Isolation, Structural Elucidation, and Inhibitory Effects of Terpenoid and Lipid Constituents from Sunflower Pollen on Epstein–Barr Virus Early Antigen Induced by Tumor Promoter, TPA

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Eight fatty acid esters of triterpene alcohols (**1**−**8**), four free triterpene alcohols (**9**, **12**, **17**, and **18**), four diterpene acids (**19**−**22**), two tocopherol-related compounds (**23** and **24**), four estolides (**25**−**28**), three *syn*-alkane-4,6-diols (**29**−**31**), one 1,3-dioxoalkanoic acid (**32**), and one aliphatic ketone (**33**), along with the mixture of free fatty acids, were isolated from the diethyl ether extract of the pollen grains of sunflower (*Helianthus annuus*). Among these compounds, 14 (**2**−**8**, **12**, **23**, **25**−**28**, and **33**) were new naturally occurring compounds, and their structures were determined on the basis of spectroscopic methods. Twenty-four terpenoids and lipids (**1**−**4**, **6**−**9**, **12**, and **19**−**33**) and six free triterpene triols (**10**, **11**, and **13**−**16**), derived from their fatty acid esters (**2**, **3**, and **5**−**8**) by alkaline hydrolysis, were evaluated with respect to their inhibitory effects on the induction of Epstein− Barr virus early antigen (EBV-EA) induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in Raji cells, which is known to be a primary screening test for antitumor promoters. Among the 30 compounds tested, 21 compounds possessing a di- or a polycyclic ring system in the molecule (**1**−**4**, **6**−**16**, and **19**−**24**) showed potent inhibitory effects on EBV-EA induction (91−100% inhibition at 1×10^3 mol ratio/TPA).

KEYWORDS: *Helianthus annuus*; Compositae; pollen; lipids; terpenoids; fatty acid esters; antitumor promoter; Epstein−Barr virus early antigen

INTRODUCTION

Helianthus annuus L. (Compositae), commonly called sunflower, is a tall annual herb, originating in North America and blooming in late summer and autumn. In the course of our search on Compositae plants to find compounds possessing potential antiinflammatory and antitumor-promoting activities, we have demonstrated that the methanol (MeOH) extract (*1*) of the flowers of *H. annuus* and sterol, triterpene alcohol, and *syn*-alkane-6,8-diol constituents isolated from the extract exhibited remarkable antiinflammatory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice (*2*, *3*). The *syn*-alkane-6,8-diols have been shown, moreover, to inhibit the tumor-promoting activity of TPA in two stage carcinogenesis in mouse skin (*4*). We have undertaken an investigation on the constituents, with special attention to terpenoid constituents, of the diethyl ether extract of *H. annuus* pollen, and we report here the isolation and characterization of 27 compounds of which 16 were terpenoid constituents, along with inhibitory effects on the induction of Epstein−Barr virus early antigen (EBV-EA) induced by TPA of 30 compounds evaluated as preliminary screens for their potent cancer chemopreventive activities.

MATERIALS AND METHODS

Crystallizations were performed in MeOH. Ultraviolet (UV) spectra were determined on a Shimadzu UV-2200 spectrometer. Infrared (IR) spectra were recorded on a Jasco IR-300 IR spectrometer in KBr disks or as liquid films. Optical rotations were measured on a Jasco DIP-370 polarimeter in CHCl$_3$ at 25 °C. Electron-impact mass spectra (EIMS) and high-resolution EIMS (HREIMS) were recorded on JEOL

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JMS-GC mate spectrometer (70 eV) using a direct inlet system. Nuclear magnetic resonance (NMR) spectra were recorded, if not otherwise specified, with a JEOL JNM LA-500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in CDCl$_3$ with tetramethylsilane (TMS; ^{1}H NMR) and CDCl$_3$ at δ 77.0 (^{13}C NMR) as internal standard. Thin-layer chromatography (TLC) on silica gel (Kieselgel 60G, Merck; 0.5 mm thick; 20 cm × 20 cm) was developed using *n*-hexane/ethyl acetate (EtOAc) (6:1, v/v). Chromatorex-ODS, 100−200 mesh octadecyl silica (Fuji Silysia Chemical, Ltd., Aichi, Japan) was used for open column chromatography. Reversed-phase preparative high-performance liquid chromatography (HPLC) was carried out on 25 cm × 10 mm i.d. Superiorex ODS S-5 μm (Shiseido Co., Ltd., Tokyo, Japan) [HPLC(A)] and Pegasil ODS II (Senshu Scientific Co., Ltd., Tokyo, Japan) [HPLC(B)] C$_{18}$ silica columns, at 25 °C with MeOH [HPLC(A)] and MeOH−H$_2$O [95:5, v/v; HPLC(B)] as the mobile phase at 4 mL/min. Normal phase HPLC was carried out on a 25 cm × 4.6 mm i.d. Senshu Pak Silica-1301N column (Senshu Scientific Co., Ltd.) [HPLC(C)] at 25 °C with *n*-hexanes−EtOAc as the mobile phase. A refractive index detector was used for both reversed- and normal phase HPLC. Gas−liquid chromatography (GLC) for fatty acid methyl esters was run on a model GC-17A instrument (Shimadzu Co., Kyoto, Japan) using a 25 m × 0.25 mm i.d. Quadrex 23 fused-silica column (Quadrex Co., New Haven, CT), column temperature 180 °C, and N$_2$ as a carrier gas (60 mL/min; split ratio 60:1). Hydrolysis of the fatty acid esters of triterpene alcohols was performed with 5% (w/v) KOH in MeOH under reflux for 2 h. Methyl ester derivatives of fatty acids were prepared by refluxing fatty acids with 1% (w/v) H$_2$SO$_4$ in MeOH for 1 h.

Chemicals and Materials. *H. annuus* L. (cultivar: Russian sunflower) was cultivated at an herbal garden of Toho University (Chiba, Japan) in 1997, and pollen grains were collected from the flowers. A voucher specimen was deposited in the herbarium of the School of Pharmaceutical Sciences, Toho University. The pollen grains were stored in a freezer until extraction and isolation. (−)-2-Methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) and (+)-MTPA chlorides and *N,N*-dimethyl-1,3-propanediamine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TPA was purchased from ChemSyn Laboratories (Lenexa, KS). Reference fatty acid methyl esters were obtained from Sigma Chemical Co. (St. Louis, MO). Three triterpene monols, helianol [3,4-*seco*-19(10→9)-*abeo*-8α,9β,10α-tirucalla-4,24-dien-3β-ol; **9**], Δ^7-tirucallol [tirucalla-7,24-dien-3β-ol; **17**], and α-amyrin [urs-12-en-3β-ol; **18**] were used as the reference compounds (*5*).

Extraction and Isolation. The pollen grains of *H. annuus* (336 g) were extracted with Et$_2$O in a Soxhlet apparatus for 48 h, and the solvent was evaporated to give an extract (28 g). The extract was chromatographed on a silica gel (silica gel 60, 230−400 mesh; Merck; 600 g) column with a stepwise gradient of *n*-hexanes−EtOAc [1:0 (2.4 L), 9:1 (3.9 L), 1:1 (2.4 L), 0:1 (1.0 L); v/v] as eluant, which yielded four fractions containing triterpenes: fractions I (R_f 0.66; 2.9 g), II (R_f 0.53; 2.0 g), III (R_f 0.43; 2.4 g), and IV (R_f 0.23; 3.2 g), in addition to three other fractions. On Chromatorex-ODS column chromatography (eluant: MeOH), fraction I yielded a purified fraction (1.0 g), which, upon preparative HPLC(A), yielded seven compounds: helianyl octanoate (**1**; 130 mg, retention time (t_R) 81.8 min), 4α,5α-epoxyhelianyl octanoate (**4**; 39 mg, t_R 39.5 min), 18-(hexadecanoyloxy)octadecenoic acid methyl ester (**25**; 45 mg, t_R 44.8 min), 18-(hexadecanoyloxy)-octadecenoic acid ethyl ester (**26**; 57 mg, t_R 49.1 min), 18-(octadecanoyloxy)octadecenoic acid methyl ester (**27**; 10 mg, t_R 71.3 min), 18-(octadecanoyloxy)octadecenoic acid ethyl ester (**28**; 11 mg, t_R 76.3 min), and (3*E*)-tricos-3-en-5-one (**33**; 40 mg, t_R 16.7 min). Fraction II, upon Chromatorex-ODS column chromatography (eluant: MeOH), gave grandiflorolic acid 15-angelate [*ent*-15β-hydroxykaur-16-en-19-oic acid 15-angelate; grandiflorolic acid angelate, **19**; 116 mg, t_R 5.9 min on HPLC(A)], a mixture of *ent*-kaur-16-en-19-oic acid (**20**) and *ent*-trachyloban-19-oic acid (**22**) [483 mg, t_R 7.3 min on HPLC(A)], and a mixture (420 mg) of (24*S*)-4α,5α:24,25-diepoxyhelianyl octanoate (**5**), (24*R*)-4α,5α:24,25-diepoxyhelianyl octanoate (**6**), (5*S*)-3α-acetyl-2,3,5-trimethyl-7α-hydroxy-5-(4,8,12-trimethyltridecanyl)-1,3α,5,6,7,7α-hexahydro-4-oxainden-1-one (**23**), and 3α-acetyl-2,3,5-trimethyl-7α-hydroxy-5-(4,8,12-trimethyltridecanyl)-1,3α,5,6,7,7α-hexahydro-4-oxainden-1-one (**24**). HPLC(B) of a portion (32 mg) of the mixture of

