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Corymbulosins I–W, Cytotoxic Clerodane Diterpenes from the Bark of *Laetia corymbulosa*

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Abstract

The isolation studies of a crude MeOH/CH₂Cl₂ (1:1) extract (N005829) of the bark of *Laetia corymbulosa* yielded 15 new clerodane diterpenes, designated corymbulosins I–W (**1–15**), as well as four known diterpenes, **16–19**. The structures of **1–15** were characterized on the basis of extensive 1D and 2D NMR and HRMS analyses. The absolute configurations of newly isolated compounds **1–15**, as well as known **16–19**, which were reported previously with only relative configurations, were determined through ECD experiments, X-ray analysis, chemical methods, including Mosher esterification, and comparison of their spectroscopic data. The isolated compounds were evaluated for cytotoxicity against human cancer cell lines. Flow cytometric and immunocytochemical observations of cells treated with cytotoxic clerodanes demonstrated that the chromatin was fragmented and dispersed with formation of apoptotic microtubules.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

INTRODUCTION

The family Salicaceae, formerly placed in Flacourtiaceae, is known to produce isozuelanin-type clerodane diterpenes.¹ In particular, *Zuelania* and *Casearia* genera typically yield such diterpenes, including esculentin A and zuelaguidins A–D and F from *Z. guidonia*,² caseanigrescens A–D from *C. nigrescens*,³ argutins A–H from *C. arguta*,⁴ caseamembrins A–U from *C. membranacea*,^{5–9} caseabalansin A–G and balanspene A–H from *C. balansae*,^{10,11} and caseagrewiifolins A and B from *C. grewiifolia*.¹² In these compounds, the tricyclic system normally is formed from a cis-fused decalin with a disubstituted tetrahydrofuran connected at C-4–C-5. Further characteristic features are two trans-configured methyls at C-8 and C-9 and a 3-methylenepent-4-en-1-yl side chain at C-9. In general, the individual compounds differ in the substituents and stereochemistries at C-2, C-6, and C-7. Although the relative configurations of the eight stereocenters in isolated compounds were usually determined by 2D-NMR experiments, such as NOE, the absolute configurations were not determined in most cases. In many reported studies, isozuelanin-type clerodanes have exhibited potent cytotoxicity.

The genus *Laetia* belongs to the family Salicaceae, but only limited phytochemical studies have been reported so far on plants from this genus.^{13–15} Previous studies of *L. corymbulosa* reported the isolation of eight isozuelanin-type clerodanes, corymbulosins A–H.^{16,17} In our continuing phytochemical investigation of rainforest plants, 15 new corymbulosins I–W (1–15) and four known 2-esterified tricyclic clerodane diterpenes were isolated from *L. corymbulosa* collected in Peru. Herein, the determination of the absolute configurations of all isolated diterpenes and their biological activities is described.

RESULTS AND DISCUSSION

The MeOH/CH₂Cl₂ (1:1) extract of *L. corymbulosa* (N005829) was provided by the U.S. National Cancer Institute (NCI, Frederick, MD, USA). The EtOAc soluble part was separated through a combination of column chromatography, preparative HPLC, and preparative TLC using silica gel and octadecylsilane (ODS).

Compound 1 (Figure 1) was obtained as an optically active colorless amorphous solid: $[\alpha]_D^{25} + 2.3$ (c 0.38, CHCl₃). The HRFABMS data revealed a molecular formula of C₂₈H₄₀O₈ from the ion at m/z 527.2631 [M + Na]⁺. The ¹H NMR spectrum (Table 1) contained signals attributable to two methyls [δ_H 0.93 (3H, d, J = 7.2 Hz, H-17), 0.92 (3H, s, H-20)], two

vinyl protons [δ_{H} 5.98 (1H, dd, J = 4.2, 1.8 Hz, H-3), 6.44 (1H, dd, J = 17.4, 10.2 Hz, H-14)], four methylenes protons [δ_{H} 5.17 (1H, d, J = 17.4 Hz, H-15a), 5.03 (1H, d, J = 10.2 Hz, H-15b), 5.06 and 4.94 (each, 1H, s, H₂-16)], two acetoxy methyls [δ_{H} 2.08 (3H, s), 1.90 (3H, s)] at C-18 and C-19, two acetal-acyloxy methine protons [δ_{H} 6.75 (1H, t, J = 1.8 Hz, H-18), 6.47 (1H, s, H-19)], and a methine proton [δ_{H} 2.33 (1H, dd, J = 12.0, 5.4 Hz, H-10)], which suggested the presence of an 18,19-di-*O*-acetyl-18,19-epoxycyclohexa-3,13-(16),14-triene, the parent skeleton of corymbulosins A–H isolated from the same plant.^{16,17} Further observation of the NMR spectra (Tables 1 and 2) revealed an oxymethine [δ_{H} 3.80 (1H, m)/ δ_{C} 73.0, C-6], an aliphatic moiety [δ_{H} 2.63 (1H, sep, J = 7.2 Hz), 1.22 and 1.20 (each, 3H, d, J = 7.2 Hz)/ δ_{C} 34.7, 19.2 and 18.7], and an additional carbonyl carbon (δ_{C} 176.4, C-1') in the structure of **1**. COSY and HMBC experiments (Figure 2) supported the presence of hydroxy and isobutanoyloxy groups at C-6 and C-2, respectively, on the parent skeleton of **1**. The observation of NOESY correlations between H-1/H-6, H-6/H-8, H-7/H-19, H-10/H-12, and H-11/H-19 as well as a ROESY correlation for H-18/H-19 suggested the relative configuration as shown in Figure 3. The absolute configuration was established by the modified Mosher ester method. The hydroxy group at the chiral C-6 was esterified using (*R*)- and (*S*)- α -methoxy- α -trifluoromethylphenylacetyl chloride (MTPA-Cl) to generate the related esters. The distribution of the positive and negative δ_{H} (*S*–*R*) values of the MTPA esters indicated an *S*-configuration of the C-6 chiral center (Figure 4). The experimental electronic circular dichroism (ECD) spectra are shown in Figure 5. Therefore, compound **1**,¹⁸ given the trivial name corymbulosin I, was assigned as (2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,–18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-6-hydroxy-2-isobutanoyloxycyclohexa-3,13(16),14-triene.

Compound **2** has the identical molecular formula, C₂₈H₄₀O₈, as **1**, based on the peak at m/z 527.2631 [M + Na]⁺ in the HRFABMS. The ¹H and ¹³C NMR spectra of **2** were very similar to those of **1**, except for H-1 at δ_{H} 2.15/1.71 (each 1H, m), H-2 at δ_{H} 5.59 (1H, m), and C-2 at δ_{C} 70.4. The NOESY correlation between H-2 and H-10 (Figure 3) indicated that compound **2** was the C-2 epimer of **1**. The ECD spectrum (Figure 5) of **2** also exhibited a different Cotton effect from that of **1** and a similar effect to that of **18**, in which H-2 has α -orientation. Therefore, compound **2** (corymbulosin J) was assigned as (2*S*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,–19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-6-hydroxy-2-isobutanoyloxycyclohexa-3,13(16),14-triene.

Compound **3** was obtained as an optically active colorless oil: $[\alpha]_{\text{D}}^{25} + 10.4$ (c 0.23, CHCl₃). The HRFABMS data showed a molecular formula of C₂₉H₄₂O₈ from the peak at m/z 541.2777 [M + Na]⁺. The ¹H and ¹³C NMR spectra of **3** were similar to those of **1** (Tables 1 and 2), but the differences in chemical shifts for H/C-6 and H/C-7 between **3** and **1**, as well as additional peaks at δ_{H} 3.30 (3H, s) and δ_{C} 57.5 in **3**, indicated the presence of a methoxy group at C-6. These results implied that compound **3** is a methoxy analogue of **1**, which was verified from COSY and HMBC experiments (Figures S94 and S95). The NOESY correlations of **3** were similar to those of **1**, with an additional correlation found between H-18 and the C-6 methoxy protons in **3** (Figure 3). Comparison of all NMR data and the optical rotations of **3** and **1** showed that **3** and **1** have the same absolute configuration, which was confirmed from ECD spectra as shown in Figure 5. On the basis of the above findings,

compound **3** (corymbulosin K) was determined as (2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-6-methoxy-2-isobutanoyloxycleroda-3,13(16),14-triene.

Compound **4**, optically active colorless oil with $[\alpha]_{\text{D}}^{25} + 12.3$ (*c* 0.05, CHCl₃), gave a sodiated peak $[M + Na]^+$ at *m/z* 527.2608 in the HRFABMS, in agreement with a molecular formula of C₂₈H₄₀O₈. The ¹H and ¹³C NMR spectra (Tables 1 and 2) indicated that compound **4** is an analog of **3** with a different ester group at C-2. The combination of a three-proton triplet (*J* = 7.2 Hz) at δ_{H} 1.19 and a two-proton quartet (*J* = 7.2 Hz) at δ_{H} 2.41 suggested the presence of a propionyl group at C-2 in **4** rather than the isobutanoyl ester found in **3**. The final structure assignment and relative configuration were confirmed by various 2D NMR analyses of **4** (Figures S94 and S95). The absolute configuration was determined from an ECD spectrum (Figure 5), and optical rotation is the same as that of **3**. Thus, compound **4** (corymbulosin L) was assigned as (2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-6-methoxy-2-propionyloxycleroda-3,13(16),14-triene.

