Enzymatic Approach to Syntheses of Unnatural Beta-Lactams

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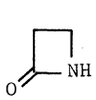
Summary. Four enzymes associated with the transformation of the peptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) into the beta-lactam antibiotic desacetylcephalosporin C have been isolated from the prokaryotic organism *Streptomyces clavuligerus* and immobilized. Appropriate choice of the cofactors allows continuous and quantitative conversion of the peptide into either penicillins or cephalosporins at room temperature. The overall process includes four oxidations, two ring closures, and one epimerization. In contrast, cell-free transformations with the eukaryotic organism *Cephalosporium acremonium* do not proceed beyond the oxidation level of penicillin. The amino acids of the natural peptide ACV can be altered by chemical means; several of the resulting peptides are converted into novel antibiotics by the enzymes of *Streptomyces clavuligerus.*

The four-membered ring **1**, containing an amide bond, is known in organic chemistry as the beta-lactam ring. This ring is the common structural feature of a family of natural products whose members are of medicinal interest as antibacterial agents. Although recent research suggests that these antibacterial properties have long been known (*1*), and were almost certainly known in the 19th century (*2*), the modern history of research on beta-lactam compounds began with the work of Fleming (*3*). This modern history has developed through six scientific eras (with some overlap).

The first era, which focused on *Penicillium* spp., began in 1929 (*3*), reached a climax in 1945 (*4*), and led to the clinical introduction of penicillin V in 1954 (*5*). This was the first important "unnatural" (that is, not occurring in nature) beta-lactam compound. The beta-lactam compounds produced by *Penicillium* spp. are penicillins (**2**). These compounds are bicyclic, with a beta-lactam ring fused to a five-membered (thiazolidine) ring, and an acylamino side chain. Penicillin V is one of a number of unnatural (biosynthetic) penicillins that can be prepared (*6*) by addition of the side chain carboxylic acid corresponding to R to the fermentation medium. However, this type of structural modification leading to unnatural products is restricted to monosubstituted derivatives of acetic acid, and it has so far been observed only in *Penicillium* spp.



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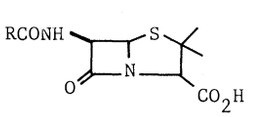

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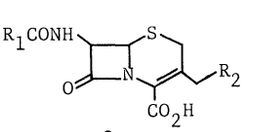
The second era, which focused on *Cephalosporium* spp., began in 1945 when Brotzu, in Cagliari, Italy, isolated a novel organism (*7*). This era reached a climax in 1959 with the proof of the structure of the antibiotic cephalosporin C (*8*) and led, in the mid-1960's, to the introduction of the antibiotics cephalothin and cephaloridine (*9*). These two unnatural semisynthetic cephalosporins were the first clinically important antibiotics of this structural type.

The cephalosporins (**3**), contain a bicyclic nucleus, in which the beta-lactam ring is fused to a six-membered (dihydrothiazine) ring, and two peripheral chains, shown as R_1 and R_2 in structure **3**, that can be modified by chemical means to produce products that do not occur in nature.



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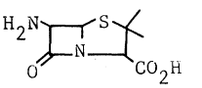
The third era began in 1959 with the discovery that 6-aminopenicillanic acid (6-APA) (**4**), the amino acid nucleus of penicillin, is present in *Penicillium* fermentations, and can be isolated when no side chain precursor is added (*10*). Although the compound was discovered in this manner, it was later determined that 6-APA is more readily produced on an industrial scale by chemical (*11*) or enzymatic (*12*) removal of the side chain of penicillin V or penicillin G, the naturally occurring penicillin whose side chain is derived from phenylacetic acid. Of the thousands of unnatural penicillins that have been synthesized since 1959 by chemical attachment of a side chain to the amino group of 6-APA, about 15 compounds have become important in medicine, including the well-known antibiotics ampicillin, cloxacillin, carbenicillin, amoxicillin, and piperacillin.



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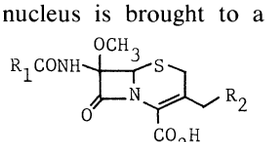
The fourth era may be termed the era of *Streptomyces* spp. It began in 1971, with the simultaneous discovery at two laboratories (*13, 14*) that certain actinomycetes, specifically *Nocardia lactamdurans, S. clavuligerus*, and *S. lipmanii*, produce a class of compounds termed cephamycins (**5**), in which the cephalosporin nucleus is brought to a higher oxidation level by oxygenation at C-7. The first clinically important compound from this period of research is the *unnatural* semisynthetic cephamycin cefoxitin, introduced in 1978 (*15*), in which the R_1 substituent has been altered chemically.



