Biosynthesis of the Macrotetrolide Antibiotics; The Incorporation of Carbon-13 and Oxygen-18 Labelled Acetate, Propionate, and Succinate

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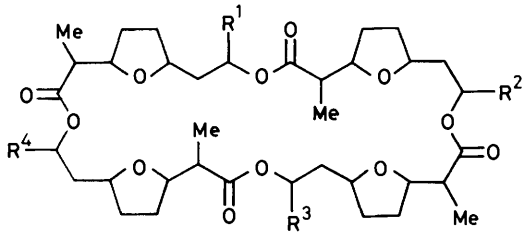
The biosynthesis of the macrotetrolide antibiotics, in particular nonactin, has been studied using carbon-13 and oxygen-18 enriched acetate and propionate, as well as carbon-13 enriched succinate, in feeding experiments with the producing organism *Streptomyces griseus*. A protocol is described which allows the separation of derivatives formed from each enantiomer of nonactic and homononactic acids. From a study of the incorporation of the labelled precursors into these derivatives it could be shown that the origins of the carbon and oxygen atoms in each enantiomer are identical. The carbon backbone of nonactic acid is assembled from two acetate, one succinate, and one propionate units, and the C–O bonds at C-8, C-6, and C-1 are derived intact from the primary precursors. Based on these data a new proposal is made to account for the biosynthesis of (±)-nonactate, and nonactin, in *S. griseus*.

The macrotetrolide antibiotics constitute a small group of macrocyclic ionophore antibiotics, which are produced in cultures of numerous strains of *Streptomyces*.[1–14] They are chemically rather inert, but exert their antibiotic effect in the presence of Na^+ or K^+ ions, against Gram positive bacteria in particular, by acting as transmembrane ion carriers.[15,16] Nine members of this class of antibiotic have been isolated to date, and they can all be represented[15] by the general structural formulae shown in Figure 1. The parent compound is nonactin,



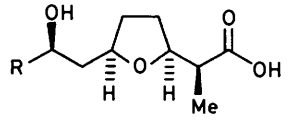
$R^1 = R^2 = R^3 = R^4 = Me$	Nonactin
$R^1 = R^3 = R^4 = Me, R^2 = Et$	Monactin
$R^1 = R^3 = Me, R^2 = R^4 = Et$	Dinactin
$R^1 = Me, R^2 = R^3 = R^4 = Et$	Trinactin
$R^1 = R^2 = R^3 = R^4 = Et$	Tetranactin

Figure 1.

whose constitution[7] and configuration[17] were first deduced by degradative and spectroscopic methods, and later substantiated by *X*-ray crystallography.[18,19] In this way it was revealed that nonactin is a cyclic tetramer, being composed of both enantiomers of a C_{10} hydroxy acid, called nonactic acid (**1**). The nonactic acid building blocks are linked together through ester bonds in a regularly alternating (+)(−)(+)(−) fashion, with the result that nonactin may be viewed as an achiral molecule possessing S_4 symmetry. The other members of the actin family are homologues of nonactin, and contain also homononactic acid (**2**) and/or bis-homononactic acid (**3**), as building blocks. Both enantiomers of each are again found, but all of the known actic acids (**1**)—(**3**) have the same relative configuration; no diastereoisomeric forms have so far been discovered.

Interest in the secondary metabolism of antibiotic producing strains of *Streptomyces* has heightened recently with the emergence of molecular genetics as a powerful new tool for the manipulation of antibiotic biosynthesis in these organisms.[20] Already there has been discussion about the possible uses of gene cloning and protein engineering to develop novel and useful antibiotic structures.[21] Whilst considerable progress has been made lately in the development of DNA cloning systems, studies of the basic biochemistry surrounding primary and secondary metabolism in Streptomycetes has lagged somewhat behind.

As an interesting model system we have sought to characterize the pathway to the macrotetrolide antibiotic nonactin. In common with other polyketide metabolites, this antibiotic is constructed from small fatty acid precursors, but the repeated use of the 10-carbon unit, nonactic acid, to generate a 32-membered macrotetrolide ring, makes it appear likely that a relatively small number of discrete enzymes will be required for the assembly of the complete antibiotic. Moreover, a fascinating aspect of this pathway, which remains to be fully elucidated, concerns the mechanisms of stereocontrol exerted at the enzymic level leading to the production of both enantiomers of nonactic acid, and the extent to which these can be manipulated to allow production of novel stereoisomeric or homologous forms of the macrotetrolides.



(+)−(2*S*, 3*S*, 6*R*, 8*R*)
(**1**) R = Me Nonacetic acid
(**2**) R = Et Homonoacetic acid
(**3**) R = Pr^i Bishomonactic acid

As an initial contribution to resolving these questions, we report here full details of feeding experiments in which carbon-13 and oxygen-18 labelled precursors have been incorporated into nonactin, using whole-cell cultures of *S. griseus* ETHA7796. This work is founded upon earlier investigations by Pape and Stahl[22,23] who, without access to modern, stable-isotope labelling techniques, made the following important discoveries: (*a*) the carbon backbones of (**1**) and (**2**) are assembled from precursors derived from acetate, propionate, and succinate, most likely in the manner shown in Figure 2, route a; (*b*) both (**1**) and (**2**) can be isolated from the culture broths of producing strains, the former mainly as the (−)-

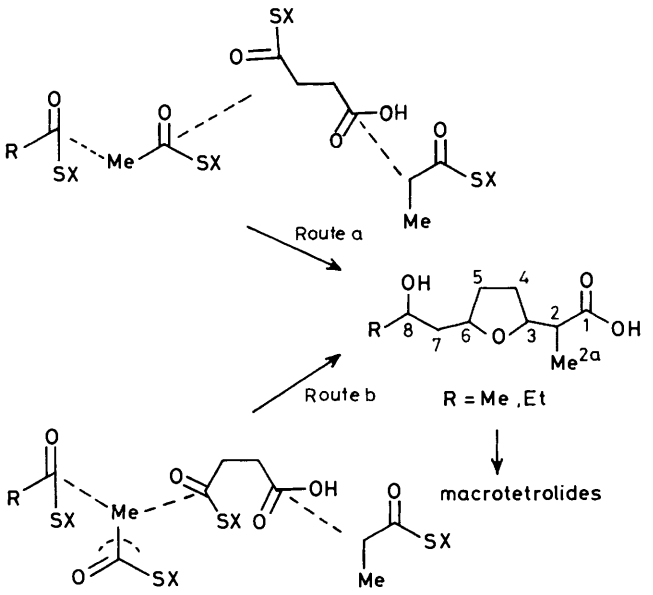


Figure 2.

