PRODUCTION OF BELLENAMINE† AND NEW METABOLITES IN A SYNTHETIC MEDIUM††

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A streptomyces antibiotic, bellenamine has been produced in a simple synthetic medium consisting of D-galactose, dextrin, ammonium sulfate and calcium carbonate. Three new minor metabolites, *N*-(aminomethyl)succinamic acid, 1′-*N*-acetylbellenamine and D-β-lysinamide have been isolated from the synthetic medium culture. They showed no antibiotic activity.

A biogenic amine, bellenamine having a unique structure and biological activities, was found in the culture filtrate of *Streptomyces nashvillensis* MD743-GF4.[1] The open-chain aldoaminal structure and the D-β-lysine moiety were first found in a natural product, and the absolute structure, (*R*)-*N*-aminomethyl-3,6-diaminohexanamide was confirmed by total synthesis.[1,2] The antibiotic inhibits the growth of Gram-positive bacteria and has activity against the human immunodeficiency virus,[3] and enhances both delayed-type hypersensitivity to sheep red blood cells and antibody formation in the mouse spleen.[1]

We have found that the strain produces bellenamine together with three minor metabolites in a synthetic medium containing only ammonium sulfate as a nitrogen source. In this paper, the culture in synthetic media and the isolation and structures of three novel metabolites, *N*-(aminomethyl)succinamic acid, 1′-*N*-acetylbellenamine and D-β-lysinamide, are reported. A major product, bellenamine has been crystallized as the sesquisulfate from an aqueous methanol solution.

Synthetic Medium Culture

When the strain was cultured at 28°C on a rotatory shaker (180 rpm) in a 500-ml baffled Erlenmeyer flask containing a medium (110 ml); D-galactose 2.0%, dextrin 2.0%, peptone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2%,[1] more than 100 μg/ml of bellenamine was produced after 5~6 days. Surprisingly, the culture in a synthetic medium with the two organic nitrogen sources from the above-mentioned medium omitted, was successful for production of bellenamine; 143 μg/ml after 14 days (Table 1). Four components, D-galactose, dextrin, $(NH_4)_2SO_4$ and $CaCO_3$ were essential for production of bellenamine, and D-galactose could not be replaced by D-glucose. Addition of L-lysine (monohydrochloride, 50~200 mg/110 ml) to the synthetic medium improved the productivity of bellenamine, but D-lysine repressed (Table 1).

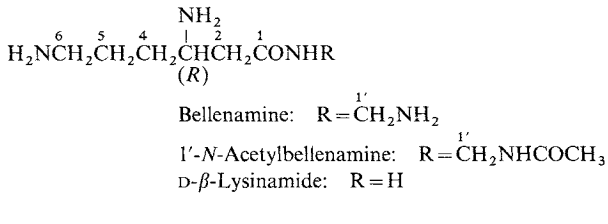
Isolation and Structures of New Metabolites

The 17-day cultured broth was filtered and the filtrate (170 ml) was passed through a column of

† Bellenamine was formerly called as D-β-lysylmethanediamine.[1]

†† This report is dedicated to the late Professor HAMAO UMEZAWA on the occasion of the 30th anniversary of the Institute of Microbial Chemistry.

Fig. 1. Structures of bellenamine and its related metabolites.



Amberlite IRC-50 (NH_4^+-H^+, 7:3). From the effluent and washings, *N*-(aminomethyl)succinamic acid (10.8 mg) was purified. The eluate with 2% aqueous ammonia was rechromatographed on Amberlite CG-50 (NH_4^+) to isolate 1'-*N*-acetylbellenamine (0.8 mg), bellenamine (18.6 mg) and D-β-lysinamide (1.6 mg). The three new metabolites exhibited no antibacterial activity at concentrations of 200 μg/ml.

Table 1. Productivity of bellenamine depending on medium components.

Medium	Productivity (bioassay, μg/ml)			
	Days			
	4	6	10	14
Basal[a]		14	106	143
+Bacto-Soytone 1%, CSL[b] 0.5%	43	121		
−D-Galactose		0	0	0
−Dextrin		0	0	0
$-(NH_4)_2SO_4$		0	0	0
$-CaCO_3$		0	0	0
+L-Lysine HCl 0.18%		16	116	153
+D-Lysine HCl 0.18%		13	31	70
+L-Lysine HCl 0.18%, D-Glucose 2%, −D-Galactose		5	14	9

[a] Basal medium consists of D-galactose 2%, dextrin 2%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2%.
[b] CSL: Corn steep liquor.

