[Chem. Pharm. Bull. 31(3) 925—933 (1983)]

Metabolic Products of *Aspergillus terreus*. VIII.[1] Astepyrone: a Novel Metabolite of the Strain IFO 4100

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(Received September 7, 1982)

A novel metabolite named astepyrone and two other new metabolites were isolated from *Aspergillus terreus* IFO 4100, and their chemical structures were determined.

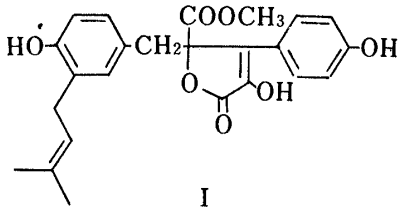
The biosynthesis of astepyrone was also studied and this metabolite was proved to be formed through the ring cleavage of orsellinic aldehyde.

Keywords——astepyrone; *Aspergillus terreus*; IFO 4100; structure; biosynthesis; orsellinic aldehyde

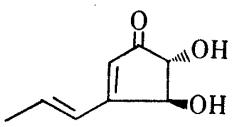
During an investigation of the meabolites of *Aspergillus terreus*, a novel metabolite named "astepyrone" and four other new metabolites were isolated from the strain IFO 4100. In this paper we describe the structures of three metabolites, including astepyrone, and the biosynthesis of astepyrone.

The fungus was cultivated stationarily on a malt extract medium at 27°C for three weeks. The culture filtrate was concentrated to one-tenth of the initial volume and extracted with AcOEt, and the extract was treated with 10% $NaHCO_3$. From this acidic fraction, terreic acid, mp 127°C, terremutin, mp 148°C, itaconic acid, mp 172°C, orsellinic acid, mp 175°C and 3,6-dihydroxybenzoquinone, mp 179°C, were isolated and identified. Astepyrone was not obtained by this procedure, but was obtained when the culture filtrate was extracted with ether and AcOEt successively and these extracts were separated by silica gel column chromatography without the $NaHCO_3$ treatment.

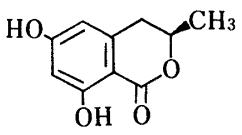
From the ether-extracted fraction, α-oxo-β-(4-hydroxyphenyl)-γ-[4-hydroxy-3-(3-methyl-2-buten-1-yl)benzyl]-γ-methoxycarbonyl-γ-butyrolactone (I),[2] mp 94—96°C, and two other new metabolites corresponding to the deprenyl derivative, mp 91—94°C, and the epoxide, mp 70°C, were isolated. The chemical structures and the biosynthesis of these butyrolactones



I



terrein



(3*R*)-3, 4-dihydro-6, 8-dihydroxy-3-methyl-isocoumarin

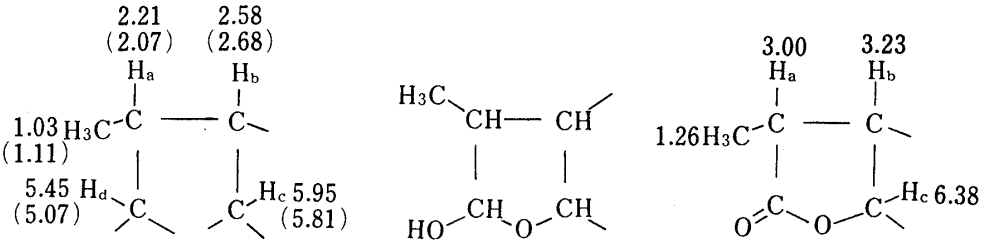
Fig. 1

will be described in the following paper (part IX).[3]

Two other isocoumarin derivatives were also isolated. One was a new compound, colorless needles (IV), mp 190—192°C, and the other was (3*R*)-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin, mp 214—215°C, the biosynthetic precursor of terrein.[4]

From the AcOEt extract, astepyrone (II), mp 133—134°C (dec.), a related metabolite (III), mp 154—156°C, and terrein, mp 127°C, were isolated.

Astepyrone was a neutral, optically active compound. It was unstable in acidic medium and more unstable in alkaline medium. The mass spectrum (MS) (M^+ 200 *m/z*) and elemental analysis gave the chemical formula as $C_9H_{12}O_5$. The ^1H and ^{13}C-nuclear magnetic resonance (NMR) spectra showed signals indicative of a 1 : 1 mixture of two compounds. The ^1H-NMR peaks of each component suggested the presence of one methyl, one methoxyl, one hydroxyl and five methine protons. One methine proton appeared as a singlet signal and the arrangement of the other four methine protons was concluded to be as in Va (Fig. 2) from spin-decoupl-



Va (δ; ppm) Vb VII (δ; ppm)

J_{a,CH_3} = 6.8 (7.1) Hz
$J_{a,b}$ = 11.2 (9.5) Hz
$J_{b,c}$ = 4.9 (5.1) Hz
$J_{a,d}$ = 5.4 (5.1) Hz

J_{a,CH_3} = 6.9 Hz
$J_{a,b}$ = 10.0 Hz
$J_{b,c}$ = 5.6 Hz

Fig. 2. The Partial Structures and ^1H-NMR (in DMSO-d_6) Parameters of Astepyrone and VII

Figures in brackets are chemical shifts and coupling constant values of the anomer.

ing experiments. II afforded a mono *p*-nitrobenzoate (VI), mp 160°C (dec.) which suggested the presence of one hydroxyl group in the molecule. Astepyrone has no aldehyde group in the molecule, but it was positive to the Tollens reagent. It was oxidized with chromic acid in AcOH to afford colorless needles (VII), mp 98—99°C. The product was negative to the Tollens reagent and had a new infrared (IR) carbonyl band (1804 cm^{-1} in $CHCl_3$; lactone) in place of the hydroxyl band (3334 cm^{-1}) of II. The ^1H and ^{13}C-NMR spectra showed the corresponding peaks to the chemical formula $C_9H_{10}O_5$. The above evidence suggested that astepyrone had a cyclic hemiacetal structure in the molecule. Thus the two components of astepyrone observed in the NMR spectrum may be anomers formed in solution. The multiplet signal of the methine proton (δ 3.00 in DMSO-d_6) coupled with methyl protons (δ 1.26) in VII changed to a doublet (J=10 Hz) on irradiation of the methyl protons, so the partial structure of Va was extended to Vb (Fig. 2).

