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Pipecolate Derivatives, Anthosamines A and B, Inducers of Larval Metamorphosis in Ascidians, from a Marine Sponge *Anthosigmella* aff. *raromicrosclera*

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Abstract: Anthosamines A and B (**1** and **2**), new pipecolate derivatives have been isolated as metamorphosis-inducers of ascidian *Halocynthia roretzi* larvae from a marine sponge *Anthosigmella* aff. *raromicrosclera*, along with a new diketopiperazine **3**, *cyclo*-(L-Arg-dehydrotyrosine). Their structures were elucidated on the basis of spectral data. Reversible isomerization between amine and iminium ion forms were observed for **1** and **2**; iminium ion forms (**1b** and **2b**) predominate in protic solvent, amine forms (**1a** and **2a**) in aprotic solvent.

INTRODUCTION

In our ongoing studies on chemical cues for larval settlement and metamorphosis, we have isolated several compounds which induce larval metamorphosis in two ascidians, *Halocynthia roretzi* and *Ciona savignyi*: urochordamines A and B[1a, 1b] from two ascidians *C. savignyi* and *Botrylloides* sp.; phlorotannins and sulfoquinovosyl diacylglycerols[1c] from the brown alga *Sargassum thunbergii*; and (*E*)- and (*Z*)-narains and related compounds from a marine sponge *Jaspis* sp.[1d, 1e] Subsequently, we found similar activity in the MeOH extract of the marine sponge *Anthosigmella* aff. *raromicrosclera* (Clionidae) collected off the Sada Peninsula, Shikoku, Japan. Bioassay-guided isolation afforded two pipecolate derivatives, anthosamines A and B (**1** and **2**), and a diketopiperazine **3**, as metamorphosis inducers on ascidian larvae. This paper describes the isolation and structural elucidation of these metabolites.

RESULTS AND DISCUSSION

The water soluble portion of the MeOH extract was partitioned between H_2O and *n*-BuOH; the *n*-BuOH layer was subjected to ODS flash chromatography with aqueous MeOH. The MeOH-H_2O (3:7) eluate, which induced metamorphosis in ascidian larvae, was further purified by reversed phase HPLC and gel filtration to afford anthosamines A (**1**, 4.16 mg, 2.1 x 10^{-4} %, wet weight) and B (**2**, 1.53 mg, 7.7 x 10^{-5} %) and a diketopiperazine **3** (40.7 mg, 2.0 x 10^{-3} %).

Structures of anthosamines A (1) and B (2)

Anthosamine A (**1**) had a molecular formula of $C_{13}H_{13}NO_3$ as determined by HRFABMS (*m/z* 232.0953, Δ 0.0 mmu). The ^1H NMR spectrum of **1** in DMSO-d_6 showed four aromatic protons [δ 6.77 (2 H, d, *J*=8.3 Hz, H-3' and 5') and 7.04 (2 H, d, *J*=8.3 Hz, H-2' and 6')], six methylene protons [δ 1.71 (2 H, m, 2 x H-5), 1.86 (1 H, m, H-4a), 2.16 (1 H, m, H-4b), and 3.06 (2 H, m, 2 x H-6)], a low field methine proton [δ 5.76 (1 H, s, H-7)], and two D_2O-exchangeable protons [δ 5.23 (1 H, s, NH) and 9.57 (1 H, br.s, OH)]. Interpretation of the ^1H-^1H COSY spectrum together with ^{13}C NMR data readily led to two partial structures: a *p*-hydroxyphenyl group (unit **a**, Fig. 1) [δ 6.77 (2 H, H-3' and 5')/7.04 (2 H, H-2' and 6'), δ 9.57 (OH); δ 158.0 (C4')] and a *n*-propylamine group (unit **b**) [δ 1.86 (H-4a)/2.16 (H-4b)/1.71 (2 x H-5)/3.06 (2 x H-6)/5.23 (NH); δ 40.7 (C6)] (Table 1 and Fig. 1). The methine proton at δ 5.76 (H-7) was long-range coupled to aromatic protons at δ 6.77 (H-3' and 5') and methylene protons at δ 1.86 (H-4a) and 2.16 (H-4b) (Fig. 1), thus revealing connectivity of two partial structures **a** and **b** through two carbons (C3 and C7); this feature was further implied by HMBC crosspeaks [δ 5.76 (H-7)/δ 126.3 (C1') and 128.9 (C3); δ 7.04 (H-2' and 6')/δ 82.7 (C7); δ 1.71 (H-5)/δ 128.9; δ 1.86 and 2.16 (H-4)/δ 128.9] (Fig. 2). The presence of a hydroxyl group at C4' was supported by the HMBC crosspeaks between the hydroxyl proton and aromatic carbons C3'-5'. The remaining C_2O_2 unit must be incorporated in an α,β-unsaturated δ-lactone of a 3-substituted pipecolic acid derivative, which was evident from HMBC crosspeaks [δ 3.06 (H-6)/δ 131.1 (C2); δ 1.86 and 2.16 (H-4)/δ 131.1; δ 5.76 (H-7)/δ 131.1 and 169.0 (C1)]. Thus, the structure of **1** (in DMSO) was determined as shown in Fig. 2.

