BENARTHIN: A NEW INHIBITOR OF PYROGLUTAMYL PEPTIDASE

III. SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS

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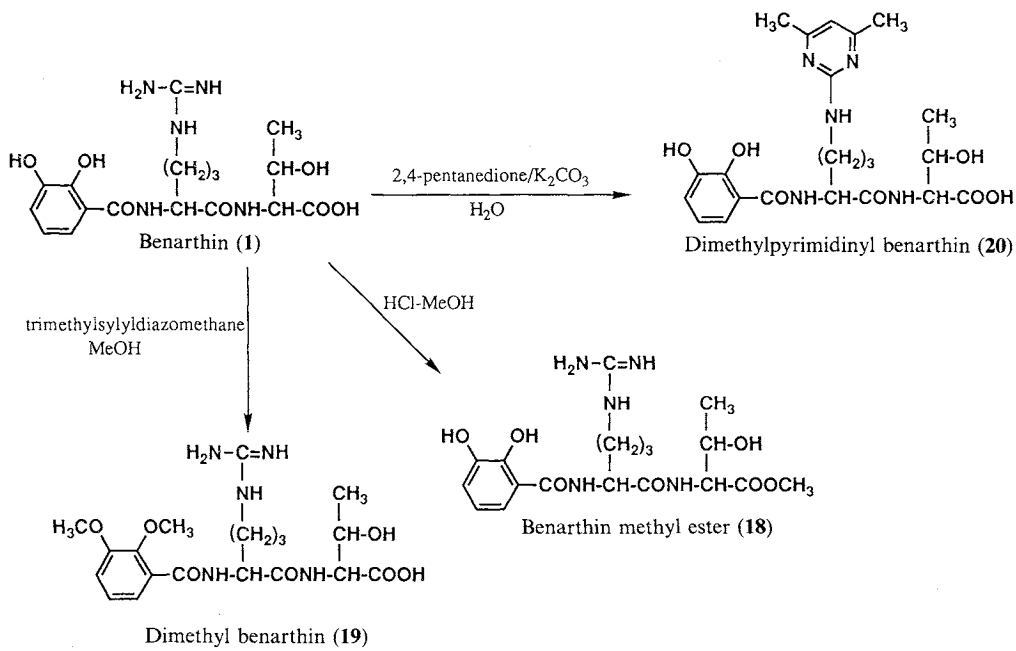
(Received for publication February 4, 1992)

Benarthin, a new inhibitor of pyroglutamyl peptidase (PG-peptidase), has been isolated from the culture filtrate of *Streptomyces xanthophaeus* MJ244-SF1. The structure of benarthin has been determined to be L-(2,3-dihydroxybenzoyl)arginyl-L-threonine. This structure was confirmed by the total synthesis of benarthin. Moreover, we synthesized benarthin derivatives to obtain information on the relationship between structure and inhibitory activity. The results indicated that the catechol group of benarthin is the essential moiety for the inhibition of PG-peptidase.

As reported in previous papers[1,2], we have described the isolation and characterization of benarthin (**1**), as well as the taxonomy and fermentation of *Streptomyces xanthophaeus*. The structure was determined to be L-(2,3-dihydroxybenzoyl)arginyl-L-threonine (Fig. 1).

In this paper, we report the synthesis of **1** and the structure-activity relationships of benarthin derivatives against pyroglutamyl peptidase (PG-peptidase).

Fig. 1. Structure of benarthin (**1**) and its derivatives (**18**, **19** and **20**).



Synthesis of Benarthin

The total synthesis of **1** is shown in Scheme 1. L-(N^{α}-Boc-N^{ω}-di-Z[†])arginyl-L-threonine benzyl ester (**4**) was synthesized by condensation of L-(N^{α}-Boc-N^{ω}-di-Z)arginine (**2**) and L-threonine benzyl ester (**3**) in dry DMF using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). Selective removal of the α-amino protective group of compound **4** was accomplished by treatment with CF_3COOH to give **5**.

