BIOSYNTHESIS OF BELLENAMINE BY *Streptomyces nashvillensis* USING STABLE ISOTOPE LABELED COMPOUNDS†

YOKO IKEDA, HIROSHI NAGANAWA, SHINICHI KONDO* and TOMIO TAKEUCHI

Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication July 6, 1992)

The biosynthesis of bellenamine was studied by feeding ^{13}C and ^{15}N labeled precursors to the synthetic medium culture of *Streptomyces nashvillensis* MD743-GF4. The high degree of incorporation of D-[1-^{13}C]β-lysine indicated that it is a direct intermediate, while supplemented L-β-lysine repressed the production of bellenamine. [2-^{13}C]Glycine was well incorporated into the C-1′ of the open-chain aldoaminal structure. All four nitrogens of bellenamine were derived from $[^{15}NH_4]_2SO_4$ present in the synthetic medium. In the addition of L-lysine and glycine, $[^{15}NH_4]_2SO_4$ was highly incorporated into CONH. The feeding experiments of ^{13}C labeled acetates suggested that the D-β-lysine moiety was derived from L-lysine by catalysis of a new 2,3-aminomutase, and L-lysine was biosynthesized from acetates *via* the TCA cycle and diaminopimelic acid pathway.

A biogenic amine, bellenamine, which was formerly called D-β-lysylmethanediamine is produced by *Streptomyces nashvillensis* MD743-GF4 and has a unique structure.[1] The open-chain aldoaminal structure and the D-β-lysine moiety were then found for the first time in a natural product. The absolute structure, (*R*)-*N*-aminomethyl-3,6-diaminohexanamide, was confirmed by total synthesis.[1,2] Bellenamine wealky inhibits growth of some Gram-positive bacteria and enhances both delayed-type hypersensitivity to sheep red blood cells and antibody formation in the mouse spleen.[1] Recently, strong inhibitory effect of bellenamine on infection of T-cell with human immunodeficiency virus was found.[3]

As reported in our previous paper,[4] bellenamine was produced in a synthetic medium containing ammonium sulfate as the sole nitrogen source, and supplement of L-lysine to the medium improved the productivity of bellenamine, but D-lysine repressed it. Our interests were the biosynthetic routes leading to the D-β-lysine and aldoaminal moieties. In this paper, the biosynthesis of bellenamine using stable isotope labeled compounds as biosynthetic precursors is reported.

Materials and Methods

Stable Isotope Labeled Compounds

Sodium [1-^{13}C]acetate (99 atom%), sodium [1,2-$^{13}C_2$]acetate (99%), [1-^{13}C]glycine (99%), [2-^{13}C]glycine (99%), Ba[$^{13}CO_3$] (99%), [^{15}N]glycine (98%) and $[^{15}NH_4]_2SO_4$ (98%) were purchased from Sigma Chemical Co., U.S.A. L-[1-^{13}C]Lysine monohydrochloride (99%) and L-[2-*amino*-^{15}N]lysine dihydrochloride (95%) were purchased from Commissariat à L'Energie Atomique, France, through Nacalai Tesque, Japan. D-[1-^{13}C]β-Lysine (10% enriched) and D-[1-^{13}C, *amide*-^{15}N]β-lysinamide (19% ^{13}C and 24% ^{15}N) were prepared from multiply labeled bellenamine.[5]

Spectral Analyses

^{13}C and ^{15}N NMR spectra were taken on a JEOL JNM-GX400 spectrometer. ^{13}C NMR spectra

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

(D_2O, pD 4.0) were obtained at 100 MHz with full proton decoupling in a 5 mm sample tube using dioxane as an internal standard ($\delta = 67.4$) and from zero filled FID (free induction decay) signals prior to Fourier transformation. ^{15}N NMR spectra (10% D_2O in H_2O, pH 4.0) were recorded at 40.5 MHz in a 10 mm sample tube using $NH_4{}^{15}NO_3$ as an external standard ($\delta = 0$) under the following conditions: pulse flip angle 45°, data points 32 K, spectral width 25 kHz, gated decoupling without NOE, delay time between scans (PD) 3 seconds and probe head temperature 24°C.

