Structure of a New Antioxidative Phenolic Acid from Oregano (*Origanum vulgare* L.)

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Five antioxidative phenolic acids were isolated from the leaves of oregano (*Origanum vulgare* L.). The structure of a new compound (5a) was determined to be 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy]phenylpropionic acid on the basis of spectroscopic evidence. Especially, phenyl glucoside (1) and 5a showed an activity comparable to that of BHA.

Much attention has been paid to spices as a resource of natural antioxidants for depressing the rancidity of fats and oils in food. Oregano (*Origanum vulgare* L.), one of the herbal spices belonging to the Family Labiatae, is known to have antioxidative activity. Chipault *et al.* examined the antioxidative effect of petroleum ether and ethyl alcohol soluble fractions extracted from many spices, and found that oregano had antioxidative properties in both fractions.¹⁾ They also reported in another article that oregano was not particularly effective in lard, but was most effective in mayonnaise and French dressing in all the tested samples.^{2,3)} As a part of a study on natural antioxidants, we have previously reported a new antioxidative glucoside (1) from this plant.⁴⁾ In this paper, we describe the isolation and structural elucidation of an additional new phenolic acid from oregano.

Dried leaves of oregano were successively extracted with dichloromethane and methanol. The ethyl acetate soluble part of the methanol extract showed strong antioxidative activity against linoleic acid in the ferric thiocyanate method.⁵⁾ This ethyl acetate soluble fraction was dissolved in 20% methanol in water and subjected to column chromatography on polyamide. Compound 1, protocatechuic acid (2) and caffeic acid (3) were isolated from the

fractions eluted with 20%, 50% and 80% methanol in water, respectively. The 0.5% formic acid in methanol fraction was further purified by silica gel and Sephadex LH-20 column

4a: R₁ = R₂ = H
4b: R₁ = H, R₂ = COCH₃
4c: R₁ = CH₃, R₂ = COCH₃

5a: R₁ = R₂ = H
5b: R₁ = H, R₂ = COCH₃
5c: R₁ = CH₃, R₂ = COCH₃

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chromatography and by repeated preparative HPLC to give compounds **4a** and **5a**. Compound **4a** was identified as rosmarinic acid on the basis of the spectral and physical data of its acetyl derivative (**4b**) and of its methyl ester (**4c**) which was synthesized from **4b**.⁶⁻⁸⁾

Compound **5a** was an off-white amorphous powder, mp 152~155°C. SI-MS of **5a** gave a molecular size of 466. The IR spectrum of this compound showed the presence of hydroxyl groups at 3500 cm⁻¹ and in the region of 3100~2400 cm⁻¹. The latter absorption and a band at 1680 cm⁻¹ suggested the presence of a conjugated carboxylic function. The ¹³C-NMR spectrum of **5a** revealed that this compound bore 25 carbons, which consisted of two methylene, one methine, two olefinic, eighteen aromatic and two carbonyl carbons. In the ¹H-NMR spectrum, two double doublets at δ 2.97 (*J*=3.4, 14.0 Hz) and 3.11 (*J*=9.7, 14.0 Hz) were attributed to the geminal protons of the benzylic methylene, which were coupled with a double doublet at δ 5.09 assignable to the oxymethine proton. Two doublets at δ 6.23 and 7.52 (*J*=15.9 Hz) and three aromatic protons [δ 6.77 (d, *J*=8.3 Hz), 6.93 (dd, *J*=2.0, 8.3 Hz), 7.03 (d, *J*=2.0 Hz)] suggested the presence of a *trans*-caffeoyl moiety, which was also supported by λ_{max} at 290, 328 nm in the UV spectrum and by ¹³C-NMR data (Table II). These data indicated that compound **5a** had a similar structure to rosmarinic acid (**4a**). In comparison with the ¹H-NMR spectrum of **4a**, two singlets at δ 6.51 and 6.75 were observed in the spectrum of **5a**, instead of the characteristic ABX-type aromatic proton signals at δ 6.62 (dd, *J*=2.0, 8.1 Hz), 6.68 (d, *J*=8.1 Hz) and 6.76 (d, *J*=2.0 Hz) in that of **4a**. It is deduced from this finding that a 1,2,4,5-tetrasubstituted aromatic ring was present in the molecule of **5a** in place of the 1,3,4-trisubstituted one of **4a**. In addition, a pair of two *ortho*-coupling protons were observed at δ 6.67 and 6.95, accompanied with diphenyl methylene protons at δ 3.89. Therefore, it was suggested that a 4-hydroxybenzylic moiety was attached to C-6' of rosmarinic acid (**4a**). This