Compound **5** was obtained as an optically active colorless oil: $[\alpha]_{\text{D}}^{25} + 28.4$ (*c* 0.02, CHCl₃). The HRFABMS data supported a molecular formula of C₂₇H₃₈O₈ from the peak at *m/z* 513.2479 $[M + Na]^+$, which indicated the loss of a methylene unit from **4**. The ¹³C and ¹H NMR spectra of **5** (Tables 1 and 2) were mostly identical to those of **4**, except for the appearance of signals for an acetyl group at δ_{H} 2.14 (3H, s), δ_{C} 170.7 (C=O) and 21.5 (CH₃), and disappearance of signals for the ethyl group at δ_{H} 1.91 (t, 3H)/ δ_{C} 9.3 and 2.41 (q, 2H)/ δ_{C} 28.0 found in the spectra of **4**. The 2D NMR (Figures S94 and S95), optical resolution, and ECD analyses (Figure 5) suggested that all chiral centers in **5** have the identical stereochemistry found in **1**, **3**, and **4**. Therefore, compound **5** (corymbulosin M) was assigned as (2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-2,18,19-tri-*O*-acetyl-18,19-epoxy-6-methoxycleroda-3,13(16),14-triene.

Compound **6** has the molecular formula C₃₀H₄₄O₈ based on the peak at *m/z* 555.2947 $[M + Na]^+$ in the HRFABMS. The ¹H and ¹³C NMR data of **6** were mostly identical with the reported data of 18,19-di-*O*-acetyl-18,19-epoxy-6-methoxy-2-(2'-methylbutanoyloxy)-cleroda-3,13(16),14-triene with the *s-cis* form of the C-9 side chain.¹⁵ However, the experimental optical rotation, $[\alpha]_{\text{D}}^{25} + 8.4$ (*c* 0.20, CHCl₃), was different from the reported value, $[\alpha]_{\text{D}} - 84$ (*c* 0.11, CHCl₃). After a thorough examination of all spectroscopic data including NOESY and ECD experiments (Figures S94, S95, and 5), we concluded that compound **6** (corymbulosin N) is (2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-6-methoxy-2-(2'-methylbutanoyloxy)-cleroda-3,13(16),14-triene with the *s-trans* form of the C-9 side chain. The absolute configuration of the isobutyl side chain was not determined, since no differences were calculated in the ECD spectra of the 2' *R* and 2' *S* isomers.

Compound **7** was isolated as an optically active colorless oil $[\alpha]_{\text{D}}^{25} - 64$ (*c* 0.07, MeOH).

HRFABMS analysis of **7** afforded a molecular ion at *m/z* 555.2921 $[M + Na]^+$ in agreement with a molecular formula of C₃₀H₄₄O₈, which was identical to that of **6**. The ¹H and ¹³C NMR spectra of **7** were similar to those of **6** (Tables 1 and 2) and identical to those of

casearlucin E.¹⁹ The β -orientation of the ester group at C-2 was supported by a 2D NOESY correlation between H-2 and H-10 in **7** (Figures S95), which indicated that **7** was the C-2 epimer of **6**. The ECD spectrum of **7** exhibited a different Cotton effect from that of **6** but similar to that of **18** (Figure 5). These observations suggested that the stereochemistry of **7** was same as that of **18**; accordingly, it was an enantiomer of casearlucin E.¹⁹ This was also supported by the different sign of the optical resolution from that of casearlucin E.¹⁹ Thus, compound **7** (corymbulosin O) was assigned as (2*S*,5*S*,6*S*,–8*R*,9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-6-methoxy-2-(2'-methylbutanoyloxy)-cleroda-3,13(16),14-triene.

On the basis of a sodiated molecular-related ion at m/z 625.3340 $[M + Na]^+$ in the HRFABMS, compound **8** has a molecular formula of $C_{34}H_{50}O_9$. The 1H and ^{13}C NMR spectra of **8** were comparable with those of **5**, except for the loss of a methoxy group at C-6 and the presence of an octanoyloxy group. Seven sp^3 carbons were present between δ_c 14.1 and δ_c 34.7, and a carbonyl carbon was found at δ_c 173.2. The 1H NMR of **8** showed 15 aliphatic protons between δ_H 4.95 and δ_H 2.31, and the oxygenated methine at C-6 was downfield shifted (δ_H 4.95, 1H, dd, $J = 11.3, 4.6$ Hz) with respect to that of **5** (δ_H 3.27, 1H, dd, $J = 12.0, 4.2$ Hz). These results strongly suggested the location of the octanoyloxy group at C-6. The absolute configuration was determined by analysis of NOESY (Figure 3) and ECD spectra (Figure 5). Thus, compound **8** (corymbulosin P) was determined as (2*R*,5*S*,6*S*, 8*R*,9*R*,10*S*,18*R*,19*S*)-2,18,19-tri-*O*-acetyl-18,19-epoxy-6-octanoyloxycleroda-cleroda-3,13(16),14-triene.

Compound **9** was obtained as an optically active colorless Oil $[\alpha]_D^{25} + 19.6$ (c 0.15, $CHCl_3$). The HRFABMS data showed a molecular formula of $C_{36}H_{54}O_9$ from the peak at m/z 653.3651 $[M + Na]^+$, which indicated two additional methylene units compared with **8**. The 1H and ^{13}C NMR spectrum of **9** (Tables 1 and 2) closely resembled those of **8**, except for the additional four protons at δ_H 1.25 (m) and two carbons at δ_c 29.19 and 29.12. Together with HMBC and COSY analyses (Figures S94), compound **9** has a decanoyloxy substituent at C-6 instead of the octanoyloxy unit in **8**. The absolute configuration of **9** was defined by comparison of various spectroscopic data, including optical rotation, NMR spectra, and calculated and experimental ECD spectra (Figure 5). Finally, compound **9** (corymbulosin Q) was assigned as (2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-2,18,19-tri-*O*-acetyl-18,19-epoxy-6-decanoyloxycleroda-cleroda-3,13(16),14-triene.

Compound **10** has the molecular formula $C_{27}H_{38}O_7$ based on the sodiated molecular-related ion at m/z 497.2511 $[M + Na]^+$ in the HRFABMS. The 1H and ^{13}C NMR spectra of **10** (Tables 1 and 2) were similar to those of **4**. Important differences included the absence of signals for an oxygenated methine and a methoxy at C-6. Instead, an unsubstituted methylene group was suggested by the presence of a multiplet centered at δ_H 1.50/1.90 and a signal at δ_c 29.4. An unsubstituted methylene group at C-6 was further supported by the HMBC correlations between H-19 and H-6, as well as the COSY correlations between H-6 and H-7 (Figure S94). The relative configuration of **10** was determined from a NOESY spectrum (Figure S95). The optical rotation, $[\alpha]_D^{25} - 27.1$ (c 0.08, $CHCl_3$), of **10** was similar to that of **13**. The absolute configuration of the latter compound was confirmed by a modified Mosher ester method as described later. On the basis of the above data together with the

ECD spectrum (Figure 5), compound **10** (corymbulosin R) was defined as (2*R*,5*S*, 8*R*,9*R*, 10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-2-propionyloxycleroda-3,13(16),14-triene.

Compound **11** was isolated as an optically active colorless oil, $[\alpha]_D^{25} -10.0$ (*c* 0.45, CHCl₃). The HRFABMS data indicated a molecular formula of C₂₈H₄₀O₇ from the peak at *m/z* 511.2681 [M + Na]⁺. The ¹H and ¹³C NMR spectra of **11** (Tables 1 and 2) were very similar to those of **1**, except for the presence of signals for an unsubstituted methylene group rather than an oxygenated methine C-6, as also seen with the NMR comparison of compounds **10** and **4**. The final structure allocation and relative configuration were determined by 2D NMR analyses of **11** (Figures S94 and S95). On the basis of an ECD spectrum (Figure 5) and optical rotation, compounds **11** and **10** have the same absolute configuration. Therefore, compound **11** (corymbulosin S) was concluded to be (2*R*,5*S*,8*R*, 9*R*,10*S*,18*R*,19*S*)-18,19-tri-*O*-acetyl-18,19-epoxy-2-isobutanoyloxycleroda-3,13(16),14-triene.

Compound **12** was obtained as an optically active colorless oil, $[\alpha]_D^{25} +0.7$ (*c* 0.35, CHCl₃). HRFABMS analysis of **12** afforded a sodiated molecular ion at *m/z* 539.2960 [M + Na]⁺, which established a molecular formula of C₃₀H₄₄O₇. The ¹H and ¹³C NMR spectra of **12** (Tables 1 and 2) were closely consistent to those of **10** but suggested the presence of a hexanoyl group at C-2 rather than the propionyl in **10**. This assignment was also supported by COSY and HMBC experiments (Figure S94). The absolute configuration of **12** was clarified from its ECD spectrum (Figure 5), which showed a similar shape to that of **11**. On the basis of all spectroscopic data, compound **12** (corymbulosin T) was thus characterized as (2*R*,5*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-2-hexanoyloxycleroda-3,13(16),14-triene.

