ISOLATION OF A BROMO ANALOG OF REBECCAMYCIN FROM *Saccharothrix aerocolonigenes*

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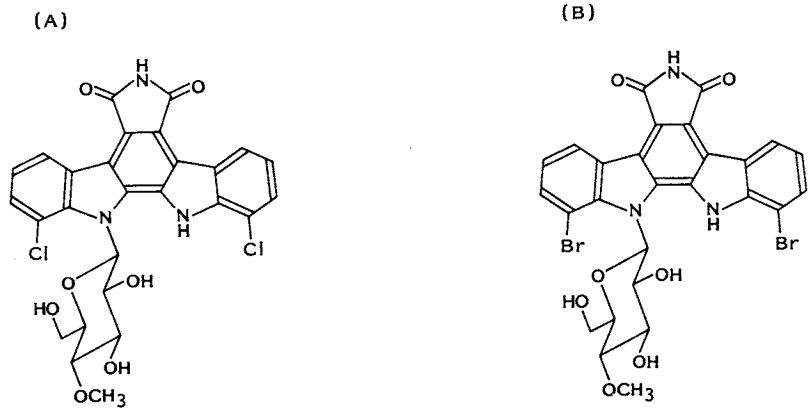
When grown in a defined medium containing 0.05% KBr, *Saccharothrix aerocolonigenes* ATCC 39243 produces a novel bromo analog of rebeccamycin. This new analog, designated bromorebeccamycin, has been isolated from the culture broth and purified by vacuum liquid chromatography and column chromatography. Spectroscopic data demonstrated that bromorebeccamycin has the same structure as rebeccamycin, except for the replacement of the two chlorine atoms by bromine atoms in the molecule. Bromorebeccamycin and rebeccamycin have a similar potency and activity against P388 leukemia in the murine model.

Rebeccamycin (Fig. 1A) is a novel antitumor antibiotic isolated from cultures of *Saccharothrix aerocolonigenes* ATCC 39243[1]. It consists of a chlorinated indolocarbazole chromophore to which is attached a methylglucose *via* an *N*-glycosidic linkage. Other natural products containing a similar indolocarbazole chromophore include staurosporine[2], K-252a, b, c, d[3~5], UCN-01 and UCN-02[6,7], acryriaflavins[8] and 6-cyano-5-methoxy-12-methylindolo[2,3-*a*]carbazole[9]. Rebeccamycin is unique among this class of metabolites because it is the only metabolite that has demonstrated a broad spectrum of *in vivo* activity in tumor-bearing murine models[1]. Bromine of inorganic origin has been found to be incorporated into chlorinated microbial metabolites chlortetracycline[10], griseofulvin[11], pyrrolinitrin[12], chloromonilicin[13] and actaplanin[14], as a result of replacing chloride with bromide in the fermentation medium. During our studies on the biosynthesis of rebeccamycin, it was decided to determine if *S. aerocolonigenes* ATCC 39243 could utilize inorganic bromine to form the bromo analog of rebeccamycin. In this paper, we describe the production and isolation of the bromo analog of rebeccamycin from cultures of *S. aerocolonigenes* supplemented with 0.05% of potassium bromide.

Fig. 1. Structure of rebeccamycin (A) and bromorebeccamycin (B).



Materials and Methods

Microorganism

The rebeccamycin-producing culture, *S. aerocolonigenes*, has been deposited with the American Type Culture Collection with the accession No. ATCC 39243.

Media and Culture Conditions

Strain ATCC 39243 was grown on slants of yeast extract - malt extract agar supplemented with $CaCO_3$. This medium consisted of glucose 0.4%, yeast extract 0.4%, malt extract 1%, $CaCO_3$ 0.15% and agar 1.5%. To prepare an inoculum for the shake flask culture, surface growth from a 5-day-old culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of vegetative medium consisting of glucose 3%, soy flour 1%, cotton-seed embryo meal 1% and $CaCO_3$ 0.3%. This vegetative culture was incubated at 28°C and 250 rpm on a rotary shaker. After 48 hours, 3-ml aliquots were transferred to a 500-ml Erlenmeyer flask containing 100 ml of a rebeccamycin-producing medium, DF-36, prepared using starch 1%, L-threonine 0.25%, $MgSO_4$ 0.1%, KH_2PO_4 0.2% and $CaCO_3$ 0.2%. For bromo analog production, this medium was supplemented with 0.05% KBr. The production culture was incubated at 28°C and 250 rpm for 6 days.

