NEW ANTIVIRAL ANTIBIOTICS, KISTAMICINS A AND B

II. STRUCTURE DETERMINATION

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The structures of antiviral antibiotics kistamicins A and B have been determined by a combination of chemical degradation and spectral analysis. They are commonly composed of D-tyrosine, 3,5-dihydrophenylglycine, a biphenyl ether bis-amino acid, and a diphenyl substituted indole tris-amino acid, forming a tricyclic ring structure. Kistamicin B possessed a phenethylamide at the amino terminal of kistamicin A. They are structurally related to the nuclei of the vancomycin group antibiotics particularly to antibiotic complestatin.

In spite of the considerable effort expended to date, only a few compounds have been discovered from natural sources that are effective against viruses. In our screening program for fermentation metabolites, we have discovered two new antiviral antibiotics, designated kistamicins A and B in the culture broth of *Microtetraspora parvosata* subsp. *kistnae* subsp. nov. collected in India. The taxonomy of the producing organism and the production, physico-chemical properties and biological activities of kistamicins A and B, have been reported in preceding paper[1]. In this report we present the structural studies on these antibiotics.

Results and Discussion

Kistamicins A (**1**) and B (**2**) were isolated from the fermentation broth of *Microtetraspora parvosata* subsp. *kistnae* subsp. nov. by 1-butanol extraction followed by column chromatography on silica gel and reversed phase silica gel[1]. They were obtained as pale yellow powders; **1**, $C_{61}H_{51}N_8O_{15}Cl$, mp >300°C (dec.), $[\alpha]_D^{25}$ −1.8° (*c* 1.0, MeOH); **2**, $C_{70}H_{60}N_9O_{16}Cl$, mp >300°C (dec.), $[\alpha]_D^{25}$ +22° (*c* 0.5, MeOH).

The degradation and spectral studies for structure determination of kistamicins were first performed on **1**. The UV spectrum of **1** exhibited absorption maxima at 231, 265, 284 and 305 nm in MeOH, which moved to 244 and 286 nm in alkaline solution. ^1H and ^{13}C NMR spectra (Table 1) assisted with COSY experiments indicated the presence of seven aromatic and seven aliphatic units shown below.

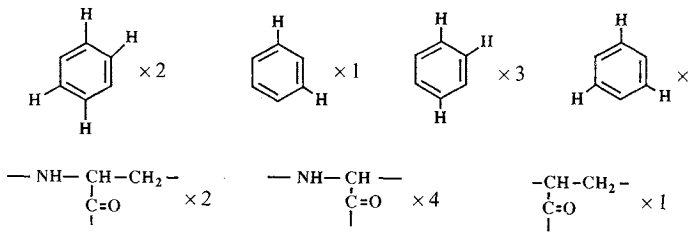

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Table 1. ^{13}C and ^{1}H NMR data for kistamicins A (**1**) and B (**2**).

