New Colchicinoids from a Native Jordanian Meadow Saffron, *Colchicum brachyphyllum*: Isolation of the First Naturally Occurring Dextrorotatory Colchicinoid

Feras Q. Alali,*,† Tamam El-Elimat,† Chen Li,‡ Amjad Qandil,† Ahmad Alkofahi,† Khaled Tawaha,§ Jason P. Burgess,‡ Yuka Nakanishi,‡ David J. Kroll,‡ Hernán A. Navarro,‡ Joseph O. Falkinham, III,⊥ Mansukh C. Wani,‡ and Nicholas H. Oberlies*,‡

Medicinal Chemistry and Pharmacognosy Department, Faculty of Pharmacy, Jordan University of Science and Technology, P.O. Box 3030, Irbid, 22110, Jordan, Natural Products Laboratory, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, North Carolina 27709-2194, Faculty of Pharmacy, University of Applied Sciences, Shafa-Badran, Amman, Jordan, and Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0406

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As part of our continuing investigation of Jordanian *Colchicum* species, the biologically active components of *Colchicum brachyphyllum* were pursued. Using bioactivity-directed fractionation, nine colchicinoids were isolated and characterized. One of these has a novel ring system, to which we have ascribed the trivial name (+)-demecolcinone (**9**), and it represents the first naturally occurring dextrorotatory colchicinoid. Another isolated compound was a new colchicinoid analogue, (−)-2,3-didemethyldemecolcine (**8**), while the remaining seven known colchicinoids were new to the species: (−)-colchicine (**1**), (−)-3-demethylcolchicine (**2**), (−)-cornigerine (**3**), β-lumicolchicine (**4**), (−)-androbiphenyline (**5**), (−)-demecolcine (**6**), and (−)-3-demethyldemecolcine (**7**). The brine shrimp lethality test was used to direct the isolation of these colchicinoids. Moreover, all pure compounds were evaluated for cytotoxicity against a human cancer cell panel, for antimicrobial activity in an array of bacteria and fungi (including yeast), and for their potential to be allosteric modulators of the γ-aminobutyric acid type A receptor.

The Hashemite Kingdom of Jordan acts as a flora bridge between the continents of Asia, Africa, and Europe, and it resides at the junction of four phyto-geographical areas, the Mediterranean, the Irano-Turanean, the Saharo-Arabian, and the Tropical or Sudanian.[1,2] These crossroads of climatic and botanic regions endow the country with a rich variety of plant life.[3,4] The Colchicaceae, a family of mainly perennial geophytes, although some vines and herbs are also included,[5] is well represented in Jordan, and in the wild, nine species of *Colchicum* have been reported, namely, *C. brachyphyllum* Boiss. & Haussk. ex Boiss., *C. crocifolium* Boiss., *C. hierosolymitanum* Feinbr., *C. ritchii*. R. Br., *C. schimperi* Janka, *C. stevenii* Kunth, *C. tauri* Siehe ex Stef., *C. triphyllum* Kunze, and *C. tunicatum* Feinbr.[6−8] The marked beneficial and poisonous effects of *Colchicum* species have been known for more than 2000 years. During the first century, Dioscorides was aware of their toxic nature, and in Arabian writings of the tenth century, they were recommended for use in gout. However, they were employed rarely during both classical and medieval times, owing to the fear inspired by their poisonous properties.[9,10]

The major cytotoxic alkaloid of *Colchicum autumnale* L., colchicine (**1**), was first isolated in 1820,[11] although its complete structure was not determined until the 1950s.[12,13] A comprehensive review on the total synthesis of **1** was published recently,[14] and there are many others on the chemistry and biological activity of the colchicinoids.[15−18] The striking effect of **1**, resulting in metaphase arrest in mitosis and meiosis and the accumulation of individual chromosomes, was first documented in 1889 by Pernice,[19] but the biological significance of this tubulin-inhibitory effect was not appreciated until the mid-1930s.[20,21] As a result, colchicine was investigated clinically as an antitumor drug, but its lack of tumor selectivity in this regard caused it to be abandoned for this indication.[22,23] Nevertheless, other colchicine analogues, such as demecolcine (**6**), have been used for chronic myelogenous leukemia and malignant lymphoma.[24] Currently, compounds with a pharmacophore that binds to the colchicine site on tubulin, such as the combretastatins, continue to be developed as potential antineoplastic agents.[25,26] Colchicine itself remains a critical agent for treatment and diagnosis of gout,[27] and it is used to combat a variety of proinflammatory disorders, such as familial Mediterranean fever,[28] Behcet's disease,[29] and usual interstitial pneumonia.[30]

