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Alachalasin A–G, new cytochalasins from the fungus *Stachybotrys charatum*

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Abstract—Alachalasin A–G (**1**–**7**), seven new cytochalasin-type of metabolites, have been isolated from cultures of an isolate of *Stachybotrys charatum*. The structures of these compounds were determined mainly by analysis of their NMR spectroscopic data, and the absolute configuration of **1** was assigned by application of the modified Mosher method. Alachalasin A (**1**) displayed inhibitory effect on HIV-1_{LAI} replication in C8166 cells with an EC₅₀ of 8.01 μM; alachalasin D (**4**) and G (**7**) showed modest antimicrobial activity.

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1. Introduction

Cytochalasins are a group of fungal secondary metabolites that display various biological activities and have been isolated frequently from fungal sources.¹ Examples include deacetylcytochalasin C and zygosporin D,² pyrichalasin H,³ phenochalasin A and B,^{4,5} cytochalasins E and H,^{6,7} cytochalasins N–R,⁸ RKS-177,⁹ aspochalasins F and G,¹⁰ 19-*O*-acetylchaetoglobosins B and D,¹¹ and cytochalasins F and T.¹² During our ongoing chemical investigations of fungal species from unique environment as sources of bioactive natural products, a subculture of an isolate of *Stachybotrys charatum* (XJ03-56-1) that was collected at high altitude (3600 m) of an unnamed glacier, Tibet, People's Republic of China, was grown in solid-substrate fermentation culture. Its organic solvent extract displayed inhibitory effect on HIV-1_{LAI} replication in C8166 cells. Bioassay-guided fractionation of

this extract led to the isolation of seven new cytochalasin-type compounds that have been named alachalasin A–G (**1**–**7**). Details of the isolation, structure elucidation, and biological activity of these compounds are reported here.

2. Results and discussion

Alachalasin A (**1**) was obtained as gum. Its molecular formula was determined to be C₂₁H₃₁NO₅ (eight degrees of unsaturation) by HRESIMS analysis [*m/z* 412.2095 (M+Na)⁺; Δ +0.1 mmu], and was supported by ¹H and ¹³C NMR data (Table 1). Analysis of ¹H, ¹³C, and HMQC NMR data for alachalasin A (**1**) revealed the presence of two exchangeable protons (δ_H 1.81 and 2.73, respectively), five methyl groups, two methylene units, seven methines (three of which are oxymethines), two quaternary carbons (one of which is oxygenated), four olefinic carbons (two of which are protonated), and two carbonyl carbons. These data accounted for all ¹H and ¹³C resonances and required compound to be tetracyclic. Analysis of the ¹H–¹H COSY NMR data led to the identification of three isolated proton spin-systems corresponding to the C-3–C-4 (including C-10),

Keywords: *Stachybotrys charatum*; Alachalasin; Anti-HIV; Antibacterial.

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Table 1. NMR spectroscopic data of alachalasin A–G (1–7)

