A STEREOISOMER OF BENZYLPENICILLIN AS SUBSTRATE AND INDUCER FOR β-LACTAMASES

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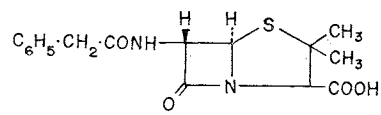
The stereoisomer (*trans*-isomer) of benzylpenicillin was examined for its antibacterial activity and properties as a substrate and inducer of β-lactamases in comparison with benzylpenicillin. The minimum inhibitory concentrations of the *trans*-isomer against *Staphylococcus aureus* FDA 209P and *Bacillus subtilis* PCI 219 were 1.6 and 12.5 μg/ml, respectively, which were about twenty-five times higher than those of benzylpenicillin. However, the *trans*-isomer was very stable to the penicillinases prepared from both *S. aureus* and *Escherichia coli* carrying an ampicillin-resistant R factor. The rates of hydrolysis of the *trans*-isomer by these pencillinases were less than 3% of the corresponding rates with benzylpenicillin as a substrate. The *trans*-isomer was a powerful inducer of the penicillinase synthesis by *S. aureus* and of the cephalosporinase synthesis by *Proteus vulgaris*.

β-Lactamases are enzymes that destroy both penicillins and cephalosporins by hydrolysis of the β-lactam ring. The enzymes are produced by a wide range of gram-positive and gram-negative bacteria, and play an important role in bacterial resistance to penicillins and cephalosporins. Penicillin β-lactamases (penicillinases) are often inducible enzymes in gram-positive bacteria, but constitutive in gram-negative bacteria[13]. Cephalosporin β-lactamases (cephalosporinases), on the other hand, are mainly produced by gram-negative bacteria and may be inducible[13]. Both penicillins and cephalosporins act as inducers of the inducible β-lactamases. Their activity as inducers is influenced by the nature of the side-chain attached to the free amino group of the nuclei of penicillin and of cephalosporin[5].

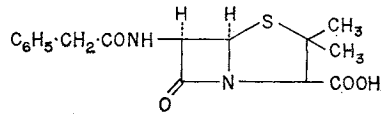
In the present study, a stereoisomer (*trans*-isomer) of benzylpenicillin (Fig. 1), was examined as a substrate and inducer of certain β-lactamases, and its antibacterial activity determined. The β-lactamases were prepared and used for this purpose, *i. e.*, penicillinase of *Staphylococcus aureus* (inducible enzyme), penicillinase of *Escherichia coli* carrying an ampicillin-resistant R factor (constitutitive enzyme) and cephalosporinase of *Proteus vulgaris* (inducible enzyme).

Fig. 1

Trans-isomer of benzylpenicillin



Benzylpenicillin (cis)



Materials and Methods

Bacterial Strain: Bacterial strains used were *S. aureus* FS108, *S. aureus* FDA 209P, *Bacillus subtilis* PCI 219, *P. vulgaris* GN76 and *E. coli* W3630 carrying an ampicillin-resistant R factor, R_{GN14}. *S. aureus* FDA 209P and *B. subtilis* PCI 219 are standard strains for the assay of antibacterial activity of drugs in this laboratory, and they are sensitive to benzylpenicillin and do not produce penicillinase. *S. aureus* FS108 and *P. vulgaris* GN76[13] are clinically isolated strains and are high producer of penicillinase and cephalosporinase, respectively. The ampicillin-resistant R factor, R_{GN14}, was obtained from a clinically isolated *E. coil* strain and successively transferred to *E. coli* W3630, a substrain of K12[6]. *E. coli* W3630 carrying R_{GN14} produces constitutively the R factor-mediated penicillinase[6,13,14].

Drugs: The *trans*-stereoisomer of benzylpenicillin was kindly provided by Toyo Jozo Co., Ltd., Tokyo and the compound used as the N,N'-dibenzylethylendiamine salt. Benzylpenicillin was obtained from Meiji Seika Co., Ltd., Tokyo and cephaloridine from Torii Pharmaceutical Co., Ltd., Tokyo.

