COMMUNICATION

ChemComm
www.rsc.org/chemcomm

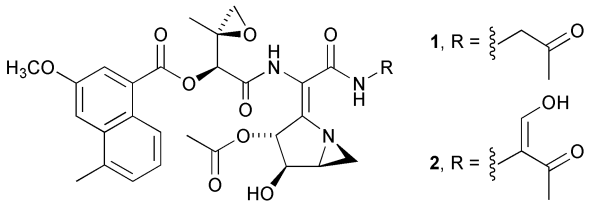
Biosynthetic studies on the azinomycins: The pathway to the naphthoate fragment†

Christophe Corre,‡ Cyrille A. S. Landreau,‡ Michael Shipman‡ and Philip A. S. Lowden*§
School of Biological and Chemical Sciences, University of Exeter, Exeter, U.K. EX4 4QD.
E-mail: p.lowden@bbk.ac.uk; Fax: +44 (0)20 76316246; Tel: +44 (0)20 70790789

Received (in Cambridge, UK) 12th July 2004, Accepted 28th August 2004
First published as an Advance Article on the web 30th September 2004

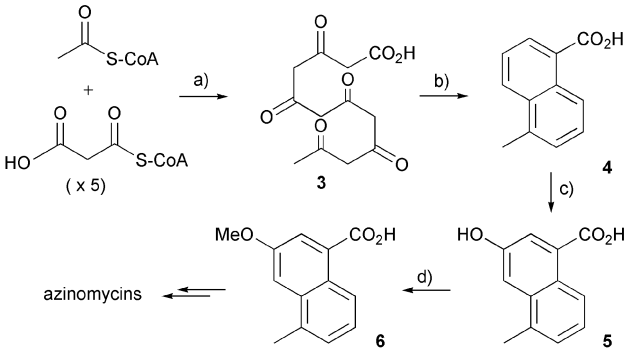
Isotopically labelled intermediates in a proposed biosynthesis of the naphthoate fragment of azinomycin B have been made and successfully incorporated by the azinomycin producing organism.

The antitumour antibiotics azinomycin A (**1**) and B (**2**) are structurally unique natural products containing the strained 1-azabicyclo[3.1.0]hexane ring system.[1] Azinomycin B (née carzinophilin) is produced by two different organisms namely *Streptomyces sahachiroi*[2] and *S. griseofuscus*.[3] These natural products possess potent *in vitro* cytotoxic activity, significant *in vivo* antitumour activity and appear to act by disruption of cellular DNA replication by interstrand cross-link (ISC) formation.[1] The epoxide and aziridine are known to be responsible for the cross-linking process which occurs *via* N7 of purine bases two base pairs apart on the complementary DNA strands.[4]

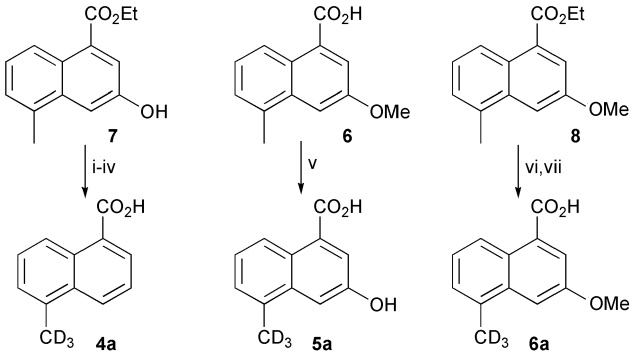


Despite considerable interest in the chemical synthesis[1,5] and mode of action[4,6] of the azinomycins, very little is known about their biosynthetic origin. Indeed, the first biosynthetic studies were reported by two of us earlier this year.[7] Incorporation of ^{13}C-labelled acetate revealed that the naphthoate fragment of azinomycin B is of polyketide origin. It was proposed that condensation of one molecule of acetyl-CoA with five of malonyl-CoA leads to linear polyketide **3**, catalysed by a polyketide synthase (PKS). Further reduction, cyclisation and aromatisation of linear polyketide **3** by the PKS leads to carboxylic acid **4**, which would be hydroxylated, methylated and incorporated into the complete azinomycin B skeleton (Scheme 1). Herein, we disclose evidence in support of this proposal using a series of isotopically labelled substrates.

