Biosynthesis of the Macrotetrolide Antibiotics: An Investigation using Carbon-13 and Oxygen-18 Labelled Acetate and Propionate

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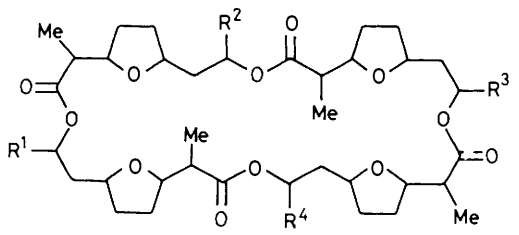
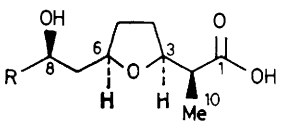
The biosynthesis of both enantiomers of nonactic acid, and of homononactic acid, has been studied using carbon-13 and oxygen-18 labelled acetate and propionate; this has shown the origin of the oxygen atoms in these building blocks and provided mechanistic information about their mode of biosynthesis and incorporation into the macrotetrolides.

The macrotetrolide ionophore antibiotics (**1**)—(**5**) are macrocyclic tetraesters constituted[1] from *both enantiomers* of nonactic acid (**6**) and homononactic acid (**7**), and these C_{10} and C_{11} hydroxyacids have been implicated[2] as intermediates on the biosynthetic pathway to (**1**)—(**5**). Although the origin of the carbon backbone of (**1**)—(**5**) from acetate, propionate, and succinate has been established, very little is known about the details of the chain building process nor how both enantiomers of each C_{10} and C_{11} building block are generated. We report here the results of feeding experiments with ^{13}C and ^{18}O labelled acetate and propionate that indicate the origins of the oxygen atoms in these antibiotics and which lead to a more detailed mechanistic scheme for macrotetrolide biosynthesis.

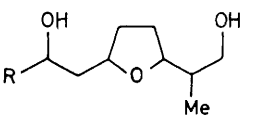
The following general procedure for following the incorporation of labels into each of the enantiomeric C_{10} and C_{11} building blocks was first established. The mixture of macrotetrolides isolated from the fermentation broth of *Streptomyces griseus* ETHA7796 usually contains nonactin (**1**) and smaller amounts of monactin (**2**).† This mixture was reduced using $LiAlH_4$ to afford the known diols[3] (±)-(**8**) and (−)-(**9**). During some of the feeding experiments larger amounts of the homologous macrotetrolides were produced which gave at this stage (±)-(**9**) as well as (±)-(**8**). This four component mixture can be converted without separation directly into the derivatives (**10**)—(**13**) by reaction with (−)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) chloride.[4] This mixture of diastereoisomers and homologues



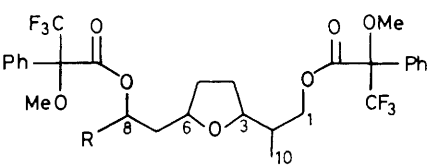
(**1**) Nonactin $R^1 = R^2 = R^3 = R^4 = Me$
(**2**) Monactin $R^1 = Et$, $R^2 = R^3 = R^4 = Me$
(**3**) Dinactin $R^1 = R^3 = Et$, $R^2 = R^4 = Me$
(**4**) Trinactin $R^1 = R^2 = R^3 = Et$, $R^4 = Me$
(**5**) Tetranactin $R^1 = R^2 = R^3 = R^4 = Et$



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

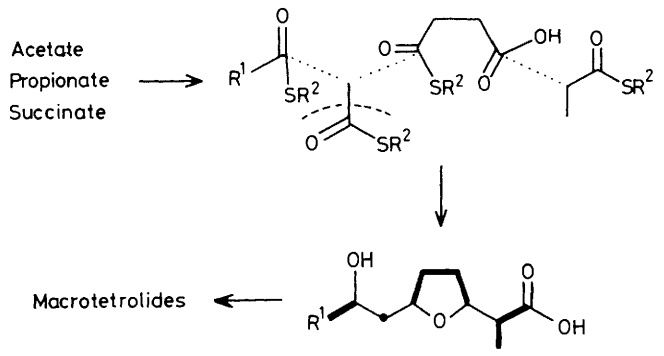


(**8**) R = Me
(**9**) R = Et



(**10**) R = Me derived from (+)-(**6**)
(**11**) R = Me derived from (−)-(**6**)
(**12**) R = Et derived from (+)-(**7**)
(**13**) R = Et derived from (−)-(**7**)

