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# Biosynthesis of Tetronasin: Part 2 Identification of the Tetraketide Intermediate Attached to the Polyketide Synthase.

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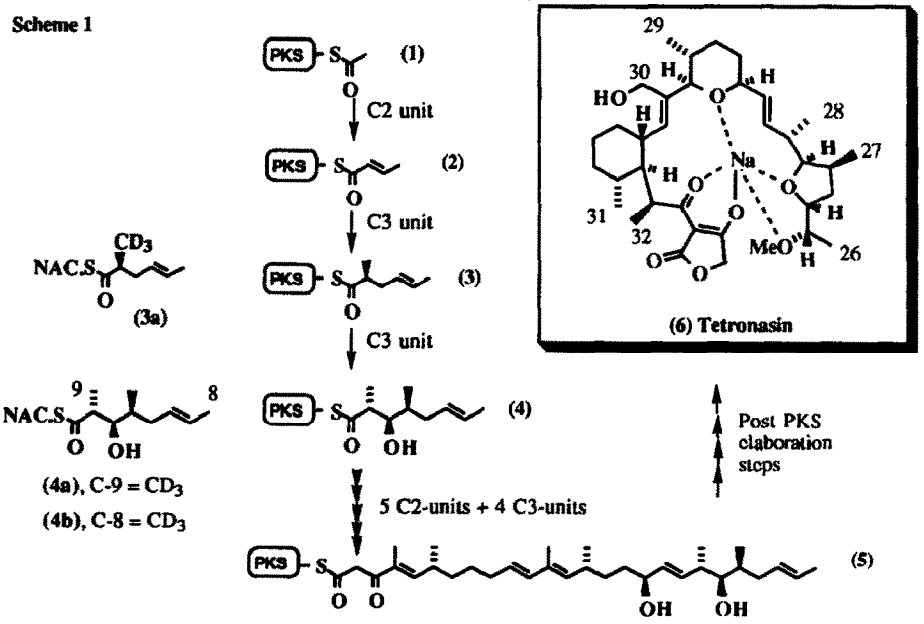
***Abstract:*** *Incorporation experiments with deuterium labelled N-acetyl cysteamine analogues (4a) and (4b) of the proposed enzyme-bound tetraketide precursor (4) show that the acyl residue is incorporated intact into tetronasin (6). Equivalent experiments with the deuterium labelled N-acetyl cysteamine analogues (7), (8) and (9), all diastereoisomers of (4), result in no intact incorporation into (6).*

According to our hypothesis for the biosynthesis of the polyketide ionophore antibiotic tetronasin (**6**), the early step of the pathway involves the formation of a linear polyketide intermediate (**5**) on a polyketide synthase (PKS).[1] All the intermediates up to this stage remain bound to the PKS through thioester links. In total, twelve chain extension cycles are required to produce (**5**). If the chain assembly steps follow the processive mode, the intermediates formed in succession at the end of the first three chain extension cycles should be (**2**), (**3**) and (**4**) as indicated in Scheme 1. In the previous paper we described incorporation experiments using synthetic acyl intermediates as *N*-acetyl cysteamine (NAC) thioesters [e.g. (**3a**)] which supported the intermediacy of the proposed triketide (**3**), and therefore by implication, that of the proposed diketide (**2**).[1] Here we describe equivalent studies which support the intermediacy of the proposed tetraketide (**4**).

**Scheme 1**



In designing our incorporation experiments, we were aware of the existence of voracious fatty acid degradation systems which can degrade added precursors to smaller isotopically-labelled primary building blocks.[2] We therefore sited our isotopic labels in NAC analogues of (4) so that fatty acid degradation would produce a *labelled* primary precursor (propionate or acetate) which would be incorporated into the metabolite with a labelling pattern predictably different from that expected following intact incorporation of the labelled tetraketide. Analysis of the distribution of deuterium in the metabolite would then give a measure of the relative importance of the alternative modes of incorporation, direct, or indirect following fatty acid degradation.

