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Structure, Conformation, and Probable Mechanism of Hydrolysis of a Spin-Labeled Penicillin Revealed by Electron Nuclear Double Resonance Spectroscopy

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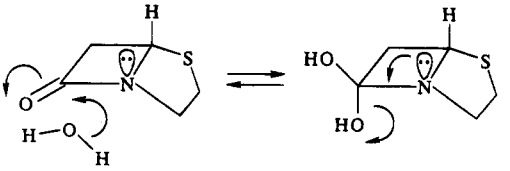
Abstract: A spin-labeled penicillin derivative has been synthesized by acylation of the amino nitrogen of 6-aminopenicillanic acid with the nitroxyl spin-label 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxylic acid and has been characterized by chemical methods. With *Bacillus cereus* β-lactamase I, the steady-state kinetic parameters $k_{cat} \approx 1810\ s^{-1}$ and $K_M \approx 118 \times 10^{-6}$ M at 22 °C and pH 7 showed that this paramagnetic substrate probe is as kinetically specific and catalytically reactive as is the classical substrate benzylpenicillin. From analysis of electron nuclear double resonance (ENDOR) spectra, the principal hyperfine coupling (hfc) components of specific protons in the fused β-lactam and thiazolidine ring were determined. The dipolar hfc components yielded electron–proton separations between the unpaired electron on the nitroxyl group and protons in the penicillin moiety. The conformation of the spin-labeled penicillin was determined on the basis of torsion angle search calculations constrained by ENDOR-determined distances. The ENDOR-assigned conformation of the spin-labeled penicillin is almost identical to the X-ray-defined structure of amoxycillin (Boles, M. O.; *et al. Acta Crystallogr., Sect. B* **1978**, *34*, 461–466). We have also determined by ENDOR the location of a methanol molecule that is hydrogen-bonded to the amide NH group, coinciding almost exactly with the position of a similarly hydrogen-bonded water molecule in the X-ray structure of amoxycillin. The location of the hydroxyl group of the methanol molecule, restricted to the "endo" side of the β-lactam ring on the basis of ENDOR distance constraints, appears as if it were poised for nucleophilic attack on the carbonyl carbon of the β-lactam. This observation is important with respect to the mechanism of hydrolysis of β-lactams because "endo" attack of a fused β-lactam compared to "exo" attack is considered to be hindered sterically but favored stereoelectronically.

Introduction

The penicillins are a family of antibiotics that have a common structural element of a β-lactam fused to a thiazolidine ring with varying side chains. While more than 50 years have passed since the discovery of penicillin, the molecular mechanism for the hydrolysis of β-lactam antibiotics still remains conjectural. Penicillins interact with two main types of bacterial enzymes: one type is the DD-peptidases which are involved in synthesis of the cell wall, against which β-lactam antibiotics have been developed; the other type is β-lactamases, which rapidly degrade penicillins and confer antibiotic resistance. It is generally accepted that both types of enzymes form acylenzyme intermediates with penicillin and that, in both the solvolytic and enzyme-catalyzed cleavage reactions of β-lactams, the approach of the nucleophile to the carbonyl carbon of the lactam group is expected to form a tetrahedral adduct.[1-3] The important question to be resolved is whether the direction of nucleophile attack is controlled by steric interactions[3b,c] or by stereoelectronic principles[4] and whether the mechanisms for the solvolytic and enzyme-catalyzed reactions differ. As illustrated in Scheme 1, the allowed direction for nucleophilic attack according to stereoelectronic principles is from the "endo" side, with the incoming nucleophilic hydroxyl group antiperiplanar to the lone pair orbital on the lactam nitrogen atom.

Scheme 1



To better understand the important mechanistic aspects of the hydrolysis of β-lactams, we have synthesized a penicillin analog whose side chain was replaced by a paramagnetic nitroxyl spin-label for structural characterization by electron nuclear double resonance (ENDOR[5]) spectroscopy. Since the unpaired electron of nitroxyl spin-labels behaves as an effective point dipole, located at about the midpoint of the N–O bond of the nitroxyl group,[6] the hyperfine (hf[5]) interactions of nearby

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(5) The following abbreviations are used: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; hf, hyperfine; hfc, hyperfine coupling; NMR, nuclear magnetic resonance, rf, radio frequency, THF, tetrahydrofuran.

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nuclei with the unpaired electron are largely dipole−dipole in character. We have shown that this hf coupling can be accurately measured by ENDOR to obtain electron−nucleus dipolar distances in the range of 3−11 Å with a precision that is exceeded only by single-crystal X-ray diffraction methods.[7] We have also shown that ENDOR of nitroxyl spin-labels in combination with molecular modeling provides a general method for determination of the structure and conformation of both small molecules and enzyme reaction intermediates in solution.[7,8]

Here, we describe the ENDOR-determined structure and conformation of the spin-labeled penicillin 6*N*-[(2,2,5,5-tetramethyl-1-oxypyrrolin-3-yl)carbonyl]penicillanic acid in solution. The conformation of the spin-labeled penicillin, as defined by ENDOR-determined distances to specific protons in the fused β-lactam ring, is almost identical to the structure of amoxycillin determined by X-ray diffraction methods.[9] We have also observed the ENDOR absorption features of a methanol molecule that is hydrogen-bonded to the NH group of the pseudo-peptide bond of the spin-labeled penicillin. This methanol molecule can be positioned only on the endo side of the β-lactam ring according to the ENDOR distance constraints and is located as if it were poised for nucleophilic attack on the carbonyl carbon of the β-lactam. It has been argued that "endo" attack of a fused β-lactam compared to "exo" attack is hindered sterically and is therefore unlikely.[3b,c] This study shows that a solvent molecule can be sterically accommodated on the endo side of the β-lactam ring and could, therefore, become the attacking nucleophile in the hydrolytic reaction to form a tetrahedral adduct. Since endo attack is favored stereoelectronically,[4] this observation is important in considering the mechanism of hydrolytic cleavage of penicillin antibiotics.

