# Shimofuridin A, a Nucleoside Derivative Embracing an Acylfucopyranoside Unit Isolated from the Okinawan Marine Tunicate *Aplidium multiplicatum*

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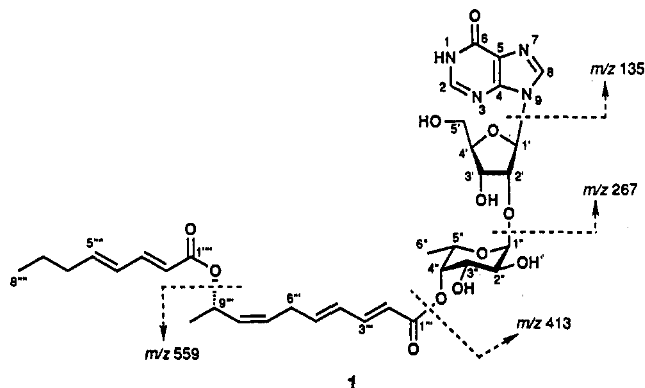
Numerous bioactive nucleoside analogs have been isolated from terrestrial and marine sources and some of them are expected to be clinically useful compounds.[1] During our continuing search for new bioactive substances from Okinawan marine organisms,[2] we recently examined extracts of the Okinawan tunicate *Aplidium multiplicatum* Sluiter and isolated a novel nucleoside derivative, shimofuridin A (1),[3] containing an acylfucopyranoside moiety and possessing cytotoxic and antimicrobial activities. This paper describes the isolation and structure elucidation of 1.

The tunicate *A. multiplicatum*, collected off Okinawa Island, was extracted with MeOH and the extract was partitioned between EtOAc and $H_2O$. The EtOAc-soluble material was subjected to silica gel flash column chromatography ($MeOH/CHCl_3$, 30:70) followed by gel filtration on Sephadex LH-20 ($MeOH/CHCl_3$, 1:1) and reversed-phase HPLC (ODS, 70% MeOH) to give shimofuridin A (1, 0.002% wet weight).

The molecular formula of shimofuridin A (1) was established as $C_{34}H_{44}O_{12}N_4$ by HRFABMS [$m/z$ 699.2896 $(M-H)^-$, $\Delta$ +1.9 mmu]. The IR absorption bands at 3400




and 1700 $cm^{-1}$ suggested the presence of hydroxyl and carbonyl groups, respectively, and the UV spectrum showed an absorption maximum at $\lambda_{max}$ 261 nm, which may be attributable to the chromophore of a purine base[4] or a conjugated dienoate functionality.[5] Interpretation of the $^1H$ and $^{13}C$ NMR data of 1 (Table 1) revealed the

**Table 1. $^1H$ and $^{13}C$ NMR Chemical Shifts and Heteronuclear Multiple Bond (HMBC) Correlations of Shimofuridin A (1) in DMSO-$d_6$**

