Inactivation of Alanine Racemase by β-Chloro-L-alanine Released Enzymatically from Amino Acid and Peptide C_{10}-Esters of Deacetylcephalothin†

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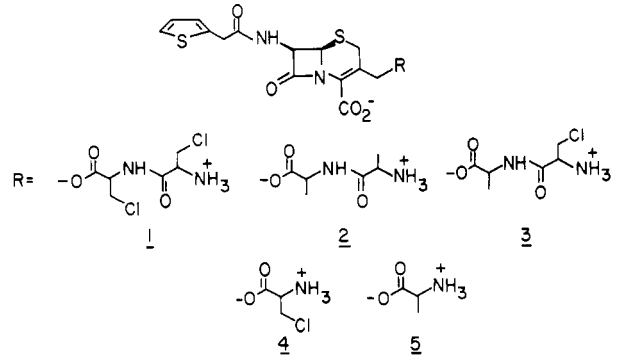
ABSTRACT: The reactions of a set of amino acid and peptidyl C_{10}-esters of deacetylcephalothin (**1**–**5**) have been examined with purified enzymes in vitro. Each of the compounds examined is a substrate for the *Escherichia coli* TEM-2 β-lactamase, and enzyme-catalyzed hydrolysis of the lactam bond gives release of an amino acid or a peptidyl fragment from a cephem nucleus. 7β-(2-Thienylacetamido)-3-[[(β-chloro-L-alanyl)oxy]methyl]-3-cephem-4-carboxylate (**4**) gives time-dependent inactivation of *E. coli* JSR-O alanine racemase in a process that requires β-lactamase for the initial liberation of β-chloro-L-alanine from the cephalosporin. Alanine racemase is similarly inactivated by 7β-(2-thienylacetamido)-3-[[[(β-chloro-L-alanyl)-β-chloro-L-alanyl]oxy]methyl]-3-cephem-4-carboxylate (**1**), but this inhibition requires the sequential action of both β-lactamase and alanine aminopeptidase. Analysis of the enzymatic transformations of 7β-(2-thienylacetamido)-3-[[[(β-chloro-L-alanyl)-L-alanyl]oxy]methyl]-3-cephem-4-carboxylate (**3**), monitored by high-field ^1H NMR, reveals that (1) β-lactamase releases the dipeptide β-chloro-L-alanyl-L-alanine from **3** and (2) leucine aminopeptidase effects stoichiometric hydrolysis of the dipeptide to β-chloro-L-alanine and L-alanine. These biochemical findings are discussed with reference to the mechanism of antibacterial action of **1** against β-lactamase-producing, penicillin-resistant microorganisms [Mobashery, S., Lerner, S. A., & Johnston, M. (1986) *J. Am. Chem. Soc. 108*, 1685].

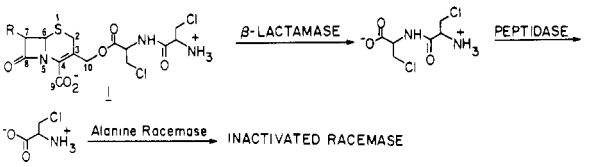
A well-documented mechanistic feature of the reaction of the β-lactamases with cephalosporins is release of a heteroatom-linked substituent from the C_{10}-position of Δ^3-cephems. Enzymatic processing, for example, of cephalothin, cephaloridine, and azidodeacetoxycephalothin gives formation of, respectively, acetate, pyridine, and azide (Sabath et al., 1965; Hamilton-Miller et al., 1970a,b; O'Callaghan et al., 1972; O'Callaghan & Muggleton, 1972; Boyd et al., 1975; Boyd & Lunn, 1979). It was originally thought that cleavage of the β-lactam bond was concerted with elimination of the functional group at C_{10}. However, recent studies have suggested that the lactamase probably acts solely to open the lactam ring of cephalosporins and that release of the leaving group occurs in a subsequent, nonenzymatic step (Faraci & Pratt, 1984; Page & Procter, 1984; Page, 1984; Grabowski et al., 1985; Mobashery & Johnston, 1986).

We have attempted to capitalize on this lactamase-initiated fragmentation of cephalosporins in the preparation of **1**, a novel



derivative of deacetylcephalothin that carries the antibacterial dipeptide β-Cl-LAla-β-Cl-LAla[1] (Cheung et al., 1983). The rationale for the design of **1** has been detailed elsewhere (Mobashery & Johnston, 1986) and is briefly summarized in Scheme I. We reasoned that lactamase-catalyzed hydrolysis of **1**, in an otherwise β-lactam-resistant microorganism, would lead to the release of the chloroalanyl dipeptide in vivo. In turn, transport of β-Cl-LAla-β-Cl-LAla from the periplasmic space to the cytosol should expose the dipeptide to the action of intracellular peptidases. As a result, β-chloro-L-alanine will accumulate and, consequently, afford inactivation of bacterial alanine racemase—an enzyme essential for peptidoglycan biosynthesis.

Scheme I



In accord with expectations, cephem **1** displays good antibacterial activity against both Gram-positive and Gram-negative lactamase-producing organisms (Mobashery et al., 1986). Upon reaction of **1** with the *Escherichia coli* TEM-2 β-lactamase, β-Cl-LAla-β-Cl-LAla is formed quantitatively (Mobashery & Johnston, 1986). Further, we have found that

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[1] The omission of the hyphen in abbreviations of amino acids (e.g., LAla) conforms with suggestions cited by the IUPAC–IUB Combined Commission on Biochemical Nomenclature (1966). The subscripts used in peptide nomenclature (e.g., α_1-H) signify the order of the amino acid residues, numbering from the amino- to the carboxy-terminal positions; thus, β_2-methyl denotes the methyl group of the second amino acid of a peptidyl structure. Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration (μg/mL); NaP_i, sodium phosphate; TFA, trifluoroacetate.

