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POLYACETYLENES AND FLAVONOIDS ISOLATED FROM FLOWERS OF *Carthamus tinctorius*

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*A new acetylene, (8*S*)-deca-4,6-diyne-1,8-di*-O-β-*D-glucopyranoside (**4**), and four known polyacetylenes (**1–3** and **5**), together with nine known flavonoids (**6–14**), were isolated from the flowers of* Carthamus tinctorius *L. The structures of the isolates were elucidated by using spectroscopic analysis, mainly 1D and 2D NMR, HR-MS, and comparison with literature data, as well as enzymatic hydrolysis and Mosher′s esterification methods. All isolated compounds were evaluated for their anti-allergic effects by analyzing the inhibition of interleukin-2 (IL-2) expression in Jurkat T cells. Among them, 6-hydroxykaempferol 3,6-diglucoside (7) inhibited IL-2 production in activated T cells.*

Keywords: *Carthamus tinctorius* L., acetylenes, flavonoids, Mosher′s esterification, anti-allergic, interleukin-2.

Carthamus tinctorius L. (Asteraceae), commonly known as safflower, has been widely used in traditional medicine to treat various diseases such as coronary heart disease, angina pectoris, gynecologic disease, stroke, and hypertension [1], or utilized as food colorant and as a natural pigment [2]. Previous phytochemistry investigation of *C. tinctorius* flowers have demonstrated the presence of quinochalcones, flavonoids, alkaloids, polyacetylenes, alkane-diol, fatty acids, steroids, and lignans [3]. Among them, polyacetylenes and flavonoids were reported to be the main constituents of this plant and to show diverse pharmacological activities, including anti-inflammatory, antioxidant, and cytotoxic effects, cerebrovascular and cardiovascular protection, and is also hepatoprotective [4]. However, no studies have reported their anti-allergic activity up to now. As a part of our ongoing investigation for new anti-allergic agents from plants, a new polyacetylene, (8*S*)-deca-4,6-diyne-1,8-di-*O*-*β*-D-glucopyranoside (**4**), and four known ones (**1–3** and **5**) along with nine known flavonoids (**6–14**), were isolated from the flowers of *C. tinctorius*. In this study, we described the isolation, structure elucidation of these compounds (**1–14**), and the evaluation of their inhibitory effects on IL-2 expression in Jurkat T cells.

Compound **4** was isolated as a colorless oil; $[\alpha]_D^{21.2}$ –48.2° (*c* 0.1, MeOH). The IR spectrum of **4** displayed absorption bands at 3344 cm^{-1} (hydroxyl group) and 2360, 2355 cm^{-1} (acetylenic). Its molecular formula was assigned as $C_{22}H_{34}O_{12}$ by HR-ESI-MS based on a quasimolecular ion at *m/z* 513.1941 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_{12}Na$, 513.1948). The ^1H NMR spectrum of **4** showed resonances for an oxygenated methine at δ 4.68 (1H, t, J = 6.6 Hz, H-8), an oxymethylene at δ 3.99 (1H, dt, J = 10.1, 6.1 Hz, H-1) and 3.68 (1H, m, overlapped, H-1), three methylene groups at δ 2.48 (2H, t, J = 7.1 Hz, H-3), 1.86 (2H, dt, J = 14.0, 7.1 Hz, H-2), and 1.78 (2H, m, H-9), and a methyl group at δ 1.04 (3H, t, J = 7.4 Hz, H-10) (Table 1). Furthermore, the ^1H and ^{13}C NMR data of **4** also indicated the presence of two *β*-D-glucopyranosyl moieties from the signals of two anomeric protons at δ 4.59 (1H, d, J = 7.8 Hz, H-1′) and 4.28 (1H, d, J = 7.8 Hz, H-1″), as well as two sets of characteristic signals (C-1′–C-6′, C-1″–C-6″) (Table 1). After enzymatic hydrolysis of **4** by *β*-glucosidase, the D-form of glucose was confirmed by comparison of its retention factor (R_f 0.31, $CHCl_3$–MeOH–H_2O, 8:5:1) on thin-layer chromatography and optical rotation $[\alpha]_D^{21.2}$ +45.3° (*c* 0.2, MeOH) with that of an authentic sample. The large coupling constant of two anomeric protons (J = 7.8 Hz, H-1′, 1″) revealed the *β*-configuration of D-glucose. Moreover, the ^{13}C NMR spectrum displayed the signals of four quaternary carbons at δ_C 81.5, 75.1, 72.1, and 65.5 assignable to acetylenic carbons.

