MADUROPEPTIN†, A COMPLEX OF NEW MACROMOLECULAR ANTITUMOR ANTIBIOTICS

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Maduropeptin, a complex of new macromolecular antitumor antibiotics, is a metabolite of *Actinomadura madurae* H710-49. The active components maduropeptins A_1, A_2 and B are acidic chromopeptides with MW of around 22,500 and composed of 14 types of amino acids and an unstable chromophore. The antibiotics are active *in vitro* against Gram-positive bacteria and highly cytotoxic to tumor cells. They produced significant prolongation of survival time of mice implanted with P388 leukemia and B16 melanoma.

In the course of screening for novel metabolites active against murine P388 leukemia in mice, we found a complex of new macromolecular antibiotics designated maduropeptin. The producing culture H710-49, isolated from a soil sample collected in Germany, was identified as *Actinomadura madurae* (ATCC 39144). Maduropeptin is an acidic macromolecular substance from its extraction behavior; the active principle was recovered from the fermentation broth by use of a basic ion exchange resin. HPLC analysis indicated that the crude solid contained at least four active components (maduropeptins A_1, A_2, B and D) and an inactive component (maduropeptin C) having similar physico-chemical properties. Upon UV irradiation in a cold room, components B and D were decomposed yielding a complex of components A_1, A_2 and C which were isolated as single entities by chromatography. The bioactive maduropeptin components showed MW's around 22,500 and characteristic UV absorption maxima at 210, 286 and 308 nm. They exhibited potent inhibitory activity against Gram-positive bacteria and tumor cells and strong *in vivo* antitumor effect against P388 leukemia and B16 melanoma implanted in mice. In this paper, we report the producing organism, production, isolation, chemical properties and biological activities of maduropeptin.

Producing Organism

An actinomycete strain, No. H710-49 (ATCC 39144), was isolated from a soil sample collected in Germany. Strain H710-49 forms both substrate and aerial mycelia. The substrate mycelium is long, branched and not fragmented into short filaments. Short spore-chains are born on the tip or monopodial branch of the aerial mycelium. The spore-chains contain 2~15 spores (mostly 4~8 spores), per chain, and are straight, hooked or spiral in shape. The spores are oval to elliptical ($0.5\sim0.6\times0.7\sim1.2\,\mu m$) with

† Maduropeptin was originally called as BBM-1644.

Fig. 1. Spore-chains of strain H710-49 (14 day-culture on inorganic salts-starch agar; 600×).

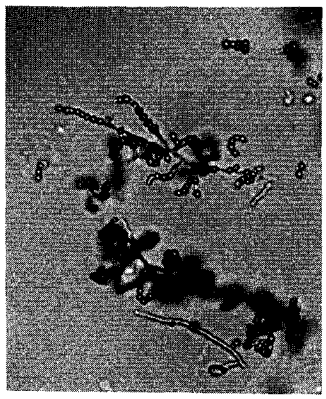


Fig. 2. Transmission electron micrograph of warty spores of strain H710-49 (14 day-culture on inorganic salts-starch agar. Bar: 1 μm).

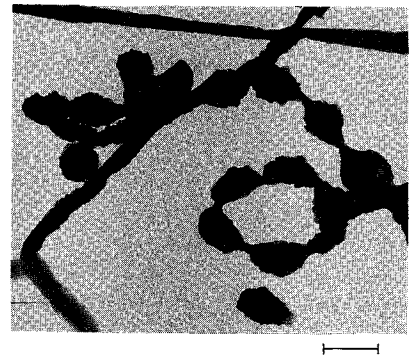


Table 1. Cultural characteristics[a] of strain H710-49.