Media: Heart infusion broth (HI broth, Nihon Eiyo Kagaku Co., Ltd., Tokyo) was used for liquid culture of bacteria. For the determination of antibacterial activity of penicillins, heart infusion agar (HI agar, Nihon Eiyo Kagaku Co., Ltd., Tokyo) and peptone water[6] were used.

Assay of Antibacterial Activity: Antibacterial activities of penicillins were determined as follows: a loopful of an overnight culture in peptone water was spotted on HI agar plates containing serial two-fold dilutions of drug. The plates were incubated for 18 hours at 37°C. The degree of antibacterial activities of drug was expressed as the minimum inhibitory concentration of bacterial growth.

Induction of β-Lactamase: Induction of β-lactamase production was carried out according to the method described in the previous paper[12]. Five ml of an overnight culture in HI broth was diluted with 200 ml of fresh HI broth and incubated with shaking at 37°C. After 2 hours of incubation, samples of the culture (20 ml) at exponential phase of growth were distributed in Erlenmeyer's flask, and varying amounts of the inducer were added to each flask. A sample without the inducer was used as control. After induction for 1.5 hours at 37°C with shaking, the production of the enzyme was terminated by addition of chloramphenicol to a concentration of 50 μg/ml and prompt chilling in an ice-water bath.

Preparation of β-Lactamase Sample: Exo-penicillinase sample of *S. aureus* was prepared from the culture of *S. aureus* FS108 in HI broth containing methicillin as an inducer for penicillinase[12]. Penicillinase sample from *E. coli* W3630 carrying R_{GN14} was prepared by the method described previously[13].

Determination of β-Lactamase Activity and Measurement of the Amount of Penicillins Hydrolyzed: β-Lactamase activities were determined iodometrically according to the method of PERRET[10]. The determination of penicillinase activities was carried out at 30°C in 0.1 M phosphate buffer (pH 5.8 for staphylococcal penicillinase and pH 7.0 for penicillinase of R factor) containing 8 mM benzylpenicillin as substrate, and one unit of the enzyme activity was defined as the activity that hydrolyzes 1 μmole of the substrate per hour under these conditions. The determination of cephalosporinase activity was carried out at pH 7.0 in the same manner as the determination of penicillinase activity except that cephaloridine was used as a substrate, and that the amount of hydrolyzed cephaloridine was calculated on the basis of 1 mole hydrolyzed substrate being equivalent to 2 moles (4 atoms) of iodine[1]. One unit of cephalosporinase activity was defined as the activity that hydrolyzes 1 μmole of the substrate per hour under the conditions mentioned above.

The assay of benzylpenicillin and its *trans*-isomer hydrolyzed by the penicillinases from *S. aureus* and *E. coli* carrying R_{GN14} was performed by the microiodometric method

of NOVICK[8,9] using 0.1 M phosphate buffer at pH 5.8 (staphylococcal penicillinase) and at pH 7.0 (penicillinase of R_{GN14}). The penicillin was dissolved in the phosphate buffer at 2 mM of concentration. One ml of the penicillin solution was mixed with 1 ml of penicillinase preparation in a centrifuge tube, and the mixture was incubated at 30°C. After 1-hour incubation, the enzymatic reaction was stopped by chilling the mixture in an ice-bath, and simultaneously, 1.0 ml of 0.15 M sodium tungstate (in 2.0 M acetate buffer, pH 4.0) was added to the mixture. The amount of the penicillin hydrolyzed was determined by the microiodometric method with the aid of the standard curves obtained by known amount of chemically hydrolyzed penicillins. Beforehand, it had been confirmed by use of the chemically hydrolyzed *trans*-isomer that the microiodometric assay method was applicable equally to the *trans*-isomer as well as to benzylpenicillin. It had been also confirmed experimentally that N,N'-dibenzylethylendiamine does not inhibit the activities of the penicillinases used.

