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YM-266183 and YM-266184, Novel Thiopeptide Antibiotics Produced by *Bacillus cereus* Isolated from a Marine Sponge

II. Structure Elucidation

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YM-266183 and YM-266184 are new antibacterial substances that have activity against drug-resistant bacteria produced by *Bacillus cereus* QN03323. These structures were elucidated by MS and NMR spectral analysis. YM-266183 and YM-266184 are the cyclic thiopeptides containing thiazole and pyridine moieties, and several unusual amino acids.

In the screening program for antibacterial substances against drug-resistant bacteria, we discovered novel substances designated YM-266183 (**1**) and YM-266184 (**2**) from the culture broth of *Bacillus cereus* QN03323 that was isolated from a marine sponge. In the preceding paper[1] we described the taxonomy, fermentation, isolation, physicochemical properties and biological activities of new antibacterial substances, **1** and **2**. These antibiotics are thiopeptide antibiotics structurally related to a known family of antibiotics whose members include thiocillins[2,3] and micrococcins[4~8]. This article describes the structural elucidations of **1** and **2** by spectroscopic studies including various two-dimensional NMR experiments.

Results and Discussion

Structure Elucidation of YM-266183

The molecular formula of **1** was determined to be $C_{48}H_{47}N_{13}O_{10}S_6$ based on positive ion mode HRMALDITOF-MS ($(M+Na)^+$, $C_{48}H_{47}N_{13}O_{10}S_6Na$, *m/z* calcd: 1180.1791, found: 1180.1795) and positive ion mode ESI-MS (*m/z* 1158 $(M+H)^+$).

The structure of **1** was elucidated by ^1H NMR, ^{13}C NMR, DEPT, DQF-COSY, TOCSY, ROESY, HSQC, HMBC and constant time HMBC[9]. Constant time HMBC (CT-HMBC) allows improvement of the resolution in the F_1 axis without influence of ^1H-^1H J-modulation. In this sample which had ^{13}C NMR signals that were very crowded, this new HMBC technique was very useful in distinguishing cross peaks of the HMBC spectrum.

The ^1H and ^{13}C NMR spectral data of **1** are shown in Table 1. The ^{13}C NMR spectrum demonstrated 48 signals which were assigned to seven methyls, one methylene, fifteen methines and twenty-five quaternary carbons by DEPT and HSQC experiments. Analysis of the 2D NMR spectra gave substructures A~D (Fig. 2).

(Substructure A (C1-C10))

A methyl signal at 2.08 ppm (H1) showed long-range correlations to the carbonyl carbon at 205.2 ppm (C2) and the methylene carbon at 49.4 ppm (C3). The relatively down-field carbonyl carbon chemical shift of C2 (205.2

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Table 1. ^{1}H and ^{13}C chemical shifts of YM-266183 (**1**) in DMSO-d_6 (26°C).

Position	^{13}C (δ)	^{1}H (δ)	Position	^{13}C (δ)	^{1}H (δ)
1	26.8	2.08 (s)	30	148.7	
2	205.2		31	125.5	8.37 (s)
3	49.4	3.89 (d, 5.9)	32	167.8	
4		8.30 (t, 5.9)	33	57.2	5.48 (d, 10.0)
5	164.4		34	71.3	
6	130.1		35	25.7	1.25 (s)
7	129.1	6.58 (q, 7.0)	36	27.4	1.22 (s)
8	13.4	1.73 (d, 7.0)	37		8.31 (d, 10.0)
9		9.56 (s)	38	159.7	
10	159.1		39	148.2	
11	150.2		40	124.6	8.25 (s)
12	125.4	8.47 (s)	41	166.7	
13	161.3		42	129.2	
14	149.3		43	128.8	6.50 (q, 6.5)
15	121.6	8.59 (s)	44	13.6	1.74 (d, 6.5)
16	168.3		45		9.65 (s)
17	149.6		46	168.5	
18	118.5	8.34 (d, 8.2)	47	56.7	4.70 (dd, 8.2, 2.4)
19	140.8	8.40 (d, 8.2)	48	68.2	4.51 (ddq, 6.5, 4.1, 2.4)
20	128.3		49	20.1	1.37 (d, 6.5)
21	151.0		50		7.57 (d, 8.2)
22	152.9		51	159.7	
23	120.6	7.99 (s)	52	149.8	
24	170.4		53	125.7	8.46 (s)
25	56.5	5.05 (dd, 8.8, 5.9)	54	164.3	
26	66.9	3.96 (ddq, 6.5, 5.9, 5.9)	55		4.69 (d, 5.9)
27	20.9	1.02 (d, 6.5)	56		5.19 (s)
28		8.36 (d, 8.8)	57		4.99 (d, 4.1)
29	160.4				

