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Epicochalasines A and B: Two Bioactive Merocytochalasans Bearing Caged Epicoccine Dimer Units from *Aspergillus flavipes*

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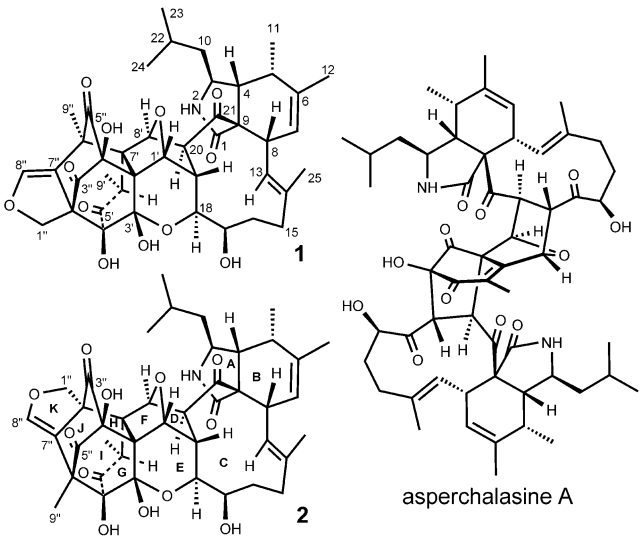
Abstract: *Two bioactive merocytochalasans, epicochalasines A (**1**) and B (**2**), a new class of cytochalasans bearing unexpected scaffolds consisting of fused aspochalasin and epicoccine dimer moieties, were isolated from the liquid culture broth of Aspergillus flavipes. Both **1** and **2** possess a hendecacyclic 5/6/11/5/6/5/6/5/6/6/5 ring system containing an adamantyl cage and as many as 19 stereogenic centers; however, the fusion patterns of **1** and **2** differ greatly, thus resulting in different carbon skeletons. The absolute configurations of **1** and **2** were determined by X-ray diffraction and calculated ECD, respectively. The biogenetic pathways of **1** and **2** are proposed to involve Diels–Alder and nucleophilic addition reactions. Both **1** and **2** induced significant G2/M-phase cell-cycle arrest. Furthermore, we found that merocytochalasans induce apoptosis in leukemia cells through the activation of caspase-3 and the degradation of PARP.*

Cytochalasans have received considerable attention from both chemists and pharmacologists over the past 50 years because of their intriguing structures and potent biological activity,[1] which is exemplified by immunomodulatory,[2] cytotoxic,[3] and nematicidal activity.[4] To date, more than 200 cytochalasans have been reported, from various fungi origins, such as *Chaetomium*, *Aspergillus*, and *Penicillium* species.[1b] Epicoccine derivatives, especially their dimers, are a class of aromatic polyketides with diverse structures that exhibit numerous forms of bioactivity, for example, antimicrobial,[5] antiviral,[6] and enzyme-inhibition activity.[5,7] Because of their considerable activity and intricate polycyclic structures, the total synthesis of epicoccine dimers (e.g., dibefurin and epicolactone) has attracted considerable attention from the synthesis community.[8]

During our continuous search for structurally intriguing and biologically valuable natural products from fungi, several fungi of different origins have been phytochemically investigated.[9] In our previous study on *Aspergillus flavipes* cultured on rice, we discovered a cytochalasan dimer (asperchalasine A, Scheme 1).[10] Considering that the fermentation of fungi under different conditions may lead to different secondary metabolites, we further investigated the metabolites of *A. flavipes* cultured in a liquid culture broth. In this study, two novel merocytochalasans, epicochalasines A (**1**) and B (**2**; Scheme 1), with high degrees of functionalization and intricate polycyclic structures, were isolated from the liquid culture broth of *A. flavipes*. Compounds **1** and **2** appear to belong to the same structural family. However, because of the 180° rotation of the oxidized epicoccine moiety **c** (Scheme 2) in **2**, their fusion patterns differ substantially, thus resulting in different carbon skeletons. Most notably, compounds **1** and **2** are the first examples of cytochalasan adducts characterized by an adamantyl cage in a hendecacyclic 5/6/11/5/6/5/6/5/6/6/5 ring system formed by the polymerization of an epicoccine dimer with a cytochalasan moiety.

