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Structure and Total Synthesis of Aspernigerin: A Novel Cytotoxic Endophyte Metabolite

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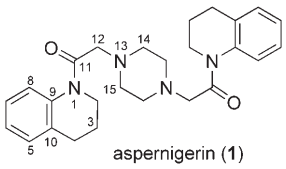
Abstract: Aspernigerin (**1**), a novel cytotoxic alkaloid consisting of an unprecedented structural framework has been isolated from the extract of a culture of *Aspergillus niger* IFB-E003, an endophyte in *Cyndon dactylon*. Its structure was elucidated on the basis of comprehensive NMR spectral analysis and confirmed by single-crystal X-ray analysis. Aspernigerin (**1**) has been shown to be cytotoxic to the tumor cell lines nasopharynyeal epidermoid KB, cervical carcinoma Hela, and colorectal carcinoma SW1116 with corresponding IC_{50} values of 22, 46, and 35 μM, respectively. A feasible total synthetic route for aspernigerin (**1**) has been established for further pharmacological research.

Keywords: aspernigerin · cytotoxic · endophyte · structure elucidation · total synthesis

Introduction

A fast-growing wealth of reports has affirmed that cancer has been and will continue to be one of the major life-threatening diseases in both developed and developing countries owing principally to the shortage of efficacious tumor-eradicating/suppressing agents. Comparable to taxol (a plant secondary metabolite detectable in most *Taxus* species), some microbe-generated natural products were proven to be quite unique in treating/preventing cancerous disorders.[1] Whilst current chemical and biological interests are being focused on early microbial secondary metabolites, such as prodigiosin derivatives,[2] it has been postulated that microorganisms collected from previously unexplored habitats may provide access to a large family of efficient producers of novel lead structures for new antitumor agents.[3] As a follow up to our characterization of tumor suppression related metabolites from marine microbes,[4] we expanded our curiosity to include endophytes, a large population of poorly investigated microbes, which have been reasoned to be unique, at least with regards to the fact that they spend their life spans in a symbiotic manner inside the living normal plant tissue where they could have "gene recombinations" with the host species.[5]

Aspergillus niger IFB-E003 was isolated as an endophyte from the healthy leaves of *Cynodon dactylon*, a salty soil borne grass belonging to the family Graminae. Previously, this endophytic strain was disclosed to be a rich source of bioactive naphtha-pyrone framed polyphenols.[6] Surprisingly, the combined mother liquor left upon fractionations of those phenolic metabolites from the extract was demonstrated to contain basic substances with pronounced cytotoxic activity. Subsequent attention to the cytotoxic fraction led to the full characterization of an active alkaloid with an unprecedented structural framework, trivially named aspernigerin (1,4-bis[2-(3,4-dihydro-2*H*-quinolin-l-yl)-2-oxoethyl]piperazine **1**). This paper is dedicated to the structure elucidation, cytotoxicity, and total synthesis of this novel antitumor endophyte metabolite.



aspernigerin (**1**)

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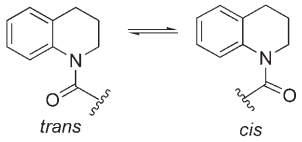
Supporting information (Figures S1–S9 and Table S1) for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

Results and Discussion

Aspernigerin (**1**) was afforded as colorless crystals. Its molecular formula $C_{26}H_{32}N_4O_2$, necessitating 13 degrees of unsaturation, was evidenced from the protonated molecular ion at *m*/*z* 433.2605 (calculated for the formula of molecular mass *m*/*z* 433.2598) in its HR (+)-ESI mass spectrum. However, the ^{13}C NMR spectrum of **1** produced a total of only twelve carbon resonance lines, implying that it was a highly symmetric molecule consisting most probably of two identical motifs, each containing a pair of equivalent carbons. A correlative interpretation of its IR, ^1H and ^{13}C NMR (Figures S1 and S2 see Supporting Information), and DEPT spectra suggested that each motif was composed of a 1,2-disubstituted benzene nucleus, an amine, an amide carbonyl, and six methylenes, two sets of which correspond altogether to ten degrees of unsaturation. The three that were left had to be compensated for by creating three more rings for the structure of **1**. With this in mind, subsequent scrutiny of its 2D NMR spectra (HMBC, HMQC, COSY, and NOESY, Figures S3–S6), allowing for the unambiguous assignment of all carbon and proton signals, generated the structural assumption for aspernigerin (**1**) on the basis of the following spectral features.

