Hyperjovinols A and B: Two New Phloroglucinol Derivatives from *Hypericum jovis* with Antioxidant Activity in Cell Cultures[1]

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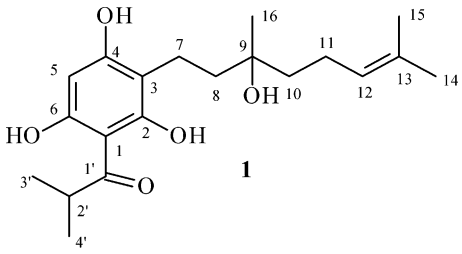
Two new and four known phloroglucinol derivatives were isolated from the dichloromethane extract of the Greek endemic plant *Hypericum jovis*. Their antioxidant activity was evaluated in vitro with the DPPH assay and in cell cultures using the DCFH-DA assay. All six compounds demonstrated significant antioxidant activity, while two of them possessed activity at a cellular level comparable to Trolox, protecting against ROS.

Plants of the genus *Hypericum* (Guttiferae), which comprises about 400 species, have been used for centuries in traditional medicine for the treatment of burns, bruises, swelling, inflammation, and anxiety, as well as bacterial and viral infections.[2] Particularly *Hypericum perforatum* (St. John's wort) has, over the course of time, been used for several disorders of the central nervous system such as insomnia, mental illness, and especially in the last years, depression. It is believed that such an effect takes place through the action of various active principles present in the plant.[3] The most common compounds isolated from plants of this genus are flavonoids, xanthones, phloroglucinol and filicinic acid derivatives, and benzopyrans less frequently. Under the great scientific interest and economic value acquired by *H. perforatum* many studies with other plants of the same genus have been carried out all around the world.[4,5]

Reactive oxygen species (ROS) are normal intermediates of metabolic processes in aerobic organisms. If these molecules are produced in excess, tissue damage can result. ROS have been proven to cause damage to the central nervous system[2] and have been implicated in the pathogenesis of various neurodegenerative disorders, including ischemia, Alzheimer's disease, Parkinson's disease, and schizophrenia.[6,7] Therefore, increased antioxidant concentration at the cellular level could provide considerable protection against ROS. Most of the known antioxidants are derived from plants, probably due to their increased capacity to defend themselves from various sources of stress. Thus, as a part of our program concerning the discovery of new antioxidant agents of natural origin, we report herein the phytochemical study and the antioxidant evaluation, in vitro and in cell cultures, of the constituents of *H. jovis* Greuter, an endemic plant of Central Crete.

Results and Discussion

Investigation of the dichloromethane extract of the air-dried aerial parts of *H. jovis* led to the isolation and structure elucidation of two new phloroglucinol derivatives, named hyperjovinol A (**1**) and hyperjovinol B (**2**), and four known phloroglucinol derivatives, **3**, **4**, **5**, and **7**. Compound **3** has been described as a mixture with incomplete assignment of ^{13}C NMR data,[8,9] while the ^{13}C NMR data of **5** and **7** have never been reported.



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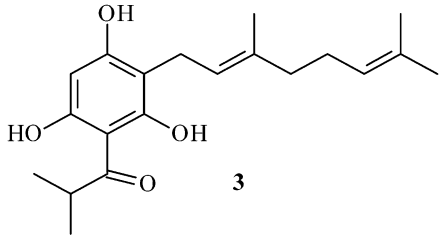

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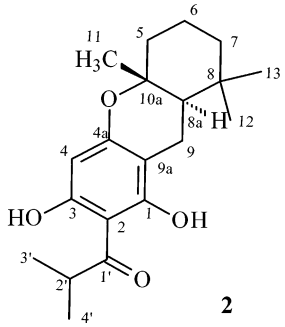

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The major constituent of the dichloromethane extract was found to be **3**, which is a phloroglucinol derivative with one geranyl and one isobutyryl chain placed in *meta* positions and directly attached to the aromatic ring. This compound has been previously described from *Helichrysum*[8] and *Esenbeckia* species,[9] with UV, IR, MS, and ^{1}H NMR data identical with those of the isolated compound. However, the previous ^{13}C NMR data were incomplete and erroneously assigned, and the correct data are given in the Experimental Section.

