BENARTHIN: A NEW INHIBITOR OF PYROGLUTAMYL PEPTIDASE

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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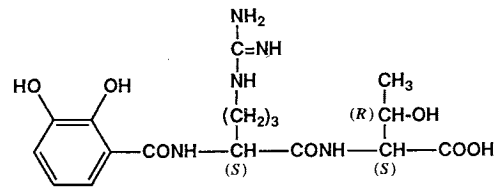
Benarthin, a new inhibitor of pyroglutamyl peptidase (PG-peptidase), has been isolated from the culture broth of *Streptomyces xanthophaeus* MJ244-SF1. The structure of benarthin was determined to be L-(2,3-dihydroxybenzoyl)argininyl-L-threonine by analysis of spectral properties and through chemical studies.

In the preceding paper[1] we have described the taxonomy and fermentation of the producing strain, as well as the purification and biological properties of benarthin. In this paper, we describe the physico-chemical properties and structure of benarthin (Fig. 1).

Physico-chemical Properties of Benarthin

The physico-chemical properties of benarthin are summarized in Table 1. The molecular formula of benarthin was determined by HRFAB-MS, ^{13}C NMR and elemental analysis. The IR spectrum of benarthin indicated the presence of NH and OH (3400 cm^{-1}) groups and peptide bonds (1660 and

Fig. 1. Structure of benarthin.



L-*N*-(2,3-Dihydroxybenzoyl)arginyl-L-threonine.

Table 1. Physico-chemical properties of benarthin.

Appearance	Colorless powder
MP	178~180°C
$[\alpha]_D^{24}$	−2.5° (*c* 1.0, H_2O)
Molecular formula	$C_{17}H_{25}N_5O_7$
Elemental analysis	Calcd for $C_{17}H_{25}N_5O_7 \cdot HCl \cdot \frac{1}{2}H_2O$:
	C 44.69, H 5.96, N 15.33, O 26.26
	Found: C 44.46, H 6.28, N 14.54, O 26.08
HRFAB-MS (*m/z*)	Calcd for $C_{17}H_{26}N_5O_7$: 412.1833
	Found: 412.1836 $(M+H)^+$
UV absorbance	$\lambda_{max}^{H_2O}$ nm (ε) 246 (8,200), 308 (2,400)
	$\lambda_{max}^{H_2O\text{-}0.1\ N\ NaOH}$ nm (ε) 252 (sh), 334 (3,800)
Color reaction (positive)	Sakaguchi, Greig-Leaback
Solubility	Soluble; H_2O, DMSO, MeOH
	Insoluble; $CHCl_3$, EtOAc, hexane
Rf[a]	0.52 (BuOH - AcOH - H_2O, 4:1:2)
	0.05 ($CHCl_3$ - MeOH - H_2O, 65:25:4)
Rm[b] (Ala = 1.0)	0.90

[a] On Silica gel TLC plate (Merck Art. No. 5715).
[b] HVPE in HCOOH - CH_3COOH - H_2O (7.5:22.5:2,700) under 800 V for 15 minutes.

Fig. 2. IR spectrum of benarthin (KBr).

