Agricultural and Biological Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb19

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Hajime Ohigashi[a], Shogo Minami[a], Hiroshi Fukui[a], Koichi Koshimizu[a], Fusao Mizutani[b], Akira Sugiura[b] & Takashi Tomana[b]
[a] Department of Food Science and Technology, Kyoto University, Kyoto 606, Japan
[b] Department of Agronomy and Horticultural Science, Kyoto University, Kyoto 606, Japan
Published online: 09 Sep 2014.

To cite this article: Hajime Ohigashi, Shogo Minami, Hiroshi Fukui, Koichi Koshimizu, Fusao Mizutani, Akira Sugiura & Takashi Tomana (1982) Flavanols, as Plant Growth Inhibitors from Roots of Peach, Prunus persica Batsh. cv. 'Hakuto', Agricultural and Biological Chemistry, 46:10, 2555-2561

To link to this article: http://dx.doi.org/10.1080/00021369.1982.10865454

Agric. Biol. Chem., **46** (10), 2555~2561, 1982

Flavanols, as Plant Growth Inhibitors from Roots of Peach, *Prunus persica* Batsh. cv. 'Hakuto'

Hajime OHIGASHI, Shogo MINAMI, Hiroshi FUKUI,[†]
Koichi KOSHIMIZU, Fusao MIZUTANI,*,[††]
Akira SUGIURA* and Takashi TOMANA*

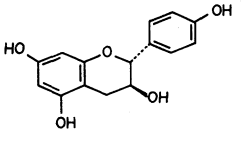
Department of Food Science and Technology, Kyoto University, Kyoto 606, Japan
**Department of Agronomy and Horticultural Science, Kyoto University, Kyoto 606, Japan*

Received May 11, 1982

Three flavanols, which inhibit root growth in the rice seedling test, have been purified. One was (+)-afzelechin (**1**) and the other two, named PIa and PIb, were new biflavanols shown by the structures **2** and **3** on the basis of chemical and spectroscopic evidence. PIa inhibited the root growth of the peach seedlings. In addition, PIa was found in the soil in the area where the peach trees were growing. The flavanols, therefore, are suggested to be one of the chemical factors causing soil sickness often encountered in peach cultivation.

The peach tree has been known as a plant which often causes soil sickness. This has been proved to result from chemicals such as hydrogen cyanide, mandelonitrile, benzaldehyde and benzoic acid formed by cyanogenesis of prunasin occurring in the root.[1,2] Recently Mizutani *et al.* suggested the presence of additional chemical factors involved in soil sickness.[2] In our preliminary experiments, using the rice seedling test,[3] the phenolic fraction inhibited root growth. Here we wish to report on the isolation, structure elucidation, and biological activities of the inhibitors.

At every step in the purification of inhibitors, the rice seedling test was used for monitoring the activity. Methanol extract from the roots (1.9 kg) was partitioned between ethyl acetate and water. The active ethyl acetate layer was chromatographed on silica gel eluted stepwise with benzene containing an



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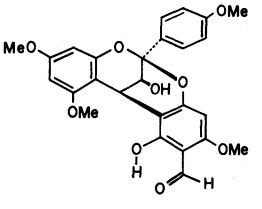

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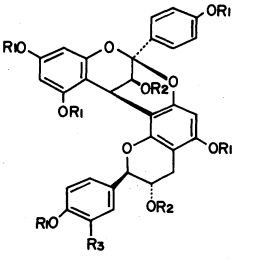

2. $R_1=R_2=R_3=H$
3. $R_1=R_2=H$, $R_3=OH$
4. $R_1=Me$, $R_2=H$, $R_3=OMe$
5. $R_1=Me$, $R_2=R_3=H$
6. $R_1=Me$, $R_2=Ac$, $R_3=H$

[†] Present address: *Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan.*
[††] Present address: *Laboratory of Horticulture, College of Agriculture, Ehime University, Matsuyama 790, Japan.*

