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SPERADINES B–E, FOUR NOVEL TETRACYCLIC OXINDOLE ALKALOIDS FROM THE MARINE-DERIVED FUNGUS *ASPERGILLUS ORYZAE*

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Abstract – Four novel tetracyclic oxindole alkaloids, namely, speradines B (**1**), C (**2**), D (**3**), and E (**4**) were isolated from the marine-derived fungus *Aspergillus oryzae*. The structures of these compounds without the absolute configurations were elucidated through 1D and 2D nuclear magnetic resonance and high-resolution mass spectrometric analyses. Among these compounds, **1** and **4** showed weak cytotoxic effects on the HeLa cell line.

Indole-terpenes are fungal and bacterial secondary metabolites with unique biological activities such as inhibitory activity of calcium-activated potassium channels,[1] antitumor activity,[2] tremorgenic activity,[3] potent and selective progesterone receptor agonistic activity,[4] and anti-MRSA activity.[5] Cyclopiazonic acid (CPA)-type alkaloids are one important group of indole-terpenes, and they usually contain three structural units: an indole, a dimethylallyl (DMA), and two acetic acids. Only six analogous natural products have been reported to date, including α-cyclopiazonic acid,[6] iso-α-cyclopiazonic acid,[7] β-cyclopiazonic acid,[6] α-acetyl-γ-(β-indolyl)methyl-tetramic acid,[8] speradine A,[9] and 3-hydroxyl speradine A.[10] In our search for novel anticancer compounds,[11] a strain of *Aspergillus oryzae* showed significant cytotoxic activity. Further chemical study led to isolation and structure elucidation of four new

CPA-type alkaloids (**1**–**4**), namely speradines B (**1**), C (**2**), D (**3**), and E (**4**). The isolation, structure elucidation, and bioactivity of compounds **1**–**4** are presented in the current study.

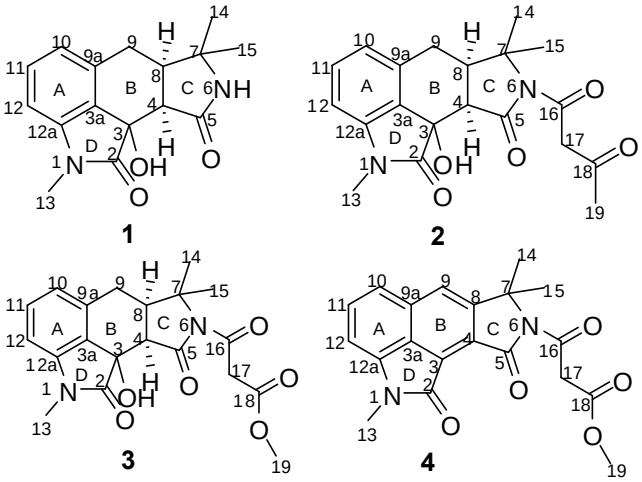

Figure 1. Structures of compounds **1**–**4**

Compound **1**, which was obtained as yellow oil, was analyzed to have the molecular formula $C_{16}H_{18}N_2O_3$ through positive high-resolution electrospray ionization mass spectroscopy (HRESIMS) (*m/z*: 287.1396 $[M + H]^+$, Calcd. for $C_{16}H_{19}N_2O_3$, 287.1390). This compound was subjected to ^1H and ^{13}C NMR (Tables 1 and 2), DEPT and HMQC spectral analyses. Results showed that **1** had sixteen carbon signals, including three methyls, one methylene, five methines, and seven quaternary carbons. The plane structure of **1** was determined using COSY and HMBC spectrum analyses (Figure 2). The COSY correlations of H-8 with H-4 and H-9, and H-11 with H-10 and H-12 demonstrated the connections from H-4 to H-9 via H-8 and from H-10 to H-12 via H-11, respectively. The HMBC correlations of H-4 with C-3 and C-3a; H-8 with C-9a; H-9 with C-3a and C-9a; H-10 with C-9 and C-3a; H-11 with C-12a; and H-12 with C-3a connected rings A and B. The HMBC correlations of H-4 with C-5; H-6 with C-4, C-7, and C-8; H-8 with C-14; and H-14 with C-7 and C-15 linked rings C and B. Since the unsaturation degree of **1** was nine, an additional ring was necessary. After carefully comparing the chemical shifts of C-2, C-3, C-3a, C-12a, and Me-13 with those of speradine A,[9] and considering the HMBC correlations from Me-13 to C-2 and C-12a, the last ring D was established and C-3 was oxidated. The NOESY correlations between H-4 and H-8 observed in **1** indicated that H-4 and H-8 were on the same side of the ring. The structure of **1** is shown in Figure 1.

