Biosynthetic Origins of the Polycyclic Xanthone Antibiotic, Citreamicin[1]

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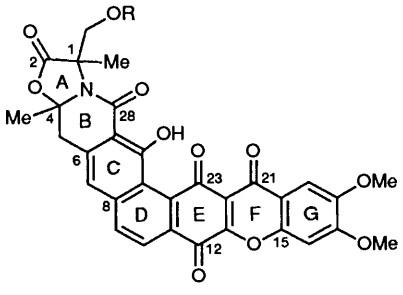
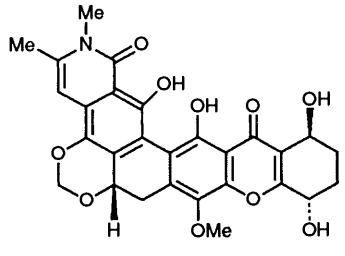
The biosynthesis of citreamicin **1** has been studied by feeding ^{13}C- and ^{18}O-labelled precursors to *Micromonospora citrea* cultures. ^{13}C-NMR spectroscopic analysis of the enriched products demonstrated a labelling pattern indicating that the antibiotic is derived from a polyketide which undergoes rearrangement by a mechanism unique for xanthone biosynthesis.

The citreamicins, *e.g.* **1**,[2,3] are polycyclic aromatic antibiotics produced by *Micromonospora citrea*. These compounds are active against a variety of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. All of the citreamicins identified from *M. citrea* share a common xanthone nucleus. Xanthones in general comprise a variety of plant, fungal and bacterial secondary metabolites, and compounds structurally related to the citreamicins include simaomicin,[4] lysolipin,[5] cervinomycin[6] and actinoplanone.[7] In order to explore possible approaches towards preparing novel and improved citreamicins by fermentation, we initiated an investigation into the biochemical origins of the citreamicins.



1; R = $COCH_2CHMe_2$



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Biosynthetic studies on xanthones have been reported. Previously we demonstrated that the antibiotic simaomicin **2** is derived from acetate, and postulated a mechanism involving a single polyketide.[8] The fungal metabolites tajixanthone and shamixanthone,[9] sterigmatocystin[10] and ravenelin[11] are all reported to be derived from single acetate-derived polyketide precursors. In higher plants it has been widely speculated that a polyketide with a shikimate-derived starter unit is the origin of a variety of xanthones; in the cases of the *Gentineacea*-xanthones,[12] mangostin[13] and mangiferin[14] the available evidence supports this hypothesis. Based upon these studies, we concluded that a probable biosynthetic route leading to the xanthone skeleton of the citreamicins would involve a single acetate-derived polyketide precursor which is suitably folded, cyclised and decarboxylated (Scheme 1a), analogous to the mechanism proposed for simaomicin.[8] However, a route involving a shikimate-derived starter unit (Scheme 1b) or a novel route involving the condensation of two discrete units (Scheme 1c) could not be excluded.

