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CHEMISTRY AND BIOCHEMISTRY OF SPHINGOSINE BASES

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The chemistry with regard to synthesis and structural analyses of long chain bases are summarized and pathways for the synthesis of their radioactively labelled optical isomers are outlined.

Aided by labelled substrates, the implication of the stereochemistry of the sphingosine bases in their catabolic and anabolic reactions and the metabolism of complex sphingolipids have been investigated.

I. Introduction

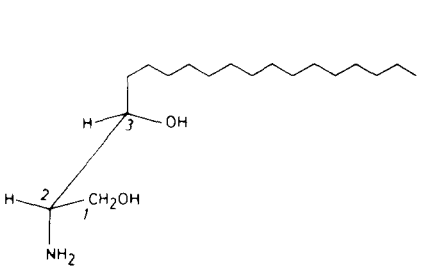
A large number of complex lipids is involved in the structure of biological membranes. The lipids most commonly present can be classified into two groups, the glycerophospholipids and the sphingolipids. The amphiphilic chemical structure of these lipid molecules is responsible for their properties and function in the membrane. Two long aliphatic chains impart the required hydrophobic (lipophilic) character, whereas a hydrophilic group is located at the interphase.

In phospholipids, either two long-chain acyl groups or an alkyl or alk-1-enyl moiety and an acyl residue are combined with the glycerol moiety of glycerophospholipids. In sphingolipids, the long-chain sphingosine base constitutes the aliphatic chain and also contains the functional groups, which accept a long-chain fatty acid bound as an amide, as well as the hydrophilic group. The basic structures of the various classes of sphingolipids are dihydrosphingosine (sphinganine, (2S, 3R) -2-amino-1,3-dihydroxy-octadecane), sphingosine (4t-sphingenine, (2S, 3R) -2-amino-1,3-dihydroxy-4t octadecene), and phytosphingosine (4D-hydroxy-sphinganine, (2S, 3R, 4R) -2-amino-1,3,4-trihydroxyoctadecane). The absolute configuration of these compounds is shown in scheme 1.

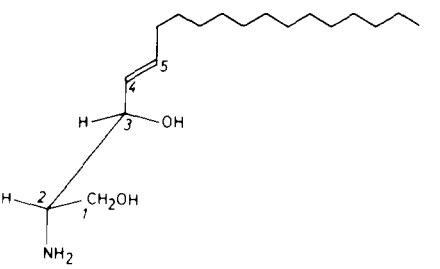
N-Acylation of the aforementioned three bases with different long-chain fatty acids results in a higly heterogeneous hydrophobic ceramide part.

Phosphodiesters and hexoses bound as glycosides as well as neuraminic acid contribute to widely varying hydrophilic molecular moieties in the individual sphingolipids. Typical examples include the sphingomyelins, ceramide mono- and polyhexosides, sulfatides, and gangliosides.

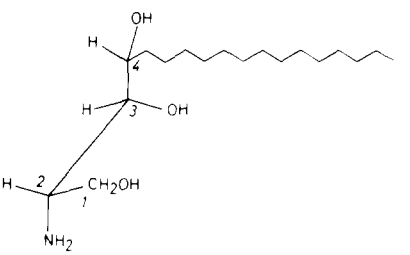
The occurrence of sphingolipids is not limited to animal cells. Anaerobic bacteria,



Dihydrosphingosine
= Sphinganine
= 2 - Amino - 1, 3 - dihydroxyoctadecane
2S, 3R = D (+) erythro



Sphingosine
= 4t - Sphingenine
= 4t - 2 - Amino - 1, 3 - dihydroxy - octadecene
2S, 3R = D (+) erythro

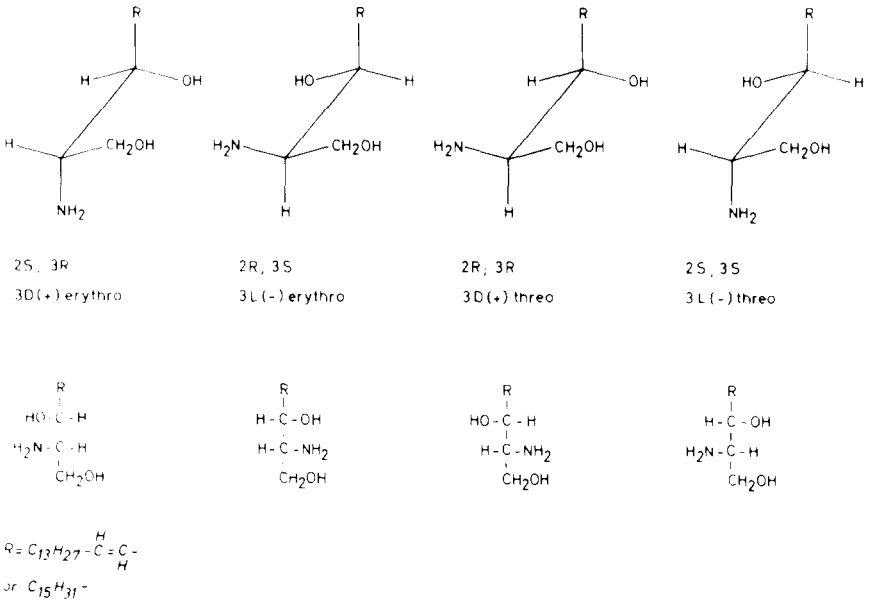


Phytosphingosine
= 4 - Hydroxysphinganine
= 2 - Amino - 1, 3, 4 - trihydroxyoctadecane
2S, 3S, 4R

Scheme 1. Absolute configuration of the long chain bases dihydrosphingosine, sphingosine and phytosphingosine.

amoebae, yeasts and fungi have been found to contain various complex sphingolipids whose constituent bases, though often varying in chain length, have the same stereochemical configuration of the functional groups [1].