| position | $^1H$ | $J$ (Hz) | $^{13}C$ | HMBC ($^1H$)[a] |
|---|---|---|---|---|
| 2 | 8.09 s | | 146.0 d | |
| 4 | | | 148.1 s | 2, 1′ |
| 5 | | | 124.5 s | 8 |
| 6 | | | 156.5 s | 2 |
| 8 | 8.36 s | | 138.8 d | 1′ |
| 1-NH | 12.03 br s | | | |
| 1′ | 6.04 d | 6.4 | 85.6 d | |
| 2′ | 4.71 dd | 5.1, 6.4 | 80.7 d | 1′, 1″ |
| 3′ | 4.31 dd | 3.4, 5.1 | 69.7 d | 3′-OH |
| 3′-OH | 5.04 | | | |
| 4′ | 4.00 ddd | 3.4 | 85.8 d | 3′, 3′-OH |
| 5′ (a) | 3.64 dd | 3.4, 12.2 | 61.2 t | 5′-OH |
| (b) | 3.68 dd | 3.4, 12.2 | | |
| 5′-OH | 5.05 | | | |
| 1″ | 4.86 d | 3.9 | 100.2 d | 2′ |
| 2″ | 3.56 dd | 3.9, 10.3 | 68.2 d | |
| 2″-OH | 5.03 | | | |
| 3″ | 3.75 dd | 3.4, 10.3 | 67.3 d | |
| 3″-OH | 5.05 | | | |
| 4″ | 4.94 t | 3.4 | 73.6 d | |
| 5″ | 3.60 dd | 3.4, 6.4 | 65.2 d | |
| 6″ | 0.49 d | 6.4 | 15.5 q | 1″ |
| 1‴ | | | 165.5 s | 4″, 2‴, 3‴ |
| 2‴ | 5.86 d | 15.2 | 119.1 d | |
| 3‴ | 7.20 m | | 145.1 d | 4‴ |
| 4‴ | 6.24 m | | 128.43 d | 6‴ |
| 5‴ | 6.21 m | | 141.82 d | 6‴ |
| 6‴ (2H) | 3.00 br s | | 30.8 t | |
| 7‴ | 5.493 m | | 131.1 d | 6‴, 9‴ |
| 8‴ | 5.485 m | | 128.4 d | 9‴ |
| 9‴ | 5.59 m | | 66.1 d | 10‴ |
| 10‴ | 1.24 d | 6.4 | 20.6 q | |
| 1⁗ | | | 166.0 s | 2⁗ |
| 2⁗ | 5.87 d | 15.6 | 119.6 d | |
| 3⁗ | 7.16 m | | 144.8 d | |
| 4⁗ | 6.26 m | | 128.38 d | |
| 5⁗ | 6.28 m | | 141.75 d | |
| 6⁗ (2H) | 2.11 m | | 34.4 t | 7⁗, 8⁗ |
| 7⁗ (2H) | 1.40 m | | 21.4 t | 6⁗, 8⁗ |
| 8⁗ | 0.86 t | 7.3 | 13.5 q | |

[a] Optimized for $^nJ_{CH}$ = 8 Hz.

presence of five $sp^2$ quaternary carbons, twelve $sp^2$ methines, ten oxymethines, one oxymethylene, three methylenes, and three methyls; this accounts for 34 carbons and 39 protons. Extensive analysis of 2D NMR spectra ($^1H$–$^1H$ COSY, HSQC,[6] HMBC,[7] NOESY, and ROESY[8] ) suggested that 1 consisted of a purine base, two sugar units, and two unsaturated fatty acids. Since the $^{13}C$ NMR chemical shifts for the purine moiety [$\delta_C$ 146.0 (d, C-2), 148.1 (s, C-4), 124.5 (s, C-5), 156.5 (s, C-6), and 138.8 (d, C-8)] corresponded well to those of the purine base of inosine,[9] the purine base of 1 was assigned as hypoxanthine; this assignment was further corroborated by the UV absorption data [$\lambda_{max}$ 244 nm (MeOH, pH 7); 252 nm (pH 2); 253 nm (pH 11)][10] of the nucleoside unit 2, which was obtained in the water-soluble fraction of the alkaline hydrolysis product of 1.

