Penicillanic Acid Sulfone: An Unexpected Isotope Effect in the Interaction of 6α- and 6β-Monodeuterio and of 6,6-Dideuterio Derivatives with RTEM β-Lactamase from *Escherichia coli*†

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Appendix: Crystal Structure of Penicillanic Acid Sulfone

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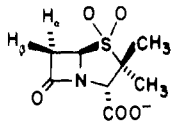
ABSTRACT: Penicillanic acid sulfone (**1**) is both a substrate and an inactivator of the RTEM β-lactamase. About 7000 hydrolytic events occur before enzyme inactivation. The 6,6-dideuterio sulfone shows a 3-fold *acceleration* of both the hydrolysis reaction and the enzyme inactivation. The kinetic and spectroscopic results are nicely accommodated by a scheme in which a transiently stable intermediate is formed in an isotopically sensitive step. The deuterated material partitions less readily toward this transiently stable intermediate by virtue of a primary isotope effect, and more enzyme is then available for the hydrolysis and inactivation pathways. Use of the stereospecifically monodeuterated sulfones shows that the 6β hydrogen is preferentially abstracted in the formation of the transiently stable intermediate and allows a detailed picture of the interaction of the sulfone and the β-lactamase to be drawn. The crystal structures of both the labeled and unlabeled compounds are reported.

Penicillanic acid sulfone [[2*S*-(2α,5α)]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide, **1**] is a semisynthetic β-lactamase inactivator first



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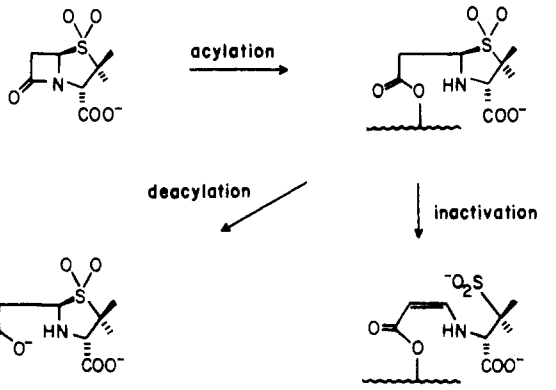
described by the Pfizer group (English et al., 1978). While this material shows only weak antibiotic activity against most bacterial species, it is a potent synergist when used together with β-lactam antibiotics against resistant bacterial strains that derive their resistance from a β-lactamase (English et al., 1978; Aswapokee & Neu, 1978; Fu & Neu, 1979). The penam sulfone evidently inhibits the β-lactamase, thereby protecting the β-lactam antibiotic from hydrolytic destruction by this enzyme.

From in vitro studies of a number of other β-lactamase inhibitors [e.g., clavulanic acid (Fisher et al., 1978; Charnas et al., 1978), 6β-bromopenicillanic acid (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b, 1980; Loosemore et al., 1980; Cohen & Pratt, 1980; Orlek et al., 1979), 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1979), and various 6-acylaminopenicillanic acid sulfones (Fisher & Knowles, 1980)], along with current views on the nature of "mechanism-based" inactivation of enzymes (Bloch, 1969; Rando, 1974; Abeles & Maycock, 1976; Walsh, 1977), a plausible scheme could be put forward for the mechanism of action of penicillanic acid sulfone [see, e.g., Fisher et al. (1980a)]. This outline is shown in Scheme I. The enzyme binds the sulfone and, in the normal first step of the catalyzed reaction (Fisher et al., 1980b), is acylated by it. The resulting acyl-enzyme intermediate can either be hydrolyzed (thus completing the β-lactamase reaction) or, by virtue of the possibility of a β-elimination reaction across C-6 and C-5, fragment to yield a more stable α,β-unsaturated acyl-enzyme, thus inactivating the enzyme. The appearance of a characteristic new chromophore at 280 nm was strongly suggestive of a β-aminoacrylic ester such as is shown in Scheme I. From preliminary studies on the rate of sulfone hydrolysis and the rate of enzyme inactivation, it appeared that several thousand hydrolytic events occurred before the enzyme activity was completely lost.

Scheme I: Simplified Pathway To Account for Enzyme-Catalyzed Hydrolysis of Penicillanic Acid Sulfone and Concomitant Inactivation of Enzyme



To test the assumptions inherent in the formulation of Scheme I, we decided to investigate the effect of deuterium substitution at C-6 of penicillanic acid sulfone on its interaction with the RTEM[1] β-lactamase from *Escherichia coli*. In the event that the putative β elimination were the rate-limiting step in the enzyme inactivation, a primary kinetic isotope effect would be expected. In contrast, the enzyme-catalyzed hy-

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[1] Abbreviations used: RTEM specifies the source of the plasmid [see Datta & Kontomichalou (1965)]; TEM-2 specifies the enzyme [see Sutcliffe (1978)]; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide.

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drolytic turnover would only be perturbed by a (presumably small) secondary isotope effect.

To our surprise, dideuteration at C-6 of penicillanic acid sulfone causes a marked *acceleration* in the rates of both the hydrolytic and the inactivation reactions. Investigation of this phenomenon led us to the synthesis of both the 6α- and the 6β-monodeuterated penicillanic acid sulfones and the further elucidation of the nature of the interaction of this simple penam sulfone with the β-lactamase.

Experimental Procedures

Materials

Benzylpenicillin and 6-aminopenicillanic acid were from Sigma Chemical Co.; β-hydroxybutyrate dehydrogenase (*Rhodopseudomonas spheroides*) was from Boehringer and had a specific activity of 3 units mg^{-1}. D_2O was from Bio-Rad Laboratories (99.8%) or from Stohler (100.0%). D_2SO_4 (99%) was from Stohler, and D_2 (99.5%) was from Matheson Gas.

6α-Bromopenicillanic acid was prepared from 6-aminopenicillanic acid by the method of Cignarella et al. (1962). Extraction of the product into methylene chloride yielded an oil (68% yield) which was used without further purification. No 6,6-dibromopenicillanic acid was detected by ^1H NMR of the product, though the mass spectrum of the trimethylsilylated material did show a trace of the dibromo compound.