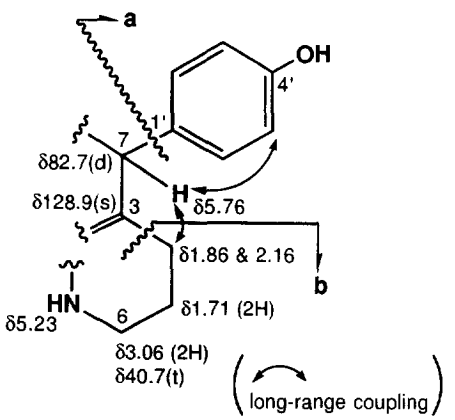


Fig. 1 Partial structure of **1** indicated by COSY (DMSO-d_6)

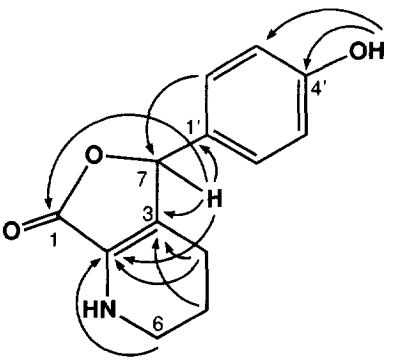
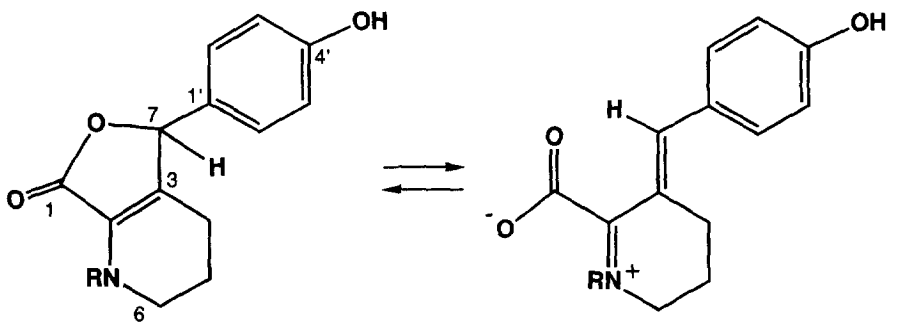


Fig. 2 Main HMBC correlations for **1** in DMSO-d_6

Table 1. ^{1}H and ^{13}C NMR Data of Anthosamine A (**1**) in DMSO-d_6

atom	^{13}C mult	^{1}H mult J (Hz)	HMBC correlations
1	169.0 s		
2	131.1 s		
3	128.9 s		
4	20.4 t	a 1.86 m	C2, C3, C5, C6
		b 2.16 m	C2, C3, C5, C6
5	20.9 t	1.71 (2H) m	C3, C4, C6
6	40.7 t	3.06 (2H) m	C2, C4
7	82.7 d	5.76 s	C1, C2, C1', C2', C6'
1'	126.3 s		
2', 6'	128.6 d	7.04 (2H) d 8.3	C7, C2', C4', C6'
3', 5'	115.5 d	6.77 (2H) d 8.3	C1', C3', C4', C5'
4'	158.0 s		
OH		9.57 br.s	C3', C4', C5'
NH		5.23 s	

