Biosynthesis of Enduracidin: Origin of Enduracididine and Other Amino Acids

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The biosynthetic origin of the amino acid moieties of enduracidin was investigated by feeding experiments with labeled compounds. Results of the incorporation and the distribution of radioactivity into the antibiotic revealed that glycine, L-serine, L-threonine, L-alanine, L-aspartic acid, L-ornithine and L-citrulline were incorporated into the corresponding amino acid moieties. Unique amino acids, enduracididine and its isomer with an imidazolidine ring, were derived from L-arginine, but not histidine. K₁ (4-hydroxyphenylglycine) and K₂ (3,5-dichloro-K₁) moieties were derived from L-tyrosine. ³⁶Cl-Sodium chloride was incorporated into the antibiotic in the early stage of fermentation.

Enduracidin, a unique peptide antibiotic^{1~3)} produced by *Streptomyces fungicidicus* No. B-5477, shows a strong bactericidal activity against gram-positive bacteria and a growth promoting effect on animals.⁴⁾ Mizuno *et al.*^{5,6)} have confirmed the chemical structure, which is composed of 12 kinds of amino acids including 17 amino acid moieties and an unsaturated fatty acid moiety as a side chain (Fig. 1). Among these amino acids, enduracididine ($\alpha(S)$ -amino- β -4(R)-(2-iminoimidazolidinyl)-propionic acid, represented as Y₁),⁷⁾ alloenduracididine ($\alpha(R)$ -amino- β -4(R)-(2-iminoimidazolidinyl)-propionic acid, represented as Y₂)⁷⁾ (these are generically represented as Y in this paper) and K₂ (3,5-dichloro-4-hydroxyphenylglycine)²⁾ are new amino acids, and K₁ (4-hydroxyphenylglycine) is rare in natural products. The aim of this investigation is to elucidate the biosynthetic origin of these four amino acids in enduracidin by the feeding experiment using radioactive compounds.

MATERIALS AND METHODS

Microorganism. *Streptomyces fungicidicus* No. B-5477, strain 71M-141 was used throughout this work.

Media and culture conditions. The seed and fermentation media are shown in Table I. A spore suspension (5×10^9 cells/ml) was prepared from a slant culture grown on glucose asparagine agar at 28°C for 10 days. An aliquot (0.5 ml) of the suspension was inoculated into 200-ml Erlenmeyer flasks containing 40 ml of seed medium and incubated at 28°C for 48 hr. One milliliter of the resultant culture was transferred to the 40-ml fermentation medium. Fermentation was carried out at 30°C for 6 days, on a rotary shaker (Gyrotory Model G25, New Brunswick Scientific Co., 200 rpm) with a ¹⁴CO₂-trapping apparatus. The radioactive compounds (10 μ Ci) were added to the culture at zero fermentation time unless otherwise mentioned.

Assay of enduracidin. The amount of enduracidin was determined by the paper disk method using *Sarcina variabilis* IFO 3067 as a test organism.¹⁾

Analysis of labeled enduracidin. The labeled enduracidin was isolated from the culture broth by the method of Sugita *et al.*⁸⁾ A mixture of radioactive enduracidin (0.2 to

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FIG. 1. Structures of Enduracidins A and B.

TABLE I. MEDIA FOR FERMENTATION

<i>Seed medium;</i>	
Corn starch	3.0%
Corn steep liquor	3.5
CaCO ₃	2.0
<i>Fermentation medium;</i>	
<i>Complex medium</i>	
Glucose	4.0%
Corn starch	2.0
Corn steep liquor	2.0
Corn gluten meal	3.0
NH ₄ Cl	0.5
NaCl	1.5
CaCO ₃	1.5
<i>Chemically defined medium</i>	
Corn starch	3.0%
Sodium glutamate	0.7
NaNO ₃	0.5
(NH ₄) ₂ SO ₄	0.1
NaCl	1.0
K ₂ HPO ₄	0.1
MnSO ₄ ·4~6H ₂ O	0.0025
FeSO ₄ ·7H ₂ O	0.0025
ZnSO ₄ ·7H ₂ O	0.0025
MgSO ₄ ·7H ₂ O	0.0025
CuCl ₂ ·2H ₂ O	0.0025
CaCO ₃	1.0

1.0 mg) and non-labeled enduracidin (5 mg) was hydrolyzed with 0.5 ml of 6N HCl at 110°C for 18 hr in a sealed tube. The amino acids in the hydrolysate were separated by two-dimensional thin-layer chromatography

on cellulose (Avicel SF) with a mixture of *n*-butanol-acetic acid-water (2:1:1, v/v) and a mixture of phenol-0.5N ammonia (80:20, v/v) as the first and the second dimensional solvents, respectively, and were detected with 0.02% ninhydrin solution. Each radioactive spot of amino acid on the thin-layer chromatogram was scraped off from the plate and mixed with a scintillation cocktail for measurement of radioactivity. The incorporation rate of radioactivity was represented by the percentage of the total radioactivity of labeled compound added. Distribution of radioactivity of individual amino acid moieties in enduracidin was represented as a percentage of the total radioactivity of all amino acid moieties in enduracidin.

