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Studies on the Biosynthesis of Blasticidin S

Part I. Precursors of Blasticidin S Biosynthesis

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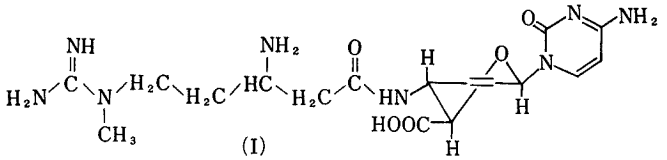
Biosynthesis of blasticidin S by the producing organism *Streptomyces griseochromogenes* has been investigated with the use of ^{14}C-labeled compounds. Studies on the incorporation of the labeled compounds demonstrated that blasticidin S was biosynthesized from D-glucose, cytosine, L-arginine and L-methionine as precursors.

Blasticidins (I)[1~5] is an antibiotic produced by *Streptomyces griseochromogenes* and the structure has been elucidated including the absolute configuration as shown in formula 1.

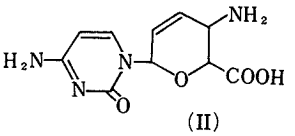
It consists of a pyrimidine nucleoside designated cytosinine (III) and a β-amino acid, blastidic acid (III) as structural components.

From the biosynthetic point of view, I is a compound of particular interest, since it contains a peculiar nucleoside II and an unusual β-amino acid III.

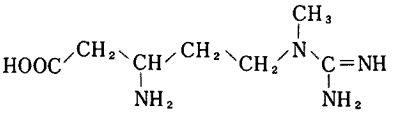
Hitherto, several paper have reported on the biosynthesis of nucleosidic antibiotics such as angustmycin A (decoyinine)[6] and angustmycin C (psicofuranine),[7] however, these studies were related to the biosynthesis of the purine nucleosides, therefore, the analogous mechanisms could not be regarded to be operating in the case of a pyrimidine nucloside. Although, the biosynthetic mechanisms of



(I)



(II)



(III)

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1) N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara *Tetrahedron Letters*, **1965**, 1405.
2) N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara, *ibid.*, **1965**, 1411.
3) N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara, *Agr. Biol. Chem.*, **30**, 126 (1966).
4) N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara, *ibid.*, **30**, 132 (1966).
5) H. Yonehara and N. Ōtake, *Tetrahedron Letters*, **1966**, 3785.
6) B. M. Chassy, T. Sugimori and R. J. Suhadolnik, *Biochim. Biophy. Acta*, **130**, 12 (1966).
7) T. Sugimori and R. J. Suhadolnik, *J. Am. Chem, Soc.*, **87**, 1136 (1965).

cytidine formation were well established, its structure is entirely different from II. Furthermore, III may be called δ-N-methyl-β-arginine, a β-amino acid found for the first time in the natural product field, so the studies of its origin seem significant.

A preliminary communication of this work has been published in a previous paper,[8] and this report concerns the details of the experiments using ^{14}C-labeled compounds to account for the biosynthetic aspects of I.

MATERIALS AND METHODS

Medium and cultures

An improved strain of *Streptomyces griseochromogenes* No. 1 was used through these studies.

Medium-1. The seed medium contained molasses 1 g, starch 1 g, meat extract 1 g and peptone 1 g in 100 ml of distilled water and the pH was adjusted to 7.2 before sterilization. The cultural medium was inoculated and incubated at 30°C on a reciprocal shaker for 48 hr and was used as an inoculum for Medium-2.

Medium-2. The synthetic medium used for the production of I contained the following: (A) sucrose 100 g and glucose 5 g in 800 ml of distilled water; (B) $(NH_4)_2HPO_4$ 10 g and KCl 3 g in 100 ml of distilled water; (C) $CaCl_2.2H_2O$ 3 g in 50 ml of distilled water; (D) $MgSO_4.7H_2O$ 2 g, $FeSO_4.7H_2O$ 40 mg, $ZnSO_4.7H_2O$ 40 mg in 50 ml of distilled water. Part A, B, C and D were sterilized separately and combined after cooling to avoid precipitation of the mineral complex. The pH of the mixed solution became 6.5.

Medium-3. When ^{14}C-glucose was used as precursor, the medium consisted of sucrose 5 g, soybean meal 2 g and NaCl 0.5 g in 100 ml of distilled water and the pH was adjusted to 6.5 before sterilization.