Interestingly, the methanolic solution of anthosamine A (**1**) exhibited a bright yellow color with an intense UV absorption at 368 nm (ε 14500), suggesting formation of a conjugated system. The ^{1}H NMR spectrum in CD_3OD revealed the presence of two entities (**1a** and **1b**) in a ratio of 3:4; in addition to signals assignable to a lactone (**1a**) as observed in DMSO-d_6, a conspicuous low field singlet at δ 7.87 (1 H, H-7) appeared. This proton showed long-range coupling to protons at δ 2.92 (2 H, m, H-4) and 6.91 (2 H, d, J=8.5 Hz, H-3' and 5') in the COSY spectrum. Further connectivities of partial units were derived by HMBC crosspeaks; δ 7.56 (2 H, d, J=8.5 Hz, H-2' and 6')/δ 153.6 (C7); δ 7.87 (1 H, s, H-7)/δ 135.6 (C2' and C6'), 123.0 (C3), 177.1 (C2), and 24.7 (C4); δ 2.92 (2 H, m, 2 x H-4)/δ 123.0,



1a: R=H
2a: R=Me

1b: R=H
2b: R=Me

Fig. 3 Structures of anthosamines A (**1**) and B (**2**)

153.6 (C7), and 177.1; δ 3.65 (2 H, m, 2 x H-6)/δ 177.1. Although a remaining carbon at δ 172.0 (s) showed no HMBC crosspeak, it must be a carboxylate carbon in consideration of the structual relationship with **1a**. The zwitterion structure was also supported by a shift of H-6 protons in CD_3OD to lower field (δ 3.19 to δ 3.65). *3E*-Geometry was deduced from a NOESY crosspeak, δ 2.92 (H-4)/δ 7.56 (H-2'). Therefore, the structure of **1b** was as shown in Fig. 3. These spectral features clearly indicated an equilibrium between lactone **1a** and zwitterion **1b**. Anthosamine A recovered from the methanolic solution showed signals for the lactone **1a** in DMSO, thus indicating that the change in geometry is reversible.

The ^1H NMR spectrum of **2** in DMSO-d_6 (Table 2) was almost superimposable on that of **1** (Table 1) except for the presence of an *N*-methyl signal at δ 2.86 (3 H, s, NMe of **2a**). HMBC crosspeaks [δ 2.86/δ 132.4 (C2) and 51.1 (C6)] implied that **2** was an *N*-methyl derivative of **1** (Fig. 1), which was supported by the positive FABMS [*m/z* 268 $(M+Na)^+$ and 246 $(M+H)^+$]. Reversible geometry was also observed for **2**; the equilibrium between **2a** and **2b** in DMSO changed from 1:4 (**2a**:**2b**) to 100:0 after 4 days, whereas the equilibrium in methanol was 1:10.

Table 2. ^1H and ^{13}C NMR Data of Anthosamine B (**2**) in DMSO-d_6

	2a		**2b**	
atom	^{13}C mult	^1H mult *J* (Hz)	^{13}C mult	^1H mult *J* (Hz)
1	168.4 s		ND	
2	132.4 s		175.9 s	
3	134.9 s		122.9 s	
4	20.4 t	a 1.83 m b 2.14 m	22.1 t	2.71 (2H) m
5	20.7 t	1.77 (2H) m	20.0 t	1.88 (2H) m
6	51.1 t	2.91 (2H) m	50.8 t	3.69 (2H) m
7	81.6 d	5.74 s	148.3 d	7.40 s
1'	125.9 s		125.3 s	
2', 6'	128.5 d	7.05 (2H) d 8.3	133.4 d	7.44 (2H) d 8.5
3', 5'	115.6 d	6.77 (2H) d 8.3	116.0 d	6.88 (2H) d 8.5
4'	158.0 s		160.0 s	
NMe	37.3 q	2.86 (3H) s	44.2 q	3.41 (3H) s
OH		9.60 s		10.30 s

ND: not detected.
HMBC correlations were shown in experimental.