fact was further supported by the presence of signals at δ 38.2, 116.2 (2C), 130.7 (2C), 133.6 and 156.4 in the ¹³C-NMR spectrum, and by the molecular size of 466 (M⁺), which was 106 mass (C₇H₆O) larger than that of rosmarinic acid. The assignments of H-2' and H-5' were confirmed by the irradiation technique in the ¹H-NMR measurement. When the methylene protons at δ 2.97 and 3.11 (H-3) were irradiated, the signal at δ 6.75 was sharpened. On the other hand, when the methylene protons at δ 3.89 (H-7''') were irradiated, the two-proton doublet at δ 6.95 (H-2''', 6''') and the singlet at δ 6.51 were sharpened. This confirmed that the singlet at δ 6.51 was attributed to H-5', and the other singlet at δ 6.75 to H-2'. The upfield shift (0.24 ppm) of H-5' compared with H-2' is considered to have been due to the shielding effect of the 4-hydroxybenzyl group at C-6'.

The ¹³C-NMR spectral data of **4a** and **5a** are listed in Table II, the assignment of signals being determined by the ¹³C-¹H COSY technique. The chemical shifts of C-1'~6' of the benzene ring revealed the substitution of the 4-

Table I. ¹H-NMR DATA OF COMPOUNDS **4a** AND **5a**
(400.5 MHz, methanol-d₄)

Proton no.	4a	5a
H-2	5.12dd	5.09dd
H-3a	2.97dd	2.97dd
H-3b	3.11dd	3.11dd
H-2'	6.76d	6.75s
H-5'	6.68d	6.51s
H-6'	6.62dd	
H-2''	7.03d	7.03d
H-5''	6.77d	6.77d
H-6''	6.92dd	6.93dd
H-7''	7.52d	7.52d
H-8''	6.26d	6.23d
H-2'''		6.95d
H-3'''		6.67d
H-5'''		6.67d
H-6'''		6.95d
H-7'''		3.89s

J(Hz) **4a**: 2, 3a=9.2; 2, 3b=3.5; 3a, 3b=14.3; 2', 6'=2.0; 5', 6'=8.1; 2'', 6''=2.0; 5'', 6''=8.2; 7'', 8''=15.9
5a: 2, 3a=9.7; 2, 3b=3.4; 3a, 3b=14.0; 2'', 6''=2.0; 5'', 6''=8.3; 7'', 8''=15.9; (2''', 6'''), (3''', 5''')=8.5

hydroxybenzyl group at C-6' and two hydroxyl groups at C-3' and C-4'. This was confirmed by the chemical shifts of the meth-

Table II. ^{13}C -NMR CHEMICAL SHIFTS OF COMPOUNDS **4a** AND **5a** (100.7 MHz, methanol- d_4)

Carbon no.	4a	5a
C-1	176.9	176.9
C-2	76.7	75.8
C-3	38.4	35.1
C-1'	130.6	127.8 ^a
C-2'	117.6	118.6
C-3'	146.0	144.9 ^b
C-4'	145.0	144.4 ^b
C-5'	116.3	118.5
C-6'	121.8	132.8 ^c
C-1''	127.9	127.9 ^a
C-2''	115.2	115.3
C-3''	146.7	146.8
C-4''	149.5	149.6
C-5''	116.5	116.5
C-6''	123.0	123.0
C-7''	147.1	147.3
C-8''	115.2	115.0
C-9''	168.9	168.7
C-1'''		133.6 ^c
C-2'''		130.7
C-3'''		116.2
C-4'''		156.4
C-5'''		116.2
C-6'''		130.7
C-7'''		38.2

