Structure elucidation of a novel family of mycolactone toxins from the frog pathogen *Mycobacterium sp.* MU128FXT by mass spectrometry†

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Structures are proposed, based on LC-ion trap MS^n analysis and high-resolution FTICR MS/MS analysis, for a novel family of mycolactone toxins isolated from the frog pathogen MU128FXT and differing from those produced by the human pathogen *M. ulcerans* MUAgy99 in having an altered polyketide side chain.

Mycolactones are cytotoxic polyketide toxins produced by the human pathogen *Mycobacterium ulcerans*, the causative agent of Buruli ulcer.[1] Mycolactones have been shown to be immunomodulatory and to induce apoptosis[1,2] and they are the only example of a polyketide-derived virulence factor in a human pathogen.[3] The structures of mycolactones A and B have been determined[1,4] and shown to be, respectively, *Z*- and *E*- isomers of a 12-membered macrocyclic polyketide to which a second highly unsaturated polyketide side chain is appended *via* an ester linkage (Fig. 1). The complete structure and its absolute configuration have been confirmed by chemical synthesis.[5,6] Further work has revealed the existence, in culture extracts of a typical strain of *M. ulcerans*, of small amounts of other mycolactones, which differ from mycolactones A and B only in the side chain,[7–9] and whose structures very largely reflect the aberrant operation of a specific cytochrome P450 hydroxylase required for mycolactone biosynthesis.[3,9,10] Recent structural analysis on mycolactones from a clinical isolate of *Mycobacterium ulcerans* MU98912 (Chinese strain) revealed additional toxin heterogeneity in the side chain which could be traced to specific changes in the relevant part of the modular polyketide synthase.[11]

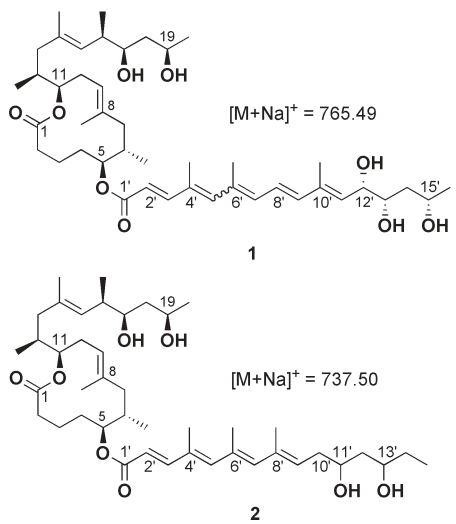

Fig. 1 The structures of mycolactone A (Z-$\Delta^{4',5'}$) and B (E-$\Delta^{4',5'}$) from the African strain MUAgy99 (**1**) and the proposed structure of a novel mycolactone from MU128FXT (**2**). Our analysis does not distinguish between different possible geometric (*E*/*Z*) isomers of (**2**).

A new *M. ulcerans*-like mycobacterium that is pathogenic for *Xenopus* frogs has been recently described as producing a mycolactone-like toxin.[12] Due to the very limited amount of sample available (a few micrograms of toxin obtained from 100 mg wet weight of cells), we have used LC-sequential mass spectrometry (LC-MS^n) performed on an ion trap mass spectrometer[9] (LCQ) to analyse the toxins produced. To characterise the detailed structural changes, MS/MS experiments on a high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometer were also carried out. This analysis allows us to propose here the structures of the novel mycolactones produced by the frog pathogen MU128FXT.‡

Preliminary LC-MS analysis (see ESI†) of a cell extract (see ESI†) showed that the typical *M. ulcerans* mycolactones, with characteristic $[M + Na]^+$ ions at *m*/*z* 765, 763, 749, and 747, were not produced by MU128FXT. Rather, two new molecular species at *m*/*z* 737 and 735 were found (Fig. 2). The major component is at *m*/*z* 737, and the component at *m*/*z* 735 is less than 10% of the total (assuming they have comparable ionization efficiency). When LC-MS/MS analyses were performed on these two ions, they showed fragmentation patterns highly similar to that of normal mycolactone A/B (see Fig. 3 and ESI†) with the characteristic ions A and B of mycolactone corresponding to the core lactone and to the polyketide side chain, respectively.[9] Evidently the compounds produced by MU128FXT are new mycolactone analogues. As in all mycolactones analysed so far, structural variations are restricted to the polyketide side chain. Multiple peaks with the same *m*/*z* were observed in the LC-MS trace (Fig. 2). LC-MS/MS showed that the MS/MS spectra of these peaks were identical, suggesting that they might be *Z*- or *E*- isomers as previously reported for mycolactone A/B.[4]

To obtain further information about the structural variations of the side chain, accurate-mass analyses (see ESI†) and deuterium exchange experiments (see ESI†) were performed on these newly-identified mycolactones. The results (see ESI†, Table 1), when compared to those from the classic mycolactones from

