PROTACTIN†, A NEW ANTIBIOTIC METABOLITE AND A POSSIBLE PRECURSOR OF THE ACTINOMYCINS

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(Received for publication March 11, 1991)

Streptomyces cucumerosporus strain L703-4 (ATCC 53784) produces a new 4-methyl-3-hydroxyanthraniloylpentapeptide lactone for which we have proposed the name protactin, in addition to several actinomycin components. Protactin is rather resistant to air oxidation but it can be converted to a new actinomycin, actinomycin Zp by ferricyanide oxidation. Actinomycin Zp possesses *in vitro* antibacterial activity and *in vivo* antitumor activity against P-388 leukemia in mice.

In our search for microbial secondary metabolites possessing antitumor activity, we found that *Streptomyces cucumerosporus* strain No. L703-4, isolated from an Indian soil sample, produced a new antitumor antibiotic designated protactin together with a complex of actinomycins.

This paper describes the production, isolation, structure and biological activity of protactin.

Fermentation of Protactin

The producing organism *Streptomyces cucumerosporus* No. L703-4 was stored and maintained on slants of yeast extract-malt extract agar consisting of glucose 0.4%, yeast extract 0.4%, malt extract 1% and agar 2%, pH 7.0. A vegetative inoculum for shake flask or submerged fermentations was prepared by transferring the mycelial growth from a slant to a 500-ml Erlenmeyer flask containing 100 ml of medium consisting of soluble starch (Nichiden Kagaku) 2%, Pharmamedia (Traders Protein) 1%, $ZnSO_4 \cdot 7H_2O$ 0.003% and $CaCO_3$ 0.4%, the pH being adjusted to 7.0 before sterilization. The flask were shaken at 28°C for 4 days on a rotary shaker (200 rpm) and 5 ml of the culture were transferred to an Erlenmeyer flask containing 100 ml of the same medium as described above. The fermentation was carried out at 28°C on a rotary shaker. The fermentation was also carried out in a 200-liter tank fermenter. In this case, 2-liter of the vegetative mycelium was inoculated into the tank containing 120 liters of the medium. Fermentation was run at 28°C with agitation at 250 rpm and aeration rate of 120 liters per minute. Antibiotic production reached a maximum of 2 μg/ml after 4 to 5 days in a flask fermentation and 50 hours in a tank fermentation.

The antibiotic titer in fermentation broths was determined by the paper disc agar diffusion assay using *Bacillus subtilis* M45 (rec$^-$) as the test organism and *in vitro* cytotoxic activity using B16 melanoma cells. The production was also monitored by TLC scanning (330 nm) (Shimadzu-Dual Wave Length TLC scanner CS-910). A concentrated ethyl acetate extract from the broth filtrate was spotted on a TLC plate RP-18 $F_{254}S$ (Merck Co.) and developed with 70% aq methanol.

Isolation and Purification

The fermentation broth (220 liters, pH 7.3) was separated into mycelial cake and supernate by a Sharples-type centrifuge (Kokusan H-600). The supernate (205 liters) was extracted with BuOH (80 liters)

† Protactin was originally called BU-3292T A or BMY-28648.

and the extract was concentrated to an aq solution (1 liter). The solution was extracted twice with ethyl acetate (1 liter each). Evaporation of the combined extracts gave a crude solid (69 g), which was dissolved in methanol and chromatographed on a column of Diaion HP-20 (Mitsubishi Chem. Industries, Tokyo, 4.0 × 75 cm), and fractions were eluted with 50% methanol (3 liters) and then with 80% methanol (5 liters). Eluate fractions were monitored by paper disc assay using *B. subtilis* M45 (rec⁻). The active fractions eluted with 80% methanol were pooled and concentrated to an oily residue. The residue was dissolved in acetone (10 ml) and the solution was added dropwise into *n*-hexane (1 liter) to deposit an oily precipitate (2.8 g). This solid was applied to a column of silica gel (2.2 × 75 cm) and developed with a methylene chloride-methanol mixture with stepwise increase of the methanol concentration (2～10%). The first active fractions eluted with 2% methanol were collected and concentrated *in vacuo* to yield the actinomycin complex. The second active fractions, eluted with 5% methanol, were combined and evaporated to yield a crude solid of protactin (1.1 g). The crude solid was chromatographed on Lichroprep RP-18 (Merck, 2.2 × 70 cm) by successive elution with 30% methanol, 50% methanol and 80% methanol. The bioactive fractions eluted with 50% methanol were combined and concentrated *in vacuo* to afford a semi-pure solid (224 mg). This solid was further purified by silica gel chromatography. Elution with methylene chloride - methanol (96 : 4) gave an active eluate which was concentrated and developed on Sephadex LH-20 (2.2 × 70 cm) with methanol. Evaporation of homogeneous active fractions afforded a solid of pure protactin (48 mg).

