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The Stereochemistry of β -Lactam Formation in Cephalosporin Biosynthesis

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^3H and ^{14}C from (2*R*,3*S*)-[U- ^{14}C ,3- $^3\text{H}_1$]cysteine and (2*R*,3*R*)-[U- ^{14}C ,2,3- $^3\text{H}_2$]cysteine were incorporated into cephalosporin C by *Cephalosporium acremonium*. Analysis of the radioactive cephalosporin C indicated that the formation of its β -lactam ring occurs stereospecifically and with retention of configuration at C-3 of cysteine.

The mechanism of the formation of the β -lactam ring of the penicillins and cephalosporins is still unknown, but information about the stereochemistry of the cyclization in the biosynthesis of benzylpenicillin by *Penicillium chrysogenum* has been obtained independently by Morecombe & Young (1975), by Aberhart *et al.* (1975) and by Young *et al.* (1977), who have shown that cysteine is incorporated into this antibiotic with retention of its 3-*pro-R*-hydrogen and loss of its 3-*pro-S*-hydrogen. The present paper describes a study of the incorporation of chirally labelled cysteines into cephalosporin C by *Cephalosporium acremonium*.

Experimental

Materials

Reagents (AnalaR grade where available) were from BDH Chemicals, Poole, Dorset, U.K. (2*R*,3*R*)-[U- ^{14}C ,2,3- $^3\text{H}_2$]Cysteine hydrochloride (^3H 9.1 μCi , 0.042 $\mu\text{Ci}/\mu\text{mol}$; ^{14}C 6.4 μCi , 0.030 $\mu\text{Ci}/\mu\text{mol}$) and (2*R*,3*S*)-[U- ^{14}C ,3- $^3\text{H}_1$]cysteine hydrochloride (^3H 7.2 μCi , 0.021 $\mu\text{Ci}/\mu\text{mol}$; ^{14}C 3.0 μCi , 0.011 $\mu\text{Ci}/\mu\text{mol}$) were synthesized by Young *et al.* (1977). The radioactive compounds were 2 years old at the time of use.

Methods

Paper electrophoresis and chromatography. Electrophoresis was carried out on Whatman no. 1 paper at pH 1.8 and 4.5 (both at 70 V/cm) as described by Smith *et al.* (1967). Paper chromatograms were run on Whatman no. 1 paper for 18 h in butan-1-ol/acetic acid/water (4:1:4, by vol.).

Measurements of radioactivity. Radioactivity determinations after paper electrophoresis and chromatography were made by combustion of the appropriate area of paper in a Packard 306 Tri-Carb Sample

Oxidizer for 0.2 min, the resulting $^3\text{H}_2\text{O}$ and adsorbed $^{14}\text{CO}_2$ being counted separately in a Nuclear-Chicago Unilux II A scintillation counter. The efficiency of counting was about 25% for ^3H and about 45% for ^{14}C . The efficiency was determined on each occasion by use of [^3H]- and [^{14}C]-hexadecane standards.

Radioactivity of cysteine hydrochlorides. A small sample of each radioactive cysteine hydrochloride was oxidized (Smith *et al.*, 1967) to cysteic acid. After electrophoresis at pH 1.8 for 1 h the $^{14}\text{C}/^3\text{H}$ ratio in the cysteic acid position was determined. The radioactive cysteic acid was located by use of a marker of unlabelled cysteic acid which was revealed by ninhydrin.

Incorporation of labelled cysteine into cephalosporin C. Washed mycelium (60 g) from a 72 h culture of *Cephalosporium acremonium* C91 was resuspended in distilled water (360 ml) and the suspension was distributed among four conical flasks with glass baffles (Smith *et al.*, 1967). The flasks were placed on a rotary shaker (200 rev./min.) at 27°C for 7 h. Equal portions of a solution of labelled cysteine hydrochloride (neutralized with 1 M-NaOH) were added after 15 min incubation and then hourly, such that the final concentration due to each addition was approx. 0.1 $\mu\text{mol}/\text{ml}$. To reduce any cystine present an approximately equivalent amount of dithiothreitol was added to the neutralized solution immediately before addition to the resuspension. After 7 h the mycelium was removed by filtration. The suspension fluid, containing about 14 mg of cephalosporin C and 17 mg of penicillin N, according to microbiological assay, was freeze-dried and the residue extracted with 70% (v/v) ethanol (Smith *et al.*, 1967). After removal of most of the ethanol *in vacuo* the solution was diluted with water and again freeze-dried.

Purification of cephalosporin C by high-pressure liquid chromatography. The water-soluble fraction of the freeze-dried extract was injected in two portions (each 1 ml) into a Waters Associates ALC-202 liquid

chromatogram equipped with a model 6000 solvent-delivery system. The column (61 cm $\times$ 7 mm diam.) was packed with Bondapak C₁₈/Porasil B reverse-phase packing material (Waters Associates, Northwich, Cheshire, U.K.). The mobile phase used was 2% (v/v) methanol in 1% (w/v) NH₄HCO₃, pH 8.4, and the flow rate was 6 ml/min. Penicillin N emerged from the column (in 18 ml) after 54 ml of eluate had been collected. Cephalosporin C, which was detected by monitoring the A_{254} of the eluate, was collected manually in 78 ml of eluate after 114 ml had emerged from the column. During the collection of eluate, CO₂ was bubbled through it to lower the pH to 7.0. The pH of the eluate containing cephalosporin C was adjusted to 4.3 by the addition of Dowex 50 (X8; H⁺ form). The resin was removed by filtration and the filtrate was freeze-dried. The cephalosporin C content of the residue, determined by measurement of A_{260} (Abraham & Newton, 1961) and by microbiological assay (Smith *et al.*, 1967), was about 10 mg.

