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The first biosynthetic studies of the azinomycins: acetate incorporation into azinomycin B†

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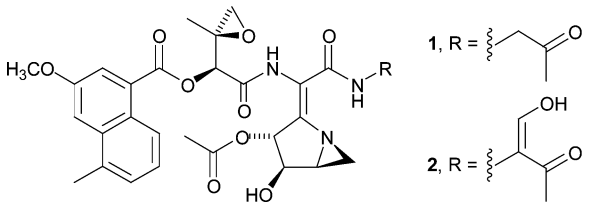
^{13}C-Labelled acetate efficiently labels the antitumour natural product azinomycin B, revealing a polyketide origin for the naphthoate fragment, that threonine is the probable precursor of the enol fragment, and that α-ketoglutarate is a probable precursor of the aziridine fragment.

The antitumour antibiotics azinomycin A (**1**) and B (**2**)[1] are remarkable natural products containing the 1-azabicyclo[3.1.0]-hexane ring system. Azinomycin B was originally isolated (named carzinophilin) from *Streptomyces sahachiroi*[2] but its structure was unknown for many years. **1** and **2** were later isolated from *S. griseofuscus* and their structures determined in 1986.[3] It was then realised that carzinophilin and **2** are identical.[4] The azinomycins form double strand crosslinks in DNA *via* alkylation of purine bases by the epoxide and aziridine rings.[5] Coleman *et al*. recently performed biochemical[6] studies that provide more details of azinomycin–DNA binding. The azinomycins have proved attractive synthetic targets and the first total synthesis of **1** was achieved by Coleman in 2001.[7] Many analogues have been prepared and tested for biological activity, in order to develop structure–activity relationships.[1]



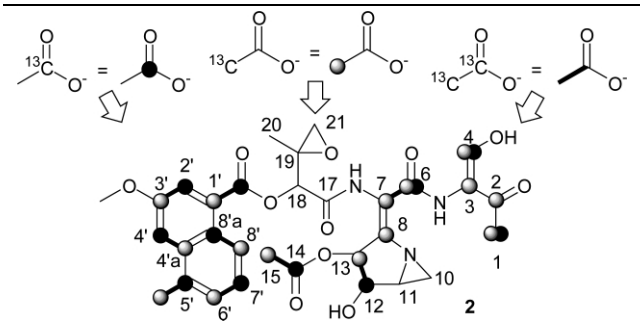
The azinomycins are exciting targets for biosynthetic studies due to the unusual azabicyclo[3,1,0]hexane ring system. The mystery of how this is generated, and the potential for biosynthetic engineering of novel antitumour agents, prompted us to initiate biosynthetic studies on these fascinating molecules. We predicted a polyketide origin for the naphthoate fragment, so we began by feeding ^{13}C-labeled acetate. Sodium acetate labeled at C1, C2 or doubly labeled with ^{13}C were each fed to *S. sahachiroi* at the onset of azinomycin production. Unambiguous enhancement of various ^{13}C resonances[3b] was observed after feeding C1- or C2-labeled acetate. Weaker labeling was observed at a few positions which cannot be assigned to specific incorporation. Doublets due to ^{13}C–^{13}C coupling with matching coupling constants were observed between several pairs of adjacent carbons when doubly labeled acetate was fed. The degree of labeling and the coupling constant values are listed in Table 1, and the labeling pattern is summarised in Scheme 1.§

The alternate labeling of the naphthoate fragment by C1- and C2-labeled acetate reveals a polyketide origin. We propose that condensation of one molecule of acetyl-CoA with five of malonyl-CoA by a polyketide synthase leads to linear polyketide **3**. This is followed by reduction, cyclisation and aromatisation to form **4**,

