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

II. STRUCTURE DETERMINATION OF BENASTATINS A AND B

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(Received for publication March 31, 1992)

Benastatins A and B, new inhibitors of glutathione *S*-transferase, have been isolated from the culture broth of *Streptomyces* sp. MI384-DF12. By X-ray crystallography, benastatin A was determined to be 8,13-dihydro-1,7,9,11-tetrahydroxy-13-dimethyl-8-oxo-3-pentyl-benzo[*a*]naphthacene-2-carboxylic acid. The structure of benastatin B was elucidated by NMR studies.

In the preceding paper[1], we have described the taxonomy, isolation, physico-chemical properties and biological activities of benastatins A and B, novel inhibitors of glutathione *S*-transferase (GST). In this paper, we describe the structure determination of benastatins A and B.

Spectroscopic Studies of Benastatin A Calcium Salt (**1**)

The molecular weight and formula of benastatin A were elucidated as $C_{30}H_{28}O_7$ (MW 500) from the FAB-MS (negative) peak at *m/z* 499 $(M-\frac{1}{2}Ca)^-$, HREI-MS (found: *m/z* 456.1921 $(M-\frac{1}{2}Ca-CO_2+H)^+$, calcd: *m/z* 456.1937 for $C_{29}H_{28}O_5$), elemental analysis after EtOAc extraction (pH 2) to remove the salt (found: C 69.22, H 6.10, O 24.55; calcd: C 69.49, H 5.83, O 24.68 for $C_{30}H_{28}O_7 \cdot H_2O$) and ^1H and ^{13}C NMR spectra of **1** (Table 1). The UV spectrum of **1** showed the maxima at 202 (log ε 4.54), 250 (4.39), 267 (4.32), 285 (4.30), 310 (4.26) and 391 (4.46) nm in EtOH, and the absorption band in alkaline solution exhibited bathochromic shifts from 379 nm in HCl - EtOH to 412 nm in NaOH - EtOH, suggesting the presence of a phenolic hydroxyl group.

The IR spectrum (KBr) of **1** showed the presence of a hydroxyl group (3430 cm^{-1}), and the presence of a carboxylate anion (1605 and 1395 cm^{-1}) and an aromatic ketone (1630 cm^{-1}) group which were supported by ^{13}C NMR signals at δ_C 179.2 (C-15) and 192.0 (C-8) ppm. Furthermore, the ^{13}C NMR spectrum of **1** revealed twenty signals of sp^2 carbons in addition to those of the two carbonyl carbons. Four of these sp^2 signals (C-1, C-7, C-9 and C-11) appeared in lower field (δ_C 165.4, 161.3, 166.9 and 166.8 ppm) indicative of oxygen-bearing carbons. The ^{13}C NMR spectrum also included eight signals of sp^3 carbons.

The ^1H NMR spectrum of **1** showed six aromatic protons, eleven aliphatic protons and a six-proton singlet at δ_H 1.72 which is ascribed to two isolated methyl groups (δ_C 35.1). An *ortho* spin-spin coupling between aromatic protons at δ_H 7.50 (5-H) and 8.24 (6-H) ppm was observed (J=9.4 Hz), and a *meta* spin-spin coupling between aromatic protons at δ_H 6.25 (10-H) and 6.65 (12-H) ppm was also observed

Table 1. ^{13}C and ^{1}H NMR data of benastatins calcium salts in CD_3OD.

