A novel fluorinated erythromycin antibiotic†

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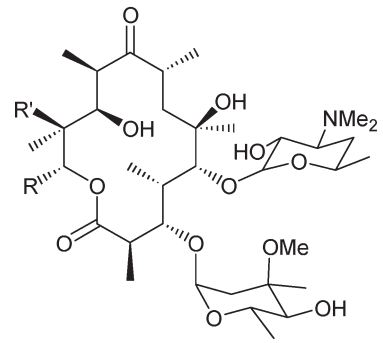
Received (in Cambridge, UK) 12th May 2005, Accepted 8th June 2005
First published as an Advance Article on the web 11th July 2005
DOI: 10.1039/b506635b

A novel fluorinated erythromycin (16-fluoroerythromycin A) has been produced by *Saccharopolyspora erythraea* ERMD1, using precursor-directed biosynthesis.

Only thirteen fluorinated natural products have been identified to date compared to over three thousand chlorinated and brominated compounds.[1,2] Fluorine is of great value in medicinal chemistry and is incorporated in a significant number of man-made drugs, among the most notable examples are prozac and mefloquin. The introduction of fluorine into a molecule can often improve the compound's bioavailability, bioactivity and stability in a number of ways.[3] The aim of this work is to assist nature to create further fluorinated natural products using precursor-directed biosynthesis, supplementing the fermentation broth with novel fluorinated starter acids. Precursor-directed biosynthesis has previously been used to generate fluorinated analogues of the polyketide derived metabolites soraphen,[4] piliformic acid[5] and enterocin,[6] however, until now no studies using precursor-directed biosynthesis to generate fluorinated analogues of the important antibiotic erythromycin had been carried out.

This work focuses on the generation of novel fluorinated erythromycin. Analogues of erythromycin in which the macrolide is fluorinated have been generated synthetically,[7] but to date only one example bearing a fluorine in the alkyl side chain has been made.[8] Propionyl coenzyme A (CoA) is the natural starter unit for erythromycin biosynthesis by *Saccharopolyspora erythraea* and its incorporation leads to the production of erythromycin A **1** (Fig. 1). The loading module of the erythromycin producing polyketide synthase (PKS) 6-deoxyethronolide B synthase (DEBS) is highly selective for propionyl CoA, though it can also accept acetyl CoA. Two different methods of increasing the breadth of starter unit specificity have been explored. The first method, employed by workers in the United States, involves the generation of a strain in which a genetic block is introduced in the first condensation step of erythromycin biosynthesis, the resultant strain is unable to produce polyketide macrolide unless *N*-acetylcysteamine (NAC) thioesters of a diketide are administered.[9] There has been a recent report of the application of this method to make 15-fluoro-6-deoxyethronolide B.[8] A second method in which, rather than disrupting DEBS, a hybrid PKS in which the natural DEBS loading module is replaced with the wide-specificity loading module of the avermectin producing polyketide synthase from *Streptomyces*



R = CH_2CH_3	R'= OH	**1**	R'= H	**1a**	*S. erythraea* W.T.
R = CH_3	R'= OH	**2**	R'= H	**2a**	
R = iso-propyl	R'= OH	**3**	R'= H	**3a**	*S. erythraea* NRRL2338/pAVLD (ERMD1)
R = sec-butyl	R'= OH	**4**	R'= H	**4a**	
R = $CH_2CH_2CH_2F$	R'= OH	**5**			

Fig. 1 Erythromycins: *S. erythraea* wild-type naturally produces erythromycin A **1** and B **1a** derived from the incorporation of propionyl CoA as the starter unit for polyketide biosynthesis. The wild-type is also able to accept acetyl CoA as the starter unit to produce **2** and **2a**. The engineered strain, ERMD1, naturally incorporates methylbutyryl CoA and isobutyryl CoA, in addition to propionyl and acetyl CoA, to produce **3** and **4**, respectively. The erythromycin B analogues of each of these compounds are also produced **1a–4a**. We have demonstrated the acceptance of potassium 4-fluorobutyrate by this strain to yield a novel fluoroerythromycin, 16-fluoro-erythromycin A, **5**.

