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SYNTHETIC STUDIES ON SPHINGOLIPIDS. XII.* SYNTHESIS OF SPHINGOSINEPHOSPHORYLCHOLINE.**

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We report a synthesis of sphingosinephosphorylcholine, which involves a mild alkaline hydrolysis of its N-trifluoroacetyl derivative. Sphingosinephosphorylcholine has been converted into N-stearoylsphingomyelin in good yield by treatment with p-nitrophenyl stearate. It is suggested that the product with an N:P ratio of 1:1, isolated by Dawson from alkaline hydrolyzates of sphingomyelin, is sphingosylvinylphosphate formed as a result of a Hoffmann type degradation.

Sphingomyelin is hydrolyzed by strong acid or alkali to the four components: sphingosine, fatty acid, phosphoric acid and choline. Under milder conditions partial hydrolysis takes place, resulting into a mixture of esters which can be separated on a preparative scale only with great difficulty. Rennkamp[1]) first isolated sphingosinephosphorylcholine as its chloride *via* the picrate by hydrolyzing sphingomyelin with dilute methanolic hydrogen chloride. Recently, Kaller[2]) obtained the chloride in good yield by using 3N aqueous-butanolic hydrochloric acid. However, although the product was chromatographically pure, it gave analytical values for carbon and chlorine which considerably deviated from the theoretical.

Because of the difficulty in controlling the hydrolysis of natural sphingomyelin, a synthesis of pure sphingosinephosphorylcholine must involve a "sphingomyelin" in which the fatty acid is replaced by an activated group capable to undergo hydrolysis under mild conditions.

An attempt was made to utilize the dichloroacetyl group which had been successfully employed for a similar purpose in the synthesis of psychosine[3]). However, when the dichloroacetyl phosphate IVa ($R' = CHCl_2$) was treated with trimethylamine, and the resulting product was debenzoylated, the amide VIa ($R' = CHCl_2$) proved to be rather stable to mild acidic or alkaline agents.

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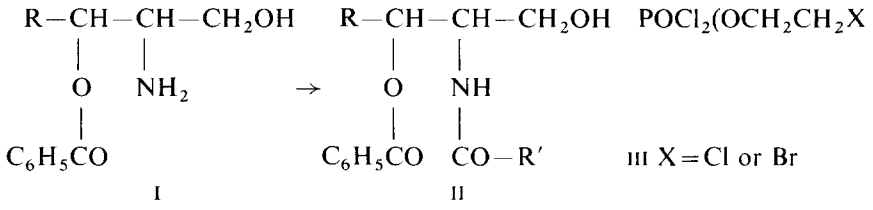
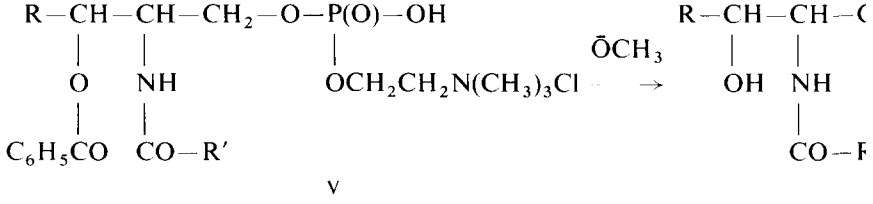
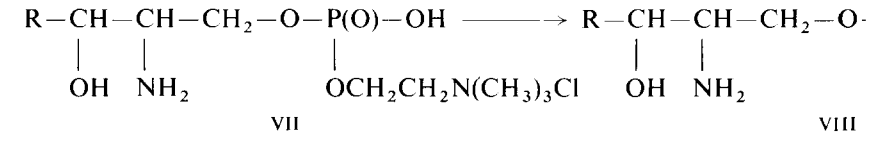
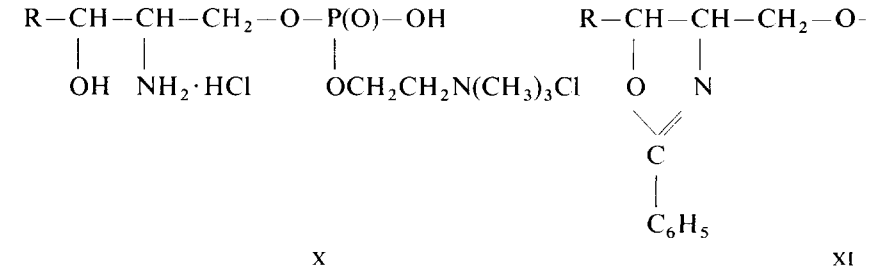