Measurement of radioactivity. Radioactivity was measured with a liquid scintillation spectrometer (Aloka, model LSC-502) using an external standard to measure efficiency. An aqueous sample solution (0.1 ml) was mixed with 10 ml of scintillation cocktail containing 12 g DPO (2,5-diphenyloxazole), 0.3 g POPOP [1,4-bis(5-phenyloxazolyl)benzene], 100 g naphthalene, 135 ml toluene, 45 ml methanol, and 720 ml dioxane.

Radiochemicals. ¹⁴C-Labeled compounds and ³⁶Cl-sodium chloride produced by Radiochemical Centre Amersham (England) were used.

RESULTS

Incorporation of various labeled compounds into enduracidin

A typical thin-layer chromatogram of

FIG. 2. Thin-layer Chromatogram of ^{14}C -Enduracidin Labeled with L-[Guanido- ^{14}C]arginine. Radioactivity was monitored with a radiochromatogram scanner (Aloka TM-2). Enduracidin was detected with 0.02% ninhydrin solution.

TABLE II. INCORPORATION OF RADIOACTIVITY INTO ENDURACIDIN

Compound	Compound fed		Enduracidin produced			
	Specific radioactivity ($\mu\text{Ci}/\mu\text{mol}$)	Total radioactivity (μCi)	Yield (μmol)	Specific radioactivity ($\mu\text{Ci}/\mu\text{mol}$)	Incorporation rate (%)	Dilution factor ^a
[U- ^{14}C]Glycine	10	10	4.58	30.3×10^{-3}	1.39	330
L-[U- ^{14}C]Alanine	10	10	1.69	6.5	0.11	1540
L-[U- ^{14}C]Serine	10	10	1.74	20.1	0.35	500
L-[U- ^{14}C]Threonine	10	10	3.50	164.9	5.77	60
L-[U- ^{14}C]Aspartic acid	10	125	6.44	66.0	0.34	150
L-[Carbamoyl- ^{14}C]citrulline	10	10	1.84	207.1	3.81	50
L-[U- ^{14}C]Ornithine	10	10	1.19	321.8	3.83	30
L-[U- ^{14}C]Arginine	10	10	2.16	482.4	10.42	20
L-[Guanido- ^{14}C]arginine	10	10	1.84	125.5	2.31	80
L-[Ring-2- ^{14}C]histidine	10	10	2.65	3.0	0.08	3330
L-[U- ^{14}C]Tyrosine	10	10	1.10	371.8	4.09	30
L-[U- ^{14}C]Phenylalanine	10	10	5.08	2.0	0.10	5000
L-[U- ^{14}C]Glutamic acid	7	10	2.33	6.4	0.15	1090
[U- ^{14}C]Starch	27.2 ^b	20	5.42	1.1 ^b	0.07	24730
D-[U- ^{14}C]Glucose	10	10	8.50	3.5	0.30	2860
[U- ^{14}C]Acetic acid	10	10	0.60	31.7	0.19	320
[1- ^{14}C]Propionic acid	10	10	7.10	2.0	0.14	5000
n-[1- ^{14}C]Butyric acid	10	10	3.20	2.8	0.09	3570
[U- ^{14}C]Oleic acid	10	10	3.70	4.9	0.18	2040

Fermentation was carried out at 30°C for 6 days using the complex medium.

^a Specific radioactivity of ^{14}C -labeled compound fed ($\mu\text{Ci}/\mu\text{mol}$)/specific radioactivity of enduracidin produced ($\mu\text{Ci}/\mu\text{mol}$).

