papers and notes on methodology

Synthesis of a naphthylvinyl-labeled glycerol ether analog of phosphatidylcholine and its use in the assay of phospholipase A_2

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Abstract The synthesis of a naphthylvinyl-labeled glycerol ether analog of phosphatidylcholine, 1-O-{12-(2-naphthyl)-dodec-11-enyl}-2-O-decanoyl-*sn*-glycerol-3-phosphocholine (NVPC), is described. This involves a Wittig reaction between 2-naphthaldehyde and a phosphonium salt which gives the *trans*-naphthylvinyl group as the predominant isomer. Lyso NVPC was prepared from NVPC by phospholipase A_2 action. NVPC absorbs strongly at 248 nm (ϵ = 58,300 M^{-1} cm^{-1}) and gives broad fluorescence emission with maxima at 343 nm and 360 nm and a quantum yield of 0.10 in ethanol. An assay for phospholipase A_2 was developed using high performance liquid chromatography with fluorescence detection to separate and quantify NVPC and lyso NVPC. Activities as low as 1–2 pmol/min in an assay volume of 0.1 ml can easily be measured. The assay was used with a pure enzyme from cobra venom and a crude enzyme from synovial fluid. Enzyme specificities for phosphatidylcholine and NVPC with cobra venom and porcine pancreatic phospholipases A_2 were compared using a titrametric assay. The use of the assay with NVPC to study the metabolism of platelet activating factor is discussed. — **Hendrickson, H. S., E. K. Hendrickson, and T. J. Rustad.** Synthesis of a naphthylvinyl-labeled glycerol ether analog of phosphatidylcholine and its use in the assay of phospholipase A_2. *J. Lipid Res.* 1987. **28:** 864–872.

Supplementary key words fluorescence • high performance liquid chromatography • platelet activating factor • synovial fluid

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the 2-acyl ester group of *sn*-3-glycerophospholipids (1). The activities of intracellular and membrane-bound phospholipases A_2 are very low. Sensitive assays are thus required to measure this activity. The most common assay involves the use of a phospholipid with a ^{14}C-labeled fatty acid in the *sn*-2 position (2, 3). The products of PLA_2 action are separated by TLC and the zones corresponding to fatty acid, phospholipid, and lyso phospholipid are scraped into vials containing scintillation fluid and counted. This is a very tedious and time-consuming procedure which requires the use of expensive radiolabeled lipids. In addition, this assay lacks specificity since PLA_1 and lysophospholipase activities, which are often present in crude cellular preparations, also result in the liberation of labeled fatty acid.

The high sensitivity of fluorescence suggests the use of a fluorescent label rather than a radiolabel. The fluorescent phospholipid, 1-acyl-2-{6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl}phosphatidylcholine (NBD-PC) has been used in an assay of PLA_2 (4). The use of this lipid gave confusing results since a product identified as lyso NBD-PC was also released by PLA_2 action, and the rate of release of NBD-caproic acid was biphasic. A more fundamental objection to the use of this lipid, however, is the potential perturbing effect of the NBD label. It is both a bulky and polar group in a position where PLA_2 might exhibit fatty acid specificity. In the hope of designing a nonperturbing fluorescent phospholipid where the fatty acid in the *sn*-2 position could be easily varied, we chose to put a naphthyl group at the end of the *sn*-1 chain. The simplest way to introduce the naphthyl group is by a Wittig reaction between 2-naphthaldehyde and a phosphonium salt. This results in a naphthylvinyl group which is nonpolar and not too bulky. In order to make a highly specific substrate for PLA_2, we chose to attach the *sn*-1 chain by an ether linkage. This would then be stable to PLA_1 and lysophospholipase action. An added advantage of the *sn*-1 ether linkage is the structural similarity to natural glycerol ether lipids metabolically related to platelet activating factor (PAF). This new fluorescent phospholipid could also be useful in the study of PAF metabolism. We present here the synthesis of naphthylvinyl phosphatidylcholine (NVPC), a glycerol ether phospholipid with the naphthylvinyl fluorescent label (**Fig. 1**). A sensi-

Abbreviations: PLA_2, phospholipase A_2; PLA_1, phospholipase A_1; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; PAF, platelet activating factor; NBD, 7-nitro-2,1,3-benzoxadiazole-4-yl; NVPC, 1-O-[12-(2-naphthyl)-dodec-11-enyl]-2-O-decanoyl-*sn*-glycerol-3-phosphocholine; lyso NVPC, 1-O-{12-(2-naphthyl)-dodec-11-enyl]-*sn*-glycerol-3-phosphocholine; PC, phosphatidylcholine.

