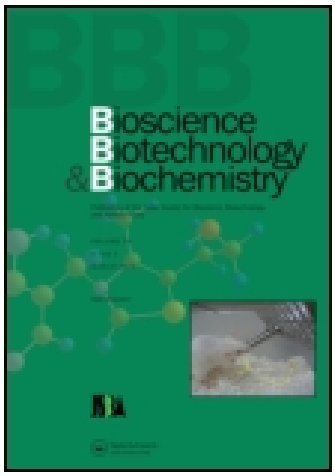

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Pyromeconic Acid and Its Glucosidic Derivatives from Leaves of Erigeron annuus, and the Siderophile Activity of Pyromeconic Acid

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Published online: 12 Jun 2014.

To cite this article: Yasuyuki Hashidoko (1995) Pyromeconic Acid and Its Glucosidic Derivatives from Leaves of Erigeron annuus, and the Siderophile Activity of Pyromeconic Acid, Bioscience, Biotechnology, and Biochemistry, 59:5, 886-890, DOI: 10.1271/bbb.59.886

To link to this article: http://dx.doi.org/10.1271/bbb.59.886

Biosci. Biotech. Biochem., **59** (5), 886–890, 1995

Pyromeconic Acid and Its Glucosidic Derivatives from Leaves of *Erigeron annuus*, and the Siderophile Activity of Pyromeconic Acid

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Received November 14, 1994

3′-*O*-Caffeylerigeroside (pyromeconic acid 3-*O*-β-D-glucoside 3′-*O*-caffeyl ester) was obtained from the leaves of *Erigeron annuus* as a new pyromeconic acid derivative, and its structure was elucidated. Together with the γ-pyrone derivative, pyromeconic acid (3-hydroxy-*4H*-pyran-4-one) and its β-glucoside (erigeroside) were also isolated from the aerial parts of *E. annuus*. The siderophile activity of pyromeconic acid was also studied.

Genus *Erigeron* is a common group of Compositae plants, and *E. annus*, *E. canadenis*, and *E. philadelphus* are now, as naturalized weeds, widely distributed throughout urban and rural areas of Japan.[1] Young leaves or floral buds of *E. annuus* and *E. philadelphus* are also known to be edible after being boiled or fried.[1,2] The author's initial observations on the chemical components of *E. annuus* arose after screening the wound-induced antifungal substances from wild weeds.[3] An antifungal spot was detected on the TLC chromatogram from the water-diffusates of physically damaged leaves of *E. annuus*,[4] while only the trace of an antifungal area was visible in the methanolic extracts of non-damaged leaves at the same scale. Subsequently, the antifungal substance inducible by wounding was identified as pyromeconic acid (3-hydroxy-*4H*-pyran-4-one, **1**) which had been first obtained from meconic acid (3-hydroxy-*4H*-pyran-4-one-2,6-dicarboxylic acid)[5] and also found as a natural product of some *Erigeron* species[6,7] and a Lauraceae.[8] Moreover, a large amount of pyromeconic acid β-glucoside (erigeroside, **2**) was obtained from the water-diffusate of comparatively less-damaged leaves. This fact suggested that compound **2** underwent hydrolytic cleavage to yield aglycon **1** in the damaged tissues. In a further investigation of the chemical constituents, a new derivative of pyromeconic acid (3′-*O*-caffeylerigeroside, **3**) was obtained from non-damaged leaves of the plant. The distribution, isolation and structural elucidation of new γ-pyrone derivative **3**, and some chemical properties of pyromeconic acid (**1**) and erigeroside (**2**) are described in this paper.

Materials and Methods

General. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JMN EX-270 instrument at 270 and 68 MHz, respectively. The COLOC spectrum was recorded by a Bruker AM-400 instrument at 400 and 100 MHz. TMS and 3-(trimethylsilyl)-propanesulfonic acid sodium salt were used as internal standards, for organic solvents ($CDCl_3$ and CD_3OD) and D_2O, respectively. FD- and EI-MS spectra were measured by JEOL JMS 01SG-2 and JEOL DX-300 instruments, respectively. UV and IR data were taken as an MeOH solution by Hitachi U-3210 and as a KBr disc by JASCO IR-700 spectrometers, respectively. Melting point (mp) values are uncorrected, and $[\alpha]_D$ values were measured at 23°C.

Plant materials and reagent. Wild *E. annuus* used in preparating the chemical constituents was collected in early June from a field in Eniwa (Hokkaido, Japan). The leaves (400 g, f.w.) were carefully detached from the stem by a razor blade. Chopped into small flaskes with the razor blade, the resulting tissues were soaked in distilled water (1500 ml) for 24 h to obtain water-solubles that were diffusing from the cut edges. Another 7.8 kg of the aerial parts of *E. annuus* were crushed with a wooden mallet, and the samples were directly extracted with acetone (20 liters). Seeds were collected from wild specimens and cultivated on vermiculite for 4 days. β-Glucosidase was purchased from Worthington Biochemicals Co.

