ASPARENOMYCINS A, B AND C, NEW CARBAPENEM ANTIBIOTICS

V. INHIBITION OF β-LACTAMASES

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Asparenomycins (ASMs) A, B and C inhibited a wide range of β-lactamases including both cephalosporinases and penicillinases usually at concentrations less than 3 μM. On studying the mechanism of inhibition of β-lactamases produced by Gram-negative bacteria by ASM A it was concluded that ASM A inhibited the β-lactamases by acylating the enzymes. This conclusion was reached from the following observations. i) The inhibition was progressive with time. ii) The inhibitor formed stable complexes with the enzyme. iii) Before completing inhibition of one molecule of the enzyme, 1.8 molecules of the inhibitor were hydrolyzed.

It is well known that β-lactamases play an important role in bacterial resistance against β-lactam antibiotics.[1] So the β-lactamase inhibitors are expected to have a synergistic effect on the β-lactamase-producing strains when used with β-lactam antibiotics sensitive to β-lactamase hydrolysis. Thus there have been many efforts to create penicillins and cephalosporins which inhibit β-lactamases.[2,3] These efforts have led to the discovery of naturally-occurring inhibitors such as clavulanic acid and the carbapenems.[4~7]

Asparenomycins (ASMs) A, B and C are new, naturally-occurring carbapenems.[8] In this study we have examined their inhibition of β-lactamases produced by Gram-negative bacteria including the mechanism by which inhibition is accomplished.

Materials and Methods

Compounds

Asparenomycins, nitrocefin, and clavulanic acid were prepared by Shionogi Research Laboratories. MC696-SY2-A, identical with MM4550,[6] was kindly supplied by S. TAKAHASHI (National Institute of Health, Japan). Other compounds were obtained from commercial sources.

Bacterial Strains

Enterobacter cloacae 214, *E. cloacae* 53, and *Escherichia coli* W3110 carrying R-plasmid RTEM were kindly supplied by M. H. RICHMOND (University of Bristol, Bristol, England) and *Klebsiella pneumoniae* GN69 and *E. coli* ML1440 carrying R-plasmid RGN238 were obtained from T. SAWAI (Chiba University, Chiba, Japan). Other strains were clinical isolates stocked in this laboratory.

Preparation of β-Lactamases

The overnight cultures of *Proteus inconstans* 31 and *P. vulgaris* 31 which produced inducible β-lactamase were diluted 10-fold with fresh nutrient phosphate broth (Nissui, Tokyo) and incubated at 37°C for 2 hours with shaking. Penicillin G was added as an inducer at 100 μg/ml and incubation was continued for an additional 2 hours. Bacteria which produced constitutive β-lactamase were grown statically overnight in nutrient phosphate broth at 37°C. Bacterial cells were harvested by centrifugation at 3,000×*g* for 15 minutes at 4°C, washed with 0.1 M potassium phosphate buffer (pH 7.0), and suspended in the same buffer. The bacteria were sonically disrupted with a Sonifier 350 (Branson Sonic Power, U.S.A.) in an ice bath. Cell debris was removed by centrifugation at 33,000×*g* for 30 minutes

at 4°C, and the supernatant was filtered through a membrane filter (0.22 μ: Millipore Corp., U.S.A.) and stored at −78°C.

The β-lactamase produced by *E. coli* W3110 (RTEM) was partially purified with a diethylaminoethyl-Sephadex A-25 column equilibrated against 0.01 M Na_2HPO_4 - KH_2PO_4 (pH 8.0). The enzyme was eluted with a convex gradient of the buffer (mixing chamber at constant volume, 180 ml of 0.01 M buffer, pH 8.0; added solution, 0.5 M buffer, pH 6.2). The β-lactamases produced by *E. coli* 6, *E. cloacae* 214, *P. vulgaris* 31, *K. pneumoniae* GN69, and *E. cloacae* 53 were partially purified with a carboxymethyl-Sephadex C-50 column equilibrated with 0.01 M Na_2HPO_4 - KH_2PO_4 (pH 6.6). The enzyme was eluted with a convex gradient of the buffer (mixing chamber at constant volume, 180 ml of 0.01 M buffer, pH 6.6; added solution, 0.5 M buffer, pH 6.2). The β-lactamase produced by *Citrobacter freundii* 19 was purified to more than 95% homogeneity as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by isoelectric focusing on a polyacrylamide gel. The detailed procedure of purification will be published elsewhere.

