K-13, A NOVEL INHIBITOR OF ANGIOTENSIN I CONVERTING ENZYME PRODUCED BY *MICROMONOSPORA HALOPHYTICA* SUBSP. *EXILISIA*

II. STRUCTURE DETERMINATION

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The structure of K-13, a potent inhibitor of angiotensin I converting enzyme (ACE), was determined to be a cyclic dipeptide composed of tyrosine and an unusual diamino dicarboxylic acid, isodityrosine, by spectral and chemical studies of K-13 and its derivatives.

K-13 is a new inhibitor of angiotensin I converting enzyme (ACE), isolated from the culture broth of *Micromonospora halophytica* subsp. *exilisia* K-13. The fermentation, isolation and biological properties of K-13 have been reported by KASE *et al.*[1] We wish to describe the structure determination of K-13 (**1**) in this paper.

K-13 (**1**) was obtained as a colorless powder, insoluble in $CHCl_3$, Et_2O, EtOAc and acetone, soluble in H_2O and freely soluble in MeOH and EtOH. It gave Rf values 0.40 ($CHCl_3$ - MeOH - EtOH - H_2O, 5:2:2:1) and 0.53 (BuOH - EtOH - $CHCl_3$ - conc NH_4OH, 4:5:2:5) on silica gel TLC.

1 showed positive color reactions with RYDON-SMITH, anisaldehyde and BCG reagents, but negative with ninhydrin and DRAGENDORFF reagents.

1 melted at 265~270°C with decomposition and was optically active: $[\alpha]_D^{19}$ −3.4° (*c* 0.6, MeOH). The high resolution fast atom bombardment mass spectrum (HRFAB-MS) indicated that **1** had the molecular formula of $C_{29}H_{29}N_3O_8$ [calcd for $C_{29}H_{30}N_3O_8$ $(M+H)^+$: 548.2033, found: 548.2028].

The IR spectrum (KBr) showed the presence of hydroxyl groups (3400 cm^{-1}) and amide groups (1650 cm^{-1}). The UV absorption spectra showed maxima at 201 (ε 43,000), 220 (sh, 22,000) and 273 nm (3,400) in water, and 201 (ε 45,000), 220 (sh, 23,000), 245 (sh, 9,900), 277 (3,700) and 295 nm (2,900) in 0.01 M NaOH - water, indicating the presence of phenol moiety in the molecule.

The ^{13}C NMR spectrum (100 MHz, CD_3OD) of **1** exhibited three benzene moieties, three amide functions, one carboxylic acid, one methyl group, three methylene groups and three methine groups (Table 2).

The partial structures, three tyrosine moieties (T-1, T-2 and T-3) and one acetyl group, were deduced from the detailed ^1H NMR decoupling experiments and the ^1H-^{13}C selective decoupling experiments, where all couplings and long range couplings within each moiety were observed. The assignments of all protons are presented in Table 1.

Acid hydrolysis of **1** gave L-tyrosine and an unusual amino acid (**3**). The ^1H and ^{13}C NMR data of **3** are presented in Tables 1 and 2. Absolute configuration of tyrosine was determined by HPLC method using 'CHIRALPAC'.

In the ^1H NMR spectrum of **1**, aromatic protons of T-2 were observed as a typical A_2X_2 system at 6.95 ppm and 6.59 ppm (5'-H_2, 6'-H_2), and that of T-1, however, showed a AMX system for 5a-H

Table 1. ^{1}H NMR data for **1**, **2** and **3**.[a,b]

	Proton	1	2	3
T-1	2-H	4.41 (dd, J=11.9, 5.4)	4.44 (dd, J=11.7, 5.5)	4.10 (X in ABX, dd, J=7.0, 6.2)
	3-H_a	2.80 (dd, J=12.3, 11.9)[c]	2.68 (dd, J=12.0, 11.7)	3.05, 3.01 (AB in ABX, J_{AB}=
	3-H_b	3.01 (dd, J=12.3, 5.4)[c]	*ca.* 2.9	14.8, J_{AX}=7.0, J_{BX}=6.2)
	5a-H	6.99 (dd, J=8.4, 2.2)[c]	6.91 (dd, J=8.3, 2.2)	7.10 (d, J=8.6)
	5b-H	7.29 (dd, J=8.3, 2.2)[c]	7.31 (dd, J=8.3, 2.2)	
	6a-H	6.69 (dd, J=8.4, 2.6)	6.62 (dd, J=8.3, 2.6)	6.79 (d, J=8.6)
	6b-H	7.06 (dd, J=8.3, 2.6)	6.93 (dd, J=8.3, 2.6)	
T-2	2′-H	4.11 (t, J=5.7)	4.16 (t, J=5.2)	
	3′-H_2	*ca.* 2.9 (AB in ABX)[c]	*ca.* 2.9	
			2.73 (dd, J=13.7, 5.2)	
	5′-H_2	6.95 (d, J=8.5)[c]	7.00 (d, J=8.7)	
	6′-H_2	6.59 (d, J=8.5)	6.70 (d, J=8.7)	
	7′-OCH_3		3.70 (s)	
T-3	2″-H	4.21 (dd, J=7.5, 3.4)	4.51 (dd, J=10.2, 2.3)	4.17 (X in ABX, dd, J=7.4, 5.7)
	3″-H_a	3.15 (dd, J=15.0, 3.4)[c]	3.05 (dd, J=16.1, 2.3)	3.14, 2.95 (AB in ABX, J_{AB}=
	3″-H_b	2.90 (dd, J=15.0, 7.5)	*ca.* 2.9	14.7, J_{AX}=7.4, J_{BX}=5.7)
	5a″-H	6.33 (X in ABX, d, J=1.7)[c]	6.44 (d, J=2.1)	6.77 (br s)
	5b″-H	6.72 (AB in ABX,	6.85 (dd, J=8.4, 2.1)	6.87 (br s)
	6b″-H	6.75 J_{AB}=8.3, J_{AX}=1.7)	7.02 (d, J=8.4)	6.87 (br s)
	7″-OCH_3		3.82 (s)	
	$COOCH_3$		3.69 (s)	
CH_3CO		2.03 (s)	1.93 (s)	