Unit	1		2	
	δ_C[a]	δ_H[b]	δ_C[b]	δ_H[b]
A-1	134.5 (s)		137.6 (s)	
2	133.0 (d)	7.72 (dd)	135.7 (d)	7.79 (dd)
3	122.4 (d)	7.56 (dd)	125.2 (d)	7.56 (dd)
4	154.2 (s)		154.8 (s)	
5	123.7 (d)	7.24 (dd)	126.2 (d)	7.20 (dd)
6	130.4 (d)	7.82 (dd)	132.5 (d)	7.77 (dd)
1′	40.3 (t)	2.92 (dd), 3.59 (m)	42.5 (t)	2.99 (br t), 3.42 (m)
2′	56.6 (d)	4.31 (m)	60.2 (d)	4.26 (dt)
3′	169.4 (s)		172.2 (s)	
NH		8.16 (d)		8.29 (d)
B-1	129.6 (s)		134.0 (s)	
2	130.8 (d)	5.70 (d)	133.3 (d)	5.70 (d)
3	131.3 (s)		136.6 (s)	
4	138.5 (s)		141.4 (s)	
5	148.7 (s)		151.5 (s)	
6	107.9 (d)	5.54 (d)	110.8 (d)	5.53 (d)
1′	54.5 (d)	6.39 (s)	57.4 (d)	6.40 (d)
2′	168.3 (s)		172.1 (s)	
NH		No		8.23 (d)
C-1		No		No
2	124.2 (d)	6.98 (s)	126.5 (d)	6.97 (s)
3	110.4 (s)		113.2 (s)	
4	125.5 (s)		129.4 (s)	
5	120.3 (d)	7.99 (d)	122.9 (d)	7.99 (d)
6	121.2 (d)	6.84 (dd)	124.1 (d)	6.84 (br d)
7	134.4 (s)		137.0 (s)	
8	114.8 (d)	7.30 (d)	117.1 (d)	7.28 (br s)
9	137.0 (s)		141.0 (s)	
1′	29.2 (t)	3.01 (m), 3.59 (m)	31.2 (t)	3.00 (br t), 3.55 (dd)
2′	52.4 (d)	6.29 (m)	55.6 (d)	6.23 (m)
3′	167.4 (s)		172.1 (s)	
NH		No		8.86 (d)
D-1	126.6 (s)		131.2 (s)	
2	119.4 (d)	7.26 (dd)	122.1 (d)	7.15 (m)
3	117.2 (d)	6.95 (d)	119.1 (d)	6.97 (d)
4	147.7 (s)		150.3 (s)	
5	144.7 (s)		147.1 (s)	
6	114.1 (d)	5.23 (d)	113.1 (d)	5.26 (d)
1′	54.3 (d)	5.41 (s)	56.6 (d)	5.64 (s)
2′	170.3 (s)		177.5 (s)	
NH		No		No
E-1	135.2 (s)		139.8 (s)	
2	133.7 (d)	7.95 (d)	135.6 (d)	7.95 (d)
3	128.1 (s)		131.4 (s)	
4	159.1 (s)		161.4 (s)	
5	125.7 (d)	6.84 (d)	128.8 (d)	6.73 (d)
6	128.9 (d)	7.37 (dd)	131.6 (d)	7.37 (dd)
1′	53.4 (d)	4.98 (d)	56.2 (d)	4.99 (d)
2′	169.9 (s)		173.0 (s)	
NH		9.52 (d)		9.54 (d)
F-1	141.0 (s)		142.6 (s)	
2, 6	103.9 (d)	6.14 (d)	106.4 (d)	6.18 (d)
3, 5	157.7 (s)		160.3 (s)	
4	101.2 (d)	5.77 (t)	103.3 (d)	5.79 (t)
1′	55.7 (d)	4.93 (d)	58.8 (d)	4.91 (d)
2′	170.6 (s)		173.4 (s)	
NH		8.74 (d)		8.73 (d)
G-1	125.2 (s)		128.1 (s)	
2, 6	129.8 (d)	6.70 (d)	132.5 (d)	6.61 (d)
3, 5	115.2 (d)	6.30 (d)	116.5 (d)	6.22 (d)
4	156.0 (s)		157.0 (s)	
1′	36.2 (t)	2.89 (t), 3.45 (dd)	40.4 (t)	2.70 (br t), 3.15 (m)
2′	54.4 (d)	4.04 (dd)	57.8 (d)	4.45 (dd)
3′	169.5 (s)		172.4 (s)	
NH (NH_2)		No		No
H-1			142.6 (s)	
2, 6			130.6 (d)	7.07 (dd)
3, 5			130.2 (d)	7.22 (t)
4			128.0 (d)	7.14 (dd)
1′			37.8 (t)	2.65 (br t), 2.65 (br t)
2′			43.5 (t)	3.22 (m), 3.26 (m)
NH				No
CO			171.5 (s)	

No: Not observed.
[a] In DMSO-d_6.
[b] In CD_3OD.

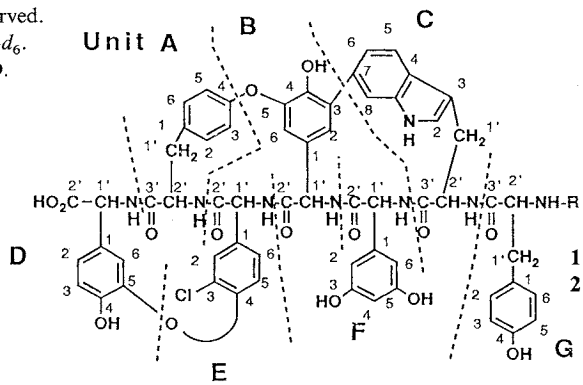
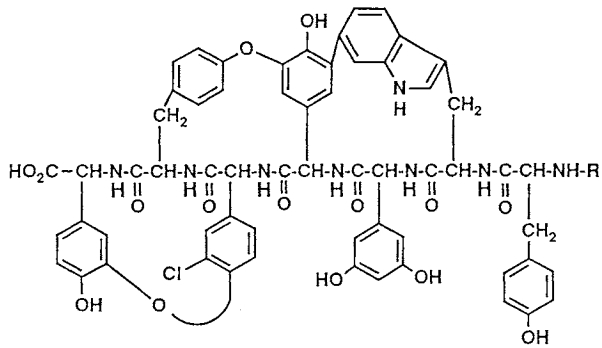
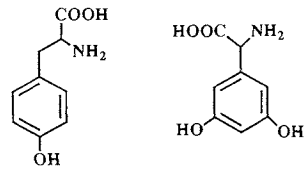