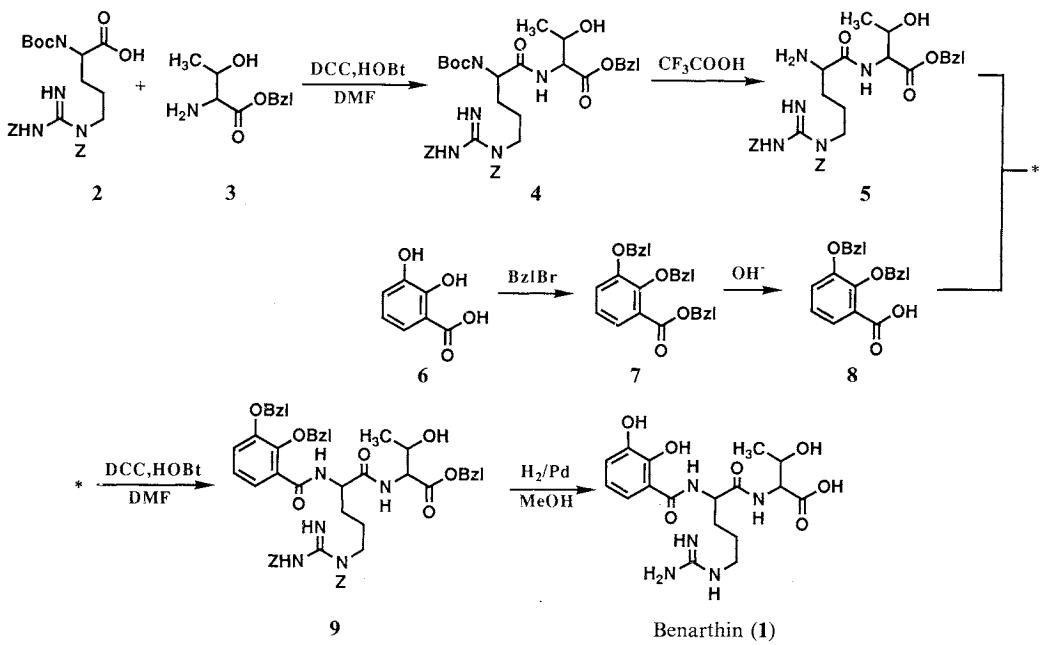
2,3-Dibenzyloxybenzoic acid (**8**) was prepared from 2,3-dihydroxybenzoic acid (**6**) by reaction with benzylbromide in dry acetone in the presence of K_2CO_3 followed by hydrolysis of the resulting benzyl ester (**7**) with sodium hydroxide.

L-(N^{ω}-Di-Z)arginyl-L-threonine benzyl ester (**5**) was condensed with 2,3-dibenzyloxybenzoic acid (**8**) in dry DMF using DCC and HOBt to form L-(N^{α}-2,3-dibenzyloxybenzoyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester (**9**). Finally, hydrogenolysis of **9** using palladium-black gave L-(2,3-dihydroxybenzoyl)arginyl-L-threonine (benarthin). The physico-chemical[2] and spectral properties of synthetic benarthin were the same as those of the natural product.

Structure-activity Relationships of Benarthin and Its Derivatives for Inhibition of PG-Peptidase

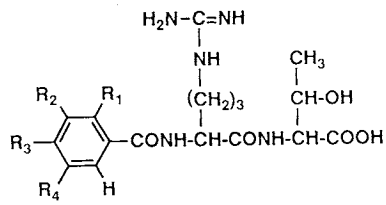
Benarthin derivatives (**10**～**17**) were prepared using the method employed in the synthesis of **1**. Benarthin methyl ester (**18**) was obtained by treatment with HCl-MeOH. Dimethyl benarthin (**19**, L-(2,3-dimethyloxybenzoyl)arginyl-L-threonine) was obtained by treatment with trimethylsylyldiazomethane in dry MeOH. Dimethylpyrimidinyl benarthin (**20**) was prepared by treatment with 2,4-pentanedione.

Scheme 1. Synthesis of benarthin.



† Z = Benzyloxycarbonyl.

Table 1. Structure-activity relationships.

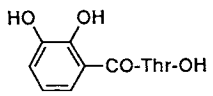


Compound	R_1	R_2	R_3	R_4	IC_{50} (μg/ml)
1	OH	OH	H	H	2.05
10	H	H	H	H	>100
11	OH	H	OH	H	>100
12	OH	H	H	OH	5.14
13	H	OH	OH	H	3.57
14	H	OH	H	OH	>100
15	OH	H	H	H	>100
19	OCH_3	OCH_3	H	H	>100

Table 2. Structure-activity relationships.

Compound	IC_{50} (μg/ml)
1	2.05
16[a]	1.79
17[b]	1.86
18	1.95
20	2.05

a



b

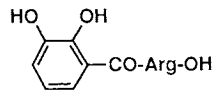


Table 3. Structure-activity relationships.

Compound	IC_{50} (μg/ml)
1	2.05
17	1.86
2,3-Dihydroxybenzoic acid	0.51
Catechol	0.65
Salicylic acid	>100
Benzoic acid	>100

Chemical shift values for ^1H NMR and physicochemical data for all derivatives are described in the Experimental section. The inhibition of PG-peptidase by the compounds synthesized was tested in order to determine structure-activity relationships. The activity was measured as reported previously[1] in order to determine the concentration of the inhibitor required for 50% inhibition (IC_{50}). The IC_{50} values of these derivatives are shown in Tables 1, 2 and 3.

The effect of hydroxyl groups on the benzoyl moiety was investigated as shown in Table 1. The compound (**13**) with the 3,4-dihydroxybenzoyl group showed almost the same activity as the compound (**1**) with the 2,3-dihydroxybenzoyl group. However, the compound (**12**) with the 2,5-dihydroxybenzoyl group showed lower activity than compound **1**. Other compounds exhibited much lower activity. These results indicated that vicinal hydroxyl groups on the benzoyl group are essential for activity.