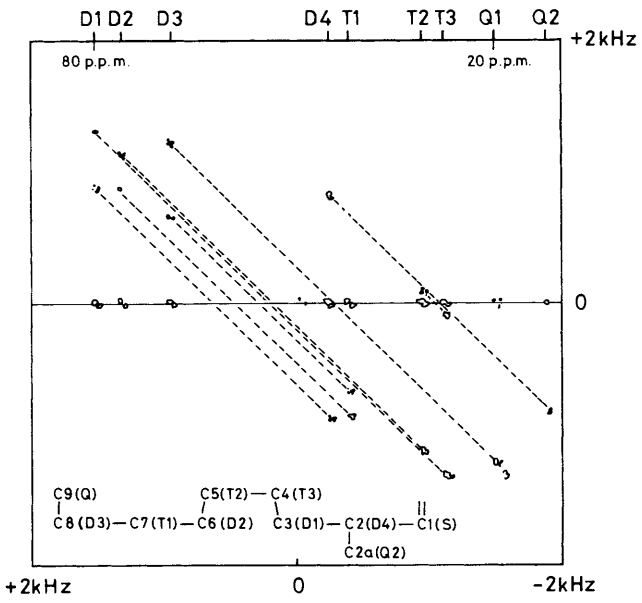


Figure 3. Part of the autocorrelation spectrum of nonactin obtained in 15 h from nonactin (*ca.* 1 g) dissolved in $CDCl_3$ (2 ml), using a Bruker CXP 200 spectrometer operating at 50.3 MHz. See text for discussion

isomer and the latter mainly as the (+)-isomer. Moreover, (**1**) in racemic and radiolabelled form was efficiently and specifically incorporated into nonactin when added to cultures of *S. griseus* and *S. griseoflavus*, suggesting strongly that (**1**) and (**2**) are biosynthetic intermediates on the pathway to the macrotetrolides.[23] The stereochemical questions inherent in nonactin biosynthesis are, therefore, indeed largely associated with the production by the micro-organism of both enantiomers of nonactic acid (**1**). Although there are several conceivable strategies for generating both (+)- and (−)-(**1**) it has been pointed out[24] that the one most likely to prevail would involve the introduction of chiral centres late in the pathway, once the construction of the carbon backbone is complete. This should at least allow the formation of (+)- and (−)-(**1**) from a common intermediate, and minimize the number of discrete enzymes necessary for the production of both.

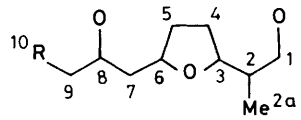
Some of the results described here have been disclosed earlier in communications.[25,26]

Results and Discussion

N.m.r. Assignments.—Feeding experiments with precursors enriched in the stable isotopes ^{13}C, ^{18}O, and ^{2}H, have been a rich source of new information about secondary metabolism in a variety of organisms. For our work it was essential from the outset to have available unambiguous assignments of $^{13}C\{^{1}H\}$ n.m.r. spectra, as well as a means of resolving the enantiomeric subunits in nonactin. In this way the incorporation of labels into (+)- and (−)-(**1**) could be followed independently.

An assignment of the $^{13}C\{^{1}H\}$ n.m.r. spectrum of nonactin made with the aid of additivity rules, chemical shift reagents, and spectra of model compounds has been reported already,[27] but with the emergence of powerful new 2D n.m.r. methods these assignments could be confirmed *unambiguously*. In particular, the analysis of scalar couplings between carbon-13 nuclei provides an extremely convenient way of relating n.m.r. spectra to molecular structure, and 2D spectroscopy offers a method for identifying all coupled resonances in a single experiment. Although the method used here[28] is not the most efficient, it is conceptually the simplest of the techniques which have been developed so far.[29] This is the 2D ^{13}C autocorrelation experiment, obtained using method B of reference 28. A part of the autocorrelation spectrum of nonactin is shown in Figure 3, in which signals from ^{13}C nuclei that are directly bonded appear in the second (vertical) dimension at half the difference of their chemical shifts shown in the first dimension. The normal proton coupled multiplet structure for each ^{13}C resonance is indicated by D = doublet, T = triplet, Q = quartet. The carbon–carbon connectivity pattern is clearly discernible; the correlated nuclei are indicated by dotted lines and reveal the carbon connectivity Q2-D4-D1-T3-T2-D2-T1-D3-Q1, and this, when compared with the constitution of nonactin, leaves no doubt over the assignment (given in Table 1).

Table 1. Assignments of the $^{13}C\{^{1}H\}$ spectra of nonactin, and derivatives (**6A**—**D**), relative to deuteriochloroform (δ 77.10)

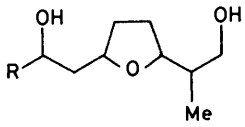


		Compound			
Carbon	Nonactin	(**6A**)	(**6B**)	(**6C**)	(**6D**)
1	174.23	68.89	68.98	68.89	69.02
2	45.28	38.29	38.36	38.21	38.38
3	80.11	80.25	80.48	80.22	80.47
4	28.20	29.03	29.02	29.03	29.05
5	31.46	31.28	31.42	31.35	31.51
6	76.40	75.48	75.49	75.53	75.68
7	42.36	42.33	42.31	39.68	39.74
8	69.10	72.25	72.33	76.72	76.76
9	20.56	20.53	20.24	27.42	27.13
10	—	—	—	9.29	9.02
2a	12.88	13.61	13.69	13.59	13.71

Resolution of the Enantiomeric Building Blocks.—*Streptomyces griseus* ETHA 7796, used in this work, normally produces nonactin together with small amounts (*ca.* 20% by weight) of monactin and dinactin. This mixture of macrotetrolides can be recrystallized, repeatedly, to afford pure nonactin, although this

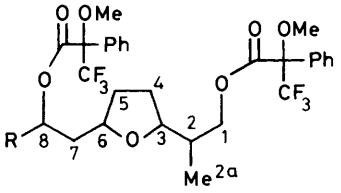
procedure is tiresomely inefficient. Alternatively, reduction with lithium aluminium hydride, without prior fractionation, affords the known[7,8] diols (±)-(**4**) and (−)-(**5**). During some feeding experiments (*vide infra*) larger amounts of the homologous macrotetrolides were produced, which gave at this stage (±)-(**5**) as well as (±)-(**4**). This four-component mixture can be converted directly into the derivatives (**6A**—**D**) by reaction with (−)-methoxy(phenyl)trifluoromethylacetyl (MPTA) chloride.[30] This mixture of diastereoisomers and homologues can then be cleanly resolved into its four pure components (>98% purity, assayed by ^1H, ^{13}C, ^{19}F n.m.r. and analytical h.p.l.c.—see Figure 4) by preparative h.p.l.c. (Si Zorbax column 25 cm × 21 mm; hexane–diethyl ether–water, 92:8:0.1 eluant; 24 ml min^{-1}) prior to analysis by ^{13}C n.m.r. spectroscopy.

The absolute configurations of the four components (**6A**—**D**) were assigned by reducing each with lithium aluminium hydride to recover the optically pure diols (**4**) or (**5**), whose optical rotations could then be compared with those values reported for the isomers of known absolute configuration by Prelog and co-workers.[8,17]



R = Me (**4**)

R = Et (**5**)



(**6A**) derived from (+)-(**1**)

(**6B**) derived from (-)-(**1**)

(**6C**) derived from (+)-(**2**)

(**6D**) derived from (-)-(**2**)

Finally, with the pure derivatives (**6A**—**D**) in hand the $^{13}C\{^1H\}$ n.m.r. spectrum of each was assigned unambiguously using the 2D autocorrelation experiment described above. The assignments are given in Table 1.