(1) Isono, K. *Pharmacol. Ther.* **1991**, *52*, 269–286 and refs cited therein.
(2) (a) Kobayashi, J.; Sato, M.; Ishibashi, M. *J. Org. Chem.* **1993**, *58*, 2645–2646. (b) Kobayashi, J.; Zeng, C.-M.; Ishibashi, M. *J. Chem. Soc., Chem. Commun.* **1993**, 79–81. (c) Kobayashi, J.; Tsuda, M.; Nakamura, T.; Mikami Y.; Shigemori H. *Tetrahedron* **1993**, *49*, 2391–2402. (d) Ishibashi, M.; Takeuchi, S.; Kobayashi, J. *Tetrahedron Lett.* **1993**, *34*, 3749–3750. (e) Kobayashi, J.; Kondo, K.; Ishibashi, M.; Wälchli, M. R.; Nakamura, T. *J. Am. Chem. Soc.* **1993**, *115*, 6661–6665.
(3) The Japanese name of this tunicate is "minami-shimofuri-boya", after which the compound is named.
(4) Kondo, K.; Shigemori, H.; Ishibashi, M.; Kobayashi, J. *Tetrahedron* **1992**, *48*, 7145–7148.
(5) Kobayashi, J.; Ishibashi, M.; Nakamura, H.; Ohizumi, Y.; Yamasu, T.; Sasaki, T.; Hirata, Y. *Tetrahedron Lett.* **1986**, *27*, 5755–5758.
(6) Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185–189.
(7) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
(8) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207–213.
(9) Kalinowski, H.-O.; Berger, S.; Braun, S. *Carbon-13 NMR Spectroscopy*; John Wiley & Sons: Chichester, 1984; p 440: inosine [$\delta_C$ 146.2 (C-2), 148.5 (C-4), 124.6 (C-5), 156.9 (C-6), and 139.1 (C-8)]; adenosine [$\delta_C$ 152.6 (C-2), 149.3 (C-4), 119.6 (C-5), 156.3 (C-6), and 140.2 (C-8)].
(10) Ikehara, M. *Chemistry for Life Science—Nucleic Acid*; Asakura Shoten: Tokyo, 1979; pp 32–33: inosine [$\lambda_{max}$ 248 nm (pH 6)]; adenosine [$\lambda_{max}$ 260 nm (pH 6.4)].

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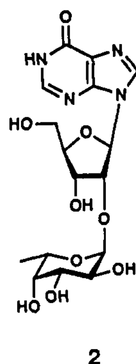
The $^1H-^1H$ COSY spectrum of 1 suggested a pento-




2

furanose and a 6-deoxyhexopyranose for the two sugar units, which were firmly identified as ribose and fucose, respectively, on the basis of GC analysis of the trimethylsilyl derivatives of the methanolysis product of the nucleoside moiety 2. The HMBC spectrum of 1 showed cross peaks due to $^1H$ - $^{13}C$ long-range correlations for H-1′/C-4 and H-1′/C-8, thus indicating that N-9 of hypoxanthine is connected to C-1′ of ribose. The $^{13}C$ NMR chemical shifts for the ribose unit of 1 (Table 1) were analogous to those for the sugar component of inosine[9] except for that of C-2′ (1: $\delta_C$ 80.7; inosine: $\delta_C$ 73.7), implying that the second sugar unit, fucose, is attached to this position, which was further confirmed by the HMBC correlations observed for H-2′/C-1″ (the anomeric position of fucose) and H-1″/C-2′. The low-field resonance ($\delta_H$ 4.94) of H-4″ of fucose implied that C-4″ was acylated and this fact was supported by the HMBC connectivity between H-4″ and C-1‴ (ester carbonyl at $\delta_C$ 165.5).

The first unsaturated fatty acid chain contained in compound 1 was inferred to be a 9-oxygenated deca-2,4,7-trienoyl group by analysis of the proton connectivities observed in the $^1H-^1H$ COSY spectrum of 1. H-9‴ of this fatty acid unit resonated at $\delta_H$ 5.59 and this chemical shift suggested that C-9‴ is further acylated by the second fatty acid chain, which was identified as an octa-2,4-dienoyl group by the $^1H-^1H$ COSY data. The double bond geometries in the two unsaturated chains were elucidated as follows. The 2‴*E*- and 2⁗*E*-configurations were deduced from the $^1H-^1H$ coupling constants ($J_{2''',3'''}$ = 15.2 Hz and $J_{2'''',3''''}$ = 15.6 Hz). The $^{13}C$ NMR chemical shifts for the C-2⁗–C-8⁗ positions of the second acyl chain agreed well with those of the corresponding positions in 2(*E*),4(*E*)-octadienoic acid.[11] Particularly for the allylic position, C-6⁗ of 1 was observed at $\delta_C$ 34.4, while C-6 of 2(*E*),4(*E*)-octadienoic acid resonates at $\delta_C$ 35.0.[11] The 4⁗*E*-configuration was established since the allylic carbons of *cis*-double bonds are known to resonate approximately at 27 ppm.[12] The homo-spin decoupling experiment irradiating at $H_2$-6‴ (the bis-allylic position of the first acyl chain) in $CD_3OD$ revealed the $J_{7''',8'''}$ value to be 10.7 Hz, implying a 7‴*Z* configuration. This finding was supported by the NOESY cross peak in $CD_3OD$ observed between $H_2$-6‴ and H-9‴. The bis-allylic carbon (C-6‴) resonated at $\delta_C$ 30.8, which suggested that C-6‴ is located between one *cis* and one *trans* olefin.[13] The $\Delta^{4''',5'''}$-double bond was therefore assigned as *E*.

