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Synthesis and biological evaluation of novel steroid-modified ether phospholipids

Haralabos C. Karantonis [a], Emmanuel N. Pitsinos [b], Smaragdi Antonopoulou [c], Elias A. Couladouros [b,d], Constantinos A. Demopoulos [a,*]

[a] *Laboratory of Biochemistry, Faculty of Chemistry, National and Kapodistrian University of Athens, Panepistimioupolis, GR-157 71 Athens, Greece*
[b] *Laboratory of Organic and Bio-Organic Chemistry, Institute of Physical Chemistry, NCSR "DEMOKRITOS", Aghia Paraskevi, P.O. Box 60228, GR-153 10 Athens, Greece*
[c] *Department of Science of Dietetics-Nutrition, Harokopio University, 70 El. Venizelou Street, GR-176 71 Athens, Greece*
[d] *Chemistry Laboratories, Agricultural University of Athens, Iera Odos 75, GR-118 55 Athens, Greece*

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Abstract

Platelet activating factor is one of the most potent inflammatory ether phospholipid mediators known and structurally modified analogues are of considerable interest as potential therapeutic preparations. Inspired by the proposed structure for a novel endogenous hydroxy-PAF analogue isolated recently from gingival crevicular fluid, we designed and prepared two novel steroid-modified ether phospholipids. These two novel compounds exhibit marked chemical and biological similarities to their endogenous prototype and they antagonize it being less active in inducing washed platelet aggregation through PAF receptors.

Keywords: Steroid ether phospolipids; Platelets; Platelet activating factor; PAF-acetylhydrolase; Periodontal disease

1. Introduction

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) occurs naturally in cell membranes and is one of the most potent inflammatory ether phospholipid mediators known (Blank et al., 1981; Demopoulos et al., 1979; Montrucchio et al., 2000). PAF (**1**, Fig. 1) plays an important role in a number of physiological and pathological processes, such as allergy, hypotension, anaphylaxis, thrombosis, ischemia, acute infections in transplantation, nephritis, gastric ulcer, etc. (Snyder, 1987). Activated inflammatory cells challenged by bacterial lipopolysaccharides produce and secrete inflammatory mediators like PAF (Jakubowski et al., 2004), which is believed to be a key regulator of various diseases like periodontal disease (Antonopoulou et al., 2003). Moreover, PAF has been shown to stimulate platelet degranulation and aggregation (Snyder et al., 1989), to cause the contraction of smooth muscles, bronchoconstriction, and coronary vasoconstriction (Snyder et al., 1989), to increase vascular permeability (Snyder et al., 1989; Shukla, 1991) and to stimulate immune response (Ruis et al., 1991; Hayashi et al., 1991; Braquet et al., 1991). A large

Abbreviations: Ac, acetyl; Bn, benzyl; LPC, lysophosphatidylcholine; PAF, platelet-activating factor; Ph, phenyl; SM, sphingomyelin; TBAF, tetrabutylammonium fluoride; TBDPS, tert-butyldiphenylsilyl; THF, tetrahydrofuran

* Corresponding author. Present address: 39 Anafis Str., GR-113 64 Athens, Greece. Tel.: +30 210 7274265; fax: +30 210 7274265.
E-mail address: demopoulos@chem.uoa.gr (C.A. Demopoulos).

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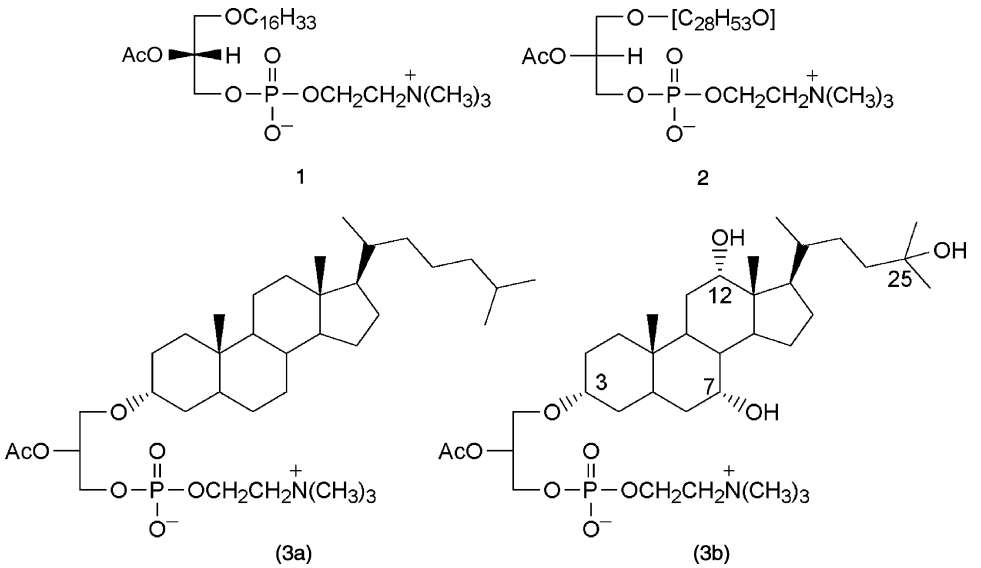


Fig. 1. Naturally occurring ether phospholipids (**1** and **2**) and designed steroid-modified analogues (**3a** and **3b**).

number of structurally modified analogues of PAF has been synthesized and exhibit diverse biological activities ranging from specific PAF antagonism (Sablina et al., 1995) and immunomodulation (O'Flaherty and Wykle, 1983; Munder et al., 1976) to selective tumor-cytotoxicity (Berdel et al., 1981; Hoffman et al., 1984) and inhibition of HIV-1 replication (Kucera et al., 1990). Because of the variety of their biological effects, ether phospholipids are of considerable interest as potential therapeutic preparations.