Incorporations of ^{13}C *Labelled Precursors.*—The incorporation of acetate, propionate, and succinate into the macrotetrolides has been investigated here using ^{13}C labelled materials. In a typical feeding protocol the ^{13}C enriched precursor, containing also a trace amount of ^{14}C label, was batch fed to several shake flask cultures of *S. griseus* ETHA 7796, over the period of active antibiotic production (days 2—6) such that the final concentration of added precursor in the broth was 20mM. After growth, the cells were collected and the macrotetrolides could be extracted from the mycelium into acetone. At this stage the nonactin was purified by flash chromatography, followed by repeated crystallization, prior to analysis by ^{13}C n.m.r. spectroscopy. Subsequently, the entire macrotetrolide extract was processed, as described earlier, to afford the derivatives (**6A**—**D**), corresponding to each enantiomer of the acids (**1**) and (**2**).

The observed enrichment of ^{13}C in (**6A**—**D**) biosynthesized from various ^{13}C labelled acetate, propionate, and succinates, are given in Table 2. The values listed for (**6A**) and (**6B**) in each

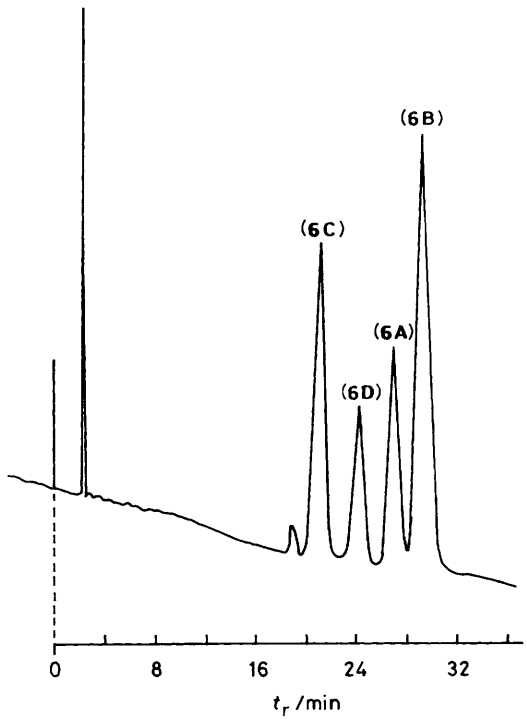


Figure 4. H.p.l.c. chromatogram of a mixture of (**6A** – **D**) eluted from a Zorbax silica column (25 cm × 4.6 mm) under isocratic conditions (hexane–diethyl ether–H_2O; 92:8:0.1) at a flow rate of 1.5 ml min^{-1}, with u.v. detection at 254 nm

experiment parallel those seen in nonactin biosynthesized from the same precursor.[25]

Of particular note are the incorporations derived from succinate. In the derivatives (**6A**) and (**6B**), enrichments occurred at C-1, C-3, and C-6, starting from [1,4-$^{13}C_2$]succinic acid; this indicated firstly that succinate may act as the direct precursor of the four carbon atoms of the tetrahydrofuran ring, and secondly that *in vivo* succinate may be converted into methylmalonyl-CoA, the direct precursor of the propionate unit at C(1)–C(2)–C(2a), with label arising at C-1 (see Figures 2 and 5). These conclusions are supported by the results of a feeding experiment with [2,3-$^{13}C_2$]succinate, which gave rise to enrichments indicating intact units at C(4)–C(5) and C(2)–C(2a) (Table 2, expt. 6).

When the incorporation of labelled acetate is examined, clear evidence emerges for its intact incorporation at C(8)–C(9). Moreover, enrichments also occur within the sections derived formally from succinate [C(3)–C(6)] and propionate [C(1)–C(2)–C(2a)], indicating again that the administered precursor has entered the primary metabolism of the cell, in this case the Krebs cycle. It is notable that consistently higher enrichments from [1-^{13}C]acetate occur at C-3, than at C-6, indicating that succinyl-CoA is removed from the pool of Krebs cycle intermediates before equilibrium with succinate can occur. Also, the C-6 centre should then be derived from C-1 of succinyl-CoA (Figure 5).

A complex pattern of enrichments could be seen in (**6A**) and (**6B**) biosynthesized from [2-^{13}C]acetate (Table 2). However, of particular note are the highest enrichments, which occur at C-7 and C-9, implying that C-7 is derived from an acetate unit which has lost its carboxy carbon during the carbon chain assembly process. Again the enrichments that appear at all positions except C-8 are consistent with the labelled acetate contributing to the pool of Krebs cycle metabolites, which also are drawn upon for antibiotic production.

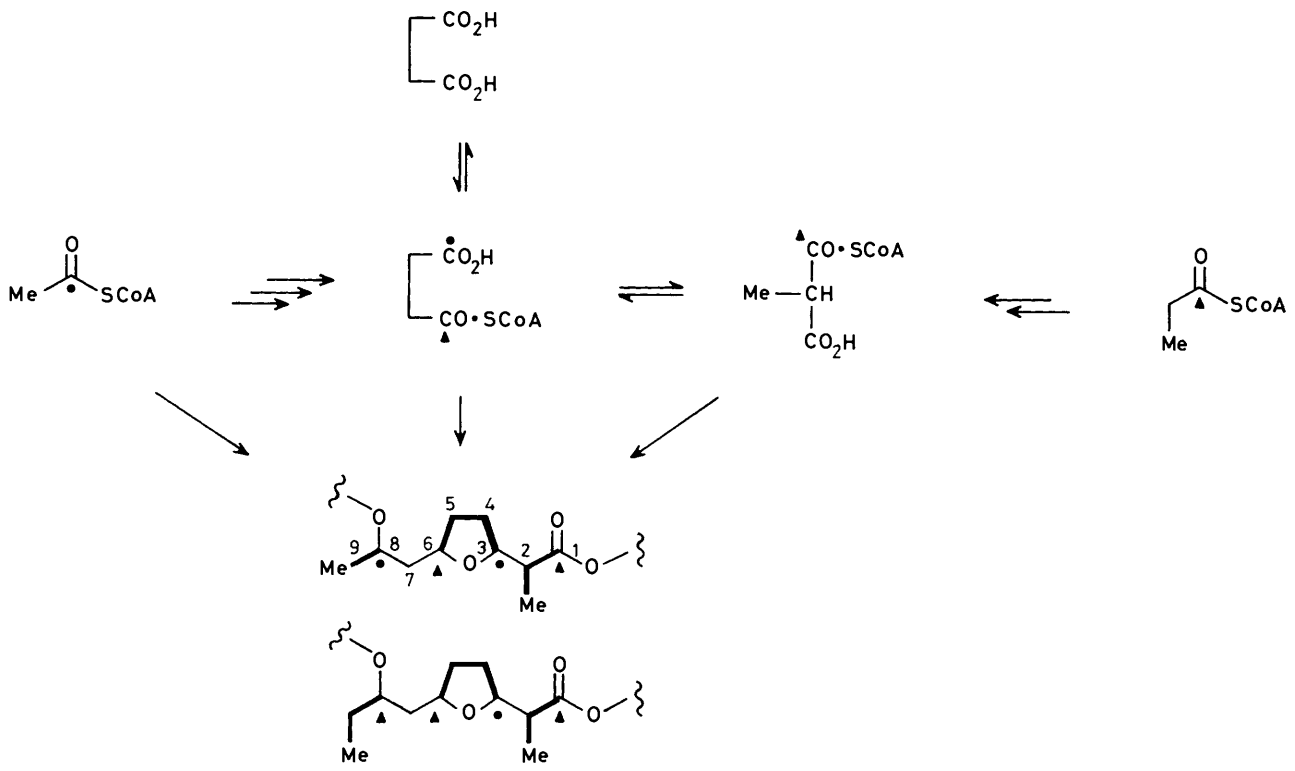
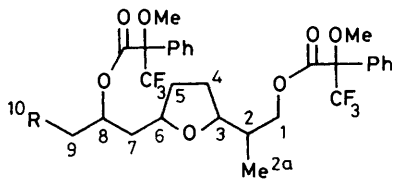


Figure 5.

