Glycerophosphocholines of the Earthworm, *Pheretima asiatica*

Ryuichiro TANAKA, Naoki NODA,* and Kazumoto MIYAHARA

Faculty of Pharmaceutical Sciences, Setsunan University, 45–1, Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan.
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The lipid composition of the earthworm, *Pheretima asiatica* (Annelida), was analyzed. Four glycerophospholipids, together with four known glycosphingolipids, were isolated in pure form. The former four were 1-alkyl 2-acyl glycerophosphocholines possessing a C17 : 0 and/or C18 : 1 fatty acid residue. Their structures, including the position and geometry of the double bond, were determined on the bases of chemical and spectral data.

Key words glycerophosphocholine; glycosphingolipid; earthworm; *Pheretima asiatica*; Annelida

Recent studies on the constituents of phylum Annelida have revealed that they contain remarkably high amounts of glycerophospholipids,[1)] together with novel neutral[2)] and amphoteric glycosphingolipids (GSLs).[3)] In continuation of the preceding study[4)] on the lipid composition of the phylum, we have examined glycerophospholipid and GSL fractions of the earthworm, *pheretima asiatica*, and have isolated and characterized four main alkylacyl glycerophosphocholines (GPCs), as well as four known GSLs.[2d)] This paper deals with the isolation and structural determination of these compounds.

The total lipid fraction obtained from the dried body walls of *P. asiatica* was subjected to a combination of silica gel, LH-20 and Cosmosil 75C_{18}-OPN with various solvent systems to give four fractions (fr. 2a—fr. 5a).

As described in the experimental section, fr. 2a gave, on TLC, a characteristic blue tailing band with Dittmer–Lester's reagent,[5)] and showed a similar *Rf* value to that of the alkylacyl GPCs fraction of the clamworm, *Marphysa sanguinea*, reported previously.[1b)] Conventional HPLC separation, followed by recycling HPLC with a reversed-phase column using $CHCl_3$–MeOH–H_2O mixture, gave four compounds, **1**—**4**.

Compound **1** exhibited the $[M+H]^+$ ion peak at *m/z* 747 in the positive ion FAB-MS, and the ^{1}H-NMR spectrum gave signals ascribable to glycerol and choline phosphate groups, together with two olefinic proton signals. In addition, signals at δ 2.34 due to methylene next being to a carbonyl group and a signal representative of a fatty acid residue were observed. The ^{13}C-NMR spectrum of **1** showed two olefinic signals and one carbonyl carbon signal, suggesting that **1** is an alkylacyl GPC with one double bond.

Treatment of **1** with 0.3% KOH in MeOH–$CHCl_3$, followed by methylation with diazomethane, gave a fatty acid methyl ester (**1a**) and a lyso product. The former was proven to be a C18 : 1 fatty acid methyl ester by electron impact (EI)-MS, and the latter was identified as 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine by comparison of its positive ion FAB-MS, ^{1}H-NMR spectrum and specific rotation with those of an authentic sample.[6)]

To clarify the position of the double bond in **1a**, it was converted into a dimethyl disulfide derivative (**1b**).[7)] Its EI-MS gave diagnostically important fragment ion peaks at *m/z* 245 and 145, which were regarded as due to fragment ion peaks produced by cleavage between the C-11 and C-12 sulfided carbons (Fig. 1). The geometry of the double bond was assigned as *cis* based on the chemical shifts of the allylic carbons C-10 and C-13 (δ 27.9, 28.1).[8)] From the results described above, the structure of **1** was concluded to be 1-*O*-hexadecyl-2-(11*Z*)-octadecenoyl-*sn*-glycero-3-phosphocholine.

