Inhibition of β -Lactamase of *Bacillus licheniformis* 749/C by Compound PS-5, a New β -Lactam Antibiotic

Yasuo FUKAGAWA, Tomoko TAKEI and Tomoyuki ISHIKURA
 Sanraku-Ocean Co., Central Research Laboratories, Fujisawa 251, Japan

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By use of a new computer-assisted u.v.-spectrophotometric assay method, the kinetic parameters of the reaction catalysed by *Bacillus licheniformis* 749/C β -lactamase were re-examined and the mode of inhibition of the enzyme by compound PS-5, a novel β -lactam antibiotic, was studied with benzylpenicillin as substrate. (1) The fundamental assay conditions for the determination of K_m and V were examined in detail with benzylpenicillin as substrate. In 0.1 M-sodium/potassium phosphate buffer, pH 6.8, at 30°C, initial substrate concentrations of benzylpenicillin above 0.7 mM were very likely to lead to substrate inhibition. The K_m value of the enzyme for benzylpenicillin at initial concentrations from 1.96 to 0.07 mM was calculated to be 97–108 μ M. (2) The K_m values of the enzyme for 6-aminopenicillanic acid, ampicillin and cephaloridine were found to be 25, 154–161 and 144–161 μ M respectively. (3) Compound PS-5 was virtually unattacked by *Bacillus licheniformis* 749/C β -lactamase. (4) The activity of the enzyme was diminished by compound PS-5, to extents depending on the duration of incubation and the concentration of the inhibitor. The rate of inactivation of the enzyme by compound PS-5 followed first-order kinetics. (5) In an Appendix, a new computer-assisted u.v.-spectrophotometric enzyme assay method, in which a single reaction progress curve of time-absorbance was analysed by the integrated Michaelis-Menten equation, was devised for the accurate and precise determination of the kinetic constants of β -lactamase. For conversion of absorbance readings into molar substrate concentrations, the initial or final absorbance reading that was independent of the reaction time was used as the basis of calculation. In calculation of K_m and V three systematic methods of data combination were employed for finer analysis of the reaction progress curve. A list of the computer program named YF6TAIM is obtainable from the author on request or as Supplementary Publication SUP 50100 (12 pages) from the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., on the terms indicated in *Biochem. J.* (1978) 169, 5.

Compound PS-5 is a novel β -lactam antibiotic having a potent inhibitory activity on various β -lactamases (Fig. 1), and is produced by several strains of *Streptomyces* (Okamura *et al.*, 1978). This compound belongs to the same group of β -lactam antibiotics as thienamycin (Albers-Schonberg *et al.*, 1978; Kahan *et al.*, 1979), *N*-acetylthienamycin (Kahan *et al.*, 1977), epithienamycins A, B and C (Stapley *et al.*, 1977; Cassidy *et al.*, 1977), compounds MM4550 and MM13902 (Brown *et al.*, 1977) and compound MM17880 (Corbett *et al.*, 1977). In comparison with traditional penicillins and cephalosporins, these new β -lactam compounds share the common property that there is no sulphur atom in the ring structure. The inhibitory activity of

compounds MM4550, MM13902 and MM17880 has been briefly reported (Brown *et al.*, 1977; Corbett *et al.*, 1977). Clavulanate is also known to inhibit various β -lactamases, though it belongs to

Fig. 1. Structure of compound PS-5

another group of β -lactam compounds (Fisher *et al.*, 1978; Labia & Peduzzi, 1978).

For the study on the mode of inhibition of β -lactamase by compound PS-5, the *Bacillus licheniformis* 749/C enzyme was chosen as a model of typical penicillinases from Gram-positive bacteria, since its enzymological properties, including the mechanism of production, amino acid composition and genetic control, have been characterized very well (Thatcher, 1975*b*).

As traditional iodometric assay methods for β -lactamase are not applicable to compound PS-5 because of its high sensitivity to iodine, a new computer-assisted u.v.-spectrophotometric assay method was devised for the quick and accurate determination of K_m and V from a single reaction progress curve. Preceding the kinetic study on the mode of inhibition of β -lactamases by compound PS-5, the fundamental assay conditions for β -lactamase were re-examined in detail with benzylpenicillin as substrate. Under routine assay conditions of macro-iodometry and acidimetry, the substrate inhibition seems ubiquitous in β -lactamases.

