Ursane- and Oleanane-Type Triterpenes from *Ternstroemia gymnanthera* Callus Tissues

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An investigation on the constituents of the callus tissues of *Ternstroemia gymnanthera* has led to the isolation of five phytosterols, 15 known triterpenoids, and four new triterpenoids (**1**−**4**). The new compounds were characterized by spectroscopic study as 3-*epi*-corosolic acid lactone (2α,3α-dihydroxyurs-11-en-13β,28-olide) (**1**), 3-*epi*-ternstroemic acid (2α,3α-dihydroxyurs-12-en-11-on-28-oic acid) (**2**), ternstroemic acid (2α,3β-dihydroxyurs-12-en-11-on-28-oic acid) (**3**), and gymntheraic acid (2α,3α-dihydroxy-11α-methoxyurs-12-en-28-oic acid) (**4**). The isolated triterpenes were compared with those from actinidiaceous plant callus tissues from a chemotaxonomic point of view.

Ternstroemia gymnanthera (Wight & Arn.) Sprague (syn. *T. japonica* Thunb.) (Theaceae) is grown in gardens as an ornamental plant in Japan, Korea, Taiwan, the People's Republic of China, and India.[1,2] The tree has little medicinal use, but the leaves have been used by the mountain people to allay malaria in Taiwan.[3] The bark and the roots possess astringent properties and are used as an antidysenteric.[3] Three antitermitic active saponins, C_1, C_2, and D, were isolated from the wood.[4,5] A new sapogenol, 22α-hydroxyerythrodiol, together with eight known oleanane triterpenes have been reported from the seeds, and four oleanane-type triterpenes and A_1-barrigenol were also reported from the leaves.[6] A procyanidin was isolated from the water-soluble components of the bark.[7] Inhibitory activity against cancer cell lines has been reported from the roots and bark of *Ternstroemia* spp.[8] During the course of the chemotaxonomic studies using the constituents from the plant callus tissues, we have previously reported the constituents produced from lardizabalaceous,[9] paeoniaceous,[10] and actinidiaceous[11] plant callus tissues.

From our studies of the constituents of the theaceous plant callus tissues, we wish to report herein the isolation and identification of triterpenes from *T. gymnanthera* callus tissues. The callus was obtained from the stem of *T. gymnanthera* and cultured on Murashige and Skoog medium (MS)[12] containing 2,4-D (1 mg/L) with kinetin (0.1 mg/L). The stored callus tissues were extracted successively with MeOH and EtOAc, and both extracts were combined. The concentrated extracts were chromatographed on a silica gel column using $CHCl_3$−MeOH and hexane−EtOAc−CH_3CN solvent systems to afford 23 compounds. These compounds were determined by analytical methods and also by comparison with published data. Four new ursane-type triterpenes (**1**−**4**) were elucidated, and 8 known ursane-type triterpenes were identified, α-amyrin, ursolic acid, corosolic acid,[13] 3-*epi*-corosolic acid,[14] ursolic acid lactone,[15] ilelatifol D,[16] 3β-hydroxy-11α-methoxyurs-12-en-28-oic acid (robustanic acid),[17] and euscaphic acid.[19] Also obtained were the lupane-type triterpene, betulinic acid, five oleanane-type triterpenes, β-amyrin, erythrodiol,[18] oleanolic acid, maslinic acid,[10] and 3-*epi*-maslinic acid[14] as minor triterpenes, and three phytosterols, sitosterol, stig-