To demonstrate the involvement of intermediates **4**–**6** in the biosynthesis of the azinomycins, trideuterated analogues **4a**, **5a** and **6a** were prepared for feeding studies. Carboxylic acid **4a** was made in four steps from naphthoic ester **7**[8] (Scheme 2). The synthetic sequence involved conversion of the alcohol into the corresponding triflate followed by its reductive removal using triethylsilane under palladium catalysis according to the method of Kotsuki.[9] Subsequent ester hydrolysis and exhaustive deuteration of the C-5 methyl group by heating with sodium hydride in d_6-DMSO[10]



Scheme 1 Proposed pathway to the naphthoate fragment a) condensation by a polyketide synthase; b) ketone reduction, aldol cyclisation, aromatisation; c) hydroxylation, possibly by cytochrome P450; d) methylation using *S*-adenosylmethionine.



Scheme 2 *Reagents and conditions*: (i) Tf_2O, Et_3N, CH_2Cl_2, 71%; (ii) *cat.* Pd(dppf)Cl_2, Et_3SiH, DMF, 60 °C, 80%; (iii) LiOH, MeOH, H_2O, 95%; (iv) NaH (3 eq.), $(CD_3)_2SO$, 140 °C, 8 h, 80%; (v) NaH (5.3 eq.), $(CD_3)_2SO$, 140 °C, 16 h, 39%; (vi) NaH (1.3 eq.), $(CD_3)_2SO$, 140 °C, 16 h, (vii) LiOH, MeOH, H_2O, 36% over 2 steps.

provided **4a** in 43% overall yield. Carboxylic acids **5a** and **6a** were made from the known naphthalenes **6** and **8**[8] using the same exhaustive deuteration reaction as the key step. In the synthesis of **5a**, concomitant demethylation of the C-3 methyl ether was achieved by using an excess of base. The extent of deuteration at the C-5 methyl group in carboxylic acids **4a**–**6a** was estimated to be ⩾90%D by ^{1}H NMR spectroscopy.¶

The sodium salts of carboxylic acids **4a**, **5a** and **6a** were separately fed to *S. sahachiroi* at a final concentration of 0.1 mM and were incorporated into azinomycin B at levels of 48%, 60% and 83% respectively,‖ as measured by electrospray mass spectrometry (Fig. 1). Analysis of labelled azinomycin B by ^{2}H NMR confirmed that deuterium incorporation occurred specifically at the C-5′ methyl group. The high levels of incorporation in each case, and the increasing efficiency of incorporation with increasing substitution, suggest that all three precursors are true biosynthetic intermediates, and that the naphthoate fragment is fully substituted before attachment to the rest of the molecule.** We tested this further

† Electronic supplementary information (ESI) available: ^{1}H and ^{2}H NMR spectra and MS data for **4a**, **4b**, **5a**, **6a**, **6b** and azinomycin B from feeding experiments. See http://www.rsc.org/suppdata/cc/b4/b410592c/

‡ Current address: Department of Chemistry, University of Warwick Coventry, CV4 7AL.

§ Current address: School of Biological and Chemical Sciences, Birkbeck College London, Malet Street, London, WC1E 7HX.

DOI: 10.1039/b410592c

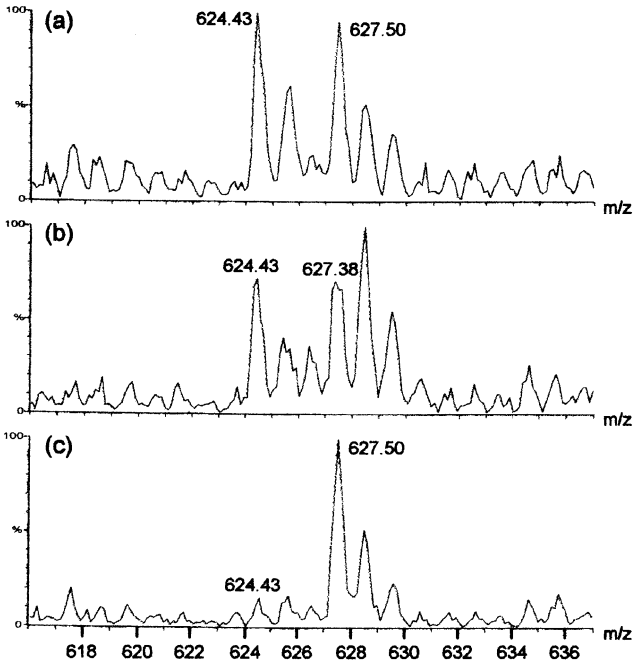
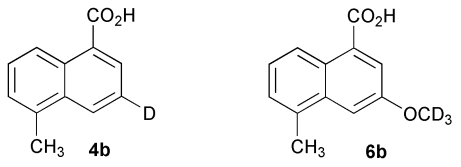