Conversion of Protactin to Actinomycin Zp

Upon standing at room temperature in solution, protactin was very slowly converted to a new yellow compound (actinomycin Zp) which showed the same UV spectrum as the actinomycins. Conversion rates under various experimental conditions are listed in Table 1.

This reaction proceeded rapidly and quantitatively upon treatment with potassium ferricyanide[1]. Details are described in the Experimental section.

Physico-chemical Properties

The physico-chemical properties of protactin and actinomycin Zp are summarized in Table 2. Protactin is a white amorphous powder, while actinomycin Zp is an orange red powder. Both antibiotics are readily soluble in dimethyl sulfoxide, *N*,*N*-dimethylformamide, methanol, ethyl acetate and chloroform but insoluble in other organic solvents and water. They gave a positive response to iodine and Rydon-Smith reagents, but negative response to Sakaguchi, ferric chloride, ninhydrin and anthrone reagents. The molecular formulae of protactin and actinomycin Zp were established as $C_{32}H_{48}N_6O_8$ and $C_{64}H_{90}N_{12}O_{16}$, respectively, based on microanalysis and mass spectra. The UV spectrum of protactin showed absorption maxima at 217, 257 and 329 nm in methanol solution suggesting an anthranilic acid type chromophore[2].

Table 1. Conversion of protactin to actinomycin Zp.

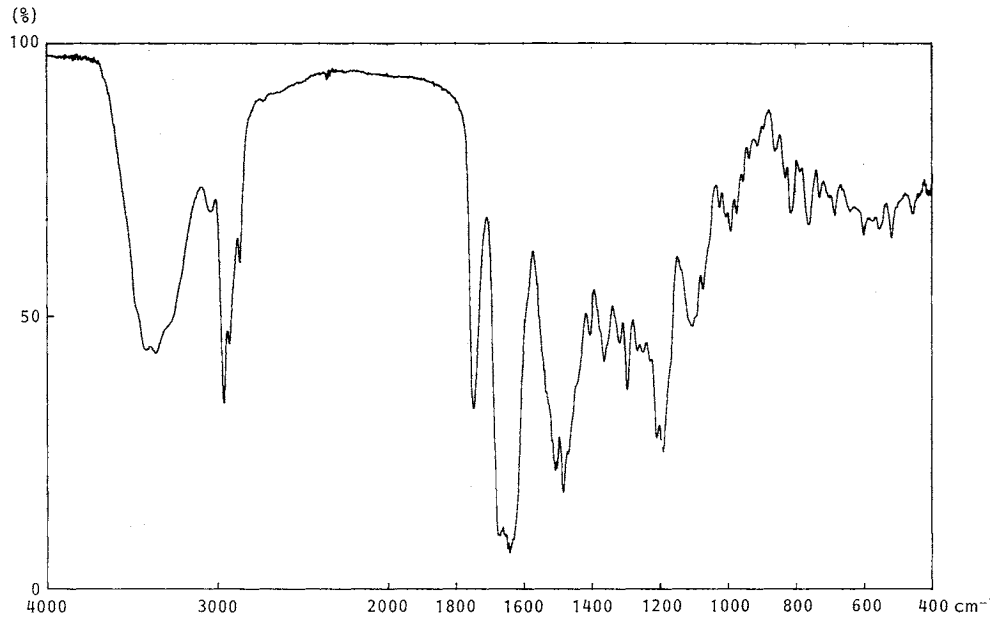
Condition	Ratio[a] (protactin/actinomycin Zp, %)		
	24 hours	48 hours	72 hours
5°C in MeOH	100/0	100/0	100/0
5°C in 10% DMSO	100/0	100/0	100/0
37°C in MeOH	100/0	100/0	97/3
37°C in 10% DMSO	97/3	92/8	90/10
37°C in nutrient[b] broth	85/15	NT	NT
37°C in Macoy 5A[c]	NT	NT	89/11

[a] Conversion rate was calculated by the peak area of HPLC analysis column: YMC-A301-2, solvent: Phosphate buffer (pH 3.5) - CH_3CN, detection: UV 254 nm.
[b] Medium for *in vitro* antimicrobial activity evaluation.
[c] Medium for *in vitro* cytotoxicity evaluation.
NT: Not tested.