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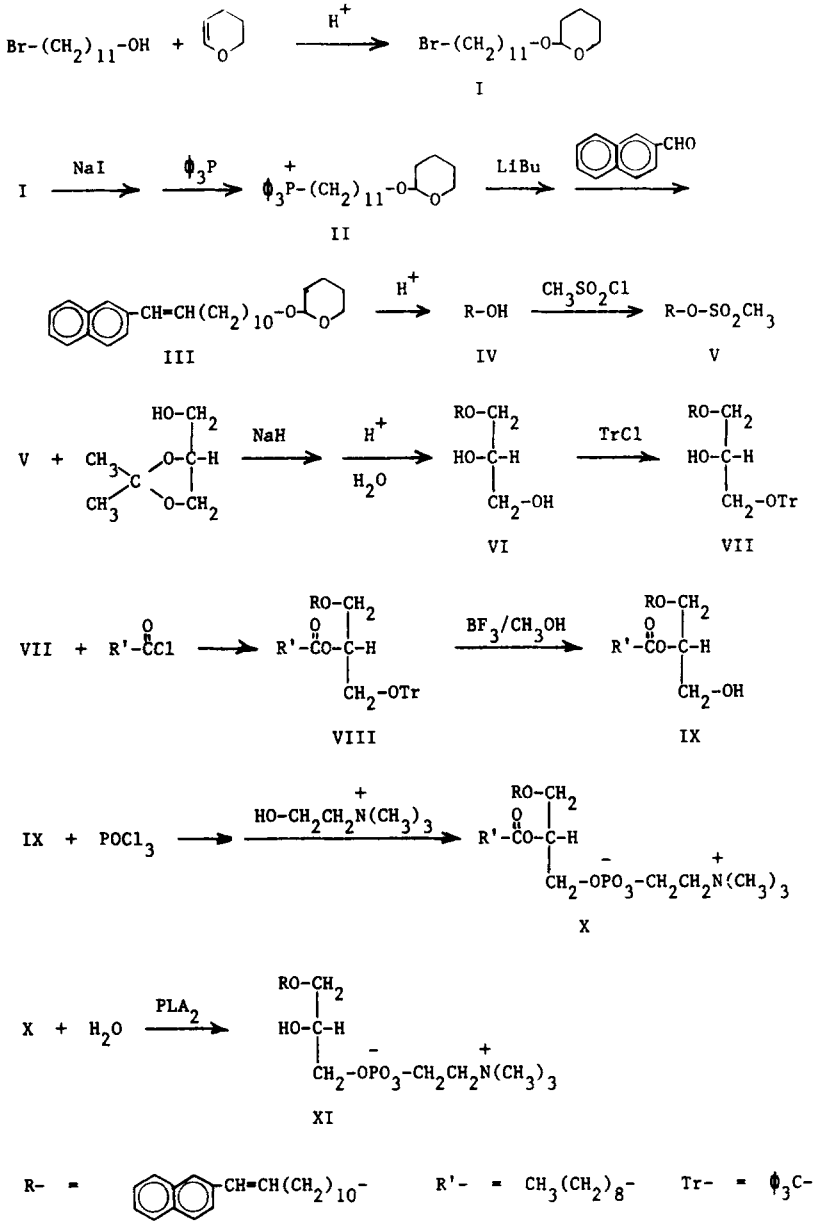

Fig. 1. Synthesis of NVPC and lyso NVPC.

tive, rapid assay for PLA_2, which utilizes high performance liquid chromatography (HPLC) with fluorescence detection to identify and quantify the products of hydrolysis, is also presented.

EXPERIMENTAL PROCEDURES

Materials

11-Bromoundecanol, *n*-butyl lithium, 2-naphthaldehyde, *p*-toluenesulfonic acid, mesyl (methanesulfonyl) chloride, sodium hydride, trityl (triphenylmethyl) chloride, decanoyl chloride, and boron trifluoride in methanol were obtained from Aldrich Chemical Co., Milwaukee, WI. 2,3-O-Isopropylidine-*sn*-glycerol was synthesized from L-serine by the procedure of Lok, Ward, and van Dorp (5). Choline *p*-toluenesulfonate was prepared according to Brockerhoff and Ayengar (6). Dry tetrahydrofuran was prepared immediately before use by distillation from a refluxing mixture of 500 ml of tetrahydrofuran, 8 g of benzophenone, and 1 g of sodium. A purple color in the refluxing mixture indicated the presence of active sodium ketyl drying agent. Benzene was dried by azeotropic distillation,

pyridine was distilled over barium oxide, and chloroform was dried by distillation over phosphorus pentoxide prior to use. All other solvents were reagent or HPLC grade. Silica gel for column chromatography (60 Ångstrom, 75–150 micron, cat. #13100) and TLC plates (cat. #01521) were obtained from Analtech, Inc., Newark, DE. PLA_2 from cobra (*Naja naja naja*) venom was obtained as a generous gift from Dr. Edward A. Dennis, University of California at San Diego. PLA_2 was purified from porcine pancreas by the method of Nieuwenhuizen, Kunze, and de Haas (7). Human synovial fluid from a rheumatoid arthritic patient was obtained from Dr. James L. Reinertsen, Park Nicollet Medical Center, Minneapolis, MN. Didecanoyl *sn*-3-phosphatidylcholine was synthesized as described by Jensen and Pitas (8).

Chromatography

TLC was carried out as described by Hendrickson, Hendrickson, and Dybvig (9). Fluorescent compounds were also visualized with a UV lamp. Fractions from preparative column chromatography were analyzed by TLC.

Analytical methods

Phosphorus was determined by the method of Eaton and Dennis (10). UV-absorption spectroscopy was performed on a Perkin-Elmer Lambda 3B spectrophotometer. Fluorescence spectra were run on a Perkin-Elmer MPF-66 spectrofluorometer. NMR spectra were recorded on a 200 MHz IBM 200-AF Fourier-transform spectrometer. Elemental analyses (C, H, N, P) were performed by Galbraith Laboratories, Inc., Knoxville, TN.

HPLC phospholipase A_2 assay

NVPC (50 μl of a 1 mM solution in $CHCl_3$) was placed in a small test tube and the solvent was evaporated under nitrogen and in vacuo. Triton X-100 (10 μl of a 10 mM solution), 190 μl of buffer (0.395 M NaCl, 66 mM Tris chloride, 13.15 mM $CaCl_2$, pH 8.0), and 250 μl of water were added to the dried lipid. The mixture was vortexed until the lipid was completely dispersed. The assay was initiated by adding 10 μl of cobra venom PLA_2 (2.4 ng) to 90 μl of the substrate mixture at room temperature. A blank contained 10 μl of water and 90 μl of substrate mixture. The final concentrations were: 0.1 mM NVPC, 0.2 mM Triton X-100, 0.15 M NaCl, 5 mM $CaCl_2$, 25 mM Tris, pH 8.0. Assays of synovial fluid were prepared in a similar manner, but using a smaller volume of more concentrated buffer so that a larger volume of synovial fluid could be used. The final concentrations of buffer components were the same.

