Synthesis and antihepatotoxic and antiproliferative activities of di- and tri-*O*-caffeoylquinic acid derivatives

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Abstract

Methyl di- and tri-*O*-caffeoylquinates were synthesized by esterification of methyl quinate with di-*O*-acetylcaffeoyl chloride, following deprotection of the acetyl groups. Moreover, 4,5-di-*O*-caffeoylquinic acid was synthesized by esterification of quinide with di-*O*-acetylcaffeoyl chloride, followed by a hydrolysis of product quinide. These synthetic compounds were tested for their hepatoprotective activity on D-galactosamine (D-GalN)/tumor necrosis factor-α (TNF-α)-induced cell death in primary cultured mouse hepatocytes, which possessed significant hepatoprotective activity concentration-dependently. The activity was enhanced by the presence of caffeoyl group. On the other hand, they showed only weak antiproliferative activities against murine colon 26-L5 carcinoma, human HT-1080 fibrosarcoma, murine B16-BL6 melanoma, and human lung carcinoma A-549 cells.

Key words caffeoylquinic acids, synthesis, hepatoprotective activity, antiproliferative activity, murine colon 26-L5 carcinoma.

Abbreviations DCC, *N,N'*-dicyclohexylcarbodiimide; D-GalN, D-galactosamine; DMAP, 4-dimethylaminopyridine; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FAB-MS, first atom bombardment mass spectrometry; 5-FU, 5-fluorouracil; HR-FAB-MS, high-resolution first atom bombardment mass spectrometry; HIV-1, human immunodeficiency virus type-1; IC_{50}, inhibition concentration-fifty; IR, infrared; LPS, lipopolisaccalide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium; NMR, nuclear magnetic resonance; *p*-TsOH, *para*-toluenesulfonic acid; TLC, thin layer chromatography; TMS, tetramethylsilane; TNF-α, tumor necrosis factor-α; XOD, xanthine oxidase.

Introduction

Caffeoylquinic acids are widely distributed[1-6] and reported to have various biological activities, such as inhibition of human immunodeficiency virus type-1 (HIV-1) protease,[7] antioxidation,[8] antiinflammation,[9] and antispasmodic activity.[10] Previously, we reported on four di-*O*-caffeoylquinic acids [3,4- and 3,5-di-*O*-caffeoylquinic acids and methyl 3,4- and 4,5-di-*O*-caffeoylquinates (**2** and **4**, respectively)] from Brazilian propolis,[11] which is a sticky plant substance collected by bees[12] and extensively used in food and beverages to improve health and prevent diseases such as inflammation, heart diseases, diabetes, and even cancer.[13] The isolated di-*O*-caffeoylquinic acids possessed strong scavenging activity towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and free-radical produced by the xanthine-xanthine oxidase (XOD) systems[11] and protective activity on CCl_4-induced cell death in primary cultured rat hepatocytes.[14] Furthermore, thirteen quinic acid derivatives were isolated from the flower buds of *Lonicera bournei* HEMSL. and showed hepatoprotective activities against hepatocyte death induced by D-galactosamine (D-GalN)/tumor necrosis factor-α(TNF-α).[15] These activities were interesting, but the meager amounts obtained led us to resign from further examination on biological activity. Thus, we tried to synthesize caffeoylquinates, to provide enough of an amount to examine further biological activities. In this paper, we report on the synthesis of caffeoylquinic acid derivatives and their hepatoprotective effect on D-GalN/TNF-α-induced cell death in primary

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cultured mouse hepatocytes. Moreover, the antiproliferative activity of all these compounds were also tested toward highly liver-metastatic murine colon 26-L5 carcinoma,[16] human HT-1080 fibrosarcoma,[17] murine B16-BL6 melanoma,[18] and human lung carcinoma A-549 cells.[19]

Materials and Methods

General : Optical rotations were measured on a JASCO DIP-140 digital polarometer at 22 ℃. IR spectra were obtained on a Shimadzu IR-408 spectrophotometer. FAB-MS and high-resolution FAB-MS (HR-FAB-MS) data were obtained by a JEOL JMS-700T spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard; chemical shifts are recorded in δ values. Column chromatography was performed by using Wakogel C-200 silica gel. TLC was carried out on precoated Merck silica gel F_{254} plates (0.25 or 0.5 mm) or RP-18 F_{254} reversed-phase plates (0.25 mm).