ppm) indicated that this carbonyl carbon attached C1 and C3 carbons, respectively. In the COSY spectrum, the H3 methylene proton was correlated to an exchangeable proton at 8.30 ppm (H4), which implied the presence of an amino acetone residue. An olefinic proton at 6.58 ppm (H7) was correlated to a vinyl methyl proton at 1.73 ppm (H8) which was coupled to a quaternary carbon at 130.1 ppm (C6). Furthermore, H7 and an exchangeable proton at 9.56 ppm (H9) were correlated to a carbonyl carbon at 164.4 ppm (C5), indicating the presence of a 2-amino-2-butanoic acid residue. Attachment of the 2-amino-2-butanoic acid residue and amino acetone residue was apparent from an HMBC correlation from the H3 methylene to the C5 carbonyl carbon. (*Z*)-Geometry for the C6-C7 olefin was implied by the ROESY correlations between H7 and H4.

(Substructure B (C11-C24-C52-C54))

The aromatic rings substructure B was established mainly by an HMBC experiment. The presence of thiazole units were deduced by comparison of the corresponding ^{1}H and ^{13}C chemical shifts with those of radamycin[10]. In the CT-HMBC spectrum, an aromatic proton signal at 8.47 ppm (H12) showed long-range couplings to carbons at 161.3 ppm (C13) and 150.2 ppm (C11), the aromatic proton signal at 8.59 ppm (H15) to carbons at 168.3 ppm (C16) and 149.3 ppm (C14), the aromatic proton signal at 7.99 ppm (H23) to carbons at 170.4 ppm (C24) and 152.9 ppm (C22), and the aromatic proton signal at 8.46 ppm (H53) to carbons at 164.3 ppm (C54) and 149.8 ppm (C52). These correlations and chemical shifts suggested the presence of four thiazole rings. Furthermore, the H15 methine proton showed a long-range correlation to C13 quaternary carbon of the thiazole, revealing the connectivity between two thiazoles. Long-range connectivities of two adjacent aromatic doublet protons at 8.34 and 8.40 ppm to carbons at 128.3, 149.6, 151.0 ppm (C20, C17, C21) revealed the presence of a 2,3,6-trisubstituted pyridine residue. An HMBC experiment optimized for a J_{CH} of 3 Hz revealed a long-range correlation of H18 methine signal to C16 of the thiazole and established the linkage of the pyridine and thiazole moieties. In addition, the long-range correlations from H19 to C54 of thiazole and from H23 to C21 of the pyridine indicated the connections between the pyridine and two thiazoles. These results revealed the presence of a thiazole-pyridine moiety of substructure B.

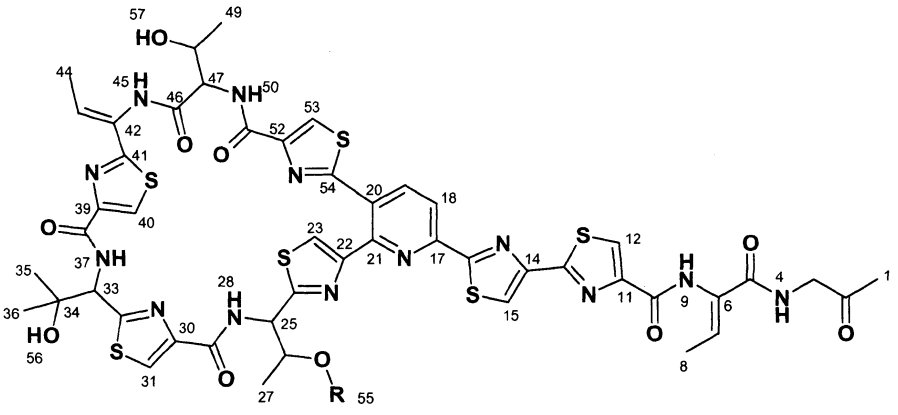
(Substructure C (C25-C38))