All of the three principal long-chain bases contain 18 carbon atoms, whereas in the brain gangliosides the C_{20}-homologues of sphingosine are present. Sphingosine was isolated by Thudichum, in 1882, from the alkaline hydrolysate of a cerebroside fraction. Seventy years elapsed there after until the stereochemistry of the sphingosines could be elucidated. The molecules contain two asymmetric carbon atoms. There is a great deal of confusion regarding the nomenclature of optical isomers of *erythro*- and *threo*-diastereomers because C–2 and C–3 are arbitrarily chosen as points of reference. The S:R-nomenclature is unequivocal, hence it should be followed. The four isomers of dihydrosphingosine are depicted in scheme 2.



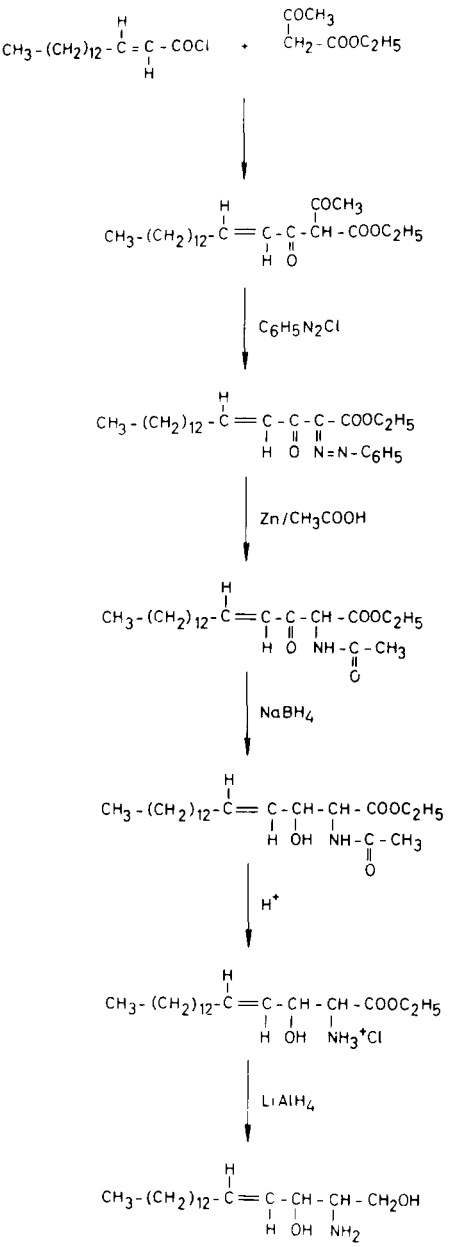
Scheme 2. Absolute configuration of the four isomeric dihydrosphingosines.

A great deal of analytical work has led to the elucidation of the structures. The results of this work were supported by total synthesis. Accordingly, sphingosine is *trans* (2S, 3R) 1,3-dihydroxy-2-amino-4-octadecene.

II. Chemical syntheses

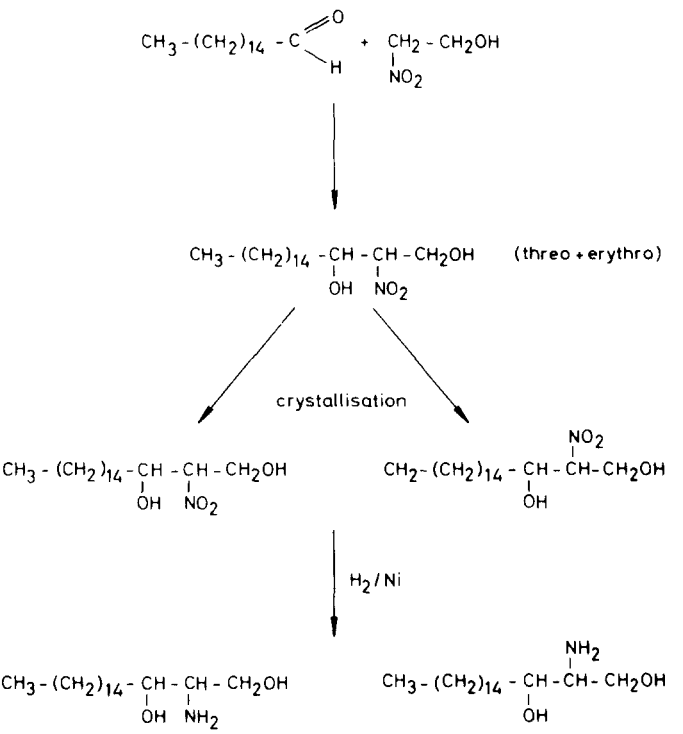
Shapiro et al. [2] reported for the first time the total synthesis of D, L-sphingosine by stepwise chain elongation, starting from myristaldehyde (scheme 3). This aldehyde is condensed with malonic acid in a Knoevenagel reaction to yield 2t-hexadecenoic acid, which is then converted to the acid chloride. Sodium acetoacetic ester is acylated with 2t-hexadecenoyl chloride to give an α, α-diacyl ester as shown in scheme 3. In a Japp–Klingemann reaction with benzenediazoniumchloride the phenylhydrazone is obtained which can be reduced with zinc/acetic acid to an acetamidoketoester. Reduction with sodium borohydride, cleavage of the amide bond with ethanolic hydrogen chloride, and reduction with lithium aluminum hydride gives a pure racemic sphingosine, whose antipodes are separated via the glutamates. The triacetyl derivative of the D-isomeric base was found to be indentical with the natural product.