Carbon	Benastatin A		Benastatin B	
	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	165.4 (s)		162.6 (s)	
2	115.1 (s)		113.5 (s)	
3	147.2 (s)		148.9 (s)	
4	121.4 (d)	7.08 (1H, s)	123.1 (d)	6.68 (1H, s)
4a	138.7 (s)		147.1 (s)	
5	127.0 (d)	7.50 (1H, d, 9.4)	20.8 (t)	2.71 (2H, m)
6	123.5 (d)	8.24 (1H, d, 9.4)	30.8 (t)	2.80 (2H, m)
6a	121.5 (s)		124.0 (s)	
7	161.3 (s)		159.5 (s)	
7a	109.7 (s)		112.8 (s)	
8	192.0 (s)		191.9 (s)	
8a	108.8 (s)		108.7 (s)	
9	166.9 (s)		166.9 (s)	
10	102.0 (d)	6.25 (1H, d, 2.4)	102.0 (d)	6.23 (1H, d, 2.4)
11	166.8 (s)		166.7 (s)	
12	107.6 (d)	6.65 (1H, d, 2.4)	107.5 (d)	6.65 (1H, d, 2.4)
12a	156.7 (s)		156.7 (s)	
13	40.4 (s)		39.9 (s)	
13a	147.7 (s)		150.0 (s)	
14	118.1 (d)	9.77 (1H, s)	119.1 (d)	8.38 (1H, s)
14a	138.2 (s)		141.6 (s)	
14b	118.5 (s)		120.7 (s)	
15	179.2 (s)		175.8 (s)	
16	37.2 (t)	3.26 (2H, br t, 7.6)	37.5 (t)	2.99 (2H, br t, 7.6)
17	33.2 (t)	1.69 (2H, m)	33.0 (t)	1.61 (2H, m)
18	33.5 (t)	1.35 (2H, m)	33.3 (t)	1.36 (2H, m)
19	23.8 (t)	1.35 (2H, m)	23.6 (t)	1.36 (2H, m)
20	14.5 (q)	0.87 (3H, t, 7.0)	14.4 (q)	0.91 (3H, t, 7.0)
21	35.1 (q)	1.72 (3H, s)	34.5 (q)	1.67 (3H, s)
22	35.1 (q)	1.72 (3H, s)	34.5 (q)	1.67 (3H, s)

(J=2.4 Hz). The ^{1}H-^{1}H COSY spectrum of **1** revealed that aliphatic protons (δ_H 0.87 (20-H), 1.35 (19-H), 1.35 (18-H), 1.69 (17-H) and 3.26 (16-H) ppm) belonged to a pentyl group.

In the HMBC (Heteronuclear Multiple Bond Connectivity) spectrum of **1**, the *gem*-dimethyl protons at δ_H 1.72 (21-H, 22-H) ppm coupled to four signals at δ_C 156.7 (C-12a), 40.4 (C-13), 147.7 (C-13a) and 35.1 (C-21 and C-22) ppm, and the aromatic proton at δ_H 6.65 (12-H) ppm coupled to four carbons at δ_C 108.8 (C-8a), 102.0 (C-10), 166.8 (C-11) and 40.4 (C-13) ppm. Consequently, the quaternary carbon at δ_C 40.4 (C-13) ppm was located at the *peri* position to the carbon at δ_C 107.6 (C-12) ppm. The methylene protons at δ_H 3.26 (16-H) ppm showed cross peaks with the signals at δ_C 115.1 (C-2), 147.2 (C-3) and 121.4 (C-4) ppm, and the aromatic proton at δ_H 7.08 (4-H) ppm showed cross peaks with the carbon signals at δ_C 115.1 (C-2), 138.7 (C-4a), 127.0 (C-5), 118.5 (C-14b) and 37.2 (C-16) ppm. The aromatic proton at δ_H 7.50 (5-H) ppm further correlated with the signals at δ_C 121.4 (C-4), 138.7 (C-4a), 123.5 (C-6), 121.5 (C-6a) and 118.5 (C-14b) ppm. Therefore, it was deduced that the pentyl group was located at the *ortho* position (C-3) to the proton at δ_H 7.08 (4-H) ppm and the 4-H was located at the *peri* position to the carbon at δ_C 127.0 (C-5) ppm.

From the above results, the presence of two partial structures (Fig. 1a and b) were revealed. But we

Fig. 1. Partial structures of benastatin A.