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) according to the method of Rozantsev.[10] 6-Aminopenicillanic acid, isobutyl chloroformate, 4-methylmorpholine, and 2-ethylhexanoic acid were also obtained from Aldrich. Potassium 2-ethylhexanoate was prepared by dissolving clean potassium metal in 1-butanol followed by the addition of 2-ethylhexanoic acid. Deuterated solvents (≥99.5% ^2H) were obtained from Cambridge Isotope Laboratories, Inc. (Woburn, MA). THF was refluxed and distilled over lithium aluminum hydride and stored over molecular sieves. All reagents were of analytical grade unless otherwise described. Deionized, distilled water was used throughout.

The potassium salt of 6*N*-benzylpenicillanic acid (hereafter referred to as benzylpenicillin) and β-lactamase I (penicillinase I, EC 3.5.2.6) of *Bacillus cereus* were obtained from Sigma Chemical Co. (St. Louis, MO). 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. The 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.[11]

6*N*-[(2,2,5,5-Tetramethyl-1-oxypyrrolin-3-yl)carbonyl]penicillanic Acid. A solution of 3.1 mL (24 mmol) of isobutyl chloroformate in 15 mL of THF was added dropwise with vigorous stirring over a period of 1 h to a solution of 4.0 g (22 mmol) of 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxylic acid in 30 mL of THF with 2.65 mL (24 mmol) of 4-methylmorpholine at 0 °C. Then, a solution of 8 g (37 mmol) of 6-aminopenicillanic acid in 50 mL of CH_2Cl_2 with 12 mL (86 mmol) of triethylamine was added dropwise with stirring over a period of 1.5 h at 0 °C. The mixture was then brought to room temperature and stirred for another 2 h. The white precipitate was filtered off, and solvent was removed *in vacuo*. Acetone was added to the residue, the white precipitate filtered off, and acetone removed *in vacuo*. The residue was then dissolved in 120 mL of water and layered with 200 mL of ethyl acetate. The pH was adjusted to 2.5 with dropwise addition of 0.1 N HCl under vigorous stirring, the ethyl acetate layer was separated, and the aqueous layer was extracted with ethyl acetate until the extracts were colorless. The combined organic extracts were washed with water to neutrality and dried over Na_2SO_4, and the solution was reduced to about 30 mL by evaporation *in vacuo*. A solution of potassium 2-ethylhexanoate (22 mmol) in 50 mL of ethyl acetate was added dropwise with stirring. The solution was concentrated to a volume of 20 mL, ether was added to complete precipitation, and the mixture was stored overnight at 4 °C. A light yellow crystalline product was collected with greater than 70% yield (mp 218−222 °C dec). Thin layer chromatography showed a single spot, and IR showed an intense band at 1772 cm^{-1} characteristic of the β-lactam group. Anal. Calcd for $C_{17}H_{23}N_3O_5SK$ (found): C, 48.55 (46.32); H, 5.51 (5.53); N, 9.99 (9.55); S, 7.62 (7.80). Mass spectrometric analysis showed the highest molecular ion peak to correspond to the expected *m/e* ratio of 420.

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 penicillins catalyzed by β-lactamase I. The change of absorbance at 232 nm for benzylpenicillin ($\Delta\epsilon$ = 1015 M^{-1} cm^{-1}) or at 230 nm for spin-labeled penicillin ($\Delta\epsilon$ = 670 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) 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.[12]

EPR and ENDOR. EPR and ENDOR spectra were recorded with use of an X-band Bruker ESP 300E spectrometer equipped with a TM_{110} cylindrical cavity, Bruker ENDOR accessory, Oxford ESR910 liquid helium cryostat, and ESP 3220 data system for computer-controlled data acquisition as previously described from this laboratory.[13] Typical experimental conditions for EPR measurements were sample temperature 20 K, microwave frequency 9.45 GHz, incident microwave power 64 μW (full power 640 mW at 0 dB), modulation frequency 12.5 kHz, and modulation amplitude 0.8 G. For ENDOR measurements they were microwave power 2 mW, rf power 50 W, rf modulation frequency 12.5 kHz, and rf modulation depth ≤8 kHz. The static laboratory magnetic field was not modulated for ENDOR. Spin-labeled samples were dissolved for EPR and ENDOR studies to a concentration of 4×10^{-3} M in perdeuterated solvents.

Molecular Modeling. Atomic coordinates of the spin-labeled penicillin 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,[14] and the penicillin moiety of the X-ray-defined structure of the amoxycillin.[9] In Scheme 2, the atomic numbering scheme is shown for purposes of discussing molecular modeling results. The C(15) and O(16) atoms of the amoxycillin were

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Scheme 2

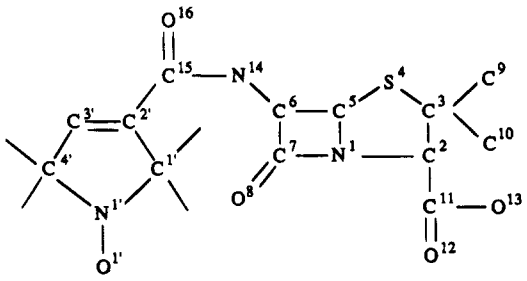

Table 1. Comparison of Steady-State Kinetic Parameters for Hydrolysis of Benzylpenicillin and Spin-Labeled Penicillin Catalyzed by β-Lactamase I of *B. cereus*

substrate	pH	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (M^{-1} s^{-1})
benzylpenicillin	7.0[a]	1730	87×10^{-6}	20×10^6
spin-labeled penicillin	7.0[a]	1810	118×10^{-6}	15×10^6

[a] At 22 °C in aqueous solution with 0.1 M NaCl buffered by 0.01 M cacodylate.

superposed onto the corresponding C and O atoms of the carboxamide group of the spin-label so that the C(15)−N(14) bond vector of the amoxycillin, and the C(2′)−C(15) bond vector of the spin-label were preserved upon joining the two fragments to generate a spin-labeled penicillin 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.