HVPE

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 the relative mobilities (Rm) of ninhydrin-positive spots to alanine were calculated.

Bioassay

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.

HPLC Analyses of the Cultured Broth

Spores of *S. nashvillensis* MD743-GF4 grown on an ISP agar slant (a stock culture of our Institute) were inoculated into a synthetic medium (110 ml, adjusted to pH 7.4 with 1 N NaOH before sterilization) containing D-galactose 2.2 g, dextrin 2.2 g, $(NH_4)_2SO_4$ 220 mg and $CaCO_3$ 220 mg in a 500-ml baffled Erlenmeyer flask, and cultured at 28°C on a rotatory shaker (180 rpm). At the start of the culture or 3 days later, L-lysine monohydrochloride (44 mg as free), D-lysine monohydrochloride (44 mg as free), D-β-lysine[2] (48 mg), L-β-lysine[2] (48 mg), D-β-lysinamide[4] (44 mg) or 1'-*N*-acetylbellenamine[4] (45 mg) was fed to each flask and the culture was continued. Each sample (*ca.* 2 ml) of 6-, 10-, 14-, 18- or 24-day

Table 1. Feeding of lysine analogs and 1'-*N*-acetylbellenamine.

Feeding	HPLC assay (μg/ml)	Cultured for (days) 6	10	14	18	24
L-Lysine[a] 44 mg	Bellenamine	29	43	62	102	
	(Bioassay)	(46)	(61)	(80)	(84)	
	L-Lysine	350	301	154	105	
	D-β-Lysinamide	<1	<1	3	5	
	AcBe	<2	2	2	3	
D-Lysine[a] 44 mg	Bellenamine	12	12	26	45	
	(Bioassay)	(40)	(37)	(16)	(46)	
	D-Lysine	392	382	392	378	
D-β-Lysine[b] 48 mg	Bellenamine	40	93	119	152	188
	(Bioassay)	(38)	(94)	(160)	(120)	(142)
	D-β-Lysine	396	386	374	329	265
	D-β-Lysinamide	12	14	11	16	23
	AcBe	2	5	8	13	15
L-β-Lysine[b] 48 mg	Bellenamine	<20	<20	<20	<20	<20
	(Bioassay)	(<30)	(<30)	(<30)	(<30)	(<30)
	L-β-Lysine	464	543	411	444	447
D-β-Lysinamide[b] 44 mg	Bellenamine	<20	<20	<20	24	44
	(Bioassay)	(<30)	(<30)	(<30)	(<30)	(<30)
	D-β-Lysinamide	354	350	332	340	373
1'-*N*-Acetyl-bellenamine[b] (AcBe) 45 mg	Bellenamine	29	21	30	24	26
	(Bioassay)	(<30)	(<30)	(<30)	(<30)	(<30)
	AcBe	451	431	445	458	476

[a] Fed on day 0 to a synthetic medium (110 ml) consisting of D-galactose 2.2 g, dextrin 2.2 g, $(NH_4)_2SO_4$ 220 mg and $CaCO_3$ 220 mg (pH 7.4).

[b] Fed on day 3.

Table 2. Preparation of ^{13}C and ^{15}N labeled bellenamines.

Expt	Addition[a] of labeled compounds	Addition of cold compounds	Cultured for (days)	Bioassay (μg/ml)	Filtrate (ml)	Bellenamine (yields, mg)
1	L-[1-^{13}C]Lys·HCl 46 mg		10	275	99	11.9
2	[1-^{13}C]AcONa 24 mg		21	204	90	6.3
3	[1,2-$^{13}C_2$]AcONa 24 mg		20	183	87	7.4
4	[1-^{13}C]Gly 43 mg		13	53	90	3.6
5	[2-^{13}C]Gly 47 mg		14	139	99	6.7
6	D-[1-^{13}C]β-Lys[b] 44 mg		14	124	97	7.0
7	D-[1-^{13}C, *Amide*-^{15}N]β-LysNH$_2$[c] 43 mg		18	36	96	1.9
8	[$^{15}NH_4]_2SO_4$[d] 193 mg		17	84	97	6.2
9	[$^{15}NH_4]_2SO_4$[d] 190 mg	L-Lys·HCl 50 mg	10	103	105	8.1
10	[$^{15}NH_4]_2SO_4$[d] 220 mg	L-LysNH$_2$·2HCl 67 mg	18	94	98	3.3
11	[$^{15}NH_4]_2SO_4$[d] 200 mg	L-Lys·HCl 50 mg, Gly 25 mg	12	107	93	4.1
12	[^{15}N]Gly 43 mg		15	141	112	11.0
13	[^{15}N]Gly 30 mg	L-Lys·HCl 50 mg	11	65	100	4.6
14	L-[2-*Amino*-^{15}N]Lys·2HCl[e] 48 mg		13	46	103	3.8

