The Oxygenation of the 3-Methyl Group of 7β-(5-D-Aminoadipamido)-3-methylceph-3-em-4-carboxylic Acid (Desacetoxycephalosporin C) by Extracts of Acremonium chrysogenum

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The final stages in the biosynthesis of cephalosporin C are thought to involve the oxidation of desacetoxycephalosporin C to desacetylcephalosporin C (Fujisawa et al., 1975). Liersch et al. (1976) showed that, in the presence of acetyl-CoA, cell-free extracts of Acremonium chrysogenum convert desacetoxycephalosporin C into cephalosporin C. They presented indirect evidence for an intermediate oxidation of the 3-methyl group on the cephalosporin nucleus by a mono-oxygenase dependent on NADH and Mn²⁺.

Using an assay which measures the conversion of [3-Me-³H]desacetoxycephalosporin C into [³H]desacetylcephalosporin C, we now show that this oxidation is in fact catalysed by a 2-oxoglutarate-linked dioxygenase similar to the enzymes described by Abbott & Udenfriend (1974).

Desacetoxycephalosporin C was prepared by hydrogenolysis of cephalosporin C by using a large excess of palladium oxide on keiselguhr (Stedman et al., 1934). [3-Me-³H]Desacetoxycephalosporin C was prepared at The Radiochemical Centre (Amersham, Bucks., U.K.) by a similar procedure but with ³H. Chromatography on DEAE-cellulose eluted with acetic acid followed by preparative t.l.c. on cellulose (Eastman Kodak, type 13254) eluted with propan-1-ol/water/acetic acid (5:2:1, by vol.) yielded a product with a specific radioactivity of about 2Ci/mmol.

A mutant of A. chrysogenum yielding high titres of cephalosporin C was grown on slopes containing 4% (w/v) maltose, 2.4% Oxoid malt extract, 1% Oxoid peptone and 2% agar for 7 days at 28°C. Mycelium from half the slope was transferred to a 250ml flask containing 40ml of 2.5% corn steep liquor, 0.55% ammonium acetate, 2.5% sucrose

Table 1. Effect of oxygenase substrates on the oxidation of desacetoxycephalosporin C

Supernatant (100 µl) was incubated for 30min at 4°C with 50 µl of water containing 0.8 nmol of [³H]desacetoxycephalosporin C (1.6 µCi) and other substrates as indicated. Desacetylcephalosporin C was then added as carrier and lactonized with 0.5M-HCl. A portion of the acidified supernatant was chromatographed on Ionex 25-SB-AC eluted with 25nm-sodium citrate buffer, pH 2.75. The lactone zone (RF 0.65) was eluted and the radioactivity it contained was corrected for the recovery of carrier. Another portion of the acidified supernatant was applied to a Dowex 1 (acetate form) column (25mmx4mm). After elution with water, conversion into desacetylcephalosporin C was estimated from the ratio of the applied to the eluted radioactivity.

<table>
<thead>
<tr>
<th>Additions to incubation</th>
<th>Conversion into desacetylcephalosporin C (relative to control without additions)</th>
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<tbody>
<tr>
<td>Compound</td>
<td>Concentration (mM)</td>
</tr>
<tr>
<td>NADPH</td>
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</tr>
<tr>
<td>NADP</td>
<td>50</td>
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<tr>
<td>NADH</td>
<td>50</td>
</tr>
<tr>
<td>FAD</td>
<td>50</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>15</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>15</td>
</tr>
<tr>
<td>2-Oxoglutarate + ascorbate</td>
<td>7.5</td>
</tr>
</tbody>
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Fig. 1. *High-pressure liquid chromatography of reaction products*

Supernatant (400 µl) prepared with 2-oxoglutarate and ascorbate was incubated for 3 h at 28°C with 300 µl of water containing 1.4 µmol of [3H]desacetoxycephalosporin C (30 µCi) and 1.4 µmol of 2-oxoglutarate. Acetic acid (1 M) was added to stop the reaction and a portion of the acidified supernatant was chromatographed at 7 MPa (1000 lb/in²) on a column (2.5 mm x 100 mm) of AS-SAX (Reeve Angel, Maidstone, Kent, U.K.). Fractions were eluted at 1 ml/min with 0.5 M-acetic acid. The *A*₅₆₀ of the eluate (-----) was measured continuously before the radioactivity (○) in 1 ml fractions was measured. 1, Solvent front; 2, ascorbate; 3, desacetoxycephalosporin C; 4, desacetylcephalosporin C.