Tryptone-yeast extract broth (ISP No. 1)	G:	Poor to moderate; floccose, sedimented and not pigmented
Sucrose-nitrate agar (CZAPEK's agar)	G:	Scant
	R:	Colorless to pale orange yellow (733)[b]
	A:	Scant; white (263)
	D:	None
Glucose-asparagine agar	G:	Poor
	R:	Yellowish white (92) to deep orange yellow (69)
	A:	Very scant; white (263)
	D:	None
Glycerol-asparagine agar (ISP No. 5)	G:	Poor to moderate
	R:	Pale yellow (89) to dark orange yellow (72)
	A:	Poor; white (263) to pale yellowish pink (31)
	D:	Brilliant yellow (83)
Inorganic salts-starch agar (ISP No. 4)	G:	Poor to moderate
	R:	Colorless to deep yellow (85)
	A:	Poor; pinkish white (9) to pale yellowish pink (31)
	D:	None
Tyrosine agar (ISP No. 7)	G:	Moderate
	R:	Brownish orange (54) to moderate reddish brown (43)
	A:	Poor; white (263) to pale yellow (89)
	D:	Strong yellow (84)
Yeast extract-malt extract agar (ISP No. 2)	G:	Moderate
	R:	Dark yellow (88) to dark brown (59)
	A:	Scant; white (263)
	D:	Light olive brown (94)
Oatmeal agar (ISP No. 3)	G:	Poor
	R:	Colorless
	A:	Poor; white (263) to pinkish white (9)
	D:	None
Peptone-yeast extract-iron agar (ISP No. 6)	G:	Poor
	R:	Grayish yellow (90) to dark grayish brown (62)
	A:	Poor; white (263)
	D:	None to moderate yellowish brown (77)

Abbreviations: G, Growth; R, reverse color; A, aerial mycelium; D, diffusible pigment.

[a] Observed after incubation at 28°C for 3 weeks.

[b] Color and number in parenthesis: ISCC-NBS color-name charts.

Table 2. Physiological characteristics of strain H710-49.

Production of:		D-Glucose	+
Amylase	+	Glycerol	+
Gelatinase	+	Inositol	−
Nitrate reductase	+	Lactose	−
Melanin	−	D-Mannitol	+
Growth in/at:		D-Mannose	−
Lysozyme, 0.001%	+	D-Melezitose	−
NaCl, 1%~7%	+	Melibiose	−
10%	−	Raffinose	−
20°C~37°C	+	L-Rhamnose	+
10°C and 41°C	−	D-Ribose	+
Utilization of[a]:		Salicin	−
D-Arabinose	−	Soluble starch	+
L-Arabinose	+	D-Sorbitol	−
Cellobiose	+	L-Sorbose	−
Cellulose	−	Sucrose	−
Dulcitol	−	Trehalose	+
D-Fructose	+	D-Xylose	+
D-Galactose	−		

[a] Basal medium: PRIDHAM-GOTTLIEB inorganic salts medium.

a round or pointed end and a warty surface. Mature spores are often separated by empty hyphae. (Figs. 1 and 2). Terminal swellings of the hyphae are occasionally observed on the substrate mycelium in CZAPEK's agar and BENNETT's agar. Motile spores, sporangia or sclerotic granules were not seen in any media examined. The cultural and physiological characteristics of strain H710-49 are shown in Tables 1 and 2, respectively.

Purified cell-wall of strain H710-49 contains *meso*-diaminopimelic acid but lacks glycine. The whole cell hydrolysate shows the presence of madurose (3-*O*-methyl-D-galactose), glucose, ribose and a small amount of mannose. The cell-wall composition and whole cell sugar components of strain H710-49 indicate that the strain belongs to cell-wall type III_B. The phospholipids contain phosphatidylinositol and phosphatidylglycerol, but not nitrogenous phospholipids, hence belong to type P-I. The menaquinone contains 68% of MK-9 (H_6) and 20% of MK-9 (H_8). The above-described characteristics of strain H710-49 resemble those of members of the genus *Actinomadura* LECHEVALIER et LECHEVALIER 1970[1]. According to the taxonomic description of known *Actinomadura* species[2~5], strain H710-49 is similar to *Actinomadura cremea*, *Actinomadura madurae* and *Actinomadura verrucosospora*. As shown in Table 3, further comparisons of the strain to the three species revealed that the strain is partially different from them, but is most similar to *A. madurae*. The strain is differentiated from *A. madurae* only in the absence of melanin formation and the lack of sucrose utilization.

Antibiotic Production

An agar slant with well-established growth of *A. madurae* H710-49 (ATCC 39144) was used to inoculate seed medium (100 ml in a 500-ml Erlenmeyer flask) containing mannitol 1%, peptone 2% and yeast extract 1%; the pH was adjusted to 7.2 before autoclaving. The seed culture was incubated at 32°C for 72 hours on a rotary shaker (250 rpm). Five ml of the mature culture was transferred to the second seed medium (100 ml) with the same composition as the first seed medium, and the seed was cultivated under the same conditions. Five ml of the inoculum growth thus prepared was employed to start fermentation in 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium composed of mannitol 2.5%,

Table 3. Characteristics of strain H710-49 and three related species of *Actinomadura*.