Preparation of ^{14}C labeled-I

A 2 ml of the inoculum described above was introduced into a 500 ml Erlenmyer flask containing 100 ml of medium-2 and incubated on a rotary shaker at 30°C. After 48 hr, the ^{14}C-labeled compounds were added aseptically to the cultures and the incubation was continued. The time course of the fermentation is presented in Fig. 1.

The yield of I produced under this condition was evaluated by the microbiological assay of the cylinder-plate agar diffusion method with the use of *Bacillus cereus* as test organism,[9] Seventy two hours after inoculation, an adequate amount of non-labeled I was added as a carrier and the fermentation broth was filtered. The filtrates were passed through a column of Amberlite IRA-410 (OH^- type) and washed successively with distilled water. The effluents and washings were directly adsorbed on a column of Dowex 50W-X1 (H^+ type) and washed with water successively followed with 5% pyridine, then the column was eluted with 1.2% ammoniacal water. Appropriate fractions showing UV absorption and ninhydrin reaction were combined and the solution was evaporated *in vacuo* as quickly as possible at a bath temperature under 45°C. The residue was redissolved in a small volume of CO_2 free redistilled water and the solution was allowed to stand overnight in a refrigerator to give crystals fo radioactive-I.

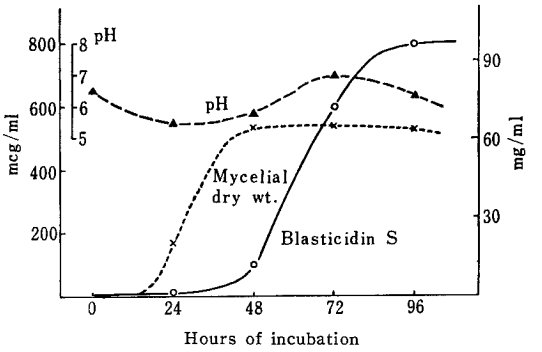

FIG. 1. Fermentation Process of *Streptomyces griseochromogenes*.

Degradation of radioactive-I and isolation of the degradation products

Hydrolysis of I was carried out in 3 N sulfuric acid solution at 90°C for 48 hr under a continuous flow of nitrogen gas which was free from CO_2. The evolved CO_2 was caught as $BaCO_3$ by passing through a saturated solution of $Ba(OH)_2$. The precipitated $BaCO_3$ was filtered on a glass filter by suction and washed subsequently with boiled distilled water, ethanol, ether in order and dried under reduced pressure.

After completion of the hydrolysis, the reaction mixture was continuously extracted with ether for 15 hr. After dehydration of the ether solution over anhydrous sodium sulfate, ether was removed by

8) H. Seto, I. Yamaguchi, N. Ōtake and H. Yonehara, *Tetrahedron Letters*, **1966**, 3793.

9) H. Iizuka, *Agr. Biol. Chem.*, **29**, 77 (1965).

evaporation under atmospheric pressure to produce a syrup, which was treated with thiosemicarbazide hydrochloride to afford the thiosemicarbazone of levulinic acid.

The aqueous layer from the ether extraction was adjusted to pH 6.0 with 0.5 N $Ba(OH)_2$ solution and the resultant precipitate of $BaSO_4$ was removed by centrifugation. The supernatant was adsorbed on a column of Amberlite IRA-410 (OH^- type) and washed thoroughly with distilled water and the column was eluted with 0.5 N aqueous CH_3COOH to afford crystals of II in substantially pure form, which was recrystallized from aqueous acetone. On the other hand, the effluent was fractionated and the appropriate fractions of pH above 7.5 showing ninhydrin reactions were combined and adsorbed on a column of Amberlite IRC-50 (H^+ type). After being washed with distilled water, the column was eluted with 0.5 N HCl and the appropriate fractions positive to ninhydrin test were combined and decolorized with activated charcoal. The solution was evaporated *in vacuo* to afford crystalline needles of III as a dihydrochloride which was recrystallized from hot ethanol.

Finally, the effluents of pH below 7.5 from the column of Amberlite IRA-410 showing UV absorption were adsorbed on a column of Dowex 50W-X1 (H^+ type) and washed with distilled water. Then the column was eluted with 5% pyridine and the eluate was concentrated *in vacuo* to give crystals of cytosine which were recrystallized from hot water. These degradation products were recrystallized until their specific radioactivity became constant.

