Altertoxins with potent anti-HIV activity from *Alternaria tenuissima*QUE1Se, a fungal endophyte of *Quercus emoryi*

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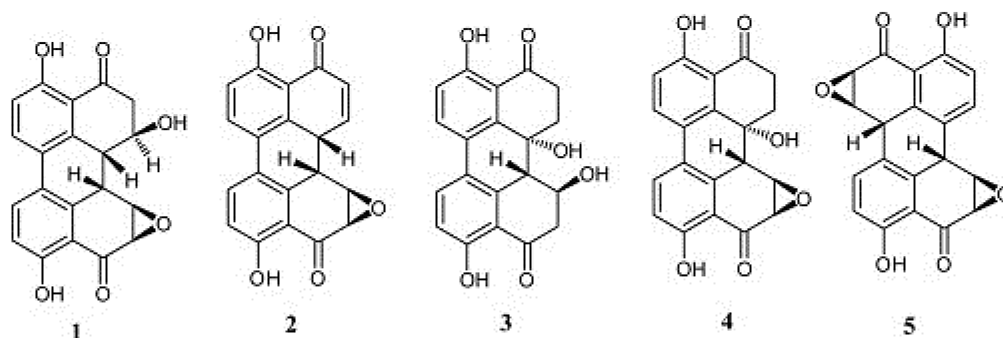
Abstract:

Screening of a small library of natural product extracts derived from endophytic fungi of the Sonoran desert plants in a cell-based anti-HIV assay involving T-cells infected with the HIV-1 virus identified the EtOAc extract of a fermentation broth of *Alternaria tenuissima* QUE1Se inhabiting the stem tissue of *Quercus emoryi* as a promising candidate for further investigation. Bioactivity-guided fractionation of this extract led to the isolation and identification of two new metabolites, altertoxins V (**1**) and VI (**2**) together with the known compounds, altertoxins I (**3**), II (**4**), and III (**5**). The structures of **1** and **2** were determined by detailed spectroscopic analysis and those of **3**–**5** were established by comparison with reported data. When tested in our cell-based assay at concentrations insignificantly toxic to T-cells, altertoxins V (**1**), I (**3**), II (**4**), and III (**5**) completely inhibited replication of the HIV-1 virus at concentrations of 0.50, 2.20, 0.30, and 1.50 μM, respectively. Our findings suggest that the epoxyperylene structural scaffold in altertoxins may be manipulated to produce potent anti-HIV therapeutics.

Graphical abstract



Keywords: Alternaria tenuissima | Endophytic fungus | Anti-HIV activity | Altertoxin V | Altertoxin VI

Article:

1. Introduction

Acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) is considered one of the most serious public health challenges on a global scale. Despite the great efforts that are being devoted to prevent, treat, and to better understand the disease, it remains one of the main causes of morbidity and mortality worldwide. It is estimated that over 35 million people were living with HIV in 2012, 2.3 million became newly infected and 1.6 million lost their lives as a result of AIDS (UNAIDS Report on the Global AIDS Epidemic 2013).[1] Of the two major types of HIV, HIV-1 and HIV-2, the former is the cause of worldwide epidemic of AIDS. HIV-1 causes the loss of $CD4^+$ T-cells by direct killing or impairing function of these cells consequently leading to the cell destruction by apoptosis.[2]

The combined therapy (highly active antiretroviral therapy, HAART) using synthetic reverse transcriptase (RT) and protease inhibitors has effectively suppressed virus replication and significantly prolonged the life of AIDS patients. However, the appearance of resistant viruses to these current drugs has created an urgent need for the discovery and development of potent anti-HIV drugs with novel modes of action.[3]