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During these eras, it was found that, among natural (naturally occurring) beta-lactam compounds, only penicillin G had sufficient efficacy to be directly useful in medicine. Structural modification of the natural products was, therefore, necessary to achieve such efficacy. These modifications, involving mainly alteration of the substituents around the peripheries of the natural ring systems, were made principally by chemists in the

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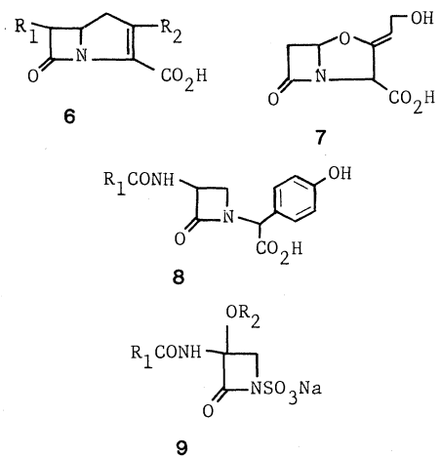
laboratories of pharmaceutical companies engaged in industrial fermentation. The concurrent role of microbiologists in these pharmaceutical laboratories was to discover or develop suitable (high producers) strains of organisms and optimum conditions for the production of the natural products.

In the fifth era of research industrial screening programs led to the discovery of a number of novel structural types. These novel beta-lactam compounds include the carbapenems (**6**) (*16*), in which the beta-lactam ring is fused to a five-membered carbon-containing (pyrroline) ring, clavulanic acid (**7**) (*17*), in which the beta-lactam ring is fused to a five-membered oxygen-containing (oxazolidine) ring, and the nocardicins (**8**) (*18*), and monobactams (**9**) (*19*), which contain a



monocyclic beta-lactam nucleus. The first clinically successful compound from this period is clavulanic acid. This compound is an antibacterial adjuvant. Although essentially devoid of antibacterial activity, clavulanic acid is an irreversible (suicide) inhibitor of beta-lactamases (*20*); admixed with amoxycillin, clavulanic acid greatly extends the antibacterial spectrum of the latter compound. This is the basis of Augmentin, introduced in 1981 (*21*).

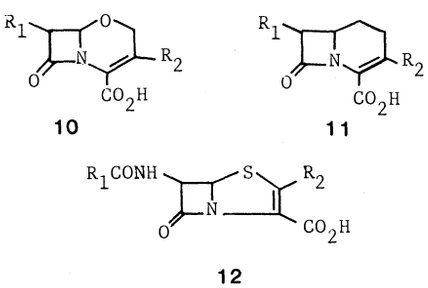
The discoveries of this fifth era led to a general realization among organic chemists that much greater structural variation within the beta-lactam–containing nucleus is possible than had previously been supposed, together with the belief that structures of interest might be economically accessible by organic synthesis.

In the sixth era, unnatural beta-lactam–containing ring systems were prepared by chemical means. The beginnings of the fifth and sixth eras coincide as the result of the chemical conversion of 6-APA into a 1-oxacephem (**10**) (*22*), and the total chemical syntheses of 1-oxacephems (*23*) and 1-carbacephems (**11**) (*24*). These early syntheses were followed by Woodward's successful conversion of penicillin V into a penem (**12**) (*25*). Many additional structural types have been reported since then (*26*). In retrospect, it is remarkable that, in the structures of clavulanic acid and the carbapenems, nature anticipated the 1-oxacephems, 1-carbacephems, and penems.

Table 1. Costs of some beta-lactam antibiotics as a function of the type of fermentation and the approximate number of chemical steps to the finished product.

Drug	Structural type	Type of fermentation	Chemical steps	Maximum dosage (g/day)*	Cost/day (dollars)†
Penicillin V	Penicillin	*Penicillium*	0	2	0.16
Ampicillin	Penicillin	*Penicillium*	4	4	0.60
Cephalexin	Cephalosporin	*Penicillium*	6	12	2.16
Cephalothin	Cephalosporin	*Cephalosporium*	2	12	32.92
Cefoxitin	Cephamycin	*Streptomyces*	2	12	59.10
Moxalactam	Oxacephem	*Penicillium*	16	12	146.45

*The highest recommended. †United States dollars.



The first clinically successful antibiotic from the sixth era of research is the 1-oxacephem termed moxalactam or latamoxef (*27*) introduced jointly in 1981 by the Shionogi Pharmaceutical Company and the Lilly Research Laboratories. The discovery and successful industrial development of latamoxef (*28*) was the culmination of more than 200 man-years of effort; although full details of the industrial process are not available, the available data suggest (*29*) that this process requires about 16 chemical steps beginning with penicillin G and proceeds in an overall yield of 20 to 25 percent. This is an outstanding achievement in organic synthesis. However, since it is generally considered that each step in a chemical process approximately doubles the cost of the finished product, it is perhaps not surprising that moxalactam is the most expensive beta-lactam antibiotic currently in use.

This point is illustrated by the data of Table 1, which provide the cost of some beta-lactam antibiotics, expressed as the wholesale price to a pharmacist in the United States for 1 day's supply (*30*). Penicillin V, ampicillin, cephalexin and moxalactam are derived from *Penicillium* fermentation products in an increasing number of chemical steps, and their costs increase progessively from $0.16 per day to $146.45 per day. The entries for cephalexin, cephalothin, and cefoxitin illustrate the inherently greater cost associated with the production of the natural products of *Cephalosporium* and *Streptomyces* fermentations compared to the cost of *Penicillium* fermentation.

