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Structural Reassignment and Absolute Stereochemistry of Madurastatin C1 (MBJ-0034) and the Related Aziridine Siderophores: Madurastatins A1, B1 and MBJ-0035

Andrew R. Tyler,[†] Hamed Mosaei,[‡] Stephanie Morton,[†] Paul G. Waddell,[†] Corinne Wills,[†] William McFarlane,[†] Joe Gray,[§] Michael Goodfellow,[⊥] Jeff Errington,[∇,‡] Nick Allenby,[∇] Nikolay Zenkin[‡] and Michael J. Hall[†]*

† School of Chemistry, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK.

‡ Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Baddiley-Clark Building, Richardson Road, Newcastle Upon Tyne, NE2 4AX, UK.

§ Pinnacle Laboratory, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne NE2 4AX, UK.

⊥ School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK.

∇ Demuris Limited, Newcastle Biomedicine Bio-Incubators, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK

ABSTRACT

The madurastatins are pentapeptide siderophores originally described as containing an unusual salicylate capped *N*-terminal aziridine ring. Isolation of madurastatin C1 (**1**) (also designated MBJ-0034), from *Actinomadura* sp. DEM31376 (itself isolated from a deep sea sediment), prompted structural reevaluation of the madurastatin siderophores, in line with the recent work of Thorson and Shaaban. NMR spectroscopy in combination with partial synthesis allowed confirmation of the structure of madurastatin C1 (**1**), as containing an *N*-terminal 2-(2-hydroxyphenyl)-oxazoline in place of the originally postulated aziridine, whilst absolute stereochemistry was determined via Harada's advanced Marfey's method. Therefore this work further supports Thorson and Shaaban's proposed structural revision of the madurastatin class of siderophores (madurastatins A1 (**2**), B1 (**3**), C1 (**1**) and MBJ-0036 (**4**)) as *N*-terminal 2-(2-hydroxyphenyl)-oxazolines.

Siderophores are key components of the bacterial endogenous secondary metabolome, facilitating intracellular uptake of Fe^{3+} and other essential metals from the surrounding environment.[1] Pathogenic bacteria also employ siderophores to sequester metals from the host organism, thus playing an important role in virulence.[2] The genus *Actinomadura*,[3] from the family *Thermomonosporaceae*, contains both environmental (predominately soil dwelling) and opportunistically pathogenic species and are a source of a number of bioactive secondary metabolites[4] including the madurastatin[5] and maduraferrin[6] siderophores.

As part of a program to identify new natural products from actinobacteria,[7] we have investigated the secondary metabolite production of a marine isolate *Actinomadura* sp. DEM31376. Along with the known cyclic heptapeptide RNAP inhibitor GE23077,[8] we isolated the siderophore madurastatin C1 (**1**)[5b] (also designated MBJ-0034).[5c] Spectroscopic analysis of isolated **1** was however not consistent with the originally reported structure. Recent work by Thorson and Shaaban proposed a structural revision for the madurastatins as containing a 2-(2-hydroxyphenyl)-oxazoline moiety, based on an analysis of the NMR data pertaining to both related bacterial siderophores and synthetic intermediates.[9] Thus as part of our investigations we compared isolated madurastatin C1 (**1**) to both aziridine and 2-(2-hydroxyphenyl)-oxazoline containing synthetic analogues, as well as examining the absolute stereochemistry of madurastatin C1 (**1**) via a Marfey's analysis.

RESULTS AND DISCUSSION

Madurastatin C1 (**1**) was first isolated from the fermentation broth of *Actinomadura sp.* DSMZ 13491 by Sosio *et al.* in 2012,[5b] and again by Shin-ya *et al.* in 2014 (as MBJ-0034 along with the related siderophore MBJ-0035 (**4**)),[5c] and was originally assigned as a linear pentapeptide

containing a salicylate capped *N*-terminal aziridine ring by spectroscopic analysis and analogy to the previously reported madurastatins A1 (**2**) and B1 (**3**) (Figure 1).

