NMR spectral analysis of cytotoxic ether lipids

Diane Dick, Scott Pluskey, Dinesh K. Sukumaran, and David S. Lawrence[1]

Department of Chemistry, State University of New York, Buffalo, NY 14214

Summary Several cytotoxic ether lipid analogs of platelet activating factor exhibit a wide range of interesting pharmacological properties. Furthermore, at least two members of this family of lipids have progressed to phase I clinical trials as potential cancer chemotherapeutic agents. In spite of the promise that these compounds hold as anticancer drugs, they remain poorly characterized. We report herein the first complete ^{1}H NMR analysis of several palmityl-based ether lipids. In addition, we report the ^{13}C NMR spectral assignments for these lipids, which are based, in part, on both the presence and magnitude of ^{31}P–^{13}C and ^{14}N–^{13}C coupling constants.—**Dick, D., S. Pluskey, D. K. Sukumaran, and D. S. Lawrence.** NMR spectral analysis of cytotoxic ether lipids. *J. Lipid Res.* 1992 **33:** 605–609.

Supplementary key words platelet activating factor

Several monoether, diether, and lyso analogs of platelet-activating factor (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) have been found to exhibit a selective cytotoxicity toward a variety of neoplasms, both in vitro and in vivo (1, 2). Intensive study of the pharmacological properties of one of these compounds, 1-O-octadecyl-2-O-methyl-*sn*-glycero-3-phosphocholine (ET-18-OMe, **1**) (**Fig. 1**), has revealed that this diether lipid analog accumulates in the plasma membrane (3) of tumor cells and, in a yet to be determined fashion, influences membrane-associated phenomena including phospholipid synthesis (4) and metabolism (5–7), as well as membrane fluidity (3).

We have initiated a program to elucidate the physical and chemical properties of Et-18-OMe and its derivatives. Such studies may provide some insight into the underlying mechanism of biological activity and may ultimately lead to the creation of even more potent antineoplastic ether lipids. Interestingly, when we commenced our efforts in this area, we were surprised to find that a complete ^{1}H NMR assignment had not been established for even one member of this 1-O-alkyl lysophospholipid family of PAF analogs, in spite of the fact that at least two PAF derivatives have progressed to clinical trails (8, 9)! Furthermore, new analogs of these cytotoxic ether lipids are being synthesized at a rapid pace, and the need to unequivocally characterize these compounds is becoming increasingly evident. We report herein the NMR spectral analysis of several palmityl-based analogs of ET-18-OMe using proton decoupling experiments in conjunction with two-dimensional correlated spectroscopy (COSY). In addition, we report the ^{13}C NMR assignments for these lipids, which are based, in part, on both the presence and magnitude of ^{31}P–^{13}C and ^{14}N–^{13}C coupling constants.

EXPERIMENTAL PROCEDURES

General methods and materials

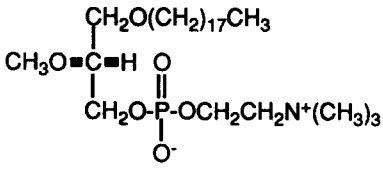
Reagent grade solvents (Fischer Scientific) were distilled prior to use. Phosphorous oxychloride and 2-bromoethanol were purchased from Aldrich and purified as needed. Thin-layer chromatography was performed on precoated silica gel 60 plates (Merck). Unless otherwise specified, the eluent used for TLC was $CHCl_3$–MeOH–H_2O 65:25:4 (v/v/v). Visualization of phospholipids was accomplished using a molybdic acid spray (10).

