Deoxy-cytochalasins from a marine-derived fungus *Spicaria elegans*

Zhen-Jian Lin, Tian-Jiao Zhu, Guo-Jian Zhang, Hong-Juan Wei, and Qian-Qun Gu

Abstract: Treatment of *Spicaria elegans* with cytochrome P-450 inhibitor resulted in two new deoxy-cytochalasins, 7-deoxy-cytochalasin Z_7 (**1**) and 7-deoxy-cytochalasin Z_9 (**2**), which were recognized as plausible precursors of cytochalasins Z_7 and Z_9, respectively. Their structures were elucidated by spectroscopic methods and the absolute configuration of **1** was determined by the conventional Mosher ester method. Their cytotoxicities against two cancer cell lines were evaluated.

Key words: cytochalasin, *Spicaria elegans*, cytochrome P-450 inhibitor, cytotoxicity.

Résumé : Le traitement du *Spicaria elegans* par l'inhibiteur du cytochrome P-450 conduit à la formation de deux nouvelles désoxycytochalasines, le 7-désoxycytochalasine Z_7 (**1**) et 7-désoxycytochalasine Z_9 (**2**) qui ont été reconnues comme des précurseurs plausibles respectivement pour les cytochalasines Z_7 et cytochalsaines Z_9. Les structures des composés **1** et **2** ont été élucidées par des méthodes spectroscopiques et la configuration absolue du produit **1** a été déterminée par la méthode conventionnelle de l'ester de Mosher. On a évalué leurs cytotoxicités contre deux séries de cellules cancéreuses.

Mots-clés : cytochalasine, *Spicaria elegans*, inhibiteur de cytochrome P-450, cytotoxicité.

[Traduit par la Rédaction]

Introduction

In our ongoing search for new bioactive secondary metabolites from marine fungi, a series of cytochalasins has been isolated from the marine-derived fungus *Spicaria elegans*, some of which exhibited interesting cytotoxicities against the A-549 cell line,[1,2] which attracted our continuous attentions. Given that P450 mono-oxygenases are believed to catalyze the oxidative polyketide tailoring reactions of cytochalasins,[3] it is possible to use the inhibitors to obtain various biosynthetic precursor products. The achievement of this was realized by the discovery of 7-deoxy-cytochalasin Z_7 (**1**) and 7-deoxy-cytochalasin Z_9 (**2**) by the addition of metyrapone, an inhibitor of P-450 dependent monooxygenases, in the culture of fungus *S. elegans*. We describe herein the isolation, structure elucidation, and biological activities of **1** and **2**.

Results and discussion

Previous study had shown that the strain *S. elegans* produced 10 phenyl-cytochalasins (Z_7–Z_{15})[1,2] as the major components, which were all oxidized at the 7-position. Metyrapone (1 mmol/L), a cytochrome P-450 inhibitor, was added on the sixth day after inoculation to the culture of *S. elegans*. After an additional 14 days of static fermentation, the metabolites were extracted from the mycelium. Compared with nontreated samples, two new 7-deoxy-cytochalasins (**1** and **2**) were recognized by HPLC-MS analysis and isolated by semiprepared HPLC.

