Isolation and Characterization of Seven Lyso Platelet-Activating Factors and Two Lyso Phosphatidylcholines from the Crude Drug "Suitetsu" (the Leech, *Hirudo nipponica*)

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Nine lyso glycerophospholipids were isolated in the pure state from the crude drug "Suitetsu", which is the dried body of the leech, *Hirudo nipponica* (Hirudidae). They were identified as 1-*O*-hexadecyl-(1), 1-*O*-octadecyl-(2), 1-*O*-tetradecyl-(3), 1-*O*-9-*cis*-hexadecenyl-(4), 1-*O*-hexadecanoyl-(5), 1-*O*-pentadecyl-(6), 1-*O*-15-methylhexadecyl-(7), 1-*O*-octadecanoyl-(8) and 1-*O*-heptadecyl-*sn*-glycero-3-phosphocholine (9). Two of them (5 and 8) are lysophosphatidylcholines and the other seven are lyso platelet-activating factors. One of them has an alkenyl carbon chain.

Keywords Suitetsu; leech; *Hirudo nipponica*; Hirudidae; lyso platelet-activating factor; alkenyl lyso platelet-activating factor

The live leech, *Hirudo medicinalis*, is known to contain a peptide, hirudin, which is a thrombin inhibitor and is expected to have anticoagulant activity. The structure and the inhibition mechanism of hirudin have been investigated by many research groups.[1] Interestingly, the crude drug "Suitetsu", dried whole body of another species of leech, *Hirudo nipponica*, has been used for the treatment of congestion,[2] but no report on its constituents has so far appeared. Recently, we found that a crude drug "Jiryu", prepared from the earthworm, *Pheretima asiatica*, contains large quantities of 1-*O*-alkyl-*sn*-glycero-3-phosphocholines (lyso platelet-activating factors, lyso PAFs), and eight lyso PAFs were isolated and characterized.[3] Because the leech belongs to the same phylum as earthworm, we were interested in the lipid composition of the crude drug "Suitetsu",[2] and now we have obtained seven lyso PAFs together with two lyso phosphatidylcholines. This paper deals with the isolation and the structure elucidation of these compounds.

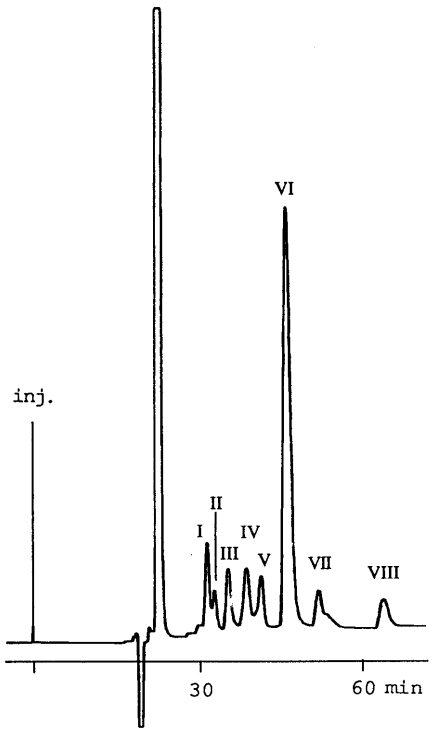
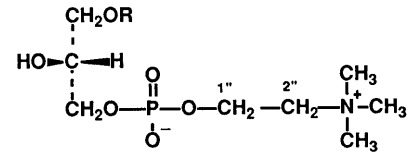


Fig. 1. HPLC Separation of Glycerophospholipids

Column, Inertsil Prep-ODS (20.0 × 250 mm); solvent, 97% MeOH; flow rate, 3.5 ml/min; chart speed, 12 cm/h; detector, RI.

The MeOH extract of the commercial crude drug, "Suitetsu" (500 g) was shaken with $CHCl_3$–MeOH–H_2O (1 : 2 : 1, v/v) and the bottom layer was evaporated to give a total lipid fraction (15.0 g). It was subjected to silica gel and Cosmosil 75C_{18} column chromatographies with various solvent systems to yield a crude phospholipid fraction (fr.-c, 0.6 g), which was further separated by means of preparative high performance liquid chromatography (HPLC). The chromatogram showed at least eight peaks (Fig. 1) and six fractions (fr.-III—VIII) corresponding to the peaks (III—VIII) were collected.