Molecular modeling was carried out with programs FRODO[15] and SYBYL[16] as previously described.[7,8] With the program package SYBYL, a systematic conformational analysis was carried out with SEARCH, which checks van der Waals contacts among nonbonded atoms by scanning all possible torsion 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 2° increments of torsion angle rotation. 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 earlier described.[6]

Results and Discussion

A. Steady-State Kinetic Studies of Hydrolysis of Penicillins Catalyzed by β-Lactamase I. To establish the specificity of catalytic reactivity of the spin-labeled penicillin compared to that of benzylpenicillin, we have carried out steady-state kinetic studies. Kinetic parameters governing the hydrolysis of benzylpenicillin and spin-labeled penicillin catalyzed by β-lactamase I of *B. cereus* were determined on the basis of initial velocity data and are compared in Table 1. The values of k_{cat} and K_M for benzylpenicillin in Table 1 are similar to those reported by other researchers.[17] The kinetic parameters for these two penicillins show that the spin-labeled penicillin is as kinetically specific and catalytically reactive as is benzylpenicillin.

B. ENDOR of Spin-Labeled Penicillin: Assignment of Resonance Features and Estimation of Electron−Proton Distances. ENDOR transitions occur as a result of the electron and nuclear spins interacting with simultaneously applied microwave and rf fields. Microwave power saturation of the central feature of the EPR spectrum (termed setting B) of a spin-label in frozen solutions selects for ENDOR essentially all orientations of the spin-label with respect to the laboratory magnetic field, while 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.[7] When g anisotropy is small compared to the average g value and nuclear hf couplings are axially symmetric, as for nitroxyl spin-labels, the maximum hf interaction energy occurs for a field oriented along the electron−nucleus vector (A_z axis), and the minimum occurs when the field is in the A_x,A_y plane.[18] We have previously described the stratagem of employing the magnetic field dependence of the EPR absorption of nitroxyl spin-labels for selection of molecular orientation in ENDOR studies.[6−8]

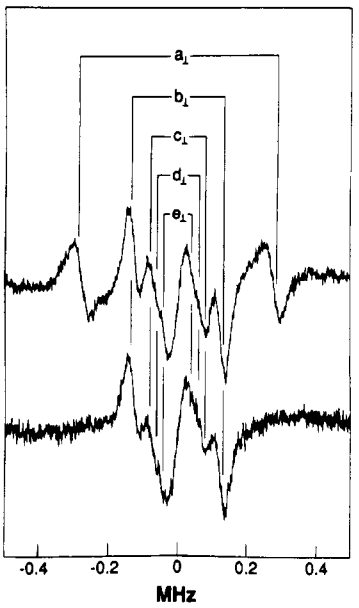

Figure 1. Proton ENDOR spectra of spin-labeled penicillin in 50:50 (v/v) [$^2H_6$]DMSO:[$^2H_4$]methanol. The static magnetic field was at setting A of the EPR spectrum. For the top spectrum, the sample was first dissolved in DMSO and then rapidly mixed with [$^2H_4$]methanol and frozen in liquid nitrogen. The bottom spectrum was obtained after thawing the sample to allow exchange of the amide proton H(14). Four 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 free proton Larmor frequency).

Figure 1 illustrates proton ENDOR spectra of spin-labeled penicillin with $\mathbf{H}_0$ at the low-field turning point of the EPR spectrum. Only the perpendicular hfc components for protons in the penicillin moiety are observed. By comparing the two spectra in Figure 1, the ENDOR features labeled $a_\perp$ are attributed to the perpendicular hfc component of H(14). With respect to the hf splitting and its relationship in the spectrum to $a_\perp$, the pair of resonance features labeled $b_\perp$ is identical to that of the H^α of amino acids similarly labeled at the amino nitrogen.[7b−d] Therefore, we assign $a_\perp$ features to H(14) and the $b_\perp$ features to H(6). Correspondingly, the parallel hfc components of H(14) and H(6) were assigned from ENDOR spectra taken with $\mathbf{H}_0$ at setting B, under which conditions both parallel and perpendicular hfc components are observed. Three other resonance features labeled $c_\perp$, $d_\perp$, and $e_\perp$ are seen in Figure 1, but their parallel hfc components cannot be resolved because

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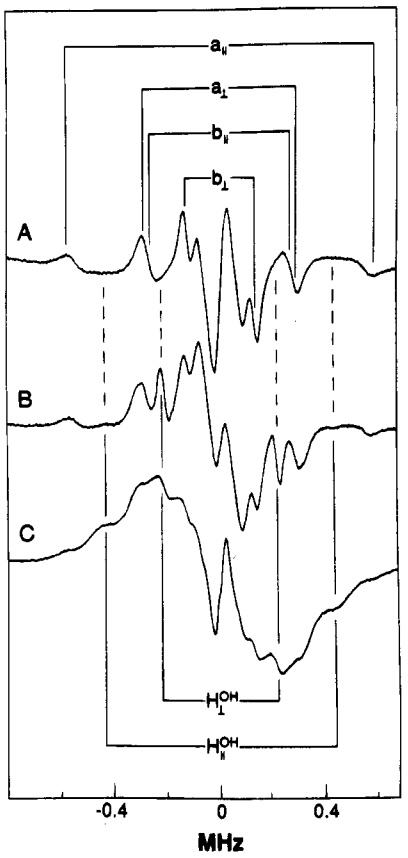

Figure 2. Proton ENDOR spectra of spin-labeled penicillin in different solvents to illustrate a tightly bound methanol molecule: (A) 50:25:25 (v/v) [$^2H_6$]DMSO:[$^2H_8$]toluene:[^2H]chloroform to which [$^2H_4$]methanol was added in 1:1 stoichiometry with spin-labeled penicillin; (B) the same ternary solvent system to which a 1:1 molar equivalent of [$^2H_3$]-methanol was added; (C) pure [$^2H_3$]methanol. 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 spectra B and C. Other line pairs are assigned to H(14) and H(6), as listed in Table 2. In order to observe the new proton resonances assigned to a hydrogen-bonded methanol molecule as shown in spectrum B, it was necessary to avoid all possible sources of adsorbed moisture because of its broadening effect. All perdeuterated aprotic solvents were of highest grade (≥99.6% ^2H), and vials of solvents were freshly opened only immediately prior to use. Acid-washed EPR sample tubes were kept in a desiccator at least overnight prior to use, and samples were prepared under nitrogen and immediately frozen and stored in liquid nitrogen until use.

they probably overlap with the more intense resonance features of $b_{\perp}$. These three resonance features are likely to derive from the three other classes of protons in the penicillin moiety, namely, H(5), H(2), and the methyl protons. The assignments of these three pairs of resonance features will be discussed later.