Structure of a diketopiperazine 3.

Compound **3** showed a molecular formula of $C_{15}H_{19}N_5O_3$, as deduced by the positive HRFABMS (*m/z* 318.1562, Δ -0.4 mmu). The ^1H NMR spectrum (DMSO-d_6) (Table 3) revealed seven D_2O-exchangeable protons [δ 7.10 (3 H, br.s, 6-NH and NH_2), 7.56 (1 H, br.s, 5-NH), 8.39 (1 H, s, 2-NH), and 9.76 (2 H, br.s, 1'-NH and OH)]. The ^1H-^1H COSY and HMQC spectra showed a partial structure $CH(NH)CH_2CH_2CH_2NH$ [δ 8.39 (1 H, s, 2-NH); δ 3.99 (1 H, m, H-2)/δ 54.5 (d, C2); δ1.72 (2 H, m, 2 x H-3)/δ 31.1 (t, C3); δ1.53 (2 H, m, 2 x H-4)/δ 23.9 (t, C4); δ3.10 (2 H, m, 2 x H-5)/δ 40.3 (t, C5); δ 7.56 (1 H, br.s, 5-NH)]. The presence of an arginine moiety was readily implied by HMBC crosspeaks; 5-

NH/C6 at δ 156.6 (s); H-2, H-3 and 2-NH/C1 at δ 166.7 (s). The acid hydrolysate (6 N HCl, 105 °C, 19 h) of **3** was subjected to a chiral HPLC analysis which resulted in identification of L-arginine. The presence of a *p*-hydroxyphenyl group [δ 6.78 (2 H, d, *J*=8.5 Hz, H-6' and 8'), 7.34 (2 H, d, *J*=8.5 Hz, H-5' and 9'), and 9.76 (OH)] linked to an olefin proton [δ 6.61 (1 H, s, H-3')] was revealed by a homoallyl coupling δ 6.78/δ 6.61. Further interpretation of the HMBC spectrum led to a connectivity C2H-NH-C1'(=O)-C2'=C3'H-phenyl [δ 3.99 (H-2)/δ 160.8 (C1'); δ 8.39 (2-NH)/δ 124.0 (C2'); δ 6.61 (H-3')/δ 160.8 (C1')]. The residual NH unit must be accommodated between C1 and C2', which was substantiated by NOESY crosspeak δ 9.76 (NH)/δ 7.34 (H-5' and 9'). Thus, the diketopiperazine **3** is composed of L-Arg and dehydrotyrosine (ΔTyr) with *2'Z*-geometry as shown in Fig. 4.

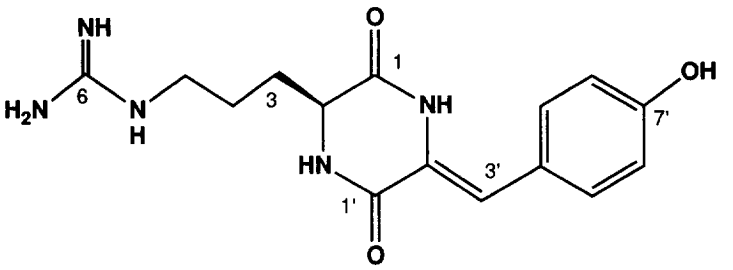


Fig. 4 Structure of a diketopiperazine **3**

CONCLUSION

Anthosamines A (**1**) and B (**2**) are a rare example of a reversible change in geometry from an amino lactone to a zwitterionic iminium carboxylate. Although the ring closure from **1b** (**2b**) to **1a** (**2a**) may occur via an *p*-quinone transition state because of favored 5-*Exo-Trig* process (the Baldwin rules[2]), signals attributable to quinone carbons were not observed in the NMR spectra.

