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Musanahol: a new aureonitol-related metabolite from a *Chaetomium* sp.

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Abstract—Two antibacterial furano-polyenes, (−)-musanahol (**1**) and 3-*epi*-aureonitol (**5**), and a fatty acid, linoleic acid (**8**) were isolated from the laboratory cultures of a *Chaetomium* sp. accessed from tomato fruits, and grown on YMG medium (yeast extract, glucose, malt extract and water) at pH 5.8–6.0. The structure of compound **1**, a new furano-polyene, was elucidated by spectroscopic methods that include extensive 2D NMR experiments, double resonance experiments, Mosher's method and PM3 calculations. (−)-Musanahol (**1**) and 3-*epi*-aureonitol (**5**) were present in the culture filtrate of the fungus. 3-*epi*-Aureonitol (**5**) completely inhibited the growth of *Streptococcus pyogenes* at 15.63 μg/mL and *Escherichia coli*, *Staphylococcus aureus*, *Salmonella choleraesuis* and *Corynebacterium diphtheriae* at 31.25 μg/mL, whereas (−)-musanahol (**1**) lacked the antimicrobial potency of compound **5** in spite of the similarities in their structures. Linoleic acid (**8**) was isolated from the mycelia of the fungus; it inhibited the growth of *S. aureus* and *Bacillus subtilis* at a minimum concentration of 15.62 μg/mL.
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1. Introduction

As part of our efforts to discover bioactive agents from organisms that flourish in unusual environments, we have initiated the screening of fungi isolated from desert plants and hypersaline soils in Oman for antimicrobial compounds. One of the fungi selected for further studies is *Chaetomium* sp. IMI no. 394226, an endophyte accessed from the fruits of tomato cultivated at Musanah in Oman.

Chaetomium is an Ascomycota of the family Chaetomiaceae. It is a large genus comprising over 100 species.[1] Several strains of *Chaetomium* are found in the soil, plants' debris and endophytic habitats, where they suppress the growth of bacteria and fungi through direct competition, mycoparasitism and antibiosis.[2] They are also well-known sources of bioactive compounds. For instance, antifungal chaetoatrosin A and fuscoatroside have been isolated[3,4] from *Chaetomium atrobrunneum* F449 and *Chaetomium* sp. no. 217, antibacterial chaetochalasin A from *Chaetomium brasiliense*,[5] antitumor differenisole A from strain RB-001 of *Chaetomium*[6] and antibiotic chaetomin from *Chaetomium cochlioides*.[7] Novel and diverse compounds with unknown biological activity have also been obtained from the fungus *Chaetomium*. Chaetochiversins A and B are coumarins from *Chaetomium chiversii*,[8] chaetoindicins A–C are alkaloids from *Chaetomium indicum*,[9] aureonitol is a furano-polyene[10] from *C. cochlioides* and colletodiol is a polyoxo macrolide from *Chaetomium funicola*.[11]

Chaetomium globosum is about the most cosmopolitan species of the genus *Chaetomium* and several bioactive compounds such as cytotoxic chaetoglobosins C, D, E, F, Q, R, T[12,13] and globosumones A–C,[14] antifungal prenisatin[15] and chaetoviridins A and B[2] have been isolated from the laboratory cultures of this fungus. Our strain of *Chaetomium* sp. produced sparse, septate, hyaline mycelia and distinctive hyphal aggregations on potato carrot agar (Oxoid UK) and tap water agar (Agar no. 1, mycological) but no sporulation was evident, even when incubated at different temperatures or on exposure to alternate cycles of UV–visible light.[16] The EtOAc extracts of the mycelia and culture filtrate of this strain demonstrated potent activity against several human microbial pathogens in the well diffusion antimicrobial assay.[17] In this paper, we report the isolation of bioactive compounds **1**, **5** and **8** from the laboratory cultures of a *Chaetomium* sp.; compound **1** is new, and **5** is an epimer of *Helichrysum* aureonitol.[18] The structural elucidation of **1** and the activity of compounds **1**, **5** and **8** against a battery of clinically important microbes are also reported.

Keywords: Polyketide; Ascomycota; *Chaetomium* sp; Absolute configuration; Mosher ester; Musanahol; Antibacterial.
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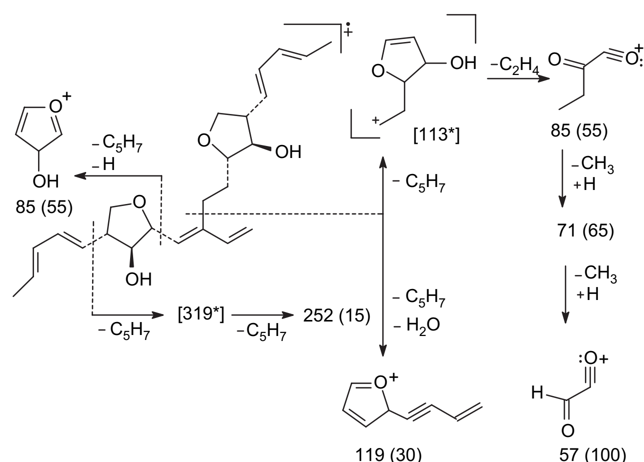
2. Results and discussion

Our strain of the fungus *Chaetomium* showed 98% homology with *C. globosum* strain no. AFTOL-ID 217 at CABI Bioscience Culture Collection based on partial sequence of 18S and complete sequence of 28S ribosomal RNA gene at CABI, UK.