Compound **13**, optically active colorless oil with $[\alpha]_D^{25} -28.7$ (*c* 0.07, CHCl₃), displayed a peak at *m/z* 441.2218 [M + Na]⁺ in the HRFABMS analysis, showing good agreement with a molecular formula of C₂₄H₃₄O₆. The ¹H and ¹³C NMR spectra of **13** (Tables 1 and 2) also closely resembled those of **10**. The lack of propionyl signals implied the presence of a hydroxy group at C-2, which was confirmed by the COSY correlations of H-2 to H-1 and H-3 (Figure S94). Applying the modified Mosher ester method clarified the absolute configuration at the C-2 position as *R* (Figure 4). Therefore, compound **13** (corymbulosin U) was established as (2*R*,5*S*,8*R*,9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-2-hydroxycleroda-3,13(16),14-triene.

HRFABMS of compound **14** showed a molecular formula C₃₀H₄₄O₈ with a sodiated molecular ion at *m/z* 555.2925 [M + Na]⁺. The ¹H and ¹³C NMR spectra of **14** (Tables 1 and 2) were mostly identical to those of corymbulosin D¹⁷ except for the absence of two methylene groups in the ester at C-6, which was confirmed by HMBC and COSY data (Figure S94). The absolute configuration of **14** was determined by comparisons of 2D NMR data (Figures S94 and S95) and experimental ECD spectra (Figure 5). Accordingly, this compound (corymbulosin V) was defined as (2*R*,5*S*,6*S*,8*R*, -9*R*,10*S*,18*R*,19*S*)-18,19-di-*O*-acetyl-18,19-epoxy-2-hydroxy-6-hexanoyloxycleroda-3,13(16),14-triene.

Compound **15** was obtained as an optically active colorless oil, $[\alpha]_{\text{D}}^{25} - 62.4$ (c 0.075, CHCl_3). The HRFABMS data supported a molecular formula of $\text{C}_{32}\text{H}_{46}\text{O}_8$ from the peak at m/z 581.3075 $[\text{M} + \text{Na}]^+$. 1D NMR spectra and MS data indicated the loss of two methylene units from the structure of a 2-oxo-clerodane, corymbulosin H,¹⁷ suggesting the presence of an octanoyl substituent at C-6 rather than the decanoyl ester in corymbulosin H. The HMBC and COSY spectra (Figure S94) also supported this conclusion. The NOESY (Figure S95) and ECD experiments (Figure 4) suggested that all stereochemical centers in **15** were identical with those in corymbulosin H. Thus, compound **15** (corymbulosin W) was assigned as (5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,-19*S*)-18,19-di-*O*-acetyl-6-octanoyloxy-18,19-epoxycleroda-3,13-(16),14-trien-2-one.

The structures of the four known compounds were identified as caseamembrin S (**16**),⁹ 18,19-di-*O*-acetyl-18,19-epoxy-6-hydroxy-2-(2'-methylbutanoyloxy)cleroda-3,13-(16),14-triene (**17**),¹⁵ caseamembrin E (**18**),⁵ and corymbulosin A (**19**)¹⁶ from comparison of their spectroscopic data with reported values. However, the absolute configurations of **16–19** and even the relative configuration of **19** were not determined.

Compound **16** was characterized as caseamembrin S with identical HRMS and optical rotation data to the reported values;⁹ however, the ^1H and ^{13}C NMR assignments for H-18, H-19, H-3', C-7, C-8, C-9, and C-10 deviated slightly from the literature values.⁹ Careful investigations using COSY, HMBC, HMQC, as well as DEPT methods supported the structure of **16** as caseamembrin S. The revised NMR data are shown in the Supporting Information (Table S1). The absolute configurations (2*R*, 5*S*, 6*S*, 8*R*, 9*R*, 10*S*, 18*R*, 19*S*) in **16** were established convincingly by ECD (Figure 5) and the comparison of optical rotation and NMR data with the other compounds, including **6**.

Compound **17** was assigned as the reported compound 18,19-di-*O*-acetyl-18,19-epoxy-6-hydroxy-2-(2'-methyl-butanoxy)cleroda-3,13(16),14-triene. The absolute configuration of **17** was first determined by the modified Mosher method and X-ray crystal structural analysis. After MTPA esterification of the C-6 alcohol, the distribution of calculated δ_{H} (*S* – *R*) values indicated the *S*-form at C-6 (Figure 4); therefore, the absolute configuration of **17** was established as 2*R*, 5*S*, 6*S*, 8*R*, 9*R*, 10*S*, 18*R*, and 19*S*. The *S* configuration of the C-2' stereocenter was further determined by X-ray crystal structural analysis as shown in Figure 6 (CCDC 1529690, Figure S94).²⁰

Similarly, compound **18** was identified as caseamembrin E from the agreement of optical rotation and various spectroscopic data with the reported values, except for revised ^1H NMR assignments as shown in the Supporting Information. Its absolute configuration was assigned as 2*S*, 5*S*, 6*S*, 8*R*, 9*R*, 10*S*, 18*R*, and 19*S* from the ECD experiment.

The structure elucidation of **19** was reported previously,¹⁶ however, no stereochemistry, even relative configuration, was determined. The HRMS, $^1\text{H}/^{13}\text{C}$ NMR spectra, and optical activity matched well with the reported values. In the 2D NOESY experiment (Figure 7), correlations between H-2/H-10, H-6/H-8, H-10/H-12, H-11/H-19, H-7/H-19, and H-18/H-19 were observed. From the comparison of the ECD spectrum (Figure 5) and optical rotation

values with those of **2** and **18**, the absolute configuration of **19** was concluded as 2*S*, 5*S*, 6*S*, 8*R*, 9*R*, 10*S*, 18*R*, and 19*S*.

The antiproliferative activities of the isolated compounds were evaluated using five human tumor cell lines, A549 (lung carcinoma), MCF-7 (estrogen receptor-positive and HER2-negative breast cancer), MDA-MB-231 (triple negative breast cancer), KB (originally isolated from epidermoid carcinoma of the nasopharynx), and KB-VIN (a KB-subline showing MDR phenotype with overexpression of P-gp) (Table 3). Except for compound **3** with a 2-isobutanoyloxy ester, the compounds with a 6-methoxy group generally exhibited lower antiproliferative activity than compounds with a 6-hydroxy group [compare **4** (6-OMe) vs **16** (6-OH) and **6** (6-OMe) vs **17** (6-OH)]. The stereochemistry at C-2 seemed to affect the activity against A549 and KB-VIN (**17** vs **18**). The related compounds isolated and evaluated in our prior study¹⁷ generally exhibited similar potencies to **4** and **6** in the present study. The substitution patterns in the prior corymbulosins¹⁷ included a C-2 hydroxy group and various C-6 ester moieties, such as octanoyl-, decanoyl-, and dodecanoyl-oxy. On the basis of the present data, the combination of C-2 ester and C-6 hydroxy groups, such as in **1**, **16**, and **17**, led to more potent antiproliferative activity against human tumor cell lines than the combination of C-2 hydroxy and C-6 ester groups.

The effects of the potent compounds **1**, **3**, **16**, **17** and **19** on cell cycle progression in the triple-negative breast cancer (TNBC) cell line MDA-MB-231 were further investigated by flow cytometry (Figure 8). All compounds showed no effect on cell cycle progression, while significant accumulations of sub-G1 cells were observed in a dose-dependent manner, except with compound **19**. When cells were treated with higher concentrations of compound **1**, **16**, or **17**, propidium iodide (PI) signals decreased unusually and dramatically in all cells. These observations suggested that the test compounds efficiently induced either cytolysis or apoptosis. Occasionally, the cytolytic activity of cytotoxic compounds can induce hemolysis in vivo. Accordingly, we tested the hemolytic activity of selected compounds using horse's blood (Table 4). Among them, compound **19** with a long aliphatic chain at C-2 showed the most potent activity but only at the unusually high concentration of 500 μ M, suggesting that all tested compounds have no hemolytic activity.

The effects of compounds on cell morphology by using immunocytochemistry were next evaluated. MDA-MB-231 cells were treated with compounds for 24 h followed by staining with antibody to α -tubulin for microtubules and 4',6-diamidino-2-phenylindole (DAPI) for chromatin (Figure 9). Cells treated with compound **3** at a lower concentration (1.5 μ M) showed normal morphology with intact nuclei and a clear microtubule network, as also found in the control cells. However, most cells treated with compound **3** at 4.9 μ M exhibited fragmented and dispersed chromatin (arrowhead) with an apoptotic microtubule array (arrow)²¹ (Figure 9A). These observations implied that compound **3** induced apoptosis in a dose-dependent manner. Similar phenotypes were observed in cells treated with compounds **1**, **16**, and **17**. These data were quite consistent with the results from flow cytometric analysis. In conclusion, newly isolated corymbulosins efficiently induce chromatin fragmentation with formation of apoptotic microtubules in TNBC MDA-MB-231 cells.

CONCLUSIONS

Fifteen new clerodane diterpenes, designated corymbulosins I–W (**1–15**), together with four known diterpenes, **16–19**, were isolated from a crude MeOH/CH₂Cl₂ (1:1) extract (N005829) of the bark of *L. corymbulosa*. The structures of **1–15** were elucidated on the basis of extensive 1D and 2D NMR and HRMS analyses. The absolute configurations of newly isolated compounds **1–15** as well as known **16–19**, which were reported previously with only relative configurations, were determined through ECD experiments, X-ray analysis, modified Mosher ester method, and comparison of their spectroscopic data. The isolated compounds were evaluated for antiproliferative activity against human cancer cell lines. Among all, compounds **1**, **3**, **16**, **18**, and **19** showed potent activity against all tested human tumor cell lines, including MDR subline, with IC₅₀ values of 0.4–1.0 μ M. A structure–activity relationship study revealed that the combination of C-2 ester and C-6 hydroxy groups led to potent antiproliferative activity. Flow cytometric and immunocytochemical observations of cells treated with cytotoxic clerodanes demonstrated that the chromatin was fragmented and dispersed with formation of apoptotic microtubules.

