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Metabolic Products of *Aspergillus terreus*. IX.¹⁾ Biosynthesis of Butyrolactone Derivatives isolated from Strains IFO 8835 and 4100

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Two new derivatives of butyrolactone I were isolated from *Aspergillus terreus* IFO 4100, and the structures were determined. A metabolic pathway from phenylalanine to butyrolactone I was established by administration of potential intermediates to growing or resting cells of *Aspergillus terreus* IFO 8835 and by enzymatic studies. Three enzymes involved in the biosynthesis were identified.

Keywords—*Aspergillus terreus*; IFO 4100; IFO 8835; biosynthesis; enzyme; butyrolactone derivative

In a previous paper,²⁾ a butyrolactone derivative (I), α -oxo- β -(*p*-hydroxyphenyl)- γ -(*p*-hydroxy-*m*-3,3-dimethylallylbenzyl)- γ -methoxycarbonyl- γ -butyrolactone (hereafter abbr. as butyrolactone I), was isolated from *Aspergillus terreus* IFO 8835, and the chemical structure was determined. Other butyrolactone derivatives, II (mp 91—94°C) and III (mp 70°C) were isolated from *Aspergillus terreus* IFO 4100 as reported in Part VIII¹⁾ of this series. In this paper, we describe the structures of these new metabolites and the biosynthesis of butyrolactone I.

Butyrolactone derivative (II) (abbr. as butyrolactone II) was isolated from the culture broth of *Aspergillus terreus* IFO 4100, and the chemical formula, C₁₉H₁₆O₇ was assigned from the mass spectrum (MS) and elemental analysis. The derivative had optical activity ($[\alpha]_D^{25} +85^\circ$), and its ultraviolet (UV) and infrared (IR) spectra resembled those of butyrolactone I. However, no signals of the 3,3-dimethylallyl (hereafter abbr. as prenyl) group were apparent in the proton magnetic resonance (PMR) spectrum, so butyrolactone II may be a de-prenyl derivative of butyrolactone I.

Butyrolactone II was synthesized by the method reported by Sakan³⁾ and Hemmerle.⁴⁾ The obtained compound, mp 206—208°C, was identical with the natural compound (II) as regards the PMR spectrum, but there were differences in the IR spectra and melting point. Thus, each compound was converted to the de-methoxycarbonyl derivative, mp 246°C ($[\alpha]_D^{25} 0^\circ$), by treatment with pyridine hydrochloride.²⁾ The products were identical on the basis of IR spectral comparison and mixed melting point determination. From these results, the structure of butyrolactone II was determined as α -oxo- β -(*p*-hydroxyphenyl)- γ -(*p*-hydroxybenzyl)- γ -methoxycarbonyl- γ -lactone.

Another butyrolactone derivative (III) (abbr. as butyrolactone III) was obtained as a slightly yellow substance. The chemical formula, C₂₄H₂₄O₈ was determined by high resolution MS (440.1481), $[\alpha]_D^{25} +80^\circ$. The IR, UV, and PMR spectra suggested an epoxide structure.

Butyrolactone III was synthesized from I by epoxidation of the prenyl group with *m*-chloroperbenzoic acid. The presence of the epoxy group was confirmed by the formation of a benzofuran derivative, mp 220—222°C. $[\alpha]_D^{27} +78^\circ$, on heating with pyridine hydrochloride (cf. aspulvinones D and F⁵⁾).

