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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · MARCH 2007

Impact Factor: 3.8 · DOI: 10.1021/np060534z · Source: PubMed

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J Nat Prod. 2007 March ; 70(3): 372–377.

Cytotoxic Constituents from the Fruiting Branches of *Callicarpa Americana* Collected in Southern Florida[⊥]

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Abstract

Bioassay-guided fractionation of the combined fruits, leaves, and twigs (fruiting branches) of *Callicarpa americana*, collected from a plot in a forested area in southern Florida, led to the isolation of six new clerodane diterpenes **(1–6)** and eight known compounds. The structures of **1–6** [12(*S*), 16ξ-dihydroxycleroda-3,13-dien-15,16-olide (**1**), 12(*S*)-hydroxy-16ξ-methoxycleroda-3,13-dien-15,16-olide (**2**), 12(*S*)-hydroxycleroda-3,13-dien-15,16-olide (**3**), 16 ξ-hydroxycleroda-3,11 (*E*),13-trien-15,16-olide (**4**), 3β,12(*S*)-dihydroxycleroda-4(18),13-dien-15,16-olide (**5**), and 12(*S*)-hydroxycleroda-3,13-dien-16,15-olide (**6**)] were elucidated by interpretation of spectroscopic data and chemical methods. The absolute configuration at C-12 in **1** and **3** was ascertained using the Mosher ester technique. The cytotoxicity of all isolates was tested against a panel of human cancer cell lines, and compounds **1**, **4**, and **6**, and the known compounds genkwanin, 16ξ-hydroxycleroda-3,13-dien-15,16-olide, and2-formyl-16ξ-hydroxy-3-A-norcleroda-2,13-dien-15,16-olide were active (ED_{50} <5 μg/mL). However, **1** was found to be inactive against human cancer cells implanted in mice using a hollow-fiber tumor model.

Callicarpa americana L. is a shrub native to the southeastern United States.[2] Although the genus has been considered traditionally as a member of the plant family Verbenaceae, molecular and micro-morphological observations have prompted contemporary taxonomic authorities to reclassify *Callicarpa* as a member of the family Lamiaceae.[3–5] Preparations of the bark of *C. americana* have been used to treat fever,[6] the leaves to treat dropsy,[7] and the roots to alleviate colic,[8] dysentery,[9] and skin cancer.[10] The roots and branches have been used in preparations intended to relieve malaria, rheumatism, and fever.[8] The leaf essential oils of *C. americana* have antialgal and mosquito-deterrent properties, and numerous essential oil components have been identified from the leaves of *C. americana*.[11–12]

As part of an ongoing effort to discover novel anticancer agents from plants,[13] a chloroform-soluble extract of the combined fruits, leaves, and twigs of *C. americana* was investigated, using cytotoxicity against hormone-dependent prostate cancer cells (LNCaP) to guide the isolation of active constituents. This plant material was obtained from a forest plot in southern Florida using a plot-based collection method (briefly reviewed in ref.[13]).

[⊥]Dedicated to the late Dr. Kenneth L. Rinehart of the University of Illinois at Urbana-Champaign for his pioneering work on bioactive natural products.

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Results and Discussion

The dried fruits, leaves, and twigs of *Callicarpa americana* L. were extracted with methanol and partitioned following a previously described protocol.[14] Based on the cytotoxic activity of the chloroform-soluble portion, bioassay-guided fractionation using a number of chromatographic techniques was carried out, guided by activity against LNCaP cells. This investigation resulted in the isolation of six new clerodane-type diterpenes **(1–6)**, the structures of which were elucidated using a range of spectroscopic techniques, including ID and 2D NMR and accurate mass measurement. Additionally, eight known compounds were identified by comparison of their measured spectroscopic data with literature values. The known compounds comprised three substances that were previously reported to occur in the genus *Callicarpa* [calliterpenone,[15,16] euscaphic acid,[17,18] and salvigenin[19,20]], and five new to the taxon [genkwanin,[21] 3β,16ξ-dihydroxycleroda-4(18), 13-dien-15,16-olide,[22] 16ξ-hydroxycleroda-3,13-dien-15,16-olide,[23] 5-hydroxy-7,4′-dimethoxyflavone,[24] and 2-formyl-16ξ-hydroxy-3-A-norcleroda-2,13-dien-15,16-olide[25,26]].

A molecular formula of $C_{20}H_{30}O_4$ and six degrees of unsaturation were determined for **1** based on accurate mass measurement. Comparison of the ^{13}C NMR and DEPT135 NMR spectra for **1** indicated the presence of four methyl groups, five methylenes, three aliphatic methines, two olefinic methines, one methine with a chemical shift typical of a hemiacetal (δ_c 98.4), and five quaternary carbons. The ^1H NMR spectrum for **1** contained signals for four downfield methines and four methyl groups, as well as a number of signals attributable to methylene protons.

HMBC NMR correlations from the vinylic methyl (H-18) to C-3, C-4, and C-5, from H-19 to C-4, C-5, C-6, and C-10, from the doublet methyl (H-17) to C-7, C-8, and C-9, from the H-20 methyl singlet to C-8, C-9, and C-10, from the H-10 bridgehead methine to C-2 and C-19, and from the H-3 proton to C-1, C-2, and C-5 helped to establish the structure of the substituted $\Delta^{3(4)}$-decalin ring system, accounting for 14 carbon signals and three of the six degrees of unsaturation. Although the bridgehead proton signal overlapped the vinylic methyl (δ_H 1.58) signal, the correlations for these two proton resonances could be distinguished in the HMBC NMR spectrum because of the width of the H-10 multiplet signal. The C-1 to C-10 linkage was evident from a cross-peak in the ^1H-^1H COSY NMR spectrum between H-l and H-10. Additional ^1H-^1H couplings were observed between H-3 and H-18 (vinylic) and H-2, and between H-17 and a buried multiplet for H-8.