20 and **22** gave **20** (9 mg; t_R 15.6 min) and **22** (7 mg; t_R 16.7 min). A portion (270 mg) of the mixture of **5**, **6**, **23**, and **24** was subjected to HPLC(A), which yielded a mixture (t_R 14.6 min) of **5** and **6** and a mixture (t_R 19.0 min) of **23** and **24**. Isolation of the individual compounds was undertaken by normal phase HPLC: (24*S*)-**5** (3 mg, t_R 26.4 min) and (24*R*)-**6** (7 mg, t_R 28.8 min) [HPLC(C); *n*-hexanes−EtOAc (92:8, v/v; 1.0 mL/min) as the mobile phase] and (5*S*)-**23** (23 mg, t_R 20.0 min) and (5*R*)-**24** (18 mg, t_R 16.6 min) [*n*-hexanes−EtOAc (20:1, v/v; 1.0 mL/min) as the mobile phase]. Fraction III was dissolved in Et$_2$O and treated with 5% aqueous NaOH solution, and the aqueous NaOH layer was neutralized with 1 M HCl and extracted with Et$_2$O, which eventually yielded neutral lipid (fraction III-1; 1.6 g) and acidic lipid (fraction III-2; 0.8 g) fractions. A portion (500 mg) of fraction III-1, upon HPLC(B), afforded four triterpene monols, **9** (233 mg, t_R 18.5 min), 4α,5α-epoxyhelianol (**12**; 26 mg, t_R 11.6 min), **17** (4 mg, t_R 24.7 min), and **18** (4 mg, t_R 26.3 min). Fraction III-2 was a mixture of fatty acids consisting of lauric acid (12.0%), myristic acid (5.0%), palmitic acid (45.3%), stearic acid (5.7%), oleic acid (6.1%), linoleic acid (3.1%), and linolenic acid (22.1%). A portion (1.0 g) of fraction IV, on HPLC(B), gave nine compounds: a mixture (t_R 21.6 min) of (24*S*)-24,25-dihydroxyhelianyl octanoate (**2**) and (24*R*)-24,25-dihydroxyhelianyl octanoate (**3**), a mixture (t_R 11.1 min) of (24*S*)-24,25-dihydroxy-4α,5α-epoxyhelianyl octanoate (**7**) and (24*R*)-24,25-dihydroxy-4α,5α-epoxyhelianyl octanoate (**8**), grandiflorolic acid (**21**; 31 mg, t_R 4.2 min), *syn*-nonadecane-4,6-diol (**29**; 21 mg, t_R 6.0 min), *syn*-henicosane-4,6-diol (**30**; 13 mg, t_R 12.6 min), *syn*-docosane-4,6-diol (**31**; 17 mg, t_R 18.1 min), and 14,16-dioxopentacosanoic acid (**32**; 50 mg, t_R 15.3 min). HPLC(C) [*n*-hexanes−EtOAc (7:3, v/v; 1.0 mL/min) as the mobile phase] of the mixture of **2** and **3** and the mixture of **7** and **8** enabled the isolation of **2** (27 mg, t_R 21.5 min) and its (24*R*)-isomer (**3**; 7 mg, t_R 22.6 min) and **7** (13 mg; t_R 31.5 min) and its (24*R*)-isomer (**8**; 17 mg, t_R 33.3 min), respectively. All isolated compounds were over 95% pure as estimated by ^{1}H NMR.

Preparation of (*R*)- and (*S*)-MTPA Esters of 2 and 3. A solution of **2** (or **3**) (6 mg, 12 μmol) in dried pyridine (50 μL) was treated with (+)-MTPA chloride (13 μL, 67 μmol), and the mixture was allowed to stand overnight at room temperature. *N,N*-Dimethyl-1,3-propanediamine (13 μL, 101 μmol) was added and allowed to stand for 10 min, and the residue obtained after evaporation of the solvent under the stream of N$_2$ was subjected to HPLC(A) to give pure (*R*)-MTPA ester of **2** (or **3**) (6 mg). Treatment of **2** (or **3**) with (−)-MTPA chloride in the same manner as above gave the (*S*)-MTPA ester.

Conformational Analysis. One thousand step systematic Monte Carlo conformation searches were carried out for **12** and (5*S*)-**23** with the extended molecular mechanics 3 (MM3*) force field as implemented in MacroModel Version 6.0 to predict the fully optimized lowest energy structure (*6*).

Identification and Characterization. *Fatty Acid Esters of Triterpene Alcohols and Their Hydrolysis Products.* Among eight fatty acid esters of triterpene alcohols, **1**−**8**, isolated, seven, **2**−**8**, were new compounds. Identification of a known compound, **1**, was performed by MS and ^{1}H NMR comparison with literature data (*7, 8*). Alkaline hydrolysis of seven triterpene esters, **2**−**8**, with 5% KOH/MeOH gave corresponding free alcohols (24*S*)-24,25-dihydroxyhelianol (**10**), (24*R*)-24,25-dihydroxyhelianol (**11**), **12**, (24*S*)-4α,5α:24,25-diepoxyhelianol (**13**), (24*R*)-4α,5α:24,25-diepoxyhelianol (**14**), (24*S*)-24,25-dihydroxy-4α,5α-epoxyhelianol (**15**), and (24*R*)-24,25-dihydroxy-4α,5α-epoxyhelianol (**16**), respectively. Characterization of the esters, **2**−**8**, and their hydrolysis products, **10**−**16**, was performed on the basis of IR, MS, ^{1}H NMR, and ^{13}C NMR data. Stereochemistry at C-24 of **2** and **3** was determined by measuring the ^{1}H NMR spectra of their MTPA esters (Mosher's method) (*9*). The MS and, when available, IR data along with some physical characteristics of **2**−**8** and **10**−**16** and the ^{1}H NMR data of the (*R*)-MTPA esters, **2*R*** and **3*R***, and (*S*)-MTPA esters, **2*S*** and **3*S***, are shown below. The ^{1}H NMR data of **2**−**8** and **10**−**16** are given in **Table 1**.

Compound 2. Amorphous gum; [α]$_D$ +1.0° (*c* 0.8, CHCl$_3$). EIMS *m/z* (%): 588 (M$^+$, 15), 570 (14), 555 (14), 530 (4), 512 (3), 484 (2), 443 (22), 427 (2), 411 (2), 385 (3), 348 (5), 335 (5), 321 (25), 308 (30), 303 (85), 145 (9), 127 (66), 95 (100). HREIMS *m/z*: 588.5118 [calcd for C$_{38}$H$_{68}$O$_4$ (M$^+$) 588.5118].

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Table 1. ¹H NMR Spectral Data (δ Values; 500 MHz; CDCl₃) of 14 Triterpenes[a]

