BENASTATINS C AND D, NEW INHIBITORS OF GLUTATHIONE *S*-TRANSFERASE, PRODUCED BY *Streptomyces* sp. MI384-DF12

PRODUCTION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITIES

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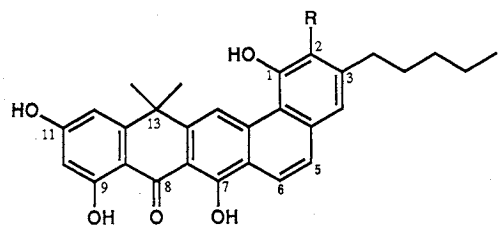
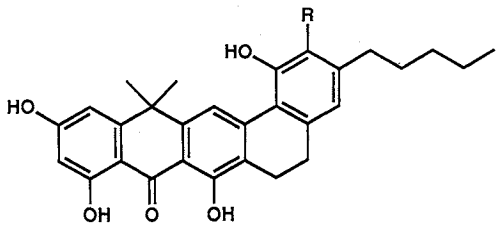
Benastatin C, a new member of the benastatins, has been isolated from the culture broth of *Streptomyces* sp. MI384-DF12. The structure of benastatin C was elucidated as 2-decarboxybenastatin A by NMR studies. Benastatin D, 2-decarboxybenastatin B, was derived from benastatin B. Benastatins C and D showed inhibitory activities against human pi class glutathione *S*-transferase (GST π) and excellent stimulatory activities on the murine lymphocyte blastogenesis *in vitro*.

We have previously reported that benastatins A (**1**) and B (**2**) were isolated from the culture broth of *Streptomyces* sp. MI384-DF12 as novel inhibitors of glutathione *S*-transferase (GST, EC 2.5.1.18)[1,2]. The biosynthetic studies showed that **1** was derived from a tetradecaketide and two methionine units[3]. In our successive study to find a new analogue in the culture broth of the benastatin producing strain by using a HPLC system equipped with the photodiode array detector, benastatin C (**3**), 2-decarboxybenastatin A, was discovered (Fig. 1). Furthermore, benastatin D (**4**) was prepared by decarboxylation of **2**. In this communication we report the production, isolation and structure determination of **3**, preparation of **4** and biological activities.

Fig. 1. Structures of benastatins.



Benastatin A (**1**) R=COOH
Benastatin C (**3**) R=H



Benastatin B (**2**) R=COOH
Benastatin D (**4**) R=H

Materials and Methods

Chemicals

Chemicals employed were as follows: Silanised silica gel 60, Silica gel 60 and TLC-plate Silica gel F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG; YMC GEL (ODS-A60-200/60) from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; packed column of Capcell Pak C_{18} from Shiseido Co., Tokyo, Japan; Quinoline from Aldrich Chemical Company, Inc., Milwaukee, U.S.A.; Glutathione, reduced form (GSH) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; 1-chloro-2,4-dinitrobenzene (CDNB) from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. All other chemicals were of analytical grade.

Analytical Instruments

HPLC was performed by a GILSON's system equipped with a Waters 991J photodiode array detector. Melting points were taken using a Yanaco MP-S3 apparatus and were uncorrected. UV spectra were recorded on a Hitachi U-3210 spectrophotometer, and IR spectra on a Hitachi 260-10 spectrophotometer. Mass spectra were obtained on a Jeol JMS-SX 102 mass spectrometer. NMR spectra were recorded on a Jeol JNM-GX400 NMR spectrometer with ^1H NMR at 400 MHz and ^{13}C NMR at 100 MHz.

Production of 3

A loopful of slant culture of *Streptomyces* sp. MI384-DF12 (FERM P-11270) was inoculated into 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto Soytone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 30°C for 3 days on a rotary shaker (180 rpm). Two ml of this seed culture were inoculated into 110 ml of the production medium consisting of glycerol 2.0%, soy bean meal (Ajinomoto Co., Inc.) 1.5%, K_2HPO_4 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005% (pH 6.2 adjusted with 1 M KH_2PO_4 before sterilization) in a 500-ml Erlenmeyer flask and cultured at 27°C for 4 days on a rotary shaker (180 rpm).