The six remaining carbon signals in the ^{13}C NMR spectrum of **1** comprised a six-carbon side chain [a methylene (δ_C 43.0, C-11), a carbinol (δ_C 64.6, C-12), a hemiacetal methine (δ_C 98.4, C-16), an olefinic methine (δ_C 116.8, C-14), a carbonyl (δ_C 172.0, C-15), and a quaternary olefinic carbon (δ_C 174.3, C-13)], which could be connected to the decalin ring system at C-9. HMBC correlations from the H-14 proton (δ_H 6.01) to C-16 (δ_C 98.4) and C-15 (δ_C 172.0) and from the H-12 carbinol proton (δ_H 4.72) to C-9 (δ_C 39.8), C-11 (δ_C 43.0), C-13 (δ_C 174.3), C-14 (δ_C 116.8), and C-16 (δ_C 98.4), as well as the ^1H-^1H COSY cross-peaks observed between H-12 (δ_H 4.72) and the H-11 protons, suggested the side chain structure to consist of an α, β-unsaturated γ-lactone ring connected to the decalin ring by a two-carbon linker. This side-chain moiety, plus the mono-unsaturated decalin ring system, accounted for the six degrees of unsaturation indicated by the molecular formula.

The ^1H NMR chemical shifts of the C-5 methyl (H-19, δ_H 1.01) and the C-9 methyl (H-20, δ_H 0.75) in **1** indicated the ring junction to have a 5α,10β-*trans* relative configuration.[27] This was also supported by consideration of the ^{13}C NMR chemical shift of the C-19 methyl group, which resonated upfield relative to the predicted value for a *cis*-clerodane.[28] The ^1H and ^{13}C NMR chemical shifts for the C-8 methyl (CH_3-17) and C-6 (^{13}C NMR shift) are indicative of

the relative configuration of CH_3-17 in *trans*-clerodanes,[29] and the corresponding chemical shifts in **1** indicate that the CH_3-17 has an α (equatorial) relative configuration.

The absolute configuration at C-12 of **1a** (the 16-*O*-acetyl derivative of **1**) was determined using the method first described by Mosher and colleagues[30,31] and further elaborated by other research groups.[32–34] Comparison of relevant ^{1}H NMR chemical shift differences between the *R*- and S-MTPA esters of **1a** [H-3 (−0.150), H-17 (−0.039), H-18 (−0.073), H-19 (−0.042), H-20 (−0.047), H-14 (+0.312), H-16 (+0.169), OAc-16 (−0.007)] showed that the ^{1}H NMR signals associated with the decalin ring system in the *R*-MTPA ester were shifted downfield relative to those of the S-MTPA ester, and the opposite was true for the resonances of the lactone ring moiety (with the exception that essentially no difference in relative shift was observed for the protons of the acetyl group). This allowed **1** to be assigned structurally as 12(*S*),16ξ-dihydroxycleroda-3,13-dien-15,16-olide.

A molecular formula of $C_{21}H_{32}O_4$ and six degrees of unsaturation were determined for **2** based on accurate mass measurement. The ^{1}H NMR and ^{13}C NMR spectra were nearly superimposable with those of **1**, with the exception of an additional carbon signal at δ_C 57.5 and a proton signal at δ_C 3.59 in the spectra for **2**, and the C-16 methine signal, which resonated at δ_C 98.4 in **1**, was shifted to δ_C 103.1 in **2**. The inference that **2** contains a 16-*O*-methyl ether was confirmed by the observation of an HMBC correlation (three-bond) from the methoxy methyl protons (δ_H 3.59) to the carbon resonance at δ_C 103.1 (C-16). The assignment of the ^{1}H NMR and ^{13}C NMR data was carried out by analysis of its ^{1}H-^{1}H COSY, HMBC, and HMQC NMR spectra. The absolute configuration of OH-12 was assigned as *S* on biogenetic grounds by analogy with **1** and **3** (see below). Thus **2** was assigned structurally as 12(*S*)-hydroxy-16ξ-methoxycleroda-3,13-dien-15,16-olide.

A molecular formula of $C_{20}H_{30}O_3$ and six degrees of unsaturation were determined for **3** based on accurate mass measurement. The IR spectrum indicated the presence of hydroxyl and α,β-unsaturated carbonyl resonances [3426 (br) and 1743 cm^{-1}]. The ^{1}H, ^{13}C, and DEPT135 NMR spectra for **3** suggested that this isolate is based on a clerodane diterpene skeleton, similar to **1**. Comparison of the ^{13}C NMR and DEPT135 NMR spectra indicated signals for four methyl carbons, six methylene carbons [δ_C 20.3, 27.6, 28.7, 37.9, 45.1, and 72.9 (oxymethylene)], two aliphatic, an oxymethine (& 65.8), two olefinic methines (δ_C 114.1, and 122.0), and five quaternary carbons (δ_C 39.5, 40.9, 144.9, 176.5, and 179.0). The ^{1}H NMR spectrum showed the presence of three quaternary methyl groups, one secondary methyl, three methine resonances in the downfield region of the spectrum, and one pair of oxymethylene protons (δ_H 4.96). The assignment of the ^{1}H NMR signals was carried out by analysis of the ^{1}H NMR, ^{1}H-^{1}H COSY, and HSQC NMR spectra, and selective proton decoupling experiments, with the latter experiments being instrumental in determining the splitting patterns for the ^{1}H NMR signals. Key ^{1}H-^{13}C correlations in the HMBC NMR spectrum of **3** were observed from H-12 to C-11, C-14, and C-16, and from H-16 to C-13 and C-14, thus confirming the structure of the side chain as being composed of an α, β-unsaturated butyrolactone with a two-carbon linker attached at the β-position.

Analysis of the ^{1}H-^{1}H *J*-values for the protons on the decalin ring system and ^{1}H-^{1}H correlations observed in the NOESY NMR of **3** indicated the same relative configuration as determined for **1**. The absolute configuration at C-12 in **3** was determined using a modified Mosher technique as described for **1**. The protons associated with the lactone ring portion of **3** (H-14, H-16a, and H-16b) showed a positive relative difference in chemical shift [Δ_δ ($\delta_S - \delta_R$], and signals associated with the decalin ring system (H-3, H-17, H-18, H-19, and H-20) showed a negative relative difference in chemical shift, thus, the absolute configuration of C-12 was determined as *S*, and the structure of **3** was established as 12(*S*)-hydroxycleroda-3,13-dien-15,16-olide.

