

SUBSTRATE SPECIFICITY OF PENICILLIN ACYLASE OF *E. COLI*

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The hydrolysis of several phenylacetylamino compounds was studied using a purified preparation of *E. coli* penicillin acylase. The L-isomers of phenylacetyl amino acids were cleaved much faster than the D-isomers. The same observation was made for some phenylacetylamino β -lactams. When the β -lactam ring is incorporated in a penam or cephem ring system, the D-isomers were hydrolysed somewhat faster than the L-isomers. We also confirmed that benzylpenicillins with an hydroxy- or an amino-group in α -position of the side chain were hydrolysed, both in the normal and the 6-epi-series.

In 1960¹⁻⁴) it was reported by several laboratories that bacterial enzymes could remove the side chain from benzylpenicillin. This method is now used in the commercial production of 6-aminopenicillanic acid (6-APA). The distribution of this enzyme has been studied⁵) and its properties have been described in some recent reviews^{6,7}).

Penicillin acylase from *Escherichia coli* preferentially cleaves phenylacetamidopenicillanic acid (benzylpenicillin). Penicillins with related side chains such as thienylacetic, furylacetic acids^{8,9}) and phenylacetic acid substituted in the α position with an hydroxy- or an amino-group⁸) are also split. Propoxy- and isobutoxyacetic acids are removed, although with lower yield, but penicillins with phenoxyacetic acid or with fatty acids as side chains are essentially uncleaved⁸).

Deacylation of several acylamino acids by the same enzyme was also reported^{10,11}). These observations were confirmed and extended by several workers¹²⁻¹⁵). The general conclusion was that this enzyme cleaved mainly or exclusively acylamino groups attached to a carbon atom with the L-configuration. The preparation of 6-epibenzylpenicillin^{16,17}) where the carbon atom bearing the phenylacetamido side chain has the D-configuration, induced us to examine this substance as substrate for the *E. coli* enzyme. Several other related products were also studied.

Materials and Methods

Penicillin derivatives

The preparation of 6-epibenzylpenicillin^{16,17}) and of 5-epibenzylpenicillin¹⁸) has already been described. Benzylpenicillin(S) sulfoxide was obtained by oxidation of benzylpenicillin with periodate^{16,19}) and benzylpenicillin sulfone by oxidation with permanganate²⁰). The preparation of 6-epi-ampicillin (IX), of α -hydroxybenzylpenicillin and of 6-epi- α -hydroxybenzylpenicillin (X) is given in reference²¹). 7-Phenylacetamido-3-cephemcarboxylic acid (XI) was prepared from 7-amino-3-desacetoxycephalosporanic acid²²) and the 7-epimer from 6-epibenzylpenicillin sulfoxide²³). The preparation of 1-(1'-carboxy-1'-isobutenyl)-3 β -phenylacetamido-4 β -methylthioazetidinone-2 and of 1-(1'-carboxy-1'-isobutenyl)-3 α -phenylacetamido-4 β -methylthioazetidinone-2 (VII) will be described elsewhere²⁴).

Desthiobenzylpenicillin (VI) was prepared by hydrogenolysis of benzylpenicillin with RANEY nickel²⁵), m.p. 95~100°C. IR ν_{\max} (KBr) 3330, 1658, 1540 (CONH), 1735 (β -lactam), 1630 (COOH) cm^{-1} ; NMR (CDCl_3 - DMSO d_6 10 : 1) δ 1.02 (d, J=6.5 Hz, two CH_3), 1.80~2.50 (m, 2'-H), 3.44

