Conformational Changes in Spin-Labeled Cephalosporin and Penicillin upon Hydrolysis Revealed by Electron Nuclear Double Resonance Spectroscopy

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Abstract: We have synthesized spin-labeled analogs of cephalosporin and penicillin and of their solvolytic methyl ester products for structural characterization by electron nuclear double resonance (ENDOR) spectroscopy. With β-lactamase I of *Bacillus cereus* 569/H/9 and the metallo-β-lactamase of *Bacillus cereus* 5/B/6, the spin-labeled penicillin and cephalosporin were shown to be as kinetically specific and catalytically active as the classical substrates benzylpenicillin and cephalosporin-C, respectively. Conformations of the spin-labeled antibiotics and of their methanolysis products have been compared on the basis of structure determination by ENDOR and molecular modeling. While the conformation of spin-labeled penicillin has been shown previously to be identical to that of amoxycillin (Mustafi, D.; Makinen, M. W. *J. Am. Chem. Soc.* **1995**, *117*, 6739−6746), the ENDOR-assigned conformation of spin-labeled cephalosporin is essentially identical to the X-ray-defined structure of cephaloglycine (Sweet, R. M.; Dahl, L. F. *J. Am. Chem. Soc.* **1970**, *92*, 5489−5507). We have also determined by ENDOR the location of a methanol molecule that is hydrogen-bonded to the pseudopeptide NH group of the spin-labeled cephalosporin. The ENDOR results assign the location of the methanol hydroxyl group on the sterically hindered *endo* side of the β-lactam ring. This position suggests that the hydroxyl group of the methanol molecule is poised for nucleophilic attack on the carbonyl carbon of the β-lactam ring. We have also compared the ENDOR-determined structures of the free β-lactam antibiotics and of their methanolysis products with the X-ray-defined structure of the benzylpenicilloyl acylenzyme intermediate of the E166N mutant of TEM-1 β-lactamase (Strynadka *et al., Nature* **1992**, 359, 700−705). The results showed that hydrolysis relieves geometrical strain in the fused β-lactam ring, as measured by the [N(1)−C(6)−C(7)−C(8)] dihedral angle in cephalosporin or by the corresponding [N(1)−C(5)−C(6)−C(7)] dihedral angle in penicillin. While the value of this dihedral angle is *eclipsed* at ∼0° for the intact antibiotic substrates and *staggered* at −35° for the benzylpenicilloyl intermediate, the methanolysis products exhibited *trans* conformations at −160°. The results illustrate how hydrolysis of the β-lactam bond in penicillin or cephalosporin antibiotics progresses favorably from a geometrically strained to a less strained conformation in the acylenzyme reaction intermediate and finally to an inherently conformationally stable product.

Introduction

The cephalosporins are a family of antibiotics that share the structural element of a β-lactam ring fused to a thiazoline ring. Both cephalosporin and the related penicillin antibiotics are hydrolyzed rapidly by β-lactamases, conferring to bacteria the basis of β-lactam antibiotic resistance.[2,3] It is generally accepted that β-lactamases form acylenzyme intermediates with penicillins and cephalosporins and that nucleophilic attack on the lactam carbonyl carbon results in formation of a tetrahedral adduct in both the solvolysis and enzyme-catalyzed reactions.[4,5] Whether the approach of the nucleophile is governed by purely steric interactions[5] or by stereoelectronic principles[6] is not clarified for the solvolytic and enzyme-catalyzed reactions. Stereoelectronic principles predict nucleophilic attack from the sterically constrained *endo* side with the incoming nucleophilic hydroxyl group antiperiplanar to the lone pair orbital on the lactam nitrogen atom.[6] On the other hand, nucleophilic attack by the active site serine residue in class A[7] and class C[8]

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β-lactamases is from the *exo* side, constituting an apparent contradiction to these requirements.

To better understand the important mechanistic aspects of the hydrolysis of β-lactams, we have synthesized cephalosporin and penicillin antibiotics and their solvolytic methanolysis products acylated at the amino nitrogen position by a paramagnetic nitroxyl spin-label for structural characterization by electron nuclear double resonance (ENDOR[9]) spectroscopy. Since the unpaired electron on the nitroxyl spin-label behaves like a point dipole located near the midpoint of the N−O bond of the nitroxyl group,[10] the hyperfine coupling between the unpaired electron and nearby magnetic nuclei can be measured accurately by ENDOR to obtain dipolar electron-nucleus distances over a 3−11 Å range with a precision that is exceeded only by that of X-ray diffraction methods.[10−13] This methodology provides an effective means for determination of structure and conformation of both small molecules and enzyme reaction intermediates in solution.

In this study, ENDOR analysis and molecular modeling were used to determine the structure and conformation of the spin-labeled cephalosporin 7-*N*-(2,2,5,5-tetramethyl-1-oxypyrrolinyl-3-carboxyl)cephalosporanic acid. The ENDOR-determined distances to specific protons in the fused thiazoline ring system describe a conformation that is essentially identical to the X-ray determined structure of cephaloglycine.[14] We have also determined the structure and conformation of the methanolysis products of spin-labeled cephalosporin, methyl 7-*N*-(2,2,5,5-tetramethyl-1-oxypyrrolinyl-3-carboxyl)cephalosporoate, and of spin-labeled penicillin, methyl 6-*N*-(2,2,5,5-tetramethyl-1-oxypyrrolinyl-3-carboxyl)penicilloate. By comparing the ENDOR-determined structures of spin-labeled cephalosporin and penicillin[12] and of their methyl ester solvolysis products with the X-ray defined structure of the benzylpenicilloyl acylenzyme intermediate of the E166N mutant of TEM-1 β-lactamase,[7a] the results illustrate the extent to which hydrolysis relieves geometrical strain in the fused β-lactam ring. While the C(6)−C(5) bond of the four-membered β-lactam ring was shown to be *eclipsed* in the free antibiotics and *staggered* in the benzylpenicilloyl acylenzyme, the methanolysis products exhibited a *trans* conformation. This latter conformation consequently must be associated with low potential energy relative to the conformers of the free substrate and acylenzyme reaction intermediate, and the corresponding change in potential energy must be an important driving force for cleavage of the lactam bond in both solvolytic and enzyme catalyzed reactions.

Experimental Procedures

General Materials. The parent spin-label 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxylic acid was obtained by hydrolysis of 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxamide (Aldrich Chemical Co., Inc., Milwaukee, WI 53233) according to the method of Rozantsev.[15] 7-aminocephalosporanic acid and 2-ethylhexanoic acid were obtained from Aldrich. The sodium salt of CEP-C[9] was obtained from Sigma Chemical Co. (St. Louis, MO 63178). Potassium 2-ethylhexanoate was prepared by dissolving clean potassium metal in *n*-butanol followed by the addition of 2-ethylhexanoic acid. Deuterated solvents (≥ 99.5% ^{2}H) were obtained from Cambridge Isotope Laboratories, Inc., (Woburn, MA 01801). All reagents were of analytical grade unless otherwise described. Deionized, distilled water was used throughout.

7-*N*-(2,2,5,5-Tetramethyl-1-oxypyrrolinyl-3-carboxyl)cephalosporanic Acid. The acid chloride of 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxylic acid (1.5 g, 8.2 mmol) was prepared, as previously described, to yield a dark, red oil.[11e] The oil was suspended in 20 mL of dry CH_2Cl_2 and added dropwise over a period of 1 h to a solution mixture of 7-aminocephalosporanic acid (3.5 g, 12.8 mmol) and 5.3 mL of triethylamine (38.6 mmol) in 40 mL of CH_2Cl_2 at 0 °C. The mixture was then brought to room temperature and stirred for another 2 h. The solvent was removed by evaporation *in vacuo*, and another 100 mL of CH_2Cl_2 was added and again removed. The residue was then dissolved in 40 mL of water and layered with 80 mL of ethyl acetate. The pH was adjusted to 2.3 with dropwise addition of 0.1 M HCl under stirring, and the ethyl acetate layer was separated and quickly washed with cold water. The organic extract was then dried over Na_2SO_4, and the organic layer reduced to 20 mL by rotary evaporation. A solution of potassium 2-ethylhexanoate (8.2 mmol) in 30 mL of ethyl acetate was added dropwise with stirring. The solution was concentrated to ∼4 mL, ether was added to complete precipitation, and the mixture was stored for 5 h at 4 °C. A light yellow, crystalline product was collected, generally with greater than 75% yield. Thin layer chromatography (TLC) showed a single spot [$R_f = 0.53$ in *n*-butanol:ethanol:water::4:1:5 (v/v)], and IR showed an intense band at 1765 cm^{-1} characteristic of the cyclic β-lactam group. Anal. Calcd for $C_{19}H_{23}N_3O_7SK$ (found): C, 47.89 (47.51); H, 4.86 (4.93); N, 8.82 (8.70); S 6.73 (6.64). Fast atom bombardment mass spectrometric analysis showed the highest molecular ion peak to correspond to the expected *m/e* ratio of 477.