Penicillanic acid was synthesized according to Clayton (1969), by reduction of 6α-bromopenicillanic acid. The resulting yellow solid (obtained in 83% yield) was used in subsequent syntheses without further purification. For kinetic studies, the sodium salt of this material was recrystallized from water–acetone to give white needles, mp 293–295 °C.

Penicillanic acid sulfone was prepared by the permanganate oxidation (Johnson et al., 1963) of penicillanic acid, as colorless plates [70% yield, mp 148 °C (dec)] after crystallization from ethyl acetate–hexane: $[\alpha]^{25}_D$ +197° (*c* 1.0, potassium salt in 100 mM potassium phosphate buffer, pH 7.0); IR (film) ν 1769, 1320, and 1123 cm^{-1}; ^1H NMR (CD_3OD) δ 1.45 (s, 3 H), 1.57 (s, 3 H), 3.25 (dd, 1 H, J = 2.1 and 16.3 Hz), 3.59 (dd, 1 H, J = 4.2 and 16.3 Hz), 4.32 (s, 1 H), 4.85 (dd, 1 H, J = 2.1 and 4.2 Hz); ^{13}C NMR (H_2O with internal D_2O lock) δ 20.20 (q, J = 130.6 Hz), 21.86 (q, J = 132.8 Hz), 39.38 (t, J = 147.1 Hz), 63.50 (d, J = 178.5 Hz), 66.02 (s), 67.58 (d, J = 147.6 Hz), 175.05 (s), 177.27 (s); mass spectrum (methyl ester) m/z 247 (M^+) and 183 ($M^+ - SO_2$). Anal. Calcd for $C_8H_{11}NO_5S$: C, 41.20; H, 4.72; N, 6.01; S, 13.73. Found: C, 41.14; H, 4.84; N, 6.01; S, 13.50.

6α-Bromo[6β-^2H]penicillanic acid was prepared analogously to the protio compound, except that 2.5 N D_2SO_4 in D_2O under N_2 was used [see McMillan & Stoodley (1968)]. The mass spectrum (trimethylsilyl derivative) showed M^+ and $M^+ + 2$ at m/z 352 and 354. The preparation comprised 97% d_1 and 3% d_0 material.

[6,6-$^2H_2$]Penicillanic acid was synthesized analogously to the unlabeled material. The catalyst was preequilibrated with D_2 in D_2O, evaporated to dryness in vacuo, and then resuspended in D_2O, before the addition of 6α-bromo[6β-^2H]-penicillanic acid (potassium salt) in D_2O. The mass spectrum (trimethylsilyl derivative) showed $M^+ + 2$ at m/z 275. The preparation comprised 95% d_2 and 5% d_1 material. It was used directly for further synthesis, or converted to the sodium salt and crystallized from water–acetone (mp 288–291 °C): ^1H NMR ($CDCl_3$) δ 1.56 (s, 3 H), 1.69 (s, 3 H), 4.45 (s, 1 H), 5.29 (s, 1 H).

[6,6-$^2H_2$]Penicillanic acid sulfone was prepared by oxidation of the corresponding penam. The product was recrystallized from ethyl acetate–hexane: mp 150 °C (dec); $[\alpha]^{25}_D$ +194° (*c* 1.0, potassium salt in 100 mM potassium phosphate buffer, pH 7.0); IR (film) ν 1770, 1318, and 1122 cm^{-1}; ^1H NMR (CD_3OD) δ 1.45 (s, 3 H), 1.57 (s, 3 H), 4.32 (s, 1 H), 4.84 (s, 1 H); mass spectrum (methyl ester) m/z 249 (M^+) and 185 ($M^+ - SO_2$). The preparation comprised 95% d_2 and 5% d_1 material. Anal. Calcd for $C_8H_9D_2NO_5S$: C, 40.85; N, 5.96; S, 13.62. Found: C, 40.74; N, 5.87; S, 13.70.

[6α-^2H]Penicillanic acid was synthesized from 6α-bromo-[6β-^2H]penicillanic acid by hydrogenolysis at 4 °C with H_2 in H_2O. The ^1H NMR was consistent with the ^2H being mainly in the 6α position (see below).

[6α-^2H]Penicillanic acid sulfone was made by oxidation of the corresponding penam. The ^1H NMR showed the relative intensities of signals for the protons at the 3, 6α, and 6β positions were 1:0.30:0.78. Assignments for the C-6 protons were made according to Green et al. (1965), Johnson et al. (1968), and McMillan & Stoodley (1966). Cis protons at C-5 and C-6 have J = 4.5–5.9 Hz, and trans protons have J = 2.2–2.8 Hz. The mass spectrum (methyl ester) showed that the material comprised 91.9% d_1 and 8.1% d_0 compounds. The composition of this sample was therefore 6α-^2H, 69.9%, 6β-^2H, 22.0%, and unlabeled, 8.1%.

[6β-^2H]Penicillanic acid was synthesized from the unlabeled 6α-bromo derivative by hydrogenolysis at 4 °C with D_2 in D_2O. A second sample of this material was made by reduction of 6α-bromo[6β-^2H]penicillanic acid (1.20 g) with tri-*n*-butyltin hydride (6.60 g, 5.3 equiv) and 2,2′-azobis(isobutyronitrile) (0.5 g) in dry benzene (200 mL), refluxed under N_2 for 8 h. The mixture was then cooled, and the solution was concentrated to 60 mL by evaporation under reduced pressure. The product was extracted into cold aqueous $KHCO_3$ (1 M). This solution was washed with ether and the aqueous layer acidified to pH 2 with concentrated HCl at 0 °C, before extraction of the free acid into ether. This solution was washed with brine and dried over $MgSO_4$, and the ether was removed by evaporation. The resulting yellow solid (71% yield) had a ^1H NMR consistent with the ^2H being mainly at the 6β position (see below).

[6β-^2H]Penicillanic acid sulfone was produced from the two samples of penam. After recrystallization, the material from the catalytic hydrogenolysis route had ^1H NMR signals for the protons at the 3, 6α, and 6β positions in the ratio 1:0.80:0.24. The mass spectrum (methyl ester) showed the material to be 95.6% d_1 and 4.4% d_0. This sample therefore comprised 6α-^2H, 20.0%, 6β-^2H, 75.6%, and unlabeled, 4.4%.

