Natural Products

International Edition: DOI: 10.1002/anie.201506264
German Edition: DOI: 10.1002/ange.201506264

Asperchalasine A, a Cytochalasan Dimer with an Unprecedented Decacyclic Ring System, from *Aspergillus flavipes*

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Abstract: *Asperchalasine A (**1**), the first cytochalasan dimer featuring a unique decacyclic 5/6/11/5/5/6/5/11/6/5 ring system consisting of 20 chiral centers, was isolated from the culture broth of Aspergillus flavipes. Three biogenetically related intermediates, asperchalasines B–D (**2**–**4**), were also isolated. Their structures, including their absolute configurations, were elucidated using a combination of HRESIMS, NMR, ECD, molecular modeling, and single-crystal X-ray diffraction techniques. Compound **1**, which possesses an unprecedented 13-oxatetracyclo[7.2.1.1^{2,5}.0^{1,6}]tridec-8,12-dione core structure, is the first example of a dimeric cytochalasan alkaloid. The biogenetic pathways of **1**–**4** were described starting from the co-isolated compounds **5** and **6**. More importantly, **1** induced significant G1-phase cell cycle arrest by selectively inhibiting cyclin A, CDK2 and CDK6 in cancerous, but not normal, cells, highlighting it as a potentially selective cell cycle regulator against cancer cells.*

Cytochalasans are a well-known class of alkaloids, bearing a perhydro-isoindolone core fused with a macrocyclic ring.[1] These compounds exhibit diverse bioactivities, such as immunomodulatory,[2] cytotoxic,[3] and nematicidal activities.[4] Since the discovery of the first cytochalasans (cytochalasins A and B) in 1966,[5] the structures and biological activities of this compound class have attracted great interest from the synthetic and pharmacological communities.[6] In recent years, studies on cytochalasans have achieved historically unprecedented progress in new structure exploration, total synthesis, and biosynthesis.[3a,7] To date, the cytochalasan alkaloids, produced by various types of fungi, consist of more than 200 members with diverse structures.

In our previous research, two cytotoxic cytochalasans with novel skeletons had been isolated from the symbiotic fungus *Chaetomium globosum*.[8] In the current study, the fermentation broth of *Aspergillus flavipes* was phytochemically investigated, leading to the isolation and characterization of four novel cytochalasan alkaloids (asperchalasines A–D, **1**–**4**; Figure 1). Asperchalasine A (**1**), which is the first example of a cytochalasan dimer generated by the fusion of two cytochalasan molecules to an epicoccine, possesses an unprecedented 13-oxatetracyclo[7.2.1.1^{2,5}.0^{1,6}]tridec-8,12-dione core containing as many as 20 chiral centers. Structurally, the dimeric and polymeric features of **1** are sufficient to make it stand out not only from the large family of cytochalasans, but also from the naturally occurring alkaloids. Meanwhile, asperchalasines B–D (**2**–**4**) seem to be the biosynthetic intermediates of **1** and are a probable biogenetic pathway for **1** from monomeric cytochalasan.

Many cytochalasans have been reported to exhibit promising cytotoxic activities. Asperchalasines A–D (**1**–**4**) were evaluated for their cytotoxic activities, and **1** was further studied for its cell cycle regulatory activity; it induced