Alkaline hydrolysis followed by methylation, in the same manner as for **1**, for each of compounds **2**, **3** and **4** gave fatty acid methyl esters possessing a C17 : 0, C18 : 1 or C18 : 1 carbon chain, respectively. The former was identified as methyl *n*-heptadecanoate by GC and EI-MS, and the latter two were characterized as the same compound, methyl (11*Z*)-octadecenoate, as that of **1** by EI-MS of their dimethyl disul-

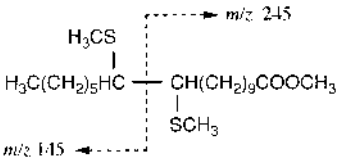

Fig. 1

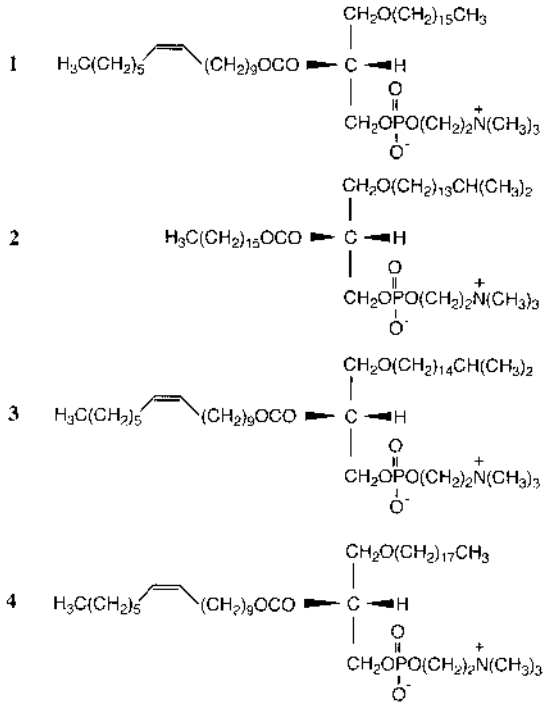

Fig. 2. Structures of **1**—**4**

∗ To whom correspondence should be addressed.

fide derivatives. Each lyso product liberated from **2**, **3** and **4** was identified as 1-*O*-14-methylpentadecyl-, 1-*O*-15-methylhexadecyl- or 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine, respectively, by comparison of their positive ion FAB-MS, NMR spectral data and specific rotation with those of the corresponding authentic samples.[6] From these data, their structures were defined as shown in Fig. 2.

On the other hand, four compounds (**5**—**8**) obtained from fr. 3a were found to be neutral GSLs which had already been isolated from the earthworm, *Pheretima aspergillum*, by Sugita *et al*.[2d] Figure 3 illustrates their structures and Table 1 shows assignments of proton and carbon signals.

Recently, Sugiura and co-workers used a biological method to examine the phospholipids of another earthworm, *Eisenia foetida*,[9] and found a substantial quantity of alkylacyl glycerophospholipids as well as platelet-activating factor (PAF), and its analogues had a *sn*-**2** short chain fatty acid residue. The main alkylacyl GPCs isolated in this study consist of a long carbon chain, and we could not obtain PAF or its analogues.

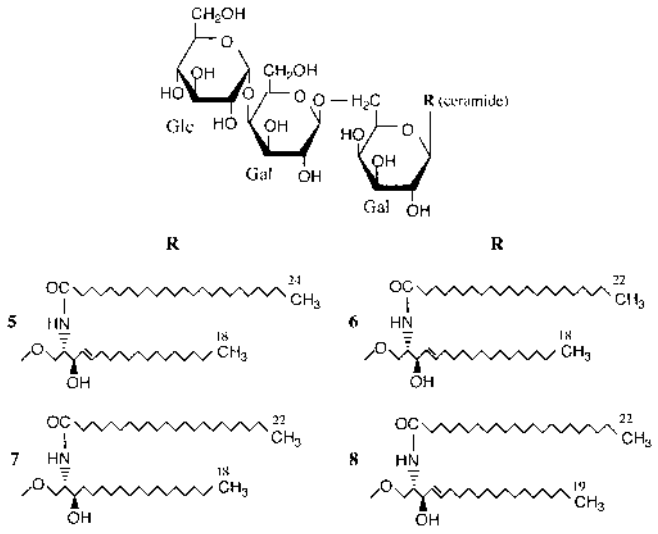

Fig. 3. Structures of **5**—**8**

Table 1. ^{13}C- and ^{1}H-NMR Spectral Data for GSLs (C_5D_5N)

