PEPTICINNAMINS, NEW FARNESYL-PROTEIN TRANSFERASE INHIBITORS PRODUCED BY AN ACTINOMYCETE

II. STRUCTURAL ELUCIDATION OF PEPTICINNAMIN E

KAZURO SHIOMI, HONG YANG,[†] JUNJI INOKOSHI, DIDIER VAN DER PYL,[††] AKIRA NAKAGAWA,[†††] HIDEO TAKESHIMA and SATOSHI ŌMURA*

Research Center for Biological Function, The Kitasato Institute,
5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan
[†††]School of Science and Engineering, Teikyo University,
1-1 Toyosatodai, Utsunomiya, Tochigi 320, Japan

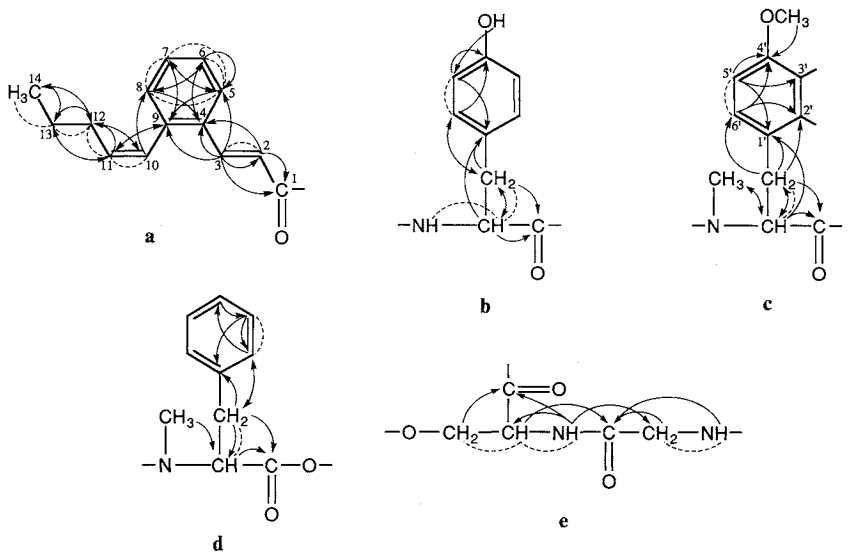
(Received for publication September 28, 1992)

Structure of novel farnesyl transferase inhibitor, pepticinnamin E, is elucidated by NMR study. Pepticinnamin E is composed of five amino acids and *o*-pentenylcinnamic acid, having a molecular weight of 907. *C*-terminal glycylserine of the compounds is in the cyclized diketopiperazine form.

Pepticinnamins are novel farnesyl transferase inhibitors isolated from the cultured broth of *Streptomyces* sp. OH-4652.[1] Here, we report on the structural elucidation of pepticinnamin E (**1**, Fig. 2), a major component of pepticinnamins.

Fig. 1. Partial structures **a**, **b**, **c**, **d**, and **e** for **1** elucidated by ^{1}H-^{1}H COSY and HMBC.

---- ^{1}H-^{1}H COSY
←— HMBC



Present address:
[†] North China Pharmaceutical Corporation, 4 Heping Road, Shijiazhuang, Hekei PR, China.
[††] Rhône-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville, 13 quai Jules Guesde, 94403 Vitry Sur Seine, France.

Table 1. ^{1}H and ^{13}C NMR assignments for **1**.

