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Cytotoxic Constituents of the Lichen *Diploicia canescens*

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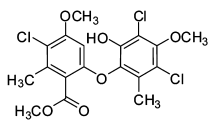
A new diphenyl ether (**1**), along with 12 known compounds, was isolated from the lichen *Diploicia canescens*. The structure of compound **1** was elucidated by spectroscopic data analysis, and the biosynthetic origin of this product is discussed. Secalonic acids B (**7**), D (**8**), and F (**9**) were isolated for the first time from *D. canescens*. The cytotoxic activities of **1**−**3**, **6**−**8**, and **10** against the B16 murine melanoma and HaCaT human keratinocyte cell lines were evaluated.

In the course of chemical studies of lichens from the seashore of Brittany, France, as potential sources of photoprotective metabolites, several crustose lichens were collected. *Diploicia canescens* (Dicks.) Massal. (Physciaceae) was selected after a TLC screen of lichens using a spectrophotodensitometer, with UV-absorbing spots indicative of photoprotective compounds. *D. canescens* is very common on basic and nutrient-enriched trees, rocks, and walls. This lichen is known to produce many chlorinated depsidones such as diploicin (**2**), dechlorodiploicin (**3**), 4-*O*-methyldiploicin (**4**), 4-*O*-methyldechlorodiploicin (**5**), scensidin (**6**), and 3-*O*-demethylscensidin.[1,2] Two depsides, atranorin and chloroatranorin, were also isolated.[1] Sala and Sargent reported two new phthalide metabolites, buellolide and canesolide, from an Australian specimen.[3] Elix et al. also reported the presence of bisxanthone derivatives (secalonic acids A−C) from another Australian specimen.[4]



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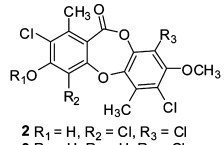
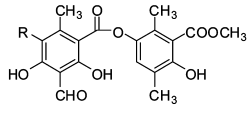
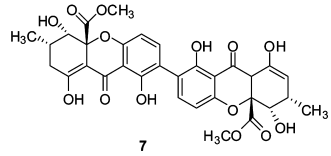


2 R_1 = H, R_2 = Cl, R_3 = Cl
3 R_1 = H, R_2 = H, R_3 = Cl
4 R_1 = CH_3, R_2 = Cl, R_3 = Cl
5 R_1 = CH_3, R_2 = H, R_3 = Cl
6 R_1 = CH_3, R_2 = H, R_3 = H



atranorin R = H
chloroatranorin R = Cl



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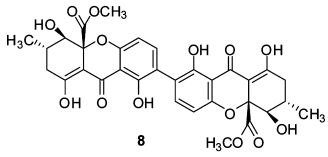

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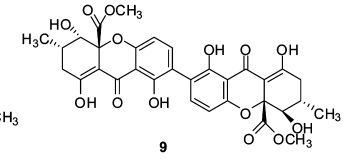

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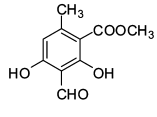

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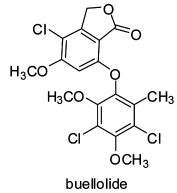

buellolide

Lichen thalli (155 g) were extracted successively in a Soxhlet apparatus with solvents of increasing polarity (*n*-heptane, CH_2Cl_2, and THF). Isolation and purification of compounds from *D. canescens* were performed using combinations of various chromatographic techniques (Sephadex LH-20 and silica gel columns, circular chromatography, preparative TLC). The extraction yield of the CH_2Cl_2 extract (5.9%) was quite high due to the presence of diploicin (**2**) in a high amount (120 mg), together with dechlorodiploicin (**3**) (34 mg), atranorin (not isolated), and chloroatranorin (14 mg). Secalonic acids B (**7**) (30 mg), D (**8**) (5 mg), and F (**9**) (11 mg) were also present in lower quantities. The *n*-heptane extract (1.6%) contained three known depsidones, 4-*O*-methyldiploicin (**4**) (3 mg), 4-*O*-methyldechlorodiploicin (**5**) (5 mg), and scensidin (**6**) (36 mg), the monoaromatic compound methyl β-orcinol carboxylate (**10**)[5] (62 mg), and a new compound (**1**) (23 mg). The THF extract contained mainly the polyol D-arabitol.