Compounds **1** (from fr.-VI) and **2** (fr.-VIII) each showed a single $(M+H)^+$ ion peak at *m/z* 482 and 510, respectively, in their positive ion fast atom bombardment mass spectra (FAB-MS). Compound **1** was identified as 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine and **2** as 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine by comparison of their physical data and proton as well as carbon nuclear magnetic resonance (^{1}H-, ^{13}C-NMR) spectra with those of authentic samples.[3] The substances in the other fractions exhibited a main $(M+H)^+$ ion peak along with quasimolecular ion



	R		R
1	$-(CH_2)_{15}CH_3$	2	$-(CH_2)_{17}CH_3$
3	$-(CH_2)_{13}CH_3$	4	$-(CH_2)_8-CH=CH-(CH_2)_5CH_3$
5	$-CO(CH_2)_{14}CH_3$	6	$-(CH_2)_{14}CH_3$
7	$-(CH_2)_{14}CH(CH_3)_2$	8	$-CO(CH_2)_{16}CH_3$
9	$-(CH_2)_{16}CH_3$		

Fig. 2. Structures of **1**—**9**

peaks such as $(M+H\pm14)^+$ or $(M+H\pm28)^+$ due to homologes, in their FAB-MS, and they were all regarded as being mixtures of homologes. Subsequently, we applied the recycling HPLC technique to separate the components in each fraction, and compounds **3** and **4** from fr.-III, **5** from fr.-IV, **6** from fr.-V, **7**, **8** and **9** from fr.-VII were isolated in the pure state. Among them, compounds **3**, **6**, **7** and **9** were identified as 1-*O*-tetradecyl-, 1-*O*-pentadecyl-, 1-*O*-15-methylhexadecyl- and 1-*O*-heptadecyl-*sn*-glycero-3-phosphocholine, respectively, by comparison of their pisitive ion FAB-MS, ^{1}H- and ^{13}C-NMR spectra and optical rotation values with those reported previously.[3]

Compound **4** exhibited a pseudo-molecular ion peak at *m/z* 480, which was 2 mass units less than that of **1** (Fig. 3). It gave a ^{1}H-NMR spectrum quite similar to those of the lyso PAFs such as **1** and **2**, but, when the spectrum was closely compared with that of **1**, additional signals at δ 2.03 (4H, m) due to allylic protons and at δ 5.34 (2H, m) ascribable to olefinic protons were observed (Fig. 4). The ^{13}C-NMR spectrum of **4** showed signals at δ 130.81 ($\times$2) due to olefinic carbons (Fig. 5). Taking these spectral data into account, **4** is considered to be a phosphorylglyceryl-ether carrying a C18:1 unsaturated carbon chain. With respect to the position of the double bond, the method reported by Scribe *et al.*,[4] failed to give any diagnostic information, but, when **4** was subjected to ozonolysis, a product (**4a**) was obtained. It showed in the ^{1}H-NMR spectrum neither methyl nor olefinic proton signals due to the alkenyl group, and its positive ion FAB-MS exhibited an $(M+H)^+$ ion peak at *m/z* 398. Therefore, it was considered that **4a** is 1-*O*-8-formyloctyl-*sn*-glycero-3-phosphocholine, and that the double bond in **4** was located at C9. The carbon signals at δ 27.9 and 28.1 were assigned to the C8 and C11 allylic carbons, respectively, by nuclear Overhauser effect and ^{1}H–^{13}C heteronuclear shift correlated two-dimensional spectra, indicating[5] the *Z* form of the double bond at C9. Hydrogenation of **4** gave **1** as identified on the basis of the ^{1}H- and ^{13}C-NMR spectra and specific rotation values. Therefore, the configuration at C2 of the glycerol moiety is *R*.

Consequently, **4** was defined as 1-*O*-(9*Z*)-octadecenyl-*sn*-glycero-3-phosphocholine, the so-called alkenyl lyso PAF (Fig. 2).

