HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF NATURAL PRODUCTS V[1]

THE USE OF HPLC IN THE CELL-FREE BIOSYNTHETIC CONVERSION OF α-AMINOADIPYL-CYSTEINYL-VALINE (LLD) INTO ISOPENICILLIN N

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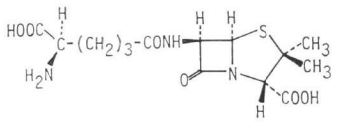
(Received for publication January 11, 1982)

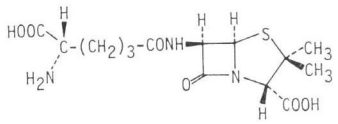
Isopenicillin N (**I**) and penicillin N (**II**) were separated effectively by reversed phase HPLC as derivatives of chiral 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (**III**). This permitted identification of isopenicillin N isolated by HPLC during a biosynthetic cell-free experiment using α-L-aminoadipyl-L-cysteinyl-D-valine (**IV**) as precursor.

The utility of HPLC in the course of the biosynthetic cell-free conversion of penicillin N (**II**) to deacetoxy cephalosporin C (**V**) has been recently reported from the Lilly Research Laboratories.[1] We now wish to describe yet another example of the application of HPLC in biosynthetic studies.

The biosynthetic cell-free cyclization reaction of α-L-aminoadipyl-L-cysteinyl-D-valine (**IV**) (ACV, ARNSTEIN's tripeptide) into isopenicillin N (**I**) has been shown independently by two groups.[2,3] When we carried out this reaction, and subjected the reaction mixture at different time intervals to HPLC analysis, we isolated a few micrograms of isopenicillin N (**I**) (Fig. 1). The antibiotic was formed in the course of cyclization from the tripeptide (**IV**), and its formation could be observed using the same HPLC system described earlier[1] for the isolation of penicillin N (reversed phase C-18 Microbondapak, pyridine - acetic acid - water, 0.4: 0.4: 99.2). The collected antibiotic was then reacted with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC, **III**). This reagent and the corresponding arabinosyl derivative, have been recently reported to be useful in the HPLC separation of enantiomeric amino acids.[4] Isopenicillin N isolated in our experiment was reacted with the chiral reagent to give reaction



I



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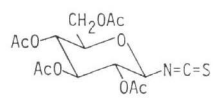
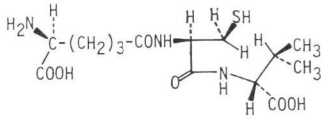
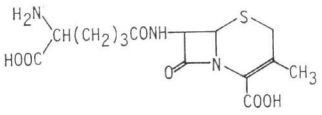


