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Inhibition of β-Lactamase-I by 6-β-Sulfonamidopenicillanic Acid Sulfones: Evidence for Conformational Change Accompanying the Inhibition Process

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A number of 6-β-sulfonamidopenicillanic acid sulfones were examined for their ability to inhibit *Bacillus cereus 569*/H β-lactamase I. Among these, 6-β-trifluoromethane sulfonamidopenicillanic acid sulfone was found to be the most potent inhibitor, effecting rapid and irreversible inactivation of the enzyme. Optical rotatory dispersion and differential scanning calorimetry were employed to probe the possible conformational changes accompanying the inactivation of *B. cereus* 569/H β-lactamase I by 6-β-trifluoromethane sulfonamidopenicillanic acid sulfone. Optical rotatory dispersion measurements indicated the presence of approximately 29 and 17% helical structure in the native and inactivated enzyme, respectively. Differential scanning calorimetry determinations revealed that the inactivated enzyme was less thermostable than the native β-lactamase. The temperatures of maximum heat absorption were 48.4(±0.5) and 57.4(±0.1)°C for the inactivated and the native enzyme, respectively. Extensive conformational changes accompanying the interaction of the enzyme with the inhibitor may be responsible for the irreversible loss in the catalytic activity.

INTRODUCTION

The demonstration that simple β-lactams such as clavulanic acid (*1–3*), 6-β-bromopenicillanic acid (*4, 5*), and various penicillanic acid sulfones (PAS) (*6–10*) are effective inhibitors of β-lactamases not only offers new hope for the control of infectious diseases caused by penicillin-resistant microorganisms but also may lead to new insight into the nature of the active site of β-lactamases and perhaps into the mechanism of their action on penicillins. The inhibition of β-lactamases by penicillanic acid sulfones, **I** (Scheme 1), has been examined in greatest detail by Knowles and co-workers (*6–8*). Sound evidence has been presented to support the contention that the inhibition process is accompanied by covalent modification (very likely acylation) of a specific serine residue (serine 70 of RTEM β-lactamase). The generation of a chromophore at 290 nm upon inhibition of the enzyme

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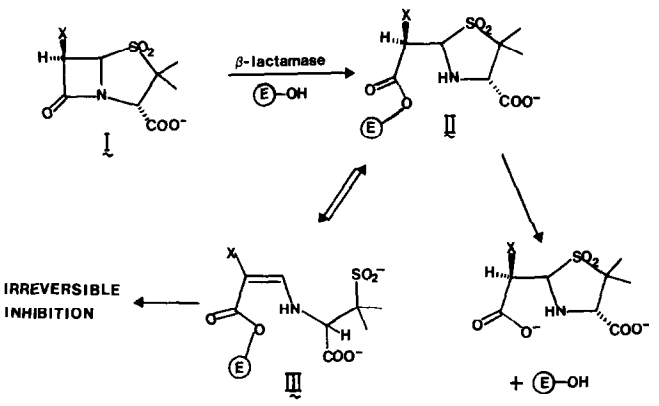
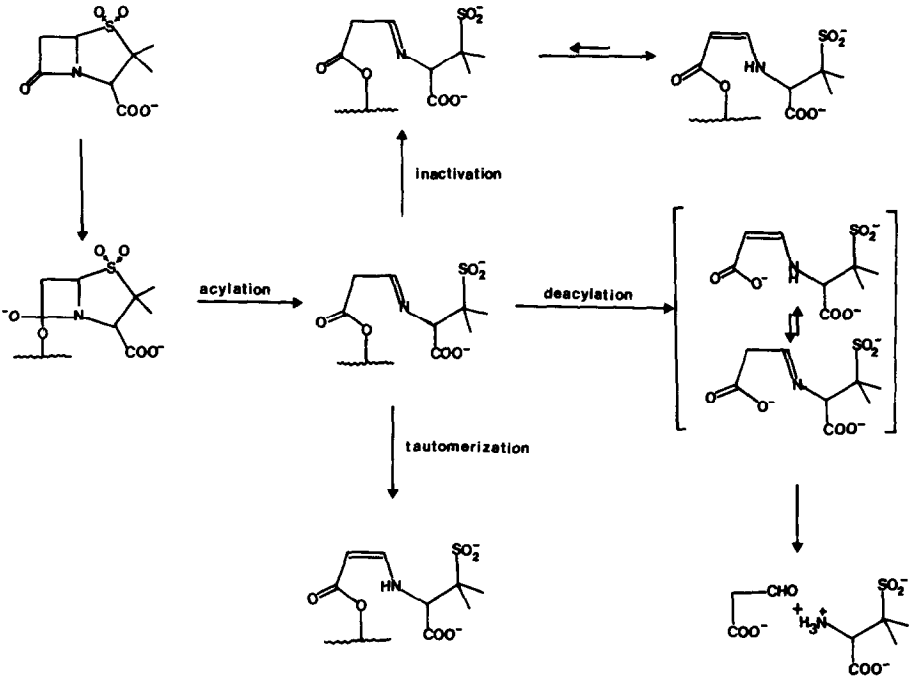


SCHEME 1.

is compatible with the assumption that the acyl enzyme, **II**, undergoes an elimination process by loss of a proton from C_6 and a sulfinate at C_5 to yield the β-aminoacrylate system, **III**. The fact that β-aminoacrylic esters are known to be much less susceptible to hydrolysis than simple esters (*11*) offered a chemically reasonable rationale for the inhibition process.

