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YM-216391, a Novel Cytotoxic Cyclic Peptide from *Streptomyces nobilis*

II. Physico-chemical Properties and Structure Elucidation

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Abstract YM-216391, a novel cytotoxic cyclic peptide, has been isolated from the cultured mycelium of *Streptomyces nobilis* JCM 4274. The planar structure of YM-216391 was assigned on the basis of 1D and 2D NMR spectroscopic techniques. The absolute configuration of the amino acid residues in YM-216391 was determined by Marfey's analysis and chiral HPLC analysis of its acid hydrolysate.

Keywords YM-216391, cyclic peptide, trisoxazole, cytotoxic

Introduction

Streptomyces nobilis JCM 4274 produced a novel cyclic peptide YM-216391 (**1**, Fig. 1), which showed potent cytotoxicity against human cancer cell lines *in vitro*. The fermentation, isolation, and biological activities of **1** have been reported in the preceding paper [1]. This paper describes the physico-chemical properties and structure elucidation of **1**.

Results

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 1. The positive and negative ion FAB-MS spectra of **1**

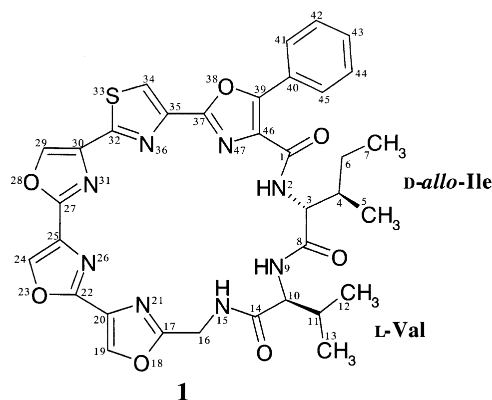

Fig. 1 Structure of YM-216391 (**1**).

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showed $[M+H]^+$, $[M+Na]^+$, and $[M-H]^-$ at *m/z* 697, 719, and 695, respectively, suggesting a molecular weight of 696. The molecular formula of **1** was established as $C_{34}H_{32}N_8O_7S$ by HRESI-MS. The IR spectrum of **1** exhibited absorption bands at 3280, 1660, 1570, and 1510 cm^{-1}, indicating a peptidic moiety in the molecule. The acid hydrolysate of **1** yielded three amino acids, glycine (Gly), valine (Val), and isoleucine (Ile). The Gly was probably derived from the oxazole-2-methylamino moiety of **1** during the acid hydrolysis.

Table 1 Physico-chemical properties of YM-216391 (**1**).

	YM-216391 (**1**)
Appearance	White powder
Melting point	>238°C dec
Molecular formula	$C_{34}H_{32}N_8O_7S$
FAB-MS (*m/z*)	
positive	697 $[M+H]^+$, 719 $[M+Na]^+$
negative	695 $[M-H]^-$
HRESI-MS (*m/z*)	
found	697.2205 $[M+H]^+$
calcd	697.2193
Optical rotation	$[\alpha]_D^{25}$ +48° (*c* 0.10, MeCN)
UV λ_{max} nm (ε) in MeCN	261 (31,000), 273 (31,200), 287 (32,000), 322 (8,700, sh)
IR ν_{max} cm^{-1} (KBr)	3280, 2960, 1660, 1570, 1510

