Analogs of natural lipids. III. Nonequivalence of methyl groups in methylated phospholipids[1]

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Abstract The dimethyl esters of a series of diastereoisomeric cyclopentanoid analogs of phosphatidic acid (A. J. Hancock, M. H. Stokes, and H. Z. Sable. 1977. *J. Lipid Res.* **18:** 81–92.) have been studied by proton NMR spectroscopy at 60, 100, and 300 MHz. The signals of the P–O–CH_3 protons near δ 3.80 show the expected doubling due to the ^{31}P–^{1}H coupling. In addition, the spectra of three of the isomers show additional multiplicity, the line separation (in Hz) being proportional to the frequency of the spectrometer. This multiplicity is due to the nonequivalence of the two methoxyl groups on phosphorus, predictable from their diastereotopic nature. The same explanation is proposed for similar observations on other compounds made by other authors. The practical utility of symmetry considerations in lipid chemistry is discussed briefly.

Supplementary key words phosphatidic acid · methylation · lipid stereochemistry · NMR spectroscopy · chirality of phosphorus

The reaction of phospholipids with diazomethane to yield methyl phosphate esters of phospholipids is commonly used in phospholipid characterization (1–4). Proton NMR spectroscopy of methylated phospholipids usually shows a doublet at δ 3.7–3.8, which is assigned to the P–O–CH_3 resonance (1, 4–6). Resonances due to C–O–CH_3 and S–O–CH_3 groups have chemical shifts upfield and downfield, respectively, from P–O–CH_3 resonances (5, 6), and are, therefore, easily distinguished. The P–O–CH_3 resonance is further identifiable since it appears as a doublet due to heteronuclear coupling of the methyl protons with phosphorus. This coupling constant, $J_{^{31}P-O-C-^{1}H}$, is found to be 11.2 ± 0.5 Hz (5). During the course of our investigation of lipid analogs (7–9), phosphates *1a–5a* (**Fig. 1**) were methylated with diazomethane as a means of characterization. These substances are analogs of phosphatidic acid, in that the glycerol moiety of diacylglycerophosphoric acid is replaced by each of the three isomeric cyclopentane-1,2,3-triols (8). Proton NMR spectroscopy of *1b–5b* at 60 MHz showed unexpected multiplicity of the P–O–CH_3 doublet in some cases (**Fig. 2**). NMR spectra of *1b–5b* at 100 and 300 MHz show that the origin of the multiplicity is nonequivalence of the methyl ester groups. The utility of this observation in the interpretation of lipid NMR spectra is briefly discussed.

METHODS

Compounds *1a–5a* were freed of metal ions by a modification of the method of Bligh and Dyer (10) and then methylated, according to the procedure of Renkonen (4), with diazomethane produced from "Diazald"[4]. NMR spectra were recorded on samples in $CDCl_3$ on Varian A60, A60A, HA-100, or HR-300 spectrometers (Varian Assoc., Palo Alto, CA).

RESULTS

Additional multiplicity of the P–O–CH_3 doublet was observed in 60 MHz spectra of *1b*, *2b*, and *3b* but not in those of *4b* or *5b*. The magnitude of the smaller doubling, measured in Hz, increased when the spectra were recorded at 100 and 300 MHz (**Table 1**), and the line separation was proportional to field strength (i.e., frequency of the spectrometer) within experimental error. On the other hand, the major doubling of the CH_3 signals, assigned to ^{31}P–O–C–^{1}H coupling, was unaffected when the field strength was changed (Table 1). Since the magnitude of coupling

Abbreviations: NMR, nuclear magnetic resonance; TMS, tetramethylsilane; PA, phosphatidic acid; PG, phosphatidyl glycerol; PGP, phosphatidyl glycerophosphate; DPI, diphosphoinositide; TPI, triphosphoinositide.

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[4] Trade name for *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide obtained from the Aldrich Chemical Company, Milwaukee, WI.

