Inhibitory Effects on HIV-1 Protease of Constituents from the Wood of *Xanthoceras sorbifolia*

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From a methanolic extract of the wood of *Xanthoceras sorbifolia*, two new compounds, 29-hydroxy-3-oxotirucalla-7,24-dien-21-oic acid (**3**, xanthocerasic acid) and epigallocatechin-(4β→8,2β→*O*-7)-epicatechin (**6**), were isolated, together with 11 known compounds. Of the isolated compounds, 3-oxotirucalla-7,24-dien-21-oic acid (**2**), oleanolic acid (**4**), and **6** were found to be inhibitory substances against human immunodeficiency virus (HIV-1) protease, with their 50% inhibitory concentrations (IC_{50}) being 20, 10, and 70 μg/mL, respectively. Condensed tannins of high molecular weights with epicatechin and epiafzelechin as the main extender units were found to be the most active principles of this plant (IC_{50} values ca. 6.0 μg/mL).

The human immunodeficiency virus (HIV), which is the causative agent of AIDS, continues to spread in the world population at an alarming rate. Currently two kinds of therapeutic approaches are used for treatment of AIDS. One is to attack HIV reverse transcriptase, which is responsible for the viral genome transcription. The other is to inhibit HIV protease (HIV-1 PR), which is essential for the processing of viral proteins and is regarded as one of the most promising targets for development of anti-HIV agents.[1] Drug combinations based on these approaches can reduce the blood virus to an undetectable level. A small amount of virus, however, may still lurk inside the immune cells in a dormant state.[2,3] Another major obstacle of long-term treatment of the disease is the remarkable tendency of HIV to mutate. Most, if not all, of the clinical chemotherapeutic agents for AIDS have shortcomings in terms of clinical resistance by HIV. Their high cost and severe side-effects further reduce the desirability of the currently available anti-HIV drugs. New anti-AIDS chemotypes should be of low toxicity and of potentially low cost.

We have previously isolated and evaluated several anti-HIV agents from natural sources and have screened various Chinese and Mongolian herbal drugs for their inhibitory activity against HIV-1 PR. An extract from the wood of *Xanthoceras sorbifolia* Bunge (Sapindaceae) was found to have moderate activity at a concentration of 200 μg/mL. This species is a shrub found in the Inner Mongolia region of the People's Republic of China. The wood of the plant is used there as an herbal drug ("Wen Guan Mu" in Chinese and "Shen Deng" in Mongolian) for the treatment of rheumatism, gout, and other diseases. Although many saponins have been isolated from the fruits[4,5]of this plant, only a few compounds have been reported from the wood.[6,7] The present paper describes the isolation and identification of a new triterpene (**3**) and a new A-type proanthocyanidin dimer (**6**), as well as inhibitory activities of other isolated compounds and fractions against HIV-1 PR.

Results and Discussion

The chopped wood of *X. sorbifolia* was extracted with MeOH, and the extract was subjected to column chromatography over Sephadex LH-20 eluted with EtOH (fractions 1 and 2) and then MeOH (fraction 3). Fractions 1−3 were tested for HIV-1 PR inhibitory activity. Moderate activity was found for fractions 1 and 3, with their inhibition being 63 ± 1% and 62 ± 2%, respectively, at 100 μg/mL. Repeated column chromatography of fraction 1 afforded a new compound named xanthocerasic acid (**3**), together with 24-methylenecycloartan-3-ol (**1**),[8] 3-oxotirucalla-7,24-dien-21-oic acid (**2**),[9] and oleanolic acid (**4**).[10] Fraction 3 was purified by a combination of column chromatographic methods to give procyanidin A-2 (**5**),[11] **6**, and an unseparable subfraction (fraction 3-2). Although fraction 2 showed only a weak inhibitory effect against HIV-1 PR, it accounted for the largest amount of the MeOH extract by weight. Repeated column chromatography of this fraction afforded dihydromyricetin,[7] dihydroquercetin,[6] naringenin,[12] myricetin,[7] epigallocatechin,[7] epicatechin,[7] epiafzelechin,[13] and **5**.

The identification of the known compounds was performed by comparing their spectral data with those reported and with authentic samples. Compounds **3** and **6** are new natural products.

