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Cytotoxic Barrigenol-like Triterpenoids from an Extract of *Cyrilla racemiflora* Housed in a Repository

Yulin Ren[†], Andrew VanSchoiack[‡], Hee-Byung Chai[†], Michael Goetz[§], and A. Douglas Kinghorn^{†,*}

[†]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH 43210, United States

[‡]Mass Spectrometry and Proteomics Facility, Campus Chemical Instrument Center, The Ohio State University, Columbus, OH 43210, United States

[§]Natural Products Discovery Institute, Baruch S. Blumberg Institute, Doylestown, PA 18902, United States

Abstract

Two new [(+)-cyrellins A (**1**) and B (**2**)] and four known barrigenol-like triterpenoids (**3–6**), along with betulinic acid and (+)-3 β -*O*-*trans*-feruloylbetulinic acid, were isolated from a sample-restricted CH₂Cl₂-soluble extract of the bark of *Cyrilla racemiflora*, collected in Dominica. The structures of the new compounds were elucidated by interpretation of their spectroscopic data, and the absolute configuration of the cyclic 1,2-diol unit of (+)-cyrellin A (**1**) was ascertained by analysis of the electronic circular dichroism (ECD) spectrum induced with [Mo₂(OAc)₄]. In the case of (+)-cyrellin B (**2**), which was found to contain a di-angeloylated glucose residue, the structure proposed was supported by analysis of its MS² and MS³ spectra. All compounds isolated were evaluated for their cytotoxicity against HT-29 human colon cancer cells, and the known compound, (+)-barringtogenol B (**3**), was found to be the most potent, exhibiting an IC₅₀ value of 1.7 μ M. This compound also showed inhibitory activity toward the CCD-112CoN human normal colon cell line, with an IC₅₀ value of 5.9 μ M, indicating a lack of cytotoxic selectivity.

Cyrrillaceae, a small plant family in the order Ericales, comprises two genera, *Cliftonia* and *Cyrilla*, with each containing a single species, *Cliftonia monophylla* Britton and *Cyrilla racemiflora* L., respectively.^{1,2} There have been no reports on the chemical components of either of these species. When isopropyl alcohol extracts of the leaves from 19 plants collected in northern South America were screened against the HT-29 human colon, A549 human lung, and MDA-MB-231 human breast cancer cell lines, an extract of *C. racemiflora* was found to show weak cytotoxic activity towards all these cell lines.³ Consistent with this

*Corresponding Author. Tel.: +1 614 247-8094. Fax: +1 614 247-8642. kinghorn.4@osu.edu.

ASSOCIATED CONTENT

Supporting Information. Mass and NMR spectra of compounds **1** and **2**; structures, UV, ECD, and ICD spectra of all isolates; diagrams of COSY, the key HMBC and selected NOESY correlations, the assignments of the ¹H and ¹³C NMR spectroscopic and other analytical data of the known compounds isolated from *C. racemiflora*. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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previous study, and in an extension of a search for new natural product anticancer agents from diverse organisms,⁴ a small-scale sample of a dichloromethane partition of an ethanol-soluble extract of the bark of *C. racemiflora*, collected in Dominica, was found to be cytotoxic against the HT-29 human colon cancer cell line. This extract was obtained from a repository donated to the Natural Products Discovery Institute (NPDI), Baruch S. Blumberg Institute (BSBI), Doylestown, PA, by Merck Research Laboratories, Rahway, NJ. Using column chromatography guided by cytotoxicity to HT-29 cells, several new and known barrigenol-like oleanane-type triterpenoids, together with two known lupane-type triterpenoids, were isolated from the extract of *C. racemiflora* provided. All compounds characterized were evaluated for their cytotoxicity against HT-29 human colon cancer cells, with the most potently cytotoxic compound, **3**, being tested against the CCD-112CoN human normal colon cell line.⁵

RESULTS AND DISCUSSION

Silica gel chromatographic separation of a small-scale sample from a repository extract of *C. racemiflora* yielded two new compounds, (+)-cyrellins A (**1**) and B (**2**), and six known compounds, (+)-barringtogenol B (**3**),⁶ (+)-22-*O*-acetylbarringtogenol B (**4**),⁷ (+)-22-*O*-angeloyl-A1-barrigenol (**5**),⁸ (+)-22-*O*-angeloyl-R1-barrigenol (**6**),⁹ (+)-3 β -*O*-*trans*-feruloylbetulonic acid,¹⁰ and (+)-betulonic acid.¹¹