[a] Labeled compounds added to a basal medium (110 ml) consisting of D-galactose 2.2 g, dextrin 2.2 g, $(NH_4)_2SO_4$ 220 mg and $CaCO_3$ 220 mg, at the start of the culture.
[b] 10% ^{13}C.
[c] 19% ^{13}C and 24% ^{15}N.
[d] $[^{15}NH_4]_2SO_4$ replaced $(NH_4)_2SO_4$ as a nitrogen source.
[e] 50% ^{15}N.

cultured broth was filtered by disposable sterile syringe filter (25 mm, 20 micron, Corning, U.S.A.) and the filtrate (1 ml) was charged to a column (5 mm in diameter) of Amberlite CG-50 (NH_4^+, 0.5 ml). After washing with H_2O (1 ml), the column was eluted with 2% aq ammonia (10 ml) and the eluate was concentrated to dryness. The residue was dissolved in H_2O (0.5 ml) and 10 μl of the solution was injected to the column of HPLC.[4] Analysis 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. As a mobile phase, 0.36% $HClO_4$ (pH 1.5) was used and UV absorbance was monitored at 200 nm (Table 1). Retention times (Rt, minutes) were as follows, L-lysine: 11.9, bellenamine: 11.0, D-lysine: 10.4, 1′-*N*-acetylbellenamine: 8.5, L-β-lysine: 8.4, D-β-lysine: 8.0 and D-β-lysinamide: 7.0.

Isolation of Labeled Bellenamine

S. nashvillensis MD743-GF4 was cultured in a synthetic medium (110 ml, pH 7.4) containing D-galactose 2.2 g, dextrin 2.2 g, $(NH_4)_2SO_4$ or $[^{15}NH_4]_2SO_4$ 220 mg and $CaCO_3$ 220 mg in a 500-ml baffled Erlenmeyer flask at 28°C for 10∼21 days, as described above. At the start of the culture, each stable isotope labeled compound was added (Table 2). The cultured broth was filtered by a filter paper (Toyo Roshi Kaisha, Japan, No. 2) and the filtrate (87∼112 ml) was passed through a column of Amberlite CG-50 (NH_4^+, 10 ml). After washing with H_2O (20 ml), the column was eluted with 1.5% aq ammonia (100 ml). Fractions of 3 ml were collected and each fraction was monitored by HVPE. Single ninhydrin-positive fractions (Rm 2.50) were collected and concentrated to yield pure labeled bellenamine (Table 2).

Results

Feeding of Lysine Analogs and 1′-*N*-Acetylbellenamine

As shown in Table 1, D-β-lysine in the synthetic medium culture was efficiently converted into bellenamine, but D-β-lysinamide and 1′-*N*-acetylbellenamine were slightly produced. L-Lysine supplemented to the synthetic medium was metabolized and produced bellenamine. In feeding experiments of D-lysine, L-β-lysine, D-β-lysinamide and 1′-*N*-acetylbellenamine, production of bellenamine did not improve.

Incorporation of Stable Isotope Labeled Precursors

Incorporation of ^{13}C and ^{15}N labeled compounds into bellenamine was analyzed by ^{13}C and ^{15}N NMR spectra, as shown in Tables 3, 4 and 5.

