AT2433-A1, AT2433-A2, AT2433-B1, and AT2433-B2 NOVEL ANTITUMOR ANTIBIOTIC COMPOUNDS PRODUCED BY *ACTINOMADURA MELLIAURA*

TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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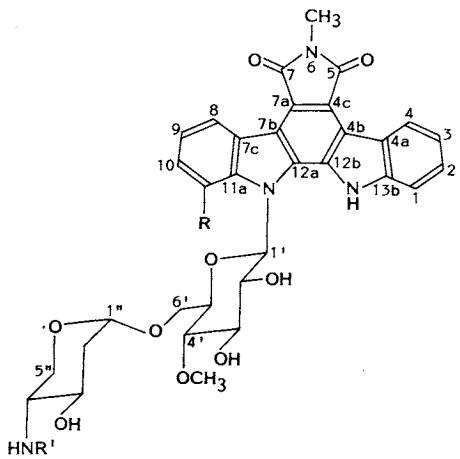
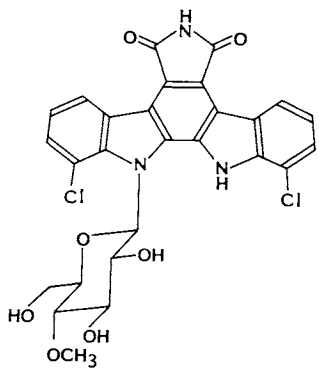
Compounds AT2433-A1 (**A1**), AT2433-A2 (**A2**), AT2433-B1 (**B1**), and AT2433-B2 (**B2**) were isolated from the cultured broth of *Actinomadura melliaura* sp. nov. (SCC 1655). Structurally these materials are closely related to rebeccamycin (**1**), an indolocarbazole antitumor antibiotic. **A1**, **A2**, **B1**, and **B2** were active against *Staphylococcus aureus* A9537, *Streptococcus faecalis* A20688, *Streptococcus faecium* (ATCC 9790), *Micrococcus lutea* (ATCC 9341), *Bacillus subtilis* (ATCC 6633). **A1** and **B1** were active against P388 leukemia in mice.

Under a cooperative antitumor screening agreement between the Schering Plough Corporation and the Bristol-Myers Company, the culture *Actinomadura melliaura* (SCC 1655) was selected for further evaluation. This led to the discovery of four novel, biologically active compounds, AT2433-A1 (**A1**),

Fig. 1. Structures of AT2433-A1, -A2, -B1, and -B2.



AT2433-A1 R=Cl R′=CH_3
AT2433-A2 R=Cl R′=H
AT2433-B1 R=H R′=CH_3
AT2433-B2 R=H R′=H



Rebeccamycin (**1**)

AT2433-A2 (**A2**), AT2433-B1 (**B1**), and AT2433-B2 (**B2**). Structurally, **A1**, **A2**, **B1**, and **B2** are closely related to rebeccamycin (**1**), an indolocarbazole antitumor antibiotic isolated from *Nocardia areocligenes*[1,2].

This paper describes the taxonomy of the producing strain, the fermentation, the purification and biological properties of **A1**, **A2**, **B1**, and **B2**. Details of the structure determination will be reported elsewhere[3].

Taxonomy of the Producing Organism

The producing culture, SCC 1655, was isolated from a soil sample collected in Bristol Cove, California by plating soil suspensions on water agar containing 75 μg/ml cephalosporin C. Morphological properties were observed on water agar and inorganic salts-starch agar after 21 days incubation at 30°C. The culture in filamentous. The substrate mycelia are moderately branched; spores are not observed. Aerial mycelia are formed and are approximately 0.5 to 0.8 μm in diameter. Spore chains are straight to slightly flexous, some hooks are observed. The spore chains, containing approximately 4 to 15 spores per chain, are usually born on branched, verticillate aerial sporophores. In the chains the spores vary in shape from orval (0.8 to 1.0 μm) and appear to be within a sheath. The spores are not motile.

Whole cell analysis by the procedures of BECKER *et al.*[4], and LECHEVALIER[5] indicates the presence of *meso*-diaminopimelic acid and madurose.

