Biosynthesis of the Macrolide Antibiotic Tylosin. Origin of the Oxygen Atoms in Tylactone

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All of the oxygen atoms in tylactone, a biosynthetic precursor of tylosin, are derived intact with their attached carbon atoms from C(1) of acetate, propionate, and butyrate, during its biosynthesis in cultures of a blocked mutant of *Streptomyces fradiae*.

Tylosin is a sixteen membered ring macrolide antibiotic whose branched and highly functionalized carbon backbone is constructed from building blocks derived from acetate, propionate, butyrate, and succinate.[1] The lack of detailed knowledge about the biosynthesis of such macrolide rings has recently been highlighted,[2] as has the value of experiments

with ^{13}C and ^{18}O labelled precursors which define the origins of the oxygen atoms in these and related antibiotics.[3] We report below the results of our experiments using [1-^{13}C,-$^{18}O_2$] labelled acetate, propionate, and butyrate which demonstrate that the oxygen atoms in tylactone (**1**), a biosynthetic precursor of tylosin, are derived intact with their attached carbon atoms from these precursors. This provides mechanistic information about the formation of the macrolide ring and further extends the general conclusions about such metabolic pathways already accrued through biosynthetic studies on the propionate derived fourteen membered ring macrolide antibiotic erythromycin,[2] the avermectins,[4] and the polyether ionophore antibiotics lasalocid[5] and monensin.[6]

The first detectable intermediate on the tylosin biosynthetic pathway is tylactone[7] (**1**), although the steps between tylactone and tylosin have now been well characterized,[8] and ^{13}C labelling experiments[1] have established the origins of the carbon atoms of the macrolide ring from acetate, propionate, butyrate, and succinate (see Figure 1). Our experiments have been carried out with a blocked mutant[7] of *Streptomyces fradiae*, which accumulates tylactone instead of tylosin. Using this mutant the incorporation of labels into the macrolide ring can be followed without the complications arising from its further functionalization and the addition of three sugar moieties.

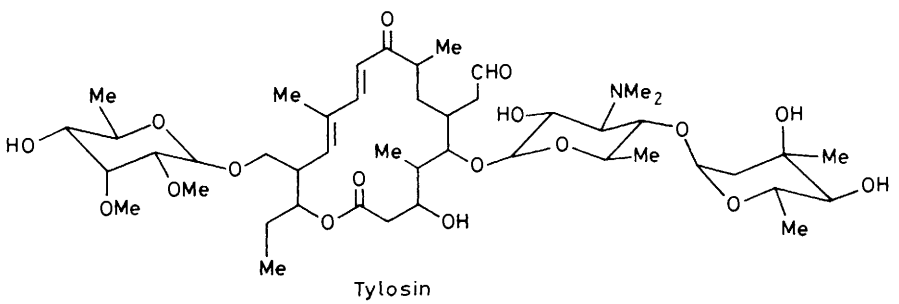
As a first step we sought to assign unambiguously the ^{13}C n.m.r. spectrum of tylactone and establish a feeding regime that would allow the incorporation of ^{13}C label from acetate, propionate, and butyrate into tylactone. The first task was accomplished by using a combination[9] of heteronuclear 2D-*J* spectroscopy,[10] to establish carbon–proton connectivities, and ^{13}C homonuclear 2D autocorrelation spectroscopy[11] to provide the carbon–carbon bond connectivities, and the assignment that followed from these spectra is given in Table 1. A suitable feeding regime involved adding batchwise [1-^{13}C] labelled acetate, propionate, or butyrate separately to shake flask cultures (100 ml) of the *S. fradiae* mutant A252, over the period of antibiotic production, so that their final concentration in the broth was 20 mM. The rigorously purified and labelled tylactones, isolated after extraction from the whole broth into chloroform, showed enriched singlets in their ^{13}C n.m.r. spectra (2—11 fold enhancements) at the positions expected on the basis of the established precursor–product relationships (Figure 1). It is of interest also that [1-^{13}C]butyrate produced substantial enrichments (6 fold) of the signals assigned to C(3), C(7), C(11), C(13), and C(15), as well as C(5), (11 fold), indicating the *in vivo* metabolism of butyrate[1] to methylmalonyl-CoA and propionyl-CoA, presumably *via* succinyl-CoA.