In a previous paper,²⁾ the following results were reported. ¹⁴C-Phenylalanine and ¹⁴C-tyrosine were incorporated well into butyrolactone I in growing cells of IFO 8835 (Phe, 37%; Tyr, 17%). ¹⁴C-Mevalonate was also incorporated into the prenyl group of butyrolactone I

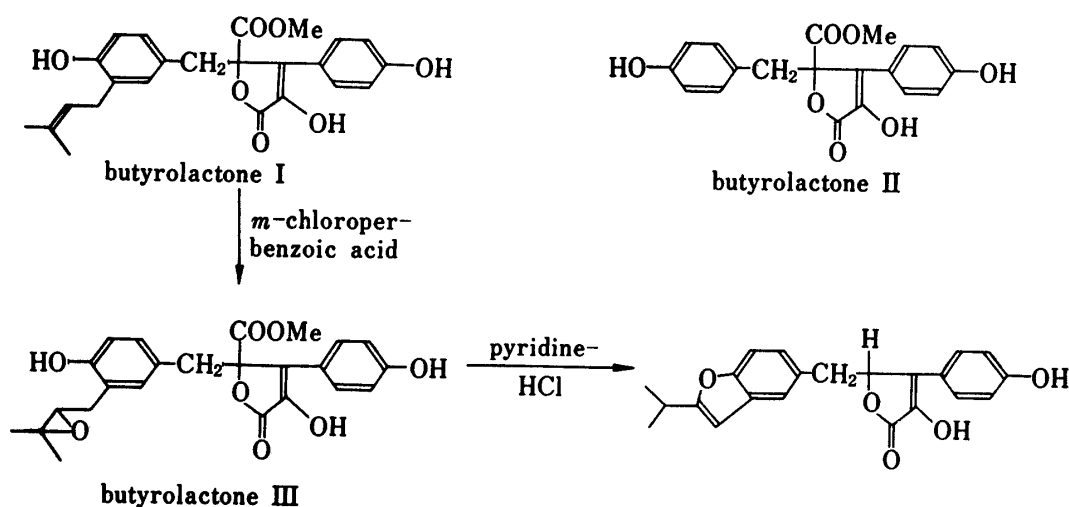
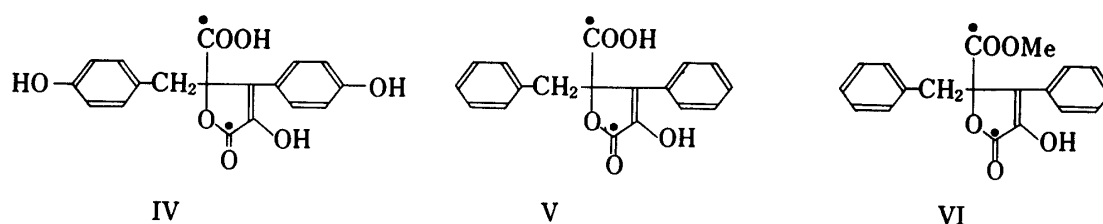


Chart 1

(7.9%). The distribution pattern of ^{14}C suggested that butyrolactone I was biosynthesized by intact condensation of two phenylpropanoid molecules between the 2 and 3' positions, followed by lactonization and prenylation. This pathway must include several other steps, *i.e.* deamination, hydroxylation, methylation, condensation, and others. However, the sequence of the biosynthetic reactions remained obscure. Thus, the pathway was studied in detail by administration of potential intermediates to living or cell-free preparations of IFO 8835.

It is reasonable to presume that Phe and/or Tyr are deaminated to yield phenylpyruvic acid (PPA) and/or *p*-hydroxyphenylpyruvic acid (HPPA), which are condensed to yield butyrolactone metabolites. Thus, radioactive PPA and HPPA were administered to the resting cells. Both compounds were incorporated well into butyrolactone I (42 and 49%, respectively; *cf.* Phe, 4.9%; Tyr, 16%). The corresponding methyl esters were also incorporated into butyrolactone I in the resting cells, but the incorporation ratios were not high (1.6 and 1.5%, respectively).



• position of ^{14}C in synthetic compounds.

Next, four radioactive potential intermediates (II, IV, V, and VI) were synthesized (racemic form) and administered. Butyrolactone II was incorporated into butyrolactone I in growing cells (9.9%), and in resting cells (2.6%), but the other compounds (IV, V, and VI) were not incorporated. From these results, it was suggested that prenylation was the last step in the biosynthesis of butyrolactone I, as in the case of aspulvinones,⁵⁾ and butyrolactone II must be the last intermediate.