Two analogues of the tetraketide (4) were prepared, each labelled with a $CD_3$ group, one at C-9, (**4a**), and the other at C-8, (**4b**). The syntheses of (**4a**) and (**4b**) are described in the following paper.[3] These two analogues would specifically label the metabolite at C-27 and C-26 respectively following intact incorporation of the precursor. Fatty acid degradation of the acyl residue of the NAC analogues of (4) would be expected to take place by essentially a reversal of the biosynthetic steps shown in Scheme 1. The products would therefore be two units of propionate and two of acetate. Degradation of (**4a**) would be expected to produce $CD_3$-labelled propionate, which would in turn label all the six propionate-derived branching carbons numbered successively C-26 to C-31. The second labelled form of the tetraketide, (**4b**), is predicted to produce $CD_3$-labelled acetate. We knew from earlier experiments using $CD_3CO_2H$ as precursor that the deuterium would be incorporated *via* a roundabout route to produce the same labelling pattern predicted to follow degradation of (**4a**).

Feeding experiments were carried out using cultures at two stages of growth, four days after inoculation, at the onset of rapid production of tetronasin, and after eleven days, when the cultures became senescent. With NAC analogues of the tetraketide (4), unlike those of the triketide (3), we sometimes observed specific incorporation even with younger cultures. This behaviour follows the trend observed in the earlier work with analogues of (2) and (3) where the balance between direct and indirect incorporation shifted towards the direct route as the precursor becomes more committed.[1] In experiments with senescent cultures, any incorporation of label from analogues of (4) was always specific, but the incorporation level was often very low and in some experiments there was no detectable incorporation whatsoever. With younger cultures the levels of enrichment were much higher, but the balance between direct and indirect incorporation of label varied unpredictably. The variability of the results from experiment to experiment with younger cells can be attributed to natural variations in the rate of development of individual cultures.

The derived samples of tetronasin were analysed by $^2H$ NMR. Three representative spectra are shown in Figure 1.

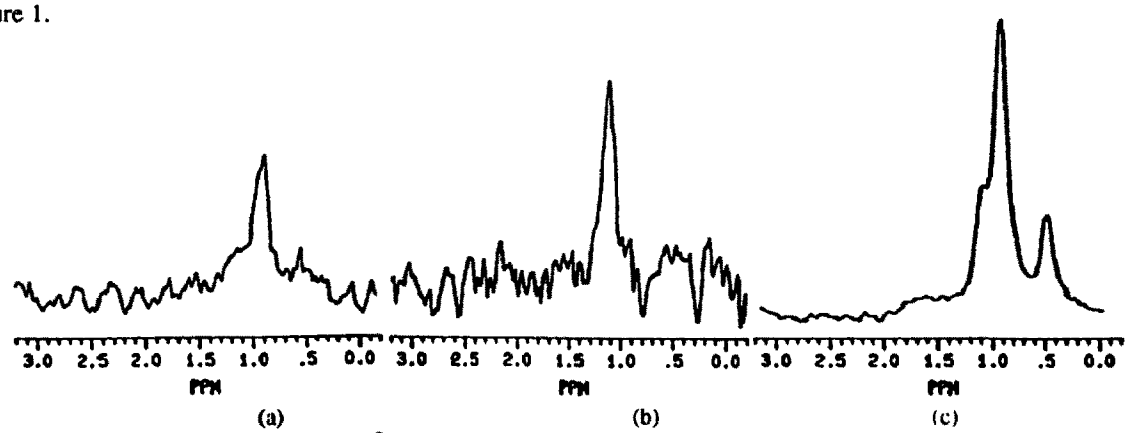


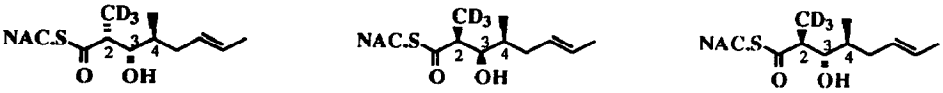

**Figure 1:** Methyl Region of the $^2H$ NMR spectra of Tetronasin
(a) Following incorporation of (**4a**)
(b) Following incorporation of (**4b**)
(c) Following incorporation of $CD_3CH_2CO_2H$

