Synthesis and Spectral Properties of Chemically and Stereochemically Homogeneous Sphingomyelin and its Analogues*

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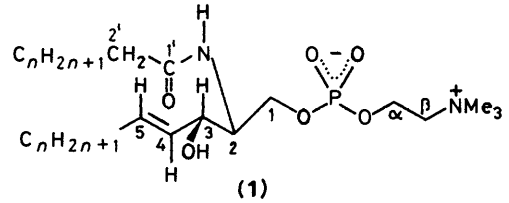
2-*N*-Stearoylsphingosyl-1-phosphocholines of D-*erythro* (2*S*,3*R*) and L-*threo* (2*S*,3*S*) configurations and their phosphorothioyl analogues were obtained by a purely synthetic approach using *O*-methyl-*N*,*N*-di-isopropylaminophosphorochloridite as the phosphitylating reagent for the formation of the phosphodiester linkage. Final products were obtained pure in yields of 70—75%. The structure and the purity of synthesized compounds was determined using ^{1}H, ^{13}C, and ^{31}P n.m.r. spectroscopy. With respect to the structure of the sphingosine long-chain base the synthetic D-*erythro*-SPM was found to be identical with the natural sphingomyelin from bovine brain whereas the semisynthetic *N*-palmitoylsphingomyelin obtained *via* a deacylation–reacylation pathway comprises a mixture of D-*erythro*- and L-*threo*-SPM. The phosphorothioyl analogues of sphingomyelin synthesized by addition of elemental sulphur to intermediate phosphite were separated into individual diastereoisomers having opposite configuration at phosphorus. The absolute configurations of diastereoisomers at phosphorus were assigned based on the known stereospecificity of phospholipase C in the hydrolysis of phosphorothioyl analogues of phospholipids.

^{1}H N.m.r. data of synthesized compounds suggest that the configuration at C-3 of the sphingosine influences the average conformation of sphingomyelin with respect to the angle of rotation about the C(1)–C(2) bond.

Sphingomyelin (**1**; SPM)† is one of the most abundant components of biological membranes and blood plasma lipoproteins.[1] Although the specific function of the sphingomyelin in membranes is not well understood, it has been suggested that it forms stable complexes with cholesterol[2] and strong intermolecular hydrogen bonds with other phospholipids.[3]



(1)

While most of biochemical studies can be performed with naturally occuring sphingomyelin, which exists as a multicomponent mixture of chemically distinct species differing in their fatty acid composition and sphingosine structure,[1] biophysical studies usually require homogeneous sphingomyelin samples.

Three different types of synthetic sphingomyelin have been used for these studies. Semisynthetic SPM is obtained by the acidic hydrolysis of natural sphingomyelin (typically from bovine brain) followed by the acylation of the resulting sphingosylphosphorylcholine (Scheme 1) with the appropriate acylating reagent obtained from the desired fatty acid.[4–7] Fully synthetic (±)-*erythro*-sphingomyelin was frequently used to examine the thermal behaviour of sphingomyelin bilayers.[8–9] No biophysical studies of the chemically and stereochemically homogeneous sphingomyelin have been reported to date, presumably owing to rather lengthy synthetic procedures leading to this lipid.[10] Only very recently several improved synthetic procedures for the synthesis of D-*erythro*-sphingosine were published.[11–18] Several other methods applicable to the synthesis of D-*erythro*-dihydrosphingosines or (±)-*erythro*-sphingosine have also been reported.[19–21] For the purpose of this work the synthetic method of Tkaczuk and Thornton[22] is best suited, since it produces two readily separable D-*erythro*-(2*S*,3*R*) and L-*threo*-derivatives (2*S*,3*S*). In this method, the formation of the C(2)–C(3) linkage of sphingosine is achieved by the addition of *trans*-vinylalane to the carbonyl group of the aldehyde derivative of the protected L-serine[22,23] (Scheme 2).

The conversion of *N*-acylsphingosine (ceramide) into sphingomyelin was carried out by phosphorylation of the 1-hydroxy group of *N*-acylsphingosine with 2-chloroethyl phosphorochloridate followed by hydrolysis of the remaining chloride at the phosphorus atom and quaternization of trimethylamine with the chloroethyl phosphodiester derivative so formed.[10,24] Although the yields reported were in the range 60—70%, this method when applied to the stereospecific synthesis of the (+)- and (−)-stereoisomers of *erythro*-SPM is called by Barenholz and Gatt[1] the low yield procedure. The alternative approach was described by Evstigneeva *et al.*[24] who have used phosphodiester method of Aneja and Davies.[25] Ceramide phosphate obtained in the first step of their synthesis was subsequently converted into sphingomyelin or other sphingophospholipid by condensation of the phosphate monoester with the appropriate hydroxy compound.

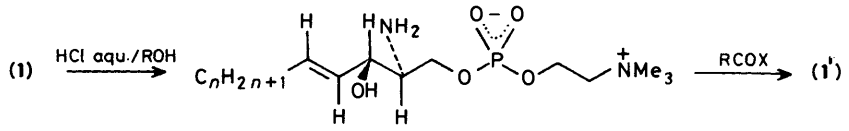
Results

Synthesis of Sphingomyelins.—This report is concerned with the synthesis of the natural sphingomyelin and its analogues of high chemical and stereochemical purity. We chose to prepare

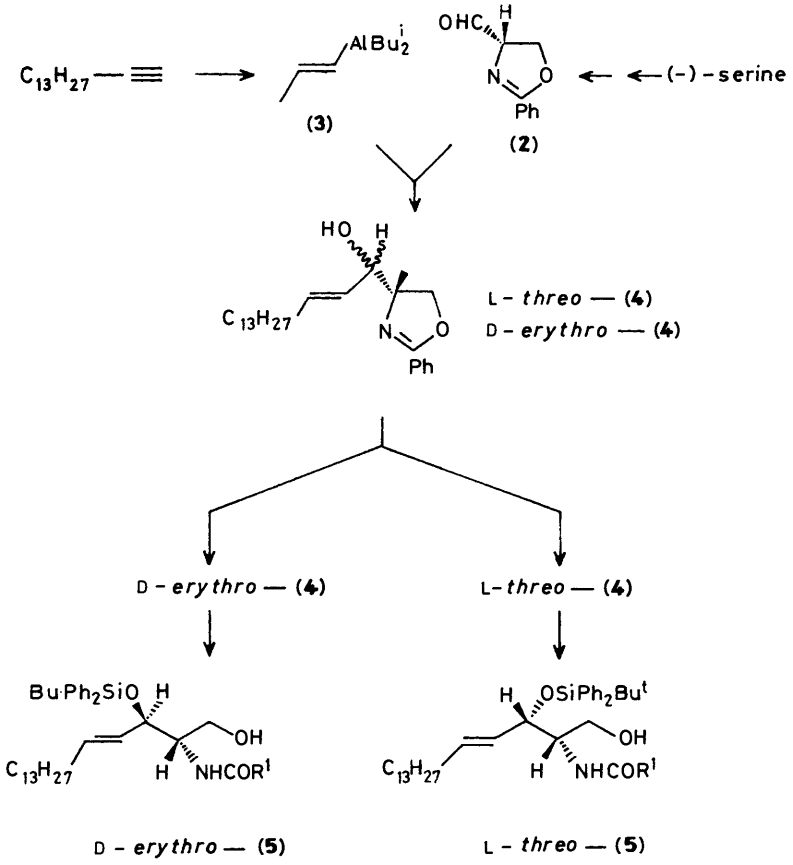
* For preliminary results see: K. S. Bruzik, *J. Chem. Soc., Chem. Commun.*, 1986, 331.

