THE INACTIVATION OF *BACILLUS CEREUS* 569/H β-LACTAMASE BY 6-β-(TRIFLUOROMETHANE-SULFONYL)AMIDOPENICILLANIC ACID SULFONE: pH DEPENDENCE AND STOICHIOMETRY

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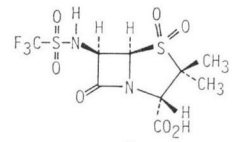
β-Lactamases have attracted considerable attention in recent years in view of their role in the ever-increasing incidences of penicillin resistance in microorganisms[1]. Efforts to identify the functional groups essential for the catalytic function by the chemical modification of the enzymes with group specific reagents have not been successful[2]. The finding that clavulanic acid, a naturally occurring β-lactam, could inactivate β-lactamases[3~5] provided an impetus for the development of several β-lactam analogs capable of serving as suicide substrates for these enzymes. These include β-bromopenicillanic acid[6,7], α-chloropenicillanic acid sulfone[8], penicillanic acid sulfone[9] and quinacillin sulfone[10]. The mechanism of action of these compounds has been elucidated[11~14]. The basic requirements for a penicillin analog to serve as a β-lactamase inactivator have been shown to include the presence of an acidic proton at C_6 and/or a good leaving group at C_5 (of the β-lactam nucleus), features that would favor elimination across the C_6–C_5 bond of the molecule. In the current study, such features were achieved by the conversion of 6-aminopenicillanic acid to 6-β-(trifluoromethanesulfonyl)amidopenicillanic acid sulfone, **I**, which proved to be a highly potent inhibitor of β-lactamase I from *Bacillus cereus* 569/H.

Homogeneous preparations of *B. cereus* 569/H β-lactamase I were obtained by the method reported earlier from this laboratory[15]. Enzymatic activity was determined by the spectrophotometric procedure using benzyl penicillin (1 mM) as substrate[16]. Inhibition studies were performed by the treatment of 0.5~1.0 ml 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 (10~20 μl) taken at regular intervals. Acetate buffers (100 mM) were used between pH 3.6 to 5.5 and phosphate (100 mM) was employed in the pH range of 6.0 to 7.5. Radioactivity measurements were performed using a Beckman LS 133 scintillation counter.

The synthesis and characterization of 6-β-(trifluoromethanesulfonyl)amidopenicillanic acid sulfone, **I**, unlabelled as well as [^{3}H]labelled in β-methyl group, has been recently described[17] and its structure is shown in Fig. 1.

Initial experiments pertained to the incubation of *B. cereus* 569/H β-lactamase I at pH 6.8 for 1 hour with different concentration of **I** to achieve a molar ratio ranging from 1 to 1,000 over that of the protein. Examination for the residual activity revealed negligible inhibition at the lowest concentration while complete loss of activity was noted at 1,000 fold molar excess of the compound. Extrapolation from a plot of percent residual activity *versus* the ratio of concentrations of **I**

Fig. 1. Structure of 6-β-(trifluoromethanesulfonyl)-amidopenicillanic acid sulfone.



I

Fig. 2. Inactivation of *B. cereus* 569/H β-lactamase I(1.67 μM) by **I** (1.68 mM) at pH 6.8 (○) and 7.5 (●).

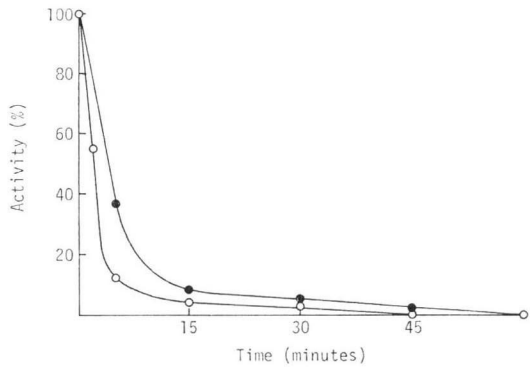


Table 1. First-order rate constants of inactivation of *B. cereus* 569/H β-lactamase I by **I**.

pH	k (sec^{-1})
3.58	2.10×10^{-2}
3.95	1.23×10^{-2}
4.25	8.00×10^{-3}
4.65	4.80×10^{-3}
5.10	1.10×10^{-3}
6.00	2.10×10^{-4}
6.80	7.60×10^{-5}