Compound **3** was obtained as an amorphous powder. Its methyl ester showed a $[M]^+$ ion at *m/z* 484.3576 ($C_{31}H_{48}O_4$) by HREIMS. The ^{1}H NMR spectrum of **3** exhibited two olefinic protons at δ 5.09 and 5.28; two protons on an oxygenated carbon at δ 3.60 (d, J = 11.1) and 4.08 (d, J = 11.1); and six singlet methyls at δ 0.89, 0.97, 1.00, 1.16, 1.58, and 1.68, of which the two methyls at relatively low field could be assigned to a terminal isopropylidene group. The ^{13}C NMR spectrum, analyzed with the aid of the DEPT technique, showed signals for two pairs of olefinic carbons at δ 118.1, 123.7, 132.1, and 145.8; two carbonyl carbons at δ 179.0 and 217.0; and a hydroxymethyl carbon at δ 65.6, which was correlated with the two protons at δ 3.60 and 4.08 in the HMQC spectrum. Compound **3** has eight degrees of unsaturation calculated from its molecular formula, indicative of four rings besides the two double bonds and two carbonyls. These findings, in combination with fragment ions at *m/z* 455 $[M - Me]^+$, 440 [M −

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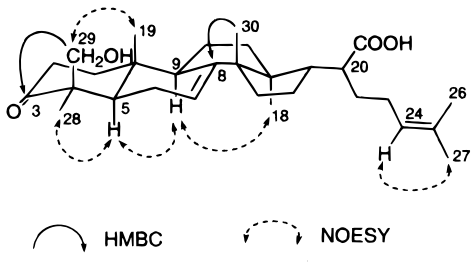


Figure 1. Characteristic correlations observed by HMBC and NOESY NMR for compound **3**.

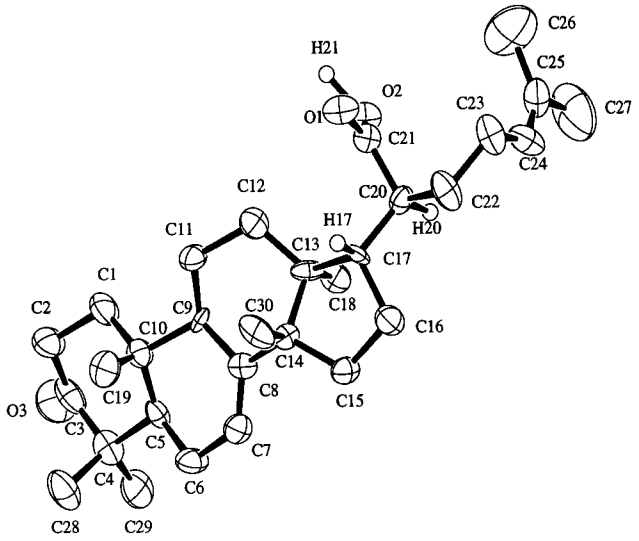


Figure 2. Computer-generated perspective drawing of the X-ray model of **2**.

$CH_2O]^+$, 425 [M − CH_2O − Me] $^+$, and 283 [M − CH_2O − Me − $C_8H_{14}O_2]^+$(side chain), suggested that **3** has a tetracyclic triterpene skeleton possessing a hydroxymethyl group and a C_8-side-chain with an isopropylidene group. Further analysis of the COSY, HMQC, and HMBC data enabled the structure of **3** to be determined as 29-hydroxy-3-oxotirucalla/eupha/lanosta-7,24-dien-21-oic acid. To establish the stereostructure of **3**, a NOESY experiment was undertaken. Compound **3** showed significant NOE correlations between H-29 and H-19 (β-face), H-28 and H-5, H-5 and H-9, H-9 and H-18 (α-face), as well as H-24 and H-27 of the molecule. Therefore, the lanostane type, whose CH_3-18 group is oriented to the β-face, was excluded. The last unsolved stereochemistry was that of C-20, which led to the assignment of **3** into the euphane or tirucallane types. Because **3** and several of its derivatives did not provide crystals good enough for X-ray analysis, we compared the ^{1}H and ^{13}C NMR spectra of **2** and **3**, with the former being confirmed as a tirucallane-type triterpene by X-ray analysis in the present investigation (Figures 1 and 2).

The NMR data of **2** and **3** were almost the same in their D rings and side chains, so their stereochemistry at C-20 was concluded to be the same. From the above evidence, the structure of **3** was formulated as 29-hydroxy-3-oxotirucalla-7,24-dien-21-oic acid (Table 1).

Compound **6**, an amorphous powder, was assigned a molecular formula of $C_{30}H_{24}O_{13}$, as established by HR-FABMS. Its ^{1}H NMR data were the same as those of procyanidin A-2 (**5**) in the region higher than 6.50 ppm. The differences between **6** and **5** in their ^{1}H NMR spectra were that **6** had a singlet signal at δ 6.76 that integrated for two protons, instead of an ABX-type signal as in **5**. The molecular weight of **6** was 16 mass units higher than that of **5**, indicating an additional hydroxyl group in **6**. Thus, it