proton(s)	2[b]	3[b]	4[b]	5	6	7[b]	8[b]	10	11	12	13	14	15	16
1								1.55 (2H)	1.52 (2H)	1.27, 1.51	1.21, 1.50	1.21, 1.50	1.47 (2H)	1.47 (2H)
2								1.32, 1.53	1.32, 1.53	1.47,1.74	1.46, 1.71	1.46, 1.71	1.48, 1.73	1.47, 1.72
3	4.03 (t) (6.3)	4.03 (t) (6.3)	4.01 (2H)	4.02 (2H)	4.02 (2H)	4.01 (2H)	4.01 (2H)	3.62 (t) (6.4)	3.62 (t) (6.4)	3.61 (t) (5.5)	3.61 (m)	3.61 (m)	3.60 (2H,ddd) (2.0, 5.0, 7.1)	3.60 (2H,ddd) (2.5, 5.0, 7.1)
6								2.06 (β), 2.27 (α)	2.06 (β), 2.27 (α)	1.72 (2H)	1.73	1.73	1.73 (2H)	1.72 (2H)
7								1.32 (α), 1.53 (β)	1.32 (α), 1.53 (β)	1.46 (2H)	1.46 (2H)	1.46 (2H)	1.46 (2H)	1.47 (2H)
8								1.55	1.56	1.47	1.60	1.60		1.60
10								2.27	2.27	1.79	1.76	1.76	1.75	1.78
11								1.50 (2H)	1.50 (2H)	1.61 (2H)	1.34 (α), 1.62 (β)	1.34 (α), 1.62 (β)	1.34 (α), 1.60 (β)	1.34 (α), 1.60 (β)
12								1.48 (α), 1.70 (β)	1.50 (α), 1.70 (β)	1.56 (α), 1.73 (β)	1.57 (α), 1.72 (β)	1.57 (α), 1.72 (β)	1.57 (α), 1.75 (β)	1.58 (α), 1.72 (β)
15								1.12 (2H)	1.12 (2H)	1.17 (2H)	1.18 (2H)	1.18 (2H)	1.19 (2H)	1.17 (2H)
16								1.26 (α), 1.91 (β)	1.29 (α), 1.89 (β)	1.30 (α), 1.91 (β)	1.32 (α), 1.92 (β)	1.32 (α), 1.92 (β)	1.31 (α), 1.94 (β)	1.34 (α), 1.92 (β)
17								1.48	1.48	1.47	1.45	1.45	1.46	1.47
18 (13α)	0.80 (s)	0.80 (s)	0.87 (s)	0.88 (s)	0.88 (s)	0.88 (s)	0.87 (s)	0.81 (s)	0.80 (s)	0.87 (s)	0.88 (s)	0.88 (s)	0.87 (s)	0.87 (s)
19 (9β)	0.89 (s)	0.89 (s)	0.86 (s)	0.86 (s)	0.86 (s)	0.85 (s)	0.86 (s)	0.91 (s)	0.91 (s)	0.86 (s)	0.86 (s)	0.86 (s)	0.85 (s)	0.85 (s)
20								1.43	1.44	1.46	1.48	1.50	1.47	1.47
21	0.88 (d) (6.8)	0.90 (d) (8.3)	0.91 (d) (5.5)	0.91 (d) (4.9)	0.91 (d) (5.6)	0.91 (d) (5.4)	0.92 (d) (4.6)	0.91 (d) (6.2)	0.91 (d) (5.9)	0.91 (d) (6.0)	0.92 (d) (6.2)	0.92 (d) (6.2)	0.90 (d) (5.5)	0.91 (d) (5.7)
22								1.25, 1.46	0.98, 1.75	1.04, 1.43	1.21, 1.50	1.09, 1.62	1.26, 1.44	1.00, 1.77
23								1.37 (2H)	1.12, 1.58	1.85, 2.04	1.38, 1.62	1.43, 1.55	1.36 (2H)	1.14, 1.58
24	3.34 (t) (6.6)	3.28 (dd) (2.0, 8.8)	5.10 (t) (7.0)	2.69 (t) (6.1)	2.69 (t) (5.8)	3.35 (t) (4.0)	3.29 (d) (9.7)	3.34 (t) (6.6)	3.28 (dd) (2.1, 10.0)	5.10 (tt) (1.2, 7.0)	2.69 (t) (6.2)	2.69 (t) (6.2)	3.34 (t) (6.6)	3.28 (dd) (2.1, 10.1)
26	1.16 (s)	1.16 (s)	1.69 (s)	1.27 (s)	1.27 (s)	1.16 (s)	1.16 (s)	1.16 (s)	1.16 (s)	1.69 (s)	1.27 (s)	1.27 (s)	1.16 (s)	1.16 (s)
27	1.21 (s)	1.21 (s)	1.61 (s)	1.31 (s)	1.31 (s)	1.21 (s)	1.21 (s)	1.21 (s)	1.21 (s)	1.61 (s)	1.31 (s)	1.31 (s)	1.21 (s)	1.21 (s)
28	1.64 (s)	1.65 (s)	1.32 (s)[c]	1.32 (s)[c]	1.32 (s)[c]	1.32 (s)	1.32 (s)	1.66 (s)	1.66 (s)	1.34 (s)[c]	1.34 (s)[c]	1.34 (s)[c]	1.33 (s)	1.33 (s)
29	1.66 (s)	1.66 (s)	1.37 (s)[c]	1.37 (s)[c]	1.37 (s)[c]	1.37 (s)	1.37 (s)	1.66 (s)	1.66 (s)	1.41 (s)[c]	1.41 (s)[c]	1.41 (s)[c]	1.40 (s)	1.40 (s)
30 (14β)	0.83 (s)	0.83 (s)	0.81 (s)	0.82 (s)	0.82 (s)	0.81 (s)	0.81 (s)	0.84 (s)	0.84 (s)	0.81 (s)	0.81 (s)	0.81 (s)	0.80 (s)	0.80 (s)
Me–CH₂–	0.88 (t) (6.8)	0.88 (t) (6.8)	0.88 (t) (7.0)	0.88 (t) (6.3)	0.88 (t) (6.3)	0.88 (t) (6.3)	0.87 (t) (7.6)							
–CH₂–	1.25 (br s)	1.25 (br s)	1.25 (br s)	1.25 (br s)	1.25 (br s)	1.25 (br s)	1.25 (br s)							
–CH₂–COO–	2.28 (t) (7.6)	2.28 (t) (7.3)	2.28 (t) (7.6)	2.28 (t) (7.6)	2.28 (t) (7.6)	2.28 (t) (7.6)	2.28 (t) (7.6)							

[a] Figures in parentheses denote *J* values (hertz). Signals not specified by a splitting pattern are multiplets. [b] Determined at 400 MHz. [c] Values bearing the same superscript in each column are interchangeable.

(R)-MTPA (2R) and (S)-MTPA (2S) Esters of 2. **2R**: ¹H NMR: δ 0.78 (3H, s, H-18), 0.81 (3H, s, H-30), 0.84 (3H, d, *J* = 6.2 Hz, H-21), 0.89 (3H, s, H-19), 1.13 (3H, s, H-26), 1.17 (3H, s, H-27), 1.64 (3H, s, H-28), 1.66 (3H, s, H-29), 4.03 (2H, t, *J* = 6.6 Hz, H-3), 4.97 (1H, dd, *J* = 2.3, 9.6 Hz, H-24). **2S**: ¹H NMR: δ 0.76 (3H, s, H-18), 0.78 (3H, d, *J* = 6.2 Hz, H-21), 0.81 (3H, s, H-30), 0.89 (3H, s, H-19), 1.16 (3H, s, H-26), 1.22 (3H, s, H-27), 1.64 (3H, s, H-28), 1.66 (3H, s, H-29), 4.03 (2H, t, *J* = 6.6 Hz, H-3), 4.95 (1H, dd, *J* = 2.3, 9.8 Hz, H-24).

Compound 3. Amorphous gum; [α]₀ +13.2° (*c* 0.4, CHCl₃). EIMS *m/z* (%): 588 (M⁺, 9), 570 (8), 555 (8), 530 (3), 512 (3), 443 (16), 348 (4), 321 (21), 303 (66), 145 (9), 127 (60), 95 (100). HREIMS *m/z*: 588.5121 [calcd for C₃₈H₆₈O₄ (M⁺) 588.5118].

(R)-MTPA (3R) and (S)-MTPA (3S) Esters of 3. **3R**: ¹H NMR: δ 0.74 (3H, s, H-18), 0.80 (3H, s, H-30), 0.84 (3H, d, *J* = 6.2 Hz, H-21), 0.88 (3H, s, H-19), 1.16 (3H, s, H-26), 1.22 (3H, s, H-27), 1.64 (3H, s, H-28), 1.66 (3H, s, H-29), 4.03 (2H, t, *J* = 6.4 Hz, H-3), 4.94 (1H, dd, *J* = 2.3, 9.8 Hz, H-24). **3S**: ¹H NMR: δ 0.77 (3H, s, H-18), 0.81 (3H, s, H-30), 0.88 (3H, d, *J* = 7.3 Hz, H-21), 0.89 (3H, s, H-19), 1.13 (3H, s, H-26), 1.17 (3H, s, H-27), 1.64 (3H, s, H-28), 1.66 (3H, s, H-29), 4.03 (2H, t, *J* = 6.6 Hz, H-3), 4.94 (1H, dd, *J* = 2.3, 9.8 Hz, H-24).

Compound 4. Amorphous gum; [α]₀ −6.2° (*c* 2.5, CHCl₃). EIMS *m/z* (%): 570 (M⁺, 28), 555 (18), 537 (4), 527 (38), 510 (7), 495 (5), 484 (4), 469 (3), 457 (3), 441 (3), 426 (6), 408 (12), 393 (4), 384 (11), 369 (26), 295 (21), 145 (10), 127 (25), 69 (100). HREIMS *m/z* (%): 570.5009 [calcd for C₃₈H₆₆O₃ (M⁺) 570.5012].

Compound 5. Amorphous gum; [α]₀ +1.4° (*c* 0.43, CHCl₃). EIMS *m/z* (%): 586 (M⁺, 75), 571 (12), 553 (12), 543 (37), 525 (31), 511 (16), 495 (4), 471 (5), 459 (7), 442 (17), 424 (20), 400 (41), 385 (100), 359 (20), 345 (10), 327 (5), 315 (7), 295 (59), 145 (26), 127 (58). HREIMS *m/z*: 586.4957 [calcd for C₃₈H₆₆O₄ (M⁺) 586.4961].

Compound 6. Amorphous gum; [α]₀ −1.1° (*c* 0.4, CHCl₃). EIMS *m/z* (%): 586 (M⁺, 22), 571 (25), 553 (3), 526 (9), 511 (5), 472 (10), 442 (10), 400 (21), 385 (50), 359 (10), 343 (5), 327 (5), 295 (39), 145 (45), 127 (80), 95 (100). HREIMS *m/z*: 586.4964 [calcd for C₃₈H₆₆O₄ (M⁺) 586.4961].

Compound 7. Amorphous gum; [α]₀ −3.6° (*c* 0.2, CHCl₃). EIMS *m/z* (%): 604 (M⁺, 6), 589 (8), 530 (3), 513 (3), 496 (3), 475 (6), 460 (4), 446 (12), 430 (12), 418 (10), 403 (36), 385 (10), 369 (3), 337 (3), 329 (3), 313 (10), 145 (19), 127 (33), 95 (76), 57 (100). HREIMS *m/z*: 604.5064 [calcd for C₃₈H₆₈O₅ (M⁺) 604.5067].

