Cytotoxic and Apoptosis-Inducing Activities of Steviol and Isosteviol Derivatives against Human Cancer Cell Lines

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Seventeen steviol derivatives, *i.e.*, **2**–**18**, and 19 isosteviol derivatives, *i.e.*, **19**–**37**, were prepared from a diterpenoid glycoside, stevioside (**1**). Upon evaluation of the cytotoxic activities of these compounds against leukemia (HL60), lung (A549), stomach (AZ521), and breast (SK-BR-3) cancer cell lines, nine steviol derivatives, *i.e.*, **5**–**9** and **11**–**14**, and five isosteviol derivatives, *i.e.*, **28**–**32**, exhibited activities with single-digit micromolar IC_{50} values against one or more cell lines. All of these active compounds possess C(19)-*O*-acyl group, and among which, *ent*-kaur-16-ene-13,19-diol 19-*O*-4′,4′,4′-trifluorocrotonate (**14**) exhibited potent cytotoxicities against four cell lines with IC_{50} values in the range of 1.2–4.1 μM. Compound **14** induced typical apoptotic cell death in HL60 cells upon evaluation of the apoptosis-inducing activity by flow-cytometric analysis. These results suggested that acylation of the 19-OH group of kaurane- and beyerane-type diterpenoids might be useful for enhancement of their cytotoxicities with apoptosis-inducing activity.

1. Introduction. – Stevioside (**1**) is a sweet-tasting diterpenoid glycoside occurring abundantly in the leaves of *Stevia rebaudiana* Bertoni (Asteraceae), which has been popularly used as a sugar substitute in Japan, Korea, and Brazil for decades [1][2]. Hydrolysis of **1** (*cf. Scheme 1*) with mineral acid affords isosteviol (**19**; 16-oxobeyeran-19-oic acid; *cf. Scheme 4*), a beyerane-type diterpenoid [3]. On the other hand, treatment of **1** with $NaIO_4$ and KOH produces steviol (**2**; (5β,8α,9β,10α,13α)-13-hydroxykaur-16-en-18-oic acid) [4], a kaurane-type diterpenoid which constitutes an aglycone moiety of **1**. Several studies have suggested that, besides sweetness, **1**, along with related compounds, which include rebaudioside A (second most abundant sweet principle of *S. rebaudiana* leaf), **2**, and **19**, may also offer therapeutic benefits, as they have antihyperglycemic, antihypertensive, anti-inflammatory, antitumor, antidiarrheal, diuretic, and immunomodulatory properties [2]. In addition, compounds **1**, **2**, and **19** have been suggested to be valuable chemopreventive agents against chemical carcinogenesis [5][6]. On the other hand, a number of derivatives of **2** and **19** have been prepared and evaluated for their bioactivities which included anti-inflammatory [7–9], cytotoxic [10–12], antibacterial [10][13], glycosidase-inhibitory [14][15], plant-growth-regulation [16], antihyperglycemic [17], and glucocorticoid-agonist activities [18]. We have reported the inhibitory effects of **2**, **19**, and some of their derivatives against DNA polymerase, DNA topoisomerase, and *Epsterin–Barr* virus early antigen (EBV-EA) acitivation [3][19]. In a continuing study on the evaluation of the biological potential of kaurane- and beyerane-type diterpenoids, we have prepared 36 derivatives,

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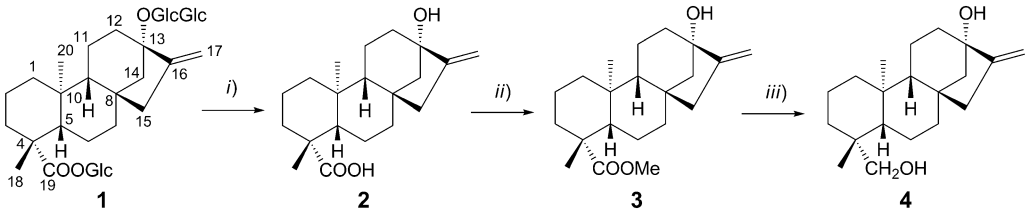
i.e., **2**–**37**, from the natural diterpenoid glycoside **1**. Here, we describe the preparation of these derivatives and evaluation of their cytotoxic activities against four human cancer cell lines. Synthesis and induction of apoptosis-signaling pathway of the derivatives of kaurenoic acid, a kaurane-type diterpenoid, has recently been reported [20].

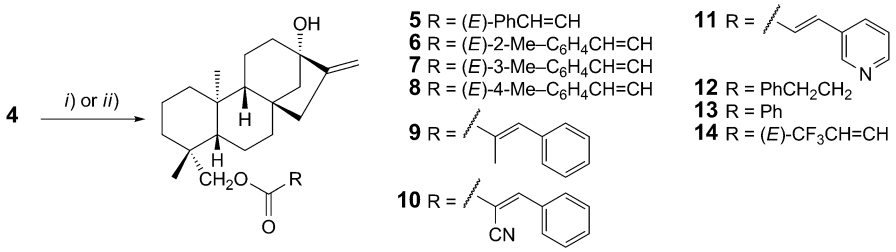
2. Results and Discussions. – *Preparation of Steviol Derivatives.* Starting from stevioside (**1**), treatment with $NaIO_4$ and KOH led to the aglycone steviol (**2**) [4]. Esterification of **2** with diazo(trimethylsilyl)methane (TMS–CHN_2) provided steviol methyl ester (**3**). Reduction of **3** with $LiAlH_4$ gave the desired alcohol **4** (*Scheme 1*). Compound **4** was acylated with di(pyridin-2-yl) thionocarbonate (DPTC) in the presence of cinnamic acid analogs, crotonic acid analogs, and PhCOOH to yield a series of 19-*O*-acylated derivatives, **5**–**14** (*Scheme 2*).

Scheme 1



i) $NaIO_4$, KOH, H_2O; 26%. *ii*) TMS–CHN_2, benzene, MeOH; 96%. *iii*) $LiAlH_4$, Et_2O; 77%.

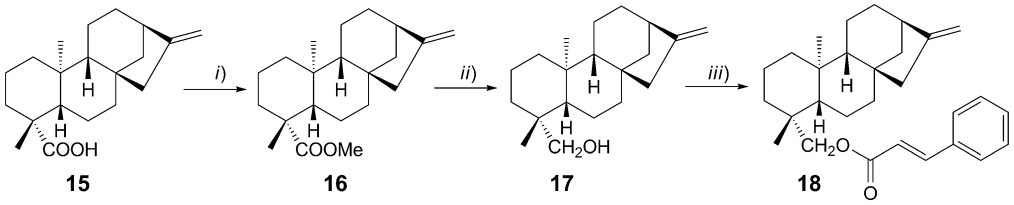
Scheme 2



i) RCOOH, di(pyridin-2-yl)thionocarbonate (DPTC), 4-(dimethylamino)pyridine (DMAP), toluene; 19–70% (for **5**–**12**, **14**). *ii*) Benzyl chloride (BnCl)/pyridine; 9% (**13**).

As will be discussed below, *ent*-kaur-16-ene-13,19-diol 19-*O*-cinnamate (**5**), a steviol derivative, exhibited potent cytotoxicity against three cancer cell lines, A549, AZ521, and SK-BR-3. To clarify the efficacy of the 13-OH group on the cytotoxicity, we prepared *ent*-kaur-16-en-19-ol 19-*O*-cinnamate (**18**), the 13-deoxy analog of **5**, from ent-kaur-16-en-19-oic acid (**15**), which has been isolated previously from the pollen of *Helianthus annuus* [21]. Thus, as shown in *Scheme 3*, compound **15** was converted to its methyl ester with TMS–CHN_2 (→**16**) and then reduced with $LiAlH_4$ to yield **17**. The alcohol **17** was acylated with cinnamic acid to yield the cinnamate **18**.

