Inhibition of Phospholipase Cγ1 and Cancer Cell Proliferation by Triterpene Esters from *Uncaria rhynchophylla*

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Investigation of the hooks of *Uncaria rhynchophylla* resulted in isolation of six phospholipase Cγ1 (PLCγ1) inhibitors (**1**−**6**). The structures of these compounds were elucidated as pentacyclic triterpene esters by spectroscopic and chemical analysis. Three of them, namely uncarinic acids C (**1**), D (**2**), and E (**3**), are newly reported as natural products. All the compounds showed dose-dependent inhibitory activities against PLCγ1 in vitro with IC_{50} values of 9.5−44.6 μM and inhibited the proliferation of human cancer cells with IC_{50} values of 0.5−6.5 μg/mL.

Phosphatidylinositol-specific phospholipase C (PI-PLC) plays a key role in intracellular signal transduction by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) to give two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which lead to a series of events that culminate in DNA synthesis and cell proliferation. PLC-mediated PI-turnover is crucially involved in the regulation of cell proliferation.[1−3] Many reports have suggested that an inhibitor of PLC, especially the γ isoform, would be a useful tool for development of anticancer agents.[4−7] Our groups have studied PLCγ1 inhibitors from medicinal plants for the development of anticancer agents.[8−12]

In the course of screening medicinal plants, the $CHCl_3$ extract of the hooks of *Uncaria rhynchophylla* (Miq.) Miq. ex Havil. (Rubiaceae) showed potent inhibitory activity against PLCγ1. *U. rhynchophylla* is a tree distributed in the People's Republic of China, where hooks have been used for hypertension, infantile convulsion, and so on in oriental traditional medicine.[13] A number of alkaloids have been reported as antihypertensive principles from the genus *Uncaria*,[14,15] but none of them has been evaluated for inhibitory activity against PLCγ. In this paper, we report the isolation and characterization of six triterpene esters (**1**−**6**) as PLCγ1 inhibitors, including three new compounds. Furthermore, we discuss the structure−activity relationships, inhibitory activities of PLC-mediated PI-turnover, and antiproliferative activities on several human cancer cell lines for these triterpene esters, as well as two previously reported triterpene esters.[12]

Results and Discussion

The $CHCl_3$−MeOH (2:1) extract from the hooks of *U. rhynchophylla* was partitioned between H_2O and $CHCl_3$, and the $CHCl_3$ extract exhibited inhibitory activity against PLCγ1 (35.4% at 250 μg/mL). Fractions 1−6, obtained by Si gel column chromatography, inhibited PLCγ1 by 24.8, 10.5, 8.6, 48.7, 15.3, and 19.4% at the 100 μg/mL level, respectively. Therefore, fraction 4 was subjected to sequential column chromatography over Si gel, Sephadex LH-20, and reversed-phase HPLC to furnish six triterpene esters (**1**−**6**).

The HRFABMS of compound **1** showed its molecular formula to be $C_{40}H_{56}O_7$. The IR spectrum of **1** showed characteristic bands at 3422 (OH), 1700 (C=O), 1630 (olefinic C=C), and 1509 (aromatic C=C) cm^{-1}. The EIMS showed prominent peaks at *m*/*z* 194.0585 ($C_{10}H_{10}O_4$, M^+ − 454) and at *m*/*z* 454.3441 ($C_{30}H_{46}O_3$, M^+ − 194), but not a molecular ion peak. The ^{13}C NMR spectrum shows signals for 30 carbons, including six methyls and 10 aromatic and conjugated double-bond carbons. The ^1H, ^{13}C, DEPT, and ^1H−^{13}C COSY spectral data suggested that **1** possesses an ester linkage between a pentacyclic triterpene acid and a phenylpropanoic acid.