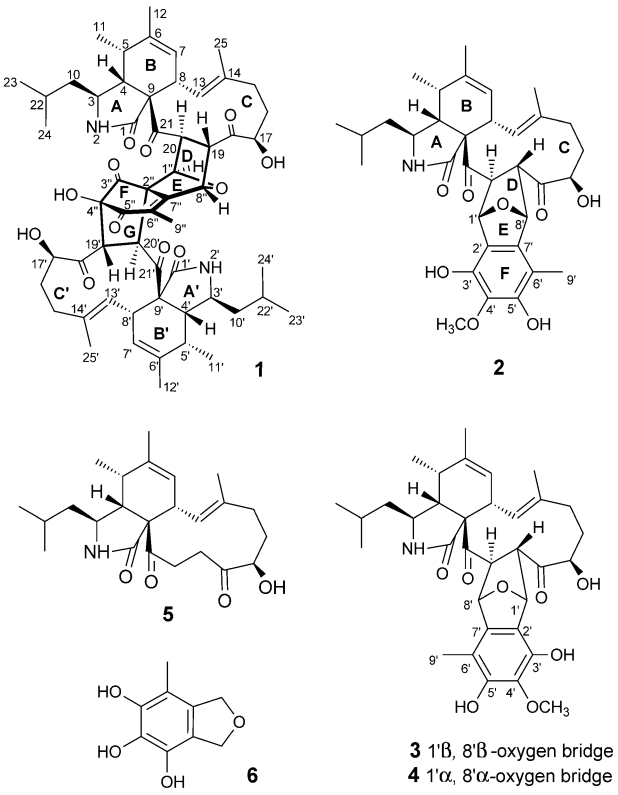

Figure 1. Structures of **1**–**6**.

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Supporting information for this article, experimental procedures, 1D and 2D NMR, MS, UV, and IR spectra for **1**–**4**, X-ray crystallographic data of **1**, **2**, and **5** in CIF format are available on the WWW under http://dx.doi.org/10.1002/anie.201506264.

significant G1-phase cell cycle arrest in four cancer cell lines and showed no influence on two normal cell lines.

Herein, we report the isolation, the structural elucidation, and the putative biogenetic pathways of **1** and its biogenetically related intermediates **2**–**4**. Moreover, the G1-phase cell cycle arrest activity of **1** on cancer cell lines is shown.

Asperchalasine A (**1**) was obtained as colorless crystals. Its molecular formula, $C_{57}H_{72}N_2O_{12}$, was established on the basis of its high-resolution ESI MS (HRESIMS) spectrum ($[M+H]^+$, m/z 977.5149; calcd. for $C_{57}H_{73}N_2O_{12}$, 977.5164), indicating 23 degrees of unsaturation. The ^{1}H NMR spectrum (Supporting Information, Table S1) of **1** displayed resonances attributable to 11 methyl groups ($\delta_H = 0.92$ (d, $J = 6.4$ Hz), 0.98 (d, $J = 6.4$ Hz) × 2, 1.04 (d, $J = 6.5$ Hz), 1.22 (d, $J = 7.0$ Hz), 1.25 (d, $J = 7.2$ Hz), 1.31 (s), 1.42 (s), 1.75 (s), 1.78 (s), 2.01 ppm (s)), four olefinic methines ($\delta_H = 5.21$ (br s), 5.31 (br s), 6.06 (d, $J = 9.3$ Hz), 6.09 ppm (d, $J = 10.7$ Hz)), and four oxygenated methine protons ($\delta_H = 4.06$ (dd, $J = 6.3$, 1.5 Hz), 4.32 (dd, $J = 7.2$, 6.5 Hz), 5.17 (d, $J = 5.1$ Hz), 5.33 ppm (s)). The ^{13}C NMR (Supporting Information, Table S1) and DEPT spectra of **1** exhibited 57 resonances, including six carbonyls, two amide carbonyls, 10 quaternary carbons (including six olefinic carbon), 22 methine (including four olefinic carbons), six methylene, and 11 methyl groups. The aforementioned spectroscopic data, along with the coexistence of aspochalasin P (**5**),[9] implied that **1** might be an dimeric cytochalasan alkaloid.

All of the protons were assigned to their respective carbons by careful analysis of the HSQC, and the dimeric nature of **1** was further confirmed by the paired signals of the aspochalasin alkaloids. Preliminary analysis of the 2D NMR spectra of **1** (Figure 2) revealed two identical aspochalasin monomers (units A and C) comparable to **5** by ^{1}H-^{1}H COSY correlations 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, and H-19/H-20, and by HMBC interactions from H-4 to C-1, C-9, and C-21, from Me-12 to C-5, C-6, and C-7, from Me-25 to C-13, C-14, and C-15, from H-17 to C-18 and C-19, and from H-19 and H-20 to C-21. For unit C, the ^{1}H-^{1}H COSY and HMBC interactions were almost identical to those of unit A.