No.		**5**		**6**		**7**		**8**	
		^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Cer	1	70.5	4.21, dd (7.0, 10.0) *ca*. 4.75[a]	70.4	4.20, dd (7.0, 10.0) *ca*. 4.74[a]	70.2	4.20, dd (7.0, 10.0) *ca*. 4.78[a]	70.0	4.24, dd (7.0, 10.0) *ca*. 4.78[a]
	2	55.0	*ca*. 4.75[a]	55.0	*ca*. 4.74[a]	54.2	*ca*. 4.78[a]	54.5	*ca*. 4.78[a]
	3	72.5	*ca*. 4.75[a]	72.5	*ca*. 4.74[a]	72.1	*ca*. 4.78[a]	72.0	*ca*. 4.78[a]
	4	132.7	5.97, dd (7.0, 15.0)	132.6	5.95, dd (7.0, 15.0)	—	1.80	131.5	6.00, dd (7.0, 15.0)
	5	132.0	5.88, ddd (7.0, 8.0, 15.0)	132.0	5.86, ddd (7.0, 8.0, 15.0)	—	—	130.8	5.91, ddd (7.0, 8.0, 15.0)
	6	32.2	2.06	32.1	2.05	—	—	31.5	2.09
	7	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	8	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	9	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	10	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	11	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	12	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	13	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.33	—	*ca*. 1.40
	CH_3	14.3	0.88, 3H, t (7.0)	14.3	0.88, 3H, t (7.0)	14.5	0.88, 3H, t (7.0)	13.1	0.90, 3H, t (7.0)
FA	CO	173.3	—	173.3	—	173.5	—	172.9	—
	2″	37.0	2.41, 2H, t (7.0)	37.0	2.39, 2H, t (7.0)	36.8	2.45, 2H, t (7.0)	36.5	2.44, 2H, t (7.0)
	CH_3	14.3	0.88, 3H, t (7.0)	14.3	0.88, 3H, t (7.0)	14.5	0.88, 3H, t (7.0)	13.1	0.90, 3H, t (7.0)
Gal	1	106.2	4.79, d (7.0)	106.2	4.78, d (7.0)	105.4	4.84, d (7.0)	105.1	4.82, d (7.0)
	2	72.6	4.44, dd (7.0, 7.3)	72.6	4.44, dd (7.0, 7.3)	72.6	4.44, dd (7.0, 7.3)	72.1	4.47, dd (7.0, 7.3)
	3	75.1[b]	4.04[a]	75.1[b]	4.02[a]	75.1[b]	4.09[a]	74.6[b]	4.07[a]
	4	69.9	4.37, dd (0.7, 4.0)	69.9	4.37, dd (0.7, 4.0)	69.9	4.38[a]	69.2	4.40, dd (0.7, 4.0)
	5	75.0[b]	4.08	75.0[b]	4.08	75.0[b]	4.09	74.5[b]	4.11
	6	69.7	4.33[a] 4.52, dd (4.7, 10.5)	69.7	4.32[a] 4.52, dd (4.7, 10.5)	69.7	4.35[a] 4.52, dd (4.7, 10.5)	69.0	4.36[a] 4.54, dd (4.7, 10.5)
Gal′	1	105.7	4.84, d (7.0)	105.7	4.82, d (7.0)	104.8	4.86, d (7.0)	104.5	4.88, d (7.0)
	2	72.9[c]	4.33[a]	72.9[c]	4.32[a]	71.7[c]	4.38[a]	71.3[c]	4.36[a]
	3	72.3[c]	4.14[a]	72.3[c]	4.14[a]	71.4[c]	4.15[a]	71.0[c]	4.18[a]
	4	80.0	4.65, dd (0.7, 4.0)	79.9	4.62, dd (0.7, 4.0)	78.9	4.70, dd (0.7, 4.0)	78.2	4.68, dd (0.7, 4.0)
	5	75.7	4.04[a]	75.7	4.02[a]	74.9	4.09[a]	74.3[b]	4.07[a]
	6	60.6	4.22 4.54	60.6	4.22 4.54	59.8	4.22 4.54	59.7	4.24 4.57
Glc	1	102.8	5.73, d (4.3)	102.7	5.69, d (4.3)	101.9	5.73, d (4.3)	101.2	5.77, d (4.3)
	2	74.2	4.16	74.2	4.16	73.4	4.16	73.0	4.19
	3	75.2[b]	4.57	75.2[b]	4.58	75.2[b]	4.57	74.6[b]	4.60
	4	75.1[b]	4.14[a]	75.1[b]	4.14[a]	75.1[b]	4.15[a]	74.5[b]	4.17[a]
	5	74.9[b]	4.88, m	74.9[b]	4.86, m	74.9[b]	4.88, m	74.2[b]	4.90, m
	6	62.7	4.28, dd (4.3, 10.5) 4.47, dd (3.0, 10.5)	62.6	4.28, dd (4.3, 10.5) 4.47, dd (3.0, 10.5)	61.8	4.28, dd (4.3, 10.5) 4.47, dd (3.0, 10.5)	60.9	4.31, dd (4.3, 10.5) 4.50, dd (3.0, 10.5)