The fungus was maintained on potato dextrose agar (PDA, Oxoid, UK) slants at 4 °C. Contact bioautography analysis[19] of the crude EtOAc extracts of the mycelia and culture filtrate of the fungus showed that the metabolites with TLC bands at R_f 0.6 (petroleum ether–EtOAc 7:3) in the mycelia, and 0.8 (petroleum ether–EtOAc 6:4) in the culture filtrate inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* or *Bacillus subtilis* in vitro.

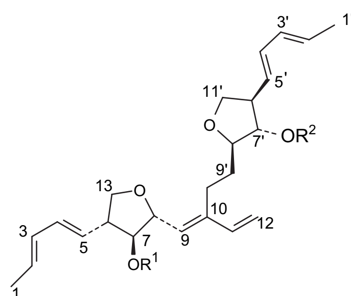
To generate sufficient quantities of the bioactive metabolites, the fungus was grown in 500 mL YMG medium (yeast extract 4.0 g, glucose 4.0 g, malt extract 10.0 g and distilled water 1.0 L, pH 5.8–6.0) in 1.0 L Erlenmeyer flasks. Freshly harvested wet mycelia or culture filtrate was separately extracted with ethyl acetate (EtOAc) and subjected to bioassay-guided fractionation by gradient chromatography over silica gel.

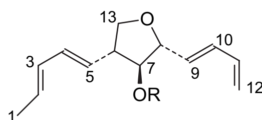
Compound **1** named musanahol was analysed for $C_{24}H_{34}O_4$ from HREIMS data and showed a molecular ion peak at 386.2494 [M$^+$]. It has prominent peaks at *m/z* 371, 353 and 338 in the EIMS spectrum that were accounted for by sequential losses of methyl, water and methyl groups; there is also an important peak at *m/z* 252 [M−134]$^+$ that derived from the loss of two penta-1,3-dienyl chains, which flank two hydroxylated tetrahydrofuran rings (see Fig. 1) in compound **1**. The peaks at *m/z* 119, 85, 71 and the base peak at *m/z* 57 were also accounted for by the major fragmentation of compound **1** (see the Scheme 1).



Scheme 1. Main fragmentation of compound **1**. *Peak not observed. Peak relative intensity is given in parenthesis.

The IR of compound **1** showed a broad absorption at 3250 cm^{-1} for hydroxyl groups, and an intense stretching absorption for conjugated double bonds at 1650 cm^{-1} and strong bands of ethers and *E*-alkenes at 1280 and 990 cm^{-1}. The ^{1}H NMR spectra of compounds **1** and **5** are remarkably similar in signal envelopes and multiplicity. Compound **1** in contrast to **5** displayed prominent A_2B_2M pattern in the ^{1}H NMR spectra arising from a 2H triplet at 1.67 (J=6.0 Hz), a 2H multiplet at 1.42 and a 1H multiplet at δ 4.15 that were readily assigned to H-10′, H-9′ and H-8′, respectively (Table 1). The ^{13}C and DEPT NMR spectra of **1** revealed one sp^2 quaternary, one terminal methylene, two methylene, two methyl, nine vinyl, two oxymethylene and four oxymethine carbons, two of which were confirmed to be secondary alcohols on acetylation. The conversion of compound **1** to **2** by acetylation simplified the near isochronous cluster of signals observed between δ 3.70 and 3.80 for H-7 (H-7′), H-13$_B$ and H-11$_{B'}$ in the ^{1}H NMR spectrum of **1**. Compound **2** displayed two acetyl signals at δ 2.32 and 2.30 as 3H singlets and two 1H triplets at δ 4.19 (J=7.3 Hz) and 4.17 (J=7.3 Hz) for H-7 and H-7′ in the ^{1}H NMR due to near identical couplings of H-7 (H-7′) to H-6 (H-6′) and H-8 (H-8′), respectively. It also showed prominent acetate signals at δ 22.6, 21.7, 168.0 and 167.0 in the ^{13}C NMR spectrum. The C-7 and C-7′ resonances of **2** were observed at δ 120.0 and 119.0, respectively (see Table 1). From the HSQC and DEPT spectral data, six double bonds are present in compound **1**. However, the molecular formula $C_{24}H_{34}O_4$ of **1** implied the presence of eight double bond equivalents; the remaining two must be due to ring forms. The HSQC and H′/H′ COSY spectra of **1** revealed the connection of carbon atoms C-13 (C-11′)–C-6 (C-6′)–C-5 (C-5′) or C-7 (C-7′)–C-8 (C-8′)–C-9 (C-9′) in compound **1**. The connectivity between C-8 (C-8′)–C-9 (C-9′)–C-10′ and C-10–C-11–C-12 was further established by HMBC experiments (Table 1).