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exposure of *E. coli* JSR-O to the haloalanyl dipeptide leads to reduction of alanine racemase activities in vivo. In this paper, we report on the in vitro reactions of **1**, and of several other amino acid and peptidyl esters (**2**–**5**) of deacetylcephalothin, with the enzymes of Scheme I. The results presented further support our premise that lactamase-promoted cleavage of an appropriately substituted cephalosporin is a biochemically reasonable method for "delivery" of mechanism-based inactivators of essential microbial enzymes.

Materials and Methods

Materials. L-Alanine dehydrogenase, alanine aminopeptidase, leucine aminopeptidase, NAD^+, and pyridoxal 5′-phosphate were Sigma products. The NAD^+ cofactor was frozen and lyophilized before use in order to remove traces of water, ethanol, and acetone. *Escherichia coli* TEM-2 β-lactamase was purchased from Applied Microbiology and Research, Porton Down, U.K. Thin-layer chromatography was performed on silica gel HLF plates from Analtech. All other reagents were of the best grade commercially available.

Amino Acid and Peptidyl Cephem Esters. The peptidyl (**1**–**3**) and amino acid (**4** and **5**) C_{10}-esters of deacetylcephalothin were prepared by methods previously reported (Mobashery et al., 1986; Mobashery & Johnston, 1986). Complete synthetic details, together with analytical data for each compound, will be given elsewhere.

Purification of Alanine Racemase. E. coli JSR-O, a laboratory strain of *E. coli* K-12, was cultivated at 37 °C on minimal medium (Davis & Mingioli, 1950) supplemented with 50 μg/mL L-methionine, 50 μg/mL-proline, 0.5% DL-alanine, and 1% glucose. Cultures were harvested in late logarithmic phase, and the cells were isolated by centrifugation at 4 °C. The cell pellet (~30 g) was resuspended in 50 mM NaP_i buffer, pH 8.0, containing 1.4 mM 2-mercaptoethanol and 10 μM pyridoxal 5′-phosphate, and the suspension was recentrifuged. The washed cells were resuspended in about 5 volumes of the same buffer and sonicated (ten 30-s bursts separated by 45-s intervals; on ice). Following sonic disruption, the cell lysates were centrifuged, and the resulting supernatant fluid was used for enzyme purification.

Alanine racemase was isolated according to the procedures developed for purification of the *Salmonella* enzyme (Wasserman et al., 1984). This method, applied to purification of the *E. coli* JSR-O racemase, afforded an enzyme that was approximately 70% homogeneous, as evidenced by polyacrylamide gel electrophoresis, and that had a specific activity of 2.0 μmol min^{-1} (mg of protein)$^{-1}$.

Alanine Racemase Assay. Alanine racemase activity was measured in the D to L direction with the L-alanine dehydrogenase coupled, spectrophotometric assay of Wang and Walsh (1978). The reaction was followed by monitoring NADH production (340 nm, $\epsilon = 6200\ M^{-1}\ cm^{-1}$) as product L-alanine was converted to pyruvate and ammonia in the presence of excess dehydrogenase. A typical 1-mL assay contained 0.1 unit of alanine racemase, 1.1 units of L-alanine dehydrogenase, 10 mM D-alanine, and 10 mM NAD^+ in 100 mM CHES buffer, pH 9.0, 37 °C. Spectrophotometric measurements were made on a Perkin-Elmer 559 spectrometer. Protein concentrations were determined by the Lowry method (Lowry et al., 1951).

Kinetics of β-Lactamase Reactions. Kinetic constants were determined for the reactions of *E. coli* TEM-2 β-lactamase with the amino acid and peptidyl cephalosporin esters **1**–**5**. A typical 1.0-mL reaction contained 0.12–0.45 mM substrate and 0.5 μg of enzyme in 100 mM NaP_i buffer, pH 7.0, 37 °C. The reaction was followed by monitoring the loss of absorbance at 267 nm of the α,β-unsaturated carboxylate chromophore of the cephem nucleus ($\epsilon = 6.5 \times 10^3\ M^{-1}\ cm^{-1}$). Protein concentrations were determined by the Lowry method (Lowry et al., 1951).

Inactivation of Alanine Racemase. Time-dependent inactivation of alanine racemase was measured according to the procedures of Wang and Walsh (1978). In order to evaluate the ability of the amino acid cephem esters **4** and **5** to afford lactamase-dependent inactivation of alanine racemase, the following protocol was used. To a 10 mM solution of **4** or **5**, in 100 mM NaP_i buffer, pH 7.0, was added 4 μL of a 5 mg/mL solution of *E. coli* TEM-2 β-lactamase; the cephem/lactamase preparation was incubated for ~20 min at 37 °C. Subsequently, 50 μL of an alanine racemase preparation (0.1 unit, in 50 mM NaP_i buffer, pH 8.0) was added to a 600-μL aliquot of the above cephem/lactamase solution, and the mixture was further incubated at 37 °C. At timed intervals, 50-μL aliquots were removed and diluted into 950 μL of the standard alanine racemase assay mixture (vide supra). In control experiments, 10 mM solutions of **4** and **5** were preincubated in 100 mM NaP_i buffer, pH 7.0, but β-lactamase was omitted. These solutions were similarly incubated with alanine racemase, and time-dependent loss of racemase activity was measured as described for experimentals.

