Chemistry and physics of lipids 13 (1974) 109–116

SYNTHESIS OF D,L-ERYTHRO-SPHINGOMYELINS

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Received December 19, 1973
Accepted March 22, 1974

This paper describes an improved procedure for the reaction of the primary hydroxyl group of 3-*O*-benzoylceramides with β-bromoethylphosphoryldichloride and for the subsequent reaction with trimethylamine (fig. 1). Column chromatography of the resulting reaction mixtures gives sphingomyelins and the corresponding ceramides in high purity. The procedure is generally applicable for the synthesis of sphingomyelins with saturated, unsaturated, and 2-hydroxy fatty acid chains.

I. Introduction

Artificial bimolecular membranes made from defined phospholipids are useful model systems for the study of transport mechanisms and structure–function relations of lipids [1]. In the lecithin series model substances are easily available by partial synthesis, whereas defined sphingomyelins which are uniform with regard to chain length of the acyl and the long chain base must be prepared by a total synthesis.

The synthesis of dihydrosphingomyelin and sphingomyelin (fig. 2a, b) has been first described by Shapiro [2a,b, 3]. In both cases the synthesis leads after several steps to 3-*O*-benzoylceramides in which the secondary hydroxyl groups are protected. The introduction of the phosphorylcholine group is performed by the method of Hirt and Berchtold [4], which was first employed in the synthesis of lecithin. As a phosphorylating agent the authors used β-chloroethyl-phosphoryldichloride [3] or β-bromoethylphosphoryldichloride [5], the latter being more reactive in the subsequent quaternization with trimethylamin. Cleavage of the benzoyl group by alcoholysis in the presence of sodium methylate leads then to the sphingomyelins.

The main difficulties which we encountered in applying Shapiro's synthesis were the reaction of 3-*O*-benzoylceramides with β-haloethylphosphoryldichloride in the presence of pyridine and the subsequent purification of the reaction products. Our results show that the phosphorylation proceeds slowly under the reported conditions and is accompanied by side reactions. Separation of the desired phorphoric acid diesters by fractional crystallization of the barium salts is unsatisfactory or even impossible when both the long chain base and the acyl rest are unsaturated, as

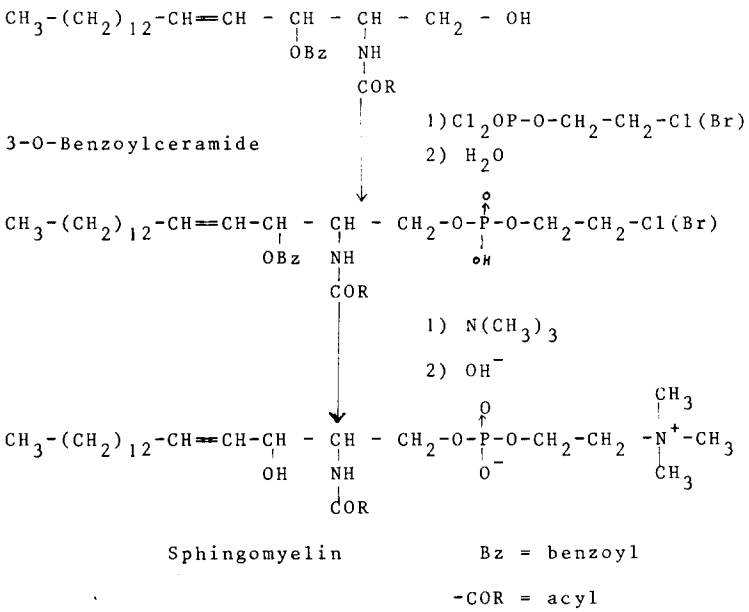

Fig. 1. Introduction of the phosphorylcholine group in 3-*O*-benzoylceramides.

this leads to a considerable increase of solubility. Thin layer chromatography of the sphingomyelins, which are obtained after reaction with trimethylamine always revealed the presence of several by-products.