The stereochemistries of the anomeric positions of the sugar moieties were assigned as $\beta$ for the ribofuranose and $\alpha$ for the fucopyranose, respectively, on the basis of the one-bond $^1H-^{13}C$ coupling constants[14] recorded by an INEPT[15] experiment of 1 in $CD_3OD$ (C-1′, $^1J_{C,H}$ = 150.7 Hz[16]; C-1″, $^1J_{C,H}$ = 174.6 Hz[17]). This finding for the $\beta$-ribofuranose unit was consistent with the ROESY correlation observed between H-1′ and H-4′ in DMSO-$d_6$ solution of 1. The absolute configurations of the ribose and fucose units were determined as D and L, respectively, by chiral HPLC followed by EIMS analysis of the collected (UV absorbing) analytes[18] using *O*-benzoyl derivatives of the methanolysis product of the nucleoside portion (2).

The EtOAc-soluble fraction of the alkaline hydrolysate of 1, containing a mixture of fatty acid units, was treated with ozone followed by $NaBH_4$ reduction. Trimethylsilyl (TMS) derivatives of the ozonolysis product were subjected to chiral GC analysis (Chirasil-Val) to give a peak identical with the TMS derivative of (*S*)-1,2-propanediol. Consequently, the absolute configuration of C-9‴ position of 1 was assigned as *S*.

From all of these results the structure of shimofuridin A was deduced as inosine 2′-α-L-4″-[9‴(*S*)-(2⁗(*E*),4⁗-(*E*)-octadienoyloxy)-2‴(*E*),4‴(*E*),7‴(*Z*)-decatrienoyl]fucopyranoside (1). This structure was fully consistent with the fragment ions observed in the negative FABMS (see supplementary material) at $m/z$ 559 (M − octadienoyloxy group)$^-$, 413 (inosine α-L-fucopyranoside − H)$^-$, 267 (inosine − H)$^-$, and 135 (hypoxanthine − H)$^-$. The conformation of the inosine unit of 1 was deduced to be *syn* from the cross peak for H-8/H-1′ observed in both the ROESY in DMSO-$d_6$ and the NOESY in $CD_3OD$.

Shimofuridin A (1) exhibited cytotoxicity against murine lymphoma L1210 cells with an $IC_{50}$ value of 9.5 μg/mL in vitro and antimicrobial activity against fungus *Trichophyton mentagrophytes* (MIC value, 133 μg/mL) and Gram-positive bacterium *Sarcina lutea* (MIC, 66 μg/mL). Compound 1 also showed inhibitory activity against protein kinase C ($IC_{50}$ 20.0 μg/mL).[19]

## Experimental Section

**Collection, Extraction, and Isolation.** The tunicate *Aplidium multiplicatum* Sluiter (order Enterogona, suborder Aplousobranchia, family Polyclinidae, subfamily Polyclininae) was collected off Seragaki Beach, Okinawa Island, in May 1992 and kept frozen until used. The voucher specimen (TN-512) was deposited at the Faculty of Pharmaceutical Sciences, Hokkaido University. The tunicate (1.0 kg wet weight) was

(11) Frighetto, N.; Silveira, C. L. P.; Reis, F. A. M.; Magalhães, E. G.; Rúveda, E. A. *Chem. Phys. Lipids* **1978**, *22*, 115–120: $^{13}C$ NMR chemical shifts for 2(*E*),4(*E*)-octadienoic acid, $\delta_C$ (DMSO-$d_6$) 118.3, 147.1, 128.2, 145.5, 35.0, 21.8, and 13.5 (C-2–C-8, respectively).