Aetate and L-lysine

Stable isotopes of L-[1-^{13}C]lysine and L-[2-*amino*-^{15}N]lysine were highly incorporated into C-1 and 3-NH_2 of bellenamine (Tables 3 and 5). In experiments with sodium [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate (Expt 2 and 3), ^{13}C - ^{13}C spin coupling analyses showed that three sets of two carbons, C-1 - C-2, C-4 - C-3 and C-4 - C-5 in bellenamine were derived from C-1 - C-2 of acetates, and C-6 bellenamine was derived from C-2 of acetate (Table 3, Fig 1).

Glycine

[2-^{13}C]Glycine was highly incorporated into C-1′ of bellenamine, while low incorporation of

Table 3. Incorporation of ^{13}C-labeled L-lysine and acetates into bellenamine.

Carbon	δ	Intensity[a] %	L-[1-^{13}C]Lys (Expt 1)	[1-^{13}C]AcONa (Expt 2)	[1,2-$^{13}C_2$]AcONa (Expt 3)		
			Enrichment ratio[b]	Enrichment ratio[b]	Enrichment ratio[b]	J_{CC} (Hz)	^{13}C-^{13}C Coupling ratio (%)[c]
1	173.9	36.4	91.7	1.6	3.3[d]	49.2	25
3	48.8	79.1	1.0	0.9	3.1[d]	36.5	28
1′	46.0	75.4	1.0	1.0	1.0		
6	39.7	77.7	1.3	1.1	4.2[d]	36.6	7
2	36.9	95.8	1.0[d]	0.9	2.1[d]	49.2	28
4	29.8	100	0.7	2.0	2.9[d]	35.1 (36.5)[e]	35
5	23.7	79.9	1.3	1.3	4.2[d]	35.1	27

[a] NMR spectra were measured at PD 1.5 seconds.
[b] Enrichment ratio was calculated from the relative intensity of C-1′ as 1.0.
[c] Ratio (%) was relative to intensities of ^{13}C-^{13}C spin coupling peaks and of whole peaks.
[d] Value included intensity of ^{13}C-^{13}C spin coupling peaks.
[e] Coupling was not clear.

Table 4. Incorporation of ^{13}C-labeled amino acids into bellenamine.

Carbon	δ	Intensity[a] %	Enrichment ratio[b]			
			[1-^{13}C]Gly (Expt 4)	[2-^{13}C]Gly (Expt 5)	D-[1-^{13}C]β-Lys (Expt 6)	D-[1-^{13}C, *Amide*-^{15}N]-β-LysNH$_2$ (Expt 7)
1	173.9	42.1	1.6	0.05	6.8	5.0[e]
3	48.8	86.6	0.9	0.07[c]	0.9	1.0
1′	46.0	71.1	1.0	1.0	1.0	1.0
6	39.7	83.5	0.8	0.09[c]	0.9	0.9
2	36.9	100	1.0	0.06[c]	0.8[d]	0.9[e]
4	29.8	98.2	0.9	0.06	0.9	0.8
5	23.7	76.6	1.1	0.11[c]	1.1	1.1

[a] NMR spectra were measured at PD 3.0 seconds.
[b] Enrichment ratio was calculated from the relative intensity of C-1′ as 1.0.
[c] Value included intensity of ^{13}C-^{13}C spin coupling peaks.
[d] 10% of ^{13}C-^{13}C spin coupling peaks were observed.
[e] ^{13}C-^{13}C Spin coupling in C-2 ($<10\%$) was observed, but no ^{13}C-^{15}N spin coupling in C-1.

Table 5. Incorporation of ^{15}N-labeled compounds into bellenamine.

Nitrogen	$[^{15}NH_4]_2SO_4$ (Expt 8) δ	$[^{15}NH_4]_2SO_4$ (Expt 8) Intensity %	$[^{15}NH_4]_2SO_4$ +L-Lys (Expt 9)	$[^{15}NH_4]_2SO_4$ +L-$LysNH_2$ (Expt 10)	$[^{15}NH_4]_2SO_4$ +L-Lys, Gly (Expt 11)	$[^{15}N]$Gly (Expt 12)	$[^{15}N]$Gly +L-Lys (Expt 13)	L-[2-*Amino*-^{15}N]-Lys (Expt 14)
			Enrichment ratio[a]					
CONH	−258.4	43.9	2.3	1.3	2.3	1.3	<0.1	<0.1
3-NH_2	−332.3	100	0.3	0.3	<0.1	0.8	0.1	1.0
1'-NH_2	−333.6	55.7	1.6	1.8	0.7	1.4	1.8	<0.1
6-NH_2	−341.9	90.0	0.3	0.3	<0.1	1.1	0.1	<0.1