Isolation of pyromeconic acid. The resulting aqueous layer that contained the hydrophilic constituents diffusing from the leaf flaskes was extracted with EtOAc (800 ml), and the EtOAc-soluble (212 mg) were chromatographed in a silica gel column (10 g of Wako-gel C-200 in $CHCl_3$; 20 mm i.d.). The column was successively eluted with 2, 5, 7.5 and 15% of MeOH in $CHCl_3$ (40 ml each). Since compound **1** was eluted with 2–7.5% MeOH/$CHCl_3$, these fractions were combined and concentrated to yield an amorphous powder. Accordingly, *ca.* 130 mg of colorless plates were obtained by re-crystallization from $CHCl_3$/MeOH. From the whole aerial parts that had been physically damaged (7.8 kg), fine plates of **1** (1.7 g) were further obtained by silica gel column chromatography and crystallization. A large amount of **1** was also detected in germinating seedlings of *E. annuus*.

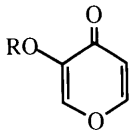
Identification and structural analyses of pyromeconic acid (**1**). Regarding the carbon signal with an upfield shift (δ_H 173.3) as that of a carboxyl carbon gave an erroneous 5-hydroxy-*2H*-pyran-2-one (5-hydroxy-α-pyrone) structure based on the COLOC data of the methylated product (**1b**).[9] The correct structure for **1** was obviously 3-hydroxy-*4H*-pyran-4-one (pyromeconic acid) for following reasons: i) the IR spectrum for the carbonyl group (1645 cm^{-1}) was most reasonable for an α-enolic ketone,[10] ii) three carbons each showing a downfield shift are most attributable to the *4H*-pyran-4-one structure that also satisfied the COLOC data, and iii) Zhang *et al.* have reported pyromeconic acid (3-hydroxy-*4H*-pyran-4-one) from another *Erigeron* species (*E. breviscapus*).[6]

Pyromeconic acid (**1**). Mp 117.5–118.5°C. R_f 0.32 in $CHCl_3$–MeOH = 10 : 1. FD-MS (*m/z*, %): 112 (100). EI-MS (*m/z*, %): 112 (M^+, 100), 84 (44), 71 (32), 69 (32), 58 (32), 55 (72), 43 (33) and 42 (58). EI-HR-MS: $C_5H_4O_3$ (found 112.014, calcd. 112.016). UV λ_{max} (MeOH): 210 (ε 22,000) and 271 nm (ε 16,700). IR ν cm^{-1} (KBr): 3200 (OH) and 1645 (C=O). ^{1}H-NMR δ ($CDCl_3$): 7.88 (d, J = 1.0 Hz, 2-H), 7.70 (dd, J = 5.3 and 1.0 Hz, 6-H), 6.59 (d, J = 5.3 Hz, 5-H). ^{13}C-NMR δ (CD_3OD, CH-COSY): 173.3 (4-C), 155.6 (6-CH), 146.7 (3-C), 140.1 (2-CH) and 114.3 (5-CH).

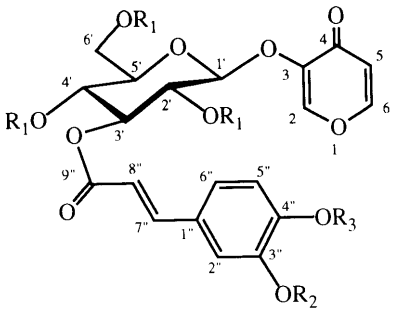
Chemical derivatives of pyromeconic acid. Acetylation and methylation of **1** were accomplished by conventional methods.

Pyromeconic acid acetate (**1a**). 91% yield R_f 0.51 in $CHCl_3$–MeOH = 10 : 1. A colorless syrup. EI-MS (*m/z*, %): 154 (M^+, 3.1), 112 ($M^+ - 42$, 63), 84 (11), 60 (26), 55 (12), 45 (42), 44 (45), 43 (100) and 40 (53). ^{1}H-NMR δ ($CDCl_3$): 7.92 (d, J = 0.7 Hz, 2-H), 7.77 (dd, J = 5.6 and 0.7 Hz, 6-H), 6.50 (d, J = 5.6 Hz, 5-H) and 2.33 (s, 3-OCO$\underline{CH_3}$). ^{13}C-NMR δ ($CDCl_3$, CH-COSY): 172.5 (4-C), 168.1 (3-O$\underline{C}$OCH$_3$), 155.4 (6-CH), 148.9 (3-C), 142.2 (2-CH), 118.2 (5-CH) and 20.7 (3-OCO$\underline{CH_3}$).

