MACBECINS I AND II, NEW ANTITUMOR ANTIBIOTICS

II. ISOLATION AND CHARACTERIZATION

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New antitumor antibiotics, macbecins I and II, were isolated from the culture broth of *Nocardia* sp. No. C-14919. Macbecins I and II belong to the ansamycin group and have a benzoquinone and hydroquinone nucleus, respectively. Both showed antitumor activity against murine leukemia P 388 *in vivo*.

In the course of our screening for new antibiotics showing antibacterial, antifungal, antiprotozoal or antitumor activity, two new antibiotics macbecins I (**1**) and II (**2**) were isolated from the fermentation broth of *Nocardia* sp. No. C-14919[1]. They were active against Gram-positive bacteria, fungi, and protozoa *in vitro*[1] and, in addition, showed good antitumor activity against murine leukemia P 388 and melanoma B 16 *in vivo*[2].

This paper describes their isolation, characterization and antitumor properties.

Isolation

Activity against *Candida albicans*[1] and thin-layer chromatography (TLC) were employed to monitor the isolation of macbecins from the culture broth of *Nocardia* sp. No. C-14919.

Both antibiotics were obtained from the broth filtrate and the mycelium of the producing organism. Since they are lipophilic and almost neutral substances, they were isolated by the usual methods for such products (Chart 1).

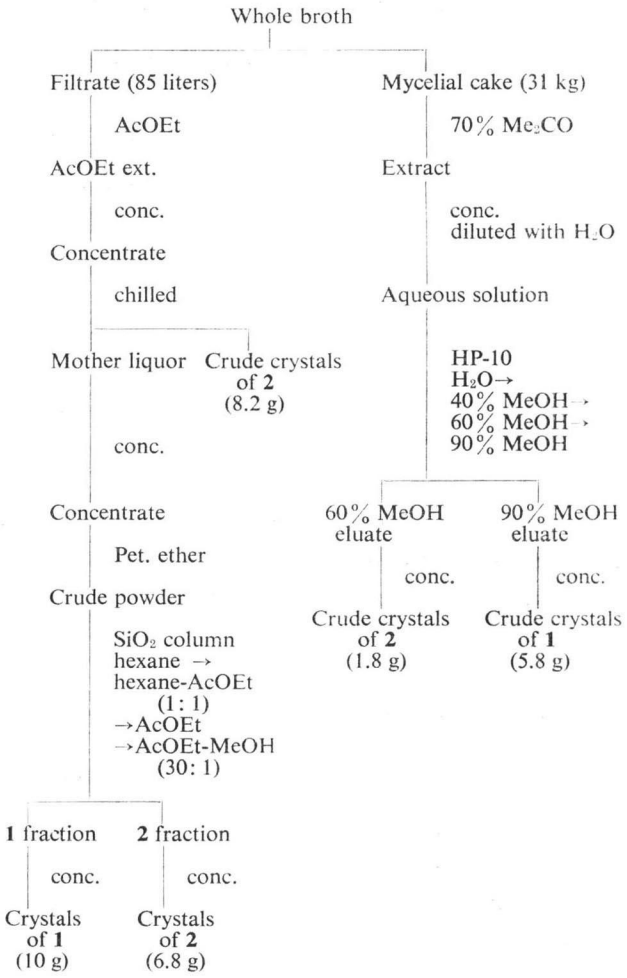
From the culture filtrate the macbecins were extracted at neutral pH with ethyl acetate. Concentration of the extract gave crude crystals of **2**. From the mother liquor **1** and additional **2** were recovered by concentration and addition of petroleum ether. This crude product was chromatographed on a column of silica gel developed successively with hexane, hexane - ethyl acetate (1: 1), ethyl acetate and ethyl acetate - methanol (30: 1). Crude crystalline **1** and **2** were obtained from appropriate eluates.

The active ingredients in the mycelium were extracted with 70% aqueous acetone. The extract, concentrated *in vacuo* to remove acetone and diluted with water, was applied to a column of nonionic adsorption resin. After washings with water and 40% aqueous methanol, the column was developed with 60% aqueous methanol to elute **2** and 90% aqueous methanol to elute **1**. Each fraction was concentrated *in vacuo* and chilled to give crude crystals. Crude crystalline **1** and **2** obtained by these procedures were recrystallized from methanol - water to afford pure yellow crystals of **1** and substantially colorless crystals of **2**.

