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Studies on Metabolites of *Macrophoma commelinae*. III. Isolation of New Metabolites and Biosynthesis of Macommelin Group[1)]

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Three new metabolites, named macommelinal, macrophin and macrophic acid, were isolated from the culture broth of *Macrophoma commelinae* IFO 9570. Their structures were determined to be 2-(4-methoxy-6-methyl-2-oxo-2*H*-pyran-5-yl)ethanal, 3-methyl-2-butenoic acid (*E*)-[5-hydroxymethyl-4-methoxy-6-(2-methoxycarbonylethenyl)-2-oxo-2*H*-pyran-3-yl]methyl ester and 3-(4-methoxy-3,5-dimethyl-2-oxo-2*H*-pyran-6-yl)-2-propenoic acid, respectively. 4-Acetyl-3-methoxy-5-methylbenzoic acid, now named macrophomic acid, was also isolated from a large-scale culture.

The biosynthesis of the macommelin group, novel 5-substituted 2-pyrone metabolites, has been investigated by feeding experiments with [1-^{13}C]-, [1,2-$^{13}C_2$]- and [1-^{13}C, 2-$^{2}H_3$]acetate and [2-^{13}C]malonate. It was concluded that all these metabolites originated from a single straight tetraketide chain. Furthermore, the biogenetic mutual relationships of these metabolites including potential intermediates were established through incorporation experiments with ^{14}C-labeled compounds.

Keywords——*Macrophoma commelinae* IFO 9570; fungi; metabolic product; macommelinal; macrophin; macrophic acid; macrophomic acid; biosynthesis; macommelin group; 2-pyrone

Macrophoma sp., one of the phytotoxic fungi, causes the black rotting of chestnut tree root and the fruit rot diseases of apple and some other plants.[2)] Previously, we reported the isolation of four novel metabolites (**1**—**4**), generically named macommelins,[3)] and rosellisin (**8**)[4)] from the culture broth of *Macrophoma commelinae* (IFO 9570) and presented the structures of these metabolites.[1a)] In this paper, we describe the isolation and the structures of three new metabolites, and also the biosynthesis of the macommelin group.

The culture filtrate of *M. commelinae* was concentrated *in vacuo* and extracted with ethyl acetate (AcOEt). The AcOEt extract was chromatographed on a silica gel column using $CHCl_3$–methanol (MeOH) as described in part I of this series.[1a)] Three new metabolites named macommelinal (**5**), macrophin (**6**) and macrophic acid (**7**), were newly isolated. The yields of these minor metabolites, **5**—**7**, were about 30, 70 and 20 mg from 6 l of the culture broth, respectively.

Macommelinal (**5**), colorless needles, mp 143—146 °C (from CCl_4), had the molecular formula $C_9H_{10}O_4$, indicating two hydrogen atoms less than macommelinol (**3**), and the ultraviolet (UV) spectrum was similar to those of **1**—**4**. The presence of an aldehyde group was indicated from the infrared (IR) (2833, 2721 and 1714 cm^{-1}) and proton nuclear magnetic resonance (^{1}H-NMR) (δ 9.45) spectra, and confirmed by derivatizing it to a semicarbazone (**10**, mp 208.5—210.5 °C). Reduction of **5** with $NaBH_4$ in ethanol (EtOH) yielded **3**, and **5** was recovered from **3** by oxidation with dimethyl sulfoxide (DMSO)/dicyclohexylcarbodiimide (DCC) or pyridinium chlorochromate (PCC). From these results the structure of **5** was determined to be 2-(4-methoxy-6-methyl-2-oxo-2*H*-pyran-5-yl)ethanal.

Macrophin (**6**), colorless microcrystals, mp 118—121 °C (from ether–petroleum ether), had the molecular formula $C_{17}H_{20}O_8$ as determined by elemental and mass spectral (MS)

