Biosynthesis of Nonactin. The Role of Acetoacetyl-CoA in the Formation of Nonactic Acid

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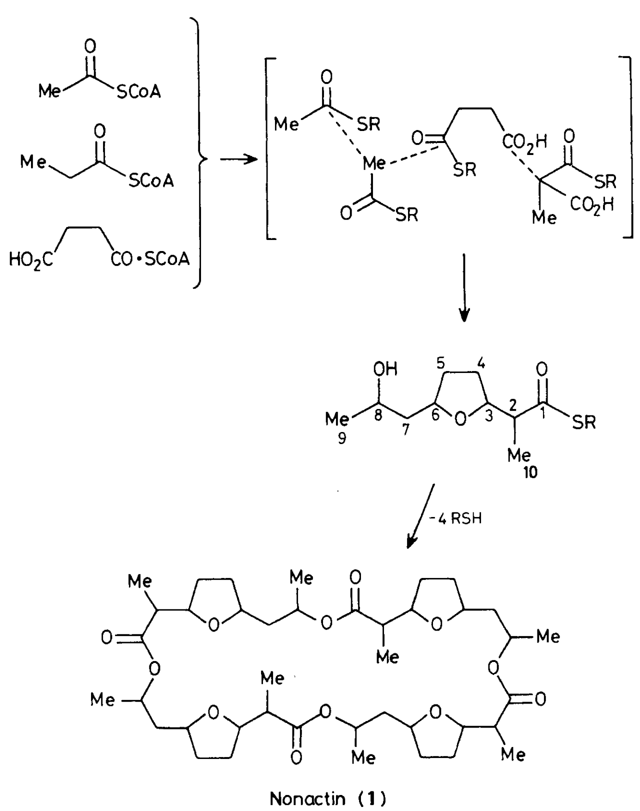
The incorporation of ethyl [1,2,3,4-$^{13}C_4$]- and ethyl [1,3-$^{13}C_2$]-acetoacetate into nonactic acid in cultures of *Streptomyces griseus* revealed the incorporation of several C_2 units derived from acetyl-CoA, as well as a single intact C_3 unit at C(1)–C(2)–C(10).

The carbon skeleton of nonactin (**1**) is assembled in *Streptomyces griseus* from two units of acetyl-CoA, one of succinyl-CoA, and one of propionyl-CoA, as revealed by ^{13}C labelling experiments.[1,2] The familiar concept of a 'starter unit' is, however, obscure in this case because of the unusual role of succinate as a precursor of the complete tetrahydrofuran ring in each nonactic acid subunit[2] (see Scheme 1). Thus chain assembly could be initiated with acetyl-CoA, or with succinyl-CoA. We decided to investigate the order in which the building blocks are assembled and focussed initially on the possibility that two units of acetyl-CoA might be combined to afford acetoacetyl-CoA. This C_4 unit could then combine with succinyl-CoA in a process involving an overall decarboxylative condensation. Finally, methylmalonyl-CoA might be added, again *via* decarboxylative condensation, to complete the assembly process (see Scheme 2). To test these ideas the incorporation of labelled acetoacetate into nonactin (**1**) has been studied. In particular, we wished to show whether or not an intact C_3 unit from C(2)—C(4) of acetoacetyl-CoA could be introduced at C(9)—C(7) in nonactic acid.

Firstly, therefore, ethyl [1,3-^{14}C]acetoacetate was administered to cultures of *S. griseus* to a final concentration of 20 mM. The specific incorporation of this labelled material was 9.9% based on the assumption that four moles of acetoacetate are incorporated into each mole of nonactin. This result indicated that the precursor, presumably in the form of a free acid, released by the action of esterases in the fermentation broth, can enter the cells, become activated as a coenzyme-A thioester, and participate efficiently in the biosynthesis. In order to demonstrate an intact incorporation, two feeding experiments were then performed using ethyl [1,2,3,4-$^{13}C_4$]acetoacetate (prepared from [1,2-$^{13}C_2$]acetate, each site 90 atom % ^{13}C) diluted firstly 1:3, and secondly 1:5, with unlabelled material.



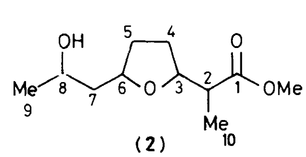
Nonactin (**1**)

Scheme 1

The nonactin isolated from each experiment was then converted by methanolysis ($MeOH–H_2SO_4$) into methyl nonactate (**2**), which could be rigorously purified by chromatography. An analysis of the ^{13}C n.m.r. spectra of these labelled samples revealed in each an identical pattern of enrichments, but not that expected based on our earlier considerations.