No.	^{13}C chemical shift		^{1}H chemical shift	
3-[2-(1-(*Z*)-Pentenyl)phenyl]-2(*E*)-propenoic acid moiety (**a**)				
1	163.9	s		
2	122.7	d	6.51	d (15.6)
3	136.7	d	7.564	d (15.6)
4	132.9	s		
5	125.6	d	7.565	d (7.0)
6	127.3	d	7.31	dd (7.0, 7.0)
7	129.04	d	7.35	dd (7.0, 7.0)
8	129.8	d	7.18	d (7.0)
9	137.2	s		
10	127.0	d	6.57	d (11.7)
11	134.2	d	5.82	dt (11.7, 7.3)
12	30.0	t	1.95	dt (7.3, 7.0)
13	22.1	t	1.33	tq (7.0, 7.3)
14	13.7	q	0.77	t (7.3)
Tyrosine moiety (**b**)				
NH			8.27	d (6.5)
C=O	170.6	s		
α	49.6	d	4.82	ddd (6.5, 6.6, 8.0)
β	36.8	t	2.53	dd (8.0, 13.4),
			2.67	dd (6.6, 13.4)
γ	127.4	s		
δ	130.2×2	d	6.99×2	d (8.3)
ε	115.1×2	d	6.67×2	d (8.3)
ζ	156.0	s		
ζ-OH			9.26	br s
N-Methyl-3-(2-chloro-3-hydroxy-4-methoxyphenyl)-alanine moiety (**c**)				
NMe	29.3	q	2.38	s
C=O	169.6	s		
α	52.4	d	5.39	dd (4.9, 9.3)
β	31.6	t	2.58	dd (9.3, 14.2),
			2.97	dd (4.9, 14.2)
1′	127.2	s		
2′	120.5	s		
3′	142.4	s		
3′-OH			9.07	br s
4′	146.9	s		
4′-OMe	55.4	q	3.46	s
5′	109.1	d	6.42	d (8.8)
6′	120.6	d	6.38	d (8.8)
N-Methylphenylalanine moiety (**d**)				
NMe	32.0	q	2.29	s
C=O	169.8	s		
α	58.5	d	5.24	dd (4.4, 12.0)
β	33.6	t	2.87	dd (12.0, 14.6),
			3.19	dd (4.4, 14.6)
γ	137.0	s		
δ	128.98×2	d	7.07×2	m
ε	128.3×2	d	7.24×2	m
ζ	126.6	d	7.23	m
Serine moiety (**e**)				
NH			8.28	br s
C=O	165.2	s		
α	53.7	d	4.15	dd (2.9, 3.9)
β	65.7	t	4.24	dd (2.9, 11.2),
			4.42	dd (3.9, 11.2)
Glycine moiety (**e**)				
NH			8.20	br s
C=O	165.8	s		
α	44.3	t	3.73	d (17.3),
			3.82	d (17.3)

Solvent: DMSO-d_6. The coupling constants (Hz) are in parentheses.

The molecular formula of **1** was established to be $C_{49}H_{54}N_5O_{10}Cl$ by HRFAB-MS.[1] IR spectrum of **1** showed amide carbonyl (1670 cm^{-1}) absorbances,[1] suggesting that the compound may be a peptide. Amino acid analysis of **1** gave tyrosine, serine, and glycine at the molecular ratio of 1.01 : 0.76 : 1, indicating that these amino acids were contained at an equal ratio in **1**.

Chemical shifts in the ^{1}H NMR and ^{13}C NMR of **1** are shown in Table 1. The DEPT spectra revealed 4 methyl, 7 methylene, 19 methine, and 15 quarternary carbons. Although the molecular formula showed 49 carbons, only 45 signals were observed in ^{13}C spectra. Because ^{13}C signals for four methines (δ 130.2, 128.98, 128.3, and 115.1) in ^{13}C NMR exhibited higher intensity comparing with other methine signals, it is reasonable to assume that each of those methine signals is derived from two carbons.

The presence of the partial structures **a**, **b**, **c**, **d**, and **e** for **1** was deduced from ^{1}H-^{1}H COSY ^{13}C-^{1}H COSY, and HMBC as shown in Fig. 1. The partial structure **a** corresponded to the cinnamic acid derivative containing in a cyclic peptide, WS-9326A, which has been isolated as a tachykinin antagonist.[2,3] Comparing coupling constants of two olefinic moieties ($J_{2,3}$ = 15.6 Hz, $J_{10,11}$ = 11.7 Hz) of **a** with those of WS-9326A, the configuration of the olefins was elucidated as 2*E*,10*Z*.

The partial structure **b** was elucidated as tyrosine moiety, which was detected by amino acid analysis. Two of four intensive methine signals in ^{13}C NMR described above were assigned to δ (δ 130.2) and ε (δ 115.1) carbons of tyrosine.