In order to evaluate the ability of the haloalanyl peptide esters **1** and **2** to afford alanine racemase inactivation, the following experimental protocol was used. A 4-μL aliquot of a 5 mg/mL solution of *E. coli* TEM-2 β-lactamase was added to a 10 mM solution of **1** or **2** in 100 mM NaP_i buffer, pH 7.0. The cephem/lactamase preparation was incubated for ~20 min at 37 °C. β-Lactamase was then removed by ultrafiltration, and 0.12 unit of alanine aminopeptidase (in 100 mM NaP_i buffer, pH 7.0) was added to the filtrate. This hydrolytic reaction, incubated at 37 °C, was monitored for appearance of β-Cl-LAla (or LAla) by thin-layer chromatography.[2] Complete hydrolysis of the peptides, liberated from **1** and **2** by the previous lactamase treatment, required from 1.0 to 1.5 h. Subsequently, 0.1 unit of alanine racemase (in 50 mM NaP_i buffer, pH 8.0) was added to 600 μL of the peptidase reaction mixture, and this solution was further incubated at 37 °C.[3] At timed intervals, 50-μL aliquots were removed and diluted into 950 μL of the standard alanine racemase assay mixture (vide supra). In control experiments, 10 mM solutions of **1** and **2** were incubated in 100 mM NaP_i buffer, pH 7.0, but β-lactamase and alanine aminopeptidase were omitted. Alanine racemase was subsequently added to these solutions, and the racemase activity was measured in time as described above for experimentals.

Analysis of Reactions of **3** *by Proton NMR.* Proton NMR spectra were obtained at 500 MHz on a DS-1000 instrument, equipped with a Nicolet 1180 computer, with sodium 3-(trimethylsilyl)propionate in D_2O as the internal reference. Chemical shift values are given in ppm.

A 500-μL solution of the TFA salt of **3** (10 mM; previously lyophilized twice from deuterium oxide; Aldrich, 99.8 atom % ^2H) was prepared in 100 mM NaP_i buffer, pD 7.0, and placed in a 5-mm NMR tube. It was necessary to mix the

[2] Silica gel; 1-butanol–H_2O–acetic acid (4:1:1) for the reaction involving hydrolysis of β-Cl-LAla-β-Cl-LAla and 1-butanol–H_2O–acetic acid (4:1:3) for the reaction involving hydrolysis of LAla-LAla. Visualization was with ninhydrin.

[3] We found it necessary to preincubate the peptide solution with the aminopeptidase prior to addition of the racemase. Coincubation of a haloalanyl peptide with the peptidase *and* alanine racemase gave substantial loss of racemase activity, resulting both from proteolysis and from inactivation specifically by β-chloroalanine.

Table I: Kinetic Data for Reactions of *E. coli* TEM β-Lactamase with Amino Acid and Peptidyl Cephalosporin Esters **1–5**[a]

substrate	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})
1	0.32	338[b]
2	0.33	338[b]
3	0.33	340
4	0.12	240
5	0.32	250

[a] Data were obtained at 37 °C in 100 mM NaP_i buffer, pH 7.0. Other experimental details are given in the text. [b] These data were reported previously (Mobashery et al., 1986).

solution with gentle warming to completely dissolve **3**. After acquisition of an initial spectrum, a 2-μL solution of *E. coli* TEM β-lactamase (5 mg/mL, prepared in 100 mM NaP_i buffer, pH 7.0) was added to the substrate solution. Spectra were then acquired at timed intervals with a one pulse-sequence experiment (43° flip angle, 500-μs delay, 16K data points, 40 acquisitions). Total acquisition time for each spectrum was approximately 40 s. The probe temperature was maintained at 36 °C.

The spectral data indicated that lactamase-catalyzed fragmentation of **3**, to give the dipeptide β-Cl-LAla-LAla (vide infra), was complete after approximately 15 min. At this point, 10 units of leucine aminopeptidase[4] (in 100 mM NaP_i buffer, pD 7.0) was added to the NMR tube, and spectra were again acquired at timed intervals, with the NMR parameters outlined above. Hydrolysis, to afford β-Cl-LAla and LAla (vide infra), was complete within approximately 30 min. Spectra of authentic samples of β-Cl-LAla-LAla and of an equimolar mixture of β-Cl-LAla and LAla were also obtained, under the same conditions described for the enzymatic experiments.

Results

Kinetics of β-Lactamase Reactions with Cephem Esters. The kinetic constants for the reactions of *E. coli* TEM β-lactamase with the peptidyl and amino acid cephalosporin esters **1–5** are reported in Table I. The data reveal that each compound of Table I is an excellent substrate for the TEM enzyme. In fact, the V_{max} rates obtained for the amino acid and peptidyl cephem esters are all substantially greater than that measured for reaction of cephalothin (K_M = 0.18 mM, V_{max} = 78 μmol min^{-1} mg^{-1}; Mobashery & Johnston, 1986), which is among the best cephalosporin substrates for the TEM-2 lactamases. Further, the data of Table I reveal that the peptidyl esters **1–3** elicit greater V_{max} values than their amino acid ester cognates **4** and **5**.