Results

Antibacterial Activity of the *Trans*-Isomer

The minimum inhibitory concentrations of the *trans*-isomer for *S. aureus* FDA 209P and *B. subtilis* PCI 219 on HI agar plate were 1.6 μg/ml and 12.5 μg/ml, respectively. Under the same experimental conditions, the corresponding values for benzylpenicillin were 0.1 units (0.065 μg)/ml and 0.8 units (0.52 μg)/ml, respectively. These results indicate that the stereochemical change in the nucleus of penicillin does not destroy the antibacterial activity of the drug, though it does cause a great decrease in the level of activity.

Stability of the *Trans*-Isomer to Penicillinases from *S. aureus* and *E. coli* Carrying R_{GN14}

The rates of hyrolysis of the *trans*-isomer by the penicillinases from *S. aureus* FS108 and *E. coli* R_{GN14} could be measured at sufficiently high concentrations of the substrate. The results, together with those with benzylpenicillin, are shown in Table 1. The *trans*-isomer was very stable to both penicillinases when compared with benzylpenicillin. Such stability of the *trans*-isomer is comparable to those of semisynthetic penicillins such as methicillin and cloxacillin which are known as penicillinase-resistant penicillins.

The *Trans*-Isomer as an Inducer for β-Lactamases

Tables 2 and 3 show the amounts of the specific penicillinase and cephalosporinase activities formed by *S. aureus* FS108 and *P. vulgaris* GN76, respectively, during 1.5 hours of cultivation in the presence of varying concentrations of the *trans*-isomer and of benzylpenicillin. In *S. aureus*, the *trans*-isomer was a more

Table 1. Hydrolysis of benzylpenicillin and its *trans*-isomer by penicillinases from *S. aureus* FS108 and *E. coli* R_{GN14}.

Substrate	Source of enzyme	Added enzyme activity (units)	Hydrolyzed substrate ($\times 10^{-3}$ μmoles)	Relative rate of hydrolysis/ enzyme activity (%)
Benzyl-penicillin	*S. aureus*	0.17	168	100
	E. coli R_{GN14}	0.45	448	100
Trans-isomer of benzyl-penicillin	*S. aureus*	17.0	368	2.2
	E. coli R_{GN14}	45.0	376	0.8

Table 2. Induction of penicillinase by benzylpenicillin and its *trans*-isomer in *S. aureus* FS108.

Inducer	Conc. of inducer (units or μg/ml)	Specific penicillinase activity (units/mg dry weight of bacteria)
None	0	11.2
Benzylpenicillin	1 (u/ml)[a)]	15.7
	2	17.6
	10	41.4
Trans-isomer of benzylpenicillin	2 (μg/ml)	22.8
	5	55.0
	10	117
	20	280

a) The potency of benzylpenicillin used is 1,536 units/1,000 μg.

Table 3. Induction of cephalosporinase by benzylpenicillin and its *trans*-isomer in *P. vulgaris* GN76.

Inducer	Conc. of inducer (units or μg/ml)	Specific cephalosporinase activity (units/mg dry weight of bacteria)
None	0	17.0
Benzylpenicillin	20 (u/ml)[a)]	75.4
	100	257
Trans-isomer of benzylpenicillin	100 (μg/ml)	67.6
	500	330

a) The potency of benzylpenicillin used is 1,536 units/1,000 μg.

effective inducer than benzylpenicillin. Benzylpenicillin induced the formation of cephalosporinase in *P. vulgaris* at a lower concentration than did the *trans*-isomer, but the cephalosporinase activity in optimum concentrations of both inducers is about the same.

Discussion

During the past ten years, a number of semisynthetic penicillins have been introduced for clinical use. Some of them are very resistant to penicillinases, especially to staphylococcal penicillinase. These penicillins were obtained by means of modification of the side-chain attached to the free amino group of the penicillin nucleus, 6-aminopenicillanic acid. In the present work, it was shown that steric modification of the penicillin nucleus also resulted in high stability of the resulting drug to the penicillinases from both gram-positive and gram-negative bacteria. Though the antibacterial activity of the *trans*-isomer is lower than that of benzylpenicillin, the minimum inhibitory concentration of the *trans*-isomer against *S. aureus*, which was determined as 1.6 μg/ml, does not exceed the practical levels for clinical use. It is expected, however, that modification of the side-chain of the *trans*-isomer will offer another route for the discovery of new semisynthetic penicillins.

