Asperflavipine A: A Cytochalasan Heterotetramer Uniquely Defined by a Highly Complex Tetradecacyclic Ring System from Aspergillus flavipes QCS12

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Abstract: Asperflavipines A (1) and B (2), two structurally complex merocytochalasans, were isolated from Aspergillus flavipes. Asperflavipine A (1), which contains two cytochalasan moieties and two epicoccine moieties, is the first cytochalasan heterotetramer to be discovered. It is uniquely defined by a highly complex tetradecacyclic ring system with three continuous bridged ring systems. Asperflavipine B (2) is a cytochalasan heterotrimer containing a cytochalasan and two epicoccine moieties with a nonacyclic ring system. The hypothetical biosynthesis of 1 and 2 is proposed to involve Diels–Alder and [3+2] cycloaddition reactions as key steps and reveals unparalleled plasticity in the biosynthesis of merocytochalasans. The existence of 1 adds a new dimension to the diversity of the cytochalasan family. Compound 1 showed moderate cytotoxicity and induced apoptosis in Jurkat, NB4, and HL60 cells through the activation of caspase-3 and degradation of poly(ADP-ribose) polymerase (PARP).

Cytoskeletal proteins are a class of fungal metabolites characterized by high structural diversity and a broad spectrum of bioactivity, including cytotoxicity, immunomodulatory and antiviral activity. An extensive literature investigation revealed that more than 300 cytochalasans have been identified to date, and a number of excellent studies have been reported in recent years. In previous studies, we discovered several bioactive merocytochalasans (see Figure S1 in the Supporting Information), including asperchalsines A–D and epicochalasines A and B. Recently, the synthesis of epicoccine derivatives and their dimers has become a hot topic in organic chemistry. From this viewpoint, the incorporation of epicoccines with cytochalasans to form structurally complex and biologically active merocytochalasans is extremely interesting. Inspired by the broad prospects, we continued our studies on Aspergillus flavipes of various origins.

Our study of an A. flavipes derived from soil (Qichun County, China) led to the isolation of two structurally complex merocytochalasans, asperflavipines A (1) and B (2; Figure 1). Asperflavipine A (1) is the first cytochalasan heterotetramer to be discovered and is uniquely defined by an unprecedented 5/6/11/5/6/5/6/5/6/5/11/6/5 fused tetradecacyclic ring system with 26 stereogenic centers. The existence of 1 adds a new dimension to the diversity of the cytochalasan family. Asperflavipine B (2) is a cytochalasan heterotrimer containing one cytochalasan and two epicoccine moieties. Its unexpected coupling patterns indicated a unique skeleton. Herein, we report the isolation, structure elucidation, and bioactivity evaluation of asperflavipines A (1) and B (2). Moreover, a hypothetical biosynthesis of 1 and 2 is proposed that involves a Diels–Alder reaction and [3+2] cycloaddition (Scheme 1).

The molecular formula of asperflavipine A (1), C_{66}H_{80}N_{2}O_{17}, with 28 degrees of unsaturation, was deduced...
from its high-resolution (HR) ESIMS spectrum with a molecular ion at \( m/z \) 1195.5338 (\([M+Na]^+\) calcd for \( C_{66}H_{46}N_2O_{17}\Na: 1195.5355\)). Detailed analysis of the \(^1\)H, \(^13\)C, DEPT, and HSQC NMR spectra of I revealed the presence of 66 carbon resonances: 12 CH\(_2\) (\( \delta_c = 24.7, 24.4, 23.3, 21.6, 20.2, 19.7, 18.3, 17.4, 16.3, 14.1, 13.9, \) and 12.4 ppm), 6 CH\(_3\) (\( \delta_c = 52.1, 51.3, 37.8, 32.6, 32.4, \) and 31.6 ppm), 24 CH (including four C(sp\(^3\)), \( \delta_c = 127.0, 126.6, 124.6, \) and 123.8 ppm, and 6 oxygenated C(sp\(^3\)), \( \delta_c = 85.8, 83.4, 81.6, 79.6, 77.9, \) and 74.6 ppm), 16 quaternary carbons (including 8 C(sp\(^3\)), \( \delta_c = 161.0, 148.7, 141.0, 140.5, 138.9, 138.2, 133.9, \) and 125.1 ppm, and 3 oxygenated C(sp\(^3\)), \( \delta_c = 100.8, 94.6, \) and 92.2 ppm), 6 carbonyl carbons (\( \delta_c = 215.9, 212.7, 207.3, 195.3, 194.2, \) and 187.2 ppm), and two amide carbonyl carbon signals (\( \delta_c = 177.1 \) and 176.3 ppm; see Table S1 in the Supporting Information). All hydrogen atoms were further assigned to carbon centers on the basis of an HSQC spectrum. These data suggested that compound I is an unusual merocytochalasan.