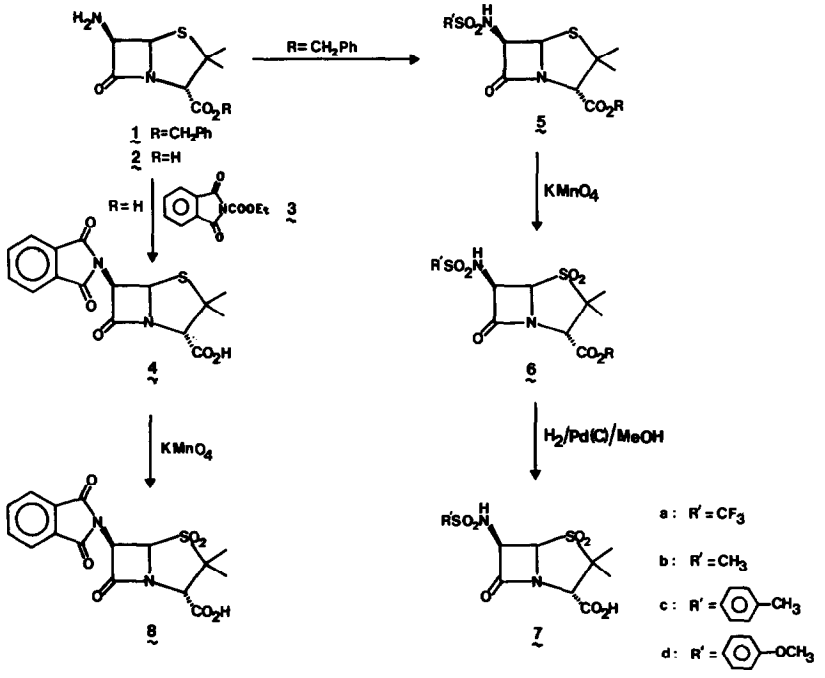
However, the acceleration in the rates of both the deacylation and irreversible inactivation observed with 6,6-dideuteropenicillanic acid sulfone led to a refine-



SCHEME 2.

ment of the mechanism above which allowed the channelling of the acyl enzyme intermediate in three ways: deacylation, irreversible inactivation and the formation of a transiently inhibited species as shown in Scheme 2 (*8*). In this mechanism, both the deacylation and the irreversible inactivation were unaffected by the isotope (at C_6) while the formation of the transiently inhibited species was an isotope-sensitive step. Thus, the abstraction of the C_6 proton (presumably by a base present in the enzyme), an obligatory requirement for the formation of a stable enamine (β-aminoacrylate ester) intermediate, was slower with deuterated PAS than with the undeuterated analog. An intramolecular crosslink formation in the enzyme by a transimination process (involving the participation of an ε-NH_2 group of the protein) accompanied by the concomitant release of penicillamine sulfinic acid was suggested to provide the basis for the irreversible inactivation.

The initial work in this area in our laboratories was based on the simple hypothesis that a more effective inhibitor based on the PAS structure might be generated by introduction of a 6-β-amino group bearing a strong electron-withdrawing group. It was felt that a 6-β-substituent might lead to more efficient binding of the inhibitor while the electron-withdrawing properties might enhance the acidity of the C_6–H, leading to a more rapid elimination to generate a β-aminoacrylate system. Among the structures considered to test this hypothesis were 6-β-phthalimidopenicillanic acid sulfone, **8** (Scheme 3), and the *N*-sulfonyl derivatives, **7**, of 6-β-aminopenicillanic acid sulfone.



SCHEME 3.

We report herein the details of our investigation of the inhibition of β-lactamase-I from *Bacillus cereus* by these compounds.

EXPERIMENTAL PROCEDURES

Synthesis. Melting points were determined on a Fisher Mel-temp melting point apparatus and are uncorrected. Infrared spectra were recorded on a Beckman IR-10 spectrometer and the frequencies (cm^{-1}) of significant peaks are reported. Nuclear magnetic resonance spectra were recorded on a Brüker WP-80 spectrometer and chemical shifts are reported on the δ scale, followed in parentheses by an account of the multiplicity, number of protons concerned, coupling constant where appropriate, and assignment to a structural feature when possible. Elemental analyses were performed by Guelph Chemical Laboratories or Galbraith Laboratories (Tennessee).

Synthesis of 6-β-toluenesulfonamidopenicillanic acid sulfone **7c**. To an ice-cold solution of 6-β-aminopencillanic acid benzyl ester, **1**, (0.50 g, 1.63 mmol) in methylene chloride (35 ml) was added dry pyridine (1.1 equivalents) and freshly recrystallized *p*-toluenesulfonylchloride (1.0 equivalents). After being stored at −15°C for 48 hr, the mixture was washed with 3% aqueous hydrochloric acid and ice water, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo* to yield **5c** as a pale yellow foam (0.659 g) which was oxidized directly without further purification.