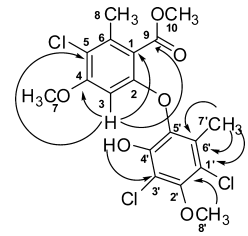

Figure 1. Selected HMBC correlations (H→C) of buellin (**1**).

Compound **1** was obtained as a yellow solid and gave a molecular formula of $C_{18}H_{17}O_6Cl_3$, as determined from the HREIMS, which showed a typical molecular ion cluster at *m*/*z* 434.0105 (calcd 434.0091). In the UV spectrum of **1**, absorption maxima were observed at 236 (log ϵ 4.56) and 287 (log ϵ 3.83) nm, revealing the presence of a conjugated system. The ^{1}H NMR spectrum indicated the presence of signals for one aromatic proton at δ 6.08, two methyl groups at δ 2.34 and 2.45, three methoxy groups at δ 3.75, 3.93, and 4.03, and a phenolic hydroxy group at δ 8.49. The complete structure of **1** was established using HMBC correlations (Figure 1), with the following connectivities observed: methyl protons CH_3-7′ (δ 2.34) with C-1′ (δ 119.7), C-5′ (δ 138.1), and C-6′ (δ 129.6); methyl protons CH_3-8 (δ 2.45) with C-1 (δ 116.6), C-5 (δ 118.4), and C-6 (δ 136.9). The *O*-methyl protons at δ 3.75, 3.93, and 4.03 correlated, respectively, in the HMBC spectrum with C-5 (δ 118.3), C-2′ (δ 151.0), and C-7 (δ 169.1). The H-3 aromatic proton (δ 6.08) correlated with C-1 (δ 116.6), C-2 (δ 154.2), C-4 (δ 157.4), C-5 (δ 118.4), and C-9 (δ 168.9). These data were used to define compound **1** (buellin) as a new chlorinated diphenyl ether derivative structurally close to the depsidone 4-*O*-methyldechlorodiploicin and the phthalide buellolide.

Compounds **7**−**9** were isolated as light yellow needles from the CH_2Cl_2 extract using silica gel column chromatography and preparative TLC. NMR studies when combined with optical properties were used to clearly characterize these compounds,

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Scheme 1. Biogenetic Scheme with Buellin (**1**) as an Intermediate toward Buellolide

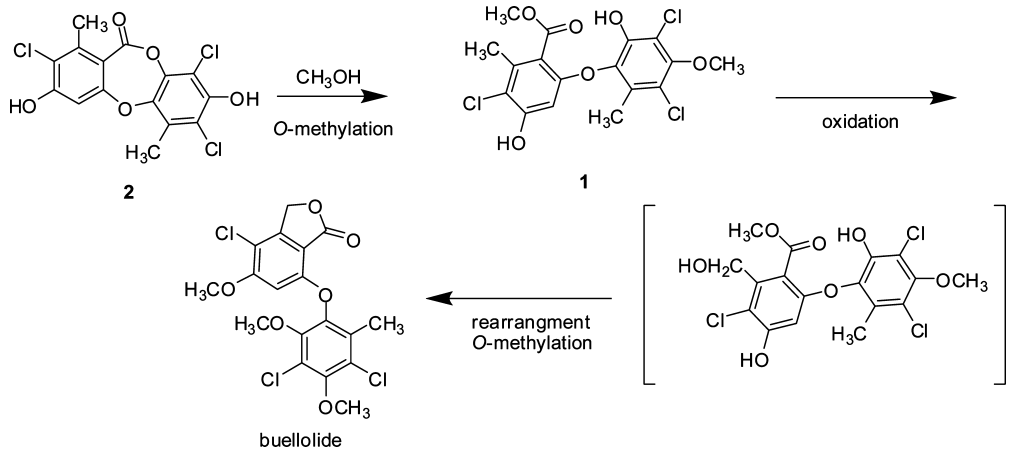

Table 1. Cytotoxic Activities (IC_{50} μM) of Compounds **1**−**3** and **6**−**8** against B16 Murine Melanoma Cells and HaCaT Human Keratinocyte Cells