Plant-associated microorganisms, especially endophytic fungi, are rich sources of novel and bioactive secondary metabolites.[4] As a part of our studies on arid land plants and their associated microorganisms for novel and/or biologically active small-molecule natural products,[5] and encouraged by the reports of the occurrence of metabolites with anti-viral activity in endophytic fungi,[6] we screened a small library of 100 extracts derived from endophytic fungal strains inhabiting the Sonoran desert plants for their anti-HIV activity. Our strategy involved culturing of these fungi in a variety of culture media, extraction of cultures with EtOAc, screening of the resulting extracts for cytotoxicity to T-lymphocytes (A3.01 cells). Those extracts showing $\leqslant$25% toxicity were evaluated for their effects on viral replication in an assay using A3.01 cells infected with the HIV-1_{LAV} strain (lymphadenopathy-associated virus) in the presence of the extracts. Of the extracts evaluated by the application of this strategy, an EtOAc extract derived from the

endophytic fungus *Alternaria tenuissima*, isolated from the living stem tissue of *Quercus emoryi* (Emory oak), exhibited promising activity and was selected for further investigation. Bioactivity-guided fractionation of this extract as described previously [7] and outlined in the Experimental section resulted in the isolation of two new metabolites, named altertoxin V (**1**) and altertoxin VI (**2**) in addition to previously known altertoxins I (**3**), II (**4**), and III (**5**) (Fig. 1). Herein we describe the structure elucidation of **1** and **2**, and anti-HIV activities of **1** and**3**–**5**.

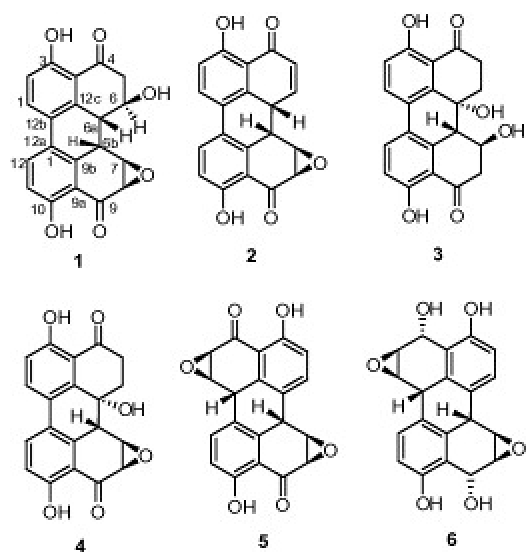


Figure 1. Structures of altertoxins V (**1**), VI (**2**), I (**3**), II (**4**), III (**5**), and IV (**6**) occurring in some strains of endophytic *Alternaria tenuissima*.

Alternaria tenuissima is a saprophytic fungal pathogen inhabiting various plant species. [8] It is found widespread in the environment. [9] Previous chemical investigations of *A. tenuissima* [10, 11, 12 and 13] have focused on the isolation and characterization of its toxic metabolites alternariol, [10 and 12] alternariol monomethyl ether, [10 and 12] altenuene, [10 and 12] tenuazonic acid, [10, 11 and 13] caseinolytic protease enzyme,[12] and beta-1,3-glucanases.[13] Many *Alternaria* sp. have been reported to contain perylene oxides and alterperylenols [14, 15, 16, 17, 18 and 19] which are known to possess antibacterial, [16] antifungal, [17] mutagenic,[14 and 15] and phytotoxic [17, 18 and 19] activities. A recent report described the isolation of altertoxin II (4) and altertoxin IV (6) from an endophytic strain of *A. tenuissima* occurring in the medicinal plant, *Tribulus terrestris*. [20]

2. Results and discussion

Altertoxin V (**1**), obtained as a brownish yellow solid, was analyzed for $C_{20}H_{14}O_6$ by a combination of HRFAB-MS and ^{13}C NMR spectroscopy. Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **1** with known epoxyperylenes, altertoxins **3**–**5** (Table 1 and Table 2, respectively), also encountered in this work suggested the presence of a common carbon skeleton in all these metabolites. The presence of ^{1}H and ^{13}C NMR signals at δ 3.87/53.5 (CH-8),

