Diketene Analogs as β-Lactamase Inhibitor

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Compounds having a structural analogy with diketene have been synthesized and their potencies as β-lactamase inhibitors have been studied. Among six compounds so far tested, α-phenyl-β-benzylidene-3-propanolide was shown to be an irreversible inhibitor of the enzyme. The availability of simple monocyclic compounds as β-lactamase inhibitors is discussed.

Keywords β-lactamase inhibitor; active site; acyl enzyme; diketene analog; cross-linking; suicide inhibitor

The resistance of microorganism to β-lactam antibiotics is caused by the secretion of β-lactamase, which catalyzes rapid hydrolysis of the β-lactam ring. Consequently, there is considerable interest in β-lactamase inhibitors. Considerable numbers of inhibitors of both synthetic and natural origin have been reported and their mechanisms of action have been investigated. Inhibition mechanisms reported are mostly of the "suicide" or "mechanism-based" type.[1] In connection with the design of new inhibitors, this mechanism seems most suited to hydrolytic enzymes having a large turnover number, such as β-lactamases.

Compounds which afford stable acyl enzyme intermediates are also expected to be good candidates as inhibitors for such enzymes. This approach is based on the transient inhibition of the enzyme activity by means of substrate analogs which carry out specific acylation of the catalytic residue to afford the acyl enzyme.[2] In our previous work, specific and efficient production of acyl enzymes from trypsin and trypsin-like enzymes was achieved by means of "inverse substrates.[3] The concept of "inverse substrates" itself is not applicable to β-lactamase since its substrates are different from those of proteases, which are composed of unit residues (amino acids) in a sequential chain. However, it seems a promising approach to design compounds that can carry out efficient acylation at the β-lactamase catalytic residue through a complementary structure to the active site.

Diketene is commonly used as a chemical modification reagent for proteins,[4] and its molecular shape, a four membered lactone ring, seems advantageous for the design of β-lactamase inhibitors. Compounds **1**—**6** were designed with this in mind (Chart 1). Inhibition experiments were performed by incubating 100 nM solution of β-lactamase I (*Bacillus cereus*) with each diketene derivative (final concentration; 100 μM). Determination of the residual activity was carried out spectrophotometrically using PADAC β-lactamase substrate, 7-(thienyl-2-acetamide)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium methyl]-3-cepham-4-carboxylic acid.[5] In Fig. 1 the residual activities of the enzyme toward **1** are shown as a function of the incubation period. The inactivation reaction was completed within one minute. At pH 7 and 8 the extents of the inactivation were 65 and 56%, respectively and at pH 5.5 the extent was 40%. Readdition of **1** caused further inhibition. The observation suggests that **1** is rapidly hydrolyzed in aqueous media, though it acts as an effective inhibitor for β-lactamase. The rate constant for the spontaneous hydrolysis of **1** was determined to be $7.6 \times 10^{-3}\ s^{-1}$ at pH 7.0. The presence of cephalosporin C, a competitive inhibitor of the enzyme, prevented the inhibition reaction as shown in Table I. This suggested that the inhibition was attributable to an active site directed specific reaction. In a control experiment, diketene itself was shown to have no inhibitory effect under the same conditions. Recovery of the enzyme activity was not observed after standing for several days. Furthermore, treatment of the inactive enzyme with 1 M hydroxylamine did not lead to the recovery of the activity. Therefore, the observed inactivation may not be simply due to the transient inhibition as a result of acyl enzyme formation but might be due to covalent bond formation, though we have no evidence for this at the present stage. Compound **1** was found to be ineffective against chymotrypsin. Diketene derivatives (**2**—**4**) and their