	1	**2**	**3**	**6**	**7**[a]	**8**[a]	etoposide
B16	0.25 ± 0.1	4.0 ± 1.0	1.7 ± 0.2	0.4 ± 0.1	2.8 ± 0.3	0.28 ± 0.02	0.28 ± 0.08
HaCaT	>10	>10	4.3 ± 0.8	6.1 ± 0.6	>10	3.7 ± 0.5	0.55 ± 0.15

[a] Compounds **7** and **8** were evaluated after 48 h (B16) or 72 h (HaCaT) of incubation at 37 °C, as already reported for these compounds.[14,15]

respectively, as (+)-secalonic acids B,[6] D,[7] and F.[7] Elix and his co-workers reported the co-occurrence of (−)-secalonic acid A and (+)-secalonic acids B and C, based on the correlation of TLC and HPLC profiles in comparison with authentic samples.[4] In this study, a variation was shown of the concentration of these pigments with the locality of collection and described them as "typically accessory substances" without taxonomic value. Despite several previous publications on *D. canescens* (seven papers from 1934 to 2008), secalonic acids B (**7**), D (**8**), and F (**9**) were isolated for the first time from this lichen in the present investigation. These substances can be named also ergochromes BB, DD, and BD, respectively, following their first description by Frank et al. from *Claviceps purpurea*.[8] Secalonic acid D (**8**) has been isolated from *Penicillium oxalicum*[9] and described along with secalonic acid F from *Aspergillus aculeatus*.[7] The isolation of these isomers in the lichen *D. canescens* is not surprising because of their biogenetic relationships. The association of two monomeric tetrahydroxanthone precursors, (+)-B or (+)-D, led to the formation of ergochromes BB and DD, respectively.[10] Secalonic acid F (**9**) is a "hybrid dimer" containing monomeric units of both secalonic acids B (**7**) and D (**8**). The tetrahydroxanthone components of secalonic acids produced by *D. canescens* have the same relative configuration at position C-10a, indicating that the cyclization is catalyzed by a specific enzyme giving a unique type of product. Diphenyl ethers are relatively rare in lichens when compared to depsides and depsidones. Nevertheless, compound **1** is the third example of a diphenyl ether found in *D. canescens*. This compound is closely related to the depsidone dechlorodiploicin (**2**) and the diphenyl ether buellolide.

The co-occurrence of depsidones and diphenyl ethers in the same lichen suggests a biosynthetic relationship between these compounds. Depsidone biosynthesis is still under active consideration. Sala and Sargent postulated that benzophenone may lead to the formation of a depsidone through a grisadienedione intermediate.[11] A more recent hypothesis suggested that a depsidone could ensue from oxidation of a *para*-depside by dioxygenase followed by cyclization of a dihydroxydihydrobenzene intermediate.[12] The diphenyl ether **1** could thus be afforded either by methanolysis of dechlorodiploicin (as already described)[11] followed by *O*-methylation (Scheme 1) or by direct methanolysis from 4-*O*-methyldechlorodiploicin. We suggest **1** is a genuine metabolite of *D. canescens*, as it was present in the *n*-heptane extract before initiating the purification process. The formation of the phthalide derivative, buellolide, involves the oxidation of the 1-methyl group of dechlorodiploicin, subsequent fission of the depside bond, concomitant recyclization with the formation of the more stable five-membered lactone ring, and ultimate *O*-methylation.[4] Elix and his collaborators have reported distinct taxonomic entities of *D. canescens* following their chemical composition. The Australian taxon that contained the phthalide derivatives was considered to be biochemically more advanced than the European taxon that produced depsidones alone.[4] Herein, our Brittany taxon could be considered as an intermediate entity with a diphenyl ether that can be considered as a biosynthetic precursor prior to cyclization of the lactone ring of buellolide (Scheme 1). The environmental conditions or collection period could also account for the different chemical compositions of these taxa.