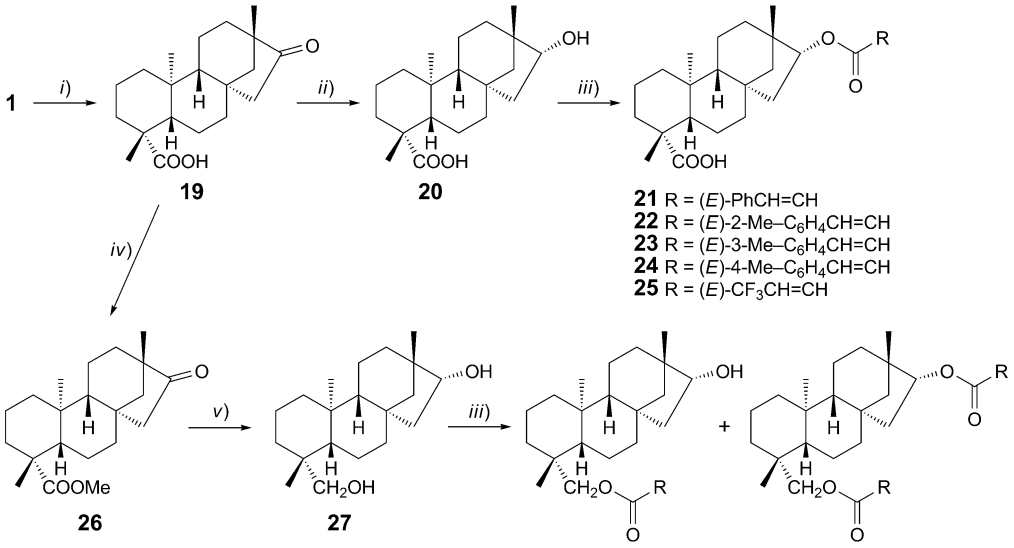
Scheme 3



i) TMS–CHN_2, benzene, MeOH; 97%. *ii*) $LiAlH_4$, Et_2O; 88%. *iii*) Cinnamic acid, DPTC, DMAP, toluene; 32%.

Preparation of Isosteviol Derivatives. Isosteviol (**19**) was prepared by the treatment of **1** with HCl. Eighteen derivatives, *i.e.*, **20**–**37**, were prepared from **19** as outlined in *Scheme 4*. The C(16)=O group of **19** was reduced with $NaBH_4$ to afford the alcohol **20** [22]. Compound **20** was then acylated with five carboxylic acids, *i.e.*, cinnamic acid, 2-, 3-, and 4-methylcinnamic acids, and 4,4,4-trifluorocrotonic acid, to yield the corresponding acyl derivatives **21**–**25**, respectively. In addition, **19** was converted to its (→ **26**) methyl ester and reduced with $LiAlH_4$ to afford **27**. The alcohol **27** was acylated with five carboxylic acids to yield the mixtures of 16-*O*-monoacylated and 16-*O*,19-*O*-

Scheme 4



i) HCl, H_2O; 98%. *ii*) $NaBH_4$, benzene, MeOH; 39%. *iii*) RCOOH, DPTC, DMAP, toluene; 14–88%. *iv*) TMS–CHN_2, benzene, MeOH; 99%. *v*) $LiAlH_4$, Et_2O; 82%.

diacylated derivatives. Each mixture of acylated derivatives was separated by TLC on SiO_2 to afford **28**–**37**. Their structures were confirmed by ^{1}H-NMR, high-resolution (HR) mass, and IR spectra.

Cytotoxic Activity. The cytotoxic activities of compounds **2**–**37** against leukemia (HL60), lung (A549), stomach (AZ521), and breast (SK-BR-3) cancer cell lines were determined by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-3*H*-tetrazolium bromide (MTT) assay. Cisplatin, which is one of the most effective and widely used chemotherapeutic drugs employed in the treatment of human cancers, was selected as a positive control. The IC_{50} values were used to determine the growth inhibition in the presence of test compounds, and the results are compiled in the *Table*.

Nine steviol, *i.e.*, **5**–**9** and **11**–**14**, and five isosteviol derivatives, *i.e.*, **28**–**32**, exhibited potent activities with single-digit micromolar IC_{50} values against one or more cell lines. Among the steviol derivatives, whereas the 19-OH compound **4** exhibited no activity, its 19-*O*-acyl derivatives exhibited cytotoxicities against four cancer cell lines. The cytotoxicities of Me cinnamates **6**–**8** were almost same as that of cinnamate **5**, which implied that Me substitution of the Ph group of cinnamate **5** has no influence on the activity. Me or CN substitution at the prop-2-enoyl C=C bond, and hydrogenation of the prop-2-enoyl C=C bond of cinnamate **5** decreased the activity against A549, AZ521, and SK-BR-3 (compounds **9**, **10**, and **12** *vs.* **5**). On the other hand, replacement of the Ph group of cinnamate **5** by a CF_3 group, leading to compound **14**, enhanced cytotoxicity substantially (IC_{50} 2.9, 4.1, 1.7, and 1.2 μM against HL60, A549, AZ521 and SK-BR-3, resp.). Furthermore, since the cinnamate **18**, prepared from *ent*-kaur-16-en-19-oic acid (**15**), exhibited no cytotoxicity against four cancer cell lines, the OH group at C(13) seems to play an important role for the 19-*O*-acylated kaurane-type diterpenoids to exhibit potent cytotoxicity against the cancer cell lines used in this study.

Among the isosteviol derivatives, acylation of the 16-hydroxybeyerane **20** (→**21**–**25**) increased cytotoxicity moderately against HL60, AZ521, and SK-BR-3 cells. On the other hand, acylation of the 16,19-dihydroxybeyerane **27** was effective only in the case of the 19-*O*-monocinnamoylation, which yielded **28** (IC_{50} 8.2, 1.2, and 2.4 μM against A549, AZ521, and SK-BR-3, resp.), for the enhancement of cytotoxicity. As has been observed on the steviol derivatives, the 19-*O*-trifluorocrotonyl derivative isosteviol **32** exhibited potent cytotoxicity (IC_{50} 6.6, 5.7, and 4.2 μM against HL60, A549, and AZ521, resp.).

Apoptosis-Inducing Activity of Compound **14**. Compound **14**, the potent cytotoxic compound against four human cancer cell lines, was then evaluated for its induction of early apoptosis in HL60 cells. Exposure of the membrane phospholipids, phosphatidylserine, to the external cellular environment is one of the earliest markers of apoptotic cell death [23]. Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the cell surface. Propidium iodide (PI) does not enter whole cells with intact membranes, and was used to differentiate between early apoptotic (annexin V positive, PI negative), late apoptotic (annexine V, PI double positive), or necrotic cell death (annexin V negative, PI positive). The ratio of early apoptotic cells (*Fig.*, lower right) was increased after treatment with 6 μM of **14** in HL60 for 24 h (from 4.4 to 33.7%), and that of late apoptotic cells (upper right) was increased after 24 h (from 1.0 to 33.7%) and 48 h

Table. *Cytotoxic Activities* ($IC_{50}\pm$S. D. [μM]) *of Steviol and Isosteviol Derivatives* **2**–**37** *against Four Human Cancer Cell Lines*[a])

Compound	HL60 (Leukemia)	A549 (Lung)	AZ521 (Stomach)	SK-BR-3 (Breast)
2	>100	>100	>100	>100
3	>100	>100	>100	>100
4	>100	>100	>100	>100
5	64.2±1.1	10.5±1.0	9.0±0.1	2.4±0.2
6	5.3±2.3	7.2±1.0	3.1±0.2	1.7±0.3
7	15.9±4.4	6.0±2.0	2.9±0.2	4.7±0.8
8	8.8±0.5	2.9±0.6	1.1±0.1	2.2±0.4
9	56.2±4.3	17.0±3.2	29.0±2.7	9.4±1.2
10	27.7±0.3	>100	>100	37.6±3.8
11	>100	2.5±0.4	27.2±3.7	1.6±0.1
12	12.9±0.2	22.1±5.2	38.0±1.0	5.8±0.5
13	41.3±0.4	4.3±2.0	2.5±1.0	2.2±0.8
14	2.9±0.4	4.1±1.2	1.7±1.4	1.2±0.1
15	53.7±5.6	>100	>100	11.0±1.0
16	80.1±4.3	48.9±5.8	47.4±1.8	15.5±0.6
17	31.3±3.4	14.7±3.6	63.0±3.9	16.4±4.0
18	>100	>100	>100	>100
19	75.0±4.7	>100	>100	>100
20	>100	>100	>100	>100
21	22.7±5.7	>100	77.6±4.1	15.2±2.7
22	81.6±5.0	>100	27.1±1.2	72.6±4.6
23	57.5±4.6	>100	91.0±1.7	31.0±3.6
24	32.8±6.6	>100	12.0±1.2	20.6±4.3
25	71.5±5.7	38.7±1.7	74.6±2.8	15.8±3.1
26	>100	>100	>100	>100
27	>100	>100	>100	>100
28	24.0±2.7	8.2±6.3	1.2±0.3	2.4±0.7
29	64.4±3.6	>100	9.0±2.1	25.0±4.2
30	20.3±2.5	>100	19.8±2.2	2.9±0.6
31	13.2±4.9	20.9±3.3	2.8±0.8	3.9±0.8
32	6.6±2.1	5.7±1.6	4.2±0.6	25.2±0.5
33	>100	>100	>100	>100
34	>100	>100	47.8±3.7	>100
35	>100	>100	>100	>100
36	>100	>100	>100	>100
37	82.8±1.6	>100	39.4±5.3	23.1±1.4
Cisplatin[b])	1.9±0.3	24.9±4.3	7.9±0.6	13.7±1.7

[a]) Cells were treated with compounds (1×10^{-4}–1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. IC_{50} Values based on triplicate experiments. [b]) Reference compound.