Table 2. Enrichments of carbon-13 in derivatives (**6A**—**D**) biosynthesized from ^{13}C-enriched acetate, propionate, and succinate



R = H (**6A**) derived from (+)-(**1**)
(**6B**) derived from (−)-(**1**)
R = Me (**6C**) derived from (+)-(**2**)
(**6D**) derived from (−)-(**2**)

Expt.	Precursor[a]	MPTA-derivative	Enrichments[b] of ^{13}C in derivatives (**6A**, **B**, **C**, and **D**) C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-2a
1	[1,4-$^{13}C_2$]Succinate	(**6A**)	3.2	—	3.2	—	—	2.7	—	—	—	—
		(**6B**)	3.3	—	3.2	—	—	3.4	—	—	—	—
2	[1-^{13}C]Acetate	(**6A**)	2.7	—	4.3	—	—	2.7	—	6.2	—	—
		(**6B**)	3.0	—	4.6	—	—	2.8	—	6.5	—	—
		(**6C**)	2.9	—	4.2	—	—	2.5	—	1.8	—	—
3	[2-^{13}C]Acetate	(**6A**)	3.6	5.1	2.2	5.8	6.0	3.1	7.7	—	6.7	4.4
		(**6B**)	3.9	5.4	2.3	7.0	5.4	3.3	8.2	—	8.1	5.2
4	[1-^{13}C]Propionate	(**6A**)	3.6	—	1.1	—	—	1.2	—	—	—	—
		(**6B**)	8.3	—	1.3	—	—	2.1	—	—	—	—
		(**6C**)	23.7	—	3.1	—	—	5.5	—	28.2	—	—
		(**6D**)	50.1	—	6.3	—	—	11.4	—	57.3	—	—
			Coupling constant (Hz) of enriched ^{13}C–^{13}C doublets in nonactin									
5	[1,2-$^{13}C_2$]Acetate		—	—	35	35	34	34	—	39	39	—
6	[2,3-$^{13}C_2$]Succinate		—	34	—	32	32	—	—	—	—	34

[a] Each site 90 atom % enriched: doubly labelled precursors were diluted at least 1:1 with unlabelled material. [b] Enrichment = relative height of carbon-13 resonance in labelled material/relative height of same resonance at natural abundance, measured under the same spectrometer conditions.

During the experiments described so far only very small amounts of homologues (**6C**) and (**6D**) were recovered. However, upon addition of labelled propionate to cultures of *S. griseus* the production of homologous macrotetrolides was strongly stimulated and substantial amounts of (**6C**) and (**6D**) could then be isolated. Indeed, the highest enrichments of [1-^{13}C]propionate occur into (**6D**), at C-1 and C-8. The secondary enrichments at C-3 and C-6 are consistent with the *in vivo* conversion of added propionate *via* propionyl-CoA → methylmalonyl-CoA → succinyl-CoA → succinate (Figure 5). The higher observed level of enrichment at C-6 is again consistent with the insertion of succinyl-CoA into the carbon

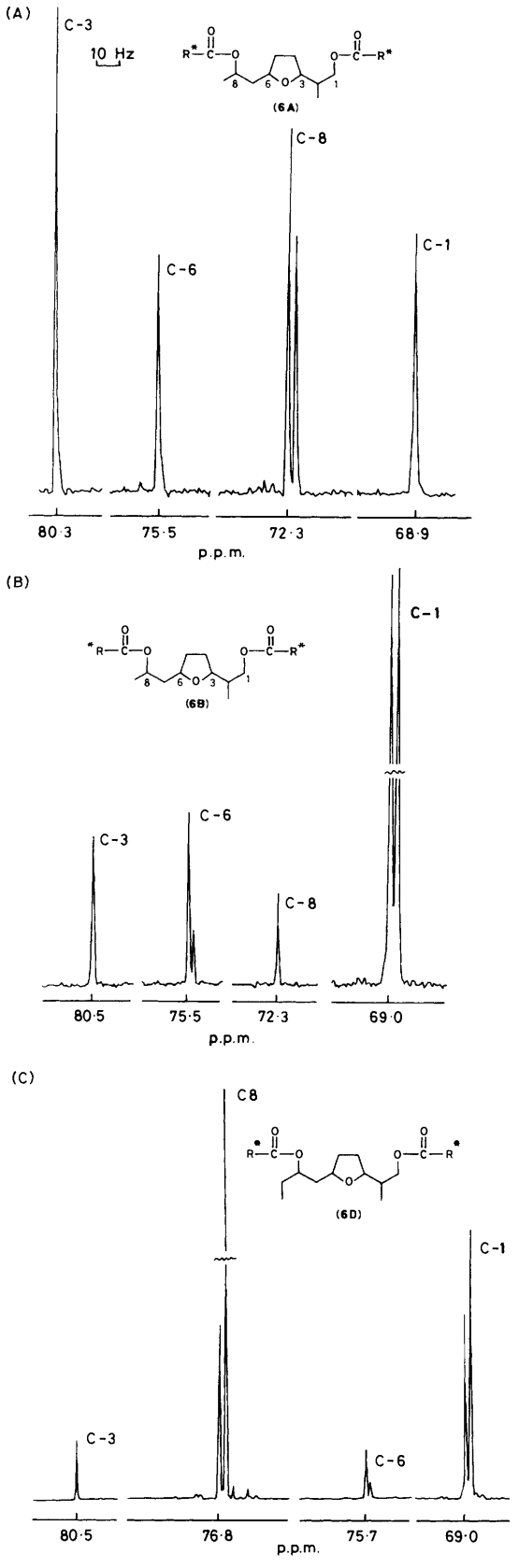


Figure 6. Portions of the $^{13}C\{^1H\}$ n.m.r. spectra of: (A), MPTA-derivative (**6A**) derived from sodium [1-^{13}C, $^{18}O_2$]acetate; (B), MPTA-derivative (**6B**) derived from sodium [1-^{13}C, $^{18}O_2$]propionate; (C), MPTA-derivative (**6D**) derived from sodium [1-^{13}C, $^{18}O_2$]propionate

backbone in the orientation shown in Figure 2, with the carbon of the thioester providing C-6.