Astepyrone had a methoxyl group (δ_H 3.74 (or 3.76)) located at a quaternary carbon (δ_C 172.4 (or 172.9)), as confirmed by selective irradiation at the methoxyl proton frequency. It showed carbonyl bands at 1675, 1663 cm^{-1} and a double bond band at 1630 cm^{-1} in the IR spectrum, and its ultraviolet (UV) maximum appeared at 235 nm (log ε 4.06). These results suggest the existence of a β-methoxyl-α,β-unsaturated-δ-lactone moiety[5] in astepyrone.

Astepyrone was reacted with ethanedithiol in the presence of BF_3-etherate to afford colorless prisms (VIII), mp 157—159°C. The chemical formula of VIII was $C_{13}H_{20}O_3S_4$ and the ^1H-NMR spectrum showed that two molecules of ethanedithiol had reacted. This suggested the existence

of two acetal moiety in II. VIII was an acid and the UV (λ_{max} 238 nm) and IR (ν_{max} 1668 cm^{-1}; C=O and 1590 cm^{-1}; C=C) spectra suggested that it was α,β-unsaturated. Desulfurization of VIII with Raney-Ni gave an oil (IX). The formula of IX was $C_9H_{18}O_3$ and its structure was deduced from the ^{1}H-NMR spectrum, which still contained all the carbons of astepyrone. IX was an acid and afforded a *p*-bromophenacyl ester (X).

By combining the above experimental results, the chemical structure of astepyrone was constructed as II (Chart 1). This structure was also supported by the result of treatment of II with aq. ammonia solution at room temperature. Colorless needles (XI), mp 117—119°C, were obtained and the chemical formula was assigned as C_7H_9NO. The ^{1}H-NMR spectrum showed the presence of one methyl, one methoxyl, two ring protons and one NH group. This compound was positive to Ehrlich reagent (purple) and gave a mono semicarbazone (XII), mp 195°C. The UV spectrum of XI resembled that of 3-acetyl-4-methylpyrrole, and not that of 2-acetyl-4-methylpyrrole.[6] From the melting points of XI and XII, XI was identified as 3-acetyl-4-methylpyrrole (ref. mp 117—119°C;[7] semicarbazone, mp 195°C[8]).

Astepyrone was also reacted with two moles of 2,4-dinitrophenylhydrazine to afford a corresponding phenylhydrazone (XIII), mp 295—296°C. The reaction of astepyrone are summarized in Chart 1.

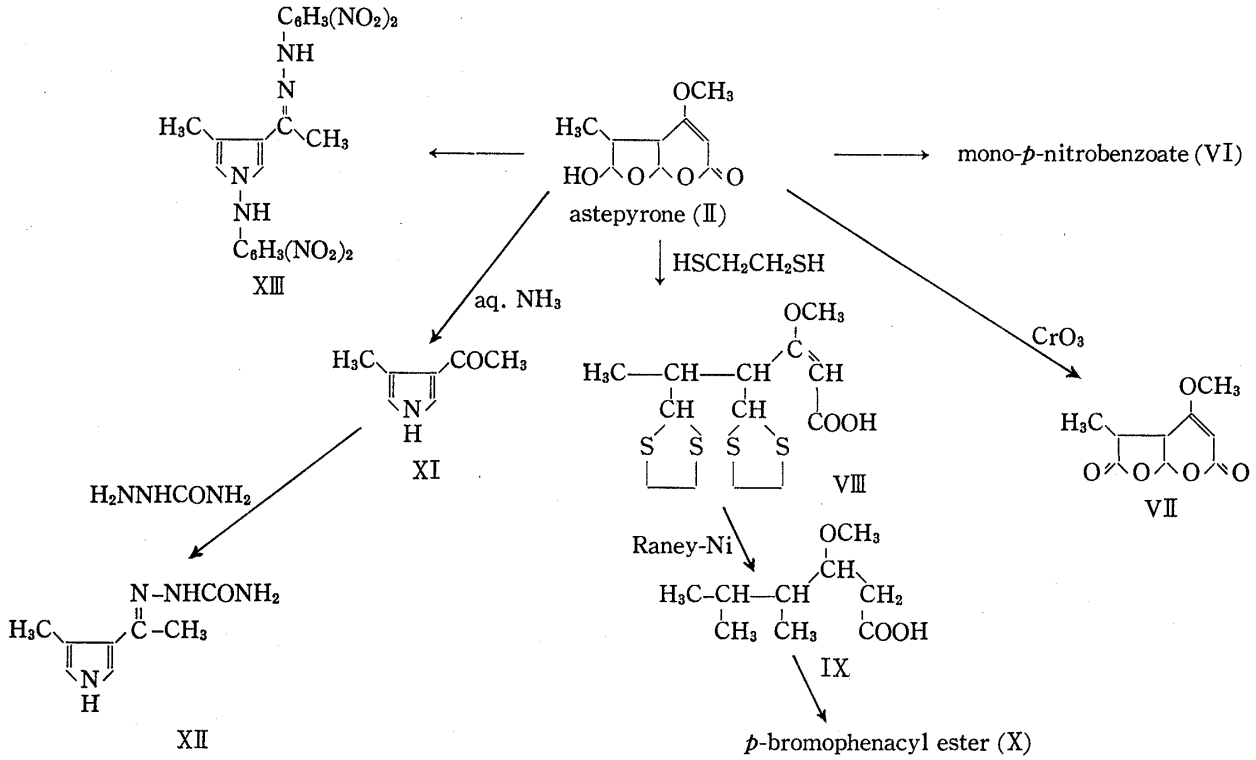
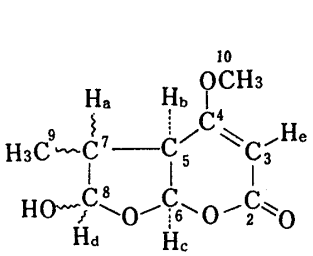
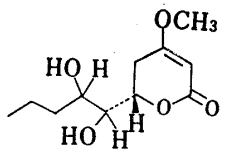