The COSY experiment gave straightforward connectivities from an amide proton at 8.36 ppm (H28) to a methyl proton at 1.02 ppm (H27). The methine carbon (C26) was inferred to be oxygenated as judged by its ^{13}C chemical shift at 66.9 ppm. In addition, an exchangeable proton at 4.69 ppm (H55) gave HMBC correlation to C27, indicating hydroxy group attached to C26. In the CT-HMBC spectrum, the H25 methine proton and H28 exchangeable proton were correlated to a carbonyl carbon at 160.4 ppm (C29). An aromatic proton signal at 8.37 ppm (H31) showed long-range couplings to the carbons at 167.8 ppm (C32) and 148.7 ppm (C30), indicating the presence of a thiazole ring. Furthermore, an HMBC experiment optimized for a J_{CH} of 3 Hz revealed a long-range correlation of the H31 methine signal to the C29 carbonyl carbon and established the linkage between thiazole and amide. The structure of the 3-hydroxyvaline moiety was revealed by CT-HMBC correlations of two singlet methines at 1.22 and 1.25 ppm (H36, H35) to a methine carbon at 57.2 ppm (C33), CT-HMBC correlation of an exchangeable proton at 5.19 ppm (H56) to an oxygenated carbon at 71.3 ppm (C34) and COSY correlation between a methine proton at 5.48 ppm (H33) and an amide proton at 8.31 ppm (H37).

(Substructure D (C39-C51))

An aromatic proton signal at 8.25 ppm (H40) showed long-range couplings to the carbons at 166.7 ppm (C41) and 148.2 ppm (C39), indicating the presence of a thiazole ring. The 2-amino-2-butenoic acid unit (C42-C44) was assigned from the COSY and CT-HMBC data, which provided correlations between the vinyl methyl signal at

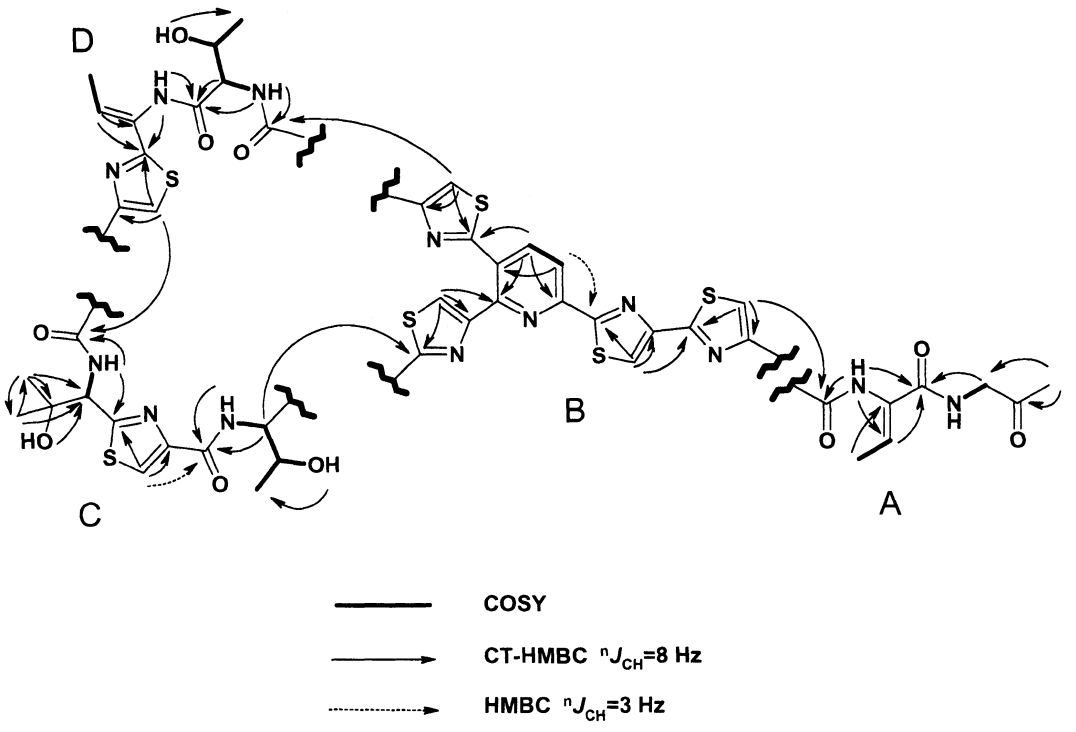
Fig. 1. Structures of YM-266183 (**1**) and YM-266184 (**2**).



YM-266183 (1) R = H

YM-266184 (2) R = CH_3

Fig. 2. Substructures of **1** elucidated by CT-HMBC, and their connectivities.



1.74 ppm (H44) and the olefinic proton signal at 6.50 ppm (H43) that was in turn correlated to the two quaternary carbons at 166.7 ppm (C41) and 129.2 ppm (C42). In addition, HMBC correlation from an amide proton at 9.65 ppm (H45) to C41 established the 2-amino-2-butenoic acid unit. ROESY correlation between the signal at 1.74 ppm (H44) and the amide signal at 9.65 ppm (H45) indicated the (*Z*)-geometry for the 2-amino-2-butenoic acid unit. COSY correlations (H47-H50, H47-H48, H48-H57 and H48-H49) allowed us to assign a threonine moiety. The long-range correlation from the amide protons (H45) and (H50) to the carbonyl carbon at 168.5 ppm (C46) indicated the connection of this threonine and the 2-amino-2-butenoic acid unit.