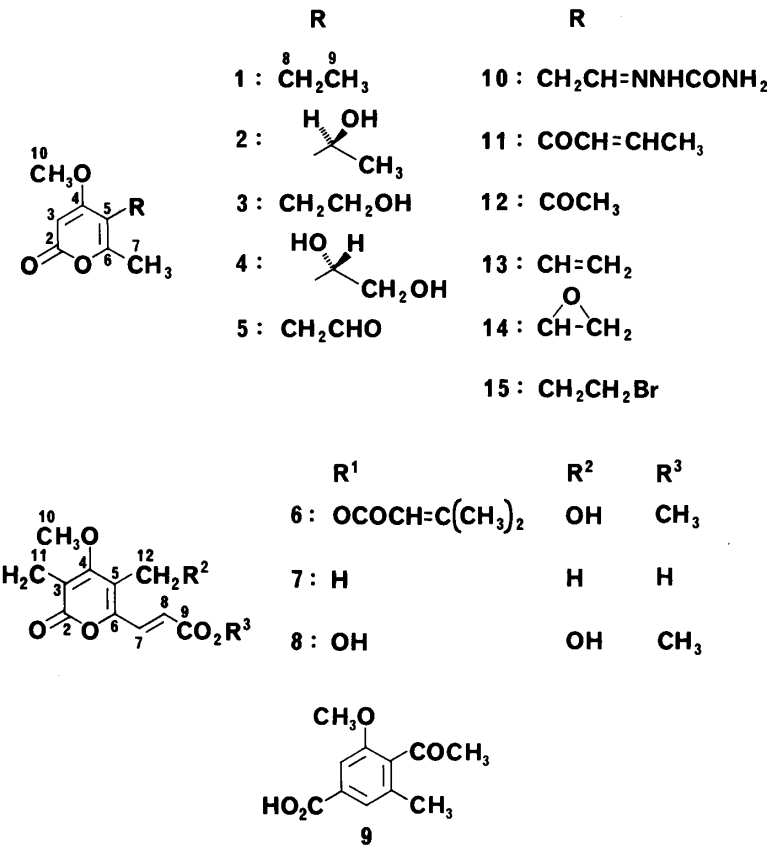

Chart 1

analyses. Its UV and IR spectra were similar to those of rosellisin (**8**, $C_{12}H_{14}O_7$).[4] In the ^{1}H-NMR spectrum, one of the methylene protons (δ 4.52) of **8** was shifted to lower field (δ 4.99). Further, the additional signals at δ 1.85 (3H, d, J=1.6 Hz), 2.12 (3H, d, J=1.2 Hz) and 5.57 (1H, m) suggested the presence of a $(CH_3)_2C=CHCO$ moiety. This partial structure was strongly supported by the peaks at m/z 83 ($[(CH_3)_2C=CHCO]^+$) and 269 ($[M^+-(CH_3)_2C=CHCO]$) in the MS. From these data, **6** was indicated to be the mono-3-methyl-2-butenoate of **8**. The position of the 3-methyl-2-butenoyl moiety was determined by ^{13}C-{^{1}H} long-range selective proton decoupling experiments.[5] Low-power irradiation of the $-C\underline{H}_2OH$ protons (δ 4.52) or $-CO_2CH_2-$ protons (δ 4.99) enhanced the intensity of the carbon signal at δ 118.1 (C-5) or 109.6 (C-3), respectively; these assignments were based on the literature values.[6] These results suggested that the 3-methyl-2-butenoyl moiety was substituting the $-CH_2O-$ group at C-3 of the pyrone ring. This conclusion was confirmed from the observation of the nuclear Overhauser effect (NOE) between $-C\underline{H}_2OH$ (δ 4.52) and 7-H (δ 7.46). Thus, the structure of **6** was determined to be as shown in Chart 1.

Macrophic acid (**7**), mp 200.5—203.5 °C, was obtained as colorless microcrystals from $CHCl_3$. Its molecular formula, $C_{11}H_{12}O_5$, was determined by elemental and MS analyses. The UV absorption spectrum was similar to that of **8** and the IR spectrum suggested the existence of a carboxyl group. In the ^{1}H-NMR spectrum, the signals of methoxyl (δ 3.78) and olefinic (δ 6.27 and 7.27) protons of **8** were observed. In addition, two methyl protons (δ 1.96 and 2.04) and one carboxyl proton (δ 12.3, exchangeable with D_2O) were recognized instead of two hydroxymethyl and one methoxycarbonyl protons in **8**. From these data, **7** was determined to be 3-(4-methoxy-3,5-dimethyl-2-oxo-2*H*-pyran-6-yl)-2-propenoic acid.

A trace amount (5 mg) of a benzoic acid derivative named macrophomic acid (**9**, mp 178.5—182 °C) was isolated from a large-scale culture (36 l). This acid was identified by

comparison with an authentic sample biotransformed from **12** as reported by us.[1b]

The isolated metabolites of macommelins (**1**—**5**) have the unique 5-substituted 2-pyrone skeleton in common, indicating a close biogenetic relationship. Yamamura *et al.*[7] proposed that citreopyrone (=pyrenocine A) (**11**), a congener of the macommelin group, is derived from two polyketide chains. On the other hand, Turner and Aldridge[8] claimed that it must be derived from a single polyketide chain.

To verify the biosynthetic origin of macommelins, feeding experiments with [1-^{13}C]- and [1,2-$^{13}C_2$]acetate were performed. Sodium [1-^{13}C]acetate (90 atom%; 1.1 g) was administered to stationary cultures (1 l) of *M. commelinae* on the 8th day after inoculation. On the 21st day, the cultures were harvested and the metabolites were isolated by AcOEt extraction followed by silica gel chromatography. In the proton-noise-decoupled carbon-13 nuclear magnetic resonance (p.n.d. ^{13}C-NMR) spectrum of the labeled macommelinol (**3**), the signals of C-2, C-4, C-6 and C-8 were enhanced approximately five-fold, as shown in Table I.

