VERUCOPEPTIN†, A NEW ANTITUMOR ANTIBIOTIC ACTIVE AGAINST B16 MELANOMA

I. TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

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(Received for publication January 28, 1993)

A new antitumor antibiotic verucopeptin was isolated from the culture broth of *Actinomadura verrucosospora* Q886-2. It showed potent cytotoxicity and specific *in vivo* activity against B16 melanoma. Maximum T/C value (162%) was obtained by Q1D × 1 treatment schedule.

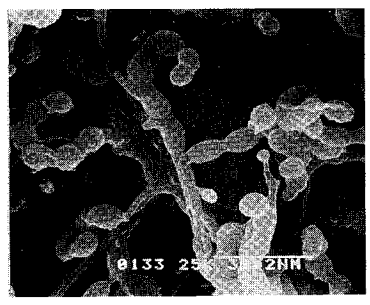
In our systematic search for the microbial metabolite with antitumor activity, *Actinomadura verrucosospora* Q886-2 isolated from a soil sample of the Philippines was found to produce a new antibiotic which showed specific activity against B16 melanoma. The antibiotic designated verucopeptin was recovered from the fermentation broth with butanol and purified by a series of chromatographies. Verucopeptin demonstrated potent cytotoxicity against various tumor cells but weak antimicrobial activity. It exhibited significant prolongation of life-span in mice transplanted with B16 melanoma. This paper describes the taxonomy, production, isolation, physico-chemical properties and biological activity of verucopeptin.

Taxonomy

Strain Q886-2 was isolated from a soil sample collected in Batangas, Luzon Island, the Philippines. Substrate mycelium was well-branched and non-fragmentary. Aerial mycelium was poorly formed, and bore monopodially hook-shaped spore chains which contained 3 to 10 spores per chain. The spores were oval ($0.6 \sim 1.0 \times 1.0 \sim 1.5\,\mu m$) and had smooth or warty surface (Fig. 1).

Fig. 1. Spore chains of strain Q886-2 on ISP medium No. 9 supplemented with fructose.

Bar represents $2\,\mu m$.



The cultural and physiological characteristics[1] of strain Q886-2 are shown in Tables 1 and 2, respectively. The growth was moderate on most descriptive media including ISP media No. 2 to No. 7[2]. The color of aerial mycelia was uniformly white and none of the mycelial or diffusible pigments was produced. Growth temperature ranged from 18 to 50°C.

The whole-cell hydrolsate contained *meso*-diaminopimelic acid, glucose and madurose[3]. The

† Originally called BU-3983T or BMY-28782.

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Table 1. Cultural characteristics of strain Q886-2.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Poor	None	Colorless	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate floccose, not turbid	None	Colorless	None
Yeast extract-malt extract agar (ISP No. 2)	Moderate	Moderate; white (263)	Colorless	None
Oatmeal agar (ISP No. 3)	Poor	Poor; white (263)	Colorless	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	Poor; white (263)	Pale yellow (89)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	Very scant; white (263)	Yellowish white (92)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Good	None	Pale yellow (89)	None
Tyrosine agar (ISP No. 7)	Moderate	Very scant; white (263)	Yellowish white (92)	None
Glucose-asparagine agar	Poor	None	Colorless	None

Observation after incubation at 28°C for 3 weeks.
Color name used: ISCC-NBS Color-Name Charts.

Table 2. Physiological characteristics of strain Q886-2.

Decomposition of:	
Adenine	−
Casein	+
Huppric acid	+
Hypoxanthine	+
Tyrosine	+
Xanthine	−
Decarboxylation of:	
Benzoate	−
Citrate	−
Mucate	−
Succinate	−
Tartrate	−
Production of:	
Amylase	+
Esculinase	+
Gelatinase	+
Nitrate reductase	+
Tyrosinase	−
Urease	−
Growth in:	
Lysozyme 0.01%	−
NaCl 1~7%	+
NaCl 9%	−
pH 5.0~12.0	+
18~50°C	+
16 and 53°C	−