HMBC of the partial structure **c** showed couplings of NCH_3 (δ 2.38)/C-α (δ 52.4), α-H (δ 5.39)/NCH_3 (δ 29.3), α-H/C-β (δ 31.6), β-H_2 (δ 2.58, 2.97)/C-α, α-H/C=O (δ 169.6), and β-H_2/C=O. Couplings were observed between α-H and β-H_2 in ^1H-^1H COSY. Therefore, the arrangement of NCH_3, C=O, C-α, and C-β was revealed. Cross peaks from α-H to C-1′ (δ 127.2) and from β-H_2 to C-1′, C-2′ (δ 120.5), and C-6′ (δ 120.6) were observed in HMBC. *Ortho* coupling (J=8.8 Hz) was observed between 6′-H (δ 6.38) and 5′-H (δ 6.42) in ^1H NMR. These data suggested the arrangement of C-1′, C-2′, C-5′, and C-6′ in phenyl ring. ^1H-^{13}C long-range couplings of 5′-H/C-3′ (δ 142.4), 5′-H/C-4′ (δ 146.9), 6′-H/C-4′, and 4′-OCH_3 (δ 3.46)/C-4′ in HMBC revealed *p*-methoxyphenyl moiety. One position at C-2′ and one position at C-3′ were still unoccupied.

The partial structure **d** was elucidated to be *N*-methylphenylalanine moiety, which was detected in chiral HPLC as described below. Remaining two intensive methine signals in ^{13}C NMR were assigned to δ (δ 128.98) and ε (δ 128.3) carbons.

The partial structure **e** was deduced to be glycylseryl moiety, which was detected by amino acid analysis as glycine and serine.

The interconnection of partial structures **a**, **b**, **c**, **d**, and **e** was elucidated from HMBC spectral data, as shown in Fig. 2. Appearance of the cross peaks in HMBC from NH (δ 8.27) and α (δ 4.82) protons of **b** to C-1 (δ 163.9) of **a** revealed the amide bond between **a** and **b**. The cross peaks from NCH_3 (δ 2.38) and α protons (δ 5.39) of **c** to carbonyl carbon (δ 170.6) of **b** indicated the amide bond between **b** and **c**. Similarly, the cross peaks from NCH_3 (δ 2.29) and α protons (δ 5.24) to **d** to carbonyl carbon (δ 169.6) of **c** indicated the amide bond between **c** and **d**. The cross peaks from α proton (δ 3.73, 3.82) of glycine to carbonyl carbon (δ 165.2) of serine and from NH (δ 8.20) of glycine to α carbon (δ 53.7) of serine suggested that glycylserine was cyclized to form diketopiperazine in the partial structure **e**. The cross peak from β proton (δ 4.24, 4.42) of serine to carbonyl carbon (δ 169.8) of **d** suggested the ester bond between **d** and **e**.

According to the molecular formula, there are three remaining atoms; one hydrogen (δ 9.07), one oxygen, and one chlorine. Those bonding positions were defined by mass fragment patterns of **1** and its diacetate derivative (Fig. 3), and ^{13}C chemical shifts of **1**. FAB-MS of **1** exhibited fragment ion peaks at m/z 199 $(\mathbf{a})^+$, 362 $(\mathbf{a}+\mathbf{b})^+$, 603 $(\mathbf{a}+\mathbf{b}+\mathbf{c})^+$, and 764 $(\mathbf{a}+\mathbf{b}+\mathbf{c}+\mathbf{d})^+$, indicating that the arrangement of

Fig. 2. Structure of pepticinnamin E (**1**) and its elucidation by HMBC experiment.