Elucidation of the structure of I was initiated by strong HMBC and \(^1\)H–\(^1\)H COSY correlations, which led to the construction of four gross units A–D (Figure 2). Units A and D were aspochalasin moieties with a 5/6/11 ring system, whereas units B and C were highly functionalized epicoccine moieties. Further analysis of HMBC and \(^1\)H–\(^1\)H COSY spectra led to the elucidation of the fusion patterns of units A–D that constructed the planar structure of I. HMBC cross-peaks of H-19 with C2, H-20 with C8' and C7', and H-8 with C21, together with a \(^1\)H–\(^1\)H COSY correlation between H-19 and H-1' (Figure 3), and consideration of the chemical shifts of C18 (\( \delta_c = 100.8 \) ppm) and C3' (\( \delta_c = 92.2 \) ppm), suggested the fusion of units A and B through C18/O/C3', C19/C1', and C20/C8'. Meanwhile, the connection of units C and D was determined by HMBC interactions between H-19'' and C7'', H-20'' and C2', H-1'' and C19'', and H-8'' and C20'', as well as a \(^1\)H–\(^1\)H COSY cross-peak for H-8'/H-19'' (Figure 3). Moreover, HMBC correlations from H-8' and Me-9' to C2'' and from H-1'' to C7' (Figure 3) revealed the connection of units B and C through a C7'/C2'' bond. The C2'/C4'' bond was constructed to satisfy the molecular formula and the quaternary nature of C2' (\( \delta_c = 53.2 \) ppm) and C4'' (\( \delta_c = 94.6 \) ppm).

The fascinating connections of units A/B, B/C, and C/D indicate that I possesses an unusual ring-fusion pattern with three continuous bridged ring systems involving rings D/F, H/ I, and J/K.

Detailed analysis of the NOESY spectrum, including H-3/ Me-11, H-4/H-10, H-5/H-8, H-8/Me-25, H-13/H-17, H-17/H- 20, and H-19/Me-25 correlations (Figure 4), together with the large coupling constant between H-8 and H-13 (\( J = 11.1 \) Hz), disclosed the relative configuration of unit A. Moreover, the hydroxy group at C18 was determined to be \( \beta \)-oriented according to the NOESY correlation between H-17 and H-20. Owing to the caged skeleton of rings D–G, the NOESY correlation H-20/Me-9' revealed the \( \beta \)-orientation of the 1',8'-oxygen bridge, which was consistent with the lack of coupling observed for H-20/H-8' because their dihedral angle was approximately 90°. Furthermore, the caged rings D–G also ensured the relative configuration of C2' and C7' of unit B, and therefore, unit C could only be located on the left side of unit B. The relative configuration of units C/D, including the \( \beta \)-oriented oxygen bridge C1'–O–C8', was elucidated from the NOESY spectrum, as discussed for units A/B (Figure 4). However, no evidence existed from which the relative configuration of C2'' and C4'' could be determined directly. Further considering the bridged rings H and I, the configuration of C2'' and C4' should be R,S or S,R. Therefore, the remaining two stereogenic centers C2'' and C4'' in unit C led...
to two candidate stereoisomers 1a and 1b, and only 1a satisfied the NOESY correlations of H-8'/H-1', Me-9'/H-1', and Me-9'/H-8', as shown in the Chem3D molecular modeling simulation (Figure 5). Thus, the structure of 1 was established with its relative configuration.

![Figure 5](image)

Figure 5. Two possible stereoisomers of 1 (1a and 1d) showing the moiety composed of units B and C (including key NOESY correlations and atom distances calculated with Chem3D).