Samples of this material (125 μ g) were analysed by electrophoresis at pH 4.5 for 35 min, followed by chromatography in the second dimension. Cephalosporin C was detected as a dark spot when viewed in u.v. light (Abraham & Newton, 1961) and the radioactivity in this spot was determined.

Degradation of labelled cephalosporin C. The labelled material was degraded to δ -(D- α -aminoadipyl)glycine by the procedure of Trown *et al.* (1963), except that preparative electrophoresis at pH 4.5 for 45 min was used to separate the acidic and neutral components of the material obtained after mild hydrolysis and oxidation. The acidic material was hydrolysed in 6M-HCl at 108°C for 18 h to yield α -aminoadipic acid and glycine. Unlabelled glycine (5 μ g) was added to the hydrolysate and the mixture was subjected to electrophoresis on paper at pH 1.8 for 35 min. The α -aminoadipic acid and glycine were made visible with ninhydrin, and appropriate areas of the paper were cut out for determinations of radioactivity.

Results and Discussion

The two stereospecifically labelled cysteines were fed separately to suspensions of washed mycelium

of *C. acremonium* and the quotient $^{14}\text{C}/^3\text{H}$ was determined in the cephalosporin C produced. The results, which are summarized in Table 1, indicate poor retention of ^3H from (2*R*,3*S*)-[3- $^3\text{H}_1$]cysteine (I) and substantial retention of ^3H from (2*R*,3*R*)-[2,3- $^3\text{H}_2$]cysteine (II). The labelled cephalosporin C (III) obtained from the experiment in which the latter cysteine was used was degraded by the method of Trown *et al.* (1963) to give C-6 and C-7 of cephalosporin C as C-1 and C-2 respectively of glycine. Hydrolysis of cephalosporin C with hot 0.1M-HCl, followed by oxidation with silver oxide, gave δ -(D- α -aminoadipyl)glycine. The hydrogen atom attached to C-6 of cephalosporin C was lost during the degradation. The zwitterionic nature of the α -aminoadipyl residue precluded the precipitation of a crystalline derivative of *N*- δ -(α -aminoadipyl)aminoacetaldehyde, which is formed, together with CO₂ and other products, on mild hydrolysis of cephalosporin C. Although the yield of δ -(α -aminoadipyl)glycine from the cephalosporin C was low, the glycine obtained from it on hydrolysis had sufficient radioactivity to show that the value for $^{14}\text{C}/^3\text{H}$ was about 2.22. No radioactivity was found in the α -aminoadipic acid. The value of $^{14}\text{C}/^3\text{H}$ for the glycine implies that about 18% of the total ^3H in the cysteine precursor is retained at C-7 in cephalosporin C. Since the distribution of ^3H between C-2 and C-3 in the cysteine is uniform (Young *et al.*, 1977), and since 50% of this ^3H was retained in cephalosporin C (Table 1), it follows that there is approx. 65% retention of ^3H at C-6 and 35% retention of ^3H at C-7 of cephalosporin C from C-3 and C-2 respectively of (2*R*,3*R*)-[2,3- $^3\text{H}_2$]cysteine.

The low retention of ^3H at C-7 of cephalosporin C could be due to the involvement of cysteine in transamination. From a comparison of the results obtained with the different chirally labelled cysteines it appears that cysteine is incorporated into cephalosporin C with retention of the 3-*pro-R*-hydrogen and loss of the 3-*pro-S*-hydrogen and therefore with retention of configuration at C-3. This finding parallels that of Young *et al.* (1977) for the incorporation of cysteine into benzylpenicillin by *P. chrysogenum* and indicates that the β -lactam ring formation occurs with the same stereochemistry in penicillin and cephalosporin biosynthesis.

Table 1. Incorporation of ^3H from chirally labelled cysteines into cephalosporin C

Cephalosporin C was produced by suspensions of *C. acremonium* in the presence of the labelled precursors as described in the text. Cysteine was determined as cysteic acid.

Precursor	$^{14}\text{C}/^3\text{H}$		Retention of ^3H in cephalosporin C (%)
	Cysteine	Cephalosporin C	
(2 <i>R</i> ,3 <i>R</i>)-[U- ^{14}C ,2,3- $^3\text{H}_2$]Cysteine	0.59	1.18	50
(2 <i>R</i> ,3 <i>S</i>)-[U- ^{14}C ,3- $^3\text{H}_1$]Cysteine	0.48	2.63	18

As with its incorporation into benzylpenicillin (Young *et al.*, 1977; Aberhart *et al.*, 1975), the stereospecificity of the incorporation of cysteine into cephalosporin C (determined from $^{14}\text{C}/^3\text{H}$ values) did not appear to be complete. The retention of 18% of the ^3H from (2*R*,3*S*)-[U- ^{14}C ,3- $^3\text{H}_1$]cysteine in cephalosporin C compares with corresponding values of 14% (Young *et al.*, 1977) and 21% (Aberhart *et al.*, 1975) for benzylpenicillin. The incorporation of some ^3H in these cases might be due to incomplete stereospecificity in the chemical syntheses, or to a biochemical loss of stereochemical homogeneity of the cysteine in the mycelial cells. The retention in cephalosporin C of 65% of the ^3H

at C-3 of the (2*R*,3*R*)-cysteine compares with corresponding values for benzylpenicillin of 76% (Aberhart *et al.*, 1975) and 86% (Young *et al.*, 1977). A secondary isotope effect might contribute to this incomplete retention. It is noteworthy that secondary isotope effects after isotopic substitution of the hydrogen of a carbon-hydrogen bond that is not broken in a reaction have been correlated with the formation of carbonium ion intermediates (Richards, 1970). One hypothetical mechanism for the closure of the β -lactam ring could involve the production of a carbonium ion at C-3 of the cysteine precursor.

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