The effects of the arginine and threonine moieties were investigated as shown in Table 2. It was apparent that the guanidino group of arginine and the free carboxylic acid group of threonine had relatively little effect on activity. The peptide moiety of **1** appeared to be of little importance in the inhibition of PG-peptidase. Furthermore, as shown in Table 3, catechol itself and 2,3-dihydroxybenozic acid showed much more inhibitory activity than **1**, however 2-hydroxybenzoic acid and benzoic acid showed no such activity. The results indicated that the vicinal hydroxyl function on the benzene ring was important for inhibition of PG-peptidase.

A variety of catechol siderophore compounds such as azotochelin[3], aminochelin[4] and myxochelin A[5] have been described from bacterial sources. These compounds are produced as iron chelators under iron deprived conditions, however, the biosynthesis of benarthin was not necessarily a result of iron deficiency and iron had no effect on the inhibitory activity of benarthin against PG-peptidase.

Vanoxonin[6,7] which is structurally related to **1** was found to be an inhibitor of thymidylate synthetase. Vanoxonin is also a catechol siderophore compound. The vicinal hydroxyl groups, like those of **1**, are essential for the inhibitory activity of vanoxonin against thymidylate synthetase. But, unlike **1**, the peptide moieties of vanoxonin are also important for inhibitory activity against thymidylate synthetase. In addition, vanadium was found to be a specific activator of vanoxonin, but the metal had no effect on the inhibitory activity of **1** against PG-peptidase. It would be interesting to resolve the mechanisms of PG-peptidase[8] and thymidylate synthetase[9] inhibition by benarthin and vanoxoin, respectively.

Experimental

General

NMR spectra were recorded on a Joel JNM-GX400 NMR spectrometer and mass spectra were measured using a Joel JMS-SX102 spectrometer. UV spectra were recorded on a Hitachi U-3210 spectrometer and IR spectra on a Hitachi I-5020 FT-IR spectrometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter and melting points on a Yanaco SP-S3 and MP-500D.

L-(N^{α}-Boc-N^{ω}-di-*Z*)arginyl-L-threonine Benzyl Ester (**4**)

HOBt (205 mg) and DCC (208 mg) were added to an ice bath-cooled solution of L-(N^{α}-Boc-N^{ω}-di-*Z*)arginine (**2**, 505 mg) and L-threonine benzyl ester (**3**, 220 mg) in dry DMF (2 ml) while stirring. After stirring for 5 hours at room temperature, the undissolved material was removed by filtration and the filtrate was evaporated and dissolved in EtOAc (30 ml). This material was washed with 5% aq $NaHCO_3$, 1% aq citric acid and saturated aq NaCl, dried with Na_2SO_4, filtered and evaporated. The residue was dissolved in $CHCl_3$ and was applied to silica gel column (2.5×30 cm) and eluted with $CHCl_3$-MeOH (100:1). The main fractions were evaporated to give a colorless powder (**4**, 408 mg, 61%): Rf 0.41 ($CHCl_3$-MeOH, 50:1), 0.57 (toluene-EtOAc, 1:1); MP 120~121°C; $[\alpha]_D^{24}$ +3.8° (*c* 1.0, $CHCl_3$); FAB-MS *m/z* 734 $(M+H)^+$, 600 $(M-COOBzl)^+$; *Anal* Calcd for $C_{38}H_{47}N_5O_{10}$: N 9.54, found: 9.61.

2,3-Dibenzyloxybezoic Acid (**8**)

Benzylbromide (1.2 ml) was added dropwise to a solution of 2,3-dihydroxybenzoic acid (**6**, 505 mg) and K_2CO_3 (1,850 mg) in dry acetone (12 ml). The reaction mixture was refluxed for 14 hours at 65°C. The undissolved material was removed by filtration and the filtrate was evaporated. The residue was dissolved in dioxane (5 ml) and 2 N NaOH (4 ml) was added. After refluxing for 40 minutes at 110°C, 2 N HCl (4 ml) was added and the reaction mixture was evaporated. $CHCl_3$ (50 ml) was added to the residue which was washed with H_2O, dried with Na_2SO_4, filtered and evaporated. Crystallization from EtOAc gave colorless prisms (**8**, 813 mg, 76%): Rf 0.49 (toluene-EtOAc, 1:1); MP 126~128°C; FAB-MS *m/z* 335 $(M+H)^+$, 244 $(M+H-C_7H_7)$; *Anal* Calcd for $C_{21}H_{18}O_4$: C 74.48, H 5.51, found: C 74.51, H 5.26.

