Coumarins from *Opopanax chironium*. New Dihydrofuranocoumarins and Differential Induction of Apoptosis by Imperatorin and Heraclenin

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Opopanax chironium is a rich source of furano- and dihydrofuranocoumarins, whose accumulation in all plant parts and especially the roots is presumably responsible for the poisonous properties of the species. The presence of two distinct chemotypes was evidenced, with the one from Sicily affording the new dihydrofuranocoumarins **5d** and **5e**, while extracts from the Sardinian chemotype showed powerful apoptotic activity, which was traced to the prenylated furanocoumarins heraclenin (**2a**) and imperatorin (**2b**). Despite a close structural similarity, compounds **2a** and **2b** induced apoptosis in Jurkat leukemia cells in mechanistically different ways.

Opopanax chironium (L.) Koch (Apiaceae) is a large plant indigenous to the Western Mediterranean area.[1] Wounding of the stem and the voluminous roots causes the exudation of a yellowish latex with a pleasant and persistent smell, reminiscent of licorice. The plant is avoided by animals because of its poisonous properties, but finds occasional use in medicine, not, however, having a specific indication, with opopanax meaning "all-healing juice".[2] The term "opopanax" is a source of confusion in pharmacognosy, since three different products bear this name. Thus, *O. chironium* is not to be confused with perfumery's opopanax, a gum-resin obtained from *Commiphora erytraea* var. *glabrescens* Engler, a tree endemic to the Horn of Africa.[3] This "bursa-opopanax", or bisabol-myrrh, is the only opopanax commercially available nowadays, since "umba-opopanax", a bitter medicinal product, is no longer an article of commerce.[2] This opopanax does not produce a sweet odor on burning and was probably of Persian origin, possibly obtained from *Opopanax persicum*.[3] On the other hand, the vague description of the ancient authors makes it impossible to identify the exact source of the so-called "true opopanax", the opopanax mentioned in the Greek and Latin treatises of medicine.[4] This gum resin was probably obtained from a variety of umbelliferous plants including, besides *O. chironium* and *O. hispidum* (Friv.) Griseb., also plants from the genus *Ferula*, *Peucedanum*, *Laserpitium*, and *Heracleum*.[2]

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Despite the rich history of opopanax and its poisonous properties, *O. chironium* has been largely overlooked in terms of chemical investigations. Apart from a series of C-17 acetylenes,[5] various phthalides were identified in the roots,[6] a surprising finding for a member of the Peucedaneae tribe of Apiaceae. As part of a study on medicinal plants from the Mediterranean area, we have investigated the constituents of various collections of *O. chironium* from Sardinia and Sicily. The lack of phthalides and the accumulation of very large amounts of coumarin derivatives were evidenced, but remarkable differences were noticed between the samples collected in Sardinia and those of Sicilian origin. No single coumarin was shared by the two populations, while only extracts from the Sardininan chemotype showed apoptotic activity. We report here the phytochemical characterization of the two chemotypes and the identification of the apoptotic principles of the plants of Sardinian origin.

Results and Discussion

The Sardinian collection of *O. chironium* consisted of roots, stems, and fruits of plants collected in the southern part of the island. An acetone extract from the roots was fractionated by gravity column chromatography to afford five crystalline fractions, containing the coumarins umbelliprenin (**1a**)[7] (0.020%), imperatorin (**2a**)[8] (0.47%), a mixture of xanthotoxin (**3a**)[9] and bergaptene (**3b**)[10] (3.4%), heraclenin (**2b**)[11] (0.40%), and heraclenol (**2c**)[12] (0.042%).