7-Deoxy-cytochalasin Z_7 (**1**) was isolated as a white powder. Its molecular formula was established as $C_{28}H_{35}NO_4$ by HR-ESI-MS (*m/z* 450.2652 $[M + H]^+$, anal. calcd. for $C_{28}H_{36}NO_4$: 450.2644) and indicated twelve degrees of unsaturation. The IR spectrum had bands due to NH and (or) OH (3392 cm^{-1}), an α,β-unsaturated ester/lactone carbonyl (1698 cm^{-1}), and an amide carbonyl (1680 cm^{-1}). The ^1H NMR spectrum (Table 1) showed the presence of an amide NH (δ_H: 5.79), three methyl doublets (δ_H: 0.97, 1.05, and 1.17), a methyl singlet (δ_H: 1.72), and 11 methine and (or) methylene protons. Ten olefinic protons (δ_H: 5.10, 5.34, 5.66, 5.96, and 7.16–7.33), suggesting a monosubstituted benzene ring, were also observed. The ^{13}C NMR spectrum of **1** displayed signals for 28 carbon atoms and confirmed the presence of an α,β-unsaturated ester/lactone carbonyl (δ_C: 167.4) and an amide carbonyl (δ_C: 172.5) (Table 1). Analysis of the DEPT spectrum revealed the presence of four methyl, two methylene, 17 methine, and three quaternary carbons, in addition to the two carbonyls. All of these 1D NMR features of **1** suggested it was closely related to an analog of cytochalasins. Accurate inspection of its 1D NMR spectra revealed, with respect to that of cytochalasin Z_7, the absence of exocyclic olefinic methylene (12-CH_2). Whereas a trisubstituted double bond (δ_H: 5.34, δ_C: 123.9, 140.0) and a methyl goup (12-Me, δ_H: 1.72, δ_C: 19.8) was present. These findings indicated that the 7-OH and the exocyclic olefinic methylene were missing, instead, a trisubstituted double bond was located between C-6 and C-7. The structure of **1** was further supported by its molecular formula ($C_{28}H_{35}NO_4$), with one fewer oxygen than cytochalasin Z_7. Compound **1** possessed a 12-membered macrocyclic ring, the same as that in cytochalasin Z_7. Accurate inspection of the ^1H and ^{13}C NMR data, including ^1H NMR coupling con-

Received 14 November 2008. Accepted 10 December 2008. Published on the NRC Research Press Web site at canjchem.nrc.ca on 11 February 2009.

Z. Lin, T. Zhu, G. Zhang, H. Wei, and Q. Gu.[1] Key Laboratory of Marine Drugs, Chinese Ministry of Education, Institute of Marine Drugs and Food, Ocean University of China, Qingdao 266003, PR China.

[1]Corresponding author (e-mail: guqianq@ouc.edu.cn).

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data for **1** and **2** in $CDCl_3$

Compound position	**1**		**2**	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		172.5 (s)		172.1 (s)
2-NH	5.79 (brs)		5.89 (brs)	
3	3.22 (m)	55.9 (d)	3.20 (m)	55.9 (d)
4	3.04 (m)	50.8 (d)	2.91 (m)	51.5 (d)
5	3.04 (m)	33.8 (d)	2.91 (m)	34.1 (d)
6		140.0 (s)		139.9 (s)
7	5.34 (brs)	123.9 (d)	5.36 (brs)	123.8 (d)
8	3.47 (m)	45.4 (d)	3.38 (m)	46.7 (d)
9		88.0 (s)		87.7 (s)
10	2.89 (dd, J = 13.2, 4.1), 2.70 (d, J = 13.7)	45.1 (t)	2.89 (m), 2.85 (m)	44.8 (t)
11	1.17 (d, J = 6.8)	13.8 (q)	1.14 (d, J = 6.6)	13.8 (q)
12	1.72 (brs)	19.8 (q)	1.74 (brs)	19.8 (q)
13	5.96 (ddd, J = 15.1, 10.0, 2.0)	125.9 (d)	5.91 (ddd, J = 15.1, 10.0, 2.7)	126.3 (d)
14	5.10 (ddd, J = 15.1, 10.1, 4.6)	137.2 (d)	5.28 (ddd, J = 15.1, 10.1, 2.7)	137.1 (d)
15	2.08 (m)	42.0 (t)	2.10 (brd, J = 14.3), 1.97 (ddd, J = 14.3, 10.4, 10.4)	43.5 (t)
16	1.62 (m)	32.5 (d)	1.39 (m)	32.5 (d)
16-CH_3	0.97 (d, J = 7.3)	18.4 (q)	1.05 (d, J = 7.1)	22.2 (q)
17	3.87 (dd, J = 3.2, 4.1)	77.3 (d)	1.77 (dd, J = 13.6, 5.5), 1.64 (dd, J = 13.6, 2.2)	53.2 (d)
18	2.72 (m)	42.8 (d)		72.8 (s)
18-CH_3	1.05 (d, J = 6.4)	8.7 (q)	1.34 (s)	27.1 (q)
19	7.16–7.33 (m)	157.5 (d)	7.45 (d, J = 16.0)	159.5 (d)
20	5.66 (dd, J = 16.0, 1.9)	120.5 (d)	5.75 (d, J = 16.0)	118.4 (d)
21		167.4 (s)		167.4 (s)
1′		137.4 (s)		137.7 (s)
2′	7.16–7.33 (m)	128.8 (d)	7.18–7.33 (m)	128.9 (d)
3′	7.16–7.33 (m)	129.0 (d)	7.18–7.33 (m)	129.0 (d)
4′	7.16–7.33 (m)	127.0 (d)	7.18–7.33 (m)	126.9 (d)
5′	7.16–7.33 (m)	129.0 (d)	7.18–7.33 (m)	129.0 (d)
6′	7.16–7.33 (m)	128.8 (d)	7.18–7.33 (m)	128.9 (d)

Fig. 1. Structures of compounds **1** and **2**.