In our continuing studies on Jordanian *Colchicum* species,[31,32] the colchicinoids of *Colchicum brachyphyllum* Boiss. & Haussk. ex Boiss. (Colchicaceae) were pursued, as, to the best of our knowledge, this species has not been investigated previously for bioactive constituents. *C. brachyphyllum* is found flowering from December to February in Northern Jordan, usually in damp basalt soil and stony habitats, and characterized as a perennial herb with corms covered by brown, membranous, smooth scales.[6−8] From extracts of the corms, flowers, leaves, roots, and stems, nine colchicinoids were isolated and characterized. One of these has a novel ring system, to which we have ascribed the trivial name (+)-demecolcinone (**9**), and it represents the first naturally occurring dextrorotatory colchicinoid. Another isolated compound was a new colchicinoid analogue, (−)-2,3-didemethyldemecolcine (**8**), while the remaining seven known colchicinoids were new to the species: (−)-colchicine (**1**), (−)-3-demethylcolchicine (**2**), (−)-cornigerine (**3**), β-lumicolchicine (**4**), (−)-androbiphenyline (**5**), (−)-demecolcine (**6**), and (−)-3-demethyldemecolcine (**7**). The structures of all compounds were elucidated using a series

* To whom correspondence should be addressed. Tel: (962) 2-720100, ext. 23523. Fax: (962) 2-7095019. E-mail: falali@just.edu.jo (F.Q.A.). Tel: (919) 541-6958. Fax: (919) 541-6499. E-mail: oberlies@rti.org (N.H.O.).
† Jordan University of Science and Technology.
‡ Research Triangle Institute.
§ University of Applied Sciences.
⊥ Virginia Polytechnic Institute and State University.

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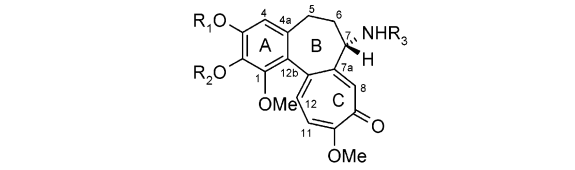
Table 1. ^{1}H, ^{13}C, DEPT-135, and HMBC NMR Data (in $CDCl_3$) for Compounds **8** and **9**

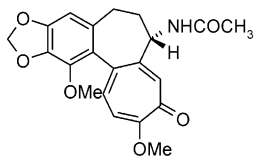
	(−)-2,3-didemethyldemecolcine (**8**)				(+)-demecolcinone (**9**)			
position	δ_C	DEPT	δ_H, mult (*J* in Hz)	HMBC (H → C)	δ_C	DEPT	δ_H, mult (*J* in Hz)	HMBC (H → C)
1	144.2	C			140.5	C		
2	136.0	C			180.2	C		
3	134.8	C			133.6	C		
4	110.6	CH	6.58 s	1[a], 3, 5, 12b	90.4	CH	5.25 s	1[a], 2, 3, 4a
4a	131.7	C			51.0	C		
5	29.6	CH_2	2.31 m/2.40 m	4, 4a, 5, 6, 7, 12b	26.3	CH_2	1.69 m/2.43 m	1[a], 4a, 6, 7, 12a[a], 12b
6	38.7	CH_2	1.63 m/2.15 m	4a, 5, 7, 7a, NCH_3[a]	23.4	CH_2	1.85m/2.04 m	4a, 5, 7, 7a
7	62.9	CH	3.31 dd (6, 6)	5, 6, 7a, 8, 12, 12a, NCH_3	65.6	CH	4.31 dd (2, 2)	5, 6, 7a, 8, 12a, 12b[a], NCH_3
7a	150.6	C			145.8	C		
8	132.5	CH	7.72 s	7, 7a, 9, 10, 12[a], 12a	130.6	CH	7.16 s	7, 7a, 9, 10, 12[a], 12a
9	179.8	C			179.4	C		
10	164.2	C			164.5	C		
11	111.7	CH	6.79 d (11)	9, 10, 12a	111.6	CH	6.66 d (11)	9, 10, 12, 12a, 10-OCH_3[a]
12	133.6	CH	7.24 d (11)	7a, 10, 12a, 12b	129.9	CH	7.11 d (11)	4a[a], 7a, 8[a], 10, 12a
12a	137.5	C			137.4	C		
12b	123.6	C			163.0	C		
1-OCH_3	60.4	CH_3	3.47 s	1	60.1	CH_3	4.14 s	1
10-OCH_3	56.3	CH_3	4.00 s	10, 11[a]	56.3	CH_3	3.93 s	9[a], 10
NCH_3	34.5	CH_3	2.21 s	7	37.0	CH_3	3.03 s	4[a], 7, 12b[a]
NH			4.15 br s					

[a] Four-bond HMBC correlations.