Psoralens such as **3a** and **3b** are the mainstay of photodynamic therapy and are currently produced by low-yielding syntheses, since no abundant natural source is known.[13] The presence of conspicuous (ca. 1%) amounts of these compounds also in the leaves suggests that the Sardinian chemotype of *O. chironium* may be a renewable source of psoralens for photodynamic therapy. Along with

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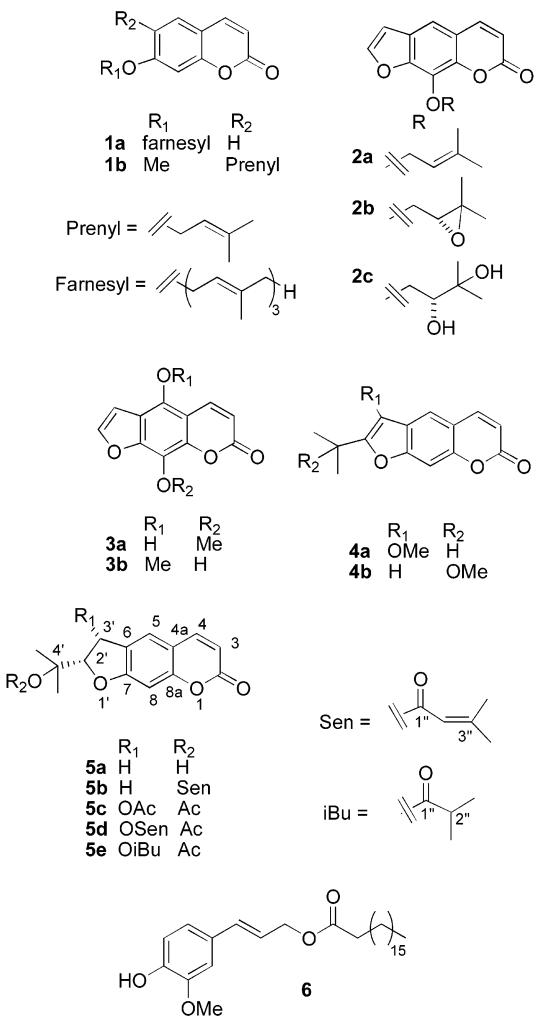
2a, **3a**, and **3b**, the fruits further afforded the prenylated coumarin suberosin (**1b**).[14]



In addition, the Sicilian collection of *O. chironium* (roots and seeds) contained large amounts of coumarin derivatives. The major constituent (peucedanin, **4a**)[15] was isolated in 0.56% yield, along with four known [marmesin (**5a**),[16] dehydromarmesin methyl ether (**4b**),[17] prantschimgin (**5b**),[18] smirniorin (**5c**)[19]] and two new (**5d** and **5e**) furanocoumarins. Compounds **5d** and **5e** were closely related dihydrofuranocoumarins, only differing in their esterification pattern. The molecular formula of **5d** was established as $C_{21}H_{22}O_7$ by ERMS. The ^{1}H NMR spectrum (Table 1) was relatively simple, containing two pairs of mutually coupled doublets and three singlets between δ 5.10 and 7.70 and five methyl singlets in the high-field region of the spectrum. The paucity of aliphatic carbons was confirmed by the ^{13}C NMR spectrum (Table 1), which showed 13 signals in the sp^2 region of the spectrum and only eight aliphatic carbons, five of which were methyl groups. The NMR data were translated in structural terms by using heteronuclear 2D NMR experiments (HMQC, HMBC), which identified a coumarin core linearly fused to a dihydrofuran ring, in agreement with the UV absorptions at λ_{max} 321, 295, and 221 nm, typical for this type of compound.[21−23] The singlets at δ 7.51 and 6.82 were assigned to H-5 and H-8 and the mutually coupled doublets at δ 6.22 and 7.62 to H-3 and H-4, respectively. The doublets at δ 5.13 and 6.40 were instead assigned to two dihydrofuran methines (H-2′ and H-3′) on the basis of diagnostic HMBC correlations (H-2′/C-7, H-3′/C-5). The HMBC spectrum also revealed that their corresponding carbons were bound to an acyl group (C-3′, cross-peak H-3′/C-1″) and to an α-acyloxyisopropyl (C-2′, cross-peaks H-2′/C-4′, H-2′/C-5′). The two acyl substituents were easily identified as an acetyl and a senecioyl (3-methyl-2-butenoyl). Their relative location was assessed by the observation of HMBC correlation of both H-3′ and H-2″ with the ester carbonyl at δ 164.2, requiring binding of the senecioyl group to C-3′ and therefore the location at C-4′ of the acetoxyl. Compound **5e** ($C_{20}H_{22}O_7$, HRMS) had NMR spectra similar to those of **5d**, with differences that could be rationalized in terms of replacement of the senecioyl group by an isobutyrate (^{1}H NMR: methine heptet at δ 2.51, methyls at δ 2.15 and 2.12; ^{13}C NMR: δ 176.6, 34.2, and 18.6). The combined analysis of HMQC and HMBC correlations unambiguously located the isobutyryl at C-3′ (correlation of the carbon signal at δ 176.6 with both H-3′ and the signal at δ 2.51 (H-2″)).