Isolation of 3

The culture broth was filtered and separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with methanol; the extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was combined with the culture filtrate and extracted with an equal volume of ethyl acetate. The extract was concentrated to dryness under reduced pressure. Since the UV spectrum of **3** was similar to that of **1**, the following purification steps were performed using the photodiode array detector of HPLC system. The dried material was chromatographed on a column of silanised silica gel ($\times$ 50 W/W powder) with a linear gradient 40 to 100% aq MeOH and on a column of YMC GEL ($\times$ 100 W/W powder) with the same linear gradient, successively. The fractions containing **1**, **2** and **3** were collected and evaporated to give a brownish powder. The powder was suspended in a solvent mixture of $CHCl_3$-MeOH (90:10), charged to a silica gel column ($\times$ 100 W/W powder), and eluted with $CHCl_3$-MeOH (85:15). The eluate was concentrated under reduced pressure to give a yellowish powder. The crude powder was further purified by a reversed phase HPLC using a Capcell Pak C_{18} column (2.0 $\times$ 25 cm, flow rate 8 ml/minute) with a solvent mixture of CH_3CN-H_2O-AcOH (78:22:1). The fractions to appear first are composed of **2**, the fractions coming next comprise **3**, and the fractions coming last composed of **1**. Each eluate was evaporated to dryness to obtain a yellow powder.

Decarboxylation of 2

The solution of **2** (203 mg), $CuSO_4$ (5.1 mg) and quinoline (1 ml) was stirred at 180°C for 30 minutes under argon. After the reaction, the mixture was diluted with ethyl acetate and washed with 1 N HCl to eliminate quinoline. The organic layer was dried over $MgSO_4$, and filtered. The filtrate was concentrated to dryness under reduced pressure and was chromatographed on a column of silica gel, which was eluted with hexane-ethyl acetate (2:1) to give yellow powder (172.9 mg) of 2-decarboxybenastatin B (**4**).

4: Rf 0.80 ($CHCl_3$-MeOH, 4:1); mp 234~236°C (dec.); FAB-MS *m/z* 457 $(M-H)^-$; UV λ_{max}^{MeOH} nm ($\log\varepsilon$) 212 (4.78), 223 (4.75), 264 (4.02), 274 (3.98), 305 (4.09), 388 (4.69); IR (KBr) cm^{-1} 3430, 2940, 1614, 1503, 1468, 1373, 1324, 1283, 1210, 1170, 1033, 833; ^1H NMR (400 MHz, DMSO-d_6) δ 0.88 (3H,

t, J=7.0 Hz), 1.31 (4H, m), 1.57 (2H, m), 1.63 (6H, s), 2.49 (2H, br t, J=7.6 Hz), 2.70 (4H, m), 6.28 (1H, d, J=2.2 Hz), 6.62 (1H, br s), 6.70 (1H, br s), 6.72 (1H, d, J=2.2 Hz), 8.34 (1H, s), 10.06 (1H, br s), 12.90 (1H, s), 12.96 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.9, 19.9, 22.0, 28.9, 30.2, 31.0, 33.8, 34.9, 38.4, 101.0, 106.7, 110.8, 115.1, 117.5, 119.4, 122.1, 140.6, 140.9, 144.4, 148.1, 154.9, 155.7, 157.8, 164.9, 165.5, 189.6.

Enzymes

Partially purified GST was prepared from rat liver as described by WU *et al.*[4]. Human pi class GST (GST π) was kindly provided by Dr. K. TAKAHASHI, University of Tokyo.

Assay for Glutathione *S*-transferase (GST) and Inhibitory Activity

GST activity was measured by a modification of the method of HABIG *et al.*[5]. The reaction mixture (total 2.0 ml) consisted of 100 mM potassium phosphate buffer (pH 7.4 for partially purified GST and pH 6.5 for GST π), 1.0 mM GSH, 1.0 mM CDNB, partially purified GST or GST π and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, following incubation at 30°C for 5 minutes. The conjugate was determined spectrophotometrically at 340 nm by monitoring changes in Beckman DU-8 spectrophotometer, corrections were made for chemical reactivity.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the conjugate by the enzyme in the system without an inhibitor and B is that with an inhibitor. IC_{50} value shows the concentration of inhibitor at 50% inhibition of enzyme activity.