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† Electronic supplementary information (ESI) available: Experimental procedures and additional MS data. See http://dx.doi.org/10.1039/b506835e

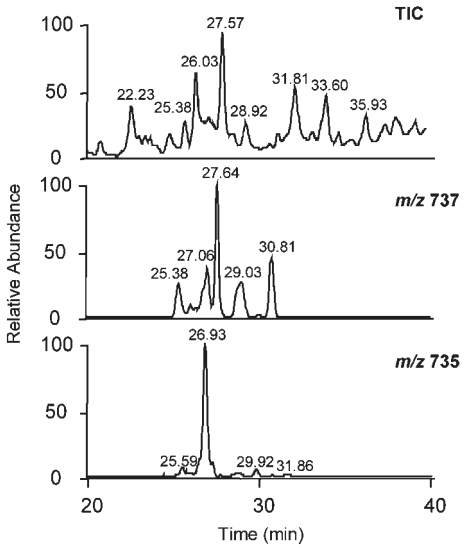

Fig. 2 LC-MS analysis of the lipid extract from MU128FXT: (a) total ion current; (b) ion trace for *m/z* 737; and (c) ion trace for *m/z* 735.

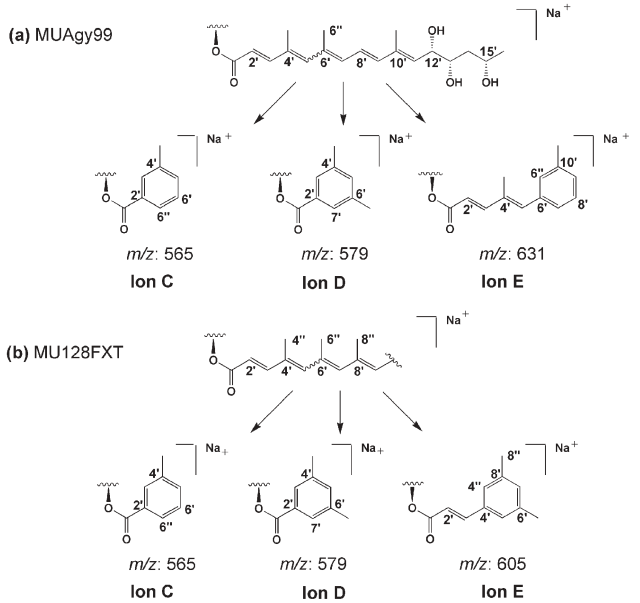

Fig. 4 The proposed structures of fragment ions C, D and E from the African strain MUAgy99 and of the corresponding fragment ions from the MU128FXT.

Mycobacterium ulcerans MUAgy99 (African strain) clearly showed that mycolactones from MU128FXT have one oxygen and one carbon atom less than their counterparts from the MUAgy99. The new mycolactones have one exchangeable proton less and one degree of unsaturation less compared to their counterparts, indicating one hydroxy group less and one double bond less in the side chain.

Detailed comparisons were carried out between the MS/MS spectrum of *m/z* 765 from MUAgy99 and the MS/MS spectrum of *m/z* 737 from MU128FXT (Fig. 3). We have previously assigned the ions C, D and E, based on the deuterated MS/MS analysis, as structures arising from the conjugated double bonds part of the side chain.[9,11] High-resolution MS/MS analysis obtained using FTICR (see ESI†, Table 2) further confirmed their identities (Fig. 4). Ion C at *m/z* 565 and ion D at *m/z* 579 are both present in the MS/MS of 765 and MS/MS of 737, suggesting that the structure of the side chain from C2′ to C7′ is conserved between both strains. However, ion E at *m/z* 631 in the MS/MS of 765 shifts to *m/z* 605 in the MS/MS of 737, 26 Da more than fragment ion D (*m/z* 579) and 26 Da less than fragment ion E (*m/z* 631) of the MS/MS 765. Therefore, the fragment ion at *m/z* 605 is the result of deletion of a CH=CH moiety between C8′ and C9′ of mycolactone-765. This result agrees with the unsaturation suggested from the molecular formula. In addition, the three consecutive methyl substitutions at C4′, C6′ and C8′ of mycolactone-737 match the formation of the significant fragment ion at *m/z* 617, due to the loss of the C4′ to C9′ unit, as a 1,3,5-trimethyl benzene neutral molecule from the side chain, as confirmed by both deuterated MS/MS and high-resolution MS/MS analysis (see ESI†, Table 2). In comparison, in the MS/MS spectrum of mycolactone-765, the corresponding fragment ion is *m/z* 659, which results from the loss of a 1,3-di-methyl benzene molecule arising from the methyl substitution pattern of the conjugated double bonds. Therefore, the structure of the part of the side chain of mycolactone-737 housing the conjugated double bonds is