Lactamase-Dependent Inactivation of Alanine Racemase by the Chloroalanyl Cephem Ester (**4**). Figure 1A shows that purified *E. coli* alanine racemase experiences time-dependent loss of activity on incubation with a 10 mM solution of **4** that had been previously treated with *E. coli* TEM β-lactamase ($t_{1/2}$ ~ 12 min). Inactivation by 10 mM β-Cl-LAla alone gave inhibition with a $t_{1/2}$ = 8 min (data not included). Figure 1A shows that in the absence of the lactamase **4** gives only ~10%

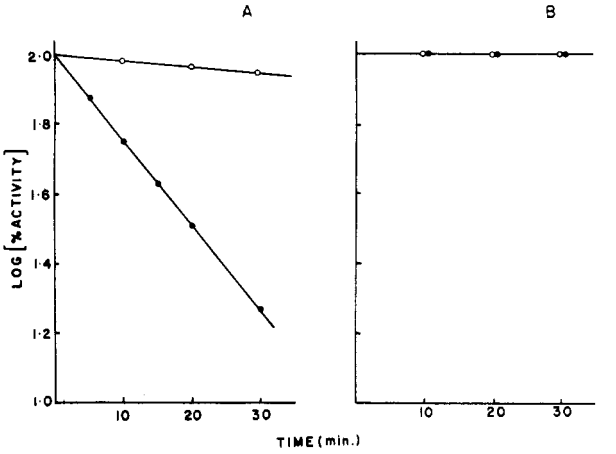


FIGURE 1: Lactamase-dependent inactivation of *E. coli* JSR-O alanine racemase by chloroalanyl (**4**) and alanyl (**5**) cephem esters. (A) Time-dependent loss of alanine racemase activity was measured after treatment with a 10 mM solution of **4** (○) and with a 10 mM solution of **4** preincubated with *E. coli* TEM-2 β-lactamase (●). (B) Time-dependent loss of alanine racemase activity was monitored after treatment with a 10 mM solution of **5** (○) and in the presence of 10 mM **5** preincubated with *E. coli* TEM-2 β-lactamase (●). Experimental conditions are given in the text.

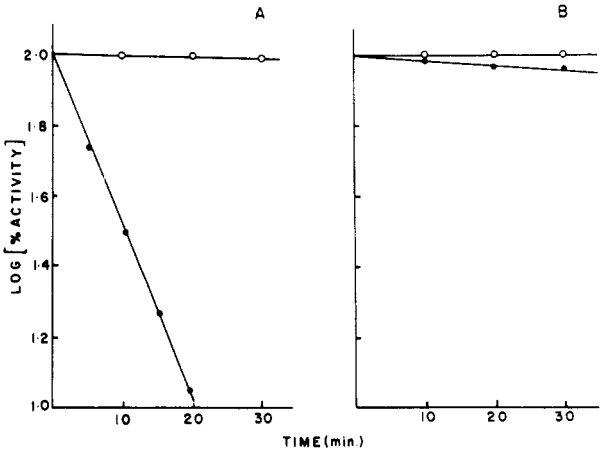


FIGURE 2: Lactamase- and peptidase-dependent inactivation of *E. coli* JSR-O alanine racemase by the peptidyl cephem esters **1** and **2**. (A) Time-dependent loss of alanine racemase activity was monitored after treatment with a 10 mM solution of **1** (○) and with a 10 mM solution of **1** incubated with *E. coli* TEM-2 β-lactamase and alanine aminopeptidase (●). (B) Time-dependent loss of alanine racemase activity was monitored after treatment with 10 mM solution of **2** (○) and with a 10 mM solution of **2** incubated with *E. coli* TEM-2 β-lactamase and alanine aminopeptidase (●). Experimental conditions are given in the text.

inactivation of the racemase in 30 min. We attribute this small loss of racemase activity to nonenzymatic release of β-Cl-LAla from **4**, as a consequence of intermolecular aminolysis (see Discussion). This process may also account for the differences in the half-times observed for inactivation by **4** and by β-Cl-LAla alone (vide infra).

The alanyl cephem ester **5** was designed to serve as a control for the chloroalanyl compound **4**. Note from Figure 1B that neither in the presence nor in the absence of β-lactamase did **5** occasion inactivation of alanine racemase. This finding further suggests that racemase inhibition afforded by **4** results from lactamase-dependent release of β-Cl-LAla from the cephem.

Lactamase- and Peptidase-Dependent Inactivation of Alanine Racemase by the Peptidyl Cephem (**1**). In Figure 2 are reported the results of experiments, analogous to those shown in Figure 1, using the peptidyl cephem esters **1** and **2** as po-

[4] We found that the aminopeptidases used in these experiments lose activity at differing rates in deuterium oxide buffers. Leucine aminopeptidase retains about 90% of its native activity (assayed in protio buffers) after approximately 40 min of incubation in deuteriated NaP_i, but alanine aminopeptidase loses nearly all activity after 1–2 min in deuteriated NaP_i buffer. Thus, while we used alanine aminopeptidase for the kinetic inactivation studies outlined above, NMR analysis in D_2O buffer required the use of the more stable leucine aminopeptidase. We have previously shown that both of these enzymes readily effect hydrolysis of chloroalanyl dipeptides (Boisvert et al., 1986).