General procedure for synthesis of methyl bis- and tris-O-(di-O-acetylcaffeoyl)quinates ***1a-4a*** *(Table I, run 5)* : To a stirred suspension of 3,4-di-*O*-acetylcaffeic acid (365 mg, 1.38 mmol) in toluene (20 ml), oxalyl chloride (582 μl, 6.90 mmol) was added, and the mixture was stirred at room temperature under Ar atmosphere for 24 h. After removal of the solvent at reduced pressure, the 3,4-di-*O*-acetylcaffeic acid chloride (**7**) was dissolved in benzene (20 ml) and added to a suspension of methyl quinate (113 mg, 0.46 mmol) in benzene (10 ml). Pyridine (111 μl, 1.38 mmol) was added dropwise, and the mixture was refluxed for 1 h under Ar atmosphere with a Dean-Stark apparatus. Then this was poured into 1N HCl, and the product was extracted with AcOEt. The AcOEt layer was washed with H_2O, dried over Na_2SO_4, and evaporated. The crude product was subjected to silica gel column chromatography using $CHCl_3$-MeOH (98:2) to give five fractions. Fraction 3 (337 mg) was subjected to preparative TLC with $CHCl_3$-MeOH (97:3) to give methyl 3,4,5-tris-*O*- (di-*O*-acetylcaffeoyl) quinate (**1a**) (75.0mg, 15.9%), methyl 3,4-bis-*O*- (di-*O*-acetylcaffeoyl) quinate (**2a**) (68.2 mg, 19.5%) and methyl 3,5-bis-*O*-(di-*O*-acetylcaffeoyl) quinate (**3a**) (38.9 mg, 11.1%), while preparative TLC of fraction 4 (94.5 mg) with $CHCl_3$-MeOH (95:5) gave methyl 4,5-bis-*O*- (3,4-di-*O*-acetylcaffeoyl) quinate (**4a**) (35.0 mg, 10.0%).

Methyl 3,4,5-Tris-*O*- (di-*O*-acetylcaffeoyl) quinate (**1a**): Colorless powder, $[\alpha]_D$-174.4° (*c* = 1.02, MeOH). IR υ_{max} ($CHCl_3$) cm^{-1}: 3500, 1770, 1720, 1640, 1505, 1430, 1375. FAB-MS *m/z*: 967 $[M+Na]^+$. HR-FAB-MS *m/z*: 967.2255 $[M+Na]^+$ (calcd for $C_{47}H_{44}O_{21}Na$: 967.2273). ^{1}H-NMR ($CDCl_3$) δ: 7.68 (1H, d, *J* = 15.9 Hz), 7.58 (2H, d, *J* = 15.9 Hz), 7.45-7.10 (9H, m), 6.46 (1H, d, *J* = 15.9 Hz), 6.31 (1H, d, *J* = 15.9 Hz), 6.30 (1H, d, *J* = 15.9 Hz), 5.78(1H, dt, *J* = 9.9, 4.3 Hz), 5.69 (1H, dt, *J* = 3.6, 3.4 Hz), 5.32 (1H, dd, *J* = 9.9, 3.4 Hz), 3.81 (3H, s), 2.31 (3H, s), 2.30 (3H, s), 2.29 (3H, s), 2.28 (3H, s), 2.26 (3H, s), 2.26 (3H, s), 2.47-2.13 (4H, m). ^{13}C-NMR ($CDCl_3$) δ: 174.70 (s), 167.98 (s), 167.94 (s), 167.86 (s), 167.78 (s), 165.65 (s), 165.37 (s), 144.10 (d), 143.81 (d), 143.73 (d), 143.69 (s), 143.67 (s), 142.45 (s), 142.44 (s), 142.40 (s), 126.76 (d), 126.68 (d), 126.54 (d), 123.91 (d), 122.95 (d), 122.82 (d), 122.77 (d), 118.91 (d), 118.59 (d), 118.25 (d), 74.05 (s), 72.19 (d), 69.08 (d), 67.54 (d), 53.34 (q), 39.05 (t), 35.88 (t), 20.64 (q), 20.62 (q), 20.57 (q), 20.54 (q).