Position	Alachalasin A (1)				Alachalasin B (2)		Alachalasin C (3)		Alachalasin D (4)	
	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{b}}$ (mult.)	HMBC (H → C#)	NOE	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{b}}$ (mult.)	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{b}}$ (mult.)	$\delta_{\text{H}}^{\text{c}}$ (J in Hz)	$\delta_{\text{C}}^{\text{d}}$ (mult.)
1		174.5, qC				174.5, qC		174.4, qC		175.9, qC
2	6.25, br s		3, 4, 9		5.74, br s		5.59, br s			
3	3.41, br q (6.6)	52.7, CH	1, 4, 5	11	3.41, br q (6.5)	52.7, CH	3.41, br q (6.6)	52.6, CH	3.29, dq (6.5, 1.5)	48.4, CH
4	3.14, br s	50.9, CH	1, 9, 21	8, 10	3.13, br s	51.5, CH	3.18, br s	51.1, CH	2.73, dd (6.0, 1.5)	50.2, CH
5		126.6, qC				126.7, qC		126.6, qC	2.82, dq (6.5, 1.5)	32.9, CH
6		132.4, qC				132.5, qC		132.4, qC		150.9, qC
7	4.07, d (9.3)	69.4, CH		12	4.11, d (8.8)	69.5, CH	4.11, d (9.3)	69.5, CH	4.03, d (9.5)	72.8, CH
8	2.08, dd (11, 9.3)	53.3, CH	4, 6, 7, 9, 13, 14	4	2.08, dd (10, 8.8)	53.4, CH	2.07, dd (11, 9.3)	53.3, CH	2.46, dd (10, 9.5)	52.4, CH
9		62.5, qC				62.1, qC		62.7, qC		64.8, qC
10	1.07, d (6.6)	23.0, CH ₃	3, 4	4	1.10, d (6.5)	23.1, CH ₃	1.09, d (6.6)	23.0, CH ₃	1.03, d (6.5)	23.8, CH ₃
11	1.69, s	14.3, CH ₃	4, 6	3	1.71, s	14.3, CH ₃	1.72, s	14.3, CH ₃	1.14, d (6.5)	13.2, CH ₃
12a	1.69, s	17.7, CH ₃	5, 7	7	1.71, s	17.7, CH ₃	1.72, s	17.7, CH ₃	5.20, s	114.3, CH ₂
12b									5.08, s	
13	6.11, dd (15, 11)	127.8, CH	8, 15		6.08, dd (15, 10)	127.4, CH	6.17, ddd (15, 11, 1.3)	127.0, CH	5.96, ddd (15, 10, 1.5)	129.7, CH
14	5.23, ddd (15, 11, 3.5)	136.3, CH	8, 15, 16		5.26, ddd (15, 11, 3.3)	136.8, CH	5.32, ddd (15, 11, 3.7)	136.8, CH	5.19, ddd (15, 10, 3.5)	136.1, CH
15a	2.01, br dd (14, 11)	36.4, CH ₂	13, 14, 16, 17, 22		2.03, br dd (14, 11)	36.5, CH ₂	2.03, m	36.5, CH ₂	2.03, br dd (14, 10)	37.3, CH ₂
15b	2.09, br dd (14, 3.5)		13, 14, 16, 17		2.14, br dd (14, 3.3)		2.16, m		2.11, br dd (14, 3.5)	
16	1.41, br dq (9.2, 6.6)	34.8, CH	14, 15, 17		1.41, br dq (9.4, 6.6)	34.8, CH	1.34, br dq (8.9, 6.7)	34.9, CH	1.42, m	36.0, CH
17	2.50, d (9.2)	70.9, CH	16, 18, 19, 22, 23	22	2.34, d (9.4)	69.5, CH	2.39, d (8.9)	70.3, CH	2.51, d (9.5)	71.6, CH
18		65.5, qC				65.3, qC		62.8, qC		67.0, qC
19a	3.73, d (6.5)	68.2, CH	18, 20, 21, 23		3.73, d (6.5)	76.2, CH	1.99, dd (14, 9.4)	22.8, CH ₂	3.75, d (6.5)	68.7, CH
19b							1.52, dd (14, 9.2)			
20a	2.24, dd (19, 6.5)	46.1, CH ₂	9, 19, 21		2.27, dd (19, 6.5)	46.9, CH ₂	2.27, dd (19, 9.4)	37.5, CH ₂	2.10, dd (20, 6.5)	47.7, CH ₂
20b	3.80, dd (19)		18, 21		3.72, d (19)		3.47, dd (19, 9.2)		3.93, d (20)	
21		207.2, qC		17		207.2, qC		208.8, qC		209.7, qC
22	1.13, d (6.6)	21.7, CH ₃	15, 16, 17		1.16, d (6.6)	21.8, CH ₃	1.15, d (6.7)	21.6, CH ₃	1.13, d (6.5)	21.7, CH ₃
23	1.28, s	16.9, CH ₃	17, 18, 19		1.26, s	17.5, CH ₃	1.26, s	24.8, CH ₃	1.25, s	17.2, CH ₃
24					3.48, s	58.1, CH ₃				
7-OH	1.81, br s				1.57, d (4.0)		1.57, br s			
19-OH	2.73, br s									

^a Recorded at 400 MHz in CDCl₃.^b Recorded at 100 MHz in CDCl₃.^c Recorded at 500 MHz in CD₃OD.^d Recorded at 125 MHz in CD₃OD.

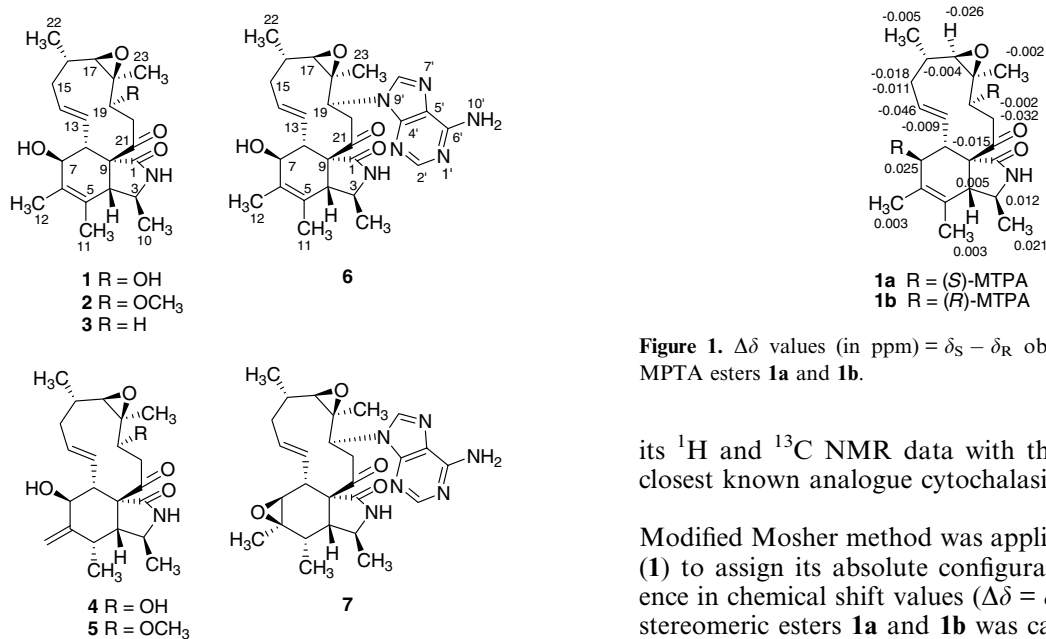


Figure 1. $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ obtained for (*S*)- and (*R*)-MPTA esters **1a** and **1b**.

its ¹H and ¹³C NMR data with those reported for its closest known analogue cytochalasin G.¹⁵

Modified Mosher method was applied for alachalasin A (**1**) to assign its absolute configuration.^{20,21} The difference in chemical shift values ($\Delta\delta = \delta_S - \delta_R$) for the diastereomeric esters **1a** and **1b** was calculated in order to assign the absolute configuration at C-7 and C-19 (Fig. 1). Calculations for all of the relevant signals suggested the *S* absolute configuration at C-7 and C-19. Ultimately, the 3*S*, 4*R*, 7*S*, 8*R*, 9*R*, 16*S*, 17*R*, 18*S*, and 19*S* absolute configuration was proposed for **1** on the basis of the results summarized in Figure 1.

