GE3, a Novel Hexadepsipeptide Antitumor Antibiotic Produced by *Streptomyces* sp.

II. Structure Determination

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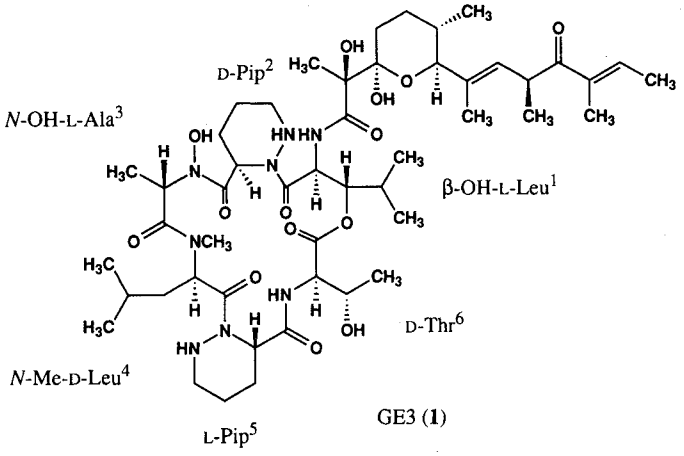
(Received for publication April 14, 1997)

As reported in the preceding paper[1], in the study of antitumor antibiotics, an antibiotic which inhibited progression of the cell cycle from G1 to S phase has been discovered in actinomycetes culture filtrates and named GE3. We also isolated GE3B from the same culture filtrate, which completely lost the biological activities (antimicrobial activity and cytotoxicity) found in GE3. In this paper, the structure elucidation of these antibiotics are described.

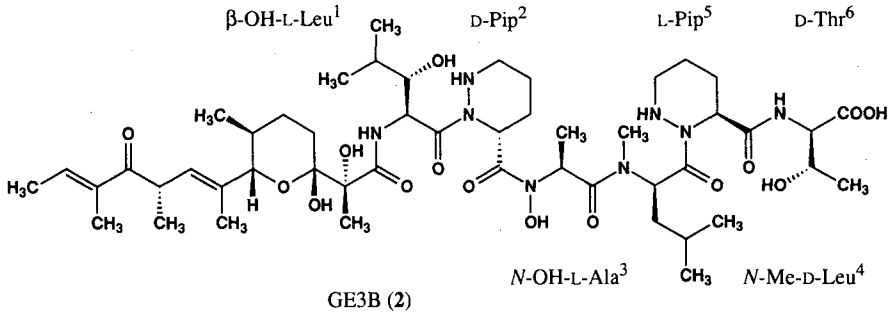
GE3 (**1**) has a molecular formula of $C_{49}H_{80}N_8O_{14}$ which was determined by high resolution (HR) FAB-MS (*m/z* 1003.5720 $[M-H]^-$, Δ+0.4 mmu). The ^{13}C NMR spectrum of **1** (Table 1) contains 49 carbon signals, including seven amide/ester carbonyl carbons (δ 176.6, 173.9, 173.7, 172.3, 170.8, 170.3, 169.6) and six α-methine carbons (δ 56.4, 55.0, 52.4, 51.8, 50.7, 49.7) characteristic of α-amino acids, indicating that **1** contains six α-amino acids and one carboxylic acid residue. The peptidic nature of **1** was further supported by the IR data (3419, 1645 cm^{-1}). An amino acid analysis of usual acid hydrolysate (110°C, 21 hours) showed the presence of threonine (Thr), alanine (Ala) (ratio 1 : 0.2) and some unidentified amino acids.

The structure elucidation of **1** was mainly based on 2D-NMR analyses. The NMR analyses including double quantum filtered (DQF) COSY, homonuclear Hartmann-Hahn (HOHAHA), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC) spectra, led to an easy assignment of almost all the proton and carbon signals except the amide proton signal of Ala. GE3 (**1**) was positive in the $FeCl_3$ color test characteristic of a hydroxamate, indicating the presence of an *N*-hydroxy amino acid. Instead of an Ala amide proton signal, the low field shifted signal (δ 9.88 br s) assignable to the *N*-hydroxy proton was observed. Therefore, the Ala detected in low yield in the amino acid analysis, was derived from *N*-hydroxyalanine (*N*-OH Ala) during

Fig. 1. Structures of GE3 (**1**) and GE3B (**2**).



GE3 (**1**)



GE3B (**2**)

Table 1. ^{13}C and ^{1}H NMR data for GE3 (**1**) in $CDCl_3$.