Extraction and Analytical Methods

The production of rebeccamycin and its bromo analog in the fermentation was monitored by HPLC using a C-18 reversed-phase column (μBondapak, 3.9 × 300 mm, Waters Associates). The solvent system was 0.1 M ammonium acetate - methanol - acetonitrile (4 : 3 : 3) at a flow rate of 1.5 ml/minute with the detector wavelength set at 313 nm. The fermentation extracts for HPLC assay were prepared by centrifuging 3 ml of fermentation broth at $1,500 \times g$ for 15 minutes. The supernatant was discarded. The mycelium was extracted with 1 ml of acetone. Fifty μl of the extract was used for HPLC analysis.

Isolation

Fermentation broth (40 liters) was filtered with diatomaceous earth (Dicalite). The resulting mycelial mat was resuspended in acetone - THF (2 : 1), stirred for 1 hour and filtered again. The Dicalite was further rinsed with acetone until it no longer fluoresced yellow under UV light. The combined filtrates were concentrated under reduced pressure to yield 4.9 g of crude extract. The crude extract was processed by vacuum liquid chromatography using 50 g of Silica gel H (E. Merck, 10 ~ 40 μm) dry packed in a 150-ml sintered glass funnel (medium porosity). After equilibrating the absorbent with isopropyl ether, the extract (in THF) was applied and pulled into the bed. The bed was sucked dry and then eluted in a step-wise fashion with isopropyl ether - THF mixtures. The major yellow band eluted with isopropyl ether - THF (1 : 1) and was concentrated to yield 370 mg residue. This residue was further fractionated on 50 g Sephadex LH-20 preswollen in THF (bed height 45 cm). The column was eluted with THF at a flow rate of 1 ml/minute. Fractions containing the yellow fluorescing band were pooled. Slow addition of hexane to this solution caused precipitation of a bright yellow solid (90 mg) designated as bromorebeccamycin.

Antitumor Assay

The test for inhibition of P388 leukemia in mice was performed using a previously described procedure[15,16].

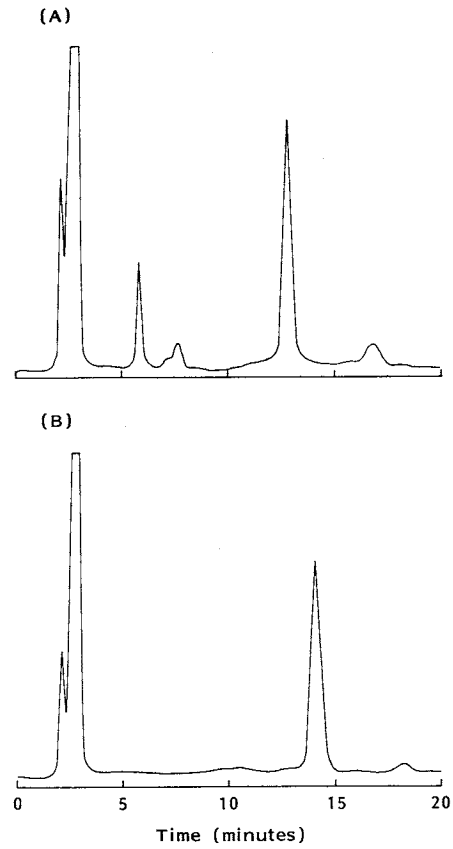
Results

Production of Bromorebeccamycin by *S. aerocolonigenes* ATCC 39243

During the course of our studies on the regulation and biosynthesis of rebeccamycin by strain ATCC 39243[17,18], a defined medium, DF-36, was developed for the production of rebeccamycin. The titer of rebeccamycin by strain ATCC 39243 in medium DF-36 was 18 μg/ml. The major metabolite produced, analyzed by HPLC, was rebeccamycin with a Rt of 12.4 minutes (Fig. 2A). Supplementing medium DF-36