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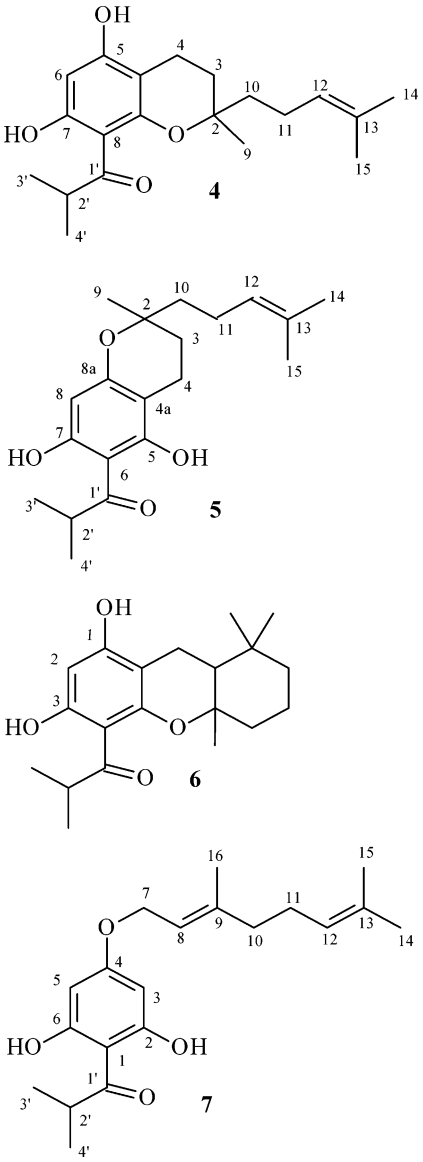

Hyperjovinol A (**1**) was isolated as a yellow amorphous, optically active compound, and its molecular formula was determined by HRMS as $C_{20}H_{30}O_5$. Its IR spectrum showed the presence of hydroxyl (3261 cm^{-1}) and carbonyl groups (1622 cm^{-1}), and its UV spectrum (288.5, 332 nm) indicated an aromatic moiety. Its ^1H NMR spectrum showed one singlet at 5.85 ppm (H-5), one triplet at 5.08 ppm (H-12), one septet at 3.90 ppm (H-2′ of the isobutyryl group), and several peaks between 1.14 and 2.65 ppm corresponding to four methylene and five methyl groups. Two of the methyl groups were relatively deshielded (1.58 ppm, Me-15 and 1.65 ppm, Me-14), indicating that they were attached to a double bond, and two other methyl groups appeared as a doublet at 1.14 ppm (Me-3′, Me-4′).

The ^{13}C NMR spectrum showed the presence of a ketone (210.9 ppm, C-1′), one double bond (123.9 ppm, C-12 and 132.5 ppm, C-13), and one pentasubstituted aromatic ring with three oxygenated aromatic carbons. Additionally there was a characteristic signal of one oxygenated quaternary aliphatic carbon (74.8 ppm, C-9). The DEPT spectrum confirmed the presence of one aliphatic methine, four methylenes, and five methyls constituting the side chains of the molecule.

In the HMBC spectrum the carbonyl carbon was correlated with the methine at 3.90 ppm (H-2′) and with the two methyls at 1.14 (Me-3′, Me-4′), which appeared as a doublet due to their correlation with the aforementioned methine. All these correlations indicated the presence of an isobutyryl group. This group was directly attached to the aromatic ring because the methine showed a ^3J correlation in the HMBC spectrum with a quaternary aromatic carbon. That carbon was also correlated with the aromatic proton at 5.85 ppm (H-5), indicating the *meta* placement of this proton relative to the isobutyryl group. The aromatic proton also showed a ^3J correlation with the other quaternary aromatic nonoxygenated carbon, obviously carrying the second side chain. This side chain was constituted of 10 carbons, specifically three methyl groups, four methylenes, one oxygenated quaternary aliphatic carbon, and one double bond with one olefinic proton, which was obviously consistent with an oxygenated geranyl group.