Compound **2**, which was obtained as yellow oil, was analyzed to have the molecular formula $C_{20}H_{22}N_2O_5$ through positive HRESIMS (*m/z*: 371.1606 $[M + H]^+$, Calcd. for $C_{20}H_{23}N_2O_5$, 371.1601). The ^1H and ^{13}C NMR (Tables 1 and 2), DEPT, and HMQC spectral analyses revealed that **2** had twenty carbon signals, including four methyls, two methylenes, five methines, and nine quaternary carbons. The 1D-NMR data

of **2** indicated a structure immensely similar to **1**, except for the presence of an additional acylamino carbonyl (δ_C 168.0), a methylene (δ_C 53.6 and δ_H 3.34, 3.63), a ketone carbonyl (δ_C 200.5), and a methyl (δ_C 29.7 and δ_H 1.89). The HMBC correlations of H-17 with C-16 and C-18, and H-19 with C-17 demonstrated the connection of a four-carbon chain from C-16 to C-19. The HMBC correlations from H-15 to C-16 linked C-16 to N-6, which was consistent to the molecular formula of **2**. The similar NOESY correlations suggested that the configuration of **2** was similar to that of **1**. The structure of **2** is shown in Figure 1.

Table 1. ^{1}H NMR data (500 MHz, *J* in Hz and δ in ppm) of compounds **1**–**4** in $CDCl_3$

postion	**1**	**2**	**3**	**4**
4	3.81 (1H, d, 8.2)	4.03 (1H, d, 8.6)	3.93 (1H, d, 8.6)	
6	5.60 (1H, brs)			
8	2.96 (1H, dd, 8.3, 8.2)	2.90 (1H, dd, 8.7, 8.6)	2.91 (1H, dd, 8.8, 8.6)	
9-1	3.11 (1H, dd, 15.4, 8.3)	3.11 (1H, dd, 15.7, 8.7)	3.15 (1H, dd, 15.8, 8.8)	8.03 (1H, s)
9-2	2.85 (1H, d, 15.4)	3.03 (1H, d, 15.7)	3.04 (1H, d, 15.8)	
10	6.85 (1H, d, 7.7)	6.88 (1H, d, 7.7)	6.88 (1H, d, 7.7)	7.57 (1H, d, 7.5)
11	7.24 (1H, dd, 7.8, 7.7)	7.27 (1H, dd, 7.8, 7.7)	7.28 (1H, dd, 7.8, 7.7)	7.59 (1H, dd, 7.5, 6.0)
12	6.64 (1H, d, 7.8)	6.65 (1H, d, 7.8)	6.67 (1H, d, 7.8)	6.89 (1H, d, 6.0)
13	3.18 (3H, s)	3.17 (3H, s)	3.22 (3H, s)	3.46 (3H, s)
14	1.30 (3H, s)	1.60 (3H, s)	1.61 (3H, s)	1.91 (3H, s)
15	1.19 (3H, s)	1.52 (3H, s)	1.54 (3H, s)	1.91 (3H, s)
17-1		3.34 (1H, d, 16.3)	3.44 (2H, s)	4.21 (2H, s)
17-2		3.63 (1H, d, 16.3)		
19		1.89 (3H, s)	3.51 (3H, s)	3.76 (3H, s)

Compound **3**, which was obtained as yellow oil, was analyzed to have the molecular formula $C_{20}H_{22}N_2O_6$ through negative HRESIMS (*m/z*: 385.1399 $[M - H]^-$, Calcd. for $C_{20}H_{21}N_2O_6$, 385.1400). The ^{1}H and ^{13}C NMR (Tables 1 and 2), DEPT, and HMQC spectral analyses revealed that **3** had twenty carbon signals, including four methyls, two methylenes, five methines, and nine quaternary carbons. The 1D-NMR data of **3** indicated that **3** was nearly similar to **2**, except for the appearance of a methoxy group (δ_H 3.51 and δ_C 52.1) and a carbonyl (δ_C 167.0), and for the disappearance of an acetyl group (δ_H 1.89 and δ_C 29.7,

200.5). The HMBC correlations from H-17 and H-19 to C-18 confirmed the substitution of the acetyl group by an ester group. The NOESY correlations between H-4 and H-8 suggested that the configuration of **3** was similar to those of **1** and **2**.

