POROTHRAMYCIN†, A NEW ANTIBIOTIC OF THE ANTHRAMYCIN GROUP: PRODUCTION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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A new antitumor antibiotic porothramycin was produced by a new strain of *Streptomyces albus*[1,2]. The antibiotic was isolated in two active forms, the natural free hydroxyl form (porothramycin A) or the crystalline methyl ether form (porothramycin B) depending upon the isolation process used. Structural studies established that porothramycin is a new member of the pyrrolo[1,4]benzodiazepine group antibiotics having only one substituent on the benzene ring. The antibiotic exhibited antimicrobial activity against Gram-positive bacteria and anaerobes and significantly prolonged the survival times of mice implanted with experimental tumors.

In the course of a screening program for new antitumor antibiotics, a strain of actinomycete (K731-113) classified as *Streptomyces albus* was found to produce a novel antitumor antibiotic, which was designated as porothramycin. The antibiotic was recovered from the fermentation broth by using organic solvent or Diaion HP-20 and purified by column chromatographies. When methanol was used in the isolation or purification process, the antibiotic was obtained as the crystalline methyl ether form (porothramycin B). While the natural hydroxyl form of the antibiotic (porothramycin A) was recovered by the procedure without using methanol. Both porothramycins A and B were active against Gram-positive bacteria and anaerobes but inactive against Gram-negative bacteria and fungi. They exhibited strong antitumor activity against P388 and L1210 leukemia and B16 melanoma in mice. This paper reports the discovery, production, isolation, structure determination and biological activity of porothramycins A and B.

Production

A well grown agar slant of *S. albus*, K731-113, was used to inoculate a vegetative medium consisting of soluble starch 2%, beet molasses 1%, fish meal 1% and $CaCO_3$ 0.5%, the pH being adjusted to 7.0 before sterilization. The vegetative medium was incubated at 28°C for 3 days on a rotary shaker (250 rpm) and 5 ml of the growth was transferred into 500-ml Erlenmeyer flasks containing 100 ml each of the fermentation medium having the same composition as the vegetative medium. The fermentation was carried out at 28°C for 7 to 8 days on a rotary shaker. The antibiotic activity in the fermentation broth was determined by the paper-disc agar diffusion method using *Bacillus subtilis* M45 (Rec$^-$ mutant) and *Bacteroides fragilis* as the test organisms. After 7 days cultivation, the antibiotic activity reached a maximum potency of 60~100 μg/ml.

† Porothramycin was originally called as BMY-28121 or BU-2916T.

Isolation and Purification

The fermentation broth (15 liters) was separated into mycelial cake and broth supernatant by using a Sharples centrifuge. The antibiotic activity in the mycelial cake was extracted twice with 1 liter each of methanol. The broth supernatant was stirred with Diaion HP-20 (1 liter). The resin was washed with water and then eluted twice with 90% aqueous acetone (3 liters each). The acetone and methanol extracts were combined and concentrated *in vacuo* to an aqueous solution which was extracted with butanol (2 liters). Evaporation of the butanol extract afforded 2.21 g of crude solid. The solid was dissolved in 40% aqueous methanol (40 ml) and charged on a column of Diaion HP-20 (270 ml) which was developed with 80% aqueous methanol. The eluate was monitored by the bioassay against *B. subtilis* M45. The appropriate eluates were pooled and concentrated *in vacuo* to give light-brown powder (330 mg) which was chromatographed on a silica gel column (Wakogel C-200, 140 ml) with a mixture of chloroform - methanol (20 : 1). The active fractions were evaporated, and the residue was crystallized from an ethyl acetate - methanol mixture (20 : 1) to deposit yellow needles of pure porothramycin B (90 mg). Following a similar isolation and purification process using acetone instead of methanol, porothramycin A was obtained as an amorphous pale yellow powder.