Another path of synthesis was developed by Grob and coworkers [3], who condensed 2-hexadecinal with 2-nitroethanol. The *threo*- and *erythro*-diastereoisomers



Scheme 3. Synthesis of D, L-sphingosine according to Shapiro et al. [2].

were separated by crystallization, and the nitro group was reduced with aluminium amalgam or with zinc/hydrochlorid acid. The corresponding acetylenic compounds could be reduced selectively and stereo specifically with sodium in butanol, or with



Scheme 4. Synthesis of dihydrosphingosine according to Grob and Jenny [3].

lithium aluminum hydride, to the *trans*-base, and with Lindlar catalyst to *cis-erythro*- and *threo*-bases respectively. Similar to the synthesis of Δ^4-*trans*-unsaturated sphingosines, the dihydrosphingosines can be synthesized from palmitoyl chloride or palmitic aldehyde as starting materials [4], as shown in scheme 4. Dihydrosphingosine is obtained, of course, by catalytic reduction of sphingosine.

Considerable confusion arose earlier because the dihydrosphingosines obtained by Seydel [5] by acidic hydrolysis (15% sulfuric acid in 50% methanol) of natural cerebroside and catalytic hydrogenation were taken as reference substances. It is known today that the products prepared by the aforesaid author were artifacts, which were formed by the inversion of configuration at C--2 by N → 0 acyl migration via an intermediate oxazoline.

In the course of our work, we have attempted to prepare and characterize the four isomers, viz., D-*threo* (2R, 3R), L-*threo* (2S, 3S), L-*erythro* (2R, 3S) and the D-*erythro* (2S, 3R) compounds [6]. Table 1 summarizes the properties of these isomers without detailed reference regarding the methods of preparation of these compounds and the separation of racemates.

The following reactions were carried out to proof the different structures: oxidation of N-acetyl derivatives of A and D as well as B and C yielded identical

Table 1
Physical properties of isomeric dihydrosphingosines

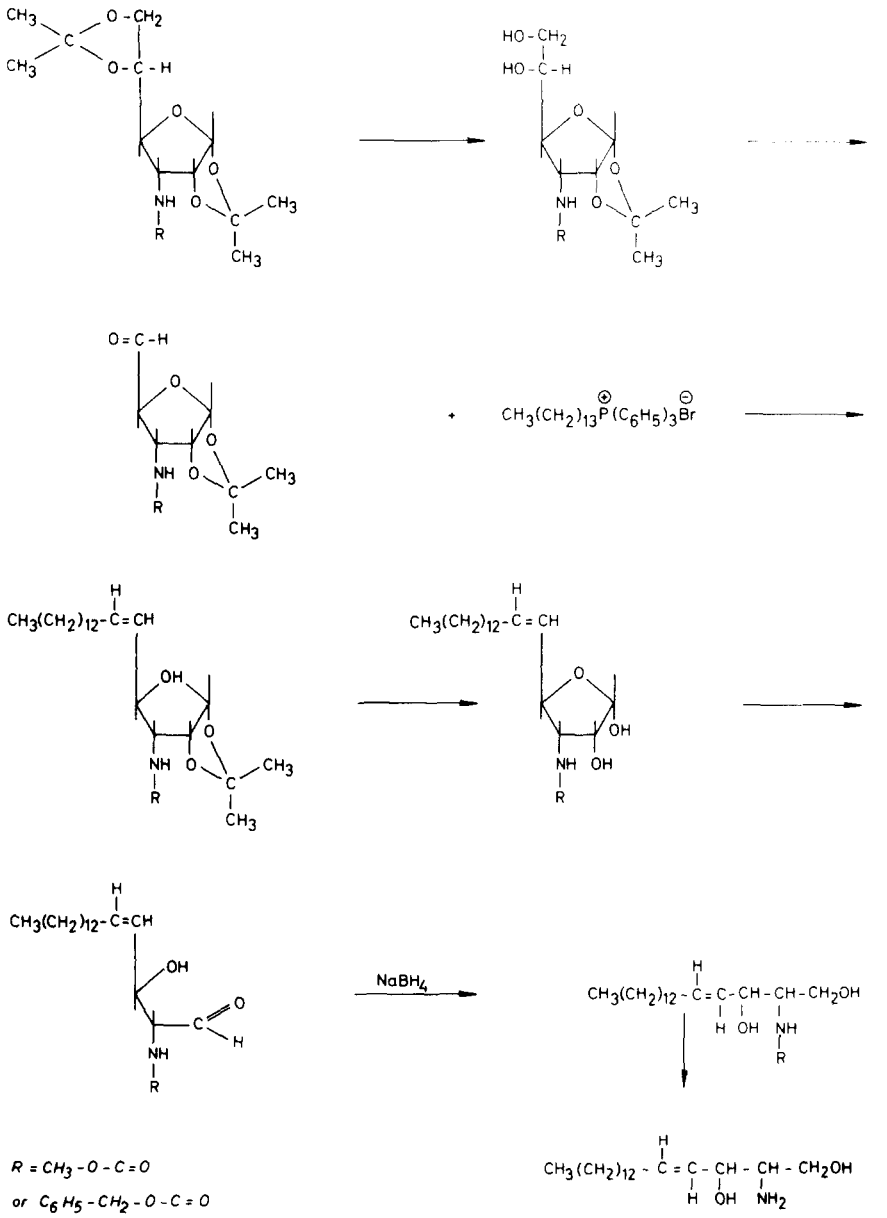
Compound	M.P. (°C)	$[\alpha]_{546}^{28}$	
A D(+) *erythro*-Dihydrosphingosine (2D, 3D(+) *erythro*-; 2S, 3R)	79 felted leaflets	+ 6.0°	(in chloroform–methanol 10 : 1)
B L(−) *erythro*-Dihydrosphingosine (2L, 3L(−) *erythro*-; 2R, 3S)	79 felted leaflets	− 6.0°	"
C D(+) *threo*-Dihydrosphingosine (2L, 3D(+) *threo*-; 2R, 3R)	108 leaflets	+ 13°	"
D L(−) *threo*-Dihydrosphingosine (2D, 3L(−) *threo*-; 2S, 3R)	108 leaflets	−13°	"
N-Acetyl derivatives of			
A	124–125	+ 7.7°	(in hexane–ethanol 10 : 1)
B	124–125	− 8.0°	
C	96–97	+ 6.2°	"
D	96–97	− 6.5°	"
Triacetyl derivatives of			
A	96–97	+ 21°	"
B	96–97	−21°	"
C	46	+ 8.0°	(in pentane)
D	46	− 8.0°	(in pentane)

2-N-acetamido-3-oxo-octadecanols, and the hydrolysis of the acetyl derivatives led to identical hydrochlorides of 2-amino-3-oxo-octadecanol [9]. These results are summarized in table 2.