Table 2. Physico-chemical properties of protactin and actinomycin Zp.

	Protactin	Actinomycin Zp
Nature	White amorphous powder	Orange red powder
MP (°C)	193~195 (dec)	248~250
Optical rotation	$[\alpha]_D^{24}$ −21°±2° (*c* 0.5, MeOH)	$[\alpha]_D^{25}$ −223°±2° (*c* 0.23, MeOH)
UV λ_{max} nm ($E_{1cm}^{1\%}$)		
in MeOH	217 (493), 257 (sh, 111), 329 (56)	206 (408), 237 (227), 422 (sh, 137), 442 (146)
in 0.01 N HCl-MeOH	207 (635), 249 (sh, 131), 290 (42)	206 (408), 237 (227), 422 (sh, 137), 442 (146)
in 0.01 N NaOH-MeOH	210 (429), 246 (299), 277 (sh, 90), 343 (50)	206 (408), 237 (227), 422 (sh, 137), 442 (146)
Microanalysis		
Calcd for	$C_{32}H_{48}N_6O_8$:	$C_{64}H_{90}N_{12}O_{16}\cdot 2H_2O$:
C	59.61	58.25
H	7.50	7.18
N	13.03	12.74
Found:		
C	59.75	58.42
H	7.58	7.17
N	11.10	11.43
MS (*m/z*)	644 (M^+, EI)	1,283 ($M^+ + 1$, SI-MS)
HREI-MS	$C_{32}H_{48}N_6O_8$ (M^+; Obsd: *m/z* 644.3489, Calcd: *m/z* 644.3532)	
TLC SiO_2 EtOAc-MeOH (4:1)	Rf 0.55	Rf 0.58
CH_2Cl_2-MeOH (9:1)	Rf 0.43	Rf 0.64

Fig. 1. IR spectrum of protactin.

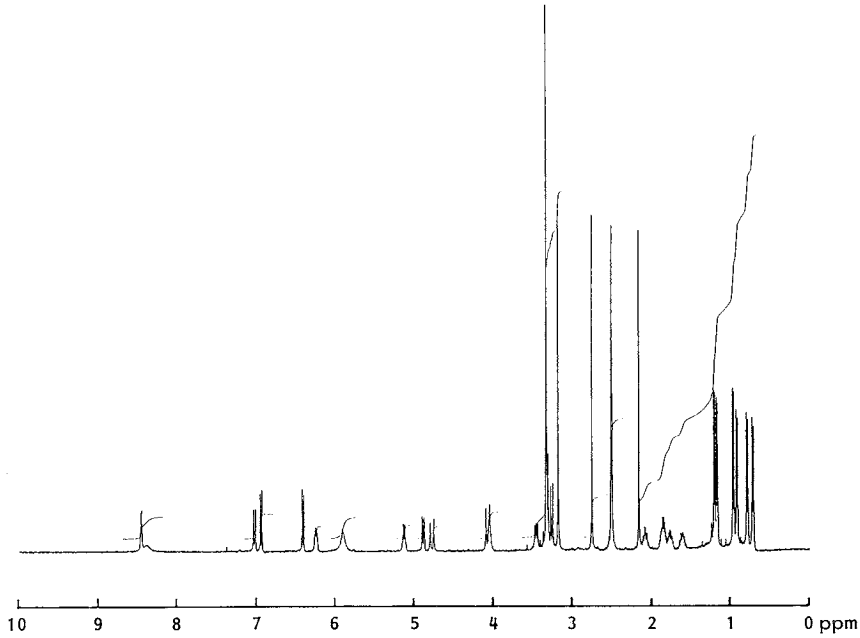


Actinomycin Zp exhibited a UV spectrum similar to that of the actinomycins. The IR and ^1H NMR of protactin are illustrated in Figs. 1 and 2, respectively.