Incorporations of Carbon-13 and Oxygen-18 Enriched Precursors.—The biosynthetic origins of the oxygen atoms in nonactin could be established by feeding experiments with ^{13}C and ^{18}O labelled acetate and propionate. These experiments rely upon the appearance of the well established[31–33] upfield ^{18}O isotope-induced shift of those ^{13}C resonances directly bonded to ^{18}O in the labelled product.

Accordingly, when [1-^{13}C, $^{18}O_2$]acetate was added to cultures of *S. griseus* labelled macrotetrolides were produced and these were converted, as before, into the bis-MTPA derivatives, affording predominantly (**6A**), (**6B**), and (**6C**). Of these, only the enriched singlets assigned to C-8 in the $^{13}C\{^1H\}$ spectra of (**6A**) or (**6B**) were accompanied by a second signal 0.04 p.p.m. upfield, due to ^{13}C still attached to ^{18}O (Figure 6A). On the other hand, when sodium [1-^{13}C, $^{18}O_2$]propionate was batch fed to cultures of *S. griseus* the production of homologous macrotetrolides was again stimulated, and samples of all four bis-MTPA derivatives (**6A**—**D**) were obtained (see Table 3).

Very high levels of ^{13}C enrichment were observed (3—70 fold) particularly in (**6D**), which is derived from (−)-homononactic acid. In addition, the signals assigned to C-1, C-6, and C-8 in the spectra of derivatives (**6C**) and (**6D**) (see Figure 6C), and those assigned to C-1 and C-6 in the spectra of (**6A**) and (**6B**) (see Figure 6B), appeared as 'doublets' due to the presence of ^{13}C–^{18}O signals upfield by 0.022—0.045 p.p.m. from the normal ^{13}C–^{16}O resonances (Table 3) whereas all other peaks in the spectra were sharp singlets at their normal positions. The high levels of ^{13}C enrichment, and the correspondingly low levels of ^{18}O exchange in this experiment, are of particular importance and indicate again a very close coupling of the primary metabolic processes utilizing propionate, to the generation of precursors required for antibiotic biosynthesis.

These results reveal clearly that in both enantiomers of nonactic acid the oxygen at C-8, and the carbonyl oxygen at C-1 are derived intact from acetate and propionate, respectively, whereas in both enantiomers of homononactic acid all these oxygens are derived intact from propionate. The appearance in all of these intermediates of an intact ^{13}C-^{18}O bond at C-6, derived from labelled propionate is, however, of special interest, and requires further comment.

As outlined earlier, both acetate and propionate can be metabolized *in vivo* to succinyl-CoA prior to incorporation into the antibiotics (Figure 5); [1-^{13}C,$^{18}O_2$]acetate would give rise to ^{16}O·^{18}O·^{13}C·CH_2·CH_2·COSCoA *via* the Krebs cycle, whereas [1-^{13}C,$^{18}O_2$]propionate can afford O_2C·CH_2CH_2·$^{13}C^{18}O$·SCoA *via* the methylmalonyl-CoA mutase reaction. These labelled succinyl-CoA's may then be incorporated into the tetrahydrofuran ring with the ^{13}C label arising either at C-3 or at C-6. It is apparent from the spectral data in Table 3, that only when the label enters C-6 in each of the four intermediates does the ^{13}C–^{18}O bond remain intact. Any ^{18}O label incorporated with C-3 would, therefore, be lost, and indeed no such shifted resonance is observed in the derivatives. It follows that the closure of the tetrahydrofuran ring in these antibiotics should occur by formation of the C(3)–O bond, most likely *via* a Michael-type addition process.

Finally, the retention of ^{13}C–^{18}O over ^{13}C–^{16}O at C-1 in the derivatives (**6A**—**D**) biosynthesized from [1-^{13}C,$^{18}O_2$]-propionate is over 50%, based on the integrated intensities of the corresponding ^{13}C resonances (Table 3). It follows, therefore, that free nonactic and homononactic acids are not obligatory intermediates in the biosynthesis, despite their occurrence in the fermentation broth of the producing organism. Rather, the direct intermediate is most probably a thiol ester derivative which can either react to generate an ester bond by direct

Table 3. Enrichments of carbon-13 and oxygen-18 in derivatives (**6A—D**) biosynthesized from sodium [1-^{13}C,$^{18}O_2$]-acetate and -propionate

Expt.	Precursor[c]	MPTA derivative	Enrichment[a] of ^{13}C and ^{18}O[b]							
			C-1	$O^{16}:O^{18}$	C-3	$O^{16}:O^{18}$	C-6	$O^{16}:O^{18}$	C-8	$O^{16}:O^{18}$
1	[1-^{13}C]Acetate	**(6A)**	2.7	—	4.3	—	2.7	—	6.2	—
		(6B)	3.0	—	4.6	—	2.8	—	6.5	—
		(6C)	2.9	—	4.2	—	2.5	—	1.8	—
2	[1-^{13}C,$^{18}O_2$]Acetate	**(6A)**	2.1	—	2.5	—	2.0	—	3.8	48:52
		(6B)	2.2	—	3.5	—	2.0	—	4.8	45:55
		(6C)	2.0	—	3.3	—	2.0	—	1.8	—
3	[1-^{13}C]Propionate	**(6A)**	3.6	—	1.1	—	1.2	—	—	—
		(6B)	8.3	—	1.3	—	2.1	—	—	—
		(6C)[d]	23.7	—	3.1	—	5.5	—	28.2	—
		(6D)[d]	50.1	—	6.3	—	11.4	—	57.3	—
4	[1-^{13}C,$^{18}O_2$]Propionate	**(6A)**	16.1	59:41	2.2	—	3.0	80:20	1.3	—
		(6B)	21.0	52:48	2.3	—	3.4	79:21	1.1	—
		(6C)[d]	29.1	50:50	3.1	—	4.4	75:25	42.5	25:75
		(6D)[d]	61	45:55	5.8	—	9.4	71:29	84	25:75

[a] Enrichment = integrated signal intensity at labelled positions ÷ that at unlabelled position in same spectrum. With ^{18}O and ^{16}O labelled centres both ^{13}C–^{16}O and ^{13}C–^{18}O signals were summed to calculate the total ^{13}C enrichment. [b] The ^{16}O:^{18}O ratios are the ratios of the integrated signal intensities. It is assumed that ^{13}C signal intensities are not *significantly* altered by the substitution of ^{16}O for ^{18}O. [c] Singly labelled precursors are *ca.* 90 atom % ^{13}C. Doubly labelled precursors are *ca.* 80% $^{13}C^{18}O_2$ + 10% $^{13}C^{18}O_1$. [d] Enrichment levels supported also by mass spectral examination.

displacement of the thiol activating group with the C-8 hydroxy of another C_{10} or C_{11} (enantiomeric) building block, or in a competitive process, undergo attack by water to release free nonactic acid.

Conclusions

The method described above, which allowed the resolution of the enantiomeric and homologous C_{10} and C_{11} building blocks derived from nonactic (**1**) and homononactic (**2**) acids, has provided for the first time an opportunity to examine the assembly of these metabolites from carbon-13 and oxygen-18 enriched acetate, propionate, and succinate. As a result, several important new observations have been made concerning the biosynthesis of the macrotetrolides.