Methyl 7-*N*-(2,2,5,5-Tetramethyl-1-oxypyrrolinyl-3-carboxyl)-cephalosporoate and Methyl 6-*N*-(2,2,5,5-Tetramethyl-1-oxypyrrolinyl-3-carboxyl)penicilloate. The methyl ester solvolysis products of SLCEP and of SLPEN[12] were prepared by refluxing 0.05 g of the corresponding cephalosporin or penicillin in 10 mL of dry methanol for 7 h according to the procedure described by Bicknell and Waley.[16] The solid product was obtained by removal of the solvent *in vacuo*. Upon completion of the reacton, only one spot for the product was observed by TLC. Fast atom bombardment mass spectrometric analysis showed the highest molecular ion peak to correspond to the expected *m/e* ratio of 509 for spin-labeled methyl cephalosporoate and 453 for spin-labeled methyl penicilloate. The observation of a single spot by TLC of the reaction product and the expected *m*/*e* ratio for the cephalosporate indicated that the cephalosporin nucleus was not degraded under these refluxing conditions.

We have similarly synthesized the [$^2H_3$]methyl ester derivatives of spin-labeled cephalosporin and penicillin using perdeuteriated methanol as the refluxing solvent. Each methanolysis product showed a single TLC spot identical to the corresponding protiated methyl ester.

Preparation of β-Lactamase I. This enzyme, isolated from *Bacillus cereus* 569/H/9, is a class A serine hydrolase and was purified as described by Myers and Shaw.[17] Stock solutions of the enzyme were prepared by dissolving the lyophilized enzyme slowly in 0.1 M NaCl buffered with 0.01 M sodium cacodylate to pH 7, followed by dialysis against the same buffer at 4 °C. Protein concentration was measured spectrophotometrically at 595 nm using Coomassie Brilliant Blue G-250 dye (Sigma) with bovine serum albumin and human albumin (Sigma) as standards[18] and on the basis of the molar absorptivity coefficient of

(9) The following abbreviations are used: CEP-C, cephalosporin-C; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; hf, hyperfine; hfc, hyperfine coupling; MOPS, 3-[*N*-morpholino]-propanesulfonic acid; rf, radiofrequency; SLCEP, spin-labeled cephalosporin; SLMeCEP, spin-labeled methyl cephalosporoate; SLMePEN, spin-labeled methyl penicilloate; SLPEN, spin-labeled penicillin; THF, tetrahydrofuran.

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26 000 M^{-1} cm^{-1} at 280 nm.[19] The difference in the determination of protein concentration using these two methods was less than 1%, indicating high homogeneity of the enzyme preparation.

Preparation of the Metallo-β-lactamase of *B. cereus* 5/B/6. This is a Zn^{2+}-metalloenzyme. Although the enzyme is normally produced by *B. cereus* 5/B/6, molecular genetics methods have been applied to clone the metallo-β-lactamase structural gene into an *Escherichia coli* expression vector, and this metallo-β-lactamase was isolated and purified as described previously.[20] The electrophoretic mobilities of the recombinant enzyme and of the *B. cereus* enzyme showed them to be identical in both native and sodium dodecyl sulfate gel electrophoresis. Protein concentration was measured spectrophotometrically on the basis of the molar absorptivity coefficient of 22 000 M^{-1} cm^{-1} at 280 nm.[16]

Enzyme Kinetics. Initial velocity data were collected spectrophotometrically to determine the steady-state kinetic parameters k_{cat} and k_{cat}/K_M for hydrolysis of CEP-C and SLCEP catalyzed by both types of β-lactamases. The change in absorbance at 264 nm for CEP-C ($\Delta\epsilon$ = 7344 M^{-1} cm^{-1}) or for SLCEP ($\Delta\epsilon$ = 3589 M^{-1} cm^{-1}) due to hydrolysis of the β-lactam group was followed with a Cary 15 spectrophotometer modified by On-Line Instrument Systems, Inc. (Jefferson, GA 30549) for microprocessor controlled data acquisition. Initial velocity data were evaluated with use of the algorithm ENZKIN provided by Professor J. Westley of the Department of Biochemistry and Molecular Biology at the University of Chicago, as previously described.[21]

EPR and ENDOR. EPR and ENDOR spectra were recorded with use of an X-band Bruker ESP 300E spectrometer equipped with a TM110 cylindrical cavity, Bruker ENDOR accessory, Oxford ESR910 liquid helium cryostat, and ESP 3220 data system for computer controlled data acquisition, as previously described.[22] Typical experimental conditions for ENDOR measurements were as follows: sample temperature, 20 K; microwave frequency, 9.45 GHz; incident microwave power, 2 mW; modulation frequency, 12.5 kHz; and rf modulation depth ≤ 8 kHz. The static laboratory magnetic field was not modulated for ENDOR. Spin-labeled samples for EPR and ENDOR studies were dissolved to a concentration of 4×10^{-3} M in perdeuteriated solvents.

Molecular Modeling. The atomic numbering schemes of spin-labeled cephalosporin and of the two methyl ester solvolysis products are illustrated in Figure 1. Atomic coordinates of the SLCEP were constructed on the basis of X-ray defined molecular fragments. Coordinates of non-hydrogen atoms of the spin-label moiety were taken from the X-ray defined structure of 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxamide,[23] and coordinates of the cephalosporin moiety were taken from the X-ray defined structure of cephaloglycine.[14] Construction of the molecular model of SLCEP followed that described for SLPEN.[12] The C(19) and O(20) atoms of the cephaloglycine were superimposed onto the corresponding C and O atoms of the carboxamide group of the spin-label so that the C(19)−N(18) bond vector of the cephaloglycine and the C(3′)−C(19) bond vector of the spin-label were preserved upon joining the two fragments to generate the SLCEP molecule. Positions of hydrogen atoms were calculated for idealized geometries with C−H and N−H bond lengths of 1.08 and 1.00 Å, respectively.

For molecular modeling of SLMeCEP and SLMePEN, idealized valence angles and bond lengths were used to convert the four-membered cyclic β-lactam ring into the corresponding methyl ester derivative. In the cephalosporoate, the values of the C(6)−C(7)−C(8) and C(7)−C(6)−N(1) valence angles were set at 112.0° and 109.9°, respectively, compared to their values of ∼90° in the intact β-lactam ring. Also, in the cephalosporoate, values of the valence angles of

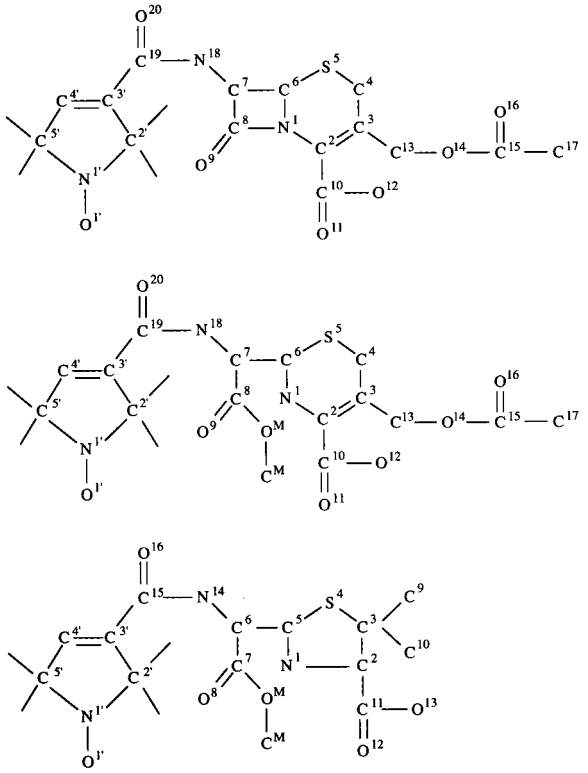

Figure 1. Comparison of the chemical bonding structures and atomic numbering schemes of SLCEP (upper), SLMeCEP (middle), and SLMePEN (lower).

104.4° for C(6)−N(1)−H(1), 116.0° for C(7)−C(8)−O(M), and 109.0° for C(8)−O(M)−C(M) were used. The SLMePEN molecule was similarly constructed according to idealized valence angles and bond lengths.

