Heptaketides from *Corynespora* sp. Inhabiting the Cavern Beard Lichen, *Usnea cavernosa*: First Report of Metabolites of an Endolichenic Fungus[1]

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Two new heptaketides, corynesporol (**1**) and 1-hydroxydehydroherbarin (**2**), along with herbarin (**3**) were isolated from an endolichenic fungal strain, *Corynespora* sp. BA-10763, occurring in the cavern beard lichen *Usnea cavernosa*. The structures of **1**−**3** were elucidated from their spectroscopic data. Aerial oxidation of corynesporol (**1**) yielded herbarin (**3**). Acetylation of **1** afforded the naphthalene derivative **4**, whereas acetylation of **3** gave the corresponding naphthoquinone **6** and dehydroherbarin (**5**). All compounds were evaluated for their cytotoxicity and ability to inhibit migration of human metastatic breast and prostate cancer cell lines MDA-MB-231 and PC-3M, respectively. Dehydroherbarin (**5**) inhibited migration of both cell lines at concentrations not toxic to these cell lines. This is the first report of metabolites from an endolichenic fungus.

Lichens are symbiotic associations of algae or cyanobacteria (photobiont) and filamentous fungi (mycobiont). The photobiont provides photosynthate to the mycobiont, which in turn makes up the bulk of the lichen thallus and provides the alga with access to water and minerals. Mycobionts also frequently produce secondary metabolites to protect lichens against harmful radiation, herbivores, and microbial infections.[2] Although viewed traditionally as bipartite or tripartite symbioses, lichens frequently harbor diverse fungi in addition to the fungal mycobiont. Incidental fungi on thallus surfaces, as well as lichenicolous fungi (relatively obligate associates that fruit from lichen thalli), have been recognized for some time,[3] and recent work has shown that lichens also harbor numerous endolichenic fungi, fungi that live within the asymptomatic lichen thallus much the same way as endophytic fungi live within healthy plant tissues.[4] For example, 1086 fungal strains have been isolated from the exterior and interior of the lichen *Letharietum vulpinae* (Parmeliaceae; Lecanorales).[5] Similarly, isolates of 640 endolichenic fungi were recovered from the lichen *Peltigera neopolydactyla* (Peltigeraceae; Peltigerales), in sites ranging from tropical rainforests to Arctic tundra, suggesting that endolichenic fungi are at least as diverse as endophytic fungal communities and, at higher latitudes, are more abundant than endophytes given the same sampling effort.[6] These findings, combined with the occurrence of over 600 species of lichens belonging to over 140 genera in the Sonoran Desert,[7] provide an opportunity of harvesting numerous endolichenic fungi from this biogeographic region.

In a continuation of our studies on arid land plants and microorganisms,[1,8] we have investigated an endolichenic fungal strain collected in the Sonoran desert of the U.S. Southwest. Although a number of lichens,[9] their fungal mycobionts,[10] and a lichenicolous fungus[11] from different geographic locations have been subjected to chemical investigations, leading to the isolation and characterization of a variety of metabolites, to the best our knowledge this constitutes the first report of secondary metabolites from an endolichenic fungus. The endolichenic fungus used in this study, *Corynespora* sp. BA-10763, was isolated from the lichen *Usnea cavernosa* (cavern beard lichen; Parmeliaceae; Lecanorales), collected in early 2005 from Coronado National Forest, Arizona. An EtOAc extract of *Corynespora* sp. BA-10763 cultured in potato dextrose broth (PDB) exhibited significant cell migration inhibitory activity in a wound-healing assay (WHA)[8] using the metastatic prostate cancer cell line, PC-3M, at a noncytotoxic concentration of 5 μg/mL. Herein we report the bioassay-guided isolation of two new heptaketides, corynesporol (**1**) and 1-hydroxydehydroherbarin (**2**), together with herbarin (**3**), the chemical transformation of **1**−**3** yielding compounds **4**−**9**, and evaluation of all compounds for their PC-3M and MDA-MB-231 (metastatic human breast cancer) cell migration inhibitory activities. Herbarin (**3**) and dehydroherbarin (**5**), with weak antimicrobial and antiamebic activities, have previously been reported from the dematiaceous fungus *Torula herbarum*.[12]

Results and Discussion

Bioassay-guided fractionation of a WHA-active EtOAc extract of a liquid culture supernatant of *Corynespora* sp. BA-10763, involving normal-phase and reversed-phase column chromatography and preparative TLC, furnished **1**−**3**. Corynesporol (**1**), isolated as a white solid, was determined to have the molecular formula $C_{16}H_{20}O_6$ by a combination of HRFABMS and ^{13}C and DEPT NMR data and indicated seven degrees of unsaturation. Its IR spectrum with absorption bands at 3413 and 1681 cm^{-1} suggested the presence of OH and conjugated carbonyl groups. The ^1H NMR spectrum of **1** when analyzed with the help of DQF-COSY indicated the presence of two spin systems in addition to three 3H singlets due to two OCH_3 groups (δ 3.96 and 3.89) and a CH_3 (δ 1.42) on a quaternary carbon. The spin system consisting of two 1H doublets was shown to be due to *meta*-coupled protons [δ 7.07 and 6.90 (J = 2.2 Hz)]. The second spin system was found to be complex and consisted of two ABX spin systems connected to each other. One of these spin systems in **1** contained three signals centered at δ 4.14 (1H, t, J = 11.0 Hz), 3.91 (1H, dd, J = 11.0 and 4.5 Hz), and 2.95 (1H, ddd, J = 13.0, 11.0, and 4.5 Hz). The chemical shifts and coupling constants of the A and B portions of this spin system were indicative of nonequivalent methylene protons of a carbon bearing an oxygen atom. The second ABX spin system in **1** contained three signals centered at δ 2.22 (1H, dd, J = 13.0 and 3.5 Hz), 1.62 (1H, dd, J = 13.0 and 11.0 Hz), and 3.35 (1H, ddd, J = 13.0, 11.0, and 3.5 Hz). The DQF-COSY spectrum of **1** also