The cytotoxic activities of compounds **1**−**3**, **6**−**8**, and **10** against the B16 murine melanoma and HaCaT human keratinocyte cell lines were evaluated using the MTT assay, with etoposide as positive control (IC_{50} = 0.28 and 0.55 μM for B16 and HaCaT, respectively) (Table 1).[13] Most of the compounds tested showed significant and selective cytotoxicity against B16 cells when compared with HaCaT cells. Only compound **10** was inactive (IC_{50} >10 μM for both cell lines), suggesting the importance of the chlorine atom for the activity of these aromatic derivatives. The most highly cytotoxic compounds against B16 cells were secalonic acids B (**7**) and D (**8**) and buellin (**1**). Compounds **7** and **8** have been already reported as either cytostatic[14] or cytotoxic[15] compounds, and their biological activities differ according to their relative configuration.

Experimental Section

General Experimental Procedures. Melting points were measured on a hot-stage Kofler apparatus. Optical rotations were determined with a Perkin-Elmer model 341 polarimeter. UV spectra were performed on a Uvikon 931 spectrophotometer. FT-IR spectra were run on a Perkin-Elmer 16 PC IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker DMX 500 WB NMR spectrometer or at 270 and 67.5 MHz, respectively, on a JEOL GSX 270 WB NMR spectrometer, using $CDCl_3$, DMSO-d_6, and acetone-d_6 as solvents (and TMS as internal standard). HRMS measurements for exact mass determination were performed on a Varian MAT 311 mass spectrometer for electronic impact and a Micromass ZabspecTOF mass spec-

trometer for chemical ionization at the Centre Régional de Mesures Physiques de l'Ouest. Chromatographic separation was performed using column chromatography on silica gel (35−70 μm) and Sephadex LH-20 gel.

Plant Material. *Diploicia canescens* thalli were collected on the rocks of the sea coast near Dinard (48°37′09,14″ N 2°01′07,45″ W), Ille et Vilaine, France, in September 2006. Voucher specimens have been deposited at the herbarium of Pharmacognosy and Mycology, Rennes, France (REN-ABB), with the reference number JB/06/10. The identification of the lichen material was supported by thalline chemical tests (K+ yellow, UV−).

Extraction and Isolation. Thalli of *D. canescens* (155 g) were first homogenized and then successively extracted with *n*-heptane, CH_2Cl_2, THF, and MeOH, using a Soxhlet apparatus. The *n*-heptane extract (2.6 g) was chromatographed on a silica gel column with a gradient solvent system of a mixture of *n*-heptane−AcOEt (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8, 0:10), to yield 17 fractions (Fa1−Fa17). Fraction Fa2 (61 mg), obtained with cyclohexane−EtOAc (95:5), was chromatographed with cyclohexane−CH_2Cl_2 mixtures to yield compounds **5** (5 mg) and **4** (3 mg). Fraction Fa4 was chromatographed on a silica gel column with a cyclohexane−CH_2Cl_2 gradient solvent system to afford six fractions (Fa4a−Fa4f). Fraction Fa4c was chromatographed by circular chromatography using cyclohexane−CH_2Cl_2 to furnish 36 mg of an amorphous yellow powder (compound **6**). Fraction Fa6 (480 mg) was chromatographed on a Sephadex LH-20 gel column with CH_2Cl_2−acetone (85:15) to afford three fractions (Fa6a−Fa6c). Fraction Fa6a was purified by preparative TLC using cyclohexane−MeOH (95:5) to yield 23 mg of compound **1**. Fraction Fa7 (348 mg) was purified by flash chromatography with cyclohexane−CH_2Cl_2 (80:20) to yield 70 fractions of 20 mL each. Fractions 12 to 36 contained 62 mg of a pure compound, **10**. On cooling the CH_2Cl_2 extract and gentle evaporation of the solvent, 5.04 g of a white-brown precipitate was obtained. The purification of 100 mg by flash chromatography with a mixture of cyclohexane−EtOAc−THF−MeOH (14:3:3:1) afforded 34 mg of compound **3**, 120 mg of compound **2**, and 14 mg of chloroatranorin. Silica gel column chromatography of the CH_2Cl_2 filtrate (2.02 g) with a gradient solvent system of *n*-heptane−AcOEt (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 0:10), 100 mL of each solvent, yielded 50 further fractions (Fb1−Fb21). In fractions Fb14 and Fb17, a yellow precipitate was formed and was separated from the supernatant by centrifugation. Altogether, 5 mg of compound **8** and 30 mg of compound **7** were obtained. Compound **9** (11 mg) was obtained by preparative TLC in toluene−EtOAc−formic acid (139:83:8). The THF extract was treated with a mixture of *n*-BuOH and water (2:1) and contained 186 mg of D-arabitol.