To a solution of the entire crude product, **5c**, in 80% aqueous acetic acid (40 ml) was added a solution of potassium permanganate (0.50 g, 2.2 equivalents) in water (25 ml) gradually over a period of 10 min. After 1 hr the mixture was decolorized by dropwise addition of 30% aqueous hydrogen peroxide and poured into water (150 ml). The white precipitate which formed was collected, washed with water, and recrystallized from a mixture of methylene chloride and methanol to give the sulfone **6c** (0.343 g, 49% from **1**) mp 138–139°C.

Anal. Calcd. for $C_{22}H_{22}N_2O_7S_2$: C, 53.65; 1 H, 4.91; N, 5.69. Found: C, 53.76, H, 5.04; N, 5.60. ^{1}H NMR: 1.17 (s, 3, C_2-CH_3), 1.49 (s, 3, C_2-CH_3), 2.44 (s, ArCH_3), 4.44 (s, 1, C_3H-CO_2Bz), 4.58 (d, 1, J = 4, C_5-*H*), 5.20 (AB quartet, 2, J_{AB} = 12, -CO_2CH_2Ph), 5.38 (dd, 1, J = 4, 11, C_6-*H*-NHSO_2Ar), 6.18 (d, 1, J = 11, C_6H-N*H*SO_2Ar), 7.15–7.90 (m, 9, ArH). ir: 3340 (ArSO_2N*H*), 1800 (β-lactam), 1740 (ester).

Hydrogenolysis of the benzyl ester **6c** (0.383 g, 0.78 mmol) in a mixture of absolute methanol (30 ml) and ethyl acetate (10 ml) on a Parr hydrogenator at 47 psi over 5% palladium on charcoal (0.5 g) for 1 hr, followed by filtration through celite to remove the catalyst and removal of solvent *in vacuo*, gave the free acid **7c** (0.355 g) as a colorless oil which was converted to the more stable crystalline sodium salt (64%) by lyophilization of a solution of **7c** in water (10 ml) containing one equivalent of sodium bicarbonate. ^{1}H NMR (D_2O) 1.22 (s, 3, C_2-CH_3), 1.40 (s, 3, C_2-CH_3), 2.34 (s, 3, Ar-CH_3), 4.17 (s, 1, C_3*H*-CO_2Bz) 4.84 (d, 4 Hz, 1, C_5-H) 5.42 (d, 4 Hz, 1, C_6-H), 7.25–7.85 (m, 4, Ar-H).

Synthesis of 6-β-p-methoxybenzenesulfonamidopenicillanic acid sulfone **7d**.

The sulfone benzyl ester **6d** was prepared from **1** as described for **6c** in 42% yield after recrystallization from a mixture of methylene chloride and methanol. (mp 149–150°C).

Anal. Calcd. for $C_{22}H_{24}N_2O_8S_2$: C, 51.96; H, 4.76; N, 5.51. Found: C, 51.97; H, 4.81; N, 5.51. ^{1}H NMR 1.18 (s, 3, C_2-CH_3), 1.49 (s, 3, C_2-CH_3), 3.87 (s, 3, Ar-O-CH_3), 4.43 (s, 1, C_3H-CO_2Bz), 4.49 (d, 1, J = 4, C_5-H), 5.20 (AB quartet, 2, $J \approx 12$, -CO_2CH_2Ph), 5.33 (dd, J = 4, 11, C_6-*H*-NHSO$_2$Ar), 6.12 (d, J = 11, C_6-H-N*H*SO$_2$Ar), 7.00 (apparent doublet, 9 Hz spacing, Ar-H *ortho* to OCH_3), 7.36 (s, 5, Ar-H of benzyl group), 7.83 (apparent doublet, 9 Hz spacing, Ar-H *ortho* to SO_2-N). ir: 3340 (ArSO$_2$-N-H), 1800 (β-lactam), 1740 (ester).

The sodium salt of **7d** was prepared from **6d** as described in the preparation of the sodium salt of **7c**. ^{1}H NMR (D_2O) 1.23 (s, 3, C_2-CH_3), 1.40 (s, 3, C_2-CH_3), 3.83 (s, 3, Ar-OCH_3), 4.18 (s, 1, C_3-H-CO_2^-), 4.87 (d, 1, J = 4, C_5-H), 5.47 (d, 1, J = 4, C_6H), 7.09 (apparent doublet, 9 Hz, spacing, Ar-H *ortho* to OCH_3), 7.82 (apparent doublet, 9 Hz spacing, Ar-H *ortho* to -SO_2-N).