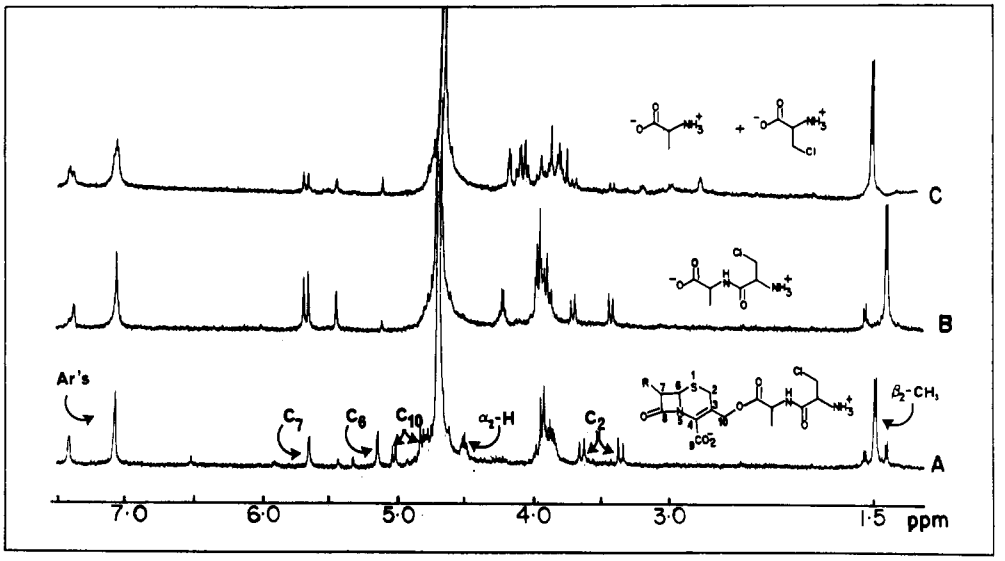


FIGURE 3: The 500-MHz ^{1}H NMR spectra of enzymatic reactions of **3** with β-lactamase and leucine aminopeptidase. (A) This spectrum of **3** was acquired prior to the addition of *E. coli* TEM-2 β-lactamase. The aromatic protons of the thienyl group of **3** are indicated by Ar's. (B) This spectrum was acquired after a 15-min incubation of **3** with the lactamase. (C) This spectrum was acquired following a 30-min incubation of the solution of spectrum B with leucine aminopeptidase. Experimental conditions are given in the text.

tential racemase inactivators. As shown in Figure 2A, alanine racemase was not found to be inactivated on incubation with **1** alone. However, when the racemase was exposed to a 10 mM solution of **1** that had been preincubated with both the TEM lactamase *and* alanine aminopeptidase, time-dependent loss of enzyme activity was observed. The half-time for inactivation in this case was approximately 7 min. Inactivation of purified *E. coli* alanine racemase by 20 mM β-Cl-LAla alone occurs with $t_{1/2}$ = 7.5 min (Boisvert et al., 1986). Thus, it would appear that the inactivation of the racemase shown in Figure 2A results from accumulation of a 20 mM concentration of chloroalanine, produced by the action first of β-lactamase on the peptidyl cephem **1** and then of alanine aminopeptidase on the released dipeptide β-Cl-LAla-β-Cl-LAla.

Figure 2B shows that the dialanyl cephem ester **2** incubated with alanine racemase does not, as expected, afford enzyme inactivation. Moreover, only a 5% loss of racemase activity is observed in 30 min when the enzyme is exposed to a 10 mM solution of **2** that had been treated, like **1**, with both β-lactamase and alanine aminopeptidase. The small reduction in racemase activity, obtained under the conditions used for the experiments described in Figure 2, is attributed to the proteolytic action of the aminopeptidase on alanine racemase (Boisvert et al., 1986).

Proton NMR Analyses. We had hoped to be able to monitor the in vitro enzymatic transformations outlined in Scheme I by ^{1}H NMR spectrometry and, further, to correlate spectral events with the inactivation data reported in Figure 2. The proton resonances of the peptidyl (β-Cl-LAla-β-Cl-LAla) fragment of **1** are poorly resolved, however, in deuterium oxide buffers (Mobashery & Johnston, 1986). By contrast, a majority of the proton signals of the C_{10}-peptidyl (β-Cl-LAla-LAla) substituent of **3** are resolved at 500 MHz in deuteriated buffers. Thus, **3** was selected for a general analysis by ^{1}H NMR spectrometry of the enzymatic processing of peptidyl cephem esters. We have reported elsewhere on the products that arise from the enzymatic transformations on **1** (Mobashery & Johnston, 1986) and on the dipeptide β-Cl-LAla-β-Cl-LAla (Boisvert et al., 1986).

Figure 3A shows the 500-MHz ^{1}H NMR spectrum of **3** prior to reaction with the lactamase. Among the structurally diagnostic resonances of **3** are the two methine doublets at 5.65 and 5.14 ppm, given by the C_7- and C_6-hydrogens, respectively, of the β-lactam ring. The C_{10}-methylene hydrogens each appear as a doublet at 5.01 and 4.82 ppm, respectively; the upfield resonance is slightly obscured by the HOD signal in Figure 3A. The quartet at 4.51 ppm is given by the α-hydrogen of the alanyl residue of the peptide. The pair of doublets at 3.64 and 3.35 ppm are the signals for the C_2-methylene group of the cephem. The methyl doublet at about 1.5 ppm is that of the alanyl residue.[5]

The spectrum of Figure 3B was acquired after **3** had been incubated for 15 min with *E. coli* TEM-2 β-lactamase. Among the spectral changes observed when **3** is incubated with the lactamase is the disappearance of the C_6- and C_7-methine resonances (2 d, 5.14 and 5.65 ppm) along with the collapse of the C_{10}-methylene signals (2 d, 4.82 and 5.01 ppm). These resonances are replaced, concurrently, by the three broad singlets of Figure 3B at 5.45, 5.66, and 5.69 ppm. We have previously observed the same set of spectral events for the enzymatic processing of both cephalothin and the dialanyl cephem ester **2** (Mobashery & Johnston, 1986). These changes appear to be diagnostic of the reaction of a C_{10}-substituted cephalosporin with a β-lactamase.

Figure 3B also shows that the chemical shift position of the α_2-methine quartet of **3** (4.51 ppm in Figure 3A) has moved upfield by approximately 0.3 ppm (4.22 ppm in Figure 3B). This change is that expected for a methine proton initially α to a carboxyl ester (as in **3**) and subsequently α to a carboxylate (as in β-Cl-LAla-LAla). Comparison of spectra A and B of Figure 3 also reveals minor changes in the chemical shift positions of the C_2-methylene hydrogens (2 d at 3.35 and 3.64 ppm in Figure 3A) and an upfield movement of the alanyl methyl doublet (from ~1.5 ppm in Figure 3A to ~1.4 ppm in Figure 3B).