with 0.05% KBr, strain ATCC 39243 did not produce any rebeccamycin but another rebeccamycin-like metabolite (Rt 13.6 minutes) as demonstrated by HPLC analysis (Fig. 2B). The new metabolite produced in the KBr-supplemented medium had the same UV absorption spectrum as rebeccamycin by photodiode array detector scan (Hewlett-Packard). The MS analysis of the new metabolite collected from HPLC showed that the new metabolite contained bromine. The above data suggested that the production of a bromo analog of rebeccamycin by strain ATCC 39243 in the KBr-supplemented medium. Ninety mg of pure new metabolite, designated as bromorebeccamycin, was isolated from a 40-liter shake flask culture of strain ATCC 39243.

Fig. 2. HPLC analysis of the mycelial extract of *Saccharothrix aerocolonigenes* ATCC 39243 grown in medium DF-36 (A) and medium DF-36 supplemented with 0.05% KBr (B).



Physico-chemical Properties of Bromorebeccamycin

The physico-chemical properties of bromorebeccamycin are summarized in Table 1. Bromorebeccamycin was soluble in dimethyl sulfoxide, *N*,*N*-dimethylformamide and tetrahydrofuran but only sparingly soluble in acetone. Observed in the FAB mass spectrum for bromorebeccamycin was an $(M+H)^+$ ion cluster (*m*/*z* 660) with isotopic ratios consistent with two bromine atoms. The mass

Table 1. Physico-chemical properties of bromorebeccamycin.

Appearance	Bright yellow amorphous solid
MW	559
Molecular formula	$C_{27}H_{21}Br_2N_3O_7$
FAB-MS (*m*/*z*)	660 $(M+H)^+$, 483 $(M-176)^+$
UV λ_{max}^{MeOH} nm (ε)	206 (402), 238 (491), 294 (366), 316 (618), 390 (58)
IR (cm^{-1})	3348, 3087, 2932, 2888, 1755, 1708, 1577, 1561, 1494, 1467, 1412, 1381, 1324, 1271, 1212, 1191, 1141, 1108, 1078, 1050, 955, 902, 801, 789, 759, 739, 729, 704, 656, 632, 587, 562, 496
^1H NMR (360 MHz) Obsd chemical shifts (ppm)	11.32 (1H, s), 10.49 (1H, s), 9.23 (1H, d), 9.04 (1H, d), 7.83 (2H, d), 7.35 (1H, t), 7.34 (1H, t), 6.89 (1H, d), 5.43 (1H, br d), 5.27 (1H, br t), 4.96 (1H, br d), 4.10 (1H, dd), 4.03 (1H, d), 3.85 (1H, m), 3.66 (1H, m), 3.59 (3H, s), 3.56 (2H, m)
^{13}C NMR (90 MHz) Obsd chemical shifts (ppm)	170.3, 170.1, 139.4, 138.5, 133.2, 129.9, 129.9, 129.6, 125.4, 124.5, 124.0, 123.0, 122.7, 122.4, 120.6, 119.4, 117.7, 117.7, 104.1, 104.1, 83.9, 81.1, 79.8, 77.7, 72.0, 60.7, 60.0
TLC[a] (Rf)	0.15

[a] Silica gel plates (Silica gel 60, Merck): $CHCl_3$ - MeOH (9 : 1).

Fig. 3. ^{1}H NMR spectrum of bromorebeccamycin (360 MHz, DMSO-d_6).