masterol, and chondrialsterol, along with stigmasteryl β-D-glucopyranoside and chondrialsteryl β-D-glucopyranoside.[20,21]
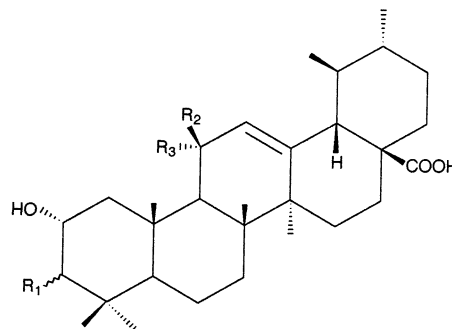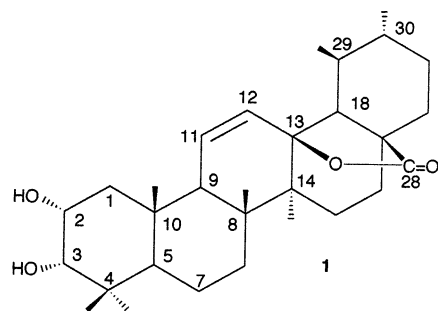
Compound **1** showed peaks at 1760 cm^{-1} (γ-lactone) in its IR spectrum and a molecular ion peak at *m/z* 470 [M]$^+$. The ^{1}H NMR spectrum showed five tertiary methyl signals at δ 0.85, 0.92, 1.0, 1.15, and 1.21 and two secondary methyl signals at δ 0.78 (3H, d, *J* = 6.0 Hz, Me-29) and 0.90 (3H, d, *J* = 6.0 Hz, Me-30). The two hydroxy proton signals appeared at δ 3.75 (1H, brs, H-3β) and 4.32 (1H, br, *J* = 11.0 Hz, H-2β), and two olefinic protons appeared at δ 5.59 (1H, d, *J* = 10.0, 3.5 Hz, H-11) and 6.07 (1H, dd, *J* = 10.0, 1.0 Hz, H-12), which were coupled with a proton at δ 2.15 (1H, brs) due to H-9. By comparison of the ^{13}C NMR data of **1** with those of 3-*epi*-corosolic acid and ilelatifol D, compound **1** was thus established as 2α,3α-

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Table 1. ^{13}C NMR Spectral Data (δ) for Compounds **1**−**4**^{a}

position	1	2	3	4
1	42.5	43.2	48.5	43.8
2	65.6	65.7	68.3	66.2
3	79.1	78.9	83.5	79.3
4	38.6	38.7	40.0	39.7
5	47.8	48.2	55.1	48.8
6	17.6	17.5	17.9	18.5
7	31.7	33.4	33.3	33.8
8	42.0	44.3	44.1	38.7
9	53.1	61.8	62.1	52.8
10	37.6	38.6	38.8	43.1
11	133.4	199.7	200.0	76.5
12	129.2	130.8	127.9	125.2
13	89.1	163.9	170.6	143.2
14	42.0	45.2	45.3	42.6
15	25.5	28.9	28.5	28.8
16	22.9	24.5	24.7	24.8
17	44.9	47.6	48.6	47.6
18	60.2	53.4	53.7	53.8
19	37.8	38.9	39.1	39.3
20	40.0	38.7	39.0	39.1
21	30.7	30.6	30.0	30.9
22	31.3	36.6	36.7	37.3
23	29.7	29.6	29.3	29.7
24	21.3	22.3	17.9	22.4
25	19.0	17.8	17.6	18.5
26	19.2	19.3	19.5	19.1
27	15.8	21.0	21.2	23.1
28	179.2	179.4	180.3	179.8
29	17.6	17.1	17.9	17.3
30	18.7	21.0	21.2	21.5
OCH$_3$				54.7

^{a} Measured in pyridine-d_5.

dihydroxyurs-11-en-13β,28-olide. This is the first report of the isolation of **1** from a natural source, and it was designated as 3-*epi*-corosolic acid lactone.