^{a,b,c} Assignments are interchangeable.

ylene carbons of C-3 and C-7'''. A comparison of **5a** with **4a** showed an upfield shift (3.3 ppm) of C-3 due to the steric compression effect from the adjacent alkyl group.⁹⁾ On the contrary, if a hydroxyl group was located at an *ortho* position to an alkyl substituent on the aromatic ring, an upfield shift of more than 5 ppm of the benzylic carbon would have been observed.¹⁰⁾ In respect of C-7''', the chemical shift value (δ 38.2) was close to that of diphenylmethane.¹¹⁾ Thus, it must be concluded that C-6' was substituted by the 4-hydroxybenzyl group.

Compound **5a** showed a specific rotation of $+76.7^\circ$ (methanol), which was the same behavior as that of rosmarinic acid ($[\alpha]_D^{25}$: $+70.8^\circ$). Therefore, the stereochemistry at C-2 is proposed to have been of *R* configuration.¹²⁾

Acetylation of **5a** with acetic anhydride and pyridine gave a pentaacetate (**5b**). The IR spectrum newly showed an absorption band of acetates at 1760 cm^{-1} , with disappearance of the peak at 3500 cm^{-1} in that of **5a**. The signals of five phenolic acetates were observed at δ 2.22, 2.24 (OAc $\times$ 2), 2.29 and 2.30 in the ^1H -NMR spectrum, and five carbonyl carbons of acetates were obtained at 168.5, 168.6 (2C), 168.7 and 169.6 in the ^{13}C -NMR spectrum. The ^1H -NMR and ^{13}C -NMR data supported the structure of **5b**.

Fig. 1. Mass Fragmentation Pattern of Compound **5c**.

Fig. 2. Antioxidative Activity of Compounds 1~5a Measured by the Ferric Thiocyanate Method (conc. 0.02%).

□, 1; ○, 2, 4a; ■, 3; ▲, 5a; ●, BHA; △, α -tocopherol; —, control.

Methylation of compound **5b** with ethereal diazomethane afforded compound **5c**. The signal of a methyl group newly appeared at δ 3.74 in the $^1\text{H-NMR}$ spectrum and at δ 52.5 in the $^{13}\text{C-NMR}$ spectrum. The IR spectrum of **5c** revealed the loss of a hydroxyl group in the region of $2400\sim 3100\text{ cm}^{-1}$. The mass fragmentation pattern of **5c** is shown in Fig. 1. The cleavage at bond **a** gave the 4-acetoxybenzyl cation (m/z 149) and its deacetyl ion (m/z 107), and the cleavage at bond **b** produced the benzyl cation (m/z 229) as a main ion peak. The other major fragmentation pathway (**c**) yielded the acylium ion (m/z 205 and 163), and m/z 440, 402, 360 and 318. A McLafferty rearrangement gave the fragments of m/z 426 and 180, the former being degraded stepwise by deacetylation to a stable ion of m/z 300.

Based on all the above data, the structure of **5a** was established to be 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy]phenylpropionic acid.

The antioxidative activity of the five phenolic compounds isolated from oregano was measured by the ferric thiocyanate method (Fig. 2). All of them were more active than α -

tocopherol, a common natural antioxidant. Among them, two new compounds (**1** and **5a**) had an activity comparable with that of BHA, a synthetic antioxidant. It is noteworthy that **1** showed stronger activity than protocatechuic acid (**2**), which was composed of the acyl moiety of **1**, and that **5a** seemed to be more effective than rosmarinic acid (**4a**), which occupied the most part of the structure of **5a**.

