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NOTE

Characterization of madurastatin C1, a novel siderophore from *Actinomadura* sp.

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Actinomycetes are known for their large genetic potential to produce several distinct classes of secondary metabolites.[1] While cultivating *Actinomadura sp.* DSMZ 13491, the producer of the cyclic heptapeptide GE23077,[2] we observed it produced an antibacterial activity. Despite being a potent and selective inhibitor of bacterial RNA polymerase, GE23077 is devoid of activity against bacterial cells, except for marginal activity against some *Moraxella* spp.[2] Here we report on the isolation, structure elucidation and properties of madurastatin C1 (**1**), the antimicrobial compound produced by this actinomycete.

A 10-ml seed culture of *Actinomadura* sp. DSMZ 13491, prepared by inoculating a frozen cell stock into 30 ml of AFT medium (2% glucose, 0.2% yeast extract, 0.8% soybean meal, 0.5% tryptone, 0.1% NaCl, 0.4% $CaCO_3$, adjusted to pH 7.2 with 0.1 N NaOH before sterilization) and incubating for 72–96 h, was transferred into 200 ml of fresh AFT medium. The production of antibacterial activity was monitored over time by depositing 10 µl of a microbial extract, prepared as described by Pozzi *et al.*,[3] onto Muller Hinton Agar (Difco Laboratories, Detroit, MI, USA) plates, previously inoculated with 10^5 cfu ml^{-1} *Staphylococcus aureus* ATCC 6538P or *Micrococcus luteus* ATCC 10240. An inhibition halo was clearly observed on *M. luteus* plates after 24 h of cultivation, whereas only a weak activity was visible on the *S. aureus* plates. An HPLC analysis of the culture extract is shown in Figure 1a. A major peak was observed at 2.6 min with a m/z of 592 $[M+H]^+$, whereas the two major congeners of GE23077, with m/z of 804 and 806, were observed at 1.8 min, as expected.[2] Minor peaks were also observed at 2.1 min, with m/z of 610 and 645. The 2.6-min metabolite was purified by medium pressure chromatography on a reverse-phase C18 RediSep RF column, using a CombiFlash RF Teledyne Isco Medium Pressure Chromatography System (Teledyne ISCO, Lincoln, NE, USA). The column was previously conditioned with a mixture of phase A (50 mM $HCOONH_4$), phase B (CH_3CN) 95:5 (v/v), and eluted with a 15-min linear gradient from 5 to 95% phase B. The active fractions were collected, concentrated under vacuum and lyophilized, yielding 13 mg of purified **1** (estimated at least 95% pure by NMR).

NMR and MS analyses of **1** indicated high similarity with the siderophores named madurastatins.[4] ^{1}H- and ^{13}C-NMR data (Table 1) indicated the presence of an *ortho* disubstituted benzene, as confirmed by bidimensional experiments showing four aromatic protons with a TOCSY correlation. In particular, a doublet at 7.61 p.p.m. had a COSY correlation with a pseudo-triplet at 6.82 p.p.m., and a doublet at 6.89 p.p.m. correlated with a pseudo-triplet at 7.34 p.p.m., whereas the pseudo-triplet signals intercorrelated. HMBC experiments demonstrated the disubstituted benzene to be a salicylic acid residue. Five aliphatic spin systems were also observed by using homo- and hetero-nuclear 2D-NMR experiments. COSY and TOCSY correlations evidenced the presence of an aziridine group and of a six-membered lactam at the C-terminus. A strong HMBC correlation between the methyl group at 2.54 p.p.m. and the α-carbon at 61.3 p.p.m. confirmed the presence of an *N*-methyl group. NMR data demonstrated that **1** is a pentapeptide carrying a salicylate at the *N*-terminus. Thus, **1** is highly related to madurastatin A1, except for the lack of the C-16 methyl, which probably originates from incorporation of glycine instead of alanine. This is confirmed by the occurrence of a signal at 3.82 p.p.m., integrating two protons, corresponding to the CH_2 at position 16. The structure of **1** as established by NMR is also supported by MS^n analysis. Its fragmentation pathway, as described in Figure 1b, showed a typical peptide fragmentation, identical to that reported for madurastatin A1,[4] except for the difference at C-16, whereas **1** produced fragments of m/z 219 and 403, ions of m/z 233 and 417 were reported for madurastatin A1.[4]

As described for madurastatin A1,[4] a characteristic bathochromic shift (from 254 and 310 nm to 280 and 445 nm) was observed when 1 mg was added to 1 ml 0.6 M $FeCl_3$ (data not shown), confirming the high chelating affinity for ferric ions.