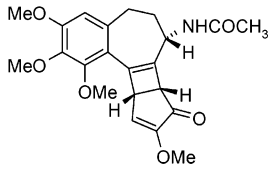
of spectrometric and spectroscopic techniques. The brine shrimp lethality test (BST) was used to direct the isolation of these colchicinoids. Moreover, all pure compounds were evaluated for cytotoxicity against a human cancer cell panel, for antimicrobial activity in an array of bacteria and fungi (including yeast), and for their potential to be allosteric modulators of the γ-aminobutyric acid type A ($GABA_A$) receptor.



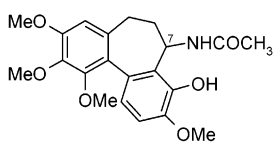
(-)-colchicine (**1**) R_1=R_2=CH_3, R_3= $COCH_3$
(-)-3-demethylcolchicine (**2**) R_1=H, R_2=CH_3, R_3= $COCH_3$
(-)-demecolcine (**6**) R_1=R_2=CH_3, R_3= CH_3
(-)-3-demethyldemecolcine (**7**) R_1=H, R_2=CH_3, R_3= CH_3
(-)-2,3-didemethyldemecolcine (**8**) R_1=R_2=H, R_3= CH_3



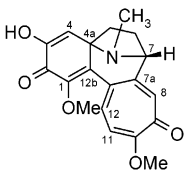
(-)-cornigerine (**3**)



β–lumicolchicine (**4**)



(-)-androbiphenyline (**5**)



(+)-demecolcinone (**9**)

Results and Discussion

The corms, flowers, leaves, roots, and stems of *Colchicum brachyphyllum* were treated individually throughout the purification processes, and each plant part was extracted in an analogous manner. The concentrated alkaloid fraction (fraction C) of each plant part was more potent in the BST than any other fraction (LC_{50} values that ranged from 1.1 to 7.4 μg/mL), and a high concentration of colchicinoids was noted in the TLC of fraction C by distinctive yellow spots after spraying with 5% phosphomolybdic acid in EtOH. Seven known colchicinoids were isolated from fraction C, and their structures were identified by 1- and 2D-NMR, mass spectra analyses, and comparisons to literature data. From all plant parts, **1**,[33] **2**,[34] and **3**[34] were isolated; from the corms, flowers, and roots, **4**[33] was isolated; from the corms **5**[35] was isolated; and from the leaves and stems, **6**[34,36] and **7**[34] were isolated. Although the structures of these compounds are well established, the ^{1}H NMR data for **1**−**4**, **6**, and **7**, and the ^{13}C NMR data for **1**−**3**, **6**, and **7** are given in Supporting Information Tables 1 and 2, respectively, to update the literature and to collect all of these data in one place. It was not surprising that the yield of **1** was high, representing at least 0.14% w/w in each of the stems, roots, and corms; however, **6** was isolated in the highest yield of all compounds in the leaves, representing at least 0.29% w/w.

Compound **8** (77.6 mg) was obtained as a yellowish powder from fraction C of both the roots and leaves. The molecular formula was determined as $C_{19}H_{21}NO_5$ by HR-FABMS, and the complete ^{1}H, ^{13}C, DEPT-135, and HMBC data sets are shown in Table 1. The 1D-NMR data suggested structural similarities with the aforementioned colchicinoids, especially compounds **2**, **6**, and **7**. In particular, compound **8** differed from 3-demethyldemecolcine (**7**) by the absence of one methoxy group. A broad singlet at δ_H 7.72 (H-8), an AB pattern at δ_H 7.24 and 6.79 (d, J = 11 Hz; H-12 and H-11, respectively), and an upfield-shifted ketone carbonyl at δ_C 179.8 (C-9) were characteristic for a tropolonic C-ring, whose presence was confirmed by corresponding HMBC data (Table 1). The methoxy signal at δ_H 4.00 showed HMBC correlations with C-10 and C-11, which placed this moiety at C-10. The other methoxy group in the molecule (δ_H/δ_C 3.47/60.4) displayed an HMBC correlation to C-1, confirming the connection of this methoxy to the C-1 position, as is typical in colchicinoids. The remaining signals verified free hydroxyl groups at the C-2 and C-3 positions, thereby establishing the structure of **8** as (−)-2,3-didemethyldemecolcine, a new colchicine analogue. The stereochemistry at position C-7 was presumed to be *S* on the basis of the well-established biosynthetic

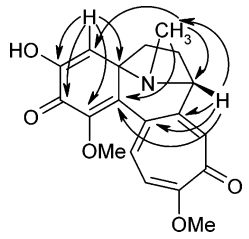


Figure 1. Key HMBC correlations for (+)-demecolcinone (**9**).

pathway of colchicinoids[37−40] and the close structural similarities with **7**, including a negative α_D value of the same magnitude.