1-O-Hexadecyl-2-O-methyl-*rac*-glycerophosphocholine (2)

The lipid alcohol **6** (200 mg, 0.61 mmol) (11, 12) was dissolved in 2 ml of trichloroethylene and the resulting solution was subsequently added, over a 10-min period, to an oven-dried 5 ml round-bottomed flask containing freshly distilled β-bromoethylphosphodichloridate (280 mg, 1.16 mmol) and triethylamine (0.25 ml, 1.79 mmol) maintained at 0°C under a N_2 atmosphere. The reaction mixture was stirred for 1 h at 0°C and then 1 h at room temperature. The precipitated $Et_3N \cdot HCl$ was filtered from solution and the solvent was subsequently removed in vacuo. The residue was dissolved in 20 ml of THF–H_2O 3:1 (v/v) and the resulting solution was stirred for 3 h at room temperature. Removal of the solvents under reduced pressure afforded the β-bromoethylphosphodiester intermediate as a white amorphous solid (TLC; R_f 0.48). This material was immediately dissolved in 30 ml of chloroform–isopropyl alcohol–acetonitrile 3:5:5 (v/v/v) and the solution was subsequently treated with 8 ml of 25% aqueous Me_3N. The solution was stirred at 60°C until TLC indicated the complete absence of β-bromoethylphosphodiester intermediate (~15 h). The residue obtained upon removal of the solvent was

Abbreviations: PAF, platelet activating factor; TLC, thin-layer chromatography.

[1]To whom correspondence should be addressed.



1

Fig. 1.

chromatographed on silica gel (elution gradient: 200 ml $CHCl_3$, 200 ml $CHCl_3$–MeOH 3:1 (v/v), 200 ml $CHCl_3$–MeOH 2:1 (v/v), $CHCl_3$–MeOH–H_2O 65:25:4 (v/v/v) to yield the desired lipid **2** as a hygroscopic white powder (254 mg, 83.2%). FAB MS *m/z* calcd for $C_{22}H_{53}NO_5P$ $(M + H)^+$ 496.37, obsd 496.49.

1-O-Hexadecyl-3-O-[[2-(trimethylamine) ethyl]phosphoryl]propanediol (3)

Alcohol **7** (300 mg, 1.0 mmol) (13) afforded lipid **3** (283 mg, 60.9%) as a crystalline white solid (R_f 0.15). FAB MS *m/z* calcd for $C_{22}H_{53}NO_5P$ $(M + H)^+$ 466.36, obsd 466.49.

1-O-Hexadecyl-2-O-benzyl-*rac*-glycerophosphocholine (4)

The protocol for the conversion of alcohol **8** (11,14) to lipid **4** was similar to that indicated above except that CCl_4 was used in place of trichloroethylene and that the β-bromoethylphosphodiester intermediate was dissolved $CHCl_3$–CH_3CN 1:1 (v/v) prior to treatment with aqueous triethylamine (15). Lipid **4** was obtained (489 mg, 70.3%) as a waxy white solid (R_f 0.23). FAB MS *m/z* calcd for $C_{22}H_{53}NO_5P$ $(M + H)^+$ 572.40, obsd 572.43.

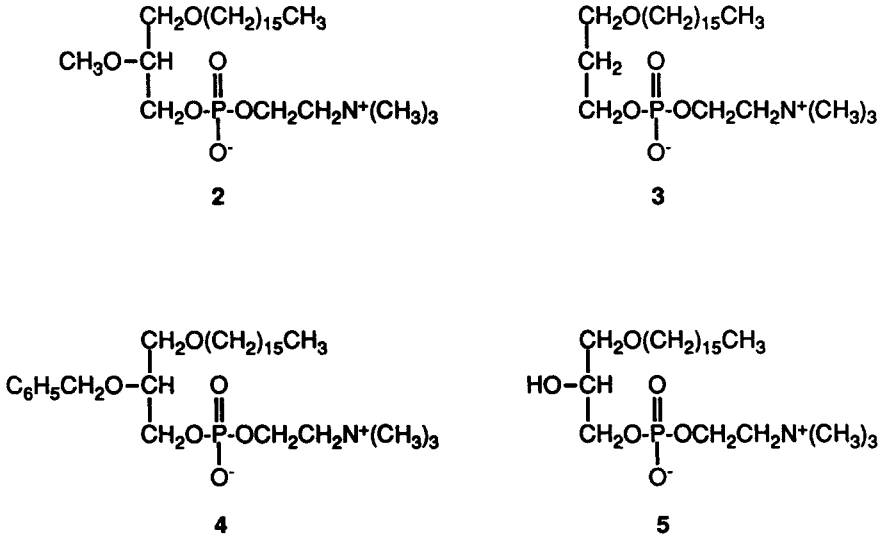
1-O-Hexadecyl-*rac*-glycerophosphocholine (5)

