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A Novel Aspochalasin with HIV-1 Integrase Inhibitory Activity from *Aspergillus flavipes*

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Abstract A novel aspochalasin, aspochalasin L (**1**), was isolated from the fermentation broth of a soil-derived fungal culture identified as *Aspergillus flavipes* (Deuteromycota). Structure elucidation of **1** was accomplished by detailed spectroscopic data analyses and by comparison with related cytochalasins. Aspochalasin L demonstrated activity against HIV integrase with an IC_{50} of 71.7 μM.

Keywords *Aspergillus flavipes*, aspochalasin, HIV integrase, cytochalasins

Aspochalasins are a subset of the fungal metabolites known as the cytochalasins. Cytochalasins are characterised by a macrocyclic 11, 13 or 14 membered carbocyclic ring system that may include ester functionality. The aspochalasins are defined by the 2-methyl-propyl group at the C-3 position of the perhydroisoindol-1-one moiety [1]. The majority of aspochalacians have been isolated from species of *Aspergillus*: aspochalasins A~D from *Aspergillus microcysticus* [2]; aspochalasins C, D and H from *Aspergillus* sp. AJ117509 [3, 4]; aspochalasins G and F from *Aspergillus* sp. [5], TMC-169 from *Aspergillus flavipes* strain TC1446 [6] and aspochalasins I~K from *Aspergillus flavipes* [7]. However, they have also been identified from a species of *Phoma* (phomacins A~C) [8] and from an unidentified fungus (aspochalasins C and E) [9].

As part of a campaign to identify natural products for the treatment of HIV infection, 100,000 natural product extracts were screened with the objective to find inhibitors of the HIV integrase enzyme. The integrase enzyme is essential for virus replication and disease progression, therefore, inhibitors of this enzyme may be valuable in the treatment of AIDS.

The extract derived from the fermentation broth of a soil-derived fungal culture of *Aspergillus flavipes* was found to be active. Bioassay guided fractionation led to the isolation of two active compounds, the novel compound aspochalasin L (**1**) and the known cyclohexapeptide

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WIN66306 (**3**) [10, 11]. We wish to report here the structural elucidation of the novel aspochalasin and the biological activities of both active compounds.

Material and Methods

Instrumental Analysis

Chiroptical measurements ($[\alpha]_D$) were obtained on a JASCO DIP-1000 digital polarimeter in a 100 by 2 mm cell. Ultraviolet (UV) absorption spectra were recorded using a Hitachi Model 150-20 double beam spectrophotometer, while infrared (IR) spectra were obtained using a Bio-Rad FTS 165 FT-IR spectrometer under PC control running Bio-Rad Win-IR software. Low-resolution ESIMS data were acquired using a Thermo-Finnigan LCQ mass spectrometer, while high-resolution ESIMS measurements were obtained on a Bruker BioApex 47E FT mass spectrometer at a cone voltage of 100 kV. Both ^{1}H and ^{13}C NMR spectra were obtained on a Varian Unity Inova 500 MHz spectrometer, in the solvents indicated and referenced to residual ^{1}H signals in the deuterated solvents.

Microorganism

Of the 100,000 extracts screened approximately 50% were methanolic extracts of microbial fermentation broths. These fermentations had been prepared from 10 ml fermentations and aliquoted into 384 well plates for high throughput screening. One of these was selected for further study. The producing fungus was isolated from a soil sample collected on Mauhipua Island and supplied to Cerylid Biosciences by PANLABS

Fermentation

The seed culture was produced by static incubation at 25°C for three days with a three hour shake on day two. The seed medium consisted of malt extract (2.1 g/litre), glycerol (4 g/litre), Oxoid peptone L34 (1 g/litre) and agar (2 g/litre).

Scale up to 3 litres: Fermentation medium consisted of yeast extract (Difco, 9 g/litre), glucose (20 g/litre), KH_2PO_4 (1 g/litre), $MgSO_4 \cdot 7H_2O$ (0.5 g/litre) and peptone (1.8 g/litre). Fermentation was static at 25°C for eight days.

