PROTHRACARCIN, A NOVEL ANTITUMOR ANTIBIOTIC

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A novel antibiotic, prothracarcin was isolated from the culture broth of *Streptomyces umbrosus* subsp. *raffinophilus* DO-62. The antibiotic has the molecular formula of $C_{14}H_{14}N_2O$ and belongs to the pyrrolo [1,4]benzodiazepine antibiotics. Its structure has been elucidated by mass and NMR spectra. It is active against Gram-positive and Gram-negative bacteria and experimental murine tumor sarcoma 180 and leukemia P388.

In the course of our screening program for novel antitumor antibiotics, an actinomycete strain DO-62 identified as *Streptomyces umbrosus* subsp. *raffinophilus* DO-62 was found to produce a novel antibiotic named prothracarcin that was previously designated DC-62.[1]

Prothracarcin was active against Gram-positive and Gram-negative bacteria and murine tumors. In this paper, the taxonomy of the producing strain, the fermentation, the isolation and characterization, the structure elucidation and the biological activities are described.

Taxonomy

The taxonomic characterization was carried out according to the method used in the International Streptomyces Project (ISP),[2] since the prothracarcin-producing strain was found to represent a species of *Streptomyces*.

Microscopic observation showed abundant aerial mycelium with straight to flexious spore chains. The mature spore chains were generally long with 10 to 30 or more spores per chain. The spores were oval in shape ($0.4\sim0.5\ \mu\times0.8\sim1.2\ \mu$) and possessed a smooth surface as seen by the electron microscope.

The aerial mycelia were white (colorless) to gray colored on agar media, thus a member of the color series "gray". The beige brown color occurred in aged cultures on inorganic salts - starch agar (ISP No. 4) and the reverse color of mycelia was golden to brown. Melanoid pigments were produced on some agar media including peptone - yeast extract - iron agar and tyrosine agar. These cultural characteristics are summarized in Table 1.

The physiological characteristics of strain DO-62 and utilization of carbon sources are shown in Tables 2 and 3. The temperature range for growth and the pH range for growth were observed after cultivation of two days and the action upon milk and decomposition of cellulose were observed after one month. All other observations were made after 20 days. Cell wall analysis revealed the presence of LL-diaminopimeric acid.

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Table 1. Cultural characteristics of DO-62.

Medium	Color of colony*			Growth and color	
	Growth	Surface	Reverse	Aerial mycelium	Pigment
Czapeck agar (Waksman No. 1)	Good Flat	Oak brown (4pi)	Deep brown (3ni)	Poor Sand (3cb)	Dull gold (2ng)
Glucose - asparagine agar (Waksman No. 2)	Moderate Flat	Spice brown (4ni)	Tile red (5ne)	Poor Pearl (3ba)	Gold (1 1/2 nc)
Yeast extract - malt extract agar (ISP No. 2)	Good Flat	Golden brown (3pi)	Mustard gold (2pg)	Good White (a)	Mustard gold (2pg)
Oatmeal agar (ISP No. 3)	Good Raised	Silver gray (3fe)	Dark covert gray (2ih)	Good Ashes (5fe)	Light beige (3ec)
Inorganic salts - starch agar (ISP No. 4)	Good Flat	Black plum (10po)	Deep brown (4pl)	Good Beige brown (3ig)	Light brown (4ng)
Glycerol - asparagine agar (ISP No. 5)	Good Flat	Rose beige (4ge)	Dark luggage tan (4pg)	Poor Sand (3cb)	Yellow maple (3ng)
Peptone - yeast extract - iron agar (ISP No. 6)	Poor Flat	Beaver gray (3ml)	Clove brown (3ni)	None	Dark brown (5pn)
Tyrosine agar (ISP No. 7)	Good Flat	Ebony brown (8pn)	Black plum (10po)	Good Natural (3dc)	Dark rose brown (7pn)
Nutrient agar	Poor Flat	Pearl pink (3ca)	Gold (2lc)	Poor White (a)	Topaz (3ne)

* Color designation from Color Harmony Manual, 4th Edition, Container Corporation of America, 1958.