L-(N^{α}-2,3-Dibenzoyloxybenzoyl-N^{ω}-di-*Z*)arginyl-L-threonine Benzyl Ester (**9**)

Trifluoroacetic acid (2 ml) was added to an ice bath-cooled solution of compound **4** (169 mg) in dry dichloromethane (0.5 ml) while stirring. Stirring was continued for 50 minutes at room temperature and the dichloromethane was evaporated. The residue was extracted with EtOAc (10 ml), washed with 5% aq $NaHCO_3$ and saturated aq NaCl, dried with Na_2SO_4 and concentrated to give a ninhydrin positive compound **5** (172 mg). HOBt (47 mg) and DCC (55 mg) were added to an ice bath-cooled solution prepared from compound **5** (148 mg) and compound **8** (81 mg) in dry DMF (3 ml). After stirring for 19 hours at room temperature, the resulting precipitate was removed by filtration. EtOAc (30 ml) was added to the filtrate, and the mixture was washed with 5% aq $NaHCO_3$, 1% aq citric acid and saturated aq NaCl. This

material was dried with Na_2SO_4 and evaporated to give a colorless powder (222 mg). The residue was dissolved in $CHCl_3$, applied to a silica gel column (2.5 × 10 cm) and was eluted with $CHCl_3$. Concentration of the main fractions gave a colorless oily residue (**9**, 147 mg, 67%); Rf 0.40 ($CHCl_3$ - MeOH, 40 : 1), 0.22 (hexane - EtOAc, 1 : 1); MP 50~52°C; $[\alpha]_D^{24}$ −2.9° (*c* 1.0, $CHCl_3$); FAB-MS *m/z* 950 $(M+H)^+$, 842 $(M-OBzl)^+$, 798 $(M-COOBzl-OH)^+$; *Anal* Calcd for $C_{54}H_{55}N_5O_{11}\cdot\frac{1}{2}H_2O$: C 67.63, H 5.89, found: C 67.87, H 6.18.

L-(2,3-Dihydroxybenzoyl)arginyl-L-threonine (Benarthin, **1**)

Palladium black (5 mg) was added to a solution prepared from compound **9** (72 mg) in MeOH (4 ml). The atmosphere was replaced with hydrogen at atmospheric pressure and stirring was continued for 48 hours at room temperature. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue (36 mg) was dissolved in MeOH and was purified by Sephadex LH-20 column chromatography (3 × 200 cm) ultimately yielding a colorless powder (25 mg). Further purification by centrifugal partition chromatography (CPC) was performed using a CPC apparatus model NMF (Sanki Engineering Limited). The following conditions were employed: BuOH - AcOH - H_2O (upper phase stationary, 750 : 50 : 750), 4 ml/minute, 1,000 rpm, 20°C, detection 254 nm. The main fractions were collected and concentrated to small volumes and were lyophilized to give benarthin acetate (21 mg, 68%) as a colorless powder. The benarthin·HCl salt was obtained by lyophilization of a 0.1 N HCl solution of benarthin. Rf 0.50 (BuOH - AcOH - H_2O, 4 : 1 : 2), 0.03 ($CHCl_3$ - MeOH - H_2O, 65 : 25 : 2); MP 177~179°C; $[\alpha]_D^{24}$ −2.9° (*c* 1.0, H_2O); FAB-MS *m/z* 412 $(M+H)^+$, 396 $(M+H-OH)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 245 (8,200), 306 (2,400), $\lambda_{max}^{H_2O-NaOH}$ (ε) 250 (sh), 334 (3,800) nm: IR *ν* (KBr) cm^{-1} 3400, 1660, 1600, 1540, 1470, 1400, 1360, 1280; ^1H NMR (400 MHz, DMSO-d_6) δ 0.96 (3H, d, *J*=5.5 Hz), 1.53 (2H, m), 1.76 (1H, m), 1.84 (1H, m), 3.08 (2H, br), 3.96 (1H, dq), 4.00 (1H, dd, *J*=6.5 and 5.5 Hz), 4.59 (1H, m), 6.59 (1H, dd, *J*=7.7 and 7.3 Hz), 6.86 (1H, d, *J*=7.7 Hz), 7.35 (1H, d, *J*=7.3 Hz), 7.70 (NH, d, *J*=6.5 Hz), 8.12 (NH, br), 9.26 (NH, br); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.6 s, 170.7 s, 168.2 s, 157.0 s, 149.8 s, 146.5 s, 118.5 d, 117.6 d, 116.8 d, 116.0 s, 66.4 d, 57.9 d, 52.5 d, 40.0 t (in DMSO), 28.9 t, 24.7 t 19.6 q; *Anal* Calcd for $C_{17}H_{25}N_5O_7\cdot HCl\cdot H_2O$: C 44.01, H 6.08, N 15.09, found: C 43.99, H 5.99, N 14.66.