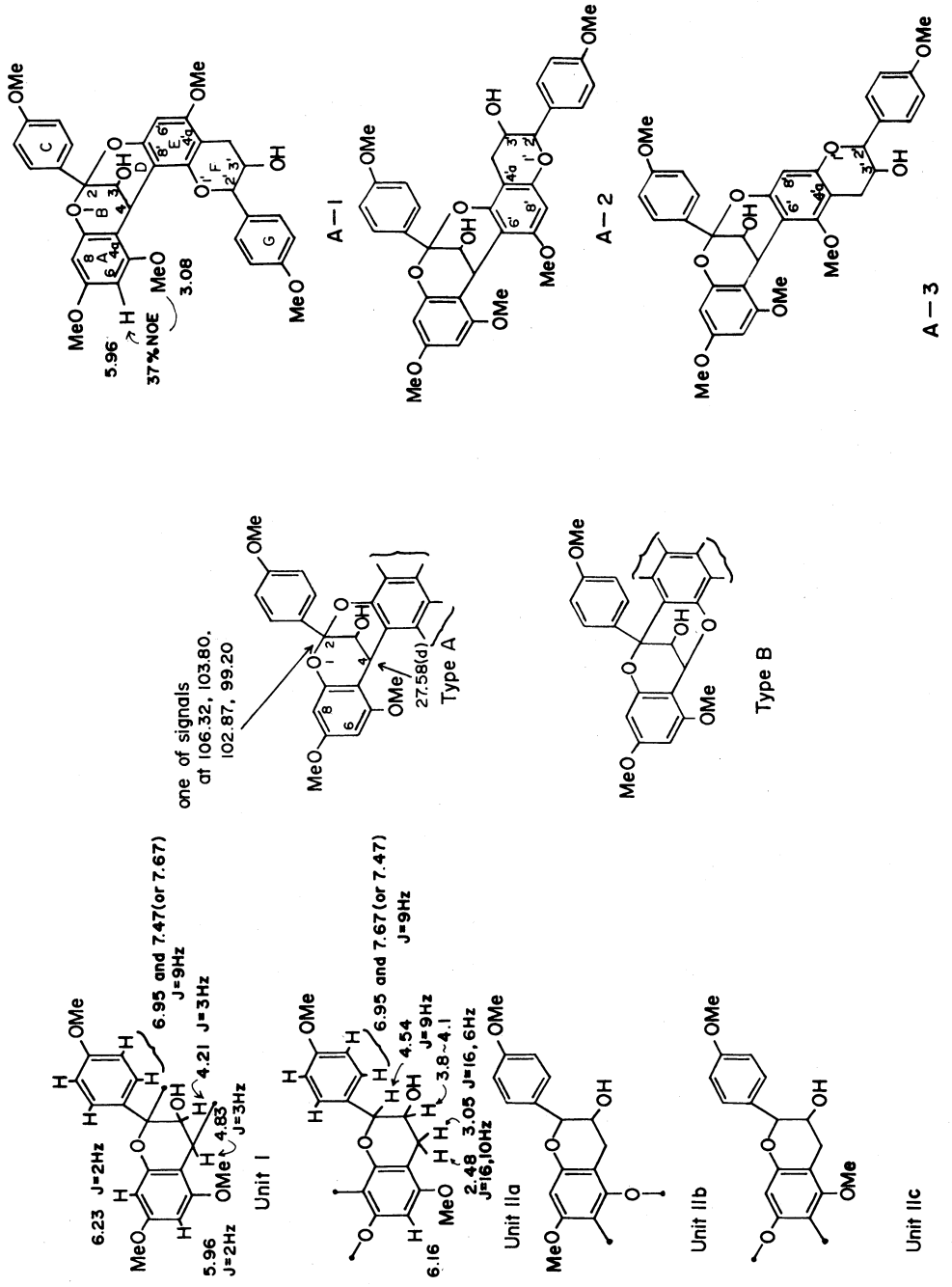

FIG. 1. The Structure Elucidation of PIa–Pentamethyl Ether (**5**).

increasing ratio of ethyl acetate. The fractions eluted with 60 and 80% ethyl acetate in benzene were active. Further purification of each fraction on silica gel afforded active compounds, **1** (207 mg) and **2** (920 mg). A related compound **3** was purified in its derived form, a hexamethyl ether (**4**) (330 mg).

