HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF NATURAL PRODUCTS. IV*

THE USE OF HPLC IN BIOSYNTHETIC STUDIES OF CEPHALOSPORIN C IN THE CELL-FREE SYSTEM**

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A new cepham metabolite has been isolated from the filtered broth of *Cephalosporium acremonium* by high performance liquid chromatography (HPLC) and identified as 7β-(5-D-amino-adipamido)-3β-hydroxy-3α-methyl-cepham-4α-carboxylic acid (**I**). Pure penicillin N was prepared using HPLC in the analytical mode. When **I** was added in place of penicillin N as substrate for the cell-free biosynthetic of cephalosporin, no formation of deacetoxycephalosporin C (**II**) was observed. A synthetic cepham derivative, 7β-(5-D-aminoadipamido)-3-exomethylene-cepham-4α-carboxylic acid (**III**) was also tested in the cell-free system as a possible intermediate. The compound **III** was shown to be an inhibitor of the ring expansion enzyme that converts penicillin N to deacetoxycephalosporin C.

We have shown earlier[3] that using HPLC one can isolate metabolites directly from the fermentation broth. Modification of appropriate stationary, and liquid phases, employed in the course of that first investigation, led to the isolation[1] of new tripeptides from the broth of *P. chrysogenum* (Fig. 1).

When the same system was applied to the present investigation of the broth of *C. acremonium* (Fig. 2), a new cepham derivative (**I**) was obtained. Its structure was suggestive of a possible relationship to deacetoxycephalosporin C (DAC, deacetoxy ceph C) (**II**) and, therefore, we examined its role in biosynthesis of DAC in a cell-free system derived from *C. acremonium*.

KOHSAKA and DEMAIN[4] were first to describe a cell-free system from *C. acremonium* CW-19 that converted penicillin N into a penicillinase-resistant cephalosporinase-sensitive material. YOSHIDA *et al.*,[5] have shown this compound to be **II** by paper electrophoresis and paper and TLC chromatography. More recently, in another cell-free system derived from *C. acremonium* mutant M-0198, this finding was further confirmed[6] using the same HPLC system we introduced in the examination of the broth of *C. acremonium*.[3] In all these experiments however impure penicillin N was used as the substrate for the cell-free synthesis of DAC. We are able to prepare essentially pure penicillin N for use in the cell-free experiment.

The process of the ring expansion from penicillin N to DAC was followed by injection into the HPLC system of aliquots of 50 microliters of the reaction mixture. The UV absorbance profile at 254 nm of authentic DAC was compared to profiles obtained from aliquots of the reaction mixture observed at 0 time, then at 20-minute intervals after addition of penicillin N to a total of 60 minutes. At that

* For paper III in this series see Reference 1.

** A portion of this work has been presented earlier by J. P. MCDERMOTT at the Second ASM Conference on Genetics and Molecular Biology of Industrial Microorganisms, Indiana University, Bloomington, Indiana, U.S.A., October 5~8, 1980[2]

Fig. 1. HPLC of *P. chrysogenum* broth.

Penicillin V production broth
△RI attenuation: 8×, Chart speed: 5 mm/minute,
Flow rate: 1 ml/minute, Column size: 4×300 mm,
Packing: μ-Bondapak C_{18},
Solvent: HCOOH-CH_3OH-H_2O (0.5:0.5:99),
P.S.I.: 1000

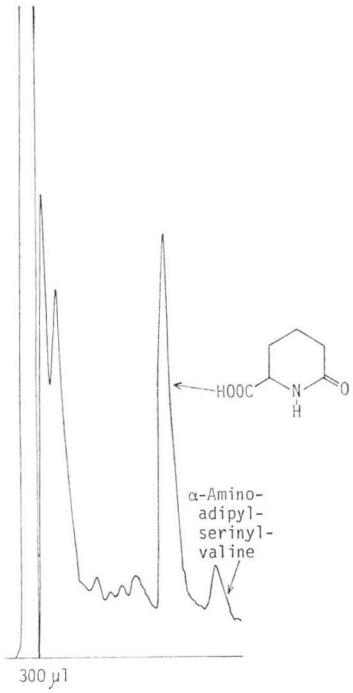
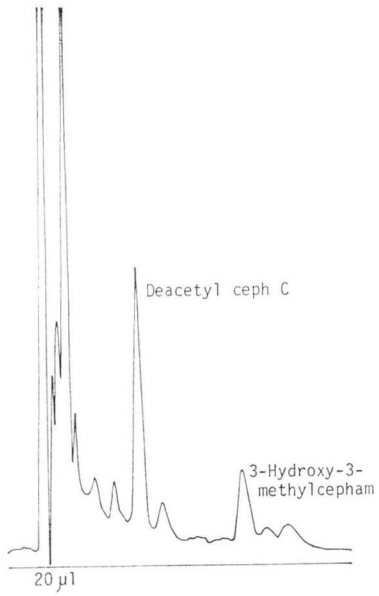
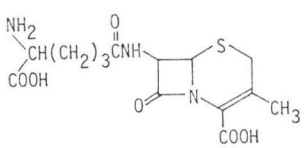


