PREPARATION OF ^{13}C AND ^{15}N LABELED BELLENAMINE AND ITS DEGRADATION PRODUCTS†

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(Received for publication July 6, 1992)

A streptomyces metabolite, bellenamine, has been converted into D-β-lysinamide and cyclized bellenamine in an acidic solution at 75°C. The structure of the new cyclized compound was assigned as (*R*)-6-(3-aminopropyl)-1,3-diazacyclohexan-4-one by spectral analyses. [1-^{13}C,*Amide*,1'-$^{15}N_2$]bellenamine, which has been isolated from the culture by feeding both L-[1-^{13}C]lysine and $[^{15}NH_4]_2SO_4$ to a synthetic medium, was degraded under acidic condition to obtain the stable isotope labeled D-β-lysine, D-β-lysinamide and cyclized bellenamine. These labeled compounds were analyzed by ^{13}C and ^{15}N NMR spectra, and will be used for the biosynthetic study on bellenamine.

A biogenic amine, bellenamine (D-β-lysylmethanediamine), having unique structure and biological activities, was isolated from the culture filtrate of *Streptomyces nashvillensis* MD743-GF4.[1] The open-chain aldoaminal structure and the D-β-lysine moiety first ever found in a natural product were reported before.[1] The absolute structure, (*R*)-*N*-aminomethyl-3,6-diaminohexanamide (Fig. 1), was confirmed by total synthesis.[2] Bellenamine weakly inhibits growth of some Gram-positive bacteria[1] and strongly combats infection by the human immunodeficiency virus (HIV).[3]

In this paper, the preparation of some degradation products of bellenamine and their stable isotope labeled compounds are reported.

Preparation and Structures of Degradation Products

Bellenamine (2 mg/ml) in aqueous solutions at pH 3.7, 7.2 and 10.6 was stable in the cold room at

Table 1. Stability of bellenamine in aqueous solution (2,000 μg/ml).

Day	Compound (μg/ml)[a]	pH 10.6		pH 7.2		pH 3.7	
		7°C	37°C	7°C	37°C	7°C	37°C
0	Bellenamine	2,000	2,000	1,880	1,880	1,900	1,900
7	Bellenamine	2,060	1,660	1,980	940	1,840	980
	Cyclized compound	<50	60	<50	600	<50	250
	D-β-Lysinamide	<50	150	<50	nd	<50	880
14	Bellenamine	1,940	1,160	1,960	80	1,980	220
	Cyclized compound	<50	400	<50	1,600	<50	420
	D-β-Lysinamide	<50	nd	<50	nd	<50	1,560
21	Bellenamine	2,000	660	1,940	80	1,900	80
	Cyclized compound	<50	660	<50	1,910	<50	410
	D-β-Lysinamide	<50	nd	<50	nd	<50	1,790

[a] Compounds were determined by HPLC analysis.
nd: Not detectable.

† Dedicated to the late Professor HAMAO UMEZAWA on the occasion of the 30th anniversary of the Institute of Microbial Chemistry.

7°C for 3 weeks, but unstable at 37°C as shown in Table 1. HPLC analysis showed that bellenamine was converted into a new cyclized compound at pH 7.2 and mainly into D-β-lysinamide at pH 3.7. These degradation products have been isolated from the hydrolysate of bellenamine in an acidic solution (pH 3.6) at 75°C for 8 hours. D-β-Lysinamide was also found in the culture broth as a minor component.[4)]

The cyclized compound was formed by deamination of 1′-NH_2 from bellenamine in a neutral aqueous solution with heating (Fig. 1). Since 3-NH in the cyclized compound derived from [1-^{13}C, *amide*, 1′-

Fig. 1. Formation of D-β-lysinamide and cyclized bellenamine from bellenamine.