Table 2. Physiological properties of strain DO-62.

Property	Result
Liquefaction of gelatin	Negative
Liquefaction of milk	Positive
Peptonization of milk	Negative
Decomposition of cellulose	Weakly positive
Hydrolysis of starch	Positive
Formation of tyrosinase	Positive
Formation of melanoid pigments	Positive
Optimum growth temperature	28 ~ 38°C
Optimum growth pH	6.6 ~ 7.5

Table 3. Utilization of carbohydrates by strain DO-62.

D-Arabinose	±	Sucrose	+
D-Xylose	−	*m*-Inositol	++
D-Glucose	+	Raffinose	++
D-Fructose	+	L-Rhamnose	+
D-Mannitol	−		

These characteristics of strain DO-62 shown in Tables 1, 2 and 3 place the organism in the genus *Streptomyces* and resemble closely those of *Streptomyces umbrosus*.[3] Although *Streptomyces umbrosus* does not utilize sucrose and raffinose,[4] we concluded that strain DO-62 could be designated as *Streptomyces umbrosus* subsp. *raffinophilus* DO-62.

Fermentation

Seed flasks were inoculated with stock cultures maintained in a deep freezer (−70°C) and grown for 48 ~ 72 hours at 28°C. The seed medium consisted of 4 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 5 g corn steep liquor, 20 g sucrose, 10 g glucose, 10 g fructose and 20 g $CaCO_3$ per liter of tap water. A 5% vegetative seed was used to inoculate the fermentation medium consisting of 0.3 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g corn steep liquor, 30 g soybean meal and 40 g soluble starch per liter of tap water. The pH of media was adjusted to 7.0 prior to sterilization.

The time course of the fermentation is shown in Fig. 1 where total antibacterial activity reached a maximum at 48 hours of incubation. As described in the later section, strain DO-62 coproduced pretomaymycin along with prothracarcin and total activity included both the pretomaymycin and pro-

Fig. 1. Time course of prothracarcin fermentation.

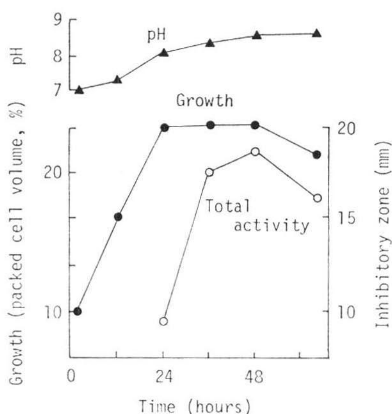
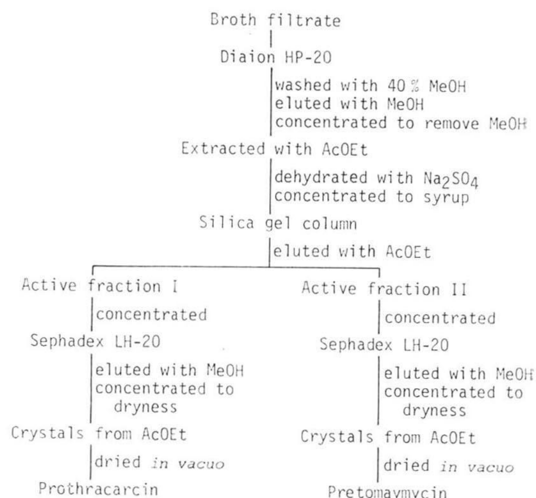


Fig. 2. Procedure for isolation.



thracarcin. No attempt was made to measure the two compounds separately. The total activity was determined by disc method on nutrient agar using *Bacillus subtilis* as the test organism and was expressed as diameters of growth inhibitory zones. Growth was expressed as packed cell volume that was determined by centrifugation at $1,200\times g$ for 10 minutes.

