EPOXOMICIN, A NEW ANTITUMOR AGENT OF MICROBIAL ORIGIN

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An actinomycete strain No. Q996-17 produced a novel compound, epoxomicin, which exhibited *in vivo* antitumor activity against B16 melanoma. Structural studies indicated that it is a new member of the epoxy-β-aminoketone group, and is closely related to eponemycin.

In our continuous search for antitumor agents showing specific activity against B16 murine melanoma, an unidentified actinomycete strain No. Q996-17 was found to produce a new compound epoxomicin. It was extracted by *n*-butanol from the fermentation broth and purified by various chromatographies. Structural studies revealed that epoxomicin has an epoxy-β-aminoketone moiety in the structure and is a new member of the eponemycin[1] group. Epoxomicin exhibited strong *in vitro* cytotoxicities against various tumor cell lines, whereas it did not exhibit anti-bacterial and anti-fungal activities. It showed strong *in vivo* inhibitory activity against B16 melanoma but moderate activity against P388 mouse leukemia.

In this report, we describe the production, isolation, physico-chemical characterization, structural elucidation and biological activities of epoxomicin.

Producing Organism

The producing strain No. Q996-17 was isolated from a soil sample collected at Andhra Pradesh State, India.

Strain No. Q996-17 formed non-fragmental substrate mycelium and sparsely rudimental aerial mycelium which turned into a coremium-like with tapered end. Sporulation was not observed at any site. The colony was colorless, yellow or olive brown. Melanoid pigments were not produced. The growth occurs between 19°C and 45°C, and up to at 3% NaCl. The cell chemistry was as follows; cell wall type IIIc, MK-9 (H_4) and MK-10 (H_4) as the major menaquinones, mycolate-less and a glycolate-less. Strain No. Q996-17 probably belongs to *Thermomonospora* and related genera[2], but remains as an unidentified actinomycete.

Antibiotic Production

A well grown agar slant of strain No. Q996-17 was inoculated to a 500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of soluble starch 2%, soybean meal 1% and $CaCO_3$ 0.5% (pH 7.0 before autoclaving). The flask was incubated at 32°C for four days on a rotatory shaker (200 rpm). A two-liter portion of the seed culture was transferred into a 200-liter tank fermenter containing 120 liters of production medium having the same composition as the seed medium. Fermentation was run at 27°C with agitation at 250 rpm and aeration rate of 120 liters per minute.

Epoxomicin was first reported as BU-4061T.

The antibiotic production in the fermentation broth was monitored by the *in vitro* cytotoxic activity against B16-F10 murine melanoma cells. The production reached a maximum after 138 hours of cultivation and the cytotoxic activity reached $\times$ 1,024 dilution (approximately 5 μg/ml) of the broth in terms of minimum effective concentration.

Isolation and Purification

The fermentation broth (45 liters, pH 8.3) was extracted with *n*-butanol (16 liters) under vigorous stirring. The organic layer was concentrated under reduced pressure. The concentrate (1 liter) was then extracted twice with 0.7 liter each of ethyl acetate. The combined ethyl acetate extracts were evaporated under reduced pressure to an oily residue which was added dropwise to 2 liters of *n*-hexane under stirring. The precipitate which deposited was collected by filtration and dried to give a crude solid of epoxomicin (29.9 g). The solid suspended in water (100 ml) was applied on a column of HP-20 (4.0 i.d. $\times$ 70 cm), which was developed with water (1 liter), 30% aqueous methanol (1 liter), 50% aqueous methanol (1 liter) and then 80% aqueous methanol. Eluates were monitored by cytotoxicity against B16-F10 melanoma cells. Active fractions eluted by 80% aqueous methanol were collected, evaporated under reduced pressure and the residue (4.48 g) was chromatographed on a silica gel column eluting with methylene chloride - methanol (98 : 2 v/v). The active fractions were pooled and concentrated *in vacuo* to afford a pale yellow solid (442 mg). This solid was dissolved in small volume of ethyl acetate and charged on a silica gel column (2.2 i.d. $\times$ 50 cm) which was developed with ethyl acetate to give a semi-pure solid. It was further chromatographed on reversed phase silica gel (C-18, 2.2 i.d. $\times$ 30 cm) with 55$\sim$60% aqueous methanol elution to afford nearly a homogeneous solid of epoxomicin (124 mg). Final purification was carried out by Sephadex LH-20 chromatography with methanol elution. Evaporation of relevant fractions gave pure epoxomicin as a white powder (100 mg).

