Multiflorane Triterpene Esters from the Seeds of *Trichosanthes kirilowii*

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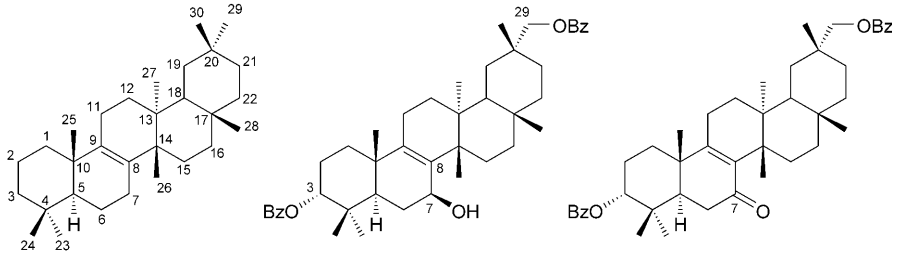
Two new multiflorane triterpene esters, (3α,7β)-3,7,29-trihydroxymultiflor-8-ene-3,29-diyl dibenzoate (**2**) and (3α)-3,29-dihydroxy-7-oxomultiflor-8-ene-3,29-diyl dibenzoate (**3**) have been isolated from the $CHCl_3$ extract of the seeds of *Trichosanthes kirilowii*, together with two known compounds, (3α,5α,22*E*)-24-ethylcholesta-7,22,25(27)-trien-3-ol and darutigenol. The structures of the new compounds were elucidated on the basis of extensive NMR experiments, and by means of an X-ray crystallographic analysis of **2**. A preliminary cytotoxicity assay showed that **2** and **3** are basically inactive against the K562 cell line.

1. Introduction. – The seeds of *Trichosanthes kirilowii* MAXIM. (Cucurbitaceae), widely cultivated in China, are reputedly used in traditional Chinese medicine for the treatment of inflammation, cough, and phlegm [1]. Species of *Trichosanthes* are well-known for their production of biologically active triterpenes [2], which have been isolated from the highly polar fraction of the non-saponifiable lipids of saponified *T. kirilowii* seed extracts [3 – 11]. Multiflorane-type triterpenes such as (3α)-multiflora-7,9(11)-diene-3,29-diol, known as karounidiol (**1**; see the *Scheme* below), and its 3-*O*-benzoate were identified as major components of the saponified extract of *T. kirilowii* seeds [4]. The former compound was found to suppress tumour production in mice, as induced by 12-*O*-tetradecanoylphorbol-13-acetate [12], and it showed cytotoxic activities against human cancer cell lines, especially renal-cancer cells [13].

Studies reported so far largely focused on compounds extracted from non-saponifiable materials of saponified *T. kirilowii* seed extracts. However, for the purpose of obtaining information on the quality of seeds of *T. kirilowii*, it would be beneficial to study the extracts of untreated or unsaponified seeds. This would avoid any unwanted chemical reactions during the saponification process, and would give a true analytical profile of the naturally present components.

To obtain pure standard compounds for qualitative and quantitative analysis, we have carried out a detailed investigation of the $CHCl_3$ extract of non-saponified *T. kirilowii* seeds. Herein, we report the isolation and structural elucidation of the new multiflorane triterpenes **2** and **3**, the chemical stability of **2** in MeOH soln. containing 5% of KOH, and their cytotoxic activities towards the K562 cell line.

2. Results and Discussion. – The crude $CHCl_3$ extract of the seeds of *Trichosanthes kirilowii* was found to be cytotoxic against the K562 cell line, with an inhibition of more



than 50% at a concentration of 230 μg/ml. Repeated column chromatographic separation on silica gel and *Sephadex LH-20* support afforded the two new multiflorane triterpenes (3*α*,7*β*)-3,7,29-trihydroxymultiflor-8-ene-3,29-diyl dibenzoate (**2**) and (3*α*)-3,29-dihydroxy-7-oxomultiflor-8-ene-3,29-diyl dibenzoate (**3**). We also isolated a known sterol, (3*α*,5*α*,22*E*)-24-ethylcholesta-7,22,25(27)-trien-3-ol [14], and the known diterpene darutigenol [15], the latter being the first diterpene isolated from the genus *Trichosanthes*. In a preliminary assay, compounds **2** and **3** showed no marked cytotoxicities against the K562 cell line.