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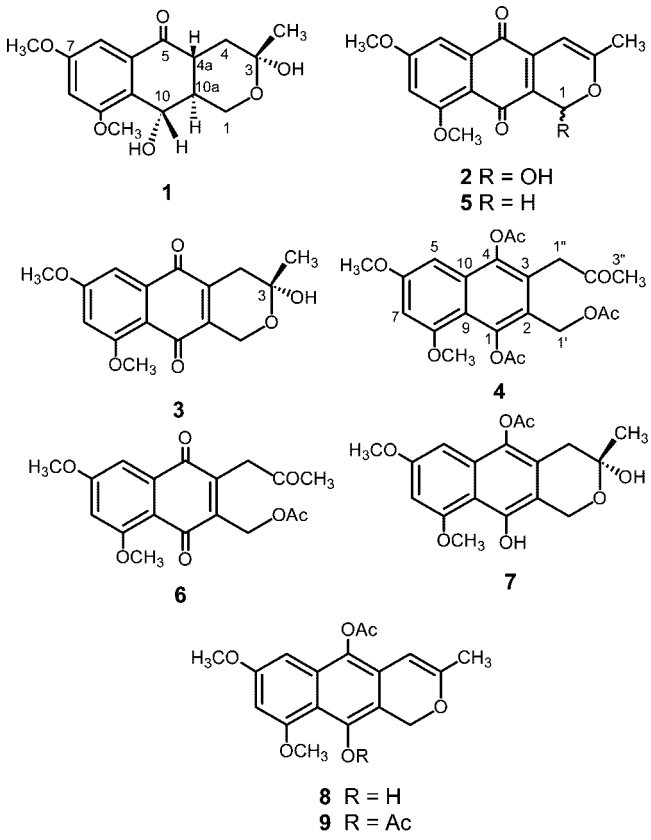


indicated that the two ABX spin systems were connected to each other through carbons bearing protons at δ 2.91 and 3.35 ($J = 13.0$ Hz), suggesting the presence of a $CH_2-CH-CH-CH_2$ moiety in **1** as part of a cyclic system. The ^{13}C NMR spectrum of **1** when analyzed with the help of the HSQC and DEPT spectra showed the presence of a conjugated ketone carbonyl (δ 197.5), six aromatic carbons, of which two were oxygenated (δ 165.0 and 161.6), and two protonated (δ 105.2 and 102.4) and one dioxygenated quaternary carbon (δ 95.7), two OCH_3 (δ 56.4), two CH_2 (δ 60.5 and 34.9), of which one was oxygenated, an oxymethine carbon (δ 56.8), a methylene (δ 34.9), and a methyl (δ 30.5) carbon. On the basis of its ^{13}C NMR data, the carbon skeleton of corynesporol (**1**) was suspected to be similar to that of the heptaketide scytalol A.[13] In its HMBC spectrum (Figure 1), one of the *meta*-coupled protons (δ_H 7.07) of **1** showed a correlation to the carbonyl carbon at δ_C 197.5 (C-5), suggesting that this carbon is attached to the aromatic ring and the remaining proton (δ_H 6.90) should be attached to C-8. These HMBC correlations together with ^1H and ^{13}C NMR data established the partial structure A ($C_9H_8O_3$) for **1** (Figure 1). The cross-peaks between δ_H 3.91 (H-1α) and δ_C 56.8 (C-10); δ_H 4.14 (H-1β) and δ_C 45.4 (C-10a); δ_H 4.65 (H-10) and δ_C 60.5 (C-1); and δ_H 3.91 (H-1α) and δ_C 95.7 (C-3) in the HMBC spectrum and the ^1H and ^{13}C NMR data for the remainder of the molecule established the presence of the partial structure B ($C_7H_{12}O_3$) in **1** (Figure 1). Aerial oxidation of corynesporol afforded herbarin (**3**),[12] suggesting that the partial structures A and B of **1** are linked to each other as shown in Figure 1. The relative stereochemistry of **1** was determined with the help of ^1H NMR coupling constants and NOE data (Figure 1). A pulse-field gradient NOE experiment showed enhancements of ^1H NMR signals at δ 2.22 (H-4β), 4.14 (H-1β), and 4.65 (H-10) on irradiation of the signal at δ 3.35 (H-4a), indicating that these protons are on the same side of the ring. The enhancement of the ^1H NMR signals at δ 3.91 (H-1α) and 1.62 (H-4α) on irradiation of the signal at δ 2.91 (H-10a) indicated that the protons H-1α, H-4α, and H-10a have the same orientations. Enhancement of the signals at δ_H 4.65 (H-10), 2.22 (H-4β), and 4.14 (H-1β) on irradiation of the signal at δ_H 1.49 (CH_3-3) suggested

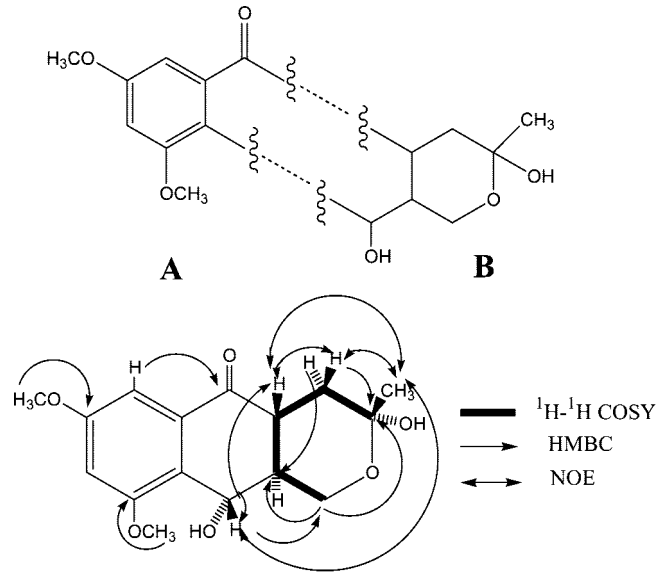


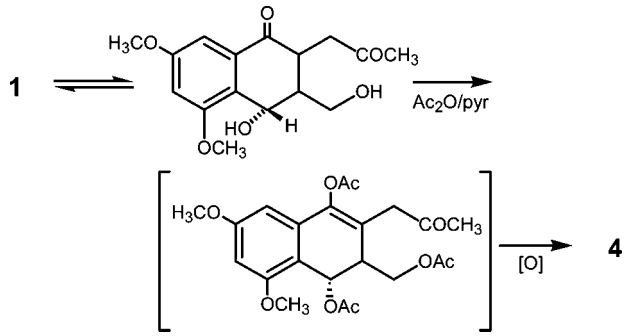
Figure 1. Partial structures and selected $^1H-^1H$ COSY, HMBC, and NOE correlations for **1**.

that these protons have the same orientation (Figure 1). The relative orientations of H-10 and H-10a were determined by the application of the relationship between dihedral angle and coupling constant for vicinal protons (the vicinal Karplus correlation).[14] The ^1H NMR coupling constant of 1.80 Hz for H-10/H-10a suggested a dihedral angle of about 90° for these two protons. Thus, the structure of corynesporol was assigned as 4,10-tetrahydro-3α,10α-dihydroxy-7,9-dimethoxy-3β-methylnaphtho[2,3-*c*]pyran-5-one (**1**).