Another route for the synthesis of dihydrosphingosine ought to be mentioned

Table 2
Physical properties of derivatives of dihydrosphingosines

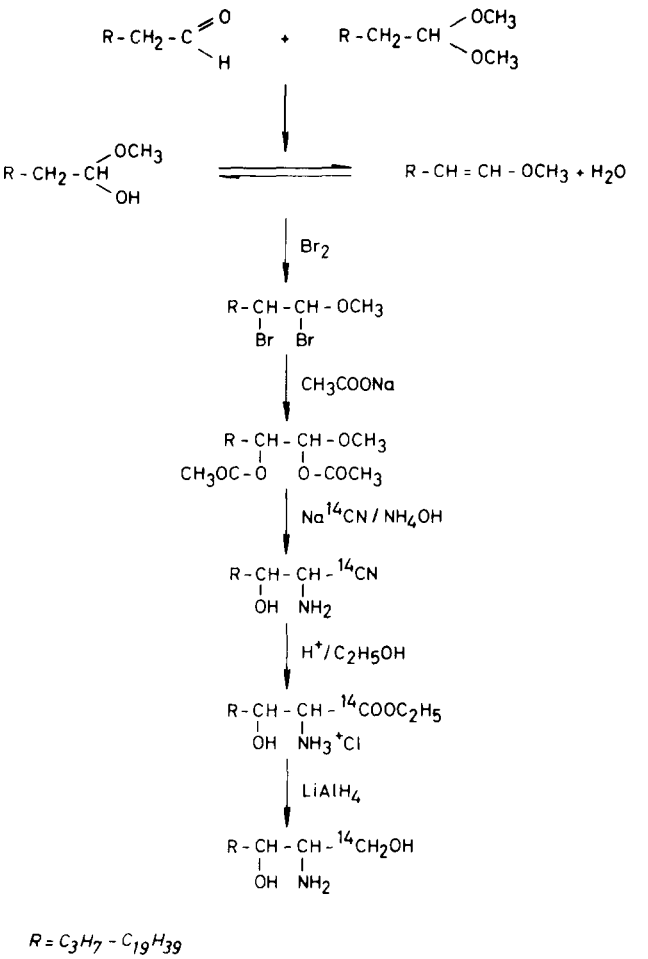
Compound	M.P. (°C)	$[\alpha]_D$
N-Acetyl-3-dehydrosphinganine of		
A (D(+) *erythro*)	105–106	+ 59°
B (L(−) *erythro*)	105–106	− 58°
C (D(+) *threo*)	105–106	− 57°
D (L(−) *threo*)	105–106	+ 57°
3-Dehydrosphinganine hydrochloride of		
A (D(+) *erythro*)	89–92	+ 26°
B (L(−) *erythro*)	90–92	− 26°
C (D(+) *threo*)	88–91	− 26.5°
D (L(−) *threo*)	88–91	+ 28°



Scheme 5. Synthesis of dihydrosphingosine and sphingosine according to Reist and Christie [7,8].

because of its stereospecificity (Reist and Christie [7,8]); this synthesis is outlined in scheme 5.

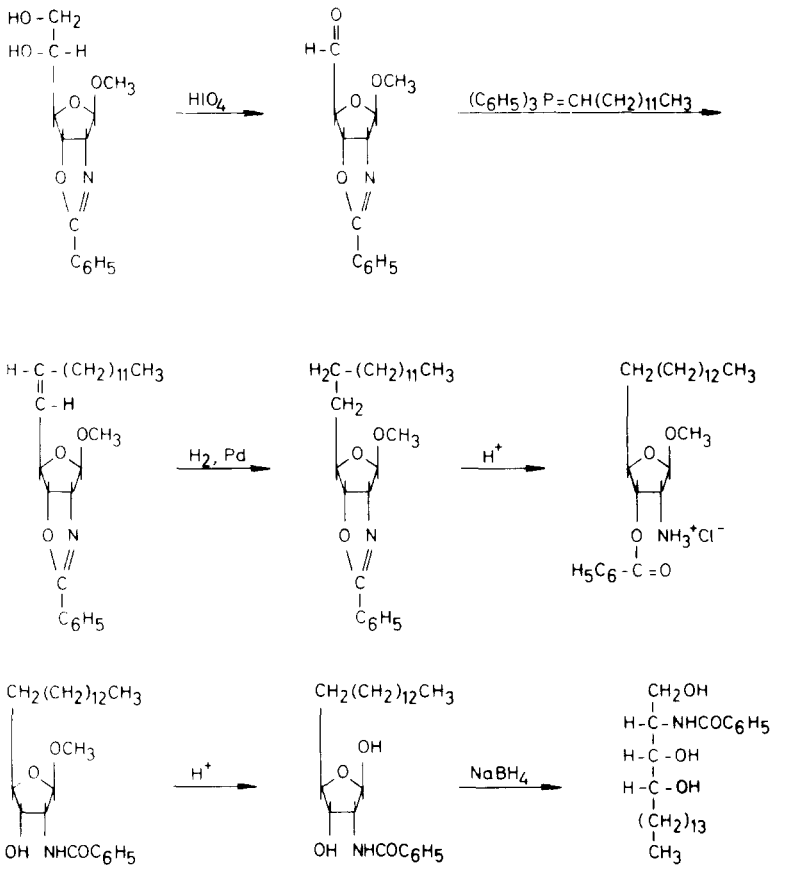
Starting from 3-amino-3-desoxy-1,2 : 5,6-di-*O*-isopropylidene-α-D-allofuranose,



Scheme 6. Synthesis of dihydrosphingosine according to Stoffel et al. [10].