Structure of Protactin

The ^{13}C NMR spectrum of protactin (Table 3) demonstrated 32 carbons including seven C-CH_3, two

Fig. 2. ^{1}H NMR spectrum of protactin in DMSO-d_6.

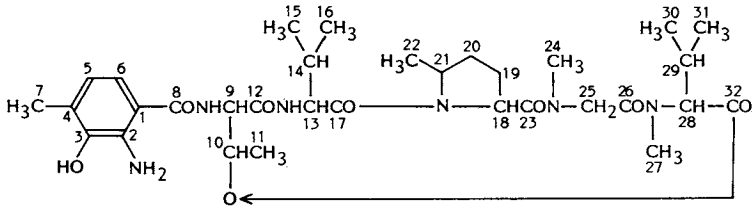


N-CH_3, three methylene, eight methine, two –CH=, four =C< and six >C=O carbons which supported the molecular formula of $C_{32}H_{48}N_6O_8$ assigned by microanalysis and mass spectrum. Nine methyls (δ 0.70 d, 0.77 d, 0.90 d, 0.95 d, 1.16 d, 1.19 d, 2.16 s, 2.75 s, 3.18 s), two adjacent aromatic protons (δ 6.40 d, 6.93 d), two amide protons (δ 7.01 d, 8.45 d), two other D_2O-exchangeable protons (δ 5.89 br s, 8.35 br s) were observed in ^{1}H NMR (Fig. 2). The IR spectrum exhibited strong absorption at 1750 and 1640 cm^{-1} suggesting that protactin has a depsipeptide structure. This was confirmed by formation of a free acid derivative (m/z 685 $(M+Na)^+$) upon mild alkaline treatment of protactin. The UV absorption maximum at 329 nm in methanol shifted to 290 nm in acidic solution and to 343 nm in alkaline solution indicating the presence of a hydroxy anthranilic acid moiety in protactin. The presence of one amino and one hydroxyl group in protactin was evidenced by the fact that acetylation in pyridine afforded the *N,O*-diacetyl derivative (m/z 729 $(M+H)^+$). When hydrolyzed with 6 N hydrochloric acid, protactin gave L-threonine, D-valine, sarcosine, *N*-methyl-L-valine and an unidentified amino acid as determined by amino acid analysis, TLC and HPLC with a chiral column. All of the protons and carbons observed in the ^{1}H and ^{13}C NMR spectra of protactin were unambiguously assigned using ^{1}H-^{1}H, ^{13}C-^{1}H, ^{13}C-^{1}H long range COSY NMR spectra (Table 3). The long range COSY revealed that the chromophore and unidentified amino acid were 4-methyl-3-hydroxyanthranilic acid and 5-methylproline, respectively (Fig. 3). The spectrum also clarified the connectivities of the amino acids and the chromophore establishing the complete structure as shown in Fig. 4. Actinomycin Zp was assigned the structure in Fig. 4 since it was prepared by potassium ferricyanide oxidation of protactin and has physico-chemical and spectral properties similar to those of the known actinomycins.

Antimicrobial Activity

The antimicrobial activity of protactin and actinomycin Zp against aerobic and anaerobic bacteria and fungi was determined by a serial 2-fold agar dilution method. Actinomycin D was used as reference

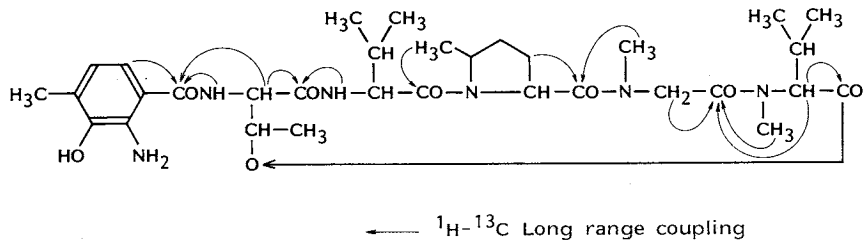
Table 3. ^{13}C and ^{1}H NMR assignments of protactin in DMSO-d_6.