Table 1. ^{1}H and ^{13}C NMR Data of **5d** and **5e**

	5d		**5e**	
position	δ_C (mult.)	δ_H (int., mult., *J* in Hz)	δ_C (mult.)	δ_H (int., mult., *J* in Hz)
2	160.6 (C)		160.6 (C)	
3	113.0 (CH)	6.22 (1H, d, 9.56)	113.2 (CH)	6.26 (1H, d, 9.60)
4	143.6 (CH)	7.62 (1H, d, 9.56)	144.1 (CH)	7.62 (1H, d, 9.60)
4a	113.4 (C)		113.4 (C)	
5	126.6 (CH)	7.51 (1H, s)	126.4 (CH)	7.52 (1H, s)
6	124.1 (C)		124.1 (C)	
7	163.2 (C)		163.0 (C)	
8	99.1 (C)	6.82 (1H, s)	98.9 (C)	6.86 (1H, s)
8a	157.1 (C)		157.0 (C)	
2′	88.2 (CH)	5.13 (1H, d, 6.62)	88.2 (CH)	5.16 (1H, d, 6.62)
3′	71.4 (CH)	6.40 (1H, d, 6.62)	71.6 (CH)	6.35 (1H, d, 6.62)
4′	82.2 (C)		81.7 (C)	
5′	24.1 (CH_3)	1.71 (3H, s)	24.7 (CH_3)	1.74 (3H, s)
6′	22.3 (CH_3)	1.65 (3H, s)	23.2 (CH_3)	1.68 (3H, s)
1″	164.2 (C)		176.6 (C)	
2″	116.1 (CH)	5.57 (1H, s)	34.2 (C)	2.51 (1H, hep, 7.3)
3″	159.2 (C)		18.6 (CH_3)	1.17 (3H, d, 7.3)
4″	27.5 (CH_3)	2.21 (3H, s)	18.6 (CH_3)	1.14 (3H, d, 7.3)
5″	20.2 (CH_3)	1.89 (3H, s)		
Ac	170.3 (C)		170.5 (C)	
	22.6 (CH_3)	2.00 (3H, s)	22.2 (CH_3)	2.03 (3H, s)

J values and NOE correlations are per se not very diagnostic to assign the relative configuration of substituted cylopentanes. Nevertheless, some empirical correlations have emerged from the wealth of data available for 2′,3′-disubstituted dihydrofuranocoumarins. Thus, values around 6.5 Hz for $J_{H-2'/H-3'}$ are diagnostic of a *cis* configuration, while smaller values (ca. 4.5 Hz) are observed in their *trans* isomers.[20] This empirical observation was rationalized and backed up by calculations showing that the H-2′/H-3′ dihedral angle has a value of 20° in the *cis* isomer and 135° in the *trans* isomer.[20] These data strongly suggested that **5d** and **5e** belong to the *cis*-series ($J_{H-2'/H-3'}$ = 6.62 Hz for both compounds). The absolute configuration of the new compounds was assigned tentatively as 2′*S*, 3′*R* by comparison of their $[\alpha]_D$ sign with those reported for the closely related analogues smirniorin (**5c**, a 3′-acyl analogue)[19] and 3′-senecioyloxymarmesin,[21] the 4′-deacetyl analogue of **5d**.