The media of SHIRLING and GOTTLIEB[6], and WAKSMAN[7] were used for cultural characteristics. Colors were determined by comparison with color chips from the Color Harmony Manual (4th Ed., Container Corp. of America, Chicago, 1958). The data are presented in Table 1. The aerial mycelia, en mass, are white. Substrate mycelial pigments range from tan to gold to brown. Diffusible pigments are not formed. Good growth occurs at 30 to 35°C poor to no growth occurs at 10 to 45°C. Carbohydrate utilization and physiological properties are present in Table 2.

Based on morphological and whole cell analysis, SCC 1655 is placed in the genus *Actinomadura*.

A comparison of strain SCC 1655 with *Actinomadura* species described in the literature indicated, based on micromorphology, that SCC 1655 could be related to either *Actinomadura yumaensis*[8] or *Actinomadura fastidiosa*[9]. SCC 1655 differs from *A. yumaensis* in the formation of diffusible pigments; utilization of adonitol, L-arabinose, D-mannitol, L-rhamnose, sucrose and D-xylose; and hydrolysis of xanthine and casein. SCC 1655 differs from *A. fastidiosa* in color of the aerial mycelia; growth at 45°C;

Table 1. Cultural properties of *Actinomadura melliaura* SCC 1655.

Agar medium	Growth	Texture and surface color	Aerial mycelium	Soluble pigment
BENNETT'S	Moderate	Flat to granular; g 2gc, bamboo	None	None
Yeast extract-malt extract	Good	Raised, folded; g 3le, yellow maple	None	None
ATCC 172[a]	Good	Raised, convoluted; g 3le, yellow maple	None	None
CZAPEK'S sucrose	Poor to fair	Flat, smooth; g 2gc, bamboo	Present, white	None
Glycerol-asparagine	Moderate	Raised granular; g 2le, mustard	None	None
EMERSON'S	Good	Raised, folded; g 2ic, honey gold	None	None
Oatmeal	Good	Flat, smooth; g 2ic, honey gold	Present white	None
Potato-glucose	Moderate	Raised, folded; g 3lg, adobe brown	None	None
Inorganic salts-starch	Fair	Flat, granular; g 2ic, honey gold	Present, white	None

[a] COTE, R. (*Ed.*): ATCC Media Handbook, 1st Ed., 1984. American Type Culture Collection, Rockville, Maryland, U.S.A.

Table 2. Physiologic properties of *Actinomadura melliaura* SCC 1655.

Carbohydrate utilization:		Sucrose	+++
Adonitol	+++	D-Trehalose	+++
D-Arabinose	+++	D-Xylose	+++
L-Arabinose	+++	Formation of:	
Dulcitol	−	Melanin	−
D-Fructose	+++	Hydrogen sulfide	−
D-Galactose	+++	Hydrolysis of:	
Glucose	+++	Adenine	+
Glycerol	+++	Hypoxanthine	+
i-Inositol	+++	Tyrosine	+
Lactose	−	Xanthine	−
Maltose	+++	Starch	+
D-Mannitol	+++	Breakdown of:	
D-Mannose	+++	Urea	−
L-Raffinose	−	Allantoin	−
L-Rhamnose	+++	Nitrate to nitrite	+

+++: Strong utilization, +: positive result, −: negative result.

utilization of L-arabinose, D-raffinose and D-xylose; and, formation of nitrate reductase. Based on these differences SCC 1655 is considered to be a new species of *Actinomadura* designated *A. melliaura* nov. sp. (LECHEVALIER and LECHEVALIER[10]) HORAN and BRODSKY.

The culture has been deposited in the American Type Culture Collection, Rockville, Maryland, under accession No. ATCC 39691.

Fermentation

The culture *A. melliaura* SCC 1655 was maintained as frozen whole broth at −20°C in vegetative medium containing 12% sucrose solution. The initial inocula of a two stage inoculum was prepared by adding 2 ml (5%) of freshly thawed whole broth to 70 ml of vegetative medium in a 300-ml Erlenmeyer flask. The vegetative media consisted of beef extract 3 g, Tryptone 5 g, Cerelose 1 g, potato starch 24 g, yeast extract 5 g and $CaCO_3$ 2 g in 1 liter tap water, pH 7.5 with NaOH. The flask was incubated at 30°C for 72 hours on a 5-cm stroke gyrotary shaker (300 rpm). The second stage inoculum was prepared by adding 25 ml (5%) of first stage inocula to 500 ml of vegetative medium in a 2-liter Erlenmeyer flask. The flask was incubated as above.