(58.0%; *Fig.*). A small number of necrotic cells (upper left) also were detected after 24 h (from 0.4 to 4.6%). These results revealed that most of the cytotoxicity of **14** to HL60 cells is due to inducing apoptotic cell death.

Conclusions. – In summary, 36 derivatives, *i.e.*, **2**–**37**, were prepared from stevioside **1**, and their cytotoxic activities were evaluated against leukemia (HL60), lung (A549),

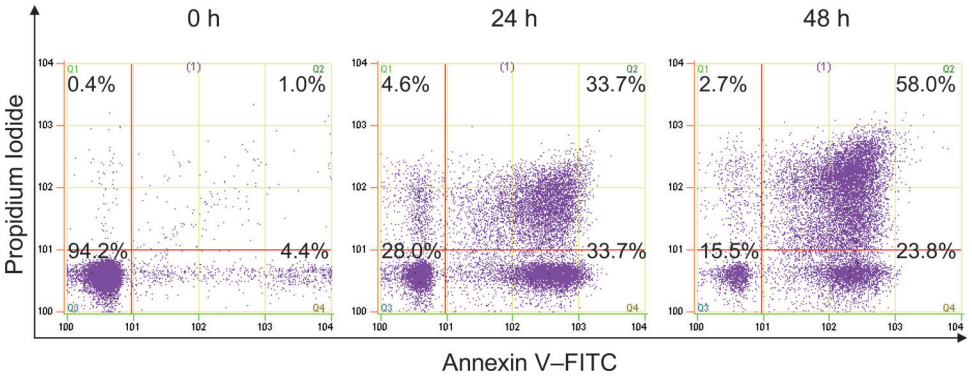


Figure. *Detection of* **14***-induced early and late apoptotic cells by annexin V/propidium iodide double staining in HL60 cell.* The HL60 cells were cultured with 6 μM of **14** for 24 and 48 h. Each value is the mean of three experiments.

stomach (AZ521), and breast (SK-BR-3) cancer cell lines. Among the derivatives, the 19-*O*-acylated derivatives, especially *ent*-kaur-16-ene-13,19-diol 19-*O*-4′,4′,4′-trifluorocrotonate (**14**), exhibited potent cytotoxicity against the cancer cell lines used. Furthermore, **14** was revealed to induce apoptotic cell death in HL60 cells. These results suggested that acylation of the 19-OH group of kaurane- and beyerane-type diterpenoids might be useful for the enhancement of their cytotoxicities with apoptosis-inducing activity. The results of this study will be of value for further utilization of stevioside in the product application in pharmaceutical field in the future.

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Experimental Part

General. Column chromatography (CC): silica gel *60* (SiO_2, 230–400 mesh, *Merck & Co. Inc.,* D-Darmstadt). TLC: silica gel *60G* (for TLC, *Merck & Co. Inc.*). LC: Reversed-phase (RP) prep. high-performance liquid chromatography (HPLC): octadecyl silica column (*Pegasil ODS II* column, 25 cm × 10 mm i.d.; *Senshu Scientific Co., Ltd.*, Tokyo, Japan) at 25° with MeOH/H_2O 19:1 (flow rate, 2.0 ml/min). Crystallizations were performed in MeOH. M.p.: *Yanagimoto Micro Mp* apparatus; uncorrected. IR Spectra: *Perkin-Elmer Spectrum One* FT-IR spectrophotometer in KBr disks; $\tilde{\nu}$ in cm^{-1}. ^{1}H-NMR Spectra: *JEOL ECX-400* spectrometer, $CDCl_3$; δ in ppm, *J* in Hz. HR-ESI-MS: *Agilent 1100 LC/MSD TOF* (time-of-flight) system (ionization mode, positive or negative; cap. voltage, 3000 V; fragmentor voltage, 225 V). HR-APCI-MS: *Agilent 1100 LC/MSD TOF* system (ionization mode, positive; cap. voltage, 3000 V; fragmentor voltage, 40 V; corona current, 2.0 μA). Microplate reader: *Sunrise-Basic* (*Tecan Japan Co., Ltd.*, Kawasaki, Japan).

Chemicals and Materials. Compounds were purchased as follows: MTT, *Dulbecco*'s modified *Eagle*'s medium (D-MEM), *Eagle*'s minimal essential medium (MEM), and DMSO from *Sigma-Aldrich Japan Co.* (Tokyo, Japan), stevioside, carboxylic acids, TMS–CHN_2 (0.6 mol/l hexane soln.), 4-(dimethylamino)pyridine (DMAP), DPTC, $NaBH_4$, and $LiAlH_4$ from *Tokyo Chemical Industry Co., Ltd.* (Tokyo, Japan), *RPMI-1640* medium, fetal bovine serum (FBS), antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), and non-essential amino acid (NEAA) from *Invitrogen Co.* (Auckland, NZ), *rh annexin V/FITC* kit (*Bender MedSystems*®) from *Cosmo Bio Co. Ltd.* (Tokyo, Japan), and cisplatin from *Wako Pure Chemical Industries Ltd.* (Osaka, Japan). All other chemicals and reagents were of anal. grade. ent-

Kaur-16-en-19-oic acid (= *(5β,8α,9β,10α,13α)-kaur-16-en-18-oic acid*; **15**) was isolated from the pollen of *Helianthus annuus* L. [21].

Cell Cultures. Cell lines HL60 (leukemia), A549 (lung), AZ521 (stomach), and SK-BR-3 (breast) were obtained from *Riken Cell Bank* (Tsukuba, Ibaraki, Japan). Two cell lines, HL60 and SK-BR-3, were grown in *RPMI-1640* medium, while A549 and AZ521 cell lines were grown in D-MEM and in 90% D-MEM + 10% MEM + 0.1 mM NEAA, resp. The medium was supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were incubataed at 37° in a 5% CO_2 humidified incubator.

Preparation of Steviol Derivatives. Steviol (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-oic Acid*; **2**). To a soln. of *stevioside* (**1**; 4.1 g, 5.1 mmol) in H_2O (300 ml), $NaIO_4$ (5.9 g, 27.6 mmol) was added and stirred at r. t. for 16 h. Then, to the mixture was added KOH (3.0 g, 53.6 mmol) and refluxed for 1 h. H_2O (300 ml) was added and pH was adjusted to 3–4 with AcOH; then the mixture was extracted with AcOEt (2 × 200 ml). The AcOEt fraction was washed with H_2O (2 ×), dried (Na_2SO_4), and evaporated under reduced pressure to yield **2** (415 mg, 26%), which was identified by spectral comparison with literature data [19].