The active compound **1** was determined to be (+)-afzelechin[4] by spectral data.

The active compound **2**, named PIa (Prunus Inhibitor a), gave a pentamethyl ether (**5**) ($C_{35}H_{34}O_{10}$) which further formed a diacetate (**6**), indicating the presence of five phenolic and two alcoholic hydroxyls. The similarity of the UV spectrum of **2** (λ_{max}^{MeOH}: 222 nm, sh., and 273 nm) to that of **1** showed the presence of a 5,7-oxygenated-flavan unit (or units). The ^{1}H-NMR signals ($CDCl_3+D_2O$) (see Fig. 1) of **5** indicated a pair of one-proton doublets (δ 5.96 and 6.23) with meta coupling ($J=2$ Hz), two pairs of two-proton doublets (δ 6.95 and 7.47, and 6.95 and 7.67) with ortho coupling ($J=$ 9 Hz) and a one-proton singlet (δ 6.16). Further ^{1}H-NMR study with decoupling experiments*[1] revealed that **5** was composed of two flavanol units, I and II (IIa or IIb or IIc), through either A or B type inter-flavan linkage as shown in Fig. 1, in which partial and possible structures to be discussed below are summarized along with the significant NMR data.

^{13}C-NMR study ($CDCl_3$) of **5** (see experimental section), which supported well the presence of the two flavanol units,[5] was effective in limiting the type of linkage. An important feature in the spectrum was a doublet at δ 27.58 which could be assigned to C-4 in structures of Type A. The signal due to C-2 in the structures had to be one of four singlets at δ 106.32, 103.80, 102.87 and 99.20. These observations led unequivocally to three possible structures, A-1, A-2 and A-3, for **5**.

In the ^{1}H-NMR spectrum of **5**, one of five methoxy groups appeared at an abnormally high field (δ 3.08). The methoxy group was confirmed to be that at C-5, because 37% NOE was observed between the methoxy protons and H-6 at δ 5.96.*[2] Treatment of **5** with active manganese dioxide caused oxidative cleavage on ring G to yield 4-methoxybenzaldehyde and, a residual fragment, phenol–aldehyde (**7**). The ^{1}H-NMR of **7** showed the methoxy group resonating within the normal field (δ 3.7). Hence the upfield shift of the methoxy group was explained by a shielding effect resulting from the aromatic ring G of Unit II. The molecular model indicated that only structure A-1 carried such a long range shielding effect.

The relative stereochemistry between the proton at C-2′ and C-3′ in Unit II of **5** was established to be *trans* by the coupling constant ($J=9$ Hz), similar to that of catechin and afzelechin.[6] Determination of the stereochemical relationship between the substituents at C-2 and C-3 on ring B was performed by the same method as utilized for proanthocyanidin-A_2-octamethyl ether,[7] structurally related to **5**. The relative change in chemical shift in $CDCl_3$ as compared with d_5-pyridine for H-6 and H-8 (ΔH-$6_{(\delta d_5\text{-pyridine}-\delta CDCl_3)}$: Δ H-$8_{(\delta d_5\text{-pyridine}-\delta CDCl_3)}$) was found to be as 1 : 1.33 in **5**, which was close to that reported for (+)-catechin–tetramethyl ether (1 : 1.26), and not for (−)-epicatechin–tetramethyl ether (1 : 1.66). This indicated that the aromatic ring C is situated in *trans* relation to the hydroxyl at C-3. The absolute configuration at C-3 was determined as *S* by the Horeau-Brooks method,[8] as applied to **7**, in which 3% increment of (+)-(*R*)-α-phenylethylamide of (−)-(*R*)-α-phenylbutyric acid, as compared with the control experiment,

*[1] Irradiation at δ 4.21 collapsed the doublet at δ 4.83 to a singlet. Furtheremore, irradiation at δ 3.9 collapsed the doublet at δ 4.54 to a singlet, and the double doublets at δ 2.48 and 3.05 to AB-type doublets with $J=16$ Hz.