L-(Benzoyl)arginyl-L-threonine (**10**)

Palladium black (7.8 mg) was added to a solution prepared from L-(N^α-benzoyl-N^ω-di-*Z*)arginyl-L-threonine benzyl ester (90 mg), $CHCl_3$ (1 ml), and MeOH (1.5 ml). The atmosphere was replaced with hydrogen at atmospheric pressure and stirring was continued for 24 hours at room temperature. The catalyst was removed by filtration and the filtrate was concentrated to dryness. Purification was performed by CPC, employing the following conditions: BuOH - AcOH - H_2O (upper phase stationary, 750 : 50 : 750), 4 ml/minute, 900 rpm, 20°C, detection 254 nm. The main fractions were collected, concentrated to a small volume and were lyophilized to give a colorless powder of L-(benzoyl)arginyl-L-threonine acetate (**10**) (40 mg, 84%). Rf 0.50 (BuOH - AcOH - H_2O, 4 : 1 : 2), 0.17 ($CHCl_3$ - MeOH - H_2O, 65 : 25 : 2); MP 123~125°C; $[\alpha]_D^{24}$ −1.6° (*c* 2.0, MeOH); FAB-MS *m/z* 380 $(M+H)^+$, 335 $(M+H-COOH)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 228 (11,200) nm, $\lambda_{max}^{H_2O-NaOH}$ (ε) 225 (sh) nm; IR *ν* (KBr) cm^{-1} 3400, 1740, 1660, 1580, 1540, 1490; ^1H NMR (400 MHz, DMSO-d_6) δ 1.07 (3H, d, *J*=6.2 Hz), 1.57 (2H, m), 1.76 (1H, m), 1.86 (1H, m), 3.12 (2H, m), 4.14 (1H, m), 4.20 (1H, dd, *J*=8.7 and 3.0 Hz), 4.59 (1H, m), 7.47 (2H, dd, *J*=7.7 and 7.2 Hz), 7.54 (1H, dd, *J*=7.7 and 7.2 Hz), 7.88 (2H, d, *J*=7.7 Hz), 7.70 (NH, br), 7.86 (NH, br), 8.60 (NH, br).

L-(2,4-Dihydroxybenzyl)arginyl-L-threonine (**11**)

L-(N^α-2,4-Dibenzoyloxybenzyl-N^ω-di-*Z*)arginyl-L-threonine benzyl ester (54 mg) was treated using the same procedure as employed with L-(N^α-2,3-dibenzoyloxybenzyl-N^ω-di-*Z*)arginyl-L-threonine benzyl ester in the synthesis of **1** yielding a colorless powder of L-(2,4-dihydroxybenzyl)arginyl-L-threonine acetate (**11**) (17 mg, 71%). Rf 0.48 (BuOH - AcOH - H_2O, 4 : 1 : 2), 0.06 ($CHCl_3$ - MeOH - H_2O, 65 : 25 : 2); MP 170~172°C; $[\alpha]_D^{24}$ +5.9° (*c* 1.0, H_2O); FAB-MS *m/z* 412 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 218 (26,600), 260 (10,400), 302 (5,500) nm, $\lambda_{max}^{H_2O-HCl}$ (ε) 258 (13,000), 294 (5,900) nm, $\lambda_{max}^{H_2O-NaCl}$ (ε) 224 (26,900), 275 (sh), 282 (8,500), 318 (10,000) nm; IR *ν* (KBr) cm^{-1} 3400, 1740, 1660, 1550, 1500, 1450, 1400; ^1H NMR (400 MHz, DMSO-d_6) δ 0.96 (3H, d, *J*=6.7 Hz), 1.51 (2H, m), 1.77 (1H, m), 1.85 (1H, m), 3.07 (2H, m), 3.95 (1H, m), 3.99 (1H, dd, *J*=8.8 and 4.0 Hz), 4.58 (1H, m), 6.26 (1H, d, *J*=1.9 Hz), 6.29 (1H, dd, *J*=1.9 and 8.3 Hz), 7.70 (NH,

d, J=7.8 Hz), 7.77 (1H, d, J=8.3 Hz), 8.02 (NH, br), 8.68 (NH, d, J=7.1 Hz).

L-(2,5-Dihydroxybenzyl)arginyl-L-threonine (**12**)

L-(N^{α}-2,5-Dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester (58 mg) was treated using the same procedure as employed with L-(N^{α}-2,3-dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester in the synthesis of **1** yielding a colorless powder of L-(2,5-dihydrobenzyl)arginyl-L-threonine acetate (**12**) (37 mg, 86%). Rf 0.48 (BuOH - AcOH - H_2O, 4:1:2), 0.06 ($CHCl_3$ - MeOH - H_2O, 65:25:2); MP 145~147°C; $[\alpha]_D^{24}$ +4.5° (c 1.0, H_2O); FAB-MS m/z 412 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 245 (sh), 325 (3,000) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 220 (sh), 360 (3,200) nm; IR ν (KBr) cm^{-1} 3350, 1740, 1670, 1550, 1450, 1400, 1320, 1210; ^1H NMR (400 MHz, DMSO-d_6) δ 1.03 (3H, d, J=6.0 Hz), 1.52 (2H, m), 1.73 (1H, m), 1.83 (1H, m), 3.11 (2H, m), 4.07 (1H, m), 4.13 (1H, dd, J=8.3 and 3.1 Hz), 4.68 (1H, m), 6.75 (1H, d, J=8.4 Hz), 6.82 (1H, dd, J=8.4 and 2.0 Hz), 7.33 (1H, d, J=2.0 Hz), 7.71 (NH, br), 7.99 (NH, d, J=8.0 Hz), 8.89 (NH, d, J=7.8 Hz).