	δ_C (ppm)	δ_H (ppm)	J_{H-H} (Hz)
Acyl side chain			
1	176.6 s		
2	77.0 s		
3	99.6 s		
4	28.0 t	1.75 m (2H)	
5	27.3 t	1.65 m (2H)	
6	32.6 d	1.46 m	
7	82.2 d	3.96 d	10.3
8	133.1 s		
9	129.5 d	5.58 dd	1.2, 9.1
10	38.5 d	4.06 dq	9.1, 6.9
11	203.1 s		
12	137.7 s		
13	136.7 d	6.72 dq	1.3, 7.0
14	14.9 q	1.85 d	7.0
15	20.2 q	1.37 s	
16	17.7 q	0.72 d	6.6
17	12.3 q	1.58 d	1.2
18	19.2 q	1.12 d	6.9
19	11.5 q	1.78 d	1.3
2-OH		2.98 s	
3-OH		6.37 s	
β-OH Leu[1]			
NH		8.24 d	10.7
α	55.0 d	4.84 t	10.7
β	78.6 d	5.41 dd	2.3, 10.7
γ	29.6 d	1.75 m	
δ	15.0 q	0.84 d	6.8
	19.0 q	0.75 d	6.9
C=O	170.8 s		
Pip[2]			
NH		4.40 dd	2.0, 12.8
α	51.8 d	4.93 dd	2.7, 10.7
β	24.2 t	1.94 m	
		2.28 m	
γ	21.3 t	1.47 m	
		1.64 m	
δ	46.1 t	2.89 m	
		3.16 d	13.5
C=O	173.9 s		
N-OH Ala[3]			
N–OH		9.88 br s	
α	50.7 d	5.15 q	7.1
β	13.5 q	1.49 d	7.1
C=O	173.7 s		
N-Me Leu[4]			
N–CH_3	29.7 q	3.01 s	
α	49.7 d	6.23 t	7.4
β	36.6 t	1.47 m	
		1.75 m	
γ	25.0 d	1.44 m	
δ	22.8 q	0.958 d	6.4
	22.9 q	0.963 d	6.4
C=O	172.3 s		
Pip[5]			
NH		3.96 d	10.3
α	52.4 d	5.19 dd	1.8, 5.9
β	24.5 t	1.71 m	
		2.57 d	11.7
γ	21.5 t	1.57 m (2H)	
δ	47.9 t	2.63 m	
		3.32 d	12.8
C=O	169.6 s		
Thr[6]			
NH		6.19 d	8.5
α	56.4 d	4.53 d	8.5
β	64.9 d	4.80 q	6.5
γ	19.0 q	1.07 d	6.5
OH		4.54 s	
C=O	170.3 s		

acid hydrolysis. Further analysis of DQF-COSY and HOHAHA spectra showed the existence of two isopropyl functional groups assigned to β-hydroxyleucine (β-OH Leu) and *N*-methylleucine (*N*-Me Leu). Two pairs of methyl proton signals (β-OH Leu: δ 0.84, 0.75; *N*-Me Leu: δ 0.963, 0.958) correlated to the methine and methylene carbon signals (β-OH Leu: δ 29.6 d, 78.6 d; *N*-Me Leu: δ 25.0 d, 36.6 t) in the HMBC spectrum, respectively. The long range coupling between *N*-methyl proton signal (δ 3.01) and an α-methine carbon signal (δ 49.7) confirmed the presence of an *N*-Me Leu residue. Two remaining amino acid residues were identified as piperazic acids (Pips) by HOHAHA correlations of two exchangeable proton signals (δ 4.40, 3.96). These findings suggested that **1** had a structure closely related to azinothricin-type cyclohexadepsipeptides[2~12].

The sequence from C-4 to C-7 in the acyl side chain was elucidated by the combined analyses of DQF-COSY and HOHAHA spectra. HMBC correlations from methyl proton signal (2-Me, δ 1.37) to oxygen-bearing quaternary carbon (C-2, δ 77.0), hemiketal carbon (C-3, δ 99.6), and carbonyl carbon (C-1, δ 176.6) signals established three quaternary carbons' sequence. The linkage around the olefinic carbons and carbonyl carbon were elucidated by HMBC correlations of methyl proton signals (8-Me/C-7, 8, 9; 10-Me/C-9, 10, 11; 12-Me/C-11, 12, 13; 13-Me/C-12, 13). The geometric configurations at C-8 and C-12 were determined to be both *E* by the high field chemical shifts of 8- and 12-methyl carbons (δ 12.3, 11.5) and the NOESY spectrum showing cross peaks between the following pairs of protons: 8-Me/10-H and 12-Me/13-Me.