⟵ HMBC

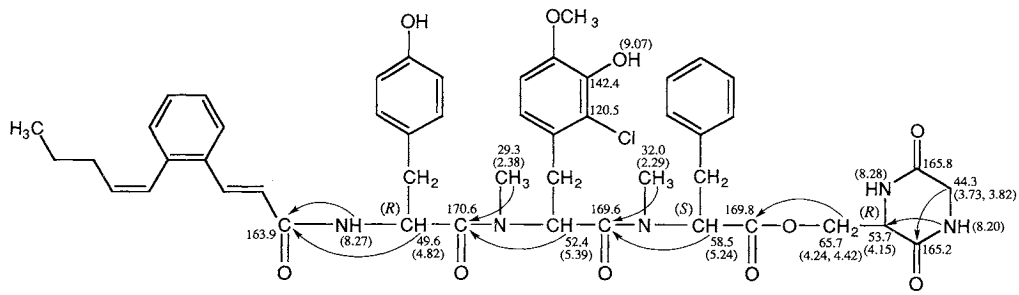
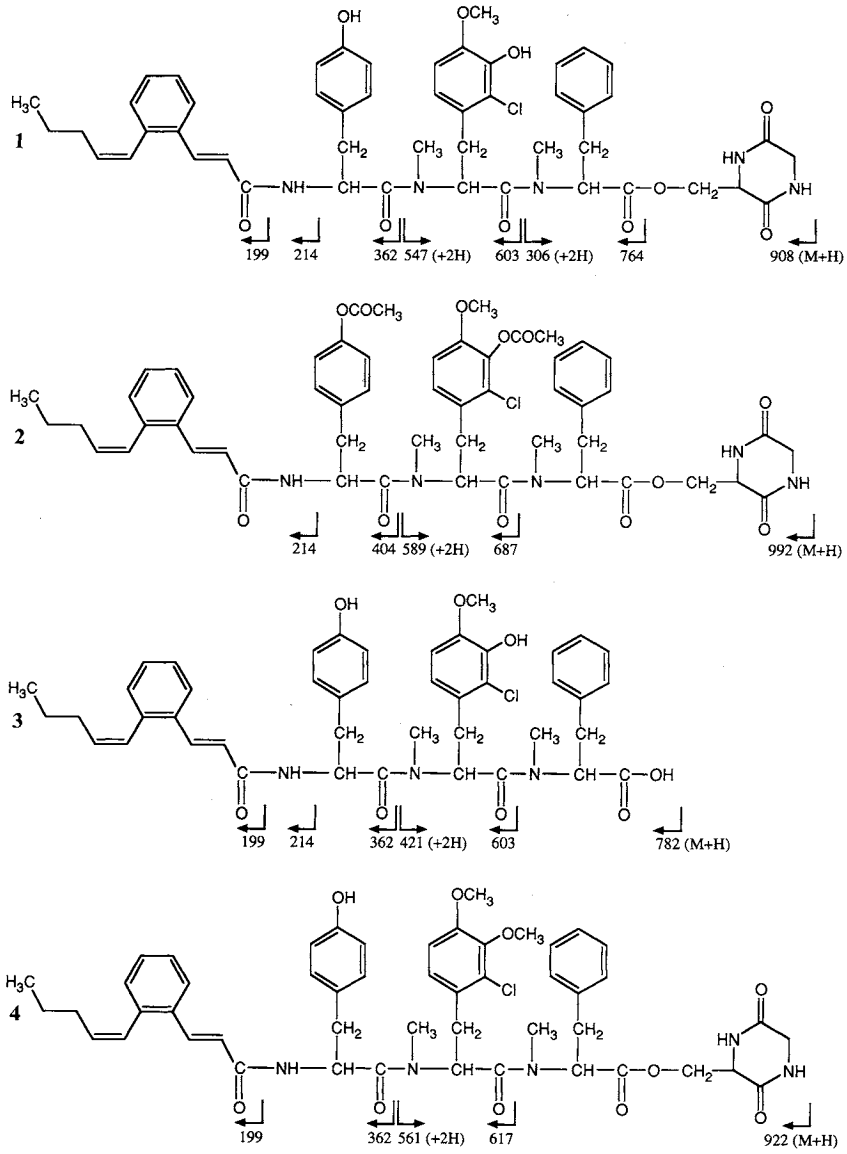


Fig. 3. FAB-MS fragmentation of **1**, **2**, **3**, and **4**.