† Abbreviations: SPM = sphingomyelin; SPsM = 2-*N*-acylsphingosyl-1-thiophosphocholine; SDC = sodium deoxycholate; PLC = phospholipase C; DCC = dicyclohexylcarbodi-imide; DIBAL = di-isobutylaluminium hydride; h.p.t.l.c. = high performance thin layer chromatography.

Throughout this paper the stereochemical nomenclature recommended by IUB[45] is used. Thus, (2*S*,3*S*)-2-amino-octadecane-1,3-diol derivatives are designated L-*threo* as opposed to Tkaczuk and Thornton[22] and our recent communication where these compounds are named D-*threo*.



Scheme 1.



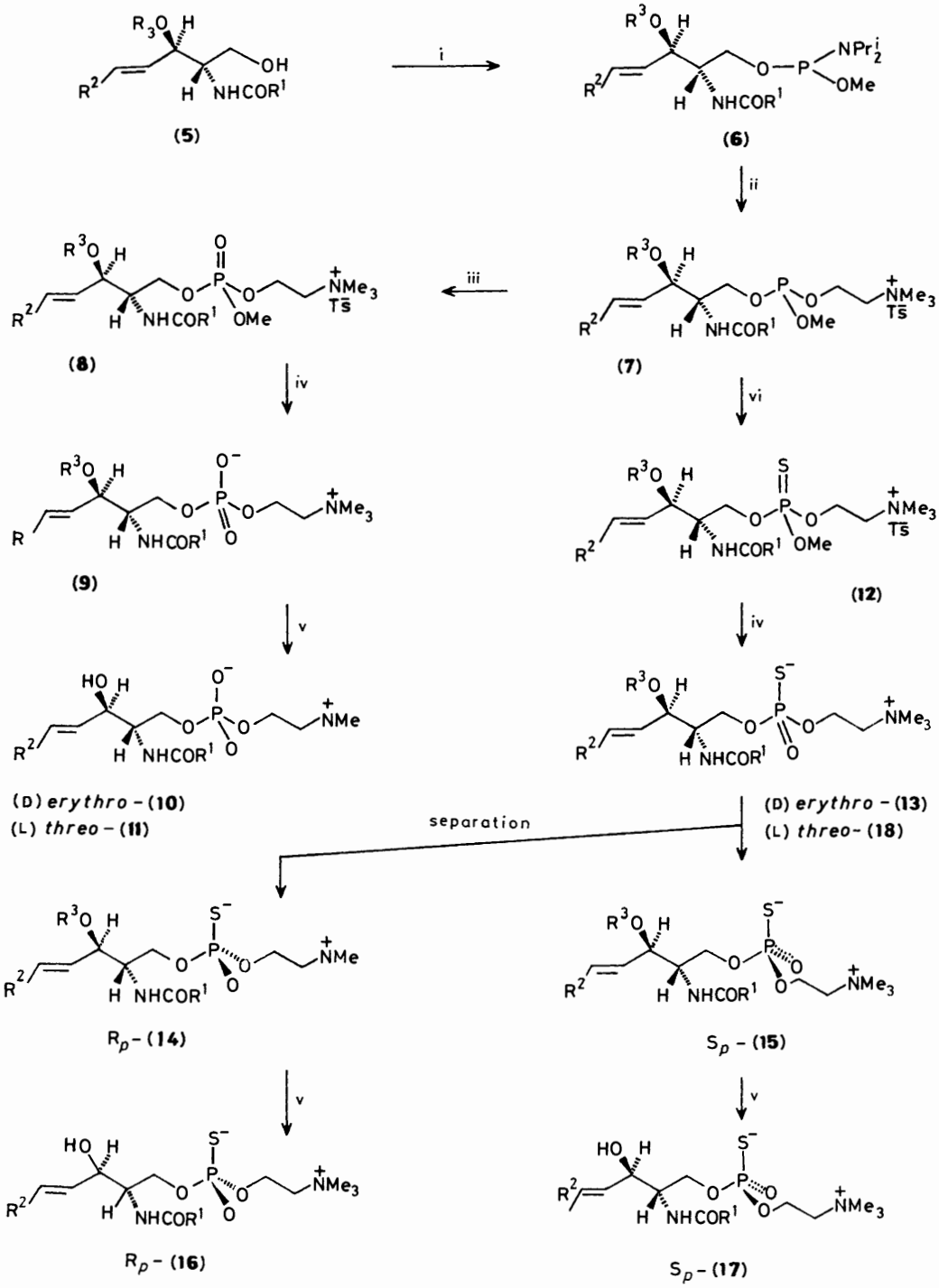
Scheme 2. *Reagents and conditions:* i, HCl–H_2O; ii, $C_{17}H_{35}CO_2C_6H_4NO_2$-*p*; iii, $ClSiPh_2Bu^t$; iv, MeOH–MeONa

the 2-*N*-stearoyl derivatives since D-*erythro*-2-*N*-stearoylsphingosyl-1-phosphocholine is the most widely distributed molecular species of sphingomyelin in animals.[1]

In order to evaluate the importance of the configuration at C-3 of the sphingosine and to obtain model compounds to study the influence of the structure of the head group on the aggregational properties of sphingomyelin, the following structural analogues of natural sphingomyelin were synthesized in addition to D-*erythro*-2-*N*-stearoylsphingosyl-1-phosphocholine, D-*erythro*-(**10**): (Rp)- and (Sp)-D-*erythro*-2-*N*-stearoylsphingosyl-1-thiophosphocholines (**16**) and (**17**) (SPsM); L-*threo*-2-*N*-stearoylsphingosyl-1-phosphocholine [L-*threo*-(**11**)] and L-*threo*-2-*N*-stearoylsphingosyl-1-thiophosphocholine [L-*threo*(**19**)].