Utilization of and acid production from:	Utilization	Acid
Adonitol	NT	+
D-(−)-Arabinose	−	−
L-(+)-Arabinose	+	+
Cellobiose	+	+
Cellulose	−	−
Dulcitol	−	−
Erythritol	NT	−
D-Fructose	+	NT
D-Galactose	+	NT
D-Glucose	+	+
Glycerol	+	+
Inositol	−	−
Lactose	−	−
D-Mannitol	+	+
D-Mannose	±	−
D-(+)-Melezitose	−	−
Melibiose	−	−
Methyl-α-glucoside	NT	−
Raffinose	−	−
L-Rhamnose	+	+
D-Ribose	−	NT
Salicine	+ (w)	NT
Soluble starch	+	NT
D-Sorbitol	−	−
L-Sorbose	−	−
Sucrose	+ (w)	NT
Trehalose	+	+
D-Xylose	+ (w)	+

+ (w), weakly positive; ±, marginal; NT, not tested.

phospholipids contained phosphatidylinositol but not nitrogenous phospholipids[4]. Therefore, strain Q886-2 belonged to cell wall type III_B and phospholipid pattern PI. The menaquinones contained 51% of MK-9(H_6), 34% of MK-9(H_8), 10% of MK-9(H_4) and 5% of MK-9(H_{10})[5,6].

Based on the taxonomic properties described above, strain Q886-2 was assigned to the genus *Actinomadura* Lechevalier and Lechevalier 1970[7]. Subsequent comparison of the strain with the *Actinomadura* species[2,7,8] indicated that it could be closely related to *Actinomadura madurae* or *A. verrucosospora*. Strain Q886-2 was further similar to the latter species in the absence of melanoid pigment formation and the lack of inositol and mannose utilization, although it was different in the production of nitrate reductase, the resistance to 7% NaCl and the growth at 47°C. Therefore, strain Q886-2 was identified as a new strain of *Actinomadura verrucosospora*.

Fermentation

A loopful spores from a well sporulated slant culture of *A. verrucosospora* Q886-2 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the vegetative medium consisting of glycerol 2%, soybean meal 1% and $CaCO_3$ 0.5%, the pH adjusted to 7.0 before sterilization. The flask was shaken at 32°C for 4 days on a rotary shaker (200 rpm) and 5 ml of the culture was transferred to an Erlenmeyer flask containing 100 ml of the same medium as described above. The fermentation was carried out at 28°C for 6 days on a rotary shaker. The scale-up fermentation was also carried out in a 20-liter stir jar. A 500 ml of the vegetative culture was inoculated into the jar containing 12 liters of the fermentation medium. The jar fermenter was operated at 28°C with agitation of 250 rpm and aeration rate of 12 liters/minute. The antibiotic production was monitored by *in vitro* cytotoxicity against B16 melanoma cells, and it reached maximum of 20 μg/ml after 90 hours fermentation.

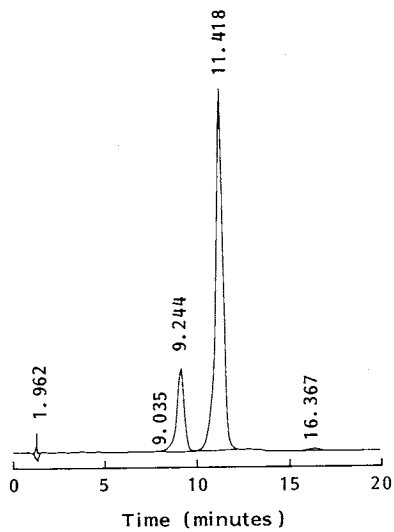
Isolation and Purification

The fermentation broth (26 liters, pH 7.3) was stirred with butanol (15 liters) for 1 hour. The organic

Table 3. Physico-chemical properties of verucopeptin.

Nature	White amorphous powder
MP	155~158°C
$[\alpha]_D^{26}$	$-36.6° \pm 0.1$ (*c* 1.0, MeOH)
Molecular formula	$C_{43}H_{73}N_7O_{13}$
FAB-MS Positive *m/z*	918 $(M+Na)^+$, 878 $(M+H-H_2O)^+$, 846
Negative *m/z*	894 $(M-H)^-$
HRFAB-MS	
Found:	*m/z* 894.5217 $(M-H)^-$
Calcd for $C_{43}H_{72}N_7O_{13}$:	894.5188
Elemental analysis	
Found:	C 56.74, H 8.31, N 10.78
Calcd for $C_{43}H_{73}N_7O_{13}\cdot H_2O$:	C 56.50, H 8.27, N 10.73
TLC	CH_2Cl_2-MeOH (9:1)
(SiO_2, Merck F_{254})	Rf 0.42, 0.54^{trace}
	n-BuOH-AcOH-H_2O (3:1:1)
	Rf 0.40, 0.53^{trace}
HPLC	Rt 9.2 minutes (18%), 11.4 minutes (82%)

Fig. 2. HPLC profile of verucopeptin.