(dd, $J=2.5$ and 5.5 Hz, 4β -H), 3.56 (s, $C_6H_5CH_2$), 3.90 (t, $J=5.5$ Hz, 4α -H), 4.15 (d, $J=7.5$ Hz, $1'$ -H), 4.70~5.00 (m, 3-H), 5.62 (COOH), 7.25 (s, C_6H_5), 7.95 (d, $J=8$ Hz, CONH). 6-Epidesthiobenzylpenicillin was prepared by the same method²⁵⁾ from 6-epibenzylpenicillin. Evaporation of the aqueous solution did not yield a pure product. Even chromatography on a column of silica gel using dichloromethane-ethyl acetate as eluent gave a mixture of two substances. For this reason, the product was transformed into the methyl ester with diazomethane. Chromatography on a column of silica gel using dichloromethane and dichloromethane - acetone (95 : 5) as eluent, gave pure 6-epidesthiopenicillin methylester, m.p. 90~100°C, $[\alpha]_D^{25} + 7.2$ (c 0.5, acetone), Rf 0.22 (tlc, silica gel, benzene - acetone (80 : 20)), mass spectrum m/e 259 ($M - COOCH_3$), IR ν_{max} (KBr) 3310, 1680, 1599 (CONH), 1745 (β -lactam), 1212 (ester) cm^{-1} , NMR ($CDCl_3$, TMS), δ 0.93 (d, $J=7$ Hz, two CH_3), 1.70~2.40 (m, $2'$ -H), 3.40 (dd, $J=2$ and 6 Hz, 4-H), 3.54 (s, $C_6H_5CH_2$), 3.71 (s, OCH_3), 3.75 (m, 4-H), 4.14 (d, $J=8$ Hz, $1'$ -H), 5.03 (m, 3-H), 6.39 (m, CONH), 7.27 (s, C_6H_5). Hydrolysis of the methyl ester with sodium hydroxide (0.1 N) and acidification gave 6-epidesthiobenzylpenicillin, m.p. 120~140°C, Rf 0.78 (tlc, acetone - acetic acid (95 : 5)), IR ν_{max} (KBr) 3420, 1665, 1517 (CONH), 1760 (β -lactam), 1710 (sh, COOH) cm^{-1} .

Amino acid derivatives

These derivatives were synthesized by reaction of the amino acids in sodium carbonate solution with phenylacetyl chloride²⁶⁾. Phenylacetyl-glycine, m.p. 145~146°C; phenylacetyl-L-alanine, m.p. 110~111°C, $[\alpha]_D^{20} - 19.9$ (c 1.0, acetone); L- α -phenylacetamidobutyric acid, m.p. 112~113°C, $[\alpha]_D^{20} - 14.1$ (c 1.0, 0.1 M $NaHCO_3$); phenylacetyl-L-valine, m.p. 139~140°C, $[\alpha]_D^{20} - 12.9$ (c 1.0, 0.1 M $NaHCO_3$).

Enzyme preparation

Fermentation of *Escherichia coli* NCIB 8743 was carried out essentially as described by COLE and SAVIDGE²⁷⁾. A suspension of cells obtained from an agar-trypticase slope was used for inoculation of the Erlenmeyer flasks. The medium (100 ml per flask of 300 ml) consisted of cornsteep liquor solids (1.2%), ammonium sulfate (0.1%), ammonium phenylacetate (0.1%), peptone (0.5%) and soya oil (0.1%) adjusted to pH 6.5 with NaOH. After incubation for 15 hours at 24°C on a rotary shaker, a sterile solution of 8% ammonium phenylacetate (1 ml per flask) was added at 15, 18 and 21 hours. After 28 hours, when a pH of 8 had been reached, the cells were killed with butyl acetate (1 ml per flask). The cells were collected by centrifugation of the broth (3,750 ml) at 9,000 rpm, washed and resuspended in 375 ml of 0.1 M phosphate buffer pH 7.5. The total activity as determined by the biochromatographic method⁶⁾ was 760 u. The resuspended cells were disrupted by sonication and the cell debris were centrifuged off at 15,000 rpm. The supernatant contained 462 u. The nucleic acids were precipitated by adding streptomycin sulfate to obtain a concentration of 0.7%²⁸⁾. After removal of the precipitate by centrifugation, ammonium sulfate (39 g per 100 ml) was added to the supernatant. The precipitate was redissolved in 0.01 M phosphate buffer pH 8.0 and dialysed at 5°C against 0.001 M phosphate pH 8.0. The solution was passed through a cooled (5°C) column of Sephadex G 25 (2.6 \times 100 cm), and eluted with 0.01 M phosphate pH 8.0. The elution was followed by the absorbance of the effluent at 280 nm and by biochromatography. The appropriate fractions were dialysed against water and lyophilized, yielding 1,760 mg of an enzyme preparation containing 0.13 u/mg (total activity 229 u). The unit of enzyme activity is defined as the amount required to produce 1 μ mole of 6-APA from benzylpenicillin per minute at 37°C and in 0.1 M phosphate buffer pH 7.5.

Assay

1) pH Stat method^{8,9)}

The rate of consumption of alkali (NaOH 0.1 N) corresponds to the rate of formation of phenylacetic acid. This method was used for all penicillins except ampicillin. A "Radiometer" pH-Stat, comprising a pH meter, an automatic titrator TTT 11, a titrigrath SBR 2 and an autoburet ABU 12 (0.25 ml) was used.