D-*erythro*- and L-*threo*-2-*N*-Stearoylsphingosines (**5**) prepared as described earlier[22] (Scheme 2, Experimental section) were used as starting materials. The target sphingomyelins were synthesized by using an elegant method to form the phosphodiester linkage recently devised by Letsinger and Caruthers for the synthesis of deoxyribonucleotides on a solid-phase support.[26–28] The applicability of the phosphoramidite approach to the synthesis of glycerophospholipids has recently been demonstrated.[29]

The synthesis of D-*erythro*-SPM and its analogues is outlined in Scheme 3. D-*erythro*- and L-*threo*-3-*O*-(Diphenyl-t-butylsilyl)-2-*N*-stearoylsphingosine [D-*erythro*-(**5**) and L-*threo*-(**5**)], were separately treated with *O*-methyl *N*,*N*-di-isopropylamino-phosphorochloridite in the presence of triethylamine in chloroform. The resulting phosphoramidites (**6**) were treated with a mixture of choline tosylate and tetrazole in the acetonitrile–THF. The phosphites (**7**) obtained in this way, were either oxidized with t-butyl hydroperoxide in THF or treated with elemental sulphur in toluene to give the corresponding phosphates (**8**) or phosphorothioates (**12**). The desired phosphodiesters (**9**) and (**13**) were obtained by demethylation of their respective triesters (**8**) and (**12**) with anhydrous trimethylamine in toluene. The final products D-*erythro*- and L-*threo*-SPMs, (**10**) and (**11**), were formed in the desilylation reaction of 3-*O*-silyl-protected derivatives (**9**) with tetrabutylammonium fluoride. The synthesis of (**9**) and (**13**) was carried out by a one-pot procedure without isolation of the intermediate compounds. When the progress of the reactions was followed by ^{31}P n.m.r.



Scheme 3. Only compounds of (D)-*erythro*-(2*S*,3*R*)-configuration are shown for simplicity. *Reagents and conditions:* i, $ClP(OMe)NPr^i_2$, NEt_3–$CHCl_3$; ii, $HO(CH_2)_2NMe_3$, Ts^-–tetrazole in MeCN–THF (1:1); iii, Bu^tOOH; iv, Me_3N; v, Bu_4NF; vi, S_8 in THF

spectroscopy, no side-products could be detected. The total yield of isolated compounds (**9**) or (**13**) exceeded 75% in all cases. Similarly, the deprotection of the 3-*O*-hydroxy group with Bu_4NF was a high yield process.

The yields of the phosphorothioyl analogues (Rp)-(**16**) and (Sp)-(**17**) were, however, lower owing to repeated chromatographic runs necessary to separate the diastereoisomers (**13**). The dealkylation of the phosphorothioate (**12**) with trimethylamine produced a mixture of diastereoisomeric phosphodiesters (**13**) of the opposite configuration at the phosphorus atom. The diastereoisomeric mixture (**13**) was separated into individual compounds by column chromatography on silica gel using acetone–methanol–water (80:20:1.5) as the eluting solvent.* The separated diastereoisomers (**14**) and (**15**) were converted

* The diastereoisomeric mixture of L-*threo*-(**18**) was also separated by chromatography on silica gel (Merck, thin layer grade) using the same solvent system. However, owing to the low yield obtained in the separation these compounds were not fully characterized and, therefore, their description is omitted here.

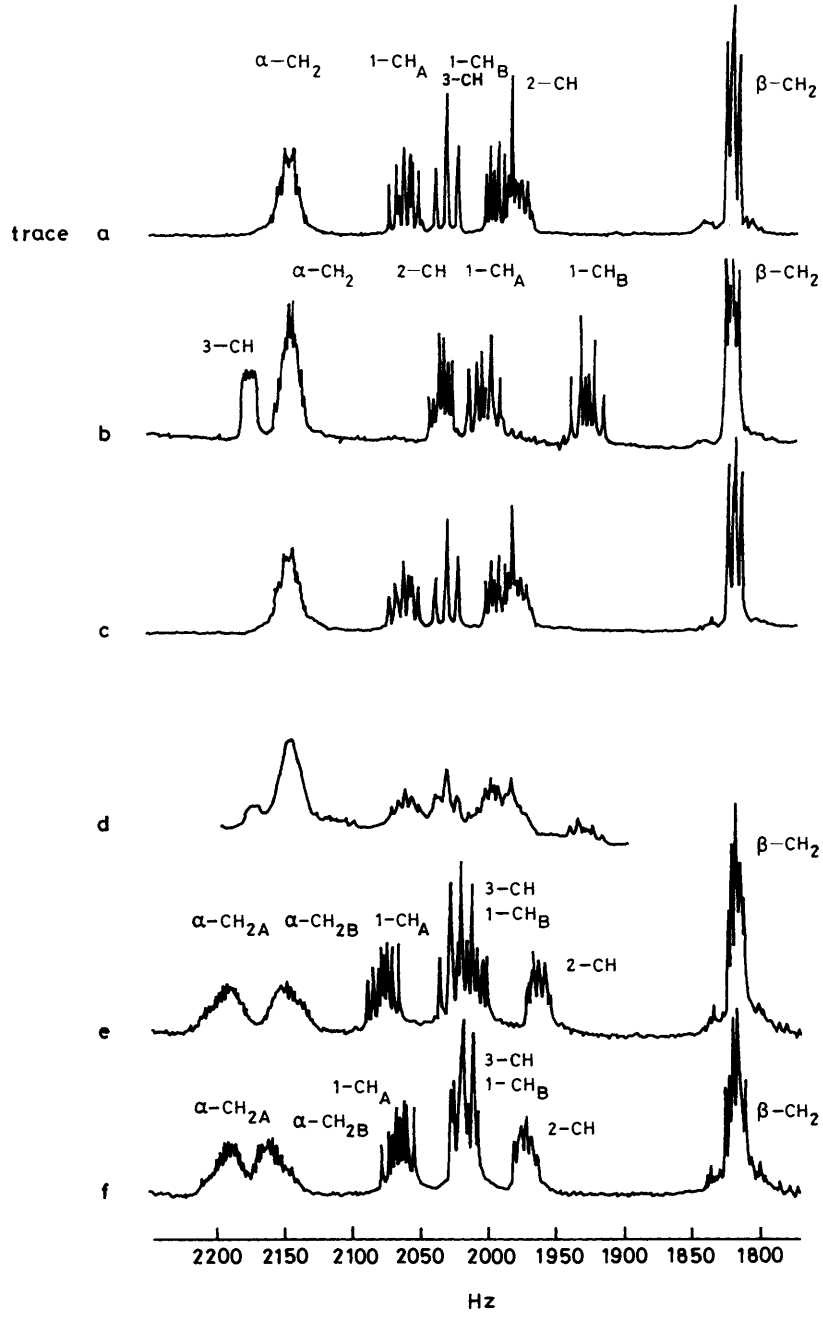


Figure 1. Partial 500 MHz ^{1}H n.m.r. spectra of sphingomyelins in [$^2H_4$]methanol solution (4 mM): trace (a) synthetic D-*erythro*-(**10**), (b) L-*threo*-(**11**), (c) bovine brain SPM, (d) semisynthetic *N*-palmitoylsphingosyl-1-phosphocholine, (e) (Rp)-SPsM (**16**), and (f) (Sp)-SPsM (**17**)

into isomeric thiosphingomyelins by a similar desilylation reaction as mentioned above. No separation of phosphorothioate diastereoisomers of opposite configurations at phosphorus was possible at the stage of compounds (**16**) and (**17**) which have a free hydroxy function, nor could a distinction be made between compounds of the D-*erythro* and L-*threo* series by applying chromatographic criteria.

