Two 1-Alkyl-2-acyl Choline Glycerophospholipids Having an Arachidonoyl or Eicosapentaenoyl Group, from the Clam Worm (*Marphysa sanguinea*)

Ryuichiro TANAKA, Atsutoshi HASHIMOTO, Naoki NODA,* and Kazumoto MIYAHARA

Faculty of Pharmaceutical Sciences, Setsunan University, 45–1, Nagaotoge-cho, Hirakata, Osaka 573–01, Japan.
Received July 26, 1994; accepted August 26, 1994

Ten choline glycerophospholipids (CGP) were isolated from the marine annelid, *Marphysa sanguinea*. Among them, six compounds were lyso platelet-activating factors (PAFs) and two were 1-hexadecyl-2-arachidonoyl- and 1-hexadecyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphocholines, which are regarded as stored precursor forms of PAF, so-called PAF-like lipid. These two compounds comprised 28% of the alkylacyl CGP fraction and are considered to be the main components of PAF-like lipid in this material.

Keywords choline glycerophospholipid; lyso platelet-activating factor; annelid; *Marphysa sanguinea*

The naturally occurring alkyl ether phospholipids have attracted much attention in recent years because of their interesting biological activities. We have recently found that members of the phylum Annelida, such as the earthworm (*Pheretima asiatica*)[1] and the leech (*Hirudo nipponica*),[2] contain remarkably high amounts of 1-alkyl-*sn*-glycero-3-phosphocholines, which are known to be precursors of platelet-activating factor (PAF). Recently, Sugiura and co-workers surveyed[3] the distribution of alkyl and alkenyl ether-linked phospholipid and PAF-like lipid, which is regarded as a stored precursor form of PAF, in various species of invertebrates, and demonstrated that members of Annelida, such as the earthworm (*Pheretima* sp.) and some clam worms, contain considerable amounts of alkylacyl phospholipid, though their structures were not identified.

This study was undertaken to elucidate the structure of choline glycerophospholipids (CGPs) in the fresh material, *Marphysa sanguinea*. By application of the recycling HPLC technique[4] for isolation of intact phospholipids from a complex mixture, six lyso PAFs, two β-lysophosphatidylcholines and two 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholines were obtained in the pure state. This paper deals with the isolation and structure of these compounds.

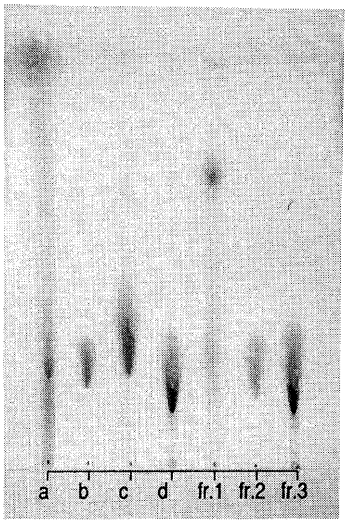

Fig. 1. TLC Profile of the Total Lipid Fraction Obtained from *M. sanguinea*

a, total lipid fraction; b, $C_{16:0}$ lyso PAF; c, $C_{16:0}$ PAF; d, glycosphingolipid fraction. solvent, $CHCl_3$–MeOH–H_2O (6:4:1). Spots were visualized with 5% H_2SO_4 in MeOH (by heating).

Live materials (3 kg) were soaked in $CHCl_3$–MeOH (1:1), then in MeOH. The combined extract was concentrated, and the residue was shaken with $CHCl_3$–MeOH–H_2O (1:2:1). The lower phase was evaporated to give 49.6 g of the extractive, which was subjected to a combination of silica-gel and Cosmosil 75C_{18}-OPN chromatographies to afford two phospholipid fractions, which showed a tailing band on spraying with Dittmer–Lester's reagent.[5] The more polar fraction was further separated into three fractions, fr. 1—fr. 3 (Fig. 1).