The presence of a *trans*-ferulic substituent was supported by characteristic spectroscopic data, for example, 1,2,4-trisubstituted aromatic, *trans*-oriented vinylic [δ 6.14 and 7.48 (each d, J = 15.9 Hz)], and aromatic methoxy in the ^1H NMR spectrum and characteristic peaks at *m*/*z* 194, 177, 149, and 137 in the EIMS. This substituent was confirmed by comparison with spectral data in the literature.[12]

In the EIMS, peaks at *m*/*z* 408 (454 − COOH) and 436 (454 − H_2O) implied the presence of a free carboxylic acid, and a free hydroxy in the triterpenoid part of **1**. In addition, significant peaks at *m*/*z* 201 (246 − COOH) and 189 (207 − H_2O), resulting from the sequential cleavage of the *retro*-Diels−Alder fragments at *m*/*z* 246 and 207, indicated a Δ^{12}-unsaturated triterpene ester with a hydroxyl group in ring A or B and a free carboxylic acid unit in ring D or E.[16,17]

By comparison with ursolic acid, the triterpenoid carbon skeleton was inferred as 27-oxidized Δ^{12}-ursene[18] from six methyl signals, including two secondary methyls, oxidized methylene signals, and a trisubstituted double bond [δ 130.0 (12) and 133.0 (13)]. In addition, the HMBC spectrum (Figure 1) showed characteristic two- and three-bond correlations of the olefinic (H-12, C-12, and C-13) and oxidized methylene (H-27 and C-27) to neighboring protons and carbons. These significant correlations supported a ferulic acid moiety linked to the C-27 position of **1** and were confirmed by comparison of the spectral data with literature values of the triterpene part of 27-*p*-(*Z*)-coumaroyloxy ursolic acid.[19]

The spectral data of **2** closely resembled those of **1**. The ^1H NMR spectrum of **2** exhibited signals for the *cis*-ferulic substituent, including a *cis*-oriented vinylic with a coupling constant of 12.8 Hz. This indicated that compound **2** is an isomer of **1**. As a result, from the above data, the structures of **1** and **2** were elucidated as 3β-hydroxy-27-(*E*)-feruloy-

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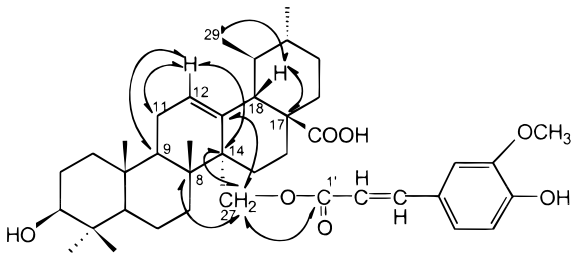


Figure 1. Significant HMBC correlations of compound **1**.

loxyurs-12-en-28-oic acid (**1**) and 3β-hydroxy-27-(*Z*)-feruloyloxyurs-12-en-28-oic acid (**2**).

The FABMS data of **3** and **4** supported the molecular formula of $C_{39}H_{54}O_6$, and the ^{1}H and ^{13}C NMR spectral data indicated that these compounds were also triterpene esters possessing a Δ^{12}-oleanene skeleton. The presence of a 27-oxidized Δ^{12}-oleanene was established by comparison with data for the triterpenoid portion reported in the literature.[12] The presence of a *p*-coumaric acid moiety was implied by signals for the 1,4-disubstituted aromatic and *trans*- or *cis*-oriented vinylic protons in the ^{1}H NMR spectrum and characteristic peaks at *m/z* 164.0475 ($C_9H_8O_3$, $M^+ - 454$) in the EIMS.[20] Chemical shifts and coupling constants were in agreement with the *E*-(**3**) or *Z*-(**4**) configuration for the 2′,3′ double bond. Consequently, the structures of these compounds were determined as 3β-hydroxy-27-*p*-(*E*)-coumaroyloxyolean-12-en-28-oic acid (**3**), and 3β-hydroxy-27-*p*-(*Z*)-coumaroyloxyolean-12-en-28-oic acid (**4**).[21,22]