^b Specific radioactivity represents as $\mu\text{Ci}/\text{mg}$.

radioactive enduracidin isolated from the culture broth is shown in Fig. 2 and the results of the incorporation of various labeled com-

pounds into enduracidin are in Table II. Among the radioactive amino acids tested, glycine, threonine, tyrosine, and ornithine

TABLE III. DISTRIBUTION OF RADIOACTIVITY TO THE AMINO ACIDS OBTAINED BY HYDROLYSIS

Compound fed	Gly	Ala	Ser	Thr	Asp	Cit	Orn	Y ^a	K ₁	K ₂ (%)
[U- ¹⁴ C]Glycine	38.4	0	52.9	3.9	4.7	0	<1	0	0	0
L-[U- ¹⁴ C]Alanine	1.0	17.9	1.6	19.6	8.0	5.3	12.8	32.0	1.3	<1
L-[U- ¹⁴ C]Serine	19.3	0	60.0	20.0	0	0	<1	0	0	0
L-[U- ¹⁴ C]Threonine	3.4	1.0	1.8	91.1	<1	<1	<1	0	0	0
L-[U- ¹⁴ C]Aspartic acid	2.0	<1	1.1	45.6	14.4	6.4	6.8	22.9	0	0
L-[U- ¹⁴ C]Ornithine	0	0	0	0	0	26.8	20.0	53.5	0	0
L-[Carbamoyl- ¹⁴ C]citrulline	0	0	0	3.0	0	25.0	5.3	66.7	0	0
L-[U- ¹⁴ C]Arginine	0	0	0	1.9	<1	17.9	18.5	60.0	<1	<1
L-[Guanido- ¹⁴ C]arginine	<1	<1	<1	<1	<1	12.9	1.8	83.2	<1	0
L-[U- ¹⁴ C]Tyrosine	0	0	0	0	0	0	0	<1	81.8	17.9
[³⁶ Cl]Sodium chloride	<1	<1	<1	0	<1	<1	<1	<1	<1	97.9

^a Enduracididine and alloenduracididine.

TABLE IV. INCORPORATION OF ³⁶Cl-SODIUM CHLORIDE INTO ENDURACIDIN

Exp.	³⁶ Cl-NaCl added		Enduracidin produced			
	Specific radioactivity (μCi/μmol)	Time (hr)	Yield (μmol)	Specific radioactivity (μCi/μmol)	Incorporation rate (%)	Dilution factor
I ^a	0.001	0	0.05	74.1 × 10 ⁻³	0.08	<1
II ^b	193.6	0	0.20	220.6 × 10 ⁻³	0.90	880
	193.6	24	0.27	237.0	1.28	820
	193.6	48	0.29	97.2	0.56	1990
	193.6	72	0.20	14.7	0.06	13170

Fermentation was carried out at 30°C for 6 days using the chemically defined medium.

³⁶Cl-Sodium chloride (5 μCi) was added to the medium.

^a The chemically defined medium containing 1% of NaCl (6840 μmol) was used.

^b Sodium chloride was omitted from the medium.

cycle-membered amino acids were efficiently incorporated (1.4 to 10.4%), but alanine, serine, aspartic acid, histidine, and phenylalanine were hardly incorporated (0.1 to 0.4%). Organic acids, fatty acids, and carbohydrates were also barely incorporated.

Distribution of radioactivity among amino acid moieties

The distribution of radioactivity among the amino acids obtained from acid hydrolysis of ¹⁴C-enduracidin is shown in Table III. ¹⁴C-Ornithine, citrulline, and arginine were incorporated mainly into the Y moiety and parts of them directly into the ornithine and citrulline moieties. ¹⁴C-Threonine was incorporated

predominantly into the threonine moiety. About 50 and 40% of ¹⁴C-glycine incorporated into enduracidin was distributed to the serine and glycine moieties, respectively, and 60% of ¹⁴C-serine to the serine moiety and 40% of the radioactivity to the glycine and threonine moieties. ¹⁴C-Tyrosine was specifically incorporated into the K₁ and K₂ moieties and the radioactivity distributed to them was 82 and 18%, respectively. About half the radioactivity of ¹⁴C-aspartic acid incorporated into the antibiotic was distributed to the threonine moiety and the remaining activity was randomized to other amino acids. ¹⁴C-Alanine was also distributed widely to the Y, threonine, and other amino acid moieties.

Incorporation of ^{36}Cl -sodium chloride into enduracidin

As shown in Table IV, when ^{36}Cl -sodium chloride was added to the fermentation medium in the presence of sodium chloride, it was hardly incorporated into the antibiotic, but when it was added to the chloride-free chemically defined medium, radioactive sodium chloride was incorporated into enduracidin at the growth phase. The incorporation rate rapidly decreased at the production phase. Almost all incorporated radioactive chloride was distributed to the K_2 moiety (Table III).