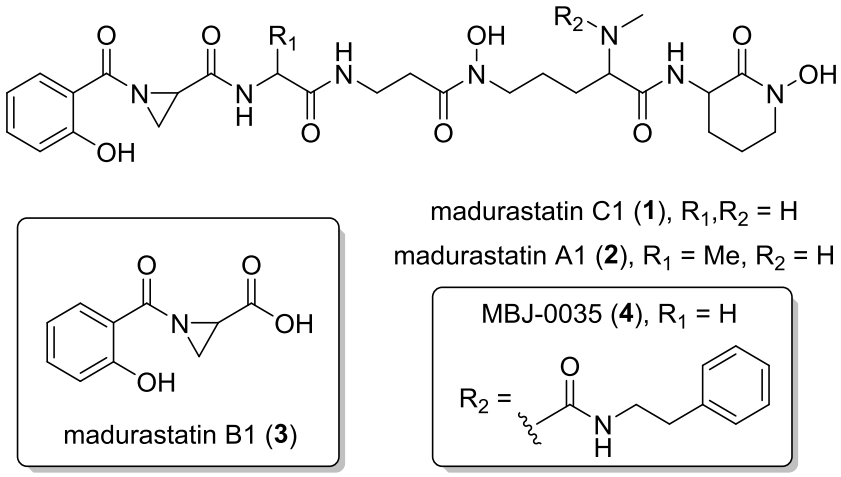


Figure 1. Previously proposed structures of the "aziridine" containing madurastatin siderophores

In our hands, madurastatin C1 (**1**) was reisolated from *Actinomadura* sp. DEM31376, a strain originally isolated from deep sea marine sediment (Canary Basin, Atlantic Ocean).[10] Based on 16S rRNA analysis, strain DEM31376 was recovered in the genus *Actinomadura*, forming a subgroup with the type strains of *A. mexicana*, *A. citrea* and *A. scrupli*. Although closely related to both *A. mexicana* and *A. citrea* (99.4% 16S rRNA gene similarity) taxonomic analysis suggests that strain DEM31376 may form the nucleus of a novel species within the genus *Actinomadura* (Figure 2).[11]

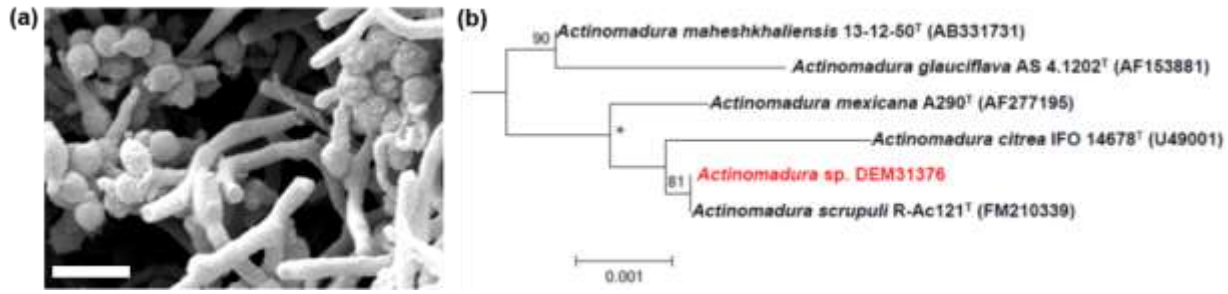


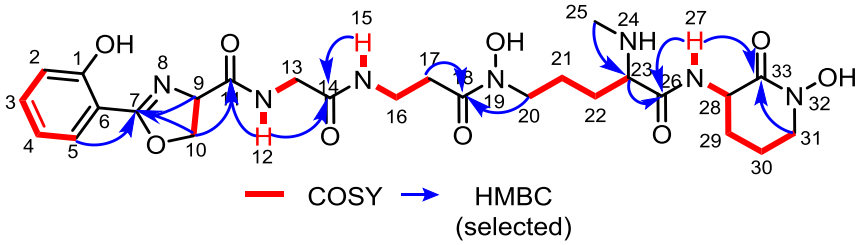
Figure 2. (a) Scanning electron micrograph of strain DEM31376 showing spiral chains of warty ornamented spores following growth on ISP3 agar, bar indicates 2 μm. (b) Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain DEM31376 and closely related type strains of *Actinomadura* species. Asterisks indicate branches also recovered using maximum likelihood and maximum parsimony tree making methods. Numbers at nodes indicate bootstrap values based on neighbor joining analysis of 1000 resampled datasets.