EXPERIMENTAL SECTION

General Experimental Procedures.

Optical rotations were measured on a JASCO P-2200 digital polarimeter. CD spectra were recorded on JASCO J-820 spectrometer. Infrared spectra (IR) were obtained with a Thermo Fisher Scientific NICOLET iS5 FT-TR spectrometer from samples in CH₂Cl₂. NMR spectra were measured on JEOL JMN-ECA600 and JMN-ECS400 spectrometers with tetramethylsilane as an internal standard, and chemical shifts are stated as δ values. HRMS data were recorded on a JMS-700 MStation quadrupole (FAB) or JMS-T100TD TOF (DART) mass spectrometer. A crystal of the compound was measured on a R-Axis RAPID II (Rigaku). Analytical and preparative TLC were carried out on precoated silica gel 60F₂₅₄ and RP-18F₂₅₄ plates (0.25 or 0.50 mm thickness; Merck). MPLC was performed on Combiflash Rf (Teledyne Isco) with silica gel and C18 cartridges (Biotage, Uppsala Sweden). Preparative HPLC was operated on a GL Science recycling system using an InertSustain C18 column (5 μ m, 20 $\times$ 250 mm).

Plant Material.

The crude MeOH/CH₂Cl₂ (1:1) extract (N005829) of bark of *L. corymbulosa* collected in Peru was provided by NCI/NIH (Frederick, MD, U.S). A voucher specimen (voucher no. QT65T0390) was deposited at the Smithsonian Institution (Washington, DC, and voucher extracts were deposited at the NCI (Frederick, MD) and Kanazawa University (Kanazawa, Japan). The crude organic extract of N005829 was evaluated for cytotoxicity by NCI with an in vitro 60-cell tumor screening panel as reported previously.^{17,22}

Extraction and Isolation.

The crude extract N005829 (12.8 g) was partitioned between H₂O and EtOAc to obtain H₂O-soluble (2.2 g) and EtOAc-soluble portions (7.4 g). The latter portion was subjected to silica gel MPLC (RediSep Rf GOLD High Performance 120 g) with a gradient system [*n*-

hexane–EtOAc 90:10 (600 mL) → 80:20 (40 mL) → 75:25 (120 mL) → 65:35 (80 mL) → 60:40 (120 mL) → 55:45 (80 mL) → 45:55 (240 mL) → 30:70 (520 mL) → 10:90 (440 mL) → EtOAc–MeOH 90:10 (120 mL) → MeOH (1400 mL)] to yield 11 fractions, F1–F11.¹⁷

F5 (1249.9 mg) was further fractionated by silica gel column chromatography (CC) eluted with *n*-hexane/EtOAc (5:1 to 0:1) to afford 12 subfractions 5a–l. Subfraction 5g (384.9 mg) was purified by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O/ MeOH (1:6), followed by repeated recycling preparative HPLC with H₂O/MeOH (1:7) to yield compound **12** (2.0 mg). Subfraction 5h (279.1 mg) was purified by repeated recycling preparative HPLC with H₂O/MeOH (1:9) to provide compounds **10** (2.0 mg), **11** (9.0 mg), and **15** (1.5 mg). Subfraction 5i (98.0 mg) was purified by repeated recycling preparative HPLC with H₂O/MeOH (1:9) to afford compounds **7** (1.5 mg) and **9** (7.1 mg). Subfraction 5j (183.2 mg) was purified by repeated recycling preparative HPLC with H₂O/MeOH (1:9) to provide compounds **10** (2.0 mg), **11** (9.0 mg), and **15** (1.5 mg). Subfraction 5i (98.0 mg) was purified by repeated recycling preparative HPLC with H₂O/MeOH (1:9) followed by preparative TLC using *n*-hexane/EtOAc (4:1) to yield compound **8** (3.3 mg).

F6 (430.8 mg) was subjected to silica gel CC eluted with *n*-hexane/CH₂Cl₂ (1:1 to 0:1), CH₂Cl₂/EtOAc (19:1), EtOAc, followed by MeOH to yield eight subfractions 6a–h. Subfraction 6f (125.9 mg) was subjected to silica gel CC eluted with *n*-hexane/ CH₂Cl₂ (1:0 to 57:4) followed by MeOH to obtain seven subfractions, 6f1–7. Subfraction 6f6 (15.5 mg) was purified by ODS preparative TLC developing three times using H₂O/MeOH (1:4 × 2 and 1:6) to afford compound **6** (5.7 mg). Subfraction 6g (179.5 mg) was subjected to silica gel CC eluted with *n*-hexane/ EtOAc (1:0 to 0:1) to obtain six subfractions 6g1–6. Subfraction 6g3 (78.0 mg) was purified by repeated recycling preparative HPLC with H₂O/ MeOH (1:4) to provide compounds **6** (1.7 mg), **18** (16.9 mg), and **19** (8.0 mg, 0.00062%).

F7 (3.52 g) was subjected to silica gel CC eluted with CH₂Cl₂/EtOAc (6:1 to 0:1) followed by MeOH to yield 10 subfractions 7a– j. Subfraction 7d (155.9 mg) was subjected to silica gel CC eluted with CH₂Cl₂/EtOAc (1:0 to 4:1) followed by MeOH to obtain seven subfractions, 7d1–7. Subfraction 7d4 (60.0 mg) was purified by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O/ MeOH (1:4), followed by repeated recycling preparative HPLC with H₂O/MeOH (1:4) to provide compounds **3** (5.1 mg) and **4** (1.0 mg).

Subfraction 7e (603.7 mg) was subjected to silica gel CC eluted with *n*-hexane/CH₂Cl₂ (1:1 to 0:1) followed by CH₂Cl₂/EtOAc (2:1) to obtain 10 subfractions, 7e1-10. Subfraction 7e6 (385.9 mg) was purified by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O/ MeOH (3:7 to 1:6), followed by recycle preparative HPLC with H₂O/MeOH (1:6) to afford compounds **4** (0.7 mg) and **5** (0.4 mg).

Subfraction 7f (830.1 mg) was subjected to silica gel CC eluted with CH₂Cl₂/EtOAc (1:0 to 0:1) followed by MeOH to obtain six subfractions, 7f1–6. Subfraction 7f3 (107.0 mg) was purified by MPLC on ODS-25 (YMC-DispoPack AT 12 g) with H₂O/MeOH (1:4) followed by recycle preparative HPLC with H₂O/MeOH (1:4) to afford compounds **2** (1.2 mg) and **18**

(10.4 mg), which was combined with 18 (16.9 mg) obtained from F6 (total 27.3 mg, 0.00213%).

n-Hexane/EtOAc (3:1) was added to subfraction 7h (192.6 mg), and the resulting insoluble material (75.7 mg) was purified by repeated recycle preparative HPLC with H₂O/MeOH (1:4) to afford compounds 1 (16.9 mg), 7 (2.3 mg), and 17 (43.5 mg, 0.0033%).

F8 was subjected to silica gel CC eluted with CH₂Cl₂/EtOAc (1:0 to 0:1) followed by MeOH to give 14 subfractions, 8a–n. Compounds 13 (1.5 mg), and 14 (11.9 mg) were obtained by repeated recycling preparative HPLC of subfraction 8f (169.1 mg) with H₂O/MeOH (1:5).

Corymbulosin I (1).—16.9 mg, 0.0013%; colorless amorphous solid $[\alpha]_D^{25} + 2.3$ (c 0.38, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 3500, 2970, 2932, 2879, 1754, 1731, 1596, 1469, 1373; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 527.2631 [M + Na]⁺ (calcd for C₂₈H₄₀O₈Na, 527.2621).

Corymbulosin J (2).—1.2 mg, 0.00007%; colorless amorphous solid; $[\alpha]_D^{25} - 88.0$ (c 0.01, MeOH); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 3463, 2957, 2923, 2853, 1730, 1595, 1458, 1376; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 527.2624 [M + Na]⁺ (calcd for C₂₈H₄₀O₈Na, 527.2621).

Corymbulosin K (3).—5.1 mg, 0.00039%; colorless oil; $[\alpha]_D^{25} + 10.4$ (c 0.23, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2970, 2936, 2879, 1755, 1731, 1596, 1469, 1373, 1227; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 541.2777 [M + Na]⁺ (calcd for C₂₉H₄₂O₈Na, 541.2777).

Corymbulosin L (4).—1.0 mg, 0.00007%; colorless oil; $[\alpha]_D^{25} + 12.3$ (c 0.05, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2961, 2927, 2880, 1755, 1732, 1456, 1373, 1224; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 527.2608 [M + Na]⁺ (calcd for C₂₈H₄₀O₈Na, 527.2621).

Corymbulosin M (5).—0.4 mg, 0.00003%; colorless oil; $[\alpha]_D^{25} + 28.4$ (c 0.02, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2926, 1740, 1373, 1224; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 513.2479 [M + Na]⁺ (calcd for C₂₇H₃₈O₈Na, 513.2464).