6-carbobenzoxy-amino-2, 2-dimethyl-5-formyl-3a, 5, 6, 6a-tetrahydro-D-ribofuro 2, 3 dioxalane was prepared in three steps. Following a Witting reaction with tetradecyl–triphenyl phosphonium bromide, the latter compound gave a mixture of *cis*- and *trans*-olefins. Hydrogenation using palladium on carbon removed the protecting group and simultaneously saturated the olefinic bond to give D-*erythro*-dihydrosphingosine. Sphingosine was synthesized in an analogous manner. The reaction condition, especially, the concentration of phenyl lithium, was chosen such that the formation of *cis*-isomers was suppressed. Thus, the *trans*-isomer was obtained in 60% yield. The protecting ethoxy group was removed with barium hydroxide.

A relatively simple method for the preparation of saturated long-chain bases has been developed by us for the synthesis of homologous dihydrosphingosines [10]; it is shown in scheme 6.

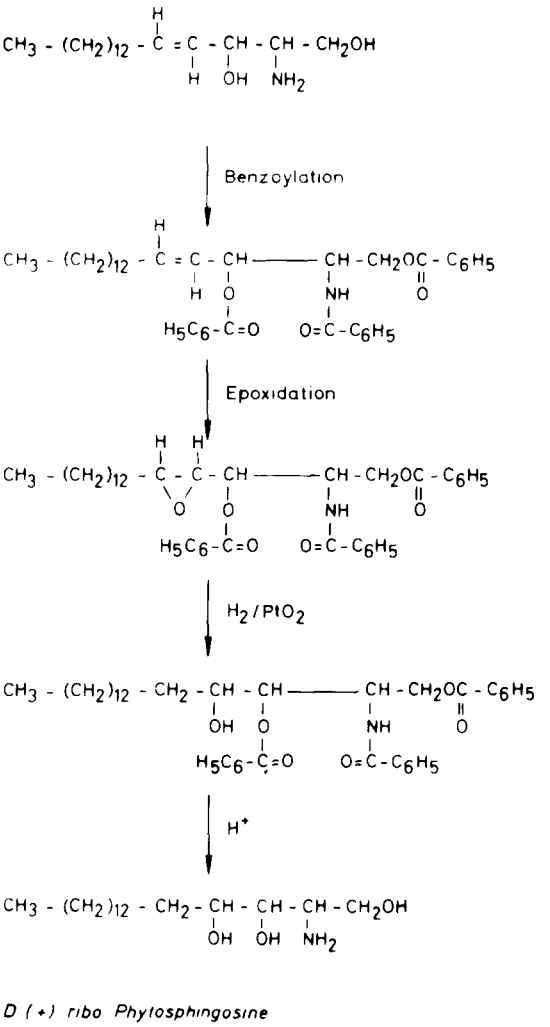


Scheme 7. Stereospecific synthesis of phytosphingosine according to Gigg et al. [11].

The corresponding hemiacetal is brominated and converted to 1-methoxy-1,2-diacetoalkane using sodium acetate. Replacement of the methoxy group by cyanide, in the presence of NH_4-ions, leads to an 1-cyano-2-amino-3-hydroxyalkane. Subsequent hydrolysis of the nitrile and reduction of the ester group with lithium aluminum hydride yields a racemic mixture of the long-chain base whose diastereomeric forms can be separated by established procedures.

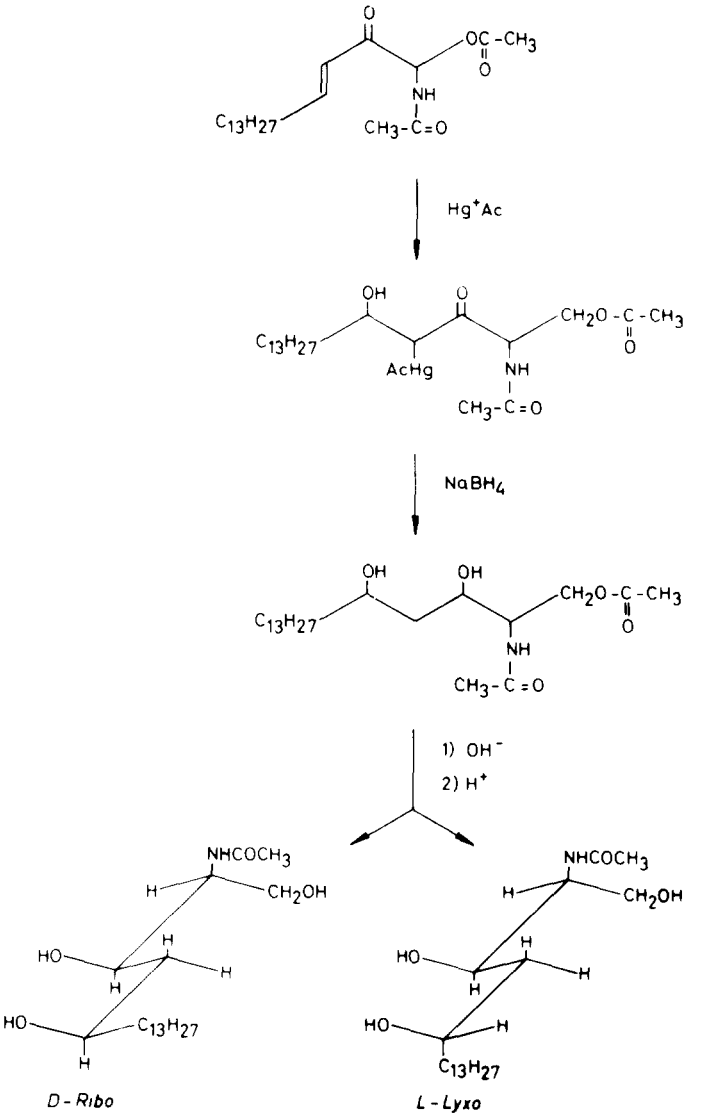
Apart from the two aforesaid bases which occur most commonly in the animal cell, phytosphingosine as well has been prepared by total synthesis. Starting from the furanoside form of galactosamine, Gigg [11–13] obtained the ribo-form of phytosphingosine, as shown in Scheme 7.