III



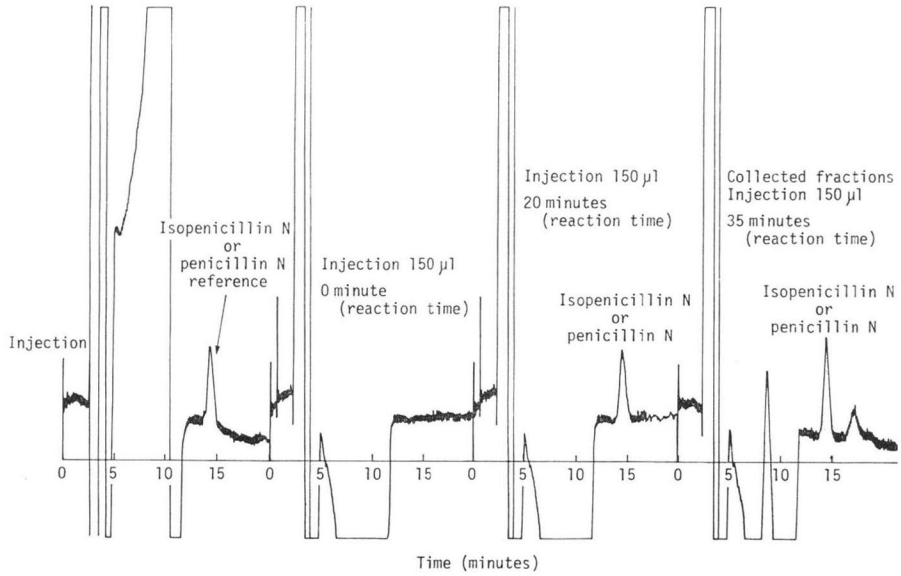
IV

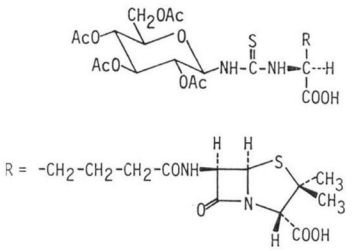


V

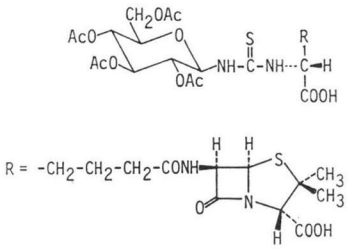
Fig. 1. HPLC of the cell-free biosynthetic experiment with ACV.

ΔRI attenuation: 1×, chart speed 5 mm/minute. Flow rate: 1.0 ml/minute. Column size: 4×300 mm. Packing: Microbondapak C-18 (Waters). Solvent: Pyridine - AcOH - H_2O (0.4: 0.4: 99.2). Pressure: ~70 Kg/cm^2.





VIa



VIb

product **VIa**. When the latter was injected into the HPLC system (Microbondapak C-18, methanol - acetonitrile - acetic acid - water, 36: 7: 2: 55), it had an identical retention time as an authentic sample of isopenicillin N reacted with GITC (Fig. 2). This sample of isopenicillin N was isolated by HPLC from an enriched fraction obtained from the broth of *Penicillium chrysogenum* (Fig. 3) analogously to procedures used for the preparation of penicillin N from *Cephalosporium acremonium* fermentation.[1] The NMR spectrum indicated a purity of 90~95% as judged by integration (Fig. 4). Both penicillins, when derivatized with GITC, have distinctly different retention times and can be separated easily when present as a mixture (Fig. 5).

Materials and Methods

All chromatograms were obtained using M6000 A pump, U6K septumles injector, Model 440 Absorbance Detector with absorbance expressed in absorbance units full scale, (A.u.f.s.), Differential Refractometer R401, all from Waters Associates, Milford, Mass. In addition, a Fisher Omniscribe strip chart recorder (Fisher Scientific, Cincinnati, Ohio) was used. Spectrally pure solvents were obtained

Fig. 2. HPLC of the GITC reaction product of isopenicillin N from the biosynthetic cyclization experiment.

All conditions are the same as in Fig. 5 with the exception of sensitivity of 0.02 A.u.f.s. in this experiment.

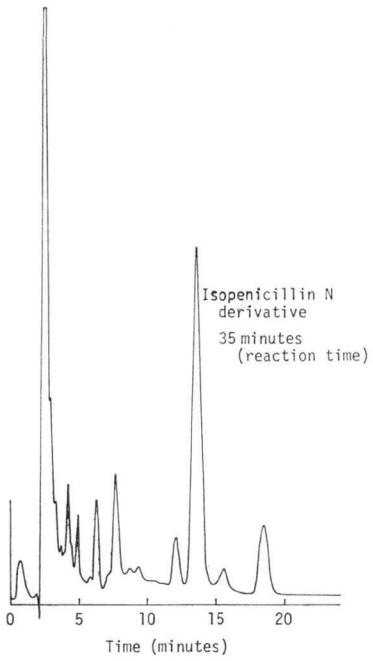


Fig. 3. Final purification of isopenicillin N.

ΔRI Attenuation: 16×. Chart speed: 5 mm/minute. Flow rate: 1 ml/minute. Column size: 4×300 mm. Packing: Microbondapak C-18 (Waters). Solvent: Pyridine - AcOH - H_2O (0.4: 0.4: 99.2). Pressure: 56 Kg/cm^2.

