A NEW BROAD-SPECTRUM AMINOGLYCOSIDE ANTIBIOTIC COMPLEX, SPORARICIN

IV. SPORARICINS C AND D

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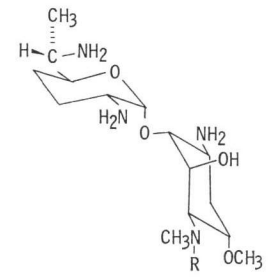
Two new aminoglycoside antibiotics, sporaricins C and D have been isolated from the culture broth of *Saccharopolyspora hirsuta* subsp. *kobensis*, which produced sporaricins A and B. The structures of sporaricins C and D have been determined to be 4-*N*-carbamoylglycylsporaricin B and 4-*N*-formylglycylsporaricin B, respectively. Sporaricins C and D are active against Gram-positive and Gram-negative bacteria including aminoglycoside-resistant strains.

In the previous papers[1~3], we reported that *Saccharopolyspora hirsuta* subsp. *kobensis* KC-6606 produced sporaricins A and B. Upon further investigation, it has been found that two new aminoglycoside antibiotics, sporaricins C and D are produced simultaneously as minor products. In this paper the isolation, properties and structural elucidation of these two minor components are described.



	R
Sporaricin A	$COCH_2NH_2$
Sporaricin B	H
Sporaricin C	$COCH_2NHCONH_2$
Sporaricin D	$COCH_2NHCHO$

Isolation

Sporaricin complex[1] (8.0 g) in crude state extracted from the fermentation broth of *Saccharopolyspora hirsuta* subsp. *kobensis* using the column chromatographic method reported previously was adsorbed on a column of Amberlite CG-50 (NH_4^+, 3×150 cm) and eluted by gradient elution between 0.05 N NH_4OH and 0.5 N NH_4OH. After elution of some minor components, sporaricin D was eluted, followed by sporaricin C. Further purification of sporaricin C or D was accomplished by CM-Sephadex C-25 (NH_4^+ form) column chromatography developed with aqueous ammonia with a gradient concentration from 0.05 N to 0.3 N, and the pure sporaricins C or D was obtained as a colorless solid.

Physico-Chemical Properties

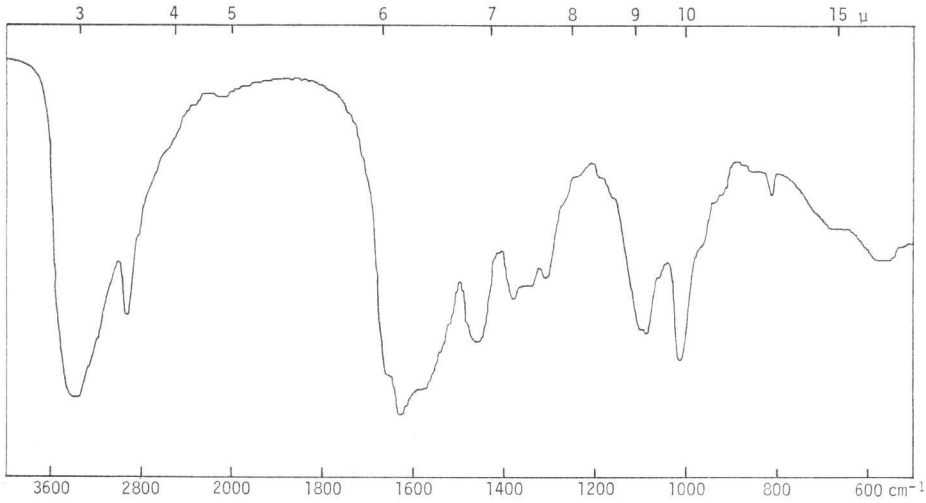
The physico-chemical properties of sporaricins C and D are listed in Table 1. They show no definite melting and decomposition point. These antibiotics are soluble in water and methanol and give

Table 1. Physico-chemical properties of sporaricins C and D.

