Volume 250, number 2, 218–220 FEB 07260 July 1989

N-Nitroso-β-lactams as β-lactamase inhibitor

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Received 1 April 1989

N-Nitroso-β-phenyl-β-lactam has been found to be a specific inhibitor of β-lactamase. *N*-Nitroso-α-phenyl-β-lactam, by contrast, was virtually ineffective although a transient inhibition of short duration was observed. The acyl enzyme derived from the β-phenyl isomer is presumably involved in a cross-linking reaction, whereas that from the α-phenyl isomer was quenched by spontaneous hydrolysis without formation of a covalent bond. No inhibitory effect of the β-phenyl isomer on chymotrypsin has been observed.

Lactamase inhibitor, β-; Acyl enzyme; Mechanism-based inhibitor; Nitroso-β-lactam, *N*-; Enzyme active site

1. INTRODUCTION

A number of β-lactamase inhibitors, either synthetic or natural, have been reported [1]; several of them are applied in the clinical field. These inhibitors are mostly classified as 'mechanism-based'. Design of inhibitors of this category seems most promising for enzymes having large turnover numbers, such as β-lactamase. *N*-Nitroso-δ-lactam was proposed as a mechanism-based inhibitor for chymotrypsin [2]. It was observed that the enzymatic hydrolysis of the lactam ring initiates spontaneous formation of a carbonium cation capable of covalently labeling the enzyme. This report prompted us to study the behavior of *N*-nitroso-β-lactams (I and II) with β-lactamases.

2. METHODS AND RESULTS

2.1. *Materials*

PADAC β-lactamase substrate, 7-(thienyl-2-acetamide)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium methyl]-3-cepham-4-carboxylic acid [3], was a product from Hoechst, Calbiochem. Cephalosporin C was obtained from Sigma. Benzoyl-L-tyrosine-*p*-nitro-anilide (BTNA) was obtained from Peptide Research Foundation, Osaka. Chymotrypsin was a Cooper Biomedical product.

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2.2. *Synthesis of* N-*nitroso-β-phenyl-β-lactam* (I)

β-Phenyl-β-lactam (I′) was synthesized as in [4]. Nitrosoation of I′ was carried out as reported [5]. Recrystallized from *n*-hexane. Pale yellow fine needles, m.p. 78.5–82.5°C (46% yield). Anal. Calcd. for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90. Found: C, 61.37; H, 4.59; N, 15.82.

2.3. *Synthesis of* N-*nitroso-α-phenyl-β-lactam* (II)

Nitrosoation of α-phenyl-β-lactam (II′) [6] was carried out as above. Pale yellow viscous oil was obtained (25% yield). IR (nujol): 1810, 1740, 1640 cm^{-1}. TLC on silica gel (benzene) gave a single spot.

2.4. *Inhibition of β-lactamase with* I

In fig.2 the residual activity of the enzyme as a function of the incubation period with I was shown. The inactivation reaction was completed within a minute (the limit of the experimental procedure). At pH 5.5, 7 and 8, the extent of the inactivation was 40, 81 and 75%, respectively. Readdition of I caused further inhibition and nearly complete inactivation. The inactivation reaction was prevented by the presence of cephalosporin C, an active site specific compound. Both I′ and the hydrolysate of I did not cause inhibition (table 1).

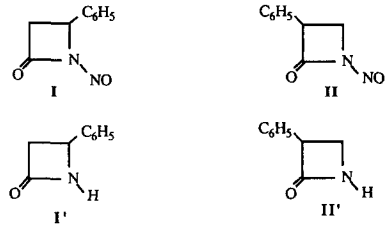

Fig.1. Structure of β-lactam derivatives.

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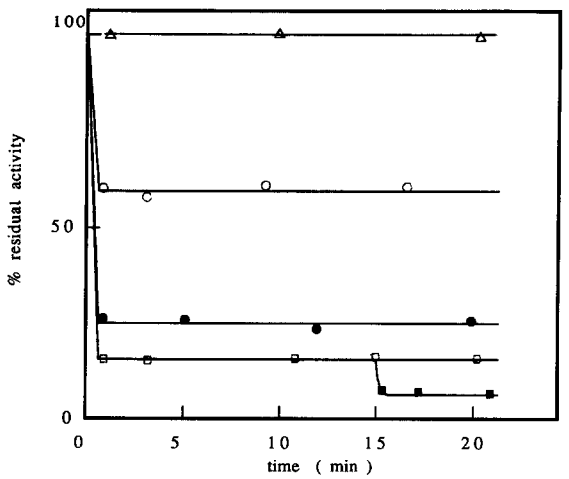

Fig.2. Time course of inhibition of *B. cereus* β-lactamase by **I**. β-Lactamase (100 nM) was incubated with **I** (1 mM) at 25°C in 0.1 M citrate, pH 5.5 (○), 0.1 M phosphate, pH 7.0 (□) and 0.05 M Tris, pH 8.0 (●). Effect of readdition of **I** (total concentration, 2 mM) (■). Control experiment: in the absence of **I** at pH 7 (△). The residual activity was determined using PADAC substrate in 0.1 M phosphate at pH 7.0, 25°C monitoring the absorbance at 570 nm taking $\Delta\epsilon$ 4000. To 3 ml of PADAC solution (50 μM), 50 μl aliquot of the incubate (containing 5×10^{-12} mol of enzyme) was added. Concentration of β-lactamase was determined based on the reported rate constant, 3500 s^{-1} for penicillin G at pH 7.0 [10].