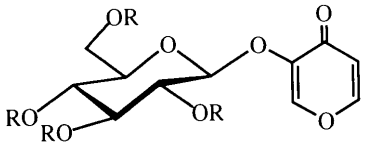
3-O-Methyl pyromeconate (**1b**). 68% yield. R_f 0.50 in $CHCl_3$–MeOH = 10 : 1. Fine colorless plates from $CHCl_3$/*n*-hexane, mp 92–93°C. EI-MS (*m/z*, %): 127 ($M^+ + 1$, 32), 126 (M^+, 100), 108 (42), 97 (30), 96 (51), 68 (52), 55 (65) and 39 (30). ^{1}H-NMR δ ($CDCl_3$): 7.60 (d, J = 0.7 Hz,



1: R=H, pyromeconic acid
1a: R=Ac
1b: R=Me



3: R_1=R_2=R_3=H, 3′-*O*-caffeylerigeroside
3a: R_1=R_2=R_3=Ac
3b: R_1=H, R_2=R_3=Me
3c: R_1=H, R_2=H, R_3=Me



2: R=H, erigeroside
2a: R=Ac

Fig. 1. Chemical Structures of Pyromeconic Acid Derivatives from *E. annuus*.

2-H), 7.73 (dd, J=5.6 and 0.9 Hz, 6-H), 6.43 (d, J=5.6 Hz, 5-H) and 3.78 (s, 3-OCH$_3$). ^{13}C-NMR δ (CDCl$_3$, CH-COSY): 173.6 (4-C), 154.5 (6-CH), 149.5 (3-C), 138.3 (2-CH), 116.0 (5-CH) and 56.3 (3-OCH$_3$). COLOC data (400/100 MHz, CDCl$_3$), proton→(carbons): 7.73→(173.6, 138.3 and 116.0), 7.60→(173.6, 154.5 and 149.5), 6.43→(149.5) and 3.78 (149.5). Cross peaks with direct C H-correlation that were observed by CH-COSY are not recorded in the COLOC data.

*Isolation of pyromeconic acid 3-O-β-*D*-glucoside (erigeroside,* **2**). The water-diffusates were concentrated and re-dissolved in a small volume of water/MeOH (1/10). After filtration to remove insoluble materials, the filtrate was concentrated to give 14.6 g of a dark brown syrup. A part of this syrup (450 mg) was subjected to silica gel column chromatography (80 g of Wako-gel C-200), eluting with 15% MeOH/CHCl$_3$ (600 ml) and then with 30% MeOH/CHCl$_3$ (300 ml). Compound **2** was eluted with 30% MeOH/CHCl$_3$, precipitated as a powder, and re-crystallized from MeOH (about 50 mg). Silica gel column chromatography on a larger scale enabled about 2 g of crude **2** to be obtained from 14.2 g of the syrup. Mp 195–196.5°C. R_f 0.17 in CHCl$_3$–MeOH=5:1. $[\alpha]_D$ −102° (H$_2$O, c=0.24). UV λ_{max} (MeOH): 212 (ε 12,300) and 261 nm (ε 10,400). FD-MS (m/z, %): 275 (M^++1, 68), 163 (14) and 112 (100). ^{1}H-NMR δ (CD$_3$OD, HH-COSY): 8.32 (br. s, 2-H), 8.11 (dd, J=5.4 and 1.0 Hz, 6-H), 6.53 (br. d, J=5.4 Hz, 5-H), 4.74 (d, J=6.6 Hz, 1′-H), 3.92 (br. d, J=12.2 Hz, 6′-Ha), 3.67 (dd, J=12.2 and 6.3 Hz, 6′-Hb), 3.54 (m, 5′-H), 3.45 (m, 2′- and 4′-H) and 3.31 (m, 3′-H). ^{13}C-NMR δ (CD$_3$OD, DEPT and CH-COSY): 176.1 (4-C), 158.1 (6-CH), 148.1 (3-C), 146.7 (2-CH), 117.0 (5-CH), 104.5 (1′-CH), 78.5 (5′-CH), 77.1 (3′-CH), 74.6 (2′-CH), 71.2 (4′-CH) and 62.5 (6′-CH$_2$).

*Acetylation of pyromeconic acid 3-O-β-*D*-glucoside.* Compound **2** (17.3 mg) was acetylated by Ac$_2$O/pyridine at 70°C, and the major product (25.3 mg) was obtained by preparative TLC.