In the present paper, precise assay conditions for β -lactamase are described with benzylpenicillin as substrate in the initial concentration range of 0.03–3.47 mM by the new computer-assisted u.v.-spectrophotometric assay method. Reasonable K_m and V values of the enzyme for benzylpenicillin, 6-aminopenicillanic acid, ampicillin and cephaloridine were obtained. With benzylpenicillin as substrate, the mode of inhibition of the enzyme by compound PS-5 was studied.

Experimental

Materials

Antibiotics were obtained from commercial sources as follows: benzylpenicillin (penicillin G, potassium salt; Meiji Seika Kaisha, Tokyo, Japan), 6-aminopenicillanic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.), ampicillin (sodium ampicillin Toyama for injection; Toyama Chemical Co., Tokyo, Japan) and cephaloridine (keflodin; Shionogi and Co., Osaka, Japan).

Compound PS-5 (sodium salt) has a u.v.-absorption maximum at 301 nm in 0.1 M-sodium/potassium phosphate buffer, pH 6.8 (ϵ 8687 M⁻¹·cm⁻¹). Its molar differential absorption coefficient ($\Delta\epsilon$) was determined to be 8120 M⁻¹·cm⁻¹ at 301 nm in the same buffer by using β -lactamases of *Bacillus cereus* 569 (Y. Fukagawa, T. Takei & T. Ishikura, unpublished work). The purity of compound PS-5 was routinely checked by measuring the A_{301}/A_{245} ratio; a sample of compound PS-5 above 99% purity gives a ratio above 3.8.

Other reagents were of analytical grade.

Preparation of β -lactamase of *Bacillus licheniformis* 749/C

By using the method of Yamamoto & Lampen (1976), *Bacillus licheniformis* 749/C (A.T.C.C. 25972) was cultured under shaking at 28°C for 18 h in twenty 500 ml Erlenmeyer flasks containing 100 ml each of casamino acid medium (Pollock, 1965). After the pH of the culture broth had been adjusted to 9.0 with 1 M-NaOH, the flasks were shaken at 28°C for a further 30 min. The supernatant solution (1.9 litres) was collected by centrifugation at 3000 rev./min (900 g). The activity of β -lactamase assayed with benzylpenicillin was about 2.2 units/ml (1 unit of enzyme in the present paper is the amount of enzyme that can hydrolyse 1 μ mol of benzylpenicillin/min at 30°C in 0.1 M-sodium/potassium phosphate buffer, pH 6.8). The clear solution was cooled to 5°C, 20 g (wet wt.) of Whatman phosphocellulose P1 (pretreated with dil. HCl and washed with water to neutrality) was added and the mixture was stirred overnight. The Whatman phosphocellulose P1 was recovered by centrifugation, washed twice with 300 ml each of cold 10 mM-potassium phosphate buffer, pH 5.0, and poured into a glass chromatographic column. The enzyme was eluted with 0.2 M-sodium/potassium phosphate buffer, pH 7.6, and the pooled active eluate (about 150 ml) was dialysed overnight at 5°C against 8 litres of 10 mM-sodium acetate buffer, pH 4.8. The dialysis residue was condensed to about 35 ml by ultrafiltration with a membrane filter (TOYO UH-1; Toyo Roshi Kaisha, Tokyo, Japan; molecular weight over 1000 retained). The total yield of β -lactamase was 2.7×10^3 units (65%). A portion of the enzyme condensate (2 ml; 1.6×10^2 units) was applied on a column (0.8 cm $\times$ 15 cm) of cephalixin-CH-Sepharose 4B that was prepared by a method similar to that of Ogawara & Umezawa (1975). After being washed with 1 ml of 10 mM-sodium acetate buffer, pH 4.8, the column was subjected to elution with 0.1 M-sodium/potassium phosphate buffer, pH 6.8. The active eluate (8 ml) had a β -lactamase specific activity of 13 units/ml.

U.v.-spectrophotometric assay of β -lactamase

Varied concentrations of substrate (2.99–2.95 ml) in 0.1 M-sodium/potassium phosphate buffer, pH 6.8, were poured into a 1.0 cm quartz cuvette (3 ml volume) and maintained at 30°C in a Hitachi double-beam u.v.-visible spectrophotometer 200–20 (Hitachi, Tokyo, Japan) (slit width 1 nm; medium response). The assay was initiated by adding 10–50 μ l (0.13–0.66 unit) of the preparation of *Bacillus licheniformis* 749/C β -lactamase to make the final volume of reaction to 3 ml, and then the decrease in absorption at the assay wavelength was read at 15 s intervals against a reference cuvette containing only