Molecular modeling was carried out with programs FRODO,[24] INSIGHT,[25] and SYBYL,[26] as previously described.[10−13] With the program package SYBYL, torsion angle calculations were carried out with SEARCH,[26] which checks van der Waals contacts among nonbonded atoms by scanning all possible torsional angles around rotatable bonds. It then identifies within van der Waals allowed conformational space those conformations that are compatible with ENDOR-determined electron-nucleus distances as added constraints, together with their respective uncertainties. Calculations were performed in 5° increments of torsion angle rotation. For SLCEP, the search calculations were applied for rotation around the C(3′)−C(19), C(19)−N(18), and N(18)−C(7) as well as the four bonds in the acetyloxymethyl group attached to the thiazoline ring at C(3). For spin-labeled methyl cephalosporoate, the search calculations were applied for rotation around the C(3′)−C(19), C(19)−N(18), N(18)−C(7), C(6)−C(7), C(7)−C(8), C(8)−O(M), and O(M)−C(M) bonds together with the four bonds in the acetyloxymethyl group attached to C(3). For the spin-labeled methyl penicilloate, the search calculations were applied for rotation around the seven bonds corresponding to those indicated above for the spin-labeled cephalosporoate molecule. The rotations were applied simultaneously to all bonds indicated above for each set of calculations to ensure that "frozen" parts of the molecule did not sterically constrain the allowed range of conformations. The effective position of the unpaired spin density of the nitroxyl group as a point dipole was applied as the reference position for distance constraints, as previously described.[10]

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Table 1. Comparison of Steady-State Kinetic Parameters of Hydrolysis of CEP-C and SLCEP Catalyzed by β-Lactamase I of *B. cereus* 569/H/9 and the Metallo-β-lactamase of *B. cereus* 5/B/6

substrate	enzyme	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (M^{-1} s^{-1})
CEP-C	β-lactamase I[a]	8	8.2×10^{-5}	9.7×10^{4}
SLCEP	β-lactamase I[a]	10	2.9×10^{-4}	3.5×10^{4}
CEP-C	metallo-β-Lactamase[b]	288	4.0×10^{-4}	7.2×10^{5}
SLCEP	metallo-β-Lactamase[b]	104	2.9×10^{-4}	3.6×10^{5}

[a] At 24 °C in aqueous solution containing 0.1 M NaCl buffered with 0.01 M cacodylate to pH 7.0. [b] At 24 °C in aqueous solution containing 0.001 M $ZnSO_4$ buffered with 0.025 M MOPS to pH 7.0.

Results

A. Steady-State Kinetic Studies. Kinetic parameters governing the hydrolysis of CEP-C and SLCEP catalyzed by both the class A serine hydrolase β-lactamase I and the class B metallo-β-lactamase were determined on the basis of initial velocity data and are compared in Table 1. The values of k_{cat} and K_M for CEP-C in Table 1 are similar to those reported by others.[27] Comparison of the kinetic parameters for hydrolysis of SLCEP to those for CEP-C catalyzed by either enzyme shows that SLCEP is as kinetically specific and catalytically reactive as is CEP-C. We have previously reported that the kinetic reactivity of spin-labeled penicillin with β-lactamase I is equivalent to that of benzylpenicillin.[12]

Since the hydrolysis of cephalosporin derivatives by β-lactamase I is kinetically sluggish compared to penicillin derivatives, we have determined the pH and pD dependence of the kinetic parameters k_{cat} and k_{cat}/K_M that govern enzyme catalyzed hydrolysis of SLPEN. The results showed that k_{cat} and k_{cat}/K_M are governed by two ionizing groups in the enzyme with pK_a values of ~4.17 and ~8.72. A kinetic shift of +0.30 units is observed for the ionization with pK_a ~4.17 in the presence of ^2H_2O, while the alkaline ionization is not altered. These results are in good agreement with the pK_a values of 4.85 and 8.6 reported for the β-lactamase I catalyzed hydrolysis of benzylpenicillin.[28]

B. ENDOR of Spin-Labeled β-Lactam Antibiotics. 1. SLCEP. For nitroxyl spin-labels, the g_z component of the $\mathbf{g}_e$ matrix is coincident with the molecular *z* axis and perpendicular to the molecular *x,y* plane. In spin-labels, *g* anisotropy is very small, *A* anisotropy is axially symmetric, and the $\mathbf{g}_e$ and **A** axes are coincident. Therefore, separate application of $\mathbf{H}_0$ at different EPR absorption features, namely along the molecular *z* axis and along the *x,y* plane, provides a means of selection of different molecular orientations for ENDOR with correspondingly distinguishable hfc components. Microwave power saturation of the low-field (or high-field) region, termed *setting A*, selects molecules for which the five-membered oxypyrrolinyl ring of the spin-label is perpendicular to the applied field while saturation of the central feature of the EPR spectrum of spin-labels in frozen solutions, termed *setting B*, selects for ENDOR essentially all orientations of the spin-label with respect to the applied magnetic field. We have previously described this stratagem of employing the magnetic field dependence of the EPR absorption of nitroxyl spin-labels for selection of molecular orientation in ENDOR studies.[10−13]

Figure 2 illustrates proton ENDOR spectra of SLCEP with $\mathbf{H}_0$ at the low-field turning point of the EPR spectrum. With this setting of $\mathbf{H}_0$, only the perpendicular hfc components for protons in the cephalosporin moiety are observed. By comparing the two spectra in Figure 2, the ENDOR features labeled $a_\perp$ are attributed to the perpendicular hfc component of H(18). With respect to the hfc splitting and its relationship in the spectrum to $a_\perp$, the pair of resonance features labeled $b_\perp$ is identical to the resonances attributed to H(6) in spin-labeled penicillin[12] and to H^α of amino acids[11b−d] similarly spin-labeled at the amino nitrogen. Since these are chemically and structurally equivalent hydrogens, we assign $b_\perp$ features to H(7). Two other pairs of resonance features $c_\perp$ and $d_\perp$ are also seen in Figure 2. These resonance features most likely originate in the two other classes of hydrogens in the cephalosporin moiety, namely, H(6) and H(4). The assignments of these features are discussed later.

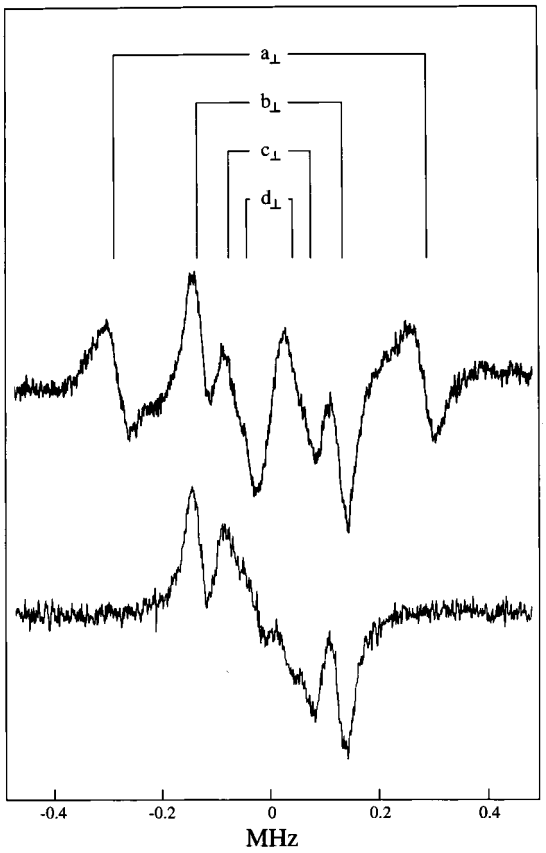

Figure 2. Proton ENDOR spectra of SLCEP in 50:50 (v/v) [$^2H_6$]-DMSO:[$^2H_4$]methanol. The magnetic field was at setting A of the EPR spectrum. For the top spectrum, the sample was first dissolved in DMSO and then rapidly mixed with [$^2H_4$]methanol and frozen in liquid nitrogen to prevent complete exchange of H(18) with solvent deuterons. The lower spectrum was obtained after thawing the sample to allow exchange of the amide proton. Three other line pairs are identified in the stick diagram and are assigned to the perpendicular hfc components of specific protons (see text). The abscissa indicates the ENDOR shift (measured ENDOR frequency minus the proton Larmor frequency).