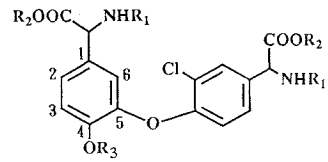
Fig. 1. Structures of kistamicins A (**1**) and B (**2**).



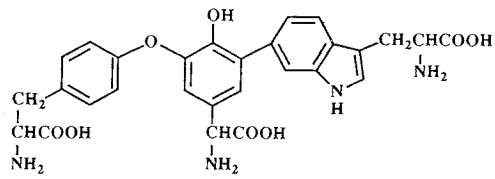
1 R=H
2 R=CO-NH-CH_2-CH_2-C_6H_5



3 **4**



5 R_1=H R_2=H R_3=H
6 R_1=$COCH_3$ R_2=CH_3 R_3=CH_3
7 R_1=$COCH_3$ R_2=H R_3=CH_3



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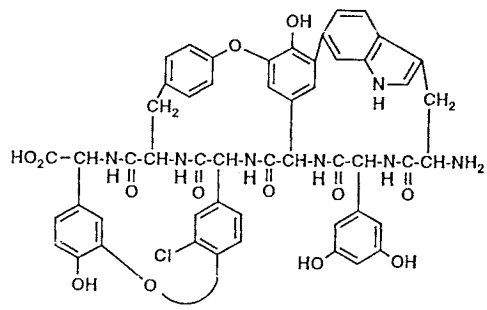
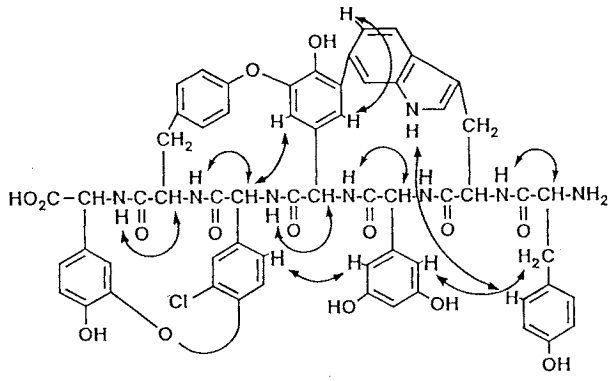


9

Acid hydrolysis (6 M HCl, 110°C, 24 hours) of **1** gave three Ninhydrin positive, water-soluble products (**3**, **4** and **5**), and two of them were identified as D-tyrosine (**3**) and 3,5-dihydroxyphenylglycine (**4**)[2] based on their physico-chemical and spectral data, and amino acid analysis results. The stereochemistry of **4** was deduced to be "D" by a comparison of its specific optical rotational value ($[\alpha]_D^{25}$ −85°, *c* 0.5, 1 M HCl) with those of D-4-hydroxyphenylglycine[3] and D-3,5-dimethoxyphenylglycine[4].

Upon *N*-acetylation in MeOH followed by methylation with diazomethane, **5** afforded the di-*N*-acetyl-tri-*O*-methyl derivative (**6**), which showed a molecular ion peak at *m/z* 492 together with an isotope ion peak at *m/z* 494 indicating the presence of a chlorine in the molecule. Two phenyl rings were demonstrated by its ^1H and ^{13}C NMR spectra. An ether linkage and substituents on these two rings of **6** were assigned based on the calculated ^{13}C chemical shift values of the aromatic carbons and COLOC ex-

Fig. 2. NOEs observed for kistamicin A (**1**).



periment ($^2J_{C-H}$ and/or $^3J_{C-H}$ of the quaternary carbons). The structure was further confirmed by NOESY experiment on di-*O*-demethyl-**6** (**7**) which was obtained by mild saponification of **6**. Clear NOEs were observed between 4-methoxy protons (δ 3.70) and the *ortho* (δ 7.01, 3-H) and *meta* (δ 7.14, 2-H) protons.