Some metabolites derived from amino acids were isolated from marine sponges of the order Clionidae; dehydrotyrosine-containing peptides have been reported.[3] Phospholipids comprising isoprenoid fatty acids were isolated from *Anthosigmella varians*;[4] this is the second report of metabolites from marine sponges of the genus *Anthosigmella*.

Anthosamines A (**1**) and B (**2**) induced larval metamorphosis on the ascidian *Halocynthia roretzi* at a concentraion of 50 μM completely, while the diketopiperazine **3** (50 μM) induced metamorphosis of 50 % of larvae.

Table 3. ^{1}H and ^{13}C NMR Data of the Diketopiperazine **3** in DMSO-d_6

	atom	^{13}C mult	^{1}H mult *J* (Hz)	HMBC correlations
Arg	1	166.7 s		
	2	54.5 d	3.99 (1H) m	C1, C3, C4, C1'
	3	31.1 t	1.72 (2H) m	C1, C2, C4, C5
	4	23.9 t	1.53 (2H) m	C2, C3, C5
	5	40.3 t	3.10 (2H) m	
	6	156.6 s		
	2-NH		8.39 s	C1, C2'
	5-NH		7.56 (1H) br.s	C6
	6-NH, NH_2		7.10 (3H) br.s	
ΔTyr	1'	160.8 s		
	2'	124.0 s		
	3'	115.1 d	6.61 s	C1', C5', C9'
	4'	124.2 s		
	5', 9'	130.9 d	7.34 (2H) d 8.5	C3', C5', C7', C9'
	6', 8'	115.5 d	6.78 (2H) d 8.5	C4', C6', C7', C8'
	7'	157.5 s		
	NH		9.76 br.s	
	OH		9.76 br.s	

EXPERIMENTAL

General.

Optical rotations were determined with a JASCO DIP-1000 polarimeter in MeOH. UV spectra were recorded on a Hitachi U-2000 spectrometer in MeOH or DMSO. Infrared spectra were measured on a JASCO IR-700 spectrometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 27 °C in methanol-d_4 or DMSO-d_6. Residual CHD_2OD (3.35 ppm), CD_3OD (49.0 ppm), CHD_2SOCD_3 (2.49 ppm), or CD_3SOCD_3 (39.5 ppm) signals were used as internal standards. FAB mass spectra were measured on a JEOL SX-102 mass spectrometer.

Bioassay.

Fifteen newly hatched larvae were placed in each well of polystyrene plates; each well contained 4 mL of artificial seawater (460 mM NaCl, 10.1 mM KCl, 9.2 mM $CaCl_2$, 35.9 mM $MgCl_2 \cdot H_2O$, 17.5 mM $MgSO_4 \cdot 7H_2O$, 10 mM Tris-HCl, pH 8.2). To each well was added 10 μL of a test sample dissolved in DMSO and the plates were incubated at 13.2 °C in the dark; 10 μL of DMSO was added as control. The larvae which had undergone metamorphosis, *i.e.*, shortening of tail, were counted under a microscope.

Isolation of ***1-3****.*

The sponge (2.0 kg, wet weight) was collected using scuba at a depth of 20 m off the Sada Peninsula, Shikoku, 600 km southwest of Tokyo. It was extracted with MeOH (3 x 1 L), and the extract was concentrated under reduced pressure at 40 °C. The aqueous residue (500 mL) was extracted with ether (3 x 700 mL), then *n*-BuOH (3 x 700 mL). The *n*-BuOH soluble portion (11.06 g), which induced

