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Aspergillines A−E, Highly Oxygenated Hexacyclic Indole−Tetrahydrofuran−Tetramic Acid Derivatives from *Aspergillus versicolor*

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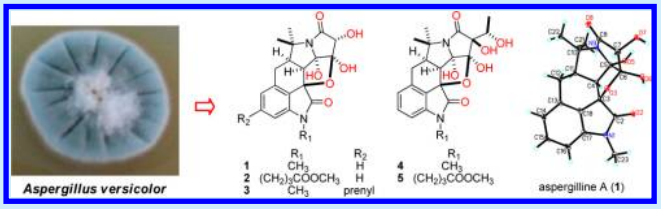
Supporting Information

ABSTRACT: Aspergillines A−E (**1**−**5**) are highly oxygenated cyclopiazonic acid (CPA)-derived alkaloids bearing a rigid and sterically congested hexacyclic indole−tetrahydrofuran−tetramate scaffold, isolated from the endophytic fungus *Aspergillus vesicolor*. Apergillines A−C represent a new subclass of CPA-derived alkaloids, and aspergillines B and E possess a butanoic acid methyl ester moiety. The structures, including absolute configuration, were elucidated by interpretation of the NMR, X-ray crystallographic, and circular dichroism data. All compounds displayed anti-TMV and cytotoxic activities.



Prenylated indole alkaloids comprise a family of natural products distributed in both terrestrial and marine organisms, including plants, fungi, actinomyces, and cyanobacteria.[1] Among them, indole−tetramic acid alkaloids are often regarded as a small but unique subtype.[1] These alkaloids are structurally characterized by a pentacyclic skeleton and the unique heterocyclic pyrrolidine-2,4-dione (tetramic acid) or pyrrolidine-2-one core. They are considered to be biosynthesized from three precursors, i.e., a tryptophan residue, two units of acetic acid, and dimethylallyl diphosphate (DMAPP).[2] It is noteworthy that tetramic acid or pyrrolidine-2-one is often referred to be a pharmacophore to interact with various biological targets.[3] These natural products thus exhibit a wide range of biological activities, such as antibacterial (e.g., reutericyclin, streptolydigin, and cryptocin),[4] antiviral (e.g., tenuazonic acid and integramycin),[5] and antitumor (e.g., tenuazonic acid and melophlins) activities.[5a,6]

Cyclopiazonic acid (CPA) is a representative indole−tetramic acid alkaloid first isolated from *Penicillium cyclopium* as a toxic metabolite.[7] It is also produced by many species of *Aspergillus* and *Penicillium*.[2b] Interestingly, CPA is one of the few inhibitors of the sarcoendoplasmic reticulum Ca^{2+}-ATPases, other examples being artemisinin (antimalarial), thapsigargin (antitumor), and speradine A (inhibitor of histone deacetylase with antibacterial activity).[8] For this reason, the CPA and its analogues have drawn widespread attention as potential drug candidates. The biosynthetic pathway and total synthesis of CPA have also been described.[2,9]

In the present study, five highly oxygenated CPA-related alkaloids, aspergillines A−E (Figure 1), all bearing a rigid hexacyclic (6/5/6/5/5/5) indole−tetrahydrofuran−tetramic acid scaffold, were isolated from *Aspergillus versicolor* (Vuillemin) Tiraboschi. The fungus was isolated from the rhizome of *Paris polyphylla* var. *yunnanensis* and identified by the internal transcript spacer (ITS) sequence (Genbank accession no. KJ801852).

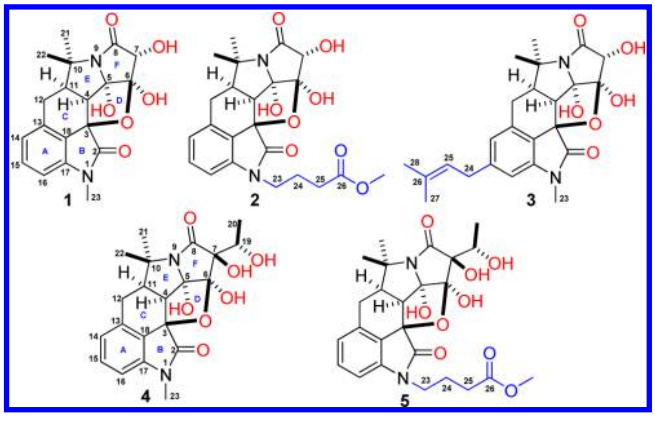


Figure 1. Structures of compounds **1**−**5**.