The COSY correlations of the H-3 quintuplet with a pair of triplets produced by H-2 and H-4 revealed the $-CH_2CH_2CH_2-$ moiety isolated by heteroatom(s) and/or quaternary carbon(s). However, the magnitude of the C-2/H-2 shift values ($\delta_C/\delta_H = 43.4/3.80$ ppm) confirmed the anchorage of 2-methylene on a more electron-poor nitrogen atom. Along with the discernible NOE effect and allyl-like coupling of H-4 with H-5, the HMBC correlations of H-3 to C-10, of H-4 to C-5, and of H-5 to C-4 highlighted the connection of 4-methylene to C-10 ($\delta_C = 133.0$ ppm) in the benzene nucleus. These observations, in conjunction with the HMBC correlations of H-2 to C-9 and C-11 ($\delta_C = 139.0$ and 169.0 ppm, respectively), disclosed the presence of the *N*-acyl-1,2,3,4-tetrahydroquinoline residue. Subtracting signals due to the residue from the ^1H and ^{13}C NMR spectra of **1** produced only two methylene resonances at $\delta_C/\delta_H = 60.7/3.28$ (s) and 53.0/2.56 ppm (s) with an integration ratio of 1:2. These spectral data, along with its molecular formula and the remaining unsaturation degrees (see above), could only be explained by assuming the presence of a piperazine ring with both nitrogen atoms bridged through a methylene group to the carbonyl of the *N*-acyl-1,2,3,4-tetrahydroquinoline residue. Moreover, this assumption was reinforced by the HMBC correlations of H-12 to C-11 and C-14/15, and the NOE cross-peak between H-12 and H-14/15. Thus, the structure of the cytotoxic alkaloid was determined to be 1,4-bis[2-(3,4-dihydro-2*H*-quinolin-l-yl)-2-oxoethyl]piperazine (**1**). For simplicity, we have trivially named the alkaloid aspernigerin.

Amide-containing molecules might show *trans*/*cis* equilibriums in solution, which could be assigned by NMR techniques.[7,8] As for aspernigerin (**1**), a broadened H-8 singlet at $\delta = 7.45$ ppm meant that the amide C=O must be oriented preferably towards the phenyl ring (Scheme 1).[8c] Furthermore, the splitting pattern of the 2,3,4-methylene proton pairs, which appeared as triplet, quintuplet, and triplet, respectively, highlighted that the reduced ring of the tetrahydroquinoline moieties was not in a rigid half-chair conformation, but in a rapidly inverting half-chair conformation.[9] The anticipated conformation of **1** in solution was contrary to that of some previously recognized analogues.[8c,d]



Scheme 1. Amide-bond rotation of aspernigerin (**1**).

Accordingly, X-ray crystallographic analysis of this metabolite was highly desired for the structural and conformational assignment of **1**. A colorless single crystal of **1** obtained from its MeOH solution was found to be suitable for the X-ray diffraction analysis and the data subsequently obtained confirmed the structure elucidated for **1** (Figures 1 and S9). Moreover, this crystallographic determination revealed that the piperazine at the center of the molecule was in a chair-conformation with the two carbonyl groups stretching towards two opposite directions.

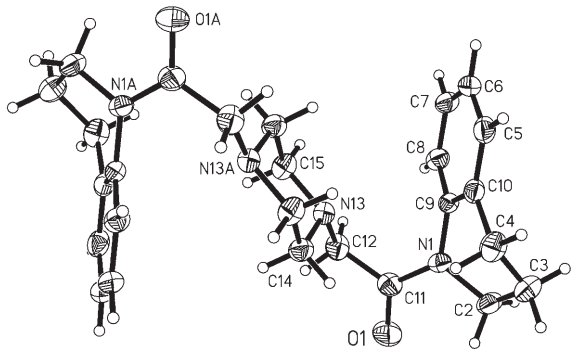


Figure 1. X-ray crystallographic structure of **1**.

