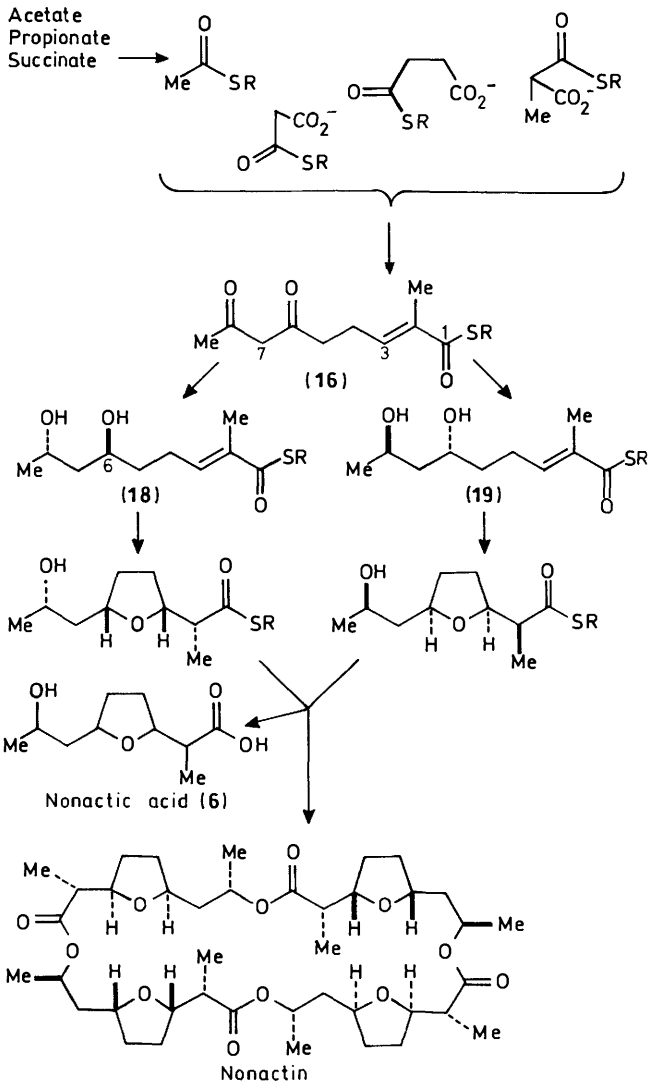
On the Biosynthetic Origins of the Hydrogen Atoms in the Macrotetrolide Antibiotics: and their Mode of Assembly Catalysed by a Nonactin Polyketide Synthase

Doreen M. Ashworth, Carole A. Clark, and John A. Robinson *
Chemistry Department, The University, Southampton SO9 5NH, U.K.

The biosynthetic origins of the hydrogen atoms in the macrotetrolide antibiotic nonactin have been studied by following the incorporations of deuterium and carbon-13 enriched acetate, propionate, and succinate into the antibiotic, using whole cell cultures of *Streptomyces griseus*. Based on these and other data two plausible mechanisms are proposed to explain the formation of (+)- and (−)-nonactic acids from these precursors, catalysed by a putative nonactin polyketide synthase multienzyme complex.

The macrotetrolide antibiotic nonactin (**1**) is a well known product[1] of the secondary metabolism of numerous strains of *Streptomyces*, and yet its constitutional simplicity, arising from its formal derivation by tetramerization of (+)- and (−)-nonactic acids (**6**) [in a stereospecific (+)(−)(+)(−) fashion], belies several interesting problems concerning its biosynthesis from the primary metabolites acetate, propionate, and succinate. Of special interest is the mechanism by which these building blocks are assembled to generate the carbon backbone of (**6**) on a putative multienzyme template, which we refer to here as the nonactin polyketide synthase (PKS) complex. The pattern of this assembly, indicated by isotopic labelling experiments[1–3] with carbon-13 enriched precursors, as well as a plausible biosynthetic pathway, are shown in Scheme 1. This involves the rare use of succinate as an intact four-carbon fragment, to provide the carbon framework of the tetrahydrofuran ring, in addition to the derivation of a three-carbon unit, C(7)-C(9), from two molecules of acetate. On the other hand, no information is available on the order in which the precursors are joined together, nor on the mechanisms of assembly, although labelling experiments with carbon-13 and oxygen-18 enriched precursors[1,3] did show that the C(3)–O bond is formed during closure of the tetrahydrofuran ring, consistent with the Michael addition by a 6-hydroxy group onto an enone system, at some stage in the biosynthesis. As a means of probing these assembly processes we have adopted an approach, well established in other systems,[4] that involves the incorporation of specifically *deuteriated* precursors into the antibiotic, in order to define the biosynthetic origins of the hydrogen atoms, and the details of this work are described and discussed below.

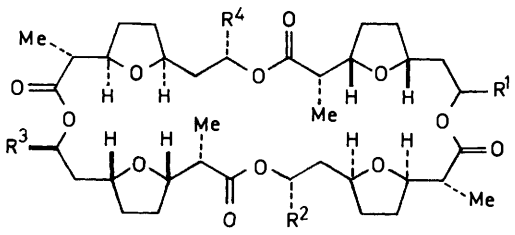


Scheme 1. The proposed biosynthetic pathway to nonactin. SR = SCoA or S-Enzyme *in vivo* see Schemes 2 and 3