Results and Discussion

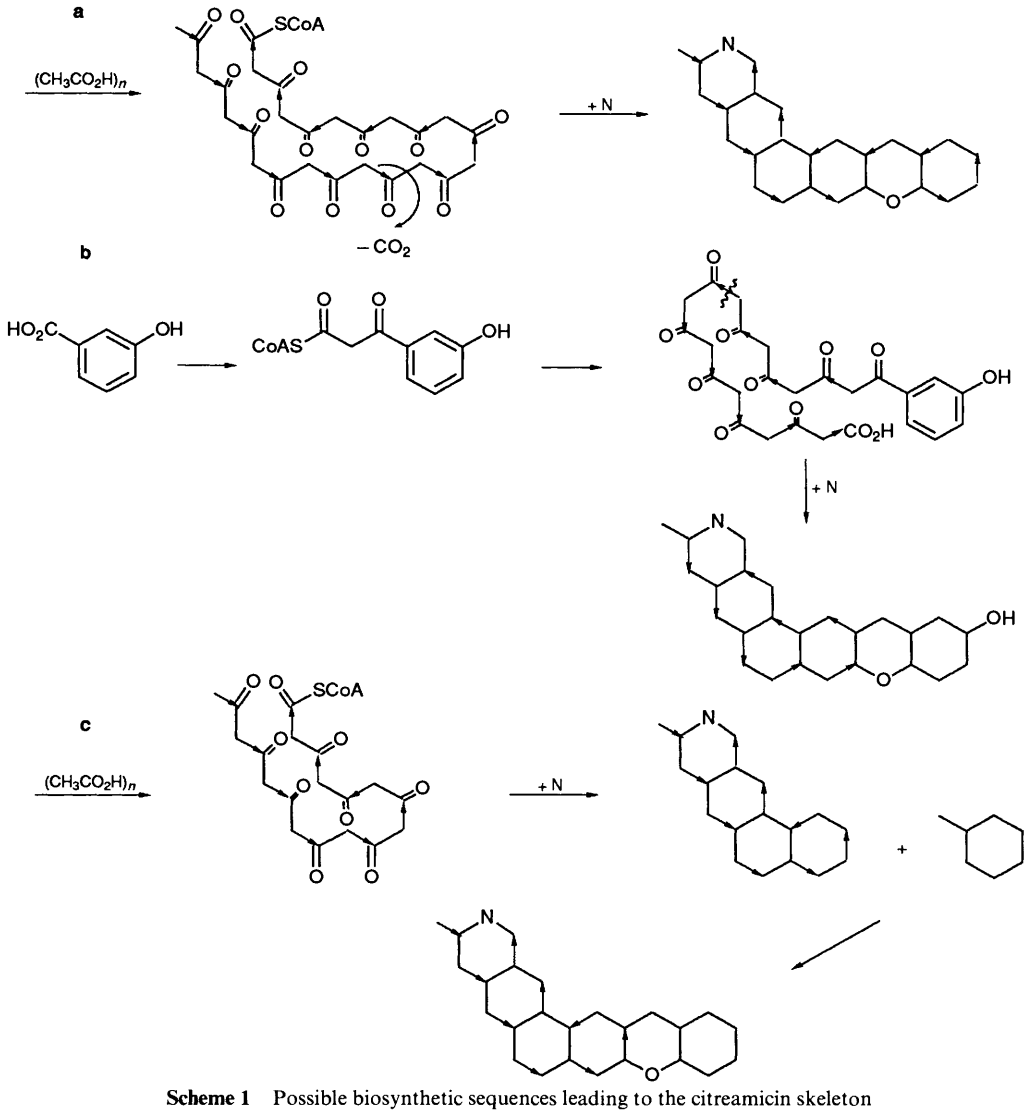
When citreamicin *alpha* **1** was isolated from a *M. citrea* fermentation supplemented with [1-^{13}C]- or [2-^{13}C]-acetate, the antibiotic was enriched in the carbon atoms shown in Table 1 and summarized in Scheme 2. When the antibiotic was produced in the presence of [1,2-$^{13}C_2$]acetate, intact units were incorporated as defined by reasonably clear pairs of coupling constants (Table 1). Completely unambiguous evidence for these units was obtained from an INADEQUATE experiment performed on the antibiotic enriched with double labelled acetate (Fig. 1). In Fig. 1, the coupled pairs of signals are connected with horizontal lines for clarity. Twelve well-defined pairs of signals correspond to the acetate units shown by arrows in Scheme 2.

When citreamicin *alpha* was isolated from the fermentation broth to which [^{13}C-*methyl*]methionine was added, the antibiotic was enriched with ^{13}C in both methoxy groups (Table 1 and Scheme 2). This suggests that the usual methylation route involving *S*-adenosyl methionine is employed by the organism.