The spectral data of **5** and **6** were almost identical with those of **1** and **2** in the triterpenoid part and those of **3** and **4** in the phenylpropanoic acid part. Therefore, structures of these compounds were identified as 3β-hydroxy-27-*p*-(*E*)-coumaroyloxyursan-12-en-28-oic acid (**5**) and 3β-hydroxy-27-*p*-(*Z*)-coumaroyloxyursan-12-en-28-oic acid (**6**) and were validated by comparison to the literature.[21,22]

Of the six isolated compounds, **1**−**3** are reported here for the first time as natural products and are named uncarinic acids C, D, and E, respectively. Furthermore, this is the first report of the occurrence of compounds **4**−**6** in Rubiaceae.

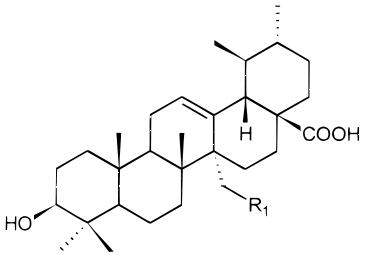
Compounds **1**−**6** were evaluated for their ability to inhibit PLCγ1 activities *in vitro* (Table 2). All compounds, including uncarinic acids A (**7**) and B (**8**),[12] exhibited dose-dependent inhibition of PLCγ1, with the IC_{50} of 9.5−44.6 μM. From these results, some preliminary structure−PLCγ1 inhibitory activity relationships of these new class inhibitors could be deduced as follows. The compounds having an ursane moiety are more active than those having an oleanane moiety (**1**>**7**, **2**>**8**, **5**>**3**, and **6**>**4**). Furthermore, the compounds possessing a *trans* configuration are more effective than those possessing a *cis* configuration (**1**>**2**, **3**>**4**, **5**>**6**, and **7**>**8**), and the compounds containing a *p*-coumaroyloxy group are more potent than those containing a feruloyloxy group (**5**>**1**, **6**>**2**, **3**>**7**, and **4**>**8**). In other words, compound **5**, which contains an ursane moiety, a trans configuration, and a *p*-coumaroyloxy moiety, shows the best inhibitory activity, with an IC_{50} of 9.5 μM.

To determine antiproliferative effects at the cellular level, these compounds were also examined for inhibitory activities against PLC-mediated PI-turnover. PLCγ1 overexpressing NIH3T3 fibroblasts (NIH3T3γ1) produce a severalfold increase in inositol phosphates (IP_t) formation compared to normal NIH3T3 cells when stimulated by platelet-derived growth factor (PDGF).[23] For that reason, these cells were used for this assay system. Compounds

Table 1. ^{13}C NMR Chemical Shifts of the Triterpene Esters **1**−**3** and **5**

position	1	2	3	5
1	39.1	38.5	38.4	38.6
2	26.6	26.8	26.6	26.6
3	78.5	78.6	78.5	78.5
4	37.0	37.1	38.5	37.0
5	55.0	55.1	55.0	55.1
6	18.0	18.1	18.2	18.1
7	33.3	33.3	32.9	33.4
8	39.9	40.1	39.8	40.0
9	48.3	48.3	48.5	48.3
10	38.5	38.5	37.0	38.5
11	23.3	23.7	23.8	23.4
12	130.0	130.4	126.7	130.1
13	133.0	132.8	137.5	133.1
14	45.3	45.3	45.1	45.4
15	24.0	24.0	23.4	24.1
16	23.5	23.5	22.7	23.6
17	47.3	47.3	46.1	47.4
18	52.6	52.0	40.9	52.3
19	38.8	38.4	44.7	38.4
20	38.9	38.9	30.7	39.0
21	30.0	30.1	33.0	30.1
22	36.6	36.7	32.3	36.7
23	27.7	27.8	27.8	27.8
24	15.4	15.5	15.4	15.5
25	15.5	15.6	15.4	15.6
26	17.3	17.4	17.9	17.3
27	65.8	65.7	65.7	65.9
28	181.3	180.8	181.2	181.3
29	17.7	17.9	32.8	17.7
30	20.8	21.0	23.4	20.9
1′	167.3	166.5	167.5	167.5
2′	116.6	114.1	114.8	115.7
3′	144.7	143.5	144.8	144.8
4′	125.5	125.5	125.9	125.8
5′	113.0	113.2	129.8	129.8
6′	146.2	146.3	115.8	114.9
7′	147.2	147.2	159.4	159.1
8′	114.1	116.5	115.8	114.9
9′	126.9	127.0	129.8	129.8
−OCH_3	55.0	55.9		