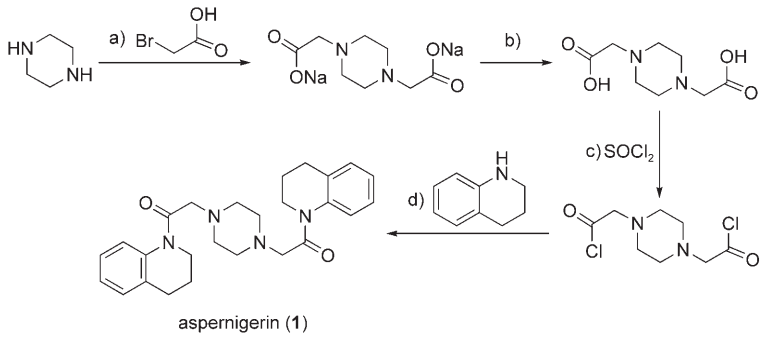
Abstract in Chinese:

从狗牙根内生真菌*Aspergillus niger* IFB-E003的发酵物提取液中分离得到1个新骨架的生物碱aspernigerin (**1**)，其结构通过波谱尤其是1D和2D NMR解析及X-单晶衍射得到确证。Aspernigerin (**1**)对人的口腔表皮样癌细胞KB、宫颈癌细胞Hela、结肠癌细胞SW1116表现出较强的活性，其IC_{50}值分别为22, 46 和 35 μM。此外，为进行进一步的药理实验还进行了aspernigerin (**1**)的全合成。

The conformation of the amide in **1** determined by its NMR spectral data acquired in $CDCl_3$ was not completely consistent with that from its single-crystal X-ray diffraction, highlighting that its conformation is highly state-dependent. This is a reasonable assumption as the *trans*/*cis* rotation equilibrium codepends on temperature[8a,10] and the solvent.[7a,8b,11] Obviously, the $CDCl_3$ used in this case might be the appropriate solvent for the preferred rotamer (corresponding to that with carbonyls orienting towards the phenyl ring) of aspernigerin (**1**).

The in vitro cytotoxicity of aspernigerin (**1**) was evaluated against three human tumor cell lines, and as a result it was found to be significantly cytotoxic against the human tumor cell lines, nasopharynyeal epidermoid KB, cervical carcinoma Hela, and colorectal carcinoma SW1116, with the corresponding IC_{50} (the inhibition concentration at which 50% survival of cells was allowed) values of 22, 46, and 35 μM, respectively. The IC_{50} values of 5-fluorouracil (a clinically prescribed antitumor drug coassayed as a positive control) against the three tumor cell lines were 14, 115, and 42 μM, respectively.

To develop a feasible route both for the construction of the novel framework and for enough material of **1** required for the pharmacological research, total synthesis of aspernigerin (**1**) was carried out based on the retrosynthetic analysis illustrated in Scheme 2. Starting from commercially available piperazine and 1,2,3,4-tetrahydroquinoline, **1** could be synthesized in four steps in good yields. The synthetic material was identical to the natural metabolite (**1**, Figure S8). Regarding the manipulation of the reaction, it was noteworthy that addition of a catalytic amount of DMAP was required, which allowed the completion of the reaction at room temperature within 3–4 h.



Scheme 2. Total synthetic route for aspernigerin (**1**). Reagents and conditions: a) $BrCH_2COOH$, aqueous NaOH solution (10%), RT, 1 h, 80%; b) HCl (37%, several drops), RT, stirring, 10 min, 74.6%; c) $SOCl_2$, DMF, CH_2Cl_2, reflux, 4 h; d) 1,2,3,4-tetrahydroquinoline, DMAP, pyridine, CH_2Cl_2, 0 °C→RT, 4 h, 47.3% (steps c and d). DMF = *N*,*N*-dimethylformamide; DMAP = 4-dimethylaminopyridine.

Conclusion

We have characterized a novel potent antitumor metabolite, aspernigerin (**1**), from *A. niger* IFB-E003, an endophytic fungus residing in the healthy leaves of *C. dactylon*. For a chemical generation of the novel framework and a scaled-up production of this endophyte metabolite, we have also developed a feasible total synthetic route for **1**. In addition to its antitumor activity, comparable to that of 5-fluorouracil, aspernigerin (**1**) possesses a unique structural framework, not yet encountered in any presently available anticancer drug or investigated cytotoxic chemicals. Accordingly, this work is of particularly high significance for the research and development of a new family of antitumor drugs with such structural character, the biosynthetic pathway of which provides a big mystery for biochemists.

