NOVEL CARBON-CARBON BOND CLEAVAGE REACTION OF PENEM ANTIBIOTIC BY CLASS C β-LACTAMASES

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Penem antibiotics show potent antimicrobial activities with broad spectra both in Gram-positive and -negative bacteria including various β-lactamase producing species.[1~3] β-Lactamases are classified into penicillinase and cephalosporinase according to their substrate specificity, whereas based on the amino acid sequence homology they are categorized into class A through D.[4] Among these classes, class A and C β-lactamases are major lactamases, which have an essential serine residue at their active sites. Penicillinases convert penicillins into penicilloic acids and cephalosporinases hydrolyze cephalosporins to give 5-exo-methylene-1,3-thiazine structure *via* expulsion of leaving groups at C-3 position.

Although there are not so many examples for hydrolytic products of penicillins by other penicillin-interacting proteins such as penicillin-binding proteins and D,D-carboxypeptidases of *Streptomyces* species, it has been shown that the latter enzymes afford *N*-phenylacetylglycine as the C-5~C-6 bond cleaved product of penicillin G.[5]

A recent study of the reaction of a penem derivative (**1**) with class A enzyme *E. coli* TEM-1 β-lactamase has demonstrated not only its hydrolytic action to give a β-lactam ring cleaved product, a penemoic acid,[2,6] but also a new role of the enzyme on degradation of the penemoic acid.[6]

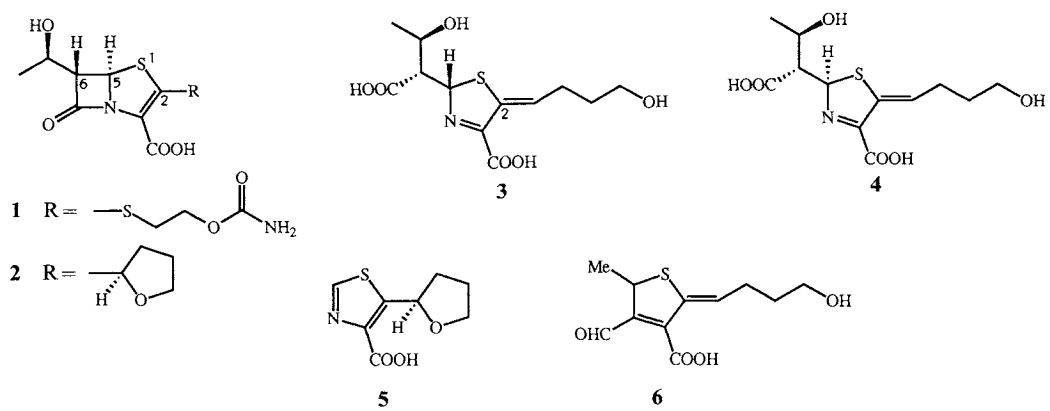
We have already reported our studies on chemical degradation of a penem derivative SY5555 (SUN-5555) (**2**) in photolysis and alkaline hydrolysis.[7,8] It was also found that the alkaline hydrolysis products were the same with those derived from hydrolytic action of a renal dehydropeptidase (DHP-I), a mammalian metalloenzyme, which destroys penems and carbapenems but not penicillins and cephalosporins.[8,9] Since class A and C β-lactamases are a family of serine proteases, the hydrolytic mechanisms must be different from that of DHP-I.

The reaction of SY5555 with class C β-lactamases from *Citrobacter freundii* GN7391[10,11] and *Enterobacter cloacea* GN7471[10,11] in 0.1 M phosphate buffer (pH 7.0) at room temperature was slow but afforded three major products (**3~5**).

UV, MS, HPLC and ^{1}H NMR analysis of these products suggested that the compounds **3** and **4** are the same with the products derived by alkaline hydrolysis through ring opening and reclosure at C-5~S-1 bond[8] whereas the compound **5** is the thiazole derivative resulted from the cleavage of C-5~C-6 bond. This thiazole (**5**) was identical with the thiazole previously obtained by photolysis.[7] This is the first observation of the C-5~C-6 bond cleavage of β-lactam antibiotics in hydrolytic action of β-lactamases. Similarity of the mechanism of this reaction to that of C-5~C-6 bond cleavage of penicillin by D,D-carboxypeptidase is suggested.