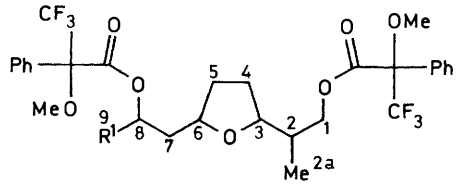
Results

Sodium [1-^{13}C, 2-$^{2}H_3$]- *and* [2-^{13}C, 2-$^{2}H_3$]-*Acetates*.—A standard protocol for feeding experiments with the nonactin producing organism has been established in our earlier work,[1] and involves the administration of labelled precursor to the fermentation broth, followed later by extraction of the antibiotic, mainly as a mixture of nonactin (**1**), monactin (**2**), dinactin (**3**) with smaller amounts of trinactin (**4**) and tetranactin (**5**). This mixture is reduced by $LiAlH_4$, and the diols formed are converted into the bis-MTPA * derivatives (**8**)—(**11**), each of which can be isolated in pure form by h.p.l.c. on silica columns.[1] In separate experiments, sodium[2-^{13}C, 2-$^{2}H_3$]- and [1-^{13}C,2-$^{2}H_3$]-acetates were fed to shake cultures of *S. griseus* ETHA7796, batchwise during the period of antibiotic

* MTPA = MeO(Ph)CF_3CCO.



$R^1 = R^2 = R^3 = R^4 = Me$	Nonactin	(1)
$R^1 = Et$, $R^2 = R^3 = R^4 = Me$	Monactin	(2)
$R^1 = R^3 = Et$, $R^2 = R^4 = Me$	Dinactin	(3)
$R^1 = R^2 = R^3 = Et$, $R^4 = Me$	Trinactin	(4)
$R^1 = R^2 = R^3 = R^4 = Et$	Tetranactin	(5)



R^1 = Me (**8**) derived from (+)-(**6**)
R^1 = Me (**9**) derived from (−)-(**6**)
R^1 = Et (**10**) derived from (+)-(**7**)
R^1 = Et (**11**) derived from (−)-(**7**)

to ^{13}C attached to ^{2}H rather than ^{1}H, the so-called α-isotope induced shift (given in Table 1).

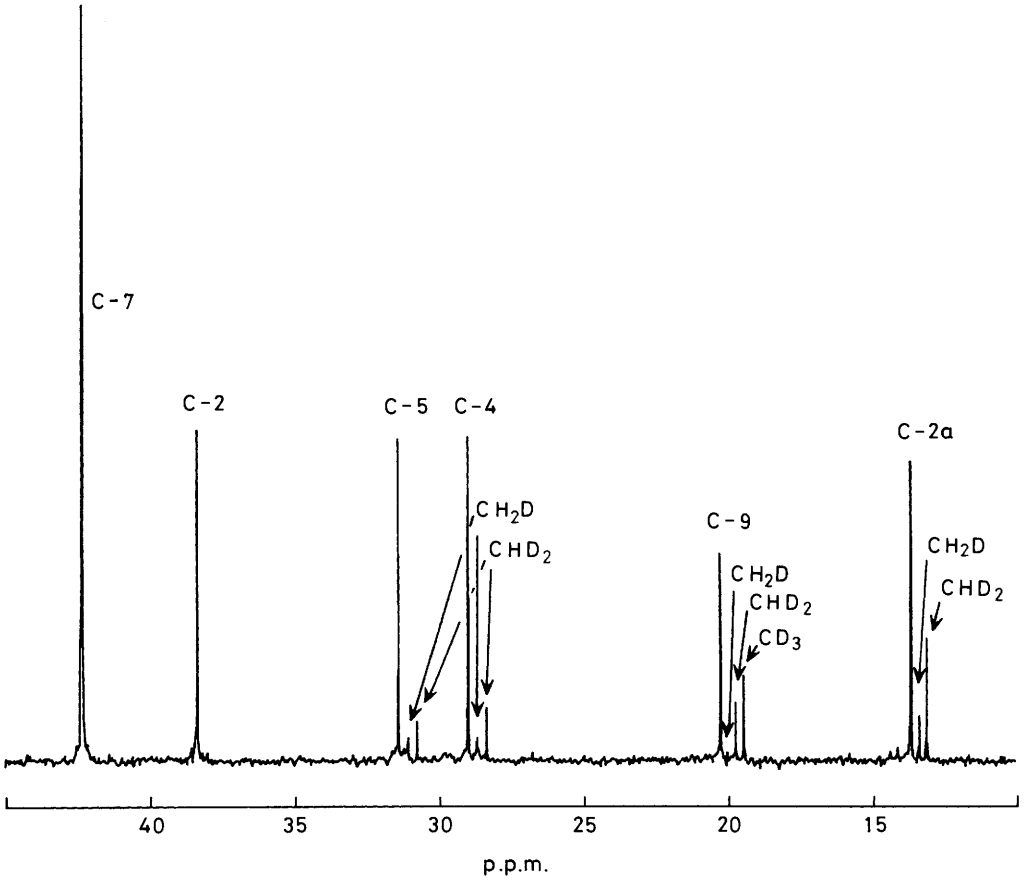
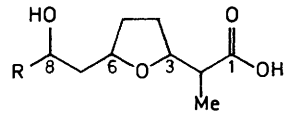


Figure 1. A section of the $^{13}C\{^{1}H,^{2}H\}$ n.m.r. spectrum of (**8**), derived from the feeding experiment with [2-^{13}C, 2-$^{2}H_3$]acetate. The natural abundance ^{13}C signals were assigned in earlier work.[1] For acquisition and processing details, see Experimental section and Table 1



(**6**) R = Me Nonactic acid
(**7**) R = Et Homononactic acid

formation, following the protocol described later (see Experimental section).