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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectroscopic Data of 1−3 in Pyridine-d_5 and 4 and 5 in CD_3OD (δ ppm)

	1		2		3		4		5	
no.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2	178.4 s		178.5 s		178.6 s		179.8 s		179.2 s	
3	82.9 s		82.1 s		82.2 s		84.7 s		84.3 s	
4	54.8 d	3.25 d (8.6)	54.0 d	3.23 d (8.6)	54.2 d	3.20 d (8. 6)	54.9 d	3.11 d (8.4)	54.3 d	3.11 d (8.3)
5	106.1 s		106.0 s		106.0 s		103.8 s		103.2 s	
6	107.2 s		107.1 s		107.0 s		109.0 s		109.4 s	
7	76.7 d	4.71 s	77.0 d	4.70 s	77.0 d	4.68 s	83.1 s		83.2 s	
8	174.5 s		173.2 s		175.0 s		174.6 s		173.0 s	
10	67.8 s		67.8 s		67.1 s		69.3 s		68.3 s	
11	56.4 d	2.36 m	56.3 d	2.34 m	55.5 d	2.32 m	56.0 d	2.40 m	55.6 d	2.24 m
12	28.0 t	2.65 d (8.4)	28.2 t	2.65 d (8.6)	29.2 t	2.61 d (8.6)	28.6 t	2.73 dd (5.6, 13.0), 2.59 t (13.0)	28.3 t	2.71 dd (5.6, 13.0), 2.50 t (13.0)
13	138.2 s		138.0 s		136.0 s		139.6 s		138.7 s	
14	122.0 d	6.99 d (7.8)	122.1 d	6.98 d (7.8)	124.9 d	6.17 d (1.8)	123.1 d	6.96 d (7.8)	122.0 d	6.92 d (7.8)
15	131.8 d	7.33 t (7.8)	131.2 d	7.32 t (7.8)	134.3 s		133.0 d	7.33 t (7.8)	133.1 d	7.30 t (7.8)
16	107.2 d	6.69 d (7.8)	107.4 d	6.69 d (7.8)	118.2 d	6.95 d (1.8)	108.3 d	6.85 d (7.8)	107.2 d	6.81 d (7.8)
17	143.2 s		142.6 s		144.8 s		144.0 s		143.1 s	
18	122.9 s		123.0 s		119.8 s		123.4 s		123.0 s	
19							69.9 d	4.09 q (6.5)	69.5 d	4.09 q (6.5)
20							17.6 q	1.31 d (6.6)	18.0 q	1.29 d (6.6)
21	30.5 q	1.71 s	30.9 q	1.70 s	31.0 q	1.68 s	30.8 q	1.51 s	30.8 q	1.52 s
22	21.8 q	1.91 s	21.9 q	1.89 s	22.0 q	1.90 s	22.0 q	1.78 s	22.0 q	1.80 s
23	26.2 q	2.98 s	46.7 t	3.94 t (8.3)	26.6 q	2.92 s	27.0 q	3.21 s	47.4 t	3.90 t (8.3)
24			25.2 t	2.14 m	27.1 t	3.60 d (6.8)			25.0 t	2.10 m
25			30.0 t	2.55 t (6.3)	122.9 d	5.15 t (6.8)			30.2 t	2.60 t (6.3)
26			174.4 s		133.0 s				174.9 s	
27					18.9 q	1.60 s				
28					25.6 q	1.81 s				
OMe			52.5 q	3.67 s					52.2 q	3.69 s

Although CPA-related alkaloids are widely distributed in fungal species, no more than 10 analogues, all showing only minor structural variations, have been discovered.[7,8c,10] Unlike the known CPA derivatives, aspergillines A−C are presumably derived from only one acetyl-CoA unit, leading to the absence of a C_2 side chain at the C-7 position. On the other hand, aspergillines B and E possess a butanoic acid methyl ester chain at the N-1 position, whereas aspergilline C has an additional prenyl group attached to C-15 on ring A. Compounds **1**−**5** displayed anti-TMV and cytotoxic activities in our test models. Here, the isolation and structure elucidation of aspergillines A−E, as well as their biological properties, are described.