The molecular formula of alachalasin B (**2**) was established as C₂₃H₃₃NO₅ (eight degrees of unsaturation) by analysis of its HRESIMS [*m/z* 426.2237 [M+Na]⁺; $\Delta -1.4$ mmu] and NMR data (Table 1), which is 14 mass units more than that of compound **1**. Analysis of the ¹H and ¹³C NMR data of **2** revealed the presence of structural features similar to those found in **1**, except that one hydroxy group (δ_H 2.73) was replaced by a methoxy unit (δ_H/δ_C 3.48/58.1) in the NMR spectra of **2**. Therefore, the gross structure of alachalasin B was established as shown in **2**. The absolute configuration of alachalasin B (**2**) was determined to be the same as that of **1** by analysis of the proton coupling constants and NOESY data.

Alachalasin C (**3**) was assigned the molecular formula of C₂₂H₃₁NO₄ (eight unsaturations) on the basis of its HRESIMS [*m/z* 396.2135 [M+Na]⁺; $\Delta -1.0$ mmu] and NMR data (Table 1), which is 16 mass units less than that of compound **1**. Analysis of the ¹H and ¹³C NMR data of **3** revealed that the oxymethine unit (δ_H/δ_C 3.73/68.2) in **1** was replaced by a methylene unit (δ_H 1.99, 1.52; δ_C 22.8) in the NMR spectra of **3**. Analysis of the ¹H–¹H COSY NMR data for **3** indicated that this newly observed methylene unit was directly connected to C-20. On the basis of these data, the gross structure of alachalasin C was established as depicted in **3**, and its relative and absolute configurations were also proposed by analogy to alachalasin A (**1**).

The molecular formula of alachalasin D (**4**) was determined as C₂₂H₃₁NO₅ (eight unsaturations) on the basis of HRESIMS analysis [*m/z* 412.2084 (M+Na)⁺; $\Delta -1.0$ mmu] and NMR data (Table 1). Detailed analysis

C-7–C-17 (including C-22, but excluding C-9), and C-19–C-20 subunits of structure **1**. HMBC correlations from H-4 and H-8 to one quaternary carbon C-9 (δ_C 62.5) and two carbonyl carbons (δ_C 174.5 and 207.2, respectively) indicated that C-1, C-4, C-8, and C-21 were all connected to C-9, and correlations from H₃-11 to C-4 and C-6, and H₃-12 to C-5 and C-7 led to the completion of a cyclohexene unit, with C-11 attached to C-5 and C-12 attached to C-6. Correlations of H₃-23 with C-17, C-18, and C-19, H-20a with C-9, C-19, and C-21, and H-20b with C-18 and C-21 established the C-17–C-21 substructure with C-23 attached to C-18, and permitted completion of the cycloundecenone subunit. Correlations from H-3 to C-1, and from the exchangeable proton (δ_H 6.25) to C-3, C-4, and C-9 revealed the presence of a pyrrolinone subunit in **1**. The chemical shifts of C-7 (δ_C 69.4) and C-19 (δ_C 68.2) indicated that they were both attached to hydroxy groups. On the basis of these data, the planar structure of alachalasin A was established as shown in **1**.

Previous studies on other cytochalasins have indicated that the cyclohexene and the pyrrolinone subunits were *cis*-fused to each other at C4/C9 ring junction, and *trans*-fused between the cyclohexene and the cycloundecenone rings at C8/C9 bridge.^{13,14} In addition, the cyclohexene ring adopted slightly twisted boat conformation, and the cycloundecenone moiety containing an *E*-olefin subunit was arranged in chair-like conformation.^{13–15} Key NOESY correlations from H-3 to H-11, H-4 to H-8 and H₃-10, H-7 to H₃-12, and H-17 to H₃-22 revealed the same relative configuration between alachalasin A (**1**) and the precedents described in the literature.^{13–15}

Biosynthetic studies of cytochalasins have demonstrated that the amino acid residue presented in this class of metabolites possesses *L*-configuration.^{16–19} Therefore, alachalasin A could possess the *S*-configuration at C-3 corresponding to the naturally predominant *L*-Ala, and this conclusion was supported by comparison of

of the ^1H and ^{13}C NMR data indicated that signals for the C-6–C-12 unit in **1** were replaced by those for a terminal olefin unit, and an olefinic carbon (δ_{C} 126.6) was replaced by a methine unit ($\delta_{\text{H}}/\delta_{\text{C}}$ 2.82/32.9) in the NMR spectra of **4**, indicating the C-5/C-6 double bond appearing in compounds **1–3** was reduced in **4**. HMBC correlations from the terminal olefinic protons (H₂-12) to C-5, C-6, and C-7 further supported this conclusion. On the basis of these data, the planar structure ofalachalasin D was established as shown in **4**.