Sodium [1,2-$^{13}C_2$]acetate (91 : 90 atom%; 200 mg) was administered to the cultures (400 ml) after dilution with equivalent non-labeled sodium acetate. In the ^{13}C-NMR spectrum of the obtained **3**, all carbon atoms except C-10 showed the ^{13}C–^{13}C doublet together with the natural abundance singlet; the doublets could be paired unambiguously on the basis of their coupling constants (Table I) and the resulting labeling pattern is consistent with a tetraketide origin as shown in Chart 2. Similar results were obtained with macommelindiol (**4**) (Table II).

To make clear whether macommelins were derived from a straight or a branched tetraketide chain, the starter of the polyketide chain was examined by cultivation with diethyl [2-^{13}C]malonate.[9] The spectrum of the resulting **3** showed high enrichment in three positions, C-3, C-5 and C-7, while that at C-9 was distinctly low (Table I). Thus, the acetate 'starter' effect is clearly observed in only one position (C-9), indicating that macommelinol is biosynthesized through an intermediate consisting of a single straight tetraketide chain as proposed by Turner, followed by the oxidative cleavage of the benzene ring and rearrangement (Chart 2).

Sodium [1-^{13}C,2-$^{2}H_3$]acetate (90 atom% ^{13}C, 99.1 atom% ^{2}H; 900 mg) was administered to the culture medium (1.2 l). Metabolites **2**, **3** and **4** were isolated from the culture broth and the ^{13}C-NMR spectra were measured. As shown in Fig. 1a, C-8 of **2** showed three isotopically shifted signals ($\Delta\delta$ −0.040, −0.094 and −0.140), which suggested the presence of one, two and three deuterium atoms on the adjacent methyl carbon, respectively. Similarly, C-8 of **4** showed two β-shifted signals ($\Delta\delta$ −0.047 and −0.100) (Fig. 1b). On the other hand, C-8 of **3** consisted of one β-shifted singlet ($\Delta\delta$ −0.094), one α-shifted triplet ($\Delta\delta$ −0.308, $^{1}J_{CD}$=

TABLE I. ^{13}C-NMR Data for Labeled Macommelinol (**3**)

Carbon No.	δ[a] (ppm)	Enrichment[b]		$J_{C\text{-}C}$ (Hz)
		Sodium [1-^{13}C]acetate	Diethyl [2-^{13}C]malonate[c]	Sodium [1,2-$^{13}C_2$]acetate
2	164.7	5.3	0.8	77.9
3	87.8	0.9	2.7	77.9
4	170.9	4.6	0.7	60.3
5	108.3	0.8	2.3	60.3
6	159.8	4.8	0.7	52.9
7	17.6	1.1	2.3	52.9
8	27.8	6.6	0.8	36.8
9	61.3	1.0	1.8	36.8
10	56.2	1.0	1.0	—

a) Relative to internal Me_4Si in $CDCl_3$. *b*) Ratio of the signal intensity for enriched and natural abundance that is normalized for the C-10 signal. *c*) Average value of three experiments.

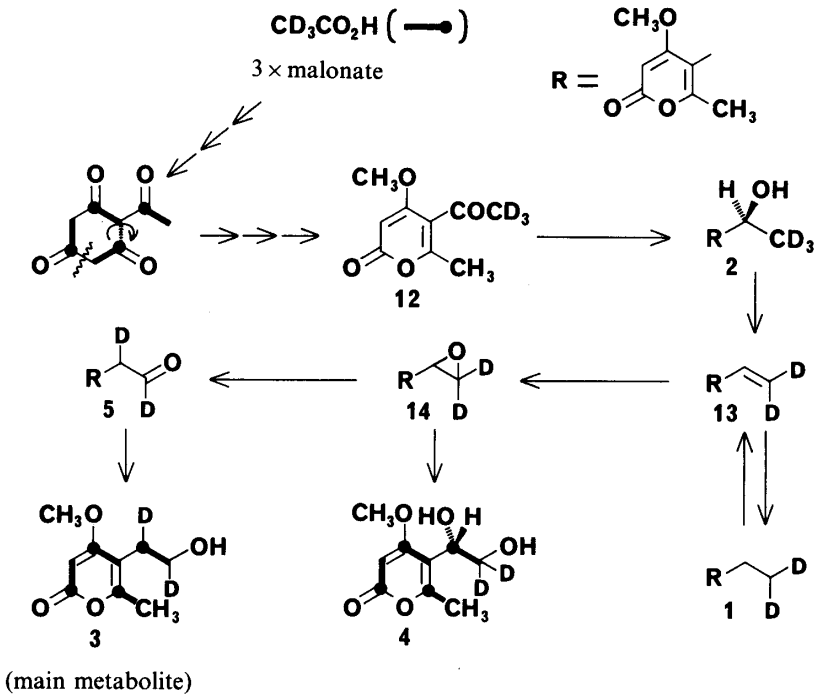

Chart 2

TABLE II. ^{13}C-NMR Data for Labeled Macommelindiol (**4**)