Strain DEM31376 was cultivated in ISP2 media in a 20 L bioreactor. Absorption of the fermentation broth onto Amberlite® XAD16N beads (elution MeOH), solid phase capture on C_8 SPE cartridges (elution with 50-100% MeOH), and finally reverse phase column chromatography (gradient elution, H_2O to MeOH, Biotage® Isolera™ SNAP C_{18}) provided 238 mg of **1**.[12] ESI-HRMS showed an $[M+H]^+$ ion at 592.2732 m/z, confirming molecular formula ($C_{26}H_{37}O_9N_7$). MS/MS gave fragments at 462, 434, 403, 349 and 318 m/z in line with previous analysis,[5b] whilst HPLC-ESI-MS showed the presence of a related molecule at 610 m/z, formed via hydrolysis of the parent ion. ^{13}C NMR revealed 26 carbon signals, six corresponding to an *ortho*-disubstituted benzene and six to carbonyl-like carbons, three of which were shown to be primary amides though HMBC correlations with exchangeable protons at 8.52, 7.93 and 8.11 ppm. Thus, the remaining two degrees of unsaturation are the due to the presence of a further two ring systems. ^1H NMR, COSY and ^{15}N-^1H HSQC revealed five amino acid spin systems, namely: glycine, β-alanine, a modified serine and two modified ornithines. Indirect measurement of the natural abundance ^{15}N spectra by ^1H-^{15}N HMBC showed the presence of seven nitrogen atoms in four different chemical shift ranges indicative of three amides, two hydroxamic acids, one amine and an unusual imine-like nitrogen. The role of **1** as a hexadentate siderophore was

confirmed through chelation with both $Fe(acac)_3$ and $Ga(acac)_3$, HPLC-ESI-MS showing signals at 645.2 and 658.2 m/z corresponding to the protonated 1:1 complexes of 1 with Fe^{3+} and Ga^{3+} respectively. Interestingly chelation with $Fe(acac)_3$ gave rise to an additional compound (663.2 m/z) demonstrating that hydrolysed **1** is also a competent ligand for Fe^{3+}. Furthermore **1** showed growth reduction against *Bacillus subtilis* (disk diffusion assay) presumably through an inhibition of Fe^{3+}uptake.

The NMR data of **1** did however highlight a number of discrepancies with the originally proposed structure. In particular the ^{13}C NMR shifts for the α- and β-carbons of the serine residue are observed at 67 and 69 ppm, highly atypical of an aryloyl substituted aziridine ring (shift range 25-45 ppm).[13] Thorson and Shaaban recently proposed that the madurastatin siderophores contain a *N*-terminal 2-(2-hydroxyphenyl)-oxazoline ring,[9] a bidentate coordination moiety present in a number of other mixed ligand siderophores (e.g. acinetobactin,[14] the amamistatins,[15] the amychelins,[16] brasilibactin A,[17] gobichelin A and B,[18] nocardichelins A and B,[19] transvalencin Z[20] and the spoxazomicins A-C[21]), in which the corresponding ^{13}C shifts typically occur from 65 to 72 ppm (Table 1).

Table 1. Revised structure and NMR data for madurastatin C1 (DMSO-*d*6, 298 K, 700 MHz)



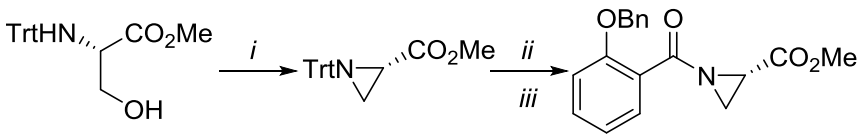
position	δ_C, type	δ_H (*J* in Hz)	δ_N[c]
1	159.1, C	-	-
2	116.6, CH	7.01, dd (8.3, 1.1)	-

3	134.1, CH	7.47, ddd (8.7, 7.3, 1.8)	-
4	119.1, CH	6.95, td (7.5, 1.1)	-
5	128.1, CH	7.65, dd (7.8, 1.8)	-
6	109.9, C	-	-
7	165.9, C	-	-
8	-	-	208.2
9	67.4, CH	5.01, dd (10.4, 7.7)	-
10	69.4, CH_2	4.65, dd (10.5, 8.4)	-
		4.52, t (8.0)	
11	170.2, C	-	-
12	-	8.52, t (5.9)	109.2
13	42.2, CH_2	3.75, dd (16.5, 6.1)	-
		3.67, dd (16.5, 5.7)	
14	168.4, C	-	-
15	-	7.93, t (5.8 Hz)	112.5
16	34.6, CH_2	3.28 – 3.22, m	-
17	32.0, CH_2	2.53 – 2.51, m	-
18	170.9, C	-	-
19	-	-	175.9
20	47.0, CH_2	3.51 – 3.42, m[b]	-
21	22.8, CH_2	1.63 – 1.53, m	-
22	30.2, CH	1.50 – 1.45, m	-
		1.43 – 1.38, m	
23	63.7, CH	2.87, t (6.6)	-
24	-	-	29.1
25	34.2, CH_3	2.21, s	-
26	173.5, C	-	-