HMBC as solid-line arrows and proton spin couplings as dotted-line arrows.

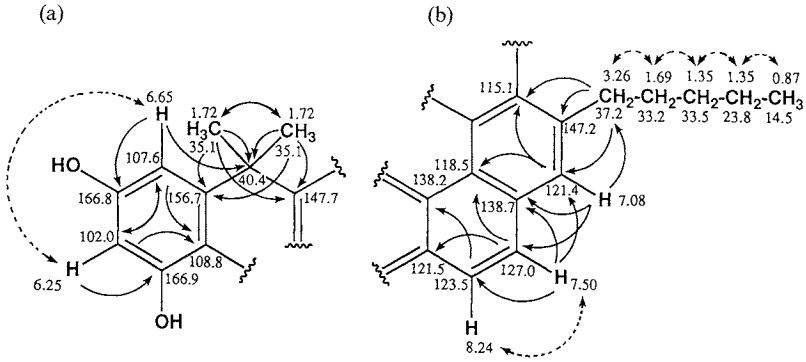
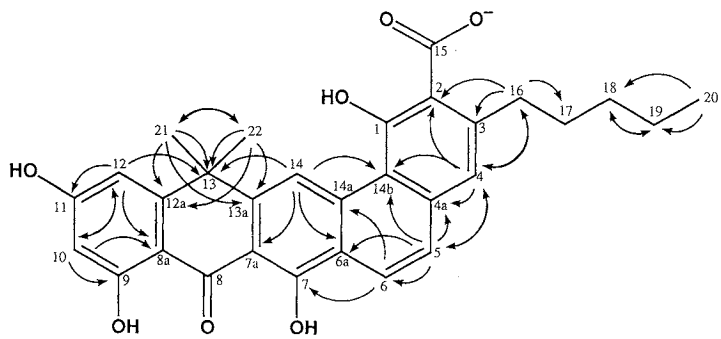


Fig. 2. ^{1}H-^{13}C correlation by HMBC experiment.



could not deduce the complete structure of **1**, since the aromatic proton at δ_H 9.77 (14-H) ppm observed at very low field and an aromatic ketone δ_C 192.0 (C-8) ppm did not show any correlations with other signals in the HMBC spectrum. The complete structure was determined by an X-ray analysis of **1** described later. After the structure determination, the ^{1}H and ^{13}C NMR assignments were performed by the aid of the HMBC spectrum as shown in Table 1 and Fig. 2.

X-Ray Crystallographic Analysis of Benastatin A

Crystals of benastatin A calcium salt were grown in an ethyl ether solution as yellow needles. The X-ray specimen of approximate dimensions $0.02 \times 0.08 \times 0.21$ mm was cut and mounted on a Philips PW1100 diffractometer. Intensities were measured by using graphite monochromated CuKα radiation. The crystal and experimental data are listed in Table 2 along with the summaries of structure determination.

The present crystal contains two formula units in the unit cell of space group $P\bar{1}$ so that the calcium ion must lay on a center of symmetry. The structure was solved by direct methods and refined by the method of least-squares with block-diagonal matrix approximations. Most of the hydrogen atoms were located on the difference electron density map, but two of the solvent water molecule, five of the ethyl group of ethyl ether molecule were missing. One of the terminal methyl of this ethyl group were disordered and distributed in two sites as can be seen in Fig. 3. The rather high value of R may have been caused by the disorder of the solvent molecule. The calcium ion is coordinated by six oxygen atoms: O7 × 2 at