The NOE observed between 3-OH (δ 6.37) and 7-H (δ 3.96) indicated the ether linkage between C-3 and C-7 belonged to the pyranose ring. The ^{13}C NMR spectral data of this acyl moiety were similar to those of the corresponding moiety in azinothricin[2]/citropeptin[7]-type cyclohexadepsipeptides.

HMBC correlations from α-, amide, and *N*-methyl protons to carbonyl carbons determined partial sequences of acyl side chain-β-OH Leu^1, *N*-OH Ala^3-*N*-Me Leu^4, and Pip^5-Thr^6. The low field chemical shift of the β-proton (δ 5.41) in β-OH Leu suggested it to be esterified and HMBC correlation between this proton and the carbonyl carbon signal of Thr confirmed the ester linkage of these two amino acids. NOESY correlations supported partial sequences obtained from HMBC spectrum. Furthermore, partial sequences of β-OH Leu^1-Pip^2-*N*-OH Ala^3 and *N*-Me Leu^4-Pip^5 were determined by NOESY peaks (β-OH Leu^1 β-H/Pip^2 NH; Pip^2 α-H/*N*-OH Ala^3 *N*-OH; *N*-Me Leu^4 α-H/Pip^5 NH). Thus the total sequence was determined and key HMBC and NOESY correlations are summarized in Fig. 2. The total planar structure of **1** was closely related to that of citropeptin[7], in which the *N*-hydroxy-*O*-methylserine (*N*-OH-*O*-Me Ser) moiety in citropeptin was replaced by an *N*-OH Ala.

Based on the similar specific rotation ($[\alpha]_D^{25}$ +111.5°, *c* 0.08, $CHCl_3$) and ^{13}C chemical shifts (Table 1) of **1** to those of azinothricin ($[\alpha]_D^{25}$ +117.65°, *c* 0.6, $CHCl_3$)[2] and A83586C ($[\alpha]_D^{25}$ +116.1°, *c* 0.2, $CHCl_3$)[4], the stereochemistry including the absolute configuration of **1**, is probably identical with those of azinothricin and A83586C. The coupling constants between 6-H (δ 1.46) and 7-H (δ 3.96) were 10.3 Hz, indicating equatorial configuration of methyl on C-6 and alkenyl group on C-7. NOESY correlations (2-Me/3-OH; 3-OH/7-H; 7-H/6-Me) suggested that the relative configuration of the tetrahydropyranyl portion are as shown in Fig. 2, which was identical with those of corresponding portions of azinothricin and A83586C. Based on the comparison of ^{13}C chemical shift values at C-10 (δ 38.5) and 10-Me (δ 19.2) of **1** with those of A83586C (C-10: δ 38.33; 10-Me: δ 19.55)[4], the stereochemistry of the acyl group in **1** was concluded to be as shown in Fig. 2. Absolute configurations of amino acids in **1** were deduced to be the same as those of azinothricin and A83586C from the comparison of specific rotation and ^{13}C chemical shift values as mentioned above. In order to determine the stereochemistry of amino acids, **1** was hydrolyzed by 6 N HCl and subjected to the reaction with isopropyl alcohol-acetyl chloride followed by trifluoroacetic anhydride. Chiral GC-MS analysis (Chirasil-Val) of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolysate revealed that Ala (*N*-OH Ala) and Thr had the L- and D-configurations, respectively. Stereochemical assignment of *N*-Me Leu was unsuccessful by GC-MS analysis. The absolute configuration of β-OH Leu was determined to be *erythro*-L-form by comparison of synthetic standards (*erythro*- and *threo*-form)[13], using the chiral GC-MS system described above. Attempts to determine the configuration of Pip residues were carried out as follows; hydrogenolysis (H_2/PtO_2/AcOH, 50°C, 6 days) converted the Pip and *N*-OH Ala residues in **1** to ornithine (Orn) and Ala residues, followed by hydrolysis (6 N HCl) and *N*-trifluoroacetyl isopropyl ester

Fig. 2. Summary of HMBC and NOESY data for GE3 (**1**).