Conditions: Column, M & S pack C18 (M & S Instruments); solvent, CH_3CN-H_2O (75:25); flow rate, 1 ml/minute; detection, UV 215 nm.

layer was separated by a Sharples type centrifuge, and concentrated to dryness under reduced pressure. The residue (54 g) was applied on a column of silica gel (Wako gel C-200, 4.0 i.d. × 50 cm), and developed with methylene chloride-methanol (100:0~90:10, v/v). The fractions were monitored by cytotoxicity against B16 and color reaction with ammonium molybdate-sulfuric acid on TLC plate. Active eluates were combined, concentrated *in vacuo* and charged again on a column of silica gel (3.5 i.d. × 50 cm). Elution was performed by ethyl acetate-methanol (50:1) and the pooled active eluate were concentrated. The residue was further purified by Sephadex LH-20 chromatography with methanol elution. Active fractions were combined, evaporated *in vacuo* and lyophilized to yield a pure solid of verucopeptin (518 mg).

Physico-chemical Properties

Physico-chemical properties of verucopeptin are summarized in Table 3. The pure compound showed two interchangeable spots on TLC and also interchangeable peaks in HPLC analysis as shown in Fig. 2,

Fig. 3. IR spectrum of verucopeptin.

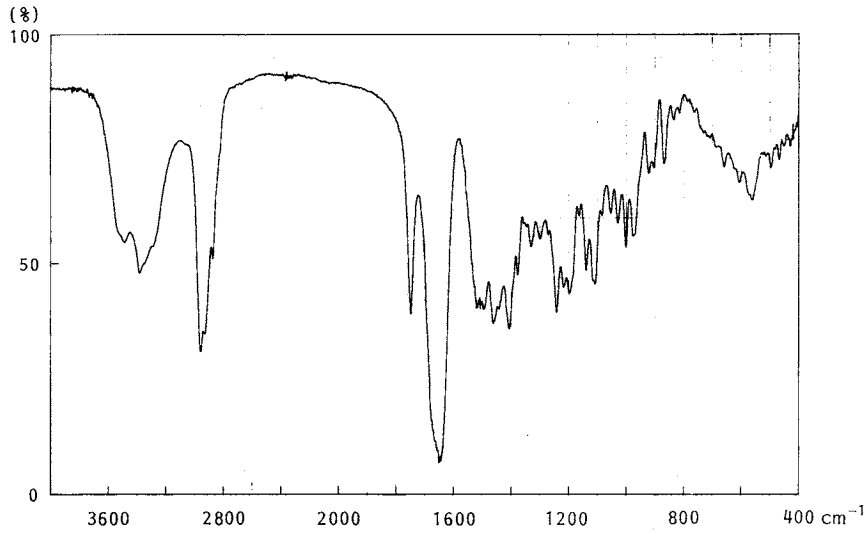
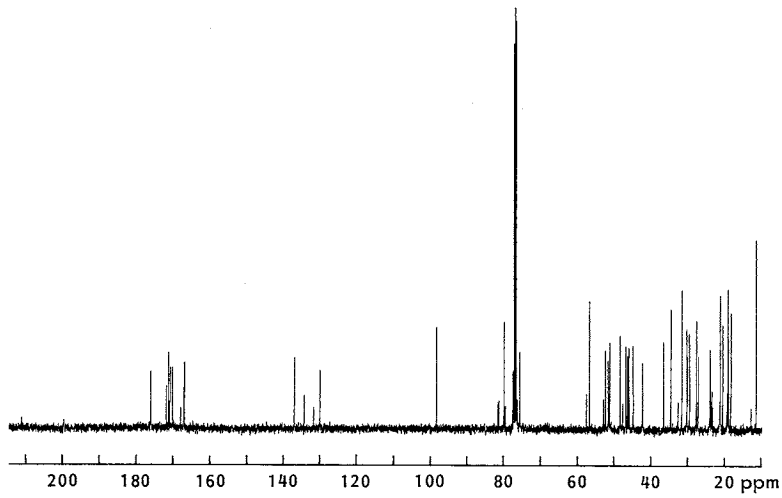