In addition to the direct economic factors that influence the cost of the finished product when the cost of a multistep chemical sequence is added to the cost of the primary fermentation product, other factors inhibit the transfer of laboratory-scale chemical syntheses of novel and potentially valuable beta-lactam systems to an industrial scale. For example, toxic reagents or solvents are common in the laboratory but cannot be used in an industrial process. In the laboratory, many reactions of interest proceed most effectively at temperatures below −70°C; but temperatures below −20°C are too costly to maintain in an industrial-scale process. Chromatographic purification of reaction mixtures is regarded as routine in the laboratory but adds considerably to the cost of an industrial process.

These and other considerations indicate that the opportunities that had emerged during the sixth period of research could not be exploited fully through chemical and microbiological research programs pursued independently. Accordingly, after several years of preparatory chemical studies (*31–33*), we initiated the collaborative effort summarized in the remainder of this article.

Basis of Current Research

Beta-lactam compounds are formed in nature as secondary metabolites of both eukaryotic and prokaryotic organisms. Examples of eukaryotic beta-lactam–producing organisms are the fungi *Penicillium chrysogenum* and *Cephalosporium acremonium*. Examples of prokaryotic

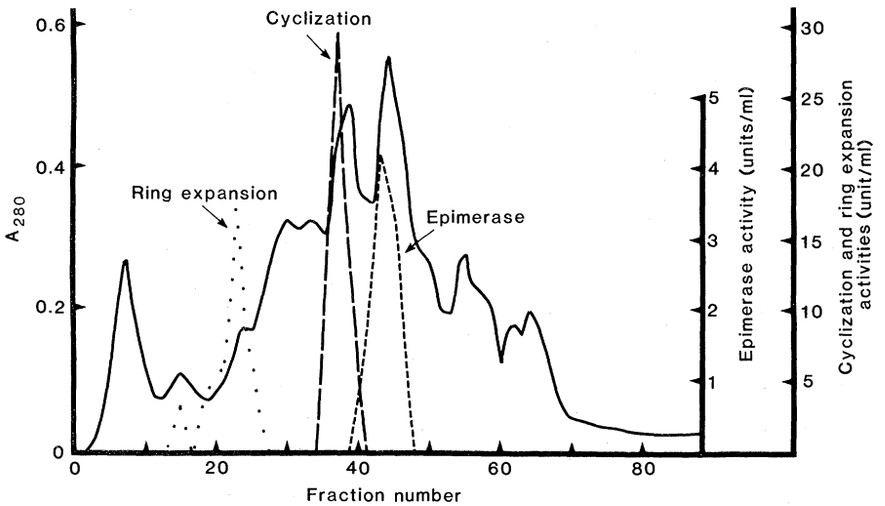


Fig. 1. DEAE-trisacryl chromatography of salt-precipitated cell-free extract of *S. clavuligerus*.

beta-lactam–producing organisms include the actinomycetes *S. clavuligerus, S. lipmanii, S. cattleya*, and *N. lactamdurans*, and a few species of nonfilamentous bacteria of the genera *Agrobacterium, Chromobacterium, Gluconobacter, Pseudomonas*, and *Serratia* (*34*).

The prokaryotes exhibit an inherently greater synthetic versatility than the eukaryotes for production of beta-lactam compounds. For example, *P. chrysogenum* synthesizes the tripeptide ACV (**13**), and converts this peptide exclusively to a penicillin as the sole stable beta-lactam–containing end product. Also, *C. acremonium* synthesizes this peptide, and converts it sequentially to a penicillin and a cephalosporin. In contrast, *S. clavuligerus* synthesizes a penicillin, a cephalosporin, and a cephamycin from one peptide precursor (*35, 36*) and, at the same time, it synthesizes clavulanic acid from a different precursor (*37*). *Streptomyces cattleya* synthesizes a penicillin, a cephalosporin, and also a carbapenem. Nonfilamentous bacteria produce both monocyclic and bicyclic beta-lactam antibiotics.

The gross features of the amino acid origins of the different classes of beta-lactam natural products are known. In the cases of the penicillins, cephalosporins, and cephamycins, all of the atoms of the bicyclic ring systems are derived from the amino acids cysteine and valine (*38*). The five-membered ring of the carbapenems is derived from glutamic acid, and the remaining two carbon atoms of the bicyclic nucleus are derived from acetate (*39*). The three carbon atoms of the beta-lactam ring of clavulanic acid are derived from glycerol or phosphoenolpyruvate; the nitrogen atom and all other carbon atoms are derived from glutamic acid (*37*). In the nocardicins and monobactams, the carbon atoms of the beta-lactam ring are derived from serine (*40*).

The diversity of the naturally occurring beta-lactam structures, and the amino acid or peptide precursors to these structures, suggested that more than one pathway exists in nature for the conversion of peptides to beta-lactam compounds. On the basis of this hypothesis, it appeared that a combined chemical and microbiological approach to novel structures could begin at the oxidation level of the amino acid or peptide precursors of the different structural classes. If the enzymes responsible for the transformations of these precursors had, or could be made to have sufficiently broad specificity to accept unnatural substrates, then the natural processes themselves would serve to synthesize unnatural beta-lactam–containing ring systems.

