Prunusols A and B, Novel Antioxidative Tocopherol Derivatives Isolated from the Leaf Wax of *Prunus grayana* Maxim.

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Novel antioxidative phenylpropanoid-substituted tocopherol derivatives, prunusols A and B, were isolated from the leaf wax of *Prunus grayana* Maxim., and their structures were fully characterized by spectroscopic and synthetic methods. Prunusols A and B were found to be the conjugates of γ -tocopherol and *p*-coumaric acid, which are diastereoisomers of each other. They showed almost the same antioxidative activity as α -tocopherol in a water/alcohol system measured by thiocyanate and TBA methods.

Recently, much attention has been focused on the antioxidative defence mechanisms in living cells against damage caused by active oxygen and free radicals.^{1,2)} In plants, endogenous natural antioxidants have been speculated to play an important role in non-enzymatic protections in addition to enzymatic inactivation by antioxidative enzymes such as superoxide dismutase, catalase and glutathione-peroxidase. Many different types of natural antioxidants have already been found in numerous plants, although the chemical properties and physiological roles of active components are not fully understood.³⁾

The authors have been involved in isolating and identifying natural antioxidants from plant materials,⁴⁾ in particular, from plant leaf wax, and the β -diketone type of antioxidant was isolated and identified in leaf wax extracted from *Eucalyptus globulus*.^{5,6)} Recently, we have screened various species of plant leaf waxes for an evaluation of antioxidative activity,⁷⁾ and the leaf wax of *Prunus grayana* Maxim. was suggested to contain antioxidative compounds that were different from tocopherols. In this paper, we report the structural elucidation of novel antioxidative tocopherol derivatives named prunusols A and B, which may play an important role in the antioxidative defence systems of plant leaf wax.

Materials and Methods

Instruments for structural determination. Spectroscopic measurements were carried out by using the following instruments: JASCO DIP-4 ($[\alpha]_D$), Hitachi 200-10 (UV), JASCO A-3 (IR), JEOL DX-705L (MS), JEOL FX 200 (NMR), JEOL GX 500 (NMR) and JASCO J-40A (CD).

Extraction of the leaf wax. *Prunus grayana* Maxim. leaves (35 kg) that had been collected in Takayama City, Gifu, Japan, in July 1986, were extracted twice with 150 l of chloroform. The chloroform extract was filtered and concentrated *in vacuo* to give 400 g of leaf wax. A part of the leaf wax (c. 140 g) was charged in a silica gel column (Fuji-Davison BW-300, 100 ϕ $\times$ 600 mm) and eluted with hexane–ethyl acetate by a stepwise increase in the volume of ethyl acetate. Five fractions eluted with hexane–ethyl acetate (9:1, 8:2, 6:4, 5:5 and 4:6, v/v) showed antioxidative activity in a water/alcohol system measured by the thiocyanate method.⁸⁾ The fractions eluted with 9:1 (v/v) and 8:2 (v/v) hexane–ethyl acetate were confirmed to contain γ -tocopherol; however, the fractions eluted with the other hexane–ethyl acetate ratios (6:4, 5:5 and 4:6, v/v) were considered to contain unknown antioxidative compounds whose *R_f* values were lower than those of tocopherols by a TLC analysis. These fractions were combined (48.9 g), charged in a silica gel column (Fuji-Davison BW300, 80 ϕ $\times$ 600 mm), and the fractions eluted with 8:2 and 7:3, v/v of benzene–ethyl acetate were found to show strong antioxidative activity. The active fraction (310 mg) was further purified by preparative TLC (Merck 60 F₂₅₄, 1 mm thickness) with CHCl₃–MeOH (98:2, v/v) as the solvent system, and repurified by preparative HPLC (Develosil SI 60, 20 ϕ $\times$ 250 mm) with hexane–ethyl acetate (85:15, v/v) as the solvent system.

The active compounds (prunusols A and B) were obtained as oily substances, the yield of prunusol A (**1**) being 20.9 mg and prunusol B (**2**) being 15.1 mg.