Carbon No.	δ [a] (ppm)	Enrichment [b]	$J_{C\text{-}C}$ (Hz)
		Sodium [1-^{13}C]acetate	Sodium [1,2-$^{13}C_2$]acetate
2	162.9	5.5	78.0
3	87.1	1.1	78.0
4	170.1	8.1	61.8
5	111.7	0.8	61.8
6	160.3	4.1	51.5
7	18.1	1.3	51.5
8	66.2	6.8	41.2
9	64.2	0.9	41.2
10	56.4	1.0	—

a) Relative to internal Me_4Si in DMSO-d_6. *b*) Ratio of the signal intensity for enriched and natural abundance that is normalized for the C-10 signal.

19.5 Hz) and one α- and β-shifted triplet ($\Delta\delta$ −0.401, $^1J_{CD}$ = 19.8 Hz), which correspond to the $^{13}CH_2CHD$, $^{13}CHDCH_2$ and $^{13}CHDCHD$ moieties, respectively (Fig. 1c). These α- and β-shift values and the coupling constants are compatible with the reported data.[10] In the signals of C-2 and C-6 of the above three metabolites, the shapes were almost the same as those of the natural abundance ones, so the presence of the shifted signal was obscure. These results suggest that the epoxide **14** acts as an intermediate on which a 1,2-hydride shift occurs in the pathway from **14** to **3**, as reported in the metabolism of styrene and rishitin.[11,12]

The mutual relationship of the metabolites of *M. commelinae* and postulated intermediates was clarified by the following incorporation experiments with ^{14}C-labeled compounds.

[Methoxy-^{14}C] **12** was chemically prepared by using ^{14}C-methyl iodide (see Experi-

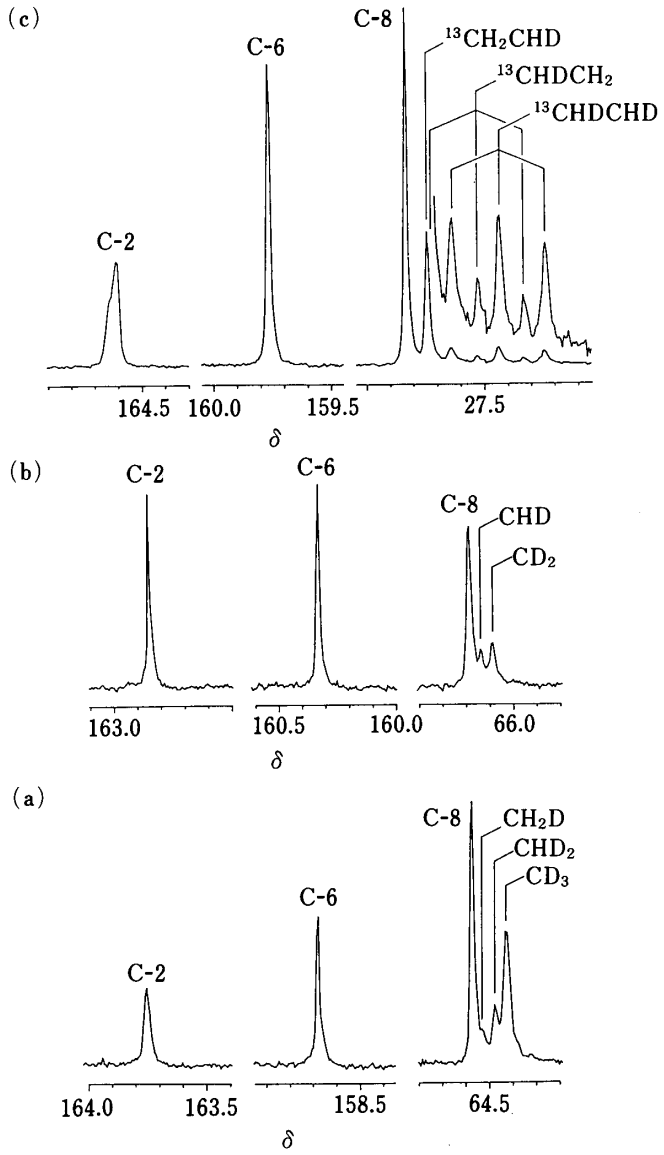

Fig. 1. Signals from p.n.d. ^{13}C-NMR Spectra of (a) **2**, (b) **4** and (c) **3** Derived from [1-^{13}C, 2-$^{2}H_3$]Acetate

mental). The ^{14}C-labeled **2**—**4** were obtained by cultivation with sodium [1-^{14}C]acetate. Four other compounds (**1**, **5**, **13** and **14**) were derived from ^{14}C-labeled **3**. Each labeled compound was administered on the 10th day after inoculation and harvested on the 21st day. The incorporation ratios into metabolites **2**, **3** and **4** are summarized in Table III. Compounds **3** and **4** both showed high recoveries and were not converted to any other metabolite, indicating that these were terminal compounds in this metabolic pathway.

