BMY-28438† (3,7-DIHYDROXYTROPOLONE), A NEW ANTITUMOR ANTIBIOTIC ACTIVE AGAINST B16 MELANOMA

I. PRODUCTION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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A new antitumor antibiotic BMY-28438 was isolated from the cultured broth of *Streptomyces tropolofaciens* No. K611-97. The antibiotic showed potent cytotoxicity against cultured B16 melanoma cells and remarkable prolongation of life span of mice bearing B16 melanoma. The structure of BMY-28438 was determined as 3,7-dihydroxytropolone on the basis of spectral analyses and direct comparison with the authentic compound synthesized.

In our search for the microbial secondary metabolites having antitumor activity, we found that new actinomycete, *Streptomyces tropolofaciens* sp. nov.[1] No. K611-97 produced a substance with specific inhibitory activity against mouse B16 melanoma. The producing organism was identified to be a new species of genus *Streptomyces* by the taxonomical studies and was named as *S. tropolofaciens*. The active principle, which was designated BMY-28438, was recovered from the fermentation broth by use of porous polymer resin and purified by a series of chromatographies. The physico-chemical data and spectral analysis disclosed that BMY-28438 is 3,7-dihydroxytropolone, and verification of the structure was secured by a direct comparison with an authentic sample synthesized from tropolone. BMY-28438 exhibited weak activity against a variety of Gram-positive and Gram-negative bacteria and fungi, and strong cytotoxicity against B16 melanoma cells. It produced significant prolongation of survival time of mice transplanted with B16 melanoma, but no antitumor activity was observed against P388 leukemia. This report describes production, isolation, physico-chemical and biological properties and structure of BMY-28438.

Antibiotic Production

A loopful of *S. tropolofaciens* No. K611-97, taken from a mature slant culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisted of soybean meal 3%, corn starch 2%, $MgSO_4 \cdot 7H_2O$ 0.33% and $CaCO_3$ 1%, the pH being adjusted to 7.0 before sterilization. The flask was then incubated at 28°C for 4 days on a rotary shaker (200 rpm) and 5 ml of the culture was transferred into 500-ml Erlenmeyer flask containing 100 ml of a fermentation medium having the same composition as the seed medium. The fermentation was carried out at 28°C for 6 days on a rotary shaker. The antibiotic production was monitored by the paper-disc agar diffusion method against *Cryptococcus neoformans* IAM 4514 as the test organism. For a large scale production, the

† This antibiotic was originally designated BU-3129T.

fermentation studies were carried out in the stainless steel fermentors. The seed culture was prepared in twenty 500-ml Erlenmeyer flasks incubated at 32°C for 4 days on a rotary shaker. The resultant culture was transferred into a 200-liter tank fermentor containing 120 liters of a production medium. The seed and production media consisted of soybean meal 3%, glucose 3%, Pharmamedia 0.5%, yeast extract 0.1% and $CaCO_3$ 0.3%. Tank fermentation was carried out at 28°C with stirring at 250 rpm and aeration at 120 liters/minute. The production reached a maximum after 115 hours.

Isolation and Purification

The cultured broth (208 liters, pH 9.06) was separated into the mycelial cake and the supernate with the aid of a continuous centrifuge (Kokusan H-600). The supernate (pH 9.0) was passed through a column of Diaion HP-20 (10 liters) to adsorb some impurities. The passed supernate was adjusted to pH 4.0 with 6 N HCl and applied again on a Diaion HP-20 column (15 liters). After washing with water (20 liters), the column was developed with a mixture of acetone - 0.1 N NH_4OH (1 : 1, 40 liters). The eluate was monitored by *in vitro* cytotoxicity against B16 melanoma cells and paper-disc assay against *C. neoformans* IAM 4514. The active fractions were pooled and evaporated *in vacuo* to afford 370 g of crude solid. A 37-g sample of this solid was charged on a column of Sephadex G-25 (8.0×110 cm, 5 liters) which was developed with water. Evaporation of the active fractions gave a semi-pure sample of BMY-28438 (21 mg). The solid was further chromatographed on Sephadex LH-20 (3.0×62 cm, 100 ml) with methanol elution to yield a nearly homogeneous

Fig. 1. UV spectrum of BMY-28438.

