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**Linking Sphingosine and Phytosphingosine Formation:
Cryptoregiochemistry of Dihydroceramide Δ^4 Desaturase**

by

Christopher K. Savile, B. Sc.

A thesis submitted to the Faculty of Graduate Studies in
partial fulfillment of the requirements for the degree of
Master of Science

to

The Faculty of Graduate Studies and Research
Department of Chemistry
Carleton University
Ottawa, Canada
February 2001

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the Faculty of Graduate Studies and Research
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Cryptoregiochemistry of Dihydroceramide Δ^4 Desaturase**

Submitted by
Christopher K. Savile

in partial fulfillment of the requirements
for the degree of Master of Science

Chair, Department of Chemistry

Thesis Supervisor

Carleton University

Abstract

The intermolecular primary deuterium isotope effects on the individual C-H bond cleavage steps involved in dihydroceramide Δ^4 desaturation have been determined by incubating rat liver microsomes with a 1:1 mixture of non-labelled substrate and the appropriate regiospecifically dideuterated analogues. Analysis of the enzymatic products via gas chromatography coupled to mass spectrometry showed that the introduction of the (*E*)- double bond between C-4 and C-5 occurs in two discrete steps: Cleavage of the C4-H bond was found to be very sensitive to isotopic substitution ($k_H/k_D = 7.8 \pm 0.2$), while a negligible isotope effect ($k_H/k_D = 1.05 \pm 0.03$) was observed for the C5-H bond breaking step. According to our mechanistic model, these results suggest that initial oxidation for this desaturation reaction is at C-4. This finding correlates nicely with the observation that 4-hydroxylated products are produced by a closely related enzyme in other aerobic life forms. Taken together, these data substantiate the notion that hydroxylation and desaturation are intimately linked at the mechanistic level and represent two reaction pathways which branch after a common initiation step.

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Chapter 1 Introduction

Dihydroceramide Δ^4 Desaturase: The Missing Link in Sphingolipid Biosynthesis

1.1 Structure of Sphingolipids

Sphingolipids are a large class of eukaryotic lipids that exist as structural components of cell membranes in animals, plants and some microbial systems (1). Sphingolipids are structurally diverse, but possess a common hydrophobic component, generically referred to as ceramide, which is composed of a long-chain base (sphingoid base) carrying *N*-acylated fatty acids of 16-24 carbon atoms (2). The sphingoid base is formed from three units: a basic amino alcohol with the 2*S*, 3*R* configuration, a long chain aliphatic backbone, and a polar head group (Figure 1.1).

Figure 1.1 - Key structural features of a sphingoid backbone

Sphinganine, 4-sphingenine (sphingosine), and 4-hydroxysphinganine (phytosphingosine) (Figure 1.2) are the most abundant sphingoid bases in the sphingolipids of the animal and plant kingdoms (3). However, the sphingoid base is a highly variable component of sphingolipids, with diverse differences in the structure of the long alkyl chain.

Figure 1.2 - Most common sphingoid bases

Depending on the source, the basic structure can be modified by differences in chain length, degree of unsaturation, methyl branching, and insertion of additional hydroxy groups (3,4). Addition of various sugar residues and other polar head groups to the ceramide give complex sphingolipids, such as glycosylceramide and sphingomyelin (Figure 1.3) (3).

Figure 1.3 - Complex sphingolipids

As a result, there are currently over 300 known sphingolipids with distinct head groups all containing the sphingoid base (5).

1.2 Biochemistry of Sphingolipids

In addition to their structural roles, sphingolipids are potent biologically active molecules that are involved in regulation of important biological processes, such as cell growth, cell differentiation, cell-cell recognition and apoptosis (programmed cell death) (6,7,8,9). Ceramide is also an important regulator of cellular responses to stress (10). Sphingosine-1-phosphate is a potent mitogen that regulates mobilization of calcium, cell growth, survival and motility (10). For most of these biological activities, the hydrophobic portion of sphingolipids, ceramide (Scheme 1.1), requires a desaturated sphingoid base backbone (sphingosine, Figure 1.2) (11). Until recently, very little was known about the introduction of this 4,5-*trans* double bond into the sphingoid base backbone during *de novo* biosynthesis (12,13).

1.2.1 Biosynthesis of Ceramide

A detailed pathway for sphingolipid biosynthesis is shown in Scheme 1.1. In animal cells, the initial steps from the condensation of serine and palmitoyl-CoA through the formation of ceramide take place in the endoplasmic reticulum. Subsequent biosynthesis of glycosphingolipids and sphingomyelin takes place in the Golgi apparatus. Degradation of complex sphingolipids occurs in the lysosomes, endosomes, and the plasma membrane, with degradation of free sphingoid bases occurring in the cytosol (3,5).

De novo sphingolipid biosynthesis is currently believed to occur as follows (Scheme 1.2) (3,5,14,15): Condensation of L-serine and palmitoyl-CoA gives 3-ketosphinganine. This compound is immediately reduced by 3-dehydrosphinganine reductase yielding D-*erythro*-sphinganine. Whether this sphingoid base was first dehydrogenated to sphingosine and then acylated to yield ceramide or first acylated then dehydrogenated remained unclear for sometime.

CCCCC(C)C(OR)C(OR)C(C)C(O)CCCC(O)C(O)C(N)C

$$R = \text{COCH}_2\text{CH}(\text{COOH})\text{CH}_2\text{COOH}$$

Figure 1.4 - Fumonisin B₁

Although the first three enzymes involved in sphingolipid biosynthesis are well characterized, the enzymatic step responsible for introduction of the 4,5-*trans* double bond was not well understood until recently (12,13,19). Michel *et al.* demonstrated that conversion of dihydroceramide into ceramide required reducing equivalents of NAD(P)H and was dependent on molecular oxygen (13). This suggested that the reaction was catalyzed by a desaturase (Scheme 1.3) and not a “dehydrogenase” as was generally believed (12,13). Dehydrogenase enzymes formally remove a proton and hydride ion from adjacent activated carbon atoms (ie. α -, and β - to a carbonyl) and are O_2 -independent. In contrast, desaturases remove hydrogen atoms from an unactivated position in an aliphatic chain and require O_2 as the electron acceptor.

Scheme 1.3 – Desaturation of dihydroceramide

Fatty acid desaturases consist of a series of coupled reactions that transport electrons from NAD(P)H to a terminal desaturase which reduces oxygen and dehydrogenates a substrate (reviewed in Ref. 20). The components and intermediary reactions of the desaturase were shown to be very similar to that of the well-characterized microsomal Δ^9 stearoyl-CoA desaturase (20) with regard to cofactors and inhibitors (12,13). These results allowed for the assignment of the name dihydroceramide desaturase to the enzyme that catalyzes this reaction, and provided further proof that

introduction of the 4,5-*trans* double bond of sphingosine occurs at the level of dihydroceramide (Scheme 1.3).

This not only filled the “gap” in the pathway of sphingolipid biosynthesis, but it also provided information on a possible site of regulation. That is, since not only ceramide derived from the sphingomyelin pool but also that from *de novo* synthesis is involved in cellular responses (Scheme 1.1) (21,22), dihydroceramide desaturase may play an important role in cell regulation (13).

1.3 Fatty Acid Desaturases: Structure and Biochemistry

Fatty acid desaturases are a ubiquitous group of enzymes that catalyze the dehydrogenation (desaturation) of unactivated fatty acyl hydrocarbon chains. The prototypical reaction, first discovered by Bloch in 1958 (23), is the oxidation of stearoyl-CoA to give oleoyl-CoA as it occurs in *Saccharomyces cerevisiae*, an organism more commonly known as baker’s yeast (Scheme 1.4). This process features the four electron reduction of O_2 to give two molecules of H_2O . Two electrons come from the *pro*-(*R*) hydrogens at C-9 and C-10; the other two electrons are provided by NADH.

Scheme 1.4 – Desaturation of stearoyl-CoA

A simplified nomenclature for desaturases specifies the position of the double bond in the product relative to the carboxyl group (ie. Δ^9 desaturase (Scheme 1.4)).

The desaturation of fatty acids occurs in a wide variety of biological species, including plants and animals, and plays an important role in the proper functioning of these organisms (reviewed in Ref. 20). Two specific classes of fatty acid desaturases exist: enzymes which are membrane bound and those that are soluble. These two desaturase systems share many common features but differ with respect to the acyl derivatives that will serve as substrates, the components which are necessary for electron transfer and the kinetic mechanism by which substrate oxidation proceeds (24,25).

1.3.1 Membrane-bound Fatty Acid Desaturases

Membrane bound fatty acid desaturases were first studied by Bloch *et al.* in *S. cerevisiae* for the Δ^9 desaturation of various fatty acids (26). It was found that reducing equivalents were provided by NADH or NADPH and that oxygen is an obligatory electron acceptor (26). These requirements have been demonstrated in a wide variety of membrane-bound systems in different species (ie. animal, bacteria, yeast, fungi, plants) (27). Further work on mammalian systems has shown that these desaturases are specific for acyl-CoA or acyl-lipid derivatives of fatty acid substrates. (27,28).

The initial breakthrough in understanding the membrane-bound desaturases came from the studies of hen- and rat-liver microsomes by Holloway *et al.* (29) and Sato *et al.* (30). They demonstrated that the mammalian Δ^9 desaturase system consisted of three major proteins (Figure 1.5): NADH-cytochrome b_5 reductase, cytochrome b_5 and a terminal desaturase component (29,30). The latter protein was inhibited by CN and was thus labelled the “cyanide-sensitive” factor. Cytochrome b_5 reductase and cytochrome b_5 are amphipathic proteins containing two domains: one hydrophilic and one hydrophobic (31,32). The hydrophilic portions are involved in electron transfer, while the hydrophobic areas serve to anchor the proteins in the phospholipid membrane (32).

Figure 1.5 – Schematic representation of the desaturase complex

The reductase delivers the reducing equivalents of NADH to the heme-iron centre of cytochrome b_5 which in turn reduces the non-heme iron containing desaturase enzyme at appropriate times in the catalytic cycle (33, 34). The desaturase protein is then capable of binding molecular oxygen and long chain saturated fatty acyl-CoA derivatives and catalyzing the dehydrogenation of the substrate with concomitant reduction of O_2 to $2\text{H}_2\text{O}$. Analysis of the Δ^6 desaturase from rat-liver microsomes demonstrated an electron transport protein system similar to that of the Δ^9 (35).

Recent hydropathy analyses (36) indicate that the enzyme contains at least four long hydrophobic domains which would be long enough to span the membrane bilayer twice (Figure 1.6). As well, Shanklin and Fox demonstrated by comparative sequence analysis that membrane-bound desaturase proteins possess eight highly-conserved histidine residues (37). Site-directed mutagenesis experiments have indicated that these amino acids are essential for catalytic activity and it has therefore been postulated that these residues act as ligands for the iron atom(s) contained in these enzymes (37). It is interesting to note that alkane ω -hydroxylase from *Pseudomonas oleovorans* also contains a multi-histidine motif similar to the membrane desaturases and has been shown by Mössbauer spectroscopy to contain a diiron cluster (38).

Figure 1.6 – Model of the topology of membrane-bound desaturases

Since the requirements for desaturation are closely related to those involved in hydroxylation reactions, it has long been suspected that desaturases may have protein fold and active site features, including a diiron core, similar to those in hydrocarbon hydroxylases (37,38). Unfortunately, progress in understanding the 3-D structure of these systems has stalled because of the instability of membrane-bound desaturase systems upon purification.

1.3.2 Soluble Fatty Acid Desaturases

Soluble fatty acid desaturases, found exclusively in plants, are highly specific for stearyl-ACP and require NADPH, NADPH-ferredoxin oxido-reductase, ferredoxin and molecular oxygen for desaturation (Scheme 1.5) (39).

Scheme 1.5 – Electron flow in stearyl-ACP desaturation

The soluble desaturases have proved much more amenable to characterization. Unlike the unstable membrane-bound desaturases, soluble desaturases can be purified via carefully controlled processes (40). Purification of a soluble plant Δ^9 desaturase from *Ricinus communis* (castor) seed, by Shanklin *et al.*, revealed the protein to be a homodimer (ca 70 kDa) with no substantial homology with the corresponding membrane-bound enzymes (41). Each subunit was demonstrated to have two atoms of catalytically essential iron, by Mössbauer (42) and Resonance Raman spectroscopy (43), which reside in a μ -oxo diiron cluster with properties similar to that found in methane monooxygenase (MMO), an enzyme that converts methane and small hydrocarbons to alcohols (44).

An important milestone in the study of fatty acid desaturases has been the crystallization of the Δ^9 stearoyl-ACP desaturase from castor seed (45) followed by its X-ray structure determination (46). The structure of this protein (Figure 1.7) confirms the presence of a diiron cluster as predicted by spectroscopic methods. Computer modelling of stearic acid in the presumed substrate binding pocket places C-9 in the vicinity of one of the irons thought to bind to molecular oxygen.

The two iron atoms are bound in a highly symmetrical environment (Figure 1.8), with one of the irons forming interactions with the side chains of E (glutamate) 196 and H (histidine) 232 and the second iron with the side chains of E105 and H146. Two additional glutamic acid side chains, from E143 and E229, are also within coordination distance. A water molecule is found within the second coordination sphere of one of the iron atoms. The lack of electron density corresponding to a μ -oxo bridge suggested that the enzyme is in reduced form due to photochemical reduction by X-ray radiation (46).

Figure 1.7 – X-ray crystal structure of the soluble Δ^9 desaturase

Figure 1.8 – View of diiron centre in Δ^9 desaturase (reduced form) with ligands

1.3.3 Dihydroceramide Desaturase

As previously discussed, the components and intermediary reactions of dihydroceramide desaturase were shown to be very similar to that of the well-characterized microsomal Δ^9 stearoyl-CoA desaturase (20) with regard to cofactors and inhibitors (12,13). The enzyme required NAD(P)H, molecular oxygen and displayed sensitivity to various desaturase inhibitors, including cyanide (CN^-), and redox effectors that shunt the electron flow away from the desaturase system (12,13). By analogy, a dihydroceramide desaturase complex has been postulated (Scheme 1.6) (12).

Scheme 1.6 – Postulated dihydroceramide desaturase complex

1.4 Stereochemistry of Dihydroceramide Desaturation

The stereochemistry of fatty acid desaturation has been studied extensively for enzymes that catalyze the formation of (*Z*)-unsaturated products (47,48,49). The collective evidence suggests that these desaturases, irrespective of their origin, employ a mechanism featuring *syn*-elimination of the *pro*-(*R*) hydrogens. In contrast, only one investigation has been published on the enzymes that catalyze the introduction of (*E*)-double bonds (50).

In the moth, *Spodoptera littoralis*, myristic acid was converted into (*Z*)-11-tetradecenoic acid and (*E*)-11-tetradecenoic acid. Formation of (*Z*)-11-tetradecenoic acid occurred via *syn*-removal of the *pro*-(*R*) C11-H and *pro*-(*R*) C12-H, in agreement with previously reported stereospecificities of other (*Z*)-desaturases (47,48,49). Formation of

(*E*)-11-tetradecenoic acid occurred by *syn*-removal of the *pro*-(*R*) C11-H and *pro*-(*S*) C12-H (Scheme 1.7) (50).

Scheme 1.7 – Formation of (*E*)- and (*Z*)-11-tetradecenoic acid from myristic acid in *S. littoralis*

By analogy, one would predict that formation of the (*E*)-double bond of sphingosine would occur with a similar stereospecificity. This indeed seems to be the case. Stoffel *et al.* (51), following the incorporation of stereospecifically labelled monotrinitiated palmitic acid in sphingolipids of rat liver, concluded that the (4 *R*) and (5 *S*) hydrogen atoms of sphinganine are *syn*-removed during introduction of the double bond (Scheme 1.8).

Interestingly, Polito *et al.* (52) concluded that phytosphingosine (4-hydroxy-sphinganine) was also formed, with retention of configuration, via removal of the *pro*-(*R*) hydrogen of sphinganine (dihydroceramide) (Scheme 1.9).

Scheme 1.8 - Stereochemistry of dihydroceramide Δ^4 desaturation

Scheme 1.9 – Stereochemistry of 4-hydroxylation of sphinganine (dihydroceramide)

1.5 Mechanism of Desaturation: Relationship to Hydroxylases

As alluded to in previous sections, desaturases and hydroxylases are closely linked at the structural level (Scheme 1.10). It is therefore not surprising that the modern view of desaturation mechanisms is usually discussed in terms of the mechanism of the well-characterized hydroxylation process.

Scheme 1.10 – Overall stoichiometry of hydroxylation and desaturation reactions

Three major classes of biological iron-based hydroxylases have been found: heme-containing cytochrome P-450 (53,54); non-heme diiron methane monooxygenase (MMO) (55); and non-heme diiron alkane ω -hydroxylase (38).

1.5.1 Heme Iron-containing Cytochrome P-450 Hydroxylase

The cytochrome P-450 monooxygenase system consists of three protein components: a cytochrome P-450, an electron transfer protein and a reductase (flavoprotein) (56,57). The classification of a protohemoprotein as a P-450 is defined by its absorption spectrum; the Fe(II)-CO complex has a characteristic absorption maximum near 450 nm due to coordination of a sulfhydryl sulfur atom (cysteine thiolate) to the heme iron of the enzyme (58). A large family of related proteins have been discovered, not only in hepatic microsomes and mitochondria but also in bacteria (59).

The catalytic mechanism of cytochrome P-450 mediated monooxygenation can be considered in two parts: oxygen activation and substrate oxidation. The proposed catalytic cycle is outlined in Scheme 1.11. It is thought that an iron(IV)oxo porphyrin cation species is the likely oxidant in these reactions (53,54), but the hydroperoxo-iron species may also be involved during substrate oxidation.

Scheme 1.11 – Formation of iron-oxo intermediate in cytochrome P-450

The substrate oxidation mechanism has remained unclear due to the instability of the intermediates in the catalytic cycle and the lack of simple model systems with similar reactivity. Early mechanistic studies of P-450 mediated oxidations showed substrate stereochemistry appeared to be conserved and the C-H bond cleavage was subject to low kinetic isotope effects. (60). This suggested that oxygen atom delivery to the substrate occurred via a concerted direct insertion of oxygen (Scheme 1.12).

Scheme 1.12 – Concerted “insertion” pathway for hydrocarbon hydroxylation

In 1978, Groves *et al.* showed that hydrogen removal for hydroxylation of norbornane-*d*₄ 1 (Figure 1.9) by cytochrome P-450 was accompanied by a very large isotope effect ($k_H/k_D = 10\text{--}12$) (61). His group also reported that the oxidation event

occurs with significant loss of stereochemistry at the oxidized carbon. Thus, they proposed, a stepwise, homolytic path for hydroxylation which involves initial hydrogen abstraction by an iron-oxo species followed by hydroxyl rebound (Scheme 1.13).

Scheme 1.13 – Hydroxyl rebound mechanism for hydrocarbon hydroxylation

Figure 1.9 – Some typical substrates employed in the mechanistic study of cytochrome P-450 reactions

Ortiz de Montellano and Stearns also provided strong support for the production of a short-lived radical intermediate in the finding that some ring opening occurred during the P-450 catalyzed hydroxylation of bicyclo[2.1.0]pentane **2** (Figure 1.9) at the C2 position, whereas no ring opening occurred during hydroxylation of the methyl group in methylcyclopropane **3** (Figure 1.9) (62). Hydroxylations at cyclopropylcarbinyl

positions as in **3** without rearrangement excluded obligate cationic intermediates. However, other experimental results indicated that if a radical intermediate was formed it must be short-lived. For example, Fretz *et al.* found a very high level of stereospecificity (>90 % retention) in the terminal methyl group hydroxylations of both enantiomers of geraniol **4** (Figure 1.9), chiral by virtue of isotopic substitution on that methyl group (63).

In 1991, Ingold *et al.* estimated the lifetime of the radical intermediate **2** using the “radical clock” method (64). Using the rate constant for ring opening of this radical and the product distribution found in P-450 catalyzed oxidation of **2** they computed a rate constant for the oxygen rebound step of $k_{OH} = 1.4 \times 10^{10} \text{ s}^{-1}$. A quantitative value for the rebound step was now available, but unfortunately this value proved to vary with substrate structure. Ingold employed a series of fast-clock substrates and calculated k_{OH} values which varied from 10^{11} to 10^{12} s^{-1} (65). The high values were interpreted as being most consistent with the possibility that the steric effects at the enzyme active site might slow the radical clocks and yield overestimated k_{OH} values. Ingold’s group also proposed that the hydroxyl rebound step might be viewed as a bimolecular homolytic substitution (S_H2) (Scheme 1.13).

Newcomb *et al.* examined the Ingold postulate that the enzyme constrained the substrates and slowed the radical rearrangement by employing a rigid bicyclic **5** (Figure 1.9) that was less susceptible to steric effects (66). P-450 catalyzed hydroxylation of **5** gave several products, including those from oxidation of the methyl group. From the ratio of rearranged to unrearranged products and the rate constant for radical ring opening, the computed rebound rate constant at 37 °C was $k_{OH} = 1.3 \times 10^{13} \text{ s}^{-1}$. Unfortunately, this rate constant exceeds that calculated for a reaction with no activation barrier at this temperature, an impossible result if a discrete radical intermediate was involved in the oxidation. They proposed that the origins of rearranged products are uncertain and the variable k_{OH} values might be a reflection of ionic rearrangements that cannot be discerned from radical rearrangements (66).

To test for cation intermediates, Newcomb’s group designed a probe **6** (Scheme 1.14) that could discriminate between radical and cation intermediates and still maintain hypersensitivity (67,68). The major product from oxidation of **6** (Scheme 1.14) was

unrearranged alcohol, but minor amounts of radical-derived and cation-derived rearrangement products were found (Scheme 1.14). The calculated oxygen rebound rate constants (k_{OH}) for these probes were very similar to that determined for 5, and from this value they determined the lifetime of the “radical” of 6 to be in the range of 70-200 fs. They propose that these lifetimes (69) are similar or below those of a transition state and do not constitute the formation of a true radical intermediate. Thus, the mechanism of P-450 catalyzed hydroxylation must involve asynchronous concerted insertions with a small amount of ring opening occurring in competition with collapse of the transition state (Figure 1.10).

Scheme 1.14 – Mechanistic probes employed for distinguishing between radical and carbocation intermediates

Figure 1.10 – Nonsynchronous, concerted model for cytochrome P-450 hydroxylation

The production of cations would explain why the results with other hypersensitive probes were so scattered. The rearranged products from the other probes were likely derived from both the fast ring openings that compete with collapse of the transition state structure and any cationic intermediates formed. This conclusion resolves one puzzle, but also presents another. How are the cationic products formed?

Newcomb *et al.* first reasoned that oxidation of the “radical” in the transition state might occur to give a cation (70), but very recently they demonstrated that the first-formed product of the hydroxylation reaction might be a protonated alcohol, produced by insertion of OH^+ (68). They showed that two electrophilic oxidants, one a preferential epoxidizing agent and the other a preferential hydroxylating agent, are produced in the natural course of P-450 oxidation reactions and that both forms can effect hydroxylation reactions (68,71) (Scheme 1.15).

Scheme 1.15 – Multiple oxidants generated by cytochrome P-450 enzymes

Newcomb concluded that hydroxylations by both the hydroperoxo-iron and iron-oxo species occur by insertion reactions. The hydroperoxo-iron species inserts the elements of OH^+ (Scheme 1.16); the iron-oxo species reacts by insertion of oxygen (68,71). Insertion of OH^+ into the C-H bond results in the formation of a protonated alcohol which can undergo a solvolysis-type reaction to give cationic rearrangement products. The mechanistic conclusions presented by Newcomb remain controversial.