Methyl 3,4-Bis-*O*- (di-*O*-acetylcaffeoyl) quinate (**2a**): Colorless powder, $[\alpha]_D$-122.7° (*c* = 0.14, MeOH). IR υ_{max} ($CHCl_3$) cm^{-1}: 3500, 1770, 1715, 1640, 1505, 1430, 1375. FAB-MS *m/z*: 721 $[M+Na]^+$. HR-FAB-MS *m/z*: 721.1741 $[M+Na]^+$ (calcd for $C_{34}H_{34}O_{16}Na$: 721.1745). ^{1}H-NMR ($CDCl_3$) δ: 7.64 (1H, d, *J* = 15.9 Hz), 7.57 (1H, d, *J* = 15.9 Hz), 7.38-7.31 (4H, m), 7.19 (2H, d, *J* = 8.2 Hz), 6.42 (1H, d, *J* = 15.9 Hz), 6.30 (1H, d, *J* = 15.9 Hz), 5.73 (1H, dt, *J* = 10.7, 4.8 Hz), 5.16 (1H, dd, *J* = 10.7, 3.4 Hz), 4.40 (1H, m), 3.83 (3H, s), 2.29 (6H, s), 2.28 (6H, s), 2.50-2.06 (4H, m). ^{13}C-NMR ($CDCl_3$) δ: 174.30 (s), 167.99 (s), 167.87 (s), 166.01 (s), 165.61 (s), 144.00 (s), 143.71 (d), 143.68 (d), 143.65 (d), 142.44 (s), 133.05 (s), 126.58 (s), 126.55 (d), 123.93 (s), 122.92 (d), 122.82 (d), 118.64 (d), 118.59 (d), 75.54 (s), 75.21 (d), 68.97 (d), 67.37 (d), 53.45 (q), 39.25 (t), 37.32 (t), 20.63 (q), 20.58 (q).

Methyl 3,5-Bis-*O*-(di-*O*-acetylcaffeoyl) quinate (**3a**): Colorless powder, $[\alpha]_D$-72.9° (*c* = 0.20, MeOH). IR υ_{max} ($CHCl_3$) cm^{-1}: 3500, 1770, 1710, 1640, 1520, 1445, 1390. FAB-MS *m/z*: 721 $[M+Na]^+$. HR-FAB-MS *m/z*: 721.1741 $[M+Na]^+$ (calcd for $C_{34}H_{34}O_{16}Na$: 721.1745). ^{1}H-NMR ($CDCl_3$) δ: 7.69 (1H, d, *J* = 15.9 Hz), 7.65 (1H, d, *J* = 15.9 Hz), 7.45-7.36 (4H, m), 7.23 (2H, dd, *J* = 8.2, 1.2 Hz), 6.47 (1H, d, *J* = 15.9 Hz), 6.40

(1H, d, J = 15.9 Hz), 5.58 (1H, dt, J = 10.4, 4.3 Hz), 5.48 (1H, dd, J = 7.2, 3.8 Hz), 3.91 (1H, m), 3.79 (3H, s), 2.31 (3H, s), 2.31 (3H, s), 2.30 (3H, s), 2.30 (3H, s), 2.50-2.06 (4H, m). ^{13}C-NMR ($CDCl_3$) δ: 174.84 (s), 167.98 (s), 167.89 (s), 166.42 (s), 166.10 (s), 143.82 (s), 143.74 (d), 143.66 (d), 142.47 (d), 142.44 (s), 133.13 (s), 133.05 (s), 126.55 (d), 126.43 (s), 123.96 (d), 123.90 (d), 122.95 (d), 122.86 (d), 118.91 (d), 118.77 (d), 74.16 (s), 72.33 (d), 71.92 (d), 70.57 (d), 53.24 (q), 38.60 (t), 35.52 (t), 20.62 (q), 20.58 (q).

Methyl 4,5-Bis-*O*-(3,4-di-*O*-acetylcaffeoyl) quinate (**4a**): Light yellow powder, $[\alpha]_D$-72.0° (c = 0.13, MeOH). IR υ_{max} ($CHCl_3$) cm^{-1}: 3500, 1770, 1720, 1640, 1505, 1430, 1375. FAB-MS *m/z*: 721 $[M+Na]^+$. HR-FAB-MS *m/z*: 721.1726 $[M+Na]^+$ (calcd for $C_{34}H_{34}O_{16}Na$: 721.1745). ^{1}H-NMR ($CDCl_3$) δ: 7.64 (1H, d, J = 15.9 Hz), 7.63 (1H, d, J = 15.9 Hz), 7.42-7.33 (4H, m), 7.20 (2H, d, J = 8.2 Hz), 6.40 (1H, d, J = 15.9 Hz), 6.38 (1H, d, J = 15.9 Hz), 5.64 (1H, dd, J = 7.0, 3.4 Hz), 5.02 (1H, dd, J = 9.4, 3.4 Hz), 4.49 (1H, dt, J = 9.4, 4.6 Hz), 3.83 (3H, s), 2.30 (3H, s), 2.29 (3H, s), 2.28 (3H, s), 2.28 (3H, s), 2.35-2.07 (4H, m). ^{13}C-NMR ($CDCl_3$) δ: 175.35 (s), 167.98 (s), 167.89 (s), 166.14 (s), 165.58 (s), 144.04 (s), 143.69 (d), 142.44 (d), 133.13 (s), 132.97 (s), 126.63 (d), 126.59 (s), 123.91 (d), 122.85 (d), 122.78 (d), 118.92 (d), 118.38 (d), 75.77 (s), 74.29 (d), 69.00 (d), 65.13 (d), 53.34 (q), 41.20 (t), 36.22 (t), 20.61 (q), 20.56 (q).