Compound **1** was isolated as an amorphous colorless powder. A C₃₀H₄₆O₅ molecular formula was deduced from the sodiated molecular ion peak at *m/z* 509.3271 (calcd 509.3243) observed in the HRESIMS, in conjunction with the ¹³C NMR spectroscopic data. The UV (λ_{max} 228 nm) and IR (ν_{max} 3419, 1713, and 1668 cm⁻¹) spectra were consistent with the absorption characteristics of hydroxy and cyclic enone groups.¹² The ¹H NMR spectrum displayed resonances for seven tertiary methyl groups at δ_{H} 0.92, 0.94, 1.04, 1.07, 1.11, 1.22, and 1.49 (singlets, 3H each), an oxygen-bearing methylene group at δ_{H} 3.29 and 3.47, three oxygenated methine groups at δ_{H} 3.79, 3.95, and 4.43, two coupled olefinic protons as doublets at δ_{H} 5.74 and 7.17, an olefinic proton at δ_{H} 5.38, and several methylene and methine groups in the range δ_{H} 1.42–2.52 (Table 1).^{12,13} Consistent with these observations, the ¹³C NMR spectrum showed 30 carbon resonances comprising seven methyls, six methylenes, nine methines (three oxygenated), seven quaternary carbons, and a carbonyl carbon. Also, an enone and a trisubstituted olefinic group were apparent from the signals at δ_{C} 125.5 (CH), 159.6 (CH) and 204.3 (C), and the signals at δ_{C} 123.1 (CH) and 144.4 (C) (Table 1), respectively.¹² In addition, a pentacyclic system was inferred from eight indices of hydrogen deficiency implied by the molecular formula and the unsaturated groups indicated by the NMR spectroscopic data. Therefore, compound **1** was assigned as an oleandiene derivative with five oxy substituents.

An enone group in **1** was proposed at the C-1 position in ring A, as supported by the HMBC correlations between H-1 and C-3 and C-5 and H-2 and C-4 and the ¹H-¹H COSY correlation between H-1 and H-2 (Figure 1). A trisubstituted alkenyl group could be located at the C-12 position, as supported by a ¹H-¹H COSY sequence observed between H-9-H-11-H-12. Two pairs of geminal tertiary methyl groups connected to C-4 and C-20, respectively, were indicated by the HMBC correlations between H-23 and H-24 and C-3 and C-5 and

H-29 and H-30 and C-19 and C-21. Three additional tertiary methyl groups (C-25, C-26, and C-27) attached at C-8, C-10, and C-14, respectively, were implied by the HMBC correlations between H-25 and C-1 and C-9, between H-26 and C-9 and C-14, and between H-27 and C-8 and C-15. The substitution of four hydroxy groups at the C-16, C-21, C-22, and C-28 positions of the D and E rings was supported by the HMBC correlations between H-30 and C-21 and H-22 and C-28 and the ^1H - ^1H COSY correlation between H-21 and H-22 and between H-15 and H-16. Comparison of the ^1H and ^{13}C NMR data of **1** (Table 1) with those of barringtogenol C showed that both compounds share the same B-, C-, D-, and E-ring substitution patterns, with differences observed for the A-ring.⁶ Further comparison of the ^1H and ^{13}C NMR data of **1** with those of 15 α ,16 α ,21 β ,22 α ,28-pentahydroxyolean-1,12-dien-3-one showed that both compounds exhibit closely comparable data, except for C-15.¹² The signals for C-15 of **1** appeared at δ_{C} 34.0 and δ_{H} 1.92 and 1.45, but those of the known reference compound were reported to resonate at δ_{C} 67.1 and δ_{H} 4.42,¹² indicating that **1** is 16,21,22,28-tetrahydroxyolean-1,12-dien-3-one.

The relative configuration of **1** was determined by analysis of its 2D NOESY NMR spectrum (Figure 2),¹⁴ with the absolute configuration of the 21,22-diol unit being established using Sneath's method.^{15–17} The *trans* di-equatorial 21,22-diol unit of **1** was defined by the large coupling constant (9.5 Hz) observed between H-21 and H-22¹⁸ and by the NOESY correlations found between H-21 and H-29 and between H-22 and H-30. The ECD spectrum induced by $[\text{Mo}_2(\text{OAc})_4]$ (IECD) of **1** showed a negative Cotton effect at 333 nm (Figure 3A), and a negative O-C-C-O dihedral angle (Figure 3B) was indicated by the di-equatorial *trans* 21,22-diol moiety. Following an experimental procedure used in the literature,^{15–17} a (21*R*, 22*R*) absolute configuration could be assigned for **1**. Thus, the structure of compound **1** [(+)-cyrrillin A] was defined as 16 α ,21 β ,22 α ,28-tetrahydroxyolean-1,12-dien-3-one, and this is one of a very limited member of barrigenol-like triterpenoids containing a ring A enone group. A strong positive Cotton effect (CE) at 235 nm and weak negative CEs at 208 and 341 nm were observed in the ECD spectrum of **1** (Figure S8, Supporting Information), and these CEs could be regarded as an indication of exciton coupling arising from the enone system linked at C-10 and the ^{12,13} olefinic functionality for a 10*R* configuration for **1**, as supported by the UV absorption at 228 nm due to the cyclic α,β -unsaturated carbonyl group (Figure S9, Supporting Information).¹² Consistent with the previous observations,¹⁸ the chemical shifts of the equatorial methyl groups, C-23 (δ 28.2) and C-29 (δ 30.6) were found to be more deshielded than those of the axial substituents at C-24 (δ 22.0) and C-30 (δ 19.0).

