# Oxytetracycline Biosynthesis: Origin of the Carboxamide Substituent

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Biosynthetic labelling studies using $^{13}C$ n.m.r. spectroscopy have demonstrated the direct incorporation of [1,2,3-$^{13}C_3$]malonate into carbons-1,2 and the carboxamide substituent of oxytetracycline.

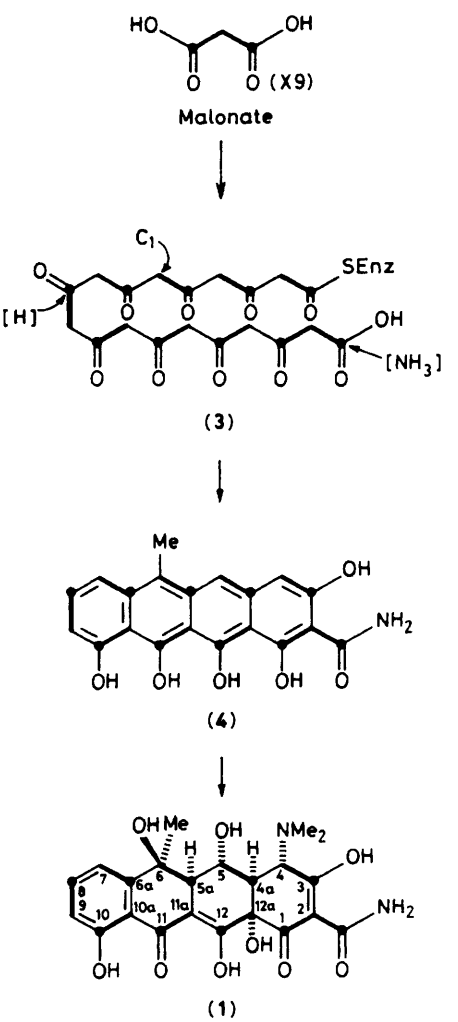
The complete polyketide origin of the tetracyclic nucleus of oxytetracycline (1) has recently been established using [1-$^{13}C$]- and [1,2-$^{13}C_2$]-acetate[1] and in the present communication we describe an investigation of the origin of the carboxamide substituent using [1,2,3-$^{13}C_3$]malonate.

Gatenbeck originally reported labelling of the amide carbon from [1-$^{14}C$]acetate, apparently involving prior conversion into $^{14}CO_2$.[2] On the basis of this study, he proposed the derivation of the tetracycline carbon skeleton from nine molecules of malonyl coenzyme A with retention of all three carbon atoms of a primer malonate unit (*cf.* Scheme 1).

The implicit role of malonamyl coenzyme A as a nonaketide starter unit was subsequently examined in an unsuccessful attempt to incorporate [1,3-$^{14}C_2$]malonamic acid into chlortetracycline.[3] The non-incorporation of this candidate precursor may have been due to cell wall impermeability; furthermore, this observation does not exclude the possible utilisation of malonic acid *via* an alternative biosynthetic intermediate.

[1,2,3-$^{13}C_3$]Malonic acid was fed to growing cultures of *Streptomyces rimosus* under the conditions described previously for the incorporation of [1,2-$^{13}C_2$]acetate,[1] and the resulting labelled oxytetracycline was similarly recovered as its crystalline hydrochloride.

The proton noise decoupled $^{13}C$ n.m.r. data (Table 1) show a coupling pattern which only differed significantly from that of the [1,2-$^{13}C_2$]acetate-derived hydrochloride in respect of the C-2 ($\delta$ 95.8 p.p.m.) and carboxamide ($\delta$ 172.1 p.p.m.) signals (Figure 1). In contrast to the spectrum of [1,2-$^{13}C_2$]acetate labelled (1) in which the major components of the C-2 signal consisted of a singlet and a doublet ($J_{2,1}$ 62.6 Hz), the spectrum of the [1,2,3-$^{13}C_3$]malonate-derived product exhibited a C-2 multiplet which included a double doublet indicating coupling with both C-1 ($J_{2,1}$ 62.7 Hz) and the carboxamide ($J_{2,\text{amide}}$ 63.8 Hz). The double doublet was accompanied by a doublet ($J_{2,1}$ 62.7 Hz) of similar intensity, resulting from incorporation of [1,2-$^{13}C_2$]acetate formed through decarboxylation of the [1,2,3-$^{13}C_3$]malonate precursor, and a dominant singlet, the relative intensity of which masked the inner satellite peaks of the double doublet. This assignment of the C-2 multiplet was supported by the triplet