Compound 8. Amorphous gum; [α]₀ +3.1° (*c* 0.3, CHCl₃). EIMS *m/z* (%): 604 (M⁺, 7), 589 (12), 571 (3), 553 (2), 543 (6), 525 (3), 511 (3), 460 (2), 442 (3), 418 (9), 403 (26), 385 (9), 343 (3), 307 (3), 145 (15), 127 (65), 95 (100), 57 (60). HREIMS *m/z*: 604.5070 [calcd for C₃₈H₆₈O₅ (M⁺) 604.5067].

Compound 10. Amorphous gum. IR *v*ₘₐₓ (cm⁻¹): 3399 (OH). EIMS *m/z* (%): 462 (M⁺, 44), 447 (12), 444 (6), 429 (15), 411 (4), 404 (6), 321 (30), 308 (33), 303 (66), 145 (8), 117 (3), 103 (1), 95 (100), 59 (60). HREIMS *m/z*: 462.4065 [calcd for C₃₀H₅₄O₃ (M⁺) 462.4073].

Compound 11. Amorphous gum. IR *v*ₘₐₓ (cm⁻¹): 3367 (OH). EIMS *m/z* (%): 462 (M⁺, 44), 447 (9), 429 (16), 411 (4), 404 (6), 386 (4), 345 (4), 317 (23), 308 (29), 303 (66), 145 (8), 117 (3), 103 (1), 95 (100), 59 (37). HREIMS *m/z*: 462.4069 [calcd for C₃₀H₅₄O₃ (M⁺) 462.4073].

Compound 12. Amorphous gum; [α]₀ +6.7° (*c* 0.3, CHCl₃). IR *v*ₘₐₓ (cm⁻¹): 3426 (OH). EIMS *m/z* (%): 444 (M⁺, 28), 429 (11), 411 (7), 462 (9), 384 (24), 371 (24), 358 (8), 343 (8), 287 (7), 169 (100). HREIMS *m/z*: 444.3970 [calcd for C₃₀H₅₂O₂ (M⁺) 444.3967].

Compound 13. Amorphous gum. IR *v*ₘₐₓ (cm⁻¹): 3437 (OH). EIMS *m/z* (%): 460 (M⁺, 10), 445 (6), 427 (7), 418 (5), 401 (22), 385 (14), 273 (16), 221 (25), 203 (18), 177 (24), 169 (90), 156 (75), 127 (66),

111 (95), 95 (100). HREIMS *m/z*: 460.3918 [calcd for $C_{30}H_{52}O_3$ (M$^+$) 460.3916].

Compound 14. Amorphous gum. IR ν_{max} (cm^{-1}): 3445 (OH). EIMS *m/z* (%): 460 (M$^+$, 16), 445 (8), 427 (7), 418 (7), 401 (22), 387 (20), 385 (19), 273 (20), 221 (30), 203 (21), 169 (100), 156 (84), 127 (60), 121 (55), 111 (64), 95 (73). HREIMS *m/z*: 460.3920 [calcd for $C_{30}H_{52}O_3$ (M$^+$) 460.3916].

Compound 15. Amorphous gum. IR ν_{max} (cm^{-1}): 3427 (OH). EIMS *m/z* (%): 460 ([M − H$_2$O]$^+$, 3), 445 (32), 419 (47), 401 (38), 383 (13), 359 (6), 343 (3), 315 (7), 145 (10), 117 (5), 111 (100), 103 (2), 59 (45). HREIMS *m/z*: 460.3923 (calcd for $C_{30}H_{52}O_3$ [M − H$_2$O]$^+$ 460.3916).

Compound 16. Amorphous gum. IR ν_{max} (cm^{-1}): 3388 (OH). EIMS *m/z* (%): 478 (M$^+$, 6), 463 (7), 445 (5), 421 (7), 405 (10), 385 (7), 377 (5), 345 (3), 333 (1), 315 (1), 145 (47), 117 (5), 111 (93), 103 (3), 95 (100), 59 (60). HREIMS *m/z*: 478.4018 [calcd for $C_{30}H_{54}O_4$ (M$^+$) 478.4022].

Free Triterpene Monols. Among the four triterpene monols isolated (**9**, **12**, **17**, and **18**), three (**9**, **17**, and **18**) were known compounds and these were identified by chromatographic (HPLC and GLC) and spectroscopic (MS and ^{1}H NMR) comparison with reference compounds. A new triterpene alcohol, **12**, was identified as 4α,5α-epoxyhelianol by spectroscopic comparison with data for **12**, which was obtained from **4** by alkaline hydrolysis as described above.

Diterpene Acids. Four known diterpene acids, **19**−**22**, were isolated, and their identification was done by MS and ^{1}H NMR comparison with literature values (*10*−*12*).

Tocopherol-Related Compounds. A new compound, (5*S*)-**23**, was isolated along with known (5*R*)-**24**, of which ^{1}H NMR data were given below for comparison. Compound **24** was identified by MS and ^{1}H NMR comparison with literature data (*13*). Characterization of **23** was performed on the basis of IR, MS, ^{1}H NMR, and ^{13}C NMR data.

Compound 23. Amorphous gum; [α]$_D$ −122.5° (*c* 1.1, CHCl$_3$). UV λ_{max}: 250 nm (ethanol). IR ν_{max} (cm^{-1}): 3427, 1714, 1652, 1461, 1378. EIMS *m/z* (%): 462 (M$^+$, 6), 419 (100), 402 (48), 237 (4), 191 (4), 177 (3), 153 (15), 137 (22), 125 (7), 109 (7). HREIMS *m/z* (%): 462.3703 [calcd for $C_{29}H_{50}O_4$ (M$^+$) 462.3709]. ^{1}H NMR: δ 0.84 and 0.85 (each 3H and d, *J* = 5.7 Hz, H-14″ and H-15″), 0.87 (6H, d, *J* = 6.6 Hz, H-13″ and H-16″), 1.32 (3H, s, H-5′), 1.78 (2H, m, H-6), 1.82 (3H, d, *J* = 0.9 Hz, H-3′), 1.84 (3H, d, *J* = 0.9 Hz, H-4′), 1.87 (1H, ddd, *J* = 4.3, 6.0, 8.5 Hz, H-7α), 2.02 (3H, s, H-2′), 2.37 (1H, ddd, *J* = 6.0, 6.0, 6.0 Hz, H-7β), 4.69 (1H, s, 7a-OH). ^{13}C NMR: δ 205.1 (C-1), 139.5 (C-2), 163.1 (C-3), 89.4 (C-3a), 87.2 (C-5), 36.8 (C-6), 33.4 (C-7), 92.6 (C-7a), 206.9 (C-1′), 24.8 (C-2′), 11.8 (C-3′), 8.7 (C-4′), 42.1 (C-1″), 22.4 (C-2″), 37.3 (C-3″), 32.7 (C-4″), 37.4, 37.5, 37.6 (C-5″, C-7″, C-9″), 24.5 (C-6″), 32.8 (C-8″), 24.8 (C-10″), 39.4 (C-11″), 28.0 (C-12″), 22.6, 22.7 (C-13″, C-16″), 19.6, 19.8 (C-14″, C-15″).

Compound 24. UV λ_{max}: 250 nm (ethanol). ^{1}H NMR: δ 0.84 (3H, d, *J* = 6.8 Hz) and 0.85 (3H, d, *J* = 7.6 Hz) (H-14″ and H-15″), 0.87 (6H, d, *J* = 6.8 Hz, H-13″ and H-16″), 1.06 (3H, s, H-5′), 1.83 (3H, s, H-3′), 1.84 (3H, s, H-4′), 2.02 (3H, s, H-2′), 4.73 (1H, s, 7a-OH).

Estolides. Among the four estolide type aliphatic esters (*14*), **25**−**28**, isolated, two were identified as **25** and **27** by MS comparison with the corresponding semisynthetic known compounds cited in the literature (*7*). Characterization of two new estolides, **26** and **28**, was done by MS and ^{1}H NMR comparison with those of **25** and **27** and literature data for the relevant compounds (*7*).

Compound 26. Amorphous gum. EIMS *m/z* (%): 564 (M$^+$, 15), 518 (37), 500 (12), 322 (7), 308 (12), 280 (26), 262 (32), 257 (13), 239 (42), 109 (51), 95 (79), 81 (82), 55 (100). HREIMS *m/z*: 564.5121 [calcd for $C_{36}H_{68}O_4$ (M$^+$) 564.5118]. ^{1}H NMR: δ 0.88 (3H, t, *J* = 7.3 Hz), 1.25 (3H, t, *J* = 7.3 Hz), 1.26 (brs), 1.61 (4H, t, *J* = 7.0 Hz), 2.01 (4H, dd, *J* = 6.4, 11.9 Hz), 2.28 (4H, t, *J* = 7.3 Hz), 4.05 (2H, t, *J* = 6.7 Hz), 4.12 (2H, dd, *J* = 7.3, 14.3 Hz), 5.34 (2H, t, *J* = 4.6 Hz).