Lipid **4** (72 mg, 0.13 mmol) and 10% Pd/C (60 mg) was introduced into a pressure bottle containing 4 ml of methanol. Hydrogenolysis was conducted at 50 psi on a parr apparatus. Upon completion of the reaction (monitored by TLC, ~18 h), the catalyst was filtered off and the methanol was removed in vacuo to furnish **5** (58 mg, 95.4%) as a hygroscopic white powder (TLC: $CHCl_3$–MeOH–H_2O 4:6:1 (v/v/v; R_f 0.21). FAB MS *m/z* calcd for $C_{22}H_{53}NO_5P$ $(M + H)^+$ 482.35, obsd 482.31.

RESULTS AND DISCUSSION

Synthesis of alkyllysophospholipids 2–5 (Fig. 2)

In general, the chemical synthesis of glycerophospholipids reserves, for the final step, the incorporation of the phosphatidylcholine side chain (16). Arnold, Weltzien, and Westphal (11) described the synthesis of a number of ether lipid analogs, including ET-18-OMe, in which this side chain is incorporated via the phosphorylating agent, β-bromoethylphosphodichloridate (17). Subsequent treatment of the phosphorylated intermediate with aqueous trimethylamine provides the desired phosphatidylcholine. With this background in mind, we prepared the racemic alcohol-containing precursors to lipids **2–5**. These precursors **6, 7**, and **8** (**Fig. 3**) were synthesized according to standard literature protocols.



2 3 4 5

Fig. 2.

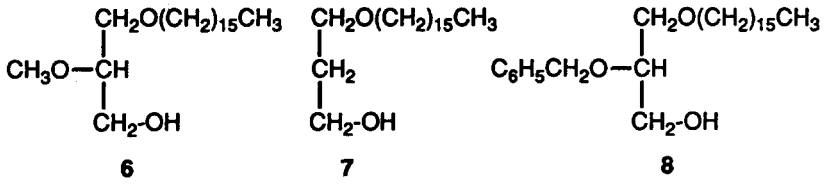


Fig. 3.

Alcohols **7** and **8** were treated with β-bromoethylphosphodichloridate, followed by displacement of the bromide on the intermediate β-bromophosphodiester with aqueous trimethylamine. The desired lipids **3** and **4** were obtained in 60.9% and 70.3% yield, respectively. Interestingly, we were able to convert the 2-methoxy-substituted alcohol **6** to the desired lipid **2** in 83.2% yield. This exceeds the yields described by Arnold, Weltzien, and Westphal (11) in their original paper. In short, in our hands, this reaction produces the requisite lipids **2–4** in sufficient quantity and purity for our needs. Hydrogenolysis of the benzyl ether-containing lipid **4** provided the hydroxyl-bearing derivative **5** in 95.4% yield.

^{1}H NMR assignment of alkyllysophospholipids 2–5

Due to their amphiphilic nature, phospholipids often assume a multitude of aggregation states in solution, leading to poorly resolved NMR spectra. For example, in $CDCl_3$, the expected resonance for the three methyl groups on the choline side chain of lipid **2** is very broad and therefore rather ill-defined, which might lead one to conclude that conversion of **6** to **2** had failed. Fortunately, a few binary and ternary solvent systems have been described to circumvent the difficulties associated with aggregation, including $CDCl_3$–CD_3OD (18) and $CDCl_3$–CD_3OD–D_2O 1:1:0.3 (v/v/v) (19, 20). We used both solvent systems in this study (in the binary system a 2:1 ratio of $CDCl_3$–CD_3OD was used).