**Scheme 1**

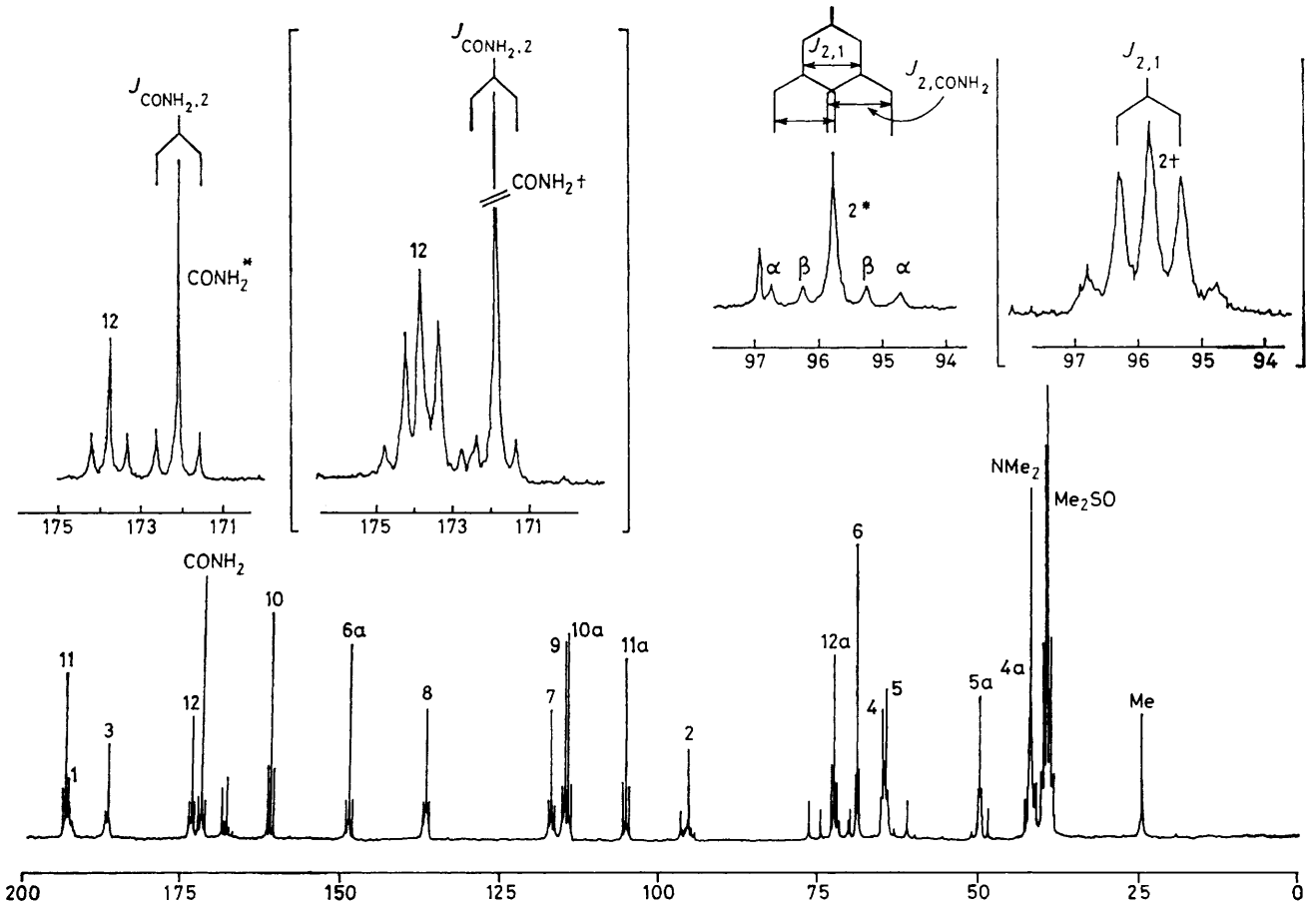
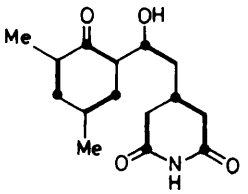



**Figure 1.** 62.9 MHz Proton noise decoupled $^{13}C$ n.m.r. spectrum of [1,2,3-$^{13}C_3$]malonate-derived oxytetracycline (**1**). Chemical shifts in p.p.m. relative to midline of $(CD_3)_2SO$. * Expansion of $CONH_2$ and C-2 regions: the C-2 signal shows two peaks (α) corresponding to the outer satellites of a double doublet, $J_{2,1}$ 62.7, $J_{2,amide}$ 63.8 Hz, and two peaks (β) corresponding to a doublet $J_{2,1}$ 62.7 Hz resulting from incorporation of [1,2-$^{13}C_2$]acetate formed by prior decarboxylation of [1,2,3-$^{13}C_3$]malonate. † Expansion of corresponding regions of the spectrum of [1,2-$^{13}C_2$]acetate-derived oxytetracycline (outer minor satellite peaks of the C-2 signal result primarily from low frequency incorporation of adjacent doubly labelled acetate[1]).

character of the carboxamide signal, comprising a singlet flanked by a doublet ($J_{2,amide}$ 65.3 Hz).