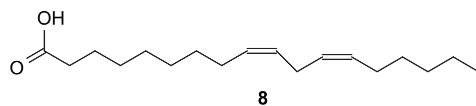


Figure 1. Structures of compounds **1**–**8**.

Table 1. ^{13}C and ^{1}H NMR data of compounds **1** and **2**, in $CDCl_3$

Position	Musanahol (**1**)				Compound **2**	
	δ_C (mult analysis)		δ_H (int, mult, *J*)	HMBC (C–H)	δ_C	δ_H (int, mult, *J*)
1	18.4	(CH_3)	1.74 (3H, dd, 6.7, 1.3)	2,3,4	19.2	1.74 (3H, dd, 6.7, 1.3)
2	129.7	(CH)	5.70 (1H, dq, 14.8, 6.7)	1,4	129.1	5.75 (1H, dq, 14.8, 6.7)
3	130.8	(CH)	6.04 (1H, ddd, 12.1, 10.5, 1.5)	1	130.8	6.07 (1H, ddd, 12.1, 10.5, 1.5)
4	133.6	(CH)	6.16 (1H, dd, 14.8, 15.0)	2,6	132.8	5.48 (1H, dd, 15.0, 8.7)
5	128.6	(CH)	5.42 (1H, dd, 15.0, 8.7)	3,6,7,13_B	128.7	6.11 (1H, dd, 14.8, 15.0)
6	51.8	(CH)	2.85 (1H, dddd, 8.1, 8.2, 8.2, 8.2)	4,5,7,13_A,13_B	50.0	2.93 (1H, dddd, 8.1, 8.2, 8.2, 8.2)
7	81.9	(CH)	3.76 (1H, dd, 7.3, 2.7)	4,5,6,8,13_A	122.0	4.19 (1H, dd, 7.3, 2.7)
8	85.1	(CH)	4.20 (1H, m)	7,10,13_A	77.6	4.32 (1H, m)
9	131.9	(CH)	5.74 (1H, obsc)	11	131.2	5.76 (1H, obsc)
10	168.2	(C)			168.0	
11	136.1	(CH)	6.35 (1H, m)	8,9	135.6	6.36 (1H, m)
12	118.5	(CH_2)	5.25 ($1H_A$, dd, 15.6, 8.7)	9,11	118.0	5.24 ($1H_A$, dd, 15.6, 8.7)
			5.12 ($1H_B$, d, 9.6)	9		5.20 ($1H_B$, d, 9.6)
13	71.4	(CH_2)	4.13 ($1H_A$, dd, 9.0, 7.9)	6,7,8	68.5	3.98 ($1H_A$, dd, 9.0, 7.9)
			3.73 ($1H_B$, dd, 8.6, 8.6)	5,6		3.81 ($1H_B$, dd, 8.6, 8.6)
1′	18.8	(CH_3)	1.74 (3H, dd, 6.7, 1.3)	2′,4′	19.5	1.74 (3H, dd, 6.7, 1.3)
2′	129.2	(CH)	5.70 (1H, dq, 14.8, 6.7)	1′,4′	129.1	5.73 (1H, dq, 14.8, 6.7)
3′	131.2	(CH)	6.04 (1H, ddd, 12.1, 10.5, 1.5)	1′	130.8	6.00 (1H, ddd, 12.1, 10.5, 1.5)
4′	132.8	(CH)	5.42 (1H, dd, 15.0, 8.7)	3′,6′,7′,11_A′	132.8	5.46 (1H, dd, 15.0, 8.7)
5′	128.6	(CH)	6.16 (1H, dd, 14.8, 15.0)	2′,6′	128.7	6.01 (1H, dd, 14.8, 15.0)
6′	51.2	(CH)	2.85 (1H, dddd, 8.1, 8.2, 8.2, 8.2)	4′,5′,7′,11_A′,11_B′	50.1	2.93 (1H, dddd, 8.1, 8.2, 8.2, 8.2)
7′	81.9	(CH)	3.77 (1H, dd, 7.3, 2.7)	6′,8′,11_A′	119.0	4.17 (1H, dd, 7.3, 2.7)
8′	85.1	(CH)	4.15 (1H, m)	7′,11_A′	77.6	4.31 (1H, m)
9′	28.9	(CH_2)	1.42 (2H, m)	10′,11_A′	29.7	1.45 (2H, m)
10′	30.3	(CH_2)	1.67 (2H, t, 6.0)	9′	30.1	1.68 (2H, t, 6.0)
11′	68.6	(CH_2)	4.12 ($1H_A$, dd, 9.0, 7.9)	4′,6′,7′,8′	65.9	3.97 ($1H_A$, dd, 9.0, 7.9)
			3.71 ($1H_B$, dd, 8.6, 8.6)	5′,6′		3.79 ($1H_B$, dd, 8.6, 8.6)
*CH_3*CO					22.6, 21.7	2.32 (3H, s), 2.30 (3H, s)
CH_3*CO*					168.0, 167.0	

