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Tyropeptins A and B, New Proteasome Inhibitors Produced by *Kitasatospora* sp. MK993-dF2

II. Structure Determination and Synthesis

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The structures of tyropeptins A and B, new proteasome inhibitors produced by *Kitasatospora* sp. MK993-dF2, were determined by analysis of various NMR experiments. The ^{1}H and ^{13}C NMR of tyropeptins were complicated due to the presence of an aldehyde group. Therefore, tyropeptins were converted to their alcohols by sodium borohydride. These alcohol derivatives gave assignable NMR spectra. The stereochemistry of tyropeptins were determined by analysis of acid hydrolysis products from tyropeptins, and further confirmed by the total synthesis. The structures of tyropeptins A and B were found to be isovaleryl-L-tyrosyl-L-valyl-DL-tyrosinal and *n*-butyryl-L-tyrosyl-L-leucyl-DL-tyrosinal, respectively.

The ubiquitin-proteasome pathway is the principle pathway for intracellular protein degradation, and is involved in many biological processes[1].

Tyropeptins A (**1**) and B (**4**), new proteasome inhibitors, were isolated from the culture broth of *Kitasatospora* sp. MK993-dF2 (Fig. 1). In the preceding paper, the taxonomy of the producing strain, isolation, physico-chemical properties and biological activities of **1** and **4** were reported[2]. We describe herein the structure determination and synthesis of **1** and **4**.

Results and Discussion

Structure Determination of Tyropeptin A (1)

The UV spectrum of **1** showed absorption maxima at 225.2 and 277.6 nm in MeOH. The absorption bands in alkaline solution exhibited characteristic bathochromic shifts.

Compound **1** gave a positive color reaction with Rydon-Smith and 2,4-dinitrophenylhydrazine reagents. The IR spectrum of **1** showed the presence of aldehyde and amide carbonyl groups at 1730, and 1640 and 1520 cm^{-1}, respectively. These observations indicated that **1** related to a peptidyl aldehyde compound. The molecular formula of **1** was established as $C_{28}H_{37}N_3O_6$ by HRFAB-MS data [found *m/z* 512.2761 $(M+H)^+$, calcd. *m/z* 512.2751 for $C_{28}H_{38}N_3O_6$] and NMR spectra. The ^{1}H and ^{13}C NMR spectra of **1** were complicated due to the racemization at the asymmetric center of the tyrosinal moiety (Table 1). Reduction of **1** with sodium borohydride gave two separable diastereomers **2** and **3** (Fig. 1). These derivatives gave assignable NMR spectra and the results are shown in Table 1.

The ^{1}H, ^{13}C NMR, DEPT and HMQC spectra of **2** revealed the presence of twenty eight carbon atoms, assigned to four primary, four secondary, thirteen tertiary and seven quaternary carbon atoms. The information from ^{1}H-^{1}H COSY and HMBC experiments on **2** are summarized in Fig. 2. The analysis of NMR spectra of **2** indicated the presence of an isovaleryl moiety, tyrosine, valine and tyrosinol residues in the molecule of **2**. Acid hydrolysis of **2**

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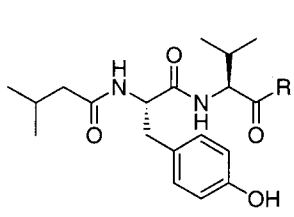
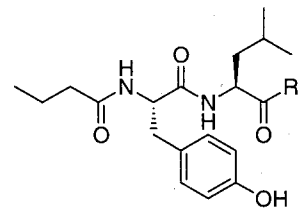
Fig. 1. The structures of tyropeptins and their derivatives.



Tyropeptin A (**1**) R = DL-Tyrosinal
(**2**) R = L-Tyrosinol
(**3**) R = D-Tyrosinol



Tyropeptin B (**4**) R = DL-Tyrosinal
(**5**) R = L-Tyrosinol
(**6**) R = D-Tyrosinol

Table 1. The ^{13}C and ^{1}H NMR assignments of **1**, **2** and **3** in CD_3OD.

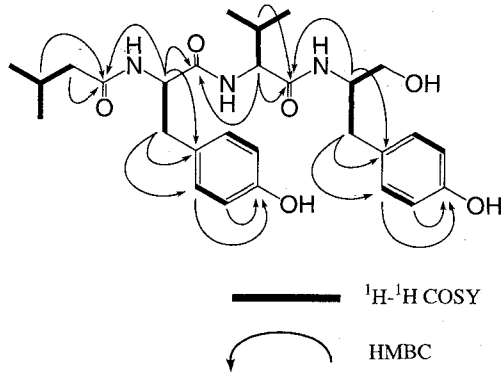
Position	**1**			**2**			**3**		
	δ_C (mult.)		δ_H	δ_C (mult.)		δ_H (mult., *J* (Hz))	δ_C (mult.)		δ_H (mult., *J* (Hz))
Isovaleryl									
CH_3	22.6	q	0.75	22.6	q	0.77 (d, 6.6)	22.6	q	0.77 (d, 6.6)
	22.7	q	0.81	22.7	q	0.82 (d, 6.6)	22.7	q	0.82 (d, 6.6)
CH	27.4	d	1.90	27.4	d	1.92 (m)	27.4	d	1.89 (m)
CH_2	46.1	t	1.99	46.1	t	2.01 (d, 7.4)	46.1	t	2.01 (d, 6.8)
C=O	175.6	s		175.6	s		175.5	s	
Tyrosyl									
α-CH	56.1	d	4.59	56.1	d	4.59 (dd, 5.2, 9.8)	56.1	d	4.59 (dd, 5.2, 9.6)
β-CH_2	37.8	t	2.72	37.7	t	2.73 (dd, 9.8, 14.0)	37.8	t	2.72 (dd, 9.6, 14.2)
			3.00			2.99 (dd, 5.2, 14.0)			3.03 (dd, 5.2, 14.2)
γ-C	129.4	s		129.2	s		129.2	s	
δ-CH	131.3	d	7.03	131.2	d	7.06 (d, 8.4)	131.2	d	7.06 (d, 8.4)
ε-CH	116.2	d	6.67	116.2	d	6.67 (d, 8.4)	116.2	d	6.68 (d, 8.4)
ζ-C	157.2	s		157.3	s		157.3	s	
C=O	173.9	s		173.9	s		173.9	s	
Valyl									
α-CH	60.3	d	4.12	60.3	d	4.10 (d, 7.0)	60.0	d	4.09 (d, 6.8)
CH	32.3	d	1.98	32.2	d	1.99 (m)	32.3	d	1.89 (m)
CH_3	18.6	q	0.86	18.6	q	0.87 (d, 6.6)	18.2	q	0.73 (d, 6.8)
	19.7	q	0.89	19.7	q	0.88 (d, 6.6)	19.7	q	0.73 (d, 6.8)
C=O	173.1	s		173.0	s		173.0	s	
Tyrosinal or Tyrosinol									
α-CH	56.6	d	4.05	54.4	d	4.01 (m)	54.5	d	4.06 (m)
β-CH_2	34.8	t	2.61	37.0	t	2.63 (dd, 9.8, 13.8)	37.1	t	2.57 (dd, 9.4, 13.8)
			2.89			2.79 (dd, 6.4, 13.8)			2.85 (dd, 5.8, 13.8)
γ-C	129.9	s		130.3	s		130.5	s	
δ-CH	131.3	d	7.03	131.3	d	7.04 (d, 8.4)	131.2	d	7.04 (d, 8.4)
ε-CH	116.2	d	6.67	116.2	d	6.68 (d, 8.4)	116.2	d	6.68 (d, 8.4)
ζ-C	156.8	s		156.9	s		157.0	s	
CH_2				63.9	t	3.47 (d, 5.4)	64.5	t	3.51 (d, 5.2)
CHO	98.7	d	4.45 (hemiacetal)						