Compounds **2** and **3** showed the same molecular formula, C$_{30}$H$_{46}$O$_5$, as confirmed by HREIMS. Compound **2** showed a λ_{max} at 250 nm in its UV spectrum, while peaks at 1732 and 1653 cm^{-1} were observed in its IR spectrum. The ^{1}H NMR spectrum exhibited five tertiary methyl signals at δ 0.89, 1.18, 1.23, 1.26, and 1.33 and two secondary methyl signals as doublets at δ 0.80 (3H, d, J = 6.5 Hz) and 0.84 (3H, d, J = 6.5 Hz). It showed also a one-proton doublet at δ 2.65 (1H, d, J = 11.5 Hz, H-18) and an olefinic proton signal at δ 5.97 (1H, s, H-12), which correlated with the signal at δ_C 130.8 ppm in the HMQC spectrum. Quaternary carbon signals at δ_C 199.7 (C=O, C-11) and 163.9 (C-13) were observed in the ^{13}C NMR spectrum. The EIMS of **2** showed a molecular ion peak at m/z 486 [M]$^+$ together with prominent fragment peaks at m/z 303 and 262. The fragment peaks were consistent with the presence of a ketone group at C-11 and further suggested the presence of an α,β-unsaturated ketone system in ring C.[22,23] In the COSY spectrum, the hydroxyl protons were correlated to the protons at δ 4.41 (1H, m) and 3.77 (1H, d, J = 2.0 Hz), which were ascribable to the 2β- and 3β-protons on the carbons bearing a hydroxyl function, respectively. The other assignments were also supported by COSY, HMQC, and HMBC experiments and further coincided with those of 3-*epi*-corosolic acid except for the B/C rings in the ^{13}C NMR spectrum (Table 1). Thus, compound **2** was established as 2α,3α-dihydroxyurs-12-en-11-on-28-oic acid. This is the first report of the isolation of **2** from a natural source, and it was designated as 3-*epi*-ternstroemic acid.

Compound **3** showed a λ_{max} at 249 nm in the UV spectrum and peaks at 1724 and 1658 cm^{-1} in its IR spectrum. The EIMS showed the same peaks as compound **2**. The ^{1}H NMR spectrum of **3** exhibited signals at δ 5.99

(1H, s, H-12), and on the basis of the correlation of the COSY spectrum, the hydroxyl protons were correlated to the protons at δ 4.23 (1H, m) and 3.43 (1H, d, J = 9.5 Hz), which were ascribable to the 2β- and 3α-protons on the carbon bearing a hydroxyl function, respectively. Furthermore, signals for **3** coincided with those of **2** except for the A ring and also with those of ring A of corosolic acid in the ^{13}C NMR spectrum (Table 1). Thus, compound **3** was confirmed as 2α,3β-dihydroxyurs-12-en-11-on-28-oic acid. This is the first report of the isolation of **3** from a natural source, and it was designated as ternstroemic acid.

The IR spectrum of compound **4** showed carboxyl (1710 cm^{-1}) and olefinic (1630 cm^{-1}) absorption bands. The EIMS of **4** showed a molecular ion peak at m/z 502 [M]$^+$, which was 16 mass units more than that of robustanic acid, together with a prominent peak at m/z 278 (100%) obtained through cleavage of ring C. The ^{1}H NMR spectrum of **4** exhibited five tertiary methyl signals at δ 0.90, 1.04, 1.07, 1.16, and 1.26 and two secondary methyl signals at δ 0.89 (3H, d, J = 6.5 Hz, Me-29) and 1.00 (3H, d, J = 6.5 Hz, Me-30). The ^{13}C NMR spectrum (Table 1) was comparable with that of 3-*epi*-corosolic acid, except for ring C, and **4** showed a singlet peak at δ_H 3.22 and at δ_C 54.7 for the methoxyl group. The olefinic proton at δ 5.69 (1H, d, J = 3.5 Hz, H-12) and the H-11 proton at δ 3.83 (1H, dd, J = 8.0, 3.5 Hz) were correlated to H-12 at δ 5.69 (1H, d, J = 3.5 Hz) and H-9 at 2.09 (1H, d, J = 8 Hz) in the COSY spectrum. The position and the stereochemistry of the methoxyl group were determined by comparison with the data published for 2α,3β,7β-trihydroxy-11α-methoxyurs-12-en-28-oic acid isolated from *Eucalyptus camaldulensis*[24] such that the methoxyl geminal proton H-11 and the methoxyl group at these positions are β-axial and α-equatorial, respectively. Thus, the structure of **4** was established as 2α,3α-dihydroxy-11α-methoxyurs-12-en-28-oic acid. This is the first time that **4** has been reported from a natural source, and it was designated as gymnantheraic acid.