Compound **5** exhibited, in the positive ion FAB-MS, the same pseudo-molecular ion peak at *m/z* 496 as those of **7** and **9**. But the ^{1}H-NMR spectrum was different from those of lyso PAFs such as **1**—**4**. It showed 2H triplet signals (J=7 Hz) at δ 2.35 due to methylene protons next to

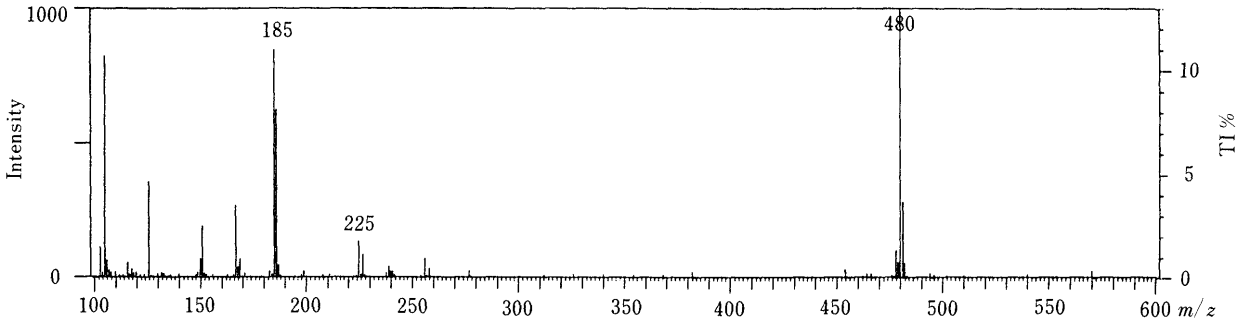


Fig. 3. Positive Ion FAB-MS of **4**

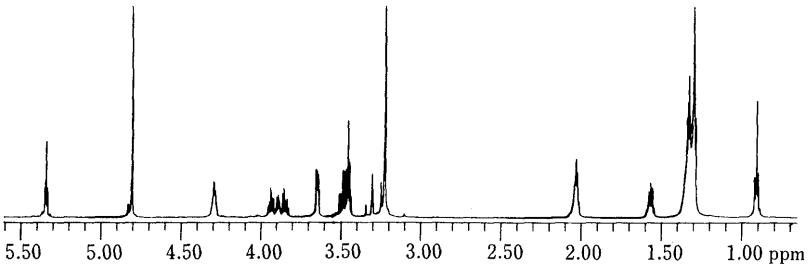


Fig. 4. ^{1}H-NMR Spectrum of **4** (600 MHz, CD_3OD)

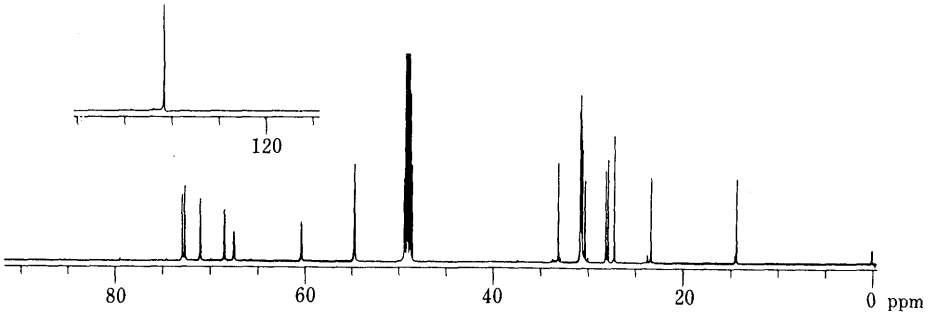


Fig. 5. ^{13}C-NMR Spectrum of **4** (600 MHz, CD_3OD)

the carboxyl group along with a 1H multiplet at δ 3.97 assignable to 2-H of the glycerol moiety. In its ^{13}C-NMR spectrum, the carboxyl carbon signal appeared. Therefore, **5** was presumed to have the hexadecanoyl group ester-bonded to glycerophosphocholine. Methanolysis of **5** with 7.5% HCl gave a fatty acid methyl ester identical with an authentic sample of methyl *n*-hexadecanoate in gas chromatography (GC). The specific rotation value of **5** ($[\alpha]_D$ +3.5°) was in good agreement with that of an authentic sample (Sigma). Accordingly, **5** was assigned the structure 1-*O*-hexadecanoyl-*sn*-glycero-3-phosphocholine (Fig. 2).