Preparative HPLC of fr. 2 gave eight compounds, **1**—**8**, all of which showed similar behavior on TLC to that of $C_{16:0}$ lyso PAF. Among them, compounds **1**—**6** were identified as $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$ (iso), $C_{17:0}$ and $C_{18:0}$ lyso PAFs, respectively, by comparison of their positive ion fast atom bombardment-mass spectra (FAB-MS), ^{1}H-NMR spectra and optical rotation values with those reported previously.[1]

Compound **7** ($[\alpha]_D$ +2.4°) exhibited the $[M+H]^+$ ion peak at *m/z* 542, while **8** ($[\alpha]_D$ +4.4°) gave the $[M+H]^+$ ion peak at *m/z* 568, in the positive ion FAB-MS. ^{1}H- and ^{13}C-NMR spectroscopic analyses revealed that both compounds are β-lyso phosphatidylcholines carrying a polyunsaturated fatty acid group.

Methanolysis of **7** with 5% methanolic HCl gave a fatty acid methyl ester, of which the ^{1}H-NMR and electron impact-mass spectra (EI-MS) were identical with those of authentic methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosapentaenoate.

Compound **8** produced a methyl ester, which was proved to be methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-docosahexaenoate. Since the signs of their specific rotations were the same as those of 1-acyl-*sn*-glycero-3-phosphocholines obtained previously,[2] **7** and **8** were revealed to be 1-eicosapentaenoyl- and 1-docosahexaenoyl-*sn*-glycero-3-phosphocholines, respectively.

We next surveyed the composition of alkylacyl CGP. ^{1}H-NMR spectroscopic analysis suggested that fr. 1 contains a mixture of ether-linked CGPs. Conventional HPLC separation followed by recycling HPLC of fr. 1 with a reversed-phase column using $CHCl_3$–MeOH–

© 1995 Pharmaceutical Society of Japan

NII-Electronic Library Service

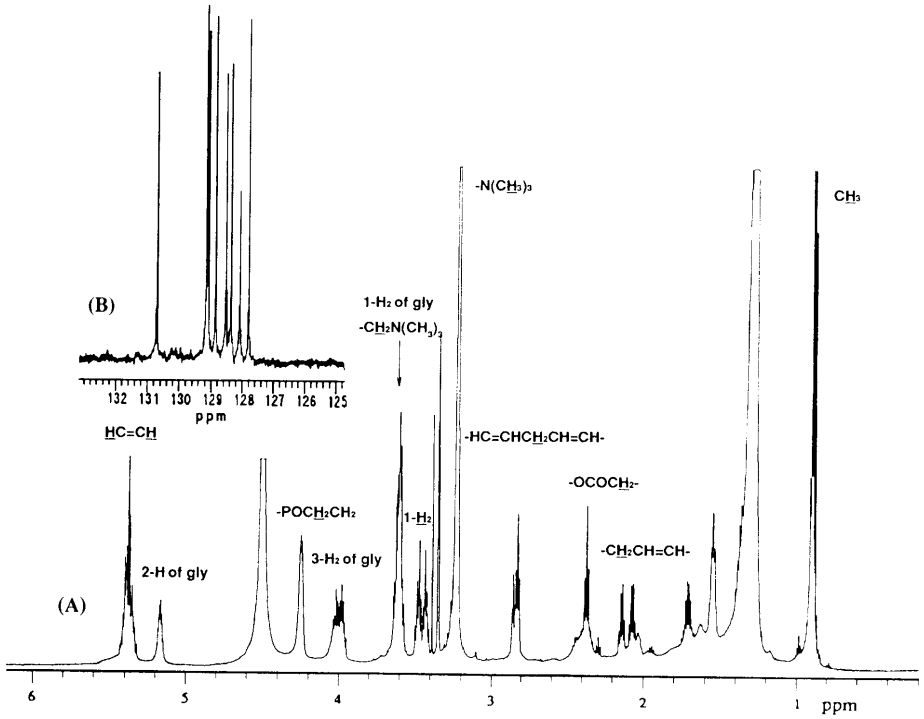

Fig. 2. ^{1}H-(A) and Partial ^{13}C-NMR (B) Spectra of **9** (in $CD_3OD:CDCl_3$, 1:2)
gly, glycerol unit.