Experimental

Melting points (mp) were measured with a Yanagimoto micro-melting point apparatus and were uncorrected. UV absorption spectra were determined on a Hitachi 220 spectrophotometer, and IR spectra were recorded by a Jasco IR-S. $^1\text{H-NMR}$ (400.5 MHz) and $^{13}\text{C-NMR}$ (100.7 MHz) spectra were determined on a JEOL GX-400 using TMS as an internal standard. MS were obtained on a Hitachi M-2000, and optical rotation values were measured with a Union PM-101. Column chromatography was performed using Wako polyamide C-200, Merck silica gel 60 (70~230 mesh) and Pharmacia Sephadex LH-20, and TLC was carried out using silica gel GF-254. HPLC was carried out with a Shimadzu LC-4A for analysis and a Shimadzu LC-6A for preparation.

Extraction and isolation. The MeOH extract (88.0 g) of dried leaves of oregano (1 kg) was separated into CH_2Cl_2 , AcOEt and H_2O soluble fractions. The AcOEt soluble part (7.5 g) was dissolved in 20% MeOH aq. and subjected to polyamide column chromatography, which was successively eluted with 20%, 50% and 80% MeOH aq. and MeOH. Compounds **1** (2.33 g), **2** (132 mg) and **3** (106 mg) were isolated from the 20%, 50% and 80% MeOH aq. fractions, respectively. Finally, the column was eluted with a mixture of MeOH-HCOOH (995:5). This fraction was rechromatographed on silica gel using AcOEt-MeOH- H_2O (21:4:3 v/v) as an eluent to separate into four fractions.

Compound 4a. The third fraction (1.14 g) was rechromatographed on a Sephadex LH-20 column using 80% EtOH aq. as an eluent to afford an off-white amorphous powder (790 mg). This compound was identified as rosmarinic acid,^{6~8)} mp $194\sim 195^\circ\text{C}$. UV λ_{max} (MeOH) nm (log ϵ): 291 (4.01), 327 (4.08). $[\alpha]_{\text{D}}^{25}$: $+70.8^\circ$ ($c=0.24$, MeOH). IR ν_{max} (Nujol) cm^{-1} : 3600, 3400~2400, 1685, 1600, 1515. SI-MS: 453 ($\text{M}+\text{H}+\text{G}$) $^+$, 361 ($\text{M}+\text{H}$) $^+$ 198.

Tetraacetyl rosmarinic acid. To a solution of 30 mg of compound **4a** in pyridine (1 ml), acetic anhydride (1 ml) was added, and the mixture was allowed to stand overnight at room temperature. The reaction mixture was poured into cold 2N-HCl and then extracted with AcOEt.

The organic layer was washed with H_2O , dried over anhydrous $MgSO_4$ and evaporated to dryness. Recrystallization of this residue from benzene gave colorless needles of compound **4b** (28 mg), mp 119–120°C. UV λ_{max} (MeOH) nm (log ϵ): 279 (4.41). $[\alpha]_D^{25}$: +46.2° ($c=0.26$, MeOH). IR ν_{max} (Nujol) cm^{-1} : 3400–2600, 1760, 1720, 1640, 1610, 1505. ^1H -NMR (CD_3COCD_3) δ : 2.24 (3H, s), 2.25 (3H, s), 2.28 (3H, s), 2.29 (3H, s), 3.22 (1H, dd, $J=8.5$, 14.5 Hz), 3.33 (1H, dd, $J=4.2$, 14.5 Hz), 5.34 (1H, dd, $J=4.2$, 8.5 Hz), 6.59 (1H, d, $J=16.1$ Hz), 7.18 (1H, d, $J=8.1$ Hz), 7.27 (1H, d, $J=2.0$ Hz), 7.29 (1H, dd, $J=2.0$, 8.1 Hz), 7.32 (1H, d, $J=9.0$ Hz), 7.62 (1H, d, $J=2.0$ Hz), 7.63 (1H, dd, $J=2.0$, 9.0 Hz), 7.68 (1H, d, $J=16.1$ Hz). ^{13}C -NMR (CD_3COCD_3) δ : 170.6, 168.64 (2C), 168.59, 168.48, 166.1, 145.1, 144.5, 143.9, 143.2, 142.3, 136.1, 133.9, 128.1, 127.4, 125.6, 124.9, 124.2, 124.1, 119.3, 73.3, 37.2, 20.5 (4C). MS m/z : 528 (M^+ , $C_{26}H_{24}O_{12}$), 486, 468, 444, 426, 402, 384, 282, 264, 247, 240, 222, 205, 198, 180, 163, 123.

