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A Simple Chemical Method for Labelling Phosphatidylcholine and Sphingomyelin in the Choline Moiety

WILHELM STOFFEL, DAC LEKIM and TSCHAE SANG TSCHUNG

*Institut für Physiologische Chemie der Universität Köln**

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Summary: A simple method for the chemical synthesis of 3-*sn*-phosphatidylcholine (lecithin) and sphingomyelin labelled in the choline moiety is described. Lecithin and sphingomyelin are first demethylated to phosphatidyl-*N*,*N*-dimethylethanolamine and ceramide 1-phosphoryl-*N*,*N*-dimethylethanolamine by reaction with sodium benzenethiolate. The dimethylamino derivatives of the two lipids have been characterized thoroughly. Quaternization of the nitrogen with methyl iodide yields the starting phosphatidylcholine and sphingomyelin. Using [^{14}C]methyl iodide for the methylation and combining the two methods facilitates the chemical synthesis of lecithin and sphingomyelin, labelled in the hydrophilic part of the molecule, in high yield.

Zusammenfassung: *Eine einfache chemische Methode zur Markierung von Phosphatidylcholin und Sphingomyelin am Cholinrest.* Es wird eine einfache Methode für die chemische Synthese von 3-*sn*-Phosphatidylcholin (Lecithin) und Sphingomyelin, die in einer Methylgruppe des Cholins markiert sind, beschrieben. Lecithin und Sphingomyelin werden zuerst zu Phosphatidyl-*N*,*N*-dimethyläthanolamin und Ceramid-1-phosphoryl-*N*,*N*-dimethyläthanolamin mit Natriumthiophenolat demethyliert. Die beiden Demethylierungsprodukte wurden durch die Analyse, IR-, NMR- und Massenspektroskopie charakterisiert.
Quaternisierung des Stickstoffs mit Methyljodid ergibt wieder die Ausgangsverbindungen. Verwendet man [^{14}C]Methyljodid für die Methylierung, so erhält man in guter Ausbeute auf einfache Weise ein Phosphatidylcholin und Sphingomyelin von beliebig hoher Radioaktivität in der hydrophilen Gruppe dieser beiden Moleküle.

The availability of labelled complex phospholipids is essential for numerous studies on the metabolism and the functions of these lipids as well as for studies on NIEMANN-PICK disease. The labelling of the acyl residues of the hydrophobic part of the phospholipids can be achieved by a number of suitable chemical or biochemical methods[1]. It is however more difficult to insert the label by chemical methods into the choline moiety of phosphatidylcholine and sphingomyelin. The biochemical synthesis gains only a low yield, particularly in the case of sphingomyelin.

We wish to describe a method which allows the demethylation of phosphatidylcholine and sphingomyelin to phosphatidyl-*N*,*N*-dimethylethanolamine and ceramide 1-phosphoryl-*N*,*N*-dimethylethanolamine in very high yield and the subsequent quaternization of the dimethylamino derivatives with methyl iodide. The use of labelled methyl iodide resulted in the synthesis of choline labelled lecithin and sphingomyelin. Conditions were established by which very satisfactory yields were also obtained in this reaction.
HABERLI[2] and SHAMMA, DENO and REMAR[3] con-

* *Address:* Professor Dr. Dr. W. STOFFEL, D-5 Köln 41, Joseph-Stelzmann-Straße 52.

[1] A. J. SLOTBOOM and P. P. H. BONSEN, Chem. Physics Lipids **5**, 301 [1970].

[2] J. HABERLI, Ph. D. thesis, Brown University, Providence, R. I. 1960, Univ. Microfilms No. 62-5746.

[3] M. SHAMMA, N. C. DENO and J. F. REMAR, Tetrahedron Letters **13**, 1375 [1966].