The material from the tin hydride reduction was subjected, after oxidation, to ion-exchange chromatography at 4 °C on a column of DEAE-cellulose (Whatman, DE-52) equilibrated with 5 mM potassium phosphate buffer, pH 7.0, and eluted with a linear gradient (5–100 mM) of the same buffer. Fractions containing the sulfone were pooled and concentrated under reduced pressure. The solution was acidified (pH 2) with 85% phosphoric acid and the product extracted into ethyl acetate. After recrystallization, the ^1H NMR signals for the protons at 3, 6α, and 6β positions were in the ratios 1:1.0:0.07. The mass spectrum (methyl ester) showed that the material was 92.8% d_1 and 7.2% d_0. This sample therefore comprised 6α-^2H, ~0%, 6β-^2H, 92.8%, and unlabeled, 7.2%.

β-Lactamase was from *E. coli* W3310 carrying the RP4 plasmid (Matthews & Hedges, 1976). The TEM-2 enzyme[1] was purified essentially as described earlier (Fisher et al., 1980b) and had a specific activity of 4300 units/A_{280nm} (enzyme absorption). A unit of activity is that amount which will catalyze the hydrolysis of 1 μmol of benzylpenicillin/min at

30 °C in 100 mM potassium phosphate buffer, pH 7.0. The enzyme was >95% homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970). Enzyme concentrations were determined on the basis of $A_{280nm}^{0.1\%} = 1.0$.

Methods

Ultraviolet measurements were made on a Perkin-Elmer 554 instrument. Infrared measurements were made on a Perkin-Elmer 457A spectrometer or on a Nicholet 7199 Fourier transform infrared instrument. NMR spectra were recorded on Varian XL-100 or CFT 20 instruments. Chemical shifts are reported relative to tetramethylsilane or sodium 3-(trimethylsilyl)-1-propanesulfonate. Mass spectra were measured on an AEI MS-9 machine, samples being derivatized either with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce) in pyridine or with diazomethane.

Enzyme-Catalyzed β-Lactam Hydrolysis. All kinetic runs were done in 100 mM potassium phosphate buffer, pH 7.0, 30 °C, except where otherwise noted. β-Lactam cleavage was measured in three ways: by ultraviolet absorption changes, by the decrease in the β-lactam carbonyl absorption in the infrared, and by ^1H NMR changes.

The incubation of penicillanic acid sulfone with the enzyme results in the formation of a chromophore having a λ_{max} at 225 nm. The reaction is followed conveniently at 235 nm, where the $\Delta\epsilon$ is 1780 (the intact sulfone has ϵ_{235nm} of 570). Typical concentrations were as follows: penicillanic acid sulfone, 0.8 mM; β-lactamase, 2 μM. The reaction was also followed in 100 mM deuterated potassium phosphate buffer, in D_2O, pD 7.4. [Reported pD values are 0.4 unit higher than the pH meter reading (Glasoe & Long, 1960).] The incubation of penicillanic acid with the enzyme results in a decrease at A_{235nm} having a $\Delta\epsilon$ of 470 (the intact penam has ϵ_{235nm} of 650).

Fourier transform infrared measurements were made at 2-cm^{-1} resolution in deuterated buffer in D_2O at 25 °C, the solution being contained in a thermostated cell with CaF_2 windows and a path length of 0.108 mm. Each spectrum was the average of 120 interferograms (total acquisition time, 3.0 min). The β-lactam carbonyl absorption is at 1771 cm^{-1} (unlabeled sulfone) or 1767 cm^{-1} (dideuterio sulfone). "Double-beam" difference spectra were obtained from the ratio of single-beam spectra at various times during the reaction and the single-beam spectrum of the same sample after the reaction was complete. Typical concentrations were as follows: penicillanic acid sulfone, 1.9 mM; β-lactamase, 3.4 μM.

Proton NMR changes were measured at 29 °C. To obtain accurate integrals for kinetic measurements, we measured the relaxation times for a solution of penicillanic acid sulfone (100 mM) in D_2O by a 180°–τ–90° T_1 experiment (Vold et al., 1978). For kinetic experiments, the spectrum of penicillanic acid sulfone (200 μL of a 152 mM solution) with β-lactamase (20 μL of a 0.38 mM solution) in 100 mM potassium phosphate buffer in D_2O, pD 7.4, in a 5-mm tube was measured. Spectra were obtained in successive 2-min blocks of 15 scans each with a 500-Hz spectral width, collected into an 8K memory.

Enzyme Inactivation. The inactivation of the β-lactamase by penicillanic acid sulfone was followed either by assay of the remaining enzyme activity after gel filtration or by dilution of an enzyme sample directly into an assay solution of benzylpenicillin. For the gel filtration assay, enzyme (25 μL of a 52 μM solution) was added to penicillanic acid sulfone (300 μL of a 25 mM solution). At appropriate intervals, portions of the incubation mixture were diluted into buffer (300 μL) at 0 °C and immediately subjected to gel filtration at 4 °C on a column (1.0 × 36 cm) of Sephadex G-50. Fractions were assayed for residual enzyme activity. Control experiments showed that the recovery of enzyme activity was >95%. For the dilution assay, portions (10 μL) of a similar incubation were first diluted into a solution (500 μL) of buffered benzylpenicillin (3 mM). A portion (25 μL) of this solution was then mixed in a 10-mm optical cuvette with a buffered solution (3.0 mL) of benzylpenicillin (3 mM), and the hydrolysis of the benzylpenicillin was followed at 240 nm. The final concentration of penicillanic acid sulfone was <3.5 μM.

K_i Determination. The K_i values for the penicillanic acid sulfones were obtained from double-reciprocal plots of the initial velocities of the hydrolysis of benzylpenicillin (140 μM) catalyzed by the enzyme (0.5 mM) in the presence of varying amounts of inhibitor (0.1–20 μM). Under these conditions, <2% of the sulfone is hydrolyzed during the reaction, and there is no detectable loss of enzyme activity. The K_m value for benzylpenicillin under the conditions used is 20 μM.