metamorphosis in the tadpole larvae of *H. roretzi*, was subjected to ODS flash column chromatography (ODS-A 60-400/230, 8 x 13 cm, YMC Co., Ltd.) with aqueous MeOH. A portion of the fraction eluted with 30 % MeOH-H_2O was subjected to HPLC [Asahipak ODP-50, 5 μm, 20 x 300 mm, Asahi Chemical Industry Co., Ltd.; linear gradient with solvent A, 15 % CH_3CN-H_2O (0.01 % TFA), and solvent B, 50 % CH_3CN-H_2O (0.01 % TFA); 100 % A in 0-10 min and 100 % A→100 % B in 10-40 min; flow rate, 3.0 mL/min] to obtain two active fractions. The first active fraction (T_R 56 min) was further purified by gel filtration on Toyopearl HW-40 (2.2 x 43 cm, Tosoh Corporation) with 90 % MeOH-H_2O (0.01 % TFA) (2.0 mL/min) to afford a diketopiperazine **3** (40.7 mg, 2.0 x 10^{-3} %, wet weight) and another active fraction, which yielded anthosamine B (**2**) (1.53 mg, 7.7 x 10^{-5} %) after HPLC purification [YMC-Pack ODS, 5 μm, 10 x 250 mm, YMC Co., Ltd.; 12 % CH_3CN-H_2O (0.01 % TFA); 2.0 mL/min]. The second active fraction (T_R 60 min) was purified by HPLC [YMC-Pack ODS, 5 μm, 10 x 250 mm; 15 % CH_3CN-H_2O (0.01 % TFA); 2.0 mL/min] to afford anthosamine A (**1**) (4.16 mg, 2.1 x 10^{-4} %).

1. yellow solid. $[\alpha]_D^{24}$ 0.0° (*c* 0.40, MeOH). IR ν_{max} (KBr) 3300, 1680, 1640, and 1570 cm^{-1}. UV λ_{max} (MeOH) 229 (ε 7300), 249 (6400), 283 (4200), and 368 nm (14500). UV λ_{max} (DMSO) 287 nm (ε 700). ^1H and ^{13}C NMR (DMSO-d_6) for **1a** see Table 1. ^1H NMR (CD_3OD) for **1a** δ: 1.85 (2 H, m, 2 x H-5), 1.96 (1 H, m, H-4a), 2.21 (1 H, m, H-4b), 3.19 (2 H, m, 2 x H-6), 5.73 (1 H, s, H-7), 6.79 (2 H, d, *J*=8.5 Hz, H-3' and 5'), and 7.06 (2 H, d, *J*=8.5 Hz, H-2' and 6'). ^1H NMR (CD_3OD) for **1b** δ: 2.01 (2 H, m, 2 x H-5), 2.92 (2 H, m, 2 x H-4), 3.65 (2 H, m, 2 x H-6), 6.91 (2 H, d, *J*=8.5 Hz, H-3' and 5'), 7.56 (2 H, d, *J*=8.5 Hz, H-2' and 6'), and 7.87 (1 H, s, H-7). ^{13}C NMR (CD_3OD) for **1a** δ: 21.8 (t, C4), 22.5 (t, C5), 42.5 (t, C6), 85.5 (d, C7), 116.7 (d, C3' and 5'), 127.9 (s, C1'), 129.7 (d, C2' and 6'), 132.8 (s, C2), 133.4 (s, C3), 159.6 (s, C4'), and 165.8 (s, C1). ^{13}C NMR (CD_3OD) for **1b** δ: 20.9 (t, C5), 24.7 (t, C4), 43.9 (t, C6), 117.2 (d, C3' and 5'), 123.0 (s, C3), 127.1 (s, C1'), 135.6 (d, C2' and 6'), 153.6 (d, C7), 162.7 (s, C4'), 172.0 (s, C1), and 177.1 (s, C2). HMBC cross peaks (CD_3OD) for **1a**: H4a/C2, C3, C5, and C6; H-4b/C2 and C3; H-5/C3, C4, and C6; H-6/C2 and C5; H-7/C2, C3, C1', C2', and C6'; H-2' and 6'/C7, C2', C4', and C6'; H-3' and 5'/C1', C3', C4', and C5'. HMBC cross peaks (CD_3OD) for **1b**: H-4/C2, C3, C5, C6, and C7; H-5/C3, C4, and C6; H-6/C2, C4, and C5; H-7/C2, C3, C4, C2', and C6'; H-2' and 6'/C7, C2', C4', and C6'; H-3' and 5'/C1', C3', C4', and C5'. FABMS (positive, NBA matrix) *m/z* 232 $(M+H)^+$ and 214 $(M+H-H_2O)^+$. HRFABMS (positive, PEG+NBA matrix) *m/z* 232.0953 (calcd for $C_{13}H_{14}NO_3$, Δ 0.0 mmu).

