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An antibacterial hydroxy fusidic acid analogue from *Acremonium crotocinigenum*

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Abstract

A fusidane triterpene, 16-deacetoxy-7-β-hydroxy-fusidic acid (**1**), was isolated from a fermentation of the mitosporic fungus *Acremonium crotocinigenum*. Full unambiguous assignment of all ^{1}H and ^{13}C data of **1** was carried out by extensive one- and two-dimensional NMR studies employing HMQC and HMBC spectra.

Compound **1** was tested against a panel of multidrug-resistant (MDR) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains and showed minimum inhibitory concentration values of 16 μg/ml.

Keywords: *Acremonium crotocinigenum*; Fusidane triterpene; Fusidic acid; Antibacterial; MRSA; MDR; *Staphylococcus aureus*

1. Introduction

Our studies on the production of metabolites by taxa of tropical rainforest fungi in fermentation, have led to the isolation and characterisation of a new metabolite, designated 16-deacetoxy-7β-hydroxy-fusidic acid (**1**), which is structurally related to the commercial antibiotic, fusidic acid, a widely used therapeutic for methicillin-resistant *Staphylococcus aureus* (MRSA) infections which is still of interest as a template for antibiotic activity improvement (Søtofte and Duvold, 2001). The metabolite is a prominent component of fermentation liquors from shake cultures of an isolate of the mitosporic fungus *Acremonium crotocinigenum*, cultured from rotting wood in Rio Palenque Forest Reserve, Pichincha Province, Ecuador in 1986, and currently held in the University of Westminster culture collection. *Acremonium* is a polyphyletic genus, often confused with *Cephalosporium* and is related to a number of ascomycete teleomorphs (Gams, 1971). It contains some 105 species, including a number which have been shown to produce biologically active metabolites (Kirk et al., 2001). Previous studies on *A. crotocinigenum* found sesquiterpenoid compounds of the isocrotonic acid type (Gyimesi and Melera, 1967).

The detection of **1** was part of a programme for screening tropical fungi for new antibiotics with activity against MRSA. There is currently an acute need for new effective antibiotics for MRSA treatment, especially since the appearance of vancomycin resistant (VRSA) strains (Centers for Disease Control and Prevention, 2003; Chang et al., 2003). Liquid fermentation was used in conjunction

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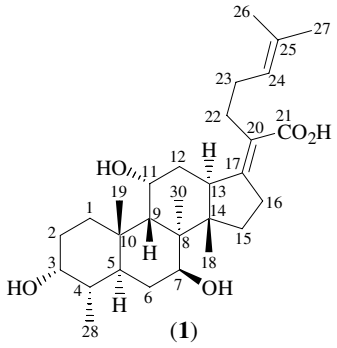
with bioautography, to qualitatively indicate the presence of antibacterial compounds, facilitating the isolation of compound **1** by vacuum liquid chromatography.



(**1**)

2. Results and discussion

Bioautography of the Diaion HP20 resin extract of the fermentation filtrate led to the isolation of compound **1** as a white solid. High-resolution ESI-TOFMS in the positive mode suggested a molecular formula of $C_{29}H_{46}O_5$. Signals in the ^1H and ^{13}C NMR spectra (Table 1) for five methyl singlets, one methyl doublet, four olefinic carbons and a carbonyl of a carboxylic acid (δ_C 173.8), were indicative of a fusidane class triterpene of the fusidic acid type (Rastup-Andersen and Duvold, 2002).

Table 1
^1H (400 MHz) and ^{13}C NMR (100 MHz) spectral data and ^1H–^{13}C long-range correlations of **1** recorded in $CDCl_3$

Position	^1H	^{13}C	^2J	^3J
1	1.50 *m*, 2.23 *m*	30.0		
2	1.75 *m*, 1.81 *m*	29.9		
3	3.73 *bq*	71.4	C-2	C-5
4	1.54 *m*	37.3		
5	2.31 *m*	36.2		
6	1.45 *m*, 1.67 *m*	34.1		
7	3.99 *t* (8.0)	70.9	C-6	C-14, C-30
8	–	45.6	–	–
9	1.52 *m*	50.8	–	–
10	–	36.7	–	–
11	4.37 *bs*	68.7	–	C-8
12	1.75 *m*, 2.39 *m*	36.4		
13	3.05 *bd* (12.1)	46.0	C-14, C-17	C-15, C-20
14	–	49.6	–	–
15	1.54 *m*, 1.77 *m*	33.4	–	–
16	2.68 *m*, 2.86*m*	33.0	C-15, C-17	C-20
17	–	160.4	–	–
18	0.89 *s*	15.9	C-14	C-8, C-13, C-15
19	0.95 *s*	24.4	C-10	C-5, C-1, C-9
20	–	125.0	–	–
21	–	173.8	–	–
22	2.44 *m*	28.5	C-20, C-23	C-17, C-21
23	2.02 *m*, 2.17 *m*	29.4	C-22, C-24	C-25
24	5.12 *t*, (7.2)	124.0	C-23	C-26, C-27
25	–	132.2	–	–
26	1.61 *s*	18.0	C-25	C-24, C-27
27	1.67 *s*	25.9	C-25	C-24, C-26
28	0.93 *d* (6.8)	16.0	C-4	C-3, C-5
30	1.36 *s*	14.6	C-8	C-7, C-9, C-14