*Pyromeconic acid 3-O-β-*D*-glucoside tetraacetate* (**2a**). 91% yield. A colorless syrup. R_f 0.65 in CHCl$_3$–MeOH=10:1. FD-MS (m/z, %): 443 (M^++1, 66), 442 (M^+, 31), 331 (M^+−aglycon, 82), 154 (16), 112 (100) and 111 (30). ^{1}H-NMR δ (CDCl$_3$, HH-COSY): 7.90 (d, J=1.0 Hz, 2-H), 7.71 (dd, J=5.8 and 1.0 Hz, 6-H), 6.43 (br. d, J=5.8 Hz, 5-H), 5.31 (d, J=7.6 Hz, 1′-H), 5.29 (dd, J=9.2 and 9.2 Hz, 3′-H), 5.17 (dd, J=9.2 and 7.6 Hz, 2′-H), 5.13 (dd, J=9.9 and 9.2 Hz, 4′-H), 4.26 (dd, J=12.4 and 4.5 Hz, 6′-Ha), 4.12 (dd, J=12.4 and 2.5 Hz, 6′-Hb), 3.72 (ddd, J=9.9, 4.5 and 2.5 Hz, 5′-H), 2.14 (s, OAc), 2.07 (s, OAc), 2.032 (s, OAc) and 2.027 (s, OAc). ^{13}C-NMR δ (CDCl$_3$, DEPT and CH-COSY): 173.7 (4-C), 170.5 (OCOCH$_3$), 170.1 (OCOCH$_3$), 169.9 (OCOCH$_3$), 169.4 (OCOCH$_3$), 154.5 (6-CH), 148.7 (3-C), 144.8 (2-CH), 118.1 (5-CH), 98.9 (1′-CH), 72.4 (3′-CH), 72.1 (5′-CH), 70.1 (2′-CH), 68.2 (4′-CH), 61.5 (6′-CH$_2$), 20.8 (OCOCH$_3$), 20.7 (OCOCH$_3$) and 20.6 (OCOCH$_3$ × 2).

Hydrolysis of **2** *with β-glucosidase.* Compound **2** (25.8 mg) dissolved in 20 ml of β-glucosidase/distilled water (0.6 mg of enzyme, 1.4 unit) was kept at 25°C for 24 h. The reaction solution was diluted with water and extracted with 50% EtOAc/*n*-BuOH (70 ml) to obtain aglycon **1** (4.7 mg, 45% yield). Solids from the aqueous layer contained unchanged **2** (8.0 mg, 31% soluble in EtOH) and free sugar (10.2 mg, 66%, soluble in 70% MeOH/H$_2$O). The sugar was dextrorotatory ($[\alpha]_D$ +27°, c=0.10 in H$_2$O), and its pentaacetate showed a good accordance with that of D-glucose in their ^{1}H-NMR spectra.

Detection and isolation of 3′-O-caffeylerigeroside (**3**). The chemical constituents of excised leaves of *E. annuus* (15 g) without any physical damage, which had been prepared by directly extracting the leaves with MeOH, showed three major quenching spots (under UV$_{254\,nm}$) by TLC (Merck Kieselgel 60 F_{254}, 0.25 mm thickness) developed in CHCl$_3$–MeOH=3:1 [top (R_f 0.77), second (R_f 0.45) and third (R_f 0.27)]. The top and third substances were identical with **1** and **2**, respectively. The second compound, however, arose neither in the water-solubles from the chopped leaves nor in the MeOH-solubles of the mechanically damaged leaves; therefore, this compound that was throught to be unstable in the damaged tissues was isolated. The extract was chromatographed in a dry silica gel column (20 g, Wako-gel C-200) and eluted with 25% MeOH/CHCl$_3$ to obtain fractions of 20 ml in volume. The target compound was eluted in the fractions from 100 to 140 ml, and the mixture obtained (56 mg) was further purified by preparative TLC (CHCl$_3$–MeOH=3:1). Subsequently, a pale yellow syrup (30 mg) was obtained.

3′-O-Caffeylerigeroside (**3**). FD-MS (m/z, %): 460 (M^++Na+1, 35), 459 (M^++Na, 100), 437 (M^++1, 64), 275 (64), 112 (65). UV λ_{max} (MeOH): 214, 250, 300 (shoulder) and 328 nm. ^{1}H-NMR δ (D$_2$O, HH-COSY): 8.32 (s, 2-H), 8.12 (d, J=5.6 Hz, 6-H), 7.68 (d, J=15.8 Hz, 7″-H), 7.16 (br. s, 2″-H), 7.10 (br. d, J=8.3 Hz, 6″-H), 6.91 (d, J=8.3 Hz, 5″-H), 6.59 (d, J=5.6 Hz, 5-H), 6.44 (d, J=15.8 Hz, 8″-H), 5.18 (dd, J=9.2 and 9.2 Hz, 3′-H), 5.06 (d, J=7.6 Hz, 1′-H), 3.96 (br. d, J=12.2 Hz, 6′-Ha), 3.83 (dd, J=9.2 and 7.6 Hz, 2′-H), 3.8–3.7 (4′ and 6′-Hb, overlapped) and 3.65 (m, 5′-H). δ (CD$_3$OD, HH-COSY): 8.34 (s, 2-H), 8.10 (d, J=5.6 Hz, 6-H), 7.61 (d, J=15.8 Hz, 7″-H), 7.07 (d, J=2.0 Hz, 2″-H), 6.97 (dd, J=8.2 and 2.0 Hz, 6″-H), 6.78 (d, J=8.2 Hz, 5″-H), 6.53 (d, J=5.6 Hz, 5-H), 6.35 (d, J=15.8 Hz, 8″-H), 5.13 (dd, J=9.5 and 9.2 Hz, 3′-H), 4.88 (overlapped with CD$_3$OH peak, J=7.9 Hz, 1′-H), 3.93 (dd, J=12.5 and 2.4 Hz, 6′-Ha), 3.71 (dd, J=12.5 and 4.9 Hz, 6′-Hb), 3.68 (dd, J=9.2 and 7.9 Hz, 2′-H), 3.60 (dd, J=9.5 and 9.4 Hz, 4′-H) and 3.57 (m, 5′-H). ^{13}C-NMR δ (CD$_3$OD, DEPT): 176.0 (4-C), 169.0 (9″-C), 158.0 (6-CH), 149.6 (4″-C), 148.1 (3-C), 147.1 (7″-CH), 146.9 (3″-C), 146.7 (2-CH), 127.9 (1″-C), 123.0 (6″-CH), 117.1 (5-CH), 116.5 (8″-CH), 115.4 (2″-CH), 115.2 (5″-CH), 103.4 (1′-CH), 78.5 (5′-CH), 77.9 (3′-CH), 73.2 (2′-CH), 69.5 (4′-CH) and 62.3 (6′-CH$_2$).