Fig. 2. HPLC of *C. acremonium* broth.

Ceph C production broth
△RI attenuation: 8×, Chart speed: 5 mm/minute,
Flow rate: 1 ml/minute, Column size: 4×300 mm,
Packing: μ-Bondapak C_{18},
Solvent: HCOOH-CH_3OH-H_2O (0.5:0.5:99),
P.S.I.: 600

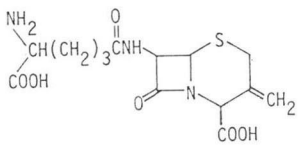


time the synthesis of DAC seemed to have stopped (Fig. 5). Boiling the cell extract prior to use in the cell-free experiment destroyed the ability of the extract to convert penicillin N to DAC (Fig. 6). Deletion of the cell-free extract from the reaction mixture prevented any formation of DAC from penicillin N (Fig. 7).



II

Deacetoxycephalosporin C



III

Exomethylenecephalosporin C

The new metabolite, 7β-(5-D-aminoadipamido)-3β-hydroxy-3α-methylcepham-4-carboxylic acid (**I**) was tested as a substrate for the synthesis of DAC. The reaction mixture and all conditions were identical as above except, of course, for the substitution of penicillin N by **I** at 0.9 mM concentration. This time there was no evidence whatever of the formation of **II** (Fig. 8). This can easily be explained

on the basis of the *cis* stereochemistry of OH and H at C-3 and C-4, respectively. Most hydratases eliminate H and OH from adjacent carbons when these elements are arranged in a *trans* configuration, *e.g.*, fumarate dehydratase.[7,8] Therefore, the new compound **I** is presumably a shunt metabolite. Its formation can be rationalized on the basis of earlier chemical work of KUKOLJA.[9] In accordance with his hypotheses, we suggest that an episulfonium ion may be involved in the formation of the 3β-hydroxycepham compound **I** and in the formation of deacetoxycephalosporin C. The episulfonium ion could arise by two alternative routes: a route involving penicillin N sulfoxide, or a route involving 2β-hydroxymethylpenicillin N. The 3β-hydroxycepham (**I**) could arise by direct attack on the episulfonium ion by hydroxide ion shown in Scheme 1, or by hydration of a carbonium ion depicted in Scheme 2. The ease of chemical transformation of 7β-(5-aminoadipamido)-3-exomethylene cepham-4α-carboxylic acid (**III**) to DAC (**II**) prompted us to test it as a substrate for penicillin N in the cell-free system. In a series of experiments conducted as above, no conversion to DAC was observed. Interestingly, when a 1 mM concentration of **III** was added to the complete reaction mixture containing 0.9 mM penicillin N the formation of DAC from penicillin N was inhibited.

Fig. 3. HPLC of an enriched fraction containing penicillin N.

Final purification of penicillin N
△RI attenuation: 16×, Chart speed: 5 mm/minute,
Flow rate: 1 ml/minute, Column size: 4×300 mm,
Packing: μ-Bondapak C_{18},
Solvent: Pyridine-AcOH-H_2O (0.4:0.4:99.2),
P.S.I.: 800

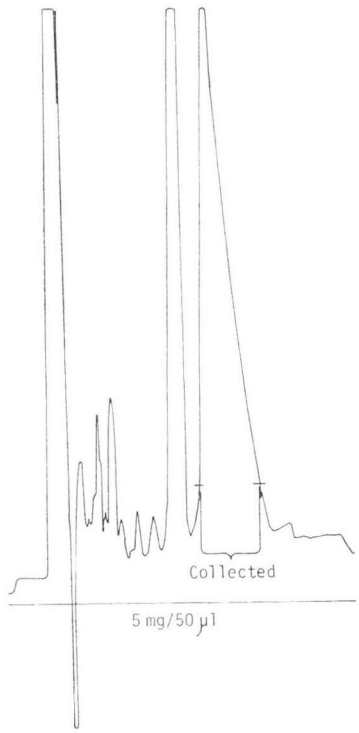


Fig. 4. NMR of penicillin N (in D_2O).