Steviol Methyl Ester (= *Methyl (5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-oate*; **3**). To a soln. of **2** (202 mg, 0.6 mmol) in dried benzene (20 ml) and MeOH (10 ml), TMS–CHN_2 (1.2 ml) was added and stirred at r.t. for 1 h. Then, the mixture was evaporated to give **3** (203 mg, 96%), which was identified by spectral comparison with literature data [24].

ent-*Kaur-16-ene-13,19-diol* (= *(5β,8α,9β,10α,13α)-Kaur-16-ene-13,18-diol*; **4**). To a soln. of **3** (204 mg, 0.6 mmol) in dried Et_2O (20 ml), $LiAlH_4$ (126 mg, 3.3 mmol) was added, and the mixture was refluxed for 6 h under N_2. To the mixture was added 1M HCl (5 ml) and H_2O (5 ml), and stirred for a few min, then extracted with Et_2O (2 × 20 ml). The Et_2O extract was washed with aq. $NaHCO_3$ and H_2O, dried (Na_2SO_4), and concentrated under reduced pressure to furnish **4** (143 mg, 77%), which was identified by spectral comparison with literature data [25].

ent-*Kaur-16-ene-13,19-diol 19-*O*-Cinnamate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-3-Phenylprop-2-enoate*; **5**). To the soln. of **4** (22 mg, 0.07 mmol) in dried toluene (1 ml), DMAP (20 mg, 0.2 mmol), DPTC (30 mg, 0.1 mmol), and cinnamic acid (26 mg, 0.2 mmol) were added. After 24 h, H_2O was added (30 ml), and the mixture was extracted with Et_2O (2 × 30 ml). The Et_2O fraction was washed with H_2O (2 ×), dried (Na_2SO_4), and evaporated under reduced pressure to afford a mixture (36 mg), which was subjected to TLC (hexane/AcOEt 7:3) to furnish **5** (14 mg, 45%). White solid. M.p. 119–123°. IR (KBr): 3403, 2929, 2854, 1709, 1663, 1612, 1450, 1330, 1271, 1181, 1091. ^{1}H-NMR (400 MHz): 1.02 (*s*, 3 H); 1.07 (*s*, 3 H); 4.01 (*d*, *J* = 11.4, 1 H); 4.37 (*d*, *J* = 11.4, 1 H); 4.82 (br. *s*, 1 H); 4.98 (br. *s*, 1 H); 6.43 (*d*, *J* = 16.0, 1 H); 7.37–7.39 (*m*, 3 H); 7.52–7.54 (*m*, 2 H); 7.66 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.-ion mode): 457.2707 ($[M+Na]^+$, $C_{29}H_{38}NaO_3^+$; calc. 457.2719).

Twenty-five acyl derivatives, *i.e.*, **6**–**14**, **18**, **21**–**25**, and **28**–**37**, described below were prepared from the corresponding alcohols by similar ways as described above for the preparation of **5**.

ent-*Kaur-16-ene-13,19-diol 19-*O*-2-Methylcinnamate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-3-(2-Methylphenyl)prop-2-enoate*; **6**). Treatment of **4** (31 mg, 0.1 mmol) with 2-methylcinnamic acid (35 mg, 0.2 mmol) gave **6** (16 mg, 35%). White solid. M.p. 151–155°. IR (KBr): 3487, 2929, 2858, 1694, 1634, 1600, 1458, 1392, 1323, 1187, 1106. ^{1}H-NMR (400 MHz): 1.02 (*s*, 3 H); 1.08 (*s*, 3 H); 2.43 (*s*, 3 H); 4.04 (*d*, *J* = 11.0, 1 H); 4.35 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.98 (br. *s*, 1 H); 6.35 (*d*, *J* = 16.0, 1 H); 7.19 (*d*, *J* = 7.3, 1 H); 7.20 (*t*, *J* = 7.3, 1 H); 7.25–7.29 (*m*, 1 H); 7.56 (*d*, *J* = 7.8, 1 H); 7.97 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 471.2867 ($[M+Na]^+$, $C_{30}H_{40}NaO_3^+$; calc. 471.2875).

ent-*Kaur-16-ene-13,19-diol 19-*O*-3-Methylcinnamate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-3-(3-Methylphenyl)prop-2-enoate*; **7**). Ttreatment of **4** (40 mg, 0.1 mmol) with 3-methylcinnamic acid (46 mg, 0.3 mmol) yielded **7** (18 mg, 30%). White solid. M.p. 149–155°. IR (KBr): 3401, 2928, 2849, 1709, 1639, 1605, 1446, 1368, 1320, 1273, 1236, 1179, 1091. ^{1}H-NMR (400 MHz): 1.02 (*s*, 3 H); 1.07 (*s*, 3 H); 2.37 (*s*, 3 H); 4.01 (*d*, *J* = 11.0, 1 H); 4.36 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.98 (br. *s*, 1 H); 6.42 (*d*, *J* = 16.0, 1 H); 7.19 (br. *d*, *J* = 7.3, 1 H); 7.27 (br. *t*, *J* = 7.8, 1 H); 7.33 (br. *d*, *J* = 6.9, 1 H); 7.34 (*s*, 1 H); 7.63 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 471.2885 ($[M+Na]^+$, $C_{30}H_{40}NaO_3^+$; calc. 471.2875).

ent-*Kaur-16-ene-13,19-diol 19-*O*-4-Methylcinnamate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-3-(4-Methylphenyl)prop-2-enoate*; **8**). Treatment of **4** (31 mg, 0.1 mmol) with 4-methylcin-

namic acid (32 mg, 0.2 mmol) furnished **8** (17 mg, 37%). White solid. M.p. 145–150°. IR (KBr): 3393, 2925, 2853, 1708, 1637, 1608, 1457, 1386, 1309, 1272, 1179. ^{1}H-NMR (400 MHz): 1.01 (*s*, 3 H); 1.07 (*s*, 3 H); 2.37 (*s*, 3 H); 4.00 (*d*, *J* = 11.0, 1 H); 4.36 (*d*, *J* = 11.0, 1 H); 4.81 (br. *s*, 1 H); 4.98 (br. *s*, 1 H); 6.38 (*d*, *J* = 16.0, 1 H); 7.18 (*d*, *J* = 8.2, 2 H); 7.42 (*d*, *J* = 8.2, 2 H); 7.63 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 471.2881 ($[M+Na]^+$, $C_{30}H_{40}NaO_3^+$; calc. 471.2875).

ent-*Kaur-16-ene-13,19-diol 19*-O-*α-Methylcinnamate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-2-Methyl-3-phenylprop-2-enoate*; **9**). Treatment of **4** (38 mg, 0.1 mmol) with α-methylcinnamic acid (39 mg, 0.2 mmol) afforded **9** (39 mg, 70%). Amorphous solid. IR (KBr): 3339, 2925, 2849, 2627, 1706, 1682, 1634, 1575, 1449, 1261, 1203, 1122. ^{1}H-NMR (400 MHz): 1.04 (*s*, 3 H); 1.08 (*s*, 3 H); 2.13 (br. *s*, 3 H); 4.00 (*d*, *J* = 11.0, 1 H); 4.37 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.99 (br. *s*, 1 H); 7.31–7.35 (*m*, 3 H); 7.39–7.43 (*m*, 2 H); 7.67 (br. *s*, 1 H). HR-ESI-MS (pos.): 471.2853 ($[M+Na]^+$, $C_{30}H_{40}NaO_3^+$; calc. 471.2875).

ent-*Kaur-16-ene-13,19-diol 19*-O-*2-Cyanocinnamate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-2-Cyano-3- phenylprop-2-enoate*; **10**). Treatment of **4** (50 mg, 0.2 mmol) with 2-cyano-3-phenylacrylic acid (50 mg, 0.3 mmol) yielded **10** (14 mg, 19%). Amorphous solid. IR (KBr): 3414, 2927, 2851, 2223, 1727, 1606, 1450, 1270, 1205, 1189, 1108, 1092. ^{1}H-NMR (400 MHz): 1.07 (*s*, 3 H); 1.08 (*s*, 3 H); 4.12 (*d*, *J* = 11.0, 1 H); 4.47 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.99 (br. *s*, 1 H); 7.49–7.53 (*m*, 2 H); 7.55–7.57 (*m*, 1 H); 8.00 (br. *d*, *J* = 7.3, 2 H); 8.24 (*s*, 1 H). HR-APCI-MS (pos.): 460.2878 ($[M+H]^+$, $C_{30}H_{38}NO_3^+$; calc. 460.2852).

ent-*Kaur-16-ene-13,19-diol 19*-O-*3-(Pyridin-3-yl)acrylate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-3-(Pyridin-3-yl)prop-2-enoate*; **11**). Treatment of **4** (20 mg, 0.07 mmol) with 3-(pyridin-3-yl)acrylic acid (20 mg, 0.1 mmol) afforded **11** (17 mg, 59%)). White solid. M.p. 132–138°. IR (KBr): 3352, 2924, 2853, 1710, 1646, 1613, 1587, 1457, 1419, 1288, 1193, 1090, 1025. ^{1}H-NMR (400 MHz): 1.02 (*s*, 3 H); 1.07 (*s*, 3 H); 4.03 (*d*, *J* = 11.0, 1 H); 4.39 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.99 (br. *s*, 1 H); 6.51 (*d*, *J* = 16.1, 1 H); 7.35 (*dd*, *J* = 4.9, 7.7, 1 H); 7.65 (*d*, *J* = 16.1, 1 H); 7.86 (*dt*, *J* = 1.5, 7.7, 1 H); 8.61 (*dd*, *J* = 1.5, 4.9, 1 H); 8.76 (*d*, *J* = 1.5, 1 H). HR-APCI-MS (pos.): 436.2879 ($[M+H]^+$, $C_{28}H_{38}NO_3^+$; calc. 436.2852).