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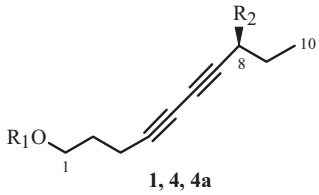
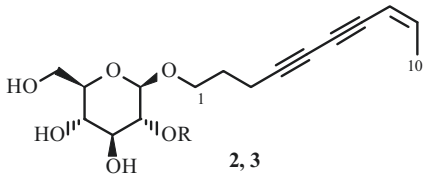
TABLE 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compound **4** (CD_3OD, δ, ppm, J/Hz)

C atom	δ_H	δ_C	C atom	δ_H	δ_C
1	3.99 (dt, J = 10.1, 6.1); 3.68 (m)[a]	69.2	2′	3.21 (m)[a]	75.1[b]
2	1.86 (dt, J = 14.0, 7.1)	29.7	3′	3.40 (m)[a]	78.0[c]
3	2.48 (t, J = 7.1)	16.5	4′	3.31 (m)[a]	71.7[d]
4	–	81.5	5′	3.31 (m)[a]	78.1[e]
5	–	65.5	6′	3.89 (m)[a]; 3.68 (m)[a]	62.8[f]
6	–	72.1	1″	4.28 (d, J = 7.8)	104.5
7	–	75.1	2″	3.21 (m)[a]	74.9[b]
8	4.68 (t, J = 6.6)	69.6	3″	3.40 (m)[a]	77.9[c]
9	1.78 (m)	29.8	4″	3.31 (m)[a]	71.6[d]
10	1.04 (t, J = 7.4)	9.8	5″	3.31 (m)[a]	78.1[e]
1′	4.59 (d, J = 7.8)	101.2	6″	3.89 (m)[a]; 3.68 (m)[a]	62.7[f]

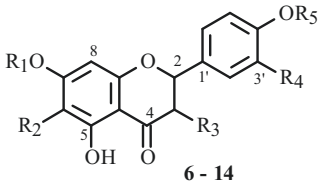
[a] Overlapped. [b–f] May be interchangeable.



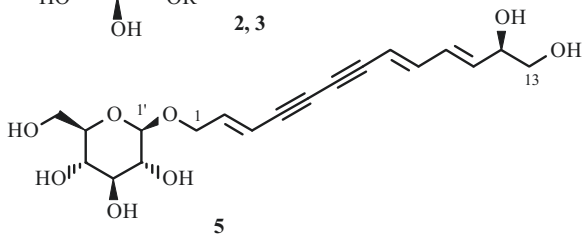
1, 4, 4a



2, 3



6 - 14

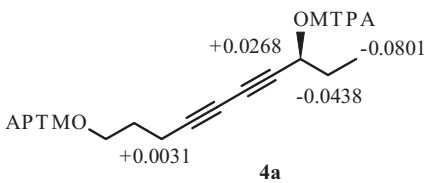


5

1: R_1 = Glc, R_2 = H; **2:** R = H; **3:** R = GlcA; **4:** R_1 = Glc, R_2 = OGlc; **4a:** $R_1 = R_2$ = H; **6:** $R_1 = R_4 = R_5$ = H, R_2 = OH, R_3 = OGlc
7: $R_1 = R_4 = R_5$ = H, $R_2 = R_3$ = OGlc; **8:** $R_1 = R_4 = R_5$ = H, R_2 = OH, R_3 = ORut; **9:** $R_1 = R_2 = R_4 = R_5$ = H, R_3 = OSop
10: $R_1 = R_2 = R_4 = R_5$ = H, R_3 = ORut; **11:** R_1 = GlcA, $R_2 = R_3 = R_4 = R_5$ = H; **12:** R_1 = GlcA, $R_2 = R_3 = R_4$ = H, $R_5 = CH_3$
13: R_1 = Glc, $R_2 = R_5$ = H, $R_3 = R_4$ = OH; **14:** $R_1 = R_2 = R_5$ = H, R_3 = ORut, R_4 = OH

GlcA: β-D-Glucopyranuronosyl; Rut: α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranosyl
Sop: β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl

The ^{1}H–^{1}H COSY correlations between H-2 with H-1 and H-3 and between H-9 with H-8 and H-10 are assumed to be due to two partial structures of C-1/C-2/C-3 and C-8/C-9/C-10. The connection of these parts with acetylenic carbons was deduced by HMBC correlations between H-3 with C-4 and C-5 and between H-8 with C-6 and C-7. The locations of the glucosyl moieties at C-8 and C-1 were verified by the HMBC correlations between H-1′ with C-8 and between H-1″ with C-1, respectively. The absolute configuration of the chiral center (C-8) in **4** was elucidated by using enzymatic hydrolysis and Mosher's esterification of aglycone **4a**. Analysis of the ^{1}H NMR chemical shift differences between δ_S (*S*)-MTPA ester and δ_R (*R*)-MTPA ester demonstrated the absolute configuration of C-8 of **4a** as *S* (Fig. 1). Therefore, the structure of **4** was determined as (8*S*)-deca-4,6-diyne-1,8-di-*O*-β-D-glucopyranoside.



4a

Fig. 1. $\Delta\delta$ ($\delta_S - \delta_R$) values of MTPA esters of compound **4a**.

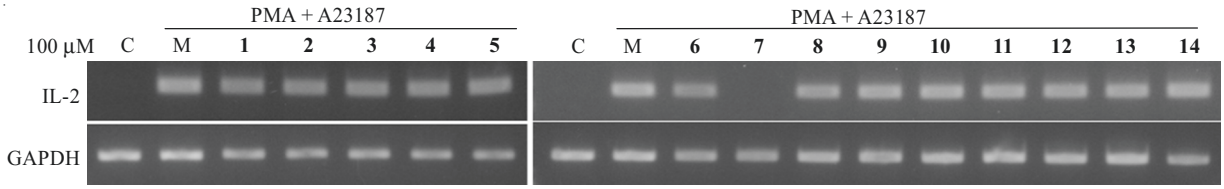


Fig. 2. Inhibition of IL-2 expression by polyacetylenes (**1**–**5**) and flavonoids (**6**–**14**) isolated from *C. tinctorius* L. Jurkat T cells (1×10^6) were treated with 100 μM concentrations of **1**–**14** and stimulated with PMA (100 nM)/A23187 (1 μM) for 6 h. After incubation, cells were harvested and total RNA was isolated from harvested cells. Human IL-2 mRNA levels were detected by conventional PCR. C: blank, M: control.

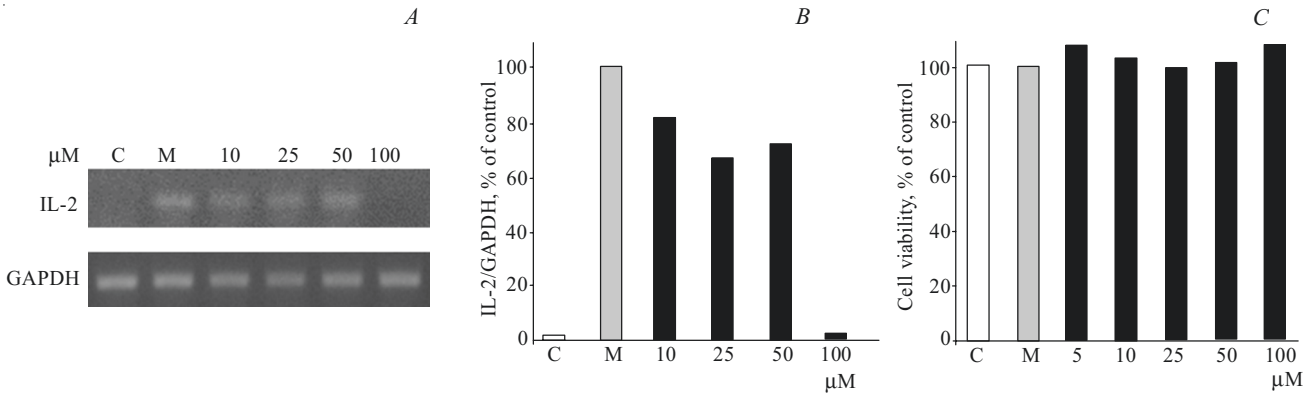


Fig. 3. 6-Hydroxykaempferol 3,6-diglucoside (**7**) inhibits IL-2 production in activated T cells. (A) Jurkat T cells (1×10^6) were pre-incubated for 30 min with or without indicated concentrations of 6-hydroxykaempferol 3,6-diglucoside (**7**) (0–100 μM), and then the cells were stimulated for 6h with PMA (100 nM)/A23187 (1 μM). IL-2 mRNA levels were detected by PCR. (*B*) The supernatants were collected from the cells treated with PMA (100 nM)/A23187 (1 μM) for 24h and IL-2 secretion was measured by ELISA. Results are expressed as mean ±SEM of three independent experiments. *$P < 0.05$ *versus* vehicle-treated control. (*C*) Viability of Jurlat cells determined by MTT-assay after 48h exposure to 6-hydroxykaempferol 3,6-diglucoside (**7**) at indicated concentrations. The data are expressed as mean ±SEM of three independent experiments.

