Semisynthetic Preparation of 1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (Platelet Activating Factor)

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1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (platelet activating factor, PAF), (1.9 μmol) was prepared from the total lipid extract of the protozoan *Tetrahymena pyriformis* 9×10^7 cells. The procedure involved mild alkaline hydrolysis of the total lipids, followed by acetylation and purification of the product by preparative TLC and HPLC. The yield was 60% with respect to the content of 1-O-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine in the total lipids, determined after preparative TLC. The alkyl side chain of the semisynthetic PAF was composed of hexadecyl residue. Our product was identified as PAF according to its biological activity, the chromatographic behaviour on TLC and HPLC, the physicochemical properties and the behaviour under treatment with PLA_2 and Lipase from *Rhizopus arrhizus*. The above procedure is proposed as a facile, inexpensive and convenient method.

Introduction

Platelet activating factor (PAF), the potent mediator in inflammatory and allergic reactions with an impressive spectrum of potency [1−4], has been identified as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine [5−8].

Several methods for semisynthetic preparation of PAF have been described starting from bovine heart [5, 7], which produce PAF having mainly an octadecyl alkyl-chain [8]. Also, from ratfish liver oil [9], a mixture of platelet activating factors containing different alkyl-chains, is obtained. Procedures including multiple steps have been previously used for the total synthesis of pure PAF [10, 11] or racemic mixtures [12, 13].

Here, we report a facile method for the semisynthetic preparation of PAF involving mild alkaline hydrolysis and acetylation of the total lipids of the protozoan *Tetrahymena pyriformis,* which contain large amounts of the glyceryl ether analog of phosphatidylcholine, mainly with an hexadecyl side chain [14].

Materials and Methods

Materials

Bovine heart-derived PAF was a gift of Dr. C. A. Demopoulos. 1-O-(hexadecyl-1′,2′-^{3}H)-2-acetyl-sn-glyceryl-3-phosphorylcholine (59.5 Ci/mmol) was purchased from Dupont (U.S.A.), and 1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine was purchased from Bachem (Switzerland). Standard lipids, bovine serum albumin, phosphocreatine and phosphocreatine kinase were obtained from Sigma. "Pro-analysis" and "HPLC grade" solvents were products of Fluka (Switzerland). Proteose-peptone was purchased from Oxoid (England) and yeast extract from Merck. Sodium pentobarbital (Nembutal) was a product of Abbot. Acetyl-salicylic acid as a lysine soluble salt was a product of Galenica (Athens, Greece). Phospholipase A_2 was prepared from pig pancreas (Boehringer Ingelheim). Lipase from Rhizopus arrhizus and standard phospholipids were products of Sigma (St. Louis, U.S.A.).

Culture conditions and lipid extraction

Tetrahymena pyriformis strain W was grown axenically at 25 °C in a 400 ml culture medium containing 2% (w/v) proteose-peptone, 0.5% (w/v) D(+)glucose

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and 0.2% (w/v) yeast extract. Cells were harvested at the late log-phase by centrifugation for 10 min at $1000 \times g$, 4 °C and washed once with the following buffer solution, pH 7: 50 mM NaCl, 2.5 mM KCl, 1 mM MgCl$_2$·6H$_2$O, 5 mM D(+)glucose, 0.1 mM KH$_2$PO$_4$ and 0.4 mM K$_2$HPO$_4$. Finally the cells were resuspended in 30 ml of this same buffer.

Cell density counted in a hemocytometer was found to be 3×10^6 cells/ml. Protein determination of sonicated cells was done by the method of Lowry [15].

Total lipids were extracted by the method of Bligh-Dyer [16]. Lipid phosphorus was determined using the method of Bartlett [17], as modified by Marinetti [18].

Determination of glyceryl-ether content of phosphatidylcholine fraction

The phospholipid classes were separated by preparative TLC with chloroform-acetic acid–methanol–water (75:25:5:1.5), (v/v/v/v), as developing solvent system [19]. The phosphatidylcholine band was scraped off the plate and extracted according to Bligh-Dyer. The glyceryl ether content corresponding to 0.2 μmol of lipid phosphorus was determined by the chromotropic acid colorimetric method after periodate oxidation of the free glyceryl ethers, obtained by acetolysis and saponification [20].

