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Dinogunellins A–D: Putative ichthyootoxic phospholipids of northern blenny *Stichaeus grigorjewi* eggs*

Shigeki Matsunaga[1], Nobutaka Takahashi[1], and Nobuhiro Fusetani[1,2,‡]

[1]Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan; [2]Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

Abstract: Dinogunellins A–D, ichthyootoxic phospholipids, have been isolated from the mature eggs of the northern blenny *Stichaeus grigorjewi*. Their structures were determined based on spectral and chemical methods as a mixture of 2'- or 3'-acylated adenosine 5'-phosphate linked to the α-carboxyl group of L-asparagine through a P–N bond.

Keywords: marine toxins; fish; eggs; phospholipids; adenosine.

INTRODUCTION

More than 50 species of freshwater and marine fishes are known or speculated to cause gastrointestinal disorders, including nausea, vomiting, abdominal pain, and diarrhea, in humans from ingestion of their mature eggs, a condition referred to as ichthyootoxin [1,2]. Proteinaceous toxins have been suggested as causative agents for egg poisonings of such freshwater species as the pike *Esox lucius* [3], barbell *Barbus fluviatus* [3], and gar *Lepisosteus* spp. [4–6], although their detailed properties have not been reported. More recently, a protein toxin having an LD_{50} value of 13 μg/kg (mice, ip) was purified and characterized from eggs of the freshwater lamprey *Lampetra japonica* [7]. It has a molecular mass of 36 kD consisting of 28 and 10 kD subunits and is rich in Ser, Glx, and Gly.

Perhaps the most well-studied ichthyootoxin was dinogunellin, a novel lysophospholipid isolated from eggs of the northern blenny *Stichaeus grigorjewi* inhabiting northern Japan [8–10]. A lipoprotein named lipostichaerin was isolated from *S. grigorjewi* roe and implicated as the causative agent [8], but has subsequently been shown to comprise a complex of vitellogenin and a mixture of toxic phospholipids, named as dinogunellin [9]. The structure of dinogunellin was proposed as unusual phospholipids (**1**) containing adenosine and 2-aminosuccinamide in 1976 [10]. However, its complete structure remained to be elucidated. Dinogunellin have been also detected in another blenny *S. nozawai* [11], cabezon (or marbled sculpin) *Scorpaenichthys marmoratus* [12], and killifish *Fundulus heteroclitus* [11].

Fortunately, we were able to procure the mature eggs of *S. grigorjewi* in Usujiri, southern Hokkaido, from which were isolated four compounds related to the previously described dinogunellin,

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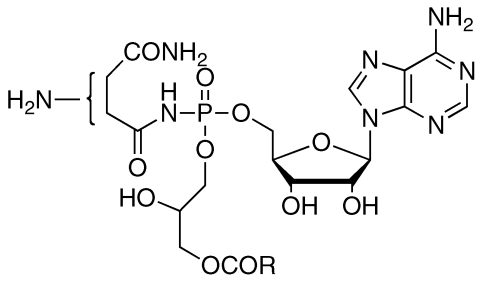
‡Corresponding author: Tel. & Fax: +81-138-40-8884; E-mail: anobu@fish.hokudai.ac.jp

and named dinogunellins A–D (**2–5**). Surprisingly, their structures differed from that proposed in the earlier work. This paper describes the isolation and structure determination of these four new compounds.



1: R = a mixture of fatty acid esters

RESULTS AND DISCUSSION

The frozen eggs (300 g, wet weight) of *S. grigorjewi* were homogenized and extracted with $CHCl_3$/MeOH (2:1). The $CHCl_3$ layer obtained after solvent partitioning was purified by silica gel column chromatography and reverse-phase high-performance liquid chromatography (HPLC) to obtain dinogunellins A–D. However, the isolated dinogunellin A (**2**) gave two peaks corresponding to dinogunellins A and B when analyzed by HPLC, whereas dinogunellin B (**3**) also gave two peaks corresponding to dinogunellins A and B (Fig. 1). Dinogunellins C and D showed the similar feature. Therefore, it was concluded that dinogunellins A and B as well as dinogunellins C and D were interconvertible to each other. The mixture of dinogunellins A and B was further purified by HPLC using a gel-filtration column to afford a mixture of dinogunellins A and B (41.3 mg, 13.8×10^{-3} % yield based on wet wt), while 3.3 mg of the mixture of dinogunellins C and D (1.1×10^{-3} %) was similarly prepared.