The known compounds were determined as 4,6-decadiyn-1-yl *β*-D-glucopyranoside (**1**) [5], (8*Z*)-8-decene-4,6-diyn-1-yl *β*-D-glucopyranoside (**2**) [6], (8*Z*)-8-decene-4,6-diyn-1-yl 2-*O*-*β*-D-glucopyranuronosyl-*β*-D-glucopyranoside (**3**) [7], (2*E*,8*E*,10*E*,12*R*)-12,13-dihydroxy-2,8,10-tridecatriene-4,6-diyn-1-yl-*β*-D-glucopyranoside (**5**) [7], 6-hydroxykaempferol 3-glucoside (**6**) [8], 6-hydroxykaempferol 3,6-diglucoside (**7**) [9], 6-hydroxykaempferol 3-rutinoside (**8**) [10], kaempferol 3-sophoroside (**9**) [11], kaempferol 3-rutinoside (**10**) [12], apigenin 7-glucuronide (**11**) [13], acacetin 7-glucuronide (**12**) [14], quercetin 7-glucoside (**13**) [15], and quercetin 3-rutinoside (**14**) [16] by comparison with reported data.

All isolated compounds (**1**–**14**) were evaluated for their inhibitory effect on IL-2 expression in T cells. Among them, 6-hydroxykaempferol 3,6-diglucoside (**7**) showed a considerable decrease in IL-2 expression, as shown in Fig. 2. However, it inhibits IL-2 transcription and secretion in PMA/23187-stimulated Jurkat T cells in a concentration-independent manner (Fig. 3, *A*, *B*). Furthermore, we determined whether the inhibition of IL-2 expression in Jurkat T cells by 6-hydroxykaempferol 3,6-diglucoside (**7**) is related to cytotoxic effects using the MTT assay. Treatment of 6-hydroxykaempferol 3,6-diglucoside (**7**) for 48 h exhibited no cytotoxicity in Jurkat T cells even at a concentration of 100 μM (Fig. 3, *C*). Thus, the inhibition of 6-hydroxykaempferol 3,6-diglucoside (**7**) is not related to cytotoxicity.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured using a Jasco P-1020 polarimeter. The UV spectra were recorded using an Agilent 8453 UV-visible spectrophotometer. The IR spectra were recorded using a Bruker IFS-66/S Fourier transform (FT)-IR spectrometer. ^{1}H (500 MHz), ^{13}C (125 MHz) nuclear magnetic resonance (NMR), and 2D NMR spectra were recorded on a Bruker Avance Digital 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany, TMS).

HR-ESI-MS spectra were recorded using a Micromass QTOF2-MS mass spectrometer in the Korea Basic Science Institute, Ochang, Korea. Silica gel 60 (Merck, 230–400 mesh) and reversed-phase (RP)-C_{18} silica gel (Merck, 75 mesh) were used for column chromatography (CC). TLC was performed using Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates. HPLC was performed using a Waters 600 Controller system with a UV detector and a YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan), and HPLC solvents were from Burdick & Jackson, USA.

Plant Material. The flowers of *C. tinctorius* were purchased from Kyungdong traditional market in Seoul (June 2013), identified by Prof. Byung Sun Min at Daegu Catholic University, and deposited at the Pharmacognosy Laboratory (voucher No. 15ACT) in the College of Pharmacy, Kyungpook National University, Korea.