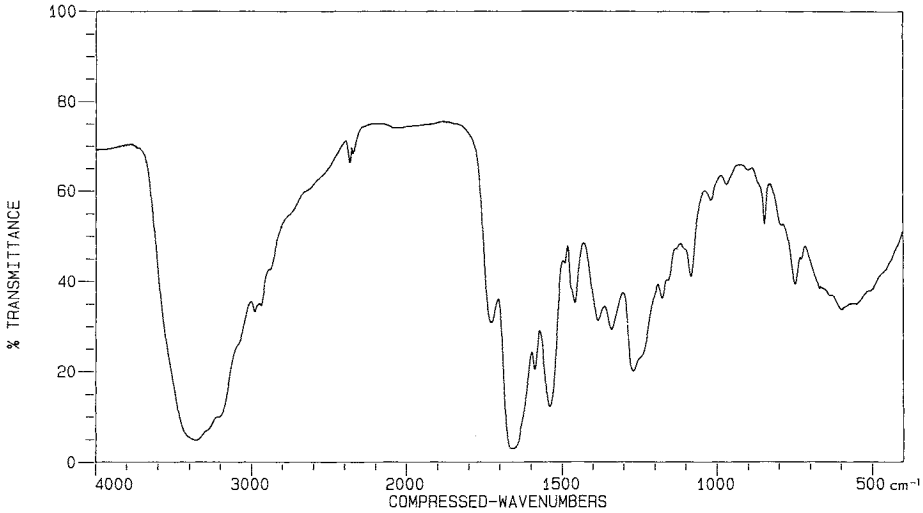
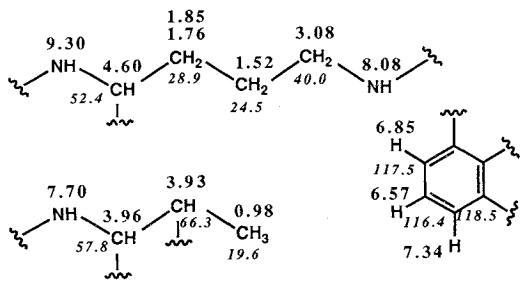


Table 2. ^{13}C and ^{1}H NMR data of benarthin in DMSO-d_6.

Assignment	^{13}C[a]	M	^{1}H[b] (J=Hz)
Threonine			
1	173.4	s	—
2	57.8	d	3.96 m
2-NH	—	—	7.70 d (8.0)
3	66.3	d	3.93 m
4	19.6	q	0.98 d (5.8)
Arginine			
1′	170.6	s	—
2′	52.4	d	4.60 m
2′-NH	—	—	9.30 br
3′	28.9	t	1.76 m, 1.85 m
4′	24.5	t	1.52 m
5′	40.0	t	3.08 br
5′-NH	—	—	8.08 br
6′	157.0	s	—
2,3-Dihydroxybenzoic acid			
1″	168.1	s	—
2″	115.9	s	—
3″	149.9	s	—
4″	146.5	s	—
5″	117.5	d	6.85 dd (7.9, 1.0)
6″	116.4	d	6.57 dd (7.9, 8.0)
7″	118.5	d	7.34 dd (8.0, 1.0)

[a] 100 MHz; δ in ppm.
[b] 400 MHz; δ in ppm.
M: Multiplicity.

Fig. 3. Partial structures of benarthin.

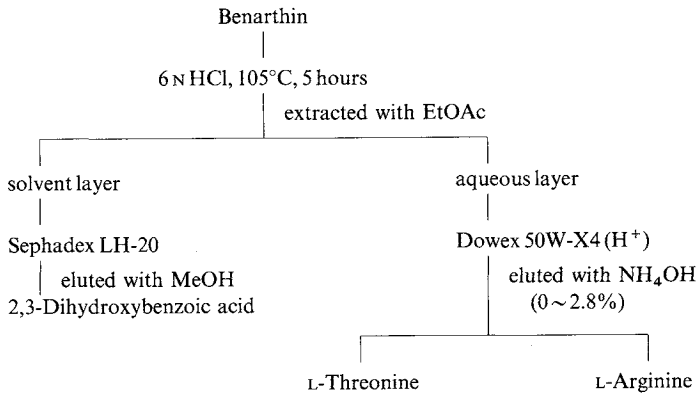


1520 cm^{-1}). The IR spectrum is shown in Fig. 2. Benarthin is soluble in H_2O, MeOH, EtOH, DMSO, but insoluble in $CHCl_3$, EtOAc and hexane. Spots on silica gel TLC plates were visualized using Sakaguchi, Greig-Leaback[2] and H_2SO_4 reagents.