Preparation of methyl di- and tri-O-caffeoylquinates ***1-4*** : To a stirred solution of **1a** (45.1 mg, 0.048 mmol) and phenolphthalein (2.0 mg) in MeOH (2.0 ml), 0.5 N $Ba(OH)_2$ (20 μl, 4.8 μmol), was added and the mixture was stirred at 50 ℃ until the red color of phenolphthalein disappeared (1 h). After evaporation of the solvent, the residue was extracted by AcOEt, and the AcOEt layer was washed with H_2O, dried over Na_2SO_4, and evaporated. The crude product was subjected to reversed-phase preparative TLC with MeOH-H_2O (2:1) to give methyl 3,4,5-tri-*O*-caffeoylquinate (**1**) (27.0 mg, 82.0%). By a similar procedure, methyl di-*O*-caffeoylquinates **2-4** were also prepared.

*Synthesis of 1-O-ethoxycarbonyl-4,5-bis-O-(di-O-acetylcaffeoyl)quinide (**8a**)* : To a stirred suspension of 3,4-di-*O*-acetylcaffeic acid (81.9 mg, 0.31 mmol) in toluene (5 ml), oxalyl chloride (131 μl, 1.55 mmol) was added. The mixture was stirred at room temperature under Ar atmosphere for 24 h. After removal of the solvent under reduced pressure, the residue **7** was dissolved in benzene (3 ml) and added to a suspension of **8** (15.3 mg, 0.062 mmol) in benzene (2 ml). Pyridine (25 μl, 0.31 mmol) was added dropwise to the mixture, and the mixture was stirred at 80 ℃ under Ar atmosphere for 12 h. The reaction mixture was poured into 1N HCl and extracted with AcOEt. The AcOEt layer was washed with H_2O, dried over Na_2SO_4, and evaporated. The residue was subjected to preparative TLC with $CHCl_3$-MeOH (97.5:2.5) to give 1-*O*-ethoxycarbonyl-4,5-bis-*O*-(di-*O*-acetylcaffeoyl) quinide (**8a**) (26.4 mg, 57.7%) and 1-*O*-ethoxycarbonyl-5-*O*-(di-*O*-acetylcaffeoyl) quinide (**8b**) (13.2 mg, 42.3%).

1-*O*-Ethoxycarbonyl-4,5-bis-*O*-(di-*O*-acetylcaffeoyl) quinide (**8a**): Colorless powder, $[\alpha]_D$+97.9° (c = 0.21, MeOH). IR υ_{max} ($CHCl_3$) cm^{-1}: 1810, 1755, 1720, 1640, 1505, 1430, 1375. FAB-MS *m/z*: 739 $[M+H]^+$. HR-FAB-MS *m/z*: 739.1877 $[M+H]^+$ (calcd for $C_{36}H_{35}O_{17}$: 739.1874). ^{1}H-NMR ($CDCl_3$) δ: 7.67 (1H, d, J = 15.9 Hz), 7.58 (1H, d, J = 15.9 Hz), 7.44-7.14 (6H, m), 6.45 (1H, d, J = 15.9 Hz), 6.27 (1H, d, J = 15.9 Hz), 5.69 (1H, br t, J = 4.6 Hz), 5.35 (1H, ddd, J = 11.8, 7.0, 4.6 Hz), 4.99 (1H, br t, J = 5.8 Hz), 4.25 (2H, q, J = 7.2 Hz), 3.21 (1H, ddd, J = 11.8, 5.8, 2.4 Hz), 2.65 (1H, d, J = 11.8 Hz), 2.49 (1H, ddd, J = 11.8, 7.0, 2.4 Hz), 2.41 (1H, br t, J = 11.8 Hz), 2.31 (3H, s), 2.31 (3H, s), 2.28 (3H, s), 2.27 (3H, s), 1.34 (3H, t, J = 7.2 Hz). ^{13}C-NMR ($CDCl_3$) δ: 170.72 (s), 168.03 (s), 168.01 (s), 167.87 (s), 164.80 (s), 164.59 (s), 152.51 (s), 145.00 (d), 144.38 (d), 144.04 (s), 143.81 (s), 142.60 (s), 142.49 (s), 132.84 (s), 132.63 (s), 126.86 (d), 126.68 (d), 124.13 (d), 124.02 (d), 122.90 (d), 122.78 (d), 117.76 (d), 117.55 (d), 77.56 (s), 73.64 (d), 66.06 (d), 65.03 (d), 64.97 (t), 33.89 (t), 33.81 (t), 20.64 (q), 20.56 (q), 14.10 (q).