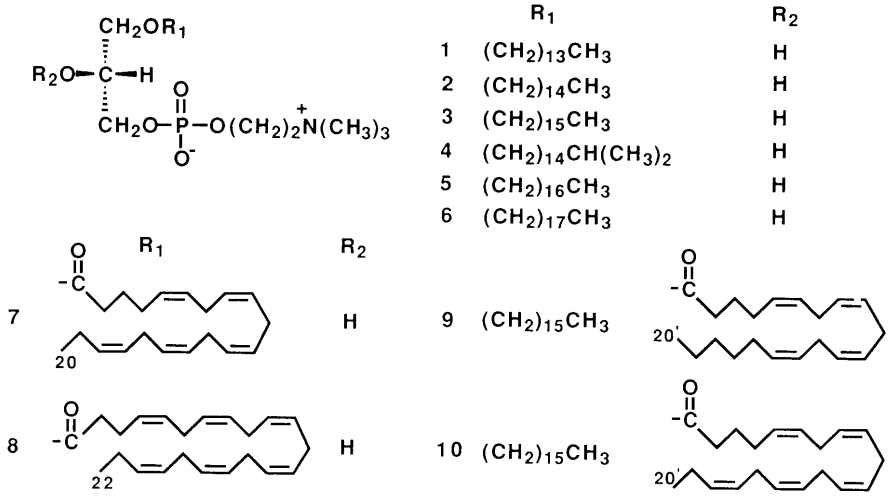

Fig. 3

H_2O mixtures gave **9** and **10** as the main components, which accounted for as much as 28% of fr. 1.

Compound **9** exhibited the $[M+H]^+$ ion peak at *m/z* 768 in the positive ion FAB-MS, and the ^{1}H-NMR spectrum showed, when compared with those of **7** and **8**, a considerable downfield shift of 2-H (δ 5.18), and upfield shifts due to 1-H_2 (δ 3.60) of the glycerol unit together with additional signals assignable to methylene groups (Fig. 2), while the ^{13}C-NMR spectrum gave eight olefinic (Fig. 2) and one carboxyl carbon signals. From these findings, **9** is considered to be a CGP having a polyunsaturated carbon chain. Treatment of **9** with 0.15% KOH in MeOH–$CHCl_3$ followed by methylation with diazomethane gave a fatty acid methyl ester and a lyso product (**9a**). The former was identified as methyl arachidonate by gas chromatography (GC), ^{1}H-NMR and EI-MS, and the latter was proved to be 1-hexadecanyl-*sn*-glycero-3-phosphocholine by comparison of the physical data with those of authentic $C_{16:0}$ lyso PAF. Thus, the structure of **9** is defined as 1-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Fig. 3).

Compound **10** gave the $[M+H]^+$ ion peak at *m/z* 766, and it showed a quite similar ^{1}H-NMR spectrum to that of **9**. Alkaline hydrolysis of **10** followed by methylation gave methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosapentaenoate and $C_{16:0}$ lyso PAF (**9a**). Accordingly, **10** is 1-hexadecyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphocholine (Fig. 3).

The present study revealed that the marine annelid, *M. sanguinea*, contains large amounts of lyso PAF and alkylacyl CGPs, and that the main CGPs (PAF-like lipid)

NII-Electronic Library Service

are 1-hexadecyl-2-arachidonoyl- and 1-hexadecyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphocholines, which account for approximately 15% and 13%, respectively, of the total alkylacyl CGP fraction.