Structure Elucidation

The ^{13}C NMR spectrum of **1** showed 32 resolved peaks corresponding to 34 carbons, which were classified as four methyls, one methylene, two methines, one aminomethylene, two aminomethines, nine sp^2 methines, twelve sp^2 quaternary carbons, and three carbonyl carbons by analysis of the DEPT spectrum. The ^1H NMR spectrum of **1** exhibited 32 proton signals, including twelve protons at δ_H 7.52~9.07 as well as four protons from one aminomethylene and two aminomethines at δ_H 4.19~5.05. An HMQC experiment established all direct ^1H-^{13}C connectivities (Table 2). Three protons at δ_H 8.67, 8.57, and 8.22, which were not connected to carbons, were ascribed to amide protons. A COSY experiment revealed the following proton spin networks (Fig. 2): (**a**) from the amide proton H-2 (δ_H 8.22) to H-5 (δ_H 0.95) and H-7 (δ_H 0.91) through H-3 (δ_H 4.82), H-4 (δ_H 2.09), and H-6 (δ_H 1.66, 1.09), (**b**) from the amide proton H-9 (δ_H 8.57) to H-12 (δ_H 0.97) and H-13 (δ_H 0.93) through H-10 (δ_H 4.60) and H-11 (δ_H 2.14), (**c**) from the amide proton H-15 (δ_H 8.67) to H-16 (δ_H 5.05, 4.19), and (**d**) from H-41/45 (δ_H 8.35) to H-43 (δ_H 7.52) through H-42/44 (δ_H 7.57). The relationship of the spin networks **a**, **b**, and **c** was established from ^1H-^{13}C long-range correlations in the HMBC spectrum and yielded a partial structure, **A**, including Val and Ile residues (Fig. 2). Key HMBC correlations used to construct this partial structure are as follows: from H-3 and the amide proton H-2 to carbonyl carbon C-1 (δ_C 160.3); from H-3, H-10, and the amide protons H-2 and H-9 to carbonyl carbon C-8 (δ_C 170.2); and from H-10, H-11, H-16, and the amide proton H-15 to carbonyl carbon C-14 (δ_C 170.8). The spin network **d** containing the five aromatic protons, together with the HMBC correlation from H-42/44 to the sp^2 quaternary carbon C-40 (δ_C 126.7), proved the presence of a monosubstituted benzene ring (partial structure **C**, Fig. 2).

Partial structures **A** and **C** account for the elemental composition of the $C_{19}H_{28}N_3O_3$ portion of **1**, leaving $C_{15}H_4N_5O_4S$ to be elucidated. The presence of heteroaromatic rings in the $C_{15}H_4N_5O_4S$ portion was deduced from the high degree of unsaturation, the singlet signals of the remaining four aromatic protons (δ_H 9.07, 8.98, 8.89, and 8.65), and the remaining fifteen sp^2 carbons (δ_C 122.2~163.0). In addition, the three aromatic protons H-19 (δ_H 8.89), H-24 (δ_H 8.98), and H-29 (δ_H 9.07) were connected to carbons at δ_C 139.6, 139.1, and 139.4, respectively, based on the HMQC correlations, and their $^1J_{CH}$ values obtained from the INEPT spectrum were 213.9 Hz (C-19), 216.0 Hz (C-24), and 211.8 Hz (C-29), which suggests three oxazole rings [2~4]. Furthermore, the aromatic proton H-34 (δ_H 8.65) was connected to a carbon at δ_C 122.2, and its $^1J_{CH}$ value was 193.4 Hz, indicating a thiazole ring[4]. The relationship of the three oxazole rings and the thiazole ring was determined by interpretation of the HMBC spectrum to yield partial structure **B** (Fig. 2). Key HMBC correlations used to construct this partial structure are as follows: from H-19 to C-17 (δ_C 163.0), C-20 (δ_C 129.1), and C-22 (δ_C 155.5); from H-24 to C-22 and C-25 (δ_C 129.9); from H-29 to C-25, C-27 (δ_C 155.0), C-30 (δ_C 135.6), and C-32 (δ_C 157.5); and from H-34 to C-30, C-32, and C-35 (δ_C 141.5). The HMBC correlations H-19/C-22, H-29/C-25, and H-29/C-32 were observed when the delay for the evolution of long-range couplings was set to 0.15 seconds ($^nJ_{CH}$=3.3 Hz), but not when set to 0.06 seconds ($^nJ_{CH}$=8.3 Hz), because of their small $^nJ_{CH}$ values.

