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Analysis of Penicillin N Ring Expansion Activity from *Streptomyces clavuligerus* by Ion-Pair High-Pressure Liquid Chromatography

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An ion-pair, reversed-phase, high-pressure liquid chromatographic method for the analysis of penicillin N ring expansion activity has been developed which allows simultaneous measurement of both substrate and product. The high-pressure liquid chromatography conditions were as follows: stationary phase, C_{18}; flow rate, 2 ml/min; detection, 220 nm. The stationary phase was preconditioned with 4.5 mM tetrabutylammonium bromide in 0.05 M KH_2PO_4 (pH 4.0)–methanol (85:15, vol/vol) and then equilibrated with 0.06 mM tetrabutylammonium bromide in 0.05 M KH_2PO_4 (pH 4.0)–methanol (95:5, vol/vol) for analysis of reaction mixtures. These conditions separated authentic samples of penicillin N and desacetoxycephalosporin C and allowed cell-free studies of the ring expansion of penicillin N to desacetoxycephalosporin C by a partially purified enzyme from *Streptomyces clavuligerus* to be followed conveniently.

The microbial conversion of penicillin N to desacetoxycephalosporin C (A → B) (Fig. 1) is catalyzed by an oxidative ring expansion enzyme which requires α-ketoglutarate as a cosubstrate. This reaction has been demonstrated in cell-free extracts from both the cephalosporin C-producing fungus *Cephalosporium acremonium* (3, 7, 8) and the cephamycin C-producing bacterium *Streptomyces clavuligerus* (4). Ring expansion activity has typically been monitored in a microbiological assay system which follows the formation of penicillinase-resistant (desacetoxycephalosporin C) antibiotic. Ring expansion activity has also been demonstrated by high-pressure liquid chromatography (HPLC) in cell-free reaction mixtures from *C. acremonium* (8a, 9, 10). The HPLC system relies on the detection of UV light-absorbing compounds at 254 nm. Since this wavelength is close to the 260-nm absorption maximum of the cephalosporin and well removed from the end absorption exhibited by the penicillin, the method primarily detects cephalosporin. Recently, an HPLC system has been developed for the determination of clavulanic acid, a β-lactam compound which, like penicillin N, has low absorption at 254 nm (1). This latter method employs a UV light-transparent mobile phase containing the ion-pairing reagent tetrabutylammonium bromide (TBAB), which allows good retention and detection of clavulanic acid.

The present work describes a modification of this ion-pair–HPLC system which makes possible the separation of penicillin N from desacetoxycephalosporin C on a standard reverse-phase column. The UV light transparency of the mobile phase also permits the simultaneous detection of both compounds at 220 nm.

MATERIALS AND METHODS

Materials. Penicillin N was synthesized chemically as described previously (4). Desacetoxycephalosporin C and desacetylcephalosporin C standards were gifts from D. Hook, Bristol Laboratories, Syracuse, N.Y. TBAB was purchased from Sigma Chemical Co., St. Louis, Mo. DEAE-Trisacryl was purchased from LKB Instruments, Inc., Rockville, Md.

Media and culture conditions. The media and conditions used to grow *S. clavuligerus* NRRL 3585 have been described previously (5). Cells were harvested after 40 h of growth by filtration through Whatman no. 2 filter paper. The cells were washed once with TDE buffer (0.05 M Tris-hydrochloride buffer [pH 7.0]–0.1 mM dithiothreitol–0.01 mM EDTA) and then resuspended to 1/10 of the original culture volume.

Ring expansion enzyme preparations. Washed cell suspensions of *S. clavuligerus* were disrupted by sonication for 2 × 15 s at a maximum intensity of 300 W (Biosonic III; Bronwill Scientific Inc., Rochester, N.Y.). Broken cell suspensions were centrifuged for 30 min at 27,000 × *g* to separate cell-free extract from cell envelopes and unbroken cells.

