OF4949, NEW INHIBITORS OF AMINOPEPTIDASE B

III. BIOSYNTHESIS

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To elevate production of OF4949 by *Penicillium rugulosum* OF4949 and to elucidate the pathway of its biosynthesis, mutants were selected on the basis of their resistance to growth inhibition by phenylalanine analogs. A mutant resistant to *m*-fluorophenylalanine, strain No. M414, had 3-fold the production of the parent. In a study of the biosynthesis of OF4949-**I** and **II**, several ^{14}C-labeled compounds were examined as possible precursors of OF4949. L-[^{14}C]Tyrosine and L-[^{14}C]asparagine were incorporated efficiently. Most of the radioactivity of L-[^{14}C]tyrosine was found in the 4-methylisodityrosine ($\mathbf{B_2}$) or isodityrosine ($\mathbf{B_1}$) moieties, and that of L-[^{14}C]asparagine was in the β-hydroxyasparagine moiety.

The OF4949 complex, which inhibits aminopeptidase B, was obtained from the culture broth of *Penicillium rugulosum* OF4949. It consists of at least four components, the major ones being OF4949-**I** and **II**.[1] Component **I** can be divided into two moieties, 4-methylisodityrosine ($\mathbf{B_2}$) and β-hydroxyasparagine, as can **II**, into isodityrosine ($\mathbf{B_1}$) and β-hydroxyasparagine.[2] The structures of $\mathbf{B_1}$ and $\mathbf{B_2}$ suggested to us that they might be biosynthesized from two moles of aromatic amino acids, L-tyrosine or L-phenylalanine. To elevate the production of these inhibitors, and to elucidate the pathway of their biosynthesis, we decided to try to find a mutant resistant to growth inhibition by phenylalanine or tyrosine analogs. To study the biosynthetic origin of these inhibitors, we examined the incorporation and distribution of several ^{14}C-labeled compounds into **I** and **II** in replacement culture.

Here, we report on the biosynthesis of OF4949-**I** and **II**.

Materials and Methods

Derivation of Mutant

Conidia of *P. rugulosum* OF4949 in sterile 0.05 M Tris - malate buffer, pH 6.0, (10^6 conidia/ml) were exposed to *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) at the concentration of 400 μg/ml for 40 minutes with shaking. After this treatment, the conidia were spread on a CZAPEK's agar plate containing 10 μg/ml *m*-fluorophenylalanine (MFP) or 100 μg/ml *p*-fluorophenylalanine (PFP), and the plates were incubated at 27°C for 6 days. Each colony was cultured for 6 days in a medium that contained glucose 3.0% and soybean meal 1.5%, and then checked for productivity by an enzyme inhibition assay.[1] The minimum inhibitory concentration against *P. rugulosum* OF4949 of MFP was 2.0 μg/ml and that of PFP, 31.2 μg/ml.

Conditions of Replacement Culture

Strain No. M414, one of the MFP-resistant mutants, was used for the biosynthetic studies. Seed cultures were grown in medium containing maltose 7.0% and Bacto Soytone 1.0%, at 27°C for 7 days on a rotary shaker at 220 rpm. The mycelia were harvested by filtration, washed three times with sterile physiological saline, and homogenized in a Hiscotron (Nippon Seimitsu Co.). The mycelial homogenate (300 mg/ml mycelia, dry weight) was resuspended in a chemically defined medium con-

sisting of maltose 2.0%, NH_4NO_3 1.0%, KH_2PO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 5.0). Replacement culture was done with vigorous shaking at 30°C for 24~72 hours.

Incorporation of Radioactivity into OF4949

^{14}C-Labeled compounds (0.5 μCi) were added to the replacement culture at the time of inoculation. After 24 hours of incubation, the culture broth was extracted with MeOH and chromatographed on a Diaion HP-20 column with 50% EtOH. Fractions containing the OF4949 complex were concentrated and chromatographed on Toyo No. 50 filter paper with PrOH - 28% NH_4OH (2:1) or 0.05 M sodium acetate - EtOH (2:1). OF4949-**I** and **II** were detected by their color reaction with ninhydrin.