[a] 400 MHz; chemical shifts in ppm, coupling constants in Hz.

[b] **1** in CD_3OD, **2** in DMSO-d_6 - CD_3OD with TMS as an internal standard, **3** in acidic D_2O with 3-(trimethylsilyl)propane sulfonic acid, sodium salt (DSS) as an internal standard.

[c] Small, unresolved long range couplings were ascertained between 3-H_a and 5a-H, 3-H_b and 5b-H, 3′-H_2 and 5′-H_2, and 3″-H_a and 5a″-H, by the decoupling experiments.

(6.99 ppm, dd, J=8.4 and 2.2 Hz), 5b-H (7.29 ppm, dd, J=8.3 and 2.2 Hz), 6a-H (6.69 ppm, dd, J=8.4 and 2.6 Hz) and 6b-H (7.06 ppm, dd, J=8.3 and 2.6 Hz), which implies that the free rotation of aromatic ring in T-1 is restricted and then T-2 seems to be L-tyrosine. The unusual amino acid (**3**) consists of T-1 and T-3. The aromatic resonance pattern of T-3 of **1** showed typical ABX system with protons for 5a″-H (6.33 ppm, d, J=1.7 Hz), 5b″-H and 6b″-H (6.72 and 6.75 ppm, AB in ABX, J_{AB}=8.3 Hz, J_{AX}=1.7 Hz). These data suggest that a diphenyl ether linkage is present between T-1 and T-3 through the oxygen atom of T-1.

The position of the diphenyl ether linkage between T-1 and T-3 was defined by long range selective proton decoupling (LSPD) and nuclear Overhauser effect (NOE) experiments of trimethyl derivative of **1** (**2**) which was obtained by methylation of **1** with diazomethane. C-6a″ carbon (147.9 ppm) exhibited ^{1}H-^{13}C long range couplings with 5a″-H ($^2J_{CH}$) and 6b″-H ($^3J_{CH}$), and C-7″ carbon (149.4 ppm) coupled with 5a″-H, 5b″-H and methyl protons (3.82 ppm). The NOE between 7″-OCH_3 and 6b″-H and between 6a-H and 5a″-H were observed in the nuclear Overhauser effect spectroscopy (NOESY) spectrum of **2**, however, there is no NOE between 7″-OCH_3 and 5a″-H. These facts suggest that methoxyl group is attached to C-7″ and the ether bond is located between C-7 and C-6a″. This unusual amino acid is identical with isodityrosine known as the component of plant cell-wall glycoprotein.[2,3]

Table 2. ^{13}C NMR data for **1**, **2** and **3**.[a,b]

Carbon		1	2	3
T-1	C-1	172.3	170.3[d]	172.0[f]
	C-2	57.6	55.4	54.9[g]
	C-3	37.4	37.8	35.61[h]
	C-4	133.0	132.2	129.3
	C-5a	132.1	131.1[e]	131.9
	C-5b	131.2	130.6[e]	131.9
	C-6a	121.3	119.3	118.3
	C-6b	122.5	120.4	118.3
	C-7	158.2[c]	156.3	157.8
T-2	C-1′	170.9	170.0[d]	
	C-2′	56.5	53.5	
	C-3′	39.1	37.8	
	C-4′	128.4	128.7	
	C-5′	132.0	131.1	
	C-6′	116.0	113.4	
	C-7′	157.1[c]	158.3	
	7′-OCH_3		55.0	
T-3	C-1″	177.4	172.1	171.9[f]
	C-2″	56.7	51.7	54.8[g]
	C-3″	38.9	35.0	35.57[h]
	C-4″	131.5	130.7	127.6
	C-5a″	119.4	117.5	123.2
	C-5b″	125.6	124.3	127.5
	C-6a″	148.0	147.9	148.0
	C-6b″	117.3	113.3	118.7
	C-7″	147.1	149.4	144.1
	1″-OCH_3		52.3	
	7″-OCH_3		56.0	
CH_3CO		172.9	167.9[d]	
		22.4	22.4	

[a] 100 MHz; chemical shifts in ppm.