At various time intervals, 10 μl of assay mixture or control was removed and added to 90 μl of hexane–isopropanol–acetic acid 6:8:1.6 to quench enzyme activity. The quenched sample was either filtered through a 0.45-μm Nylon-66 filter or centrifuged for 2 min at 15,600 *g* (microcentrifuge), and 20 μl was analyzed by HPLC with a 15-cm silica gel column (#85774, Waters Associates, Milford, MA). A flow rate of 1 ml/min of hexane–isopropanol–water 6:8:1.6 was maintained with a Kratos Spectroflow 400 pump. Fluorescence was detected (excitation = 250 nm, emission > 320 nm) with a Kratos Spectroflow 980 detector and recorded on a Spectra-Physics SP 4290 integrator-plotter. The amount of lyso NVPC released, n, was calculated by the equation: n = (lyso NVPC area)(initial NVPC moles)/(lyso NVPC area + NVPC area).

Titrametric phospholipase A_2 assay

PLA_2 was assayed using a pH-stat by the method of Deems and Dennis (11). Assays were run at 38°C in the presence of 0.1 M KCl and 10 mM $CaCl_2$. The substrates were prepared as mixed micelles with Triton X-100 or sodium cholate at the concentrations indicated.

Chemical syntheses

Preparation of 11-bromo-undecyl tetrahydropyranyl ether (I) (Fig. 1). 11-Bromo undecanol (20 g, 0.08 mol) was converted to the tetrahydropyranyl ether using a procedure described by Bernady et al. (12). The crude product was applied to an 80-g silica gel column in two equal portions and eluted with hexane–ethyl acetate 12:1. A pure product (24.8 g of a pale yellow oil) was obtained in 92.5% yield. TLC with hexane–ethyl acetate 9:1: R_f = 0.50.

Preparation of 11-(2-tetrahydropyranoxy)-undecanyl triphenylphosphonium iodide (II). The 11-bromo-undecyl tetrahydropyranyl ether (28.6 g, 0.16 mol) was converted to the iodo compound and then to the phosphonium salt using the procedure described for the preparation of 8-carbethoxyoctyl triphenylphosphonium iodide by Bergelson (13). Crystals of the phosphonium salt (43.0 g, mp 48°C) were obtained in 85% yield.

Preparation of 1-(2-naphthyl)-12-(2-tetrahydropyranoxy)-dodec-1-ene (III). Approximately 12.5 g of compound II was evaporated with 30 ml of benzene to yield 12.46 g (19.3 mmol) of dry phosphonium salt. The salt was stirred under nitrogen with 30 ml of tetrahydrofuran that had been dried previously over sodium ketyl. When the salt dissolved, 20 mmol of *n*-butyl lithium was added slowly. The solution quickly turned a deep orange because of the presence of the ylide. The solution was stirred for 15 min, and then 2.01 g (12.9 mmol) of 2-naphthaldehyde was added. The reaction was stirred under nitrogen for an additional 2 hr, deactivated with water, and taken up in 50 ml of methylene chloride. This was added to 100 ml of ethyl ether and washed three times with 50 ml of water. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to give a yellow oil (13.8 g). The oil was extracted twice in an ice bath with 50-ml portions of ethyl ether, and the ether extracts were evaporated to give a lemon yellow oil containing colorless crystals of triphenyl-

phosphonium oxide (combined weight: 9.67 g). The oil was poured off and the crystals were washed twice with cold hexane. The hexane washes were added to the oil and the solvent was evaporated. This crude product was applied to a 120-g silica gel column and eluted with 5% ethyl acetate in hexane. Pure product was obtained as a pale yellow oil (4.42 g, 87% yield). TLC with hexane–ethyl acetate 9:1: R_f = 0.53. IR: aromatic C–H at 3056 cm^{-1} and 3009 cm^{-1}; *trans*–CH=CH– at 964.5 cm^{-1}

Preparation of 12-(2-naphthyl)-dodec-11-en-1-ol (IV). The product of the last reaction (III, 4.49 g, 11.4 mmol) was taken up in 216 ml of ethanol and stirred for 15 min in a 60°C water bath. *p*-Toluene sulfonic acid monohydrate (2.16 g, 11.4 mmol) was added to the solution and stirring at 60°C was continued for 15 min. The solution was evaporated to near dryness, taken up in 250 ml of ethyl ether, washed three times with 75-ml portions of water, dried over $MgSO_4$, and evaporated to give white crystals. Crystallization from hexane gave white crystals of pure product (3.08 g, 87% yield) (mp 81–83°C). TLC with hexane–ethyl acetate 9:1: R_f = 0.11. The IR spectrum showed a large O–H peak at 3350 cm^{-1}. NMR (200 MHz) ($CDCl_3$): 1.27–1.58 ppm (m, C-2 to C-9 $-CH_2-$); 2.25 ppm (q (7Hz), *trans* isomer C-10 $-CH_2-$); 2.40 ppm (q (7Hz), *cis* isomer C-10 $-CH_2-$); 3.61 ppm (t (7 Hz), C-1 $-CH_2-$); 5.74 ppm (d (12) Hz) of t (7 Hz), *cis* isomer C-11 =CH–); 6.34 ppm (d (16 Hz) of t (7 Hz), *trans* isomer C-11 =CH–); 6.54 ppm (d (16 Hz), *trans* isomer C-12 –CH=); 6.55 ppm (d (11 Hz), *cis* isomer C-12 –CH=); 7.35–7.82 ppm (m, aromatic C–H).

Preparation of 12-(2-naphthyl)-dodec-11-enyl mesylate (V). To a solution of 6.12 g (19.7 mmol) of 12-(2-naphthyl)-dodec-11-en-1-ol (IV) in 15 ml of dry pyridine and 11 ml of hexane, 2.71 g (23.7 mmol) of mesyl chloride was added slowly with stirring in a water bath at room temperature. Stirring was continued and the water bath was warmed to 40°C. After 1 hr, TLC (hexane–acetone 7:3: product R_f = 0.53) showed the reaction to be complete. The reaction mixture was diluted with 60 ml of hexane–benzene 1:1 and washed successively with water, 0.5 N HCl until acidic, water, 5% $NaHCO_3$, and water (twice). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. Crystallization from hexane–acetone gave 6.04 g (79% yield) of white crystals, mp 65–69°C.