The configurations of the double bonds at C-2 (C-2′) and C-4 (C-4′) in compound **1** were shown to be trans,trans based on the observed coupling constant values of 14.8 (14.8) Hz between H-2 (H-2′) and H-3 (H-3′) and 15.0 (15.0) Hz between H-4 (H-4′) and H-5 (H-5′) (Table 1). The conjugated dienes in compound **1** adopted *s*-cis orientation in $CHCl_3$ as evidenced from the λ_{max} absorption at 249 and 275 nm in the UV.[20]

Irradiation of the signals for H-6 (H-6′) at δ 2.85 leads to the suppression or even negative signals for H-7 at δ 3.76 and enhancement of the signals for H-8 and H-13_A (H-$11_{A'}$). Therefore, H-6 and H-7 are trans and are connected by a significant coupling constant, whereas H-6 and H-8 are cis. The $[\alpha]_D^{25}$ value for compound **1** was −4.3 in $CHCl_3$, and the absolute configurations of the hydroxyl groups in **1** were determined as C-7 (*S*) and C-7′ (*S*) by advanced Mosher's methodology[21] (see Table 2). Taken together, the relative configurations of the stereogenic centres in compound **1** are C-6 (*R*), C-6′ (*R*), C-7 (*S*), C-7′ (*S*), C-8 (*R*) and C-8′ (*R*). The configuration and conformation of **1** suggested by NOE, UV and coupling constant data are in good agreement with the most stable conformer obtained (Fig. 2) based on PM3 semi-empirical calculations.[22] The starting point for the conformational search was the structure assigned on the basis of spectroscopic data. Rotations about C(3)–C(4), C(3′)–C(4′) and C(10)–C(10′) were then systematically investigated.

Table 2. $\Delta\delta_H=(\delta_S-\delta_R)$ Data for relevant protons of the (*S*)- and (*R*)-MTPA Mosher ester derivatives of **1**

Position	$\Delta\delta_H=(\delta_S-\delta_R)$	Assigned configuration
H-11′A (H-13_A)	−0.04	
H-11′B (H-13_B)	−0.30	
H-6 (H-6′)	−0.15	
H-7 (H-7′)	−0.15	
H-8 (H-8′)	−0.03	
		C-7*S*
		C-7′*S*
H-9′	+0.03	
H-10′	+0.02	
H-1 (H-1′)	0.00	

Compounds **5** and **1** were isolated from the EtOAc extract of the culture filtrate. Compound **5** has molecular composition $C_{13}H_{18}O_2$ and identical ^{13}C NMR spectroscopic data (Table 3) with (2*S*,3*R*,4*S*)-2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran, the aureonitol isolated by Abraham and Arfmann[10] from *C. cochlioides*. However, there are distinct differences between this aureonitol and compound **5**. The aureonitol isolated[10] from *C. cochlioides* was a solid (mp=65 °C) with $[\alpha]_D^{27}$ value of −7.8. The absolute configuration[10] of the hydroxyl group at position 3 was determined as C-3 (*R*) by Mosher's method, and no biological activity has been reported in the literature for aureonitol. Compound **5**, however, was obtained as a semi-solid with $[\alpha]_D^{25}$ value of +2.5 in $CHCl_3$. The absolute configuration of the hydroxyl group at position 3 was determined as C-3 (*S*) by Mosher's method.[21] UV spectroscopic data also suggested *s*-cis orientation in $CHCl_3$ for the conjugated diene moiety of compound **5** as in **1**. Double resonance NMR experiments and the absolute configuration of the hydroxyl group enabled compound **5** to be described as

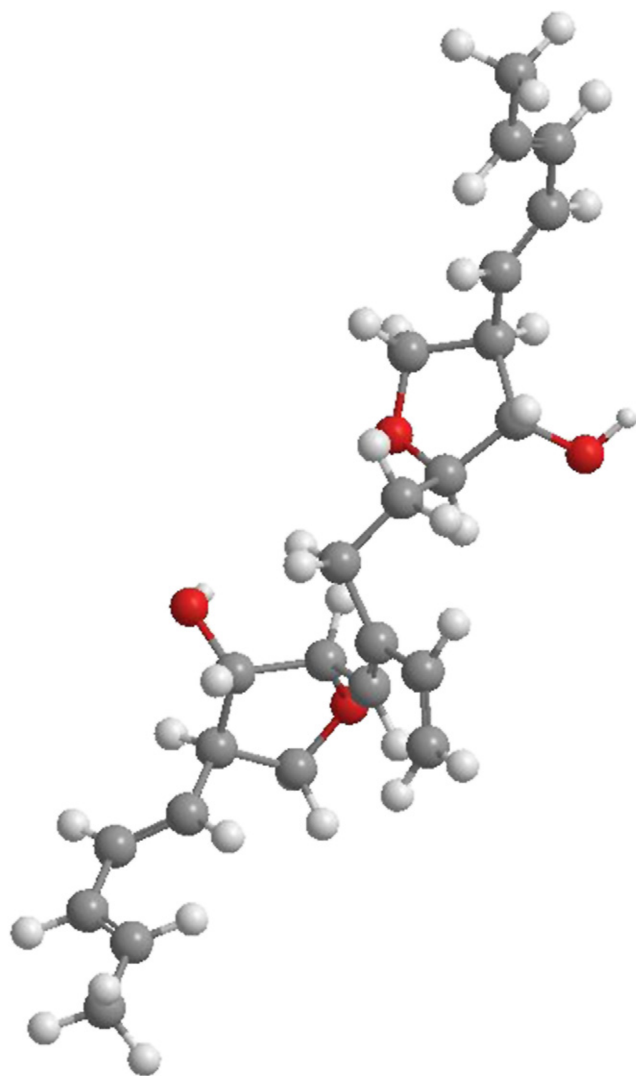


Figure 2. The molecular structure of the most stable conformer of **1** based on PM3 semi-empirical calculations.