Coupling constants (*J*) in Hz are given in parentheses. Cer, ceramide unit; FA, fatty acid unit; Gal, galactose unit. *a*) Signals are overlapping. *b*, *c*) The assignments may be interchanged.

Experimental

Melting points (mp) were determined on a Yanako MP-S3 apparatus and are uncorrected. The NMR spectra were recorded on a GE NMR OMEGA 600 instrument at 600 MHz (^{1}H) and 150 MHz (^{13}C), and on a JEOL JNM GX-400 instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C) at 35 °C using tetramethylsilane (TMS) as an internal reference. The abbreviations used are as follows: s, singlet; t, triplet; dd, double-doublet; m, multiplet. 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, glycerin; collision gas, Xe). Optical rotations were measured at 25 °C with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel Al sheets (Merck Art. 5556). 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—8 The MeOH extracts (75 g) of the dried body walls (1 kg) of the commercial crude drug "Jiryu" (*P. asiatica*) (purchased from Tochimoto Tenkaido) were treated with $CHCl_3$–MeOH–H_2O (1 : 2 : 1, 900 ml), and the lower phase was concentrated to give a brown extract (27 g). This was subjected to column chromatography on silica gel with the solvent $CHCl_3$–MeOH (8 : 2→7 : 3→6 : 4)→$CHCl_3$–MeOH–28% NH_4OH (6 : 4 : 1) to give fractions 1 (13 g), 2a (7 g) and 3 (7 g). Fraction 2a (1.1 g) (positive to Dittmer–Lester's reagent) was applied to a Cosmosil 75C_{18}-OPN column and eluted with MeOH to provide a crude glycerophospholipid fraction (0.2 g). Chromatography of this fraction by LH-20 (MeOH), followed by separation by HPLC with an Inertsil Prep-ODS column using $CHCl_3$–MeOH–H_2O (5 : 20 : 1) as the eluent, gave four fractions (2a-1—2a-4). Each fraction was further separated by HPLC in a recycling mode to give compounds **1** (18 mg from fr. 2a-1), **2** (14 mg from fr. 2a-2), **3** (14 mg from fr. 2a-3) and **4** (14 mg from fr. 2a-4). Fraction 3 was subjected to column chromatography on Cosmosil 75C_{18}-OPN to give fr. 3a (1 g), fr. 4a (4 g) and fr. 5a (2 g). Fraction 3a was separated by HPLC with an L-column (Chemicals Inspection & Testing Institute; 5 mm, 1 cm×25 cm×2; solvent, $CHCl_3$–MeOH–H_2O, 5 : 20 : 1) to give compounds **5** (22 mg), **6** (25 mg), **7** (3.7 mg) and **8** (4.4 mg).