Compound **2** was isolated as an amorphous colorless powder, having a molecular formula of $\text{C}_{46}\text{H}_{72}\text{O}_{11}$, as deduced from a sodiated molecular ion peak at m/z 823.4948 (calcd 823.4972) observed in the HRESIMS, and from the ^{13}C NMR data (Table 2). The IR spectrum of **2** showed hydroxy (3445 cm^{-1}), ester (1714 cm^{-1}), and alkenyl (1645 cm^{-1}) group absorption bands.¹⁹ The ^1H and ^{13}C NMR spectra of **2** displayed several pairs of signals at δ_{H} 6.07 (dq, 6.9, 1.3) and 6.12 (dq, 7.2, 1.4), 1.81 (s) and 1.82 (s), and 1.91 (s), and at δ_{C} 167.5 and 167.1, 128.8 and 128.3, 137.9 and 139.1, 20.6, and 15.8 (Table 2). These signals could be assigned to two angeloyl groups,²⁰ as indicated by the HMBC correlations observed between H-4''a and C-1''a and C-3''a, between H-5''a and C-2''a, and

between H-4^b and C-1^b and C-3^b, between H-5^b and C-2^b, and supported by the NOESY correlations between H-3^a and H-4^a and H-5^a and between H-3^b and H-4^b and H-5^b (Figures 1 and 2). In addition, the ¹H and ¹³C NMR spectra of **2** exhibited signals due to six oxygenated carbons, at δ_{H} 3.51, 5.33, 5.08, 3.73 and δ_{C} 73.5, 75.9, 69.8, 75.4 for four methine groups, at δ_{H} 3.61 m and 3.67 m and δ_{C} 62.1 for a hydroxymethyl group, and at δ_{H} 4.70 d (7.7) and δ_{C} 105.9 for an anomeric proton and carbon (Table 2). These carbons could be assigned to a hexose saccharide unit,^{21,22} as evidenced by a ¹H-¹H COSY sequence of H-1'-H-2'-H-3'-H-4'-H-5'-H-6', and from the HMBC correlations between H-2' and C-4', between H-4' and C-6', and between H-5' and C-1' (Figure 1). This saccharide moiety in **2** was substituted by two angeloyl groups at C-3' and C-4', as implied by the deshielded ¹H NMR signals (δ around 1.0) for H-3' and 0.5 ppm for H-4', when compared with those of aesculioside IIe,²¹ and by the HMBC correlations observed between H-3' and C-1^a and between H-4' and C-1^b (Figure 1).

An elemental formula of C₃₀H₄₉O₃ could be proposed for the aglycone of **2**, based on its molecular formula and the sugar unit mentioned above. Seven sharp singlets observed in the ¹H NMR spectrum could be assigned as tertiary methyl groups attached at C-4, C-8, C-10, C-14, and C-20, as supported by the HMBC correlations between H-23 and H-24 and C-3 and C-5, between H-25 and C-1 and C-9, between H-26 and C-9 and C-14, also between H-27 and C-8 and C-15, and between H-29 and H-30 and C-19 and C-21. A trisubstituted double bond was proposed at the C-12 position, as indicated by the ¹³C NMR signals at δ_{C} 123.6 (CH) and 144.4 (C) and the HMBC correlations between H-12 and C-9 and C-18 (Figure 1). A hydroxymethyl group was deduced at C-17 by the HMBC correlations between H-28 and C-16 and C-22, with two secondary hydroxy groups linked at the C-3 and C-16 positions, as supported by the HMBC correlations observed between H-1, H-23, and H-24 and C-3, and between H-28 and C-16. Six indices of hydrogen deficiency could be indicated from these elucidations, and the aglycone of **2** could be assigned as an olean-12-ene unit substituted with three hydroxy groups at C-3, C-16, and C-28, and an oxygen substituent at C-22, for which the ¹³C NMR signal was deshielded to δ 86.1.

Comparison of the ¹³C NMR spectroscopic data of the aglycone of **2** with those of camelliagenin A^{23,24} showed the NMR data of these two compounds to be closely comparable except for C-22. For **2**, the C-22 resonance assigned at δ_{C} 69.5 for camelliagenin A²⁴ occurred at δ_{C} 86.1, indicating substitution by the saccharide unit at this position.²² This unit could be assigned as the di-angeloylated sugar moiety referred above, as supported by the HMBC correlation between H-22 and C-1' (Figure 1). Also, this was corroborated by comparison of the ¹³C NMR data of **2** with those of napoleogenin B.^{22,25} The signals at δ_{C} 92.1, 64.6, and 16.4 assigned to C-21, C-24, and C-6' of napoleogenin B,²² respectively, were replaced by those at δ_{C} 44.8 (C-21), 16.3 (C-24), and 62.1 (C-6') for **2** (Table 2), indicating that this compound contains a C-21 methylene, a C-24 methyl, and a C-6' hydroxy group.