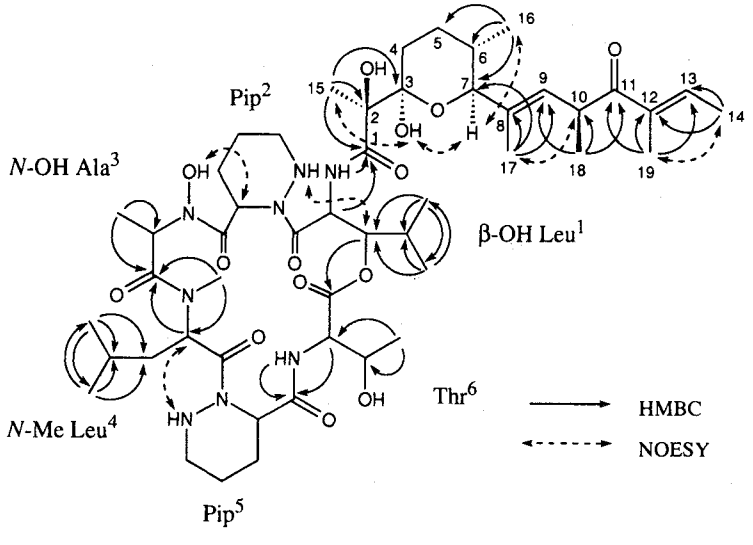
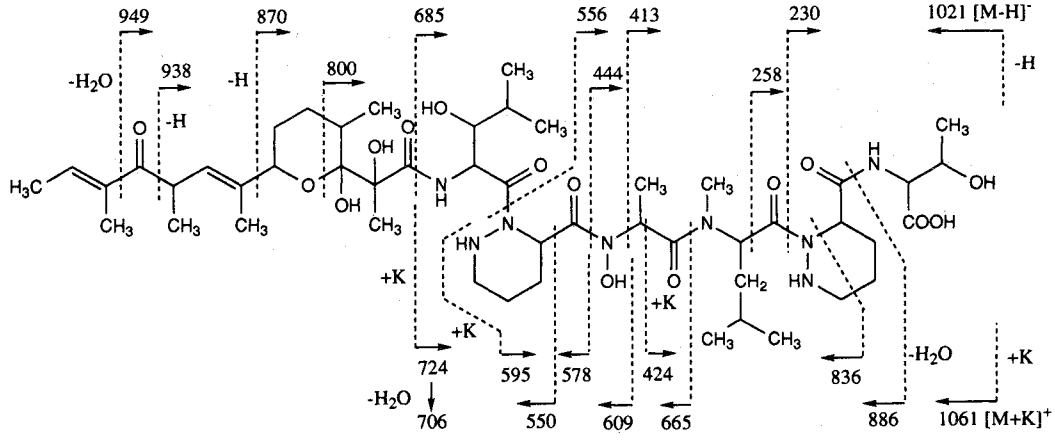


Fig. 3. Fragment ion peaks observed in FAB-MS/MS of GE3B (**2**).



derivatization (isopropyl alcohol/acetyl chloride; trifluoroacetic anhydride). Chiral GC-MS analysis showed that two molecules of Orn and Ala were L-, D-, and L-form respectively, indicating that **1** contains L-Pip, D-Pip, and *N*-OH L-Ala. Though stereochemical assignment of two Pip residues (Pip^2 and Pip^5) and *N*-Me Leu^4 were unsuccessful, structural similarity of **1** with other known cyclohexadepsipeptides[2~12] indicated that Pip^2, *N*-Me Leu^4 and Pip^5 were likely to be D-, D- and L-forms, respectively. Thus the structure of **1**, including absolute configuration, were concluded to be as shown in Fig. 1.

The molecular formula of GE3B (**2**) was determined to be $C_{49}H_{82}N_8O_{15}$ by HRFAB-MS (*m/z* 1021.5849 $[M-H]^-$, $\Delta+2.8$ mmu) which differed from those of GE3 (**1**) simply by the addition of one oxygen and two hydrogen atoms. Although IR spectrum of **2** showed the amide carbonyl absorption (1635 cm^{-1}), a weak absorption owing to ester carbonyl group of **1** (1734 cm^{-1}) was no longer found in **2**, indicating that **2** was the hydrolysate of ester bond in **1**. GE3B (**2**) gave broad and complicated ^1H NMR spectrum ($CDCl_3$) which made it difficult to analyze. This may be due to multiple conformations of **2** in contrast to **1** which has a rigid conformation by intramolecular hydrogen bonds. The amino acid analysis of **2** showed that **2** had the same amino acid composition as **1**. Application of the tandem FAB-MS (FAB-MS/MS) experiment to the sequential assignments of amino acids was carried out and the results are shown in Fig. 3. Negative ion FAB-MS/MS of $[M-H]^-$ ion of **2** as a precursor ion gave the ion derived from cleavage of the acyl side chain (*m/z* 685 $[M-C_{19}H_{29}O_5]^-$). Other useful ion peaks to the structure assignment are summarized in Fig. 3, and these data disclosed the linear peptide structure for **2** as shown in Fig. 3.

GE3 (**1**) and GE3B (**2**) were thus proved to be cyclic and linear hexadepsipeptides, respectively. Complete loss of biological activities in **2** suggested that cyclic and three-dimensional structure is essential for expression of biological activities.

Acknowledgments

We wish to thank Mr. K. HONDA in our laboratory for GC-MS operations.

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