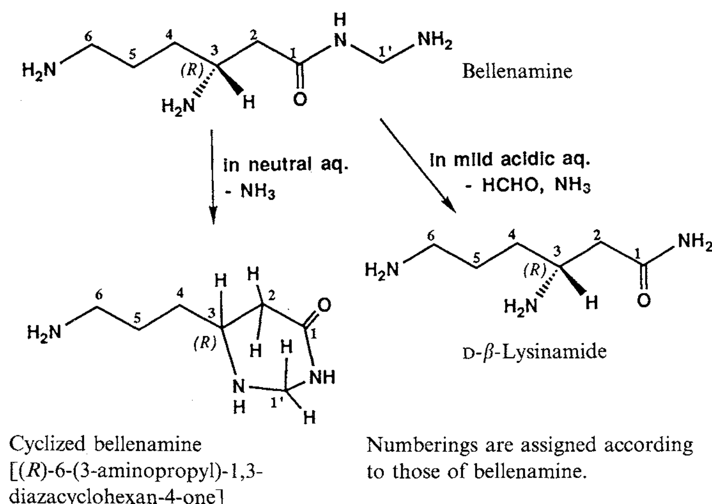

Table 2. ^{13}C NMR spectra of stable isotope labeled compounds in D_2O (pD 4.0).

Carbon	[1-^{13}C,*Amide*,1′-$^{15}N_2$]bellenamine			D-[1-^{13}C,*Amide*-^{15}N]β-lysinamide		
	δ (ppm)	Relative intensity (%)	J (Hz)	δ (ppm)	Relative intensity (%)	J (Hz)
C-1	173.91 d	100	$J_{CN}=13.7$	175.40 d	100	$J_{CN}=16.8$
C-3	48.81	1.6		49.20	2.0	
C-1′	46.02 dd	2.6	$J_{CN}=6.1, 12.2$			
C-6	39.75	1.9		39.86	2.0	
C-2	36.98 dd	2.5	$J_{CN}=7.6$ $J_{CC}=48.8$	36.80 dd	2.5	$J_{CN}=7.6$ $J_{CC}=47.3$
C-4	29.90 d	3.1	$J_{CC}=3.1$	29.97 d	2.0	$J_{CC}=3$
C-6	23.73	2.9		23.80	3.3	

Carbon	[1-^{13}C,*Amide*-^{15}N]cyclized bellenamine			D-[1-^{13}C]β-lysine		
	δ (ppm)	Relative intensity (%)	J (Hz)	δ (ppm)	Relative intensity (%)	J (Hz)
C-1	174.14 d	100	$J_{CN}=15.9$	176.87	100	
C-3	51.48	2.0		49.17	4.2	
C-1′	57.05 d	2.7	$J_{CN}=7.3$			
C-6	40.12	2.3		39.76	3.2	
C-2	36.99 d	1.5	$J_{CC}=46.4$	37.81 d	3.3	$J_{CC}=53.4$
C-4	32.42 d	3.0	$J_{CC}=3.7$	29.85 d	5.0	$J_{CC}=3$
C-5	24.07	3.2		23.74	4.0	

Table 3. ^{15}N NMR spectra of stable isotope labeled compounds in 10% D_2O (pD 4.0).

Nitrogen	[1-^{13}C,*Amide*,1'-$^{15}N_2$]-bellenamine			D-[1-^{13}C,*Amide*-^{15}N]-β-lysinamide			[1-^{13}C,*Amide*-^{15}N]cyclized bellenamine		
	δ (ppm)	Relative intensity (%)	J (Hz)	δ (ppm)	Relative intensity (%)	J (Hz)	δ (ppm)	Relative intensity (%)	J (Hz)
CONH	−258.60	21.0		−261.46	29.1				
^{13}CONH	−258.66 d	100	$J_{CN}=13.7$	−261.50 d	100	$J_{CN}=15.3$	−248.28 d	100	$J_{CN}=15.2$
3-NH_2	−332.14	12.6		−332.29	10.7		−330.03	28.1	
1'-NH_2	−333.64	90.2							
6-NH_2	−341.74	14.0		−341.93	10.9		−342.08	34.1	

$^{15}N_2$]bellenamine was not enriched with ^{15}N, C-3 retained the configuration. On the NMR spectrum in a 1:1 mixture of pyridine-d_5 and D_2O, 3-H at δ 2.97 was coupling to 2-H at δ 2.32 with $J=11$ Hz and to another 2-H at δ 2.61 with $J=4.4$ Hz. NOE between 3-H and 1'-H (δ 4.38) was observed by NOE difference spectroscopy experiment. Therefore, the conformational structure of cyclized bellenamine was determined to be (*R*)-6-(3-aminopropyl)-1,3-diazacyclohexan-4-one, having a half-chair form with 3,1'-pseudodiaxial protons (Fig. 1). Although cyclized bellenamine does not show antibacterial activity, it may be an important compound for biological activities of bellenamine.

