Biosynthesis of norsolorinic acid and averufin: substrate specificity of norsolorinic acid synthase

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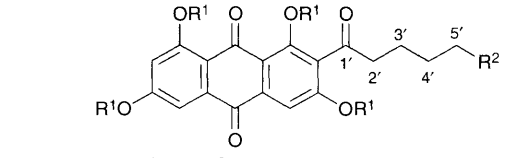
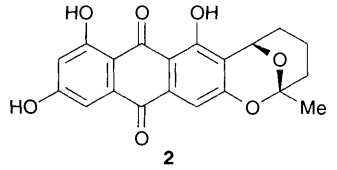
The *N*-acetylcysteamine thioesters of pentanoic and 6-fluorohexanoic acids are metabolised by cultures of *Aspergillus parasiticus* ATCC 24690 to produce side chain modified analogues or norsolorinic acid in which the normal hexanoyl side chain is replaced by pentanoyl and 6-fluorohexanoyl moieties respectively.

Norsolorinic acid **1** is the first isolable intermediate on the biosynthetic pathway[1] leading *via* averufin **2** to the potent hepatocarcinogenic mycotoxin aflatoxin B_1. It is produced as the first enzyme-free intermediate of the corresponding polyketide synthase, norsolorinic acid synthase (NSAS). While norsolorinic acid can formally be regarded as a decaketide formed by successive condensations of an acetate starter unit with nine malonates,[2] the demonstration that [1-^{13}C]-hexanoic acid was incorporated intact into averufin provided compelling evidence that averufin and, by implication, norsolorinic acid were in fact octaketides formed by condensation of a hexanoate starter with seven malonates.[3] On the basis of more recent studies which have shown the efficient incorporation of ^{18}O label from [1-^{13}C, $^{18}O_2$]-hexanoate and ^{13}C label from [1-^{13}C]-3-oxo-octanoate into averufin it has been proposed that the initial hexanoate starter unit is generated by a specialised fatty acid synthase (FAS) that provides this unit either separately to NSAS or as part of a larger FAS/NSAS fusion (Scheme 1). As attempts to obtain a cell-free preparation demonstrating NSAS activity have so far proved unsuccessful, we have investigated the substrate specificity of NSAS by carrying out incorporation studies with a variety of analogues of the essential hexanoate starter unit in cultures of *Aspergillus parasiticus* ATCC 24690.



1 R^1 = H; R^2 = Me **9** R^1 = Me; R^2 = Me
7 R^1 = R^2 = H **10** R^1 = Me; R^2 = H
8 R^1 = H; R^2 = CH_2F **11** R^1 = Me; R^2 = CH_2F



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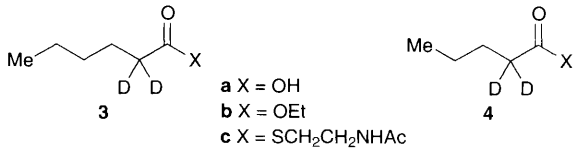
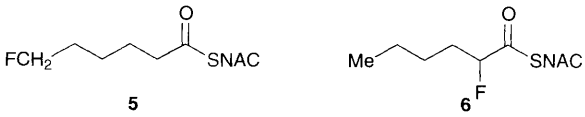


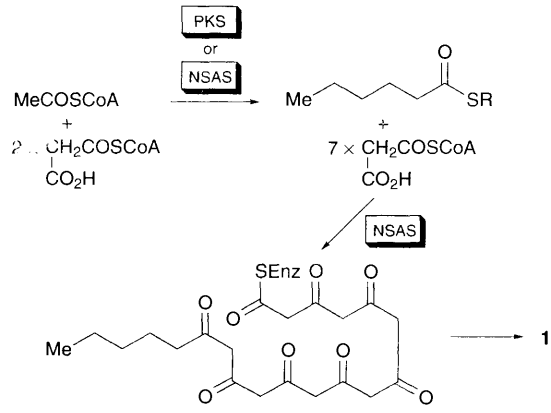
3 **a** X = OH **b** X = OEt **c** X = SCH_2CH_2NHAc **4**