Degradation of ^{14}C-OF4949

The area containing **I** or **II** labeled with a ^{14}C-precursor was cut out of the paper and extracted in a small amount of water. The extract was hydrolyzed with 6 N HCl at 100°C for 15 hours. The hydrolysate was put on Toyo No. 50 filter paper and developed with PrOH - 28% NH_4OH (2:1). Each spot of the hydrolyzed products, **A**, **B**$_1$ and **B**$_2$, was cut out from the chromatogram, and the radioactivity was counted.

Labeled Compounds

L-[*U*-^{14}C]Aspartate, L-[*U*-^{14}C]asparagine, L-[*U*-^{14}C]glutamate, [*U*-^{14}C]glycine, L-[*U*-^{14}C]isoleucine, L-[*U*-^{14}C]threonine, L-[*U*-^{14}C]phenylalanine, L-[*U*-^{14}C]tyrosine (each 10 mCi/m atom of carbon labeling), L-[*U*-^{14}C]malate (22 mCi/mmol), and [2.3-^{14}C]succinate (57 mCi/mmol) were purchased from Amersham Japan. [*U*-^{14}C]Fumarate (80~100 mCi/mmol) was purchased from Commissariat a l'Energie Atomique, France.

Measurement of Radioactivity

A liquid scintillation counter (Beckman LS3801) was used to measure radioactivity. Radiochromatograms were scanned with a paper chromatogram scanner (Aloka JPC-213) and a thin-layer chromatogram scanner (Aloka JPC-203).

Results

Strain Improvement

The best mutant strain, No. M414, isolated as a single colony, accumulated 58.8 μg/ml OF4949, about three times the accumulation of the parent (Table 1). The mutant could grow on agar with 20 μg/ml MFP. Maximum production of this strain was 125 μg/ml when cultured with a modified production medium[1] with high aeration.

Biosynthetic Studies

Various amino acids and organic acids were incubated with the mycelial homogenate for 72 hours. Among the amino acids tested, L-aspartate, L-asparagine, L-glutamate, L-isoleucine, glycine, L-phenylalanine and L-tyrosine stimulated production (Table 2). Addition of organic acids of the tricarboxylic acid cycle also stimulated production.

The incorporation of ^{14}C-labeled compounds into OF4949 was examined. A typical paper chromatogram of radioactive **I** and **II** isolated from culture broth is shown in Fig. 1. Of the radioactive amino acids and organic acids tested, L-[*U*-^{14}C]tyrosine was most efficiently incorporated into **I** and **II**, followed by L-[*U*-^{14}C]asparagine and L-[*U*-^{14}C]phenylalanine (Table 3). Less L-[*U*-^{14}C]aspartate, [*U*-^{14}C]glycine, L-[*U*-^{14}C]isoleucine, and [*U*-^{14}C]fumarate were incorporated than the labeled L-asparagine. L-[*U*-^{14}C]Glutamate, L-[*U*-^{14}C]malate and [2.3-^{14}C]succinate were hardly incorporated at all.

To examine the distribution of radioactivity in **I** and **II**, OF4949 labeled with ^{14}C-amino acid pre-

Table 1. Productivity of OF4949 of analog-resistant mutants.

Strain No.	Character	OF4949 (μg/ml)
OF4949	Parent	17.4
M158	MFP^r	27.9
M206	MFP^r	29.8
M287	MFP^r	27.2
M300	MFP^r	29.4
M304	MFP^r	30.9
M414	MFP^r	58.8
P85	PFP^r	25.8
P86	PFP^r	24.5
P87	PFP^r	25.3

MFP^r: Resistant to *m*-fluorophenylalanine.
PFP^r: Resistant to *p*-fluorophenylalanine.

Table 3. Incorporation of ^{14}C-labeled substrate into OF4949-I and **II**.

Labeled compound	Efficiency (% incorporation)*		
	I	II	I+II
[*U*-^{14}C]Fumarate	0.3	1.4	1.7
L-[*U*-^{14}C]Malate	0.5	0.4	0.9
[2.3-^{14}C]Succinate	0.1	0.3	0.4
L-[*U*-^{14}C]Asparagine	3.0	3.6	6.6
L-[*U*-^{14}C]Aspartate	1.3	1.9	3.2
L-[*U*-^{14}C]Glutamate	0.6	0.5	1.1
[*U*-^{14}C]Glycine	1.5	1.4	2.9
L-[*U*-^{14}C]Threonine	0.4	0.4	0.8
L-[*U*-^{14}C]Isoleucine	1.7	0.9	2.6
L-[*U*-^{14}C]Phenylalanine	2.0	2.3	4.3
L-[*U*-^{14}C]Tyrosine	4.2	7.3	11.5

Compounds (0.5 μCi/ml) labeled with ^{14}C were added to chemically defined medium with strain No. M414 mycelium homogenate at 30°C for 24 hours.