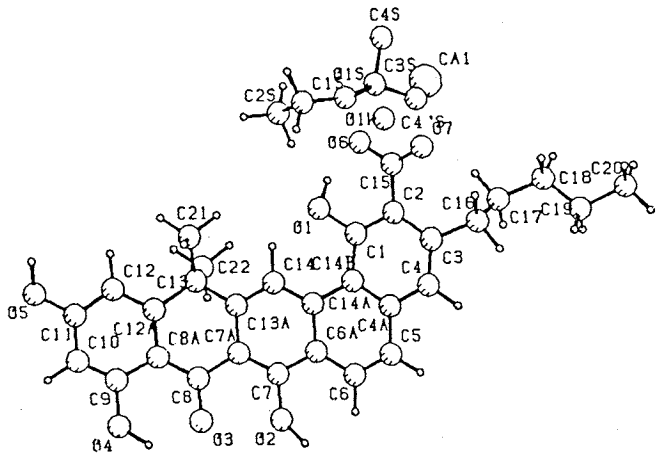
Table 2. Crystal and experimental data.[a]

Compound name:	Benastatin A calcium salt water and ethyl ether solvate	
Compound formula (including solvent of crystallization):	$C_{30}H_{27}O_2 \cdot \frac{1}{2}Ca \cdot H_2O \cdot (C_2H_5)_2O$	
Formula weight:	611.2	
Crystal system:	Triclinic	
Space group:	$P\bar{1}$	
Unit cell constants:	a = 12.325(9) Å,	$\alpha = 90.98(6)°$,
	b = 15.567(10) Å,	$\beta = 93.10(7)°$,
	c = 8.559(6) Å,	$\gamma = 109.89(8)°$
Cell volume:	1541 Å^3	
No. of molecules/cell:	2	
Calculated density:	1.317 g cm^{-3}	
μ for CuKα radiation:	14.6 cm^{-1}	
No. of measured reflections:	1,998	
Measured in the 2θ range:	6° through 120°	
No. of possible reflections in the same 2θ range:	4,570	
No. of C, O and Ca atoms refined anisotropically:	45	
No. of H atoms found on the difference electron density map and refined with isotropic temperature factor:	32	
Final R value	0.103	

[a] The parameters, bond lengths and angles have been sent to the Cambridge Crystallographic Data Centre.

Fig. 3. X-ray molecular structure of benastatin A drawn by PLUTO program[5].

The calcium ion and the molecules of solvent of crystallization are also shown.

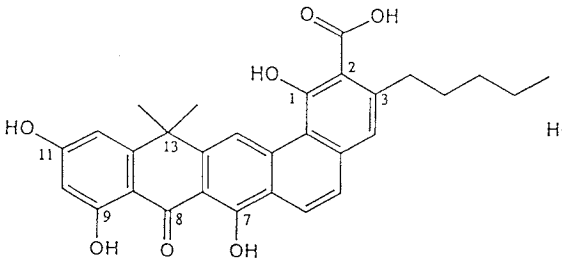


2.291(9) Å, O1W × 2 at 2.356(11) Å and O5 × 2 at 2.403(7) Å. The structure of the molecule is illustrated in Fig. 3. Therefore, the structure of benastatin A was determined to be 8,13-dihydro-1,7,9,11-tetrahydroxy-13-dimethyl-8-oxo-3-pentyl-benzo[*a*]naphthacene-2-carboxylic acid (Fig. 4).

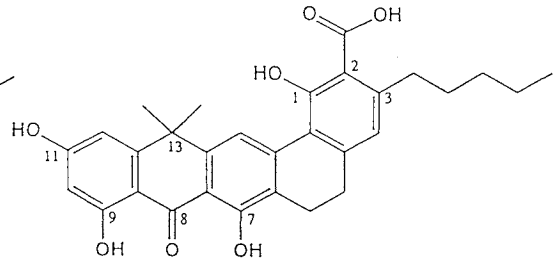
Structure of Benastatin B

The molecular weight and formula of benastatin B were elucidated as $C_{30}H_{30}O_7$ (MW 502) from the data of FAB-MS (negative) peak at *m/z* 501 $(M-\frac{1}{2}Ca)^-$, elemental analysis after EtOAc extraction (pH 2) to remove the salt (found: C 70.51, H 6.29, O 23.50; calcd: C 70.44, H 6.11, O 23.46 for $C_{30}H_{30}O_7 \cdot \frac{1}{2}H_2O$)

Fig. 4. Structures of benastatins A and B.