Herein, the isolation, structural elucidation, and postulated biosynthetic pathways of **1** and **2** are described. Moreover, **1** and **2** were observed to induce significant G2/M-phase cell-cycle arrest in HL60 and apoptosis in leukemia cells through the activation of caspase-3 and the degradation of poly(ADP-ribose) polymerase (PARP).

Epicochalasine A (**1**) was initially isolated as a colorless amorphous powder. The molecular formula $C_{42}H_{49}NO_{12}$ with



Scheme 1. Structures of epicochalasines A (**1**) and B (**2**) and asperchalasine A.

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Supporting information for this article, including experimental procedures, 1D and 2D NMR, MS, UV, and IR spectra for **1** and **2**, and X-ray crystallographic data of **1** in CIF format, is available on the WWW under http://dx.doi.org/10.1002/anie.201511315.

19 degrees of unsaturation was established by the presence of a pseudo-molecular-ion peak at m/z 782.3180 in the high-resolution ESIMS spectrum, in combination with the ^{13}C NMR spectrum. The ^{1}H NMR spectroscopic results, together with the HSQC spectra of **1**, suggested the presence of three olefinic hydrogen atoms at $\delta_H = 6.48$ (s), 6.16 (d, $J =$ 10.9 Hz), and 5.36 ppm (br s); four oxygenated methine hydrogen atoms at $\delta_H = 5.25$ (d, $J = 6.3$ Hz), 4.62 (br s), 4.34 (s), and 3.93 ppm (dd, $J = 10.4$, 7.4 Hz); two oxygenated methylene hydrogen atoms at $\delta_H = 4.76$ (d, $J = 11.0$ Hz) and 4.62 ppm (d, $J = 11.0$ Hz); seven methyl groups at $\delta_H = 1.77$ (s), 1.52 (s), 1.43 (s), 1.28 (d, $J = 7.2$ Hz), 1.18 (d, $J = 7.1$ Hz), 0.95 (d, $J = 6.5$ Hz), and 0.94 ppm (d, $J = 6.5$ Hz); and 14 aliphatic hydrogen atoms between $\delta_H = 1.20$ and 4.79 ppm. The ^{13}C NMR and DEPT spectra of **1** indicated 42 carbon resonances ascribed to four free carbonyl groups, an amide carbonyl group, three olefinic groups, eight oxygenated carbon atoms (including three quaternary, four methine, and a methylene), 16 aliphatic carbon atoms, and seven methyl groups. Considering the NMR and MS data for previously reported secondary metabolites of *A. flavipes*, epicochalasine A (**1**) was speculated to be an aspochalasin adduct.