The remaining units, three sp^2 quaternary carbons (δ_C 154.1, 150.6, and 130.8), a nitrogen atom, and an oxygen atom, were connected to yield an oxazole ring based on the high degree of unsaturation and the chemical shift values of these carbons. The relationship of this oxazole ring and partial structures **A**, **B**, and **C** were deduced from the interpretation of the HMBC spectrum as described below

Table 2 ^{1}H and ^{13}C NMR assignments for YM-216391 (**1**).

No.	δ_C	δ_H (multiplicity; *J* in Hz)	HMBC
1	160.3		
2		8.22 1H (d; 6.5)	C-1, C-3, C-8, C-46*
3	57.1	4.82 1H (dd; 6.5, 3.5)	C-1, C-4, C-5, C-6, C-8, C-46*
4	38.8	2.09 1H (m)	C-3*, C-5, C-6*, C-7*
5	14.7	0.95 3H (d; 7.5)	C-3, C-4, C-6
6	25.6	1.66 1H (m)	C-3, C-4, C-5, C-7
		1.09 1H (m)	C-3, C-4, C-5, C-7
7	12.1	0.91 3H (t; 7.5)	C-4, C-6
8	170.2		
9		8.57 1H (d; 9.0)	C-8, C-10, C-11
10	57.5	4.60 1H (dd; 9.0, 4.5)	C-8, C-11, C-12, C-13, C-14
11	31.5	2.14 1H (m)	C-10, C-12, C-13, C-14
12	17.4	0.97 3H (d; 6.5)	C-10, C-11, C-13
13	19.7	0.93 3H (d; 6.5)	C-10, C-11, C-12
14	170.8		
15		8.67 1H (dd; 9.0, 2.0)	C-14, C-16*
16	35.2	5.05 1H (dd; 17.0, 9.0)	C-14, C-17, C-20*
		4.19 1H (dd; 16.5, 2.5)	C-14, C-17
17	163.0		
19	139.6	8.89 1H (s)	C-17, C-20, C-22*
20	129.1		
22	155.5		
24	139.1	8.98 1H (s)	C-22, C-25
25	129.9		
27	155.0		
29	139.4	9.07 1H (s)	C-25*, C-27, C-30, C-32*
30	135.6		
32	157.5		
34	122.2	8.65 1H (s)	C-30, C-32, C-35, C-37
35	141.5		
37	154.1		
39	150.6		
40	126.7		
41/45	127.5	8.35 1H (d; 7.5)	C-39, C-43
42/44	128.6	7.57 1H (t; 7.0)	C-39*, C-40, C-43*
43	130.0	7.52 1H (t; 7.0)	C-41/45, C-42/44*
46	130.8		

* These correlations were observed only when the delay was set to 0.15 seconds.

(Fig. 3). Partial structure **A** was connected at C-16 to C-17 of partial structure of **B** by the correlations from H-16 (δ_H 5.05, 4.19) to C-17 and from H-16 (δ_H 5.05) to C-20. The correlation from H-34 to C-37 (δ_C 154.1) determined the linkage between C-35 of partial structure **B** and C-37 of the oxazole ring. C-40 of partial structure **C** was linked to C-39 of the oxazole ring by the correlations from H-41/45 and H-42/44 to C-39 (δ_C 150.6). The correlations from H-2 and H-3 to C-46 (δ_C 130.8) established the linkage between C-1 of partial structure **A** and C-46 of the oxazole ring. The HMBC correlations H-2/C-46, H-3/C-46, H-16/C-20, and H-42/44/C-39 were observed only when the delay was set to 0.15 seconds.