Recently, a new endogenous phospholipid was identified in gingival crevicular fluid (Antonopoulou et al., 1998). This molecule induced washed rabbit platelet aggregation in a dose-dependent manner with PAF-like aggregation tracing. Its EC_{50} value was in the order of 0.1 μM, final concentration, being less active than PAF, which induces aggregation with EC_{50} value in the order of 0.1 nM, final concentration. Furthermore, it was shown that this phospholipid acts through PAF receptors. Enzymatic and chemical treatments along with ESMS analysis demonstrated that the phospholipid has a glyceryl ether backbone with an acetyl group at the *sn*-2 position and at least one free hydroxyl group at the *sn*-1 carbon chain, which can be acetylated under mild acetylation conditions. Unfortunately, the very small amount of material isolated hindered full structural elucidation of this endogenous hydroxy-PAF analogue. Nevertheless, the available structural information led to the proposed structure **2** (Fig. 1) leaving ambiguous the exact nature of the ether side chain.

The biological activity of this new ether phospholipid and its proposed structure attracted our attention. In particular, the molecular formula $C_{28}H_{54}O_2$ (Mr 422.7) proposed for the hydroxylated ether unit intrigued us since fatty acids or alcohols of such length are not common in mammals. Thus, attempting to speculate on plausible structures for this unit, based on the available chemical and spectroscopic data, was haphazard.

Nevertheless, consideration of alternative molecular formulae for the ether segment indicated the formula $C_{26}H_{46}O_4$ (Mr 422.6). This formula could accommodate the presence of four ring systems, an observation that led us to consider derivatives of a steroid alcohol, such as cholesterol or cholestanol, as alternative ether units. The fact that, to the best of our knowledge, no steroid-modified ether phospholipid has been previously reported (Lal et al., 1994; Lebeau et al., 1991) along with the important and diverse biological activity of ether phospholipids prompted us to investigate such novel PAF analogues despite the lack of any indication that they might occur in nature.

Herein, we report the synthesis and some biological properties of two steroid-modified ether phospholipid analogues (**3a** and **3b**). Apart from cholestanol derivative **3a**, we opted to investigate the phospholipid derivative of 24-nor-5β-cholestane-3α,7α,12α,25-tetrol (**3b**). This C_{26}-analogue of naturally occurring C_{27} bile alcohols (Dayal et al., 1976) not only fits the available ESMS spectroscopic data for **2** but also, we reasoned that due to the steric demand of the neo-pentyl (C-12) and ter-

tiary (C-25) hydroxyls, only the C-7 hydroxyl would be readily acetylated, thus accommodating the observed chemical reactivity of the new hydroxy-PAF analogue.