*[2] In the ^{1}H-NMR of 3,5,7-flavan-triols, such as catechin and epicatechin, H-6 was reported to resonate at a higher field than H-8.[6] Furthermore, while an NOE was observed only in the proton at δ 5.96 on irradiation at δ 3.08, irradiation at δ 3.6 showed NOEs in both the doublets at δ 6.23 (20 ~ 22%) and 5.96 (3 ~ 5%). Thus the doublets at δ 5.96 and 6.23 were assigned to H-6 and H-8, respectively, and hence the signal at δ 3.08 was attributed to the methoxy group at C-5.

TABLE I. THE EFFECT OF COMPOUND **1** AND **2** ON THE ROOT GROWTH IN THE RICE SEEDLING TEST

Compound	% Growth (100 × wt. of roots treated/wt. of control)				
	Concentration (ppm)				
	1,000	500	250	125	62.5
(+)-Afzelechin (**1**)	71.2	102.8	107.3	114.7	100.0
PIa (**2**)	58.2	65.5	93.8	94.4	107.3

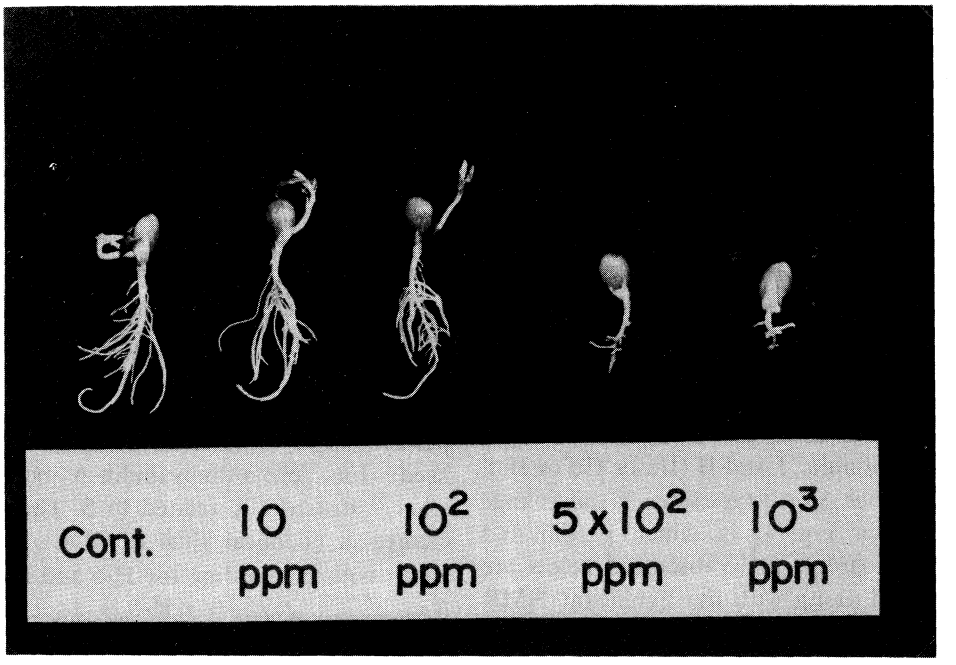

FIG. 2. The Inhibitory Activity of PIa (**2**) against the Root Growth of Peach Seedlings.

was detected, similar to the case of (+)-menthol((3*S*)-configuration) (3.5% increment of the amide). The result matched with our expectation based on the fact that (+)-afzelechin, having (3*S*)-configuration,[9] was isolated. The configuration at C-3′ must be *S* based on the biogenetical backgrounds.

Compound **4** ($C_{36}H_{36}O_{11}$), a hexamethyl ether of PIb (**3**),*3 was closely related to **5** spectroscopically. The ^{1}H-NMR spectrum in the aromatic proton region showed one of the flavanol units was derived from catechin (or epicatechin). On oxidation of **4** with active manganese dioxide, 3,4-dimethoxybenzaldehyde and **7** were obtained. The result, together with the coupling constant of H-2′ (J= 9 Hz), indicated that Unit II of **4** originated from catechin. The absolute configuration at C-3′ was supposed to be *S*, because of the coexistence of (+)-catechin in a phenolic fraction of the root extract.

