Biosynthesis of a 7- α -Methoxycephalosporin

INCORPORATION OF MOLECULAR OXYGEN

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(Received 19 September 1978)

A 7- α -methoxycephalosporin containing a carbamoyloxymethyl substituent at C-3 (cephamycin C) has been isolated from the extracellular fluid of an aqueous suspension of *Streptomyces clavuligerus* shaken in the presence of $^{18}\text{O}_2$. The cephalosporin has been converted into its *N*-acetyl dimethyl ester and the distribution of ^{18}O in the latter determined by chemical-ionization mass spectrometry. The results indicate that the oxygen atom of the methoxy group, as well as that linked to the exocyclic methylene group at C-3, is derived from molecular O_2 .

The oxygen atom attached to the exocyclic methylene at C-3 of cephalosporin C (I, R = OCOCH_3 , X = H) produced by *Cephalosporium acremonium* is derived from molecular O_2 (Stevens *et al.*, 1975). In biosynthesis, this O_2 is introduced during the conversion of deacetoxycephalosporin C (I, R = H, X = H) into deacetylcephalosporin C (I, R = OH, X = H), the reaction being catalysed by a dioxygenase (Brewer *et al.*, 1977).

A cephalosporin (cephamycin C) produced by *Streptomyces clavuligerus* contains a carbamoyl group in place of the acetoxy group of cephalosporin C and also a 7- α -methoxy group (I, R = OCONH_2 , X = OCH_3) (Nagarajan *et al.*, 1971).

(I)

The experiments described in this paper were designed to determine whether the oxygen atom of the methoxy group of cephamycin C, as well as that linked to the exocyclic methylene group at C-3, is derived from molecular O_2 . A brief preliminary account of the work has been reported previously (Abraham *et al.*, 1978).

Materials and Methods

Materials

Oxoid yeast extract powder (code L21) was from Oxoid, London E.C.4, U.K. Hi-Soy (a soya flour)

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was from the British Arkady Co., Old Trafford, Manchester M16 0NJ, U.K. Casamino acids and Bacto-Tryptone were from Difco Laboratories, Detroit, MI, U.S.A. An authentic sample of 7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethylceph-3-em-4-carboxylic acid and its 7-methoxy analogue (cephamycin C) were kindly provided by Eli Lilly and Co., Indianapolis, IN, U.S.A. $^{18}\text{O}_2$ (99% atoms ^{18}O excess) was from British Oxygen Co., London SW19 3UF, U.K. DL-[1- ^{14}C]Valine was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Silica gel HF₂₅₄ was obtained from E. Merck, Darmstadt, Germany, and supplied by BDH Chemicals, Poole, Dorset, U.K. Other reagents (AnalaR grade where available) were from BDH.

Culture and growth conditions

S. clavuligerus (A.T.C.C. 27064) was maintained on Bennett's modified agar (Whitney *et al.*, 1972) and sporulated agar slants were used to inoculate liquid media.

To a baffled Erlenmeyer flask (500ml) was added 50ml of a medium with the following composition: glycerol, 10g; sucrose, 20g; Bacto-Tryptone, 5g; yeast extract, 1g; Hi-Soy flour, 15g; K_2HPO_4 , 0.2g; and water to 1 litre. The medium was inoculated with a loopful of spores from a sporulated agar slant to give a concentration of 10^3 – 10^4 viable units/ml, and the flask shaken on a rotary shaker at 200rev./min at 27°C for 48 h.

A sample (5ml) of the resulting culture was added to 50ml of a chemically defined medium in a 500ml baffled Erlenmeyer flask. The defined medium was composed of: glycerol, 15g; sucrose, 20g; proline, 0.5g; sodium glutamate, 1.5g; NaCl, 5g; KH_2PO_4 ,

2g; CaCl_2 , 0.4g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.1g; ZnCl_2 , 0.05g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1g; in 1 litre of water. The flask was shaken at 200 rev./min at 27°C for 24h, and the mycelium then harvested by centrifugation at 6000g for 5 min and washed by resuspension in water (25 ml) and centrifugation as before.

For production of the 7- α -methoxycephalosporin (cephamycin C) in the presence of $^{18}\text{O}_2$ the washed mycelium was suspended in water (1 g wet wt./10 ml).

