Isolation and Synthesis of a New Bioactive Ellagic Acid Derivative from *Combretum yunnanensis*

Yukihiro Asami,[†] Takashi Ogura,[‡] Noboru Otake,[†] Toshio Nishimura,[†] Yao Xinsheng,[§] Takashi Sakurai,[⊥] Hiromichi Nagasawa,[⊥] Shohei Sakuda,*[,⊥] and Kuniaki Tatsuta*[,‡]

Department of Biosciences, School of Science and Technology, Teikyo University, 1-1 Toyosatodai, Utsunomiya 320-8551, Japan, Graduate School of Science and Engineering, Waseda University, Shinjuku-ku, Tokyo 169-8555, Japan, Department of Natural Products, Shenyang Pharmaceutical University, No. 103, Wenhua Road, Shenhe District, Shenyang 110015, China, and Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

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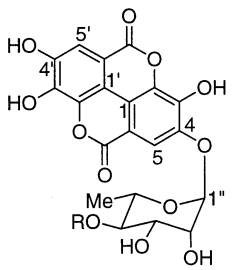
A new ellagic acid derivative (**1**) was isolated from the branches of *Combretum yunnanensis*, and its structure was assigned as 4-(4″-*O*-acetyl-α-rhamnopyranosyl)ellagic acid by analysis of its spectral data. Total synthesis of **1** was achieved by the glycosylation of 3,3′-di-*O*-benzylellagic acid (**5**) with 4-*O*-acetyl-2,3-di-*O*-benzyl-L-rhamnose (**4**) as a key reaction, and the absolute configuration of **1** was determined. Compound **1** showed weak inhibitory activity against the growth of various tumor cells and inhibited HIV-1 protease.

In the course of our screening for new bioactive natural products among Chinese plants distributed in Yunnan Province,[1] we found that the 60% EtOH extract of the branches of *Combretum yunnanensis* Exell (Combretaceae) inhibited the growth of P388/ADM (adriamycin-resistant mouse leukemia) cells. This finding prompted us to isolate and characterize the active principle involved in the plant, a new ellagic acid derivative (**1**). In this paper, we describe the structural determination, total synthesis, and some biological activities of **1**.

The 60% EtOH extract (35 g) of the branches of *C. yunnanensis* was applied on an HP-20 column. Growth inhibitory activity toward P388/ADM cells was observed in the 100% MeOH eluate from the column. The MeOH fraction was further purified by LH-20 column chromatography and reversed-phase HPLC, successively. Three active fractions were obtained, and these afforded two known compounds, ellagic acid (4.9 mg, **3**) and 4-(α-rhamnopyranosyl)ellagic acid (8.8 mg, **2**),[2] and a new ellagic acid derivative (8.6 mg, **1**).



R = Ac **1**
R = H **2**

The molecular formula of **1** was determined as $C_{22}H_{18}O_{13}$ by analysis of its HRFABMS and ^{13}C NMR spectra. The presence of an ellagic acid moiety in **1** was easily revealed by comparison of the ^{13}C NMR of **1** with that of **2** or **3**. Further analysis of the NMR spectra showed that **1** consisted of an ellagic acid moiety, a rhamnopyranosyl residue, and an acetyl group. In the NOESY spectrum of **1**, NOE was observed between the anomeric proton and H-5 of the ellagic acid moiety, which clarified that the rhamnose residue was present at C-4 of the ellagic acid moiety similarly to the case of **2**. The small $^3J_{H-1'',H-2''}$ value indicated the α-glycoside.[2] The position of the acetyl group was determined to be at C-4 of the sugar residue from the chemical shift of H-4 and long-range coupling between H-4 and the ester carbon observed in the HMBC spectrum of **1**. Thus, the structure of **1** was assigned as 4-(4″-*O*-acetyl-α-rhamnopyranosyl)ellagic acid.