DISCUSSION

These studies on the biosynthetic origin of the amino acid moieties of enduracidin indicated that radioactive amino acids such as glycine, alanine, serine, threonine, aspartic acid, citrulline, and ornithine supplied exogenously were incorporated into the corresponding amino acid moieties directly or *via* their metabolic pathways. We concluded that these common amino acid moieties of enduracidin were biosynthesized through well known metabolic pathways of amino acids as follows: 1) Glycine-serine-threonine pathway; serine $\rightleftharpoons$ glycine $\rightleftharpoons$ threonine, 2) Aspartate pathway; threonine $\leftarrow$ aspartate $\leftarrow$ tri-carboxylic acid (TCA) cycle $\rightarrow$ glutamate $\rightarrow$ ornithine $\rightarrow$ citrulline, 3) Alanine pathway; alanine $\rightarrow$ pyruvate $\rightarrow$ acetyl-CoA $\rightarrow$ TCA cycle $\rightarrow$ the same metabolic pathway as that of aspartate.

As shown in Fig. 1, enduracidin has six kinds of D-amino acids: D-alanine, D-serine, D-ornithine, D- K_1 , D- Y_2 , and D-allothreonine. Although the biosynthetic mechanisms for these moieties are unclear, the D-amino acid moieties are probably derived from L-isomers by an enzymatic racemization⁹ or epimerization^{10,11} during the biosynthesis of enduracidin, as happens in the case of other known peptide antibiotics.¹²

It is assumed that the biosynthetic origin of enduracididine and its isomer (Y) is arginine or histidine since it has an imidazolidine ring.

FIG. 3. Proposed Biosynthetic Pathway of Y Moiety (Enduracididine and Alloenduracididine).

The radioisotopic experiments revealed that arginine, ornithine, and citrulline were efficiently incorporated into the Y moiety, but histidine was not incorporated (Table II). This finding suggests that the imidazolidine ring of Y moiety is formed by the cyclization between the carbon at the γ -position and the amino group of the guanido moiety of arginine. The proposed biosynthetic pathway of enduracididine and its isomer (Y) is shown in Fig. 3.

Two kinds of aromatic amino acid moieties, K_1 and K_2 , were derived from tyrosine, but not from phenylalanine (Table II). Tyrosine is probably metabolized to 4-hydroxyphenylglyoxalic acid through a similar pathway as that in nocardicin biosynthesis,¹³ and converted to 4-hydroxyphenylglycine (K_1) by a transaminase.

The chlorination of the aromatic ring might occur at an earlier intermediate, probably a metabolite of tyrosine, prior to the formation of the peptide, because the radioactive chloride was efficiently incorporated into enduracidin only at the early stage (zero to 48 hr) of fermentation at which the antibiotic was not yet being produced.

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REFERENCES

- 1) E. Higashide, K. Hatano, M. Shibata and K. Nakazawa, *J. Antibiot.*, **21**, 126 (1968).
 - 2) M. Asai, M. Muroi, N. Sugita, H. Kawashima, K. Mizuno and A. Miyake, *J. Antibiot.*, **21**, 138 (1968).
 - 3) K. Tsuchiya, M. Kondo, T. Oishi and T. Yamazaki, *J. Antibiot.*, **21**, 147 (1968).
 - 4) K. Takeda, T. Kobayashi, T. Kanekiyo and Y. Hamada, *Nippon Yoton Kenshi*, **11**, 280 (1974).
 - 5) K. Mizuno, M. Asai, S. Horii, M. Hori, H. Iwasaki and J. Ueyanagi, *Antimicrob. Agent Chemother.*, **1970**, 6 (1971).
 - 6) H. Iwasaki, S. Horii, M. Asai, K. Mizuno, J. Ueyanagi and A. Miyake, *Chem. Pharm. Bull.*, **21**, 1184 (1973).
 - 7) S. Horii and Y. Kameda, *J. Antibiot.*, **21**, 665 (1968).
 - 8) N. Sugita, K. Naito, M. Asai, T. Suzuki, E. Higashide and K. Mizuno, *J. Takeda Res. Lab.*, **31**, 313 (1972).
 - 9) M. Yamada and K. Kurahashi, *J. Biochem.*, **63**, 59 (1968).
 - 10) M. Bodanszky and D. Perlman, *Nature*, **218**, 291 (1968).
 - 11) M. Bodanszky and D. Perlman, *Science*, **163**, 352 (1969).
 - 12) K. Kurahashi, "Antibiotics IV, Biosynthesis," ed. by J. W. Corcoran, Springer-Verlag, 1981, pp. 325.
 - 13) J. Hosoda, N. Tani, T. Konomi, S. Ohsawa, H. Aoki and H. Imanaka, *Agric. Biol. Chem.*, **41**, 2007 (1977).
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