The spectral observations of spectra A and B of Figure 3 indicate that β-lactamase hydrolysis of **3** generates the di-

[5] The thienylacetamido side-chain methylene hydrogens of **3**, as well as the β_1-methylene and α_1-methine protons of the chloroalanyl fragment, are not resolved in the spectrum of Figure 3A. The four methylene resonances appear as a broad multiplet between 4.0 and 3.8 ppm, and the α_1-hydrogen is buried under the HOD signal. The α_1-methine resonance of β-Cl-LAla-LAla, trifluoroacetate salt, in D_2O appears at 4.45 ppm (Cheung et al., 1983).

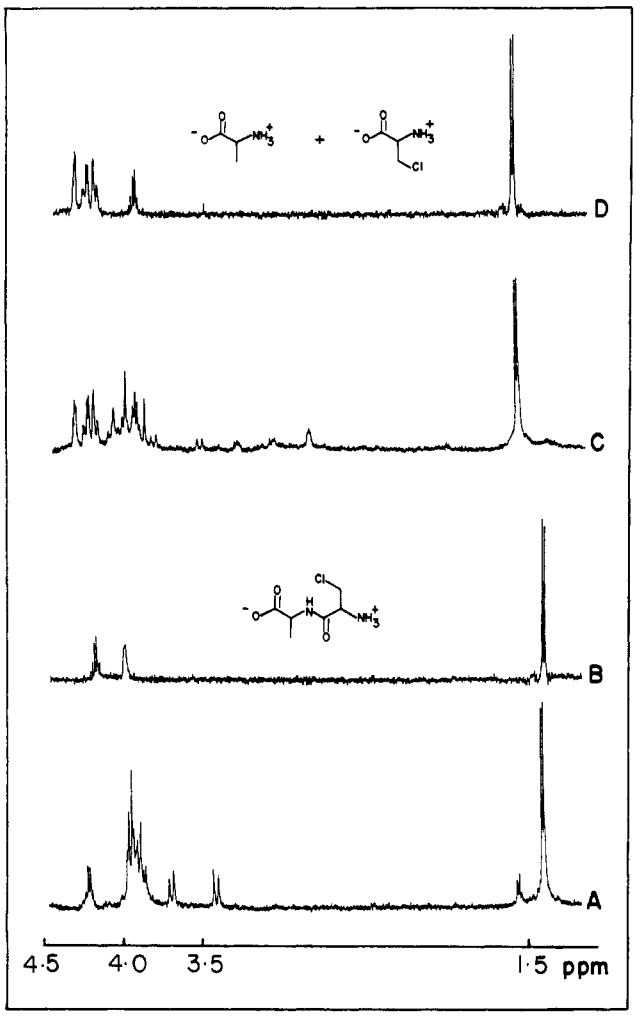


FIGURE 4: ^{1}H NMR spectra of reaction products formed on treatment of **3** with β-lactamase and leucine aminopeptidase together with authentic samples of β-Cl-LAla-LAla, β-Cl-LAla, and LAla. (A) This is a reproduction of the upfield region of the spectrum of Figure 3B. (B) This is the spectrum of a 10 mM solution of β-Cl-LAla-LAla. (C) This is a reproduction of the upfield region of the spectrum of Figure 3C. (D) This is the spectrum of authentic samples of β-Cl-LAla and LAla (each at 10 mM). Experimental conditions are given in the text.

peptide β-Cl-LAla-LAla. The data presented in spectra A and B of Figure 4 confirm this conclusion. Figure 4A reproduces the proton spectrum, upfield of the HOD signal, shown in Figure 3B; Figure 4B presents the ^{1}H NMR spectrum of an authentic sample of β-Cl-LAla-LAla. The β_1-methylene and α_1-methine hydrogens of the dipeptide are not resolved in the spectrum of Figure 4B. However, note that the α_2-hydrogen of β-Cl-LAla-LAla (4.22 ppm in Figure 4B) coincides with the methine quartet of Figure 4A and that the alanyl methyl doublet (1.40 ppm in Figure 4B) is superimposed on the methyl doublet of Figure 4A.

After the spectrum of Figure 3B was obtained, leucine aminopeptidase was added to the NMR tube, and following a 30-min incubation period, the spectrum of Figure 3C was recorded. A number of the resonances that appear in the spectrum of Figure 3C are worthy of comment. First of all, there is a now well-resolved doublet of doublets (4.07 ppm, dd, 1 H; 4.13 ppm, dd, 1 H) indicative of the β-methylene hydrogens of β-Cl-LAla (Cheung et al., 1983). Second, there is a single-proton multiplet centered at about 4.25 ppm, which represents the α-methine hydrogen of chloroalanine. Note that the α-methine hydrogen of alanine has shifted downfield (3.82 ppm, q, 1 H; Figure 3C) from its position in the peptide (4.22 ppm, q, 1 H; Figure 3B). Similarly, the β-methyl resonance of Figure 3B (1.40 ppm, d, 3 H) is slightly downfield in Figure 3C (1.52 ppm, d, 3 H).

The spectrum of Figure 3C, upfield of the water signal, is reproduced in Figure 4C. Note that the major resonances of Figure 4C coincide with the spectrum of a 1:1 mixture of LAla and β-Cl-LAla (Figure 4D). Thus, we conclude that treatment of the reaction products of Figure 3B with leucine aminopeptidase gives stoichiometric hydrolysis of the dipeptide β-Cl-LAla-LAla. The resonances of Figures 3C and 4C that are not attributable to the amino acids can be ascribed to products of fragmentation of cephem **3** (Mobashery & Johnston, 1986).