4.85/55.7 (CH-7), and 4.57/66.1 (CH-6) suggested that **1** contained an epoxide ring and an aliphatic carbon bearing an OH group. The position of this OH group was determined to be at C-6 based on the multiplicity of H-6 at δ 4.57 (ddd, $J_{6,5ax}$ = 12.0; $J_{6,5eq}$ = 4.0; $J_{6,6a}$ = 9.0 Hz) as a result of ^{1}H–^{1}H coupling with vicinal H_{ax}-5 at δ 3.13 (dd, J = 12.0, 15.0 Hz), H_{eq}-5 at δ 2.93 (dd, J = 4.0, 15.0 Hz), and H-6a at δ 3.82 (dd, J = 9.0, 2.5 Hz). It was further confirmed by the ^{1}H–^{1}H COSY correlations of H-6 (δ4.57) with H-5 (δ 3.13, 2.93), H-6a (δ 3.82), and 6-OH (δ 5.85), and H-6a with H-6b (δ 4.38) (Fig. 2). This coupling pattern and COSY correlations confirmed the connectivity of the two cyclohexane rings (E and B) and two biphenyl rings (A and D). The presence of a [1,1′-biphenyl]-4,4′-diol moiety in **1** was further suggested by its UV data (see Experimental) which were found to be in the typical range of this chromophore for related epoxyperylenes. [14, 17, 18 and 19] The absence of vicinal coupling between H-6b (δ 4.38) and H-7 (δ4.85) suggested that the dihedral angle between these protons to be approximately 90° indicating β-configuration for the oxirane ring in **1**. [14, 15 and 16] This was further supported by the similarities between the^{13}C and ^{1}H NMR chemical shifts of 7,8-epoxytetralone moiety of **1** with similar moieties of the known epoxyperylenes **4** and **5** (Table 1 and Table 2; Fig. 3); the downfield shift observed for C-6b (δ 45.0) of **4** may be attributed to the deshielding effect of C_{6a}-OH. Furthermore, the vicinal coupling between H-6 and H-6a ($J_{6,6a}$ = 9.0 Hz) suggested that H-6 and H-6a in **1** have axial orientations. The *cis*-orientation of H-6a and H-6b was inferred from the small vicinal coupling ($J_{6a,6b}$ = 2.5 Hz) between them. Since H-6 and H-6a are*trans*-diaxial, the configuration of 6-OH should be β-equatorial. It was supported by the presence of almost superimposable ^{13}C and ^{1}H NMR peaks due to the 6-hydroxytetralone portion of **1** and the corresponding 7-hydroxytetralone portion of **3** as well as the reported data for related perylene oxides (Table 1 and Table 2;Fig. 3). [6] Based on the foregoing evidence, the structure of altertoxin V was established as (6*R*,6a*R*,6b*S*,7*R*,8*S*)-3,6,10-trihydroxy-4,9-dioxo-4,5,6,6a,6b, 7,8,9-octahydro-7,8-epoxyperylene (**1**).

Table 1.^{1}H NMR (500 MHz) data for compounds **1–5**

Position	**1**[a] δ_H (mult. J in Hz)	**2**[b] δ_H (mult.J in Hz)	**2**[c] δ_H (mult.J in Hz)	**3**[a] δ_H (mult. J in Hz)	**3**[b] δ_H (mult. J in Hz)	**4**[b] δ_H (mult. J in Hz)	**5**[a] δ_H (mult. J in Hz)
1	8.01, d (8.5)	8.01, d (8.5)	8.12, d (8.5)	7.81, d (9.0)	8.05, d (8.0)	7.89, d (9.0)	4.87, d (3.5)
2	6.88, d (8.5)	7.10, d (8.8)	7.07, d (8.5)	7.06, d (8.5)	7.03, d (8.0)	7.09, d (9.0)	3.85, d (3.5)
5_{ax}	3.13, dd (12.0, 15.0)	6.58, d (10.3)	6.52, d (9.5)	3.15, ddd (5.0, 14.5, 17.5)	3.07, ddd (4.8, 14.4, 19.2)	3.23, ddd (5.0, 14.5, 14.5)	6.87, d (8.5)
5_{eq}	2.93,dd			2.68, ddd	2.57, ddd	2.82, m	