It is of biogenetic interest that ursane- and oleanane-type triterpenoids may be produced in a stepwise fashion. Callus tissues offer the possibility of producing new sources of natural products. A biogenetic sequence for these ursane-type triterpenes in which ursolic acid proceeds through corosolic acid to 3-*epi*-corosolic acid can be hypothesized based on the co-occurrence of these constituents at different degrees of oxidation at C-2. For the oleanane-type compounds from callus tissues it may be also suggested that the oxidation of β-amyrin proceeds through erythrodiol, oleanolic acid, and maslinic acid to 3-*epi*-maslinic acid. The two 3-epimeric pairs (corosolic acid−3-*epi*-corosolic acid and maslinic acid−3-*epi*-maslinic acid) may be formed from corosolic acid and maslinic acid, respectively, via 3-ketones, as has been shown for 3-*epi*-maslinic acid.[24] Furthermore, the three 13β,28-olide compounds may be biosynthesized by dehydration of intermediate allylic compounds according to a mechanism analogous to that proposed by Ikuta et al.[10] and Siddiqui et al.,[25] so the presence of 11α-methoxyl triterpenes (gymnantheraic acid **4** or robustanic acid) is important from a biogenetic point of view. The hypothetical biosynthesis of the three 13β,28-olide compounds in this callus tissue was proved finally by feeding experiments with [U-^{14}C]-labeled ursane-type compounds.[26] The triterpenoid compounds and the metabolic pathway produced from the *T. gymnanthera* callus tissues were analogous to those produced from three actinidiaceous plants' callus tissues (*Actinidia aruguta*, *A. chinensis,* and *A. polygama*).[11] However, both the metabolic pathways of the triterpenoids between the *T. gymnanthera* and the actinidia-

ceous plants of the callus tissues diverged into three different pathways. Corosolic acid is the key compound, as shown in Scheme S1. In the former, corosolic acid was biosynthesized into 3-*epi*-corosolic acid and furthermore each of the three 13β,28-olide compounds may be derived from ursolic acid, corosolic acid, and 3-*epi*-corosolic acid, respectively. In contrast, in the latter, there were differences in the biosynthetic abilities among the three actinidiaceous plant callus tissues (routes A and B) (Scheme S1). Therefore, the relationship of the biosynthetic pathways of the triterpenoid compounds of both the callus tissues may be considered to be closely related. Analogous results have also been reported for the triterpenoid production from the paeoniaceous[27] and lardizabalaceous plant callus tissues.[28] The Theaceae, Actinidiaceae, and Paeoniaceae belong to the subclass Dilleniidae.[29] Therefore, the results suggest that the comparison of the secondary products and of the metabolic pathways such as for triterpenes[27,28] and alkaloids[30] produced from callus tissues makes it possible to distinguish one group of plants from another. It may be helpful to interpret the chemotaxonomic or phylogenetic significance using the analytical results of the secondary products of callus tissues and hence may enhance the development of plant chemotaxonomy.

Experimental Section

General Experimental Procedures. The UV and IR spectra were recorded on UV-3000 (Shimadzu) and Valor-III (JASCO) instruments, respectively. The optical rotations were recorded on a P 1010 (JASCO) instrument. The ^{1}H and ^{13}C NMR spectra were obtained at 500 and 125 MHz (JEOL GSX-500), respectively, at room temperature, in pyridine-d_5. Chemical shifts are given in δ (ppm) with residual solvent signals (δ 7.55 and 135.5 ppm, respectively) as internal standards, and coupling constants (*J*) are in Hz. Multiplicities for the ^{13}C NMR spectra were determined by DEPT experiments at 90° and 135°, and the NMR assignments were determined by H−H COSY, HMQC, and HMBC experiments (Varian Unity-400 spectrometer). EIMS were recorded with a direct inlet probe at 70 eV (JEOL JMS-SX102A) and HREIMS measured on a JEOL JMS-SX102A mass spectrometer. Medium-pressure liquid chromatography was performed with a CIG column system (22 × 150 mm, Kusano Scientific Co., Tokyo). HPLC was carried out on a Waters 600; HPLC column CAPCELL PAK C$_{18}$ AG120, 5 μm, 4.6 × 250 nm (Shiseido); flow rate 0.5 mL min^{-1}; detection UV 210 nm.