Figure 2 compares proton ENDOR spectra of spin-labeled penicillin dissolved in neat methanol and in an aprotic ternary solvent system to which methanol was added in 1:1 stoichiometry. In the top spectrum, the parallel and perpendicular hfc components for H(14) and H(6), labeled $a_{\|}$, $a_{\perp}$ and $b_{\|}$, $b_{\perp}$, respectively, are assigned. In the middle spectrum, two line pairs are seen that are not present in the top spectrum 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 broken-line stick diagram. The lowermost spectrum was recorded for the sample dissolved in pure CD_3OH. There is noticeable line broadening with loss of resolution due to the presence of hydroxyl protons of bulk solvent. Nonetheless, all of the resonance features observed in the middle spectrum are also evident in this spectrum. We conclude that the new features in the middle spectrum must derive from a tightly bound methanol OH group because of its sharp, intense resonance features.

Table 2. Summary of hfc Components (A, MHz) and Estimated Electron–Proton Distances (r, Å) in 6*N*-[(2,2,5,5-Tetramethyl-1-oxypyrrolin-3-yl)carbonyl]penicillanic Acid

proton	$A_{\|\|}$	$A_{\perp}$	A_{iso}	$A_{\|\|}^D$	$A_{\perp}^D$	r^a
penicillin[b]						
H(14)	1.173	0.580	0.004	1.169	−0.584	5.13 ± 0.03
H(6)	0.535	0.265	0.002	0.533	−0.267	6.67 ± 0.04
H(5)[c]	–	0.153	–	–	−0.153	8.01 ± 0.16
H(2)[c]	–	0.121	–	–	−0.121	8.68 ± 0.30
H(CH_3)[c]	–	0.085	–	–	−0.085	9.77 ± 0.45
solvent						
H^{OH}	0.874	0.436	0.001	0.873	−0.437	5.67 ± 0.04

[a] The uncertainty in the frequency of 0.010–0.015 MHz due to the line width of each ENDOR absorption is included in the calculation of electron–proton distances. [b] See the atomic numbering scheme (in Scheme 2) for atomic designations. [c] For these three classes of protons, electron–proton distances are calculated from perpendicular hfc components alone. Assignments of these dipolar distances to specific protons are based on molecular modeling studies, as discussed in the text.

Table 3. Comparison of Dihedral Angles of the X-ray-Defined Reference Molecular Model[a] and of the ENDOR-Constrained Conformations of 6*N*-[(2,2,5,5-Tetramethyl-1-oxypyrrolin-3-yl)-carbonyl]penicillanic Acid

		ENDOR	
dihedral angle	X-ray[a]	conformer **1**	conformer **2**
[C(3′)═C(2′)–C(15)–N(14)]	−30°	−35 ± 5°	−35 ± 5°
[C(2′)–C(15)–N(14)–C(6)]	−172°	−168 ± 3°	−168 ± 3°
[C(15)–N(14)–C(6)–C(7)]	−129°	−118 ± 14°	−67 ± 13°

[a] References 9 and 14.

In Table 2 we have summarized the principal hfc components of specific protons of spin-labeled penicillin identified in the ENDOR spectra. The relative signs of the methyl and vinyl protons of the spin-label moiety and of H^N and H^{α} of spin-labeled amino acids have been determined.[6,7b–d] Under the conditions $|A_{iso}| \ll |A_{\|}|$, $|A_{\perp}|$ 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 r calculated on the basis of the dipolar equation. As seen in Table 2, the isotropic contributions of H(14), H(6), and H^{OH} are vanishingly small. Since the other three classes of protons in the penicillin moiety are even more distant than H(14) and H(6), as evident from their line splittings in Table 2, we have calculated corresponding values of r on the basis of only their observed perpendicular hfc components.

C. Conformation of Spin-Labeled Penicillin. To determine the conformation of spin-labeled penicillin, we have carried out a torsion angle search analysis to identify conformers that accommodate the ENDOR-based values of r within van der Waals allowed conformational space. The general methodology of application of torsion angle search calculations to define molecular conformation constrained by ENDOR-determined electron–proton distances has been described in earlier publications.[7,8] The results of torsion angle search calculations carried out within van der Waals hard-sphere limits[16b] and constrained by the ENDOR-determined electron–proton separations to H(14) and H(6) and their uncertainties listed in Table 2 are summarized in Table 3. The search calculations were applied for simultaneous rotation around the C(2′)–C(15), C(15)–N(14), and N(14)–C(6) bonds.

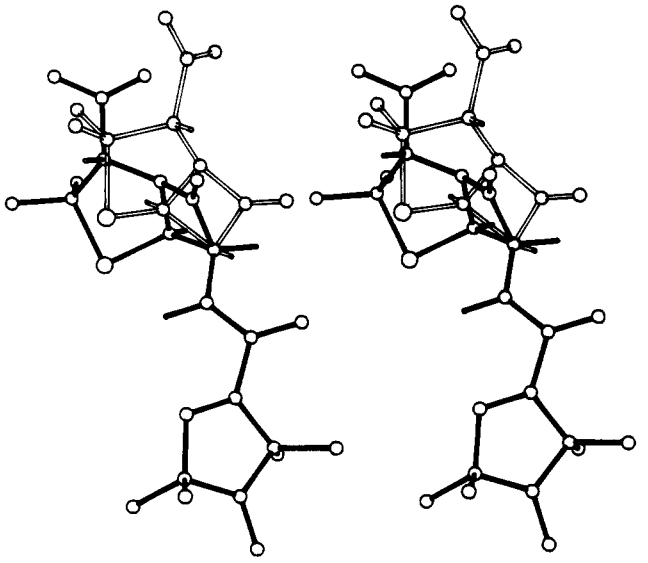

Figure 3. Stereo diagram of the ENDOR-determined conformations of spin-labeled penicillin. The structures were drawn according to the mean values of dihedral angles listed in Table 3. The solid bonds correspond to conformer **1** of Table 3 while the open-bond structure is that of conformer **2**. The ENDOR-active hydrogen positions for H(14), H(6), H(5), and H(2) are shown in addition to the positions of all non-hydrogen atoms.