a, $R=CH_3(CH_2)_{14}$
b, $R=CH_3(CH_2)_{12}-CH=CH$







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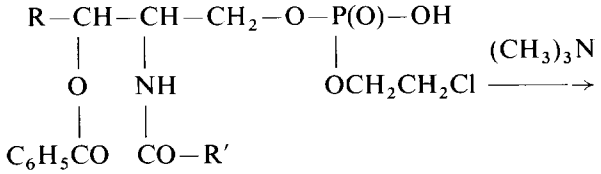
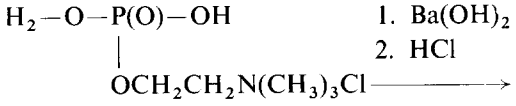


IV (Table 1)



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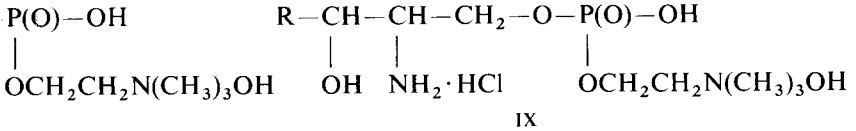
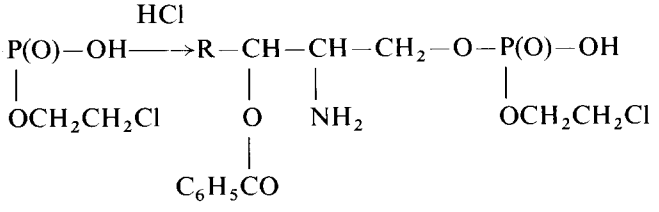


IX



XII

ne 1.

A further difficulty arose from the simultaneous partial quaternization of the dichloroacetyl group during treatment with trimethylamine (IV→V), a result which hampered the continuation of the synthesis. Applying the theoretical amount of trimethylamine to the more reactive bromoethyl derivative IVa ($R' = CHCl_2$; Br instead of Cl) did not prevent this side reaction. Likewise unsuccessful was an attempt with the benzyloxycarbonyl group, since its removal from VI ($R' = C_6H_5CH_2OCO$) by warm trifluoroacetic acid[4]) led to a simultaneous cleavage of the phosphate bond.

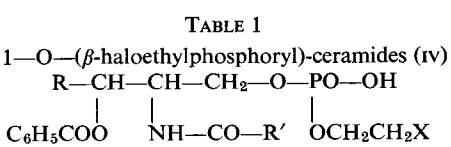
The use of the trifluoroacetyl group provided a method for a practical synthesis of sphingosinephosphorylcholine. The amido ester II (a and b, $R' = CF_3$) was phosphorylated with β-chloroethylphosphoryldichloride (III)[5]) and the resulting phosphate IV was quaternized with trimethylamine. The chromatographically pure product V was debenzoylated by the Zemplén method[6]) with barium methoxide, and the amide VI was easily hydrolyzed with 0.5 N aqueous methanolic barium hydroxide at room temperature. The crude monochloride VII obtained on acidification with hydrochloric acid could be converted without further purification into N-stearoyl-sphingomyelin by means of p-nitrophenyl stearate. The hitherto unknown sphingosinephosphorylcholine base (VIII)* was prepared by treating VII with an anion exchange resin. The analytically pure chloride VIIa was obtained directly from VIa, while VIIb was prepared by adding alcoholic hydrogen chloride to a solution of VIIIb in isopropanol, adjusting the *p*H to 5. At a lower *p*H a phosphate of higher chlorine content was obtained. On the other hand, we were unable to isolate a product with a chlorine value corresponding to the chloride hydrochloride (X). Even with a large excess of hydrochloric acid, the maximum chlorine content amounted to approximately 1.5 equivalents. This behavior must be ascribed to the tendency of X to undergo partial solvolysis. The lability of the chlorine atom of sphingomyelin chloride (VII, primary amine acylated with fatty acid) in alcoholic solution was already observed by Thudichum[7]), and quite recently Ansell and Spanner[8]) showed that the halogen can be quantitatively removed by shaking a chloroform solution with methanol–water. Kaller[2]) obtained preparations of sphingosinephosphorylcholine chloride with varying chlorine contents, probably resulting from the different degree of concentration in the two phase system applied.