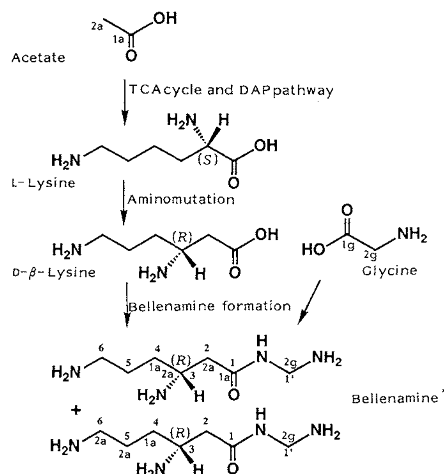
[a] Enrichment ratio was derived from comparing with intensity % of each ^{15}N in bellenamine prepared by addition of $[^{15}NH_4]_2SO_4$.

$[1\text{-}^{13}C]$glycine into C-1 was observed (Table 4). $[^{15}N]$Glycine was incorporated into all nitrogen atoms, but feeding $[^{15}N]$glycine with L-lysine (Expt 13) showed incorporation of the ^{15}N into only 1'-NH_2 (Table 5). The ^{13}C of carbonate salts were not incorporated into C-1' (data not shown).

Ammonium sulfate

Since $(NH_4)_2SO_4$ is a sole nitrogen source in the synthetic medium, the ^{15}N of $[^{15}NH_4]_2SO_4$ was incorporated into all four nitrogen atoms of bellenamine. When L-lysine or L-lysinamide was added, incorporation of the ^{15}N into both CONH and 1'-NH_2 was observed, while, the addition of both L-lysine and glycine increased markedly incorporation of $[^{15}NH_4]_2SO_4$ into CONH (Table 5).

Fig. 1. Biosynthesis of bellenamine.



All nitrogens were derived from $(NH_4)_2SO_4$.
1a and 2a: Carbons from acetate. 1g and 2g: Carbons from glycine.
* A mixture of two kinds of labeled bellenamines was obtained by feeding $[1,2\text{-}^{13}C_2]$acetate, as same as reported in biosynthesis of streptothricin F.[9,10]

D-β-Lysine and its amide

As described above, addition of D-$[1\text{-}^{13}C]$β-lysine markedly improved the production of bellenamine and the ^{13}C was highly incorporated into C-1 of bellenamine (Table 4). Supplement of D-$[1\text{-}^{13}C$, *amide*-$^{15}N]$β-lysinamide did not improve the poductivity, but the $[1\text{-}^{13}C]$ was incorporated into C-1 of bellenamine, while, the [*amide*-^{15}N] was hardly incorporated into CONH. Because ^{13}C - ^{15}N spin coupling at C-1 of the labeled bellenamine was not observed (Table 4).

Discussion

The feeding experiments using lysine analogs and 1'-*N*-acetylbellenamine in the synthetic medium culture suggested that D-β-lysine is a direct intermediate of bellenamine biosynthesis. However, both D-β-lysinamide and 1'-*N*-acetylbellenamide, which were isolated from the synthetic medium culture as minor products,[4] were not direct intermediates. Furthermore, feeding of D-lysine[4] as well as of L-β-lysine repressed the productivity of bellenamine.