Compound **1** was obtained as colorless crystals, optically active ($[\alpha]_D$ −52.6). The molecular formula $C_{19}H_{20}N_2O_6$ was established on the basis of HRESIMS (m/z at 359.1212 $[M + Na]^+$), requiring 11 degrees of unsaturation. The ^1H NMR spectrum displayed signals of *N*-methyl protons (δ_H 2.98, s, H_3-23), an oxymethine proton (δ_H 4.71, s, H-7), and a 1,2,3-trisubstituted benzene ring (δ_H 6.99, d, J = 7.8 Hz, H-14; 7.33, t, J = 7.8 Hz, H-15; 6.69, d, J = 7.8 Hz, H-16). The ^{13}C NMR and DEPT data (Table 1) disclosed the presence of two ester and/or amide groups, three methyls (including one *N*-methyl group), one methylene, six methine groups (including one oxygenated and three sp^2 methines), and seven quaternary carbons (including an oxygenated quaternary carbon and two diheteroatom-bearing carbons at δ_C 106.1 and 107.2). Among them, two ester and/or amide groups and six olefinic carbons account for five degrees of unsaturation. These data suggested that **1** is an alkaloid with a hexacyclic ring system. The gross structure of **1** was elucidated by careful analysis of its 1D and 2D NMR spectra. Thus, the HMBC correlations between H_3-23 and C-2/C-17, together with the $^4J_{CH}$ HMBC correlations between H-14/H-16 and C-3, indicated the presence of a 1-*N*-methyloxindole (rings A and B). The presence of a six-membered ring (ring C) was established by the $^1H-^1H$ COSY correlations of H_2-12/H-11/H-4, along with the HMBC correlations between H_2-12 and C-14/C-18 and between H-4 and C-2/C-3. These findings, together with the HMBC correlations of H_2-12 with C-10 (a quaternary carbon attached to N-9), of H_3-21 and H_3-22 with C-10, and of H-11 and H-4 with C-5, led to the establishment of the planar subunit **1a** (Figure 2).

It followed that the HMBC cross-peaks between H-7 and C-6/C-8 allowed the construction of subunit **1b** (Figure 2). The direct linkage between **1a** and **1b** at C-5 and C-6 could be readily established by the key HMBC correlations observed for H-4/H-7 and C-5. However, since C-3, C-5, C-6, and C-8 are fully substituted carbons, it was difficult to determine the connections among them by interpreting the NMR data alone. Three partial structures (i, ii, and iii as shown in Figure 2) were thus proposed, based on different carbon−nitrogen connections among C-5, C-6, and/or C-8 with N-9. Among these possible substructures, carbon

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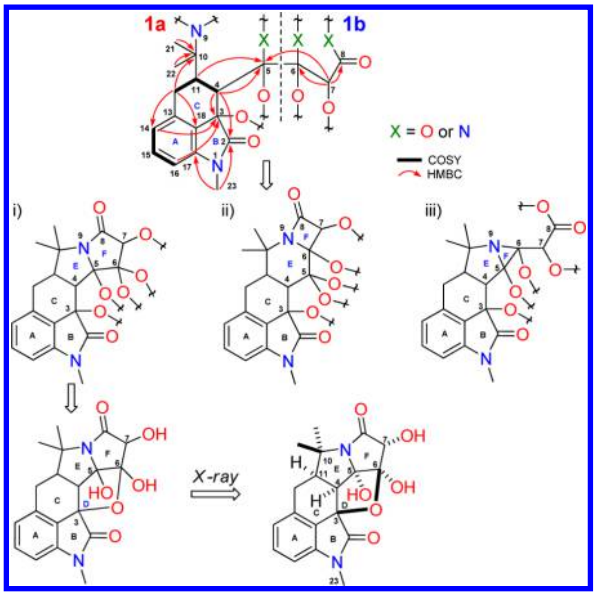

Figure 2. Structural elucidation of **1** based on NMR and X-ray crystallographic analyses.

skeleton i (formed by C-5−N-9 and C-8−N-9 linkages) is similar to that of CPA,[7] except for the absence of a 1-hydroxyethyl side chain (C-19 and C-20) attached to C-7. Subsequent comparison of the NMR data of **1** with those of CPA clearly showed the presence of characteristic signals for this type of alkaloids, including proton signals of H-4, H-11, H_2-12, H-14, H-15, and H-16, and carbon signals of C-8, C-10, C-11, and C-12.

Finally, the presence of a tetrahydrofuran ring (ring D) between the hemiketal carbon (δ_C 107.2, C-6) and C-3 was proposed instead of an epoxide group between C-6 and C-5 or C-7, taking into consideration the downfield shifts of C-3, C-5, and C-7 signals at δ_C 82.9, 106.1 and 76.7, respectively.