*Preparation of 1-O-{12-(2-naphthyl)-dodec-11-enyl}-*sn*-glycerol (VI).* 2,3-O-Isopropylidine-*sn*-glycerol (1.4 g, 10.71 mmol) was slowly added to a solution of 0.6 g of NaH in 35 ml of dry benzene with stirring at room temperature. The mesylate (V)(3.75 g, 9.65 mmol) was added and the reaction was heated to reflux at about 80°C for 4 hr. After cooling in an ice bath, methanol was added to the reaction mixture to destroy the excess NaH, and the mixture was neutralized with 20% acetic acid in methanol. The reaction mixture was diluted with 40 ml of benzene and washed twice with equal volumes of methanol–water 1:1. The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness. The solid was dissolved in 5 ml of methanol. Twenty ml of 10% conc. HCl in methanol was added and the mixture was heated on a steam bath for 20 min. The mixture was evaporated to about ⅓ its volume, diluted with 50 ml of $CHCl_3$, and washed twice with methanol–water 1:1. The organic phase was evaporated and dried under vacuum. The crude product was applied to a 30-g silica gel column in 10% acetone in hexane, and eluted stepwise with 10%, 20%, and 30% acetone in hexane. The product (1.75 g, 47% yield) was a pale yellow solid. TLC in hexane–acetone 7:3: R_f = 0.41. Mesylate starting material (V) (0.84 g) was recovered in 22% yield.

*Preparation of 1-O-{12-(2-naphthyl)-dodec-11-enyl}-3-O-trityl-*sn*-glycerol (VII).* Compound VI (3.59 g, 9.3 mmol) was added to 3.12 g (11.2 mmol) of trityl chloride in 30 ml of dry pyridine and stirred overnight at room temperature. The reaction mixture was poured into 30 ml of ice and water, and extracted with 80 ml of ethyl ether. The ether phase was washed with 0.5 M HCl until acidic, water, 2% $NaHCO_3$, and twice with water. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. Benzene and ethanol were added and evaporated to remove traces of water. The dried residue was applied to a 28-g silica gel column in 7% acetone in hexane. The column was eluted stepwise with 7%, 15%, and 30% acetone in hexane. The product was obtained (5.43 g, 93% yield) as an oil. TLC in hexane–acetone 7:1: R_f = 0.34.

*Preparation of 1-O-{12-(2-naphthyl)-dodec-11-enyl}-2-O-decanoyl-3-O-trityl-*sn*-glycerol (VIII).* Compound VII (8.66 g, 13.8 mmol) was dissolved in 15 ml of dry pyridine and 30 ml of hexane and stirred in an ice bath. A solution of 3.95 g (20.7 mmol) of decanoyl chloride in 10 ml of hexane was added with stirring over 5 min. The ice bath was removed and stirring was continued at room temperature for 30 min. TLC (hexane–acetone 7:1: product R_f = 0.48) showed the reaction to be complete. The reaction mixture was diluted with 100 ml of benzene and washed twice with 0.5 M NH_3 in methanol–water 3:1 followed by two washes with methanol–water 1:1. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was applied to an 80-g silica gel column in 15% ethyl ether in hexane and eluted in the same solvent. A clear oil was obtained (8.46 g, 78% yield).

*Preparation of 1-O-{12-(2-naphthyl)-dodec-11-enyl}-2-O-decanoyl-*sn*-glycerol (IX).* Compound VIII (8.46 g, 10.8 mmol) was detritylated with BF_3/methanol using the method described by Hendrickson et al. (9). The crude product was applied to an 80-g silica gel column in 15% ethyl ether in hexane and eluted stepwise with 15%, 30%, and 50% ethyl ether in hexane. A pure product (1.88 g, mp 25–27°C, TLC in hexane–ether 7:3: R_f = 0.17) was obtained along with a fraction (2.63 g) which contained a small impurity with a slightly larger R_f value. The combined yield (4.51 g) was 77%.

*Preparation of 1-O-{12-(2-naphthyl)-dodec-11-enyl}-2-O-decanoyl-*sn*-glycerol-3-phosphoryl choline (X).* The pure detritylated compound (IX) (1.88 g, 3.49 mmol) was converted to the choline phosphate by treatment with $POCl_3$ followed by choline tosylate using the method described by Hendrickson et al. (9) with slight modification. The alcohol was added to $POCl_3$ in chloroform and pyridine at 0°C, followed by stirring at room temperature for 1 hr. Choline tosylate in pyridine was added and the mixture was stirred at room temperature overnight. The crude product was applied to a 30-g silica gel column in chloroform–methanol 3:1 and eluted with the same solvent followed by stepwise elution with chloroform–methanol–conc. ammonia–water mixtures (65:25:0.5:0.5, 65:25:1:1, and 65:35:2:2). A pure product (1.12 g, 45.5% yield) was obtained as a white fluorescent solid. TLC in chloroform–methanol–conc. ammonia–water 95:54:5.5:2: R_f = 0.30. This compound gave a single peak (fluorescence detection: ex = 250 nm, em > 320 nm) upon HPLC on a silica gel column in hexane–isopropanol–water 6:8:1.6. NMR (200 MHz)($CDCl_3/CD_3OD$, 9/1): 0.72 ppm (t (7Hz), acyl-CH_3); 1.11–1.13 ppm (m, -CH_2-); 1.30–1.48 ppm (m, β-acyl -CH_2-); 2.10 ppm (q (7 Hz) alkyl C-10 -CH_2-); 2.17 ppm (t (7 Hz), α-acyl -CH_2-CO); 3.04 ppm (s, $N(CH_3)_3$); 3.25–3.29 ppm (m, -CH_2-N); 3.40–3.44 ppm (m, glycerol *sn*-3 -CH_2- and alkyl C-1 -CH_2-); 3.78–3.85 ppm (m, glycerol *sn*-1 -CH_2-); 4.00–4.13 ppm (m, choline P-O-CH_2-); 4.93–5.04 ppm (m, glycerol *sn*-2-CH-); 5.59 ppm (d (12 Hz) of t (7 Hz), *cis* isomer alkyl C-11 =CH-); 6.20 ppm (d (16 Hz) of t (7 Hz), *trans* isomer alkyl C-11 =CH-); 6.39 ppm (d (16 Hz), *trans* isomer alkyl C-12 -CH=); 6.40 ppm (d (10 Hz), *cis* isomer alkyl C-12-CH=); 7.19–7.67 ppm (m, aromatic C-H). Anal. calcd. for $C_{40}H_{66}PNO_7 \cdot H_2O$: C, 66.54; H, 9.49; N, 1.94; P, 4.29. Found: C, 64.69; H, 9.19; N, 2.04; P, 4.92.