Buellin (1): yellow solid (CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 236 (4.56), 287 (3.83) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.34 (3H, s, CH_3-7′), 2.45 (3H, s, CH_3-8), 3.75 (3H, s, OCH_3-7), 3.93 (3H, s, OCH_3-8′), 4.03 (3H, s, OCH_3-10), 6.08 (1H, s, H-3), 8.49 (1H, s, OH-4′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.0 (C-9), 157.4 (C-4), 154.2 (C-2), 151.0 (C-2′), 145.9 (C-4′), 138.1 (C-5′), 136.9 (C-6), 129.6 (C-6′), 119.7 (C-1′), 118.3 (C-5), 116.6 (C-1), 115.2 (C-3′), 97.4 (C-3), 60.7 (C-8′), 56.4 (C-7), 53.0 (C-10), 17.9 (C-8), 14.2 (C-7′); EIMS *m*/*z* 434 $[M]^+$ 402 $[M - MeOH]^+$ 367 $[M - Cl]^+$; HREIMS *m*/*z* 434.0105 $[M]^+$ (calcd for $C_{18}H_{17}O_6Cl_3$ 434.0091); R_f 0.87 (toluene−EtOAc−formic acid, 6:3:0.3).

Diploicin (2): white powder (acetone); UV (MeOH) λ_{max} (log ε) 207 (4.65), 320 (4.04) nm; ^{1}H and ^{13}C NMR (acetone-d_6, 270 MHz), data comparable to published data;[16−18] EIMS *m*/*z* 422 $[M]^+$ 387 $[M - Cl]^+$; HREIMS *m*/*z* 421.9267 $[M]^+$ (calcd for $C_{16}H_{10}O_5Cl_4$ 421.9282); R_f 0.67 (toluene−EtOAc−formic acid, 7:2:0.5).

Dechlorodiploicin (3): white powder (CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 207 (4.67), 314 (3.78) nm; ^{1}H NMR ($CDCl_3$, 270 MHz), data comparable to published data;[3] EIMS *m*/*z* 388 $[M]^+$, 352 $[M - Cl]^+$; HREIMS *m*/*z* 387.9674 $[M]^+$ (calcd for $C_{16}H_{11}O_5Cl_3$ 387.9672); R_f 0.87 (toluene−EtOAc−formic acid, 7:2:0.5).

4-*O*-Methyldiploicin (4): white powder (CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 229 (4.25), 256 (2.43) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.94 (3H, s, OCH_3-9), 3.87 (3H, s, OCH_3-8′), 2.64 (3H, s, CH_3-7′), 2.52 (3H, s, CH_3-8); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 160.3 (COO, C-7), 156.4 (C, C-4), 156.2 (C, C-2), 150.9 (C, C-2′), 146.4 (C, C-5′), 140.1 (C, C-4′), 140.0 (C, C-6), 129.1 (C, C-6′), 128.7 (C, C-5), 126.6 (C, C-1′), 119.1 (C, C-1), 118.7 (C, C-3), 118.3 (C, C-3′), 60.8 (OCH_3, C-8), 60.7 (OCH_3, C-8′), 18.7 (CH_3, C-9), 15.3 (CH_3, C-7′); EIMS *m*/*z* 436 $[M]^+$, 401 $[M - Cl]^+$, 373 $[M - CO - Cl]^+$; HREIMS *m*/*z* 435.9419 $[M]^+$ (calcd for $C_{17}H_{12}O_5Cl_4$ 435.9439); R_f 0.80 (toluene−EtOAc−formic acid, 7:2:0.5).