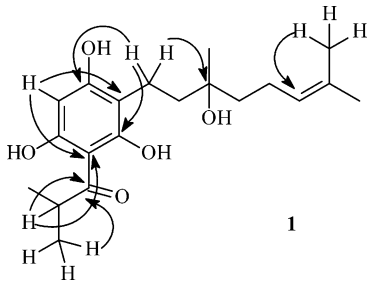

Figure 1. Important HMBC correlations of **1** optimized for $J = 8$ Hz.

The two protons of the methylene that was directly attached to the aromatic ring were identified by their correlation with two of the three oxygenated aromatic carbons. These two protons were also correlated with the oxygenated aliphatic carbon, revealing the position of the oxygenation of the geranyl group (Figure 1). All these data in combination with the MS spectrum revealed that **1** was a hydrated derivative of **3** with a hydroxy group attached at the C-9 position. However, the configuration at C-9 could not be established.

Two compounds very closely related to **1** were identified as **4** and **5**. These two compounds have been reported as semisynthetic derivatives of **3**,[8] and very recently **4** was reported as a natural constituent of *H. amblycalyx*,[10] an other endemic *Hypericum* species of Crete. Compound **5** is reported herein for the first time as a natural product, along with its ^{13}C NMR data.

Hyperjovinol B (**2**) was isolated as a yellow amorphous, optically active compound, and its molecular formula was determined by HRMS as $C_{20}H_{28}O_4$. Its ^1H NMR and ^{13}C NMR spectra showed similarities with all the aforementioned compounds, but in this case there was no double bond. This observation in combination with the molecular weight, which was identical with those of **4** and **5**, indicated that this compound should contain an additional ring. Its highly overlapping ^1H NMR spectrum surprisingly became very clear when it was recorded in C_6D_6, facilitating the structure elucidation. A key observation was the correlation in the HMBC spectrum between C-8a (47.8 ppm) and H-5 (0.99, 1.15 ppm), H-11 (0.76 ppm), and H-12 (0.62 ppm). This observation indicated that **2** was a derivative of **4** or **5** in which C-10a was joined with C-8a, forming a fused cyclohexane ring. The type of fusion between the pyrano and the aromatic ring and the discrimination between **2** and the alternative structure **6** were based on the NMR spectra performed in d_6-DMSO. In this solvent, the clear observation of the two free phenolic OH groups made evident the observation of their ^3J correlations in the HMBC spectrum. Indeed, C-4 was found to be correlated only with one OH group, which was identified as OH-3 by

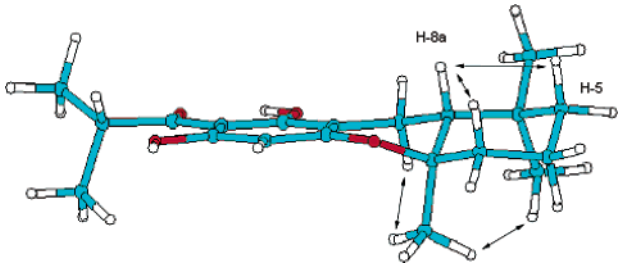

Figure 2. Important NOESY correlations of **2** measured in C_6D_6 (depicted for relative configuration of the minimized energy structure derived from MM+ force field).

Table 1. Free-Radical Scavenging Activity

extract	DPPH absorbance IC_{50}, μg/mL[a]
H. jovis extr. H_2O	73.0 (±12.0)
H. jovis extr. MeOH	48.7 (±23.9)
H. jovis extr. CH_2Cl_2	104.5 (±29.5)
Pycnogenol	36.5 (±9.2)

[a] Values are means of three experiments (standard deviation is given in parentheses).

its correlation with C-2. Additionally, the second free OH group was correlated with C-2 and C-9a. These correlations were compatible only with the structure of **2** because in the case of **6** C-2 should be correlated with both OH-1 and OH-3.