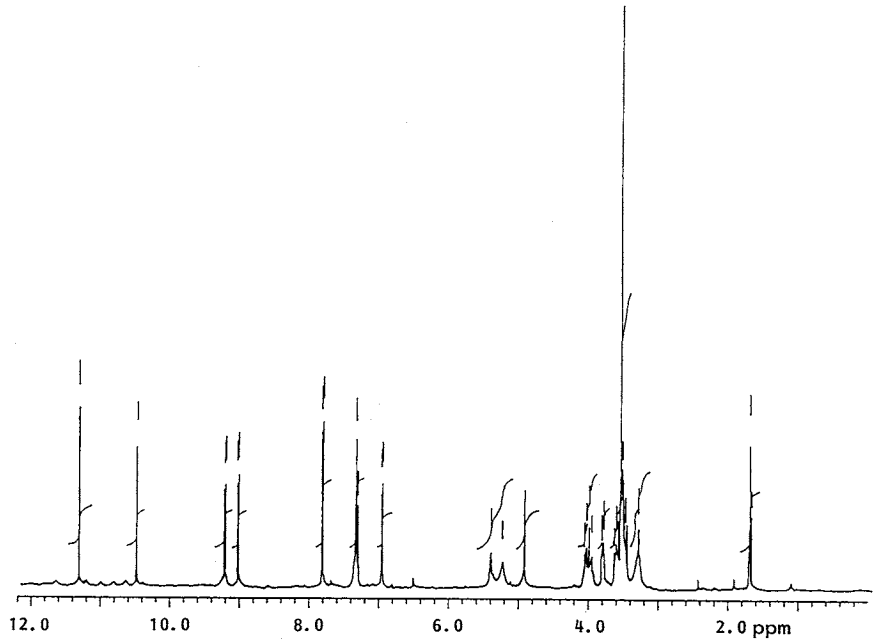
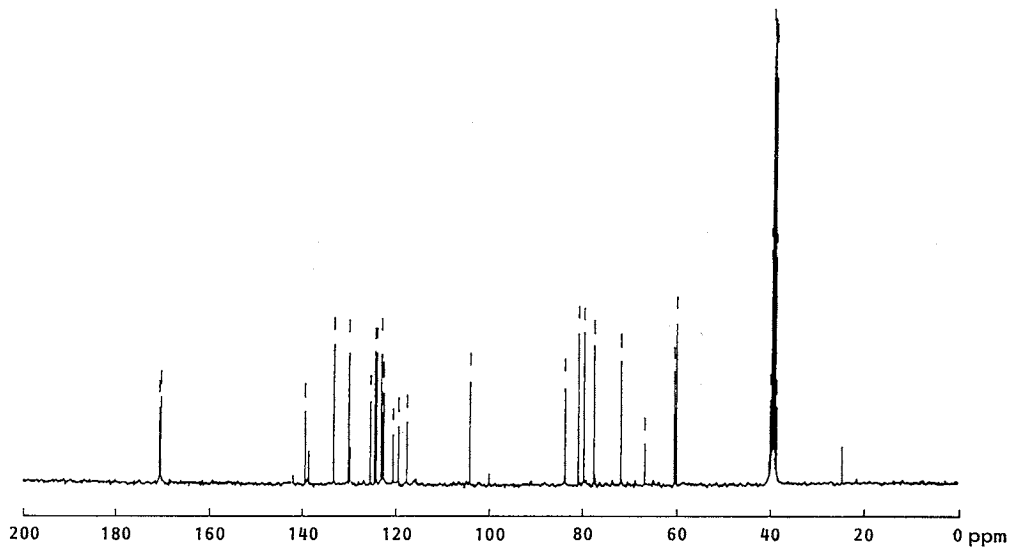
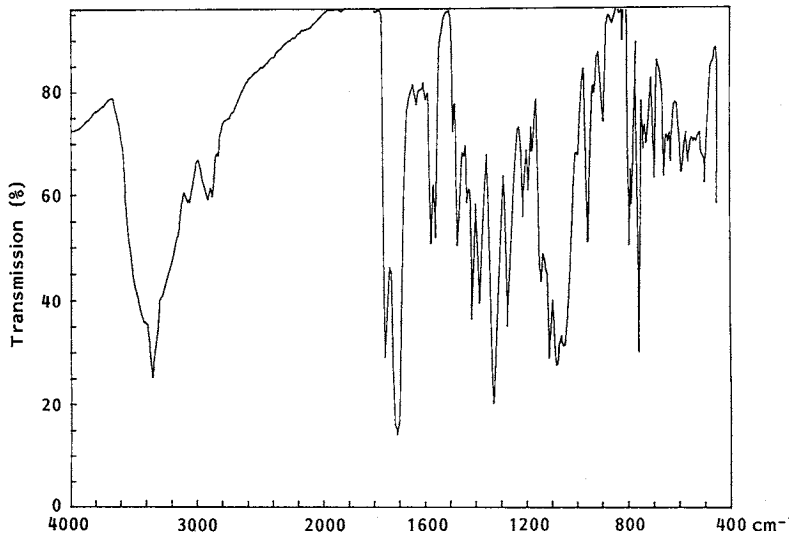