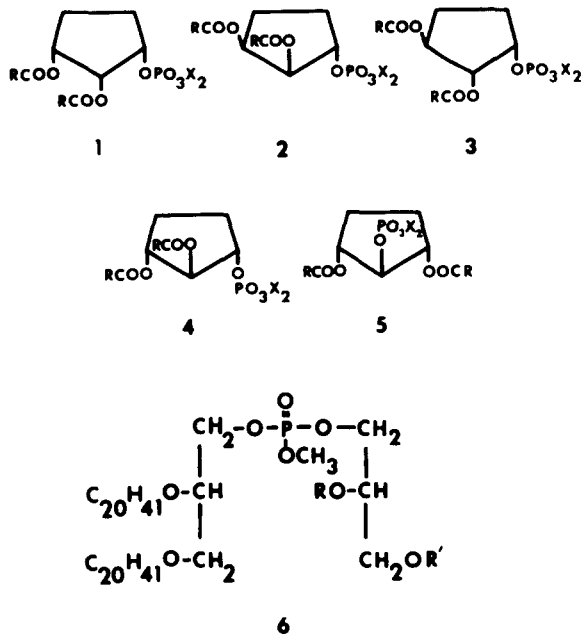

Fig. 1. Configurational and positional designation of cyclopentanoid analogs of phosphatidic acid. *1*, DL-(1,2,3/0)-cyclopentane-1,2,3-triol-1-phosphate; *2*, DL-(1,2/3)-cyclopentane-1,2,3-triol-3 phosphate; *3*, DL-(1,2/3)-cyclopentane-1,2,3-triol-1-phosphate; *4*, DL-(1,3/2)-cyclopentane-1,2,3-triol-1-phosphate; *5*, (1,3/2)-cyclopentane-1,2,3-triol-2-phosphate. For *1–5*, the following letter designations are used in the text: *a*, X = H; *b*, X = CH_3; R = $C_{15}H_{31}$. No. *6* represents 2,3-di-*O*-phytanyl-*sn*-glycerol-1-phosphoryl-1′-*sn*-glycero-2′- and 3′-mono- and disulfates, with the following letter designations used in the text: *a*, R = SO_3CH_3, R′ = H; *b*, R = H, R′ = SO_3CH_3; *c*, R = R′ = SO_3CH_3.

constants is independent of field strength (11), this result shows that the unexpected multiplicity was not due to spin–spin coupling between the P–O–CH_3 protons and some other proton in the molecule. When symmetry factors are considered (12–15), the methyl groups of *1b–4b* are seen to be diastereotopic, since they represent paired ligands attached to a prochiral center, i.e., the phosphorus atom of the –O–PO–$(OCH_3)_2$ group, in a chiral molecule[5]. As a result the methyl groups of *1b–4b* may give separate NMR signals. In the case of *4b*, the difference in chemical shift between the diastereotopic methyl groups must be very small since no additional multiplicity of the P–O–CH_3 doublet is observed even at 300 MHz (Table 1). A similar result is predicted for PA (dimethyl ester), since in this case, also, the methyl groups are diastereotopic but appear to have coincident proton chemical shifts at 60 MHz (4). The methyl groups of *5b* are enantiotopic, so additional multiplicity of the P–O–CH_3 doublet is neither expected nor observed (Table 1).

DISCUSSION

The observation and explanation of the additional splitting of the P–O–CH_3 resonance given here for the PA analogs stresses the importance of stereochemical principles in the interpretation of NMR spectra of lipids and, in addition, shows that NMR can be a useful tool in the characterization of phospholipids if proper precautions are exercised. Besides the PA analogs discussed in this report, the methyl groups of all dimethylated phosphate groups of phospholipids, such as PGP, DPI, and TPI, are in theory diastereotopic and, under certain circum-

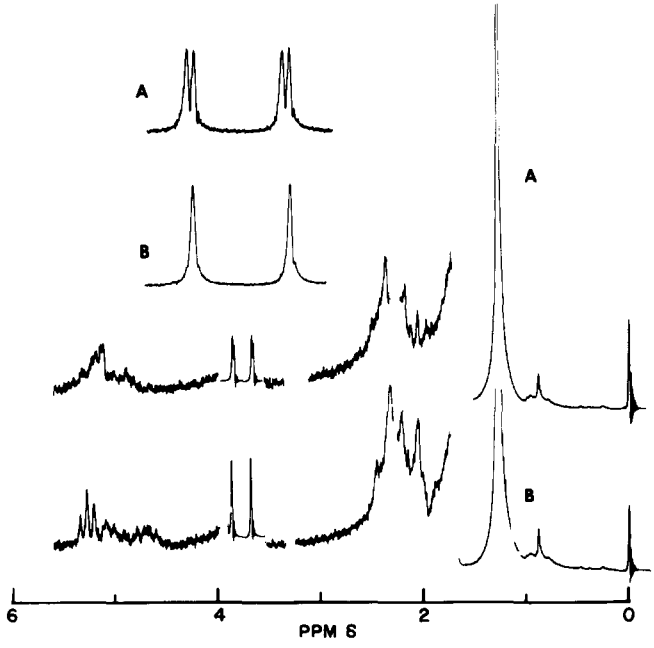

Fig. 2. Proton magnetic resonance spectra (60 MHz) of dimethyl esters of (1,2/3)-1P analog *3b* and (1,3/2)-1P analog *4b*. Spectra A are of compound *3b* and spectra B are of compound *4b*. The lower spectra were obtained at 500 Hz sweep width and 500 sec sweep time, and the insets above are the signals of the methoxyl groups at δ 3.80, recorded at 100 Hz sweep width and 500 sec sweep time.