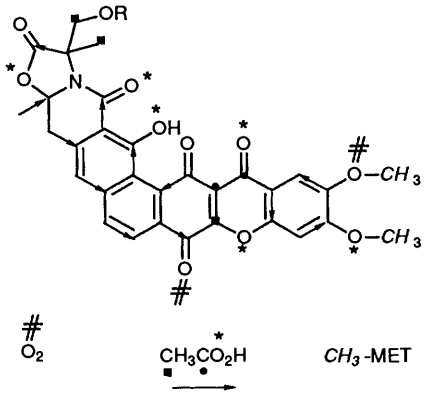
Citreamicin *alpha* produced in the presence of [1-^{13}C,$^{18}O_2$]-acetate was shown to contain oxygen derived from acetate at positions 4, 15, 17, 21, 26 and 28 (Table 1 and Scheme 2) by measuring the isotope-induced shifts by ^{13}C NMR spectroscopy. When citreamicin *alpha* was produced in the presence of [1-^{13}C]- and [2-^{13}C]-acetate together with an atmosphere of approximately 50% $^{18}O_2$, antibiotic was produced which contained ^{18}O at positions 12 and 18 (Table 1 and Scheme 2). These approaches failed to disclose the origin of the oxygen at position 23. With both sets of experiments, the possibility of the incorporation of oxygen-18 followed by loss through chemically or biochemically induced exchange cannot be excluded.

Results from our labelling studies are summarised in Scheme 2. The incorporation of acetate clearly indicates the polyketide nature of the citreamicins, although the labelling pattern demonstrates considerable complex rearrangement. The almost total acetate origins of the citreamicin nucleus excludes a pathway of the type proposed for mangostin biosynthesis involving a shikimate derivative.[13]

If the citreamicin xanthone were derived from one polyketide chain with simply a single carbon being excised and replaced with oxygen as is postulated to occur in simaomicin[8] and



Scheme 1 Possible biosynthetic sequences leading to the citreamicin skeleton



Scheme 2 Structure and biosynthetic origin of citreamicin *alpha*

illustrated in Scheme 1a, then doubly-labelled acetate should have been incorporated throughout the molecule, with one unit forming carbons 21–22, except in the case of carbon 13 which would be derived from the methyl of an acetate unit. In contrast, our results which exclude a simaomicin-like biosynthetic pathway, demonstrate carbons 21 and 22 are both derived from carbon 1 of acetate.

The simaomicin-like route is further excluded by the labelling pattern observed in ring G, with carbons 15 and 20 originating from carbons 1 and 2 of the same acetate unit, respectively. This cannot be explained by a simple model involving rotation around bond 20–21 in the decarboxylated intermediate (as indicated in Scheme 3a) since this latter mechanism predicts the head-to-tail condensation of acetate units in the opposite direction to that observed in ring G.

The two rearrangement processes outlined in Schemes 3b and 3c lead to the observed acetate incorporation patterns. Each of these processes begins with the excision of a single carbon, presumably through oxidative decarboxylation. However, instead of simple ring closure, a reactive intermediate is formed, which subsequently rearranges forming the xanthone ring system. Spiro intermediates, as in **3b**, have been proposed to account for the oxygen substitution patterns found in xanthones derived from higher plants.[15] The cyclopropyl alternative shown in 3c is also feasible. Both of these mechanisms predict that carbons 13 and 21 are derived from the same acetate unit. If this were the case, two-bond coupling could be observable between carbons 13 and 21, in the material derived from [1,2-$^{13}C_2$]acetate. We were not able to detect any such coupling. Since the predicted coupling is small (0–3 Hz[16]), it is possible that one of the rearrangement processes is occurring and that the two-bond coupling is too small to be detected.