Apart from coumarins, a mixture of saturated long-chain coniferates was also obtained from the Sicilian collection of *O. chironium*. Crystallization from hexane afforded the major component, coniferyl stearate (**6**), whose structure was confirmed by synthesis, capitalizing on a recent straightforward protocol for the chemoselective esterification of phenolic alcohols.[22] While the occurrence of mixtures

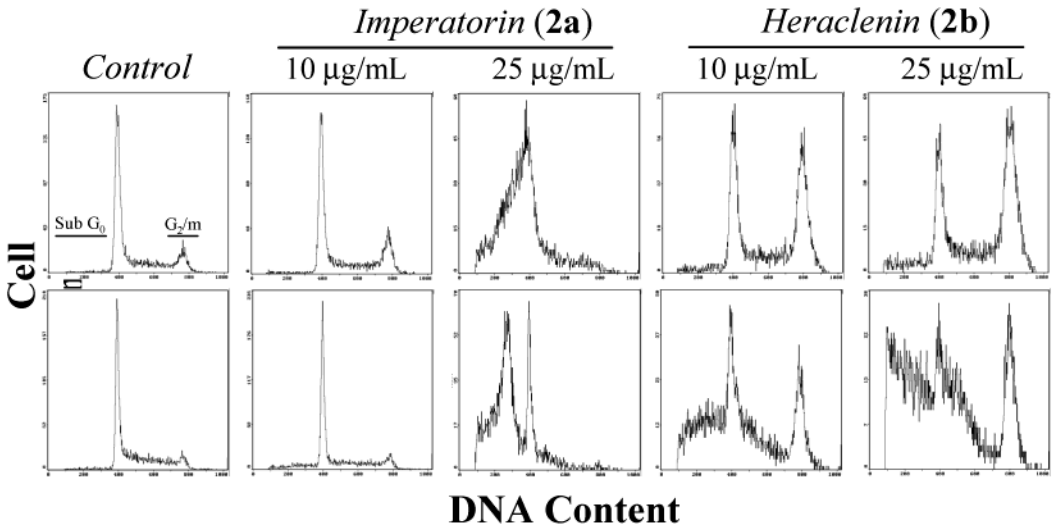


Figure 1. Differential effects of imperatorin (**2a**) and heraclenin (**2b**) on the cell cycle. Jurkat cells were treated with the indicated concentrations of the compounds for 48 h (upper panel) or 72 h (lower panel), and the cell cycle was studied by flow cytometry and PI staining. Results are representative of three different experiments.

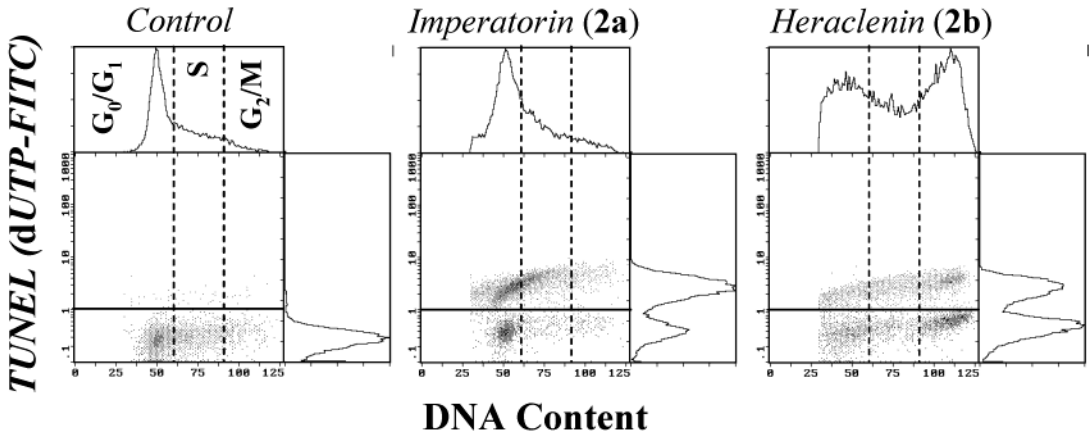


Figure 2. Cell cycle phase dependence for apoptosis induced by imperatorin (**2a**) and heraclenin (**2b**). Jurkat cells were stimulated with a solution of 25 mg/mL of **2a** and **2b** for 72 h, and the cell cycle and the DNA strand breaks were analyzed by the TUNEL method using flow cytometry. Results are representative of three different experiments.

of fatty acid esters of coniferol is not uncommon, **6** has apparently never been characterized before as a pure compound.

