Chemistry of Natural Compounds, Vol. 45, No. 5, 2009

iso-α-CYCLOPIAZONIC ACID, A NEW NATURAL PRODUCT ISOLATED FROM THE MARINE-DERIVED FUNGUS *Aspergillus flavus* C-F-3

Ai-Qun Lin, Lin Du, Yu-Chun Fang, Fa-Zuo Wang, Tian-Jiao Zhu, Qian-Qun Gu*, and Wei-Ming Zhu*

UDC 547.94+615.33

A new natural product, iso-α-*cyclopiazonic acid (**1**), together with its isomer* α-*cyclopiazonic acid (**2**); three mycotoxins: aflatoxin* B_1 *(*AFB_1*) (**3**), aflatoxin* Q_1 *(*AFQ_1*) (**4**), and* O-*methylsterigmatocystin (OMST) (**5**); two diketopiperazine alkaloids: ditryptophenaline (**6**) and 3-[(1H-indol-3-yl)methyl]-6-benzylpiperazine-2,5-dione (7), were isolated from the marine-derived fungus* Aspergillus flavus. *Their structures were determined by analysis of spectroscopic data. The cytotoxicities of compounds **1** and **2** were studied using HL-60, MOLT-4, A-549, and BEL-7402 cell lines.*

Key words: *Aspergillus flavus*, cyclopiazonic acid, cytotoxic activity.

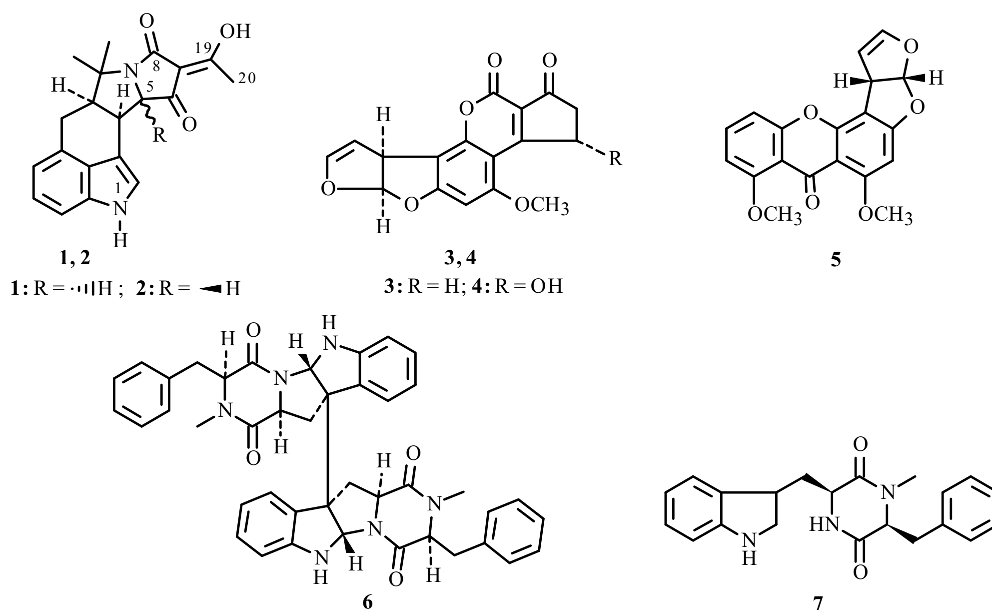
α-Cyclopiazonic acid (α-CPA) was a mycotoxic metabolite first isolated from *Penicillium cyclopium* Westling [1]. Since then, several other *Penicillium* and *Aspergillus* strains, including *P. camembert* and *A. flavus* and *A. tamarii*, were also found to produce α-CPA [2–4]. It has a range of biological activities, most notably Ca^{2+}-ATPase inhibition, and can be used as a tool for the elucidation of intracellular Ca^{2+} influx [5–7]. In the course of our search for new anticancer compounds from marine microorganisms, *iso*-α-cyclopiazonic acid (*iso*-α-CPA) (**1**) and α-CPA (**2**), together with three mycotoxins: aflatoxin B_1 (AFB_1) (**3**), aflatoxin Q_1 (AFQ_1) (**4**), and *O*-methylsterigmatocystin (OMST) (**5**); two diketopiperazine alkaloids: ditryptophenaline (**6**) and 3-[(1H-indol-3-yl)methyl]-6-benzylpiperazine-2,5-dione (**7**), were isolated from the *A. flavus* strain (c-f-3) isolated from marine algae, collected in Putian Pinghai, China. Compound **1** was first synthesized by Holzapfel in 1968 and isolated as a natural product for the first time. In this paper, we report the isolation and structure elucidation of **1**, and the cytotoxic activities and structure–activity relationships of **1** and **2**.