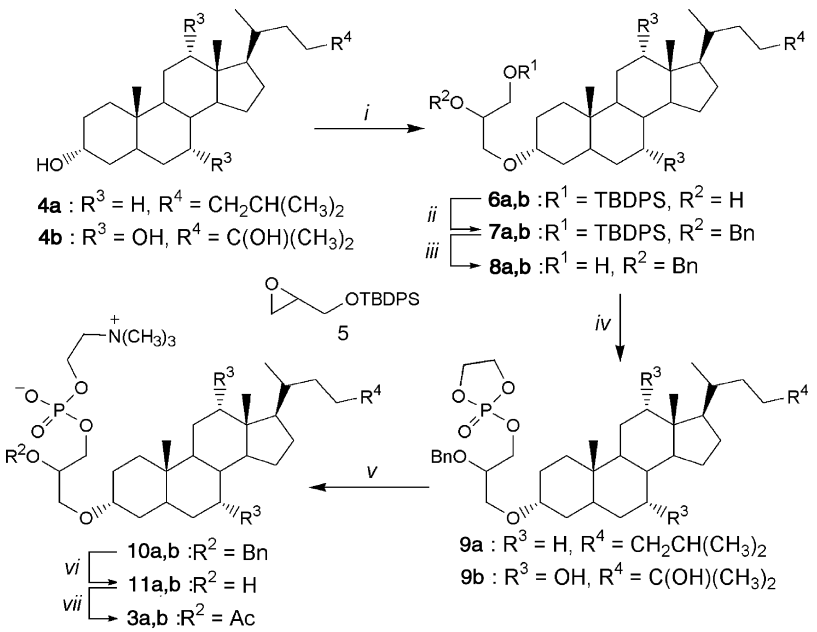
2. Experimental

2.1. Materials and methods

All commercially available chemicals employed (Merck, Darmstadt, Germany, or Aldrich, Steinheim, Germany) were of reagent-grade and used without further purification. All reactions were carried out under a dry Ar atmosphere with anhydrous, freshly distilled solvents under anhydrous conditions unless otherwise noted. All reactions were magnetically stirred with Teflon stir bars, and temperatures were measured externally. THF and toluene were freshly distilled over sodium benzophenone ketyl. Reactions were monitored by TLC carried out on 0.25 mm precoated glass plates Merck Silica gel 60 F_{254}. Visualisation was affected with UV light (254 nm), iodine vapour chamber, 7% ethanolic phosphomolybdenic acid-heat or *p*-anisaldehyde solution (2.5% *p*-anisaldehyde, 3.4% sulfuric acid, 1% acetic acid in 95% ethanol) and heat as developing agent. Preparative column chromatography: silica gel (Merck Si 60; 40–63 μm). Chromatographic material used for TLC lipid isolation was silica gel H-60 (Merck, Darmstadt, Germany). The solvents used for HPLC were purchased from Rathburn (Walkerburn, Peebleshire, UK). All standards were obtained from Sigma (St. Louis, MO, USA). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (Demopoulos et al., 1979). PAF-acetylhydrolase from human serum has been purified according to Stafforini (Stafforini et al., 1987). Bovine serum albumin (BSA) and BN 52021 were obtained from Sigma. Chrono-lume Reagent and Chrono-lume ATP standard were purchased from Chrono-log Corporation (Havertown, PA, USA).

2.2. Preparation of steroid-modified ether phospholipids

For the preparation of the targeted modified ether phospholipids, Bittmans's synthetic strategy was employed (Guivisdalsky and Bittman, 1989a,b). Thus, highly regioselective opening of the epoxide ring of racemic, *tert*-butyldiphenylsilyl-protected glycidol **5** by cholestanol (**4a**) was catalyzed by BF_3-etherate to provide alcohol **6a** in 53% isolated yield – based on the starting material used – (Scheme 1) (Erukulla et al., 1995). In preparation for the introduction of the phosphocholine moiety, benzylation followed by cleavage of the silyl-ether with TBAF furnished alcohol **8a** (72% yield over two steps). Phosphorylation, using 2-chloro-2-oxo-1,3,2-dioxaphospholane in benzene in the presence



Scheme 1. Synthesis of steroid-modified ether phospholipids **3a** and **3b**. **a** series: R^3 = H, R^4 = $CH_2CH(CH_3)_2$; **b** series: R^3 = OH, R^4 = $C(OH)(CH_3)_2$. Reagents and conditions: (i) **5**, $BF_3{\cdot}Et_2O$, CH_2Cl_2; (ii) $PhCH_2Br$, NaH, THF, 0–25 °C; (iii) TBAF, THF; (iv) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N, toluene, 0 °C; (v) Me_3N, CH_3CN, 65–70 °C; (vi) H_2, $Pd(OH)_2/C$, $MeOH/H_2O$ (9:1, v/v); (vii) Ac_2O, $CHCl_3$, 25 °C.

of 1 equivalent triethylamine, followed by treatment of the phosphate thus obtained with excess anhydrous trimethylamine in dry acetonitrile furnished phosphocholine **10a** (75% yield over two steps) (Thuong and Chabrier, 1974). Finally, the *lyso*-PAF analogue **11a**, obtained by hydrogenolysis of the benzyl ether moiety, was acetylated (Ac_2O, $CHCl_3$, rt, 12 h) to provide the targeted cholestanyl-ether phospholipid **3a** in 35% yield.

The reported selective glucuronidation of 24-nor-5β-cholestane-3α,7α,12α,25-tetrol (**4b**) at C-3 (Dayal et al., 1993) fuelled our hopes that this tetrahydroxylated steroid could be used in the BF_3-etherate catalyzed epoxide ring opening step without prior protection of the hydroxyls at C-7, -12 and -25. Indeed, when glycidol **5** was reacted with the above tetrol, alcohol **6b** was obtained in 45% yield. Further transformation of this alcohol to the targeted ether phospholipid **3b** was accomplished in analogy to the preparation of ether phospholipid **3a** from alcohol **6a** described above.

2.3. Analytical data for selected new compounds

Compound **6a**: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.75–7.65 (m, 4 H, C_6H_5SiO); 7.46–7.31 (m, 6 H, C_6H_5SiO); 3.89–3.80 (m, 1 H, C*H*OH); 3.71 (d, *J* = 5.21 Hz, 2 H, C*H*$_2$OTBDPS); 3.68–3.47 (m, 2 H, C*H*$_2$OCH); 3.28–3.15 (m, 1 H,CH_2OCH); 2.52 (broad d, *J* = 5.21 Hz, 1 H, CHO*H*); 2.04–0.55 (m, 55 H, *steroid*+$(CH_3)_3CSiO$).

Compound **6b**: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.60–7.56 (m, 4 H, C_6H_5SiO); 7.36–7.26 (m, 6 H, C_6H_5SiO); 3.87 (bs, 1 H, *steroid*-$H_{12β}$); 3.78–3.72 (broad m, 2 H, C*H*OH + *steroid*-$H_{7β}$); 3.63–3.58 (m, 2 H, C*H*$_2$OTBDPS); 3.55–3.37 (m, 3 H, C*H*$_2$OCH+*steroid*-$H_{3β}$); 3.09–2.95 (broad m, 1 H, O*H*); 2.85–2.69 (broad m, 1 H, O*H*); 2.32–0.76 (m, 25 H, *steroid*); 1.10 (s, 6 H, *steroid*-26-CH_3 + 27-CH_3); 0.98 (s, 9 H, $(CH_3)_3CSiO$); 0.91 (d, *J* = 6.33 Hz, 3 H, *steroid*-21-CH_3); 0.79 (s, 3 H, *steroid*-19-CH_3); 0.58 (s, 3 H, *steroid*-18-CH_3).