ent-*Kaur-16-ene-13,19-diol 19*-O-*3-Phenylpropionate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl 3-Phenylpropanoate*; **12**). Treatment of **4** (31 mg, 0.1 mmol) with 3-phenylpropanoic acid (34 mg, 0.2 mmol) afforded **12** (25 mg, 56%). Amorphous solid. IR (KBr): 3524, 2931, 2853, 1724, 1664, 1444, 1394, 1373, 1292, 1248, 1176, 1106. ^{1}H-NMR (400 MHz): 0.90 (*s*, 3 H); 1.01 (*s*, 3 H); 2.63 (*t*, *J* = 7.8, 2 H); 2.95 (*t*, *J* = 7.8, 2 H); 3.86 (*d*, *J* = 11.0, 1 H); 4.22 (*d*, *J* = 11.0, 1 H); 4.81 (br. *s*, 1 H); 4.97 (br. *s*, 1 H); 7.18–7.21 (*m*, 3 H); 7.26–7.28 (*m*, 2 H). HR-ESI-MS (pos.): 459.2854 ($[M+Na]^+$, $C_{29}H_{40}NaO_3^+$; calc. 459.2875).

ent-*Kaur-16-ene-13,19-diol 19*-O-*Benzoate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl Benzoate*; **13**). To a soln. of **4** (50 mg, 0.2 mmol) in dried pyridine (5 ml), PhCOCl (54mg, 0.4 mmol) was added. After 24 h, H_2O (30 ml) was added, and the mixture was extracted with Et_2O (2 × 30 ml). The Et_2O fraction was washed with H_2O (2 ×), dried (Na_2SO_4), and evaporated under reduced pressure to give a mixture (139 mg), which was subjected to TLC (hexane/AcOEt 7:3) and HPLC (t_R 10.4 min) to furnish **13** (6 mg, 9%). White solid. M.p. 117–122°. IR (KBr): 3339, 2928, 2850, 1715, 1663, 1603, 1451, 1282, 1121. ^{1}H-NMR (400 MHz): 1.08 (*s*, 3 H); 1.09 (*s*, 3 H); 4.12 (*d*, *J* = 11.0, 1 H); 4.49 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.99 (br. *s*, 1 H); 7.45 (br. *t*, *J* = 7.8, 2 H); 7.56 (br. *t*, *J* = 7.4, 1 H); 8.04 (br. *d*, *J* = 7.1, 2 H). HR-ESI-MS (pos.): 431.2554 ($[M+Na]^+$, $C_{27}H_{36}NaO_3^+$; calc. 431.2562).

ent-*Kaur-16-ene-13,19-diol 19*-O-*4′,4′,4′-Trifluorocrotonate* (= *(5β,8α,9β,10α,13α)-13-Hydroxykaur-16-en-18-yl (2*E*)-4,4,4-Trifluorobut-2-enoate*; **14**). Treatment of **4** (31 mg, 0.1 mmol) with 4,4,4-trifluorocrotonic acid (44 mg, 0.3 mmol) yielded **14** (16 mg, 37%). White solid. M.p. 95–99°. IR (KBr): 3401, 2929, 2854, 1731, 1458, 1330, 1268, 1136, 1120. ^{1}H-NMR (400 MHz): 0.98 (*s*, 3 H); 1.04 (*s*, 3 H); 4.01 (*d*, *J* = 11.0, 1 H); 4.39 (*d*, *J* = 11.0, 1 H); 4.82 (br. *s*, 1 H); 4.98 (br. *s*, 1 H); 6.49 (*dq*, *J* = 1.8, 15.6, 1 H); 6.75 (*dq*, *J* = 6.4, 15.6, 1 H). HR-APCI-MS (pos.): 409.2346 ($[M+H-H_2O]^+$, $C_{24}H_{32}F_3O_2^+$; calc. 409.2354).

Methyl ent-*Kaur-16-en-19-oate* (= *Methyl (5β,8α,9β,10α,13α)-Kaur-16-en-18-oate*; **16**). As described for the preparation. of **3**, treatment of **15** (119 mg, 0.4 mmol) with TMS–CHN_2 (1.2 ml, 0.7 mmol) gave **16** (121 mg, 97%), which was identified by spectral comparison with literature data [26].

ent-*Kaur-16-en-19-ol* (=*(5β,8α,9β,10α,13α)-Kaur-16-en-18-ol*; **17**). As described for the preparation of **4**, treatment of **16** (100 mg, 0.3 mmol) with $LiAlH_4$ (210 mg, 5.5 mmol) afforded **17** (80 mg, 88%), which was identified by spectral comparison with literature data [27].

ent-*Kaur-16-en-19-ol 19*-O-*Cinnamate* (=*(5β,8α,9β,10α,13α)-Kaur-16-en-18-yl (2*E*)-3-Phenylprop-2-enoate*; **18**). Treatment of **17** (52 mg, 0.2 mmol) with cinnamic acid (58 mg, 0.4 mmol) afforded **18** (24 mg, 32%). White solid. M.p. 133–145°. IR (KBr): 2923, 2869, 1717, 1660, 1642, 1449, 1383, 1328, 1288, 1254, 1205, 1178, 1035, 1008. ^{1}H-NMR (400 MHz): 1.02 (*s*, 3 H); 1.07 (*s*, 3 H); 4.02 (br. *d*, *J*=11.0, 1 H); 4.38 (*d*, *J*=11.0, 1 H); 4.74 (br. *s*, 1 H); 4.80 (br. *s*, 1 H); 6.44 (*d*, *J*=16.0, 1 H); 7.37–7.39 (*m*, 3 H); 7.52–7.54 (*m*, 2 H); 7.66 (*d*, *J*=16.0, 1 H). HR-ESI-MS (pos.): 441.2760 ($[M+Na]^+$, $C_{29}H_{38}NaO_2^+$; calc. 441.2769).

Isosteviol (=*16-Oxobeyeran-19-oic Acid*; **19**). To the soln. of **1** (8.0 g, 10.0 mmol) in H_2O (300 ml), 12M HCl (6 ml) was added, and the mixture was refluxed for 2 h. Then, the mixture was extracted with AcOEt (2 × 200 ml). The AcOEt fraction was washed with H_2O (2 ×), dried (Na_2SO_4), and evaporated under reduced pressure to yield **19** (3.1 g, 98%), which was identified by spectral comparison with literature data [19].

(16α)-16-Hydroxybeyeran-19-oic Acid (**20**). To a soln. of **19** (103 mg, 0.3 mmol) in benzene (5 ml) and MeOH (2.5 ml), $NaBH_4$ (219 mg, 5.8 mmol) was added, and the mixture was stirred at r.t. for 5 h. Then, 1M HCl (5 ml) was added, and the mixture was stirred for few min, then extracted with Et_2O (2 × 20 ml). The Et_2O fraction was washed with H_2O, dried (Na_2SO_4), and concentrated under reduced pressure to furnish **20** (40 mg, 39%), which was identified by spectral comparison with literature data [15][22].

(16α)-16-Hydroxybeyeran-19-oic Acid 16-O-*Cinnamate* (=*(16α)-16-{[(2*E*)-3-Phenylprop-2-enoyl]oxy}beyeran-19-oic Acid*; **21**). Treatment of **20** (30 mg, 0.1 mmol) with cinnamic acid (61 mg, 0.4 mmol) gave **21** (37 mg, 88%). White solid. M.p. 100–106°. IR (KBr): 2947, 2848, 1711, 1694, 1638, 1451, 1311, 1172. ^{1}H-NMR (400 MHz): 0.80 (*s*, 3 H); 0.95 (*s*, 3 H); 1.22 (*s*, 3 H); 4.86 (*dd*, *J*=5.0, 9.6, 1 H); 6.44 (*d*, *J*=16.0, 1 H); 7.35–7.36 (*m*, 3 H); 7.51–7.53 (*m*, 2 H); 7.64 (*d*, *J*=16.0, 1 H). HR-ESI-MS (pos.): 473.2677 ($[M+Na]^+$, $C_{29}H_{38}NaO_4^+$; calc. 473.2668).