In Table I the inhibitory effect of **1** and **2** on the root growth in the rice seedling test is shown. They exhibit activity at a concentration

*3 PIb (**3**) was a free phenol, because no methoxy signal was detected by the ^{1}H-NMR spectrum of the fraction before methylation.

of 1000 and 500 ppm, respectively. By reason of the contamination of small amounts of impurity, it was unsuccessful to measure the precise activity of PIb (**3**). It must, however, be active, because the fraction mainly composed of PIb showed no less activity than PIa (**2**). PIa, the main flavanol among the three, also inhibited the growth of peach seedlings at 500 ppm as shown in Fig. 2.

In an attempt to examine the accumulation of the flavanols in soil, we isolated PIa from the extract of the soil collected in the peach growing area.*4 Therefore it may be concluded that the flavanols excreted from the root are one of the chemical factors which cause soil sickness.

EXPERIMENTAL

Melting points were measured on a hot stage and are uncorrected. The ^{1}H-NMR and the ^{13}C-NMR were recorded on a Hitachi Model R-22 (90 MHz) and a JEOL Model JMN FX-60 (15 MHz) spectrometer, respectively. Chemical shifts are expressed in ppm (δ) from TMS as an internal standard, and singlet, doublet, double doublet, triplet, quartet and multiplet are abbreviated to s, d, dd, t, q and m, respectively. MS, IR and UV spectra were obtained on a Hitachi RMU-6L and an M-80 mass spectrometer with EI ionization, a Hitachi EPI-G3 infrared spectrometer and a Shimadzu UV-200, respectively. Optical rotations were measured with a Jasco Model J-5 recorder. GLC was performed on a Hitachi 063 Gas Chromatograph with an FID.

Bioassay. The rice seedling test was conducted by the method previously reported.[3] The inhibitory activity of the compounds against root growth was measured by the relative weight of the roots against that of the control group. The peach seedlings were performed as follows. A peach seed previously germinated under stratification was sowed on an agar medium (Murashige and Skoog basal medium[10] containing 1% agar) with specific amounts of the inhibitor, and it was cultivated at 28°C under continuous fluorescent light for 6 days.

Isolation of active compounds. The roots of *P. persica* (1.9 kg) were extracted with MeOH, and the solvent was evaporated *in vacuo* to afford an aqueous extract which was further extracted with EtOAc. The EtOAc soluble part (70 g) was chromatographed on Wako gel C-100 (500 g) eluted stepwise with benzene containing increasing amounts of EtOAc. Inhibitory activity was found in fractions eluted with 60 and 80% EtOAc in benzene.

The 60% EtOAc eluate (800 mg) was further chromatographed on silica gel H (type 60, Merck Co.) eluted with 7.5% MeOH in chloroform to give a yellow resin. The resin (160 mg) was recrystallized from MeOH-chloroform to yield **1** (72 mg) as colorless needles, mp 208~210°C. $[\alpha]_D^{20}$ +18.0° (c=1.33, water : acetone=1 : 1), UV λ_{max}^{MeOH} nm (ε): 222 (29,000, sh.), 272 (6500), IR ν_{max}^{KBr} cm^{-1}: 3600, 3000, 1620, 1525, 1475, 1145, ^{1}H-NMR (d_6-acetone+D_2O) δ: 7.27 (2H, d, J=9 Hz), 6.85 (2H, d, J=9 Hz), 6.02 (1H, d, J=2 Hz), 5.91 (1H, d, J=2 Hz), 4.63 (1H, d, J=8 Hz), 4.0~4.2 (1H, m), 2.92 (1H, dd, J=16 and 6 Hz), 2.61 (1H, dd, J=16 and 8 Hz).