* Percentage incorporation, (Total dpm of [^{14}C]-OF4949-I or II)/(Total dpm of [^{14}C]compound added)×100.

Table 2. Effects of amino acids or organic acids on OF4949 production in replacement culture with chemically defined medium.

Added (10 mM)	OF4949 production (μg/ml)	
	Strain No. OF4949	Strain No. M414
Amino acid		
Glycine	3.0	16.2
L-Alanine	2.5	11.6
L-Valine	2.4	13.5
L-Leucine	2.2	9.7
L-Isoleucine	3.5	17.6
L-Serine	2.5	9.5
L-Threonine	2.4	9.7
L-Phenylalanine	3.4	17.2
L-Tyrosine	3.7	16.4
L-Tryptophan	2.6	7.0
L-Cysteine	1.5	3.0
L-Methionine	1.9	6.2
L-Proline	3.2	15.7
L-Aspartate	3.8	20.3
L-Asparagine	3.4	17.2
L-Glutamate	4.3	19.2
L-Glutamine	2.7	11.7
L-Histidine	2.9	12.1
L-Lysine	2.7	9.5
L-Arginine	2.8	11.7
Casamino acid	3.4	16.5
Organic acid		
Acetate	4.2	14.4
Pyruvate	—	15.7
Propionate	3.1	14.1
Butyrate	0.6	2.5
Citrate	4.3	14.1
Oxalacetate	—	14.1
L-Malate	4.3	20.0
Maleate	4.1	15.3
Fumarate	3.9	20.0
Succinate	4.5	16.4
α-Ketoglutarate	—	16.0
None	1.9	9.2

cursors was degraded by acid hydrolysis. L-[*U*-^{14}C]Tyrosine was incorporated mainly into the **B** moiety, whereas most of the radioactivity derived from L-[*U*-^{14}C]asparagine appeared in the A moiety (Table 4).

During flask culture of strain No. M414, the ratio of **I** to **II** produced in the early maximum production phase (Day 12) was about 1:1.5; that of the late maximum production phase (Day 18) was 2.5:1. This suggested that the biosynthetic methylation of a phenolic hydroxyl group of **II** may give **I**. So, [^{14}C]**I** and [^{14}C]**II** obtained when L-[*U*-^{14}C]tyrosine was used were isolated and added to replacement culture. The radioactivity of the [^{14}C]**I** was incorporated only negligibly into **II**, whereas about 30% of the radioactivity of **II** was found in **I** after 18 hours of incubation (Table 5).

Fig. 1. Paper chromatography of [^{14}C]OF4949 labeled with [^{14}C]tyrosine (a) or with [^{14}C]asparagine (b). Radioactivity was monitored with a paper chromatogram scanner (Aloka JPC-213). OF4949-I and II were detected by their color reaction with ninhydrin.

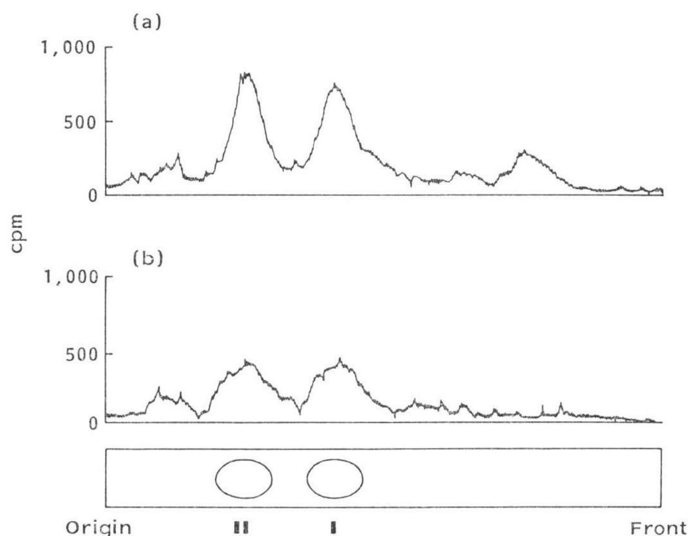


Table 4. Distribution of radioactivity among the degradation products of [^{14}C]OF4949 labeled with different ^{14}C-labeled precursors.