The question then arose whether sphingosinephosphorylcholine monochloride should be formulated as VII or IX. To test the stability of the primary amine hydrochloride, an attempt was made to prepare the hydrochloride of XII by a ring scission of the oxazoline phosphate XI, in a manner described previously[9]). However, in contradistinction to I which forms stable salts, a

* The "sphingosinephosphorylcholine" reported in the literature refers to the monochloride.

TABLE 1

1—O—(β-haloethylphosphoryl)-ceramides (IV)



R	R′	X	m.p. (°C)	Formula	Calculated C	H	N	Cl	P	Found C	H	N	Cl	P
$CH_3(CH_2)_{14}$	$CHCl_2$	Cl	105	$C_{29}H_{47}Cl_3NO_7P$	52.88	7.20	2.13	16.14		53.0	7.76	2.48	15.85	
	$CHCl_2$	Br	118	$C_{29}H_{47}BrCl_2NO_7P$	49.54	6.73	2.0			50.0	6.90	2.34		
	CF_3	Cl	118	$C_{29}H_{46}ClF_3NO_7P$					4.81					4.82
	CF_3	Br	129	$C_{29}H_{46}BrF_3NO_7P$	50.62	6.75	2.03			51.21	7.28	2.24		
	$C_6H_5CH_2O$	Cl	85	$C_{35}H_{53}ClNO_8P$	61.61	7.83		5.19		62.06	7.44		4.88	
$CH_3(CH_2)_{12}—CH=CH$	$CHCl_2$	Cl		$C_{29}H_{45}Cl_3NO_7P$	53.01	6.90	2.13	16.18		52.57	6.97	2.52	16.20	
	CF_3	Cl		$C_{29}H_{44}ClF_3NO_7P$*	54.24	6.90	2.20		4.82	54.12	7.40	2.40		4.81
	$C_6H_5CH_2O$	Cl	102°	$C_{35}H_{51}ClNO_8P$			2.06	5.21	4.55			1.94	5.65	4.68

* The analytically pure product was obtained by elution from a silicic acid column with chloroform–methanol 9:1, after separation of the impurities with chloroform, and chloroform–methanol 30:1.

compound containing one chlorine atom was obtained which obviously was XII. Although this particular result appears to favor formula VII, we nevertheless incline to formulate the monochloride as IX on account of its carbon analysis which agreed well with the theoretical value (found: C, 53.14; $C_{23}H_{52}ClN_2O_6P$ requires C, 53.20; $C_{23}H_{50}ClN_2O_5P$ (VII) requires C, 55.10). That the sphingomyelin molecule in its solid state has the hydrated rather than the zwitterionic structure has been shown in previous work[9]).

Conflicting results have been reported in the literature concerning the alkaline hydrolysis of sphingomyelin. Rouser *et al.*[10]) identified sphingosine-phosphorylcholine chromatographically in such hydrolyzates, while Dawson[11]) was unable to confirm this result. The latter author isolated a product of resinous texture with an N:P ratio of 1:1. It was considered by the present authors that an alkaline hydrolysis of sphingomyelin provides the conditions for a Hoffmann degradation of a tetraalkyl ammonium hydroxide which should result in the liberation of trimethylamine and formation of sphingosylvinylphosphate. This was, indeed, shown to be the case. When a solution of sphingomyelin in 1 N methanolic potassium hydroxide was heated in a sealed tube at 100 °C, the hydrolyzate gave a picrate which was identified by thin layer chromatography with that of trimethylamine. The solid product isolated from the hydrolyzate on cooling could not be purified owing to its resinous nature characteristic of compounds of this type. These results suggest that Dawson's compound might be impure sphingosylvinylphosphate or its hydrochloride.

Experimental

3-O-Benzoylceramides (II). These amido esters were prepared by acylation of I as previously described[3]).

3-O-Benzoyl-N-benzyloxycarbonylsphingosine. The ceramide crystallized readily from acetonitrile; m.p. 60 °C

Calcd. for $C_{33}H_{47}NO_5$:	C 73.71 H 8.81 N 2.60
Found:	C 73.59 H 9.12 N 2.99

3-O-Benzoyl-N-trifluoroacetylsphingosine. The crude compound was purified by a column of 30 parts of alumina (Merck, acid-washed), and eluted first with benzene, then with benzene–ether (1:1). It was recrystallized from nitromethane; m.p. 75 °C.

