A NEW BROAD-SPECTRUM AMINOGLYCOSIDE ANTIBIOTIC COMPLEX, SPORARICIN

III. THE STRUCTURES OF SPORARICINS A AND B

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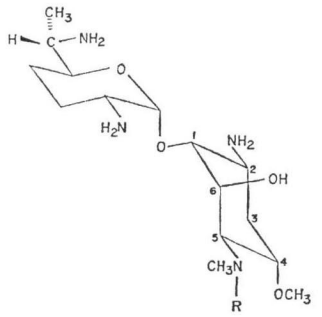
The structures of sporaricins A and B have been determined to be 2-amino-1-O-(2,6-diamino-2,3,4,6,7-pentadeoxy-β-L-*lyxo*-heptopyranosyl)-2,3,5-trideoxy-5-(N-glycyl-N-methyl-amino)-4-O-methyl-D-*chiro*-inositol and 2-amino-1-O-(2,6-diamino-2,3,4,6,7-pentadeoxy-β-L-*lyxo*-heptopyranosyl)-2,3,5-trideoxy-4-O-methyl-5-methylamino-D-*chiro*-inositol, respectively.

Two new antibiotics, sporaricins A and B are major components of an aminoglycoside antibiotic complex isolated from the cultured broth of *Saccharopolyspora hirsuta* subsp. *kobensis*[1]. Sporaricin A exhibits a broad antibacterial spectrum and marked chemotherapeutic effects against infections in mice[2]. In this paper, structural elucidations of sporaricins A and B are reported.

Sporaricins A and B (**1** and **2**) have the formulae $C_{17}H_{35}N_5O_5$ and $C_{15}H_{32}N_4O_4$, respectively, which are derived from the elemental analysis, ^{13}C NMR spectra and mass spectrometry. Both antibiotics are basic colorless solids having no characteristic absorption in their ultraviolet spectra and give positive ninhydrin and RYDON-SMITH reactions. The former (**1**) shows $[\alpha]_D^{25}$ +104° (*c* 1, H_2O) and the latter (**2**) shows $[\alpha]_D^{25}$ +139.5° (*c* 1, H_2O). The mass spectrometry of **1** or **2** showed a highly intense peak at *m/e* 143 assigned to a purpurosamine B moiety[3]. The infrared spectrum of **1** showed a band at 1628 cm^{-1} (amide I) in a KBr tablet which was not observed in that of **2**. The ^1H NMR spectrum of **1** in D_2O indicated a pseudodisaccharide and the presence of three methyl groups, 3.88 ppm (singlet, OCH_3), 3.52 ppm (singlet, NCH_3), 1.50 ppm (doublet, J=7.0 Hz., CCH_3). The ^1H NMR spectrum of **2** was similar to that of **1**, but the methylene group signal at 4.01 ppm was not observed. The most significant difference between **1** and **2** is the marked deshielding of the N-methyl resonance from 2.81 ppm in the latter to 3.52 ppm in the former.



Sporaricin A (1) R = NH_2CH_2CO-
Sporaricin B (2) R = H

Alkaline hydrolysis of **1** afforded **2** and an amino acid (**3**) which was confirmed to be identical with glycine by amino acid analysis and tlc.

Tetra-N-acetylsporaricin B (**4**) prepared from **2** with acetic anhydride in methanol was treated

with 6 N hydrogen chloride in anhydrous methanol followed by re-N-acetylation with acetic anhydride in methanol. The products were chromatographed on a silica gel column which was packed with chloroform and developed with chloroform-acetone and then chloroform-methanol. A methyl glycoside (**5**) was eluted first and thereafter another methyl glycoside (**6**). After elution of unreacted **4**, an N-acetylaminocyclitol (**7**) was eluted. Methyl glycoside (**5**) was confirmed to be identical with methyl 2,6-bis-(acetamido)-2,3,4,6,7-pentadeoxy-β-L-*lyxo*-heptopyranoside (methyl 2,6-di-N-acetyl-6-*epi*-α-purpurosaminide B[4]); the other methyl glycoside (**6**) was the α-anomer. These glycosides were obtained by methanolysis of fortimicin B followed by re-N-acetylation[4].

Fig. 1. Alkaline hydrolysis of sporaricin A.