Compound **8a**: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.36–7.30 (m, 5 H, $C_6H_5CH_2O$); 4.67 (AB_q, Δν = 21.02 Hz, *J* = 11.71 Hz, 2 H, $C_6H_5CH_2O$); 3.81–3.48 (m, 5 H, C*H*$_2$C*H*C*H*$_2$); 3.29–3.17 (m, 1 H, CH_2OCH); 2.40 (broad t, *J* = 7.44 Hz, 1 H, CH_2OH); 1.99–0.56 (m, 46 H, *steroid*).

Compound **8b**: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.36–7.28 (m, 5 H, $C_6H_5CH_2O$); 4.66 (AB_q, Δν = 20.84 Hz, *J* = 11.91 Hz, 2 H, $C_6H_5CH_2O$); 3.95 (broad s, 1 H, *steroid*-$H_{12β}$); 3.81–3.46 (m, 7 H, C*H*$_2$C*H*C*H*$_2$ + *steroid*-$H_{3β}$ + *steroid*-$H_{7β}$); 3.17–3.07 (broad m, 1 H, O*H*); 2.98–2.36 (broad m, 2 H, 2 × O*H*); 2.31–0.84 (m, 25 H, *steroid*); 1.18 (s, 6 H, *steroid*-26-CH_3 + 27-CH_3); 0.99 (d, *J* = 6.33 Hz, 3 H, *steroid*-21-CH_3); 0.88 (s, 3 H, *steroid*-19-CH_3); 0.67 (s, 3 H, *steroid*-18-CH_3).

Compound **3a**: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 5.14–5.02 (broad m, 1 H, C*H*OAc); 4.28 (broad s, 2 H, *choline*-CH_2); 3.98–3.87 (broad m, 2 H, C*H*$_2$OPO$_3$); 3.78 (broad s, 2 H, *choline*-CH_2); 3.62–3.48 (broad m, 2 H, C*H*$_2$OCH); 3.33 (s, 9 H, $N^+(CH_3)_3$); 3.24–3.12 (m, 1 H, CH_2OCH); 2.06 (s, 3 H, CH_3COO); 1.99–0.54 (m, 46 H, *steroid*). MS (ESI+) *m*/*z* for $C_{37}H_{68}NNaO_7P(M + Na^+)$, calc.: 692.5; found: 692.7

Compound **3b**: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 5.11–4.90 (broad m, 1 H, C*H*OAc); 4.30 (broad s, 2 H, *choline*-CH_2); 3.93–2.94 (broad m, 9 H, *steroid*-$H_{12β}$, $H_{3β}$, $H_{7β}$ + C*H*$_2$OPO$_3$ + *choline*-CH_2 + C*H*$_2$OCH); 3.36 (broad s, 9 H, $N(CH_3)_3$); 2.31–0.86 (m, 25 H, *steroid*); 2.06 (s, 3 H, CH_3COO); 1.15 (s, 6 H, *steroid*-26-CH_3 + 27-CH_3); 0.99 (d, *J* = 5.58 Hz, 3 H, *steroid*-21-CH_3); 0.86 (s, 3 H, *steroid*-19-CH_3); 0.65 (s, 3 H, *steroid*-18-CH_3). MS (ESI+) *m*/*z* for $C_{36}H_{67}NO_{10}P$ ($M + H^+$), calc.: 704.4; found 704.2.

2.4. Purification of synthetic phospholipids

Botth diastereomers of compound **3a** (1-*O*-cholestanol-2-acetyl-*sn*-glycero-3-phosphocholine and 1-phosphocholine-2-acetyl-*sn*-glycero-3-*O*-cholestanol) and compound **3b** (1-*O*-24-nor-5β-cholestane-3α,7α,12α,25-tetrol-2-acetyl-*sn*-glycero-3-phosphocholine and 1-phosphocholine-2-acetyl-*sn*-glycero-3-*O*-24-nor-5β-cholestane-3α,7α,12α,25-tetrol) referred as (D,L)-**3a** and (D,L)-**3b**, respectively, based on the absolute configuration of the glycerol moiety, were further purified.

After synthesis the lipid compounds were extracted according to Bligh–Dyer (Bligh and Dyer, 1959). The extracts were evaporated to dryness under a stream of nitrogen and re-dissolved in a small volume of chloroform/methanol (1:1, v/v) and were further purified by TLC and HPLC.

2.4.1. TLC purification

TLC plates were 20 cm × 20 cm glass plates coated with silica gel H and activated by heating at 120 °C for 1 h. Their thickness was 0.5 mm (analytical). The chromatogram was developed in chloroform/methanol/glacial acetic acid/water (100:65:20:15, v/v) (Kates, 1975). Detection of compounds was performed with iodine vapour. Both (D,L)-**3a** (RF = 0.42) and (D,L)-**3b** (RF = 0.32) migrated between sphingomyelin (SM; RF = 0.51) and lysophosphatidylcholine (LPC; RF = 0.30).