27	-	8.11, d (8.3)	118.8
28	49.4, CH	4.32, ddd (10.9, 8.2, 5.3)	-
29	27.8, CH_2	1.96 – 1.82, m[b]	-
		1.67 qd (12.5, 4.3)	
30	20.4, CH_2	1.96 – 1.82, m[b]	-
31	51.2, CH_2	3.51 – 3.42, m[b]	-
32	-	-	171.8
33	165.0, C	-	-

[a]HMBC performed at 10 Hz, [b]signals overlapping, [c]^{15}N shifts measured indirectly by ^{1}H-^{15}N HMBC performed at both 8 and 12 Hz, referenced against liq. NH_3

To test this hypothesis, we synthesized the salicylate containing fragment of **1** as both an aziridine **5** and as an oxazoline **6** for comparison. *N*-Trityl-L-serine methyl ester **7** was activated with mesyl chloride followed by base catalysed intramolecular ring closure to give methyl (*S*)-1-tritylaziridine-2-carboxylate **8**, confirmed by single crystal X-ray analysis.[22] Trityl deprotection with TFA/Et_3SiH in DCM[23] followed by *in situ* reaction with 2-(benzyloxy)benzoyl chloride gave the desired aziridine containing fragment **5** (Scheme 1).

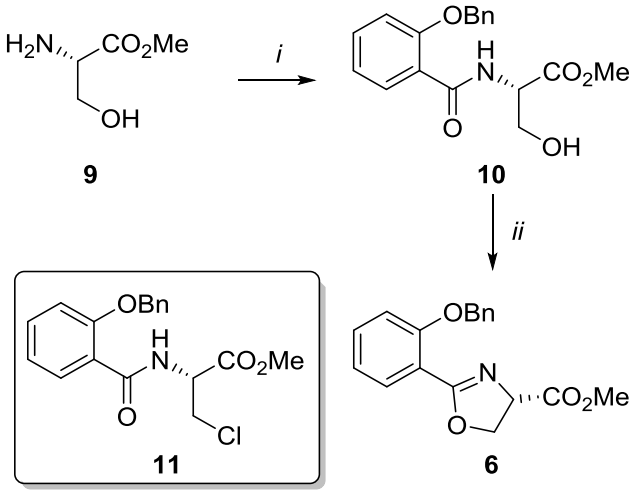


7 **8** **5**

Scheme 1. (i) MsCl, Et_3N, DCM, 0 °C to RT, 18 h, then Et_3N, THF, reflux, 16 h, 70%. (ii) Et_3SiH, Et_3N, DCM, 0 °C to RT, 5 h. (iii) 2-(benzyloxy)benzoyl chloride, Et_3N, -78 °C to RT, 48 h, 35% over 2 steps.

Synthesis of the oxazoline containing fragment **6** involved reaction of L-serine methyl ester **9** with 2-(benzyloxy)benzoyl chloride to give amide **10**. Attempted oxazoline formation with thionyl chloride gave only low yields of **6** alongside an unwanted chlorinated derivative **11**.[24]

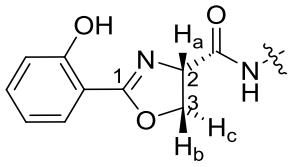
Treatment of **10** with diethylaminosulphur trifluoride (DAST) provided the desired oxazoline **6** in 79% yield.[25] Confirmation of the structure of **6** was given by single crystal X-ray analysis,[22] including comparison to the closely related X-ray structure of spoxazomicin C (Scheme 2).[21a]



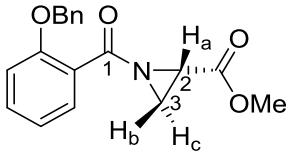
Scheme 2. (i) 2-(benzyloxy)benzoyl chloride, Et_3N, -78 °C to RT, 18 h, 95%. (ii) DCM, DAST, -78 °C to RT, 2 h, 79%.