† Nonactin contains both enantiomers of (**6**) combined in a (+)(−)(+)(−) order, whereas in monactin one of the (+) nonactic acid units has been replaced by a (+)-homononactic acid unit. The latter compound upon reduction affords (−)-diol (**9**), ref. 3.



Scheme 1

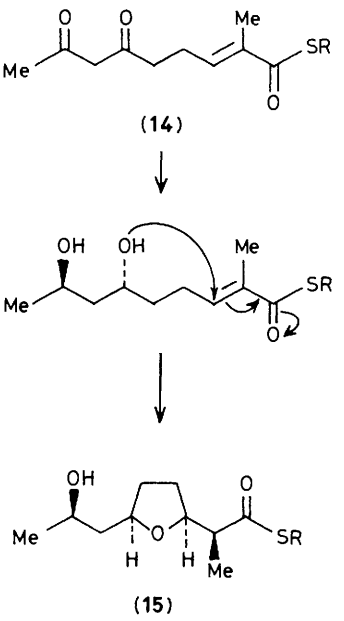
can then be cleanly resolved into the four pure components (>98% purity based on ^{1}H, ^{13}C, ^{19}F n.m.r. and analytical h.p.l.c.) by preparative h.p.l.c. (Si-Zorbax column 25 cm × 21 mm; hexane–diethyl ether–H_2O 92:8:0.1 eluant; 24 ml min^{-1}) prior to analysis by ^{13}C n.m.r. spectroscopy at 100 MHz. At this stage feeding experiments using ^{13}C labelled acetate, propionate, and succinate confirmed that the origin of the carbon atoms in each enantiomer of the C_{10} and C_{11} building blocks was identical and occurred as deduced earlier[5] (Scheme 1).

When sodium [1-^{13}C,$^{18}O_2$]acetate was added to cultures of *S. griseus* doubly labelled nonactin and monactin were produced and intact ^{13}C–^{18}O bonds were subsequently detected in the ^{13}C spectra of the derivatives (**10**)—(**12**) by looking for the well established[6] upfield ^{18}O isotope induced shift of the ^{13}C resonances. Of the three compounds examined only the enriched singlets (4 fold enhancement) assigned to C(8) in the ^{13}C spectra of (**10**) and (**11**) were accompanied by a second signal 4.1 Hz upfield due to ^{13}C still attached to ^{18}O (see Table 1). 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 stimulated and samples of all four bis-MTPA derivatives (**10**)—(**13**) derived from (+)- and (−)-C_{10} and (+)- and (−)-C_{11} building blocks, respectively, were obtained.

Very high levels of ^{13}C enrichment were observed (3—70 fold) particularly in (**13**) derived from the (−)-homononactate unit, which is not normally generated in significant amounts by this micro-organism unless propionate is added to the fermentation during the period of antibiotic production. In addition, the signals assigned to C(1), C(6), and C(8) in the spectra of (**12**) and (**13**), and to C(1) and C(6) in those of (**10**) and (**11**), appeared as 'doublets' due to the presence of ^{13}C–^{18}O signals upfield by 2.2—4.5 Hz from the normal ^{13}C–^{16}O signals (Table 1), 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 a very close coupling of the primary metabolic processes utilizing propionate, to the generation of precursors required for antibiotic biosynthesis. Both acetate and propionate can be metabolized to succinate prior to incorporation; [1-^{13}C,$^{18}O_2$]acetate is converted first into $^{18}O^{16}O^{13}C{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}SCoA$ *via* the citric acid cycle,‡ whereas [1-^{13}C,$^{18}O_2$]propionate can afford $O_2C{\cdot}CHMe{\cdot}$-$^{13}C^{18}O{\cdot}SCoA$ and $O_2C{\cdot}CH_2{\cdot}CH_2{\cdot}^{13}C^{18}O{\cdot}SCoA$ *via* reaction on methylmalonyl-CoA mutase. The labelled succinyl-CoA's can then be incorporated into the tetrahydrofuran ring with ^{13}C label arising at C(3) or at C(6).