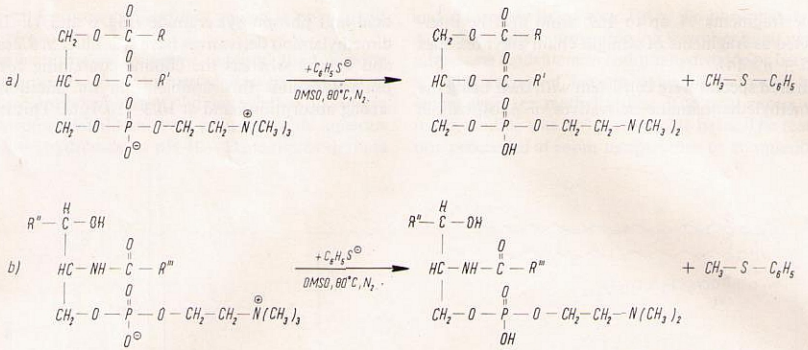

Fig. 1. Reaction scheme of the demethylation of phosphatidylcholine (a) and spingomyelin (b). Reaction time: 3 h (a) and 6 h (b).

verted quaternary to tertiary amines using sodium benzenethiolate. JENDEN, HAMIN and LAMB[4] described the microestimation of acetylcholine and related compounds by gas chromatography after the selective demethylation of the quaternary ammonium salt. Their demethylation procedure was based on this method. The ester linkage of acetylcholine remained unattacked when the reaction was carried out at low temperature and under anhydrous conditions.

This observation led us to establish conditions for the demethylation of phosphatidylcholine, a diester of glycerophosphorylcholine, and sphingomyelin, which possesses phosphodiester and amide linkages. The reaction is shown in Fig. 1.

The *demethylation* can be carried out giving high yields under the conditions described under "Experimental". It is essential for the quantitative quaternization of the dimethyl derivatives that no water or dimethylsulfoxide is present. We freed the reaction product from dimethylsulfoxide by dialysis of the chloroform solution against water.

The products phosphatidyl-*N,N*-dimethylethanolamine and ceramide 1-phosphoryl-*N,N*-dimethylethanolamine, were shown to be pure by thin-layer chromatography (solvent system: chloroform/methanol/10% NH_4OH 60:35:8).

Sphingomyelin isolated from brain lipids and its demethylation product separated into two bands due to the long chain (lower band) and shorter chain fatty acid residues (upper bands) in the ceramide species. The demethylation products have been further characterized. Microphosphorus determinations[5] proved to be satisfactory. The NMR spectrum of the phosphatidyl-*N,N*-dimethylethanolamine (Fig. 2) exhibited the signals characteristic for the $N^{\oplus}(CH_3)_2$ at 2.9 ppm, $CH_2-N^{\oplus}$ at 3.3, CH_2-O Pat 4.4, $-CH_2-CO-O-$ at 2.3 and $CH_2-O-CO-$ at 4.2 ppm and was identical with that of a synthetic reference compound[6]. The NMR spectrum of demethylated sphingomyelin (Fig. 3) showed the following typical protons: $N(CH_3)_2$ 2.8 ppm; NHCO 7.3, CH_2OP 4.0 ppm.

Mass spectrometry was carried out on the two unaltered dimethylethanolamino derivatives and sphingomyelin. Typical fragments are present in the mass spectrum of phosphatidyl-*N,N*-dimethylethanolamine (Fig. 4) at *m/e* 71 $\cdot CH-CH_2-N(CH_3)_2$, *m/e* 58 $CH_2=N^{\oplus}(CH_3)_2$, *m/e* 169 $HO-CH_2-CH_2-O-P^{\oplus}(OH)_2-O-CH_2-CH_2$, and of the deacylated tris(trimethylsilyl)-*sn*-glycerol-3-phosphoryl-*N,N*-dimethylethanolamine at *m/e* 58, 71, 72; M—15 444; M—90 369 and M—103 356. The latter was closely similar to that given by DUNCAN *et al.*[7]. The mass spectrum of ceramide 1-phosphoryl-*N,N*-dimethylethanolamine (Fig. 5) showed among others the typical fragments *m/e* 58 and 71.

[4] D. J. JENDEN, I. HAMIN and S. I. LAMB, Analytic. Chem. **40**, 125 [1968].