Spectral Data.—^{1}H *N.m.r. spectra.* The structural assignments of all synthesized compounds were supported by high resolution ^{1}H, ^{13}C, and ^{31}P n.m.r. spectral results obtained under nonaggregating conditions. It was of particular importance to compare the ^{1}H and ^{13}C n.m.r. data for natural and synthesized sphingomyelin in order to assess the validity of the previous stereochemical assignments.[22,23] Because of recently published results on the epimerization of the sphingosine at C-3 during acidic hydrolyses of sphingolipids,[30] commercial, semisynthetic SPM produced by the deacylation–reacylation process shown in the Scheme 1 was also included in this study.*

^{1}H N.m.r. spectra for the polar entity of sphingomyelins are shown in Figure 1. Assignments of resonances to particular proton positions are based on homodecoupling experiments. As

* The commercial 2-*N*-palmitoylsphingosyl-1-phosphocholine was manufactured according to the procedure described in the ref. 7.

might be expected, the spectra of the synthetic D-*erythro*-SPM (**10**) and natural SPM (traces a and c, respectively) are virtually superimposable. The only additional peaks seen at 5.42 p.p.m. (not shown) in the spectrum of bovine brain SPM are due to a low content (*ca.* 25%) of unsaturated fatty acid. Interestingly, the inversion of configuration at C-3 of the sphingosine in L-*threo*-SPM causes a downfield shift for 3-H of 0.3 p.p.m. (trace b). This change of chemical shift is accompanied by a decrease in the vicinal coupling constant $^3J_{2,3}$ from 8.7 to 3.1 Hz (for a complete spin–spin coupling analysis see Table 1). The differences in chemical shifts for other protons are less dramatic. The coupling constants for 1-H, 2-H of D-*erythro*-(**10**) and L-*threo*-(**11**) are indicative of different conformations of these compounds with respect to the angle of rotation about the C(1)–C(2) bond.

It is evident from the spectrum of semisynthetic sphingomyelin that it comprises a 2:1 mixture of D-*erythro*- and L-*threo*-SPM (trace d).

The ^{1}H n.m.r. spectra of the phosphorothioyl analogues (**16**) and (**17**) show no major changes in coupling constants as compared with these of the parent compound (traces e, f of Figure 1 and Table 1). This is in agreement with the ^{1}H and ^{13}C n.m.r. data obtained for phosphorothioyl derivatives of glycerophospholipids.[31–33] Differences in the ^{1}H n.m.r. spectra of (Sp)-(**16**) and (Rp)-(**17**) and of D-*erythro*-(**10**) arise from (1) changes in the chemical shifts of protons vicinal to the phosphorus atom as a result of the altered electronegativity of the phosphorus atom in the former compounds; and (2) the chemical nonequivalence of the methylene protons of the choline group induced by the new chiral centre at the phosphorus atom.

Table 1. ^{1}H N.m.r. coupling constants of protons of polar group of sphingomyelins in CD_3OD

Coupling constant	D-*erythro*-(**10**)	L-*threo*-(**11**)	(Rp)-(**16**)	(Sp)-(**17**)
$^3J_{PO.\alpha_A\text{-}H}$	6.90	6.79	8.23	8.23
$^3J_{PO.\alpha_B\text{-}H}$	6.90	7.02	7.01	7.03
$^3J_{\alpha\text{-}H_A.\beta\text{-}H_B}$	7.08	7.02	7.26	7.26
$^3J_{\alpha\text{-}H_B.\beta\text{-}H_A}$	2.45	2.49	2.27	2.27
$^2J_{\alpha\text{-}H_A.\alpha\text{-}H_B}$	14.0	14.0	13.83	13.83
$^2J_{\beta\text{-}H_A.\beta\text{-}H_B}$	14.0	14.0	13.88	13.88
$^3J_{PO.1\text{-}H_A}$	6.53	6.40	8.09	7.8
$^3J_{PO.1\text{-}H_B}$	5.77	6.45	6.6	7.3
$^2J_{1\text{-}H_A.1\text{-}H_B}$	10.50	10.16	10.54	10.60
$^3J_{1\text{-}H_A.2\text{-}H}$	4.61	6.40	4.46	4.93
$^3J_{1\text{-}H_B.2\text{-}H}$	3.17	6.45	3.22	3.15
$^3J_{2\text{-}H.3\text{-}H}$	8.0	3.07	8.15	7.95
$^3J_{3\text{-}H.4\text{-}H}$	7.97	6.08	7.60	7.55
$^3J_{4\text{-}H.5\text{-}H}$	15.32	15.42	15.3	15.2
$^4J_{4\text{-}H.6\text{-}H}$	1.4	1.34	1.4	1.4
$^4J_{3\text{-}H.5\text{-}H}$	0.5	1.22	0.7	<1
$^3J_{^{14}N.\alpha\text{-}H_A}$	2.63	2.50	2.59	2.66
$^3J_{^{14}N.\beta\text{-}H_B}$	2.75	2.50	2.87	2.92

[a] All couplings were calculated from the 500 MHz ^{1}H n.m.r. spectra taken at 300 K in CD_3OD at a 4 mM concentration of sphingomyelin.

^{13}C *N.m.r. Spectra.*—^{13}C N.m.r. spectra obtained for synthetic and natural sphingomyelins are summarized in Table 2. Resonance assignment in these spectra was based on the proton–carbon coupling pattern and on comparison of the spectral data obtained with those for lecithins.[34,35] Synthetic D-*erythro*-(**10**) had a spectrum virtually identical with that of bovine brain sphinomyelin except for minor peaks at 130.8 and 28.1 p.p.m. in that of the latter, which may be accounted for by the presence of small amounts of unsaturated fatty acids. Major differences in the spectra of SPMs (**10**) and (**11**) are observed in the chemical shifts of the carbonyl carbon (0.5 p.p.m.) and carbon atoms C-5 (0.58 p.p.m.), C-4 (1.53 p.p.m.), C-3 (1.65 p.p.m.), and C-1 (0.75 p.p.m.) of the sphingosine backbone. Chemical shift differences for C-4 and C-5 [δ(C-4) − δ(C-5)] = 5 p.p.m. have recently been proposed as an indication of the *erythro* configuration of sphingolipid, while $\Delta\delta$ = 4.0 p.p.m. indicates the *threo* configuration.[30b] These results, however, were obtained for solutions of sphingolipids in chloroform–methanol (2:1 or 4:1). In the present work it is also found that for D-*erythro*-(Rp)-(**16**) and D-*erythro*-(Sp)-(**17**) in chloroform solution $\Delta\delta$ = 4.8 p.p.m. This parameter is clearly lower for sphingomyelins in methanol solution but the relationship $\Delta\delta_{4,5\text{-}erythro} > \Delta\delta_{4,5\text{-}threo}$ still holds.