A section of the $^{13}C\{^{1}H,^{2}H\}$ n.m.r. spectrum of (**8**), derived from the first feeding experiment with [2-^{13}C, 2-$^{2}H_3$]acetate, is shown in Figure 1. This, and the corresponding spectra from (**9**), reveals the intact incorporations of ^{13}C–^{2}H bonds, through the well known[4] displacement upfield of those resonances due

Starting with the resonance for C-9 in the putative starter units (*vide infra*), three α-shifted signals are clearly seen in the spectra for (**8**) and (**9**), and the magnitude of the upfield shifts is indicative of the presence in the population of molecules having retained in increasing proportions, one, two, and three ^{2}H atoms. Also, the two upfield signals at each of the resonances for C-4, C-5, and C-2a, indicate the intact incorporation of one or two ^{2}H atoms attached to ^{13}C at these positions, there being no discernible signal, however, corresponding to $^{13}C^{2}H_3$ at C-2a. It is significant that no deuterium retention is apparent at C-7, which is a methylene group originating from C-2 of acetate, although the intensity of the ^{13}C resonance for this carbon indicates that the centre is highly enriched in ^{13}C. There is a small signal 0.08 p.p.m. upfield of the enriched C-7 signal (see Figure 1), which although too small to be an α-shift, may correspond to a β-deuterium isotope shift[5] arising from the

Table 1. Isotope shifted ^{13}C resonances in compounds (**8**)—(**10**) upon incorporation of sodium [2-^{13}C, $^{2}H_3$]acetate;* see Figure 1 also

^{13}C Enrichments and α-deuterium shifts after incorporation of Na[2-^{13}C, $^{2}H_3$]acetate											
Carbon;	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-2a	C-10
Enrichment[a]											
(**8**)	2.42	2.41	1.23	2.21	1.90	1.45	5.20	1.00	1.79	1.92	—
(**9**)	3.16	2.89	1.60	2.82	2.81	1.91	6.56	1.00	1.91	2.61	—
(**10**)	1.36	1.48	0.71	1.18	1.10	0.99	3.17	1.00	1.43	1.09	0.70

Compound (**8**)

α-Shifts[b] for	C-4	C-5	C-9	C-2a
CH_2D	0.31(8)	0.31(5)	—	0.28(12)
CHD_2	0.66(10)	0.66(9)	0.55(16)	0.55(30)
CD_3	—	—	0.82(23)	—

Compound (**9**)

α-Shifts[b] for	C-4	C-5	C-9	C-2a
CH_2D	0.31(8)	0.35(7)	—	0.28(15)
CHD_2	0.65(17)	0.67(12)	0.54(28)	0.55(41)
CD_3	—	—	0.81(40)	—

Compound (**10**)

α-Shifts[b] for	C-4	C-5	C-2a	C-10
CH_2D	0.31(9)	0.35(7)	0.28(16)	0.27(10)
CHD_2	0.66(19)	0.67(15)	0.55(33)	0.55(29)
CD_3	—	—	—	—

* Insufficient of the derivative (**11**) was recovered in these experiments to allow a full analysis by ^{13}C n.m.r. [a] Each enrichment given is the relative height of the carbon-13 resonance in labelled material, compared to the relative height of the same resonance at natural abundance, measured under the same spectrometer conditions. [b] The isotope induced shifts are given as Δ(p.p.m.) upfield from the natural abundance singlet. The intensity of the shifted signal as a percentage of the intensity of the non-shifted signal, at the natural abundance chemical shift, is given in each case in parentheses.

Table 2. Isotope shifted resonances in compounds (**8**)—(**10**) after incorporation of sodium [1-^{13}C, 2-$^{2}H_3$]acetate*

		β-Shifts[a] for compound		
Carbons	Species	(**8**)	(**9**)	(**10**)
C(3),C(4)	^{13}C-CHD	0.06(42)	0.06(52)	0.06(32)
	^{13}C-CD_2	0.12(61)	0.12(145)	0.12(116)
C(6),C(5)	^{13}C-CHD	N.d.	0.06(40)	0.06(17)
	^{13}C-CD_2	0.11(40)	0.11(92)	0.11(75)
C(8),C(9)	^{13}C-CH_2D	N.d.	0.04(13)	—
	^{13}C-CHD_2	0.07(34)	0.07(36)	—
	^{13}C-CD_3	0.11(102)	0.11(155)	—

* See footnote * in Table 1. [a] The isotope induced β-shifts are given as Δ(p.p.m.) upfield from the natural abundance singlet. The intensity of the shifted signal as a percentage of the intensity of the non-shifted signal, at the natural abundance chemical shift, is given in each case in parentheses. N.d. = Not detected.

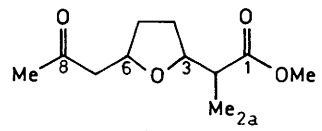
presence of ^{2}H at either C-8, or C-6, in those molecules enriched also in ^{13}C at C-7 (*vide infra*).

Complementary and supporting data were obtained in the second feeding experiment with sodium [1-^{13}C, 2-$^{2}H_3$]acetate. Now the incorporation of intact ^{13}C–C–^{2}H units are detected in the $^{13}C\{^{1}H\}$ n.m.r. spectrum of the products due to upfield β-isotope induced shifts, which are typically in the range 0—0.1 p.p.m. The three bis-MTPA derivatives (**8**)—(**10**) were prepared from the macrotetrolide mixture isolated from this experiment, and the β-isotope induced shifts [5] are recorded in Table 2. Three signals are shifted upfield of the C-8 resonance, in the spectra of (**8**) and (**9**), due to the intact incorporation of one, two or three deuteriums at C-9, with the preponderance being of $C^{2}H_3$–^{13}C labelled molecules. The signals for C-3 in the spectra of (**8**), (**9**), and (**10**), showed β-shifted resonances 0.06 and 0.12 p.p.m. upfield, indicating a mixture of molecules mono- and di-deuteriated at C-4, and a similar pattern at the C-6 resonance indicates mono- and di- deuteriation at C-5 also.

It follows from these experiments that all the hydrogens at C-4, C-5, and C-9 in each enantiomer of the nonactic acid subunits are incorporated intact, from the precursors acetate and succinate, whereas both of the hydrogens at C-7 do not arise intact from acetate, but instead most likely are derived from the medium as a result of exchange processes occurring during the biosynthesis.