In our previous ENDOR study of SLPEN, we demonstrated that a tightly bound solvent molecule is hydrogen bonded to H(14) of the pseudopeptide NH group.[12] Figure 3 compares proton ENDOR spectra of SLCEP dissolved in an aprotic solvent to which methanol was added in 1:1 stoichiometry. In spectrum I, the parallel and perpendicular hfc components are assigned for H(18) and H(7), labeled $a_\parallel$, $a_\perp$, and $b_\parallel$, $b_\perp$, respectively. In spectrum II, two line pairs are seen that are not present in spectrum I and are assigned to the parallel and perpendicular hfc components of the hydroxyl proton of the methanol molecule. These new features are designated by the stick diagram in the lower part of the figure. Because of the sharp and intense resonance features for the solvent hydroxyl

(27) (a) Buckwell, S. C.; Page, M. I.; Longridge, J. L.; Waley, S. G. *J. Chem. Soc., Perkin Trans. II* **1988**, 1823−1827. (b) Gibson, R. M.; Christensen, H.; Waley, S. G. *Biochem. J.* **1990**, *272*, 613−619. (c) Murphy, B. P.; Pratt, R. F. *Biochem. J.* **1989**, *258*, 765−768.

(28) Waley, S. G. *Biochem. J.* **1975**, *149*, 547−551.

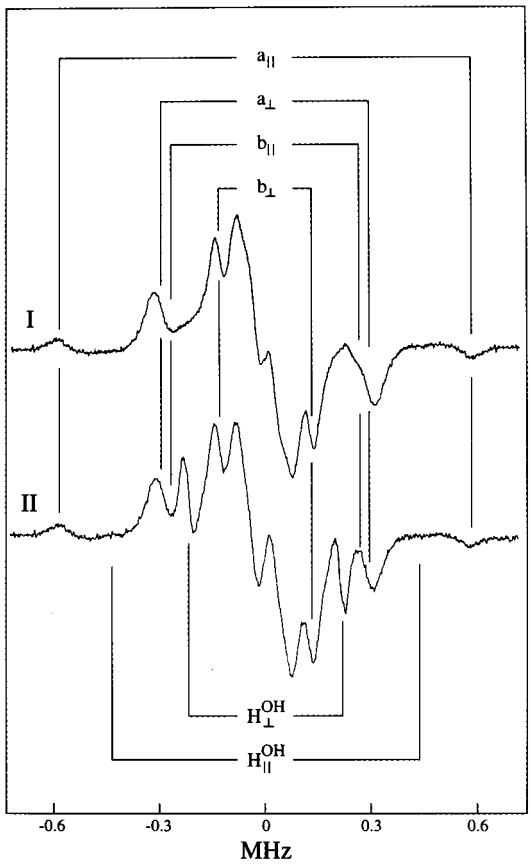

Figure 3. Proton ENDOR spectra of SLCEP in different solvents to illustrate a tightly bound methanol molecule. Solvent conditions are (I), 50:25:25 (v/v) [$^2H_6$]DMSO:[$^2H_8$]toluene:[^{2}H]chloroform to which [$^2H_4$]methanol was added in 1:1 stoichiometry (with respect to SLCEP) and (II), 50:25:25 (v/v) [$^2H_6$]DMSO:[$^2H_8$]toluene:[^{2}H]chloroform to which [$^2H_3$]methanol was added in 1:1 stoichiometry. These spectra were taken with $\mathbf{H}_0$ at setting B of the EPR spectrum. Both parallel and perpendicular hfc components of the methanol hydroxyl proton are identified in spectrum II. Other line pairs labeled *a* and *b* are assigned to H(18) and H(7), respectively, as described in the text. To detect the proton resonances assigned to the hydrogen-bonded methanol molecule, as shown in spectrum II, it was necessary to avoid all possible sources of adsorbed moisture. Therefore, only perdeuteriated solvents of the highest grade (99.6% ^{2}H) were used and vials of solvents were freshly opened only immediately prior to use. Acid-washed EPR sample tubes were kept in a desiccator prior to use.

proton, we conclude that these features must derive from a tightly bound methanol OH group, similar to that observed in SLPEN.[12]

In Table 2 are summarized the principal hfc components of specific protons of SLCEP identified in the ENDOR spectra of Figures 2 and 3. Under the conditions $|A_{iso}| << |A_{||}|$, $|A_{\perp}|$ and $A_{||}^{D} > 0 > A_{\perp}^{D}$, the dipolar hfc components $A_{||}^{D}$ and $A_{\perp}^{D}$ have been calculated using the constraint $(A_{||} + 2A_{\perp}) = 3A_{iso}$. In Table 2 we have listed the values of isotropic and dipolar hfc components for protons together with corresponding electron−proton separations calculated on the basis of the dipolar equation. Since the isotropic contributions of distant protons are small, as seen for H(18), H(7), H(4), and H^{OH}, we have calculated the value of *r* for H(6) solely on the basis of its observed perpendicular hfc component.

2. Proton ENDOR Spectra of the Methanolysis Reaction Product of SLCEP. Figure 4 compares proton ENDOR spectra of SLMeCEP in deuteriated solvents to identify resonance features of exchangeable protons. In spectrum I, only the perpendicular hfc component for H(7) is clearly observed in addition to the largely unresolved resonances of thiazoline

Table 2. Summary of hfc Components (*A*, MHz) and Estimated Electron−Proton Distances (*r*, Å) in SLCEP

proton	$A_{||}$	$A_{\perp}$	A_{iso}	$A_{||}^{D}$	$A_{\perp}^{D}$	r^a
			Cephalosporin[b]			
H(18)	1.167	0.581	0.002	1.165	−0.583	5.13 ± 0.02
H(7)	0.537	0.268	0.000	0.537	−0.268	6.66 ± 0.03
H(6)	—	0.150	—	—	−0.150	8.08 ± 0.14[c,d]
H(4)	0.166	0.085	−0.001	0.167	−0.084	9.77 ± 0.22[d]
			Solvent			
H^{OH}	0.875	0.439	−0.001	0.876	−0.438	5.66 ± 0.03

[a] Uncertainty in frequency of 0.010−0.015 MHz due to each ENDOR absorption is included in the calculation of electron−proton distances. [b] See the atomic numbering scheme in Figure 1 for atom designations. [c] For H(6), the electron−proton distance is calculated from its perpendicular hfc component alone. [d] Assignments of the dipolar distances to H(6) and H(4) are based on molecular modeling studies, as discussed in the text.

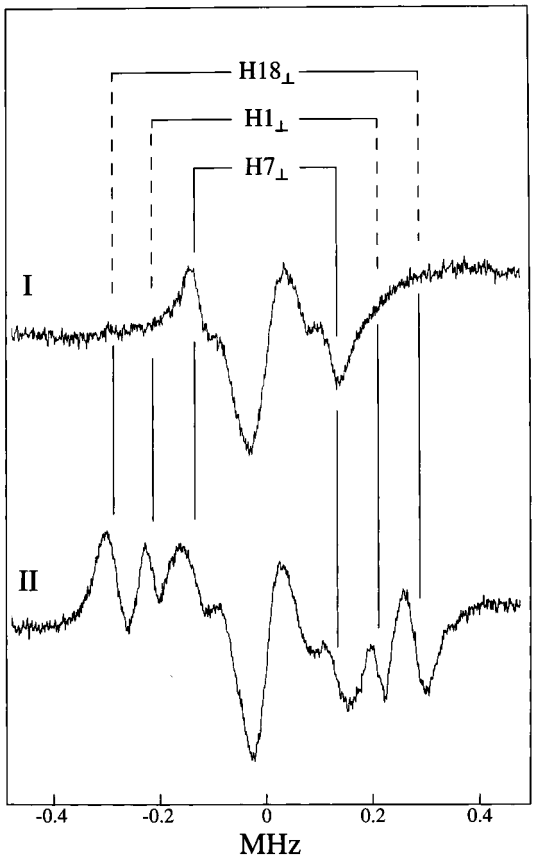

Figure 4. Proton ENDOR spectra of spin-labeled [$^2H_3$]methyl cephalosporoate with $\mathbf{H}_0$ at setting A. Spectrum I, [$^2H_4$]methanol. Spectrum II, 50:25:25 (v/v) [$^2H_6$]DMSO:[^{2}H]chloroform:[$^2H_8$]toluene. The ENDOR line pairs are identified in the stick diagram and are assigned to specific exchangeable protons H(18) and H(1) as well as to H(7), as discussed in the text.