The *cis* or *trans* fusion of the pyrano and the cyclohexane rings was studied via the ^{1}H NMR and NOESY spectra in C_6D_6. In the ^{1}H NMR spectrum, H-8a was observed at 1.45 ppm as a doublet of doublets (13.3, 4.9 Hz). The big coupling constant with one of the two H-9 protons showed that H-8a was in an axial position. In the NOESY spectrum H-8a was found to be correlated with H-5 ax and not with H-13, proving that the type of fusion was *trans* (Figure 2).

Finally, compound **7** was identified as a previously described compound isolated from *Helichrysum gymnoconum*.[11] Its ^{1}H NMR data were identical with the literature, but its ^{13}C NMR data have never been reported.

To evaluate the antioxidant properties of *H. jovis*, we first examined the ability of the plant extracts to scavenge the stable free radical DPPH. As deduced from Table 1, *H. jovis* extracts are able to scavenge DPPH; especially the methanol extract possesses antioxidant activity comparable to that of a known—and widely accepted[12] standard—procyanidin-rich pine bark aqueous extract (Pycnogenol). However, the DPPH assay, like many of the commonly used antioxidative assays, is performed in a cell-free system. This approach does not consider cellular mechanisms that influence the net response to a substance.[13] For instance, the possibility that the cell membrane can impede the endocytosis of a potent free-radical scavenger cannot be ruled out. Consequently, we used the DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) assay. The latter is nonpolar and diffuses into the cell, where it is hydrolyzed by cellular esterases to form dichlorodihydrofluorescein, which, upon reaction with a broad range of oxidizing species, yields the highly fluorescent dichlorofluorescein.[14] All extracts of *H. jovis*, even at concentrations lower than their IC_{50} at the DPPH assay, inhibited the basal levels of reactive oxygen species (ROS) production in normal human skin fibroblasts. The inhibitory effect of the dichloromethane extract was comparable to that of Pycnogenol (Table 2).

Furthermore, when we tested the ability of the purified active constituents of the dichloromethane extract of the plant to inhibit endogenous cellular ROS production in the same assay (Table 3), we found that the activities of **1** and **3** were comparable to that of the known antioxidant Trolox (water-soluble form of vitamin E). Moreover, **1** also has the ability to prevent the exogenous stimulation of ROS production by H_2O_2, although in this case it was slightly less effective than Trolox (Figure 3).

Table 2. Intracellular ROS Levels

extract (100 μg/mL)	DCF-fluorescence (% of control)[a]
H. jovis extr. H_2O	64.8 (±3.4)
H. jovis extr. MeOH	50.3 (±1.1)
H. jovis extr. CH_2Cl_2	41.9 (±0.9)
Pycnogenol	41.3 (±1.4)

[a] Values are means of three experiments, standard deviation is given in parentheses.

Table 3. Intracellular ROS Levels

	DCF-fluorescence (% of control)[a] at concentration =	
compound	10 μM	100 μM
1	76.1 (±5.4)	49.2 (±4.1)
2	89.7 (±10.1)	57.1 (±2.5)
3	79.4 (±2.3)	50.8 (±4.5)
4	81.5 (±0.5)	73.0 (±0.4)
5	94.1 (±2.2)	62.4 (±10.2)
7	92.2 (±2.1)	61.5 (±4.4)
Trolox	81.4 (±19.2)	55.2 (±6.0)

[a] Values are means of three experiments; standard deviation is given in parentheses.

