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JM47, a cyclic tetrapeptide HC-toxin analogue from a marine *Fusarium* species

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Abstract

The known metabolite, enniatin B, and a cyclic tetrapeptide, JM47, which is a new natural product, were extracted from brown rice cultures of a marine fungus, identified as a *Fusarium* species, isolated from the marine alga *Codium fragile*. NMR studies, including ^{15}N HMQC and ^{15}N HMBC, established the structure of JM47 as cyclo(Ala-Ala-Aoh-Pro), where Aoh is the amino acid, (2*S*,9*S*)-2-amino-8-oxo-9-hydroxydecanoic acid. The absolute stereochemistry of the Aoh side chain carbinol centre was determined using Mosher ester methodology. Analysis of NOESY data assisted by molecular modelling revealed an alternating L-, D-, L-, D-configuration for the tetrapeptide core. The absolute stereochemistry of the core was determined by acidic hydrolysis and chiral TLC analysis of the proline residue. JM47 belongs to the HC-toxin family of cyclic tetrapeptides which possess a 2-amino-8-oxo-9,10-epoxydecanoic acid residue in place of the Aoh unit. This is the first report of an analogue of HC-toxin from a marine *Fusarium* species.

Keywords: *Fusarium* species; *Codium fragile*; Enniatin B; JM47; HC-toxin analogue

1. Introduction

Over the past 60 years fungi have proven to be a valuable resource for the discovery of novel natural products, many of them potential targets for agrochemical and biomedical development (Turner, 1971; Turner and Aldridge, 1983; Biabani and Laatsch, 1998). HC-toxins are a group of fungal compounds which contain the unusual amino acid, 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe), and were originally isolated from *Cochliobolus carbonum* (anamorph *Helminthosporium carbonum* or *Bipolaris zeicola*) (Walton et al., 1982; Liesch et al., 1982; Kim et al., 1985; Tanis et al., 1986). The HC-toxins are host-selective toxins and act as inhibitors of histone deacetylase, a key nuclear enzyme involved in transcriptional control (Taunton et al., 1996). The antiprotozoal activity of a range of cyclic tetrapeptides related to the HC-toxins has been reported by Merck scientists (Darkin-Rattray et al., 1996).

The world's oceans represent a resource of huge dimension for natural products chemists, however, the chemistry of marine fungi has until recently been a relatively neglected area of marine natural products chemistry. In our search for new and biologically active natural products from marine organisms, we have isolated more than 700 surface associated bacterial and fungal strains. The screening of culture extracts of these isolates has led to the discovery of a number of strains exhibiting significant antibiotic activity (Burgess et al., 1999). One such marine fungal strain selected for further investigation, MOBCOF-1, produced a range of secondary metabolites when grown on a brown rice medium. The residue from the ethyl acetate extract of this fungal culture was partitioned between hexane and 90% aqueous methanol. Silica gel column chromatography of the active polar fraction led to the isolation of a cyclodepsipeptide, enniatin B (**1**), and an HC-toxin analogue, JM47 (**2**).

2. Results and discussion

The marine fungus, MOBCOF-1, was isolated from the alga *Codium fragile* subsp. *atlanticum* collected off

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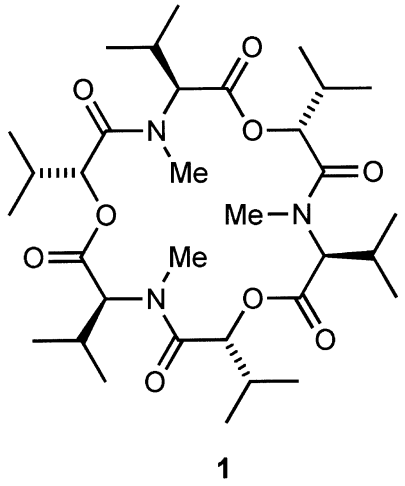
the east coast of Scotland (Dunbar). Microscopic examination of MOBCOF-1 conidia showed that this strain was a *Fusarium* species. The ethyl acetate extract of a brown rice culture of the fungus was partitioned between hexane and 90% aqueous methanol. The polar fraction was subjected to silica gel column chromatography eluting with a chloroform–methanol gradient. Further chromatography, using Sep-Pak silica gel cartridges and a hexane–acetone gradient, yielded two pure compounds (**1** and **2**, Fig. 1).