1−**8** also reduced PDGF-induced IP_t formation in the NIH3T3γ1 cells, with IC_{50} values of 24.3−78.7 μM. These inhibitory activities on PLC-mediated PI-turnover parallel closely the in vitro inhibition of enzyme levels (Table 2).

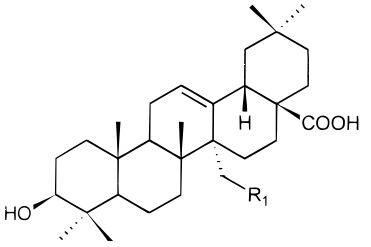


1 R_1=*E*-feruloyl

2 R_1=*Z*-feruloyl

5 R_1=*p*-*E*-coumaroyl

6 R_1= *p*-*Z*-coumaroyl



3 R_1= *p*-*E*-coumaroyl

4 R_1= *p*-*Z*-coumaroyl

7 R_1=*E*-feruloyl

8 R_1=*Z*-feruloyl

Several reports have suggested that PLCγ1 also plays a key role in proliferation and progression of human cancer.[4−7] Accordingly, antiproliferative activities of these compounds were also evaluated in several human cancer cell lines

Table 2. Inhibitory Effects of Compounds **1**−**8** against PLCγ1 in Vitro and PDGF-Stimulated IP_t Formation in NIH3T3γ1 Cells

	IC_{50} value (μM)[a]	
compound	PLCγ1	IP_t formation
1	14.3 ± 1.0	78.7 ± 2.9
2	44.1 ± 2.1	39.9 ± 2.3
3	22.0 ± 2.0	32.5 ± 1.7
4	27.7 ± 1.0	76.5 ± 1.7
5	9.5 ± 0.9	24.3 ± 1.0
6	24.5 ± 1.4	78.7 ± 2.3
7	35.7 ± 1.1	31.4 ± 2.6
8	44.6 ± 2.5	39.4 ± 1.7
amentoflavone	29.0 ± 1.5	9.2 ± 0.9

[a] Data were expressed as mean ± SE of three experiments.

Table 3. Antiproliferative Effects of Compounds **1**−**8** on Human Cancer Cell Lines

	IC_{50} value (μg/mL)			
compound	A-549	HCT-15	MCF7	HT-1197
1	6.5	1.9	5.9	5.8
2	2.8	2.5	0.6	4.4
3	4.1	0.5	1.2	2.3
4	2.0	1.3	1.3	1.1
5	2.4	2.8	0.6	3.2
6	4.7	2.7	0.9	4.5
7	0.7	1.4	2.0	3.5
8	1.8	1.4	2.6	2.3
adriamycin	0.7	13.4	0.01	0.03

(Table 3). All eight compounds inhibited the growth of HCT-15 (colon), MCF-7 (breast), A549 (lung), and HT-1197 (bladder) overexpressing PLCγ1, with IC_{50} values of 0.5−6.5 μM. From the above results, these compounds seem to be worthy candidates for further development as potential anticancer agents.