Asperflavipine B (2) was determined to have the molecular formula C_{42}H_{38}O_{12} on the basis of the HRESIMS ion at m/z 778.2827, which together with the 1H and 13C NMR data suggested that it is also a merocytochalasan. Extensive analysis of the 1D and 2D NMR spectra of 2 uncovered the gross structures of units A–C (see Figure S2), and further analysis of the HMBC and 1H–1H COSY spectra revealed the fusion patterns of units A/B and B/C (see Figure S2). A 1H–1H COSY cross-peak for H-20/H-1' and HMBC interactions between H-19 and C7', H-20 and C2', and H-8' and C18 suggested that unit B was turned over in 2 as compared with that of 1. Furthermore, HMBC correlations between H-1' and C4', H-8' and C6', and Me-9' and C7' established the linkage of units B/C through C2/C4' and C7/C6' bonds. The relative configuration of unit A and the 1α,8α-α-oxygen bridge of 2 were determined by analysis of its NOESY spectrum and coupling constants. The relative configuration of units B and C of 2 was further elucidated by detailed analysis of the NOESY spectrum, as aided by molecular modeling with Chem3D (see Figure S3). The key NOESY correlations between Me-9' and H-19 revealed that ring G is oriented vertically and above the plane of the rings to which is fused, and indirectly confirmed the relative configuration of C2' and C7'. Finally, NOESY correlations H-8'/Me-9' and Me-9'/H-8' determined the relative configuration of C4' and C6' in unit C (see Figure S3).

To further confirm the elucidated structures of asperflavipines A (1) and B (2), we performed computational predictions of their 13C NMR spectra (see Table S2). The calculated 13C NMR data showed good agreement with the experimental values, with average absolute deviations of 2.7 ppm for both compounds 1 and 2 (see Table S2). The absolute configurations of 1 and 2 were finally determined by comparison of their experimental electronic circular dichroism (ECD) spectra with the computed spectra (Figure 6).

![Figure 6](image)

Figure 6. Experimental and calculated ECD spectra of 1 (top) and 2 (bottom).

It is always challenging to determine the configuration of structurally complex natural products, regardless of the relative or absolute configuration. Merocytochalasans, such as asperchalasines A–D[6] and compounds 1 and 2, with the configuration of unit A fixed as shown, always possess an oxygen bridge in unit B that is formed by a Diels–Alder reaction. Their relative configurations were assigned cautiously by analyzing the dihedral angles and coupling constants of H-20/H-8' (or H-1') and H-19/H-1' (or H-8'). In this study, to facilitate the determination of the configuration of these compounds, we extensively analyzed the splitting patterns and coupling constants of these related protons. Owing to the changeable positions of C1' and C8' (front or back sides) and the uncertainty in the type of carbon atom present as C18 (CH, C=O, or C), H-1', H-8', and H-19 are not suitable as markers to determine the configuration of the oxygen bridge. However, the splitting pattern of H-20 is ideal for determining the configuration of the oxygen bridge because C21 is always a carbonyl carbon atom and H-20 is only adjacent to H-19 and H-1' (or H-8'). When the oxygen bridge is β-oriented, the dihedral angle between H-20 and H-1' (or H-8') is approximately 90°, and H-20 only couples with H-19 and appears as a doublet; when the oxygen bridge is α-oriented, the dihedral angle of H-20 and H-1' (or H-8') is no longer 90°, and H-20 couples with both H-19 and H-1' (or H-8'), and it appears as a double doublet or a triplet. Therefore, the splitting pattern of H-20 can serve as a benchmark to determine the configuration of the oxygen bridge. In short, a doublet for H-20 indicates a β-oriented oxygen bridge, and a double doublet or triplet suggests an α-oriented oxygen bridge in these merocytochalasans. To the best of our knowledge, all cytochalasans for which the absolute config-
uration has been determined share the same absolute configuration in the core structure (at C3, C4, C5, C8, and C9). This consistency may be largely due to the stereoselectivity of enzymes and the use of L-amino acids as precursors in biosynthetic pathways[9] and should always be considered during efforts to determine the absolute configuration of cytochalasans.