Although the fundamental roles of acetate, succinate, and propionate in the biosynthesis is in accord with the earlier studies of Pape and Stahl,[22,23] the results shown in Table 2 indicate that the manner in which these precursors are assembled is best formulated by route b, shown in Figure 2. Thus, succinate should provide all four carbon atoms of the tetrahydrofuran rings, whereas three carbons [C(7)—C(9)] in the backbone of nonactic acid (**1**) are furnished from two acetate units, with the carboxy carbon of one being lost during the process of carbon-chain assembly. It is unusual to see succinate used in this way for antibiotic biosynthesis in Streptomycetes. The majority of macrolide, polyether, and related polyketide-type antibiotics from Streptomycetes are assembled from building blocks derived principally from acetate, propionate, and butyrate,[34] and carbon-chain assembly appears to proceed using chemistry that is closely related to that catalysed by the condensing enzyme of fatty acid synthetase,[35] although this remains to be rigorously established. At present no information can be gleaned concerning the order in which the various building blocks are assembled into the backbones of nonactate and homononactate.

Further examination of the results in Table 2 reveal other interesting observations which merit additional comment. In particular, the effect of added propionate on the production of homologous macrotetrolides, and the variations in the levels of incorporation of [1-^{13}C]propionate into (+)- and (−)-(**1**), may be rationalized in several ways. Arguably the most plausible scenario, although certainly not the only one, would involve the action of a single enzyme system for carbon-chain assembly, which can incorporate at the terminal position acetyl-CoA, propionyl-CoA, or isobutyryl-CoA. When [1-^{13}C]-propionate is administered to the fermentation the *in vivo* pool of propionyl-CoA, which is normally low, is dramatically increased, and this enzyme complex then becomes saturated with the labelled precursor, thereby leading to the formation of enriched *homologue* rather than *nonactin*. As the concentration of propionyl-CoA falls, so nonactin production returns to normal levels, giving at first labelled and later unlabelled material, as labelled precursor pools are diluted. The mixture of macrotetrolides isolated, therefore, comprises highly enriched homologue, plus labelled nonactin diluted with unlabelled material. Moreover, the highest enrichment occurs at C-8 in (−)-homononactate, which for reasons that are not clear, is not normally produced at significant levels during the fermentation.

The incorporations of ^{13}C and ^{18}O labelled acetate and propionate have established clearly the origins of the oxygen atoms in nonactic and homononactic acids. Indeed, taken with the earlier ^{13}C labelling experiments it is apparent that the origins of the carbon and oxygen atoms in each enantiomer of (**1**) and (**2**) *are identical*. This points to a common mode of assembly for each enantiomeric species, and the retentions of ^{18}O provides also mechanistic information about the closure of the tetrahydrofuran rings (*vide supra*). Based on these data we propose the pathway shown in Figure 7 as a likely *in vivo* biosynthetic route leading firstly to the enantiomeric C_{10} building blocks, and subsequently to the macrotetrolide nonactin. Thus, the intermediate (**7**) may represent the direct product of a carbon chain synthetase, which should contain all of the component enzymes necessary to assemble the backbone from acetyl-CoA, malonyl-CoA, succinyl-CoA, and methylmalonyl-CoA. This intermediate is centrally placed to afford *via* stereospecific reductions the diols (**8**) and (**9**). These diols may then afford, *via* a stereospecific *syn*-Michael addition on the *E*-enone, the stereoisomeric nonactates, still activated at the carboxy carbon as thiol esters. These intermediates may react further to generate an ester bond by the direct displacement of the thiol with the 8-hydroxy group of another enantiomeric C_{10} or C_{11} building block. In this way, the entire biosynthesis may proceed without the intervention of free unactivated intermediates, possibly by direct transfers between the active sites of one or more components of an antibiotic synthase complex. Whilst the

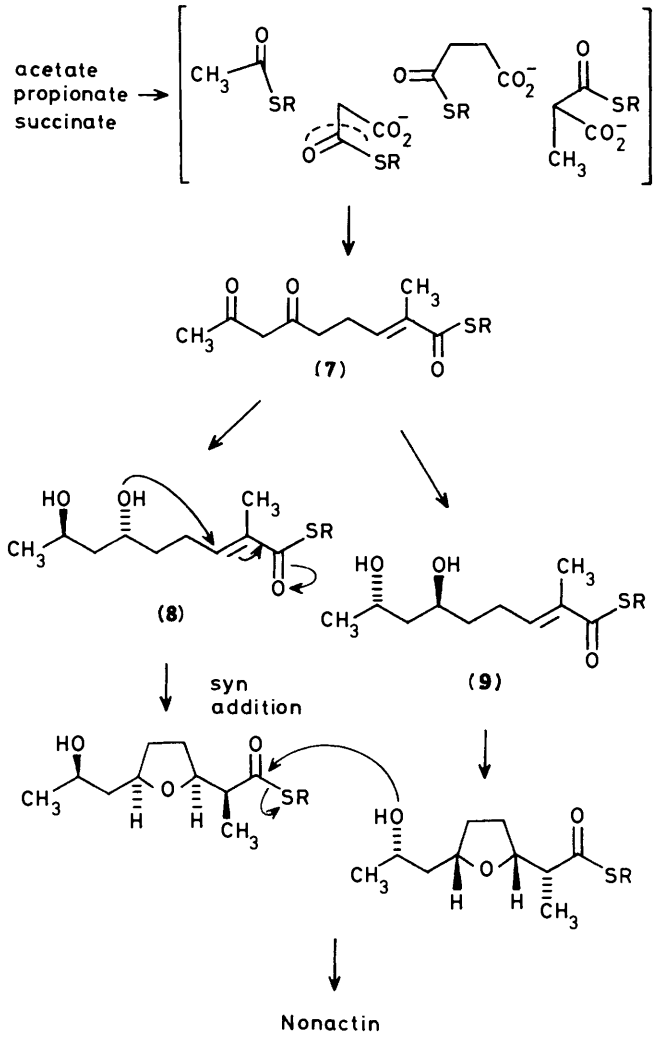


Figure 7. The proposed biosynthetic pathway to nonactin

route shown in Figure 7 may be regarded as speculation, its formulation at least allows the design of further experiments to test its correctness, and such work is currently underway.

Experimental

Melting point determinations were carried out on a Reichert hot-stage apparatus, and are uncorrected. N.m.r. spectra were recorded on XL-100, WH-400, or AM 360 spectrometers and are referenced using the residual solvent signal (7.27 for ^1H in $CHCl_3/CDCl_3$; 77.1 for $^{13}C\{^1H\}$ in $CDCl_3$). The measurements of mass spectra were made on a Kratos MS30 instrument. Separations by h.p.l.c. were completed on a Dupont 8800 instrument with an in-line u.v. detector, using Zorbax silica columns. The analytical t.l.c. plates used were of Merck silica GF_{254}, and flash chromatography was performed by the method of Still[36] using Kieselgel-60 (230—400 mesh).