Macbecins I and II were produced together under the usual fermentation conditions. To simplify the isolation procedure, advantage was taken of the reversible interconversion of **1** and **2** by oxidation

or reduction. For instance, two phases were formed from the concentrated ethyl acetate extract of the broth filtrate by adding methanol, water and hexane. The lower layer was separated, oxidized with dilute aqueous $FeCl_3$ solution, and chilled to give crude crystals of **1**. The mother liquor was again extracted with ethyl acetate and the concentrated extract yielded crude **1** by adding petroleum ether. The crude **1** was purified by column chromatography on silica gel to give crystals of **1** as described above.

Chart 1. Isolation procedure for macbecins I and II.



Physicochemical Properties of Macbecins I and II

Macbecin I was obtained as yellow needles or prisms from methanol - water or ethyl acetate, while **2** crystallized as substantially colorless needles or prisms from the same solvents.

Pure **1** is readily soluble in dimethylsulfoxide; soluble in ethyl acetate, chloroform, butanol, ethanol, methanol, methylisobutylketone and acetone; slightly soluble in diethyl ether and benzene; practically insoluble in petroleum ether, hexane and water. Likewise, **2** is readily soluble in dimethylsulfoxide; soluble in ethyl acetate, butanol, ethanol, methanol, methylisobutylketone and acetone; slightly soluble in diethyl ether, benzene, chloroform and water; practically insoluble in petroleum ether and hexane.

The physicochemical properties of **1** and **2** are summarized in Table 1.

Both antibiotics gave negative color reactions with ninhydrin, EHRLICH, GREIG-LEABACK and magnesium acetate reagents. Whereas **1** showed a negative reaction to BARTON's reagent[3], **2** was positive.

The UV and visible spectra of **1** have λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 274 (455) and 397 (43) and **2** show absorption maxima at 255 (290) and 308 (sh).

The IR spectrum of **1** has characteristic absorption bands at ν_{max}^{KBr} cm^{-1} 1740, 1692, 1660, 1645, 1605, 1100, 1085 and 1025 attributable to ester C=O, quinonoid C=C and C=O, amide C=O and ether linkage (Figs. 1, 2).

As described in the preceding section, **1** was also obtained by oxidation of **2** with mild oxidizing agents such as $FeCl_3$, while **1** was reversibly converted to **2** with reducing agents such as $Na_2S_2O_4$. This

relationship and other physicochemical properties such as UV, visible and IR spectra indicate that **1** is a benzoquinonoid and **2** is its quinol form.

The presence of the quinonoid ring protons at δ6.41 and 6.81 in the ^{1}H NMR spectrum of **1** and benzenoid protons at δ 6.35 and 6.45 in that of **2** (Figs. 3 and 4) support the conclusion that **1** is a disubstituted benzoquinone and **2** is its hydroquinone.

The molecular formula of **1** was assigned as $C_{30}H_{42}N_2O_8$ from the molecular ion peak at *m/e* 558.2951 (calcd. for $C_{30}H_{42}N_2O_8$: 558.2941) in the high-resolution mass spectrum.

Both antibiotics are relatively stable in neutral and weakly acidic solutions, but unstable in alkaline solutions. Chromatographic data are presented in Table 2.

Table 1. Physicochemical properties of macbecins I and II.

	Macbecin I	Macbecin II
Appearance	yellow needles or prisms	colorless needles or prisms
Melting point	187°C~188° (dec.)	148°C (dec.)
Optical rotation	$[\alpha]_D^{25}$ +350° (*c* 0.5, MeOH)	$[\alpha]_D^{25}$ +62° (*c* 0.5, MeOH)
Elemental analysis Found	C 64.85% H 7.62 N 5.01	C 62.32% H 8.43 N 4.82
Calcd. for	$C_{30}H_{42}N_2O_8$ C 64.49 H 7.58 N 5.01	$C_{30}H_{44}N_2O_8 \cdot H_2O$ C 62.26 H 8.01 N 4.84
UV $\lambda_{max}^{MeOH-N\ HCl(9:1)}$ nm ($E_{1cm}^{1\%}$)	274 (455), 240 (sh) 397 (43)	255 (295), 308 (sh)
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	274 (455), 240 (sh) 397 (43)	255 (290), 308 (sh)
$\lambda_{max}^{MeOH-N\ NaOH(9:1)}$ nm ($E_{1cm}^{1\%}$)	236 (585), 265 (500) 550 (56)	236 (505), 265 (420) 550 (50)
IR ν_{max}^{KBr} (cm^{-1})	3430, 3340, 2950, 2910, 1740, 1692, 1660, 1645, 1605, 1500, 1375, 1315, 1120, 1100, 1085, 1060, 1020	3480, 3250, 2980, 1685, 1625, 1598, 1472, 1390, 1370, 1315, 1207, 1090, 1065, 1042, 1030,
Color reaction	positive: $KMnO_4$ negative: ninhydrin, EHRLICH, GREIG-LEABACK, Mg acetate, BARTON[3]	positive: $KMnO_4$, BARTON[3] negative: ninhydrin, EHRLICH, GREIG-LEABACK, Mg acetate

Fig. 1. IR spectrum of macbecin I (KBr).