*Preparation of 1-O-{12-(2-naphthyl)-dodec-11-enyl}-*sn*-glycerol-3-phosphoryl choline (XI).* Compound X (0.66 g, 0.94 mmol) was added to 100 ml of ethyl ether–methanol 49:1 and 3 ml of 0.1 M sodium borate buffer (containing 4 mg/ml of calcium acetate, pH = 7.4) was added. Phospholipase A_2 (300 μl containing 176 units of cobra venom enzyme, #P-6139, Sigma Chemical Co., St. Louis, MO) was added and the mixture stirred vigorously at 30°C in a glass-stoppered flask overnight. The mixture was evaporated to dryness and the residue was applied to a 30-g silica gel column in chloroform–methanol 3:1. The column was eluted stepwise with chloroform–methanol–conc. ammonia–water mixtures (65:25:1:1, 20:14:0.5:0.5, and 20:14:1:1). The lyso product (0.43 g) was obtained in 83% yield. This compound gave a single peak (fluorescence detection: ex = 250 nm, em > 320 nm) upon HPLC on a silica gel column in hexane–isopropanol–water 6:8:1.6. Anal. calcd. for $C_{30}H_{48}PNO_6 \cdot H_2O$: C, 63.47; H, 8.87; N, 2.46; P, 5.42. Found: C, 64.19; H, 8.70; N, 2.38; P, 5.46.

RESULTS

The synthesis of 12-(2-naphthyl)-dodec-11-en-1-ol (IV) was adapted from the synthesis of 12-(9-anthryl)-11-*trans*-dodecenoic acid as reported by Bergelson (13). Bergelson used sodium ethoxide as the base for his Wittig reaction. This proved too weak a base for a Wittig reaction, so we utilized a much stronger base, *n*-butyl lithium, and a protecting group, the tetrapyranyl ether, which was stable with this strong base. The Wittig reaction proceeds with good yield (87%), and gives the *trans* isomer as the major product as evidenced by its IR peak at 964.5 cm^{-1} and NMR spectrum. Spin-spin coupling constants for vinyl protons are in the range of 12–18 Hz for *trans* and 6–12 Hz for *cis* (14). The NMR spectrum of the naphthylvinyl alcohol (**Fig. 2**) shows splitting for the vinyl protons of about 11–12 Hz for the *cis* isomer and 16 Hz for the *trans* isomer. The C-11 vinyl proton gives a doublet (16 Hz) of triplets (7 Hz) at 6.34 ppm for the *trans* isomer, and a doublet (12 Hz) of triplets (7 Hz) at 5.74 ppm for the *cis* isomer. The C-12 proton gives a doublet (16 Hz) at 6.54 ppm for the *trans* isomer, and a doublet (11 Hz) at 6.55 ppm for the *cis* isomer. Other peak assignments are given in the Experimental section under Chemical syntheses. Integration of the C-11 proton peaks from both isomers indicate about 81% *trans* isomer.

Incorporation of the naphthylvinyl alcohol into NVPC was accomplished by standard synthetic procedures. NVPC was converted to lyso NVPC by treatment with PLA_2. HPLC analyses during the course of this reaction showed a small amount of material with an R_f similar to that of NVPC which was resistant to hydrolysis. This could be due to a *sn*-2 phosphocholine isomer resulting from some acyl migration prior to phosphorylation. Since PLA_2 is specific for only the *sn*-1 position of such isomers, this isomer with an ether linkage at the *sn*-1 position would not be hydrolyzed. The lyso NVPC should thus be devoid of any such positional isomer.

The UV absorption spectrum of NVPC shows a strong peak at 248 nm (ϵ = 58,300 M^{-1} cm^{-1}) and weaker peaks at 256 nm, 276 nm, 286 nm, and 297 nm. Excitation of NVPC at 248 nm gives broad fluorescence emission with maxima at 343 nm and 360 nm in ethanol and hexane, and 346 nm and 363 nm in water (**Fig. 3**). The quantum yield of NVPC in ethanol was determined to be 0.10 using quinine as a reference (quantum yield for quinine bisulfate in 1 N H_2SO_4 = 0.55 (15)). This is similar to the quantum yield of 0.10 for naphthalene (15). Lyso NVPC had the same molar extinction coefficient and quantum yield as NVPC.