Degradation of III

III was degraded in 0.5 N $Ba(OH)_2$ solution to

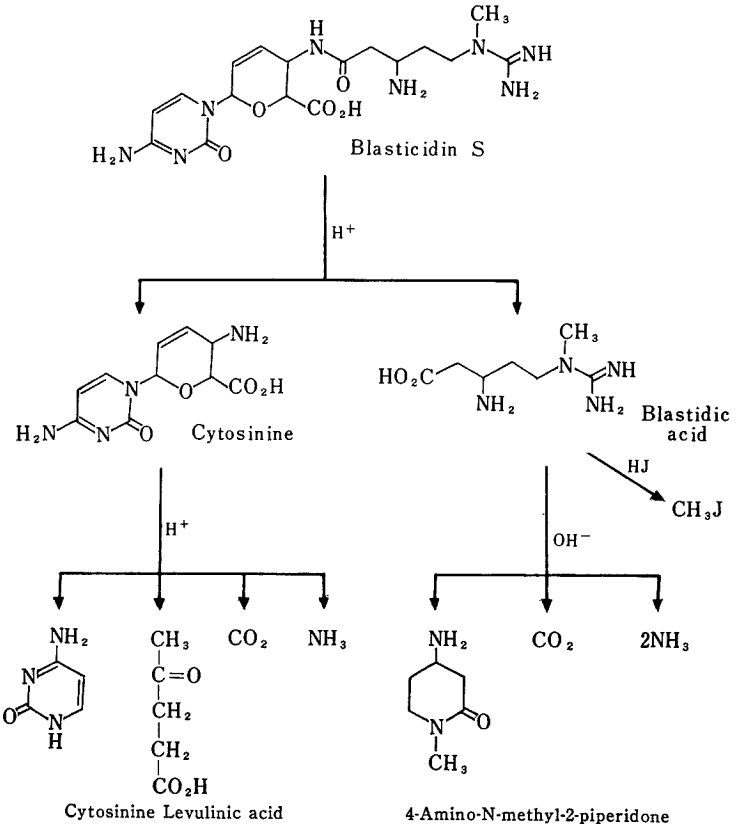

FIG. 2. Degradation Scheme of Radioactive Blasticidin S.

afford CO_2 and 4-amino-N-methyl-2-piperidone[10] under a stream of CO_2 free nitrogen gas. CO_2 was precipitated as $BaCO_3$ and purified as mentioned above. 4-Amino-N-methyl-2-piperidone was purified by vacuum distillation. N-Methyl group of III was converted into CH_3J by the action of HJ, which was absorbed in toluene and the radioactivity was directly measured by scintillation counting. The degradation scheme of I is summarized in Fig. 2.

Isolation and identification of L-ornithine in the fermentation broths

A filtered broth of *St. griseochromogenes* cultured under the same condition was passed through a column of active carbon and washed with distilled water. Effluent and washings were adsorbed on a column of IRA-410 (OH^- type). Then the column was eluted with 0.5 N CH_3COOH and the eluate was concentrated *in vacuo*. The residue was dissolved in distilled water and adsorbed on Dowex 50W-X1 (H^+ type). The column was washed with 5% pyridine and then eluted with 1.2% ammonia. Appropriate fractions positive to ninhydrin test were collected and concentrated to afford a crystalline mass which was applied as a sample for paper chromatographic identification.

Radioactivity measurement

Radioactivities were measured by a Packard liquid scintillation spectrometer in dioxane-naphthalene solution (naphthalene 1 g, PPO 100 mg and POPOP 2.5 mg in 10 ml dioxane). $BaCO_3$ was suspended in toluene scintillation solution (PPO 40 mg and POPOP 1 mg in 10 ml toluene) by the aid of Cab-O-Sil (400 mg).

^{14}C-Labeled compounds used

The following ^{14}C-labeled compounds were used in this work. D-Glucose-U-^{14}C, D-glucose-1-^{14}C, D-glucose-6-^{14}C, L-arginine-U-^{14}C, glycine-U-^{14}C, L-aspartic acid-U-^{14}C, L-alanine-U-^{14}C, L-glutamic acid-U-^{14}C and sodium acetate-U-^{14}C (from Daiichi Chemical); L-methionine-methyl-^{14}C, cytosine-2-^{14}C and cytidine-U-^{14}C (from Radio Chemical Center); and β-alanine-3-^{14}C, (from New England Nuclear).

RESULTS AND DISCUSSION

Incorporation of various compounds into blasticidin S

The incorporation ratio of ^{14}C-labeled compounds into the antibiotic is presented in Table I.