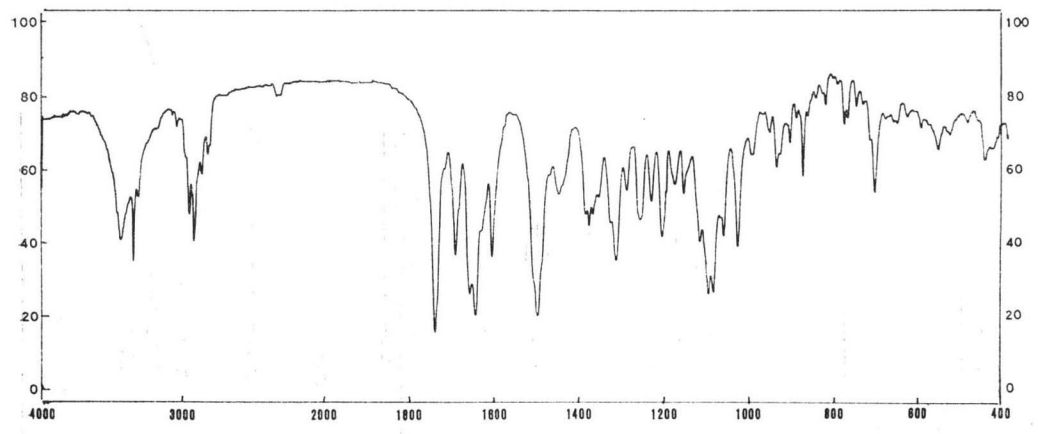


Fig. 2. IR spectrum of macbecin II (KBr).

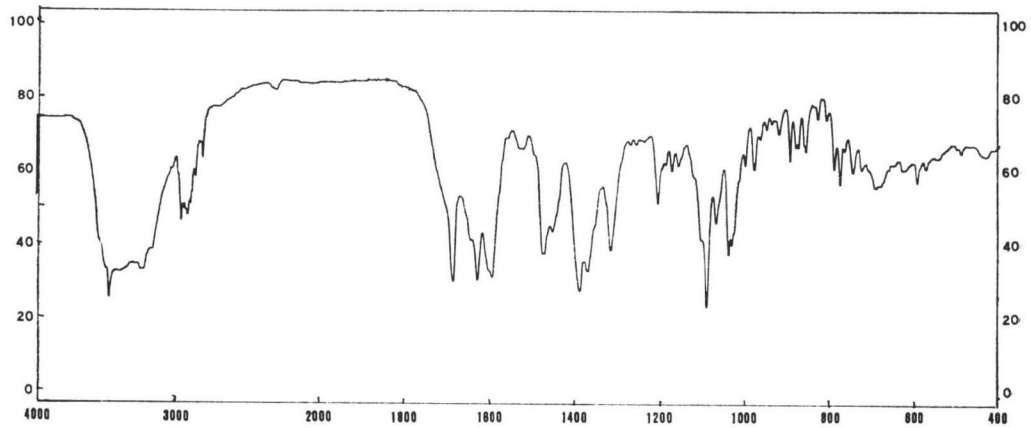


Fig. 3. NMR spectrum of macbecin I.