Experimental Section

General Experimental Procedures. IR spectra were obtained with a Perkin-Elmer 1710 spectrophotometer. The NMR spectra were taken on either a JEOL GSX 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) or a JEOL LA 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer. EIMS were obtained on a VG Trio-2 spectrometer, and HRFABMS were taken on a JMS−SX 102A spectrometer (JEOL, Japan). TLC was carried out on Si gel 60 F_{254} and $RP_{18}F_{254}$ plates (Merck, Darmstadt, Germany). Column chromatography was performed over Si gel 60 (Merck, particle size 230−400 mesh or 15 μm) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Plant Material. The hooks of *U. rhynchophylla* (Miq.) Miq. ex Havil. (Rubiaceae) were purchased from the Korean Export and Import Federation of Drugs, Seoul, Korea, and identified by Dr. Y. S. Chang, School of Pharmacy, China Medical College, Taichung, Republic of China. A voucher specimen (SNUPH-0051) has been deposited in the College of Pharmacy herbarium, Seoul National University in Korea.

Extraction and Isolation. The dried hooks (20 kg) were extracted three times with $CHCl_3$−MeOH (2:1) in an ultrasonic apparatus for 3 h. The combined solution was evaporated in vacuo to yield a total extract (800 g), which was fractionated with $CHCl_3$ and H_2O. Separation of inhibitory principles from the $CHCl_3$ extract was carried out by bioactivity guided-separation. Column (12 × 45 cm) chromatography over Si gel (3 kg, 230−400 mesh) using $CHCl_3$−MeOH mixtures with increasing polarity (30:1→2:1) gave six fractions. Column chromatography of fraction 4, which showed potent activity, over Si gel (4.5 kg, 12 × 60 cm, 230−400 mesh, $CHCl_3$−acetone, 30:1) yielded six subfractions (fractions 7−12) and continuous vacuum column chromatography of fraction 10 (250 g, 15 μm, 12 × 15 cm, $CHCl_3$−MeOH−HOAc, 50:1:0.1) also gave six subfractions (fractions 13−18).

Compounds **1** and **2** were isolated from fraction 14 by vacuum column chromatography using Si gel (80 g, 2 × 30 cm, 15 μm, *n*-hexane−$CHCl_3$−^{i}PrOH, 23:1:1) and then Sephadex LH-20 (2.5 × 80 cm, *n*-hexane−CH_2Cl_2−^{i}PrOH, 3:4:1). Final purification was achieved by semipreparative HPLC (YMC J'sphere, ODS−H80, 4 μm, 10 × 250 mm, 254 nm, 2 mL/min) on RP_{18} eluted with H_2O−CH_3CN−MeOH, 15:62:23, to yield compounds **1** (93.3 mg, t_R 30.11 min) and **2** (115.4 mg, t_R 36.94 min). Compounds **3**−**6** were separated and purified from fraction 15 by the same isolation procedure as **1** and **2** except for the LC solvent system (H_2O−CH_3CN−MeOH, 17:61:22). Retention times were 29.10 (**3**, 35.5 mg), 32.88 (**4**, 70.0 mg), 30.95 (**5**, 43.0 mg), and 34.59 min (**6**, 135.1 mg).