	Strain H710-49	*A. cremea*	*A. madurae*	*A. verrucosospora*
Morphology:				
Spore chains	Hook or spiral	Hook or spiral	Hook or spiral	Hook or spiral
(No. of spores per chain)	(2~15)	(3~8)	(3~12)	(5~12)
Spore surface	Warty	Warty	Warty	Warty
Cultural property[a]:				
ISP media				
Aerial mycelium	w, p, y	w, y, p	w, p	w, p, bl
Substrate mycelium	d, y, o, b	d, b	d, p. b, w, gy	y, o, p
Soluble pigment	—, y, b	—	—	—
Physiological characteristics:				
Melanin production	−	+	+	−
Nitrate reduction	+	+	+	−
Starch hydrolysis	+	−	+	+
Growth at 7% NaCl (w/v)	+	−	v[b]	−
Utilization of:				
Cellobiose	+	−	+	+
D-Galactose	−	−	v	+
Sucrose	−	+	+	+
Trehalose	+	−	v	+

[a] Abbreviations: −, None; d, colorless; w, white; y, yellow; o, orange; p, pink; b, brown; gy, gray; bl, blue.
[b] v: 11~89% of strains are positive.

glucose 0.5%, soybean meal 1%, peptone 0.5%, meat extract 1%, $CaCO_3$ 0.3% and sodium chloride 0.2%. Fermentation was carried out at 28°C on a rotary shaker at 250 rpm agitation. The antibiotic production was monitored by the paper-disc agar diffusion assay using *Bacillus subtilis* M45 (rec$^-$ mutant)[6] as the test organism. The antibiotic activity in the culture broth gradually increased and reached a maximum of 300 μg/ml after 6~7 days.

Fermentation was also carried out in a tank fermenter (200 liters). The second seed culture (5,000 ml) described above was inoculated to the fermenter containing 120 liters of the production medium composed of corn starch 5.0%, soybean meal 1.0%, Pharmamedia 1.0%, yeast extract 1.0% and $CaCO_3$ 1.0%, the pH was adjusted to 7.0 after sterilization. The tank fermenter was operated at 28°C at 250 rpm agitation and an aeration rate of 120 liters/minute. The pH of the culture broth gradually rose with the progress of fermentation and reached 7.8 after 72 hours when a peak antibiotic potency of 500 μg/ml was obtained.

Isolation and Purification

Extraction

The harvested broth (110 liters) was centrifuged and the filtrate was stirred with 5.5 liters of Trisacryl DEAE for 1 hour. The resin was washed with water (100 liters) and eluted three times with 5-liter portions of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.3 M sodium chloride. The eluates were combined, concentrated to 3 liters, and dialyzed against running water at 5°C overnight. The retentate solution contained *ca.* 54 g of crude maduropeptin based on lyophilization of a portion of the solution.

Isolation of Maduropeptins A_1, B, C

The above solution was applied to a Trisacryl DEAE column (4.0 × 80 cm) which had been pre-washed with 0.01 M Tris-HCl buffer (pH 7.4) at 5°C in a dark cold room. After washing with the buffer, the column was developed with the same buffer solution containing an increasing gradient of sodium chloride (0.1 M→0.15 M). The eluate was monitored by UV absorption at 210 nm, bioassay against *B. subtilis* M45,