It is well known that methicillin is the most active inducer and that benzylpenicillin, on the other hand, is a poor inducer of staphylococcal penicillinase[5,12]. However, the *trans*-isomer of benzylpenicillin was very good inducer for staphylococcal penicillinase and its inducibility was found to be equivalent to that of methicillin. In contrast to the situation with staphylococcal penicillinase, benzylpenicillin is more active as an inducer of cephalosporinase in gram-negative bacteria than is methicillin[2,3,7,11]. The *trans*-isomer showed about the same activity as an inducer of cephalosporinase in *P. vulgaris* as did benzylpenicillin. It has been suggested by several workers that a repressor, analogous to that of β-galactosidase, exists in the staphylococcal penicillinase synthesis system[4,12]. However, the regulatory system of cephlosporinase synthesis in gram-negative bacteria has so far been unsolved. As shown in this paper, the *trans*-isomer is a powerful inducer of both penicillinase and cephalosporinase. Though this property is not a desirable one with respect to the use of this penicillin as a drug, the *trans*-isomer appears to be an interesting substance for the study on the regulatory systems of β-lactamase production.

References

1) Alicino, J. F. : Iodometric assay of natural and synthetic penicillins, 6-aminopenicillanic acid and cephalosporin C. Anal. Chem. 33 : 648~649, 1961

2) AYLIFFE, G. A. J. : Induction of cephalosporinase and penicillinase in *Proteus* species. Nature 201 : 1032, 1964
3) AYLIFFE, G. A. J. : Cephalosporinase and penicillinase activity of gram-negative bacteria. J. Gen. Microbiol. 40 : 119～126, 1965
4) COHEN, S.; H. SWEENEY & F. LEITNER : Temperature-sensitive repression of staphylococcal penicillinase. Science 149 : 877～879, 1965
5) CROMPTON, B.; M. JAGO, K. CRAWFORD, G. G. F. NEWTON & E. P. ABRAHAM : Behavior of some derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid as substrates, inhibitors and inducers of penicillinases. Biochem. J. 83 : 52～63, 1962
6) EGAWA, R.; T. SAWAI & S. MITSUHASHI : Drug resistance of enteric bacteria. XII. Unique substrate specificity of penicillinase produced by R factor. Jap. J. Microbiol. 11 : 173～178, 1967
7) HENNESSEY, T. D. : Inducible β-lactamase in Enterobacteria. J. Gen. Microbiol. 49 : 277～285, 1967
8) NOVICK, R. P. : Micro-iodometric assay for penicillinase. Biochem. J. 83 : 236～240, 1962
9) NOVICK, R. P. : Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. J. Gen. Microbiol. 33 : 121～136, 1963
10) PERRET, C. J. : Iodometric assay of penicillinase. Nature 174 : 1012～1013, 1954
11) SABATH, L. D.; M. JAGO & E,P. ABRAHAM : Cephalosporinase and penicillinase activities of a β-lactamase from *Pseudomonas pyocyanea*. Biochem. J. 96 : 739～752, 1965
12) SAWAI, T.; H. HASHIMOTO, S. MITSUHASHI & S. YAMAGISHI : Drug resistance of staphylococci. V. Two types of high-resistance to penicillin G in *Staphylococcus aureus*. Jap. J. Microbiol. 11 : 179～188, 1967
13) SAWAI, T.; S. MITSUHASHI & S. YAMAGISHI : Drug resistance of enteric bacteria. XIV. Comparison of β-lactamases in gram-negative rod bacteria resistant to α-aminobenzylpenicillin. Jap. J. Microbiol. 12 : 423～434, 1968
14) YAMAGISHI, S.; K. O'HARA, T. SAWAI & S. MITSUHASHI : The purification and properties of penicillin β-lactamases mediated by transmissible R factors in *Escherichia coli*. J. Biochem. 66 : 11～20, 1969