(16α)-16-Hydroxybeyeran-19-oic Acid 16-O-*2-Methylcinnamate* (=*(16α)-16-{[(2*E*)-3-(2-Methylphenyl)prop-2-enoyl]oxy}beyeran-19-oic Acid*; **22**). Treatment of **20** (19 mg, 0.06 mmol) with 2-methylcinnamic acid (21 mg, 0.1 mmol) afforded **22** (9.2 mg, 33%). White solid. M.p. 89–95°. IR (KBr): 2926, 2851, 1714, 1635, 1456, 1313, 1262, 1172. ^{1}H-NMR (400 MHz): 0.81 (*s*, 3 H); 0.96 (*s*, 3 H); 1.23 (*s*, 3 H); 2.43 (*s*, 3 H); 4.87 (*dd*, *J*=5.5, 9.6, 1 H); 6.36 (*d*, *J*=15.8, 1 H); 7.17–7.20 (*m*, 2 H); 7.24–7.27 (*m*, 1 H); 7.56 (br. *d*, *J*=7.3, 1 H); 7.97 (*d*, *J*=15.8, 1 H). HR-ESI-MS (pos.): 487.2798 ($[M+Na]^+$, $C_{30}H_{40}NaO_4^+$; calc. 487.2824).

(16α)-16-Hydroxybeyeran-19-oic Acid 16-O-*3-Methylcinnamate* (=*(16α)-16-{[(2*E*)-3-(3-Methylphenyl)prop-2-enoyl]oxy}beyeran-19-oic Acid*; **23**). Treatment of **20** (21 mg, 0.07 mmol) with 3-methylcinnamic acid (21 mg, 0.1 mmol) furnished **23** (14 mg, 46%). White solid. M.p. 98–105°. IR (KBr): 2928, 2849, 1713, 1695, 1635, 1456, 1312, 1269, 1236, 1176. ^{1}H-NMR (400 MHz): 0.81 (*s*, 3 H); 0.95 (*s*, 3 H); 1.22 (*s*, 3 H); 2.36 (*s*, 3 H); 4.85 (*dd*, *J*=5.0, 9.6, 1 H); 6.43 (*d*, *J*=16.0, 1 H); 7.17 (br. *d*, *J*=7.8, 1 H); 7.26 (br. *t*, *J*=9.1, 1 H); 7.32 (br. *d*, *J*=8.7, 1 H); 7.34 (br. *s*, 1 H); 7.62 (*d*, *J*=16.0, 1 H). HR-ESI-MS (pos.): 487.2824 ($[M+Na]^+$, $C_{30}H_{40}NaO_4^+$; calc. 487.2824).

(16α)-16-Hydroxybeyeran-19-oic Acid 16-O-*4-Methylcinnamate* (=*(16α)-16-{[(2*E*)-3-(4-Methylphenyl)prop-2-enoyl]oxy}beyeran-19-oic Acid*; **24**). Treatment of **20** (20 mg, 0.06 mmol) with 4-methylcinnamic acid (22 mg, 0.1 mmol) afforded **24** (9 mg, 31%). White solid. M.p. 95–100°. IR (KBr): 2926, 2850, 1710, 1695, 1635, 1456, 1310, 1270, 1168. ^{1}H-NMR (400 MHz): 0.80 (*s*, 3 H); 0.95 (*s*, 3 H); 1.22 (*s*, 3 H); 2.37 (*s*, 3 H); 4.85 (*dd*, *J*=5.0, 9.6, 1 H); 6.40 (*d*, *J*=15.8, 1 H); 7.17 (*d*, *J*=7.8, 2 H); 7.42 (*d*, *J*=7.8, 2 H); 7.62 (*d*, *J*=15.8, 1 H). HR-ESI-MS (pos.): 487.2827 ($[M+Na]^+$, $C_{30}H_{40}NaO_4^+$; calc. 487.2824).

(16α)-16-Hydroxybeyeran-19-oic Acid 16-O-*Trifluorocrotonate* (=*(16α)-16-{[(2*E*)-4,4,4-Trifluorobut-2-enoyl]oxy}beyeran-19-oic acid*; **25**). Treatment of **20** (30 mg, 0.09 mmol) with 4,4,4-trifluorocrotonic acid (31 mg, 0.2 mmol) afforded **25** (11 mg, 27%). White solid. M.p. 90–96°. IR (KBr): 3448, 3020, 2929, 2852, 1724, 1693, 1666, 1519, 1467, 1440, 1308, 1261, 1215, 1137, 1052. ^{1}H-NMR (400 MHz): 0.79 (*s*,

3 H); 0.93 (*s*, 3 H); 1.22 (*s*, 3 H); 4.84 (*dd*, *J* = 5.0, 10.1, 1 H); 6.51 (*dq*, *J* = 1.8, 15.8, 1 H); 6.74 (*dq*, *J* = 6.4, 15.8, 1 H). HR-ESI-MS (neg.): 441.2235 ($[M-H]^-$, $C_{24}H_{32}O_4F_3^-$; calc. 441.2253).

Methyl Isosteviol (= *Methyl 16-Oxobeyeran-19-oate*; **26**). As described for the preparation of **3**, treatment of **19** (145 mg, 0.5 mmol) with TMS–CHN_2 (2 ml, 1.2 mmol) gave **26** (150 mg, 99%), which was identified by spectral comparison with literature data [19].

(16α)-Beyerane-16,19-diol (**27**). As described for the preparation of **4**, treatment of **26** (148 mg, 0.4 mmol) with $LiAlH_4$ (374 mg, 9.8 mmol) yielded **27** (112 mg, 82%), which was identified by spectral comparison with literature data [16].

16α,19-Dihydroxybeyerane 19-O-*Cinnamate* (= *(16α)-16-Hydroxybeyeran-19-yl (2*E*)-3-Phenyl-prop-2-enoate*; **28**) *and 16α,19-Dihydroxybeyerane Di*-O-*cinnamate* (= *(16α)-Beyerane-16,19-diyl (2*E*,2′*E*)-Bis(3-phenylprop-2-enoate)*; **33**). Treatment of **27** (32 mg, 0.1 mmol) with cinnamic acid (66 mg, 0.4 mmol) afforded a crude product (74 mg), which was subjected to TLC (hexane/AcOEt 7:3) to furnish **28** (22 mg, 48%) and **33** (16 mg, 27%).

Data for **28**. White solid. M.p. 135–140°. IR (KBr): 3436, 2931, 2845, 1704, 1640, 1450, 1387, 1284, 1253, 1205, 1182. ^{1}H-NMR (400 MHz): 0.91 (*s*, 3 H); 0.95 (*s*, 3 H); 1.01 (*s*, 3 H); 3.86 (*dd*, *J* = 4.6, 11.0, 1 H); 3.98 (*d*, *J* = 11.0, 1 H); 4.39 (*d*, *J* = 11.0, 1 H); 6.43 (br. *d*, *J* = 16.0, 1 H); 7.36–7.38 (*m*, 3 H); 7.51–7.53 (*m*, 2 H); 7.65 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 459.2879 ($[M+Na]^+$, $C_{29}H_{40}NaO_3^+$; calc. 459.2875).

Data for **33**. Amorphous solid. IR (KBr): 2929, 2849, 1711, 1638, 1450, 1310, 1203, 1173. ^{1}H-NMR (400 MHz): 0.94 (*s*, 3 H); 0.96 (*s*, 3 H); 1.02 (*s*, 3 H); 3.99 (*d*, *J* = 11.0, 1 H); 4.38 (*d*, *J* = 11.0, 1 H); 4.88 (*dd*, *J* = 5.0, 9.6, 1 H); 6.42 (br. *d*, *J* = 15.6, 1 H); 6.47 (br. *d*, *J* = 15.6, 1 H); 7.37–7.39 (*m*, 6 H); 7.51–7.56 (*m*, 4 H); 7.65 (*d*, *J* = 15.6, 1 H); 7.67 (*d*, *J* = 15.6, 1 H). HR-ESI-MS (pos.): 589.3299 ($[M+Na]^+$, $C_{38}H_{46}NaO_4^+$; calc. 589.3294).

16α,19-Dihydroxybeyerane 19-O-*2-Methylcinnamate* (= *(16α)-16-Hydroxybeyeran-19-yl (2*E*)-3-(2-Methylphenyl)prop-2-enoate*; **29**) *and 16α,19-Dihydroxybeyerane Di*-O-*2-methylcinnamate* (= *(16α)-Beyerane-16,19-diyl (2*E*,2′*E*)-Bis[3-(2-methylphenyl)prop-2-enoate]*; **34**). Treatment of **27** (31 mg, 0.1 mmol) with 2-methylcinnamic acid (61 mg, 0.4 mmol) gave **29** (8.7 mg, 19%) and **34** (9.3 mg, 15%).

