CEPHALOSPORIN FORMATION BY CELL-FREE EXTRACTS FROM *STREPTOMYCES CLAVULIGERUS*

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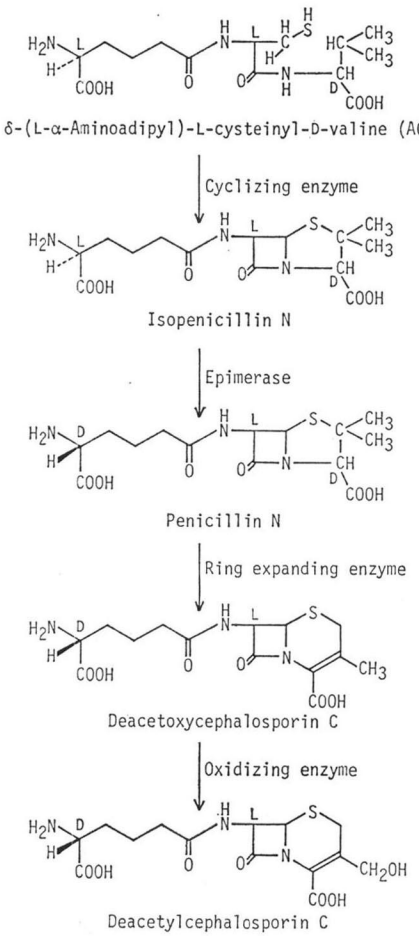
Cell-free extracts of *Streptomyces clavuligerus* convert δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV) into an antibiotic product which is 30~50% penicillinase-insensitive. Thin-layer chromatography resolves this antibiotic product into one major penicillinase-sensitive component and one major and one minor penicillinase-resistant component. The major and minor penicillinase-resistant antibiotics co-chromatograph with deacetoxycephalosporin C and deacetylcephalosporin C, respectively. Ring expansion of a penicillin intermediate, as evidenced by the production of penicillinase-resistant antibiotic, shows an absolute requirement for α-ketoglutarate, while ATP, K^+ and Mg^{2+} have lesser effects. Ring expansion activity is not sedimented by high speed centrifugation and is unaffected by membrane-disrupting treatments. Penicillin N and ACV (presumably *via* penicillin N) are the only substrates so far accepted by the ring expanding enzyme. New syntheses of penicillin N and isopenicillin N are described.

Organisms which synthesize cephalosporin antibiotics appear to do so by oxidative ring expansion of penicillin N, itself formed by oxidative cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), followed by L to D epimerization in the aminoadipyl moiety (Scheme 1)[8,14]. A number of workers have demonstrated ring expansion activity in cell-free systems of *Cephalosporium acremonium*[7,11,17,20]. However, until recently the extent of ring expansion observed was low and difficult to reproduce[4]. In addition, there is some uncertainty concerning the necessary cofactors. Thus, the stimulating effects of ascorbic acid, O_2, ferrous ions and ATP are clear, but the role of α-ketoglutarate is not. Most workers observe α-ketoglutarate to have no effect or to afford only slight stimulation[7,17]. On the other hand, FELIX *et al.* have recently shown a 3-fold increase in ring expansion activity in the presence of α-ketoglutarate[4]. Clarification of this point is desirable, because a requirement for α-ketoglutarate, together with the other known cofactor requirements, would suggest that the ring expansion enzyme is an intermolecular dioxygenase, and allow its behaviour to be compared to that of other intermolecular dioxygenases such as proline hydroxylase[13] and thymine hydroxylase[1].

Streptomyces clavuligerus is a prokaryotic organism which produces a variety of β-lactam compounds including penicillin N, cephamycin C and clavulanic acid[16]. Since cephamycin antibiotics are variations of the basic cephalosporin structure, it has been assumed, although not yet demonstrated, that they arise from a pathway which involves the same type of ring expansion enzyme as found in *C. acremonium*. In an earlier report concerning the cyclization of ACV by cell-free extracts of *S. clavuligerus*[10], the formation of a penicillin product was demonstrated. The antimicrobial spectrum of this penicillin product indicated it to be a mixture of isopenicillin N and penicillin N. Since penicillin N is the normal substrate for the ring expansion enzyme, this result suggested that a multistep cell-free conversion of ACV

Scheme 1.



into deacetoxycephalosporin C should be possible, provided that ring expansion activity is also present in these cell-free extracts. The present study is concerned with this multistep process and the cofactor requirements for ring expansion activity as it occurs in cell-free extracts from *S. clavuligerus*.