Table 4. Comparison of ENDOR-Determined Electron-Proton Distances (Å) in Spin-Labeled Penicillin with Distances Calculated for Conformers **1** and **2**

proton	ENDOR	conformer **1**[a]	conformer **2**[a]
H(14)	5.13 ± 0.03	5.11	5.11
H(6)	6.67 ± 0.04	6.70	6.70
H(5)	8.01 ± 0.16	7.95	8.30
H(2)	8.68 ± 0.30	8.90	9.00
$H(CH_3)$[b]	9.77 ± 0.45	9.95	10.12
		10.39	10.74

[a] Conformers **1** and **2** were derived from torsion angle search calculations based on the electron−proton distance constraints to H(14) and H(6), as illustrated in Figure 3. [b] The geometrically averaged values of idealized hydrogens are compared.

In Table 3 we have also compared the values of corresponding dihedral angles of the X-ray-determined structures[9,14] from which the model of spin-labeled penicillin was constructed. The distance constraints to H(14) and H(6) limited the [C(3′)=C-(2′)−C(15)−N(14)] and [C(2′)−C(15)−N(14)−C(6)] dihedral angles to values of −35 ± 5° and −168 ± 3°, respectively. The resultant conformation of the spin-label moiety, as defined by the torsion angle around the C(2′)−C(15) bond, is in excellent agreement with the structure of the parent spin-labeled carboxamide.[14] Also, these results assign a planar *trans* conformation to the pseudo-peptide C(15)−N(14) bond. On the other hand, torsion angle search calculations around the N(14)−C(6) bond yielded two distinct families of conformers compatible with the electron-to-proton distances to H(14) and H(6).

In Figure 3 we have illustrated the molecular structures of the two ENDOR-compatible conformations of spin-labeled penicillin. These structures correspond to the mean values of the dihedral angles listed in Table 3. In Table 4 we have compared ENDOR-based electron−proton distances with their corresponding values derived from conformers **1** and **2**. As discussed in the text, the conformational search calculations were based on the ENDOR-determined distance constraints only to H(14) and H(6). As seen in Table 4 for conformer **2**, the predicted distances to H(5), H(2), and $H(CH_3)$ are not compatible with the corresponding ENDOR-determined values of *r*. On the other hand, conformer **1** is entirely compatible with all of the ENDOR-determined distances. In a recent study of the molecular structure of benzylpenicillin using both solid-state NMR and single-crystal X-ray diffraction methods, Fattah *et al.* have found four different conformations of benzylpenicillin.[19] While the principal differences among the four conformations pertain to the orientation of the benzyl side chain with respect to the fused β-lactam moiety, the values of dihedral angles around the C(15)−N(14) and N(14)−C(6) bonds in the four conformers are almost identical to those listed for conformer **1** in Table 3.

D. Location of the Methanol Molecule Hydrogen-Bonded to Spin-Labeled Penicillin. The ENDOR results in Figure 2 demonstrate that a methanol molecule is tightly bound to the spin-labeled penicillin. Assignment of the location and orientation of the methanol molecule was made on the basis of three constraints: (i) the sharpness of the resonance features assigned to the methanolic H^{OH} indicates a tightly bound molecule hydrogen-bonded to a group on the spin-labeled penicillin, (ii) the H^{OH} of the methanol molecule must lie on the surface of a sphere of radius 5.67 Å centered on the effective position of the point dipole of the nitroxyl group;[6] and (iii) the H^{OH} lies in or is very nearly coincident with the plane of the oxypyrrolinyl ring to account for the hfc patterns dependent on the $\mathbf{H}_0$ setting. Since hydrogen bonding is the most likely basis for tight binding, we furthermore invoked the structural parameters observed in strongly hydrogen-bonded groups containing oxygen and nitrogen atoms, namely, a D−H···A distance of ~2.75 Å and a D−H···A angle of ≥135°, as observed for maximum stabilization.[20] We were unable to identify a location for the methanol OH group that would allow hydrogen bonding simultaneously to the β-lactam carbonyl O and the amide NH group under the three constraints listed above. Also, hydrogen bonding to only the β-lactam O(8) was ruled out on the basis of the electron-to-proton distance of 5.67 Å. While hydrogen bonding of the methanol OH group to O(16) could satisfy the 5.67 Å requirement for the electron-to-proton distance, we could not find other additional intermolecular interactions that could be considered as stabilizing to account for the sharpness of the ENDOR features. In addition, in the case of spin-labeled amino acids which exhibit similar geometry around the pseudo-peptide bond with essentially identical chemical groups, we have not been able to detect solvent molecules hydrogen-bonded to the carbonyl oxygen of the pseudo-peptide bond.[7b-d,21] We consequently searched for a position of the methanol OH group satisfying the above three constraints and hydrogen-bonded only to the amide NH group of the spin-labeled penicillin molecule that was accommodated by the solvent accessible surface[22] calculated for conformer **1** since this conformer best accounts for the ENDOR-defined distances to protons on the penicillin moiety listed in Table 4.

Figure 4 illustrates the results of the modeling analysis. The calculated solvent accessible surface of conformer **1** accommodates the methyl group and the hydroxyl group of a methanol molecule so that the $N-H\cdots O_{MeOH}$ non-hydrogen atom distance

(19) Fattah, J.; Twyman, J. M.; Heyes, S. J.; Watkin, D. J.; Edwards, A. J.; Prout, K.; Dobson, C. M. *J. Am. Chem. Soc.* **1993**, *115*, 5636−5650.