Source of radioactivity	[^{14}C]-OF4949	% Incorporation[a]	% Distribution of ^{14}C[b]			Recovery of radioactivity from OF4949[c]
			A	B_1	B_2	
L-[*U*-^{14}C]-	I	3.3	5.9	16.4	77.7	70.5
Tyrosine	II	7.2	8.3	91.7	—	69.5
L-[*U*-^{14}C]-	I	2.1	84.7	14.6	0.7	73.4
Asparagine	II	3.3	89.2	10.8	—	68.5

[a] (Total dpm of [^{14}C]OF4949-**I** or **II**)/(Total dpm of ^{14}C-labeled compound added)×100.
[b] (Total dpm of ^{14}C-labeled degradation product)/(Total dpm of [^{14}C]**A**+[^{14}C]**B**)×100.
[c] (Total dpm of [^{14}C]**A**+[^{14}C]**B**)/(Total dpm of [^{14}C]OF4949 added)×100.

Table 5. Incorporation of [^{14}C]OF4949-**II** into OF4949-**I**.

Percentage of total activity[a]				Recovery of radioactivity[b]
[^{14}C]OF4949 added		[^{14}C]OF4949 yielded		
I	II	I	II	
85.1	14.9	88.4	11.6	70.8
6.4	93.6	33.4	66.6	79.3

[a] (Total dpm of [^{14}C]OF4949-**I** or **II**)/(Total dpm of [^{14}C]OF4949-**I**+**II**)×100.
[b] (Total dpm of [^{14}C]OF4949 yielded)/(Total dpm of [^{14}C]OF4949 added)×100.

Discussion

The efficiency of L-tyrosine incorporation into **I** and **II** and its distribution pattern in these inhibitors suggested that the **B** moiety of **I** and **II** is derived from L-tyrosine. Isodityrosine of plant cell walls is biosynthesized by oxidative coupling *via* peroxidase-generated free radicals with two tyrosine units.[3,4] Similarly, ascaris cuticle contains isotrityrosine, an oxidatively coupled trimer of tyrosine

Fig. 2. Paper chromatography of [^{14}C]*erythro*-β-hydroxyasparagine labeled with [^{14}C]asparagine.

The culture broth with [^{14}C]asparagine was extracted with MeOH and passed through a column of Diaion HP-20. The fractions were purified on a Dowex 50W (H^+) column. The eluate was chromatographed on a Toyo No. 50 filter paper with EtOH - 28% NH_4OH (2 : 1).