Benastatin A



Benastatin B

and ^{1}H and ^{13}C NMR spectra of benastatin B calcium salt (**2**). UV and IR spectra of **2** were similar to those of **1**. Though the ^{1}H and ^{13}C NMR spectra of **2** were almost coincident with those of **1**, the chemical shifts differed to some extent (Table 1). **1** showed two protons 5-H (δ_H 7.50 ppm) and 6-H (δ_H 8.24 ppm), but these corresponding signals were not observed in the ^{1}H NMR spectrum of **2**. Instead of these aromatic proton signals, methylene proton signals at δ_H 2.71 (5-H) and 2.80 (6-H) ppm were present. These were seen coupling with each other in the ^{1}H-^{1}H COSY spectrum of **2**. Also in the ^{13}C NMR spectrum of **2**, two methylene carbon signals at δ_C 20.8 (C-5) and 30.8 (C-6) ppm were observed and no analogous signals to the two aromatic carbon signals at δ_C 127.0 (C-5) and 123.5 (C-6) ppm in the spectrum of **1** could be found. Therefore, the structure of benastatin B was determined to be 5,6,8,13-tetrahydro-1,7,9,11-tetrahydroxy-13-dimethyl-8-oxo-3-pentyl-benzo[*a*]naphthacene-2-carboxylic acid (Fig. 4).

Structures of benastatins A and B contain a benzo[*a*]naphthacene skeleton. Natural products with this ring system were reported previously, for example, G-2N, G-2A[2] and KS-619-1[3,4], but these compounds contain a benzo[*a*]naphthacene quinone skeleton exactly. Thus, the benastatins which do not have a quinone moiety are unique.

Experimental

General

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 Hitachi M-80H mass spectrometer and a Jeol JMS-SX 102 mass spectrometer. NMR spectra were recorded on a Jeol JNM-GX400 NMR spectrometer with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz.

Mass Sepctrometry

The FAB-MS of **1** and **2** were obtained under following conditions; ion acceleration voltage-10 kV, resolution power 1500, xenon gas and glycerol matrix.

NMR Spectrometry

A sample (10.2 mg) of **1** was dissolved in 0.55 ml of CD_3OD. The HMBC spectrum of **1** resulted from the following parameters; the spectrum width was 3,751 Hz in F2 (^{1}H resonance), data points 1 K, 20 kHz in F1 (^{13}C resonance), 256 FIDs (zero-filled to 512), and $\Delta 1$ and $\Delta 2$ durations of 3.7 and 60.0 mseconds, respectively.

X-Ray Crystallographic Analysis

Crystals of benastatin A were grown in an ethyl ether solution as yellow needles. An X-ray specimen

of approximate dimensions 0.02 × 0.08 × 0.21 mm was cut and mounted on a Philips PW1100 diffractometer.

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) GERBER, N. N. & M. P. LECHEVALIER: Novel benzo[*a*]naphthacene quinones from an actinomycete, *Frankia* G-2 (ORS 0260604). Can. J. Chem. 62: 2818~2821, 1984
3) MATSUDA, Y. & H. KASE: KS-619-1, a new inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase from *Streptomyces californicus*. J. Antibiotics 40: 1104~1110, 1987
4) YASUZAWA, T.; M. YOSHIDA, K. SHIRAHATA & H. SANO: Structure of a novel Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase inhibitor KS-619-1. J. Antibiotics 40: 1111~1114, 1987
5) MOTHERWELL, W. D. S. & W. CLEGG: PLUTO. A Program for Drawing Molecular and Crystal Structures. Cambridge Crystallographic Data Center, University Chemical Laboratory, Cambridge, 1983