Table 1 Incorporation of ^{13}C-labeled acetate into azinomycin B

	δ_C/ppm		Relative enhancement[a] by [1-^{13}C]-acetate[b]	Relative enhancement[a] by [2-^{13}C]-acetate[c]	$^1J_{C-C}$/Hz observed with [1,2-$^{13}C_2$]-acetate
	$CDCl_3$	CD_2Cl_2			
C1	24.5	24.4	**7.3–8.6**[d]	**3.4–4.7**[d]	
C2	191.5	191.3	2.7–3.9[d]	**3.0–5.3**[d]	
C3	118.6	118.6	2.7–3.9[d]	**3.1–5.0**[d]	
C4	150.8	150.6	**4.7–5.7**[d]	**2.8–6.0**[d]	
C6	162.0	162.2	**7.3**	**5.4**	70.8[b]
C7	119.3	119.3	0.9–1.5[d]	**7.8**	70.8, 82.9[b]
C8	153.0	154.1	0.8–1.2[d]	**6.5**	
C10	36.7	36.9	2.2	1.9	
C11	46.4	46.9	1.3	1.0	
C12	77.4	76.9	**166**[e]	3.0	39.9[c]
C13	84.4	84.5	1.8	**9.2**	39.9, 49.0[c]
C14	173.0	172.8	**5.2**	3.1	58.8[b]
C15	21.0	20.9	2.4	**4.1**	58.8[b]
C17	164.0	164.3	2.2	1.5	
C18	77.1	77.0	Low[e]	2.4	
C19	56.2	56.1	0.8–1.2[d]	1.4	
C20	17.2	17.1	1.1	2.9	
C21	53.9	53.8	2.9	Low[e]	
C1′	128.1	128.4	2.0	**11.4**	76.1[b]
C1′ CO	165.7	165.8	**23.8**	2.2	76.1[b]
C2′	122.3	122.3	**41.3**	2.4	65.6[b]
C3′	156.0	156.2	0.5–0.7[d]	**18.4**	65.6[b]
C3′ OCH_3	55.7	55.8	1.7	1.9	
C4′	108.5	108.4	**39.9**	1.9	59.6[b]
C4′a	134.5	134.6	1.4	**12.9**	58.8[b]
C5′	133.3	133.7	**27.0**	1.7	45.2[b]
C5′ CH_3	20.3	20.1	1.0	**10.0**	44.5[b]
C6′	127.9	127.9	1.6	**10.4**	53.5[b]
C7′	125.4	125.3	**42.3**	1.7	53.5[b]
C8′	123.9	123.8	1.1	**11.9**	56.5[b]
C8′a	127.0	126.9	**29.5**	1.4	56.5[b]

[a] [1-^{13}C]-acetate was fed in 8 1 mM batches, [2-^{13}C]-acetate in 5 2 mM batches, and [1,2-$^{13}C_2$]-acetate in 5 1 mM batches, all at regular intervals. Spectra were run in the solvent indicated. Relative enhancements were determined by calculating the ratios of all peak heights to those of unlabeled azinomycin B and normalising to the peak with the lowest ratio that could be accurately measured. Enhancements likely to be due to specific incorporation are highlighted in bold. [b] Measured in $CDCl_3$. [c] Measured in CD_2Cl_2. [d] Signal intensities in labeled and/or unlabeled spectrum are low so enhancements cannot be measured precisely. [e] Signals obscured by solvent resonances so enhancements cannot be measured precisely.



Scheme 1 Observed incorporation of labeled acetate into azinomycin B.

† Electronic supplementary information (ESI) available: experimental procedures for fermentation of *S. sahachiroi* and isolation of azinomycin B, NMR data from labeling experiments. See http://www.rsc.org/suppdata/cc/b4/b400093e/

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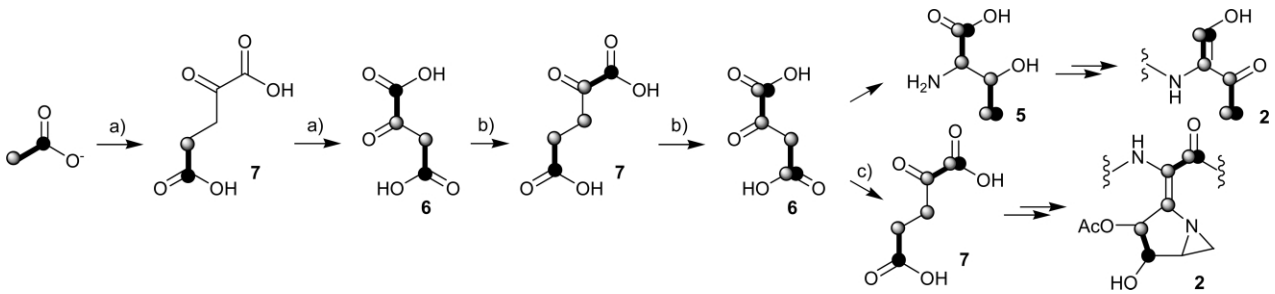
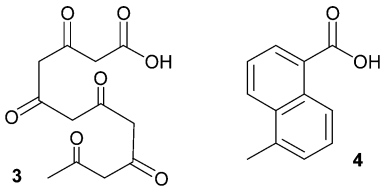


Scheme 2 Predicted labeling of the enol and aziridine fragments, *via* oxaloacetate (**6**) and threonine (**5**) for the enol fragment, and *via* α-ketoglutarate (**7**) for the aziridine fragment. a) 1st pass through Krebs cycle; b) 2nd pass through Krebs cycle; c) 3rd and subsequent passes through Krebs cycle.

which is then hydroxylated, methylated and elaborated to azinomycin B. As expected, feeding of methyl-^{13}C-labeled methionine (3×0.1 mM at regular intervals) resulted in strong labeling (43%) of only the OCH_3 carbon. Experiments are underway with advanced precursors to test this pathway further.