	Sporaricin C		Sporaricin D	
Nature	Basic colorless solid		Basic colorless solid	
$[\alpha]_D^{23}$	+126°		+125°	
UV	End absorption		End absorption	
Elementary analysis	$C_{18}H_{36}N_6O_6 \cdot H_2CO_3 \cdot H_2O$		$C_{18}H_{35}N_5O_6 \cdot \frac{1}{2}H_2CO_3 \cdot H_2O$	
(%)	Found	Calcd.	Found	Calcd.
C	44.18	44.52	47.36	47.63
H	7.67	7.87	7.88	8.21
N	16.54	16.40	15.25	15.01
MW (Mass)	432 (FD)		417	
IR (KBr) cm^{-1}	3,350, 2,930, 1,650(sh), 1,625, 1,575		3,350, 2,930, 1,665, 1,630, 1,575	
^1H NMR (D_2O) ppm*				
C-CH_3	1.57		1.52	
N-CH_3	3.58		3.60	
O-CH_3	3.89		3.89	
-NCH_2CO	4.55		4.71	
anomeric H	5.44		5.43	

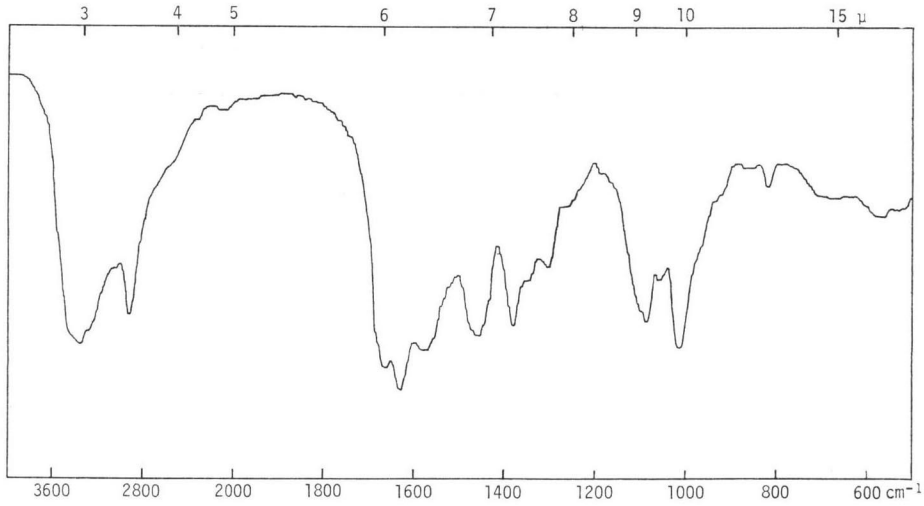
* TMS as external reference.

Fig. 1. IR spectrum of sporaricin C.



positive ninhydrin and RYDON-SMITH reactions. Sporaricins C and D show $[\alpha]_D^{23}$ +126° and +125°, respectively. Molecular formulae of $C_{18}H_{36}N_6O_6$ (*m/z* 432) and $C_{18}H_{35}N_5O_6$ (*m/z* 417) for sporaricins C and D, respectively, were calculated from mass spectrometric and microanalytical data. In their IR spectra, absorption of an amide function was observed at 1625 and 1650 (sh) cm^{-1} in sporaricin C (Fig. 1) and at 1630 and 1665 cm^{-1} in sporaricin D (Fig. 2). Their ^1H NMR spectra showed the presence of one anomeric proton, three methyl groups assigned to C-CH_3, N-CH_3 and O-CH_3 and

Fig. 2. IR spectrum of sporaricin D.



three methylene groups assigned to C-CH_2-C, respectively (Table 3). In the spectrum of sporaricin D, but not sporaricin C, a one proton singlet was observed at 8.71 ppm attributable to the *N*-formyl hydrogen of the former. From these properties sporaricins C and D are considered to be new aminoglycoside antibiotics.

Biological Properties

The antibacterial spectra of sporaricins C and D compared with sporaricin A are shown in Table 2. The antibacterial activity of sporaricins C and D are less active than that of sporaricin A.

Intravenous acute LD_{50} of both sporaricins C and D in mice were 200~300 mg/kg.

Structures of Sporaricins C and D

The molecular formulae $C_{18}H_{36}N_6O_6$ and $C_{18}H_{35}N_5O_6$ were established for sporaricins C and D (**1** and **2**) by FD and EI mass spectra and elementary analysis, respectively. From their ^1H NMR spectra, which were very similar to that of sporaricin A including the chemical shift of the N–CH_3 singlet, both **1** and **2** were presumed to be 4-*N*-acylsporaricins B. However, the methylene proton signal of the glycyl group which was observed at δ 4.01 ppm in sporaricin A, was present at lower field (δ 4.55 and 4.71 ppm in **1** and **2**, respectively (Table 3)). Moreover, an unprecedented signal at δ 8.71 ppm (1H, singlet) was observed only in the spectrum of **2**.