In order to isolate the remaining portion of **1**, which was presumed to contain an indole nucleus by the NMR studies, **1** was hydrolyzed with 6 M HCl containing thioglycolic acid at 110°C for 24 hours[5]. From the hydrolysate, a new amino acid (**8**) positive to EHRLICH's reagent was isolated along with **3**, **4** and **5**. The struture of **8** was assigned by its ^{1}H and ^{13}C NMR spectra as shown above.

Linkage of these four amino acids (**3**, **4**, **5** and **8**) by six peptide bonds accounts for the molecular formula of **1**. The linkages were established by the HETCOR and long range HETCOR (8 Hz) experiments of **1**. In addition, the NOE correlations between the protons of these amino acids, particularly those between α-methines and/or amide protons were analyzed as shown in Fig. 2 assigning the total structure of **1**. The location of the tyrosine at *N*-terminal was confirmed by Edman degradation of **1**. After first cycle of degradation, **1** gave PTH-tyrosine together with hexapeptide (**9**). The aromatic ring system of the upper half of **1** (amino acid **8**) was also confirmed by ^{13}C chemical shifts comparison with that of complestatin[3], a protease inhibitor of complement in the human complement system[6]. The corresponding ^{13}C signals of amino acid **8** moiety in **1** and complestatin agreed well with each other except the presence of an additional *N*-methyl signal in the latter. Thus the structure of **1** was determined as shown in Fig. 1.

The physico-chemical properties and ^{1}H and ^{13}C NMR spectra of **2** (Table 1) are very similar to those of **1**. The NMR spectra and molecular formula of **2** indicated the presence of an additional set of signals which were assignable to a 2-phenethylaminocarbonyl residue. The presence of this group was confirmed by recovery of 2-phenethylamine (**10**, EI-MS *m/z* 121, M^+) upon total acid hydrolysis of **2**. In the NOESY of **2**, the proton signals of the 2-phenethylamino moiety did not show NOEs with any protons of other constituents suggesting that this group was a terminal unit of the molecule. As stated before, degradation of **1** gave a PTH-tyrosine together with **9**, on the other hand **2** was unreactive to the reaction. These data indicated that 2-phenethylamine was substituted *via* an ureide bond at the *N*-terminus in **2** as shown in Fig. 1.

Kistamicins A and B are structurally related to the glycopeptide antibiotics such as vancomycin, ristocetin and teicoplanin. Kistamicins A and B are however distinct in possessing an indole nucleus in place of the modified tyrosine or β-hydroxytyrosine contained in the latter group of antibiotics. Kistamicins

A and B are closely related to antibiotic complestatin[6]. It is interesting to note that **9** which lacks only the *N*-terminal tyrosine residue to **1** was completely inactive against the influenza virus in our test. The vancomycin aglycone prepared by the method of NAGARAJAN and SCHABEL[7] also did not show activity against the virus in our assay.

Experimental

General Procedure

TLC was performed on precoated silica gel plates (Silica gel 60 F_{254}, Merck, 0.25 mm thick). The IR spectra were determined on a Jasco IR-810 spectrometer and the UV spectra on a Jasco UVIDEC-G10C spectrometer. The ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 spectrometer operated in the Fourier transform mode using tetramethylsilane and/or dioxane as the internal standard. EI-MS and FAB-MS were run with a JEOL JMS-DX303 HF spectrometer. Optical rotation was determined with a Jasco Model DIP-140. Amino acid analysis was carried out on a PICO TAG amino acid analysis system (Waters).

Total Acid Hydrolysis of 1

Kistamicin A (**1**, 520 mg) was heated in 6 M HCl (30 ml) at 110°C for 24 hours in a sealed container. The hydrolysate was dried *in vacuo* and the residue was dissolved in water and applied on a column of Dowex 50W × 8 (H^+) which was developed with dil. HCl. The eluate was monitored by TLC and the fractions containing homogeneous amino acids were pooled and concentrated. **4** (73 mg) was eluted with 0.6 M HCl, **3** (90 mg) with 1 M HCl and **5** (96 mg) with 6.5 M HCl.

3: $[\alpha]_D^{25}$ +9.1° (*c* 0.5, 5 M HCl), the stereochemistry was determined as "D" by HPLC (column: MCl GEL CRS10W, 4.6 i.d. × 50 mm; mobile phase: 2 mM $CuSO_4$; flow rate: 1 ml/minute; detection: UV 254 nm; Rt 14.4 minutes, Rt of L-Tyr 24.9 minutes).