A number of ellagic acid glycosides have been isolated from natural sources, but their syntheses have not been reported because of the difficult *O*-glycosylation. We attempted the synthesis of **1** to confirm its structure including the absolute configuration as follows. The starting material, 4-*O*-acetyl-2,3-di-*O*-benzyl-L-rhamnose (**4**), was selectively synthesized from L-rhamnose according to Koto's procedure.[3] The other starting material, 3,3′-di-*O*-benzylellagic acid (**5**), was derived from ellagic acid in four steps according to reported procedures.[4,5] Although the glycosylation of **5** with **4** was attempted under various known conditions,[6] rhamnoside **6** was not produced in a high yield. The best result was obtained by our developed method using Tsunoda's conditions (tri-*n*-butylphosphine and *N,N,N′,N′*-tetramethylazodicarboxamide)[7,8] to give exclusively the desired α-rhamnoside (**6**) in a reasonable yield. The α-rhamnoside was expected to be preferentially produced, because the formation of *β*-rhamnoside was well known to be restricted by the instability factor due to the 1,2-*cis* configuration in the transition state.[9] De-*O*-benzylation of **6** gave 4-*O*-(4″-*O*-acetyl-α-L-rhamnopyranosyl)ellagic acid (**1**), which was identical in all respects with the natural product, confirming the absolute structure.

Growth inhibitory activity of **1** on tumor cells was tested with seven cell lines, and the IC_{50} values obtained are listed in Table 1. The activity of **2** was also tested. The growth inhibitory activity of **1** was weak when compared to a strong well-known inhibitor such as adriamycin, which showed IC_{50} values of 0.04 and 1.10 *μ*g/mL to K562 and

* To whom correspondence should be addressed. (S.S.) Tel: +81-3-5841-5133. Fax: +81-3-5841-8022. E-mail: asakuda@mail.ecc.u-tokyo.ac.jp. (K.T.) Tel: +81-3-3203-4141. Fax: +81-3-3200-3203. E-mail: tatsuta@mn.waseda.ac.jp.
[†] Teikyo University.
[‡] Waseda University.
[§] Shengyang Pharmaceutical University.
[⊥] University of Tokyo.

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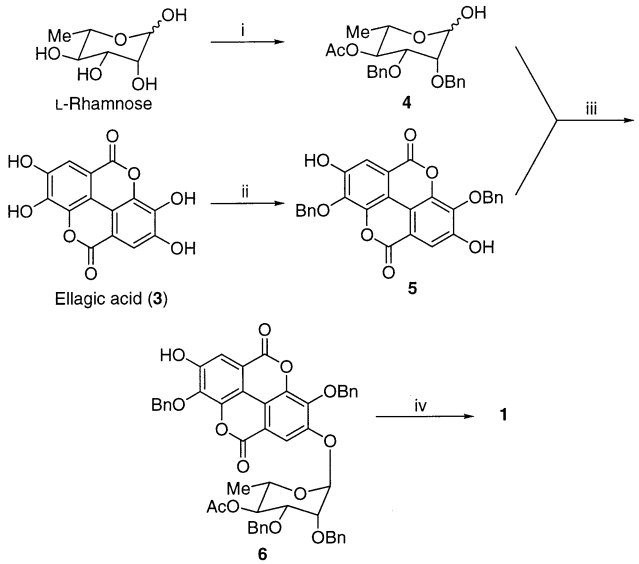
Scheme 1[a]



[a] (i) ref 3; (ii) refs 4 and 5; (iii) *n*-Bu_3P, TMAD, DMF, rt, 2 h, 64%; (iv) H_2, $Pd(OH)_2$, 1,4-dioxane, rt, 3 h, 86%.

Table 1. Growth Inhibitory Activity of **1** and **2** on Tumor Cells

	IC_{50} (μg/mL)	
cell line[a]	**1**	**2**
P388	52.0	62.0
P388/ADM	19.0	53.0
K562	80.0	82.0
K562/ADM	56.0	67.0
B16	52.0	88.0
HeLa	76.0	86.5
KB	61.0	98.0

[a] See Experimental Section for details.

K562/ADM cells, respectively. However, interestingly, **1** inhibited the growth of two adriamycin-resistant cells (P388/ADM and K562/ADM) more strongly than their parent cells (P388 and K562). Compound **1** showed stronger growth inhibitory activity toward all cells tested than **2**. Compounds **1** and **2** also showed inhibitory activity on HIV-1 protease with IC_{50} values of 11.0 and 4.8 μg/mL, respectively. Synthetic **1** showed no toxicity to mice at a dose of 100 mg/kg by intraperitoneal administration.