An HPLC assay was developed for PLA_2 using NVPC as a substrate and the cobra venom enzyme. NVPC and lyso NVPC are cleanly separated by HPLC on a silica gel column with hexane–isopropanol–water 6:8:1.6 as a solvent

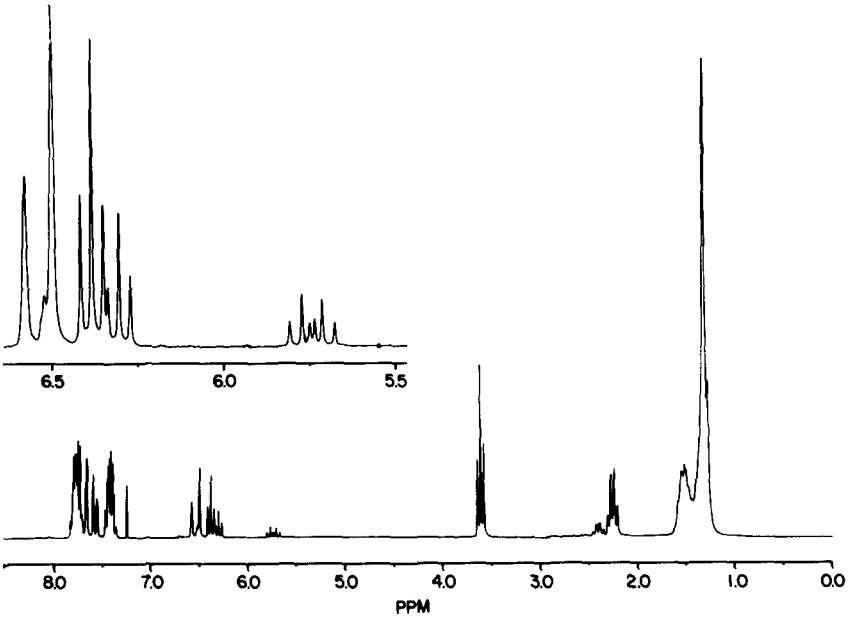

Fig. 2. NMR spectrum of naphthylvinyl alcohol (IV) at 200 MHz. Insert: vinyl protons, 5.7–6.6 ppm. Solvent: $CDCl_3$.

(**Fig. 4**). The amount of lyso NVPC released was calculated from the area ratio of lyso NVPC over NVPC plus lyso NVPC. A linear release of lyso NVPC was observed over a period of time up to 4% hydrolysis (**Fig. 5A**).

The specificities of PLA_2 from cobra venom and porcine pancreas for PC and NVPC were compared using a titrametric assay. The results, shown in **Table 1,** indicate that the cobra venom enzyme is about half as active towards NVPC as compared with PC in mixed micelles with Triton X-100. The pancreatic enzyme, however, showed about the same activity towards both substrates in mixed micelles with sodium cholate.

A sample of human synovial fluid from a rheumatoid arthritic patient was analyzed for PLA_2 activity using the HPLC assay with a 2:1 ratio of Triton X-100 to NVPC. A typical time course is shown in Fig. 5B. An activity of

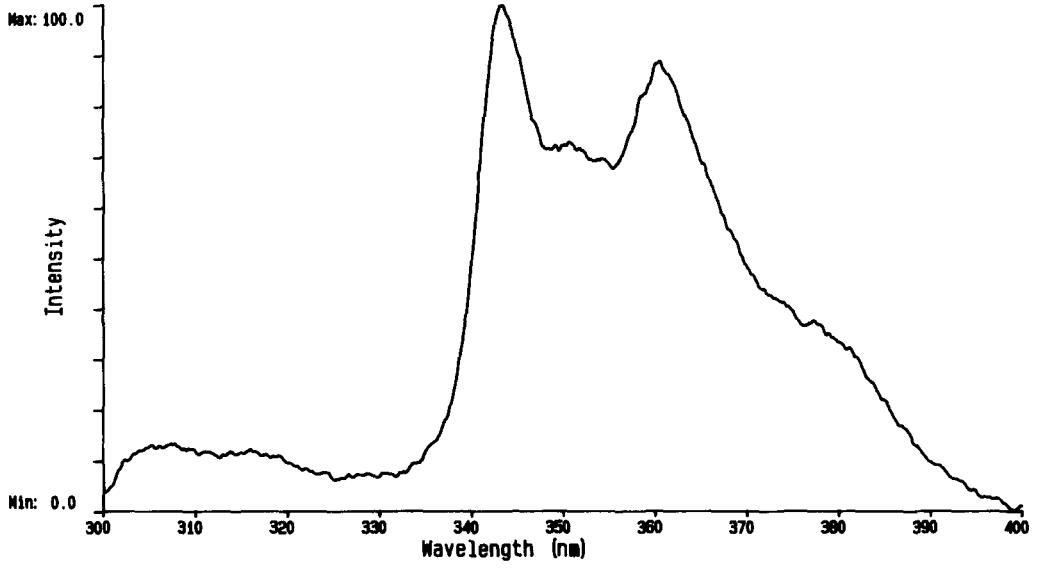

Fig. 3. Fluorescence spectrum of NVPC in ethanol.

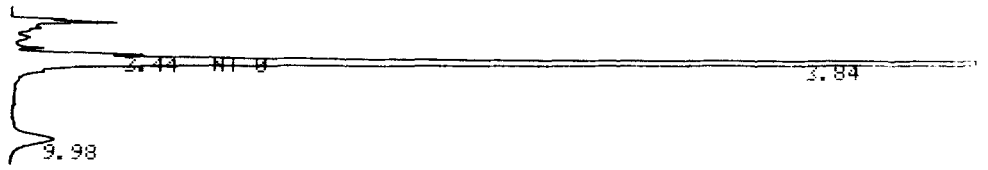

Fig. 4. HPLC analysis of NVPC and lyso NVPC from PLA_2 assay. Solvent: hexane–isopropanol–water 6:8:1.6, 1 ml/min. Fluorescence detection: excitation = 250 nm, emission > 320 nm. Retention times: NVPC, 3.84 min; lyso NVPC, 9.98 min.