3β-Hydroxy-27-(*E*)-feruloyloxyurs-12-en-28-oic acid, uncarinic acid C (1): white amorphous powder; mp 263−266 °C (dec); $[\alpha]^{26.9}_D$ +5.06° (*c* 0.5, ^{i}PrOH); UV (^{i}PrOH) λ_{max} (log ε) 204 (4.21), 236 (3.94), 327 (4.18) nm; IR (KBr) ν_{max} 3422, 1700, 1630, 1509 cm^{-1}; ^{1}H NMR ($CDCl_3$−CD_3OD, 9:1, 300 MHz) δ 7.48 (1H, d, *J* = 15.9 Hz, H-3′), 7.02 (1H, dd, *J* = 8.5 Hz, H-9′), 6.99 (1H, s, H-5′), 6.76 (1H, d, *J* = 8.5 Hz, H-8′), 6.14 (1H, d, *J* = 15.9 Hz, H-2′), 5.50 (1H, br s, H-12), 4.26 (1H, d, *J* = 12.7 Hz, H-27b), 4.10 (1H, d, *J* = 12.7 Hz, H-27a), 3.82 (3H, s, −OCH_3), 3.09 (1H, dd, *J* = 8.1, 7.4 Hz, H-3), 2.22 (1H, dd, *J* = 11.5 Hz, H-18), 0.89 (3H, s, H-23), 0.86 (3H, s, H-25), 0.83 (3H, d, *J* = 5.9 Hz, H-30), 0.79 (3H, d, *J* = 6.1 Hz, H-29), 0.75 (3H, s, H-26), 0.69 (3H, s, H-24); ^{13}C NMR ($CDCl_3$−CD_3OD, 9:1, 75 MHz), see Table 1; EIMS *m*/*z* (rel int) 454 (M^+ − $C_{10}H_{10}O_4$, 15), 421 (8), 246 (8), 207 (8), 201 (18), 194 (M^+ − $C_{30}H_{46}O_3$, 100), 189 (12), 179 (38), 177 (33), 77 (60); HRFABMS (positive) *m*/*z* 671.3950 (calcd for $C_{40}H_{56}O_7Na$: 671.3924);

3β-Hydroxy-27-(*Z*)-feruloyloxyurs-12-en-28-oic acid, uncarinic acid D (2): white amorphous powder; mp 195−197 °C (dec); $[\alpha]^{27.5}_D$ +2.16° (*c* 0.5, ^{i}PrOH); UV (^{i}PrOH) λ_{max} (log ε) 204 (4.37), 236 (shoulder), 327 (4.14) nm; IR (KBr) ν_{max} 3436, 1693, 1660, 1640, 1044 cm^{-1}; ^{1}H NMR ($CDCl_3$−CD_3OD, 9:1, 300 MHz) δ 7.63 (1H, s, H-5′), 7.10 (1H, d, *J* = 8.5 Hz, H-9′), 6.82 (1H, d, *J* = 8.5 Hz, H-8′), 6.73 (1H, d, *J* = 12.8 Hz, H-3′), 5.69 (1H, d, *J* = 12.8 Hz, H-2′), 5.54 (1H, t, *J* = 3.2 Hz, H-12), 4.29 (1H, d, *J* = 12.5 Hz, H-27b), 4.13 (1H, d, *J* = 12.5 Hz, H-27a), 3.88 (3H, s, −OCH_3), 3.13 (1H, dd, *J* = 8.2, 7.6 Hz, H-3), 2.32 (1H, d, *J* = 10.8 Hz, H-18), 1.04 (1H, m, H-19), 0.94 (3H, s, H-23), 0.90 (3H, s, H-25), 0.86 (3H, d, *J* = 5.9 Hz, H-30), 0.83 (3H, d, *J* = 6.1 Hz, H-29), 0.74 (3H, s, H-26), 0.72 (3H, s, H-24); ^{13}C NMR ($CDCl_3$−CD_3OD, 9:1, 75 MHz), see Table 1; EIMS *m*/*z* (rel int) 454 (M^+ − $C_{10}H_{10}O_4$, 20), 441 (5), 375 (3), 285 (15), 239 (12), 201 (18), 194 (M^+ − $C_{30}H_{46}O_3$, 100), 177 (45), 133 (62); HRFABMS (positive) *m*/*z* 671.3951 (calcd for $C_{40}H_{56}O_7Na$: 671.3924).