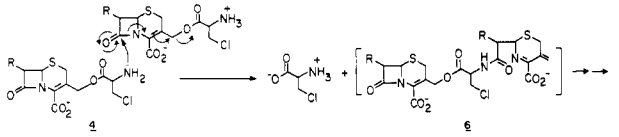
Discussion

Resistance to the β-lactam antibiotics continues to be a major problem in the chemotherapy of infectious diseases. Medicinal chemistry has, nonetheless, frequently achieved notable success in its attempts to meet the challenge of the penicillin-resistance problem. These efforts have included both the isolation and the design of new β-lactams with improved stability to the action of the β-lactamases. Among this group of compounds are the semisynthetic penems (Doyle & Naylor, 1964), the cephamycins (Nagarajan et al., 1971; Otsuka et al., 1981), thienamycin (Komatsu & Nishikawa, 1980), and the monobactams, such as azthreonam (Sykes et al., 1982). Fisher (1984) has provided a recent and comprehensive review of this topic. An alternate approach has centered around the clinical concept of simultaneous administration of an effective—although perhaps lactamase sensitive—β-lactam antibiotic and a lactamase inactivator. Lactamase inhibitors which are candidates for this strategy include clavulanic acid (Brown et al., 1976), the carbapenems (Brown et al., 1977; Maeda et al., 1977; Okamura et al., 1980), the penicillanic acid sulfones (English et al., 1978; Labia et al., 1980), the penem and cephem sulfenimines (Gordon et al., 1980), and the 6-halopenicillinates (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b; Cartwright & Coulson, 1979). Two combinations, amoxicillin/clavulanate (Augmentin) and ticarcillin/clavulanate (Timentin), have come into clinical use recently.

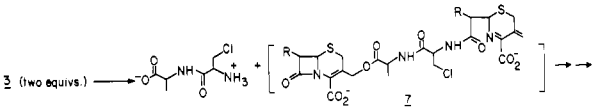
The peptidyl cephem ester **1** was conceived on the basis of our suspicion that the β-lactamases need not be circumvented but might actually be conscripted for delivery of a known cytotoxic agent, such as a chloroalanine-containing peptide. As outlined in Scheme I, inactivation of the putative intracellular target enzyme, alanine racemase, requires three sequential microbial transformations: (1) lactamase-catalyzed hydrolysis of the cephalosporin to generate the cytotoxic dipeptide in the periplasmic space; (2) transport of the peptide across the inner plasma membrane; (3) hydrolysis of the internalized peptide by bacterial peptidases. We have previously documented the role played by microbial peptidases in the mechanism of action of haloalanyl peptides (Boisvert et al., 1986). The work described in this paper was undertaken in order to provide an in vitro evaluation of these essential biochemical predictions.

Alanine Racemase Inactivations. The data of Figure 1 show that *E. coli* alanine racemase suffers time-dependent inactivation on exposure to the chloroalanyl cephem ester **4**, in a process that is clearly dependent on the action of β-lactamase. This result alone appears to underscore our general proposition that the β-lactamases are biochemically competent for release of mechanism-based enzyme inactivators from appropriately C_{10}-substituted cephalosporin substrates. Racemase inactivation is also achieved with the dichloroalanyl cephem ester **1**. This process, as predicted by Scheme I and documented by the results of Figure 2, requires the sequential action of

Scheme II



Scheme III



β-lactamase and alanine aminopeptidase; omission of either enzyme does not give inhibition of alanine racemase by **1**.

We attempted to visualize the steps involved in the inactivation reported in Figure 2 by ^{1}H NMR spectrometry, using **3** as a model for **1**. The results, shown in Figures 3 and 4, clearly demonstrate that β-lactamase promotes the stoichiometric release of β-Cl-LAla-LAla from the cephem **3** and that, in turn, leucine aminopeptidase effects hydrolysis of the chloroalanyl dipeptide to its component amino acids. On the basis of these findings, we conclude that **1** may be considered, as it were, a "pre-proinactivator" of alanine racemase and that inactivation is minimally a two enzyme dependent process. Hydrolysis of the cephem, catalyzed by β-lactamase, first releases the antibiotic dipeptide β-Cl-LAla-β-Cl-LAla, and this must be cleaved further, in a peptidase-dependent step, before racemase inactivation can take place.

We do not presume to suggest that the observations reported here, using purified enzymes in vitro, constitute proof that the events of Scheme I explain the mechanism of antibacterial action of **1** in vivo. The results of these biochemical experiments, however, correlate well with our microbiological findings. In this regard, we have discovered that **1** is a much more potent antibacterial agent against lactamase-producing organisms than against isogenic parental strains that lack the β-lactamase enzyme. For example, the MIC for **1** against *E. coli* JSR-O is 12.5 μg/mL, while the MIC for *E. coli* JSR-O (pBR322) is 1.56 μg/mL; *E. coli* JSR-O (pBR322) contains the ampicillin resistance (TEM-2 β-lactamase) gene. Similarly, both **1** and **2** are effective antibiotics against penicillin- and methicillin-sensitive strains of *Staphylococcus aureus*, presumably because these organisms, in general, are sensitive to cephalosporins. Against methicillin-resistant (lactamase-producing) *S. aureus*, however, only the dichloroalanyl ester **1** is active. The MIC for **1** is 0.85 μg/mL while the MIC for **2** is >200 μg/mL (Mobashery et al., 1986). We have also shown, in independent studies (Boisvert et al., 1986), that the dipeptide β-Cl-LAla-β-Cl-LAla leads to a reduction of in vivo alanine racemase activities in both Gram-positive and Gram-negative microorganisms. It is perhaps reasonable, therefore, to assume that the events predicted in Scheme I—and now documented by the in vitro studies reported in this paper—may extend to the in vivo processing of **1** and of other peptidyl cephalosporin esters like it.