Table 1. ^{13}C NMR Spectral Data of Compounds **2** and **3** and **7**−**10**[a]

position	**2**	**3**	**7**	**8**	**9**	**10**
1	38.7	38.1	37.2	31.2	36.9	39.3
2	35.2	35.8	27.5	25.3	27.5	35.8
3	217.5	217.0	79.1	70.3	80.4	213.3
4	48.1	53.8	38.9	42.4	41.7	54.3[b]
5	52.5	53.8	50.6	45.5	51.4	52.1[b]
6	24.6	24.9	23.9	23.7	23.6	25.1
7	118.1	118.1	118.0	118.0	118.1	118.0
8	145.6	145.8	145.1	145.7	145.3	148.8
9	48.4	48.3	48.5	48.5	48.5	48.5
10	35.1	35.3	34.9	34.7	34.4	35.4
11	17.8	18.2	17.6	17.7	17.4	18.3
12	30.3	30.3	30.1	30.4	30.0	30.2
13	43.4	43.4	43.3	43.3	43.0	43.5
14	51.1	51.2	50.9	51.1	50.7	51.2
15	33.7	33.7	33.4	33.6	33.2	33.7
16	27.4	27.4	27.2	27.3	27.0	27.4
17	49.9	49.9	49.7	49.8	49.4	50.0
18	21.9	22.0	21.6	21.7[b]	21.6[b]	22.0
19	12.9	13.8	13.0	13.8	13.6	13.9
20	47.6	47.6	47.1	47.7	47.4	47.5
21	179.5	179.0	180.8	179.0	179.0	181.8
22	32.6	32.6	32.3	32.6	32.2	32.6
23	26.2	26.2	26.0	26.2	25.7	26.3
24	123.7	123.7	123.3	123.7	123.5	123.6
25	132.1	132.1	132.0	132.0	131.8	132.4
26	17.9	17.8	17.6	17.7	17.2	17.9
27	25.9	25.9	25.6	25.8	25.3	26.0
28	24.7	20.4	27.2	21.5[b]	21.3[b]	20.1
29	21.8	65.6	14.7	65.2	63.6	66.9
30	27.5	27.6	27.5	27.3	26.8	27.6
CH$_3$CO						21.1
CH$_3$*CO*						171.0

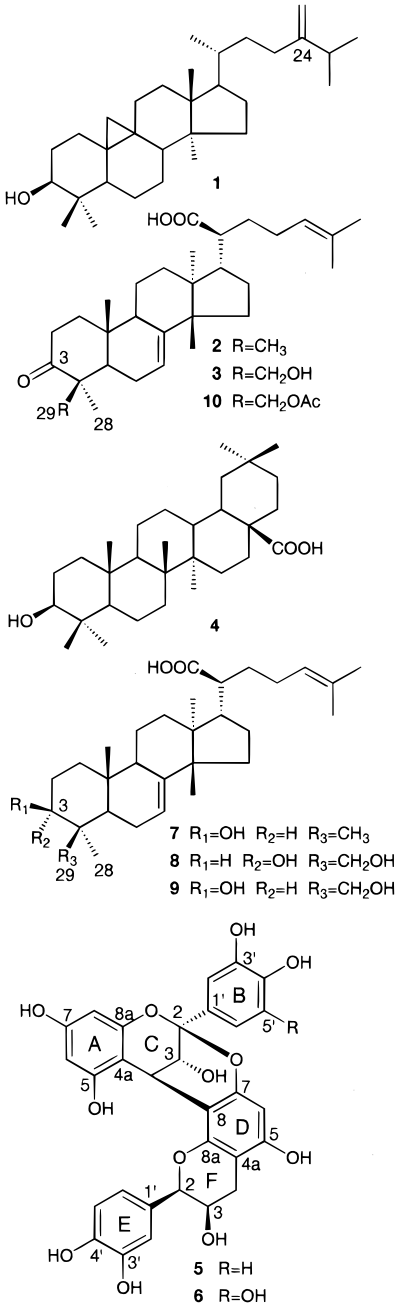
[a] Spectra run in $CDCl_3$−CD_3OD (8:1) at 75 MHz except for **9**, run at 100 MHz. [b] Assignments in the same column may be reversed.

was evident that **6** is a doubly linked proanthocyanidin dimer with epigallocatechin as one of its flavan-3-ol units, whose H-B2′ and H-B6′ protons were responsible for the singlet signal observed at δ 6.76. The appearance of a broad singlet due to H-F2 suggested the presence of a flavan-3-ol moiety with 2,3-cis (epicatechin-type) stereochemistry. The chemical shift of H-F2 (δ 4.97) was the same as that of procyanidin **5** (δ 4.97) and obviously more downfield than that of the 4,6-linked procyanidins A-6 and A-7,[14] thus suggesting that the two flavan units are linked through the C-C4 and C-D8 positions. The order of the two flavan-3-ol units was determined, and the inter-flavan linkage sites were confirmed by HMBC. Thus, the H-B2′,-B6′ signals of epigallocatechin at δ 6.76 were correlated with the characteristic ether-linked C-C2 at δ 98.2. In this manner, epigallocatechin was assigned as the upper unit, and the whole structure of **6** was determined as epigallocatechin-(4β→8,2β→*O*-7)-epicatechin.