Bioassays

Antibiotics were assayed by the hole-plate method (Brownlee *et al.*, 1948) against *Salmonella gallinarum* (strain X142 from Eli Lilly and Co.) and a strain of *Escherichia coli* (kindly provided by Professor A. L. Demain, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.) that is supersensitive to β -lactam antibiotics (Aoki *et al.*, 1976). Cephalosporin C and cephamycin C could be assayed in concentrations of 20 and 15 $\mu\text{g}/\text{ml}$ respectively. With the supersensitive strain of *E. coli* the corresponding concentrations were 0.5 and 1 $\mu\text{g}/\text{ml}$ respectively.

Assays of culture filtrates and the extracellular fluid of suspensions of washed mycelium were also carried out under conditions in which any penicillin N present would have been selectively destroyed. β -Lactamase I from *Bacillus cereus* 569/H9 was added to the sample tested, or to the agar medium of the test plate, in sufficient quantity to destroy 20 μg of penicillin N/ml under the conditions used (Smith *et al.*, 1967).

Electrophoresis and chromatography

Electrophoresis on Whatman no. 1 paper was carried out at 70 V/cm at pH 6.5 and 1.8 as described by Fawcett *et al.* (1976). T.l.c. was carried out on 0.1 mm-thick silica-gel HF₂₅₄ plates. Descending paper chromatography was carried out on Whatman no. 1 paper in a solvent system consisting of (A) propan-1-ol/acetic acid/acetonitrile/pyridine/water (40:9:40:30:36, by vol.) (Whitney *et al.*, 1972), or (B) butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase, or 4:1:4, by vol., upper phase). Spots were located by Cd/ninhydrin (Heilman *et al.*, 1957), or by starch/ I_2 (Thomas, 1961), or by examination under u.v. (240–250 nm) light.

Bioautography

This was carried out after electrophoresis of samples on paper at pH 6.5 followed by chromatography in system (A) or (B) at 4°C . The supersensitive strain of *E. coli* was used as the test organism.

Production of cephamycin C in the presence of $^{18}\text{O}_2$

A suspension (50 ml) of mycelium of *S. clavuligerus* was placed in a 100 ml flask (A), the base of which was 10 cm in diameter, with a centre cup containing 0.5 ml of 2M-KOH and a stopper connected to a three-way tap (B). The tap carried connections to a water pump (C) and a water-containing reservoir (D) (Fig. 1). Air was removed from the flask by the pump and the flask then connected to a 250 ml vial of $^{18}\text{O}_2$ (E) that was broken at one end by scoring the glass tip and breaking it after the tubing from the reservoir (D) had been connected; the other tip was broken by a magnetically operated plunger (F). The $^{18}\text{O}_2$ which entered the system was kept under slight positive pressure, as measured by the water manometer (G), by adjusting the level of the reservoir (D). The flask was placed on a rotary shaker (200 rev./min)

Fig. 1. Apparatus for the production of cephamycin C in the presence of $^{18}\text{O}_2$

The diagram is not to scale. Air is evacuated from the flask (A) through a three-way tap (B) by the use of a water pump (C). The lower tip of vial (E) is heavily scored before attaching it to the water reservoir (D) and then broken inside the connecting tubing. $^{18}\text{O}_2$ in the vial is released by breaking the fragile tip with a magnet (F). The $^{18}\text{O}_2$ entering flask (A) is kept under slight positive pressure by adjusting the level of water in (D); the pressure is measured with a water manometer (G).

at 27°C. After 6 h the O₂ supply was turned off at (B) and shaking continued for a further 1 h. In all, about 50 ml of O₂ was used by the mycelium.

The mycelium was separated from the extracellular fluid by centrifugation (6000g for 5 min) and washed once by centrifugation after resuspension in 25 ml of water. Samples of the pooled supernatants were used for bioassay and measurements of u.v.-absorption spectra, and the remaining solution was freeze-dried.