Physico-chemical Properties

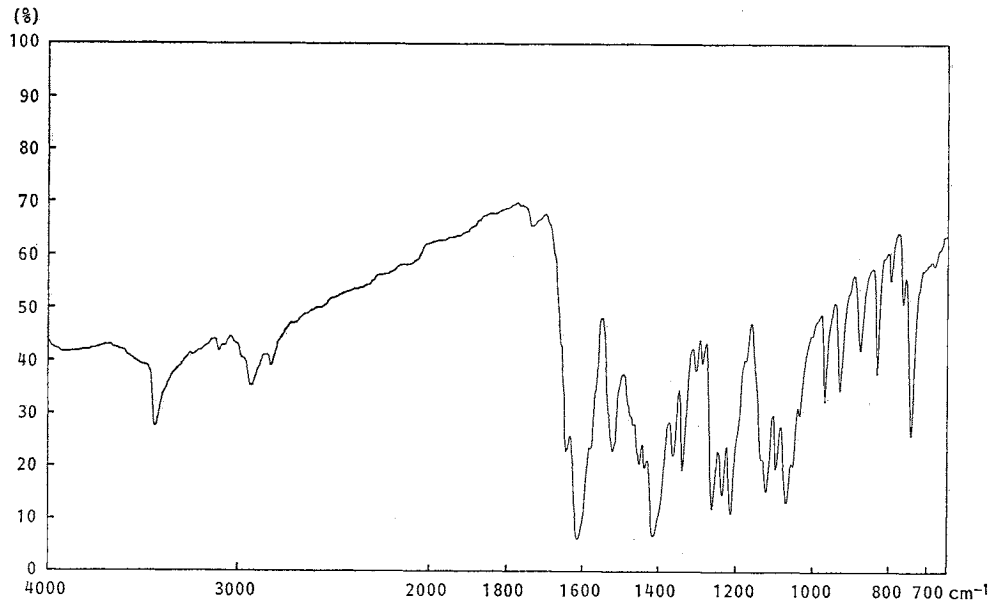
Porothramycins A (**I**) and B (**II**) are readily soluble in methanol, chloroform and pyridine, slightly soluble in ethyl acetate and ethyl ether but practically insoluble in *n*-hexane and water. They gave a positive response to Dragendorff and Rydon-Smith reagents but were negative to ninhydrin, ferric chloride and anthrone reactions. The molecular formulae of **I** and **II** were determined to be $C_{18}H_{21}N_3O_4$ and $C_{19}H_{23}N_3O_4$, respectively, based on the microanalysis and mass spectral results. Their physico-chemical data are summarized in Table 1. The UV spectra of **I** and **II** were similar, exhibiting the maxima at around 214, 235 and 335 nm in methanol and no shift was observed in acidic or alkaline solution. The IR and ^1H NMR spectra of **II** are shown in Figs. 1 and 2, respectively. The spectral data of **I** and **II** were very similar to each other but they differed clearly in the ^1H and ^{13}C NMR spectra. The spectrum of **II** showed two OCH_3 signals at δ 3.36 and 3.90 ppm while **I**

Table 1. Physico-chemical properties of porothramycins A (**I**) and B (**II**).

	I	**II**
Nature	Pale yellow powder	Yellow needles
MP (°C, dec)	140~150	164~166
$[\alpha]_D^{27}$ ($CHCl_3$)	+432° (*c* 0.46)	+669° (*c* 0.62)
Molecular formula	$C_{18}H_{21}N_3O_4$	$C_{19}H_{23}N_3O_4$
Elementary analysis (%)	Calcd: Found:	Calcd: Found:
C	62.96, 62.57,	63.85, 63.42,
H	6.16, 6.05,	6.49, 6.71,
N	12.24 11.70	11.76 11.62
MS (*m/z*)	325 ($M^+ - H_2O$), 281, 252	325 ($M^+ - MeOH$), 281, 252
UV λ_{max}^{MeOH} nm (ε)	214 (22,000), 235 (20,300), 335 (45,500)	213 (27,800), 236 (27,100), 338 (57,500)
Rf EtOAc - Me_2CO (1 : 1)*	0.14	0.14
Rf EtOAc - MeOH (9 : 1)	0.20	0.20
Rf $CHCl_3$ - MeOH (9 : 1)	0.53	0.53

* TLC, SiO_2.

Fig. 1. IR spectrum of porothramycin B (II) (KBr).



lacked the higher-field OCH_3 signal (δ 3.36). This difference was substantiated by their ^{13}C NMR spectra (Table 2). The spectrum of **II** indicated the presence of an OCH_3 carbon at δ 54.5 ppm which was absent in that of **I**. These findings suggested that **II** is a methyl ether of **I**. In fact, when **I** was crystallized from ethyl acetate - methanol, yellow needles of **II** was obtained. They could not be differentiated by three TLC systems examined.