Compound **2** was obtained as colourless needles. The compound analysed for $C_{44}H_{58}NaO_5$, as determined by HR-MALDI-MS (*m*/*z* 689.4196 ($[M+Na]^+$) and ^{13}C-NMR (DEPT) experiments. The IR absorptions at 1720 and 1702, and at 1602, 1585, and 1453 cm^{-1}, respectively, provided evidence of C=O and Ph groups. The ^{1}H-NMR spectrum of **2** indicated seven angular Me groups (*singlet*s at *δ*(H) 0.95, 1.02, 1.03, 1.13, 1.14, 1.17, and 1.35) and four CH_nO moieties ($n=1$ or 2) at *δ*(H) 4.86 (br. *s*, 1 H), 4.11 (*d*, $J=10.7$, 2 H), and 4.42 (*m*, 1 H), as well as ten aromatic signals at *δ*(H) 7.47–8.03. The ^{13}C-NMR and DEPT spectra of **2** (see *Table* in the *Exper. Part*) displayed twelve aromatic signals, two C=O signals, and 30 signals for the triterpene core, including two quaternary olefinic signals at *δ*(C) 139.52 and 139.49, and three signals due to oxygenated C-atoms at *δ*(C) 78.80, 70.36 and 74.10. These observations indicated that **2** was a trihydroxylated triterpenoid substituted with two benzoyl (Bz) groups. The three OH groups were determined to be in positions 3, 29 and 7, as deduced from HMBC correlations between H–C(3) and C(24), H–C(29) and C(30), and H–C(7) and C(9), respectively (*Fig. 1*). The C=C bond was placed between C(8) and C(9) based on HMBC correlations between H–C(26) and C(8), and between H–C(25) and C(9). The positions of the Bz groups were confirmed by HMBC cross-peaks between H–C(3) and C(3a), and between H–C(29) and C(29a), respectively. A comparison between the NMR data of **2** and its analogue with a (4-aminobenzoyl)oxy rather than a BzO group in position 3, isolated from the pumpkin seeds of Cucurbitaceae [16], revealed that the signals of the triterpene core of the two molecules were identical, as well as the two benzoate ester linkages at C(3) and C(29).

A single-crystal X-ray diffraction study of **2** was undertaken to determine its relative configuration. A perspective view of **2** is depicted in *Fig. 2*, supporting the preceding deductions concerning its structure. H–C(3), Me(24), Me(25), Me(26), Me(28), and Me(30) are all *β*-configured, and H–C(7), Me(23), Me(27), and Me(29) are *α*-configured, which is in agreement with the reported configuration of the above 4-aminoben-