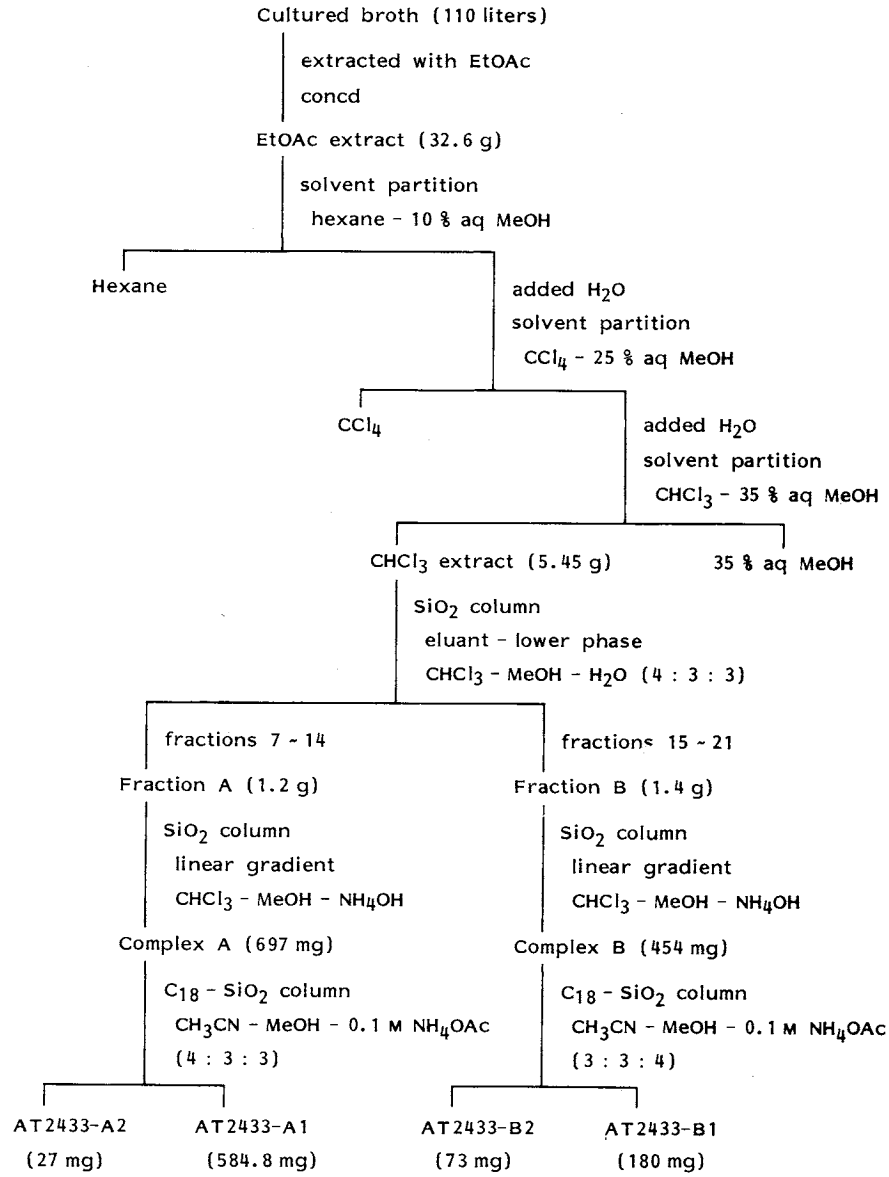
Fermentation for extraction was prepared by adding 70 ml (5%) of second stage inocula to 10 liters of production medium. The production medium consisted of yeast extract 5 g, Cerelose 10 g, soluble starch 20 g, NZ-Amine (Difco) 5 g, $CaCO_3$ 4 g, $CoCl_2$ 0.4 mg in 1 liter tap water, pH 7.0. The fermentation was carried out at 30°C with an agitation rate of 350 rpm and an air flow of 3.5 liters/minute. The fermentations were harvested for extraction at 96 hours.

Isolation and Purification

The isolation procedure for **A1**, **A2**, **B1**, and **B2** is illustrated in Fig. 2. The ethyl acetate extract was first resolved into an A subfraction and B subfraction by solvent partition and medium pressure column chromatography. These subfractions were purified to pure A complex and B complex, respectively. Resolution of each complex was achieved by medium pressure reversed phase column chromatography. The sequence was repeated five times to get enough **A2** for chemical and biological evaluation.