2. yellow solid. $[\alpha]_D^{24}$ 0.0° (*c* 0.15, MeOH). IR ν_{max} (KBr) 3200, 1680, 1650, and 1570 cm^{-1}. UV λ_{max} (MeOH) 249 (ε 7100), 369 (18400), and 453 nm (1900). UV λ_{max} (DMSO) 285 (ε 6000), 357 (2400), and 486 nm (3600). ^1H and ^{13}C NMR (DMSO-d_6) for **2a** and **2b** see Table 2. HMBC cross peaks (DMSO-d_6) for **2a**: H-4a/C2, C3, C5, and C6; H-4b/C3 and C5; H-5/C3, C4, and C6; H-6/C4 and C5; H-7/C2, C3, C1', C2', and C6'; H-2' and 6'/C7, C2', C4', and C6'; H-3' and 5'/C1', C3', C4', and C5'; NMe/C2 and C6; OH/C3', C4', and C5'. HMBC cross peaks (DMSO-d_6) for **2b**: H-5/C3; NMe/C2 and C6; H-3' and 5'/C1'; H-2' and 6'/C2', C4', and C6'; OH/C3' and 5'. ^1H NMR (CD_3OD) for **2a** δ: 1.88 (2 H, m, 2 x H-5), 1.93 (1 H, m, H-4a), 2.20 (1 H, m, H-4b), 2.94 (3 H, s, NMe), 2.97 (2 H, m, 2 x H-6), 5.67 (s, H-7), 6.79 (2 H, d, *J*=8.5 Hz, H-3' and 5'), and 7.06 (2 H, d, *J*=8.5 Hz, H-2' and 6').

^{1}H NMR (CD_3OD) for **2b** δ: 2.04 (2 H, m, 2 x H-5), 2.85 (1 H, m, 2 x H-4), 3.54 (3 H, s, NMe), 3.78 (2 H, m, 2 x H-6), 6.90 (2 H, d, *J*=8.5 Hz, H-2' and 6'), 7.52 (2 H, d, *J*=8.5 Hz, H-3' and 5'), and 7.56 (1 H, s, H-7). FABMS (positive, NBA matrix) *m/z* 268 $(M+Na)^+$ and 246 $(M+H)^+$. HRFABMS could not be obtained due to weak intensity of ion peaks.

3. yellowish oil. $[\alpha]_D^{24}$ -78.8° (*c* 1.00, MeOH). IR ν_{max} (KBr) 3340, 3185, 1675, 1425, 1205, 1175, and 1135 cm^{-1}. UV λ_{max} (MeOH) 223.5 (ε 4700) and 318 nm (6000). ^{1}H and ^{13}C NMR see Table 3. FABMS (positive, thioglycerol matrix) *m/z* 318 $(M+H)^+$. HRFABMS (positive, PEG matrix) *m/z* 318.1562 (calcd for $C_{15}H_{20}N_5O_3$, Δ -0.4 mmu).

Determination of stereochemistry of arginine.

Compound **3** (0.1 mg) was dissolved in 6 N HCl (0.5 mL, PIERCE), and the mixture was kept at 105 °C for 19 h. After evaporation, the hydrolysate was analyzed by HPLC [CROWNPAK CR(+) (4.0 x 150 mm, DAICEL CHEMICAL INDUSTRIES, LTD.); aq. $HClO_4$ (pH 1.5); 0.5 mL/min; UV 200 nm]: retention time (5.0 min) of arginine in **3** was identical with that of the authentic L-isomer; T_R 4.0 min for the authentic D-isomer.

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