The occurrence of large amounts of psoralens presumably underlies the poisonous properties of the Sardinian chemotype of *O. chironium*. Since furanocoumarins can display light-independent apoptotic properties,[23] we investigated by flow cytometry the relative percentage of hypodiploidy (death cells) and arrest in different phases of the cycle induced in Jurkat cells at 48 or 72 h from the treatment. Only the acetone extract from the Sardininan collection showed powerful apoptotic activity, traced back to the furanocoumarins imperatorin (**2a**) and heraclenin (**2b**). Interestingly, the other coumarins from this collection were only marginally active. Figure 1 shows that heraclenin (**2b**) could arrest cells at the G_2/M phase of the cell cycle, as evident after 48 h of treatment. Hypodiploidy was only detected after 72 h of treatment, suggesting that heraclenin does not induce apoptosis directly, but rather deregulates progression of the cell cycle, arresting cells in mitosis and triggering DNA fragmentation and apoptosis. This type of secondary apoptotic pathway is the hallmark of the inhibitors of microtubule assembly such as paclitaxel (Taxol) and podophyllotoxin.[24,25] On the other hand, heraclenin showed only marginal activity in assays of tubulin depolymerization/polymerization activity,[26] suggesting that its effects on the cell cycle are cell-type specific. While further research is necessary to identify the mechanism(s) by which heraclenin (**2b**) arrests cells in the G_2/M phase, it is nevertheless interesting to note that its deoxygenated analogue imperatorin (**2a**) failed to induce cell cycle arrest and showed potent apoptotic activity only at the higher doses tested (25 μg/mL). To confirm that **2b** and **2a** induce apoptosis in different phases of the cell cycle, we performed double staining experiments with propidium iodide (PI) and fluorescein isothiocyanate-12-deoxy-2-uridine triphosphate (FITC-dUTP). This technique makes it possible to identify the cell cycle phase when DNA fragmentation occurs.[27] Figure 2 shows that, in Jurkat cells treated for 72 h with heraclenin, most of the DNA fragmentation occurs at the G_2/M phase of the cell cycle. In contrast, DNA fragmentation in imperatorin-treated cells occurred mainly at the G_1/S transition phases of the cell cycle. These results show that apoptosis induced by these furanocoumarins is primarily directed to growing cells, an observation that suggests **2a** and **2b** are interesting leads for the development of drugs selectively toxic to cancer cells.

The hallmark of *O. chironium* is the accumulation of large amounts of coumarins, but the detection of chemical polymorphisms is also worth noting and parallels similar observations made for *Ferula communis* L.[28] and *Thapsia garganica* L.[29] This suggests that Mediterranean umbel-

liferous plants characterized by a wide area of distribution can evolve into a variety of chemotypes to meet different environmental conditions. While warning against the taxonomic use of phytochemical data obtained from a single plant collection, this also increases the opportunities of discovery associated with plant prospecting, by augmenting the chemical diversity obtainable from a single species.

Experimental Section

General Experimental Procedures. Melting points were measured on a Büchi SMP-20 apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. The IR (KBr) spectra were obtained on a Perkin-Elmer 681 spectrophotometer. UV spectra were obtained in MeOH using a Beckman DU70 spectrophotometer. The ^{1}H, ^{13}C, HMQC, and HMBC NMR spectra were recorded on a Bruker AMX-500 spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0). LRMS and HRMS were recorded on a VG Prospect (FISONS) mass spectrometer. Silica gel 60 (70−230 mesh) was used for gravity column chromatography. HPLC in isocratic mode was performed on a Beckman apparatus equipped with a refractive index detector and using 250 × 4 mm LUNA SI60 columns. Known compounds were identified by comparison of the spectroscopic (^{1}H and ^{13}C NMR) and physical (mp, $[\alpha]_D$) data with those of the literature.