1-*O*-Ethoxycarbonyl-5-*O*-(di-*O*-acetylcaffeoyl)quinide (**8b**): Colorless powder, $[\alpha]_D$+27.7° (c = 0.20, MeOH). IR υ_{max} ($CHCl_3$) cm^{-1}: 3500, 1805, 1755, 1720, 1640, 1505, 1430, 1375. FAB-MS *m/z*: 493 $[M+H]^+$. HR-FAB-MS *m/z*: 493.1340 $[M+H]^+$ (calcd for $C_{23}H_{25}O_{12}$: 493.1346). ^{1}H-NMR ($CDCl_3$) δ: 7.66 (1H, d, J = 15.9 Hz), 7.42-7.35 (2H, m), 7.23 (1H, d, J = 8.2 Hz), 6.38 (1H, d, J = 15.9 Hz), 5.11 (1H, ddd, J = 11.3, 7.0, 4.3 Hz), 4.88 (1H, br t, J = 5.8 Hz), 4.40 (1H, br t, J = 4.3 Hz), 4.23 (2H, q, J = 7.2 Hz), 3.10 (1H, ddd, J = 11.3, 5.8, 2.9 Hz), 2.71 (1H, d, J = 11.3 Hz), 2.42 (1H, ddd, J = 11.3, 7.0, 2.9 Hz), 2.33 (1H, br t, J = 11.3 Hz), 2.30

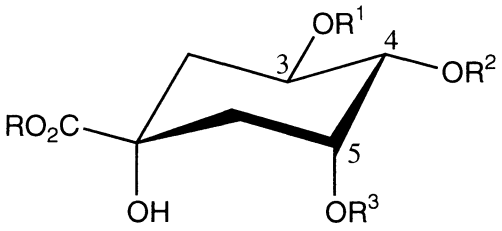
(3H, s), 2.30 (3H, s), 1.33 (3H, t, J = 7.2 Hz). ^{13}C-NMR ($CDCl_3$) δ: 171.34 (s), 168.03 (s), 167.92 (s), 164.69 (s), 152.45 (s), 144.55 (d), 143.88 (s), 142.52 (s), 132.73 (s), 126.54 (d), 124.05 (d), 122.89 (d), 117.79 (d), 77.68 (s), 75.69 (d), 68.50 (d), 64.80 (d), 64.34 (t), 33.31 (t), 32.62 (t), 20.60 (q), 20.58 (q), 14.05 (q).

*Conversion of 1-O-ethoxycarbonyl-5-O-(di-O-acetylcaffeoyl) quinide (**8b**) to 1-O-ethoxycarbonyl-4,5-bis-O-(di-O-acetylcaffeoyl)quinide (**8a**)* : To a stirred suspension of the 3,4-di-*O*-acetylcaffeic acid (81.9 mg, 0.31 mmol) in toluene (5 ml), oxalyl chloride (131 μl, 1.55 mmol) was added, and the mixture was stirred at room temperature under Ar atmosphere for 24 h. After removal of the solvent under reduced pressure, the residue **7** was dissolved in benzene (3 ml) and added to a suspension of **8b** (57.0 mg, 0.116 mmol) in benzene (2 ml). Pyridine (62 μl, 0.775 mmol) was added dropwise to the mixture, and the mixture was stirred at 80 ℃ under Ar atmosphere for 18 h. The reaction mixture was poured into 1N HCl and extracted with AcOEt. The AcOEt layer was washed with H_2O, dried over Na_2SO_4, and evaporated. The residue was subjected to preparative TLC with $CHCl_3$-MeOH (97.5:2.5) to give 1-*O*-ethoxycarbonyl-4,5-bis-*O*-(3,4-di-*O*-acetylcaffeoyl)quinide (**8a**)(57.4 mg, 67.0%).

*Preparation of 4,5-di-O-caffeoylquinic acid (**5**)* : Quinide **8a** (1.6 g, 2.2 mmol) was stirred with H_2O (100 ml) under reflux for 48 h. After removal of the solvent under reduced pressure, the residue was subjected to reversed-phase silica gel column chromatography using CH_3CN-H_2O-AcOH (10:90:1) to give 4,5-di-*O*-caffeoylquinic acid[20] (**5**) (294 mg, 26.7%).