β-Lactamase Assay and Determination of Inhibition Constant, *Ki* Value

β-Lactamase activity was determined by a spectrophotometric method which was based on the decrease of the optical density specific to the β-lactam ring upon hydrolysis of the substrates.[9]

The enzyme preparations were diluted with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.001% gelatin. The enzyme solution of 0.2 ml was added to 2 ml of the same buffer containing both the substrate and the inhibitor at 30°C. The reaction mixture was incubated in a cuvette and the initial hydrolysis rate was followed with a spectrophotometer (Hitachi model 200–20) with circulation of water at 30°C. Apparent MICHAELIS constant was calculated from LINEWEAVER-BURK plots[10] and inhibition constant, *Ki*, was obtained from plots of apparent MICHAELIS constant *versus* concentration of an inhibitor.

Spot Test for Measurement of Inhibition

Spot test was carried out as described previously.[11] An inhibitor was serially diluted 2- or 4-fold in 0.1 M potassium phosphate buffer (pH 7.0) and 0.025 ml of the inhibitor solution was placed in each well of a microtiter plate (Cooke Engineering, U. S. A.). The enzyme in 0.025 ml of the same buffer was added to each well and the mixture was incubated for 10 minutes at room temperature. A 0.05-ml of nitrocefin[12] (100 μg/ml in the same buffer) was then added to each well and incubated for another 30 minutes at room temperature. The amount of the enzyme added was that which hydrolyzed completely nitrocefin in 0.1 ml of the buffer (50 μg/ml) in the well in 30 minutes. The minimum effective concentration for inhibition was defined as the lowest concentration of the inhibitor at which nitrocefin gave no color change.

Analytical Isoelectric Focusing

Analytical isoelectric focusing on thin-layers of polyacrylamide gels was carried out with LKB Multiphor apparatus according to the manufacture's instruction. Samples were run on a LKB Ampholine PAG plate (pH range: 3.5 ~ 9.5) for 2 hours at the constant power of 25 watts.

Results

Inhibition of β-Lactamases

Minimum effective concentrations for inhibition of ASM A, ASM B and ASM C were shown in Table 1 and were compared with those of MC696-SY2-A and clavulanic acid.[4] Asparenomycins inhibited both cephalosporinases and penicillinases. This inhibition character was different from that of clavulanic acid which did not inhibit cephalosporinases[13] with an exception of *P. vulgaris* enzyme which was reported to have some penicillinase properties.[14] Among ASMs, ASM B was the most effective and inhibited β-lactamases at concentrations of less than 1 μM in most cases. ASMs were, however, less effective than MC696-SY2-A.

Table 1. Inhibitory activity of β-lactamase inhibitors[a].

Source of β-lactamase[b]	Substrate specificity[c]	Minimum effective concentration (μM)				
		Asparenomycin A	Asparenomycin B	Asparenomycin C	MC696-SY2-A	Clavulanic acid
Escherichia coli 6	C	2.9	1.5	0.77	0.039	1300
Proteus morganii 7	C	12	1.5	3.1	0.61	>1300
Proteus inconstans 31	C	0.74	0.049	0.77	0.61	>1300
Enterobacter cloacae 92	C	2.9	0.77	3.1	0.15	>1300
Proteus vulgaris 31	C	0.003	0.012	1.5	0.010	5.0
Klebsiella sp. 363	P	0.19	0.39	190	0.002	0.32
Enterobacter cloacae 53	P	0.74	0.19	49	0.002	0.32
Escherichia coli W3110 (RTEM)	P	0.047	0.025	3.1	0.002	0.32
Escherichia coli ML1410 (RGN238)	P	0.047	0.012	12	0.010	20

a) Inhibitory activities were determined by spot test and are expressed as minimum effective concentration as defined in Materials and Methods section.
Asparenomycins A, B and C were serially diluted 2-fold, and MC696-SY2-A and clavulanic acid were diluted 4-fold.

b) β-Lactamase solutions used were crude extracts.

c) C and P indicate cephalosporinase and penicillinase, respectively.

Fig. 1. LINEWEAVER-BURK plots of the initial hydrolysis rate of nitrocefin in the presence of ASM A.

Partially purified β-lactamase from *K. pneumoniae* GN 69 was used. a. Enzyme was added to the mixture of the substrate, nitrocefin, and ASM A at final concentrations of 0 μM (●), 0.06 μM (▽), 0.12 μM (■), and 0.18 μM (○). b. Enzyme was preincubated for 10 minutes at 30°C with ASM A of 0 μM (●), 0.005 μM (▽), 0.010 μM (■), or 0.015 μM (○). Then the substrate, nitrocefin, was added.