It was known that cell-free experiments would probably be necessary for this work because peptides, or species more complex than peptides, are not accepted by intact eukaryotic beta-lactam–producing organisms (*41*). During our work, analogous behavior was anticipated and subsequently observed with prokaryotic beta-lactam–producing organisms. Once this requirement had been determined, it was necessary to select an organism or organisms for the study. We chose to study the *Streptomyces* spp. because of their demonstrated greater biosynthetic versatility and, at the same time, compare the behavior of cell-free preparations from *Streptomyces* spp. and from the eukaryote *C. acremonium* (*42*).

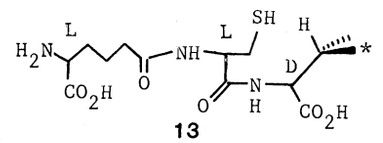
The most economical protocol for the cell-free synthesis of beta-lactam compounds would commence with amino acids. However, although a cell-free synthesis of the peptide precursor of the penicillins and cephalosporins from its constituent amino acids has been reported (*43*), this work depended on the use of a mutant of *Cephalosporium* that did not produce a beta-lactam. Since analogous observations with penicillin and cephalosporin-producing organisms have not yet been made, it seemed until quite recently (see below) that cell-free syntheses of "unnatural" structures of interest would require peptides as the starting materials.

Penicillin-Cephalosporin Biosynthetic Pathway

All organisms that produce penicillin and cephalosporin synthesize and utilize the common precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (**13**) (ACV). This peptide is formed from L-α-aminoadipic acid (Aad), L-cysteine (Cys), and L-valine (Val) through the steps

$$\text{Aad} + \text{Cys} \rightarrow \text{Aad-Cys} + \text{Val} \rightarrow \text{ACV}$$

with the valine moiety undergoing epimerization during its incorporation (*38*). The primary penicillin is **14**, isopenicillin N, synthesized from ACV through the action of a ferrous ion and ascorbate requiring cyclization enzyme (cyclase) (*44*). This oxidative process proceeds with the loss of four hydrogen atoms, of which two are carbon-bound, namely,



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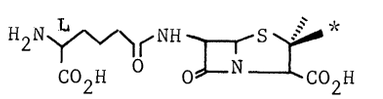

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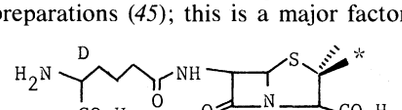
the beta hydrogen of the valinyl moiety (*33*), and the pro-S hydrogen of the cysteinyl moiety (*38*). Loss of these hydrogens accompanies S-C and N-C bond formation, with retention of configuration at each carbon atom. The S-C bond–forming stereochemistry has some consequences, as is discussed below.

Penicillin biosynthesis and cephalosporin biosynthesis are linked through the action of an epimerase, which accomplishes L- to D- isomerization in the side chain of isopenicillin N to form penicillin N (**15**). The epimerase has been isolated from *Streptomyces* preparations but not from *Cephalosporium* preparations (*45*); this is a major factor



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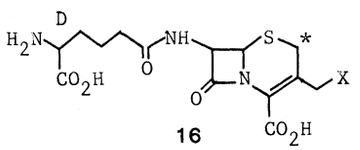
leading to greater synthetic versatility of systems derived from prokaryotic beta-lactam–producing organisms.

The primary cephalosporin, desacetoxycephalosporin C (**16**, X=H), is formed by oxidative expansion of the thiazolidine ring of penicillin N (*46–49*).



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In the course of this process, the beta-methyl group (see *) of penicillin N becomes C-2 of the six-membered ring (*38*); this stereochemical result also has consequences. The ring expansion of penicillin N is achieved through the action of a ferrous ion, ascorbate (*50*), and α-ketoglutarate requiring ring expansion enzyme (expandase), which has been isolated from both eukaryotic and prokaryotic systems (*47–49, 51*). Under the conditions of ring expansion, further hydroxylation of **16** to desacetylcephalosporin C (**16**, X is OH) occurs. This is the next to the last intermediate in the biosynthesis of cephalosporin C (**16**, where X is OAc) by *C. acremonium* (*52, 53*). With the *Streptomyces*, oxygenation at C-7 leading to cephamycins occurs after the formation of **16** (where X is OAc) or **16** (where X is $OCONH_2$) (*54*).

Before the viability of our proposal could be tested, it was necessary to determine how many of the above processes can be achieved efficiently from ACV under cell-free conditions. The pioneering studies of two groups of investigators (*55, 56*) aided considerably in defining the techniques that would have to be developed.

Since a biological sequence beginning with amino acids is not yet feasible, a chemical synthesis of the peptide was necessary. In the synthesis of this compound (*31*), we used *t*-butoxycarbonyl protection for amino groups, trityl protection for sulfur, and benzhydryl protection for carboxyl groups; this process leads, after removal of the protecting groups (*57*), to the dimer ACV disulfide, a stable crystalline compound, in more than 50 percent yield from the individual amino acids. This disulfide is converted quantitatively to ACV on treatment with dithiothreitol (*57*). The strategy developed for the synthesis of ACV has been extended without difficulty to the syntheses of more than 25 analogs of the natural peptide. A general synthesis of penicillin analogs of **14** and **15** has also been devised (*36, 57*) to facilitate the identification of products resulting from the cyclization of ACV analogs containing variations of the aminoadipyl moiety.