Materials and Methods

Materials

Penicillinase (Bacto-penase concentrate, 10^7 units/ml) was from Difco Laboratories, Detroit. Monosodium α-ketoglutarate, disodium ATP, and 6-aminopenicillanic acid were from Sigma Chemical Co., St. Louis. Penicillin G, penicillin V, deacetoxycephalosporin C, and deacetylcephalosporin C were generously provided by D. HOOK, Bristol Laboratories, Syracuse. Penicillin K and dihydropenicillin F were prepared by condensation of 6-aminopenicillanic acid with octanoic acid and hexanoic acid, respectively, according to the ethyl chloroformate procedure (method B) of PERRON *et al.*[15]. The compounds were isolated as the crystalline potassium salts by treatment of the free acids with potassium 2-ethylhexanoate. Bis-ACV was chemically synthesized as previously described[19].

Synthesis of Penicillin N

Benzyl *N*-Succinimidylcarbonate: A solution of *N*-hydroxysuccinimide (1.15 g, 10.0 mmole) and triethylamine (1.39 ml, 1.01 g, 10.0 mmole) in methylene chloride (20 ml) was stirred at 0°C and treated dropwise with a solution of benzyl chloroformate (1.43 ml, 1.71 g, 10.0 mmole) in methylene chloride (20 ml). The addition required 10 minutes and a white solid precipitated towards the end. The cooling bath was then removed and the reaction mixture allowed to warm to room temperature over 30 minutes to form a clear solution. This solution was washed successively with water (3×30 ml) and saturated brine (20 ml), and dried over sodium sulfate. Evaporation of the solvent afforded a colorless syrup which solidified, after brief drying under vacuum, to a white solid, 2.43 g (97.6%). Recrystallization from ethyl acetate-hexane gave colorless needles, mp 76~79°C, IR ($CHCl_3$): 1814, 1790, 1744 (s, carbonate and succinimidyl C=O) cm^{-1}, ^1H NMR ($CDCl_3$) δ: 2.75 (4H, s, CH_2–CH_2), 5.38 (2H s, Ph-CH_2), 7.53 (5H, s, Ph). *Anal.* Calcd. for $C_{12}H_{11}NO_5$: C 57.83, H 4.45, N 5.62. Found: C 58.05, H 4.66, N 5.68.

D-α-Aminoadipic Acid: Crude *N*-chloroacetyl D-α-aminoadipic acid, obtained as a byproduct from the resolution of α-aminoadipic acid[19] was recrystallized twice from ethyl acetate - hexane to give colorless rosettes, mp 105~107°C (Reference 5. mp 99~100°C), $[\alpha]_D^{25}$ −2.94°, $[\alpha]_{365}^{25}$ −16.2° (*c* 1.0, CH_3-OH).

The product (2.5 g) was dissolved in a mixture of concentrated hydrochloric acid (20 ml), formic acid (20 ml) and water (20 ml) and refluxed gently for 7 hours. After cooling to room temperature the solution was concentrated, under reduced pressure, to a small volume. The resultant syrup was transferred to a 300-ml beaker using water (to give a total volume of 20 ml), and the pH was adjusted to 2.9 with

3.0 N sodium hydroxide (24 ml) to precipitate a white solid. The mixture was diluted with ethanol (60 ml) and cooled at 4°C. The product was collected by filtration, washed with ethanol and dried to give 1.64 g, mp 194~196°C, $[\alpha]_D^{22}$ −24.3° (*c* 2.0, 6 N HCl), (Reference 5, $[\alpha]_D^{23}$ −25.0° (*c* 2.0, 6 N HCl)).

N-Carbobenzoxy-D-α-Aminoadipic Acid α-Benzhydryl Ester Dicyclohexylamine Salt: A suspension of D-α-aminoadipic acid α-benzhydryl ester[1] (654.0 mg, 2.0 mmole) in methanol (21.0 ml) and dimethoxyethane (9.0 ml) was treated with dicyclohexylamine (0.42 ml, 2.11 mmole) to form a cloudy solution. Benzyl *N*-succinimidylcarbonate (498.0 mg, 2.0 mmole) was next added followed, after 30 minutes, by a further portion of dicyclohexylamine (0.42 ml, 2.11 mmole). After 18 hours stirring at room temperature, the solvent was evaporated and the residual syrup was partitioned between water (25 ml) and methylene chloride (30 ml). The organic layer was washed successively with 10% potassium bisulfate (3×10 ml), water (20 ml), saturated brine (10 ml), dried over sodium sulfate and evaporated to leave a golden syrup (755 mg). The crude product was dissolved in dry ether (15 ml) and treated with dicyclohexylamine (0.33 ml, 1.0 equiv.). Hexane was next added and the product precipitated as an oil, but crystallized over several days. The product was collected by filtration, washed with hexane - ether and dried under high vacuum to give 632 mg (49%) of the dicyclohexylamine salt, mp 94~108°C raised to 108~110°C after recrystallization from ether - hexane containing a trace of ethyl acetate. $[\alpha]_D^{21}$ +15.0°, $[\alpha]_{365}^{21}$ +47.7° (*c* 1.0, CH_3OH); IR (Nujol): 3335 (s, amide NH), 2800~1900 (br, NH_2^+), 1715, 1710 (s, urethane, ester C=O), 1635, 1540 (s, carboxylate C=O) cm^{-1}. *Anal.* Calcd. for $C_{39}H_{50}N_2O_6$: C 72.87, H 7.84, N 4.36. Found: C 72.30, H 7.98, N 4.54.