Calcd. for $C_{27}H_{40}F_3NO_4$:	N, 2.80
Found:	N 2.79

1-O-(β-haloethylphosphoryl)-ceramides (IV). To a stirred solution of β-chloro-(or β-bromo-[12])-ethylphosphoryl dichloride (0.02 mole) in dry

chloroform (15 ml) cooled to −12 °C was added dropwise dry pyridine (0.02 mole). A solution of the appropriate 3-O-benzoylceramide (0.01 mole) in dry chloroform (15 ml) was then added during 5–10 min, and the mixture was stirred at +5 °C for 4–5 hr.

The clear reaction product was added in a thin stream to a slight excess of a vigorously stirred solution of 5% barium hydroxide containing a few drops of phenolphthalein. Stirring was continued for one hr at 10 °C, whereby the *p*H was maintained above 7 by adding more barium hydroxide, if necessary. Cold ether (100 ml) was then added slowly and stirring was continued allowing the temperature to raise during one hr to 20–23 °C. The upper layer was washed once with water, shaken several times with cold 2N hydrochloric acid and then with distilled water to a *p*H of about 4, and evaporated to dryness at 30–40 °C. The phosphates crystallized readily from ethyl acetate or acetone, except for the trifluoroacetyl derivative of sphingosine which was obtained as a thick oil and refused to crystallize. The latter phosphate was separated from the starting material by passing a chloroform solution through a silica acid column. The product eluted with chloroform-methanol (19:1, and 9:1) was shown to be pure by a thin layer chromatogram on silica gel, developed with methanol–chloroform–acetic acid–water (100:50:16:8). The saturated benzyloxycarbonyl derivative solidified under ether in the cold and melted at 84–85 °C. The yields amounted to 50–60%.

The decomposition of excess of the phosphorylating agent could also be effected by using pyridine instead of barium hydroxide. The reaction product was poured into a mixture of ice–water, pyridine (10 ml) and ether (50 ml) and stirred for 3 hr. This operation was followed by treatment with hydrochloric acid as above.

3-O-benzoyl-N-dichloroacetyl-1-O-(β-chloroethylphosphoryl)-dihydrosphingosine barium salt. The free phosphoric acid was dissolved in a few ml. of chloroform, ether was added and the solution was titrated with 5% aqueous barium hydroxide (phenolphthalein). The mixture was stirred for 30 min until no more base was consumed. The organic layer was washed once with water and evaporated in *vacuo* at 35 °C. The residue was recrystallized from methanol. m.p. 82–84 °C.

Calcd. for $(C_{29}H_{46}Cl_3NO_7P)_2Ba$: C 47.93 H 6.38 N 1.93
Found: C 48.19 H 6.52 N 1.91

Quaternization of IV*a, b* ($R' = CF_3$). A solution of the β-chloroethyl phosphate (3.7 g) in dry toluene (10 ml) was treated with trimethylamine (5 ml) as described previously[5]). After evaporation of the solvent and complete removal of the excess of trimethylamine in *vacuo*, the product was purified by a silicic acid column. Any unchanged material was removed by chloro-

form, and the quaternized product (v) was eluted with chloroform-methanol 1:1. The separation was pursued by a TLC system consisting of chloroform–methanol–acetic acid–water (100:50:16:8). The chromatographically pure product v obtained in 50–60% yield was dissolved in absolute methanol (75 ml), 1 N methanolic barium methoxide was added to a *p*H slightly above 7 (approximately 5 ml), and the solution was allowed to stand overnight at room temperature. After acidification to *p*H 1–2, the solvent was evaporated in *vacuo* without warming. The residue was dissolved in isopropanol, the inorganic salts were filtered off from the concentrated solution and the product VI precipitated by addition of acetonitrile. For hydrolysis of the acyl group the crude amide VI (1.0 g) was dissolved in a mixture of 1 N methanolic barium methoxide (15 ml), methanol (12.5 ml) and water (3.5 ml) and the slightly turbid solution was stirred overnight at ambient temperature. The mixture was acidified with methanolic hydrogen chloride to *p*H 5, filtered from the inorganic salts and the filtrate evaporated in *vacuo* at 30–40 °C to dryness*, the residue weighing 500–600 mg. After elution from a silicic acid column with methanol–chloroform (4.1) the monochloride VII crystallized from a mixture of isopropanol and acetonitrile.