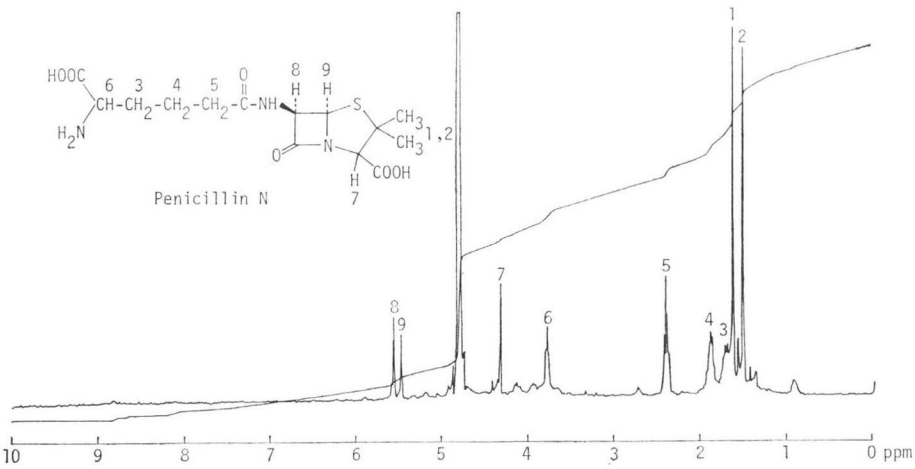


Fig. 5. Cell-free experiment with penicillin N.

(1) UV Range: 254 (0.2), Chart speed: 5 mm/minute, Flow rate: 4 ml/minute, Column size: 4×300 mm, Packing: μNH_2 (Dupont), Solvent: AcOH-CH_3OH-CH_3CN-H_2O (2:4:7.5:86.5), P.S.I.: 4500

(2) UV Range: 254 (0.2), Chart speed: 5 mm/minute, Flow rate: 4 ml/minute, Column size: 4×300 mm, Packing: μNH_2 (Waters), Solvent: AcOH-CH_3OH-CH_3CN-H_2O (2:4:7.5:86.5), P.S.I.: 4000

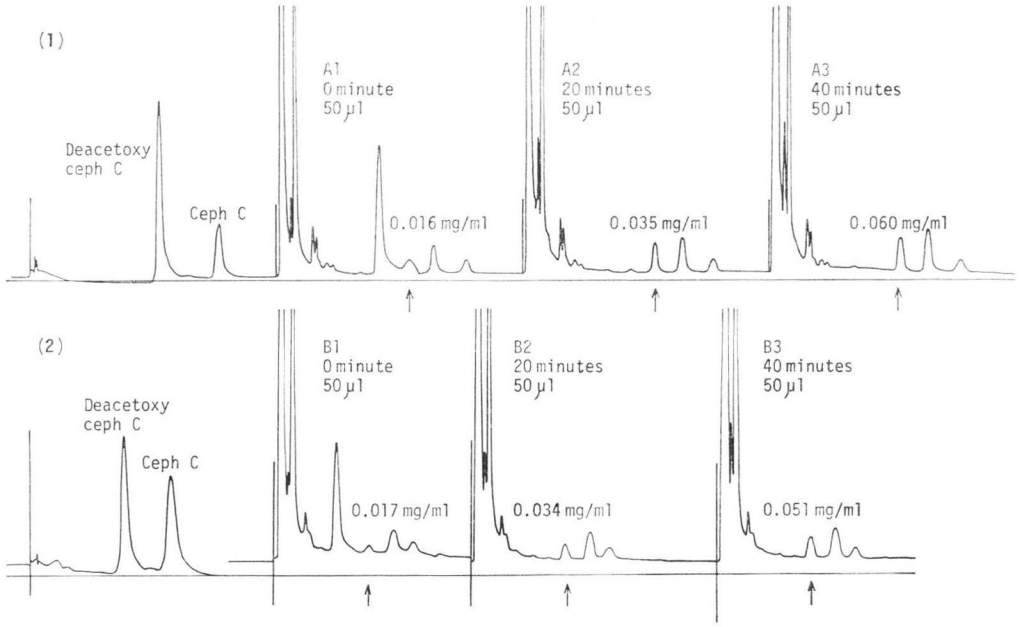


Fig. 6. Cell-free experiment with boiling cell extract.