Chart 1. Reactions of Astepyrone

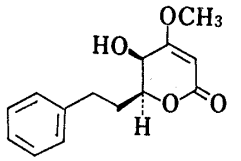
The stereochemistry of astepyrone was elucidated as IIa for the following reasons. The small coupling constant between H_b and H_c (*ca.* 5 Hz in Va and VII) showed that two rings are *cis*-fused, since a larger coupling would be expected for a *trans* junction. The large coupling between H_a and H_b (9—11 Hz in Va and VII) dose not rigidly establish the relationship of these two protons, since two cases (parallel and antiparallel) are possible for a five-membered ring. Whatever the configuration of the C-7-CH_3 group, the absolute stereochemistry at C-6 can be deduced from the n-π^* Cotton effect of the α,β-unsaturated lactone system.[9] Astepyrone (II) and the lactone derivative (VII) showed $\Delta\varepsilon$+19.6 and +21.0 at 247 nm, respectively, indicating *R*-configuration by comparison with compounds of known configura-



IIa



LL-p880β

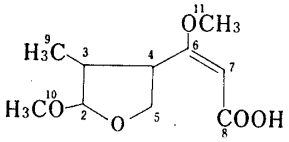


dihydrokawain-5-ol

Fig. 3

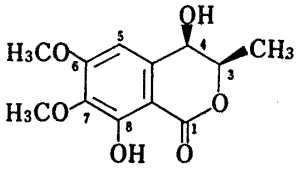
tion, dihydrokawain-5-ol ($\Delta\varepsilon$+11.5 at 247 nm[10]) and LL-p880β ($\Delta\varepsilon$ −12.5 at 247 nm[11]), both of which are believed to have half-chair conformation at the pentenolide system.[9]

Colorless needles (III), mp 154—156°C, were obtained by crystallization from *n*-hexane, $[\alpha]_D^{29}$ +79°. The MS and elemental analysis gave the chemical formula $C_{10}H_{16}O_5$. It was an acid (ν_{max} 1680 cm^{-1}; C=O) and dissolved in 10% $NaHCO_3$. The UV maximum at 233 nm indicated the existence of an α,β-unsaturated acid moiety in III. Two methoxyl groups were recognized in the ^{1}H-NMR spectrum and selective decouplings in the ^{13}C-NMR spectrum suggested that one methoxyl group was located on a quaternary carbon (δ 175.3), as in astepyrone. The relationships of five carbons were determined by ^{1}H-NMR spin-decoupling. This partial structure was very similar to that of astepyrone, though III had one more methoxyl group. III was negative to Tollens reagent and did not react with CrO_3. Consequently, the structure III was proposed (Fig. 4).



III

Fig. 4



IV

Fig. 5

Another new isocoumarin derivative (IV), mp 190—192°C, was isolated from the fraction after the butyrolactone (I)-epoxide on chromatography. It was crystallized from ether as colorless needles. The chemical formula was assigned as $C_{12}H_{14}O_6$ from the mass spectrum (M^+ 254 *m/z*) and elemental analysis. The UV spectrum (λ_{max} 219, 228 (shoulder), 273 and 307 nm) was very similar to those of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin and 3,4-dihydro-6-methoxy-8-hydroxy-3-methylisocoumarin.[12] IV had two methoxyl groups, one ring proton, and two hydroxyl groups, one of which was phenolic and located at the *o*-position relative to a carbonyl group (1648 cm^{-1} in IR, violet with $FeCl_3$). IV was refluxed with iodine and red phosphorus in AcOH to obtain the dehydrate (XIV), mp 193—195°C (ref. 193°C[12]), which was identified as 6,7-dimethoxy-8-hydroxy-3-methylisocoumarin by comparing the melting point and IR spectrum with those of an authentic sample. IV had a –CH(OH)–CH(CH_3)–O– moiety in the molecule and these two methine protons were coupled with J=2 Hz, which suggested *cis*-configuration.[13] The absolute configuration at C-3 was determined to be *R* from the negative Cotton effect at 273 nm.[14] From these results, the chemical struc-

ture of IV was determined to be (3*R*, 4*R*)-3,4-dihydro-4,8-dihydroxy-6,7-dimethoxy-3-methyl-isocoumarin (Fig. 5).

Astepyrone may be biosynthesized *via* the polyketide route. After the administration of [2-^{14}C]sodium acetate, the radioactivity was incorporated mainly in fatty acid (56.2% of the added activity; 70% of the activity in mycelium) and the incorporation ratio into astepyrone was unexpectedly low (0.5%) (*cf.* terrein 5.6%, butyrolactone (I) 0.85%). Determination of the distribution of ^{14}C-labelled carbons by the degradation method would therefore be very difficult.