Assay for Malonsemialdehyde. Malonsemialdehyde was assayed by using β-hydroxybutyrate dehydrogenase and NADH. A sample of penicillanic acid sulfone (25 μL of a solution of 4 mM) was completely hydrolyzed by β-lactamase (10 μL of a solution of 85 μM), in the presence of NADH (1 mL of a solution of 0.32 mM), and β-hydroxybutyrate dehydrogenase (30 μL of an ammonium sulfate suspension containing 2 nmol) was added. The fall in absorbance at 340 nm provided a measure of the aldehyde present. Control experiments showed that the addition of penicillanic acid sulfone, β-lactamase, penicilloic acid, or acetaldehyde resulted in no change in the NADH absorption. Authentic malonsemialdehyde [prepared according to Den et al. (1959) from ethyl β,β′-diethoxypropionate (Straus & Voss, 1926)] responds quantitatively in this assay.

Results and Discussion

Penicillanic acid sulfone (**1**) is both a substrate and an inactivator of the β-lactamase, and on the basis of studies of other β-lactam derivatives that also result in enzyme inactivation (Fisher et al., 1978; Charnas et al., 1978; Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b; Cartwright & Coulson, 1979), the mechanistic outline of Scheme I was proposed (Fisher et al., 1980a). It was the purpose of the present work to probe the adequacy of Scheme I as a description of the interaction between the β-lactamase and penicillanic acid sulfone, which is structurally one of the simplest of the β-lactamase inactivators [see also Labia et al. (1980)].

The enzyme-catalyzed hydrolysis of penicillanic acid sulfone can be followed by ultraviolet, infrared, and NMR spectroscopy. In the ultraviolet, an increase in absorbance at 225 nm is observed which shows clean isosbestic points over the whole conversion of substrate to products. The reaction rate is independent of substrate concentration at levels higher than 50 μM, and a first-order rate constant (k_{cat}) of 1.99 s^{-1} can be derived (Table I). That this absorbance change is monitoring the hydrolytic cleavage of the β-lactam ring is confirmed by direct measurement in the infrared of the rate of disappearance of the carbonyl absorbance at around 1770 cm^{-1}. The rate of β-lactam cleavage can also be followed by ^1H NMR, by measuring the signal intensities of the *gem*-dimethyl groups at C-2 in the parent sulfone and its hydrolysis product. In each case, the first-order rate constants are within 5% of that measured in the ultraviolet in D_2O.

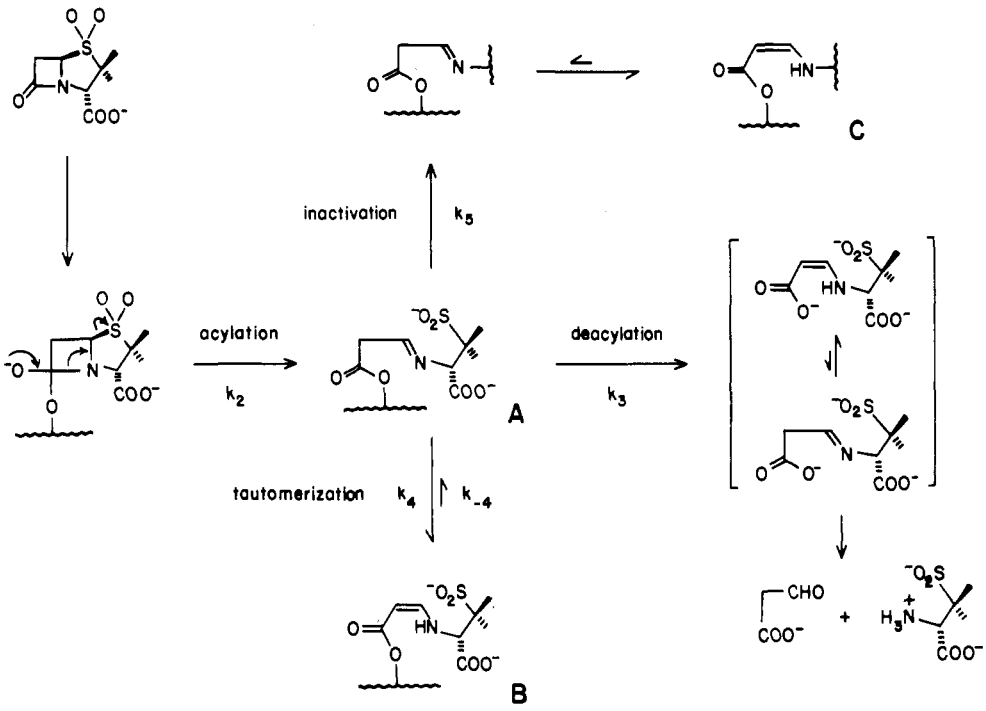
By analogy with other substrates for the β-lactamase, we expect that the enzyme catalyzes the cleavage of the β-lactam ring—via the intermediacy of an acyl-enzyme (Fisher et al.,

Table I: Kinetic Parameters for the Interaction of Labeled and Unlabeled Penicillanic Acid Sulfone with β-Lactamase

	k_{cat}(obsd) (s^{-1})	$k_{cat}/k_{cat}^{h_2}$	k_{inact}(obsd) (s^{-1})	$k_{inact}/k_{inact}^{h_2}$
sulfone sample				
unlabeled (h_2)	1.99	1.00	2.8×10^{-4}	1.0
6,6-dideuterio	6.00	3.02	7.3×10^{-4}	2.61
6α-monodeuterio	2.60 (2.32)[a]	1.17[b]		
6β-monodeuterio	4.47[c] (4.94)[a]	2.48[b]		
unlabeled + dideuterio (1:1 mixture)	2.98			
sulfide sample				
unlabeled	45.0			
6,6-dideuterio	44.8			

[a] Value calculated for a sample of 100% isotopically pure material. [b] Based on the calculated value for k_{cat}. [c] Sample prepared by the tin hydride route (see Experimental Procedures). The sample prepared by catalytic hydrogenolysis has a k_{cat}(obsd) of 3.86 s^{-1}, which gives 5.1 s^{-1} when allowance is made for the isotopic distribution.