N-Carbobenzoxy-D-α-aminoadipic Acid α-Benzhydryl Ester: A suspension of *N*-carbobenzoxy-D-α-aminoadipic acid α-benzhydryl ester dicyclohexylamine salt (120.0 mg, 0.202 mmole) in ethyl acetate (20 ml) was washed with 10% sodium bisulfate (3×10 ml) followed by water (5 ml). The organic layer was dried over sodium sulfate and evaporated to leave a colorless syrup 92.7 mg (99.5%) after drying under high vacuum; IR ($CHCl_3$): 3435 (m, NH), 3600~2400 (br, CO_2H), 1720 (s, br, urethane, ester, carboxylic acid C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.38~1.98 (4H, m), 2.23 (2H, br, s, aminoadipyl CH_2-CO), 4.50 (1H, m, aminoadipyl methine), 5.04 (2H, s, PhCH_2), 6.53 (1H, d, J=8 Hz, urethane N*H*), 6.86 (1H, s, benzhydryl methine), 7.28 (15H, s, Ph), 8.43 (1H, br s, CO_2H).

Benzhydryl 6-Aminopenicillanate *p*-Toluenesulfonic Acid Salt: A suspension of 6-aminopenicillanic acid (216 mg, 1.0 mmole), in methylene chloride (4.0 ml) and methanol (2.0 ml), was stirred for 2.5 hours at 24°C with diphenyldiazomethane (400 mg, 2.06 mmole). The resultant clear, red solution was evaporated to dryness. The residue was dissolved in methylene chloride (20 ml) and evaporated to dryness. This process was repeated with more methylene chloride (20 ml) to leave a red syrup, which was dried under high vacuum for 0.5 hour. The crude product was dissolved in dry ether (15 ml) and stirred vigorously while a solution of anhydrous *p*-toluenesulfonic acid (172 mg, 1.0 mmole) in dry acetone (2.5 ml) was added. The toluenesulfonic acid salt precipitated as a white solid, and was collected after cooling overnight at 4°C, and dried under high vacuum to give 498 mg (90% yield), mp 162~164°C (dec.) (Reference12, mp 166~168°C), $[\alpha]_D^{25}$ +123.3°, $[\alpha]_{365}^{25}$ +457.3° (*c* 1.5, CH_3OH); IR (KBr): 3680~2300 (br), 2660, 2593 (m, NH_3^+), 1784 (s, β-lactam C=O), 1734 (s, ester C=O) cm^{-1}, ^1H NMR ($CDCl_3$/DMSO-d_6) δ: 1.24 (3H, s, CH_3), 1.62 (3H, s, CH_3), 2.32 (3H, s, ArCH_3), 4.55 (1H, s, C3 methine), 4.97 (1H, d, J=4.3 Hz), 5.54 (1H, d, J=4.3 Hz, β-lactam protons), 6.91 (1H, s, ester methine), 7.13 (~2H, d, J=8 Hz, tosyl H3 and H5) overlapping with 7.33 (~10H, s, Ph), 8.35 (~2H, d, J=8 Hz, tosyl H2 and H6) overlapping with 6.57~8.50 (~3H, br, exch. with D_2O, NH_3^+).

Fully Protected Penicillin N: A solution of *N*-carbobenzoxy D-α-aminoadipic acid α-benzhydryl ester (92.7 mg, 0.201 mmole) in methylene chloride (1.0 ml) was treated with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 55.0 mg, 0.22 mmole) and stirred for 5 minutes. To this mixture was added, in methylene chloride (3×1.0 ml), a solution of 6-aminopenicillanic acid benzhydryl ester (80.9 mg, 0.21 mmole) which had been freshly liberated from the *p*-toluenesulfonic acid salt (115.0 mg, 0.21 mmole). After 45 hours the solvent was evaporated, and the residual syrup was dissolved in ethyl acetate (5 ml) and washed with 10% sodium bisulfate (3×3 ml) and water (3 ml). The organic layer was dried over sodium sulfate and evaporated to leave a pale yellow foam (168.6 mg). Purification by HPLC using toluene - ethyl acetate (4: 1) afforded a colorless foam (72.4 mg, 44%); $[\alpha]_D^{23}$ +92.96°, $[\alpha]_{365}^{23}$ +343.4° (*c* 0.724, methylene chloride); IR ($CHCl_3$): 3430 (m, NH), 1785 (s, β-lactam C=O), 1737, 1724,