and HPLC (TSK Gel DEAE 3SW column, 0.01 M phosphate buffer pH 7.0 containing 0.13 M sodium sulfate elution). Four components, maduropeptin C (Rt 12.4 minutes), B (Rt 19.5 minutes), D (Rt 21.9 minutes) and A_1 (Rt 24.9 minutes) were eluted. Maduropeptins A_1, B and D were bioactive while C was bioinactive. The first, bioinactive, fractions were pooled, concentrated below 40°C and dialyzed against deionized water at 5°C in a dark room for 18 hours. Concentration of the retentate yielded a semi-pure solid of maduropeptin C. The second UV-absorbing fractions were pooled and worked up as above to yield a solution of semi-pure maduropeptins A_1, B and D mixture (estimated weight 4.78 g). One third of the solution was re-chromatographed on a column of Trisacyl DEAE (4.0 × 25 cm). Elution was carried out with 0.01 M Tris-HCl buffer (pH 7.4) containing sodium sulfate (starting from 0.05 M up to 0.15 M). Upon monitoring by bioassay and HPLC, two active peak fractions were eluted. They were concentrated and dialyzed to give solutions of maduropeptin A_1 (calcd weight 105 mg) and maduropeptin B (calcd weight 326 mg). The eluates between the above two peaks contained mostly maduropeptin D and small amount of maduropeptins A_1 and B. The semi-pure solution of maduropeptin A_1 (calcd weight 70 mg) was further purified by semi-preparative HPLC: column, TSK 545 DEAE (21.5 × 150 mm, LKB) and mobile phase, 0.01 M Tris-barbital buffer, pH 7.0 containing 0.2 ~ 0.25 M sodium sulfate (linear gradient). The eluate was monitored by HPLC and appropriate fractions were combined and dialyzed against deionized water to obtain a solution containing nearly pure maduropeptin A_1 (14 mg weight). The semi-pure maduropeptin B solution (70 mg equivalent) was similarly purified to yield a solution containing 27 mg of nearly pure maduropeptin B.

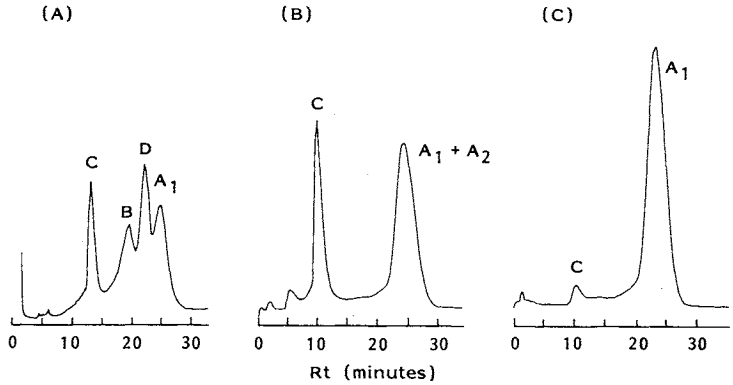
Purification of Photo-degradation Products

The natural maduropeptin components A_1, B, C and D, were found to be reasonably stable at 5°C in the dark but components B and D were shown by HPLC to be readily decomposed by light to a mixture of maduropeptins A_1 and C (Fig. 3). When the solution was allowed to stand at room temperature under light, maduropeptin A_1 was also decomposed giving only bioinactive maduropeptin C. Purification and characterization of the major products was attempted, utilizing photo-degradation to simplify the problem.

A solution of components A_1, B, C and D (ratio, 29 : 23 : 35 : 21) was gently stirred under a fluorescent

Fig. 3. HPLC chromatograms of maduropeptin mixture, photo-decomposed products and maduropeptin A_1.

(A) Maduropeptin mixture, (B) photo-decomposed products, (C) maduropeptin A_1.

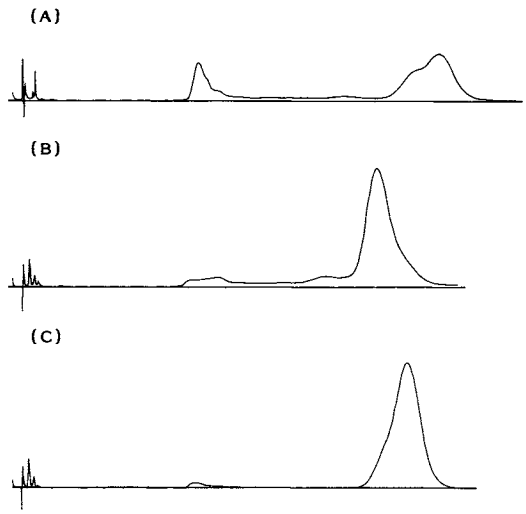


(A): Component ratio of A_1, 29; B, 23; C, 35; D, 21. (B): component ratio of $A_1 + A_2$, 62; C, 38. (C): component A_1 97% purity. HPLC column: TSK Gel DEAE 3SW (7.5 × 75 mm, Toyo Soda Manufacturing Co., Tokyo); solvent: 0.01 M phosphate buffer (pH 7.0) + 0.13 M Na_2SO_4; detection: UV (210 nm); flow rate: 1 ml/minute.

lamp (15 W) at 5°C for 65 hours. The resulting reaction solution appeared to contain only components A_1 and C (ratio 62 : 38) by HPLC analysis. The component A_1 in the solution was, however, found to be a mixture of maduropeptins A_1 and A_2 as described later. The solution was charged on a column of Trisacryl DEAE (2.0 × 95 cm). Elution was carried out first with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.11 M sodium chloride and then with a gradient of sodium chloride from 0.12 M to 0.15 M. The bioinactive UV fractions and bioactive UV fractions were pooled and desalted by dialysis to give maduropeptin C solution (calcd weight, 25 mg; purity, 99%) and A_1 solution (71 mg; purity, 90%).