protons that are clustered near the Larmor frequency, as in Figure 2. Spectrum II was recorded in a ternary perdeuteriated aprotic solvent system with the same magnetic field setting as for spectrum I, under which conditions exchangeable protons remain bonded to the molecule. Two prominent resonance features are observed in spectrum II that are not observed in spectrum I. In spectrum II the perpendicular hfc component labeled H18$_{\perp}$ has a similar relationship to H7$_{\perp}$, as between $a_{\perp}$ and $b_{\perp}$ in Figure 2, identifying its origin as H(18). The second prominent resonance feature in spectrum II due to an exchangeable proton is assigned to the perpendicular hfc component of H(1). The intensity and the line width of the resonance features of H(1) in Figure 4 are clearly different from the ENDOR

Table 3. Summary of hfc Components (in MHz) and Estimated Electron−Proton Distances in SLMeCEP and in SLMePEN

proton	$A_{\|\|}$	$A_{\perp}$	A_{iso}	$A_{\|\|}^{D}$	$A_{\perp}^{D}$	r^a (Å)
			Cephalosporoate			
H(18)	1.162	0.582	−0.001	1.163	−0.581	5.14 ± 0.02
H(7)	0.541	0.274	−0.002	0.543	−0.272	6.63 ± 0.04
H(1)	0.855	0.429	−0.001	0.856	−0.428	5.70 ± 0.04
			Penicilloate			
H(14)	1.170	0.584	0.001	1.170	−0.585	5.13 ± 0.03
H(6)	0.540	0.272	−0.001	0.541	−0.271	6.64 ± 0.05
H(1)	0.854	0.429	−0.001	0.855	−0.428	5.70 ± 0.04

[a] Uncertainty in frequency of 0.010−0.015 MHz due to line width of each absorption is included in the calculation of electron−proton distances.

Table 4. Values of Dihedral Angles of the ENDOR Constrained Conformations of SLCEP, SLMeCEP, SLPEN,[a] and SLMePEN

dihedral angle	β-lactam antibiotic[b] (deg)		methyl ester product (deg)
	Cephalosporin		
[C(4′)═C(3′)-C(19)-N(18)]	−35 ± 3	(−33)	−30 ± 3
[C(3′)-C(19)-N(18)-C(7)]	−170 ± 3	(175)	−172 ± 2
[C(19)-N(18)-C(7)]	−118 ± 4	(−138)	−135 ± 10
[C(8)-C(7)-C(6)-N(1)]	2	(2)	−153 ± 10
	Penicillin		
[C(4′)═C(3′)-C(15)-N(14)]	−35 ± 5	(−30)	−40 ± 5
[C(3′)-C(15)-N(14)-C(6)]	−168 ± 3	(−172)	−165 ± 5
[C(15)-N(14)-C(6)-C(7)]	−118 ± 14	(−129)	−135 ± 15
[C(7)-C(6)-C(5)-N(1)]	9	(9)	160 ± 10

[a] Values of dihedral angles for spin-labeled penicillin are taken from ref 12. [b] Values of dihedral angles for cephalosporin and penicillin molecules given in parentheses are derived from X-ray defined structures of cephaloglycine[14] and amoxycillin,[29] respectively.

features observed for the methanol hydroxyl proton in Figure 3. The parallel hfc components for H(1) and H(18) were also assigned from ENDOR spectra with $\mathbf{H}_0$ setting at B (data not shown) together with their corresponding perpendicular hfc components, as shown here. No evidence for tightly bound solvent molecules could be found for SLMeCEP comparable to the H^{OH} resonance features assigned in Figure 3 for SLCEP. For SLMePEN, we have assigned similarly both parallel and perpendicular hfc components for H(1), H(6), and H(14), and no evidence for a tightly bound solvent molecule was observed. Also, we have not been able to resolve the resonance features of the ester methyl protons in SLMeCEP or in SLMePEN.

In Table 3 we have summarized the principal hfc components of specific protons of SLMeCEP and SLMePEN identified in ENDOR spectra. We have also listed the values of isotropic and dipolar hfc components for protons together with their corresponding electron−proton separations calculated on the basis of the dipolar equation. As seen in Table 3, electron−proton distances for these three classes of protons in SLMeCEP are virtually identical to those of the corresponding protons in SLMePEN.

C. Conformation of Spin-Labeled β-Lactam Antibiotics. The general methodology of applying torsion angle search calculations to define molecular conformation constrained by ENDOR-determined electron−proton distances has been described by us previously.[10−13] The results of torsion angle search calculations constrained by ENDOR-determined electron−proton distances, and their uncertainties within van der Waals hard-sphere limits are summarized in Table 4. While for SLCEP only two ENDOR determined distance constraints to H(18) and H(7) were employed, in SLMeCEP a third distance constraint to H(1) could be applied, in addition to those of H(18) and H(7). For purposes of comparison we have included, in addition to the results for SLCEP, SLMeCEP, and SLMePEN, the corresponding values for SLPEN, the latter having been determined in a previous study.[12] For SLCEP and SLPEN we have also compared the value of each dihedral angle with those derived from X-ray defined structures of cephaloglycine[14] and amoxycillin,[29] respectively, from which the molecular models of the spin-labeled antibiotics were constructed.

As evident in Table 4, the conformation of the spin-labeled moiety in SLCEP defined by the torsion angle of −35° ± 3° around the C(3′)−C(19) bond is in excellent agreement with the structure of the parent spin-labeled carboxamide. These results also assign a planar *trans* conformation to the pseudopeptide C(19)−N(18) bond, as seen earlier in the ENDOR analyses of SLPEN[12] and of spin-labeled amino acids.[11b−d] The [C(19)−N(18)−C(7)−C(8)] dihedral angle of −118° ± 4° in SLCEP is identical to that in SLPEN and is comparable to the corresponding X-ray defined dihedral angle in cephaloglycine.[14] Also, as seen in Table 4, comparison of the dihedral angles of SLMeCEP and SLMePEN shows that their ENDOR defined conformations are very nearly identical. The only significant difference in the dihedral angles between the free substrates and their respective methyl ester solvolysis products is that associated with rotation around the C(7)−C(6) bond and the C(6)−C(5) bond, respectively. As seen in Table 4, upon hydrolysis of the β-lactam group, the four-membered β-lactam ring changes its orientation from *eclipsed* to an essentially *trans* structure. This difference is illustrated in Figure 5 in which we have compared the ENDOR-derived conformations of SLCEP and of its methyl ester product.

In Figure 5, three ENDOR-defined hydrogen atoms H(1), H(7), and H(18), to which the ENDOR determined distances specify the conformation of SLMeCEP, are indicated in addition to non-hydrogen atoms. We note that H(1) bonded to N(1) makes an angle of 40° with the plane of the oxypyrrolinyl ring at the position of the effective point dipole of the N−O group. The broader resonance features of H(1), as seen in Figure 4, compared to those of the hydroxyl group of the hydrogen bonded methanol molecule in Figure 3, are, thus, consistent with its greater out-of-plane position. Essentially identical structures were found for the corresponding penicillinoyl derivatives as shown for cephalosporoyl derivatives in Figure 5.

In Table 5, we have compared ENDOR-determined electron−proton distances with corresponding values in the resultant conformer of SLCEP assigned through constrained torsion angle search calculations. Corresponding results were similarly compared also for SLPEN.[12] As discussed above, the conformational search calculations were performed with the ENDOR-determined distance constraints only to H(18) and H(7). The predicted distances from the nitroxyl group to the methylene and methyl protons of the acetyloxymethyl group attached to the thiazoline ring are found to be ∼11 and 14 Å, respectively. The absorption features of these protons appear near the Larmor frequency in the ENDOR spectra, and, therefore, could not be adequately resolved for structure determination. On the other hand, as seen in Table 5, the model derived distances to H(6) and H(4) are in excellent agreement with the corresponding ENDOR-determined values of *r*, and only one very small family of conformers is compatible with the ENDOR data. In contrast, the distance constraints for assignment of the structure of SLPEN resulted in a 2-fold conformational degeneracy.[12] In that case, however, the ENDOR features of three classes of protons in the thiazolidine ring were resolved, and comparison of the corresponding ENDOR estimated electron−proton distances to their model derived counterparts ruled out one of the conformers for structure assignment.

(29) Boles, M. O.; Girven, R. J.; Gane, P. A. C. *Acta Crystallogr., Sect. B* **1978**, *34*, 461−466.