The relative configuration ofalachalasin D was assigned by analysis of its proton coupling constants and by comparison of its NMR data with those ofalachalasin A (**1**). The small coupling constant (1.5 Hz) observed between H-4 and H-5 indicated that both protons adopted pseudoequatorial orientation with respect to corresponding six-membered ring. A 9.5-Hz coupling constant between H-7 and H-8 indicated that the relative configuration between these protons remained the same in **4** comparing to that in **1–3**. The absolute configuration ofalachalasin D (**4**) was again assigned as shown by analogy toalachalasin A (**1**).

The molecular formula ofalachalasin E (**5**) was established as C₂₃H₃₃NO₅ (eight unsaturations) by analysis of its HRESIMS [m/z 426.2238 [M+Na]⁺; Δ -1.3 mmu] and NMR data (Table 2), which is 14 mass units more than that of compound **4**. Analysis of the ^1H and ^{13}C NMR data for **5** revealed the presence of essentially the same structural features as those found in **4**, except that the resonances for an additional methoxy group ($\delta_{\text{H}}/\delta_{\text{C}}$ 3.50/58.0) were observed in its NMR spectra. HMBC correlations from this methoxy protons to C-19 revealed the connectivity of this methoxy group to C-19. Therefore, the gross structure ofalachalasin E was established as shown in **5**. The configuration ofalachalasin E was assigned as shown in **5** by analogy toalachalasin D (**4**).

Alachalasin F (**6**) was assigned the molecular formula of C₂₇H₃₄N₆O₄ (14 unsaturations) on the basis of HRESIMS analysis [m/z 529.2550 (M+Na)⁺; Δ -0.7 mmu] and NMR data (Table 2). Analysis of the ^1H and ^{13}C NMR data of **6** revealed the presence of the same core structure as that found in **1**, except that additional resonances for two aromatic protons (δ_{H} 8.12 and 8.22, respectively) and five sp² carbons (δ_{C} 120.4, 143.9,

Table 2. NMR spectroscopic data ofalachalasins E and G (**5** and **7**) in CDCl₃ andalachalasin F (**6**) in CD₃OD

Position	Alachalasin E (5)		Alachalasin F (6)		Alachalasin G (7)	
	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{b}}$ (mult.)	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{c}}$ (mult.)	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{c}}$ (mult.)
1		173.6, qC		176.0, qC		174.2, qC
2	5.48, br s				5.81	
3	3.26, br d (6.3)	46.7, CH	3.39, q (6.6)	54.1, CH	3.60, q (6.4)	47.5, CH
4	2.78, br d (5.4)	48.7, CH	2.98, br s	52.2, CH	2.63, br d (6.2)	50.2, CH
5	2.87, m	31.7, CH		127.9, qC	1.82, m	36.7, qC
6		148.8, qC		134.4, qC		57.7, qC
7	4.16, d (9.9)	71.4, CH	4.02, d (5.8)	70.5, CH	2.98, d (5.8)	61.4, CH
8	2.50, ddd (9.9, 9.8, 3.3)	51.5, CH	2.10, m	54.2, CH	2.16, m	50.2, CH
9		62.5, qC		64.5, qC		65.2, qC
10	1.06, d (6.3)	24.0, CH ₃	0.87, d (6.6)	22.5, CH ₃	0.99, d (6.4)	24.9, CH ₃
11	1.12, d (6.6)	12.9, CH ₃	1.70, s	14.5, CH ₃	1.15, d (7.2)	12.7, CH ₃
12a	5.28, br s	114.6, CH ₂	1.70, s	17.7, CH ₃	1.25, s	19.6, CH ₃
12b	5.09, br s					
13	6.04, dd (15, 9.8)	127.3, CH	6.21, ddd (16, 11, 1.5)	130.6, CH	6.34, ddd (15, 11)	128.2, CH
14	5.31, ddd (15, 10, 3.3)	136.4, CH	5.34, ddd (16, 11, 3.6)	135.3, CH	5.30, ddd (15, 11, 3.4)	134.3, CH
15a	2.05, br dd (14, 10)	36.2, CH ₂	2.12, m	37.5, CH ₂	2.14, m	36.3, CH ₂
15b	2.14, br dd (14, 3.3)		2.29, m		2.32, m	
16	1.43, m	34.8, CH	1.80, m	36.2, CH	1.60, m	35.7, CH
17	2.35, d (9.3)	67.5, CH	2.66, d (9.2)	73.2, CH	2.61, d (9.0)	71.6, CH
18		65.2, qC		65.8, qC		65.0, qC
19	3.40, d (6.6)	76.3, CH	4.58, d (9.2)	56.7, CH	4.37, d (9.0)	56.2, CH
20a	2.19, dd (19, 6.6)	46.5, CH ₂	4.50, dd (19, 9.2)	41.5, CH ₂	4.58, dd (19, 9.0)	40.7, CH ₂
20b	3.89, d (19)		4.05, d (19, 9.2)		4.11, d (19)	
21		207.9, qC		208.9, qC		207.7, qC
22	1.17, d (6.6)	21.7, CH ₃	1.21, d (6.6)	21.9, CH ₃	1.26, d (6.7)	21.9, CH ₃
23	1.27, s	17.4, CH ₃	1.34, s	19.1, CH ₃	1.35, s	19.0, CH ₃
24	3.50, s	58.0, CH ₃				
7-OH	1.57, br s					
2'			8.12, s	153.1, CH	8.18, s	152.1, CH
4'				151.9, qC		151.3, qC
5'				120.4, qC		120.4, qC
6'				157.2, qC		155.5, qC
8'			8.22, s	143.9, CH	7.89, s	142.8, CH
10'					5.54, br s	

^a Recorded at 400 MHz.