Preparation of ^{13}C and ^{15}N Labeled Compounds

Bellenamine was produced by strain MD743-GF4 in a synthetic medium consisting of D-galactose, dextrin, ammonium sulfate and calcium carbonate, and the productivity was improved by addition of L-lysine.[4] Stable isotopes of L-[1-^{13}C]lysine and $[^{15}NH_4]_2SO_4$ was highly incorporated into bellenamine, and [1-^{13}C,*amide*,1'-$^{15}N_2$]bellenamine was obtained. The multiply labeled bellenamine was degraded under mild acidic condition described above to obtain D-[1-^{13}C,*amide*-^{15}N]β-lysinamide, D-[1-^{13}C,*amide*-^{15}N]cyclized bellenamine and [1-^{13}C]β-lysine. These ^{13}C and ^{15}N labeled compounds were analyzed by ^{13}C and ^{15}N NMR spectra (Tables 2 and 3). At C-4 of all 1-^{13}C labeled compounds, small ^{13}C-^{13}C spin couplings ($^3J_{CC}=\sim 3$ Hz) with 1-^{13}C were observed (Table 2). D-[1-^{13}C,*Amide*-^{15}N]β-lysinamide was used for biosynthetic studies on bellenamine.[5]

Experimental

General

MP's were determined with an Electrothermal IA9100 digital melting point apparatus and were not corrected. MS were measured on a JEOL JMS-SX102 mass spectrometer in a FAB mode. IR spectra were taken on a Hitachi 260-10 spectrophotometer.

^{1}H, ^{13}C and ^{15}N NMR spectra were taken on a JEOL JNM-GX400 spectrometer. ^{1}H NMR spectra were recorded at 400 MHz in a 5 mm sample tube using D_2O ($\delta=4.80$) as an internal standard. ^{13}C NMR spectra were recorded at 100 MHz with full proton decoupling in a 5 mm sample tube using dioxane ($\delta=67.4$) as an internal standard. ^{15}N NMR spectra were recorded at 40.5 MHz in 10 mm sample tube using $NH_4{}^{15}NO_3$ ($\delta=0$) as an external standard.[5]

TLC was carried out on silica gel plates (E. Merck, Art. 5715) developed with $CHCl_3$ - MeOH - 25% aq ammonia (2:2:1) and Rf values of ninhydrin-positive spots were calculated. High-voltage paper electrophoresis (HVPE) was performed on a CAMAG HVE system at 3,300 V for 10 minutes, using HCOOH - CH_3COOH - H_2O (25:75:900, pH 1.8) as an electrolyte solution and the relative mobilities (Rm) of ninhydrin-positive spots to alanine were calculated.[6] HPLC was performed on a Waters 600E

system using Waters Optipak CE column (3.9 × 150 mm) with a guard column (Optipak CE, 3.9 × 35 mm) at 15.0°C and a flow rate of 0.4 ml/minute. 0.36% $HClO_4$ (pH 1.5) was used as a mobile phase and UV absorbance was monitored at 200 nm.[4] Retention times (Rt, minutes) were as follows, bellenamine: 11.7, D-β-lysinamide: 7.1 and cyclized bellenamine: 6.7.

Mild Acid Hydrolysis of Bellenamine

A solution (pH 3.6) of bellenamine (177.1 mg) in H_2O (13 ml) and 1 N HCl (2.4 ml) was heated at 75°C for 8 hours. After neutralization with aq ammonia, the solution was passed through a column of Amberlite CG-50 (NH_4^+, 100 ml). The column was washed with H_2O (200 ml) and eluted with 1.5% aq ammonia (1,500 ml). Fractions of 11 ml were collected.