Physico-chemical Properties

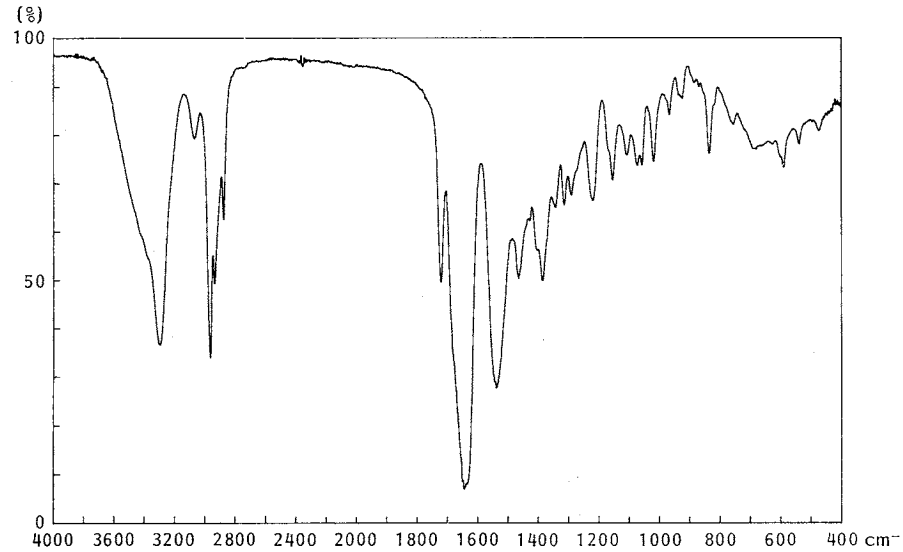
Physico-chemical properties of epoxomicin are summarized in Table 1. It is readily soluble in methanol, methylene chloride and ethyl acetate and practically insoluble in water. Epoxomicin gave positive response to iodine vapor, ammonium molybdate-sulfuric acid solution and Rydon-Smith reagent, but negative response to ninhydrin and anthrone reagent.

Table 1. Physico-chemical properties.

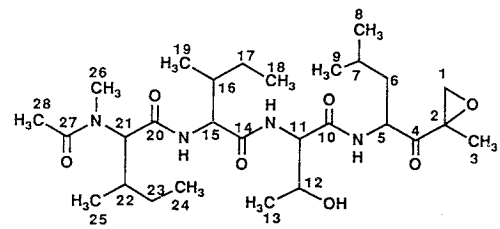
Nature	Colorless powder
Melting point	107$\sim$109°C
$[\alpha]_D^{24.5}$	$-66.1 \pm 0.4°$ (*c* 0.5, MeOH)
UV λ_{max} (MeOH)	End absorption
SI-MS observed *m/z*	577 $(M+Na)^+$, 555 $(M+H)^+$
Microanalysis	Calcd for $C_{28}H_{50}N_4O_7$: C 60.62, H 9.09, N 10.10
	Found: C 60.45, H 9.15, N 10.18
IR ν_{max}^{KBr} (cm^{-1})	3300, 2950, 1720, 1640, 1540
^{13}C-NMR	208.3 (s), 172.1 (s), 171.7 (s), 170.8 (s) 170.6 (s), 66.5 (d),
(100 MHz, $CDCl_3$)	61.5 (d), 59.2 (s), 58.0 (d), 56.4 (d), 52.4 (t), 50.6 (d),
δ ppm (multiplicity)	39.5 (t), 36.2 (d), 32.1 (q), 31.9 (d), 25.1 (d), 24.7 (t), 24.6 (t),
	23.3 (q), 22.1 (q), 21.1 (q), 17.8 (q), 16.8 (q), 15.6 (q), 15.5 (q),
	11.1 (q), 10.5 (q)
Acid hydrolysis	L-Threonine, L-isoleucine, *N*-methylisoleucine
TLC* (Rf)	0.60 (CH_2Cl_2 - MeOH, 9 : 1)
	0.27 (hexane - acetone, 1 : 1)

* Merck Kieselgel 60 F_{254}.