Compound **1** was identified as enniatin B by comparison of its NMR and EI mass spectral data with those in the literature (Tomoda et al., 1992). This compound has been previously reported as a metabolite of various *Fusarium* species (Audhya and Russell, 1974; Deol et al., 1978).

Compound **2** was assigned the molecular formula $C_{21}H_{34}N_4O_6$, with seven double bond equivalents, on the basis of its mass and NMR spectra. Interpretation of the NMR data (especially 2D COSY, HMQC and HMBC) suggested a structure derived from discrete amino acid units. Three amide carbonyl resonances (*r*, *s* and *t*) were identified in the ^{13}C NMR spectrum (δ 170–174), Table 1. However, the intensity of the most downfield signal (carbon *t* at δ_C 173.68) was approximately double the other two, suggesting the presence four peptidic carbonyl groups in total. Consistent with the carbonyl analysis, four amino acid α-methine groups—*m*, *n*, *o* and *p*- were identified (respectively at δ_C 47.36, 48.03, 51.86, and 57.82) and correlated through their one bond couplings in the HMQC spectrum to the corresponding ^1H NMR signals (at δ_H 4.55, 4.45, 4.76 and 4.68). The peptidic nitrogen atoms, (Table 2) were observed through ^{15}N HMQC and ^{15}N HMBC spectra; three of the nitrogens—*v* (δ_N 127.8), *w* (δ_N 123.6) and *x* (δ_N 133.5)—were secondary while the fourth and most downfield (*y* at δ_N 136.5) was tertiary. The three secondary nitrogens were correlated to their respective hydrogens at δ_H 7.20, 7.35 and 7.40 through the HMQC spectrum. Three of the four amino acids were readily identified through analysis of COSY and HMBC spectra. Two of these three were shown to be alanyl units by connection of their α-methines (*m* and *n*) to methyl groups (respectively *a* and *b*) which manifested doublet resonances in the ^1H NMR spectrum. The third amino acid possessed an α-methine (*p*) linked to a contiguous series of three methylenes (*f*, *g* and *l*) and was, therefore, shown to be a prolyl unit. A $^3J_{H,N}$ coupling was observed between one of the CH_2-*f* methylene hydrogens and the tertiary prolyl nitrogen centre (*y*) in the



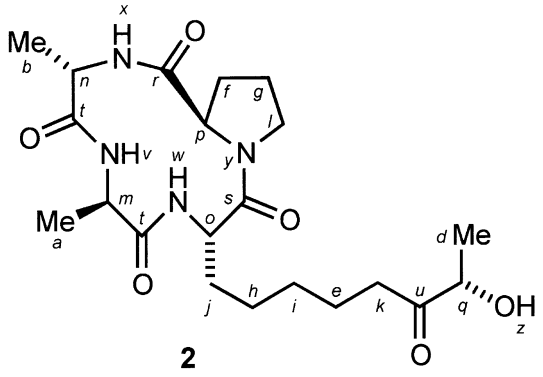
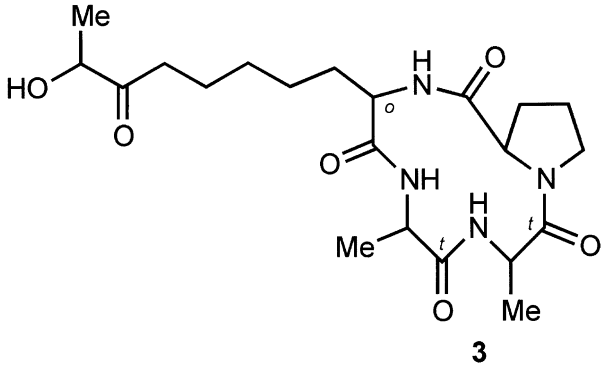


Fig. 1. Structures of enniatin B (**1**) and JM47 (**2**).



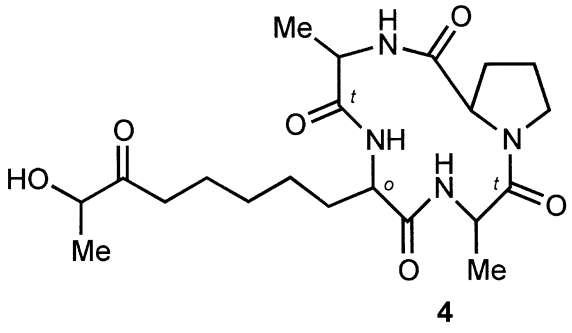


Fig. 2. Candidate structures (**3** and **4**) for the tetrapeptide core of JM47 that were eliminated by analysis of HMBC, COSY and NOESY data.