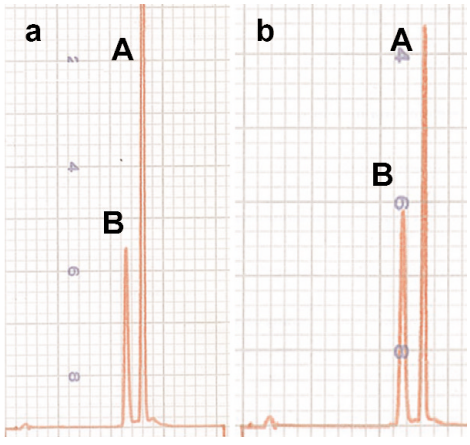
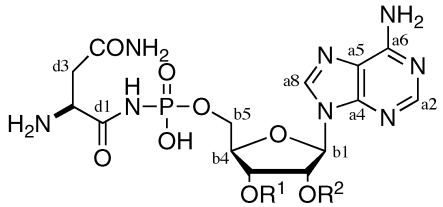


Fig. 1 Analytical HPLC of the isolated dinogunellins A (a) and B (b). Column ODS (4.6 × 250 mm); mobile phase MeCN-20 mM KH_2PO_4 (45:55); flow 1.5 ml/min; detection at 254 nm. Retention times of dinogunellins A and B are 13.0 and 11.0 min, respectively.



2: R^1 = X, R^2 = H
3: R^1 = H, R^2 = X
4: R^1 = Y, R^2 = H
5: R^1 = H, R^2 = Y

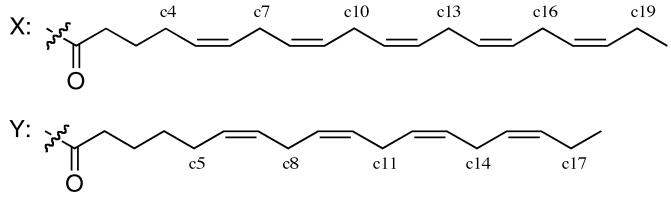


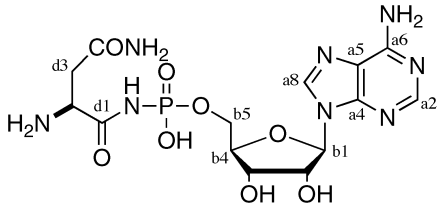
Table 1 NMR data of dinogunellins A (**2**) and B (**3**).

Number	**2**			**3**		
	δ_C[a]	δ_H[b]	HMBC (#C)[b]	δ_C[a]	δ_H[b]	HMBC (#C)[b]
a2	154.0	8.17	a4, a6	154.0	8.16	a4
a4	151.2			151.0		
a5	120.1			120.0		
a6	157.3					
a8	141.3	8.62	a4, a5	141.0	8.55	a4, a5
b1	88.2	6.08	a4, a8, b3	87.2	6.23	a4, a8
b2	74.9	4.94		79.8	5.6	c1
b3	75.0	5.41	b1, c1	70.6	4.65	
b4	83.8	4.31	b3	85.7	4.2	
b5	66.5	4.15/4.10				
c1	174.6			174.2		
c2	34.4	2.47	c1	34.2	2.39	c1
c3	25.9	1.74	c1	25.8	1.64	c1
c4	27.6	2.16		27.5	2.05	
c5	130.0	5.39		129.4		
c6	127–129	5.25–2.40				
c7, c10, c13, c16	26.5	2.80–2.83				
c8, c9, c11, c12, c14, c15, c17	127–129	5.25–2.40				
c18	132.3	5.53	c18, c20			
c19	21.5	2.07	c19, c18			
c20	14.7	0.94	d4			
d2	53.0	3.88	d4			
d3	37.8	2.75/2.56				
d4	178.0		d3			
NH_2		7.69/6.94				

[a]In CD_3OD.
[b]In CD_3OH.