Sodium [$^{2}H_3$]*Acetate,* [2-$^{2}H_2$]*Propionate, and* [$^{2}H_4$]-*Succinate.*—Additional data on the fate of substrate hydrogens came from the incorporation of deuterium labelled precursors, using ^{2}H n.m.r. to detect the sites of enrichment in nonactin and relevant derivatives.

The incorporation of sodium [$^{2}H_3$]acetate, in a standard feeding experiment, gave rise to nonactin, whose ^{1}H and ^{2}H n.m.r. spectra indicated substantial deuterium enrichments at all positions except C-2 and C-7. This labelled nonactin was then converted into the ketone (**12**) (obtained as a racemate), by hydrolysis and oxidation (i, MeOH, H_2SO_4; ii, Jones



(**12**)

oxidation), and the ^{1}H and ^{2}H n.m.r. spectra of this labelled (**12**) are also shown in Figure 2. These spectra show very clearly the deuterium enrichments at every protonated position in (**1**) and (**12**), again with the exception of C-2 and the two heterotopic positions at C-7. This again confirms the complete loss of deuterium from C-2 of acetate, during the creation of the C-7 centre in (**1**). The symmetrical distribution of deuterium enrichments around the four carbon atoms of the tetrahydrofuran ring in (**12**) is consistent with the origin of these atoms from succinate, derived *in vivo* from acetate *via* the Krebs cycle, with the enrichments at C-3, C-6 as well as C-8, arising presumably due to concomitant passage of deuterium from acetate to the C-4′ position of NAD(P)H, with transfer back to a biosynthetic intermediate catalysed by one or more NAD(P)-dependent dehydrogenases specific to the nonactin pathway.

In a similar way, [$^{2}H_4$]succinic acid was incorporated into nonactin, and this labelled nonactin was converted into the ketone (**12**). The ^{2}H n.m.r. spectra of (**1**) and (**12**) obtained in this way revealed substantial deuterium enrichments, at all positions except C-2, C-7, and C-9; the four stereoheterotopic positions at C-4 and C-5 were enriched to equal extents, as were C-3, C-6, and C-8.

Finally, an incorporation experiment with [2-^{3}H, 2-^{14}C]-propionate ($^{3}H/^{14}C$ = 31.4) indicated the substantial loss of ^{3}H label during the incorporation of this precursor into nonactin. Although the specific incorporation of ^{14}C label was, as in earlier experiments,[1] very high (10.0%), only a very low retention of the ^{3}H label was detected; the methyl nonacetate derived from this labelled material, recrystallised as its naphthylurethane derivative (m.p. 89—92 °C), showed $^{3}H/^{14}C$ = 0.25. Moreover, these low levels of ^{3}H retention fell

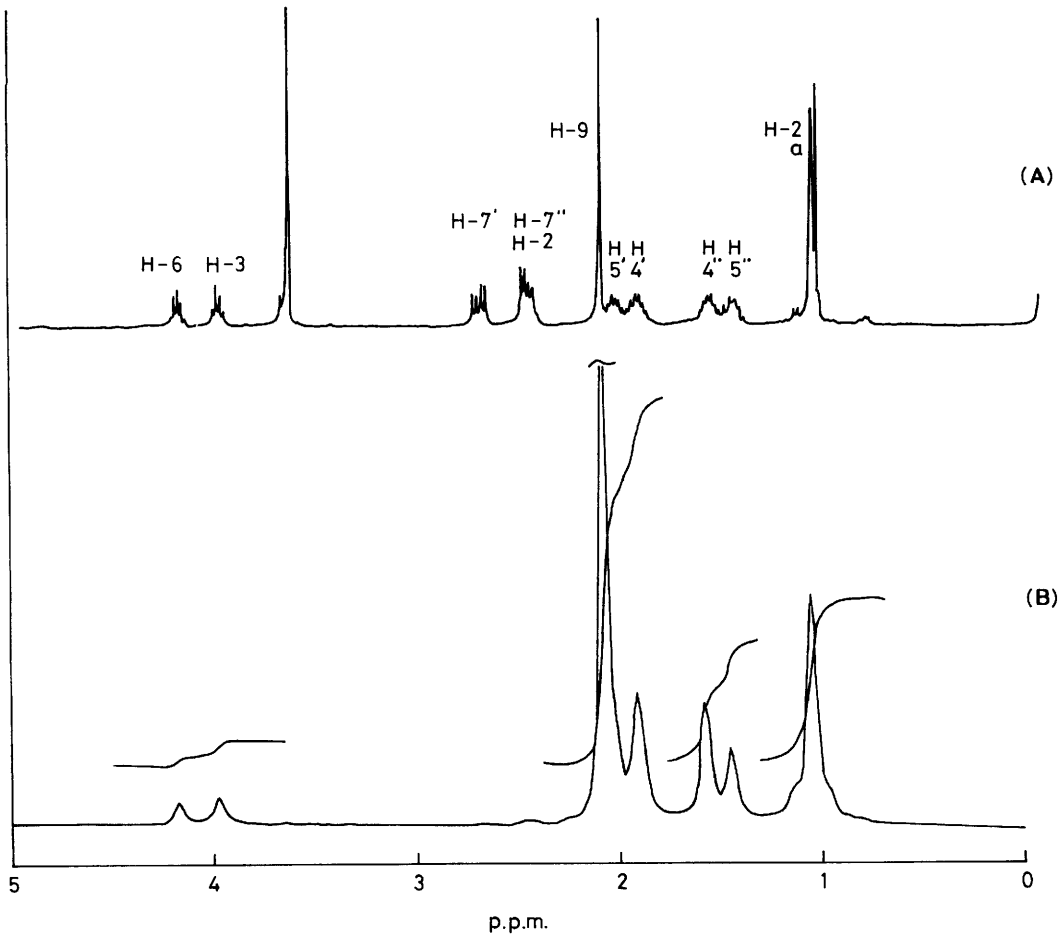


Figure 2. ^{1}H (**A**) and $^{2}H\{^{1}H\}$ (**B**) N.m.r. spectra of methyl 8-oxononactate (**12**), derived after biosynthesis from sodium [$^{2}H_3$]acetate. See Experimental section