6-β-Phthalimidopenicillanic acid sulfone **8**. A solution of 6-β-aminopenicillanic acid **2** (10.0 g) in water (80 ml) containing sodium carbonate (5 g) was stirred vigorously at room temperature with *N*-ethoxycarbonylphthalimide **3**, for 3.5 hr. The mixture was filtered, acidified to pH2 with 10% aqueous phosphoric acid, and extracted with methylene chloride. The organic extract was washed with water and saturated aqueous sodium chloride. Removal of solvent *in vacuo* from the dried organic extract gave **4** as an amorphous solid (11.2 g, 69%). Anal. Calcd. for $C_{16}H_{14}N_2O_5S$: C, 54.48, H, 4.07; N, 8.09. Found: C, 54.84; H, 4.23; N, 8.26. ^{1}H NMR (DMSO-d_6) 1.61 (s, 3, C_2-CH_3), 1.82 (s, 3, C_2-CH_3), 4.62 (s, 1, C_3H-CO_2H), 5.59 (d, $J \approx 4$, C_5 or 6-H), 5.67 (d, $J \approx 4$, C_6 or 5 -H), 7.65–8.0 (m, 4, Ar-H).

The triethylammonium salt of the free acid, formed by evaporating to dryness a solution of the acid (1.62 g) and 1 equivalent of triethylamine in methylene chloride, was dissolved in water (55 ml). The pH of the solution was adjusted to and maintained at neutrality by treatment with 10% aqueous sodium hydroxide solution or 10% aqueous phosphoric acid as required during the addition at ice-bath temperature of a solution of potassium permanganate (1.0 g) in water containing phosphoric acid (0.6 ml). After 25 min excess permanganate was destroyed with sodium bisulfite and the mixture was filtered through celite, acidified, and extracted with methylene chloride. Removal of solvent *in vacuo* from the dried organic extract gave the sulfone **8**, as a homogeneous, colorless solid (0.81 g). Recrystallization from aqueous acetone gave colorless plates (mp 190–192°C dec.) Anal. Calcd. for $C_{16}H_{14}N_2O_7S$: C, 50.79; H, 3.73; N, 7.40. Found: C, 50.78; H, 3.60; N, 7.11. ^{1}H NMR (acetone-d_6) 1.47 (s, 3, C_2-CH_3), 1.64 (s, 3, C_2-CH_3), 4.71 (s, 1, C_3H), 5.14 (d, 1, J = 4, C_5-H), 5.95 (d, 1, J = 4, C_6-H), 7.93 (s, 5, Ar-H).

6-β-Methanesulfonamidopenicillanic acid sulfone **7b**. To an ice-cold solution of 6-β-aminopenicillanic acid benzyl ester, **1** (1.59 g, 5.2 mmol), and triethylamine (1.67 ml, 12 mmol) in methylene chloride (9 ml) was added, dropwise over 30 min, a solution of methane sulfonyl chloride (0.4 ml, 5.2 mmol) in methylene chloride (5 ml). The mixture was stirred at room temperature for 1.5 hr and washed with water (30 ml), dilute (0.2 N) aqueous hydrochloric acid, and saturated aqueous sodium chloride (30 ml). Removal of solvent *in vacuo* from the dried organic phase

gave **5b** in quantitative yield. ^{1}H NMR: 1.39 (s, 3, C_2-CH_3), 1.57 (s, 3, C_2-CH_3), 3.03 (s, 3, CH_3-SO_2-NH-), 4.35 (s, 1, C_3-H), 5.06 (s, 2, -CO_2CH_2Ph), 5.08 (d, 1, J = 4, C_5-H), 5.43 (d, 1, J = 4, C_6-H), 7.3 (s, 5, Ar-H).

The sulfone **6b** was prepared in 55% yield by oxidation of **5b** with potassium permanganate as described in the preparation of **6c**, except that isolation of the product involved dilution of the oxidation mixture with water followed by extraction with methylene chloride and removal of solvent *in vacuo*. Anal. Calcd. for $C_{16}H_{20}N_2O_7S_2$: C, 46.15; H, 4.84; N, 6.73. Found: C, 46.28; H, 4.66; N, 6.80. ^{1}H NMR: 1.25 (s, 3, C_2-CH_3), 1.53 (s, 3, C_2-CH_3), 3.12 (s, 3, CH_3SO_2NH-), 4.50 (s, 1, C_3-H), 4.80 (d, 1, J = 4, C_5-H), 5.23 (s, 2, CO_2CH_2Ph), 5.40 (d, 1, J = 4, C_6-H), 7.3 (s, 5, Ar-H).

Hydrogenolysis of **6b** as described for **6c** gave the free acid **7b** in 58% yield. Anal. Calcd. for $C_9H_{14}N_2O_7S_2$: C, 33.13, H, 4.32; N, 8.58. Found: C, 33.36; H, 4.46; N, 8.41. ^{1}H NMR (DMSO-d_6) 1.35 (s, 3, C_2-CH_3), 1.47 (s, 3, C_2-CH_3), 3.06 (s, 3, CH_3SO_2NH), 4.10 (s, 1, C_3-H), 5.25 (d, 1, J = 4, C_5-H), 5.45 (d, 1, J = 4, C_6-H).