In a paper on the synthesis of lecithins, Eibl [6] also reported that the phosphorylation method of Hirt and Berchtold leads to a non-uniform product. In the following it is shown that a modification of the procedure developed by Eibl can also be employed in the phosphorylation of 3-*O*-benzoylceramides.

In the procedure developed in our laboratory the 3-*O*-benzoylceramides are treated with β-bromoethylphosphoryldichloride in the presence of triethylamine. The resulting reaction mixtures are hydrolyzed and reacted with trimethylamine without preceding purification. After cleavage of the benzoyl group the resulting mixture of ceramide, sphingomyelin and by-products is separated by column chromatography.

After finishing this work we learned from a paper by Evstigneeva [7], that the yields of the phosphorylation reaction are further improved by lowering the reaction temperature and increasing the amount of the phosphorylating agent.

II. Results and comments

The described procedure is generally suitable for the synthesis of dihydrosphingo-

myelin (fig. 2a) and sphingomyelin (fig. 2b) with different acyl chains. By introduction of 2-acetoxypalmitic acid in 3-*O*-benzoylceramide it is possible to prepare a sphingomyelin with a 2-hydroxy fatty acid in the acyl rest (fig. 2d). The protecting acetyl group in position 2 of the acyl rest is removed in the last reaction step together with the benzoyl group. By introduction of a trans-2-hexadecenoic acid a dihydrosphingomyelin (fig. 2c) with a double bond in position 2, 3 of the acyl rest was prepared. The distance of this double bond from the polar head is comparable with that of the more common sphingomyelin (fig. 2b), which contains a trans double bond in position 4, 5 of the long chain base. Compound c is possibly of interest as a model substance, because it is more readily available than compound b which contains the sensitive allylic system.

The infrared spectra of the present compounds (fig. 3) show the typical absorptions of sphingomyelin which were reported earlier [2b]. In spectrum 3 the carbonyl absorption at 6.08 μ is split into two peaks which is probably due to the double bond in the α-position to the carbonyl group.

Of particular interest for the preparation of artificial membranes is the solubility of the lipids in organic solvents. We found considerable differences between lecithins and sphingomyelins. At 20°C dipalmitoyl and distearoyl lecithin are fairly soluble in many polar and non-polar solvents. Palmitoyldihydrosphingomyelin (fig. 2a) which contains comparable chain lengths of the hydrophobic rests is practically insoluble in chloroform and methanol at this temperature. It is only soluble in polar mixtures such as chloroform–methanol–water. The introduction of a double bond in the neighborhood of the polar head has a considerable effect on solubility. Sphingomyelin with the allylic system in the long chain base (fig. 2b), and dihydrosphingomyelin with a double bond in position 2 of the acyl rest (fig. 2c), are fairly soluble in methanol and chloroform but rather insoluble in non-polar solvents. In decane which is often employed as a solvent for the preparation of membranes, the sphingomyelins readily form a gel.

Since the molecular structures of lecithin and sphingomyelin both contain the phosphorylcholine end-group and are rather similar with regard to the hydrophobic chains, probably the middle part of the molecule is responsible for the different solubility. Both the hydroxyl and the amide group are able to participate in hydrogen bonding which may lead to a cross-linking of the molecules. The presence of strong hydrogen bonds is also demonstrated by the infrared spectrum [2b].

III. Methods and materials

D,L-3-*O*-benzoylceramides were prepared by the method of Shapiro [3]. Myristaldehyde was purchased as a 50% solution in dioctylphthalate from Merck-Schuchardt, Darmstadt, Germany. Fatty acid chlorides were purchased from Fluka AG, Buchs, Switzerland. β-Bromoethylphosphoryldichloride was prepared by the method of Renshaw and Hopkins [8].