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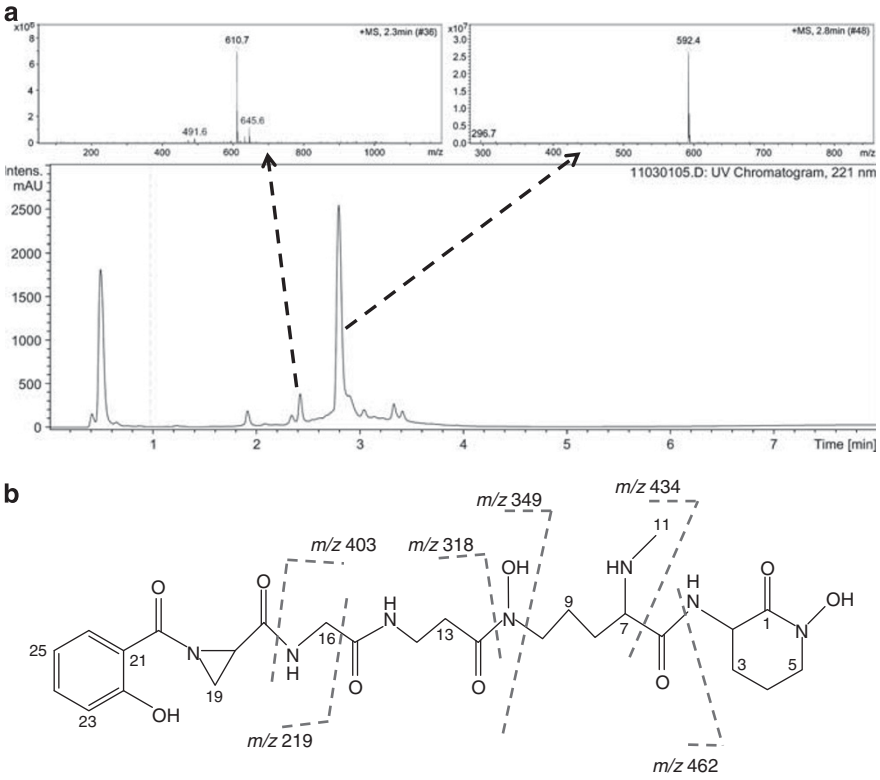

Figure 1 (**a**) HPLC profile of the methanol extract from *Actinomadura* sp. DSMZ 13491. Extraction from liquid culture was performed as described.[3] (**b**) Assignment of the fragment ions produced by **1**. MS^n spectra were measured using a Bruker Esquire3000 plus ion trap mass spectrometer equipped with an ESI source operating in the positive-ion mode. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 ^{1}H- and ^{13}C-NMR chemical shifts of 1 measured at 400 MHz in CD_3OD

Position	δ_H, *J (Hz)*	δ_C
1		164.9
2	4.43 m	50.1
3	1.72–1.76 m	27.0
4	1.94 m	20.3
5	3.54 m	51.2
6		173.6
7	3.66 m	61.3
8	1.71–1.78 m	21.5
9	1.55 m	22.8
10	3.52–3.63 m	46.7
11	2.54 s	30.7
12		170.9
13	2.64 m	31.7
14	3.39 m	34.8
15		170.8
16	3.86 d (16)–3.78 d (16)	42.1
17		169.2
18	4.97 dd (9.83, 8.38)	67.9
19	4.53 t (8.38)–4.60 t (9.83)	69.3
20		167.0
21		109.7
22		159.5
23	6.89 dd (8.3, 0.6)	116.2
24	7.34 dt (8.2, 1.1)	133.7
25	6.82 dt (7.4, 0.5)	118.7
26	7.61 dd (7.7, 1.1)	128.0

By analogy with madurastatin A1,[4] we can infer that m/z 610 and 645 present in the 2.1-min peak (Figure 1a) correspond to **1** hydrolyzed at the aziridine ring (position C-19) and to the **1**-Fe^{+3} complex, respectively. Consistently, the compound at m/z 610 yields an MS fragment at 480 amu, instead of 462 amu as seen with **1** (data not shown). Moreover, the conversion of the m/z 592 to 645 was observed upon addition of **1** to the $FeCl_3$ aqueous solution. Although inhibition halos of 25 and 5 mm were observed with *M. luteus* and *S. aureus*, respectively, when spotting 50 µg of **1** onto Muller Hinton Agar plates, neither **1**-hydrolyzed nor **1**-Fe^{3+} complex showed antimicrobial activity when tested with *M. luteus*, confirming the important role of the aziridine ring for the biological activity.

In conclusion, *Actinomadura* sp. DSMZ 13491 can produce a second bioactive metabolite in addition to GE23077. Earlier experiments had indeed shown that the genome of this strain encodes at least 12 nonribosomal peptide modules.[5] Although we do not know the relatedness between the *Actinomadura* strain described by Harada *et al.*[4] and ours, it is remarkable that a sputum- and a soil-derived strain produce very similar compounds without the need for iron limited media, as seen instead with other actinomycetes.[6–9]

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1 Baltz, R. H. Antimicrobials from actinomycetes: back to the future. *Am. Soc. Microbiol.* **2,** 125–131 (2007).
2 Ciciliato, I. *et al.* Antibiotics GE23077, novel inhibitors of bacterial RNA polymerase. I. Taxonomy, isolation and characterization. *J. Antibiot.* **57,** 210–217 (2004).
3 Pozzi, R. *et al.* The genus *Actinoallomurus* and some of its metabolites. *J. Antibiot.* **64,** 133–139 (2010).
4 Harada, K. *et al.* Isolation and structural characterization of siderophores, madurastatins, produced by a pathogenic *Actinomadura madurae*. *J. Antibiot.* **57,** 125–135 (2004).
5 Sosio, M., Bossi, E., Bianchi, A. & Donadio, S. Multiple peptide synthetase gene clusters in actinomycetes. *Mol. Gen. Genet.* **264,** 213–221 (2000).
6 Imbert, M., BGchet, M. & Blondeau, R. Comparison of the main siderophores produced by some species of Streptomyces. *Curr. Microbiol.* **31,** 129–133 (1995).
7 Neilands, J. B. Siderophores: structure and function of microbial iron transport compounds. *Biol. Chem.* **270,** 26723–26726 (1995).
8 Marcus Miethke, M. & Marahiel, M. A. Siderophore-based iron acquisition and pathogen control. *Microbiol. Mol. Biol. Rev.* **71,** 413–451 (2007).
9 Matsuo, Y. *et al.* Streptobactin, a tricatechol-type siderophore from marine-derived *Streptomyces* sp. YM5-799. *J. Nat. Prod.* **74,** 2371–2376 (2011).