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Structures of Amamistatins A and B, Novel Growth Inhibitors of Human Tumor Cell Lines from an Actinomycete

Kiyotake Suenaga, Susumu Kokubo, Chikara Shinohara,† Tomoko Tsuji,† and Daisuke Uemura*

Department of Chemistry, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan

†Sagami Chemical Research Center, Nishi-Ohnuma 4-4-1, Sagamihara 229-0012, Japan

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Abstract: Two new lipopeptides, amamistatins A (**1**) and B, were isolated from an actinomycete. The absolute stereostructure of **1** was determined by spectroscopic and chemical analyses. Amamistatins A (**1**) and B showed growth inhibition for human tumor cell lines.

Keywords: peptides; oxazoles; degradation; stereochemistry

In our screening for growth inhibitors of human tumor cell lines derived from microorganisms, amamistatins A (**1**)[1] and B[2] were isolated from an actinomycete collected on the Amami Island in the Kagoshima Prefecture, Japan. Amamistatins were purified from the mycelial cake of the culture broth through a series of processes: EtOAc extraction, silica gel column chromatography, and reversed-phase HPLC. The details of the taxonomy, fermentation, isolation, and biological activities will be reported in a separate paper.[1]

The molecular formula of **1** was determined to be $C_{37}H_{55}N_5O_{11}$ by HRFABMS [*m/z* 746.3981, calcd for $C_{37}H_{56}N_5O_{11}$ $(M + H)^+$ 746.3976]. The IR spectrum showed bands at

[1] $[\alpha]_D^{26}$ –9.8 (*c* 0.61, MeOH); UV (MeOH) λ_{max} 335 (ε 11000), 272 (ε 22000) nm; IR ($CHCl_3$) 3340, 1740, 1660, 1500 cm^{-1}.

[2] Amamistatin B is the demethoxy derivative of **1**. The stereochemical assignment of amamistatin B is underway. Amamistatin B: $[\alpha]_D^{28}$ –8.2 (*c* 0.47, MeOH); UV (MeOH) λ_{max} 307 (ε 12000), 266 (ε 22000) nm; IR ($CHCl_3$) 3350, 1740, 1665, 1510 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ 8.27 (s, 1H) [7.93 (s, 1H)], 7.85 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.40 (ddd, *J* = 8.3, 7.3, 1.5 Hz, 1H), 7.03 (dd, *J* = 8.3, 0.7 Hz, 1H), 6.99 (dd, *J* = 7.9, 7.3, 0.7 Hz, 1H), 5.17 (dd, *J* = 10.2, 2.1 Hz, 1H), 4.67 (dd, *J* = 9.9, 5.1 Hz, 1H) [4.64 (dd, *J* = 9.9, 5.1 Hz, 1H)], 4.52 (d, *J* = 10.3 Hz, 1H), 3.92 (dd, *J* = 15.9, 11.8 Hz, 1H), 3.65 (dd, *J* = 15.9, 3.8 Hz, 1H), 3.52 (t, *J* = 6.2 Hz, 2H) [3.62 (m, 1H), 3.54 (m, 1H)], 2.71 (s, 3H), 2.02 (m, 1H), 1.93 (m, 1H), 1.91 (m, 1H), 1.88 (m, 1H), 1.77 (m, 1H), 1.76 (m, 1H), 1.76 (m, 1H), 1.70 (m, 1H), 1.56 (m, 1H), 1.56 (m, 1H), 1.55 (m, 1H), 1.55 (m, 1H), 1.48 (m, 1H), 1.45 (m, 1H), 1.32 (m, 2H), 1.28 (m, 1H), 1.25 (m, 2H), 1.24 (m, 1H), 1.20 (m, 2H), 1.20 (m, 2H), 1.18 (s, 3H), 1.18 (s, 3H), 0.84 (dd, *J* = 7.1, 6.1 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 177.3, 173.3 [173.2], 171.1, 164.5 [159.9], 164.0, 160.3, 158.5, 154.9, 134.4, 130.3, 128.1, 121.4, 118.7, 112.3, 80.9 [80.8], 54.6 [54.5], 54.4 [54.5], 53.5, 51.5 [47.7], 48.0, 33.3, 32.3, 32.2 [32.1], 31.6 [31.5], 30.8, 30.7, 29.2, 28.1, 27.7, 27.3, 24.7 [24.6], 24.1, 23.6 [23.3], 22.2 [22.0], 14.8, 12.2. The counterparts of the doubled signals in brackets; HRFABMS *m/z* 716.3901, Calcd for $C_{36}H_{54}N_5O_{10}$ $(M + H)^+$ 716.3871.