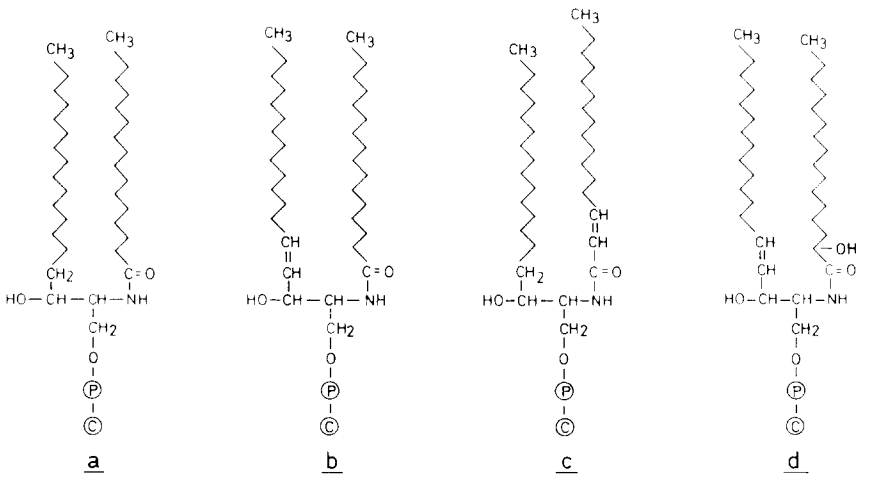

Fig. 2. Structure of sphingomyelins; –Ⓟ–Ⓒ stands for phosphorylcholine. a = N–(Palmitoyl)-dihydrosphingomyelin; b = N-(palmitoyl)-sphingomyelin; c = N-(*trans*-2-hexadecenoyl)-dihydro-sphingomyelin; d = N-(2-hydroxypalmitoyl)-sphingomyelin.

D,L-2-hydrosypalmitic acid was purchased from Roth, Karlsruhe, Germany. Analytical solvents were used for all operations. Column chromatography was performed with Silicar CC 7, 100–200 mesh, from Mallinckrodt, St. Louis, USA. Thin-layer chromatography was performed on TLC plates precoated with silica gel 60 from Merck, Darmstadt. Infrared spectra were recorded from KBr-disks on a Perkin Elmer spectro-photometer 621.

IV. Experimental

A. Introduction of the phosphorylcholine group

To a solution of 1.5 g (0.0062 mole) of β-bromoethylphosphoryldichloride in 15 ml of absolute chloroform (freshly prepared by distillation over phosphorus pentoxide) is added dropwise, with stirring and cooling, 1.25 g (0.0125 mole) of absolute triethylamine. A solution of 2 g (0.0031 mole) of 3-*O*-benzoylceramide in 10 ml of absolute chloroform is added dropwise to the reaction mixture at 0°C over 30 min. After stirring for a further hour at 0°C the mixture is kept for 48 hr at 20°C. The solution then has a red-brown colour.

The solvent is removed in vacuum at 20–25°C and the residue dissolved in 150 ml of dioxan. At 0°C 25–30 ml of water are added dropwise with stirring the pH being maintained at 8.0–8.5 by adding diluted ammonia solution. After the mixture has been kept at about 20°C for 5 hr under nitrogen, 250 ml of vold water are added and the solution is acidified to pH 1–2 with 2 N sulphuric acid. The turbid solution is