Table 2. ^{13}C NMR chemical shift (ppm) of porothramycins A (**I**) and B (**II**) (in $CDCl_3$).

Carbon No.	I	II
C-1	34.4 t	34.7 t
C-2	126.4 s	127.0 s
C-3	135.9 d	136.1 d
C-5	163.9 s	163.7 s
C-5a	121.0 s	121.2 s
C-6	117.1 d	117.2 d
C-7	112.1 d	111.9 d
C-8	115.5 d	115.6 d
C-9	147.1 s	146.9 s
C-9a	133.0 s	133.5 s
C-11	86.0 d	87.8 d
C-11a	56.1 d	56.1 d
C-12	135.3 d	135.2 d
C-13	124.7 d	124.6 d
C-14	167.0 s	166.8 s
$N(CH_3)_2$	36.1 q, 36.4 q	35.9 q, 37.3 q
11-OCH_3	—	54.5 q
9-OCH_3	59.3 q	59.1 q

Structure Determination

The physico-chemical properties and biological activity of **II** suggested a resemblance to those of the pyrrolo[1,4]benzodiazepine group of antibiotics[3]. In particular, its strong UV absorption at around 335 nm indicated a close similarity to those of anthramycin[4] and mazethramycin[5]. However, unlike these antibiotics, porothramycin did not show bathochromic shift of the UV absorption in alkaline solution. A distinct difference to the known antibiotics of the pyrrolo[1,4]benzodiazepine family was seen in the ^1H NMR spectrum (Table 3) which showed two NCH_3 (δ 3.04 and 3.10), two OCH_3 (δ 3.36 and 3.90) and three aromatic protons (δ 6.77, 6.89 and 7.63). The multiplicity of the aromatic protons was evidently assignable to the three contiguous aromatic protons which have never been found in the known antibiotics of this group.

Fig. 2. ^{1}H NMR spectrum of porothramycin B (**II**) (360 MHz in $CDCl_3$).

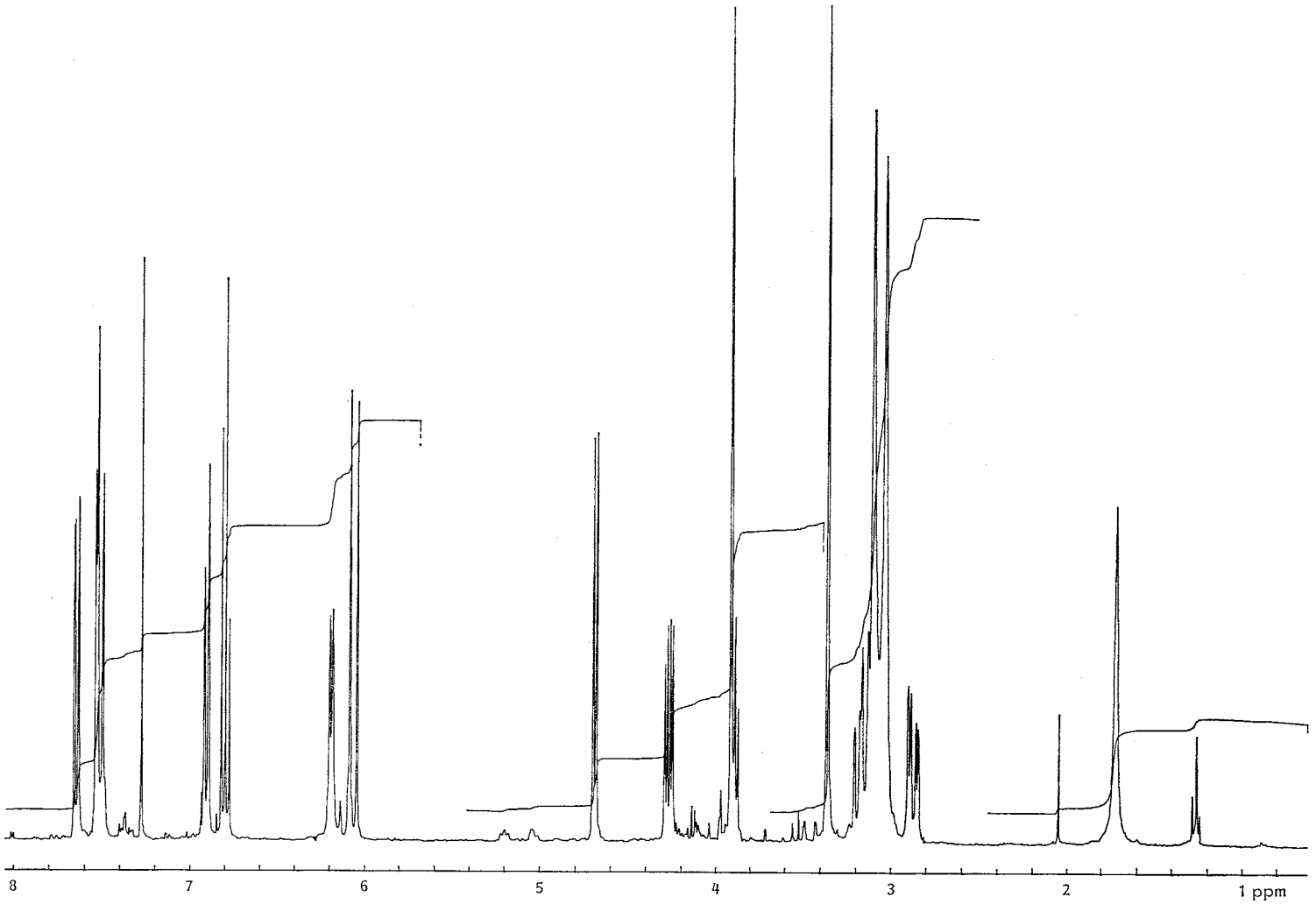
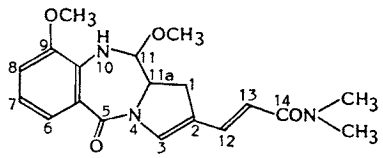
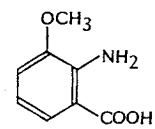