Isolation of Compounds 1 and 3

The fermentation broth (3 litres) was extracted with methanol (3 litres), filtered and concentrated *in vacuo* to yield a dark oily residue. The crude extract was fractionated by C_{18} solid-phase extraction (SPE) (20% stepwise gradient elution from 100% H_2O to 100% MeOH). The active fraction was further purified by C_{18} preparative reversed-phase HPLC [10 ml/minute linear gradient elution from 50% H_2O/50% CH_3CN/0.1% FA to 100% CH_3CN/0.1% FA in 30 minutes through a 5 μm Varian Dynamax C_{18} (ODS) 250×21.4 mm column] and semi-preparative reversed phase phenyl HPLC [4 ml/minute linear gradient elution from 100% H_2O/0.1% FA to 100% CH_3CN/0.1% FA in 20 minutes through a 5 μm Alltech Alltima phenyl 150×10 mm column] with PDA detection at 210 and 254 nm to afford aspochalasin L (**1**) and cyclohexapeptide WIN66306 (**3**).

Physicochemical Properties of 1

Aspochalasin L (**1**): yellowish glassy solid; $[\alpha]_D^{20}$ −84.7° (*c* 0.005, MeOH); IR (film) ν_{max} 3343 (br), 2956, 2931, 2256, 1688, 1508~1560, 1385, 1006~1049 cm^{-1}; UV (PDA) (CH_3CN/H_2O) λ_{max} 209, 281 nm; ^{1}H NMR and ^{13}C NMR data see Table 1; ESI(+)MS (20 kV) *m/z* 472 $(M+Na)^+$, 921 $(2M+Na)^+$; HRESI(+)MS 472.2674 $[(M+Na)^+$, $C_{25}H_{39}NO_6Na$ requires 472.2675].

HIV-1 Integrase Assays

Compounds **1** and **3** were tested against HIV integrase enzyme in a combined 3′-processing/strand transfer assay as described by Ovenden *et al.* [12]. Briefly, the assay measured the ability of compounds to inhibit both 3′-processing and strand transfer activities of integrase. The assay included BSA to minimize the identification of promiscuous inhibitors [13] and secondary screens were conducted to rule out non-specific inhibitors [12]. An authentic integrase inhibitor L-731,988 was included to validate the assays [14]. Compounds **1** and **3** inhibited HIV integrase with IC_{50}s of 71.7 and 32.1 μM respectively.

Biological Activity

Compounds **1** and **3** were assayed for their cytotoxicity and their ability to inhibt HIV-1_{NL4-3} in the HuT78 T-cell line. Cytotoxicity was assessed by incubating HuT78 cells in medium containing compound at concentrations up to 100 μM and cell viability determined after 6 days as described by Ovenden *et al.* [12]. Compound **1** gave a CC_{50} value of 70 μM and compound **3** a CC_{50} of 75 μM.

In assays to assess the ability of compounds **1** and **3** to inhibit HIV-1 replication in HuT78 cells, cells were infected in the presence of compound and incubated for 6 days. After 6 days, cell culture supernatants were harvested and the level of p24 determined as described by Ovenden *et al.* [12].

Compounds **1** and **3** had minimal effect on virus replication with neither compound showing an effect at concentrations less than their CC_{50}s.

Table 1 NMR data (500MHz) for aspochalasin L (**1**) in d_6-DMSO

No.	^{13}C (ppm)[a]	^{1}H (δ, m, J Hz)	gHMBC correlations
1	174.3	—	—
2	—	8.19 br s	C-1, C-3, C-9
3	53.5	3.15 m	C-1, C-4, C-9, C-10
4	50.8	2.81 br s	C-1, C-3, C-5, C-6, C-8, C-9, C-21
5	125.4	—	—
6	133.9	—	—
7	67.9	3.75 br m	—
8	46.5	2.45 dd, 10.5, 10.5	C-4, C-7, C-9, C-13, C-14, C-21
9	61.5	—	—
10	44.9	1.07 m	C-3
11	16.7	1.65 s	C-4, C-5, C-6
12	14.0	1.61 s	C-5, C-6, C-7
13	123.4	5.80 d 10.5	C-7, C-8, C-9, C-14, C-15, C-25
14	136.9	—	—
15	37.6	1.96 m	C-13, C-14, C-16, C-17, C-25
16	28.8	1.30 m	C-14
		1.48 m	
17	69.9	3.49 br d 3.6	C-15, C-16
18	77.2	3.36 br d 9.0	C-16, C-17, C-19
19	77.0	2.92 br d 9.0	C-18, C-20, C-21, 19-OMe
20	42.6	1.96 m	C-9, C-19, C-21
		3.69 br d 19.0	
21	209.3	-	—
22	23.6	1.57 m	C-10, C-23, C-24
23	21.2	0.83 d 6.6	C-10, C-22, C-24
24	22.3	0.86 d 6.6	C-10, C-22, C-23
25	15.0	1.33 s	C-13, C-14, C-15
19-OMe	56.6	3.32 s	C-19
7-OH	—	4.55 br s	—