Extraction and Isolation. The dried flowers of *C. tinctorius* (10.0 kg) were extracted and refluxed with ethanol (10 L × 3) at 60°C for 3 h. The ethanol-soluble part was concentrated *in vacuo* to give a residue (2.4 kg), which was suspended in H_2O and partitioned with *n*-hexane and ethyl acetate, successively. The EtOAc extract (280.0 g) was subjected to silica gel vacuum liquid chromatography (VLC) (63–200 μm, Merck) and eluted with a gradient mixture of CH_2Cl_2–methanol (20:1–1:0) to give six fractions (CTE1–6). Fraction CTE2 (71.0 g) was chromatographed on MCI gel CC and eluted with a gradient mixture of MeOH–H_2O (1:1.5–1.5:1) to give seven fractions (CTE2.1–2.7). Fraction CTE2.5 (11.0 g) was resubjected to silica gel, CC and eluted with a gradient mixture of EtOAc–MeOH (1:0–0:1) to yield eight fractions (CTE2.5.1–2.5.8). Fraction CTE2.5.4 (2.0 g) was separated on a C18 gel CC and eluted with a gradient mixture of MeOH–H_2O (1:4–1:0) to afford compound **1** (29.0 mg). Fraction CTE4 (39.5 g) was subjected to silica gel CC and eluted with a gradient mixture of MC–MeOH (9:1–0:1) and continuously chromatographed on MCI gel using a stepwise elution mixture of MeOH–H_2O (1:1.5 and 1:1) to give eight fractions (CTE4.1–4.8). Fraction CTE4.2 (498.3 mg) was isolated on silica gel and eluted with a gradient mixture of EtOAc–MeOH–H_2O (9:1:0.1–0:1:0.1) to yield compound **13** (15.0 mg). Fraction CTE5 (30.5 g) was subjected to MCI gel CC using a gradient elution mixture of MeOH–H_2O (1:3–1:0) to give eight fractions (CTE5.1–5.8). Fraction CTE5.3 (1.2 g) was chromatographed on C18 gel and eluted with a gradient mixture of MeOH–H_2O (1:5–1:1.5) to give compounds **8** (13.0 mg), **4** (8.3 mg), and **7** (13.0 mg). Fraction CTE5.4 (1.1 g) was recrystallized to yield compound **6** (79.0 mg) and a soluble part (CTE5.4M). Fraction CTE5.4M (1.0 g) was subjected to silica gel CC with a gradient elution of EtOAc–MeOH–H_2O (9:1:0.1–0:1:0.1) to obtain six fractions (CTE5.4M.1–5.4M.6). Fraction CTE5.4M.2 (216.0 mg) was isolated by preparative HPLC with a gradient elution mixture of MeOH in water with 0.5% formic acid (40–50%, 6 mL/min, 60 min) to obtain compounds **5** (2.0 mg, t_R = 20.0 min), **9** (49.0 mg, t_R = 24.9 min), and **14** (57.0 mg, t_R = 34.3 min). Fraction CTE5.5 (2.0 g) was chromatographed on silica gel eluting with a gradient mixture of EtOAc–MeOH–H_2O (9:1:0.1–0:1:0.1) to give compounds **10** (560.0 mg) and **3** (32.7 mg). Fraction CTE5.6 (1.2 g) was subjected to silica gel CC using a gradient elution mixture of EtOAc–MeOH–H_2O (9:1:0.2–0:1:0.2) to give seven fractions (CTE5.6.1–5.6.7). Compounds **12** (3.0 mg, t_R = 30.5 min) and **11** (5.0 mg, t_R = 20.0 min) were purified by preparative HPLC with a gradient mixture of MeOH in water with 0.5% formic acid (50–70%, 6 mL/min, 60 min) from Frs. CTE5.6.1 (144.0 mg) and CTE5.6.3 (250.0 mg), respectively. The water-soluble layer (1.7 kg) was chromatographed on an HP dianion and eluted with a gradient stepwise mixture of MeOH–H_2O (0:1–1:0) to obtain four fractions (CTW1–CTW4). Fraction CTW4 (19.0 g) was applied to MCI gel to give six fractions (CTW4.1–4.6). Fraction CTW4.6 (11.0 g) was continuously subjected to silica gel CC eluting with a gradient mixture of EtOAc–MeOH–H_2O (9:1:0.1–0:1:0.1) to afford five fractions (CTW4.6.1–4.6.5). Fraction CTW4.6.2 (672.0 mg) was purified by preparative HPLC with a gradient solution mixture of MeOH–H_2O (50–70%, 6 mL/min, 60 min) to give compound **2** (6.4 mg, t_R = 34.4 min).

(8*S*)-Deca-4,6-diyne-1,8-di-*O*-*β*-D-glucopyranoside (4). Colorless oil; $[\alpha]_D^{21.2}$ –48.2° (*c* 0.1, MeOH). UV (λ_{max}, nm) (log ε): 209 (3.34), 266 (2.91), 282 (2.85), 293 (2.74), 313 (2.61). IR (ν_{max}, cm^{-1}): 3344, 2942, 2837, 2360, 2355, 1076, 1036, 1027. For ^1H and ^{13}C NMR, see Table 1. HR-ESI-MS *m/z* 513.1941 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_{12}Na$, 513.1948).