UV Range: 254 nm, Chart speed: 5 mm/minute, Column size: 4×300 mm, Solvent: AcOH-CH_3OH-CH_3CN-H_2O (2:4:7.5:86.5), Flow rate: 4 ml/minute, Packing: μNH_2 (Waters), P.S.I.: 4500

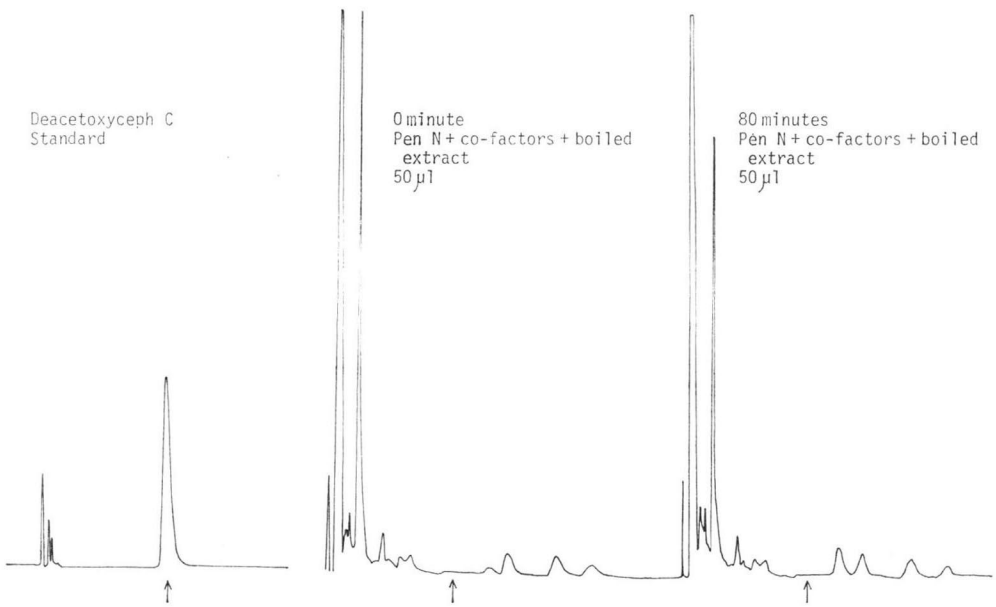


Fig. 7. Cell-free experiment without cell extract.

UV Range: 254 nm, Chart speed: 5 mm/minute, Flow rate: 4 ml/minute, Column size: 4×300 mm, Packing: μNH_2 (Waters), Solvent: AcOH-CH_3OH-CH_3CN-H_2O (2:4:7.5:86.5), P.S.I.: 4500