Isolation of cephamycin C

The freeze-dried material obtained from the extracellular fluid described above was stirred with 70% (v/v) ethanol at 4°C. Insoluble material was separated by centrifugation, and the supernatant freeze-dried after concentration *in vacuo* in a rotary evaporator to remove most of the ethanol. The resulting material was dissolved in 0.05M-acetic acid (1 ml) and the solution applied to a column (2.4 cm $\times$ 1 cm diam.) of Dowex-50 (X4; H⁺ form; 200–400 mesh). Elution was carried out with water at a flow rate of 7 ml/min and 2.5 ml fractions were collected. Fractions 4–10 showed antibacterial activity and a u.v.-absorption spectrum similar to that of authentic cephamycin C (Nagarajan *et al.*, 1971). These fractions were pooled and freeze-dried to yield 6 mg of product.

N-Acetylcephamycin C dimethyl ester

The freeze-dried preparation of cephamycin C was dissolved in 3% NaHCO₃ (2 ml), the solution was cooled in ice and acetone (2 ml) was added. To the cooled and stirred solution acetic anhydride (100 μ l) was added in 5 μ l portions, the pH reading being maintained between 8 and 9 by dropwise addition of 0.5M-NaOH. After the reaction mixture had been kept for 1 h at 0°C, acetone was removed in a rotary evaporator and the resulting solution was freeze-dried after adjustment of its pH to 6.5. The product was dissolved in water (2 ml) and the solution added to a column (8 cm $\times$ 1 cm diam.) of Dowex 50 (X4; H⁺ form). Elution was carried out with water at a flow rate of 7 ml/min and 2.5 ml fractions were collected. Measurements of u.v.-absorption spectra showed that all the *N*-acetylcephamycin C was in fractions 1–5. These fractions were pooled and freeze-dried.

To the preparation of *N*-acetylcephamycin C in methanol (1 ml) at 0°C was added dropwise an ethereal solution of diazomethane, prepared from *N*-methyl-*N*-nitrosoguanidine in the apparatus described by Fales *et al.* (1973), until the yellow colour was no longer discharged. The cooled mixture was kept for 15 min; 1 drop of 0.1M-acetic acid was then added and the solvent was removed *in vacuo*. The product, which was ninhydrin-negative, but reacted with starch/I₂, was shown by paper electrophoresis to have no net charge at pH 6.5. Its u.v.-absorption

spectrum showed maxima at 242 and 264 nm. When tested by t.l.c. on silica gel HF₂₅₄ in methanol/chloroform (1:9, v/v) it gave a spot with blue fluorescence and *R_F* 0.64, corresponding to that given by the *N*-acetyl dimethyl ester of authentic cephamycin C.

Mass spectrometry

Isobutane and isobutane/ethylenediamine/ethanol-amine chemical-ionization spectra were obtained by using a VG micromass 16F mass spectrometer (VG Organic, Altrincham, Cheshire, U.K.) with an indicated source pressure of 8 mPa (*m/e* 57:43, 7:1). The temperature of the source was 200°C and that of the sample was 60°C; ethylenediamine and ethanol-amine (50 μ l) were introduced via the septum inlet (180°C); spectra were recorded on u.v. paper and with a Digico-Carrick data system (Digico, Stevenage, Herts., U.K.).

Results

Antibiotic production by S. clavuligerus

S. clavuligerus (A.T.C.C. 27064) has been reported to produce penicillin N (Higgins & Kastner, 1971), deacetoxycephalosporin C (Higgins *et al.*, 1974) and the 3-carbamoyloxymethyl analogue of cephalosporin C as well as cephamycin C (Nagarajan *et al.*, 1971). In addition, it produces the β -lactam antibiotic, clavulanic acid (Brown *et al.*, 1976), and also holomycin (Kirby, 1978).

In the fermentation described in the present paper penicillin N was not produced in sufficient quantity (5–15 μ g/ml) to be detected with confidence by bioassay of the culture filtrate before and after treatment with β -lactamase I. However, small amounts of penicillin N were apparently produced, since oxidation with performic acid (as described by Fawcett *et al.*, 1976) of the radioactive material obtained after DL-[1-¹⁴C]valine had been added to the suspension of washed mycelium yielded [¹⁴C]penicillaminic acid. The only antibiotic detected by bioautography after paper electrophoresis and chromatography of a sample of the culture filtrate was cephamycin C. Bioassay of the culture filtrate indicated that this substance (identified by comparison with an authentic sample) was present in a concentration of about 150 μ g/ml. The failure to detect other β -lactam antibiotics in the procedures used indicated that they were not present in concentrations greater than about 15 μ g/ml.