Enzyme preparation. β-Lactamase I was isolated from a constitutive β-lactamase producing strain of *B. cereus* 569/H. The enzyme, purified as previously described (*12*), was greater than 98% homogeneous as estimated by polyacrylamide gel electrophoresis either in the absence (*13*) or the presence of sodium dodecyl sulfate (*14*).

Protein concentrations of β-lactamase I samples were estimated from the previously established $E^{1\%}_{280\,nm}$ value of 8.34 (*15*). Assays of enzymatic activity were performed spectrophotometrically (*16*) with benzylpenicillin (1 mM) as substrate using a Beckman Model 35 recording spectrophotometer.

Inhibition studies. Inhibition studies were performed by the treatment of 0.1 ~ 0.5 ml of enzyme solution (0.3–1.0 μM in appropriate buffer) with an aliquot of an aqueous solution of the compound (1 ~ 10 mM) to achieve the desired molar excess over that of the protein. The progress of the reaction was monitored by measurement of enzymatic activity using aliquots (10–25 μl) taken at regular intervals. Acetate buffers (100 mM) were used between pH 4.0 and 5.5, and sodium phosphate was employed in the pH range of 6.0 to 7.5. The amount of the compound required to effect complete inactivation of the enzyme was estimated by extrapolation of the plot of percentage remaining activity at the end of 180 min of incubation versus the ratio of molar concentration of the inhibitor to that of the enzyme.

Differential scanning calorimetry. A Perkin–Elmer DSC-2 equipped with an intracooler accessory was used to perform differential scanning calorimetry on native and inactivated β-lactamases dissolved in 50 mM sodium phosphate (pH 6.8). Aliquots of 75 μl (2% protein) were contained in stainless-steel large-volume sample pans, and the differential heat capacity was measured against the same volume of buffer in the reference cell at a programmed scan rate of 2.5°K min^{-1} from 290 to 350°K. Denaturation curves for the enzyme solutions were recorded using a range sensitivity of 0.2 mcal sec^{-1}. Maximum baseline noise was ±0.025 mcal deg^{-1} and the temperature reproducibility was ±0.1°K (Indium standard).

The required use of disposable sample capsules in the DSC instrument placed severe constraints on sample to sample baseline reproducibility, resulting in an

inability to accurately measure the absolute partial heat capacity of the protein as a function of temperature. In addition, the presence of baseline drift above 355°K did not allow the accurate estimation of the change in heat capacity accompanying the transition (10% total height). The enthalpy of denaturation, ΔH_{cal}, could, however, be accurately determined from the area under the transition peak.

Thermograms were also obtained on a Dupont 1090 DSC employing the interactive DSC V2.0 program. The protein was dissolved in 50 mM sodium phosphate, pH 6.80, and was contained in stainless-steel large-volume sample pans for measurements. Samples were heated at 2.5°K min^{-1} from 285 to 350°K. Maximum baseline irregularities were ±0.1 mcal deg^{-1} g^{-1}, and the peak temperature reproducibility was ±0.4°K.

Optical rotatory dispersion (ORD). ORD spectra of the native and the inactivated β-lactamase solutions (6.92 μM in 50 μM sodium phosphate, pH 6.8) were recorded with the aid of a Benderic Model 560 C polarmatic recording spectropolarimeter using a 1.8-mm slit width. The percentage α-helical content was determined by the depth of the trough at 233 nm using the expression, % helix = $([M']_{233} + 1900)/138$, according to the method of Ismande *et al.* (*17*).

RESULTS AND DISCUSSION

The first penicillanic acid sulfone prepared in our laboratory was 6-β trifluoromethanesulfonamidopenicillanic acid sulfone **7a**. The synthesis of **7a** involved triflation of 6-β-aminopenicillanic acid benzyl ester **1**, to give **5a**, followed by oxidation with potassium permanganate and hydrogenolysis. We were strongly encouraged in this endeavour by the observation that **7a** was substantially more potent than any of the known penicillanic acid sulfones as an inhibitor of the β-lactamase from *Escherichia coli RTEM* or *B. cereus* 569/H (see Table 1). Further investigation has now revealed that the *N*-methanesulfonyl derivative, **7b**, is also a reasonably effective inhibitor but is significantly lower in potency than **7a**.

Since the sizes of the N-substituent in **7a** and **7b** are comparable, it is reasonable to assume that the higher potency of **7a** relative to **7b** can be attributed to the strong electron-withdrawing ability of the trifluoromethyl group.

It is interesting to note that the aryl sulfonamides **7c** and **7d** are somewhat less potent than **7b**. It has been reported that sulfones of good substrates of β-lactamase such as the sulfone of penicillin V (**7**) are substrates but not inhibitors of β-lactamase. This has been assumed to indicate that deacylation of the initial acyl enzyme is more rapid than the secondary reactions which lead to formation of a stabilized acyl enzyme and inhibition of the β-lactamase. It is possible that the aryl sulfonamides are somewhat better analogs of normal substrates for the β-lactamase than are the other sulfones listed in Table 1 and, as a consequence, the initial acyl enzymes produced from **7c** and **7d** may deacylate more rapidly.