The structures of three metabolites (Fig. 1) were confirmed by MS and NMR analyses. *N*-(Aminomethyl)succinamic acid was acetylated with acetic anhydride in methanol to give the *N*-acetyl-methyl ester. D-β-Lysinamide was identical with a hydrolysis product derived from bellenamine in all respects.[4] The acetylated position of 1'-*N*-acetylbellenamine was confirmed by the HMBC experiment; 1'-methylene protons at δ 4.58 were correlated to two carbons at δ 172.7 (C-1) and 175.4 (acetyl carbonyl). The chirality was determined to be *R*-configuration by the optical rotation of D-β-lysine ($[\alpha]_D^{23}$ −26° in 1 N HCl) obtained from the hydrolysate of 1'-*N*-acetylbellenamine. Consequently, the absolute structure was shown to be (*R*)-*N*-(acetamidomethyl)-3,6-diaminohexanamide.

It is not clear whether these metabolites are biosynthetic intermediates of bellenamine. The biosynthetic studies will be reported in due course.[5]

Experimental

General

MP's were determined with a Electrothermal IA9100 digital melting point apparatus and were uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. MS were measured on Hitachi RMU-6M (EI) and M80H (SI) mass spectrometers. IR spectra were taken on a Hitachi 260-10 spectrophotometer. ^1H and ^{13}C NMR spectra in D_2O (pD 4~5) were recorded on a JEOL JNM-GX400 spectrometer. High-voltage paper electrophoresis[6] (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 detected with ninhydrin reagent. The relative mobilities (Rm) to alanine were calculated. Antibiotic activities in a phosphate buffer (pH 8.0) were determined by ordinary cylinder-plate assay using *Bacillus subtilis* PCI219 as a test organism and crystalline bellenamine sesquisulfate (542 μg/mg) as an assay standard.

Culture Conditions

Spores of *S. nashvillensis* MD743-GF4 on ISP-4 agar slant were inoculated into a synthetic medium (110 ml, adjusted to pH 7.4 with 1 N NaOH) consisting of D-galactose (Wako Pure Chemical Industries) 2.0%, dextrin (Wako) 2.0%, $(NH_4)_2SO_4$ 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). Bacto-Soytone (Difco), corn steep liquor (Ajinomoto), D-glucose (Wako), L- or D-lysine monohydrochloride (Peptide Institute, Inc.) was used as a medium component (Table 1).

Isolation of *N*-(Aminomethyl)succinamic Acid

The 17-day culture in the synthetic medium (2 flasks) was filtered. The filtrate (pH 5.6, 170 ml, bioassay: 196 μg/ml) was charged to a column of Amberlite IRC-50 (Rohm and Haas, NH_4^+ - H^+, 7 : 3, 10 ml), and the column was washed with H_2O (20 ml). The effluent and washings were combined, and passed through a column of Amberlite IR-120 (H^+, 10 ml). After washing with H_2O (30 ml), the column was eluted with 2% aqueous ammonia and the eluate was concentrated to dryness (39.2 mg). The residue was purified by preparative HVPE and a ninhydrin-positive compound showing Rm 1.30 was extracted with H_2O to yield *N*-(aminomethyl)succinamic acid (10.8 mg) as the colorless powder. MP 119~121°C (dec); SI-MS *m/z* 147 $(M+H)^+$; IR ν_{max} (KBr) cm^{-1} 3310, 3100, 2950, 1670, 1560, 1470, 1420, 1390, 1280, 1230; ^1H NMR δ 2.57 (4H, m, CH_2CH_2). 4.51 (2H, s, NCH_2N); ^{13}C NMR δ 31.9 (CH_2), 32.1 (CH_2), 46.1 (NCH_2N), 177.6 (CONH), 180.8 (COOH).