Experimental Section

General: Melting points were measured on a Boetius micromelting point apparatus and are uncorrected. IR spectra were recorded on KBr disks by using a Nexus 870 FTIR spectrometer, and UV spectra on a U-3000 UV-VIS spectrometer. NMR spectra were acquired in $CDCl_3$ on Bruker DRX-500 and DPX-300 spectrometers by using solvent signal and TMS as internal standards. The EIMS spectrum was recorded on a VG-ZAB-HS mass spectrometer, and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) spectrum on a Mariner Mass 5304 instrument. ELISA plate reader was obtained from Sunrise (USA). Silica gel (200–300 mesh) for column chromatography and silica GF_{254} for TLC were produced by Qingdao Marine Chemical Company (China). Sephadex LH-20 was purchased from Pharmacia Biotech (Sweden). All chemicals used in the study were of analytical grade.

Microorganism: The isolation and identification of the endophytic fungal strain *A. niger* IFB-E003 have been described in our previous paper.[6]

Extraction and isolation: Cultivation of strain IFB-E003 and fractionation for naphtha-pyrones have been previously detailed.[6] The combined mother liquor left therefrom, after having been tested for recognition of cytotoxic compounds, was rechromatographed by using silica-gel column chromatography (200 g silica gel; $CHCl_3$/MeOH 100:0→100:16). By using TLC monitoring, the elutes produced (500 mL each) were combined into six fractions, which were assessed for cytotoxicity. The active fraction (fraction 2) was further fractionated over silica gel ($CHCl_3$/MeOH 100:0→100:16) to afford three parts (fractions 2–1–fractions 2–3). Repeated gel filtration of fractions 2–2 (cytotoxic) over Sephadex LH-20 ($CHCl_3$/MeOH 1:1) produced aspernigerin (**1**, 93 mg). Colorless crystal; m.p. 186–188 °C; for ^1H and ^{13}C NMR data ($CDCl_3$) see Table 1; IR (KBr): $\tilde{\nu}$ = 2950, 2805, 1639, 1582, 1490, 1454, 1391, 1015, 845, 778 cm^{-1}; UV/Vis (MeOH): λ_{max} (log ε) = 246 (4.23), 215 nm (4.37); EIMS (70 eV): *m*/*z* (%): 432 $[M]^+$ (9), 300 (3), 272 (100), 217 (9), 172 (62), 111 (79), 56 (46), 42 (51); HR-ESI-MS: *m*/z: calcd for $C_{26}H_{32}N_4O_2$: 433.2598; found: 433.2605 $[M+H]^+$.

Crystal structure measurement of aspernigerin (1): Crystal structure determination was carried out on a Nonius CAD4 diffractometer equipped with graphite-monochromated $Mo_{K\alpha}$ (λ = 0.71073) with Lorentz polarization and absorption corrections for a crystal (0.25 × 0.20 × 0.20 mm) of the title compound. The intensities were collected at 293 K by using ω scan mode with variable scan speed. A total of 11639 reflections were collected in the range of θ = 2.37–25.99°, of which 2238 were independent. The 1831 observable reflections with $I \geq 2\sigma(I)$ were used in the structure solution and refinements. The structure was solved by direct methods and refined on F^2 by full-matrix least-squares methods by using SHELX-97.[12]

Table 1. NMR spectral data for aspernigerin (**1**) in $CDCl_3$.

C no.	δ_C [ppm]	δ_H [ppm], mult., int., J [Hz]	COSY	Key HMBC correlations
2/2′	43.4 CH_2	3.80, t, 4H, 6.5	H-3	C-3/3′, 4/4′, 9/9′, 11/11′
3/3′	24.0 CH_2	1.96, q, 4H, 6.5	H-2, H-4	C-2/2′, 4/4′, 10/10′
4/4′	26.8 CH_2	2.72, brt, 4H, 6.5	H-3, H-5	C-3/3′, 5/5′, 9/9′
5/5′	128.5 CH	7.13, m, 2H	H-4	C-4/4′, C-7/7′
6/6′	125.3 CH	7.09, m, 2H		C-5/5′, 8/8′,10/10′
7/7′	126.1 CH	7.14, m, 2H		C-5/5, 8/8′, 9/9′
8/8′	124.4 CH	7.45, brs, 2H		
9/9′	139.0 C			
10/10′	133.0 C			
11/11′	169.0 C			
12/12	60.7 CH_2	3.28, s, 4H		C-11/11′, 14,14′/15,15′
14/14′, 15/15′	53.0 CH_2	2.56, brs, 8H		