Barrigenol-like triterpenoids containing a di-angeloylated glycosylated residue are unusual natural products discovered previously from *Xanthoceras sorbifolia*.^{22,25,26} Among these, the structure of napoleogenin B has been confirmed by analysis of its single-crystal X-ray diffraction data,²⁵ and the structure of a compound called M-C as determined by analysis of

its NMR spectroscopic data, was supported by abundant ions observed in the mass spectrum for the triterpenoid aglycone, di-angeloylated fucose, and angeloyl residue.²² This indicates that the major MS fragment ions could provide important information supportive of the structure determination of this compound type, as supported by an investigation for the MS¹–MS³ and MS⁴ spectra of a number of oleanene-type triterpenoid saponins, in which the potential fragmentation pathways were analyzed, with predominant fragment ions found to be formed from the cleavage of the glycosidic and acyl bonds, and from removal of a CH₂O unit from C-14.²⁷ Consistent with these previous studies, the MS² spectrum of **2** exhibited fragment ions at *m/z* 723.5 (C₄₁H₆₄O₉Na), 623.4 (C₃₆H₅₆O₇Na), 479.4 (C₃₀H₄₈O₃Na), and 367.1 (C₁₆H₂₄O₈Na), which could originate from the removal of one or two neutral angelic acid units (C₅H₈O₂, *m/z* 100) from the molecular [M+Na]⁺ ion at *m/z* 823.6 (C₄₆H₇₂O₁₁Na) or cleavage of the C-O bond between C-22 and C-1', respectively (Figure S3c and Scheme S1, Supporting information). The MS³ spectra of the selected peaks at *m/z* 723.5, 623.4, and 367.1 all displayed the closely similar fragmentation pathway as in the MS² spectrum, and that at *m/z* 479.2 for the aglycone exhibited a major sub-fragment ion at *m/z* 436.4, for which a C₃H₇ or C₂H₃O (*m/z* 43) unit was removed presumably through the cleavage of several bonds. Both the MS² and MS³ spectra confirmed that two angeloyl groups were connected to the glycosyl residue but not at the triterpenoid aglycone core of **2**.

The same relative configuration as 3-*O*-acetyloleanolic acid¹⁴ could be characterized for the triterpenoid skeleton of **2** by the NOESY correlations shown in Figure 2. The sugar moiety of **2** was assigned as a β-glucose unit, as evidenced by the cross correlations observed between H-1' and H-22 and H-3', H-3' and H-5', and H-2' and H-4' in the 2D NOESY NMR spectrum and confirmed by 1D NOE experiments. Upon irradiation of the signal at δ 4.70 for H-1', the signals at δ 4.12 (H-22), δ 5.33 (H-3'), and δ 3.73 (H-5') were enhanced slightly. However, irradiation of the signals at δ 3.51 (H-2') and δ 5.08 (H-4') did not result in any other signals being enhanced (Figure S4i, Supporting Information). The D-configuration of the glucose unit could be inferred from biogenetic considerations. Furthermore, the ¹³C NMR resonances of the axial C-24 methyl groups of the oleanane-type triterpenes have been found to be influenced strongly by the orientation of the hydroxy group at C-3.^{28,29} Inversion of the C-3 configuration results in a shielding of about 5 ppm for an axial methyl group at C-4.²⁸ Also, such an effect was observed for the geminal methyl groups linked at C-20 with a hydroxy group at the C-21 position of the oleanane-type triterpenes.³⁰ In addition, the equatorial geminal methyl groups of these compounds were found to be less shielded than the axial ones.¹⁸ A (21*R*, 22*R*) absolute configuration was determined for barringtonol C by chemical reactions, comparison of the NMR spectroscopic data with those of the epimers, and analysis of Dreiding models,³¹ and the ¹³C NMR signals due to the axial C-24 and C-30 methyl groups of this compound displayed at δ 16.5 and 19.4, respectively (Table S2, Supporting Information).⁶ Based on this evidence, a 3*S* absolute configuration could be proposed for **2** by the chemical shifts for its axial C-24 methyl group at δ 16.3 and its equatorial C-23 methyl group at δ 28.7 and the same relative configuration as those of 3-*O*-acetyloleanolic acid indicated by its NOESY correlations (Figure 2). Therefore, the structure of compound **2** [(+)-cyrrillin B] was defined as 22α-*O*-(3,4-di-*O*-angeloyl-β-D-glucopyranosyl)-3β,16α,28-trihydroxyolean-12-ene.

The identities of the known compounds (Figure S5, Supporting Information) isolated from *C. racemiflora* were determined by analysis of their spectroscopic data and by comparison of these data with reference values,^{6–11} with the full assignments of their ¹H and ¹³C NMR spectroscopic data being listed in Tables S1–S3 (Supporting Information). The absolute configuration of the 1,2-diol unit of **5** and **6** was confirmed by Snatzke's method,^{15–17,32} and the overall absolute configurations of the known compounds were corroborated by comparison of their NOESY NMR and ECD spectra with those of reference compounds (Figures S7, S8, and S10, Supporting Information).

Barrigenol-like triterpenoids are a small group of olean-12-enes hydroxylated at the C-3, C-15, and/or C-16, C-22 and/or C-21, and C-28 positions,³¹ occurring mainly in the families Apocynaceae (*Gymnema sylvestre*),³³ Lecythidaceae (*Barringtonia acutangula*¹⁸ and *Barringtonia asiatica*),¹⁹ Pittosporaceae (*Pittosporum tobira*),³⁴ Sapindaceae (*Aesculus hippocastanum*,⁶ *Aesculus pavia*,²¹ *Harpullia pendula*,⁸ and *Xanthoceras sorbifolium*),^{22,25} and Theaceae (*Camellia sinensis*).³⁵ Some of these compounds were identified as the main active components of *Barringtonia asiatica*, for which the seeds have been used as fish poison by indigenous Polynesian populations,³⁶ and of *Gymnema sylvestre*, with its leaves having been found to have sweetness inhibitory properties.³³