Fig. 4. ^{13}C NMR spectrum of verucopeptin (100 MHz, in $CDCl_3$).



which indicated that verucopeptin existed as a tautomeric mixture in solution. It is readily soluble in acetonitrile, methanol, dimethyl sulfoxide and dimethylformamide, slightly soluble in ethanol, ethyl acetate and chloroform, but practically insoluble in *n*-hexane and water. Color reactions are as follows: positive response to iodine vapor and ammonium molybdate-sulfuric acid and negative to ninhydrin, anthrone and Sakaguchi reagents. The molecular formula was assigned as $C_{43}H_{73}N_7O_{13}$ based on the HRFAB-MS (*m/z* 894.5217 $(M-H)^-$) and elemental analysis. Verucopeptin showed end absorption in the UV spectrum. The IR spectrum in KBr is shown in Fig. 3. The ^1H and ^{13}C NMR (Fig. 4) spectra of verucopeptin gave broad double peak signals possibly due to tautomerization. Structural studies provided a novel hexadepsipeptide structure for this compound. Detail of the structure determination is reported in the companion paper[9].

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of verucopeptin was determined against various bacteria and fungi by the serial two-fold agar dilution method. Nutrient agar (Eiken) was used for aerobic bacteria, GAM (Nissui) for anaerobic bacteria and Sabouraud dextrose agar (Difco) for

Table 4. *In vitro* cytotoxicity against murine and human tumor cells.

Compound	IC_{50} (μg/ml)			
	B16-F10[a]	P388[b]	Moser[a]	HCT-116[c]
Verucopeptin	0.004	0.08	0.4	0.04

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

Table 5. Antitumor activity of verucopeptin against B16 melanoma (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST[a] (day)	T/C[b] (%)	Body weight change on day 5 (g)
Verucopeptin	8	Q1D × 1	17.5	135	−5.3
	4	Q1D × 1	19.0	146	−4.3
	2	Q1D × 1	21.0	162	−4.8
	1	Q1D × 1	16.5	127	−4.0
	0.5	Q1D × 1	17.0	131	−1.0
	0.25	Q1D × 1	17.0	131	0.0
	0.13	Q1D × 1	14.0	108	+0.5
	2	Q4D × 3	18.0	138	−4.0
	1	Q4D × 3	17.5	135	−2.3
	0.5	Q4D × 3	19.0	146	−0.5
	0.25	Q4D × 3	16.0	123	0.0
	0.13	Q4D × 3	15.0	115	+0.0
	0.063	Q4D × 3	14.0	108	+0.5
Mitomycin C	2	Q4D × 3	27.5	212	+0.5
	1	Q4D × 3	22.0	169	+0.8
	0.5	Q4D × 3	18.0	138	+0.6
	0.25	Q4D × 3	15.5	119	+0.8
Vehicle	—	Q4D × 3	13.0	—	+0.8
Verucopeptin	1	Q1D × 9	Tox	Tox	—
	0.5	Q1D × 9	20.0	148	−4.0
	0.25	Q1D × 9	19.5	138	−1.0
	0.13	Q1D × 9	19.0	131	−0.3
	0.063	Q1D × 9	17.5	121	+1.0
	0.031	Q1D × 9	16.0	110	+1.0
Vehicle	—	Q1D × 9	14.5	—	+0.5

[a] Median survival time.
[b] Significant antitumor effect (T/C ≥ 125%).

fungi. Verucopeptin showed marginal inhibitory activity against *Streptococcus faecalis* and *Bacillus subtilis* with MIC value of 100 μg/ml but no activity against other Gram-positive, Gram-negative and anaerobic bacteria and fungi at 100 μg/ml.