The 80% eluate (14.1 g) was chromatographed on Wako gel C-100 (300 g) eluted with chloroform containing increasing amounts of acetone to afford active fractions, 50 and 60% acetone eluate. Separation of the 50% acetone eluate (7.9 g) on silica gel H (type 60) eluted with chloroform–MeOH (92.5 : 7.5) gave three active fractions. After evaporation of the solvent, two of three gave solids which were recrystallized from MeOH–chloroform to give **1** (135 mg) and PIa (**2**) (920 mg), respectively. The third fraction (350 mg) obtained here was combined with the 60% acetone eluate (3.9 g) and it was chromatographed on Florisil (300 g) eluted with EtOAc–MeOH (95 : 5), followed by preparative TLC on silica gel developed with chloroform–MeOH (4 : 1), to give an active fraction (380 mg), mainly composed of PIb (**3**). Then the fraction was methylated in the same manner as described below to give PIb–hexamethyl ether (**4**) (330 mg). PIa (**2**), colorless needles, mp 256~257°C (decomp.), $[\alpha]_D^{16}$ −102.9° (c=0.88, MeOH), UV λ_{max}^{MeOH} nm (ε): 222 (57,000, sh.), 273 (4000), IR ν_{max}^{KBr} cm^{-1}: 3400, 1625, 1520, 1240, 1150, ^{1}H-NMR (d_6-DMSO+D_2O) δ: 7.42 (2H, d, J=9 Hz), 7.33 (2H, d, J=9 Hz), 6.82 (4H, d, J=9 Hz), 6.02 (1H, s), 5.92 (1H, d, J=2 Hz), 5.88 (1H, d, J=2 Hz), 4.68 (1H, d, J=8 Hz), 4.21 (1H, d, J=4 Hz), 3.99 (1H, d, J=4 Hz), 3.8~4.1 (1H, m), 2.88 (1H, dd, J=16 and 6 Hz), *ca.* 2.5 (1H, m, overlapped with DMSO). PIb–hexamethyl ether (**4**), colorless needles from MeOH, mp 159~161°C, $[\alpha]_D^{16}$ −53.1° (c=0.32, chloroform), UV λ_{max}^{MeOH} nm (ε): 223 (53,000, sh.), 272 (5700), IR ν_{max}^{KBr} cm^{-1}: 3450, 1615, 1520, 1465, 1120, MS m/z: 644 (M^+), ^{1}H-NMR ($CDCl_3$+D_2O) δ: 7.64 (2H, d, J=9 Hz), 7.06 (1H, d, J=8 Hz), 7.00 (1H, s), 6.94 (2H, d, J=9 Hz), 6.90 (1H, d, J=8 Hz), 6.23 (1H, d, J=2 Hz), 6.12 (1H, s), 5.93 (1H, d, J=2 Hz), 4.84 (1H, d, J=4 Hz), 4.53 (1H, d, J=9 Hz), 4.22 (1H, d, J=4 Hz), 4.1~3.8 (1H, m), 3.88 (3H, s), 3.86 (3H, s), 3.78 (3H, s), 3.70 (3H, s), 3.68 (3H, s), 3.12 (3H, s), 3.11 (1H, dd, J=16 and 6 Hz), 2.51 (1H, dd, J=16 and 10 Hz).

The other fraction (180 mg) obtained by column chromatography on Florisil was methylated with dimethyl sulfate. The methylated compounds were purified on

*4 The soil sample was collected at a different place from the root sample. In this experiment, 7 mg of PIa was obtained from 1 kg of the dried soil. Contents of (+)-afzelechin (**1**) and **3** were too small to be isolated.

Wako gel C-100 (20 g) eluted with benzene–EtOAc, followed by preparative TLC on silica gel developed with benzene–acetone (9 : 1), to give a tetramethyl ether (16 mg) of (+)-cateching $[\alpha]_D^{16}$ −11.9° (c=1.34, chloroform).