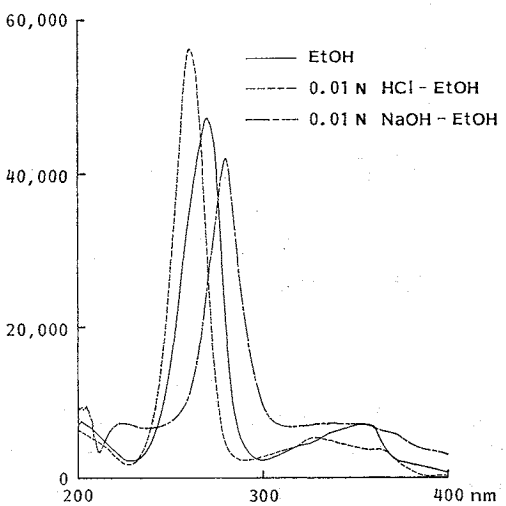
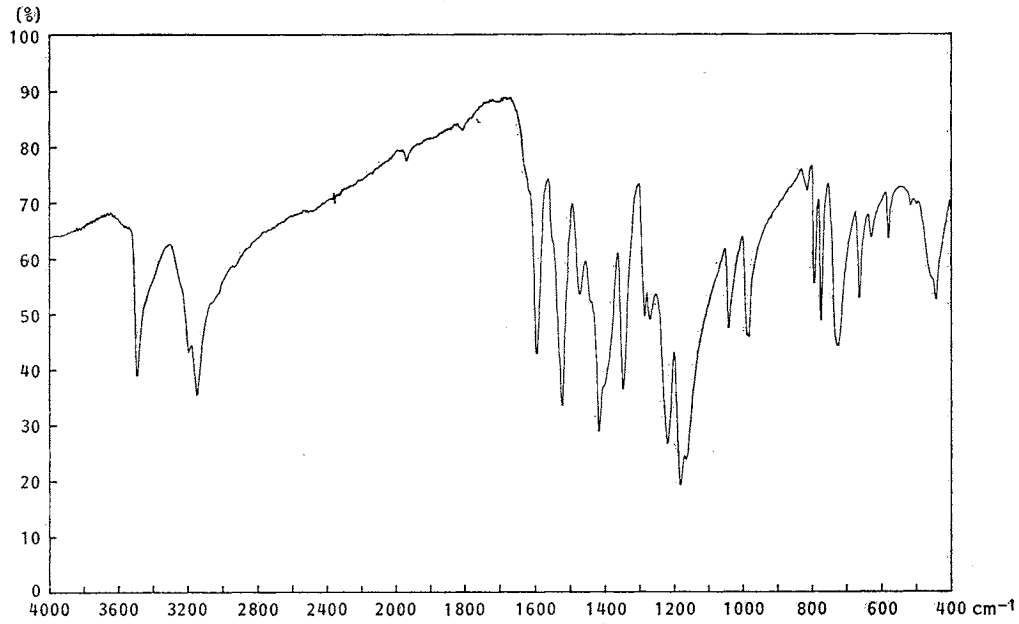


Table 1. Physico-chemical properties of BMY-28438.

Nature:	Pale yellow needles
MP	Sublimes at >166°C
UV λ_{max} nm (ε)	
in EtOH:	273 (46,816), 340 (sh, 6,083), 351 (6,853), 359 (6,699)
in 0.01 N HCl - EtOH:	265 (55,902), 329 (5,313), 366 (3,542)
in 0.01 N NaOH - EtOH:	224 (7,161), 283 (41,657), 340 (7,084), 354 (7,007)
Microanalysis:	
Calcd for $C_7H_6O_4$:	C 54.55, H 3.92.
Found:	C 53.99, H 3.68.
Mass spectrum (HR electron impact (EI)-MS):	
Observed *m/z*	154.0232 (M^+, $C_7H_6O_4$ calcd 154.0265),
	126.0294 (M^+-CO, $C_6H_6O_3$ calcd 126.0317),
	108.0170 (M^+-CO-H_2O, $C_6H_4O_2$ calcd 108.0211),
	80.0273 ($M^+-2CO-H_2O$, C_5H_4O calcd 80.0262)
^1H NMR[a]:	7.02 (br s)
^{13}C NMR[a]:	117.5, 127.1, 156.5, 156.7

[a] δ in ppm (DMSO-d_6).

Fig. 2. IR spectrum of BMY-28438 (KBr).



sample of the antibiotic. Crystallization of the solid from ethanol deposited fine needles of pure BMY-28438 (4.0 mg).

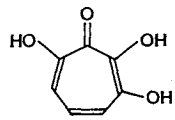
Physico-chemical Properties

BMY-28438 is weakly acidic, and readily soluble in dimethylformamide and dimethyl sulfoxide, slightly soluble in water, methanol and ethanol but practically insoluble in other organic solvents. It showed positive response to ferric chloride and negative to ninhydrin, anthrone and Fehling reaction. BMY-28438 crystals did not exhibit definite mp and gradually sublimed over 170°C. The molecular formula of $C_7H_6O_4$ was assigned for the antibiotic based on its high resolution mass spectral (HR-MS) data (M^+ m/z 154.0232) and microanalysis. The antibiotic exhibited strong UV absorption at 273, 340 (sh), 351 and 359 nm in ethanol, at 265, 329 and 366 nm in acidic ethanol and at 224, 283, 340 and 354 nm in alkaline ethanol (Fig. 1). The physico-chemical data of BMY-28438 are summarized in Table 1, and IR spectrum in KBr pellet is shown in Fig. 2.