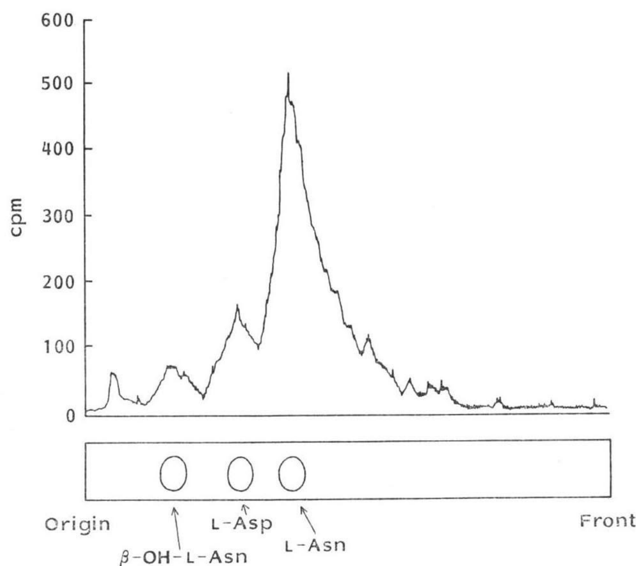
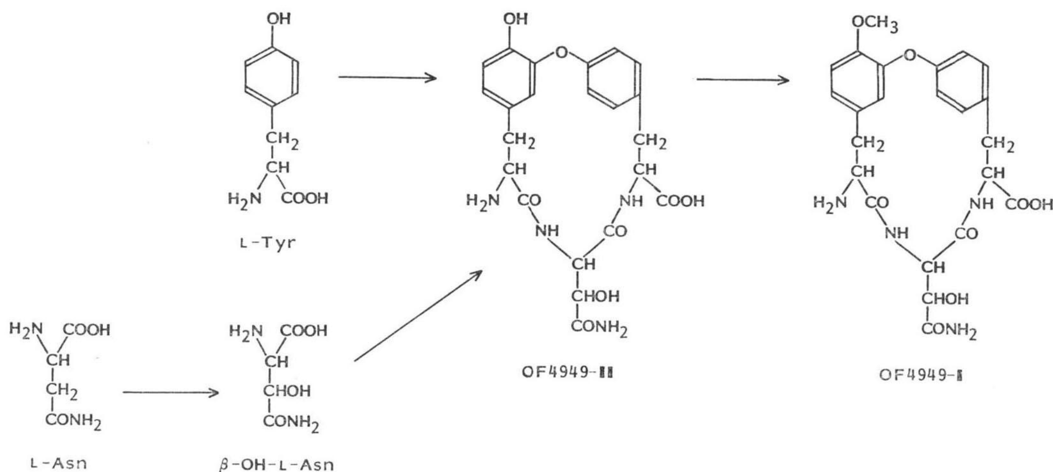


Fig. 3. Tentative pathway for biosynthesis of OF4949-I and II.



with one biphenyl and one diphenyl ether bridge.[5] It seems likely that the **B** moiety of **I** and **II** is formed from two molecules of tyrosine by the action of a peroxidase.

The L-*erythro*-β-hydroxyasparagine moiety may be biosynthesized by one of the following suggested mechanisms. (1) The moiety may be derived from β-hydroxyaspartate by ammonia ligation. (2) L-Asparagine may be β-oxygenated to give β-hydroxyasparagine. (3) β-Hydroxyasparagine may be synthesized from another pathway. Investigations of the biosynthesis of L-*erythro*-β-hydroxyaspartate showed that this amino acid is synthesized from one molecule of glycine and one molecule of glyoxalate by enzymatic condensation by 3-hydroxyaspartate aldolase (EC 4.1.3.14),[6,7] or from dihydroxyfumarate by transamination and hydration with aspartate aminotransferase (EC 2.6.1.1).[8] However, the low incorporation of [^{14}C]glycine and the ^{14}C-labeled organic acids of the tricarboxylic

acid cycle into OF4949 suggested to us that β-hydroxyaspartate is not a direct precursor of the L-*erythro*-β-hydroxyasparagine moiety. The high rate of uptake and the distribution of [^{14}C]asparagine indicated that the L-*erythro*-β-hydroxyasparagine moiety could be derived from L-asparagine. The formation of β-hydroxyasparagine from L-asparagine can be explained by the assumption that β-hydroxylation of L-asparagine occurs. In preliminary experiments, a few radioactive peaks corresponding to L-*erythro*-β-hydroxyasparagine derived from [^{14}C]asparagine were detected in culture broth by paper chromatography (Fig. 2). Similar aliphatic oxygenation systems have been reported in microorganisms and in animals involving 6-*N*-trimethyl-L-lysine hydroxylase,[9,10] 4-trimethylbutyric acid (γ-butyrobetaine) hydroxylase (EC 1.14.11.1),[11,12] collagen prolyl-4-hydroxylase (EC 1.14.11.2),[13,14] and collagen lysyl hydroxylase (EC 1.14.11.4);[15,16] these are all dioxygenases dependent on α-ketoglutarate. In the experiment reported here, the enhancement of OF4949 production by the organic acids in the tricarboxylic acid cycle suggests that such a system is involved.

Furthermore, the ratio of **I** to **II** produced during culture and the incorporation of ^{14}C-labeled **II** into **I** suggested that *O*-methylation of the phenolic hydroxyl group may occur after cyclization. *O*-Methyl transferases catalyzing the transmethylation of phenolic hydroxyl groups are widely distributed in nature.[17~19]

These results suggested to us the tentative biosynthetic pathway shown in Fig. 3.

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