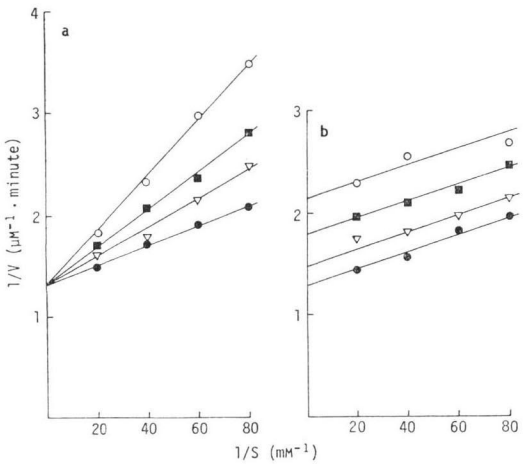
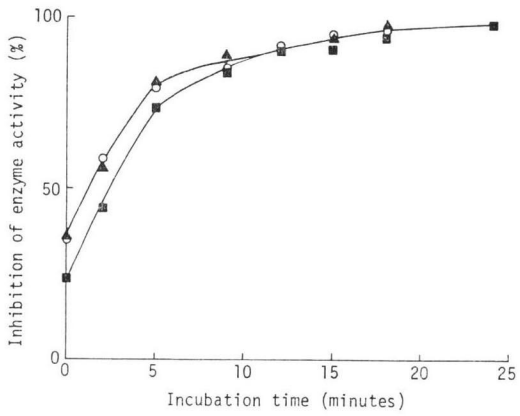


Fig. 2. Time dependence of β-lactamase inhibition by ASM A.

Enzyme in 0.1 M potassium phosphate buffer (pH 7.0) was mixed with the excess of ASM A and incubated at 30°C. At the indicated times, 0.2 ml of cephaloridine (1.1 mM) was added to a 2-ml portion of the mixture. Enzyme activity was obtained from the initial hydrolysis rate of cephaloridine. Inhibition is expressed as a percentage of decrease of enzyme activity with ASM A as compared to that without ASM A. Concentrations of ASM A employed were 0.0025 μM for *P. vulgaris* 31 enzyme (■), 2.5 μM for *E. coli* 6 enzyme (○), and 0.1 μM for *K. pneumoniae* GN 69 enzyme (▲).



Stability to β-Lactamase

By spectrophotometric method using change of absorbance at 281 nm ASM A was found to be very stable to β-lactamases under the condition that the substrate concentration was much higher than that of the enzyme (more than 500-fold excess).

Progressive Manner of ASM A Inhibition

When the β-lactamase was added to the mixture of ASM A and the substrate, LINEWEAVER-BURK plot was characteristic of the enzyme being competitively inhibited. When the enzyme was, however, incubated with ASM A for 10 minutes at 30°C prior to the addition of the substrate, LINEWEAVER-BURK plot no longer showed the character of competitive inhibition. The example with *K. pneumoniae* GN69 enzyme was shown in Fig. 1. In connection with this fact, β-lactamase inhibition by ASM A was progressive with time. The examples were shown in Fig. 2.

Table 2. Inhibition constant of asparenomycin A to β-lactamases.

Source of β-lactamase[a]	Substrate	Inhibition constant *Ki*[b] (μM)
Escherichia coli 6	cephalexin	3.4
Enterobacter cloacae 214	cephalexin	3.2
Citrobacter freundii 19	cephalothin	2.4
Proteus vulgaris 31	nitrocefin	0.003
Klebsiella pneumoniae GN69	nitrocefin	0.12
Enterobacter cloacae 53	nitrocefin	0.55
Escherichia coli W3110 (RTEM)	nitrocefin	0.028

[a] Enzyme from *C. freundii* 19 was purified and those from the other strains were partially purified.

[b] Determined by spectrophotometric assay.

Determination of Inhibition Constant, *Ki*

As shown in Fig. 1a, ASM A inhibited β-lactamases competitively when the enzyme was added to the mixture of the substrate and the inhibitor. So the inhibition constants were calculated as described in Materials and Methods section (Table 2). *Ki* values for each enzyme were similar to minimum effective concentrations (Table 1). *Ki* values for penicillinases and *P. vulgaris* enzyme were smaller than those for cephalosporinases indicating that ASM A had higher affinity for penicillinase and *P. vulgaris* enzyme.