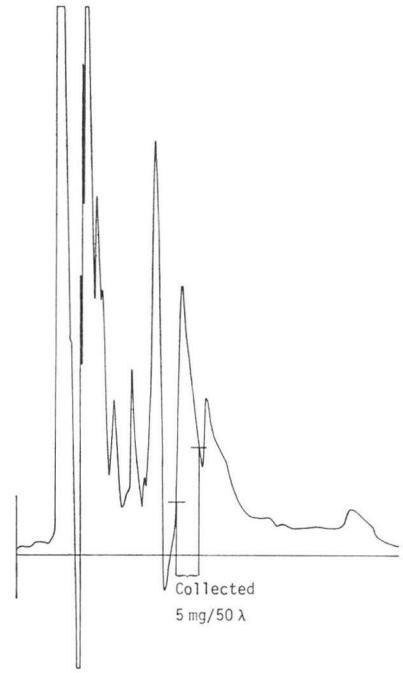
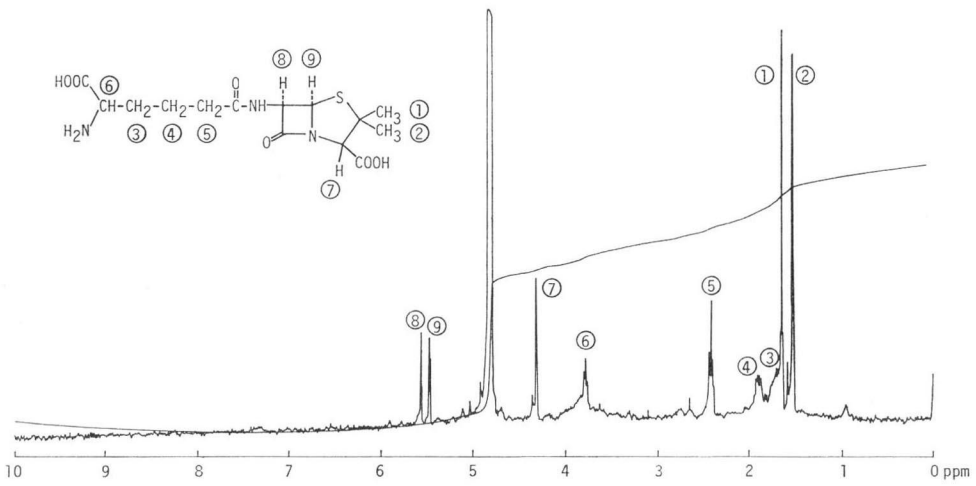


Fig. 4. NMR spectrum of isopenicillin N in D_2O.

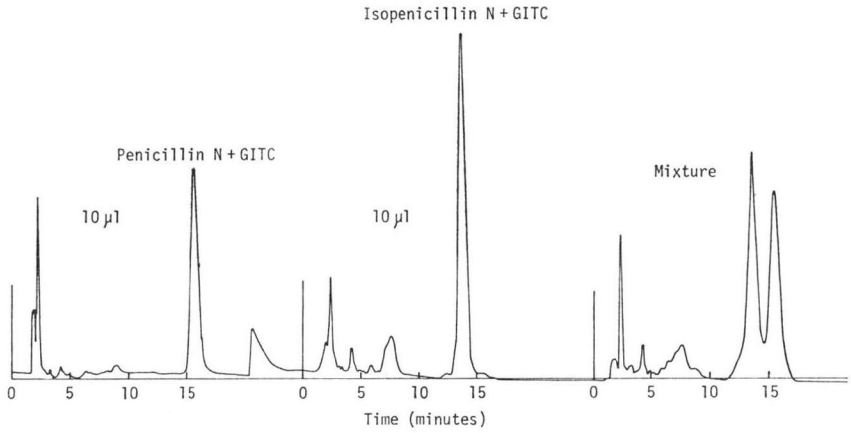


from Burdick E. Jackson, Muskegon, Mich. or J. T. Baker. Only deionized and glass distilled water was used. NMR spectra were recorded using a Bruker WH360 NMR spectrometer.

HPLC column characteristics for isopenicillin N derivative are as follows:

Fig. 5. HPLC of GITC reaction products of penicillin N and isopenicillin N; separation of a mixture of the two compounds.

Chart speed: 5 mm/minute. Flow rate: 1.2 ml/minute. Column size: 4×300 mm. Packing: Microbondapak C-18 (Waters). Solvent: MeOH - CH_3CN - HOAc - H_2O (36:7:2:55). Pressure: 140 Kg/cm². UV range: 254 nm, 0.2 A.u.f.s. for penicillin N+GITC; 0.1 A.u.f.s. for isopenicillin N+GITC, and the mixture.