[5] G. R. BARTLETT, J. biol. Chemistry **234**, 466 [1959].

[6] D. CHAPMAN and A. MORRISON, J. biol. Chemistry **241**, 5044 [1966].

[7] I. H. DUNCAN, W. J. LENNARZ and C. C. FENSELAU, Biochemistry [Washington] **10**, 927 [1971].

The fragments 71 up to 169 could also be interpreted as fragments of straight chain alkyl residues ($15 + n \times 14$).

Infrared spectra were consistent with their being the dimethylethanolamine derivatives of phosphatidic acid and phosphorylceramide (Fig. 6 and 7). The dimethylamino derivatives have a doublet at 9.2 μm and 9.5 μm whereas the choline containing compounds exhibit this doublet and an additional strong absorption band at 10.3—10.4 μm. This is a

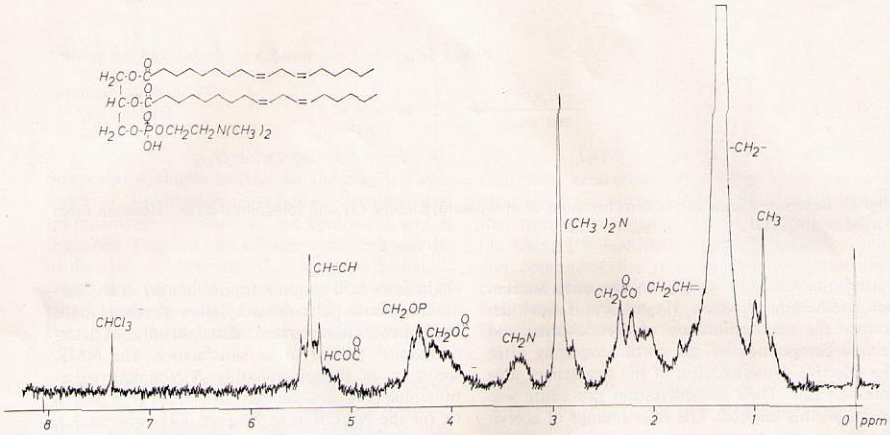

Fig. 2. NMR spectrum of phosphatidyl-*N*,*N*-dimethylethanolamine. Solvent: C^3HCl_3.

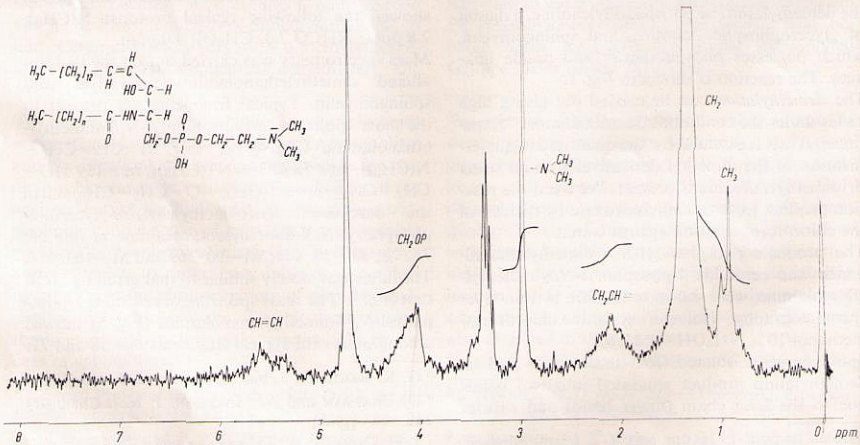

Fig. 3. NMR spectrum of ceramide 1-phosphoryl-*N*,*N*-dimethylethanolamine. Solvent: $C^3H_3O^3H(H)$.

typical feature for quaternary amines as pointed out by NELSON[8].