Experimental

The NMR spectra were recorded on a JEOL JNM GX-400 instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C) at a probe temperature of 35 °C using tetramethylsilane (TMS) as an internal reference. The abbreviations used are as follows: s, singlet; t, triplet; dd, double-doublet; q, quartet; m, multiplet. The signals marked with asterisks appear as doublets (J=6—8 Hz) owing to coupling with ^{31}P. MS were acquired on a JEOL JMS DX-300 spectrometer (EI-MS: ionization voltage, 30 eV; accelerating voltage 3—10 kV, positive ion FAB-MS: accelerating voltage 3 kV; matrix, glycerol; collision gas, Xe). Optical rotations were measured at 25 °C with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel HPTLC with Al sheets (Merck Art. 5556). Spots were visualized with 5% H_2SO_4 in MeOH (by heating). Column chromatography was carried out on Merck Silica gel (230—400 mesh, Art. 9385), and Cosmosil 75C_{18}-OPN (Nacalai Tesque). Preparative HPLC was conducted over Inertsil Prep-ODS (10 μm, 20×250 mm, GL Sciences) on a JASCO 880-PU equipped with a JASCO 830-RI. Recycling HPLC was carried out on a JASCO 880-PU equipped with a JASCO preparative recycle valve.

Isolation of Compounds 1—10 Sold as commercial bait for fishing, *M. sanguinea* (3 kg) was purchased from Meitokuya (Aichi prefecture, June 1993). The live materials were soaked in 5 l of $CHCl_3$–MeOH (1:1), then in 5 l of MeOH, each for 10 d at room temperature. The extracts were combined and precipitates were removed by filtration. The filtrate was evaporated *in vacuo* to dryness to give an extractive (190 g), which was shaken with 900 ml of $CHCl_3$–MeOH–H_2O (1:2:1). The lower phase gave, on evaporation, a total lipid fraction (49.6 g), which was placed on a silica gel column and eluted successively with the following: $CHCl_3$–MeOH (8:2→7:3)→$CHCl_3$–MeOH–H_2O (7:3:0.5→6:4:1→5:5:1). The eluates were monitored by HPTLC (mobile phase: $CHCl_3$–MeOH–H_2O (6:4:1) and those showing a positive tailing band on spraying with Dittmer–Lester's reagent[5] were combined and evaporated to give a phospholipid fraction (10.4 g). This was subjected to chromatography on a 75C_{18}-OPN column using MeOH→$CHCl_3$–MeOH (1:1) as the eluent to afford two fractions, fr. a (2.0 g) and fr. b (8.1 g). Fraction b was further separated by column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (7:3:0.5→6:4:1) to give three fractions, fr. 1 (3.5 g), fr. 2 (1.8 g) and fr. 3 (2.8 g). A part (300 mg) of fr. 2 was subjected to preparative HPLC using 97% MeOH as the eluent to give compounds **1** (8 mg), **2** (9 mg), **3** (102 mg), 4 (5 mg), **5** (6 mg), **6** (16 mg), **7** (10 mg) and **8** (12 mg). A part (917 mg) of fr. 1 was separated by preparative HPLC in a recycling mode by use of $CHCl_3$–MeOH–H_2O (2:10:0.5) to give **9** (138 mg) and **10** (116 mg). Compounds **1**—**6** were identified respectively as $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$ (iso), $C_{17:0}$ and $C_{18:0}$ lyso PAFs by comparison of their positive ion FAB-MS, ^{1}H-NMR spectra and optical rotation values with those obtained from the earthworm, *Pheretima asiatica*.