A molecular formula of $C_{20}H_{28}O_3$ and seven degrees of unsaturation were determined for **4** based on accurate mass measurement. The IR spectrum for this isolate displayed a broad absorption at 3426 cm^{-1} and an additional absorption at 1743 cm^{-1}, indicative of the presence of hydroxyl and carbonyl functionalities (with the wavelength of the latter being consistent with an α,β-unsaturated γ-lactone ring).[35] The NMR spectra for **4** were obtained in deuterated methanol (MeOD). However, signal crowding in the downfield region of the ^1H NMR spectrum of **4** obtained in MeOD made assignment of the associated signals difficult. Thus, NMR spectra for **4** were also measured in pyridine-d_5, making use of the phenomenon of pyridine-induced chemical shift modification.[36] Comparison of the ^{13}C NMR and DEPT135 NMR spectra for **4** indicated the presence of four methyl groups, four methylenes, seven methines, and an additional five quaternary carbons.

The ^1H NMR and HMQC NMR spectra obtained for **4** in pyridine-d_5 exhibited a pair of *trans*-coupled methine doublets at δ_H 6.39 and 6.52 (H-12 and H-11, J = 16.4 Hz), corresponding to an asymmetrically substituted olefinic bond, and these ^1H NMR signals correlated in the HMQC NMR spectrum with ^{13}C NMR resonances at δ_C 156.1 and 119.9 (C-11 and C-12, respectively). Analysis of the HMBC and HMQC NMR spectra confirmed that the connectivity and relative configuration of **4** were consistent with those of **1–3.** Thus, **4** was assigned as 16ξ-hydroxycleroda-3,11,13-trien-15,16-olide.

A molecular formula of $C_{20}H_{30}O_4$ and six degrees of unsaturation were determined for **5** based on accurate mass measurement. Comparison of the ^{13}C NMR and DEPT135 NMR spectra for **5** indicated the presence of 20 carbons, including three methyls, seven methylenes, five methines, and five quaternary carbons. Visual inspection of the ^1H, DEPT135, and ^{13}C NMR spectra suggested a close homology between **5** and **1–4**. HMBC NMR correlations for **5** were observed from H-14 to C-15 and C-16, from H-16 to C-13 and C-14, and from H-11 to C-9, C-12, and C-13, establishing the side chain structure as being the same as that of **3**. Additional HMBC correlations from H-18 to C-5, C-3, and C-4, from H-17 to C-7 and C-9, from H-19 to C-3, C-4, C-5, and C-10, and from H-20 to C-8, C-9, C-10, C-11, and C-12 ($4J_{C\text{-}H}$) helped to determine the structure of the decalin ring moiety. The assignment of C-1, C-2, and C-6 was complicated by the nearly overlapping ^{13}C NMR signals for C-1 and C-19 and for C-2, C-6, and C-8. The ^1H-^{13}C assignments for these signals are thus based on interpretation of the DEPT135, HMBC, and HMQC NMR spectra, and comparison with published values for 3β, 16ξ-dihydroxycleroda-4(18), 13-dien-15,16-olide.[22]

A similar compound, pentandranoic acid C, differing from **5** in the composition of the side chain, was reported by Xu and colleagues from *Callicarpa pentandra* Roxb.[26] The orientation of OH-3 in pentandranoic acid C was determined as α, based on NOESY NMR data and X-ray crystallographic analysis. The H-3 chemical shift of **5** (δ_H 4.33, $CDCl_3$) was in close agreement with that of pentandranoic acid C (δ_H 4.30, $CDCl_3$), but the C-3 chemical shift was significantly different from that of pentandranoic acid C (δ_C 69.7 in **5**, rather than δ_C 74.6). Furthermore, in **5** the ^1H-^1H splitting pattern for H-3 was a doublet of doublets (J = 5.4, 11.6 Hz), whereas Xu et al. reported a triplet (J = 3.0 Hz).[26] The relative configuration of OH-3 in 3β,16ξ-dihydroxycleroda-4(18),13-dien-15,16-olide was determined by Ma et al.[22] as β, based on the observation of a dd splitting pattern (J = 5.8, 12 Hz) for H-3, implying an α-axial orientation for H-3. Based on these considerations, OH-3 was assigned as β for **5**. Thus, the structure of **5** was determined as 3′,12(*S*)-dihydroxycleroda-4(18),13-dien-15,16-olide.

A molecular formula of $C_{20}H_{30}O_3$, indicating six degrees of unsaturation, was determined for **6** based on accurate mass measurement. The ^1H NMR and DEPT135 NMR spectra of **6** were nearly identical to those of **3**, and HMQC and HMBC experiments confirmed that, with the exception of the atoms associated with the γ-lactone ring, **6** was identical with **3**. Two olefinic methines were observed in the DEPT135 NMR spectrum. One of these was assigned as the

C-3 methine (δ_C 120.7), and this correlated with H-3 in the HMQC spectrum. The ^{13}C NMR signal at δ_C 143.7 exhibited an apparent correlation with the residual solvent peak at δ_H 7.26 in the HMQC spectrum, and cross-peaks were observed in the ^1H-^1H COSY and HMBC NMR spectra between H-14 and the chloroform peak, indicating that a proton signal was overlapped with the residual solvent peak. After optimizing the window functions relating to Gaussian multiplication, two signals were resolved, revealing a methine at δ_H 7.28 (dd, J= 3.1, 1.6 Hz). HMBC NMR correlations indicated two- and three-bond coupling between H-15 and C-13 and C-14, and showed weak coupling with the carbonyl signal at δ_C 173.1 (C-16). The H-14 proton displayed an HMBC correlation with C-15.