A pool of fractions 9 and 10 showing Rm 2.00 (HVPE) and Rf 0.19 (TLC) gave D-β-lysine[1,2] (16.7 mg); FAB-MS (positive) *m/z* 147 $(M+H)^+$.

Fractions 18 ~ 25 showing Rm 2.10 and Rf 0.35 were concentrated to obtain the cyclized compound (48.9 mg) as a colorless hygroscopic powder; $[\alpha]_D^{24}$ −58° (*c* 1.0, H_2O); FAB-MS (positive) *m/z* 158 $(M+H)^+$; IR ν_{max} (KBr) 3400, 3300, 2970, 1655, 1585, 1505, 1405, 1340, 1160, 830, 730; ^1H NMR (D_2O, pD 6.0) δ 1.60 (2H, m, 4-H_2), 1.77 (2H, m, 5-H_2), 2.12 (1H, dd, *J* = 10.6, 16 Hz, 2-H), 2.48 (1H, dd, *J* = 5.3, 16 Hz, 2-H), 3.04 (3H, m, 3-H, 6-H_2), 4.25 (2H, ABq, *J* = 12.8 Hz, 1'-H_2); ^1H NMR (pyridine-d_5 - D_2O, 1 : 1) δ 1.78 (2H, m, 4-H_2), 2.09 (2H, m, 5-H_2), 2.32 (1H, dd, *J* = 11, 18 Hz, 2-H), 2.61 (1H, dd, *J* = 4.4, 18 Hz, 2-H), 2.97 (1H, m, 3-H), 3.36 (2H, m, 6-H_2), 4.38 (1H, d, *J* = 12 Hz, 1'-H), 4.56 (1H, d, *J* = 12 Hz, 1'-H); ^{13}C NMR (D_2O, pD 6.0) δ 174.1 (C-1), 57.0 (C-1'), 51.7 (C-3), 40.3 (C-6), 37.1 (C-2), 32.4 (C-4), 24.2 (C-5).

The pool of fractions 50 ~ 62 showing Rm 2.50 and Rf 0.21 led to the recovery of bellenamine (12.8 mg).

The pool of fractions 70 ~ 94 showing Rm 2.24 and Rf 0.22 gave D-β-lysinamide (68.9 mg) as a colorless powder; MP 89 ~ 93°C (dec); $[\alpha]_D^{25}$ −3.8° (*c* 0.5, H_2O); FAB-MS (positive) *m/z* 146 $(M+H)^+$; IR ν_{max} (KBr) 3410, 3210, 2960, 1680, 1640, 1590, 1490, 1390, 1320, 1270, 1205, 1180, 1170, 830, 730; ^1H NMR (D_2O, pD 4.0) δ 1.80 (4H, m, 4-H_2, 5-H_2), 2.65 (1H, dd, *J* = 7.7, 16.8 Hz, 2-H), 2.77 (1H, dd, *J* = 5.2, 16.8 Hz, 2-H), 3.05 (2H, m, 6-H_2), 3.68 (1H, m, 3-H); ^{13}C NMR (D_2O, pD 4.0) δ 175.4 (C-1), 49.2 (C-3), 39.9 (C-6), 36.8 (C-2), 30.0 (C-4), 23.8 (C-5).

Culture with Stable Isotope Labeled Compounds

Spores of *S. nashvillensis* MD743-GF4 on an ISP-4 agar slant were inoculated into a synthetic medium (110 ml, adjusted to pH 7.4 with 1 N NaOH) containing D-galactose 2.0%, dextrin 2.0%, $[^{15}NH_4]_2SO_4$ (98% enrichment, Sigma Chemical Co., U.S.A.) 0.2% and $CaCO_3$ 0.2% in a 500-ml baffled Erlenmeyer flask and cultured at 28°C on a rotatory shaker (180 rpm). Three days later, 43 mg of L-[1-^{13}C]lysine monohydrochloride (99% enrichment, Commissariat à L'Energie Atomique, France through Nacalai Tesque, Japan) was added to each flask and the culture was continued for 9 days. The culture broth in 7 flasks was filtered to yield 710 ml of the filtrate (pH 6.4).