The gross structure of **1** was established by 2D NMR spectroscopy and by comparison with the related compounds asperchalasines B and C.[10] The ^{1}H–^{1}H COSY cross-peaks (Figure 1a) of Me-11/H-5/H-4/H-3/H-10/H-22/Me-23, H-7/H-8/H-13, H-15/H-16/H-17/H-18, and H-19/H-20 and HMBC interactions (Figure 1a) from H-4 to C1, C9, and C21; from Me-12 to C5, C6, and C7; from H-8 to C4 and C9; from Me-25 to C13, C14, and C15; from H-18 to C20; and from H-19 and H-20 to C21 established the tricyclic aspochalasin scaffold (unit A). Aside from those of unit A, 18 carbon signals remained, which were preliminarily attributed to two epicoccine units (units B and C) on the basis of our reported adducts of cytochalasin and epicoccine (asperchalasines B and C).[10] The gross structures of units B and C were elucidated by HMBC correlations and carbon chemical shifts (Figure 1b,c), as follows: H-1′ to C7′ and C8′; H-8′ to C1′ and C2; Me-9′ to C5′, C6′, and C7′; H-6′ to C8′; H-1″ to C2″, C3″, C7″, and C8″; H-8″ to C1″, C2″, and C7″; and Me-9″ to C5″, C6″, and C7″. In addition, the HMBC correlations (Figure 1d) from H-18 to C3′, H-19 to C2′, H-1′ to C20, and H-8′ to C21 revealed the connectivity between units A and B through the newly formed furo[3,2-*c*]pyran ring system (rings D and E). Furthermore, the HMBC correlations (Figure 1d) from H-6′ and H-8′ to C6″, Me-9″ to C7′, and H-1″ to C4′ suggested the connection of units B and C through C4′/C2″ and C7′/C6″ bonds, and the linkage of C2′/C4″ was proposed to satisfy their quaternary nature and chemical shifts as well as the aforementioned molecular formula. Thus, the gross planar structure of **1** was established.

The relative configuration of unit A, which is similar to those of asperchalasines B and C,[10] was verified by a NOESY experiment (Figure 1e). The NOESY cross-peaks of H-5 to H-4 and H-8, H-4 to H-10, and H-8 to Me-25 and the interactions between Me-25, H-16β, and H-19 suggested that these moieties are cofacial and β-oriented. Additionally, the NOESY correlations from H-3 to Me-11 and H-13 to H-15α, H-18, and H-20, as well as the correlations between H-15α, H-17, and H-18, established their α orientations. Moreover, the key NOESY correlation between H-20 and H-6′ not only suggested a β-oxygen bridge between C1′ and C8″ but also indicated an α configuration for H-6′. Given the adamantyl cage rings D–G and the aforementioned NOESY correlation of H-20/H-6′, unit C could only be located on the left side of unit B. With respect to the conformation of the rigid cagelike carbon framework of rings D–J, the relative configurations at C3′, C4′, C2″, C4″, and C6″ were finally established as shown in Scheme 1. Thus, the structure of **1** with its relative configuration was tentatively established.

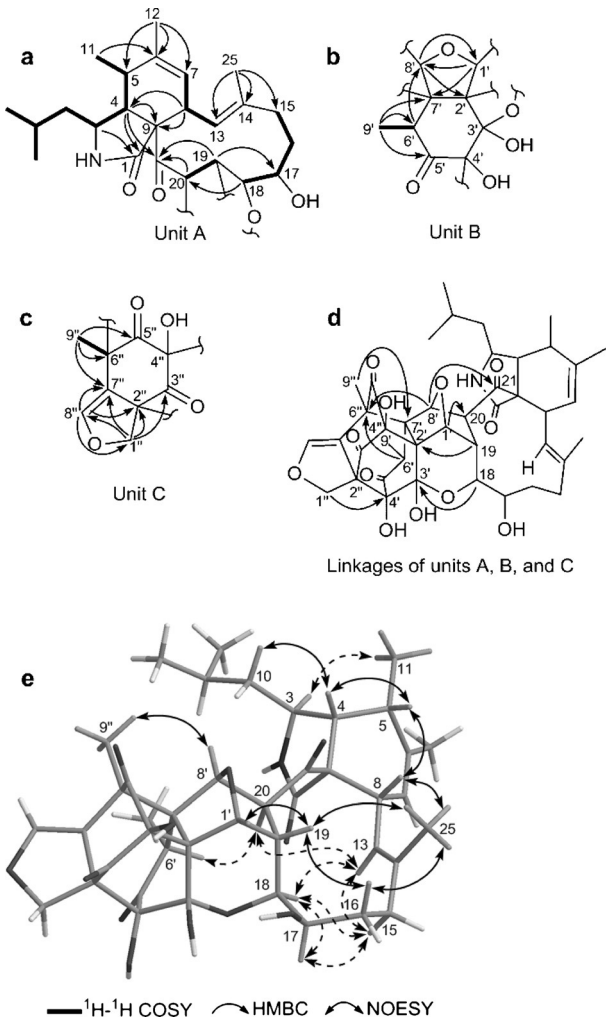


Figure 1. Key ^{1}H–^{1}H COSY, HMBC, and NOESY correlations for epicochalasine A (**1**).