Scheme 1.16 – Mechanism for hydroxylation by the P-450 hydroperoxo-iron species

The relevance of P-450 catalyzed oxidation reactions to the desaturase mechanism was heightened by the recent confirmation that the Δ^{22} desaturation of sterols is catalyzed by a cytochrome P-450 catalyst (72). In addition, 4,5-desaturation of valproic acid (2-propylpentanoic acid) is catalyzed by hepatic cytochrome P-450, an enzyme which also catalyzes 4- and 5-hydroxylation of valproic acid (73).

1.5.2 Non-heme Diiron-containing MMO Hydroxylase

Despite the structural differences between cytochrome P-450 and MMO, the reaction chemistries they catalyze are very similar. Therefore, it is possible to compare the proposed mechanism of P-450 with that of the structurally dissimilar non-heme diiron systems (74,75).

The soluble MMO system consists of three protein components: a hydroxylase (MMOH), a reductase and a coupling protein, all three of which are required for enzymatic activity (76). The crystal structures, in both the reduced and oxidized form, of MMOH from two different sources have been determined (77,78). Each iron in the MMOH cluster is six coordinate, having one nitrogen ligand (Histidine) and five oxygen ligands comprised of glutamic acid residues, bridging hydroxides, and a terminal water (Figure 1.11).

Figure 1.11 – Schematic representation of the diiron centre of MMOH in reduced and oxidized forms

Recently, there has been significant progress in understanding the oxygen activation mechanism of non-heme diiron containing MMOH. It has been shown that the catalytic cycle of MMOH proceeds through several discrete intermediates (79,80) (Scheme 1.17).

Scheme 1.17 – Proposed mechanism of oxygen activation in MMOH

It is postulated that the cycle is first initiated with the reaction of oxygen with MMOH_{red} to form intermediate P, which is spontaneously converted to the intermediate Q. "Q" then reacts with substrate to afford hydroxyl product and MMOH_{ox} , the diiron(III) form. On the basis of their spectroscopic properties, "P" and "Q" are thought to be a (μ -peroxo)diiron(III) and bis(μ -oxo)diiron(IV) species, respectively (81). The latter intermediate is also depicted as a bis(oxo)iron(IV) species which resembles the iron(IV)oxo(porphyrin⁺) species that has been postulated for cytochrome P-450. The second iron can be thought of as fulfilling the role of the heme of P-450 as an electron storage site to reduce the oxidation level of its partner from V to IV.

The substrate oxidation mechanism by MMO was first discussed in terms of the Groves “hydroxyl rebound” mechanism proposed for cytochrome P-450 (Scheme 1.13) (82). Various “radical clock” mechanistic probes have been employed in studies with two well-characterized MMO systems (*Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b), but the results for MMO are less consistent than those reported for cytochrome P-450.

Lipscomb *et al.* (83) reported that the *M. trichosporium* OB3b sMMO system oxidized methylcubane **7** at all positions and that the major alcohol product derived from rearrangement of the cubylcarbinyl radical, which implicated a radical-based mechanism (Scheme 1.18).

Scheme 1.18 – Products of MMO catalyzed methylcubane oxidation

However, Newcomb and co-worker’s investigation of methylcubane **7** (Scheme 1.18) oxidation by both sMMO systems gave contrasting results (84). They reported that all positions of the methylcubane were indeed functionalized by both sMMO’s, but the major rearranged alcohol product was shown to be 1-homocubanol, which derives not from a radical intermediate but from a cationic rearrangement process. In addition, use of the hypersensitive cyclopropane-based mechanistic probe **6**, designed to distinguish between “radical” and “cationic” species, was successfully oxidized by *M. capsulatus* (Bath) MMOH, and a cationic rearrangement product was detected as a minor product. Newcomb proposed, by analogy to P-450, that these products could only be formed if a hydroperoxo-iron species existed (Scheme 1.19). Hydroxylation of the substrate would then occur via insertion of “OH⁺” into the C-H bond to give a protonated alcohol (68,84).

Scheme 1.19 – Possible intermediates produced in MMOH-catalyzed oxidations

The implication of a cationic component in MMO hydroxylations complicates the mechanistic picture, but it reinforces some similarities between P-450 and MMO hydroxylation reactions. Future mechanistic studies of MMO hydroxylations of alkanes will have to focus on further assessment of the oxidizing properties of the peroxo-iron and iron-oxo intermediates.

1.5.3 Non-heme Diiron-containing Alkane ω -Hydroxylase

Alkane ω -hydroxylases are non-heme diiron containing enzymes that catalyze the terminal hydroxylation of simple alkanes ($\text{C}_5\text{-C}_{12}$) (85). Groves *et al.* recently demonstrated that the alkane monooxygenase of *Pseudomonas oleovorans* (AlkB), hydroxylates via a substrate radical intermediate (86). The group demonstrated that AlkB-catalyzed oxidation of norcarane (bicyclo[4.1.0]heptane) **8** (Scheme 1.20) and 2-methyl-1-phenylcyclopropane gave rearranged products as a result of a carbon-centered radical intermediate (lifetime = ca. 1 ns).

They propose that the nanosecond lifetime observed for the radical intermediate is long enough to qualify this species as a distinct intermediate and supports their hypothesis that non-heme iron enzymes can participate in an oxygen rebound mechanism (87). No products of cationic rearrangement were observed (Scheme 1.20). It is clear that the mechanism for non-heme diiron catalyzed reactions remains highly speculative.

Scheme 1.20 – Mechanism of norcaradiene hydroxylation by AlkB

1.5.4 Mechanism of Fatty Acid Desaturation

Despite all of the advances mentioned so far, very little information has emerged for the mechanism by which fatty acid desaturation occurs. Based on the requirement of molecular oxygen and NAD(P)H, Bloch *et al.* first proposed that the reaction occurred by way of a hydroxy-intermediate in a stepwise process (88,89). By comparing release of tritium from the four possible tritium-labelled substrates in their studies of bacterial Δ^9 desaturation (*C. diphtheriae*), a substantial primary tritium kinetic isotope effect at C-9 position but not at the C-10 position was deduced (47,89). This suggested that the rate-limiting step in Δ^9 desaturation was removal of the (9*R*)-hydrogen atom. They proposed that desaturation may be viewed as a monooxygenase reaction, in which the first step was hydroxylation at the 9-position followed by dehydration (Scheme 1.21).

Scheme 1.21 – Hydroxylation / dehydration mechanism for Δ^9 desaturation

In an attempt to support this hypothesis, they prepared and incubated the four possible 9- and 10- hydroxy fatty acids and their CoA / ACP derivatives with a Δ^9 desaturase system from *S. cerevisiae* (88). However, synthetic 9- and 10-hydroxystearate, either as the free acid or the thioester, were not substrates for oleate-forming enzyme systems (88,90).

In contrast, Morris *et al.* claimed that a substantial kinetic isotope effect was operating at both the C-9 and C-10 positions for Δ^9 desaturation in *C. vulgaris*, suggesting a mechanism that involved the simultaneous concerted removal of the hydrogens instead of a stepwise process (91). These results have subsequently been shown to be in error (92)

Strittmatter *et al.* demonstrated isotopic discrimination in their studies with the reconstituted rat liver stearoyl-CoA desaturase by comparing $V_{\max}$ of completely deuterated stearoyl-CoA, 9,10-tetradeuterated stearoyl-CoA and stearoyl-CoA. The magnitude of k_H/k_D values ($k_H/k_D = 6$) was within the range expected for a primary isotope effect (31). Thus, they proposed that hydrogen removal is the rate-limiting step in enzymatic desaturation as suggested by Bloch *et al.* for the whole cell bacterial system. However, they did not distinguish between the presence of an isotope effect at one or both carbons (C-9 and C-10).

The Buist group, by analogy to the presented work, have proposed a mechanistic scheme relating hydroxylation and desaturation (49) (Scheme 1.22). Initial hydrogen abstraction occurs by a highvalent iron-oxo species, similar to MMO, which generates a carbon-centred radical intermediate. This species could collapse to an olefin via a rapid Lewis acid (Fe^{3+})-catalyzed dehydration of an alcohol intermediate (pathway A), simple disproportionation (pathway B), or by a one electron oxidation/deprotonation sequence (pathway C). An alternative route to a carbocation intermediate may be insertion of OH^+ in the C-H bond followed by rapid loss of water from a protonated alcohol intermediate, as proposed by Newcomb (68,84). The formation of an organo-iron intermediate followed by reductive elimination is also possible (Scheme 1.23) (93).

Scheme 1.22 – Proposed mechanistic pathways for desaturation of fatty acids

Scheme 1.23 – Pathway involving the formation of an organo-iron intermediate

Buist *et al.* have primarily focused on studying the site of initial oxidative attack in desaturation (cryptoregiochemistry) using convenient *in vivo* yeast expression systems. They have developed three complementary methods, based on the mechanistic considerations depicted in Scheme 1.22, to decipher the cryptoregiochemistry of desaturation (49,94,95).

Scheme 1.24 - Sulfoxidation of thia fatty acids in the Δ^9 desaturase of *S. cerevisiae*

The first method developed by the Buist group was the “thia-test”. This method monitors the efficiency of oxo-transfer to sulfide as a function of sulfur position in appropriate thia substrate analogues (Scheme 1.24) (49,96). They demonstrated that the Δ^9 desaturase could be induced to act as a highly enantioselective oxygenase in *S. cerevisiae* and sulfoxidation of a number of thia-fatty analogues by Δ^9 desaturase was most efficient when sulfur replaces C-9 of stearic acid (49,97). Substantially less sulfoxide was obtained when sulfur was at the C-10 position, and sulfoxidation at the C-8 and C-11 positions was negligible. The stereochemistry of sulfoxidation at the C-9 and

C-10 positions was consistent with that reported for hydrogen removal during desaturation (*R*-configuration of sulfoxide). These observations were recently extended to the Δ^6 desaturase, where only 6-thiaoleate was significantly converted to the predicted enantiomerically pure sulfoxide (96).

The second method utilizes monofluorinated fatty acids to probe the cryptoregiochemistry of desaturation and test for the possible involvement of discrete hydroxylated and carbocationic intermediates (pathway A and C in Scheme 1.22) (95,98). They demonstrated with the Δ^9 desaturase of *S. cerevisiae* that only fluoroolefinic products were formed when either methyl (*R*, *S*)-9- or 10-fluoropalmitate (stearate) were incubated with the enzyme (Scheme 1.25) (95). The α - and β -fluorine substituent effects at C-9 and C-10 were determined to be $k_H/k_F = 6.2$ and $k_H/k_F = 2.4$, respectively. The magnitude of the observed fluorine substituent effects were deemed consistent with initial oxidation at C-9, as previously determined (94). As well, they rationalized that because no evidence for fluorohydrin formation was found and the observed α - and β -fluorine substituent effects were of relatively low magnitude, desaturation could not involve a discrete alcohol or carbocation intermediate (Scheme 1.25). Thus, by a process of elimination, a disproportionation mechanism (pathway B, Scheme 1.22) has been favoured, where initial oxidation occurs at C-9.

Scheme 1.25 - Fluoro fatty acids as probes for carbocation and hydroxyl intermediates

The final and most robust technique developed by the Buist group for determining cryptoregiochemistry is the kinetic isotope effect (KIE) approach, which can be used to detect the first, energetically difficult and hence isotopically sensitive C-H cleavage step (Appendix A). This was first demonstrated with the Δ^9 desaturase in *S. cerevisiae*, where a large isotope effect was observed for C-H cleavage at C-9 while a negligible isotope effect was obtained for hydrogen abstraction at C-10 (94) (Scheme 1.26). This methodology has now been extended to various other desaturase systems and unambiguous assignments have been made for a number of membrane-bound fatty acid desaturases (Δ^5 , Δ^6 , Δ^{11} , Δ^{12} , ω -3) (99,96,100,101,102). A pattern of kinetic isotope effects (one large, one negligible) has been found for all of these membrane-bound desaturases and without exception it has been found that initial oxidation always occurs at the carbon closest to the polar head group.

Scheme 1.26 - KIE test for cryptoregiochemistry of Δ^9 desaturase

1.6 Deciphering the Cryptoregiochemistry of Dihydroceramide Δ^4 Desaturation

As previously discussed, the activities of mammalian sphingolipids depend critically (103) on the presence of an (*E*)-4,5-double bond in the long chain base structure

(9, Scheme 1.27). Interestingly, bioactive yeast sphingolipids are derived from 4-hydroxysphinganine (phytosphingosine) (104). These two distinct groups of sphingolipids seem to arise via a bifurcation in the biosynthetic pathway at the step in which the saturated sphinganine backbone is oxidatively modified. Thus, the animal sphingosines are formed by the highly regio- and stereoselective introduction of the double bond via an enzyme known as dihydroceramide desaturase (13), while a 4-hydroxylase (104) is responsible for the biosynthesis of the phytoanalogues (10, Scheme 1.27).

Scheme 1.27 – Dihydroceramide Δ^4 desaturation and 4-hydroxylation of sphinganine derivatives

This particular dichotomy in oxidation reaction pathway is not unprecedented. A number of cases are now known where alcohol and alkene are formed as mixtures by a bifunctional enzyme (73,105,106) or separately by two closely related proteins (106,107). Minor changes in substrate structure can also effect a switch in reaction pathway (108). On the basis of the proposed mechanistic scheme (Scheme 1.22) relating hydroxylation and desaturation and the results with other membrane-bound proteins, one would predict that for any given desaturase / hydroxylase pair of enzymes, the (cryptic) site of initial oxidative attack leading to unsaturated product should match the regiochemistry of hydroxylation. Therefore, it is postulated that oxidation by dihydroceramide Δ^4 desaturase should be initiated at C-4...a testable hypothesis.

1.7 Research Goals

The purpose of this thesis was to investigate the cryptoregiochemistry (site of initial oxidation) of dihydroceramide Δ^4 desaturase through use of primary deuterium kinetic isotope effect (KIE) measurements. This approach requires the diastereoselective synthesis of *N*-octanoyl-D-*erythro*-sphinganine (Figure 1.12) and the corresponding 4,4-dideuterated (Figure 1.13) and 5,5-dideuterated analogues (Figure 1.14), followed by determination of the intermolecular kinetic isotope effect on C-H bond cleavage at C-4 and C-5. The KIE can be evaluated via direct competition between the labelled and unlabelled substrates. In this manner, evidence for or against the hypothesis that 4-hydroxylation and 4,5-desaturation are mediated by closely related enzymes could be obtained, linking phytosphingosine and sphingosine formation.

Figure 1.12 – *N*-Octanoyl-D-*erythro*-sphinganine 11

Figure 1.13 – *N*-Octanoyl-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine [4,4- $^2\text{H}_2$]-11

Figure 1.14 – *N*-Octanoyl-D-*erythro*-[5,5- $^2\text{H}_2$]-sphinganine [5,5- $^2\text{H}_2$]-11

Chapter 2

Deciphering the Cryptoregiochemistry of Dihydroceramide Δ^4 Desaturase: A Kinetic Isotope Effect Study

2.1 Experimental Approach

A major reason for determining kinetic isotope effects (KIE's) in enzymology has been to determine the “rate-determining step” of enzymatic reactions (109). It is now well understood that if cleavage of a C-H bond is the slow, rate determining step in an enzyme-catalyzed reaction, then substitution of hydrogen with a heavier deuterium atom should in principle decrease the reaction rate (Appendix A). This decrease in the rate is called a “deuterium isotope effect” and it is expressed as the ratio between the rate constants for the reactions involving hydrogen and deuterium isotopes (k_H/k_D), (Appendix A) (110). One problem with the determination of isotope effects for enzymatic reactions is that the isotopically sensitive step (ie. C-H bond cleavage) is not always the only rate-determining step in the reaction sequence. Often one observes suppressed values resulting from additional rate limitations imposed by steps such as electron transfer, substrate binding, product release etc. The net result is that the “intrinsic isotope effect” (full effect originating from a single isotopically sensitive step) will be masked, and a reduced “apparent isotope effect” will be observed (111). Masking can be minimized by use of intramolecular isotope effects, where both isotopes in equivalent positions within the same substrate compete in a reaction (111).

We decided to probe the cryptoregiochemistry of the dihydroceramide Δ^4 desaturase enzyme by measuring the deuterium kinetic isotope effect (KIE) on the two C-H cleavage steps involved in desaturation. In this study, we took advantage of the fact that, according to our mechanistic scheme (Chapter 1, Scheme 1.22), initial hydrogen abstraction should be slow relative to second C-H cleavage and therefore more sensitive to the isotope effect. The enzymatic isotope effects in our study were determined via intermolecular experiments where both labelled and unlabelled substrates were present

and compete in the same incubation (112). This competitive method has several advantages: (a) exactly the same conditions are provided for the reactions of both labelled and unlabelled substrates; (b) time as well as the absolute concentration of labelled and unlabelled substrates do not have to be measured accurately; and (c) the same purity of labelled and unlabelled substrates is not required (112). Thus, if complete masking does not occur, the results obtained from this study will provide the closest estimate of the observed isotope effect, provided conditions are adjusted to allow for low % conversion of substrate (112). An intramolecular method could not be used for our kinetic isotope effect (KIE) studies, as the desaturase enzyme is selective for only one methylene hydrogen (ie. *pro-R* or *pro-S*).

Our experimental approach involved incubating a roughly equimolar mixture of the appropriate regiospecifically dideuterated ($-CD_2-$) substrate analogue and the non-deuterated parent compound with a convenient source of the desaturase. Naturally occurring long-chain dihydroceramides are poorly soluble in aqueous environments, which hamper the study of their metabolism (12). To alleviate this problem, the *N*-acyl group was substituted with a short chain (octanoyl) analogue which is known to be taken up by the cells and converted into desaturated short-chain ceramides (19). Comparison of the d_0/d_1 ratio of sphingosine products derived from a ca. 1:1 mixture of *N*-octanoyl-D-*erythro*-sphinganine 11 and *N*-octanoyl-D-*erythro*-[4,4- 2H_2]-sphinganine ([4,4- 2H_2]-11) with that obtained from a ca. 1:1 mixture of *N*-octanoyl-D-*erythro*-sphinganine 11 and *N*-octanoyl-D-*erythro*-[5,5- 2H_2]-sphinganine ([5,5- 2H_2]-11) allowed us to compute the competitive deuterium kinetic isotope effect (KIE) for each C-H bond rupture.

2.2 Results and Discussion

Part 1: Synthesis of Dihydroceramide Substrates

2.2.1 Strategies for Sphingolipid Synthesis

The vast spectrum of sphingolipid biological activity has prompted well over 50 syntheses of sphingosine and its isomers (113). As discussed, the activities of

mammalian sphingolipids depend critically on the presence of an (*E*)-4,5-double bond in the long chain base structure (103). For this reason, most synthetic strategies have focussed on the synthesis of sphingosine-based sphingolipids; very few methodologies have been developed for the synthesis of sphinganine. However, many of the sphingosine-based strategies can be adopted for saturated sphingolipid preparation. The four major strategies are briefly introduced and discussed in terms of their applicability to the synthesis of regiospecifically dideuterated sphinganines.

In the first strategy, carbohydrates are used as the source of chirality (114). 2,4-*O*-Protected D-threoses are subject to Wittig olefination with a triphenylphosphonium ylide derived from hexadecenylbromide (Scheme 2.1). A highly efficient method for the synthesis of sphingosine, but one that cannot be used to prepare the sphinganines required for this study.

Scheme 2.1 – Carbohydrate strategy for synthesis of sphingosine

The second strategy features introduction of chirality through Sharpless asymmetric epoxidation (115) (Scheme 2.2); a method which could have been utilized to prepare the desired dideuterated compounds.

Scheme 2.2 – Chirality through catalysis approach for synthesis of sphinganine

The third approach is based on the aldol reaction (116). The boron enolate of chiral *N*-acyloxazolidinone is reacted with hexadecenal in an aldol reaction to construct

the sphingosine skeleton. This is a highly selective strategy that could have also been used to prepare the necessary dideuterated sphinganine substrates (Scheme 2.3).

Scheme 2.3 – Relayed asymmetric induction approach for synthesis of sphinganine

The fourth approach relies on the amino acid serine as the source of chirality (Scheme 2.4) and is the simplest and most logical strategy for the synthesis of the regiospecifically dideuterated sphinganines (117-122).

Scheme 2.4 – Chirality from serine approach for synthesis of sphinganine

Amino acids are one of nature's largest pools of chiral molecules and have been used for the greatest number of sphingosine syntheses (113). This approach generally features the diastereoselective addition of alkynyl, alkenyl or alkyl nucleophiles centred on lithium, magnesium or zinc to a chiral aldehyde derived from L-serine (123). In hindsight this is only logical, as serine is the biosynthetic precursor. Synthetically it is also a rational choice because, along with the correct *S*-configuration at C-2, it provides a C-1 hydroxyl and an easily manipulated acid functionality in the correct orientations. As well, since this strategy is convergent, the incoming nucleophile is prepared independently and can be altered as required. However, the first syntheses encountered problems with loss of chiral integrity and lack of diastereoselectivity during nucleophilic addition (117).

It was not until 1988, when four papers by Nimkar *et al.* (119), Garner *et al.* (120), Radunz *et al.* (121) and Herold (122) were published almost simultaneously, that serine-based syntheses started to emerge. All of this activity was inspired by a short and efficient synthesis of the enantiopure serine aldehyde **12** (Figure 2.1) by Garner and coworkers (124). The most versatile synthesis is that of Herold (122), which illustrated how careful selection of reaction conditions enables one to steer the reaction so that all possible isomers can be selectively synthesized.

Figure 2.1 – Fully protected L-serine aldehyde **12**

In Herold's synthesis, diastereomeric carbamate protected sphingosine was prepared from the fully protected serine aldehyde **12** by alkynylation. Deketalization, reduction of the triple bond and Boc-group removal afforded sphingosine and its isomers (122).

Following Herold's convergent approach, a modified synthetic strategy was developed for the synthesis of the dihydroceramide probes required for this study (Scheme 2.5). Nucleophilic addition of the appropriate alkyl chain to the serine aldehyde **12** gives the protected sphinganine derivative, which can be easily deprotected (122) and acylated (125) to give the dihydroceramide probe. It was hoped that we could alter the reaction conditions to favour *erythro* diastereoselectivity during nucleophile addition and preclude formation of the unnatural *threo* diastereomer. A strategy that is conceptually simple, yet experimentally difficult.

Scheme 2.5 – Synthetic strategy for preparation of *N*-octanoyl-*D*-erythro-sphinganine 11

2.2.2 Synthesis of 1,1-Dimethylethyl (*S*)-4-formyl-2,2-dimethyloxazolidine-3-carboxylate (12)

As described in section 2.2.1, the simplest and most logical strategy for the preparation of dideuterated dihydroceramide is a convergent synthesis starting with a serine-derived aldehyde and a regiospecifically dideuterated acyl chain. Garner and co-workers (124) synthesized a fully protected serine-aldehyde, 1,1-dimethylethyl 2,2-dimethyl-4-(*S*)-formyloxazolidine-3-carboxylate **12** (Figure 2.1), that fulfilled all the necessary requirements for sphingolipid synthesis. Those requirements included: (a) large-scale availability; (b) configuration and chemical stability; (c) ability to exert stereocontrol during addition reactions; and (d) easy subsequent deprotection and manipulation. Using a similar but improved methodology (126) to that of Garner and co-workers, the oxazolidine aldehyde, 1,1-dimethylethyl (*S*)-4-formyl-2,2-

dimethyloxazolidine-3-carboxylate **12**, was synthesized in 47 % overall est. yield as outlined in Scheme 2.6 from commercially available L-serine.