Chemical shift considerations and HMBC NMR data were used to establish that the α,β-unsaturated lactone ring of **6** is attached to C-12 at the α-carbon, rather than at the β-carbon, thus differing in this respect from **1–5**. In **6**, the ^1H NMR and ^{13}C NMR chemical shifts associated with C-14 (δ_C 143.7) were significantly downfield (average $\Delta_\delta = -34.3$) and the ^{13}C NMR resonance for C-13 (δ_C 138.3) was considerably upfield (average $\Delta^\delta = +28.0$) relative to the analogous signals in **1–5**. These differences of NMR chemical shifts for **6** can be explained as resulting from the electron-withdrawing (deshielding) effect of the conjugated carbonyl on the β-carbon (and proton in **6**). NMR data for several compounds having this structural feature exhibit similar ^{13}C and ^1H NMR patterns.[37,38] Thus, the structure of **6** was elucidated as 12(*S*)-hydroxycleroda-3,13-dien-16,15-olide, assuming the same absolute configuration for OH-12 as determined for **1** and **3** on biogenetic grounds.

The isolates obtained in this investigation of *C. americana* were tested for cytotoxicity in a panel of human cancer cell lines. Cytotoxic activity (ED_{50} < 5 μg/mL) was observed in at least one cell line for **1**, **4**, **6**, genkwanin, 16ξ-hydroxycleroda-3,13-dien-15,16-olide, and 2-formyl-16ξ-hydroxy-3-A-norcleroda-2,13-dien-15,16-olide (Table 1). A structure-activity relationship trend was observed for these clerodane diterpene isolates, in which compounds lacking a free hydroxy group at the 16-position were less cytotoxic than compounds with a γ-hydroxy group. The activity of the 3β-hydroxy-4(18)-exomethylene analogs was weaker compared with the $\Delta^{3(4)}$-clerodane diterpenes. Indeed, **5**, which lacks an OH-16 group, was not active in the cell line panel. These trends suggest that the γ-OH in the α,β-unsaturated γ-lactone ring structure is necessary for activity, but that the structure of the decalin ring system also contributes to the cytotoxic potency.

Owing to its initial cytotoxicity and the relatively large amount isolated, compound **1** was tested in vivo against LNCaP, Lul, and MCF-7 cells in a hollow fiber antitumor model[39,40] at 6.25, 12.5, 25, and 50 mg/kg in mice. No activity was observed at either the i.p. or s.c. sites in any of the three cell lines, even at the highest dose tested (50 mg/kg), and two of the three mice died at each the two highest doses (25 mg/kg and 50 mg/kg) (data not shown).

The co-occurrence of clerodane diterpenes and methoxylated flavones was recently observed in an investigation of the cytotoxic constituents from *Premna tomentosa*,[41] and the co-occurrence of these two structural classes in *Callicarpa americana* lends support to the previously noted close relationship with the genus *Premna*[4] Furthermore, the occurrence of salvigenin, a 6-methoxylated flavonoid suggests a close alliance with Lamiaceae, as flavonoid substitution at C-6 is usually associated with members of this family.[19,42] This is in agreement with the current taxonomic thinking that *Callicarpa* and several other closely related genera should be included in the Lamiaceae, rather than in the Verbenaceae (see disscussion above).[43]

Experimental Section

General Experimental Procedures

Melting points of the isolates were determined on a Thomas Hoover capillary melting point apparatus (Unimelt, Philadelphia, PA) and are uncorrected. Optical rotations of the isolates were measured using a Perkin-Elmer Model 241 polarimeter (Germany). Ultraviolet (UV) absorption spectra were recorded using a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). Optical rotatory dispersion (ORD) measurements (for calliterpenone) were performed using a JASCO J-810 spectropolarimeter (Tokyo, Japan). Infrared absorption spectra (IR) of the isolates were recorded on a Nicolet Protegé 460 FTIR spectrophotometer (Thermo, Waltham, MA). The NMR spectra were obtained on Bruker Avance DPX-300 (300 MHz), DPX-360 (360 MHz), DRX-400 (400 MHz), and DRX-600 (600 MHz) NMR spectrometers. Spectroscopic-grade deuterated solvents (Sigma-Aldrich, St. Louis, MO) were used for all NMR experiments, and tetramethylsilane (TMS) was used as internal standard for all samples in $CDCl_3$, and the residual solvent peak was used to calibrate samples in pyridine-d_5 and MeOD (δ_H 8.71 and 3.31, respectively, for the corresponding solvent peaks). Spectrum (^{1}H NMR) optimization for **6** was carried out using Bruker-provided Gaussian multiplication window function used LB = −0.98 and GB = 0.145. Low- and high-resolution (accurate mass) electrospray-ionization time-of-flight (ESITOF) mass spectra were recorded on a Micromass LCT (Milford, MA). Chemical ionization (CI), electron impact (EI), and fast-atom bombardment (FAB) mass spectra were obtained on a Finnigan MAT 90 mass spectrometer (Finnigan A.G., Bremen, Germany). Silica gel (Merck, Darmstadt, Germany) and polystyrene polymer (styrene-divinylbenzene; MCI Gel®, Sigma-Aldrich) were used for low-pressure chromatography. HPLC was carried out on a Waters HPLC system with two Waters 515 pumps and a Waters 2487 dual wavelength detector (Waters, Milford, MA), using an octadecylsilane (ODS) column (Waters, Sunfire 19 × 150 mm, 5 μm) and HPLC-grade solvents at a flow rate of 8 mL/min. Thin later chromatography (TLC) was performed on silica gel 60 F_{254} on glass plates (Merck) using various solvent systems.

Plant Material

The fruiting branches of *Callicarpa americana* L. (Lamiaceae) were collected from a forested area of Matheson Hammock, near Miami, Florida (permit #0014 from Natural Areas Management, Miami-Dade County Park and Recreation Department), and the plant material was air dried prior to milling. Voucher specimens (TL-71 and TL-93) were deposited at the Fairchild Tropical Garden herbarium in Florida and the herbarium of The Field Museum of Natural History in Chicago, IL. Assistance in identifying the plant material was provided by Mr. Roger Hammer, Director of Castellow Hammock Preserve and Nature Center, Miami, FL.