Acetylation of **1** and **2** with acetic anhydride in methanol gave tri-*N*-acetyl compounds of **1** and **2**, respectively. Alkaline hydrolysis of **1** with 1 N $Ba(OH)_2$ at 100°C for 1 hour gave sporaricin B and hydantoic acid. Similar alkaline hydrolysis of **2** gave sporaricin B and glycine. When the free base of **2** in water was heated at 100°C for 1 hour, sporaricin B and formylglycine were detected.

From the results mentioned above, the structures of **1** and **2** were suggested to be 4-*N*-carbamoylglycyl- and 4-*N*-formylglycylsporaricin B, respectively.

In order to prove the structures of **1** and **2**, 4-*N*-carbamoylglycyl- and 4-*N*-formylglycylsporaricin B were synthesized. Sporaricin B was treated with *N*-(benzyloxycarbonyloxy)succinimide in the presence

Table 2. Comparison of the antimicrobial spectra of sporaricins C and D with that of A.

Organisms	MIC (mcg/ml)		
	Sporaricin C	Sporaricin D	Sporaricin A
Staphylococcus aureus FDA 209P	3.13	6.25	0.39
Bacillus anthracis	0.78	1.56	0.20
Bacillus cereus	6.25	12.5	1.56
Bacillus subtilis ATCC 6633	0.78	1.56	0.20
Streptococcus faecalis	>25	>50	25
Escherichia coli NIHJ	12.5	25	1.56
Escherichia coli K-12 ML1410	>25	50	3.13
Escherichia coli K-12 ML1410 R-81[1]	25	>50	12.5
Escherichia coli K-12 ML1410 R-82[2]	12.5	50	3.13
Escherichia coli K-12 ML1410 R-101[3]	25	50	3.13
Proteus vulgaris OX-19	6.25	6.25	1.56
Proteus inconstans[4]	25	50	1.56
Klebsiella pneumoniae PCI 602	12.5	12.5	1.56
Pseudomonas aeruginosa Shibata	25	25	3.13
Pseudomonas aeruginosa A3	25	50	3.13
Pseudomonas aeruginosa GN-315[5]	>25	>50	6.25
Serratia marcescens	6.25	12.5	1.56
Mycobacterium smegmatis ATCC 607	>25	12.5	0.39

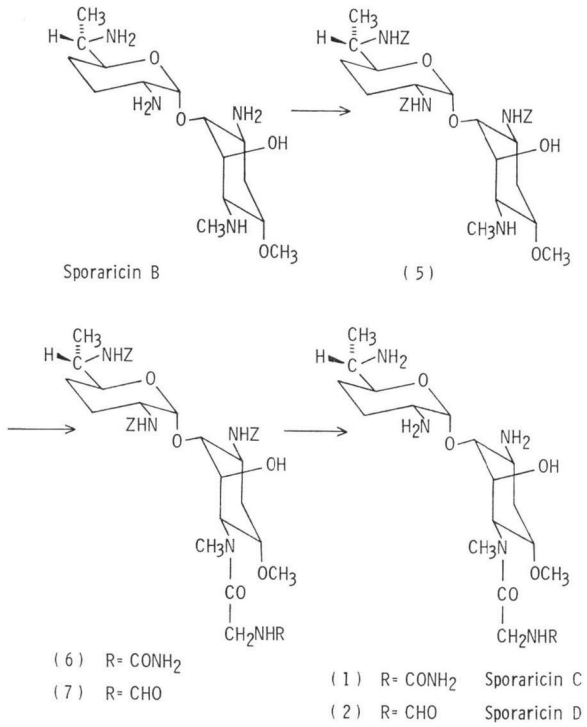
[1] APH(3′)-I. [2] APH(3′)-II. [3] AAD(2″). [4] AAC(2′). [5] AAC(6′)-IV.

Table 3. Chemical shifts and coupling constants of ^{1}H NMR spectra of sporaricins C and D.