Crude ring expansion enzyme was prepared by the gradual addition of streptomycin sulfate to the cell-free extract to a final concentration of 1% (wt/vol). The supernatant resulting from centrifugation at 17,000 × *g*

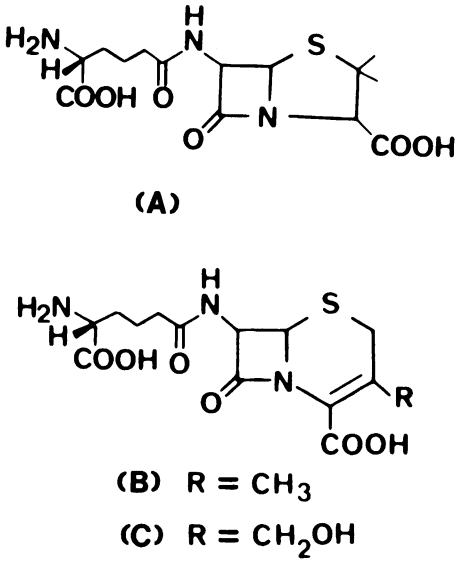

FIG. 1. Structures of penicillin N (A), desacetoxycephalosporin C (B), and desacetylcephalosporin C (C).

for 15 min was supplemented with solid ammonium sulfate to 40% saturation. After centrifugation was carried out as described above, additional ammonium sulfate was added to the supernatant to give 70% saturation. The pellet recovered from centrifugation was resuspended to 1/100 of the original culture volume in TDE buffer and then washed twice by ultrafiltration (PM-10 filter; Amicon Corp., Lexington, Mass.).

Partially purified ring expansion enzyme was prepared from crude enzyme by a modification of the ion-exchange chromatographic procedure described by Kupka et al. (8). Crude enzyme (2.5 ml) was applied to a DEAE-Trisacryl column (1.6 by 15 cm) which was equilibrated with TDE buffer. The column was washed with 50 ml of TDE buffer and eluted with a linear gradient of 150 ml each of normal TDE buffer and TDE buffer containing 0.4 M Tris-hydrochloride. Fractions of 50 drops (2.5 ml) were collected, and ring expansion activity was located by assay of 0.3-ml samples. Ring expansion enzyme-containing fractions were pooled, concentrated to 2.5 ml, and washed with TDE buffer by ultrafiltration.

Ring expansion assay system. Ring expansion activity was measured in reaction mixtures containing 0.48 mM penicillin N (actual concentration as determined by the chemical assay of Holm [2]), 2.8 mM sodium ascorbate, 0.045 mM $FeSO_4$, 1 mM α-ketoglutarate, 7.5 mM KCl, 7.5 mM $MgSO_4$, 0.05 M Tris-hydrochloride buffer (pH 7.0), and 0.05 to 0.30 ml of crude or partially purified enzyme in a final volume of 0.4 ml. Reaction mixtures were incubated at 20°C, and reactions were terminated by the addition of 0.4 ml of methanol. Reaction mixtures were then centrifuged for 5 min at 12,000 × *g* and analyzed by either thin-layer chromatography or HPLC.

Thin-layer chromatography of reaction mixtures and standards. Five-microliter amounts of ring expansion reaction mixtures prepared with crude or partially purified ring expansion enzyme were spotted onto a cellulose thin-layer chromatography sheet (Eastman Kodak Co., Rochester, N.Y.). Desacetoxycephalosporin C (0.05 μg) and desacetylcephalosporin C (0.05 μg) were also applied to the cellulose sheet. The chromatogram was developed in *n*-butanol–acetic acid–water (3:1:1), dried thoroughly, and then placed face-down on an agar slab inoculated with *Escherichia coli* Ess and containing penicillinase at 2 × 10^5 U/ml. *E. coli* Ess is a supersensitive indicator organism which was generously provided by A. L. Demain, Massachusetts Institute of Technology, Cambridge. After 30 min at 21°C, the cellulose sheet was removed and the agar slab was incubated overnight at 37°C. The agar slab was then flooded with 5% trichloroacetic acid to improve visualization of the inhibition zones.

