zaheer, A.; Sahu, S. K.; Ryken, T. C.; Traynelis, V. C. *Neurochem. Res.* **2007**, *32*, 115-124.
104. Shanklin, J.; Cahoon, E. B. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 611.
105. Brenner, R. R. *Prostaglandins, Leukotrienes Essent. Fatty Acids*, **2003**, *68*, 71.
106. Breslow, R. *Chem. Soc. Rev.* **1972**, *1*, 553.
107. Bloomfield, D. K.; Bloch, K. *Biochem. Biophys. Acta*, **1958**, *30*, 220.
108. Bloomfield, D. K.; Bloch, K. *J. Biol. Chem.* **1960**, *235*, 337.
109. Schroepfer, G. J.; Bloch, K. *J. Biol. Chem.* **1965**, *240*, 54.
110. Behrouzian, B.; Buist, P. H. *Curr. Opin. Chem. Biol.* **2002**, *6*, 577.
111. Behrouzian, B.; Fauconnot, L.; Daligault, F.; Nugier-Chauvin, C.; Patin, H.; Buist, P. H. *Eur. J. Biochem.* **2001**, *268*, 3545.
112. Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1998**, *120*, 871.

113. Fox, B. G.; Shanklin, J.; Ai, J.; Loehr, T. M.; Sanders-Loehr, J. *Biochemistry*, **1994**, *33*, 12776.
114. Shanklin, J.; Whittle, E.; Fox, B. G. *Biochemistry*, **1994**, *33*, 12787.
115. Shanklin, J.; Achim, C.; Schmidt, H.; Fox, B. G.; Munck, E. *Proc. Natl. Acad. Sci. USA*, **1997**, *94*, 2981.
116. Whittle, E. J.; Tremblay, A. E.; Buist, P. H.; Shanklin, J. *Proc. Natl. Acad. Sci. USA*, **2008**, *105*, 14738.
117. Guy, J. E.; Whittle, E. J.; Kumaran, D.; Lindqvist, Y.; Shanklin, J. *J. Biol. Chem.* **2007**, *282*, 19863.
118. Lindqvist, Y.; Huang, W.; Schneider, G.; Shanklin, J. *EMBO J.* **1996**, *15*, 4081.
119. Moche, M.; Shanklin, J.; Ghoshal, A.; Lindqvist, Y. *J. Biol. Chem.* **2003**, *278*, 25072.
120. Dyer, D. H.; Lyle, K. S.; Rayment, I.; Fox, B. G. *Protein Sci.* **2005**, *14*, 1508.
121. Cahoon, E. B.; Shanklin, J.; Ohlrogge, J. B. *Proc. Natl. Acad. Sci. USA*, **1992**, *89*, 11184.
122. Cahoon, E. B.; Becker, C. K.; Shanklin, J.; Ohlrogge, J. B. *Plant Physiol.* **1994**, *106*, 807.
123. Cahoon, E. B.; Cranmar, A. M.; Shanklin, J.; Ohlrogge, J. B. *J. Biol. Chem.* **1994**, *269*, 27519.
124. Shanklin, J.; Somerville, C. *Proc. Natl. Acad. Sci. USA*, **1991**, *88*, 2510.
125. Thompson, G. A.; Scherer, D. E.; Aken, S. F.; Kenny, J. W. *Proc. Natl. Acad. Sci. USA*, **1991**, *88*, 2578.
126. Baik, M. H.; Newcomb, M.; Friesner, R. A.; Lippard, S. J. *Chem. Rev.* **2003**, *103*, 2385.
127. Behrouzian, B.; Savile, C. K.; Dawson, B.; Buist, P. H.; Shanklin, J. *J. Am. Chem. Soc.* **2002**, *124*, 3277.
128. Strittmatter, P.; Spatz, L.; Corcoran, D.; Rogers, M. J.; Setlow, B.; Redline, R. *Proc. Natl. Acad. Sci. USA*, **1974**, *71*, 4565.