A crude extract of the fermentation broth (110 liters) was prepared by ethyl acetate extraction (2 × 110

Fig. 2. Isolation and purification procedures for AT2433-A1, -A2, -B1, and -B2.



liters). The pooled extracts were evaporated *in vacuo* to yield 32.6 g of extract. This was partitioned between hexanes (3 × 200) and 10% aqueous methanol (22.2 ml water - 200 ml methanol). The methanol layer was diluted (44 ml water) to 25% aqueous methanol and partitioned against pre-equilibrated carbon tetrachloride (3 × 200 ml). The methanol layer was diluted (42 ml water) to 35% aqueous methanol and partitioned against pre-equilibrated chloroform (3 × 200 ml). The chloroform extracts were pooled and evaporated *in vacuo* to yield 5.45 g of residue.

The chloroform residue from above was subjected to medium pressure column chromatography on silica gel (Woelm, 140 g, 32~63 μm) with the lower phase of a chloroform - methanol - water mixture (4:3:3) as eluant. The flow rate was 21 ml/minute and the following fractions were collected; fraction 1

180 ml, fraction 2 100 ml, fraction 3 50 ml, and fractions 4～33 75 ml. Fractions 7～14 were combined and concentrated *in vacuo* to yield 1.2 g of subfraction A. Similarly, fractions 15～21 were combined to yield 1.4 g of subfraction B.

Subfraction A was subjected to column chromatography on silica gel (Woelm, 37 g, 32～63 μm) using a 2-liter linear gradient of chloroform saturated with concd ammonium hydroxide (1%) to 10% methanol in chloroform saturated with conc ammonium hydroxide (1%). Forty 50-ml fractions were collected. The chromatography was monitored at 405 nm with a UV detector. Fractions 31～34 were combined and concentrated *in vacuo*. The residue was dissolved in chloroform - methanol (2 : 1) (50 ml) and precipitated with hexane (1,700 ml) to yield complex A (697 mg).

Chromatography of subfraction B (500 mg) was carried out exactly as described above for subfraction A. fractions 24～34 gave complex B (454 mg) after concentration and precipitation.

Complex A (360 mg) was subjected to reversed-phase chromatography on C_{18} bonded-phase silica (Baker, column 330 ml, 40 μm particles). The column was eluted with acetonitrile - methanol - 0.1 M ammonium hydroxide (4 : 3 : 3). The flow rate was 19 ml/minute and 50 ml fractions were collected. The chromatography was monitored at 405 and 435 nm with a UV detector. Fractions 17～24 were combined and extracted with chloroform (500 ml). The extract was concentrated *in vacuo*. The resulting residue was dissolved in chloroform - methanol (2 : 1) (2 ml) and precipitated with hexane (500 ml) to yield AT2433-A1 (**A1**, 51.8 mg). Fractions 12～16 were combined with comparable fractions from four separate experiments (experiments 2, 3, 4, and 5 began with 100, 167, 160, and 188 mg of complex A, respectively) and extracted with chloroform (1,000 ml). The extract was concentrated *in vacuo*. The resulting residue was dissolved in chloroform - methanol (2 : 1) (5 ml) and precipitated from hexane (500 ml) to yield AT2433-A2 (**A2**, 27 mg). (Experiments 2, 3, 4 and 5 yielded 54, 162, 155 and 162 mg of **A1**, respectively.)

Complex B (360 mg) was chromatographed using the same reversed-phased system with acetonitrile - methanol - 0.1 M ammonium acetate (3 : 3 : 4) as eluant. Fractions 14～16 yielded AT2433-B2 (**B2**, 73 mg). The residue obtained by chloroform (500 ml) extraction of fractions 17～24 was rechromatographed under the same conditions and yielded AT2433-B1 (**B1**, 180 mg).

Table 3. Physico-chemical properties of AT2433-A1 (**A1**), AT2433-A2 (**A2**), AT2433-B1 (**B1**), and AT2433-B2 (**B2**).