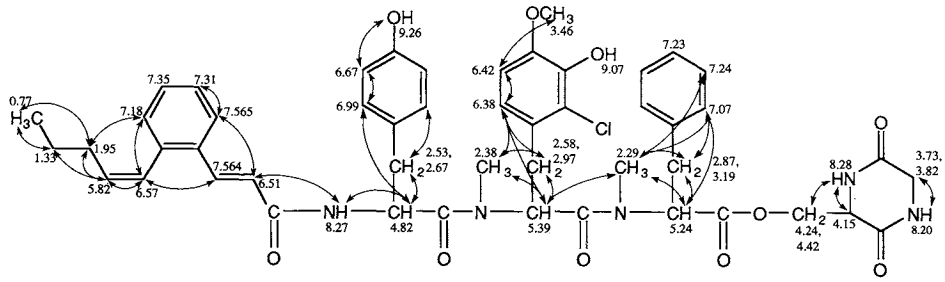


partial structures **a**, **b**, **c**, **d**, and **e** was reasonable and partial structure **c** contained all remaining atoms. Treatment of **1** with acetic anhydride in pyridine gave the diacetate **2** (MW 991), suggesting that two hydroxyl groups existed in **1**. One hydroxyl group is native one in tyrosine residue. According to the mass fragmentation of **1**, another hydroxyl group should connect to partial structure **c**. The mass fragmentation of **2** showed that partial structure **c** was monoacetylated indicating the existence of one hydroxyl group. ^{13}C chemical shifts of partial structure **c** revealed that the hydroxyl group (δ 9.07) and chlorine atom combined to phenyl-3 (δ 142.4) and phenyl-2 (δ 120.5), respectively. Thus partial structure **c** was elucidated as *N*-methyl-3-(2-chloro-3-hydroxy-4-methoxyphenyl)alanine, which was a new derivative of dopa.

Further structural confirmation for **1** was obtained from the ROESY experiment (Fig. 4). The cross

Fig. 4. ROESY experiments of **1**.

⟷ ROESY



peaks were observed between each α proton and NH or *N*-methyl proton of amino acids. Protons at β-methylene also had couplings with δ-methines in aromatic amino acids. The cross peaks between 2-H (δ 6.51) and 5-H (δ 7.565) and between 8-H (δ 7.18) and 12-H_2 (δ 1.95) of **a** confirmed the stereoisomerism of cinnamic acid derivative.

A short peptide **3** obtained by mild alkaline hydrolysis of **1** had MW 781, which was coincided with MW of **1**–lacking diketopiperazine generated by cleaving at the ester bond. The chemical structure of **3** was verified by the mass fragment pattern (Fig. 3). Methylation of **1** gave monomethylated compound **4** and its mass fragmentation revealed that phenyl-3-OH of dopa derivative was methylated but not tyrosine hydroxyl.

Table 2. HPLC data for amino acid in **1** on a Chiralpak WH column.

Amino acids	Retention time (minutes)	
	Authentic amino acids	Amino acids in **1**
D-Serine	9.7	9.2
L-Serine	14.1	—
D-*N*-Methylphenylalanine	12.1	—
L-*N*-Methylphenylalanine	54.6	53.4
D-Tyrosine	12.6	12.9
L-Tyrosine	41.9	—

—: Not detected.

Stereochemistry of the amino acids of **1** was elucidated by chiral HPLC. Acid hydrolysate of **1** was purified to each amino acid, and their retention times in HPLC with Chiralpak WH column were compared with those of authentic amino acids (Table 2). The absolute configurations of tyrosine and serine were D and that of *N*-methylphenylalanine was L, while the dopa derivative was not tested because it decomposed during purification. Thus the structure of **1** was elucidated as shown in Fig. 2.

Experimental

NMR spectra were recorded on a JEOL JNM-EX400 spectrometer in DMSO-d_6. The mixing time in ROESY was 250 milliseconds. FAB-MS were recorded on a JEOL JMS DX-300 spectrometer.

Amino acid analysis was done as follows: the sample was hydrolyzed with 6N HCl at 110°C for 22 hours and the resulting hydrolysate was examined in an amino acid autoanalyzer (Hitachi 835).

Acetylation of **1**

A mixture of **1** (3.0 mg) and acetic anhydride (0.1 ml) in pyridine (0.9 ml) was kept at room temperature for 19 hours. The reaction mixture was concd to dryness and developed on a preparative TLC ($CHCl_3$-MeOH, 20:1). The major UV positive zone was collected and extracted with MeOH. The extract was concd and purified by Sephadex LH-20 (8 ml) eluted with MeOH to give **2** (1.5 mg) as a colorless powder. FAB-MS *m/z* 214, 404, 589, 687, 992 $(M+H)^+$, 1,014 $(M+Na)^+$.

Mild Alkaline Hydrolysis of **1**

A mixture of **1** (2.4 mg) in MeOH (0.5 ml) and 1 N NaOH (0.17 ml) was stirred magnetically at room temperature for 20 hours. The reaction mixture was neutralized with 1 N HCl, concd to dryness, dissolved in H_2O, and extracted with EtOAc. The extract was concd to dryness to give **3** (2.2 mg) as a colorless powder. FAB-MS *m/z* 199, 214, 362, 421, 603, 782 $(M+H)^+$, 804 $(M+Na)^+$.