the enzyme in the buffer. The following assay wavelengths and absorption constants were employed in the present work: for benzylpenicillin, $\Delta\epsilon$ $636\text{M}^{-1}\cdot\text{cm}^{-1}$ at 240nm and $336\text{M}^{-1}\cdot\text{cm}^{-1}$ at 245nm; for 6-aminopenicillanic acid, $\Delta\epsilon$ $1510\text{M}^{-1}\cdot\text{cm}^{-1}$ at 223nm; for ampicillin, $\Delta\epsilon$ $840\text{M}^{-1}\cdot\text{cm}^{-1}$ at 235nm; for cephaloridine, $\Delta\epsilon$ $9600\text{M}^{-1}\cdot\text{cm}^{-1}$ (ϵ $14540\text{M}^{-1}\cdot\text{cm}^{-1}$) at 255nm and $3680\text{M}^{-1}\cdot\text{cm}^{-1}$ (ϵ $4040\text{M}^{-1}\cdot\text{cm}^{-1}$) at 285nm.

Computation of K_m and V by the integrated Michaelis-Menten equation

The observed absorbance readings were converted into molar substrate concentrations by the final-absorbance method that used as zero the final absorbance reading at completion of reaction or by the initial-absorbance method that used the corrected initial absorbance reading as the basis of conversion. Then the time-(molar substrate concentration) data were combined by any one of the constant-difference, constant-point and all-combination methods of data combination to calculate V and K_m by the integrated form of the Lineweaver-Burk plot. The detailed procedure of the computer-assisted u.v.-spectrophotometric assay method of β -lactamase by the integrated Michaelis-Menten equation is explained in the Appendix (Fukagawa, 1980).

Inactivation of the β -lactamase by compound PS-5 and the calculation of the rate constant of inactivation

Varied concentrations of compound PS-5 (final concentration 0.0266–0.0837mM) were dissolved in 2.88ml of 0.1M-sodium / potassium phosphate buffer, pH6.8, in 1cm quartz cuvettes and maintained at 30°C. The β -lactamase (20 μ l; 0.26 unit) was added and the mixture was incubated at 30°C for the indicated periods of time. The residual activity

of the enzyme was measured by adding 100 μ l of 10mM-benzylpenicillin in the same phosphate buffer (final concentration of substrate 0.32mM), and the fall of absorbance at 240nm was followed under the above-specified assay conditions. In zero-time tests, compound PS-5 and benzylpenicillin were mixed in the phosphate buffer before the enzyme was added to start the hydrolysis of benzylpenicillin. The residual activity of the treated enzyme was read as the difference in absorption at 240nm for 60s from 30s to 90s after the reaction began.

For calculation of the rate constant of inactivation (k_a), the following first-order equation proposed by Zyk & Citri (1967) was employed:

$$k_a = \ln(E_0/E_t)/t$$

where t is the period of preincubation of the enzyme with compound PS-5, E_0 is the enzyme activity of the control (zero-time tests) and E_t is the enzyme activity remaining after time t of preincubation with compound PS-5.

The conformational response constant (K_{cr}) defined by Zyk & Citri (1967) was graphically determined by plotting reciprocals of k_a and concentration of compound PS-5.

Results

Fundamental assay conditions for Bacillus licheniformis 749/C β -lactamase with benzylpenicillin as substrate

Time courses of hydrolysis were followed for a range of initial benzylpenicillin concentrations (0.03–3.47mM). With the highest concentrations (3.47 and 1.96mM), which are within the range routinely employed for macro-iodometry and acidimetry, a clear acceleration phase was observed. With 3.47mM-benzylpenicillin, the rate of hydrolysis (expressed as $-\Delta A_{245}/0.5\text{min}$) increased from 0.028

Table 1. Time courses of hydrolysis of benzylpenicillin by *Bacillus licheniformis* 749/C β -lactamase
Time-absorbance assay data of 0.03–3.47mM-benzylpenicillin were arranged by the reaction velocity ($-\Delta A/0.5\text{min}$) to show three phases of acceleration, constant velocity and deceleration.