NMR spectra were obained on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR.

gave tyrosine and valine, which were identified by cellulose TLC analysis (*n*-PrOH - Pyridine - H_2O - CH_3COOH, 15 : 10 : 12 : 3). The sequence of acyl group and amino acid residues in **2** was established from the HMBC spectral data on **2**. The carbonyl carbon of the isovaleryl moiety at δ_C 175.6 was coupled to a α-methylene proton of the tyrosine residue at δ_H 4.59. The carbonyl carbon of the tyrosine moiety at δ_C 173.9 was coupled to an α-methylene proton of the valine residue at δ_H 4.10. The carbonyl carbon of the valine residue at δ_C 173.0 was coupled to an α-methylene proton of the tyrosinol residue at δ_H 4.01.

To determine the configurations of amino acid residues in **2**, the free amino acids obtained from acid hydrolysis of **2** were coupled with Boc-L-Phe-OSu to form one of the diastereomeric dipeptides, Boc-L-Phe-D-amino acid or Boc-L-Phe-L-amino acid. After removal of the Boc group, the [L-Phe]dipeptide diastereomers of each of the amino acids contained in **2** analyzed by LC/MS. Table 2 shows the elution time of each the [L-Phe]dipeptide diastereomers. All amino acids and amino alcohol in **2** have L-configurations. The structure of **2**, therefore, was determined to be isovaleryl-L-tyrosyl-L-valyl-L-tyrosinol.

Fig. 2. Summary of ^1H-^1H COSY and HMBC experiments of **2**.



Compound **3** had the same molecular formula as **2**. The ^1H and ^{13}C NMR spectra of **3** were very similar to those of **2** (Table 1). In fact, the results of NMR spectral analysis of **3** indicated that its planar structure was the same as that of **2**. However, the chemical shifts of ^1H and ^{13}C NMR data of **3** were slightly different with those of **2**. Probably **3** was a stereoisomer of **2** at the asymmetric center of the tyrosinol moiety. As a consequence of the analysis of amino acid residues in **3** (Table 2), the structure of **3** was determined to be isovaleryl-L-tyrosyl-L-valyl-D-tyrosinol. These alcohols **2** and **3** did not inhibit the chymotrypsin-like activity of 20S proteasome at a concentration of 100 μg/ml.

From all data described above, the structure of **1** was concluded to be isovaleryl-L-tyrosyl-L-valyl-DL-tyrosinal.

Structure Determination of Tyropeptin B (4)

The physico-chemical properties of **4** were very similar to that of **1**. The molecular formula of **4** was established as $C_{28}H_{37}N_3O_6$ by HRFAB-MS data [found *m/z* 512.2757 $(M+H)^+$, calcd. *m/z* 512.2751 for $C_{28}H_{38}N_3O_6$] and was found to have the same molecular formula as **1**. The ^1H and

Table 2. Determination of the stereochemistries of amino acid in **2**, **3**, **5** and **6** by LC/MS analysis using [L-Phe] dipeptides method.

Amino acids	Retention time of [L-Phe]-amino acid (minutes)				
	Authentic amino acids	Amino acids in			
		2	**3**	**5**	**6**
L-Valine	13.5	13.6	13.3	-	-
D-Valine	24.6	-	-	-	-
L-Leucine	21.7	-	-	21.8	21.1
D-Leucine	30.0	-	-	-	-
L-Tyrosine	16.1	16.2	16.2	16.2	16.0
D-Tyrosine	22.8	-	-	-	-
L-Tyrosinol	23.6	23.6	-	23.6	-
D-Tyrosinol	27.1	-	26.8	-	26.8

- : Not detected.

Table 3. The ^{13}C and ^{1}H NMR assignments of **4**, **5** and **6** in CD_3OD.