Compound **9** (45.5 mg) was isolated from fraction C of the leaves, also as a yellowish powder. The HREIMS data (obsd *m/z* 341.1260 for $[M]^+$) revealed the molecular formula as $C_{19}H_{19}NO_5$, which corresponded to an index of unsaturation of 11. Characteristic signals for a tropolonic C-ring were evident, as described above for compound **8** (H/C-8 through H/C-12; Table 1), and this included a methoxy substituent at the C-10 position (10-OCH_3), observed via HMBC correlations from δ_H 3.93 to C-9 and C-10. The other methoxy singlet (δ_H/δ_C 4.14/60.1) showed an HMBC correlation to δ_C 140.5 (C-1), and this established a 1-OCH_3 group, again, as would be expected for a colchicinoid.

To this point the assignments were consistent with those observed with compounds **1**−**8**. Yet, many of the remaining unassigned NMR signals were somewhat peculiar for a colchicinoid. For example, the downfield signals consisted of one, rather upfield-shifted, olefinic methine group (δ_H/δ_C 5.25/90.4, H-4/C-4), three olefinic quaternary carbons (the forth of which, C-1, was assigned above), and another upfield-shifted ketone moiety, which was potentially α,β-unsaturated, especially given the corresponding IR data at 1572 cm^{-1}. There was also a relatively downfield-shifted quaternary carbon (δ_C 51.0). H-4 displayed HMBC correlations both to the neighboring hydroxylated olefinic carbon (δ_C 133.6, C-3) and to the ketone carbonyl (δ_C 180.2, C-2) (Table 1; Figure 1). The chemical shift of C-12b (δ_C 163.0) supported its position β to the ketone, and an HMBC correlation from H-4 to the quaternary and nonaromatic C-4a (δ_C 51.0) suggested that the A-ring had adopted a cyclohexadienone configuration.

Thus far, 9 of the 11 degrees of unsaturation had been assigned (rings A and C, five double bonds, and two ketone moieties), and as there were no remaining olefinic signals, ring B was proposed to have a bicyclic structure. Examination of the COSY data disclosed an isolated proton spin system comprising H_2-5/H_2-6/H-7, which was inserted between C-4a and C-7a; several HMBC correlations, such as H_2-5 to C-4a and C-12b, and H-7 to C-7a, C-8, and C-12a, supported this arrangement to form ring B, which was consistent with other colchicine analogues. An N-CH_3 group was construed from δ_H 3.03. This tertiary N was part of a piperidine ring via connection to both C-7 and C-4a, and several HMBC correlations supported this bicyclic ring structure as noted in Table 1 and Figure 1.

The trivial name demecolcinone (**9**) was ascribed to this novel colchicinoid due to its structural similarities to demecolcine (**6**); Supporting Information Figure 1 illustrates a postulated biosynthesis of **9** that starts with **6** and proceeds through 2,3-didemethyldemecolcine (**8**). Of note in the HMBC spectra for **9** was the presence of several four-bond correlations. Although these may be considered unusual, such long-range correlations have been reported,[41,42] especially in constrained ring systems;[43,44] an energy-minimized representation of **9** illustrating the constrained rings is shown in Supporting Information Figure 2. ROESY analysis was attempted to determine the relative configuration of positions C-4a and C-7; however, the data were inconclusive. Molecular modeling of the potential isomers did not reveal any definitive through-space correlations that would define the 3D-configuration of **9**. However, if the biosynthetic mechanism is correct (Supporting Information Figure 1), it may be safe to presume that position C-7 remains *S*. Finally, the optical rotation of **9** was found to be positive, which was surprising given that all known colchicinoids have a negative α_D value. Thus, (+)-demecolcinone (**9**) represents the first naturally occurring dextrorotatory colchicinoid.

Table 2. Human Cancer Cell Panel and BST Results for Compounds **1**−**9**

	human cancer cell panel[a]			
compound	MCF-7	H460	SF268	BST[b]
1	0.016	0.030	0.025	3.7
2	0.18	0.51	0.28	74.6
3	0.061	0.059	0.097	7.7
4	1.5	2.1	3.2	74.6
5	0.20	0.26	0.37	48.8
6	0.60	0.69	6.2	61.0
7	0.79	1.1	3.2	71.5
8	130.1	215.9	80.2	88.2
9	28.7	31.3	28.4	79.3
camptothecin[c]	0.072	0.0069	0.059	nt
colchicine[c]	nt	nt	nt	2.8

[a] Cytotoxicity results are expressed as EC_{50} values (μM; concentration to inhibit growth by 50%) derived from single experiments using 11 data points, each run in triplicate. [b] BST results are expressed as LC_{50} values (μg/mL; concentration to kill 50% of the brine shrimp) derived from single experiments using four data points, each run in triplicate. [c] Positive controls; nt = not tested.