The compounds isolated from fractions 1 and 3 were tested for their inhibitory activity against HIV PR. Compounds **2**, **4**, and **6** were found to be active, with IC_{50} values of 20, 10, and 70 μg/mL, respectively. Oleanolic acid (**4**) has been reported to have inhibitory activity on HIV and its protease,[15,16] in agreement with the present result. Compound **2** is the first example of a tirucallane-type triterpene having a significant HIV PR inhibitory effect.

Because small variations in the structures of **2** and **3** resulted in big differences in their inhibitory activity against HIV-protease, a few structural modifications were made for **2** and **3** to study this phenomenon further. Only a 3β-hydroxylated product (**7**) was obtained by reduction of the 3-oxo group of **2**. Both the 3α- and 3β- products (**8** and **9**) were obtained by reduction of **3**. Acetylation of **3**

gave a 29-*O*-acetylated product (**10**). All these synthesized analogues, **7**−**10**, were novel compounds. Among these analogues, **7** and **9** showed moderate inhibitory activity against HIV-1 PR, with an IC_{50} value of 100 μg/mL. However, it is hard to draw any conclusions on the structure−activity relationship of these tirucallane triterpenoids from *X. sorbifolia* due to the relatively small number of compounds studied to date (Table 2).



The most potent inhibitory activity was observed in a subfraction, fraction 3−2, which could not be separated using conventional chromatographic techniques. This fraction turned dark blue with ferric chloride reagent and orange-red with anisaldehyde−sulfuric acid reagent, suggesting the presence of condensed tannins. Fraction 3−2 was fractionated by ultrafiltration with 10, 30, and 100 kDa molecular-weight cutoffs to give the corresponding fractions I−IV (IC_{50} values 100, >100, >100, 6.0 μg/mL, respectively). Although, for tannins, these cutoffs may not reflect the molecular sizes accurately, they most probably reflect the correct order of molecular size. The most potent activity was found in fraction IV, which was obtained in the largest amount and showed the largest molecular weights. To investigate its constituents, negative ion APIMS of fraction IV was carried out. Groups of peaks were found with $[M - H]^-$ at *m*/*z* A 289; B 559, 575, 591; C 847, 863, 879; and D 1135, 1149, 1151, corresponding to flavan-3-ol monomers, dimers, trimers, and tetramers, with each of these oligomers containing A-type units. Because all of the condensed tannin oligomers measured in this laboratory to date (data not shown) give APIMS patterns of smaller oligomers, the APIMS peaks of fraction IV were thought to be generated by fragmentation of the polymers. The flavan-3-ol units of the oligomers included epiafzelechin, epicatechin, and epigallocatechin. Accordingly, it was inferred that group B was composed of epiafzelechin−epicatechin, epicatechin−epicatechin, and epicatechin−epigallocatechin dimers (*m*/*z* 559, 575, and 591, respectively). Group C was composed of three epicatechins, two epicatechins with one epiafzelechin or with one epigallocatechin (*m*/*z* 863, 847, and 879, respectively). However, group D contained no epigallocatechin but epicatechin units only (*m*/*z* 1151 and 1149 for four epicatechins with one or two A-type units, respectively) or three epicatechins with one epiafzelechin (*m*/*z* 1135). The intensity of these peaks, which contained epigallocatechin, were less than that of those containing no epigallocatechin. In the higher molecular group, group D, no epigallocatechin-containing peaks were detected. This may suggest that epigallocatechin units are less abundant in fraction IV. The average molecular weight of fraction IV could not be estimated by APIMS, because the high polymers gave neither singly charged nor multi-charged molecular ions. However, the fragment peaks did give some hint of the structural nature of the extender flavan units of the polymers in this fraction. To confirm the result derived from APIMS and to exclude other possibilities, thiolytic degradation was carried out. Thiolytic degradation of fraction IV followed by column chromatography led to the isolation of benzylthioepicatechin and benzylthioepiafzelechin. Therefore, the major extender flavan units of the polymers were concluded in fraction IV to be epicatechin and epiafzelechin (Scheme 1).