(20) Jeffrey, G. A.; Saenger, W. *Hydrogen Bonding in Biological Structures*; Springer-Verlag: Berlin, 1991; p 569.

(21) In the course of these studies, we have also reinvestigated the ENDOR properties of spin-labeled amino acid systems, taking corresponding precautions to avoid sources of adsorbed moisture, as described in Figure 2 for spin-labeled penicillin. As earlier, we have been unable to find resonance features that could be ascribed to methanol or water molecules hydrogen-bonded to the carbonyl O or the NH of the pseudo-peptide bond.

(22) Lee, B.; Richards, F. M. *J. Mol. Biol.* **1971**, *55*, 379−400.

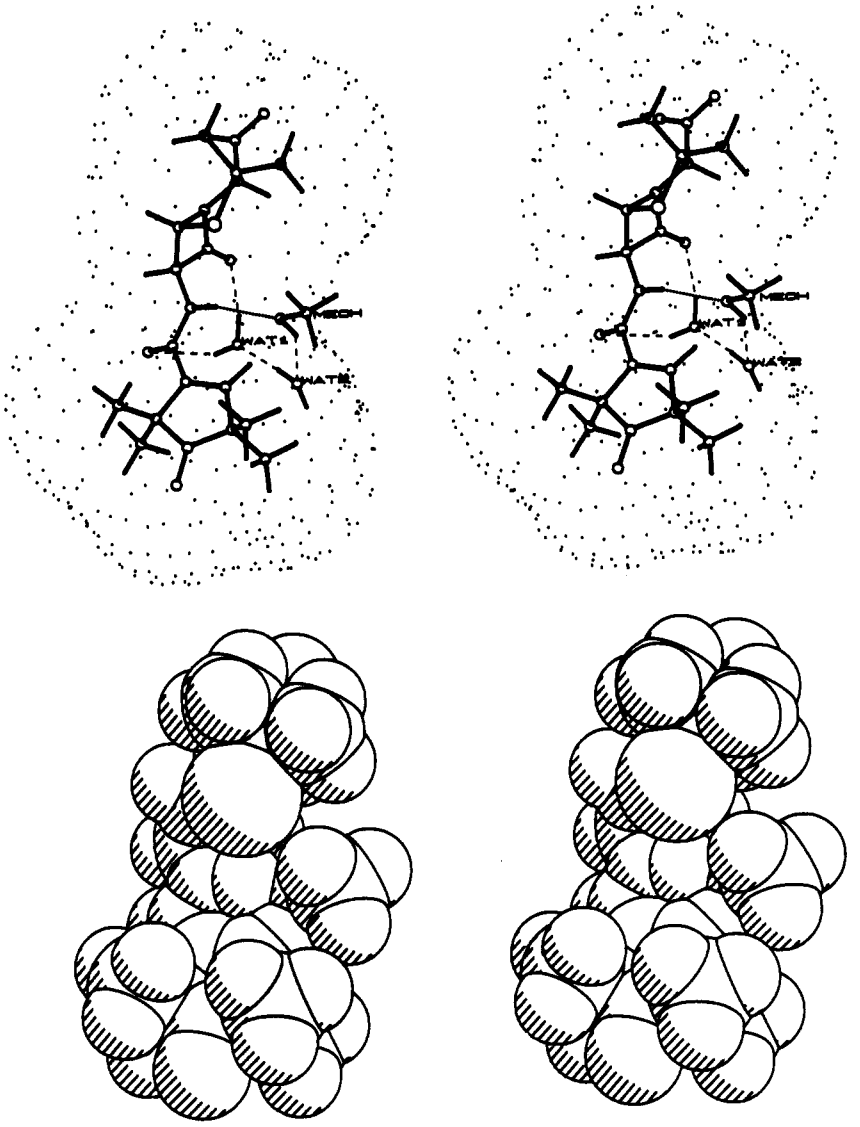

Figure 4. Stereo diagram of the ENDOR-determined conformation of conformer **1** of spin-labeled penicillin and the hydrogen-bonded methanol molecule. In the upper diagram the solvent accessible surface of the spin-labeled penicillin, calculated according to the Lee and Richards algorithm,[22] is shown by the dotted surface. Two water molecules are also illustrated in this diagram with open-bond structures labeled as WAT1 and WAT2. Their positions are defined by X-ray crystallographic studies of amoxycillin trihydrate.[9] The third water molecule is not included here for purposes of clarity because its oxygen atom would be almost exactly superposed onto the hydroxyl oxygen of the hydrogen-bonded methanol molecule identified by ENDOR. The possible hydrogen bonds between these two water molecules and other hydrogen-bonding donors and acceptors in this complex are indicated by dashed lines while the hydrogen bond between the pseudo-peptide NH and O_{MeOH} is indicated by the dotted line. In the lower diagram, the methanol−spin-labeled penicillin complex is drawn in the same projection as in the upper diagram with van der Waals radii of 1.54 Å (C), 1.4 Å (O), 1.2 Å (H), 1.5 Å (N), and 1.75 Å (S). The X-ray-defined water molecules are not included in the lower diagram.