	(4.0, 15.0)			(3.0, 3.0, 17.5)	(3.0, 3.0, 17.4)		
6_{ax}	4.57, ddd (4.0, 9.0, 12.0)	7.45, d (10.3)	7.84, d (10.5)	2.41, ddd (4.0, 14.5, 14.5)	2.30, ddd (4.2, 14.4, 14.4)	2.38, m	7.82, d (8.5)
6_{eq}				3.0, ddd (3.0,5.0,14.5)	3.05–2.93, m	2.86, m	
6a	3.82, dd (2.5, 9.0)	2.56, br s	2.77, br s				
6b	4.38, d (2.5)	3.79, br s	3.88, br s	3.05, d (8.5)	3.05–2.93, m	3.52, s	4.87, d (3.5)
7	4.85, d (3.5)	4.25, d (3.5)	4.58, d (3.5)	4.74, ddd (5.0, 9.0, 12.0)	4.52, m	4.21, d (3)	4.48, s
8_{ax}	3.87, d (3.5)	3.72, d (3.5)	3.75, d (3.5)	2.90, dd (12.0, 16.0)	3.05–2.93, m	3.69, d (3)	3.85, d (3.5)
8_{eq}				3.07, dd (5.0, 16.0)	2.85, dd (4.8, 15.6)		
11	6.88, d (8.5)	7.04, d (8.8)	7.00, d (8.5)	7.0, d (9.0)	6.93, d (8.0)	7.04, d (8.5)	6.87, d (8.5)
12	7.80, d (8.5)	7.89, d (8.7)	8.12, d (8.5)	7.81, d (9.0)	7.99, d (8.0)	7.83, d (9.0)	7.82, d (8.5)
12b							4.48, s
3-OH	11.83, s		12.38, s	12.68, s	12.72, s	12.70, s	
4-OH							11.36, s
6-OH							
6a-OH	5.85, d (5.5)				5.27, s		
7-OH					5.36, d (5.4)	12.10, s	
10-OH	11.50, s		12.15, s	12.31, s	12.32, s		11.36, s

[a]Measured in DMSO-d_6. [b]Measured in $CDCl_3$. [c]Measured in acetone-d_6.

Table 2. ^{13}C NMR (125 MHz) data for compounds **1**, **3–5**

Position	**1**[a]	**3a**	**4**[b]	**5b**
1	134.8, CH	132.6, CH	132.9, CH	55.9, CH
2	114.7, CH	119.5, CH	119.8, CH	53.6, CH
3	160.0, C	162.3, C	163.3, C	196.8, C
3a	115.4, C	116.9, C	113.5, C	112.3, C
4	203.3, C	205.0, C	204.5, C	159.7, C
5	46.9,CH_2	33.9, CH_2	32.1, CH_2	114.5, CH
6	66.1, CH	34.5, CH_2	33.3, CH_2	132.1, CH
6a	45.2, CH	69.2, C	68.3, C	128.8, C
6b	36.7, CH	51.9, CH	45.0, CH	37.5, CH
7	55.7, CH	66.1, CH	55.7, CH	55.9, CH
8	53.5, CH	47.7, CH_2	52.8, CH	53.6, CH
9	197.1, C	202.1, C	196.6, C	196.8, C
9a	111.5, C	113.8, C	114.6, C	112.3, C
9b	143.8, C	139.1, C	138.8, C	143.0, C
10	158.8, C	162.0, C	162.6, C	159.7, C
11	114.3, CH	117.5, CH	118.0, CH	114.5, CH
12	131.3, CH	132.4, CH	132.5, CH	132.1, CH
12a	130.6, C	124.1, C	123.9, C	128.8, C
12b	128.5, C	122.7, C	122.4, C	37.5, CH
12c	142.0, C	135.5, C	133.5, C	143.0, C

aMeasured in DMSO-d_6 . bMeasured in $CDCl_3$.