Position	δ_C	δ_H	Position	δ_C	δ_H
Chromophore			MePro		
1	139.6 (s)		18	56.7 (d)	6.24 (dd, 4.3, 8.6)
2	127.3 (s)		19	29.70 (t)	1.76 (m)
3	142.1 (s)				2.08 (m)
4	112.9 (s)		20	30.8 (t)	1.61 (m)
5	117.3 (d)	6.40 (d, 8.1)			1.85 (m)
6	118.4 (d)	6.93 (d, 8.1)	21	53.5 (d)	4.04 (dd, 6.0, 12.8)
7	16.5 (q)	2.16 (s)	22	18.8 (q)	1.19 (d, 6.0)
8	168.5 (s)		23	172.9 (d)	
2-NH_2		5.89 (br s)	Sar		
3-OH		8.35 (br s)	24	34.38 (q)	2.75 (s)
Thr			25	51.25 (t)	4.06 (d, 18.0)
9	53.7 (d)	4.88 (dd, 1.9, 9.1)			4.77 (d, 18.0)
10	72.6 (d)	5.12 (dq, 1.9, 6.2)	26	167.1 (s)	
11	16.6 (q)	1.16 (d, 6.2)	MeVal		
12	169.4 (s)		27	38.5 (q)	3.18 (s)
9-NH		7.01 (d, 9.1)	28	69.5 (d)	3.26 (d, 9.4)
Val			29	26.4 (d)	2.50 (m)
13	57.8 (d)	3.45 (dd, 10.2, 5.5)	30	18.8 (q)	0.77 (d, 6.7)
14	26.4 (d)	2.50 (m)	31	21.0 (q)	0.95 (d, 6.7)
15	18.8 (q)	0.70 (d, 6.7)	32	168.1 (s)	
16	18.8 (q)	0.90 (d, 6.7)			
17	170.8 (s)				
13-NH		8.45 (d, 5.5)			

δ ppm (multiplicity, J=Hz).

Fig. 3. Amide and ester bond assignment in protactin.

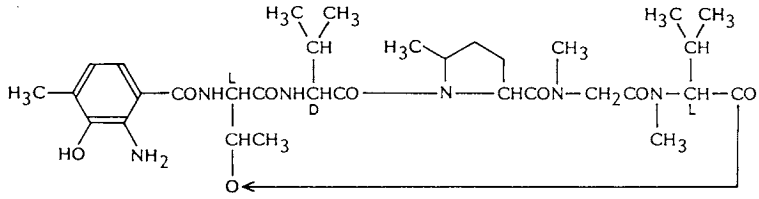


with the results shown in Table 4. They are active against aerobic Gram-positive bacteria, weakly active against anaerobic Gram-positive bacteria and inactive against Gram-negative bacteria and fungi. They are about one fourth as active as actinomycin D.

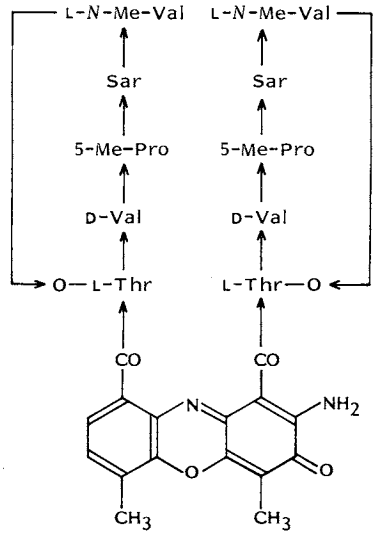
Antitumor Activity

Protactin and actinomycin Zp were tested for *in vitro* cytotoxicity against murine and human tumor

Fig. 4. The structures of protactin and actinomycin Zp.



Protactin



Actinomycin Zp

Table 4. Antimicrobial activity.

Test organisms	Test medium	MIC (μg/ml)		
		Protactin	Actinomycin Zp	Actinomycin D
Staphylococcus aureus FDA 209P	A	1.6	1.6	0.4
S. aureus Smith	A	1.6	1.6	0.4
Streptococcus faecalis A9612	A	0.8	0.8	0.1
Micrococcus luteus 1001	A	0.4	0.4	0.2
Bacillus subtilis PCI 219	A	0.4	0.8	0.2
Escherichia coli NIHJ	A	>100	>100	>100
Klebsiella pneumoniae D11	A	>100	100	25
Pseudomonas aeruginosa A9930	A	>100	>100	100
Proteus vulgalis A9436	A	>100	>100	>100
Bacteroides fragilis A22693	B	>100	>100	>100
Clostridium difficile A21675	B	>100	>100	25
Candida albicans IAM 4888	C	>100	>100	>100
Cryptococcus neoformans IAM 4519	C	100	>100	>100
Aspergillus fumigatus IAM 2530	C	>100	>100	>100

A: Mueller-Hinton agar (Eiken), B: GC medium, C: GAM agar (Nissui).