The process of biosynthesis was further studied in detail by using cell-free preparations. The crude enzyme mixture was prepared by grinding the mycelia in a mortar with sea sand in 0.01 M Tris buffer (pH 7.0) containing mercaptoethanol, magnesium chloride, and phenylmethylsulfonylfluoride (PMSF). These additives, particularly PMSF, were essential for preserving the enzyme activities. The homogenate was centrifuged at 12000 *g*, and the

supernatant was fractionated by 40% saturation with ammonium sulfate. The precipitates were desalted by gel filtration on Sephadex G-25 with 0.01 M Tris buffer (pH 7.0). The first turbid fraction containing protein was centrifuged at 100000 g, and the supernatant was used for the following experiments as the crude enzyme mixture. It showed the following activities in preliminary tests.

1) Prenylation of butyrolactone II to butyrolactone I in the presence of dimethylallylpyrophosphate (DMAPP).

2) Methylation of compound IV to butyrolactone II in the presence of S-adenosylmethionine (SAM).

3) Formation of butyrolactone II by condensation of HPPA in the presence of SAM.

These activities were lost by heating or freezing, and the half-life of the activities was about one day at 4°C in 0.01 M Tris buffer. This crude enzyme mixture had activity to form asterriquinone derivatives from indolepyruvic acid,⁶⁾ but no indole-derived butyrolactone derivative was obtained.

¹⁴C-Labeled substrates were incubated with the crude enzyme mixture and DMAPP in 0.05 M Tris buffer (pH 8.0). When the potential intermediates, II, IV, V, and VI were incubated, butyrolactone II was converted to butyrolactone I. Compound V and VI were not changed to any other compound, that was also the case in growing or resting cells.

In these experiments, butyrolactone I produced from butyrolactone II was optically active ($[\alpha]_D^{17} +86^\circ$; natural butyrolactone I, $[\alpha]_D^{15} +100^\circ$) in spite of the use of racemic substrate. The unreacted substrate (butyrolactone II) recovered from the reaction mixture was levorotatory ($[\alpha]_D^{18} -31^\circ$). These results showed that only the *d*-form of butyrolactone II was available as a substrate for butyrolactone I.

Compound IV was methylated by the crude enzyme mixture in the presence of SAM to yield butyrolactone II ($[\alpha]_D^{15} +33^\circ$), and IV was further converted to butyrolactone I in the presence of both SAM and DMAPP. Compound V was not methylated to compound VI under the same conditions.

HPPA was metabolized by the crude enzyme mixture in the presence of SAM to yield butyrolactone II ($[\alpha]_D^{15} +76^\circ$; *cf.* pure II, $+85^\circ$). No product (including IV) was detected by TLC or radioisotopic assay under these conditions without SAM. From these results, the participation of IV was excluded. Namely, the pathway HPPA→IV→II does not operate in this fungus.

No modification of PPA (including the formation of V or VI) occurred with the crude enzyme mixture with or without SAM. Compound VI was also not metabolized. These results suggested that the introduction of a hydroxyl group into the phenyl ring of PPA or Phe preceded the condensation and lactone ring formation. HPPA methyl ester was changed to butyrolactone II ($[\alpha]_D^{17} +26^\circ$) by the crude enzyme mixture even without SAM. Thus, it was concluded that HPPA methyl ester was the real substrate at the step of condensation, and that HPPA could be metabolized to butyrolactone II after its conversion to the methyl ester. HPPA methyl ester also afforded butyrolactone II under weakly alkaline conditions without enzymes, but butyrolactone II thus obtained was optically inactive. Thus, this condensation process certainly took place enzymatically in the fungus.