Enzymatic Hydrolysis. A solution of compound **4** (5.0 mg) and *β*-glucosidase (10.0 mg) in water (2 mL) was let to stand for 20 h at 37°C. After the reaction was completed, the mixture was extracted with EtOAc, and the EtOAc layer was dried using airflow overnight to afford aglycone **4a**. The aglycone **4a** was then identified by the ^1H NMR spectrum in $CDCl_3$. The aqueous layer was concentrated and purified to obtain the sugar moieties and then analyzed by TLC ($CHCl_3$–MeOH–H_2O, 8:5:1) and compared with an authentic sample. The optical rotation of sugar was measured and confirmed to be D-glucose (positive $[\alpha]_D$) [17].

4,6-Decadiyne-1,8-diol (4a). ^1H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.36 (1H, t, J = 6.2, H-8), 3.75 (2H, t, J = 6.1, H-1), 2.43 (2H, t, J = 7.0, H-3), 1.79 (2H, m, H-2), 1.74 (2H, m, H-9), 1.02 (3H, t, J = 7.4, H-10).

Determination of Configuration of the Hydroxyl Group by Mosher's Esterification. Aglycone **4a** (0.5 mg) and 4-(dimethylamino)-pyridine (0.2 mg) were transferred into each vial, and this mixture was dried under reduced vacuum.

R-(–) and *S*-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (6.0 μL) was added to each vial in pyridine-d_5 (0.6 mL) immediately; then the vial was sealed, shaken, and heated to mix the sample and MTPA-Cl evenly. The vial was permitted to stand at room temperature for 1 h. After reaction, (*S*)- and (*R*)-MTPA [α-methoxy-α-(trifluoromethyl)phenylacetyl] esters of aglycone **4a** were obtained. These esters were analyzed by ^{1}H NMR to calculate the chemical shift differences between δ_S and δ_R [18].

(*S*)-MTPA Ester of 4a. ^{1}H NMR (500 MHz, C_5D_5N, δ, ppm, J/Hz): 5.8718 (1H, t, J = 6.4, H-8), 4.4644 (2H, m, H-1), 2.3718 (2H, t, J = 7.0, H-3), 1.8430 (2H, m, H-2), 1.8178 (2H, m, H-9), 0.8927 (3H, t, J = 7.4, H-10).

(*R*)-MTPA Ester of 4a. ^{1}H NMR (500 MHz, C_5D_5N, δ, ppm, J/Hz): 5.8450 (1H, t, J = 6.4, H-8), 4.4644 (2H, m, H-1), 2.3687 (2H, t, J = 7.0, H-3), 1.8430 (2H, m, H-2), 1.8616 (2H, m, H-9), 0.9728 (3H, t, J = 7.4, H-10).

Cell Culture. Jurkat T cells (ATCC TIB-152, Manassas, VA) were grown in RPMI medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). The cells were cultured at 37°C in a humidified incubator containing 5% CO_2 and 95 % air.

Reverse Transcription PCR and Conventional PCR. Jurkat T cells (1×10^6) were incubated with the indicated concentrations of compounds for 30 min at 37°C. Incubated cells were stimulated with PMA (100 nM) and A23187 (1 μM) for 6 h for PCR. Cells were harvested and total RNAs were isolated with TRIZOL reagent (JBI, Korea). Reverse transcription of the RNA was performed using RT PreMix (Enzynomics, Korea). For conventional PCR, the primers and PCR conditions for each gene were as follows: human IL-2, 5′-CAC GTC TTG CAC TTG TCA C-3′ and 5′-CCT TCT TGG GCA TGT AAA ACT-3′; human GAPDH, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ and 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′. The amplification profile was composed of denaturation at 94°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 40 s. The 30 cycles were preceded by denaturation at 72°C for 7 min. All experiments were performed at least three times unless otherwise indicated.

Cell Viability Assay. Jurkat T cells (3×10^5) were seeded in a 24 well-plate and incubated with isolates (**1–14**) for 24 h. After incubation, MTT solution (20 μL, 5 mg/mL) was added to the cells (180 L). After 2 h of incubation at 37°C in the incubator, the cells were centrifuged and the supernatants were taken out. Then 150 μL of DMSO was added and the whole incubated for 15 min at room temperature. After incubation, absorbance was detected at 590 nm wavelength.

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Thi Men Ngo and Thi Oanh Vu contributed equally to this work.

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