The molecular formula of **2** was determined as $C_{16}H_{14}O_6$ from its HRFABMS and ^{13}C NMR data and indicated 10 degrees of unsaturation. Its UV spectrum exhibited peaks at 447 and 421 nm, characteristic of a pyranonaphthoquinone,[12a] and its IR absorption bands at 3450 and 1680 cm^{-1} suggested the presence of OH and quinone carbonyl functions. The ^1H NMR spectrum of **2** indicated the occurrence of two *meta*-coupled aromatic protons (δ 7.28 and 6.72, $J = 2.2$ Hz), three 3H singlets, of which two were due to OCH_3 groups (δ 3.94 and 3.93) and the third due to a CH_3 group on a quaternary carbon (δ 2.12), and two 1H singlets at δ 6.64 and 6.03, accounting for 13 out of 14 protons in **2**; the remaining proton was suspected to be that of an OH group. The ^{13}C NMR spectrum assigned with the help of the DEPT data contained signals due to two quinone carbonyl carbons (δ 183.3 and 180.9), 10 aromatic/olefinic carbons, of which three are oxygenated (δ 164.3, 161.7, and 160.2) and three protonated (δ 104.6, 103.9, and 93.0), two methoxy carbons (δ 56.4 and 55.9), one methyl carbon (δ 20.9), and a methine carbon (δ 87.8). These ^{13}C NMR data closely resembled those reported for dehydroherbarin (**5**)[12c] except that the chemical shift and the multiplicity of C-1 in **2** (δ 87.8 d) was different from that of **5** (δ 63.3 t), suggesting that the OH group in **2** should be attached to this carbon. Compound **2** was thus identified as 1-hydroxydehydroherbarin. The failure of **2** to yield an MTPA ester under a variety of conditions, probably due to the instability of the hemiacetal under these reaction conditions, precluded stereochemical assignment of the OH group at C-1. Compound **3** was identified as herbarin by comparison of its ^1H NMR spectroscopic data with those reported in the literature.[12c] However, as the ^{13}C NMR spectrum of herbarin has not been assigned previously, it was done with the help of DEPT, HSQC, and HMBC data (see Experimental Section).

Acetylation of corynesporol (**1**) afforded **4** as a colorless crystalline solid. The molecular formula of **4** was determined as $C_{22}H_{24}O_9$ from its HRFABMS and ^{13}C NMR data. The ^1H NMR spectrum of **4** indicated the presence of six 3H singlets consisting of signals due to two OCH_3 (δ 3.85 and 3.88), three $OCOCH_3$ (δ

Scheme 1



2.12, 2.36, and 2.42), and one $COCH_3$ (δ 1.98), two *meta*-coupled aromatic protons [δ 6.52 and 6.51 (each d, J = 2.0 Hz)], a methylene group [δ 3.82 (brs)], and an oxygenated methylene group [δ 5.28 (1H, d, J = 12.0 Hz) and 5.07 (1H, d, J = 12.0 Hz)]. The ^{13}C NMR spectrum of **4** when analyzed with the help of the DEPT data confirmed the presence of a ketone carbonyl (δ 204.9), three ester carbonyls (δ 170.6, 169.7, and 168.7), 10 aromatic carbons, of which two are oxygenated (δ 159.7 and 157.0) and two protonated (δ 100.2 and 92.5), and two methylene carbons, of which one was sandwiched by an aromatic carbon and a ketone carbonyl (δ 43.0) and the other between an aromatic carbon and an oxygen atom of an acetyl group (δ 58.3). On the basis of these data, **4** was identified as 2-acetoxymethyl-1,4-diacetoxy-3-(2′-oxopropyl)-6,8-dimethoxynaphthalene. A possible reaction sequence for the formation of **4** from corynesporol (**1**) is depicted in Scheme 1.

Compounds **1**–**5** were evaluated in our in-house bioassays for potential anticancer activity including the inhibition of cancer cell proliferation (MTT assay),[15] metastatic cancer cell migration (wound-healing assay; WHA),[8] and induction of heat shock response.[16] Of the compounds tested, dehydroherbarin (**5**) showed significant inhibition of PC-3M (metastatic prostate cancer) and MDA-MB-231 (metastatic breast cancer) cell migration (Figure 2) at 5 μM; herbarin (**3**) exhibited similar activity toward the PC-3M cell line but at a higher concentration (10 μM) and therefore was not subjected to further biological evaluation. The extent of cell migration inhibition when determined using NIH ImageJ software[17] suggested that dehydroherbarin (**5**) caused about 50% inhibition of migration of both PC-3M and MDA-MB-231 cells at a concentration of 5 μM (Figure 3). However, the concentrations of **5** required for 50% (IC_{50}) and 25% (IC_{25}) inhibition of PC-3M cell proliferation/survival as measured by the MTT assay[15] at 40 h (time used for WHA with PC-3M) were determined to be 3.5 and 2.2 μM, respectively. As PC-3M and MDA-MB-231 cell migration inhibitions occurred at a concentration above their IC_{50} values as determined by the MTT assay, and because the cells appeared completely viable on microscopic inspection after exposure to dehydroherbarin (**5**) in the cell migration assay, it was thought necessary to determine the toxicity of **5** in other cell viability assays not dependent on cell proliferation. As shown in Figure 4 when tested in a flow cytometry-based cell viability assay,[18] both PC-3M and MDA-MB-231 cells after exposure to WHA with **5** were found to be completely viable. To verify the viability of these cells after WHA, a microscopy-based live/dead assay kit was used to label live cells (metabolically active) and dead cells (metabolically inactive but able to incorporate ethidium homodimer 1) after drug treatment.[19] In this assay both types of cells were found to be completely viable (Figure 5), further suggesting that the cell migration inhibitory activity exhibited by dehydroherbarin (**5**) is not associated with cytotoxicity. The phosphatidylinositol (PtdIns) 3-kinase inhibitor, LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one], was used as a positive control in both assays, as it is known to exhibit antiproliferative and antimigratory activity without any cellular toxicity.[20]