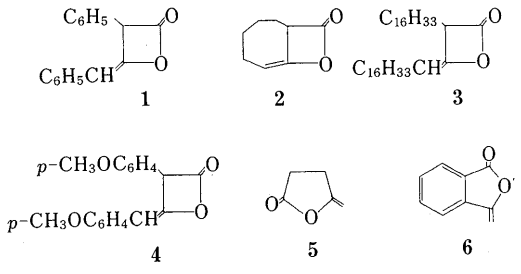

Chart 1

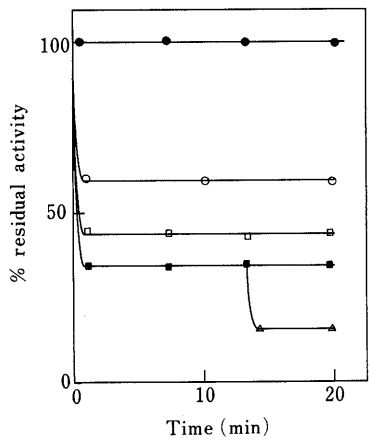

Fig. 1. Time Course of Inhibition of *B. cereus* β-Lactamase by **1**

β-Lactamase (100 nM) was incubated with **1** (100 μM) at 25 °C in 0.1 M citrate at pH 5.5 (○), 0.1 M phosphate at pH 7.0 (■) and 0.05 M Tris at pH 8.0 (□). The effect of readdition of **1** (total concentration, 200 μM) after 13 min is shown (△). The control experiment was carried out in the absence of **1** at pH 7.0 (●). The residual activity was determined by measuring the catalytic activity of an aliquot drawn from the incubation mixture. The activity was determined using PADAC β-lactamase substrate in 0.1 M phosphate at pH 7.0 as described in the text.

TABLE I. Effect of Inhibitor **1** and Related Compounds on β-Lactamase Activity

Inhibitor[a]	% enzymatic activity[b]
None[c]	100
1	30
1[d]	102
1 + cephalosporin C[e]	104
Diketene	100

a) Added in 10^3-fold molar excess. The solution contained 0.5% DMSO in 0.1 M phosphate buffer at pH 7.0. Incubation was carried out for 15 min at 25 °C. *b*) Determined by using PADAC β-lactamase substrate. *c*) Control experiment: in the absence of **1**. *d*) Compound **1** was completely hydrolyzed. Prior to the addition, **1** was treated for 10 min at 100 °C in 0.1 M phosphate buffer, pH 7.0. *e*) The enzyme was incubated for 15 min with cephalosporin C (50 mM) prior to the addition of **1**.

analogs having a five membered ring (**5**,**6**) showed no inhibitory activity under similar conditions. The structure of **1** may fit well to the enzyme active site, and acyl enzyme formation and subsequent cross-linking reaction may take place. Formation of the Schiff base between a lysine residue in the enzyme and the β-keto residue produced be the enzymatic catalysis of **1** is one possible explanation for the cross-linking.

In the search for new β-lactam antibiotics, considerable numbers of antibiotics carrying novel ring systems have been discovered. Monocyclic β-lactam, monobactam,[6] is a typical example of this category. Furthermore, several compounds which lack a β-lactam ring were proposed to exhibit antibacterial activity in a similar manner to β-lactam antibiotics. These non-β-lactam antibiotics (β-lactam antibiotic family) include γ-lactam[7] and isoxazolidone[8] derivatives. These examples encouraged us to consider diketene analogs as inhibitors of β-lactamase. Diketene analogs could be useful as β-lactamase inhibitors, though the stability of **1** is not satisfactory. Improvement of the stability in aqueous media by means of structural modification will be the subject of further study.

Experimental

Materials PADAC β-lactamase substrate was purchased from Hoechst, Calbiochem (lot 506302). Benzoyl-L-tyrosine-*p*-nitroanilide was obtained from the Peptide Research Foundation, Osaka. *Bacillus cereus* β-lactamase (lot P 0839) and cephalosporin C were obtained from Sigma. Chymotrypsin was a product of Cooper Biomedical (lot CDI).