Spectroscopic investigation of the *B. cereus* β-lactamase-I inhibited by **7a** revealed the presence of a chromophore at 310 nm, suggesting a similarity between this inhibition process and that observed upon treatment of the β-lactamase from *E. coli RTEM* with penicillanic acid sulfone and quinacillin sulfone (*7,18*). How-

TABLE 1

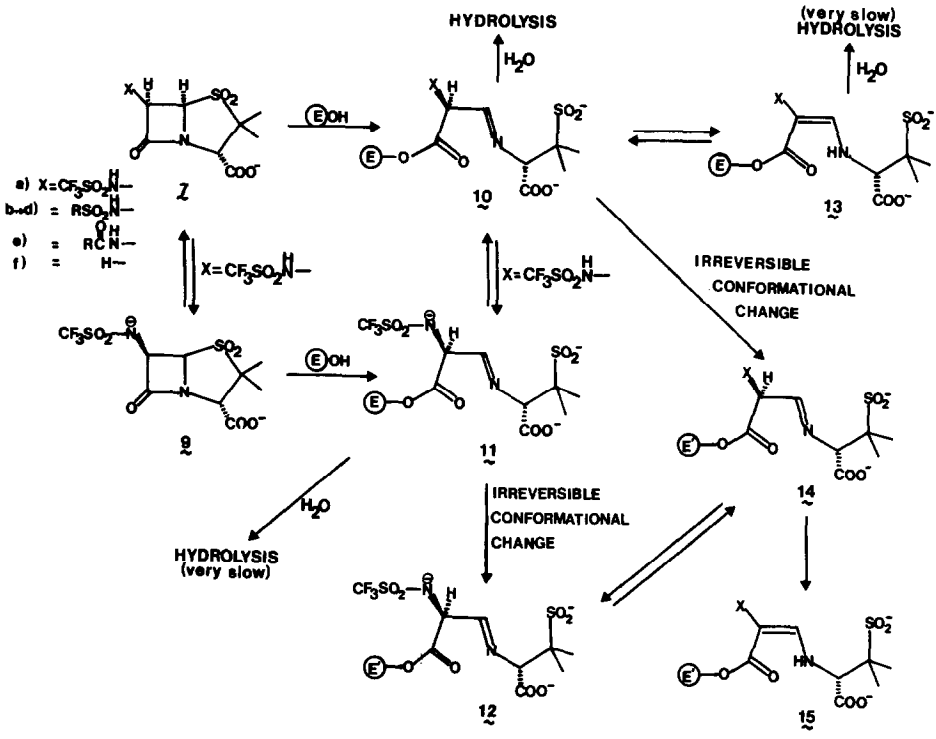
NUMBER OF HYDROLYTIC EVENTS BEFORE INACTIVATION

	B. cereus	E. coli RTEM
Penicillanic acid sulfone	20,000[b]	7000[a]
Quinacillin sulfone	1,300[b]	400
7a	120[b]	60
7b	18,000	n.d.
7c	24,000	n.d.
7d	28,000	n.d.
8[c]	8,000	n.d.

[a] From Ref. (*7*).
[b] From Ref. (*18*).
[c] Patented by Ajinomoto Company, Inc. (JP 82 16, 889, Jan. 1982; *CA 97:* P6064y) for anti-β-lactamase activity. n.d., not determined.

ever, it soon became clear that the inhibition process being observed in our laboratory with β-lactamase-I was not following the hypothetical scheme proposed by Knowles and co-workers for the inhibition of β-lactamase from *E. coli RTEM*. Treatment of β-lactamase-I with **7a** bearing a tritium label in the β-methyl group at C_2 yielded an irreversibly inhibited enzyme which retained 82% of the tritium label expected if 1 mol equivalent of **7a** were covalently attached (*19*). These observations are clearly incompatible with a cross linking mechanism which would predict a loss of penicillamine sulfinic acid and no retention of the tritium label. Furthermore, amino acid analysis of the inhibited enzyme after complete hydrolysis indicated the presence of an amino acid with the same retention time as penicillamine sulfinic acid. Enzymatic degradation of completely inhibited β-lactamase yielded a peptide which retained the chromophore at 310 nm with an amino acid composition compatible with the sequence FAFASTYK containing serine 70 of β-lactamase-I (*20*), which is homologous to the serine of RTEM β-lactamase modified by quinacillin sulfone (*7*).

Further investigations of the kinetics of inhibition of β-lactamase-I by **7a** revealed other significant differences in this inhibition process as compared with those effected by PAS and quinacillin sulfone. The inhibitory potency of **7a** was markedly greater at low pH values than at neutral pH whereas the potency of other penicillanic acid sulfones was insensitive to pH (*19*). Since the pK_a of the triflamide group of **7a** was estimated to be approximately 3.6 (*20*), it was clear that, while significant amounts of the form **7a** would be present at pH $\approx$ 4, the dianionic form **9** would predominate at neutral pH (see Scheme 4). A reasonable rationale for the observed pH dependence of inhibition by **7a** would be available if it could be assumed that **9** which predominates at neutral pH is a much poorer substrate for the β-lactamase than **7a** which is present in significant amount only at lower pH (*20*). Support for such an argument comes from the recent study which dem-



SCHEME 4.

onstrates that, both the α-COOH and α-COO^- forms of carbenicillin are hydrolyzed by the *B. cereus* β-lactamase-I, but that the latter is a much poorer substrate (*21*).