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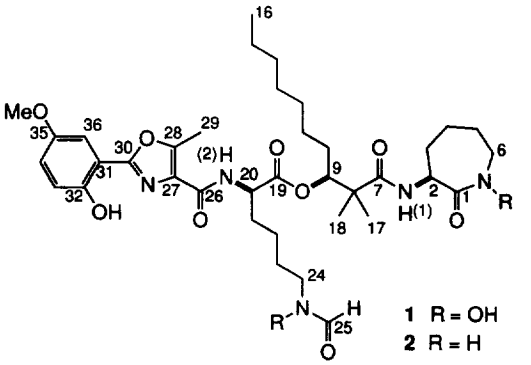


1740, 1660 cm^{-1} that were assigned to ester and amide groups. A $FeCl_3$ test of **1** indicated the presence of a phenol group. The NMR data for **1** are summarized in Table 1. The ^1H NMR spectrum of **1** showed the presence of two amide NH groups (δ 8.93 and 7.48), three hydroxyl groups (δ 9.93, 9.77, and 9.50), a 1,2,4-trisubstituted benzene ring (δ 7.25, 7.04, and 7.00) and a methoxy group (δ 3.76). In the ^{13}C NMR and DEPT spectra, 37 carbon signals were observed, including four carbonyl carbons (δ 173.3, 171.6, 168.6, and 160.8), nine aromatic carbons (δ 157.1, 152.4, 152.1, 150.2, 128.5, 120.0, 118.4, 110.1, and 109.3), one oxymethine carbon (δ 77.9), one oxymethyl carbon (δ 55.6), and four nitrogen-bearing carbons (δ 52.3, 52.0, 50.9, and 48.9). Duplicate signals due to a formyl group (δ_C 161.6, 157.0; δ_H 8.22, 7.88) were also observed. Due to restricted rotation about the formamide moiety, the NMR signals for several protons and carbons were doubled at 23 °C as shown in Table 1. The doubled NMR signals broadened at 60 °C and 90 °C, and coalesced at 120 °C. The remaining carbon signals were assigned to four methyls, 12 methylenes, and one quaternary carbon. A detailed analysis of the phase-sensitive DQF-COSY and HOHAHA spectra of **1** gave three partial structures, two lysine residues [NH(1)-C2-C6 and NH(2)-C20-C24] and C9-C16 (Figure 1). In addition, the presence of a methyloxazole group in **1** was disclosed by correlations in the HMBC spectrum, H-29/C27 and H-29/C28, as well as by

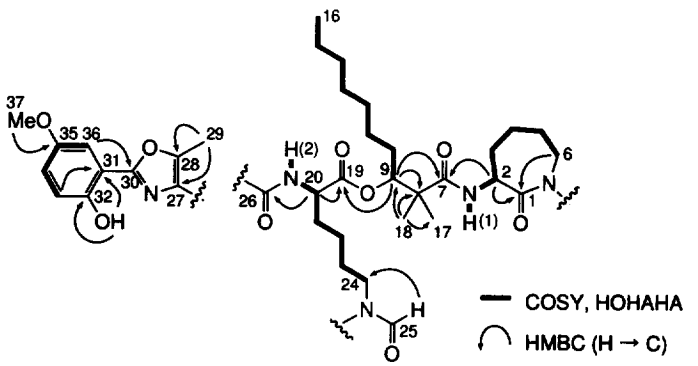


Figure 1. Partial structures of amamistatin A (**1**) based on the phase-sensitive DQF-COSY and HOHAHA spectra and selected HMBC correlations.

Table 1 NMR Data for amamistatin A (**1**) in DMSO-d_6 at 23 °C

position	^{1}H	^{13}C	position	^{1}H	^{13}C
1		[b]168.6	21	1.88 m	29.9
2	[a]4.40 br d (11.5)	50.9	22	1.35 m	22.6
3	1.74 m, 1.39 m	30.0	23	1.56 m	26.4
4	1.78 m, 1.63 m	27.0	24	3.37 m [3.43 m][c]	48.9 [45.4][c]
5	1.66 m, 1.39 m	25.5	25	8.22 s [7.88 s][c]	161.6 [157.0][c]
6	3.87 dd (15.8, 11.7)	52.3	26		160.8
	3.47 dd (15.8, 3.8)		27		128.5
7		173.3	28		152.4
8		45.6	29	2.65 s	11.4
9	5.09 d (9.0)	77.9	30		157.1
10	1.43 m, 1.37 m	29.4	31		110.1
11	1.18 m, 1.12 m	25.6	32		150.2
12	1.15 m	28.5	33	7.00 d (9.0)	118.4
13	1.23 m	28.6	34	7.04 dd (9.0, 2.9)	120.0
14	1.15 m	31.1	35		152.1
15	1.23 m	22.0	36	7.25 d (2.9)	109.3
16	0.81 t (7.1)	13.9	37	3.76 s	55.6
17	1.09 s	19.7 [19.6][c]	C32-OH	9.93 br s	
18	1.04 s	22.9	OH	9.77 br s	
19		171.6	OH	9.50 br s [9.93 br s][c]	
20	4.49 m [4.47 m][c]	52.0	NH(1)	7.48 br d (6.2)	
			NH(2)	8.93 br d (8.2) [8.89 br d (8.2)][c]	

[a] Recorded at 600 MHz. Coupling constants (Hz) are in parentheses.
[b] Recorded at 150 MHz.
[c] Observed as doubled signals.