Hepatoprotective and antiproliferative activities : Hepatoprotective activities of compounds **1-5** were tested at 10, 50 and 100 μM according to the previous reports.[21] Silymarine was used as a positive control; its hepatoprotective activity was examined at 25 and 50 μM.

Antiproliferative assay was carried out, using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium (MTT) method,[22] by the procedure reported previously.[23] 5-Fluorouracil, a clinically used drug, was used as a positive control. The cultured cells were treated with the compounds at five different concentrations ranging from 0.01 to 100 μg/ml, while for the positive control, concentrations ranging from 0.001 to 10 μg/ml were used. The assay was performed in quadruplicate, and results are expressed as IC_{50} values (μM).



1: R = Me, $R^1 = R^2 = R^3$ = caffeoyl
2: R = Me, $R^1 = R^2$ = caffeoyl, R^3 = H
3: R = Me, $R^1 = R^3$ = caffeoyl, R^2 = H
4: R = Me, R^1 = H, $R^2 = R^3$ = caffeoyl
5: R = R^1 = H, $R^2 = R^3$ = caffeoyl

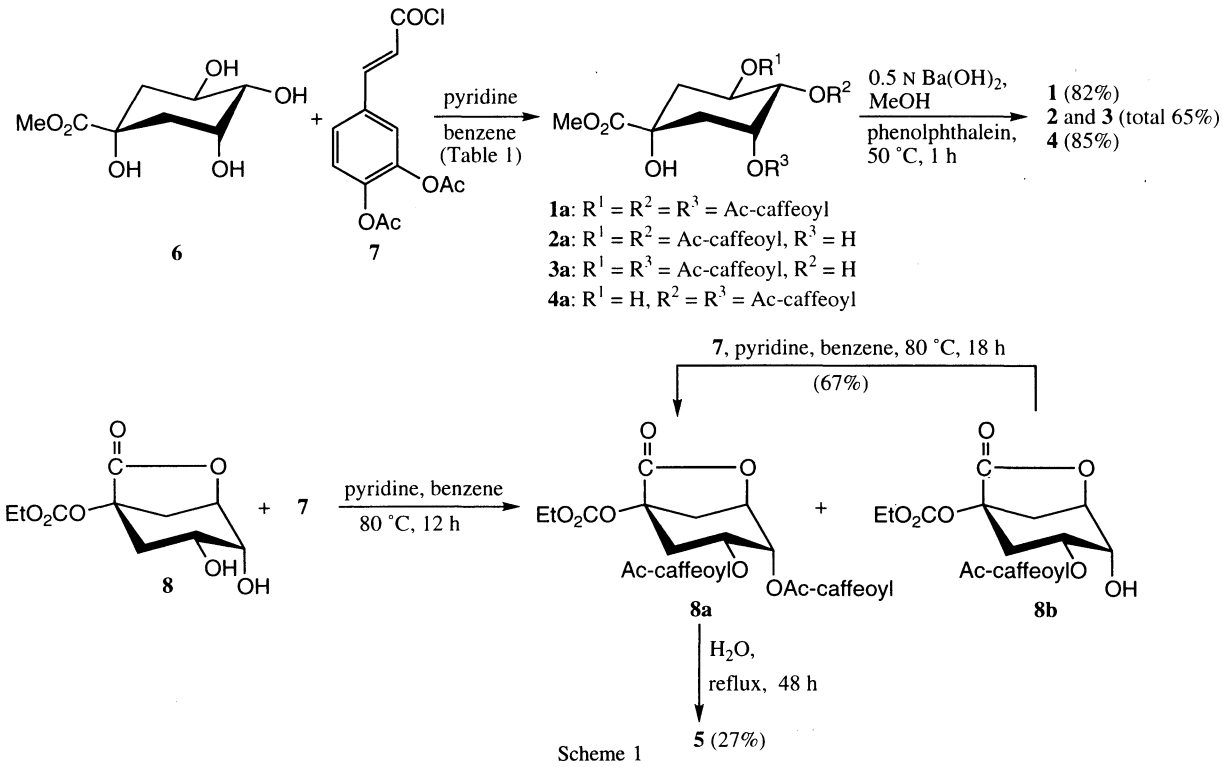
Fig. 1

Results and Discussion

Chemistry

Synthesis of di- and tri-*O*-caffeoylquinic acids have not been reported, except for the enzymatic conversion of chlorogenic acid to 3,5-di-*O*-caffeoylquinic acid.[24] We first examined direct esterification of quinic acid and caffeic acid with *N,N'*-dicyclohexylcarbodiimide (DCC)/4-dimethylaminopyridine (DMAP), thionyl chloride ($SOCl_2$), or *p*-toluenesulfonic acid (*p*-TsOH). These direct esterification reactions resulted in the formation of complex mixtures, due to the presence of many polar functional groups, and this could not be improved by using methyl quinate (**6**) and 3,4-di-*O*-acetylcaffeic acid. The esterification with pyridine in benzene,[25] however, succeeded to give a mixture of **1a-4a** (Scheme 1, Table I). Methyl quinate (**6**) was first reacted with **7** in the presence of three equivalents of pyridine to give only **2a** and **3a** in low yields (7.3 and 2.2%, respectively) (run 1). As the reaction temperature was raised, diester **4a** was converted to triester **1a** (run 2 and 3), and elongation of the reaction time resulted in the selective production of **1a** (run 4). On the other hand, the use of Dean-Stark apparatus accelerated the reaction and raised the total yield of **1a-4a** (run 5), while use of five equivalents of **7** and pyridine gave **1a** selectively (run 6). The di- and triesters **1a-4a** were deprotected with 0.5 N $Ba(OH)_2$ in MeOH using phenolphthalein as an indicator,[26] to give the methyl quinates **1-4**.

4,5-Di-*O*-caffeoylquinic acid (**5**), on the other hand,