129. Enoch, H. G.; Catala, A.; Strittmatter, P. *J. Biol. Chem.* **1976**, *251*, 5095.

130. Mason, H. S. *Adv. Enzymol.* **1957**, *19*, 79.
131. Shanklin, J.; Achim, C.; Schmidt, H.; Fox, B.; Munck, E. *Proc. Natl. Acad. Sci. USA*, **1997**, *94*, 2981.
132. Sperling, P.; Heinz, E. *Eur. J. Lipid Sci. Technol.* **2001**, *103*, 158.
133. Thiede, M.; Ozols, J.; Strittmatter, P. *J. Biol. Chem.* **1986**, *261*, 13230.
134. Stukey, J. E.; McDonough, V. M.; Martin, C. E. *J. Biol. Chem.* **1990**, *265*, 20144.
135. Mayer, K. M.; McCorkle, S. R.; Shanklin, J. *BMC Bioinformatics*, **2005**, *2*, 284.
136. Heinz, E. *Biosynthesis of Polyunsaturated Fatty Acids. In Lipid Metabolism in Plants*; T. S. Morre, Jr., Ed.; CRC Press: Boca Raton, **1993**; pp 33-89.
137. Schwartzbeck, J. L.; Jung, S.; Abbott, A. G. et al *Phytochemistry*, **2001**, *57*, 643.
138. Covello, P. S.; Reed, D. W. *Plant Physiol.* **1996**, *111*, 223.
139. Pinilla, A.; Camps, F.; Fabrias, G. *Biochemistry*, **1999**, *38*, 15272.
140. Morris, L. J.; Harris, R. V.; Kelly, W.; James, A. T. *Biochem. J.* **1968**, *19*, 673.
141. Shu, L.; Nesheim, J. C.; Kauffmann, K.; Munck, E.; Lipscomb, J. D.; Que, L. Jr., *Science* **1997**, *275*, 515.
142. Cook, G. K.; Mayer, J. M. *J. Am. Chem. Soc.* **1994**, *116*, 1855.
143. Light, R. J.; Lennarz, W. J.; Bloch, K. *J. Biol. Chem.* **1962**, *237*, 1793.
144. Brett, D.; Howling, D.; Morris, L. J.; James, A. T. *Arch Biochem Biophys.* **1971**, *143*, 535.
145. Stoffel, W.; Assmann, G.; Bister, K. *Hoppe-Seyler's Z. Physiol. Chem.* **1971**, *352*, 1531.
146. Navarro, I. F.; Fabrias, G.; Camps, F. *J. Am. Chem. Soc.* **1997**, *119*, 11335.
147. Rawlings, L.; Reese, P. B.; Ramer, S. E.; Vederas, J. C. *J. Am. Chem. Soc.* **1989**, *111*, 3382.
148. McInnes, A. G.; Walter, J. A.; Wright, J. L. C. *Tetrahedron*, **1983**, *39*, 3515.
149. Buist, P. H.; Marecak, D. M. *J. Am. Chem. Soc.*, **1991**, *113*, 5877.

150. Buist, P. H.; Marecak, D. M. *Can. J. Chem.* **1994**, *72*, 176.
151. Buist, P. H.; Marecak, D. M. *J. Am. Chem. Soc.* **1992**, *114*, 5073.
152. Pirkle, W. H.; Beare, S. D.; Muntz, R. L. *Tett. Lett.* **1974**, *26*, 2295.
153. Tremblay, A. E.; Tan, N.; Whittle, E.; Hodgson, D. J.; Dawson, B.; Buist, P. H.; Shanklin, J. *Org. Biomol. Chem.* **2010**, *8*, 1322.
154. Seco, J. M.; Latypov, S.; Quinoa, E.; Riguera, R. *Tett. Lett.* **1994**, *35*, 2921.
155. Lao, K. Y. Y.; Hodgson, D. J.; Dawson, B.; Buist, P. H. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2799.
156. Tremblay, A. E.; Lao, K. Y. Y.; Hodgson, D. J.; Dawson, B.; Buist, P. H. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5146.
157. Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1996**, *118*, 6295.
158. Reed, D. W.; Savile, C. K.; Qiu, X.; Buist, P. H.; Covello, P. S. *Eur. J. Biochem.* **2002**, *269*, 5024.
159. Beckmann, C.; Rattke, J.; Oldham, N. J.; Sperling, P.; Heinz, E.; Boland, W. *Angew. Chem. Int. Ed.* **2002**, *41*, 2298.
160. Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 2171.
161. Behrouzian, B.; Buist, P. H.; Shanklin, J. *Chem. Commun.* **2001**, 401.
162. Lyle, K. S.; Haas, J. A.; Fox, B. *Biochemistry*, **2003**, *42*, 5857.
163. Fauconnot, L.; Buist, P. H. *J. Org. Chem.* **2001**, *66*, 1210.
164. Buist, P. H.; Marecak, D. M.; Dawson, B.; Black, B. *Can. J. Chem.* **1996**, *74*, 453.
165. Hodgson, D. J.; Lao, K. Y. Y.; Dawson, B.; Buist, P. H. *Helv. Chim. Acta*, **2003**, *86*, 3688.
166. Behrouzian, B.; Hodgson, D.; Savile, C. K.; Dawson, B.; Buist, P. H.; Shanklin, J. *Mag. Res. Chem.* **2002**, *40*, 524.
167. Gakh, Y. G.; Gakh, A. A.; Gronenborn, A. M. *Mag. Res. Chem.*, **2000**, *38*, 551.
168. Carvalho, F.; Gauthier, L. T.; Hodgson, D. J.; Dawson, B.; Buist, P. H. *Org. Biomol. Chem.* **2005**, *3*, 3979.

169. Hodgson, D. J.; Buist, P. H. *Tetrahedron Asymmetry*, **2003**, *14*, 641.
170. Zhou, J.; Kelly, E. L.; Bachmann, B. O.; Gunsior, M.; Townsend, C. A.; Solomon, E. I. *J. Am. Chem. Soc.* **2001**, *123*, 7388.
171. Rettie, E.; Rettenmeier, A. W.; Howald, W. N.; Baillie, T. A. *Science*, **1987**, *235*, 890.
172. Broun, P.; Shanklin, J.; Whittle, E.; Somerville, C. *Science*, **1998**, *282*, 1315.
173. Crombie, L.; Heavers, A. D. *J. Chem. Soc. Perkin Trans. 1*, **1992**, 1929.
174. Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217.
175. Durand, S.; Parrain, J.; Santelli, M. *J. Chem. Soc. Perkin Trans. 1*, **2000**, 253.
176. Jeffery, T.; Gueugnot, S.; Linstrumelle, G. *Tett. Lett.* **1992**, *33*, 5757.
177. Caruso, T.; Spinella, A. *Tetrahedron*, **2003**, *59*, 7787.
178. Tedeschi, C.; Saccavini, C.; Maurette, L.; Soleilhavoup, M.; Chauvin, R. *J. Organomet. Chem.* **2003**, *670*, 151.
179. Fujita, K.; Yorimitsu, H.; Shinokubo, H.; Oshima, K. *J. Am. Chem. Soc.* **2004**, *126*, 6776.
180. Fu, Y.; Weng, Y.; Hong, W.; Zhang, Q. *Synlett.* **2011**, (6), 809.
181. Jacquot, C.; McGinley, C. M.; Plata, E.; Holman, T. R.; van der Donk, W. A. *Org. Biomol. Chem.* **2008**, *6*, 4242.
182. Savile, C. K.; Reed, D. W.; Meesapyodsuk, D.; Covello, P. S.; Buist, P. H. *J. Chem. Soc., Perkin Trans. 1*, **2001**, 1116.
183. Mancuso, A. J.; Huang, S.; Swern, D. *J. Org. Chem.* **1978**, *43*, 2480.
184. Rawlings, B. J.; Reese, P. B.; Ramer, S. E.; Vederas, J. C. *J. Am. Chem. Soc.* **1989**, *111*, 3382.
185. Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352; Midland, M. M.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1977**, *99*, 5211.