Assay of Mitogen Induced Murine Lymphocyte Blastogenesis *In Vitro*

The tissue culture medium employed was a complete medium designated Roswell Park Memorial Institute (RPMI)-1640 supplemented with 20% fetal calf serum, 25 mM Hepes buffer, 100 μg/ml of streptomycin sulfate and 100 units/ml of benzylpenicillin. Lipopolysaccharide (LPS) or concanavalin A (Con A) were utilized to stimulate unseparated spleen cells or spleen T cells at final concentrations of 100 and 5 μg/ml, respectively.

After spleens were aseptically removed from BALB/c mice, single cell suspension of the spleen cells was prepared and subjected to a hyper shock treatment to remove erythrocytes therefrom. Spleen T cells were separated from the spleen cells by passing through a Nylon column. Two $\times 10^5$ spleen cells or spleen T cells were mixed with the prescribed concentration of the test compound (total 0.2 ml) in each well of a microplate (COSTAR) with a flat bottom, and were incubated at 37°C in a humidified atmosphere (95% air, 5% CO_2) for 72 hours. Before 8 hours from the completion of cultivation, 37 KBq of [^{3}H]thymidine was added to each well. The resultant cells were harvested and measured for [^{3}H]-thymidine incorporation into DNA.

Results and Discussion

Production and Isolation of 3

The strain of *Streptomyces* sp. MI384-DF12 was cultured in Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. As shown in Fig. 2, **3** was isolated from the culture broth (30 liters) by chromatography of reversed-phase silica gel, silica gel and Capcell Pak C_{18} (HPLC) followed by solvent extraction and the total yield was 42.5 mg. The purity of each preparation was confirmed by TLC and HPLC.

Structure of 3

The physico-chemical properties of **3** are summarized in Table 1. **3** is soluble in dimethyl sulfoxide, sparingly soluble in methanol, acetone and ethyl acetate, but insoluble in water. The molecular weight and formula of **3** were elucidated as $C_{29}H_{28}O_5$ (MW 456) from the FAB-MS peaks at m/z 457 $(M+H)^+$

Fig. 2. Isolation procedure for benastatins.

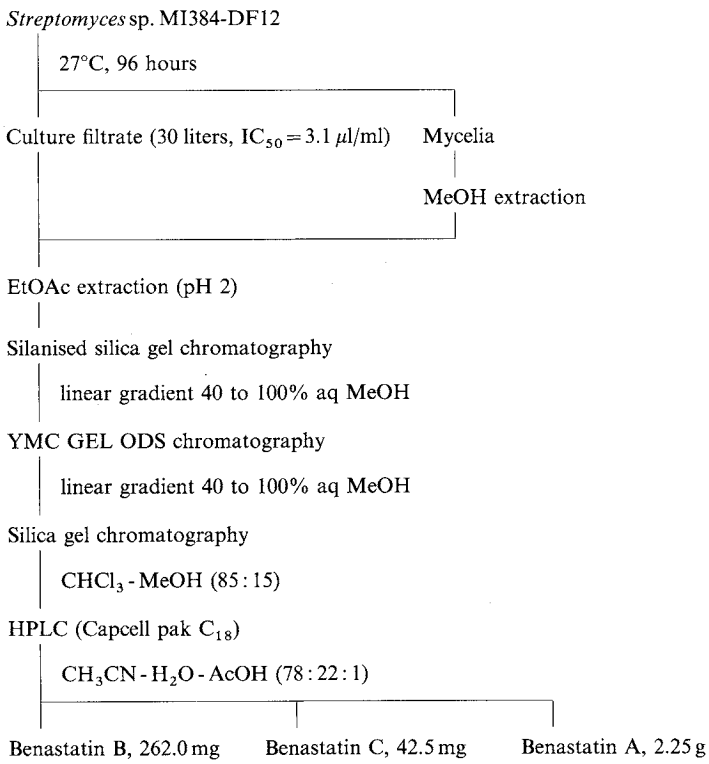


Table 1. Physico-chemical properties of benastatin C.