From these results, the biosynthesis of butyrolactone I from phenylalanine was determined to take the route shown in Chart 2. It is probable that the activity for prenylation of II to I is so strong in the strain IFO 8835 that no accumulation of II occurs.

Purification of the enzymes was also undertaken. The crude enzyme mixture was fractionated on a column of hydroxyapatite by gradient elution with phosphate buffer (pH 7.0) containing mercaptoethanol and magnesium chloride. The first protein peak had no enzymatic activity. The second peak had both methylation and condensation activities, and the third peak had the prenylation activity. The prenylation enzyme had an optimum pH at 8.0 (in Tris buffer), and its K_m value was 0.15 mM for butyrolactone II.

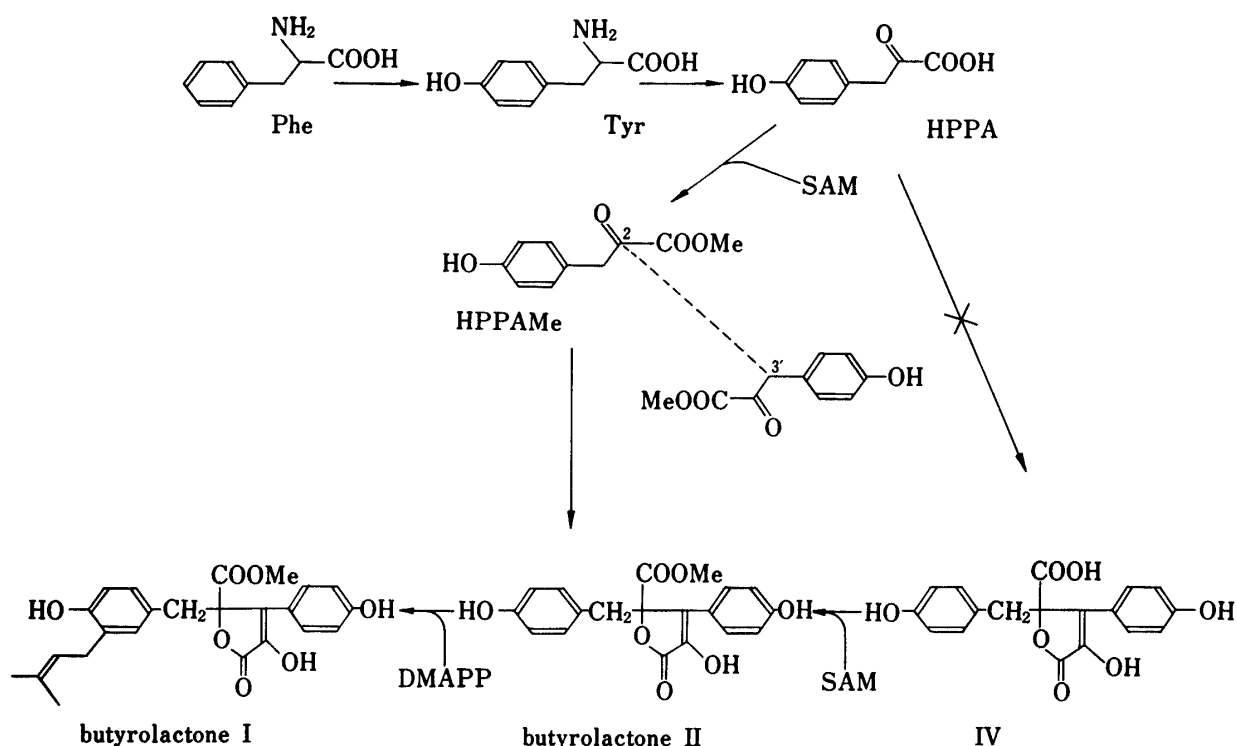


Chart 2. Biosynthesis of Butyrolactone I by *Aspergillus terreus* IFO 8835

Each fraction was further chromatographed on DEAE Sephadex A-25 by gradient elution with sodium chloride in Tris buffer (pH 7.0). In this chromatography, the prenylation activity appeared at the second fraction as a single peak, and the activities of methylation and condensation were found in the third fraction; the latter two activities were not separated clearly. The condensation activity had an optimal pH at 7.5 (in Tris buffer), and its K_m value was 0.77 mM for HPPA. The methylation activity had an optimal pH at 7.6 (in Tris buffer) and the K_m value was 0.25 mM for IV. Further purification and characterization were not attempted because of the instability of the enzyme activities.