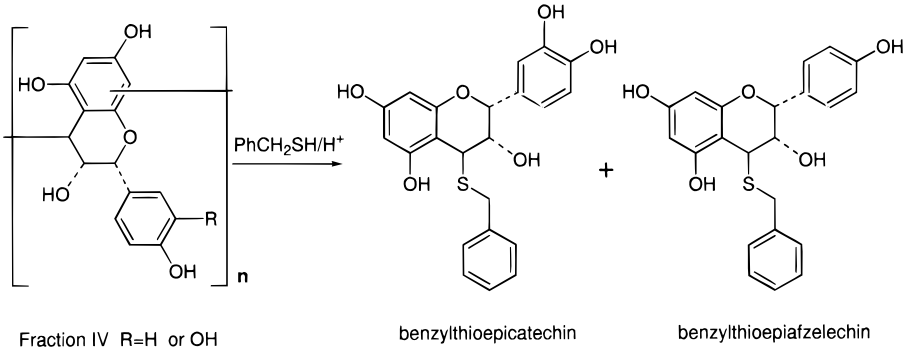
Table 2. Inhibitory Activity of Constituents of *X. sorbifolia* against HIV-1 Protease

compound	% inhibition ± S.D.[a] (100 μg/mL)	IC_{50} (μg/mL)
1	19 ± 8	>100
2	90 ± 8	20
3	14 ± 5	>100
4	96 ± 1	10
5	−2 ± 9	>100
6	89 ± 6	70
7	50 ± 5	100
8	26 ± 1	>100
9	53 ± 3	100
10	26 ± 1	>100
acetyl pepstatin	100 ± 0	0.15

[a] Average of triplicate experiments.

Tannins are well-known to have inhibitory activity against many enzymes, which are often considered to be due to their common property of interacting with proteins. However, as separation technology has improved, many pure tannin compounds have been isolated and identified. Previous investigations on the enzyme inhibitory activity of tannins have shown that pure tannin compounds may have some specificity on a given enzyme.[17] The present results demonstrated that, of the two dimers (**5** and **6**) isolated from *X. sorbifolia*, only one (**6**) showed inhibitory

Scheme 1. Thiolytic Degradation of Fraction IV



activity against HIV-1 PR. Further investigations on large numbers of pure tannin compounds are necessary before a conclusion on structure−activity relationships can be drawn.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-360 automatic polarimeter. IR spectra were measured with a JASCO FT-IR-230 infrared spectrometer. ^{1}H and ^{13}C NMR spectra were measured with either a Varian Gemini 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz), a Varian Unity 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz), or a JEOL JNA-LA 400WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz) NMR spectrometer, with chemical shifts being represented in parts per million (ppm) and TMS used as an internal standard. EIMS were measured with a JEOL JMS-AX 505 HAD mass spectrometer at an ionization voltage of 70 eV. Atmospheric pressure ionization APIMS were measured with a Perkin-Elmer SCIEX API−III biomolecular mass analyzer. HR-FABMS were measured with a JEOL SX-102A mass spectrometer with a resolution of 5000, and *m*-nitrobenzyl alcohol being used as a matrix.

HIV Protease Assay. An HIV protease assay kit (Bachem Feinchemikalien AG, Bubendorf, Switzerland; lot no. PR D-00070) was used. The HIV protease (HIV PR) inhibition assay was performed, and inhibitory activity was calculated as described previously.[15] Acetyl pepstatin from the same assay kit was used as a positive control, with its IC_{50} being 0.24 μM (0.15 μg/mL).

Plant Material. The wood of *X. sorbifolia* was purchased from Yaocaigongyingzhan of Huhhot, Inner Mongolia, People's Republic of China, in August of 1996. The plant material was identified by Associate Professor Katsuko Komatsu and Dr. Hirotoshi Fushimi of the Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University. A voucher specimen (TMPW19180) is stored at the Museum of Materia Medica, Toyama Medical and Pharmaceutical University, Japan.

Extraction and Isolation. The wood of *X. sorbifolia* (2.8 kg) was chopped into small pieces and extracted with MeOH under reflux (9 L × 3, each 2 h). The MeOH extract was evaporated to give 140 g of an extract. This extract was subjected to Sephadex LH-20 column chromatography to give fractions 1 (14 g) and 2 (101 g) from the EtOH eluates, as well as fraction 3 (14 g) from the MeOH eluate. Fractions 1−3 showed 63% ± 1, 16% ± 2, and 62% ± 2 inhibition against HIV-1 PR, respectively.

Fraction 1 was flash chromatographed on Si gel 60 silanized (RP-2) with 60% MeOH (9.4 g) and then MeOH (3.8 g). The MeOH fraction was chromatographed on a column of Si gel, eluted with *n*-hexane−$CHCl_3$ (1:1) containing increasing amounts of $CHCl_3$ (fractions 1-1 and 1-2) and then with $CHCl_3$−MeOH (fraction 1-3). Fraction 1-1 was further chromatographed on a Si gel open column with *n*-hexanes−EtOAc (9:1) to afford **1** (40 mg). Fraction 1-2 was purified on chromatorex-ODS DM1020T (Fuji Silysia Chemical Ltd., Aichi, Japan; RP_{18}) open column with MeOH−H_2O (9:1) to afford **2** (50 mg). Fraction 1-3 was subjected to Si gel medium-pressure liquid chromatography (MPLC) with *n*-hexanes−EtOAc (6:4) to afford **3** (30 mg) and **4** (2 mg).