Appearance	Yellow powder
MP (°C)	214~216 (dec)
FAB-MS (*m/z*)	Positive: 457 $(M+H)^+$, Negative: 455 $(M-H)^-$
MW	456
Molecular formula	$C_{29}H_{28}O_5$
UV nm (log ε)	
λ_{max}^{EtOH}:	209 (4.53), 267 (4.39), 284 (4.38), 311 (4.30), 333 (4.21), 386 (4.44)
$\lambda_{max}^{EtOH-HCl}$:	209 (4.50), 267 (4.39), 284 (4.37), 311 (4.30), 333 (4.21), 386 (4.44)
$\lambda_{max}^{EtOH-NaOH}$:	280 (4.49), 323 (4.07), 369 (4.21), 422 (4.47)
IR ν_{max}^{KBr} cm^{-1}	3456, 2927, 1623, 1600, 1466, 1376, 1339, 1300, 1238, 1172, 1039, 862
Rf value on TLC	0.80 ($CHCl_3$-MeOH=4:1, silica gel)
Color reaction	Phosphomolybdate-H_2SO_4, $FeCl_3$
Solubility	
Soluble:	DMSO, MeOH, Me_2CO, EtOAc
Insoluble:	H_2O

and *m/z* 455 $(M-H)^-$ and ^1H and ^{13}C NMR spectra of **3** (Table 2). UV and IR spectra of **3** were similar to those of **1**. Though the ^1H and ^{13}C NMR spectra of **3** were almost coincident with those of **1**, there were some differences. **1** showed a carbonyl carbon signal at δ_C 171.6 (C-15) ppm, but this corresponding signal was not observed in the ^{13}C NMR spectrum of **3**. Instead of this carbon signal, an additional aromatic proton signal appeared at δ_H 7.03 (2-H) ppm and showed a coupling constant of 1.8 Hz with an aromatic proton at δ_H 7.26 (4-H) ppm in the ^1H NMR spectrum of **3**. Therefore, it was deduced that the aromatic proton located at *meta* position (C-2) to the 4-H. Furthermore, in the HMBC (Hetero-nuclear Multiple Bond Connectivity) spectrum of **3** (Fig. 3), the aromatic proton at δ_H 7.03 (2-H) ppm coupled to four carbons at δ_C 156.9 (C-1), 119.2

Fig. 3. ^{1}H-^{13}C correlation for **3** by the HMBC experiment.

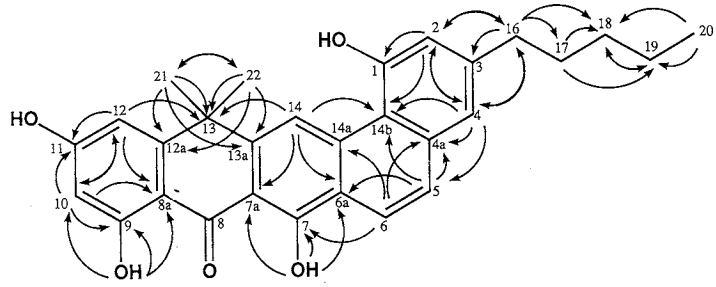


Table 2. ^{13}C and ^{1}H NMR data of benastatins A and C in DMSO-d_6.