^{31}P *N.m.r. Spectra.*—Under nonaggregating conditions the ^{31}P n.m.r. spectra of phospholipids offer the simplest means of differentiating between the diastereoisomers of opposite configurations at phosphorus. Indeed, chemical shifts of (Rp)-(**16**) and (Sp)-(**17**) samples in $CDCl_3$ are 57.3 and 57.1 p.p.m.; respectively. The dispersion of diastereoisomers of SPsM in

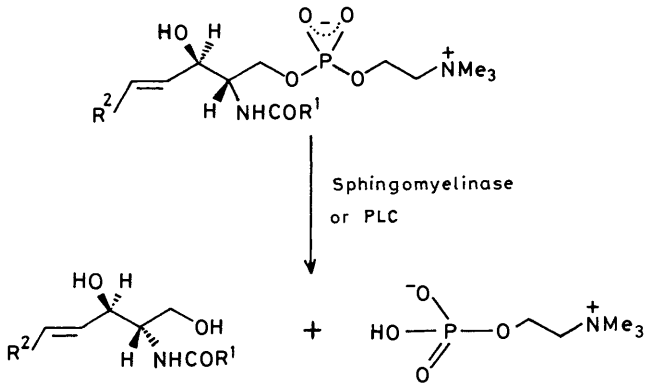
Table 2. ^{13}C N.m.r. data for synthetic sphingomyelins

Carbon atom (function)	D-*erythro*-(**10**)[a,c]	L-*threo*-(**11**)[b,d]	D-*erythro*-(**16**)[b,c]	D-*erythro*-(**17**)[b,c]
1′(C=O)	175.86	176.35	175.86	175.74
4(HC=)	135.1	133.57	135.00	134.96
5(=CH)	131.3	130.72	131.2	131.08
3CH(OH)	72.57	70.92	72.67	72.71
($CH_2N^+Me_3$)	67.5 (8.0)	67.5 (7.1)	67.38 (8.3)	67.33 (8.1)
(CH_2OP)	60.42 (5.0)	60.43 (5.0)	60.79 (4.7)	60.75 (4.7)
1(CH_2OP)	65.88 (5.7)	65.13 (5.6)	66.12 (6.1)	64.05 (6.0)
2(CHNH)	55.3 (7.3)	55.23 (8.0)	55.19 (7.5)	55.10 (7.7)
6(CH_2CH=)	33.5	33.44	33.46	33.42
Me_3N^+	54.7 (3.8)	54.72 (3.7)	54.9 (3.8)	54.84 (3.5)
2′(CH_2C=O)	37.4	37.26	37.47	37.42
3′(CH_2)	27.2	27.72	27.14	27.10
16′,16(CH_2)	33.1 (33.08)	33.07	33.07	33.02
17′,17(CH_2)	23.75 (23.73)	23.72	23.72	23.67
18′,18(CH_3)	14.47 (14.46)	14.43	14.45	14.47
4′—15′,7—15(CH_2)	30.9—30.4	30.79—30.4	30.86—30.45	30.76—30.42

[a] Obtained with Bruker AM-500 at 125 MHz. [b] Obtained with Bruker CXP-300 at 75 MHz. [c] CD_3OD solution at 0.05M concentration of SPM. [d] 0.01M solution in CD_3OD.

water–deoxycholate shows two ill-resolved peaks at 56.7 and 56.4 p.p.m. corresponding to (Sp)-(**17**) and (Rp)-(**16**), respectively. Parallelling the behaviour of the phosphorothioate analogues of lecithin,[32] transfer of the mixture of the diastereoisomers (**16**) and (**17**) from chloroform to aqueous medium (the change from reverse to normal micelles) causes the reversal of the diastereoisomer peak positions in the ^{31}P n.m.r. spectra of these compounds. Also, the chemical shifts of the D-*erythro*-(**10**) and L-*threo*-(**11**) in methanol solution are 0.18 p.p.m. apart (1.61 and 1.43 p.p.m., respectively). The spectrum of semisynthetic SPM (Figure 2) consisting of two resonances at the above positions in a ratio of 2:1 is in complete agreement with the ^1H n.m.r. results (trace d, Figure 1).

Enzymatic Hydrolysis.—There are two kinds of phosphodiesterases capable of hydrolysing the phosphodiester bond of sphingomyelin: sphingomyelinase (SMase) and phospholipase C (PLC). Both enzymes cleave the phosphorus–oxygen bond producing, thereby, phosphocholine and ceramide (Scheme 4).



Scheme 4.

The activity of sphingomyelinase is generally much lower than that of PLC and with staphylococcal enzyme only the natural substrate was found to undergo hydrolysis. No reaction was observed when the analogues (**16**) and (**17**) were used as substrates.

Bacterial phospholipases C were found to degrade stereospecifically the (Sp)-diastereoisomers of phosphorothioate analogues of phospholipids derived from L-glycerol 3-phosphate[32] (Scheme 5). Two previously tested enzymes[32] were

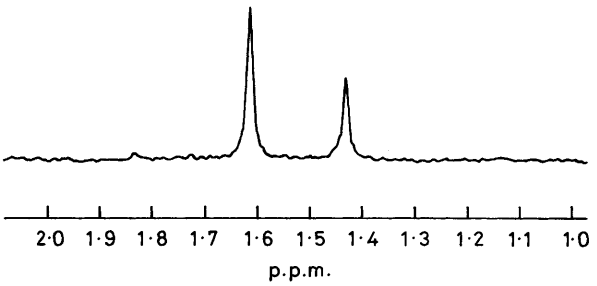
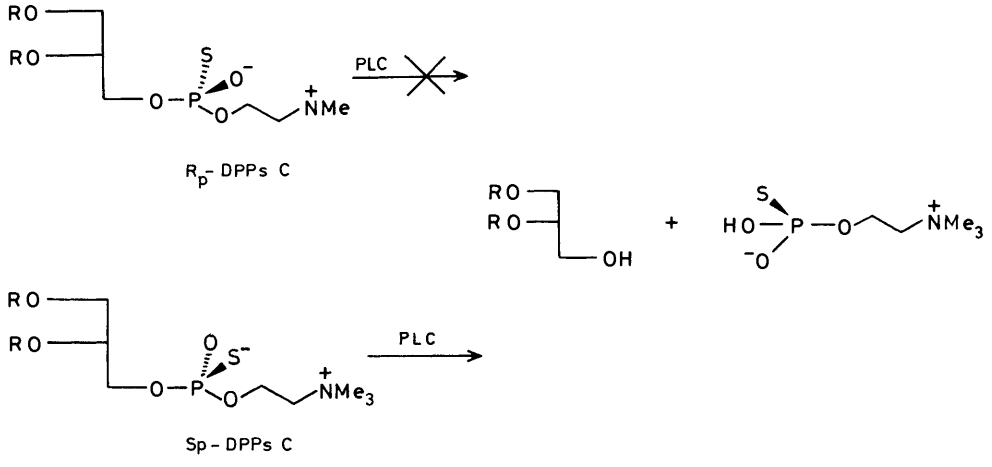