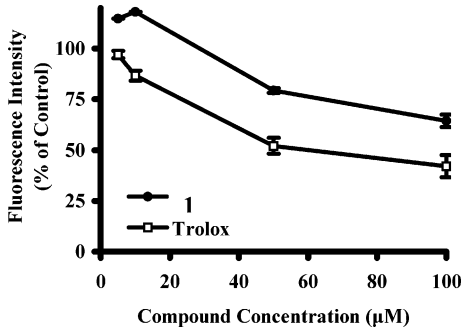

Figure 3. Inhibition of ROS production in human skin fibroblasts. Cells were pretreated with hyperjovinol A (**1**) or Trolox for 4 h and then loaded with 10 μM DCFH-DA and challenged with 100 μM H_2O_2, as described in the Experimental Section. Data were calculated as percent of control DCF-fluorescence (the fluorescence of a culture not treated with antioxidant prior to H_2O_2 challenge). Values represent the mean of three independent experiments (error bars indicate standard deviation).

Concerning some structure−activity relationships, it is clear that the presence of three free hydroxyl groups, as in the case of **1** and **3**, enhances the antioxidant activity, at least at 100 μM, in comparison with **2**, **4**, **5**, and **7**, which have only two free hydroxyl groups.

In conclusion, the significant antioxidant activity of the Greek endemic species *H. jovis* was identified, and its active principles were isolated and characterized. These active constituents have the ability to exert their antioxidative action intracellularly, blocking endogenous ROS production, as shown by the DCFH-DA assay.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Shimadzu-160A spectrophotometer. IR spectra were recorded on a Perkin-Elmer Paragon 500 instrument. ^{1}H NMR spectra were measured on a Bruker DRX-400 (400 MHz) spectrometer and ^{13}C NMR on a Bruker

AC-200 (50 MHz). Chemical shifts are given in δ values with TMS as an internal standard. Coupling constants (J) are given in Hz. COSY, NOESY (mixing time = 1000 ms), HMQC, and HMBC data were were performed using standard Bruker microprograms. FABMS were obtained using a ZAB HF instrument, in glycerol matrix with NaCl as additive for positive ion mode. HRMS were obtained on a AEI MS-902 mass spectrometer. EIMS were determined on a HP-6890 spectrometer. Column chromatography was conducted using Merck silica flash gel 60 (40−63 μm), with an overpressure of 300 mbar.

Plant Material. The aerial parts of *H. jovis* were collected at Zaros gorge in central Crete, Greece, in July 2001. A voucher sample (No. KL126) has been deposited in the herbarium of the Laboratory of Pharmacognosy, Department of Pharmacy, University of Athens.

Extraction and Isolation. The air-dried aerial parts of the plant (300 g) were pulverized using a laboratory mill and successively extracted with CH_2Cl_2, MeOH, and H_2O (3 × 1.5 L for each solvent). The CH_2Cl_2 extract was evaporated under reduced pressure, and the residue (33 g) was submitted to vacuum column chromatography using CH_2Cl_2−cyclohexane (50:50 to 100:0) and CH_2Cl_2−MeOH (100:0 to 98:2) gradient solutions, which finally gave fractions A1−10. Fraction A7 was pure **3** (1.2 g). Fraction A6 was submitted to flash chromatography using a CH_2Cl_2−cyclohexane gradient and gave fractions B1−15. Fraction B7 was submitted to flash chromatography with CH_2Cl_2−cyclohexane (10:90 to 30:70) and gave **2** (35 mg) and **7** (20 mg). Fraction B8 was submitted to flash chromatography with CH_2Cl_2−cyclohexane (20:80 to 50:50) and gave **4** (252 mg) and **5** (56 mg). Fraction A9 was submitted to flash chromatography with CH_2Cl_2−MeOH (99:1) and gave **1** (34 mg).

The relative configuration of these compounds and the complete assignment of ^{1}H and ^{13}C NMR spectra were done via 2D NMR techniques.