124 pmol/min per ml of fluid was calculated. An activity of 840 pmol/min per ml of fluid was observed in the assay of synovial fluid using a sonicated dispersion of NVPC (0.1 mM) in the absence of Triton X-100. Thus the PLA_2 activity in synovial fluid appears to be inhibited by Triton X-100. This assay has proven suitable in a preliminary study of PLA_2 activity in synovial fluid (Hendrickson, H. S., and M. E. Benton, unpublished results).

A sample of lyso NVPC was acetylated using acetic anhydride and pyridine. The naphthylvinyl PAF analog thus produced was sent to Dr. Donald J. Hanahan for biological assay. It gave an ED_{50} value (16) for platelet aggregation of 1.0×10^{-9} M compared to a value of 1.33×10^{-10} M for synthetic *sn*-3 hexadecyl acetyl glycerol ether phosphatidylcholine (D. J. Hanahan, University of Texas Health Science Center at San Antonio, personal communication).

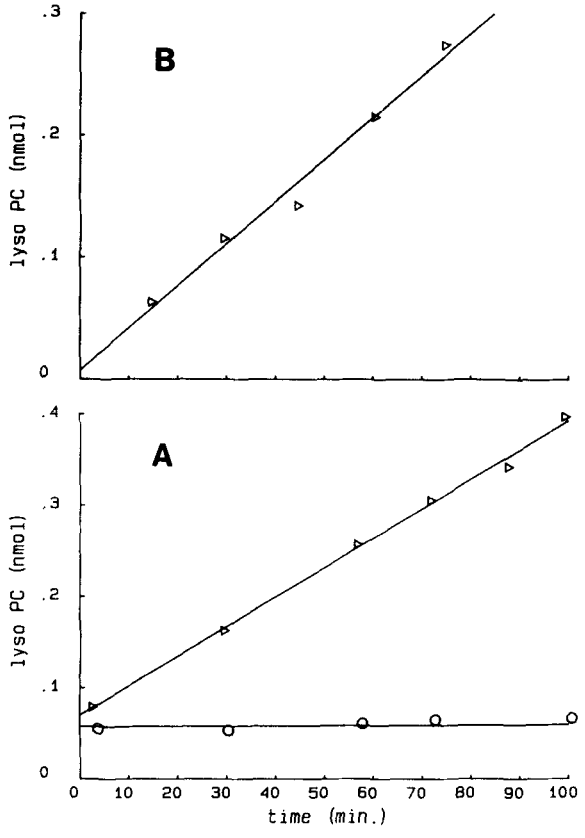

Fig. 5. Assay of PLA_2. Release of lyso NVPC as a function of time. Initial amount of substrate, NVPC, is 10 nmol/0.1 ml (0.1 mM); 0.2 mM Triton X-100. A. Control (no enzyme), ○; 2.4 ng of cobra venom PLA_2, Δ. pH = 8. Calculated PLA_2 activity: 3.2 pmol/min. B. Human synovial fluid (27.8 μl). pH = 6.8. Calculated PLA_2 activity: 3.45 pmol/min.

DISCUSSION

NVPC was synthesized primarily as a substrate for an HPLC assay of PLA_2. The naphthylvinyl group serves as a nonpolar, nonperturbing fluorescent label. A molecular model of NVPC shows that the naphthylvinyl group is only about 50% larger in cross-section diameter than a saturated hydrocarbon chain. The *trans* isomer, which is the major product of the Wittig reaction, is favored because of greater steric hindrance in the *cis* isomer. The low quantum yield for the naphthylvinyl group (0.10) is compensated by its very large extinction coefficient (58,300 M^{-1} cm^{-1}). The fluorescence sensitivity, which is the product of quantum yield and extinction coefficient, compares favorably with other fluorescent probes.

The ether linkage at the *sn*-1 position of NVPC was chosen to eliminate any PLA_1 and lysophospholipase activities. These phospholipases are not known to catalyze the hydrolysis of a saturated ether linkage (1). Other enzyme activities (phospholipase C, for example) can easily be differentiated from phospholipase A_2 and measured by observing products with different retention times. The

TABLE 1. Specific activities of different phospholipases A_2 toward PC and NVPC in mixed micelles by titrametric assay

Enzyme Source	Specific Activity[a] (μmol min^{-1} mg^{-1}) PC[b]	NVPC
Naja naja naja	1378 ± 79	732 ± 99
Porcine pancreas	808 ± 185	686 ± 46

[a] Conditions: 1 mM phospholipid in mixed micelles. 10 mM Triton X-100 for the *Naja naja naja* enzyme, and 5 mM sodium cholate for the pancreatic enzyme. Temperature, 38°C. Mean ± standard deviation (n = 5). Initial rates are reported.

[b] Didecanoyl phosphatidylcholine.

assay with NVPC is thus highly specific for PLA_2 in the presence of other phospholipases. Activities as low as 1–2 pmol/min in an assay volume of 0.1 ml can easily be measured. This assay seems to be about as sensitive as a radioisotope assay using PC labeled in the *sn*-2 position with ^{14}C-labeled fatty acid. It is, however, much simpler and less time-consuming; samples from the assay mixture can be analyzed in 15–20 min. Different fatty acyl NVPC substrates can readily be synthesized from lyso NVPC so that fatty acid specificity at the *sn*-2 position can be determined.

PLA_2 activity has been reported to occur in human synovial fluid (17–19). Accordingly, we subjected synovial fluid from a rheumatoid arthritic patient to the HPLC assay and found that we could easily determine this activity. The physical state of the substrate is quite important depending on the source of enzyme. Initial assays of synovial fluid were run with mixed micelles of NVPC and Triton X-100. A sonicated dispersion of NVPC in the absence of Triton X-100, however, gave much higher activity. It is important, therefore, to find the optimal physical state of the substrate for the enzyme being studied. This assay has thus proven quite effective with crude enzyme preparations.