1700 (br, s, urethane, ester, amide C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.26 (3H, s, CH_3), 1.61 (3H, s, CH_3), overlapping with 1.60~2.01 (4H, m, aminoadipyl methylene), 2.23 (2H, m, CH_2CO), 4.52 (1H, m, aminoadipyl methine) overlapping with 4.53 (1H, s, *H*-C3), 5.11 (2H, m, PhCH_2), 5.50 (1H, d, J=9 Hz, urethane NH) overlapping with 5.57 (1H, d, J=5.4 Hz, *H*-C5), 5.64 (1H, q, J=5.4, 9 Hz, H-C6), 6.27 (1H, d, J=9 Hz, amide N*H*), 6.90 (1H, s) and 6.96 (1H, s, benzhydryl methines), 7.23~7.47 (25 H, m, Ar*H*).

Penicillin N Monosodium Salt: A solution of the fully protected penicillin N (41.2 mg, 0.050 mmole) and sodium bicarbonate (4.3 mg, 0.051 mmole) in freshly distilled tetrahydrofuran (4.0 ml) and water (3.0 ml) was contained within a 25-ml round bottom flask. To this solution was added 10% palladium on charcoal (82.0 mg) and the flask was fitted with a rubber septum. The flask was then purged with nitrogen followed by hydrogen. A small balloon was next attached to the septum using a syringe needle and the apparatus was filled with hydrogen so that the balloon was partially inflated. Vigorous stirring was then commenced and continued for 45 minutes. After this time the reaction mixture was filtered through Celite. The solids were washed with water (10 ml), ethanol (5 ml) and water (10 ml) and the combined filtrates and washings were lyophilized to leave a dark grey fluffy solid (19.5 mg). The product was redissolved in ice-cold water (5 ml) and washed with ethyl acetate (2 ml). The aqueous layer was filtered through a small tissue plug and again lyophilized to leave a light grey solid (18.2 mg (95%)), (65% pure by chemical assay[6]); IR (KBr): 3700~2400 (s, br, NH_3^+), 1770 (s, β-lactam C=O), 1660, 1600, 1525 (s, br, amide, carboxylate C=O) cm^{-1}; ^1H NMR (D_2O) δ: major component: 1.49 (3H, s, CH_3), 1.62 (3H, s, CH_3), 1.57~2.20 (4H, m, aminoadipyl methylenes), 2.62 (2H, t, J=7 Hz, aminoadipyl CH_2CO), 3.80 (1H, t, J=6 Hz, aminoadipyl methine), 5.67 (1H, d, J=4 Hz) and 5.71 (1H, d, J=4 Hz, β-lactam protons).

Synthesis of Isopenicillin N: This was performed exactly as described above, but with L-α-aminoadipic acid in place of the D-enantiomer.

Organisms and Culture Conditions

S. clavuligerus NRRL 3585 was maintained and cultured for preparation of cell-free extracts as described previously[10]. *Escherichia coli* Ess is a supersensitive mutant generously provided by A. L. DEMAIN, Massachusetts Institute of Technology, Cambridge. It was cultivated on Trypticase soy broth or Trypticase soy +2% agar at 37°C.

Preparation of Broken-cell Suspensions and Cell-free Extracts

Cell-free extracts were prepared by sonication of washed cell suspensions as described previously[10]. The normal period of sonication was 2×15 seconds at maximum intensity (300 watts, Biosonic III, Bronwill Scientific, Rochester) and resulted in cell-free extracts containing 5~10 mg protein/ml. To study the effect of sonication upon ring expansion activity, sonication times were varied from 15 seconds to 2 minutes. In all cases cell suspensions were cooled in ice-water while sonication was performed in 15 seconds bursts, with 30 seconds intervals between bursts for machine cooling. The sonicated cell suspensions were then centrifuged for 10 minutes at 3,500×g to sediment unbroken cells. The resulting supernatant was designated "broken-cell suspension". "Cell-free extract" was prepared by further centrifugation of the broken-cell suspension for 1 hour at 100,000×g. Broken-cell suspensions and cell-free extracts were stored as aliquots at −20°C.