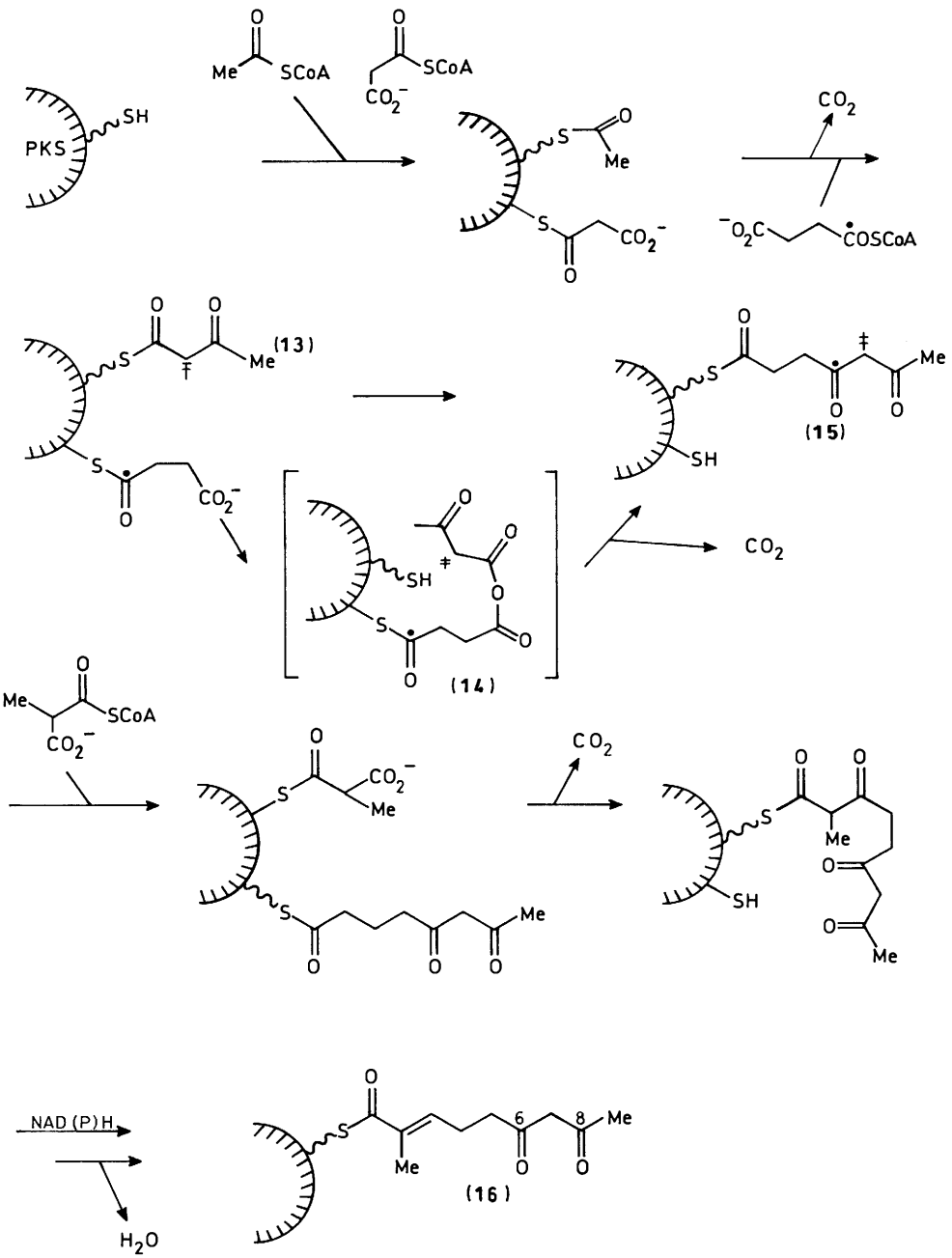
further during repeated rounds of purification, indicating the presence in these samples of small amounts of the homologues (**2**)—(**5**) very highly enriched in both ^{14}C and ^{3}H. As a final check, sodium [2-$^{2}H_2$]propionate was administered to shake cultures of *S. griseus* and the nonactin produced was degraded to the derivatives (**8**) and (**9**), which after exhaustive purification were completely devoid of deuterium enrichment, as judged by ^{2}H n.m.r. spectroscopy, whereas the homologues (**10**) and (**11**) from the same experiment were highly enriched, and only at C-9 in the starter unit.

Discussion

The assembly of the carbon backbone of nonactic acid cannot be rationalised straightforwardly, according to the classical concepts of polyketide biosynthesis,[6] since instead of the usual head-to-tail coupling of acetate or propionate units, the union is required of two acetate units, with propionate and succinate moieties, to derive a branched ten-carbon segment, as implied by the results from carbon-13 labelling experiments,[1,2] and illustrated in Scheme 1. Nevertheless, the new deuterium labelling experiments described above are helpful when formulating possible pathways.