^b Recorded at 125 MHz.

^c Recorded at 100 MHz.

151.9, 153.1, and 157.2) were observed in the NMR spectra of **6**. The molecular formula for **6** suggested the presence of five more nitrogen atoms in addition to the one that was incorporated into the core structure, indicating that the possible presence of an adenine moiety in **6**. HMBC correlations from H-2' (δ_{H} 8.12) to C-4' (δ_{C} 151.9) and C-6' (δ_{C} 157.2), and H-8' (δ_{H} 8.22) to C-4' and C-5' (δ_{C} 120.4) further confirmed the presence of such a moiety. Key HMBC correlations from H-19 of the core structure to C-4' and C-8' (δ_{C} 143.9) of the adenine unit led to the connection of the adenine moiety to C-19, thereby completing the gross structure ofalachalasin F (**6**) as shown. The relative and absolute configurations ofalachalasin F (**6**) were proposed as shown by analogy toalachalasin A (**1**).

The molecular formula of the final compoundalachalasin G (**7**) was established as $\text{C}_{27}\text{H}_{34}\text{N}_6\text{O}_4$ (14 unsaturations) on the basis of HRESIMS analysis [m/z 529.2549 (M+Na) $^+$; Δ -0.6 mmu] and NMR data (Table 2), which is the same as that of compound **6**. Analysis of the ^1H and ^{13}C NMR data for **7** revealed the presence of structural features similar to those found in **6**, except that the two olefinic carbons (δ_{C} 127.9 and 134.4, respectively) were replaced by an oxygenated quaternary carbon (δ_{C} 57.7) and a methine carbon (δ_{C} 36.7) in the NMR spectra of **7**. HMBC correlations from the methyl protons H₃-12 to C-5, C-6, and C-7 revealed the same connectivities for the subunit from C-5 to C-7, indicating that the C-5/C-6 double bond appearing in **6** was reduced. Considering the unsaturation requirement foralachalasin G, the two oxygenated sp^3 carbons C-6 and C-7 in **7** have to be connected to the oxygen atom to form an epoxide moiety. On the basis of these data, the planar structure ofalachalasin G was established as shown in **7**.

The relative configuration ofalachalasin G (**7**) was established by analysis of ^1H - ^1H NMR coupling constants, NOESY data, and by comparison of its ^1H NMR data with those ofalachalasin F (**6**), and its closest related known analogue cytochalasin G.¹⁵ The coupling constant of 5.8 Hz observed between H-7 and H-8 indicated that these protons adopted pseudoaxial orientation, and were *trans* to each other with respect to the cyclohexane ring, whereas no coupling was observed between H-4 and H-5, indicating the vicinal angle between these protons was close to 90°. The absolute configuration ofalachalasin G (**7**) was assigned by analogy toalachalasin F (**6**) and the known precedent cytochalasin G.¹⁵

Alachalasin A (**1**) was tested for *in vitro* activity against HIV-1_{LAI} in C8166 cells, and it showed anti-HIV-1 activity with EC₅₀ and CC₅₀ values of 8.01 μM and 101.55 μM , respectively (selectivity index: 12.68; positive control indinavir showed EC₅₀ value of 8.18 nM). Alachalasin A–G (**1**–**7**) were also evaluated for activity against a panel of bacteria, including *Staphylococcus aureus* (ATCC 6538), *Streptococcus mutans* (ATCC 25175), *Micrococcus luteus* (ATCC 9431), *Enterococcus faecalis* (ATCC 19433), and *Sarcina lutea* (CMCC B28001), and fungi *Geotrichum can-*

didum (AS2.498), *Candida albicans* (ATCC 10231), and *Aspergillus fumigatus* (ATCC 10894). Alachalasin D (**4**) showed activity against *M. luteus* and *S. aureus* affording zones of inhibition of 9 and 7 mm at 100 $\mu\text{g}/\text{disk}$, respectively. Alachalasin G (**7**) showed modest activity against *S. aureus*, affording a zone of inhibition of 15 mm at the same level. Alachalasin G (**7**) was evaluated further and displayed a MIC value of approximately 99 μM . However, none of these compounds showed antimicrobial activity against *S. mutans*, *E. faecalis*, and *S. lutea*, or antifungal activity against *G. candidum*, *C. albicans*, and *A. fumigatus* at 100 $\mu\text{g}/\text{disk}$.

A few cytochalasin type of metabolites have been described previously with anti-HIV activities.^{22–24} For example, cytochalasin A and L-696,474 were reported as HIV-1 protease inhibitors,^{22,23} and aspochalasin L showed HIV-1 integrase inhibitory activity.²⁴ Based on structural similarity between **1** and above-mentioned compounds,alachalasin A might target the same enzymes as its known precedents, but further study is still needed in order to elucidate the mechanism for its anti-HIV-1 activity.