186. Lorca, M.; Kuhn, D.; Kurosu, M. *Tett. Lett.* **2001**, *42*, 6243.

187. Chambez, E. M.; Horning, E. C. *Anal. Letters*, **1968**, *1*, 201; Klebe, J. F. *J. Am. Chem. Soc.* **1966**, *88*, 3390.
188. Midland, M. M.; McDowell, D. C.; Hatch, R. L.; Tramontano, A. *J. Am. Chem. Soc.* **1980**, *102*, 867.
189. Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543-2549; Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512.
190. Spinella, A.; Caruso, T.; Martino, M.; Sessa, C. *Synlett*. **2001**, (12), 1971.
191. Presser, A.; Hüfner, A. *Monatshefte für Chemie*, **2004**, *135*, 1015.
192. Gunstone, F. D.; Pollard, M. R.; Scrimgeour, C. M.; Vedanayagam, H. S. *Chem. Phys. Lipids*, **1977**, *18*, 115.
193. Hallgren, B.; Ryhage, R.; Stenhagen, E. *Acta. Chem. Scand.* **1959**, *13*, 845.
194. Gunstone, F. D.; Lie Ken Jie, M.; Wall, R. T. *Chem. Phys. Lipids*, **1969**, *3*, 297.
195. Nagel, Z. D.; Klinman, J. P. *Chem. Rev.* **2006**, *106*, 3095.
196. Fletcher, S.; Ahmad, A.; Perouzel, E.; Heron, A. et al *J. Med. Chem.* **2006**, *49*, 349.
197. Frost, D. J.; Gunstone, F. D. *Chem. Phys. Lipids*, **1975**, *15*, 53.
198. Kuroda, H.; Hanaki, E.; Izawa, H.; Kano, M.; Itahashi, H. *Tetrahedron*, **2004**, *60*, 1913.
199. Melander, L.; Saunders, W. H. Jr. *Reaction Rates of Isotopic Molecules*, 2nd Ed.; Wiley and Sons: Hoboken, **1979**, pp 91-129.
200. Fritsche, K.; Hornung, E.; Peitzsch, N.; Renz, A.; Feussner, I. *FEBS Letters*, **1999**, *462*, 249.
201. Northrop, D. B. *Annu. Rev. Biochem.* **1981**, *50*, 103.
202. Schomaker, J. M.; Pulgam, V. R.; Borhan, B. *J. Am. Chem. Soc.* **2004**, *126*, 13600.
203. Iwasaki, G.; Sano, M.; Sodeoka, M.; Yoshida, K.; Shibasaki, M. *J. Org. Chem.* **1988**, *53*, 4864.
204. Griffith, W. P.; Kwong, E. *Synth. Commun.* **2003**, *33*, 2945.

205. Ashby, E. C.; Goel, A. B. *J. Org. Chem.* **1981**, *46*, 3936.
206. Parker, D. *J. Chem. Soc. Perkin Trans. II*, **1983**, 83.
207. Maskill, H. *The Physical Basis of Organic Chemistry*, Oxford University Press, USA, **1985**, pp 367-404.
208. Dussault, P. H.; Eary, C. Y.; Woller, K. R. *J. Org. Chem.* **1999**, *64*, 1789.
209. Olah, G. A.; Prakash, G. K. S.; Arvanaghi, M. *Synthesis*, **1984**, (3), 228.
210. Ashby, E. C.; Goel, A. B. *J. Org. Chem.* **1981**, *46*, 3936.
211. Savile, C. K.; Reed, D. W.; Buist, P. H. et al *J. Chem. Soc. Perkin Trans. I*, **2001**, 1116.
212. Sakai, T.; Hamamoto, H.; Mori, K. *Agri. Biol. Chem.* **1986**, *50*, 1621.
213. Dauenpen, M.; Reed, D. W.; Savile, C. K.; Buist, P. H. et al *Biochemistry*, **2000**, *39*, 11948.