The ^{1}H NMR assignments for lipids **2–5** are provided in **Table 1**. The nearly identical chemical shifts exhibited by several of the heteroatom-bearing methylene and methine groups, even at 400 MHz, proved to be a major obstacle in obtaining these assignments. Due to the compact nature of the proton resonances contained within the COSY spectra of lipids **2–5**, we found it necessary to confirm all assignments by stepwise frequency–specific single irradiation experiments as well. The chemical shifts for the protons situated at sites *a, b, c, j,* and *h* were assigned on the basis of literature precedent (21). In addition, the *h*-protons are coupled to the protons at position *i* and, via long range, to the protons at position *g*. COSY and single irradiation studies also established the chemical shift values for the protons positioned at site *d*. The *e-f-g* triad is a relatively isolated spin system with slight spin-spin leakage form *g* to *h*. Assignments for this triad were also based upon COSY and single irradiation experiments. However, it is important to note that severe overlap of proton resonances occurs in a number of instances, with several regions of each NMR spectrum exhibiting decidedly non-first order behavior. For comparison, the previously determined chemical shift assignments for PAF are provided in Table 1 as well (21).

^{13}C NMR assignment of alkyllysophospholipids 2–5

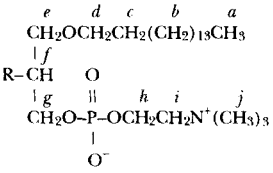
These assignments were based on comparisons to literature values for phosphatidylcholines (22) and on both the presence and magnitude of ^{31}P–^{13}C and ^{14}N–^{13}C couplings (20). Three bond carbon–phosphorus couplings in phospholipids (i.e., between P and $^{\beta}$C) tend to be ≥ 7.5 Hz, which is indicative of an averaged rotomer population in which the O–P bond is oriented anti to the $^{\beta}$C–$^{\alpha}$C bond. In contrast, two bond carbon–phosphorus couplings in phospholipids (i.e., between P and $^{\alpha}$C) are between 4 to 6 Hz in magnitude (20). In addition, due to the presence of nitrogen–phosphorus couplings, the split ^{13}C resonances in the 66.4–66.5 ppm and 54.1–54.4 ppm ranges were assigned to carbons *i* and *j*, respectively. The ^{13}C spectral shift values for lipids **2–5** are provided in **Table 2**.

In summary, we have prepared several palmityl-based analogs of ET-18-OMe. The chemical shift assignments reported herein represent the first complete NMR analysis of this important class of medicinally useful lipids. Such data should be of decided assistance in the characterization of other highly modified analogs of these cytotoxic ether lipids.

We are pleased to acknowledge the generous financial support of the National Institutes of Health (grant CA 47356).

Manuscript received 6 September 1991 and in revised form 27 December 1991.

TABLE 1. ^{1}H NMR chemical shift assignments (ppm) for lipids **2–5** ($CDCl_3$–CD_3OD 2:1, v/v)



	2	3	4	5	PAF
a	0.79	0.80	0.80	0.80	0.89
	t	t	t	t	t
b	1.20	1.20	1.20	1.20	1.28
	m	m	m	m	m
c	1.47	1.48	1.50	1.48	1.54
	brd t	t	t	brd t	t
d	3.36	3.34	3.38	3.38	3.46
	t	t	t	o/*e*	t
e	3.41	3.46	3.52	3.39	3.59
	dist t	t	m	o/*d*	d
f	3.48	1.82	3.74	3.86	5.13
	o/*i*	p	m	brd	p
g	3.81;3.88	3.86	3.87;3.95	3.96	4.01
	m	q	m	m	m
h	4.15	4.14	4.10	4.29	4.27
	brd	brd	brd	brd	brd m
i	3.50	3.50	3.35	3.60	3.64
	o/*f*	brd t	brd t	brd	p
j	3.12	3.14	3.02	3.14	3.22
	s	s	s	s	s
R	3.38		4.64;7.25		2.06
	a		dd;m		s