Acetylation of 3′-O-caffeylerigeroside. A part of crude **3** was acetylated by Ac$_2$O/pyridine at 50°C, and the major product of the reaction mixture was purified by TLC.

Pentaacetate of **3** (**3a**). A colorless syrup. R_f 0.21 in CHCl$_3$–MeOH=49:1. ^{1}H-NMR δ (CDCl$_3$, HH-COSY): 7.91 (d, J=1.0 Hz, 2-H), 7.71 (dd, J=5.6 and 1.0 Hz, 6-H), 7.69 (d, J=15.8 Hz, 7″-H), 7.41 (dd, J=8.3 and

2.0 Hz, 6″-H), 7.36 (d, J=2.0 Hz, 2″-H), 7.23 (d, J=8.3 Hz, 5″-H), 6.44 (br. d, J=5.6 Hz, 5-H), 6.32 (d, J=15.8 Hz, 8″-H), 5.42 (dd, J=9.6 and 9.6 Hz, 3′-H), 5.37 (d, J=8.3 Hz, 1′-H), 5.25 (dd, J=9.6 and 7.9 Hz, 2′-H), 5.21 (dd, J=9.6 and 9.6 Hz, 4′-H), 4.27 (dd, J=12.5 and 4.6 Hz, 6′-Ha), 4.14 (dd, J=12.5 and 2.6 Hz, 6′-Hb), 3.72 (ddd, J=9.9, 4.6 and 2.6 Hz, 5′-H), 2.31 and 2.30 (both s, 4″- and 5″-OAc), 2.10 (s, OAc), 2.08 (s, OAc) and 2.00 (s, OAc). ^{13}C-NMR δ ($CDCl_3$): 173.7 (4-C), 170.6 ($O\underline{C}OCH_3$), 169.9 ($O\underline{C}OCH_3$), 169.4 ($O\underline{C}OCH_3$), 168.1 and 167.9 (3″- and 4″-$O\underline{C}OCH_3$), 165.4 (9″-C), 154.5 (6-CH), 148.7 (3-C), 144.8 (4″-C), 144.6 (2-CH), 143.9 (3″-CH), 142.5 (7″-CH), 132.8 (1″-C), 126.7 (6″-CH), 124.0 (2″-CH), 123.0 (5″-CH), 118.1 (5-CH), 117.6 (8″-CH), 98.9 (1′-CH), 72.5 (3′-CH), 72.1 (5′-CH), 70.9 (2′-CH), 68.2 (4′-CH), 61.6 (6′-CH_2), 20.71 ($OCO\underline{CH_3}\times 2$), 20.66 ($OCO\underline{CH_3}$) and 20.6 ($OCO\underline{CH_3}\times 2$).

Methylation of 3′-O-caffeylerigeroside. Methylation of **3** was performed with Me_2SO_4/K_2CO_3/acetone (15 μl/100 mg/2 ml) at room temperature for 24 h. To avoid spontaneous hydrolytic cleavage, the resulting solution concentrated under dried N_2 gas was directly applied on TLC (in $CHCl_3$–MeOH=3:1). The major product detected at R_f 0.87 was identified as dimethyl derivative **3b**.