L-(3,4-Dihydroxybenzyl)arginyl-L-threonine (**13**)

L-(N^{α}-3,4-Dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester (53 mg) was treated using the same procedure as employed with L-(N^{α}-2,3-dibenzoyloxybenyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester in the synthesis of **1** yielding a colorless powder of L-(3,4-dihydroxybenzyl)arginyl-L-threonine acetate (**13**) (32 mg, 86%). Rf 0.45 (BuOH - AcOH - H_2O, 4:1:2), 0.04 ($CHCl_3$ - MeOH - H_2O, 65:25:2); MP 147~149°C; $[\alpha]_D^{22}$ +4.3° (c 1.0, H_2O); FAB-MS m/z 412 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 257 (8,500), 292 (4,400) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 240 (sh), 288 (sh), 315 (8,800) nm; IR ν (KBr) cm^{-1} 3400, 1740, 1670, 1550, 1520; ^1H NMR (400 MHz, DMSO-d_6) δ 1.05 (3H, d, J=6.5 Hz), 1.52 (2H, m), 1.71 (1H, m), 1.82 (1H, m), 3.10 (2H, m), 4.14 (1H, m), 4.19 (1H, dd, J=8.5 and 3.0 Hz), 4.52 (1H, m), 6.77 (1H, d, J=8.2 Hz), 7.25 (1H, dd, J=8.2 and 2.0 Hz), 7.31 (1H, d, J=2.0 Hz), 7.65 (NH, m), 7.79 (NH, d, J=8.5 Hz), 8.21 (NH, d, J=8.0 Hz).

L-(3,5-Dihydroxybenzyl)arginyl-L-threonine (**14**)

L-(N^{α}-3,5-Dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester (30 mg) was treated using the same procedure as employed with L-(N^{α}-2,3-dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester in the synthesis of **1** yielding a colorless powder of L-(3,5-dihydroxybenzyl)arginyl-L-threonine acetate (**14**) (20 mg, 66%). Rf 0.45 (BuOH - AcOH - H_2O, 4:1:2), 0.03 ($CHCl_3$ - MeOH - H_2O, 65:25:2); MP 124~126°C; $[\alpha]_D^{25}$ −3.6° (c 1.0, H_2O); FAB-MS m/z 412 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 246 (5,700), 295 (1,800) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 235 (sh), 277 (3,000), 328 (2,200) nm; IR ν (KBr) cm^{-1} 3400, 1740, 1660, 1600, 1550, 1450; ^1H NMR (400 MHz, DMSO-d_6) δ 1.06 (3H, d, J=6.4 Hz), 1.52 (2H, m), 1.71 (1H, m), 1.82 (1H, m), 3.11 (2H, m), 4.14 (1H, dq, J=6.4 and 2.9 Hz), 4.19 (1H, dd, J=8.0 and 2.9 Hz), 4.51 (1H, m), 6.39 (1H, dd, J=2.0 and 2.0 Hz), 6.70 (2H, d, J=2.0 Hz), 7.67 (NH, br), 8.31 (NH, d, J=8.0 Hz).

L-(2-Hydroxybenzyl)arginyl-L-threonine (**15**)

L-(N^{α}-2-Benzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester (37 mg) was treated using the same procedure as employed with L-(N^{α}-2,3-dibenzoyloxybenyl-N^{α}-di-Z)arginyl-L-threonine benzyl ester in the synthesis of **1** yielding a colorless powder of L-(2-hydroxybenzyl)arginyl-L-threonine acetate (**15**) (38 mg, 87%). Rf 0.50 (BuOH - AcOH - H_2O, 4:1:2), 0.13 ($CHCl_3$ - MeOH - H_2O, 65:25:2); MP 173~175°C; $[\alpha]_D^{26}$ +10.6° (c 1.0, 50% MeOH); FAB-MS m/z 396 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 240 (11,400), 296 (4,000) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 240 (sh), 330 (6,800) nm; IR ν (KBr) cm^{-1} 3400, 1740, 1660, 1550, 1500, 1450, 1380, 1320, 1250; ^1H NMR (400 MHz, DMSO-d_6) δ 0.96 (3H, d, J=6.2 Hz), 1.52 (2H, m), 1.77 (1H, m), 1.87 (1H, m), 3.07 (2H, m), 3.93 (1H, m), 3.99 (1H, dd), 4.64 (1H, m), 6.85 (1H, dd, J=7.8 and 7.9 Hz), 6.91 (1H, d, J=8.4 Hz), 7.34 (1H, ddd, J=8.4, 7.8 and 1.5 Hz), 7.74 (NH, d, J=7.9 Hz), 7.93 (1H, dd, J=7.9 and 1.5 Hz), 8.15 (NH, br), 9.30 (NH, br).