The incorporation of [1-^{13}C,$^{18}O_2$] labelled acetate, propionate, and butyrate was then completed in an identical fashion. The results of these experiments are given in Table 2. They demonstrate that the carbonyl oxygens at C(1) and C(9)

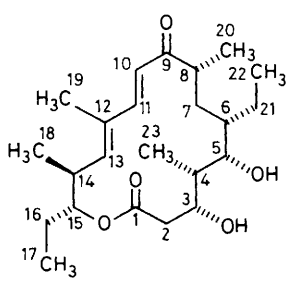
Table 1. Assignment of the ^{13}C n.m.r. spectrum of tylactone in $CDCl_3$ at 100 MHz.

Assignment	δ/p.p.m.[a]
C(1)	174.6
C(2)	39.5
C(3)	66.9
C(4)	40.0
C(5)	73.0
C(6)	38.2
C(7)	32.9
C(8)	45.1
C(9)	203.8
C(10)	118.7
C(11)	147.7
C(12)	133.6
C(13)	145.4
C(14)	38.8
C(15)	78.8
C(16)	24.8
C(17)	9.7
C(18)	16.2
C(19)	13.1
C(20)	17.9
C(21)	23.0
C(22)	11.8
C(23)	9.4
$CDCl_3$	77.1000

[a] Chemical shifts are relative to the centre line of $CDCl_3$.



Tylosin



Tylactone (1)

Table 2. Incorporations of [1-^{13}C,$^{18}O_2$] labelled acetate (Ac), propionate (Pr) and butyrate (Bu) into tylactone; the ^{18}O induced shifts of the ^{13}C resonances, and the ^{16}O:^{18}O ratios are given.

	Δδ/Hz[a]			
C atom	[1-$^{13}C^{18}O_2$]Ac[b]	[1-$^{13}C^{18}O_2$]Pr[b]	[1-$^{13}C^{18}O_2$]Bu[b]	^{16}O:^{18}O[c]
1	3.8	—	—	53:47
3	—	1.9	(1.9)	46:54 (60:40)
5	—	—	2.9	54:46
9	4.9	—	—	54:46
15	—	3.8	(3.9)	47:53 (66:34)

[a] All spectra were recorded at 100 MHz; spectral width 25000 Hz, 60° pulse, pulse delay 2.4 s, 64K data points; with resolution enhancement. [b] Doubly labelled precursors are approximately 70% ($^{13}C^{18}O_2$) + 14% ($^{13}C^{18}O_1$), with 90% ^{13}C enrichment at C(1). [c] ^{16}O:^{18}O ratios are the ratios of the integrated signal intensities.

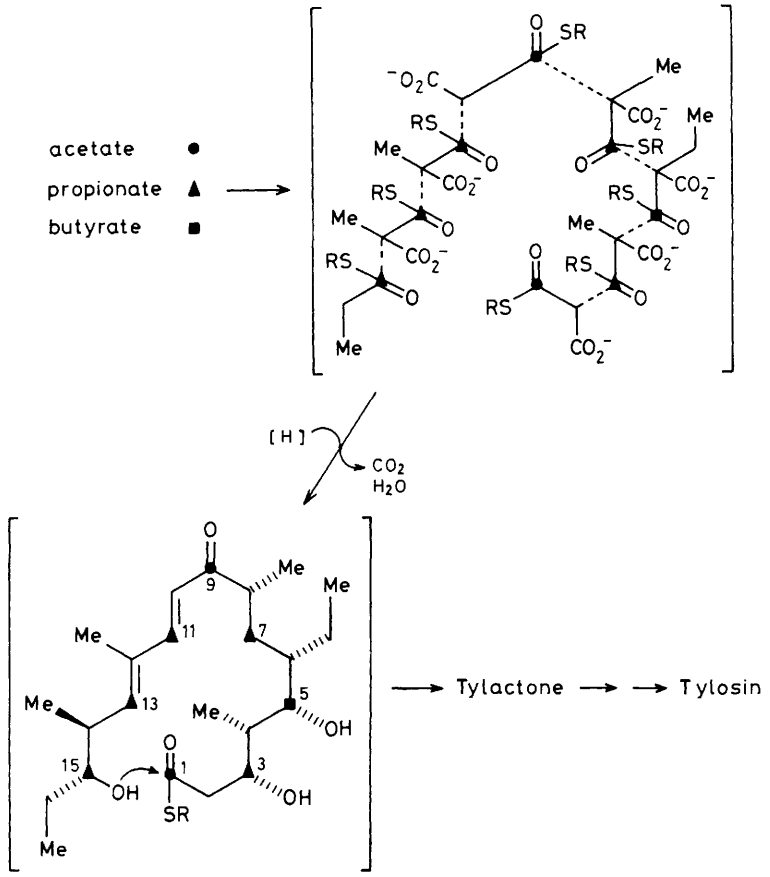


Figure 1

can be derived intact with C(1) of acetate, that the C(15)–O and C(3)–O bonds remain intact from propionate, and that the C(5)–O as well as the C(15)–O and C(3)–O bonds remain intact with C(1) of butyrate. The high levels of ^{13}C–^{18}O retention over ^{13}C–^{16}O at the two carbonyl groups is noteworthy, and is high enough to be inconsistent with the occurrence of free carboxylate groups at these carbons in intermediates during the biosynthesis. These data thus provide direct support for the closure of the macrolide ring as the last step in tylactone formation by the direct nucleophilic displacement of a thiol activating group, possibly that used during carbon chain extension, by a hydroxy group at C(15) (Figure 1).