Cell Lines and Reagents. Jurkat cells (ATCC, Rockville, MD) were maintained in exponential growth in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, and the antibiotics penicillin and streptomycin (Gibco BRL-Life Technologies, Barcelona, Spain). FITC-dUTP and terminal deoxynucleotide transferase (TdT) were from Boehringer Mannheim (Germany). All other reagents not cited above or mentioned below were from Sigma Chemical Co. (Barcelona, Spain).

Plant Material. *Opopanax chironium* used for the phytochemical characterization of the two chemotypes was collected in Paulilatino (NU, Sardinia) in January 2001 (roots and leaves) and June 2001 (seeds) and near Capo Milazzo (ME, Sicily) in June 2000 (roots and seeds). The plant material was identified by Prof. M. Ballero (Sardinian collection) and by Dr. A. Bader (Sicilian collection). A voucher specimen is kept at the Dipartimento di Scienze Botaniche (Sardinian collection, #001-41) and at the Novara Laboratories (Sicilian collection, #00-02).

Extraction and Isolation. (a) Sardinian collection: Dried and powdered roots (500 g) were extracted with acetone (1 × 3 L; 2 × 1 L), obtaining, after removal of the solvent, 72 g of a semisolid residue, which was fractionated by gravity column chromatography on silica gel (300 g, petroleum ether−EtOAc as eluant). Fractions eluted with petroleum ether−EtOAc (95:5) afforded 103 mg (0.020%) of umbelliprenin (**1a**), 2.37 g (0.47%) of imperatorin (**2a**), 17.0 g (3.4%) of a ca. 1:1 mixture of xanthotoxin (**3a**) and bergapten (**3b**), 2.05 g (0.40%) of heraclenin (**2b**), and 210 mg (0.042%) of heraclenol (**2c**). In a similar manner, from 700 g of dry leaves, the following compounds were obtained: umbelliprenin (**1a**, 311 mg, 0.043%), imperatorin (**2a**, 128 mg, 0.018%), a mixture of xanthotoxin (**3a**) and bergapten (**3b**) (0.12%), and heraclenin (**2b**, 146 mg, 0.020%). An acetone extract from the fruits (100 g) gave 108 mg of **1c** (0.011%), 61 mg (0.0061%) of **2a**, and 398 mg of a mixture of **3a** and **3b**. (b) Sicilian collection: dried and powdered roots (250 g) were extracted with acetone (1 × 1.3 L and then 1 × 0.6 L). Evaporation of the solvent left 24 g of a dark oil, which was fractionated by gravity silica gel chromatography (160 g, petroleum ether−EtOAc gradient). Fractions eluted with petroleum ether−EtOAc (9:1) afforded 865 mg of a mixture of coniferyl esters and fatty acids and 7.06 g of crude peucedanin (**4a**). After crystallization from petroleum ether−ether, 1.43 g (0.56%) of pure **4a** was obtained, while the fractions containing the coniferyl esters were further fractionated by gravity column chromatography with silica gel (35 g) using petroleum ether−EtOAc (95:5) as eluant. After crystallization from petroleum ether, 25 mg of coniferyl stearate (**6**) was obtained. The fractions eluted with petroleum ether−EtOAc (4:6) were further chromatographed on silica gel and then alumina, to afford 35 mg (0.014%) of dehydromarmesin methyl ether (**4b**), 18 mg (0.0071) of marmesin (**5a**), 51 mg (0.020%) of prantschimgin (**5b**), 15 mg (0.006%) of smirniorin (**5c**), and a mixture of **5d** and **5e** (298 mg). These compounds could be separated by HPLC on silica gel (*n*-hexane−EtOAc, 75:25, as eluant), eventually obtaining 24 mg (0.0090%) of **5d** and 16 mg (0.0064%) of **5e**.