While these results support the existence of a mechanism for the direct incorporation of an intact malonate unit into carbons-1,2 and the carboxamide substituent of (**1**), a possible alternative interpretation based on the selective re-incorporation of metabolically derived $^{13}CO_2$ into the amide moiety might also appear to account for the observed spectral data. This would parallel the pathway involving mediation of $^{13}CO_2$ which was previously invoked to explain two features of the carboxamide signal of [1,2-$^{13}C_2$]acetate-derived (**1**),[1] namely the appreciably enhanced intensity of the natural abundance singlet and the presence of a low intensity doublet due to coupling with $^{13}C$-2. A qualitatively similar spectrum would result from the incorporation of [1,2,3-$^{13}C_3$]malonate if accompanied by extensive decarboxylation and efficient re-utilisation of the resulting $^{13}CO_2$. However, quantitative comparison of the carboxamide signals in the spectra of the [1,2,3-$^{13}C_3$]malonate and [1,2-$^{13}C_2$]acetate-derived products (Figure 1), shows that, whereas in malonate-labelled (**1**), the carboxamide doublet is far more intense than the corresponding doublet of the acetate-labelled product, the observed relative enrichment of the natural abundance singlet is considerably reduced (malonate:acetate = *ca.* 1:2.7).

It follows that re-incorporation of $CO_2$ formed *via* malonate decarboxylation is less efficient than that of $CO_2$ derived from

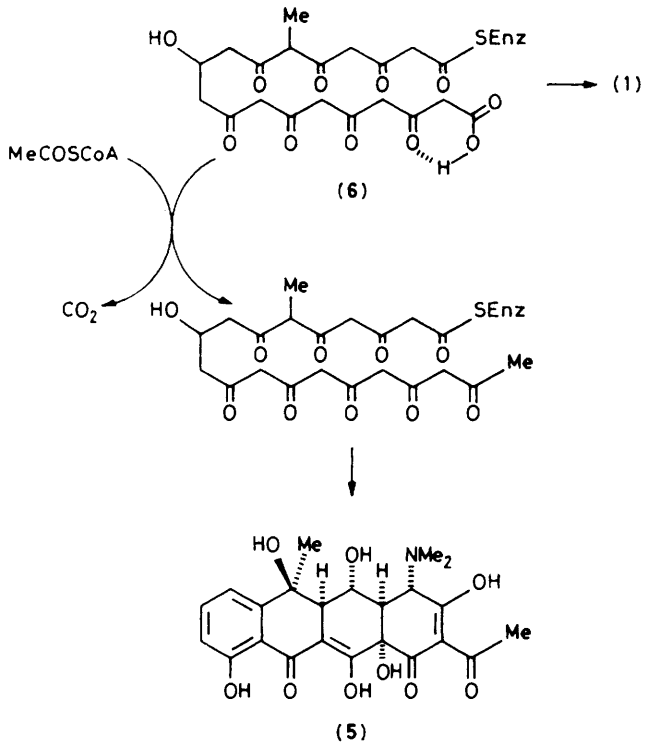



(**2**)

acetate. Consequently, the observation that only the [1,2,3-$^{13}C_3$]malonate-derived product exhibits significant coupling of carbon-2 with both C-1 and the $CONH_2$ substituent confirms the operation of a pathway for the incorporation of an intact malonate unit, as originally suggested by Gatenbeck.

An analogous direct incorporation of [1,2,3-$^{13}C_3$]malonate, again with partial decarboxylation, was recently reported in a study of the biosynthesis of the *Streptomyces* antibiotic cycloheximide (**2**).[4] In this polyketide, malonate appears to function stereospecifically as a primer unit in the biosynthesis of the glutarimide ring.

The stage at which the amide bond is formed in (**1**) remains to be determined. While nitrogen insertion may occur early in the sequence requiring a malonamate primer unit, an equally acceptable scheme would involve formation of the amide moiety subsequent to the assembly of a nonaketide acid




Scheme 2

intermediate (**3**) which is then released from the enzyme following cyclisation to 6-methylpretetramid (**4**). Similarly, although it is generally assumed that the biosynthesis of the 2-acetyldecarboxamidotetracycline series, *e.g.* (**5**) requires the sequential assembly of a decaketide based on an acetate primer unit, the presently available data do not exclude a mechanism involving subsequent displacement by acetyl coenzyme A of the carboxy moiety of an exclusively malonate-derived nonaketide. In this event, the carboxamido and acetyl series of tetracyclines would be derived from a common advanced polyketide intermediate such as (**6**), (*cf.* Scheme 2).

**Table 1.** 62.9 MHz $^{13}C$ N.m.r. data for oxytetracycline (**1**) [in $(CD_3)_2SO$] derived from [1,2,3-$^{13}C_3$]malonate.

| Coupled carbons | $J_{CC}$/Hz |
|---|---|
| $CONH_2$, 2 | 65.3, 63.8[a] |
| 2, 1 | 62.7,[b] 62.4 |
| 12a, 12 | 52.4, 53.0 |
| 11a, 11 | 56.1, 56.0 |
| 10a, 10 | 63.0, 63.2 |
| 9, 8 | 58.2, 56.9 |
| 7, 6a | 57.6, 61.1 |
| 6, 5a | 38.0, 38.2 |
| 5, 4a | 34.1 33.3 |
| 4, 3 | 40.8, 40.6 |

[a] Doublet. [b] Double doublet (*cf.* Figure 1).

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