Next, [1-^{13}C]sodium acetate was administered to the culture medium and culture was continued for two weeks. The ^{13}C-enriched astepyrone was oxidized to the corresponding lactone (VII) to produce a simpler NMR spectrum (see above). The obtained ^{13}C-NMR spectrum of enriched lactone was compared with the natural abundance ^{13}C-NMR of the lactone (VII). Each signal was assigned on the basis of the chemical shift values, the coupling patterns and selective decoupling experiments (*e.g.*, C-2 and C-4 were distinguished by comparing the chemical shifts and coupling patterns with those of β-methoxy-α,β-unsaturated-δ-lactone,[15] C-4 and C-8 were confirmed by selective decouplings at 9-CH_3 and 10-OCH_3 protons, respectively).

As shown in Fig. 6, four of the eight skeleton carbons were enriched in an alternating manner, which showed that astepyrone was biosynthesized by the polyketide route.

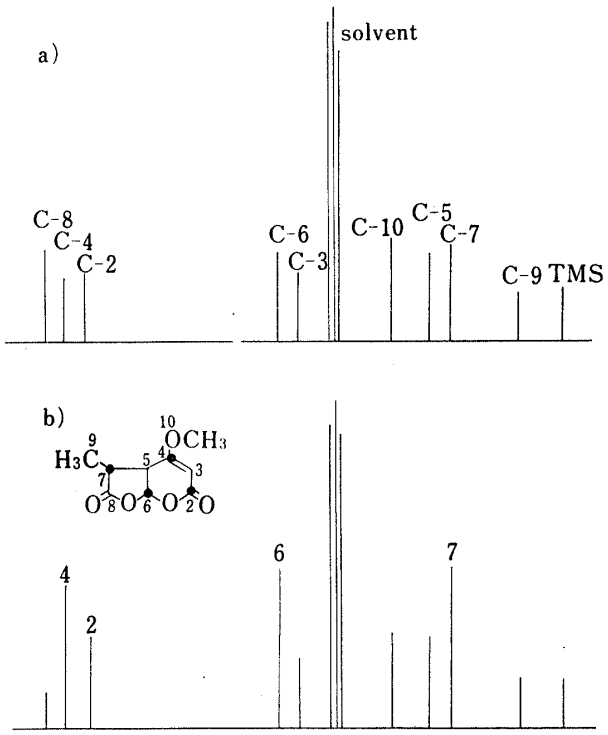
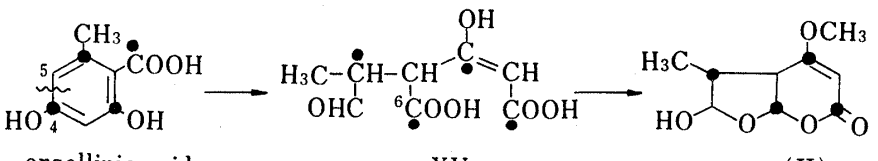


Fig. 6. Proton Noise-decoupled ^{13}C-NMR Spectra of VII (in $CDCl_3$)

a) Natural abundance.
b) Enriched with [1-^{13}C]-sodium acetate (●; enriched site).

From the structure and the distribution of ^{13}C-labelled carbons in astepyrone, it seemed likely that orsellinic acid might be a precursor; it is present in this culture medium (see above). If astepyrone were formed by oxidative cleavage of an orsellinic acid derivative at C-4-C-5, the pattern of ^{13}C-enriched carbon in astepyrone would be expected to coincide with the above experimental result. If orsellinic acid is a precursor, astepyrone must be biosynthesized *via* a rather unstable β-ketoacid (enol form of XV, Fig. 7), theoretically. In order to avoid decarboxylation at this step, a methoxyl group might be introduced before the ring-opening. Namely, orsellinic acid 2-methyl ether is also a potential precursor. Further, the C-6 carbonyl group might be derived from an aldehyde group, so orsellinic aldehyde might be another attractive precursor. These three radioactive aromatic compounds were prepared as follows.

[Aldehyde-^{14}C]orsellinic aldehyde was synthesized by the condensation of orcinol with



orsellinic acid XV astepyrone (II)

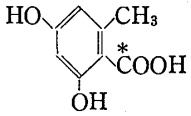
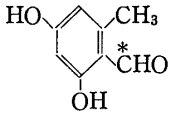
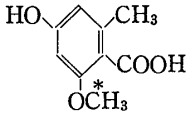
Fig. 7

[^{14}C]NaCN and $Zn(CN)_2$ according to Thomas's procedure.[16] [Carboxyl-^{14}C]orsellinic acid was synthesized by the oxidation of the above radioactive aldehyde by Hoesch's method.[17] [2-Methoxy-^{14}C]orsellinic acid 2-methyl ether was prepared by the reaction of 4-carboethoxyorsellinic acid ethyl ester with [^{14}C]CH_3I and then hydrolysis.

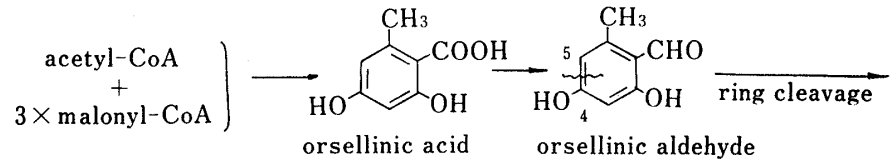
These compounds were each administered to the 5 (or 10 or 15)-day-old culture medium and cultures were continued for three weeks in total. After the harvest, the metabolites were isolated and assayed for radioactivity incorporated. The results are shown in Table I. Orsellinic acid 2-methyl ether was not incorporated to astepyrone, which suggested that methylation occurred after ring cleavage. The incorporations of orsellinic acid and aldehyde were almost the same and were high, which confirmed that these compounds could both act as precursors of astepyrone. When radioactive orsellinic acid was fed in the culture broth, the liberated CO_2 was radioactive. However, in the case of orsellinic aldehyde, the CO_2 had no radioactivity. This result showed that orsellinic aldehyde was not converted to orsellinic acid.