Compound 28. Amorphous gum. EIMS *m/z* (%): 592 (M$^+$, 41), 546 (79), 528 (22), 322 (12), 308 (18), 285 (33), 280 (32), 267 (47), 262 (37), 123 (31), 109 (45), 95 (62), 81 (71), 55 (100). HREIMS *m/z*: 592.5431 [calcd for $C_{38}H_{72}O_4$ (M$^+$) 592.5431]. ^{1}H NMR: δ 0.88 (3H, t, *J* = 6.3 Hz), 1.25 (3H, t, *J* = 7.3 Hz), 1.26 (br s), 1.62 (4H, t, *J* = 6.6 Hz), 2.01 (4H, dd, *J* = 6.3, 11.7 Hz), 2.29 (4H, t, *J* = 7.6 Hz), 4.05 (2H, t, *J* = 6.6 Hz), 4.12 (2H, dd, *J* = 7.1, 14.2 Hz), 5.34 (2H, t, *J* = 4.6 Hz).

syn-Alkane-4,6-diols. Three known *syn*-alkane-4,6-diols, **29**−**31**, were isolated, and their identification was done by MS and ^{1}H NMR comparison with literature values (*7*, *15*−*17*). The absolute configurations at C-4 and C-6 (*R*,*S* and/or *S*,*R*) of these alkanediols remained undetermined.

1,3-Dioxoalkanoic Acid. A known isolated 1,3-dioxoalkanoic acid, **32**, was identified by MS and ^{1}H NMR comparison with literature values (*7*).

Aliphatic Ketone. Characterization of a new isolated aliphatic ketone, **33**, was done by IR, MS, and ^{1}H NMR comparison with literature data for the relevant compound (*18*).

Compound 33. Amorphous gum. UV λ_{max}: 220 nm (*n*-hexane). IR ν_{max} (cm^{-1}): 1693, 1468, 1375. EIMS *m/z* (%): 336 (M$^+$, 15), 293 (51), 153 (6), 125 (12), 112 (100), 97 (63), 83 (12), 69 (15), 55 (30). HREIMS *m/z*: 336.3393 [calcd for $C_{23}H_{44}O$ (M$^+$) 336.3392]. ^{1}H NMR: δ 0.88 (3H, t, *J* = 6.3 Hz, H-23), 0.94 (3H, t, *J* = 7.3 Hz, H-1), 1.26 (br s), 2.19 (2H, dd, *J* = 6.6, 13.9 Hz, H-2), 2.52 (2H, t, *J* = 7.3 Hz, H-6), 6.09 (1H, d, *J* = 15.9 Hz, H-4), 6.82 (1H, dt, *J* = 6.8, 15.6 Hz, H-3).

Free Fatty Acids. Identification and determination of the composition of free fatty acids were performed as their methyl ester derivatives by GLC.

In Vitro EBV-EA Activation Experiment. The inhibition of EBV-EA activation was assayed using Raji cells (EBV genome-carrying human lymphoblastoid cells; nonproducer type), cultivated in 10% fetal bovine serum RPMI-1640 medium (Sigma). The indicator cells (Raji cells; 1 ×10^6 cells/mL) were incubated in 1 mL of the medium containing 4 mM *n*-butyric acid as an inducer, 32 pM TPA (20 ng/mL in dimethyl sulfoxide), and a known amount (32, 16, 3.2, and 0.32 nmol) of the test compound at 37 °C in a CO$_2$ incubator. After 48 h, the cell suspensions were centrifuged at 1000 rpm for 10 min, and the supernatant was removed. The activated cells were stained with high-titer EBV-EA positive sera from nasopharyngeal carcinoma patients, and the conventional indirect immunofluorescence technique was employed for detection. In each assay, at least 500 cells were counted and the experiments were repeated three times. The average extent of EA induction was determined and compared with that on positive control experiments in which the cells were treated with *n*-butyric acid plus TPA where the extent of EA induction was ordinarily more than around 40%. The viability of treated Raji cells was assayed by the Trypan Blue staining method (*19*).

RESULTS

Eight fatty acid esters of triterpene alcohols, four free triterpene alcohols, four diterpene acids, two tocopherol-related compounds, four estolides, three *syn*-alkane-4,6-diols, one 1,3-dioxoalkanoic acid, and one aliphatic ketone, in addition to free fatty acids, were isolated and characterized from the Et$_2$O extract of *H. annuus* pollen in this study. Structural determination of the new compounds is described below.

Fatty Acid Esters of Triterpene Alcohols and Free Triterpene Alcohols. Structures of seven triterpene esters, **2**−**8**, and seven triterpene alcohols, **10**−**16**, and stereochemistry at C-24 of **2** and **3** by means of Mosher's method were determined as described below.

Compounds 2, 3, 10, and 11. The MS of **2** and **3** showed a M$^+$ at *m/z* 588 ($C_{38}H_{68}O_4$), and characteristic ions at *m/z* 145 and 127 pointed to octanoate ester (*7*). Alkaline hydrolysis of compounds **2** and **3** gave compounds **10** and **11**, respectively. The mass spectrum of **10** showed a M$^+$ at *m/z* 462 ($C_{30}H_{52}O_3$). Compound **10** has one oxymethine (δ_H 3.34), two olefinic methyls (2 × CH$_3$; δ_H 1.66), one secondary methyl (δ_H 0.90), and five tertiary methyls (δ_H 0.81, 0.84, 0.91, 1.16, and 1.21). By comparison of ^{1}H and ^{13}C NMR data with those of **9**, **10** was found to have the same 3,4-*seco*-triterpene structure with a C$_8$ side chain possessing two hydroxyl groups. The resonances

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Table 2. ^{13}C NMR Spectral Data (δ Values; CDCl$_3$) of Seven Triterpenes

carbon(s)	10	11	12	13	14	15	16
1	32.2	32.2	21.2	21.4	21.4	21.4	21.4
2	26.4	26.4	34.8	34.8	34.8	34.9	34.9
3	63.9	63.9	62.4	62.4	62.4	62.4	62.4
4	122.3	122.3	63.7	63.6	63.6	63.6	63.7
5	134.3	134.2	68.8	68.5	68.5	68.8	68.6
6	25.2	25.1	23.0	23.0	23.0	23.0^a	23.0^a
7	23.7	23.7	22.8	22.9	22.9	22.8^a	22.8^a
8	44.1	44.1	40.5	40.5	40.5	40.5	40.4
9	38.7	38.6	40.5	40.5	40.5	40.5	40.4
10	55.0	55.0	57.2	57.2	57.2	57.3	57.3
11	39.2	39.2	34.8	34.8	34.8	34.9	34.9
12	30.4	30.4	30.0	30.0	30.0	30.1	30.1
13	46.2	46.2	46.2	46.2	46.2	46.3	46.3
14	47.9	47.9	48.1	48.1	48.1	48.2	48.1
15	34.2	34.2	34.2	34.2	34.2	34.3	34.3
16	28.2	28.1	28.0	28.1	28.0	28.2	28.1
17	50.7	50.6	50.3	50.2	50.3	50.6	50.4
18 (13α)	15.5	15.5	15.8	15.9	15.9	15.9	15.9
19 (9β)	18.2	18.2	17.4	17.4	17.4	17.5	17.5
20	35.9	36.4	35.7	35.7	35.8	35.8	36.3
21	18.6	18.9	18.6	18.5	18.6	18.5	18.8
22	33.3	33.8	36.4	32.7	32.9	33.2	33.7
23	28.5	28.8	24.9	25.6	25.9	28.5	28.8
24	78.8	79.7	125.2	64.8	64.9	78.8	79.7
25	73.2	73.3	131.0	58.4	58.1	73.2	73.3
26	23.3	23.3	25.7	18.8	18.7	23.3	23.3
27	26.6	26.6	17.6	25.0	25.0	26.7	26.6
28	20.9	20.9	23.7^a	23.7^a	23.7^a	23.7^a	23.8^a
29	20.9	20.9	22.6^a	22.6^a	22.6^a	22.7^a	22.7^a
30 (14β)	19.0	19.0	19.4	19.4	19.4	19.4	19.4

^{a} Values bearing the same superscript in each column are interchangeable.

of two methyl singlets at δ_H 1.16 and 1.21, attributed to H-26 and H-27, suggested that one of the hydroxyl groups in the side chain was present at C-25 (*20*). This was supported by heteronuclear multiple bond correlation (HMBC) spectroscopy, which provided cross-correlations for δ_H 1.16 (with C-24, C-25, and C-27) and δ_H 1.21 (with C-24, C-25, and C-26). The presence of cross-correlations for δ_H 3.34 (H-24) with C-22, C-23, C-25, C-26, and C-27 in the HMBC spectrum suggested that the other side chain hydroxyl group was located at C-24, which was supported by the diagnostic MS fragment ions at m/z 145 (C$_8$H$_{17}$O$_2^+$; side chain), 117 (C$_6$H$_{13}$O$_2^+$, C$_{22}$−C$_{27}$), 103 (C$_5$H$_{11}$O$_2^+$, C$_{23}$−C$_{27}$), and 59 (C$_3$H$_7$O$^+$, C$_{25}$−C$_{27}$). Further analysis of the ^{13}C DEPT, ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H detected multiple quantum coherence (HMQC), and nuclear Overhauser enhancement (NOE) and exchange spectroscopy (NOESY) spectra and comparison of the ^{1}H (**Table 1**) and ^{13}C NMR spectral data (**Table 2**) with those of relevant compounds (*8, 20*) revealed the structure of **10** to be 24,25-dihydroxyhelianol [3,4-*seco*-19(10→9)-*abeo*-8α,9β,10α-tirucall-4-ene-3β,24,25-triol]. Close similarity of the MS, ^{1}H, and ^{13}C NMR data of **11** with those of **10** suggested that **11** was a stereoisomer at C-24 of **10**. The absolute configuration at C-24 was determined by application of the modified Mosher's method (*9*) for (*R*)-MTPA (**2R**) and (*S*)-MTPA (**2S**) esters of **2**. As shown in **Figure 2**, the Δδ ($\delta_S − \delta_R$) values for H-26 and H-27 (Δδ = 0.03 and 0.05) were found to be positive, whereas those for the H-18 (Δδ = −0.02) and H-21 (Δδ = −0.06) were negative, which unequivocally demonstrated that **2** possesses 24*S* stereochemistry (*9*). On the other hand, **3** showed almost the opposite Δδ values for the corresponding signals of (*R*)-MTPA (**3R**) and (*S*)-MTPA (**3S**) esters from those of the MTPA esters of **2** (**Figure 2**) indicating that **3** is a 24*R* stereoisomer. The combined evidence confirmed that compounds **2**, **3**, **10**, and **11** were (24*S*)-24,25-dihydroxyhelianyl octanoate, (24*R*)-

24,25-dihydroxyhelianyl octanoate, (24*S*)-24,25-dihydroxyhelianol, and (24*R*)-24,25-dihydroxyhelianol, respectively.