[1] 1-(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-Eicosapentaenoyl-*sn*-glycero-3-phosphocholine (**7**): $[\alpha]_D$ +2.4° (c=3.6, MeOH). Positive ion FAB-MS m/z: 542 $(M+H)^+$. ^{1}H-NMR (CD_3OD, 400 MHz) δ: 0.97 (3H, t, J=7.6 Hz, H_3-20), 1.69 (2H, m, H_2-3), 2.12 (2H, m, H_2-4), 2.10 (2H, m, H_2-19), 2.37 (2H, t, J=7.3 Hz, H_2-2), 2.65 (8H, m, H-7, H-10, H-13, H-16), 3.23 (9H, s, $N(C\underline{H}_3)_3$), 3.65 (2H, m, $C\underline{H}_2N$), 3.90 (2H, m, H_2-3 of glycerol), 3.97 (1H, m, H-2 of glycerol), 4.09 (1H, dd, J=6.0, 11.0 Hz, H-1 of glycerol), 4.17 (1H, dd, J=5.0, 11.0 Hz, H-1 of glycerol), 4.30 (2H, m, $POC\underline{H}_2$), 5.30—5.40 (10H, olefinic protons). ^{13}C-NMR (CD_3OD, 100 MHz) δ: 14.65, 21.50, 25.91, 26.46—26.58, 27.57, 34.37, 54.78, 60.43*, 66.30, 67.55*, 67.88*, 69.90*, 128.20, 128.95, 129.14 (×2C), 129.25 (×2C), 129.49, 129.90, 130.04, 132.83, 175.11. 1-(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-Docosahexaenoyl-*sn*-glycero-3-phosphocholine (**8**): $[\alpha]_D$ +4.4° (c=0.2, MeOH). Positive ion FAB-MS m/z: 568 $(M+H)^+$. ^{1}H-NMR (CD_3OD, 400 MHz) δ: 0.97 (3H, t, J=7.6 Hz, H_3-22), 2.08 (2H, quintet, J=7.0 Hz), 2.40 (4H, m), 2.80 (10H, m), 3.22 (9H, s, $N(C\underline{H}_3)_3$), 3.64 (2H, m, $C\underline{H}_2N$), 3.90 (2H, m, H_2-3 of glycerol), 3.97 (1H, m, H-2 of glycerol), 4.11 (1H, dd, J=6.0, 11.0 Hz, H-1 of glycerol), 4.18 (1H, dd, J=5.0, 11.0 Hz, H-1 of glycerol), 4.29 (2H, m, $POC\underline{H}_2$), 5.3—5.4 (12H, olefinic protons). ^{13}C-NMR (CD_3OD, 100 MHz) δ: 14.66, 21.54, 23.80, 26.50—26.64, 34.97, 54.79, 60.47*, 66.42, 67.64*, 67.91*, 69.95*, 128.26, 129.00, 129.2—129.3 (×7C), 129.54, 130.36, 132.89, 174.71. 1-Hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (**9**): $[\alpha]_D$ +3.27° (c=1.1, $CHCl_3$). Positive ion FAB-MS m/z: 768 $(M+H)^+$. ^{1}H-NMR (CD_3OD:$CDCl_3$, 1:2, 400 MHz) δ: 0.90 (6H, t, J=7.6 Hz, H_3-16, H_3-20′), 2.06 (2H, q, J=5.0 Hz), 2.13 (2H, q, J=5.0 Hz), 2.39 (2H, t, J=7.0 Hz, H_2-2′), 2.82 (6H, m), 3.22 (9H, s, $N(C\underline{H}_3)_3$), 3.43 (2H, m, H_2-1), 3.60 (2H, m, H_2-1 of glycerol), 3.60 (2H, m, $C\underline{H}_2N$), 4.00 (2H, m, H_2-3 of glycerol), 4.22 (2H, m, $POC\underline{H}_2$), 5.18 (1H, m, H-2 of glycerol), 5.30—5.45 (8H, olefinic protons). ^{13}C-NMR (CD_3OD:$CDCl_3$, 1:2, 100 MHz) δ: 14.1, 25.1, 25.8—34.0, 54.5, 59.1*, 64.3*, 66.9*, 69.5, 72.0, 72.3*, 127.8, 128.1, 128.4, 128.5, 128.8, 129.1, 129.2, 130.7, 173.9. 1-Hexadecyl-2-(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosapentaenoyl-*sn*-glycero-3-phosphocholine (**10**): $[\alpha]_D$ +6.3° (c=0.8, $CHCl_3$). Positive ion FAB-MS m/z: 766 $(M+H)^+$. ^{1}H-NMR (CD_3OD:$CDCl_3$, 1:2, 400 MHz) δ: 0.88 (6H, t, J=7.6 Hz, H_3-16, H_3-20′), 2.06 (2H, q, J=5.0 Hz), 2.14 (2H, q, J=5.0 Hz), 2.38 (2H, t, J=7.3 Hz, H_2-2′), 2.83 (8H, m), 3.23 (9H, s, $N(C\underline{H}_3)_3$), 3.45 (2H, m, H_2-1), 3.59 (2H, m, H_2-1 of glycerol), 3.62 (2H, m, $C\underline{H}_2N$), 4.00 (2H, m, H_2-3 of glycerol), 4.24 (2H, m, $POC\underline{H}_2$), 5.18 (1H, m, H-2 of glycerol), 5.30—5.45 (10H, olefinic protons). ^{13}C-NMR (CD_3OD:$CDCl_3$, 1:2, 100 MHz) δ: 14.2, 25.2, 25.8—34.1, 54.5, 59.2*, 64.4*, 66.9*, 69.6, 72.1, 72.3*, 127.3, 128.2, 128.4, 128.5, 128.6 (×2C) 128.9, 129.2 (×2C), 132.3, 173.9.