Scheme 2.6 – Synthesis of 1,1-dimethylethyl (*S*)-4-formyl-2,2-dimethyloxazolidine-3-carboxylate **12**

Esterification (127) of L-serine with methanol / acetyl chloride under Fischer (128) conditions gave methyl serinate. The ester was then converted into the carbamate in 85 % overall yield using di-*tert*-butyl dicarbonate at a pH > 10 in THF and triethylamine (129), effectively protecting the amine from mild acids, hydrides and oxidants (130). Protection of the remaining hydroxy and amide functionalities was achieved by formation of the 2,2-dimethyloxazolidine ring. Reaction with 2,2-dimethoxypropane in acetone under Lewis acid-catalyzed conditions (131) gave clean formation of the mixed-ketal ester with an 80 % yield. Controlled reduction (124) of the oxazolidine ester with ca. 1 equivalent of diisobutylaluminum hydride (DIBALH) in toluene at –78 °C followed by quenching with anhydrous MeOH at the same temperature led to formation of the oxazolidine aldehyde **12** with a trace amount of the corresponding alcohol, as a result of over-reduction. Purification on flash silica gave pure **12** in a 69 % yield.

Garner *et al.* determined the enantiomeric purity of **12**, as well as the antipode, via ^1H and ^{19}F NMR on the Mosher derivatives of the corresponding alcohol, HPLC and optical rotation (124). The optical rotation of **12** was determined to be $[\alpha]_{\text{D}} -88.3^\circ$ ($c=0.48$, CHCl_3) as compared to $[\alpha]_{\text{D}} -91.7^\circ$ ($c=1.34$, CHCl_3) obtained by Garner and co-worker's (124). Thus, **12** was synthesized in high enantiomeric excess and was configurationally stable to purification and manipulation, as judged by optical rotation measurements.

The IR data of **12** was consistent with the values reported by Garner *et al.* (124). Absorptions corresponding to the $\text{C}=\text{O}$ (aldehyde) and $\text{C}=\text{O}$ (carbamate) were observed at 1739 cm^{-1} and 1710 cm^{-1} , respectively (Figure 2.2).

The ^1H NMR spectrum (Figure 2.3) of **12** showed two sets of signals at ambient temperature. It seems the oxazolidine derivatives exist as slowly interconverting rotomers on the NMR time-scale (124). This has been proposed to result from a combination of conformational changes associated with the substituted oxazolidine ring and the restricted rotation associated with the amide bond because of its partial double bond character (124, 132). Upon raising the probe temperature to 340 K the signals merged into one set, confirming the existence of a dynamic equilibrium. Two doublets corresponding to $\text{C}(5)\text{-H}_a$ and $\text{C}(5)\text{-H}_b$ were observed at 3.56 ppm and 3.80 ppm, respectively, and a broad singlet for the enantiomeric hydrogen was observed at 3.93 ppm. A singlet at 9.37 ppm for the aldehyde proton confirmed the DIBALH-mediated reduction. All other signals were in accord with the assigned structure. As expected, cooling the sample restored the spectrum to its original condition.

The ^{13}C NMR (298 K, CDCl_3) spectrum (Figure 2.4) was in accord with the assigned structure of **12**. The carbon signal at 198.3 ppm, confirmed the presence of the aldehyde group. It is of interest to note that in this case satisfactory resolution of ^{13}C signals was achieved only at ambient temperature.

Figure 2.2 – IR spectrum of 1,1-dimethylethyl (*S*)-4-formyl-2,2-dimethyloxazolidine-3-carboxylate 12

Figure 2.3 – ^1H NMR (C_6D_6 , 340 K) spectrum of 1,1-dimethylethyl (*S*)-4-formyl-2,2-dimethyloxazolidine-3-carboxylate 12

Figure 2.4 – ^{13}C NMR (CDCl_3 , 298 K) spectrum of 1,1-dimethylethyl (*S*)-4-formyl-2,2-dimethyloxazolidine-3-carboxylate **12**

2.2.3 Synthesis of *N*-Octanoyl-*D*-erythro-sphinganine (**11**)

As described in 2.2.1, the simplest strategy for sphingolipid synthesis is that which was developed by Newman *et al.*, wherein a long-chain carbon nucleophile is added to a protected serine-derived aldehyde (**117**). This approach was generally effective but suffered from poor diastereoselectivity. Garner and co-workers with the synthesis of the chiral oxazolidine aldehyde **12** rectified this problem. The fully protected aldehyde has been shown to participate stereoselectively in addition reactions (120,122,137). Two models predict the diastereoselectivity of the nucleophilic addition reaction: the Felkin-Anh model (138) and the Cram chelation model (139).

The Felkin-Anh model predicts a conformation that features nucleophilic attack from the less hindered *re* face (Scheme 2.7). Addition to the *re* face gives the natural

erythro-diastereomer as the major adduct, where the hydroxyl and the amino group are in an *anti* configuration (120,140).

Scheme 2.7 – Felkin-Anh model illustrating attack from the *re* face

The Cram chelation model predicts a conformation that features chelation of the aldehyde and urethane carbonyl with the metal counterion of an organometallic nucleophile (Scheme 2.8). Thus, attack will occur on the *si* face resulting in preferential formation of the unnatural *threo*-diastereomer (120,140), where the hydroxyl and amino group are in the *syn* configuration.

Scheme 2.8 – Cram chelation model illustrating attack from the *si* face

It is evident that stereocontrol could be achieved during these organometallic additions to the oxazolidine aldehyde **12** by varying the reagent type so as to preclude or favour one of the pathways.

There are several experimental factors that can affect the diastereoselectivity of addition reactions to chiral oxazolidine aldehyde **12**, but the most dramatic results are obtained by varying the metal counterion of the nucleophile (123). For example, lithium reagents favour formation of the *anti* diastereomer (> 90 % ds), as predicted by the non-chelated Felkin-Anh model (120,122,123). In contrast, zinc reagents tend to behave as chelating Lewis acids and generally favour formation of the *syn* diastereomer (> 90 % ds), which can be rationalized in terms of the Cram chelation model (123). There have

been various diastereoselectivities reported when magnesium reagents are utilized as the incoming nucleophile (122,123,140). Williams *et al.* reported in 1996 that the steric bulk of the incoming nucleophile had a drastic effect on the diastereoselectivity of magnesium reagents (140). Additions of methyl and phenyl Grignard reagents favoured formation of the *anti* diastereomers (Felkin-Anh non-chelation pathway), whereas *i*-propyl and *t*-butyl Grignard reagents gave the *syn* diastereomers (Cram chelation pathway). They rationalized that the reactions with methyl or phenyl Grignard reagents were kinetically faster, and could occur before chelation takes place. Thus, a non-chelation pathway is followed and formation of the *anti*-diastereomer is favoured. In contrast, the bulky Grignard reagents react at a much slower rate. This allows chelation to occur, which favours formation of the *syn*-diastereomer (140).

The coordinating ability of a solvent can also have a drastic effect on the stereoselectivity of nucleophilic addition reactions to oxazolidine aldehyde **12**. A more coordinating solvent will tend to prevent chelation and will favour the non-chelation Felkin-Anh pathway, whereas a weakly coordinating solvent will promote chelation and favour the Cram chelation pathway (140,141). As well, addition of chelating Lewis acids (ie. ZnCl_2 , MgCl_2) will prevent formation of the products resulting from the non-chelation pathway (122,123).

Researchers have utilized these observations for syntheses of *D*-erythro-sphingosine and its isomers by using a lithiated alkynyl nucleophile. Addition of lithium pentadecyne to oxazolidine aldehyde **12** favours formation of the *anti* diastereomer (> 95 % ds), as predicted by the non-chelation Felkin-Anh model (120,122). Reduction of the triple bond, purification and hydrolytic deprotection (122) affords *D*-erythro-sphingosine. This would be an effective strategy for the synthesis of the unlabelled sphinganine probe. However, preparation of the regiospecifically dideuterated substrates would require addition of a lithiated alkyl reagent; reagents that have been demonstrated to be very poor nucleophiles and give extremely low yields in additions to chiral oxazolidine aldehyde **12** (123). Therefore, in order to synthesize the necessary dideuterated probes a compromise between diastereoselectivity and reaction yield would have to be adopted.

Alkyl magnesium reagents are very potent reagents for nucleophilic addition reactions to **12** (123). Williams *et al.* have shown that the kinetics of 1° alkyl Grignard

additions to **12** are such that chelation can be minimized during reaction and moderate selectivity for the *erythro* diastereomer can be demonstrated (140). Given the low yields obtained with alkylolithium reagents (123), it was decided to attempt to add pentadecyl magnesium bromide to chiral oxazolidine aldehyde **12**.

N-Octanoyl-D-*erythro*-sphinganine **11** was synthesized in 5 % overall est. yield from chiral oxazolidine aldehyde **12** as outlined in Scheme 2.5. Nucleophilic addition (140) of magnesium pentadecylbromide to chiral oxazolidine **12** gave an inseparable diastereomeric mixture of the fully protected sphinganine derivatives, *tert*-butyl [4*S*, 1' (*R**, *S**)]-2,2-dimethyl-4-(1'-hydroxyhexadecyl)-oxazolidine-3-carboxylate, with a 50 % yield. All spectral data were in accord with the assigned structures (142) (see Experimental, section 3.4.1).

The diastereoselectivity of the addition reaction can be assessed via two methods. The first method features transketalization of the fully protected sphinganine derivatives under acid-catalyzed conditions to give the corresponding 1,3-dioxanes followed by ¹H NMR analysis (143) (Scheme 2.9).

Scheme 2.9 – Formation of 1,3-dioxanes

Distinct coupling constants ($^3J_{4,5}$) corresponding to the *trans*-diaxial arrangement ($^3J_{4,5}$ = 8-10 Hz) of the *erythro* diastereomer and the *gauche* coupling ($^3J_{4,5}$ = 1-2 Hz) of the *threo* diastereomer are observed (143), but recent results indicate that this method may not be ideal (144). Koskinen *et al.* demonstrated that the *erythro* diastereomer does not undergo transketalization as readily as the *threo* diastereomer (144). Thus, previously reported *erythro* / *threo* selectivities, where this method was utilized, are questionable (123,143).

The second method is a much simpler procedure for determining the relative configuration of the products (11) and was utilized to determine the diastereoselectivity for the addition of magnesium pentadecylbromide to 12.

^1H NMR analysis of the corresponding known C2-dihydroceramides (*N*-acetyl sphinganine), prepared by hydrolytic deprotection with 3 N HCl and acylation with acetic anhydride, revealed distinct signal and coupling differences for the C1-, C2- and C3-protons of each diastereomer (Figure 2.5).

Figure 2.5 – Partial ^1H NMR spectrum of diastereomeric C2-dihydroceramide

The ratio of the intensities for the C3 protons of the *erythro*- and *threo*-diastereomers revealed the addition of magnesium pentadecylbromide to aldehyde 12 proceeded with moderate *anti* selectivity to give a ca. 2:1 mixture of *erythro*- and *threo*-diastereomers. This does not necessarily imply that under the reaction conditions aldehyde 12 adopted the predicted Felkin-Anh conformation over the Cram chelation conformation in a 2:1 ratio. According to the Curtin-Hammett principle (145,146), the conformers of the reactant may interconvert at a much faster rate than that at which the competing reactions occur (Figure 2.6).

Figure 2.6 – Effect of conformation on product distribution

The product ratio is therefore not determined by the conformational energy barrier but instead primarily on the relative energy of the two transition states leading to the products. Thus, perhaps under these reaction conditions the Felkin-Anh conformation provides access to a slightly lower energy transition state favouring formation of the *anti*-diastereomer. It should be noted, however, that if reaction conditions preclude formation of a particular conformer, the reaction product of that conformer would not be formed. For example, the Felkin-Anh conformer may provide access to the lowest energy transition state under initial non-chelation conditions, but addition of a Lewis acid (magnesium) could prevent its formation and its resulting product. This would allow the

Cram chelation conformer to dominate, which would favour attack on the *si* face and formation of the *syn*-diastereomer. If this process were to occur during the reaction a mixture of diastereomers would be obtained.

Another explanation for the observed diastereoselectivity of Grignard addition arises from the possibility that an alternate conformation of **12** may exist. That is, a non-chelated intermediate which allows unhindered attack from both faces, but slightly favouring *re* attack, may dominate during reaction (Scheme 2.10).

Scheme 2.10 – Proposed Non-chelated intermediate

In an attempt to separate the diastereomers, the fully protected diastereomeric sphinganine derivatives were selectively deprotected. Deketalization (**122**) with *Amberlyst 15*, a non-aqueous acidic resin, gave the diastereomeric *N*-Boc sphinganines, which displayed separation on silica TLC. Purification using flash chromatography afforded the *erythro*- (225 mg, 42 %) and *threo*- (132 mg, 24 %) diastereomers in a 1.70:1 ratio, confirming the ^1H NMR C2-dihydroceramide spectral analysis.

Interestingly, upon examination it was discovered that while both diastereomers were hydrophobic by water spray (one more slightly than the other), only one could be detected by exposure to iodine vapour. In fact, it was this characteristic difference that facilitated identification and separation of the diastereomers. The least polar compound, later identified as the *threo* adduct, was extremely hydrophobic, but did not stain with iodine. In contrast, the more polar *erythro* adduct was less hydrophobic and more iodine active. Given the geometries of the respective diastereomers, it was suggested that an intramolecular hydrogen bond might exist between the secondary hydroxyl group and urethane carbonyl of the *threo* diastereomer. This hydrogen bond would increase the molecule's hydrophobic character, increase the R_f on TLC, and possibly withdraw

electron density from the amide nitrogen preventing coordination with iodine. Recently, Williams et al. (140) demonstrated by X-ray analysis that a strong intramolecular hydrogen bond did exist in an analogue of the *threo* diastereomer, but not in the *erythro* diastereomer (Figure 2.7). While this finding does not confirm the existence of an intramolecular hydrogen bond between the hydroxyl and urethane carbonyl groups of the *threo* *N*-Boc sphinganine diastereomer, it does substantiate the hypothesis.

Figure 2.7 – ORTEP representation of *threo*- and *erythro*- diastereomer analogues

Cleavage of the carbamate moiety with 1 N HCl in dioxane gave the *erythro*- and *threo*- sphinganines (122). Their relative configurations were established by ^1H NMR analysis of the corresponding C2-dihydroceramides (Figure 2.8 and 2.9). Distinct signal and coupling differences were observed for the C1-, C2- and C3- protons of each diastereomer. The C2 and C3 protons of D-*erythro*-C2-dihydroceramide displayed signal differences of ca. 0.15 ppm compared to L-*threo*-C2-dihydroceramide. As well, the ^1H NMR spectrum showed $^3J_{2,3} = 6.6$ Hz, a value characteristic of *trans* coupling (Figure 2.8). Conversely, the ^1H NMR spectrum of L-*threo*-C2-dihydroceramide showed $^3J_{2,3} = 2.9$ Hz, a value characteristic of *gauche* coupling (Figure 2.9) (11,147). All ^1H NMR signals were in accord with the assigned structures (11) (see Experimental, section 3.4.3(a)).

In addition, a partial assignment of the ^{13}C NMR spectrum (Figure 2.10) was carried out using literature values (123) and those obtained for the labelled substrate intermediates. Distinct signals were observed for the amide carbonyl, C1, C2, C3, C4 and C5 at 173.4, 62.2, 57.0, 72.3, 34.8 and 26.9 ppm, respectively. All ^{13}C NMR signals were in accord with the assigned structure (see Experimental, section 3.4.3(a)).

Figure 2.8 – Partial ^1H NMR spectrum of D-*erythro*-C2-dihydroceramide

Figure 2.9 – Partial ^1H NMR spectrum of L-*threo*-C2-dihydroceramide

Figure 2.10 – ^{13}C spectrum of D-*erythro*-C2-dihydroceramide

The enantiomeric purity of D-*erythro*-sphinganine was determined from the ^1H NMR spectrum of the (S)- α -methoxy- α -(trifluoromethyl)phenylacetate (MTPA) derivatives (Mosher derivatives) (148,149). Analysis of the methoxy proton signals of synthetic (S)-MTPA-D-*erythro*-sphinganine and comparison to the MTPA derivative of commercially available DL-*erythro*-sphinganine clearly revealed that all reactions with serine aldehyde had occurred with no detectable racemization (Figure 2.11 and 2.12). Thus, it can be concluded that D-*erythro*-sphinganine was synthesized with a high enantiomeric purity. The intense signal at 3.44 ppm for the DL-sphinganine is due to overlapping methoxy signals.

Figure 2.11 – Partial ^1H NMR spectrum of D-*erythro*-sphinganine (S)-MTPA derivative

Figure 2.12 – Partial ^1H NMR spectrum of DL-*erythro*-sphinganine (S)-MTPA derivative

As discussed in Chapter 1 (Section 1.2.1), introduction of the (*E*)-4,5-double bond occurs at the level of dihydroceramide (13,19). The most potent desaturase activity *in vitro* has been demonstrated to occur when the *N*-acyl group is replaced with a short-chain octanoyl substituent (13). Therefore, the *N*-octanoyl derivative of *D*-*erythro*-sphinganine was utilized for this cryptoregiochemical study.

N-octanoyl-*D*-*erythro*-sphinganine **11** was prepared similar to that of Kashiwagi and co-workers (125). Reaction of *D*-*erythro*-sphinganine with octanoyl chloride using a two-phase system composed of a mild aqueous alkaline solution and diethyl ether followed by purification on silica, using CHCl₃/MeOH (15:1) as eluent, gave **11** with a 56 % yield. The ¹H NMR (Figure 2.13), and MS (Figure 2.15) spectra were in accord with the assigned structure (11,125). The purity of **11** was deemed suitable for biological experiments, as determined by GC (Figure 2.14).

The ¹H NMR spectrum (Figure 2.13) displayed all the characteristic signals associated with the sphingoid base (11). The triplet at 2.25 ppm for the methylenes α to the amide confirmed acylation.

The mass spectrum (Figure 2.15) of O-trimethylsilylated **11** displayed the following characteristic fragment ions (125): *m/z* 556 ($M^{+•}-CH_3$); 468 ($M^{+•}-(CH_3)_3Si-O-CH_2$); 313 ($((CH_3)_3Si-O-CH(CH_2)_{14}CH_3)^{+}$); and 258 ($((CH_3)_3Si-O-CH_2CH-NH-C(O)(CH_2)_6CH_3)^{+}$), which are consistent with the assigned structure (125).

Figure 2.13 - ^1H NMR spectrum of *N*-octanoyl-D-erythro-sphinganine 11

Figure 2.14 - GC of *N*-octanoyl-D-erythro-sphinganine 11

Figure 2.15 - MS of *N*-octanoyl-*D*-erythro-sphinganine 11

2.2.4 Synthesis of Deuterium Labelled Grignard Precursor, [1,1-²H₂]-1-Bromopentadecane (13)

[1,1-²H₂]-1-Bromopentadecane 13 was synthesized in 67 % overall est. yield as outlined in Scheme 2.11. Reduction of pentadecanoic acid using LiAlD₄ (133) gave the corresponding 1,1-dideuterated alcohol. Subsequent bromination (134) with HBr gave 13.

The trimethylsilyl (TMS) derivative of [1,1-²H₂]-1-pentadecanol revealed the compound was obtained with high deuterium content (essentially 100 % within experimental error) as determined by MS. The MS displayed the characteristic fragment ion, m/z 287 ($M^{+}-CH_3$), which is consistent with the assigned structure.

The title compound 13 was essentially pure by GC. The ¹H NMR was in accord with the assigned structure; the absence of a signal at 3.5 ppm confirmed the position of the deuterium label at C-1 (see Experimental, section 3.3.2).

Scheme 2.11 – Synthesis of [1,1-²H₂]-1-bromopentadecane 13

2.2.5 Synthesis of Deuterium Labelled Grignard Precursor, [2,2-²H₂]-1-Bromopentadecane (14)

[2,2-²H₂]-1-Bromopentadecane 14 was synthesized in 57 % overall est. yield as outlined in Scheme 2.12. BF₃-catalyzed methylation (135) of pentadecanoic acid gave pentadecanoate. Deuterium was introduced α to the carboxyl group by base-catalyzed exchange with sodium metal and MeOD (136), giving the corresponding 2,2-dideuterated methyl ester. Subsequent reduction (133) and bromination (134) gave 14.

[2,2-²H₂]-Pentadecanoate was obtained with high deuterium content (ca. 95-97 %) as determined by MS. The MS confirmed the molecular weight of pentadecanoate and displayed the following characteristic fragment ions: *m/z* 258 (M⁺) and 227 [(M⁺-OCH₃)], which are consistent with the assigned structure. A small (M-1)⁺ peak (ca. 5 %) corresponding to the mono-deuterated ester was also observed.

The title compound 14 was essentially pure by GC. The ¹H NMR was in accord with the assigned structure, the absence of a signal at 1.8 ppm confirmed the position of the deuterium label at C-2 (see Experimental, section 3.3.6).

Scheme 2.12 – Synthesis of [2,2- $^2\text{H}_2$]-1-bromopentadecane 14

2.2.6 Synthesis of *N*-Octanoyl-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine ([4,4- $^2\text{H}_2$]-11)

N-Octanoyl-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine [4,4- $^2\text{H}_2$]-11 was synthesized in 4 % overall est. yield from chiral oxazolidine aldehyde 12 and the deuterium labelled Grignard precursor 13 as outlined in Scheme 2.13.

Nucleophilic addition (140) of magnesium [1,1- $^2\text{H}_2$]-pentadecyl-1-bromide to chiral oxazolidine aldehyde 12 gave an inseparable diastereomeric mixture of the fully protected sphinganine derivatives, *tert*-butyl [4*S*, 1'(*R*^{*}, *S*^{*})]-2,2-dimethyl-4-(1'-hydroxy[2',2'- $^2\text{H}_2$]-hexadecyl) oxazolidine-3-carboxylate, with a 53 % yield. All spectral data were in accord with the assigned structure (142) (see Experimental, section 3.5.1).

^1H NMR analysis of the corresponding C2-[4,4- $^2\text{H}_2$]-dihydroceramides (*N*-acetyl-[4,4- $^2\text{H}_2$]-sphinganine), revealed the addition of magnesium [1,1- $^2\text{H}_2$]-pentadecyl-1-bromide 13 to aldehyde 12 proceeded with moderate *anti* selectivity to give a ca. 2:1 mixture of *erythro*- and *threo*- diastereomers.

Deketalization (122) with *Amberlyst* 15, gave the diastereomeric *N*-Boc sphinganine. Purification using flash chromatography afforded the *erythro*- (272 mg, 48 %) and *threo*- (157 mg, 28 %) diastereomers in a 1.73:1 ratio, confirming the ¹H NMR C2-[4,4-²H₂]-dihydroceramide spectral analysis.

Cleavage of the carbamate moiety with 1 N HCl in dioxane gave the respective sphinganine (122). Their relative configuration was established, similar as described in section 2.2.3, by analysis of the ^1H NMR spectra of the corresponding C2-dihydroceramides (Figure 2.16 and 2.17). The C2- and C3- protons of *D-erythro*-C2-[4,4- $^2\text{H}_2$]-dihydroceramide displayed signal differences of ca. 0.15 ppm compared to *L-threo*-C2-[4,4- $^2\text{H}_2$]-dihydroceramide. In addition, the ^1H NMR spectrum showed $^3J_{2,3} = 6.7$ Hz, a value characteristic of *trans* coupling (Figure 2.16), whereas the ^1H NMR spectrum of *L-threo*-C2-[4,4- $^2\text{H}_2$]-dihydroceramide showed $^3J_{2,3} = 2.5$ Hz, a value characteristic of *gauche* coupling (Figure 2.17) (11,147). All ^1H NMR signals were in accord with the assigned structures (11) (see Experimental, section 3.5.3(a)).