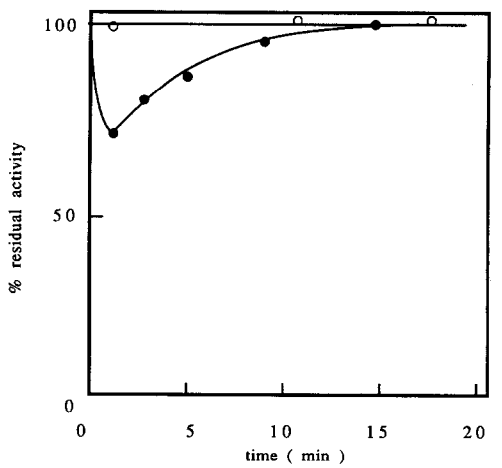

Fig.3. Time course of interaction of *B. cereus* β-lactamase by **II**. The hydrolytic rate of PADAC lactamase substrate (50 μM) in the presence of enzyme (100 nM) and **II** (1 mM) in situ was determined in 0.1 M phosphate at pH 7.0, 25°C (●). In the absence of **II** (○).

2.5. *Interaction of* II *with β-lactamase*

Analysis was carried out by a direct measurement method: the hydrolysis of PADAC substrate was determined in the presence of **II** in situ. The velocity change was analyzed as a function of the incubation period. As shown in fig.3, compound **II** exhibited transient inhibition; rapid decrease and slow recovery of activity.

Table 1

Effect of inhibitors and related compounds on β-lactamase activity[a]

Inhibitor	Catalytic activity (%)
None[b]	100
I	20
I[c]	100
I + cephalosporin C[d]	102
I′	100

[a] Enzyme (1 nM) was incubated with **I** or **I**′ (1 mM) for 15 min at 25°C, pH 7 (0.1 M phosphate containing 0.5% dimethyl sulfoxide)

[b] Control experiment

[c] Hydrolysate of **I** was added. Prior to the addition, **I** was treated for 10 min at 100°C in 0.1 M phosphate buffer, pH 7.0

[d] Enzyme was incubated for 15 min with cephalosporin C (50 mM) prior to the addition of **I**

2.6. *Interaction of* I *with chymotrypsin*

Irreversible inactivation of chymotrypsin did not happen with **I**. The observation is quite different from that of β-lactamase. In the same manner as described in section 2.4, the effect of compounds **I** (100 μM) on chymotrypsin (100 nM) was analyzed using BTNA [7] as a substrate in 0.05 M Tris, pH 8.0, at 25°C. Transient inhibition of chymotryptic activity, essentially identical to that of fig.3, was observed.

2.7. *Inhibition of β-lactamases of different origin*

The extent of inhibition is dependent on the enzyme source (table 2). **I** is superior to one of the most potent inhibitors, clavulanic acid, toward *E. coli* TEM R^+ and *P. aeruginosa* U 31. β-Lactamases used are those cited in the literature [8].

Table 2

Inhibition of β-lactamases from various origins

Enzyme source	% inhibition[a]	
	I	Clavulanic acid
B. cereus	<5	74
P. vulgaris	30	90
E. cloacae GN 4413	44	34
E. coli TEM R^+	50	<5
P. aeruginosa U 31	91	37

[a] Enzyme was incubated with inhibitor (10 mM) in 0.05 M phosphate buffer at pH 7.0, 25°C for 10 min. Residual activity of the incubate was measured using nitrocefin as a substrate [11]

3. DISCUSSION

I interacts presumably to give an acyl enzyme and subsequently brings about covalent modification of the enzyme. The formation of the covalent bond was suggested from the fact that no enzyme activity was recovered after treatment of the inhibited enzyme with 1 M hydroxylamine. By contrast, acyl-β-lactamase from **II** might be hydrolyzed spontaneously without formation of the covalent bond. Chemically both **I** and **II** are comparable. The rate constant for spontaneous hydrolysis of **II** (0.063 s^{-1} at pH 7.0) was not much different from that of **I** (0.14 s^{-1} at pH 7.0). The structural difference between acyl enzymes from **I** and **II** may cause the difference in the proximity of the respective reactive species to the enzyme nucleophile. Discrimination of **I** between chymotrypsin and β-lactamase may be caused by structural differences of the respective active sites interacting with the reactive species from **I**.

In the search for new β-lactam antibiotics, considerable numbers of antibiotics carrying a novel ring system have been discovered. Monocyclic β-lactam, monobactam [9] is just one example which encouraged us to design nitroso-β-lactams as possible inhibitors of β-lactamase. Although compound **I** has been shown to be effective in vitro, its stability in aqueous media is not sufficient enough for practical purposes. Nitroso-β-lactam, however, could be proposed as a new key compound for β-lactamase inhibitors. Further work on the improvement of the stability by designing appropriate substituents is in progress.

Acknowledgements: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture. We are grateful to Drs N. Katayama and S. Harada, Takeda Chemical Ind. Ltd., for their kind help in the analysis of inhibitory activity.

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