Experimental⁷⁾

Cultivation—IFO 8835 or 4100 was cultivated in 500 ml Roux flasks containing 200 ml of a malt extract medium as reported in a previous paper.²⁾

Isolation of Butyrolactones II and III—The culture broth of IFO 4100 was concentrated in vacuum and extracted with ether. The ether extract was chromatographed on silica gel (Wakogel C-200, benzene-AcOEt, 10:1—10:3). Butyrolactone I, mp 95°C (dec.), butyrolactone II, and butyrolactone III were eluted in that order. Butyrolactone II was crystallized from CH_2Cl_2 as colorless plates, mp 91—94°C. *Anal.* Calcd for $\text{C}_{19}\text{H}_{16}\text{O}_7$: C, 64.04; H, 4.53. Found: C, 63.43; H, 4.52. MS m/z , Calcd for $\text{C}_{19}\text{H}_{16}\text{O}_7$: 356.0894. Found: 356.0878. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3390, 3290, 1760, 1740 (sh), 1730, 1660. PMR (acetone- d_6) δ : 3.43 (2H, s, CH_2), 3.71 (3H, s, OCH_3), 6.43 (2H, d, $J=9$ Hz, aromatic H), 6.63 (2H, d, $J=9$ Hz, aromatic H), 6.85 (2H, d, $J=9$ Hz, aromatic H), 7.51 (2H, d, $J=9$ Hz, aromatic H), 8.4 (3H, 3OH). $[\alpha]_D^{25} +85^\circ$ ($c=1$, EtOH).

Butyrolactone III was recrystallized from CH_2Cl_2 to afford a yellowish mass which could not be obtained as crystalline form due to its hygroscopicity, mp 70°C (not sharp), MS m/z : Calcd for $\text{C}_{24}\text{H}_{24}\text{O}_8$: 440.1482. Found: 440.1481. MS m/z : 440 (M^+), 396, 191, 173, 155. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3370, 1740, 1610. PMR (acetone- d_6) δ : 1.15, 1.20 (each 3H, s, CH_3), 2.66 (2H, m), 3.4 (2H, s, CH_2), 3.7 (3H, s, OCH_3), 4.1 (1H, m), 6.4 (3H, m), 6.85 (2H, d, $J=8$ Hz), 7.5 (2H, d, $J=8$ Hz), 7.85 (1H, s, OH), 8.8 (2H, OH). $[\alpha]_D^{25} +80^\circ$ ($c=0.7$, EtOH).

Synthesis of Butyrolactone II—The method of Sakan³⁾ and Hemmerle⁴⁾ was applied with some modifications. HPPA (1 g) was methylated with a large excess of CH_2N_2 in ether for 5 min. The reaction mixture was concentrated, and the residue was treated with saturated Na_2CO_3 (30 ml) under vigorous shaking at room temperature for 1 h. The reaction mixture was acidified with HCl and extracted with ether. The ether solution was concentrated, and the residue was chromatographed on silica gel (Kanto, for chromato-

graphy) with benzene-AcOEt (9: 1). The main fraction was collected and recrystallized from benzene-EtOH as colorless needles, mp 206–208°C (dec.), yield 530 mg. *Anal.* Calcd for $C_{19}H_{16}O_7$: C, 64.04; H, 4.53. Found: C, 64.15; H, 4.27. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400, 3380, 3290, 1750, 1710. MS m/z : 312 ($M^+ - 44$), 280, 252, 223, 177, 107. PMR (acetone- d_6) δ : 3.46 (2H, s, CH_2), 3.78 (3H, s, OCH_3), 6.56 (2H, d, $J=9$ Hz, aromatic H), 6.80 (2H, d, $J=9$ Hz, aromatic H), 6.90 (2H, d, $J=9$ Hz, aromatic H), 7.57 (2H, d, $J=9$ Hz, aromatic H), 8.6 (3H, br, 3OH).