Extraction and Isolation

The milled plant material (634 g) was extracted with MeOH (overnight, 3 × 2.5 L, at room temp.), and the concentrated MeOH extract was suspended in 90% MeOH (500 mL). A series of previously described liquid/liquid partitioning steps[13,14] (petroleum ether-90% MeOH; $CHCl_3$-20% MeOH; $CHCl_3$-aqueous 1% NaCl) were used to prepare a $CHCl_3$ extract (15.5 g), defatted and essentially free of tannins. This $CHCl_3$ extract was chromatographed over Si gel, eluting with a stepped gradient of MeOH in $CHCl_3$, resulting in nine major fractions F01-F09. Of these, fractions F03-F06 were active against LNCaP cells.

Fraction F03 was chromatographed over MCI gel (eluting with 100% MeOH) resulting in six pooled fractions (F20-F25). F20 was chromatographed over Si gel (eluting with hexane/acetone), resulting in six combined fractions (F26-31). F26 (90% hexane) was separated by reversed-phase HPLC (80% MeOH) yielding **2**, **3**, **4**, and **6** (t_R 28.9, 25.3, 26.6, and 23.8 min, respectively), as well as 16ξ-hydroxycleroda-3,13-dien-15,16-olide (t_R 31.1 min). 5-

Hydroxy-7,4′-dimethoxyflavone was purified from F28 (hexane-acetone, 4:1) by preparative TLC (hexane-EtOAc-acetone, 7:3:1). F31 was chromatographed over silica gel (eruting with hexane-EtOAc, 1:1), and **5** and 30,16ξ-dihydroxycleroda-4(18),13-dien-15,16-olide were isolated by further purification of the subfractions obtained. 2-Formyl-16ξ-hydroxy-A-norcleroda-2,13-dien-15,16-olide was obtained by semi-preparative HPLC of F22 (77% MeOH, t_R 10.7 min).

Fractions F04 and F05 were combined, and the residue (7.8 g) was chromatographed over coarse Si gel, eluting with a stepped gradient of increasing polarity from 100% $CHCl_3$ to 40% MeOH in $CHCl_3$, affording 64 fractions of 200 mL each, which were combined according to TLC profiles into ten pooled fractions (F10-F19). Of these, F14 yielded genkwanin, and calliterpenone crystallized from F15 on standing. Repeated Si gel chromatography of F15 resulted in the isolation of a fraction containing impure **1**, which was further purified by re versed-phase HPLC (70% CHCN, t_R 27 min). Calliterpenone, euscaphic acid, genkwanin, and salvigenin were also obtained from the combined fractions F04 and F05.

Fraction F06 (1.0 g) was chromatographed over MCI gel, but the resulting pooled fractions were essentially inactive, and further purification of the active principles was not performed.

12(*S*),16ξ-Dihydroxycleroda-3,13-dien-15,16-olide (1)—$[\alpha]^{20}_D$ -67 (*c* 0.18, MeOH); UV (MeOH) $\lambda_{max;}$ (log ε) 209 (3.17) nm; IR (dried film) υ_{max} 3394 (broad), 2926, 1748, 1133, 949 $cm^{-1;}$ ^1H NMR ($CDCl_3$, 360 MHz) δ 6.13 (1H, br, H-16), 6.01 (1H, s, H-14), 5.19 (1H, s, H-3), 4.72 (1H, d, *J* = 8.0 Hz, H-12), 2.10 (2H, br, H-2), 1.87 (1H, m, H-11a), 1.77 (1H, m, H-1a), 1.74 (1H, m, H-6a), 1.60 (1H, m, H-10), 1.58 (3H, s, H-18), 1.50 (1H, m, H-8), 1.49 (1H, m, H-11b), 1.48 (1H, m, H-1b), 1.39–1.51 (2H, m, H-7), 1.22 (1H, m, H-6b), 1.01 (3H, s, H-19), 0.80 (3H, d, *J* = 6.1 Hz, H-17), 0.75 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 174.3 (C, C-13), 172.0 (C, C-15), 144.2 (C, C-4), 120.8 (CH, C-3), 116.8 (CH, C-14), 98.4 (CH, C-16), 64.6 (CH, C-12), 47.1 (CH, C-10), 43.0 (CH_2, C-11), 39.8 (C, C-9), 38.3 (C, C-5), 36.9 (CH, C-8), 36.5 (CH_2, C-6), 27.5 (CH_2, C-7), 26.6 (CH_2, C-2), 20.1 (CH_3, C-19), 19.2 (CH_2, C-1), 18.1 (CH_3, C-18), 17.6 (CH_3, C-20), 16.1 (CH_3, C-17); ESIMS *m/z* 333 $[M-H]^-$; HRCIMS *m/z* 352.2475 $[M+NH4]^+$ (calcd 352.2488 for $C_{20}H_{34}O_4$). **1a**-12-(*R*)-MTPA ester ^1H NMR (pyridine-d_5, 400 MHz) δ 7.357 (1H, s, H-16), 6.407 (1H, s, H-14), 6.219 (1H, dd, *J* = 7.2, 4.0 Hz, H-12), 5.173 (1H, s, H-3), 2.249 (1H, dd, *J* = 16.5, 7.2 Hz, H-11a), 2.180 (3H, s, OAc-16), 1.948 (1H, dd, *J* = 16.5, 4.0 Hz, H-11b), 1.538 (3H, s, H-18), 0.938 (3H, s, H-19), 0.807 (3H, d, *J* = 6.5 Hz H-17), 0.704 (3H, s, H-20). **1a**-12-(*S*)-MTPA ester ^1H NMR (pyridine-d_5, 400 MHz) S 7.533 (1H, s, H-16), 6.719 (1H, s, H-14), 6.192 (1H, dd, *J* = 7.5, 3.9 Hz, H-12), 5.024 (1H, s, H-3), 2.265 (1H, dd, *J* = 16.4, 7.5 Hz, H-11a), 2.174 (3H, s, OAc-16), 1.909 (1H, dd, *J* = 16.4, 3.9 Hz, H-11b), 1.465 (3H, s, H-18), 0.895 (3H, s, H-19), 0.768 (3H, d, *J* = 6.4 Hz H-17), 0.657 (3H, s, H-20).

