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Biosynthesis of Tetronasin: Part 5. Novel Fluorinated and Non-fluorinated Analogues of Tetronasin *via* Intact Incorporation of Di-, Tri- and Tetraketide Analogue Precursors.

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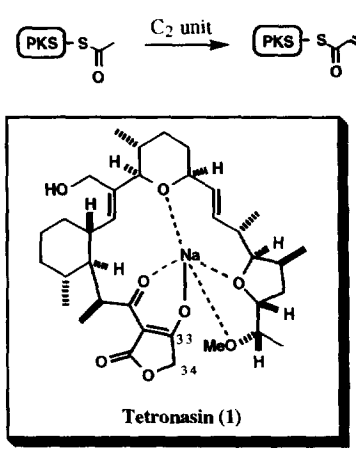
Abstract: *Incorporation experiments with both fluorine-labelled and unlabelled N-acetylcysteamine analogues (6), (7), (8), (9), (11), (13), (14) and (17) of the proposed di-, tri- and tetraketide precursors showed that the acyl residues are incorporated intact into tetronasin (1). Equivalent experiments with N-acetylcysteamine analogues (12), (15), (16) and (18) resulted in no detected intact incorporation into (1).* Copyright © 1996 Elsevier Science Ltd

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.

Preliminary biosynthetic studies,[3] using primary precursors labelled variously with the stable isotopes ^{13}C, ^{2}H and ^{18}O, have 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. The *O*-methyl group arises from methionine, but the origin of the C_2-fragment C-33 + C-34 remains unclear.



Tetronasin (1)

Scheme 1

A biosynthetic pathway has been proposed in which a typical linear polyketide intermediate (**2**) is assembled on a polyketide synthase (PKS) *via* a sequence of enzyme bound intermediates. In total twelve extension cycles are required to produce (**2**). The acyl residue of (**2**) is then released from the enzyme, probably as the free acid or a thioester, and, in subsequent stages, cyclisations and other modifications take place to produce tetronasin (**1**).

If the chain assembly steps follow the processive mode, in which, after each condensation, the attendant reductions and dehydrations occur prior to the next chain extension, then the intermediates formed in succession at the end of the first three chain extension cycles should be (**3**), (**4**) and (**5**), as indicated in Scheme 1. In two previous papers of this series[4] we described successful incorporation experiments, using ^{2}H-labelled synthetic acyl intermediates as *N*-acetylcysteamine (NAC) thioesters. These support the intermediacy of the proposed tri- and tetraketide intermediate structures.

It was decided to probe whether the PKS and subsequent enzymes involved in the tetronasin biosynthetic pathway are capable of elaborating structural analogues of the normal substrates to corresponding analogues of tetronasin. To this end, a selection of di-, tri- and tetraketide analogue precursors (**6**)-(**18**) were synthesised as their NAC-thioesters.[5] Each analogue was characterised by its own particular modification, which may have included the replacement of one its methyl groups by an ethyl, *iso*-propyl or benzyl moiety.

In order to inhibit voracious fatty acid degradation systems from degrading added precursors,[6] a further structural modification was devised for a selection of the analogues: the α-hydrogen was replaced by fluorine, which is capable of replacing hydrogen without noticeable stereochemical consequences. This modification was designed to prevent α, β-dehydrogenation of triketide analogues - the first step in the fatty acid degradation sequence. In addition, it was hoped that the detection of any metabolite, which had incorporated a fluorinated precursor, would be facilitated by the high sensitivity of fluorine in ^{19}F n.m.r. spectroscopy.

Feeding experiments were carried out using cultures at two stages of growth. The 'early' stage was at three days after inoculation, at the onset of rapid production of tetronasin, and the 'late' stage after ten days, when the cultures became senescent. 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 in order to produce the acetate building blocks for biosynthesis of the specialised fatty acids) may also have become less active at this stage, thus increasing the chances of specific incorporation of our analogue precursor into tetronasin.

Each analogue precursor was administered to an intact culture of *S. longisporoflavus* as its 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.[7] Just prior to precursor administration the pH of the culture was raised slightly, to pH 8, by the addition of 10% sodium hydroxide. This has been shown to increase the amount of incorporation,[4] probably by increasing the porosity of the cell membranes. Other techniques were employed in attempts to increase intact incorporation, including the co-feeding of 2,6-*O*-dimethyl-β-cyclodextrin and co-feeding the β-oxidation enzyme inhibitors 3-(tetradecylthio)propanoic acid and 4-pentynoic acid.[8] Three days after administering the precursors, the cells were harvested and tetronasin extracted.