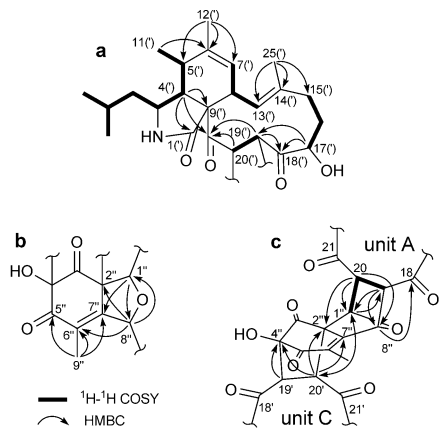


Figure 2. Key HMBC and ^{1}H-^{1}H COSY correlations of **1** (**a**: units A and C; **b**: unit B; **c**: fusions of units A and C to B).

On the basis of those analyses, the remaining signals were assigned to two carbonyls ($\delta_C = 197.1$, 207.9 ppm), four quaternary carbons ($\delta_C = 69.3$, 89.0, 127.4, 162.0 ppm), two methines ($\delta_C = 83.3$, 83.6 ppm; $\delta_H = 5.33$ (s), 5.17 ppm (d, 5.1 Hz)), and a methyl ($\delta_C = 11.9$ ppm; $\delta_H = 2.10$ ppm (s)). The ^{13}C NMR resonances of $\delta_C = 197.1$, 127.4, and 162.0 ppm suggested the presence of an α,β-unsaturated ketone moiety containing a trisubstituted double bond, which was further confirmed by HMBC cross-peaks of Me-9″ to C-5″, C-6″, and C-7″. Furthermore, HMBC correlations from H-1″ to C-7″ and C-8″, and from H-8″ to C-1″, C-2″, and C-6″, together with the presence of the free carbonyl group ($\delta_C =$ 207.9 ppm), preliminarily suggested a gross structure of unit B with a carbon skeleton similar to that of the co-isolated compound **6** (epicoccine, 1,3-dihydro-7-methyl-4,5,6-trihydroxy-isobenzofuran).[10] However, the structural determination of this moiety still requires more convincing evidence.

The ^{1}H-^{1}H COSY cross-peak of H-20/H-1″ and the HMBC interactions from H-19 to C-1″, C-7″, and C-8″, from H-20 to C-1″, C-2″, and C-8″, from H-1″ to C-19, and from H-8″ to C-18 suggested the connection of units A and B through C-19/C-8″ and C-20/C-1″ C–C bonds. Thus, an unusual 7-oxabicyclo[2.2.1]heptane moiety (rings D and E) connected units A and B. Furthermore, HMBC correlations from H-19′ to C-4″ and from H-20′ to C-1″, C-2″, and C-4″ verified the conjunction of units B and C through the C-19′/C-4″ and C-20′/C-2″ linkages to construct a bicyclo[3.2.1]octane motif (rings F and G), which together with the aforementioned rings D and E, constituted the unique core structure of 13-oxatetracyclo[7.2.1.1^{2,5}.0^{1,6}]tridec-8,12-dione. Therefore, the gross structure of compound **1** was elucidated to be an unprecedented epicoccine-containing dimeric cytochalasan alkaloid featuring a decacyclic 5/6/11/5/5/6/5/11/6/5 ring system.