L-(2,3-Dihydroxybenzyl)threonine (**16**)

L-(2,3-Dibenzoyloxybenzyl)threonine benzyl ester (84 mg) was treated using the same procedure as employed with L-(N^{α}-2,3-dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine benzyl ester in the synthesis of L-(2,3-dibenzoyloxybenzyl-N^{ω}-di-Z)arginyl-L-threonine yielding a colorless powder of L-(2,3-dibenzoyloxybenzyl)threonine acetate (**16**) (26 mg, 63%). Rf 0.68 (BuOH - AcOH - H_2O, 4:1:2), 0.15 ($CHCl_3$ - MeOH -

H_2O, 65:25:2); MP 77~79°C; $[\alpha]_D^{26}$ +22.2° (*c* 1.0, H_2O); FAB-MS *m/z* 256 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 248 (27,400), 310 (8,200) nm $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 260 (sh), 338 (12,700) nm; IR *v* (KBr) cm^{-1} 3400, 1740, 1650, 1600, 1540, 1500, 1480, 1340, 1250; ^1H NMR (400 MHz, DMSO-d_6) δ 1.12 (3H, d, *J*=6.5 Hz), 4.21 (1H, m), 4.41 (1H, dd), 6.72 (1H, dd, *J*=7.8 and 1.5 Hz), 6.94 (1H, dd, *J*=8.1 and 7.8 Hz), 7.37 (1H, dd, *J*=8.1 and 1.5 Hz), 8.61 (NH, d, *J*=8.0 Hz).

L-(2,3-Dihydroxybenzyl)arginine (**17**)

L-(N^α-2,3-Dibenzoyloxybenyl-N^ω-di-*Z*)arginine benzyl ester (100 mg) was treated using the same procedure as employed with L-(N^α-2,3-dibenzoyloxybenyl-N^ω-di-*Z*)arginyl-L-threonine benzyl ester in the synthesis of **1** yielding a colorless powder of L-(2,3-dihydroxybenzyl)arginine acetate (**17**) (35 mg, 97%). Rf 0.53 (BuOH-AcOH-H_2O, 4:1:2), 0.08 ($CHCl_3$-MeOH-H_2O, 65:25:2); MP 132~134°C; $[\alpha]_D^{24}$ +9.0° (*c* 2.0, H_2O); FAB-MS *m/z* 311 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 248 (14,300), 310 (4,400) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 225 (sh), 250 (sh), 340 (7,100) nm; IR *v* (KBr) cm^{-1} 3400, 1740, 1660, 1580, 1550, 1480, 1450, 1320, 1300; ^1H NMR (400 MHz, DMSO-d_6) δ 1.54 (2H, m), 1.78 (1H, m), 1.86 (1H, m), 3.09 (2H, m), 4.29 (1H, m), 6.59 (1H, dd, *J*=7.7 and 7.7 Hz), 6.85 (1H, dd, *J*=8.4 and 1.3 Hz), 7.32 (1H, dd, *J*=7.7 and 1.3 Hz), 8.41 (NH, br), 8.98 (NH, br).

L-(2,3-Dihydroxybenzyl)arginyl-L-threonine Methyl Ester (**18**)

5% HCl-MeOH (3 ml) was added to a solution of **1** (60 mg) in dry MeOH (2 ml). After the reaction mixture was stirred for 10 hours at room temperature, it was concentrated and was purified twice using CPC, employing the following conditions: BuOH-AcOH-H_2O (upper phase stationary, 750:50:750), 4 ml/minute, 900 rpm, 20°C, detection 254 nm. The main fractions were collected and concentrated to a small volume and were lyophilized to give a colorless powder of L-(2,3-dihydroxybenzyl)arginyl-L-threonine methyl ester (**18**) (62 mg, 86%); Rf 0.60 (BuOH-AcOH-H_2O, 4:1:2), 0.25 ($CHCl_3$-MeOH-H_2O, 65:25:2); MP 161~163°C; $[\alpha]_D^{24}$ −12.5° (*c* 1.0, H_2O); FAB-MS *m/z* 426 $(M+H)^+$, 412 $(M+H-Me)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 248 (14,300), 310 (4,400) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 260 (sh), 338 (7,100) nm; IR *v* (KBr) cm^{-1} 3400, 1740, 1680, 1600, 1550, 1450, 1400, ^1H NMR (400 MHz, DMSO-d_6) δ 1.05 (3H, d, *J*=6.6 Hz), 1.55 (2H, m), 1.65 (1H, m), 1.81 (1H, m), 3.13 (2H, m), 3.61 (3H, s), 4.11 (1H, dq, *J*=6.6 and 3.1 Hz), 4.27 (1H, dd, *J*=8.3 and 3.1 Hz), 4.63 (2H, m), 6.18 (1H, dd, *J*=7.8 and 7.4 Hz), 6.61 (1H, dd, *J*=7.4 and 1.6 Hz), 7.16 (1H, dd, *J*=7.8 and 1.6 Hz), 7.82 (NH, br), 8.02 (NH, d, *J*=8.2 Hz), 10.92 (NH, br); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.5 s, 171.0 s, 168.7 s, 156.7 s, 156.5 s, 148.4 s, 118.8 d, 115.0 s, 112.9 d, 110.9 d, 66.2 d, 57.7 d, 51.8 d, 51.8 q, 40.4 t (in DMSO), 29.2 t, 25.1 t, 20.1 q.