The reaction of penem **2** with class A β-lactamases

Fig. 1.



of *Bacillus cereus*†, *Escherichia coli* 205†, and *Proteus vulgaris* GN76[10,11] was more rapid than that with class C β-lactamases and provided two products (**3** and **4**). However, no thiazole derivative **5** was detected. The results are summarized in Table 1. In a prolonged reaction(>40 hours) with class A and C β-lactamases, another minor product appeared in the reaction medium.

The ^{1}H NMR spectrum (500 MHz, CD_3CN) of this minor product indicated the presence of an aldehyde (δ 9.91 ppm (s)) and the methyl protons (δ 1.51 ppm (d)) adjacent to a methine proton (δ 4.70 ppm (q)) which showed the NOE with the aldehyde proton, suggesting a close proximity of these groups to the aldehyde; also the same moiety with that of the compounds **3** and **4** at C-2 position (δ 1.63 (m), 2.12 (m), 3.50 (t), 6.10 (t))[8] were noted. These are consistent with the ^{13}C NMR spectrum observed for three methylene carbons at 28.3, 31.1, and 60.2 ppm, and one methyl carbon at 22.4 ppm. The methine carbon for the hydroxyethyl moiety at C-6 of penem **2**, however, disappeared in the spectrum and instead a characteristic carbon for sulfide methine was observed at 46.0 ppm. This indicates the substitution of the hydroxyl group with a sulfide group. The strong UV absorption with λ_{max} of 358, 275, 215 nm (H_2O) is correlated to a conjugated diene structure connected to carbonyl group for which structure four vinylic carbons at 126.9 ppm (d), 148.0 ppm (s), 139.7 ppm (s) and 145.0 ppm (s) and an aldehyde carbon at 187.8 ppm and a carboxylate carbon at 164.3 ppm (s) were observed in the ^{13}C NMR spectrum. The FAB-MS spectrum showed its parent ion at 243 m/z $(M+1)^+$, suggesting loss of the nitrogen atom and a CO_2 unit. The absence of nitrogen atom in the product was also confirmed by elementary analysis. Hence, we can conclude that the minor compound has the structure **6** as depicted in Fig. 1. A similar product was reported in the reaction of a penem derivative in acidic media.[12] It is assumed that this compound **6** was derived from the compounds **3** and **4** in nonenzymic fashion, since the compounds **3** and **4** were converted to **6** upon standing in the phosphate buffer (pH 7.0) without β-lactamases and this conversion reaction was not accelerated with β-lactamases.[6] A plausible mechanism for this conversion is shown in Scheme 1.

Table 1. The ratios* of the degradation products of the penem **2** by β-lactamases.

β-Lactamases from	Product		
	3	**4**	**5**
Citrobacter freundii[a]	15	19	66
Enterobacter cloacea[a]	25	25	50
Bacillus cereus[b]	71	29	0
Escherichia coli[b]	70	30	0
Proteus vulgaris[b]	71	29	0

* The ratios were determined from HPLC-area at 240 nm; column: Inertsil ODS-2 (4.6 × 250 mm, flow rate 1.5 ml/minute; eluent A: 5 mM Na_2HPO_4, 45 mM KH_2PO_4 and 5mM n-Bu_4NBr; eluent B: 50% eluent A in CH_3CN; A : B, 85 : 15 to 50 : 50 linear gradient for 30 minutes.

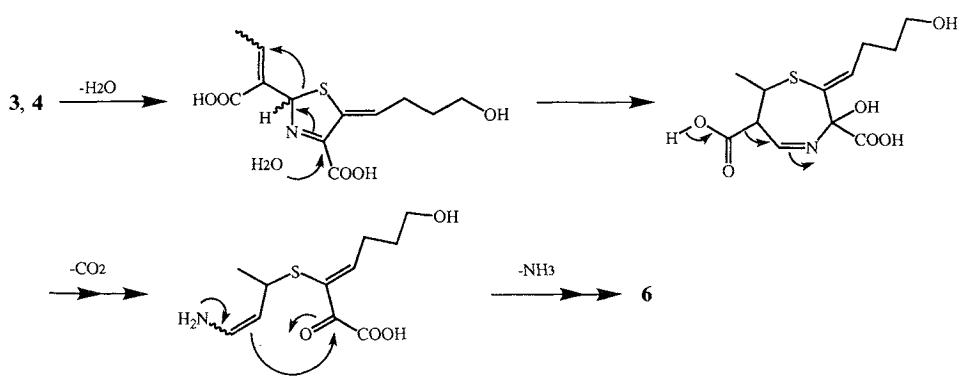
[a] Class C β-lactamase.