Table 1
^{1}H NMR spectral data [δ (ppm) in $CDCl_3$ (400 MHz)][a] and ^{13}C NMR spectral data [δ (ppm) in $CDCl_3$ (101 MHz)] for JM47 (**2**)

Position	^{1}H	^{13}C	HMBC	NOESY
a	1.26 *d* (6.9)	14.06	H-*m*, *v*	*m*, *v*
b	1.30 *d* (6.9)	14.65	H-*n*	*n*, *x*
d	1.36 *d* (7.1)	19.83	H-*q*, *z*	*k*, *q*
e	1.61 *m*	23.24	H-*i*, *k*	*i*, *k*
f_1	1.84 *m*	24.92	H-g_1, g_2, l_1, l_2, *p*	f_2, *p*
f_2	2.38 *m*	24.92	H-g_1, g_2, l_1, l_2, *p*	f_1, *p*
g_1	1.92 *m*	25.09	H-f_1, f_2, l_1, l_2, *p*	g_2, l_1
g_2	2.28 *m*	25.09	H-f_1, f_2, l_1, l_2, *p*	g_1, l_2
h	1.30 *m*	25.45	H-*i*, j_1, j_2, *o*	*i*, j_1, j_2
i	1.30 *m*	28.80	H-*e*, *h*, *k*	*e*, *h*
j_1	1.55 *m*	29.08	H-*i*, *h*, *o*	*h*, j_2, *o*, *w*
j_2	1.65 *m*	29.08	H-*i*, *h*, *o*	*h*, j_1, *w*
k	2.47 *m*	37.23	H-*e*, *i*	*e*, *l*
l_1	3.49 *ddd* (10.1, 7.5, 4.9)	47.08	H-f_1, f_2, g_1, g_2, *p*	g_1, l_2, *o*
l_2	3.95 *ddd* (10.1, 8.5, 4.9)	47.08	H-f_1, f_2, g_1, g_2, *p*	g_2, l_1, *o*
m	4.55 *dq* (9.8, 6.9)	47.36	H-*a*, *v*	*a*, *w*
n	4.45 *dq* (10.3, 6.8)	48.03	H-*b*, *x*	*b*, *v*
o	4.76 *dt* (10.4, 7.6)	51.86	H-*h*, j_2, *w*	*h*, j_1, l_1, l_2
p	4.68 *dd* (7.9, 2.1)	57.82	H-f_1, f_2, g_1, g_2, l_1, l_2	f_1, f_2, *x*
q	4.21 *dq* (4.5, 7.1)	72.59	H-*d*, *z*	*d*
r	–	171.37	H-f_1, f_2, *n*, *p*, *x*	–
s	–	173.19	H-j_1, *o*	–
t	–	173.68	H-*a*, *b*, *m*, *n*, *o*, *v*, *w*	–
u	–	212.36	H-*d*, *e*, *k*, *q*, *z*	–
v	7.20	–	–	*a*, *n*
w	7.35	–	–	*m*
x	7.40	–	–	*b*, *p*
z	7.35	–	–	–

[a] Coupling constants (*J*) in Hz are given in parentheses.

Table 2
^{15}N NMR spectral data [δ (ppm) in $CDCl_3$ (41 MHz)] for JM47

Position	^{15}N	HMQC	HMBC
v	127.8	H-*v*	H-*a*, *m*
w	123.6	H-*w*	H-*o*
x	133.5	H-*x*	H-*b*, *n*
y	136.5		H-f_2

^{15}N HMBC spectrum. Inspection of the remaining NMR data disclosed the presence of 5 methylene units (*e*, *h*, *i*, *j* and *k*), a strongly deshielded carbonyl centre (δ_C 212.36), a methyl group (*d*, doublet resonance at δ_H 1.36), and a carbinol-like methine centre (*q* at δ_C 72.59). The latter methine, manifest in the ^{1}H NMR spectrum as a double quartet ($J = 4.5$ and 7.1, δ_H 4.21), was found to be coupled to methyl group *d* and a hydroxyl group (*z*) in the COSY spectrum. The HMBC spectral data were consistent only with the presence of a 7-hydroxy-6-oxooctyl chain connected to the α-methine centre (*o*) of the fourth amino acid, which was therefore identified as 2-amino-8-oxo-9-hydroxydecanoic acid (Aoh).