On the basis of these findings, it was of interest to evaluate derivatives of **3** and **5** for their cell migration inhibitory activity. Treatment of herbarin (**3**) with Ac_2O/Et_3N/DMAP afforded the dehydration product, dehydroherbarin (**5**), and the naphthoquinone, **6**, formed by acetylation of the hydroxy-ketone resulting from opening of the pyrano-hemiacetal ring under the basic conditions used for this reaction. Reduction of herbarin (**3**) with $NaBH_4$ gave an unstable compound that on acetylation afforded the stable dihydropyranonaphthalene, **7**. Reductive acetylation[21] of **3** yielded the corresponding pyranonaphthalene, **8**, which on acetylation afforded its acetate, **9**. Deacetylation of **9** under mild conditions gave dehydroherbarin (**5**). The structures of these derivatives were established with the help of their spectroscopic parameters and comparison of these data with those of **3**–**5** (see above) and those reported for related naphthoquinones[22] and naphthalenes.[23] When tested, compounds **7**–**9** were found to be inactive in the WHA, suggesting the possible requirement of the central quinone moiety in naphthopyrans for cell migration inhibitory activity. Lack of activity of 1-hydroxyherbarin (**2**) in this assay may be due to the instability of its hemiketal moiety under the conditions utilized.

Experimental Section

General Experimental Procedures. Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO Dip-370 digital polarimeter using $CHCl_3$ or MeOH as solvent. UV spectra were recorded on a Shimadzu UV-1601 UV–vis spectrophotometer. IR spectra for KBr disks were recorded on a Shimadzu FTIR-8300 spectrometer. NMR spectra were recorded in $CDCl_3$ or d_6-acetone with a Bruker DRX-500 instrument at 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR, using residual $CHCl_3$ or acetone as internal standard. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. Low-resolution and high-resolution MS were recorded, respectively, on a Shimadzu LCMS-8000 QPα and a JEOL HX110A mass spectrometer. Whatman LRP-2 was used for reversed-phase column chromatography, and reversed-phase preparative TLC was performed on Merck RP-18 F_{254S} precoated aluminum sheets.

Isolation and Identification of the Fungal Strain. The lichen host, the cavern beard lichen, *Usnea cavernosa,* was collected from tree branches ca. 1.5 m above the forest floor in mixed coniferous forest (Douglas fir, *Pseudotsuga menziesii*; Ponderosa pine, *Pinus ponderosa*) at 2438 m elevation in Coronado National Forest in Arizona in September 2005. The lichen was identified at the University of Arizona Robert L. Gilbertson Mycological Herbarium by one of the authors (A.E.A.) following Bungartz et al.[7b] To isolate endolichenic fungi, a healthy thallus of *U. cavernosa* was washed in running tap water for 30 s and then surface-sterilized by sequential immersion in 96% ethanol for 10 s, 0.5% sodium hypochlorite for 2 min, and 70% ethanol for 2 min. Following treatment, the thallus was cut into small pieces (ca. 1 cm in length) and transferred under sterile conditions to 2% malt extract agar (MEA), which encourages growth of diverse endolichenic fungi.[6] Plates were sealed with Parafilm[6] and incubated up to two months at room temperature under ambient light. One of the emergent fungi was subcultured on 2% MEA, photographed, and deposited as a living voucher in the Robert L. Gilbertson Mycological Herbarium at the University of Arizona under the accession number BA-10763. Since endolichenic fungi frequently lack reproductive structures in culture, it could not be identified beyond the level of phylum on the basis of morphology alone. The fungal strain was therefore identified by analysis of the nuclear internal transcribed spacer region (ITS) as described previously.[4b] ITS data for the focal isolate, BA-10763, were integrated into existing alignments for the Dothideomycetes (Ascomycota) based on highest-affinity matches obtained through BLAST searches of the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) and FASTA searches implemented through the Fungal Metagenomics Project (http://biotech.inbre.alaska.edu/fungal-metagenomics). Subsequent phylogenetic analyses following our previously reported method[4a] indicated the taxonomic placement of BA-10763 within the mitosporic genus *Corynespora* (incertae sedis, Ascomycota).

Extraction and Isolation. A seed culture of *Corynespora* sp. BA/10763 grown on PDA for two weeks was used for inoculation. Mycelia were scraped out and mixed with sterile PDB (150 mL) and filtered through a 100 μm filter to separate spores from the mycelia. Absorbance

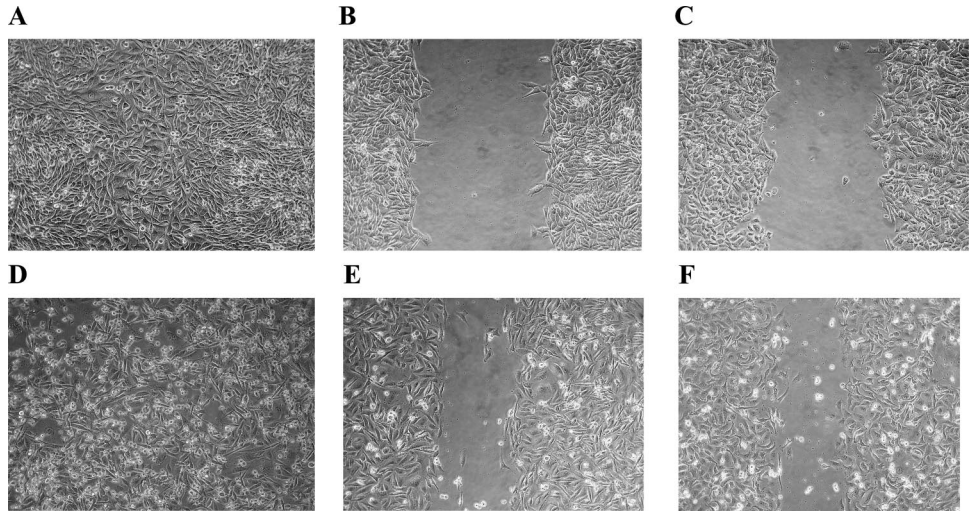


Figure 2. Wound-healing assay on dehydroherbarin (**5**) with metastatic cancer cells PC-3 M (A−C) and MDA-MB-231 (D−F). (A and D) DMSO control (negative). (B and E) LY294002 control (positive) at 7.5 μM. (C and F) Dehydroherbarin at 5.0 μM.