[b] Class A β-lactamase.

The major products were thus different in the hydrolytic reaction of the penem **2** with class A and class C β-lactamases, which difference may reflect distinct mechanisms of class A and C β-lactamases.

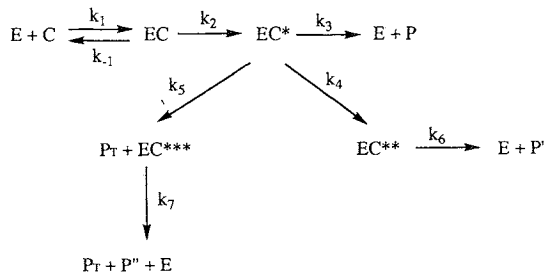
In reaction of β-lactam antibiotics with β-lactamase, Michaelis complex (EC) reversibly forms

Scheme 1.



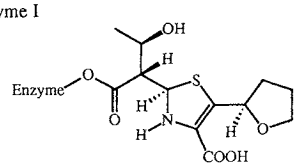
† *Bacillus cereus* Type I and *E. coli* 205 TEM R^+(566) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Scheme 2.

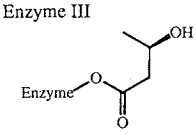


P': compounds **3** + **4**
Pr: thiazole **5**
P'': 3-hydroxybutyric acid
E: β-lactamase, C: β-lactam antibiotic
EC: Michaelis Complex

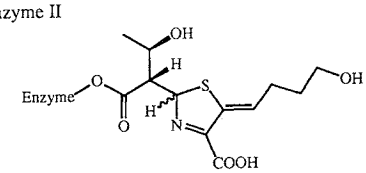
EC*: Acyl Enzyme I



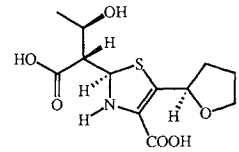
EC***: Acyl Enzyme III



EC**: Acyl Enzyme II



P: Penemoic Acid



first, then this Michaelis complex turns into an acylated β-lactamase (EC*) through reaction of β-lactam ring with serine hydroxyl group at active site. The acyl enzyme I (EC*) generates product (P) and catalytically active β-lactamase (E) through hydrolysis of the ester bond, as shown in Scheme 2.[4] In the acyl enzyme I of class A, the tetrahydrofuran ring-cleavage would occur very rapidly to convert acyl enzyme I to II (EC**) which would be quickly hydrolyzed to **3** and **4**. Since the epimers **3** and **4** were not interconvertible in reaction media (pH 7.0, phosphate buffer) with or without β-lactamases, and no penemoic acid derivative was detected in the enzymic reactions (*i.e.* $k_3=0$), ring opening of the tetrahydrofuran moiety and the epimerization at C-5 would occur during the conversion from the acyl enzyme I to II. Significant difference between Class C and class A β-lactamases is that acyl enzyme I (EC*) is more stable in class C than in class A.[4] Hence, the stable acyl enzyme I can be converted not only to the acyl enzyme II, but also to the acyl enzyme III (EC***) through liberation of the thiazole derivative **5**. This is compatible with the results that D,D-carboxypeptidase exclusively gives C-5~C-6 bond-cleaved product(P'') in its action on benzylpenicillin.[5]

Proteus vulgaris β-lactamase, like class C enzymes, and unlike other class A enzymes, prefers cephalosporins to penicillins. This enzyme did not give the C-C bond cleaved product **5** from penem **2**, and hence is thought to have an intrinsic character of penicillinase. The hydrolytic pattern of penem **2** is thus related to the classification of β-lactamases by the amino acid sequence homology rather than to that by the substrate specificity.

In conclusion, we have observed the first example for the C-5~C-6 bond cleavage of β-lactam antibiotics by class C β-lactamases. We have also found a correlation between the amino acid sequence homology and the products of the enzymes in the hydrolysis of the penem antibiotic **2**. Further kinetic analysis will reveal more details of the role of these enzymes in their hydrolytic actions on penem antibiotics.

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