All chromatographically and spectroscopically pure compounds (purity >95%) isolated from *C. racemiflora* in the present study were tested in terms of their cytotoxicity against the HT-29 human colon cancer cell line, using paclitaxel as the positive control. A known compound, (+)-barringtogenol B (**3**), showed potent cytotoxicity toward HT-29 cells, with an IC₅₀ value of 1.7 μM (Table 3). Two known compounds, (+)-22-*O*-acetylbarringtogenol B (**4**) and (+)-22-*O*-angeloyl-A1-barrigenol (**5**), were less active, showing an IC₅₀ value of 7.8 and 8.7 μM, respectively. The potentially cytotoxic isolate, **3**, was also tested against CCD-112CoN human normal colon cell line,⁵ and it was found to be active, showing an IC₅₀ value of 5.9 μM (Table 3), indicating a lack of selective inhibitory activity toward cancerous and non-cancerous human colon cells.

Investigation of the cytotoxicity of the isolates from the present study showed that an angeloyl group attached at either C-21 or C-22 enhanced cytotoxicity toward HT-29 cells. Acetylation of the C-22 hydroxy group of **3** and substitution of **5** with a hydroxy group at the C-21 position decreased the potency of these compounds. Such observations are consistent with those indicated from previous studies.^{20,26,37–39} Also, an earlier study has shown that the presence of a hydroxy group at the C-24 position can enhance the cytotoxicity of some barrigenol-like triterpenoids, but replacing the C-21 angeloyl group with an epoxy group or introducing a hydroxy group at the C-15 position resulted in the potency being decreased.⁴⁰ Several related compounds containing a 3,7-dimethyl-2,6-octadienoyl group at C-21 with a glycosylated group at C-3, or a di-angeloylated glycosylated residue at C-21, were found to be cytotoxic against a small panel of human tumor cell lines, with some analogues showing selectivity.^{20–22,35,41} When tested for acute toxicity to mice, a crude saponin mixture (CIDI, containing mainly barrigenol-like triterpenoids) showed LD₅₀ values of 25 mg/kg and 1275 mg/kg in ip and oral administration, respectively.³⁴ Interestingly, CIDI showed activity when evaluated in a clinical study for the treatment of human cutaneous basaloma by topical administration.³⁴

Mechanistically, a barrigenol-like triterpenoid was found to inhibit the proliferation of human melanoma A375.S2 cells by the downregulation of IGF-1R/Raf/MEK/ERK cascades.⁴¹ Therefore, barrigenol-like triterpenoids seem worthy of further investigation for the development of new anticancer agents.

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations were measured on a Perkin-Elmer model 343 polarimeter. UV spectra were recorded on a Hitachi U2910 UV spectrophotometer. ECD measurements were performed on a JASCO J-810 spectropolarimeter. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer. ¹H and ¹³C, DEPT, HSQC, HMBC, NOESY, and COSY NMR spectra were recorded on a Bruker Avance DRX-400 or a DRX-800 MHz NMR spectrometer. ESIMS and HRESIMS were measured on a Q-TOF mass spectrometer. MS² and MS³ spectra were recorded on a Bruker amaZon ETD instrument. All mass spectrometric data were obtained in a positive-ion mode using an ESI ion source, with scan ranges (*m/z*) from 100 to 1000. For MSⁿ measurements, a dilute sample (around 1 μM in MeOH) was introduced via a syringe pump at a flow rate of 3 μL/min, and the fragment ions were isolated with a 4.0 Da window. Column chromatography was conducted using silica gel (65 × 250 or 230 × 400 mesh, Sorbent Technologies, Atlanta, GA). Analytical thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Sorbent Technologies, Atlanta, GA). Sephadex LH-20 was purchased from Amersham Biosciences, Uppsala, Sweden. For visualization of TLC plates, sulfuric acid reagent was used. Fluorescence was tested using a Spectroline (model ENF-260C) UV light source. All procedures were carried at room temperature using solvents purchased from commercial sources and employed without further purification.

Plant Material

A bark sample of *Cyrilla racemiflora* was collected and identified by Dr. Lee Kendall at St. Mark Parish (15° 14' N; 61° 21' W), Soufriere Mound, Sulfurspring, Dominica, in July 1992, and a voucher herbarium specimen representing this collection has been deposited at the New York Botanical Garden, Bronx, NY, under accession number KL00017.

Extraction and Isolation

The ground dried bark of *C. racemiflora* was extracted exhaustively with EtOH at room temperature, and the solvent was evaporated in vacuo. The resultant dried EtOH extract was suspended in 10% H₂O in MeOH and partitioned with *n*-hexane three times. The spent aqueous layer was concentrated under a vacuum to approximately 40% aqueous MeOH and extracted three times with equal volumes of CH₂Cl₂ to yield a CH₂Cl₂-soluble extract, which showed cytotoxicity toward HT-29 cells with an IC₅₀ value of 5.3 μg/mL. A small aliquot of the CH₂Cl₂ extract (0.5 g; the initial material available to this investigation) was subjected to passage over a silica gel column (2.5 × 25 cm), and eluted with gradient mixtures of *n*-hexane–acetone (100:1→1:3; 50 mL each). Fractions were pooled by TLC analysis to yield 13 combined fractions. Of these, fractions 4–11 were deemed cytotoxic

toward the HT-29 cell line (IC₅₀ values of ca. 10 µg/mL for fractions 4 and 11 and ca. 5 µg/mL for fractions 5–10).