From these results, we concluded that orsellinic aldehyde was a more closely related intermediate than orsellinic acid, and the biosynthetic pathway of astepyrone was proposed to be

Table I. Incorporations of ^{14}C-Labelled Compounds to Astepyrone

Administered compound	Day of administration	Amount added ($\times 10^6$ dpm)	Incorporation into astepyrone		Distribution of radioactivity (%)			
			Amount (mg/l)	(%)	Culture broth	Mycelium	Respiratory CO_2	Total
*CH_3COONa	5	108	195	0.5	18	70	9	97
	5	1.56	308	8.2	53	4	24	81
	10	1.41	240	18.1	70	3	23	96
	15	1.64	186	15.3	51	5	30	86
	5	2.22	290	4.5	77	10	0	87
	10	2.10	266	22.2	90	7	0	97
	15	2.23	220	16.8	82	13	0	95
	5	2.10	266	0.5	85	3	0	88
	10	1.88	155	0.1	89	3	0	92
	15	1.78	88	0.4	90	2	0	92

* Labelled site.



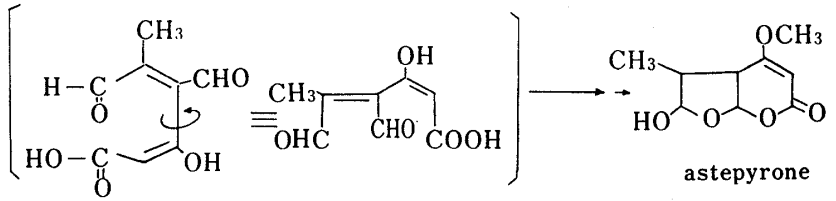


Chart 2. Biosynthesis of Astepyrone

as shown in Chart 2.

Astepyrone had anti-ulcerogenic activity in the rat, but also showed considerable toxicity.[18]

Experimental[19]

Culture Conditions——*Aspergillus terreus* IFO 4100 strain was cultivated stationarily in 500 ml Roux flasks containing 200 ml of the culture medium: glucose; 20 g, malt extract (Difco); 20 g, polypeptone (Daigo-Eiyo); 1 g, tap water; 1 l. After cultivation at 27°C for three weeks, the culture broth was filtered. The filtrate was concentrated to one-tenth of the initial volume under reduced pressure and extracted with organic solvent.

Metabolites from the AcOEt Extract——The AcOEt extract was concentrated and kept at room temperature to give colorless needles (astepyrone; II), mp 133—134°C (yield 50—100 mg/l), and terrein was obtained by further concentration of the mother liquor (yield 160—300 mg/l). Another crop of colorless needles (III), mp 154—156°C (yield 1—2 mg/l) was isolated by silica gel column chromatography (Wako gel C-200; eluting solvent, benzene–AcOEt (9: 1 v/v)) of the above extract.

Astepyrone (II)——This product was easily crystallized from benzene to afford colorless needles. *Anal.* Calcd for $C_9H_{12}O_5$: C, 53.99; H, 6.04. Found: C, 54.07; H, 6.06. MS *m/z*: 200 (M^+). UV λ_{max}^{EtOH} nm (log ε): 235 (4.06). IR ν_{max}^{KBr} cm^{-1}: 3334, 2884, 1675, 1663, 1630. ^{1}H-NMR (DMSO-d_6) δ: 3.7 , 3.76 (s, OCH_3), 5.11, 5.18 (s, C_3-H), 6.70 (br s, OH) and Fig. 2. ^{13}C-NMR (DMSO-d_6) δ: 11.9, 15.1 (q, 9-CH_3), 42.0, 44.9 (d, C-7), 44.9, 46.4 (d, C-5), 56.5, 56.7 (q, 10-CH_3), 88.5, 88.8 (d, C-3), 100.1, 101.0, 101.3 and 105.9 (each d, C-6 and C-8), 163.3 (s, C-2), 172.4, 172.9 (s, C-4). $[\alpha]_D^{14}$ +80.7° (c=0.9, pyridine). CD (c=0.03, EtOH) $\Delta\varepsilon^{20}$: +19.6 (247) (positive maximum).

***p*-Nitrobenzoate (VI) of Astepyrone**——A mixture of astepyrone (100 mg) and *p*-nitrobenzoyl chloride (140 mg) in pyridine (2 ml) was warmed on a water bath for 15 min. The product was crystallized from ether to obtain pale yellowish prisms (VI; 79 mg), mp 160°C (dec.). *Anal.* Calcd for $C_{16}H_{15}NO_8$: C, 55.01; H, 4.33; N, 4.01. Found: C, 55.03; H, 4.27; N, 3.87. MS *m/z*: 349 (M^+). $[\alpha]_D^{18}$ +65° (c=0.4, EtOH).