Position	**4**			**5**		**6**	
	δ_C (mult.)		δ_H	δ_C (mult.)	δ_H (mult., *J* (Hz))	δ_C (mult.)	δ_H (mult., *J* (Hz))
n-Butyryl							
CH_3	13.9 q		0.80	13.9 q	0.83 (t, 7.6)	13.9 q	0.82 (t, 7.6)
CH_2	20.3 t		1.49	20.3 t	1.53 (m)	20.2 t	1.51 (m)
CH_2	38.7 t		2.10	38.7 t	2.13 (t, 7.6)	38.7 t	2.12 (t, 7.4)
C=O	176.1 s			176.2 s		176.2 s	
Tyrosyl							
α-CH	56.0 d		4.55	56.3 d	4.53 (dd, 5.6, 9.4)	56.1 d	4.53 (dd, 5.4, 9.4)
β-CH_2	37.9 t		2.74	37.7 t	2.77 (dd, 9.4, 14.0)	37.8 t	2.74 (dd, 9.4, 14.0)
			3.00		3.00 (dd, 5.6, 14.0)		3.01 (dd, 5.4, 14.0)
γ-C	129.2 s			128.9 s		129.1 s	
δ-CH	131.3 d		7.03	131.3 d	7.06 (d, 8.4)	131.3 d	7.04 (d, 8.2)
ε-CH	116.2 d		6.67	116.2 d	6.68 (d, 8.4)	116.1 d	6.68 (d, 8.2)
ζ-C	157.3 s			157.6 s		157.3 s	
C=O	173.8 s			173.9 s		173.8 s	
Leucyl							
α-CH	53.4 d		4.30	53.5 d	4.31 (dd, 5.8, 9.2)	53.3 d	4.23 (dd, 5.6, 9.0)
CH_2	42.2 t		1.47	42.0 t	1.49 (m)	42.1 t	1.31 (m)
CH	25.7 d		1.55	25.7 d	1.56 (m)	25.6 d	1.35 (m)
CH_3	22.2 q		0.85	22.0 q	0.87 (d, 6.4)	22.1 q	0.81 (d, 6.2)
	23.4 q		0.90	23.5 q	0.90 (d, 6.4)	23.4 q	0.83 (d, 6.2)
C=O	174.3 s			174.2 s		174.2 s	
Tyrosinal or Tyrosinol							
α-CH	56.6 d		4.03	54.5 d	3.98 (m)	54.5 d	4.03 (m)
β-CH_2	34.9 t		2.88	36.9 t	2.64 (dd, 7.6, 13.8)	37.1 t	2.56 (dd, 9.2, 14.0)
			2.61		2.78 (dd, 6.6, 13.8)		2.84 (dd, 5.6, 14.0)
γ-C	130.4 s			130.2 s		130.4 s	
δ-CH	131.3 d		7.03	131.3 d	7.03 (d, 8.4)	131.3 d	7.02 (d, 8.2)
ε-CH	116.2 d		6.67	116.3 d	6.68 (d, 8.4)	116.2 d	6.68 (d, 8.2)
ζ-C	156.9 s			157.1 s		157.0 s	
CH_2				63.8 t	3.48 (dd, 1.8, 5.4)	64.5 t	3.51 (d, 5.4)
CHO	98.7 s		4.46 (hemiacetal)				

NMR spectra were obained on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR.

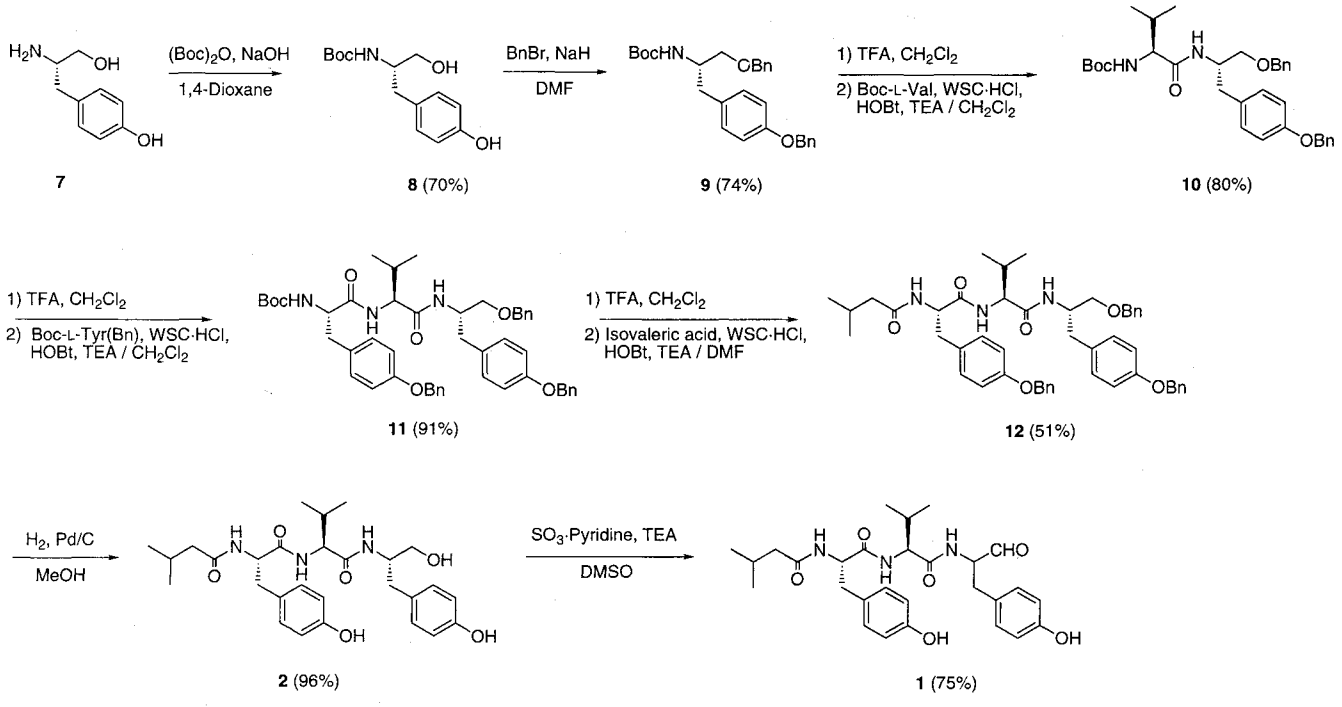
^{13}C NMR data of **4** are shown in Table 3. Because of the racemization, the NMR spectra of **4** were also complicated like in the case of **1**. Therefore, **4** was converted to its alcohol derivatives **5** and **6** (Fig. 1). The ^{1}H and ^{13}C NMR data of **5** and **6** are shown in Table 3.