Alachalasin A–G (**1**–**7**) are new members of the cytochalasin class of compounds. In all probability, the biosynthesis of **1**–**7** proceeds in a manner similar to that of other cytochalasins.^{16–19} The molecules may be derived from Ala, acetate, and three units of Met, and the plausible biogenetic pathway was that Ala was attached to an acetate–malonate derived C₁₆-polyketide unit that contains two methyl substituents originating from the methyl of Met.^{3–12} Most of the known cytochalasins incorporated Phe, Tyr, Try, Leu, and Val as structural subunits, butalachalasin A–G (**1**–**7**) incorporated Ala instead of above-mentioned amino acids. Alachalasin A–G also differ from most known cytochalasins by virtue of the presence of a C-17–C-18 epoxide moiety rather than an *exo*-cyclic unit.^{2–12} Most noticeably, the presence of an adenine unit inalachalasin G (**6**) and H (**7**) is unprecedented in this class of compounds. Alachalasin A–G are the first secondary metabolites to be reported from *S. charatum*.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on Shimadzu Biospec-1601 spectrophotometer. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ^1H and ^{13}C NMR data were acquired with a Bruker Avance-400 and Varian Inova-500 spectrometer using solvent signals (CDCl_3 ; $\delta_{\text{H}}/\delta_{\text{C}}$ 7.26/77.0) and (CD_3OD ; $\delta_{\text{H}}/\delta_{\text{C}}$ 3.30/49.5) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000^{plus} spectrometer. HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

3.2. Fungal material

The culture of *S. charatum* was isolated by Dr. Bin Liu from a sample that was collected at 3,600 m of an unnamed glacier, Tibet, on August 3, 2003. The isolate was identified by Dr. Bin Liu, and assigned the Accession No. XJ03-56-1 in Professor X. Liu's culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 25 °C for 10 days. Fermentation was carried out in four 500-mL Fernbach flasks each containing 100 g of rice. Spore inoculum was prepared by suspension in sterile distilled H₂O to give a final spore/cell suspension of 1×10^6 /mL. Distilled H₂O (100 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in². for 30 min.¹⁸ After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at 25 °C for 40 days.

3.3. Extraction and isolation

The fermented rice substrate was extracted repeatedly with MEK (3 × 500 mL), and the organic solvent was evaporated to dryness under vacuum to afford 2.8 g of crude extract. The extract was fractionated by Silica gel VLC using *n*-hexane–CH₂Cl₂–MeOH gradient elution. The fractions that were eluted with 100:1 (45 mg), 100:2 (80 mg), 100:3 (105 mg), and 100:4 CH₂Cl₂–MeOH (90 mg) were further separated by semi-preparative reversed-phase HPLC (Kramasil C₁₈ column; 10-μm; 10 × 250 mm, 2 mL/min). Purification of these fractions using different gradients afforded alachalasin A (**1**; 24 mg, *t*_R 12.0 min; 45% MeOH in H₂O over 5 min, 45–100% over 20 min), B (**2**; 2.6 mg, *t*_R 28.1 min; 40% MeOH in H₂O over 5 min, 40–50% over 5 min, 50–65% MeOH in H₂O over 20 min), C (**3**; 2.5 mg, *t*_R 27.3 min; 20% MeOH in H₂O over 5 min, 20–50% over 5 min, 50–90% over 30 min), D (**4**; 6.1 mg, *t*_R 10.5 min; same gradient as in purification of **1**), E (**5**; 4.8 mg, *t*_R 26.2 min; same gradient as in purification of **2**), F (**6**; 2.1 mg, *t*_R 14.1 min; 50% MeOH in H₂O over 5 min, 50–100% over 20 min), G (**7**; 1.5 mg, *t*_R 18.2 min; same gradient as in purification of **6**).

3.3.1. Alachalasin A (1). Gum; [α]_D +5.0 (*c* 1.3, CH₃OH); UV (CH₃OH) λ_{\max} 218 (ϵ 1900) nm; IR (heat) ν_{\max} 3386 (br), 2967, 2929, 1692, 1380, 890 cm⁻¹; ¹H, ¹³C NMR, HMBC, and NOESY data, see Table 1; HRESIMS obsd *m/z* 412.2095 [M+Na]⁺, calcd for C₂₂H₃₁NO₅Na, 412.2094.

3.3.2. Alachalasin B (2). Amorphous powder; [α]_D +10 (*c* 0.41, CH₃OH); UV (CH₃OH) λ_{\max} 220 (ϵ 1700) nm; IR (heat) ν_{\max} 3493, 3389, 3260, 2978, 2925, 1715, 1677, 1451, 1103 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRESIMS obsd *m/z* 426.2237 [M+Na]⁺, calcd for C₂₃H₃₃NO₅Na, 426.2251.

3.3.3. Alachalasin C (3). Amorphous powder; [α]_D -8.0 (*c* 0.20, CH₃OH); UV (CH₃OH) λ_{\max} 220 (ϵ 1300) nm; IR (heat) ν_{\max} 3354 (br), 2966, 2928, 1695, 1378, 1031 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRE-

SIMS obsd *m/z* 396.2135 [M+Na]⁺, calcd for C₂₂H₃₁NO₄Na, 396.2145.