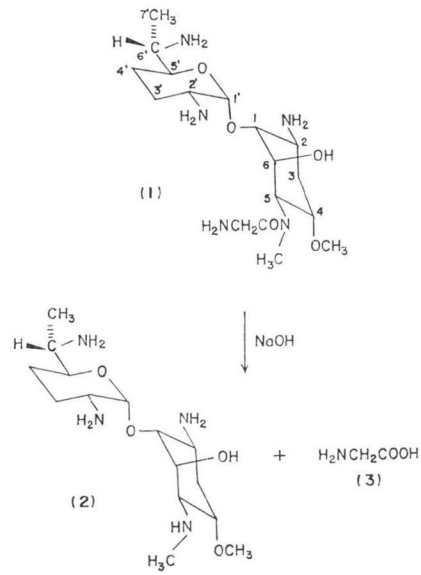
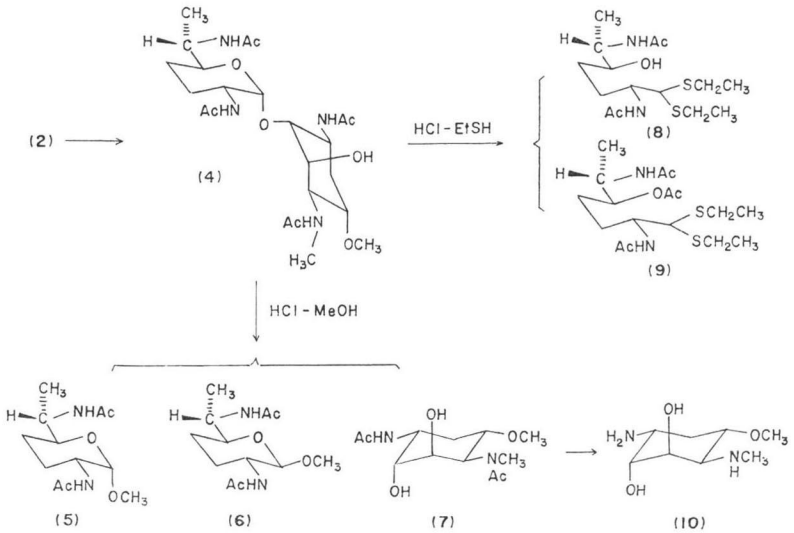


Fig. 2. Methanolysis and mercaptolysis of tetra-N-acetyl sporaricin B (**4**).



The di-N-acetyl-6-*epi*-purpurosamine B diethyl dithioacetal (**8**) and peracetyl-6-*epi*-purpurosamine B diethyl dithioacetal (**9**), which were derived from **4** were identical with mercaptolysis products of fortimicin[4] in their optical rotations and ^{1}H NMR spectra.

The mass spectrum of **7** gave a molecular ion at *m/e* 274 (M^+ for $C_{12}H_{22}N_2O_5$). Colorless needles of the aminocyclitol (**10**) were prepared by alkaline hydrolysis of **7** with 4 N NaOH at 110°C for 6 hours followed by column chromatography on a cation-exchanger, CM-Sephadex C-25 (NH_4^+). The mass spectrum of **10** gave a molecular ion at *m/e* 191 ($M^+ + 1$ for $C_8H_{18}N_2O_3$) and **10** showed mp 74~75°C and $[\alpha]_D^{25}$ +75° (*c* 1, H_2O). In the ^{1}H NMR spectrum of **10** (Tables 1 and 2), singlet signals

Table 1. Chemical shifts of ^1H NMR spectra.

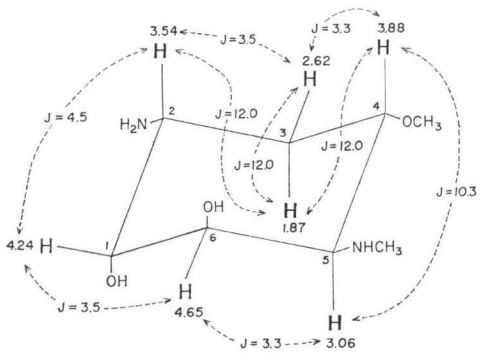
Proton	Chemical shifts (ppm)			
	Sporaricin A (1)	Aminocyclitol (10)	Sporaricin B (2)	
			free base	sulfate
1′	5.42		5.39	5.88
2′	3.25		3.25~	4.05~
3′	1.7 ~ 2.4		1.7 ~ 2.7	1.8 ~ 2.6
4′				
5′	4~		4~	3.9~
6′	3.32		3.27	4.2
7′	1.5		1.49	1.73
1	4.24	4.34	4.30	4.72
2	3.65	3.54	3.59	4.3~
3ax.	2.05	1.87	1.94	2.20
3eq.	2.81	2.62	2.61	2.94
4	4.39	3.88	3.8~	4.1~
5	4.96	3.06	2.98	3.68
6	4.69	4.65	4.77	5.12
N–CH_3	3.52	2.84	2.81	3.24
O–CH_3	3.88	3.90	3.86	3.88
gly.-CH_2	4.01			