(2*R*,3*S*,4*R*)-2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran. Compound **5** is neither a monomer of **1** nor an enantiomer of aureonitol reported by Abraham and Arfmann,[10] rather it is a 3-epimer of *Helichrysum* aureonitol that was previously described[18] as (2*R*,3*R*,4*R*)-2-(1,3-butadienyl)tetrahydro-4-(1,3-pentadienyl)-3-furanol, aureonitol in the chemical index.

Table 3. ^{13}C NMR data for compounds **5** and **8**, aureonitol and linoleic acid

Position	5	Aureonitol	8	Linoleic acid
1	18.1	18.0	178.9	180.6
2	129.4	128.3	33.8	34.2
3	130.8	130.9	24.6	24.8
4	133.2	133.1	29.0	29.2
5	128.2	129.3	29.1	29.2
6	51.5	51.6	29.1	29.2
7	81.5	81.7	29.7	29.7
8	84.7	84.8	27.2	27.3
9	133.3	133.2	130.0	130.0
10	131.5	131.0	128.0	128.2
11	136.1	136.2	25.6	25.8
12	118.2	118.1	127.9	128.0
13	71.0	71.1	130.0	130.2
14			27.2	27.3
15			29.3	29.5
16			31.5	31.6
17			22.6	22.7
18			14.1	14.1

Table 4. Minimum inhibitory concentrations of compounds **1**, **5** and **8**

Organisms	1	5	8	Gentamycin	Miconazole
	MIC (μg/mL)				
Escherichia coli ATCC 9637	125.0	31.3	NA[b]	2.0	NT[c]
Klebsiella pneumoneae ATCC 10031	NA[b]	NA[b]	NA[b]	1.0	NT
Pseudmonas aeruginosa ATCC 27853	NA[b]	NA[b]	NA[b]	0.5	NT
Staphylococcus aureus ATCC 29213	250.0	31.3	15.62	0.5	NT
Salmonella choleraesuis ATCC 14028	125.0	31.3	NA[b]	1.0	NT[c]
Candida albicans ATCC 10231	250.0	62.5	NA[b]	250.0	2.0
Bacillus subtilis[a]	250.0	125.0	15.62	0.5	2.0
Corynebacterium diphtheriae	125.0	31.3	NA[b]	3.9	NT[c]
Enterobacter cloacae	125.0	125.0	NA[b]	2.0	NT[c]
Enteropathogenic *E. coli*	250.0	250.0	NA[b]	250.0	NT[c]
Methicillin resistant *S. aureus*	125.0	125.0	NA[b]	7.8	NT[c]
Proteus mirabilis	250.0	250.0	NA[b]	2.0	NT[c]
Shigella flexneri	250.0	250.0	NA[b]	3.9	NT[c]
Streptococcus pyogenes	125.0	15.6	31.3	3.9	NT[c]

[a] Clinical isolate.
[b] Not active.
[c] Not tested.

Compound **8** was the major bioactive compound (yield 8%) isolated from the mycelia extract. It is an unsaturated fatty acid identified as linoleic acid (**8**) based on spectral correlation with a known compound[23] (Table 3).

Compound **8** was antibacterial to *S. aureus* and *B. subtilis* at a concentration of 31.25 μg/mL. *cis*-Type long chain unsaturated fatty acids are well known for their strong antibacterial activity[24,25] and high toxicity (LD_{50} 5 μg/mL) towards saprophytic nematodes.[26] Compound **5** inhibited the growth of methicillin resistant *S. aureus* at MIC value of 125 μg/mL, and of *E. coli*, *S. aureus*, *Salmonella choleraesuis* and *Corynebacterium diphtheriae* at 31.25 μg/mL. It also inhibited the growth of *Streptococcus pyogenes* at 15.63 μg/mL. Compound **5** is superior to **1** in antibacterial activity against clinically important microbes (Table 4).

3. Conclusion

Compound **8** must have contributed significantly to the observed antibacterial properties of the EtOAc extract of the mycelia of our strain of *Chaetomium* sp. Linoleic acid and linoleic acid-derived compounds are sporogenic natural products[27] controlling the ratio of asexual to sexual spores in some organisms. The role of mycelia linoleic acid in the activation of sporulation[27] is rather uncertain in this fungus because *Chaetomium* sp. used in this study did not sporulate. However, the abundance of linoleic acid in the mycelia of *Chaetomium* sp. may be of interest in the biological control of plant parasitic nematodes.[26]

Biogenetically, compounds **1** and **5** may be obtained from similar precursors. It is unlikely that compound **5** will convert to **1** during the isolation process.