Decarboxymethylation of II—The natural butyrolactone II (70 mg) was heated with freshly prepared pyridine-HCl (2.5 g) at 200–210°C for 70 min. The reaction mixture was diluted with H_2O , acidified with HCl, and extracted with ether. The ether extract was concentrated, and the residue was recrystallized from CHCl_3 -MeOH to provide a colorless powder, mp 246–249°C, yield 30 mg. The same compound was obtained from the synthetic butyrolactone II. *Anal.* Calcd for $C_{17}H_{14}O_5$: C, 68.45; H, 4.73. Found: C, 67.55; H, 4.68. MS m/z : Calcd for $C_{17}H_{14}O_5$: 298.0841. Found: 298.0859. MS m/z : 298 (M^+), 107. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3255, 1725, 1675, 1610, 1595. $[\alpha]_D^{25} 0^\circ$ ($c=1$, EtOH).

Synthesis of the Epoxide (III) from I—*m*-Chloroperbenzoic acid (660 mg) was added to a solution of butyrolactone I (1 g) in CHCl_3 (25 ml), and the mixture was kept at 4°C for 3 d. The solvent was evaporated off, and the residue was chromatographed on silica gel (Wakogel C-200) with benzene-AcOEt. The fraction with the same *Rf* value as natural butyrolactone III was further purified by preparative thin-layer chromatography (TLC) (silica gel 60 PF₂₅₄; benzene-AcOEt, 1: 1). The product showing *Rf* 0.42 was indistinguishable from III, yield, 190 mg, $[\alpha]_D^{27} +78^\circ$ ($c=0.5$, EtOH).

Benzofuran Derivative from III—Butyrolactone III (630 mg) was heated with pyridine-HCl (3 g) at 200–210°C for 50 min. The reaction mixture was poured into ice water, acidified with HCl, and extracted with ether. The extract was fractionated by preparative TLC with benzene-AcOEt (3: 1). The main fraction was recrystallized from benzene to yield a colorless powder (110 mg), mp 220–222°C. *Anal.* Calcd for $C_{22}H_{20}O_5$: C, 72.51; H, 5.53. Found: C, 72.53; H, 5.21. MS m/z : 364 (M^+), 346, 318, 173. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3540, 3500, 1755, 1705, 1590. PMR (DMSO- d_6) δ : 1.3 (6H, d, $J=6.5$ Hz, 2CH_3), 2.9 (1H, not clear, overlapped with signals at δ : 3.2, CH), 3.2 (2H, center of AB part of ABX type, $J=14, 6$, and 4 Hz, CH_2), 5.6 (1H, dd, $J=6, 4$ Hz, X part of ABX type, $-\dot{\text{C}}\text{H}-\text{O}-$), 6.25 (1H, s, $-\text{CH}=\text{}$), 6.8–7.6 (7H, aromatic H), 8.5 (2H, br s, 2OH).

Administration Experiments—In the experiments using growing cells, the fungus was cultivated for 4–6 d, and ^{14}C -labelled compounds were administered under the mycelia mat. The fungus was cultivated for further 8–10 d.

In the experiments using resting cells, the culture broth was discarded after cultivation for 4–6 d, and the mycelia mat was gently washed three times with 200 ml of 0.1 M phosphate buffer (pH 6.0), then incubated with ^{14}C -labeled compounds in 0.1 M phosphate buffer (200 ml). After incubation at 27°C for 8–10 d, the resting cells were harvested.