Methylation of compound 4b. A solution of compound **4b** (10 mg) in ether (2 ml) was treated with ethereal diazomethane at 4°C for 5 min. After the excess diazomethane had been decomposed with a few drops of acetic acid, the reaction mixture was extracted with aq. saturated $NaHCO_3$ solution. The ethereal layer was washed with water, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude methylated product (8 mg) was recrystallized from MeOH to give colorless needles of compound **4c**, mp 99–101°C. UV λ_{max} ($CHCl_3$) nm (log ϵ): 284 (4.35). $[\alpha]_D^{25}$: +35.0° ($c=0.2$, $CHCl_3$). IR ν_{max} (Nujol) cm^{-1} : 1760, 1720, 1680, 1630, 1500, 1200. ^1H -NMR ($CDCl_3$) δ : 2.270 (3H, s), 2.274 (3H, s), 2.30 (3H, s), 2.31 (3H, s), 3.18 (1H, dd, $J=5.1$, 14.8 Hz), 3.23 (1H, dd, $J=7.6$, 14.8 Hz), 3.74 (3H, s), 5.35 (1H, dd, $J=5.1$, 7.6 Hz), 6.41 (1H, d, $J=15.9$ Hz), 7.10–7.15 (3H, m), 7.23 (1H, d, $J=8.5$ Hz), 7.38 (1H, d, $J=2.0$ Hz), 7.42 (1H, dd, $J=2.0$, 8.5 Hz), 7.63 (1H, d, $J=15.9$ Hz). ^{13}C -NMR ($CDCl_3$) δ : 169.8, 168.2, 168.1, 168.0, 167.9, 165.6, 144.3, 143.8, 142.5, 142.0, 141.2, 134.6, 133.1, 127.3, 126.6, 124.5, 123.9, 123.4, 123.0, 118.2, 76.7, 52.5, 36.8, 20.63 (2C), 20.59 (2C). MS m/z (%): 542 (M^+ , $C_{27}H_{26}O_{12}$), 500, 458 (5), 440, 416, 398, 278 (5), 247 (4), 236 (17), 222 (5), 205 (19), 194 (100), 180 (23), 163 (48), 123 (17).

Compound 5a. The second fraction (70 mg) was rechromatographed on a Sephadex LH-20 column using 80% EtOH as an eluent to afford an amorphous powder, which was homogeneous on a TLC plate with a solution of AcOEt–MeOH– H_2O (21:4:3 v/v) as the developing solvent, but was revealed to consist of three components by HPLC analysis: column, develosil 5-ODS (Chemco), 150 $\times$ 4.6 mm; solvent, H_2O –MeOH–HOAc (27:23:1 v/v); flow rate, 0.5 ml/min; pressure, 120 kg/cm²; detection, absorption at 290 nm. These three constituents were separated by repeated preparative HPLC using the following system: column, develosil 5-ODS (Chemco), 250 $\times$ 10 mm;

solvent, H_2O –MeOH–HOAc (27:23:1 v/v); flow rate, 1.5 ml/min; pressure, 130 kg/cm². The major component (**5a**) was isolated as an off-white amorphous powder (21 mg), mp 152–155°C. UV λ_{max} (MeOH) nm (log ϵ): 290 (4.07), 328 (4.08). $[\alpha]_D^{25}$: +76.7° ($c=0.30$, MeOH). IR ν_{max} (Nujol) cm^{-1} : 3500, 3100–2400, 1680, 1620, 1600, 1510. SI-MS: 559 ($M+H+G$)⁺, 467 ($M+H$)⁺, 163, 107.