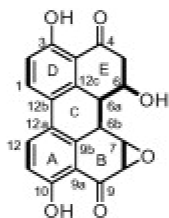


Figure 2. ^{1}H–^{1}H COSY correlations (bold lines) for **1**.

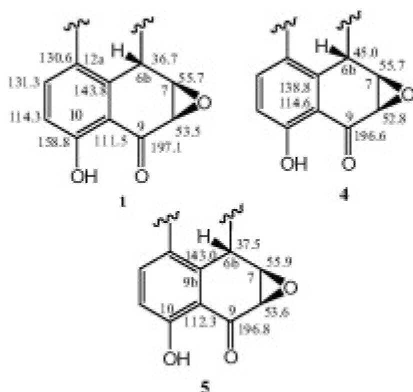


Figure 3. ^{13}C NMR data for 7,8-epoxytetralone moieties of **1**, **4**, and **5**.

Altertoxin VI (**2**) was obtained as an unstable yellow solid. Its APCI-MS suggested that it had a molecular weight of 18 mu less than that of **1**. The high instability of **2** in solution precluded obtaining its HRMS and ^{13}C NMR data. The ^{1}H NMR data for **2** (Table 1) was found to be very similar to those of **1** except that **2** had additional signals at δ 7.45 (d, J = 10.3 Hz, H-6) and 6.58 (d, J = 10.3 Hz, H-5) which were attributable to a*cis*-double bond.

The position (C-5/C-6) of this double bond was evident from the downfield chemical shifts observed for H-6a to δ 2.56 (br s) and H-6b to δ 3.79 (br s) as compared with **1** which contains an OH group at C-6. The ^{1}H NMR resonances of the rest of the protons of **1** and **2** were very similar (Table 1). No vicinal coupling between H-6b (δ 3.79) and H-7 (δ 4.25) was observed which required the dihedral angle between these protons to be approximately 90° suggesting a β-configuration for the oxirane ring in **2**. [14, 15 and 16] Thus the structure of altertoxin VI was determined as (6a*R*,6b*R*,7*R*,8*S*)-3,10-dihydroxy-4,9-dioxo-4,6a,6b,7,8,9-hexahydro-7,8-epoxyperylene (**2**). The remaining metabolites were identified as altertoxins I (**3**), [14] II (**4**),[14 and 20] and III (**5**), [14] by comparison of their spectroscopic data with those reported for these compounds.

All metabolites encountered were evaluated for their ability to inhibit HIV-1 viral replication in A3.01 infected cells. Based on their cytotoxicity to A3.01 cells, compounds **1**–**3** and **5** were tested at a concentration of 1.5 µg/mL whereas **4** was tested at 0.5 µg/mL. At these concentrations **1** and **3**–**5** inhibited viral replication almost completely (97–99%) while **2** caused only 33% inhibition on the peak day of virus production. The reduced effectiveness of **2** was

probably due to its instability in the cell culture medium. Remarkably, the inhibition exhibited by active compounds **1** and **3**–**5** was similar or better than to that shown by AZT at 20 μM, the positive control used for this assay (Fig. 4). In order to determine the minimum concentration required for complete inhibition of viral replication, each compound was tested at decreasing concentrations until viral replication was observed. Anti-viral activity was determined 9 days post-infection, the day of peak viral replication in the untreated control (see Fig. 4). As shown in Figure 5, altertoxin V (**1**), one of the new metabolites encountered in this work displayed the lowest 50% inhibitory concentration (IC_{50}) of 0.09 μM, with almost complete inhibition of viral replication at 0.50 μM. The remaining HIV-active metabolites, altertoxins I (**3**), II (**4**), and III (**5**) exhibited higher IC_{50} values of 1.42, 0.21 and 0.29 μM, with near complete inhibition of viral replication occurring at 2.20, 0.30, and 1.50 μM, respectively. These limited structure–activity data implicated the requirement of epoxytetralone and [1,1′-biphenyl]-4,4′-diol moieties for the observed antiviral activity of altertoxins at insignificantly cytotoxic concentrations. Although some altertoxins are known to be mutagenic,[14 and 15] the fact that their mutagenicity was found to occur only at cytotoxic concentrations,[21] coupled with the observation of no negative effects on cell morphology or viability at concentrations used in our studies suggest that they have the potential to be developed as effective anti-HIV agents. Using a combination of the IC_{50} and cytotoxicity data, the therapeutic indexes for altertoxins I (**3**), II (**4**), III (**5**), and V (**1**) were determined to be 3, 6.5, 15 and 50, respectively. While these indexes are low, they do provide a narrow window for further development and suggests that epoxyperylene structure may serve as a promising scaffold that could be further manipulated to afford potent and non-toxic anti-HIV therapeutics.