Compounds 4 and 12. The MS of compound **4** showed a M$^+$ at m/z 570 (C$_{38}$H$_{66}$O$_3$), and characteristic fragment ions at m/z 145 and 127 pointed to an octanoate ester (*7*). Alkaline hydrolysis of **4** gave a compound **12**, which showed a M$^+$ at m/z 444 (C$_{30}$H$_{52}$O$_2$) in the MS and IR absorptions due to a hydroxyl group (3426 cm^{-1}) and a trisubstituted double bond (821 cm^{-1}). Compound **12** has one olefinic methine (δ_H 5.10), one oxymethylene (2H; δ_H 3.61), two olefinic methyls (δ_H 1.61, 1.69), one secondary methyl (δ_H 0.91), and five tertiary methyls (δ_H 0.81, 0.86, 0.87, 1.34, and 1.41). Compound **12**, in its MS, displayed peaks at m/z 333 [M$^+$ − C$_8$H$_{15}$ (side chain)], 302 [M$^+$ − C$_6$H$_{11}$ (C$_{23}$−C$_{27}$) − CH$_2$=CHCH$_2$OH], 275 [m/z 333 − CH$_2$=CHCH$_2$OH], 274 [M$^+$ − C$_{10}$H$_{18}$O$_2$ (*seco*-A and B rings)], and 163 (m/z 274 − C$_8$H$_{15}$), which suggested a 3,4-*seco*-triterpene structure and a C-24 monounsatureted C$_8$ side chain (*8*). A further fragment ion at m/z 169 (C$_{10}$H$_{17}$O$_2^+$, *seco*-A and B rings; base peak) suggested the presence of an epoxy group in the ring system located most probably at C-4. This was supported by HMBC spectroscopy, which provided cross-correlations for H-28 (with C-4, C-5, and C-29) and H-29 (with C-4, C-5, and C-28). Further analysis of the ^{13}C DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra and comparison of the ^{1}H and ^{13}C NMR data (**Tables 1** and **2**) with those of compound **9** and relevant compound (*8*) revealed the structure of **12** to be 4α,5α-epoxyhelianol. The most stable conformation of **12** with minimum steric energy was simulated using MacroModel. The results of the calculations are shown in **Figure 3** together with the significant NOEs (←→). The conformation of **12** was fairly consistent with the result from the NOE experiment carried out in solution. Compound **12** exhibited definite NOE correlations between [H-28 (4-Me)−H-19 (9β-Me)−H-30 (14β-Me)−H-17−H-21] on the β-face of molecule in the NOESY, which indicated that the epoxy group (C-4) was oriented to the α-face of the ring system. Thus, we concluded that compound **12** is 4α,5α-epoxyhelianol [4α,5α-epoxy- 3,4-*seco*-19(10→9)-*abeo*-8α,9β,10α-tirucall-4-en-3β-ol], and **4** is 4α,5α-epoxyhelianyl octanoate.

Compounds 5, 6, 13, and 14. The MS of **5** and **6** showed a M$^+$ at m/z 586 (C$_{38}$H$_{66}$O$_4$), and characteristic fragment ions at m/z 145 and 127 pointed to octanoate ester (*7*). Alkaline hydrolysis of **5** and **6** gave compounds **13** and **14**, respectively, both of which showed a M$^+$ at m/z 460 (C$_{30}$H$_{52}$O$_3$). Compounds **13** and **14** displayed almost the same ^{1}H NMR signals with each other: one oxymethine (δ_H 2.69), one oxymethylene (2H, δ_H 3.61), one secondary methyl (δ_H 0.92), and seven tertiary methyls (δ_H 0.81, 0.86, 0.88, 1.27, 1.31, 1.33, and 1.41). The ^{1}H and ^{13}C NMR signals (**Tables 1** and **2**) arising from triterpene ring systems of **13** and **14** were almost indistinguishable from those of the corresponding signals of **4**, suggesting that **13** and **14** possess a 4α,5α-epoxyhelianol structure. The ^{1}H NMR signals of the oxymethine (δ_H 2.69) and the tertiary methyls (δ_H 1.27, 1.31) suggested the presence of an epoxy group at C-24 in the side chain (*21*), which was supported by HMBC experiment in which cross-correlations for H-24 (with C-22, C-23, C-25, C-26, and C-27), H-26 (with C-24, C-25, and C-27), and H-27 (with C-24, C-25, and C-26) were observed. The ^{13}C NMR chemical shift differences for the side chain ^{13}C signals made possible the stereochemical assignment at C-24 of **13** and **14**. Thus, 24*R* and 24*S* epimers of 24,25-epoxycycloartanol (24,25-epoxycycloartan-3β-ol) exhibited ^{13}C NMR chemical shift differences ($\delta_R − \delta_S$) for the side chain ^{13}C signals as C-20 ($\delta_R − \delta_S$ = 0.1), C-21 (−0.1), C-22 (0.2), C-23 (0.3), C-24
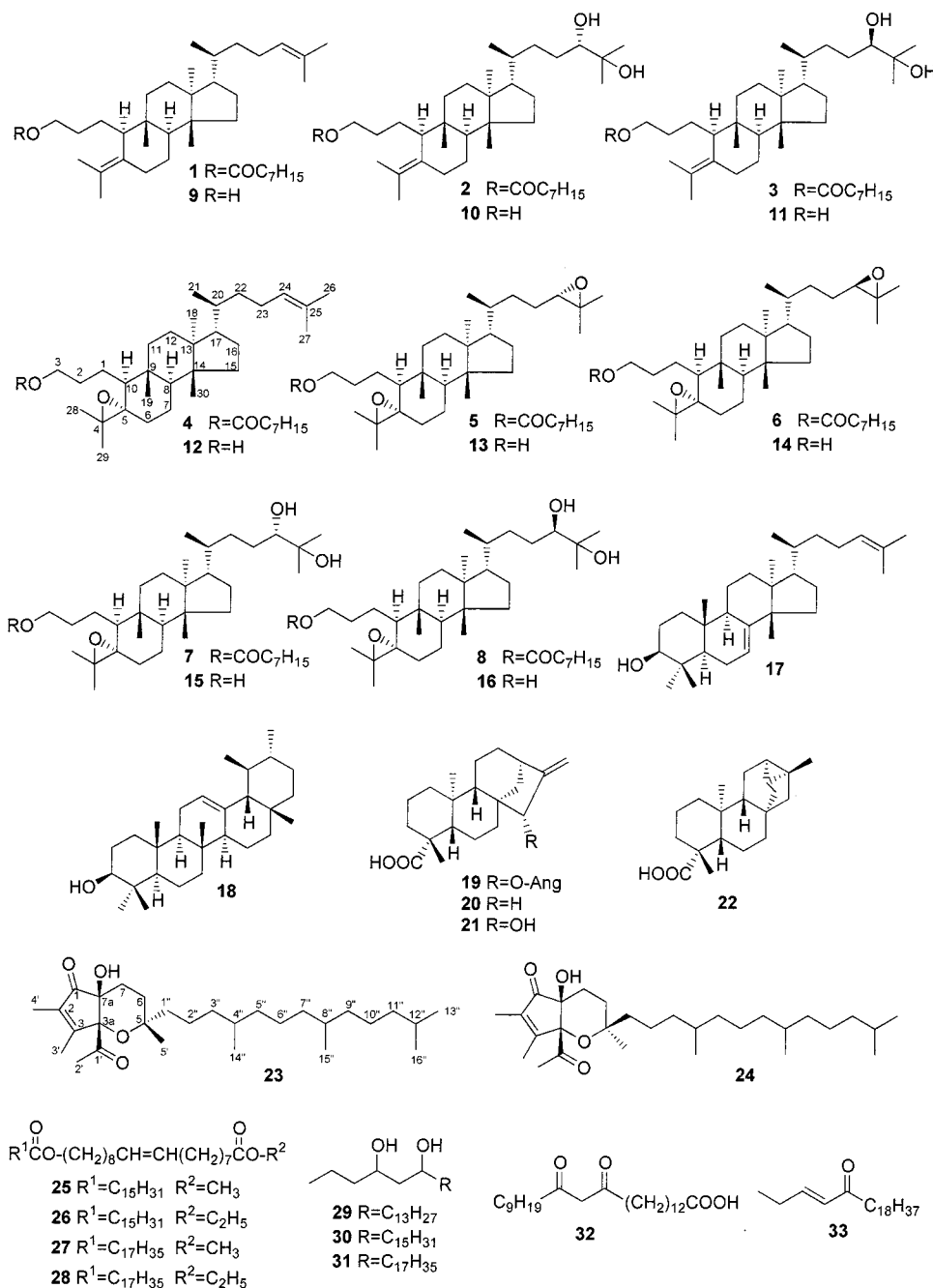
Figure 1. Structures of compounds in this study (**19**: Ang = angeloyl).