Isolation and Purification

Activity against *Bacillus subtilis* and thin-layer chromatography were used to monitor prothracarcin and pretomaymycin during isolation from the culture broth. Since prothracarcin and pretomaymycin are lipophilic, they were isolated by the usual methods for such compounds (Fig. 2). The whole broth (18 liters) was filtered with aid of 10% Celite. The solid cake was discarded and the culture filtrate was applied on a column (1 liter) of non-ionic porous resin, Diaion HP-20 (Mitsubishi Chemical Ind.). After washing with water (2 liters) and then with aqueous methanol (2: 3, v/v, 2 liters) to remove impurities, the column was eluted with 5 liters of methanol. The eluate was concentrated to remove methanol and the residual aqueous layer was extracted with ethyl acetate. The solvent layer was dehydrated with Na_2SO_4 and was concentrated to leave a syrup. The syrup was carefully put on a column (300 ml) of silica gel (Wakogel C-200, Wako Junyaku, Japan) and eluted with ethyl acetate.

The active fraction I containing prothracarcin was eluted first and then the active fraction II containing pretomaymycin was eluted. The active fraction I was concentrated to dryness and dissolved in a small amount of methanol. This concentrate was applied on a column of Sephadex LH-20 (Pharmacia Fine Chemicals Inc., Sweden) and eluted with methanol. A small amount of pretomaymycin was eluted first and then main fractions containing prothracarcin were eluted. Fractions containing prothracarcin were concentrated to dryness and crystallized from ethyl acetate. Crystals were dried *in vacuo* to obtain prothracarcin. The active fraction II containing pretomaymycin was treated similarly redundant to obtain crystals of pretomaymycin.

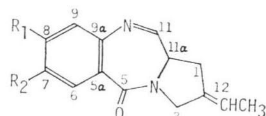
By the above procedures, the amounts of pretomaymycin and prothracarcin obtained were 10 mg and 23 mg, respectively.

Physico-chemical Properties and Structure Elucidation

The identification of pretomaymycin obtained in the previous section was made by a high resolution

mass measurement that gave the molecular formula $C_{15}H_{18}N_2O_3$ and by NMR spectra as shown in Tables 4 and 5. Signals appeared in the PMR spectrum were in good agreement with those of pretomaymycin.[5,6] The UV absorption spectrum of pretomaymycin in methanol was identical to that of tomaymycin (Fig. 3).[7]

Table 4. PMR spectra of prothracarcin and pretomaymycin.

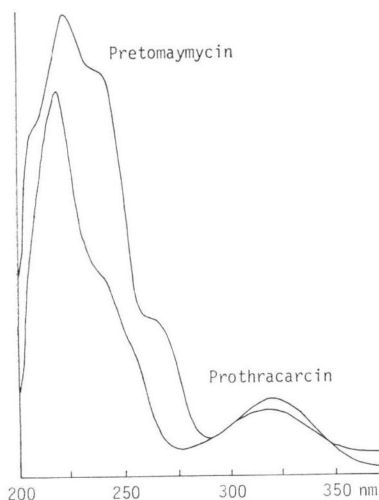


Prothracarcin: $R_1=R_2=H$
Pretomaymycin: $R_1=OH$, $R_2=OCH_3$

	Prothracarcin	Pretomaymycin
1-H	2.97	2.98
3-H	4.28	4.26
6-H	7.99~8.09	7.49
7-H		
8-H	7.33~7.63 (7-H, 8-H, 9-H)	
9-H		6.89
11-H	7.78	7.65
11a-H	3.91	3.83
12-H	5.61	5.58
7-OCH_3		3.92
12-CH_3	1.75	1.74

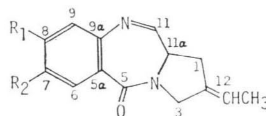
δ (ppm) in $CDCl_3$

Fig. 3. UV Absorption spectra in MeOH.



Most of pyrrolo [1,4]benzodiazepine antibiotics are known to form methanol adducts in MeOH and thus these spectra are actually of tomaymycin (methanol adduct of pretomaymycin) and of methanol adduct of prothracarcin.