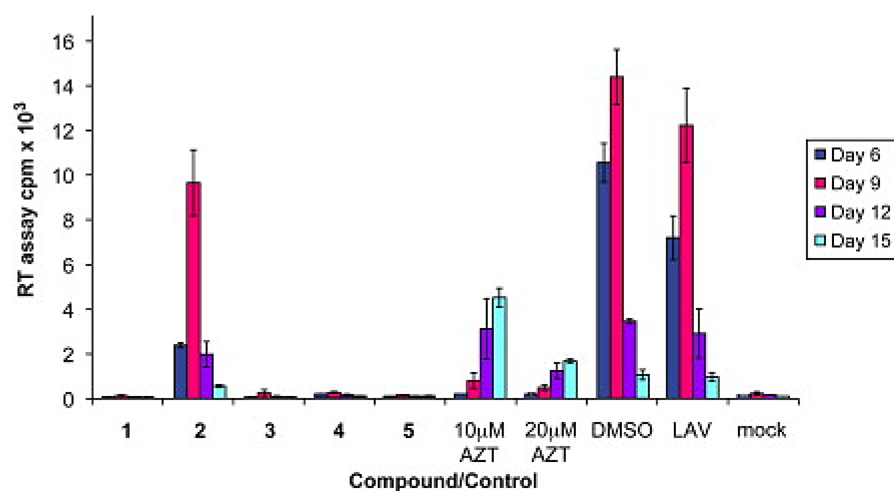


Figure 4. Anti-HIV activity of altertoxins **1**–**5** as determined by the reverse transcriptase (RT) assay. The effect of **1**–**5** on HIV-1 replication was determined by infecting A3.01 cells with HIV-1_{LAV} in the presence of test compounds. Compounds **1**–**3** and **5** were tested at 1.5 μg/mL whereas **4** was tested at 0.5 μg/mL. Controls used included DMSO (negative control; same concentration present in the wells containing **1**–**5**), AZT (positive control at 10 and 20 μM),

HIV-1$_{LAV}$ infected cells with no treatment (LAV), and cells only (mock). Virus levels in all culture supernatants were determined by the RT assay.[7]