Spectrum (c) is a control spectrum derived from tetronasin following incorporation of $CD_3CH_2CO_2H$ and it shows the pattern of peaks to be expected when all six branching methyl groups are labelled as a result of indirect incorporation of label from either (**4a**) or (**4b**) following fatty acid degradation. Spectrum (a), derived from tetronasin which had incorporated (**4a**) in a *senescent* culture, shows a strong peak at δ 0.9 ppm as would be expected for specific incorporation of a $CD_3$ group at C-27; the level of enrichment is low but other methyl signals, including the isolated singlet at δ 0.5 ppm which would have been clearly visible following indirect incorporation, are not detected. Spectrum (b), derived from tetronasin which had incorporated (**4b**) in *young* cells, is similarly consistent with specific incorporation, with the $CD_3$ group now sited at C-26 leading to a signal at δ 1.1 ppm; the incorporation level is significantly higher and again there is no evidence for indirect incorporation of any $CD_3$ residues into other methyl sites. The complementary nature of these two experiments, in which two different sites of tetronasin become specifically labelled, strengthens the evidence for specific and intact incorporation of the two labelled analogues of the tetraketide (**4**). The intact incorporation of (**4**) also confirms the evidence for intact incorporation of (**3**) which was presented in the previous paper, and by implication the role of crotonate (**2**) as the diketide intermediate on the PKS.

These results therefore demonstrate that the first three chain extension cycles carried out by the tetronasin PKS, leading up to the tetraketide, follow a processive mode of operation. Only one other PKS has been investigated methodically through several successive chain extension cycles (the aspyrone PKS) and it also followed the processive mode.[4] The evidence is mounting therefore that this mode is probably general for PKS systems which produce aliphatic polyketide chains.

To establish the validity of the incorporations seen with (**4a**) and (**4b**) we also tested as precursors 'wrong' diastereoisomers of the tetraketide (**4**). This check is essential because there are recognised biosynthetic reactions which can interconvert stereoisomers of a β-hydroxy ester residue: reversible interconversion of the carbinol site (C-3) of the NAC analogue of (**4**) with the corresponding ketone could epimerise the chiral centre at C-3 leading to (**7**); the chiral centre at C-2 can also be epimerised as a further consequence of this oxidation by enolisation of the transient keto acid. The possible interconversion of the hydroxy ester with the corresponding keto ester was recognised by Cane in his recent studies of the erythromycin PKS in which he incorporated a deuterium label at the carbinol carbon to rule out any reversible redox reaction.[5] However this approach does not eliminate an alternative epimerisation mechanism for epimerisation at both C-2 and C-3 — interconversion of the β-hydroxy ester intermediate with an α,β-unsaturated ester by reversible dehydration, with different stereochemistries of reaction in the forward and reverse directions. Any of these epimerisation processes could occur to the test compound by adventitious interaction with suitable enzymes before it becomes attached to the polyketide synthase. Accordingly, three stereoisomers of (**4**) were synthesised, (**7**), (**8**) and (**9**), which cover all possible stereochemical variations at C-2 and C-3 (Scheme 2). The remaining chiral centre at C-4 could be epimerised in a transient β-ketoester intermediate by enolisation towards that site, but this is energetically less favoured than enolisation towards C-2, and so is considered unlikely to occur in the mild environment of the cell.