The spectral evidence discussed above strongly suggested **1** being a highly oxygenated alkaloid with a sterically congested hexacyclic indole−tetrahydrofuran−tetramic acid scaffold. ROESY experiments were used to reveal the partial relative configuration of **1**, in which the cross peaks of H-4/Me-21 and H-11/Me-21 demonstrated that H-4, H-11, and Me-21 possessed the same orientation (Figure S1, Supporting Information). After numerous attempts, a bunch of tiny colorless needle crystals settled in the brown mother liquor of compound **1** by vapor-exchange method of methanol and water in a closed tube, placed at room temperature for nearly 1 month. An appropriate crystal was selected for X-ray crystallography analysis. Through structural refinement, the Hooft parameter is 0.12(13) for 1016 Bijvoet pairs,[11a] allowing an explicit assignment of the absolute structure as 3*R*,4*R*,5*S*,6*R*,7*R*,11*R* (CCDC 1016366) (Figure 3).

Aspergilline B (**2**) displayed ^{13}C NMR patterns similar to that of **1**, but the molecule is 86 mass units higher, as deduced from the molecular formula $C_{23}H_{26}N_2O_8$ (HRESIMS *m*/*z* 481.1581 $[M + Na]^+$). The major difference was that the 1-*N*-methyl group in **1** is now replaced by a butanoic acid methyl ester moiety in **2**, which could be confirmed by the $^1H-^1H$ COSY correlations of H_2-23/H_2-24/H_2-25, along with the HMBC correlations between H_2-23 and C-2/C-17, between H_2-24 and C-26, as well as between H_3-27 and C-26. The almost identical carbon and proton chemical shifts of **2** compared with those of **1** (Table 1) indicated that compound **2** had the same relative configurations of all stereocenters in rings C−F as those of **1**. In the CD spectra of both **1** and **2**, the Cotton effects were negative at 205 nm and positive at 225 nm, suggesting the same absolute configuration for these two compounds. Thus, the structure of aspergilline B (**2**) was proposed as shown in Figure 1.

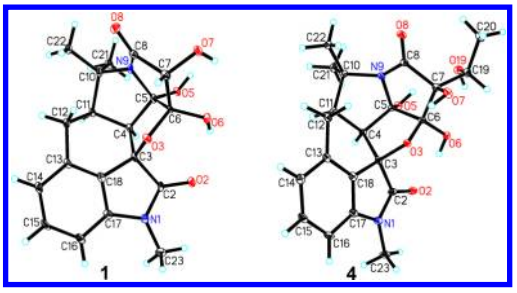

Figure 3. X-ray crystal structures of **1** and **4**.

The molecular formula of aspergilline C (**3**) was deduced to be $C_{24}H_{28}N_2O_6$ by positive HRESIMS at *m*/*z* 463.1839 $[M + Na]^+$. It is therefore 68 mass units higher than that of **1**. A direct comparison of the NMR data between **3** and **1** indicated that both structures shared the same skeleton. A significant difference was that **3** possesses an additional prenyl group attached to C-15 in ring A. The HMBC correlations between H_2-24 and C-14/C-16, and between H_3-27/H_3-28 and C-25, together with the $^1H-^1H$ COSY correlations of H_2-24/H-25, provided further evidence for the structural assignment. The relative configuration of **3** was also identical to that of **1** by comparison of NMR data (Table 1). Again, negative Cotton effects at 207 nm and positive at 225 nm were observed for **3** in the CD spectrum, indicating **1**−**3** had the same configuration.

Aspergilline D (**4**) was obtained as colorless crystals. Its molecular formula $C_{21}H_{24}N_2O_7$ was established by HRESIMS (*m*/*z* at 439.1486 $[M + Na]^+$) and the ^{13}C NMR data. In comparison with **1**, compound **4** possesses an additional 1-hydroxyethyl side chain [δ_C 69.9 (C-19) and 17.6 (C-20)] attached to C-7. Such an assignment was supported by the HMBC correlations between H_3-20 and C-7 and between H-19 and C-6, as well as the $^1H-^1H$ COSY correlation of H-19/H_3-20. The relative stereochemistry of partial chiral centers in **4** was established by ROSEY experiment. Correlations of H-4/Me-21, H-4/H-11, and H-11/Me-21 indicated that H-4, H-11, and Me-21 were in the same orientation (Figure S1, Supporting Information). Finally, by the same method as for **1**, the crystal of **4** was obtained and the diffraction experiment was carried out. According to the refined Flack parameter value 0.03(17) and Hooft parameter value 0.12(5) for 1295 Bijvoet pairs,[11] the ORTEP drawing for **4** (CCDC 1016367) (Figure 3) revealed the absolute configuration as 3*R*,4*R*,5*S*,6*R*,7*S*,11*R*,19*S* with a probability of 1.000.