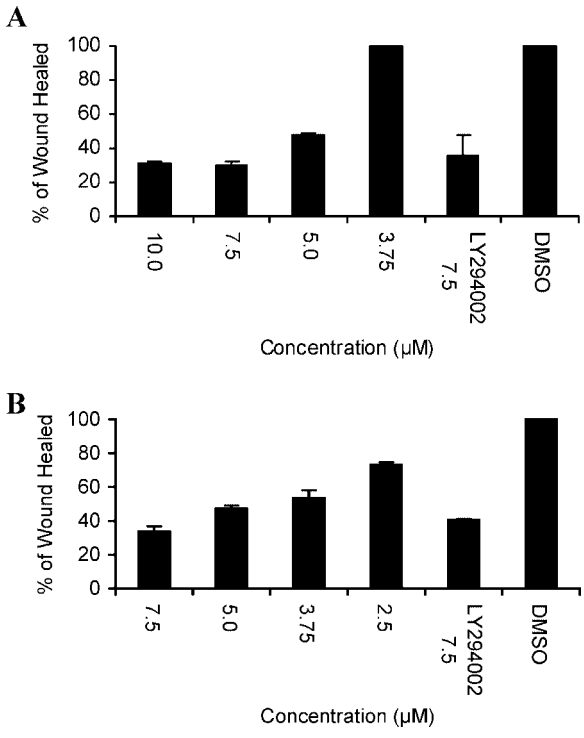


Figure 3. Quantitative wound-healing assay data for dehydroherbarin (**5**). (A) In metastatic prostate cancer (PC-3M) cells. (B) In metastatic breast cancer (MDA-MB-231) cells.

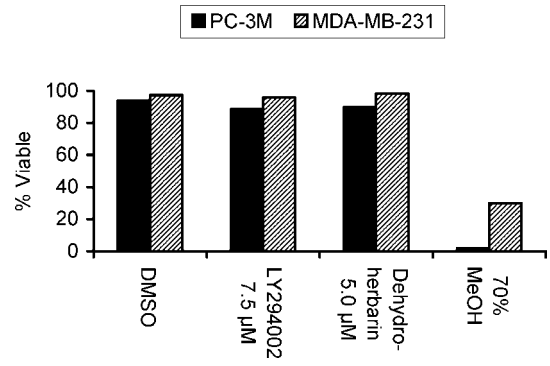


Figure 4. Flow cytometry-based cell viability assay of dehydroherbarin (**5**) in metastatic prostate cancer (PC-3M) and breast cancer (MDA-MB-231) cells. DMSO control shows background viability of cells, and 70% methanol control shows cell death. LY294002 is a known antiproliferative agent that inhibits migration at nontoxic concentrations.

of the spore solution was measured and adjusted to between 0.5 and 0.3. This spore solution (10 mL) was used to inoculate 10 500 mL Erlenmeyer flasks, each containing 200 mL of the medium and incubated at 160 rpm and 28 °C. After 4 days of inoculation, the glucose level in the medium was monitored using the glucose strips (URISCAN glucose strip), and on day 12, the strip gave a green color for the glucose test, indicating a very low level of glucose in the medium. Mycelia were separated from the supernatant by filtering through Whatman No. 1 filter paper. The supernatants (pH 4.56) were neutralized to pH 7 with 0.5 M NaOH and extracted with EtOAc (10 × 500 mL), and the combined EtOAc extracts were evaporated under reduced pressure to afford a dark brown semisolid (1.1 g) that was found to be active in a wound-healing assay (WHA)[8] using the PC-3 M cell line at 5 μg/mL. A portion (1.0 g) of this extract was subjected to column chromatography on LiChroprep DIOL (30.0 g) made up in hexane (100 mL) and eluted with hexane containing increasing amounts of CH_2Cl_2, CH_2Cl_2, followed by CH_2Cl_2 containing increasing amounts of MeOH, and finally MeOH. Sixteen fractions (F_1−F_{16}; 100 mL each) were collected and combined on the basis of their TLC profiles to obtain F_1−F_8 (25.6 mg), F_9 (134.5 mg), F_{10}−F_{15} (627 mg), and F_{16} (230 mg), of which the combined fraction F_{10}−F_{15} was found to contain the major compound active in the WHA. A portion (173 mg) of the combined fraction F_{10}−F_{15} was further fractionated by medium-pressure liquid chromatography on reversed-phase silica gel (5.2 g) (MeOH−H_2O linear gradient 50−100%) to give 45 fractions (10 mL each), which were combined on the basis of their TLC patterns, yielding fractions A (1.9 mg), B (118 mg), and C (51.7 mg). A portion (30 mg) of the WHA-active fraction B was purified by reversed-phase preparative TLC (eluant: 35% H_2O in MeOH) to give **1** (3.9 mg; R_f 0.4), **2** (1.2 mg; R_f 0.2), and **3** (18.6 mg; R_f 0.3). A portion (130 mg) of F_9 was subjected to column chromatography over reversed-phase silica gel (3.9 g) made up in 55% MeOH in H_2O and eluted with increasing amounts of MeOH. Nine fractions (10 mL each) were collected, and of these, fraction 3 (F_{9C}; 98.5 mg) was found to contain **1** as the major compound. A portion (10.0 mg) of F_{9C} was further purified by reversed-phase preparative TLC (eluant: 35% H_2O in MeOH) to obtain an additional quantity of **1** (9.2 mg; R_f 0.4).

Corynesporol (1): white, crystalline solid; mp 132−134 °C; $[\alpha]_D^{25}$ −13.9 (*c* 0.09, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 283 (4.72), 255 (4.83) nm; IR ν_{max} 3413, 1681, 1600, 1458, 1319, 1161, 848, 802 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ 7.07 (1H, d, J = 2.2 Hz, H-6), 6.90 (1H, d, J = 2.2 Hz, H-8), 4.65 (1H, d, J = 1.8 Hz, H-10), 4.14 (1H, t, J = 11.0 Hz, H-1β), 3.96 (3H, s, OCH_3-7), 3.91 (1H, dd, J =11.0 and 4.5 Hz, H-1α), 3.89 (3H, s, OCH_3-9), 3.35 (1H, ddd, J = 13.0, 11.0 and 3.5 Hz, H-4a), 2.95 (1H, ddd, J = 13.0, 11.0 and 4.5 Hz, H-10a), 2.22 (1H, dd, J = 13.0 and 3.5 Hz, H-4β), 1.62 (1H, dd, J =