Chemical shifts of ^1H NMR spectra were measured in D_2O using TMS as the external reference.

Table 2. Coupling constants of ^1H NMR spectra.

Coupling	Coupling constants (Hz.)			
	Sporaricin A (1)	Aminocyclitol (10)	Sporaricin B (2)	
			free base	sulfate
$J_{1',2'}$	3.6		3.4	3.6
$J_{5',6'}$	7.0		6.5	
$J_{6',7'}$	7.0		6.5	6.5
$J_{1,6}$	3.8	3.5	3.5	3.5
$J_{1,2}$	3.5	4.5	4.0	3.5
$J_{2,3ax.}$	13.0	12.0	11.6	11.0
$J_{2,3eq.}$	3.5	4.5	4.5	4.5
$J_{3ax.,3eq.}$	11.8	12.0	12.0	12.0
$J_{3ax.,4}$	12.0	12.0	11.6	11.0
$J_{3eq.,4}$	4.5	4.5	4.5	4.5
$J_{4,5}$	11.5	10.3	10.0	10.0
$J_{5,6}$	2.8	3.3	3.2	3.0

Fig. 3. Spectral analysis of ^1H NMR of the aminocyclitol (**10**).



for OCH_3 (3.90 ppm) and NCH_3 (2.84 ppm) groups were observed. A cyclic array of one methylene (1.87 and 2.62 ppm) and five methine groups (3.06, 3.54, 3.88, 4.34 and 4.65 ppm) were indicated by application of spin decoupling methods. The relationship of protons in a series of CH_3O–$\underline{C}\underline{H}$–, CH_3N–$\underline{C}\underline{H}$–, HO–$\underline{C}\underline{H}$–, HO–$\underline{C}\underline{H}$– and NH_2–$\underline{C}\underline{H}$– was carefully analyzed as shown in Fig. 3. Coupling constants indicated axial protons at C_2, C_4 and C_5 and no substitution at C_3. The $\Delta[M]_{436}$TACu[5] value of **10** was $-2120°$. Thus, the absolute structure of **10** was determined to be 2-amino-2,3,5-trideoxy-4-O-methyl-5-methylamino-D-*chiro*-inositol as shown in Fig. 3.

In ^{13}C NMR spectra, chemical shifts in **2** and **10** were assigned by the selective proton decoupling technique as shown in Table 3. The significant change in assignment for C_1 in **2** (77.2 ppm) and **10** (69.1 ppm) was reasonably explained by the deshielding effect of glycosidation. From this result, it was concluded that the position of linkage of 6-*epi*-purpurosamine B to the aminocyclitol was C_1 in **2**. The coupling constant ($J_{1',2'}=3.4$ Hz.) of the anomeric proton in **2** showed it to be a *β*-L-*lyxo*-heptopyranoside. Therefore, the structure of sporaricin B was proposed as 2-amino-1-O-(2,6-diamino-2,3,4,6,7-pentadeoxy-*β*-L-*lyxo*-heptopyranosyl)-2,3,5-trideoxy-4-O-methyl-5-methylamino-D-*chiro*-inositol.

In the ^1H NMR spectra **1** and **2** (Table 1), down-field shifts were observed at the C_5 and NCH_3

protons. Thus, the structure of sporaricin A was proposed as 2-amino-1-O-(2,6-diamino-2,3,4,6,7-pentadeoxy-β-L-*lyxo*-heptopyranosyl)-2,3,5-trideoxy-5-(N-glycyl-N-methylamino)-4-O-methyl-D-*chiro*-inositol.