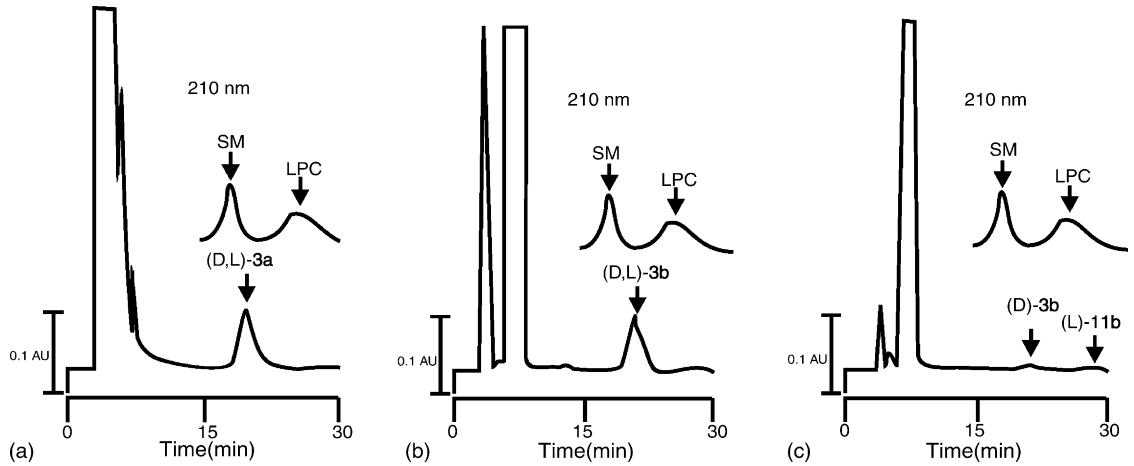


Fig. 2. HPLC purification of compounds **3a**, **3b**, (L)-**11b** and (D)-**3b**. (a) HPLC purification of (D,L)-**3a** after TLC purification. (b) HPLC purification of (D,L)-**3b** after TLC purification. (c) HPLC separation of (L)-**11b** and (D)-**3b** after PAF-acetylhydrolase treatment.

2.4.2. HPLC purification

HPLC was performed at room temperature on a HP Series 1100 liquid chromatography model (Hewlett-Packard, Waldbronn, Germany) equipped with a 100 μL loop Rheodyne (7725 i) loop valve injector, a degaser G1322A, a quat gradient pump G1311A and a HP UV spectrophotometer G1314A as a detection system. The spectrophotometer was connected to a Hewlett-Packard model HP-3395 integrator-plotter. A Sphereclone 5u NH2 normal phase column (250 mm × 4.6 mm i.d.) from Phenomenex, (Hurdsfield, Cheshire, UK) with a NH2 silica (20 mm × 4.0 mm i.d.) precolumn cartridge was used. This step of purification of the synthetic compounds was performed with an isocratic elution system consisted of acetonitrile/methanol (75:25, v/v) at 210 nm. Both synthetic lipids eluted between SM and LPC (Fig. 2).

2.5. Biological evaluation

PAF-induced aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer coupled to a Chrono-Log recorder (Havertown, PA, USA). The synthetic compounds were tested for their biological activity toward washed rabbit platelets according to the method of Demopoulos et al. (1979). The purified synthetic compounds were dissolved in 2.5 mg bovine serum albumin per mL saline. The platelet aggregation induced by PAF (5×10^{-10} M, final concentration) was measured as PAF-induced aggregation, in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various concentrations of the examined compounds. Desensitization experiments (Lazanas et al., 1988) with PAF and experiments with its specific inhibitor BN 52021 (0.1 μM) were also performed. ATP secretion was measured by a sensitive luminescent (firefly luciferin–luciferase) assay for extracellular ATP. Luminescence is created by chrono-lume reagent (firefly luciferin–luciferase) reacting with ATP secreted by dense granules in washed platelets. The luminescence technique is calibrated during the testing procedure by use of chrono-lume ATP standard (2 nmol). Chrono-lume reagent was added (0.09 μM, final concentration) 2 min before the addition of the aggregating agent or the chrono-lume ATP standard and luminescence was measured simultaneously with aggregation.

2.6. PAF-acetylhydrolase assay

A volume of 40 μL of Tris buffer solution (50 mM, pH 7.5) and 37 μL of the examined compound in BSA (2.5 mg/mL saline), corresponding to a quantity as much as five times the quantity capable of inducing platelet aggregation, were added and mixed vigorously to a pre-warmed at 37 °C test tube. From this mixture the aggregatory activity of the one-fifth is tested and defined as the activity at zero time. Afterwards, human serum PAF-acetylhydrolase was added and the whole mixture is incubated at 37 °C. At different time intervals, aliquots were taken to test the ability, of the possibly partially catabolized lipid sample, to induce washed rabbit platelet aggregation. As control the same procedure is carried out with PAF (80% C-16PAF and 20% C-18PAF) – in an amount that induces platelet aggregation equivalent to that of the examined compound – instead of the lipid compound.