The observed retention of three deuterium atoms from acetate at C-9 in both enantiomers of nonactic acid, supports the notion that the C(8)–C(9) acetate acts as a starter unit for the chain assembly process. The occurrence of homologues, including (**2**)—(**5**), then requires in these cases that propionate may act instead as the starter unit,[1] presumably due to a less than strict substrate specificity exhibited by the polyketide synthase (PKS). In the case of nonactin, one model of the assembly is shown in Scheme 2, where the acetyl-CoA, once loaded onto the putative PKS, undergoes a condensation with an enzyme bound malonate unit, to afford acetoacetate (**13**), again linked to the enzyme as a thiol ester. In the next stage, it is necessary to couple this fragment to succinate, lose carbon-1 from the acetoacetate, and leave the new seven-carbon intermediate activated, most plausibly as a thiol ester, ready for the final condensation step with methylmalonyl-CoA. These changes could be achieved, as shown in Scheme 2, by allowing succinyl-CoA, or an equivalent enzyme bound form, to generate first the mixed anhydride (**14**), and then, by a formal transacylation-decarboxylative condensation, the required new seven-carbon intermediate (**15**). In this way, not only is the desired connectivity established, but also no additional ATP is required to activate the carboxy group of succinyl-CoA. The final steps should then comprise a comparatively straightforward sequence involving decarboxylative-condensation, reduction and dehydration, to afford the enone (**16**); an ideal precursor for the formation of the two enantiomeric forms of nonactic acid. These may be derived by stereospecific reductions of the carbonyl groups at C-6 and C-8, and a formal syn-Michael addition of the 6-hydroxy group onto the α,β-unsaturated thiol ester[1] (Scheme 1).

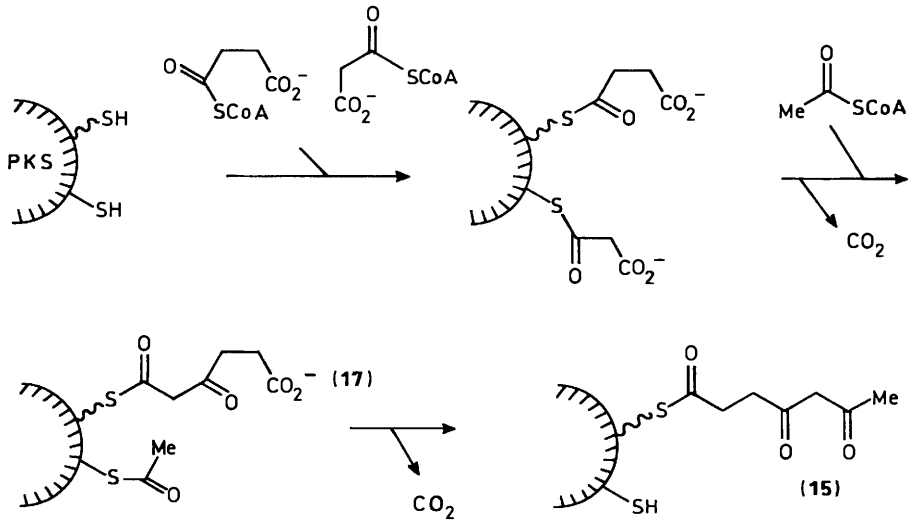
If all these reactions are catalysed by a single unique multienzyme complex, an acyl carrier protein (*cf.* fatty acid synthase[7]) may be required to transfer the substrate, anchored to the pantetheinyl moiety as a thiol ester, from one active site to



Scheme 2. A model sequence to account for the assembly of nonactate, catalysed by the putative nonactin polyketide synthase complex. See also Scheme 1

the next. In the final steps a stereospecific cyclotetramerisation generates the macrotetrolide, which is excreted by the micro-organism into the extracellular medium. These steps may also form part of the repertoire of the same nonactin PKS; oxygen-18 labelling experiments indicated [1,3] that neither nonactic acid, nor any other free carboxylic acid, is an obligatory intermediate, rather that nonactate should remain activated as a thiol ester as it is transferred to the active site(s) responsible for the cyclotetramerisation. The labelling experiments described above are at least consistent with this scheme, and may explain also the complete absence of deuterium at C-7, upon incorporation of deuteriated acetate, since washout of the label can occur at several stages in the pathway, where this carbon is doubly activated to enolisation. Whether or not a single multicomponent polyketide synthase complex is responsible for the synthesis of nonactin, a key aspect of the proposal involves the occurrence of a common intermediate (**16**) that is channelled along two stereochemically enantiocomplementary pathways, in order to generate mirror image forms of a nonactic acid (thiol ester) derivative; it is not clear *a priori* how many distinct active sites should be involved in this, nor how the reactions are regulated to ensure that both enantiomeric units are on hand for formation of the macrotetrolide.

The transfer of deuterium label from acetate to the nicotinamide coenzymes, detected in these experiments, is well precedented and can occur as acetyl-CoA enters the Krebs cycle, a major pathway of primary metabolism in the cell. The incorporation of deuterium label from acetate into positions that formally arise by the reduction of carbonyl groups has been seen previously during studies of both fatty acid [8] and polyether antibiotic biosynthesis.[9–11] The participation here of these coenzymes in reductions at C-3, C-6, and C-8, therefore,



Scheme 3. An alternative model for the early stages of nonactate assembly. See also Scheme 1

implicates NAD(P)-dependent dehydrogenases as components of the nonactin PKS complex. The formation of deuterium enriched succinyl-CoA, and of methylmalonyl-CoA *via* the methylmalonyl-CoA mutase reaction, upon feeding deuterium labelled acetate, also accounts for the secondary enrichments at C-2a, C-4, and C-5, seen during these experiments.