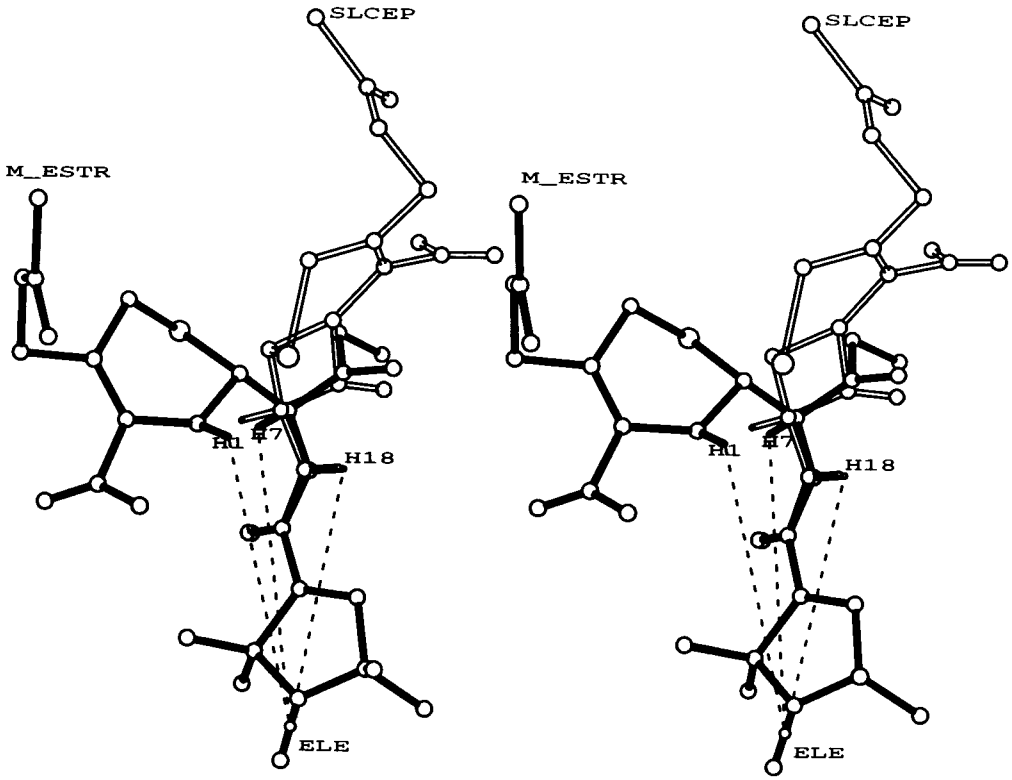

Figure 5. Stereodiagram comparing the ENDOR-determined conformations of spin-labeled cephalosporin, illustrated by open bonds and labeled SLCEP, and of spin-labeled methyl cephalosporoate illustrated by solid bonds and labeled M_ESTR. The conformation of the product is defined by the ENDOR-determined electron−proton distances to H(1), H(7), and H(18), indicated by broken lines. The effective position of the electronic point dipole is labeled ELE. The two molecules are superpositioned onto each other by the five atoms of the oxypyrrolinyl ring and the carboxyl C(19) atom.

Table 5. Comparison of ENDOR Determined Electron−Proton Distances (in Å) in SLCEP, with Model Distances Obtained by Torsion Angle Search Calculations

proton	ENDOR	conformer found[a]
H(18)	5.13 ± 0.02	5.12
H(7)	6.66 ± 0.03	6.66
H(6)	8.08 ± 0.14	8.08
H(4)[b]	9.77 ± 0.22	9.58
H(13)[b]	—	10.76
H(15)[b]	—	13.89

[a] ENDOR-determined conformer was derived from torsion angle search calculations based on the electron−proton distance constraints to H(18) and H(7) only. [b] The geometrically averaged values of idealized hydrogens are compared. See atomic numbering scheme for atom designations.

D. Location of a Methanol Molecule Hydrogen-Bonded to SLCEP. The ENDOR results in Figure 3 demonstrated that a methanol molecule is tightly bound to SLCEP. The location and orientation of the methanol molecule were defined on the basis of three constraints: (*i*) the sharp and intense resonance features assigned to H^{OH} indicate that the methanol molecule is tightly bound to a group on the spin-labeled cephalsoporin; (*ii*) H^{OH} must lie on the surface of a sphere of radius 5.66 Å centered on the effective point dipole of the nitroxyl group;[10] and (*iii*) H^{OH} is exactly or nearly exactly coincident with the plane of the oxypyrrolinyl ring because of the dependence of its ENDOR-determined hfc patterns on the settings of the applied magnetic field. Similar spectroscopic constraints correspondingly define the position of a tightly bound methanol molecule in the case of SLPEN.[12]

These three constraints are satisfied by hydrogen bonding of the hydroxyl group only to the pseudopeptide NH group of SLCEP. As shown in Figure 6, the methanol molecule is located on the *endo* or concave side of the β-lactam group within a cleft formed by the solvent accessible surface. The calculated solvent accessible surface of the SLCEP molecule neatly accommodates the methyl group and the hydroxyl group of the methanol molecules so that an $N-H\cdots O_{MeOH}$ donor−acceptor distance of ∼2.84 Å and an $N-H\cdots O_{MeOH}$ angle of ∼139° is realized, consistent with structural parameters of nitrogen and oxygen containing groups for optimal stabilization by hydrogen bonding.[31] In the X-ray defined structure of cephaloglycine, a water molecule is similarly observed hydrogen bonded to the pseudopeptide NH group.[14] The donor-to-acceptor D−H···A distance between the oxygen atom of the hydrogen-bonded water molecule and the peptide nitrogen atom of cephaloglycine was reported as 2.72 Å.[14] Essentially identical relationships are observed for methanol hydrogen bonded to the pseudopeptide NH group of SLPEN[12] and hydrogen bonded water molecules forming the crystal structure of amoxycillin.[29]

Discussion

A. Mechanism of Solvolysis of β-Lactam Antibiotics: Stereoelectronic Considerations. Despite the extreme geometrically strained structure of the fused, four-membered ring of β-lactam antibiotics, they are remarkably stable in aqueous solution. Since nucleophilic attack from the *exo* side must be viewed as sterically facile, the chemical stability of the β-lactam group in solution is, therefore, enigmatic. As originally pointed out by R. B. Woodward, the fused β-lactam ring is chemically stable because the sterically preferred *exo* mode of attack is not stereoelectronically allowed while the stereoelectronically favored *endo* attack is sterically hindered.[6c] In the case of the β-lactam group, stereoelectronic principles require that the

(30) Lee, B.; Richards, F. M. *J. Mol. Biol.* **1971**, *55*, 379−400.
(31) Jeffrey, G. A.; Saenger, W. *Hydrogen Bonding in Biological Structures*; Springer Verlag: Berlin, 1991; p 569.

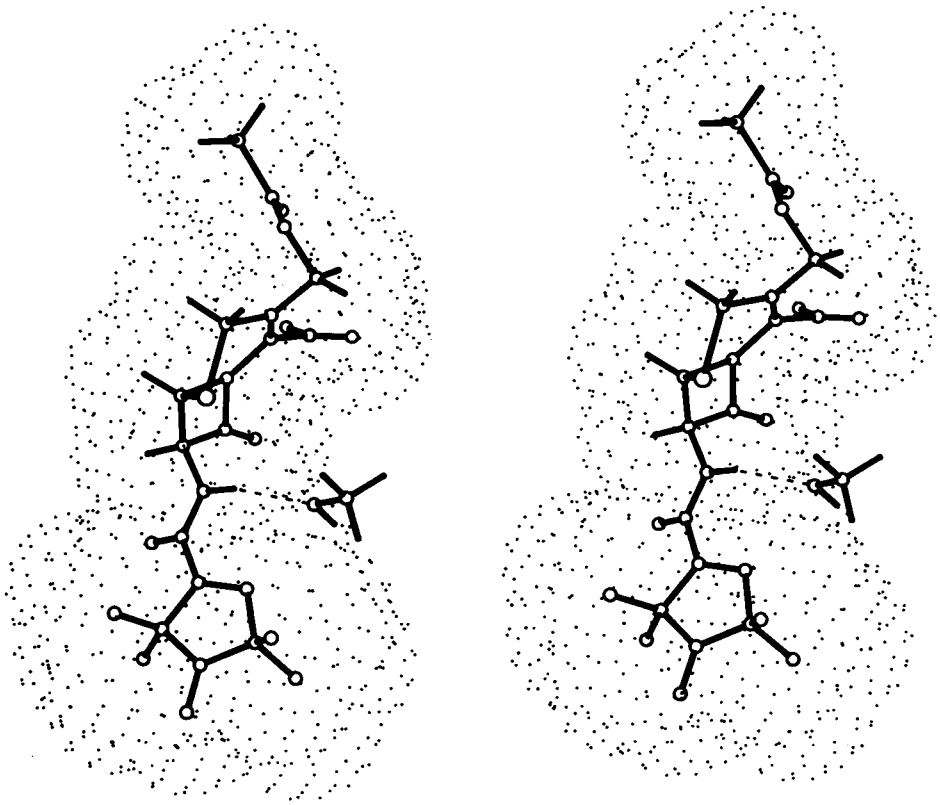

Figure 6. Stereodiagram of the ENDOR-determined conformation of SLCEP and the hydrogen-bonded methanol molecule. The solvent accessible surface of the spin-labeled cephalosporin, calculated according to the Lee-Richards algorithm,[30] is shown by the dotted surface. The hydrogen bond between the pseudopeptide NH and O_{MeOH} is indicated by the broken line.