(0.2), C-25 (−0.4), C-26 (−0.2), and C-27 (0.0) (*20*), which were almost consistent with those observed between **13** and **14**: C-20 ($\delta_{14} - \delta_{13} = 0.1$), C-21 (0.1), C-22 (0.2), C-23 (0.3), C-24 (0.1), C-25 (−0.3), C-26 (−0.1), and C-27 (0.0), as calculated from the ^{13}C NMR data in **Table 2**. Compounds **13** and **14** were, therefore, attributed to the 24*S* and 24*R* epimers of 4α,5α:24,25-diepoxyhelianol [4α,5α:24,25-diepoxy- 3,4-*seco*-19(10→9)-*abeo*-8α,9β,10α-tirucallan-3β-ol], respectively. Analysis of the ^{13}C DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra supported the proposed structure. Fatty acid esters **5** and **6** were, hence, concluded to have the structures (24*S*)-4α,5α:24,25-diepoxyhelianyl octanoate and (24*R*)-4α,5α: 24,25-diepoxyhelianyl octanoate, respectively.

Compounds 7, 8, 15, and 16. The MS of **7** and **8** showed a M$^+$ at *m/z* 604 (C$_{38}$H$_{68}$O$_5$) and characteristic fragment ions at *m/z* 145 and 127 suggesting these to be octanoate esters (*7*). Alkaline hydrolysis of **7** and **8** gave compounds **15** and **16**,

respectively, both of which displayed [M − H$_2$O]$^+$ at *m/z* 460 (C$_{30}$H$_{52}$O$_3$) in the MS. A close similarity of the ^{1}H and ^{13}C NMR signals (**Tables 1** and **2**) for the ring systems of **15** and **16** with the corresponding signals of compounds **12**−**14** suggested that compounds **15** and **16** possess the 4α,5α-epoxyhelianol ring system structures. On the other hand, compounds **15** and **16** exhibited the C$_8$ side chain signals in the ^{1}H and ^{13}C NMR spectra very close to those of compounds **10** and **11** (**Tables 1** and **2**), which suggested that compounds **15** and **16** possess a 24,25-dihydroxylated C$_8$ side chain. Thus, compounds **15** and **16** are proposed to have the structure of 24,25-dihydroxy-4α,5α-epoxyhelianol [24,25-dihydroxy-4α,5α-epoxy-3,4-*seco*-19(10→9)-*abeo*-8α,9β,10α-tirucallan-3β-ol], which was supported from the analysis of their MS, ^{13}C DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra. Compounds **15** and **16** exhibited the signals of H-24 [**15**: δ_H 3.34 (t, *J* = 6.6 Hz); **16**: δ_H 3.28 (dd, *J* = 2.1, 10.1 Hz)] and C-24
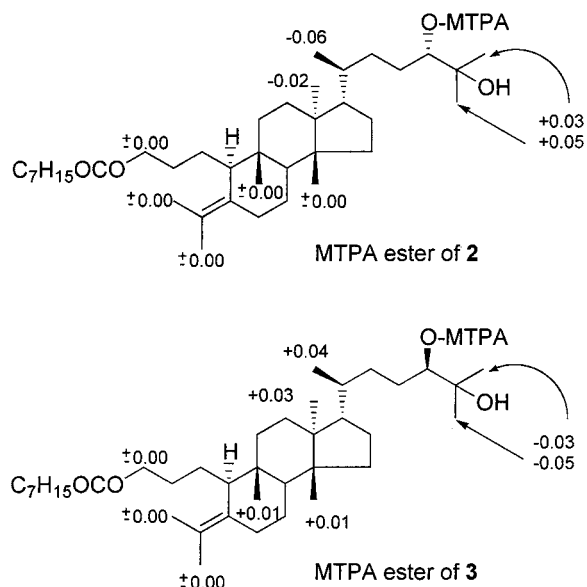
Figure 2. Chemical shift differences ($\Delta\delta$) between (*S*)-MTPA and (*R*)-MTPA esters of (24*S*)-(**2**) and **3**.
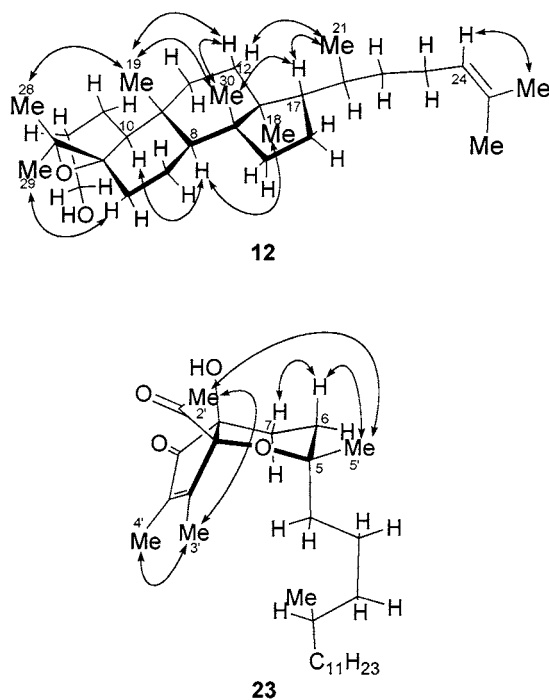

Figure 3. Energy-minimized conformations and some representative NOE correlations ($\leftarrow\rightarrow$) for **12** and (5*S*)-**23**.

(**15**: δ_C 78.8; **16**: δ_H 79.7), which were consistent with those of (24*S*) (**10**) and (24*R*) (**11**) epimers of 24,25-dihydroxyhelianol (**Tables 1** and **2**), respectively, indicating that **15** has a 24*S* configuration while **16** has a 24*R* configuration. In conclusion, compounds **7**, **8**, **15**, and **16** were established to possess the structures of (24*S*)-24,25-dihydroxy-4α,5α-epoxyhelianyl octanoate, (24*R*)-24,25-dihydroxy-4α,5α-epoxyhelianyl octanoate, (24*S*)-24,25-dihydroxy-4α,5α-epoxyhelianol, and (24*R*)-24,25-dihydroxy-4α,5α-epoxyhelianol, respectively.

Tocopherol-Related Compound. The MS of compound **23** showed a M$^+$ at *m/z* 462 (C$_{29}$H$_{50}$O$_4$). Compound **23** possesses a hydroxyl group (OH; δ_H 4.69), an acetyl methyl (δ_H 2.02), two olefinic methyls (δ_H 1.82, 1.84), a tertiary methyl (δ_H 1.32), and four secondary methyls (δ_H 0.84, 0.85, 0.87, and 0.87) as

shown by ^{1}H NMR spectroscopy. The IR, MS, and ^{1}H NMR spectral data of **23** were almost superimposable with those of known (5*R*)-**24** (*11*), with one exception for the H-5′ methyl signal. Compound **23** exhibited it at δ_H 1.32 whereas **24** exhibited it at a distinctively higher field, δ_H 1.06, suggesting that **23** was the stereoisomer of **24** at C-5. The most stable conformation for the proposed structure of **23** with minimum steric energy was simulated using MacroModel, and the results are shown in **Figure 3** together with some representative NOEs ($\leftarrow\rightarrow$). Compound **23** exhibited definite NOE correlations between [H-2′ (3a-acetyl)−H-5′ (5β-Me)−H-6β−H-7β] and [H-2′ (3a-acetyl)−H-3′ (3-Me)−H-4′ (2-Me)] of the molecule in the NOESY, which supported the proposed structure, i.e., the methyl group at C-5 of **23** is oriented to the β-face of the ring system. Therefore, the structure of **23** was assigned as (5*S*)-3a-acetyl-2,3,5-trimethyl-7a-hydroxy-5-(4,8,12-trimethyltride-canyl)-1,3a,5,6,7,7a-hexahydro-4-oxainden-1-one. Analysis of ^{13}C DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra confirmed the proposed structure. Stereochemistry at C-4″ and C-8″ of **23** remained undetermined.