Compound **1** shows a quasi-molecular ion peak at *m/z* 337 $[M+H]^+$ in the positive ESI-MS, and together with the ^1H and ^{13}C NMR spectra, supported the molecular formula of **1** ($C_{20}H_{20}N_2O_3$) as **2**. ^1H and ^{13}C NMR data (Table 1) disclosed the existence of eight sp^2 quaternary carbons, four sp^2 methines, three sp^3 methines, one sp^3 quaternary carbon, one sp^3 methylene, and three methyl groups. The ^{13}C NMR spectrum of **1** showed the characteristic carbon signals assignable to amide (δ_C 173.2), ketone (δ_C 195.0), and enol (δ_C 183.1, 107.1), indicating that **1** possessed a tetramic acid ring. Four lower-field proton signals at δ_H 6.89 (s), 7.11 (d, J = 8.0 Hz), 7.02 (dd, J = 8.0, 7.1 Hz), and 6.78 (d, J = 7.1 Hz), together with eight olefinic carbons (δ_C 123.5 d, 109.1 s, 129.9 s, 116.3 d, 120.8 d, 108.7 d, 134.6 s, 128.4 s), suggested the presence of a disubstituted indole ring. Comparison of the NMR spectral data (Table 1) of **1** with those of **2** showed that **1** has the same planar structure as **2**. The ^1H NMR data of **1** differed from those of **2** only in the chemical shifts of H-4 (δ_H 3.78), H-5 (δ_H 4.64), and H-11 (δ_H 3.27), which shifted 0.10, 0.53, and 0.67 ppm more downfield than **2**, respectively; and H-21 (δ_H 0.85) and H-22 (δ_H 1.48), which shifted 0.75 and 0.2 ppm more upfield than **2**; further comparison of their CD data suggested that the only difference was the stereochemistry of C-5, viz **2** has *S* configuration, while **1** has *R*. So **1** was identified as *iso*-α-cyclopiazonic acid, in agreement with the reported NMR and CD data in the literature [1, 8].

Key Laboratory of Marine Drugs, Chinese Ministry of Education; Institute of Marine Drugs and Food, Ocean University of China, Qingdao 266003, P. R. China, fax: 0086 532 82033054, e-mail: guqianq@ouc.edu.cn and weimingzhu@ouc.edu.cn.
Published in Khimiya Prirodnykh Soedinenii, No. 5, pp. 569–571, September–October, 2009. Original article submitted March 11, 2008.

TABLE 1. ^1H and ^{13}C NMR Data for **1** and **2** (600 and 150 MHz, Acetone-d_6, TMS, δ, ppm)

C atom	**1**		**2**	
	δ_H (J/Hz)	δ_C	δ_H (J/Hz)	δ_C
2	6.89 (1H, s)	123.5 d	7.14 (1H, s)	123.1 d
3		109.1 s		110.2 s
4	3.78 (1H, t, J = 5.0)	36.5 d	3.68 (1H, dd, J =11.0, 5.9)	36.9 d
5	4.64 (1H, br.s)	72.3 d	4.11 (1H, d, J =11.0)	72.6 d
6		195.0 s		195.3 s
7		107.1 s		106.4 s
8		173.2 s		175.4 s
10		62.9 s		63.8 s
11	3.27 (1H, m)	53.5 d	2.60 (1H, m)	53.9 d
12	3.06 (2H, m)	26.8 t	3.02 (1H, dd, J = 15.9, 5.9) 3.06 (1H, t, J = 14.9)	27.0 t
13		129.9 s		129.7 s
14	7.11 (1H, d, J = 8.0)	116.3 d	7.19 (1H, d, J = 8.3)	116.6 d
15	7.02 (1H, dd, J = 8.0, 7.1)	120.8 d	7.04 (1H, dd, J = 8.2, 6.9)	121.9 d
16	6.78 (1H, d, J = 7.1)	108.7 d	6.81 (1H, d, J = 6.9)	109.5 d
17		134.6 s		134.5 s
18		128.4 s		127.0 s
19		183.1 s		183.5 s
20	2.40 (3H, s)	19.1 q	2.38 (3H, s)	19.0 q
21	0.85 (3H, s)	21.7 q	1.60 (3H, s)	24.6 q
22	1.48 (3H, s)	30.5 q	1.68 (3H, s)	26.4 q



Biosynthesis studies have suggested that **2** is derived from *L*-tryptophan, acetic acid, and mevalonic acid, and its direct precursor is *β*-cyclopiazonic acid [9]. For **1** and **2**, the only difference is in the configuration of C-5, which is *S* in **2** and *R* in **1**, and we deduce that **1** should be derived from D-tryptophan.