By similar experiments as described for compounds **2** and **3**, the structures of **5** and **6** were determined to be *n*-butyryl-L-tyrosyl-L-leucyl-L-tyrosinol and *n*-butyryl-L-tyrosyl-L-leucyl-D-tyrosinol, respectively. The structure of **4** was concluded to be *n*-butyryl-L-tyrosyl-L-leucyl-DL-tyrosinal.

Synthesis of Tyropeptin A (**1**)

Tyropeptin A (**1**) was synthesized as shown in Scheme 1. Two hydroxyl groups of **7** were protected with benzyl groups to give **9**. Compound **9** was treated with trifluoroacetic acid (TFA), and then coupled with Boc-L-Val using water soluble carbodiimide hydrochloride (WSC·HCl) and 1-hydroxybenzotriazole (HOBt) to yield dipeptide **10**. The deprotected **10** was coupled with Boc-L-Tyr(Bn) to give tripeptide **11**, which was deprotected and coupled with isovaleric acid to form acyl tripeptide **12**. Hydrogenolysis of **12** with palladium on charcoal gave isovaleryl-L-tyrosyl-L-valyl-L-tyrosinol (**2**). The spectroscopic data and physico-chemical properties of the synthetic compound were identical to those of **2**, alcohol derivative of natural **1**. Compound **2**, synthesized here, was oxidized using sulfur trioxide-pyridine complex (SO_3·Pyridine) to give peptidyl aldehyde. The NMR spectra of synthetic aldehyde were also complicated due to the racemization of α-position of aldehyde group as those of **1**. The spectroscopic data, the physico-chemical properties and the proteasome inhibitory activity of the

Scheme 1. Synthesis of tyropeptin A (**1**).



synthetic aldehyde were identical to those of natural **1**.

Synthesis of Tyropeptin B (**4**)

Tyropeptin B (**4**) was synthesized as shown in Scheme 2. Compound **4** was synthesized by the similar procedure as described in the case of **1**, which gave a 15.6% yield from **9**. The structure of synthetic **4** was identical to the natural **4** by comparisons of its spectroscopic data, physico-chemical properties and proteasome inhibitory activities.

Some proteasome inhibitors were known previously. Peptidyl aldehydes such as MG-132[3] are substrate-related inhibitors. Peptidyl boronic acids such as PS-341[4] demonstrated to have high degree of enzyme selectivity, hence was inactive against many common proteases. Lactacystin is a natural product that inhibits cell cycle progression[5]. Studies on modes of action have shown that lactacystin is a selective and irreversible proteasome inhibitor[6].

We discovered two new proteasome inhibitors, tyropepeins A (**1**) and B (**4**) produced by *Kitasatospora* sp. MK993-dF2. Our structural studies demonstrated that the tyropeptins belong to a family of substrate-related inhibitor. The structures of **1** and **4** were found to be isovaleryl-L-tyrosyl-L-valyl-DL-tyrosinal and *n*-butyryl-L-tyrosyl-L-leucyl-DL-tyrosinal, respectively.

Experimental

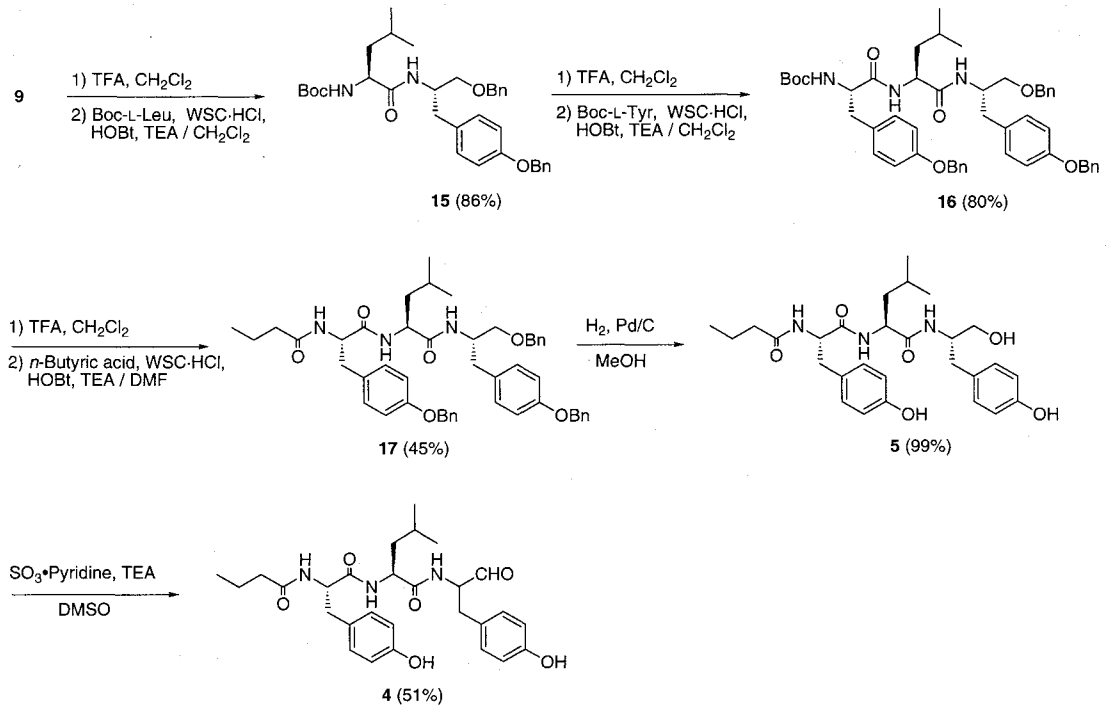
General

NMR spectra were obtained on a JEOL JNM-A 500 spectrometer at 500 MHz and a JEOL JNM-EX 400 spectrometer at 400 MHz. Chemical shifts were given in ppm using TMS as an internal standard. UV absorption spectra were determined using a Hitachi U-3210 spectrophotometer. FAB-MS and HRFAB-MS were measured using a JEOL JMS-SX 102 spectrometer. LC/MS and APCI-MS spectra were measured using a Hitachi M-1200H spectrometer. Optical rotations were determined using a Perkin-Elmer 241 polarimeter.