	A1	A2	B1	B2
Molecular formula	$C_{34}H_{35}N_4O_9Cl$	$C_{33}H_{33}N_4O_9Cl$	$C_{34}H_{36}N_4O_9$	$C_{33}H_{34}N_4O_9$
MW	678	664	644	630
FD-MS	679 $(M+H)^+$ [a]	664 $(M)^+$, 687 $(M+Na)^+$	644 $(M)^+$, 667 $(M+Na)^+$	630 $(M)^+$, 653 $(M+Na)^+$
UV λ_{max}^{MeOH} nm (ε)	200 (30,781), 235 (39,934), 283 (34,307), 316 (45,562), 395 (3,865)	198 (30,478) 234 (38,645), 286 (33,598), 314 (43,890) 394 (3,453)	202 (28,980), 234 (41,280), 284 (33,617), 316 (46,948), 400 (4,057)	201 (30,807), 233 (41,076), 282 (33,390), 315 (46,935), 400 (3,969)
IR (KBr) cm^{-1}	3425, 3362, 2940, 1750, 1696, 1581, 768, 759	3420, 3355, 2930, 1745, 1691, 1575, 765, 755	3363, 2940, 1750, 1692, 1575, 750	3365, 2940, 1750, 1692, 1578, 750
Rt (minutes)[b]	4.2	3.33	3.00	2.50

[a] Chemical ionization MS, methane gas.

[b] Retention time (Rt) on reversed phase HPLC (μBondapak C-18, Alltech, eluant acetonitrile - methanol - 0.1 M ammonium acetate (4 : 3 : 3), 254 nm, 2 ml/minute).

FD: Field desorption.

Reversed-phase HPLC was used to guide the processing and chromatographic steps. A μBondapak C_{18} column (10 μm, 0.46 × 25 cm, Alltech) was used. The eluant consisted of acetonitrile 40%, methanol 30%, and 0.1 M ammonium hydroxide 30% and was pumped at a 2 ml/minute. Detection was by UV absorbance at 254 nm.

Physico-chemical Properties

A1, **A2**, **B1**, and **B2** were each obtained as bright yellow solids. These substances were readily soluble in DMSO and chloroform - methanol mixtures (5 ~ 33% MeOH), sparingly soluble in ethyl acetate and THF, and insoluble in hexanes and water. Listed in Table 3 are additional physico-chemical data.

The UV, IR, ^{1}H NMR and ^{13}C NMR spectra for **A1** are reproduced in Figs. 3, 4, 5 and 6, respectively.

Fig. 3. UV spectrum of AT2433-A1 in MeOH.

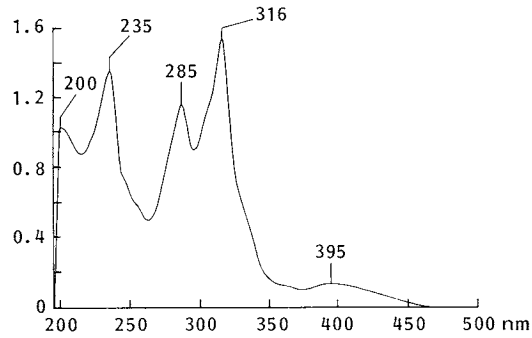


Fig. 4. IR spectrum (KBr) of AT2433-A1.

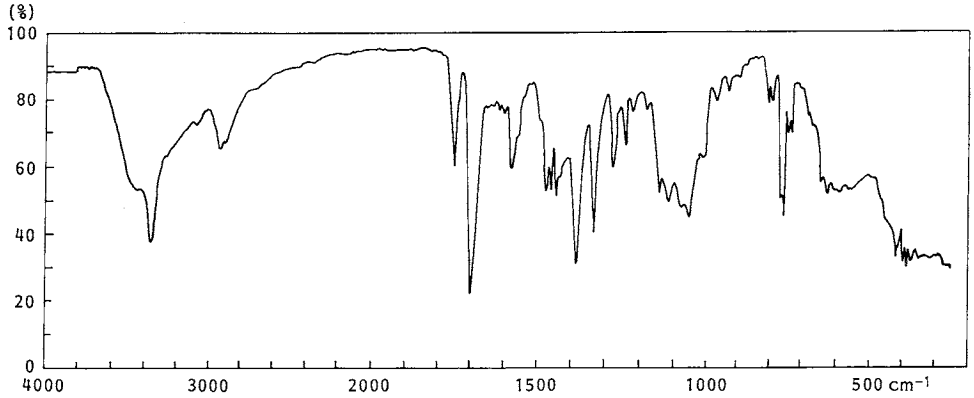


Fig. 5. The ^{1}H NMR spectrum of AT2433-A1 in DMSO.