2.7. PAF-acetylhydrolase treatment and acetylation

The examined compound was dissolved in BSA (0.25 mg/mL saline) and 800 μL of Tris buffer 50 mM, pH 7.4 were added as well as 100 μL of human serum PAF-acetylhydrolase. The whole mixture was maintained at 37 °C for 45 min. Afterwards, lipid compounds were extracted according to Bligh–Dyer, evaporated to dryness under a stream of nitrogen, redissolved in a small volume of chloroform/methanol (1:1, v/v) and subjected to TLC separation. Acetylation of the products was performed by the addition of 1 mL acetic anhydride and incubation at 60 °C for 45 min. After that, the reaction mixture was evaporated and extracted according to Bligh–Dyer (Bligh and Dyer, 1959) and tested for its ability to induce washed rabbit platelet aggregation. Acetylation of the initial compound, without human serum PAF-acetylhydrolase was also performed using the same procedure.

3. Results and discussion

Compound (D,L)-**3a** at a final concentration of 2.5×10^{-8} M, based on phosphorus determination (Bartlett, 1959), exhibited biological activity smaller than control PAF (5×10^{-10} M, final concentration) causing reversible platelet aggregation (a, Fig. 3). Pretreatment with PAF-acetylhydrolase and subsequent TLC analysis indicated two products. Based on the known substrate selectivity of the enzyme (Demopoulos and Antonopoulou, 1996), the first one (RF = 0.18) was attributed to (L)-**11a**, while the second one (RF = 0.42), which resisted further treatment with PAF-acetylhydrolase, was attributed to compound (D)-**3a**. Compound (D)-**3a** did not cause platelet aggregation.

Compound (D,L)-**3b** was less active than control PAF. Thus, a final concentration of 1×10^{-4} M, based on phosphorus determination, was required to achieve biological activity equal to that of 2.5×10^{-11} M PAF (final concentration), causing reversible platelet aggregation and 81% desensitization of control PAF (b, Fig. 3). Furthermore, compound (D,L)-**3b** (1×10^{-4} M, final concentration) acts through PAF receptors since BN 52021 inhibits its activity at 96%. It also causes ATP secretion (0.31 nmol) as PAF (2.5×10^{-11} M, final concentration) does (0.21 nmol).

In order to gain additional information, an amount of (D,L)-**3b** was subjected to acetylation giving (D,L)-**3b**Ac, while a quantity as much as two times the above was subjected to PAF-acetylhydrolase treatment giving (L)-**11b** and unaffected (D)-**3b**. The products were extracted according to the Bligh–Dyer method and isolated by HPLC (c, Fig. 2). Acetylation of (L)-**11b** gave (L)-**11b**Ac. All the above compounds in a final concentrat-

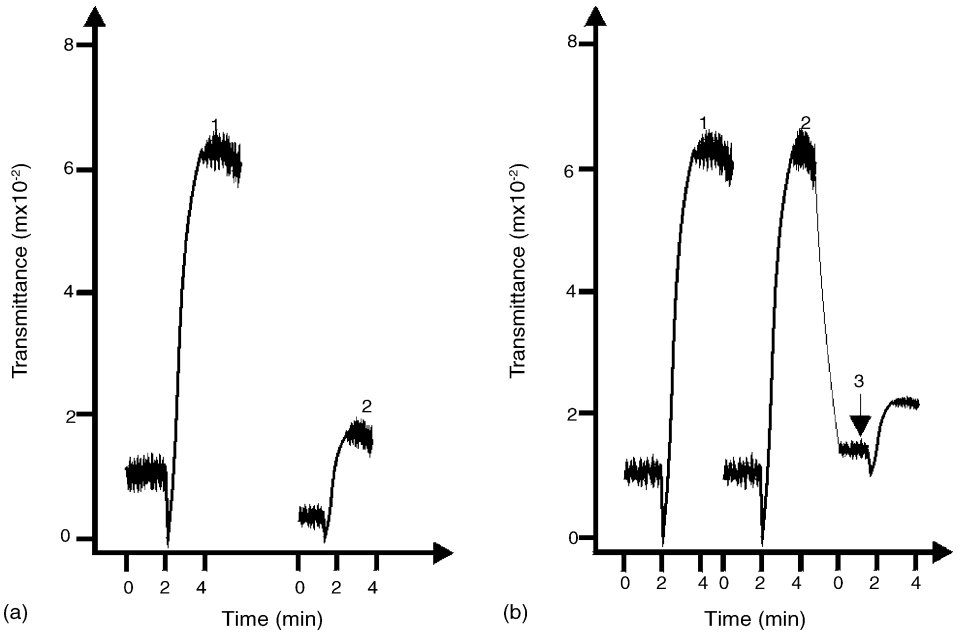


Fig. 3. Biological activity of compounds **3a** and **3b**. (a) Biological activity of compound (D,L)-**3a** toward washed platelets: 1, PAF (5×10^{-10} M, final concentration); 2, compound (D,L)-**3a** (2.5×10^{-8} M, final concentration). (b) Biological activity of compound (D,L)-**3b** toward washed platelets: 1, PAF (2.5×10^{-11} M, final concentration); 2, compound (D,L)-**3b** (1×10^{-4} M, final concentration); 3, addition of PAF (2.5×10^{-11} M, final concentration) for desensitization.