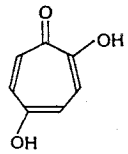
Structural Studies

BMY-28438 was given the molecular formula of $C_7H_6O_4$ by mass spectrum and microanalysis. The HR-MS produced strong fragment ions caused by loss of CO and H_2O from the molecular ion: m/z 126.0294 (M^+-CO), m/z 108.0170 (M^+-CO-H_2O) and m/z 80.0273 ($M^+-2CO-H_2O$). The IR spectrum exhibited strong absorption at 3490, 1590, 1520, 1410, 1210 and 1180 cm^{-1}. The ^1H NMR showed only one signal at 7.02 ppm (br s) and the ^{13}C NMR four carbon signals at 117.5, 127.1, 156.5 and 156.7 ppm indicating a tautomeric structure.

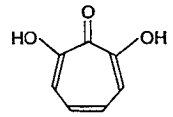
These data combined with its characteristic UV spectrum strongly suggested 3,7-dihydroxytropolone[2] for BMY-28438. In order to confirm the proposed structure, 3,7-dihydroxytropolone was synthesized by persulfate oxidation of tropolone[3]. The reaction mixture was subjected to a Craig counter-current distribution machine (chloroform - toluene - methanol - H_2O, 15 : 15 : 23 : 7) and



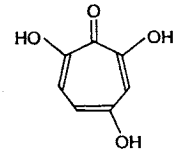
BMY-28438 (3,7-Dihydroxytropolone) (1)



5-Hydroxytropolone



7-Hydroxytropolone



4,7-Dihydroxytropolone

Table 2. Antimicrobial activity of BMY-28438 and reference tropolones by agar dilution method.

Test organisms	MIC (μg/ml)			
	BMY-28438 (3,7-Dihydroxy-tropolone)	4,7-Dihydroxy-tropolone	7-Hydroxy-tropolone	Tropolone
Staphylococcus aureus FDA 209P	25	100	12.5	3.1
S. aureus Smith	25	100	12.5	3.1
S. epidermidis D153	25	100	12.5	1.6
Streptococcus faecalis A9612	50	>100	25	3.1
Micrococcus luteus PCI 1001	25	>100	12.5	50
Bacillus subtilis PCI 219	25	>100	12.5	3.1
Escherichia coli NIHJ	12.5	>100	6.3	50
Klebsiella pneumoniae D11	50	>100	50	50
Pseudomonas aeruginosa D15	50	>100	100	100
Candida albicans IAM 4888	50	>100	100	100
Cryptococcus neoformans D49	25	>100	100	100
Aspergillus fumigatus IAM 2530	100	>100	100	50
Trichophyton mentagrophytes D155	25	>100	100	50

3,7-dihydroxytropolone was separated from coproduced 5-hydroxy, 7-hydroxy and 4,7-dihydroxy analogs. It was further purified by Sephadex LH-20 chromatography to a homogeneous sample. BMY-28438 was identical with 3,7-dihydroxytropolone synthesized in all respects.

Biological Properties

In Vitro Antimicrobial Activity

The MICs of BMY-28438 were determined against various bacteria and fungi by the 2-fold agar dilution method. Tropolone and two tropolone derivatives, 7-hydroxy and 4,7-dihydroxytropolones coproduced in the synthesis of 3,7-dihydroxytropolone were comparatively tested. Nutrient agar medium was used for Gram-positive and Gram-negative bacteria and Sabouraud dextrose agar medium for fungi. As shown in Table 2, BMY-28438 showed very weak inhibitory activity against Gram-positive and Gram-negative bacteria, while tropolone and 7-hydroxytropolone[4] showed much higher activity against Gram-positive bacteria. In contrast to 3,7-dihydroxytropolone, 4,7-dihydroxytropolone did

Table 3. *In vitro* cytotoxicity and inhibition of macromolecule biosynthesis.

Compound	Cytotoxicity vs. B16 (IC_{50}: μg/ml)	Inhibition of macromolecule biosynthesis vs. L1210 (IC_{50}: μg/ml)		
		DNA	RNA	Protein
BMY-28438 (3,7-Dihydroxytropolone)	0.04	9.4	14	11
4,7-Dihydroxytropolone	0.38	39	>100	>100
7-Hydroxytropolone	0.30	21	41	>100
5-Hydroxytropolone	2.4	>100	>100	100
Tropolone	1.8	2.2	20	9.9

Table 4. Antitumor activity against P388 leukemia and B16 melanoma in mice.