Estolides. Compound **26** showed a M$^+$ at *m/z* 564 (C$_{36}$H$_{68}$O$_4$) in the MS and fragment ions at *m/z* 322 [M$^+$ − C$_{14}$H$_{28}$ − C$_2$H$_5$OH], 308 [C$_{17}$H$_{31}$COOC$_2$H$_5$$^+$], 280 [M$^+$ − C$_{14}$H$_{28}$ − C$_2$H$_5$OCOH=CH$_2$], 262 [M$^+$ − C$_{14}$H$_{28}$ − C$_2$H$_5$COCH=CH$_2$ − H$_2$O], 257 [C$_{15}$H$_{31}$COOH$_2$$^+$], and 239 [C$_{15}H_{31}CO^+$]. The ^{1}H NMR spectrum of **26** was very close to that of **25** with the exception for the presence of ethoxy signals [δ_H 1.25 (3H, t) and 4.12 (2H, q)] in the spectrum of **26** instead of a methoxy signal [δ_H 3.67 (3H, s)] of **25**. This indicated that **26** was an ethyl ester homologue of **25**, i.e., **26**. Compound **28** showed a M$^+$ at *m/z* 592 (C$_{38}$H$_{72}$O$_4$) in the MS along with fragment ions at *m/z* 322 [M$^+$ − C$_{16}$H$_{32}$ − C$_2$H$_5$OH], 308 [C$_{17}$H$_{31}$COOC$_2$H$_5$$^+$], 285 [C$_{17}H_{35}$COOH$_2$$^+$], 280 [M$^+$ − C$_{16}$H$_{32}$ − C$_2$H$_5$OCOH=CH$_2$], 267 [C$_{17}$H$_{35}$CO$^+$], and 262 [M$^+$ − C$_{16}$H$_{32}$ − C$_2$H$_5$COCH=CH$_2$ − H$_2$O]. It, in connection with the good agreement of the ^{1}H NMR spectrum of **28** with that of **26**, was concluded that **28** is 18-(octadecanoyloxy)octadecenoic acid ethyl ester.

Aliphatic Ketone. The MS of **33** showed a M$^+$ at *m/z* 336 (C$_{23}$H$_{44}$O) and fragment ions at *m/z* 293 [C$_{18}$H$_{37}$COC$^+$], 112 [C$_2$H$_5$CH=CHCOC$_2$H$_5$$^+$], 97 [C$_2H_5$CH=CHCOCH$_2$$^+$], 83 [C$_2H_5$CH=CHCO$^+$], and 55 [C$_2H_5$CH=CH$^+$]. The IR spectrum of **33** showed the presence of an α,β-unsaturated ketone (ν_{max} 1693 cm^{-1}), which is consistent with the ^{1}H NMR resonances at δ_H 6.09 (d, *J* = 15.9 Hz) and 6.82 (dt, *J* = 6.8, 15.6 Hz) (−CH=CHCO−). The above evidence, coupled with the large coupling constants (*J* ≈ 16 Hz) of the olefinic methine signals, suggested that compound **33** possesses the structure (3*E*)-tricos-3-en-5-one.

Inhibitory Effect of Sunflower Pollen Terpenoid and Lipid Constituents on EBV-EA Induction. The inhibitory effects on the induction of EBV-EA induced by TPA were examined as a preliminary evaluation of the potential antitumor-promoting activities for 24 sunflower pollen constituents, **1**−**4**, **6**−**9**, **12**, and **19**−**33**, and six triterpene alcohols, **10**, **11**, and **13**−**16**, derived from their fatty acid esters by alkaline hydrolysis. The inhibitory effects (**Table 3**) were compared with those of reference compound, β-carotene, a vitamin A precursor that has been studied extensively in cancer chemoprevention using animal models (*22*). Among the 30 compounds tested, 21 compounds possessing a di- or a polycyclic ring system in the molecule (**1**−**4**, **6**−**16**, and **19**−**24**) exhibited potent inhibitory effects (91−100% inhibition at 1 × 10^3 mol ratio/TPA and 56−81% inhibition at 5 × 10^2 mol ratio/TPA) on EBV-EA

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Table 3. Percentage of EBV-EA Induction in the Presence of 30 Compounds with Respect to a Positive Control (100%)[a]

compd	concentration (mol ratio/TPA)			
	1000	500	100	10
1	2.6 (70)	26.4	75.8	100
2	0 (60)	21.6	66.7	92.4
3	0 (60)	22.7	67.9	93.9
4	9.3 (70)	35.0	77.0	100
6	3.1 (70)	31.3	78.1	100
7	0 (70)	30.0	73.5	98.6
8	0 (60)	25.4	70.0	95.7
9	0 (70)	23.8	70.2	93.0
10	0 (60)	19.1	63.5	82.7
11	0 (60)	18.5	62.3	81.6
12	0 (70)	28.6	73.8	95.8
13	0 (70)	23.4	69.3	95.7
14	0 (70)	22.5	68.3	94.4
15	0 (60)	23.2	64.4	85.0
16	0 (60)	21.3	65.3	83.9
19	0 (60)	21.6	70.0	94.0
20	2.5 (60)	23.7	71.1	95.3
21	2.0 (60)	21.5	70.0	92.1
22	0 (60)	22.6	69.5	92.1
(5S)-23	6.9 (60)	41.0	75.2	100
(5R)-24	7.2 (60)	43.6	76.0	100
25	18.6 (70)	51.4	82.7	100
26	18.9 (70)	52.4	82.8	100
27	20.7 (70)	53.6	83.5	100
28	21.1 (70)	55.6	84.7	100
29	22.3 (70)	57.0	85.0	100
30	23.5 (70)	57.9	85.3	100
31	23.7 (70)	58.1	86.1	100
32	14.7 (70)	48.6	82.1	100
33	19.2 (70)	51.1	84.3	100
β-carotene[b]	8.6 (70)	34.2	82.1	100

[a] Values represent relative percentages to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cell. [b] Reference compound.

induction by TPA with preservation of the high viability (60−70%) of the Raji cells. The other nine aliphatic compounds (**25**−**33**) showed, however, not so potent effects (75−85% inhibition at 1×10^3 mol ratio/TPA).

DISCUSSION

Our investigation on the constituents of the pollen extract of *H. annuus*, with special attention to terpenoid constituents, led to the isolation and characterization of 27 compounds consisting of four triterpene monol fatty acid esters (**1**, **4**, **5**, and **6**), four triterpene triol monoesters (**2**, **3**, **7**, and **8**), four free triterpene monols (**9**, **12**, **17**, and **18**), four diterpene acids (**19**−**22**), two tocopherol-related compounds (**23** and **24**), four estolides (**25**−**28**), three *syn*-alkane-4,6-diols (**29**−**31**), one 1,3-dioxoalkanoic acid (**32**), and one aliphatic ketone (**33**) among which 14 (**2**−**8**, **12**, **23**, **25**−**28**, and **33**) were new naturally occurring compounds. Two estolide methyl esters, **25** and **27**, have recently been prepared by semisynthesis from the corresponding acids (*7*). Characterization of **1**, as one of the most predominant triterpene constituents, and *syn*-alkane-4,6-diols in this study is consistent with the recent observation on the hexane extract of *H. annuus* pollen (*7*). In addition, this study has demonstrated that **1** occurs in a significant amount as a free form, **9**, in the *H. annuus* pollen extract. Compound **9** occurs as the most predominant triterpene constituent in the nonsaponifiable lipid fraction of the tubular flower extracts of *H. annuus* and other Compositae plants (*2*). The possibility that several epoxidized (**4**−**8**) and 24,25-dihydroxylated (**2**, **3**, **7**, and **8**) compounds characterized in this study are artifacts derived

from **1** and **9** during storage of the pollen and extraction and isolation procedures cannot be excluded. Whereas the *H. annuus* pollen extract contains both C-5 stereoisomers of the tocopherol-related compounds, (5S)-**23** and (5R)-**24**, the 5R epimer (**24**) has previously been isolated from *Ficus pumila* (Moraceeae) leaves (*13*). In addition to the compounds characterized in this study, *H. annuus* var. Saturn pollen has been reported to contain various β-diketones, β-hydroxyketones, 1,3-dioxoalkanoic acids, and alkanes in the hexane extract (*7*). The major component reported in the pollen lipids (*n*-hexane extract) of *H. annuus* var. Saturn was the *seco*-triterpene **1**, followed by β-diketones as the second major group of compounds (*7*). **Table 3** shows the inhibitory effects on an in vitro assay of the induction of EBV-EA induced by TPA in Raji cells of the 30 compounds and the reference substance, β-carotene. The inhibitory effects of 21 di- or polycyclic compounds (**1**−**4**, **6**−**16**, and **19**−**24**) were almost equivalent to or stronger than that of β-carotene. Among them, four free 3,4-*seco*-triterpene triols, **10**, **11**, **15**, and **16**, showed the most potent inhibitory effect on EBV-EA induction, 15−18% inhibition even at 1×10 mol ratio/TPA, while their C-3 fatty acid esters exhibited a slightly reduced effect as has been observed for **2**, **3**, **7**, and **8** (1−8% inhibition at 1×10 mol ratio/TPA). This suggests that hydroxylation at C-24 and C-25 in the side chain and deesterification at C-3 in the ring system enhance the inhibitory effects as far as helianol type compounds are concerned. The similar structure−activity relationship has been observed also in the cucurbitane type triterpenes in our recent study (*23*). The inhibitory effects against EBV-EA induction have been demonstrated to be closely parallel with those against tumor promotion in vivo (*24, 25*), and the diethyl ether extract of sunflower pollen grains and the terpenoid and lipid constituents of the extract are, therefore, suggested to be potent cancer chemopreventive agents as antitumor promoters.

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