Initial concn. (mM)	Amount of enzyme (unit/3 ml)	Reaction velocity phase (min)		
		Acceleration	Constant velocity	Deceleration
3.465	0.66	0–8.0	8.0–11.5	11.5–
1.962	0.66	0–3.0	3.0–6.0	6.0–
1.475	0.66	0–1.0	1.0–4.0	4.0–
0.970	0.66		0–2.0	2.0–
0.656	0.66		0–1.0	1.0–
0.330	0.66			0–
0.326	0.26			0–
0.191	0.26			0–
0.095	0.26			0–
0.070	0.26			0–
0.098	0.13			0–
0.033	0.13			0–

at 0.5 min to 0.032 at 8 min, and for 1.96 mM-benzylpenicillin the rate (expressed as $-\Delta A_{240}/0.5 \text{ min}$) increased from 0.060 at 0.5 min to 0.061 at 3 min. The durations of the acceleration, constant-velocity and deceleration phases of the time course for each substrate concentration are summarized in Table 1.

Progressive relief from substrate inhibition would be an obvious explanation for the observed acceleration. In Fig. 2 the initial reaction velocity (extrapolated value of $-\Delta A_{240}/0.5 \text{ min}$ from 0 to 0.5 min) is plotted against substrate concentration, showing clearly that substrate inhibition indeed occurs. It appears that, under the assay conditions employed in the present work, substrate inhibition must be taken into account with benzylpenicillin concentrations greater than 0.4 mM.

In addition, the duration of the constant-velocity phase of hydrolysis seems too long at 3.47, 1.96, 1.48 and 0.98 mM. Crabbe & Bardsley (1976) indicated that the Scatchard space was best suited to reveal clearly the possible deviation of enzyme assay data from the Michaelis-Menten equation. Here, as an example of the hydrolysis curves, the assay data for 1.96 mM-benzylpenicillin were chosen. Twenty-four pairs of time-absorbance assay data from this single time course were converted into time-(molar concentration) data by the final-absorbance method, and then combined at a constant difference of 1 (Yun & Suelter, 1977) for the calculation of K_m

Fig. 2. Initial velocity of hydrolysis of 0.03–3.47 mM-benzylpenicillin by *Bacillus licheniformis* 749/C β -lactamase

For the observed time-absorbance assay data, the initial velocity of hydrolysis ($-\Delta A/0.5 \text{ min}$ from 0 to 0.5 min per 220 units of the enzyme/l) was calculated by extrapolation. The ideal curve was drawn by using the K_m value of 0.105 mM and the V value of 0.22 mmol/min per 220 units of the enzyme (see Table 2). ●, Observed curve; ○, ideal curve.

and V by the integrated form of the Scatchard plot (Y. Fukagawa, unpublished work) (Fig. 3).

It is evident that the first 11 data pairs belonging to the acceleration and constant-velocity phases deviate significantly from Michaelian kinetics because of substrate inhibition. Moreover, the last three data pairs also do not fit the Michaelis-Menten equation, presumably because of measurement error and/or the cessation of steady-state conditions.

In conclusion, it seems advisable to employ only the deceleration phase or the non-linear portion of the reaction progress curve for the accurate and precise determination of the kinetic parameters, after omission of a last few data pairs with impermissible errors.

Now, after preliminary trimming in the Scatchard space, the non-linear portions of all the hydrolysis curves, except at 3.47 mM, in Table 1 were subjected to computation of K_m and V by the all-combination method [see the Appendix (Fukagawa, 1980)] (Table 2).

It seems highly probable that β -lactamase of *Bacillus licheniformis* 749/C has a K_m value of 97–108 μM for 1.96–0.07 mM-benzylpenicillin under the specified assay conditions (r was always above 0.99). The hydrolysis curve for 0.03 mM-benzylpenicillin proved to be unreliable in the Scatchard space.

For comparison, the data in Fig. 2, except those at 3.47 and 0.03 mM, were used for the calculation of K_m and V by the traditional initial-velocity method, in which $1/[S]_0$ was plotted against $1/v$ [$=1/(-\Delta A_{240}/0.5 \text{ min})$]. The obtained kinetic para-

Fig. 3. Scatchard plot of the assay data for 1.96 mM-benzylpenicillin combined at a constant difference of 1. For checking of the non-Michaelian behavior, 24 pairs of time-absorbance data were combined at a constant difference of 1 and plotted by the integrated form of the Scatchard plot (Y. Fukagawa, unpublished work).

Table 2. K_m and V values of *Bacillus licheniformis* 749/C β -lactamase calculated for various initial concentrations of benzylpenicillin

After preliminary trimming by the integrated form of the Scatchard plot (Y. Fukagawa, unpublished work), the deceleration phases of the assay data, except at 3.47 mM, in Table 1 were used for computation of K_m and V by the all-combination method of data combination. V values are expressed in $\mu\text{mol/min}$ per 220 units of the enzyme.