Assays

Cyclization Assay: Cyclization of ACV was measured in reaction mixtures containing: bis-ACV 0.9 mM, dithiothreitol 4 mM, Na-ascorbate 2.8 mM, $FeSO_4$ 45 μM, tris-HCl buffer (pH 7.0) 0.05 M, cell-free extract 0.3 ml, final volume 0.4 ml. The mixtures were incubated for 1 hour at 20°C unless otherwise indicated, and the reactions terminated by addition of 0.4 ml of methanol. Antibiotic product was estimated by bioassay using *E. coli* Ess as indicator organism as described previously[10]. One unit of antibiotic is defined as that amount which gives a zone of inhibition equivalent to 1 μg of cephalosporin C with *E. coli* Ess as indicator organism.

Epimerase Assay: Epimerase activity was followed using the cyclization assay system described above but 40 μg of isopenicillin N (62% pure) was used as substrate in place of bis-ACV. All other constituents and conditions were the same as for the cyclization assay. Penicillin N (product) was distinguished from isopenicillin N (substrate) by bioassay using *E. coli* Ess as the indicator organism.

E. coli Ess is approximately 30 × more sensitive to penicillin N than to isopenicillin N and shows no zone of inhibition due to isopenicillin N at the concentration used in the epimerase assay.

Ring Expansion Assay: Ring expansion activity was followed using the cyclization assay system described above but supplemented with ATP 0.5 mM, α-ketoglutarate 1 mM, KCl 7.5 mM, and $MgSO_4$ 7.5 mM. Total volume and incubation conditions were the same as for the cyclization assay. The antibiotic product formed was again estimated by bioassay using *E. coli* Ess as indicator organism. Bioassays were also performed using agar plates supplemented with penicillinase at 2×10^5 units/ml, to distinguish penicillin from cephalosporin-type antibiotics. Authentic deacetoxycephalosporin C (1 μg) was not affected by penicillinase at this concentration. Again 1 unit of antibiotic is that amount which gives a zone of inhibition equivalent to 1 μg of cephalosporin C.

Protein Assay: The protein content of the cell-free extracts was estimated by the method of BRADFORD[8]. Bovine gamma globulin was used as the standard.

Thin-layer Chromatography of Reaction Mixtures

Cyclization and ring expansion reaction mixtures for thin-layer chromatography were incubated for 2 hours at 20°C. After termination, the reaction mixtures were centrifuged for 5 minutes at 12,000 × *g*, and 5 μl amounts of supernatant were spotted onto two separate cellulose thin-layer chromatography (TLC) sheets (Eastman Kodak Co., Rochester). Deacetoxycephalosporin C (0.05 μg) and deacetylcephalosporin C (0.05 μg) were also applied to the TLC sheets, both as separate spots and as over spots on 5 μl amounts of ring expansion reaction mixture. Chromatograms were developed completely (about 4 hours) in *n*-butanol - acetic acid - water (3 : 1 : 1). One dried chromatogram was placed face down on an agar slab inoculated to 2% with *E. coli* Ess. The duplicate chromatogram was placed on a similar agar slab containing penicillinase at 2×10^5 units/ml. After 30 minutes at 21°C, the TLC sheets were removed and the agar slabs were incubated overnight at 37°C. Slabs were then flooded with 5% trichloroacetic acid to improve visualization of the zones of inhibition.

Results

In a normal cyclization assay, cell-free extracts of *S. clavuligerus* cyclized ACV into penicillinase-sensitive antibiotic (Table 1). Penicillinase-resistant antibiotic was not produced under these conditions. When the cyclization assay system was supplemented with Mg^{2+} and K^+ ions, ATP and α-ketoglutarate, *i.e.*, factors associated with ring expansion activity in *C. acremonium*, the ring expansion assay revealed conversion of ACV into an antibiotic product which was 30 ~ 50% penicillinase resistant.

The reaction mixtures were also examined by TLC (Fig. 1). Bioassay of developed TLC sheets on agar slabs inoculated with *E. coli* Ess (Fig. 1a) resolved the antibiotic produced in ring expansion reaction mixtures into two main spots (Rf 0.48 and 0.54). A third faint spot at Rf 0.32 was occasionally observed, and is seen in Fig. 1. In contrast, cyclization reaction mixtures produced only one spot (Rf 0.54). Bioassay of the developed TLC sheets on agar supplemented with penicillinase (Fig. 1b) revealed the spot (Rf 0.54) seen in both ring expansion and cyclization reaction mixtures to be penicillinase-sensitive, and the second spot (Rf 0.48) seen only in ring expansion reaction mixtures to be penicillinase-resistant. The third faint spot (Rf 0.32) was also penicillinase-resistant. Chromatography of authentic standards led to ambiguous results, because other components of the reaction mixtures influenced the mobilities of the antibiotics. To overcome this problem, the standards were spotted on top of ring expansion reaction mixtures. Under these conditions, deacetoxycephalosporin C co-chromatographed with the penicillinase-resistant spot at Rf 0.48, and deacetylcephalosporin C co-chromatographed with the faint spot occasionally seen at Rf 0.32.