Scheme II: Pathways That Account for Interaction of Penicillanic Acid Sulfone with β-Lactamase



1980b)—to the corresponding penicilloic acid. It is evident, however, that the penicilloic acid (if formed) fragments further, and the ^1H NMR spectrum of the products from hydrolysis in D_2O shows peaks at δ 1.09 (s, 3 H), 1.17 (s, 3 H), and 4.0 (s, 1 H), consistent with the sulfinate of penicillamine, along with signals at δ 5.32 and 9.69, indicative of an aldehyde in equilibrium with its hydrate. Since the ^1H NMR spectrum of the products from the hydrolysis of the 6,6-dideuteriopenicillanic acid sulfone is identical with that from the unlabeled material, both of the protons at C-6 must exchange with the D_2O solvent during the reaction. No exchange of the C-6 protons of the intact β-lactam can be detected. These data suggest that, consequent upon (or synchronously with) β-lactam cleavage, the thiazolidine ring opens and the resulting imine hydrolyzes to yield malonsemialdehyde and the sulfinate of penicillamine (Scheme II). This is supported directly by the fact that 92% of the expected amount of malonsemialdehyde can be assayed by using β-hydroxybutyrate dehydrogenase. The formation of malonsemialdehyde also accounts for the new ultraviolet chromophore at 225 nm and for the fact that the two protons at C-6 of the original penicillanic acid sulfone are lost during the hydrolysis. The fragmentation of the molecule into two parts after β-lactam hydrolysis is consistent with the hydrolytic behavior of the sulfone of phenoxymethylpenicillin. In this case, the products are phenoxyacetylglycinaldehyde and the sulfinate of penicillamine [C. Kemal, experiments quoted in Fisher et al. (1981)], the aldehyde moiety in this case having suffered decarboxylation.

The K_m value for penicillanic acid sulfone is most easily found by measuring the inhibition of benzylpenicillin hydrolysis by the sulfone, over times short enough that negligible amounts of sulfone have been hydrolyzed and insignificant enzyme inactivation has occurred. Measurements of K_i for the sulfone under these conditions yield a value of 0.8 μM. This agrees with the value of 0.8 μM determined by Labia et al. (1980). As a substrate, therefore, penicillanic acid sulfone has a k_{cat} of 2.0 s^{-1} and a K_m of 0.8 μM.

Penicillanic acid sulfone also functions as an irreversible inactivator of the β-lactamase in a process that is much slower than the hydrolytic reaction. The inactivation is followed by measuring the progressive loss of catalytic activity, the rate constant of which, k_{inact}, is 2.8×10^{-4} s^{-1} (Table I). The inactivation reaction is irreversible, in the sense that negligible amounts of enzyme activity return after gel filtration or after exhaustive dialysis against neutral buffer. From the ratio of k_{cat} to k_{inact}, it is evident that ~7000 molar equiv of sulfone are hydrolyzed before the enzyme is inactivated. This value was confirmed by examination of the fraction of enzyme ac-

tivity remaining after long times (>4 h), from a series of incubations of enzyme with lower molar ratios (<7000) of sulfone to enzyme.

The above results are consistent with the pathways outlined in Scheme I, in which the first-formed acyl-enzyme partitions between a hydrolytic reaction (the deacylation step of the normal catalytic process) and a β-elimination reaction across C-6 and C-5 (that leads to a more stable acyl-enzyme). This scheme also accommodates the observation that the inactivated protein possesses a new chromophore at 280 nm having an ϵ of 16 000, which is consistent with the β-aminoacrylate ester chromophore shown in Scheme I (Ostercamp, 1970; Bell et al., 1970). It transpires, however, that Scheme I is *not* adequate to account for all the features of the interaction of β-lactamase and penicillanic acid sulfone. These features will now be discussed.

When a portion of an incubation of sulfone with the enzyme is diluted into a solution of benzylpenicillin, the rate of benzylpenicillin hydrolysis *accelerates* during the first minute or so, before reaching a steady value that depends on the extent of enzyme inactivation. This acceleration shows that there is a transiently inhibited form of the enzyme that decomposes (regenerating active enzyme) with a rate constant of 0.05 s^{-1}. This value is independent of the time of preincubation of sulfone with the enzyme and of the extent of irreversible enzyme inactivation. The kinetic importance of this transiently inhibited form of the enzyme is strikingly illustrated by the behavior of the 6-deuterated penicillanic acid sulfones.

When the interaction of 6,6-dideuteriopenicillanic acid sulfone (**1**, $H_\alpha = H_\beta = {}^2H$) with the β-lactamase is investigated, it is found that both the rate of hydrolysis of the sulfone (k_{cat}) and the rate of irreversible inactivation of the enzyme (k_{inact}) are *accelerated* by ~3-fold (Table I). The value of $k_{cat}^{h_2}/k_{cat}^{d_2}$ is 0.33, or 0.58/deuterium. The value of $k_{inact}^{h_2}/k_{inact}^{d_2}$ is 0.38 (0.62/deuterium). This surprising result is unaffected by further recrystallization of both labeled and unlabeled sulfones. Moreover, X-ray crystallographic analyses of both materials show no discernible differences (see Appendix). Since both hydrolysis and inactivation are equally accelerated by deuterium substitution, it seemed possible that a secondary β-deuterium isotope effect could be responsible for the more rapid acylation of the enzyme, which would lead in turn to faster hydrolysis and faster inactivation. Secondary β-deuterium isotope effects may have several origins. First, the lower steric bulk of deuterium can, in hindered situations, lead to small kinetic effects (Carter & Melander, 1973), but even if the hydrogens on C-6 are subject to steric compression in the intact penam (and from the crystal structures reported under Appendix any such compression is at most marginal), this would give a k^h/k^d larger than 1. Secondly, electronic effects, which have often been ascribed to hyperconjugation (Shiner et al., 1968) could in principle yield an effect in the right direction, but such effects are small, and their relevance to the ground state of a β-lactam is dubious. A third cause of a β-deuterium isotope effect is if, during the reaction, the hybridization at the relevant carbon atom changes. Thus, a change from sp^2 to sp^3 could give a value of k^d/k^h of up to 1.25 (Kirsch, 1977). Yet the change in the carbon–hydrogen bond strength at C-6 on β-lactam ring opening is not expected to be large, and our observed values of k^d/k^h of 1.6 are well outside the range of secondary effects. Indeed, when the hydrolysis of the corresponding labeled and unlabeled sulfide (i.e., the parent penams) was studied, *no* secondary isotope effect was detected (Table I). To confirm that there was no discrimination between labeled and unlabeled sulfones in the essentially irreversible acylation step, we ran competition experiments with 1:1 mixtures of labeled and unlabeled sulfones. From ^1H NMR measurements, the rates of disappearance of the labeled and unlabeled materials were equal, which rules out any preferential consumption of the dideuterio sulfone. Further indication that the origin of the different k_{cat} values for labeled and unlabeled materials is not due to a difference in the rates of enzyme acylation comes from the fact that the observed k_{cat} for a 1:1 mixture of the two materials is 2.98 s^{-1}, which is different from the average for the two species [(1.99 + 6.02)/2 = 4.0 s^{-1}].