Corymbulosin N (6).—7.4 mg, 0.00057%; colorless oil; $[\alpha]_D^{25} + 8.4$ (c 0.20, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2966, 2932, 1755, 1729, 1337, 1227; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 555.2947 [M + Na]⁺ (calcd for C₃₀H₄₄O₈Na, 555.2934).

Corymbulosin O (7).—3.8 mg, 0.00029%; colorless oil; $[\alpha]_D^{25} - 64.0$ (c 0.07, MeOH); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2955, 2926, 2881, 1756, 1730, 1373, 1226; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 555.2921 [M + Na]⁺ (calcd for C₃₀H₄₄O₈Na, 555.2934).

Corymbulosin P (8).—3.3 mg, 0.00025%; colorless oil; $[\alpha]_D^{25} + 0.9$ (c 0.20, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2964, 2928, 2860, 1751, 1733, 1373, 1224; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 625.3340 [M + Na]⁺ (calcd for C₃₄H₅₀O₉Na, 625.3353).

Corymbulosin Q (9).—7.1 mg, 0.00055%; colorless oil; $[\alpha]_D^{25} + 19.6$ (c 0.15, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2955, 2926, 2854, 1738 1373, 1220; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 653.3651 [M + Na]⁺ (calcd for C₃₆H₅₄O₉Na, 653.3666).

Corymbulosin R (10).—2.0 mg, 0.00015%, colorless oil; $[\alpha]_D^{25} - 27.1$ (c 0.08, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2958, 2926, 2855, 1755, 1734, 1374, 1226; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 497.2511 [M + Na]⁺ (calcd for C₂₇H₃₈O₇Na, 497.2515).
Corymbulosin S (11).

Corymbulosin R (11).—9.0 mg, 0.0007%; colorless oil; $[\alpha]_D^{25} - 10.0$ (c 0.45, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2967, 2941, 2870, 1749, 1730, 1373, 1226; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 511.2681 [M + Na]⁺ (calcd for C₂₈H₄₀O₇Na, 511.2672).

Corymbulosin T (12).—2.0 mg, 0.00015%; colorless oil; $[\alpha]_D^{25} + 0.7$ (c 0.35, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2961, 2935, 2870, 1757, 1733, 1374, 1224; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 539.2960 [M + Na]⁺ (calcd for C₃₀H₄₄O₇Na, 539.2985).

Corymbulosin U (13).—1.5 mg, 0.00011%; colorless oil; $[\alpha]_D^{25} - 28.7$ (c 0.07, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 3473, 2955, 2933, 1750, 1374, 1227; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 441.2218 [M + Na]⁺ (calcd for C₂₄H₃₄O₆Na, 441.2253).

Corymbulosin V (14).—11.9 mg, 0.00092%; colorless oil; $[\alpha]_D^{25} - 18.8$ (c 0.28, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2961, 2930, 1735, 1374, 1225; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 555.2925 [M + Na]⁺ (calcd for C₃₀H₄₄O₈Na, 555.2934).

Corymbulosin W (15).—1.5 mg, 0.00011%; colorless oil; $[\alpha]_D^{25} - 62.4$ (c 0.28, CHCl₃); IR $\nu_{\max}$ (CH₂Cl₂) cm⁻¹ 2961, 2927, 2855, 1761, 1374, 1220; ¹H and ¹³C NMR, Tables 1 and 2; HRFABMS *m/z* 581.3075 [M + Na]⁺ (calcd for C₃₂H₄₆O₈Na, 581.3090).

General Procedure for Esterification with (S/R)-MTPA-Cl.—To a solution of 1 (0.7 mg, 1.4 μmol) in anhydrous CH₂Cl₂ (0.25 mL) were added Et₃N (6.9 μL, 49.5 μmol), DMAP (3.8 mg, 31.1 μmol), and (S)-MTPACl (6.9 μL, 36.9 μmol). The mixture was stirred at rt for 3.5 h, followed by direct purification using preparative TLC with CH₂Cl₂ to afford the (R)-MTPA ester (0.9 mg, 90%). The corresponding (S)-MTPA ester (1.2 mg, 93%) was obtained by the same procedure using (R)-MTPACl.

(R)-MTPA ester of 1: 90% yield. ¹H NMR (CHCl₃, 600 MHz) δ_H 7.51–7.37 (5H, m, aromatic protons), 6.52 (1H, s, H-19), 6.43 (1H, dd, *J* = 18.0, 11.4 Hz, H-14), 5.86 (1H, brd, *J* = 3.6 Hz, H-3), 5.76 (1H, t, *J* = 1.8 Hz, H-18), 5.43 (1H, m, H-2), 5.17 (1H, d, *J* = 18.0 Hz,

H-15), 5.06 (1H, m overlap, H-6), 5.06 (1H, s, H-16), 5.03 (1H, d, $J = 11.4$ Hz, H-15), 4.95 (1H, s, H-16), 3.62 (3H, s, OMe), 2.61 (1H, sep, $J = 7.2$ Hz, H-2'), 2.37 (1H, dd, $J = 13.8$, 3.6 Hz, H-10), 2.08 (2H, m, H-12), 2.03 (1H, m, H-1 β), 1.94 (3H, s, 18-OAc), 1.85 (3H, s, 19-OAc), 1.75 (1H, q, $J = 12.6$ Hz, H-7 α), 1.20 (3H, d, $J = 7.2$ Hz, H-3'), 1.18 (3H, d, $J = 7.2$ Hz, H-3'), 0.96 (3H, d, $J = 7.2$ Hz, H-17), 0.95 (3H, s, H-20).

(*S*)-MTPA ester of 1: 93% yield. ^1H NMR (CHCl_3 , 600 MHz) δ_{H} 7.53–7.41 (5H, m, aromatic protons), 6.47 (1H, s, H-19), 6.46 (1H, dd, $J = 1.8$, 1.2 Hz, H-18), 6.43 (1H, dd, $J = 18.0$, 11.4 Hz, H-14), 5.98 (1H, brd, $J = 3.6$ Hz, H-3), 5.47 (1H, m, H-2), 5.16 (1H, d, $J = 18.0$ Hz, H-15), 5.09 (1H, dd, $J = 13.2$, 4.2 Hz, H-6), 5.05 (1H, s, H-16), 5.03 (1H, d, $J = 11.4$ Hz, H-15), 4.92 (1H, s, H-16), 3.46 (3H, s, OMe), 2.62 (1H, sep, $J = 7.2$ Hz, H-2'), 2.40 (1H, dd, $J = 13.8$, 3.0 Hz, H-10), 2.08 (2H, m, H-12), 2.03 (1H, m, H-1 β), 2.02 (3H, s, 18-OAc), 1.87 (3H, s, 19-OAc), 1.80 (1H, dt, $J = 13.2$, 4.2 Hz, H-7 β), 1.65 (1H, q, $J = 13.2$ Hz, H-7 α), 1.22 (3H, d, $J = 7.2$ Hz, H-3'), 1.19 (3H, d, $J = 7.2$ Hz, H-3'), 0.94 (3H, s, H-20), 0.93 (3H, d, $J = 6.6$ Hz, H-17).

(*R*)-MTPA ester of 17: 99% yield. ^1H NMR (CHCl_3 , 600 MHz) δ_{H} 7.51–7.38 (5H, m, aromatic protons), 6.52 (1H, s, H-19), 6.44 (1H, dd, $J = 17.4$, 10.8 Hz, H-14), 5.89 (1H, brd, $J = 3.6$ Hz, H-3), 5.76 (1H, dd, $J = 1.8$, 1.2 Hz, H-18), 5.43 (1H, m, H-2), 5.16 (1H, d, $J = 17.4$ Hz, H-15), 5.07 (1H, dd, $J = 13.2$, 4.2 Hz, H-6), 5.06 (1H, s, H-16), 5.03 (1H, d, $J = 10.8$ Hz, H-15), 4.95 (1H, s, H-16), 3.62 (3H, s, OMe), 2.44 (1H, m, H-2'), 2.39 (1H, dd, $J = 13.8$, 3.6 Hz, H-10), 2.08 (2H, m, H-12), 2.03 (1H, m, H-1), 1.93 (3H, s, 18-OAc), 1.84 (3H, s, 19-OAc), 1.75 (1H, q, $J = 13.2$ Hz, H-7 α), 1.16 (3H, d, $J = 7.2$ Hz, H-5'), 0.96 (3H, d, $J = 6.6$ Hz, H-17), 0.95 (3H, s, H-20), 0.94 (3H, t, $J = 7.2$ Hz, H-4').

(*R*)-MTPA ester of 13: 40% yield. ^1H NMR (CHCl_3 , 600 MHz) δ_{H} 7.56–7.44 (5H, m, aromatic protons), 6.72 (1H, t, $J = 1.7$ Hz, H-18), 6.36 (1H, dd, $J = 17.2$, 10.6 Hz, H-14), 6.30 (1H, s, H-19), 6.02 (1H, brd, $J = 4.5$ Hz, H-3), 5.64 (1H, m, H-2), 5.09 (1H, d, $J = 17.2$ Hz, H-15), 4.99 (1H, s, H-16), 4.97 (1H, d, $J = 10.6$ Hz, H-15), 4.88 (1H, s, H-16), 2.33 (1H, dd, $J = 14.0$, 2.6 Hz, H-10), 2.05 (3H, s, 18-OAc), 2.00 (2H, m, H-12), 1.75 (1H, m, H-6), 1.73 (3H, s, 19-OAc), 1.46 (1H, m, H-6), 0.91 (3H, s, H-20), 0.88 (3H, d, $J = 6.7$ Hz, H-17).