Figure 2.16 – Partial ^1H NMR spectrum of *D*-erythro-C2-[4,4- $^2\text{H}_2$]-dihydroceramide

Figure 2.17 – Partial ^1H NMR spectrum of *L*-threo-C2-[4,4- $^2\text{H}_2$]-dihydroceramide

The ^1H and ^{13}C NMR spectra were consistent with the location of the deuterium label of D-*erythro*-C2-[4,4- $^2\text{H}_2$]-dihydroceramide. The ^1H NMR spectrum (Figure 2.16) revealed the absence of a signal at 1.51 ppm for C(4)-H₂ and the ddd signal at 3.58 ppm for C(3)-H of the unlabelled material collapsed to a doublet ($^3J_{2,3} = 6.7$ Hz). The signal assigned to C-4 at 34.83 ppm had disappeared in the ^{13}C NMR spectrum of D-*erythro*-C2-[4,4- $^2\text{H}_2$]-dihydroceramide (Figure 2.18). An upfield β -isotope shift of 0.11 ppm and 0.19 ppm was observed for the resonances at C-3 and C-5 respectively, and the signal assigned to C-2 experienced a γ -isotope shift (0.02 ppm) (150).

Figure 2.18 – Partial ^{13}C NMR spectrum of D-*erythro*-C2-[4,4- $^2\text{H}_2$]-dihydroceramide

The enantiomeric purity of D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine was determined from the ^1H NMR spectrum of the (*S*)- α -methoxy- α -(trifluoromethyl)phenylacetate (MTPA) derivatives (148,149), as described in section 2.2.3. Analysis of the methoxy proton signals of synthetic (*S*)-MTPA-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine clearly revealed that all reactions with serine aldehyde had occurred with no detectable racemization (Figure 2.12 and 2.19).

Figure 2.19 – Partial ^1H NMR spectrum of D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine (S)-MTPA derivative

N-octanoyl-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine [4,4- $^2\text{H}_2$]-11, was prepared similar to that described in 2.2.3 (125). The purity of [4,4- $^2\text{H}_2$]-11 was deemed suitable for biological experiments by GC (Figure 2.20) and consisted entirely of dideuterated species (within experimental error) as determined by MS (Figure 2.21). The mass spectrum of O-trimethylsilylated [4,4- $^2\text{H}_2$]-11 displayed the following characteristic fragment ions: m/z 558 ($\text{M}^{+\bullet} - \text{CH}_3$); 470 ($\text{M}^{+\bullet} - (\text{CH}_3)_3\text{Si-O-CH}_2$); 315 ($((\text{CH}_3)_3\text{Si-O-CHCD}_2(\text{CH}_2)_{13}\text{CH}_3)^+$); and 258 ($((\text{CH}_3)_3\text{Si-O-CH}_2\text{CH-NH-C(O)(CH}_2)_6\text{CH}_3)^+$), which are consistent with the assigned structure (125).

The ^1H NMR spectrum (Figure 2.22) displayed all the characteristic signals associated with the sphingoid base (11). The triplet at 2.25 ppm for C(2')-H₂ confirmed acylation.

Figure 2.20 - GC of *N*-octanoyl-*D*-erythro-[4,4-²H₂]-sphinganine [4,4-²H₂]-11

Figure 2.21 - MS of *N*-octanoyl-*D*-erythro-[4,4-²H₂]-sphinganine [4,4-²H₂]-11

Figure 2.22 - ^1H NMR spectrum of *N*-octanoyl-*D*-erythro-[4,4- $^2\text{H}_2$]-sphinganine [4,4- $^2\text{H}_2$]-11

2.2.7 Synthesis of *N*-Octanoyl-*D*-erythro-[5,5- $^2\text{H}_2$]-sphinganine ([5,5- $^2\text{H}_2$]-11)

N-Octanoyl-*D*-erythro-[5,5- $^2\text{H}_2$]-sphinganine [5,5- $^2\text{H}_2$]-11 was synthesized in 5 % overall est. yield from chiral oxazolidine aldehyde 12 and the deuterium labelled Grignard precursor 14 as outlined in Scheme 2.14.

Nucleophilic addition (140) of magnesium [2,2- $^2\text{H}_2$]-pentadecyl-1-bromide to chiral oxazolidine aldehyde 12 gave an inseparable diastereomeric mixture of the fully protected sphinganine derivatives, *tert*-butyl [4*S*, 1'(*R*^{*}, *S*^{*})]-2,2-dimethyl-4-(1'-hydroxy[3',3'- $^2\text{H}_2$]-hexadecyl) oxazolidine-3-carboxylate, with a 55 % yield. All spectral data were in accord with the assigned structure (142) (see Experimental, section 3.6.1).

Scheme 2.14 – Synthesis of *N*-octanoyl-D-erythro-[5,5-²H₂]-sphinganine [5,5-²H₂]-11

¹H NMR analysis of the corresponding C2-[5,5-²H₂]-dihydroceramides (*N*-acetyl [5,5-²H₂]-sphinganine), revealed the addition of magnesium [2,2-²H₂]-pentadecyl-1-bromide 14 to aldehyde 12 proceeded with moderate *anti* selectivity to give a ca. 2:1 mixture of *erythro*- and *threo*- diastereomers.

Deketalization (122) with *Amberlyst* 15 gave the diastereomeric *N*-Boc sphinganines. Purification on flash silica afforded the *erythro*- (261 mg, 37 %) and *threo*- (147 mg, 21 %) diastereomers in a 1.78:1 ratio, confirming the ¹H NMR C2-[5,5-²H₂]-dihydroceramide spectral analysis.

Cleavage of the carbamate moiety with 1 N HCl in dioxane gave the respective sphinganines (122). The relative configuration of the deuterium labelled sphinganines was established, similar as described in 2.2.3, by analysis of the ¹H NMR spectra of the corresponding C2-dihydroceramides (Figure 2.23 and 2.24).

Figure 2.23 – Partial ¹H NMR spectrum of D-*erythro*-C2-[5,5-²H₂]-dihydroceramide

Figure 2.24 – Partial ¹H NMR spectrum of L-*threo*-C2-[5,5-²H₂]-dihydroceramide

The C2- and C3- protons of D-*erythro*-C2-[5,5-²H₂]-dihydroceramide displayed signal differences of ca. 0.15 ppm compared to L-*threo*-C2-[5,5-²H₂]-dihydroceramide. The ¹H NMR spectrum of D-*erythro*-C2-[5,5-²H₂]-dihydroceramide also showed ³J_{2,3} = 6.5 Hz, a value characteristic of *trans* coupling (Figure 2.23), whereas the ¹H NMR spectrum of L-*threo*-C2-[5,5-²H₂] dihydroceramide showed ³J_{2,3} = 2.6 Hz, a value characteristic of *gauche* coupling (Figure 2.24) (11,147). All ¹H NMR signals were in accord with the assigned structures (11) (see Experimental, section 3.6.3(a)).

The ¹H and ¹³C NMR spectra were consistent with location of the deuterium label of D-*erythro*-C2-[5,5-²H₂]-dihydroceramide. The ¹H NMR spectrum (Figure 2.23) revealed the collapse of a multiplet signal (ca. 1.50 ppm) for the diastereotopic hydrogens at C-4 of the unlabelled compound to two sets of broadened doublet of doublets (1.37 ppm and 1.51 ppm). The signal assigned to C-5 at 26.85 ppm had disappeared in the ¹³C NMR spectrum of D-*erythro*-C2-[5,5-²H₂]-dihydroceramide (Figure 2.25). An upfield β-isotope shift of 0.19 ppm was observed for the resonance at C-4 and the signal assigned to C-3 experienced a γ-isotope shift (0.02 ppm) (150).

Figure 2.25 – Partial ¹³C NMR spectrum of D-*erythro*-C2-[5,5-²H₂]-dihydroceramide

The enantiomeric purity of D-*erythro*-[5,5- $^2\text{H}_2$]-sphinganine was determined from the ^1H NMR spectrum of the (S)- α -methoxy- α -(trifluoromethyl)phenylacetate (MTPA) derivatives (148,149), as described in section 2.2.3. Analysis of the methoxy proton signals of (S)-MTPA-D-*erythro*-[5,5- $^2\text{H}_2$]-sphinganine revealed that all reactions with serine aldehyde occurred with no detectable racemization (Figure 2.12 and 2.26).

Figure 2.26 – Partial ^1H NMR spectrum of D-*erythro*-[5,5- $^2\text{H}_2$]-sphinganine (S)-MTPA derivative

N-octanoyl-D-*erythro*-[5,5- $^2\text{H}_2$]-sphinganine [5,5- $^2\text{H}_2$]-11, was prepared similar to that described in 2.2.3 (125). The purity of [5,5- $^2\text{H}_2$]-11 was deemed suitable for biological experiments by GC (Figure 2.27) and consisted primarily of dideuterated species (95-97 %) as determined by MS (Figure 2.28). The mass spectrum of O-trimethylsilylated [5,5- $^2\text{H}_2$]-11 displayed the following characteristic fragment ions: m/z 558 ($\text{M}^+ - \text{CH}_3$); 470 ($\text{M}^+ - (\text{CH}_3)_3\text{Si-O-CH}_2$); 313 ($(\text{CH}_3)_3\text{Si-O-CHCH}_2\text{CD}_2(\text{CH}_2)_{12}\text{CH}_3$) $^+$; and 258 ($(\text{CH}_3)_3\text{Si-O-CH}_2\text{CH-NH-C(O)(CH}_2)_6\text{CH}_3$) $^+$, which are consistent with the assigned structure (125).

The ^1H NMR spectrum (Figure 2.29) displayed all the characteristic signals associated with the sphingoid base. The triplet at 2.25 ppm for the methylenes α to the amide confirmed acylation.

Figure 2.27 - GC of *N*-octanoyl-*D*-erythro-[5,5- $^2\text{H}_2$]-sphinganine [5,5- $^2\text{H}_2$]-11

Figure 2.28 - MS of *N*-octanoyl-*D*-erythro-[5,5- $^2\text{H}_2$]-sphinganine [5,5- $^2\text{H}_2$]-11

Figure 2.29 - ¹H NMR spectrum of *N*-octanoyl-*D*-erythro-[5,5-²H₂]-sphinganine [5,5-²H₂]-11

Part 2: Trial Incubation Experiments

Trial incubation experiments were performed in order to: (a) determine if the synthetically prepared compounds were suitable substrates; and (b) examine the formation of unlabelled and labelled sphingosine products (*N*-octanoyl-*D*-erythro-sphingosine 15, *N*-octanoyl-*D*-erythro-[4-²H₁]-sphingosine [4-²H₁]-15 and *N*-octanoyl-*D*-erythro-[5-²H₁]-sphingosine [5-²H₁]-15) (Scheme 2.15) from the corresponding substrates.

Thus, 10 nmol of lipid substrate (11, [4,4-²H₂]-11 and [5,5-²H₂]-11) was complexed with bovine serum albumin (BSA) (151) and incubated for 30 minutes at 37 °C with 30 μL of rat liver microsomes (300 μg) containing the dihydroceramide Δ⁴ desaturase (see Experimental, section 3.10) (13). Previous protein concentration experiments, using BSA as standard protein, revealed the stock microsomal suspension to contain ca. 10 μg/μL of protein (152). The reaction was terminated with the addition of

CHCl_3 . Centrifugation and extraction with CHCl_3 (2 x 500 μL) gave the lipid extracts and reaction with *bis*(trimethylsilyl)-trifluoroacetamide (BTFA) gave the trimethylsilyl ceramide derivatives for GC/MS analysis.

Scheme 2.15 – Δ^4 Desaturation of 11, [4,4- $^2\text{H}_2$]-11 and [5,5- $^2\text{H}_2$]-11

The trimethylsilyl derivatives give characteristic fragments in their mass spectra, with diagnostic ions arising from cleavage of the C2-C3 bond (125) (Scheme 2.16). For incubations using non-labelled *N*-octanoyl-*D*-erythro-sphinganine 11, the identity of both substrate 11 and product 15 was confirmed by mass spectral analysis. Diagnostic fragment ions were m/z 258 and 313 (trimethylsilylated substrate), m/z 258 and 311 (trimethylsilylated product) (Figures 2.30 to 2.32). Control experiments, which lacked the necessary reductant (NADH), were also performed to confirm that product 15 formation was as a result of *N*-octanoyl-*D*-erythro-sphinganine 11 desaturation (Figure 2.33).

Scheme 2.16 - Δ^4 Desaturation of *N*-octanoyl-*D*-erythro-sphinganine 11 and structure of the trimethylsilylated substrate (11-TMS) and product (15-TMS) derivatives

Figure 2.30 – SIM-GC/MS traces of the reaction substrate 11 and product 15 trimethylsilyl derivatives (*-TMS-cholesterol)

Figure 2.31 – Mass spectrum of reaction substrate trimethylsilyl *N*-octanoyl-*D*-erythro-sphinganine 11-TMS

Figure 2.32 – Mass spectrum of reaction product trimethylsilyl *N*-octanoyl-*D*-erythro-sphingosine 15-TMS

Figure 2.33 – TIC-GC/MS displaying NADH dependence

When performing intermolecular competition KIE experiments one must take care to maintain the level of conversion below 15 %, as enrichment of the d_2/d_0 ratio with labelled material may cause a reduction in the primary KIE (112) (Appendix B). Analysis of unlabelled lipid extracts revealed the level of substrate 11 conversion to product 15 was approximately 10 %, a reasonable value for preventing enrichment of the d_2/d_0 ratio of the starting materials (Table 2.1).

GC/MS analysis of the $[4,4-^2H_2]$ -11 lipid extract (Figure 2.34) revealed a dramatic decrease in the percent desaturation relative to 11 (10-fold) and $[5,5-^2H_2]$ -11 (9-fold), which pointed to a possible large isotope effect at C-4 (Table 2.1). The slight decrease (1.2-fold) in percent desaturation of $[5,5-^2H_2]$ -11 relative to 11 cannot be interpreted as a β -secondary isotope effect (109), with any degree of confidence (Figure 2.35). These observations demonstrate the importance of using a competitive approach in our intermolecular kinetic isotope effect studies.

Table 2.1 – Conversion of *N*-octanoyl-D-*erythro*-sphinganine **11**, *N*-octanoyl-D-*erythro*-[4,4-²H₂]-sphinganine [4,4-²H₂]-**11** and *N*-octanoyl-D-*erythro*-[5,5-²H₂]-sphinganine [5,5-²H₂]-**11**^a

Experiment	Substrate Incubated	Product	% Desaturated ^b
1	11	15	10 %
2	[4,4- ² H ₂]- 11	[4- ² H ₁]- 15	1 %
3	[5,5- ² H ₂]- 11	[5- ² H ₁]- 15	9 %

^a Each entry represents an average value based on four experiments (standard deviation = 5.0 %)

^b % Desaturation refers to the ratio: [(product / (product + substrate) x 100]

The deuterated sphingosine products ([4-²H₁]-**15** and [5-²H₁]-**15**) consisted entirely of monodeuterated species (within experimental error) as determined by GC/MS. The mass spectra were in accord with the proposed structures (125): for the unlabelled TMS derivative *N*-octanoyl-D-*erythro*-sphingosine **15** (Figure 2.32): *m/z* 554 (M⁺-CH₃); *m/z* 311 ((CH₃)₃Si-O-CHCH=CH-(CH₂)₁₂CH₃)⁺; *m/z* 258 ((CH₃)₃Si-O-CH₂CHNHC(O)(CH₂)₆CH₃)⁺, for the labelled TMS derivative *N*-octanoyl-D-*erythro*-[4-²H₁]-sphingosine ([4-²H₁]-**15**) (not shown): *m/z* 555 (M⁺-CH₃); *m/z* 312 ((CH₃)₃Si-O-CHCD=CH-(CH₂)₁₂CH₃)⁺; *m/z* 258 ((CH₃)₃Si-O-CH₂CHNHC(O)(CH₂)₆CH₃)⁺, and for the TMS derivative of *N*-octanoyl-D-*erythro*-[5-²H₁]-sphingosine ([5-²H₁]-**15**) (not shown): *m/z* 555 (M⁺-CH₃); *m/z* 312 ((CH₃)₃Si-O-CHCH=CD-(CH₂)₁₂CH₃)⁺; *m/z* 258 ((CH₃)₃Si-O-CH₂CHNHC(O)(CH₂)₆CH₃)⁺.

Figure 2.34 – SIM-GC/MS traces of trimethylsilyl *N*-octanoyl-D-*erythro*-[4,4-²H₂]-sphinganine reaction substrate ([4,4-²H₂]-11-TMS) and product ([4-²H₁]-15-TMS)

Figure 2.35 – SIM-GC/MS traces of trimethylsilyl *N*-octanoyl-D-*erythro*-[5,5-²H₂]-sphinganine reaction substrate ([5,5-²H₂]-11-TMS) and product ([5-²H₁]-15-TMS)

Part 3: Intermolecular Kinetic Isotope Effect Studies

For determination of the competitive intermolecular isotope effect for Δ^4 desaturation of dihydroceramide (112), a mixture consisting of approximately equal amounts of each deuterated and non-deuterated substrate was prepared (ca. 100 μg). Each incubation was carried out three times and the six starting mixtures were individually prepared. A small portion of each mixture was derivitized to the corresponding TMS-sphinganine and examined by GC/MS to determine the d_0/d_2 ratio. 10 nmol of the remaining portion was complexed with BSA (151) and administered to the rat liver microsomes (see Experimental, section 3.10). After 30 minutes the reaction was terminated and the lipid extracts were derivitized with BTFA and the deuterium content of the sphingosine products (d_0/d_1) was assessed by SIM-GC/MS. As discussed in part 2, the trimethylsilyl derivatives give characteristic fragments in their mass spectra, with diagnostic ions arising from cleavage of the C2-C3 bond (125) (Scheme 2.16). Diagnostic fragment ions, which were used to determine isotopic ratios, were m/z 311 (unlabelled trimethylsilylated product) and 312 (labelled trimethylsilylated product).

Analysis of extract 11, [4,4- $^2\text{H}_2$]-11 and 11, [5,5- $^2\text{H}_2$]-11 revealed their average overall conversion to sphingosine products (15, [4- $^2\text{H}_1$]-15 and 15, [5- $^2\text{H}_1$]-15) to be ca. 4 % and 7 %, respectively. Since the overall percent conversion of substrate to product was less than 10 % for each incubation, the intermolecular kinetic isotope effects (k_H/k_D) were measured via the following equation (Appendix B) (112):

$$\frac{K_H}{K_D} = \frac{P_H / P_D}{S_H^0 / S_D^0}$$

where, P_H / P_D is the ratio of the nondeuterated *N*-octanoyl-D-*erythro*-sphingosine 15 to monodeuterated *N*-octanoyl-D-*erythro*-sphingosine ([4- $^2\text{H}_1$]-15, [5- $^2\text{H}_1$]-15) and S_H^0 / S_D^0 is the ratio of nondeuterated *N*-octanoyl-D-*erythro*-sphinganine 11 to dideuterated *N*-octanoyl-D-*erythro*-sphinganine ([4,4- $^2\text{H}_2$]-11, [5,5- $^2\text{H}_2$]-11). Thus,

$$\frac{K_H}{K_D} = \frac{(\%d_0)_P}{(\%d_1)_P} / \frac{(\%d_0)_S}{(\%d_2)_S}$$

It should be noted that k_H/k_D can be calculated as the ratio of isotopic species in the products or in the unchanged substrates relative to the ratio in the original substrate mixture. However, it is self-evident that the measurement of isotopic species in the unreacted substrates for small amounts of conversion does not constitute an accurate method of determining rate ratios (111,112)

The analytical data is displayed in Table 2.2. A large primary deuterium isotope effect (7.8 ± 0.2 , average of 3 experiments $\pm$ standard deviation) was determined for the carbon-hydrogen cleavage at C-4, while the C5-H bond-breaking step was essentially insensitive to deuterium substitution ($KIE = 1.05 \pm 0.03$, average of 3 experiments $\pm$ standard deviation). The observed isotope effect at C-4 is close to the theoretical maximum value for a primary deuterium isotope effect at 30 °C ($k_H/k_D = 7.2$) (110). However, it should be noted that a small proportion (<10 %) of the observed isotope effect may be due to an α -secondary isotope effect (109). On the other hand, the magnitude of the intrinsic primary deuterium isotope effect at C-4 may well be higher since our estimated value is derived via an intermolecular competition experiment and is subject to partial masking by other enzymatic steps (109). Large intrinsic primary deuterium isotope effects are not uncommon for biological hydrocarbon activation thought to involve iron-oxo species (153).

Table 2.2 – Intermolecular isotopic discrimination in dihydroceramide Δ^4 desaturation of *N*-octanoyl-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine [4,4- $^2\text{H}_2$]-11 and *N*-octanoyl-D-*erythro*-[5,5- $^2\text{H}_2$]-sphinganine [5,5- $^2\text{H}_2$]-11

Isotopic Composition (% mol) ^a							
Substrates ^b				Products			
Expt	d ₀	4,4-d ₂	5,5-d ₂	d ₀	4-d ₁	5-d ₁	KIE
1	50.3	49.7		88.8	11.2		7.8
2	51.8	48.2		89.2	10.8		7.7
3	52.9	47.1		90.0	10.0		8.0
							(7.8 ± 0.2) ^c
1	64.4		35.6	65.9		34.1	1.07
2	50.7		49.3	51.8		48.2	1.05
3	51.3		48.7	51.5		48.5	1.01
							(1.05 ± 0.03) ^d

^a The % mol of each isotopic species is given as an average value based on two to three GC/MS runs. ^b The six starting mixtures were individually prepared. ^c The average KIE at C-4 ± standard deviation. ^d The average KIE at C-5 ± standard deviation.

The results presented in this thesis provide the first clues as to the mechanism of the dihydroceramide Δ^4 desaturase. The KIE data clearly indicate that C-H cleavage at C-4 but not C-5 is kinetically important. According to our model (Chapter 1, Scheme 1.22), this implies that initial oxidative attack occurs at C-4 in a relatively slow, isotopically sensitive step. The second C-H cleavage at C-5 appears to be fast as might be expected for the collapse of a radical or carbocation intermediate (Chapter 1, Figure 1.22). Thus, dihydroceramide Δ^4 desaturation involves stepwise, non-synchronous removal of two hydrogens.

These results also strengthen the notion that the production of phytosphingosine via 4-hydroxylation and formation of sphingosine via 4,5-desaturation are closely linked at the mechanistic level. The stereochemical evidence also points to a common site of attack since it is the *pro*-(*R*) hydrogen at C-4, which is removed in both enzymatic reactions (51,52). This picture is very strongly reminiscent of the connection which has been established between the structurally related castor oleate 12-hydroxylase and microsomal Δ^{12} oleate desaturase (101) (Scheme 2.17). Using a KIE approach, it was

shown that the latter enzyme initiated oxidation at the same carbon which is attacked by the corresponding 12-hydroxylase (KIE, Δ^{12} oleate desaturase: $k_H/k_D = 7.3$ (C-12); $k_H/k_D = 1.05$ (C-13)). In addition, the membrane-bound oleate 12-hydroxylase and the membrane-bound Δ^{12} desaturase have also been shown to have many biochemical similarities. These similarities include: (a) substrate requirements: oleate esterified to PC (48,154); (b) stereoselectivity of the reactions: removal of *pro-R* hydrogens from C-12(13) (48); (c) b_5 electron-transport system (155); (d) inhibition with CN^- but not with CO_2 (156,157); and (e) homology in amino acid sequence (108).

This trend in KIE's has also been observed in the cytochrome P450-catalyzed 4-desaturation of the anti-epileptic drug, valproic acid (2-propylbutanoic acid) – a process which is accompanied by the formation of a 4-hydroxylated metabolite as the major product (73).