On the basis that the rates of sulfone hydrolysis and of enzyme inactivation are both accelerated by 6,6-dideuterio substitution by a factor larger than can be ascribed to a secondary isotope effect, we require a mechanistic pathway that uses a *primary* deuterium isotope effect to produce the observed kinetic accelerations. This is not as paradoxical as it sounds, for an enzyme-catalyzed reaction. In Scheme II, we illustrate the simplest pathway that can accommodate the results presented. The crux of Scheme II is that *an enzyme–substrate intermediate can partition in an isotope-sensitive step to a transiently stable complex*. If the main pathway for substrate hydrolysis *and* the pathway for enzyme inactivation are insensitive to deuterium substitution, then these processes will both be speeded up if less enzyme partitions into the transiently stable complex (B, Scheme II). Provided that the deuteron removed in the k_4 step is rapidly lost (either directly or via an enzyme base) to the solvent, the k_{-4} step will be isotopically insensitive. Deuterium substitution will then lead, by the primary kinetic isotope effect on k_4, to lower steady-state levels of the transient complex B, leaving more of the enzyme "available" for the other processes (hydrolysis and inactivation). Scheme II illustrates these points and also contains the proposed chemical structures that explain the kinetic and spectroscopic observations with penicillanic acid sulfone. Nucleophilic attack by the hydroxyl group of serine-70 (Fisher et al., 1980b, 1981) on the β-lactam carbonyl group leads to a tetrahedral intermediate that leads to the imine A in which both rings of the penicillanic acid sulfone have been opened (Scheme II). This acyl-enzyme suffers three fates, only one of which is sensitive to isotopic substitution at C-6. First, deacylation of the kind that occurs with normal substrates leads to the observed products of the reaction: malonsemialdehyde and the sulfinate of penicillamine. (It is possible, of course, that the imine hydrolyzes first, and this is followed by deacylation and loss of malonsemialdehyde from the enzyme. Our results do not bear on this point.) Secondly, the imine may tautomerize in an *isotopically sensitive step* to the more stable enamine B. This is the transiently inhibited form of the enzyme. The enamine B could in principle deacylate (more slowly than A, since it is an α,β-unsaturated ester) or tautomerize back to A (after loss of the deuteron) and deacylate normally. Either pathway is consistent with the data presented here, though results favoring the latter route are presented in the following paper (Kemal & Knowles, 1981). Finally, the enzyme may suffer inactivation. We propose that this involves a transimination of the imine A by a lysine residue of the protein, leading to a cross-linked inactive enzyme, C, having the β-aminoacrylate chromophore that is observed in the fully inactivated enzyme. Kinetically, the first-formed acyl-enzyme A must partition most favorably toward deacylation, allowing a number of hydrolytic turnovers as the enzyme accumulates into B, which is a "waiting room" from which free enzyme is more slowly generated. Since the formation of B is governed by a primary kinetic isotope effect involving a proton at C-6, slower formation of B will allow a

greater flux of A through the direct deacylation pathway, and the increase in the hydrolytic turnover rate will be a direct consequence of the primary isotope effect between A and B. Inactivation (A → C) is a relatively rare event that occurs only once every 7000 turnovers or so. It, too, will be accelerated by a reduction in the rate of A → B and will show a similar inverse isotope effect to that observed for the hydrolysis reaction.

The above is a qualitative description of how Scheme II accommodates the observed phenomena. More precisely, steady-state treatment of Scheme II (at short times, when enzyme inactivation can be neglected) provides

$$k_{cat} = k_2k_3k_{-4}/(k_3k_{-4} + k_2k_4 + k_2k_{-4}) \quad (1)$$

and (for longer incubations)

$$k_{inact} = k_5k_{-4}/k_4$$

At saturating substrate concentrations ($[S]_0 \gg K_m$) and since $k_2 > k_3 > k_4 > k_{-4} \gg k_5$ (see the arguments above), then provided that k_4 is the only isotopically sensitive step

$$k_{cat}^h/k_{cat}^d = k_4^d/k_4^h$$

and

$$k_{inact}^h/k_{inact}^d = k_4^d/k_4^h$$

That is, both k_{cat} and k_{inact} should reflect the same isotope effect, which is the inverse of the isotope effect on k_4. This is what is observed.

Finally, the rates of hydrolysis of the 6α- and the 6β-*mono*deuterated sulfones are revealing. The rate for the pure 6β-deuterio compound (4.9 s^{-1}) is lower than that for the dideuterio material (6.0 s^{-1}), and the rate for the pure 6α-deuterio compound (2.3 s^{-1}) is higher than that for the unlabeled species (2.0 s^{-1}). These data may be interpreted in two ways. First, the abstraction of protons from the 6 position in the A–B tautomerization (Scheme II) may not be stereospecific. On the basis of a primary deuterium kinetic isotope effect at each position (α and β) of 3.0 and a negligible secondary deuterium isotope effect, one may conclude from the results of Table I that abstraction of what was the β proton (in **1**) is preferred over abstraction of the α proton by a factor of between 4 and 5. Alternatively, the proton abstraction from the 6 position of the substrate moiety in A may be completely stereospecific, only the β proton being lost. In this case, the observed isotope effect for the 6β-deuterated sulfone would derive from a primary isotope effect of ~2.5, and that for the 6α-deuterated sulfone would be caused by a secondary isotope effect of ~1.2. Together, these would result in a combined effect of 3.0 for 6,6-dideuterio sulfone, as observed.