Because of the lack of certain key 2D NMR correlations and literature precedent for a dimeric epicoccine-containing cytochalasan alkaloid possessing such a complex scaffold with multiple quaternary carbon atoms, NMR spectroscopy alone was insufficient for the determination of the structure and relative configuration of **1**. We therefore turned to single-crystal X-ray analysis. After numerous attempts with different solvent systems, a tiny crystal of **1** was obtained from MeOH–acetone (10:1) with a drop of water by slow evaporation in a closed tube at 4 °C. An X-ray diffraction experiment (Figure 2) confirmed the elucidated planar structure and relative configuration of **1** and also unambiguously deter-

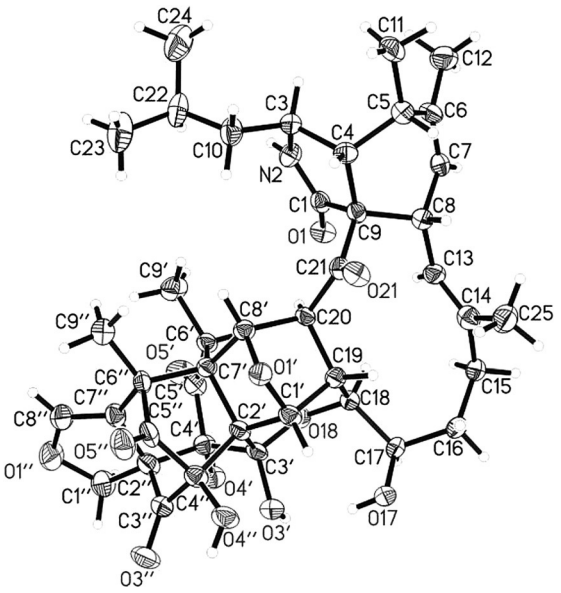


Figure 2. X-ray crystal structure of epicochalasine A (**1**).

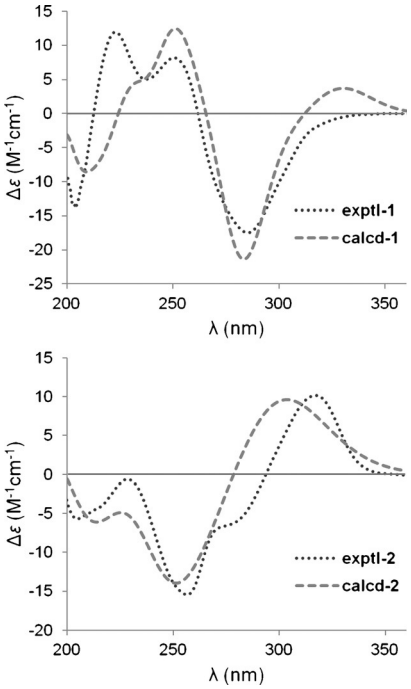


Figure 3. Experimental and calculated ECD for **1** and **2**.

mined the absolute configuration of **1** to be 3*S*,4*R*,5*S*,8*S*,9*S*,17*R*,18*S*,19*S*,20*S*,1′*R*,2′*R*,3′*S*,4′*R*,6′*S*,7′*R*,8′*S*,-2″*R*,4″*R*,6″*R* (Flack parameter: 0.0(3); Hooft parameter: −0.07(8)). The absolute configuration of **1** was consistent with that of other members of the aspochalasin family.