Fraction 2, 10 g, was chromatographed on a Si gel open column ($CHCl_3$−MeOH, 10:0−7:3) followed by RP_{18} (30−100% MeOH) or MCI gel CHP20P (Mitsubishi Chemical Corporation, Tokyo, Japan; 20−100% MeOH) column chromatography to afford dihydromyricetin (50 mg), dihydroquercetin (15 mg), naringenin (2 mg), myricetin (200 mg), epigallocatechin (80 mg), epicatechin (80 mg), epiafzelechin (3 mg), and **5** (30 mg).

Fraction 3 (14.3 g) was applied to MCI gel CHP20P eluted with H_2O (3.9 g) and MeOH (7.2 g). The MeOH eluate was then subjected to Sephadex LH-20 column chromatography eluted with 50% MeOH (fraction 3-1, 2.5 g) and MeOH (fraction 3-2, 3.1 g). Fraction 3-1 was further chromatographed over RP_{18} and MCI gel CHP20P to afford **5** (20 mg) and **6** (3 mg). A portion of fraction 3-2 (500 mg) was fractionated by ultrafiltration to give four fractions whose molecular sizes increased in the order of I−IV (19 mg, 60 mg, 3 mg, and 357 mg, respectively) [devices used: (a) Centriprep-10; (b) Centriprep-30 (Amicon, Beverly, MA); (c) Centricon Plus-20 Biomax-100 (Millipore, Bedford, MA), corresponding to 10, 30, and 100 kDa molecular-weight cutoffs, respectively].

29-Hydroxy-3-oxotirucalla-7,24-dien-21-oic acid (xanthocerasic acid, 3): amorphous powder; $[\alpha]^{25}_D$ −48.4° (*c* 0.23, $CHCl_3$); IR (KBr) ν_{max} 3600−3200, 2960, 2880, 2850, 1700, 1640, 1445, 1382, 1042 cm^{-1}; ^{1}H NMR [$CDCl_3$−CD_3OD (8:1), 500 MHz] δ 0.89 (3H, s, H-18), 0.97 (3H, s, H-19), 1.00 (3H, s, H-30), 1.16 (3H, s, H-28), 1.58 (3H, s, H-26), 1.68 (3H, s, H-27), 2.70 (1H, dt, J = 14.4, 5.8 Hz, H-2a), 3.60 (1H, d, J = 11.1 Hz, H-29a), 4.08 (1H, d, J = 11.1 Hz, H-29b), 5.09 (1H, br t, J = 7.0 Hz, H-24), 5.28 (1H, br s, H-7); ^{13}C NMR, see Table 1; EIMS *m/z* 470 $[M]^+$(1), 455 $[M - Me]^+$(1), 440 $[M - CH_2O]^+$(35), 425 $[M - CH_2O - Me]^+$(100), 407 (70), 283 $[M - CH_2O - Me - C_8H_{14}O_2]^+$(90).

Methylation of 3. Compound **3** (5 mg) in benzene−MeOH (1:1, 1 mL) was treated with 10% trimethylsilyldiazomethane dissolved in hexane (0.3 mL) to give a methyl ester of **3**, whose HREIMS showed a molecular ion at *m/z* 484.3576 (calcd for $C_{31}H_{48}O_4$, 484.3554).

Epigallocatechin-(4*β*→8,2*β*→*O*-7)-epicatechin (6): amorphous powder; $[\alpha]^{25}_D$ +51.2° (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ϵ) 280 (3.80) nm; IR (KBr) ν_{max} 3300, 2920, 1620, 1450, 1140, 1010 cm^{-1}; ^{1}H NMR (CD_3OD, 300 MHz) δ 2.80 (1H, dd, J = 17.0, 2.3 Hz, H-F4a), 2.99 (1H, dd, J = 17.0, 5.4 Hz, H-F4b), 4.08 (1H, d, J = 3.8 Hz, H-C3), 4.28 (1H, br s, H-F3), 4.44 (1H, d, J = 3.3 Hz, H-C4), 4.97 (1H, s, H-F2), 6.04 (1H, d, J = 2.7 Hz, H-A6), 6.10 (1H, d, J = 2.7 Hz, H-A8), 6.13 (1H, s, H-D6), 6.76 (2H, s, H-B2′,-B6′), 6.85 (1H, d, J = 8.2 Hz, H-E5′), 7.02 (1H, dd, J = 8.2, 2.2 Hz, H-E6′), 7.19 (1H, d, J = 2.2 Hz, H-B2′); ^{13}C NMR (CD_3OD, 75 MHz) δ 27.4 (C-C4), 28.0 (C-F4), 65.1 (C-F3), 66.2 (C-C3), 79.8 (C-F2), 94.6 (C-D6, A8), 96.3 (C-A6), 98.2 (C-C2), 100.4 (C-D4a), 102.3 (C-A4a), 105.2 (C-D8), 105.5 (C-B2′,-B6′), 113.9 (C-E2′), 114.0 (C-E5′), 118.3 (C-E6′), 129.2 (C-E1′), 129.8 (C-B1′), 132.4 (C-B4′), 144.0 (C-E4′), 144.3 (C-B3′,-E3′,-B5′), 150.0 (C-D8a), 150.2 (C-D5), 152.1 (C-A5), 154.5 (C-D7), 154.9 (C-A8a), 156.0 (C-A7); positive ion APIMS *m/z* 615 $[M + Na]^+$ (70), 594 $[M + H]^+$ (72), 475 (70),