Each metabolite was isolated and the radioactivity was measured with a liquid scintillation spectrometer in a dioxane scintillator. The specific radioactivity and incorporation ratio were used as indexes of incorporation.

Synthesis of VI—VI was prepared from PPA (1 g) by the same methods as described for butyrolactone II, yield 420 mg. It was crystallized from benzene-petr. benzin as colorless needles, mp 156–158°C. *Anal.* Calcd for $C_{19}H_{16}O_5$: C, 70.36; H, 4.98. Found: C, 70.46; H, 4.80. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3300, 1740. MS m/z : 324 (M^+), 292, 279, 191, 91.

Synthesis of IV from II—II (100 mg) was dissolved in 2 N NaOH (2 ml), and kept for 24 h at room temperature. The reaction mixture was acidified with HCl, and allowed to stand for 24 h. The resulting crystals were washed with H_2O , and dried in a desiccator, mp 195–198°C (dec.). *Anal.* Calcd for $C_{18}H_{14}O_7$: C, 63.16; H, 4.12. Found: C, 63.23; H, 4.09. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3500, 3400, 3200, 1730.

Compound V was prepared from compound VI by the same methods, mp 152–155°C (dec.). *Anal.* Calcd for $C_{18}H_{14}O_5$: C, 69.67; H, 4.55. Found: C, 69.77; H, 4.45.

Synthesis of ^{14}C -Compounds—1- ^{14}C -HPPA and 1- ^{14}C -PPA were synthesized from 1- ^{14}C -glycine *via* hydantoin.⁸⁾ ^{14}C -labeled II, IV, V, and VI were synthesized from ^{14}C -HPPA or ^{14}C -PPA as described above. The specific radioactivities of these ^{14}C -compounds were adjusted to 1–2 $\times 10^5$ dpm/mg before use.

Preparation of Crude Enzyme Mixture—All procedures were carried out at 4°C. Buffer solution containing 10 mM each of mercaptoethanol and MgCl_2 was used in all experiments. The mycelia (10 g), 6–7 d old, were dried until damp, and ground in a mortar with sea sand (10 g) in 30 ml of 0.01 M Tris buffer (pH 7.0) containing 20.9 mg of PMSF (final concentration, 4 mM). The homogenate was centrifuged at 12000 *g* for 30 min. The supernatant (*ca.* 25 ml; protein content, 3 mg/ml⁹⁾) was fractionated by 40% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitates were collected by centrifugation, and re-dissolved in a minimum volume of 0.01 M Tris buffer (pH 7.0). The solution was chromatographed on a column of Sephadex G-25 (2.5 $\times$ 25 cm) with 0.01 M Tris buffer (pH 7.0). The turbid protein fraction was centrifuged at 100000 *g* for 60 min. The clear supernatant (35 ml; protein content, 0.6 mg/ml) was used as the crude enzyme mixture.

Assay of Enzymatic Reactions—For the prenylation reaction, ^{14}C -butyrolactone II (2.5 mg; final concentration, 1 mM) was dissolved in 1% Na_2CO_3 (0.1 ml) and diluted with 0.05 M Tris buffer (pH 8.0) to a total volume of 2 ml. To this solution, DMAPP (6.0 mg; final, 2 mM), the enzyme solution (5 ml), and KF (final, 10 mM) were added. After incubation at 30°C for 30 min, the reaction mixture was acidified with HCl,

and extracted with ether. The extract was fractionated on TLC (Merck, Silica gel 60 F₂₅₄, benzene-AcOEt, 1: 1). The spots of ¹⁴C-compounds were detected by TLC-radioscanning, and each spot was extracted with MeOH and assayed by the use of a liquid scintillation spectrometer.

For studies of the methylation and condensation activities, compound IV and HPPA (each final concentration, 1 mM) were used as substrates, and the activities were assayed under the same conditions as described above.