(12) Gunstone, F. D.; Pollard, M. R.; Scrimgeour, C. M.; Vedanayagam, H. S. *Chem. Phys. Lipids* **1977**, *18*, 115–129.

(13) The $^{13}C$ NMR chemical shifts for bis-allylic carbons between *cis–cis*, *cis–trans*, and *trans–trans* olefins are approximately 25, 30, and 35 ppm, respectively.[12]

(14) Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293–297.

(15) Morris G. A.; Freeman, R. *J. Am. Chem. Soc.* **1979**, *101*, 760–762.

(16) C-1′ of adenosine (β-anomer): $^1J_{C,H}$ = 150.7 Hz; C-1′ of α-adenosine (α-anomer): $^1J_{C,H}$ = 145.2 Hz. These data were recorded by us in $CD_3OD$ solutions. α-Adenosine was purchased from Sigma Chemical Co.

(17) $^1J$ (C, equatorial H) is usually around 170 Hz and $^1J$ (C, axial H) around 160 Hz in hexopyranoses, the former corresponding to α-anomer and the latter to β-anomer: Hansen, P. E. *Prog. NMR Spectrosc.* **1981**, *14*, 175–296.

(18) The chiral HPLC afforded fractions containing *O*-benzoyl derivatives of methyl D-ribose and methyl L-fucose, which were collected and subjected to EIMS analysis. The EIMS fragmentation patterns of these fractions proved to be identical with those of *O*-benzoyl/Me derivatives prepared from authentic D-ribose and L-fucose, respectively.

(19) Kikkawa, U.; Go, M.; Koumoto, J.; Nishizuka, Y. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 636–643.

extracted with MeOH (1.5 L × 2). The MeOH extract was partitioned between EtOAc (500 mL × 3) and 1 M NaCl (500 mL). A portion (1.4 g) of the EtOAc-soluble material (2.7 g) was subjected to column chromatography on silica gel (3.0 × 45 cm), eluting with 10–100% MeOH in $CHCl_3$. The fraction eluting with $CHCl_3$/MeOH (70:30) was separated by gel filtration on a Sephadex LH-20 column (Pharmacia, 2.0 × 100 cm) with $CHCl_3$/MeOH (1:1) to give a fraction (125–160 mL), which was then passed through a Sep-Pak $C_{18}$ cartridge (Waters, 10 × 10 mm). The fraction eluting with 40–70% MeOH was finally purified by reversed-phase HPLC (YMC-Pak ODS, 5 μm, 10 × 250 mm, 70% MeOH; flow rate, 3.0 mL/min) to give shimofuridin A (1, $t_R$ 29.2 min, 8.9 mg, 0.002% wet weight).

**Shimofuridin A (1):** colorless solid; mp 210 °C (MeOH); $[\alpha]^{19}_D$ −186° (c 1.4, pyridine); UV (MeOH) $\lambda_{max}$ 310 (ε 5000) and 261 nm (44000); IR (KBr) $\nu_{max}$ 3400, 2920, 1700, 1640, 1140, 1080, 1040, and 1000 $cm^{-1}$; $^1H$ and $^{13}C$ NMR (see Table 1); FABMS (negative, diethanolamine matrix) m/z 699 $(M - H)^-$, 559, 413, 267, and 135; HRFABMS m/z 699.2896 (M − H; calcd for $C_{34}H_{43}O_{12}N_4$, 699.2875).