Comparison of the ^1H and ^{13}C NMR spectra of **1**, **5** and **6** confirmed the presence of an oxazoline in the natural product. Both the ^{13}C NMR shifts of the ring carbons (C-2 and C-3) and carbonyl-like carbon (C-1) and the ^1H NMR shifts for H_a, H_b and H_c of **1** align well with those of synthetic oxazoline **6**. In addition, coupling constant analysis of **6** shows J_{cis}. J_{trans} and J_{gem} of 10.5, 7.6 and 8.8 Hz respectively,[26] corresponding closely to those observed in **1** (note that in **5**, J_{cis} = 5.3, J_{trans} = 3.1 and J_{gem} = 1.3 Hz, demonstrating the small geminal coupling characteristic of an aziridine).[27] Analysis of the literature pertaining to the madurastatin siderophores A1 and MBJ-0036 reveals that the ^1H and ^{13}C NMR data of these compounds also closely match those of oxazoline **6**, suggesting that in agreement with Thorson and Shaaban,[9] these structures should also be similarly revised as containing an *N*-terminal 2-(2-hydroxyphenyl)-oxazoline (Table 2).

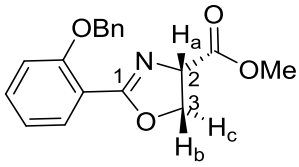
Table 2. ^1H and ^{13}C NMR comparison of **1** with synthetic compounds **5** and **6**.



1



5



6

position	**1**	**5**	**6**
1, δ_C	165.9	178.1	168.1
2, δ_C	67.4	33.1	69.3
3, δ_C	69.4	37.5	70.8
H_a, δ_H (*J* in Hz)	5.05 dd (10.7, 8.1)	3.23 dd (5.3, 3.1)	4.97 dd (10.5, 7.6)
H_b, δ_H (*J* in Hz)	4.69 dd (10.7, 8.5)	2.63 dd (5.3, 1.3)	4.62 dd (10.5, 8.8)
H_c, δ_H (*J* in Hz)	4.61 t (8.3)[b]	2.62 dd (3.1, 1.3)	4.67 dd (8.8, 7.6)

[a](700 MHz, CD_3OD), [b]signal is an apparent triplet due to similar values of J_{trans} and J_{gem}

Finally Harada's advanced Marfey's method was employed to determine the absolute stereochemistry of madurastatin C1 (**1**).[28] Hydrolysis of **1** with concentrated HI[29] and derivatization with *N*α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide was followed by analysis by HPLC-ESI-MS. Comparison to pre-prepared amino acid standards revealed the presence of D-serine, L-*N*-methyl-ornithine and L-ornithine allowing absolute stereochemical determination of all three stereocentres (Figure 3).

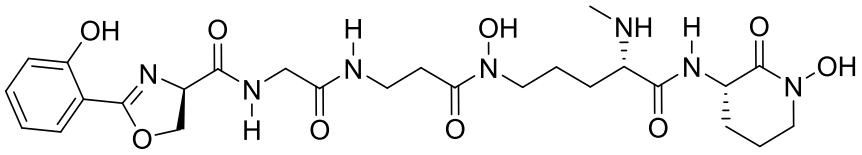


Figure 3. Revised structure of madurastatin C1 (**1**) including absolute stereochemistry.

In conclusion, we have confirmed Thorson and Shaaban's revised structure of madurastatin C1 (**1**) as an oxazoline containing mixed ligand *N*-terminal 2-(2-hydroxyphenyl)-oxazoline hexadentate siderophore and have determined its absolute stereochemistry. Based on NMR analysis, similar structural revisions should also be applied across the madurastatin family of natural products including madurastatin A1, B1 and MBJ-0035.[5,9]

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXX.

Characterization details for DEM31376, isolation procedures and analysis (including 1D and 2D NMR spectra) for madurastatin C1 (**1**), experimental details for the synthesis and characterization of compounds **5**, **6**, **8** and **10**, crystal data for **6**, **8** and **10** (PDF).

AUTHOR INFORMATION

Corresponding Author

* Tel (M. J. Hall): +44 (0) 191 208 7321. Fax: +44 (0) 191 208 6929. Email: michael.hall@newcastle.ac.uk

ORCID

Michael J. Hall: 0000-0001-6475-9161; Jeff Errington: 0000-0002-6977-9388;

Notes

The authors declare no competing financial interest

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11) The 16s rRNA sequence for *Actinomadura* sp. DEM31376 was deposited with the NCBI, GenBank accession number KY512569.

12) Production of madurastatin C1 from *Actinomadura* sp. DEM31376 was estimated at 19 mg/L (bioreactor, ISP2 media), in comparison to the reported 65 mg/L from *Actinomadura* sp. DSMZ 13491 (shake flask, AFT media) [ref 5b] and 32 mg/L from *Streptosporangium* sp. 32552 (shake flasks, custom media) [ref 5c].

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* Corresponding author. Telephone: +44 (0) 191 208 7321. Fax: +44 (0) 191 208 6929. E-mail: michael.hall@newcastle.ac.uk

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