At least two similar compounds with a positive α_D value, although lacking the colchicinoid-ring system of 6−7−7 and termed androcymbine-type alkaloids, have been isolated from another Jordanian *Colchicum* species.[34] Interestingly, those authors[34] noted that colchicinoids are derived biosynthetically from a levorotary and *S* androcymbine-type precursor, and more recent studies support that argument.[37] Moreover, they suggested that enzymes that convert an *R*-configuration, dextrorotary androcymbine, which they isolated in that study,[34] to an *R*-configuration, dextrorotary colchicinoid may not exist, since dextrorotary and *R* colchicinoids were unknown at that time. Indeed, in this study, as suggested (Supporting Information Figure 1), the dextrorotary **9** may have been generated from the levorotary **8**, which was previously synthesized from a levorotary androcymbine, all the while retaining an *S*-configuration at C-7 in each molecule. If this was the case, the positive α_D value of **9** was not due to a change in the configuration of C-7. Rather, the observed change in the rotation of plane-polarized light was possibly a result of the constrained ring system generated by the piperidine ring across the B-ring. Studies are ongoing to generate a crystal of **9** suitable for X-ray analysis to verify the presumed *S*-stereochemistry at position C-7.

Compounds **1**−**9** were tested for general toxicity against the BST, for anticancer activity against a human cancer cell panel, and for antimicrobial activity against bacteria and fungi, including yeast. In the BST (Table 2), compounds **1** and **3** were by far the most toxic, with all other colchicinoids being approximately an order of magnitude less toxic. Indeed, this structure activity relationship held against the human cancer cell panel, with **1** and **3** exhibiting activity on the same order of magnitude as the positive

control, camptothecin. This correlation illustrates the power of the BST to identify strong anticancer correlations. However, the other seven compounds should not be discounted for their anticancer activity, as the BST is limited in its predictive capacity to distinguish between strong-to-moderate and weak potency anticancer compounds. The other known colchicinoids, compounds **2** and **4**−**7**, all had EC_{50} values in the human cancer cell panel of approximately 1 μM, even though their LC_{50} values in the BST were all greater than 40 μg/mL. The two new compounds, **8** and **9**, were essentially equipotent to **2** and **4**−**7** in the BST, but their EC_{50} values against the human cancer cell panel were lower. Therefore, the BST represents a quick initial screen for potent cytotoxins, but a finer level of discrimination for anticancer activity required the human cancer cell panel. Against the antimicrobial assays (data not shown), none of the compounds demonstrated any activity, even against the eukaryotic fungi and yeast, all having MIC values > 500 μg/mL.

The compounds were also assayed for their ability to modulate the $GABA_A$ ligand gated chloride channel, since colchicine has been shown to be a $GABA_A$ inhibitor.[45,46] GABA is the major inhibitory neurotransmitter in the brain, and positive modulators of this receptor hold promise in several areas of CNS research, including, but not limited to, anxiety, epilepsy, and sleep disorders.[47] For this, two functional binding assays, [^{35}S]TBPS and [^{3}H]flunitrazepam, which are designed to identify positive $GABA_A$ allosteric modulators, were used. These assays are complimentary because their endpoints are opposite of each other: decreased binding for [^{35}S]TBPS and increased binding for [^{3}H]flunitrazepam. Thus, this minimizes the possibility of identifying a false positive, a problem often found with natural products screened in binding assays. The positive control, allopregnanolone, at 10 μM completely inhibited [^{35}S]TBPS binding in rat cerebral cortical homogenates and caused a 2-fold increase in [^{3}H]flunitrazepam binding (data not shown). Initial screening of the nine compounds isolated in this study at 10 μM identified two of the colchicinoids, compounds **3** and **5**, as potential positive allosteric modulators since they displayed approximately 50% of the allopregnanolone activity in both assays. However, dose−response experiments with **3** and **5** failed to confirm this activity and instead revealed these two compounds as weak partial agonists with maximum responses only about 25% of that elicited by alloprenganolone. Colchicine (**1**) was negative in both binding assays (not shown), in keeping with its reported $GABA_A$ antagonist activity.[45,46]

In summary, a compound with a novel colchicine ring system (**9**) and a new colchicine derivative (**8**), along with seven known colchicinoids (**1**−**7**), were isolated from a native Jordanian meadow saffron, *C. brachyphyllum*. These compounds possessed cytotoxic properties in keeping with their structures, and two compounds (**3** and **5**) had some activity at the $GABA_A$ receptor. Compound **9** possessed a constrained novel structure that is unprecedented in nature. In addition, to the best of our knowledge, this compound represents the first naturally occurring dextrorotatory colchicinoid, making a very interesting addition to the well-known colchicine family of compounds.