Fig. 4. ^{13}C NMR spectrum of bromorebeccamycin (90 MHz, DMSO-d_6).



spectrum also showed a fragment ion (*m/z* 483) resulting from loss of 4-methylglucose. The same facile cleavage was observed in the mass spectrum of rebeccamycin. Readily apparent in the ^{1}H NMR spectrum (Fig. 3) were two downfield aromatic resonances (9.04 and 9.23 ppm) that are characteristic for this class. These positions (*para* to halogens) are deshielded by the imide carbonyls. The ^{13}C NMR spectrum for bromorebeccamycin is shown in Fig. 4. The chemical shifts of the carbons bearing the bromine atoms (resonances degenerate at 104.1 ppm) are shielded relative to the corresponding chlorinated positions of

Fig. 5. IR spectrum of bromorebeccamycin (KBr).



rebeccamycin. The IR spectrum is presented in Fig. 5. Based on the spectral data, it was readily determined that bromorebeccamycin has the structure shown in Fig. 1(B).

Antitumor Activity

The effect of bromorebeccamycin on P388 leukemia implanted in mice is shown in Table 2. Prolongation of survival of leukemic mice was observed at dose levels ranging from 16 ~ 128 mg/kg. The maximum effect (65% increase in life span) was achieved at a dose of 64 mg/kg. The potency and activity of bromorebeccamycin on P388 leukemia in the murine model are similar to those of rebeccamycin[1].

Table 2. Effect of bromorebeccamycin on P388 leukemia.

Dose (mg/kg/injection)	MST (days)	Effect (% T/C)
128	15.5	155
64	16.5	165
32	16.0	160
16	14.0	140
8	12.0	120
4	11.5	115
Control	10.0	100

Tumor inoculum: 10^6 ascites cells, ip.
Host: CDF_1 mice.
Evaluation: MST.
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C ≥ 125 considered significant antitumor activity.
Control mice were given saline injections.

Discussion

A novel analog of rebeccamycin, bromorebeccamycin, was isolated from cultures of *S. aerocolonigenes* ATCC 39243 grown in a defined medium supplemented with 0.05% KBr. The development of the defined medium, DF-36, was important for the discovery of bromorebeccamycin. Addition of 0.05% KBr to this defined medium not only supports the production of bromorebeccamycin, but also suppresses the production of rebeccamycin, thereby significantly facilitating the purification steps. A simple and reproducible purification procedure was also developed for the isolation of rebeccamycin class compounds. This purification scheme is suitable for scale-up isolation of rebeccamycin and its analogs. Bromorebeccamycin and rebeccamycin have the similar potency and activity against P388 leukemia implanted in mice. Addition of potassium fluoride and potassium iodide to medium DF-36 did not lead to production of the corresponding fluoro- and iodo-analogs of rebeccamycin. For production of fluoro- and iodo-analogs of rebeccamycin, different feeding strategies are required.

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