(*S*)-MTPA ester of 13: 28% yield. ^1H NMR (CHCl_3 , 600 MHz) δ_{H} 7.51–7.37 (5H, m, aromatic protons), 6.69 (1H, t, $J = 1.7$ Hz, H-18), 6.39 (1H, dd, $J = 17.5$, 10.8 Hz, H-14), 6.28 (1H, s, H-19), 6.02 (1H, brd, $J = 3.7$ Hz, H-3), 5.61 (1H, m, H-2), 5.08 (1H, d, $J = 17.5$ Hz, H-15), 5.01 (1H, s, H-16), 4.98 (1H, d, $J = 10.8$ Hz, H-15), 4.90 (1H, s, H-16), 2.31 (1H, d, $J = 12.0$ Hz, H-10), 2.04 (3H, s, 18-OAc), 2.02 (2H, m, H-12), 1.78 (3H, s, 19-OAc), 1.74 (1H, m, H-6), 1.47 (1H, m, H-6), 0.94 (3H, s, H-20), 0.89 (3H, d, $J = 6.7$ Hz, H-17).

(*S*)-MTPA ester of 17: 71% yield. ^1H NMR (CHCl_3 , 600 MHz) δ_{H} 7.53–7.42 (5H, m, aromatic protons), 6.47 (1H, s, H-19), 6.46 (1H, t, $J = 1.2$ Hz, H-18), 6.43 (1H, dd, $J = 17.4$, 10.8 Hz, H-14), 6.00 (1H, brd, $J = 4.2$ Hz, H-3), 5.46 (1H, m, H-2), 5.16 (1H, d, $J = 17.4$ Hz, H-15), 5.10 (1H, dd, $J = 12.0$, 4.2 Hz, H-6), 5.05 (1H, s, H-16), 5.03 (1H, d, $J = 10.8$ Hz, H-15), 4.93 (1H, s, H-16), 3.46 (3H, s, OMe), 2.46 (1H, m, H-2'), 2.42 (1H, dd, $J = 13.2$, 4.2 Hz, H-10), 2.08 (2H, m, H-12), 2.01 (3H, s, 18-OAc), 1.91 (1H, m, H-8), 1.86 (3H, s, 19-

OAc), 1.81 (1H, dt, $J = 13.2, 4.2$ Hz, H-7 β), 1.18 (3H, d, $J = 6.6$ Hz, H-5'), 0.96 (3H, t, $J = 7.2$ Hz, H-4'), 0.94 (3H, s, H-20), 0.93 (3H, d, $J = 6.6$ Hz, H-17).

Assay for Antiproliferative Activity.

Antiproliferative activity of the compounds was determined by the sulforhodamine B (SRB) assay as described previously.^{22,23}

Cell Cycle Analysis.

Cell cycle distribution was analyzed by measurement of cellular DNA content by staining with propidium iodide (PI)/RNase (BD Biosciences) as described previously.²⁴ Stained cells were analyzed by flow cytometry (BD LSRFortessa).

In Vitro Hemolytic Assay.

The assay was performed using the method reported by Vo et al.²⁵ with some modifications. Preserved horse's blood (defibrinated) was purchased from Cosmo Bio (Japan). After the blood was centrifuged for 5 min at 232g, supernatant was discarded. Hemocytes-enriched pellets were washed three times with PBS and suspended in saline at 0.5% (v/v) concentration. A 250 μ L amount of the hemocytes was mixed with a 250 μ L of test compound. After 30 min incubation at 37 °C, supernatant was recovered from the mixture by centrifugation for 10 min at 232g. The absorbance of the resultant supernatant was measured at 570 nm using a microplate reader (SPARK 10M, Tecan, Switzerland). Saponin Quillaja sp. (S4521, SIGMA) was used as a positive control, for which PBS containing 50%, 20%, or 4% DMSO was used as a negative control. The percentage of hemolysis induced by compound was calculated as (absorbance of test sample – absorbance of negative control) / (absorbance of positive control – absorbance of negative control) $\times$ 100%. Experiments were conducted three times for each compound at each concentration.

Immunocytochemistry.

MDA-MB-231 cells were seeded in the 8-well chamber slide (Lab-Tech) 24 h prior to treatment with compounds. After 24 h treatment, cells were fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.5% Triton-X100. Cells were then probed with antibody to α -tubulin (B5-1-2, Sigma) followed by FITC-conjugated antibody to mouse IgG (Sigma). Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). Microtubules were detected using a confocal microscope (Zeiss, LSM700) controlled by ZEN software (Zeiss). Final images were reconstructed from 12–18 sections acquired at 0.6–1 μ m intervals and merged using ZEN (black edition) software. Experiments were repeated at least twice for each compound at each concentration. Final images were prepared using Adobe Photoshop CS6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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	R	R'		R	R'
Corymbulosin I (1)		OH	Corymbulosin S (11)		H
Corymbulosin J (2)		OH	Corymbulosin T (12)		H
Corymbulosin K (3)		OMe	Corymbulosin U (13)	HO	H
Corymbulosin L (4)		OMe	Corymbulosin V (14)	HO	OC(O)(CH ₂) ₄ CH ₃
Corymbulosin M (5)		OMe	Corymbulosin W (15)	O=	OC(O)(CH ₂) ₆ CH ₃
Corymbulosin N (6)		OMe			
Corymbulosin O (7)		OMe	Caseamembrin S (16)		OH
Corymbulosin P (8)		OC(O)(CH ₂) ₆ CH ₃	17		OH
Corymbulosin Q (9)		OC(O)(CH ₂) ₈ CH ₃	Caseamembrin E (18)		OH
Corymbulosin R (10)		H	Corymbulosin A (19)		OH

Figure 1.
Structures of isolated diterpenes from *L. corymbulosa*.

Figure 2.
Selected HMBC correlations (arrows in red); COSY connectivities (bold lines) for 1.

Figure 3.
Key NOESY (red) and ROESY (blue) correlations for 1– 3 and 8.

Figure 4.

δ_{H} (*S*–*R*) values (ppm) calculated from *O*-MTPA esters of compounds **1**, **13**, and **17**.

Figure 5.
Experimental ECD spectra of compounds 1–19 in acetonitrile.

Figure 6.
ORTEP of compound **17** [ellipsoid contour probability 50%].

Figure 7.
Key NOESY correlations for **19**.

Figure 8.

Effects of selected clerodane diterpenes, **1**, **3**, **16**, **17**, and **19** on cell cycle progression in MDA-MB-231 cells. MDA-MB-231 cells were treated with **1**, **3**, **16**, **17**, or **19** for 24 h at the indicated concentrations. DMSO was used as a vehicle control. Cell cycle distributions of treated cells were analyzed by flow cytometry after staining with propidium iodide (PI). A tubulin polymerization inhibitor Combretastatin A-4 (CA-4) was used as a control for accumulation of G2/M phase.

Figure 9.

Induction of chromatin fragmentation and formation of apoptotic microtubules in MDA-MB-231 cells. (A) Dose-dependent induction of chromatin fragmentation and apoptotic microtubules. Cells were treated for 24 h with 0.1% DMSO (left panels) or Corymbulosin K (3) at 1.5 μM (3-fold IC_{50}) (middle panels) or 4.9 μM (10-fold IC_{50}) (right panels). Fixed cells were stained with DAPI (upper panels) for chromatin (blue) and antibody to α -tubulin (lower panels) for microtubules (green). Represented image is a projection of 12–18 sections acquired at 0.6–1 μm intervals by confocal laser-scanning microscopy. Same experiments were done using compounds as indicated at 3-fold IC_{50} (1.5 μM Corymbulosin I (1), 1.4 μM Caseamembrin S (16), or 1.6 μM 17) (B). Arrows, cells with apoptotic microtubules. Arrow heads, fragmented and dispersed chromatin. Bar, 25 μm .

Table 1.