Among the compounds tested, D-glucose-U-^{14}C, D-glucose-1-^{14}C, D-glucose-6-^{14}C, cytosine-2-^{14}C, cytidine-U-^{14}C, L-methionine-methyl-^{14}C, L-arginine-U-^{14}C and L-arginine-guanidino-^{14}C were incorporated very effectively into I. But other amino acids and fatty acid such as glycine, L-alanine, β-alanine, L-aspartic acid and acetic acid were essentially not incorporated. The minor incorporation ratio observed in the case of L-arginine-U-^{14}C as compared with that of L-arginine-guanidino-^{14}C might be ascribed to the transamidination between labeled arginine and unlabeled ornithine which was produced during fermentation.

Distribution of radioactivity in the degradation products of I

In order to establish the distribution of radioactivity in the labeled antibiotic, I was degraded according to the scheme as shown in Fig. 2. The results are presented in Table II.

On the basis of these experimental results

TABLE I. INCORPORATION RATIO OF ^{14}C-COMPOUNDS INTO BLASTICIDIN S

	(%)		(%)
D-Glucose-(U)-^{14}C	3.7	L-Arginine-(U)-^{14}C	30.3
D-Glucose-1-^{14}C	4.0	*L-Aspartic acid-(U)-^{14}C	0.5
D-Glucose-6-^{14}C	4.9	β-Alanine-1-^{14}C	0.6
Cytosine-2-^{14}C	95.1	Acetic acid-(U)-^{14}C	0.5
Cytidine-(U)-^{14}C	15.3	Glycine-(U)-^{14}C	1.1
L-Methionine-(methyl)-^{14}C	38.3	L-Alanine-(U)-^{14}C	0.5
L-Arginine-(guanidino)-^{14}C	51.2		

* Unlabeled cytosine was added simultaneously.

10) T. Endō, N. Ōtake, S. Takeuchi and H. Yonehara, *J. Antibiotics*, **17A**, 172 (1964).

TABLE II. DISTRIBUTION OF RADIOACTIVITY IN THE DEGRADATION PRODUCTS OF BLASTICIDIN S

Compound \ Precursor	Cytosine-2-^{14}C		Cytidine-(U)-^{14}C		D-Glucose-(U)-^{14}C		D-Glucose-1-^{14}C		D-Glucose-6-^{14}C		L-Methionine-(methyl)-^{14}C		L-Arginine-(U)-^{14}C		L-Arginine-(guanidino)-^{14}C	
	dpm/μMol	%	dpm/μMol	%	dpm/μMol	%	dpm/μMol	%	dpm/μMol	%	dpm/μMol	%	dpm/μMol	%	dpm/μMol	%
Blasticidin S	4909	100	853	100	335	100	350	100	831	100	1574	100	3019	100	2691	100
Blastidic acid 2HCl	0	0	5	0.5	57	16.9	52	14.8	221	26.6	1547	98.3	2958	98.0	2634	97.9
CO_2	—	—	—	—	—	—	—	—	—	—	—	—	744	24.5	2677	0
4-Amino-N-methyl-2-piperidone	—	—	—	—	—	—	—	—	—	—	—	—	2097	69.2	0	0
CH_3J	—	—	—	—	—	—	—	—	—	—	1599	101.6	0	0	—	—
Cytosinine	4768	97.1	838	98.2	280	83.6	312	89.2	621	74.6	3	0.1	45	1.5	73	2.7
Cytosine	4711	96.1	835	97.9	76	22.8	76	21.6	198	23.8	3	0.1	35	1.1	71	2.6
Levulinic acid thiosemicarbazone	0	0	2	0.2	174	52.0	221	62.9	0	0	—	—	—	—	—	—
CO_2	0	0	3	0.3	38	11.3	23	6.7	380	45.7	—	—	—	—	—	—

the following conclusions are confirmed:

i) Cytosine is incorporated intact into the cytosine part of I in high yield and randomization does not take place.

ii) When cytidine is supplemented as precursor, almost all of radioactivity locates in the cytosine nucleus of I, but only negligible amounts in the sugar portion, indicating the cleavage occurred in the glycosidic linkage. A similar result was also reported in the case of the biosynthesis of angustmycin C.[7]

iii) When I is labeled with glucose as precursor, most of the radioactivity locates in the sugar portion, but in this case, a considerable randomization is observed. The reason for this remarkable randomization is explicable based on the incorporation of ^{14}C-carbamyl phosphate[11] formed from ^{14}C-labeled glucose in the course of the fermentation. Indeed, the C_2 of cytosine nucleus and guanidino carbon of III are radioactive.