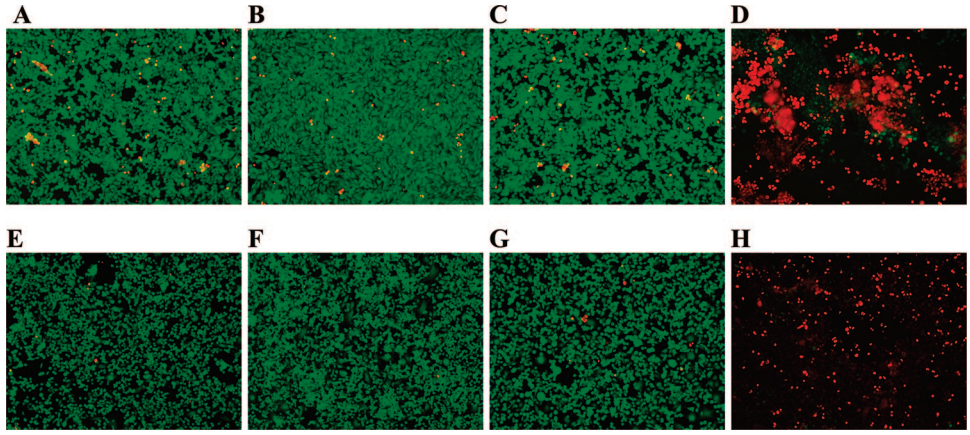


Figure 5. Microscopy-based cell viability assay of dehydroherbarin (**5**) with metastatic cancer cells PC-3 M (A−D) and MDA-MB-231 (E−H). (A and E) DMSO control. (B and F) LY294002 at 7.5 μM. (C and G) Dehydroherbarin at 5.0 μM. (D and H) 0.1% saponin for 10 min. DMSO (negative) control shows background viability of cells, and 0.1% saponin (toxicity) control provided in live/dead assay kit results in nearly complete cell death. LY294002 (cell viability) control is antimigratory and antiproliferative but not cytotoxic.

13.0 and 11.0 Hz, H-4α), 1.42 (3H, s, CH_3-3); ^{13}C NMR (125 MHz, acetone-d_6) δ 197.5 (C, C-5), 165.0 (C, C-7), 161.6 (C, C-9), 124.5 (C, C-5a), 118.4 (C, C-9a), 105.2 (CH, C-6), 102.4 (CH, C-8), 95.7 (C, C-3), 60.5 (CH_2, C-1), 56.8 (CH, C-10), 56.4 (CH_3, OCH_3-7 and OCH_3-9), 49.1 (CH, C-4a), 45.4 (CH, C-10a), 34.9 (CH_2, C-4), 30.5 (CH_3, CH_3-3); HRFABMS *m/z*289.1079 [M − (2H + H_2O)]$^+$ (calcd for $C_{16}H_{17}O_5$, 289.1076).

1-Hydroxydehydroherbarin (2): orange, amorphous solid; $[\alpha]_D^{25}$ −12.1 (*c* 0.06, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 447 (4.21), 421 (4.13), 337 (4.29), 278 (4.82) nm; IR ν_{max} 3450, 2920, 1680, 1585, 1323, 1161, 1064, 837 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) δ 7.28 (1H, d, J = 2.2 Hz, H-6), 6.72 (1H, d, J = 2.2 Hz, H-8), 6.64 (1H, s, H-4), 6.03 (1H, s, H-1), 3.94 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 2.12 (3H, s, CH_3-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.3 (C, C-5), 180.9 (C, C-10), 164.3 (C, C-9), 161.7 (C, C-7), 160.2 (C, C-3), 135.6 (C, C-5a), 133.8 (C, C-9a), 123.6 (C, C-4a), 114.6 (C, C-10a), 104.6 (CH, C-6), 103.9 (CH, C-8), 93.0 (CH, C-4), 87.8 (CH, C-1), 56.4 (CH_3, OCH_3), 55.9 (CH_3, OCH_3), 20.9 (CH_3, CH_3-3); HRFABMS *m/z*303.0867 [M + 1]$^+$ (calcd for $C_{16}H_{15}O_6$, 303.0869).

Herbarin (3): yellow, crystalline solid; mp 191–193 °C (lit.[12a] 190−192 °C); $[\alpha]_D^{25}$ +4.8 (*c* 0.06, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 411 (4.47), 267 (5.11) nm; IR ν_{max} 3325, 2920, 2356, 1643, 1319, 852 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.22 (1H, d, J = 2.4 Hz, H-6), 6.68 (1H, d, J = 2.4 Hz, H-8), 4.73 (1H, ddd, J = 18.8, 3.4, 1.9 Hz, H-1a), 4.66 (1H, dt, J = 18.8, 3.7 Hz, H-1b), 3.93 (3H, s, OMe), 3.92 (3H, s, OMe), 2.80 (1H, ddd, J = 17.6, 3.7, 1.9 Hz, H-4a), 2.52 (1H, dt, J = 17.6, 3.4 Hz, H-4b), 1.59 (3H, s, CH_3-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.8 (C, C-10), 181.7 (C, C-5), 164.6 (C, C-7), 161.0 (C, C-9), 143.0 (C, C-5a), 137.0 (C, C-4a), 135.7 (C, C-10a), 113.0 (C, C-9a), 103.7 (CH, C-6), 103.6 (CH, C-8), 93.9 (C, C-3), 60.5 (CH_2, C-1), 56.4 (CH_3, OCH_3-7), 55.8 (CH_3, OCH_3-9), 31.8 (CH_2, C-4), 28.4 (CH_3, CH_3-3); HRFABMS *m/z*305.1027 [M + 1]$^+$ (calcd for $C_{16}H_{17}O_6$, 305.1025).

Arial Oxidation of Corynesporol (1) to Herbarin (3). Corynesporol (1.0 mg) dissolved in CH_2Cl_2 (0.2 mL) was streaked on a reversed-phase TLC plate (RP-18), the plate was exposed to air for 5 h and eluted with MeOH−H_2O (65:35), and the yellow band that appeared on the TLC plate (R_f, 0.4) was scraped out and extracted with MeOH (10 mL × 3) and evaporated under reduced pressure. The product (0.8 mg) formed was found to be identical (TLC, LC-MS, and ^{1}H NMR) with herbarin (**3**) obtained above.