There is, however, one line of evidence which encourages the consideration of alternative models to explain these assembly processes. We have shown recently [12] that ethyl [1,2,3,4-$^{13}C_4$]- and [1,3-$^{13}C_2$]-acetoacetates, when fed to shake cultures of *S. griseus*, lead to several enrichments in nonactin. Most importantly, the C_3 unit comprising C(1)–C(2)–C(2a), can be derived *intact* from this precursor, without cleavage of acetoacetyl-CoA *in vivo* into acetyl-CoA units. This observation is almost certainly accounted for by the metabolism of acetoacetyl-CoA to butyryl-CoA, isomerisation of the latter to isobutyryl-CoA, with oxidation then giving methylmalonyl-CoA, the precursor of the relevant propionate unit in nonactin. The occurrence of this pathway in other Streptomycetes is now well documented,[13] and the key enzyme in the sequence, an isobutyryl-CoA mutase, has been partially purified[14] from *S. cinnamonensis*. However, in regard to nonactin biosynthesis, the lack of any observable *intact* incorporation of a C_3 unit at C(7)–C(9) in nonactic acid, either indicates that the $^{13}C_4$-labelled acetoacetyl-CoA *in vivo* cannot donate an acetoacetate unit to the nonactin PKS, or that such a unit as (**13**) does not occur as a discrete intermediate in the assembly process; questions that are difficult to resolve in this whole cell system. This becomes relevant since alternative models of the assembly can be formulated, and one is shown in Scheme 3. Here succinyl-CoA is the first substrate to be accepted by the PKS enzyme, and the first condensation may occur with malonyl-CoA, leading to (**17**). In this case, the assembly is then completed by further rounds of elongation, as shown in Scheme 3. Again, this alternative mechanism is consistent with all the labelling experiments completed to date.

New information must now be sought in other ways, and one attractive possibility involves feeding to whole cell cultures putative intermediates, activated as thiol ester derivatives. We have recently shown [15] that the diols (**18**) and (**19**), in the form of caprylcysteamine thiol esters, when presented to shake cultures of *S. griseus*, are taken up efficiently and stereospecifically incorporated into nonactin. Clearly, not only can these materials enter the cells, they are also suitable substrates for those enzymes acting in the late stages of the pathway. This establishes the strategy as a viable one for detecting enzyme bound intermediates, and immediately provides support for the involvement of the diketone (**16**) (*cf.* Scheme 1) as a common precursor to the enantiomeric nonactic acids. Similar experiments with other thiol ester activated materials may allow a distinction to be made between the mechanisms of chain assembly shown in Schemes 2 and 3.

Attempts have been made in this work to detect the summed activities of the nonactin PKS in cell free extracts of *S. griseus*. These involved adding to an extract of soluble proteins prepared by sonication of whole cells, acetyl-CoA, malonyl-CoA, succinyl-CoA, and [1,3-^{14}C]methylmalonyl-CoA, as well as NAD(P), NAD(P)H, and ATP. However, the turnover of ^{14}C activity into nonactic acid (or nonactin), extracted from the assay cocktail after mild base hydrolysis of all thiol esters and dilution with unlabelled material, was very low (2.2%). Some years ago, some success was achieved [16] in the detection of an ATP and coenzyme-A dependent nonactic acid activating activity, as well as an activity catalysing the condensation of nonactic acids to afford macrotetrolides, both as membrane associated proteins in extracts of *S. griseus*. However, it is most likely that until assays for specific reactions in the assembly process can be developed, and until the biosynthetic enzymes can be over-expressed, their purification and characterisation will remain difficult to achieve. Indeed, we have now embarked upon experiments to clone the biosynthetic genes for nonactin, using the array of powerful new methods [17] for gene cloning in the antibiotic producing Streptomycetes, developed over the past few years. From this approach we may expect in the first instance to derive important information about the primary structure of the nonactin polyketide synthase from the sequence of its cloned gene(s), a prerequisite to the overexpression of the gene products by genetic engineering methods.

Experimental

N.m.r. spectra were recorded on a Bruker AM360 spectrometer in chloroform. The $^{13}C\{^1H\}$ spectra occupied 32 K data points, were accumulated using a 60° pulse, and no relaxation delay, and were transformed following 0.5 Hz line broadening by exponential multiplication. The $^{13}C\{^1H,^2H\}$ spectra were recorded and processed in the same way, with 1 W ^1H and 3 W ^2H broadband decoupling; the spectrometer was used in unlocked mode, with the drift compensation circuit on the spectrometer enabled. ^2H N.m.r. spectra were also run unlocked with 2 K data points, and transformed after 1 Hz line broadening by exponential multiplication. Other analytical

procedures were identical to those described previously.[1] Sodium [1-^{13}C, 2-$^{2}H_3$]- and [2-^{13}C, 2-$^{2}H_3$]-acetates (90 atom % ^{13}C, 98 atom % ^{2}H) were purchased from B.O.C. Prochem.

Growth of Streptomyces griseus DSM 40695 = ETH A7796.—The conditions for the maintenance and growth of this strain in shake flask cultures has been described previously.[1]

Feeding Experiments with Sodium [2-^{13}C, 2-$^{2}H_3$]- *and* [1-^{13}C, 2-$^{2}H_3$]-*acetates.*—(a) Sodium [1-^{14}C]- and [1-^{13}C, 2-$^{2}H_3$]-acetate (164 mg; 1.38×10^7 d.p.m./mmol) in distilled water (2 ml) at pH 7, was sterilised at 121 °C for 15 min, and then added batchwise, in two equal portions, to each of two 100-ml shake flask fermentation cultures of *Streptomyces griseus*, 48 and 72 h after inoculation. After 6 days growth, the macrotetrolides were isolated by extraction of the whole broth, and purified by flash chromatography, following the procedure described earlier.[1] The nonactin was recrystallised from methanol, m.p. 146—147 °C (9.0×10^6 d.p.m./mmol; 16% specific incorporation). The mixture of labelled macrotetrolides were converted into the bis-MTPA derivatives (**8**)—(**11**), which could be resolved by h.p.l.c., again following our published methods.[1]*

(b) Sodium [2-^{13}C, 2-$^{2}H_3$]acetate (250 mg) with unlabelled sodium acetate (750 mg), in distilled water (10 ml) at pH 7, was sterilised at 121 °C, and added batchwise to a single 100-ml shake flask culture, in equal portions after 72, 96, and 120 h. After 6 days growth the macrotetrolides were extracted and processed, as described in the previous experiment.