In this study, we isolated and synthesized a new ellagic acid rhamnoside (**1**). This is the first case of efficient total synthesis of an ellagic acid glycoside. Until now, only seven ellagic acid rhamnosides have been isolated from plant sources; these are 4-*O*-rhamnopyranosyl-3-*O*-methylellagic acid,[10] 4-*O*-(α-L-rhamnopyranosyl)-3,3′-di-*O*-methylellagic acid,[11] 4-(α-rhamnopyranosyl)ellagic acid (**2**),[2] 3-*O*-(2″-*O*-acetyl-α-rhamnopyranosyl)-3-*O*-methylellagic acid,[12] 3-*O*-(3″-*O*-acetyl-α-rhamnopyranosyl)-3-*O*-methylellagic acid,[12] 3-*O*-(4″-*O*-acetyl-α-rhamnopyranosyl)-3-*O*-methylellagic acid,[12] and 3-*O*-(α-rhamnopyranosyl)-3-*O*-methylellagic acid.[12] Antioxidant activity of the last four compounds was reported,[12] but no other biological activity of ellagic acid rhamnosides is known. The activity of **1** and **2** toward tumor cells and HIV protease is novel information. Work to test the effects of **1** in in vivo bioassay systems is now in progress.

Experimental Section

General Experimental Procedures. Melting points were measured on a micro hot-stage Yanaco MP-S3 and were uncorrected. Optical rotations were measured with a JASCO DIP-360 photoelectric polarimeter. Mass spectra were recorded on a JEOL SX102A or JEOL JMS-700T spectrometer, elemental analyses were performed on a Perkin-Elmer PE2400II elemental analyzer, and ^{1}H NMR spectra were recorded on Bruker Avance 600 or JEOL JMN-A500 spectrometers. Silica gel TLC and column chromatography were performed on Merck TLC 60F254 and Fuji silysia BW-820MH, respectively. Chromatograms were visualized by spraying with a solution of sodium phosphomolybdate hydrate in EtOH or *p*-anisaldehyde in HOAc−EtOH−H_2SO_4 or under ultraviolet light of wavelength 254 nm.

Plant Material. The branches of *C. yunnanensis* were collected in Yunnan Province of China in 1998. Voucher specimens are deposited in the Kunming Institute of Botany Academia Sinica.