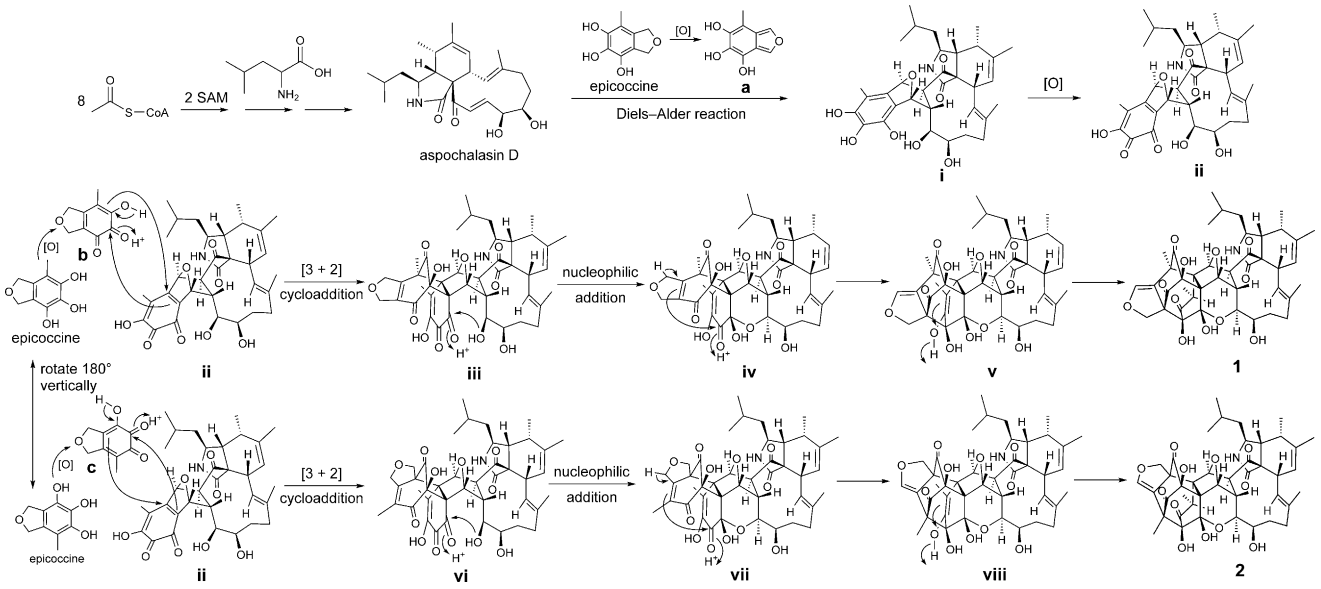
The ^{1}H and ^{13}C NMR data of epicochalasine B (**2**) were superimposable with those of **1**, with the exception of slight changes in certain resonances assignable to units B and C. Extensive analyses of the 2D NMR data (see Figure S1 in the Supporting Information) verified the identical structure of units A–C, and HMBC correlations from H-1′ to C4″, H-8′ to C2″, H-1″ to C7′ and Me-9″ to C4′ suggested a different fusion pattern for units B and C because of the linkages C2′/C4″, C4′/C6″, and C7′/C2″. Thus, **2** was determined to be an isomer of **1** with a different carbon scaffold. The relative configuration of **2** was established by a NOESY experiment (see Figure S1) in the same manner as that of **1**.

To determine the absolute configuration of **2**, we performed an electronic circular dichroism (ECD) calculation by using time-dependent density functional theory (TDDFT). The calculated ECD curve of **2** demonstrated good agreement with the experimental curve (Figure 3), which confirmed the absolute configuration of **2**, as shown in Scheme 1. An ECD calculation was also performed for **1**, and the result was consistent with that of the X-ray diffraction experiment.