A stereospecific proton abstraction in the A → B conversion could be due to the fortuitous positioning of a base at the active site that catalyzes the tautomerization, but it is equally possible that only one of the C-6 protons is accessible to solvent and that proton is abstracted by water or hydroxide ion. The option of abstracting this (the 6β) proton is not open, of course, to sulfones of 6β-aminoacylpenicillanic acids such as quinacillin and methicillin (Fisher et al., 1981), and these compounds must lose their proton from the other side (i.e., the proton that was 6α in the original sulfone). However, since the imine A has lost the steric constraints of the intact penam, the partial or complete stereochemical preference for the β proton of the unsubstituted sulfone may only reflect the steric exigences of the acyl-enzyme. The sufficiency of Scheme II in describing the interaction of penicillanic acid sulfone with the β-lactamase is supported by the results reported in the following paper (Kemal & Knowles, 1981) in which the kinetic and spectroscopic details of the reactions are further defined.

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Appendix: Crystal Structure of Penicillanic Acid Sulfone

The crystal structures of the free acids of both unlabeled penicillanic acid sulfone and of its 6,6-dideuterio analogue have been determined. The structure of the unlabeled molecule is shown in Figure A1, the bond lengths and bond angles for both compounds are listed in Tables AI and AII, and the positional parameters are listed in Table AIII. There are no significant differences between the two structures, and bond distances and angles of the β-lactam and thiazolidine rings agree with those of other penams (Boles & Girven, 1976; Blanpain et al., 1977; Csöregh & Palm, 1977). The sulfur–oxygen bond lengths are very close to the standard S=O double bond distance of 1.43 Å (Sime & Abrahams, 1960; *Tables of Interatomic Distances and Configurations in Molecules and Ions*, 1965). The thiazolidine rings adopt a nearly envelope conformation with the atoms C_2, C_3, N, and C_5 approximately in one plane. The distance of S from this plane is 0.86_6 Å (unlabeled sulfone) and 0.87_6 Å (dideuterio sulfone). A similar conformation was observed in ampicillin, in which the sulfur atom is 0.84 Å out of the plane (Boles & Girven, 1976).

An important structural feature for the biological activity is the pyramidal character of the β-lactam nitrogen (Woodward, 1980). The distance of the nitrogen atom from the plane of its three attached carbon atoms is 0.377 Å (unlabeled sulfone) and 0.373 Å (dideuterio sulfone), and these values are compared with those for other penams in Table AIV.

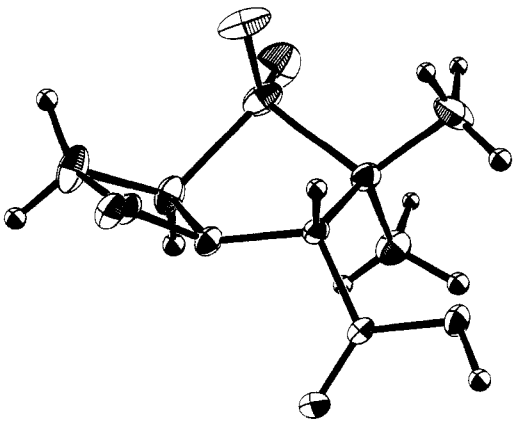

FIGURE A1: Perspective drawing of the structure of penicillanic acid sulfone.

Table AI: Bond Distances (Å) of Penicillanic Acid Sulfone[a]

bond	unlabeled sulfone[b]	6,6-dideuterio sulfone[c]
$S-O_9$	1.44_2	1.44_2
$S-O_8$	1.43_7	1.43_9
S–C	1.82_3	1.82_3
C_2-C_{10}	1.52_4	1.52_3
C_2-C_{11}	1.52_7	1.52_0
C_2-C_3	1.55_4	1.55_8
C_3-C_{13}	1.52_3	1.52_0
$C_{13}-O_{15}$	1.32_7	1.32_7
$C_{13}-O_{14}$	1.18_4	1.18_4
C_3-N	1.47_3	1.47_0
$N-C_5$	1.47_4	1.47_6
C_5-S	1.79_8	1.80_1
$N-C_7$	1.38_8	1.38_9
C_7-O_{12}	1.20_2	1.19_7
C_6-C_7	1.51_8	1.52_5
C_5-C_6	1.54_5	1.54_7

[a] C_{10} is the C_β methyl group; C_{11} is the C_α methyl group; C_{13} is the carboxyl group; O_8 is the β-sulfone oxygen; O_9 is the α-sulfone oxygen; O_{12} is the β-lactam carboxyl oxygen; O_{14} is the α-carboxyl oxygen; O_{15} is the β-carboxyl oxygen. [b] Estimated standard deviations: 0.005–0.007 Å. [c] Estimated standard deviations: 0.004–0.006 Å.

Table AII: Bond Angles (deg) of Penicillanic Acid Sulfone[a]

angle	unlabeled sulfone[b]	6,6-dideuterio sulfone[c]
O_9-S-O_8	118.8	119.0
O_9-S-C_2	108.0	108.2
O_8-S-C_5	114.3	113.8
C_5-S-C_2	93.2	93.2
C_5-S-O_9	107.7	108.2
O_8-S-C_2	111.6	111.4
$S-C_2-O_{10}$	109.1	108.6
$O_{10}-C_2-O_{11}$	111.5	112.4
$S-C_2-C_3$	101.0	100.8
$O_{11}-C_2-C_3$	113.1	113.1
$S-C_2-O_{11}$	108.4	107.9
$C_3-C_2-O_{10}$	113.2	113.1
C_2-C_3-N	106.1	105.9
$C_2-C_3-C_{13}$	114.8	114.6
$N-C_3-C_{13}$	111.0	111.2
$C_3-C_{13}-O_{15}$	110.4	110.9
$C_3-C_{13}-O_{14}$	124.1	124.2
$O_{14}-C_{13}-O_{15}$	125.5	124.9
C_3-N-C_5	119.1	119.4
C_3-N-C_7	127.5	127.3
C_7-N-C_5	92.5	92.9
$N-C_7-O_{12}$	131.7	130.9
$N-C_7-C_6$	93.0	92.8
$O_{12}-C_7-C_6$	136.2	136.4
$C_7-C_6-C_5$	85.0	85.1
C_6-C_5-N	88.6	88.5
C_6-C_5-S	118.1	117.2
$N-C_5-S$	99.3	99.0
$C_2-C_3-C_{13}-O_{15}$	59	59

[a] See footnote *a* of Table AI. [b] Estimated standard deviations: 0.6–0.8°. [c] Estimated standard deviations: 0.5–0.7°.