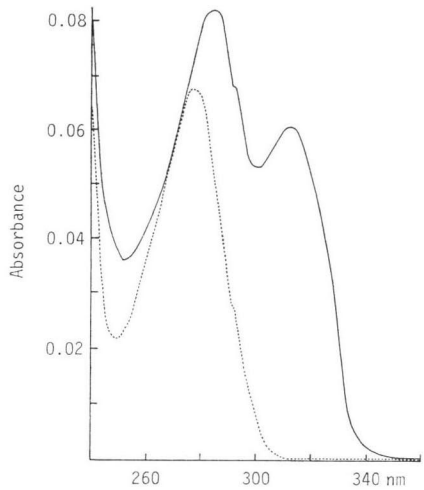
β-Lactamase I (631 nM); compound **I** (6.31 μM).
pH of the reaction as indicated. Enzymatic activity monitored with benzylpenicillin as substrate[16].

to the enzyme indicated that total loss of enzymatic activity could be achieved at 120 fold molar excess of the compound. The rapid inactivation of the enzyme upon incubation with 1,000 fold molar excess of I at pH 6.8 and 7.5 is shown in Fig. 2.

The pH dependence of the reaction was investigated by treatment of the enzyme with **I** at a concentration to provide a 10 fold molar excess of the compound over that of the protein. Enzyme solutions under identical conditions except for the omission of **I**, served as controls. The reaction was monitored periodically by the measurement of enzymatic activity[16] using 1 mM benzyl penicillin (in the same buffer as that in the reaction medium or in a buffer at pH 6.8) as substrate. In all instances, the inactivation of the enzyme was found to obey pseudo first-order kinetics. The rate of inactivation increased with the decrease in the pH of the reaction medium (Table 1). Consequently, complete inactivation of the enzyme at low pH values could be achieved by using concentrations of **I** lower than that required at high pH. No loss of activity was noted in the control samples.

The stoichiometry of the reaction leading to the inhibition of enzyme was investigated by incubating *B. cereus* 569/H β-lactamase I at pH 6.8 with a 1,000 fold molar excess of [^{3}H]labelled **I** for 30 minutes. Chromatography of the reaction mixture on a 2×36 cm column of Sephadex G-25 (100 mM phosphate, pH 6.8, serving as equilibration and elution medium) resulted in a clear separation of the protein from the excess of I and other products formed during the reaction. Examination of the recovered protein fractions for the [^{3}H]label and subsequent quantitation revealed the presence of 0.82 moles of **I** per mole of the protein. These results suggest that the inactivation process is accompanied by a stoichiometric incorporation of approximately one mole of the inhibitor per mole of the enzyme. The retention of the radioactive label even after prolonged dialysis or gel filtration of the inactive enzyme is indicative of a covalent modification of the enzyme. The inactive enzyme was characterized by a strong absorption band with a λ_{max} at 311 nm (Fig. 3). No regeneration of activity could be detected upon incubation of the inactive protein at pH 6.8 or treatment with 10 mM NH_2OH, pH 7.0.

Fig. 3. Absorption spectra of *B. cereus* 569/H β-lactamase I inactivated by **I**.
Enzyme (3.5 μM) and **I** (3.5 mM) in one ml of 100 mM phosphate buffer, pH 6.8 were allowed to react for 30 minutes. The inactive enzyme was recovered by chromatography in a column of 2×36 cm Sephadex G-25 with 100 mM phosphate, pH 6.8 serving as equilibration and elution medium.
—— inactive enzyme; ------ native enzyme.



The data presented in this study demonstrate the high potency of **I** to serve as an irreversible inhibitor of β-lactamases. The introduction of a strong electron-withdrawing trifluoromethanesulfonyl group was adopted to enhance the acidity of the C_6 α-proton of the β-lactam. The parent compound, 6-β-(trifluoromethanesulfonyl)amidopenicillanic acid, was neither a good substrate nor a potent inhibitor of the enzyme. Its sulfone derivative (**I**) serves as a very effective inhibitor of β-lactamase, an observation analogous to that noted with sulfones derived from

penicillin analogs which are poor substrates[10]. The initial step in the interaction of a suicide substrate with β-lactamase has been shown to involve acylation of the enzyme[10,14] to yield an acyl enzyme intermediate. In the case of penicillanic acid sulfone, proposals for a partitioning of the acyl-enzyme species (*via* an imine intermediate) into three different pathways have been advanced to explain the kinetic and spectroscopic results observed during the interaction[14]. Concerning **I**, it is not inconceivable that the mechanism of action is similar to that proposed for penicillanic acid sulfone. The formation of a chromophore during the inactivation process is consistent with this view. However, the pH dependence of inactivation with **I** is distinct from that observed with penicillanic acid sulfone, which fails to exhibit such behaviour over the pH range studied. Regardless of the mechanism involved, 6-β-(trifluoromethanesulfonyl)amidopenicillanic acid sulfone has proved to be an extremely potent, irreversible inhibitor of *B. cereus* 569/H β-lactamase.

Acknowledgements

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