By careful analysis of the HMBC, HMQC and COSY spectra it was possible to show that **1** was a new fusidic acid analogue. Assuming that the methyl doublet was C-28 of the fusidane skeleton, the protons of this group coupled to a methine proton (δ_H 1.54, H-4) in the COSY spectrum. H-4 formed part of a spin system with a deshielded methine (δ_H 3.73, H-3) and two methylene groups (at C-2 and C-1). In the HMBC spectrum, C-1 was coupled to by the protons of methyl-C19 (δ_H 0.95) which showed further couplings to C-10 (^2J), C-9 (^3J) and C-5 (^3J). In the COSY spectrum, H-5 (δ_H 2.31 *m*) coupled to both protons of a methylene moiety (C-6, δ_H 1.45, 1.67), which further coupled to a deshielded oxymethine proton (C-7, δ_H 3.00, *t*). Inspection of the HMBC spectrum showed that the carbon associated with this deshielded proton was coupled to by the protons of a further angular methyl singlet (C-30), which showed additional couplings to a methine carbon (C-9) and two quaternary carbons (C-8, δ_C 45.6 and C-14, δ_C 49.6). This completed the resonances for the A and B rings of compound **1**. Inspection of the COSY spectrum showed that the proton associated with C-9 (H-9) formed part of a CH–CH–CH_2–CH spin system which allowed identification of positions C-9, C-11, C-12 and C-13, respectively. C-11 was deshielded (δ_C 68.7, δ_H 4.37) indicating that an oxygen should be placed here. Furthermore, H-13 (delineated by inspection of the HMQC spectrum) was also deshielded (δ_H 3.05) suggesting that it was allylic and that an olefinic carbon (C-17) should be placed at the neighbouring carbon, which is typical for fusidic acid metabolites (Rastup-Andersen and Duvold, 2002). The protons of a methyl group (C-18) coupled to C-13 (^3J), C-14 (^2J) and to a methylene carbon (C-15, ^3J). CH_2-15 coupled to a deshielded allylic methylene group (δ_H 2.68, 2.86 (CH_2-16)) which again was supportive of being alpha to an olefinic carbon (C-17, δ_C 160.4). This completed rings C and D of **1**. H-13 and H_2-16 both gave a ^2J coupling to C-17 and a ^3J coupling to C-20, suggesting a C-17,20 double bond. In the HMBC spectrum C-17 was also coupled to by the protons of an allylic methylene (C-22, δ_H 2.44) which also coupled to a carbonyl carbon of a carboxylic acid group (C-21) and an olefinic methine carbon (C-24, δ_C 124.0). A further methylene (C-23) could be placed between C-22 and C-24 by couplings observed in the COSY spectrum. Finally, two deshielded geminal methyl groups could be placed on an olefinic carbon (C-25) *via* their HMBC correlations to this carbon and to the olefinic partner C-24 finalising the C-24–C-25 double bond. These resonances completed the eight carbon chain of the fusidane triterpene skeleton. HRESI-MS of **1** suggested a molecular formula of $C_{29}H_{46}O_5$ $[M]^+$ (475.3422). From the chemical shift values of H-3, H-7 and H-11, hydroxyl groups must be placed at these positions. From the molecular formula and chemical shift of the C-21 carbon, a car-

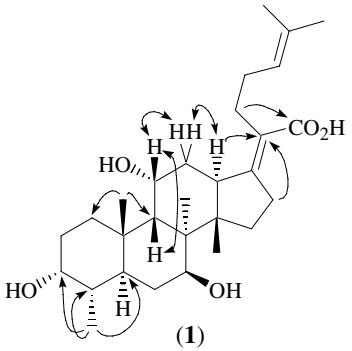


Fig. 1. Key COSY (double headed arrow) and HMBC (single headed arrow) correlations for compound **1**.

boxylic acid must be placed at C-21 and this is identical to that seen in fusidic acid (see Fig. 1).