*Methylation of PIa (**2**).* To PIa (**2**) (200 mg) in dry acetone (20 ml) anhydrous potassium carbonate (16 g) and dimethyl sulfate (2 ml) were added, and it was refluxed for 6 hr. After filtration, the solvent was evaporated to give an oil which was partitioned between EtOAc and water. The EtOAc layer was dried over anhydrous sodium sulfate, and concentrated. The residue was chromatographed on Wako gel C-100 with benzene–acetone to give a crude solid which was recrystallized from MeOH to afford PIa–pentamethyl ether (**5**) (140 mg) as colorless needles, mp 162~163°C. $[\alpha]_D^{14}$ −60.0° (c=0.35, chloroform), MS m/z: 614.21669 (M^+, $C_{35}H_{34}O_{10}$), UV $\lambda_{max}^{CHCl_3}$ nm (ε): 273 (4200), IR ν_{max}^{KBr} cm^{-1}: 3400, 1620, 1520, 1465, 1255, 1125, ^{1}H-NMR ($CDCl_3+D_2O$) δ: besides the signals shown in Fig. 1 four additional methoxy signals appeared at 3.82, 3.79, 3.72 and 3.69, ^{1}H-NMR (d_5-pyridine) δ: 8.13 (2H, d, J= 9 Hz), 7.73 (2H, d, J=8 Hz), 7.09 (2H, d, J=9 Hz), 7.04 (2H, d, J=8 Hz), 6.59 (1H, d, J=2 Hz), 6.44 (1H, s), 6.23 (1H, d, J=2 Hz), 5.38 (1H, d, J=3 Hz), 4.89 (1H, d, J= 9 Hz), 4.71 (1H, d, J=3 Hz), 4.6~4.1 (1H, m), 3.73 (3H, s), 3.70 (6H, s), 3.64 (3H, s), 3.50 (1H, dd, J=16 and 6 Hz), 3.24 (3H, s), 2.96 (1H, dd, J=16 and 10 Hz), ^{13}C-NMR ($CDCl_3$) δ: 160.02 (s), 159.70 (s), 159.59 (s), 158.58 (s), 157.07 (s), 153.20 (s), 151.51 (s), 151.15 (s), 130.74 (s), 130.52 (s), 128.49 (2×d), 128.19 (2×d), 113.82 (2×d), 113.53 (2×d), 106.32 (s), 103.80 (s), 102.87 (s), 99.20 (s), 92.97 (d), 92.53 (d), 91.82 (d), 81.36 (d), 68.67 (d), 67.18 (d), 55.42 (q), 55.25 (3×q), 54.52 (q), 28.58 (t), 27.58 (d).

*Acetylation of PIa-pentamethyl ether (**5**).* PIa-pentamethyl ether (**5**) (25 mg) was acetylated with pyridine (1 ml) and acetic anhydride (1 ml). The usual treatment gave a diacetyl–PIa–pentamethyl ether (**6**) (25 mg) as a colorless resin, $[\alpha]_D^{16}$ −16.7° (c=0.18, MeOH), UV $\lambda_{max}^{CHCl_3}$ nm (ε): 270 (3100), IR ν_{max}^{KBr} cm^{-1}: 1750, 1620, 1520, 1250, ^{1}H-NMR ($CDCl_3$) δ: 7.63 (2H, d, J=9 Hz), 7.38 (2H, d, J=9 Hz), 6.91 (4H, d, J=9 Hz), 6.24 (1H, d, J=3 Hz), 6.18 (1H, s), 5.98 (1H, d, J=3 Hz), 5.49 (1H, d, J=4 Hz), 5.14 (1H, m), 4.87 (1H, d, J=8 Hz), 4.83 (1H, d, J=4 Hz), 3.80 (6H, s), 3.72 (3H, s), 3.70 (3H, s), 3.22 (3H, s), 3.06 (1H, dd, J=16 and 6 Hz), 2.59 (1H, dd, J=16 and 9 Hz), 1.88 (3H, s), 1.72 (3H, s).

*Oxidation of PIa-pentamethyl ether (**5**) with active MnO_2.* Active MnO_2 was prepared as follows. Potassium permanganate (9.6 g) was dissolved in hot water (60 ml) to which manganese sulfate tetrahydrate (11.1 g) in 150 ml water was added and it was stirred for 2 hr. The precipitated MnO_2 was collected by filtration, washed with water and dried at 100~120°C for 4 hr.