Fig. 1. IR spectrum of epoxomicin.



Its molecular formula was established as $C_{28}H_{50}N_4O_7$ on the basis of microanalysis, ^{13}C NMR and SI-MS (*m/z*: 555 $(M+H)^+$, 577 $(M+Na)^+$).



Epoxomicin (**1**)

It did not exhibit characteristic UV absorption. In the IR spectrum (Fig. 1), epoxomicin showed strong absorption bands at 3300 (OH and/or NH), 1720 (ketone) and 1640 and 1540 cm^{-1} (amide) indicating a peptide structure for epoxomicin. ^{13}C NMR spectrum of epoxomicin demonstrated the presence of 28 carbons including 10 methyl (δ: 10.5, 11.1, 15.5, 15.6, 16.8, 17.8, 21.1, 22.1, 23.3, 32.1), four methylene (δ: 24.6, 24.7, 39.5, 52.4), eight methine (δ: 25.1, 31.9, 36.2, 50.6, 56.4, 58.0, 61.5, 66.5), one quaternary (δ: 59.2) and five carbonyl carbons (δ: 170.6, 170.8, 171.7, 172.1, 208.3).

Structural Studies

The IR (Fig. 1) and ^1H and ^{13}C NMR spectral data (Table 2) of epoxomicin (**1**) suggested a peptide structure.

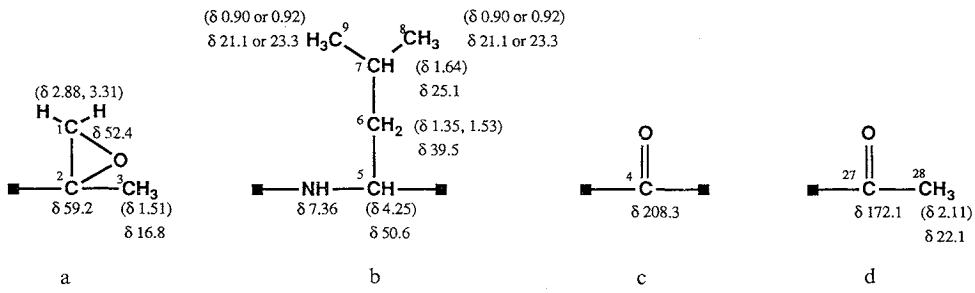
Acid hydrolysis of **1** (200 mg in 10 ml 6 N HCl) gave three ninhydrin positive substances by TLC. To determine these ninhydrin positive substances, the hydrolysate was chromatographed on Dowex 50WX4 (H^+ type, 1.5 i.d. × 20 cm). The column was developed with increasing concentrations of hydrochloric acid. Threonine (**2**, 12 mg) was eluted with 0.06 N hydrochloric acid and a mixture of two ninhydrin positive substances was eluted with 0.3 N HCl. The mixture was separated by Dowex 50WX4 (pyridine type, 2.0 i.d. × 75 cm) column developed with pyridine-formic acid buffer. Elution with 0.1 M pyridine-formic acid buffer (pH 3.1) afforded *N*-methylisoleucine (**3**, 12.0 mg), whose structure was confirmed by ^1H and ^{13}C NMR and SI-MS spectrum (*m/z*: 168 $(M+Na)^+$, 146 $(M+H)^+$). The next ninhydrin positive fraction was eluted by 0.2 M pyridine-formic acid buffer and was identified as isoleucine (**4**, 5.0 mg). The chirality of **2** and **4** was determined to be L configuration using chiral HPLC (TSK gel ENANTIO L1).

Table 2. ^{13}C and ^{1}H NMR of epoxomicin in $CDCl_3$.