Antitumor Activity

Verucopeptin was tested for *in vitro* cytotoxicity and inhibition of macromolecule biosynthesis by the method described in the preceding paper[10]. B16-F10 (murine melanoma), P388 (murine lymphocytic leukemia), Moser (human colorectal carcinoma) and HCT-116 (human colon carcinoma) cells were grown to the logarithmic phase in the media shown in Table 4. The cytotoxicity against tumor cells was determined colorimetrically after staining viable cells with neutral red (B16-F10, Moser and HCT-116 cells, at 540 nm) or XTT (P388 cells, at 450 nm). Verucopeptin showed relatively specific cytotoxicity against B16-F10 cells with IC_{50} value of 0.004 μg/ml, which was 20, 100 and 10 times more potent than those against P388, Moser and HCT-116 cells, respectively (Table 4).

The inhibitory effects of verucopeptin on macromolecule (DNA, RNA and protein) biosynthesis were determined in cultured B16-F10 melanoma cells. Labeled precursor, ^{3}H-thymidine, ^{14}C-uridine or ^{3}H-leucine was added to the cultured cells with test materials and incubated. After washing with chilled 5% trichloroacetic acid solution, the radioactivity incorporated into the acid-insoluble fraction of the tumor cells was determined by a liquid scientillation counter. Verucopeptin inhibited DNA and RNA

Table 6. Antitumor activity of verucopeptin against P388 and L1210 leukemias (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST[a] (day)	T/C[b] (%)	Body weight change on day 4 (g)
P388 leukemia					
Verucopeptin	8	Q1D×1	11.5	115	−2.5
	4	Q1D×1	11.0	110	−2.3
	2	Q1D×1	10.0	100	−2.0
	1	Q1D×1	10.0	100	−1.5
	0.5	Q1D×1	10.0	100	−0.5
	0.25	Q1D×1	11.0	110	+0.3
Mitomycin C	4	Q1D×1	16.5	165	−0.8
	2	Q1D×1	15.5	155	+0.5
	1	Q1D×1	14.0	140	+2.0
	0.5	Q1D×1	13.0	130	+1.5
	0.25	Q1D×1	11.5	115	+1.0
Vehicle	—	Q1D×1	10.0	—	+1.0
L1210 leukemia					
Verucopeptin	8	Q1D×1	Tox	Tox	—
	4	Q1D×1	9.5	119	−2.5
	2	Q1D×1	9.0	113	−2.0
	1	Q1D×1	8.0	100	−2.0
	0.5	Q1D×1	8.0	100	−0.5
	0.25	Q1D×1	8.0	100	+0.8
Mitomycin C	8	Q1D×1	12.5	156	−0.3
	4	Q1D×1	11.0	138	+0.5
	2	Q1D×1	10.5	131	+1.0
	1	Q1D×1	10.0	125	+1.5
	0.5	Q1D×1	10.0	125	+1.5
Vehicle	—	Q1D×1	8.0	—	+1.6

[a] Median survival time.
[b] Significant antitumor effect (T/C≥125%).

syntheses with IC_{50} values of 0.26 and 0.29 μg/ml, respectively, whereas it showed only weak inhibition of protein synthesis at 1.0 μg/ml.

In vivo antitumor activity was tested in the experimental mouse tumor systems. Male BDF_1 mice were intraperitoneally inoculated with 0.5 ml of 10% melanotic melanoma brei, and female CDF_1 mice were intraperitoneally inoculated with 0.4 ml of diluted ascitic fluid containing 10^6 P388 cells. Mitomycin C was used as a reference compound. Test compounds were intraperitoneally administered to the mice by the following treatment schedules: once day 1 only (Q1D × 1), once a day on days 1, 5 and 9 (Q4D × 3) or on days 1 to 9 consecutively (Q1D × 9). Verucopeptin showed significant therapeutic activity against B16 melanoma with maximum T/C of 146~162% by various treatment schedules (Table 5). It exhibited the best chemotherapeutic activity by Q1D × 1 treatment schedule. The compound showed no significant prolongation of life-span in P388 leukemia system (Table 6).

Discussion

Our antitumor screening directed to solid tumors has discovered new antibiotics 3,7-dihydroxytropolone[11], eponemycin[10] and epoxomicin[12]. Verucopeptin is another new antibiotic which showed specific antitumor activity against B16 melanoma. It demonstrated the highest T/C value and the widest active dose-range the range between minimum and maximum effective doses, among these antibiotics against B16 melanoma in mice. Chemical studies provided a new 19-membered cyclodepsipeptide structure for verucopeptin.

The specific activity and a novel structure of this antibiotic may give us a possibility to develop unique therapeutic agents effective against human solid tumors.

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