Fig. 1 Electrospray mass spectra of azinomycin B isolated after feeding (a) **4a**; (b) **5a** and (c) **6a**. Unlabelled azinomycin B has $MH^+ = 624.43$.

using competition experiments. If **6** is *not* a true biosynthetic intermediate and is incorporated due to some flexibility in the biosynthetic enzymes, then its incorporation would be expected to be reduced by an equivalent concentration of the true substrate **4** or **5**. If it *is* a real intermediate, then it would be expected that incorporation of earlier intermediates would be reduced by an equivalent concentration of **6**. Precursor **6b** labelled at the *O*-methyl group with deuterium was prepared from ester **7** in two steps [(i) NaH, CD_3I, DMF; (ii) LiOH, MeOH, H_2O, 95% over 2 steps]. This material was co-fed at 0.1 mM with an approximately equal concentration of either **4a** or **5a**. Relative incorporation levels were compared by measurement of the CD_3 peak intensities in the ^2H NMR spectrum of the produced azinomycin B (Fig. 2). The relative incorporations of deuterium (normalised to the relative amounts of each precursor) at the C-5′ methyl and C-3′ *O*-methyl signals were 35 : 65 and 36 : 64 after co-feeding of **6b** with **4a** and **5a** respectively. These data are consistent with the idea that the naphthoate **6** is fully assembled prior to attachment to the rest of the azinomycin skeleton.



In addition, the mechanism of oxidation of **4** to **5** was investigated by feeding of labelled precursor **4b**. This material was made in a similar way to **4a** using Et_3SiD for reduction of the triflate (the step involving deuteration of the C-5′ methyl group being omitted). After feeding **4b** (0.1 mM), incorporation of the substrate into azinomycin B was observed by ESMS at 60%, comparable to that seen with **4a**. ^1H and ^2H NMR revealed that azinomycin B was labelled with deuterium specifically at C-4′. These data can be interpreted in terms of the 'NIH shift' mechanism for aromatic ring hydroxylation,[11] also observed in the biosynthesis of the fungal polyketide aflatoxin.[12]

To conclude, our studies using deuterium labelled derivatives of **4**, **5** and **6** provide strong support for the pathway proposed in Scheme 1, in which **6** is a pivotal biosynthetic intermediate. Studies are now in progress in our laboratory on the genetics and enzymology of azinomycin biosynthesis.

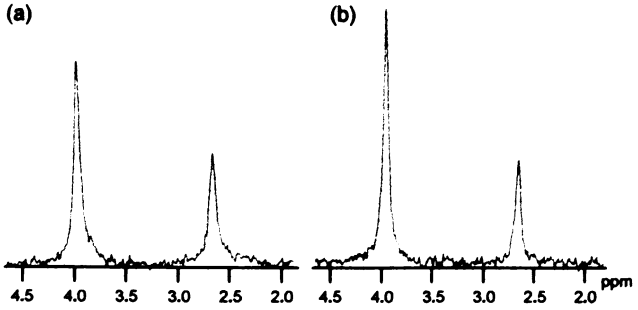


Fig. 2 ^2H NMR spectra of azinomycin B produced by competitive feeding of (a) **6b** : **4a** (8 : 9 ratio) and (b) **6b** : **5a** (4 : 3 ratio). The C-5′ methyl group is at 2.68 ppm, the C-3′ *O*-methyl group is at 3.96 ppm.

We are indebted to the EPSRC (GR/N63529/01) and BBSRC (B15997) for financial support of this work. We are grateful to our colleague Alison Hill for helpful discussions.