Oxidation of Astepyrone with Chromic Acid——A solution of astepyrone (100 mg) in AcOH (2 ml) was treated with CrO_3 (130 mg) in AcOH (2 ml), and the mixture was kept at room temperature for one hour. After decomposition of the excess of CrO_3 with MeOH, the reaction mixture was diluted with water and extracted with AcOEt. The extract was concentrated and the residue was crystallized from isopropyl ether to obtain colorless needles (VII, 95 mg), mp 98—99°C. *Anal.* Calcd for $C_9H_{10}O_5$: C, 54.54; H, 5.09. Found: C, 54.61; H, 5.06. MS *m/z*: 198 (M^+). UV λ_{max}^{EtOH} nm (log ε): 234 (4.09). IR ν_{max}^{KBr} cm^{-1}: 1804, 1726, 1636. ^{1}H-NMR (DMSO-d_6) δ: 1.26 (3H, d, J=6.9 Hz, CH_3), 3.00 (1H, dt, J=10, 6.9 Hz, C_7-H), 3.23 (1H, dd, J=10, 5.6 Hz, C_5-H), 3.79 (3H, s, OCH_3), 5.33 (1H, s, C_3-H), 6.38 (1H, d, J=5.6 Hz, C_6-H). ^{13}C-NMR ($CDCl_3$) δ: 14.7 (q, C-9), 38.6 (d, C-7), 44.3 (d, C-5), 56.8 (q, C-10), 89.3 (d, C-3), 97.0 (d, C-6), 161.7 (s, C-2), 170.4 (s, C-4), 175.2 (s, C-8). $[\alpha]_D^{27}$ +89° (c=0.7, EtOH). CD (c=0.02, EtOH) $\Delta\varepsilon^{20}$: −12.6 (226) (negative maximum), +21.0 (247) (positive maximum).

Reaction of Astepyrone with Ethanedithiol——BF_3-etherate (2 ml) was added to a solution of astepyrone (200 mg) in ethanedithiol (4 ml) under ice-cooling and the mixture was kept overnight at 4°C, then diluted with water and extracted with $CHCl_3$. The extract was separated by preparative thin-layer chromatography (TLC) (silica gel; benzene–AcOEt (3: 1 v/v)) and the product was crystallized from CCl_4 to obtain colorless prisms (VIII, 165 mg), mp 157—159°C. *Anal.* Calcd for $C_{13}H_{20}O_3S_4$: C, 44.29; H, 5.72. Found: C, 44.13; H, 5.63. MS *m/z*: 352 (M^+), 190, 132. UV λ_{max}^{EtOH} nm (log ε): 238 (4.01). IR ν_{max}^{KBr} cm^{-1}: 1668, 1590. ^{1}H-NMR ($CDCl_3$) δ: 1.12 (3H, d, J=6.8 Hz, CH_3), 2.46 (1H, m, –CH–CH_3), 3.13, 3.23 (each 4H, br s, –CH_2–CH_2–), 3.68 (3H, s, OCH_3), 4.39 (1H, dd, J=11, 4.2 Hz, –CH–), 4.68 (1H, d, J=5.2 Hz, –CH–), 4.73 (1H, d J=11 Hz, –CH–), 5.18 (1H, s, C=CH–). $[\alpha]_D^{18}$ −61° (c=1.1, EtOH).

Reduction of VIII with Raney-Ni——VIII (165 mg) was dissolved in EtOH (50 ml) and the solution was refluxed with W_2-Raney-Ni (prepared from 10 g of alloy) for 4 h. The Raney-Ni was collected and washed with EtOH. Since the product was found to be absorbed on the Raney-Ni, the latter was dissolved in 10% HCl and extracted with $CHCl_3$. The solvent was evaporated off and the residue was purified by preparative TLC (silica gel, benzene–AcOEt (4: 1 v/v)) to yield a pale yellow oil (IX, 45 mg). *Anal.* Calcd for $C_9H_{18}O_3$: C, 62.04; H, 10.41. Found: C, 61.92; H, 10.69. MS (In-beam EI method[20]) *m/z*: 175.1336 (Calcd for $C_9H_{19}O_3$ (M^++1), 175.1333), 159 (M^+−CH_3), 142 (M^+−OCH_3), 115 (M^+−CH_2COOH), 103 ($CH(OCH_3)CH_2COOH$), 70 ($C(CH_3)CH(CH_3)CH_3$), 43 (CH_3CHCH_3). ^{1}H-NMR ($CDCl_3$) δ: 0.77—0.98 (12H, m, 4×CH_3), 1.61 (2H, m, 2×CH), 2.48 (2H, m, –CH_2–), 3.36 and 3.38 (3H, each s, OCH_3), 3.69 (1H, m, –CH–), 10.17 (1H, br s, OH). Two methoxy peaks were recognized in the NMR spectrum; this suggested that IX was a mixture of diastereoisomers (ratio 65: 45). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1717. $[\alpha]_D^{29}$ +39° (c=0.8, EtOH).

***p*-Bromophenacyl Ester (X) of IX**——A mixture of IX (30 mg) and *p*-bromophenacyl bromide (47 mg) in EtOH (2 ml) was refluxed for one hour. The solvent was evaporated off and the residue was extracted with ether. A pale yellow oil (X, 48 mg) was obtained by preparative TLC (silica gel, benzene). MS (In-

beam EI method) *m/z*: 373, 371, 301, 299, 198, 196, 186, 184, 175, 115, 85. IR ν_{max}^{KBr} cm^{-1}: 1735, 1698.

Treatment of Astepyrone with aq. Ammonia——Astepyrone (200 mg) was dissolved in 30% NH_4OH (8 ml) and the solution was kept at room temperature for 20 min, then acidified with 10% HCl and extracted with ether. The solvent was removed and the residue was recrystallized from ligroin to obtain colorless prisms (XI, 29 mg), mp 117—119°C. *Anal.* Calcd for C_7H_9NO: C, 68.27; H, 7.37; N, 11.37. Found: C, 68.24; H, 7.34; N, 11.26. MS *m/z*: 123 (M^+), 108, 80. UV λ_{max}^{EtOH} nm (log ε): 249 (3.92), 280 (shoulder) (3.40). IR ν_{max}^{KBr} cm^{-1}: 3200, 1625. ^{1}H-NMR ($CDCl_3$) δ: 2.32 (3H, d, J=1. Hz, CH_3), 2.40 (3H, s, $-COCH_3$), 6.55 (1H, m, C_2-H), 7.35 (1H, dd, J=3.5, 2.0 Hz, C_5-H), 8.59 (1H, br s, NH).