Table 3. ^{1}H NMR of porothramycin B (II) ($CDCl_3$, 360 MHz).



Chemical shift δ (ppm)	Number of proton	Multiplicity (J=Hz)	Assignment
2.87	1H	dd (6.0, 17.0)	1-H
3.04	3H	s	$N(CH_3)_2$
3.10	3H	s	
3.17	1H	dd (11.0, 17.0)	1'-H
3.36	3H	s	11-OCH_3
3.90	3H	s	9-OCH_3
4.27	1H	dd (6.0, 11.0)	11a-H
4.70	1H	d (6.0)	11-H
6.06	1H	d (15.2)	13-H
6.19	1H	d (6.0)	NH
6.77	1H	t (8.0)	7-H
6.89	1H	dd (2.5, 8.0)	8-H
7.50	1H	d (15.2)	12-H
7.51	1H	s	3-H
7.63	1H	dd (2.5, 8.0)	6-H

II was refluxed with 1 N NaOH to afford two UV-absorbing fragments (**III** and **IV**). Compound **III** was obtained as colorless needles and its molecular formula was determined to be $C_8H_9NO_3$ by microanalysis and mass spectrum. The ^{1}H NMR spectrum indicated that **III** contained a 1,2,3-trisubstituted benzene nucleus with one of the substituents being the OCH_3 group of the parent antibiotic. Further analysis of its spectral data disclosed that **III** is 2-amino-3-methoxybenzoic acid. NYC and MITCHELL[6] reported a synthesis of the acid, whose melting point and UV spectrum were found to be identical with those of **III**.



III

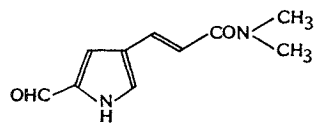
Compound **IV** was analyzed as $C_{10}H_{12}N_2O_2$ by mass spectral data (m/z 192 (M^+)) and microanalysis. The ^{1}H NMR spectrum of **IV** indicated two NCH_3 groups (δ 3.08 (6H, s)), two *trans*-olefinic protons (6.63 (d, J=15.2 Hz) and 7.56 (d, J=15.2 Hz)), two aromatic protons (7.10 (br s) and 7.27 (br s)), a formyl proton (9.50 (s)) and a NH-proton on aromatic ring (10.1 (br s)). The resonance patterns suggested a resemblance to those of 4-allyl-2-formylpyrrole[7] which was obtained by alkaline hydrolysis of sibiromycin. Spectral comparison of the two compounds revealed that **IV** differed from 4-allyl-2-formylpyrrole in having $CON(CH_3)_2$ group in place of the CH_3 group of the latter. Thus, **IV** was assigned to be 2-formylpyrrole-4-*N*,*N*-dimethylacrylamide.

