Pergamon

Tetrahedron Letters, Vol. 35, No. 2, pp. 307-310, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $6.00+0.00

0040-4039(93)E0215-6

Biosynthesis of Tetronasin: Part 1 Introduction and Investigation of the Diketide and Triketide Intermediates Bound to the Polyketide Synthase.

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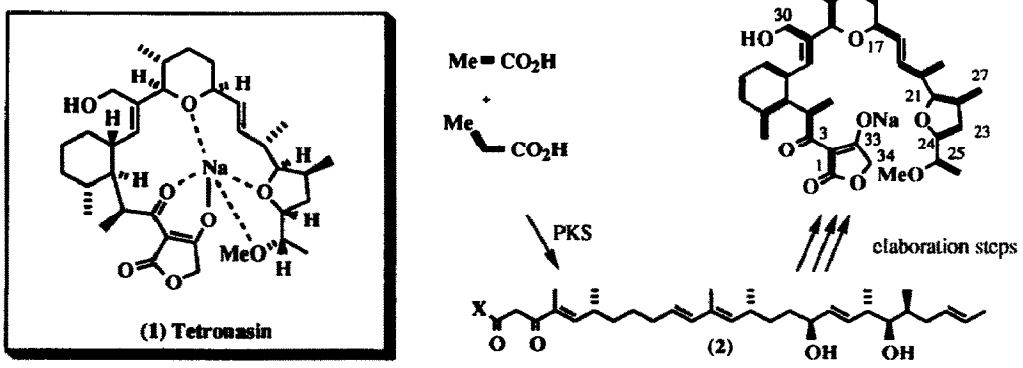
Abstract: *Incorporation experiments with deuterium labelled precursors show that the N-acetyl cysteamine analogues of the proposed diketide (4) and triketide (5) suffer degradation before incorporation into tetronasin (1) when administered early in the growth cycle. However intact incorporation of the triketide precursor into (1) was observed when it was administered to senescent cells.*

Tetronasin (ICI M139603) (1),[1] an antibiotic produced by *Streptomyces longisporoflavus*, is of commercial interest as a growth accelerator in cattle and as an antiparasitic agent.[2] As an ionophore, it shows a strong affinity for sodium ions which may be the basis of its action as an antibiotic.

In preliminary biosynthetic studies using primary precursors labelled variously with the stable isotopes ^{13}C, ^{2}H and ^{18}O, it has been shown that the carbon skeleton of (1) is derived from seven acetate and six propionate units linked head to tail in a way consistent with a polyketide pathway, as is indicated in Scheme 1.[3a] The O-methyl group arises from methionine, but the origin of the C_2-fragment C-33 + C-34 remains unclear. Experiments with precursors enriched with ^{18}O demonstrated that two C-O bonds of tetronasin are derived intact from the carboxy of acetate (at C-1, and C-17) and two from propionate (at C-3 and C-21).[3b] A further key piece of evidence, from experiments with deuterated precursors, is that the C-30 CH_2 group can be derived intact from the primary precursor pool showing that this residue is not oxidised to a carbonyl group at any stage of the biosynthesis.[3c]

On the basis of these results, the biosynthetic pathway outlined in Scheme 1 has been proposed.

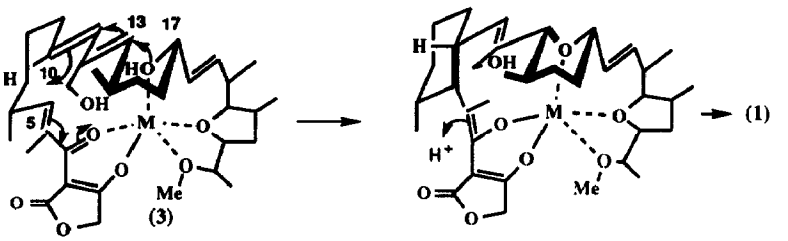
Scheme 1



In the early stages of this pathway, a typical linear polyketide intermediate (2) is assembled on a polyketide synthase (PKS) *via* a sequence of enzyme-bound intermediates. The acyl residue of (2) is then released from the enzyme, probably as the free acid or a thioester. In subsequent stages four rings are formed:

the tetrahydrofuran and tetronic acid residues at the methyl and carboxyl termini respectively, and the tetrahydropyran and cyclohexane rings in the middle regions of the chain. The formation of the first two rings can be accounted for by well-documented biosynthetic reactions but the generation of tetrahydropyran and cyclohexane residues poses a more intriguing problem.[3] A reaction has been proposed which leads to the simultaneous formation of both of these rings. This is illustrated in Scheme 2 for a possible late intermediate (3) in which the tetrahydrofuran and tetronic acid residues are already formed. The proposed reaction is without biochemical precedent, but a stereoelectronic analysis of the reaction suggests that it is mechanistically plausible. Thus examination of a model of (3) indicates that the various groups can be folded into a low energy conformation in which the centres to be linked are within bonding distance and the axes of the relevant orbitals are parallel and correctly aligned for simultaneous cyclisation of both six-membered rings. Moreover, this conformation of (3) could be stabilised by binding a metal ion (M) in a central cavity, which resembles that found in the antibiotic. In presenting this mechanism in Scheme 2 it is assumed for convenience that the formation of the tetrahydrofuran and tetronic acid rings precedes the concerted cyclisation, but the timing of the various elaboration steps is undetermined, and a variety of derivatives of (2) less elaborate than (3) could well undergo the proposed concerted cyclisation.