Compound	Dose qd 1→9, ip (mg/kg/day)	MST (days)	T/C (%)	Average weight change on day 5 (g)
vs. P388 leukemia				
BMY-28438	2.5	10.0	95	−0.2
	1.3	11.0	105	+0.4
	0.63	10.0	95	+0.4
	0.31	11.0	105	+1.2
	0.16	11.0	105	+0.0
Mitomycin C	1.3	21.0	200	+0.2
	0.63	21.0	200	+0.2
	0.31	16.0	152	+0.8
	0.16	15.0	143	+0.8
	0.08	12.0	114	+1.2
Vehicle	—	10.5	—	+0.7
vs. B16 melanoma: Expt 1				
BMY-28438	5.0	Toxic	—	—
	2.5	12.0	75	−0.8
	1.3	29.0	181	+0.4
	0.63	23.0	144	+1.0
	0.31	18.0	113	+1.2
	0.16	16.0	100	+0.4
Mitomycin C	1.0	27.0	169	+0.6
	0.3	22.0	138	+0.4
	0.1	16.0	100	+1.0
Vehicle	—	16.0	—	+1.3
vs. B16 melanoma: Expt 2				
Tropolone	30	18.0	106	+2.0
	10	18.0	106	+3.3
	3.0	17.5	103	+2.5
	1.0	19.0	112	+2.3
Vehicle	—	17.0	—	+1.9

MST: Median survival time.

not show activity against all test strains. BMY-28438 exhibited significant activity against fungi, whereas other analogs were nearly inactive.

In Vitro Cytotoxicity and Inhibition of Macromolecule Biosynthesis

BMY-28438, tropolone and hydroxytropolones were tested for *in vitro* cytotoxicity against logarithmically growing murine B16-F10 melanoma cells in suspension culture. B16-F10 cells (4×10^4 cells) in the enriched EAGLE's minimum essential medium containing 10% calf serum and the test samples

solution were planted into the wells of 96-well microtiter plates and incubated at 37°C for 72 hours with 5% CO_2 under high humidity condition. After staining with neutral red, cytotoxicity of the test samples was determined colorimetrically. As shown in Table 3, BMY-28438 showed the most potent cytotoxicity followed by 7-hydroxytropolone, 4,7-dihydroxytropolone, tropolone and 5-hydroxytropolone. BMY-28438 was about 8~60 times more cytotoxic than tropolone and the hydroxytropolones. The inhibitory effects on macromolecule biosynthesis were examined in cultured L1210 leukemia cells. L1210 cells (5×10^5 cells) were exposed to radioactive precursors ([*methyl*-^{3}H]thymidine 0.05 μCi/ml, [2-^{14}C]uridine 0.05 μCi/ml and L-[4,5-^{3}H]leucine 0.2 μCi/ml) for 60 minutes after treatment with each test compound for 15 minutes in the above medium. The incorporated radioactivities into the acid-insoluble fraction were measured by using a liquid scintillation counter and the results were shown in Table 3. BMY-28438 showed non-specific inhibitory effects on DNA, RNA and protein biosynthesis with the IC_{50} values of 9 to 14 μg/ml.

In Vivo Antitumor Activity

The antitumor activity of BMY-28438 was determined in male BDF_1 mice against P388 leukemia and B16 melanoma. P388 leukemia and B16 melanoma were inoculated by the intraperitoneal injection at 10^6 cells and 0.5 ml of a 10%-tumor brei per mouse, respectively. Test material was administered to mice intraperitoneally once daily through 1 to 9 days after tumor implantation. Tropolone and mitomycin C were comparatively tested in the experiments. As shown in Table 4, BMY-28438 exhibited significant prolongation of life span in mice bearing B16 melanoma, while no activity was observed against P388 leukemia. Tropolone was inactive against B16 melanoma even at the maximum dose tested.

Discussion

A new actinomycete strain, *S. tropolofaciens* No. K611-97 produced a new antitumor antibiotic BMY-28438. The antibiotic isolated from the fermentation broth was identified as 3,7-dihydroxytropolone by a direct comparison with the authentic sample synthesized. 3,7-Dihydroxytropolone has been synthesized previously[2,3], but this is the first example of the isolation of 3,7-dihydroxytropolone from the natural source.

Several natural and synthetic tropolones have been found to show antitumor activity against P388 murine leukemia[5~9]. However, the antitumor activity of 3,7-dihydroxytropolone has never been reported. It should particularly be noted that BMY-28438 exhibited antitumor activity against B16 melanoma in mice but it was inactive against P388 leukemia. Other hydroxytropolones and tropolone did not exert antitumor effect on B16 melanoma in our test. It is interesting that, unlike other tropolone derivatives, BMY-28438 showed only weak activity against bacteria and fungi, but strong cytotoxicity against B16 melanoma cells. These observations make BMY-28438, 3,7-dihydroxytropolone, distinct in the hydroxytropolone derivatives so far tested and justify further investigation on its antitumor activity and preparation of its analogs.

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