453 (72), 442 (69), 303 (100); negative ion APIMS *m/z* 591 [M − H]$^-$ (100); HRFABMS *m/z* 593.1293 (calcd for $C_{30}H_{25}O_{13}$, 593.1295 [M + H]$^+$).

Reduction of 2 and 3. Compound **2** (5 mg) was treated with $NaBH_4$ (20 mg) in MeOH (25 mL) for 1 h. The reaction mixture was then concentrated to dryness and subjected to RP_{18} column chromatography eluted with H_2O−MeOH to give **7** (3 mg). Compound **3** (10 mg) was treated in the same manner to give **8** (3 mg) and **9** (6 mg).

3*β*-Hydroxytirucalla-7,24-dien-21-oic acid (7): amorphous powder; ^{1}H NMR [$CDCl_3$, 300 MHz] δ 0.73, 0.85, 0.89, 0.97, 0.97 (each 3H, s, H-18, 19, 30, 28, 29), 1.58 (3H, s, H-26), 1.68 (3H, s, H-27), 3.23 (1H, dd, J = 4.2, 10.7 Hz, H-3), 5.09 (1H, br t, J = 7.0 Hz, H-24), 5.25 (1H, br s, H-7); ^{13}C NMR, see Table 1; EIMS *m/z* 456 [M]$^+$(20), 441 (40), 423 (45).

3α,29-Dihydroxytirucalla-7,24-dien-21-oic acid (8): amorphous powder; ^{1}H NMR [$CDCl_3$−CD_3OD (8:1), 500 MHz] δ 0.71, 0.89, 0.96, 1.04 (each 3H, s, H-18,-19,-30,-28), 1.58 (3H, s, H-26), 1.68 (3H, s, H-27), 3.57 (1H, d, J = 11.2 Hz, H-29a), 3.79 (1H, d, J = 11.2 Hz, H-29b), 3.90 (1H, t, J = 3.0 Hz, H-3), 5.09 (1H, br t, J = 7.0 Hz, H-24), 5.22 (1H, br s, H-7); ^{13}C NMR, see Table 1; EIMS *m/z* 472 [M]$^+$(1).

3*β*,29-Dihydroxytirucalla-7,24-dien-21-oic acid (9): amorphous powder; ^{1}H NMR [$CDCl_3$−CD_3OD (8:1), 400 MHz] δ 0.69, 0.88, 0.95, 1.19 (each 3H, s, H-18,-19,-30,-28), 1.58 (3H, s, H-26), 1.68 (3H, s, H-27), 3.38 (1H, dd, J = 5.6, 14.1 Hz, H-3), 3.48 (1H, d, J = 11.1 Hz, H-29a), 4.25 (1H, d, J = 11.1 Hz, H-29b), 5.09 (1H, br t, J = 7.0 Hz, H-24), 5.23 (1H, br s, H-7); ^{13}C NMR, see Table 1; EIMS *m/z* 472 [M]$^+$(10).

Acetylation of 3. Compound **3** (10 mg) was treated with Ac_2O (0.5 mL) in pyridine (0.5 mL) for 12 h and then worked up in the usual manner to give **10** (10 mg).

29-*O*-Acetyl-3-oxotirucalla-7,24-dien-21-oic acid (10): amorphous powder; ^{1}H NMR [$CDCl_3$−CD_3OD (8:1), 300 MHz] δ 0.88, 1.00, 1.03, 1.14 (each 3H, s, H-18,-19,-30,-28), 1.58 (3H, s, H-26), 1.67 (3H, s, H-27), 2.00 (3H, s, CH_3CO) 2.79 (1H, dt, J = 14.2, 5.2 Hz, H-2a), 4.06 (1H, d, J = 11.2 Hz, H-29a), 4.66 (1H, d, J = 11.2 Hz, H-29b), 5.08 (1H, br t, J = 6.6 Hz, H-24), 5.27 (1H, br s, H-7); ^{13}C NMR, see Table 1; EIMS *m/z* 512 [M$^+$]-(35), 497 [M − Me] $^+$(10), 452 [M − CH_3COOH]$^+$ (30), 437 (40), 419 (60), 355 [M − Me − $C_8H_{14}O_2$]$^+$(95), 98 (100).