Synthesis

Total lipids of *Tetrahymena pyriformis* were dissolved in 15 ml chloroform-methanol (1:4), (v/v) and treated with 1 ml 1.2 N NaOH in 50% methanol, at 60 °C, for 20 min. After cooling, the alkali stable lipids were extracted with a mixture of 30 ml chloroform-methanol (9:1), (v/v), 15 ml iso-butanol and 30 ml water. The chloroform phase was washed three times with 15 ml of water and evaporated to dryness in a flash evaporator, with repeated additions of absolute ethanol for the complete elimination of water [21]. The dry residue was dissolved in 4 ml pyridine and treated with 4 ml acetic anhydride in the dark overnight, at room temperature. The solvents were completely eliminated in a flash evaporator by repeated additions of absolute ethanol [22].

Purification

The PAF derived was isolated by preparative TLC with chloroform–methanol–water (65:35:6),

(v/v/v), as developing solvent system [5]. The region between standard lipids sphingomyeline and lysophosphatidylcholine, where the biologically active compound migrates, was scrapped off the plate, extracted according to Bligh-Dyer and subjected to lipid phosphorus analysis.

HPLC separation for further purification of PAF was performed on a Varian LC (5000), equipped with a variable wavelength UV-Spectrophotometer and a Micropak Si-10 column. The column was eluted isocratically at a flow rate of 1 ml/min with acetonitrile–methanol–75% phosphoric acid (130:5:1.5), (v/v/v). 1 ml/min fractions were collected, extracted according to Bligh-Dyer and the chloroform phase was washed 3-times with the upper phase for the elimination of the phosphoric acid [23]. The retention time of standard [^{3}H] PAF was determined with a Liquid Scintillation Counter (Tri-Carb, Packard), applying the scintillation fluid Insta-gel (Packard).

The HPLC fractions containing the purified PAF were subjected to lipid phosphorus analysis and to alkyl chain analysis by GLC.

Alkyl chain analysis

A portion of the purified PAF product was submitted to acetolysis and saponification [24], followed by extraction of the free glyceryl ethers with diethylether. The extract was washed 3-times with an equal volume of water, evaporated to dryness and treated with 1 ml acetone plus 50 μl of 72% HClO$_4$ at room temperature, for 45 min [25]. The isopropylidene derivatives of the glyceryl ethers were extracted three times with petroleum ether (b.p. 30–60 °C), dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. The residue was redissolved in petroleum ether and analyzed by GLC on a Varian Aerograph 3700, equipped with a flame ionization detector and connected to a CDS III Varian Integrator.

Column: 15% DEGS on Chromosorb W/AW 80/100. Temperatures of column, injector and detector: 200 °C 220 °C and 220 °C respectively. Flow-rate: 40 ml/min.

Characterization of PAF

Our product was identified as PAF by comparison with bovine heart-derived PAF, according to the following criteria. 1. The biological activity; 2. the

chromatographic behaviour on TLC and HPLC; 3. the physicochemical properties as described by Pinckard *et al.* [30] and 4. the behaviour under treatment with PLA_2 and lipase from *Rhizopus arrhizus*, according to Benveniste *et al.* [31].

Biological tests

In vitro, the biological activity of the purified semisynthetic PAF, bovine heart derived PAF and PAF (alkyl chain, C_{16}) was determined from the aggregation of ASA treated washed rabbit platelets, prepared according to the method of Ardlie *et al.* [26] as modified by Benveniste *et al.* [27]. Tyrode-gelatine-EGTA (TG-EGTA), buffer solution, pH 6.5, was used for washing the rabbit platelets and Tyrode-gelatine-Ca^{2+} (TG-Ca^{2+}) buffer solution, pH 7.4 was used for aggregation buffer [28]. In the aggregation buffer, 1 mM CP and 10 U/ml CPK were added [29], followed by the addition of the PAF samples suspended in 2.5 mg BSA/ml saline solution. Aggregation was measured with a Chronolog Corp. Aggregometer.

The *in vivo* biological activity was determined by measuring the effect of the purified semisynthetic PAF on the systolic blood pressure (SBP) of normotensive male 2 months old (180−250 b.w.) Wistar rats, anesthetized with intraperitoneal injection of sodium pentobarbital (40 mg/Kg b.w.). The results were compared to those of bovine heart-derived PAF. PAF samples suspended in 2.5 mg BSA/ml saline solution were administered through the tail vein. SBP was measured with the M.K. III physiograph, equipped with a pneumatic pulse transducer and the programmed electrosphygmomanometer PE-300 (Narco biosystems).