Methanolysis of 7 and 8 Each (5 mg) of **7** and **8** was treated with 5% methanolic HCl at 90 °C for 1 h. The fatty acid methyl ester liberated was extracted with *n*-hexane and analyzed by GC (fused silica capillary column Bonded MPS-50, Quadrex, 0.25×50 m, column temperature 230 °C (hold, 12 min) −240 °C at 1 °C/min). t_R (min): 15.9 (methyl-all-*cis* 5,8,11,14,17-eicosapentaenoate), 23.9 (methyl-all-*cis* 4,7,10,13,16,19-docosahexaenoate). Their EI-MS and ^{1}H-NMR spectra were superimposable on those of corresponding authentic samples.

Alkaline Hydrolysis of 9 and 10 Each (20 mg) of **9** and **10** was treated with 0.15% KOH in MeOH–$CHCl_3$ (10 ml, 1:1) at 90 °C for 2 h. The reaction mixture was neutralized with 0.1 N HCl and evaporated to dryness. The residue was shaken with *n*-hexane and MeOH (1:1, 10 ml), the *n*-hexane layer was treated with diazomethane in ether, and the product was analyzed by GC (under the same conditions as described above). t_R (min): 14.7 (methyl arachidonate) from **9**, 15.9 (methyl-all-*cis* 5,8,11,14,17-eicosapentaenoate) from **10**. Their EI-MS and ^{1}H-NMR spectra were in agreement with those of corresponding authentic samples. The MeOH layer was concentrated to give a residue, and this was subjected to column chromatography on silica gel using $CHCl_3$–MeOH–H_2O (6:4:1) to yield a lyso form (**9a**, 9 mg). **9a**: $[\alpha]_D$ −3.5° (c=0.5, MeOH), positive ion FAB-MS m/z: 482 $(M+H)^+$. Its ^{1}H-NMR spectrum was in accord with that of authentic $C_{16:0}$ lyso PAF.

References

1) N. Noda, S. Tsunefuka, R. Tanaka, K. Miyahara, *Chem. Pharm. Bull.*, **40**, 1349 (1992); *idem*, *ibid.*, **40**, 2756 (1992).
2) N. Noda, R. Tanaka, M. Nishi, S. Inoue, K. Miyahara, *Chem. Pharm. Bull.*, **41**, 1366 (1993).
3) T. Sugiura, T. Fukuda, T. Miyamoto, K. Waku, *Biochim. Biophys. Acta*, **1126**, 298 (1992).
4) N. Noda, R. Tanaka, K. Miyahara, T. Kawasaki, *Biochim. Biophys. Acta*, **1169**, 30 (1993); N. Noda, R. Tanaka, K. Tsujino, Y. Takasaki, M. Nakano, M. Nishi, K. Miyahara, *J. Biochem.* (Tokyo), **116**, 435 (1994).
5) J. C. Dittmer, R. L. Lester, *J. Lipid Res.*, **5**, 126 (1964).

NII-Electronic Library Service