In addition to the normal one-dimensional ^{13}C n.m.r. spectra, and in a desire to separate clearly the effects of chemical shift differences from the effects of coupling, the two-dimensional ^{13}C homonuclear *J*-resolved spectrum of the labelled methyl nonactate was also recorded. Submatrix columns taken from the contour plot, at the chemical shifts corresponding to specific resonances in the ^{13}C n.m.r. spectrum, are shown in Figure 1. Clearly an intact C_2 unit has been incorporated at C(9)–C(8), at C(3)–C(4), and at C(5)–C(6), which is entirely consistent with the breakdown of acetoacetyl-CoA to acetyl-CoA, processing of the latter *via* the Krebs cycle to succinate, and assembly of these units into nonactic acid, as indicated in Scheme 1. However, the spectrum also reveals an intact C_3 unit at C(1)–C(2)–C(10). The singlet due to C(2) is surrounded by a doublet of doublets (*J* 57 and 34 Hz) as well as two doublets ($J_{1,2}$ 57, $J_{2,10}$ 34 Hz). Moreover, each of the signals for C(1) and C(10) also show a doublet of doublets due to one-bond ($J_{2,10}$ 34, $J_{1,2}$ 57 Hz) and two-bond ($J_{1,10}$ 3 Hz) coupling.

This pattern is consistent with a mixture composed of doubly labelled molecules, *and* a large proportion of molecules containing three contiguous ^{13}C labels at these positions. This latter result cannot be explained by any process involving the cleavage of acetoacetyl-CoA into acetyl-CoA. We, therefore, conducted another feeding experiment using ethyl [1,3-$^{13}C_2$]acetoacetate diluted 1:4 with unlabelled material. The ^{13}C n.m.r. spectrum of the derived methyl nonactate revealed two enriched doublets† at C(1) and C(2)



(**2**)

† The height of these signals was approximately 10% that of the natural abundance singlets.