3.3.4. Alachalasin D (4). Amorphous powder; [α]_D +7.0 (*c* 0.5, CH₃OH); UV (CH₃OH) λ_{\max} 203 (ϵ 2400) nm; IR (heat) ν_{\max} 3361 (br), 3220 (br), 2967, 2929, 1710, 1684, 1381, 1024 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HMBC data (CD₃OD, 500 MHz) H-3 → C-1, 4, 5, 9; H-4 → C-1, 3, 5, 6, 8, 9, 10, 11, 21; H-5 → C-3, 4, 6, 7, 11, 12; H-7 → C-5, 6, 8, 12, 13; H-8 → C-1, 4, 7, 9, 13, 14, 21; H-10 → C-3, 4; H-11 → C-4, 5, 6; H-12a → C-5, 6, 7; H-12 b → C-5, 6, 7; H-13 → C-7, 8, 14, 15; H-14 → C-8, 15, 16; H-15a → C-13, 14, 16, 17, 22; H-15b → C-13, 14, 16, 17; H-16 → C-14, 15, 17, 22; H-17 → C-16, 18, 19, 22, 23; H-19 → C-17, 18, 20, 21, 23; H-20a → C-9, 18, 19, 21; H-20b → C-18, 21; H-22 → C-15, 16, 17; H-23 → C-17, 18, 19; HRESIMS obsd *m/z* 412.2084 [M+Na]⁺, calcd for C₂₂H₃₁NO₅Na, 412.2094.

3.3.5. Alachalasin E (5). Amorphous powder; [α]_D +29 (*c* 0.15, CH₃OH); UV (CH₃OH) λ_{\max} 210 (ϵ 2700) nm; IR (heat) ν_{\max} 3347 (br), 3293 (br), 2964, 2933, 1693, 1379, 1102 cm⁻¹; ¹H and ¹³C NMR, see Table 2; HMBC data (CDCl₃, 500 MHz) *N*-H → C-3, 4, 9; H-3 → C-4, 5; H-4 → C-1, 3, 5, 6, 9, 10, 21; H-5 → C-3, 4, 6, 11, 12; H-7 → C-5, 6, 12, 13; H-8 → C-1, 4, 7, 9, 13, 14, 21; H-10 → C-3, 4; H-11 → C-4, 5, 6; H-12a → C-5, 6, 7; H-12b → C-5, 6, 7; H-13 → C-8, 15; H-14 → C-8, 16; H-15a → C-14; H-15b → C-13, 14, 16, 17; H-16 → C-14, 15, 17, 22; H-17 → C-16, 22, 23; H-19 → C-17, 18, 20, 21, 23, 24; H-20a → C-19, 21; H-20b → C-18, 21; H-22 → C-15, 16, 17; H-23 → C-17, 18, 19; H-24 → C-19; HRESIMS obsd *m/z* 426.2238 [M+Na]⁺, calcd for C₂₃H₃₃NO₅Na, 426.2251.

3.3.6. Alachalasin F (6). Amorphous powder; [α]_D +28 (*c* 0.1, CH₃OH); UV (CH₃OH) λ_{\max} 205 (ϵ 2600), 264 (ϵ 1900) nm; IR (heat) ν_{\max} 3345 (br), 3221 (br), 2968, 2930, 1692, 1645, 1601, 1380 cm⁻¹; ¹H and ¹³C NMR, see Table 2; HMBC data (CD₃OD, 500 MHz) H-3 → C-1, 9; H-4 → C-1, 5, 6, 9, 21; H-8 → C-1, 7, 9, 13, 14; H-10 → C-3, 4; H-11 → C-4, 5, 6; H-12 → C-5, 6, 7; H-13 → C-15; H-15a → C-17; H-15b → C-13, 14, 17; H-16 → C-14, 15, 17, 22; H-17 → C-16, 22, 23; H-19 → C-17, 18, 20, 21, 23, 4', 8'; H-20a → C-18, 21; H-20b → C-18, 21; H-22 → C-15, 16, 17; H-23 → C-17, 18, 19; H-2' → C-4', 6'; H-8' → C-4', 5'; HRESIMS obsd *m/z* 529.2550 [M+Na]⁺, calcd for C₂₇H₃₄N₆O₄Na, 529.2543.

3.3.7. Alachalasin G (7). Amorphous powder [α]_D -4.0 (*c* 0.06, CH₃OH); UV (CH₃OH) λ_{\max} 204 (ϵ 1900), 264 (ϵ 1100) nm; IR (heat) ν_{\max} 3346 (br), 3225 (br), 2965, 2928, 1692, 1642, 1602, 1381 cm⁻¹; ¹H and ¹³C NMR, see Table 2; HMBC data (CDCl₃, 500 MHz) H-3 → C-1, 4, 5, 9; H-4 → C-1, 3, 5, 6, 9, 10, 21; H-5 → C-3, 4, 6, 11, 12; H-7 → C-6, 8, 12, 13; H-8 → C-1, 7, 9, 13, 14, 21; H-10 → C-3, 4; H-11 → C-4, 5, 6; H-12 → C-5, 6, 7; H-13 → C-15; H-15a → C-13, 14; H-15b → C-13, 14, 16, 17; H-16 → C-17; H-17 → C-22, 23; H-19 → C-17, 18, 20, 21, 23, 4', 8'; H-20a → C-21; H-20b → C-18, 21; H-22 → C-15, 16, 17; H-23 → C-17, 18, 19; H-

2' → C-4', 6'; H-8' → C-4', 5'; HRESIMS obsd *m/z* 529.2549 [M+Na]⁺, calcd for C₂₇H₃₄N₆O₄Na, 529.2543.