**Alkaline Hydrolysis of 1.** Shimofuridin A (1, 1 mg) was dissolved in MeOH (1 mL) and 0.5 N KOH (0.5 mL) and stirred at room temperature for 2 h. After addition of saturated NaCl (3 mL), the aqueous layer was extracted with EtOAc (3 mL × 3). The EtOAc-soluble fraction contained a mixture of fatty acid compounds. The aqueous layer was passed through an adsorption column of Amberlite XAD-2 (0.5 × 5 cm) eluted with $H_2O$ (5 mL) and then with MeOH (5 mL). The fraction eluted with MeOH afforded the nucleoside unit 2.

**Sugar Analysis by GC.** The nucleoside moiety (2, 0.9 mg) was dissolved in 0.5 M HCl/MeOH (0.5 mL) and heated at 65 °C for 15 h in a sealed tube. After evaporation of the solvent by a stream of nitrogen, the residue was dissolved in pyridine (50 μL) and treated with hexamethyldisilazane (10 μL) and trimethylsilyl chloride (5 μL) at room temperature for 30 min. Solvent was removed by a nitrogen stream and the residue dissolved in hexane was used for GC analysis [1.5% OV-17 glass column (3 mm × 2 m); $N_2$ as a carrier gas; the program rate: 120–200 °C at 0.5 °C/min] showing peaks at $t_R$ 4.0, 4.2, 5.3, and 6.9 min, which corresponded to those of ribose (4.2 min) and fucose (4.0, 5.3, and 6.9 min), respectively. The TMS/Me derivatives of 6-deoxyhexoses (fucose and rhamnose) and aldopentoses (ribose, arabinose, and xylose) were prepared as authentic specimens.

**Determination of the Stereochemistry of the Sugar Units by Chiral HPLC.** The methanolysis product of 2 (0.9 mg) obtained by the same procedures as described above was dissolved in $CH_2Cl_2$ (1 mL) and treated with benzoyl chloride (2.5 μL) in the presence of $Et_3N$ (4.3 μL) at room temperature for 3 h. Evaporation of the solvent under reduced pressure afforded a residue, which was subjected to chiral HPLC analysis using SUMIPAK OA-4000 (Sumika Chemical Analysis Service, 4.6 × 250 mm; flow rate, 1.0 mL/min; UV detection at 254 nm) to show a peak at $t_R$ 6.5 min on elution with hexane/EtOH (70:1) and a peak at $t_R$ 10.8 min on elution with hexane/EtOH (995:5). Retention times of the O-benzoyl/Me derivatives of D- and L-fucose, eluted with hexane/EtOH (70:1), were 6.0 and 6.5 min, respectively, while the retention times of the O-benzoyl/Me derivatives of D- and L-ribose eluted with hexane/EtOH (995:5) were 10.8 and 11.3 min, respectively.

**Analysis of C-9‴ Configuration.** A solution of the EtOAc-soluble fraction of the alkaline hydrolysate (0.5 mg) of 1 in MeOH (0.5 mL) was bubbled with $O_3$ at −78 °C for 1 min. After the removal of excess ozone by a stream of nitrogen, a solution of $NaBH_4$ (5 mg) in MeOH (200 μL) was added and the whole mixture was stirred for 45 min at 0 °C. 1 M Potassium phosphate buffer (pH 7.0) was added and the reaction mixture was evaporated under reduced pressure. The residue was treated with pyridine (50 μL), hexamethyldisilazane (10 μL), and trimethylsilyl chloride (5 μL) for 30 min at room temperature. After evaporation of the reagents, the mixture was dissolved in hexane and used for chiral GC analysis [Chirasil-Val column (Alltech, 0.32 mm × 25 m); nitrogen as a carrier gas; the program rate: 30–40 °C at 0.5 °C/min] to show a peak at $t_R$ 2.4 min. The TMS derivatives were prepared from authentic specimens of racemic 1,2-propanediol and (S)-1,2-propanediol (Kanto Chemicals) by the same procedures as described above. Retention times of TMS derivatives of (S)- and (R)-1,2-propanediol were found to be 2.4 and 5.4 min, respectively.

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**Supplementary Material Available:** 2D NMR and other spectra of 1 (11 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.