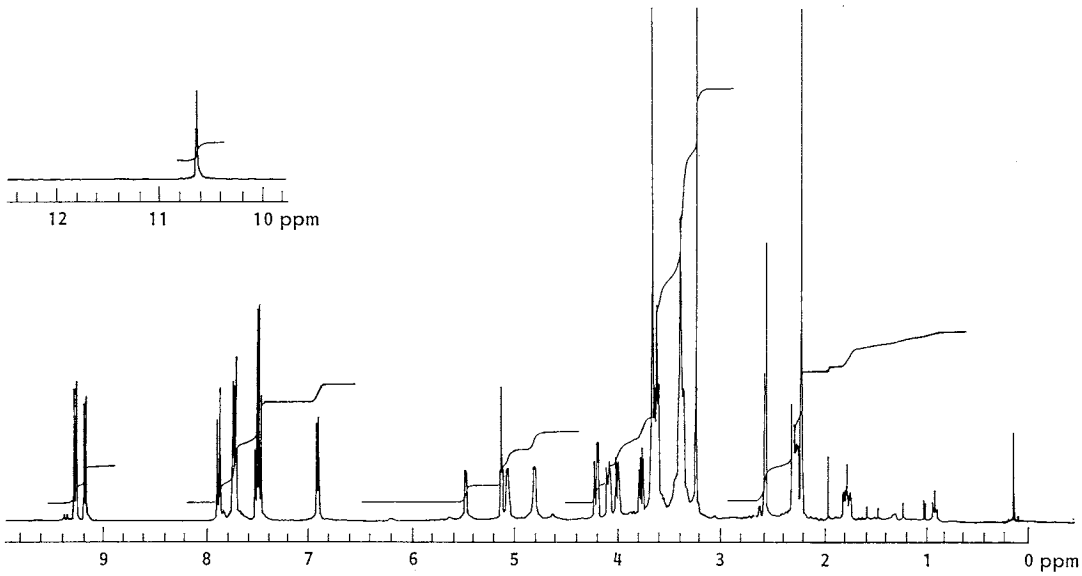


Fig. 6. The ^{13}C NMR spectrum of AT2433-A1 in DMSO.

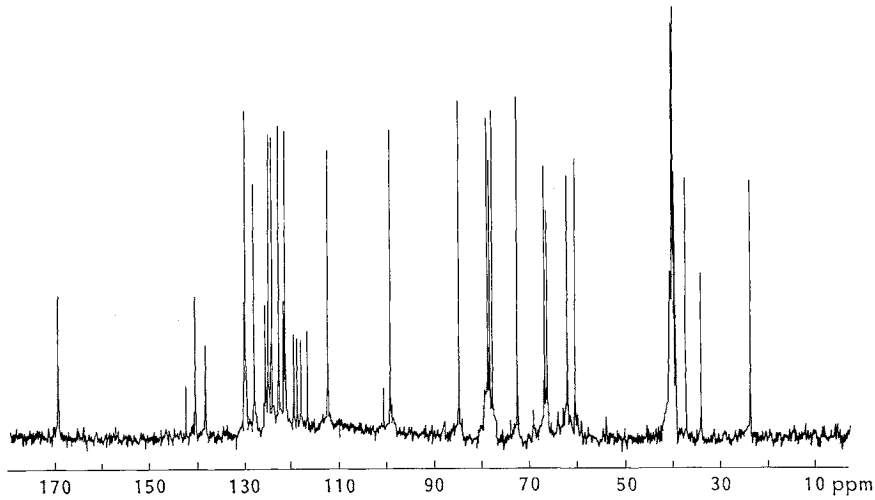


Table 4. Antimicrobial activity of AT2433-A1 (**A1**), -A2 (**A2**), -B1 (**B1**), and -B2 (**B2**).