nucleophile approach the carbonyl carbon antiperiplanar to the lone pair orbital on the N(1) atom.[6a,b]

Since hydrolysis of β-lactam antibiotics occurs via formation of a tetrahedral species, it is insightful to relate the ENDOR-determined structure of SLCEP with its hydrogen bonded methanol molecule to mechanistic aspects of the hydrolytic breakdown of the β-lactam ring. We have inspected the van der Waals constraints of the methanol:SLCEP complex to determine the steric limitations of small shifts in the position of the methanol molecule. Through detailed consideration of the van der Waals radii of atoms comprising the fused ring system, and, in particular, D−H···A distance constraints for hydrogen bonding to the pseudopeptide NH, the carbonyl O, and the thiazoline S, we found that migration of the hydrogen bonded methanol OH group from its ENDOR-defined location is sterically feasible. Thus, through displacement or migration of the methanol molecule, its hydroxyl group could become an attacking nucleophile in the hydrolytic reaction, forming a tetrahedral adduct at the lactam carbonyl carbon through a general base catalyzed mechanism. A cluster of water or solvent molecules hydrogen bonded to each other and to the pseudopeptide NH group and the β-lactam carbonyl oxygen, as observed in the crystal structure of amoxycillin,[29] would provide the basis for the base catalyzed process, as has been suggested by Sinnott.[5c]

Although sterically hindered nucleophilic attack from the *endo* side of the β-lactam group of penicillin and cephalosporin has been viewed as unlikely,[5] the steric compatibility for nucleophilic approach by the hydrogen bonded methanol molecule to the carbonyl carbon provides a strong argument in favor of *endo* attack in view of similarly positioned solvent molecules hydrogen bonded to the pseudopeptide NH group. Nucleophilic attack by the methanol molecule initially hydrogen bonded to the pseudopeptide NH group would then occur antiperiplanar to the lone pair of the lactam nitrogen, approaching the lactam amide π-electron structure at a nearly perpendicular angle. This approach is consistent with structural data defining the path of nucleophilic O···C═O attack.[32] These structural considerations are, in fact, not only compatible with stereoelectronic rules but also would be compatible with the principle of least nuclear motion that has also been invoked to explain chemical reactivity of the β-lactam group.[5b,c] We, consequently, favor the interpretation that solvolysis of β-lactam antibiotics proceeds via nucleophilic attack on the *endo* side of the molecule. We have discussed these relationships previously in the context of ENDOR studies of SLPEN.[12]

B. Comparison of Structures of β-Lactam Antibiotics in Solution to the Acylenzyme of TEM-1 β-Lactamase. It is insightful to relate the ENDOR-determined structures of the spin-labeled derivatives of penicillin and cephalosporin substrates and of their methyl ester products to the X-ray defined structure of the benzylpenicilloyl moiety in the acylenzyme reaction intermediate of TEM-1 β-lactamase.[7a] This acylenzyme species was rendered to be sufficiently chemically stable for structure analysis through site directed mutagenesis by converting an important residue in deacylation Glu-166 into Asn.

In Figure 7, we compare the ENDOR determined structures of SLPEN and SLMePEN with the X-ray defined structure of the benzylpenicilloyl moiety of the acylenzyme intermediate. The structures are superposed on the basis of the C(15), N(14), and C(6) atoms common to both benzylpenicillin and SLPEN and SLMePEN. The salient result evident from this comparison is that the change in conformation from the substrate to the acylenzyme intermediate and finally to the product can be

(32) (a) Bürgi, H. B.; Dunitz, J. D.; Shafter, E. *Acta Crystallogr.* **1974**, *1330*, 1517−1527. (b) Bürgi, H. B.; Dunitz, J. D; Lehn, J. M.; Wipff, G. *Tetrahedron* **1974**, *30*, 1563−1572. (c) Dunitz, J. D. *Phil. Trans. R. Soc. Lond. Series B* **1975**, *272*, 99−108.

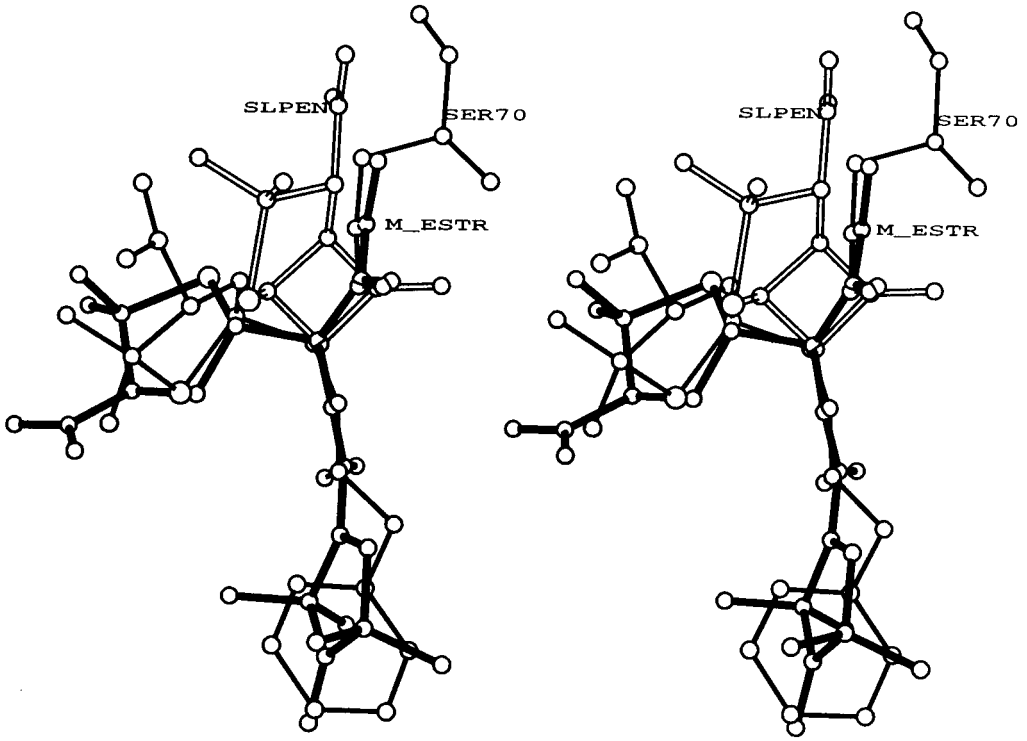

Figure 7. Stereodiagram comparing the ENDOR-determined structures of SLPEN (open bonds) and SLMePEN (solid bonds) with the X-ray defined structure of the benzylpenicilloyl moiety of the acylenzyme intermediate of TEM-1 β-lactamase (thin bonds). The molecules are labeled SLPEN, M_ESTR, and SER70, respectively.

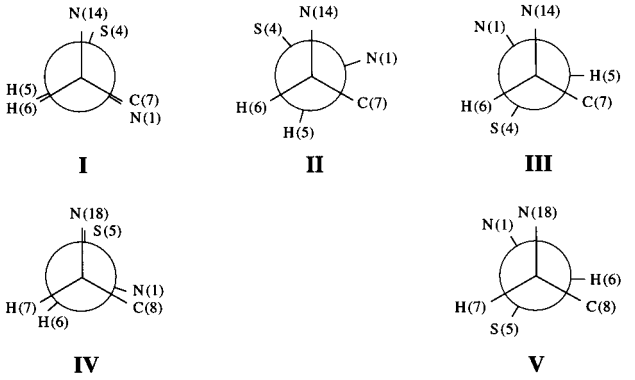

Figure 8. In the upper part, Newman diagrams illustrating the projection down the C(6)−C(5) bond for spin-labeled penicillin (**I**), the benzylpenicilloyl acylenzyme intermediate of TEM-1 β-lactamase (**II**), and spin-labeled methyl penicilloate (**III**). In the lower part, Newman diagrams illustrating the projection down the C(7)−C(6) bond for SLCEP (**IV**) and spin-labeled methyl cephalosporoate (**V**). The dihedral angles are drawn according to the ENDOR-determined structures of **I**, **III**, **IV**, and **V** and the X-ray defined structure of **II**.[7a]

viewed as occurring in a unidirectional manner. In Figure 7 comparison of the three structures to demonstrate conformational changes is facilitated by their common basic penicillin structure. However, similiar comparison of the ENDOR determined structures of the cephalosporin analogs SLCEP and SLMeCEP with the X-ray defined structure of the benzylpenicilloyl acylenzyme intermediate of TEM-1 showed comparable changes, as seen in Figure 7 for penicillin derivatives.