4-*O*-Methyldechlorodiploicin (5): white powder (CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 231 (4.54), 261 (4.16), 326 (3.82) nm; ^{1}H ($CDCl_3$, 500 MHz), data comparable to published data;[3] ^{13}C NMR ($CDCl_3$, 125 MHz) δ 160.9 (COO, C-7), 160.6 (C, C-2), 158.9 (C, C-4), 150.6 (C, C-2′), 146.3 (C, C-5′), 142.4 (C, C-6), 140.6 (C, C-4′), 128.1 (C, C-6′), 126.4 (C, C-1′), 122.0 (C, C-5), 118.5 (C, C-3′), 114.5 (C, C-1), 101.3 (CH, C-3), 60.8 (OCH_3, C-8′), 56.7 (OCH_3, C-8), 18.5 (CH_3, C-9), 13.8 (CH_3, C-7′); EIMS *m*/*z* 402 $[M]^+$, 367 $[M - Cl]^+$, 352 $[M - CH_3 - Cl]^+$; HREIMS *m*/*z* 401.9830 $[M]^+$ (calcd for $C_{17}H_{13}O_5Cl_3$ 401.9829); R_f 0.85 (toluene−EtOAc−formic acid, 7:2:0.5).

Scensidin (6): yellow powder (CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 231 (4.60), 263 (4.10), 285 (3.94) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) data comparable to published data;[19] ^{13}C NMR ($CDCl_3$, 125 MHz) δ 162.3 (COO, C-7), 161.3 (C, C-2), 158.7 (C, C-4), 152.7 (C, C-2′), 142.9 (C, C-4′, C-5′), 142.1 (C, C-6), 130.3 (C, C-6′), 121.5 (C, C-5), 119.3 (C, C-1′), 114.9 (C, C-1), 101.9 (C, C-3′), 101.3 (CH, C-3), 56.6 (OCH_3, C-8), 56.5 (OCH_3, C-8′), 18.5 (CH_3, C-9), 13.8 (CH_3, C-7′); EIMS *m*/*z* 368 $[M]^+$, 333 $[M - Cl]^+$, 318 $[M - CH_3 - Cl]^+$, 305 $[M - CO - Cl]^+$; HREIMS *m*/*z* 368.0217 $[M]^+$ (calcd for $C_{17}H_{14}O_5Cl_2$ 368.0218); R_f 0.69 (toluene−EtOAc−formic acid, 7:2:0.5).

Cytotoxicity Assay. Each experiment was repeated at least three times, and three different wells were used for each concentration. The cytotoxic activity of compounds **1**−**3**, **6**−**8**, and **10** was determined against B16 cells seeded at 15 000 cells/mL in RPMI 1640 with 5% fetal calf serum (FCS) and against HaCaT cells seeded at 25 000 cells/mL in DMEM with 10% FCS at day 0 in a 48-well plate. The medium was removed after 5 h to RMPI with 1% FCS or to DMEM with 2% DMEM. Compounds were serially diluted in appropriate medium at days 1, 2, and 3 with concentrations ranging from 0.25 to 100 μM. Incubation was performed at 37 °C in an atmosphere of 10% CO_2. After 72 h of incubation, cell growth and viability were measured at day 4, using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as previously described.[20] The cytotoxic activity of compounds **7** and **8** was determined in the same way except in a 96-well plate on B16 cells seeded at 30 000 cells/mL in RPMI 1640 with 10% FCS and HaCaT cells seeded at 15 000 cells/mL in DMEM with 10% FCS at day 0. Compounds were diluted at day 1. Incubation was performed at 37 °C for B16 and HaCaT cells in an atmosphere of 10% or 5% CO_2, respectively. Cell growth and viability were measured after 48 or 72 h of incubation for B16 and HaCaT cells, respectively.

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Supporting Information Available: NMR data of buellin (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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