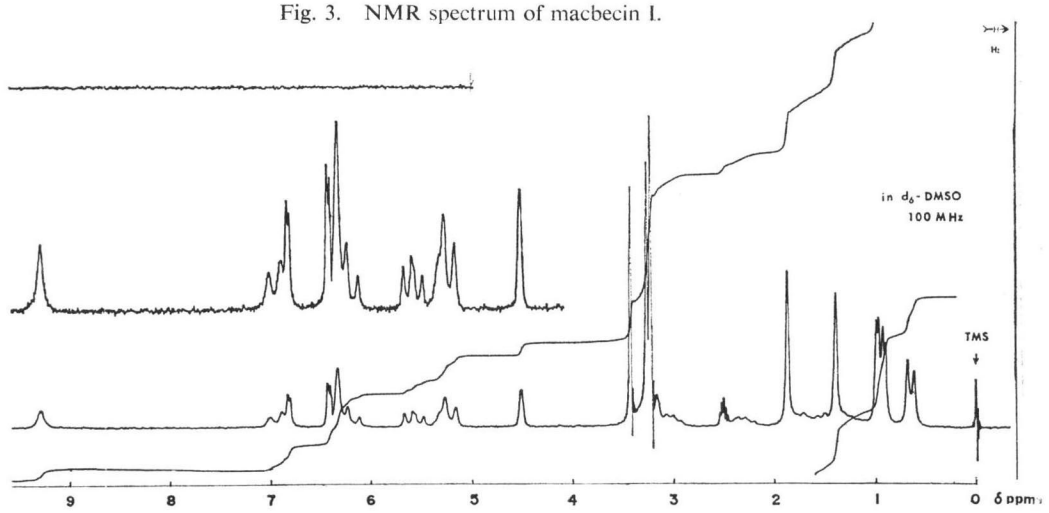


Fig. 4. NMR spectrum of macbecin II.

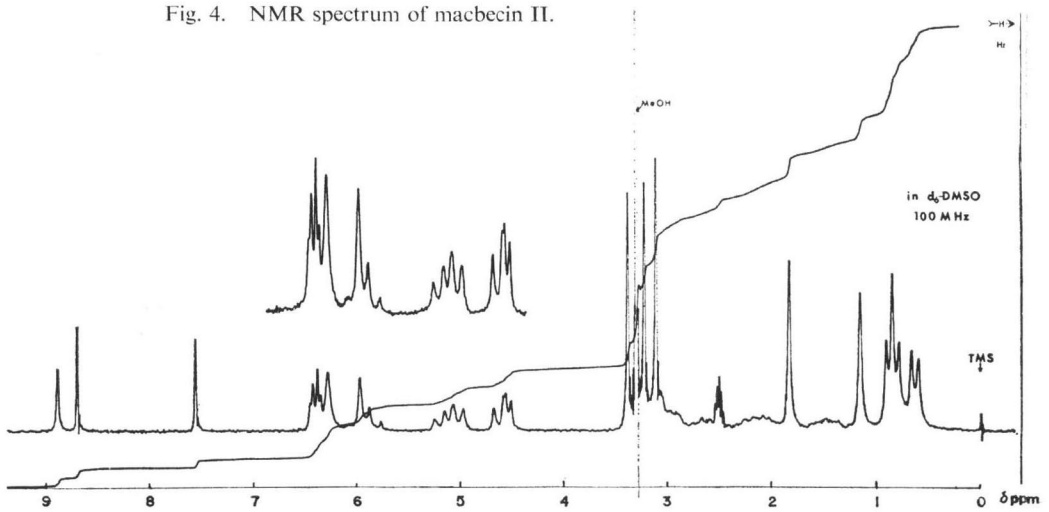


Table 2. Thin-layer chromatographic properties*.

	Macbecin I	Macbecin II
$CHCl_3$ - MeOH (9: 1)	0.85	0.50
$CHCl_3$ - MeOH (19: 1)	0.77	0.22
AcOEt - Me_2CO (9: 1)	0.80	0.50
AcOEt - MeOH (9: 1)	0.85	0.78

* Silica gel spot film, Tokyo Kasei Co., Japan.

Antitumor activity of macbecins I and II*

The activity of the macbecins against mouse leukemia P 388 (P 388) was assayed by the method of GERAN *et al.*[4] C57BL/6×DBA/2 F_1 (BDF_1) mice weighing 18~22 g were inoculated intraperitoneally (i.p.) with 1×10^6 cells of P 388. The mice were treated according to three schedules; daily on Days 1 to 9, every 4th day on Days 1, 5 and 9 and once on Day 1. Macbecins were suspended in 0.85% NaCl solution containing 0.2% Tween 80. As shown in Table 3, macbecin I exhibited significant antitumor activity against P 388. For a variety of treatment schedules the survival times in treated groups were more than 150% as long as those of the control group and **2** was nearly as active as **1** (data not shown). Maximum T/C values with **1** were 171% for daily treatment (Days 1 to 9), 160% for treatment every 4th day (Days 1, 5 and 9) and 154% for single treatment (Day 1 only). Thus the therapeutic effectiveness was slightly higher for daily than for single or intermittant treatment.