These assignments are referenced relative to tetramethylsilane (TMS). Two-dimensional correlated (COSY) spectra were collected at 400 MHz using a Varian-400S spectrometer at 25°C. Lipid samples were 20 mM in $CDCl_3$–CD_3OD–D_2O 1:1:0.3 (v/v/v). COSY spectra were obtained in the absolute value mode with the pulse sequence: relaxation delay –90°–t_1–90°–t_2 (23). Free induction decays were sampled for 256 t_1 values ranging from 0.0 to 0.341 sec in steps of 1.33 msec. The relaxation delay was 1.8 sec and the 90° pulse on our spectrometer was 26.4 μsec. For each t_1, a total of 64 scans was collected. In our experiments the data size in the time domain was 2048 × 256. After zerofilling and 2D-FT, the frequency domain spectra was 2048 × 2048. The spectral resolution was improved by multiplication of the free induction decays with a sine bell function. The data were symmetrized. Single irradiation experiments were performed at 400 MHz and the binary solvent system $CDCl_3$–CD_3OD 2:1 (v/v) was used to give a final lipid concentration of 15–20 mM. The chemical shift data for PAF are taken from Heymans et al. (21), which were obtained at 250 MHz in CD_3OD. Abbreviations: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; p, pentent; m, multiplet; brd, broad; dist, distorted; o/, overlap with.

TABLE 2. ^{13}C NMR chemical shift assignments (ppm) for lipids **2–5** (40–50 mM in $CDCl_3$–CD_3OD–D_2O 1:1:0.3, v/v/v)

	2	3	4	5
a	13.94	13.73	13.78	13.70
b	22.55	22.56	22.55	22.55
	26.00	25.97	25.97	25.91
	29.26	29.26	29.25	29.25
	29.46	29.47	29.45	29.44
	29.57	29.59	29.60	29.49
	29.61	30.38	29.85	29.59
c	31.84	31.86	31.85	31.86
d	70.48 or 71.86	71.32	70.63 or 71.91	71.66 or 72.02
e	70.48 or 71.86	67.42	70.63 or 71.91	71.66 or 72.02
f	79.87 ($J_{C\text{-}P}$ = 7.62)	30.64 ($J_{C\text{-}P}$ = 7.13)	obscured by $CDCl_3$	69.73 ($J_{C\text{-}P}$ = 7.26)
g	64.92 ($J_{C\text{-}P}$ = 6.36)	63.16 ($J_{C\text{-}P}$ = 5.47)	65.50 ($J_{C\text{-}P}$ = 5.88)	67.53 ($J_{C\text{-}P}$ = 5.85)
h	59.26 ($J_{C\text{-}P}$ = 5.21)	59.64 ($J_{C\text{-}P}$ = 5.09)	59.12 ($J_{C\text{-}P}$ = 4.71)	59.43 ($J_{C\text{-}P}$ = 4.96)
i	66.43[a]	66.55[a]	66.43[a]	66.47[a]
j	54.43 ($J_{C\text{-}N}$ = 3.62)	54.10 ($J_{C\text{-}N}$ = 3.56)	54.05 ($J_{C\text{-}N}$ = 3.44)	54.12 ($J_{C\text{-}N}$ = 3.69)
R	57.78		72.44	
			128.10	
			128.38	
			128.71	
			138.81	

These assignments are referenced relative to TMS. Conducted at 75.46 MHz on a Varian Gemini-300 spectrometer at 25°C. The ^{13}C NMR chemical shift assignments for compound **2** have been previously reported (21).

[a]Due to the complexity of the signal for ^{1}C, the $J_{C\text{-}P}$ and $J_{C\text{-}N}$ values could not be accurately measured.