The NOESY correlations for rings A–C (or A′-C′) (Supporting Information, Figure S1) closely resembled those of **5**, which implied the *cis* and *trans* fusion for rings A/B (or A′/B′) and B/C (or B′/C′), respectively. Moreover, the boat confirmation of ring B (or B′) was determined by NOESY correlation of H-5 and H-8 (or H-5′ and H-8′). Furthermore, NOESY interactions among H-8/H-19 (H-8′/H-19′), H-8/Me-25 (H-8′/Me-25′), and H-19/Me-25 (H-19′/Me-25′) were observed, suggesting the β-orientation of H-19 (H-19′). Consequently, NOESY correlations between H-13/H-20 (H-13′/H-20′) and H-13/H-17 (H-13′/H-17′) indicated the α-orientations of H-17 (H-17′) and H-20 (H-20′). Therefore, the relative configurations of units A and C were determined.

Although no useful NOESY signals were observed for H-1″ and H-8″, there are two possible stereoisomers with respect to the previously determined units A and B featuring opposite configurations of the oxygen bridge (**I** and **II**, Figure 3), and molecular modeling studies were performed on both stereoisomers of the A-B fusion unit. The protons of H-19 ($\delta_H =$ 3.09 ppm (d, $J = 5.5$ Hz)) and H-8″ ($\delta_H = 5.33$ (s)) showed neither ^{1}H-^{1}H coupling nor a ^{1}H-^{1}H COSY correlation with each other in **1**, which is consistent with a dihedral angle of approximately 90° between those protons. Careful inspection of the molecular models revealed that only **I**, with a 1″α, 8″α-oxygen bridge, satisfied the aforementioned condition. Thus,

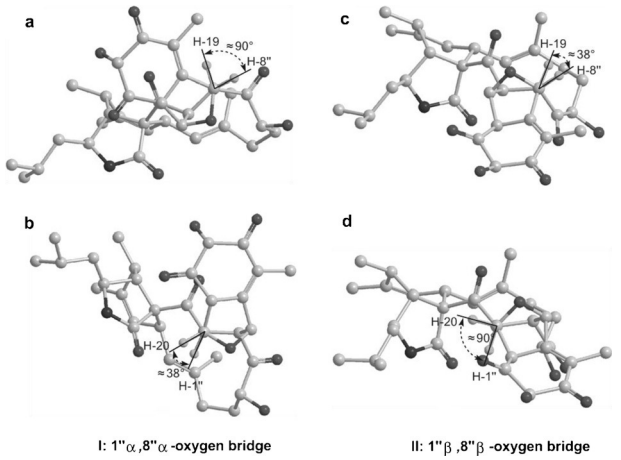


Figure 3. Molecular models of **I** (1″α,8″α-oxygen bridge) and **II** (1″β,8″β-oxygen bridge) showing the dihedral angles of H-19/H-8″ and H-20/H-1″ in units A and B (**a** and **b** for H-19/H-8″ and H-20/H-1″ in **I**; **c** and **d** for H-19/H-8″ and H-20/H-1″ in **II**).

the fusion pattern for units A and B in **1** was preliminarily determined as configuration **I** (Figure 3).

Owing to the lack of NOESY correlations, the relative configuration of the remaining quaternary carbons, C-2″ and C-4″, could not be determined. Therefore, further evidence, such as single-crystal X-ray diffraction analysis, was necessary to confirm both the planar structure and the stereochemistry of **1**. After many attempts at recrystallization with various solvent systems and temperature conditions, a single crystal of **1** suitable for X-ray analysis was successfully obtained from $MeOH/CH_2Cl_2/H_2O$ (20:1:1) in a closed tube at room temperature for more than one month. The X-ray diffraction analysis, conducted with Cu Kα radiation (Flack parameter of 0.11(2), Figure 4),[11] unambiguously confirmed the full structure and absolute configurations of all of the asymmetric carbon centers of **1** as 3(3′)*S*,4(4′)*R*,5(5′)*S*,8(8′)*S*,9(9′)*S*,17(17′)*R*,19(19′)*S*,20(20′)*S*,1″*R*,2″*R*,4″*R*,8″*S*. Despite the high degree of stereochemical complexity, **1** was determined to be the first dimeric cytochalasan alkaloid that contained an epicoccine moiety and was uniquely defined by the decacyclic 5/6/11/5/5/6/5/11/6/5 ring system.