Isolation of [1-^{13}C,*Amide*,1'-$^{15}N_2$]bellenamine

The broth filtrate (710 ml) was passed through a column of Amberlite CG-50 (NH_4^+, 40 ml). After washing with H_2O (150 ml), the column was eluted with 1.5% aq ammonia (500 ml) and fractions of 5 ml were collected. Fractions 8 ~ 10 showing Rm 1.49 were concentrated to recover L-[1-^{13}C]lysine (48.7 mg). The pool of fractions 20 ~ 39 showing Rm 2.50 gave the ^{13}C and ^{15}N labeled bellenamine (26.3 mg). ^1H NMR (D_2O, pD 4.0) δ 1.80 (4H, m, 4-H_2, 5-H_2), 2.72 (1H, ddd, *J* = 5.8*, 8.2, 16.8 Hz, 2-H), 2.85 (1H, ddd, *J* = 4.9, 6.0*, 16.8 Hz, 2-H), 3.05 (2H, m, 6-H_2), 3.73 (1H, m, 3-H), 4.53 (2H, m, 1'-H_2). (* showed couplings with ^{13}C.) The ^{13}C and ^{15}N NMR spectra are shown in Tables 2 and 3, respectively. Enrichment ratio of 1-^{13}C was calculated to be 85% from intensities of ^{13}C-^{13}C coupling peaks ($^1J_{CC}$) at 2-^{13}C.

Mild Acid Hydrolysis of [1-^{13}C,*Amide*,1'-$^{15}N_2$]bellenamine

The ^{13}C and ^{15}N labeled bellenamine (26.3 mg) was hydrolyzed by the similar condition mentioned above. By column chromatography of Amberlite CG-50 (NH_4^+, 30 ml), the following labeled compounds were isolated.

D-[1-^{13}C]β-Lysine (0.8 mg), ^1H NMR (D_2O, pD 4.0) δ 1.80 (4H, m, 4-H_2, 5-H_2), 2.61 (1H, ddd, *J* = 6.8*, 7.8, 17.1 Hz, 2-H), 2.73 (1H, ddd, *J* = 4.9, 6.8*, 17.1 Hz, 2-H), 3.06 (2H, m, 6-H_2), 3.63 (1H, m, 3-H).

[1-^{13}C,*Amide*-^{15}N]cyclized bellenamine: 8.4 mg. ^{1}H NMR (D_2O, pD 6.0) δ 1.59 (2H, m, 4-H_2), 1.78 (2H, m, 5-H_2), 2.12 (1H, ddd, J=6.6*, 10.6, 17.5 Hz, 2-H), 2.48 (1H, ddd, J=4.9, 7.5*, 17.5 Hz, 2-H), 3.04 (3H, m, 3-H, 6-H_2), 4.20 (1H, dd, J=<1**, 12 Hz, 1'-H), 4.25 (1H, dd, J=3.6**, 12 Hz, 1'-H).

[1-^{13}C,*Amide*,1'-$^{15}N_2$]bellenamine: 3.2 mg were recovered.

D-[1-^{13}C,*Amide*-^{15}N]β-lysinamide: 10.3 mg. ^{1}H NMR (D_2O, pD 4.0) δ 1.82 (4H, m, 4-H_2, 5-H_2), 2.68 (1H, ddd, J=6.1*, 7.9, 16.5 Hz, 2-H), 2.80 (1H, ddd, J=5.2, 5.2*, 16.5 Hz, 2-H), 3.08 (2H, m, 6-H_2), 3.70 (1H, m, 3-H). (* and ** showed couplings with ^{13}C and ^{15}N, respectively.)

The ^{13}C and ^{15}N NMR spectra of stable isotope labeled compounds are shown in Tables 2 and 3, respectively.

Acknowledgment

The authors thank Professor YASUO SUZUKI, University of Shizuoka, for his kind suggestion in performing this work.

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