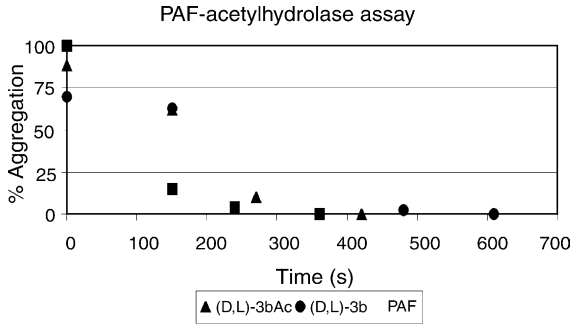


Fig. 4. Biological activity of **3b** and related compounds. PAF-acetylhydrolase assay for (D,L)-**3b** (0.6×10^{-6} M, final concentration), (D,L)-**3b**Ac (0.6×10^{-6} M, final concentration) and PAF (5×10^{-10} M, final concentration).

ing, based on phosphorus determination (Bartlett, 1959), equal to 0.6×10^{-4} M for (D,L)-**3b** and (D,L)-**3b**Ac and 0.3×10^{-4} M for (L)-**11b**, (L)-**11b**Ac and (D)-**3b**, were tested for their ability to cause platelet aggregation. Compound (D,L)-**3b** exhibited a biological activity that gave signal at the aggregometer equal to 7.5×10^{-2} m. This bioactivity disappeared upon PAF acetylhydrolase treatment (i.e. (L)-**11b** and (D)-**3b** were not active). Acetylation of (D,L)-**3b** and (L)-**11b** resulted to products that exhibit greater biological activity than (D,L)-**3b** that gave signal to the aggregometer equal to 11.0×10^{-2} and 11.3×10^{-2} m, respectively. The greater biological activity reveals that further acetyl group(s) was/were introduced on the molecules that were subjected to further acetylation, revealing also a physiological role of acetyl groups concerning the exhibition of biological activity of this kind of biomolecules.

Furthermore, we examined the effect of human serum PAF-acetylhydrolase on the ability of compounds (D,L)-**3b** and (D,L)-**3b**Ac to induce platelet aggregation. PAF-acetylhydrolase treatment decreased in time the activity of these compounds but in a slower rate than control PAF (Fig. 4).

These results indicate that, concerning its biological activity, the synthetic compound (L)-**3b** resembles to the natural hydroxy-PAF analogue that has been isolated from gingival crevicular fluid (Antonopoulou et al., 1998); both of them act through PAF-receptors, desensitize platelets against PAF and are deactivated by PAF-acetylhydrolase. Moreover, the synthetic compound seems to antagonize its endogenous prototype since it causes washed platelet reversible aggregation through PAF receptors with EC_{50} value 0.1 mM based on phosphorus determination, being less active than the natural one, which induces aggregation with EC_{50} in the order of 0.1 μM.

These compounds may be useful in inflammatory conditions treatment, since PAF antagonists are potential therapeutic agents. Rupatadine (Izquierdo et al., 2003), for example, is a new agent, which exhibits strong antagonist activity toward, both, histamine H_1 and PAF receptors. This agent is used for the management of diseases with allergic inflammatory conditions and it is clinically effective in relieving symptoms in patients with seasonal and perennial allergic rhinitis in which PAF plays a pivotal role (Labrakis-Lazanas et al., 1988). Moreover, it is more effective than agents that exhibit only antihistaminic effect (Izquierdo et al., 2003).

References

Antonopoulou, S., Demopoulos, C.A., Argyropoulos, D., Baltas, G., Kotsifaki, H., Diamanti-Kipioti, A., 1998. Identification of a new endogenous platelet-activating factor-like molecule in gingival crevicular fluid. Biochem. J. 330, 791–794.

Antonopoulou, S., Tsoupras, A., Baltas, G., Kotsifaki, H., Mantzavinos, Z., Demopoulos, C.A., 2003. Hydroxyl-platelet-activating factor exists in blood of healthy volunteers and periodontal patients. Mediators Inflamm. 12 (4), 221–227.

Bartlett, G.R., 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466–468.

Berdel, W.E., Bausert, W.R.E., Fink, U.F., Rastetter, J., Munder, P.G., 1981. Anti-tumor action of alkyl-lysophospholipids. Anticancer Res. 1, 345–352.

Blank, M.L., Lee, T.C., Fitzgerald, V., Snyder, F., 1981. A specific acetylhydrolase for 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (a hypotensive and platelet-activating lipid). J. Biol. Chem. 256, 175–178.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Braquet, P., Hosford, D., Koltz, P., Guilbaud, J., Paubert-Braquet, M., 1991. Effect of platelet-activating factor on tumor necrosis factor-induced superoxide generation from human neutrophils—possible involvement of G-proteins. Lipids 26, 1071–1075.

Dayal, B., Salen, G., Padia, J., Shefer, S., Tint, G.S., Sasso, G., Williams, T.H., 1993. Bile alcohol glucuronides: regioselective *O*-glucuronidation of 5β-cholestane-3α,7α,12α,25-tetrol and 24-nor-5β-cholestane-3α,7α,12α,25-tetrol. Carbohydr. Res. 240, 133–142.

Dayal, B., Shefer, S., Tint, G.S., Salen, G., Mosbach, E.H., 1976. C_{26}-Analogs of naturally occurring C_{27} bile alcohols. J. Lipid Res. 17, 478–484.

Demopoulos, C.A., Antonopoulou, S., 1996. A discovery trip to compounds with PAF-like activity. Adv. Exp. Med. Biol. 416, 59–63.

Demopoulos, C.A., Pinckard, R.N., Hanahan, D.J., 1979. Platelet-activating factor. Evidence for 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). J. Biol. Chem. 254, 9355–9358.