Prunusol A (1). $[\alpha]_D^{25} + 15$ ($c=0.2$, CHCl_3); UV λ_{max} (EtOH): 285 nm ($\epsilon=5900$); IR ν_{max} (CHCl_3) cm^{-1} : 3600, 3300, 2900, 1750, 1620, 1510, 1460, 1380, 1340, 1200, 1140, 1100, 960, 830, and 700; $^1\text{H-NMR}$ δ (500 MHz, CDCl_3): 1.20 (3H, s, 2- CH_3), 1.62 (1H, ddd, $J=16.3, 7.5$ and 6.9 Hz, $\text{H}_{3\alpha}$), 1.79 (1H, ddd, $J=16.3, 7.5$ and 6.9 Hz, $\text{H}_{3\beta}$), 2.15 (3H, s, 8- CH_3), 2.25 (3H, s, 7- CH_3), 2.29 (1H, ddd, $J=16.9, 7.5$ and 6.9 Hz, $\text{H}_{4\alpha}$), 2.64 (1H, ddd, $J=16.9, 7.5$ and 6.9 Hz, $\text{H}_{4\beta}$), 2.94 (1H, dd, $J=16.3$ and 2.5 Hz, $\text{H}_{11\alpha}$), 2.99 (1H, dd, $J=16.3$ and 6.3 Hz, $\text{H}_{11\beta}$), 4.25 (1H, dd, $J=6.3$ and 2.5 Hz, H_{12}), 5.65 (1H, br. s, 4'-OH), 6.66 (2H, d, $J=7.5$ Hz, $\text{H}_{3'}$ and $\text{H}_{5'}$) and 6.87 (2H, d, $J=7.5$ Hz, $\text{H}_{2'}$ and $\text{H}_{6'}$); EI-MS m/z : 562 (M^+), 354, 284, 256, 236; TLC (Merck 60F₂₅₄, 0.25 mm thickness) $R_f=0.31$ (hexane-ethyl acetate, 3:1); CD λ (EtOH) nm ($\Delta\epsilon$): 283 (−2.1), 255 (+4.2), 232 (+18.8). Data for the $^{13}\text{C-NMR}$ spectrum are shown in Table I.

Prunusol B (2). $[\alpha]_D^{25} + 10$ ($c=0.2$, CHCl_3); UV λ_{max} (EtOH): 283 nm ($\epsilon=5700$); IR ν_{max} (CHCl_3) cm^{-1} : 3600, 3300, 2900, 1750, 1620, 1510, 1460, 1380, 1340, 1200, 1140, 1100, 960, 830 and 700 cm^{-1} ; $^1\text{H-NMR}$ δ (200 MHz, CDCl_3): 1.20 (3H, s, 2- CH_3), 1.70 (2H, t, $\text{H}_{3\alpha}$ and $\text{H}_{3\beta}$), 2.15 (3H, s, 8- CH_3), 2.25 (3H, s, 7- CH_3), 2.97 (2H, d, $J=11$ Hz, $\text{H}_{11\alpha}$ and $\text{H}_{11\beta}$), 4.25 (1H, t, $J=11$ Hz, H_{12}), 5.65 (1H, br. s, 4'-OH), 6.66 (2H, d, $J=7.5$ Hz, $\text{H}_{3'}$ and $\text{H}_{5'}$), 6.87 (2H, d, $J=7.5$ Hz, $\text{H}_{2'}$ and $\text{H}_{6'}$); EI-MS m/z : 562 (M^+), 354, 284, 256, 236; TLC (Merck 60F₂₅₄, 0.25 mm thickness) $R_f=0.28$ (hexane-ethyl acetate, 3:1); CD λ (EtOH) nm ($\Delta\epsilon$): 283 (+1.2), 255 (−2.4), 232 (−10.9). Data for the $^{13}\text{C-NMR}$ spectrum are shown in Table I.