Experimental Section

General Experimental Procedures. Optical rotations, UV spectra, and IR spectra were measured with a Rudolph Autopol III polarimeter, a Varian Cary 3 UV−vis spectrophotometer, and a Nicolet Avatar 360 FT-IR, respectively. All NMR experiments were performed in $CDCl_3$ with TMS as an internal standard; gs-COSY, ROESY, gs-HSQC, gs-HMBC, and ^{1}H NMR spectra were run on a Varian Unity Inova-500 instrument using a 5 mm broad-band inverse probe with *z*-gradient, while a Bruker DPX-300 instrument was utilized for the some of the ^{1}H, ^{13}C NMR and DEPT-135 NMR spectra using a Bruker 5 mm QNP probe. Low-resolution ESIMS and APCIMS were determined on an Applied Biosystems/MDS Sciex API 150 EX single quadrupole LC/MS system (Applied Biosystems, Foster City, CA); high-resolution FABMS and EIMS were measured with a Micromass Autospec mass spectrometer (Manchester, UK) and a Finnigan MAT 95Q hybrid-sector instrument (ThermoFinnigan, San Jose, CA), respectively. Molecular modeling was performed using Sybyl v.6.9.1 (Tripos Associates Inc., St. Louis, MO) on an SGI Octane. Energy-minimized structures were obtained using 10 cycles of simulated annealing by heating to 700 K for 1000 fs and cooling to 200 K for 1000 fs using an exponential annealing function. HPLC was performed on a Lachrom Merck-Hitachi (Tokyo, Japan), equipped with a quaternary gradient L-7150 pump, L-7455 diode-array detector, L-7200 autosampler, and D-7000 interface. The preparative HPLC column was a Hibar Merck prepacked column RT 250-25, Lichrosorb RP-18 (7 μm). PTLC was carried out on 20 × 20 cm plates with silica gel F_{254} (Merck KGaA, Germany). Column chromatography was carried out using silica gel 60 (0.06−0.2 mm; 70−230 mesh), and TLC utilized silica gel 60 with gypsum and pigment addition for UV-visualization (both from Scharlau Chemie S.A., Barcelona, Spain). TLC spots were visualized by UV (Vilber Lourmat, 4 W-254 nm tube) or made visible by spraying the developed plates with 5% phosphomolybdic acid in EtOH.

Plant Material. Corms, flowers, leaves, roots, and stems of *C. brachyphyllum* were collected during the flowering stage in February 2003 in the northern part of Jordan from al-Mazzah in al-Mafraq. A voucher specimen (PHC-106) was deposited in the herbarium of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan.

Extraction and Isolation. Each plant part was processed individually throughout the purification process, starting with drying at room temperature and grinding into a powder using a laboratory mill. Extracts were generated via infusion by soaking the plant materials (900 g of corms, 238 g of flowers, 500 g of leaves, 111 g of roots, and 135 g of stems, all dry weights) in MeOH at room temperature for 6 days with intermittent shaking, followed by filtration to separate the marc; this process was repeated six times for 36 total days of extraction. The filtrates were combined and dried under reduced pressure to yield five MeOH extracts (272 g of corms, 119 g of flowers, 167 g of leaves, 8.5 g of roots, and 66 g of stems), and each extract was fractionated based on the method of Šimánek and co-workers.[48,49] Briefly, these extracts were dissolved in 5% acetic acid and extracted with light petroleum (fraction A), and then, the aqueous acid residues were re-extracted three times with diethyl ether (fraction B). The acidic aqueous residues were made alkaline (pH 9) with 10% NH_4-OH followed by extraction three times with CH_2Cl_2 (fraction C). All fractions were dried under vacuum. Fraction C's of the corms and leaves (4.2 and 11.2 g, respectively) were subjected to chromatography over silica gel using a gradient of 100% hexane to 100% CH_2Cl_2 to 2.4% MeOH in CH_2Cl_2 to yield 21 pools from the former and 28 pools from the latter. Further purifications of fraction C's of the flowers (0.4 g) and stems (1.2 g) were carried out via PTLC developed with $CHCl_3$/MeOH (9:1). Pure compounds were isolated via preparative HPLC from fraction C of the root, from four PTLC zones of the purification of the stems, from four PTLC zones from the purification of the flowers, from 15 alkaloid-rich pools from the purification of the corms, and from 25 alkaloid-rich pools from the purification of the leaves, all using a gradient solvent system of CH_3CN and 3% acetic acid in water (10:90 to 60:40 over 30 min) with a 10 mL/min flow rate, monitoring at 245 nm, and injecting between 75 and 175 mg of material dissolved in 2 mL of MeOH and mobile phase in a 1:1 ratio. The purities

of the isolated compounds were checked by TLC developed with either $CHCl_3$/MeOH [9:1] or CH_2Cl_2/acetone/diethylamine [12:6:2].