The *methylation procedure* required the following preparations: Prior to methylation the solution of ceramide phosphoryl-*N,N*-dimethylethanolamine in chloroform/methanol was adjusted with aqueous sodium hydroxide to pH 10—11, taken to dryness and rigorously dried over P_2O_5. To phosphatidyl-*N,N*-dimethylethanolamine, on the other hand, was added one equivalent of sodium hydroxide or better a 200% excess of barium oxide in methanol together with the methyl iodide. The final excess of methyl iodide was 50% on a molar basis. The reaction proceeded at room temperature to completion

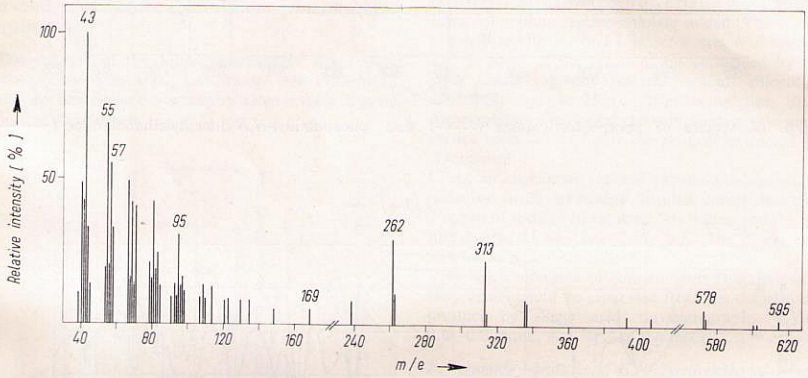

Fig. 4. Mass spectrum of phosphatidyl-*N,N*-dimethylethanolamine. 300 μA, 70 eV, 250°C.

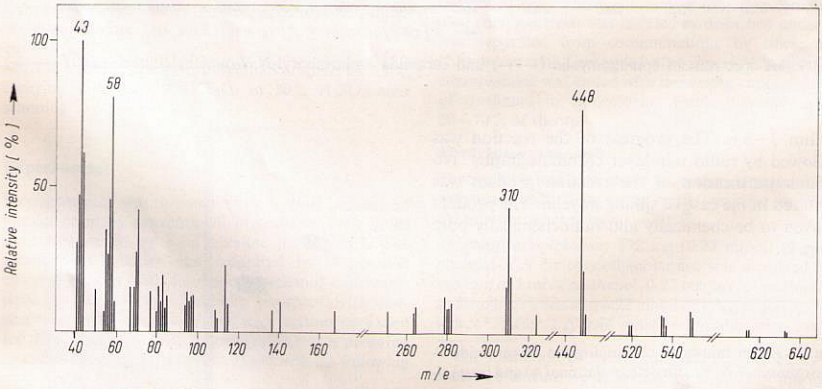

Fig. 5. Mass spectrum of ceramide 1-phosphoryl-*N,N*-dimethylethanolamine.

[8] G. J. NELSON, Lipids **3**, 104 [1967].

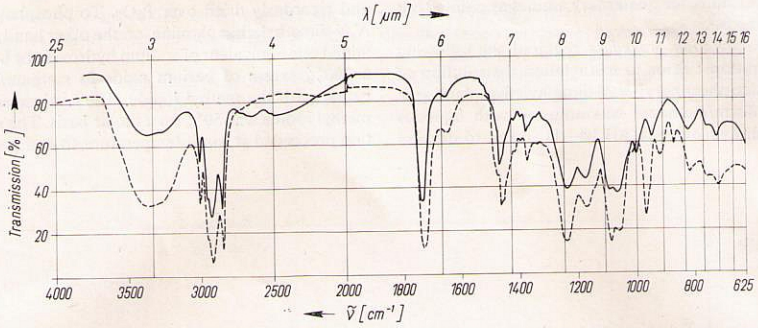

Fig. 6. IR spectra of phosphatidylcholine (-----) and phosphatidyl-*N,N*-dimethylethanolamine (———).