Reduction of Tyropeptin A (**1**)

To a solution of **1** (8.3 mg) in MeOH (2 ml) was added sodium borohydride (8.3 mg) at 0°C. After stirring at room temperature for 2 hours, to the reaction mixture was added acetone (1 ml). The solvent was removed, and then the residue was purified using reverse phase HPLC under the

Scheme 2. Synthesis of tyropeptin B (**4**).



following conditions: column; Capcell Pak UG (Shiseido, 20.0×250 mm), mobile phase; MeOH - 2 mM ammonium carbonate (40 : 60), flow rate; 10.0 ml/minute, detection; UV at 220 nm. Fractions containing **2** or **3** were separately collected and concentrated under reduced pressure to give **2** (3.3 mg) and **3** (2.3 mg), respectively.

2: IR (KBr) ν_{max} 3280, 2960, 1640, 1620, 1550, 1510, 1450, 1390, 1230. UV λ_{max}^{MeOH} nm (log ε) 225 (4.17), 277 (3.41). $[\alpha]_D^{22}$ −32.7° (*c* 0.3, MeOH). HRFAB-MS (*m/z*) calcd for $C_{28}H_{40}O_6N_3$, 514.2917; found, 514.2913 $(M+H)^+$.

3: IR (KBr) ν_{max} 3280, 2960, 1630, 1550, 1520, 1460, 1380, 1240. UV λ_{max}^{MeOH} nm (log ε) 225 (4.06), 277 (3.30). $[\alpha]_D^{23}$ 0° (*c* 0.2, MeOH). HRFAB-MS (*m/z*) calcd for $C_{28}H_{40}O_6N_3$, 514.2917; found, 514.2913 $(M+H)^+$.

Reduction of Tyropeptin B (**4**)

To a solution of **4** (7.0 mg) in MeOH (2 ml) was added sodium borohydride (7.0 mg) under cooling. Using a similar procedure as described for **1**, reduction of **4** gave **5** (3.3 mg) and **6** (2.3 mg).

5: IR (KBr) ν_{max} 3300, 2960, 1650, 1520, 1450, 1360, 1240. UV λ_{max}^{MeOH} nm (log ε) 225 (4.18), 278 (3.42). $[\alpha]_D^{22}$ −30.8° (*c* 0.23, MeOH). HRFAB-MS (*m/z*) calcd for $C_{28}H_{40}O_6N_3$, 514.2917; found, 514.2905 $(M+H)^+$.

6: IR (KBr) ν_{max} 3430, 2630, 1640, 1520, 1450, 1380, 1240. UV λ_{max}^{MeOH} nm (log ε) 225 (4.06), 277 (3.30). $[\alpha]_D^{23}$ 0° (*c* 0.1, MeOH). HRFAB-MS (*m/z*) calcd for $C_{28}H_{40}O_6N_3$, 514.2917; found, 514.2908 $(M+H)^+$.

Preparation of L-Phenylalanyl Amino Acid Diastereomer

Tyropeptins derivative (0.2 mg) was hydrolyzed with 6 N hydrochloric acid (0.5 ml) at 105°C for 18 hours. Preparation of L-phenylalanyl amino acid diastereomer was carried out using the procedure described by MITCHELL *et al.*[7] and NAGAI *et al.*[8]. A solution of sodium bicarbonate (50 μl, 20 mg/ml) was added to the acid hydrolysate of tyropeptin derivative or known free amino acid (0.2 mg). To the solution was added the 50 μl of *tert*-butyloxycarbonayl-L-phenylalanine-*N*-hydroxysuccinimide ester (Boc-L-Phe-Osu) (40 mg/ml) in 1,4-dioxane. The mixture was allowed to stand at room temperature for 18 hours, and then evaporated to dryness *in vacuo*. The residue was dissolved in 50 μl of TFA and allowed to stand at room temperature for 1 hour to remove the Boc group. The TFA was removed by evaporation *in vacuo*. The resulting material was dissolved in 30 μl of H_2O and then the dipeptide solution was analyzed by LC/MS.

Determination of L- and D- Amino Acid by LC/MS for Diastereometric Dipeptide

The analysis of the dipeptides was performed using LC/MS under the following conditions: column; Pegasil ODS (Senshu Scientific Co., Ltd., 4.6×150 mm), flow rate; 1.0 ml/minute, solvent system; liner gradient of solvent A in solvent B, 0~100% in 60minutes (solvent A: 15% aq ammonium acetate - CH_3COOH - H_2O - CH_3CN, 80 : 1 : 880 : 720; solvent B: 80 : 1 : 1600 : 0), detection; UV at 254 nm. Hitachi M-1200H Mass spectrometer was used for detection in an APCI (positive) mode.

Synthesis of Tyropeptins A (**1**)

8: To a solution of L-tyrosinol hydrochloride (**7**) (5.03 g, 24.8 mmol) in 50 ml of 1,4-dioxane was added 49.6 ml of 1 N NaOH (49.6 mmol) at 0°C. Then, $(Boc)_2O$ (5.5 g, 25.2 mmol) was added to the reaction mixture at 0°C, and the solution was stirred for 3 hours at room temperature. The solvent was evaporated, and then the resulting residue was dissolved in EtOAc and washed with 5% aq. citric acid. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (*n*-Hexane-EtOAc, 1 : 1) to give **8** (4.59 g) as a colorless oil in 70% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (9H, s), 2.74 (2H, d, J=7.6 Hz), 3.54 (1H, m), 3.64 (1H, m), 3.81 (1H, br), 4.80 (1H, m), 6.01 (1H, br), 6.74 (2H, d, J=8.4 Hz), 7.03 (2H, d, J=8.4 Hz). APCI-MS *m/z* 266 $(M-H)^-$.