Position	^{1}H NMR δ (multiplicity, J=Hz)	^{13}C NMR δ (multiplicity)	Position	^{1}H NMR δ (multiplicity, J=Hz)	^{13}C NMR δ (multiplicity)
1	2.88 (d, 5.1)	52.4 (t)	15	4.26 (m)	58.0 (d)
	3.31 (d, 5.1)		15-NH	7.28 (d, 8.1)	—
2	—	59.2 (s)	16	1.96 (m)	36.2 (d)
3	1.51 (s)	16.8 (q)	17	0.84 (m)[b]	24.7 (t)[b]
4	—	208.3 (s)		0.95 (m)[b]	
5	4.52 (m)	50.6 (d)	18	0.86 (m)[c]	15.5 (q)[c]
5-NH	7.36 (d, 7.7)	—	19	0.84 (m)[d]	11.1 (q)[d]
6	1.35 (m)	39.5 (t)	20	—	170.6 (s)
	1.53 (m)		21	4.68 (d, 11.4)	61.5 (d)
7	1.64 (m)	25.1 (d)	22	2.10 (m)	31.9 (d)
8	0.92 (d, 6.5)[a]	23.3 (q)[a]	23	1.11 (m)[b]	24.6 (t)
9	0.90 (d, 6.5)[a]	21.1 (q)[a]		1.35 (m)[b]	
10	—	170.8 (s)	24	0.83 (m)[c]	15.6 (q)[c]
11	4.45 (dd, 2.9, 7.7)	56.4 (d)	25	0.85 (m)[d]	10.5 (q)[d]
11-NH	6.93 (d, 7.7)		26	2.98 (s)	32.1 (q)
12	4.24 (dq, 2.9, 6.5)	66.5 (d)	27	—	172.1 (s)
13	1.10 (d, 6.5)	17.8 (q)	28	2.11 (s)	22.1 (q)
14	—	171.7 (s)			

[a~d] May be interchanged.

Fig. 2. Partial structures of epoxomicin.



Analysis of ^{1}H-^{1}H and ^{1}H-^{13}C COSY spectra of **1**, confirmed the presence of these three amino acids (**2**, **3** and **4**), and in addition, showed following four structural fragments (a to d) as shown in Fig. 2. The connectivity of the fragments was elucidated by analyzing the ^{13}C-^{1}H long range COSY spectrum. The ketone carbon (fragment c) displayed contours with methyl protons of fragment a. In fragment a, C-3 methyl carbon showed contours with C-1 methylene protons and C-1 methylene carbon with C-3 methyl protons. The presence of epoxy ring in the fragment a was supported by measuring $J_{C\text{-}H}$ of C-1 methylene (δ_C 52.4 and δ_H 2.88 and 3.31). The observed $J_{C\text{-}H}$ (180 Hz) was characteristic to that for epoxy ring. Thus the structure of unknown unit of epoxomicin was clarified to be 1,2-epoxy-2-methyl-4-amino-6-methyl-heptane-3-one. This fragment could not be obtained by acid hydrolysis of **1**. The carbonyl carbon in fragment d displayed contours with *N*-methyl protons of the *N*-methylisoleucine fragment, establishing *N*-acetyl-*N*-methylisoleucine terminal of **1**.

The ^{13}C-^{1}H long range COSY spectrum also showed the linkage of three amino acids and epoxy-β-amino-ketone fragment. The amide carbons exhibited contours to NH protons of their adjacent amino acid, leading to the total structure of epoxomicin (Fig. 3). The sequence of these fragments was

Fig. 3. ^{13}C-^{1}H long range COSY and MS of epoxomicin.

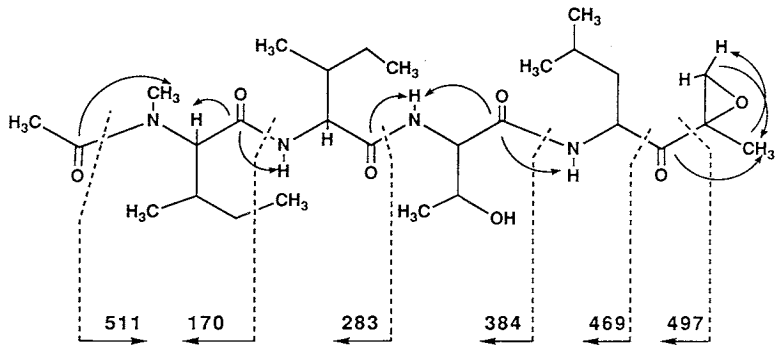


Table 3. *In vitro* cytotoxicity.