avermitilis was developed by the Staunton and Leadlay group in Cambridge, UK.[10] *S. avermitilis* incorporates 2-methylbutyric acid and isobutyric acid as its natural starter units, however its acceptance of a wide range of alternate starter acids has been demonstrated.[11] The Staunton–Leadlay loading module replacement strain, *S. erythraea* NRRL2338/pAVLD (ERMD1), has been shown to be capable of producing a wide range of erythromycin analogues when supplemented with appropriate starter acids.[10,12] It has been demonstrated the avermectin PKS is able to accept small starter acids containing cyclic and branched hydrocarbons, however, a lack of tolerance for polar functionality such as hydroxyl groups has been noted.[11] As the incorporation of a fluorine atom into the starter unit of erythromycin would be desirable we decided to investigate whether a starter acid bearing a carbon fluorine bond could be accepted by ERMD1.

Basing our series of starter acids on the short chain and branched compounds for which the avermectin loading module has shown tolerance, we administered the potassium salts of 2-fluoroisobutyrate,‡ 3-fluoropropionate, 4-fluorobutyrate and 4-fluoropentanoate to *S. erythraea* wild-type and ERMD1 strains. Potassium 3-fluoropropionate, 4-fluorobutyrate and 4-fluoropentanoate were initially synthesised by fluorinating 2-phenylethanol,

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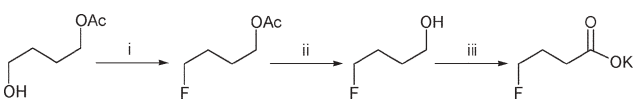
† Electronic supplementary information (ESI) available: Additional MS data. See http://dx.doi.org/10.1039/b506635b

3-phenylpropan-1-ol, or 4-phenylbutan-2-ol, respectively, with diethylaminosulfur trifluoride (DAST), then oxidising the resultant corresponding monofluoroalkyl benzenes.[13] Whilst it was possible to use this route to generate small quantities of 3-fluoropropionate and 4-fluoropentanoate; 4-fluorobutyrate was more readily synthesised on a multigram scale through an alternate route starting by mono protecting 1,4-butanediol as its acetyl ester.[14] The unprotected hydroxyl group was then displaced by reaction with DAST, the ester reduced using $LiAlH_4$, and the exposed alcohol oxidised under Sharpless conditions[15] (Scheme 1).

Fluorinated starter acids were fed in triplicate to 45 ml shake cultures of both wild-type *S. erythraea* and ERMD1. The cultures were supplemented with the potassium salts to a final concentration of 6 and 12 mM.§ Three control cultures of *S. erythraea* wild-type and ERMD1 were grown in parallel, these were pulse fed with deionised water. The crude extracts from the cultures were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).¶ Erythromycin A **1** was produced in the unfed control cultures at a level comparable to that of the cultures fed to a concentration of 6 and 12 mM with the potassium salt of the alternative starter unit, suggesting that substrate toxicity was not a problem.

Potassium 4-fluorobutyrate, when administered at a final concentration of 12 mM, was incorporated by the ERMD1 strain to yield 16-fluoroerythromycin A **5** at approximately 10% of the total erythromycins produced. The LC-MS trace exhibits peaks corresponding to erythromycins **1**, **2**, **3**, **3a**, **4** and **4a** resulting from the incorporation of the natural starter acids (Fig. 2). The trace shows that 16-fluoroerythromycin A **5** co-elutes with eythromycin A **1**. An as yet unidentified compound with mass of 765.2 Da and retention time of 12.38 min was observed in the fed and control cultures of *S. erythraea* wild-type and ERMD1, from the MS/MS fragmentation patterns it is possible that this compound is an analogue of erythromycin A bearing two additional hydroxyl moieties in the aglycone core. No incorporation of potassium 4-fluorobutyrate by the wild-type strain was observed, nor were any partially processed aglycones bearing the fluorinated moiety observed in extracts of either *S. erythraea* wild-type or ERMD1. Potassium 3-fluoropropionate, 4-fluoropentanoate and 2-fluoro-isobutyrate were not incorporated by either *S. erythraea* wild-type or ERMD1.

The formula of fluoroerythromycin **5** was revealed by high-resolution mass spectrometric analysis¶ ($[M + H]^+$: 766.4785 (obs.) and 766.4753 (calc.)). The structure of this molecule was further confirmed by comparison of its protonated and sodiated MS/MS spectra¶ with the corresponding spectra of erythromycin A. The fragmentation patterns of both the protonated and sodiated ions are consistent with those for erythromycin A, and demonstrate the presence of 4-fluorobutyrate as the starter unit.