Combination of above two fragments **III** and **IV** allowed us to propose 1,10,11,11a-tetrahydro-9,11-dimethoxy-5*H*-pyrrolo[2,1-c][1,4]benzodiazepine-5-one-2-*N*,*N*-dimethylacrylamide for the structure of **II**. As analyzed in Table 3, the absence of coupling between 11-H and 11a-H indicated the dihedral angle of H(11)-C(11)-C(11a)-H(11a) approximately 90°. Taking into consideration of the

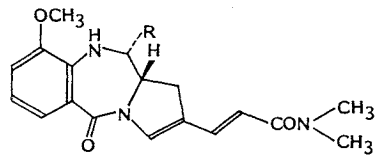
absolute stereochemistry of the known pyrrolo[1,4]benzodiazepine group antibiotic[8,9], 11(*R*) and 11a(*S*) configuration is estimated for **II**. The spectral difference between **I** and **II** indicated that **I** has a free hydroxyl group at C-11. Therefore, the structures of **I** and **II** are described as below.

Biological Activity

The antibacterial spectra of porothramycins A and B for various bacteria are shown in Table 4. MICs were determined by the agar dilution method using nutrient agar medium (Eiken) for aerobic bacteria and GAM agar medium (Nissui) for anaerobic bacteria. The inoculum size was adjusted to 10^4 cfu/ml for aerobic bacteria and $10^7 \sim 10^8$ for all anaerobic bacteria. Porothramycins A and B exhibited moderate antibacterial activity against aerobic Gram-positive bacteria and all anaerobic bacteria. They did not show activity against aerobic Gram-negative bacteria. Antitumor activity of porothramycins A and B was determined in mice (male BDF_1 strain). Lymphocytic leukemia P388 and lymphoid leukemia L1210 were implanted by intraperitoneal injection of 0.4 ml diluted ascitic fluid containing 10^6 and 10^5 cells, respectively. Melanotic melanoma B16 was implanted 0.5 ml of a 10%-tumor brei. The antibiotics were dissolved in 0.9% saline containing 10% dimethyl sulfoxide



IV



Porothramycin A (**I**) R = OH
Porothramycin B (**II**) R = OCH_3

Table 4. Antibacterial activity of porothramycins A (**I**) and B (**II**).

Organism	MIC (μg/ml)	
	I	**II**
Escherichia coli NIHJ	>50	>50
Pseudomonas aeruginosa A9930	>50	>50
Staphylococcus aureus FDA 209P	3.1	3.1
Streptococcus faecalis A9612	12.5	6.3
Bacillus subtilis PCI 219	1.6	1.6
Bacteroides fragilis A22053	0.8	0.4
Clostridium difficile A21675	6.3	3.1
C. perfringens A22787	0.8	0.4

Table 5. Antitumor activity of porothramycins A (**I**) and B (**II**).

	T/C (%) of MST*							
	Dose in mg/kg/day, ip							
	1	0.5	0.25	0.13	0.063	0.031	0.016	0.008
P388 Leukemia								
I		100	231	231	180	140	130	110
II			210	200	170	160	140	120
Mitomycin C	≧450	340	180	150	140	120		
L1210 Leukemia								
II			129	129	129	106	94	
Mitomycin C	141	141	129	129	106			
B16 Melanoma								
II			131	113	106	100		
Mitomycin C	191	164	136	124				

* Ratio of median survival time of test and control animals, values ≧125% indicate significant antitumor effect.

and graded doses of them were administered to mice intraperitoneally once daily for 9 days 24 hours after tumor inoculation. Mitomycin C was comparatively tested as a reference compound in the experiments. As shown in Table 5, porothramycins A and B exhibited potent antitumor activity against leukemia P388 and L1210, but were marginally effective against melanoma B16.

The acute toxicity of porothramycin B was determined in *dd*Y mice by single intraperitoneal administration, the LD_{50} being 0.81 mg/kg.