Fraction 4 was chromatographed over a silica gel column (1.5 × 15 cm) and eluted with gradient mixtures of *n*-hexane–acetone (10:1→1:1, 25 mL each), with the sub-fractions were pooled after TLC analysis to obtain a combined fraction. From this, a precipitate formed was purified by separation over a Sephadex LH-20 column (2.5 × 25 cm), eluted with CH₂Cl₂-MeOH (1:1, 20 mL each), to afford betulinic acid (6 mg). Combined fractions 5–7 were chromatographed over a silica gel column (2.5 × 25 cm), that was eluted with gradient mixtures of *n*-hexane–acetone (10:1→1:1; 20 mL each) to yielded four sub-fractions. Of these, the first sub-fraction was purified over a Sephadex LH-20 column (2.5 × 25 cm), eluted with CH₂Cl₂-MeOH (1:1, 20 mL each), to yield (+)-3β-*O*-*trans*-feruloylbetulinic acid (1 mg). The second sub-fraction was separated over a silica gel column (1.5 × 15 cm), eluted with gradient mixtures of *n*-hexane–acetone (10:1→1:1; 20 mL each), and then further separated using a Sephadex LH-20 column (2.5 × 25 cm), eluted with CH₂Cl₂-MeOH (1:1, 20 mL each), affording (+)-22-*O*-angeloyl-A1-barrigenol (**5**, 3 mg), (+)-barringtogenol B (**3**, 1 mg), and (+)-22-*O*-acetylbarringtogenol B (**4**, 1 mg). Combined fractions 8–10 were chromatographed over a silica gel column (2.5 × 25 cm), that was eluted with gradient mixtures of *n*-hexane–acetone (10:1→1:1; 20 mL each), with the sub-fractions pooled by TLC analysis. The pooled sub-fractions were further separated over a Sephadex LH-20 column (2.5 × 25 cm), eluted with CH₂Cl₂-MeOH (1:1, 20 mL each), to yield (+)-cyrillin B (**2**, 4 mg) and (+)-22-*O*-angeloyl-R1-barrigenol (**6**, 1 mg). Fractions 11 was chromatographed over a silica gel column (2.5 × 25 cm), and eluted with gradient mixtures of *n*-hexane–acetone (5:1→1:1; 30 mL each), with the sub-fractions pooled by TLC analysis. The pooled sub-fractions were purified by separation over a Sephadex LH-20 column (2.5 × 25 cm), eluted with CH₂Cl₂-MeOH (1:1, 20 mL each), to afford cyrillin A (**1**, 3 mg).

(+)-Cyrillin A (1)—Amorphous colorless powder; [α]_D²⁰ +58.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 228 (3.93) nm; ECD (MeOH, nm) λ_{max} (ε) 341 (−1.60), 235 (+9.87), 208 (−2.43); IR (dried film) ν_{max} 3419, 2965, 1713, 1668, 1362, 1221, 1108, 822 cm^{−1}; ¹H and ¹³C NMR data, see Table 1; positive-ion HRESIMS *m/z* 509.3271, calcd for C₃₀H₄₆O₅Na, 509.3243.

(+)-Cyrillin B (2)—Amorphous colorless powder; [α]_D²⁰ +17.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.27) nm; ECD (MeOH, nm) λ_{max} (ε) 227 (+2.02), 210 (+2.46), 204 (+2.25); IR (dried film) ν_{max} 3445, 2927, 1714, 1645, 1456, 1385, 1234, 849 cm^{−1}; ¹H and ¹³C NMR data, see Table 2; positive-ion HRESIMS *m/z* 823.4948, calcd for C₄₆H₇₂O₁₁Na, 823.4972.

Determination of Absolute Configuration of the 1,2-Diol Unit of 1—The ECD spectra of **1** (0.10 mM in a DMSO solution) and [Mo₂(OAc)₄] (0.12 mM in a DMSO solution), and the IECD spectrum of **1** [0.10 mM in a [Mo₂(OAc)₄] (0.12 mM) DMSO solution for 20 min) were measured on a JASCO J-810 spectropolarimeter in a 0.5 cm cell from 500 nm to 261 nm under identical conditions. The IECD spectrum of **1** was subtracted

from the ECD spectrum of **1** (0.10 mM in DMSO) and then subtracted consecutively from the ECD spectrum of $[\text{Mo}_2(\text{OAc})_4]$ (0.12 mM in DMSO), and the relation between the absolute configuration of the 1,2-diol moiety of **1** and the sign of the O-C-C-O dihedral angles was performed following a reported procedure.^{15–17} $[\text{Mo}_2(\text{OAc})_4]$ (Sigma-Aldrich) and DMSO (Sigma Aldrich, anhydrous 99.9%) were commercially available and used without further purification. Using the same procedure, the IECD spectra of compounds **2–6** were measured, and the absolute configuration of the 1,2-diol units of compounds **5** and **6** was confirmed.