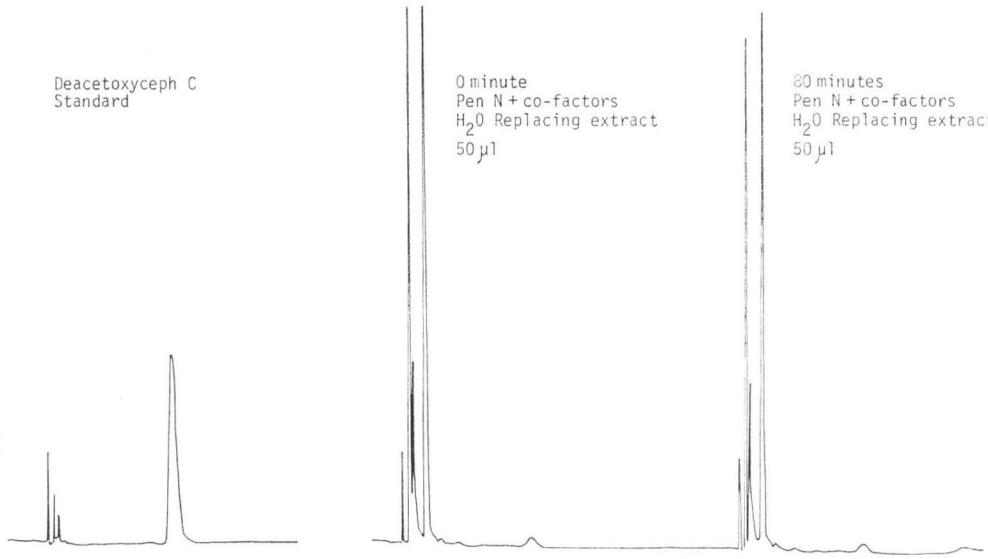
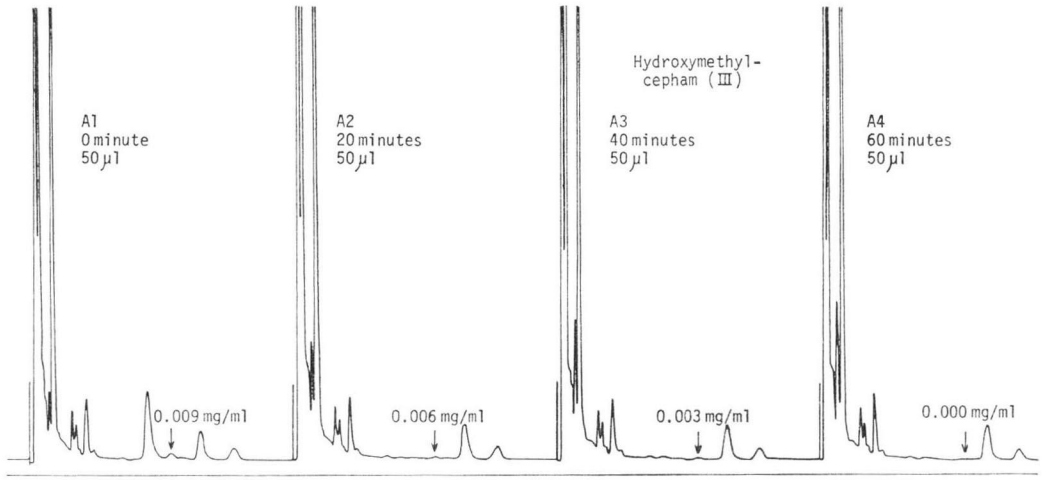


Fig. 8. Cell-free experiment with 3-CH_3-3-OH-cepham.

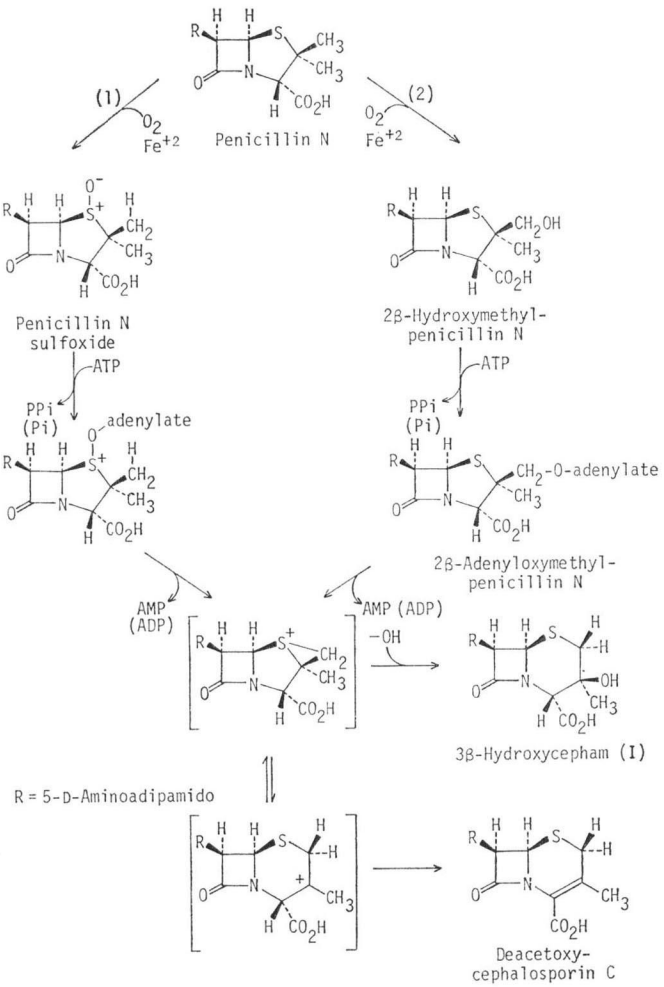
UV Range: 254 (0.2), Chart speed: 5 mm/minute, Flow rate: 4 ml/minute, Column size: 4×300 mm, Packing: μNH_2 (Dupont), Solvent: AcOH-CH_3OH-CH_3CN-H_2O (2:4:7.5:86.5), P.S.I.: 4500