The cytotoxic activities of compounds **1** and **2** were evaluated by the MTT method [10] using HL-60 and MOLT-4 cell lines, and by the SRB method [11] using A-549 and BEL-7402 cell lines.

Compound **1** displayed cytotoxic activitives against the four cell lines with IC_{50} values (Table 2) of 90.0, 68.6, 42.2, >100 μM, respectively, and **2** with IC_{50} values of 2.4, 12.3, 21.5, 17.5 μM, respectively. Compound **2** was most active on HL-60 cell lines and much more active than **1** on all four cell lines.

The structure of **1** differed from **2** only in the ring junction stereochemistries at carbon C-5, hence **2** has *S* configuration, while **1** has *R*. The above results indicate that the *S* configuration of C-5 in CPA appears to be an important determinant of biological activities.

TABLE. 2. Cytotoxicity of Compounds **1–2** against HL-60, MOLT-4, BEL-7402, and A-549 (IC_{50}, μM)

Compound	HL-60	MOLT-4	BEL-7402	A-549
1	90.0	68.6	>100	42.2
2	2.4	12.3	17.5	21.5

EXPERIMENTAL

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. ^1H, ^{13}C NMR, and DEPT spectra were recorded on a JEOL Eclips-600 spectrometer using TMS as an internal standard, and chemical shifts were recorded as δ in ppm. ESI-MS was measured on a Q-TOF Ultima Global LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (YMC-Pack ODS-A, 10×250 mm, 5 μm, 4 mL/min).

Microorganism. The *A. flavus* strain was separated from a marine algae *Enteromorpha tubulosa*, collected at Putian Pinghai, China, in August, 2005. It was identified by Prof. Hong Kui. Working stocks were prepared on potato dextrose agar slants stored at 4°C.

Fermentation. The fungus was grown under static conditions at 24°C for 40 days in 167 × 1000 mL conical flasks containing liquid medium (300 mL/flask) composed of glucose (1%), maltose (2%), and yeast extract (0.3%), mannitol (2%), monosodium glutamate (1%), KH_2PO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.03%), corn plasm (0.1%), and artificial seawater after adjusting its pH to 6.5.

Extraction and Isolation. The fermented whole broth (50 L) was filtered through cheesecloth to separate into supernatant and mycelia. The supernatant was extracted with ethyl acetate, while the mycelia were extracted with 70% aqueous acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution, which was extracted with ethyl acetate. Both ethyl acetate solutions were combined and concentrated under reduced pressure to give a crude extract (77.7 g). The crude extract was separated into eight fractions on a silica gel column using a step gradient elution of $CHCl_3$–MeOH. Fraction 5 was chromatographed on a silica gel column using a step gradient elution of petroleum ether–Me_2CO to give eight fractions, and the fifth fraction was purified by semipreparative HPLC (75% MeOH–0.3%TFA) to give compounds **1** (108.8 mg) and **2** (163.2 mg). The sixth fraction was chromatographed on a Sephadex LH-20 column with 50% MeOH–$CHCl_3$ and purified by semipreparative HPLC (60% MeOH) to give compound **3** (25 mg). The seventh fraction was chromatographed on a Sephadex LH-20 column with MeOH and purified by semipreparative HPLC to give compounds **4** (3 mg), **5** (8 mg), **6** (126.8 mg), and **7** (6.8 mg).

***iso*-α-Cyclopiazonic Acid** (**1**). Yellow amorphous solid; $[\alpha]_D^{20}$ +323.5° (*c* 0.150, $CHCl_3$); ESI-MS (positive ion) *m/z:* 337 ($[M+H]^+$, $C_{20}H_{20}N_2O_3$; CD (MeOH) λ 318 (Δε+4.44), 298 (+29.60), 285 (0), 272 (–20.13), 258 (0), 217 (+20.11), 204 (+42.30); ^1H and ^{13}C NMR (600 MHz and 150 MHz, acetone-d_6) see Table 1.

α-Cyclopiazonic Acid (**2**). Yellow amorphous solid; $[\alpha]_D^{20}$ –92.1° (*c* 0.100, $CHCl_3$); ESI-MS (positive ion) *m/z* 337 ($[M+H]^+$, $C_{20}H_{20}N_2O_3$); CD (MeOH) λ 308 (Δε+2.04), 294 (0), 260 (–1.34), 226 (–13.66), 220 (–27.60); ^1H and ^{13}C NMR (600 MHz and 150 MHz, acetone-d_6) see Table 1.