The presence of seven double bond equivalents, together with four peptidic carbonyl resonances, suggested that the four amino acids were connected in a cyclic tetrapeptide structure. The order of connectivity was deduced by analysis of the HMBC, COSY and NOESY spectra. One of the three possible combinations, cyclo (Ala-Ala-Pro-Aoh), structure **3** (Fig. 2), was readily excluded by observation of a cross peak between the Aoh α-H (*o*) and one of the alanyl carbonyl carbons (*t*) in the HMBC spectrum. Such a cross peak could only be observed with a cyclo(Ala-Ala-Aoh-Pro) or cyclo (Ala-Aoh-Ala-Pro) combination as in structures **2** and **4** respectively. More detailed analysis of the COSY and NOESY cross peaks between the NHs and α-CHs (Table 1) permitted, with the aid of molecular modelling, unequivocal assignment of structure **2** to JM47 (Fig. 3). The NOESY data, for example the cross peak between CH-*o* and CH_2-*l*, between CH-*n* and NH-*v* and between CH_3-*b* and NH-*x*, could only be accommodated by assigning alternating configurations to the amino acids as in cyclo(L-Ala-D-Ala-L-Aoh-D-Pro) or its enantiomer. Comparison of the energy minimised conformations for the various diastereomeric forms of the cyclic tetrapeptide also suggested that structure **2**, with its alternating configurations, is the lowest energy diastereomer.

The cyclo(Ala-Ala-Aoh-Pro) structure of JM47 identified it as a member of the HC-toxin group of fungal compounds which contain D-proline. This suggested that the absolute stereochemistry for the core tetrapeptide of JM47 is that of structure **2** rather than its enantiomer,

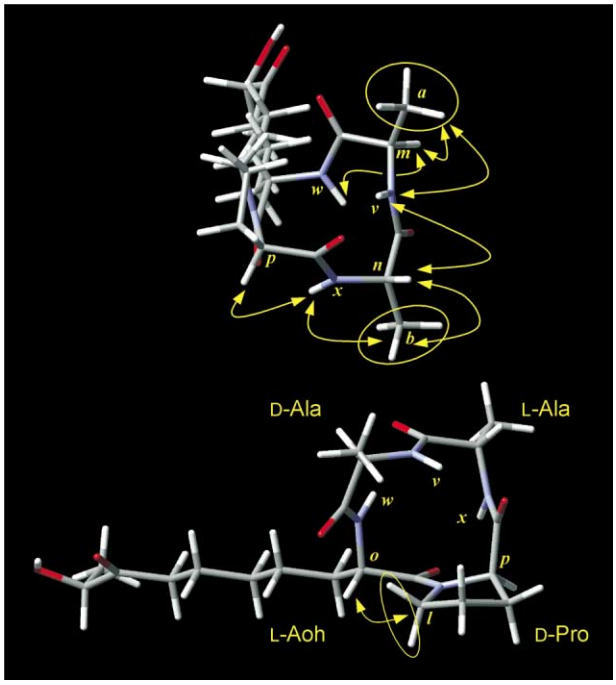


Fig. 3. Two views of the molecular mechanics energy-minimised JM47 structure showing key NOE correlations.

cyclo(D-Ala-L-Ala-D-Aoh-L-Pro). Acidic hydrolysis and chiral TLC analysis of the resulting hydrolysate using L-Pro and D-Pro reference samples confirmed the absolute stereochemical assignment given to the core structure of **2**. The side chain carbinol centre (*q*) in the Aoh residue, however, was too distant from the core to allow it to be related to the tetrapeptide stereocentres in NMR experiments. Consequently the absolute stereochemistry of this centre was determined using Mosher ester methodology (Dale and Mosher, 1973; Ohtani et al., 1991). Thus, the diastereomeric (*R*)- and (*S*)-MTPA esters of **2** (**2r** and **2s**) were prepared and the Aoh side chain resonances located with the assistance of COSY. Chemical shift differences ($\Delta\delta_{\mathbf{2s-2r}}$) for corresponding signals in the ^{1}H NMR spectra were negative for methyl-*d* and positive for methylenes-*k* and -*e* (Table 3), indicating that the carbinol centre (*q*) is *S*-configured in JM47.