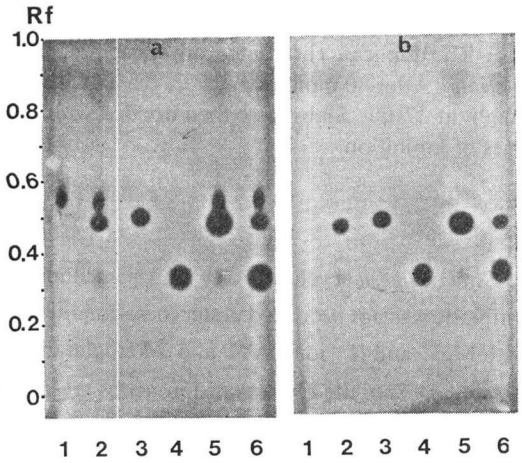
Since the multi-step conversion of ACV into cephalosporins is presumed to proceed *via* an epimerase (Scheme 1), cell-free extracts of *S. clavuligerus* were examined specifically for epimerase activity. The

Fig. 1. Examination of antibiotic products of cyclization and ring expansion activities by thin-layer chromatography (TLC).

Two cellulose TLC sheets were spotted with the following samples:

1. 5 μl of cyclization reaction mixture. 2. 5 μl of ring expansion reaction mixture. 3. 0.05 μg deacetoxycephalosporin C. 4. 0.05 μg deacetylcephalosporin C. 5. 5 μl of ring expansion reaction mixture +0.05 μg deacetoxycephalosporin C. 6. 5 μl of ring expansion reaction mixture +0.05 μg deacetylcephalosporin C.

Both cyclization and ring expansion assay systems employ bis-ACV as substrate. Chromatograms were developed 4 hours in *n*-butanol - acetic acid - water (3: 1: 1) then bioassayed as indicated in Materials and Methods. One TLC sheet (a) was bioassayed on inoculated agar; the second (b) on inoculated agar supplemented with penicillinase at 2×10^5 units/ml.



epimerase assay system was a normal cyclization assay system in which ACV was replaced as substrate by 40 μg of isopenicillin N preparation (62% pure). Twenty microliter amounts of epimerase reaction mixtures were bioassayed using *E. coli* Ess as indicator organism (Table 2). *E. coli* Ess is insensitive to isopenicillin N at the concentration used in the epimerase assay system. In contrast, *E. coli* Ess is quite sensitive to penicillin N at the same concentration. Cell-free extracts of *S. clavuligerus* converted isopenicillin N into a penicillinase-sensitive product which had antibacterial activity against *E. coli* Ess. This

Table 1. Cyclization and ring expansion activities of cell-free extracts of *S. clavuligerus*.

Reaction mixture tested*	Bioactivity** [units/mg protein]	
	no penicillinase	+penicillinase
Cyclization assay system	2.10	0
Ring expansion assay system	2.36	1.07

* Both cyclization and ring expansion assay systems employ bis-ACV as substrate.

** Bioactivity [units/mg protein] is the amount of antibiotic (detected by bioassay) formed under standard assay conditions by 1 mg of cell-free extract protein; +penicillinase indicates that the bioassay was performed in the presence of penicillinase.

Table 2. Epimerase activity of cell-free extracts of *S. clavuligerus*.

Preparation tested	Diameter of zone inhibition (mm)	
	no penicillinase	+penicillinase
1 μg isopenicillin N*	0	0
1 μg penicillin N**	17.5	0
20 μl epimerase reaction mixture (1 hour incubation)	13.0	0
20 μl epimerase reaction mixture (0 hour control)	0	0

* Isopenicillin N preparation was 62% pure.

** Penicillin N preparation was 65% pure.

Table 3. Effect of assay system components on ring expansion activity.

Omissions from ring expansion assay system*	Bioactivity** [units/mg protein]	
	no penicillinase	+penicillinase
None (normal ring expansion assay system)	1.78	0.51
K^+	2.34	0.48
Mg^{2+}	1.41	0.33
ATP	2.22	1.27
α-Ketoglutarate	1.58	0
K^+ and ATP	2.34	0.64
K^+, Mg^{2+}, ATP and α-ketoglutarate (normal cyclization assay system)	2.34	0

* The ring expansion assay system employs bis-ACV as substrate.

** See footnote ** of Table 1.

conversion was not observed in reaction mixtures stopped at zero time or reaction mixtures containing heat-inactivated cell-free extract.