Scheme 1

Table I Reaction of methyl quinate (**6**) with **7**.

run	**7** (eq)	pyridine (eq)	temp.	time (h)	yield (%)			
					1a[a)]	**2a**[a)]	**3a**	**4a**
1	3	3	50℃	17	-	7.3	2.2	-
2	3	3	80℃	6	10.4	17.2	6.1	14.1
3	3	3	reflux	3	16.0	11.5	6.6	3.1
4	3	3	reflux	11	15.5	-	-	-
5	3	3	reflux	1	15.9	19.5	11.1	10.0
6	5	5	reflux	0.5	25.7	-	-	-

a: Yields were obtained from the ^{1}H-NMR spectra of mixtures of **2a** and **3a**.

was synthesized from quinide **8**[27] through an esterification with **7** (Scheme 1). Esterification of **8** with **7** (5 eq) gave a diester **8a** and a mono ester **8b** in 58% and 42% yield, respectively. The former diester **8a** was hydrolyzed to 5 in 27% yield, while the latter mono ester **8b** was converted to the diester **8a** in 67% yield.

Hepatoprotective activity

The protective activity of di-*O*-caffeoylquinic acids isolated from Brazilian propolis was reported on CCl_4-induced cell death in primary cultured rat hepatocytes.[14] However, the majority of human hepatitis is triggered by an immunological response to viral infection, endotoxin, or autoantigen.[28,29] In the D-GalN/lipopolisaccalide (LPS)-induced liver failure model in mice, TNF-α is secreted from LPS-stimulated macrophages and strongly induces hepatocyte apoptosis, which triggers an inflammatory reaction and massive hepatocyte necrosis.[30,31] Thus, D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes is used as an *in vitro* model of immunological liver failure. Hepatoprotective activities of caffeoylquinic acids **1-5** were tested on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes (Table II). Almost all compounds showed a significant hepatoprotective activity at 50 and 100 μM concentrations, with a cell survival rate ranging from 40.4 to 88.6% as compared to that of control (37.6%), while neither caffeic acid nor quinic acid[15] showed any protective activity. Among them, tricaffeoyl derivative **1** exhibited the strongest hepatoprotective activity at 100 μM concentration with cell survival rate equal to 88.6%. On di-*O*-caffeoylquinic acid derivatives, free acid **5** possessed stronger protective activity (IC_{50}, 78 μM) than the corresponding methyl ester **4** (IC_{50}, 86 μM). Chlorogenic acid (*i.e.*, 3-*O*-caffeoylquinic acid) revealed only a weak hepatoprotective activity. These results indicate that the increase of the number of the caffeoyl group could enhance the hepatoprotective activity, which is

Table II Hepatoprotective activities of di- and tri-*O*-caffeoylquinic acids **1-5** on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes.