Materials and Methods

All chromatograms were obtained using Waters M6000 pump, U6K septumless injector (Waters Associates, Milford, Mass.) with Schoeffel Model 770 UV detector (Schoeffel Instruments, Westwood, N.J.) and Fisher omniscribe recorder (Fisher Scientific, Cincinnati, Ohio). Spectrally pure solvents were obtained from Burdick E. Jackson, Muskegon, Mich., or J. T. Baker. Only deionized water

Scheme 1.

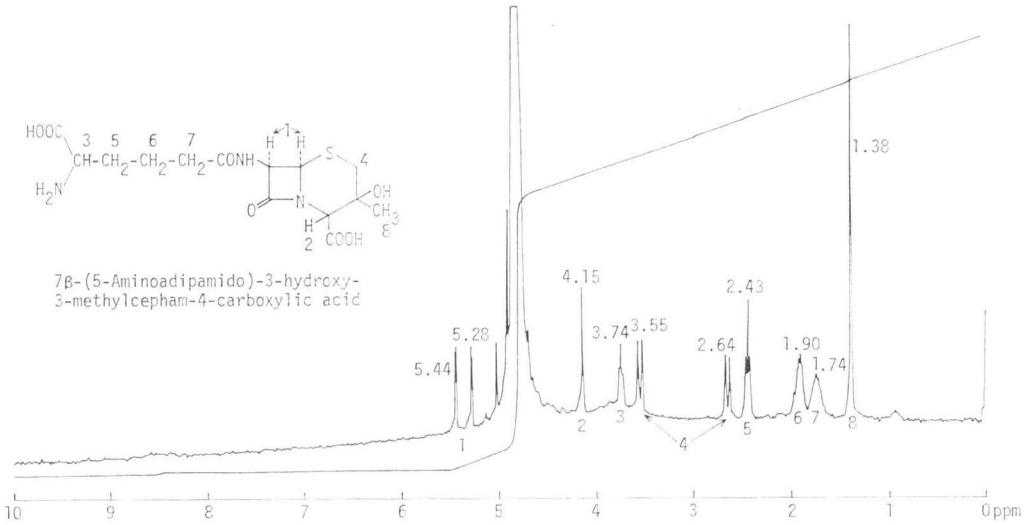


was used. When indicated, a differential refractometer (Waters Associates) was used. NMR spectra were recorded using a Bruker WH360 NMR spectrometer. Mass spectra were recorded using Varian-MAT Model 731 mass spectrometer. Preparative HPLC was performed using Waters Associates Prep LC/System 500 with Prep Pak 500 compression chamber.

Isolation of Penicillin N

Three hundred ml of filtered broth of *C. acremonium* (approx. 6 mg/ml of penicillin N) were chromatographed over 600 ml of slurried resin (Duolite ES861 nonfunctional resin) on a column (50×600 mm). As soon as the contents settled, the column was washed with 1,200 ml of water. Two column volumes were collected and the fractions lyophilized (A). Ten gram of this material were chromatographed on the Waters Prep LC/System 500 with one Prep Pak silica cartridge. An acetonitrile-water (80: 20) mixture was used as the mobile phase. Fractions were first selected on the basis of the agar disk assay using *P. solanaceareum* alone and with penicillinase. Those fractions which lost the antibacterial activity upon treatment with penicillinase were considered to be rich in penicillin N. Fractions that appeared to contain the largest amount of penicillin N with a smaller amount of DAC were immediately lyophilized. The typical yield of crude penicillin N was 750 mg from 10 g of crude "A" from lyophilized broth. Aliquots (15 mg) of the crude penicillin N fraction were then purified on the analytical

Fig. 9. NMR of 3-methyl-3-hydroxy-cepham (in D_2O).