Figure 2. 202 MHz ^{31}P N.m.r. spectrum of semisynthetic 2-*N*-palmitoyl-sphingosyl-1-phosphocholine, 1 mg in 2 ml of CD_3OD, 1 600 transients

also used in this work to determine the absolute configurations of the synthesized thiosphingomyelin isomers. In addition to PLC from *Clostridium perfringens* and Zn^{2+} PLC from *Bacillus cereus*, Co^{2+} PLC from the same source was used. The activity of each enzyme towards diastereoisomers of SPsM (**16**) and (**17**) was assayed by t.l.c. monitoring the formation of *N*-stearoylsphingosine. The results are, therefore, only qualitative but they allow the tentative assignment of the configuration of SPsM isomers at the phosphorus atom. The reactions were carried out with thiosphingomyelin substrates as comicelles using detergents such as SDC or Triton X-100 for solubilization of the phospholipid. In the case of PLC from *Bacillus cereus* it was reported that only the enzyme in which Co^{2+} ion was substituted for Zn^{2+} ion is active against sphingomyelin.[36] The new enzyme thus obtained (Co^{2+} PLC) was checked for possible reversal of stereospecificity using the diastereoisomeric mixture of DPPsC as a substrate. The stereospecificity of PLC was not altered by metal-ion exchange since Co^{2+}-PLC readily hydrolysed, a (Rp + Sp)-DPPsC mixture while no reaction could be detected under the same conditions with (Sp)-DPPsC alone. The product of the enzyme-catalyzed hydrolysis of thiosphingomyelins-stearoylsphingosine was isolated by chromatography and its structure was established by t.l.c. and ^1H n.m.r. spectroscopy. All three PLCs studied were active against the diastereoisomer of SPsM which was obtained from the 3-*O*-silylated derivative (**15**) characterized by lower mobility on a silica gel chromatographic column (designated 'slow', Scheme 3). No hydrolysis was detected when the second isomer of SPsM obtained from diastereoisomer (**14**) (designated as 'fast') was incubated with PLCs. All enzymes showed much higher activity with either bovine brain sphingomyelin or synthetic D-*erythro*-(**10**).



Scheme 5.

Discussion

The aim of the present study was to provide a method for the synthesis of chemically and stereochemically homogeneous sphingomyelin and its analogues suitable for biophysical studies of phospholipid bilayers. The vast majority of studies concerning thermal phase behaviour, *X*-ray diffraction, and ^{31}P n.m.r. of sphingomyelin bilayers, sphingomyelin–lecithin, and sphingomyelin–cholesterol interactions utilized synthetic (±)-*erythro*-SPM[8,9,37–39] or semisynthetic D-*erythro*-SPM.[6,7] Because the biophysical characteristics of lipids are, to a large extent, affected by the presence of foreign molecular species it is advisable to use homogeneous lipid samples to establish the reference behaviour of the given phospholipid.

Although it has been demonstrated that in the case of glycerophospholipids the stereochemistry of these molecules has only a minor effect on the results of biophysical experiments,[40,41] the case of sphingomyelin is clearly different. Its aggregation involves interactions of molecules having two chiral centres bearing functions capable of hydrogen bonding,[3] the efficiency of which is more likely to be dependent upon stereochemistry than pure hydrophobic interactions. In biophysical studies of sphingomyelin bilayers, therefore, it is necessary to establish the reference behaviour of aggregates composed of sphingomyelin of well-defined structure. Our recent results of differential scanning calorimetry of sphingomyelin dispersions[42] clearly show that it is essential that the model compounds studied are stereochemically pure.

The preparation of the semisynthetic sphingomyelin by deacylation of natural SPM under acidic conditions followed by reacylation of the thus formed sphingosylphosphocholine leads to a product the sphingosine of which is substantially epimerized at C-3. In spite of the results of Cohen *et al.*,[7] who reported a stereospecific semisynthesis of sphingomyelins, the problem of the stereospecificity of the hydrolysis step still remains unsolved as shown recently.[30] The highly acidic conditions of *N*-acyl bond cleavage are likely to cause epimerization at the configurationally labile allylic carbon bearing the hydroxy group. The epimerization at C-3 *via* a carbonium ion pathway can be also facilitated by the neighbouring group participation of the amino or acylamino function at the adjacent C-2 position. The extent to which this process occurs during acidolysis varies in the range 20—70% depending upon the conditions of the deacylation reactions.[30] Since chromatographic separation of D-*erythro*-(**10**) from the corresponding L-*threo*-(**11**) proved difficult, the semisynthetic route to homogeneous sphingomyelin preparations is of questionable value.

The synthetic method presented in this report affords D-*erythro*-SPM of high chemical and stereochemical purity. It allows also for the preparation of numerous sphingomyelin analogues which may be useful in studying stereochemical aspects of phospholipid aggregation.*

The entire synthesis using phosphoroamidite reagent is carried out under mild conditions and affords product in high yield. Because of the cleanliness of consecutive steps of the synthesis a product of high purity is obtained after only single column chromatography on silica gel.

^1H N.m.r. spectra obtained under nonaggregating conditions allowed the identification of the synthesized compounds. Because of major differences in the chemical shifts of 3-H and 2-H D-*erythro*-SPM and L-*threo*-SPM can be easily distinguished.

Preparation of pure sphingomyelin and its analogues affords model compounds which allow a study of both stereochemical effects within the phospholipid bilayer and some aspects of lipid–lipid and lipid–protein interactions. The number of enzymes known to interact specifically with sphingomyelin can be now studied for possible sites of these interactions and specificity with respect to substrate stereochemistry. In the light of preferential SPM–cholesterol interactions it would be particularly interesting to use the synthesized analogues for the investigations of stereochemical effects in the interactions of these lipids.

* The oxidation of the phosphite (**7**) with H_2O/iodine/pyridine or addition of elemental selenium makes it possible to prepare phospholipids containing ^{17}O or ^{77}Se.

Experimental

All organic solvents used for the synthesis were of analytical grade and were stored in ampoules over appropriate drying reagents. Phosphitylation, coupling, and oxidation were carried out using a vacuum line technique. The following chemicals were purchased from Sigma: pentadec-1-ene, stearic acid (>99%), bovine brain sphingomyelin, and semisynthetic *N*-palmitoylsphingosyl phosphocholine. Di-isobutylaluminium hydride, tetrabutylammonium fluoride, and tetrazole were obtained from Aldrich. Chloro-*N*,*N*-di-isopropylaminomethoxyphosphine was synthesized from phosphorus trichloride by a literature method.[43] Phospholipases C from *Bacillus cereus* and from *Clostridium perfringens* were obtained from Calbiochem. Sphingomyelinase from *Staphylococcus aureus* was purchased from Boehringer. All enzymes were used without further purification.