Isolation of 1. Sixty percent EtOH extracts (35 g) of the branches of *C. yunnanensis* Exell were dissolved in H_2O (40 L), and the solution was applied on a Diaion HP-20 column (3 L). After washing with H_2O (9 L), the column was eluted stepwise with 50% MeOH (9 L), 100% MeOH (9 L), and 100% acetone (9 L). The activity was detected in the 100% MeOH eluate, and the solution was concentrated in vacuo. The residue (3.41 g) was chromatographed on a Sephadex LH-20 column (20 × 750 mm, MeOH), and two active fractions (elution volume: 400−450 and 460−500 mL) were obtained. The former fraction (118.1 mg) was further purified by reversed-phase HPLC (column, Senshu pak Pegasil ODS, 10 × 250 mm; mobile phase, gradient elution of 20−100% MeOH in H_2O in 30 min; flow rate 4 mL/min). The peaks with retention times of 16.8 and 21.7 min afforded **2** (8.8 mg) and **1** (8.6 mg), respectively. The latter fraction from the LH-20 column was purified by HPLC under the same conditions to afford ellagic acid (retention time, 19.0 min; 4.9 mg). **1**: $[\alpha]_D^{27}$ −84° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 258 (4.52), 352 (4.02); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.70 (1H, s, H-5), 7.44 (1H, s, H-5′), 5.54 (1H, s, H-1″), 4.89 (1H, dd, J = 10, 10 Hz, H-4″), 4.09 (1H, dd, J = 10, 3 Hz, H-3″), 4.04 (1H, s, H-2″), 3.75 (1H, dq, J = 10, 6 Hz, H-5″), 2.05 (3H, s, COCH_3), 1.01 (3H, d, J = 6 Hz, H-6″); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 170.0 (s, *C*OCH$_3$), 159.2 (s, C-7 or 7′), 159.1 (s, C-7 or 7′), 148.9 (s, C-4′), 146.1 (s, C-4), 142.0 (s, C-3), 140.5 (C-3′), 136.7 (s, C-2 or 2′), 136.6 (s, C-2 or 2′), 114.8 (s, C-6), 111.9 (s, C-6′), 111.6 (d, C-5), 110.0 (d, C-5′), 107.4 (s, C-1′), 106.8 (s, C-1), 99.6 (d, C-1″), 73.6 (d, C-4″), 69.8 (d, C-2″), 67.6 (d, C-3″), 67.4 (d, C-5″), 21.0 (q, CO*C*H$_3$), 17.5 (q, C-6″); ^{1}H NMR (MeOH-d_4, 500 MHz) δ 7.75 (1H, s, H-5), 7.40 (1H, s, H-5′), 5.58 (1H, s, H-1″), 5.06 (1H, dd, J = 10, 10 Hz, H-4″), 4.25 (1H, s, H-2″), 4.18 (1H, dd, J = 10, 3 Hz, H-3″), 3.91 (1H, dq, J = 10, 6 Hz, H-5″), 2.11 (3H, s, COCH_3), 1.56 (3H, d, J = 6 Hz, H-6″); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 172.5 (s, *C*OCH$_3$), 161.0 (s, C-7 or 7′), 161.0 (s, C-7 or 7′), 150.0 (s, C-4′), 147.7 (s, C-4), 143.0 (s, C-3), 141.0 (C-3′), 137.8 (s, C-2′), 137.7 (s, C-2), 116.1 (s, C-6), 113.5 (d, C-5), 113.1 (s, C-6′), 111.8 (d, C-5′), 109.7 (s, C-1′), 108.8 (s, C-1), 101.5 (d, C-1″), 75.2 (d, C-4″), 71.8 (d, C-2″), 70.0 (d, C-3″), 69.1 (d, C-5″), 21.0 (q, CO*C*H$_3$), 17.9 (q, C-6″); FABMS (negative, glycerol matrix) m/z 489 [M − H]$^-$; HRFABMS (positive, glycerol matrix) m/z 491.0847 [M + H]$^+$ (calcd for $C_{22}H_{19}O_{13}$, 491.0826).

Synthesis of 4-*O*-(4″-*O*-Acetyl-2″,3″-di-*O*-benzyl-α-L-rhamnopyranosyl)-3,3′-di-*O*-benzylellagic Acid (6). To a suspension of **4**[3] (247 mg, 641 μmol), **5**[4,5] (618 mg, 1.28 mmol), and tri-*n*-butylphosphine (950 μL, 3.85 mmol) in DMF (14 mL) was added a solution of *N*,*N*,*N*′,*N*′-tetramethylazodicarboxamide[7,8] (663 mg, 3.85 mmol) in DMF (10 mL), and the solution was stirred at room temperature for 2 h. The solution was evaporated in vacuo. The residue was chromatographed on silica gel (120 g) with toluene−ethyl acetate (8:1) to give, after recrystallization from acetone, colorless rosettes of **6** (348 mg, 64%): R_f 0.33 (toluene−ethyl acetate, 6:1); mp 182−185 °C; $[\alpha]_D^{28}$ −13° (*c* 0.96, CHCl3); $[\alpha]_D^{27}$ −8.2° (*c* 0.98, THF); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.90 (1H, s), 7.56 (1H, s), 7.25−7.55 (20H, m), 5.99 (1H, d, J = 2.0 Hz), 5.35 (2H, s), 5.30 (2H, s), 4.99 (1H, dd, J = 10.0, 10.0 Hz), 4.79 (1H, d, J = 12.0 Hz), 4.71 (1H, d, J = 12.0 Hz), 4.53 (1H, d, J = 12.0 Hz), 4.37(1H, d, J = 12.0 Hz), 4.13 (1H, dd, J = 3.0, 2.0 Hz), 3.83 (1H, dd, J

= 10.0, 3.0 Hz), 3.60 (1H, dq, J = 10.0, 6.0 Hz), 2.05 (3H, s), 0.99 (3H, d, J = 6.0 Hz); HRFABMS m/z 851.2741 $[M + H]^+$ (calcd for $C_{50}H_{43}O_{13}$, 851.2704). Anal. Found: C, 70.27; H, 4.81. Calcd for $C_{50}H_{42}O_{13}$: C, 70.58; H, 4.98.