Structure **2** (lacking stereochemical detail) was included in a Merck patent filed in 1997 claiming anti-protozoal activity (Meinke et al., 1997). However, this is the first report of the compound as a natural product. To the best of our knowledge, this is also the first report of an HC-toxin analogue from a marine *Fusarium* species.

Enniatin B exhibited antibiotic activity against *Staphylococcus aureus* and vancomycin resistant enterococci VRE788, with inhibition zones of 8 and 9 mm, respectively (2.5 mg/ml, 30 μl), but not against methicillin resistant *Staphylococcus aureus* MRSA 4 and *Escherichia coli*. JM47 showed no antibiotic activity against the species tested.

Table 3
Partial ^{1}H NMR spectral data [δ (ppm) in $CDCl_3$ (400 MHz)] for Mosher ester derivatives **2s** and **2r**

Position	**2s**	**2r**	$\Delta\delta_{\mathbf{2s-2r}}$
d	0.40	1.40	−1.00
q	4.84	5.05	*S*[a]
k	2.44	2.36	+0.08
e	1.65	1.55	+0.10

[a] Absolute configuration of the carbinol centre.

3. Experimental

3.1. General experimental procedures

Column chromatography was carried out on either Matrex silica 60 (Fisher) or Sep-Pak Vac 6cc (1 g) silica cartridges (Waters). Whatman F_{254} silica gel plates were used for analytical TLC. NMR spectra were recorded on Bruker AC200 and DPX400 spectrometers. 2D NMR spectra (COSY, NOESY, HMQC, HMBC and HMQC–TOCSY) were recorded on the Bruker DPX400 instrument using standard gradient-assisted pulse sequences. COSY experiments were recorded using a double-quantum filter. NOESY experiments used a 1 s mixing time and the HMQC–TOCSY experiments used an MLEV-17 mixing sequence duration of 75 ms. For ^{13}C–^{1}H HMQC experiments the echo delays were matched to $^1J_{CH}=135$ Hz, and for ^{15}N–^{1}H HMQC experiments they were matched to $^1J_{NH}=85$ Hz. HMBC experiments were carried out using a low-pass filter with delays matched to similar values as the HMQC experiments. The echo delay required for the long-range $^nJ_{CH}$ was matched to either 5 Hz or 7 Hz, while that for the long-range $^nJ_{NH}$ was matched to 2.5 Hz. EI-mass spectra were recorded using a Thermoquest Automass instrument. Molecular mechanics modelling was undertaken using the MM2 force field as implemented in Chem3D® (CambridgeSoft).

3.2. Marine fungus isolation and culture conditions

The marine fungus MOBCOF-1 was isolated from the surface of the alga *Codium fragile* collected off the east coast of Scotland (Dunbar) and was identified as a *Fusarium* species. The fungus was cultivated on a medium which consisted of sodium tartrate (0.01 g), yeast extract (0.02 g), brown rice (20 g), KH_2PO_4 (0.01 g) and sea water (40 ml).

3.3. Extraction and isolation of the fungal metabolites

Marine fungus MOBCOF-1 was cultivated on the brown rice medium (12 conical flasks, 20 g brown rice

and 40 ml sea water per flask) for 30 days at 28 °C. Each culture was extracted with ethyl acetate (200 ml) overnight. The combined extracts were filtered and evaporated to dryness. The residue was dissolved in 90% aqueous methanol (100 ml) and extracted with hexane (5×50 ml) to remove fatty acids. The aqueous methanol extract was evaporated to dryness (0.97 g) and subjected to column chromatography (silica gel, 25 g; 49/1→1/1 chloroform-methanol gradient eluant). Individual column fractions were combined into six aggregate fractions according to their TLC profiles. Further purification on a Sep-Pak Vac 6cc (1 g) silica cartridge column eluting with a gradient hexane-acetone solvent mixture (3/1→1/1) yielded a waxy crystalline solid (enniatin B, 48 mg) and a white powder (JM47, 8.2 mg).