Hyperjovinol A (1): yellow amorphous solid; $[\alpha]_D$ +5° (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} nm (log ϵ) 288.5 (3.95), 332 sh; IR ($CHCl_3$) ν_{max} cm^{-1} 3261, 2970, 1622, 1234, 1140; ^{1}H NMR ($CDCl_3$/TMS, 400 MHz, δ ppm, J in Hz) 1.14 (6H, d, J = 6.4 Hz, Me-3′, Me-4′), 1.22 (3H, s, Me-16), 1.55 (2H, t, J = 8.2 Hz, H-10), 1.58 (3H, s, Me-15), 1.65 (3H, s, Me-14), 1.73 (2H, t, J = 6.9 Hz, H-8), 2.03 (2H, dt, J = 6.8, 8.2 Hz, H-11), 2.60 (H, dt, J = 14.0, 6.9 Hz, H-7a), 2.65 (H, dt, J = 14.0, 6.9 Hz, H-7b), 3.90 (H, sept, J = 6.4 Hz, H-2′), 5.08 (H, t, J = 6.8 Hz, H-12), 5.85 (H, s, H-5); ^{13}C NMR ($CDCl_3$/TMS, 50 MHz, δ ppm) 15.9 (C-7), 17.6 (C-15), 19.3 (C-3′, C-4′), 22.9 (C-11), 25.7 (C-14), 26.6 (C-16), 39.1 (C-2′), 39.7 (C-8), 41.9 (C-10), 74.8 (C-9), 95.4 (C-5), 104.1 (C-1), 108.7 (C-3), 123.9 (C-12), 132.5 (C-13), 159.6 (C-6), 160.6 (C-4), 162.8 (C-2), 210.9 (C-1′); FABMS m/z 373 $[M + Na]^+$; HRFABMS m/z 373.1994 (calcd for $C_{20}H_{30}O_5Na$, 373.1991).

Hyperjovinol B (2): yellow amorphous solid; $[\alpha]_D$ −2° (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} nm (log ϵ) 292.5 (3.98), 349 (sh); IR ($CHCl_3$) ν_{max} cm^{-1} 3252, 2938, 1625, 1227, 1146; ^{1}H NMR (C_6D_6/TMS, 400 MHz, δ ppm, J in Hz) 0.62 (3H, s, Me-12), 0.76 (3H, s, Me-11), 0.99 (H, td, J = 13.3, 4.4, H-5ax), 1.07 (3H, s, Me-13), 1.15 (H, ddd, J = 13.3, 4.8, 3.4, H-5eq), 1.22 (3H, d, J = 6.8, Me-4′), 1.23 (3H, d, J = 6.8, Me-3′), 1.29 (2H, m, H-6), 1.45 (H, dd, J = 13.3, 4.9, H-8a ax), 1.56 (H, td, J = 12.6, 4.1, H-7ax), 1.89 (H, ddd, J = 12.6, 4.8, 3.4, H-7eq), 2.28 (H, dd, J = 16.6, 13.3, H-9ax), 2.87 (H, dd, 16.6, 4.9, H-9eq), 3.89 (H, sept, J = 6.8, H-2′), 5.48 (H, s, H-4); ^{13}C NMR (C_6D_6/TMS, 50 MHz, δ ppm) 17.7 (C-9), 19.5 (C-4′), 19.6 (C-3′), 19.9 (C-6), 19.9 (C-13), 20.5 (C-12), 31.9 (C-11), 33.4 (C-8), 39.1 (C-2′), 39.5 (C-7), 41.5 (C-5), 47.8 (C-8a), 79.0 (C-10a), 95.5 (C-4), 103.8 (C-2), 103.8 (C-9a), 157.9 (C-3), 159.9 (C-4a), 165.4 (C-1), 210.3 (C-1′); EIMS m/z 332 (40), 289 (100), 209 (20), 165 (40); HREIMS m/z 332.1982 (calcd for $C_{20}H_{28}O_4$, 332.1988).

3: ^{13}C NMR ($CDCl_3$/TMS, 50 MHz, δ ppm) 16.0 (C-16), 17.6 (C-15), 19.2 (C-3′, C-4′), 21.4 (C-7), 25.6 (C-14), 26.9 (C-11), 39.1 (C-2′), 39.6 (C-10), 95.4 (C-5), 104.1 (C-1), 106.2 (C-3), 121.6 (C-8), 123.7 (C-12), 131.8 (C-13), 138.8 (C-9), 160.1 (C-6), 161.3 (C-4), 162.7 (C-2), 211.5 (C-1′).