Semicarbazone (XII) of XI——A mixture of XI (66 mg), semicarbazide·HCl (96 mg) and NaOAc (74 mg) in EtOH was refluxed for 2 h. The reacted solution was extracted with AcOEt and the extract was concentrated. The residue was washed with ether and the insoluble part was crystallized from MeOH to obtain pale yellowish needles (XII, 31 mg), mp 193—195°C. *Anal.* Calcd for $C_8H_{12}N_4O$: C, 53.32; H, 6.71; N, 31.09. Found: C, 53.22; H, 6.54; N, 30.81. MS *m/z*: 180 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3490, 3420, 3200, 1683.

Reaction of Astepyrone with 2,4-Dinitrophenylhydrazine (2,4-DNP)——Astepyrone (100 mg) was dissolved in EtOH and 2,4-DNP–H_2SO_4 reagent (2,4-DNP 0.4 g, conc. H_2SO_4 2 ml, EtOH 10 ml, H_2O 3 ml) (6 ml) was added. The mixture was kept at room temperature for several hours. The precipitated crystals were collected by filtration and recrystallized from MeOH–THF to obtain red prisms (XIII, 106 mg), mp 295—296°C. *Anal.* Calcd for $C_{19}H_{16}N_8O_8$: C, 47.11; H, 3.33; N, 23.13. Found: C, 47.34; H, 3.35; N, 22.86. MS *m/z*: 484 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3450, 1615.

Metabolite III——Colorless needles from *n*-hexane, mp 154—156°C. *Anal.* Calcd for $C_{10}H_{16}O_5$: C, 55.54; H, 7.46. Found: C, 55.35; H, 7.68. MS *m/z*: 216 (M^+), 185, 127, 114. IR ν_{max}^{KBr} cm^{-1}: *ca.* 3000 (COOH), 1680 (C=O), 1600 (C=C). UV λ_{max}^{EtOH} nm (log ε): 233 (4.06). ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, d, J=7.1 Hz, CH_3), 2.44 (1H, ddq, J=10, 4.6, 7.1 Hz, C_3-H), 3.36 (3H, s, OCH_3), 3.69 (3H, s, C_{11}-OCH_3), 3.80 (1H, dd, J=7.6, 7.6 Hz, C_5-H), 4.15 (1H, dd, J=9, 7.6 Hz, C_5-H), 4.41 (1H, ddd, J=10, 9, 7.6 Hz, C_4-H), 4.85 (1H, d, J=4.6 Hz, C_2-H), 5.14 (1H, s, C_7-H). ^{13}C-NMR (The coupling constants were obtained from the spectrum measured by non decoupling with NOE technique) ($CDCl_3$) δ: 11.6 (qm, J_{CH}=125.5 Hz, C-9), 42.8 (dm, J_{CH}=132.4 Hz, C-3), 44.8 (dm, J_{CH}=133.3 Hz, C-4), 54.8 (qd, J_{CH}=143.0 Hz, C-10), 56.1 (q, J_{CH}=145.0 Hz, C-11), 69.8 (tm, J_{CH}=148.5 Hz, C-5), 92.9 (d, J_{CH}=147.5 Hz, C-7), 106.7 (dm, J_{CH}=167.7 Hz, C-2), 172.6 (s, C-8), 175.3 (sm, C-6). $[\alpha]_D^{29}$ +79° (c= 0.45, EtOH).

(3*R*,4*R*)-3,4-Dihydro-4,8-dihydroxy-6,7-dimethoxy-3-Methylisocoumarin (IV)——This metabolite was isolated by silica gel column chromatography as the fraction after the epoxy derivative of butyrolactone (I). It was crystallized from ether as colorless needles, mp 190—192°C. *Anal.* Calcd for $C_{12}H_{14}O_6$: C, 56.69; H, 5.55. Found: C, 56.62; H, 5.67. MS *m/z*: 254 (M^+). UV λ_{max}^{EtOH} nm (log ε): 219 (4.38), 228 (shoulder) (4.34), 273 (4.12), 307 (3.63). IR ν_{max}^{KBr} cm^{-1}: 3480, 1648. ^{1}H-NMR (acetone-d_6) δ: 1.49 (3H, d, J=6.5 Hz, CH_3), 3.76 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.62 (1H, d, J=2 Hz, C_4-H), 4.70 (1H, br s, alcoholic OH), 4.73 (1H, qd, J=6.5, 2 Hz, C_3-H), 6.75 (1H, s, C_5-H), 11.24 (1H, s, phenolic OH). CD (c=0.04, EtOH) $\Delta\varepsilon^{18}$: −1.42 (273) (negative maximum).

Dehydration of IV——Iodine (5 mg) and red phosphorus (5 mg) in AcOH (1 ml) were added to a solution of IV (10 mg) in AcOH (1 ml), and the mixture was refluxed for 2 h, then cooled. The solution was filtered and concentrated, and the residue was crystallized from ether to obtain colorless needles (XIV, 6 mg), mp 193—195°C (ref. mp 192—193°C[21]). The acetate, mp 184—186°C (ref. 185—186°C[21]), of XV was obtained by treatment of XIV with Ac_2O and pyridine.