12(S)-Hydroxy-16ξ-methoxycleroda-3,13-dien-15,16-olide (2)—$[\alpha]^{20}_D$ -94 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 264 (3.41), 204 (4.23) nm; IR (dried film) υ_{max} 3442 (broad), 2955, 2928, 2353 1756 $cm^{-1;}$ ^1H NMR ($CDCl_3$, 300 MHz) δ_H 6.07 (1H, d, *J* = 0.94 Hz, H-14), 5.71 (1H, d, *J* = 0.94 Hz, H-16), 5.20 (1H, br s, H-3), 4.71 (1H, br d, *J* = 8.0 Hz, H-12), 3.59 (3H, s, MeO-16), 1.59 (3H, br s, H-18), 1.53 (1H, m, H-8), 1.02 (3H, s, H-19), 0.81 (3H, d, *J* = 6.1 Hz, H-17), 0.76 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C 171.2 (C, C-13), 170.0 (C, C-15), 144.1 (C, C-4), 120.7 (CH, C-3), 117.5 (CH, C-14), 103.1 (CH, C-16), 65.0. (CH, C-12), 57.5 (CH_3, MeO-16), 47.3 (CH, C-10), 43.4 (CH_2, C-11), 39.8 (C, C-9), 38.4 (C, C-5), 37.0 (CH, C-8), 36.5 (CH_2, C-6), 27.5 (CH_2, C-7), 26.7 (CH_2, C-2), 20.1 (CH_3, C-19), 19.2 (CH_2, C-1), 18.0 (CH_3, C-18), 17.6 (CH_3, C-17), 16.0 (CH_3, C-20); ESITOFMS *m/z* 371.2204 $[M + Na]^+$ (calcd 371.2198 for $C_{21}H_{32}O_4Na$).

12(*S*)-Hydroxycleroda-3,13-dien-15,16-olide (3)—$[\alpha]^{20}_{D}$ -38 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (&log ε) 206 (4.22) nm; IR (dried film) υ_{max} 3426 (broad), 2958, 2922, 1743 $cm^{-1;}$ ^{1}HNMR (MeOD, 600 MHz) 85.93 (1H, dt, *J* = 1.8, 1.2 Hz, H-14), 5.17 (1H, br s, H-3), 4.96 (2H, d, *J* = 1.8 Hz, H-16), 4.71 (1H, dd, *J* = 8.4, 1.8 Hz, H-12), 2.08 (1H, ddddq, *J* = 17.6, 9.3, 6.8, 2.9, 1.5 Hz, H-2β), 1.95 (1H, dddq, *J* = 17.6, 4.6, 4.6, 1.4 Hz, H-2α), 1.89(1H, dd, 7=16.4, 8.4 Hz, H-11a), 1.83 (1H, dd, *J* = 12.5, 6.8 Hz, H-1β), 1.74 (1H, ddd, *J* = 12.8, 3.2, 3.2 Hz, H-6α), 1.72 (1H, dd, 11.8, 1.5 Hz, H-10), 1.60 (1H, dqd, 11.2, 6.7, 4.6 Hz, H-8), 1.58 (3H, m, H-18), 1.53 (1H, dd, *J* = 15.9, 1.8 Hz, H-11b), 1.47 (2H, m, H-7), 1.44 (1H, m, H-1α), 1.24 (1H, ddd, *J* = 12.8, 12.8, 4.5 Hz, H-6p), 1.04 (3H, s, H-19), 0.83 (3H, d, *J* = 6.7 Hz, H-17), 0.78 (3H, s, H-20); ^{13}C NMR (MeOD, 75 MHz) δ 179.0 (C, C-13), 176.5 (C, C-15), 144.9 (C, C-4), 122.0 (CH, C-3), 114.1 (CH, C-14), 72.9 (CH_2, C-16), 65.8 (C, C-12), 48.3 (CH, C-10), 45.1 (CH_2, C-11), 40.9 (C, C-9), 39.5 (C, C-5), 38.2 (CH, C-8), 37.9 (CH_2, C-6), 28.7 (CH_2, C-7), 27.6 (CH_2, C-2), 20.7 (CH_3, C-19), 20.3 (CH_2, C-1), 18.3 (CH_3, C-18), 18.2 (CH_3, C-20), 16.5 (CH_3, C-17); ESITOFMS *m/z* 341.2085 $[M + Na]^+$ (calcd 341.2093 for $C_{20}H_{30}O_3Na$).