Test cell		IC_{50} (μg/ml)	
		Epoxomicin	Eponemycin
Mouse melanoma	B16-F10[a]	0.002	0.002
Human colon carcinoma	HCT-116[b]	0.005	0.010
Human colorectal carcinoma	Moser[a]	0.044	0.012
Mouse leukemia	P388[c]	0.002	0.005
Human myelogenous leukemia	K562[c]	0.037	0.061

Medium
[a] EAGLE's minimum essential medium + 10% FCS.
[b] McCOY's 5A medium + 10% FCS.
[c] RPMI1640 medium + 10% FCS.

further confirmed by EI-MS spectrum. The spectrum exhibited ion peaks (*m/z*: 170, 283, 384, 469, 497 and 511) which are derived by rupture between CO–NH or CO–C bonds as shown in Fig. 3. Thus the total structure of epoxomicin was elucidated as **1**.

Biological Activities

Epoxomicin did not demonstrate antibacterial or antifungal activities by paper disc-agar diffusion assay at the concentration of 1 mg/ml.

Epoxomicin was tested for its *in vitro* cytotoxicities and *in vivo* antitumor activities by the method described in the preceding paper[1]. Eponemycin and/or mitomycin C were tested comparatively as reference compounds. For *in vitro* test the cells were harvested in their logarithmic phase, implanted to 96-well microtiter plates and incubated in the media as shown in Table 3 with 10% fetal calf serum (FCS). The test cells used were three solid type tumor cells (B16-F10, HCT116 and Moser) and two leukemia cells (P388 and K562). Epoxomicin showed quite potent cytotoxicities against all of the cells tested showing IC_{50} values of 0.002 to 0.044 μg/ml (Table 3). The cytotoxicities of epoxomicin against solid tumor cells and leukemia cells were nearly equal to each other. Epoxomicin and eponemycin showed almost equal cytotoxicities.

The *in vivo* antitumor activity was determined in tumor-bearing mice. Male BDF_1 mice were intraperitoneally inoculated with 0.5 ml of 10% melanotic melanoma B16 brei and female CDF_1 mice were intraperitoneally inoculated with 0.4 ml of diluted ascitic fluid containing 10^6 lymphocytic leukemia

Table 4. Antitumor activity against B16 melanoma (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST[a] (days)	T/C[b] (%)	Average weight change on day 5 (g)
Epoxomicin	1.0	Q1D×9	9.0	Toxic	−3.8
	0.5	Q1D×9	21.0	140	−1.0
	0.25	Q1D×9	20.5	137	−0.2
	0.13	Q1D×9	19.5	130	+0.8
	0.063	Q1D×9	17.5	117	+1.3
Eponemycin	1.0	Q1D×9	6.5	Toxic	−3.8
	0.5	Q1D×9	26.0	173	−2.5
	0.25	Q1D×9	23.5	157	+0.5
	0.13	Q1D×9	19.0	127	+1.5
	0.063	Q1D×9	17.5	117	+1.3
Mitomycin C	1.0	Q1D×9	30.5	203	−0.5
	0.3	Q1D×9	23.5	157	0.0
	0.1	Q1D×9	16.5	110	+1.8
Vehicle[c]	—	Q1D×9	15.0	—	+0.5

[a] Median survival time.
[b] T/C (MST of treatment mice/MST of control mice × 100) ≥ 125% is considered significant antitumor effect.
[c] Physiological saline containing 10% DMSO.