The final consideration was to assign stereochemistry of hydroxyl groups at C-3, C-7 and C-11. The hydrogens at C-3 and C-11 were assigned as equatorial (rel β) on the basis of no large discernable couplings for these signals, which would make the hydroxyl groups at these positions both α and axial. The coupling constant for H-7 (δ 3.99, *t*) was 8.0 Hz indicating an axial-axial interaction with the axial partner of CH_2-6. This would make H-7 axial (α) and the OH at this position therefore equatorial (β). This was further supported by an NOE between H-7 and CH_3-30 indicating that they are both on the alpha face of the fusidane skeleton.

Fusidic acid and all known analogues to date have no substitution on carbon 7. Compound **1** possesses an hydroxyl at this position. *A. crotocinigenum* has also been found to produce analogues with an hydroxyl on carbon 16 and **1** is the first member of this class to be completely unsubstituted at the C-16 position.

Compound **1** was tested against a battery of drug-resistant bacteria and where active, possessed a minimum inhibitory concentration of 16 μg/ml, which although occasionally more active than erythromycin and norfloxacin, was significantly less potent than the fusidic acid comparator.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants (*J* values) are given in Hertz. Accurate mass measurements were determined on a Micromass Q-TOF Ultima Global Tandem Mass Spectrometer. The sample was run under electrospray ionisation mode using 50% acetonitrile in water and 0.1% formic acid as solvent. [Glu]-fibrinopeptide B peptide was used as an internal standard, $[M+2H]^{2+} = 785.8426$.

IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer and UV spectra on a Thermo Electron Corporation Helios spectrophotometer.

3.2. Fungal strain

Cultures were maintained on malt extract agar (Oxoid) and for long term storage on malt extract agar plugs submerged in sterile distilled water at room temperature, as part of the University of Westminster culture collection (Culture No. cc56). The isolate was identified as *A. crotocinigenum* by David Brayford, initially through DNA sequencing of a PCR product amplified from the variable ITS (internal transcribed spacer) region of the ribosomal RNA locus using the conserved primers ITS1F and ITS4 (White et al., 1990; Gardes and Bruns, 1993). A search of DNA sequence databases with 508 bp of DNA sequence from this PCR product using the FASTA algorithm (Pearson and Lipman, 1988; http://www.ebi.ac.uk/fasta/) revealed the most closely related sequence accession to be AJ621773 (*Acremonium crotocinigenum*), which showed 98.2% identity over a 513 bp overlap. This *A. crotocinigenum* strain was isolated from the basidiome of *Trametes versicolor* in a Hungarian coal mine (Schol-Schwarz, 1965) and was named by Gams (1971). Comparisons of the conidia, chlamydospores and colony appearance with those described in Schol-Schwarz (1965) and Gams (1971) were used to confirm that cc56 was indeed morphologically the same as *A. crotocinigenum*. Further confirmation was obtained by direct comparison of cc56 with strain CABI 112775 (syn. CBS 129.64) kindly supplied by the International Mycological Institute, Egham, UK. The ITS sequence for isolate cc56 has been deposited in the GenBank database (accession number DQ882846).

3.3. Fermentation and extraction

Inoculum for the fermentation was prepared by vigorously shaking twenty 8 mm diameter plugs, excised from an actively growing culture of *A. crotocinigenum* on 2% malt extract agar (Oxoid), in 10 ml of sterile distilled water containing 2–3 ml of glass beads (VWR). The resulting mycelial suspension (2 ml) was added to each of twenty 1000 ml conical flasks, containing 200 ml of sterilised 2% potato dextrose broth (Difco). The flasks were incubated on a rotary shaker (200 rpm) for two weeks at 26 °C.

Biomass was removed from the culture broth by filtering through muslin prior to filtration through a Whatman No. 1 filter paper. The filtrate was then extracted with Diaion HP20 resin (400 ml; Mitsubishi) which had previously been washed with HPLC grade methanol (Merck) and thoroughly conditioned with distilled water. The resin was removed, washed with distilled water (2×1000 ml) and eluted with HPLC grade methanol (2×1000 ml). The methanolic eluent was evaporated to dryness.

3.4. Isolation

The crude HP20 resin extract of the culture filtrate was dissolved in methanol (5 ml) and combined with an equivalent mass of silica gel (flash chromatography grade; BDH; 1.6 g) and evaporated. The slurry was packed in a pre-column cartridge assembled in a Biotage™ chromatography apparatus along with a 40 mm diameter silica gel column.