REFERENCES

1. Westphal, O. 1987. Ether lipids in oncology—welcoming address. *Lipids.* **22**: 787–788.
2. Berdel, W. E., W. R. E. Bausert, U. Fink, J. Rastetter, and P. G. Munder. 1982. Anti-tumor action of alkyl-lysophospholipids. *Anticancer Res.* **1**: 345–352.
3. Van Blitterwsijk, W. J., H. Hilkman, and G. A. Storme. 1987. Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids.* **22**: 820–823.
4. Daniel, L. W., L. A. Etkin, B. T. Morrison, J. Parker, S. Morris-Natsche, J. Surles, and C. Piantadosi. 1987. Ether lipids inhibit the effects of phorbol diester tumor promoters. *Lipids.* **22**: 851–855.
5. Modolell, M., R. Andreesen, W. Pahlke, U. Brugger, and P. G. Munder. 1979. Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospholipid. *Cancer Res.* **39**: 4681–4686.
6. Herrmann, D. B. J., and H. A. Neumann. 1986. Cytotoxic ether lipids. *J. Biol. Chem.* **261**: 7742–7747.
7. Parker, J., L. W. Daniel, and M. Waite. 1987. Evidence of protein kinase C involvement in phorbol diester-stimulated arachidonic acid release and protaglandin synthesis. *J. Biol. Chem.* **262**: 5385–5393.
8. Herrmann, D. B. J., H. A. Neumann, W. E. Berdel, M. E. Heim, M. Fromm, D. Boerner, and U. Bicker. 1987. Phase 1 trial of the thioether phospholipid analogue BM 41.440 in cancer patients. *Lipids.* **22**: 962–966.
9. Berdel, W. E., U. Fink, and J. Rastetter. 1987. Clinical phase 1 pilot study of the alkyl lysophospholipid derivative ET-18-OCH_3. *Lipids.* **22**: 967–969.
10. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5**: 126–127.
11. Arnold, D., H. U. Weltzien, and O. Westphal. 1967. Uber die Synthese von Lysolecithinen und ihren Atheranaloga. *Justus Liebigs Ann. Chem.* **709**: 234–239.
12. Tsushima, S., Y. Yoshioka, S. Tanida, H. Nomura, S. Nojima, and M. Hozumi. 1982. Syntheses and antimicrobial activities of alkyl lysophospholipids. *Chem. Pharm. Bull.* **30**: 3260–3270.
13. Katti, S. S., M. V. Natekar, S. E. Sansare, S. Pathak, and S. D. Pradhan. 1969. Preparation of *n*-long chain oxypropanols and oxybutanols and their performance as water evaporation retardants. *Indian J. Tech.* **7**: 93–96.
14. Verkade, P. E., and J. D. van Roon. 1942. Uber die α γ-Benzylidenglycerole. *Rec. Trav. Chim.* **61**: 831–834.
15. Prakash, C., S. Saleh, D. F. Taber, and I. A. Blair. 1989. Synthesis of trideuterated *O*-alkyl platelet activating factor and lyso derivatives. *Lipids.* **24**: 786–792.
16. Eibl, H. 1980. Synthesis of glycerolipids. *Chem Phys. Lipids.* **26**: 405–429.
17. Eibl, H., D. Arnold, H. U. Weltzien, and O. Westphal. 1967. Zur Synthese von α- und β-Lecithinen und ihren Atheranaloga. *Justus Liebigs Ann. Chem.* **709**: 226–230.
18. Bhatia, S. K., and J. Hajdu. 1988. Stereospecific synthesis of ether and thioether phospholipids. The use of L-glyceric acid as a chiral phospholipid precursor. *J. Org. Chem.* **53**: 5034–5039.
19. Murari, R., and W. Baumann. 1981. Quadrupolar ^{13}C–^{14}N couplings and ^{14}N relaxations in aggregated and nonaggregated choline phospholipids. *J. Am. Chem. Soc.* **103**: 1238–1240.
20. Murari, R., M. A. A. El-Rahman, Y. Wedmid, S. Parthasarathy, and W. J. Baumann. 1982. Carbon-13 nuclear magnetic resonance spectroscopy of phospholipids in solution. Spectral and stereochemical assignments based on ^{13}C–^{31}P and ^{13}C–^{14}N couplings. *J. Org. Chem.* **47**: 2158–2163.
21. Heymans, F., E. Michel, M-C. Borrel, B. Wichrowski, J-J. Godfroid, O. Convert, E. Coeffier, M. Tence, and J. Benveniste. 1981. New total synthesis and high resolution ^1H NMR spectrum of platelet activating factor, its enantiomer and racemic mixtures. *Biochim. Biophys. Acta.* **666**: 230–237.
22. Bridsall, N. J. M., J. Feeney, A. G. Lee, Y. K. Levine, and J. C. Metcalfe. 1972. Dipalmitoyl-lecithin:assignment of the ^1H and ^{13}C nuclear magnetic resonance spectra and conformational studies. *J. Chem. Soc. Perkin Trans.* **2**: 1441–1445.
23. Aue, W. P., E. Bartholdi, and R. R. Ernst. 1976. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *J. Chem. Phys.* **64**: 2229–2246.