Acetylation of compound 5a. Acetylation of **5a** (15 mg) was carried out as already mentioned. The crude product was purified by column chromatography on Sephadex LH-20 using isopropyl alcohol as an eluent to give a colorless viscous gum (**5b**). UV λ_{max} (MeOH) nm (log ϵ): 276 (4.29). $[\alpha]_D^{25}$: +45.5° ($c=0.11$, MeOH). IR ν_{max} (Nujol) cm^{-1} : 3400–2600, 1760, 1720, 1700, 1680, 1635, 1590, 1505, 1200. ^1H -NMR (CD_3COCD_3) δ : 2.22 (3H, s), 2.24 (6H, s), 2.29 (3H, s), 2.30 (3H, s), 3.20 (1H, dd, $J=9.5$, 14.8 Hz), 3.37 (1H, dd, $J=3.9$, 14.8 Hz), 4.19 (2H, s), 5.26 (1H, dd, $J=3.9$, 9.5 Hz), 6.57 (1H, d, $J=16.1$ Hz), 7.00 (1H, s), 7.06 (2H, d, $J=8.5$ Hz), 7.24 (2H, d, $J=8.5$ Hz), 7.29 (1H, s), 7.33 (1H, d, $J=8.8$ Hz), 7.62 (1H, d, $J=2.0$ Hz), 7.63 (1H, dd, $J=2.0$, 8.8 Hz), 7.68 (1H, d, $J=16.1$ Hz). ^{13}C -NMR (CD_3COCD_3) δ : 170.8, 169.6, 168.7, 168.6 (2C), 168.5, 166.1, 150.4, 145.1, 144.5, 143.8, 142.2, 141.6, 138.8, 138.3, 134.3, 133.9, 130.5 (2C), 127.4, 126.6, 125.8, 124.9, 124.1, 122.7 (2C), 119.2, 73.1, 38.0, 34.3, 20.9, 20.5 (4C). SI-MS: 677 ($M+H$)⁺, 163, 149, 123, 107.

Methylation of compound 5b. Methylation of compound **5b** (10 mg) was carried out as already mentioned to give a colorless viscous gum (9 mg). UV λ_{max} (MeOH) nm (log ϵ): 279 (4.36). $[\alpha]_D^{25}$: +22.9° ($c=0.70$, $CHCl_3$). IR ν_{max} (film) cm^{-1} : 1760, 1730, 1710, 1680, 1630, 1500, 1200. ^1H -NMR ($CDCl_3$) δ : 2.24 (3H, s), 2.25 (3H, s), 2.28 (3H, s), 2.30 (3H, s), 2.31 (3H, s), 3.13 (1H, dd, $J=9.0$, 14.7 Hz), 3.21 (1H, dd, $J=4.6$, 14.7 Hz), 3.74 (3H, s), 4.07 (2H, s), 5.24 (1H, dd, $J=4.6$, 9.0 Hz), 6.37 (1H, d, $J=16.1$ Hz), 6.90 (1H, s), 7.02 (2H, d, $J=8.5$ Hz), 7.11 (1H, s), 7.14 (2H, d, $J=8.5$ Hz), 7.23 (1H, d, $J=8.3$ Hz), 7.36 (1H, d, $J=2.0$ Hz), 7.40 (1H, dd, $J=2.0$, 8.3 Hz), 7.62 (1H, d, $J=16.1$ Hz). ^{13}C -NMR ($CDCl_3$) δ : 170.0, 169.5, 168.2, 168.1, 168.0, 167.9, 165.7, 149.3, 144.3, 143.8, 142.5, 141.0, 140.5, 137.7, 136.8, 133.1, 132.9, 129.9 (2C), 126.6, 125.3, 125.2, 123.9, 123.0, 121.8 (2C), 118.1, 72.4, 52.5, 37.7, 33.9, 21.1, 20.7 (2C), 20.6 (2C).

Antioxidative assay. Antioxidative activity was measured by the ferric thiocyanate method as previously described.⁵⁾

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