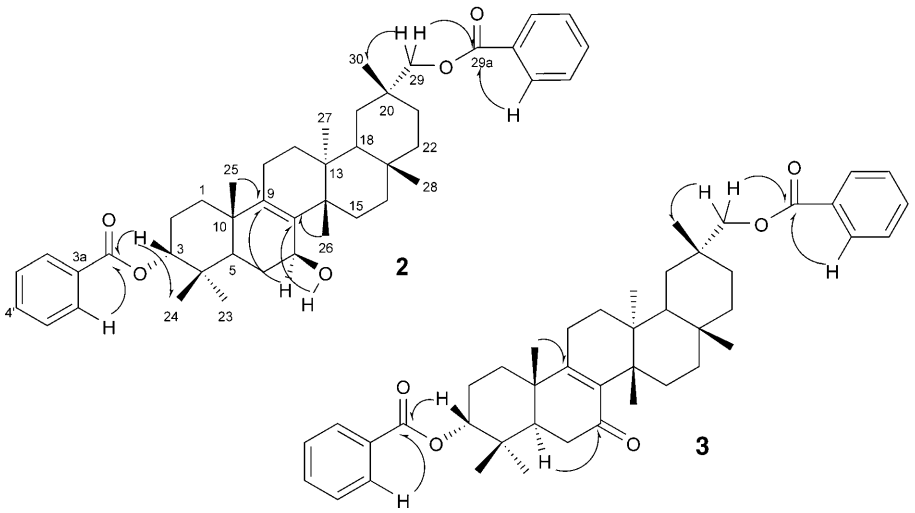


Fig. 1. *Selected HMBC correlations for compounds* **2** *and* **3**

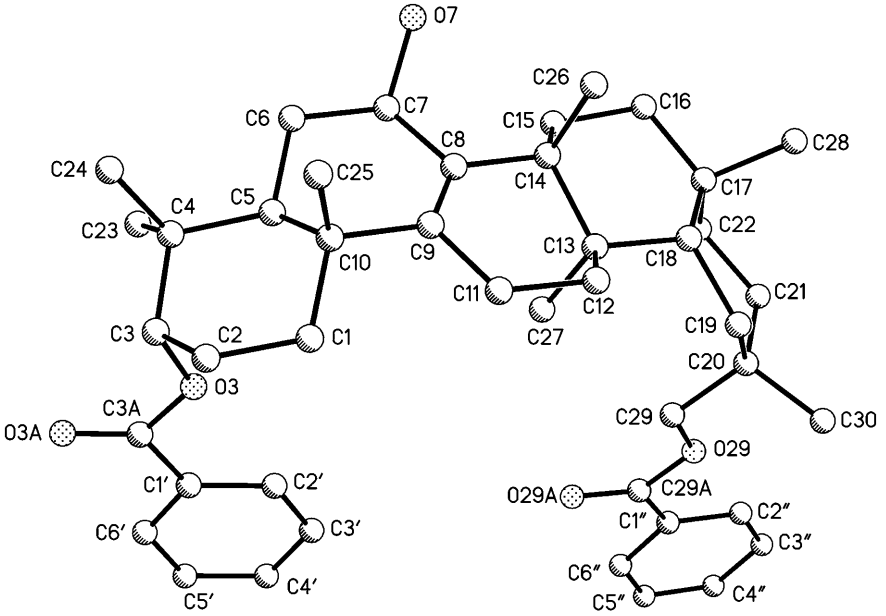
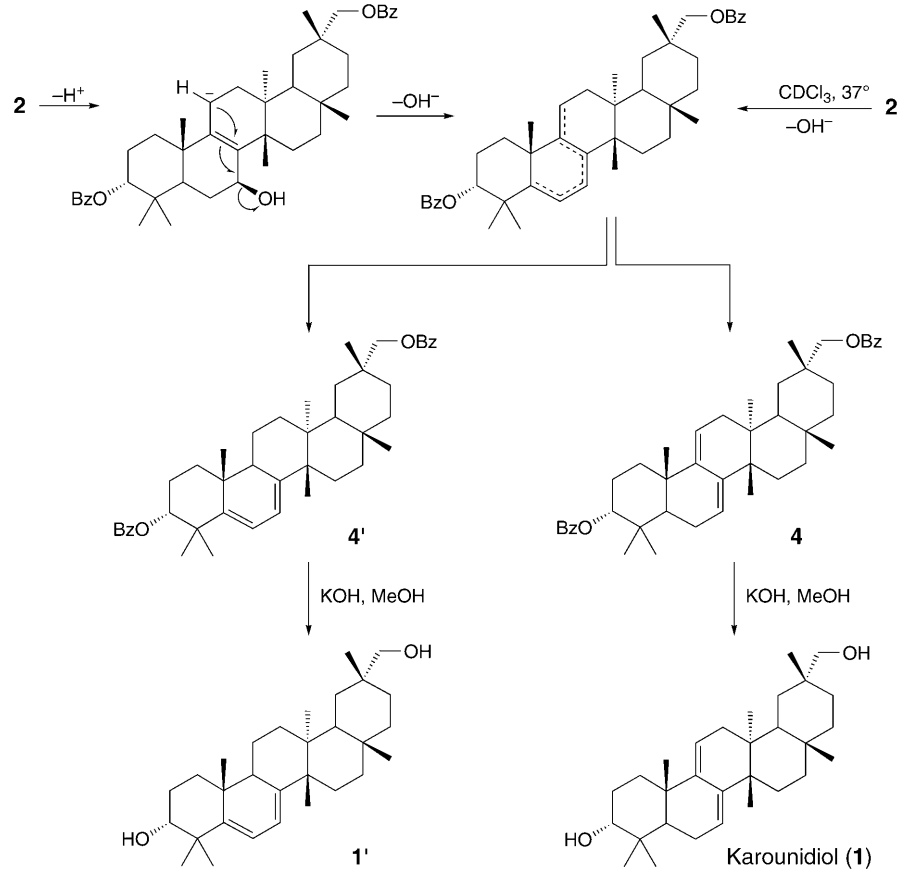


Fig. 2. *X-Ray crystal structure of* **2**

zoyl analogue [16]. From these data, the structure of **2** could be fully elucidated. The molecule is composed of five six-membered rings with *trans*-fused *A*/*B* and *C*/*D* rings, and *cis*-fused *D*/*E* rings.