Aflatoxin Q_1 (AFQ_1) (**4**). Yellow amorphous solid; ESI-MS (positive ion) *m/z:* 351 $[M+Na]^+$, $C_{17}H_{12}O_7$; ^1H NMR (600 MHz, DMSO-d_6, δ, J/Hz): 3.16 (2H, d, J = 5.0, H-2), 4.12 (H, dd, J = 10.6, 5.0, H-3), 6.78 (1H, s, H-7), 6.97 (1H, d, J = 6.9, H-9); 6.75 (1H, dd, J = 2.9, 2.3, H-10), 5.42 (1H, dd, J = 2.8, 2.1, H-11), 4.81 (1H, dt, J = 6.9, 2.3, H-12).

***O*-Methylsterigmatocystin** (**OMST**) (**5**). Yellow amorphous solid; ESI-MS (positive ion) *m/z:* 339 $[M+H]^+$, 361 $[M+Na]^+$, $C_{19}H_{14}O_6$; ^1H NMR (600 MHz, DMSO-d_6, δ, J/Hz): 6.96 (1H, d, J = 8.2, H-4), 7.51 (1H, dd, J = 8.7, 8.2, H-5), 6.77 (1H, d, J = 8.3, H-6), 6.40 (1H, s, H-11), 6.81 (1H, d, J = 7.3, H-14), 4.80 (1H, ddd, J = 6.8, 2.3, 1.8, H-15), 5.45 (1H, dd, J = 2.8, 2.2, H-16), 6.49 (1H, dd, J = 2.8, 2.3, H-17), 3.93 (3H, s, H-18), 3.97 (3H, s, H-19); ^{13}C NMR (150 MHz, DMSO-d_6, δ): 181.4 (C-1), 109.0 (C-2), 163.3 (C-3), 105.9 (C-4), 135.7 (C-5), 111.2 (C-6), 154.9 (C-7), 154.0 (C-8), 106.5 (C-9), 164.6 (C-10), 102.5 (C-11), 162.3 (C-12), 102.5 (C-13), 90.5 (C-14), 48.0 (C-15), 113.2 (C-16), 145.4 (C-17), 56.8 (C-18), 56.8 (C-19).

Ditryptophenaline (**6**). White crystal; $[\alpha]_D^{21}$ –283.6° (*c* 0.150, $CHCl_3$); ESI-MS (positive ion) *m/z:* 693 $[M+H]^+$, 715 $[M+Na]^+$, 1385 $[2M+H]^+$, $C_{42}H_{40}N_6O_4$; ^1H NMR (600 MHz, $CDCl_3$, δ, J/Hz): 5.85 (2H, s, NH-1, 1′), 5.17 (2H, s, H-2, 2′), 7.03 (2H, dd, J = 7.8, 1.4, H-5, 5′), 6.66 (2H, td, J = 7.3, 1.0, H-6, 6′), 7.03 (2H, t, J = 6.8, H-7, 7′), 6.62 (2H, d, J = 8.3, H-8, 8′), 3.57 (2H, dd, J = 11.8, 4.6, H-11, 11′), 1.93 (2H, dd, J = 12.4, 5.0, H-12a, 12a′), 1.63 (2H, t, J = 12.4, H-12b,12b′), 4.39 (2H, t, J = 5.0, H-15, 15′), 3.29 (2H, dd, J = 14.2, 5.0, H-17a, 17a′), 3.52 (2H, dd, J = 14.2, 2.3, H-17b, 17b′), 7.18 (4H, d,

J = 7.8, H-19, 23, 19′, 23′), 7.56 (4H, t, J = 7.8, H-20, 22, 20′, 22′), 7.41 (2H, t, J = 7.3, H-21, 21′), 2.98 (6H, s, H-24, 24′); ^{13}C NMR (150 MHz, DMSO-d_6, δ): 79.3 (C-2, 2′), 60.0 (C-3, 3′), 127.8 (C-4, 4′), 126.1 (C-5, 5′), 118.6 (C-6, 6′), 130.1 (C-7, 7′), 109.9 (C-8, 8′), 152.3 (C-9, 9′), 59.0 (C-11, 11′), 37.7 (C-12, 12′), 165.6 (C-13, 13′), 63.5 (C-15, 15′), 164.6 (C-16, 16′), 36.6 (C-17, 17′), 136.4 (C-18, 18′), 130.1 (C-19, 23, 19′, 23′), 129.9 (C-20, 22, 20′, 22′), 128.4 (C-21, 21′), 32.3 (C-24, 24′).