MS² and MS³ Spectra of 2—MS² m/z 823.6, $[\text{M} + \text{Na}]^+$ (rel. int. %) 823.6 (8.7), 723.5 (24.4), 623.4 (100), 479.4 (2.6), 367.1 (2.6). All peaks from MS² spectrum were selected by the mass spectrometer for MS³ measurements. MS³ m/z (723.5 of 823.6, rel. int. %) 723.6 (2.6), 721.6 (5.8), 623.5 (100), 593.5 (2.6), 525.5 (5.2), 497.5 (11.0), 479.5 (14.9), 267.2 (15.5), 221.2 (2.6). MS³ m/z (623.4 of 823.6, rel. int. %) 623.4 (0), 605.4 (9.6), 593.5 (76.3), 575.5 (9.0), 523.5 (4.5), 497.5 (27.1), 479.5 (100), 447.4 (3.4), 400.3 (1.1). MS³ m/z (479.4 of 823.6, rel. int. %) 479.2 (37.8), 462.4 (7.1), 449.5 (7.1), 436.4 (100), 382.0 (35.2), 368.0 (9.0), 275.4 (9.6), 182.1 (10.9), 160.2 (4.4). MS³ m/z (367.1 of 823.6, rel. int. %) 367.1 (1.3), 349.2 (3.2), 267.2 (100), 245.2 (3.2), 167.1 (7.1).

Cytotoxicity against HT-29 Cells

Cytotoxicity of the samples was evaluated against HT-29 human colon cancer cells using a reported procedure.⁵ The cells were cultured under standard conditions and trypsinized. Then, the harvested cells were added to 96-well plates and treated by the test samples dissolved in DMSO at different concentrations, the positive control, and the negative control (DMSO). The plates were incubated at 37 °C in 5% CO₂ for three days, and then the cells were fixed, incubated at room temperature for 30 min, washed with tap water, dried at room temperature overnight, and dyed using sulforhodamine B. After the dyed cells were lysed in tris-base buffer, the plates were read at 515 nm with an ELISA plate reader. Paclitaxel [Sigma-Aldrich, 95% (HPLC), powder] was used as a positive control, and the IC₅₀ values of the test samples in serial dilutions were calculated using nonlinear regression analysis (Table Curve2Dv4; AISN Software, Inc., Mapleton, OR). Measurements were performed in triplicate and are representative of three independent experiments.

Cytotoxicity against CCD-112CoN Cells

Following a previous procedure⁵ and the method for screening cytotoxicity towards HT-29 cells mentioned above, the cytotoxicity of compound **3** and paclitaxel was screened against CCD-112CoN normal human colon cells. Briefly, non-cancerous human colon CCD-112CoN cells were cultured in MEME medium supplemented with 10% FBS, PSF, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acid, and 1.5 g/L sodium bicarbonate. The cells were trypsinized, and the harvested cells were added to 96-well plates and treated by the test samples dissolved in DMSO at different concentrations, the positive control (paclitaxel), and the negative control (DMSO). The plates were incubated at 37 °C in 5% CO₂ for three days, and then the cells were fixed by the addition of cold 20% trichloroacetic acid, followed by washing with tap water, drying at room temperature overnight, and dying with sulforhodamine B. After the dyed cells were lysed in tris-base buffer, the plates were

read at 515 nm with a Bio-Tek μ Quant microplate reader. The IC_{50} values were calculated using nonlinear regression analysis (Table Curve2Dv4; AISN Software, Inc., Mapleton, OR) from three independent measurements, with each performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
COSY (— , $^1\text{H} \rightarrow ^1\text{H}$) and key HMBC (— , $^1\text{H} \rightarrow ^{13}\text{C}$) correlations of **1** and **2**.

Figure 2.
Selected NOESY ($^1\text{H} \rightarrow ^1\text{H}$) correlations of **1** and **2**.

Figure 3.

A. ECD spectrum induced by $[Mo_2(OAc)_4]$ of **1** {0.10 mM in a 0.12 mM DMSO solution of $[Mo_2(OAc)_4]$ }. Data were measured between 261 and 500 nm and subtracted from the spectra of the sample (0.10 mM) in DMSO and then from that of $[Mo_2(OAc)_4]$ (0.12 mM) in DMSO recorded under identical conditions. B. Relationship between the absolute configuration of C-21 and C-22 and the sign of the O-C-C-O dihedral angle of **1**.