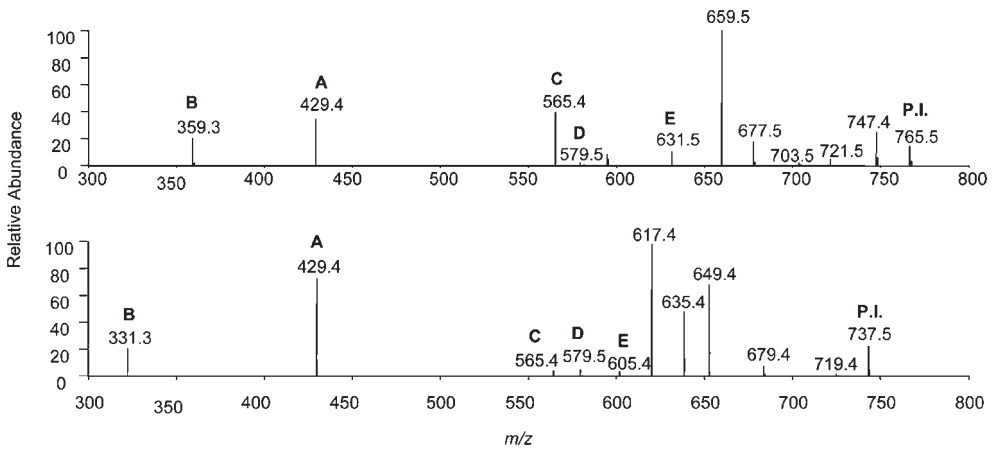

Fig. 3 The MS/MS spectra of mycolactone precursor ions at *m/z* 765 (MUAgy99) and at *m/z* 737 (MU128FXT). (P.I.: precursor ion.)

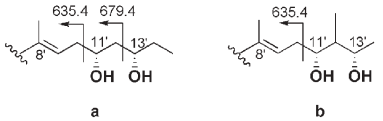

Fig. 5 Two possible structures of the distal (hydroxy) end of the side chain of mycolactone-737 from MU128FXT. Both structures can generate a fragment ion at *m*/*z* 635.4, but only structure **a** can produce a fragment ion at *m*/*z* 679.4.

established as shorter than its counterpart in MUAgy99 as the result of an apparent CH=CH moiety (between C8′ and C9′) deletion in the side chain of mycolactone-765.

Having assigned the double bond part of the side chain, the structure around the distal hydroxy end remained to be established. From the deuterium exchange experiment, it was clear that the side chain of mycolactone-737 bears two hydroxy groups. No oxidative cleavage was observed on mycolactone-737 with either sodium periodate or lead tetra-acetate under conditions where mycolactone-765 was fully converted to its aldehyde oxidation product at *m*/*z* 675 ($[M + Na]^+$). This result showed that the two hydroxyl groups of mycolactone-737 do not form a vicinal diol, but more likely a 1,3-diol. The accurate mass of the key fragment ion at *m*/*z* 635 from the MS/MS spectrum of mycolactone-737 as well as the MS^3 spectrum of *m*/*z* 635 strongly suggest it is due to the loss of a $C_5H_{10}O_2$ (102 Da) unit from the hydroxy (distal) end of the side chain. This fragment ion is formed through a McLafferty rearrangement. This low-energy rearrangement also causes the loss of a $C_4H_8O_2$ (88 Da) unit from the core lactone,[9] a common feature in the MS/MS spectra of all the mycolactones. This common fragmentation is demonstrated here by the formation of the significant fragment ion at *m*/*z* 649 in the MS/MS spectrum of 737, and at *m*/*z* 677 in the MS/MS spectrum of 765. Therefore, based on the fragmentation mechanism, a hydroxy group is placed at C11′. Together with the results of the oxidative degradation experiments and biosynthetic considerations, only two arrangements remain plausible for the hydroxy end of the side chain (Fig. 5). To distinguish between them, we focused on the fragment ion at *m*/*z* 679. The accurate mass of this fragment ion suggested that it is formed by loss of a C_3H_6O (58 Da) unit. To confirm this, an MS^3 experiment was performed on *m*/*z* 679 using the FTICR mass spectrometer. The MS^3 spectrum clearly showed that the ion at *m*/*z* 679 still contained the intact core lactone (the presence of ion A and characteristic loss of $C_4H_8O_2$ unit), confirming that the C_3H_6O unit can be lost from the side chain. A similar loss can also be observed in the MS/MS spectrum of 765, in which a C_2H_4O (44 Da) unit (acetaldehyde) is lost from the side chain to form the fragment ion at *m*/*z* 721. Scrutiny of structures **a** and **b** of Fig. 5 shows that only structure **a** could lose a C_3H_6O unit as propionaldehyde. Therefore, all our results strongly support the structure of mycolactone-737 from MU128FXT to be **2**, shown in Fig. 1. For the second component at *m*/*z* 735, we know from comparison of its formula that it might derive from mycolactone-737 by replacing one of the two hydroxy groups of the side chain by a keto group. Indeed, the MS/MS spectra of 735 contains the same ions C, D and E as those in the MS/MS spectra of 737, confirming that part of the side chain containing the conjugated double bonds is the same as that of mycolactone-737. A significant feature of the MS/MS of 735 is that it generates a very strong fragment ion at *m*/*z* 663 (base peak). Because of its high intensity, the formation of this fragment very likely results from the loss of a 2-butanone molecule (C_4H_8O unit, 72 Da) from the side chain *via* a McLafferty rearrangement, therefore suggesting that the keto group is located at the C13′ position.