4: mp 202~204°C; HRFAB-MS *m/z* 184.0616 $(M+H)^+$, Δ0.6 mmu for $C_8H_{10}NO_4$; UV in 0.01 N HCl λ_{max} nm (ε) 202 (30,200), 226 (sh, 9,200), 280 (3,300), in 0.01 N NaOH λ_{max} nm (ε) 215 (28,800), 295 (4,000); ^1H NMR in D_2O δ 4.91 (1H, s), 6.47 (1H, t, J=2.2 Hz), 6.52 (2H, d, J=2.2).

5: mp >240°C (dec.); $[\alpha]_D^{25}$ +4° (*c* 0.5, 1 M HCl); HRFAB-MS *m/z* 367.0699 $(M+H)^+$, Δ0.2 mmu for $C_{16}H_{16}N_2O_6Cl$; UV in 0.01 N HCl λ_{max} nm (ε) 204 (58,100), 232 (sh, 23,900), 275 (6,200), 282 (sh, 5,600), in 0.01 N NaOH λ_{max} nm (ε) 212 (44,700), 233 (26,600), 287 (8,100), 298 (sh, 7,600); ^1H NMR in D_2O δ 4.75 (1H, s), 4.84 (1H, s), 6.97 (1H, d, J=8.6 Hz), 7.06 (1H, d, J=2.1 Hz), 7.14 (1H, d, J=8.6 Hz), 7.22 (1H, dd, J=8.3, 2.1 Hz), 7.32 (1H, dd, J=8.3, 2.1 Hz), 7.63 (1H, d, J=2.1 Hz); ^{13}C NMR in D_2O δ 58.0 (d), 58.2 (d), 118.9 (d), 119.4 (d), 121.2 (d), 124.7 (s), 126.8 (d), 127.2 (s), 129.0 (d), 130.7 (s), 131.3 (d), 143.7 (s), 149.0 (s), 154.0 (s), 173.2 (s), 173.4 (s).

Di-*N*-acetyl-tri-*O*-methyl-5 (6)

5 (6 mg) was dissolved in MeOH (1 ml) and acetylated with acetic anhydride (0.2 ml) at room temperature for one hour, and the reaction solution was evaporated *in vacuo*. The residue was treated with an excess CH_2N_2 in ether. The usual work-up followed by purification of the product by preparative TLC developing with toluene - MeOH (4 : 1) yielded pure **6** (3.7 mg): mp 77~79°C; HREI-MS *m/z* 492.1316 (M^+), Δ1.6 mmu for $C_{23}H_{25}N_2O_8Cl$; IR ν_{max} (KBr) 1745, 1660, 1510, 1490, 1275, 1240 cm^{-1}; ^1H NMR in $CDCl_3$ δ 2.03 (3H, s), 2.06 (3H, s), 3.72 (3H, s), 3.76 (3H, s), 3.80 (3H, s), 5.49 (1H, d, J=7.3 Hz), 5.52 (1H, d, J=7.3 Hz), 6.43 (1H, d, J=7.3 Hz), 6.50 (1H, d, J=7.3 Hz), 6.65 (1H, d, J=8.6 Hz), 6.95 (1H, d, J=2.1 Hz), 6.98 (1H, d, J=8.6 Hz), 7.13 (1H, dd, J=8.6, 2.1 Hz), 7.18 (1H, dd, J=8.6, 2.1 Hz), 7.43 (1H, d, J=2.1 Hz).

Di-*N*-acetyl-mono-*O*-methyl-5 (7)

6 (3 mg) was dissolved in 0.1 M KOH - MeOH (2 ml) and refluxed for 30 minutes. The reaction solution was diluted with water (10 ml), acidified to pH 3.0 and then extracted with EtOAc (5 ml). Purification of the extract by preparative TLC with $CHCl_3$ - MeOH - AcOH (14 : 6 : 1) followed by Sephadex LH-20 column chromatography (MeOH) afforded **7** (1.6 mg): mp >280°C (dec.); HRFAB-MS *m/z* 465.1066 $(M+H)^+$,

Δ0.1 mmu for $C_{21}H_{22}N_2O_8Cl$; IR ν_{max} (KBr) 1720, 1650, 1510, 1490, 1275, 1240 cm^{-1}; ^1H NMR in DMSO-d_6 δ 1.84 (3H, s), 1.87 (3H, s), 3.70 (3H, s), 4.70 (1H, d, J=6.8 Hz), 4.73 (1H, d, J=6.8), 6.43 (1H, d, J=8.6 Hz), 6.93 (1H, br s), 7.01 (1H, d, J=8.6 Hz), 7.09 (1H, dd, J=8.6, 2.1 Hz), 7.14 (1H, dd, J=8.3, 2.1 Hz), 7.37 (1H, d, J=2.1 Hz), 7.77 (1H, d, J=6.8 Hz), 7.83 (1H, d, J=6.8 Hz).