Acetylation of Corynesporol (1). To a solution of **1** (8.0 mg) in CH_2Cl_2 (1.0 mL) were added Ac_2O (0.1 mL), Et_3N (0.3 mL), and DMAP (1.0 mg), and the reaction mixture was stirred overnight, after which it was poured into brine and extracted with ether (3 × 10 mL). The ether extracts were combined, washed with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure, and the product was purified by preparative TLC (silica gel) using 2% *i*-PrOH in CH_2Cl_2 as eluant to give 2-acetoxymethyl-1,4-diacetoxy-3-(2′-oxopropyl)-6,8-dimethoxynaphthalene (**4**) (5.5 mg; R_f, 0.6) as a white, crystalline solid: mp 128−130 °C; UV (EtOH) λ_{max} (log ε) 389 (3.45), 304 (3.39), 242 (3.30) nm; IR ν_{max} 2939, 1762, 1735, 1627, 1581, 1373, 1207, 1184, 1056, 833 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.52 (1H, d, J = 2.0 Hz, H-8), 6.51 (1H, d, J = 2.0 Hz, H-6), 5.28 (1H, d, J = 12.0 Hz, H-1′a), 5.07 (1H, d, J = 12.0 Hz, H-1′b), 3.88 (3H, s, OCH_3), 3.82 (2H, s, H-1′), 3.85 (3H, s, OCH_3), 2.44 (3H, s, OAc), 2.36 (3H, s, OAc), 2.12 (3H, s, OAc), 1.98 (3H, s, $COCH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 203.3 (C, C-2″), 170.6 (C, O*C*OCH$_3$), 169.7 (C, O*C*OCH$_3$), 168.7 (C, O*C*OCH$_3$), 159.7 (C, C-6), 157.0 (C, C-8), 144.6 (C, C-4), 142.4 (C, C-1), 130.9 (C, C-3), 125.7 (C, C-10), 121.9 (C, C-2), 115.2 (C, C-9), 100.2 (CH, C-7), 92.5 (CH, C-5), 58.3 (CH_2, C-1′), 56.3 (CH_3, OCH_3), 55.2 (CH_3, OCH_3), 43.0 (CH_2, C-1″), 29.1 (CH_3, C-3″), 20.8 (CH_3, OCO*C*H$_3$), 20.7 (CH_3, OCO*C*H$_3$), 20.5 (CH_3, OCO*C*H$_3$); HRFABMS *m/z*373.1287 [MH − CH_3COOH]$^+$ (calcd for $C_{20}H_{21}O_7$, 373.1272).

Acetylation of Herbarin (3). To a solution of **3** (10.0 mg) in CH_2Cl_2 (1.0 mL) were added Ac_2O (0.1 mL), Et_3N (0.3 mL), and DMAP (0.1 mg), and the reaction mixture was stirred at 25 °C until the starting material disappeared (TLC control), after which it was poured into brine and extracted with ether (3 × 10 mL). The ether extracts were combined, washed with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure and the two major products were separated on preparative TLC (silica gel) using 2% *i*-PrOH in CH_2Cl_2 as eluant to give dehydroherbarin (**5**) (6.2 mg; R_f, 0.8) and **6** (2.2 mg; R_f, 0.5). The ^{1}H and ^{13}C NMR, and MS data of **5** were consistent with those reported[12a,c] for dehydroherbarin.

2-Acetoxymethyl-6,8-dimethoxy-3-(2′-oxopropyl)-1,4-naphthoquinone (6): yellow, crystalline solid; mp 138−140 °C, UV (EtOH) λ_{max} (log ε) 362 (3.62), 343 (3.59), 264 (3.48), 245 (3.45), 220 (3.40) nm; IR ν_{max} 3294, 2923, 2360, 1747, 1654, 1596, 1362, 1018, 848 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.20 (1H, d, J = 2.2 Hz, H-5), 6.72 (1H, d, J = 2.2 Hz, H-7), 5.05 (2H, s, H-1′), 3.95 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.86 (2H, s, H-1″), 2.29 (3H, s, OAc), 1.99 (3H, s, $COCH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 203.3 (C, C-2″), 184.8 (C, C-4), 181.1 (C, C-1), 170.6 (C, O*C*OCH$_3$), 164.7 (C, C-6), 161.9 (C, C-8), 142.9 (C, C-2), 141.3 (C, C-3), 135.0 (C, C-9), 114.0 (C, C-10), 104.5 (CH, C-5), 103.2 (CH, C-7), 57.6 (CH_2, C-1′), 56.5 (CH_3, OCH_3), 55.9 (CH_3, OCH_3), 41.1 (CH_2, C-1″), 30.1 (CH_3, OCO*C*H$_3$), 20.7 (CH_3, 3″-CH_3); HRFABMS *m/z* 347.1125 [M + 1]$^+$ (calcd for $C_{18}H_{19}O_7$, 347.1131).

Reduction of Herbarin (3) Followed by Acetylation. To a solution of herbarin (**3**) (5.5 mg) in EtOAc (1.0 mL) containing the antioxidant, 2,6-di*tert*-butyl-4-methylphenol (BHT) (11.0 mg) was added saturated $Na_2S_2O_4$(0.5 mL). The biphasic mixture was stirred vigorously until the yellow solution turned colorless. The organic layer was separated and dried over anhydrous Na_2SO_4 and was filtered through a cotton plug into a clean vial under a nitrogen atmosphere. The EtOAc solution containing the hydroquinone was then acetylated with Ac_2O/Et_3N/DMAP as described above. The product was separated on preparative silica gel TLC using 2% *i*-PrOH in CH_2Cl_2 as eluant to give 5-acetoxy-3,4-dihydro-3α-hydroxy-7,9-dimethoxy-3β-methyl-1*H*-naphtho[2,3-*c*]pyran-10-ol (**7**) (2.8 mg; R_f, 0.4) as a white, crystalline solid: mp 165−167 °C; $[\alpha]_D^{25}$ −8.6 (*c* 0.07, CH_3OH); UV (EtOH) λ_{max} (log ε)

344 (3.26), 329 (3.24), 305 (3.21), 293 (3.19), 241 (3.11) nm; IR ν_{max}3413, 2931, 2364, 1743, 1620, 1589, 1369, 1211, 1157, 1037, 937, 725 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 9.21 (1H, s, OH-10), 6.50 (1H, d, $J = 2.2$ Hz, H-6), 6.42 (1H, d, $J = 2.2$ Hz, H-8), 4.95 (2H, s, H-1), 3.99 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 2.81 (2H, d, $J = 13.0$ Hz, H-4), 2.42 (3H, s, OAc), 1.59 (3H, s, CH_3-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.3 (C, O*C*OCH$_3$), 157.9 (C, C-7), 157.5 (C, C-9), 147.1 (C, C-10), 135.6 (C, C- 5), 128.5 (C, C-4a), 124.3 (C, C-5a), 112.8 (C, C-10a), 109.4 (C, C-9a), 97.8 (CH, C-8), 94.5 (C, C-3), 92.3 (CH, C-6), 59.1 (CH_2, C-1), 56.3 (CH_3, OCH_3), 55.2 (CH_3, OCH_3), 34.5 (CH_2, C-4), 29.2 (CH_3, CH_3-3), 20.6 (CH_3, OCO*C*H$_3$); HRFABMS *m/z* 331.1163 $[MH - H_2O]^+$ (calcd for $C_{18}H_{19}O_6$, 331.1182).