Table I. $^{13}\text{C-NMR}$ DATA FOR γ -TOCOPHEROL, **1** AND **2**^a

	γ -Tocopherol	1	2
2	75.4 (s) ^d	75.3 (s) ^d	75.5 (s) ^d
2- CH_3	24.2 (q)	24.2 (q)	23.3 (q)
3	31.5 (t)	30.8 (t)	30.9 (t)
4	22.4 (t)	19.7 (t)	19.8 (t)
4a	118.2 (s)	119.8 (s)	119.8 (s)
5	112.1 (d)	115.3 (s)	115.5 (s)
6	146.2 (s)	148.1 (s)	148.3 (s)
7	121.6 (s)	123.6 (s)	123.8 (s)
7- CH_3	12.1 (q)	12.0 (q)	12.2 (q)
8	125.6 (s)	125.4 (s)	125.5 (s)
8a	144.0 (s)	143.0 (s)	143.2 (s)
8- CH_3	12.0 (q)	11.8 (q)	11.9 (q)
10	—	168.5 (s)	168.6 (s)
11	—	37.9 (t)	38.0 (t)
12	—	36.9 (d)	37.0 (d)
1'	40.1 (t)	39.3 (t)	40.7 (t)
2'	21.1 (t)	21.0 (t)	20.8 (t)
3',4',7',9'	37.5 (t)	37.4 (t)	37.4 (t)
4'	32.8 ^b (d)	32.7 ^b (d)	32.8 ^b (d)
4'- CH_3	19.8 ^c (q)	19.6 ^c (q)	19.7 ^c (q)
6'	24.5 (t)	24.4 (t)	24.5 (t)
8'	32.7 ^b (d)	32.6 ^b (d)	32.5 ^b (d)
8'- CH_3	19.7 ^c (q)	19.2 ^c (q)	19.4 ^c (q)
10'	24.9 (t)	24.7 (t)	24.9 (t)
11'	39.4 (t)	39.1 (t)	39.4 (t)
12'	28.0 (d)	27.9 (d)	28.0 (d)
12'- CH_3 , 13'	22.8 (q)	22.6 (q)	22.8 (q)
1''	—	131.7 (s)	131.8 (s)
2''	—	127.9 (d)	128.0 (d)
3''	—	115.7 (d)	115.8 (d)
4''	—	154.7 (s)	154.9 (s)
5''	—	115.7 (d)	115.8 (d)
6''	—	127.9 (d)	128.0 (d)

^a Measured at 50 MHz in CDCl_3 .

^{b,c} Assignments may be interchanged.

^d Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet.

Acetylation of 1. A mixture of **1** (1.2 mg), acetic anhydride (0.2 ml) and anhydrous pyridine (0.2 ml) was kept at room temperature for 24 hr. The excess acetic anhydride was decomposed with methanol, and the solvent was removed under reduced pressure. The acetylated **1** was purified by preparative HPLC [Develosil SI-60-5 ($8\phi \times 250$ mm); hexane-EtOAc (85:15)] to give 1.0 mg of an oily compound. Monoacetate of **1**: $^1\text{H-NMR}$ δ (200 MHz, CDCl_3): 2.15 (3H, s), 2.25 (6H, s), 2.97 (2H, d, $J=11$ Hz), 4.25 (1H, t, $J=11$ Hz), 6.90 (2H, d, $J=7.5$ Hz) and 6.95 (2H, d, $J=7.5$ Hz); EI-MS: m/z 604 (M^+).

Syntheses of 1 and 2. A mixture of 0.24 mmol of γ -tocopherol (100 mg), 0.33 mmol of *p*-coumaric acid (60 mg) and 0.12 mmol of *p*-toluenesulfonic acid (20 mg) in dry dioxane was heated for 3 hr in a boiling water bath.⁹ The reaction products, after removing the solvent by evaporation, were purified by preparative TLC and HPLC to afford 6.6 mg and 6.9 mg of two products (about 10% yield each). The spectral data and chromatographic behavior of each of synthetic compounds were identical with those of **1** and **2**.

Antioxidative assays

(1) **Thiocyanate method.** Each sample (16 μmol) was added to a mixed solution (25 ml) of linoleic acid/99.0% ethanol/0.2 M phosphate buffer. The mixed solution in a conical flask was incubated at 40°C, and the peroxide value was determined by reading the absorbance at 500 nm after a coloring reaction with FeCl_2 and thiocyanate.⁸

(2) **Thiobarbituric acid (TBA) method.** A mixed solution was prepared and incubated as just described. The formation of malonaldehyde was measured by reading the absorbance at 532 nm after a reaction with thiobarbituric acid.¹⁰