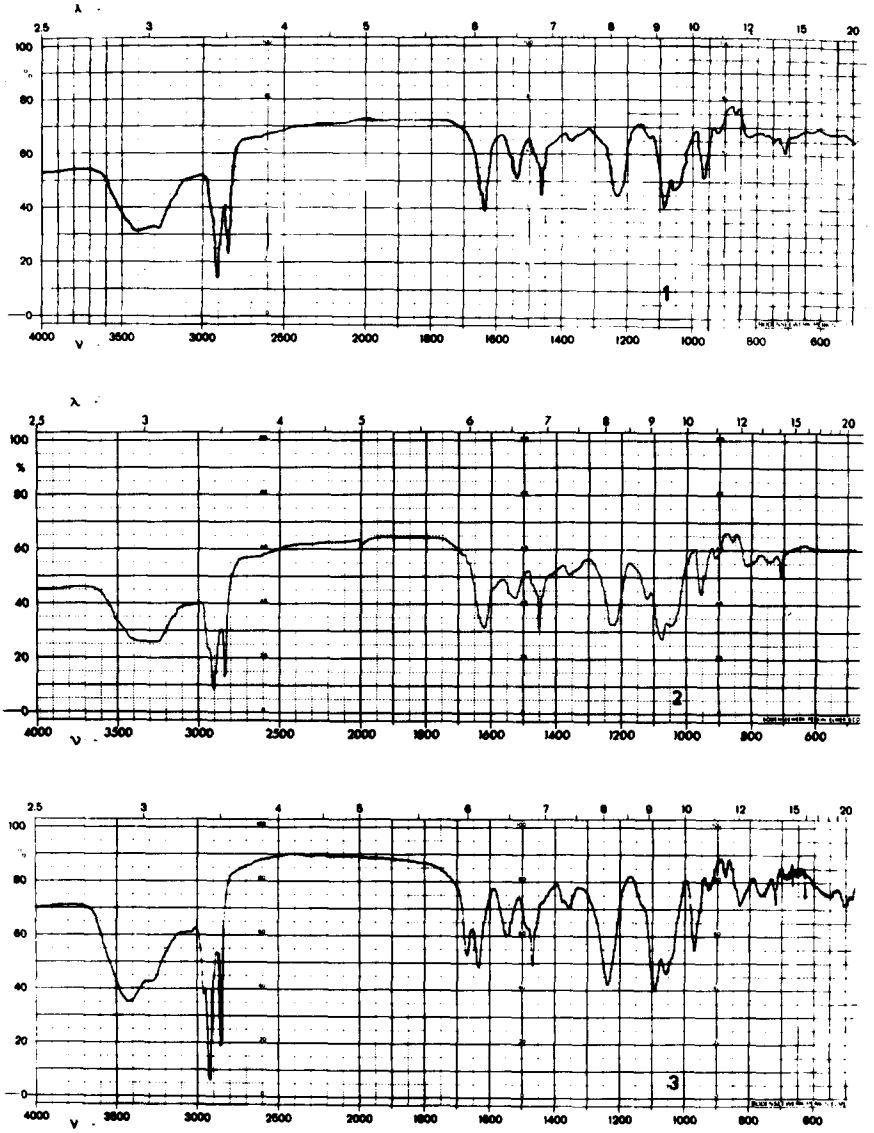

Fig. 3. IR-spectra of (1) natural sphingomeylin from ox-brain; (2) N-(2-DL-hydroxypalmitoyl)-DL-sphingomyelin; (3) N-(*trans*-2-hexadecenoyl)-DL-dihydrosphingomyelin. All spectra have been recorded from KBr disks (0.6 mg of sphingomyelin/200 mg of KBr).

immediately extracted three times with 100 ml portions of diethyl ether. The combined ether layers are washed three times with water and then dried over sodium sulfate. After evaporation of ether a yellow oil (2.8 – 3.1 g) remains.

The oil is dried for 2 days in vacuum over phosphorus pentoxide. Three grammes of the dried product are dissolved in a mixture of absolute benzene–trimethylamine (3 : 2) and the solution is heated for 48 hr in a sealed tube at 60–65°C. Benzene and unreacted trimethylamine are removed in vacuum at 30°C and the residue is kept under vacuum for a further hour in order to remove the remaining trimehylamine.

The residue is dissolved in 40 ml of absolute methanol and 4 ml of 1 N methanolic sodium methylate solution are added. The solution is kept under nitrogen for 5 hr at 20–25°C, thereafter 6 ml of 1 N hydrochloric acid, and 50 ml of chloroform are added. The solution is then washed several times with methanol–water (2 : 3) in order to remove all the inorganic salts. This procedure has to be carried out exactly as described, otherwise emulsions will be formed. The lower phase is evaporated to dryness at a temperature not exceeding 25–30°C. Foaming at the end of the distillation can be prevented by addition of methanol and further evaporation without heating.