Prostenik et al. [14] epoxidized the double bond of tribenzoyl sphingosine and subsequently opened the epoxide ring by catalytic reduction. After resolution of the racemate, the ribo-phytosphingosine was obtained via the oxalate, scheme 8.



Scheme 8. Synthesis of phytosphingosine according to Prostenik et al. [14].

In the course of biochemical studies we had to consider 5-hydroxydihydrosphingosine as a possible intermediate in the conversion of dihydrosphingosine to sphingosine. It was possible to synthesize both the ribo- and the lyxo-form of 5-hydroxydihydrosphingosine [15], as is shown in the scheme 9.



Scheme 9. Synthesis of 5-hydroxydihydrosphingosine [15].

III. Analysis and proof of structure

It was of crucial importance in our studies to determine whether the compounds were pure – chemically, radiochemically and stereochemically. Only thus it was possible to make reliable statements regarding enzyme specificities. Simple methods for

the determination of purity are thin-layer chromatography and gas chromatography, as well as the combination of gas chromatography and mass spectrometry.

A comparison of mass spectra of *threo*- and *erythro*-derivatives of dihydrosphingosine reveals that the fragmentation yields various ion intensities. The *threo*-derivative is fragmented easily between C–1 and C–2, which results in an ion peak at m/e = 384 (M–103). Other typical fragments are as follows: m/e = 472 (M–15); m/e = 428 (M–59); m/e = 397 (M–90); m/e = 328 (M–15–90); m/e = 313 (M–174) (cleavage between C–2 and C–3); m/e = 294 (M–103–90); m/e = 247 (cleavage between C–2 and C–3 along with transfer of the TMS-group from oxygen at C–3 to nitrogen at C–2); m/e = 157 (M–247–90) (loss of trimethyl silanol of the fragment m/e = 247).

Information regarding the length and structure of the long aliphatic chain is obtained unequivocally by periodate oxidation and gas chromatography of the resulting aldehydes, which represent the base from C–3 upto the terminal methyl group. Correspondingly, the phytosphingosines on periodate oxidation yield aldehydes representing the chain from C–4 up to the terminal methyl group.

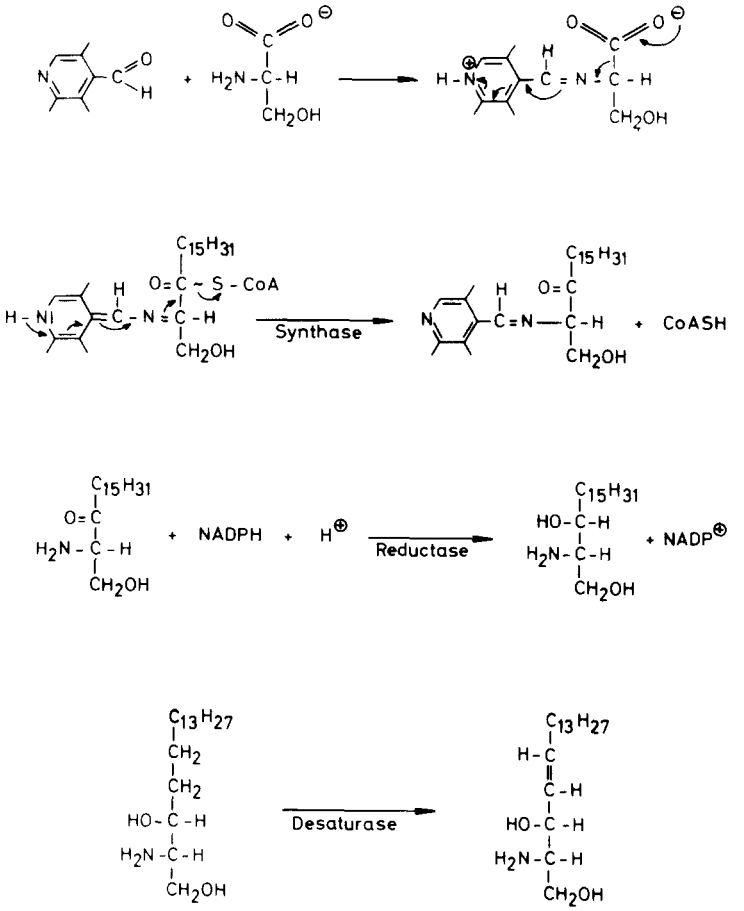
IV. Biochemistry of sphingosine bases

The biosynthesis and degradation of long-chain bases have been exposed by us during the past years; reference is made to a pertinent review [16]. Instead of following the chronological order the following treatise is written to show summarily the pathways by which the cell synthesizes and degrades these compounds (scheme 10).