Results and Discussion

The molecular formula of **1** was confirmed by the high-resolution mass spectrum to give a molecular ion peak at m/z 562.4053 (562.4022; calcd. for $C_{37}H_{54}O_4$). In the 1H -NMR spectrum of **1**, two doublets [δ 6.66 (2H, $J=7.5$ Hz) and δ 6.87 (2H, $J=7.5$ Hz)] in the aromatic region suggested the presence of 1,4-disubstituted benzene ring ($H_{2''}$, $H_{3''}$, $H_{5''}$ and $H_{6''}$). Moreover, ABX-type signals at δ 2.94 ($H_{11\alpha}$, dd, $J=16.3$, 2.5 Hz), δ 2.99 ($H_{11\beta}$, dd, $J=16.3$, 6.3 Hz) and δ 4.25 (H_{12} , dd, $J=6.3$, 2.5 Hz) were observed, and the coupling of these protons was confirmed by 1H - 1H COSY NMR spectrum. Two methylene protons at δ 1.62 and δ 1.79 ($H_{3\alpha}$, ddd, $J=16.3$, 7.5, 6.9 and $H_{3\beta}$, ddd, $J=16.3$, 7.5, 6.9) were coupled with the adjacent methylene protons at δ 2.29 and δ 2.64 ($H_{4\alpha}$, ddd, $J=16.9$, 7.5, 6.9, and $H_{4\beta}$, ddd, $J=16.9$, 7.5, 6.9). As shown in the ^{13}C -NMR spectrum of **1** (Table I), it was strongly suggested that **1** contained an γ -tocopherol moiety as the partial structure of **1**. All signals due to chroman ring and side chain of γ -tocopherol were observed: however, the 5-carbon in the chroman ring of **1** was observed at δ 115.3 as a singlet, although the 5-carbon of γ -tocopherol at δ 112.1 was a doublet under the off-resonance condition. This data suggested that the 5-position of the chroman ring was substituted in **1**. The methine

proton (H_{12}) at δ 4.25 was found to be attached to the carbon (C-12) at δ 36.9 by the ^{13}C - 1H selective decoupling technique. Similarly, the methylene protons at δ 2.94 ($H_{11\alpha}$) and δ 2.99 ($H_{11\beta}$) were observed to be attached to the carbon at δ 37.9 (C-11). A C=O absorption at 1750 cm^{-1} in the IR spectrum and a carbonyl carbon at δ 168.5 in the ^{13}C -NMR spectrum of **1** suggested the presence of a δ -lactone ring.

Acetylation of **1** gave a monoacetate, and it was indicated that only the aromatic protons ($H_{2''}$, $H_{3''}$, $H_{5''}$ and $H_{6''}$ in **1**) were shifted downfield by acetylation; therefore, a hydroxy group was assumed to be in the p -position of the benzene ring. From these results, it was assumed that prunusol A had one of two possible chemical structures, A or B as shown in Fig. 1. In the NOE difference spectrum of **1**, a reasonable increase was observed at H_{12} (δ 4.25) upon irradiating at $H_{4\beta}$ (δ 2.64):

Fig. 1. Two Possible Structures for **1**.

Fig. 2. Structures of **1** and **2**.

therefore, it is assumed that structure A is the preferred structure of **1**. By comparing the spectroscopic data of prunusol B with those of prunusol A, it was strongly suggested that prunusol B was the diastereoisomer of prunusol A, because of the difference in the values of their $[\alpha]_D^{25}$ and CD spectra.

In order to confirm the structure of these prunusols, chemical syntheses of **1** and **2** were carried out by the coupling reaction of

γ -tocopherol with *p*-coumaric acid in the presence of *p*-toluenesulfonic acid, although the yield was only 10%. Both **1** and **2** had the same absolute configuration (*R*) as D- γ -tocopherol at the 2-positions;¹¹⁾ therefore, the additional chiral center in **1** and **2** was assumed to exist at β -carbon of the δ -lactone ring. Recently, Nonaka *et al.* have confirmed the stereochemical structures of cinchonains, phenylpropanoid-substituted epicatechins.⁹⁾ The absolute configuration of the β -carbons of prunusols A and B can be established as shown in **1** and **2** (Fig. 2), respectively, by comparing their CD spectra with those of cinchonains.

In the *Prunus* leaf wax, a large amount of γ -tocopherol (about 1 mg/g of leaf wax) has been detected,⁷⁾ and *p*-coumaric acid is also widely distributed in the plant leaves.¹²⁾ Therefore, it is probable that phenylpropanoid-substituted tocopherols such as prunusols A and B are produced between γ -tocopherol and *p*-coumaric acid in the leaves.

The antioxidative activity of prunusols A and B was examined by the thiocyanate and TBA methods as shown in Fig. 3. Both **1** and **2** showed almost the same antioxidative activity as α -tocopherol, but they showed less antioxidative activity than γ -tocopherol.

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Fig. 3. Antioxidative activity of **1** and **2**.

(a) Determined by the thiocyanate method.

(b) Determined by the thiobarbituric acid (TBA) method.

—○—, control; —●—, BHA; —△—, α -tocopherol; —▲—, γ -tocopherol; —□—, **1**; —■—, **2**.

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