Compounds	Conc. (μM)	Cell survival rate (% of normal)	Inhibition[a] (%)	IC50 (μM)
normal		100.0 ± 5.6	100.0	
control		37.6 ± 2.4	0.0	
1	100	88.6 ± 6.2**	81.7	67
	50	58.3 ± 6.0**	33.2	
	10	37.3 ± 3.3	-0.5	
2	100	49.8 ± 5.5*	19.6	
	50	40.4 ± 2.5	4.5	
	10	41.7 ± 2.0	6.6	
3	100	63.0 ± 4.3**	40.7	
	50	48.6 ± 7.2*	17.6	
	10	37.5 ± 4.9	-0.2	
4	100	77.1 ± 10.6**	63.3	86
	50	48.7 ± 5.4**	17.8	
	10	38.4 ± 2.8	1.3	
5	100	75.7 ± 6.2**	61.1	78
	50	58.5 ± 2.4**	33.5	
	10	51.9 ± 2.4**	22.9	
caffeic acid	100	51.1 ± 5.8*	21.6	
	50	47.6 ± 5.6*	16.0	
	10	46.4 ± 5.1*	14.1	
chlorogenic acid	100	51.8 ± 3.9**	22.8	
	50	44.0 ± 4.3*	10.3	
	10	42.1 ± 5.7	7.2	
Silymarine[b]	50	82.9 ± 5.1**	72.6	37
	25	55.6 ± 2.7**	28.8	

Data are expressed as mean ± S.D., n = 4; for normal and control, n = 8.
*: $p < 0.01$, **: $p < 0.001$, significant difference from control.
a) : Inhibition (%) was calculated by the formula [(% cell viability of sample - % cell viability of control)/(% cell viability of normal - % cell viability of control)] × 100. b) : Silymarin is a mixture of three conformational isomers (silybinin, silydianin and silychristin) with the same molecular weight ($C_{25}H_{22}O_{10}$, 482.4), and thus the IC_{50} value was calculated with this value.

similar to our previous report.[15] The natural dicaffeoylquinic acids **1-5** possessed strong scavenging activity toward DPPH radical and free radical produced by the xanthine-XOD systems,[11] indicating their strong antioxidative activity. On the other hand, increasing evidence indicates that the total cellular balance between reactive oxygen species and antioxidants possibly affects the signaling mechanisms of various responses to TNF-α.[32,33] Thus, the hepatoprotective activity of these compounds may be due to their antioxidative activity.

Table III Antiproliferative activities of di- and tri-*O*-caffeoylquinic acids **1-5**.

Compounds	IC_{50} (μM)			
	Colon 26-L5	HT-1080	B16-BL6	A-549
1	29.4	47.8	38.3	148
2	37.7	116	127	>190
3	52.4	119	122	>190
4	76.3	157	105	>190
5	44.5	>195	>195	>195
chlorogenic acid	149	>280	>280	>280
caffeic acid	111	337	314	387
quinic acid	>520	>520	>520	>520
5-FU	0.050	0.640	4.40	5.20

Antiproliferative activity

Antiproliferative activities of caffeoylquinic acids **1-5** and of caffeic, quinic, and chlorogenic acids were tested towards four cell lines; murine colon 26-L5 carcinoma, human HT-1080 fibrosarcoma, murine B16-BL6 melanoma, and human lung carcinoma A-549 cells (Table III). They showed only a weak activity, but interestingly, their activities were selective toward highly liver metastatic murine colon 26-L5 carcinoma cells. Like the hepatoprotective activity, methyl 3,4,5-tri-*O*-caffeoylquinate (**1**) exhibited the most potent activity. This antiproliferative activity also might be correlated to its antioxidative activity.

Conclusion

We synthesized five caffeoylquinic acids **1-5** and examined their hepatoprotective activity on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes. They possessed a significant hepatoprotective activity, which was enhanced by the increase of the number of caffeoyl group. Moreover, they were found to have a weak antiproliferative activity against four cancer cell lines. These activities seemed to be correlated with their antioxidative activities.

和文抄録

メチルジおよびトリカフェオイルキネート化合物 1–4 は，メチルキネート 6 をジアセチルカフェオイルクロライド 7 でエステル化した後，アセチル基の脱保護によって合成した。さらに，4,5-ジカフェオイルキナ酸 5 は，

キニド 8 を 7 とエステル化して生成したキニド 8a の加水分解により合成した。これらの合成した化合物 1-5 を用いてマウス初代培養肝細胞の D-GalN/TNF-α 誘発肝細胞死における肝保護活性の試験を行ったところ，濃度依存的な肝保護活性が認められた。その活性はカフェオイル基の存在により増強された。一方，これらの化合物 1-5 はマウス 26-L5 大腸癌腫，ヒト HT-1080 繊維芽肉腫，マウス B16-BL6 黒色種，及びヒト A-549 肺癌腫に対し弱い細胞増殖抑制活性を示した。

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