3″,4″-Di-O-methyl product of **3** (**3b**). Colorless solid, 5.6 mg (55% yield). FD-MS (*m/z*, %): 487 (M^++Na, 13), 465 (M^++1, 30), 464 (M^+, 100), 352 (16), 208 (13) and 112 (17). ^{1}H-NMR δ (CD_3OD): 8.34 (d, J=0.7 Hz, 2-CH), 8.10 (dd, J=5.6 and 0.7 Hz, 6-CH), 7.69 (d, J=15.8 Hz, 7″-H), 7.25 (d, J=2.0 Hz, 2″-H), 7.20 (dd, J=8.3 and 2.0 Hz, 6″-H), 6.99 (d, J=8.3 Hz, 5″-H), 6.53 (br. d, J=5.6 Hz, 5-H), 6.51 (d, J=15.8 Hz, 8″-H), 5.14 (dd, J=9.6 and 9.2 Hz, 3′-H), approx. 4.86 (overlapped with $CD_3O\underline{H}$ peak, 1′-H), 3.93 (dd, J=12.2 and 1.6 Hz, 6′-Ha), 3.875 and 3.869 (both s, 3″- and 4″-OCH_3) and 3.74–3.52 (4H, m, 2′-, 4′-, 5′-H and 6′-Hb). ^{13}C-NMR δ (CD_3OD): 176.0 (4-C), 168.7 (9″-C), 158.1 (6-CH), 152.8 (4″-C), 150.8 (7″-CH), 149.7 (3″-C), 148.2 (3-C), 146.6 (2-CH), 129.0 (1″-C), 124.1 (6″-CH), 117.1 (5-CH), 116.7 (8″-CH), 112.6 (2″-CH), 111.6 (5″-CH), 103.4 (1′-CH), 78.5 (5′-CH), 77.9 (3′-CH), 73.2 (2′-CH), 69.5 (4′-CH), 62.3 (6′-CH_2), 58.5 (4″-OMe) and 56.5 (3″-OMe). The minor product at R_f 0.78 was also obtained as a colorless syrup (1.8 mg, 18%).

4″-O-Methyl product of **3** (**3c**). FD-MS (*m/z*, %): 451 (M^++1, 90), 450 (M^+, 100), 338 (17), 194 (16) and 112 (35). ^{1}H-NMR δ (CD_3OD): 8.34 (s, 2-CH), 8.10 (d, J=5.6 Hz, 6-CH), 7.63 (d, J=15.8 Hz, 7″-H), 7.10 (br. s, 2″-H), 7.08 (dd, J=8.3 and 2.0 Hz, 6″-H), 6.95 (d, J=8.3 Hz, 5″-H), 6.53 (br. d, J=5.6 Hz, 5-H), 6.41 (d, J=15.8 Hz, 8″-H), 5.13 (dd, J=9.6 and 9.3 Hz, 3′-H), approx. 4.80 (overlapped with $CD_3O\underline{H}$ peak, 1′-H), 3.92 (br. d, J=12 Hz, 6′-Ha), 3.89 (s, 4″-OCH_3) and 3.72–3.56 (4H, m, 2′-, 4′-, 5′-H and 6′-Hb).

Hydrolytic cleavage of **3b**. Compound **3b** (2.0 mg) was dissolved in 2 ml of MeOH/triethylamine/H_2O (2:1:1) and kept at room temperature for 2 h. The reaction resulted in two products, 3,4-di-*O*-methylcaffeic acid (0.6 mg, R_f 0.53 in $CHCl_3$) and a glucosidic pyrone (1.1 mg) identical with **2** by a ^{1}H-NMR comparison.

Solubilization of iron(III) with pyromeconic acid. A solution of 0.2 M iron(II) sulfate (10 ml) was adjusted pH 9.0 with NaOH, and an excess of 30% H_2O_2 was then added to the solution to precipitate iron(III) hydroxide (ferric hydroxide). The pale brown precipitated paste was hardly water-soluble and was washed several times with distilled water. The ferric hydroxide thus obtained suspended in water was adjusted to a total volume of 10 ml. The original suspension (500 μl) diluted with 9.0 ml of distilled water was used as the substrate solution. To this substrate solution, compound **1** as a 500 μl aqueous solution (0, 1, 10 and 100 mM) was added (the final concentrations of **1** were 0, 0.05, 0.5 and 5 mM, respectively, and of iron(III) was 10 mM) and the mixture kept for 24 h. Like other compounds that have an α-enolic ketone,[11,12] the solution of **1** with iron(III) also showed rapid coloration to a deep reddish-brown. When the colored supernatants were concentrated, a reddish brown solid was obtained. The solid was hardly soluble in alcohols but soluble in water was likely to have been an iron(III)-pyromeconic acid complex, although no chemical proof of this was obtained.

Quantitative analyses of the solubilized iron(III). No characteristic absorption peak of the supernatant was visible in the range from 350 to 550 nm, because the large UV absorption of the pyrone ring (270 nm) made the absorbance curve relatively flat. On the other hand, the transmittance curve for the supernatant of 0.5 mM pyromeconic acid/iron(III) hydroxide showed a valley at 480 nm (Fig. 2). For quantitative measurement of the solubilized iron(III) by UV absorbance, 480 nm was therefore chosen as an appropriate wavelength.