Initial concn. (mM)	Range of concns. used (mM)	No. of data pairs	V	K_m (μM)	V/K_m
1.962	0.745–0.054	10	217	108	2.01
1.475	0.766–0.013	12	214	103	2.08
0.970	0.546–0.041	12	214	107	2.00
0.656	0.478–0.022	12	209	101	2.07
0.330	0.290–0.015	18	207	104	1.99
0.326	0.288–0.022	13	215*	98	2.19
0.191	0.169–0.012	11	221*	105	2.10
0.095	0.085–0.009	12	201*	97	2.07
0.070	0.052–0.015	7	216*	107	2.02
0.098	0.091–0.019	12	216*	104	2.08
0.033	0.027–0.004	14	162*	35	4.63

* Corrected by a factor of 2.5 or 5 (r was over 0.99 except at 0.033 mM).

Table 3. K_m and V of *Bacillus licheniformis* 749/C β -lactamase for ampicillin

Under the same assay conditions as were used for benzylpenicillin, the hydrolysis curves of 0.65 mM- and 0.32 mM-ampicillin were followed at 235 nm ($\Delta\epsilon$ 840 $\text{M}^{-1}\text{cm}^{-1}$). After preliminary check by the integrated form of the Scatchard plot (Y. Fukagawa, unpublished work), K_m and V were calculated by the final-absorbance method and the all-combination method of data combination. V values are expressed in $\mu\text{mol/min}$ per 220 units of the enzyme.

Initial concn. (mM)	Amount of enzyme (unit/3 ml)	Range of concns. used (mM)	No. of data pairs	V	K_m (μM)	V/K_m
0.652	0.66	0.579–0.020	21	163	155	1.05
0.328	0.66	0.277–0.020	13	170	161	1.06
0.324	0.26	0.296–0.045	17	169*	154	1.10

* Corrected by a factor of 2.5 (r was over 0.99 in all the assays).

meters were $K_m = 137 \mu\text{M}$ and $V = 211 \mu\text{mol/min}$ per 220 units of enzyme ($r = 0.9850$). If $[S]_0$ were replaced by $[S]_i$ according to the final-absorbance method (see the Appendix) and v were expressed in $-\Delta A_{240}/10\text{s}$, K_m and V would be $107 \mu\text{M}$ and $205 \mu\text{mol/min}$ per 220 units of enzyme ($r = 0.9987$) respectively (Y. Fukagawa, unpublished work).

Determination of the kinetic parameters of Bacillus licheniformis 749/C β -lactamase for 6-aminopenicillanic acid, ampicillin and cephaloridine

(a) *6-Aminopenicillanic acid*. At an initial concentration of 0.071 mM the hydrolysis of 6-aminopenicillanic acid by 0.58 unit of the enzyme/3 ml was followed at 232 nm for 35 min. After non-Michaelian data pairs corresponding to substrate inhibition and the cessation of steady-state conditions were preliminarily deleted in the Scatchard space, 15 data pairs from 2 to 16 min were combined by the all-combination method to give the K_m and V values of $25 \mu\text{M}$ and $8.4 \mu\text{mol/min}$ per 193 units of enzyme respectively ($r = 0.9998$). K_m reported in the literature is $16.6 \mu\text{M}$ (Thatcher, 1975b).

(b) *Ampicillin*. Three hydrolysis curves of ampicillin recorded at initial concentrations of 0.65 and 0.32 mM yielded the results shown in Table 3 when analysed by the final-absorbance method and the all-combination method of data combination.

It is noteworthy that, in contrast with 6-aminopenicillanic acid, ampicillin did not show substrate inhibition at 0.65 mM. Though its relative rate of hydrolysis has been reported (Thatcher, 1975b), no K_m value of the enzyme for ampicillin is available in the literature.

(c) *Cephaloridine*. As the final absorbance reading was less reliable because of spectrophotometric instability of breakdown products from cephaloridine, both the final-absorbance method and the initial-absorbance method were employed for computation of K_m and V . After the result for substrate inhibition at 0.47 mM was removed, the assay data were combined by the all-combination method to provide the kinetic constants shown in Table 4.