Carbon	Benastatin A		Benastatin C	
	δ_C ppm (100 MHz)	δ_H ppm (J in Hz, 400 MHz)	δ_C ppm (100 MHz)	δ_H ppm (J in Hz, 400 MHz)
1	169.2 (s)	10.96 (1-OH, br)[a]	156.9 (s)	10.87 (1-OH, br)[a]
2	114.3 (s)		114.1 (d)	7.03 (1H, d, 1.8)
3	146.4 (s)		143.5 (s)	
4	116.8 (d)	6.89 (1H, s)	119.2 (d)	7.26 (1H, d, 1.8)
4a	136.4 (s)		136.0 (s)	
5	126.5 (d)	7.56 (1H, d, 9.4)	126.7 (t)	7.69 (1H, d, 9.4)
6	121.1 (d)	8.12 (1H, d, 9.4)	120.0 (t)	8.12 (1H, d, 9.4)
6a	118.9 (s)		119.5 (s)	
7	159.3 (s)	13.78 (7-OH, s)	159.3 (s)	13.81 (7-OH, s)
7a	107.7 (s)		108.2 (s)	
8	189.2 (s)		189.8 (s)	
8a	106.9 (s)		106.8 (s)	
9	165.0 (s)	12.92 (9-OH, s)	165.1 (s)	12.86 (9-OH, s)
10	100.9 (d)	6.28 (1H, d, 2.2)	101.0 (d)	6.29 (1H, d, 2.2)
11	165.6 (s)	10.96 (11-OH, br)[a]	165.8 (s)	10.87 (11-OH, br)[a]
12	106.8 (d)	6.78 (1H, d, 2.2)	106.9 (d)	6.76 (1H, d, 2.2)
12a	155.1 (s)		154.9 (s)	
13	39.0 (s)		40.4 (s)	
13a	145.7 (s)		145.8 (s)	
14	116.5 (d)	10.12 (1H, s)	116.5 (d)	9.59 (1H, s)
14a	137.6 (s)		135.8 (s)	
14b	117.5 (s)		116.2 (s)	
15	171.6 (s)			
16	35.8 (t)	3.20 (2H, br t, 7.6)	34.9 (t)	2.68 (2H, br t, 7.6)
17	31.3 (t)	1.60 (2H, m)	30.2 (t)	1.65 (2H, m)
18	31.7 (t)	1.32 (2H, m)	30.9 (t)	1.31 (2H, m)
19	22.2 (t)	1.32 (2H, m)	21.9 (t)	1.31 (2H, m)
20	14.0 (q)	0.87 (3H, t, 7.0)	13.9 (q)	0.86 (3H, t, 7.0)
21	34.4 (q)	1.74 (3H, s)	34.3 (q)	1.72 (3H, s)
22	34.4 (q)	1.74 (3H, s)	34.3 (q)	1.72 (3H, s)

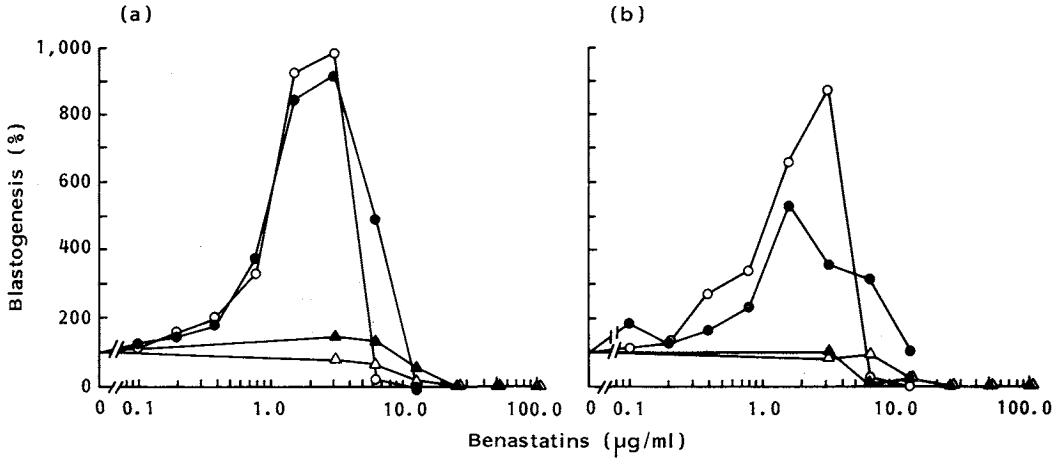
[a] The intensity of this broad signal corresponded to two hydroxy groups.

(C-4), 116.2 (C-14b) and 34.9 (C-16) ppm. Consequently, it was determined that **3** was a new analogue of benastatins (2-decarboxybenastatin A) and the structure of **3** was 8,13-dihydro-1,7,9,11-tetrahydroxy-13,13-dimethyl-8-oxo-3-pentylbenzo[*a*]naphthacene (Fig. 1).

After the structure determination of **3**, 2-decarboxybenastatin B was prepared by decarboxylation from **2** as described in the experimental section. The derived compound which was 5,6,8,13-tetra-

Fig. 4. Effect of benastatins on mitogen induced murine lymphocyte blastogenesis *in vitro*.

(a) LPS induced, (b) Con A induced, △ benastatin A, ▲ benastatin B, ○ benastatin C, ● benastatin D.



hydro-1,7,9,11-tetrahydroxy-13,13-dimethyl-8-oxo-3-pentylbenzo[*a*]naphthacene was designated benastatin D (**4**).