Protons	Sporaricin C (1)		Sporaricin D (2)	
	ppm	*J* (Hz)	ppm	*J* (Hz)
1′	5.44	3.5	5.43	3.5
2′	3.35~	3.5	3.35~	3.5
3′	1.8		1.8	
4′	≀ 2.5		≀ 2.5	
5′	4.09	6.3	4.09	6.3
6′	3.34	6.3, 6.3	3.35	6.3, 6.3
7′	1.57	6.3	1.52	6.3
1	3.69	12.0, 4.0, 3.5	3.69	11.5, 4.0, 3.5
2 ax.	2.06	12.0, 11.5, 12.0	2.06	11.5, 11.5, 12.0
2 eq.	2.82	4.5, 4.0, 12.0	2.82	4.0, 4.0, 12.0
3	4.42	4.5, 11.5, 11.5	4.42	4.0, 11.5, 11.0
4	4.97	3.0, 11.5	4.98	3.0, 11.0
5	4.73	3.0, 3.5	4.75	3.0, 3.5
6	4.27	3.5, 3.5	4.28	3.5, 3.5
N-CH_3	3.58		3.60	
O-CH_3	3.89		3.89	
Gly. CH_2	4.55		4.71	
N-CHO			8.71	

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

Fig. 3. Syntheses of sporaricins C and D from sporaricin B.



of nickel (II) acetate tetrahydrate[4] to give 1,2′,6′-tris-*N*-benzyloxycarbonylsporaricin B (**5**). The 4-*N*-free compound (**5**) was coupled with the *N*-hydroxysuccinimide ester of hydantoic acid to give the 4-*N*-hydantoyl compound (**6**) and with that of formylglycine to give 4-*N*-formylglycyl compound (**7**). Catalytic hydrogenation of **6** and **7** gave 4-*N*-carbamoylglycyl- and 4-*N*-formylglycylsporaricin B in overall yields of 50% and 55%, respectively. These 4-*N*-substituted sporaricin B were identical with natural sporaricins C and D in specific rotation, IR and ^{1}H NMR spectra and biological properties.

Experimental

The spectrometric data were obtained by the following instruments. Optical rotation; digital polarimeter DIP-4 of Japan Spectroscopic Co., Ltd., infrared spectra; Model DS-403G of Japan Spectroscopic Co., Ltd., mass spectra; Model JMS-01SG (FD) of Japan Electron Optics Lab. and Model RMU-6MG of Hitachi, ^{1}H NMR spectra; Model JNM-MH-100 of Japan Electron Optics Lab. and ^{13}C NMR spectra; Model JNM-FX-100 of Japan Electron Optics Lab. The chromatography were performed with the following reagents. Silica gel; Wako gel C-200 for column chromatography, E. Merck Kieselgel PF_{254} nach Stahl for preparative thin-layer chromatography and E. Merck Kieselgel DC-Alufolien 60 F_{254} for thin-layer chromatography.

Isolation of Sporaricins C and D (**1** and **2**)

The sporaricin complex[1] (8.0 g) was adsorbed on a column of Amberlite CG-50 (NH_4^+ form, 3×150 cm) and eluted by gradient elution between 0.05 N NH_4OH (5 liters) and 0.5 N NH_4OH (5 liters), and the eluate was cut into 25-ml fractions. Fractions were monitored by biological activity using a standard disc assay against *Bacillus subtilis* ATCC 6633 and were detected by ascending paper chromatography developed with a lower phase of $CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1). After elution

of some minor components, **2** (fraction Nos. 168~192) was eluted, followed by **1** (fraction Nos. 193~210). Each fractions of **1** and **2** were concentrated and lyophilized to give colorless crude solids (**1**: 400 mg, **2**: 40 mg, respectively). The crude powder of **1** (400 mg) was further chromatographed on a CM-Sephadex C-25 (NH_4^+ form) column (1.2×60 cm). After washing with deionized water the column was eluted by gradient elution between 0.05 N NH_4OH (300 ml) and 0.3 N NH_4OH (300 ml), and the eluate was cut into 5-ml fractions. Fractions containing **1** were combined and lyophilized to afford a colorless solid (20 mg) of pure **1**; $[\alpha]_D^{23}$ +126° (*c* 1, H_2O); MS (FD); *m/z* 432 (M^+); ^1H NMR (D_2O) δ: 1.57 (3H, d, *J*=6.3 Hz, CCH_3), 3.58 (3H, s, NCH_3), 3.89 (3H, s, OCH_3), 5.44 (1H, d, *J*=3.5 Hz, H-1′).