Irreversible Interaction between the β-Lactamase and ASM A

Using purified enzyme from *C. freundii* 19 we further studied the interaction between the β-lactamase and ASM A in more detail. When the β-lactamase and ASM A were mixed at molar ratio of 0.6 or 1.2 (ASM A/enzyme), the remaining enzyme activity reached a constant value at about 5-minute and a recovery of the enzyme activity was not observed for at least 60 minutes after mixing (Fig. 3). A second addition of the enzyme to the mixture of the enzyme and ASM A after 60-minute incubation showed no more inhibition. This indicates that ASM A in the mixture was consumed entirely. A plot of the remaining activity *versus* the molar ratio of ASM A to the β-lactamase extrapolated to 1.8 (Fig. 4). This indicates that ASM A of 1.8-fold molar excess was needed for complete inhibition of the enzyme.

Results of analytical isoelectric focusing of the mixture of ASM A and the enzyme on a polyacrylamide thin-layer is shown in Fig. 5. As the ratio of ASM A/β-lactamase was increased, two new bands (a major and a minor band) appeared with concomitant disappearance of the native enzyme band. This showed the formation of two kinds of stable complexes of ASM A and the β-lactamase.

Incubation of ASM A (4.23 μM) with β-lactamase (3.79 μM) led to the drastic change of ultraviolet absorption of ASM A (Fig. 6). This fact indicates that ASM A was no longer an intact form in the complex with the β-lactamase.

Fig. 3. Time dependence of β-lactamase activity in the presence of limiting quantities of ASM A.

Purified β-lactamase from *C. freundii* 19 in 0.1 M potassium phosphate buffer (pH 7.0) (containing 0.01% of gelatin) was used. The enzyme was mixed with ASM A at molar ratio (inhibitor/enzyme) of 0.6 (●) and 1.2 (○) (final concentration of the enzyme was 9.5 μM) and incubated at 30°C. At the indicated times 10-μl aliquots were diluted with 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) and the enzyme activities were determined by spectrophotometric method using 100 μM of cephaloridine as a substrate. Remaining enzyme activity is expressed as a percentage of the enzyme activity of nontreated control.

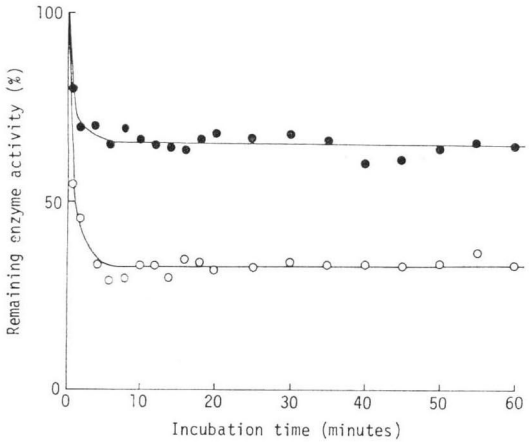


Fig. 4. Plots of remaining enzyme activity *versus* molar ratio of ASM A/β-lactamase.

Purified β-lactamase from *C. freundii* 19 was used. Each value of remaining activity was obtained from the same type of experiments as performed in Fig. 3.

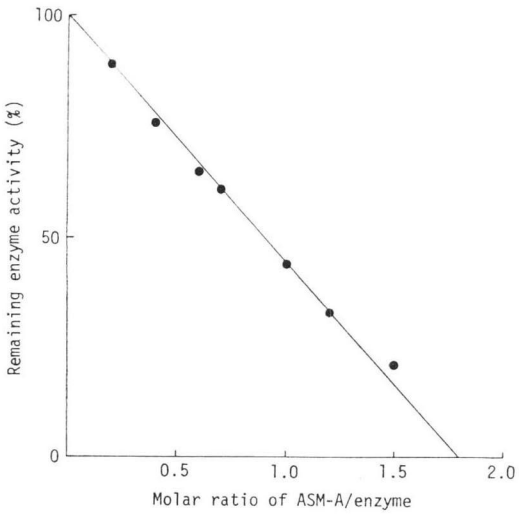


Fig. 5. Isoelectric focusing of inactivated *C. freundii* 19 β-lactamase on a thin-layer of polyacrylamide gel.

The enzyme (final concentration of 13.2 μM) was mixed with ASM A at molar ratio (ASM A/enzyme) of 0 (A), 10 (B), 3 (C), 1 (D), and 0.5 (E). The pH range of the gel was approximately 9.5 (top) to 3.5 (bottom). The pI of native enzyme is 9.2.