Ring expansion activity of cell-free extracts was observed only when cyclization reaction mixtures were supplemented with K^+, Mg^{2+}, ATP and α-ketoglutarate. The importance of each of these components was investigated by preparation of ring expansion reaction mixtures from which one or more of these components was deleted (Table 3). Alpha-ketoglutarate was the only component which markedly affected ring expansion activity, and no penicillinase-resistant antibiotic was formed in the absence of this cofactor. Deletion of Mg^{2+} ions appeared to depress ring expansion activity somewhat, but K^+ ions had no effect, and deletion of ATP caused enhancement of ring expansion activity. When a range of ATP concentrations from 0.04~0.8 mM was tested, no concentration was found which stimulated ring expansion activity (data not shown). A number of *α-keto* acids including pyruvate, oxaloacetate, α-ketobutyrate, α-ketoisovalerate and α-ketoadipate were tested as possible substitutes for α-ketoglutarate in ring expansion reaction mixtures. None of these was found to support ring expansion activity (data not shown).

Ring expansion activity in *C. acremonium* is stimulated by Triton X-100 and by sonication, an observation which suggests that the activity is membrane-associated[18]. In contrast, ring expansion activity in *S. clavuligerus* cell-free extracts is not sedimented by centrifugation at 100,000×*g* for 1 hour, an observation which suggests that the activity is not particulate. The effects of Triton X-100 and sonication on ring expansion activity in *S. clavuligerus* cell-free extracts were, therefore, investigated. Ring expansion reaction mixtures were prepared with and without Triton X-100 at a final concentration of 0.1% (v/v). Both cell-free extracts and broken cell suspensions (see Materials and Methods) were used as enzyme sources to ensure that membrane-associated effects, if any, would be detected. Triton X-100 depressed ring expansion activity somewhat when either cell-free extract or broken-cell suspension was used as the enzyme source (Table 4). In a similar experiment using the same enzyme sources, Triton X-100 had no effect on cyclization activity (data not shown).

The effect of sonication on ring expansion activity was determined by exposure of cell suspensions to various periods of sonication, followed by preparation of cell-free extracts. Specific ring

Table 4. Effect of Triton X-100 on ring expansion activity in *S. clavuligerus* cell-free extracts.

Enzyme preparation tested*	Bioactivity** [units/mg protein]	
	no penicillinase	+penicillinase
Cell-free extract	2.36	1.07
Broken cell suspension	2.62	1.12
Cell-free extract +0.1% Triton X-100	1.87	0.72
Broken cell suspension +0.1% Triton X-100	2.62	0.48

* See footnote ** of Table 3.

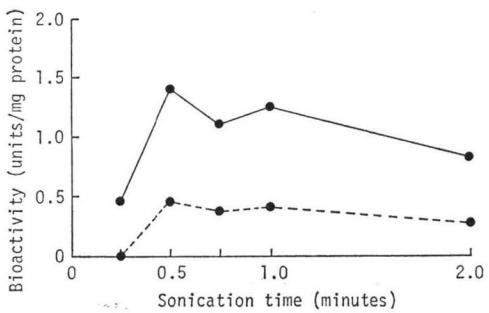
** Based on the protein content of the cell-free extract for both cell-free extract and for broken cell suspension.

Fig. 2. Effect of sonication on ring expansion activity of resulting cell-free extracts.

Cell-free extracts were prepared from washed cell suspensions which had been sonicated for varying lengths of time. Ring expansion activity of each extract was determined under standard assay conditions using bis-ACV as substrate.

Total antibiotic formed in the ring expansion assay was determined by bioassay, ——.

Penicillinase-insensitive antibiotic was determined by bioassay in the presence of penicillinase, ------.



expansion activity was highest in cell-free extracts prepared by 30 seconds of sonication, the procedure normally used to prepare cell-free extracts (Fig. 2). With a 15-second sonication, cell breakage was incomplete and the yield of enzyme activity was low. Longer periods of sonication improved cell breakage, but ring expansion activity began to decrease when the sonication time reached 2 minutes. There was no apparent stimulation of ring expansion activity by sonication in excess of that needed to provide good cell breakage. The cyclization activity of these same cell-free extracts was also not stimulated by over-sonication (data not shown).

Table 5. Ring expansion activities of various substrates.

Substrate	Bioactivity* [units/mg protein]	
	no penicillinase	+penicillinase
ACV	2.44	1.11
6-Aminopenicillanic acid	0.45	0
Penicillin V	99.2	0
Penicillin G	175.0	0
Penicillin N	17.2	2.44
Penicillin K	124.3	0
Dihydropenicillin F	155.5	0

* See footnote ** of Table 1.