Anal. Calcd. for $C_{18}H_{36}N_6O_6 \cdot H_2CO_3 \cdot H_2O$: C, 44.52; H, 7.87; N, 16.40.
Found: C, 44.18; H, 7.67; N, 16.54.

The crude powder (40 mg) of **2** was chromatographed on a CM-Sephadex C-25 (NH_4^+ form) column in a similar manner described above to give a colorless solid (20 mg) of **2**; $[\alpha]_D^{23}$ +125° (*c* 1, H_2O); MS: *m/z* 417 (M^+); ^1H NMR (D_2O) δ: 1.52 (3H, d, *J*=6.3 Hz, CCH_3), 3.60 (3H, s, NCH_3), 3.89 (3H, s, OCH_3), 5.43 (1H, d, *J*=3.5 Hz, H-1′), 8.71 (1H, s, formyl).

Anal. Calcd. for $C_{18}H_{35}N_5O_6 \cdot \frac{1}{2}H_2CO_3 \cdot H_2O$: C, 47.63; H, 8.21; N, 15.01.
Found: C, 47.36; H, 7.88; N, 15.25.

1,2′,6′-Tri-*N*-acetylsporaricin C (**3**)

To a solution of **1** (5 mg) in methanol (0.2 ml), acetic anhydride (0.02 ml) was added and the solution was allowed to stand overnight at room temperature. The solution was concentrated and the residue was chromatographed on a column of silica gel with $CHCl_3$ - MeOH (4: 1) to give **3** (6 mg) as a colorless solid; $[\alpha]_D^{23}$ +106° (*c* 0.5, H_2O); ^1H NMR (D_2O) δ: 1.58 (3H, d, *J*=6.7 Hz, CCH_3), 2.44, 2.46, 2.50 (each 3H, s, $COCH_3$), 3.55 (3H, s, NCH_3), 3.88 (3H, s, OCH_3), 5.43 (1H, d, *J*=3.33 Hz, H-1′).

Anal. Calcd. for $C_{24}H_{42}N_6O_9 \cdot \frac{3}{2}H_2O$: C, 49.22; H, 7.75; N, 14.55.
Found: C, 48.83; H, 7.27; N, 14.26.

1,2′,6′-Tri-*N*-acetylsporaricin D (**4**)

2 (5 mg) was treated with acetic anhydride in a similar manner as described for the preparation of **3** to give **4** (5.5 mg) as a colorless solid; $[\alpha]_D^{23}$ +108° (*c* 0.5, H_2O); ^1H NMR (D_2O) δ: 1.57 (3H, d, *J*=6.7 Hz, CCH_3), 2.43, 2.47, 2.50 (each 3H, s, $COCH_3$), 3.57 (3H, s, NCH_3), 3.89 (3H, s, OCH_3), 5.43 (1H, d, *J*=3.5 Hz, H-1′), 8.63 (1H, s, formyl).

Anal. Calcd. for $C_{24}H_{41}N_5O_9 \cdot \frac{3}{2}H_2O$: C, 50.51; H, 7.77; N, 12.27.
Found: C, 50.66; H, 7.28; N, 12.07.

Alkaline Hydrolysis of **1**

1 (2 mg) was dissolved in 1 N aqueous barium hydroxide (0.2 ml) and the solution was heated in a sealed tube at 100°C for 1 hour. The hydrolyzate was neutralized with carbon dioxide. On thin-layer chromatogram with $CHCl_3$ - MeOH - CH_3COOH (10: 5: 1), the reaction mixture showed a spot at Rf 0.32 concurrent with hydantoic acid. Sporaricin B was detected with the solvent systems reported previously[1].

Hydrolysis of **2**

i) **2** (2 mg) was hydrolyzed in a similar manner as described for alkaline hydrolysis of **1** and the reaction was monitored by TLC examination. On TLC with $CHCl_3$ - MeOH - 17% NH_4OH (1: 8: 2), the reaction mixture gave two spots of sporaricin B (Rf 0.32) and glycine (Rf 0.57).

ii) A solution of free base of **2** (2 mg) in water (0.2 ml) was heated in a sealed tube at 100°C for 1 hour. On TLC with $CHCl_3$ - MeOH - CH_3COOH (10: 5: 1), the reaction mixture gave a spot at Rf 0.47 concurrent with formylglycine. Sporaricins B and D, and glycine were also observed on TLC with $CHCl_3$ - MeOH - 17% NH_4OH (1: 8: 2).