All the nonhydrogen atoms were refined anisotropically. All the hydrogen atoms were placed in calculated positions and were assigned fixed isotropic thermal parameters at 1.2 times the equivalent isotropic U of the atoms to which they are attached and allowed to ride on their respective parent atoms. The contributions of these hydrogen atoms were included in the structure-factors calculations. The refinement gave the final $R_1 = 0.042$ with $w = [\sigma^2(F_o)^2 + (0.1(\max(0,F_o^2) + 2F_c^2)/3)^2]^{-1}$. Some crystallographic and experimental data for aspernigerin (**1**, H_2O) are listed in Table S1.[13]

Cytotoxicity assay: The cytotoxicity was evaluated as described elsewhere[14] with some modifications. Briefly, target tumor cells were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. After diluting to 2×10^4 cells mL^{-1} with the complete medium, the obtained cell suspension (100 μL) was added to each well of 96-well culture plates. The subsequent incubation was permitted at 37°C, 5% CO_2 atmosphere for 24 h before the cytotoxicity assessments. Tested samples at preset concentrations were added to 6 wells with 5-fluorouracil coassayed as a positive reference. After 48 h exposure period, PBS (40 μL containing 2.5 mg mL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) was added to each well. After 4 h the medium was replaced by DMSO (150 μL) to solubilize the purple formazan crystals produced. The absorbance at 570 nm of each well was measured on an ELISA plate reader. The IC_{50} value was defined as the concentration at which 50% survival of cells was allowed.

Total synthetic procedure for aspernigerin (1): Solvents were dried and refined by the conventional procedure prior to use. DMF was distilled under reduced pressure from magnesium sulfate, dried twice over molecular sieves (4 Å), and stored over molecular sieves (4 Å). CH_2Cl_2 was distilled from P_2O_5. Pyridine was distilled from NaOH.

N,N′-1,4-Piperazinediacetic acid: Piperazine (150 mg, 1.74 mmol) and bromoacetic acid (496 mg, 3.57 mmol) were dissolved in NaOH (10%, 10 mL) and stirred for 1 h at room temperature. Colorless needles (namely, disodium *N,N′*-1,4-piperazinediacetate, 343 mg, 80% yield) were formed and collected by filtration. The obtained needles were dissolved in water and treated with several drops of HCl (37%) whilst stirring for 10 min at room temperature. The white precipitate produced was collected by filtration and dried in vacuo to give *N,N′*-1, 4-piperazinediacetic acid (210 mg, 74.6%). M.p. 250–252°C; ^1H NMR (D_2O): $\delta = 3.81$ (s, 4H; $2\times CH_2$), 3.58 ppm (s, 8H; $4\times CH_2$) (see Figure S7); ESIMS: m/z: 203.1 $[M+H]^+$.

*Aspernigerin (**1**)*: DMF (2 drops) and $SOCl_2$ (0.22 mL, 2.96 mmol) were added sequentially to a suspension of *N,N′*-1,4-piperazinediacetic acid (150 mg, 0.74 mmol) in CH_2Cl_2 (10 mL), followed by stirring under reflux until no more HCl was produced. Removal of the solvent and excess $SOCl_2$ in vacuo (<60°C) provided *N,N′*-1,4-piperazinediacetyl chloride which was then suspended in CH_2Cl_2 (10 mL). DMAP (40 mg, 0.3 mmol) and pyridine (1.2 mL, 14.8 mmol) were added sequentially to the suspension, kept in an ice bath, followed by the dropwise addition of 1,2,3,4-tetrahydroquinoline (394 mg, 2.96 mmol). The reaction mixture was stirred for 4 h at room temperature. After evaporation of the solvent, the remaining mixture was subjected to silica-gel column chromatography ($CHCl_3$/MeOH 100:1→100:8). After evaporation of the solvent from the elute, the afforded residue was subjected to gel filtration over Sephadex LH-20 ($CHCl_3$/CH_3OH 1:1) to give **1** (152 mg, 47.3% yield, two steps).

Acknowledgements

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