Notes and references

¶ Interestingly, a small amount of additional deuterium incorporation ($\leqslant$30%D) at the C-2 and C-4 positions of **4a–6a** was also observed. This could be quantified using a combination of ^1H and ^2H NMR spectroscopy along with ES-MS. The levels of additional incorporation were most pronounced in **5a** (see supporting information†). This extra labelling was taken into account when interpreting the feeding results.

‖ Labelling by **4a**, **5a**, and **6a** was reproducible, but absolute incorporation levels were variable. To allow comparison of relative incorporation levels, these data were obtained from experiments conducted at the same time, using the same batch of production medium and using the same starter culture of *S. sahachiroi*.

** We cannot discount that the different levels of incorporation reflect differences in cell permeability or diversion into other metabolic pathways. Conclusive proof for this hypothesis must await mechanistic studies on purified enzymes.

1 For a review, see T. J. Hodgkinson and M. Shipman, *Tetrahedron*, 2001, **57**, 4467.
2 T. Hata, F. Koga, Y. Sano, K. Kanamori, A. Matsumae, R. Sunagawa, T. Hoshi, T. Shima, S. Ito and S. Tomozawa, *J. Antibiot. Ser. A*, 1954, **7**, 107.
3 K. Nagaoka, M. Matsumoto, J. Onoo, K. Yokoi, S. Ishizeki and T. Nakashima, *J. Antibiot.*, 1986, **39**, 1527; K. Yokoi, K. Nagaoka and T. Nakashima, *Chem. Pharm. Bull.*, 1986, **34**, 4554; S. Ishizeki, M. Ohtsuka, K. Irinoda, K.-I. Kukita, K. Nagaoka and T. Nakashima, *J. Antibiot.*, 1987, **40**, 60.
4 J. W. Lown and K. C. Majumdar, *Can. J. Biochem.*, 1977, **55**, 630; R. W. Armstrong, M. E. Salvati and M. Nguyen, *J. Am. Chem. Soc.*, 1992, **114**, 3144; T. Fujiwara, I. Saito and H. Sugiyama, *Tetrahedron Lett.*, 1999, **40**, 315; J. A. Hartley, A. Hazrati, L. R. Kelland, R. Khanim, M. Shipman, F. Suzenet and L. F. Walker, *Angew. Chem. Int. Ed.*, 2000, **39**, 3467; R. S. Coleman, R. J. Perez, C. H. Burk and A. Navarro, *J. Am. Chem. Soc.*, 2002, **124**, 13008.
5 R. S. Coleman, J. Li and A. Navarro, *Angew. Chem. Int. Ed.*, 2001, **40**, 1736; J.-Y. Goujon and M. Shipman, *Tetrahedron Lett.*, 2002, **43**, 9573; M. Hashimoto, M. Sugiura and S. Terashima, *Tetrahedron*, 2003, **59**, 3063.
6 H. Zang and K. S. Gates, *Biochemistry*, 2000, **39**, 14968; S. Alcaro and R. S. Coleman, *J. Med. Chem.*, 2000, **43**, 2783; R. S. Coleman, C. H. Burk, A. Navarro, R. W. Brueggemeier and E. S. Diaz-Cruz, *Org. Lett.*, 2002, **4**, 3545; S. Alcaro, F. Ortuso and R. S. Coleman, *J. Med. Chem.*, 2002, **45**, 861; K. Miyashita, M. Park, S. Adachi, S. Seki, S. Obika and T. Imanishi, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 1075.
7 C. Corre and P. A. S. Lowden, *Chem. Commun.*, 2004, 990.
8 M. Shibuya, *Tetrahedron Lett.*, 1983, **24**, 1175; H. J. Bryant, C. Y. Dardonville, T. J. Hodgkinson, M. B. Hursthouse, K. M. A. Malik and M. Shipman, *J. Chem. Soc., Perkin Trans. 1*, 1998, 1249.
9 H. Kotsuki, P. K. Datta, H. Hayakawa and H. Suenaga, *Synthesis*, 1995, 1348.
10 M. E. Cass, T. M. Garrett and K. N. Raymond, *J. Am. Chem. Soc.*, 1989, **111**, 1677 and references therein.
11 G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop and S. Udenfriend, *Science*, 1967, **157**, 1524.
12 T. J. Simpson, A. E. de Jesus, P. S. Steyn and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1983, 338.