1,2′,6′-Tris-*N*-benzyloxycarbonylsporaricin B (**5**)

To a solution of sporaricin B (205 mg) in methanol (6 ml), nickel (II) acetate tetrahydrate (450 mg) was added, and the reaction mixture was stirred at room temperature for 30 minutes to give a clear greenish solution. *N*-(Benzyloxycarbonyloxy)succinimide (514 mg) was added and the solution was stirred at room temperature for 2 hours. After addition of concentrated ammonium hydroxide (6 ml),

the reaction mixture was evaporated and the residue was extracted with chloroform. The organic layer was washed with 3 N ammonium hydroxide and water, dried with Na_2SO_4 and concentrated to give a syrupy residue (505 mg). The residue was chromatographed on a short column of silica gel with $CHCl_3$ - MeOH (10: 1) to give **5** as a colorless solid, 410 mg (90%); $[\alpha]_D^{20}$ +56° (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 1.07 (3H, d, J=6 Hz, CCH_3), 2.40 (3H, s, NCH_3), 3.35 (3H, s, OCH_3).

1,2′,6′-Tris-*N*-benzyloxycarbonyl-4-*N*-(*N*-carbamoylglycyl)sporaricin B (**6**)

To a solution of hydantoic acid (118 mg) and *N*-hydroxysuccinimide (115 mg) in dimethylformamide (2 ml), dicyclohexylcarbodiimide (206 mg) was added and the mixture was stirred at room temperature for 2 hours. The resulting colorless crystal was filtered off and the filtrate was concentrated to give a syrup which was dissolved in a solution of tris-*N*-benzyloxycarbonylsporaricin B (**5**, 240 mg) and triethylamine (0.5 ml) in dioxane (7.5 ml), and the solution was heated at 70°C for 3 hours. The reaction mixture was concentrated to give a syrup which was dissolved in chloroform and washed with water, dried with Na_2SO_4 and concentrated to give a syrup. The syrup was purified with preparative TLC ($CHCl_3$ - MeOH, 10: 1) to give **6** as a colorless syrup, 207 mg (73%); $[\alpha]_D^{24}$ +47° (*c* 2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 1.09 (3H, CCH_3), 3.30 (3H, s, OCH_3).

1,2′,6′-Tris-*N*-benzyloxycarbonyl-4-*N*-(*N*-formylglycyl)sporaricin B (**7**)

Tris-*N*-benzyloxycarbonylsporaricin B (**5**, 300 mg) was treated with *N*-hydroxysuccinimide ester of formylglycine in a similar procedure as described for the preparation of **6** to give **7**, 319 mg (95%); $[\alpha]_D^{24}$ +41° (*c* 2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 1.08 (3H, d, J=6 Hz, CCH_3), 2.97 (3H, s, NCH_3), 3.34 (3H, s, OCH_3).

4-*N*-(*N*-Carbamoylglycyl)sporaricin B (**1**, Sporaricin C)

A solution of **6** (205 mg) in acetic acid (3 ml) was hydrogenated with 10% palladium on carbon under an atmospheric pressure of hydrogen. After filtration, the filtrate was concentrated to about 1 ml. The concentrate was diluted with 200 ml of water and neutralized with aqueous ammonia. The solution was charged on a column of CM-Sephadex C-25 (NH_4^+ form) and eluted with a linear gradient elution between 0.05 N NH_4OH (100 ml) and 0.5 N NH_4OH (100 ml). The eluate containing **1** was lyophilized to give a colorless solid of **1**, 93 mg (77%); $[\alpha]_D^{24}$ +126° (*c* 1, H_2O).

4-*N*-(*N*-Formylglycyl)sporaricin B (**2**, Sporaricin D)

A solution of **7** (314 mg) in acetic acid (5 ml) was hydrogenated with 10% palladium on carbon. Purification in a similar manner as described for preparation of **1** gave **2** as a colorless solid, 115 mg (64%); $[\alpha]_D^{24}$ +127° (*c* 1, H_2O).

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