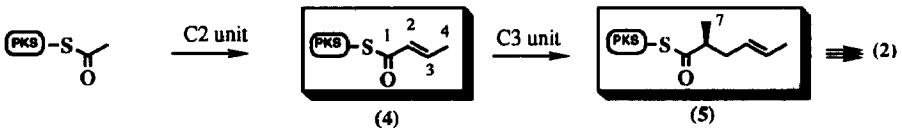
Scheme 2



In this and the succeeding papers we describe a series of collaborative studies aimed at testing these ideas on two fronts. Our first aim was to determine the structure of the product from the polyketide synthase by administering isotopically labelled analogues of shorter acyl chains which it was speculated might exist as enzyme-bound intermediates. Our second aim was to synthesise (3) and appropriate structural analogues to test the feasibility of achieving the proposed concerted biosynthetic cyclisation as a biomimetic synthesis *in vitro* without the relevant enzymes.

The proposed structure (2) for the final PKS product is one of many consistent with the strategy of the outlined biosynthesis. It was selected as the first candidate for investigation in our biosynthetic studies because it requires the fewest steps for elaboration to the final antibiotic. The array of structural features in (2) could reasonably be formed on a PKS, if it operates in an analogous way to a fatty acid synthase.[4] If the chain extension processes follow the processive mode, in which the functionality of each chain extension unit is completely established prior to the next extension cycle, the successive products of the first two chain extension cycles on the PKS will be crotonate (4) and the hexenoate (5) as shown in Scheme 3. In this paper we describe the results of our biosynthetic investigations of these two precursors.

Scheme 3



Firstly, crotonate doubly-labelled with ^{13}C at C-1 and C-2 was investigated as a precursor. It was administered to an intact culture of *S. longisporoflavus* as its *N*-acetyl cysteamine (NAC) thioester to aid transfer of the labelled acyl residue onto the appropriate thiol binding site of the tetronasin PKS, a ploy that has proved successful with a number of PKS systems operating in intact organisms.[5] After 3 days further growth the cells were harvested and the tetronasin extracted. Intact incorporation of the precursor should lead to selective enrichment at C-23 and C-24, and hence to the appearance of ^{13}C-^{13}C doublets together with the natural abundance singlets for those carbons in the ^{13}C nmr spectrum. Instead, all the carbons derived from

acetate gave rise to ^{13}C-^{13}C doublets with no selective enrichment at the expected sites. This pattern of enrichment is consistent with degradation of the test precursor, crotonate, to two units of acetate one of which would be doubly labelled with ^{13}C. Such degradation of crotonate is an essential feature of primary metabolism in which fatty acyl chains are degraded stepwise by way of β-oxidation to produce acetate units for a variety of purposes. The resulting acetate units are released as a coenzyme A esters, and so are correctly derivatised for incorporation by the PKS into the various acetate-derived units of tetronasin.

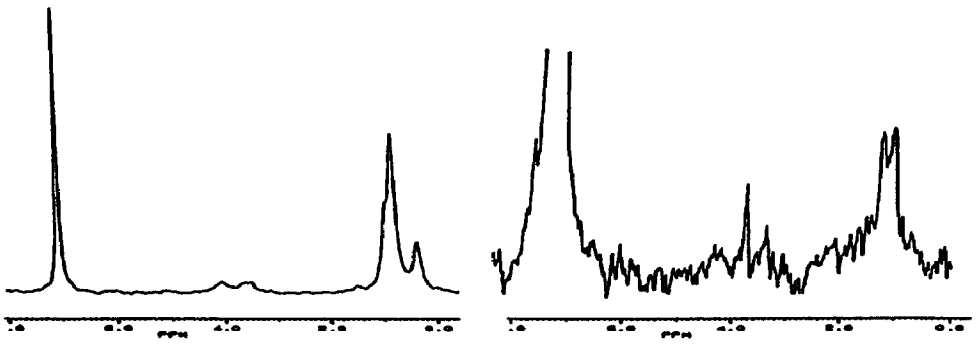
The failure of crotonate to be incorporated specifically at a level of enrichment high enough for detection by ^{13}C-^{13}C couplings arising from the two predicted carbons of tetronasin does not rule out the idea that this compound is an intermediate on the PKS. We therefore sought alternative protocols for the experiment which might produce a more favourable balance in the competition between random incorporation of isotope following fatty acid degradation of the test precursor to primary building blocks, and specific incorporation resulting from direct incorporation of an intact C_4 unit. In the interests of economy and simplicity of detection of isotopic labelling (by ^2H nmr) we switched to the use of deuterium as the isotopic label. Accordingly the NAC-thioester of [4-$^2H_1$, 2-$^2H_1$] crotonate was synthesised and administered to the organism at various stages of growth. The outcome in every experiment was the same: tetronasin was produced which showed a pattern of deuterium enrichment consistent with degradation of the precursor to deuterium labelled acetate; there was no evidence for selective enrichment with ^2H at C-25 and C-27 as would be expected for direct incorporation.