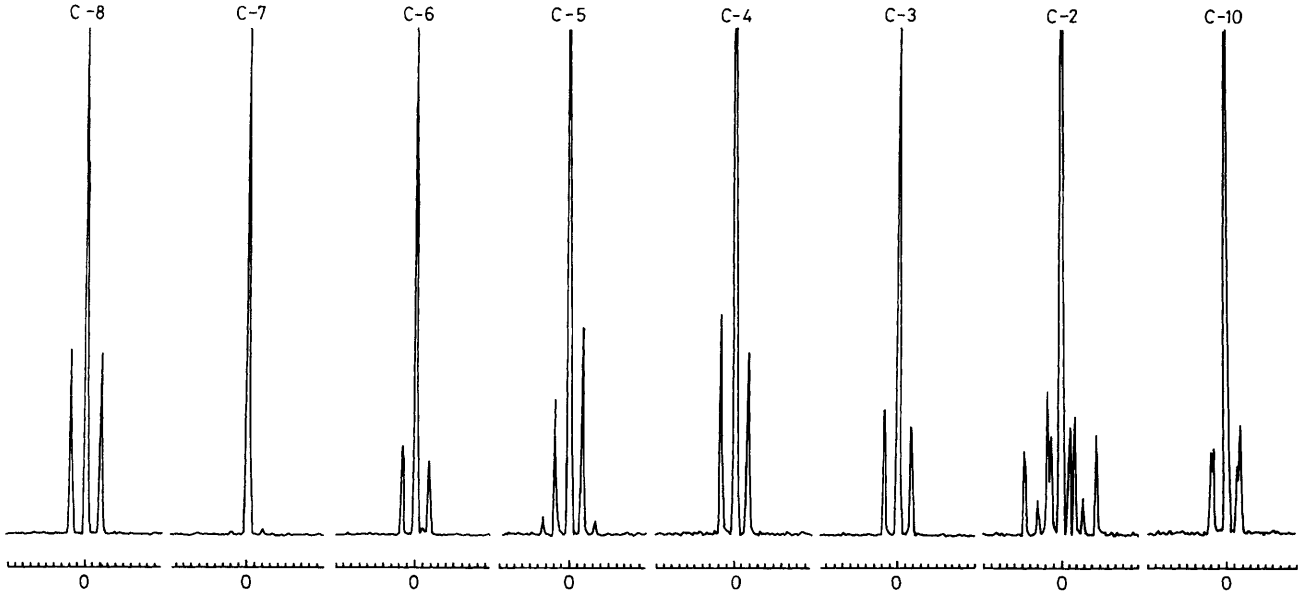
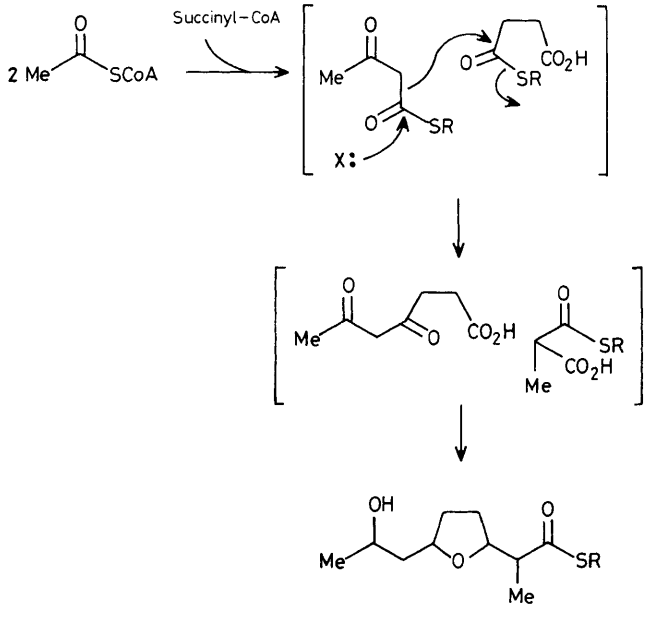
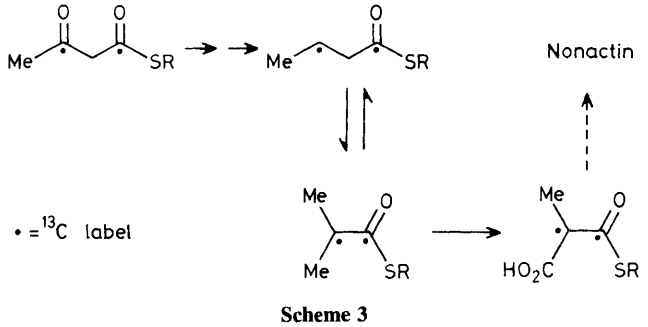


Figure 1. Submatrix columns from the contour plot of a ^{13}C-homonuclear *J*-resolved spectrum of labelled methyl nonactate (**2**) biosynthesized from ethyl [1,2,3,4-$^{13}C_4$]acetoacetate. Spec. freq. = 90.5 MHz, SW2 = 7246 Hz, SW1 = 113 Hz, spectral size (F_2) = 8K, spectral size (F_1) = 64 W zero filled to 128 W. Transformed with unshifted sine-bell function. Resonances due to C(8), C(7), C(2), C(10), C(3), C(4), C(5), and C(6) are shown. Plot limits ± 100 Hz. Scale divisions represent 10 Hz.



Scheme 2



Scheme 3

($J_{1,2}$ 57 Hz), indicating that the ^{13}C labels are now directly bonded, and therefore that an intramolecular rearrangement of the linear C_4 unit had occurred *in vivo*.

Whilst at present we cannot define the stage at which a rearrangement has occurred, it is more than conceivable that these results are related to recent observations[3–5] of a metabolic pathway in other Streptomycetes which leads to the overall interconversion of n-butyryl-CoA and isobutyryl-CoA with oxidation of the latter to (*S*)-methylmalonyl-CoA, and which implicate now the direct intramolecular rearrangement of a linear C_4 and iso-C_4 fatty acid coenzyme-A thioester (Scheme 3). Further experiments are underway to test this proposal.‡

Also, the ability of acetoacetyl-CoA to enter efficiently one area of *S. griseus* metabolism without prior cleavage to acetyl-CoA, together with the complete absence of evidence for an intact C_3 unit at C(9)–C(8)–C(7) in (**2**), indicates strongly (but does not prove) that acetoacetyl-CoA is not directly involved in nonactic acid biosynthesis.

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‡ An alternative explanation could invoke the well known conversion of succinyl-CaA into methylmalonyl-CoA. However, this would require the terminal oxidation of a C_4 fatty acid, which to the authors' knowledge is without precedence to date.