The labelling pattern produced from acetate in the A ring, formed by the addition of an isobutyryl unit to the xanthone nucleus, cannot be explained in terms of the most obvious

Table 1 ^{13}C NMR spectroscopic data for citreamicin α (**1**) derived from isotopically enriched precursors[a]

Carbon	δ	^{13}C enhancement[b]			^{13}C–^{13}C coupling,[c]	^{18}O-induced shifts, Δδ[d]	
		1-^{13}C-Ac	2-^{13}C-Ac	$^{13}CH_3$-Met	^{1}J/Hz	^{18}OAc[e]	$^{18}O_2$[f]
1	62.3	0.9	6.5	—	54.8	—	—
1a	20.2	1.4	5.8	—	nc	—	—
1b	65.0	0.8	3.6	—	nc	—	—
2	171.5	2.3[g]	1.7[g]	—	54.8	—	—
4	93.4	8.3	0.8	—	43.4	0.029	—
4a	25.8	1.6	9.4	—	43.4	—	—
5	41.9	0.9	8.2	—	40.8	—	—
6	134.8	5.4	0.6	—	40.7	—	—
7	117.7	0.7	3.5	—	56.3	—	—
8	140.7	7.2	0.5	—	56.3	—	—
9	132.2	1.0	3.8	—	60.7	—	—
10	124.4	9.3	0.9	—	60.7	—	—
11	129.7	0.5	1.8	—	58.0	—	—
12	178.1	1.8	0.4	—	58.1	—	0.012
13	153.4	0.2	2.0	—	nc	—	—
15	150.8	2.0	0.6	—	64.7	0.026	—
16	100.4	0.6	2.8	—	73.4	—	—
17	155.5	2.8	0.4	—	73.4	0.016	—
17-OMe	56.8	—	—	3.8	nc	—	—
18	148.7	0.3	2.3	—	74.5	—	0.017
18-OMe	56.5	1.0	1.0	3.1	nc	—	—
19	104.9	7.3	0.7	—	74.4	—	—
20	119.5	0.5	3.2	—	64.7	—	—
21	172.1	1.9	0.3	—	nc	0.035	—
22	120.8	3.2	0.4	—	nc	—	—
23	181.2	0.2	1.3	—	55.0	—	—
24	137.7	3.3	0.4	—	55.0	—	—
25	119.9	0.3	2.4	—	67.5	—	—
26	162.1	2.7	0.6	—	67.5	0.0096	—
27	107.3	0.4	3.0	—	63.7	—	—
28	165.8	2.0	0.5	—	63.7	0.028	—
1′	171.5	2.3[g]	1.7[g]	—	nc	—	—
2′	42.9	1.5	2.7	—	nc	—	—
3′	25.5	1.7	2.2	—	nc	—	—
3′a	22.3	1.9	2.1	—	nc	—	—
4′	22.4	1.4	2.2	—	nc	—	—

[a] Experiments run in $CDCl_3$; signal assignments from ref. 2. [b] Ratio of normalized signal intensities for enriched and natural abundance samples. [c] Determined on material derived from 1,2-$^{13}C_2$-acetate experiment. [d] Isotope shifts due to ^{18}O, in ppm. [e] Determined on material derived from 1-^{13}C,^{18}O-acetate experiment. [f] Determined on material derived from $^{18}O_2$ experiment. [g] Signals for C-2 and 1′ are superimposed.

routes involving either valine or mevalonic acid. The pathway leading to the introduction of this unit remains unidentified.

The incorporation of oxygen from acetate showing carbons 4, 15, 17, 21, 26 and 28 are all attached to oxygens derived from acetate is predictable. However, the demonstration that the oxygen on carbon 12 is not from acetate, but is from molecular oxygen, is surprising. Furthermore, even though the oxygen at position 18 is derived from molecular oxygen, as expected, the lack of incorporation into position 23 was not anticipated. Given the extensive rearrangement postulated to occur, unpredicted labelling patterns are not totally unreasonable. Complex oxygen exchanges have been reported to occur in other antibiotic biosynthetic systems (for example, see ref. 17).