Reductive Acetylation of Herbarin (3). A mixture of **3** (7.3 mg), Zn dust (22 mg), and saturated anhydrous NaOAc in Ac_2O/AcOH (5:1 v/v; 1 mL) was heated under reflux for 45 min.[21] The reaction was monitored using TLC. After the disappearance of **3**, the reaction mixture was partitioned between Et_2O/H_2O and the ether layer was washed with H_2O. The combined Et_2O layer was concentrated and the product separated using preparative TLC (silica gel) with 2% *i*-PrOH in CH_2Cl_2 as eluant to give 5-acetoxy-3,4-dehydro-7,9-dimethoxy-3-methylnaphtho[2,3-*c*]pyran-10-ol (**8**) as a white, crystalline solid (6.0 mg; R_f, 0.6): mp 173−175 °C; UV (EtOH) λ_{max} (log ε) 366 (3.04), 348 (3.02), 252 (2.88), 209 (2.79) nm; IR ν_{max} 3398, 1751, 1647, 1585, 1465, 1369, 1218, 1176, 1033, 898, 821 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 9.15 (1H, s, OH-10), 6.46 (1H, d, $J = 2.1$ Hz, H-8), 6.36 (1H, d, $J = 2.1$ Hz, H-6), 5.48 (1H, s, H-4), 5.26 (2H, s, H-1), 3.98 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 2.42 (3H, s, OAc), 1.94 (3H, s, CH_3-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.6 (C, O*C*OCH$_3$), 158.3 (C, C-3), 157.6 (C, C-7), 157.4 (C, C-9), 146.5 (C, C-10), 129.9 (C, C-4a), 129.8 (C, C-10a), 123.4 (C, C-5), 109.6 (C, C-5a), 107.2 (C, C-9a), 97.1 (CH, C-6), 94.5 (CH, C-8), 92.9 (CH, C-4), 63.7 (CH_2, C-1), 56.3 (CH_3, OCH_3), 55.2 (CH_3, OCH_3), 20.6 (CH_3, 3-CH_3), 20.3 (CH_3, OCO*C*H$_3$); HRFABMS *m/z* 331.1182 $[M + 1]^+$ (calcd for $C_{18}H_{21}O_7$, 331.1181).

Acetylation of 8. To a solution of **8** (5.0 mg) in CH_2Cl_2 (1.0 mL) were added Ac_2O (0.1 mL), Et_3N (0.1 mL), and DMAP (0.5 mg), and the reaction mixture was stirred overnight, after which it was poured into brine and extracted with ether, washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure. The crude product thus obtained was separated on preparative TLC (silica gel) using 2% *i*-PrOH in CH_2Cl_2 as eluant to give 3,4-dehydro-5,10-diacetyl-7,9-dimethoxy-3-methylnaphtho[2,3-*c*]pyran (**9**) as a white, crystalline solid (4.4 mg; R_f, 0.6): mp 168−170 °C; UV (EtOH) λ_{max} (log ε) 359 (3.62), 308 (3.54), 267 (3.47), 209 (3.37) nm; IR ν_{max} 3753, 2923, 2850, 2360, 2341, 1751, 1654, 1373, 1207, 1041, 821, 671 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.51 (1H, d, $J = 2.2$ Hz, H-8), 6.39 (1H, d, $J = 2.2$ Hz, H-6), 5.53 (1H, s, H-4), 5.19 (1H, bs, H-1a), 5.02 (1H, bs, H-1b), 3.93 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 2.45 (3H, s, OAc), 2.34 (3H, s, OAc), 1.95 (3H, s, CH_3-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.4 (C, O*C*OCH$_3$), 168.9 (C, O*C*OCH$_3$), 158.9 (C, C-3), 157.3 (C, C-7), 156.9 (C, C-9), 138.9 (C, C-10), 134.8 (C, C-5), 130.6 (C, C-4a), 122.4 (C, C-10a), 116.2 (C, C-5a), 113.7 (C, C-9a), 98.8 (CH, C-6), 94.6 (CH, C-8), 92.5 (CH, C-4), 63.7 (CH_2, C-1), 56.1 (CH_3, OCH_3), 55.2 (CH_3, OCH_3), 20.6 (CH_3, 2 × OAc), 20.2 (CH_3, CH_3-3); HRFABMS *m/z* 373.1287 $[M + 1]^+$ (calcd for $C_{18}H_{21}O_7$, 373.1272).

Deacetylation of 9. To a solution of **9** (1.3 mg) in MeOH (0.5 mL) was added NaCN (0.1 mg) and the mixture stirred at 25 °C for 3 h and separated by preparative TLC (silica gel) using 2% *i*-PrOH in CH_2Cl_2 to afford dehydroherbarin (**5**) (0.7 mg; R_f, 0.8) identical (LC-MS and ^1H NMR) with the previously obtained sample.

Cytotoxicity and Wound-Healing Assays. The tetrazolium-based colorometric assay (MTT assay), wound-healing assay (WHA), and quantification of wound-healing inhibition were carried out as described previously.[8]

Flow Cytometry Assay.[18] Cells obtained directly from the wound-healing assays were collected with trypsin, washed twice with PBS, and stained with Guava PCA 96 ViaCount Flex reagent according to the manufacturer's instructions. Cells were assayed on a Guava Easy Cyte flow cytometer using EasyFit analysis to distinguish between viable and dead cells or debris.

Live/Dead Cell Assay.[19] Microscopy-based viability assays were performed according to manufacturer's instructions with a live/dead viability/cytotoxicity assay kit (Molecular Probes). Live cells were distinguished by a green fluorescence signal obtained after intracellular esterase activity enzymatically converted the nonfluorescent cell-permeable calcein AM to the fluorescent calcein. Ethidium homodimer 1 enters only cells with damaged membranes (indicative of dead or damaged cells) and produces a bright red fluorescence upon binding nucleic acids.

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