HPLC of reaction mixtures and standards. The equipment used for HPLC consisted of an M6000A

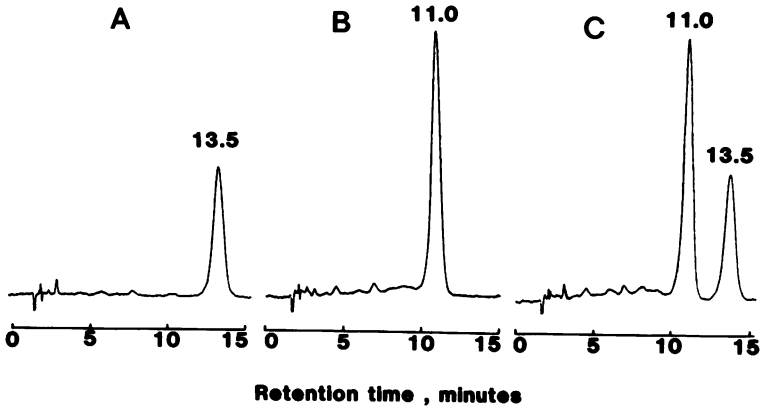

FIG. 2. Analysis by HPLC of standard solutions of penicillin N and desacetoxycephalosporin C. Weighed amounts of each standard were dissolved in water to give a final concentration of 0.1 mg/ml. Twenty-microliter amounts of penicillin N (A), desacetoxycephalosporin C (B), and a mixture of the two (C) were analyzed by HPLC.

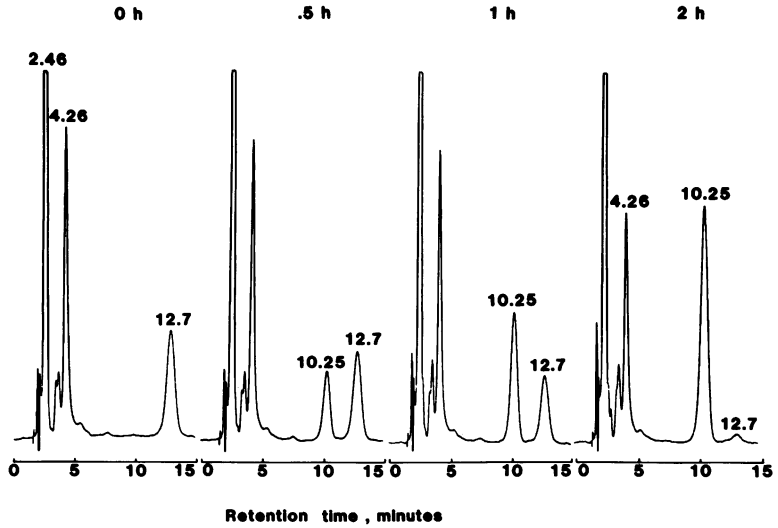

FIG. 3. Analysis by HPLC of ring expansion reaction mixtures containing partially purified enzyme. Ring expansion reaction mixtures were sampled after 0, 0.5, 1.0, and 2.0 h, and 20-μl amounts were analyzed by HPLC.

pump, a UK-6 injector, an M-480 variable wavelength detector, an M-420 data module, and a C_{18} column (Rad Pak A in a Z module). All equipment was obtained from Waters Scientific Ltd., Mississauga, Ontario, Canada. The stationary phase was preconditioned with 60 ml of 4.5 mM TBAB in 0.05 M KH_2PO_4 (pH 4.)–methanol (85:15, vol/vol) at 2 ml/min and then equilibrated with 0.06 mM TBAB in 0.05 M KH_2PO_4 (pH 4.0)–methanol, (05:5, vol/vol) for analysis of the samples. When equilibrium was reached, 20-μl amounts of reaction mixtures and standard solutions were analyzed at a flow rate of 2 ml/min with detection at 220 nm and 0.02 absorbance units full scale.