Epicochalasines A (**1**) and B (**2**) are the first examples of merocytochalasans characterized by the coupling of aspochalasin and epicoccine dimer moieties; we termed this compound class "epicochalasine". Biogenetic pathways of **1** and **2** involving a series of cycloaddition reactions were proposed to explain the formation of the carbon cage scaffolds (Scheme 2). Biosynthetically, compounds **1** and **2** could plausibly be derived from aspochalasin D, which is generated through the PKS-NRPS pathway. Thus, aspochalasin D underdoes a Diels–Alder reaction with a molecule of oxidized epicoccine to produce the intermediate **i**, which is further oxidized to yield **ii**. An additional oxidized epicoccine molecule (**b**) is fused to **ii** through a [3+2] cycloaddition reaction to generate the dimeric epicoccine-containing intermediate **iii**.[8a] Then, nucleophilic addition forms a new pyran ring to provide **iv**. Afterwards, **iv** undergoes an additional nucleophilic addition to generate the carbon cage scaffold **v**, and finally compound **1** is formed by keto–enol tautomerism. Notably, compound **2** was formed in the same manner as **1**. However, the fusion pattern of the two epicoccine moieties in **2** differs greatly from that of **1** because of the 180° rotation and because of the different oxidization position of the epicoccine moiety **c** in **2**. The [3+2] cycloaddition and subsequent nucleophilic addition are the key steps in the formation of the skeletons of the novel compound class, which may attract great attention from the total synthesis and biosynthesis communities.

The proliferation-inhibition effects of compounds **1** and **2** were investigated with HL60 and NB4 cells. Treatment with compound **1** or **2** resulted in the inhibition of cell proliferation in both cell lines (IC_{50}, **1**: 65.55 and 74.60 μM; **2**: 23.00 and 28.71 μM, respectively; see Figure S2). Compounds **1** and **2** were also tested for their influence on the cell cycle with HL60 cells. Interestingly, both compounds induced significant G2/M-phase cell-cycle arrest (Figure 4a,b).

Compounds **1** and **2** were also observed to induce apoptosis of leukemia cells. Cell morphological changes were observed with an inverted light microscope (NIKON, Tokyo, Japan) after treatment with **1** or **2**, and the results indicated that the cells shrank and detached from the culture surface, thus suggesting that these compounds probably induced apoptosis. To analyze the apoptosis-induction potential of compounds **1** and **2**, we performed an apoptosis assay by flow cytometry analysis. We found that **1** and **2** induced significant apoptosis of HL60 and NB4 cells, as compared to the control group (Figure 4c). Moreover, treatment with **1** or



Scheme 2. Plausible biosynthetic pathways of epicochalasines A (**1**) and B (**2**). CoA = coenzyme A, SAM = *S*-adenosyl-L-methionine.