‡ Assuming the operation of a 'normal' *si*-citrate synthase, ref. 7.



Scheme 2. Only one enantiomer of (**15**) is illustrated.

It is apparent from the spectra, however, that only when the label enters C(6) in each of the four building blocks does the ^{13}C–^{18}O bond remain intact. The ^{18}O label attached to C(3) must therefore be lost and indeed no shifted resonance is observed. In addition, in both enantiomers of each C_{10} and C_{11} subunit the oxygen atom at C(8) can be derived intact from acetate and propionate, respectively, and in all cases the carbonyl oxygen at C(1) can also be derived intact with C(1) from propionate.

The origins of the carbon *and* oxygen atoms in each enantiomer of (**6**) and of (**7**) from acetate, propionate, and succinate are therefore identical. This points to a common mode of biosynthesis for each enantiomer and provides mechanistic information about (a) the formation of the tetrahydrofuran rings and (b) the cyclo-tetramerization which generates (**1**)—(**5**). Thus an intermediate such as (**14**) (Scheme 2) can be transformed into both (+)- and (−)-nonactic acids by two sequences that are enantiocomplementary, each involving two carbonyl group reductions and a stereospecific *syn*-Michael addition§ by the C(6) hydroxy group to the *E*-enone. Finally, since the retention of ^{13}C–^{18}O over ^{13}C–^{16}O at C(1) in (**12**) and (**13**) derived from [1-^{13}C,$^{18}O_2$]propionate is over 50% (Table 1) it follows that (**7**), and hence most probably (**6**) also, are not obligatory intermediates on the biosynthetic pathway to the macrotetrolides. Rather an intermediate such as (**15**) can react to generate an ester bond by direct displacement of the thiol activating group with the C(8) oxygen of another C_{10} or C_{11} building block or undergo attack by H_2O to release free nonactic acid.¶ This implies that the entire biosynthesis may occur without the intervention of free unactivated intermediates, possibly by direct transfers between

§ For proven examples of similar *syn*-Michael additions see ref. 7, ch. 10.

¶ Free nonactic acid and homononactic acid have been isolated from the culture broth of *S. griseus* [the former occurring mainly as the (−)-enantiomer and the latter mainly as the (+)-enantiomer], and labelled nonactic acid can be efficiently incorporated into macrotetrolides when added to cultures of *S. griseus* and *S. griseoflavus* (see ref. 2).

Table 1. Incorporations of label in derivatives (**10**)—(**13**) derived from [1-^{13}C,$^{18}O_2$]acetate and propionate. All spectra were recorded in $CDCl_3$ at 100 MHz.

Precursor[a]	MTPA derivative	^{16}O:^{18}O Ratios[b] with isotope shifts (Δ/Hz) C(1)	C(6)	C(8)
[1-^{13}C,$^{18}O_2$]acetate	(**10**)	—	—	48:52 (4.1)
	(**11**)	—	—	45:55 (4.2)
[1-^{13}C,$^{18}O_2$]propionate	(**10**)	59:41 (3.3)	80:20 (2.4)	—
	(**11**)	52:48 (3.6)	79:21 (2.4)	—
	(**12**)	50:50 (3.2)	75:25 (2.7)	25:75 (4.5)
	(**13**)	45:55 (3.6)	71:29 (2.5)	25:75 (4.0)

[a] Doubly labelled precursors are approximately 70% $^{13}C^{18}O_2$ + 14% $^{13}C^{18}O_1$. [b] The ^{16}O:^{18}O ratios are the ratios of the integrated signal intensities.

the active sites of one or more multi-enzyme antibiotic synthetase complexes.

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