EM silica gel 60 F254 and h.p.t.l.c. plates on aluminium support were used to follow the progress of reactions and to determine the purity of the products. Silica gel plates were visualized with a 10% ethanolic solution of phosphomolybdic acid.

^1H N.m.r. spectra were recorded on Bruker AM-500 and Bruker FX-90 spectrometers. ^{13}C N.m.r. spectra were taken with Bruker AM-500, Bruker CXP-300, and Bruker FX-90 instruments. Chemical shifts reported are given in p.p.m. relative to internal $SiMe_4$. ^{31}P N.m.r. spectra were obtained with Bruker AM-500 and Bruker WP-200 machines operating at 202 and 81 MHz, respectively. Chemical shifts are referenced to external 85% aqueous H_3PO_4 as a standard.

Pentadec-1-yne was obtained from pentadec-1-ene by the addition of t-butyl hypochlorite in ethanol solution followed by the elimination of the resulting 1-chloro-2-ethoxypentadecane with a five-fold excess of butyl-lithium.[44] *p*-Nitrophenyl stearate used for the acylation of 1-benzoylsphingosines was obtained from stearic acid that was at least 99% pure by its condensation with *p*-nitrophenol in the presence of DCC.

D-*erythro*-2-*N*-Stearoylsphingosine. D-*erythro*-(**5**), and L-*threo*-2-*N*-stearoylsphingosine, L-*threo*-(**5**), were obtained according to the procedure described in the ref. 22 with the following modification. Methyl 2-phenyl-4,5-dihydro-oxazole-4-carboxylate was reduced to the corresponding 4-carbaldehyde (**2**) (Scheme 2) with DIBAL in ether solution at −77 °C. After the reaction was judged complete by t.l.c. ($CHCl_3$–MeOH; 20:1) tridecylvinylalane (**3**) in hexane was added to the reaction mixture at −15 °C. The resulting solution was stirred for 0.5 h at room temperature and then worked-up as described in the literature.[22] This procedure allows omission of the step whereby the aluminium complexes resulting from reduction of the methoxycarbonyl derivatives are decomposed and an unstable aldehyde (**2**) isolated. The yield of the protected derivative (**4**) was unaffected by this. D-*erythro*-(**5**) (Found: C, 78.1; H, 11.1; N, 1.8. $C_{52}H_{89}NO_3Si$ requires C, 78.0; H, 11.1; N, 1.5%), $[\alpha]_D^{20}$ −15.48° (*c* 2.6 in $CHCl_3$); L-*threo*-(**5**) (Found: C, 78.2; H, 11.0; N, 1.75. $C_{52}H_{89}NO_3Si$ requires C, 78.0; H, 11.1; N, 1.55%), $[\alpha]_D^{20}$ +14.6 ° (*c* 4.4 in $CHCl_3$).

Synthesis of Sphingomyelins.—Into a solution of D-*erythro*-(**5**) (1.104 g, 1.37 mmol) and triethylamine (2.7 mmol, 370 µl) in

chloroform (5 ml) was added with a syringe chloro-*N,N*-diisopropylaminomethoxyphosphine (1.64 mmol, 324 mg, 20% molar excess) at room temperature. The mixture was set aside for 0.5 h and the progress of the reaction was checked by t.l.c. (ether–hexane; 1:1). No starting ceramide (**5**) could be detected. The mixture was concentrated to dryness under vacuum and solid tetrazole (350 mg, 4.92 mmol, 3-fold excess) and choline tosylate (1.01 g, 4 mmol) were placed in the reaction flask. All solids were dissolved in acetonitrile–THF (1:1 v/v; 10 ml). The solution was stored for 3 h at room temperature and t.l.c. analysis showed completion of the reaction. The mixture was again evaporated to dryness under reduced pressure and the residue was dissolved in THF and added to t-butyl hydroperoxide (80% solution in Bu^{t}OH; 170 mg, 10% excess). The reaction mixture was then stored for 2 h after which ethyl acetate (10 ml) was added and the resulting solution washed with triethylammonium hydrogen carbonate buffer (1M; pH 7.5) to remove excess of tetrazole and choline tosylate. The organic phase was concentrated to dryness and the residue was rendered anhydrous by repeated evaporation with dry toluene. Finally, the toluene solution (10 ml) was treated with anhydrous trimethylamine (3 ml) for 12 h at room temperature. After this period of time, the demethylation was complete as judged by t.l.c. showing only one spot for the product (**9**) [(R_F 0.44, methanol–chloroform–water (40:60:2)]. Finally, crude product (**9**) was dissolved in THF (5 ml) and treated with tetrabutylammonium fluoride (520 mg, 2 mmol) at ambient temperature. The deprotection reaction proved complete in 48 h. The crude product was transferred into a centrifuge bottle and treated with acetone. The resulting precipitate was centrifuged off and the supernatant containing very little of sphingomyelin was discarded. Crude sphingomyelin was purified by column chromatography on silica gel (Merck, 40—63 μm) using chloroform–methanol–water (65:35:4) as eluant yielding chromatographically pure D-*erythro*-(**10**) (700 mg, 70% from ceramide); R_F 0.2 [chloroform–methanol–water (65:35:4)]; δ_P 1.62 (CD_3OD) and 1.49 ($CDCl_3$); δ_C values are given in Table 2; δ_H (500 MHz; 4 mM in CD_3OD, see Table 1 for coupling pattern) 5.702 (1 H, dtd, 5-H), 5.449 (1 H, ddt, 4-H), 4.27 (2 H, m, α-H), 4.106 (1 H, ddd, 1A-H), 4.046 (1 H, t, 3-H), 3.969 (1 H, ddd, 1B-H), 3.935 (1 H, m, 2-H), 3.625 (2 H, m, β-H), 3.21 (9 H, br s, N–Me), 2.180 (2 H, m, 2′-H), 2.027 (2 H, br q, 6-H), 1.58 (2 H, br sx, 3′-H), 1.378 (br sx, 4′-H), 1.288 (48 H, br s, 5′-H to 17′-H, 7-H to 17-H), and 0.898 (6 H, t, 18-H, 18′-H).