Brine Shrimp Lethality Test (BST). The BST was performed as described previously.[50,51]

Human Cancer Cell Panel. Evaluations of the cytotoxicity of natural products conducted previously by this group most commonly employed the 9KB human oral epidermoid carcinoma cell line.[52,53] In the current studies, a panel of unrelated human cancer cell lines was selected to replace the previous assay. MCF-7 human breast carcinoma (Barbara A. Karmanos Cancer Center, Detroit, MI), NCI-H460 human large cell lung carcinoma (American Type Culture Collection, Manassas, VA), and SF-268 human astrocytoma (NCI Developmental Therapeutics Program, Frederick, MD) cell lines were all adapted and maintained in RPMI-1640 medium supplemented with fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA) at 10% (v/v) and the antibiotics penicillin G (100 U/mL) and streptomycin sulfate (100 μg/mL) in a humidified 5% CO_2 atmosphere kept at 37 °C. Strict attention was paid to using cells when in the logarithmic phase of cell growth, and fresh cell stocks were expanded at the end of 20 passages to maintain continuity of results during fractionation and compound purification.

Cell suspensions were first prepared at densities of 3000 (MCF-7), 1500 (NCI-H460), or 10 000 (SF-268) cells per 50 μL of medium for each well of 96-well culture dishes and plated in triplicate for each drug concentration. Plant extracts, fractions, and pure compounds were dissolved in DMSO initially at 4 mg/mL, then diluted in culture medium at twice the intended final concentration. Fifty microliters of each 2× drug solution was then added to wells containing an equal volume of each cell suspension. For initial screening and fractionation samples, cells were exposed to fractions at final concentrations of 2 and 20 μg/mL; for EC_{50} determinations, pure compounds were diluted serially in half-log steps. In all cases, the final DMSO concentration was ≤0.5%. Blank wells and wells with media but no cells were included for background correction since TCA-precipitated proteins from serum alone result in some background SRB absorbance.

After a three-day continuous exposure, cells were fixed by addition of 25 μL of cold 50% (w/v) trichloroacetic acid (TCA) to the growth medium in each well at 4 °C for 1 h, then washed five times with water. The TCA-fixed cells were then stained for 30 min with 50 μL of 0.4% (w/v) sulforhodamine B (SRB) in 1% (v/v) acetic acid followed by five rinses with 1% (v/v) acetic acid to remove unbound dye. The fixed, stained plates were air-dried and bound dye was then solubilized by incubation with 100 μL of 10 mM Tris base for at least 5 min. Absorbance was measured at 540 nm using a Tecan Ultra multiplate reader. The percent cellular survival was calculated as the fractional corrected absorbance of drug/extract-treated samples relative to control cells treated with vehicle alone: (sample OD_{540} − media blank OD_{540}/mean control OD_{540} − media blank OD_{540}) × 100. For EC_{50} calculations, survival data were evaluated by variable slope curve-fitting using Prism 4.0 software (GraphPad, San Diego, CA).

Antimicrobial Assays. Minimal inhibitory concentrations (MICs) of pure compounds were measured by broth microdilution in 96-well microtiter dishes. Cells of strains of *Micrococcus luteus, Mycobacterium smegmatis*, *Saccharomyces cerevisiae*, and *Aspergillus niger* were grown, and suspensions prepared, as described previously.[54] The medium was one-tenth strength brain heart infusion broth (BHIB; BBL Microbiology Systems, Cockeysville, MD). A 2-fold dilution series of the test compounds was prepared in 96-well microtiter plates in a 50 μL volume of the medium, and then, the dilution series was inoculated with 50 μL of each cell suspension. The resulting inoculated dilution series was incubated at 30 °C, and growth, noted as turbidity and scored visually, was recorded daily for 3 days. The MIC of each compound was defined as the minimal concentration completely inhibiting growth as evidenced by a lack of turbidity.