Growth of Streptomyces griseus *DSM* 40695 = *ETH A7796*.—Cultures of *S. griseus* ETH A7796 were maintained on Emerson agar at 4 °C. Mycelium scraped from an agar slant was used to inoculate a three-stage fermentation. The first stage comprised tryptic soy broth (20 ml) in an Erlenmeyer flask (100 ml) having four indentations in its side walls. This was inoculated with the mycelium and incubated at 30 °C for 48 h on an orbital shaker at 125 r.p.m. The second stage comprised: Bacto-tryptone (0.8 g), yeast extract (0.4 g), $NaNO_3$ (0.3 g), and $CaCO_3$ (0.2 g) mixed in distilled water (90 ml), sterilized at 121 °C for 20 min in a 500 ml Erlenmeyer flask having four baffles. Maltose (3 g) was dissolved in water (10 ml), sterilized separately, and added to the flask before inoculation. The inoculum comprised 5% vol/vol of medium from the first-stage culture, and growth was on an orbital shaker at 30 °C for 48 h. The production medium comprised: Bacto-tryptone (0.8 g), yeast extract (0.4 g), $NaNO_3$ (0.3 g), $CaCO_3$ (0.2 g), $MnSO_4$ (0.06 g), and $ZnSO_4$ (0.005 g) dissolved in distilled water (90 ml) in a 500 ml baffled Erlenmeyer flask. The pH was adjusted to 7.2 with sodium hydroxide solution (5M). After sterilization, maltose (3 g) in water (10 ml) was added. A 5% inoculum was used and the flask was incubated on an orbital shaker at 30 °C. Nonactin production began after *ca.* 48 h and continued for *ca.* 4 days, to give final titres of *ca.* 1—1.5 mg/ml of macrotetrolides.

Isolation and Purification of Nonactin.—The cells were collected by centrifugation, freeze-dried, and then extracted with acetone (3 × 30 ml). The extracts were dried ($MgSO_4$) and the solvent was evaporated to leave a yellow oil. This was chromatographed on silica gel eluting firstly with $CHCl_3$ to remove lipid material, and then with EtOAc–$CHCl_3$ (1:2), to afford the macrotetrolide antibiotics. These were recrystallized from ethanol to give nonactin as white needles, m.p. 145—148 °C (lit.,[8] 148 °C).

2-(2-*Hydroxypropyl*)-2-*tetrahydro*-5-*furylpropan*-1-*ol* (**4**).—A solution of nonactin (170 mg, 0.23 mmol) in tetrahydrofuran (THF) (2 ml) was added dropwise to $LiAlH_4$ (170 mg, 4.5 mmol) suspended in THF (2 ml) and the mixture heated under reflux for 3 h. Saturated aqueous NH_4Cl (2 ml) was then added at 0 °C followed by water (0.5 ml). Solids were filtered off and the product was extracted (diethyl ether; 4 × 10 ml), and the extract dried ($MgSO_4$) and evaporated to leave an oil. This was purified by flash chromatography (EtOAc) to give the racemic diol (**4**) as a colourless oil; $\nu_{max.}$ 3 500 and 3 000 cm^{-1}; $\delta(CDCl_3)$ 0.83 (3 H, d, 7 Hz), 1.20 (3 H, d, 6 Hz), 1.6 (6 H, multiplet), 2.6 (2 H, br s), 3.6 (2 H, m), and 4.0 (3 H, m).

The reduction of macrotetrolide mixtures (*i.e.* nonactin with monactin, dinactin, trinactin, and tetranactin) in this way afforded (±)-(**5**) as well as (±)-(**4**).

cis-2-{3-[*Methoxy*(*phenyl*)*trifluoromethylacetoxy*]*propan*-2-*yl*}-5-{2-[*methoxy*(*phenyl*)*trifluoromethylacetoxy*]*propyl*}-*furan* (*Bis-MPTA*) Derivatives (**6A**) *and* (**6B**).—The diol (**4**) (40 mg, 0.21 mmol) and (−)-methoxy(phenyl)trifluoromethylacetyl chloride (134 mg, 0.53 mmol) in CH_2Cl_2 (0.3 ml) were mixed with pyridine (0.3 ml) and 4-dimethylaminopyridine (60 mg, 0.53 mmol) and the mixture stirred at room temperature for 2 h. It was then diluted with water (2 ml) and the product extracted with Et_2O (30 ml). The extract was washed with dilute aqueous HCl and saturated aqueous Na_2CO_3, dried ($MgSO_4$), and evaporated to yield a colourless oil. The product was purified by flash chromatography (CH_2Cl_2 as eluant) to afford the mixture of diastereoisomers (**6A**) and (**6B**) (106 mg, 80%) again as a colourless oil; $\delta_H(CDCl_3)$ 0.90 (3 H, d), 1.26 (3 H, 2 × d), 1.8 (7 H, m), 3.6 (8 H, s), 4.38 (2 H, m), 5.3 (1 H, m), and 7.42 (10 H, m); $\delta_C(CDCl_3)$ for spectral assignments see Table 1; *m/z* 638 (M + 18, NH_4^+, c.i. mode, 100%), 248 (10), and 231 (30).

The conditions for h.p.l.c. separation of (**6A—D**) are given in Figure 4, and the text. For (**6A**) $[\alpha]_D$ −64° (*c* 5.0, $CHCl_3$), for (**6B**) $[\alpha]_D$ −16° (*c* 5.0, $CHCl_3$), for (**6C**) $[\alpha]_D$ −49° (*c* 0.7, $CHCl_3$), for (**6D**) $[\alpha]_D$ −32° (*c* 0.4, $CHCl_3$).

Assignment of Absolute Configurations for (**6A—D**).—The assignments for (**6A**) and (**6B**) were achieved as follows. The derivative (34 mg, 0.046 mmol) in dry THF (2 ml) with $LiAlH_4$

(34 mg) was refluxed for 24 h. Water (0.3 ml), aq. NaOH (15% w/v solution; 0.1 ml), and then water (0.3 ml), were added sequentially, and the solid was filtered off; the filtrate was then dried (Na_2SO_4) and evaporated to leave a colourless oil. The diol (**4**) was purified by flash chromatography (EtOAc–EtOH, 19:1) (7 mg, 41%). From (**6A**) the diol (**4**) had $[\alpha]_D$ −31.5° (*c* 3.78, benzene); from (**6B**) the diol (**4**) had $[\alpha]_D$ +29.5° (*c* 4.06, benzene). Literature values[8] for (+)-(2*S*,3*R*,6*S*,8*S*)-diol (**4**), derived from (−)-(2*R*,3*R*,6*S*,8*S*)-nonactic acid (**1**), has $[\alpha]_D$ +31° (*c* 2.16, benzene), whereas (−)-(2*R*,3*S*,6*R*,8*R*)-diol (**5**), derived from (+)-(2*S*,3*S*,6*R*,8*R*)-homononactic acid (**2**) has $[\alpha]_D$ −30° (*c* 3.93, benzene).

The absolute configurations of (**6C**) and (**6D**) were achieved by reducing dinactin with $LiAlH_4$ to afford (−)-$C_{11}H_{22}O_3$ diol (**5**) and (+)-$C_{10}H_{20}O_3$ diol (**4**), according to the procedure of Prelog and co-workers.[8] These diols were converted into the corresponding bis-MPTA derivatives (**6B**) and (**6C**), which were matched by h.p.l.c. retention times. Thus (**6C**) is derived from (2*S*,3*S*,6*R*,8*R*)-(+)-homononactic acid and (**6D**) is derived from (2*R*,3*R*,6*S*,8*S*)-(−)-homononactic acid.