Table 2. ^{13}C NMR data (125 MHz, δ in ppm) of compounds **1**–**4** in $CDCl_3$

position	**1**	**2**	**3**	**4**
2	178.2 s	178.0 s	177.6 s	165.0 s
3	73.3 s	73.3 s	73.0 s	125.8 s
3a	125.8 s	125.7 s	125.4 s	126.5 s
4	50.4 d	50.4 d	50.4 d	125.5 s
5	172.1 s	173.1 s	172.6 s	164.9 s
7	57.8 s	64.9 s	64.9 s	66.2 s
8	43.1 d	42.1 d	42.0 d	152.8 s
9	26.2 t	25.2 t	25.1 t	123.3 d
9a	135.8 s	135.4 s	135.4 s	132.5 s
10	120.7 d	121.2 d	121.1 d	120.4 d
11	130.9 d	131.3 d	131.3 d	131.7 d
12	106.3 d	106.3 d	106.3 d	105.2 d
12a	143.1 s	142.9 s	143.0 s	141.8 s
13	26.5 q	26.5 q	26.6 q	26.7 q
14	32.6 q	24.0 q	23.9 q	26.9 q
15	25.2 q	28.2 q	28.0 q	26.9 q
16		168.0 s	167.1 s	167.2 s
17		53.6 t	45.4 t	45.7 t
18		200.5 s	167.0 s	168.2 s
19		29.7 q	52.1 q	52.5 q

Compound **4**, which was obtained as orange powder, was analyzed to have the molecular formula $C_{20}H_{18}N_2O_5$ through positive HRESIMS (*m/z*: 389.1108 $[M + Na]^+$, Calcd. for $C_{20}H_{18}N_2NaO_5$, 389.1113). The ^{1}H and ^{13}C NMR (Tables 1 and 2), DEPT, and HMQC spectral analyses revealed that **4** had twenty carbon signals, including four methyls, one methylene, four methines, and eleven quaternary carbons. The ^{1}H and ^{13}C NMR data of **4** and **3** were compared. The results showed four obviously downfield shifts, including C-3 (from δ_C 73.0 s to δ_C 125.8 s), C-4 (from δ_C 50.4 d to δ_C 125.5 s), C-8 (from δ_C 42.0 d to δ_C 152.8 s), and C-9 (from δ_C 25.1 t to δ_C 123.3 d). Since the degree of unsaturation of **4** was thirteen, which was two more than that of **3**, two additional double bonds were necessary. Furthermore, taking into

account one oxygen atom and four hydrogen atoms less than molecular formula of **3** and similar 2D NMR, compound **4** was deduced to be the 3,4-dehydrate and 8,9-dehydro derivative of **3**.