1-*O*-Hexadecyl-2-(11*Z*)-octadecenoyl-*sn*-glycero-3-phosphocholine (1) Powder, mp 157—158 °C, $[\alpha]_D$ +4.0° (*c*=0.9, MeOH–$CHCl_3$, 1 : 1). Positive ion FAB-MS *m/z*: 747 $[M+H]^+$. ^{1}H-NMR (CD_3OD–$CDCl_3$, 1 : 2) δ: 0.90 (6H, t, *J*=7.6 Hz, H_3-16, H_3-18), 2.06 (4H, q, *J*=5.0 Hz, H_2-10, H_2-13), 2.34 (2H, t, *J*=7.0 Hz, H_2-2′), 3.22 (9H, s, $N(CH_3)_3$), 3.45 (2H, m, H_2-1), 3.60 (1H, m, H_2-1 of glycerol), 4.00 (1H, m, H_2-1 of glycerol), 3.60 (2H, m, CH_2N), 3.39 (2H, H_2-3 of glycerol), 4.24 (2H, m, $POCH_2$), 5.16 (1H, m, H-2 of glycerol), 5.35 (2H, olefinic protons). ^{13}C-NMR (CD_3OD–$CDCl_3$, 1 : 2) δ: 14.2, 23.0, 25.4, 25.8—34.8, 54.5, 59.5, 64.5, 67.0, 69.7, 72.2, 72.4, 130.2, 130.3, 174.4.

1-*O*-14-Methylpentadecyl-2-heptadecanoyl-*sn*-glycero-3-phosphocholine (2) Powder, mp 150—154 °C, $[\alpha]_D$ +5.1° (*c*=0.7, MeOH–$CHCl_3$, 1 : 1). Positive ion FAB-MS *m/z*: 735 $[M+H]^+$. ^{1}H-NMR (CD_3OD–$CDCl_3$, 1:2) δ: 0.88 (6H, d, *J*=7.6 Hz, H_3-15, H_3-16), 0.90 (3H, t, *J*=7.6 Hz, H_3-17′), 2.38 (2H, t, *J*=7.0 Hz, H_2-2′), 3.22 (9H, s, $N(CH_3)_3$), 3.45 (2H, m, H_2-1), 3.60 (1H, m, H_2-1 of glycerol), 4.00 (1H, m, H_2-1 of glycerol), 3.60 (2H, m, CH_2N), 3.39 (2H, H_2-3 of glycerol), 4.24 (2H, m, $POCH_2$), 5.16 (1H, m, H-2 of glycerol). ^{13}C-NMR (CD_3OD–$CDCl_3$, 1 : 2) δ: 14.2, 22.8, 23.0 25.4, 25.8—34.8, 54.5, 59.6, 64.5, 67.0, 69.7, 72.2, 72.4, 174.4.