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

Compound	Dose[a] (mg/kg/day)	MST (day)	T/C[b] (%)	Body weight change on day 4 (g)
Protactin	30	Tox	Tox	—
	10	16.0	160	−3.0
	3	14.0	140	+0.3
	1	13.0	130	+0.5
	0.3	12.0	120	+1.3
	0.1	11.0	110	+1.3
	0.03	11.0	110	+1.3
Actinomycin Zp	1	Tox	Tox	—
	0.3	8.0	80	−3.3
	0.1	16.0	160	−1.0
	0.03	14.0	140	+1.0
	0.01	12.0	120	+1.0
	0.003	11.0	110	+1.0
Actinomycin D	0.3	7.0	70	−3.5
	0.1	16.0	160	−2.0
	0.03	15.0	150	+0.5
	0.01	14.0	140	+1.3
	0.003	13.5	135	+1.0
	0.001	11.0	110	+1.3
Vehicle	—	10.0	—	+1.5

[a] QD × 3, ip.
[b] Significant antitumor effect (T/C ≥ 125%).
Tox: Toxicity.

cells and for *in vivo* antitumor activity in mice. Actinomycin D was used as a reference compound in both *in vitro* and *in vivo* experiments. Cytotoxicity of the samples against B16-F10 (murine melanoma) and Moser (human colorectal carcinoma) cells was determined as described previously[3]. Both protactin and actinomycin Zp were highly cytotoxic, but the latter was more cytotoxic than the former *in vitro*. Against B16-F10 and Moser cells, the observed IC_{50} values of protactin were 0.007 and 0.09 μg/ml, those of actinomycin Zp <0.00016 and 0.06 μl/ml, and those of actinomycin D 0.002 and 0.9 μg/ml, respectively. Inhibitory effects of protactin on macromolecular synthesis (DNA, RNA and protein) were determined *in vitro*. Cultured L1210 murine leukemia cells (5×10^5 cells/ml) were incubated with test materials at 37°C for 15 minutes and then further incubated for 60 minutes after the addition of labeled precursors, [^{3}H]thymidine, [^{14}C]uridine or [^{3}H]leucine, respectively, to the cultured mixtures. After precipitation and washing with cold 5% trichloroacetic acid solution, the radioactivity incorporated into the acid-insoluble fraction of the tumor cells was determined in a liquid scintillation counter. Protactin and actinomycin D inhibited RNA synthesis stronger than DNA synthesis, whereas both the compounds showed no inhibitory effect on protein synthesis at 100 μg/ml, the highest concentration tested. *In vivo* antitumor activities of protactin and actinomycin Zp were tested in tumor-bearing female CDF_1 mice. After the intraperitoneal implantation of diluted ascitic fluid with 1.0×10^6 lymphocytic leukemia P388 cells into the mice, test compounds were administered intraperitoneally once a day on days 1, 2 and 3 (QD × 3). Both components demonstrated fairly good anti-P388 leukemic activity with maximum T/C values of 160%, similar to that of actinomycin D (Table 5).

Discussion

Streptomyces cucumerosporus No. L703-4 produced a new metabolite, protactin, along with

actinomycins. The structure of protactin was determined to be 4-methyl-3-hydroxyanthraniloyl-L-threonyl-D-valyl-5-methylprolyl-sarcosyl-L-*N*-methylvalyl lactone based on the spectral analyses. This type of molecule has been synthesized chemically[4,5]. Moreover, such a compound lacking an amino group may accumulate in the medium when actinomycin-producing Streptomyces are supplied 3-hydroxybenzoic acid or related analogs[6].