The complex solution prepared as a supernatant from a mixture of 5 mM pyromeconic acid and 10 mM iron(III) hydroxide was diluted with a 5 mM pyromeconic acid solution to each concentration. Although the diluted complex solutions contained an excess amount of free **1**, the standard curve showed a striking linear relationship (absorbance $\times$0.86=mole concentration of **1** in the original solution, with 5 mM of **1** as the blank) from zero to 2.5 mM concentration. Furthermore, the supernatant of the 0.5 mM pyromeconic acid/iron(III) hydroxide mixture was on the standard curve. It is therefore likely that the concentration of soluble iron(III) was highly dependent on the concentration of **1** in the original mixture. The absorbance of 5 mM pyromeconic acid at 480 nm (water as the blank) was almost zero, enabling all the blank solutions for quantification to be replaced with pure water.

Antifungal activities of **1** *and the iron(III)-solubilized solution of* **1**. An antifungal assay on **1** and **2** was performed by TLC-bioautography, using *Cladosporium herbarum* AHU 9262 as previously described.[5] The minimum concentration of **1** to show inhibition of fungal growth on silica gel-coated glass plates (Merck Kieselgel 60 F_{254}, 0.25 mm in thickness) was 15 μg/80 mm^2, with some retardation at 4 μg/80 mm^2, while **2** did not show any inhibition at 135 μg/80 mm^2. During a seedling assay, using radish on absorbent cotton, an unidentified fungus was naturally propagated on the aerial root tissues of the control seedlings (with water only) and of the test seedlings with 1 mM solution of **1** (7 days at room temperature; no significant effect on the growth of the test seedlings was observed). The fungus was not propagated on the plant tissues cultivated with a 1 and 0.2 mM iron(III)-solubilized solution of **1**.

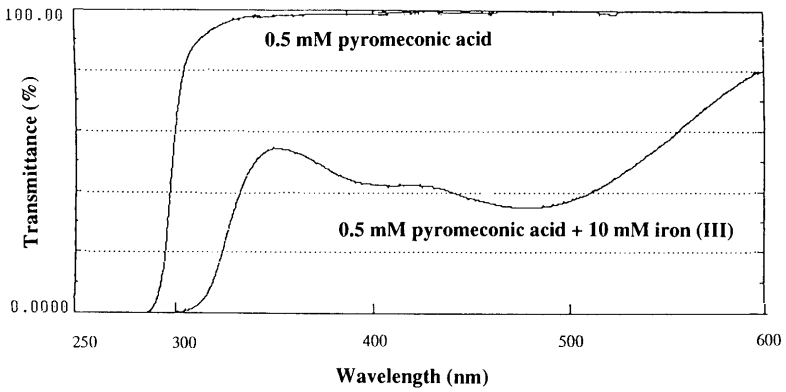


Fig. 2. Transmittance Curve for a Pyromeconic Acid Solution Solubilizing Iron(III).

Using distilled water as the blank, a transmittance curve for the supernatant from a mixture of 0.5 mM pyromeconic acid and 10 mM iron(III) hydroxide was constructed. As shown by the transmittance curve for the 0.5 mM pyromeconic acid solution alone, unchanged compound **1** rarely influenced the transmittance of the supernatant in the range from 340 to 600 nm. Moreover, supernatant of the iron(III) hydroxide suspension alone shows 100% transmittance from 250 to 600 nm. The transmittance curve for the iron(III) and pyromeconic acid mixture indicates that the coloration was due to iron(III) solubilized into the supernatant.

Results and Discussion

Like some other species of genus *Erigeron*,[6,7] *E. annuus* also contained a large amount of pyromeconic acid β-glucoside (erigeroside, **2**), and free pyromeconic acid (**1**) was released from **2**, possibly *via* hydrolytic cleavage catalyzed by a β-glucosidase when the tissue was physically damaged. As a new natural product, 3′-*O*-caffeylerigeroside (**3**) was also found in the leaves. Compound **3** was obtained from the fresh leaves of *E. annuus* by extracting with organic solvents (*e.g.*, acetone, ethanol or methanol), but was undetectable in the water-diffusates of the damaged leaves that had been prepared as described in the materials and methods section. It is most likely that compound **3** was unstable and easily cleaved enzymatically into **2** and caffeic acid in the process used to prepare the diffusates.

The structure of compound **3** was determined by spectroscopic analyses and chemical conversions. In the ^{1}H-NMR spectrum in D_2O, most proton signals assignable as those of the sugar moiety overlapped at around δ_H 3.8–3.5. However, the C-1′ anomeric proton signal was visualized as a doublet (δ_H 5.06, $J=7.6$ Hz). In contrast, all the proton signals were characterized when CD_3OD was used as the solvent instead of D_2O, except for the anomeric proton signal that was almost overlapped by the peak of $CD_3O\underline{H}$. The overlap was confirmed by HH-COSY and a homo gated decoupling experiment to suppress the solvent peak. One of the sugar proton signals at δ_H 5.13 was assignable as the proton of a methine carbon substituted with the caffeoyloxy group due to its remarkable downfield shift, and HH-COSY and the homo gated decoupling experiments to irradiate at this and the anomeric proton signals, respectively, show clear evidence for the position of the caffeoyloxy group at the C-3′.