5 **6**

In initial experiments, the efficiency of hexanoate as a starter unit was compared by feeding [2-$^2H_2$]-hexanoate in the form of the free acid **3a**, the ethyl ester **3b** and the *N*-acetylcysteamine (NAC) thioester **3c**. All of these forms have been used to study the incorporation of polyketide starter units and assembly intermediates. While thioesters have been particularly valuable for the intact incorporation of assembly intermediates,[5] no clear advantage has been generally noted for starter units. However, as shown in Fig. 1, a clear difference is found for norsolorinic acid biosynthesis. Intact incorporation is demonstrated by the incorporation of ^{2}H into the 2′-methylene signal at δ 2.75 in the ^{2}H NMR spectrum of norsolorinic acid (obtained on the derived tetramethyl ether) while indirect incorporation *via* degradation to [$^2H_2$]acetyl CoA results in enrichment of the 6′-methyl at δ 0.95. Whereas the free hexanoic acid **3a** showed a trace amount of intact incorporation and a substantial amount of indirect labelling, the NAC thioester **3c** gave a much higher level of enrichment (*ca.* 40%†) which was almost entirely due to intact incorporation. Interestingly, the ethyl ester **3b** gave a good, but lower, level of enrichment due to intact incorporation against a small but significant background of indirect incorporation. These results clearly demonstrate the preference for the NAC thioester in this case and are in good agreement with analogous experiments with averufin.[3]

To examine whether norsolorinic acid synthase displays any flexibility for the starter units it accepts, a series of parallel experiments were carried out with butyrate, pentanoate, heptanoate and octanoate, all labelled at C-2 with ^{2}H. No intact incorporation of label from butyrate, heptanoate or octanoate was observed, but the results for pentanoate incorporation were essentially identical to those observed for hexanoate. Thus it appears that NSAS can accept hexanoate or pentanoate with similar efficiency, but not shorter or longer fatty acids.



Scheme 1 Proposed assembly of the polyketide precursor of norsolorinic acid

The presence of the resulting analogue **7** along with the co-produced norsolorinic acid was evident from ions at *m/z* 412, 413 and 414 in the high resolution mass spectrum of the derived tetramethyl ethers which correspond to **7** labelled respectively with zero, one and two ^2H atoms. In addition, signals due to the C_5 side chain could be seen in the 500 MHz ^1H NMR spectrum of the tetramethyl ethers **9** and **10**. HPLC-MS analysis of the mixture showed the presence of two major components in a 3 : 2 ratio with the appropriate molecular weights for **9** and **10**. A small amount (*ca.* 1 mg) of the tetramethyl ether **10** was isolated by preparative HPLC. It showed an identical UV spectrum to **9** and the 500 MHz ^1H NMR spectrum showed the anticipated signals for the C_5 side chain. [δ_H 2.77 (2 H, t, *J* 7.5 Hz, 2′-H_2), 1.68 (2 H, pent, *J* 7.5 Hz, 3′-H_2), 1.38 (2 H, sext, *J* 7.5 Hz, 4′-H_2) and 0.93 (3 H, t, *J* 7.5 Hz, 5′-H_3).] High resolution mass spectrometry confirmed the molecular formula to be $C_{23}H_{24}O_7$. (Found, M 412.1515; calculated M 412.1522.)