Experimental

Melting points were obtained on a Yanagimoto's microscope hot stage (uncorrected). The spectrometric data were obtained by the following instruments. Optical rotation; Japan Spectroscopic Co., Ltd. Digital polarimeter DIP-4. Infrared spectra; Japan Spectroscopic Co., Ltd. Model DS-403G. Mass spectra; Japan Electron Optics Lab. Model JMS-D-100 and Hitachi Model RMU-6MG. Amino acid analyzer; Japan Electron Optics Lab. Model JLC-5AH. ^{13}C NMR spectra; Varian Model XL-100 and Japan Electron Optics Lab. Model JNM-FX-100. ^{1}H-NMR spectra; Varian Model XL-100 and Japan Electron Optics Lab. Model JNM-MH-100 (TMS as the external reference in D_2O). Chromatographies were performed with the following reagents. Silica gel; Wako gel C-200 (column chromatography), E. Merck Kieselgel GF_{254} nach Stahl (preparative layer chromatography), E. Merck DC-Alufolien 60 F_{254} (thin-layer chromatography).

Table 3. Chemical shifts of ^{13}C NMR spectra.

	Carbon	Chemical shifts (ppm)							
		Sporaricin A (1)		Aminocyclitol (10)		Sporaricin B (2)		β-shift	
1	1′	98.4	d			97.8	d	6.1	
2	2′	50.6	d			50.6	d		
3	3′	26.3	t			27.0	t	5.5	
4	4′	27.3	t			27.3	t		
5	5′	74.6	d			74.8	d	3.8	
6	6′	50.5	d			50.6	d		
7	7′	18.3	q			18.5	q	3.6	
8	1	78.2	d	69.1	d	77.2	d	5.7	
9	2	46.6	d	46.9	d	46.7	d		
10	3	31.8	t	32.4	t	33.2	t	4.7	
11	4	73.2	d	78.2	d	78.4	d	3.9	
12	5	55.8	d	60.8	d	61.3	d		
13	6	70.1	d	72.9	d	65.4	d	3.4	
14	N-CH_3	33.8	q	33.3	q	33.5	q		
15	O-CH_3	56.6	q	56.7	q	56.9	q		
16	gly.-CH_2	43.3	t						
17	gly.-CO	176.4	s						

The ^{13}C-FT NMR spectra were taken with a Varian XL-100 spectrometer.

Samples were dissolved in D_2O containing dioxane as the internal reference (67.4 ppm).

Assignments, s, d, t and q show the multiplicities in an off-resonance experiment.

Alkaline hydrolysis of sporaricin A (1)

A solution of **1** (20 mg) in 2 ml of 4 N NaOH was heated in a sealed tube at 100°C for 1 hour. The hydrolysate was neutralized with 4 N H_2SO_4 and diluted to 10 ml with deionized water and then passed through a column (1.2×2 cm) of Amberlite CG-50 (NH_4^+). After washing with deionized water, the column was eluted with 0.5 N NH_4OH. The bioactive fractions were concentrated *in vacuo* and freeze-dried to give a white powder of **2** (12 mg), identical in all respects with the product obtained from the fermentation. $[\alpha]_D^{25}$ +142° (*c* 1, H_2O).

Anal. Calcd. for $C_{15}H_{32}N_4O_4 \cdot H_2O$: C, 51.40; H, 9.78; N, 15.99.
Found: C, 51.04; H, 9.33; N, 15.53.

The Amberlite CG-50 (NH_4^+) washing were charged on a column (1.2×2 cm) of Dowex 50×2 (H^+) and washed with deionized water. The column was eluted with 0.5 N aqueous pyridine (adjusted to pH 5.0 with acetic acid). The ninhydrin-positive fractions were collected and evaporated *in vacuo*. The residue was diluted to 10 ml with deionized water and charged on a column (1.2×2 cm) of Dowex 1×2 (OH^-), washed with deionized water and then eluted with 0.1 M aqueous acetic acid. The ninhydrin-positive fractions were concentrated and freeze-dried to give a white powder of glycine (**3**). **3** was identified by tlc (*n*-BuOH - AcOH - H_2O 4:1:1, 70% EtOH) and amino acid analysis.