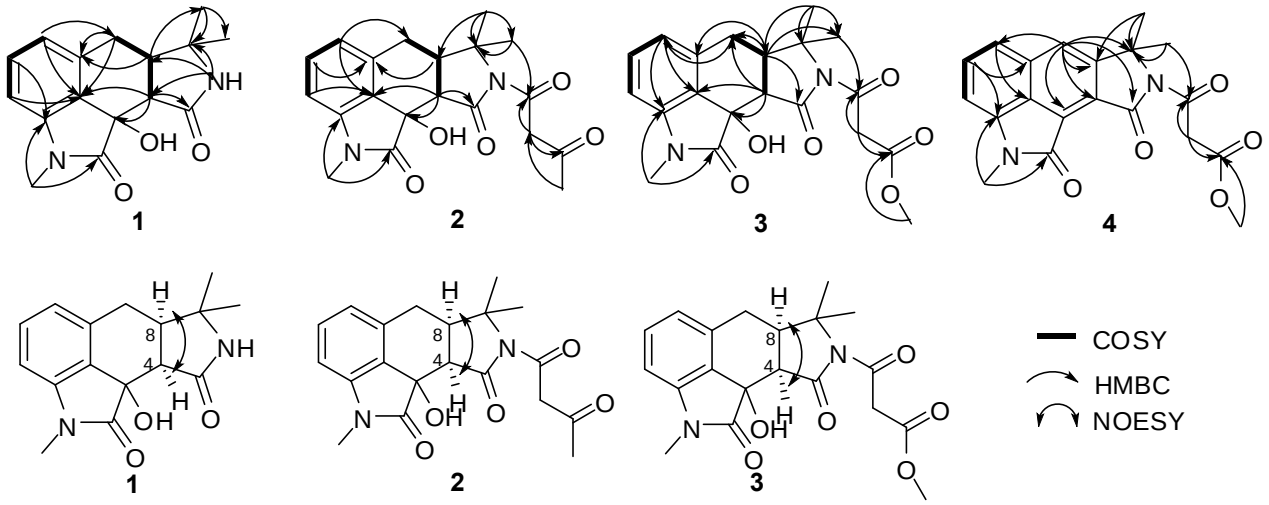

Figure 2. Key COSY, HMBC and NOESY correlations of compounds **1**–**4**

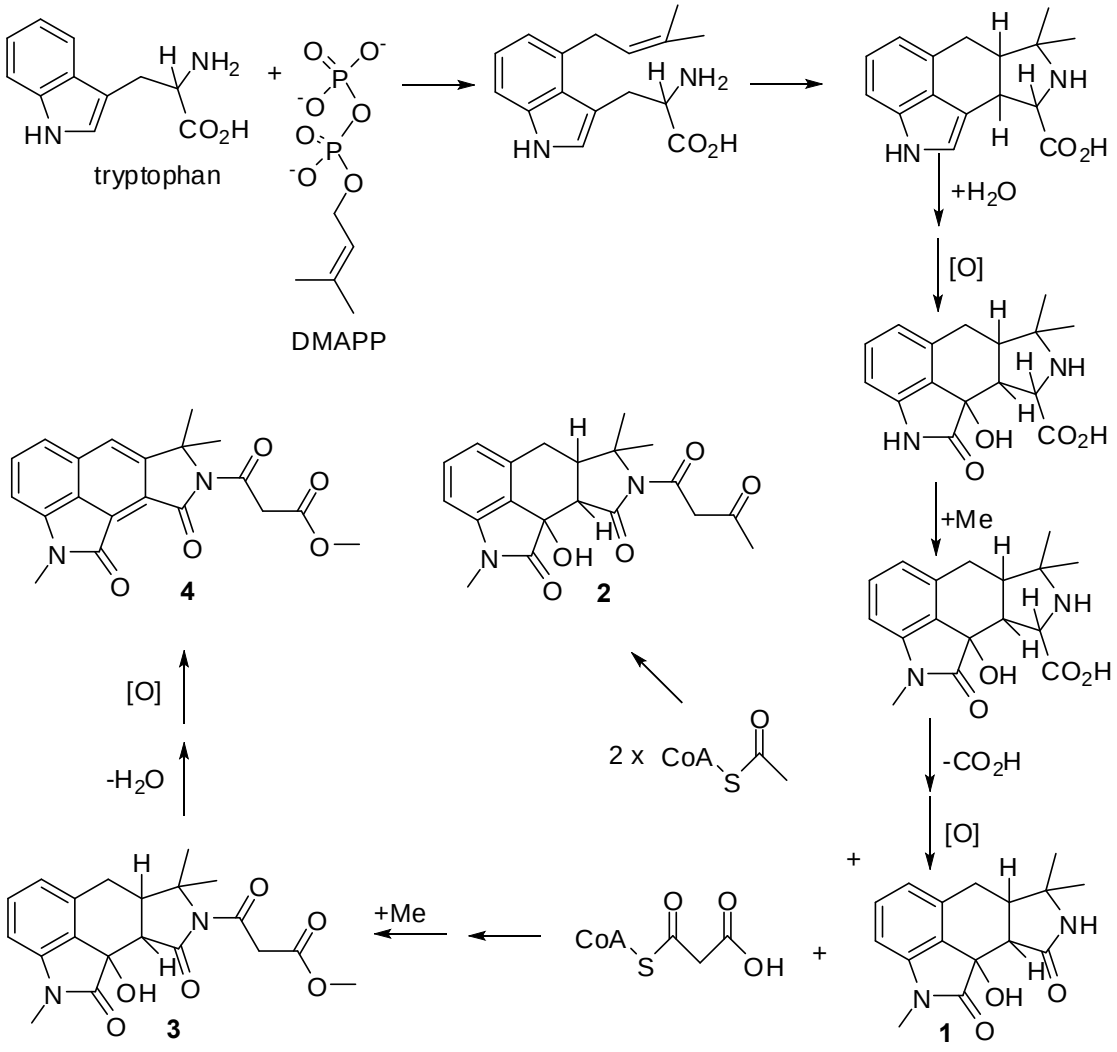

Figure 3. Plausible biosynthetic pathway of compounds **1**–**4**

To explain the biogenetic origin of speradines B, C, D, and E (**1**–**4**), a plausible biosynthetic pathway is proposed in Figure 3, which is very similar to the reported biosynthesis of CPA.[12]