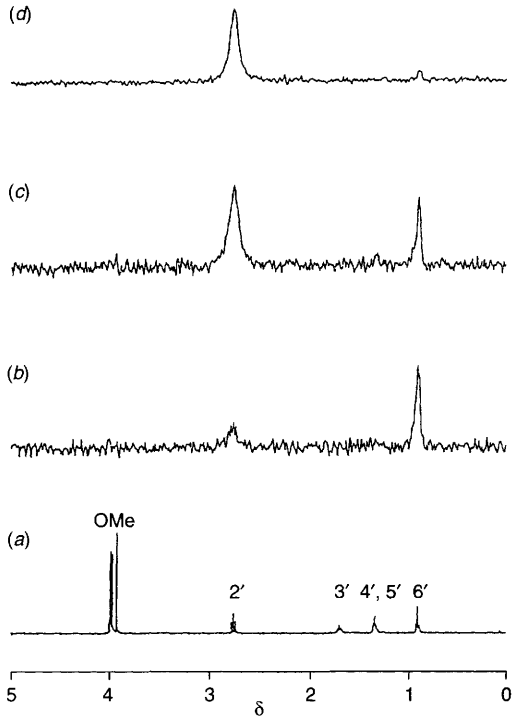


Fig. 1 0–6 ppm regions of (*a*) the 400 MHz ^1H NMR spectrum of norsolorinic acid tetramethyl ether, and the 62 MHz ^2H NMR spectra enriched from feeding [2-$^2H_2$]-hexanoate in the form of (*b*) the free acid, (*c*) the ethyl ester and (*d*) the NAC thioester.

Table 1 Incorporation of labelled thioesters in *A. parasiticus*.

Substrate[a]	Yield of **1** (mgl^{-1})	Incorporation (%)[d]
control	290	—
3c	44	40
4c	45[b]	30
5	70[c]	36
6	16	0

[a] All precursors were added at a level of 50 mg/100 ml culture. [b,c] Combined yields of **1** and **7**, and **1** and **8** respectively. [d] Estimated from mass spectra and NMR of the derived tetramethyl ethers.

Encouraged by this evidence of flexibility in NSAS with regard to the starter group, the NAC thioesters **5** and **6** of 6-fluoro and 2-fluoro-hexanoic acids were prepared (by treatment of the corresponding hydroxyacids with DAST) and fed to cultures of *A. parasiticus*. For the 6-fluorohexanoate feed, the norsolorinic acid tetramethyl ether was isolated as before and analysed by mass spectrometry. This showed the presence of major amounts of a new metabolite with a molecular weight of 444 corresponding to the tetramethyl ether **11** of 6′-fluoro-norsolorinic acid **8**. ^{19}F NMR spectroscopy showed a signal at δ −218.2 with the expected second order multiplet for the terminal CH_2F group which appeared in the ^1H NMR spectrum as a doublet of triplets (*J* 47.3 and 6.1 Hz) at δ 4.46. Integration of the ^1H NMR signals indicated that **8** was present to the extent of *ca.* 36% of the mixture. Again, this was confirmed by HPLC analysis of the isolated mixture which allowed the isolation of a small amount of **11**. High resolution mass spectrometry confirmed the molecular formula. (Found, M 444.1579; $C_{24}H_{25}O_7F$ requires M 444.1584.)

Interestingly, the 2-fluorohexanoate gave no evidence of incorporation, and a dramatic decrease in the yield of **1** is observed. This could be due to degradation of **6** to produce highly toxic 2-fluoroacetate. However, an alternative explanation could be that **6** is acting as a specific inhibitor of the synthase. In fact, while compounds **3c**, **4c** and **5** are all efficiently incorporated, in each case a marked lowering of norsolorinic acid production is observed (Table 1), suggesting that they may cause some inhibition of NSAS. Further clarification of this will require isolation of the purified synthase.

Analogous experiments are being carried out in an averufin-producing strain of *A. parasiticus* and will be reported in full elsewhere. However, analysis of the ^2H NMR spectra of averufin **2** enriched from feeding the NAC thioesters of [2-$^2H_2$]- and [4-$^2H_2$]-hexanoate indicate that both the 2′- and 4′-methylenes in **2** retain both hydrogens from the hexanoate starter. These results, along with the previously demonstrated retention of all three hydrogens from [2-$^2H_3$]acetate on incorporation into averufin,[6] rule out the possible intermediacy[7] of dehydro-derivatives of norsolorinic acid in its further conversion to averufin.

Footnote

† Estimated by mass spectral and ^1H NMR analysis of the isolated norsolorinic acid tetramethyl ether isolated after feeding [1-^{13}C, 2-$^2H_2$]-hexanoyl NAC thioester.

References

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