Since it was clear that a cross linking process was not operative in the irreversible inactivation of β-lactamase-I by **7a**, we turned our attention to the possibility that irreversible inactivation was associated with conformational alteration of the acyl enzyme intermediate. Although there is considerable evidence to indicate that conformational changes accompany the interaction of β-lactamases with poor (so-called A-type) substrates (*22, 23*), there have been no reports of conformational alteration of β-lactamases accompanying interactions with penicillanic acid sulfones. One indication that conformational alterations might be involved in the inhibition processes was the observation that β-lactamase-I was completely inhibited by 780 equivalents of 6-β-phthalimidopenicillanic acid sulfone **8**, in the presence of 10% acetonitrile, but required 8000 equivalents for complete inhibition in the absence of the cosolvent.

In order to assess the effect of cosolvents on native β-lactamase, the activity of the enzyme solution prepared in a medium containing acetonitrile at a final concentration of 10% (v/v) was determined over a period of several hours using benzyl penicillin as substrate. The initial rate of hydrolysis of benzyl penicillin was identical with that observed in the case of the enzyme preparation unexposed to the cosolvent. Furthermore, the initial rate of hydrolysis of benzyl penicillin by

the enzyme was unaffected by the inclusion of the cosolvent at a final concentration of 10% (v/v) in the assay. Since there is negligible effect on the catalytic properties of the β-lactamase by the cosolvent alone, a reasonable explanation of this phenomenon might be that the acyl enzyme produced by interaction with **8** is prone to conformational alteration to an irreversibly inhibited form, and that the conformational change is promoted by the presence of the cosolvent.

Further characterization of the β-lactamase inactivated by **7a** has now been achieved by the use of DSC and ORD techniques, and offers support for this interpretation.

The advantages of using the technique of DSC in the assessment of conformational changes in proteins due to either a change in their environment or interactions with other compounds have been recognized (*24, 25*). Since the temperature at which the unfolding occurs as well as the heat required to effect this process are dependent on the initial state of the protein, changes in parameters (T_m and ΔH_{cal}) characterizing the unfolding process reflect the degree of deviation from the native state of the protein. The results of the DSC studies on the native and the inactivated β-lactamases are shown in Fig. 1 and the thermal denaturation parameters are presented in Table 2. Native β-lactamase shows a single broad transition (ΔH_{cal} = 3.7 cal/g) with a maximum in the excess heat capacity (ΔC_D) of 0.499 occurring at 57.4 ± 0.1°C.

The thermogram for the inactivated enzyme preparation indicates the presence of at least two conformational variants. The major peak (85% of the total area) at 48.4 ± 0.5°C represents a species with considerably lower thermal stability than the native enzyme. The minor fraction (≃15% of the total area) appears to have properties similar to that of the native enzyme. The overall enthalpy for the denaturation of the inactivated preparation is 35 ± 9% lower than that of the

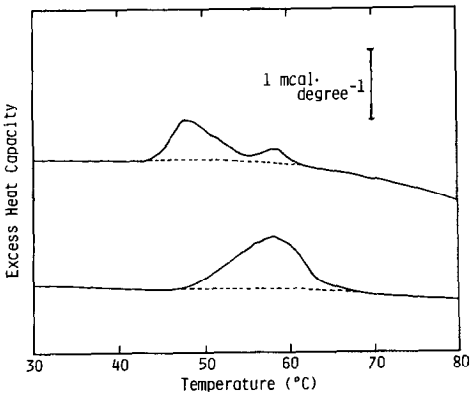


FIG. 1. DSC thermograms of native and inactivated β-lactamase. Both native and inactivated enzyme samples contained 1.58 mg protein in 75 μl 50 mM sodium phosphate, pH 6.8. Measurements were made using a Perkin–Elmer DSC-1. Lower trace, native enzyme; upper trace, inactivated enzyme; —, 1st heating; ---, 2nd heating. Previous work under similar conditions has demonstrated this transition to be kinetically rather than thermodynamically limited, leading to irreversible (within the time course of the experiment) denaturation. Hence, no peak is observed on the second heating of the sample.

TABLE 2

THERMAL DENATURATION DATA OF NATIVE AND INACTIVATED β-LACTAMASE[a]

	Native enzyme	Inactivated enzyme[d]
T_m(°K)[b]	330.5(±0.1)	321.5(±0.5); 329.1(±0.1)
ΔC_D(cal deg^{-1} g^{-1})[c]	0.499	0.447(±0.001); 0.22(±0.1)
ΔH_{cal}(cal g^{-1})[e]	3.71(±0.19)	2.43(±0.15)
(kcal mol^{-1})	111(±6)	72.8(±4.5)

[a] The values represent the means and standard deviations of results obtained with the Perkin–Elmer DSC-2 and the Dupont 1090 instruments at a heating rate of 2.5°K min^{-1}.