Compound **2** was not formed from other compounds except **12**. This suggests that **12** and **2** are located at an early step in the metabolic pathway. The radioactivities of **2** (9.7%), **5** (91.7%) and a postulated intermediate **13** (52.9%) were well incorporated into the main product **3**. The high incorporation of the aldehyde **5** showed it to be the immediate precursor of **3**. Though **5** was supposed to be biosythesized through 1,2-hydride shift of the epoxy compound **14** as described above, the radioactivity of **14** was taken into **3** at a low ratio (2.5%) and was mainly incorporated into the dihydroxy compound **4** (76.0%). This result may be caused by spontaneous hydrolysis of the epoxy ring in this feeding experiment.

Though the minor metabolite **1** seems to be biosynthesized from the potential intermediate **13**, the reduction of **13** to **1** was not detectable. Although the ω-oxidation of **1** to **3** was not recognized in the experiment with [1-^{13}C,2-$^{2}H_3$]acetate, compound **1** was mainly

TABLE III. Incorporation Ratio of ^{14}C-Labeled Compounds

Administered compound	Radioactivity (μCi)	Incorporation ratio (%)		
		2	3	4
1	4.32	u.d.	71.2	3.5
2	1.29	82.8	9.7	0.3
3	2.85	u.d.	84.5	1.0
4	2.26	u.d.	0.4	77.7
5	3.38	u.d.	91.7	3.1
12[a)]	9.41	14.7	1.6	0.3
13	2.96	u.d.	52.9	6.3
14	2.82	u.d.	2.5	76.0

u.d. = undetected. *a*) 80.9% of the radioactivity was incorporated into macrophomic acid (**9**).

converted to **3** in a high ratio (71.2%). These phenomena may be explained by reversibility between **13** and **1**.

The methyl ketone derivative **12** was mainly transformed to macrophomic acid (**9**) (80.9%) together with a reduced product **2** (14.7%), which possessed the same chirality as the metabolite isolated in the usual cultivation. In spite of having a capacity to convert the exogeneous methyl ketone **12** to macrophomic acid (**9**) in good yield, this fungus scarcely produces **9** in the normal culture. It is supposed that the endogeneous methyl ketone **12** may exist in an enzyme-bound form.

Combined with the incorporation behavior of added [1-^{13}C,2-$^{2}H_3$]acetate, the biosynthetic pathway of macommelins, including postulated intermediates, is proposed to be as shown in Chart 2.

Experimental

Melting points were taken on a Yanagimoto micro hot-stage melting point apparatus and are uncorrected. UV spectra were measured with a Hitachi 323 spectrometer, IR spectra were taken as KBr discs using a Jasco A-202 spectrometer, ^1H-NMR spectra were recorded on JNM-PMX 60 (60 MHz) and JEOL FX-100 (100 MHz) spectrometers with tetramethylsilane (Me_4Si) as an internal reference, and ^{13}C-NMR spectra were measured with JEOL FX-100 (25 MHz) and JNM-GX 400 (100 MHz) spectrometers. The following abbreviations are used: sh, shoulder; s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Optical rotations were measured with a Jasco DIP-181 digital polarimeter. MS were recorded on a Hitachi M-80 spectrometer. Wakogel C-200, Kieselgel 60 GF_{254} and Kieselgel 60 PF_{254} were used for column chromatography, thin-layer chromatography (TLC) and preparative TLC (prep. TLC), respectively. Stable isotopes and radioisotopes were purchased from MSD, Canada and New England Nuclear, respectively. ^{14}C Radioactivity was assayed with a liquid scintillation spectrometer (Aloka LSC-651; 7 g of 2,5-diphenyloxazole (PPO), 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP) and 100 g of naphthalene in 1 l of dioxane as the scintillator).