5: $[\alpha]_D$ −1° (*c* 0.1, $CHCl_3$); ^{13}C NMR ($CDCl_3$/TMS, 50 MHz, δ ppm) 15.8 (C-4), 17.6 (C-15), 19.3 (C-3′, C-4′), 22.2 (C-11), 24.0 (C-9), 25.7 (C-14), 30.2 (C-3), 39.1 (C-2′), 39.4 (C-10), 77.8 (C-2), 95.7 (C-8), 103.2 (C-6), 101.8 (C-4a), 123.9 (C-12), 131.9 (C-13), 157.6 (C-7), 160.1 (C-8a), 164.0 (C-5), 210.3 (C-1′).

7: ^{13}C NMR ($CDCl_3$/TMS, 50 MHz, δ ppm) 16.7 (C-16), 17.7 (C-15), 19.2 (C-3′, C-4′), 25.7 (C-14), 26.2 (C-11), 39.2 (C-2′), 39.5 (C-10), 65.1 (C-7), 94.5 (C-3), 94.5 (C-5), 104.0 (C-1), 118.4 (C-8), 123.6 (C-12), 131.9 (C-13), 142.1 (C-9), 164.7 (C-6), 163.3 (C-4), 164.7 (C-2), 210.2 (C-1′).

Free-Radical Scavenging Assay. The test compounds diluted in DMSO were added to a solution of the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) (Fluka) in absolute ethanol (final DPPH concentration: 0.5 mM), and the absorbance at 510 nm was monitored at various time points.[15] A DPPH solution with the corresponding concentration of the vehicle DMSO (10% v/v) was used as control. Results are expressed as IC_{50}'s, i.e., the final concentration of the test compound in the reaction mixture (in μg/mL) that reduces the absorbance to 50% of that of the vehicle.

Intracellular ROS Assay. The applied method was a modification of that described by Von Zglinicki et al.[16] Normal human skin fibroblast cultures developed in our laboratory were routinely cultured as described.[17] Cells were plated in 96-well plates at a density of 10^4 cells/well in 100 μL of Minimum Essential Medium (MEM) supplemented with 10% FBS. When the cultures reached confluency (typically, 48 h after plating), the test compounds diluted in DMSO and further diluted in culture medium were added (final volume: 100 μL). After a 4 h incubation, media were aspirated, the cell monolayers were washed with phosphate-buffered saline (PBS), and 100 μL of a 10 μM DCFH-DA (Molecular Probes) solution in PBS was added to the cells. After 45 min of incubation at 37 °C in a humidified chamber, fluorescence was determined using a Fluostar Galaxy microplate reader (BMG Labtechologies); excitation was at 485 nm, and emission was read at 520 nm. Alternatively, 30 min after DCFH-DA addition, the cell cultures received a supplementary 10 μL of a 1.1 mM H_2O_2 solution (final H_2O_2 concentration: 100 μM) for a further 30 min, and then fluorescence determination followed. After the incubation with the test compounds for the indicated time intervals, no signs of cytotoxicity were microscopically evident, nor any changes in cell-viability compared to the untreated cells (typically 95%, as determined by the Trypan Blue exclusion test). Fluorescence values were normalized according to the total protein content of each well: after solubilization of the cell monolayer with 80 μL of 0.1 N NaOH and staining with 20 μL of a protein assay dye reagent concentrate according to the manufacturer's (BioRad) protocol, absorbance was recorded at 595 nm and protein concentration was estimated by means of a reference curve, constructed using known amounts of bovine serum albumine (BSA) (Sigma).

Controls. For both assays, the vehicle (DMSO) was used as negative control, while a procyanidin-rich pine bark aqueous extract (Pycnogenol) (Vilco) and a synthetic compound (Trolox) (Fluka) with established antioxidant properties were used as positive controls.

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References and Notes

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