The selectivity factor $\alpha = \frac{\text{K' penicillin N derivative}}{\text{K' isopenicillin N derivative}}$

$\alpha = 1.17$

The capacity factor K' for the isopenicillin N derivative:

$\frac{t_R^1 - t_0}{t_0} = 4.95$ $\quad t_R^1 = 65.5$ mm

$t_R^2 = 74.5$ mm

$t_0 = 11$ mm

K' penicillin N derivative $= \frac{t_R^2 - t_0}{t_0} = 5.77$

The effective theoretical plates for the isopenicillin N derivative:

$$N = 16\left(\frac{t_R^1}{W}\right)^2 \qquad W = 6.5 \text{ mm}$$

$$= 1{,}626$$

$$N_{eff} = N\left(\frac{K'}{1+K'}\right)^2$$

$$= 1{,}125$$

Monitoring the Cell-Free Biosynthesis Experiment by HPLC

Each reaction mixture contained 850 μl of cell-free extract* (prepared from strain CW19 of *Cephalosporium acremonium* by sonication procedure, and held at −70°C until reaction time), 100 μl of precursor (1 mg of ACV disulfide and 1 mg of DTT were dissolved in 1 ml of tris buffer at pH 7.2 and allowed to react for 15 minutes at room temperature) and finally 50 μl of cofactors (10 mg $FeSO_4$ + 28 mg ascorbic acid in 10 ml of tris buffer at pH 7.2). Tris buffer was prepared by adding 0.05 M of Trizma Base (reagent grade, Sigma), 0.01 M KCl, 0.01 M $MgSO_4$ and adding 6 N HCl to pH 7.2. Flasks were kept on ice until the reaction was started by the addition of precursor; a 100 μl sample was withdrawn, diluted 1:1 with methanol and held for bioassay. The reaction mixture was incubated at 25°C on a shaker set at 250 rpm. Samples of 150 μl were withdrawn at 0-, 20- and 35-minute intervals and injected into the HPLC system. A refractive index detector was used to monitor the appearance of an isopenicil-

* The cell-free extract of CW 19 was prepared by J. KUPKA and A. L. DEMAIN at M. I. T. Details of this work will be published by these authors elsewhere.

lin N peak after first injecting into the system 5 μg of an authentic sample of the antibiotic for determination of its retention time (Fig. 1). The peak from the cell-free biosynthesis experiment was collected and lyophilized. The area of the peak corresponded to approximately 5 μg of antibiotic. The process of cyclization of ACV into penicillin was followed by a weak UV absorbance profile at 254 nm and a corresponding strong peak resulting from differential refractive index detection. These peaks had a identical retention time with those obtained from authentic samples of isopenicillin N using both detection systems.

Preparation of GITC (2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate, **III**)

This compound was synthesized by reacting α-acetobromglucose with silver thiocyanate according to NIMURA, OGURA and KINOSHITA.[4]

Isolation of Isopenicillin N (**I**)

A crude sample of isopenicillin N, containing approximately 10% of the antibiotic from the fermentation of *P. chrysogenum*, was subjected to analytical HPLC (Fig. 3), and the appropriate peak was collected and lyophilized. The NMR spectrum (Fig. 4) indicated that the compound was approximately 90~95% pure (integration).

Derivatization of Penicillin N and Isopenicillin N, Formation of **VIa** and **b**

Stock solutions of antibiotics were made in a concentration of 1 mg/kg in a mixture of acetonitrile - water (1: 1) with addition of sodium bicarbonate to pH 8.5. GITC was used in a concentration of 2 mg/ml in acetonitrile. Both were mixed in a ratio of 1:1 and allowed to react for 35 minutes. The same procedure was used for the lyophilized peak from HPLC of the cell-free reaction mixture.

Synthesis of δ-(L-α-Aminoadipyl-L-cysteinyl-D-valine) Disulfide (**IV**)

The tripeptide disulfide was prepared according to procedures described by WOLFE and JOKINEN.[5]

Acknowledgements

We thank the following members of Lilly Research Laboratories for their help. Dr. ROBERT HAMILL, a sample of crude isopenicillin N from *P. chrysogenum* fermentation; Messrs. PAUL PRANC and CHARLES S. CAMPBELL, preparation of ACV disulfide.

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