Compound **8** gave almost the same ^{1}H-NMR spectrum as that of **5**, and showed an $(M+H)^+$ ion peak at *m/z* 524, which is 28 mass units more than that of **5**. Therefore, **8** is very likely to be a homolog of **5**, that is, the *n*-hexadacenoyl group in **5** is replaced by *n*-octadecanoyl in **8**. A fatty acid methyl ester liberated by methanolysis of **8** was proved to be methyl *n*-octadecanoate by GC. Thus, **8** is 1-*O*-octadecanoyl-*sn*-glycero-3-phosphocholine (Fig. 2).

Seven lyso PAFs together with two lyso phosphatidylcholines were isolated in the pure state. Among the nine compounds, **4** is unique in that it has the C18 : 1 alkenyl group. Although the yield of lyso PAFs from "Suitetsu" was lower (*ca.* 0.08%) than that from "Jiryu" (*ca.* 0.2%), the fact that the lyso PAFs were also obtained from the leech, which belongs to the same phylum, Annelida, as the earthworm, suggests the distribution of these compounds in other annelids. Recently, Waku and co-workers reported[6] the presence of PAF-like lipid in some Annelids. We could not detect PAF itself on TLC in this study.

Experimental

The instruments and materials used are cited in the preceding paper.[3] ^{1}H- and ^{13}C-NMR spectra were recorded at 30° using TMS as an internal reference. The abbreviations used are as follows: s, singlet; d, doublet; dd, double-doublet; t, triplet; m, multiplet; br s, broad singlet. Signals marked with asterisks appear as doublets (J=5—7 Hz) because of the coupling with ^{31}P. Specific rotations were measured at 25°C.

Isolation of Compounds 1—9 The crushed powder (500 g) of the commercial crude drug "Suitetsu", *H. nipponica* purchased from Tochimoto Tenkaido was extracted with $CHCl_3$–MeOH (1 : 1, 2 l) and MeOH (2 l) successively at room temperature. The combined extract was concentrated to give a brown syrup (25 g). This was shaken with $CHCl_3$–MeOH–H_2O (1 : 2 : 1, 900 ml) and the lower phase was collected. After removal of the solvent, the residue (15.0 g) was chromatographed over silica gel ($CHCl_3$–MeOH, 8 : 2→6 : 4→$CHCl_3$–MeOH–28% NH_4OH, 6 : 4 : 1) successively to yield three fractions; fr. 1 (5.0 g), fr. 2 (5.7 g) and fr. 3 (2.1 g). Chromatography of fr. 3 on Cosmosil 75C_{18}-OPN with MeOH afforded three fractions; fr.-a (0.7 g), fr.-b (0.8 g) and fr.-c (0.6 g). Fraction-c was subjected to preparative HPLC on a reversed phase column (size, 2 × 25 cm; 10 μm, Inertsil Prep-ODS, GL Sciences Inc.) using 95% MeOH as an eluent to give fr.-I (10 mg), fr.-II (5 mg), fr.-III (18 mg), fr.-IV (13 mg), fr.-V (12 mg), fr.-VI (460 mg), fr.-VII (15 mg) and fr.-VIII (12 mg). Recrystallization of fr.-VI and VIII from AcOEt–MeOH (1 : 15) gave colorless needles, **1** (350 mg) and **2** (10 mg), respectively. Recycling HPLC was conducted with a JASCO recycling valve to give **3** (5 mg) and **4** (12 mg) from fr.-III (15 cycles), **5** (3 mg) from fr.-IV (5 cycles), **6** (6 mg) from fr.-V (5 cycles), **7** (2 mg), **8** (5 mg) and **9** (2 mg) from fr.-VII (6 cycles).

4: Powder, mp 233—240 °C (dec.), $[\alpha]_D$ −8.5° (c=1.0, MeOH). Positive ion FAB-MS *m/z* (%): 480 $(M+H)^+$ (100), 225 (15), 185 (83). ^{1}H-NMR (CD_3OD) δ: 0.89 (3H, t, J=7.0 Hz, 15-Me), 1.26—1.37 (CH_2), 1.57 (2H, m, H_2-2), 2.02 (4H, m, H_2-8 and H_2-11), 3.23 (9H, s, N-Me), 3.46 (2H, m, H_2-1), 3.48 (2H, m, H_2-1′), 3.64 (2H, m, H_2-2″), 3.83—3.96 (3H, m, H-2′ and H_2-3′), 4.29 (2H, m, H_2-1″), 5.34 (2H, m, H-9 and H-10). ^{13}C-NMR (CD_3OD) δ: 14.3 (C-16), 27.9, 28.1 (C-8, C-11), 33.1 (C-2), 54.8 (N-Me), 60.4* (C-1″), 67.6* (C-3′), 68.5* (C-2″), 71.0* (C-2′), 72.7, 73.0 (C-1′, C-1), 130.8 (C-9 and C-10).