and 0.5 % CaCO₃. After 48 h growth on an orbital shaker (220 rev./min, 5 cm throw) at 28°C, 1.25 ml was transferred to another 250 ml flask containing 25 ml of 3.6 % Bibby's soya meal, 3.8 % lactose, 1 % CaCO₃, 0.5 % (NH₄)₂SO₄, 0.06 % urea, 0.19 % L-methionine and 0.3 % maize oil. After a further 48 h on the shaker at 28°C, the mycelium was harvested (4000 g, for 2 min at 4°C), scraped off the chalk layer, washed in cold 50 mM-1,4-piperazinediethanesulphonic acid adjusted to pH 7 with KOH, and finally resuspended in the same buffer (5 ml). This suspension was added to 5 g of chilled 0.1 mm glass beads (Glen Creston, Stanmore, Middx., U.K.) in a 25 mm-diameter test tube. The mycelium was disrupted (Ranhand, 1974) at 4°C by agitating the tube vigorously for 2 min with a vortex Rotamixer (Hooke and Tucker, Croydon, Surrey, U.K.). The broken-cell suspension was decanted off the beads and centrifuged (30000 g for 5 min). The supernatant, which contains the dioxygenase, was stored at 4°C.

When the supernatant was incubated at 4°C with [3H]desacetoxycephalosporin C and was then acidified, 8 % of the substrate was converted into a product that behaved as the lactone of desacetylcephalosporin C on thin-layers of Ionex 25-SB-AC/UV254 (Camlab, Cambridge, U.K.) and on Dowex 1 (acetate form). The effect of adding a range of typical oxygenase substrates to the incubation mixture (Table 1) suggested that the reaction was dependent on 2-oxoglutarate and ascorbate. Nicotinamide nucleotides, which are substrates for mono-oxygenases (Hayashi, 1974), decreased the amount of product. This may reflect their role in the removal of any endogenous 2-oxoglutarate by enzymes such as 2-oxoglutarate dehydrogenase or glutamate dehydrogenase. When the
supernatant was desalted on Sephadex G-25 no activity was detected even in the presence of 2-oxoglutarate and ascorbate. Full activity was restored when 0.1 mM-Fe$^{2+}$ was added as well as 2-oxoglutarate and ascorbate.

The conversion into desacetylcephalosporin C was also measured without lactonization. A supernatant was prepared from mycelium broken in the presence of 2-oxoglutarate (5 mM) and ascorbate (5 mM) and incubated with $[^3]$Hdesacetoxycephalosporin C. The reaction was stopped by adding 1M-acetic acid which does not lactonize desacetylcephalosporin C. After centrifuging (600g for 2 min) the mixture, portions of the supernatant were chromatographed at high pressure on an anion-exchanger (Fig. 1). A new radioactive and u.v.-absorbing peak (4) was detected with the same retention time as a desacetylcephalosporin C standard. Conversions estimated from the $A_{260}$ and radioactive-peak areas were 25% and 24%, respectively. This demonstrates not only that the supernatant oxidized desacetoxycephalosporin C to desacetylcephalosporin C, but also that the specific radioactivity of the cephalosporin nucleus scarcely changed during the oxidation. This suggests either that there is a large isotope effect or that the $^3$H is not confined to the 3-methyl group of the desacetoxycephalosporin C.

Estimates of the oxidation were also made on this same reaction mixture after lactonization. The thin-layer and Dowex 1 assays showed conversions of 25% and 21% respectively. The destruction of a small amount of cephalosporin during lactonization could explain the lower result obtained by the Dowex 1 technique. In this assay the conversion is measured relative to the total radioactivity loaded on to the Dowex 1, whereas after t.1.c. or high-pressure liquid chromatography it is estimated after correction for the recovery of the desacetylcephalosporin C. Although assays using Dowex 1 are less accurate their simplicity makes them the technique of choice.

The enzyme which oxidizes the methyl group on the cephalosporin nucleus in our strain of *A. chrysogenum* has different properties from those deduced by Liersch *et al.* (1976). Instead, its properties and action more closely resemble those of the dioxygenase thymine-7-hydroxylase found in *Neurospora crassa* (Abbott & Udenfriend, 1974).

We thank Mr. D. I. Bilkus for his assistance with the high-pressure liquid chromatograms.


The Carbamylation of the 3-Hydroxymethyl Group of 7α-Methoxy-7β-(5-D-aminoadipamido)-3-hydroxymethylceph-3-em-4-carboxylic Acid (Desacetyl-7α-methoxycephalosporin C) by Homogenates of *Streptomyces clavuligerus*

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*Streptomyces clavuligerus* produces two O-carbamoylated cephalosporins, cephalexin C and cephalosporin C carbamate (Nagarajan *et al.*, 1971). *Streptomyces* synthesize a number of other antibiotics which contain an O-carbamate, for example, O-carbamoyl-D-serine (Tanaka & Sashikata, 1963), novobiocin (Kominek, 1972) and