9: To a solution of **8** (4.59 g, 17.0 mmol) in 45 ml of DMF was added 2.40 g of NaH (50.9 mmol) at 0°C, and the solution was stirred for 10 minutes at 0°C. Then, benzyl bromide (6.4 g, 37.4 mmol) was added to the reaction mixture at 0°C, and the solution was stirred for additional 5 hours at room temperature. The solvent was evaporated, and then the resulting residue was dissolved in EtOAc and washed with 5% aq. citric acid. The organic layer was washed with H_2O, and dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (*n*-Hexane - EtOAc, 10 : 1) to give 9 (5.59 g) as a colorless oil in 74% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (9H, s), 2.81 (2H, m), 3.38 (2H, s), 3.90 (1H, m), 4.48 (2H, dd, J=11.6, 24.0 Hz) 4.86 (1H, br), 5.03 (2H, s), 6.87 (2H, d, J=8.4 Hz), 7.08 (2H, d, J=8.4 Hz), 7.25~7.45 (10H, m). FAB-MS *m/z* 448 $(M+H)^+$.

10: To a solution of **9** (2.0 g, 4.5 mmol) in 20 ml of CH_2Cl_2 was added 5 ml of TFA at 0°C, and then the solution was stirred for 1 hour at room temperature. The solvent was evaporated, and then the residue was coevaporated with toluene twice. To the residue in 20 ml of CH_2Cl_2 was added 498 mg of triethylamine (TEA, 4.9 mmol), 1.1 g of *N*-Boc-L-Val (4.9 mmol), 1.0 g of 1-hydroxybenztriazole · H_2O (HOBt · H_2O, 1.0 g) and 1.1 g of water soluble carbodiimide hydrochloride (WSC · HCl) at 0°C. The reaction mixture was stirred for 18 hours at room temperature. Then the reaction mixture was diluted with $CHCl_3$, washed with 5% aq. $NaHCO_3$, 4% aq. citric acid and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (toluene - acetone, 50 : 1) to give **10** (1.95 g) as a white solid in 80% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 0.84 (3H, d, J=6.2 Hz), 0.91 (3H, dd, J=6.2 Hz), 1.44 (9H, s), 2.82 (2H, d, J=6.8 Hz), 3.37 (2H, s), 3.86 (1H, m), 4.23 (1H, m), 4.47 (2H, dd, J=11.8, 17.8 Hz), 5.00 (1H, br), 5.02 (2H, s), 6.20 (1H, br), 6.86 (2H, d, J=8.4 Hz), 7.07 (2H, d, J=8.4 Hz), 7.30~7.45 (10H, m). APCI-MS *m/z* 547 $(M+H)^+$.

11: To a solution of **10** (2.0 g, 3.8 mmol) in 16 ml of CH_2Cl_2 was added 4 ml of TFA at 0°C, and then the solution was stirred for 1 hour at room temperature. The solvent was evaporated, and then the residue was coevaporated with toluene twice. To the residue in 20 ml of CH_2Cl_2 was added 397 mg of TEA (3.9 mmol), 1.5 g of *N*-Boc-L-Tyr(Bn) (3.9 mmol), 823 mg of HOBt · H_2O (5.4 mmol) and 854 mg of WSC · HCl (4.6 mmol) at 0°C. The reaction mixture was stirred for 18 hours at room temperature. Then the reaction mixture was diluted with $CHCl_3$, washed with 5% aq. $NaHCO_3$, 4% aq. citric acid and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography ($CHCl_3$) to give **11** (2.6 g) as a white solid in 91% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 0.78 (3H, d, J=6.8 Hz), 0.84 (3H, d, J=6.8 Hz), 1.41 (9H, s), 2.08 (1H, m), 2.79 (2H, br), 2.98 (2H, d, J=6.4 Hz), 3.36 (2H, br), 4.13 (1H, m), 4.25 (2H, m), 4.48 (2H, dd, J=11.8, 17.8 Hz), 4.97 (2H, s), 4.99 (2H, s), 6.19 (1H, d, J=6.0 Hz), 6.49 (1H, d, J=8.0 Hz), 6.84 (2H, d, J=8.4 Hz), 6.87 (2H, d, J=8.4 Hz), 7.05 (2H, d, J=8.4 Hz), 7.09 (2H, d, J=8.4 Hz), 7.25~7.45 (15H, m). APCI-MS *m/z* 801 $(M+H)^+$.

12: To a solution of **11** (820 mg, 1.1 mmol) in 8 ml of CH_2Cl_2 was added 2 ml of TFA at 0°C, and then the solution was stirred for 1 hour at room temperature. The solvent was evaporated, and then the residue was coevaporated with toluene twice. To the residue in 10 ml of DMF was added 117 mg of TEA (1.2 mmol), 237 mg of isovaleric acid (2.3 mmol), 243 mg of HOBt · H_2O (1.6 mmol) and 252 mg of WSC · HCl (1.4 mmol) at 0°C. The reaction mixture was stirred for 18 hours at room temperature. Then the reaction mixture was diluted with $CHCl_3$, washed with 5% aq. $NaHCO_3$, 4% aq. citric acid

and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography ($CHCl_3$) to give **12** (411 mg) as a white solid in 51% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 0.77 (3H, d, J=6.8 Hz), 0.84 (3H, d, J=6.8 Hz), 0.85 (3H, d, J=6.0 Hz), 0.88 (3H, d, J=6.0 Hz), 1.98~2.09 (4H, m), 2.80 (2H, d, J=8.4 Hz), 2.97 (2H, d, J=7.2), 3.37 (2H, s), 4.12 (1H, dd, J=6.4, 8.4 Hz), 4.23 (1H, m), 4.47 (2H, s), 4.62 (1H, dd, J=7.0, 14.2 Hz), 4.96 (2H, s), 4.98 (2H, s), 5.99 (1H, d, J=7.2 Hz), 6.16 (1H, d, J=8.8 Hz), 6.50 (1H, d, J=8.8 Hz), 6.84 (2H, d, J=8.8 Hz), 6.85 (2H, d, J=8.8 Hz). 7.06 (2H, d, J=8.8 Hz), 7.07 (2H, d, J=8.8 Hz), 7.25~7.45 (15H, m). APCI-MS *m/z* 784 $(M+H)^+$.