Production of cephamycin C by suspensions of mycelium in water

Suspensions in water of the mycelium of *S. clavuligerus* harvested after 24 h (1 g wet wt./10 ml) produced cephamycin C at a linear rate for 7 h (9 μ g/ml

per h) at 27°C on a rotary shaker at 200 rev./min. When the time of harvesting was increased the production of antibiotic under these conditions declined, and was very low with mycelium harvested after 48 h.

No significant difference was detected in cephamycin C production when the mycelial suspension was shaken in O₂ instead of in air. A typical experiment in which ¹⁸O₂ was used in the apparatus shown in Fig. 1 yielded about 3 mg of cephamycin C. The extracellular fluids from four such experiments were combined and freeze-dried and extracted with 70% (v/v) ethanol. Evaporation of the extract yielded a solid (320 mg) containing 12 mg of cephamycin C by the bioassay method.

Isolation of cephamycin C and its N-acetyl dimethyl ester

Purification by chromatography on Dowex 50 (X4; H⁺ form) of the material produced in the presence of ¹⁸O gave a product with a u.v. spectrum similar to that of authentic cephamycin C (Nagarajan *et al.*, 1971). After paper electrophoresis at pH 6.5 a major ninhydrin-positive spot was revealed in a position corresponding to cephamycin C. Two minor spots, one twice as far as cephamycin C towards the anode and the other close to the origin, were also visible. Bioassay and A₂₆₄ values for the purity of this preparation of cephamycin C were about 90 and 72% respectively (Table 1). In the corresponding product obtained from experiments in which the mycelium was shaken in conical flasks in air, only one active compound corresponding to cephamycin C was revealed by bioautography after electrophoresis at pH 6.5 followed by chromatography in systems A and B respectively.

Table 1. *Preparation of the dimethyl ester of N-acetylcephamycin C produced by S. clavuligerus in the presence of ¹⁸O*

In the bioassays *S. gallinarum* was used as the test organism. Estimates from u.v. absorption were based on the value of 149 for A_{1cm}^{1%} of cephamycin C at 264 nm (Nagarajan *et al.*, 1971). — indicates no determination.

	Weight (mg)	Content of cephamycin C (mg) estimated by:	
		Bioassay	A _{1cm} ^{1%}
Extraction with 70% (v/v) ethanol	320	12	—
Dowex-50 (X4; H ⁺ form)	6	5.5	4.3
N-Acetylation and Dowex-50 (X4; H ⁺ form)	3	—	2.2
Dimethylation	3	—	2.2

N-Acetylation of the cephamycin C was accompanied by about a 5-fold decrease in activity against the supersensitive strain of *E. coli*. The N-acetyl derivative (Table 1) migrated about twice as far as cephamycin C itself towards the anode on electrophoresis and gave a spot that was ninhydrin-negative but starch/I₂-positive. Its u.v.-absorption spectrum was similar to that of cephamycin C.

Incorporation of ¹⁸O₂ into cephamycin C

The isobutane chemical-ionization mass spectrum of N-acetylcephamycin C dimethyl ester corresponded to the fragmentation shown in Fig. 2. It showed a quasimolecular ion (M+1)⁺ (Field, 1969), and fragments *a*, (b+H)⁺ and *c* were present in significant abundance at 60°C (Fig. 3), but at temperatures above 100°C the ion *d* from the α-amino-adipamido side chain attached to the β-lactam ring increased until it dominated the spectrum, whereas the abundance of none of the other ions exceeded 5%. The isobutane/ethylenediamine chemical-ionization spectra showed a 3-fold increase in the abundance of the quasimolecular ion.

The isobutane chemical-ionization mass spectrum of the dimethyl ester of the N-acetyl derivative of cephamycin C that had been produced by *S. clavuligerus* in the presence of ¹⁸O₂ revealed the presence of ions with masses 2 units higher than those of fragments *a*, *b* and *c* (Fig. 2). The shift in fragments *a* and *b* indicated that ¹⁸O was present in the 7-α-methoxy group of this preparation of cephamycin C and the shift in fragment *c* indicated that it was also present in the oxygen atom attached to the exocyclic methylene group at C-3. The ¹⁶O/¹⁸O ratios in fragments *a*, *b* and *c* are given in Table 2.