The ability of *S. clavuligerus* cell-free extracts to cause ring expansion of substrates other than ACV was examined, by substitution of such substrates for ACV in the normal ring expansion assay system. In each case 250 μg of the substrate was employed, without purification. Six aminopenicillanic acid was 98% pure as purchased. Penicillin G and penicillin V were 100% pure, as determined by D. HOOK, Bristol Laboratories, Syracuse. Penicillin N, penicillin K and dihydropenicillin F were determined to be 65%, 91% and 81% pure, respectively, according to the arsenomolybdate assay of HOLM[6], with penicillin V as standard. In these cases, ring expansion assays actually contained 162 μg, 228 μg and 202 μg of these penicillins, respectively. All of these substrates showed bioactivity in the absence of penicillinase, because of their inherent antibacterial properties, and this activity was completely destroyed by penicillinase (Table 5). Only penicillin N, the presumed natural substrate for ring expansion, was converted to a penicillinase-insensitive form by the ring expansion system.

Discussion

The results of the present work, together with those of the previous study[10], demonstrate that at least three enzymes of the penicillin-cephalosporin biosynthetic pathway are present and active in cell-free extracts of *S. clavuligerus*. Indeed, all of the steps summarized in Scheme 1 appear to occur in this system. It was suggested earlier that *S. clavuligerus* cell-free extracts cyclize ACV into isopenicillin N, which is then partially converted to penicillin N by an epimerase[10]. This conversion has now been demonstrated. In addition, the presence of α-ketoglutarate in these cell-free extracts allows the penicillin N to be acted upon by the ring expansion enzyme to yield deacetoxycephalosporin C. This compound undergoes a further oxidation to deacetylcephalosporin C.

The bioassay procedures employed in this and the preceding[10] work allow monitoring of the various processes. Thus, cyclase conversion of ACV to isopenicillin N gives an antibiotic product which inhibits *Micrococcus luteus* ATCC 9341 but not *E. coli* Ess. Epimerase conversion of isopenicillin N yields penicillin N, which inhibits both *M. luteus* and *E. coli* Ess. Ring expansion converts penicillin N into deacetoxycephalosporin C, which inhibits *E. coli* Ess in the presence of penicillinase.

Such a multistep conversion of ACV to deacetoxycephalosporin C is not normally observed in cell-free systems from *C. acremonium* unless fresh extracts are used[21], and even then only a trace of cephalosporin is formed. This represents a major difference between the *S. clavuligerus* and *C. acremonium* cell-free systems. Furthermore, in *S. clavuligerus* cell-free extracts, all three enzyme activities are stable to storage at -20°C. In contrast, epimerase activity in *C. acremonium* cell-free extracts is extremely labile.

The cofactor requirements of ring expansion activity in the two systems also appears to differ. In *C. acremonium*, ATP is strongly stimulatory, and α-ketoglutarate has no effect on cell-free extracts. Alpha-ketoglutarate does however stimulate ring expansion in permeabilized cells from *C. acremonium*[4]. In cell-free extracts from *S. clavuligerus* on the other hand α-ketoglutarate is absolutely required for ring expansion activity, while ATP has an inhibitory effect. The absolute requirement for α-ketoglutarate suggests[1,13] that ring expansion in *S. clavuligerus* involves an intermolecular dioxygenase which uses α-ketoglutarate as a substrate.

In their original studies, KOHSAKA and DEMAIN found that ring expansion activity in *C. acremonium* would pass through a 1.2 μ Millipore filter[11]. More recently, however, SAWADA *et al.* concluded that the ring expansion enzyme in *C. acremonium* is membrane bound, on the basis of the following observations: (i) ring expansion activity is reduced by Millipore filtration of enzyme preparations and will not enter a Sephadex G-100 column; (ii) ring expansion activity is increased by sonication and Triton X-100 treatment of enzyme preparations, processes which may affect membrane-bound enzymes[18]. In contrast, the ring expansion activity of *S. clavuligerus* cell-free extracts seems to be caused by a soluble enzyme since this activity is not sedimented by high speed centrifugation, and is not affected by membrane disrupting treatments. Moreover, neither sonication nor detergent treatment releases any further ring expansion activity from membrane-containing broken-cell suspensions and activity is not enhanced by either treatment. This disparity could be explained if the ring expansion preparation used by SAWADA *et al.*[18] was contaminated with unlyzed protoplasts.

Ring expansion activity in *S. clavuligerus*, like that in *C. acremonium*, shows considerable specificity as to the nature of the side chain on the penicillin substrate[11]. Only penicillin N, the presumed natural substrate, is converted to a cephalosporin.

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