The hypothetical biosynthesis of asperflavipines A (1) and B (2) is proposed to involve a Diels–Alder reaction and [3+2] cycloaddition as key steps (Scheme 1). An intermolecular Diels–Alder reaction between aspochalasin D (unit A) and an epicoccine moiety (unit B) lead to the linkage of C19/C8’ and C20/C1’ with an α-oriented oxygen bridge to give vii. Next, another epicoccine moiety (unit C) is added through [3+2] cycloaddition to give ix, and an oxidation step provides 2.

It would seem that compound 1 should be constructed unit by unit, similarly to compound 2. However, careful analysis of the structure revealed that it is impossible for the Diels–Alder reaction to take place between aspochalasin D and unit C if the fusion of units A–C is complete, because the existence of the quaternary C2” atom blocks the possibility of forming a 1”,7”-diene motif in unit C. Therefore, we propose that the [3+2] cycloaddition occurs between two merocytochalasan intermediates iii and iv, which leads to the unprecedented cytochalasan heterotrimer bearing two cytochalasan moieties and two epicoccine moieties. As shown in Scheme 1, [3+2] cycloaddition plays an important role in the construction of the merocytochalasans of asperflavipines A (1) and B (2). These pathways reveal unparalleled plasticity of the biosynthesis of merocytochalasans and may provide new insight into the inestimable chemical diversity of the merocytochalasan family.

Compound 1 was found to possess moderate cytotoxicity against seven cancer cell lines, with IC_{50} values ranging from 12.7 to 27.6 μM (Figure 7A, B; 2 was inactive up to 40 μM). Jurkat, NB4, and HL60 cells were used as models to further analyze the anticancer effects of 1. Cell shrinking and severe blebbing of the plasma membrane was observed after treatment with 1, thus suggesting that 1 probably induced apoptosis in these cells.[10] Flow cytometry analysis of cells treated with 1 showed that 1 induced apoptosis in these tested cells (Figure 7C). As compared with the control group (DMSO, <0.1%), treatment with 1 at 12 μM resulted in 45.7, 54.7, and 55.9% apoptosis incidence in Jurkat, NB4, and HL60 cells, respectively. Furthermore, treatment with compound 1 altered the expression levels of poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 (Figure 7E). Taken together, these results suggested that compound 1 induced apoptosis in these leukemia cells through the activation of caspase-3 and degradation of PARP. It is notable that compound 1 is much more cytotoxic than 2. Further studies are necessary to develop a deeper understanding of the cytotoxicity of 1 and the structure–activity relationship of the heterotetrameric and heterotrimeric merocytochalasans.

In conclusion, the first cytochalasan heterotetramer asperflavipine A (1) and an unexpected cytochalasan heterotrimer asperflavipine B (2) were isolated from a solid cultivation of A. flavipes QCS12. Compound 1 is uniquely defined by a tetradecacyclic ring system with three continuous bridged ring systems. It possesses 26 stereogenic centers (including 8 quaternary carbon centers) and consists of two cytochalasan moieties and two epicoccine moieties. The discovery of 1 adds a new dimension to the diversity of the merocytochalasan family and may attract increased interest from the biosynthetic, synthetic, and pharmacological communities for further investigation.

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Figure 7. Inhibition of proliferation and induction of apoptosis in cancer cells by compound 1. A) Dose-response viability curves for seven cancer cell lines after treatment with 1 for 48 h (mean ± standard deviation (SD) for three independent experiments). B) IC$_{50}$ value of 1 for each of the cell lines, as calculated with SPSS software. C) Cell apoptosis was determined by annexin V-FITC and propidium iodide (PI) staining and flow cytometric analysis after treatment with 1 (12 μM) for 48 h. Cells in the lower-right quadrant are early apoptotic cells, and cells in the upper-right quadrant are late apoptotic cells. D) Columns show the mean of three independent fluorescence-activated cell sorting (FACS) assays for apoptosis; bars correspond to the standard deviation; P values were calculated by a two-tailed Student t-test. E) Western blot analysis for the apoptosis marker PARP and cleaved caspase-3; β-actin was used as the loading control.

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Conflict of interest

The authors declare no conflict of interest.

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