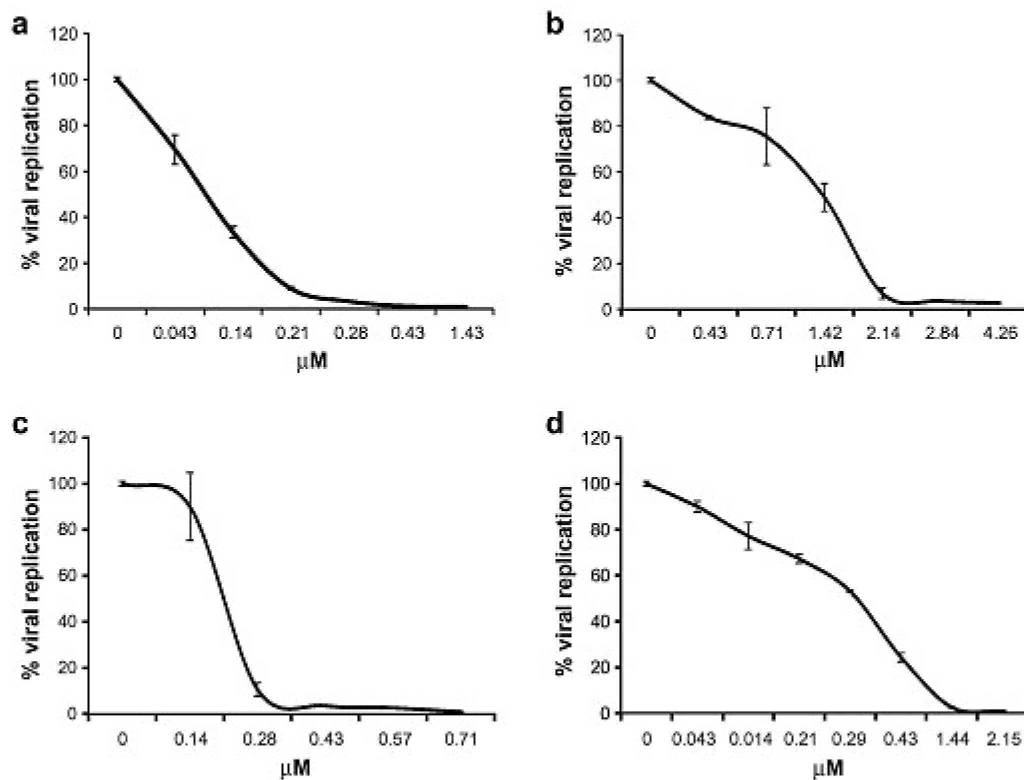


Figure 5. Dose–response curves of HIV-1 replication for the active altertoxins: (**a**) altertoxin V (**1**), (**b**) altertoxin I (**3**), (**c**) altertoxin II (**4**), and (**d**) altertoxin III (**5**). The range of concentrations at which these compounds effectively inhibit the HIV-1 replication was determined by infecting A3.01 cells with HIV-1$_{LAV}$ in the presence of decreasing concentrations of altertoxins. Virus levels in culture supernatants were determined by the RT assay. The% of viral replication was estimated by comparison with the untreated control (concentration of 0).[7] Presented are the mean and standard deviation of duplicate determinations.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a JASCO Dip-370 polarimeter using MeOH or $CHCl_3$ as solvent. 1D and 2D NMR spectra were recorded in $CDCl_3$, acetone-d_6, or DMSO-d_6 using residual solvent peaks as internal standards with a Bruker DRX-500 instrument at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. UV spectra were recorded on a Shimadzu UV-160 UV–vis spectrometer. Low resolution and high resolution MS were recorded on Shimadzu LCMS QP8000α and JEOL HX110A spectrometers, respectively.

3.2. Antiviral activity evaluation

The experimental procedures for cytotoxicity and anti-HIV assays and determinations of dose–response curves for compounds **1** and **3**–**5** have been described previously.[7]

3.3. Fungal isolation, identification and cultivation

Stem tissues of *Quercus emoryi*, collected in early 2005 in Arizona, were processed as reported before for the isolation of endophytic fungal strains.[22] The strain QUE1Se selected for further investigation was identified as *A. tenuissima* based on its morphological characteristics and partial LSU rRNA sequences compared to MicroSeq library (Microbial ID, Newark, DE), and GenBank sequence database and was assigned as *A. tenuissima* QUE1Se. The culture is deposited at the Arizona State University Biology Department and the Southwest Centre for Natural Products Research and Commercialization at the University of Arizona microbial culture collection under the accession numbers QUE1Se and CS-36-91, respectively. The strain was sub-cultured in potato dextrose agar (PDA). To produce culture medium for isolation of secondary metabolites, the endophyte was cultured in potato dextrose broth (PDB; Difco, Plymouth, MN) in 10 × 4.0 L shaker flasks, each containing 2.0 L of media, at 120 RPM, at 26 °C for 15 days.