Isolation——*Macrophoma commelinae* IFO 9570 was grown stationarily in 500 ml Roux flasks containing 200 ml of a malt extract medium consisting of malt extract (20 g, Difco Laboratories), glucose (20 g, Daiich Seiyaku), polypepton (1 g, Daigo Eiyo) and tap water (1 l) at 27 °C. The culture was harvested after 21 d. The culture filtrate (6 l) was concentrated to one-third of the initial volume *in vacuo* and extracted with AcOEt (330 ml × 3). The AcOEt layer was dried (Na_2SO_4) and evaporated. The AcOEt extract (1.8 g) was redissolved in $CHCl_3$ and applied to a silica gel column (3.7 × 33 cm), which was eluted stepwise with increasing proportions of MeOH (0, 0.5, 1, 2.5 and 5% (v/v) MeOH) in $CHCl_3$ and monitored by TLC.

The 0.5% MeOH–$CHCl_3$ eluted fraction was subjected to prep. TLC with MeOH–$CHCl_3$ (1 : 19). Recrystallization from ether–petroleum ether yielded macrophin (**6**) (70 mg) as colorless microcrystals.

The 1% MeOH–$CHCl_3$ eluted fraction was crystallized from CCl_4 to obtain macommelinal (**5**) (30 mg) as colorless needles.

The earlier fraction eluted with 2.5% MeOH–$CHCl_3$ was suspended in cold $CHCl_3$ and filtered. The $CHCl_3$-insoluble precipitate was crystallized from $CHCl_3$ to obtain macrophic acid (**7**) (20 mg) as colorless microcrystals.

Macommelinal (5)——mp 143—146 °C. MS *m/z*: 182 (M^+), 153, 111, 43. UV λ_{max}^{EtOH} nm (log ε): 285 (3.80). IR

ν_{max}^{KBr} cm^{-1}: 2833, 2721, 1714, 1700, 1648, 1565. ^{1}H-NMR ($CDCl_3$) δ: 2.17 (3H, s), 3.41 (2H, d, J=1.2 Hz), 3.75 (3H, s), 5.39 (1H, s), 9.45 (1H, t, J=1.2 Hz). *Anal.* Calcd for $C_9H_{10}O_4$: C, 59.33; H, 5.53. Found: C, 59.56; H, 5.69.

Macrophin (6)——mp 118—121 °C. MS *m/z*: 352 (M^+), 269, 193, 83, 55. UV λ_{max}^{EtOH} nm (log ε): 231 (4.52), 340 (4.16). IR ν_{max}^{KBr} cm^{-1}: 3490, 1720 (sh), 1701, 1643, 1608, 1554. ^{1}H-NMR ($CDCl_3$) δ: 1.85 (3H, d, J=1.6 Hz), 2.12 (3H, d, J=1.2 Hz), 2.31 (1H, br s, exchangeable with D_2O), 3.73 (3H, s), 4.03 (3H, s), 4.52 (2H, s), 4.99 (2H, s), 5.57 (1H, m), 6.64 (1H, d, J=15.2 Hz), 7.46 (1H, d, J=15.2 Hz). ^{13}C-NMR ($CDCl_3$) δ: 20.1 (q), 27.2 (q), 52.1 (q), 54.5 (t), 55.9 (t), 62.6 (q), 109.6 (s), 115.2 (d), 118.1 (s), 125.4 (d), 130.3 (d), 154.1 (s), 157.9 (s), 162.3 (s), 166.0 (s), 166.1 (s), 169.3 (s). *Anal.* Calcd for $C_{17}H_{20}O_8$: C, 57.95; H, 5.72. Found: C, 58.04; H, 5.79.

Macrophic Acid (7)——mp 200.5—203.5 °C. MS *m/z*: 224 (M^+), 196, 153, 97. UV λ_{max}^{EtOH} nm (log ε): 236 (4.34), 340 (4.11). IR ν_{max}^{KBr} cm^{-1}: 3187—2800, 1733, 1691, 1643, 1610, 1546. ^{1}H-NMR (DMSO-d_6) δ: 1.96 (3H, s), 2.04 (3H, s), 3.78 (3H, s), 6.27 (1H, d, J=15.1 Hz), 7.27 (1H, d, J=15.1 Hz), 12.3 (1H, br s, exchangeable with D_2O). *Anal.* Calcd for $C_{11}H_{12}O_5$: C, 58.92; H, 5.40. Found: C, 58.70; H, 5.33.

Semicarbazone of Macommelinal (5)——A solution of $H_2NNHCONH_2 \cdot HCl$ (34 mg) and anhydrous sodium acetate (33 mg) in water (2.5 ml) was added to a solution of **5** (50 mg) in EtOH (1.5 ml). The mixture was kept for 2 d at room temperature. The precipitate was collected by filtration, washed with water (2 ml × 5) and dried under vacuum. The precipitate was crystallized from EtOH to obtain the semicarbazone derivative **10** (48 mg) as colorless needles, mp 208.5—210.5 °C. MS *m/z*: 239 (M^+), 222, 196, 180, 164, 43. UV λ_{max}^{EtOH} nm: 232, 285. IR ν_{max}^{KBr} cm^{-1}: 3492, 1712, 1693 (sh), 1688, 1646, 1590, 1565. ^{1}H-NMR (pyridine-d_5) δ: 2.06 (3H, s), 3.18 (2H, d, J=5.8 Hz), 3.57 (3H, s), 5.57 (1H, s), 6.9 (2H, br s, exchangeable with D_2O), 7.25 (1H, t, J=5.8 Hz), 10.7 (1H, br s, exchangeable with D_2O). *Anal.* Calcd for $C_{10}H_{13}N_3O_4$: C, 50.20; H, 5.48; N, 17.57. Found: C, 50.39; H, 5.56; N, 17.32.