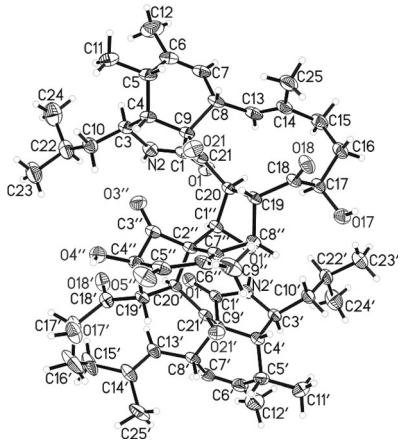
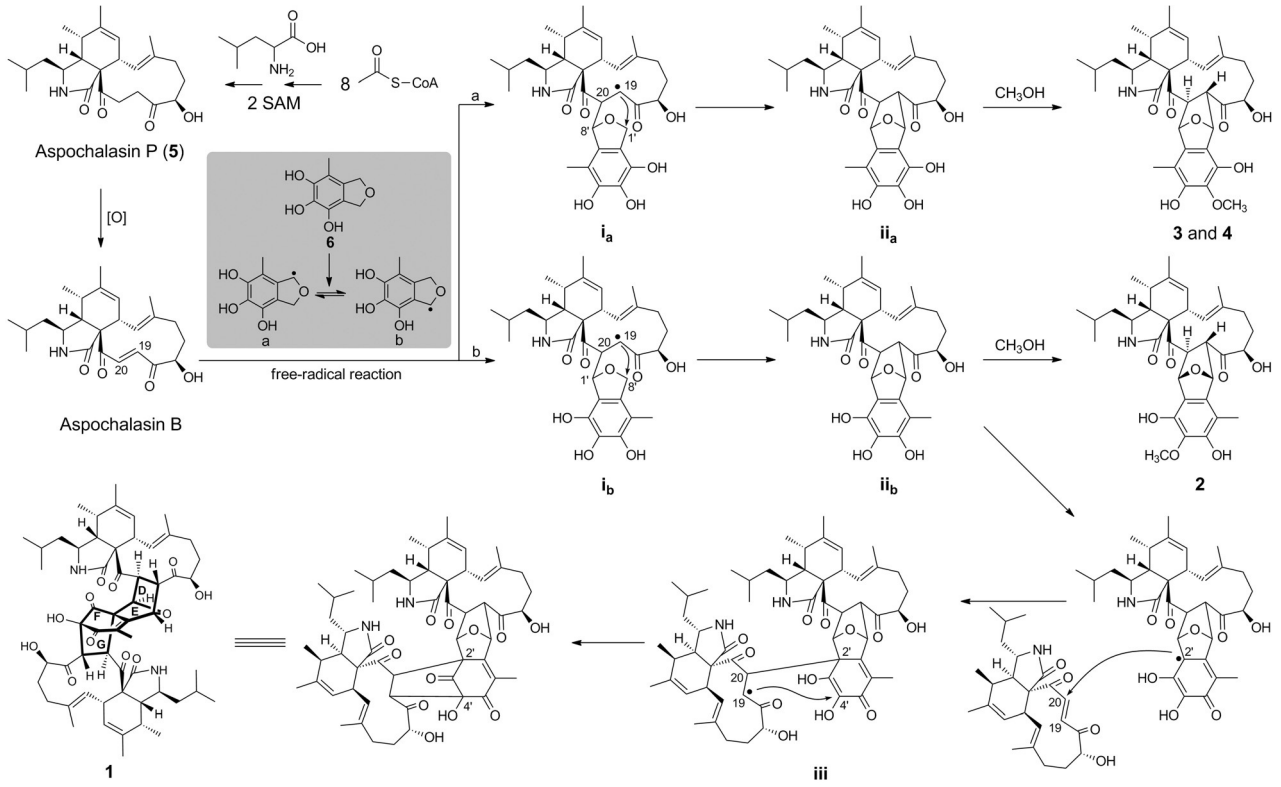


Figure 4. X-ray structure of **1**.[16] Ellipsoids are set at 30% probability.