Protactin appeares to be the first example of an anthraniloylpentapeptide lactone metabolite discovered as a natural fermentation product and it may prove to be the direct precursor used for the biosynthesis of an actinomycin molecule *in vivo*. Protactin contains 5-methyl proline which is an unusual constituent present in the peptide moiety of actinomycin Z_5[7,8] and Schering 1a and 1b[9]. By contrast, actinomycin Zp, formed by chemical oxidation is a new actinomycin that possessed two residues of 5-methylprolines in the molecule. Although protactin exhibited antimicrobial and antitumor activity under the experimental conditions employed, at least a part of this activity may be due to actinomycin Zp synthesized during incubation. Further tests are needed to clarify whether protactin *per se* has significant biological activity.

Experimental

TLC was performed on precoated silica gel plates (Kieselgel $60F_{254}$, Merck). IR and UV spectra were recorded on a Jasco IR-810 spectrophotometer and a Jasco UVIDEC-610C spectrophotometer, respectively. EI-MS and SI-MS spectra were measured on a Hitachi M80B, and HREI-MS on a Jeol JMS-DX-300 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Jeol JMN-GX 400 spectrometer operated in Fourier transform mode. Amino acid analysis was carried out using the Waters PICO-TAG system.

Conversion of Protactin to Actinomycin Zp

A solution of protactin (**1**, 20 mg) in methanol (5 ml) was added to a stirred solution of potassium ferricyanide (30 mg) in 0.067 M phosphate buffer (pH 7.1, 5 ml). After stirring at room temperature for 10 minutes, the solution was diluted with water (20 ml) and extracted 3 times with 15 ml portions of ethyl acetate. The combined extracts were dried over sodium sulfate and evaporated to afford a homogeneous solid of actinomycin Zp (17 mg).

IR (KBr) cm^{-1} 3250, 2970, 2940, 2860, 1750, 1670, 1630, 1580, 1510, 1480, 1190, 1090; ^1H NMR (400 MHz, $CDCl_3$) δ 0.74 (6H, d, J=6.6 Hz), 0.90 (3H, d, J=6.9 Hz), 0.92 (3H, d, J=6.9 Hz), 0.96 (3H, d, J=6.6 Hz), 0.98 (3H, d, J=6.6 Hz), 1.12 (3H, d, J=6.6 Hz), 1.13 (3H, d, J=6.6 Hz), 1.26 (6H, d, J=5.5 Hz), 1.48 (6H, d, J=5.9 Hz), 1.7~2.2 (6H, m), 2.25 (3H, s), 2.55 (3H, s), 2.6~2.7 (4H, m), 2.89 (6H, s), 2.93 (3H, s), 2.94 (3H, s), 3.48 (2H, m), 3.62 (1H, d, J=17.6 Hz), 3.63 (1H, d, J=17.6 Hz), 4.31 (1H, m), 4.42 (1H, m), 4.52 (1H, m), 4.67 (1H, d, J=17.6 Hz), 4.68 (1H, m), 5.23 (2H, m), 6.06 (1H, d, J=9.0 Hz), 6.13 (1H, d, J=9.0 Hz), 7.07 (1H, d, J=6.6 Hz), 7.37 (1H, d, J=8.0 Hz), 7.61 (1H, d, J=8.0 Hz), 7.62 (1H, d, J=6.6 Hz), 8.23 (1H, d, J=5.9 Hz), 8.42 (1H, d, J=5.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 7.8 (q), 15.0 (q), 17.5 (q), 17.9 (q), 18.8 (q×3), 19.1 (q×4), 19.2 (q×2), 19.3 (q), 21.5 (d), 21.6 (d), 26.7 (d), 26.8 (d), 29.5 (d), 29.7 (d), 31.8 (d), 32.1 (d), 34.9 (q×2), 39.3 (q×2), 51.5 (t×2), 54.7 (d), 55.1 (d), 55.3 (d), 55.5 (d), 58.1 (d), 58.3 (d), 59.3 (d), 59.4 (d), 71.3 (d), 71.5(d), 75.1 (d×2), 102.1 (s), 113. 4(s), 125.7 (d), 127.5 (s), 129.1 (s), 130.3 (d), 132.9 (s), 140.5 (s), 145.0 (s), 145.8 (s), 147.4 (s), 166.3 (s), 166.4 (s), 166.6 (s×2), 167.6 (s), 167.7 (s), 168.5 (s), 169.0 (s), 173.4 (s×3), 173.9 (s), 179.1 (s).