To PIa–pentamethyl ether (**5**) (100 mg) in benzene (10 ml) MnO_2 (1 g) was added and it was stirred for 3 days at room temperature. The reaction mixture was filtered and the filtrate was concentrated to yield a pale yellow resin. Column chromatography on Wako gel C-100 with n-hexane–EtOAc gave 4-methoxybenzaldehyde (4 mg) and **7** (15 mg). **7**, colorless needles from benzene–n-hexane, mp 215.5~217°C, $[\alpha]_D^{16}$ −141.8° (c=0.67, chloroform), UV λ_{max}^{MeOH} nm (ε): 224 (48,000), 297 (21,000), IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2950, 1635, 1610, 1500, 1450, MS m/z: 480 (M^+), ^{1}H-NMR ($CDCl_3$) δ: 12.74 (1H, OH, s), 10.03 (1H, s), 7.63 (2H, d, J=9 Hz), 6.94 (2H, d, J=9 Hz), 6.24 (1H, d, J=3 Hz), 6.11 (1H, d, J=3 Hz), 5.96 (1H, s), 4.81 (1H, d, J=3 Hz), 4.12 (1H, d, J=3 Hz), 3.79 (6H, s), 3.73 (3H, s), 3.71 (3H, s), 1.6 (1H, OH, broad s).

Determination of the configuration at C-3 by the Horeau–Brooks method. To **7** (5 μmol), in a sealed vial, anhydrous pyridine (3.5 μl) and (±)-α-phenylbutyric anhydride (3 μl) were added. After warming at 40°C for 1.5 hr, (+)-(*R*)-α-phenylethylamine (3 μl) was added to the reaction mixture and mixed throughly for 15 min. The mixture was analyzed by GLC to determine the relative quantity of (+)-(*R*)-α-phenylethylamide of (−)-(*R*)-α-phenylbutyric acid to that of (+)-(*S*)-α-phenylbutyric acid. Parallel experiments with (+)-menthol ((3*S*)-configuration) and (−)-menthol ((3*R*)-configuration) were carried out as positive controls. GLC analyses were performed on a stainless column (3 mm i.d. × 2 m) packed with 1% OV-17 on Gas Chrom. Q at 180°C, 20 ml/min N_2 flow rate, where (+)-(*R*)-α-phenylethylamides of (−)-(*R*)- and (+)-(*S*)-α-phenylbutyric acid were obtained at t_R 5.8 and 6.5 min, respectively. The values of the relative increment of the amide of (−)-(*R*)-acid in **7**, (+)-menthol and (−)-menthol, as compared with that in the control experiment without an alcohol, were +3.0, +3.5 and −4.3%, respectively.

*Oxidation of PIb-hexamethyl ether (**4**) with active MnO_2.* PIb-hexamethyl ether (**4**) (75 mg) was oxidized with active MnO_2 (750 mg) in the same manner as for **5** to give 3,4-dimethoxybenzaldehyde (3 mg) and **7** (6 mg).

*Isolation of PIa (**2**) from the soil sample.* The soil sample (18 kg) around the peach roots was collected at Sanyo town, Okayama, and it was extracted with MeOH. The usual treatment gave PIa (118 mg).

Acknowledgments. The authors wish to thank Dr. Akira Kato of the Faculty of Pharmaceutical Sciences of Kyoto University (presently at Niigata College of Pharmacy) and Dr. Yasuaki Esumi of The Institute of Physical and Chemical Research for their MS measurements, Dr. Tetsuro Shingu at Kobe Gakuin University for his helpful advice on NOE measurements, Dr. Tohru Himoto at JEOL Co. for taking the ^{13}C-NMR spectrum and Dr. Naohiro Otsuka at Okayama University for his help in collecting plant materials. We also wish to thank Mr. Susumu Ohno for collecting the samples and his

technical assistance, and Miss Shigeko Yamashita for her ^{1}H-NMR measurements. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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