The specificities of PLA_2 from different sources for PC and NVPC were determined using a titrametric assay. Cobra venom PLA_2 showed about half the activity with NVPC as compared with PC in mixed micelles with Triton X-100. Porcine pancreatic PLA_2, however, showed about the same activity with both substrates in mixed micelles with sodium cholate. The lower activity of the cobra venom enzyme with NVPC-Triton X-100 mixed micelles may be due to tighter packing of phospholipid in this system. Certainly the substitution of an ether for an ester group decreases the polarity of the head group in NVPC leading to less headgroup repulsion, and this could lead to tighter packing, particularly in the presence of a nonionic detergent. The activity of PLA_2 is quite dependent on surface pressure or packing density of phospholipids in monolayer systems (20). This again emphasizes the importance of the physical state of phospholipid substrate with respect to the particular PLA_2.

The similarity of lyso NVPC to lyso PAF makes this a potentially useful substrate for an HPLC assay of the acetyl transferase involved in the synthesis of PAF and also for an arachidonyl transferase which may be involved in a putative PAF cycle (21). NVPC, with an ether linkage at the *sn*-1 position, may be a better substrate than phosphatidylcholine for a study of the PLA_2 involved in PAF metabolism. Finally, the high biological activity of acetyl NVPC (naphthylvinyl PAF analog) makes this a potentially useful probe for the study of PAF receptors.

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REFERENCES

1. Dennis, E. A. 1983. Phospholipases. *In* The Enzymes. 3rd ed. Vol. 16. P. Boyer, editor. Academic Press, New York. 307–353.
2. van den Bosch, H., and A. J. Aarsman. 1979. A review on methods of phospholipase A determination. *Agents Actions.* **9:** 382–389.
3. Ross, M. I., R. A. Deems, A. J. Jesaitis, E. A. Dennis, and R. J. Ulevitch. 1985. Phospholipase activities of the $P388D_1$ macrophage-like cell line. *Arch. Biochem. Biophys.* **238:** 247–258.
4. Wittenauer, L. A., K. Shirai, R. L. Jackson, and J. D. Johnson. 1984. Hydrolysis of a fluorescent phospholipid substrate by phospholipase A_2 and lipoprotein lipase. *Biochem. Biophys. Res. Commun.* **118:** 894–901.
5. Lok, C. M., J. P. Ward, and D. A. van Dorp. 1976. The synthesis of chiral glycerides starting from D- and L-serine. *Chem. Phys. Lipids.* **16:** 115–122.
6. Brockerhoff, H. B., and N. K. N. Ayengar. 1979. Improved synthesis of choline lipids. *Lipids.* **14:** 88–89.
7. Nieuwenhuizen, W., H. Kunze, and G. H. de Haas. 1974. Phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4) from porcine pancreas. *Methods Enzymol.* **32:** 147–154.
8. Jensen, R. G., and R. E. Pitas. 1976. Synthesis of some acylglycerols and phosphoglycerides. *Adv. Lipid Res.* **14:** 213–247.
9. Hendrickson, H. S., E. K. Hendrickson, and R. H. Dybvig. 1983. Chiral synthesis of a dithiolester analog of phosphatidylcholine as a substrate for the assay of phospholipase A_2. *J. Lipid Res.* **24:** 1532–1537.
10. Eaton, B. R., and E. A. Dennis. 1976. Analysis of phospholipase C (*Bacillus cereus*) action toward mixed micelles of phospholipid and surfactant. *Arch. Biochem. Biophys.* **176:** 604–609.
11. Deems, R. A., and E. A. Dennis. 1981. Phospholipase A_2 from cobra venom (*Naja naja naja*). *Methods Enzymol.* **71:** 703–710.
12. Bernady, K. F., M. B. Floyd, J. F. Poletto, and M. J. Weiss. 1979. Synthesis of prostaglandins via conjugate addition of lithium trans-1-alkenyltrialkylalanate reagents. A novel reagent for conjugate 1,4-additions. *J. Org. Chem.* **44:** 1438–1447.
13. Bergelson, L. D. 1980. Lipid Biochemical Preparations. Elsevier/North Holland, Amsterdam. 84.
14. Silverstein, R. M., G. C. Bassler, and T. C. Morrill. 1974. Spectrometric Identification of Organic Compounds. 3rd ed. J. Wiley, New York. 226.
15. Hercules, D. M. 1966. Fluorescence and Phosphorescence Analysis: Principles and Applications. J. Wiley Interscience, New York. 60–61.
16. Tokumura, A., H. Homma, and D. J. Hanahan. 1985. Structural analogs of alkylacetylglycerophosphocholine in-

hibitory behavior on platelet activation. *J. Biol. Chem.* **260:** 12710–12714.
17. Vadas, P., and W. Pruzanski. 1984. Role of extracellular phospholipase A_2 in inflammation. *Adv. Inflammation Res.* **7:** 51–59.
18. Bromalaski, J. S., M. A. Clark, S. D. Douglas, and R. B. Zurier. 1985. Enhanced phospholipase A_2 and C activities of peripheral blood polymorphonuclear leukocytes from patients with rheumatoid arthritis. *J. Leukocyte Biol.* **38:** 649–654.
19. Fawzy, A., R. Dobrow, and R. C. Franson. 1986. Modulation of human synovial phospholipase A_2 by monovalent cations. *Federation Proc.* **45:** 1560 (abstract).
20. Hendrickson, H. S., W. M. Trygstad, T. L. Loftness, and S. L. Sailer. 1981. Phospholipase A_2 activity on mixed lipid monolayers: inhibition and activation of phospholipases A_2 from porcine pancreas and *Crotalus adamanteus* by anionic and neutral amphiphiles. *Arch. Biochem. Biophys.* **212:** 508–514.
21. Robinson, M., M. L. Blank, and F. Snyder. 1985. Acylation of lysophospholipids by rabbit alveolar macrophages. *J. Biol. Chem.* **260:** 7889–7895.