Mild Alkaline Hydrolysis

1 (5.0 mg) was dissolved in 0.1 N sodium hydroxide (0.5 ml) and kept at room temperature for 2 days. After neutralization with 0.1 N hydrochloric acid (pH 7.0), the reaction mixture was diluted with water (10 ml) and extracted with BuOH (10 ml×2). The BuOH layer was concentrated to give a colorless solid, which was purified by preparative TLC (SiO_2, CH_2Cl_2-MeOH, 50:2) to yield protactin acid (**2**, 2.7 mg). **2**: White powder; SI-MS *m/z* 685 $(M+Na)^+$, 667 $(M+Na-H_2O)^+$; IR (KBr) cm^{-1} 3400, 2940, 1640, 1470, UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 217 (478), 261 (sh, 110), 332 (46).

Acetylation

1 (10 mg) was stirred with acetic anhydride (0.5 ml) and dry pyridine (1.0 ml) for 20 hours at room temperature. The reaction mixture was diluted with ethyl acetate (20 ml), and the solution was washed

successively with dil HCl (20 ml) and water (20 ml). The organic solution was dried over sodium sulfate and evaporated *in vacuo* to yield a solid acetyl derivative (12 mg). It was purified by preparative TLC (SiO_2, CH_2Cl_2 - MeOH, 9 : 1).

Di-*N*,*O*-acetylprotactin (**3**, 6.4 mg): White powder; TLC (SiO_2, CH_2Cl_2 - MeOH, 9 : 1) Rf 0.65; SI-MS *m/z* 729 $(M+H)^+$; IR (KBr) cm^{-1} 3420, 2970, 1750, 1670, 1520,1480, 1190; ^1H NMR (400 MHz, DMSO-d_6) δ 0.70 (3H, d, J=6.6 Hz), 0.76 (3H, d, J=6.6 Hz), 0.89 (3H, d, J=6.6 Hz), 0.93 (3H, d, J=6.6 Hz), 1.13 (3H, d, J=6.2 Hz), 1.21 (3H, d, J=6.2 Hz), 1.63 (1H, m), 1.84 (3H, m), 2.02 (1H, m), 2.03 (3H, s), 2.14 (3H, s), 2.29 (3H, s), 2.50 (1H, m), 2.75 (3H, s), 3.10 (1H, d, J=9.5 Hz), 3.14 (3H, s), 3.44 (1H, dd, J=5.9 and 10.2 Hz), 4.04 (1H, dq, J=6.2 and 13.2 Hz), 4.12 (1H, d, J=18.0 Hz), 4.62 (1H, d, J=18.0 Hz), 4.93 (1H, dd, J=9.0 and 2.2 Hz), 5.08 (1H, dq, J=2.2 and 6.2 Hz), 6.26 (1H, dd, J=4.0 and 7.9 Hz), 7.27 (1H, d, J=9.0 Hz), 7.29 (1H, d, J=8.1 Hz), 7.39 (1H, d, J=8.1 Hz), 8.41 (1H, d, J=5.9 Hz), 9.62 (1H, s).

Acid Hydrolysis of **1**

1 (5.0 mg) in 6 N HCl (0.5 ml) was heated in a sealed tube at 105°C for 16 hours. The reaction mixture was diluted with water (30 ml) and washed with ethyl acetate. The aq solution was evaporated *in vacuo*, lyophilized and subjected to TLC and amino acid analysis. Threonine, valine and *N*-methylvaline were identified by a direct comparison with authentic samples obtained from actinomycin D. The configuration of these amino acids was determined to be L-threonine (Rt 4.11 minutes), D-valine (Rt 8.14 minutes) and *N*-methyl-L-valine (Rt 8.67 minutes) by chiral HPLC (Column: MCI-Gel CRS 10W, 4.6 × 50 mm, Mitsubishi Chemical Industries Limited, elution: $CuSO_4$ 0.5 mM, flow rate 0.5 ml/minute, detection: UV 254 nm, Rt: D-Thr 3.60 minutes, L-Thr 4.11 minutes, D-Val 8.14 minutes, L-Val 14.47 minutes, *N*-Me-D-Val 7.16 minutes, *N*-Me-L-Val 8.67 minutes).

Acknowledgments

The authors wish to express their thanks to Dr. H. KAWAGUCHI, the expresident and to Dr. T. OKI, the Director of the Institute for their encouragement and valuable advice during this study.

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