Scheme 2.17 – Biosynthesis of ricinoleate and linoleate

Our data also shed some light on the type of oxidative enzymes involved in 4-desaturation / hydroxylation. That dihydroceramide desaturase and the yeast sphingolipid 4-hydroxylase are both members of the membrane-bound diiron enzyme family of catalysts had previously been postulated (104) by virtue of the apparent biochemical similarities of the former enzyme to the membrane-bound Δ^9 stearoyl CoA desaturase (20,158) and the detection of the diagnostic multi-histidine diiron-binding

motifs (36, 37) for yeast 4-hydroxylase. This view is supported by the findings in this thesis. Not only do the two catalysts share a common regiochemistry of initial oxidative attack, but the relative magnitudes (one large, one negligible) of the primary deuterium isotope effects obtained in this study are similar to the pattern of values which have been obtained for a large number of membrane-bound desaturases *in vivo* (47,94,96,99,100,101,102,159). It should be noted that, this study constitutes the first *in vitro* assessment of a desaturase-mediated kinetic isotope effect and substantiates these earlier results obtained *in vivo*.

The observation that the dihydroceramide Δ^4 desaturase initiates the reaction at C-4 sustains another interesting trend which has emerged from investigations into the cryptoregiochemistry of fatty acid-type desaturations ranging from Δ^4 to ω -3 (see reference list above). Without exception, it has been demonstrated that initial oxidation always occurs at the carbon atom closest to the polar head group (Scheme 2.18). Since there can be no inherent thermodynamic basis for this regiochemical imperative, this phenomenon must be the consequence of some active site motif which is common to this class of enzymes. Further interpretation into the significance of this data must await information regarding the membrane-bound protein structure to be obtained.

Scheme 2.18 – Model representing fatty acid desaturases with three different regioselectivities

In conclusion, several studies have pointed out that the double bond at C4, C5 is crucial for the signal transduction role of sphingolipids in mammals. Despite the importance of dihydroceramide desaturase, which catalyzes the introduction of this olefinic bond, and the efforts of many laboratories around the world, this enzyme remains shrouded in mystery. The mechanistic study reported above strongly supports the idea that dihydroceramide desaturase and yeast 4-hydroxylase are linked by a common mechanism and in light of previous studies (ie. 12-hydroxylase / Δ^{12} desaturase and valproic acid), are possibly related at the molecular level. As well, the elucidation of double-bond formation in the sphingoid backbone of sphingolipids has another intriguing aspect besides its biochemical importance. Modulation of dihydroceramide Δ^4 desaturase activity would offer a method of influencing the bioactive levels of ceramide. Moreover, knowing the properties of the desaturase system and the mechanism involved, one may think of ways of obtaining cellular systems devoid of desaturated sphingolipids. This study provides the first clues into the mechanism of dihydroceramide desaturation, and offers an unprecedented opportunity to develop selective chemical inhibitors of this process.

Summary: We were the first to determine the intermolecular primary deuterium isotope effects involved in dihydroceramide Δ^4 desaturation. Our results strongly suggest that the initial oxidative attack of Δ^4 desaturase is at C-4 and that the production of phytosphingosine (4-hydroxysphinganine) **10** in yeast is carried out by a modified Δ^4 desaturase.

Savile, C.K.; Fabrias, G.; Buist, P.H. *J. Am. Chem. Soc.* **2001**, *in press*.

Chapter 3

Experimental

Part 1: Synthesis of Substrates

3.1 General Methods

^1H (200 MHz and 400 MHz) and ^{13}C NMR (50 and 100 MHz) spectra were obtained on Varian XL 200 or Brüker AMX 400 spectrometers using dilute CDCl_3 , C_6D_6 or CD_3OD solutions. Abbreviations used in assigning peaks are the following: s=singlet, d=doublet, t=triplet, q=quartet, p=pentet, m=multiplet, br=broad. All chemical shifts are expressed in ppm (δ) and are referenced to tetramethylsilane (TMS). Coupling constant (J) values are reported in Hertz (Hz).

Mass spectra of substrates were obtained by GC-MS using a Kratos Concept 1H mass spectrometer equipped with a 70 eV EI ionization chamber. Samples were introduced using a HP 5980 Series 2 gas chromatograph equipped with a J. & W. 30 m x 0.21 mm, DB-5 capillary column.

Flash chromatography using silica gel (230–400 mesh) was used to purify all intermediates. Chromatography using silica gel (70–230 mesh) was used to purify substrates. Analytical TLC was performed using Merck glass plates pre-coated with silica G/UV 254. Visualization of UV-inactive materials was accomplished by using phosphomolybdic acid (PMA) followed by charring or a combination of I_2 vapour followed by a water spray.

Unless otherwise stated, all reagents and starting materials were purchased from Aldrich Chemical Co. and used without purification. Tetrahydrofuran (THF), toluene and diethyl ether (Et_2O) were freshly distilled from Na-benzophenone ketyl. All air- and moisture-sensitive reactions were performed under N_2 . Organic extracts were typically shaken with saturated NaCl, dried over MgSO_4 , and solvents were evaporated *in vacuo* on a Büchi RE 111 Rotavapor.

3.2 Synthesis of Protected L-Serine Aldehyde (12)

3.2.1 Methyl *N*-Boc Serinate

Acetyl chloride (40 mL) was added to MeOH (250 mL) at 0 °C and stirred for 5 min. L-Serine (20.08 g, 190 mmol) was added in one portion and heated to a reflux for 2 h. The reaction mixture was cooled to RT and the solvent was evaporated under reduced pressure to give crude methyl serinate HCl as a colourless crystalline solid.

A solution of di-*tert*-butyl dicarbonate (Boc)₂O (36.01 g, 160 mmol) in anhydrous THF (100 mL) was added over 30 min., using a dropping funnel, to a suspension of methyl serinate HCl (24.98 g) in Et₃N (35.10 g, 346 mmol) and anhydrous THF (300 mL) at 0 °C. The reaction mixture was stirred overnight at RT and then heated and stirred at 50 °C until complete by TLC (6 h). The solvent was removed *in vacuo* and the residue was partitioned between Et₂O (150 mL) and dH₂O (150 mL). The ethereal layer was removed and the aqueous layer was extracted with Et₂O (2 x 100 mL). The combined organic layers were washed with 3 % HCl (100 mL), 5 % NaHCO₃ (100 mL), sat. NaCl (200 mL), dried over MgSO₄ and concentrated *in vacuo* to give methyl *N*-Boc serinate (29.67 g, 85 %) as a golden oil (124,126).

R_f 0.29 (1:1 hexane/EtOAc)

IR (neat) 3411 cm⁻¹ (OH), 1717 (br) cm⁻¹ (C=O, carbamate-, methyl-ester)

¹H NMR (200 MHz)¹²⁴ δ 1.43 (br s, 9 H, (CH₃)₃C-), 2.19 br s, 1 H, exchange with D₂O, OH), 3.76 (s, 3 H, -OCH₃), 3.85 (dd, *J* 4.0, 12.0, 1 H, C(3)-H), 3.93 (dd, *J* 4.0, 12.0, 1 H, C(3)-H), 4.34 (m, 1H, C(2)-H), 5.45 (br s, 1 H, exchange with D₂O, NH)

[α]_D -0.40° (c=0.69, MeOH) [lit. ¹²⁶ [α]_D -17.5 to -18.9°]

Note: Variable optical measurements are not uncommon for this compound (124).

3.2.2 3-(1,1-Dimethylethyl)-4-Methyl-(*S*)-2,2-dimethyloxazolidine-3,4-dicarboxylate

$\text{BF}_3 \cdot \text{OEt}_2$ (0.4 mL) was added to a solution of methyl *N*-Boc serinate (29.67 g, 136 mmol) in acetone (400 mL) and 2,2-dimethoxypropane (17.71 g, 170 mmol) and stirred until complete by TLC (5 h). The solvent was evaporated under reduced pressure and the residual oil was taken up in CH_2Cl_2 (150 mL). The organic layer was washed with 5 % NaHCO_3 / dH_2O (1:1, 100 mL), sat. NaCl (100 mL), dried over MgSO_4 and concentrated *in vacuo* to give 3-(1,1-dimethylethyl)-4-methyl-(*S*)-2,2-dimethyloxazolidine-3,4-dicarboxylate (27.85 g, 80 %) as a viscous golden liquid (124,126).

R_f 0.40 (4:1 hexane/EtOAc)

IR (neat) 1760 cm^{-1} (C=O, ester), 1710 cm^{-1} (C=O, carbamate)

^1H NMR (C_6D_6 , 400 MHz, 340 K) 124 δ 1.40 (br s, 9 H, $(\text{CH}_3)_3\text{C-}$), 1.51 (s, 3H, $\text{CH}_3\text{C(2)}$), 1.77 (s, 3H, $\text{CH}_3\text{C(2)}$), 3.37 (s, 3H, $-\text{OCH}_3$), 3.77 (dd, J 8.9, 6.9 Hz, 1H, C(5)-H), 3.80 (dd, J 8.9, 3.6 Hz, 1H, C(5)-H), 4.30 (m, 1H, C(4)-H)

^{13}C NMR (100 MHz) 140,143 δ 171.72 ($-\text{COOMe}$), 152.12 (COO-t-Bu), 95.07 (C-2), 80.91 ($\text{C}(\text{CH}_3)_3$), 66.27 (C-5), 59.28 (C-4), 52.41 ($-\text{OCH}_3$), 28.35 ($\text{C}(\text{CH}_3)_3$), 26.02 and 24.95 ($(\text{CH}_3)_2\text{C-2}$)

$[\alpha]_D -43.9^\circ$ ($c=1.3$, CHCl_3) [lit. 124 $[\alpha]_D -46.7^\circ$ ($c=1.3$)]

3.2.3 1,1-Dimethylethyl (*S*)-4-formyl 2,2-dimethyloxazolidine-3-carboxylate (12)

3-(1,1-Dimethylethyl)-4-Methyl-(*S*)-2,2-dimethyloxazolidine-3,4-dicarboxylate (4.04 g, 16 mmol) was dissolved in anhydrous toluene (32 mL) and cooled to -78°C before the addition of 1.5 M DIBALH (25 % wt/wt in toluene, 17.5 mL, 26 mmol) slowly

via syringe. Stirring was continued for 2 h at $-78\text{ }^{\circ}\text{C}$ before addition of anhydrous MeOH (8 mL) and warming to RT. The mixture was then poured into a solution of potassium sodium tartrate (36 g) in dH_2O (100 mL) and stirred for 2 h. The phases were separated and aqueous layer was extracted with Et_2O (2 x 75 mL). The combined organic layers were washed with sat. NaCl (75 mL), dried over MgSO_4 and concentrated *in vacuo* to give the crude aldehyde. Purification on flash silica, using 4:1 hexane/EtOAc as eluent, gave pure **12** (2.48 g, 69 %) as a clear viscous liquid (124,126).

R_f 0.27 (4:1 hexane/EtOAc)

IR (neat) 1739 cm^{-1} (C=O, aldehyde), 1710 cm^{-1} (C=O, carbamate)

^1H NMR (C_6D_6 , 400 MHz, 340 K) 124 δ 1.35 (br s, 9 H, $(\text{CH}_3)_3\text{C}-$), 1.44 (s, 3H, $\text{CH}_3\text{C}(2)$), 1.58 (s, 3H, $\text{CH}_3\text{C}(2)$), 3.56 (dd, J 9.3, 7.3 Hz, 1H, C(5)-H), 3.80 (dd, J 9.3, 3.6 Hz, 1H, C(5)-H), 3.93 (m, 1H, C(4)-H), 9.37 (s, 1 H, -CHO)

^{13}C NMR (100 MHz) 140,143 δ 198.28 (-CHO), 152.39 (COO-*t*-Bu), 95.12 (C-2), 80.47 ($\text{C}(\text{CH}_3)_3$), 65.12 (C-5), 63.59 (C-4), 28.14 ($\text{C}(\text{CH}_3)_3$), 26.78 and 24.80 ($(\text{CH}_3)_2\text{C}-2$)

$[\alpha]_D -88.3^{\circ}$ ($c=0.48$, CHCl_3) [lit. 124 $[\alpha]_D -91.7^{\circ}$ ($c=1.34$, CHCl_3)]

3.3 Synthesis of Deuterium Labelled Intermediates (13, 14)

3.3.1 $[1,1\text{-}^2\text{H}_2]\text{-1-Pentadecanol}$

A solution of pentadecanoic acid (4.20 g, 17.4 mmol) in Et_2O (10 mL) was added, via syringe, to a stirring suspension of LiAlD_4 (1.10 g, 26 mmol) in Et_2O (10 mL) at $0\text{ }^{\circ}\text{C}$. The reaction mixture was stirred overnight under N_2 . The solution was diluted with Et_2O (50 mL) and quenched with dH_2O (20 mL) and 3 M HCl (30 mL). The organic layer was removed and the aqueous layer was further extracted with Et_2O (2 x 50 mL). The combined organics were washed with 3 M HCl (50 mL), sat. NaCl (50 mL), dried over

MgSO₄ and concentrated *in vacuo* to give [1,1-²H₂]-1-pentadecanol (3.70 g, 93 %) as a white solid (133).

R_f 0.17 (4:1 hexane/EtOAc)

IR (KBr) 3299 cm⁻¹ (OH)

¹H NMR (200 MHz) δ 0.86 (t, *J* 6.7, 3H, CH₃CH₂-), 1.24 (br s, 24H, CH₃(CH₂)₁₂-), 1.53 (br t, *J* 7.9, 2H, -CH₂CD₂OH)

MS (EI, 70 eV, TMS derivative) *m/z* 287 (M⁺-CH₃)

3.3.2 [1,1-²H₂]-1-Bromopentadecane (13)

48 % HBr (4.02 g) was added to [1,1-²H₂]-1-pentadecanol (3.69, 16 mmol) followed by conc. H₂SO₄ (1 mL), in portions. The stirred solution was heated to a gentle reflux for 4 h under N₂. The reaction mixture was diluted with dH₂O (20 mL) and extracted with Et₂O (3 x 50 mL). The combined organic layers were washed with dH₂O, 5 % NaHCO₃ (25 mL), sat. NaCl (40 mL) and dried over MgSO₄. The Et₂O was removed under reduced pressure to give the crude bromo-alkane. Purification on silica, using Et₂O as eluent, gave pure [1,1-²H₂]-1-bromopentadecane 13 (3.37 g, 72 %) as a yellow oil (134).

R_f 0.69 (9:1 hexane/EtOAc)

¹H NMR (200 MHz) δ 0.86 (t, *J* 6.6, 3H, CH₃CH₂-), 1.24 (br s, 24H, CH₃(CH₂)₁₂-), 1.83 (br t, *J* 7.3, 2H, -CH₂CD₂Br)

3.3.3 Methyl pentadecanoate

A solution of 10 % BF_3/MeOH (150 mL) was added to pentadecanoic acid (4.38 g, 18 mmol) and refluxed under N_2 for 3 h. The reaction mixture was cooled to RT, quenched with dH_2O (100 mL) and extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers were washed with sat. NaCl (50 mL), dried over MgSO_4 and concentrated *in vacuo* to give methyl pentadecanoate (3.96 g, 86 %) as a colourless oil (135).

R_f 0.55 (4:1 hexane/EtOAc)

IR (neat) 1744 cm^{-1} ($\text{C}=\text{O}$, ester)

^1H NMR (200 MHz) δ 0.86 (t, J 6.7, 3H, CH_3CH_2-), 1.24 (br s, 22H, $\text{CH}_3(\text{CH}_2)_{11}-$), 1.60 (p, J 7.0, 2H, $-\text{CH}_2\text{CH}_2\text{COOMe}$), 2.29 (t, J 7.4, 3H, $-\text{CH}_2\text{COOMe}$), 3.65 (s, 3H, $-\text{OCH}_3$)

3.3.4 Methyl [2,2- $^2\text{H}_2$] pentadecanoate

Methyl pentadecanoate (5.16 g, 20 mmol) was added to a suspension of Na (0.11 g, 5 mmol) in CH_3OD (5 mL) and refluxed under N_2 for 1 h. The solvent was evaporated under reduced pressure and the procedure was repeated twice with fresh Na and CH_3OD . The reaction mixture was cooled to RT, acidified to pH 2 with 1:1 conc. DCl and D_2O (6 mL) and extracted with CH_2Cl_2 (3 x 40 mL). The combined organics were dried over MgSO_4 and concentrated *in vacuo* to give methyl [2,2- $^2\text{H}_2$] pentadecanoate (4.42 g, 85 %) as a colourless liquid (136).

R_f 0.54 (4:1 hexane/EtOAc)

IR (neat) 1744 cm^{-1} ($\text{C}=\text{O}$, ester)

^1H NMR (200 MHz) δ 0.86 (t, J 6.8, 3H, CH_3CH_2-), 1.24 (br s, 22H, $\text{CH}_3(\text{CH}_2)_{11}-$), 1.58 (br t, J 7.3, 2H, $-\text{CH}_2\text{CD}_2\text{COOMe}$), 3.65 (s, 3H, $-\text{OCH}_3$)

MS (EI, 70 eV) m/z 258 ($M^{+\bullet}$), 227 ($M^{+\bullet} - OCH_3$)

3.3.5 [2,2- 2H_2]-1-Pentadecanol

Methyl [2,2- 2H_2] pentadecanoate (2.70 g, 10 mmol) was reduced (133) with $LiAlH_4$ (0.61 g, 15 mmol) using a procedure similar to that described in 3.3.1 to give [2,2- 2H_2]-1-pentadecanol (2.12, 92 %) as a white solid.

R_f 0.17 (4:1 hexane/EtOAc)

IR (KBr) 3293 cm^{-1} (OH)

1H NMR (200 MHz) δ 0.86 (t, J 6.2, 3H, CH_3CH_2-), 1.24 (br s, 24H, $CH_3(CH_2)_{12}-$), 3.62 (br s, 2H, $-CD_2CH_2OH$)

3.3.6 [2,2- 2H_2]-1-Bromopentadecane (14)

[2,2- 2H_2]-1-Pentadecanol (3.49 g, 15 mmol) was brominated (134) with 48 % HBr (4.09 g) and conc. H_2SO_4 (2 mL) using a procedure similar that described in 3.3.2 to give [2,2- 2H_2]-1-bromopentadecane 14 (3.71 g, 85 %) as a yellow liquid.

R_f 0.69 (9:1 hexane/EtOAc)

1H NMR (200 MHz) δ 0.87 (t, J 6.4, 3H, CH_3CH_2-), 1.24 (br s, 24H, $CH_3(CH_2)_{12}-$), 3.38 (br s, 2H, $-CD_2CH_2Br$)

3.4 Synthesis of *N*-Octanoyl-*D*-*erythro*-Sphinganine (11)

3.4.1 *tert*-Butyl [4*S*, 1'(*R'*, *S'*)]-2,2-dimethyl-4-(1'-hydroxyhexadecyl)oxazolidine-3-carboxylate

Pentadecyl magnesium bromide was prepared by addition of bromopentadecane (3.61 g, 12 mmol) to a suspension of magnesium (0.31 g, 13 mmol) and I_2 (0.10 g) in THF (25 mL) under N_2 . After an exothermic reaction had subsided, the Grignard reagent was added dropwise, via syringe, to a stirred solution of **12** (2.80 g, 12 mmol) in anhydrous THF (25 mL) at $-78^\circ C$. The reaction mixture was stirred at $-78^\circ C$ for 1 h and then at RT until complete by TLC (24 h). The mixture was diluted with Et_2O (100 mL) and quenched with sat. NH_4Cl (60 mL). The phases were separated and the aqueous layer was extracted with Et_2O (2 x 70 mL). The combined organic layers were washed with 5 % $Na_2S_2O_3$ (70 mL), dH_2O (70 mL), sat. $NaCl$ (70 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give the crude amino alcohol. Purification on flash silica, using 9:1 hexane/ $EtOAc$ as eluent, gave an inseparable mixture of the fully protected *erythro*- and *threo*- sphinganine diastereomers (2.69 g, 50 %) as a colourless oil (140).

R_f 0.32 (4:1 hexane/ $EtOAc$)

IR (neat) 3439 cm^{-1} (OH), 1700 cm^{-1} (C=O, carbamate)

1H NMR (400 MHz) 142 δ 0.90 (t, J 6.7, 3H, $-CH_2CH_3$), 1.27 (br s, 26 H, $-(CH_2)_{13}CH_3$),

1.47 (br s, 9H, $-C(CH_3)_3$), 1.51 (br s, 6H, $-C(2)(CH_3)_2$), 1.53 (br m, 2H, $-C(1')CH_2-$),

3.50-4.12 (m, 4H, C(5)- H_2 , C(4)- H , C(1')- H), 5.21-5.40 (2 br s, 1H, OH)

$[\alpha]_D -17.2^\circ$ ($c=0.37$, $CHCl_3$) [lit. 142 $[\alpha]_D -26.9^\circ$ ($c=2.54$, $CHCl_3$) pure "*erythro*"]

MS (EI, 70 eV) m/z 398 $((CH_3)_2COCH_2CHCH(O-Si(CH_3)_3)(CH_2)_{14}CH_3)^+$, 313

$((CH_3)_3Si-O-CH(CH_2)_{14}CH_3)^+$, 310 $((O)CHCHCH(O-Si)(CH_2)_{14}CH_3)^+$

3.4.1(a) C2-Dihydroceramide Diastereomers

The fully protected sphinganine (100 mg, 0.23 mmol) was dissolved in dioxane (3 mL) and 3N HCl (1.5 mL) and then refluxed for 1 h. After cooling to RT, the reaction mixture was basicified with 2 N NaOH (1 mL) and extracted with Et₂O (2 x 20 mL). The combined organics were washed with sat. NaCl (20 mL), dried over MgSO₄ and concentrated *in vacuo* to give the corresponding free amine (28.0 mg, 41 %) (122). Acetic anhydride (0.5 mL, 5 mmol) was added to a solution of the amine (5.1 mg, 0.017 mmol) in MeOH (1 mL) and left overnight. The reaction mixture was diluted with dH₂O (1 mL) and extracted with Et₂O (3 x 1.5 mL). The combined organic extracts were evaporated under a steady stream of N₂ to give a mixture of the C2-dihydroceramide diastereomers (5.1 mg, 90 %, 37 % overall) as a white solid (160).

R_f 0.38 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.6, 3H, -CH₂CH₃), 1.28 (br s, 26H, -(CH₂)₁₃CH₃), 1.42 (m, 2H, *threo*-C(4)-H₂), 1.51 (m, 2H, *erythro*-C(4)-H₂), 1.97 (s, 3H, -C(O)CH₃), 3.54 (m, 1H, *threo*-C(1)-H), 3.59 (m, 1H, *erythro*-C(3)-H), 3.63 (m, 1H, *threo*-C(1)-H), 3.64-3.72 (m, 2H, *erythro*-C(1)-H₂), 3.76 (m, 1H, *threo*-C(3)-H), 3.81 (m, 1H, *erythro*-C(2)-H), 3.88 (m, 1H, *threo*-C(2)-H)

3.4.2 N-Boc-D-erythro-Sphinganine and N-Boc-L-threo-Sphinganine

Amberlyst 15 (1.09 g) was added to a solution of the *tert*-Butyl [4*S*, 1'(*R*^{*}, *S*^{*})]-2,2-dimethyl-4-(1'-hydroxyhexadecyl)oxazolidine-3-carboxylate (0.83 g, 2 mmol) in anhydrous MeOH (20 mL). The heterogeneous mixture was stirred at RT until complete by TLC (24 h). After filtration through Celite (1:1 hexane/EtOAc), the solvent was

evaporated *in vacuo* to give a mixture of diastereomers (0.54 g, 71 %). Separation of isomers using flash chromatography (3:1 hexane/EtOAc) gave the “*threo*” (0.13 g) and “*erythro*” (0.23 g) diastereomers as waxy solids (122). Their structures were confirmed by ^1H NMR analysis (122).