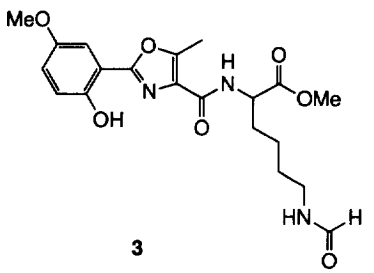
characteristic NMR signals ($\delta_{H\text{-}29}$ 2.65, δ_{C27} 128.5, δ_{C28} 152.4, and δ_{C30} 157.1) that corresponded to those of the methyloxazole group in nostocyclamide.[2] The connections among the aromatic partial structural units were clarified by the HMBC correlations (H-37/C35, H-33/C31, C32-OH/C32, C32-OH/C31, and H-36/C30). The HMBC correlations suggested the presence of a 2-aminocaprolactam structure (H-2/C1 and H-6/C1), a 2,2-dimethyl-3-hydroxydecanoic acid portion (H-9/C8, H-9/C7, H-18/C8, H-18/C9, and H-18/C17), and an *N*-formyllysine residue (H-20/C19, H-25/C24, and H-20/C26), which were connected by HMBC correlations, H-2/C7 and H-9/C19. The locations of the remaining two hydroxyl groups in **1** were determined by the following experiments. Hydrogenolysis (H_2, Pd/C, EtOH, 23 °C) of **1** gave **2**, the molecular weight of which was 32 MS unit (2 × O) less than that of **1**. In addition, the ^{1}H NMR spectrum of **2** showed the presence of two more amide NH groups (δ 6.53, 6.04) than in **1**. This information indicated that **1** had two amide N-OH groups, which were degraded to amide NH groups by hydrogenolysis. Although a connection between C26 and C27 could not be established, such connectivity was obvious based on a consideration of the molecular formula of **1**. Thus, the gross structure of amamistatin A was clarified as shown in formula **1**.

The absolute stereostructure of **1** was elucidated as follows. Hydrogenolysis of **1** followed by methanolysis gave methyl ester **3** and alcohol **4**. Acidic hydrolysis of **3** and **4** (6 M HCl, 110 °C, 24 h) followed by separation by reversed-phase HPLC gave lysines, respectively.[3]

[3] Conditions for the HPLC separation: column, Develosil ODS-HG-5 (4.6 × 250 mm) × 2; solvent, 0.05% CF_3COOH aq, flow rate,

The absolute configurations of the lysines in **3** and **4** were respectively determined to be D and L by a chiral HPLC analysis.[4] The absolute stereochemistry of C9 in **4** was determined using a modified Mosher's method.[3] The alcohol **4** was transformed into (*S*)- and (*R*)-MTPA esters, **5a** and **5b**, ^{1}H NMR signals of which were assigned based on the 2D NMR spectra, and the $\Delta\delta$ values (δ_S–δ_R, ppm) were then calculated.[5] The results established that the absolute stereochemistry of C9 in **2** was *S*. Thus, the absolute stereochemistry of amamistatin A was determined as depicted in formula **1**.

The structures of amamistatins A (**1**) and B are closely related to those of formobactin [4] and nocobactin NA.[5] Amamistatins A (**1**) and B did not show cell-killing effect but anti-proliferative effect against several kinds of human tumor cell lines. The IC_{50} values of amamistatin A (**1**) are 0.48, 0.56, and 0.24 μM against MCF-7 breast, A549 lung, and MKN45 stomach cancer cell line, respectively. Further studies on the mechanisms of the anti-proliferative activity and on the selectivity for the tumor cells are currently in progress.



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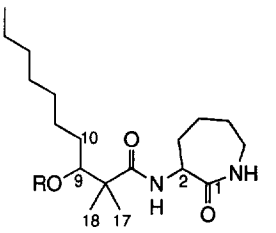

4 R = H
5a R = (*S*)-MTPA
5b R = (*R*)-MTPA

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1.0 mL/min; detection at 205 nm. The retention time of lysine was 5.6 min.

[4] Conditions for the chiral HPLC analysis: column, CROWNPAK CR(+) (Daicel Chemical Ind., Ltd) (4.0 × 150 mm); solvent, 0.13% $HClO_4$ aq. ; flow rate, 0.4 mL/min; detection at 200 nm. Retention times (min) of the authentic samples: L-Lys (5.9), D-Lys (5.4).

[5] The $\Delta\delta$ values in ppm: -0.04 (H-10), -0.02 (H-9), +0.05 (H-17), +0.04 (H-18), +0.04 (H-2)