**Scheme 2**




(**7**) (**8**) (**9**)

Synthetic routes to each of the diastereoisomers (**7**), (**8**) and (**9**) were developed to allow the introduction of a $CD_3$ label as shown and are described in the following paper.[3] The *N*-acetyl cysteamine thioesters were administered, as their *N*-acetyl cysteamine thioesters, to senescent cultures of *Streptomyces longisporoflavus* following the standard protocol discussed above. The resulting tetronasin samples were analysed by $^2H$ NMR, as before, by examination of the methyl regions of the spectra. In contrast to the results obtained with the

'correct' diastereoisomer (**4a**) and (**4b**) the $CD_3$ groups from the remaining three diasteroisomers were not incorporated specifically.

**Figure 2**

The spectrum shown in Figure 2 is of tetronasin derived from isomer (**7**) and shows a complex set of signals between δ 0.5 and 1.5 ppm corresponding to incorporation of deuterium into five methyl groups derived from the methyl group of propionate (C-27 to C-29, C-31, and C-32). This is consistent with β-oxidation of the precursor followed by cleavage to produce $CD_3$-labelled propionate, which is then incorporated into the various $C_3$-units in the usual way. The tetronasin samples produced in cells incubated with isomers (**8**) and (**9**) showed no significant signal in the $^2H$ NMR. All experiments were repeated to ensure reliability.

The results from the feeding of isomer (**7**) are most interesting. A single change in the stereochemistry at the hydroxyl centre ensures that there is a switch from intact incorporation of the acyl chain to degradation followed by indirect incorporation of deuterium through the resulting labelled propionate. The remaining results are negative which suggests that the two isomers are not susceptible to β-oxidative cleavage, at least under the conditions of the experiment. There is no evidence that any of the various stereoisomers are easily converted to (**4**) by adventitious metabolic reactions, which would have undermined the positive results obtained with this 'correct' isomer.

The failure of the three 'wrong' diastereoisomers to give a specific incorporation into tetronasin strengthens the evidence that (**4**) is the true tetraketide intermediate. According to our hypothesis there are a further nine chain extension cycles leading to the linear polyketide chain at the core of the tetronasin structure. We can therefore now proceed with greater confidence to synthesise larger proposed intermediate for further incorporation studies.

Note that these experiments with intact cells throw no significant light on the molecular basis of the stereochemical control exercised by the PKS system in carrying out the chain extension processes. It is clear that the isomers are not readily inter-converted so that only the correct one can be incorporated into the normal natural product. It is possible, however, that one of the 'wrong' diastereoisomers was indeed taken up by the PKS and converted by a sequence of chain extension cycles to a *stereoisomer* of the normal PKS product, and that this was then converted to an equivalent isomer of tetronasin. So little of any such stereoisomer would have been produced that it would be difficult to detect unless it followed the major natural product, tetronasin through its isolation procedure. It remains to be discovered, therefore, whether the PKS and subsequent enzymes involved in the tetronasin biosynthetic pathway are capable of elaborating structural and stereochemical analogues of the normal substrates to corresponding analogues of the normal product. These more searching questions relating to molecular recognition between substrate and PKS active sites will only be addressed by suitably controlled experiments carried out *in vitro* using isolated enzymes.

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***References and Notes***

1. See preceding paper: *Biosynthesis of Tetronasin: Part 1. Introduction and Investigation of the Diketide and Triketide Intermediates Bound to the Polyketide Synthase.* and references therein.
2. Numa, S. *Fatty Acid Metabolism and its Regulation;* New Comprehensive Biochemistry, 7, Elsevier: Amsterdam.1984; Chapter 5.
3. See following paper in this series: *Biosynthesis of Tetronasin: Part 3 Preparation of Deuterium Labelled Tri- and Tetraketides as Putative Biosynthetic Precursors of Tetronasin.*.
4. Jacobs, A.; Staunton, J.; Sutowski, A.C., *J. Chem. Soc. Chem. Comm.*, **1991**, 1113 and references therein.
5. Cane, D.E.; Prabhakaran, P.C.; Tan, W.; Ott, W.R., *Tetrahedron Lett.*, **1991**, 32, 5457.

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