5: Powder, $[\alpha]_D$ +3.5° (c=0.2, MeOH). Positive ion FAB-MS *m/z* (%): 496 $(M+H)^+$ (57), 518 $(M+Na)^+$ (3), 184 (100). ^{1}H-NMR (CD_3OD) δ: 0.89 (3H, t, J=7.0 Hz, 15-Me), 1.20—1.40 (CH_2), 1.61 (2H, m, H_2-3), 2.35 (2H, t, J=7.0 Hz, H_2-2), 3.22 (9H, s, N-Me), 3.64 (2H, m, H_2-2″), 3.90 (2H, m, H_2-1′), 3.97 (1H, m, H_2-2′), 4.10 (1H, dd, J=6.0, 12.0 Hz, H_2-3′), 4.18 (1H, dd, J=5.0, 12.0 Hz, H_2-3′), 4.29 (2H, m, H_2-1″). ^{13}C-NMR (CD_3OD) δ: 14.4 (Me), 23.7, 26.0, 30.2—30.8 (10C), 33.1, 34.9, 54.7 (N-Me), 60.4*, 66.3, 67.8*, 67.9*, 69.9*, 175.4 (C-1).

8: Powder, $[\alpha]_D$ +12.0° (c=0.3, MeOH). Positive ion FAB-MS *m/z* (%): 524 $(M+H)^+$ (48), 546 $(M+Na)^+$ (11), 184 (100). ^{1}H-NMR (CD_3OD) δ: 0.89 (3H, t, J=7.0 Hz, 17-Me), 1.20—1.38 (CH_2), 1.61 (2H, m, H_2-3), 2.35 (2H, t, J=7.0 Hz, H_2-2), 3.22 (9H, s, N-Me), 3.64 (2H, m, H_2-2″), 3.89 (2H, m, H_2-1′), 3.97 (1H, m, H_2-2′), 4.10, (1H, dd, J=6.0, 12.0 Hz, H_2-3′), 4.18 (1H, dd, J=5.0, 12.0 Hz, H_2-3′), 4.28 (2H, m, H_2-1″).

Ozonolysis of 4 $^1O_3$ was passed into a solution of **4** (5 mg) in MeOH (5 ml) for 15 min at −15 °C, and then dimethyl disulfide (0.5 ml) was added to the reaction mixture. After removal of the solvent, the residue was subjected to column chromatography on silica gel using $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) to give **4a** (3 mg) as a powder. Positive ion FAB-MS *m/z* (%): 398 $(M+H)^+$ (32), 184 (100). ^{1}H-NMR (CD_3OD) δ: 1.26—1.37 (CH_2), 1.57 (2H, m, H_2-2), 3.23 (9H, s, N-Me), 3.46 (2H, m, H_2-1), 3.48 (2H, m, H_2-1′), 3.64 (2H, m, H_2-2″), 3.83—3.96 (3H, m, H-2′ and H_2-3′), 4.29 (2H, m, H_2-1″).

Hydrogenation of 4 Compound **4** (5 mg) was shaken with 10% palladium carbon (20 mg, Merck) in MeOH (10 ml). The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo* to give **1** (5 mg).

Methanolysis of 5 and 8 Compound **5** (1 mg) or **8** (1 mg) was heated with 7.5% methanolic HCl at 90 °C for 1 h. The fatty acid methyl ester formed was extracted with *n*-hexane and analyzed by GC on a Shimadzu GC-8A using a glass column (2% OV-17; 3.2 mm × 3 m, column temperature, 170—230 °C at 3 °C/min; carrier gas, N_2 1.6 kg/cm^2). t_R (min): 10.6 (methyl *n*-hexadecanoate) from **5**, 15.6 (methyl *n*-octadecanoate) from **8**.

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