We observed that the parent triterpene framework of **2**, after hydrolysis and dehydration to the $\Delta^{7,9(11)}$ diene **4** (3,29-dibenzoylkarounidiol), is in equilibrium with the cor-

Scheme. *Proposed Mechanism for the Base-Catalysed Dehydration/Hydrolysis of* **2** *to Karounidiol* (**1**). The latter readily undergoes a C=C shift to the isomer **1′** in $CDCl_3$ solution at 37° (see text and *Exper. Part*).



responding $\Delta^{6,8}$ diene **4′**, when exposed to $CDCl_3$ soln. at 37° for 1 h (*Scheme*). Under these conditions, the ^{1}H-NMR spectra[1]) showed signals for both **4** [δ(H) 6.12/5.77 (2*dd*, J = 9.8, 2.5, H–C (7,11))] and **4′** [δ(H) 5.54/5.25 (2 br. *s*, H–C(6,7))]. Thereby, the $\Delta^{7,9(11)}$ isomer **1** was found to be the predominant dehydration product of **2**. A possible mechanism for this base-catalysed dehydration is presented in the *Scheme*. These observations suggests that karounidiol (**1**), the only naturally occurring triterpene with a $\Delta^{7,9(11)}$ unsaturation, could be, in fact, an artefact of the commonly applied saponification process, thus rationalizing the wide distribution of multiflorane triterpenes reported in the Cucurbitaceae family [16].

Compound **3** exhibited the molecular formula $C_{44}H_{56}O_5$, based on ^{13}C-NMR (DEPT) and HR-MALDI-MS experiments. The IR spectrum of **3** exhibited characteristic absorptions at 1718 and 1661, and at 1600, 1586, and 1451 cm^{-1}, respectively, for

[1]) (D_6)Benzene [16] or (D_6)acetone were the solvents of choice for these NMR experiments.

C=O and Ph groups. In the ^{1}H-NMR spectra, there were characteristic triterpene signals, with seven angular Me groups, and three H-atoms attached to oxygenated C-atoms [δ(H) 4.95 (br. *s*, H–C(3)); 4.03 (*s*, CH_2(29)]. In addition, ten aromatic H-atoms due to two BzO groups at C(3) and C(29) were identified by comparing the corresponding signals with those for **2**. The ^{13}C-NMR (DEPT) spectra showed twelve aromatic signals, two ester C=O resonances, and 30 triterpene signals, including those of two quaternary olefinic C-atoms (δ(C) 163.39, 142.87), a keto C-atom (δ(C) 198.37), and two oxygenated C-atoms (δ(C) 77.78, 75.19).

The ^{13}C-NMR spectra of **2** and **3** were nearly identical, except for a missing oxygenated-carbon signal (C(7)) in **3**, which was replaced with a C=O signal. Accordingly, the olefinic C(9) resonance was shifted downfield to δ(C) 163.39 due to conjugation. Significant evidence for this assignment was obtained by detailed HMQC and HMBC experiments, in which correlations between H–C(5) and C(7) were clearly observed. In addition, the NMR spectroscopic data of the triterpene skeleton agreed well with those of the known 3-benzoate of (3α,29α)-7-oxomultiflor-8-ene-3,29-diol [10]. The configurations of all angular Me groups and of H–C(3) in **3** were found to be conserved relative to those in **2**, as concluded from the NMR data.

Conclusions. – Compounds **2** and **3** are the first two secondary triterpene metabolites from *Trichosanthes* in which two benzoyl (Bz) moieties are present. Although these novel compounds were not found to be cytotoxic against K562 cells, they can be readily used as reference markers for qualitative and quantitative analyses of the seeds of *T. kirilowii*, especially **2**, which is present in relatively large amounts (300 mg per 2.5 kg of dry seeds, *i.e.*, 120 ppm). It is worth mentioning that karounidiol (**1**), obtained from the saponified part of the seeds, is not a versatile reference standard for the quality control of this herb, because this compound is not naturally occurring in the seeds, and also because saponification is time-consuming and often gives rise to irreproducible results. A more-detailed biological evaluation of **2** and its congeners is currently underway.

This work was supported by a grant from the *10th Five-Year National Key Science and Technology Project* (No. 2001BA701A11) from the *Ministry of Science and Technology of the People's Republic of China*.