Discussion

Porothramycin is a new antibiotic of the pyrrolo[1,4]benzodiazepine family. This group of antibiotics have either 8-methyl-9-hydroxy (anthramycin[4] and mazethramycin[5]), 7-methoxy-8-hydroxy (tomaymycin[10], neothramycin[11] and chicamycin[12]) or 7-sibirosamyl-8-methyl-9-hydroxy (sibiromycin[7]) in their benzene rings. Recently, two new antibiotics with no substituent on the ring (prothracarcin[13] and abbeymycin[14]) were reported. Porothramycin is the first example which has only one substituent (9-methoxy) on the benzene ring. The absence of a hydroxy group on the ring is another unique structural feature of the antibiotic.

Experimental

General Procedures

TLC was performed on precoated silica gel plates (Kieselgel $60F_{254}$, Merck, 0.25 mm thick). The IR spectra were determined on a Jasco IRA-1 spectrometer and the UV spectra on a Shimadzu UV-200 spectrometer. The ^1H and ^{13}C NMR spectra were recorded at 32°C on a Varian Model FT 80A or a Bruker WM-360 spectrometer operated in Fourier transform mode using TMS or dioxane (67.4 ppm) as the internal standard. The mass spectra were obtained by using a Hitachi RMU-6MG mass spectrometer modified with an in-beam/electron impact system.

Alkaline Hydrolysis of Porothramycin B (II)

II (240 mg) was refluxed with 31 ml of 1 N NaOH solution for 35 minutes. The reaction solution was adjusted to pH 5 with 6 N HCl and extracted with BuOH. The BuOH extract was concentrated *in vacuo* to give 285 mg of brown solid, which was subjected to silica gel column chromatography (70 ml) developed with $CHCl_3$ - MeOH (50 : 1 ~ 3 : 1). The effluent of $CHCl_3$ - MeOH (50 : 1) was pooled and evaporated to dryness and the residue was dissolved in benzene and left standing at 5°C to yield colorless needles (43 mg) of compound **III**.

Compound **III**: MP 175~176°C; MS *m/z* 167 (M^+), 149, 134, 121, 106; UV λ_{max}^{MeOH} nm (ε) 225 (27,200), 247 (sh, 7,200), 336 (5,600); IR ν_{max}^{KBr} cm^{-1} 3500, 3380, 3000~2400, 1670, 1590, 1555, 760; ^1H NMR ($CDCl_3$) 3.85 (3H, s), 6.55 (1H, t, J=8 Hz), 6.85 (1H, dd, J=2 and 8 Hz), 7.50 (1H, dd, J=2 and 8 Hz).

Anal Calcd for $C_8H_9NO_3$: C 57.48, H 5.43, N 8.38.
Found: C 57.30, H 5.25, N 8.35.

The following eluates ($CHCl_3$ - MeOH, 9 : 1) were evaporated to a residue (93 mg) which was chromatographed on a silica gel column (70 ml) with elution of $CHCl_3$ - MeOH (20 : 1) mixture. Appropriate fractions were pooled and concentrated *in vacuo* to give a yellow residue (19 mg). This was dissolved in a small amount of $CHCl_3$ and diluted with benzene - *n*-hexane (1 : 1). Upon standing in a cold room, pale-yellow crystalline solids of **IV** were deposited, yield 11 mg.

Compound **IV**: MP 155~157°C; MS *m/z* 192 (M^+), 148, 120; UV λ_{max}^{MeOH} nm (ε) 260 (16,400), 320 (15,500); IR ν_{max}^{KBr} cm^{-1} 3120, 1660, 1640, 1585, 1560, 970.

Anal Calcd for $C_{10}H_{12}N_2O_2$: C 62.49, H 6.29, N 14.57.
Found: C 62.47, H 6.26, N 13.98.

Addendum in Proof

After we reported porothramycins A and B (BMY-28121 A and B) in the Annual Meeting of the

Agricultural Chemical Society, Apr. 1~4, 1987 and in the U.S. patent, a new antibiotic A-65636 was presented at the 27th Intersci. Conf. on Antimicrob. Agents Chemother.[15,16] meeting by the Abbott Laboratories. The presented data indicated A-65636 is identical with porothramycin B.

Acknowledgment

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