Cell-Free Experiments

Crude cell-free extracts were obtained successfully by sonic oscillation of *S. clavuligerus, S. lipmanii, S. cattleya*, and *C. acremonium* cells, followed by centrifugation to remove particulate material (*35, 47*). Isopenicillin N was formed in each case on addition of these extracts to reaction mixtures containing ACV dimer, dithiothreitol, *p*H 7.0 to 7.4 tris-HCl buffer, and the essential cofactors ferrous ion and ascorbate (*58*). The reaction mixtures were incubated in air at 20° to 25°C, and the reactions were terminated, before assay, by addition of methanol. These experiments established that ACV disulfide is not a substrate for the cyclase, and that dithiothreitol functions only to convert the dimer to its biologically active monomer.

Both bioassay and high-performance liquid chromatographic (HPLC) procedures were used to monitor the cyclization. The bioassay was based on the observations (*47, 59*) that *Micrococcus luteus* is 20 times more sensitive toward isopenicillin N and penicillin N than toward cephalosporins, whereas *Escherichia coli* Ess and *Pseudomonas aeruginosa* Pss are 20 times more sensitive toward penicillin N and cephalosporins than toward isopenicillin N. The HPLC analyses (*47, 60, 61*) were based on reversed-phase columns, and mobile phases consisting of methanol and phosphate buffer with detection at 220 nm or, alternatively, mixtures of pyridine, acetic acid, and water or of acetic acid, methanol, acetonitrile, and water, with detection at 254 nm. Quantitative assay of the peptide to penicillin conversion was possible (*60*).

Evidence for epimerase activity in cell-free extracts from *C. acremonium* had been reported by three groups of investigators (*62*). However, this activity is extremely labile, difficult to demonstrate routinely, and purification has not been possible. On the other hand, cyclization reaction mixtures with crude extracts of *S. clavuligerus* contain a stable epimerase. This first became apparent on examination of the antimicrobial spectrum of the antibiotic produced in the cyclization of ACV (*35*), and the presence of penicillin N was then confirmed by the conversion of this antibiotic to cephalosporins (*36*). Although the composition of the mixture of isopenicillin N and penicillin N resulting from the action of the epimerase on isopenicillin N could not be measured directly by HPLC, details of the epimerization process became accessible when we used an analog of ACV.

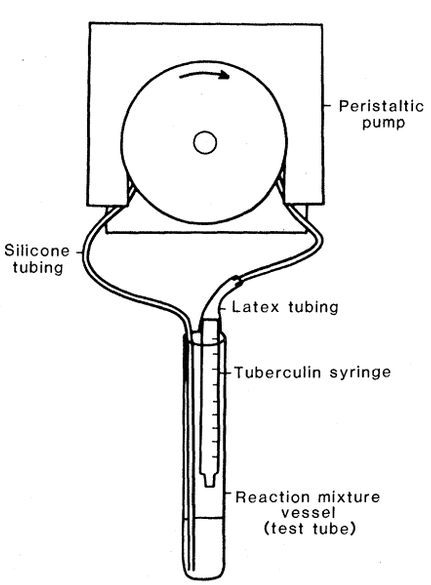


Fig. 2. Immobilized enzyme reactor.

Initial experiments on the ring expansion of penicillin N (*50, 63*) in cell-free extracts of *C. acremonium* indicated a requirement for ferrous ions and ascorbate, but not for α-ketoglutarate. In contrast, it was necessary to add α-ketoglutarate, as well as ferrous ions and ascorbate, for the conversion of ACV or penicillin N to cephalosporins in the crude cell-free extracts of *S. clavuligerus* (*36*). This difference in the behavior of the expandases from the two organisms was eventually traced to the presence of exogenous alpha-ketoglutarate in the crude extracts of *C. acremonium* (*48*).

The hydroxylation of desacetoxycephalosporin was first observed by Turner *et al.* (*52*) in cell-free extracts of both *C. acremonium* and *S. clavuligerus* and was found to be dependent on ferrous ions, ascorbate, and α-ketoglutarate as cofactors. Since the same cofactors are required for the action of the expandase, it is not surprising that cell-free experiments on the ring expansion of penicillin N led to both desacetoxy- and desacetylcephalosporin C (*48, 51*) when either *C. acremonium* or *S. clavuligerus* was used.

The experiments with crude cell-free extracts permit the following conclusions. (i) Extracts from *S. clavuligerus* contain all of the enzymes required to convert ACV into desacetylcephalosporin C, a process that involves four oxidations and one epimerization. (ii) The process can be arrested at the oxidation level of penicillin by deletion of alpha-ketoglutarate from reaction mixtures. (iii) Cell-free extracts from *C. acremonium* cannot convert ACV beyond the oxidation level of penicillin because they lack the epimerase; (iv) the presence or absence of this crucial link between penicillin biosynthesis and

cephalosporin biosynthesis represents the most significant difference between the cell-free systems derived from the prokaryotic and eukaryotic organisms.