Discussion

Under the conditions used in the present paper *S. clavuligerus* produced much more cephamycin C than other cephalosporins and penicillin N, and this facilitated its isolation in the small-scale experiments used to study the incorporation of ¹⁸O₂. Cephamycin C could be eluted from Dowex 50 (X4; H⁺ form) with water, presumably because of the strong acidity of its carboxy groups, and this relatively simple procedure yielded a product that was up to 90% pure.

Preliminary experiments showed that N-acetylcephamycin C dimethyl ester failed to give a molecular ion under electron-impact conditions in the mass spectrometer and that the abundance of key fragments was very low, in agreement with earlier findings with cephalosporins having α-amino-adipyl side chains (Nagarajan *et al.*, 1971). In contrast, the isobutane chemical-ionization spectra of the cephamycin C derivative, like that of other cephalosporins (Abraham *et al.*, 1978), exhibited a quasimolecular

Fig. 2. Fragmentation of the dimethyl ester of *N*-acetylcephamycin C by chemical-ionization mass spectrometry. In all structures $R' = [CH_2]_3CH(NHCOCH_3)CO_2CH_3$. Chemical-ionization mass spectrometry was performed with isobutane, and isobutane/ethylenediamine/ethanolamine as described in the Materials and Methods section.

Fig. 3. Isobutane chemical-ionization mass spectrum of *N*-acetylcephamycin C dimethyl ester. Chemical-ionization mass spectrometry was carried out as described in the Materials and Methods section. The relative abundance of the fragments is based on a value of 100% for m/e 456 (fragment a, Fig. 2).

Table 2. Relative amounts of ^{16}O and ^{18}O found by mass spectrometry in fragments of *N*-acetylcephamycin C dimethyl ester produced in the presence of $^{18}O_2$

Fragments a, b and c correspond to those in Fig. 2. Corrections have been made for the observed natural abundances of ^{34}S , ^{13}C and 2H in the ^{16}O sample (see Fig. 3). Each quotient is the average of three measurements.

Fragment	m/e	$^{16}O/^{18}O$ ratio
a	456, 458	3
b	287, 289	3
c	231, 233	3.5

ion $(M+1)^+$ and abundant fragments arising from fission of the β -lactam ring. This behaviour is similar

to that reported by Mitscher *et al.* (1975) for the isobutane and ammonia chemical-ionization spectra of a series of penicillin derivatives. The introduction of ethylenediamine or ethanolamine transformed the isobutane chemical-ionization spectra into the equivalent of ammonia chemical-ionization spectra, in accordance with the prediction of Bowen & Field (1974). As well as giving the expected increase in the intensity of the quasimolecular ion, this procedure also identified those fragments that contained a proton derived from the reagent gas. The mass-spectrometric analysis of *N*-acetylcephamycin C dimethyl ester that had been prepared from cephamycin C produced by *S. clavuligerus* in the presence of $^{18}O_2$ has shown that the oxygen atom of the 7- α -

methoxy group, like that of the oxygen atom attached to the exocyclic methylene group at C-3, is derived from molecular O_2 . The 3-fold dilution of ^{18}O with ^{16}O in the isolated cephamycin C may presumably be attributed to the presence of intracellular cephamycin C in the mycelium used and, in small part, to incomplete removal of $^{16}O_2$ from the system at the beginning of the experiment.

The methyl moiety of the methoxy group is known to be derived from the methyl of methionine (Whitney *et al.*, 1972). It is thus clear that two enzyme reactions, an oxygenation and a methyl transfer, are involved. A study of these reactions and the substrates involved in cell-free systems is now in progress. Attempts to find evidence for the biosynthesis of a methoxy derivative of the tripeptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine that is believed to be a precursor of penicillin N (Fawcett *et al.*, 1976) have not been successful (J. O'Sullivan & E. P. Abraham, unpublished work). It is not unlikely that the methoxy group is introduced by hydroxylation and subsequent methylation after the formation of the cephalosporin ring system.

We are grateful to the Medical Research Council and the National Research Development Corporation for financial support.

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