Tetra-N-acetylsporaricin B (4)

To a solution of **2** (228.5 mg) in 20 ml of methanol, 4 ml of acetic anhydride was added and the mixture was stirred at room temperature for 1 hour. The solvent was removed under reduced pressure to give a yellowish oil (387.7 mg) followed by chromatography on a column (1.5×30 cm) of silica gel. The column was developed with chloroform - methanol (20:1). The fractions were monitored by tlc

(detected by dil.H_2SO_4). The product thus obtained was crystallized from chloroform - methanol - ether to colorless prisms of **4** (128.7 mg), mp 139~141°C, $[\alpha]_D^{22}$ +52° (*c* 1, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3400, 3310, 3080, 1650 (amide), 1625 (amide). MS: *m/e* 500 (M^+), 303, 275, 257, 227. ^1H NMR (D_2O)δ: 1.58 (3H, d, J=7 Hz. CCH_3), 2.46, 2.47, 2.50, 2.63 (each 3H, s, $COCH_3$), 3.61 (3H, s, NCH_3), 3.90 (3H, s, OCH_3), 5.47 (1H, d, J=3.5 Hz. anomeric H).

Anal. Calcd. for $C_{23}H_{40}N_4O_8 \cdot H_2O$: C, 53.27; H, 8.16; N, 10.52.
Found: C, 52.99; H, 7.65; N, 10.52.

Mercaptolysis of tetra-N-acetylsporaricin B (**4**)

A solution of **4** (360 mg) in ethanethiol (1 ml) and concentrated hydrogen chloride (1 ml) was stirred at 30°C for 44 hours. Excess reagent was then removed by evaporation under reduced pressure and the aqueous residue was diluted with 100 ml of water. The solution was adjusted to pH 5.0 by addition of lead carbonate, filtered and evaporated *in vacuo* to leave a pale yellow paste. This material was extracted with methanol and filtered. After repeating this procedure twice, 5 ml of this methanolic solution was treated with acetic anhydride (0.5 ml) at room temperature for 1 hour. The solvent was removed under reduced pressure and the residue was charged on a column (2.2×25 cm) of silica gel. The column was eluted with chloroform.

The per-N,O-acetyl-6-*epi*-purpurosamine B diethyl dithioacetal (**9**) was eluted first. The eluates were combined, evaporated to give colorless needles (10.4 mg), mp 128~132°C, IR ν_{max}^{KBr} cm^{-1}: 3255, 3070, 1745, 1760. MS: *m/e* 393 (M^+ +1), 392 (M^+), 363, 257, 198. $[\alpha]_D^{25}$ +30° (*c* 0.2, CH_3OH).

Anal. Calcd. for $C_{17}H_{32}N_2O_4S_2$: C, 52.01; H, 8.22; N, 7.14.
Found: C, 52.42; H, 8.07; N, 7.08.

Thereafter di-N-acetyl-6-*epi*-purpurosamine B diethyl dithioacetal (**8**) was eluted with chloroform - methanol (20: 1). The combined eluates were evaporated to give a colorless oil (40 mg) of **8**. $[\alpha]_D^{25}$ 0° (*c* 0.3, CH_3OH). MS: *m/e* 351 (M^+ +1). ^1H NMR (pyridine-d_5) δ: 1.21, 1.26 (each 3H, t, J=8 Hz. CH_2CH_3), 1.33 (3H, d, J=7 Hz. CCH_3), 2.07, 2.13 (each 3H, s, $COCH_3$), 2.74 (4H, q, J=8 Hz. $CH_2CH_3 \times 2$).

Methanolysis of tetra-N-acetylsporaricin B

A solution of **4** (500 mg) with 20 ml of 6 N HCl in dry methanol was heated under reflux for 12 hours. The reaction mixture was diluted with 10 ml of dry methanol. The methanolysate was evaporated several times with dry methanol to leave a pale yellow paste. The paste was diluted with 100 ml of deionized water and passed through a column (2×10 cm) of Dowex 1×2 (OH^-). The eluate was evaporated to dryness under reduced pressure and co-evaporated with toluene to remove residual water. The residue was acetylated with 1.5 ml of acetic anhydride in 15 ml of dry methanol at room temperature overnight. The reaction mixture was evaporated under reduced pressure and chromatographed on a column (2×20 cm) of silica gel which was packed with chloroform and developed with chloroform - acetone (1: 1). The eluates were monitored by tlc (detected by dil.H_2SO_4). Initial fractions having an Rf 0.61 were combined and evaporated under reduced pressure to afford methyl 2,6-bis(acetamido)-2,3,4,6,7-pentadeoxy-β-L-*lyxo*-heptopyranoside(methyl 2,6-di-N-acetyl-6-*epi*-α-purpurosaminide B) (**5**) as a colorless solid. The product was crystallized with chloroform - ethyl acetate to colorless needles (**5**, 107 mg), mp 218~220°C, $[\alpha]_D^{24}$ +57° (*c* 1, CH_3OH). [lit.[4] mp 217~218°C, $[\alpha]_D^{24}$ +55.2° (*c* 0.77, CH_3OH)]. ^1H NMR ($CDCl_3$) δ: 1.19 (3H, d, J=7 Hz. CCH_3), 1.99 (6H, s, $COCH_3 \times 2$), 3.37 (3H, s, OCH_3), 4.61 (1H, d, J=3,5 Hz. anomeric H).