Structure Determination of Benarthin

The molecular formula of benarthin was found to be $C_{17}H_{25}N_5O_7$. All 17 carbons were visible in the ^{13}C NMR spectrum (Table 2). DEPT spectra established the presence of 10 carbons bearing protons (1 methyl, 3 methylenes and 6 methines) and the ^{1}H NMR spectrum showed 4 exchangeable proton signals (Table 2). The partial structures (Fig. 3) were deduced through the ^{1}H-^{1}H COSY spectrum. The connection of the partial structures and assigned quatenary carbons in benarthin were determined by a heteronuclear multiple-bond correlation (HMBC)[3] experiment as shown in Fig. 4. The positional arrangement of the three carbonyl carbons at

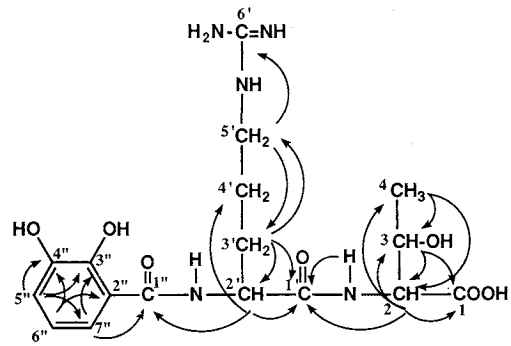
Scheme 1. Acid hydrolysis of benarthin.



δ 173.4 (C-1), 170.6 (C-1′) and 168.1 (C-1″) was established by the HMBC spectrum. The carbonyl carbon at δ 173.4 (C-1) was coupled to two methine protons at δ 3.96 (2-H) and 3.93 (3-H). The carbonyl carbon at δ 170.6 (C-1′) was coupled to two methine protons at δ 4.60 (2′-H) and 3.96 (2-H), one methylene proton at δ 1.76 and 1.85 (3′-H_2) and an exchangeable proton at δ 7.70 (2-NH). Another carbonyl carbon at δ 168.1 (C-1″) was coupled to an aromatic proton at δ 7.34 (7″-H) and a methine proton at δ 4.60 (2′-H). The quaternary carbon at δ 157.0 (C-6′) coupling to the methylene proton at δ 3.08 (5′-H_2) was assigned to a guanidine residue based on its chemical shift and positive Sakaguchi reaction for benarthin.

Fig. 4. Proton-carbon correlation map of benarthin by HMBC.

⟶ ^1H-^{13}C long range coupling.



In the basis of the results described above and benarthin's positive Greig-Leaback[2] and negative ninhydrin reactions, it was suggested that the structure of benarthin could be a peptide with a masked *N*-terminus. Hydrolysis of benarthin with 6 N HCl at 105°C for 5 hours gave one solvent soluble substance and two ninhydrin positive compounds as shown in Scheme 1. The solvent-soluble compounds was purified by column chromatography on Sephadex LH-20 in MeOH and was identified as 2,3-dihydroxybenzoic acid[4]. The two ninhydrin positive compounds were separated by column chromatography on Dowex 50W-X4 using linear gradient elution between 0~2.8% NH_4OH. One of these compounds was crystallized and the other was purified by centrifugal partition chromatography (CPC). They were identified as L-threonine and L-arginine, respectively.

The sequence of the constituents was confirmed by FAB-MS spectrum. The presence of ion peaks at *m/z* 137 $(M-Arg-Thr)^+$, *m/z* 293 $(M-Thr)^+$ and *m/z* 366 $(M-COOH)^+$ was consistent with the structural information inferred from NMR results.

Thus, the structure of benarthin was determined to be L-(2,3-dihydroxybenzoyl)arginyl-L-threonine.

In order to confirm the proposed structure, we carried out a synthesis of benarthin. The synthesis of benarthin will be described in the following paper[5].

Experimental

General

NMR spectra were recorded on a Joel JNM-GX400 NMR spectrometer and mass spectra were obtained using a Joel JMS-SX102 spectrometer. UV spectra were recorded on a Hitachi U-3210 spectrometer. IR spectra were measured on a Hitachi I-5020 FT-IR spectrometer and optical rotation was determined using a Perkin-Elmer 241 polarimeter. Melting point was measured on a Yanaco SP-S3.