The K_m value obtained at 0.065 mM-cephaloridine was found to be less reliable in the Scatchard space, because the initial substrate concentration was too low in comparison with K_m and because the absorb-

Table 4. K_m and V of *Bacillus licheniformis* 749/C β -lactamase for cephaloridine

Under the same assay conditions as were used for benzylpenicillin, the hydrolysis curves of 0.474 mM- and 0.065 mM- cephaloridine were followed at 285 nm ($\Delta\epsilon$ 3680 M⁻¹·cm⁻¹) and 255 nm ($\Delta\epsilon$ 9600 M⁻¹·cm⁻¹) respectively. The observed assay data were converted into their corresponding time-(molar concentration) data by the initial-absorbance and final-absorbance methods. After preliminary check by the integrated form of the Scatchard plot (Y. Fukagawa, unpublished work), they were combined by the all-combination method for the calculation of K_m and V . V values are expressed in μ mol/min per 220 units of the enzyme (r was always over 0.99).

Initial concn. (mM)	A		Range of concs. used (mM)	No. of data pairs	V	K _m (μM)	V/K _m
	Initial	Final					
Initial-absorbance method							
0.4738	1.914		0.334–0.036	13	53	149	0.36
0.0649	0.943		0.058–0.015	11	53	161	0.33
Final-absorbance method							
0.474		0.171	0.335–0.037	13	54	156	0.35
0.065		0.330	0.058–0.015	11	49	144	0.34

ances of substrate and its degradation products were more unstable at 255 nm (Ross & O'Callaghan, 1975) than at 285 nm (Y. Fukagawa, unpublished work).

Interaction of compound PS-5 with *Bacillus licheniformis* 749/C β -lactamase

(a) *Insusceptibility of compound PS-5 to attack by *Bacillus licheniformis* 749/C β -lactamase.* After an extremely high activity of the enzyme (88 units; estimated to be about 1 nmol) was mixed with about 15 nmol (4.8 μ g as the sodium salt) of compound PS-5 in 3.0 ml of 0.1 M-sodium/potassium phosphate buffer, pH 6.8, at 30°C, the hydrolysis of compound PS-5 was monitored at 301 nm ($\Delta\epsilon$ 8120 M⁻¹·cm⁻¹) for 30 min. During this period of incubation, no change in u.v. absorption at 301 nm was recorded, which shows no susceptibility of compound PS-5 to attack by the β -lactamase.

Complete resistance of compound PS-5 to hydrolysis by the enzyme was also confirmed by the routine agar plate-disc assay method, in which varied concentrations of compound PS-5 were disc-assayed overnight at 30°C on a nutrient agar plate seeded by *Comamonas terrigena* I.F.O. 12685 (detector organism) in the presence or absence of *Bacillus licheniformis* 749/C β -lactamase (Okamura *et al.*, 1978).

(b) *Hydrolysis of benzylpenicillin by *Bacillus licheniformis* 749/C β -lactamase in the presence of compound PS-5.* When mixtures of a fixed concentration of benzylpenicillin with varied amounts of compound PS-5 were exposed to the action of the β -lactamase, clear inhibitory effects of compound PS-5 were observed (Fig. 4).

The computer analysis indicated that none of the progress curves except the control in Fig. 4 fitted the Michaelis-Menten equation at all. But it seemed probable that the extent of inhibition of the enzyme by compound PS-5 depended not only on the concentration of the inhibitor but also on the progress of time of reaction.

Fig. 4. Hydrolysis of benzylpenicillin by *Bacillus licheniformis* 749/C β -lactamase in the presence of compound PS-5

To 0.5 mM-benzylpenicillin compound PS-5 was added at the indicated concentrations and the mixture then kept at 30°C. After 0.22 unit of the β -lactamase/ml was added, the time course of hydrolysis of benzylpenicillin was followed at 240 nm. Control ($\square$) contained no compound PS-5. The molar ratio of benzylpenicillin to compound PS-5 was 45.4 ($\bullet$), 22.7 ($\blacksquare$), 12.5 ($\triangle$) and 8.5 ($\circ$).

(c) *Effect of preincubation with compound PS-5 on the activity of *Bacillus licheniformis* 749/C β -lactamase.* By using the method of Zyk & Citri (1967), the enzyme was preincubated with com-

pound PS-5 at 30°C for various lengths of time, and then the remaining activity of the enzyme was measured by addition of benzylpenicillin as the assay substrate. Fig. 5 shows the increasing extent of inhibition of the enzyme by compound PS-5 with the period of preincubation.