Crude cell-free extracts have been subjected to extensive purification (*40–49*), resulting in complete separation of cyclase and expandase-hydroxylase activities in the case of *C. acremonium*; in the case of *S. clavuligerus*, separation of cyclase, epimerase, expandase, and hydroxylase activities has been achieved. Nucleic acids were precipitated with protamine sulfate or streptomycin sulfate, and the supernatants subjected to salt precipitation. With *C. acremonium*, the cyclase and expandase-hydroxylase activities were present in the 50 to 80 percent ammonium sulfate precipitate; with *S. clavuligerus*, the cyclase, epimerase, and expandase-hydroxylase activities were found in the 40 to 70 percent ammonium sulfate precipitate. The salt-precipitated cell-free extract from *S. clavuligerus* is a useful reagent for organic synthesis. With appropriate addition of cofactors, ACV is now converted quantitatively into penicillins or cephalosporins (or both), and HPLC assay of reaction mixtures is facilitated by the almost complete absence of baseline contamination (*60*). Furthermore, as described below, all enzyme activities can be immobilized at this stage.

The cyclase and expandase-hydroxylase activities of the salt-precipitated extract of *C. acremonium* CW-19 were separated by gel filtration, which was followed by ion-exchange chromatography on DEAE-Sepharose (*44, 47, 48*). The cyclase (molecular weight, 41,000) has been purified to homogeneity, and studies of its constitution have begun. The expandase has a molecular weight of 31,000 ± 3,000. In *C. acremonium* TR4, the expandase and hydroxylase are believed to be a single bifunctional enzyme (*49*).

In the case of *S. clavuligerus*, the salt-precipitated extract was subjected to gel filtration and ion-exchange chromatography on DEAE-trisacryl or (Fig. 1) ion-exchange chromatography alone. A 100-fold purification of the cyclase (molecular weight, 36,500) and a 35-fold purification of the epimerase (molecular weight, 60,000) were achieved (*45*). In contrast to a recent report that the expandase and hydroxylase of *C. acremonium* are a single bifunctional enzyme (*49*), we found that the expandase and hydroxylase of *S. clavuligerus* are different enzymes and can be separated completely (*46*). An HPLC assay for the ring expansion of penicillin N by the purified expandase of *S. clavuligerus* (molecular weight, 29,000) has been developed (*51*).

The enzyme transformations described so far are effected as batch processes because termination of reactions with methanol inactivates the enzymes. A continuous process would require immobilization of the enzymes. This has been achieved (*64*) with either the salt-precipitated cell-free extract or the reconstituted cyclase, epimerase, expandase, and hydroxylase of *S. clavuligerus*. This development was based on the observation that the enzymes—but none of the subtrates, products, or cofactors—adsorb to DEAE-trisacryl under conditions of low ionic strength (Fig. 1). Laboratory-scale investigations were conducted with enzyme-loaded resin packed into a plastic syringe barrel; ACV, together with the desired cofactors, was circulated continuously through this column by means of a peristaltic pump (Fig. 2). The contents of the test tube could be monitored continuously.

The methanol-phosphate HPLC system, with detection at 220 nm, is shown in Fig. 3. At the beginning of the reaction, peaks are observed at 2.0, 14.4, and 21.3 minutes, corresponding, respectively, to α-ketoglutarate, dithiothreitol, and ACV monomer. After 15 minutes, the peptide peak has diminished considerably, and a new peak is observed at 5.9 minutes. The peptide has disappeared completely after 30 minutes and, in addition to the peak at 5.9 minutes, a new peak is apparent in the 2.0-minute region. This peak continues to grow during the next 30 minutes of the process. Bioassay, HPLC assay with detection at 260 nm (Fig. 4), and thin-layer chromatography (Fig. 5) reveal that the major product after 60 minutes is desacetylcephalosporin C, and the minor product is penicillin N.

The enzyme-loaded reactors are stable to prolonged storage at −20°C, and the columns could be used several times, beginning with peptide. No difficulties were encountered in the scale-up from microgram to milligram quantities of ACV.

Thus all of the transformations from ACV to desacetylcephalosporin C that occur in intact cells of *S. clavuligerus* can be observed efficiently and routinely under controlled cell-free conditions.

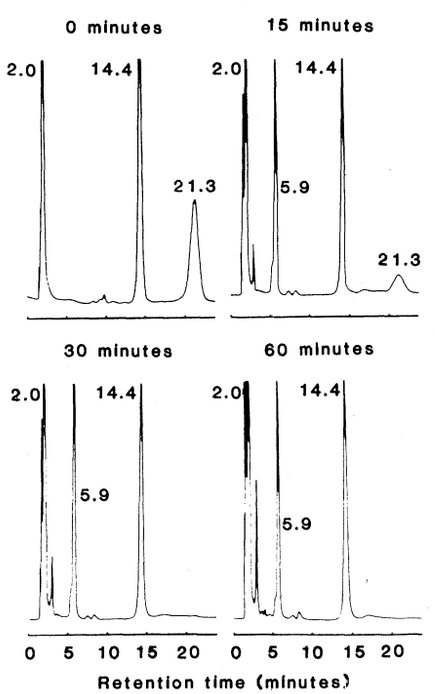


Fig. 3. HPLC analysis of the conversion of ACV to antibiotics on the immobilized enzyme reactor. The mobile phase consisted of 5 percent methanol and 95 percent KH_2PO_4 (0.05*M*, adjusted to *p*H 4 with concentrated H_3PO_4); detection at 220 nm.