Isovaleryl-L-tyrosyl-L-valyl-L-tyrosinol (**2**): To a solution of **12** (381 mg, 0.49 mmol) in 20 ml of DMF was added 100 mg of 10% palladium on charcoal (Pd/C), and the mixture was stirred under H_2 atmosphere for 18 hour at room temperature. Catalyst was filtered through a cerite layer and the filtrate was concentrated *in vacuo*. The residue was purified using silica gel column chromatography ($CHCl_3$-MeOH, 10 : 1) to give **2** (241 mg) as a white powder in 96% yield.: $[\alpha]_D^{23}$ −36.2° (*c* 0.5, MeOH). IR (KBr) ν_{max} 3280, 2960, 1640, 1620, 1550, 1520, 1450, 1390, 1230. UV λ_{max}^{MeOH} nm (log ε) 225 (4.24), 278 (3.49). ^1H NMR (500 MHz, CD_3OD) δ 0.77 (3H, d, J=6.4 Hz), 0.82 (3H, d, J=6.4 Hz), 0.87 (3H, d, J=6.4 Hz), 0.89 (3H, d, J=6.4 Hz), 1.92 (1H, m), 1.99 (1H, m), 2.01 (2H, d, J=7.4 Hz), 2.63 (1H, dd, J=7.6, 12.0 Hz), 2.74 (1H, dd, J=9.8, 14.2 Hz), 2.79 (1H, dd, J=6.4, 13.8 Hz), 3.00 (1H, dd, J=5.0, 14.0 Hz), 3.47 (1H, d, J=5.2 Hz), 4.02 (1H, m), 4.11 (1H, d, J=7.2 Hz), 4.60 (1H, dd, J=5.2, 9.6 Hz), 6.67 (2H, d, J=8.4 Hz), 6.68 (2H, d, J=8.4 Hz), 7.04 (2H, d, J=8.4 Hz), 7.06 (2H, d, J=8.4 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 18.6, 19.7, 22.6, 22.7, 27.4, 32.2, 37.0, 37.7, 46.1, 54.4, 56.1, 60.3, 63.9, 116.2, 116.2, 129.2, 130.3, 131.2, 131.3, 156.9, 157.2, 173.0, 173.9, 175.6. HRFAB-MS (*m/z*) calcd. for $C_{28}H_{40}O_6N_3$, 514.2917; found, 514.2914 $(M+H)^+$.

Isovaleryl-L-tyrosyl-L-valyl-DL-tyrosinal (**1**): To a solution of **2** (100 mg, 0.20 mmol) in 1 ml of DMSO was added 118 mg of TEA (1.17 mmol) at room temperature, and the solution was stirred for 5 minutes at 0°C. Then, 93 mg of sulfur trioxide-pyridene complex (SO_3·Pyridine, 0.6 mmol) in 0.5 ml of DMSO was added to the reaction mixture at 0°C, and the solution was stirred for an additional 1 hour at room temperature. The reaction mixture was diluted with EtOAc, and washed with 4% aq. citric acid, H_2O, 5% aq. $NaHCO_3$ and brine. The organic layer was dried ove.r Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (toluene - acetone, 2 : 1 to 1 : 1) to give **1** (74 mg) as a white powder in 75% yield. $[\alpha]_D^{22}$ −17.0° (*c* 0.5, MeOH). IR (KBr) ν_{max} 3290, 2960, 1730, 1640, 1520, 1450, 1370, 1230. UV λ_{max}^{MeOH} nm (log ε) 225 (4.18), 278 (3.46). HRFAB-MS (*m/z*) calcd for $C_{28}H_{38}O_6N_3$, 512.2761; found, 512.2759 $(M+H)^+$.

Synthesis of Tyropeptin B (**4**)

15: To a solution of **9** (3.59 g, 8.0 mmol) in 32 ml of CH_2Cl_2 was added 8 ml of TFA at 0°C, and then the solution was stirred for 1 hour at room temperature. The solvent was evaporated, and then the residue was coevaporated with toluene twice. To the residue in 32 ml of CH_2Cl_2 was added 890 mg of TEA, (8.83 mmol), 2.2 g of *N*-Boc-L-Leu·H_2O (8.8 mmol), 1.9 g of HOBt·H_2O (12.1 mmol) and 2.0 g of WSC·HCl (10.4 mmol) at 0°C. The reaction mixture was stirred for 18 hours at room temperature. Then the reaction mixture was diluted with $CHCl_3$, washed with 5% aq. $NaHCO_3$, 4% aq. citric acid, brine and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (toluene - acetone, 50 : 1) to give **15** (3.89 g) as a white solid in 86% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (6H, d, J=5.2 Hz), 1.43 (9H, s), 1.62 (3H, br), 2.81 (2H, d, J=8.0 Hz), 3.37 (2H, d, J=3.6 Hz), 4.05 (1H, m), 4.20 (1H, m), 4.48 (2H, dd, J=12.2, 20.6 Hz), 4.82 (1H, br), 5.02 (2H, s), 6.37 (1H, br), 6.86 (2H, d, J=8.2 Hz), 7.07 (2H, d, J=8.2 Hz), 7.30~7.45 (10H, m). APCI-MS *m/z* 561 $(M+H)^+$.

16: To a solution of **15** (3.89 g, 6.94 mmol) in 32 ml of CH_2Cl_2 was added 8 ml of TFA at 0°C, and then the solution was stirred for 1 hour at room temperature. The solvent was evaporated, and then the residue was coevaporated with toluene twice. To the residue in 40 ml of CH_2Cl_2 was added 771 mg of TEA (7.63 mmol), 2.83 g of *N*-Boc-L-Tyr(Bn) (7.63 mmol), 1.41 g of HOBt·H_2O (10.4 mmol) and 1.7 g of WSC·HCl (9.0 mmol) at 0°C. The reaction mixture was stirred for 18 hours at room temperature. Then the reaction mixture was diluted with $CHCl_3$, washed with 5% aq. $NaHCO_3$, 4% aq. citric acid and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography ($CHCl_3$) to give **16** (4.46 g) as a white solid in 80% yield.: ^1H NMR (400 MHz, $CDCl_3$) δ 0.87 (6H, d, J=6.4 Hz), 1.41 (9H, s), 1.47~1.62 (3H, m), 2.79 (2H, m), 2.97 (2H, d, J=6.8 Hz), 3.37 (2H, d, J=3.6 Hz), 4.18 (1H, m), 4.22 (2H, m), 4.34 (1H, m), 4.49 (2H, dd, J=12.0, 16.8 Hz), 4.92 (1H, br), 4.97 (2H, s), 4.99 (2H, s), 6.32 (1H, s), 6.34 (1H, s), 6.85 (2H, d, J=8.8 Hz), 6.87 (2H, d, J=8.8 Hz), 7.05 (2H, d, J=8.8 Hz), 7.08 (2H, d, J=8.8 Hz), 7.25~7.45 (15H, m). APCI-MS *m/z* 814 $(M+H)^+$.