The column was eluted with the following mobile phase fractions: 100% dichloromethane (100 ml), 2% methanol/dichloromethane (200 ml), 4% methanol/dichloromethane (200 ml), 6% methanol/dichloromethane (200 ml), 8% methanol/dichloromethane (200 ml) and finally 10% methanol/dichloromethane (200 ml). None of the fractions were observed to contain the desired metabolite which had correlated to a zone of inhibition in the bioautographical analysis. The column was therefore further eluted with 20% methanol/dichloromethane and a series of 30 ml volume fractions were collected. TLC analysis showed the target compound to be present in fractions 7–18. These fractions were combined, evaporated to dryness and re-dissolved in 9:1 ethyl acetate/*n*-hexane (2 ml) for further fractionation on a 10 mm diameter Biotage column, using isocratic 9:1 ethyl acetate/*n*-hexane as the mobile phase, fractionated into 7 ml fractions. The target compound was contained in fractions 5–15, these were combined, dried and reconstituted in 8% methanol/dichloromethane for further isocratic fractionation using the same solvent and a 10 mm Biotage column. Fractions of 3 ml volume were collected, TLC analysis showed the compound to be solely present in fractions 12–32. These fractions were combined and the dry weight of pure compound determined to be 17.1 mg.

3.5. Thin layer chromatography and bioautography analysis

Thin layer chromatography (TLC) separation was achieved using silica gel plates and three solvent systems (9:1 dichloromethane/methanol; 6:4 ethyl acetate/*n*-hexane and 9:1 ethyl acetate/*n*-hexane). All solvents used were HPLC grade.

Metabolites were visualised on the TLC plates by spraying with a 4% vanillin/concentrated sulphuric acid solution and heating with a hot air gun.

Bioautographic analysis was performed using *Staphylococcus aureus* (NCTC 6571) as the test organism.

S. aureus inoculum was prepared by seeding a 100 ml conical flask containing sterile nutrient broth (10 ml), the flask was shaken overnight at 200 rpm at 37 °C.

The inoculum was applied to the run TLC plates by gently dabbing with sterilised foam. The seeded plates were subsequently incubated overnight at 37 °C in a humidified chamber. The incubated plates were then sprayed with nitro-blue tetrazolium (Sigma Ltd.) in order to stain the live *S. aureus* and then re-incubated for 1 h to develop, the undeveloped areas of the plates indicating the presence of growth-inhibiting compounds.

3.6. Antibacterial assay

S. aureus strain ATCC 25923 was the generous gift of E. Udo (Kuwait University, Kuwait). *S. aureus* RN4220 containing plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein, was provided by J. Cove (Ross et al., 1989). Strain XU-212, which possesses the TetK tetracycline efflux protein, was provided by E. Udo (Gibbons and Udo, 2000). SA-1199B, which overexpresses the *norA* gene encoding the NorA MDR efflux protein was provided by G. Kaatz (Kaatz et al., 1993). All *Staphylococcus aureus* strains were cultured on nutrient agar and incubated for 24 h at 37 °C prior to MIC determination. Bacterial inocula equivalent to the 0.5 McFarland turbidity standard were prepared in normal saline and diluted to give a final inoculum density of 5×10^5 cfu/ml. The inoculum (125 μl) was added to all wells and the microtitre plate was incubated at 37 °C for 18 h. The MIC was recorded as the lowest concentration at which no bacterial growth was observed as previously described (Gibbons and Udo, 2000) (see Table 2).

3.7. 16-Deacetoxy-7β-hydroxyfusidic acid (*1*)

White powder; $[\alpha]_D^{21} - 113.64°$ (*c* 0.08, $CHCl_3$); UV (ACN) λ_{max} (log ε): 233 (3.96) nm; IR ν_{max} (thin film) cm^{-1}: 3369.62, 2924.39, 1715.97, 1696.02, 1558.27, 1436.56, 1375.42, 1255.07 1053.01, 934.01, 653.86; ^1H NMR and ^{13}C NMR ($CDCl_3$): see Table 1; HRES-MS (*m*/*z*): 475.3422 $[M+H]^+$ (calc. for $C_{29}H_{47}O_5$, 475.3418).

Table 2
MICs of **1** and standard antibiotics in μg/ml

Strain (resistance mechanism)	1	Fusidic acid	Norfloxacin	Erythromycin	Tetracycline
ATCC 25923	16	0.125	2	0.25	0.25
SA-1199B (NorA)	16	0.125	32	0.25	0.25
RN4220 (MsrA)	16	0.25	2	128	0.25
XU212 (TetK, mecA)	>64	>64	16	>256	128
EMRSA-15	16	0.125	0.5	2048	0.125
EMRSA-16 (mecA)	>64	4	128	4096	0.125

All MICs were determined in duplicate.

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