“*threo*” isomer:

mp 60-62 °C

R_f 0.18 (2:1 hexane/EtOAc) (no stain by I_2)

^1H NMR (400 MHz) 122 δ 0.90 (t, J 6.7, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26H, $-(\text{CH}_2)_{13}\text{CH}_3$), 1.47 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.52 (m, 2H, $\text{C}(4)\text{-H}_2$), 2.00 (br s, 1H, OH), 3.61 (br s, 1H, OH), 3.77 (dd, J 3.4, 11.2, 1H, $\text{C}(1)\text{-H}$), 3.85 (m, 1H, $\text{C}(2)\text{-H}$), 3.92 (m, 1H, $\text{C}(3)\text{-H}$), 4.01 (dd, J 3.4, 11.2, $\text{C}(1)\text{-H}$), 5.23 (br s, 1H, NH)

“*erythro*” isomer:

mp 80-82 °C

R_f 0.17 (2:1 hexane/EtOAc) (stain by I_2)

^1H NMR (400 MHz) 122 δ 0.90 (t, J 6.6, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26H, $-(\text{CH}_2)_{13}\text{CH}_3$), 1.47 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.55 (m, 2H, $\text{C}(4)\text{-H}_2$), 2.15 (br s, 1H, OH), 3.54 (br s, 1H, OH), 3.75-3.81 (m, 3H, $\text{C}(1)\text{-H}$, $\text{C}(2)\text{-H}$, $\text{C}(3)\text{-H}$), 4.01 (dd, J 3.4, 11.4, 1H, $\text{C}(1)\text{-H}$), 5.42 (br s, 1H, NH)

3.4.3 D-erythro-Sphinganine

A solution of *N*-Boc-*D*-erythro-sphinganine (205 mg, 0.5 mmol) in 1 N HCl (2.5 mL) and 1,4-dioxane (5 mL) was stirred at 100 °C for 30 min. After cooling to RT, 2 N NaOH (3 mL) was added to the mixture and extracted with Et_2O (3 x 15 mL). The combined Et_2O extracted were washed with sat. NaCl (20 mL), dried over MgSO_4 and

concentrated *in vacuo* to give the crude amine. Recrystallization from 1:1 hexane/EtOAc afforded pure *D-erythro*-sphinganine (88 mg, 58 %) as a white solid (122).

mp 92-94 °C (lit.¹⁶¹ 85-88 °C)

R_f 0.23 (4:1:0.1 CHCl₃/MeOH/2 N NH₄OH)

3.4.3(a) *D-erythro*-C2-Dihydroceramide

The free amine (5.1 mg, 17 μmol) was acylated (160) with Ac₂O as described in 3.4.1 (a) to give the corresponding C-2 dihydroceramide (5.2 mg, 90 %) as a white solid.

R_f 0.36 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.7, 3H, -CH₂CH₃), 1.28 (br s, 26 H, -(CH₂)₁₃CH₃), 1.51 (m, 2H, C(4)-H₂), 1.97 (s, 3H, -C(O)CH₃) 3.59 (ddd, *J* 6.6, 2.7, 8.6, 1H, C(3)-H), 3.66 (dd, *J* 6.3, 11.3, 1H, C(1)-H), 3.70 (dd, *J* 4.2, 11.3, 1H, C(1)-H), 3.81 (ddd, *J* 4.2, 6.3, 6.4, 1H, C(2)-H)

¹³C NMR (100 MHz) ¹²³ δ 173.37 (C=O), 72.40 (C-3), 62.16 (C-1), 57.03 (C-2), 34.83 (C-4), 33.08 (C-16), 30.79, 30.76, 30.48 (C-6 to C-16), 26.85 (C-5), 23.74 (C-2'), 22.77 (C-17), 14.44 (C-18)

3.4.4 *L-threo*-Sphinganine

N-Boc-*L-threo*-sphinganine (119 mg, 0.3 mmol) was deprotected (122) in 1 N HCl (1.5 mL) and 1,4-dioxane (3 mL) as described in 3.4.3 to give *L-threo*-sphinganine (40 mg, 45 %) as a white solid.

mp 106-108 °C (lit. ¹⁶² 99.5-100.5 °C)

R_f 0.20 (4:1:0.1 CHCl₃/MeOH/2 N NH₄OH)

3.4.4(a) *L*-threo-C2-Dihydroceramide

The free amine (4.5 mg, 15 μ mol) was acylated (160) with Ac₂O as described in 3.4.1(a) to give the corresponding C2-dihydroceramide (4.9 mg, 96 %) as a white solid.

R_f 0.38 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.8, 3H, -CH₂CH₃), 1.28 (br s, 26 H, -(CH₂)₁₃CH₃), 1.42 (m, 2H, C(4)-H₂), 1.98 (s, 3H, -C(O)CH₃), 3.54 (dd, *J* 6.4, 10.9, 1H, C(1)-H), 3.63 (dd, *J* 6.4, 10.9, 1H, C(1)-H), 3.76 (ddd, *J* 2.6, 6.9, 5.6, 1H, C(3)-H), 3.88 (ddd, *J* 2.6, 6.4, 6.4, 1H, C(2)-H)

3.4.5 *N*-Octanoyl-D-erythro-sphinganine (11)

D-erythro-sphinganine (1.5 mg, 5.0 μ mol) was dissolved in Et₂O (0.5 mL) followed by addition of aqueous 0.3 M NaHCO₃ (1 mL). The mixture was emulsified, and the reaction was started by addition of octanoyl chloride (10 μ L, 65 μ mol). The reaction mixture was vortexed and stored at RT until complete by TLC (2 h). The reaction was neutralized with 2N NaOH (0.5 mL) and extracted with Et₂O (3 x 2 mL). The combined ethereal layers were washed with dH₂O (3 mL), sat NaCl (3 mL), dried over Na₂SO₄ and concentrated under a steady stream of N₂. Purification on silica, using CHCl₃/MeOH (15:1) as eluent, gave *N*-octanoyl-D-erythro-sphinganine 11 (1.2 mg, 56 %) as a white solid (125).

R_f 0.43 (9:1 $\text{CHCl}_3/\text{MeOH}$)

^1H NMR (400 MHz) 11 δ 0.90 (t, J 6.6, 6H, 2 x CH_3), 1.27 (br s, 34H, $-(\text{CH}_2)_{13}\text{CH}_3$, -
C(3')- $(\text{CH}_2)_4\text{CH}_3$), 1.54 (m, 2H, C(4)- H_2), 1.65 (m, 2H, C(3')- H_2), 2.25 (t, J 7.4, 2H,
C(2')- H_2), 3.78 (dd, J 2.9, 11.2, 1H, C(1)- H), 3.82-3.87 (m, 2H, C(2)- H , C(3)- H), 4.04
(dd, J 3.3, 11.2, 1H, C(1)- H), 6.36 (d, J 7.4, 1H, NH)

MS (EI, 70 eV) m/z 556 ($\text{M}^{+\bullet}$ - CH_3), 468 ($\text{M}^{+\bullet}$ - $(\text{CH}_3)_3\text{Si-O-CH}_2$), 313 ($(\text{CH}_3)_3\text{Si-O-CH}(\text{CH}_2)_{14}\text{CH}_3$) $^+$, 258 ($(\text{CH}_3)_3\text{Si-O-CH}_2\text{CH-NH-C(O)(CH}_2)_6\text{CH}_3$) $^+$

3.5 Synthesis of *N*-Octanoyl-D-*erythro*-[4,4- $^2\text{H}_2$]-Sphinganine ([4,4- $^2\text{H}_2$]-11)

3.5.1 *tert*-Butyl [4*S*, 1'(*R'*, *S'*)]-2,2-dimethyl-4-(1'-hydroxy[2',2'- $^2\text{H}_2$] hexadecyl) oxazolidine-3-carboxylate

The Grignard reagent, magnesium [1,1- $^2\text{H}_2$]-pentadecyl-1-bromide, was prepared as described in 3.4.1 using [1,1- $^2\text{H}_2$]-1-bromopentadecane **13** (2.37 g, 8 mmol), $\text{Mg}(0)$ (0.20 g, 8 mmol), I_2 (0.10 g) and THF (15 mL).

The title compound was prepared as described in 3.4.1. Grignard addition (140) of magnesium [1,1- $^2\text{H}_2$]-pentadecyl-1-bromide (15 mL, 0.5 M) to **12** (1.80 g, 8 mmol) in THF (20 mL) at -78°C gave the crude amino alcohol. Purification on flash silica, using 9:1 hexane/EtOAc as eluent, gave an inseparable mixture of the fully protected *erythro*- and *threo*-sphinganine diastereomers (1.88 g, 53 %) as colourless oil.

R_f 0.34 (4:1 hexane/EtOAc)

IR (neat) 3439 cm^{-1} (OH), 1700 cm^{-1} (C=O, carbamate)

^1H NMR (400 MHz) 142 δ 0.90 (t, J 6.7, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26 H, $-(\text{CH}_2)_{13}-\text{CH}_3$), 1.47 (br s, 9H, $-\text{C}(\text{CH}_3)_3$), 1.51 (br s, 6H, $-\text{C}(2)(\text{CH}_3)_2$), 3.50-4.12 (m, 4H, C(5)- H_2 , C(4)- H , C(1')- H), 5.22-5.41 (2 br s, 1H, OH)

$[\alpha]_D -18.6^\circ$ ($c=0.57$, CHCl_3) [lit. 142 $[\alpha]_D -26.9^\circ$ ($c=2.54$, CHCl_3) pure "*erythro*"]

MS (EI, 70 eV) m/z 400 $((CH_3)_2COCH_2CHCH(O-Si(CH_3)_3)CD_2(CH_2)_{13}CH_3)^+$, 315 $((CH_3)_3Si-O-CHCD_2(CH_2)_{13}CH_3)^+$, 312 $((O)CHCHCH(O-Si)CD_2(CH_2)_{13}CH_3)^+$

3.5.1(a) [4,4- 2H_2]-C2-Dihydroceramide Diastereomers

The fully protected sphinganine mixture (110 mg, 0.25 mmol) was deprotected (122) and acylated (160) to the corresponding C2-dihydroceramide mixture (5.1 mg, 30 % yield overall) as described in 3.4.1(a).

R_f 0.38 (9:1 $CHCl_3/MeOH$)

1H NMR (400 MHz) 11 0.89 (t, J 6.6, 3H, $-CH_2CH_3$), 1.28 (br s, 26H, $-(CH_2)_{13}CH_3$), 1.97 (s, 3H, $-C(O)CH_3$), 3.54 (m, 1H, *threo*-C(1)-H), 3.58 (d, J 6.7, 1H, *erythro*-C(3)-H), 3.62 (m, 1H, *threo*-C(1)-H), 3.64-3.71 (m, 2H, *erythro*-C(1)-H₂), 3.75 (d, J 2.4, 1H, *threo*-C(3)-H), 3.81 (m, 1H, *erythro*-C(2)-H), 3.88 (m, 1H, *threo*-C(2)-H)

3.5.2 *N*-Boc-D-*erythro*-[4,4- 2H_2]-Sphinganine and *N*-Boc-L-*threo*-[4,4- 2H_2]-Sphinganine

Amberlyst 15 (1.02 g) was added to a solution of *tert*-Butyl [4*S*, 1'(*R*^{*}, *S*^{*})]-2,2-dimethyl-4-(1'-hydroxy[2',2'- 2H_2] hexadecyl) oxazolidine-3-carboxylate (0.85 g, 2 mmol) in anhydrous MeOH (20 mL). The heterogeneous mixture was stirred at RT until complete by TLC (24 h). After filtration through Celite (1:1 hexane/EtOAc), the solvent was evaporated *in vacuo* to give a mixture of diastereomers (0.57 g, 73 %). Separation of isomers using flash chromatography (3:1 hexane/EtOAc) gave the "*threo*" (0.16 g) and

“*erythro*” (0.27 g,) diastereomers as waxy solids (122). Their structures were confirmed by ^1H NMR analysis (122).

“*threo*” isomer:

mp 59-61 °C

R_f 0.18 (2:1 hexane/EtOAc) (no stain by I_2)

^1H NMR (400 MHz) 122 δ 0.90 (t, J 6.7, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26H, $-(\text{CH}_2)_{13}\text{CH}_3$), 1.47 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.99 (br s, 1H, OH), 3.61 (br s, 1H, OH), 3.77 (dd, J 3.4, 11.2, 1H, C(1)-H), 3.85 (m, 1H, C(2)-H), 3.92 (br s, 1H, C(3)-H), 4.01 (dd, J 3.4, 11.2, C(1)-H), 5.23 (br s, 1H, NH)

“*erythro*” isomer:

mp 78-80 °C

R_f 0.17 (2:1 hexane/EtOAc) (stain by I_2)

^1H NMR (400 MHz) 122 δ 0.90 (t, J 6.6, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26H, $-(\text{CH}_2)_{13}\text{CH}_3$), 1.47 (s, 9H, $\text{C}(\text{CH}_3)_3$), 2.19 (br s, 1H, OH), 3.51 (br s, 1H, OH), 3.75-3.85 (m, 3H, C(1)-H, C(2)-H, C(3)-H), 4.01 (dd, J 3.4, 11.4, 1H, C(1)-H), 5.42 (br s, 1H, NH)

3.5.3 D-*erythro*-[4,4- $^2\text{H}_2$]-Sphinganine

N-Boc-D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine (257 mg, 0.65 mmol) was deprotected (122) in 1 N HCl (3.5 mL) and 1,4-dioxane (6.5 mL) as described in 3.4.3. Recrystallization (1:1 hexane/EtOAc) gave D-*erythro*-[4,4- $^2\text{H}_2$]-sphinganine (58 mg, 31 %) as a white solid.

mp 89-91 °C (lit. 161 85-88 °C)

R_f 0.23 (4:1:0.1 $\text{CHCl}_3/\text{MeOH}/2\text{ N NH}_4\text{OH}$)

3.5.3(a) *D*-erythro-[4,4-²H₂]-C2-dihydroceramide

The free amine (4.4 mg, 15 μ mol) was acylated (160) with Ac₂O as described in 3.4.1(a) to give the corresponding [4,4-²H₂]-C2-dihydroceramide (4.9 mg, 95 %) as a white solid.

R_f 0.36 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.7, 3H, -CH₂CH₃), 1.28 (br s, 26 H, -(CH₂)₁₃CH₃), 1.97 (s, 3H, -C(O)CH₃), 3.58 (d, *J* 6.7, 1H, C(3)-H), 3.66 (dd, *J* 6.3, 11.3, 1H, C(1)-H), 3.70 (dd, *J* 4.2, 11.3, 1H, C(1)-H), 3.81 (ddd, *J* 4.2, 6.3, 6.5, 1H, C(2)-H)

¹³C NMR (100 MHz) ¹²³ δ 173.37 (C=O), 72.30 (C-3, upfield β -isotope shift (0.11 ppm)), 62.16 (C-1), 57.01 (C-2, upfield γ -isotope shift (0.02 ppm)), 34.83 (C-4, absent), 33.08 (C-16), 30.80, 30.76, 30.48 (C-6 to C-16), 26.65 (C-5, upfield β -isotope shift (0.19 ppm)), 23.74 (C-2'), 22.77 (C-17), 14.44 (C-18)

3.5.4 *L*-threo-[4,4-²H₂]-Sphinganine

N-Boc-*L*-threo-[4,4-²H₂]-sphinganine (143 mg, 0.4 mmol) was deprotected (122) in 1 N HCl (2 mL) and 1,4-dioxane (3.5 mL) as described in 3.4.3. Recrystallization (1:1 hexane/EtOAc) gave *L*-threo-[4,4-²H₂]-sphinganine (55 mg, 53 %) as a white solid.

mp 105-107 °C (lit. ¹⁶² 99.5-100.5 °C)

R_f 0.20 (4:1:0.1 CHCl₃/MeOH/2 N NH₄OH)

3.5.4(a) *L*-threo-[4,4-²H₂]-C2-Dihydroceramide

The free amine (4.9 mg, 16 μ mol) was acylated (160) with Ac₂O as described in 3.4.1(a) to give the corresponding [4,4-²H₂]-C2-dihydroceramide (5.3 mg, 97 %) as a white solid.

R_f 0.38 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.8, 3H, -CH₂CH₃), 1.28 (br s, 26 H, -(CH₂)₁₃CH₃), 1.98 (s, 3H, -C(O)CH₃), 3.55 (dd, *J* 6.4, 10.9, 1H, C(1)-H), 3.63 (dd, *J* 6.4, 10.9, 1H, C(1)-H), 3.75 (d, *J* 2.5, 1H, C(3)-H), 3.88 (ddd, *J* 2.6, 6.4, 6.4, 1H, C(2)-H)

3.5.5 *N*-Octanoyl-D-erythro-[4,4-²H₂]-sphinganine ([4,4-²H₂]-11)

D-erythro-[4,4-²H₂]-sphinganine (1.5 mg, 5.0 μ mol) was acylated (125) as described in 3.4.5. Purification on silica, using CHCl₃/MeOH (15:1) as eluent, gave *N*-octanoyl-D-erythro-[4,4-²H₂]-sphinganine [4,4-²H₂]-11 (1.0 mg, 46 %) as a white solid.

R_f 0.43 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.90 (t, *J* 6.6, 6H, 2 x CH₃), 1.27 (br s, 34H, -(CH₂)₁₃CH₃, -C(3')-(CH₂)₄CH₃), 1.65 (m, 2H, C(3')-H₂), 2.25 (t, *J* 7.4, 2H, C(2')-H₂), 3.78 (dd, *J* 3.0, ca. 12, 1H, C(1)-H), 3.81-3.87 (m, 2H, C(2)-H, C(3)-H), 4.04 (dd, *J* 3.4, 11.3, 1H, C(1)-H), 6.35 (d, *J* 7.4, 1H, NH)

MS (EI, 70 eV) *m/z* 558 (M⁺ - CH₃), 470 (M⁺ - (CH₃)₃Si-O-CH₂), 315 ((CH₃)₃Si-O-CHCD₂(CH₂)₁₃CH₃)⁺, 258 ((CH₃)₃Si-O-CH₂CH-NH-C(O)(CH₂)₆CH₃)⁺

3.6 Synthesis of *N*-Octanoyl-*D*-*erythro*-[5,5-²H₂]-Sphinganine ([5,5-²H₂]-11)

3.6.1 *tert*-Butyl [4*S*, 1'(*R*^{*}, *S*^{*})]-2,2-dimethyl-4-(1'-hydroxy[3',3'-²H₂] hexadecyl) oxazolidine-3-carboxylate

The Grignard reagent, magnesium [1,1-²H₂]-pentadecyl-1-bromide, was prepared as described in 3.4.1, using [2,2-²H₂]-1-bromopentadecane **14** (3.02 g, 10 mmol), Mg(0) (0.26 g, 11 mmol), I₂ (0.10 g) and THF (20 mL).

The title compound was prepared as described in 3.4.1. Grignard addition (140) of magnesium [2,2-²H₂]-pentadecyl-1-bromide (20 mL, 0.5 M) to **12** (2.26 g, 10 mmol) in THF (25 mL) at -78 °C gave the crude amino alcohol. Purification on flash silica, using 9:1 hexane/EtOAc as eluent, gave an inseparable mixture of the fully protected sphinganine *erythro*- and *threo*- diastereomers (2.43 g, 55 %) as colourless oil.

R_f 0.34 (4:1 hexane/EtOAc)

¹H NMR (400 MHz) ¹⁴² δ 0.90 (t, *J* 6.7, 3H, -CH₂CH₃), 1.27 (br s, 24 H, -(CH₂)₁₂CH₃), 1.47 (br s, 9H, -C(CH₃)₃), 1.51 (br s, 6H, -C(2)(CH₃)₂), 1.53 (br m, 2H, -C(1')CH₂CD₂-), 3.50-4.11 (m, 4H, C(5)-H₂, C(4)-H, C(1')-H), 5.22-5.40 (2 br s, 1H, OH)

[α]_D -16.5° (c=0.76, CHCl₃) [lit. ¹⁴² [α]_D -26.9° (c=2.54, CHCl₃) pure "*erythro*"]

MS (EI, 70 eV) *m/z* 400 ((CH₃)₂COCH₂CHCH(O-Si(CH₃)₃)CH₂CD₂(CH₂)₁₂CH₃)⁺, 315 ((CH₃)₃Si-O-CHCH₂CD₂(CH₂)₁₂CH₃)⁺, 312 ((O)CHCHCH(O-Si)CH₂CD₂(CH₂)₁₂CH₃)⁺

3.6.1(a) [5,5-²H₂]-C2-Dihydroceramide Diastereomers

The fully protected sphinganine mixture (90 mg, 0.2 mmol) was deprotected (122) and acylated (160) to the corresponding C2-dihydroceramide mixture (4.5 mg, 31 % overall) as described in 3.4.1(a).

R_f 0.38 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ 0.90 (t, J 6.6, 3H, -CH₂CH₃), 1.29 (br s, 24H, -(CH₂)₁₂CH₃), 1.41 (m, 1H, C(4)-H), 1.51 (m, 1H, C(4)-H), 1.98 (s, 3H, -C(O)CH₃), 3.55 (m, 1H, *threo*-C(1)-H), 3.60 (m, 1H, *erythro*-C(3)-H), 3.63 (m, 1H, *threo*-C(1)-H), 3.64-3.73 (m, 2H, *erythro*-C(1)-H₂), 3.77 (m, 1H, *threo*-C(3)-H), 3.82 (m, 1H, *erythro*-C(2)-H), 3.88 (m, 1H, *threo*-C(2)-H)

3.6.2 *N*-Boc-*D*-*erythro*-[5,5-²H₂]-Sphinganine and *N*-Boc-*L*-*threo*-[5,5-²H₂]-Sphinganine

Amberlyst 15 (1.15 g) was added to a solution of *tert*-Butyl [4*S*, 1'(*R*^{*}, *S*^{*})]-2,2-dimethyl-4-(1'-hydroxy[3',3'-²H₂] hexadecyl) oxazolidine-3-carboxylate (0.90 g, 2 mmol) in anhydrous MeOH (20 mL). The heterogeneous mixture was stirred at RT until complete by TLC (24 h). After filtration through Celite (1:1 hexane/EtOAc), the solvent was evaporated *in vacuo* to give a mixture of diastereomers (0.71 g, 87 %). Separation of isomers using flash chromatography (3:1 hexane/EtOAc) gave the “*erythro*” (0.26 g,) and “*threo*” (0.15 g) diastereomers as waxy solids (122). Their structures were confirmed by ¹H NMR analysis (122).