Though the activity of the enzyme decreased as the length of preincubation with compound PS-5 continued, it did not go down to zero. There always remained several percent of the initial enzyme activity detected even after a long period of preincubation (10 min) with a high concentration of compound PS-5. Strictly speaking, these observations of inhibition might suggest that the action of

Fig. 5. Effect of preincubation with compound PS-5 on *Bacillus licheniformis* 749/C β -lactamase

To 2.88 ml of 0.0277 mM-compound PS-5 in 0.1 M-sodium/potassium phosphate buffer, pH 6.8, 20 μ l of the β -lactamase (0.26 unit) was added and the mixture incubated at 30°C for the indicated periods of time. After preincubation, 100 μ l of 10 mM-benzylpenicillin in the buffer was added for assay of the remaining enzyme activity. ■, Control (without compound PS-5); ▲, zero-time test; preincubation with compound PS-5; ●, 30s; □, 60s; △, 90s; ○, 120s.

compound PS-5 on the β -lactamase was due, not to destructive inactivation, but to more-or-less reversible inactivation or deactivation.

(d) Calculation of the rate constant of inactivation and the conformational response constant. The rate constant (k_a) for inactivation of the enzyme by compound PS-5 was determined at 0.0266 mM-, 0.0419 mM-, 0.0630 mM- and 0.0837 mM-compound PS-5 (Fig. 6).

Zyk & Citri (1967) used the control test without inhibitor as the common starting point for inactivation curves when β -lactamases were inactivated by methicillin and oxacillin. If the control test without compound PS-5 (intact activity of the enzyme, $-\Delta A_{240}/\text{min}$ 0.038) were employed instead of the zero-time tests (benzylpenicillin plus varied concentrations of compound PS-5 without preincubation), the inactivation would appear not to follow pseudo-first-order kinetics. At least so far as the four concentrations of compound PS-5 used are concerned, it seems more reasonable to set the starting point of inactivation on the zero-time test rather than the control test without inhibitor (Fig. 6). From Fig. 6 the following rate constants of inactivation were calculated: $k_a = 0.0100/\text{s}$ at 0.0266 mM-compound PS-5; 0.0168/s at 0.0419 mM-compound PS-5; 0.0253/s at 0.0630 mM-compound PS-5; 0.0356/s at 0.0837 mM-compound PS-5.

The conformational response constant (K_{cr}) was induced by plotting the reciprocals of k_a and the concentration of compound PS-5 (Zyk & Citri, 1967) (Fig. 7).

Fig. 6. Time course of inactivation of *Bacillus licheniformis* 749/C β -lactamase by various concentrations of compound PS-5

Under the same conditions as given in Fig. 5, the time course of inactivation of the enzyme by compound PS-5 was traced at 0.0266 mM-, 0.0419 mM-, 0.0630 mM- and 0.0837 mM-compound PS-5. The remaining enzyme activity was expressed as the difference in absorption at 240 nm observed for 60 s from 30 s to 90 s after the assay substrate (benzylpenicillin) was added. As explained in the text, the zero-time tests instead of the control without compound PS-5 were used as the starting points for inactivation lines. Compound PS-5: ●, 0.0266 mM; □, 0.0419 mM; △, 0.0630 mM; ○, 0.0837 mM.

Fig. 7. Determination of K_{cr}

By using the method of Zyk & Citri (1967), the reciprocal of the concentration of compound PS-5 was plotted against the reciprocal of k_a (data from Fig. 6). From this plot, the regression line of $y = 2.7853x - 5.4070$ was obtained at $r = 0.9994$ by the least-squares method. Therefore K_{cr} was calculated to be -0.5151 .

In contrast with the results obtained by Zyk & Citri (1967), a negative value of K_{cr} (-0.515 ; $r = 0.9994$) was calculated, which will be meaningless as a kinetic constant. With respect to this negative value of K_{cr} , some plausible reasons will be considered. First of all, the starting points of inactivation lines in Fig. 6 (zero-time tests with inhibitor) differ from those used by Zyk & Citri (1967) (controls without inhibitor). It is also possible that the concentrations of compound PS-5 employed in Fig. 6 were too low to saturate the β -lactamase. Under the assay conditions specified in the present work, the limited accuracy and precision of the u.v.-spectrophotometric method did not permit the use of a higher concentration of the inhibitor.