¹H NMR Spectroscopic Data of Compounds 1–15

position	1 ^a (CDCl ₃), δ_H (J in Hz)	2 ^b (CDCl ₃), δ_H (J in Hz)	3 ^b (CDCl ₃), δ_H (J in Hz)	4 ^b (CDCl ₃), δ_H (J in Hz)	5 ^d (CDCl ₃), δ_H (J in Hz)
1	1.91 m overlap	2.15 m	1.91 m	1.92 m	1.98 m
2	5.43 m	1.71 m			1.88 m
3	5.98 dd (4.2, 1.8)	5.59 m	5.43 m	5.47 m	5.46 m
6	3.80 m	5.89 brs	5.91 dd (4.4, 1.6)	5.91 brd (3.6)	5.90 brd (3.6)
7	1.66 q (13.2)	4.00 m	3.31 dd (12.0, 4.0)	3.29 dd (9.6, 4.0)	3.27 dd (12.0, 4.2)
	1.74 dt (13.2, 3.6)	1.68 m	1.48 m	1.49 m	1.51 m
8	1.79 m	1.77 m	1.86 m	1.86 dt (12.4, 4.0)	1.86 dt (13.2, 4.2)
10	2.33 dd (12.0, 5.4)	1.80 m	1.70 m	1.71 m	1.72 m
11	1.52 m	2.35 dd (13.3, 2.3)	2.33 dd (11.2, 5.2)	2.27 dd (12.8, 3.6)	2.24 dd (13.8, 3.6)
12	1.21 m overlap	1.60 m	1.50 m	1.50 m	1.47 m
14	2.09 m	1.18 m	1.25 m	1.22 m	1.24 m
15	6.44 dd (17.4, 10.2)	2.07 m	2.08 m	2.08 m	2.08 m
	5.17 d (17.4)	6.43 dd (17.6, 10.8)	6.434 dd (17.2, 10.8)	6.43 dd (17.6, 10.8)	6.43 dd (17.4, 10.8)
16	5.03 d (10.2)	5.22 d (17.6)	5.16 d (17.2)	5.20 d (17.6)	5.21 d (17.4)
	5.06 s	5.03 d (10.5)	5.02 d (10.8)	5.03 d (10.8)	5.04 d (10.8)
	4.94 s	5.06 s	5.05 s	5.05 s	5.05 s
17	0.93 d (7.2)	4.91 s	4.94 s	4.94 s	4.94 s
18	6.75 t (1.8)	0.94 d (6.9)	0.95 d (6.8)	0.95 d (6.8)	0.95 d (7.2)
19	6.47 s	6.71 t (1.4)	6.66 dd (1.6, 1.2)	6.66 t (1.6)	6.66 dd (1.8, 1.2)
20	0.92 s	6.42 s	6.433 s	6.45 s	6.46 s
2'	2.63 des (7.2)	0.95 s	0.92 s	0.93 s	0.94 s
3'	1.22 d (7.2)	2.56 sep (7.2)	2.63 sep (6.8)	2.41 q (7.2)	
	1.20 d (7.2)	1.19 d (7.3)	1.22 d (6.8)	1.19 t (7.2)	
18-OAc	2.08 s	1.19 d (7.3)	1.20 d (6.8)		
19-OAc	1.90 s	2.07 s	2.07 s	2.09 s	2.07 s
2-OAc		1.90 s	1.88 s	1.90 s	1.91 s
6-OH	1.86 brs	1.83 brs			2.14 s

position	δ^1 (CDCl ₃), δ_H (J in Hz)	δ^2 (CDCl ₃), δ_H (J in Hz)	δ^3 (CDCl ₃), δ_H (J in Hz)	δ^4 (CDCl ₃), δ_H (J in Hz)	δ^5 (CDCl ₃), δ_H (J in Hz)
6-OMe			3.30	3.30	3.30 s
position	δ^9 (CDCl ₃), δ_H (J in Hz)	δ^7 (CDCl ₃), δ_H (J in Hz)	δ^8 (CDCl ₃), δ_H (J in Hz)	δ^6 (CDCl ₃), δ_H (J in Hz)	δ^{10} (CDCl ₃), δ_H (J in Hz)
1	1.92 m	2.15 m	1.92 m	1.93 m	1.89 m
		1.68 m	1.87 m	1.88 m	
2	5.43 m	5.59 m	5.46 m	5.46 m	5.41 m
3	5.93 dd (4.8, 1.8)	5.81 brd (3.2)	5.94 brd (3.4)	5.94 brd (3.1)	5.87 brd (4.1)
6	3.32 dd (12.4, 3.6)	3.51 dd (12.0, 3.8)	4.95 dd (11.3, 4.6)	4.95 dd (11.0, 4.4)	1.90 m
					1.50 m
7	1.47 m	1.49 m	1.61 m	1.60 m	1.42 m
	1.86 m	1.89 m	1.68 m	1.70 m	1.72 m
8	1.70 m	1.76 m	1.86 m	1.87 m	1.62 m
10	2.34 dd (9.0, 8.4)	2.35 m	2.25 m	2.29 m	2.13 dd (13.4, 3.4)
11	1.48 m	1.47 m	1.50 m	1.51 m	1.50 m
	1.25 m	1.26 m	1.25 m	1.23 m	1.28 m
12	2.08 m	2.08 m	2.11 m	2.11 m	2.09 m
14	6.44 dd (17.4, 10.8)	6.42 dd (17.5, 11.0)	6.44 dd (17.5, 10.6)	6.44 dd (17.5, 10.6)	6.43 dd (17.4, 10.5)
15	5.16 d (17.4)	5.20 d (17.5)	5.23 d (17.5)	5.22 d (17.5)	5.21 d (17.4)
	5.03 d (10.8)	5.03 d (11.0)	5.04 d (10.6)	5.05 d (10.6)	5.03 d (10.5)
16	5.05 s	5.02 s	5.05 s	5.07 s	5.05 s
	4.94 s	4.93 s	4.93 s	4.95 s	4.92 s
17	0.95 d (6.8)	0.91 d (7.2)	0.92 d (6.9)	0.92 d (6.9)	0.88 d (6.9)
18	6.66 dd (1.8, 1.2)	6.62 t (1.9)	6.51 t (1.8)	6.51 t (1.4)	6.69 t (1.4)
19	6.43 s	6.39 s	6.55 s	6.55 s	6.32 s
20	0.92 s	0.93 s	0.96 s	0.96 s	0.94 s
2'	2.46 m	2.38 m	2.29 ddd (15.7, 7.8, 2.7)	2.31 ddd (15.1, 7.7, 2.7)	2.40 dd (15.1, 7.6)
3'	1.57 m	1.44 m	1.60 m	1.58 m	1.19 t (7.6)
	1.69 m	1.69 m			
4'	0.97 t (7.2)	0.94 t (7.6)	1.28 m ^c	1.25 m ^d	
5'	1.18 d (6.6)	1.17 d (6.9)	1.28 m ^c	1.25 m ^d	

position	ν $\mathbf{1}^a$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{2}^b$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{3}^c$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{4}^d$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{5}^e$ (CDCl ₃), δ_{H} (J in Hz)
6'			1.28 m ^c	1.25 m ^d	
7'			1.28 m ^c 0.88 t (6.9)	1.25 m ^d	
8'				1.25 m ^d	
9'				1.25 m ^d	
10'				0.86 t (6.9)	
18-OAc	2.07 s	2.06 s	2.08 s	2.08 s	
19-OAc	1.87 s	1.87 s	1.93 s	1.93 s	2.07 s
2-OAc			2.14 s	2.14 s	1.90 s
6-OMe	3.31 s	3.34 s			
position	ν $\mathbf{11}^b$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{12}^b$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{13}^b$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{14}^b$ (CDCl ₃), δ_{H} (J in Hz)	ν $\mathbf{15}^b$ (CDCl ₃), δ_{H} (J in Hz)
1	1.93 m	1.97 dd (15.1, 4.4)	1.91 m	1.99 dd (13.4, 4.8)	2.53 dd (17.4, 6.8)
2	5.37 m	1.89 m	1.60 m	1.65 m	2.57 dd (17.4, 12.8)
3	5.87 dd (4.6, 1.4)	5.40 m	4.37 m	4.44 m	
6	1.71 m	5.85 brd (4.1)	5.92 brd (3.7)	6.00 brd (3.4)	6.14 brd (1.4)
7	1.50 m	1.72 m	1.71 m	4.97 dd (11.7, 4.8)	5.12 dd (11.9, 4.1)
	1.42 m	1.46 m	1.45 m		
	1.52 m	1.40 m	1.37 m	1.68 m	1.72 m
8	1.62 m	1.50 m	1.52 m		1.81 dt (13.7, 4.1)
10	2.20 t (8.2)	1.62 m	1.62 m	1.87 m	2.00 m
11	1.50 m	2.13 dd (13.4, 3.4)	2.13 dd (11.9, 5.0)	2.32 dd (13.4, 3.8)	2.86 dd (12.8, 6.4)
12	1.28 m	1.49 m	1.51 m	1.50 m	1.51 m
14	2.11 m	1.25 m	1.30 m	1.26 m	1.27 m
15	6.43 dd (17.4, 10.5)	2.06 m	2.07 m	2.09 t (8.6)	2.07 m
	5.20 d (17.4)	6.43 dd (17.5, 11.0)	6.42 dd (17.5, 10.8)	6.42 dd (17.5, 10.8)	6.43 dd (17.6, 10.9)
16	5.02 d (10.5)	5.18 d (17.5)	5.22 d (17.5)	5.22 d (17.5)	5.21 d (17.6)
	5.04 s	5.03 d (11.0)	5.02 d (10.8)	5.02 d (10.8)	5.05 d (10.9)
	4.94 s	5.05 s	5.04 s	5.04 s	5.07 s
17	0.88 d (6.9)	4.92 s	4.93 s	4.94 s	4.93 s
		0.89 d (6.8)	0.89 d (6.9)	0.91 d (6.9)	0.96 d (6.9)

position	$\delta_{\text{H}}^{\text{a}}$ (CDCl ₃), $\delta_{\text{H}}^{\text{b}}$ (J in Hz)	$\delta_{\text{C}}^{\text{a}}$ (CDCl ₃), $\delta_{\text{C}}^{\text{b}}$ (J in Hz)	$\delta_{\text{H}}^{\text{c}}$ (J in Hz)	$\delta_{\text{H}}^{\text{d}}$ (J in Hz)	$\delta_{\text{H}}^{\text{e}}$ (J in Hz)
18	6.68 t (1.4)	6.67 t (1.2)	6.68 t (1.7)	6.51 t (1.4)	6.65 d (1.4)
19	6.30 s	6.30 s	6.31 s	6.54 s	6.62 s
20	0.93 s	0.92 s	0.98 s	0.99 s	0.95 s
2'	2.62 sep (7.3)	2.36 t (7.3)		2.29 sep (3.1)	2.31 ddd (10.5, 7.8, 2.8)
3'	1.21 d (7.3)	1.65 m		1.60 m	1.59 m
	1.20 d (7.3)				
4'		1.34 m ^f		1.28 m ^g	1.27 m ^h
5'		1.34 m ^f		1.28 m ^g	1.27 m ^h
6'		0.91 t (7.4)		0.87 t (7.2)	1.27 m ^h
7'					1.27 m ^h 0.88
8'					t (6.9)
18-OAc	2.08 s	2.08 s	2.08 s	2.07 s	2.07 s
19-OAc	1.90 s	1.90 s	1.90 s	1.90 s	1.91 s

^a 600 MHz.^b 400 MHz.^{c-h} Overlapping signals.