The 18–C chain is built up from a C–16 and a C–2 unit. Palmitoyl-CoA condenses with L (=2S) serine. Due to the formation of a Schiff base around the pyridoxal phosphate, the C–2 of serine is capable of a nucleophilic attack at the carbonyl group of the thioester. According to the mechanism shown, 3-oxo-dihydrosphingosine is formed by synchronous decarboxylation under retention of the optically active center at C–2. In a stereospecific reduction which is dependent on NADPH, the hydrogen at the B-side is transferred giving rise to 3S-configuration. The *trans*-double bond is then introduced by a mechanism not known so far. We have proved by stereospecific labelling that first the 4R- and thereafter the 5S-hydrogen atom undergo a *cis*-elimination to give a 4-*trans* olefinic bond (scheme 11). Therefore, the 4R hydrogen atom is eliminated first, as shown by a strong isotopic effect. Similarly, in the synthesis of phytosphingosine the 4R-hydrogen is removed and substituted by the hydroxyl group whose origin is unknown as yet. Desaturation as well as hydroxylation take place at the oxidation level of dihydrosphingosine as could be proved by double labelling of dihydrosphingosine.

The only products of biosynthesis found in nature are the D-*erythro* (2S, 3R)-forms, due to stereospecificity of the synthetase and reductase reactions.

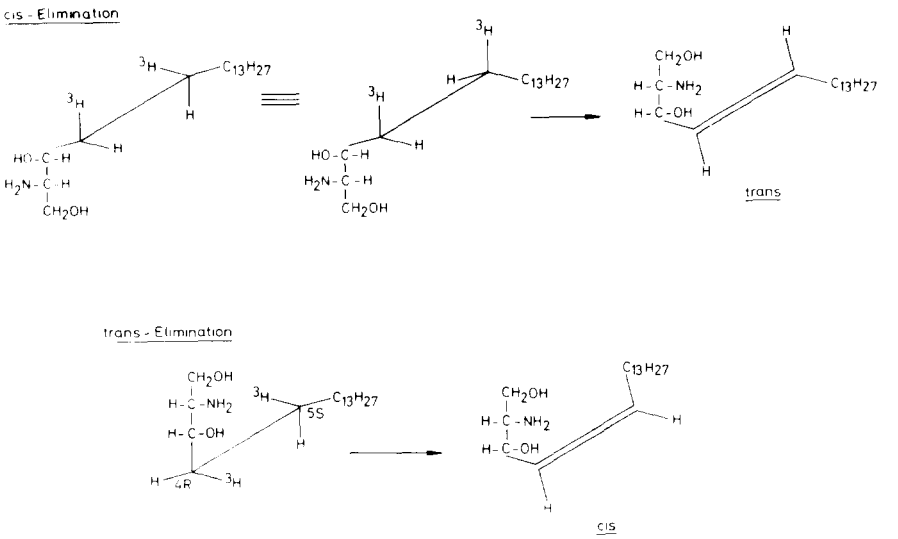
We were concerned also with the degradation of the sphingosine bases. This is of particular interest in view of the unique arrangement of the functional groups (2-



Scheme 10. Metabolic pathways of sphingosine bases.

amino-1,3-diol-system). Hydrolytic enzymes liberate sphingosine bases from the complex sphingolipids. They represent for the cell highly toxic compounds, which after N-acylation, are either immediately brought to the ceramide pool, or, in the form of Schiff base with pyridoxalphosphate, are phosphorylated at C–1 by an ATP-dependent kinase. The phosphorylated long-chain bases are cleaved between C–2 and C–3 by a microsomal aldolase which owes its function to sulfhydryl groups. Consequently, the several compounds which are formed, are shown in scheme 12.

In the case of dihydrosphingosine phosphate, palmitaldehyde and phosphoryl ethanolamine are formed from each of the three long-chain bases, 2-*trans*-hexadecenal is formed from sphingosine-1-phosphate, and 2-hydroxy-palmitaldehyde from phytosphingosine-1-phosphate. The phosphate ester of the compound listed