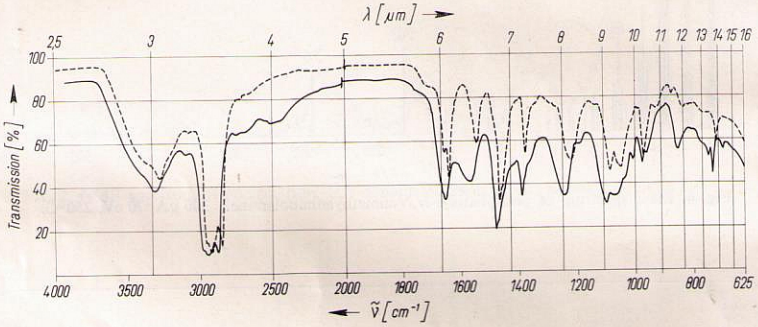

Fig. 7. IR spectrum of sphingomyelin (-----) and ceramide 1-phosphoryl-*N,N*-dimethylethanolamine (———).

within 3—5 h. The progress of the reaction was followed by radio thin-layer chromatography. No further purification of the reaction product was required in the case of sphingomyelin. The product proved to be chemically and radiochemically pure (Fig. 8).

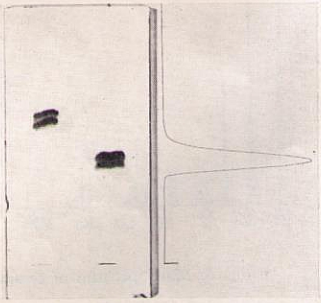

Fig. 8. Radio thin-layer chromatogram of ceramide 1-phosphoryl-*N,N*-dimethylethanolamine (A) and [*methyl*-^{14}C]sphingomyelin (B).

Solvent system: chloroform/methanol/10% NH_4OH 60:35:8; charring with 0.5% $Na_2Cr_2O_7$ in 50% H_2SO_4.

During the methylation of phosphatidyl-*N,N*-dimethylethanolamine about 5% of the lyso compound was formed. This was separated from phosphatidylcholine by silicic acid chromatography[9]. Fig. 9 represents the radio thin-layer chromatogram of [*methyl*-^{14}C]lysophosphatidylcholine (A) and phosphatidyl-*N,N*-dimethylethanolamine (B) and [*methyl*-^{14}C]phosphatidylcholine (C).

The formation of lysolecithin however can be completely avoided when an excess of barium oxide is used instead of the equivalent amount of sodium hydroxide.

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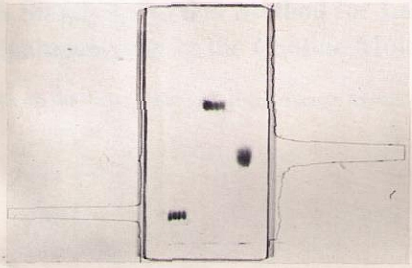

Fig. 9. Radio thin-layer chromatogram of [*methyl*-^{14}C]-lysophosphatidylcholine (A), phosphatidyl-*N,N*-dimethylethanolamine (B) and [*methyl*-^{14}C]phosphatidylcholine (C).
Solvent system: chloroform/methanol/water 65:25:4; charring with 0.5% $Na_2Cr_2O_7$ in 50% H_2SO_4 after scanning.

Experimental

Sphingomyelin was isolated from a lipid extract of human brain by conventional procedures; ester lipids were hydrolyzed by mild alkaline hydrolysis[10]. The sphingolipid mixture was separated by silicic acid chromatography with increasing methanol concentrations in chloroform. Synthetic phosphatidylcholine, prepared in this laboratory, and soya lecithin were used for these studies. Sodium benzenethiolate was prepared essentially according to JENDEN *et al.*[4] with the following modification: equivalent amounts of sodium and thiophenol were refluxed in ethanol for 1 h. On cooling the sodium salt crystallized from the clear solution. The crystalline precipitate was collected on a BÜCHNER funnel, washed with toluene and petroleum ether and stored in a desiccator over P_2O_5.