Fig. 1. a. Separation and purification of semisynthetic PAF by silica HPLC using acetonitrile−methanol−75% phosphoric acid (130:5:1.5). □ PAF in µg, quantified by the aggregation of washed rabbit platelets.
b. Separation of phospholipid standards by silica HPLC with the above solvent system. − − − [³H] PAF, in cpm. Phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lyso-phosphatidylcholine (lyso-PC), sphingomyeline (Sph), 1-O-alkyl-sn-glyceryl-phosphorylcholine (lyso-PAF).

Results

Starting from 9×10^7 *Tetrahymena pyriformis* cells containing 207 mg protein and 38.7 µmol of total lipid phosphorus, we were able to prepare 1.9 µmol of semisynthetic PAF.

The yield of the semisynthetic procedure described was calculated on the basis of lipid phosphorus determinations, after preparative TLC purification. 38.7 µmol of total lipid phosphorus were found to contain 7.2 µmol of phosphatidylcholine of which 3.2 µmol were its glyceryl-ether analog. Therefore the yield of the semisynthetic product was 60% with
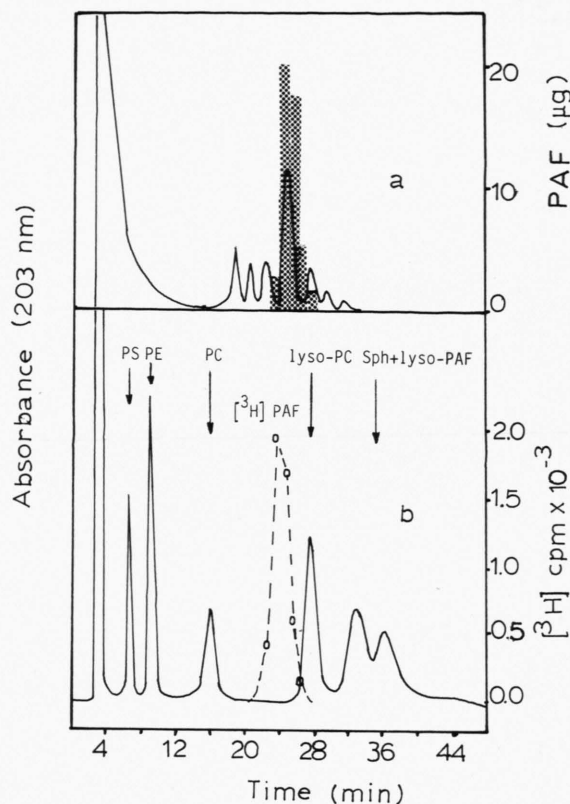
respect to the content of the glyceryl-ether analog of phosphatidylcholine.

The semisynthetic product isolated by preparative TLC gave seven distinct peaks in normal phase silica HPLC (Fig. 1a). The biologically active fractions (purified PAF) were eluted with a retention time of 24 min, identical to that of [³H] PAF (Fig. 1b). Since the peak areas recorded by the UV detector depend on the degree of unsaturation, as well as on other absorbing functional groups (32), the semisynthetic PAF quantified by phosphorus analysis was found to contain 93% of the lipid phosphorus submitted to HPLC purification.
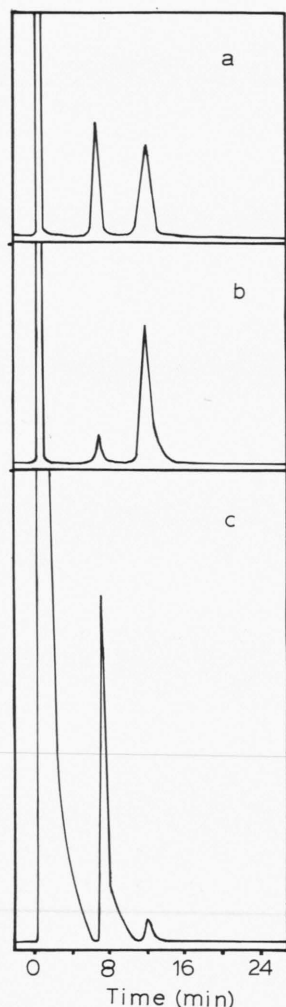
Fig. 2. GLC pattern of glycerylether isopropylidene-derivatives. (a) Chimyl and Batyl alcohol mixture, (b) Bovine heart-derived PAF. (c) *Tetrahymena pyriformis*-derived PAF.
Column: 15% DEGS; colums temperature: 200 °C; injector and detector temperatures: 220 °C; flow rate: 40 ml/min; injection volume: 1 μl.