[b] T_m is the temperature of maximum heat absorption.

[c] ΔC_D, maximum excess specific heat occurring at T_m.

[d] Two peaks were noted in the denaturation profile of the inactivated enzyme, with the higher "melting" peak contributing approximately 15% of the total area.

[e] ΔH_{cal} is the calorimetric enthalpy for the denaturation process as calculated from the area under the transition curve.

native enzyme. However, if the higher denaturing species (the minor component) in the inactivated preparation has an enthalpy similar to that of the native species, the enthalpy of the less stable species would be even lower. The decrease in T_m and ΔH_{cal} for the major fraction of the inactivated enzyme suggests a substantial alteration in the secondary structure reflected in a decreased thermal stability of the protein. Exactly the converse would have been observed if the irreversible inactivation of the enzyme involved the introduction of an intramolecular crosslink in the protein (*26*).

The results of optical rotatory dispersion measurements on the native as well as the inactivated (by **7a**) β-lactamase preparations are shown in Fig. 2. The native

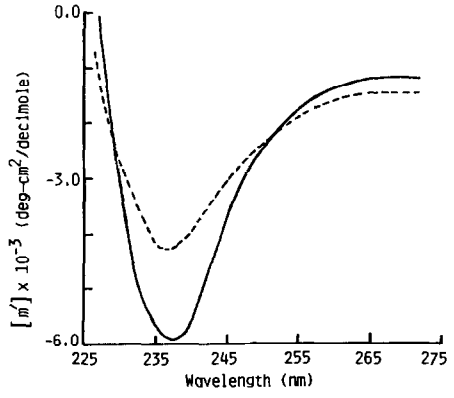


FIG. 2. ORD spectra of the native and inactivated β-lactamases. The ORD spectra of protein solutions (6.92 μM in 50 mM sodium phosphate, pH 6.8) were recorded on a Benderic Model 460C polarmatic recording spectropolarimeter using a 1.8-mm slit width. —, Native enzyme; ---, inactivated enzyme.

enzyme is characterized by a helical content (estimated from the depth of the trough at 233 nm) of approximately 29%, a value in good agreement with that documented in the literature (*17*). Similar estimations (not taking into consideration the contribution that may arise from the chromophore) show the presence of 17% helical structure in the inactivated β-lactamase. This observation is indicative of extensive conformational changes (approximately 41% diminution in helical content) accompanying the inactivation process. The decreased rotation at 200–202 nm for the inactivated enzyme (data not shown) also indicates a decreased helical content, but the noise makes the calculation of the exact magnitude of this change difficult.

The decrease in the thermal stability and the diminution in the helical content revealed in the DSC and ORD studies respectively suggest a gross disruption of the native conformation in the irreversibly inactivated β-lactamase. Thus, extensive conformational changes accompanying the interaction of the enzyme with the inhibitor may be responsible for the irreversible loss of catalytic activity.

We propose that all of the observations concerning the inhibition of β-lactamases by penicillanic acid sulfones can be accommodated by the processes illustrated in Scheme 4 which incorporates some alterations of the inhibition scheme (Scheme 2) proposed by Brenner and Knowles (*8*). For penicillanic acid sulfone **7f**, acylamido, **7e**, or sulfonamidopenicillanic acid sulfones, **7b**, **c**, and **d**, in which the amido N–H is only moderately acidic, the major processes involved are formation of the acyl enzyme, **10**, followed by hydrolysis, partitioning to a reversibly inhibited form **13**, or irreversible inhibition through conformational alteration of **10** to give **14**. Tautomerism of **14** to **15** then yields an irreversibly inhibited form of the enzyme with essentially the same chromophore as **13** but with an altered protein conformation.

For **7a**, which shows only irreversible inhibition and an unusual pH dependence, the acidity of the N–H results in the formation of **9**, which predominates at neutral pH. The acyl enzyme **10a** can be produced from **7a** but undergoes rapid deprotonation to yield **11** rather than elimination to yield the reversibly inhibited form **13a**, thus eliminating the reversible inhibition pathway. The trianion **11** might also be derived directly from **9** but at a much slower rate. The presence of the anionic site ($CF_3SO_2N^-$) near the ester carbonyl group should inhibit the hydrolytic pathway and the major pathway involves irreversible conformational change to give **12**, as supported by the ORD and DSC studies cited above. It should be noted that the potency of quinacillin sulfone as a β-lactamase inhibitor (*7*) may also be due in part to the presence of an anionic site (CO_2^-) in the C_6 sidechain.[3] Interconversion of **12** and **14a** followed by tautomerism to **15** explains the formation of the chromophore at 310 nm characteristic of inhibition by **7a**.

Although the evidence presented herein which supports the hypothesis illustrated in Scheme 4 is circumstantial, it serves as a useful starting point for the design of potentially more effective β-lactamase inhibitors which incorporate structural features expected to effectively destabilize the conformation of acyl enzyme intermediates. Such studies are now being actively pursued in these laboratories.

[3] The authors thank one of the referees for a useful comment in this regard.

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