Demethylation procedures

1. Phosphatidyl-N,N-dimethylethanolamine

a) 1 mmol phosphatidylcholine was dissolved in 50 ml peroxide free dioxane (dried over Na) at 90—100°C and 1.5mmol sodium benzenethiolate added. The mixture was refluxed for about 1 h under a stream of nitrogen. The progress of the reaction was controlled by thin-layer chromatography (solvent system: chloroform/methanol/water 65:25:4). After completion of the demethylation the cooled reaction mixture was poured on ice cold 2N HCl and the product extracted with chloroform.

b) As an alternative, 1mmol phosphatidylcholine was dissolved in 20 ml freshly distilled dimethylsulfoxide, 2 mmol of sodium benzenethiolate added and the mixture heated at 95—100°C for 2 h. The product was isolated as described under 1a.

Very small amounts of contaminants (lysoderivatives, fatty acids) could be separated from the demethylated product by silicic acid chromatography. Yield: 0.6—0.8 mmol, 60—80% of the theory.

2. Ceramide 1-phosphoryl-N,N-dimethylethanolamine

1 mmol sphingomyelin was dissolved in 20 ml dimethylsulfoxide, 5 mmol sodium thiophenolate added and the mixture heated with magnetic stirring at 95—100°C under nitrogen for a period of 2—6 h. The reaction was controlled by thin-layer chromatography. The demethylated sphingomyelin was isolated as described under 1a and separated from contaminations by silicic acid chromatography. Ceramide 1-phosphoryl-*N,N*-dimethylethanolamine was eluted with increasing concentrations of methanol in chloroform. Yield: 0.5—0.7 mmol, 50—70% of theory.

Methylation procedure

The reaction was carried out in a reaction vessel sealed with a stop cock. Before opening the vessel for sampling, the mixture was cooled to —40°C.

a) Phosphatidylcholine: 172 mg (0.22 mmol) of phosphatidyl-*N,N*-dimethylethanolamine was dissolved in a mixture of 5 ml of methanol, 0.22 mequiv. of methanolic sodium hydroxide and 0.22 mmol of [^{14}C] or [^{3}H]methyl iodide*. After 2 h 0.44 mmol non-radioactive methyl

[9] W. STOFFEL and A. SCHEID, this journal **348**, 205 [1967].
[10] R. M. C. DAWSON, Biochem. J. **75**, 45 [1960].

* The formation of lysolecithin can be avoided by an excess of barium oxide instead of equivalent amounts of sodium hydroxide, BaO is separated from the reaction mixture by filtration after the reaction has gone to completion.

iodide and 0.1 mequiv. of NaOH were added. After approximately 2 h the reaction was complete as indicated by thin-layer chromatography. The reaction mixture was acidified with 2N HCl and distributed between chloroform and water. Lysolecithin (5 mg) was separated from lecithin (114 mg; 0.146 mmol) by silicic acid chromatography as described before[9]. Total yield: 70% of theory.

b) Sphingomyelin: 200 mg (0.26 mmol) of ceramide 1-phosphoryl-*N,N*-dimethylethanolamine and 0.26 mmol NaOH were concentrated and dried over P_2O_5. The methylation was carried out as described for phosphatidylcholine. Yield: 150 mg (0.2 mmol) or 75% of theory.

Microphosphorus determinations were carried out according to BARTLETT[5].

For thin-layer chromatography 0.25 mm silica gel H thin-layer plates were used, solvent system: chloroform/methanol/10% NH_4OH 60:35:8. Phosphorus positive spots were revealed using ZINZADZE reagent[11], or by charring with 0.5% $Na_2Cr_2O_7$ in 50% H_2SO_4 at 160°C. Radiochromatograms were analyzed with a Berthold scanner, model LB 2722 or the Packard radiochromatogram scanner model 7201. NMR spectra were recorded with a Varian NMR 60 D, IR spectra with a Perkin-Elmer IR spectrometer 457, mass spectra with a Varian MAT, model CH5. Spectra were taken at a cathode current of 300 μA, an ionisation potential of 70 eV and 250°C. The deacylation and the formation of trimethylsilyl derivatives of phosphatidyl-*N,N*-dimethylethanolamine and phosphatidylcholine were carried out according to DUNCAN, LENNARZ and FENSELAU[7].

[11] J. C. DITTMER and R. L. LESTER, J. Lipid Res. **5**, 126 [1964].