Table 5. CMR spectra of prothracarcin and pretomaymycin.



Prothracarcin: $R_1=R_2=H$
Pretomaymycin: $R_1=OH$, $R_2=OCH_3$

	Prothracarcin	Pretomaymycin
1	31.1	31.2
2	119.0	119.6
3	53.7	54.0
5	164.9	164.4
5a	127.3	132.8
6	126.6*	112.7
7	126.7*	148.7
8	130.2*	141.1
9	131.4*	119.0
9a	145.8	145.7
11	164.8	163.1
11a	51.6	51.7
12	108.9	111.1
12-CH_3	14.0	14.6
7-OCH_3		56.2

δ (ppm) in $CDCl_3$
* Assignment may be reversed.

Table 6. Physico-chemical properties of prothracarcin.

M.P.	85~87°C (dec.)
Mol. Form.	$C_{14}H_{14}N_2O$
Analysis	C: 74.12 H: 6.32 N: 12.15
UV λ_{max} nm (ε) in MeOH	218 (21,000) 239 (sh 11,000) 255 (sh 7,000) 316 (4,000)
$[\alpha]_D^{22}$ (*c* 0.1, AcOEt)	+17.1°
Solubility	Soluble in DMSO, $CHCl_3$, MeOH, EtOH, AcOEt Partly soluble in ethyl ether Insoluble in H_2O, petroleum ether

The UV absorption spectrum of prothracarcin was similar to that of pretomaymycin, although its profile was shifted toward shorter wave length (Fig. 3). The molecular formula of prothracarcin was determined by a high resolution mass measurement as $C_{14}H_{14}N_2O$ that was in good agreement with the elemental analysis (Table 6).

As shown in Table 4, PMR spectrum of prothracarcin resembled closely that of pretomaymycin except signals in the aromatic region. Appearance of signals corresponding to one low field aromatic proton (7.99~8.09 ppm, m) and three aromatic protons (7.33~7.63 ppm, m) and lack of a methoxy

Fig. 4. EI-MS spectrum of prothracarcin.

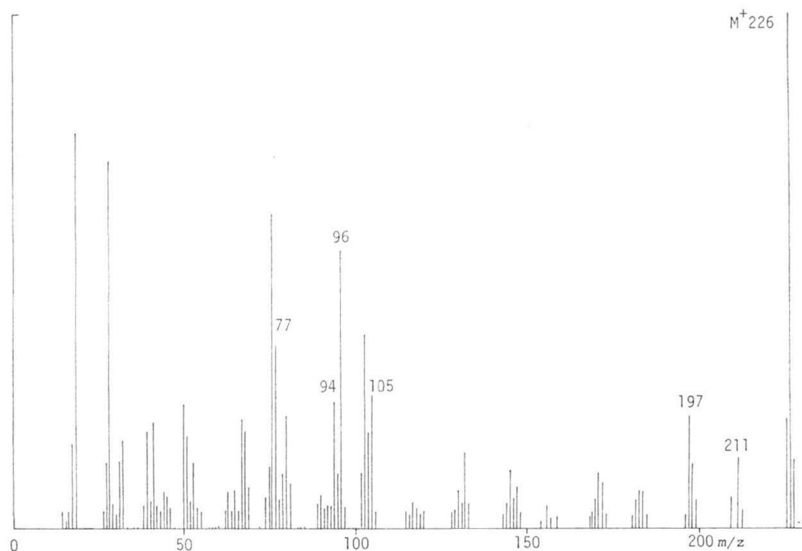
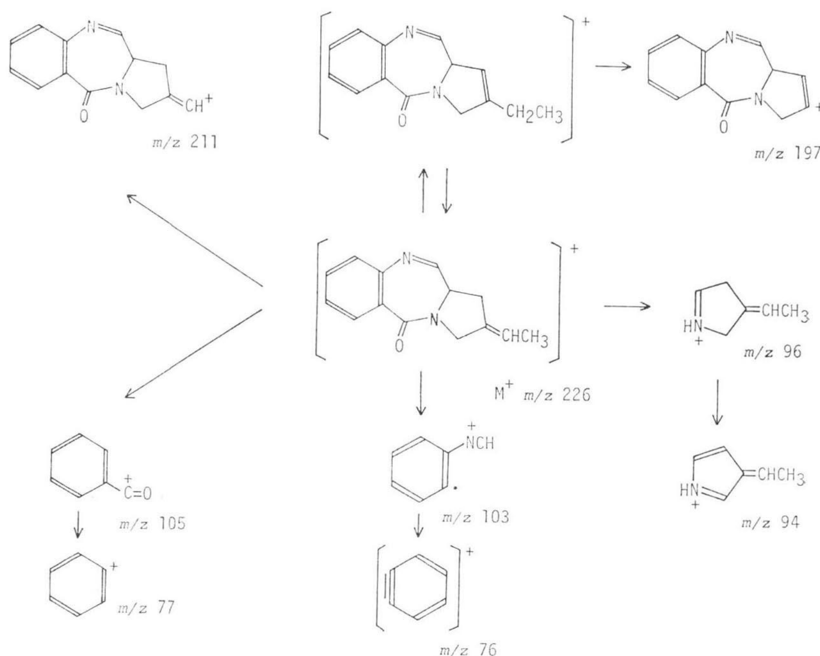