*3.3.1. Enniatin B (**1**)*

^{1}H NMR spectral data (200 MHz, $CDCl_3$): δ 0.89 (9H, *d*, $J=7.1$ Hz), 0.95 (9H, *d*, $J=6.2$ Hz), 0.98 (9H, *d*, $J=6.2$ Hz), 1.05 (9H, *d*, $J=6.2$ Hz), 2.20–2.40 (6H, *m*), 3.12 (9H, *s*), 4.50 (3H, *d*, $J=9.6$ Hz), 5.13 (3H, *d*, $J=8.7$ Hz). ^{13}C NMR spectral data (50 MHz, $CDCl_3$): δ 18.39 (CH_3), 18.63 (CH_3), 19.34 (CH_3), 20.28 (CH_3), 27.85 (CH), 29.84 (CH), 33.04 (NCH_3), 62.99 (CH), 75.66 (CH), 169.36 (C=O), 170.23 (C=O). EIMS 70 eV, *m*/*z* (rel. int.): 639 $[M]^+$ (45), 596 (18), 556 (22), 496 (12), 456 (8), 409 (21), 296 (42), 196 (64), 169 (69).

*3.3.2. Cyclo{L-alanyl-D-alanyl-[(2S,9S)-2-amino-9-hydroxy-8-oxodecanoyl]-D-prolyl}, JM47 (**2**)*

A white powder, mp 114–116 °C, $[\alpha]_D^{21}-71.4°$ ($CHCl_3$; *c* 1.43); ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (101 MHz, $CDCl_3$) spectral data: see Table 1. EIMS 70 eV, *m*/*z* (rel. int.): 438 $[M]^+$ (6), 395 (4), 393 (4), 366 (7), 326 (12), 181 (14), 172 (74), 131 (16), 70 (100). HR–EIMS 70 eV, found: *m*/*z* 438.2486 $[M]^+$, $C_{21}H_{34}N_4O_6$ requires: 438.2478.

3.4. Hydrolysis and chiral TLC analysis of JM47

JM47 (2 mg) was suspended in 6 N HCl (2 ml) and heated at 100 °C for 12 h. The reaction mixture was cooled to room temperature and evaporated to dryness before dissolving the residue to 50% aqueous methanol (2 ml). TLC analysis was carried out using a slight modification of the CHIRALPLATE® method (Günther, 1988; Lorenz et al., 1998). Samples (2 μl) of the reaction mixture and authentic samples of both D- and L-proline (1% w/v solutions) were applied to the CHIRALPLATE® and the plate eluted with MeOH:H_2O:MeCN (50:50:200). The plate was then allowed to dry and dipped in a 0.3% solution of ninhydrin in acetone. Gentle heating using a hair dryer allowed visualisation of the proline as a yellow spot in contrast to the alanine residues which were visualised as purple spots. D- and L-proline, R_F 0.32 and 0.38 respectively, could be confidently separated and assigned using this method. The proline component of the hydrolysis mixture co-migrated with D-proline and was therefore assigned accordingly.

3.5. Preparation and purification of Mosher esters of JM47

JM47 (2 mg) in dichloromethane (0.5 ml) was sequentially treated with pyridine (0.25 ml), 4-(*N,N*-dimethylamino)pyridine (1 mg) and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (20 mg). The mixture was stirred at room temperature for 4 h, monitoring by TLC. The reaction mixture was then applied to a silica gel column (0.6×6 cm) and eluted with CH_2Cl_2 (5 ml). The eluted material was diluted with CH_2Cl_2 (5 ml) and then washed with 1% $NaHCO_3$ solution (15 ml) and H_2O (2×10 ml). The organic layer was evaporated to give the *R*-Mosher ester derivative (**2r**). The *S*-Mosher ester derivative of JM47 (**2s**) was prepared in an identical manner using *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride. Partial ^{1}H NMR (400 MHz, in $CDCl_3$) spectral data for **2s** and **2r**: see Table 3.

3.6. Antibiotic assays

Antibiotic activity was assessed using the standard paper disk assay (Bergquist and Bedford, 1978). Strains used were: *Staphylococcus aureus*, vancomycin resistant enterococci (VRE788), methicillin resistant *Staphylococcus aureus* MRSA 4 and *Escherichia coli*, which were provided by the Department of Medical Microbiology, University of Edinburgh. Paper discs (Whatman, 6 mm) were saturated with a methanol solution of the pure compounds (2.5 mg/ml, 30 μl) and placed onto nutrient agar plates inoculated with the test organism. Plates were then incubated overnight at 37 °C. Inhibition zones indicative of antimicrobial activity were measured in mm and include the diameter of the disc.

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