The alkyl chain analysis of the purified product showed that the semisynthetic PAF obtained by the present method was at least 95% the hexadecyl derivative (Fig. 2).

The biological activity of various PAF preparations on washed rabbit platelets, expressed as EC_{50} were found as follows:

EC_{50} of bovine heart-derived PAF (alkyl chain; C_{18} mainly) $= (10.8 \pm 1.3) \times 10^{-11}$ M

EC_{50} of semisynthetic PAF (alkyl chain; C_{16} mainly) (present) $= (3.8 \pm 0.8) \times 10^{-11}$ M

EC_{50} of PAF (alkyl chain; C_{16}) (Bachem) $= (3.0 \pm 1.0) \times 10^{-11}$ M.
(Values represent the mean ± S.D., of five different experiments.)

The hypotensive response of Wistar rats to semisynthetic PAF was found to be similar to that of bovine heart-derived PAF (Table I).

The semisynthetic PAF and the bovine heart-derived PAF were completely inactivated after treatment with 0.5 N NaOH in methanol or PLA_2 but was almost resistant after incubation with lipase from *Rhizopus arrhizus* or treatment with 4 N HCl (Table II).

Discussion

An increasing interest concerning the biochemistry, pharmacology and the pathophysiological role of PAF has been observed during the last years. So, several synthetic and semisynthetic methods for its preparation have been described and different species of PAF, with respect to the alkyl chain length, are commercially available.

In the present work, we propose a new semisynthetic method for PAF preparation, starting from the total lipids of the easily cultured protozoan *Tet-*

Table I. Hypotensive activity of PAF. PAF solutions in BSA/saline were injected intravenously through the tail vein of anesthetized Wistar rats. SBP was measured indirectly from the tail before the injection and at various time intervals afterwards. Data represent the mean ± S.D. for five determinations.

PAF (in BSA/saline)	Dose (ng P/Kg b.w.)	Decrease of SBP %	Time of recovery [min]
PAF from bovine heart	1.5	20 ± 5	1.0 ± 0.1
PAF from bovine heart	3.0	58 ± 3	2.0 ± 0.2
PAF from *Tetrahymena pyriformis*	2.0	27 ± 4	1.3 ± 0.2
PAF from *Tetrahymena pyriformis*	4.0	59 ± 3	2.2 ± 0.3

Table II. Physicochemical characteristics of PAF. PAF from *Tetrahymena pyriformis* and from bovine heart adjusted to a concentration of 10 ng/ml were submitted to chemicals and enzymatic treatment. Washed rabbit platelet aggregation induced by 20 μl of treated samples and untreated controls were compared. Results are expressed as the percentage of activity recovered in treated samples. Values are the means ± S.D. from 3 tests.

| Treatment | Sensitivity to chemicals and enzymes (% recovered activity) | |
	PAF from *Tetra-hymena pyriformis*	PAF from bovine heart
0.5 N NaOH methanol, 25 °C, 2−3 min	0	0
4 N HCl in H_2O, 25 °C, 3 h	98.5 ± 1.3	97.8 ± 1.9
PLA_2, 0.03 mg, 37 °C, 1h	0	0
Lipase from *Rhizopus arrhizus*, 0.1 mg, 30 °C, 18 h	97.0 ± 2.5	98.0 ± 2.0

rahymena pyriformis. This protozoan contains large amounts of the glyceryl ether analog of PC [14] and the natural occurrence of PAF in its lipids has also been reported [33]. Our method has a very good yield, reaching the 60% of the glyceryl ether analog of PC content of the cell.

The alkyl chain analysis of our semisynthetic PAF has shown that its alkyl side chain is composed of hexadecyl residue. It is well established that the alkyl chain length plays an important role in the biological activity of PAF, the hexadecyl-analog being the more potent [32]. In agreement to this, the biological activity of our PAF on washed rabbit platelets was found threefold more potent than that of the bovine heart-derived, which contains mainly the octadecyl residue. Therefore, the availability PAF (alkyl chain; C_{16}) easily prepared by the present procedure would allow its beneficial use in biochemical and physiological experiments, especially in binding studies. On the other hand, for studying the biochemical effects of PAF on the protozoan, it would be more convenient to use the Tetrahymena pyriformis-derived PAF, which most probably has the same structure as the protozoans native PAF.

Our method compaired to others is more rapid and very facile, since the starting material is easily available and no special laboratory equipment (*e.g.* for hydrogenation) is required. Therefore, the procedure can be easily reproduced in every biochemical laboratory.

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