iv) It is of particular interest to consider about the formation of levulinic acid from II, the mechanism involving a disproportion reaction as shown in Fig. 3 has been proposed by Ōtake *et al.*[3] In order to determine the validity of this mechanism, the ratio of levulinic acid and CO_2 produced was reinvestigated with the aid of specifically labeled D-glucose as precursor. It could theoretically be expected that, if the randomization in sugar moiety does not take place at all, the ratio of levulinic acid and CO_2 must be 5 : 1 with the use of glucose-U-^{14}C, 1 : 0 of glucose-1-^{14}C and 0 : 1 glucose-6-^{14}C respectively. The results presented in Table II show good agreement between the experimental and theoretical values.

v) When methionine-methyl-^{14}C is used as precursor, almost all of the radioactivity is located in III with negligible amounts in II. Further degradation of III with HJ demonstrates that the methyl group of III is derived from methyl group of L-methionine.

The introduction of a methyl group into the guanidino nitrogen of III is probably comparable to creatine formation[12] from guanidino acetic acid.

vi) When I is labeled with L-arginine-guanidino-^{14}C as precursor, almost all of the radioactivity is observed in the CO_2 derived from the guanidino-carbon of III after the hydrolysis with $Ba(OH)_2$. However, when L-arginine-U-^{14}C is used as precursor, all of the radioactivity is located in carbon skeleton of III but negligible amounts in N-methyl portion. The radioactivity ratio of 4-amino-N-methyl-2-piperidone and CO_2 is found to be 3 : 1 experimentally, nevertheless, supposing that L-arginine-U-^{14}C is incorporated intact, the

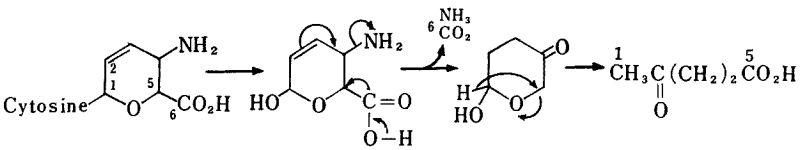

FIG. 3. Mechanism of Levulinic Acid Formation.

radioactive ratio must be 5 : 1 as theoretically expected. This deviation of experimental value from theoretical value is ascribable to the transamidination between L-arginine-U-^{14}C and L-ornithine produced in the fermentation broth.

Indeed, the presence of a significant dose of radioactive L-ornithine was confirmed in the cultures. The data clearly show that L-arginine, except its α-amino group, is a direct

11) P. Bernfeld, "Biogenesis of Natural Compounds," Pergamon Press, 1963, p. 56.

12) H. Borsook and J. W. Dubnoff, *J. Biol. Chem.*, **132**, 559 (1940).

precursor of III into which the carbon skeleton may be incorporated intact from L-arginine.

To-date, little information is available concerning the biosynthesis of β-amino acids, for example, β-lysine[13] and isotyrosine[14] were reported to be biosynthesized from their corresponding α-amino acids, nevertheless, the details of the mechanisms are not certained.

Costilow and his co-workers[6] have reported that with the use of a cell free system of an unidentified strain of *Clostridium*, β-lysine was found out to be derived from L-lysine through an interconvertible reaction which needed a catalytic amount of α-ketoglutarate or pyridoxal phosphate.

Details of the mechanism which is involved in the transfer of amino group from α to β position remain uncertain, however, one of the possible routes is presumed to be through double bond formation.

In conclusion, the evidence so far presented lends support to a scheme as shown in Fig. 4 depicting the direct incorporation of precursors in blasticidin S biosynthesis.

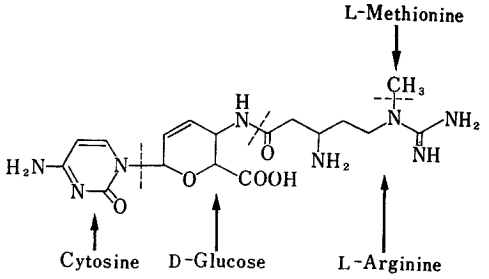

FIG. 4. Precursors of Blasticidin S Biosynthesis.

13) R. N. Costilow, O. M. Rochovansky and H. A. Barker, *J. Biol. Chem.*, **241**, 1573 (1966).
14) G. Roncari, Z. Kurylo-Borowska and L. C. Craig, *Biochem.*, **5**, 2153 (1966).