The process of carbon chain construction resembles to some extent that occurring during erythromycin biosynthesis, and fatty acid biosynthesis, except that at least four different substrates are now used (malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, and a propionyl-CoA starter unit) and additional dehydration steps must accompany the series of reductive processes required appropriately to modify the putative transient β-ketothiol esters. Of these the steric courses of the reductions which generate the C(3) and C(5) hydroxyl groups occur in the opposite sense, with respect to the substrate, to that which generates the C(15) chiral centre (see Figure 2); a change which is consistent with these reactions occurring at different active sites on distinct reductases. The ^{18}O labelling results show that this stereochemical change is most unlikely to occur through a dehydration–rehydration sequence at C(14)–C(15), *via* an enone or dienone intermediate. The relevance of these findings to the biosynthesis of other sixteen membered ring macrolides are best considered, and are reinforced, in the light of the stereochemical homologies revealed by Celmers' general model of macrolide structure and biogenesis.[12]

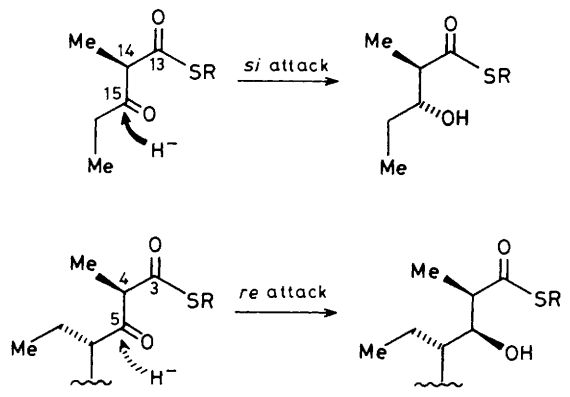


Figure 2

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References

1 S. Omura, H. Takeshima, A. Nakagawa, J. Miyazawa, F. Piriou, and G. Lukacs, *Biochemistry*, 1977, **16**, 2860.
2 D. E. Cane, H. Hasler, and T.-C. Liang, *J. Am. Chem. Soc.*, 1981, **103**, 5960.
3 J. C. Vederas, *Can. J. Chem.*, 1982, **60**, 1637.
4 D. E. Cane, T.-C. Liang, L. Kaplan, M. K. Nallin, M. D. Schulman, O. D. Henseus, A. W. Douglas, and G. Albers-Schönberg, *J. Am. Chem. Soc.*, 1983, **105**, 4110.
5 C. R. Hutchinson, M. M. Sherman, J. C. Vederas, and T. T. Nakashima, *J. Am. Chem. Soc.*, 1981, **103**, 5953.
6 D. E. Cane, T.-C. Liang, and H. Hasler, *J. Am. Chem. Soc.*, 1981, **103**, 5962; 1982, **104**, 7274; D. E. Cane, W. D. Celmer, and J. W. Westley, *ibid.*, 1983, **105**, 3594; A. A. Ajaz and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1983, 679.
7 R. H. Baltz and E. T. Seno, *Antimicrob. Agents Chemother.*, 1981, **20**, 214; N. D. Jones, M. O. Chaney, H. A. Kirst, G. M. Wild, R. H. Baltz, R. L. Hamill, and J. W. Paschal, *J. Antibiot.*, 1982, **35**, 420.
8 R. H. Baltz, E. T. Seno, J. Stonesifer, and G. M. Wild, *J. Antibiot.*, 1983, **36**, 131; S. Omura, N. Sadakane, and H. Matsubara, *Chem. Pharm. Bull.*, 1982, **30**, 223.
9 See for example, J. A. Robinson and D. L. Turner, *J. Chem. Soc., Chem. Commun.*, 1982, 148.
10 D. L. Turner and R. Freeman, *J. Magn. Reson.*, 1978, **29**, 587.
11 D. L. Turner, *J. Magn. Reson.*, 1983, **53**, 259.
12 W. D. Celmer, *Pure Appl. Chem.*, 1971, **28**, 413.