is ~2.84 Å, and the N−H···O_{MeOH} angle is ~139°, in good agreement with stabilizing hydrogen-bonding geometry observed for other similar chemical groups.[20] As seen in Figure 4, the methanol molecule is located on the endo side of the β-lactam group within a cleft formed by the solvent accessible surface. The electron-to-(hydroxyl) proton distance of 5.67 ± 0.04 Å did not permit location of the methanol molecule on the "exo" side of the β-lactam ring within allowed van der Waals interactions. In the X-ray-defined structure of amoxycillin, there are three structured water molecules forming a network of hydrogen bonds with the pseudo-peptide NH and the β-lactam carbonyl O. We have illustrated the positions of two of these water molecules in Figure 4 together with that of the ENDOR-defined methanol molecule. Assignment of the methanol OH group to hydrogen bond with the NH according to the constraints detailed above placed it almost exactly onto WAT3 of the amoxycillin trihydrate structure.[9] Therefore, for purposes of clarity in illustration, this water molecule has not been explicitly included in Figure 4. Its structural relationships to WAT1 and WAT2 are essentially equivalent to those illustrated for the methanol OH group. Given the compatibility of the positions of the X-ray-defined water molecules[9] with the solvent accessible surface of the β-lactam ring of spin-labeled penicillin, as shown in Figure 4, it is probable that water molecules would be similarly hydrogen-bonded to the methanol−spin-labeled penicillin complex under conditions of adsorbed moisture. The calculated electron-to-proton distances of these two water molecules are 5.76 and 5.77 Å for WAT1 and 6.04 and 7.12 Å for WAT2. They could, therefore, conceivably account for the broadened resonance features observed under conditions of adsorbed moisture (*cf.* Figure 2). To compare hydrogen-bonding structural parameters, we have, therefore, listed in Table 5 geometric data for the X-ray-defined water molecules[9] together with those for the ENDOR-defined methanol molecule.

E. Stereoelectronic Considerations. Since the hydrolysis of β-lactam antibiotics occurs via formation of a tetrahedral

Table 5. Comparison of Hydrogen-Bonding Geometrical Parameters in the Methanol–Spin-Labeled Penicillin Complex Defined by ENDOR and of Amoxycillin Trihydrate Defined by X-ray Crystallography[9]

donor (D)	acceptor (A)	D–A distance[a] (Å)	D–H···A angle[a] (deg)
N(14)	O_{MeOH}[b]	2.84	139
O(WAT1)	O(8)	2.91	163
O_{MeOH}[b]	O_{WAT2}	2.89	147
O_{WAT2}	O_{WAT1}	2.99	176
O_{WAT1}	O(16)	3.62	155

[a] The five hydrogen bonds listed above are shown in Figure 4. The network of hydrogen bonds is based on modeling to indicate a likely arrangement of hydrogen-bonding donor–acceptor atom pairs. It is probable that other configurations of hydrogen bonds involving the X-ray-defined positions of the water O atoms may also contribute to stabilization of structured water in the amoxycillin trihydrate complex. See Scheme 2 for the designation of atoms in spin-labeled penicillin. [b] The position of O_{MeOH} coincides almost exactly with that of WAT3 in amoxycillin trihydrate.[9]

adduct,[3] it is of direct interest to relate the ENDOR-determined structure of the methanol–spin-labeled penicillin complex to mechanistic aspects of the hydrolytic breakdown of the β-lactam ring. The hydrogen-bonding network of water molecules identified in the X-ray structure of amoxycillin trihydrate[9] superposed onto the structure of the methanol–spin-labeled penicillin complex suggests a mechanism to facilitate activation of the hydrogen-bonded methanol molecule as the attacking nucleophile on the β-lactam carbonyl C atom. As pointed out above, it is probable that, in the presence of water, the methanol molecule would find itself within a similar hydrogen-bonding network. In the hydrolysis of the β-lactam ring, polarization of the carbonyl bond is then likely to occur through hydrogen bonding with solvent water molecules. Similarly, hydrogen bonding of the methanol OH group to a water molecule will polarize the O–H bond, facilitating development of negative charge on the O atom and potentiating its nucleophilicity. In this manner the hydrogen-bonded methanol molecule could be readily converted into the attacking nucleophile on the carbonyl carbon through a base-catalyzed process involving water. The observation of a network of hydrogen-bonded water molecules,[9] as illustrated in Figure 4, suggests a feasible structural basis for such interactions. Wolfe and co-workers have shown on the basis of *ab initio* molecular orbital calculations that a catalytic water molecule in the methanolysis of a β-lactam ring can contribute at least a 10 kcal/mol lowering of the activation barrier.[23] A similar mechanism for hydrolysis of β-lactam antibiotics in aqueous solutions in the neutral pH region has been proposed by Page.[3b]

We have inspected the van der Waals constraints of the methanol–spin-labeled penicillin complex to determine the steric limitations to small shifts in the position of the methanol. We found 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 amide N, the carbonyl O, or the thiazolidine S, that migration of the methanol OH group from its ENDOR-defined location was sterically allowed so that the methanol molecule could be located as shown in Figure 5. In this position, the incoming methanol O makes an O···C(7)=O(8) angle of 82°, a O···C(7) separation of 2.84 Å, and O···C(7)–C(6) and O···C(7)–N(1) angles of 94° and 92°, respectively. These structural parameters are within the range observed through X-ray-defined structural data to define the path of beginning approach for nucleophilic O···C=O interactions.[24] Coupled molecular vibrations to tilt the lactam C=O group downward and out of the N(1)–C(7)–C(6) plane would facilitate shortening of the O_{MeOH}···C(7) interaction for bond formation.[24] The approach of the methanol molecule from its hydrogen-bonded position along the endo surface of the penicillin moiety to its position shown in Figure 5 would then form a methyl penicilloate ester. This approach for the incoming nucleophile to form a bond with the carbonyl C is antiperiplanar to the lone pair orbital on the N(1) atom and is stereoelectronically favored.[4]

Because nucleophilic approach on the endo side has been viewed as strongly sterically hindered, the application of stereoelectronic rules has been criticized with the counter proposal that exo attack is favored for hydrolysis;[3b,c] *i.e.*, the nucleophile approaches the carbonyl carbon from the sterically less hindered side. However, the detection of a hydrogen-bonded methanol molecule, as illustrated in Figure 4, together with the hydrogen-bonded network of X-ray-defined water molecules associated with the amoxycillin molecule[9] and the steric compatibility of the methanol molecule with structural data defining the path of approach of nucleophilic O···C=O interactions,[24] provides strong arguments against the assumption[3b,c] that the endo side of the penicillin molecule free in solution is too sterically crowded for chemistry. We, thus, favor the interpretation that formation of the tetrahedral adduct is governed by stereoelectronic rules for the free penicillin molecule. However, stereoelectronic rules[4] require that while the approaching nucleophile is antiperiplanar to the lone pair orbital on the N(1) atom, rigid retention of this conformation leads also to facile expulsion of the nucleophile by microscopic reversibility. Thus, within the tetrahedral adduct, a configurational change of the lone pair orbitals on the heteroatoms must occur that leads to strengthening of the O_{Nu}···C bond and labilization of the C(7)–N(1) bond. This change could occur either through inverse pyramidalization of the C(5)–N(1), C(7)–N(1), and C(2)–N(1) bonds, which is expected to be associated with a high energy barrier[25] and is, therefore, unlikely to occur, or through fast protonation of the β-lactam N(1) in the tetrahedral adduct by a nearby solvent molecule. Since the β-lactam N(1) is a stronger base than a normal amide nitrogen,[26] the latter process is likely to occur, particularly in an aqueous solvent.[27]