[b] **1** in CD_3OD, **2** in DMSO-d_6 - CD_3OD with TMS as an internal standard, **3** in acidic D_2O with DSS as an internal standard.

[c~g] These assignments may be interchangeable.

Fig. 1. The partial structure of **2** (NOE and LSPD experiments).

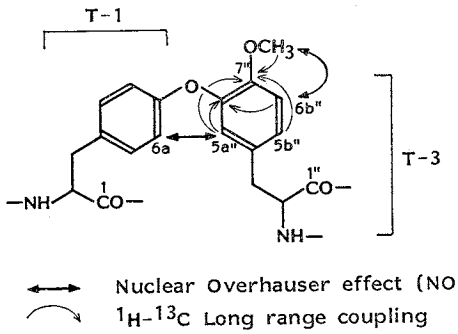
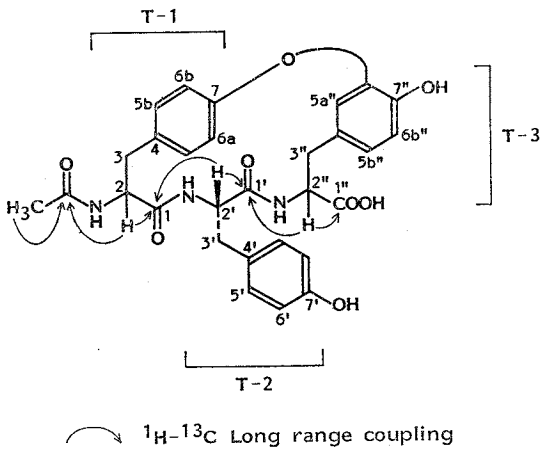


Fig. 2. The structure of K-13 (**1**).



The arrangement of the amide bonds was established by further LSPD experiments, where three-bond long range couplings were observed between 2-H and acetyl carbonyl, 2′-H and C-1, and 2″-H and C-1′ (Fig. 2).

Thus, the whole structure of K-13 was confirmed as Fig. 2. The study about absolute configuration at the remaining two centers, C-2 and C-2″, is now in progress.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on Bruker AM400 spectrometer with TMS (0 ppm), DSS (0 ppm) and dioxane (67.4 ppm) as the internal standard. IR spectra were obtained using a Shimadzu IR-27G spectrometer. UV spectra were taken with a Hitachi 200-20 spectrometer. Secondary ion mass spectra (SI-MS) and high resolution mass spectra (HR-MS) were measured on Hitachi M-80B mass spectrometer. Melting points were taken with a Yanagimoto melting point apparatus and were not corrected. Thin-layer chromatography (TLC) was performed on pre-coated plates, Merck Kieselgel 60 F_{254} and detected with iodine and ninhydrin.

Hydrolysis of **1**

K-13 (**1**, 13 mg) was suspended in 6 M hydrochloric acid (2 ml) and heated for 20 hours at 110°C in a sealed tube. The solution was evaporated and the crude products were purified on preparative TLC (Kieselgel 60 F_{254}, EtOH - H_2O - conc NH_4OH, 16 : 4 : 1) to give L-tyrosine (3 mg) and **3** (5 mg) as a colorless powder. L-Tyrosine was identified by HPLC method as follows.

HPLC Analysis of Optically Active Tyrosine

L-Tyrosine, obtained by hydrolysis of **1**, was subjected to HPLC using the following conditions; retention time (t_R) 23′50″, HPLC; Shimadzu LC-3A, column; 'CHIRALPAK'WH 4.6 i.d. ×150 mm (DAICEL Chemical Ind.), mobile phase; 0.5 mM $CuSO_4$, flow rate; 2 ml/minute, temperature; 50°C, detection; UV (254 nm). Retention times of authentic D- and L-tyrosine samples were; t_R 8′20″ and 23′50″, respectively.

Methylation of **1**

To a solution of K-13 (**1**, 10 mg) in MeOH (2 ml), etherial diazomethane (2 ml), generated from bis(*N*-methyl-*N*-nitroso)terephthalamide (9 g) in ether (40 ml), was added, and stood for 15 hours at room temp. The solution was evaporated to give **2** (10 mg) which was recrystallized from aq MeOH to afford colorless needles. **2**: MP >300°C; $[\alpha]_D^{23}$ −20° (*c* 0.1, DMF); IR (KBr) cm^{-1} 3400, 3340, 3294, 2856, 2838, 1737, 1664, 1632, 1525, 1513, 1270, 1250, 1231, 1214, 1026; SI-MS *m/z* 590 $(M+H)^+$. The ^{1}H and ^{13}C NMR data are presented in Tables 1 and 2, respectively.

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References

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