4. Experimental

4.1. General

NMR data were generated on Bruker Avance 400 MHz NMR spectrometer. All spectra were recorded in $CDCl_3$ with TMS as internal standard. UV spectra were recorded on UV–visible HP-8453 spectrophotometer. IR spectra were measured on a Nicolet FT-IR spectrometer and optical rotations were measured with Perkin Elmer Model 341 polarimeter at 25 °C. HREIMS and FABMS spectra were recorded with a JEOL JMS-SX 102 A (EIMS, 70 eV, gun high 3.0 kV; FABMS with *m*-nitrobenzoic acid as matrix, at 85.5 °C). Silica gel chromatography was carried out using EM Science silica gel 60 (70–230 mesh ASTM) and all the solvents were distilled prior to use. Whatman precoated silica gel (K6F, 60 Å) was used for TLC with compounds visualised by spraying with 20% (v/v) H_2SO_4 or 1% vanillin–H_2SO_4 followed by heating.

4.2. Fungal isolation, identification and cultivation

The fungal strain *Chaetomium* sp. was isolated from tomato fruit collected from an agricultural farm in Musanah, Sultanate of Oman. The fruit was surface sterilised with 1% NaOCl and washed with sterile distilled water. The skin of the tomato was removed and pieces of the fruit were placed on potato dextrose agar plates (PDA, Oxoid UK) supplemented with 50 mg/L chloramphenicol. The plates were incubated at 25 °C for three days. Emerging fungal mycelium was transferred to fresh PDA plates and incubated for one week and periodically checked for purity. The fungus, coded DUF 16 was maintained on PDA slants at 4 °C. It was assigned accession no. IMI 394226 by CABI Bioscience, UK and identified as *Chaetomium* sp. based on partial sequence of 18S and complete sequence of 28S ribosomal RNA gene. It showed 98% homology with *C. globosum* isolate AFTOL-ID 217.

Ten discs (5 mm diameter) taken from the periphery of the 10-day old culture on PDA plates, were inoculated in 500 mL YMG medium (yeast extract 4.0 g, glucose 4.0 g, malt extract 10.0 g and distilled water 1.0 L, pH 5.8–6.0) in 1.0 L Erlenmeyer flasks. The flasks were incubated at 25 °C for eight days (pH after the fermentation run was 7.6±0.2) in an orbital shaker (Sanyo, Gallenkamp Orbital Incubator) operating at 140 rpm.

4.3. Extraction and isolation

The culture broth was filtered through Whatman no. 1 filter paper under vacuum. The mycelia and the culture filtrate were processed separately. The wet mycelia were milled in a Waring blender. Approximately 600 g of the wet mycelia was obtained from 4.0 L of the broth. The ground mycelia were extracted with 1.2 L of ethyl acetate (EtOAc) by maceration for two days, drained and then re-soaked in 1.2 L of EtOAc for another day. The EtOAc extracts were then combined, dried over sodium sulfate, concentrated in vacuo and allowed to dry in a hood to give 660 mg of crude mycelial extract. The volume of culture filtrate obtained after filtration was 3.2 L. The culture filtrate was extracted twice with equal volumes of EtOAc. The EtOAc layer was removed, dried over sodium sulfate and concentrated in vacuo. The residues were dried in a hood to give 380 mg of the crude culture filtrate.

The crude culture filtrate extract (1.7 g) was chromatographed on a silica gel 60 column (50×2 cm) and eluted successively with petroleum ether–EtOAc step gradients, collecting fractions in 50 mL portions: petroleum ether 500 mL (58.1 mg); petroleum ether–EtOAc 9.9:0.1, 1.0 L (31.1 mg), petroleum ether–EtOAc 9.8:0.2, 2.5 L (102.7 mg), petroleum ether–EtOAc 9.7:0.3, 1.0 L (32.5 mg), petroleum ether–EtOAc 9.5:0.5, 1.5 L (34.8 mg), petroleum ether–EtOAc 9.0:1.0, 5.5 L (197 mg), petroleum ether–EtOAc 8.5:1.5, 1.5 L (41.5 mg), petroleum ether–EtOAc 8:2, 4.5 L (130.8 mg), petroleum ether–EtOAc 7:3, 5.0 L (169.3 mg), petroleum ether–EtOAc 1:1, 3.0 L (119.6 mg), petroleum ether–EtOAc 3:7, 5.0 L (45.1 mg), EtOAc 1.0 L (57 mg) followed by acetone 500 mL (176 mg) and EtOH 750 mL (543 mg).

From the fractions eluted with petroleum ether–EtOAc (9.0:1.0), compounds **1** (25.5 mg), R_f 0.68 (petroleum–EtOAc 8:2) and **5** (30 mg), R_f 0.79 (petroleum–EtOAc 6:4) were obtained.