3.4. Extraction and isolation

The culture (20.0 L) *A. tenuissima* QUE1Se obtained above was filtered through Whatman No. 1 filter paper. The yellow colored filtrate was extracted with EtOAc (16.0 L) and the combined extract was evaporated under reduced pressure to afford a dark yellow residue (1.4 g) which was found to be active in our cell-based anti-HIV assay. [5] This extract (1.4 g) was partitioned between hexanes and 80% aq. MeOH. The bioactive 80% aq MeOH fraction was diluted to 50% aq MeOH with H_2O and extracted with $CHCl_3$. Evaporation of the anti-HIV active $CHCl_3$ fraction under reduced pressure yielded a dark coloured residue (595.3 mg). A portion (550.0 mg) of this was subjected to gel-permeation chromatography on a column of Sephadex LH–20 (20.0 g) and eluted with 300 mL each of hexane–CH_2Cl_2 (1:4), CH_2Cl_2–acetone (3:2), CH_2Cl_2–acetone (1:4), CH_2Cl_2–MeOH (1:4), and MeOH. A total of six fractions were collected. Four of these fractions, A (72.2 mg), B (56.8 mg), C (102.5 mg), and D (114.8 mg), were found to have anti-HIV activity, and the remaining two fractions, E (35.8 mg), and F (10.8 mg) were anti-HIV inactive. Column chromatography of fraction A (72.2 mg) over Lichroprep diol Si gel (4.0 g) and elution with CH_2Cl_2 followed by preparative TLC on Si gel (eluant: iso-PrOH–CH_2Cl_2, 3:97) afforded altertoxin III (**5**) (1.9 mg). Purification of fraction B (56.8 mg) by column chromatography on Si gel and elution with increasing amounts of acetone in CH_2Cl_2afforded altertoxin II (**4**) (15.0 mg). Column chromatography of fraction C (102.5 mg) on Si gel (4.0 g) and elution with increasing amounts of acetone in CH_2Cl_2 followed by preparative TLC on Si gel (eluant: iso-PrOH–CH_2Cl_2, 3:97) afforded altertoxin V (**1**) (3.1 mg), altertoxin VI (**2**) (3.7 mg), and altertoxin I (**3**) (11.2 mg).

3.4.1. Altertoxin V (**1**)

Brownish yellow solid; $[\alpha]_D^{25}$ +395.60 (c 0.12, CH_3OH); UV λ_{max} (MeOH, log ε) 213.0, (5.37), 262.0, (5.23), 348.0, (4.68); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2, respectively; HRFABMS m/z 351.0884 [M+H]+ (calcd for $C_{20}H_{15}O_6$, 351.0869).

3.4.2. Altertoxin VI (**2**)

A yellow unstable solid; it was soluble in $CDCl_3$ to give yellow solution, which was found to be unstable and turned into a black solution within about an hour and deposited insoluble black particles after a few hours at 0 °C. ^{1}H NMR data see Table 1; APCIMS (−)-ve mode m/z 331.0 [M−H]+ (calcd for $C_{20}H_{12}O_5$, 332.0).

3.4.3. Altertoxin I (**3**)[14]

Yellow solid; $[\alpha]_D^{25}$ + 395.5° (c 0.39, $CHCl_3$); UV λ_{max} ($CHCl_3$, log ε) 216.0, (3.63), 259.5, (4.52), 289.5, (4.17), 353.5 (3.80); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2, respectively.

3.4.4. Altertoxin II (**4**)[14 and 20]

Yellow solid; $[\alpha]_D^{25}$ +373.8° (c 0.1, CH_3OH); UV λ_{max} (MeOH, log ε) 216.0, (5.22), 260.0, (5.24), 363.0 (4.49); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2, respectively.

3.4.5. Altertoxin III (**5**)[14]

Pale brown solid; $[\alpha]_D^{25}$ + 720.5° (c 0.04, $CHCl_3$); UV λ_{max} ($CHCl_3$, log ε) 240.0, (4.96), 268.0, (5.19), 354.0 (4.73); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2, respectively; APCIMS (−)-ve mode m/z 348.0 [M]+ (calcd for $C_{20}H_{12}O_6$, 348.0).

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