[Aldehyde-^{14}C]Orsellinic Aldehyde——A mixture of [^{14}C]NaCN (250 μCi; New England Nuclear) and $Zn(CN)_2$ (1.8 g) was added to a solution of orcinol (1.3 g) in dry ether (15 ml), and the mixture was stirred for 2 h under a dry HCl stream. The ether solution was removed by decantation, and water (10 ml) was added to the residue. This mixture was boiled for a few minutes, then cooled. The precipitate was collected by filtration and crystallized from water to obtain colorless needles (1.02 g; 4.1×10^4 dpm/mg), mp 187—189°C (ref. 181°C[16]). ^{1}H-NMR (acetone-d_6) δ: 2.45 (3H, s, CH_3), 6.12 (2H, m, aromatic-H), 9.98 (1H, s, CHO), 12.25 (2H, s, OH).

[Carboxyl-^{14}C]Orsellinic Acid——[^{14}C]Orsellinic aldehyde (800 mg) and ethyl chlorocarbonate (1.1 ml) in acetone (3.2 ml) was treated with 1 N NaOH under ice-cooling and stirring. After 40 min, the mixture was brought to room temperature and held there for several hours. The precipitate was collected by filtration and crystallized from petr. benzin to afford colorless prisms (1.07 g), mp 60—61°C (ref. 58°C[16]). $KMnO_4$ solution (0.94 g in 16 ml of water) was added dropwise to the above bis(ethoxycarbonyl) derivative (1.07 g) in acetone (9.4 ml) at 40°C and the mixture was allowed to react for 45 min. The precipitated MnO_2 was removed by filtration and the filtrate was acidified with conc. H_2SO_4 to afford colorless needles (940 mg), mp 117—119°C (ref. mp 115°C[16]). The above crude bis(ethoxycarbonyl) orsellinic acid (790 mg) was dissolved in 1 N NaOH (12.6 ml) and the solution was kept for 1.5 h at room temperature. Acidification of the reaction mixture with conc. HCl resulted in the precipitation of a white solid, which was collected and precipitated from AcOEt–benzene as a colorless powder (270 mg, 3.8×10^4 dpm/mg), mp 186—189°C (dec.) (ref. 184—185°C[16]).

[2-Methoxy-^{14}C]Orsellinic Acid 2-Methyl Ether——4-Ethoxycarbonylorsellinic acid ethyl ester (270 mg), mp 47—50°C (prepared from orsellinic acid ethyl ester and ethyl chlorocarbonate) was refluxed with [^{14}C]CH_3I

(100 μCi; Amersham International Ltd.), cold CH_3I (0.1 ml) and K_2CO_3 in acetone (5 ml) for 6 h. The reaction mixture was filtered and purified by preparative TLC (acid-washed silica gel, AcOEt–benzene (1: 30 v/v)) to afford 2-methoxy-4-ethoxycarbonylorsellinic acid ethyl ester as a colorless oil (210 mg). This was treated with conc. H_2SO_4 (0.55 ml) at 27°C for 5 h. The reaction mixture was then diluted with water and extracted with ether. The ether extract was evaporated to dryness and the residue was crystallized from petr. benzin–AcOEt to afford colorless prisms (90 mg), mp 100—104°C. The obtained 4-ethoxycarbonyl-2-methoxyorsellinic acid was dissolved in 4 N NaOH (1.4 ml) and reacted at room temperature for 2 h. The reaction mixture was acidified and the precipitate was collected and crystallized from *n*-hexane–AcOEt to yield a colorless powder (39 mg, 4.26×10^5 dpm/mg), mp 170—173°C (dec.) (ref. mp 175—177°C[21]).

Incorporation of [2-^{14}C]Sodium Acetate into Metabolites——[2-^{14}C]Sodium acetate (50 μCi; Amersham International Ltd.) in water (50 ml) was administered to 5-d old culture broth in two Fernbach flasks (800 ml each). After cultivation for 3 weeks, the metabolites were isolated and the radioactivity was assayed with a liquid scintillation spectrometer (Aloka, LSC-651) in dioxane scintillator. The radioactivity of mycelia was determined by the combustion method (Packard model 306). The yields of terrein (1.282 g) and astepyrone (313 mg) were calculated by the dilution method and the incorporations were 5.6% and 0.5%, respectively.

Incorporation of [1-^{13}C]Sodium Acetate into Astepyrone——[1-^{13}C]Sodium acetate (500 mg; 90% isotopic purity) in water (18 ml) was divided in three portions and added to the culture medium (1.8 l) at the 5th, 7th and 9th d. Culture was continued for 14 d. The isolated astepyrone (14 mg) was oxidized with CrO_3 to the lactone (VII).

Incorporation of [Aldehyde-^{14}C]Orsellinic Aldehyde into Astepyrone——[Aldehyde-^{14}C]orsellinic aldehyde (2.22×10^6 dpm) in water (20 ml) was added to the culture medium (500 ml) in a Fernbach flask at the 5th d and cultivated for 3 weeks. Astepyrone was isolated and its radioactivity was assayed. The total amount of the metabolite was calculated by the dilution method. The CO_2 liberated during the fermentation was trapped in 10% NaOH and assayed, but no radioactivity was recognized. The mycelia were extracted with ether and 90% of the radioactivity in the mycelia was extracted. However, no major radioactive compound was recognized on Radio-TLC scanning.

The results of administration to 10-d and 15-d cultures were also studied. [^{14}C]Labelled orsellinic acid and orsellinic acid 2-methyl ether were also administered to cultures by the same procedure.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, which is gratefully acknowledged. We thank the Institute for Fermentation, Osaka, for supplying the IFO strain. We also thank Misses Y. Teranishi and T. Ueda for elementary analyses and spectral measurement, and Hokuriku Pharm. Co., Ltd. for the assay of radioactivity of mycelia and bioactivity. A part of this work was carried out by Miss M. Neyama, to whom we are grateful.

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