Table 5. Antitumor activity against P388 leukemia (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST[a] (day)	T/C[b] (%)	Body weight change on day 4 (g)
Epoxomicin	1.0	Q1D×9	10.5	105	−1.3
	0.5	Q1D×9	13.0	130	−1.5
	0.25	Q1D×9	11.5	115	−0.3
	0.13	Q1D×9	11.0	110	−0.5
	0.063	Q1D×9	10.5	105	+0.3
Mitomycin C	1.0	Q1D×9	17.0	170	−0.8
	0.5	Q1D×9	15.5	155	0.0
	0.25	Q1D×9	13.0	130	+1.0
	0.13	Q1D×9	12.0	120	+1.3
	0.063	Q1D×9	11.0	110	+0.8
Vehicle[c]	—	Q1D×9	10.0	—	+0.8

[a] Median survival time.
[b] T/C (MST of treatment mice/MST of control mice × 100) ≥ 125% is considered significant antitumor effect.
[c] Physiological saline containing 10% DMSO.

P388 cells, respectively. As shown in Table 4, epoxomicin exhibited strong therapeutic activity against B16 melanoma when administered intraperitoneally once daily for nine days (Q1D×9). It showed significant antitumor effect with the minimum effective dose of 0.13 mg/kg/day which was equivalent to that of eponemycin. However, in terms of maximum T/C %, eponemycin showed superior activity (173% at 0.5 mg/kg/day) to epoxomicin (140% at 0.5 mg/kg/day). Epoxomicin showed moderate *in vivo* activity against P388 leukemia (T/C 130% at 0.5 mg/kg/day) by Q1D×9 treatment schedule as shown in Table 5.

Discussion

Our fermentation screening directed to the slow growing solid tumors using murine B16 melanoma

cells resulted in the discovery of 3,7-dihydroxytropolone[3] and eponemycin[1] followed by epoxomicin. Epoxomicin is the second compound reported so far containing an epoxy-β-amino-ketone moiety. Epoxomicin has a tripeptide side chain but eponemycin has an isooctanoyl-L-seryl side chain attached to epoxy-β-amino-ketone moiety. This difference in side chain may cause the difference in chemotherapeutic effect of the two agents against B16 melanoma. It is interesting to note that both compounds showed superior *in vivo* antitumor activities against solid tumor B16 melanoma, but moderate to no *in vivo* activity against P388 leukemia, although they showed nearly equal *in vitro* cytotoxicities against B16-F10 and P388 cells.

OIKAWA *et al.* reported the strong angiogenic inhibitory activity of eponemycin[4]. Angiogenesis is associated with tumor development, and this inhibitory activity may support the different *in vivo* activity of eponemycin against B16 and P388. The angiogenic activity of epoxomicin has not yet been tested but it is likely to have angiogenic activity from its structural and activity profile similarity to those of eponemycin.

Their mechanisms of action and structure-activity relationship studies are underway and will lead to new therapeutic agents specifically effective against solid tumors.

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References

1) SUGAWARA, K.; M. HATORI, Y. NISHIYAMA, K. TOMITA, H. KAMEI, M. KONISHI & T. OKI: Eponemycin, a new antibiotic active against B16 melanoma. I. Production, isolation, structure and biological activity. J. Antibiotics 43: 8~18, 1990
2) MCMARTHY, A. J.: Thermomonospora and related genera. *In* BERGEY's Manual of Systematic Bacteriology. Volume 4. *Ed.*, S. T. WILLIAMS *et al.*, pp. 2552~2572, Williams & Wilkins Co., 1989
3) SUGAWARA, K.; M. OHBAYASHI, K. SHIMIZU, M. HATORI, H. KAMEI, M. KONISHI, T. OKI & H. KAWAGUCHI: BMY-28438 (3,7-dihydroxytropolone), a new antitumor antibiotic active against B16 melanoma. I. Production, isolation, structure and biological activity. J. Antibiotics 41: 862~868, 1988
4) OIKAWA, T.; M. HASEGAWA, M. SHIMAMURA, H. ASHINO, S. MUROTA & I. MORITA: Eponemycin, a novel antibiotic, is a highly powerful angiogenesis inhibitor. Biochem. Biophys. Res. Commun. 181: 1070~1076, 1991