Figure 8 illustrates Newman diagrams of conformations of the three species in Figure 7 with respect to the C(6)−C(5) bond. This figure illustrates the extent to which hydrolysis relieves geometrical strain in the fused four-membered β-lactam ring. The conformations of the free substrates are eclipsed, as seen in diagrams **I** for SLPEN and **IV** for SLCEP. The conformation of the acylenzyme intermediate around the C(6)−C(5) bond is slightly more relaxed, with [N(14)−C(6)−C(5)−S(4)] and [C(7)−C(6)−C(5)−N(1)] dihedral angles near −36° and −33°, respectively. The methyl ester product, however, is the least geometrically strained, with a nearly *anti* conformation as measured by [N(14)−C(6)−C(5)−S(4)] and [C(7)−C(6)−C(5)−N(1)] dihedral angles of −145° and −160°, respectively. Figure 8 provides an illustrative example of how hydrolysis of spin-labeled penicillin or cephalosporin progresses favorably from a conformation of high potential energy, due to the eclipsed relationship with respect to the C(6)−C(5) bond in penicillin or to the C(7)−C(6) bond in cephalosporin to a less strained intermediate, and finally to an inherently stable product which is of lowest potential energy.

C. Mechanism of Hydrolysis of β-Lactam Antibiotics. The mechanism of hydrolysis of β-lactam antibiotics catalyzed by the serine hydrolase classes of β-lactamases involves formation of an acylenzyme with the nucleophilic side chain of Ser-70.[7,8,33] While the $-CH_2OH$ side chain is chemically analogous to the methanol molecule, X-ray crystallographic studies[7a] of the benzylpenicilloyl acylenzyme species of a deacylation defective mutant of TEM-1 β-lactamase shows that the nucleophile approaches the carbonyl carbon from the *exo* side, in apparent contradiction of the requirements of stereoelectronic rules. In addition to stereoelectronic considerations, the approach of a nucleophilic group with high electron density would be energetically disfavored because of the high electron density on the *exo* side of the β-lactam ring through the presence of the lone pair orbital on the nitrogen.[34] Thus, nucleophilic attack in the enzyme catalyzed reaction must circumvent this problem either through inversion of conformation around the β-lactam nitrogen, a process that we consider extremely unlikely for the intact antibiotic, or protonation of the nitrogen from the *exo* side by an active site residue, either prior to or concerted

(33) The mechanism and catalytic roles of active site residues in class B metallo-β-lactamases have not been assigned.

(34) We are indebted to Professor P. Deslongchamps for pointing out these relationships to us.

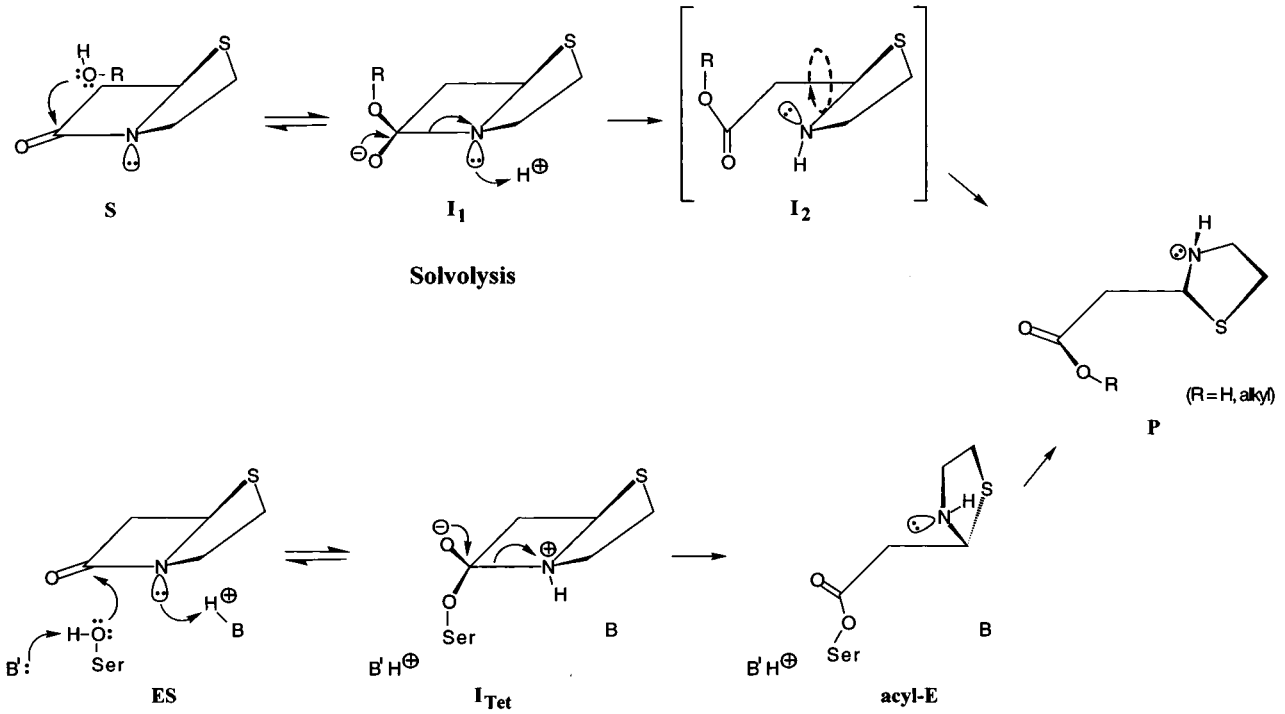

Figure 9. Schematic illustration comparing the reaction pathways for solvolytic breakdown of β-lactam antibiotics with that catalyzed by a class A β-lactamase. The species I_2 in the solvolysis pathway is indicated in brackets because it has not been identified and is likely to be extremely short-lived. For product formation, rotation around the C(5)−C(6) bond has to occur as indicated by the dashed circle with an arrow. In the pathway for enzyme catalyzed hydrolysis, BH^+ and B′ represent catalytic groups in the active site that facilitate protonation of the β-lactam N(1) and nucleophilic attack by the Ser-70 side chain, respectively. The participation of such catalytic groups is suggested by the pH profile of steady-state kinetic parameters, as discussed in the text.

with nucleophilic attack. Protonation of the nitrogen prior to hydrolysis in fact would render both *endo* and *exo* attack stereoelectronically equivalent and would simultaneously have the resultant effect of weakening the N(1)−C(8) bond. Since the β-lactam N(1) is at least as strong a base as a normal amide nitrogen,[5] protonation of the nitrogen is likely to occur, especially considering the aqueous environment of the active site of the enzyme and the possibility of a proton donor group in the active site. The pH profile of the kinetic parameters k_{cat} and k_{cat}/K_M identified two ionizing groups in the active site of β-lactamase I with pK_a values of 4.17 and 8.72. These groups are likely to be responsible for base-catalyzed nucleophilic attack and acid catalyzed protonation of the β-lactam nitrogen in the enzyme active site.

In Figure 9 we contrast the hydrolysis mechanisms of β-lactam antibiotics in solvolysis and enzyme-catalyzed reactions. The scheme is based on the ENDOR-determined structures of the (spin-labeled) substrate (S) and product (P) and on the X-ray-defined structure of the benzylpenicilloyl acylenzyme intermediate (acyl-E) of TEM-1 β-lactamase.[7a] In the case of either penicillin or cephalosporin, the four-membered ring is nearly planar, with interior angles of ∼90°. The unusual stability of β-lactam antibiotics in solution is not surprising in light of the sterically unfavorable stereoelectronic requirement of *endo* attack on the free substrate. As illustrated in Figure 9, the main difference between the two reaction pathways is protonation at N(1) in the enzyme-catalyzed reaction. Enzyme-catalyzed hydrolysis of β-lactam antibiotics is more efficient than solvolysis because it circumvents the stereoelectronic requirement for *endo* attack by protonation of the β-lactam N(1) prior to or concerted with nucleophilic attack. These considerations indicate why identification of the proton donor residue in the active site of the enzyme at present constitutes an important research objective in studies to define the mechanism of action of β-lactamase enzymes.

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