Hydrolytic cleavage of dimethyl derivative **3b** with a weakly alkaline solution resulted in 3,4-di-*O*-methylcaffeic acid and a glucosidic γ-pyrone that agreed with **2** by TLC and ^{1}H-NMR spectroscopy. Accordingly, it was unambiguously proven that compound **3** possessed a β-D-glucoside moiety. As already described, the coupling constant ($J=$ 7.6 Hz) for the anomeric proton was reasonable for the β-anomer of the D-glucose unit. Thus, the structure of **3** (Fig. 1) was thoroughly elucidated.

As a cinnamoyl derivative of sugars, the 6-*O*-caffeylglucose unit of the blue anthocyanins of several plants (*e.g.*, gentian and morning-glory)[13,14] is well known. Moreover, McNeil *et al.* have found the pyranoarabinose 3-*O*-feluryl ester unit in barley cell wall.[15] However, caffeoyl-substituted glycosides seem rare among natural products. Compound **3** is instead considered to be an analogous compound to chlorogenic acid (3-caffeylquinic acid) which is widely recognized as a chemical protector in various plants when the tissues are physically wounded.[16]

The appearance of an antifungal zone on the TLC-bioautogram of extracts of damaged and non-damaged leaves suggests the process for the accumulation of pyromeconic acid (**1**) to involve a caffeoyl esterase and then β-glucosidase being activated first in the damaged tissues according to the level of the damage, and free **1** being finally released. Free caffeic acid released first from the damaged part causes a browning response of the tissues, while **1** being highly accumulated in the severely damaged parts might play a defensive role. Indeed, **1** showed weak antifungal activity on a TLC-bioautogram (minimum inhibitory concentration of 15 μg/80 mm^2), but **2** was non-toxic. The activity of **3** has not been examined yet, but crude extracts from the non-damaged leaves likely to be rich in **3** did not show any inhibitory zone.

Zhang *et al.* first isolated pyromeconic acid β-glucoside (**2**) together with free pyromeconic acid (**1**) from *E. breviscapus*,[6] while Yatsyuk and Segal also reported **2** from the leaves of *E. acris*.[7] Thus, occurrence of **1** and **2** in *E. annuus* is chemotaxonomically reasonable. However, according to the author's chemical investigation (data not shown), neither **1** nor **2** was detected in *E. canadensis*. Moreover, no pyrone derivative was detected in the rosette leaves of *E. philadelphus*. Thus, among the genus *Erigeron* plants naturalized in Japan, only *E. annuus* is likely to produce pyromeconic acid derivatives, although it is necessary to re-investigate the floral part of *E. philadelphus*.

The characteristic partial structure of **1** is the α-enolic ketone moiety, which is a siderophilic functional group, like an *N*-hydroxycarbamoyl (hydroxamic acid) moiety.[17] Natural products possessing an α-enolic ketone group are 3-hydroxy-γ-pyrone derivatives (*e.g.* 3-hydroxy-*4H*-pyran-4-one-2,6-dicarboxylic acid),[11] flavonols (*e.g.* Kaempferol), tropolone derivatives (*e.g.* hinokitiol) and so on. Hinokitiol, for example, is also known to be siderophilic and to form an iron-complex.[12]

As was expected, a solution of **1** (5 mM) that had been added to iron(II) hydroxide instantaneously made iron(III) soluble and colored the solution a clear reddish-brown (Fig. 2). The mechanism by which **1** solubilized iron(III) is thought to be similar to that of hydroxamic acid in forming its iron complex,[17] but no chemical proof has been obtained. The siderophilic property of **1** is regarded as a key function of the γ-pyrone derivative in the plant tissues. In fact, the non-damaged floral part of *E. annuus* contained a high concentration of free **1**, unlike the other aerial parts. It is not possible to explain the high accumulation of **1** in the floral tissues only by its defensive role. Rather, the siderophilic nature of **1** is more likely to be associated with the uptake of mineral elements; therefore, the concentration of metals in *E. annuus* should be examined.

The fact that compound **1** was detected in germinating seedlings of *E. annuus* suggests that the biosynthesis of **1** had already begun at an early germination stage. The young leaves and floral parts, both with large accumulated amounts of **1** and its sugar conjugates, are edible, so that the γ-pyrone seems thoroughly non-toxic for mammals. However, Zhang *et al.* have reported a pressor effect of **1** and depressor and hypnotic effects of **2**.[6] The unique physiological activity of **1** is likely due to its water-soluble and siderophilic nature, although the activity of **2** being opposite to that of **1** cannot be explained.

Acknowledgments. The author thanks Dr. S. Tahara (Hokkaido University) for his technical advice, Mr. K. Watanabe and Mrs. E. Fukushi (Hokkaido University) for FD- and EI-MS analyses, and Mr. A. Sato (Hokkaido Tokai University) for COLOC measurements.

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