Metylation of **1**

A mixture of **1** (2.2 mg in 0.2 ml of MeOH) and trimethylsilyldiazomethane (0.5 ml, 10% hexane soln, Tokyo Kasei Kogyo Co., Ltd.) in benzene (0.8 ml) was kept at room temperature for 1 hour. The reaction mixture was concd to dryness and developed on a preparative TLC ($CHCl_3$-MeOH, 10:1). The major UV positive zone was collected and extracted with MeOH. The extract was concd and purified by Sephadex LH-20 (6 ml) eluted with MeOH to give **4** (1.3 mg) as a colorless powder. FAB-MS *m/z* 199, 362, 561, 617, 922 $(M+H)^+$, 944 $(M+Na)^+$.

Examination of the Stereochemistry of Amino Acid of **1**

Compound **1** (26.0 mg) was hydrolyzed with 1.5 ml of 6 N HCl at 110°C for 22 hours. The reaction mixture was concd to dryness. The hydrolysate was charged on Muromac AG 50W × 8 column (40 ml, Muromachi Kagaku Kogyo Kaisha, Ltd.) equilibrated with 0.1 M pyridine-formic acid buffer (pH 3.1). The column was developed with the same buffer followed by a 0.2 M pyridine-formic acid buffer (pH 3.1) and 0.2 M pyridine-acetic acid buffer (pH 4.4), in that order. Appropriate fractions were combined and concd to dryness. The amino acids were desalted on a Muromac AG 50W × 8 column (3 ml, H^+ type) with 1 N NH_4OH. Serine [2.8 mg, FD-MS *m/z* 105 $(M+H)^+$] was eluted with 0.1 M pyridine-formic acid buffer (pH 3.1). *N*-Methylphenylalanine [4.6 mg, FAB-MS *m/z* 180 $(M+H)^+$] was eluted with 0.2 M pyridine-formic acid buffer (pH 3.1). Crude tyrosine (18.0 mg) was eluted with 0.2 M pyridine-acetic acid buffer (pH 4.4). Crude tyrosine was further purified by Chiralpak WH (i.d. 4.6 × 250 mm, Daicel Chemical Industries, Ltd.) under the condition described below. The eluate was desalted on active carbon (0.5 ml) with 50% acetone (adjusted to pH 3 with TFA) to yield 3.7 mg of tyrosine [FAB-MS *m/z* 182 $(M+H)^+$].

The stereochemistry of the amino acids was examined by HPLC with a chiral column. Analytical condition–column, Chiralpak WH (i.d. 4.6 × 250 mm); mobile phase, 1 mM $CuSO_4$; flow rate, 1.6 ml/minute; detection, UV absorption at 225 nm.

Acknowledgment

We thank Dr. MARC VUILHORGNE of Rône-Poulenc Rorer for his help about structural elucidation. We also thank Dr. TOSHIO OTANI of Taiho Pharmaceutical Co. Ltd. for amino acid analysis and Mr. HIROAKI UTSUMI of JEOL Ltd. for measurements of NMR spectra.

References

1) ŌMURA, S.; D. VAN DER PYL, J. INOKOSHI, Y. TAKAHASHI & H. TAKESHIMA: Pepticinnamins, new farnesyl-protein transferase inhibitors produced by an actinomycete. I. Producing strain, fermentation, isolation and biological activity. J. Antibiotics 46: 222~228, 1993

2) HAYASHI, K.; M. HASHIMOTO, N. SHIGEMATSU, M. NISHIKAWA, M. EZAKI, M. YAMASHITA, S. KIYOTO, M. OKUHARA, M. KOHSAKA & H. IMANAKA: WS9326A, a novel tachykinin antagonist isolated from *Streptomyces violaceusniger* No. 9326. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. J. Antibiotics 45: 1055~1063, 1992

3) SHIGEMATSU, N.; M. HASHIMOTO, N. KAYAKIRI, S. TAKASE & H. TANAKA: Structure of WS9326A, a new substance P receptor antagonist isolated from a *Streptomyces*. J. Org. Chem., to submitted