The solid residue is dissolved in about 20 ml of chloroform and transferred to a column of 80 g of silica gel (Silicar CC 7, 100–200 mesh, Mallinckrodt). The mixture is chromatographed first with 500 ml of chloroform–methanol (95 : 5) and subsequently with 500 ml of chloroform–methanol–water (65 : 25 : 4). Fractions of 10–15 ml are collected and the course of separation is controlled by thin-layer chromatography. Methylbenzoate and then ceramide are obtained with chloroform–methanol (95 : 5). Chloroform–methanol–water (65 : 25 : 4) first eluates some by-

Table 1
Melting points and approximate R_F values of the synthesized ceramides

	Melting Point [a]		R_F [b]
	Observed [a]	Reported	
D,L-Ceramides			
N-(Palmitoyl)			
DL-sphinganin	103–104	104–105 [9]	0.50
N-(Oleoyl)			
DL-sphinganin	86– 87		0.52
N-(2'DL-Hydroxypalmitoyl)			
DL-sphinganin	106–108	108–114 [c] [10]	0.43
N-(*trans*-2-Hexadecenoyl)			
DL-sphinganin	109–110	110–111 [9]	0.52
N-(Palmitoyl)			
DL-sphingenin	94–95	97–98 [9]	0.53
N-(Oleoyl)			
DL-sphingenin	68–69		0.50
N-(2'DL-Hydroxypalmitoyl)			
DL-sphingenin	99–100	100–101 [10]	0.42

[a] Melting points were determined with a Kofler micro melting point apparatus.
[b] TLC on silica gel G plates. The solvent system was $CHCl_3$–CH_3OH, 93 : 7 (v/v).
[c] D-isomers.

Table 2
Melting points, approximate R_F values and analysis of sphingomyelins

	Melting point				Calcl.				Found			
	observed	reported	R_F [a]	Formula	C	H	N	P	C	H	N	P
DL-Sphingomyelins												
N-(Palmitoyl)												
DL-dihydrosphingomyelin	224–225	223–224 [3]	0.20									
N-(Oleoyl)												
DL-dihydrosphingomyelin	208–210		0.20	$C_{41}H_{85}N_2O_7P$	65.40	11.44	3.74	4.14	65.33	11.27	3.88	4.25
N-(2'DL-Hydroxypalmitoyl)												
DL-dihydrosphingomyelin	228–230		0.15	$C_{39}H_{83}N_2O_8P$	63.40	11.32	3.79	4.19	63.15	11.06	3.80	3.93
N-(*trans*-2-Hexadecenoyl)												
DL-dihydrosphingomyelin	205–206		0.20	$C_{39}H_{81}N_2O_7P$	64.96	11.33	3.89	4.30	64.68	11.56	4.11	4.15
N-(Palmitoyl)												
DL-sphingomyelin	209–210	209–211 [3]	0.20									
N-(Oleoyl)												
DL-sphingomyelin	188–190		0.20	$C_{41}H_{83}N_2O_7P$	65.95	11.20	3.75	4.15	65.88	11.33	3.80	4.35
N-(2'DL-Hydroxypalmitoyl)												
DL-sphingomyelin	219–221		0.15	$C_{39}H_{81}N_2O_8P$	63.55	11.08	3.80	4.20	63.56	11.23	3.75	4.13

[a] TLC on silica gel G plates. The solvent system was $CHCl_3$–CH_3OH–H_2O, 65 : 25 : 4 (v/v).

products and subsequently sphingomyelin. After combining the fractions of ceramide and sphingomyelin, respectively, the solutions are evaporated to dryness in vacuum at 25–30°C. The yields are 0.6–0.8 g of ceramide and 0.6–0.8 g of sphingomyelin (30–35% of the theoretical amount of sphingomyelin calculated for 3-*O*-benzoyl-ceramide) (see tables 1 and 2).

Acknowledgements

This work has been financially supported by the Deutsche Forschungsgemeinschaft. I wish to thank Professor P. Läuger who initiated this work. I am also indebted to Dr. G. Pohl and Dr. J. Southon for criticism and help in writing the manuscript.

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