Analysis of enol fragment labeling was impaired by very low signal intensities for C1 to C4, as previously noted.[3b] Although incorporation could not be seen with [1,2-$^{13}C_2$]-acetate, enhancement of signal intensity was apparent for C1 and C4 after feeding with [1-^{13}C]-acetate and at all of C1 to C4 with [2-^{13}C]-acetate. This tail-to-tail arrangement of acetate units and scrambling of C2 of acetate is characteristic of the Krebs cycle[8] (Scheme 2). This is consistent with the enol fragment arising from threonine (**5**), which in turn is derived from the Krebs cycle intermediate oxaloacetate (**6**), *via* aspartate and homoserine. We propose that the hydroxyl group of threonine is oxidised to a ketone and the carboxyl group reduced to an aldehyde.

Clear labeling was observed in the aziridine fragment, with C1 of acetate labeling C6 and C12 of azinomycin B, while C2 of acetate labeled all of C6, C7, C8 and C13. Feeding of doubly labeled acetate resulted in very distinct coupling between C6 and C7 and between C12 and C13 (Fig. 1). A smaller proportion of labeled molecules displayed additional coupling at C7 or C13 (the low intensity double doublets in Fig. 1). This can be explained by coupling to C8 as well as to C6 or C12 respectively. Low intensity doublets due to C–C coupling were also apparent at C6, C7, C8 and C13 after feeding 2-^{13}C acetate. Our labeling data are entirely consistent with α-ketoglutarate (**7**) acting as a precursor to the first five carbons of the aziridine fragment (Scheme 2). The C6/7 pair showed additional enhancement of the ^{13}C singlet, while the C12/13 pair showed labeling only in the doublet (Fig. 1). This is consistent with the C12/13 pair originating from C4/5 of α-ketoglutarate, since these carbons are introduced as an intact pair at each pass through the Krebs cycle. We cannot yet say if there is a unique pathway from α-ketoglutarate to azinomycin B or whether the aziridine fragment arises from glutamate, glutamine, arginine or proline, all of which are biosynthesised from α-ketoglutarate.

These are the first biosynthetic studies on azinomycin B. The data confirm a polyketide origin for the naphthoate fragment, are consistent with the enol fragment arising from threonine, and suggest that the aziridine fragment arises in part from α-ketoglutarate. Studies are underway with labeled glucose to determine the origin of the remaining carbon atoms. These results will inform future studies on the genetics and enzymology of azinomycin biosynthesis. The azinomycin biosynthetic gene cluster will act as a rich source of diversity for combinatorial biosynthesis of novel, bioactive natural products.

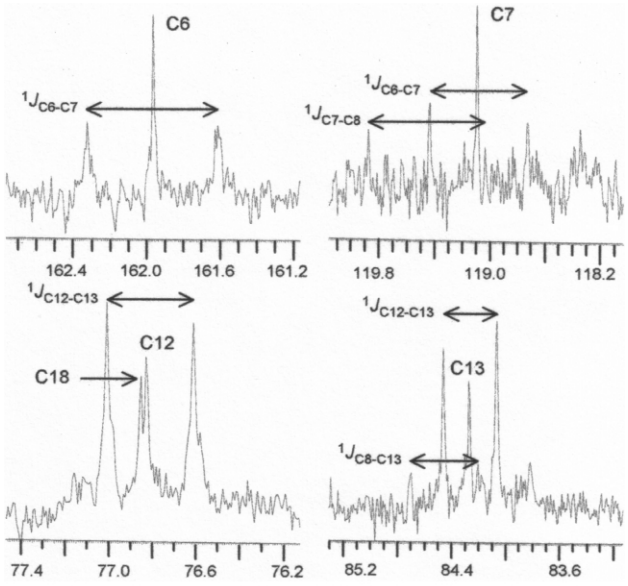


Fig. 1 ^{13}C NMR resonances in CD_2Cl_2 for C6, C7, C12, C13 of azinomycin B after feeding with 1,2-$^{13}C_2$ labeled acetate. Strong doublets are due to ^{13}C–^{13}C coupling in intact acetate units. The weak double doublets for C7 and C13 are due to additional coupling to C-8.

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Notes and references

§ It was necessary to reassign the C4′a and C5′ ^{13}C resonances,[3b] since these displayed erroneous labeling. This was achieved by isolating the labeled naphthoate fragment of azinomycin B and fully assigning its ^{1}H and ^{13}C NMR spectra using 2-D methods (see supporting information).

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