The acute toxicities (LD_{50}) of **1** and **2** by the intraperitoneal route in mice were 50~100 mg/kg and

Table 3. Activity of macbecin I against mouse leukemia P 388.

Treatment schedule		Dose (mg/kg/day)	No. of mice	Survival time (days)	MST* (days)	T/C** (%)	Body weight change (g) (Day 1~9)
Q1D	Days 1~9	20	5	10 13 19 21 26	19.5	171	−0.6
		10	5	19 19 19 20 21	19.5	171	−0.1
		5	5	15 16 16 17 22	16.5	145	0.3
Q4D	Days 1, 5 & 9	80	5	6 8 8 8 11	8.2	72	−0.7
		40	5	15 18 18 18 19	18.2	160	0.4
		20	5	13 15 16 18 19	16.5	145	0.8
	Day 1 only	160	5	4 4 4 4 5	4.1	36	—
		80	5	8 11 17 18 23	17.5	154	−1.9
		40	5	14 15 15 17 17	15.5	136	−0.7
Control			16	11 11 11 11 11 11 11 11 11 12 12 12 12 12 13 14	11.4		2.7

P 388 cells (1×10^6) were inoculated intraperitoneally on Day 0. Drug was injected intraperitoneally on the days indicated following tumor inoculation.

* MST, median survival time.

** T/C, the ratio of the median survival time of the treated group divided by that of the control group.

* We are grateful to Dr. Y. SAKURAI of the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, for preliminary tests of antitumor activities.

25 ~ 100 mg/kg, respectively.* These are relatively low for antitumor antibiotics.

Discussion

The molecular formula $C_{30}H_{42}N_2O_8$, melting point and optical rotation of **1** are different from those of geldanamycin[6], an antiprotozoal ansamycin with a benzoquinone moiety and from those of herbimycin[7] which was recently isolated as a herbicidal antibiotic (Table 4). Structural eludication of the macbecins will be reported elsewhere[5]. It is noteworthy that **2** is the first ansamycin isolated in the hydroquinone form from a fermentation broth. The macbecins showed activity against murine leukemia P 388 and melanoma B 16[2]. Since **1** is the first natural benzoquinonoid ansamycin active *in vivo* against leukemia P 388, studies on the structure-activity relationship with other ansamycins and on the mechanism of action are now expected.

Table 4. Comparison of macbecin I with known antibiotics.

	Macbecin I	Geldanamycin*	Herbimycin**
Producing organism	*Nocardia* sp. No. C-14919	*Streptomyces hygroscopicus* var. *geldanus* var. nov. (UC-5208)	*Streptomyces hygroscopicus* AM-3672
mp	187 ~ 188°C	252 ~ 255°C	230°C
Optical rotation $[\alpha]_D$	+350° (*c* 0.5, MeOH) +351° (*c* 0.1, $CHCl_3$)	+55° (*c* 0.638, $CHCl_3$)	+137° (*c* 1.0, $CHCl_3$)
Mol. formula	$C_{30}H_{42}N_2O_8$	$C_{29}H_{40}N_2O_9$	$C_{30}H_{42}N_2O_9$
MS *m/e* (M^+)	558.2951	560	574.295
UV spectrum	240 (sh)	255 (16350)	
λ_{max}^{MeOH} (ε) nm	274 (25100) 397 (2400)	304 (19300) 400 (sh 980)	270 (20900) 392.5 (1650)

* C. DEBOER *et al.*: J. Antibiotics 23: 442 (1970).
** S. ŌMURA *et al.*: J. Antibiotics 32: 255 (1979).

Experimental

Melting points were determined with a Mettler FP-5 instrument heating at 3°/min. UV spectra were recorded with a Shimadzu UV-200 double beam spectrophotometer. IR spectra were recorded with a Hitachi 285 grating infrared spectrophotometer. NMR spectra were obtained using a Varian XL-100-12 instrument; chemical shifts (δ) are reported in ppm down field from an internal TMS reference. Mass spectra were determined with a JEOL JMS-OISC spectrometer equipped with a direct inlet system. For TLC silica gel spot films (Tokyo Kasei) were used.