3-[(1H-Indol-3-yl)methyl]-6-benzylpiperazine-2,5-dione (7). White amorphous solid; ESI-MS (positive ion) *m/z:* 334 $[M+H]^+$, $C_{20}H_{19}N_3O_2$; ^{1}H NMR (600 MHz, DMSO-d_6, δ, J/Hz): 10.92 (1H, s, NH-1), 6.95 (1H, d, J = 2.3, H-2), 7.42 (1H, d, J = 7.8, H-4), 6.99 (1H, t, J = 7.3, H-5), 7.07 (1H, t, J = 7.7, H-6), 7.32 (1H, d, J = 8.2, H-7), 2.54 (1H, dd, J = 5.0, 14.2, H-10a), 2.70 (1H, dd, J = 4.1, 14.2, H-10b), 4.02 (1H, t, J = 5.5, H-11), 8.01 (1H, s, NH-12), 3.95 (1H, t, J = 6.8, H-14), 2.11 (2H, m, H-17), 6.79 (2H, d, J = 7.8, H-19, 23), 7.23 (2H, t, J = 7.3, H-20, 22), 7.18 (1H, t, J = 7.3, H-21), 2.64 (1H, s, H-24); ^{13}C NMR (150 MHz, DMSO-d_6, δ): 124.4 (C-2), 108.8 (C-3), 118.8 (C-4), 118.4 (C-5), 120.9 (C-6), 111.3 (C-7), 127.5 (C-8), 136.0 (C-9), 29.7 (C-10), 55.3 (C-11), 166.2 (C-13), 55.6 (C-14), 166.8 (C-16), 39.5 (C-17), 136.5 (C-18), 129.7 (C-19, 23), 127.5 (C-20, 22), 126.0 (C-21), 32.3 (C-24).

Biological Assay. MTT assay. The cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cell suspensions (200 μL), with a density of 5×10^4 cells mL^{-1}, were placed in 96-well microtiter plates and incubated for 24 h under the above conditions. The test compound solution (2 μL in DMSO) at different concentrations was added to each well and further incubated for 72 h in the same conditions. Then 20 μL of the MTT solution (5 mg/mL in RPMI-1640 medium) was added to each well and the mixture incubated for 4 h. The old medium containing MTT (150 μL) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

SRB assay cells were placed in 96-well plates and allowed to attach and grow for 24 h. The compounds or the vehicle (MeOH) were added and incubated with the cells for 48 h. Following drug exposure, the cells were fixed with 10% trichloroacetic acid at 4° for 1 h, and then the cell layer was stained with an SRB solution (0.4%) for 30 min. Excess stain was washed off with 1% AcOH, and the SRB was solubilized with 10 mM Tris base for 1 h on an orbital shaker. The absorbance of the SRB solution was measured at 520 nm. Dose-response curves were plotted, and the IC_{50} values calculated from the linear portion of the log dose-response curves.

ACKNOWLEDGMENT

This work was financially supported by Chinese National Programs for High Technology Research and Development (No. 2007AA092447) and the Shandong Province Natural Science Fund (No. Z2006C13). The *Aspergillus flavus* was identified by Prof. Hong Kui at the Chinese Academy of Tropical Agricultural Sciences, Hainan, China. The antitumor assay was performed at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

REFERENCES

1. C. W. Holzapfel, *Tetrahedron*, **24**, 2101 (1968).
2. A. A. El-Banna, J. I. Pitt, and L. Leistner, *Syst. Appl. Microbiol.*, **10**, 42 (1987).
3. T. Goto, D. T. Wicklow, and Y. Ito, *Appl. Environ. Microbiol.*, **62**, 4036 (1996).
4. N. Gqaleni, J. E. Smith, and J. Lacey, *Food Addit. Contam.*, **13**, 677 (1996).
5. R. T. Riley, D. E. Goeger, H. Yoo, and J. L. Showker, *Toxicol. Appl. Pharmacol.*, **114**, 261 (1992).
6. B. S. Karon, J. E. Mahaney, and D. D. Thomas, *Biochemistry*, **33**, 13928 (1994).
7. N. Demaurex, D. P. Lew, and K. H. Krause, *J. Biol. Chem.*, **267**, 2318 (1992).
8. A. A. Chalmers, C. P. Gorst-Allman, and P. S. Steyn, *J. Chem. Soc., Chem. Commun.*, 1367 (1982).
9. C. W. Holzapfel and D. C. Wilkins, *Phytochemistry*, **10**, 351 (1971).
10. T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).
11. P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, and T. Jonathan, *J. Natl. Cancer Inst.*, **82**, 1107 (1990).