Fig. 4. Preparative HPLC chromatograms of photo-decomposed maduropeptin, maduropeptins A_1 and A_2.

(A): Photo-decomposed maduropeptin ($A_1 + A_2 +$ C), (B) maduropeptin A_1, (C) maduropeptin A_2.



HPLC column: TSK Gel DEAE 3SW (7.5 × 75 mm, Toyo Soda Manufacturing Co., Tokyo), solvent: 0.01 M phosphate buffer (pH 7.0) + 0.09 M Na_2SO_4, detection: UV (210 nm), flow rate: 1 ml/minute.

Although the maduropeptin A_1 obtained showed single peak identical with that of natural maduropeptin A_1 by the above HPLC system, a modified HPLC system (0.01 M phosphate buffer, pH 7.0, containing 0.09 M sodium sulfate) revealed that it was a mixture of two components, the natural A_1 and a new component named A_2. Careful comparison of the photo-degradation products indicated that maduropeptins B and D were decomposed by light to yield component A_2. A part of the A_1 and A_2 mixture solution (39 mg) was subjected to semi-preparative HPLC (TSK Gel 545 DEAE, 21.5 × 150 mm, and 0.01 M phosphate buffer, pH 7.0, containing 0.25 M sodium chloride). The appropriate fractions were dialyzed against deionized water at 5°C to yeild component A_1 solution (2 mg, 97%) and component A_2 solution (6 mg, 99%) (Fig. 4).

Physico-chemical Properties

The four components, maduropeptins A_1, A_2, B and C were isolated as described above but maduropeptin D has not been obtained as a single entity. Maduropeptin C is very stable and A_1 and A_2 are fairly stable to isolation work-up. Maduropeptin B is, however, very unstable upon solidification and thus only limited data on this component were obtained. They are distinguished from each other by HPLC as shown in Fig. 3.

The four components are readily soluble in water but are insoluble and decompose in organic solvents resulting in complete loss of the activity. They were positive with Folin-Lowry, xanthoprotein, biuret and ninhydrin reagents but negative to Sakaguchi and anthrone reagents. Maduropeptins A_1 and A_2 exhibited UV absorption maxima at 210, *ca.* 280 and 308 nm while maduropeptin C lacked the maximum at 308 nm. Some of the physico-chemical data are shown in Table 4. The acidic nature of the four maduropeptin components was indicated by their isoelectric points. Maduropeptins A_1, A_2 and B are stable in neutral conditions at 5°C but gradually decompose at room temperature. They are unstable in acidic or alkaline solution or upon UV irradiation.

Table 4. Physico-chemical properties of maduropeptin components.

	Maduropeptin			
	A_1	A_2	B	C
Nature	White powder	White powder	White powder	White powder
MP (dec, °C)	240~244	226~230	235~238	249~252
$[\alpha]_D^{27}$	−84° (c 0.1, H_2O)	−27° (c 0.2, H_2O)	−48° (c 0.2, H_2O)	−76° (c 0.5, H_2O)
Isoelectric point	4.75	4.90	4.80	4.77
Elementary analysis (Found)				
C:	45.07	48.13	43.91	46.97
H:	6.34	6.60	6.05	7.13
N:	12.70	13.18	12.66	13.82
S:	0.98	0.98	2.24	1.12
UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	210 (129), 286 (9.8), 308 (7.8)	201 (234), 278 (10.2), 306 (7.1)	275 (7.9)	219 (55), 278 (2.8)
IR ν_{max} (KBr) cm^{-1}	3400~3200, 1640, 1530	3450~3200, 1640, 1530	3450~3200, 1640, 1530	3450~3200, 1640, 1520
MW				
Gel filtration[a]				22,500
HPLC[b]				27,000

[a] Sephadex G-75.
[b] Asahipak GS-320.