Preparation of α-Phenyl-β-benzylidene-3-propanolide (1) Treatment of mandelic acid with phosphorus pentachloride and subsequent dimerization were carried out as described by Farnum *et al.*[9] Recrystallization from *n*-hexane gave colorless granules: mp 70—70.5 °C (lit.[9] mp 63—70 °C).

Preparation of Diketene Analogs (2—6) Synthesis of 9-oxo-8-oxabicyclo[5.2.0]non-6-ene (**2**), α-hexadecyl-β-heptadecylidene-3-propanolide (**3**) α-*p*-methoxyphenyl-β-(*p*-methoxyphenyl)methylene-3-propanolide (**4**), γ-methylene-γ-butyrolactone (**5**) and 1-oxo-3-methylenephthalan (**6**) were carried out according to the reported procedures.[9-13]

General Procedure for the Measurement of β-Lactamase Activity Residual activity of β-lactamase was determined by using PADAC β-lactamase substrate[5] as a substrate in 100 mM phosphate buffer (pH 7.0) at 25 °C with monitoring of the decrease of absorbance at 570 nm, taking $\Delta\varepsilon$: 4000. To PADAC solution in buffer (3 ml) containing 0.5% dimethylsulfoxide (DMSO), 50 μl of β-lactamase solution was added. Final concentrations of substrate and enzyme were 50 μM and 1.67 nM, respectively. The concentration of *Bacillus cereus* β-lactamase I was determined based on the reported rate constant, 3500 s^{-1} for penicillin G at pH 7.0.[14]

Measurement of Spontaneous Hydrolysis of Diketene Derivatives Rates of spontaneous hydrolysis were measured at pH 5.5 (100 mM citrate buffer), pH 7.0 (100 mM citrate buffer) and pH 8.0 (50 mM Tris buffer). Decrease in the absorption band at the appropriate wavelength was monitored after rapid mixing of diketene (in DMSO) with the buffer. The final concentrations of **1** and DMSO were 130 μM and 1.6%, respectively. Hydrolysis of **1** at pH 5.5 and 7.0 was monitored at 250 nm, and for the hydrolysis at pH 8.0, the wavelength of 242 nm was used.

Inhibition of β-Lactamase by 1 Inhibition reactions were carried out in the same media as employed for the spontaneous hydrolysis. To 1 ml of β-lactamase solution (100 nM), 10 μl of inhibitor stock solution in DMSO (final concentration; 100 μM) was added. After incubation for an appropriate period at 25°C, a 50 μl aliquot was withdrawn and subjected to residual activity analysis using PADAC according to the general procedure. In the same manner, lactamase activity in the absence of inhibitor was determined as a control.

Analysis of Inhibition of Chymotrypsin The effect of **1** on chymotrypsin was analyzed by monitoring the enzyme-catalyzed hydrolysis of benzoyl-L-tyrosine *p*-nitroanilide (BTNA)[15] spectrophotometrically in the presence of **1**. To a solution of BTNA in 0.05 N Tris (pH 8.0) containing 5% DMSO (3 ml), 75 μl of chymotrypsin stock solution was added. The absorption increase at 385 nm was recorded. In the same manner, the effect of the presence of **1** on the reaction velocity was analyzed as a function of incubation time after addition of the inhibitor. The final concentrations of substrate and chymotrypsin were 100 μM and 100 nM, respectively.

Treatment of Inhibited Lactamase with Hydroxylamine β-Lactamase was reacted with **1** in 100 mM phosphate buffer (pH 7.0) at 25°C for 1 min. Concentrations of lactamase and **1** were 100 nM and 100 μM, respectively. Residual activity was 35%. The inhibited enzyme solution was mixed with an equal volume of 2 M hydroxylamine (pH 7.0) and kept at 4°C for 12 h. The resulting solution was subjected to catalytic activity analysis after dialysis against 100 mM phosphate buffer (pH 7.0). A similar procedure was performed in the absence of hydroxylamine as a control.

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