The results of the feeding experiments using stable isotope labeled compounds suggested that L-lysine biosynthesized from acetate, was converted into D-β-lysine by an aminomutase different from the known lysine 2,3-aminomutase.[7,8] GOULD *et al.*[9,10] reported that the L-β-lysine moiety in streptothricin F produced by *Streptomyces* was biosynthesized by 2,3-aminomutase from L-lysine which was derived from acetate *via* the TCA cycle and diaminopimelic acid (DAP) pathway. The known lysine 2,3-aminomutase catalized migration of 2(*S*)-NH_2 to 3(*S*)-NH_2 by an intramolecular process.[10] From measurements of enrichment ratios and ^{13}C-^{13}C spin couplings in bellenamines labeled by feeding of [1-^{13}C] and [1,2-$^{13}C_2$]acetates, the acetate incorporation to D-β-lysine was similar to that to L-β-lysine of streptothricin F,[9,10] as shown in Fig. 1. That is, decarboxylation step of *meso*-DAP in the DAP pathway, gives two labeled L-lysines,[9,10] and then a mixture of two kinds of labeled bellenamines having two sets of two ^{13}C at C-1 - C-2 and C-4 - C-3, and having one set of two ^{13}C at C-4 - C-5 and a single ^{13}C at C-6 is formed *via* D-β-lysine.

Most interestingly, C-2 and NH_2 of glycine were efficiently incorporated into C-1′ and 1′-NH_2 of bellenamine, respectively. The nitrogen atom of $(NH_4)_2SO_4$ was introduced into all four nitrogens, and in the case of feeding both L-lysine and glycine, high incorporation of ^{15}N into CONH was observed.

From these results, the biosynthetic pathway of bellenamine is proposed as shown in Fig. 1. Two interesting findings, the presence of new 2,3-aminomutase forming D-β-lysine from L-lysine and the incorporation of glycine into the open-chain aldoaminal structure of bellenamine, are reported here.

Acknowledgment

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

References

1) IKEDA, Y.; S. KONDO, D. IKEDA, K. YOKOSE, H. FURUTANI, T. IKEDA, M. HAMADA, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: D-β-Lysylmethanediamine, a new biogenetic amine produced by a *Streptomyces*. J. Antibiotics 39: 476~478, 1986
2) IKEDA, Y.; D. IKEDA & S. KONDO: Total syntheses of bellenamine and its isomers. J. Antibiotics 45: 1677~1680, 1992
3) IKEDA, R.; H. HOSHINO, Y. IKEDA, S. KONDO & T. TAKEUCHI: Activity and mechanism of bellenamine, a new anti-HIV agent. Abstracts of 40th Annual Meeting of the Society of Japanese Virologists, p. 178, Kobe, Oct. 30, 1992
4) IKEDA, Y.; S. GOMI, M. HAMADA, S. KONDO & T. TAKEUCHI: Production of bellenamine and new metabolites in a synthetic medium. J. Antibiotics 45: 1763~1766, 1992
5) IKEDA, Y.; H. NAGANAWA, S. KONDO & T. TAKEUCHI: Preparation of ^{13}C and ^{15}N labeled bellanamine and its degradation products. J. Antibiotics 45: 1925~1929, 1992
6) UMEZAWA, H. & S. KONDO: Electrophoresis of antibiotics. *In* Methods in Enzymology. Vol. 43. Antibiotics. *Ed.*, J. H. HASH, pp. 279~290, Academic Press, 1975
7) CHIRPICH, T. P.; V. ZAPPIA, R. N. COSTILOW & H. A. BARKER: Lysine 2,3-aminomutase. Purification and properties of a pyridoxal phosphate and *S*-adenosylmethionine-activated enzyme. J. Biol. Chem. 245: 1778~1789, 1970
8) ABERHART, D. J.; S. J. GOULD, H.-J. LIN, T. K. THIRUVENGADAM & B. H. WEILLER: Stereochemistry of lysine 2,3-aminomutase isolated from *Clostridium subterminale* strain SB4. J. Am. Chem. Soc. 105: 5461~5470, 1983
9) GOULD, S. J.; K. J. MARTINKUS & C.-H. TANN: Biosynthesis of streptothricin F. 1. Observing the interaction of primary and secondary metabolism with [1,2-$^{13}C_2$]acetate. J. Am. Chem. Soc. 103: 2871~2872, 1981
10) THIRUVENGADAM, T. K.; S. J. GOULD, D. J. ABERHART & H.-J. LIN: Biosynthesis of streptothricin F. 5. Formation of β-lysine by *Streptomyces* L-1689-23. J. Am. Chem. Soc. 105: 5470~5476, 1983