The reactivity of β-lactam antibiotics has been rationalized on the basis of steric strain due to the fused four- and five-membered rings comprising the penicillin nucleus.[29] However, the fused β-lactam ring of penicillin antibiotics is rather stable in aqueous solution considering the inherent ring strain that

(23) (a) Wolfe, S.; Yang, K.; Khalil, M. *Can. J. Chem.* **1988**, *66*, 2733–2750. (b) Wolfe, S.; Kim, C. K.; Yang, K. *Can. J. Chem.* **1994**, *72*, 1033–1043.

(24) (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. *Philos. Trans. R. Soc. London, B* **1975**, *272*, 99–108.

(25) Lehn, J. M.; Wipff, G. *J. Am. Chem. Soc.* **1980**, *102*, 1347–1354.

(26) Page, M. I. *Acc. Chem. Res.* **1984**, *17*, 144–151.

(27) The X-ray structure of a benzylpenicilloyl acylenzyme intermediate of an RTEM-1 β-lactamase mutagenized to be deacylation defective has been recently reported.[28] In contradistinction to our analysis for the free β-lactam antibiotic with a hydrogen-bonded methanol molecule, the nucleophilic serine residue in the enzyme has approached the β-lactam group from the exo or sterically less hindered side. The kinetic relationships for protonation of N(1) and nucleophilic attack at C(7) are not determined for the enzyme-catalyzed reaction. Protonation of N(1) prior to or concerted with nucleophilic attack in the enzyme-catalyzed reaction would greatly weaken the C(7)–N(1) bond and alter the reactivity of the β-lactam group from that in the free molecule in solution at neutral pH, and nucleophilic attack would then be sterically and stereoelectronically allowed from either side of the β-lactam group.

(28) Strynadka, N. C. J.; Adachi, H.; Jensen, S. E.; Johns, K.; Sielecki, A.; Betzel, C.; Sutoh, K.; James, M. N. G. *Nature* **1992**, *359*, 700–705.

(29) Strominger, J. L. In *Antibiotics I: Mechanism of Action*; Gottlieb, D., Shaw, P. D., Eds.; Springer-Verlag: New York, 1967; pp 705–713.

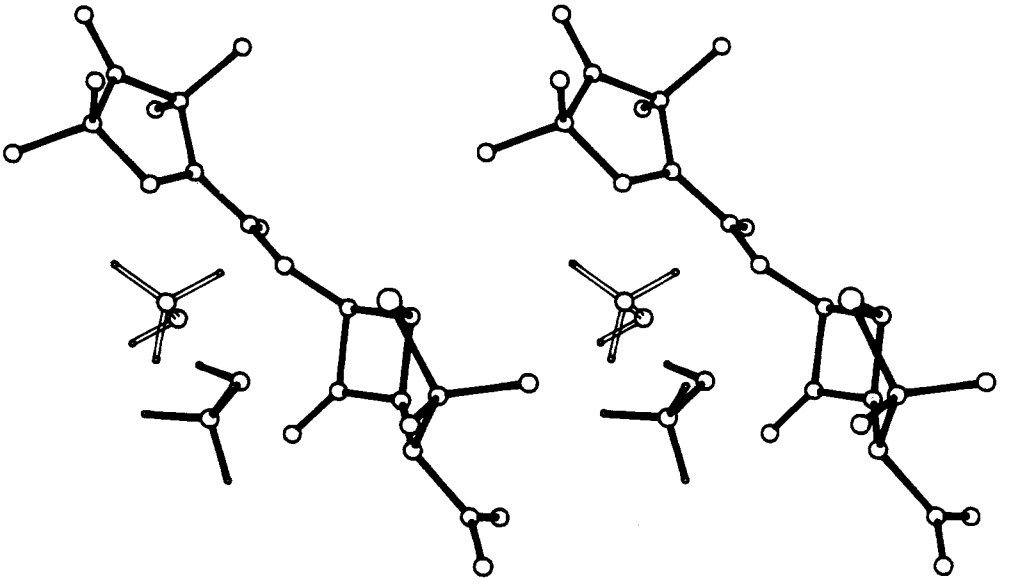

Figure 5. Stereo diagram comparing the change in the methanol molecule from its ENDOR-defined position to a sterically allowed site to act as a nucleophile attacking the β-lactam carbonyl C atom. The ENDOR-defined methanol molecule is shown with open bonds while the spin-labeled penicillin molecule and the nucleophilic methanol are shown with solid bonds. This position, viewed as the beginning approach[24] of the nucleophile to form the tetrahedral adduct, is reached by a 1.5 Å shift in the position of O_{MeOH} along a sterically allowed path.

should encourage nucleophilic attack. If the nucleophile were to approach the carbonyl C atom solely from the exo side, it might be expected that free β-lactam antibiotics would not exhibit the chemical stability that they do in solution. As originally pointed out by R. B. Woodward, the chemical stability of the fused β-lactam ring can be explained by the circumstance that the sterically preferred mode of attack, *i.e.*, exo attack, is not allowed stereoelectronically while the stereoelectronically favored mode of attack, *i.e.*, endo attack, is sterically hindered.[4c] The chemical stability of β-lactam antibiotics in aqueous solutions, thus, appears to be consistent with hydrolysis governed by stereoelectronic requirements rather than through steric reasons alone.

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