Fig. 5. Fragmentation pathways of prothracarcin in EI-MS spectroscopy.



signal at 3.92 ppm suggested that prothracarcin has a non-substituted aromatic ring. Lack of an absorption peak at about 3200 cm^{-1} in IR absorption spectrum (data not shown) confirmed the absence of phenolic group in prothracarcin. This speculation was further supported by CMR spectrum of prothracarcin (Table 5).

Fragmentation pathways of prothracarcin in EI-MS also supported the proposed structure (Figs. 4 and 5). Thus, we concluded that the structure of prothracarcin is 8-dehydroxy-7-demethoxypretomaymycin.

Other physico-chemical properties of prothracarcin are summarized in Table 6.

Biological Activities

The *in vitro* activities against various bacteria are shown in Table 7. Prothracarcin showed weak activities against Gram-positive bacteria and Gram-negative bacteria.

The acute toxicity of prothracarcin was calculated from the number of survivors at 14 days after a single intraperitoneal injection into *dd*Y mice. The LD_{50} of prothracarcin was 42 mg/kg.

Antitumor activities were examined as described in the previous paper.[8] For comparison mitomycin C was similarly administered intraperitoneally to a group of test animals. As shown in Tables 8 and 9, prothracarcin exhibited antitumor activities against murine tumors.

Table 7. Antibacterial activity of prothracarcin.

Test organism	MIC (μg/ml)
Staphylococcus aureus ATCC 6538P	50
Bacillus subtilis No. 10707	50
Escherichia coli ATCC 26	50
Salmonella typhosa ATCC 9992	>100
Shigella sonnei ATCC 9290	>100

Table 8. Antitumor activity of prothracarcin against murine sarcoma 180 (s.c.-i.p.).

Compound	Dose (mg/kg)	Tumor volume (mm^3)	T/C
Control	—	1272	—
Prothracarcin	25.0	477	0.38
	12.5	1264	0.99
Mitomycin C	6.0	237	0.19

Drugs were injected intraperitoneally 24 hours after tumor implantation. T/C represents the ratio of the median tumor volume of the treated group divided by that of the control group. T/C≤0.5 was considered active.

Table 9. Antitumor activity of prothracarcin against murine leukemia P388 (i.p.-i.p.).

Compound	Dose (mg/kg)	Survival days	ILS %
Control	—	9.0	—
Prothracarcin	25.0	11.4	27
	12.5	9.2	2
Mitomycin C	4.0	11.8	31

Drugs were injected intraperitoneally 24 hours after tumor implantation. Increase of life span (ILS %) was calculated from the average life span of the treated mice and that of the control mice. ILS≥20 was considered active.

Acknowledgements

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