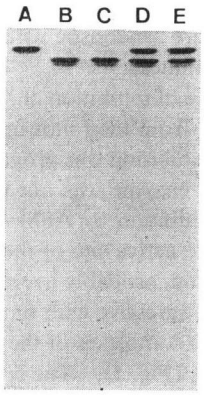
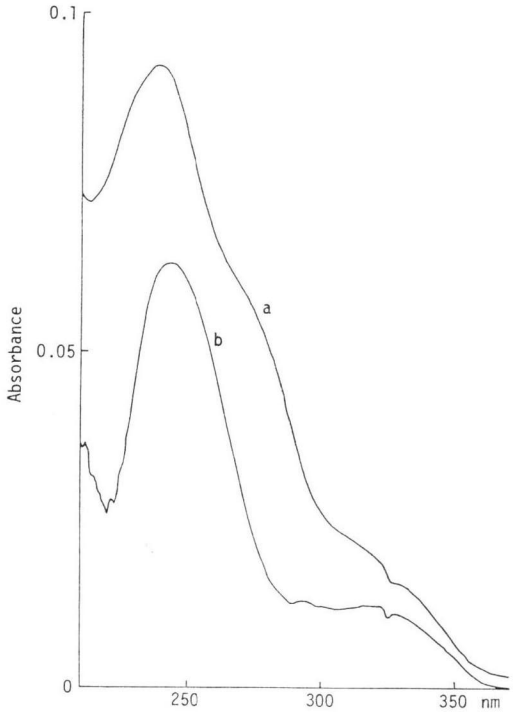


Fig. 6. Ultraviolet spectral change of ASM A after treatment with *C. freundii* 19 enzyme.

ASM A of 4.23 μM was mixed with 3.79 μM of the enzyme and the mixture was incubated at 30°C for 15 minutes. Then spectra was recorded with reference of enzyme alone. a: ASM A without treatment, b: ASM A treated with the enzyme.



Discussion

The minimum effective concentration of ASM A determined by spot test was similar to inhibition constant, *Ki*, determined by spectrophotometric method (Tables 1 and 2). Thus spot test was found to be a convenient method for estimating approximate value of *Ki*. ASM A, ASM B and ASM C as well as MC696-SY2-A gave relatively low values of minimum effective concentration for a wide range of β-lactamases. This is characteristic of these inhibitors and is different from the case of clavulanic acid which inhibited only penicillinases and *P. vulgaris* enzyme (Table 1). Because of these inhibitory activities the ASMs are synergistic with ampicillin[15] against β-lactamase-producing bacteria similar to other β-lactamase-inhibitors.[13,16]

Of the mode of inhibition of ASM A, the formation of a stable enzyme complex acylated by ASM A was most likely from the following facts.

First, the inhibition was progressive with time (Fig. 2) while the ASM A inhibited the enzymes competitively if the enzyme was not preincubated with ASM A. This implies that there are at least two stages in the inhibition by ASM A, that is, the first stage is the competition of the inhibitor with the substrate for the active site of the enzyme, and it is followed by the second stage which causes a progressive inhibition, probably irreversible inhibition. The inhibition of RTEM β-lactamase by clavulanic acid was also progressive with time and the inhibitor was suggested to acylate the enzyme.[17]

Second, the complexes of the purified *C. freundii* β-lactamase and ASM A was observed by the isoelectric focusing (Fig. 5). The complexes were very stable because they exhibited clear bands after 2-hour run and the recovery of the activity of the enzyme was not observed during 60-minute incubation of the enzyme with ASM A (Fig. 3).

Third, only 1.8-fold molar excess of ASM A was required for the complete inhibition of the enzyme (Fig. 4). Although ASM A was stable under the condition which ASM A was more than 500-fold excess, the drastic change of ultraviolet spectrum of ASM A was observed when ASM A was mixed with approximately equimolar enzyme (Fig. 6). These indicate that 1.8 molecules of ASM A were hydrolyzed with concomitant inactivation of one enzyme molecule.

One of the properties required for a good β-lactamase-inhibitor seems to be the ability to form a stable, covalently-linked complex with the enzyme as shown in this study and in other papers.[17,18] In this respect the inhibitors differ from the substrates in the reaction site and the complexes with the latter are very unstable. The structure of the moiety derived from 6β-bromopenicillanic acid, a β-lactamase inhibitor, in the complex was shown to be a dihydrothiazine.[18] However little is known about the structure of the moiety derived from the carbapenems. Identification of this structure will offer a clue to elucidate the relationship between the structures of the inhibitors and their inhibitory activity.

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