Compound **1** is the first example of a cytochalasan dimer. The biosynthetic pathways for **1**–**4** (for details of the structural elucidations of **2**–**4**, see the Supporting Information) were proposed starting from the co-isolates **5** and **6** by means of a free-radical mechanism (Scheme 1). Compound **5** underwent oxidation to give aspochalasin B, and at the same time, **6** was activated to generate the free radicals **a** and **b**. Then, aspochalasin B underwent an intermolecular free-radical reaction with **a** and **b** to give the intermediates $\mathbf{i}_a$ and $\mathbf{i}_b$, respectively, which was followed by cyclizations via an intramolecular free-radical reaction to give $\mathbf{ii}_a$ and $\mathbf{ii}_b$. After that, compounds **2**–**4** were formed by a subsequent methylation reaction. The intermediate $\mathbf{ii}_b$ then reacted with another molecule of aspochalasin B by means of repeated intermolecular and intramolecular free-radical reactions to give the complex dimeric cytochalasan **1**. It is noteworthy that both the fusion of units A/B and the fusion of units B/C generated bridged bicyclic frameworks (rings D/E and F/G).

Asperchalasines A–D (**1**–**4**) were evaluated for their cytotoxic activities by the MTS assay, and all of them showed weak inhibitory activities (Supporting Information Figure S5). Confocal fluorescence microscopy was carried out to visualize the F-Actin microfilaments of Rko cells after treatment with compound **1**. F-actin was found to be disrupted by compound **1** (20 μM; 1 h) compared with the control (Supporting Information, Figure S6), which suggested that **1** is an effective cytoskeletal inhibitor, similar to some other cytochalasans, such as chaetoglobosin A.[3b] Compound **1** was further tested for its influence on the cell cycle with four human cancer cell lines and two normal cell lines. Compound **1** induced significant G1-phase cell cycle arrest in the four tested cancer cell lines but almost no effect on the normal cell lines (Figure 5A,B). To better understand the potential mechanism, western blot analysis was preformed to investigate the effect of compound **1** on cyclin A, CDK2, and CDK6, which are involved in the G1-phase and G1 to S transition during the cell cycle.[12] Compound **1** reduced the expression of these proteins in cancer cells, but did not affect the amounts of them in normal cells (Figure 5C). Taken together, these results suggested that the selective G1 cell cycle arrest ability of **1** depends on the selective inhibition of cyclin A and CDKs in cancer but not normal cell lines. It is noteworthy that cytochalasans, such as chaetoglobosins A and K,[3b,13] have been reported to cause G2-phase cell cycle arrest, and in this case, **1** was found to be the first cytochalasan to induce significant G1-phase cell cycle arrest and showed no noteworthy effect on G2-phase cells, which might make it a novel selective G1-phase cell cycle regulator against cancer cells, or act as a sensitizer in combinations with other anticancer drugs that profit from its effects on the cell cycle.

In conclusion, this study demonstrated the isolation and characterization of a new dimeric cytochalasan alkaloid (**1**) and three biogenetically related intermediates (**2**–**4**) from *A. flavipes*. Asperchalasine A (**1**), which bears a unique and intriguing decacyclic 5/6/11/5/5/6/5/11/6/5 framework, is the first dimeric cytochalasan. As shown in the proposed biosynthetic pathways, epicoccine plays an important role in the biosynthesis of these novel cytochalasans. Epicoccine was first isolated from the fungus *Aspergillus terreus* in 1996[10] and was



Scheme 1. Plausible biogenetic pathway for **1**–**5**.

isolated as a main secondary metabolite (> 4 g) of *A. flavipes* in this case. Epicoccine and its derivatives, including epicoccine dimers, were found to be inhibitors of a variety of enzymes,[14] such as protein kinases, histone deacetylase, and calcineurin. Their special structures and remarkable biological activities have already attracted considerable interest from organic chemists for total synthesis efforts.[15] Therefore, the discovery of the epicoccine-containing dimeric cytochalasan alkaloid asperchalasine A and the merger between cytochalasans and epicoccine derivatives may have an epoch-making significance in the research field of cytochalasans, which may lead to a strong upsurge in the research of cytochalasans and attract great interest from the total synthetic, biosynthetic, and pharmacological communities.