$GABA_A$ Binding Assays. The γ-aminobutyric acid type A ($GABA_A$) assays were performed as described[55] with some modifications. Radioligands and scintillant were obtained from Perkin-Elmer Life Sciences (Boston, MA), and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Adult male rats (Charles River Laboratories, Raleigh, NC) were euthanized using excess CO_2 asphyxiation and decapitated, and the cerebral cortex was removed rapidly, snap frozen, and stored at −80 °C until needed. Cortical membranes were prepared by homogenization of several cortices in 10 volumes (w/v) of ice-cold 0.32 M sucrose using a glass/Teflon homogenizer. Homogenates were centrifuged at 1500*g* (10 min at 4 °C); the supernatants were retained and centrifuged at 10000*g* (20 min at 4 °C). The pellets were retained and resuspended in assay buffer (original volume; 50 mM sodium phosphate buffer containing 200 mM NaCl, pH 7.4) and centrifuged at 10000*g* (10 min at 4 °C). This wash step was repeated twice, and then the pellets were resuspended in one-tenth the original volume of buffer and stored at −80 °C until needed.

The binding assays were run in duplicate at rt in a 96-well plate format, using 1.4 mL polypropylene assay tubes (Matrix Technologies Corporation, Hudson, NH). In a final volume of 500 μL, each assay sample contained 50 μg of cortical homogenate protein (added last), 10 μM test compound, and either 1.0 μM GABA and 1.0 nM [^{3}H]flunitrazepam (74.1−85.4 Ci/mmol) or 5.0 μM GABA and 2.0 nM [^{35}S]TBPS (157−200 Ci/mmol). For the [^{3}H]flunitrazepam binding assay, nonspecific binding was determined in the presence of 10 μM clonazepam. For the [^{35}S]TBPS (*tert*-butylbicyclophosphorothionate) binding assay, 200 μM picrotoxin was used to determine nonspecific binding. Allopregnanolone (10 μM) was tested on each plate as a positive control. After a 1 h incubation at rt, bound radioligand was separated from free by rapid vacuum filtration (cell harvester; Brandel, Inc., Gaithersburg, MD) over GF/B filter plates (Perkin-Elmer Life Sciences, Boston, MA) presoaked for at least 20 min either in assay buffer containing 0.1% polyethylenimine ([^{3}H]flunitrazepam assay) or in 0.1% polyethylenimine and 0.15% BSA ([^{35}S]TBPS assay). Each well was washed three times with 1.0 mL of ice-cold assay buffer. Plates were allowed to dry prior to addition of 20 μL of Microscint 20 scintillation cocktail. Each well was counted for 2 min on a Topcount 12-dectector scintillation counter (Perkin-Elmer). The effect of a test compound was expressed as a percentage of 10 μM allopregnanolone, which elicits a maximum response in both assays. The net changes in binding relative to basal (total binding) were used for these calculations. Any compound giving at least 50% of the allopregnanolone response had its EC_{50} value and maximum response determined in the appropriate assay using six different concentrations of test compound.

(−)-2,3-Didemethyldemecolcine (8): yellowish powder (77.6 mg); $[\alpha]^{23}_D$ −90° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 241 nm (ϵ, 11806) IR ν_{max} 3532, 2931, 2855, 1604, 1587 (s), 1563, 1491, 1460, 1364, 1275, 1251 (base peak), 1196, 1182, 1131, 1067, 990; ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data, see Table 1; (+)-APCIMS *m/z* 344.5 $[MH]^+$, 329.5 $[M - CH_3]^+$, 312.8 $[M - NHCH_3]^+$, 286.6; HRFABMS $[M + H]^+$ *m/z* 344.1482 (calcd for $[C_{19}H_{21}NO_5 + H]^+$, 344.1498).

(+)-Demecolcinone (9): yellowish powder (45.5 mg); $[\alpha]^{23}_D$ +242° (*c* 0.3, MeOH); UV (MeOH) λ_{max} 232 nm (ϵ, 18735); IR ν_{max} 3056, 2984, 2943, 2839, 1572 (strong, shoulder), 1508, 1463, 1406, 1350, 1272, 1248, 1218, 1207, 1175, 1152, 1123, 751, 731 (base peak); ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data, see Table 1; (+)-APCIMS *m/z* 342.5 $[MH]^+$, 327.3 $[MH - CH_3]^+$, 314.5, 286.6; HREIMS *m/z* 341.1260 (calcd for $[C_{19}H_{19}NO_5]^+$, 341.1263).

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Supporting Information Available: Tables of NMR data for compounds **1**−**4** and **6**−**7**, a figure suggesting a possible biosynthetic route of compound **9**, and a figure illustrating the energy-minimized structure of **9** are available free of charge via the Internet at http://pubs.acs.org.

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