Hydrolysis of Benarthin

Benarthin (60 mg) was hydrolyzed with 6 N HCl (5 ml) at 105°C for 5 hours. After concentration to dryness, H_2O (5 ml) was added to the hydrolysate and a solvent soluble substance was extracted with EtOAc (5 ml). The EtOAc extracts were treated with Na_2SO_4 and concentrated to dryness. Further purification was performed by Sephadex LH-20 column chromatography (2×80 cm) developed with MeOH ultimately yielding 2,3-dihydroxybenzoic acid as a colorless powder (14.8 mg): Rf 0.80 (BuOH-AcOH-H_2O, 4:1:2) FAB-MS *m/z* 155 $(M+H)^+$, 137 $(M-OH)^+$, 153 $(M-H)^-$, 109 $(M-COOH)^-$; IR ν(KBr) cm^{-1} 3400, 3100, 1690, 1670, 1610, 1480, 1440, 1390; ^1H NMR (400 MHz, DMSO-d_6) δ 6.70 (1H, dd, $J=9.0$ and 9.0 Hz), 6.98 (1H, dd, $J=9.0$ and 2.0 Hz), 7.24 (1H, dd, $J=9.0$ and 2.0 Hz), 9.26 (br); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.4 s, 150.4 s, 145.9 s, 120.6 d, 119.9 d, 118.5 d, 113.2 s.

After EtOAc extraction of the hydrolysate, the aqueous layer was applied to a Dowex 50W-X4 (H^+) column (1.5×20 cm). L-Threonine and L-arginine were eluted with 0~2.8% NH_4OH using a liner gradient. Fractions of approximately 5-ml in volume were collected. The fractions (Nos. 10~15) containing L-threonine were crystallized from MeOH-H_2O (4:1) to give colorless crystals (16.4 mg): MP 248~251°C; Rf 0.25 (BuOH-AcOH-H_2O, 4:1:2), $[\alpha]_D^{24}$ −27.1° (*c* 0.5, H_2O); FAB-MS *m/z* 120 $(M+H)^+$; IR ν(KBr) cm^{-1} 3500, 3200, 3000, 1640, 1480, 1450, 1420, 1370, 1320, 1260, 1200, 1150, 1080; ^1H NMR (400 MHz, D_2O) δ 1.25 (3H, d, $J=7.2$ Hz), 4.55 (1H, m), 5.85 (1H, d, $J=5.3$ Hz); ^{13}C NMR (100 MHz, D_2O) δ 173.6 s, 66.6 d, 61.2 d, 20.2 q.

The fractions (Nos. 25~31) containing L-arginine were further purified by CPC, which was perfomed using a CPC apparatus model NMF (Sanki Engineering Limited), employing the following conditions: BuOH-AcOH-H_2O (upper phase stationary, 750:50:750), 4 ml/minute, 900 rpm, 20°C. The fractions containing L-arginine were concentrated to a small volume, were acidified with 0.1 N HCl (5 ml) and were lyophilized to give a L-arginine·HCl salt as a colorless powder (26.8 mg): MP 228~230°C; Rf 0.13 (BuOH-AcOH-H_2O, 4:1:2); $[\alpha]_D^{24}$ +19.9° (*c* 1.0, 6 N HCl); FAB-MS *m/z* 175 $(M+H)^+$; IR ν(KBr) cm^{-1} 3400, 3180, 1660, 1640, 1500, 1410, 1350, 1250, 1180, 1100, 1080; ^1H NMR (400 MHz, D_2O) δ 1.69 (1H, m), 1.74 (1H, m), 1.93 (2H, m), 3.27 (2H, t, $J=6.4$ Hz), 3.79 (1H, dd); ^{13}C NMR (100 MHz, D_2O) δ 175.1 s, 157.6 s, 55.2 d, 41.3 t, 28.3 t, 24.7 t.

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