[5] In natural phosphatidic acid, carbon 2 of the glycerol moiety is the only asymmetric center, whereas in compounds *1–4*, chiral centers exist at carbons 1, 2, and 3. Compounds *1–4*, as isolated, exist as DL mixtures. NMR spectra of DL mixtures in achiral media are identical to the spectra of either enantiomer unless the enantiomers form a molecular compound that persists in solution. In a strict sense, molecular disymmetry is neither a necessary nor sufficient condition for the presence of diastereotopic groups. A substitution criterion may be applied to test for diasteotopic groups. If substitution of each group (G) by a chiral or achiral group (G′) generates a set of diastereomers, the G groups are diastereotopic (13). Thus, *O,O*-diethylmethylphosphothiolate was found to contain two diastereotopic methylenic protons on each ethyl group, even though the molecule is achiral (15). Rapid inversion at the phosphorus atoms would lead to equivalence of the methyl groups of a —O–PO–$(OCH_3)_2$ group, but in contrast to most nitrogen compounds, tri- and tetracoordinated phosphorus compounds normally are configurationally stable (15).

stances, may give rise to separate signals or otherwise manifest their diastereotopicity.

Monomethyl phospholipids also can give two P–O–CH_3 proton NMR resonances since the unsubstituted phosphate oxygen atoms of chiral, disubstituted phospholipids such as PG and its derivatives are diastereotopic, and these compounds will be converted into a mixture of diastereomers when protonated[6] and treated with diazomethane. An example of this type of diastereoisomerism is found in the multiplicity of the P–O–CH_3 resonance of dimethyl-1-*sn*-phosphatidyl-1′-*sn*-glycerol-2′-sulfate (*6a*, Fig. 1) (6). The additional multiplicity of the P–O–CH_3 resonance that persists under conditions of ^{31}P–^{1}H-heteronuclear decoupling can be interpreted in terms of these considerations of symmetry. Methyl phosphatidylglycerol, dimethylphosphatidylglycero-3′-sulfate (*6b*), and trimethylphosphatidylglycero-2′,3′-disulfate (*6c*) do not show additional multiplicity of the P–O–CH_3 resonance at 100 MHz (5, 6), but these signals are significantly broadened in the case of *6b* and *6c*. Additional multiplicity might be observed in these cases if spectra are recorded at a higher field strength or if a different solvent is used (13). A related nonlipid example is found in the multiplicity of methyl signals of the ethyl phosphate group of dinucleotide ethyl phosphotriesters (16). In this case, the multiplicity has been shown to be strongly dependent on the nature of the solvent.

All the considerations of chirality of the phosphorus atoms in these compounds are identical to those for the carbon atoms of carbohydrates, e.g., the aldoses and *aric* acids (17, 18). These considerations have a practical significance in the metabolism of phospholipids. For example, nonequivalence of the two phosphorus atoms of cardiolipin has been demonstrated in metabolic studies (19–21). Further, the observation of two signals in ^{31}P NMR studies of cardiolipin (22) has been shown by Powell and Jacobus (23) to be a logical stereochemical consequence of the conclusion of LeCocq and Ballou (24) that in a pseudo-Fischer projection, cardiolipin must be represented in the *lyxo* configuration to account for the observed optical rotation.

The nonequivalence of the methyl groups of PA (dimethyl ester) and its analogs represents only one type of diastereotopic group in these molecules, since the methylenic hydrogens at positions 1 and 3 are also homomorphic ligands attached to a prochiral center[5] and, as a result, these hydrogens also are diastereotopic. Since cardiolipin contains six such pairs of methylenic hydrogens, a complete discussion of the stereochemistry and NMR spectrum of this molecule would have to deal with those groups also.

[6] The protonated form of a phospholipid is the usual substrate for methylation by diazomethane (1). Proton transfer between the unsubstituted phosphorus oxygen atoms is rapid, leading to methylation at either site.

TABLE 1. Chemical shift differences for diastereotopic P–O–CH_3 resonances of methyl esters of cyclopentanoid PA analogs at various spectrometer frequencies

Compound	$\Delta\delta^a$ at: 60 MHz	100 MHz	300 MHz
1b	0.6	1.0	2.8
2b	0.5	0.9	2.8
3b	0.8	1.5	4.5
4b	—[b]		
5b	—[b]		

The major doubling of each CH_3 signal, due to ^{31}P–O–C–^{1}H coupling, was 11.3 ± 0.1 Hz, as measured with the frequency counter of the HA-100 spectrometer. The value of the coupling constant measured in the 60 and 300 MHz spectra was essentially unchanged from the value measured at 100 MHz.

[a] Values in Hz; estimated error: ± 0.1 Hz.

[b] No additional multiplicity observed at any frequency.

NOTE ADDED IN PROOF

Aneja and Davies (25) recorded the 220 MHz proton NMR spectra of the dimethyl esters of 1,2-dipalmitoyl phosphatidic acid and observed a doublet of doublets with a chemical shift difference of approximately 2 Hz. No additional multiplicity was observed in the spectrum of 1,3-dipalmitoyl-glycero-2-phosphoric acid. Aneja and Davies recorded this observation in their experimental section and did not discuss it further. It seems clear to us that they are dealing with the same phenomenon that we have reported in the present study. Their observation, therefore, constitutes an example of the nonequivalence of methyl groups in a methylated derivative of a natural phosphatide.

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