Compound L-*threo*-(**11**) was synthesized in a similar fashion to compound D-*erythro*-(**10**) as described above. Starting from L-*threo*-(**5**) (400 mg) the final product was obtained in 71% (260 mg) yield; R_F 0.2 (chloroform–methanol–water (65:35:4); δ_P 1.43 (CD_3OD) and 1.40 ($CDCl_3$); δ_C values are collected in Table 2; δ_H (500 MHz; 4 mM in CD_3OD) 5.726 (1 H, dtd, 5-H), 5.452 (1 H, ddt, 4-H), 4.333 (1 H, ddd, 3-H), 4.272 (2 H, m, α-H), 4.052 (1 H, dt, 2-H), 3.991 (1 H, dt, 1-H), 3.843 (1 H, dt, 1B-H), 3.640 (2 H, m, β-H), 3.23 (9 H, br s, NMe), 2.22 (2 H, m, 2′-H), 2.033 (2 H, dt, 6-H), 1.599 (2 H, br sx, 3′-H), 1.376 (2 H, br sx, 4′-H), 1.288 (46 H, br s, 5′-H to 17′-H, 5-H to 17-H), and 0.898 (6 H, t, 18-H, 18′-H).

D-erythro-2-N-*Stearoylsphingosyl*-1-*thiophosphocholines*.—The diastereoisomeric mixture (**13**) was prepared as described for D-*erythro*-(**10**) with the following modification.

Synthesis of mixture of diastereoisomers (**13**). The phosphite (**7**) (1.37 mmol) was dissolved in benzene (5 ml) and treated with an excess of anhydrous elemental sulphur. The slurry was stirred at room temperature overnight, and the resulting mixture was worked-up as described above. The crude phosphorothioate (**12**) was dissolved in toluene (10 ml) and the anhydrous trimethylamine (3 ml) was added. After the reaction mixture have been kept in the refrigerator for 12 h the demethylation was completed. Crude product was chromatographed on silica gel [(chloroform–methanol (1:1)] yielding a 1:1 mixture of diastereoisomers (**13**) (1.0 g, 74%).

Separation of diastereoisomers. The above mixture (200—300 mg) was separated by column chromatography on a silica gel (2.5 × 30 cm column, Merck Kieselgel HR for t.l.c.). The column was eluted with acetone–methanol–water (80:20:1.5). Elution of diastereoisomers from the column required *ca.* 400 ml of the solvent: D-*erythro*-(**14**) (fast): R_F 0.27 in the above solvent, δ_P 57.8 ($CDCl_3$); D-*erythro*-(**15**) (slow): R_F 0.14 in the above solvent, δ_P 57.45 ($CDCl_3$).

Deprotection of the Diastereoisomers (**14**) *and* (**15**).—Either one of the diastereoisomers (**14**) or (**15**) (0.32 g, 325 μmol) was dissolved in THF and Bu_4NF (hydrate, 201 mg, 0.64 mmol) was added. The reaction was complete after 12 h at room temperature [t.l.c. chloroform–methanol (1:1), substrate R_F 0.34, product R_F 0.13]. The product was precipitated with acetone and centrifuged off. The pellet was chromatographed on silica gel using acetone–methanol–water (80:20:1.5).

D-*erythro*-(**16**) (fast): R_F 0.4 in chloroform–methanol–water (65:35:4); δ_P 57.3 ($CDCl_3$); $[\alpha]_D^{20}$ +9.8° (*c* 8.0, $CHCl_3$); δ_H (500 MHz, 4mM in CD_3OD): 5.699 (1 H, dt d, 5-H), 5.448 (1 H, ddt, 4-H), 4.403 (1 H, m, αA-H), 4.329 (1 H, m, αB-H), 4.162 (1 H, ddd, 1A-H), 4.032 (1 H, m, 1B-H), 4.064 (1 H, tr, 3-H), 3.933 (1 H, m, 2-H), 3.642 (2 H, m, β-H), 3.23 (9 H, br s, NMe), 2.186 (2 H, m, 2′-H), 2.026 (2 H, br q, 6-H), 1.590 (2 H, br m, 3′-H), 1.349 (br m, 4′-H), 1.288 (48 H, br s, 5′-H to 17′-H, 7-H to 17-H), 0.898 (6 H, br t, 18-H, 18′-H).

D-*erythro*-(**17**) (slow): R_F 0.4 in the above solvent; δ_P 57.1 ($CDCl_3$); $[\alpha]_D^{20}$ +3.92° (*c* 3.6, $CHCl_3$); δ_H (500 MHz, 4 mM in CD_3OD): 5.700 (1 H, dtd, 5-H), 5.452 (1 H, ddt, 4-H), 4.400 (1 H, m, αA-H), 4.330 (1 H, m, αB-H), 4.133 (1 H, ddd, 1A-H), 4.043 (1 H, m, 1B-H), 4.036 (1 H, tr, 3-H), 3.947 (1 H, m, 2-H), 3.641 (2 H, m, β-H), 3.228 (9 H, br s, NMe), 2.179 (2 H, m, 2′-H), 2.027 (2 H, br q, 6-H), 1.582 (2 H, br m, 3′-H), 1.378 (2 H, m, 4′-H), 1.288 (48 H, br s, 5′-H to 17′-H, 7-H to 17-H), and 0.898 (6 H, tr, 18-H, 18′-H).

Hydrolysis of Sphingomyelins with PLCs.—Sphingomyelin or its analogue (**16**) or (**17**) (1 mg) was dispersed in HEPES buffer (0.05M, pH 8.0, 0.4 ml) containing sodium deoxycholate (10 mM) in a 2 ml vial and the vial was shaken until an homogeneous, slightly opaque dispersion was obtained. The dispersion was treated with 10μl of PLC suspension containing *ca.* 10—50 μg of enzyme. The vial was then incubated at 37 °C, and 0.2 ml of Dole reagent was added. The upper hexane phase (10 μl) was used for ceramide detection. T.l.c. plates were developed in chloroform–methanol (14:1) and compared against a blank experiment in which no enzyme was used. The organic phases from several experiments were collected and concentrated. The residue was chromatographed on small silica gel column using the above solvent system for eleution giving 2-*N*-stearoylsphingosine: δ_H (500 MHz, CD_3OD) 5.692 (1 H, dtd, 5-H), $^3J_{5,4}$ 15.3 Hz, $^3J_{6,5}$ 5.5 Hz, 5.46 (1 H, ddt, 4-H), $^3J_{4,3}$ 7.5 Hz, $^4J_{6,4}$ 1.2 Hz, 4.039 (1 H, t, 3-H), $^3J_{3,2}$ 7.5 Hz, 3.85 (1 H, dt, 2-H), $^3J_{2,1}$ 5.1 Hz, 3.688 (2 H, d, 1-H), and 2.034 (2 H, br q, 6-H).

Acknowledgements

This work was supported by the Polish Academy of Sciences and partially by the National Institute of Health. 500 MHz and 300 MHz n.m.r. was performed mainly at the Campus Chemical Instrumentation Center of The Ohio State University. The author of this work is grateful to Dr. B. Uznanski of this Laboratory for sharing his ideas and to Prof. M.-D. Tsai for his generous support of the n.m.r. part of this work.

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Received 24*th November* 1986; *Paper* 6/2246