Lastly, a direct comparison of the NMR data of **5** and **4** suggested that both compounds shared the same skeleton. As in the case of **1** and **2**, the only difference between **4** and **5** arose from the substituted group at N-1. Thus, the *N*-methyl group in **4** is replaced by a butanoic acid methyl ester in **5**. The relative configuration of **5** was determined to be the same as those in **4** by the similar carbon and proton chemical shifts of both compounds (Table 1). The absolute configuration of **5** was identical to that of **4**, as shown by the CD spectra.

To the best of our knowledge, aspergillines A−E are new derivatives of indole−tetrahydrofuran−tetramic acid structure. Scheme S1 (Supporting Information) shows a plausible biogenetic pathway of these unusual secondary metabolites from acetic acid, tryptophan, and DMAPP. The proposed pathway consists of two phases: formation of the pentacyclic indole−tetramic acid scaffold and further structural modifications.

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In contrast to the formation of the CPA scaffold involving two molecules of acetic acid, the aspergillines A−C presumably arise from only one unit of acetic acid in the first phase of biogenesis, which thus caused loss of a C_2 fragment.[1b,2] During the second phase, a series of stereoselective oxidations at C-2, C-3, C-5, and C-7 occur, followed by ketalization and *N*-alkylation, leading to the formation of aspergillines A−E.

Since many natural products possessing a pyrrolidine-2-one core have been reported to possess antiviral and antitumor properties,[3,5,6] the isolated alkaloids were tested for antitobacco mosaic virus (anti-TMV) and cytotoxic activities.

The inhibitory activity against TMV replication was tested by the half-leaf method, using ningnamycin as positive control.[12] Compounds **1**−**5** exhibited significant activity showing IC_{50} values in the range of 15.4−48.6 μM, even more potent than ningnamycin (IC_{50} = 52.4 μM) (Table 2). The protective effect of

Table 2. Anti-TMV Activity on *Nicotiana tabacum* Leaf and Protective Effect of 1−5 on TMV Infection

compd	inhibition rate at 20 μM[a] (%)	IC_{50}[a] (μM)	Inhibition rate at 20 μM[b] (%)
1	56.4 ± 3.8	15.4	65.8 ± 4.2
2	47.3 ± 3.2	22.8	52.6 ± 3.5
3	35.6 ± 2.8	41.3	34.7 ± 3.0
4	38.9 ± 3.5	37.5	49.8 ± 3.2
5	33.6 ± 3.0	48.6	41.4 ± 3.5
ningnamycin	30.5 ± 2.8	52.4	28.6 ± 3.2

[a]Anti-TMV activity. [b]Protective effect on TMV infection.

1−**5** against TMV was also evaluated by pretreating the tobacco leaves with individual compounds for 6 h before inoculation with TMV.[12] The results (Table 2) indicated a protective effect on the host plant by these alkaloids. The inhibition rates ranged from 34.7 to 65.8% at 20 μM concentration. This finding suggests that pretreatment with the alkaloids may enhance the resistance of the host plant against TMV infection.

Compounds **1**−**5** were also screened for their cytotoxic activities against a panel of human cell lines, including NB4 promyelocytic leukemia, A549 lung epithelial carcinoma, SHSY5Y neuroblastoma, PC3 prostate cancer, and MCF7 breast adenocarcinoma cells using the MTT method as previously described with paclitaxel as a positive control.[13] As shown in Table 3, compounds **1**, **3**, and **4**

Table 3. Cytotoxic Activity of 1−5[a]

compd	NB4	A549	SHSY5Y	PC3	MCF7
1	3.8	1.2	3.4	2.6	1.5
2	7.2	>10	5.4	2.6	4.5
3	1.2	2.8	1.5	2.8	3.6
4	2.2	1.5	3.6	4.2	2.9
5	4.7	2.8	8.2	>10	6.5
Taxol	0.03	0.02	0.2	0.2	0.1

[a]Results were expressed as IC_{50} values (μM).

exhibited moderate cytotoxicity in all cell lines, with IC_{50} values ranging from 1.2 to 4.2 μM, whereas **2** and **5** displayed moderate cytotoxicity with IC_{50} values of less than 10 μM in four cell lines.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, physical−chemical properties, 1D and 2D NMR, CD spectra for compounds **1**−**5**, and X-ray crystal structures (CIF) of compounds **1** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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