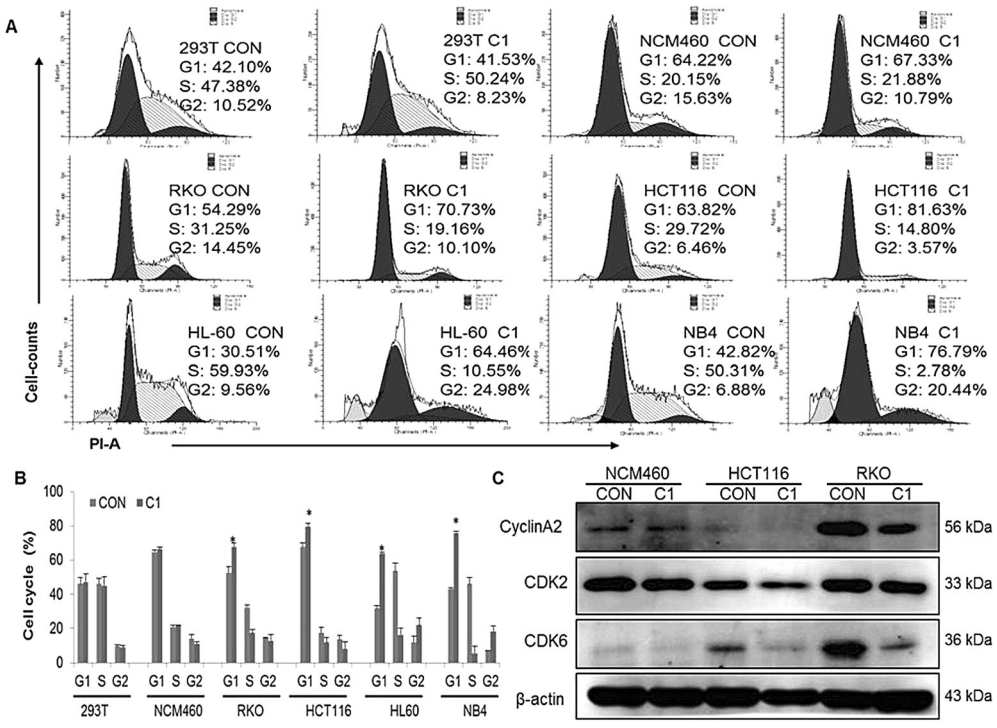


Figure 5. A, B) Effects of asperchalasine A (C1) on cell cycle progressions of four cancer cell lines (RKO, HCT116, NB4, and HL60) and two normal cell lines (293T and NCM460) at a concentration of 20 μM after 48 h treated. Columns, means of three different experiments; bars, SD, * $P < 0.01$ vs control group. C) Western blot analysis of cell cycle regulatory proteins. β-Actin was used as a loading control.

Acknowledgements

We thank the Analytical and Testing Center at Huaz-

hong University of Science and Technology for assistance in conducting ECD and IR analyses. This study was financially supported by the Program for New Century Excellent Talents in University, State Education Ministry of China (NCET-2008-0224), the National Natural Science Foundation of China (Nos. 31370372, 31200258, 31270395,81573316, and 81202423), and the National Science and Technology Project of China (Nos. 2011ZX09102-004 and 2013ZX09103001-020).

Keywords: alkaloids · Aspergillus flavipes · cytochalasan · dimers

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 13374–13378
Angew. Chem. **2015**, *127*, 13572–13576

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[16] CCDC 1039890 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

Received: July 8, 2015
Published online: September 3, 2015