1-*O*-15-Methylhexadecyl-2-(11*Z*)-octadecenoyl-*sn*-glycero-3-phosphocholine (3) Powder, mp 152—154 °C, $[\alpha]_D$ +5.0° (*c*=0.7, MeOH–$CHCl_3$, 1 : 1). Positive ion FAB-MS *m/z*: 761 $[M+H]^+$. ^{1}H-NMR (CD_3OD–$CDCl_3$, 1 : 2) δ: 0.88 (6H, d, *J*=7.6 Hz, H_3-16, H_3-17), 0.90 (3H, t, *J*=8.0 Hz, H_3-18′), 2.06 (4H, q, *J*=5.0 Hz, H_2-10, H_2-13), 2.34 (2H, t, *J*=7.0 Hz, H_2-2′), 3.22 (9H, s, $N(CH_3)_3$), 3.45 (2H, m, H_2-1), 3.60 (1H, m, H_2-1 of glycerol), 4.00 (1H, m, H_2-1 of glycerol), 3.60 (2H, m, CH_2N), 3.41 (2H, H_2-3 of glycerol), 4.10 (2H, m, $POCH_2$), 5.17 (1H, m, H-2 of glycerol), 5.35 (2H, olefinic protons). ^{13}C-NMR (CD_3OD–$CDCl_3$, 1 : 2) δ: 14.2, 22.8, 23.0, 25.4, 25.8—34.8, 54.5, 59.5, 64.7, 67.0, 69.6, 72.2, 72.4, 130.2, 130.3, 174.3.

1-*O*-Octadecyl-2-(11*Z*)-octadecenoyl-*sn*-glycero-3-phosphocholine (4) Powder, mp 154—155 °C, $[\alpha]_D$ +5.0° (*c*=0.7, MeOH–$CHCl_3$, 1 : 1). Positive ion FAB-MS *m/z*: 775 $[M+H]^+$. ^{1}H-NMR (CD_3OD–$CDCl_3$, 1 : 2) δ: 0.88 (6H, d, *J*=7.6 Hz, H_3-18, H_3-18′), 2.03 (4H, q, *J*=5.0 Hz, H_2-10, H_2-13), 2.35 (2H, t, *J*=7.0 Hz, H_2-2′), 3.22 (9H, s, $N(CH_3)_3$), 3.46 (2H, m, H_2-1), 3.61 (1H, m, H_2-1 of glycerol), 4.00 (1H, m, H_2-1 of glycerol), 3.60 (2H, m, CH_2N), 3.39 (2H, H_2-3 of glycerol), 4.25 (2H, m, $POCH_2$), 5.16 (1H, m, H-2 of glycerol), 5.35 (2H, olefinic protons). ^{13}C-NMR (CD_3OD–$CDCl_3$, 1:2) δ: 14.2, 23.0, 25.4, 25.8—34.8, 54.5, 59.4, 64.6, 67.1, 69.7, 72.2, 72.4, 130.2, 130.3, 174.4.

***N*-Tetracosanoyl-1-*O*-[α-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(4*E*)-octadecasphingenine (5)** White powder, mp 140—150 °C (dec.), $[\alpha]_D$ +18.1° (*c*=1.5, pyridine). Positive ion FAB-MS *m/z*: 1158 $[M+Na]^+$, Negative ion FAB-MS *m/z*: 1134 $[M-H]^-$. ^{1}H- and ^{13}C-NMR δ: Table 1.

***N*-Docosanoyl-1-*O*-[α-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(4*E*)-octadecasphingenine (6)** White powder, mp 140—150 °C (dec.), $[\alpha]_D$ +20.1° (*c*=1.5, pyridine). Positive ion FAB-MS *m/z*: 1130 $[M+Na]^+$, Negative ion FAB-MS *m/z*: 1106 $[M-H]^-$. ^{1}H- and ^{13}C-NMR δ: Table 1.

***N*-Docosanoyl-1-*O*-[α-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-dihydrooctadecasphingenine (7)** White powder, mp 141—148 °C (dec.), $[\alpha]_D$ +17.0° (*c*=0.4, pyridine). Positive ion FAB-MS *m/z*: 1132 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR δ: Table 1.

***N*-Docosanoyl-1-*O*-[α-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(4*E*)-nonadecasphingenine (8)** White powder, mp 141—148 °C (dec.), $[\alpha]_D$ +20.0° (*c*=0.4, pyridine). Positive ion FAB-MS *m/z*: 1144 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR δ: Table 1.

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