4′-Acetyl-3′-senecioyl 3′-hydroxymarmesin (5d): foam; $[\alpha]_D^{25}$ −130° (*c* 0.4, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 321 (3.8), 295 (3.6), 221 (4.5) nm; IR (KBr) ν_{max} 3356, 1736, 1640, 1588, 1500, 1406 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HREIMS *m*/*z* 386.1387, calcd for $C_{21}H_{22}O_7$, 386.1366.

4′-Acetyl-3′-isobutyryl-3′-hydroxymarmesin (5e): foam; $[\alpha]_D^{25}$ −112° (*c* 0.3, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 321 (3.8), 294 (3.6), 222 (4.5) nm; IR (KBr) ν_{max} 3351, 1733, 1644, 1585, 1500, 1406 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HREIMS *m*/*z* 374.1299, calcd for $C_{20}H_{22}O_7$, 374.1286.

Coniferyl stearate (6): white powder, mp 89 °C; IR (KBr) ν_{max} 3521, 1732, 1705, 1611, 1606, 1518, 1464, 1437, 1279, 1165, 1026 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.90 (3H, m, H-2′, H-5′, and H-6′), 6.58 (1H, d, J = 15.8 Hz, H-3), 6.16 (1H, dt, J = 15.8, 6.7 Hz, H-2), 5.67 (1H, s, OH), 4.71 (2H, d, J = 6.7 Hz, H-1a,b), 3.91 (3H, s, OMe), 2.35 (2H, t, J = 7.2 Hz, H-2″a,b), 1.70 (2H, m, H-3″a,b), ca. 1.26 (30 H, br s), 0.66 (3H, t, J = 6.9 Hz, H-18″); EIMS *m*/*z* 446 $[M]^+$ $[C_{28}H_{46}O_4]^+$ (2).

Synthesis of Coniferyl Stearate (7). To a cooled (0 °C) and stirred solution of coniferyl alcohol (300 mg, 1.66 mmol) and stearic acid (480 mg, 1.66 mmol, 1 molar equiv) in dry THF (25 mL) were added triphenylphosphine (437 mg, 1.66 mmol, 1 molar equiv) and diethyl axodicarboxylate (40% in toluene, 733 mL, 1.66 mmol, 1 molar equiv). The cooling bath was removed, and the reaction was left to warm to room temperature for 2 h and then worked up by evaporation. The crude residue was purified by gravity column chromatography (10 g of silica gel) packed with petroleum ether. Elution was started with petroleum ether, collecting 200 mL of eluate, and then changed to petroleum ether−EtOAc (95:5) to obtain 340 mg (49%) of **7**, identical (MS, ^{1}H NMR) with the natural product.

Determination of Nuclear DNA Loss and Cell Cycle Analysis. The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70% for 24 h at 4° C). Then, the cells were washed twice with phosphate-buffered saline (PBS) containing 4% glucose and subjected to RNA digestion (RNase, 50 U/mL) and PI (20 mg/mL) staining in PBS for 1 h at room temperature. The cell cycle was analyzed by cytofluorimetry as previously described.[27] With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells, and the subsequent staining makes it possible to determine the percentage of subdiploid cells (sub-G0/G1 fraction).

Detection of DNA Strand Breaks by the TUNEL Method. The percentage of apoptotic cells was alternatively measured by the TUNEL method as previously described,[30] with minor modifications. Briefly, cells (1×10^6) were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C, washed twice in PBS, and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 20 min. Fixed cells were washed three times in PBS and resuspended in a final volume of 50 mL of TUNEL buffer (0.3 nmol of FITC-dUTP, 3 nmol of dATP, 50 nmol of $CoCl_2$, 5 U of TdT, 200 mM potassium cacodylate, 250 mg/mL bovine serum albumine (BSA), and 25 mM Tris-HCl pH 6.6). The cells were incubated for 1 h at 37 °C and then washed twice in PBS and analyzed by flow cytometry. To determine both the DNA strand breaks and the cell cycle, TUNEL-stained cells were counterstained with PI and treated with RNase as described above prior to cytofluorimetric analysis. In this method, fixation in formaldehyde prevents the leaking of low molecular weight DNA from apoptotic cells, and thus the cell cycle distribution estimates both apoptotic and nonapoptotic cells.[27]

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