Dinogunellins A and B (**2** and **3**) exhibited UV absorption at 260 nm (ε 14 300) in MeOH, which is consistent with the value reported for dinogunellin, and gave an $[M–H]^-$ ion peak at *m/z* 743 in the negative mode fast atom bombardment mass spectrometry (FABMS) spectrum. The ^{1}H NMR spectrum indicated that the mixture contained dinogunellins A and B in a ratio of 3:1. Interpretation of the ^{1}H NMR, ^{31}P NMR, correlation spectroscopy (COSY), homonuclear Hartmann–Hahn (HOHAHA), and heteronuclear single-quantum coherence (HSQC) spectra measured separately in CD_3OD and CD_3OH readily identified one unit each of adenine, furanose, fatty acid, and asparagine, but signals corresponding to a glycerol were missing (Table 1). Furthermore, the heteronuclear multiple-bond correlation (HMBC) spectrum indicated that a fatty acid moiety was attached at 3 position of the furanose in dinogunellin A (**2**), while at 2 position in dinogunellin B (**3**), thereby revealing that the acyl group was prone to migrate between the neighboring hydroxyl groups [13,14]. The rotating frame Overhauser effect spectroscopy (ROESY) spectrum indicated that the furanose moiety was ribose.

During measuring NMR spectra of the mixture of dinogunellins A and B in DMSO-d_6, we realized that the ^{1}H NMR spectrum became simpler; it exhibited only one set of signals for adenine, ribose, and asparagine moieties; two methine protons of the ribose unit experienced high-field shifts due to deacylation. In addition, the signals assignable to fatty acid were clearly observed, indicating that spontaneous hydrolysis of the acyl group took place. Interpretation of the NMR spectra of the hydrolysates (Table 2) disclosed the presence of the deacylated compound **6** which was identical with the previously reported "compound A" obtained by acid hydrolysis [10]. Further analysis of the structure of deacyl-



6

Table 2 NMR data of **6** in DMSO-d_6.

Number	δ_C	δ_H	HMBC (#C)
a2	152.6	8.13 s	a4, a5,a 6
a4	149.7		
a5	118.8		
a6	156.0		
a8	139.6	8.45 s	a4, a5, a6
NH_2		7.28 brs	a5
b1	86.6	5.89 d	a4, a8, b2, b3, b4
b2	73.8	4.60 t	b1, b4
b3	70.8	4.20 t	b1, b4, b5
b4	84.0 ($J_{C\text{-}P}$ = 7.4 Hz)	4.01 brs	b3
b5	64.5 ($J_{C\text{-}P}$ = 5.6 Hz)	3.85 brt	b3, b4
d1	176.9($J_{C\text{-}P}$ = 7.4 Hz)		
d2	52.9 ($J_{C\text{-}P}$ = 5.5 Hz)	3.43 brs	
d3	42.0	2.46 brdd	d1, d4
		2.20 brs	d4
d4	172.9		
NH_2		6.87 brs	d4
		7.49 brs	

dinogunellin (**6**) was performed by the ^{31}P NMR, COSY, HOHAHA, HSQC, and HMBC spectra. In the ^{31}P NMR spectrum a signal was observed at –6.47 ppm which was consistent with the phosphoramidate group [15]. Interpretation of ^{1}H NMR, COSY, HOHAHA, and HSQC data again allowed us to assign three units mentioned above. Connectivity of ribose and adenine was established by HMBC cross-peaks, δ_H 5.89/δ_C 149.7 and δ_H 5.89/δ_C 139.6. The presence of C-1-substituted asparagine was established by HMBC cross-peaks δ_H 6.87/δ_C 172.9, δ_H 2.16/δ_C 172.9, and δ_H 2.46/δ_C 176.9. The ^{31}P-^{1}H HMBC spectrum showed a cross-peak between the phosphorus and H-5 on ribose (δ_H 3.85; δ_C 64.5). ^{13}C NMR data indicated that C-1 carbonyl and C-2 methine carbons (δ_C 176.9; δ_C 52.9) in the asparaginyl moiety were coupled to phosphorus ($^{2}J_{P\text{-}C}$ = 7.4, $^{3}J_{P\text{-}C}$ = 5.5 Hz). Therefore, the deacyldinogunellin (**6**) was assigned as shown. Furthermore, ^{1}H NMR data reported for compound A agreed with the data obtained for **6** [10]. Gas chromatography (GC) analysis identified eicosapentaenoic acid as the acyl moiety.

The absolute configuration of the asparaginyl moiety was determined as L by chiral GC analysis of the acid hydrolysates of **6**, whereas D-configuration of ribose was concluded from the optical rotation of adenosine obtained by treating **6** with 1 % NH_4OH. Thus, the structures of dinognellins A and B were established as **2** and **3**, respectively.