[1,4-$^{13}C_2$]*Succinic Acid.*—Potassium cyanide (90% enriched in ^{13}C; 1.0 g) was added to dichloroethane (0.76 g) and 18-crown-6 (200 mg) in acetonitrile (9 ml) and refluxed for 2 h. The mixture was then filtered and the filtrate diluted (CH_2Cl_2), washed (saturated brine), dried (Na_2SO_4), and evaporated to leave a brown wax (600 mg). This wax in methanol (6 ml) with sodium hydroxide solution (1M; 14 ml) was refluxed overnight. The solution was then evaporated to dryness and the residue redissolved in water (5 ml) and acidified (concentrated HCl) to pH 1. The product crystallized, and was collected and purified by sublimation, yield 450 mg (50%), m.p. 183—187 °C (lit.,[37] m.p. 187—190 °C); $\delta_H(D_2O)$ 2.66 (2 H, s); $\delta_C(D_2O)$ 30.05 (d, J_{C-C} 56.5 Hz), and 178.4 (s); *m/z* 103 (19%), 102 (52), and 56 (100).

[2,3-$^{13}C_2$]*Succinic Acid.*—A mixture of [1,2-$^{13}C_2$]dibromoethane (each site 90% enriched; 1 g), 18-crown-6 (100 mg), and KCN (1.4 g) were refluxed in acetonitrile (3 ml) for 3 days. After filtration the solution was added to CH_2Cl_2 (30 ml) and washed (saturated brine). The organic layer was separated, dried (Na_2SO_4), and evaporated to leave a brown wax (470 mg). This wax in MeOH (2.5 ml) and aqueous NaOH (2M; 5 ml) was refluxed for 16 h, and then evaporated to leave a white solid. This was dissolved in water and acidified to precipitate succinic acid, which was purified by sublimation, yield 271 mg (43%), m.p. 185—187 °C; $\delta_C(D_2O)$ 30.35 (s) and 178.7 (d); *m/z* 113 (13), 102 (39), 76 (50), 75 (52), 57 (98), and 45 (100).

Sodium [1-^{13}C,$^{18}O_2$]*Acetate.*—Iodomethane (2.26 g, 16 mmol), potassium [^{13}C]cyanide (1.056 g, 16 mmol), and [^{18}O]water (0.1 ml, 97%) were heated under reflux in anhydrous methanol (5 ml) for 22 h. The solution was then directly distilled to leave a solid residue. To the distillate was added a solution of potassium t-butoxide in t-butyl alcohol (1.23M; 13 ml) and [^{18}O]water (0.806 g, 97%) and the solution was heated under reflux for a further 48 h. It was then evaporated to dryness under reduced pressure and the residue taken up in water (4 ml) and acidified to pH 1 (50% H_2SO_4). The acidified solution was lyophilised, and the lyophilisate adjusted to pH 9 (10% NaOH), and freeze-dried to give sodium [1-^{13}C,$^{18}O_2$]acetate (1.01 g, 11.05 mmol) in 72% yield.

A sample of this material (4 mg) was added to an equal amount of sodium [1-^{13}C]acetate (4 mg) in deuterium oxide (D_2O). The resultant ^{13}C n.m.r. spectrum had two enriched peaks at 170.24 p.p.m. separated by 1.3 Hz corresponding to the ^{13}C–$^{16}O_2$ signal from sodium [1-^{13}C]acetate and the ^{13}C–$^{18}O_2$ signal from the synthesized material. Accurate isotopic composition was determined after preparation of the *p*-phenylphenacyl derivative.

p-*Phenylphenacyl* [1-^{13}C,$^{18}O_2$]*Acetate.*—*p*-Phenylphenacyl bromide (55 mg, 0.2 mmol) was added to sodium [1-^{13}C,$^{18}O_2$]acetate (20 mg, 0.2 mmol) with 15-crown-5 (4 mg) in benzene–acetonitrile (1:1; 2 ml) and heated under reflux overnight. The solvent was evaporated under a stream of nitrogen, and the residue chromatographed (benzene 100%) to give a white crystalline solid (40 mg, 0.114 mmol) in 76% yield; m.p. 108—109 °C; $\delta_H(CDCl_3)$ 2.25 (3 H, d, $J_{^{13}C,^1H}$ 14 Hz), 5.35 (2 H, d, $J_{^{13}C,^1H}$ 8 Hz), 7.4—8.0 (9 H, m); the ^{13}C n.m.r. spectrum showed one enhanced signal at 170.2 p.p.m. The resolution enhanced spectrum showed that this signal had three components corresponding to ^{13}C–$^{16}O_2$:^{13}C–^{18}O,^{16}O:^{13}C–$^{18}O_2$ in a ratio of 1:1:11.

Sodium [1-^{13}C,$^{18}O_2$]*Propionate.*—Iodoethane (4 g, 25.1 mmol), potassium [^{13}C]cyanide (1.625 g, 25 mmol) and [^{18}O]water (0.24 ml) were heated under reflux in anhydrous methanol (6.4 ml) for 12 h. After further heating for 33 h, the entire mixture was distilled and the distillate treated with a solution of potassium t-butoxide in t-butyl alcohol (0.85M; 29.5 ml) and [^{18}O]water (1.06 g). The solution was heated under reflux for a further 48 h and then concentrated under reduced pressure to leave a solid. The solid was taken up in water (5 ml) and the solution taken to pH 1 (50% H_2SO_4) and lyophilised. The lyophilisate was then adjusted to pH 9 (10% NaOH) and freeze-dried to give sodium [1-^{13}C,$^{18}O_2$]propionate (1.4 g, 14 mmol) in 56% yield.

Sodium [1-^{13}C]propionate (4 mg) was added to an equal quantity of the synthesized material. The resultant ^{13}C n.m.r. spectrum showed two enriched peaks at 173.76 p.p.m. separated by 1.3 Hz, corresponding to the ^{13}C–$^{16}O_2$ signal from sodium [1-^{13}C]propionate and the upfield shifted signal from ^{13}C–$^{18}O_2$ in the synthesized material.

Accurate isotopic composition was determined after preparation of the *p*-phenylphenacyl derivative.

p-*Phenylphenacyl* [1-^{13}C,$^{18}O_2$]*Propionate.*—This derivative, prepared using the procedure described above, had m.p. 99—100 °C. The resolution-enhanced ^{13}C spectrum showed one enhanced resonance at 173.75 p.p.m. comprised of three components corresponding to ^{13}C–$^{16}O_2$:^{13}C–$^{18}O^{16}O$:^{13}C–$^{18}O_2$ in the ratio 1:1.5:26.

Feeding Experiments.—Labelled precursors were dissolved in distilled water, filter sterilized, and administered to the two-day old shake flask production cultures of *S. griseus*, in equal portions over the following 2 or 3 days, to a final concentration in the broth of 20mM. Doubly ^{13}C labelled materials were diluted at least 1:1 with unlabelled material before feeding. The macrotetrolide antibiotics were isolated and processed according to the procedures described above.

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