Dihydrosphingosinephosphorylcholine hydrochloride (IX*a*). m.p. 205–210 °C

Calcd. for $C_{23}H_{54}ClN_2O_6P$:	C 53.03	H 10.4	Cl 6.8	N 5.37	P 5.95
Found:	C 53.47	H 10.72	Cl 6.74	N 5.57	P 5.9

Sphingosinephosphorylcholine hydrochloride (IX*b*). m.p. 205 °C.

Calcd. for $C_{23}H_{52}ClN_2O_6P$:	C 53.20	H 10.10	Cl 6.83	N 5.40	P 5.97
Found:	C 53.14	H 10.34	Cl 7.34	N 5.67	P 6.30

Dihydrosphingosinephosphorylcholine, base (VIII*a*). A solution of the hydrochloride (50 mg) in 80% methanol (10 ml) was stirred with Amberlite IRA-45 (1 g) until the filtrate was free of chlorine (Beilstein test). After evaporation, the residue crystallized from isopropanol–acetonitrile. The highly hygroscopic amorphous product melted at 190–195 °C.

Calcd. for $C_{23}H_{53}N_2O_6P$:	C 56.53	H 10.50	N 5.78	P 6.39
Found:	C 56.75	H 10.96	N 6.0	P 6.22

Sphingosinephosphorylcholine, base (VIII*b*). Prepared similarly, m.p. 190–195 °C.

Calcd. for $C_{23}H_{51}N_2O_6P$:	C 57.23	H 10.65	N 5.81	P 6.4
Found:	C 56.17	H 10.91	N 5.65	P 5.96

* It may be necessary to repeat this treatment in order to remove completely the inorganic salts.

p-Nitrophenyl stearate. Prepared from p-nitrophenol and stearic acid by refluxing the chloroform solution for 30 min in the presence of dicyclohexylcarbodiimide. The ester crystallized from methanol and a little chloroform, and melted at 71–72 °C.

Calcd. for $C_{24}H_{39}NO_4$: C 71.07 H 9.69
Found: C 70.89 H 9.84

Acylation of Sphingosinephosphorylcholine Hydrochloride (VII*b*). The crude monochloride VII (100 mg) was dissolved in ethanol (5 ml), p-nitrophenyl stearate (100 mg) and 5% sodium hydrogen carbonate solution (0.5 ml) were added, and the mixture was refluxed for 2 hr. The yellow solution was acidified with dilute hydrochloric acid to *p*H 2 and cooled. The precipitate was filtered and washed with cold 80% methanol. To remove the chlorine from the sphingomyelin chloride formed, the product was dissolved in 90% ethanol (5 ml), and to the solution were added a few drops of a 10% alcoholic triethylamine to *p*H 7.5. After cooling, the precipitate was dried and recrystallized from ethyl acetate and a few drops of methanol. The sphingomyelin thus obtained was identical in every respect with an authentic sample. Yield 90 mg (60%).

References

1) F. Rennkamp, Z. Physiol. Chem. **287** (1949) 215
2) H. Kaller, Biochem. Z. **334** (1961) 451
3) D. Shapiro, E. S. Rachaman and T. Sheradsky, J. Am. Chem. Soc. **86**, (1964) 4472
4) F. Weygand and W. Steglich, Z. Naturforsch. **14b** (159) 472
5) D. Shapiro, H. M. Flowers and S. Spector-Shefer, J. Am. Chem. Soc. **81** (1959) 3743
6) G. Zemplén, J. Am. Chem. Soc. **52** (1930) 2568
7) J. L. W. Thudichum, In: F. Pietzcker, ed., Die chemische Konstition des Gehirns des Menschen und der Tiere, Tübingen, 1901, p. 169
8) G. B. Ansell and S. Spanner, Biochem. J. **79** (1961) 176
9) D. Shapiro, H. M. Flowers and S. Spector-Shefer, J. Am. Chem. Soc. **81** (1959) 4360
10) G. Rouser, J. F. Berry, G. Marinetti and F. Stotz, J. Am. Chem. Soc. **75** (1953) 310
11) R. M. C. Dawson, Biochem. J. **68** (1958) 357
12) R. Hirt and R. Berchtold, Pharm. Acta Helv. **33** (1958) 349