Autoaminolysis of Amino Acid and Peptidyl Cephems. We find that our cephems are unstable to decomposition at high concentrations. At low concentrations (e.g., ~0.3–0.4 mM), the amino acid and peptidyl cephem esters **1**–**5** are stable for up to 36 h in phosphate buffer at room temperature. We atttribute the instability of these compounds at high concentrations (e.g., 10 mM and above) to intermolecular aminolysis. Intermolecular aminolytic fragmentation has been shown to occur with thienamycin (Kahan et al., 1979), a process that occasioned the synthesis of the pharmacokinetically more stable *N*-formimidoyl derivative of thienamycin, called imipenem. Autoaminolysis also occurs with cephalosporins (Indelicato et al., 1974).

A likely route for intermolecular aminolysis of **4** is shown in Scheme II. Note that the sequence of Scheme II predicts the partial, nonenzymatic release of β-Cl-LAla from **4**. This, in fact, may account for the small amount (~10%) of enzyme inactivation observed when purified alanine racemase is exposed to **4** in the absence of β-lactamase (Figure 1A). Intermolecular aminolysis would simultaneously consume, as well as liberate, chloroalanine as a lactam aminoacyl adduct (**6**) is formed. This process obviously decreases the effective concentration of chloroalanine that ultimately can be released from **4** by subsequent action of β-lactamase. This fact would also account for the observation (Figure 1A) that the half-time for alanine racemase inactivation is much longer (~12 min) than anticipated (8 min) for a solution of **4** that would otherwise generate a 10 mM solution of the amino acid in an exclusively enzymatic process.

We have suggested in Scheme II a structure (**6**) for the intermediate, fragmented cephalosporin formed upon nonenzymatic autoaminolysis of **4**. While formation of this intermediate seems reasonable on the basis of chemical intuition alone, we do not have unambiguous evidence for **6**. However, examination of the ^{1}H NMR spectra of Figures **3** and **4** provides at least partial evidence for the formation of **7**, the peptidyl cognate of 6, from **3** (Scheme III).

Examination of the spectrum of **3** prior to addition of β-lactamase (Figure 3A) reveals one major and two minor doublets in the region between 1.40 and 1.55 ppm. The ^{1}H NMR spectrum of **3** in DMSO-d_6 (data not included) lacks these multiplicities in the alanyl methyl region. We suspect that the two additional methyl resonances of Figure 3A can be accounted for by the intermolecular aminolysis shown in Scheme III. At high concentrations (10 mM) in aqueous solution, the chloroalanyl amino group of **3** accomplishes carbonyl addition to the β-lactam of another cephem. This releases, first of all, a small amount of the dipeptide β-Cl-LAla-LAla, giving rise to the alanyl methyl doublet at 1.40 ppm in Figure 3A; note the coincidence of the major methyl signal (1.40 ppm) of Figure 3B with the cognate resonance in Figure 3A. Correspondingly, the upfield doublet (1.54 ppm; Figure 3A) could represent the methyl group of the alanyl residue of the cephalosporin/cephalosporinate adduct **7**. In support of the proposition that β-Cl-LAla-LAla and **7** are formed stoichiometrically in the autoaminolytic reaction, the methyl doublets at 1.40 and 1.54 ppm had identical integral intensities (data not included).

Upon reaction of **3** with β-lactamase, the intense methyl doublet of Figure 3A (1.48 ppm) disappears and is replaced by the alanyl methyl signal of the free peptide (1.40 ppm; Figure 3B). The doulet at 1.54 ppm of Figure 3A, however, persists in the spectrum of Figure 3B, consonant with our argument that this signal is indicative of a compound (e.g., **7**) formed by nonenzymatic processes. After treatment of the solution of Figure 3B with leucin aminopeptidase, only one methyl doublet—that corresponding to the β-methyl resonance of LAla—is seen to survive. This final spectrum suggests that all peptide (and ester) bonds of both substrates and intermediates, such as **7**, are cleaved and that only LAla and β-Cl-LAla are present after treatment with the aminopeptidase. These findings suggest, in turn, that both enzymatic and autoaminolytic processes ultimately would lead to the production of 2 equiv of β-Cl-LAla from 1 equiv of the chloroalanyl cephem ester **1**. It is perhaps not surprising, therefore, to have observed (Figure 2A) that a 10 mM solution of **1** affords

alanine racemase inactivation with a $t_{1/2}$ value (~7 min) nearly identical with that obtained when the enzyme is inactivated by 20 mM β-Cl-LAla alone.

The reactivity of cephalosporins to nucleophilic addition by amines is well understood. Indeed, the chemical fragmentation of cephalosporin C was first described by Abraham and coworkers (Hamilton-Miller et al., 1970a,b) in an ammonolytic reaction. We are not surprised to discover, therefore, that our amino acid and peptidyl cephem esters are likely to suffer concentration-dependent autoaminolysis. And we are alert to the fact that any potential therapeutic utility of compounds such as **1** will be constrained by these nonenzymatic, intermolecular reactions. As imipenem improved upon the chemical stability of thienamycin, perhaps a formimidoyl—or similarly substituted—derivative of **1** is indicated. The efficacy of compounds of this sort await both synthesis and testing.

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