“*threo*” isomer:

mp 63-65 °C

R_f 0.18 (2:1 hexane/EtOAc) (no stain by I₂)

¹H NMR (400 MHz) ¹²² δ 0.90 (t, *J* 6.7, 3H, -CH₂CH₃), 1.27 (br s, 24H, -(CH₂)₁₂CH₃), 1.47 (s, 9H, C(CH₃)₃), 1.43-1.53 (m, 2H, C(4)-H₂), 2.10 (br s, 1H, OH), 3.61 (br s, 1H, OH), 3.77 (dd, *J* 3.4, 11.2, 1H, C(1)-H), 3.85 (m, 1H, C(2)-H), 3.93 (m, 1H, C(3)-H), 4.01 (dd, *J* 3.4, 11.2, C(1)-H), 5.22 (br s, 1H, NH)

“*erythro*” isomer:

mp 77-79 °C

R_f 0.17 (2:1 hexane/EtOAc) (stain by I₂)

¹H NMR (400 MHz) ¹²² δ 0.90 (t, *J* 6.7, 3H, -CH₂CH₃), 1.27 (br s, 24H, -(CH₂)₁₂CH₃), 1.47 (s, 9H, C(CH₃)₃), 1.43-1.53 (m, 2H, C(4)-H₂), 2.06 (br s, 1H, OH), 3.54 (br s, 1H, OH), 3.76-3.85 (m, 3H, C(1)-H, C(2)-H, C(3)-H), 4.01 (dd, *J* 3.4, 11.4, 1H, C(1)-H), 5.41 (br s, 1H, NH)

3.6.3 *D-erythro*-[5,5-²H₂]-Sphinganine

N-Boc-*D-erythro*-[5,5-²H₂]-sphinganine (243 mg, 0.6 mmol) was deprotected (122) in 1 N HCl (3 mL) and 1,4-dioxane (6 mL) as described in 3.4.3. Recrystallization (1:1 hexane/EtOAc) gave *D-erythro*-[5,5-²H₂]-sphinganine (63 mg, 35 %) as a white solid.

mp 88-90 °C (lit. ¹⁶¹ 85-88 °C)

R_f 0.23 (4:1:0.1 CHCl₃/MeOH/2 N NH₄OH)

3.6.3(a) *D-erythro*-[5,5-²H₂]-C2-Dihydroceramide

The free amine (5.0 mg, 17 μ mol) was acylated (160) with Ac₂O as described in 3.4.1(a) to give the corresponding [5,5-²H₂]-C2-dihydroceramide (5.6 mg, 97 %) as a white solid.

R_f 0.36 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.7, 3H, -CH₂CH₃), 1.28 (br s, 24 H, -(CH₂)₁₂CH₃), 1.37 (dd, *J* 13.8, 9.2, 1H, C(4)-H), 1.51 (dd, *J* 3.3, 13.8, 1H, C(4)-H), 1.98 (s, 3H, -C(O)CH₃) 3.59 (ddd, *J* 6.6, 3.4, 5.8, 1H, C(3)-H), 3.66 (dd, *J* 6.3, 11.4, 1H, C(1)-H), 3.70 (dd, *J* 4.2, 11.4, 1H, C(1)-H), 3.81 (ddd, *J* 4.3, 6.3, 6.5, 1H, C(2)-H)

¹³C NMR (100 MHz) ¹²³ δ 173.37 (C=O), 72.38 (C-3, upfield γ -isotope shift, (0.02 ppm)), 62.16 (C-1), 57.03 (C-2), 34.64 (C-4, upfield β -isotope shift (0.19 ppm)), 33.08 (C-16), 30.79, 30.76, 30.48 (C-6 to C-16), 26.85 (C-5, absent), 23.74 (C-2'), 22.77 (C-17), 14.44 (C-18)

3.6.4 *L-threo*-[5,5-²H₂]-Sphinganine

N-Boc-*L-threo*-[5,5-²H₂]-sphinganine (130 mg, 0.4 mmol) was deprotected (122) in 1 N HCl (2 mL) and 1,4-dioxane (3.5 mL) as described in 3.4.3. Recrystallization (1:1 hexane/EtOAc) gave *L-threo*-[5,5-²H₂]-sphinganine (46 mg, 45 %) as a white solid.

mp 106-108 °C (lit. ¹⁶² 99.5-100.5 °C)

R_f 0.20 (4:1:0.1 CHCl₃/MeOH/2 N NH₄OH)

3.6.4(a) *L*-threo-[5,5-²H₂]-C2-Dihydroceramide

The free amine (4.0 mg, 13 μ mol) was acylated (160) with Ac₂O as described in 3.4.1(a) to give the corresponding [5,5-²H₂]-C2-dihydroceramide (4.2 mg, 92 %) as a white solid.

R_f 0.38 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.7, 3H, -CH₂CH₃), 1.28 (br s, 24 H, -(CH₂)₁₂CH₃), 1.32 (not resolved, 1H, C(4)-H), 1.40 (d, *J* 6.7, 1H, C(4)-H), 1.98 (s, 3H, -C(O)CH₃), 3.55 (dd, *J* 6.4, 10.9, 1H, C(1)-H), 3.63 (dd, *J* 6.4, 10.9, 1H, C(1)-H), 3.76 (ddd, *J* 2.6, 6.9, 6.5, 1H, C(3)-H), 3.88 (ddd, *J* 2.6, 6.3, 6.4, 1H, C(2)-H)

3.6.5 *N*-Octanoyl-D-erythro-[5,5-²H₂]-sphinganine ([5,5-²H₂]-11)

D-erythro-[5,5-²H₂]-sphinganine (1.5 mg, 5.0 μ mol) was acylated (125) as described in 3.4.5. Purification on silica, using CHCl₃/MeOH (15:1) as eluent, gave *N*-octanoyl-D-erythro-[5,5-²H₂]-sphinganine [5,5-²H₂]-11 (0.5 mg, 23 %) as a white solid.

R_f 0.43 (9:1 CHCl₃/MeOH)

¹H NMR (400 MHz) ¹¹ δ 0.89 (t, *J* 6.7, 6H, 2 x CH₃), 1.27 (br s, 32H, -(CH₂)₁₂CH₃, -C(3')-(CH₂)₄CH₃), 1.56 (m, 2H, C(4)-H₂), 1.67 (m, 2H, C(3')-H₂), 2.25 (t, *J* 7.4, 2H, C(2')-H₂), 3.78 (dd, *J* 2.9, 11.4, 1H, C(1)-H), 3.82-3.87 (m, 2H, C(2)-H, C(3)-H), 4.04 (dd, *J* 3.4, 11.3, 1H, C(1)-H), 6.35 (d, *J* 7.4, 1H, NH)

MS (EI, 70 eV) *m/z* 558 (M⁺ - CH₃), 470 (M⁺ - (CH₃)₃Si-O-CH₂), 315 ((CH₃)₃Si-O-CHCH₂CD₂(CH₂)₁₂CH₃)⁺, 258 ((CH₃)₃Si-O-CH₂CH-NH-C(O)(CH₂)₆CH₃)⁺

Part 2: Determination of Sphinganine Enantiomeric Purity

3.7 Synthesis of (*S*)- α -Methoxy- α -(trifluoromethyl) phenylacetate ((*S*)-MTPA) Sphinganine Derivatives (Mosher Derivatives)

3.7.1 D-erythro-Sphinganine (*S*)-MTPA Derivative

(*S*)- α -Methoxy- α -(trifluoromethyl) phenylacetyl chloride (10 μ L, 50 μ mol) was added to a solution of D-*erythro*-sphinganine 11 (2.0 mg, 6.6 μ mol) in dry pyridine (100 μ L) followed by shaking for 5 minutes to mix the reagents. The mixture was left overnight at room temperature. When complete by TLC (12 h), the reaction was quenched with water (1 ml) and extracted with CH_2Cl_2 (3 x 1.5 mL). The combined extracts were washed with CuSO_4 (2 x 1.5 mL), dH_2O (1.5 mL), sat. NaCl (1.5 mL), dried over anhydrous Na_2SO_4 and concentrated under a steady stream of N_2 . The residue was purified on silica, using 1:1 hexane/EtOAc as eluent, to give the corresponding (*S*)-MTPA derivative (149).

R_f 0.57 (4:1 hexane/EtOAc)

^1H NMR (400 MHz) 149 δ 0.90 (t, J 6.6, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26H, $-(\text{CH}_2)_{13}\text{CH}_3$), 1.50 (m, 2H, C(4)- H_2), 3.23 (d^a, J 1.24, 3H, 2-OCH₃^b), 3.44 (d^a, J 0.84, 3H, 3-OCH₃^b), 3.54 (d^a, J 0.88, 3H, 1-OCH₃^b), 4.23 (dd, J 5.5, 11.8, 1H, C(1)- H), 4.38 (dd, J 3.6, 11.8, 1H, C(1)- H), 4.51 (m, 1H, C(2)- H), 5.17 (m, 1H, C(3)- H), 6.95 (d, J 9.1, 1H, NH), 7.3-7.5 (m, 15H, Ar- H)

^a - ¹H methoxy signals were distorted quartets (⁵J_{HF})

^b - Assignments of 1- and 3-methoxy signals are uncertain and may be interchanged

3.7.2 D-erythro-[4,4-²H₂]-Sphinganine (S)-MTPA Derivative

The (S)-MTPA derivative of D-erythro-[4,4-²H₂]-sphinganine [4,4-²H₂]-11 was prepared as described in 3.7.1 using (S)- α -Methoxy- α -(trifluoromethyl) phenylacetyl chloride (10 μ L, 50 μ mol), D-erythro-[4,4-²H₂]-sphinganine (2.0 mg, 6.6 μ mol) and dry pyridine (100 μ L) (149).

R_f 0.57 (4:1 hexane/EtOAc)

¹H NMR (400 MHz) ¹⁴⁹ δ 0.90 (t, *J* 6.6, 3H, -CH₂CH₃), 1.27 (br s, 26H, -(CH₂)₁₃CH₃), 3.23 (d^a, *J* 1.24, 3H, 2-OCH₃^b), 3.44 (br s^a, 3H, 3-OCH₃^b), 3.54 (d^a, *J* 0.90, 3H, 1-OCH₃^b), 4.23 (dd, *J* 5.5, 11.8, 1H, C(1)-H), 4.39 (dd, *J* 3.6, 11.8, 1H, C(1)-H), 4.50 (m, 1H, C(2)-H), 5.17 (d, *J* 6.0, 1H, C(3)-H), 6.95 (d, *J* 9.1, 1H, NH), 7.3-7.5 (m, 15H, Ar-H)

^a - ¹H methoxy signals were distorted quartets (⁵J_{HF})

^b - Assignments of 1- and 3-methoxy signals are uncertain and may be interchanged

3.7.3 D-erythro-[5,5-²H₂]-Sphinganine (S)-MTPA Derivative

The (S)-MTPA derivative of D-erythro-[5,5-²H₂]-sphinganine [5,5-²H₂]-11 was prepared as described in 3.7.1 using (S)- α -Methoxy- α -(trifluoromethyl) phenylacetyl chloride (10 μ L, 50 μ mol) D-erythro-[5,5-²H₂]-sphinganine (2.0 mg, 6.6 μ mol) and dry pyridine (100 μ L) (149).

R_f 0.57 (4:1 hexane/EtOAc)

^1H NMR (400 MHz, CDCl_3) 149 δ 0.90 (t, J 6.7, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 24H, $-(\text{CH}_2)_{12}\text{CH}_3$), 1.47 (m, 2H, C(4)- H_2), 3.23 (d^a , J 1.24, 3H, 2-O CH_3^b), 3.44 (d^a , J 0.96, 3H, 3-O CH_3^b), 3.54 (d^a , J 1.10, 3H, 1-O CH_3^b), 4.23 (dd, J 5.5, 11.8, 1H, C(1)- H), 4.38 (dd, J 3.7, 11.9, 1H, C(1)- H), 4.52 (m, 1H, C(2)- H), 5.17 (m, 1H, C(3)- H), 6.95 (d, J 9.1, 1H, NH), 7.3-7.5 (m, 15H, Ar- H)

^a - ^1H methoxy signals were distorted quartets ($^5J_{\text{HF}}$)

^b - Assignments of 1- and 3-methoxy signals are uncertain and may be interchanged

3.7.4 DL-erythro-Sphinganine (S)-MTPA Derivative

The (S)-MTPA derivative of DL-erythro-sphinganine (Sigma D-6908) was prepared as described in 3.7.1 using (S)- α -Methoxy- α -(trifluoromethyl) phenylacetyl chloride (10 μL , 50 μmol) DL-erythro-sphinganine (2.0 mg, 6.6 μmol) and dry pyridine (100 μL) (149).

R_f 0.57 (4:1 hexane/EtOAc)

^1H NMR (400 MHz, CDCl_3) 149 δ 0.90 (t, J 6.6, 3H, $-\text{CH}_2\text{CH}_3$), 1.27 (br s, 26H, $-(\text{CH}_2)_{13}\text{CH}_3$), 1.70 (m, 2H, C(4)- H_2), 3.23 (d^a , J 1.24, 3H, 2(D)-O CH_3^b), 3.24 (d^a , J 0.92, 3H, 2(L)-O CH_3^b), 3.44 (d^a , J 1.24, 3H, 3(D)-O CH_3^b), 3.44 (d^a , J 1.24, 3H, 1(L)-O CH_3^b), 3.54 (d^a , J 0.84, 3H, 1(D)-O CH_3^b), 3.56 (d^a , J 0.96, 3H, 3(L)-O CH_3^b), 4.05 (dd, J 5.8, 11.5, 1H, L-C(1)- H), 4.23 (dd, J 5.5, 11.8, 1H, D-C(1)- H), 4.38 (dd, J 3.6, 11.8, 1H, D-C(1)- H), 4.44 (dd, J 3.8, 11.8, 1H, L-C(1)- H), 4.48 (m, 1H, L-C(2)- H), 4.51 (m, 1H, D-C(2)- H), 5.17 (m, 1H, D-C(3)- H), 5.21 (m, 1H, L-C(3)- H), 6.95 (d, J 9.1, 1H, NH), 7.16 (d, J 9.1, 1H, NH), 7.3-7.5 (m, 15H, Ar- H)

^a - ^1H methoxy signals were distorted quartets ($^5J_{\text{HF}}$)

^b- Assignments of 1- and 3-methoxy signals are uncertain and may be interchanged

Part 3: Biological Experiments

3.8 Materials

All buffers and salts were purchased from Merck (Darmstadt, Germany), NADH, BSA and other biochemicals were purchased from Sigma-Aldrich Química (Madrid, Spain). Male Sprague-Dawley rats were supplied by the animal breeding facility of the Centro de Investigación y Desarrollo (Barcelona, Spain).

3.9 Preparation of Rat Liver Microsomes

Microsomes were prepared as previously reported (13). The male Sprague-Dawley rat (60 days old, 260 g) was killed by decapitation. The liver (13.7 g) was excised and washed with ice-cold sucrose/phosphate buffer (0.25 M sucrose, 100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4). The liver was minced with a scalpel, added to the same phosphate buffer (0.5 g of liver/mL) and homogenized with 4 up and down strokes at 160 rpm in a Braun glass homogenizer with a loose fitting Teflon pestle. The homogenate was centrifuged at 16,000 rpm for 5 min and the resulting supernatant was removed and centrifuged at 33,000 rpm for 1 h. The pellet was re-suspended in alkaline phosphate buffer (100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 8.0) (1g of liver/mL). After centrifugation at 110,000 for 1 h, the pellet was re-suspended in phosphate buffer (100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4) (2.5 g of liver/mL) and stored at -80°C .

3.10 Dihydroceramide Desaturase Assay

The procedure reported by Michel *et al.* was followed (13). The semi-truncated dihydroceramide probes were complexed with BSA as described for other lipids (151). The solution contained 10 nmol of lipid substrate, 30 nmol of BSA, 10 μL of EtOH and 90

μL of phosphate buffer (100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4). A rat liver microsomal suspension containing 300 μg of protein was added to the substrate/BSA complex and diluted to a volume of 320 μL with phosphate buffer. 1 μmol of NADH in 30 μL of phosphate buffer was added to the reaction mixture and incubated at 37 °C for 30 min. To reduce mass spectral interferences from endogenous material, the mixture was cooled to 0 °C on ice and rapidly extracted with 500 μL of hexane. The reaction was terminated with the addition of 500 μL of CHCl_3 . The phases were separated by centrifugation and the organic layer was collected. The extraction procedure was repeated twice using 250 μL of CHCl_3 . The combined organics were evaporated under a steady stream of N_2 for derivatization.

3.11 Derivatization of Lipid Extracts

For GC-MS analysis, the lipid extract was treated with 100 μL of bis(trimethylsilyl)trifluoroacetamide at 25 °C for 1 h. Excess reagent was evaporated under a stream of N_2 and the residue was diluted with 100 μL of CHCl_3 for analysis.

3.12 Mass Spectrometric Determination of Substrate and Product Deuterium Content

Mass spectra were obtained by GC-MS using a Fisons MD-800 mass spectrometer coupled to a Fisons 8000 series GC equipped with a non-polar Hewlett Packard HP-1 capillary column (30 m X 0.20 mm), temperature programmed from 150 °C to 320 °C at 6 °C/min. Analyses were carried out under selected ion monitoring (SIM) mode. The deuterium content was estimated using a dwell time of 0.02 sec which resulted in 5-8 scans per GC peak; the integrated intensities of the individual ions in the pertinent ion cluster were recorded using *Lab Base* software and have been corrected for natural isotopic abundances. Isotopic ratios were determined using the following ions: m/z 313, $((\text{CH}_3)_3\text{Si}-\text{O}-\text{CH}(\text{CH}_3)_{14}\text{CH}_3)^+$ (*N*-octanoyl-D-*erythro*-sphinganine 11) ; m/z 311, $((\text{CH}_3)_3\text{Si}-\text{O}-\text{CHCH}=\text{CH}-(\text{CH}_2)_{12}\text{CH}_3)^+$ (*N*-octanoyl-D-*erythro*-sphingosine 15).

3.13 Protein Concentration Experiments

Protein concentrations were measured by the method of Bradford (152) using bovine serum albumin (BSA) as standard protein. Determinations were carried out using 96-well ELISA plates. A calibration curve was constructed via serial dilution of a BSA solution in phosphate buffer (100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4). Protein concentrations were: 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0 $\mu\text{g/mL}$. The total volume of each well was 320 μL . Serial dilution of the microsomal suspension in phosphate buffer (100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4) gave solutions with dilution factors from 1/100 to 1/6400. The total volume of each well was 320 μL . The Bradford reagent (40 μL) was then added to each well, both BSA and microsomes, and incubated for 15 minutes. When complete, the absorbance was measured at 595 nm using a Titerek Multiscan Plus MK II reader.

Appendix A

Primary Deuterium Kinetic Isotope Effect

Primary Kinetic Isotope Effects (KIE's) originate in isotopically sensitive zero-point energy (the vibrational energy level at absolute zero ($n=0$)) differences between initial states and transition states.

Consider isotopic molecules R-H and R-D (R=massive molecule, H=hydrogen, D=deuterium) where each is subject to an elementary reaction in which the R-H or R-D bond is broken. It is a fundamental result of quantum mechanics that the vibrational energy levels of the bonds are given by,

$$E_n = (n + 1/2)h\nu$$

where ν is the frequency of the stretching vibration and n is the vibrational quantum number.

At low temperature nearly all bonds are at their lowest vibrational level and will possess the zero-point vibrational energy given by,

$$E_0 = 1/2 h\nu$$

Presuming the molecule behaves as a simple harmonic oscillator, the vibrational frequency is given by,

$$\nu = \frac{1}{2\pi} \left(\frac{k'}{\mu} \right)^{1/2}$$

where k' is the force constant and μ is the reduced mass

$$\mu = \frac{m_R m_H}{m_R + m_H}$$

where m represents the mass.

If H is replaced with D, μ will increase, whereas k' will not change, because it is determined by the electronic distribution. Therefore, $\nu_D < \nu_H$ ($\nu_D = 2200 \text{ cm}^{-1}$ and $\nu_H = 2800 \text{ cm}^{-1}$) and $E_0^H > E_0^D$ ($E_0^H = 4.3 \text{ kcal/mol}$ and $E_0^D = 3.2 \text{ kcal/mol}$). The implication of the

difference can be seen graphically (Figure A); because of the difference in zero-point energies, the bond dissociation energies of R-H and R-D are different, the energy required to break the R-D bond being greater by 1.15 kcal/mol.

Figure A - Bond dissociation profile for R-H and R-D bonds

Thus, if we assume a similar transition state for each substrate the reaction rates of each can be calculated and an approximation of the maximum primary deuterium kinetic isotope effect can be established. Using

$$\frac{k_H}{k_D} = \exp\left(\frac{\Delta E_0}{RT}\right)$$

at 298 K gives 7.2 for the maximum primary KIE, k_H/k_D , for C-H / C-D cleavage.

Appendix B

Evaluation of k_H/k_D in an Intermolecular Competition¹¹²

Assuming that two isotopic molecules, S_H and S_D , undergo analogous irreversible reactions, according to

it is possible to write the general rate expression:

$$\nu_H = -\frac{dS_H}{dt} = k_H S_H \quad \text{and} \quad \nu_D = -\frac{dS_D}{dt} = k_D S_D$$

Since dt is the same for both equations, it must consequently hold that

$$\frac{1}{k_H} \times \frac{dS_H}{S_H} = \frac{1}{k_D} \times \frac{dS_D}{S_D}$$

With the initial conditions $S_H = S_H^0$ and $S_D = S_D^0$, integration and rearrangement gives

$$\frac{k_H}{k_D} = \frac{\ln(S_H / S_H^0)}{\ln(S_D / S_D^0)}$$

Introducing the fractional amount of conversion, or fraction of reaction, F , yields:

$$\frac{k_H}{k_D} = \frac{\ln(1 - F_H)}{\ln(1 - F_D)}$$

Since the limit of $\ln(1 - x)$ as $x \rightarrow 0$ is $-x$, at low level of conversion (10 - 20%) this eq. reduces to:

$$\frac{k_H}{k_D} = \frac{F_H}{F_D}$$

F_H as a function of F_D is shown in Figure B. It is evident that for small conversion of either isotopic species the curves are almost straight lines (lower left corner). The simple meaning of all this is that the fraction of reaction of each species is proportional to the specific reaction rate of that species as long as the supply of the reactants is not appreciably decreased by consumption.

Since the fractional conversion can be measured via:

$$F_H = 1 - \frac{S_H}{S_H^0} = \frac{P_H}{S_H^0}$$

Thus, k_H/k_D can be calculated using the following equation:

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

or

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

The ratio of the two isotopic species in the product (P_H / P_D), and starting mixture (S_H^0 / S_D^0) can experimentally be obtained using a mass spectrometer.

Figure B - The relation between the fractions of reaction of two competing isotopic reactants. The figures on the curves indicate the rate-constant ratio, k_H/k_D

References

1. Kanfer, J. N.; Hakomori, S. in *"Handbook of Lipid Research Vol. 3, Sphingolipid Biochemistry"*, Hanahan, D. J., Ed., Plenum Press, New York, 1983.
2. Karlsson, K. A., *Chem. Phys. Lipids*, **1970**, *5*, 6-43.
3. Sweeley, C. C. in *"Biochemistry of Lipids, Lipoproteins and Membranes"*, Vance, D. E.; Vance, J., Eds., Elsevier, Amsterdam, 1991, pp. 327-361.
4. Sperling, P.; Zähringer, U.; Heinz, E. *J. Biol. Chem.* **1998**, *273*, 28590.
5. Bell, R. M.; Hannun, Y. A.; Merrill, Jr. A. H., Eds., *Advances in Lipid Research: Sphingolipids and their Metabolites, Vol. 25 & 26*", Academic Press, Orlando, 1993.
6. Hannun, Y. A. *J. Biol. Chem.* **1994**, *269*, 3125.
7. Mathias, S.; Kolesnick, R. *Adv. Lipid Res.* **1993**, *25*, 65.
8. Hannun, Y. A.; Obeid, L. M. *Trends. Biochem. Sci.* **1995**, *20*, 73.
9. Obeid, L. M.; Iinardic, C. M.; Karolak, L. A.; Hannun, Y. A. *Science* **1993**, *259*, 1769.
10. Dickson, R. C. *Annu. Rev. Biochem.* **1998**, *67*, 27.
11. Bielawska, A.; Crane, H. M.; Liotta, D.; Obeid, L. M.; Hannun, Y. A. *J. Biol. Chem.* **1993**, *268*, 26226.
12. Geeraert, L.; Mannaerts, G. P.; van Veldhoven, P. P. *Biochem. J.* **1997**, *327*, 125.
13. Michel, C.; van Echten-Deckert, G.; Rother, J.; Sandhoff, K.; Wang, E.; Merrill, Jr. A. H. *J. Biol. Chem.* **1997**, *272*, 22432.
14. Merrill, Jr. A. H.; Wang, E. *J. Biol. Chem.* **1986**, *261*, 3764.
15. (a) Snell, E. E.; Di Mari, S. J.; Brady, R. N. *Chem Phys. Lipid* **1970**, *5*, 116. (b) Morell, P.; Radin, N. S. *J. Biol. Chem.* **1970**, *245*, 342.
16. Wang, E.; Norred, W. P.; Bacon, C. W.; Riley, R. T.; Merrill, Jr. A. H. *J. Biol. Chem.* **1991**, *266*, 14486.
17. Merrill, Jr. A. H.; van Echten, G.; Wang, E.; Sandhoff, K. *J. Biol. Chem.* **1993**, *268*, 27299.
18. Ong, D. E.; Brady, R. N. *J. Biol. Chem.* **1973**, *248*, 3884.
19. Rother, J.; van Echten, G.; Schwarzmann, G.; Sandhoff, K. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 14.