Data for **29**. White solid. M.p. 133–142°. IR (KBr): 3488, 2929, 2847, 1700, 1632, 1458, 1321, 1276, 1180, 1163, 1001. ^{1}H-NMR (400 MHz): 0.91 (*s*, 3 H); 0.96 (*s*, 3 H); 1.01 (*s*, 3 H); 2.43 (*s*, 3 H); 3.86 (*dd*, *J* = 4.6, 10.5, 1 H); 4.00 (*d*, *J* = 11.0, 1 H); 4.38 (*d*, *J* = 11.0, 1 H); 6.34 (*d*, *J* = 15.6, 1 H); 7.18–7.22 (*m*, 2 H); 7.24–7.28 (*m*, 1 H); 7.56 (br. *d*, *J* = 7.3, 1 H); 7.96 (*d*, *J* = 15.6, 1 H). HR-ESI-MS (pos.): 473.3020 ($[M+Na]^+$, $C_{30}H_{42}NaO_3^+$; calc. 473.3032).

Data for **34**. Amorphous solid. IR (KBr): 2925, 2850, 1713, 1635, 1456, 1312, 1264, 1173, 1033. ^{1}H-NMR (400 MHz): 0.95 (*s*, 3 H); 0.98 (*s*, 3 H); 1.03 (*s*, 3 H); 2.44 (*s*, 3 H); 2.45 (*s*, 3 H); 4.01 (*d*, *J* = 11.0, 1 H); 4.37 (*d*, *J* = 11.0, 1 H); 4.90 (*dd*, *J* = 5.0, 10.1, 1 H); 6.35 (*d*, *J* = 16.0, 1 H); 6.39 (*d*, *J* = 16.0, 1 H); 7.12–7.22 (*m*, 6 H); 7.49 (*d*, *J* = 7.8, 1 H); 7.52 (*d*, *J* = 7.3, 1 H); 7.96 (*d*, *J* = 16.0, 1 H); 7.99 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 617.3606 ($[M+Na]^+$, $C_{40}H_{50}NaO_4^+$; calc. 617.3607).

16α,19-Dihydroxybeyerane 19-O-*3-Methycinnamate* (= *(16α)-16-Hydroxybeyeran-19-yl (2*E*)-3-(3-Methylphenyl)prop-2-enoate*; **30**) *and 16α,19-Dihydroxybeyerane Di*-O-*3-methylcinnamate* (= *(16α)-Beyerane-16,19-diyl (2*E*,2′*E*)-Bis[3-(3-methylphenyl)prop-2-enoate]*; **35**). Treatment of **27** (29 mg, 0.09 mmol) with 3-methylcinnamic acid (62 mg, 0.4 mmol) afforded **30** (19 mg, 45%) and **35** (13 mg, 23%).

Data for **30**. White solid. M.p. 125–132°. IR (KBr): 3436, 2930, 2846, 1706, 1628, 1452, 1309, 1284, 1253, 1205, 1181. ^{1}H-NMR (400 MHz): 0.91 (*s*, 3 H); 0.95 (*s*, 3 H); 1.01 (*s*, 3 H); 2.37 (*s*, 3 H); 3.86 (*dd*, *J* = 4.6, 10.5, 1 H); 3.97 (*d*, *J* = 11.0, 1 H); 4.38 (*d*, *J* = 11.0, 1 H); 6.41 (*d*, *J* = 16.0, 1 H); 7.18 (br. *d*, *J* = 6.9, 1 H); 7.27 (br. *t*, *J* = 7.8, 1 H); 7.33 (br. *d*, *J* = 6.9, 1 H); 7.34 (br. *s*, 1 H); 7.62 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 473.3016 ($[M+Na]^+$, $C_{30}H_{42}NaO_3^+$; calc. 473.3032).

Data for **35**. Amorphous solid. IR (KBr): 2926, 2850, 1714, 1635, 1456, 1372, 1312, 1265, 1236, 1176. ^{1}H-NMR (400 MHz): 0.93 (*s*, 3 H); 0.96 (*s*, 3 H); 1.01 (*s*, 3 H); 2.37 (*s*, 3 H); 2.38 (*s*, 3 H); 3.98 (*d*, *J* = 11.0, 1 H); 4.37 (*d*, *J* = 11.0, 1 H); 4.87 (*dd*, *J* = 5.5, 9.6, 1 H); 6.40 (*d*, *J* = 16.0, 1 H); 6.45 (*d*, *J* = 16.0, 1 H); 7.18–7.20 (*m*, 2 H); 7.25–7.27 (*m*, 2 H); 7.32–7.36 (*m*, 4 H); 7.62 (*d*, *J* = 16.0, 1 H); 7.63 (*d*, *J* = 16.0, 1 H). HR-ESI-MS (pos.): 617.3580 ($[M+Na]^+$, $C_{40}H_{50}NaO_4^+$; calc. 617.3607).

16α,19-Dihydroxybeyerane 19-O-*4-Methycinnamate* (=*(16α)-16-Hydroxybeyeran-19-yl (2*E*)-3-(4-Methylphenyl)prop-2-enoate*; **31**) *and 16α,19-Dihydroxybeyerane Di*-O-*4-methylcinnamate* (=*(16α)-Beyerane-16,19-diyl (2*E,*2′*E*)-Bis[3-(4-methylphenyl)prop-2-enoate]*; **36**). Treatment of **27** (30 mg, 0.1 mmol) with 4-methylcinnamic acid (61 mg, 0.4 mmol) gave **31** (19 mg, 43%) and **36** (9 mg, 15%).

Data for **31**. White solid. M.p. 113–118°. IR (KBr): 3421, 2932, 2843, 1705, 1636, 1453, 1254, 1181, 1026. ^{1}H-NMR (400 MHz): 0.91 (*s*, 3 H); 0.95 (*s*, 3 H); 1.00 (*s*, 3 H); 2.37 (*s*, 3 H); 3.86 (*dd*, *J*=4.6, 10.5, 1 H); 3.97 (*d*, *J*=11.0, 1 H); 4.38 (*d*, *J*=11.0, 1 H); 6.38 (*d*, *J*=16.0, 1 H); 7.18 (*d*, *J*=8.0, 2 H); 7.42 (*d*, *J*=8.0, 2 H); 7.62 (*d*, *J*=16.0, 1 H). HR-ESI-MS (pos.): 473.3011 ($[M+Na]^+$, $C_{30}H_{42}NaO_3^+$; calc. 473.3032).

Data for **36**. Amorphous solid. IR (KBr): 2925, 2850, 1714, 1635, 1456, 1310, 1257, 1168. ^{1}H-NMR (400 MHz): 0.94 (*s*, 3 H); 0.96 (*s*, 3 H); 1.02 (*s*, 3 H); 2.37 (*s*, 3 H); 2.38 (*s*, 3 H); 3.98 (*d*, *J*=11.0, 1 H); 4.38 (*d*, *J*=11.0, 1 H); 4.88 (*dd*, *J*=5.0, 9.6, 1 H); 6.38 (*d*, *J*=16.0, 1 H); 6.43 (*d*, *J*=16.0, 1 H); 7.18 (*d*, *J*=7.7, 2 H); 7.19 (*d*, *J*=8.2, 2 H); 7.43 (*d*, *J*=8.2, 2 H); 7.45 (*d*, *J*=7.7, 2 H); 7.63 (*d*, *J*=16.0, 1 H); 7.64 (*d*, *J*=16.0, 1 H). HR-ESI-MS (pos.): 617.3564 ($[M+Na]^+$, $C_{40}H_{50}NaO_4^+$; calc. 617.3607).

16α,19-Dihydroxybeyerane 19-O-*Trifluorocrotonate* (=*(16α)-16-Hydroxybeyeran-19-yl (2*E*)-4,4,4-Trifluorobut-2-enoate*; **32**) *and 16α,19-Dihydroxybeyerane Di*-O-*trifluorocrotonate* (=*(16α)-Beyerane-16,19-diyl (2*E,*2′*E*)-Bis(4,4,4-trifluorobut-2-enoate)*; **37**). Treatment of **27** (31 mg, 0.1 mmol) with 4,4,4-trifluorocrotonic acid (65 mg, 0.5 mmol) yielded **32** (18 mg, 41%) and **37** (8 mg, 14%).