Isolation of 1'-*N*-Acetylbellenamine and D-β-Lysinamide

The Amberlite IRC-50 column described above, was eluted with 2% aqueous ammonia and the eluate (100 ml) was concentrated to give a crude powder (48.3 mg). The powder was rechromatographed on a column of Amberlite CG-50 (NH_4^+, 5 ml) eluted with 2% aqueous ammonia. The first eluate (12 ml) was concentrated and purified by preparative HVPE to afford a ninhydrin-positive compound showing Rm 1.58, 1'-*N*-acetylbellenamine (0.8 mg) as the colorless powder. MP 115~119°C (dec); $[\alpha]_D^{24}$ −10.5° (*c* 1.0, H_2O), $[\alpha]_D^{24}$ −17.8° (*c* 0.5, 0.08 N HCl); SI-MS *m/z* 217 $(M+H)^+$; IR ν_{max} (KBr) cm^{-1} 3350, 3100, 3000, 2950, 2890, 1660, 1565, 1500, 1450, 1390, 1365, 1320, 1300, 1140, 720; ^1H NMR δ 1.77 (4H, m, 4-H_2, 5-H_2), 2.00 (3H, s, $NCOCH_3$), 2.61 (1H, dd, J=8.4 and 16.3 Hz, 2-H), 2.71 (1H, dd, J=5.6 and 16.3 Hz, 2-H), 3.04 (2H, m, 6-H_2), 3.67 (1H, br, 3-H), 4.58 (2H, s, 1'-H_2); ^{13}C NMR δ 175.4 (s, Ac-CO), 172.7 (s, C-1), 49.1 (d, C-3), 45.1 (t, C-1'), 39.7 (t, C-6), 37.2 (t, C-2), 29.8 (t, C-4), 23.7 (t, C-5), 22.6 (q, Ac-CH_3).

Bellenamine (18.6 mg, Rm 2.50) was obtained as the colorless syrup of free base from the second eluate (24 ml). Concentration of the third eluate (10 ml) gave D-β-lysinamide (1.6 mg, Rm 2.24) which was identical with a mild acid hydrolysis product of bellenamine.[4]

Crystalline Bellenamine Sesquisulfate

To a solution (pH 2.0) of bellenamine (free base, 123 mg) in 6 N H_2SO_4 (0.29 ml), MeOH (0.8 ml) was added. The mixture was kept in a refrigerator for 4 days to give crystalline bellenamine sesquisulfate (187 mg). MP 170~178°C (dec); $[\alpha]_D^{24}$ −11.6° (*c* 1.0, H_2O); IR ν_{max} (KBr) cm^{-1} 3450, 3080, 1690, 1620, 1560, 1510, 1410, 1270, 1240, 1190, 1175, 1130 (vs, SO_4^{2-}).

Anal Calcd for $C_7H_{18}N_4O \cdot 3/2H_2SO_4$: C 26.16, H 6.59, N 17.43, O 34.85, S 14.97.
Found: C 26.07, H 6.74, N 17.30, O 34.93, S 14.81.

N-(Acetamidomethyl)succinamic Acid Methyl Ester

N-(Aminomethyl)succinamic acid (5.3 mg) was acetylated with acetic anhydride (0.1 ml) in MeOH (0.5 ml) at room temperature for 21.5 hours to give the *N*-acetyl-methyl ester (6.5 mg); EI-MS *m/z* 202 (M^+), 159 $(M-CH_3CO)$, 127, 115, 100, 87; SI-MS *m/z* 203 $(M+H)^+$, 225 $(M+Na)^+$.

Isolation of D-β-Lysine from the Hydrolysate of 1'-*N*-Acetylbellenamine

A solution of 1'-*N*-acetylbellenamine (11 mg, 0.05 mmol) in 4 N HCl (1.5 ml) was heated at 100°C for 3 hours. The hydrolysate was concentrated to dryness and the residue was purified by Amberlite CG-50 (NH_4^+, 5 ml) column. After washing with H_2O (15 ml), the column was eluted with 1% aq ammonia. The ninhydrinin-positive eluate was collected and concentrated to give a colorless powder of D-β-lysine (6 mg, 82%), $[\alpha]_D^{23}$ $-26°$ (*c* 0.5, 1 N HCl), literature,[1] $-24.5°$.

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