20. Cook, H. W. in "Biochemistry of Lipids, Lipoproteins and Membranes" Vance, D. E.; Vance, J., Eds., Elsevier, Amsterdam, 1991, pp. 141-168.
21. Bose, R.; Verheji, M.; Haimovitz-Friedman, A.; Scotto, K.; Fuks, Z.; Koelsnick, R. *Cell* **1995**, *82*, 405.
22. Yokoyama, K.; Nojiri, H.; Suzuki, M.; Setaka, M.; Suzuki, A.; Nojima, S. *FEBS Lett.* **1995**, *368*, 477.
23. Bloomfield, D. K.; Bloch, K. *Biochem. Biophys. Acta* **1958**, *30*, 220.
24. Northrup, D. *Biochemistry* **1975**, *14*, 2644.
25. Behrouzian, B.; Buist, P. H.; Shanklin, J. *J. Chem Soc. Chem. Commun.* **2001**, *In Press*.
26. (a) Bloomfield, D. K.; Bloch, K. *J. Biol. Chem.* **1960**, *235*, 337. (b) Bloch, K.; Baronowsky, P.; Goldfine, H.; Lennarz, W. J.; Light, R.; Norris, A. T.; Scheuerbrandt, G. *Federation Proc.* **1961**, *20*, 921.
27. (a) Scheuerbrandt, G.; Bloch, K. *J. Biol. Chem.* **1962**, *237*, 2064. (b) Fulco, A. J. *Ann. Rev. Biochem.* **1974**, *43*, 215. (c) Jeffcoat, R.; Brawn, R. P.; Safford, R.; and James, A. T.; *Biochem J.*, **1977**, *161*, 431.
28. (a) Parasad, M. R.; Joshi, V. C.; *J. Biol. Chem.* **1979**, *254*, 6362. (b) Strittmatter, P.; Spatz, L.; Corcoran, D.; Roger, M. J.; Setlow, B.; Redline, R. *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 4565.
29. Shimakata, T.; Mihara, K.; Sato, R. *J. Biochem. (Tokyo)* **1972**, *72*, 1163.
30. Holloway, P. W. *Biochemistry* **1971**, *10*, 1556.
31. Enoch, H. G.; Catala, A.; Strittmatter, P. *J. Biol. Chem.* **1976**, *251*, 5095.
32. Spatz, L.; Strittmatter, P. *J. Biol. Chem.* **1973**, *248*, 793.
33. (a) Oshino, N.; Imai, Y.; Sato, R. *J. Biochem.* **1971**, *69*, 155. (b) Strittmatter, P.; Rogers, M. J.; Spatz, L. *J. Biol. Chem.* **1972**, *247*, 2768.
34. Oshino, N.; Imai, Y.; Sato, R. *Biochim. Biophys. Acta* **1966**, *128*, 13.
35. Okayasu, T.; Nagao, M.; Ishibashi, T.; Imai, Y. *Arch. Biochem. Biophys* **1981**, *206*, 21.
36. (a) Stuke, J. E.; McDonough, V. M.; Martin, C. E. *J. Biol. Chem.* **1989**, *264*, 16537. (b) Stuke, J. E.; McDonough, V. M.; Martin, C. E. *J. Biol. Chem.* **1990**, *265*, 20144.
37. Shanklin, J.; Whittle, E.; Fox, B. G. *Biochemistry* **1994**, *33*, 12787.

38. Shanklin, J.; Achim, C.; Schmidt, H.; Fox, B. G.; Münck, E. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2981.
39. (a) Nagai, J.; Bloch, K. *J. Biol. Chem.* **1968**, *243*, 4626. (b) Jaworski, G.; Stumpf, P. K. *Arch. Biochem. Biophys.* **1974**, *162*, 158. (c) Jacobson, B. S.; Jaworski, J. G.; Stumpf, P. K. *Plant Physiol.* **1974**, *54*, 484.
40. (a) McKeon, T. A.; Stumpf, P. K.; *J. Biol. Chem.* **1982**, *257*, 12141. (b) Thompson, G. A.; Scherer, D. E.; Foxall-van Akin, S.; Kenny, J. W.; Young, H. L.; Shintain, D. K.; Kridl, J. C.; Knauf, V. C. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 2578.
41. Shanklin, J.; Somerville, C. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 2510.
42. Fox, B. G.; Shanklin, J.; Somerville, C.; Münck, E. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2486.
43. Fox, B. G.; Shanklin, J.; Ai, J.; , Loehr, T.; Sanders-Loehr, J. *Biochemistry* **1994**, *33*, 12776.
44. Que, L. Jr.; True, A. E. in “*Progress in Inorganic Chemistry: Bioinorganic Chemistry*”, Ed., Lippard S. J.; Wiley, New York; Vol. 38, pp. 97-200.
45. Schneider, G.; Lindqvist, Y.; Shanklin, J.; Somerville, C. *J. Mol. Biol.* **1992**, *225*, 561.
46. Lindqvist, Y.; Huang, W.; Schneider, G.; Shanklin, J. *EMBO J.* **1996**, *15*, 4081.
47. Schroepfer, G. J.; Bloch, K. *J. Biol. Chem.* **1965**, *240*, 54.
48. (a) Morris, L. J.; Harris, R. W.; Kelly, W.; James, A. T. *Biochem. J.* **1968**, *109*, 673. (b) Morris, L. J. *Biochem. J.* **1970**, *118*, 681.
49. (a) Buist, P. H.; Marecak, D. M. *J. Am. Chem. Soc.* **1991**, *113*, 5877. (b) Buist, P. H.; Marecak, D. M. *J. Am. Chem. Soc.* **1992**, *114*, 5073.
50. Navarro, I.; Font, I.; Fabrias, G.; Camps, F. *J. Am. Chem. Soc.* **1997**, *119*, 11335.
51. Stoffel, W.; Assmann, G.; Blister, K. *Hoppe-Seyler's Z. Physiol. Chem.* **1971**, *352*, 1531.
52. Polito, A. J.; Sweeley, C. C. *J. Biol. Chem.* **1971**, *246*, 4178.
53. Sono, M.; Roach, M. P.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841. (b) Akhatar, M.; Wright, J. N. *Nat. Prod. Reports* **1991**, *8*, 527.
54. Ortiz de Montellano, P. R. Ed. “*Cytochrome P-450: Structure, Mechanism and Biochemistry*”, Sec. ed., Plenum, New York, **1995**.
55. Bradley, J. W.; Lipscomb, J. D. *Chem. Rev.* **1996**, *96*, 2625.

56. White, R. E. *Pharmacol. Ther.* **1991**, *266*, 13469.
57. Fulco, A. *Annu. Rev. Pharm. Toxicol.* **1991**, *31*, 177.
58. Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370.
59. White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315.
60. (a) MacMahon, R. E.; Sullivan, H. R.; Craig, J. C.; Pereira, W. E. *Arch. Biochem. Biophys.* **1969**, *132*, 575. (b) Heinz, E.; Tulloch, A. P.; Spencer, J. F. *J. Biol. Chem.* **1969**, *244*, 882. (c) Hamberg, M.; Björkhem, I. *J. Biol. Chem.* **1971**, *246*, 7411.
61. Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154.
62. Ortiz de Montellano, P. R.; Stearns, R. A. *J. Am. Chem. Soc.* **1987**, *109*, 3415.
63. Fretz, H.; Woggon, W. D.; Voges, R. *Helv. Chim. Acta.* **1989**, *72*, 391.
64. Browry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5687 and 5699.
65. Atkinson, J. K. Ingold, K. U. *Biochemistry* **1993**, *32*, 9209.
66. Newcomb, M.; Le Tadic M.; Putt D. A.; Holenberg, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 3312.
67. Newcomb, M.; Chestney, D. L. *J. Am. Chem. Soc.* **1994**, *116*, 9753.
68. Newcomb, M.; Shen, R.; Choi, S. Y.; Toy, P. H.; Hollenberg, P. F.; Vaz, A. D. N.; Coon, M. J. *J. Am. Chem. Soc.* **2000**, *122*, 2677.
69. (a) Le Tadic-Biadatti, M. H.; Newcomb, M. *J. Chem. Soc., Perkin Trans. 2* **1996**, 1467. (b) Newcomb, M.; Choi, S. Y.; Horner, J. H. *J. Org. Chem.* **1999**, *64*, 1225.
70. Newcomb, M.; Le Tadic-Biadatti, M.; Chestney, D. L.; Roberts, E. S.; Holenberg, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 12085.
71. Toy, P. H.; Newcomb, M.; Coon, M. J. *J. Am. Chem. Soc.* **1998**, *120*, 9718.
72. Hata, S.; Nishino, T.; Katsuki, H.; Aoyama, Y.; Yoshida Y. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 162.
73. Rettie, A. E.; Boberg, M.; Rettenmeier, A. W.; Baillie, T. A. *J. Biol. Chem.* **1988**, *263*, 13733.
74. Feig, A L.; Lippard, S. T. *Chem. Rev.* **1994**, *94*, 759.

75. (a) Colby, J.; Dalton H. *Biochem. J.*, **1978**, *171*, 461. (b) Colby, J.; Dalton H.; *Biochem. J.* **1979**, *177*, 903.
76. Woodland, M. P.; Dalton H. *J. Biol. Chem.* **1984**, *259*, 53.
77. Rosenzweig A. C.; Fridrick, C. A.; Lippard, S. T.; Nordlund, P. *Nature*, **1993**, *366*, 537.
78. Elango, N.; Radhakrishnan, R.; Froland, W. A.; Wallar, B. J.; Earhart, C. A.; Lipscomb, J. D.; Ohlendorf, D. H. *Protein Sci.* **1997**, *6*, 556.
79. (a) Lee, S. K.; Nesheim, J. C.; Lipscomb, J. D. *J. Biol. Chem.* **1993**, *268*, 21569. (b) Lee, S. K.; Fox, B. G.; Froland, W. A.; Lipscomb, J. D.; Münck, E. *J. Am. Chem. Soc.* **1993**, *115*, 6450.
80. (a) Liu, K. E.; Wang, D.; Huynh, B. H.; Edmondson, D. E.; Salifoglou, A.; Lippard, S. T. *J. Am. Chem. Soc.* **1994**, *116*, 7465. (b) Liu, K. E.; Valentine, A. M.; Qiu, D.; Edmondson, D. E.; Appelman, E. H.; Spiro, T. G.; Lippard, S. T. *J. Am. Chem. Soc.* **1995**, *117*, 4997. (c) Liu, K. E.; Wang, D.; Valentine, A. M.; Wang, D.; Huynh, B. H.; Edmondson, D. E.; Salifoglou, A.; Lippard, S. T. *J. Am. Chem. Soc.* **1995**, *117*, 10174.
81. (a) Que, L. Jr.; Dong, Y.; *Acc. Chem. Res.* **1996**, *29*, 190. (b) Shu, L.; Nesheim, J. C.; Kauffmann, K.; Münck, E.; Lipscomb, J. D.; Que, L. Jr. *Science* **1997**, *275*, 515.
82. (a) Green, J.; Dalton, H. *J. Biol. Chem.* **1989**, *264*, 17698. (b) Fox, B. G.; Borneman, J. G.; Wackett, L. P.; Lipscomb, J. D. *Biochemistry* **1990**, *29*, 6419. (c) Deighton, N.; Podmore, I. D.; Symons, M. C. R.; Wilkins, P. C.; Dalton, H. *J. Chem. Soc., Chem. Commun.* **1991**, 1086.
83. Jin, Y.; Lipscomb, J. D. *Biochemistry* **1999**, *38*, 6178.
84. Choi, S. Y.; Eaton, P. E.; Kopp, D. A.; Lippard, S. J.; Newcomb, M.; Shen, R. *J. Am. Chem. Soc.* **1999**, *121*, 12198.
85. Kok, M.; Oldenhuis, R.; van der Linden, M. P. G.; ratjees, P.; Kingma, J.; van Lelyveld, P. H.; Witholt, B. *J. Biol. Chem.* **1989**, *264*, 5435.
86. Austin, R. N.; Chang, H. K.; Zylstra, G. J.; Groves, J. T. *J. Am. Chem. Soc.* **2000**, *122*, 11747.
87. McLain, J. L.; Lee, J.; Groves, J. T. in "Biomimetic Oxidations Catalyzed by Transition Metal Complexes", Ed. Meunier, B., Imperial College Press, London, 2000, pp. 91-169.
88. Light, R.; Lennarz, W. J.; Bloch, K. *J. Biol. Chem.* **1962**, *237*, 1793.
89. Bloch, K. *Acc. Chem. Res.* **1969**, *2*, 193.

90. Gurr, M. I.; Bloch, K. *Biochem. J.* **1966**, *99*, 16C.
91. Gurr, M. I. in “*Biochemistry of Lipids*”, Ed., Goodwin, T. W., Butterworths, London, **1974**, Vol. 4, pp 221-225.
92. Fauconnot, L.; Buist, P. H. Unpublished data.
93. Siegbahn, P. E. M.; Crabtree, R. H. *J. Am. Chem. Soc.* **1997**, *119*, 3103.
94. Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1996**, *118*, 6295.
95. Buist, P. H.; Behrouzian, B.; Alexopoulos, K. A.; Dawson, B.; Black, B. *J. Chem. Soc. Perkin Trans. 1* **1997**, 2617.
96. Fauconnot, L.; Buist, P. H. *J. Org. Chem.* **2001**, *In Press*.
97. (a) Buist, P. H.; Marecak, D. M.; Dawson, B.; Black, B. *Can. J. Chem.* **1996**, *74*, 453.
(b) Buist, P. H.; Marecak, D. M. *Can. J. Chem.* **1994**, *72*, 176.
98. Buist, P. H.; Behrouzian, B.; Alexopoulos, K. A.; Dawson, B.; Black, B. *J. Chem. Soc. Chem. Commun.* **1996**, 2671.
99. Fauconnot, L.; Buist, P. H. Unpublished data.
100. Pinilla, A.; Camps, F.; Fabrias, G. *Biochemistry* **1999**, *38*, 15272.
101. Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1998**, *120*, 871.
102. (a) Meesapyodsuk, D.; Reed, D. W.; Savile, C. K.; Buist, P. H.; Ambrose, S. J.; Covello, P. S. *Biochemistry* **2000**, *39*, 11948. (b) Savile, C. K.; Meesapyodsuk, D.; Reed, D. W.; Covello, P. S.; Buist, P. H. *J. Chem. Soc., Perkin Trans. 1* **2000**, *in press*.
103. He, L.; Byun, H.; Smit, J.; Wilschut, J.; Bittman, R. *J. Am. Chem. Soc.* **1999**, *121*, 3897.
104. Grilley, M. M.; Stock, S. D.; Dickson, R. C.; Lester, R. L.; Takemoto, J. Y. *J. Biol. Chem.* **1998**, *273*, 11062.
105. Ortiz de Montellano, P. R. *Trends in Pharmacol. Sci.* **1989**, *10*, 354.
106. Broun, P.; Shanklin, J.; Whittle, E.; Sommerville, C. *Science* **1998**, *282*, 1315.
107. Hata, S.; Nishino, T.; Oda, Y.; Katsuki, H.; Aoyama, Y.; Yoshida, Y. *Tetrahedron Lett.* **1983**, *24*, 4729.
108. Van de Loo, F. J.; Broun, P.; Turner, S.; Sommerville, C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6743.

109. Walsh, C. "Enzymatic Reaction Mechanisms", Freeman, W. H. and Company, USA, 1979, pp 108-123.
110. Maskill, H. "The Physical Basis of Organic Chemistry", Oxford University Press, USA, 1985, pp 367-404.
111. (a) Northrop, D. B. *Biochemistry* **1975**, *14*, 2644. (b) Northrop, D. B. In: "Isotope-Effects on Enzyme-Catalyzed Reactions", Cleland, W. W.; O'Leary, M. H.; Northrop, D. B. Eds. University Park Press, 1976, pp 122-151.
112. Melander, L.; Saunders, W. H. Jr. "Reaction Rates of Isotopic Molecules", Wiley and Sons Inc., USA, 1979, pp 91-129.
113. Koskinen, P. M.; Koskinen, A. M. P. *Synthesis-Stuttgart* **1998**, 1075.
114. (a) Schmidt, R. R.; Zimmerman, P. *Tetrahedron Lett.* **1986**, *27*, 481. (b) Zimmerman, P.; Schmidt, R. R. *Leibigs. Ann. Chem.* **1988**, 663.
115. Julina, R.; Herzig, T.; Bernet, B.; Vasella, A. *Helv. Chim. Acta* **1986**, *69*, 368.
116. Nicolau, K. C.; Caufield, T.; Kataoka, H.; Kumazawa, T. *J. Am. Chem. Soc.* **1988**, *29*, 239.
117. Newman, H. *J. Am. Chem. Soc.* **1973**, *95*, 4096.
118. Boutin, R. H.; Rapoport, H. *J. Org. Chem.* **1986**, *51*, 5320.
119. Nimkar, S.; Menaldino, D.; Merrill, Jr. A. H.; Liotta, D. *Tetrahedron Lett.* **1988**, *29*, 3037.
120. Garner, P.; Park, J. M.; Malecki, E. *J. Org. Chem.* **1988**, *53*, 4395.
121. Radunz, H. E.; Devant, R. M.; Eiermann, V. *Leibigs Ann. Chem.* **1988**, 1103.
122. Herold, P. *Helv. Chim. Acta* **1988**, *71*, 354.
123. Villard, R.; Fotiadu, F.; Buono, G. *Tetrahedron: Asymmetry* **1998**, *9*, 607.
124. (a) Garner, P. *Tetrahedron Lett.* **1984**, *25*, 5855. (b) Garner, P.; Park, J. M. *J. Org. Chem.* **1987**, *52*, 2361.
125. Kashiwagi, M.; Mikami, T.; Chiba, M.; Chiba, S.; Matsumoto, H.; Akino, T.; Gasa, S. *Biochem. Mol. Biol. Int.* **1997**, *42*, 1071.
126. McKillop, A.; Taylor, R. J. K.; Watson, R. J.; Lewis, N. *Synthesis* **1993**, 31.
127. Branquet, E.; Durand, P.; Vo-Quang, L.; Le Goffic, F. *Synth. Commun.* **1993**, *23*, 153.

128. Jones, M. Jr. "*Organic Chemistry*", Norton, W.W. and Company, USA, 1997, pp 963-966.
129. McNulty, J.; Still, I. W. J. *Synth. Commun.* **1992**, *22*, 979.
130. Greene, T. W.; Wuts, P. G. M. "*Protective Groups in Organic Synthesis*" Vol. II, Wiley, New York, 1991, pp. 324-330.
131. Moriwake, T.; Hamano, S. I.; Saito, S.; Torii, S. *Chem. Lett.* **1987**, 2085.
132. Ibuka, T.; Habashita, H.; Otake, A.; Fujii, N. *J. Org. Chem.* **1991**, *53*, 4370.
133. Crombie, L.; Heaves, A. D. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1929.
134. Vogel, A. "*Vogel's Textbook of Practical Organic Chemistry*", Fourth Ed., Longman Group Ltd., England, 1978, pp 386-388.
135. Metcalfe, L. D.; Schmitz, A. A. *Anal. Chem.* **1961**, *33*, 363.
136. Rakoff, H. *Prog. Lipid Res.* **1982**, *42*, 225.
137. Garner, P.; Ramakanth, S. *J. Org. Chem.* **1986**, *51*, 2609.
138. Chérest, M.; Felkin, H.; Prudent, N. *Tetrahedron Lett.* **1968**, 2199.
139. Cram, D. J.; Wilson, D. R. *J. Am. Chem. Soc.* **1963**, *85*, 1245.
140. Williams, L.; Zhang, Z.; Shao, F.; Carroll, P. J.; Joullie, M. M. *Tetrahedron* **1996**, *52*, 11673.
141. Coleman, R. S.; Carpenter, A. J. *Tetrahedron, Lett.* **1992**, *33*, 1697.
142. Hoffman, R. V.; Tao, J. *J. Org. Chem.* **1998**, *63*, 3979.
143. Hafner, A.; Duthaler, R. O.; Marti, R.; Rihs, G.; Rothe-Streit, P.; Schwarzenbach, F. *J. Am. Chem. Soc.* **1992**, *114*, 2321.
144. Koskinen, A. M. P.; Koskinen, P. M. *Tetrahedron Lett.* **1993**, *34*, 6765.
145. Curtin, D. Y. *Rec.Chem. Prog.* **1954**, *15*, 111.
146. Eliel, E. L. "*Stereochemistry of Carbon Compounds*", Mc-Graw-Hill, New York, 1962, pp. 151-152, 237-238.
147. Abraham, R. J.; Fisher, J.; Loftus, P. "*Introduction to NMR Spectroscopy*", John Wiley & Sons, Chichester, 1995, pp. 43-46.
148. Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543.

149. Li, S.; Wilson, W. K.; Schroepfer, Jr. G. J. *J. Lipid Res.* **1999**, *40*, 764.
150. Wehrli, F. W.; Marchand, A. P.; Wehrli, S. "Interpretation of ^{13}C NMR Spectra", John Wiley & Sons, Chichester, 1988.
151. Pagano, R. E. *Methods Cell. Biol.* **1989**, *29*, 75.
152. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
153. Abad, J. L.; Camps, F.; Fabrias, G. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 3279.
154. Engeseth, N.; Stymne, S. *Planta* **1996**, *198*, 238.
155. (a) Kearnes, E. V.; Hugly, S.; Somerville, C. *Arch. Biochem. Biophys.* **1991**, *284*, 431. (b) Smith, M. A.; Jonsson, L.; Stymne, S.; Stobart, K. *Biochem. J.* **1992**, *287*, 141.
156. Somerville, C.; Browse, J. *Science* **1991**, *252*, 80.
157. Baumann, H.; Buhler, M.; Fochem, H.; Hirsinger, F.; Zoebelein, H.; Falbe, J. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 41.
158. Strittmatter, P.; Spatz, L.; Corcoran, D.; Rogers, M. J.; Setlow, B.; Redline, *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 4565.
159. Baenzinger, J. E.; Smith, I. C. P.; Hill, R. J. *J. Chem. Phys. Lipids* **1990**, *54*, 17.
160. Gaver, R. C.; Sweeley, C. C. *J. Am. Chem. Soc.* **1966**, *88*, 3643.
161. Wild, R.; Schmidt, R. R. *Liebigs Ann. Chem.* **1995**, 755.
162. Carter, H. E.; Shapiro, D. *J. Am. Chem. Soc.* **1953**, *75*, 5131.