The absolute configuration of the Val and Ile residues was determined by application of the Marfey's method using 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA)[5] and chiral HPLC analysis of the acid hydrolysate of **1**. Marfey's analysis revealed the presence of L-Val in the

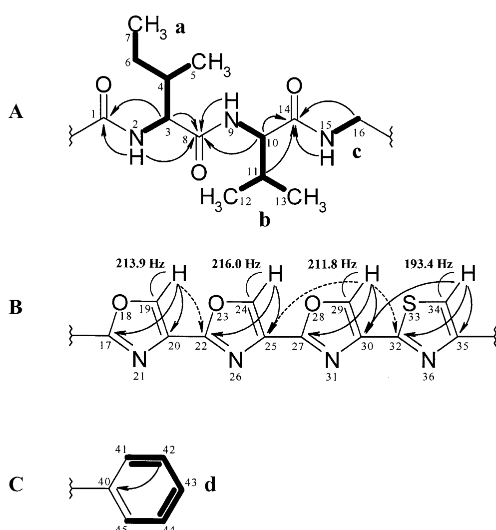

Fig. 2 Partial structures of YM-216391 (**1**). Bold lines: proton spin networks; solid arrows: HMBC correlations; and dashed arrows: HMBC correlations observed only when the delay was set to 0.15 seconds.

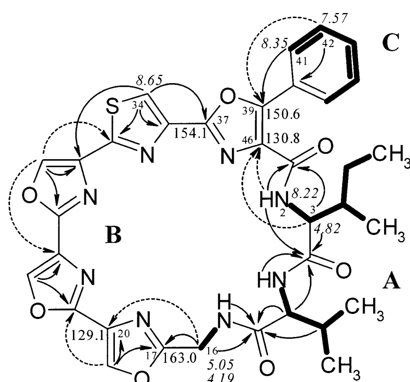

Fig. 3 COSY and HMBC correlations for YM-216391 (**1**). The bold lines, solid arrows, and dashed arrows are the same as those in Fig. 2.

acid hydrolysate of **1**, whereas it could not distinguish between D-Ile and D-*allo*-Ile because of their identical HPLC retention times. Chiral HPLC analysis successfully determined the absolute configuration of Ile to be D-*allo*. Thus, the absolute stereochemistry of **1** was determined as shown in Fig. 1.

Discussion

The complete structure of **1** was determined using spectral analyses and chemical degradation. It is structurally characterized as a 24-membered cyclic peptide composed of two amino acid residues including a D-amino acid and heteroaromatic rings, including a trisoxazole moiety. The structure of **1** is related to a 24-membered cyclic compound containing a pentaoxazole moiety, telomestatin, which was isolated from *Streptomyces anulatus* [6]. Compound **1** differs particularly from telomestatin in the presence of amino acid residues. To our knowledge, **1** is the first example of a cyclic peptide containing a sequential trisoxazole-thiazole moiety. Further pharmacological studies are now underway.

Experimental

General Procedures

UV and IR spectra were recorded on Shimadzu UV-2200 and HORIBA FT-210 spectrometers, respectively. Melting point was measured on a Yanaco MP-500D micro melting point meter. Optical rotation was obtained on a HORIBA SEPA-200 polarimeter at 589.6 nm. FAB-MS and HRESI-MS spectra were recorded on JEOL DX-300 and Micromass Q-Tof Ultima API mass spectrometers, respectively. NMR spectra were measured on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C in DMSO-d_6. Chemical shifts are given in ppm using TMS as an internal standard. The delay for the evolution of long-range couplings in the HMBC experiment was set to 0.06 and 0.15 seconds ($^{n}J_{CH}$=8.3 and 3.3 Hz, respectively).

Amino Acid Analysis

Approximately 1.0 mg of **1** was hydrolyzed with 100 μl of 6 N HCl at 110°C for 18 hours. The hydrolysate was evaporated to dryness and dissolved in 100 μl of 0.1 M borate buffer (pH 9.2). To 20 μl of this solution, 10 μl of 25 mM 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F, Dojindo) solution in ethanol was added, and the mixture was heated at 60°C for 5 minutes. The reaction mixture was then diluted with 300 μl of 10 mM citrate buffer (pH 6.2), and 20 μl of the NBD derivative solution was analyzed by HPLC. The analysis of the NBD derivatives was performed on a TSKgel ODS-80Ts (150×4.6 mm, Tosoh) column maintained at 30°C with UV detection at 470 nm. MeCN - H_2O (1 : 1) and 10 mM citrate buffer (pH 6.2) were used as mobile phases under gradient elution mode (MeCN - H_2O 6%~36% for 0~16 minutes, 36%~37% for 16~21 minutes, 37%~100% for 21~35 minutes, and 100%~100% for 35~37 minutes) at a flow rate of 1.0 ml/minute. The NBD derivatives of the acid hydrolysate were identified by comparing the retention times with NBD derivatized authentic amino acids. The retention times of