Dinogunellins C and D had a molecular weight of 718 as evidenced by an $[M–H]^-$ ion in the negative-mode FABMS spectrum. The ^{1}H NMR spectrum of this mixture was very similar to that of dinogunellins A and B. The ratio of dinogunellins C and D (**4** and **5**, respectively) was revealed to be 3:1 from the ^{1}H NMR spectrum. Interpretation of 2D NMR data and GC analysis of the fatty acid released by saponificaion revealed that 6*Z*, 9*Z*, 12*Z*, 15*Z*-octadecatetraenoic acid moiety was present in dinogunellins C and D instead of eicosapentaenoic acid moiety in dinogunellins A and B.

Dinogunellins are novel adenosine-containing phospholipids, a structural class that appears to be unprecedented in marine natural products [16]. The distinctive presence of a phosphoramidate bond in dinogunellins is a feature that is shared with phosmidosines, the antifungal nucleotide antibiotics from *Streptomyces durhameusis* [17,18]. Interestingly, dinogunellins occur as a lipoprotein, complexed with the egg protein of mature eggs, vitellogenin, an egg protein in mature eggs [3]. Since dinogunellins are present in mature eggs but not in immature eggs, the biological role that they play is an intriguing subject that invites further investigation.

EXPERIMENTAL SECTION

General procedures

NMR spectra were recorded on a JEOL alpha 600 or 500 NMR instrument. ^{1}H and ^{13}C chemical shifts were referenced to internal solvent peaks: δ_H 3.3 and δ_C 49.0 for CD_3OD and CD_3OH; δ_H 2.49 and δ_C 39.7 for DMSO-d_6, δ_H 4.60 for D_2O; and external standard δ_P 0.00 for 85 % H_3PO_4 and δ_N 0.00 for NH_3. FABMS spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using NBA as a matrix. UV spectra were recorded on a SHIMADZU Bio-Spec 1600 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-5300 spectrophotometer. GC analysis was carried out on a Shimadzu GC-9A. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter.

Collection and isolation

The mature eggs of the northern blenny *S. grigorjewi* were obtained in Usujiri, Hokkaido in April 2000 and April 2001. The eggs were frozen immediately and kept at –20 °C until used.

In the prior researches, dinogunellin was purified by mouse-assay guided fractionation after repeated normal-phase chromatography. In this study, the reported chemical properties of dinogunellin were used. Dinogunellin showed a UV maximum at 259 nm in MeOH and spots at Rf values of 0.5 and

0.24 positive to ninhydrin, Hans-Isherwood and sulfuric acid reagents on silica gel thin-layer chromatography (TLC) plates when developed with $CHCl_3$/MeOH/AcOH/H_2O (30:15:2:4), and $CHCl_3$/MeOH/H_2O (65:25:4), respectively. UV absorption at 259 nm and ninhydrin reaction together with ^{1}H NMR spectra were used as criteria for dinogunellin, throughout the present experiments.

A 300 g (wet wt) portion of the frozen roe, collected in April 2001, was homogenized and extracted with 300 ml (× 4) of $CHCl_3$/MeOH (2:1). The extracts were concentrated and partitioned between ether (300 ml × 2) and water (300 ml). The organic layer was purified on a silica gel column (Merck silica gel 60, 5 × 20 cm) using stepwise elution with $CHCl_3$, acetone, MeOH, $CHCl_3$/MeOH/H_2O (65:25:4), and $CHCl_3$/MeOH/H_2O (5:5:2). The MeOH, $CHCl_3$/MeOH/H_2O (65:25:4), and $CHCl_3$/MeOH/H_2O (5:5:2) effluents were combined and partitioned between *n*-hexane and 90 % MeOH. The 90 % MeOH layer was further partitioned between $CHCl_3$ and 70 % MeOH. The organic layer was purified by HPLC on Inertsil ODS-3 (3 × 25 cm) using a gradient elution from 50 % *n*-PrOH to 100 % *n*-PrOH. Dinogunellin-containing fractions were further purified by HPLC on Inertsil ODS-3 (2 × 25 cm) using gradient elution from 70 to 100 % MeOH. Dinogunellin-containing fractions were purified by HPLC on Inertsil ODS-3 (2 × 25 cm) with 45 % MeCN containing 20 mM KH_2PO_4. Finally, desalting each peak using an α-2500 column (2 × 25 cm) and MeOH afforded dinogunellins A and B (41.3 mg, 13.77 × 10^{-3} % yield based on wet wt), and dinogunellin C and D (3.3 mg, 1.1 × 10^{-3} % yield based on wet wt).