Plant Material. Stems of *Ternstroemia gymnanthera* (Wight & Arn.) Sprague (Theaceae) were collected in April 1992 at the Medicinal Plant Garden of the Science University of Tokyo. The plant material was identified by Dr. T. Nakamura, Faculty of Pharmaceutical Sciences, Science University of Tokyo, and a voucher specimen (number Ter-92-04) was deposited at the herbarium of our Institute.

Callus Cultures. Callus tissues from the stems of *T. gymnanthera* were established in April 1992. Murashige and Skoog[12] (MS) medium (minus glycine) containing 2,4-dichlorophenoxyacetic acid (2,4-D) (1−3 mg/L) and kinetin (KIN) (0.1 mg /L) as plant growth regulators was used for the induction of callus tissues. The callus tissues were subcultured every 5−6 weeks onto fresh MS medium containing 2,4-D (1 mg /L) and KIN (0.1 mg /L) at 25 ± 1° in the dark.

Extraction and Isolation. The *T. gymnanthera* callus tissues were harvested at five-week intervals and were stored in MeOH for phytochemical investigation. The stored callus tissues (fresh weight 2.8 kg, dry weight 34 g) were extracted with cold MeOH and EtOAc in a Waring blender. The extracts were concentrated under reduced pressure, and the residue was partitioned between CHCl$_3$ (1.5 L) and H$_2$O (0.2 L) to obtain an organic-soluble fraction. The CHCl$_3$ solution was evaporated to dryness under reduced pressure, and the extracts were chromatographed on a column of silica gel

(Merck 9385) with gradient elution using CHCl$_3$ with increasing proportions of MeOH to afford crude triterpenoid mixtures. Repeated chromatography of the mixtures on a silica gel column with hexane−EtOAc−MeCN afforded compounds **1**−**4**, the triterpenes α-amyrin, ursolic acid, corosolic acid, 3-*epi*-corosolic acid, ursolic acid lactone, ilelatifol D, 3β-hydroxy-11α-methoxyurs-12-en-28-oic acid (robustanic acid), and euscaphic acid, and the phytosterols betulinic acid, sitosterol, stigmasterol, chondrialsterol, stigmasteryl β-D-glucopyranoside, and chondrialsteryl β-D-glucopyranoside. Sephadex LH-20 was used for further purification of all triterpene compounds using CHCl$_3$−MeOH (1:1) before the measurement of their NMR spectra. β-Amyrin, erythrodiol, oleanolic acid, maslinic acid, and 3-*epi*-maslinic acid were further purified by preparative HPLC using MeOH−H$_2$O solvent systems.

2α,3α-Dihydroxyurs-11-ene-13β-28-olide (1) (3-*epi*-Corosolic acid lactone): colorless powder (23 mg); [α]$_D$ +92.5° (*c* 0.02, pyridine); IR (KBr) ν$_{max}$ 3400, 1760 cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 0.78 (3H, d, *J* = 6.0 Hz, Me-29), 0.85 (3H, s, Me-24), 0.90 (3H, d, *J* = 6.0 Hz, Me-30), 0.92 (3H, Me-25), 1.00 (3H, s, Me-27), 1.15 (3H, *s*, Me-26), 1.21 (3H, s, Me-23), 2.15 (1H, brs, H-9), 3.75 (1H, brs, 3β), 4.32 (1H, brd, *J* = 11.0 Hz, H-2β), 5.59 (1H, dd, *J* = 10.0, 3.0 Hz, H-11), 6.07 (1H, dd, *J* = 10.0, 1.0 Hz, H-12); ^{13}C NMR, see Table 1; EIMS *m/z* [M]$^+$ 470 (17), 426 (56), 189 (22); HREIMS *m/z* [M]$^+$ 470.3385 (calcd for C$_{30}$H$_{46}$O$_4$, 470.3393).