	MIC (μg/ml)					MIC (μg/ml)			
	A1	**A2**	**B1**	**B2**		**A1**	**A2**	**B1**	**B2**
Staphylococcus aureus A9537	16	16	32	32	*C. albicans* 15049	>32	>16	>16	>16
					C. krusei 15052	>32	>16	>16	>16
Streptococcus faecalis A20688	16	16	32	32	*C. tropicalis* 15051	>32	>16	>16	>16
					C. tropicalis 22493	>32	>16	>16	>16
Escherichia coli A15119	>125	>63	>63	>63	*Trichophyton tonsurans* 22833	>32	>16	>16	>16
Klebsiella pneumoniae A9664	>125	>63	>63	>63	*T. rubrum* 22789	>32	>16	>16	>16
Pseudomonas aeruginosa A9843a	>125	>63	>63	>63	*Microsporum gypseum* 22810	>32	>16	>16	>16
Proteus mirabilis A9900	>125	>63	>63	>63	*M. canis* 9872	>32	>16	>16	>16
Candida albicans A9540	>32	>16	>16	>16	*M. canis* 22494	>32	>16	>16	>16

Table 5. Antimicrobial activity of AT2433-A1 (**A1**), -A2 (**A2**), -B1 (**B1**), and -B2 (**B2**).

	MIC (μg/ml)			
	A1	**A2**	**B1**	**B2**
Micrococcus luteus ATCC 9341	<0.25	1.0	0.25	4.0
Bacillus subtilis ATCC 6633	4.0	8.0	8.0	64.0
Escherichia coli SS1431	64.0	64.0	16.0	64.0
Streptococcus faecium ATCC 9790	32.0	64.0	128.0	256.0
S. faecalis ATCC 29212	16.0	8.0	8.0	8.0

Biological Properties

AT2433-A1, AT2433-A2, AT2433-B1, and AT2433-B2 were tested against a variety of Gram-positive and Gram-negative organism. The results are shown in Tables 4 and 5. The four compounds were active against several Gram-positive organisms: *Micrococcus luteus* (ATCC 9341), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (A9537), *Streptococcus faecalis* (A20688), and *Streptococcus faecium* (ATCC 9790). **B1** was also active against the Gram-negative bacterium *Escherichia coli* SS 1431.

Table 6. Antitumor activity of AT2433-A1 and AT2433-B1 against P388 leukemia.

Compound	Treatment schedule[a]	Dose (ip)	MST[b] (days)	T/C[c] (%)	AWC[d] (g, day-5)	Survivors (day-5)
AT2433-A1	ip, Q03D × 3; 1	16	Toxic	Toxic	−3.2	2/6
		8	17.5	194	−4.1	4/6
		4	14.0	156	−2.3	5/6
		2	15.5	172	−2.2	6/6
		1	13.5	150	−0.7	6/6
		0.5	13.5	150	−0.2	6/6
AT2433-B1	Q03D × 3; 1	16	13.0	144	−0.7	6/6
		8	12.0	133	−0.3	6/6
		4	12.0	133	−0.6	6/6
		2	11.0	122	0.0	6/6
		1	10.5	117	4.0	6/6
		0.5	10.0	111	3.0	5/5
Control		Saline	9.0	100	1.2	10/10

Tumor inoculum: 10^6 ascites cells implanted ip, host: CDF_1 male mice, dose: mg/kg/injection.

[a] RTE, schedule: ip, every third day for 3 injections starting day-1.

[b] Median survival time in days.

[c] (MST days/MST days control) × 100. Criteria: % T/C ≥ 125 considered significant activity.

[d] Average host weight change in g day-5.

AT2433-A1 and AT2433-B1 were evaluated for antitumor activity against transplantable mouse leukemia P388. As shown in Table 6 both materials were effective in life prolongation of mice implanted with this tumor. At the maximum tolerated dose (no day-5 or early deaths due to toxicity) to 2 mg/kg given every third day for 3 injections the increase in life span over controls was 72% (T/C 172%) for **A1**. For **B1** at the highest dose tested (16 mg/kg) it was inhibitory to tumor growth but less effective than **A1**.

Conclusion

AT2433-A1, AT2433-A2, AT2433-B1, and AT2433-B2 are new antitumor antibiotics of the rebeccamycin chemotype. The antitumor data for AT2433-A1 suggests that it is at least 8 times more potent than AT2433-B1 in antitumor effects. This data also suggest that **A1** is most potent and active member of this class of compounds reported to date. Experiments are in progress to establish a preclinical data base for AT2433-A1.

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