The crude extract of the mycelia (1.9 g) was applied to a column (40×2 cm) packed with 40 g silica gel and eluted with petroleum ether–EtOAc step gradients, collecting fractions in 50 mL portions: petroleum ether 1.0 L (116 mg); petroleum ether–EtOAc 9.8:0.2, 8.0 L (631.5 mg), petroleum ether–EtOAc 9.6:0.4, 3.0 L (82.8 mg), petroleum ether–EtOAc 9.2:0.8, 3.0 L (54.1 mg), petroleum ether–EtOAc 9.0:1.0, 4.0 L (104.1 mg), petroleum ether–EtOAc 8.8:1.2, 4.3 L (82 mg), petroleum ether–EtOAc 8.5:1.5, 1.8 L (84.6 mg), petroleum ether–EtOAc 8.0:2.0, 1.0 L (27.8 mg), petroleum ether–EtOAc 7.0:3.0, 2.0 L (83.3 mg), EtOAc 1.0 L (288 mg). The column was then washed with EtOH (500 mL) to give a residue (866 mg). Compound **8** (151.9 mg), a fatty acid with R_f 0.64 (petroleum ether–EtOAc 7:3) was eluted with petroleum ether–EtOAc (9:1).

4.3.1. Musanahol (1). Colourless semi-solid, $[\alpha]_D^{25}$ −4.3 (*c* 0.10, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 249 (3.5), 275 (3.1) nm; IR (KBr) 3250, 2950, 1650, 1280–990 cm^{-1}; TLC R_f 0.68 (petroleum ether–EtOAc 8:2); EIMS *m/z* 386 [M^+] (35), 371 (28), 353 (24), 338 (25), 330 (30), 325 (30), 315 (25), 294 (14), 252 (15), 197 (10), 149 (18), 119 (30), 85 (55), 71 (65), 57 (100), 43 (90), 18 (80). HREIMS *m/z* 386.2494 (calcd for $C_{24}H_{34}O_4$, 386.2457); ^1H and ^{13}C NMR data: see Table 1.

4.3.2. Compound 2. Compound **1** (13 mg) was mixed with 2 mL of pyridine and 2 mL of acetic anhydride in a capped vial and left for 72 h at room temperature. Distilled water (20 mL) was added to the reaction mixture and extracted twice with 20 mL of $CHCl_3$. The combined organic layer was washed, extracted fast with 10 mL of ice cold dilute HCl once and then washed with water. The organic layer

was dried over anhydrous sodium sulfate, concentrated in vacuo and purified by prep TLC to give compound **2**, R_f 0.93 (petroleum ether–EtOAc 8:2); IR (KBr) 2970, 1740, 1660, 990–1280 cm^{-1}; EIMS *m/z* 470 [M^+] (15), 369 (5), 309 (15), 279 (20), 263 (30), 236 (100), 192 (20), 167 (40), 150 (40), 113 (25), 83 (30), 71 (35). HREIMS *m/z* 470.2693 (calcd for $C_{28}H_{38}O_6$, 470.2668); ^1H and ^{13}C NMR data: see Table 1.

4.3.3. Compound 5. Colourless semi-solid, $[\alpha]_D^{25}$ +2.5 (*c* 0.12, $CHCl_3$); UV ($CHCl_3$) 249 (3.5), 275 (3.1) nm; IR (KBr) 3400, 1650, 1280–990 cm^{-1}; TLC R_f 0.73 (petroleum ether–EtOAc 7:3); EIMS *m/z* 206 [M^+] (2.5), 178 (15), 149 (20), 85 (55), 71 (65), 57 (100). HREIMS *m/z* 206.1317 (calcd for $C_{13}H_{18}O_2$, 206.1307); ^1H NMR δ: 6.16 (1H, dd, 15.0, 14.8, H-4), 6.04 (1H, ddd, 12.1, 10.5, 1.5, H-3), 5.74 (1H, obsc, H-9), 5.70 (1H, dq, 14.8, 6.7, H-2), 5.42 (1H, dd, 15.0, 8.7, H-5), 5.25 (1H, dd, 15.6, 8.7, H-12A), 5.12 (1H, d, 9.6, H-12B), 4.13 (1H, m, H-8), 4.15 (1H, dd, 9.0, 7.9, H-13A), 3.76 (1H, dd, 7.3, 2.7, H-7), 3.71 (1H, dd, 8.6, 8.6, H-13B), 2.85 (1H, dddd, 8.2, 8.2, 8.2, 8.1, H-6), 1.74 (3H, dd, 6.7, 1.3, H-1), ^{13}C NMR data: see Table 3.

4.3.4. Preparation of the (*R*)- and (*S*)-MTPA esters of compounds 1 and 5. A mixture of compound **1** or **3** (5 mg) and millimolar equivalents of (*R*)- or (*S*)-MTPA chloride was taken in two capped vials. 4-(Dimethylamino) pyridine (1.6 mg) and two drops of pyridine were then added to each vial. The reaction mixture was allowed to stand at room temperature for four days, then separately diluted with 1 mL of hexane and chromatographed over silica gel in a disposable pipette, eluting with a gradient of CH_2Cl_2–EtOAc mixtures. The purified *R*- and *S*-Mosher esters were then analysed by ^1H NMR and ^1H–^1H COSY spectra.