Feeding Experiments with Sodium [$^{2}H_3$]*Acetate,* [2-$^{2}H_2$]-*Propionate and* [$^{2}H_4$]*Succinate.*—The sodium [$^{2}H_3$]acetate (98 atom % $^{2}H_3$), sodium [2-$^{2}H_3$]propionate (98 atom % $^{2}H_2$), and sodium [$^{2}H_4$]succinate (98 atom % $^{2}H_4$) in distilled water at pH7, were sterilised at 121 °C, and then added in 3 equal portions to a shake flask culture of *S. griseus*, until the concentration of labelled material in the broth was 20 mM. As before, the macrotetrolides were extracted after a total of six days growth, and nonactin was obtained pure after flash chromatography (Si column, eluting firstly with chloroform, and then with ethyl acetate–chloroform, 1:1) and recrystallisation from methanol.

(±)-*Methyl Nonactate and* (±)-*Methyl* 8-*Oxononactate* (**12**).—Nonactin (100 mg) in 5% H_2SO_4–MeOH was refluxed for 48 h, and then extracted into dichloromethane, washed with saturated aqueous sodium hydrogen carbonate, dried ($MgSO_4$), and evaporated. The resulting oil was chromatographed on a silica column with diethyl ether–dichloromethane (1:1) as eluant to afford pure (±)-methyl nonactate[18] as a colourless oil (100 mg, 93%); δ($CDCl_3$) 1.10 (3 H, d, *J* 7 Hz), 1.18 (3 H, d, *J* 7 Hz), 1.35 (4 H, m), 2.0 (2 H, m), 2.5 (1 H, m), 3.0 (1 H, br s), 3.7 (3 H, s), 3.9 (2 H, m), and 4.1 (1 H, m).

(±)-Methyl nonactate (50 mg) in acetone (2 ml) at 0 °C was titrated with Jones reagent [CrO_3 (35 mg), H_2SO_4 (0.29 ml), water (1 ml)]. When complete, the reaction was diluted with dichloromethane, washed (saturated sodium hydrogen carbonate), dried ($MgSO_4$), and evaporated. The resulting product was chromatographed (Si, diethyl ether–dichloromethane, 1:4) to afford (±)-methyl 8-oxononactate (**12**)[18] as a colourless oil (45 mg, 90%); δ($CDCl_3$) 1.10 (3 H, d, *J* 7 Hz), 1.50 (1 H, m), 1.65 (1 H, m), 2.0 (1 H, m), 2.15 (1 H, m), 2.20 (3 H, s), 2.50 (2 H, m), 2.75 (1 H, m), 3.70 (3 H, s), 4.05 (1 H, m), and 4.25 (1 H, m) (see Figure 2).

* Insufficient of the derivative (**11**) was recovered in these experiments to allow a full analysis by ^{13}C n.m.r.

Acknowledgements

The authors thank the S.E.R.C. for financial support of this work.

References

1 See, D. M. Ashworth, J. A. Robinson, and D. L. Turner, *J. Chem. Soc., Perkin Trans. 1*, 1988, 1719.
2 D. M. Ashworth, J. A. Robinson, and D. L. Turner, *J. Chem. Soc., Chem. Commun.*, 1982, 491.
3 D. M. Ashworth and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1983, 1327.
4 For reviews see, M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, **8**, 539; T. J. Simpson, *Nat. Prod. Rep.*, 1987, **4**, 339; *Chem. Soc. Rev.*, 1987, **16**, 123.
5 C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 856.
6 A. J. Birch and F. W. Donovan, *Austr. J. Chem.*, 1953, **6**, 360.
7 For a review see, S. J. Wakil, J. K. Stoops, and V. C. Joshi, *Ann. Rev. Biochem.*, 1983, **52**, 537.
8 R. H. White, *Biochemistry*, 1980, **19**, 9.
9 D. M. Doddrell, E. D. Laue, F. J. Leeper, J. Staunton, A. Davies, A. B. Davies, and G. A. F. Ritchie, *J. Chem. Soc., Chem. Commun.*, 1984, 1302.
10 M. M. Sherman and C. R. Hutchinson, *Biochemistry*, 1987, **26**, 438.
11 G. R. Sood, D. M. Ashworth, A. A. Ajaz, and J. A. Robinson, *J. Chem. Soc., Perkin Trans. 1*, 1988, 3183.
12 C. A. Clark and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1985, 1568.
13 K. A. Reynolds, D. O'Hagan, D. Gani, and J. A. Robinson, *J. Chem. Soc., Perkin Trans 1*, 1988, 3195.
14 G. Brendelberger, J. Retey, D. M. Ashworth, K. Reynolds, F. Willenbrock, and J. A. Robinson, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 1089.
15 Z. Spavold and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1988, 4.
16 P. O. Stahl, Ph. D. Dissertation, University of Tübingen, 1975.
17 See, D. A. Hopwood, in 'Proceedings Int. Symp. Biol. Actinomycetes,' 1986; J. T. Feyerman, *Biotechnology*, 1986, **4**, 786; R. Hutter and T. Eckhardt, in 'Actinomycetes in Biotechnology,' Academic Press, London, 1988, p. 89.
18 J. Dominguez, J. D. Dunitz, H. Gerlach, and V. Prelog, *Helv. Chim. Acta*, 1962, **40**, 129.

Received 17*th October* 1988; *Paper* 8/04118K