Synthesis of 4-*O*-(4″-*O*-Acetyl-α-l-rhamnopyranosyl)-ellagic Acid (1). To a solution of **6** (211 mg, 249 μmol) in 1,4-dioxane (21 mL) was added 20% palladium hydroxide on carbon, and the mixture was stirred vigorously under an H_2 atm at room temperature for 3 h. The palladium hydroxide on carbon was filtered off and washed with MeOH. The filtrate was evaporated in vacuo to a residue. This was chromatographed on Sephadex LH-20 (10 g) with MeOH to give a solid, which was recrystallized from EtOH−H_2O (3:1) to provide colorless crystals of **1** (104 mg, 86%): R_f 0.42 (*n*-BuOH−EtOH−$CHCl_3$−H_2O, 4:4:2:3); mp >300 °C; $[\alpha]_D^{28}$ −106° (*c* 0.34, MeOH); $[\alpha]_D^{28}$ −97° (*c* 0.91, THF); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.73 (1H, s), 7.47 (1H, s), 5.56 (1H, d, J = 2.0 Hz), 4.90 (1H, dd, J = 10.0, 10.0 Hz), 4.10 (1H, dd, J = 10.0, 3.0 Hz), 4.05 (1H, dd, J = 3.0, 2.0 Hz), 3.76 (1H, dq, J = 10.0, 6.0 Hz), 2.06 (3H, s), 1.03 (3H, d, J = 6.0 Hz); HRFABMS m/z 491.0791 $[M + H]^+$ (calcd for $C_{22}H_{19}O_{13}$, 491.0825). Anal. Found: C, 51.99; H, 4.19. Calcd for $C_{22}H_{18}O_{13}\cdot H_2O$: C, 51.97; H, 3.97.

Cell Growth Inhibitory Activity. Samples were dissolved in MeOH at appropriate concentrations, and the solution (10 μL) was mixed with RPMI1640 medium (90 μL, Nissui Pharmaceutical Co., Ltd) containing cells (1×10^4) of P388/ADM (adriamycin-resistant mouse leukemia), P388 (mouse leukemia), K562/ADM (adriamycin-resistant human leukemia), K562 (human leukemia), B16 (mouse melanoma), HeLa (human ovarian carcinoma), or KB (human oral epidermoid carcinoma). After incubating the mixture at 37 °C for 3 or 4 days, viable cell number was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[13]

HIV Protease Inhibitory Activity. HIV-1 protease assay was performed according to the method of Ma et al. as follows.[14] A sample was dissolved in MeOH and the solution (3.0 μL) was added to the assay solution containing the substrate (2.0 μg, His-Lys-Ala-Arg-Val-Nle-*p*-nitro-Phe-Glu-Ala-Nle-NH_2, Bachem Feinchemikalien AG) and the HIV-1 protease (0.07 μg, Bachem Feinchemikalien AG) in a buffer (12 μL, 20 mM NaOAc, 200 mM NaCl, 5 mM DTT, and 10% glycerol, pH 4.9). The reaction mixture was incubated for 3 h at 37 °C, and the reaction was stopped by addition of 10% trifluoroacetic acid (2.0 μL). The amount of the product (*p*-nitro-Phe-Glu-Ala-Nle-NH_2) was analyzed by reversed-phase HPLC (column, Senshu pak Pegasil ODS, 4.6 × 250 mm; mobile phase, gradient elution of 5−65% acetonitrile in 0.1% trifluoroacetic acid in 15 min; flow rate, 1 mL/min; UV 280 nm; retention time, 13.1 min). The inhibitory activity of a sample was calculated as follows: % inhibition = $(A_{control} - A_{sample}) \times 100/A_{control}$ (where A is the relative peak area of the product). Pepstatin was used as a positive control.

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Supporting Information Available: ^{1}H and ^{13}C NMR, HMQC, HMBC, and NOESY spectra of natural **1** and the ^{1}H NMR spectrum of synthetic **1** are available free of charge via the Internet at http://pubs.acs.org.

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