Scheme 2.

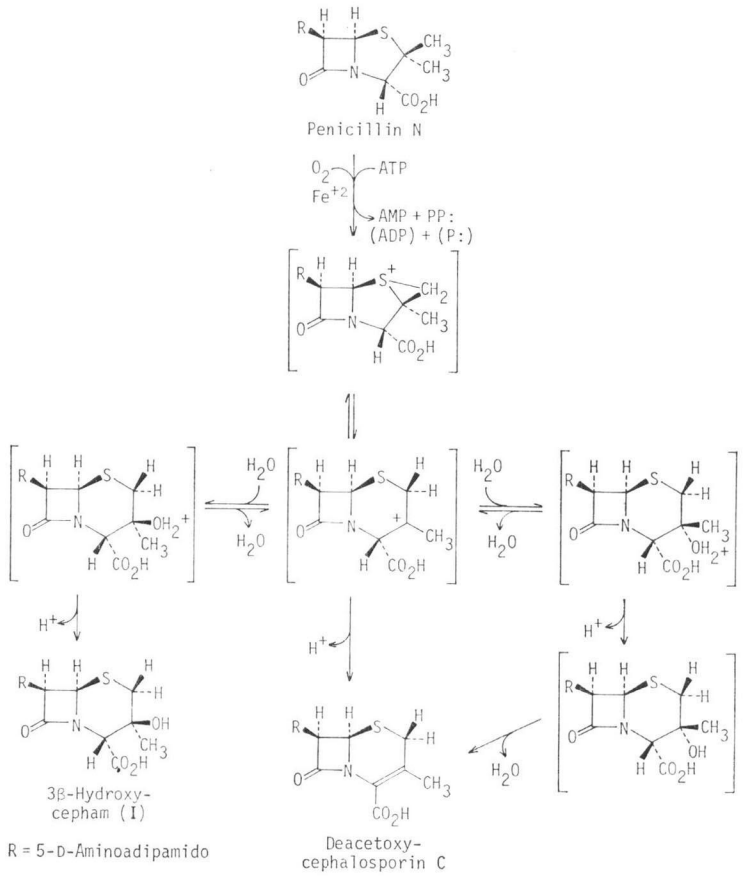
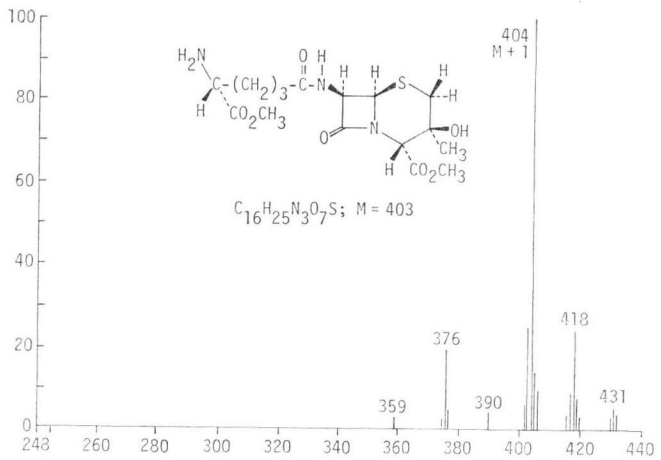
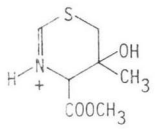


Fig. 10.

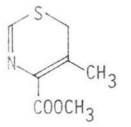
a. FD-Mass spectrum of methyl ester of 3-methyl-3-hydroxy-cepham.



b. Some fragments in the E.I. mass spectrum.



$C_7H_{12}NO_3S$
Calcd.: 190.0538
Found : 190.0538



$C_7H_9NO_2S$
Calcd.: 171.0346
Found : 171.0338

HPLC system shown in Fig. 3 and afforded 1~2 mg of penicillin N. (>90% pure, for NMR 360 MHz, see Fig. 9).