Only partial purification of the extracted tetronasin, by one flash chromatography column, was attempted in order not to lose any tetronasin analogue also present. The extracts were then analysed by high resolution electrospray mass spectroscopy[9] and the results summarised in the Table. The feeding experiments with precursor analogues (**6**), (**7**), (**8**), (**9**), (**11**), (**13**), (**14**) and (**17**) each gave a peak in the mass spectrum which corresponded to the expected analogue of tetronasin, resulting from intact incorporation (*e.g.* Figure 1). It is therefore deduced that tetronasin analogues (**19**), (**20**), (**21**), (**22**) and (**23**) have been successfully biosynthesised. The likely structures of the analogue metabolites are shown in Scheme 2 and each modification of tetronasin's natural structure is highlighted. The heights of analogue MK^+ peaks, expressed as a percentage of the natural tetronasin-K^+ peak height, suggest very low levels of incorporation. When comparing these values, it must be remembered that in the late feedings most of the tetronasin had been produced prior to administration of the analogue precursor. Measured accurate masses were in agreement with theoretical values, and the calculated deviations are listed in the Table.

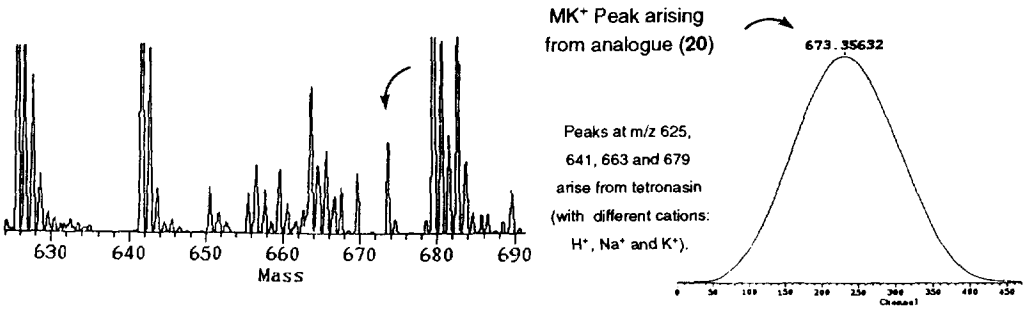
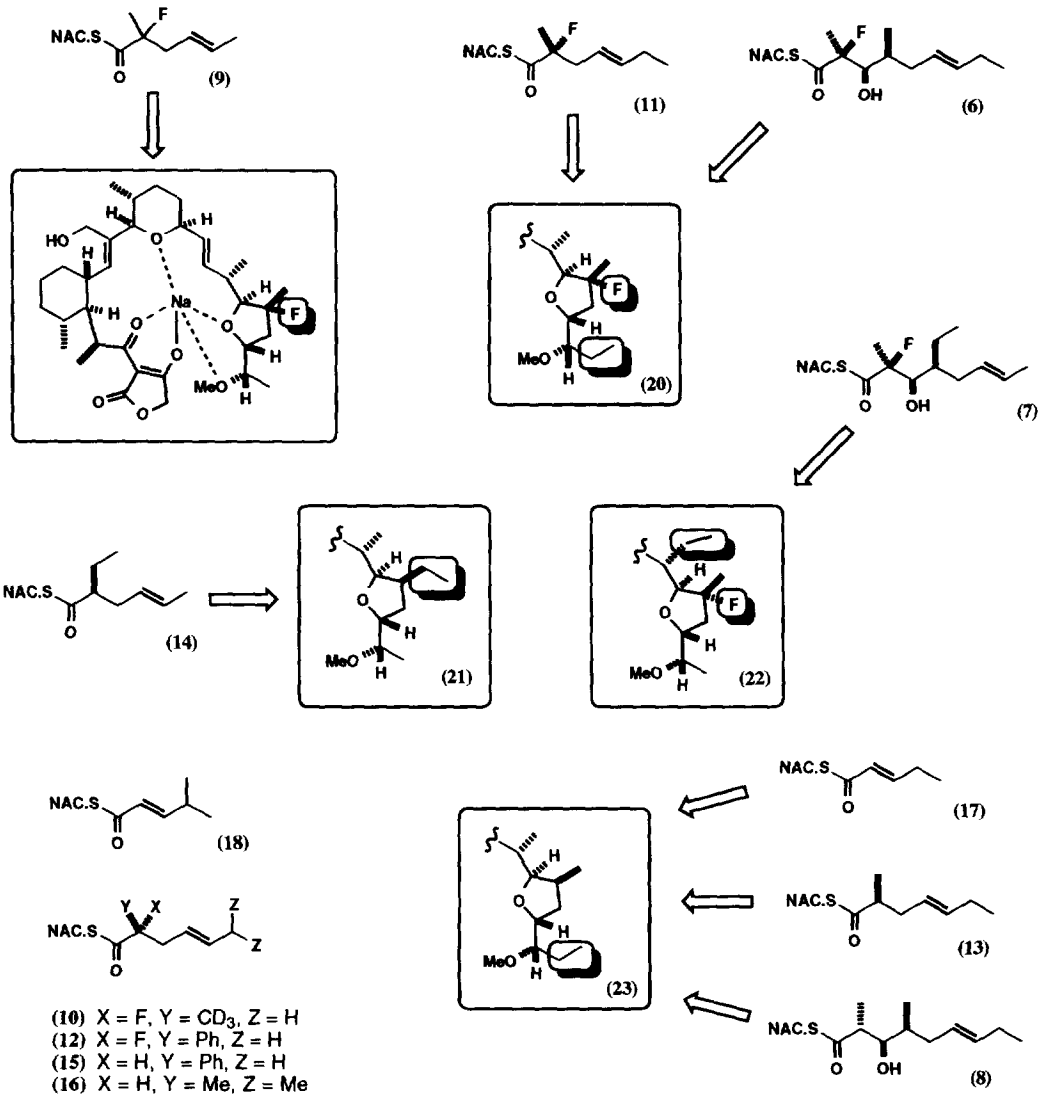