We therefore turned to the proposed triketide intermediate (**5**) in the hope that its NAC analogue would be more likely to show intact incorporation because it is a more 'committed' structure for molecular recognition by the PKS, and because its branched chain structure might make it less acceptable as a substrate for competing fatty acid degradation.

Synthetic routes, suitable for introducing deuterium at key sites appropriate for the biosynthetic study, were developed for the synthesis of this compound as a racemate and in both its (*R*)- and (*S*)- forms and are described elsewhere.[6] Initially the racemic form was employed in a survey of various feeding protocols. In choosing optimum conditions for specific incorporation we were guided by the observation that tetronasin production continues throughout the growth cycle after the usual induction period even when the cultures became senescent, whereas the production of certain fatty acid co-metabolites fell sharply as the cultures aged. We therefore reasoned that the enzymes of fatty acid degradation, which normally serve to degrade fatty chains derived from the food source to produce the acetate building blocks for biosynthesis of the specialised fatty acids, might also have become less active, thus increasing the chances of specific incorporation of our labelled PKS precursor into tetronasin.

The NAC derivative of the racemic form of (**5**), labelled with deuterium as a CD_3 group at C-7, was administered to a culture of *S. longisporoflavus* at the usual time early on in the growth cycle when tetronasin production was at a maximum (3 days after inoculation) and in a parallel experiment the same precursor was administered to senescent cells (10 days after inoculation). The two samples of tetronasin derived from these cultures gave deuterium nmr spectra shown in Figure 1. Spectrum (a), which is derived from the metabolite produced by young cells, shows a complex set of overlapping signals around δ 1 ppm. We suspected that this pattern was caused by incorporation of deuterium into all the methyl groups derived from C-3 of propionate. This could arise by efficient fatty acid degradation of the precursor to produce CD_3 labelled propionate with the label derived from C-7 of the administered precursor. This conclusion was vindicated by feeding CD_3 propionate at the same stage of growth, which produced labelled tetronasin with an essentially identical pattern of isotopic enrichment according to the ^2H nmr.

In contrast, the incorporation of the same precursor by senescent cells produced a very different result. Firstly, and not surprisingly given the low level of production of antibiotic at the time of feeding, the level of enrichment was much lower. Consequently the deuterium nmr spectrum of the labelled metabolite (spectrum (b)) was complicated by two signals at δ1.2 and 3.4 which are attributed to the natural abundance spectrum arising from the trace of ethanol in the $CHCl_3$ used as solvent. Significantly, however, instead of a complex set of signals around δ.1.0 indicating several labelled methyl groups, there was only one signal (at δ 0.9) which could be assigned to tetronasin, and this corresponded to the methyl position (C-27) predicted to be enriched following an intact incorporation of the acyl residue of (**5**). The signal at δ 0.5, which is characteristic of random incorporation and is well resolved from the ethanol signals, was absent.



(a) (b)

Figure 1: ^2H NMR of tetronasin (1)
(a) Following incorporation of NAC analogue of (5) on day 3 of growth
(b) Following incorporation of NAC analogue of (5) on day 10 of growth

Encouraged by this result we went on to test the (*R*)-isomer and (*S*)-isomers of the NAC analogues of (5) separately as potential precursors in senescent cultures. The (*S*)-isomer (which is predicted by our hypothesis to be the correct isomer for the biosynthesis) gave, as expected, positive incorporation results, similar to those observed for the racemate. The (*R*)-isomer gave a very much lower level of isotopic enrichment under equivalent conditions, showing that it is not an efficient precursor.

These results represent an important advance in our studies of tetronasin biosynthesis. Firstly, they show that the PKS follows the processive mode of chain assembly in the early chain extension cycles, as has been demonstrated for several other polyketide metabolites. Secondly, they demonstrate that senescent cultures are suitable for achieving specific incorporation with suppression of competing random incorporation caused by fatty acid degradation of precursors and so have opened the way to studies with more elaborate PKS intermediates. The senescent culture technique may prove effective with other polyketide producing organisms.

Acknowledgements: We thank the SERC for financial support.

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6. See one of the following papers in this series: *Biosynthesis of Tetronasin: Part 3 Preparation of Deuterium Labelled Tri- and Tetraketides as Putative Biosynthetic Precursors of Tetronasin.*.

(*Received in UK* 17 *August* 1993; *revised* 9 *November* 1993; *accepted* 12 *November* 1993)