Anal. Calcd. for $C_{12}H_{22}N_2O_4$: C, 55.80; H, 8.58; N, 10.84.
Found: C, 55.74; H, 8.49; N, 10.52.

Continued elution with chloroform - acetone (1: 2) gave fractions having an Rf 0.39, which were combined and evaporated under reduced pressure to afford methyl 2,6-bis(acetamido)-2,3,4,6,7-pentadeoxy-α-L-*lyxo*-heptopyranoside (methyl 2,6-di-N-acetyl-6-*epi*-β-purpurosaminide B) (**6**, 55 mg), recrystallized from chloroform - ethyl acetate to colorless needles, mp 212~213°C, $[\alpha]_D^{25}$ −114° (*c* 1, CH_3OH). [lit.[4] mp 210~211°C, $[\alpha]_D^{24}$ −111° (*c* 0.94, CH_3OH)]. ^1H NMR ($CDCl_3$) δ: 1.22 (3H, d, J=6.7 Hz. CCH_3), 2.00 (6H, s, $COCH_3 \times 2$), 3.50 (3H, s, OCH_3), 4.23 (1H, d, J=8 Hz. anomeric H).

Anal. Calcd. for $C_{12}H_{22}N_2O_4$: C, 55.80; H, 8.58; N, 10.84.
Found: C, 55.35; H, 8.45; N, 10.49.

After elution of unreacted **4**, the column was eluted with chloroform - methanol (8: 1) to afford the 2,5-di-N-acetylaminocyclitol (**7**, 170 mg), $[\alpha]_D^{25}$ +91° (*c* 1, H_2O). MS: *m/e* 274 (M^+). ^1H NMR (D_2O) δ: 2.50, 2.65 (each 3H, s, $COCH_3$), 3.61 (3H, s, NCH_3), 3.89 (3H, s, OCH_3).

Aminocyclitol (**10**)

A solution of 2,5-di-N-acetylaminocyclitol (**7**, 170 mg) in 4 N NaOH (5 ml) was heated at 110°C for 6 hours. The reaction mixture, diluted to 200 ml with water followed by neutralization with 6 N HCl, was charged on a column (2×10 cm) of CM-Sephadex C-25 (NH_4^+). The column was washed with deionized water and then eluted by gradient elution between 0.025 N NH_4OH and 0.5 N NH_4OH. The ninhydrin-positive fractions were evaporated to give colorless needles of **10** (100 mg), mp 74~75°C, $[\alpha]_D^{25}$ +75° (*c* 1, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3420, 2940, 1575, 1465. ^1H NMR (D_2O) δ: 1.87 (1H, m, $J_{2,3}=12$, $J_{3,4}=12$, $J_{3,3gem.}=12$ Hz. H-3ax.), 2.62 (1H, m, $J_{2,3}=4.5$, $J_{3,4}=4.5$, $J_{3,3gem.}=12$ Hz. H-3eq.), 2.84 (3H, s, NCH_3), 3.06 (1H, dd, $J_{4,5}=10.3$, $J_{5,6}=3.3$ Hz. H-5), 3.54 (1H, dd, $J_{1,2}=4.5$ Hz. H-2), 3.88 (1H, m, H-4), 3.90 (3H, s, OCH_3), 4.34 (1H, dd, $J_{1,6}=3.5$ Hz. H-1), 4.65 (1H, dd, H-6). MS: *m/e* 191 (M^+).

Anal. Calcd. for $C_8H_{18}N_2O_3 \cdot \frac{1}{2}H_2O$: C, 48.22; H, 9.61; N, 14.46.
Found: C, 48.28; H, 9.26; N, 14.17.

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