Table 2.

¹³C NMR Spectroscopic Data in CDCl₃ of Compounds 1–15

position	1, ^a δ _c	2, ^b δ _c	3, ^b δ _c	4, ^b δ _c	5, ^b δ _c	6, ^b δ _c	7, ^b δ _c	8, ^b δ _c	9, ^a δ _c	10, ^b δ _c	11, ^b δ _c	12, ^b δ _c	13, ^b δ _c	14, ^b δ _c	15, ^b δ _c
1	26.8	26.3	27.0	27.2	27.2	27.0	26.5	26.9	27.0	26.1	26.2	26.3	29.6	29.5	35.5
2	66.1	70.4	66.1	66.1	66.3	66.1	70.5	66.1	66.2	66.3	66.3	66.3	64.2	63.8	198.0
3	121.9	124.4	121.3	121.2	121.1	121.3	123.8	123.0	123.1	120.4	120.4	120.4	123.8	126.4	125.6
4	145.4	144.3	146.3	146.2	146.2	145.0	145.2	144.3	144.3	147.1	147.2	147.1	145.4	142.6	163.0
5	53.8	53.6	53.1	53.0	52.9	53.1	53.0	52.0	52.1	49.2	49.3	49.2	49.3	52.2	53.4
6	73.0	74.3	81.8	81.9	82.0	81.8	83.0	73.6	73.6	29.4	29.2	29.4	28.9	73.7	73.8
7	37.2	37.50	31.0	31.0	31.0	31.0	31.3	33.0	33.1	27.2	27.2	27.2	27.2	33.0	33.0
8	37.5	37.54	36.8	36.8	36.5	40.0	36.9	37.1	37.2	37.2	37.3	37.16	37.18	36.7	36.2
9	37.3	38.1	37.4	37.5	37.5	37.4	41.1	37.3	37.4	37.1	37.1	37.09	37.20	37.3	37.8
10	36.5	41.0	36.4	36.5	36.7	36.4	38.2	36.5	36.5	34.3	34.2	34.4	33.4	36.2	41.9
11	28.0	27.6	27.8	27.1	28.0	27.7	27.4	28.0	28.1	28.2	28.1	28.2	28.2	27.9	27.3
12	23.7	23.7	23.8	23.8	23.8	23.7	23.7	23.8	23.9	23.6	23.6	23.6	23.6	23.8	23.7
13	145.1	145.0	145.1	145.2	145.2	146.2	145.0	145.1	145.1	145.4	145.3	145.3	145.4	145.2	144.7
14	140.4	140.2	140.5	140.4	140.4	140.5	140.3	140.4	140.4	140.4	140.4	140.4	140.3	140.3	140.1
15	112.2	112.6	112.2	112.3	112.4	112.1	112.5	112.5	112.5	112.3	112.1	112.2	112.5	112.6	112.8
16	115.5	115.4	115.6	115.6	115.6	115.6	115.5	115.5	115.5	115.4	115.4	115.4	115.3	115.4	115.5
17	15.7	15.7	15.9	15.9	16.0	15.9	15.9	15.6	15.6	15.7	15.7	15.7	15.8	15.6	15.5
18	95.5	95.0	96.1	96.1	96.2	96.0	95.6	95.2	95.2	94.5	94.4	94.5	94.6	95.3	94.4
19	97.8	97.4	98.3	98.4	98.5	98.3	97.9	98.3	98.2	99.7	99.5	99.7	99.8	98.3	98.1
20	25.5	25.4	25.6	25.6	25.5	25.6	25.6	25.5	25.5	26.3	26.1	26.0	26.1	25.5	24.7
1'	176.4	177.1	176.5	174.0	170.7	176.0	176.5	173.2	173.2	174.0	176.4	173.4		173.3	173.0
2'	34.7	34.1	34.0	28.0	21.5	41.1	41.1	34.7	34.8	28.0	34.1	34.6		34.7	34.6
3'	19.2	18.9	19.3	9.3		27.1	26.8	24.8	24.7	9.3	19.2	24.8		24.4	24.6
4'	18.7	18.9	18.7			11.6	11.7	29.1	29.4		18.7	31.2		31.3	29.1
5'						16.6	16.6	28.9	29.23			22.3		22.3	28.8
6'								31.6	29.19			13.9		13.9	22.6
7'								22.6	29.12						31.6
8'								14.1	31.9						14.1

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position	δ^a , 1	δ^b , 2	δ^b , 3	δ^b , 4	δ^b , 5	δ^b , 6	δ^b , 7	δ^b , 8	δ^b , 9	δ^b , 10	δ^b , 11	δ^b , 12	δ^b , 13	δ^b , 14	δ^b , 15
9'						22.8									
10'						14.1									
18-OAc	C=O	170.1	170.0	170.3	170.3	170.2	170.2	170.09	170.08	170.3	170.3	170.3	170.3	170.1	169.54
	Me	21.2	21.2	21.4	21.4	21.4	21.3	21.3	21.4	21.4	21.2	21.3	21.3	21.3	21.5
19-OAc	C=O	169.7	169.9	169.8	169.9	169.9	169.9	170.08	170.07	170.1	170.0	170.1	170.1	170.0	169.47
	Me	21.4	21.5	21.6	21.7	21.8	21.6	21.7	21.7	21.3	21.3	21.4	21.5	21.7	21.0
2-OAc	C=O							170.5	170.5						
	Me							21.5	21.5						
δ 150 MHz.															
δ 100 MHz.															

Table 3.

Antiproliferative Activity of the Isolated Diterpenes

compounds	cell lines ^a (IC ₅₀ μM) ^b				
	A549	MDA-MB-231	MCF-7	KB	KB-VIN
1	0.66	0.48	0.68	0.56	0.98
3	0.47	0.49	0.50	0.45	0.49
4	4.60	4.95	4.94	5.19	4.92
6	5.04	4.90	5.82	5.23	5.19
7	4.75	3.31	4.65	4.25	4.76
8	5.98	4.93	6.39	5.16	5.03
9	40.2	20.5	31.7	19.8	39.2
11	>40	22.9	26.2	25.1	26.6
12	2.29	0.49	0.69	0.56	0.61
14	4.76	4.73	5.19	4.74	4.88
16	0.58	0.45	0.66	0.53	0.90
17	4.15	0.54	0.89	0.73	4.07
18	0.53	0.40	0.55	0.43	0.51
19	0.45	0.43	0.44	0.42	0.45
Paclitaxel (nM)	6.53	8.36	12.13	7.07	2213.34

^a A549 (lung carcinoma), MDA-MB-231 (triple-negative breast cancer), MCF-7 (estrogen receptor-positive and HER2-negative breast cancer), KB (epidermoid carcinoma of the nasopharynx), KB-VIN (P-gp-overexpressing MDR subline of KB).

^b Antiproliferative activity expressed as IC₅₀ values for each cell line, the concentration of compound that caused 50% reduction relative to untreated cells determined by the SRB assay.

Table 4.

Hemolytic Activity of Isolated Diterpenes

compound	% hemolysis ^a		
	20 μ M	100 μ M	500 μ M
1	10.6 $\pm$ 1.0	17.5 $\pm$ 3.0	22.3 $\pm$ 0.2
3	14.4 $\pm$ 1.0	16.5 $\pm$ 1.2	17.7 $\pm$ 0.8
6	15.5 $\pm$ 1.0	16.3 $\pm$ 1.4	17.0 $\pm$ 1.9
8	1.0 $\pm$ 2.1	12.2 $\pm$ 2.2	12.3 $\pm$ 2.1
9	5.1 $\pm$ 2.1	10.0 $\pm$ 2.6	50.6 $\pm$ 5.5
11	0.0 $\pm$ 2.1	3.5 $\pm$ 2.8	5.0 $\pm$ 0.9
14	16.3 $\pm$ 1.1	19.6 $\pm$ 2.5	34.3 $\pm$ 3.2
17	2.2 $\pm$ 1.0	9.5 $\pm$ 1.1	27.1 $\pm$ 1.3
19	2.0 $\pm$ 2.5	35.5 $\pm$ 3.1	118.7 $\pm$ 6.9

^aData are expressed as means $\pm$ standard deviation for three independent experiments. A saponin mixture extracted from quillaja bark (sapogenin, 20–35%, Sigma-Aldrich, USA) was used as a positive control. This control showed 100% hemolysis of red blood cells at 100 μ g/mL.