1. Isolation of macbecins

(1) The cultured broth (95 liters) of *Nocardia* sp. No. C-14919 was mixed with 2 kg of Hyflo-Supercel (Johns-Manville Products, U.S.A.). The mixture was filtered in a filter press to yield 85 liters of filtrate and 31 kg of moist mycelial cake. The filtrate was extracted twice with one-third volumes of ethyl acetate. When the ethyl acetate layer, washed with water and concentrated *in vacuo* to 200 ml, was cooled, crude crystals of **2** (8.2 g) were obtained by filtration. The filtrate was further concentrated to 50 ml and 300 ml of petroleum ether was added. The resulting precipitate (38 g) obtained by filtration was dissolved in 50 ml of methanol, and mixed with 5 g of silica gel (Merck 0.063 ~ 0.2 mm). The methanol was removed *in vacuo* and the adsorbent was placed on top of a column (500 ml) of silica gel. The column was washed with 500 ml of hexane and elution was carried out

* We are grateful to Dr. MURATA and his colleagues for their toxicological tests.

successively with 1 liter of hexane-ethyl acetate (1: 1), 1 liter of ethyl acetate and 1 liter of ethyl acetate-methanol (30: 1). Each fraction (100 ml) of effluent was tested by TLC; **1** was eluted from the column in fractions 13 to 16, while **2** was eluted in fractions 21 to 23. The fractions containing **1** were pooled and concentrated. After addition of 80% aqueous methanol and cooling, **1** was obtained as yellow crystals (10.1 g). The fractions containing **2** were also pooled, concentrated to 100 ml and cooled to give **2** (6.8 g) as substantially colorless crystals.

(2) The moist mycelial cake (31 kg) was extracted twice with 40 liters of 70% aqueous acetone and the resulting extracts were pooled and concentrated *in vacuo* to remove the acetone. The aqueous concentrate was diluted with water to 30 liters and passed through a column (1 liter) of Diaion® HP-10 (Mitsubishi Chemicals, Japan). The column was washed with 2 liters of water and 2 liters of 40% aqueous methanol. It was then eluted successively with 2.5 liters of 60% aqueous methanol and 2.5 liters of 90% aqueous methanol while 250 ml fractions were collected. Fraction 3 to 4 were combined, concentrated *in vacuo* and cooled to give crude crystals of **2** (1.8 g). Fractions 13 to 15 were pooled and concentrated *in vacuo*. After addition of 80% aqueous methanol, and cooling, crude crystals of **1** (5.8 g) were obtained. Crude **1** and **2** were recrystallized from aqueous methanol to yield:

Macbecin I: mp 187~188°C (decomp.)

Anal. Calcd. for $C_{30}H_{42}N_2O_8$: C, 64.49; H, 7.58; N, 5.01; O, 22.91%
Found: C, 64.85; H, 7.62; N, 5.01; O, 22.45

Macbecin II: mp 148°C (decomp.)

Anal. Calcd. for $C_{30}H_{44}N_2O_8 \cdot H_2O$: C, 62.26; H, 8.01; N, 4.84%
Found: C, 62.32; H, 8.43; N, 4.82

(3) The filtrate obtained as described above was extracted twice with 30 liters of ethyl acetate; the ethyl acetate layer was washed with water and concentrated to 500 ml *in vacuo*. Four liters of methanol was added to the concentrate, followed by 4 liters of water and 3 liters of hexane. After the mixture had been stirred the lower layer was separated. A solution of 20 g ferric chloride in 8 liters of water was added to the lower layer, and, with occasional stirring, the mixture was allowed to stand at room temperature for 5 hours. At the end of this time it was cooled. The crude crystals of **1** deposited were collected by filtration and dried (18 g). The filtrate was further extracted with ethyl acetate (5 liters). After the ethyl acetate extract was washed with water and concentrated to 100 ml, 1 liter of petroleum ether was added. The precipitate (13 g) consisted of crude **1**. This was purified by the chromatographic procedure described above.

2. Reduction of macbecin I

Macbecin I (400 mg) dissolved in 40 ml of ethyl acetate was shaken intensively with a solution (30 ml) of sodium hydrosulphite ($Na_2S_2O_4$) (500 mg) in a separatory funnel. The water layer was discarded. The procedure was repeated twice, then the ethyl acetate layer was thoroughly washed with water, and evaporated *in vacuo*. The residue dissolved in methanol (10 ml) and filtered, was mixed with 40 ml of water and cooled to give crystalline **2**, mp 147~148°C (decomp.).

3. Oxidation of macbecin II

Macbecin II (**2**) (400 mg) was dissolved in 60 ml of methanol and 32 ml of a 1% aqueous solution of ferric chloride was added. The mixture, allowed to stand at room temperature for 1 hour, deposited crystals of **1**. These were collected and washed with 20% aqueous methanol. mp 186~187°C (decomp.).

Acknowledgement

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