Table 3. Inhibitory activity of benastatins against GST.

Enzymes	IC_{50} (μg/ml)			
	Benastatins			
	A	B	C	D
GST[a]	2.20	0.50	24.00	65.00
GSTπ	1.25	1.10	0.16	0.70

[a] Partially purified from rat liver.

Biological Activities of 3 and 4

As shown in Table 3, **3** and **4** had no significant inhibitory activity against partially purified GST which was deduced to contain several kinds of isozymes, but showed more considerable activities against GST π than **1** and **2**, respectively. GST π is an isozyme of human pi class GST and is expressed in the process of carcinogenesis[6].

Moreover, **3** showed the inhibitory activity against esterase obtained from hog pancreas lipase (Nutritional Biochem., U.S.A.) which was located on the cellular surface[7]. Its IC_{50} value was 10.0 μg/ml. As reported previously, esterastin[8] and ebelactones A and B[9] were inhibitors of esterase, and the former suppressed and the latter enhanced immune responses. Therefore, **3** and **4** were subjected to the assay of the mitogen induced murine lymphocyte blastogenesis *in vitro*. **3** and **4** showed excellent stimulatory activities on the murine lymphocyte blastogenesis as shown in Fig. 4. **3** and **4** increased the incorporation of [^{3}H]thymidine into mitogen treated cells and did not show an effect on non-treated cells.

3 had no significant antimicrobial activity at 100 μg/ml. **4** had an activity against Gram-positive bacteria, but the activity of **4** was weaker than those of **1** and **2**. They have low toxicities; there were no deaths after ip injection of mice with 100 mg/kg.

Acknowledgment

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References

1) AOYAGI, T.; T. AOYAMA, F. KOJIMA, N. MATSUDA, M. MARUYAMA, M. HAMADA & T. TAKEUCHI: Benastatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. J. Antibiotics 45: 1385~1390, 1992
2) AOYAMA, T.; H. NAGANAWA, Y. MURAOKA, H. NAKAMURA, T. AOYAGI, T. TAKEUCHI & Y. IITAKA: Benastatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. II. Structure determination of benastatins A and B. J. Antibiotics 45: 1391~1396, 1992
3) AOYAMA, T.; H. NAGANAWA, Y. MURAOKA, T. AOYAGI & T. TAKEUCHI: Biosynthesis of benastatin A. J. Antibiotics 45: 1767~1772, 1992
4) WU, C. & K. P. MATHEWS: Indomethacin inhibition of glutathione *S*-transferases. Biochem. Biophys. Res. Commun. 112: 980~985, 1983
5) HABIG, W. H.; M. J. PABST & W. B. JACOBY: Glutathione *S*-transferases. The first step in mercapturic acid formation. J. Biol. Chem. 249: 7130~7139, 1974
6) MOSCOW, J. A.; C. R. FAIRCHILD, M. J. MADDEN, D. T. RANSOM, H. S. WIEAND, E. E. O'BRIEN, D. G. POPLACK, J. COSSMAN, C. E. MYERS & K. H. COWAN: Expression of anionic glutathione-*S*-transferase and P-glycoprotein genes in human tissues and tumors. Cancer Res. 49: 1422~1428, 1989
7) AOYAGI, T.; H. SUDA, M. NAGAI, H. TOBE, J. SUZUKI, T. TAKEUCHI & H. UMEZAWA: Release of a plasma membrane-bound triaminopeptidase activity from mammalian cells by thermolysin. Biochem. Biophys. Res. Commun. 80: 435~442, 1978
8) UMEZAWA, H.; T. AOYAGI, T. HAZATO, K. UOTANI, F. KOJIMA, M. HAMADA & T. TAKEUCHI: Esterastin, an inhibitor of esterase, produced by actinomycetes. J. Antibiotics 31: 639~641, 1978
9) UMEZAWA, H.; T. AOYAGI, K. UOTANI, M. HAMADA, T. TAKEUCHI & S. TAKAHASHI: Ebelactone, an inhibitor of esterase, produced by actinomycetes. J. Antibiotics 33: 1594~1596, 1980