The compounds **1**–**4** were tested for their cytotoxic effects on the Hela and MGC803 cell lines using the Sulforhodamine B (SRB) method.[13] Compounds **1** and **4** demonstrated weak cytotoxicity against the HeLa cell line, with IC_{50} values of 0.20 and 0.20 mM, respectively.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were obtained from an Anton Paar MCP-200 digital polarimeter. IR spectra were recorded on a Nicolet Avatar 670 spectrophotometer. ^{1}H-NMR, ^{13}C-NMR, DEPT spectra and 2D-NMR were recorded on a BRUKER BIOSPIN AVANCE III spectrometer using TMS as the internal standard. HRESIMS were obtained by an Agilent Q-TOF 6520 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (ODS-A, 10×250 mm, 5 μm) at 5 mL/min.

Fungal Material. The fungus *A. oryzae* was isolated from marine sediments collected from Langqi Island, Fujian, China. It was identified according to its morphological characteristics and ITS by Beijing Sunbiotech Co. Ltd, and preserved in our laboratory at −80 ˚C. The producing strain was prepared on Martin medium and stored at 4 ˚C.

Fermentation and Extraction. The fungus *A. oryzae* was cultured under static conditions at 28 ˚C for 30 days in 1000-mL conical flasks containing the liquid medium (400 mL/flask), composed of glucose (10 g/L), maltose (20 g/L), mannitol (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), yeast extract (3 g/L), and seawater. The fermented whole broth (40 L) was filtered through cheese cloth to separate supernatant from mycelia. The former was extracted two times with EtOAc to give an EtOAc solution, while the latter was extracted three times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted two times with EtOAc to give another EtOAc solution. Both EtOAc solutions were combined and concentrated under reduced pressure to give a crude extract (42.3 g).

Purification. The crude extract of the fungus *A. oryzae* was separated into five fractions on a silica gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction A (6.7 g) was purified on a Sephadex LH-20 ($CHCl_3$:MeOH, 1:2) to derive three subfractions. Subfraction A-2 (2.8 g) was further purified on a silica gel column through a step gradient elution of CH_2Cl_2 and MeOH to afford four subfractions. Subfraction A-2-1 (160 mg) was purified by a semipreparative HPLC (60% MeOH) to yield compound **4** (3.2 mg). Fraction C (5.4 g) was further purified on a Sephadex LH-20 ($CHCl_3$:MeOH,

1:2) to afford two subfractions. Subfraction C-1 (3.1 g) was further purified on a silica gel column using a step gradient elution of CH_2Cl_2 and MeOH to afford four subfractions. Subfraction C-1-1 (130 mg) was purified by a semipreparative HPLC (45% MeOH) to yield compounds **1** (2.9 mg), **2** (5.7 mg), and **3** (3.2 mg).

Speradine B (**1**): yellow oil ($CHCl_3$); $[\alpha]^{20}_D$ –31.1 (*c* 0.17, $CHCl_3$); IR (KBr) ν_{max} 3428, 2925, 1708, 1614, 1475, and 1368 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 287.1396 $[M + H]^+$, calcd for $C_{16}H_{19}N_2O_3$, 287.1390).

Speradine C (**2**): yellow oil ($CHCl_3$); $[\alpha]^{20}_D$ –272.9 (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3407, 2929, 1728, 1610, 1475, and 1307 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 371.1606 $[M + H]^+$, calcd for $C_{20}H_{23}N_2O_5$, 371.1601).

Speradine D (**3**): yellow oil ($CHCl_3$); $[\alpha]^{20}_D$ –40.6 (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3432, 2933, 1736, 1704, 1618, 1475, and 1340 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 385.1399 $[M – H]^–$, calcd for $C_{20}H_{21}N_2O_6$, 385.1400.

Speradine E (**4**): orange powder ($CHCl_3$); IR (KBr) ν_{max} 3444, 2921, 1732, 1699, 1634, 1458, and 1328 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 389.1108 $[M + Na]^+$, calcd for $C_{20}H_{18}N_2NaO_5$, 389.1113).

Biological Assays. The cytotoxic activity for the HeLa and MGC803 cell lines were evaluated by the SRB method. Doxorubicin was used as the reference drug.

ACKNOWLEDGEMENTS

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