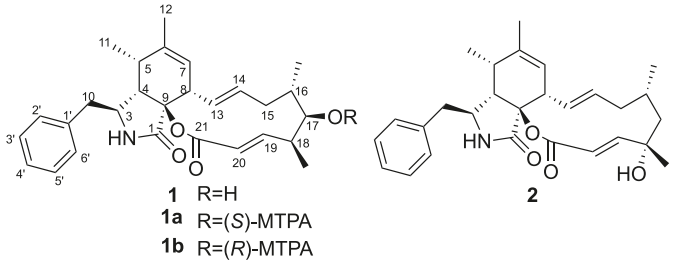
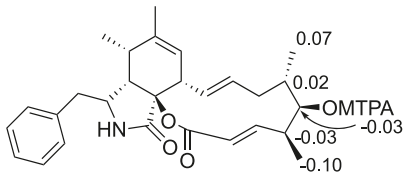


Fig. 2. The key $\Delta\delta$ values [$\Delta\delta$ (in ppm) = $\delta_S - \delta_R$] obtained for (*S*)- and (*R*)-MTPA esters of compound **1**.



stants, for compound **1** and cytochalasin Z_7 should have the most coincident relative configuration (structure from C-15 to C-19). The absolute configuration of the 17 position in **1** was established by a conventional Mosher ester method using the (*S*)- and (*R*)-α-methoxy-α-trifluoromethyl-α-phenylacetic acid (MTPA) esters (**1a** and **1b**, respectively) (Fig. 1), where the positive values of $\Delta\delta^{S-R}$ for H-16 and 16-CH_3 and negative values of $\Delta\delta^{S-R}$ for H-18 and 18-CH_3 (Fig. 2) suggested a 17-(*R*) configuration in **1**, which was the same as that of cytochalasin Z_7. Therefore, the absolute configurations of **1** could be deduced as shown in Fig. 1.

7-Deoxy-cytochalasis Z_9 (**2**) was isolated as a white powder. Its HR-ESI-MS (*m*/*z* 450.2638 $[M + H]^+$, anal. calcd. for $C_{28}H_{36}NO_4$: 450.2644) suggested that it might be a deoxy-derivative of cytochalasin Z_9, with a molecular formula of $C_{28}H_{35}NO_4$ showing one fewer oxygen atom than cytochalasin Z_9. This assumption could be confirmed by its 1D NMR spectra. Analysis of its 1D NMR spectra, with respect to that of cytochalasin Z_9, revealed that the singlet of 11-CH_3 in cytochalasis Z_9 was upfiled shifted to δ_H: 1.14 and changed to a doublet, which suggested the double bond at C-5,6 was missing in **2**. Most similarly to **1**, the 1D NMR spectra of **2** (Table 1) also showed the absence of the oxygenated methine group at C-7, whereas there was the appearance of a trisubstituted double bond (δ_H: 5.36, δ_C: 123.8, 139.9) assigned between C-6 and C-7. On the basis of these 1D NMR spectra features, the structure of **2** was elucidated as 7-deoxy-cytochalasis Z_9.

Compounds **1**and **2** were evaluated for their cytotoxicities against the A-549 and P-388 cell lines by the MTT [3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Compound **1** showed moderate cytotoxicity against the A-549 cell line with an IC_{50} value of 15.0 μmol/L, while **2** was inactive. In comparison to that of cytochalasins Z_7 and Z_9, the double bond (C-6,7) in **1** and **2** resulted in a much weaker cytotoxicity.