Behavior of Unnatural Substrates

The cyclases of *C. acremonium* and *S. clavuligerus* exhibit similar specificity toward unnatural substrates in which the aminoadipyl moiety has been altered. The L-glutamyl-, L-aspartyl-, N-acetyl-L-α-aminoadipyl-, and glycyl-L-α-aminoadipyl–containing peptides are inactive substrates (*47–49, 65*), but adipyl-L-cysteinyl-D-valine is cyclized to its corresponding penicillin, carboxybutylpenicillin.

The expandases of *C. acremonium* and *S. clavuligerus* show no activity toward penicillin G, ampicillin, *n*-pentylpenicillin, *n*-heptylpenicillin and carboxybutylpenicillin (*36, 47–49*).

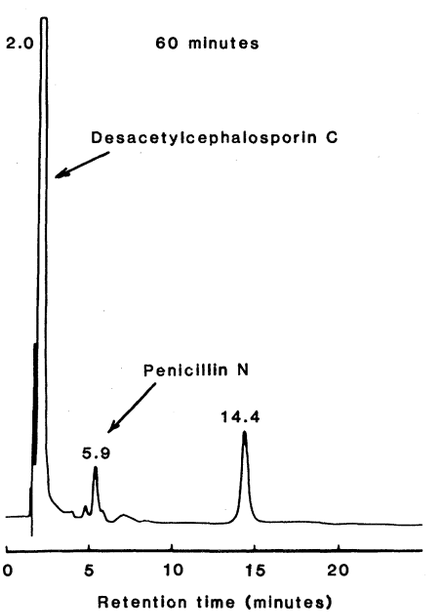
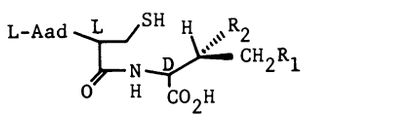


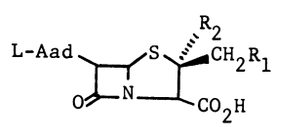
Fig. 4. HPLC analysis, with detection at 260 nm, of a 60-minute reaction of ACV on the immobilized enzyme reactor.

The behavior of aminoadipyl-modified peptides and penicillin N analogs toward cyclase and expandase, respectively, indicated that a modification of the valinyl moiety of ACV, potentially leading to a modification within the penicillin nucleus, would require that the L-α-aminoadipyl moiety be present. For a modification within the cephalosporin nucleus beginning with a peptide, the analog would require the L-α-aminoadipyl moiety, and the experimental system would require cyclase, epimerase, and expandase. The *S. clavuligerus* system was, therefore, used.

On the basis of the stereochemistry of the S-C bond–forming steps of penicillin and cephalosporin synthesis (**13** → **14**, and **15** → **16**, respectively), reviewed earlier, we anticipated that a peptide of the general structure **17** would cyclize to the isopenicillin N analog **18**, that this would epimerize to the penicillin N analog **19**, and that **19** would undergo ring expansion to **20**.



17 a: $R_1 = R_2 = H$
b: $R_1 = R_2 = CH_3$
c: $R_1 = H$; $R_2 = C_2H_5$



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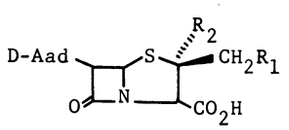

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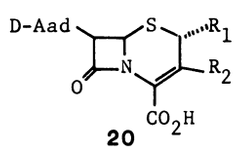


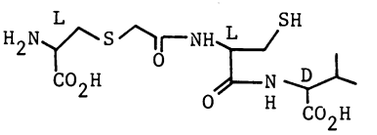
20

In accord with these expectations, biologically active penicillins were obtained after exposure of the peptides **17a**, **17b**, and **17c** to the cyclase (*66*). In each case, a single penicillinase-sensitive product was formed, and the subsequent action of the epimerase and expandase led to cephalosporins, with **17b** affording **20** ($R_1 = R_2 = CH_3$) (*66*). In the work of Abraham *et al.* with a cell-free system from *C. acremonium* (*67*), analogous cyclizations **17** → **18** have been observed. A second product of cyclization in these cases was found to be a biologically inactive cepham. Because of the absence of the epimerase, the *C. acremonium* system does not transform **18** further.

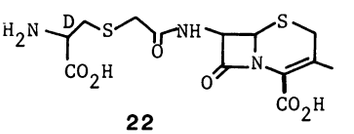
On the basis of bioassay data, the relative activities of cephalosporins **20** (containing the D-α-aminoadipyl side chain) toward *E. coli* Ess. are as follows: $R_1 = H$, $R_2 = ethyl > R_1 = H$, $R_2 = methyl > R_1 = R_2 = H \approx R_1 = R_2 = methyl$ (*66*).

A cephalosporin of type **20** with $R_1 = R_2 = H$ was introduced recently (*68*). The nucleus of this compound (ceftizoxime) is prepared by a multistep chemical sequence from the natural product cephalosporin C.