Prenylation of Butyrolactone II with Crude Enzyme Mixture—Butyrolactone II (100 mg; final concentration, 1 mM) was dissolved in 1% Na₂CO₃ (7 ml), and DMAPP (400 mg) in 0.05 M Tris buffer (pH 8.0, 90 ml) was added. KF was also added to give 10 mM final concentration. This solution was incubated with 200 ml of crude enzyme mixture prepared from 80 g of mycelia. After incubation at 30°C for 3 h, the reaction mixture was acidified with HCl, and extracted with ether. The extract was chromatographed on a column of silica gel (Wakogel C-200, 1.8 × 16 cm) with benzene-AcOEt (10: 2). The first fraction (yield, 40 mg) was recrystallized from CH₂Cl₂. It was found to be identical with I by IR, PMR, and MS comparisons. $[\alpha]_D^{17} + 86^\circ$ ($c=0.5$, EtOH).

The second fraction, containing unreacted butyrolactone II, was recrystallized from CHCl₃-MeOH, mp 103–105°C (yield, 8.5 mg). MS m/z : 356 (M⁺), 312, 280, 252, 223, 177, 107. $[\alpha]_D^{18} - 31^\circ$ ($c=0.5$, EtOH).

Methylation of IV with Crude Enzyme Mixture—A solution of IV (100 mg; final concentration, 2 mM) in 1% Na₂CO₃ (5 ml) was mixed with 0.05 M phosphate buffer (pH 8.0, 90 ml) and SAM (140 mg), then the crude enzyme mixture (150 ml, prepared from 78 g of mycelia) was added, and the mixture was incubated at 30°C for 3 h. The products were extracted with ether and chromatographed on silica gel (Wakogel C-200, 1.8 × 12 cm). The compound eluted with benzene-AcOEt (10: 2) was recrystallized from benzene-EtOH, yield, 18 mg. $[\alpha]_D^{15} + 33^\circ$ ($c=0.9$, EtOH). It was identified as butyrolactone II. The unreacted compound IV was recovered from the following fraction eluted with MeOH (yield, 80 mg). $[\alpha]_D^{15} - 6.5^\circ$ ($c=4.3$, EtOH).

Condensation Products of HPPA with the Crude Enzyme Mixture in the Presence of SAM—HPPA (72 mg; final concentration, 2 mM) was incubated with the crude enzyme mixture (180 ml, prepared from 80 g of mycelia) and SAM (180 mg) in 0.05 M Tris buffer (pH 8.0) (total incubation volume, 200 ml). The product which appeared at R_f 0.5 on preparative TLC (Silica gel 60 PF₂₅₄, benzene-AcOEt, 1: 1), was isolated and identified as butyrolactone II (yield, 10 mg). $[\alpha]_D^{17} + 76^\circ$ ($c=0.4$, EtOH). Under the same conditions except for the absence of SAM, II was not detected by TLC or radioactive assay.

Purification of the Crude Enzyme Mixture—The crude enzyme mixture (30 ml) was applied to a column of hydroxyapatite (1.5 × 20 cm) pre-equilibrated with 0.01 M phosphate buffer (pH 7.0), and eluted with a linear gradient of phosphate buffer (from 0.01 to 0.4 M, total volume, 300 ml).

Each fraction containing protein was assayed for prenylation, methylation, and condensation activities, and divided into two parts. The first active fraction (40 ml; protein content, 0.3 mg/ml) had methylation and condensation activities, and the second active fraction (45 ml; protein content, 0.2 mg/ml) had prenylation activity.

For further purification, each part (30 ml) was adjusted to pH 7.5 and adsorbed on a DEAE Sephadex A-25 column (1 × 10 cm). On gradient elution with NaCl (0–1 M in 0.01 M Tris buffer (pH 7.0)), three peaks of proteins appeared. The second peak had prenylation activity, and the third peak had methylation and condensation activities.

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