Table AIII: Positional Parameters (Å) for Penicillanic Acid Sulfone[a]

	unlabeled sulfone[b]			6,6-dideuterio sulfone[b]		
	x	*y*	*z*	*x*	*y*	*z*
S_1	0.1779 (3)	1.0722 (2)	0.6762 (1)	0.1782 (2)	1.0720 (2)	0.6762 (0)
C_2	0.1186 (9)	1.0951 (8)	0.6014 (2)	0.1185 (7)	1.0953 (6)	0.6014 (1)
C_3	0.2541 (8)	0.9291 (8)	0.5767 (2)	0.2550 (6)	0.9286 (6)	0.5767 (1)
N_4	0.2553 (7)	0.7784 (6)	0.6199 (2)	0.2556 (5)	0.7782 (4)	0.6199 (1)
C_5	0.1554 (11)	0.8164 (9)	0.6750 (2)	0.1538 (8)	0.8154 (7)	0.6749 (2)
C_6	0.3346 (11)	0.6982 (9)	0.7029 (2)	0.3348 (8)	0.6987 (7)	0.7031 (2)
C_7	0.4319 (10)	0.6921 (9)	0.6444 (2)	0.4329 (7)	0.6916 (7)	0.6443 (1)
O_8	0.0198 (8)	1.1662 (8)	0.7102 (2)	0.0190 (6)	1.1565 (6)	0.7102 (1)
O_9	0.4002 (7)	1.1190 (7)	0.6845 (2)	0.4006 (6)	1.1191 (6)	0.6846 (1)
C_{10}	0.1911 (16)	1.2911 (9)	0.5812 (3)	0.1924 (12)	1.2918 (7)	0.5817 (3)
C_{11}	−0.1213 (9)	1.0675 (9)	0.5931 (3)	−0.1207 (7)	1.0660 (9)	0.5936 (2)
O_{12}	0.5972 (7)	0.6386 (6)	0.6241 (2)	0.5980 (5)	0.6386 (4)	0.6241 (1)
C_{13}	0.1759 (9)	0.8520 (7)	0.5205 (2)	0.1761 (6)	0.8521 (5)	0.5206 (1)
O_{14}	0.1272 (7)	0.6908 (5)	0.5125 (2)	0.1268 (5)	0.6909 (4)	0.5125 (1)
O_{15}	0.1732 (6)	0.9895 (5)	0.4821 (2)	0.1730 (4)	0.9893 (4)	0.4820 (1)
H_{C3}	0.407 (11)	0.978 (9)	0.567 (2)	0.425 (9)	0.965 (7)	0.565 (2)
H_{C5}	0.002 (14)	0.780 (11)	0.682 (3)	−0.029 (11)	0.786 (9)	0.679 (2)
$H_{\alpha,C6}$	0.291 (11)	0.570 (8)	0.720 (3)	0.302 (8)	0.582 (6)	0.724 (2)
$H_{\beta,C6}$	0.430 (10)	0.762 (10)	0.728 (3)	0.432 (8)	0.779 (8)	0.729 (2)
H_{C10}	0.327 (11)	1.316 (9)	0.591 (3)	0.367 (10)	1.317 (9)	0.590 (3)
H_{C10}	0.126 (14)	1.376 (14)	0.604 (4)	0.111 (11)	1.379 (9)	0.604 (3)
H_{C10}	0.174 (14)	1.307 (11)	0.539 (3)	0.184 (9)	1.318 (7)	0.544 (2)
H_{C11}	−0.140 (10)	1.098 (9)	0.555 (2)	−0.121 (8)	1.090 (6)	0.553 (2)
H_{C11}	−0.194 (13)	1.162 (11)	0.617 (3)	−0.192 (7)	1.165 (6)	0.619 (2)
H_{C11}	−0.170 (12)	0.941 (9)	0.602 (3)	−0.196 (11)	0.929 (9)	0.605 (2)
H_{COOH}	0.168 (14)	0.945 (12)	0.450 (3)	0.167 (10)	0.937 (7)	0.448 (2)

[a] For atom numbering, see footnote *a* of Table AI. [b] Standard deviations are in parentheses.

Table AIV: Distance of Nitrogen from Plane of Attached Carbons

compound	distance (Å)
ampicillin[a]	0.35
6,6-dideuteriopenicillanic acid sulfone[b]	0.37
penicillanic acid sulfone[b]	0.38
ampicillin trihydrate[a]	0.38
benzylpenicillin diethyl carbonate ester[c]	0.38
benzylpenicillin[d]	0.40
methicillin methyl ester[e]	0.44

[a] Boles & Girven (1976). [b] This work. [c] Csöregh & Palm (1977). [d] Pitt (1952). [e] Blanpain et al. (1977).

Experimental Procedures

The crystals were orthorhombic, space group $P2_12_12_1$. The cell parameters were as follows: (unlabeled sulfone) $a = 6.257$, $b = 7.009$, $c = 23.756$ Å; $d_c = 1.487$ g cm^{-3}; (dideuterio sulfone) $a = 6.247$, $b = 6.993$, $c = 23.759$ Å; $d_c = 1.506$ g cm^{-3}. Intensity data were collected on a Picker automatic four-circle diffractometer with Zr-filtered Mo Kα radiation ($\lambda = 0.7107$ Å) in the θ–2θ mode. In the refinements, 1295 (unlabeled sulfone) and 1332 (dideuterio sulfone) reflections [$F > 2.0\sigma(F)$] were used. Structures were solved by direct methods. The positional and thermal parameters of the 15 nonhydrogen atoms were subjected to several cycles of full-matrix least-squares refinement. The eleven hydrogen atoms were found from a difference map after R had reached 0.076 (0.079 for the dideuterio compound), and inclusion of the parameters for the hydrogen atoms then lowered R to 0.0366 (0.0401 for the dideuterio compound). Refinement was terminated when all shifts for nonhydrogen atoms were <0.8 of the corresponding estimated standard deviation.

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