Although a biological synthesis of cephalosporins **20** from peptide precursors has thus been realized, the process requires a prior chemical synthesis of a peptide and, in particular, L-α-aminoadipic acid ($50 per gram). Therefore, the recent discovery that the peptide **21**, in which L-carboxymethylcysteine replaces L-α-aminoadipic acid, is an efficient substrate for the cyclase of *C. acremonium* (*69*) and is converted quantitatively to the cephalosporin **22** by the



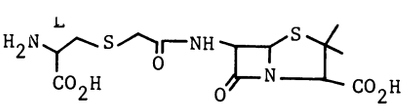
21



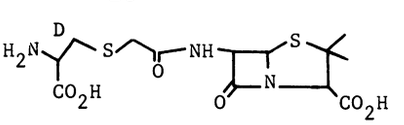
22

cyclase, epimerase, and expandase of *S. clavuligerus* (*70*), is of some importance. L-Carboxymethylcysteine is readily accessible from the inexpensive precursors L-cysteine and bromoacetic acid. At the same time, the isopenicillin N analog **23** is also readily accessible from 6-APA, bromoacetic acid and L-cysteine (*71*).

Compound **23** and its side chain epimer **24** are interconverted within minutes



23



24

to a 1:1 equilibrium mixture by the epimerase of *S. clavuligerus* (*70*) (Fig. 6). The action of epimerase and expandase on **23** therefore leads smoothly to **22**, and establishes an exceptionally simple synthesis of this compound from 6-APA that obviates a requirement for D-cysteine ($50 per gram). The penicillin (*71*) and cephamycin (*72*) having the D-carboxymethylcysteine side chain have interesting antibacterial activity, but an economical synthesis of this side chain has not yet been achieved.

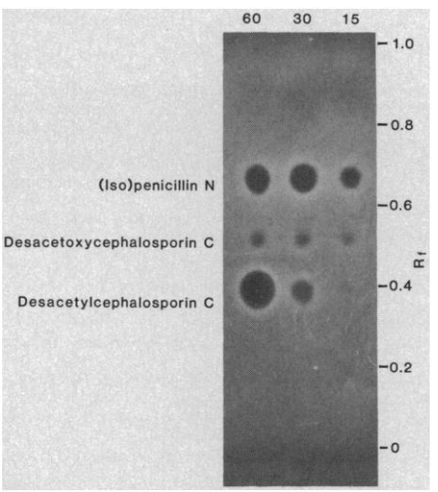


Fig. 5. TLC analysis of reaction mixtures from the immobilized enzyme reactor after 15 minutes, 30 minutes, and 60 minutes. The chromatogram was bioassayed on agar inoculated with *E. coli* Ess.

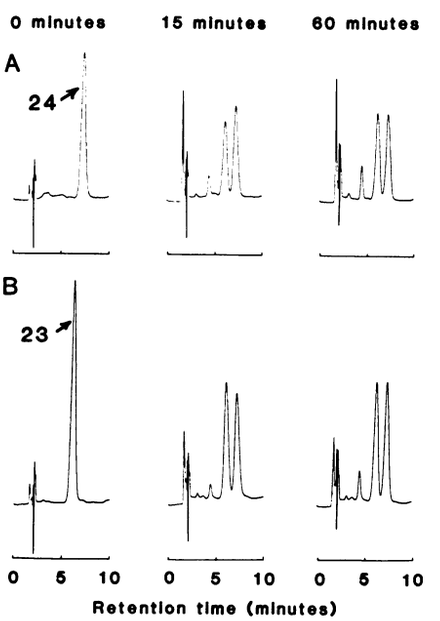


Fig. 6. HPLC analysis of epimerase reaction mixtures containing **23** and **24** as substrates. (A) Epimerase reaction mixture starting with **23**, sampled after 0, 15, and 60 minutes. (B) Epimerase reaction mixture starting with **24**, sampled after 0, 15, and 60 minutes.

Summary and Anticipated Developments

The original hypothesis of our work—namely, that the natural beta-lactam–forming processes can be used to achieve syntheses of unnatural antibiotics—has been confirmed for the penicillin-cephalosporin biosynthetic pathway. The exploitation of this pathway was a

logical first stage in the development of our strategy because the research of Arnstein (*38*) and Abraham (*41*) had already established that ACV was the natural peptide precursor of the penicillins and cephalosporins. We expect that the still unknown counterparts of ACV that represent the precursors of the carbapenems and clavulanic acid will be found eventually, together with the enzymes that promote the oxidative cyclizations of these precursors. An enzymatic synthesis of these structural types should then be feasible.

Industrial-scale production of the cyclase, epimerase, and expandase of the penicillin-cephalosporin pathway should also be feasible, either by (i) regulation of enzyme levels by carbon (*73*) or nitrogen (*74*) sources during the fermentation of the organisms; (ii) systematic screening of mutants of *S. clavuligerus* (*75*); or (iii) genetic engineering techniques (*76–79*), or combinations of these procedures.

It seems probable that Alexander Fleming's forecast (*4*) will continue to be operative: ". . . we are not at the end of the penicillin story. Perhaps we are only just at the beginning."

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