Discussion

At an early stage of chemical studies, a new β -lactam antibiotic called compound PS-5 was found to be sensitive to iodine, and thus, for enzymological studies of compound PS-5, a β -lactamase assay method other than iodometry became a prerequisite. Among available assay methods, u.v. spectrometry (Waley, 1974; Samuni, 1975) was a best candidate, because: (1) with a highly improved u.v. spectrophotometer the quantitative measurement of β -lactam compounds was accurate, precise and easy to operate; (2) no special assay reagent such as iodine was necessary; (3) it allowed the selective

measurement of the hydrolysis of a given β -lactam compound in mixtures of two or more β -lactam compounds by choice of a suitable or characteristic wavelength for the relevant compound (Citri *et al.*, 1976). In this line of approach, high efficiency of a computer system in data-processing and improved specifications of a Hitachi double-beam u.v.-visible spectrophotometer 200-20 were utilized to establish a new computer-assisted u.v.-spectrophotometric assay method in which accurate and precise values of K_m and V were speedily obtained from a single reaction progress curve of time-absorbance by the integrated form of the Lineweaver-Burk plot (Yun & Suelter, 1977).

It is well acknowledged that kinetic constants of various β -lactamases reported in the literature are often found to be less than reliable (Thatcher, 1975a). As is apparent from Fig. 2, substrate inhibition of *Bacillus licheniformis* 749/C β -lactamase by benzylpenicillin seems to begin at about 0.4 mM under the reaction conditions specified in the present paper. Considered from the viewpoint of substrate inhibition, some discrepancies in kinetic constants of a particular β -lactamase published in the literature might reasonably be explained. For example, the acidimetric-assay data of Labia *et al.* (1973) (Table 1) on the hydrolysis of ampicillin by *Escherichia coli* K12 β -lactamase seem to indicate a probable presence of substrate inhibition in the gradual increase of K_m and r values with the prolongation of the time interval of observation. Unless their assay data at low substrate concentrations were non-Michaelian, the true values of K_m and V would be higher than those listed in Table 2 (Labia *et al.*, 1973).

In addition to checking for substrate inhibition, it is also important to examine assay data for non-Michaelian kinetics at lower substrate concentrations. For this purpose the Scatchard space is best suited (Crabbe & Bardsley, 1976).

Compound PS-5 is a new analogue of the thienamycin group of β -lactam compounds, and its therapeutic utility is expected from preliminary biological studies, particularly on the treatment of β -lactamase-producing resistant pathogens. Its characteristic antimicrobial spectrum and inhibitory activity on various β -lactamases motivated our studies on the mechanism of action of compound PS-5, including inactivation of β -lactamases.

The characteristic spectrophotometric property of compound PS-5 (and probably of other thienamycin analogues) is very useful, not only for checking the purity of the antibiotic, but also for the differential assay of compound PS-5 or benzylpenicillin in mixtures (Citri *et al.*, 1976).

It is noteworthy that compound PS-5 was resistant to *Bacillus licheniformis* 749/C β -lactamase, whereas it was susceptible to β -lactamases

of *Bacillus cereus* 569, *Proteus vulgaris* GN76, *Citrobacter freundii* GN346 and *Streptomyces* sp. E750-3 (Y. Fukagawa, T. Takei & T. Ishikura, unpublished work). According to Thatcher (1975b), the proposed amino acid sequences of *Bacillus licheniformis* 749/C β -lactamase and *Bacillus cereus* 569/H type-I β -lactamase show 60% homology. Thus compound PS-5 and other thienamycin analogues might also be valuable as an active-site-directed biochemical tool for comparative study of various β -lactamases.

In agreement with the results obtained by Zyk & Citri (1967), *Bacillus licheniformis* 749/C β -lactamase was inactivated (deactivated) by compound PS-5 according to first-order kinetics (Fig. 6). But the conformational response constant (K_{cr}), which was defined by Zyk & Citri (1967) to be the concentration of inducer analogue (inhibitor) for which the rate of analogue-induced inactivation of the enzyme was half maximal, was found to be negative (Fig. 7). This negative K_{cr} value might be ascribed to difference in choice of the starting point for inactivation lines and/or to the limited precision of the u.v.-spectrophotometric assay method.

Clavulanate, which differs considerably in chemical structure from compound PS-5 and other thienamycin analogues, is also a potent inhibitor of β -lactamase. Fisher *et al.* (1978) and Labia & Peduzzi (1978) have studied the kinetics of inactivation by clavulanate of β -lactamases from several strains of *Escherichia coli*, and shown that clavulanate is destroyed by the enzymes and simultaneously inhibits the enzymes. In general, compound PS-5, like clavulanate, seems to be hydrolysed very slowly by the β -lactamases mentioned above except the enzyme of *Bacillus licheniformis* 749/C, while the enzymes are simultaneously inactivated (Y. Fukagawa, T. Takei & T. Ishikura, unpublished work).

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