MW Determination

The MW of maduropeptin C was estimated by gel filtration using a Sephadex G-75 column (22 × 685 mm) and 1/15 M phosphate buffer pH 7.0 with a flow rate of 30 ml/hour. The calibration standard kit (Pharmacia Fine Chem.) comprised of blue dextran (MW 2,000,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen (MW 25,000) and ribonuclease A (MW 13,700) was chromatographed simultaneously. Maduropeptin C was eluted just after chymotrypsinogen as monitored by HPLC and its MW was calculated to be 22,500. Upon HPLC co-chromatography with the standard kit (Asahipak GS-320, 7.6 × 500 mm, Asahi Chemical Industry Co. and 0.1 M phosphate buffer, pH 7.0, containing 0.3 M sodium chloride), maduropeptin C was eluted before chymotrypsinogen indicating a molecular weight of 27,000.

Amino Acid Analysis

Maduropeptins A_1, B and C were hydrolyzed with 6 N HCl at 110°C for 22 hours. Part of the solution was oxidized with performic acid at 110°C for 20 hours for determination of cystine and tryptophan. After concentration to dryness *in vacuo*, the residue was subjected to amino acid analysis by a Waters Pico tag amino acid analyzer (Waters type ALC/GPC 606) with the results described in Table 5. Noteworthy is that all maduropeptin components do not contain basic amino acids, which distinguish them from the known macromolecular chromoprotein antibiotics.

Non-protein Chromophore

Maduropeptins B and D are highly sensitive to UV light yielding the bioinactive apoprotein maduropeptin C. Maduropeptins A_1 and A_2 are rather refractory to UV, but upon treatment with acidic methanol, they afforded a lipophilic substance with antimicrobial and cytotoxic activity, along with maduropeptin C. Preliminary characterization indicated that the bioactive degradation product has UV absorption considerably different from that of the original antibiotics and does not show synergistic

Table 5. Amino acid composition of maduropeptin components.

	Maduropeptin				Neocarzinostatin[a]
	A_1	A_2	B	C	
Lysine	—	—	—	—	0.96
Histidine	—	—	—	—	—
Arginine	—	—	—	—	1.60
Aspartic acid	6.0	5.2	5.8	5.4	10.00
Threonine	9.9	11.4	10.1	11.3	9.67
Serine	6.3	4.3	4.8	4.3	8.35
Glutamic acid	6.2	5.3	5.5	5.5	4.15
Proline	4.1	4.3	4.8	4.3	2.38
Glycine	9.3	7.9	7.7	8.2	12.15
Alanine	8.5	8.7	8.7	8.7	13.80
1/2 Cystine	—	0.4	0.9	0.3	3.10
Valine	7.4	6.8	11.1	7.1	8.90
Methionine	0.2	0.9	—	0.7	—
Isoleucine	1.7	1.0	1.0	1.8	0.94
Leucine	(1.0)[b]	(1.0)	(1.0)	(1.0)	5.03
Tyrosine	0.8	1.1	0.9	1.0	0.74
Phenylalanine	1.0	1.6	4.5	1.6	4.64
Tryptophan	—	—	—	—	1.64

[a] Literature values (J. Antibiotics, Ser. A 19: 253~259, 1966).
[b] Content of leucine is arbitrary assigned as 1.0.

Table 6. Antimicrobial activity of maduropeptin components and neocarzinostatin.

Test organisms	MIC (μg/ml)				
	A_1	A_2	B	C	Neocarzinostatin
Staphylococcus aureus 209P	1.6	1.6	1.6	>100	0.8
S. aureus Smith	0.8	1.6	0.8	>100	0.8
S. aureus D136	3.1	3.1	1.6	>100	1.6
S. epidermidis D153	3.1	3.1	1.6	>100	0.2
Micrococcus luteus PCI 1001	3.1	1.6	1.6	>100	1.6
Bacillus subtilis PCI 219	3.1	1.6	1.6	>100	1.6
Escherichia coli Juhl	>50	>50	>25	>100	>25
Klebsiella pneumoniae D11	>50	>50	>25	>100	>25
Pseudomonas aeruginosa A9930	>50	>50	>25	>100	>25
Proteus vulgaris A9436	>50	>50	>25	>100	>25
Candida albicans A9540	>50	>50	>25	>100	>25
Cryptococcus neoformans D49	>50	>50	>25	>100	>25
Aspergillus fumigatus IAM 2530	>50	>50	>25	>100	>25
Trichophyton mentagrophytes D155	>50	>50	>25	>100	>25

antimicrobial effect with maduropeptin C[7]. The results suggested that the bioactive degradation product was not a true non-protein chromophore but rather an artifact.