[a] Carbon chemical shifts were assigned from gHSQC and gHMBC data

Results and Discussion

The structure of aspochalasin L (**1**) was determined by detailed spectroscopic investigation and also by comparison to the literature data, in particular to that of aspochalasin K (**2**) [7]. The positive ESI mass spectrum of **1** revealed a sodiated ion at *m*/*z* 472. Accurate mass measurement of the ion confirmed a molecular formula of $C_{25}H_{39}NO_6Na$ indicative of seven double bond equivalences. Investigation of the NMR data (Table 1) revealed the presence of two double bonds (δ_C 123.4, 125.4, 133.9 and 136.9) and two carbonyl moieties (δ_C 174.3 and 209.3), accounting for four of the double bond equivalences and implying three ring systems in the molecule. An exchangeable proton at δ_H 8.19, correlated to a carbon at δ_C 174.3 in the gHMBC, indicative of an amide moiety. This amide proton showed COSY and gHMBC correlations to C-3. In turn H-3 correlated into H_2-10, H-22, H_3-23 and H_3-24 in the TOCSY experiment, linking the amide to the 2-methyl-propyl group. A final TOCSY correlation to H-4 established a link to the remainder of the molecule, which was further supported by observed gHMBC correlations to C-1, C-4, C-5 and C-9. Interestingly, H-4 also showed gHMBC correlations into the amide carbon (C-1) as well as to quaternary C-9 which implied a five-membered cyclic amide residue. In addition, H-4 showed long range correlations to C-5, C-6, C-11, which placed the fully substituted double bond adjacent to C-4. The olefinic methyl resonating at δ_H 1.65 correlated into C-4, C-5 and C-6. The closely resonating methyl at δ_H 1.61 correlated to C-5, C-6 and C-7. These correlations positioned two of the olefinic methyls at C-11 and C-12. The hydroxy methine at C-7 (δ_H 3.75) coupled to H-8 and also showed gHMBC