Conversion of Macommelinal (5) to Macommelinol (3)——$NaBH_4$ (12 mg) was added to a solution of **5** (50 mg) in EtOH (10 ml). The mixture was stirred for 30 min at room temperature, concentrated to one-half of the initial volume under reduced pressure after the addition of water (10 ml), and extracted with AcOEt (15 ml × 5). The organic layer was dried (Na_2SO_4) and evaporated. The residue (48 mg) was crystallized from CCl_4 and recrystallized from benzene (C_6H_6) to obtain **3** as colorless needles, mp 115—117 °C. *Anal.* Calcd for $C_9H_{12}O_4$: C, 58.69; H, 6.57. Found: C, 58.56; H, 6.57.

Conversion of Macomelinol (3) to Macommelinal (5)——(a) Benzene (1 ml), pyridine (1 ml), phosphoric acid (0.1 ml), and DCC (2.5 g) were added to a solution of **3** (1.0 g) in DMSO (3.5 ml). After being stirred for 2 h at room temperature, the mixture was filtered and the insoluble dicyclohexylurea (2.45 g) was washed with MeOH (3 ml × 3). The filtrate and washings were combined and oxalic acid (2 g) was added. Stirring was carried out for 1 h at room temperature, then the mixture was filtered. The filtrate was poured into 10% $NaHCO_3$ solution (50 ml) and extracted with AcOEt (50 ml × 4). The AcOEt layer was dried and evaporated. The residue was dissolved in acetone (50 ml) and the solution was filtered. The filtrate was evaporated; the residue was placed on a silica gel column (2 × 10 cm) and eluted with $CHCl_3$ and then 5% MeOH–$CHCl_3$. Several crystallizations from C_6H_6 and then CCl_4 gave the aldehyde **5** (790 mg) as colorless needles, mp 145—148 °C. *Anal.* Calcd for $C_9H_{10}O_4$: C, 59.33; H, 5.53. Found: C, 59.61; H, 5.61.

(b) Pulverized PCC (2.54 g) was added to a solution of **3** (198 mg) in CH_2Cl_2 (40 ml) with stirring. Stirring was continued for 25 min at room temperature, then the dark reaction mixture was placed on a silica gel column (3 × 22 cm), and eluted with AcOEt. The eluate was evaporated to dryness *in vacuo*, and the residue was crystallized from CCl_4 to obtain the aldehyde **5** (111 mg) as colorless needles, mp 144—146 °C.

Incorporation of Stable Isotope-Labeled Compounds——(a) Sodium [1-^{13}C]acetate (1.1 g) was dissolved in water (5 ml) and fed to five culture flasks on the 8th day. The cultures were harvested on the 21st day and the metabolites were isolated as described previously.[1a] Yields of labeled **3** and **4** were 450 and 13 mg, respectively. The p.n.d.^{13}C-NMR spectra were measured and the enrichment was calculated.

(b) Sodium [1,2-$^{13}C_2$]acetate (200 mg), which was diluted with non-labeled sodium acetate (200 mg), was administered to two culture flasks, and culture was continued as above. The labeled **3** (190 mg) and **4** (9 mg) were separated by prep. TLC and purified by recrystallization.

(c) Diethyl [2-^{13}C]malonate (500 mg) in ethanol (5 ml) was added to two culture flasks and labeled **3** (60 mg) was obtained.

(d) Sodium [1-^{13}C,2-$^2H_3$]acetate (900 mg) was fed to six culture flasks and labeled **2** (11 mg), **3** (300 mg) and **4** (13 mg) were isolated. ^{13}C-NMR ($CDCl_3$) data of **2**; δ: 17.6 (C-7), 23.2 (C-9), 56.2 (C-10), 64.6 (C-8), 89.0 (C-3), 114.0 (C-5), 158.7 (C-6), 163.8 (C-2), 170.4 (C-4).