3β-Hydroxy-27-*p*-(*E*)-coumaroyloxyolean-12-en-28-oic acid, uncarinic acid E (3): white amorphous powder; $[\alpha]^{28.3}_D$ −2.56° (*c* 0.5, ^{i}PrOH); UV (^{i}PrOH) λ_{max} (log ε) 206 (4.17), 229 (4.05), 315 (4.37) nm; IR (KBr) ν_{max} 3447, 1685, 1640, 1509, 1168 cm^{-1}; ^{1}H NMR ($CDCl_3$−CD_3OD, 9:1, 300 MHz) δ 7.47 (1H, d, *J* = 15.9 Hz, H-3′), 7.32 (1H, d, *J* = 8.6 Hz, H-5′, -9′), 6.79 (1H, d, *J* = 8.6 Hz, H-6′, -8′), 6.11 (1H, d, *J* = 15.9 Hz, H-2′), 5.52 (1H, br s, H-12), 4.25 (1H, d, *J* = 12.6 Hz, H-27b), 4.18 (1H, d, *J* = 12.6 Hz, H-27a), 3.07 (1H, dd, *J* = 8.4 Hz, H-3), 2.81 (1H, dd, *J* = 10.4 Hz, H-18), 1.01 (1H, dd, *J* = 10.40 Hz, H-19), 0.83 (3H, s, H-23), 0.80 (3H, s, H-30), 0.79 (3H, s, H-25), 0.75 (3H, s, H-29), 0.72 (3H, s, H-24), 0.71 (3H, s, H-26), 0.69 (1H, m, H-19); ^{13}C NMR ($CDCl_3$−CD_3OD, 9:1, 75 MHz), see Table 1; EIMS *m*/*z* (rel int) 454.3451 (M^+ − $C_9H_8O_3$, 100), 393 (15), 246 (9), 201 (8), 189 (8), 164 0.0475 (M^+ − $C_{30}H_{46}O_3$, 85), 147 (30), 119 (15); HRFABMS (positive) *m*/*z* 641.3799 (calcd for $C_{39}H_{54}O_6Na$: 641.3818)

3β-Hydroxy-27-*p*-(*Z*)-coumaroyloxyolean-12-en-28-oic acid (4): white amorphous powder; mp 240−242 °C (dec); spectral data were in agreement with those previously reported.[21,22]

3β-Hydroxy-27-*p*-(*E*)-coumaroyloxyurs-12-en-28-oic acid (5): white amorphous powder; spectral data were in agreement with those previously reported;[21] ^{13}C NMR ($CDCl_3$−CD_3OD, 9:1, 75 MHz), see Table 1.

3β-Hydroxy-27-*p*-(*Z*)-coumaroyloxyurs-12-en-28-oic acid (6): white amorphous powder; mp 198−201 °C (dec); spectral data were in agreement with those previously reported.[21,22]

***In Vitro* PLCγ1 Assay.** The PLCγ1 assay was carried out according to the method of Rhee et al. described previously.[10,23,24]

Measurement of Intracellular Inositol Phosphates (IP_t). NIH3T3γ1 cells, 1×10^6 cells, were labeled with 1 μCi/mL of myo-[2-^{3}H]-inositol in inositol-free DMEM for 24 h. The labeled cells were washed with phosphate-buffered saline and incubated in inositol-free DMEM containing 20 mM HEPES, pH 7.5, 20 mM LiCl, and 1 mg/mL bovine serum albumin at 37 °C for 15 min, and then test compounds were added. After 20 min, cells were stimulated with 50 mg/mL of PDGF for 30 min. IP_t were extracted by 5% $HClO_4$ for 30 min in an ice bath, and the acid-soluble fraction was applied to a Bio-Rad AG 1-X8 anion exchange column. The column was then washed with 10 mL of distilled water followed by 10 mL of 60 mM ammonium formate containing 5 mM sodium tetraborate. The IP_t were eluted with a solution containing 1.0 M ammonium formate and 0.1 M formic acid.[23,25]

Antiproliferation Assay of Cancer Cell. Measurement of the ability to inhibit proliferation of cancer cells followed the method described previously.[10]

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