Antimicrobial Activity

The antimicrobial activity, measured as MIC, of maduropeptins A_1, A_2, B and C was assessed using the agar dilution assay. Nutrient broth was used for Gram-positive and Gram-negative bacteria and Sabouraud dextrose broth for fungi. The inoculum size was adjusted to $10^5 \sim 10^6$ cfu/ml for bacteria and 10^6 cfu/ml for fungi. Incubation was carried out at 28°C for 18 hours and neocarzinostatin (NCS) was used as a reference compound. The results are shown in Table 6.

Maduropeptins A_1, A_2 and B exhibited significant inhibitory activity against Gram-positive bacteria with MICs being 2~15 times greater than those of NCS. They were inactive against Gram-negative bacteria. Maduropeptin C, the apoprotein of the components A_1, A_2 and B, did not show *in vitro* activity.

Antitumor Activity

Maduropeptin components were tested for *in vitro* cytotoxicity against murine and human tumor cells and for *in vivo* antitumor activity in mice. NCS was used as a reference compound for both *in vitro* and *in vivo* experiments.

In Vitro Cytotoxicity

Murine melanoma B16-F10 cells were grown in EAGLE's minimum essential medium supplemented with fetal calf serum (FCS, 10%) and kanamycin (60 μg/ml), and human colon carcinoma HCT-116 cells in McCOY's 5A medium supplemented with FCS (10%), benzylpenicillin (100 U/ml), and streptomycin (100 μg/ml). Exponentially growing B16-F10 and HCT-116 cells were harvested, counted and suspended in the culture medium at 3×10^4 and 6×10^4 cells/ml, respectively. After planting 180 μl of cell suspension into wells of a 96-well microtiter plate with test samples (20 μl), the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 72 hours. The cytotoxicity was colorimetrically determined at 540 nm after staining the viable cells with neutral red solution. Maduropeptin components A_1, A_2 and B showed potent cytotoxicity with IC_{50} values ranging from 0.007 to 0.16 μg/ml against both tumor cells. In particular, component A_1 gave 65-fold (vs. B16-F10) and 190-fold (vs. HCT-116) more potent activity than NCS. Maduropeptin C did not show cytotoxicity at 100 μg/ml against either cell line (Table 7).

Table 7. *In vitro* cytotoxicity against murine melanoma B16 and human colon carcinoma HCT-116 cells.

Compound	IC_{50} (μg/ml)	
	B16-F10	HCT-116
Maduropeptin A_1	0.017	0.007
Maduropeptin A_2	0.043	0.16
Maduropeptin B	0.028	0.029
Maduropeptin C	>100	>100
Neocarzinostatin	1.1	1.3

In Vivo Antitumor Activity

The *in vivo* antitumor activity of maduropeptin

Table 8. Antitumor activity of maduropeptin components against P388 (ip) in female CDF_1 mice.

Compound	Treatment (Q1D × 3, ip) mg/kg/day	MST (day)	T/C (%)	Average weight change on day 4 (g)
Maduropeptin A_1	0.06	16.5	165	−1.3
	0.02	15.0	150	−0.5
	0.006	14.0	140	0.0
	0.002	11.0	110	+1.0
Maduropeptin A_2	0.06	7.0	70	−1.0
	0.02	15.5	155	−1.0
	0.006	15.0	150	−0.5
	0.002	14.0	140	+0.5
	0.0006	11.0	110	+1.8
Maduropeptin B	1	7.0	70	−2.3
	0.3	23.5	235	−3.0
	0.1	16.0	160	−2.0
	0.03	15.5	155	−0.8
	0.01	14.0	140	0.0
Vehicle	—	10.0	—	+0.8

Table 9. Antitumor activity of maduropeptin A_1 against P388 (ip) in male BDF_1 mice.