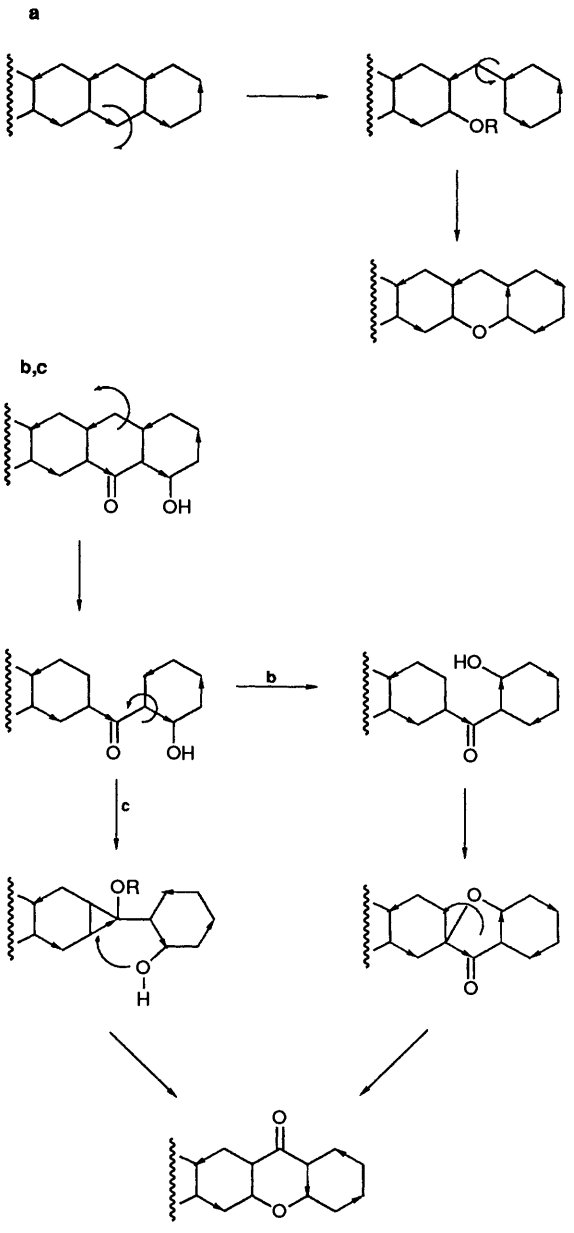
Structural elucidation of minor fermentation components, which may give further insight into the nature of the biosynthetic intermediates of the citreamicins, is currently in progress.

Experimental

Culture of Micromonospora citrea NRRL 18351.—*M. citrea* was preserved in a frozen state at −75 °C in 20% glycerol. Frozen culture (*ca.* 1.5 cm^{-3}) was used to inoculate seed medium (100 cm^3) in a 500 cm^3 Erlenmeyer flask containing (in %) yeast extract (0.5), NZ Amine (0.5), dextrin (2.0), glucose (1.0) and $CaCO_3$ (0.1). The culture was incubated at 30 °C on a rotary shaker at 200 rpm. After 3 d this culture (5 cm^3) was added to production medium (100 cm^3) in a 500 cm^3 Erlenmeyer flask consisting of either (in %) dextrin (1.25), sucrose (1.25), soy peptone (1.0) and $CaCO_3$ (0.35), or dextrin (2.5), bactopeptone (0.5), $CaCO_3$ (0.1), sucrose (1.25) and ferric ammonium citrate (0.005). Following a further 5–8 d incubation at 30 °C on a rotary shaker at 200 rpm, the antibiotic was extracted and purified as described below.

Addition of Labelled Precursors.—Aqueous solutions of labelled sodium acetate (obtained from Cambridge Isotope Laboratories, MA, USA; 98–99% enriched) and methionine (from Cambridge Isotope Laboratories; 99% enriched) were prepared and sterilized by filtration. [1,2-$^{13}C_2$]acetate, [1-^{13}C]- or [2-^{13}C]acetate (1 g) was added per dm^3 of medium at the time of inoculation; [1-^{13}C,$^{18}O_2$]acetate (200 mg) was added per dm^3 of medium following 4 d of incubation of the culture; [^{13}C-*methyl*]methionine (25 mg) was added per dm^3 of medium at the time of inoculation.