Table 1

¹H and ¹³C NMR Spectroscopic Data of 1^a

position	δ_C , ^b type	δ_H , ^c (J in Hz)
1	159.6 CH	7.17 d (10.2)
2	125.5 CH	5.74 d (10.1)
3	204.3 C	
4	41.3 C	
5	54.4 CH	α 1.60 m
6	19.7 CH ₂	1.62 m
7	33.4 CH ₂	1.45 m 1.42 m
8	42.7 C	
9	41.9 CH	α 1.95 m
10	40.1 C	
11	24.1 CH ₂	2.12 dd (11.5, 3.0)
12	123.1 CH	5.38 t (3.1)
13	144.4 C	
14	45.1 C	
15	34.0 CH ₂	α 1.45 m β 1.92 m
16	67.9 CH	β 4.43 m
17	47.1C	
18	41.8 CH	β 2.25 m
19	48.2 CH ₂	α 2.52 m β 1.07 m
20	36.2 C	
21	78.8 CH	α 3.95 d (9.5)
22	78.4 CH	β 3.79 d (9.6)
23	28.2 CH ₃	α 1.11 s
24	22.0 CH ₃	β 1.07 s
25	19.3 CH ₃	β 1.22 s
26	17.7 CH ₃	β 1.04 s
27	27.2 CH ₃	α 1.49 s
28	69.7 CH ₂	3.47 m 3.29 m
29	30.6 CH ₃	α 0.94 s
30	19.0 CH ₃	β 0.92 s
OH-16		3.50 m
OH-28		3.91 m

^a Overlapped signals were assigned from ¹H–¹H COSY, HSQC, and HMBC spectra without designating multiplicity. CH₃, CH₂, CH, and C multiplicities were determined by DEPT 90, DEPT 135, and HSQC experiments.

^b Data (δ) measured in acetone-*d*₆ at 100.61 MHz and referenced to the solvent residual peak at δ 29.84.¹³

^cData (δ) measured in acetone-*d*₆ at 400.13 MHz and referenced to the solvent residual peak at δ 2.05.¹³

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Table 2

¹H and ¹³C NMR Spectroscopic Data of 2^a

position	δ_C^b , type	δ_H^c (J in Hz)	position	δ_C^b , type	δ_H^c (J in Hz)
1	39.6 CH ₂	α 1.01 m β 1.60 m	25	16.1 CH ₃	β 0.97 s
2	28.2 CH ₂	1.57 m 1.60 m	26	17.1 CH ₃	β 0.94 s
3	78.6 CH	α 3.19 m	27	27.5 CH ₃	α 1.49 s
4	39.6 C		28	69.3 CH ₂	3.33 m 3.56 m
5	56.3 CH	α 0.82 m	29	33.8 CH ₃	α 0.90 s
6	19.1 CH ₂	α 1.57 m β 1.44 m	30	15.8 CH ₃	β 0.98 s
7	33.6 CH ₂	α 1.60 m β 1.33 m	1'	105.9 CH	β 4.70 d (7.7)
8	40.6 C		2'	73.5 CH	α 3.51 m
9	47.6 CH	α 1.72 m	3'	75.9 CH	β 5.33 d (9.6)
10	37.7 C		4'	69.8 CH	α 5.08 d (9.7)
11	24.2 CH ₂	1.89 m	5'	75.4 CH	β 3.73 m
12	123.6 CH	5.28 m	6'	62.1 CH ₂	3.61 m 3.67 m
13	144.4 C		1''a	167.5 C	
14	42.3 C		2''a	128.8 C	
15	34.4 CH ₂	1.89 m 1.85 m	3''a	137.9 CH	6.07 dq (6.9, 1.3)
16	69.3 CH	β 4.52 m	4''a	20.6 CH ₃	1.81 d (1.5)
17	45.2 C		5''a	15.8 CH ₃	1.91 d (7.2)
18	42.6 CH	β 2.19 m	1''b	167.1 C	
19	47.7 CH ₂	α 2.47 m β 1.05 m	2''b	128.3 C	
20	32.0 C		3''b	139.1 CH	6.12 dq (7.2, 1.4)
21	44.8 CH ₂	α 2.26 m β 1.78 m	4''b	20.6 CH ₃	1.82 d (1.3)
22	86.1 CH	β 4.12 m	5''b	15.8 CH ₃	1.91 d (7.2)

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position	δ_C^b type	δ_H^c (J in Hz)	position	δ_C^b type	δ_H^c (J in Hz)
23	28.7 CH ₃	α 0.99 s	28-OH		4.14 m
24	16.3 CH ₃	β 0.79 s			

^a Overlapped signals were assigned from ¹H-¹H COSY, HSQC, and HMBC spectra without designating multiplicity. CH₃, CH₂, CH, and C multiplicities were determined by DEPT 90, DEPT 135, and HSQC experiments.

^b Data (δ) measured in acetone-*d*₆ at 100.61 MHz and referenced to the solvent residual peak at δ 29.84.¹³

^c Data (δ) measured in acetone-*d*₆ at 400.13 MHz and referenced to the solvent residual peak at δ 2.05.¹³

Table 3Cytotoxicity of Compounds 1–6^a

compound	HT-29 ^b	CCD-112CoN ^c
1	>10	NT
2	>10	NT
3	1.7 ± 0.3	5.9 ± 0.3
4	7.8 ± 0.7	NT
5	8.7 ± 1.7	NT
6	>10	NT
paclitaxel ^d	0.0008 ± 0.00035	19.3 ± 4.1

^aIC₅₀ values are the concentration required for 50% inhibition of cell viability after 72 h of incubation with a given test compound and were calculated using nonlinear regression analysis with measurements performed in triplicate and representative of three independent experiments.

^bRepresented as IC₅₀ values (μM) toward the HT-29 human colon cancer cell line.

^cRepresented as IC₅₀ values (μM) toward the CCD-112CoN human normal colon cell line.

NT = not tested.

^dPositive control.