RESULTS

HPLC analysis of standards. We have recently developed an HPLC system for the analysis of ring cyclization activity in cell-free systems from *S. clavuligerus* (6). When ring expansion reaction mixtures were analyzed with this HPLC system (mobile phase, 0.05 M KH_2PO_4 [pH 4.0]–methanol [95:5, vol/vol]), penicillin N was not separated from desacetoxycephalosporin C, and both compounds eluted with a retention time of about 5 min. Incorporation of the ion-pairing agent TBAB into the mobile phase at a final concentration of 0.06 mM increased the retention time for both compounds and separated the penicillin from the cephalosporin (Fig. 2). Twenty-microliter amounts of standard solutions or mixtures at 0.1 mg/ml were analyzed by HPLC. Desacetoxycephalosporin C eluted with a retention time of 11.0 min, whereas penicillin N eluted at 13.5 min. Complete baseline separation of mixtures of these two compounds was routinely achieved with this system.

HPLC analysis of ring expansion reaction mixtures containing partially purified enzyme. Ring expansion reaction mixtures could be analyzed conveniently with this HPLC system. Reaction mixtures were prepared with 0.2 ml of partially purified ring expansion enzyme (protein concentration, 2.72 mg/ml). Samples were taken at intervals and inactivated with methanol, and 20-μl amounts were analyzed by HPLC (Fig. 3). Reaction mixtures showed a peak at 10.25 min, owing to desacetoxycephalosporin C, which increased in area during the 2-h incubation period. The penicillin N peak at 12.7 min showed a corresponding decrease in area. Two additional peaks seen in all reaction mixtures and controls were identified as α-ketoglutarate (4.26 min) and ascorbate (2.46 min).

The actual concentration of the penicillin N substrate used in ring expansion reaction mixtures was determined by chemical assay and used, together with the peak areas, to calculate the concentration of penicillin N remaining at

TABLE 1. Quantitation of penicillin N in ring expansion reaction mixtures

Sample time (h)	Penicillin N peak area (μV/s)	Penicillin N concn (mM)	Peak area (nmol)
0	1,303,210	0.240[a]	271,502
0.5	1,007,405	0.186[b]	
1.0	736,541	0.136[b]	
2.0	198,008	0.036[b]	

[a] Determined by chemical assay of stock penicillin N solution.
[b] Calculated from the penicillin N peak area.

each sample time (Table 1). Penicillin N decreased in concentration from 0.240 mM at 0 time to 0.036 mM at 2 h, representing a disappearance of 85% of the penicillin N originally present at 0 time.

The authentic desacetoxycephalosporin C standard used in this study was of unknown purity and was not available in sufficiently large amounts for determination by chemical assay. Therefore, it was not possible to determine directly the amount of desacetoxycephalosporin C produced by comparison with a known standard. However, the assumption that penicillin N is quantitatively converted to desacetoxycephalosporin C allowed the concentration of desacetoxycephalosporin C to be determined by difference for each sample time (Table 2). Thus, desacetoxycephalosporin C increased in concentration from 0 mM at 0 time to 0.204 mM at 2 h. Calculation of the peak area per nanomole for desacetoxycephalosporin C gave results which varied by less than 2% for the three sample times.