Monitoring the Cell-Free Biosynthesis Experiment by HPLC

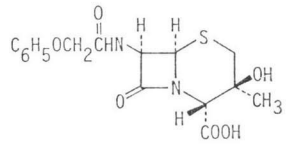
Each reaction mixture contained: cell-free extract, 2.5 ml (*ca.* 4 mg protein/ml), penicillin N, 0.9 mM; ferrous sulfate, 0.04 mM; ascorbic acid, 0.67 mM; ATP, 0.83 mM and Triton X-100, 0.1% in a final volume of 3.0 ml. The reaction was initiated by addition of enzyme. The final pH of each reaction mixture was 7.2. Reaction mixtures were shaken at 250 rpm at 25°C. Samples were withdrawn at 20 minutes intervals and rapidly filtered through cotton to remove particulates prior to injection. Aliquots of 50 μl were withdrawn from the clarified samples and were immediately injected onto the HPLC column. The UV (254 nm) absorbance profile of authentic DAC was compared to the profiles obtained from the clarified aliquots of the reaction mixture. The disappearance of penicillin N was monitored on the refractive index detector while the appearance of DAC was observed on both the refractive index and the UV 254 nm detectors. The identity of the DAC peak was established both by retention properties in HPLC and by isolating a sufficient quantity for an NMR spectrum.

A 500-ml Erlenmeyer flask containing 50 ml of fermentation medium was inoculated with 5 ml of a 72-hour vegetative culture of strain 92G-AD-11, a highly productive strain derived from strain M8650. Cells were harvested 10 hours after the onset of rapid cephalosporin C production by centrifugation of the whole broth at 3,500×*g* for 15 minutes. The supernatant layer was decanted and the lipid-like layer covering the cells was removed by a careful scraping to leave the cell layer undisturbed. The cell layer was then lifted away from the insoluble medium constituents. One gram of the cell paste was washed twice in 10 ml of ice cold water. During each wash step the cells were resuspended throughly to insure efficient washing and then were centrifuged at 3500×*g* for 15 minutes. Washed cells were resuspended in 0.05 M tris buffer, pH 8, containing 0.01M $MgSO_4$, 0.01 M KCl, 2% bovine serum albumin, and 1,000 units/ml Traysylol. Two milliliters of buffer were used for each gram of damp mycelia. Extracts were prepared by milling the cell suspensions for 2 minutes (B. Braun, Melsungen AG, Type 85 3030). Cell debris were removed by centrifugation (5,000×*g*, 10 minutes). The supernatants were filtered through 0.45 μ Nalgene Filter Units (Nalge Company, Rochester, New York) to remove any whole cells. None were observed microscopically in the filtrate. The filtered supernatants were used immediately in the cell-free reactions.

Isolation of 7β-(5-D-Aminoadipamido)-3β-hydroxy-3α-methylcepham-4α-carboxylic acid (I) from the Fermentation Broth of *C. acremonium*

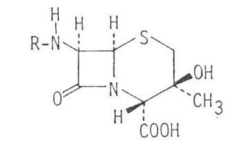
Material corresponding to the peak shown in Fig. 2 was collected through several passages of the

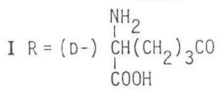
broth through the HPLC system and rechromatographed in the same system to obtain a homogeneous material. Its NMR spectrum (shown in Fig. 9) was identical with the spectrum of the synthetic material[10]. In addition, the dimethyl ester prepared from the substance by reaction with diazomethane gave a satisfactory molecular ion in the FD spectrum. Calcd. for $C_{16}H_{25}N_3O_7S$=403; Found: M+1=404 (Fig. 10a). In the EI spectrum there were two well-defined fragments (Fig. 10b) indicative of cepham nucleus of the type present in structure IV[11] synthesized several years ago in our laboratories.

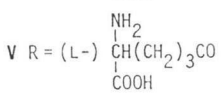


IV

Hydroxycepham of GUTOWSKI



I R = (D-) 



V R = (L-)

I. D-Hydroxycepham
V. L-Hyroxycepham

Acknowledgments

We thank Dr. D. DORMAN for the interpretation of the NMR spectra, Mr. J. OCCOLOWITZ for the mass spectra, and Dr. G. M. MACIAK for several oxidation experiments using aminooxidases. We also thank Mr. J. CHAPMAN for biosynthetic experiment using a cell-free extract. We gratefully acknowledge a sample of synthetic D-6-oxopiperidine-2-carboxylic acid from Dr. R. P. ELANDER of Bristol-Myers Company.

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