Erukulla, R.K., Byun, H.-S., Locke, D.C., Bittman, R., 1995. Stereospecific and regiospecific ring opening of glycidol with primary and secondary alcohols mediated by diisobutylaluminium hydride. J. Chem. Soc., Perkin Trans. 1, 2219–2220.

Guivisdalsky, P.N., Bittman, R., 1989a. An efficient stereocontrolled route to both enantiomers of platelet activating factor and analogues with long-chain esters at C_2: saturated and unsaturated ether glyc-

erolipids by opening of glycidyl arenesulfonates. J. Org. Chem. 54, 4643–4648.

Guivisdalsky, P.N., Bittman, R., 1989b. Regiospecific opening of glycidyl derivatives mediated by boron trifluoride. Asymmetric synthesis of ether-linked phospholipids. J. Org. Chem. 54, 4637–4642.

Hayashi, H., Kudo, I., Nojima, S., Inoue, K., 1991. Biological response of guinea pig peritoneal macrophages to platelet-activating factor. Lipids 26, 1193–1199.

Hoffman, D.R., Hajdu, J., Snyder, F., 1984. Cytotoxicity of platelet activating factor and related alkyl-phospholipid analogs in human leukemia cells, polymorphonuclear neutrophils, and skin fibroblasts. Blood 63, 545–552.

Izquierdo, I., Merlos, M., Garcia-Rafanell, J., 2003. Rupatadine: a new selective histamine H1 receptor and platelet-activating factor (PAF) antagonist. A review of pharmacological profile and clinical management of allergic rhinitis. Drugs Today (Barc.) 39 (6), 451–468.

Jakubowski, A., Olszanecki, R., Chlopicki, S., 2004. Involvement of platelet activating factor in immediate heart response to lipopolysaccharide. J. Physiol. Pharmacol. 55 (2), 409–421.

Kates, M., 1975. In: Work, T.S., Work, E. (Eds.), Techniques of Lipidology: Isolation, Analysis and Identification of Lipids, vol. 3 (Part II). North-Holland, Amsterdam, p. 435.

Kucera, L.S., Iyer, N., Leake, E., Raben, A., Modest, E.J., Daniel, L.W., Piantadosi, C., 1990. Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation. AIDS Res. Hum. Retroviruses 6, 491–501.

Labrakis-Lazanas, K., Lazanas, M., Koussissis, S., Tournis, S., Demopoulos, C.A., 1988. PAF of biological fluids in disease: blood levels in allergic rhinitis. Haematologica 73 (5), 379–382.

Lal, B., Gangopadhyay, A.K., Lakadawala, A.D., Alrega, M.V., 1994. Synthesis of labdane derived PAF antagonists. Indian J. Chem. Sect. B 33, 415–427.

Lazanas, M., Demopoulos, C.A., Tournis, S., Koussissis, S., Labrakis-Lazanas, K., Tsarouhas, X., 1988. PAF of biological fluids in desease: IV. Levels in blood in allergic reactions induced by drugs. Arch. Dermatol. Res. 280, 124–126.

Lebeau, L., Oudet, P., Mioskowski, C., 1991. Synthesis of new phospholipids linked to steroid–hormone derivatives designed for two-dimensional crystallization of proteins. Helv. Chim. Acta. 74, 1697–1706.

Montrucchio, G., Alloatti, G., Camussi, G., 2000. Role of platelet-activating factor in cardiovascular pathophysiology. Physiol. Rev. 80, 1669–1699.

Munder, P.G., Weltzein, H.U., Modolell, M., 1976. Lysolecithin analogs, a new class of immunopotentiators. In: Miescher, P.A. (Ed.), Seventh International Symposium on Immmunopathology Germany. Schwabe Publishers, Basel, Switzerland, pp. 411–424.

O'Flaherty, J.T., Wykle, R.L., 1983. Biology and biochemistry of platelet-activating factor. Clin. Rev. Allergy 1, 353–367.

Ruis, N.M., Rose, J.K., Valone, F.H., 1991. Tumor necrosis factor release by human monocytes stimulated with platelet-activating factor. Lipids 26, 1060–1064.

Sablina, M.A., Ushakova, I.P., Serebrennikova, G.A., 1995. Synthesis of modified ether phospholipids. Russ. Chem. Bull. 44, 946–950.

Shukla, S.D., 1991. Inositol phospholipid turnover in PAF transmembrane signalling. Lipids 26, 1028–1033.

Snyder, F., 1987. Platelet Activating Factor and Related Lipid Mediators. Plenum Press, New York.

Snyder, F., Lee, T.C, Blank, M.L., 1989. Platelet activating factor and related ether lipid mediators, biological activities, metabolism and regulation. In: Khachaturien, Z.S., et al. (Eds.), Annuals of New York Academy of Sciences, vol. 568. New York, 35 pp.

Stafforini, D.M., McIntyne, T.M., Carter, M.E., Prescott, S.M., 1987. Human plasma platelet-activating factor acetylhydrolase. J. Biol. Chem. 26, 4223–4230.

Thuong, N.T., Chabrier, P., 1974. Nouvelle methode de preparation de la phosphorylcholine, de la phosphorylhomocholine et leurs derives. Bull. Soc. Chim. Fr., 667–671.