2α,3α-Dihydroxyurs-12-en-11-on-28-oic acid (2) (3-*epi*-Ternstroemic acid): colorless powder (9.4 mg); [α]$_D$ +61.7° (*c* 0.0094, MeOH); UV (MeOH) λ$_{max}$ 250 nm; IR (CHCl$_3$) ν$_{max}$ 3466, 1732, 1653 cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 0.80 (3H, d, *J* = 6.5 Hz, Me-29), 0.84 (3H, d, *J* = 6.5 Hz, Me-30), 0.89 (3H, s, Me-24), 1.18 (3H, s, Me-26), 1.23 (3H, s, Me-27), 1.26 (3H, s, Me-23), 1.33 (3H, s, Me-25), 2.65 (1H, d, *J* = 11.5 Hz, H-18), 2.72 (1H, s, H-9), 3.77 (1H, d, *J* = 2.0 Hz, H-3β), 3.46 (1H, dd, *J* = 10.5, 4.5 Hz, H-1β), 4.41 (1H, m, H-2β), 5.97 (1H, s, H-12); ^{13}C NMR, see Table 1; EIMS *m/z* [M]$^+$ 486 (30), 468 (40), 453 (23), 303 (100), 262 (90), 257 (35); HREIMS *m/z* [M]$^+$ 486.3346 (calcd for C$_{30}$H$_{46}$O$_5$, 486.3349).

2α,3β-Dihydroxyurs-12-en-11-on-28-oic acid (3) (Ternstroemic acid): colorless powder (1.7 mg); [α]$_D$ +52.9° (*c* 0.0017, MeOH); UV (MeOH) λ$_{max}$ 249 nm; IR (CHCl$_3$) ν$_{max}$ 3467, 1724, 1658 cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 0.89 (6H, d, *J* = 7 Hz, Me-29 and 30), 1.08 (3H, *s*, Me-24), 1.16 (3H, *s*, Me-26), 1.27 (3H, s, Me-27), 1.35 (3H, s, Me-25), 1.38 (3H, s, Me-23), 2.64 (1H, s, H-9), 2.65 (1H, d, *J* = 7 Hz, H-18), 3.43 (1H, d, *J* = 9.5 Hz, H-3α), 3.87 (1H, dd, *J* = 12.5, 4 Hz, H-1β), 4.23 (1H, m, H-2β), 5.99 (1H, s, H-12); ^{13}C NMR, see Table 1; EIMS *m/z* [M]$^+$ 486 (15), 303 (72), 262 (100), 257 (45), 217 (33), 189 (43); HREIMS *m/z* [M]$^+$ 486.3329 (calcd for C$_{30}$H$_{46}$O$_5$, 486.3345).

2α,3α-Dihydroxy-11α-methoxyurs-12-en-28-oic acid (4) (Gymnantheraric acid): colorless powder (4.6 mg); [α]$_D$ −18.47° (*c* 0.0046, CHCl$_3$); IR (CHCl$_3$) ν$_{max}$ 3460, 1710, 1630, 1150 cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 0.89 (3H, d, *J* = 6.5 Hz, Me-29), 0.90 (3H, s, Me-24), 1.00 (3H, d, *J* = 6.5 Hz, Me-30), 1.04 (3H, s, Me-25), 1.07 (3H, s, Me-27), 1.16 (3H, s, Me-26), 1.26 (3H, s, Me-23), 2.09 (1H, d, *J* = 8 Hz, H-9), 2.62 (1H, d, *J* = 11.5 Hz, H-18), 3.22 (3H, s, OMe), 3.76 (1H, d, *J* = 2.0 Hz, H-3β), 3.83 (1H, dd, *J* = 8.0, 3.5 Hz, H-11), 4.33 (1H, m, H-2β), 5.69 (1H, d, *J* = 3.5 Hz, H-12); ^{13}C NMR, see Table 1; EIMS *m/z* [M]$^+$ 502 (42), 470 (22), 456 (36), 278 (100); HREIMS *m/z* [M]$^+$ 502.2663 (calcd for C$_{31}$H$_{50}$O$_5$, 502.3658).

Supporting Information Available: Scheme S1: Biogenetic pathway of ursane-type triterpenes in callus tissues of *Terstroemia gymnanthera* and Actinidiaceous plants. This material is available free of charge via the Internet at http//pubs.acs.org.

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