Figure 1: Mass spectrum[9] of tetronasin, containing analogue (**20**), from feeding of (**6**)

Scheme 2



(10) X = F, Y = CD_3, Z = H
(12) X = F, Y = Ph, Z = H
(15) X = H, Y = Ph, Z = H
(16) X = H, Y = Me, Z = Me

As one might expect, the precursor analogues containing the smaller unnatural groups, *i.e.* F instead of H, or ethyl in place of methyl, have undergone intact incorporation, whereas analogues of tetronasin with larger unnatural groups, such as benzyl or *iso*-propyl, are not observed.

All the extracts derived from ^{19}F-labelled precursor feedings were examined by ^{19}F n.m.r. The ^{19}F n.m.r. spectrum of extract from the precursor (**9**) feeding apparently showed a well-resolved doublet of sextets at 1 ppm upfield of the precursor. However, this signal did not fit analogue (**19**) since, at lower field n.m.r., the doublet's coupling constant appeared to decrease and the signal coalesced into a broad septet. We therefore suspect that these signals are due to impurities, possibly derived from the association of remaining precursor with an unknown chiral substrate, giving rise to the two separate sextet peaks, one for each enantiomer. Further peaks were observed in the ^{19}F spectra of other samples, but in no case were we satisfied that they could be assigned, and were probably due to impurities rather than the particular analogue metabolite. The electrospray mass spectroscopy provides strong evidence for the formation of tetronasin analogues but ^{19}F n.m.r. appears not to possess the sensitivity needed to detect the low levels produced.

Table of Results

Analogue	Stage of Growth	Total fed (mg/50ml)	Co-fed	Unnatural moieties	M.S. MK^+ (amu)*	Deviation (ppm)	Rel. peak height (%)
Tetra **6**	Early	15	–	F, Et	673.34711	-6.98	0.4
	Late	15	–		673.35046	-2.00	0.4
Tetra **7**	Late	5	–	F, Et	673.35016	-2.45	0.03
Tetra **8**	Late	8	–	Et	655.36920	+12.16	0.3
Tri **9**	Late	28	–	F	659.33148	-7.09	0.7
Tri **10**	Early	20	–	F, CD_3	no peak	–	0
	Late	20	–		no peak	–	0
	Late	20	**A, B, C**		no peak	–	0
Tri **11**	Early	25	–	F, Et	673.35156	-0.37	0.1
	Late	24	–		no peak	–	0
	Late	100	–		673.34961	-3.26	0.5
	Late	25	**A**		673.35522	+5.07	0.2
	Late	24	**C**		no peak	–	0
Tri **12**	Late	15	–	F, CH_2Ph	no peak		0
Tri **13**	Early	21	–	Et	655.36664	+8.26	0.2
	Late	21	–		655.35999	-1.89	0.3
Tri **14**	Late	15	**A, B, C**	Et	655.36346	+3.34	0.9
Tri **15**	Late	20	**A, B, C**	CH_2Ph	no peak	–	0
Tri **16**	Late	12	**A, B, C**	*i*-Pr	no peak	–	0
Di **17**	Early	21	–	Et	655.35730	-6.00	0.5
	Late	21	–		655.36200	+1.18	0.2
Di **18**	Late	12	**A, B, C**	*i*-Pr	no peak	–	0

A = 3-(tetradecylthio)propanoic acid, **B** = 4-pentynoic acid, **C** = 2,6-*O*-dimethyl-β-cyclodextrin. * M.S. tetronasin-K^+ 641.4802

In summary, the first examples of intact incorporation of structural analogues of advanced PKS-bound biosynthetic intermediates are presented. Previously, an analogue of the PKS-bound diketide precursor to avermectin has been incorporated into an avermectin analogue, *via* feeding to a blocked mutant of *S. avermitilis*.[10] Our observations of intact incorporations employing the wild-type tetronasin-producer, imply that the biosynthetic enzymes were able to load on the analogue, in competition with the endogenous substrate, and to 'turn over' all the intermediates in the analogue's subsequent bio-conversion. The estimated levels of incorporation of between 0.1 and 1 % (even for the late feedings) therefore appear significant, and have implications for our understanding of the substrate specificity of the PKS.

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