(Assembly of Substructures)

The connectivities of the substructures were also established by a CT-HMBC experiment. The amide carbon signal at 159.1 ppm (C10) showed long-range heteronuclear correlation to the amide proton at 9.56 ppm (H9) and the methine of thiazole at 8.47 ppm (H12), and allowed attachment of substructure A and B as shown. HMBC correlation from H25 to C24 connected substructure B and C. In turn, H40 and H37 correlated to C38, thus linking substructure C and D. Finally, HMBC showed a long-range correlation from the H53 methine proton of thiazole to the amide carbonyl at 159.7 ppm (C51), closing the peptide ring. Thus, the planar structure of **1** was established as shown in Fig. 1.

Structure Elucidation of YM-266184

The molecular formula of **2** was determined to be $C_{49}H_{49}N_{13}O_{10}S_6$ based on positive ion mode HRMALDITOF-MS ($(M+Na)^+$, $C_{49}H_{49}N_{13}O_{10}S_6Na$, *m/z* calcd: 1194.1948, found: 1194.1933) and positive ion mode ESI-MS (*m/z* 1172 $(M+H)^+$). The ^{1}H and ^{13}C chemical shifts of **2** are shown in Table 2. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1** except for the presence of a methoxy group (C55) instead of the hydroxy group. In the HMBC experiment, this methyl proton at 2.90 ppm (H55) showed a correlation to the methine at 75.6 ppm (C26), indicating a change from the hydroxy group of **1** to methoxy group. The structure of **2** is shown in Fig. 1.

Table 2. ^{1}H and ^{13}C chemical shifts of YM-266184 (**2**) in DMSO-d_6 (26°C).

Position	^{13}C (δ)	^{1}H (δ)	Position	^{13}C (δ)	^{1}H (δ)
1	26.8	2.08 (s)	30	148.5	
2	205.2		31	126.0	8.41 (s)
3	49.5	3.89 (d, 5.5)	32	168.2	
4		8.30 (t, 5.5)	33	57.2	5.52 (d, 10.4)
5	164.4		34	71.1	
6	130.1		35	27.4	1.24 (s)
7	129.0	6.58 (q, 6.7)	36	25.6	1.28 (s)
8	13.4	1.73 (d, 6.7)	37		8.22 (d, 10.4)
9		9.58 (s)	38	159.5	
10	159.1		39	148.1	
11	150.3		40	125.0	8.28 (s)
12	125.4	8.47 (s)	41	166.9	
13	161.3		42	129.1	
14	149.4		43	128.6	6.49 (q, 7.3)
15	121.6	8.58 (s)	44	13.9	1.72 (d, 7.3)
16	168.2		45		9.53 (s)
17	149.5		46	167.6	
18	118.9	8.38 (d, 7.9)	47	56.6	4.73 (dd, 7.3, 3.1)
19	140.5	8.46 (d, 7.9)	48	67.1	4.46 (m)
20	128.6		49	19.0	1.44 (d, 6.1)
21	151.5		50		7.57 (d, 7.3)
22	153.8		51	159.4	
23	120.0	7.97 (s)	52	150.0	
24	170.3		53	125.4	8.46 (s)
25	55.2	5.28 (dd, 9.8, 3.7)	54	164.2	
26	75.6	3.89 (dq, 6.1, 3.7)	55	55.6	2.90 (s)
27	15.5	1.04 (d, 6.1)	56		5.28 (br s)
28		8.11 (d, 9.8)	57		5.21 (br s)
29	160.4				

Experimental

Positive ion mode ESI-MS analysis was performed with the Waters Alliance HT LC/MS system. HRMALDITOF-MS was measured with an Applied Biosystems Voyager ELITE XL time-of-flight mass spectrometer using CHCA (α-cyano-4-hydroxycinnamic acid) as matrix.

NMR spectra were acquired on a VARIAN INOVA NMR spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C) and a JEOL ALPHA NMR spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C) in DMSO-d_6 at 26°C. Chemical shifts were referenced to the TMS peak at 0 ppm for ^{1}H and ^{13}C spectra. The J_{CH} long-range coupling of CT-HMBC was set to 8 Hz and it of HMBC was set to 3 Hz.

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