Compound	Treatment (Q1D × 9, ip) mg/kg/day	MST (day)	T/C (%)	Average weight change on day 4 (g)
Maduropeptin A_1	0.025	9.0	90	−1.8
	0.013	18.0	180	−1.7
	0.006	18.0	180	−0.3
	0.003	16.0	160	0.0
	0.0016	15.0	150	+1.2
	0.0008	14.0	140	+1.2
Neocarzinostatin	1	13.5	135	−0.8
	0.5	21.0	210	+0.3
	0.25	19.0	190	+1.0
	0.13	18.5	185	+1.2
	0.063	18.5	185	+1.8
	0.031	17.0	170	+1.8
	0.016	14.5	145	+1.2
	0.008	14.0	140	+1.5
Vehicle	—	10.0	—	+2.1

Table 10. Antitumor activity of maduropeptin A_1 against B16 melanoma in male BDF_1 mice (subrenal capsule assay).

Compound	Dose (Q1D × 5, ip) mg/kg/day	Δ Tumor size (OMU[a] ± SE)	% Inhibition of tumor growth
Maduropeptin A_1	0.02	0.3±1.3	97
	0.01	1.6±2.2	86
	0.005	5.3±3.2	53
	0.0025	10.6±5.5	6
Vehicle	—	11.3±2.9	—
Neocarzinostatin	1.0	3.5±2.1	84
	0.5	7.1±1.9	67
	0.25	9.2+1.9	57
	0.13	14.8±2.6	31
Vehicle	—	21.3±1.9	—

[a] Ocular micrometer units.

components was examined against P388 lymphocytic leukemia and melanoma B16. P388 cells (10^6 cells per mouse) were inoculated intraperitoneally into male BDF_1 or female CDF_1 mice. Graded doses of the test materials were administered intraperitoneally to groups of 4 female CDF_1 mice on days 1 to 3 (Q1D × 3) or to groups of 6 male BDF_1 mice on days 1 to 9 (Q1D × 9) after tumor implantation (day 0). Death or survival of the treated and non-treated animals was recorded daily during the observation period of 45 days and the median survival time (MST) was calculated for the test (T) and control (C) groups. A T/C value of ≧125% is considered a significant antitumor effect. As shown in Table 8, maduropeptin components A_1, A_2 and B gave highly potent antitumor activity with maximum T/C values of 165%, 155% and 235% (Q1D × 3 treatment), respectively, against P388 leukemia. When compared with NCS in the Q1D × 9 treatment against P388 leukemia, maduropeptin A_1 was approximately 10-fold more potent than NCS in terms of minimum effective dose (Table 9). Anti-B16 melanoma activity of maduropeptin A_1 was determined

in the mouse subrenal capsule (SRC) assay[8]. A minced fragment of the melanoma was implanted beneath the renal capsule of male BDF_1 mice and each initial graft size was measured with an ocular micrometer scale (day 0). The animals were randomized in groups of 4 and treated with graded doses of the test materials on days 1 to 5 (Q1D × 5). On day 6, the kidney was excised and the final graft size was determined. Antitumor activity was expressd as % inhibition of tumor growth and an inhibition value of $\geqq 50\%$ is considered significant antitumor activity. As shown in Table 10, maduropeptin A_1 gave dose-related inhibition of tumor growth and was approximately 50-fold more active than NCS in terms of minimum effective dose.

Acute Toxicity

The acute toxicity was determined by intraperitoneal administration of graded doses of the materials to groups of 5 normal male *dd*Y mice. The LD_{50} was calculated 10 days after administration according to the method of VAN DER WAERDEN[9]. Maduropeptin A_1 (LD_{50} 0.067 mg/kg) was approximately 50-fold more toxic than NCS (LD_{50} 3.1 mg/kg) tested as a reference.

Discussion

Five components of antitumor antibiotic maduropeptin have been isolated from the culture filtrate of *A. madurae* strain No. H710-49. They are acidic polypeptides and the bioactive components, maduropeptins A_1, A_2, B and D carry a chromophore unit.

Many chromoprotein antibiotics including neocarzinostatin[10], auromomycin[11], macromomycin[12], actinoxanthin[13], sporamycin[14], largomycin[15] and C-1027[16] have been identified as effective antitumor agents and, among them, neocarzinostatin is in clinical use. Maduropeptin appears to be a new addition to this family from its physico-chemical and biological profile, but it is distinctly different from the preceding antibiotics in not containing basic amino acids. As mentioned previously, attempts to isolate a true non-protein chromophore of maduropeptin were unsuccessful. Isolation and characterization of the chromophore are continuing and will be reported later.

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