Dimethyl Benarthin [L-(2,3-Dimethyloxybenzyl)arginyl-L-threonine] (**19**)

Trimethylsylyldiazomethane (5 ml) was added to a solution of **1** (30 mg) in dry MeOH (2 ml). After the solution was stirred for 6 hours at room temperature, the reaction mixture was concentrated and subjected to chromatography on a column of Sephadex LH-20 in MeOH. It was further purified using CPC, employing the following conditions: $CHCl_3$-MeOH-H_2O (upper phase stationary, 5:6:4), 4 ml/minute, 600 rpm, 20°C, detection 254 nm. The main fractions were collected and concentrated to give a colorless powder of L-(2,3-dimethyloxybenzyl)arginyl-L-threonine (**19**) (8.2 mg, 26%). Rf 0.54 (BuOH-AcOH-H_2O, 4:1:2), 0.27 ($CHCl_3$-MeOH-H_2O, 65:25:2); MP 130~132°C; $[\alpha]_D^{24}$ +14.6° (*c* 0.5, MeOH); FAB-MS *m/z* 440 $(M+H)^+$, 395 $(M+H-COOH)^+$; UV λ_{max}^{MeOH} (ε) 240 (sh), 295 (4,400) nm; IR *v* (KBr) cm^{-1} 3400, 1700, 1680, 1600, 1500, 1450, 1400, ^1H NMR (400 MHz, CD_3OD-d_4 + CF_3COOH) δ 1.18 (3H, d, *J*=5.5 Hz), 1.68 (2H, m), 1.92 (1H, m), 2.08 (1H, m), 3.20 (2H, m), 3.88 (3H, s), 3.92 (3H, s), 4.30 (1H, m), 4.34 (1H, m), 4.80 (2H, m), 7.15 (1H, dd, *J*=7.7 and 7.7 Hz), 7.21 (1H, dd, *J*=7.7 and 2.0 Hz), 7.46 (1H, dd, *J*=7.7 and 2.0 Hz); ^{13}C NMR (100 MHz, CD_3OD-d_4 + CF_3COOH) δ 175.8 s, 173.3 s, 167.4 s, 158.7 s, 154.4 s, 149.2 s, 127.7 s, 125.4 d, 122.8 d, 117.2 d, 68.9 d, 62.1 q, 60.5 d, 56.6 q, 54.2 d, 41.9 t, 30.8 t, 25.4 t, 20.4 q.

L-(N^α-2,3-Dihydroxybenzyl-dimethylpyrimizyl)arginyl-L-threonine (**20**)

2,4-Pentanedione (100 μl) and K_2CO_3 (100 mg) were added to a solution of **1** (100 mg) in H_2O (1.5 ml). After the mixture was stirred for 24 hours at 37°C, the reaction solution was acidified with 2N HCl to pH 4.7 and was allowed to stand for 14 hours at 4°C. The resulting precipitate was filtrated, washed with

H_2O, and was dissolved in MeOH. The solution was evaporated yielding a crude powder (42.4 mg). The powder was purified by column chromatography using Sephadex LH-20 (30 × 800 mm) in MeOH ultimately yielding a colorless powder of L-(N^{α}-2,3-dihydroxybenzyl-dimethylpyrimizyl)arginyl-L-threonine (**20**) (36.9 mg, 32%). Rf 0.45 (BuOH - AcOH - H_2O, 4:1:2), 0.37 ($CHCl_3$ - MeOH - H_2O, 65:25:2); MP 147~150°C; $[\alpha]_D^{24}$ +12.8° (*c* 1.0, MeOH); FAB-MS *m/z* 476 $(M+H)^+$; UV $\lambda_{max}^{H_2O}$ (ε) 230 (sh), 306 (11,800) nm, $\lambda_{max}^{H_2O\text{-}HCl}$ (ε) 308 (13,400) nm, $\lambda_{max}^{H_2O\text{-}NaOH}$ (ε) 227 (60,200), 306 (8,700), 340 (7,900) nm; IR *ν* (KBr) cm^{-1} 3400, 1650, 1600, 1530, 1460, 1400, 1350, 1300; ^1H NMR (400 MHz, MeOD-d_4) δ 1.18 (3H, d, *J*=6.7 Hz), 1.77 (2H, m), 1.90 (1H, m), 2.02 (1H, m), 2.23 (6H, s), 3.44 (2H, m), 4.33 (1H, m), 4.41 (1H, d, *J*=2.8 Hz), 4.74 (1H, dd, *J*=4.8 and 8.9 Hz), 6.33 (1H, s), 6.70 (1H, dd, *J*=7.6 and 8.2 Hz), 6.91 (1H, d, *J*=7.6 Hz), 7.31 (1H, d, *J*=8.2 Hz).

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