Dinogunellins A and B

Dinogunellins A and B were slightly yellowish and soluble in $CHCl_3$/MeOH, DMSO, but not soluble in $CHCl_3$, *n*-hexane, MeCN, and EtOH. After separation, they gradually become less soluble in MeOH and $CHCl_3$/MeOH. Dinognellins A and B were very unstable, and upon exposure to light and preservation at room temperature they decomposed. It showed UV absorption at 260 nm (ε 14 300) in MeOH. Dinogunellins A and B were positive to ninhydrin, H_2SO_4, and phosphomolybdic acid, but negative to Dragendorff's reagent. Rf values on silica gel 60F plates, 0.15 $CHCl_3$/MeOH/H_2O (65:25:4) and 0.42 $CHCl_3$/MeOH/AcOH/H_2O (30:15:2:4). The negative-mode FABMS spectrum revealed a $[M–H]^-$ ion peak at *m/z* 743. For ^{1}H and ^{13}C NMR data, see Table 1.

Preparation of compound A

A 1.8-mg decomposed mixture of dinogunellins A and B was partitioned between ether and H_2O. The aqueous layer was purified by HPLC on Devilosil C-30 using gradient elution from H_2O to 20 % MeOH. The compound A containing fraction was further purified by HPLC on α-2500 using MeOH to afford compound A. ^{1}H NMR (D_2O) δ 8.29 (1H, s), δ 8.05 (1H, s), δ 5.92 (1H, d, *J* = 5.8 Hz), δ 4.54 (1H, dd, *J* = 5.39 Hz), δ 4.26 (1H, dd, *J* = 5.0, 3.85 Hz), δ 4.16 (1H, mt), δ 3.94 (2H, mt), δ 3.82 (1H, br), δ 2.59 (1H, dd, *J* = 11.5, 5.0 Hz), δ 2.44 ((1H, dd, *J* = 16.16, 8.08 Hz).

Dinogunellins C and D

Dinogunellins C and D were isolated as a yellowish oil and soluble in MeOH, $CHCl_3$/MeOH, and DMSO, and showed UV maxima at 260 nm (ε 10 200) in MeOH. The negative-mode FABMS spectrum gave a $[M–H]^-$ ion peak at *m/z* 717. The TLC profiles and color reaction of dinogunellins C and D were identical with those of dinogunellins A and B.

GC analysis of EPA

GC analysis of fatty acid was carried out by using a Supelco SP^{TM}-2380 fused silica capillary column (25 mm × 30 m) and a Shimadzu gas β-chromatograph GC-9A. The initial temperature was settled at 140 °C for 5 min, and rose to 240 °C at 4/min. Derivatization of fatty acid residues was done by ether solution of diazomethane. The product was concentrated, dissolved in *n*-hexane, and subjected to analysis. Identification of fatty acids was carried out by co-injection with the standard samples.

Chiral GC analysis of asparaginyl moiety

GC analysis of asparaginyl moiety was carried out by using a Chirasil-L-Val capillary column (25 m × 0.25 mm) and Shimadzu gas chromatography GC-9A. The initial temperature was settled at 50 °C, and rose to 200 °C at 4 °C/min. A 0.1-mg decomposed mixture of dinogunellins A and B was heated in 10 % HCl in MeOH (0.1 ml) at 105 °C for 45 min in a screw-capped test tube. After removal of the methanolic HCl in a stream of N_2, CH_2Cl_2 (0.1 ml) and trifluoroacetic anhydride (0.1 ml) were added, and the mixture was kept at 105 °C for 15 min. The product was evaporated, dissolved in CH_2Cl_2, and subjected to analysis. The product peak was identified by co-injection with the standard samples of L-Asp.

Preparation of adenosine

2.0 mg of the mixture of **6** and fatty acid was treated with 1 % NH_4OH (0.15 ml) at 175 °C for 20 min in a screw-capped test tube. The solvent was removed in vacuo, reaction mixture was dissolved in H_2O, and subjected to purified by reversed-phase HPLC (Develosil C-30) with gradient elution from H_2O to 20 % MeOH to afford adenosine (0.3 mg). The optical rotation of prepared adenosine: $[\alpha]^{23}_D$ – 21.1 ° (*c* 0.02, H_2O).

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