HPLC analysis of ring expansion reaction mixtures containing crude enzyme. When ring expansion reaction mixtures were prepared with 0.1 ml of crude ring expansion enzyme (47.0 mg of protein per ml) instead of partially purified enzyme, the HPLC profiles were more difficult to interpret (Fig. 4). The 0 time sample (data not shown) gave an HPLC profile similar to that seen in Fig. 3 (0 time). After 1 h of incubation, the penicillin N had decreased markedly, but the desacetoxycephalosporin C peak was much smaller than that anticipated for the quantitative conversion of penicillin N to desacetoxycephalosporin C. After 2 h of incubation, the penicillin N peak had almost disappeared, but the desacetoxycephalosporin C peak remained small. The small size of these desacetoxycephalosporin C peaks was presumed to be due to the further conversion of desacetoxycephalosporin C to desacetylcephalosporin C. Previous studies have

TABLE 2. Quantitation of desacetoxycephalosporin C in ring expansion reaction mixtures

Sample time (h)	Desacetoxycephalosporin C peak area (μV/s)	Desacetoxycephalosporin C concn (mM)	Peak area (nmol)
0	0	0	
0.5	633,023	0.054[a]	586,132[b]
1.0	1,213,371	0.104[a]	583,351[b]
2.0	2,365,832	0.204[a]	579,861[b]

[a] Calculated by difference from the penicillin N concentration.

[b] Calculated from the peak area (as measured by HPLC) divided by the desacetoxycephalosporin C concentration (as determined by difference from the penicillin N concentration).

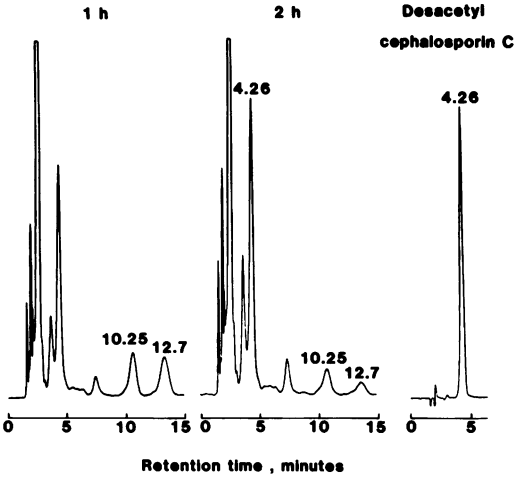

FIG. 4. Analysis by HPLC of ring expansion reaction mixtures containing crude enzyme and comparison with authentic desacetylcephalosporin C. Ring expansion reaction mixtures were sampled after 1 and 2 h, and 20-μl amounts were analyzed by HPLC. Desacetylcephalosporin C was dissolved in water to give a final concentration of 0.1 mg/ml, and 20 μl was analyzed by HPLC.

shown that desacetoxycephalosporin C undergoes oxidation to desacetylcephalosporin C (Fig. 1C) by an oxidase found in crude enzyme preparations (4). When authentic desacetylcephalosporin C was analyzed by HPLC, the material eluted with a retention time of 4.26 min, the same as α-ketoglutarate. Therefore, in ring expansion reaction mixtures, any desacetylcephalosporin C formed would comigrate with and be obscured by α-ketoglutarate. When HPLC analysis was carried out by detection at 260 nm to accentuate the presence of cephalosporin and diminish the effect of α-ketoglutarate, a large peak at 4.26 min remained (data not shown).

Thin-layer chromatography of ring expansion reaction mixtures. Ring expansion reaction mixtures were analyzed by thin-layer chromatography to confirm that desacetoxycephalosporin C underwent further oxidation to desacetylcephalosporin C in crude enzyme-containing reaction mixtures. Five-microliter amounts of 2-h reaction mixtures and samples of authentic desacetoxycephalosporin C and desacetylcephalosporin C (0.05 μg each) were spotted on cellulose thin-layer chromatography sheets. Chromatograms were developed and bioassayed in the presence of penicillinase. Reaction mixtures containing partially purified ring expansion enzyme produced only desacetoxycephalosporin C (Fig. 5). In contrast, the major antibiotic seen in crude enzyme-containing reaction mixtures was desacetylcephalosporin C.