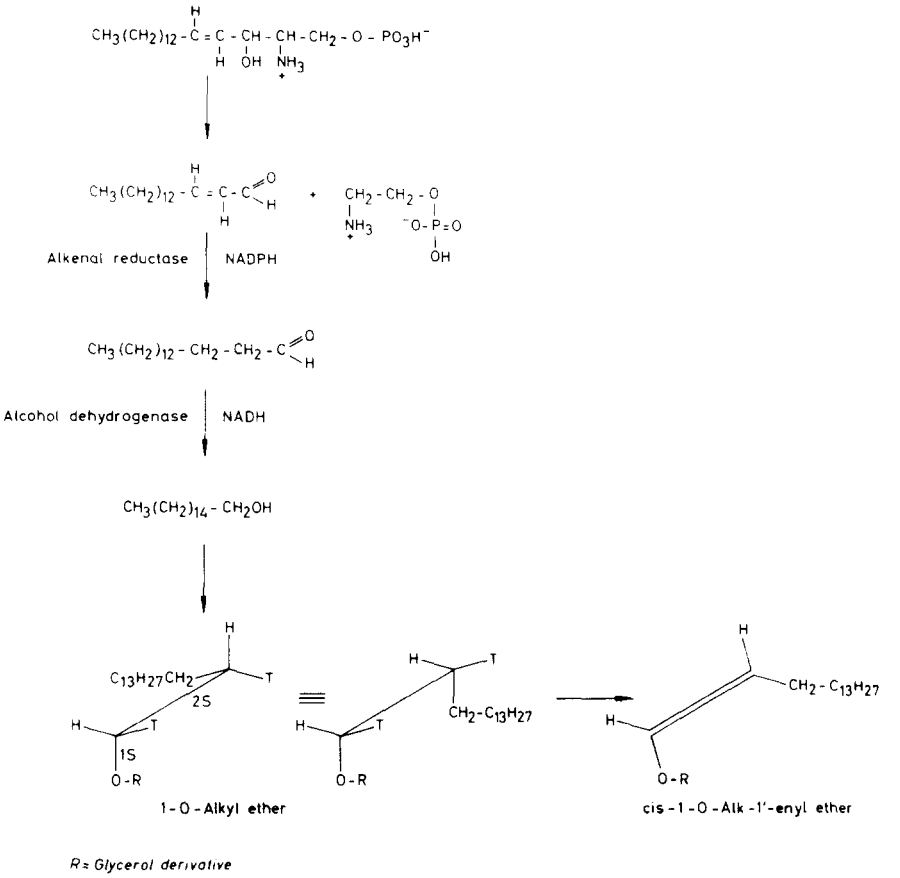


Scheme 11. Stereospecific hydrogen elimination of dihydrosphingosine to sphingosine.

first can be prepared chemically; the esters of the other two compounds can be obtained biochemically on a preparative scale using a very active sphingosine kinase present in thrombocytes.

We also wanted to determine the path which the products of degradation of sphingosine follow in metabolism. Sphingosine, the base most commonly present should be considered first. Phosphoryl ethanolamine to a large extent is utilized directly for the synthesis of the hydrophilic moiety of phosphatidyl ethanolamine, and a small part is methylated to build up the choline moiety of phosphatidyl cholines and sphingomyelins. 26-Hexadecenal is reduced by a NADPH-dependent alkenal-reductase to palmitaldeheyde, which is then either reduced to hexadecanol by a alcohol dehydrogenase, or oxidized to palmitic acid by an aldehyde oxidase. Palmitaldehyde serves as precursor for the synthesis of plasmalogens whereas palmitic acid is either degraded or esterified in acyl lipids. In connection with the substrate specificity of the aforesaid enzymes and others that are involved in the synthesis of complex sphingolipids, table 3 demonstrates how the configuration at carbon atoms 2 and 3 determine as to which reactions are feasible. We have investigated the metabolism of the four isomeric dihydrosphingosines in the rat and studied their reactions in the rat liver. The 2R, 3S = D-*erythro* dihydrosphingosine is completely degraded or converted to other substances in a very short period; 50% of the radioactivity recovered is found in palmitic and stearic acid, distributed among acyl lipids (trilycerides, ethanolamine and choline phosphoglycerides) and in acyl groups of ceramides, sphingomyelins and cerebrosides.

The 2R, 3S (L(−) *erythro*) antipode is merely acylated to ceramide and phos-



Scheme 12. Mechanism of degradation of long chain bases.

phorylated at C–1 without a cleavage of the phosphate ester by aldolase. The same result is obtained with the D(+) *threo* compound, which has the same configuration at C–2. Similar to these three isomers, the 2S, 3S = L(–) *threo*-antipode is acylated and, surprisingly, utilized for the synthesis of sphingomyelin. Thus the consistent finding that mainly in the case of 2S, 3R-isomers the unsaturated base sphingosine occurs in sphingomyelin, could again be confirmed. Roughly 30% of *threo*-sphingosine could be detected in structural analyses of the sphingomyelin formed. The configuration at C–2 and C–3 does not permit phosphorylation by the kinase, and consequently cleavage by aldolase does not occur.

Table 3
Distribution of radioactivity (%) in various lipid fractions of rat liver after administation of labelled *erythro*- and *threo*-dihydrosphingosines

Compounds isolated	Compounds administered			
	2S;3R (≡D(+) *erythro*)	2R;3S (≡L(−) *erythreo*)	2R;3R (≡D(+) *threo*)	2S;3S (≡L(−) *threo*)
	%	%	%	%
Ceramides	11.6	55.7	44.3	89.7
Bases	74.2 (64% D 18 : 1^{4T}; 36% D 18 : 0)	100 (97% D 18 : 0; 3% D 18 : 1^{4T})	100 (D 18 : 0)	100 (98.2% D 18 : 0; 1.8% D 18 : 1^{4T})
Fatty acids	25.8 (82% 16 : 0; 18% 18 : 0)	0	0	0
Sphingomyelins	38.5	0	0	10.3
Bases	90 (80% D 18 : 1^{4T}; 20% D 18 : 0)	–	–	100 (69% *threo* D 18 : 0; 31% *threo* D 18 : 1^{4T})
Fatty acids	10 (90% 16 : 0; 10% 18 : 0)	–	–	0
Cerebrosides	3.3	0	0	0
1-phosphate ester	traces	20.7	43.8 (D 18 : 0)	0 (D 18 : 0)
Ester fatty acids	46.6 (79% 16 : 0; 21% 18 : 0)	0	0	0
Substrate	0	23.6	11.9	0

R = $C_{15}H_{31}$– D 18 : 0 = Dihydrosphingosine D 18 : 1^{4T} = Sphingosine.

V. Conclusion

Significant advances have been made in the chemistry and stereochemistry of sphingosine bases which constitute the structural units of the very complex sphingolipids. The various methods of chemical synthesis and the effective means for the separation of enantiomeric forms in their optical antipodes have permitted radioactive labelling of these molecules with carbon or hydrogen atoms in specific positions. This has enabled us to tackle biochemical problems related to the biosynthesis of these compounds, their degradation by the cell, and the fate of the degradation products.

The elucidation of chemical structure of these compounds took 70 yr, whereas the methods now available permitted to expose the biochemical reactions within 5 yr. It is believed that the complex sphingolipids derived from sphingosine bases are highly significant for the function of the cell, such as the nerve cell, and pathological processes occurring in the cell. In numerous studies using labelled sphingosine bases in tissue cultures of normal and transformed cells we have found evidence of the significance of these lipids.

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