BIOSYNTHESIS OF CETOCYCLINE

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Cetocycline† is a broad spectrum antibiotic belonging to the tetracycline family. Because of its unusual structural features and biological properties,[1] a continuing interest in this antibiotic has developed. Several factors led us to explore its biosynthesis. While the biosynthetic origin of the tetracyclines has received intense study and their overall polyketide origin is beyond reasonable dispute,[2,3] the origin of relatively few of the individual carbon atoms has been established by direct experimentation.[3] The origin of the "starter" unit has been notoriously difficult to establish directly[4] and an uneven efficiency of incorporation of "extender" units has been remarked upon by several authors[3,5,6] raising the possibility that the formation of the polyketide skeleton might not occur in a concerted manner. The application of stable isotope methodology to study of the biosynthesis of cetocycline would allow for direct experimental observation of all carbons. Furthermore, the 2-acetyl moiety is more convenient for examining the origin of the "starter" unit than is the 2-carboxamide moiety of the clinically used tetracyclines. In addition, the biosynthetic pathways of *Nocardia* species have received rather less study than those of the Streptomyces.

For the incorporation experiments, *Nocardia sulphurea*, Abbott isolate S1A75B, was grown at 28°C at 250 rpm (5% vegetative inoculum) in a medium consisting of 2% soy bean meal, 0.5% Difco yeast extract, 0.2% $CaCO_3$, 0.05% citric acid, and 6% Cerelose added post sterilization at zero time. Two percent additional Cerelose was added at 144 hours. In this medium, antibiotic production began at about 96 hours and continued until at least 288 hours and neither growth nor antibiotic production were measurably affected by the quantities of propionic and acetic acid to be added in the labeling experiments. Therefore, $CH_3{}^{13}CO_2Na$ (90% atomic excess ^{13}C, Stohler) was added to the producing culture at 96, 120, 144 and 168 hours (17.7 or 8.8 mg/100 ml medium/500 ml flask) and the flasks were harvested at 240 hours, pooled, the pH adjusted to 6.5 and then frozen. After thawing and pH adjustment to 1~2 with 5 M HCl, the beers were saturated with NaCl and extracted twice with

Table 1. Incorporation of [1-^{13}C]-labeled acetate into cetocycline.[15]

Position	Chemical shift (ppm)	Enhancement ratio*
1	196.0	1.47
2**	111.3	1.05
2-*C*OCH$_3$	200.5	1.51
2-CO*C*H$_3$	26.4	1.00
3	190.9	1.62
4	50.6	0.84
4-NH*C*OCH$_3$	169.9	1.17
4-NHCO*C*H$_3$	22.5	Reference
4a	41.4***	—*
5	25.6	0.91
5a	129.8	1.31
6	121.9	1.07
6-CH$_3$	13.8	1.06
6a	136.9	1.47
7	114.4	0.89
8	135.0	1.61
9	119.1	0.98
9-CH$_3$	15.2	1.07
10	154.7	1.49
10a	108.8	0.94
11	162.5	1.73
11a**	111.3	1.05
12	201.0	1.75
12a	79.0	1.02

* Mean of two independent experiments. Determined as the ratio of enriched to natural ^{13}C abundance (area) normalized to CH_3 in 4-NHCOCH$_3$.

** Overlapping signals of C-2 and C-11a.

*** Obscured by DMSO-d_6 signal.

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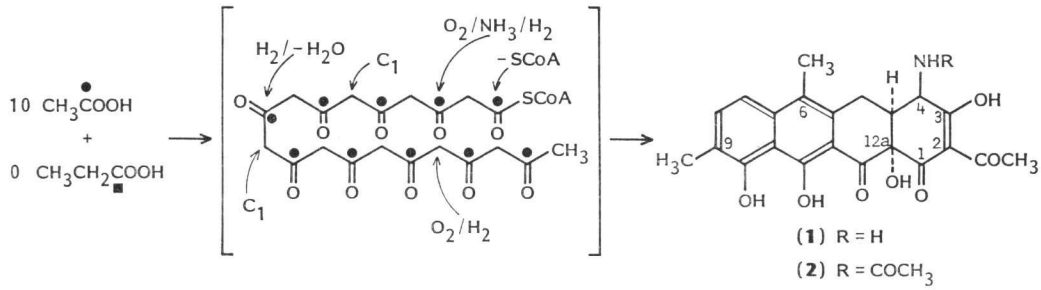
† Cetocycline has also been known as chelocardin and cetotetrin.



half the volume of BuOH. The BuOH extracts were concentrated to dryness and the hydrochloride salt taken up in MeOH. Solid K_2CO_3 and then Ac_2O were added in slight excess at room temperature and the mixture was stirred for 10 minutes. Excess water was added and the *N*-acetyl cetocycline (**2**) was isolated by extraction with CH_2Cl_2. Purification was achieved by repeated medium pressure liquid chromatography[7] on Silic AR CC-4 using 50% EtOAc in hexane or by flash chromatography on oxalic acid treated SiO_2 with EtOAc - Et_2O.

The ^{13}C NMR spectra were recorded at 20.1 MHz on a Bruker WP-80 spectrometer using Me_4Si as internal standard. Measurements were made at ambient temperature (approx. 30~35°C) and are accurate to about 0.2 ppm. Due to extensive epimerization at C-4 in DMSO-d_6, giving rise to duplicate spectral lines, the time of the experiment had to be restricted to about 5 hours. The sample was dissolved (*ca.* 100 mg in 0.4 ml) just prior to introduction in the instrument. A small pulse flip angle (16 liters), a short acquisition time (0.93 second) and about 20,000 scans were found to be suitable parameters for the time frame wanted (spectral width 4424 Hz). The spectra were interpreted in comparison with published tetracycline data, and by use of model anhydrotetracyclines, decoupling experiments and standard shift values.[8~12] The isotopic enrichment was determined as the ratio of enriched/natural ^{13}C abundance (area), normalized to the methyl group of the *N*-acetyl moiety, which served as an internal reference standard. The data are set forth in the table. From this, it is readily apparent that acetate serves as the starter unit and that the alternating enrichment pattern required by polyketide theory is observed. The variation in atomic enrichments observed are no greater overall than is seen with other polyketides, such as, for example, aklavinone.[13,14]

Experiments carried out using [1-^{13}C]-labeled propionate under otherwise identical conditions resulted in no measurable enrichment of any carbon of cetocycline. This is also consistent with polyketide theory and a methionine origin for the two *C*-methyl groups.

The starter unit for classical tetracyclines is usually suggested to be malonamyl coenzyme A.[2,3] Bioisosteric analogy would suggest that the starter unit for the 2-acetyltetracyclines, including cetocycline, should be acetoacetyl coenzyme A. While this seems intuitively unlikely, with acetyl coenzyme A being a more conventional choice for the C-2 acetyl tetracyclines and carbamoyl coenzyme A, for parallelism, the corresponding unit for the clinically important tetracyclines, work appearing after submission of this paper rules out this attractive hypothesis.[16]

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