Thiolytic Degradation of Fraction IV. Fraction IV (50 mg) was added to a mixture of EtOH (10 mL), HOAc (2 mL), and benzylthiol (1 mL). The mixture was stirred at 70 °C for 24 h, then concentrated under a vacuum. The residue was chromatographed on a column of Sephadex LH-20 with benzene and then MeOH. The MeOH eluate was further chromatographed on RP_2 with MeOH−H_2O (1:1) to give benzylthioepicatechin (2 mg) and benzylthioepiafzelechin (1 mg).

Benzylthioepicatechin: amorphous powder; ^{1}H NMR (CD_3OD) δ 7.46 (2H, br d, J = 7.0 Hz, H-2″,-6″), 7.34 (2H, br t, J = 7.0 Hz, H-3″,-5″), 7.27 (1H, br t, J = 7.0 Hz, H-4″), 6.96 (1H, d, J = 2.0 Hz, H-2″), 6.78 (1H, d, J = 8.2 Hz, H-5′), 6.71 (1H, dd, J = 8.2, 2.0 Hz, H-6′), 5.99 (1H, d, J = 2.5 Hz, H-6), 5.90 (1H, d, J = 2.5 Hz, H-8), 5.26 (1H, br s, H-2), 4.08 (1H, d, J = 2.2 Hz, H-4), 4.01 (2H, s, −SCH_2−), 3.89 (1H, br d, J = 2.2 Hz, H-3); positive ion APIMS *m/z* 435 [M + Na]$^+$ (100), 413 [M + H]$^+$ (55), 311 [epicatechin-2 + Na] $^+$ (90); negative ion APIMS *m/z* 411 [M − H]$^-$ (85), 287 [epicatechin-2 − H]$^-$ (100).

Benzylthioepiafzelechin: amorphous powder; ^{1}H NMR (CD_3OD) δ 7.46 (2H, br d, J = 7.0 Hz, H-2″, -6″), 7.34 (2H, br t, J = 7.0 Hz, H-3″,-5″), 7.26 (1H, br t, J = 7.0 Hz, H-4″), 7.23 (2H, d, J = 8.2 Hz, H-2′,-6′), 6.80 (2H, d, J = 8.2 Hz, H-3′,-5′), 5.99 (1H, d, J = 2.5 Hz, H-6), 5.92 (1H, d, J = 2.5 Hz, H-8), 5.31 (1H, br s, H-2), 4.10 (1H, d, J = 2.4 Hz, H-4), 4.01 (2H, s, −SCH_2−), 3.87 (1H, br d, J = 2.4 Hz, H-3); positive ion APIMS *m/z* 419 [M + Na]$^+$ (100), 295 [epiafzelechin-2 + Na]$^+$ (70); negative ion APIMS *m/z* 395 [M − H] (30), 271 [epiafzelechin-2 − H]$^-$ (70).

Crystal Data and X-Ray Crystal Structure Determination of 2.[18] Single crystals of **2**, suitable for X-ray analysis, were obtained by slow evaporation of a solution in $CHCl_3$−CH_3OH.

Crystal data: $C_{30}H_{46}O_3$; M_r = 454.69; orthorhombic, space group $P2_12_12_1$, a = 12.750(6) Å, b = 30.671(5) Å, c = 6.860(7) Å, V = 2682(2) Å^3, Z = 4, D_{calc} = 1.126 g/cm^3, μ(Mo Kα) = 0.07 mm^{-1}, F(000) = 1000.00, T = 296 K; colorless prismatic crystals, dimensions 0.05 × 0.07 × 0.39 mm.

Data Collection. Rigaku AFC7R diffractometer, ω scan technique, graphite-monochromated Mo Ka (λ = 0.71069 Å) radiation; 3543 reflections measured (2.08° ≤ θ ≤ 27.50°), 1029 observed [$I > 1.5\sigma(I)$].

Structure Analysis and Refinement. The crystal structure was solved by direct methods (SIR 92)[19] and refined by full-matrix least-squares on F values (TEXSAN).[20] Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were calculated at geometrical positions except those of H-17, H-20, and H-21, which were obtained from a difference Fourier synthesis. Isotropic temperature factors of the hydrogen atoms were set equal to 1.1 U_{eq} of the bonded non-hydrogen atom. The final indices were R = 0.056, R_w = 0.068, with goodness-of-fit = 1.18. The highest and the lowest peaks in the final difference Fourier map were 0.20 and −0.20 e Å^{-3}.

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(18) Crystallographic data for compound **2** have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax +44 1223 336033; e-mail *deposit@ccdc.cam.ac.uk*).
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