3.3.8. Preparation of (R)-MTPA ester (1b) and (S)-MTPA ester (1a). A solution of **1** (2.0 mg, 0.005 mmol) in CH₃OH was transferred to a clean NMR tube and dried completely under vacuum. Deuterated pyridine (0.5 mL) and (S)-MPTACl (5.0 μL, 0.026 mmol) were added into the NMR tube, and all contents were mixed thoroughly by shaking the NMR tube carefully. The reaction was performed at room temperature for 24 h. ¹H NMR data of the S-MTPA ester derivative (**1a**) were obtained without purification. Compound **1a**: ¹H NMR (pyridine-*d*₅, 400 MHz) δ 6.49 (1H, dd, *J* = 15, 11 Hz, H-13), 5.12 (1H, ddd, *J* = 15, 11, 3.5 Hz, H-14), 4.53 (1H, d, *J* = 9.3 Hz, H-7), 4.19 (1H, d, *J* = 19 Hz, H-20b), 4.19 (1H, d, *J* = 6.5 Hz, H-19), 3.44 (1H, br q, *J* = 6.6 Hz, H-3), 3.23 (1H, br s, H-4), 2.31 (1H, d, *J* = 9.2 Hz, H-17), 2.68 (1H, dd, *J* = 19, 6.5 Hz, H-20a), 1.42 (1H, br d, *J* = 14 Hz, H-15b), 2.48 (1H, dd, *J* = 11, 9.3 Hz, H-8), 1.68 (1H, br d, *J* = 14 Hz, H-15a), 1.47 (3H, s, H-11), 1.71 (3H, s, H-12), 1.93 (1H, br dq, *J* = 9.2, 6.6 Hz, H-16), 1.26 (3H, s, H-23), 0.94 (3H, d, *J* = 6.6 Hz, H-22), 0.82 (3H, d, *J* = 6.6 Hz, H-10).

Similarly, another sample of compound **1** (2.0 mg, 0.005 mmol), (R)-MPTACl (5.0 μL, 0.026 mmol), and deuterated pyridine (0.5 mL) were allowed to react in NMR tube at ambient temperature for 24 h, ¹H NMR data of the R-MTPA ester derivative (**1b**) were obtained without further purification. Compound **1b**: ¹H NMR (pyridine-*d*₅, 400 MHz) δ 6.50 (1H, dd, *J* = 15, 11 Hz, H-13), 5.17 (1H, ddd, *J* = 15, 11, 3.5 Hz, H-14), 4.50 (1H, d, *J* = 9.3 Hz, H-7), 4.22 (1H, d, *J* = 19 Hz, H-20b), 4.19 (1H, d, *J* = 6.5 Hz, H-19), 3.43 (1H, br q, *J* = 6.6 Hz, H-3), 3.23 (1H, br s, H-4), 2.29 (1H, d, *J* = 9.2 Hz, H-17), 2.68 (1H, dd, *J* = 19, 6.5 Hz, H-20a), 1.43 (1H, br d, *J* = 14 Hz, H-15b), 2.50 (1H, dd, *J* = 11, 9.3 Hz, H-8), 1.70 (1H, br d, *J* = 14 Hz, H-15a), 1.47 (3H, s, H-11), 1.71 (3H, s, H-12), 1.93 (1H, br dq, *J* = 9.2, 6.6 Hz, H-16), 1.26 (3H, s, H-23), 0.95 (3H, d, *J* = 6.6 Hz, H-22), 0.80 (3H, d, *J* = 6.6 Hz, H-10).

3.4. Antimicrobial and antifungal bioassays

Antimicrobial and antifungal bioassays were conducted according to a literature procedure.²⁵ The bacterial strains were grown on Mueller–Hinton agar, the yeasts *C. albicans* (ATCC 10231) and *G. candidum* (AS2.498) were grown on Sabouraud dextrose agar, and the fungus *A. fumigatus* (ATCC 10894) was grown on potato dextrose agar. Test compounds were absorbed onto individual paper disks (6 mm diameter) at 100 μg/disk and placed on the surface of agar. The assay plates were incubated at 25 °C for 48 h (at 37 °C for 24 h for antimicrobial activity) and examined for the presence of a zone of inhibition.

3.5. Anti-HIV assays

Anti-HIV assays include cytotoxicity and HIV-1 replication inhibition assay. The cytotoxicity was measured by MTT method described in the literature.²⁶ Cells

(3 × 10⁴/well) were seeded into a 96-well microtiter plate in the absence or presence of various concentration of test compounds in triplicate well and incubated at 37 °C in a humid atmosphere of 5% CO₂. After a 4-day incubation, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The concentration that caused the reduction of viable cells by 50% (CC₅₀) was determined. As paralleling with cytotoxicity assay, the HIV-1 replication inhibition was determined by p24 antigen capture ELISA.²⁷ C8166 cells were exposed to HIV-1_{LAI} (MOI = 0.058) at 37 °C for 1.5 h, washed with PBS to remove free viruses, and then seeded into 96-well microtiter plate at 3 × 10⁴ cells per well in the absence or presence of test compounds (indinavir sulfate was used as positive control). After 4 days, the supernatant was collected and inactivated by 0.5% Triton X-100. The supernatant was diluted three times, added to the plate coated with anti-p24 McAb (provided by Dr. Bin Yan, Wuhan Institute of Virology, China), and incubated at 37 °C for 1 h. After washing five times with PBST, the HRP labeled anti-p24 antibody (provided by Dr. Bin Yan, Wuhan Institute of Virology, China) was added and incubated at 37 °C for 1 h. Then the plate was washed 5 times with PBST, followed by adding OPD reaction mixture. The assay plate was read at 490 nm using a microplate reader within 30 min. The inhibition rate and EC₅₀ based on p24 antigen expression level were calculated, and the selective index was calculated as CC₅₀/EC₅₀.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.11.042.

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