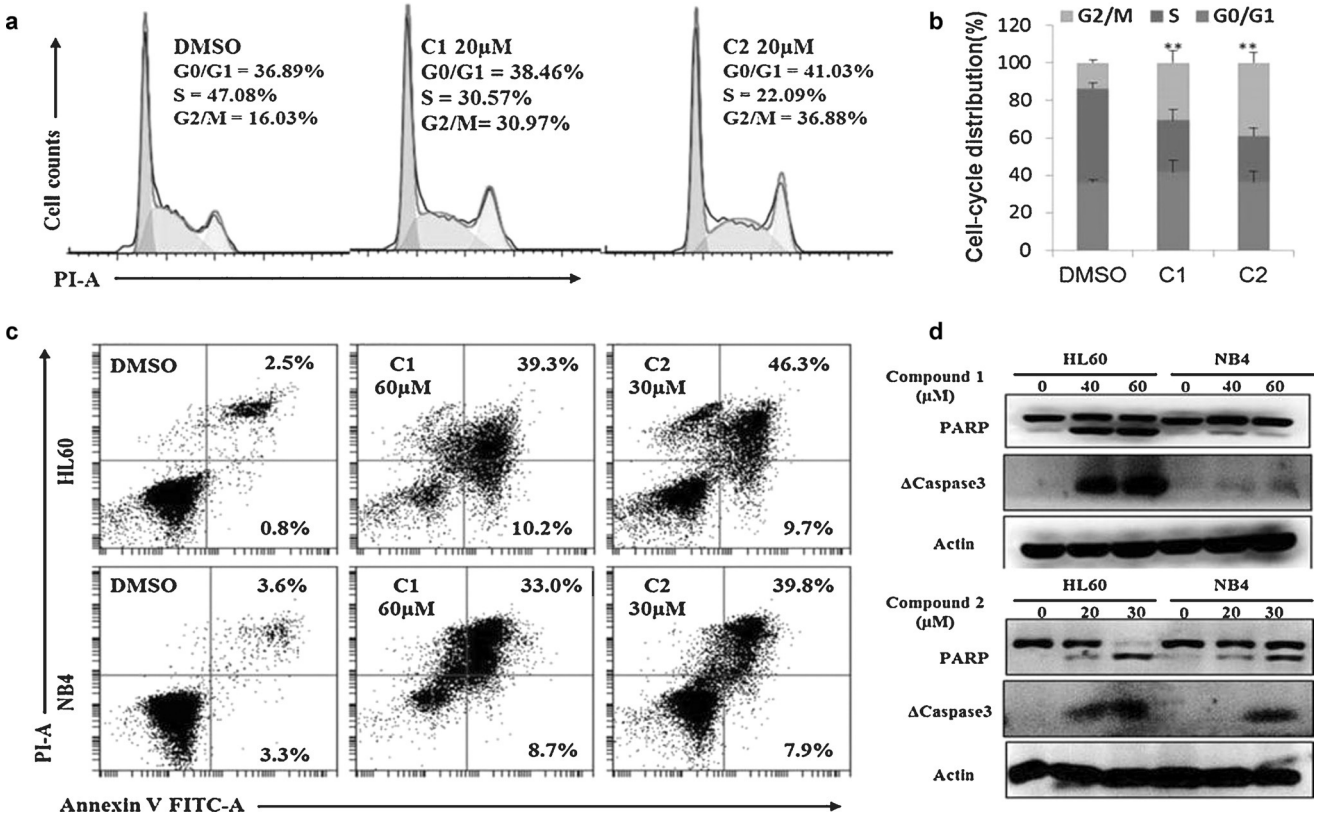


Figure 4. a) Effects of compounds **1** and **2** on HL60 cell-cycle progression at a concentration of 20 μM after 48 h of treatment. b) Columns: means of three different flow cytometry experiments; bars: standard error of the mean (SEM). $^{**}P < 0.01$ versus a control group. c) Compounds **1** and **2** induce apoptosis in leukemia cells (cells in the lower-right quadrant are early-apoptotic cells, and cells in the upper-right quadrant are late-apoptotic cells). d) Western blot analysis for PARP and caspase-3 levels. β-Actin was used as a loading control.

2 altered the expression levels of caspase-3 and PARP (Figure 4d). These data suggest that **1** and **2** induced apoptosis in leukemia cells, as mediated by the activation of caspase-3 and the degradation of PARP. This study is the first to find that merocytochalasans induce the apoptosis of cancer cells.

In conclusion, epicochalasines A (**1**) and B (**2**), two merocytochalasans possessing unprecedented hendecacyclic

5/6/11/5/6/5/6/5/6/6/5 ring systems with an adamantyl cage and G2/M-phase cell-cycle-arrest activity, were isolated from the liquid culture broth of *A. flavipes*. Structurally, **1** and **2** represent a novel class of cytochalasans characterized by the coupling of aspochalasin and epicoccine dimer moieties. To the best of our knowledge, the high degree of functionalization and the intricate polycyclic structures of **1** and **2** make these compounds unique natural products, not only within the cytochalasan family.

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