Experimental Part

General. Column chromatography (CC): silica gel (200–300 mesh; *QingDao HaiYang Co.*; China). Anal. TLC: precoated silica-gel *G60 F-254* plates (*YanTai ZiFu Co.*, China). Melting points (m.p.): *X4* micro-melting point apparatus; uncorrected. Optical rotation: *Perkin-Elmer 341* polarimeter. IR Spectra: *Bio-Rad FTS-185* spectrometer; KBr pellets; in cm^{-1}. NMR Spectra: *Bruker DRX-500* spectrometer, at 500 (^{1}H) or 125 MHz (^{13}C); δ in ppm rel. to residual solvent peaks of C_5H_5N (δ(H) 7.20, 7.57, 8.73), *J* in Hz. Assignments were supported by HMQC and HMBC experiments. HR-MALDI-MS: *Ionspec* mass spectrometer; in *m/z*.

Plant Material. The seeds of *Trichosanthes kirilowii* were collected in Bozhou, Anhui Province, P.R. China, in August 2000, and authenticated by Dr. *Gui-Xin Chou*, Shanghai R&D Center for Standardization of Chinese Medicines, Shanghai. A voucher specimen (Herbarium No. 1123) was deposited at the Herbarium of the Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, P.R. China.

Extraction and Isolation. The dried seeds (2.5 kg) of *T. kirilowii* were extracted with $CHCl_3$ (3 × 10 l), and the extract was evaporated to dryness *in vacuo* to afford a brown residue (39.5 g). The latter was subjected to CC (SiO_2; petroleum ether/AcOEt 20 : 1, 14 : 1, 9 : 1, and 2 : 1) to afford four fractions: *Fr. 1–4*. *Fr. 1* and *2* were purified by repeated CC to afford **3** (50 mg) and **2** (300 mg), respectively. *Fr. 3* was purified by CC (*Sephadex LH-20*;

Table. *^{13}C-NMR Chemical Shifts of Compounds* **2** *and* **3**. Recorded at 125 MHz in (D_6)acetone. For atom numbering, see chemical formulae and *Fig. 2*.

Position	**2**	**3**	Position	**2**	**3**
1	31.54	29.80	21	30.05	28.39
2	23.96	23.03	22	37.43	38.66
3	78.80	77.78	23	22.04	21.50
4	37.55	37.10	24	27.97	27.11
5	45.49	43.36	25	20.56	18.13
6	31.50	36.44	26	27.63	27.03
7	70.36	198.37	27	18.43	18.25
8	139.52	142.87	28	31.30	30.62
9	139.49	163.39	29	74.10	75.19
10	39.11	39.33	30	29.01	26.45
11	21.36	22.20	3a	165.97	165.81
12	31.57	29.98	29a	166.91	166.93
13	38.92	38.23	1′	131.89	130.72
14	41.82	39.23	2′,6′	130.04	129.58
15	26.72	29.80	3′,5′	133.78	128.60
16	37.43	35.94	4′	129.44	133.04
17	31.86	31.30	1″	131.53	130.77
18	44.03	41.45	2″,6″	130.00	129.60
19	30.24	30.47	3″,5″	133.78	128.49
20	32.84	32.52	4″	129.44	132.92

$CHCl_3$/MeOH 1:1) to provide darutigenol (60 mg) [15]. *Fr. 4* was further purified by CC (1. SiO_2, $CHCl_3$; 2. *Sephadex LH-20*, $CHCl_3$/MeOH 1:1) to furnish (3*α*,5*α*,22*E*)-24-ethylcholesta-7,22,25(27)-trien-3-ol (42 mg) [14].

(3α,7β)-3,7,29-Trihydroxymultiflor-8-ene-3,29-diyl Dibenzoate (**2**). Colourless needles (acetone). M.p. 162–164°. $[\alpha]_D^{20} = +16.1$ ($c = 0.44$, $CHCl_3$). IR (KBr): 3531, 2944, 1720, 1702, 1602, 1585, 1453, 1391, 1377, 1315, 1272, 1176, 1115, 1070, 1026, 962, 709. ^{1}H-NMR (500 MHz, (D_6)acetone): 0.95, 1.02, 1.03, 1.14, 1.17, 1.35 (6*s*, Me(23) – Me(28)); 1.13 (*s*, Me(30)); 4.11 (*d*, $J = 10.7$, Me (29)); 4.44–4.42 (*m*, H–C (7)); 4.86 (br. *s*, H–C (3)); 7.47–7.50 (*m*, H–C(3′, 3″, 5′, 5″)); 7.59–7.62 (*m*, H–C (4′,4″)); 7.97 (br. *d*, $J = 7.8$, H–C (2′,6′)); 8.03 (br. *d*, $J = 7.8$, H–C (2″,6″)). ^{13}C-NMR (125 MHz, (D_6)acetone): see *Table*. HR-MALDI-MS: 689.4196 ($[M+Na]^+$, $C_{44}H_{58}NaO_5^+$; calc. 689.4176).