the NBD derivatives of the acid hydrolysate were 9.9 (Gly), 20.4 (Val), and 26.4 minutes (Ile).

Marfey's Analysis

Approximately 1.0 mg of **1** was hydrolyzed with 100 μl of 6 N HCl at 110°C for 18 hours. The acid hydrolysate was evaporated to dryness and dissolved in 100 μl of 0.1 N HCl. To 50 μl of the acidic solution, 20 μl of 1 N $NaHCO_3$ and 100 μl of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA, Marfey's reagent, Pierce) solution in acetone was added, and the mixture was heated at 40°C for 1 hour. The reaction mixture was cooled to room temperature, neutralized with 1 N HCl (20 μl), and evaporated to dryness. The residue was dissolved in 1 ml of MeCN and 2 μl of the FDAA derivative solution was analyzed by HPLC. The analysis of the FDAA derivatives was performed on a TSKgel ODS-80Ts (150×4.6 mm) column maintained at 30°C with UV detection at 340 nm. MeCN and 0.05 M triethylamine phosphate buffer (pH 3.0) were used as mobile phases under linear gradient elution mode (MeCN 10%~50% for 0~40 minutes) at a flow rate of 1.0 ml/minute. The FDAA derivatives of the acid hydrolysate were identified by comparing the retention times with FDAA derivatized authentic amino acids. The retention times of the FDAA derivatives of the acid hydrolysate were 27.8 (L-Val; D-Val 31.4 minutes) and 36.1 minutes (D-Ile and D-*allo*-Ile; L-Ile and L-*allo*-Ile 31.5 minutes). This analysis could not determine whether the absolute configuration of Ile was D or D-*allo*.

Chiral HPLC Analysis

To determine the absolute configuration of Ile, 5 μl of the remaining acidic solution prepared for Marfey's analysis was analyzed by chiral HPLC on a SUMICHIRAL OA-5000 (150×4.6 mm, Sumika Chemical Analysis Service) column maintained at 30°C with UV detection at 254 nm, using 2 mM $CuSO_4$ as the mobile phase at a flow rate of 1.0 ml/minute. The retention time of the acid hydrolysate of **1** was 28.8 minutes (D-*allo*-Ile; D-Ile 35.8 minutes).

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References

1. Sohda K, Nagai K, Yamori T, Suzuki K, Tanaka A. YM-216391, a novel cytotoxic cyclic peptide from *Streptomyces nobilis*. I. Fermentation, isolation and biological activities. J Antibiot 58: 27–31 (2005)
2. Roesener JA, Scheuer PJ. Ulapualide A and B, extraordinary antitumor macrolides from nudibranch eggmasses. J Am Chem Soc 108: 846–847 (1986)
3. Matsunaga S, Fusetani N, Hashimoto K. Kabiramide C, a novel antifungal macrolide from nudibranch eggmasses. J Am Chem Soc 108: 847–849 (1986)
4. Hiemstra H, Houwing HA, Possel O, van Leusen AM. Carbon-13 nuclear magnetic resonance spectra of oxazoles. Can J Chem 57: 3168–3170 (1979)
5. Marfey P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Res Comm 49: 591–596 (1984)
6. Shin-ya K, Wierzba K, Matsuo K, Ohtani T, Yamada Y, Furihata K, Hayakawa Y, Seto H. Telomestatin, a novel telomerase inhibitor from *Streptomyces anulatus*. J Am Chem Soc 123: 1262–1263 (2001)