^{18}Oxygen (obtained from Isotec Inc., OH, USA; 52.9 atom %) feeding was carried out using a 500 cm^3 Erlenmeyer flask containing culture (100 cm^3) in an enclosed system, with *ca.* 2 dm^3 of gas being circulated by a peristaltic pump. Following 3 d of incubation [1-^{13}C]- and [2-^{13}C]-acetate (20 mg each) were added to the fermentation broth and the whole system was



Scheme 3 Mechanisms for polyketide skeleton rearrangement leading to citreamicin

charged with fresh $^{18}O_2$. Throughout the incubation period carbon dioxide was removed by passing the circulating gas through dilute aqueous sodium hydroxide.

Antibiotic Extraction and Purification.—The citreamicins were isolated by extraction of whole broth with acidified acetone (3 vol.; 5% 2 mol dm^{-3} HCl in acetone). The resulting solutions were filtered with the aid of Celite 545 (20 g/100 cm^3), and the filtrate was partitioned into two phases by the addition of methylene dichloride (1 vol.). Evaporation of the methylene dichloride phase yielded a residue which was redissolved in acetone (5 cm^3) for reverse phase chromatography. The reverse phase system consisted of a C18 column (10 u, 21.4 mm × 25 cm), eluted with a mixture of acetonitrile–ammonium acetate buffer, 0.05 mol dm^{-3}, pH 4.5 (3:2) at 10 cm^3 min^{-1}. Fractions were collected at 2 min intervals and combined on the basis of analytical HPLC.[2] Depending upon the fermentation conditions employed, 5–10 mg of pure citreamicin *alpha* per 100 cm^3 of culture was obtained.

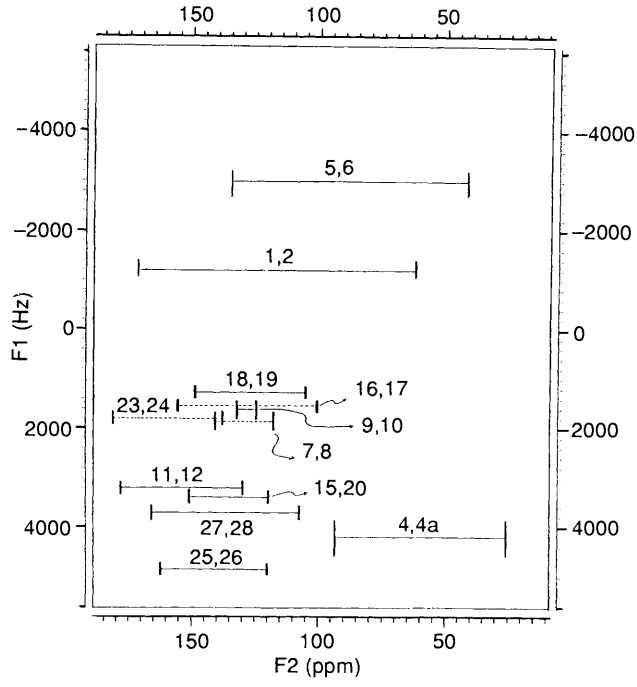


Fig. 1 Two-dimensional INADEQUATE spectrum of citreamicin derived from [1,2-$^{13}C_2$]acetate

NMR Measurements.—Spectra were obtained using a General Electric GN-500 spectrometer operating at a carbon frequency of 125.76 MHz. The two-dimensional INADEQUATE sequence used to obtain the spectrum shown in Fig. 1 is a slightly modified form of the sequence outlined by Bax *et al.*,[18] with the use of composite pulses[19] and a 45 degree read pulse. The coherence transfer time was optimized for a J_{CC} coupling constant of 45 Hz. The spectral width was 22 727 Hz (181 ppm) in both dimensions.

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