From comparisons of the proposed structure of mycolactone-737 from MU128FXT with that of its counterpart mycolactone-765 from MUAgy99 the following variations in the mycolactone biosynthetic gene complement of MU128FXT can be proposed. First, the missing additional hydroxyl on the side chain suggests the lack of a gene encoding a cytochrome P450. This would account for only two mycolactone species being produced compared to MUAgy99 (African strain) and MU91982 (Chinese strain). In MUAgy99 the MlsB PKS is responsible for side chain synthesis. Thus, the missing CH=CH moiety in mycolactone-737 compared to mycolactone-765 suggests the absence of a whole module of polyketide synthase activities in the equivalent MlsB gene of MU128FXT. Finally, there is probably an altered acyltransferase in the loading module of MlsB in MU128FXT as our structural data indicate that a propionate starter unit is used in place of acetate.

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Notes and references

‡ Since this manuscript was submitted for publication, a different candidate structure for the toxin from strain MU128FXT has been recently published (A. Mve-Obiang, R.E. Lee, K.A. Trott, T.C. Grammer, J.M.Parker, B.S. Ranger, R. Grainger, E.A. Mahrous, P.L.C. Small, *Infect. Immun.*, 2005, **73**, 3307–3312), involving (compared to MUAgy99) loss of the hydroxy group at C12′ and of the methyl group at C10′, and saturation of the C10′=C11′ double bond. This candidate structure would not fit our data.

1 K. M. George, D. Chatterjee, G. Gunawardana, D. Welty, J. Hayman, R. Lee and P. L. C. Small, *Science*, 1999, **283**, 854–857.
2 K. M. George, L. Pascopella, D. M. Welty and P. L. Small, *Infect. Immun.*, 2000, **68**, 877–883.
3 J. Rohr, *Angew. Chem., Int. Ed.*, 2000, **68**, 2847–2848.
4 G. Gunawardana, D. Chatterjee, K. M. George, P. Brennan, D. Whittern and P. L. C. Small, *J. Am. Chem. Soc.*, 1999, **121**, 6092–6093.
5 A. B. Benowitz, S. Fidanze, P. L. C. Small and Y. Kishi, *J. Am. Chem. Soc.*, 2001, **123**, 5128–5129.
6 S. Fidanze, F. Song, M. Szlosek-Pinaud, P. L. C. Small and Y. Kishi, *J. Am. Chem. Soc.*, 2001, **123**, 10117–10118.
7 L. D. Cadapan, R. L. Arslanian, J. R. Carney, S. M. Zavala, P. L. Small and P. Licari, *FEMS Microbiol. Lett.*, 2001, **205**, 385–389.
8 A. Mve-Obiang, R. E. Lee, F. Portaels and P. L. C. Small, *Infect. Immun.*, 2003, **71**, 774–783.
9 H. Hong, P. J. Gates, J. Staunton, T. Stinear, S. T. Cole, P. F. Leadlay and J. B. Spencer, *Chem. Commun.*, 2003, 2822–2823.
10 T. Stinear, A. Mve-Obiang, P. L. Small, W. Frigui, M. J. Pryor, R. Brosch, G. A. Jenkin, P. D. Johnson, J. K. Davies, R. E. Lee, S. Adusumilli, T. Garnier, S. F. Haydock, P. F. Leadlay and S. T. Cole, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 1345–1349.
11 H. Hong, J. B. Spencer, J. L. Porter, P. F. Leadlay and T. Stinear, *ChemBioChem*, 2005, **6**, 643–648.
12 A. Mve-Obiang, R. E. Lee, K. A. Trott, T. C. Grammer, J. M. Parker, B. Ranger, R. Grainger, B. D. Liflandi and P. L. C. Small, *Report of the 7th WHO Advisory Group Meeting on Buruli Ulcer*, World Health Organisation, Geneva, Switzerland, 2004.