A new generation of plausible precursors of chaetoglobosin A[4] and epoxycytochalasin H[5] had been obtained by treatment fungi with cytochrome P-450 inhibitors. Those results, as well as the experiments in this paper, indicated that the biosynthetic oxidation at the 7 position of cytochalasins was exactly due to the cytochrome P-450. More frequently, the cytochalasins, likewise cytochalasins Z_7 and Z_9, have an oxidation at the C-9 position, which was believed to be formed from the acetate-derived polyketide chain by an enzymatic 'Baeyer–Villager'-like oxidation.[6] Even though a cytochrome P-450 monooxygenase CYP85A2 had been reported to have a Baeyer–Villiger oxidation activity,[7] it was still unknown whether the Baeyer–Villager-like oxidation in cytochalasins was related to the cytochrome P-450. In our experiments, no more deoxy-cytochalasin was detected except for **1** and **2** when *S. elegans* was treated with metyrapone, therefore, the P-450 enzyme inhibitor can't block the Baeyer–Villager-like oxidation in this strain. Maybe it is not the P-450 enzyme that catalyzed the Baeyer–Villager-like oxidation in the biosynthetic pathway of cytochalasins. This warrants further investigations.

Experimental section

General

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckman DU® 640 spectrophotometer. IR spectra were taken on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^1H, ^{13}C NMR and DEPT, and 2D NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as an internal standard and chemical shifts were recorded as δ values. NOESY experiments were carried out using a mixing time of 0.5 s. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semiprepartive HPLC was performed using an ODS column (Shin-pak ODS $_H$, 10 mm × 250 mm, 5 μm, 4 mL/min).

Fungal material

The fungus *Spicaria elegans* was isolated from the marine sediments collected in Jiaozhou Bay, China. It was preserved in the China Center for Type Culture Collection (patent depositary number: KLA03 CCTCC M 205049). Working stocks were prepared on potato dextrose agar slants stored at 4 °C.

Fermentation and extraction

The fungus was incubated at 28 °C under static conditions in 30 1000 mL conical flasks containing the liquid medium (300 mL/flask) composed of glucose (20 g/L), peptone (5 g/L), malt extract (3 g/L), yeast extract (3 g/L), and sea-water after adjusting its pH to 7.0. The inhibitor metyrapone (1 mmol/L) was added on the sixth day after inoculation to the culture of *S. elegans*. After an additional 14 days of static fermentation, the fermented whole broth (9 L) was extracted three times with EtOAc to give an EtOAc solution, which was concentrated under reduced pressure to give a crude extract (5.5 g).

Purification

The crude extract (5.5 g) was separated into four fractions (Fr. 1–4) on a Si gel column using a gradient elution of $CHCl_3$:MeOH. Fr. 2 was eluted with $CHCl_3$:MeOH (100:1, 0.4 g) and was purified into 8 subfractions (Fr. 2-1 ~ Fr. 2-8) by Si gel column using stable elution of $CHCl_3$:MeOH (200:1). Subfraction Fr. 2-6 was further purified by reverse-phase column using stable elution of MeOH:H_2O (7: 3) to give 4 subfractions (Fr. 2-6-1 ~ Fr. 2-6-4), respectively. Further purification of subfraction Fr. 2-6-4 by extensive HPLC (75% MeOH, 4.0 mL/min) gave compounds **1** (3 mg) and **2** (2 mg).

7-Deoxy-cytochalasin Z_7 (1)

White powder. $[\alpha]_D^{25}$ +94.8 (*c* 0.1, MeOH). UV (MeOH) λ_{max} (nm) (log ε): 233 (2.43). IR (KBr, cm^{-1}) ν_{max}: 3368, 2937, 1700, 1444, 1214, 1076, 990. ^1H and ^{13}C NMR (see Table 1). HR-ESI-MS *m/z*: 450.2652 $[M + H]^+$. Anal. calcd. for $C_{28}H_{36}NO_4$: 450.2644.

7-Deoxy-cytochalasin Z_9 (2)

White powder. $[\alpha]_D^{25}$ +12.8 (*c* 0.1, MeOH). UV (MeOH) λ_{max} (nm) (log ε): 230 (2.00). IR (KBr, cm^{-1}) ν_{max}: 3298, 2900, 1694, 1321 1201, 999. ^1H and ^{13}C NMR (see Table 1). HR-ESI-MS *m/z*: 450.2638 $[M + H]^+$. Anal. calcd. for $C_{28}H_{36}NO_4$: 450.2644.