Scheme 1 Synthesis of potassium 4-fluorobutyrate from 1,4-butanediol. *Reagents and conditions*: (i) DAST, CH_2Cl_2, −78 °C, 3 h, 82%; (ii) $LiAlH_4$, Et_2O, 0–20 °C, 4 h, 98%; (iii) $RuCl_3$, $NaIO_4$, CCl_4–H_2O–MeCN (1:1:1), RT, 24 h, 70%.

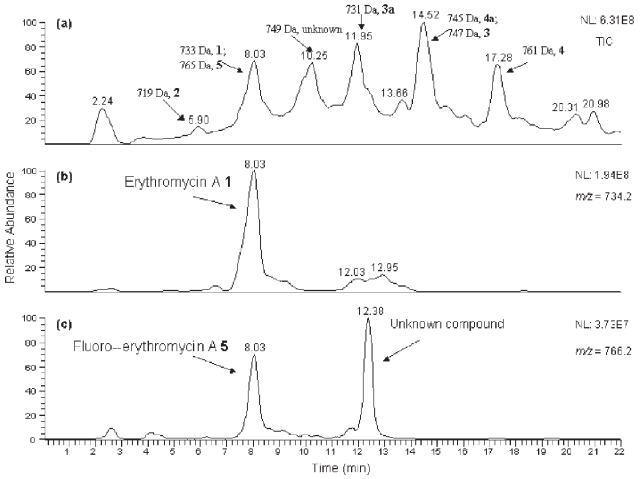


Fig. 2 LC-MS analysis of crude extract of ERMD1 strain fed with 12 mM potassium 4-fluorobutyrate. (a) Total ion current; (b) Ion trace of $[M + H]^+$ at *m/z* 734.2 for erythromycin A; (c) Ion trace of $[M + H]^+$ at *m/z* 766.2 for fluoroerythromycin A. Erythromycin A and fluoroerythromycin A co-eluted at 8.03 min. The MS/MS of the 12.38 min component reveals that it is not fluoroerythromycin A; see ESI.† This unidentified compound is also present in the unfed control.

In conclusion we have demonstrated the generation of a novel 'natural' fluorinated erythromycin. Though the avermectin loading module has been shown to be unable to accept starter acids bearing polar functional groups such as hydroxyl moieties, we have shown the surprising incorporation of a starter acid bearing a highly polar carbon–fluorine bond by the modified erythromycin PKS of ERMD1. This implies that it is not the polarity of the functional group, but its ability to hydrogen bond that prevents incorporation of these starter acids. It is evident that not only is the 4-fluorobutyrate starter unit accepted but it is fully processed (no partially processed fluoroerythromycin precursors can be observed) by post PKS enzymes to yield a novel fluorinated erythromycin A analogue.

We thank Professors J. Staunton and P. F. Leadlay for their helpful discussions relating to this work, their support and encouragement and for their provision of the two bacterial strains. The Royal Society is gratefully acknowledged for a RS BP Dorothy Hodgkin Fellowship (R. J. M. G.).

Notes and references

‡ 2-Fluoroisobutyric acid (95%) was purchased from Manchester Organics Limited.

§ Cultures of *S. erythraea* WT and ERMD1 were grown in SSDM (45 ml)[16] and incubated in sprung flasks in an orbital shaker with a 32 mm throw at 27 °C, 190 rpm. Aqueous solutions of the potassium salts of the fluorinated acids were pulse fed to resting cell cultures (48-h old cultures) at 3 × 12 h intervals to final concentrations of 6 and 12 mM. 96 h after innoculation the cultures were harvested by centrifugation. The supernatant was adjusted to pH 9 with potassium hydroxide, and extracted with ethyl acetate (3 × 50 ml). The organic phase was dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude extract containing erythromycins was analysed by LC-MS/MS.

¶ High-resolution mass analyses were performed by electrospray ionization on an API QSTAR pulsar (Applied Biosystems). The LC-MS/MS was performed on a Finnigan LCQ instrument. (ThermoFinnigan, San Jose, USA) The crude extract was eluted on a reverse phase C18 column (Phenomenex, 5 μm, 4.6 × 250 mm) with 20 mM ammonium acetate and acetonitrile. The anotated spectra are included in the ESI.†

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