[OMe-^{14}C] 12——A solution of [^{14}C]CH_3I (1 mCi) and cold CH_3I (0.23 ml) in *N*,*N*-dimethylformamide (DMF) (3 ml) was added to a mixture of 5-acetyl-4-hydroxy-6-methyl-2-pyrone[13] (300 mg) and Ag_2O (630 mg) at 0 °C. The mixture was kept overnight at that temperature with stirring, then AcOEt (60 ml) was added and filtered. The filtrate was washed with water (50 ml × 3), dried (Na_2SO_4) and evaporated to dryness. The residue was subjected to prep. TLC with MeOH–$CHCl_3$ (3:97). Recrystallization from cyclohexane yielded [OMe-^{14}C] **12** (158 mg; 2.03 μCi/mg) as colorless needles, mp 101—104 °C.

^{14}C-Labeled 2—4——Sodium [1-^{14}C]acetate (0.812 mCi), which was diluted with cold sodium acetate (1 g), was administered to five culture flasks on the 10th day and the cultures were harvested on the 21st day. The labeled

metabolites were isolated by column chromatography and prep. TLC on silica gel using MeOH–$CHCl_3$. ^{14}C-Labeled **3** (380 mg; 0.138 μCi/mg), mp 116—118 °C, and **4** (23 mg; 0.102 μCi/mg), mp 182—185 °C, were obtained on recrystallization from C_6H_6 and $CHCl_3$–MeOH, respectively. Labeled **2** (34 mg; 0.0527 μCi/mg), mp 125—128 °C, was also prepared by the washing-out method with 30 mg of cold **2**.

^{14}C-Labeled 1——Triphenylphosphine (Ph_3P) (248 mg) was added to a warm solution of ^{14}C-labeled **3** (67 mg) and CBr_4 (314 mg) in C_6H_6 (5 ml). The mixture was refluxed for 30 min on a water bath. After cooling, the resulting brown-yellow precipitate was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was subjected to prep. TLC with MeOH–$CHCl_3$ (1 : 99) and the ^{14}C-labeled bromide **15** (78 mg), mp 133—134 °C, was obtained.

$NaBH_4$ (51 mg) was added to a solution of ^{14}C-labeled **15** (78 mg) in DMSO (1.4 ml), and the mixture was heated for 10 min at 150 °C. After cooling, water (20 ml) was added and the whole was extracted with AcOEt (20 ml × 3). The AcOEt layer was dried (Na_2SO_4) and evaporated. The AcOEt extract was subjected to prep. TLC with C_6H_6–acetone (9 : 1). Crystallization from cyclohexane yielded ^{14}C-labeled **1** (39 mg, 0.149 μCi/mg) as colorless needles, mp 88—89 °C.

^{14}C-Labeled 5——^{14}C-Labeled **5** (45 mg; 0.143 μCi/mg), mp 140 °C, was synthesized by PCC oxidation of ^{14}C-labeled **3** (96 mg) as described above.

^{14}C-Labeled 13——1,8-Diazabicyclo[5.4.0]-7-undecene (DBU) (1.5 ml) was added to a solution of ^{14}C-labeled **15** (159 mg) in C_6H_6 (10 ml). The mixture was refluxed for 2 h. After cooling, 1 N H_2SO_4 (20 ml) was added and the whole was extracted with $CHCl_3$ (20 ml × 4). The $CHCl_3$ layer was washed with 10% brine (20 ml × 2), dried (Na_2SO_4) and evaporated *in vacuo*. The residue was subjected to prep. TLC with C_6H_6–acetone (4 : 1) and ^{14}C-labeled **13** (71 mg; 0.139 μCi/mg), mp 65—67 °C, was obtained.

^{14}C-Labeled 14——*m*-Chloroperoxybenzoic acid (mCPBA) (80% content; 80 mg) was added to a solution of ^{14}C-labeled **13** (50 mg) in $CHCl_3$ (7 ml). The mixture was kept for 24 h at room temperature, then $CHCl_3$ (40 ml) was added and the whole was washed successively with 1% KI, 1% $Na_2S_2O_3$, 1% $NaHCO_3$ and 10% brine (each 30 ml). The $CHCl_3$ layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was subjected to prep. TLC with C_6H_6–acetone (4 : 1) and ^{14}C-labeled **14** (24 mg; 0.115 μCi/mg), mp 112—116 °C, was obtained.

Incorporation Experiments with ^{14}C-Labeled Compounds——Each ^{14}C-labeled compound (**1**—**5**, **12**—**14**) was administered to one culture flask on the 10th day and harvested on the 21st day. The culture broth was adsorbed on a charcoal column (2 × 11 cm), which was washed with water (1 l) and eluted with acetone–water (2 : 1; 300 ml) followed by acetone (300 ml). The acetone–water and acetone eluates were combined and evaporated *in vacuo*. An aliquot of the residue was subjected to prep. TLC with C_6H_6–acetone (3 : 2). The radioactive spots of metabolites were scraped off and transferred into vials containing 1 ml of MeOH and 9 ml of scintillator for radioactivity measurement.

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References and Notes

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