Preparation of the (*S*)- and (*R*)-MTPA ester derivatives of 1

Compound **1** (2.0 mg) was divided into two aliquots and transferred into two clean reaction bottles and was dried completely under vacuum. Each solubilized in 0.5 mL of pyridine. The two samples were treated with (*R*)- and (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (1 equiv.) under a N_2 gas stream, and then stirred for 24 h at room temperature. The organic layer was then washed with water, HCl (1 mol/L), water, $NaHCO_3$(satd.), and water, then dried (Na_2SO_4) and concentrated under reduced pressure to obtain the ester. Final purification achieved by HPLC gave **1a** and **1b**, respectively. ^1H NMR data for the (*S*)-MTPA ester derivative (**1a**) of **1** (600 MHz, $CDCl_3$) δ: 5.76 (1H, brs, 2-NH), 3.24 (1H, m, H-3), 3.05 (1H, m, H-4), 3.05 (1H, m, H-5), 5.35 (1H, brs, H-7), 3.48 (1H, m, H-8), 2.90 (1H, dd, *J* = 4.1, 13.8 Hz, H-10a), 2.69 (1H, dd, *J* = 9.6, 13.8 Hz, H-10b), 1.20 (3H, d, *J* = 6.9 Hz, H-11), 1.73 (3H, brs, H-12), 6.04 (1H, ddd, *J* = 13.0, 10.6, 1.8 Hz, H-13), 5.07 (1H, m, H-14), 2.30 (1H, ddd, m, H-15a), 2.09 (1H, brd, *J* = 14.6 Hz, H-15b), 1.80 (1H, m, H-16), 0.88 (3H, d, *J* = 6.4 Hz, 16-CH_3), 5.09 (1H, m, H-17), 2.80 (1H, m, H-18), 0.97 (3H, d, *J* = 7.3 Hz, 18-CH_3), 5.68 (1H, dd, *J* = 15.5, 2.3 Hz, H-19), 7.15 ~ 7.56 (11H, m, H-20 and Ph-H). ^1H NMR data for the (*R*)-MTPA ester derivative (**1b**) of **1** (600 MHz, $CDCl_3$) δ: 5.82 (1H, brs, 2-NH), 3.24 (1H, m, H-3), 3.05 (1H, m, H-4), 3.05 (1H, m, H-5), 5.35 (1H, brs, H-7), 3.48 (1H, m, H-8), 2.90 (1H, dd, *J* = 4.1, 13.8 Hz, H-10a), 2.69 (1H, dd, *J* = 9.6, 13.8 Hz, H-10b), 1.20 (3H, d, *J* = 6.9 Hz, H-11), 1.73 (3H, brs, H-12), 6.04 (1H, ddd, *J* =

13.0, 10.6, 1.8 Hz, H-13), 5.07 (1H, m, H-14), 2.34 (1H, ddd, m, H-15a), 2.05 (1H, brd, J = 14.6 Hz, H-15b), 1.78 (1H, m, H-16), 0.81 (3H, d, J = 6.4 Hz, 16-CH_3), 5.12 (1H, m, H-17), 2.83 (1H, m, H-18), 1.07 (3H, d, J = 7.3 Hz, 18-CH_3), 5.71 (1H, dd, J = 15.5, 2.3 Hz, H-19), 7.15 ~ 7.56 (11H, m, H-20 and Ph-H).

Biological assays

Cytotoxic activity was evaluated by the MTT method using A-549 and H-L60 cell lines. 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 (tsFT210 cell line at 32 °C). An aliquot (200 μL) of those cell suspensions at a density of 5×10^4 cell mL^{-1} was plated in 96 well microtiter plates and incubated for 24 h at the above condition. Then 2 μL of the test compound solutions (in DMSO) at different concentrations was added to each well and further incubated for 72 h in the same condition. MTT solution (20 μL of 5 mg/mL in IPMI-1640 medium) was added to each well and incubated for 4 h. Old medium containing MTT (150 μL) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals that had formed. Absorbance was then determined on a SPECTRA MAX PLUS plate reader at 570 nm.

Acknowledgements

This work was financially supported by the Chinese National Natural Science Fund (No. 30772640) and the Shandong Province (No. Z2006C13). The antitumor assay was performed at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

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