16ξ-Hydroxycleroda-3,11(*E*),13-trien-15,16-olide (4)—$[\alpha]^{20}_{D}$ -56 (*c* 0.085, MeOH); UV (MeOH) λ_{max} (&log ε) 267 (4.47) nm; IR (dried film) υ_{max} 3426 (broad), 2958, 2922, 1743 $cm^{-1;}$ ^{1}H NMR (pyridine-rf_5, 300 MHz) δ 6.74 (1H, s, H-16), 6.52 (1H, d, *J* = 16.4 Hz, H-11), 6.39 (1H, d, *J* = 16.4 Hz, H-12), 6.22 (1H, s, H-14), 5.22 (1H, s, H-3), 1.89 (2H, m, H-2), 1.58 (3H, brs, H-18), 1.42 (2H, m, H-1), 1.37 (1H, H-8), 1.37 (2H, m, H-7), 1.30–1.43 (1H, m, H-10), 0.96 (3H, s, H-19), 0.86 (3H, s, H-20), 0.72 (3H, d, *J* = 4.5 Hz, H-17); ^{13}C NMR (pyridine-rf_5, 75 MHz) δ 171.8 (C, C-15), 163.3 (C, C-13), 156.1 (CH, C-11), 143.6 (C, C-4), 121.0 (CH, C-3), 119.9 (CH, C-12), 115.4 (CH, C-14), 99.3 (CH, C-16), 50.3 (CH, C-10), 45.2 (C, C-9), 41.2 (CH, C-8), 37.6 (CH_2, C-6), 36.6 (C, C-5), 27.0 (CH_2, C-2), 26.9 (CH_2, C-7), 20.4 (CH_2, C-1), 19.9 (CH_3, C-19), 18.1 (CH_3, C-18), 17.0 (CH_3, C-17), 12.1 (CH_3, C-20); ^{1}H NMR (MeOD, 300 MHz) 8 6.29 (1H, s, H-11), 6.29 (1H, s, H-12), 6.27 (1H, s, H-16), 5.93 (1H, s, H-14), 5.18 (1H, s, H-3), 1.81 (1H, m, H-2a), 1.60 (3H, brs, H-18), 1.44 (2H, m, H-1), 1.48 (1H, H-8), 1.52 (2H, m, H-7), 1.38 (1H, m, H-10), 1.06 (3H, s, H-19), 0.98 (3H, s, H-20), 0.76 (3H, d, *J* = 4.5 Hz, H-17); ^{13}C NMR (MeOD, 75 MHz) 8 173.9 (C, C-15), 164.4 (C, C-13), 157.6 (CH, C-11), 144.8 (C, C-4), 121.8 (CH, C-3), 120.5 (CH, C-12), 115.7 (CH, C-14), 100.3 (CH, C-16), 51.8 (CH, C-10), 46.2 (C, C-9), 42.4 (CH, C-8), 38.6 (C, C-5), 36.6 (CH_2, C-6), 27.9 (CH_2, C-2), 27.8 (CH_2, C-7), 21.3 (CH_2, C-1), 20.3 (CH_3, C-19), 18.2 (CH_3, C-18), 17.2 (CH_3, C-17), 12.5 (CH_3, C-20); ESITOFMS 339.1941 $[M + Na]^+$ (calcd 339.1936 for $C_{20}H_{28}O_3Na$).

3beta;,12(*S*)-Dihydroxycleroda-4(18),13-dien-15,16-olide (5)—$[\alpha]^{20}_{D}$ +18 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (&log ε) 207 (4.10) nm; IR (dried film) υ_{max} 3400 (broad), 2920, 2850, 1746, 1028 $cm^{-1;}$ ^{1}H NMR ($CDCl_3$, 300 MHz) 8 5.90 (1H, dd, *J* = 3.0, 1.7 Hz, H-14), 4.91 (1H, d, *J* = 1.2 Hz, H-18a), 4.86 (2H, dd, *J* = 1.9, 1.9 Hz, H-16), 4.73 (1H, br d, *J* = 8.0 Hz, H-12), 4.72 (1H, s, H-18b), 4.33 (1H, dd, *J* = 11.6, 5.4 Hz, H-3), 2.15–2.25 (2H, m, H-2), 1.92 (1H, m, H-1a), 1.88 (1H, dd, *J* = 15.6, 8.0 Hz, H-11a), 1.58 (1H, m, H-6a), 1.54 (1H, m, H-8), 1.54 (2H, m, H-7), 1.54 (1H, H-lb), 1.47 (1H, dd, 15.6, 1.1 Hz, H-11b), 1.38 (1H, dd, *J* = 12.1, 2.1 Hz, H-10), 1.18 (1H, dd, 12.8, 4.3 Hz, H6b), 1.05 (3H, s, H-19), 0.80 (3H, d, *J* = 5.6 Hz, H-17), 0.76 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.3 (C, C-13), 173.6 (C, C-15), 162.0 (C, C-4), 114.2 (CH, C-14), 99.6 (CH_2, C-18), 70.9 (CH_2, C-16), 69.7 (CH, C-3), 65.2 (CH, C-12), 49.2 (CH, C-10), 44.1 (CH_2, C-11), 40.4 (C, C-9), 40.2 (C, C-5), 37.4 (CH, C-8), 37.31* (CH_2, C-2), 37.30* (CH_2, C-6), 27.2 (CH_2, C-7), 21.24 (CH_3, C-19), 21.16 (CH_2, C-1), 17.6 (CH_3, C-20), 16.0 (CH_3, C-17) (* these resonances are interchangeable); ESITOFMS *m/z* 357.2051 $[M + Na]^+$ (calcd 357.2042 for $C_{20}H_{30}O_4Na$).

12(*S*)-Hydroxycleroda-3,13-dien-16,15-olide (6)—$[\alpha]^{20}_{D}$ -67 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 209 (3.87) nm; IR (dried film) υ_{max} 3436 (broad), 2955, 2928, 1744 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.28 (1H, dd, *J* = 3.1, 1.6 Hz, H-14), 5.19 (1H, br s, H-3), 4.82 (2H, dd, *J* = 1.7, 1.7 Hz, H-15), 4.69 (1H, br d, *J* = 8.4 Hz, C-12), 2.05 (2H, m, H-2), 1.87 (1H, dd, *J* = 15.6, 8.6 Hz, H-11a), 1.80 (1H, m, H-1a), 1.73 (1H, m, H-6a), 1.68 (1H, m, H-10), 1.67 (1H, dd, *J* = 15.6, 2.3 Hz, H-11b), 1.62 (1H, m, H-8), 1.59 (3H, dd, *J* = 3.4, 1.9 Hz, H-18), 1.47 (1H, d, *J* = 17.0 Hz, H-1b), 1.45 (2H, m, H-7), 1.22 (1H, m, H-6b), 1.01 (3H, s, H-19), 0.83 (3H, d, *J* = 6.6 Hz, H-17), 0.75 (3H, s, H-20); ^{13}C NMR ($CDC1_3$, 75 MHz) δ 173.1 (C, C-16), 144.2 (C, C-4), 143.7 (CH, C-14), 138.3 (C, C-13), 120.7 (CH, C-3), 70.3 (CH_2, C-15), 63.8 (CH, C-12), 47.4 (CH, C-10), 43.3 (CH_2, C-11), 39.7 (C, C-9), 38.4 (C, C-5), 37.1 (CH, C-8), 36.7 (CH_2, C-6), 27.6 (CH_2, C-7), 26.7 (CH_2, C-2), 20.2 (CH_3, C-19), 19.2 (CH_2, C-1), 18.0 (CH_3, C-18), 17.8 (CH_3, C-20), 16.1 (CH_3, C-17); ESITOFMS m/z 341.2099 $[M + Na]^+$ (calcd 341.2093 for $C_{20}H_{30}O_3Na$).