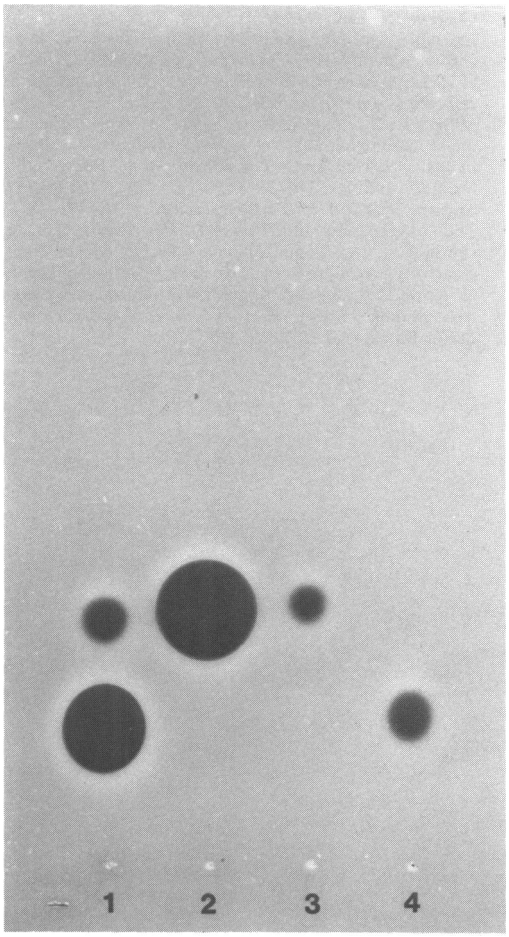

FIG. 5. Examination of antibiotic products of ring expansion reaction mixtures by thin-layer chromatography. A cellulose thin-layer chromatography sheet was spotted with the following samples: 5 μl of ring expansion reaction mixture containing crude enzyme and sampled at 2 h (lane 1); 5 μl of ring expansion reaction mixture containing partially purified enzyme and sampled at 2 h (lane 2); 0.05 μg of desacetoxycephalosporin C (lane 3); and 0.05 μg of desacetylcephalosporin C (lane 4). The chromatogram was developed for 4 h in a *n*-butanol–acetic acid–water (3:1:1) and then bioassayed on an agar slab inoculated with *E. coli* Ess and containing penicillinase at 2×10^5 U/ml.

DISCUSSION

An ion-pair HPLC system has been developed for analysis of the cell-free ring expansion of penicillin N to desacetoxycephalosporin C. Potential baseline instability associated with high concentrations of TBAB was eliminated by preconditioning the stationary phase with 4.5 mM TBAB and then switching to a mobile phase containing 0.06 mM TBAB for analysis of reaction mixtures. This protocol led to smooth baselines while retaining the improved chromatographic characteristics imparted by TBAB. When ring expansion reaction mixtures containing partially purified enzyme were analyzed, penicillin N was seen to decrease in concentration from 0.240 to 0.036 mM over the 2-h incubation period. In contrast, desacetoxycephalosporin C was seen to increase in concentration from 0 to 0.204 mM over the 2-h period, assuming a quantitative conversion of penicillin N to desacetoxycephalosporin C. This assumption is supported by the calculated peak areas per nanomole for desacetoxycephalosporin C, which are in close agreement at the various sampling times.

Ring expansion reaction mixtures containing crude enzyme could not be analyzed satisfactorily in this HPLC system because of a subsequent enzymatic oxidation of desacetoxycephalosporin C to desacetylcephalosporin C. This latter product comigrates with α-ketoglutarate and accounts for the otherwise unexpected increase in area of the α-ketoglutarate peak during incubation. Since both the ring expansion reaction and the oxidation of desacetoxycephalosporin C to desacetylcephalosporin C consume α-ketoglutarate (11), it seems likely that the peak at 4.26 min shown in Fig. 4 (2 h) is mainly due to desacetylcephalosporin C. Analysis of reaction mixtures by HPLC with detection at 260 nm further supports this hypothesis. Thin-layer chromatography of this reaction mixture confirmed the presence of desacetylcephalosporin C.

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