Preparation of Triamino Acid (**8**)

1 (390 mg) was treated with 6 M HCl (3 ml) and thioglycolic acid (1 ml). The suspension was degassed under vacuum, and heated at 110°C for 24 hours. The reaction solution was diluted with water (100 ml), adjusted to pH 5.5 with 6 M NaOH and then concentrated. The residue was charged on a column of cellulose (Merck, No. 2331). The column was washed with BuOH - AcOH - H_2O (14 : 3 : 1) to elute **3**, **4** and **5**, and then developed with the mixture of 3 : 1 : 1 ratio. By TLC examination, relevant fractions were pooled, evaporated, and chromatographed on Sephadex LH-20 (50% MeOH) to afford pure **8** as pale yellow powder (39 mg): mp 350°C (dec.); $[\alpha]_D^{25}$ +25° (c 0.5, 1 M HCl); HRFAB-MS m/z 549.1992 $(M+H)^+$, Δ0.7 mmu for $C_{28}H_{29}N_4O_8$; UV in 0.01 N HCl - MeOH λ_{max} nm (ε) 226 (sh, 38,900), 291 (10,200), in 0.01 N NaOH - MeOH λ_{max} nm (ε) 211 (42,100), 226 (40,000), 305 (sh, 8,500); ^1H NMR in D_2O - DCl δ 3.13 (1H, dd, J=12, 7.3 Hz), 3.24 (1H, dd, J=12, 5.5 Hz), 3.37 (1H, dd, J=14, 7.3 Hz), 3.44 (1H, dd, J=14, 5.1 Hz), 4.27 (1H, dd, J=7.5, 5.1 Hz), 4.33 (1H, dd, J=7.3, 5.5 Hz), 5.02 (1H, s), 7.00 (2H, d, J=8.8 Hz), 7.08 (1H, d, J=2.2 Hz), 7.15 (1H, d, J=2.0 Hz), 7.17 (1H, d, J=7.0 Hz), 7.20 (1H, d, J=2.2 Hz), 7.23 (2H, d, J=8.8 Hz), 7.24 (1H, s), 7.63 (1H, dd, J=7.0, 2.0 Hz); ^{13}C NMR in D_2O - DCl δ 26.7 (t), 35.8 (t), 54.3 (d), 55.2 (d), 57.3 (d), 107.8 (s), 119.0 (d × 2), 119.4 (d), 120.3 (d), 120.8 (d), 121.9 (s), 124.1 (d), 125.6 (s), 126.6 (d), 127.5 (d), 127.9 (s), 129.1 (s), 130.2 (s), 132.0 (d × 2), 135.1 (s), 145.4 (s), 147.3 (s), 157.3 (s), 172.1 (s), 172.4 (s), 172.8 (s).

Edman Degradation of **1**

Ta a 70% EtOH solution of **1** (63 mg) were added phenyl isothiocyanate (1 ml) and triethylamine (1 ml). The mixture was allowed to stand at 50°C for 2 hours and then concentrated to remove the solvent. The residue was dissolved in MeOH (2 ml) and added dropwise to 20 ml of benzene. The precipitate formed was collected by centrifugation, treated with TFA (2 ml) at 40°C for 2 hours and dried under stream of nitrogen. The solid was dissolved in MeOH (2 ml) and the solution was diluted with ether (20 ml). After removal of the precipitated **9**, the ethereal solution was concentrated. The residue was treated with 1 M HCl (3 ml) at 80°C for 10 minutes. The PTH-Tyr prepared was extracted with EtOAc and identified by a direct comparison with an authentic specimen. The above insoluble crude **9** was purified by preparative HPLC (column: YMC-Pack D-ODS-5, 20 i.d. × 250 mm; mobile phase: isoPrOH - CH_3CN - H_2O (7 : 3 : 30) containing 0.1% TFA; flow rate: 12 ml/minute; detection: UV 254 nm; Rt 10.0 minutes) followed by Sephadex LH-20 column chromatography (MeOH) to afford a pale yellow powder of **9**: mp 215°C (dec.); HRFAB-MS m/z 1,008.2622 $(M+H)^+$, Δ1.2 mmu for $C_{52}H_{43}N_7O_{13}Cl$.

Acknowledgment

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