17: To a solution of **16** (1.0 g, 1.23 mmol) in 8 ml of CH_2Cl_2 was added 2 ml of TFA at 0°C, and the solution was stirred for 1 hour at room temperature. The solvent was evaporated, and the residue was coevaporated with toluene twice. To the residue in 10 ml of DMF was added 136 μg of TEA (1.35 mmol), 238 mg of *n*-butyric acid (2.7 mmol), 284 mg of HOBt·H_2O (1.85 mmol) and 295 mg of WSC·HCl (1.60 mmol) at 0°C. Then, the reaction mixture was stirred for 18 hours at room temperature. The reaction mixture was diluted with $CHCl_3$, washed with 5% aq. $NaHCO_3$, 4% aq. citric acid and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography ($CHCl_3$) to give **17** (435 mg) as a white solid in 45% yield.: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (3H, d, J=6.4 Hz), 0.86 (3H, d, J=6.4 Hz), 0.87 (3H, t, J=7.2 Hz), 1.40~1.65 (5H, m), 2.12 (2H, t, J=7.6 Hz), 2.80 (2H, d, J=8.0), 2.96 (2H, d, J=6.8 Hz), 3.38 (2H, d, J=3.6 Hz), 4.20 (1H, m), 4.31 (1H, m), 4.43 (2H, dd, J=12.0, 14.4 Hz), 4.60 (1H, dd, J=7.2, 14.4 Hz), 4.95 (2H, s), 4.97 (2H, s), 6.00 (1H, d, J=8.0 Hz), 6.31 (1H, d, J=8.8 Hz), 6.40 (1H, d, J=8.0 Hz), 6.84 (2H, d, J=8.8 Hz), 6.85 (2H, d, J=8.8 Hz). 7.07 (4H, d, J=8.8 Hz) 7.25~7.45 (15H, m). APCI-MS *m/z* 784 $(M+H)^+$, 782 $(M-H)^-$.

n-Butyryl-L-tyrosyl-L-leucyl-L-tyrosinol (**5**): To a solution of **17** (405 mg, 0.52 mmol) in 8 ml of DMF was added 100 mg of 10% Pd/C, and the mixture was stirred under H_2 atmosphere for 18 hours at room temperature. Catalyst was filtered out through cerite and the filtrate was concentrated *in vacuo*. The residue was purified using silica gel column chromatography ($CHCl_3$ - MeOH, 10 : 1) to give **5** (262 mg) as a white powder in 99% yield.: $[\alpha]_D^{23}$ −36.2° (*c* 0.5, MeOH). IR (KBr) ν_{max} 3320, 2960, 1640, 1620, 1520, 1450, 1360, 1240. UV λ_{max}^{MeOH} nm (log ε) 225 (4.24), 278 (3.49). ^{1}H NMR (500 MHz, CD_3OD) δ 0.87 (3H, t, J=7.6 Hz), 0.86 (3H, d, J=6.4 Hz), 0.90 (3H, d, J=6.4 Hz), 1.49 (2H, m), 1.53 (2H, m), 1.56 (1H, m), 2.13 (2H, t, J=7.6 Hz), 2.64 (1H, d, J=7.6, 13.8 Hz), 2.77 (1H, dd, J=9.0, 13.6 Hz), 2.78 (1H, dd, J=6.4, 13.6 Hz), 3.00 (1H, dd, J=5.2, 14.0 Hz), 3.48 (2H, dd, J=1.8, 5.2 Hz), 3.98 (1H, m), 4.31 (1H, dd, J=5.8, 9.2 Hz), 4.54 (1H, dd, J=5.6, 9.2 Hz), 6.68 (4H, d, J=8.4 Hz), 7.03 (2H, d, J=8.4 Hz). 7.06 (2H, d, J=8.4 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 13.9, 20.2, 22.0, 23. 5, 25.7, 36.9, 37.7, 38.7, 42.0, 53.5, 54.5, 56.2, 63.8, 116.2, 116.2, 129.1, 130.3, 131.3, 131.3, 156.9, 157.3, 173.9, 174.2, 176.2. HRFAB-MS (*m/z*) calcd for $C_{28}H_{40}O_6N_3$, 514.2917; found, 514.2910 $(M+H)^+$.

n-Butyryl-L-tyrosyl-L-leucyl-DL-tyrosinal (**4**): To a solution of **5** (100 mg, 0.20 mmol) in 1 ml of DMSO was added 118 mg of TEA (1.17 mmol) at room temperature, and the solution was stirred for 5 minutes at 0°C. Then, 93 mg of SO_3·Pyridine (0.6 mmol) in 0.5 ml of DMSO was added to the reaction mixture at 0°C, and then the solution was stirred for additional 1 hour at room temperature. The reaction mixture was diluted with EtOAc, and washed with 4% aq. citric acid, H_2O, 5% aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (toluene - acetone, 3 : 1 to 2 : 1) to give **4** (51 mg) as a white powder in 51% yield.: $[\alpha]_D^{22}$ −14.8° (*c* 0.6, MeOH). IR (KBr) ν_{max} 3290, 2960, 1730, 1650, 1520, 1450, 1380, 1240. UV λ_{max}^{MeOH} nm (log ε) 225 (4.34), 278 (3.63). HRFAB-MS (*m/z*) calcd for $C_{28}H_{38}O_6N_3$, 512.2761; found, 512.2765 $(M+H)^+$.

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