Data for **32**. White solid. M.p. 112–119°. IR (KBr): 3443, 3019, 2929, 2851, 1727, 1667, 1455, 1391, 1372, 1308, 1261, 1215, 1138, 1053. ^{1}H-NMR (400 MHz): 0.91 (*s*, 3 H); 0.93 (*s*, 3 H); 0.97 (*s*, 3 H); 3.86 (*dd*, *J*=4.6, 10.5, 1 H); 3.98 (*d*, *J*=11.0, 1 H); 4.41 (*d*, *J*=11.0, 1 H); 6.49 (*dq*, *J*=1.8, 15.6, 1 H); 6.74 (*dq*, *J*=6.4, 15.6, 1 H). HR-APCI-MS (pos.): 411.2551 ($[M+H-H_2O]^+$, $C_{24}H_{34}F_3O_2^+$; calc. 411.2511).

Data of **37**. Amorphous solid. IR (KBr): 3020, 2928, 2854, 1726, 1667, 1458, 1442, 1371, 1308, 1262, 1215, 1139, 1050. ^{1}H-NMR (400 MHz): 0.89 (s, 3 H); 0.94 (*s*, 3 H); 0.98 (*s*, 3 H); 3.98 (*d*, *J*=11.0, 1 H); 4.38 (*d*, *J*=11.0, 1 H); 4.86 (*t*, *J*=7.5, 1 H); 6.46–6.55 (*m*, 2 H); 6.70–6.80 (*m*, 2 H). HR-ESI-MS (pos.): 573.2412 ($[M+Na]^+$, $C_{28}H_{36}F_6NaO_4^+$; calc. 573.2415).

Cytotoxicity Assay. Cytotoxicity assay was performed according to the method described in [28]. Briefly, HL60 (leukemia), A549 (lung), AZ521 (stomach), and SK-BR-3 (breast) cell lines (each 3×10^3 cells/well) were treated with compounds for 48 h, and then MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) soln. was added to the well. After incubation for 3 h, the generated blue formazan was solubilized with 0.04M HCl in ^{i}PrOH. The absorbances at 570 nm (top) and 630 nm (bottom) were measured with a microplate reader.

Annexin V/Propidium Iodide Double Staining. Apotosis was detected using an *rh Annexin V/FITC* kit [29], and HL60 (1.5×10^5 cells) was exposed to test compound (final concentration: 6 μM). To prepare the cell sample for flow cytometry, cells were washed with annexin-binding buffer and stained with annexin V–fluorecein isothiocyanate (FITC) and propidium iodide (PI) for 10 min. The cell samples were analyzed by flow cytometer (*Cell Lab Quanta SC*; *Beckman Coulter K. K.*, Tokyo, Japan) using the FL1 and FL2 ranges for annexin V-FITC and PI, resp.

REFERENCES

[1] D. D. Soejarto, A. D. Kinghorn, N. R. Farnsworth, *J. Nat. Prod.* **1982**, *45*, 590.
[2] V. Chatsudthipong, C. Muanprasat, *Pharmacol. Ther.* **2009**, *121*, 41
[3] T. Akihisa, Y. Hamasaki, H. Tokuda, M. Ukiya, Y. Kimura, H. Nishino, *J. Nat. Prod.* **2004**, *67*, 407.
[4] T. Ogawa, M. Nozaki, M. Matsui, *Tetrahedron* **1980**, *36*, 2641.
[5] K. Yasukawa, S. Kitanaka, S. Seo, *Biol. Pharm. Bull.* **2002**, *25*, 1488.
[6] M. Takasaki, T. Konoshima, M. Kozuka, H. Tokuda, J. Takayasu, H. Nishino, M. Miyakoshi, K. Mizutani, K.-H. Lee, *Bioorg. Med. Chem.* **2009**, *17*, 600.
[7] S.-F. Chang, B.-H. Chou, L.-M. Yang, F.-L. Hsu, W.-K. Lin, Y. Ho, S.-J. Lin, *Bioorg. Med. Chem.* **2009**, *17*, 6348.
[8] B.-H. Chou, L.-M. Yang, S.-F. Chang, F.-L. Hsu, C.-H. Lo, W.-K. Lin, L.-H. Wang, P.-C. Liu, S.-J. Lin, *Phytochemistry* **2009**, *70*, 759.

[9] B.-H. Chou, L.-M. Yang, S.-F. Chang, F.-L. Hsu, L.-H. Wang, W.-K. Lin, P.-C. Liu, S.-J. Lin, *J. Nat. Prod.* **2011**, *74*, 1379.
[10] L.-H. Lin, L.-W. Lee, S.-Y. Sheu, P.-Y. Lin, *Chem. Pharm. Bull.* **2004**, *52*, 1117.
[11] Y. Wu, G.-F. Dai, J.-H. Yang, Y.-X. Zhang, Y. Zhu, J.-C. Tao, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1818.
[12] J. Li, D. Zhang, X. Wu, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 130.
[13] Y. Wu, C.-J. Liu, X. Liu, G.-F. Dai, J.-Y. Du, J.-C. Tao, *Helv. Chim. Acta* **2010**, *93*, 2052.
[14] J. C. Tao, G. Q. Tian, Y. B. Zhang, Y. Q. Fu, G. F. Dai, Y. Wu, *Chin. Chem. Lett.* **2005**, *16*, 1441.
[15] Y. Wu, J.-H. Yang, G.-F. Dai, C.-J. Liu, G.-Q. Tian, W.-Y. Ma, J.-C. Tao, *Bioorg. Med. Chem.* **2009**, *17*, 1464.
[16] J. Hershenhorn, M. Zohar, B. Crammer, Z. Ziv, V. Weinstein, Y. Kleifeld, Y. Lavan, R. Ikan, *Plant Growth Regul.* **1997**, *23*, 173.
[17] J. Chen, X. Zha, M. Sun, J. Cai, W. Zhou, M. Ji, *Lett. Drug Des. Discovery* **2010**, *7*, 686.
[18] L.-M. Yang, F.-L. Hsu, S.-F. Chang, J.-T. Cheng, J.-Y. Hsu, C.-Y. Hsu, P.-C. Liu, S.-J. Lin, *Phytochemistry* **2007**, *68*, 562.
[19] Y. Mizushina, T. Akihisa, M. Ukiya, Y. Hamasaki, C. Murakami-Nakai, I. Kuriyama, T. Takeuchi, F. Sugawara, H. Yoshida, *Life Sci.* **2005**, *77*, 2127.
[20] I. Hueso-Falcón, N. Girón, P. Velasco, J. M. Amaro-Luis, A. G. Ravelo, B. de las Heras, S. Hortelano, A. Estevez-Braun, *Bioorg. Med. Chem.* **2010**, *18*, 1724.
[21] M. Ukiya, T. Akihisa, H. Tokuda, K. Koike, J. Takayasu, H. Okuda, Y. Kimura, T. Nikaido, H. Nishino, *J. Agric. Food Chem.* **2003**, *51*, 2949.
[22] V. A. Al'fonsov, G. A. Bakaleinik, A. T. Gubaidullin, V. E. Kataev, G. I. Kovylyaeva, A. I. Konovalov, I. A. Litvinov, I. Yu. Strobykina, O. V. Andreeva, M. G. Korochkina, *Russ. J. Gen. Chem.* **2001**, *71*, 1144.
[23] S. Martin, C. P. M. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. A. A. van Schie, D. M. LaFace, D. R. Green, *J. Exp. Med.* **1995**, *182*, 1545.
[24] N. P. D. Nanayakkara, J. A. Klocke, C. M. Compadre, R. A. Hussain, J. M. Pezzuto, A. D. Kinghorn, *J. Nat. Prod.* **1987**, *50*, 434.
[25] C. Subrahmanyam, B. V. Rao, R. S. Ward, M. B. Hursthouse, D. E. Hibbs, *Phytochemistry* **1999**, *51*, 83.
[26] R. Batista, J. L. Humberto, E. Chiari, A. B. Oliveira, *Bioorg. Med. Chem.* **2007**, *15*, 381.
[27] F. Piozzi, S. Passannanti, M. P. Paternostro, V. Sprio, *Phytochemistry* **1971**, *10*, 1164.
[28] M. Ukiya, T. Kikuchi, H. Tokuda, K. Tabata, Y. Kimura, T. Arai, Y. Ezaki, O. Oseto, T. Suzuki, T. Akihisa, *Chem. Biodiversity* **2010**, *7*, 1871.
[29] T. Kikuchi, E. Uchiyama, M. Ukiya, K. Tabata, Y. Kimura, T. Suzuki, T. Akihisa, *J. Nat. Prod.* **2011**, *74*, 137.

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