(3α)-3,29-Dihydroxy-7-oxomultiflor-8-ene-3,29-diyl Dibenzoate (**3**). Colourless prisms (acetone). M.p. 233–235°. $[\alpha]_D^{20} = -14.2$ ($c = 0.44$, $CHCl_3$). IR (KBr): 2959, 1718, 1661, 1600, 1586, 1451, 1381, 1367, 1315, 1274, 1174, 1114, 1069, 1027, 933, 709. ^{1}H-NMR (500 MHz, (D_6)acetone): 0.96, 1.04, 1.08, 1.09, 1.23, 1.42 (6*s*, Me(23) – Me(28)); 1.15 (*s*, Me(30)); 1.51–1.54 (*m*, CH_2(21)); 2.15 (*dd*, $J = 6.8$, 6.7, H–C(5)); 2.40–2.47 (*m*, H–C(6)); 4.03 (*s*, CH_2(29)); 4.95 (br. *s*, H–C(3)); 7.42–7.47 (*m*, H–C(3′,3″,5′,5″)); 7.88 (br. *d*, $J = 7.8$, H–C(2′,6′)); 8.05 (br. *d*, $J = 7.8$, H–C (2″,6″)). ^{13}C-NMR (125 MHz, (D_6)acetone): see *Table*. HR-MALDI-MS: 665.4250 ($[M+1]^+$, $C_{44}H_{57}O_5^+$; calc. 665.4200).

Alkaline Hydrolysis of **2**. Compound **2** (2 mg) was heated at reflux with 5% KOH in MeOH at 80° for 2 h. After cooling, the mixture was extracted with $CHCl_3$. The org. layer was concentrated, and analysed, along with authentic karounidiol (**1**), by high-pressure TLC ($CHCl_3$/MeOH 10:1). Both compounds gave a red spot with identical R_f values after spraying with 10% aq. H_2SO_4 and heating. ESI-MS Analysis of the $CHCl_3$ extract showed a signal at *m*/*z* 441 corresponding to the $[M+H]^+$ ion of **1**.

X-Ray Crystal Structure of **2**[2]. Diffraction data were collected on a *Siemens P4* diffractometer, using MoK_α monochromated radiation in the 2*θ* range 3.7–50.6°. The OH H-atoms were located from a difference map, and

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[2]) The crystallographic data of **2** have been deposited with the *Cambridge Crystallographic Data Centre* as supplementary publication number CCDC-242274. Copies of the data can be obtained, free of charge, *via* internet (http://www.ccdc.cam.ac.uk/data_request/cif), e-mail (data_request@ccdc.cam.ac.uk) or fax (+44-1223-336033).

refined isotropically with fixed positional parameters. The remaining H-atoms were placed in idealised positions, with isotropic displacement parameters equal to $1.2U_{eq}$ (or $1.5U_{eq}$ for Me groups) of the parent C-atoms. In the final cycles of the refinement, the non-H-atoms were assigned anisotropic displacement parameters. The bond lengths and angles were normal within experimental error. Crystal data: $C_{44}H_{58}O_5$; M_r 666.90 g/mol; orthorhombic system; space group $P2_12_12_1$, $a=11.883(4)$, $b=12.085(3)$, $c=26.065(5)$ Å; $Z=4$, $V=3743.2(16)$ Å^3; $D_{calc}=1.183$ g/cm^3; crystal size, $0.40\times0.26\times0.24$ mm.

Cytotoxicity Assay. The K562 cell lines were cultured in *Dulbecco*'s modified *Eagle* medium (180 µl; *Gibco BRL*) in 96-well micro-culture plates. Medium (20 µl) containing various amounts of extract was added to all wells (in triplicate). Control cells received medium containing analogous $CHCl_3$ concentrations. After incubation at 37° in a 5% CO_2 atmosphere for 3 d, Alamar Blue (20 µl) was added to each well. After incubation for another 6 h, the colour change of the soln. was measured with an ELISA micro-autoreader at 570 and 600 nm. The activation rate was calculated according to the formula provided by the Alamar Blue assay (*Nivelles*, Belgium).

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Received April 26, 2005