4.3.4.1. *C*(7),*C*(7′)-Bis-(*S*)-MTPA ester 3. HRMS (FAB) *m/z* 819.3340 (calcd for $C_{44}H_{49}F_6O_8$ $[M+H]^+$ 819.3332); TLC R_f 0.84 (petroleum ether–EtOAc 7.5:2.5).

4.3.4.2. *C*(7),*C*(7′)-Bis-(*R*)-MTPA ester 4. HRMS (FAB) *m/z* 819.3342 (calcd for $C_{44}H_{49}F_6O_8$ $[M+H]^+$, 819.3332); TLC R_f 0.88 (petroleum ether–EtOAc 7.5:2.5).

4.3.4.3. *C*(7)-(*S*)-MTPA ester 6. EIMS *m/z* 422 [M^+], HREIMS *m/z* 422.1711 (calcd for $C_{23}H_{25}F_3O_4$, 422.1705); TLC R_f 0.96 (petroleum ether–EtOAc 7:3).

4.3.4.4. *C*(7)-(*R*)-MTPA ester 7. EIMS *m/z* 422 [M^+], HREIMS *m/z* 422.1715 (calcd for $C_{23}H_{25}F_3O_4$, 422.1705); TLC R_f 0.91 (petroleum ether–EtOAc 7:3).

4.3.5. Compound 8. UV ($CHCl_3$) λ_{max} (log ε) 241 (2.8) nm; IR (KBr) 3431, 2918, 2849, 1703, 1620, 1464, 1288, 942 cm^{-1}; ^1H NMR δ: 5.36 (4H, m), 2.78 (2H, dd, J=6.4, 6.6 Hz), 2.34 (2H, t, J=7.6 Hz), 2.05 (4H, m), 1.25 (16H, m), 0.89 (3H, t, J=6.7 Hz); ^{13}C NMR data: see Table 3.

4.4. Bioassays

4.4.1. Well diffusion bioassay. The antimicrobial activities of the crude EtOAc extracts of the mycelia and culture filtrates were evaluated according to the well diffusion method[17] against *E. coli* (ATCC 9637), *Klebsiella pneumoneae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 27853), *S. aureus* (ATCC 29213), *S. choleraesuis* (ATCC 14028), *Candida albicans* (ATCC 10231), and clinical isolate of *B. subtilis*. Briefly, a loopful of test culture was inoculated in 5 mL nutrient broth tube and incubated at 37 °C for 18 h. The broth culture was diluted with normal saline to McFarland standard 0.5. Wells (5 mm) were made in the nutrient agar plates (Oxoid, UK) for bacteria or potato dextrose agar (PDA, Oxoid, UK) plates for *C. albicans* that were previously swabbed with diluted broth culture of the respective organism. The crude extract (50 μL, 500 μg/mL in DMSO) was added to each well and the plates were incubated at 37 °C and averages of observed zones of inhibition after 24 h for tests performed in three replicates were compared. In this test, gentamycin and miconazole in DMSO were used as positive controls.

4.4.2. Contact bioautography assay. Contact bioautography assay was performed essentially as described by Hamburger and Cordell.[19] Solutions of test extract in EtOAc was spotted on TLC plates (2.5×8 cm) and developed in petroleum ether–EtOAc (1:1). For each sample, two TLC plates were run, and one of the plates was sprayed with 1% vanillin. The second TLC plate was placed surface to surface on nutrient agar in a Petri dish, initially swabbed separately with *E. coli*, *S. aureus* and *B. subtilis* and then incubated at 37 °C for 24 h. The TLC plate was separated from the nutrient agar surface, sprayed with the aqueous solution of methyl thiazolyl tetrazolium chloride (MTT, 2.5 mg/mL), incubated for 30 min at 37 °C and observed for colour change. A yellow zone of inhibition is accepted as indicative of activity (inhibition of the growth of organism) and a purple colour on the TLC indicates absence of activity. The R_f value of the active compound was located from the reference plate. MTT detects the dehydrogenase activity in the growing organism. The enzyme converts MTT to formazan intermediate, which stains the plate purple, whereas when there is no growth of test organism, the colour remains yellow.[28]

4.4.3. Minimum inhibitory concentration (MIC). The MIC values for compounds **1**, **5** and **8** were determined against 14 organisms (see Table 4) according to a broth microdilution procedure.[29] A solution of each of the compounds (2000 μg/mL) in dimethyl sulfoxide (DMSO) was diluted 2-fold with nutrient broth to give a series of concentrations ranged 1000–0.425 μg/mL, respectively. Each dilution (100 μL) was dispensed in rows of microwell titre plates. Appropriately diluted (according to McFarland standard 0.5) test organism (10 μL) was added to each well. Miconazole and gentamycin solutions in DMSO were used as positive controls and nutrient broth with 10 μL DMSO in the absence of the test compound was used as negative control. The plates were incubated at 37 °C for 24 h. The lowest concentration at which the test microorganism demonstrated visible growth was recorded as the MIC.

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