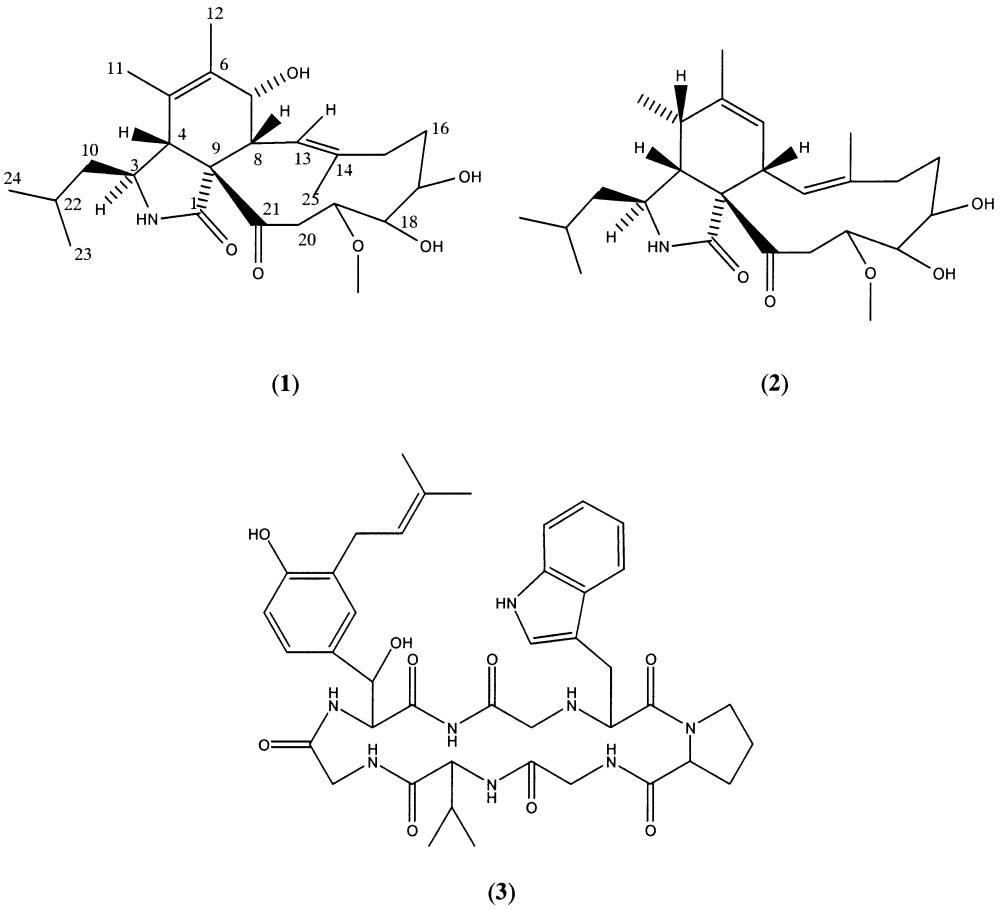

Fig. 1 Structures of aspochalasin L (**1**), aspochalasin K (**2**) and WIN66306 (**3**).

correlations into C-9, suggesting a six-membered ring was fused to the five-membered amide ring. Long range correlations from H-4 and H-8 into δ_C 209.3 placed the remaining carbonyl at C-21. Correlations from a deshielded methylene moiety, H_2-20, to C-21 and C-19 placed this group between the carbonyl and an oxy-methine (H-19, δ_H 2.92, δ_C 77.0). This H-19 methine showed correlations into C-18, C-20, C-21 as well as the methoxy ester carbon (δ_C 56.6), confirming the location of the methoxy group. The H-19 to H_2-15 spin system was established by COSY correlations and supported by the correlations observed in the gHMBC (Table 1). The C-15 methylene group was relatively deshielded (δ_H 1.96) suggesting proximity to electron withdrawing moieties. This was confirmed by gHMBC correlations from H_2-15 to the remaining tri-substituted double bond with correlations observed to C-13, C-14 and the olefinic methyl C-25. In turn the olefinic methine, H-13 showed a COSY into H-8, and also gHMBC correlations into C-7, C-8, C-9, C-14, C-15 and C-25. This allowed for the closure of the third and final ring of the molecule, and thus confirmed the planar structure of aspochalasin L (**1**) as shown. ROESY correlations were observed from H-8 to H-7 and from H-4 and H_2-10, placing H-4, H-7, H-8 and H_2-10. These observed ROESY correlations, as well as molecular modelling studies, allowed H-4, H-7, H-8 and H_2-10 to be placed on the same face of the molecule. This defines the relative stereochemistry at C-7 for **1**, and also implies that the relative stereochemistry at C-3, C-8 and C-9 for **1** is the same as for aspochalasin K (**2**). Weak ROESY correlations between H-17 and H-18 and H-18 and H-19 suggest that the oxy-methines are *syn* to each other, however this conclusion remains tentative and the stereochemistry of these centres remains unassigned.

The structure of **3** was confirmed by comparison with the literature data [11]. The compound was identified as the previously described compound WIN66306, a cyclic peptide also isolated from the fermentation broth of *Aspergillus flavipes*. It is interesting to note that during the isolation of WIN66306 (**3**), a neurokinin antagonist, the authors also isolated rosellichalasin, a cytochalasin that was first isolated in 1989 [15]. Rosellichalasin also displayed

neurokinin antagonist activity against the NK1 and NK2 receptors.

A variety of biological activities have been reported for the cytochalasins and some investigation has been undertaken to examine the structural features of the molecules that convey certain activities. For example Flashner *et al.* demonstrated that the conjugated α,β-keto unsaturated group in the macrolide moiety of cytochalasin A was important for conveying antibacterial activity against Gram-positive bacteria [16]. More recently aspochalasins I~K were found to exhibit moderate cytotoxicity against several NCI cell lines and in a separate publication 19,20-epoxycytochalsin Q was noted to be an inhibitor of CCR5 which is implicated in HIV infection [17]. Whilst the cytochalasins identified in this study are able to inhibit the activity of integrase in *in vitro* enzyme assays, in their current forms they are not able to inhibit the replication of HIV-1. This is not uncommon amongst integrase enzyme inhibitors with many unable to inhibit the enzyme within a preintegration complex or within the cell [18, 19].

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