Determination of Absolute Configuration of OH-12 in 1 and 3

Prior to the esterification of OH-12 with MTPA chloride, OH-16 of **1** was acetylated using pyridine and acetic anhydride, with reaction conditions (0 °C, 5 min) that favored the acetylation of the less hindered hydroxyl group (OH-16) rather than the more sterically crowded OH-12. After HPLC separation of the mixture of epimers at C-16 (present in approximately a 2:3 ratio), the more abundant of the epimers **1a** was selected for acylation using the *S*- and *R*-MTPA chloride (separately). The reactions were carried out in deuterated pyridine under nitrogen, using NMR tubes as the reaction vessel, as previously described.[44] Compound **3** was reacted in the same manner with MTPA chloride, but with no need for prior acetylation. [39,40]

Bioassay Evaluation

The isolates obtained were evaluated for cytotoxicity against a panel of human cancer cell lines according to established protocols.[45,46] The hollow fiber assay was performed following an established protocol,[39] with minor modification.[40] Briefly, the hollow fiber assay was performed using NCr *nu/nu* athymic mice (five to six-weeks-old), obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). LNCaP, Lu1, and MCF-7 cells were implanted at intraperitoneal and subcutaneous sites (i.p. and s.c.) enclosed in sealed, semi-porous hollow fibers. The test compound **1** was administered i.p. at 6.25, 12.5, 25, and 50 mg/kg (three mice per dose group). Each mouse received each of the three cell lines implanted at both physiological sites. The mice were handled and cared for humanely, following a protocol (ACC No. 01-124) approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago.

Acknowledgements

The research described herein was supported by grant U19-CA52956, funded by the National Cancer Institute, NIH, Bethesda, MD. We thank the staff at the Miami-Dade County Parks and Recreation Department for granting permission to collect the plant material used in this investigation, and we also thank Mr. Roger Hammer, Director of the Castellow Hammock Preserve and Nature Center, Miami, FL for assistance in plant identification. Mass spectra were obtained and processed by Dr. Keith Fagerquist, Department of Chemistry, University of Minnesota, Dr. John L. Anderson, Research Resources Center, UIC, and Dr. Kari Green-Church, Campus Chemical Instrument Center, OSU. Assistance in acquiring spectra on the Bruker Avance DRX-600 NMR was provided by Dr. Charles E. Cottrell, Campus Chemical Instrument Center, OSU. Dr. Raymond W. Doskotch is gratefully acknowledged for helpful discussions and his insight regarding the 600 MHz NMR data for compound **3**.

References and Notes

1. The results presented here have appeared in part in: JonesWPAPharmacognostic Investigation of Callicarpa Americana for Potential Anticancer AgentsPh.D. DissertationUniversity of Illinois at ChicagoChicago, IL2006

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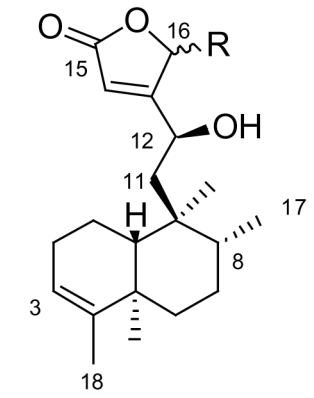
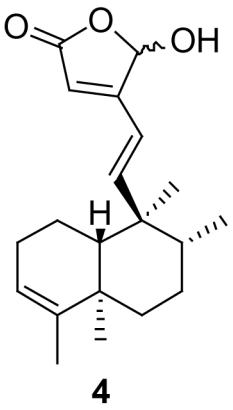
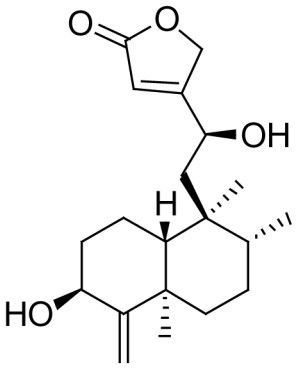


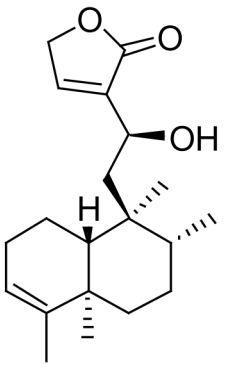
1 R = OH
1a R = OAc
2 R = OMe
3 R = H



4



5



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1. .

Table 1

Cytotoxicity Data for Isolates from *C. americana* in Several Human Cell Lines[a]

Compound Code	Cell Line[b,c]					
	MCF-7	Lu1	Col2	LNCaP	hTERT-RPE1	HUVEC
1	-	2.4	2.3	4.1	1.9	1.8
4	2.7	2.6	-	2.5	-	1.2
6	2.8	2.9	-	3.3	-	-
Genkwanin	-	>20	>20	>20	3.9	>20
16ξ-Hydroxycleroda-3,13-dien-15,16-olide	3.5	3.7	-	3.3	-	4.1
2-Formyl-16ξ-hydroxy-3-A-norcleroda-2,13-dien-15,16-olide	3.9	8.7	9.0	4.5	1.9	9.8

[a] Compounds **2**, **3**, and **5**, and the known compounds calliterpenone, 3(3,16ξ-dihydroxycleroda-4(18),13-dien-15,16-olide, euscaphic acid, 5-hydroxy-7,4′-dimethoxyflavone, and salvigenin were inactive in the cell lines tested.

[b] Cell lines: MCF-7 = breast cancer; Lu1 = lung cancer; Col2 = colon cancer; LNCaP = hormone-dependent prostate cancer; hTERT-RPE1 = human telomerase reverse-transcriptase retinal pigment epithelium; HUVEC = human umbilical vein epithelial cells.

[c] ED_{50} values are given in μg/mL, and values <5 μg/mL are considered to be active.