2) Colorimetric method

The results of the pH Stat method were checked by a colorimetric method using *p*-dimethylaminobenzaldehyde²⁸⁾. This assay is based on the formation of a SCHIFF base (λ_{max} at 415 nm) by reaction of *p*-dimethylaminobenzaldehyde with 6-APA.

3) Biochromatographic assay⁶⁾

This assay is based upon paper chromatography of 6-APA, phenoxyacetylation and bioassay and was used for the determination of the amount of enzyme during its purification.

4) Determination of the rate of hydrolysis of ampicillin and 6-epiampicillin.

It was not possible to use the pH Stat method for ampicillin. Ampicillin with pKa 2.7 and 7.25²⁹⁾ exists at the pH used partially as a zwitterion, partially as the sodium salt. The same is true for a phenylglycine (pKa 2.9 and 8.9) formed during the reaction, but the relative amount for both forms are different, which means that the amount of alkali consumed is not stoichiometric. The determination of 6-aminopenicillanic acid by chromatography and bioassay is too time-consuming for kinetic studies and it is not applicable to the transformation of 6-epiampicillin to 6-epiaminopenicillanic acid. The spectrophotometric assay using buffered copper sulfate^{30,31)} was used for ampicillin and also for 6-epiampicillin.

5) Hydrolysis of phenylacetyl amino acids and phenylacetamide.

The amount of amino acid or ammonia liberated was determined with ninhydrin³²⁾. The colorimetry was performed using a Technicon amino acid analyser with the reagent prepared by dissolving 11 g ninhydrin and 850 mg hydrindantin in 850 ml ethylene glycol monomethylether and 850 ml water, adding 310 ml ethylene glycol monomethylether and 190 ml 4M acetate buffer pH 5.5³³⁾. The extinction was measured at 570 nm and standard curves were determined using ammonium chloride or the appropriate amino acid in the range 0.01 ~ 0.1 μ mol per ml.

The phenylacetyl amino acid or phenylacetamide (0.2 mmol) was dissolved in 2 ml of 0.1 N NaOH and 6 ml 0.1 M phosphate buffer pH 7.5. A solution of 10 mg acylase preparation in 2 ml of water was added and the mixture was kept at 37°C. At different times a sample of 1 ml was taken and added to 3 ml of 5% CCl₃COOH solution. After centrifugation, 1 ml of the supernatant was diluted to 50 ml with 0.1 M acetate buffer pH 5.6 and the amount of amino acid or ammonia liberated was assayed with the amino acid analyser. The solutions of the sample (rate 0.23 ml per minute) and ninhydrin (rate 0.42 ml per minute) were mixed, heated at 95°C (duration : 20 minutes) and the extinction was determined at 570 nm.

Results

Influence of pH and Temperature

The rate of the reaction increased with temperature for benzylpenicillin, its 5- and 6-epimers up to 55°C. All other determinations were performed at 37°C in order to obtain values which were comparable to those described in the literature. The determination of the influence of pH showed an optimum at about 8 for benzylpenicillin and its 6-epimer, but at 7 for 5-epibenzylpenicillin (Fig. 1). A similar pH optimum (about 8) has been found for penicillin acylase of *E. coli* using a purified enzyme^{28,34)} or a cell suspension⁸⁾. The values (pH optimum: 7.0 and optimum temperature at 35°C) found by another author³⁵⁾ for the hydrolysis of benzylpenicillin by the *E. coli* enzyme are quite different.

V_{max} and Km

The rate of deacylation of benzylpenicillin for different substrate concentrations is given in Table 1. Km and V_{max} were determined by LINEWEAVER-BURK plots and are shown in Fig. 2. For benzylpenicillin V_{max} 0.139 μ mol/min/mg and Km 4.5×10^{-5} M were obtained. The reported values for Km are 7.4×10^{-4} M at 37°C and pH 7.5²⁸⁾ and 2×10^{-5} M at 25°C and pH 8.0³⁴⁾, and 30×10^{-8} M for the cell-bound enzyme⁸⁾. For 6-epi and 5-epibenzylpenicillins (Fig. 2) the Km value is very low, indicating a very high affinity for the enzyme.

Several values were obtained by the pH-Stat and the colorimetric method. The good agreement between two methods indicated that for the study of the substrate specificity of the enzyme it is prefer-

Fig. 1. Reaction rate V_{max} of sodium benzylpenicillin (A), potassium 6-epibenzylpenicillin (B), potassium 5-epibenzylpenicillin (C) at different pH using 0.02 mmol penicillin in 20 ml of water containing 2.5 mg (0.325 u) of acylase at 37°C. V_{max} are 0.139 for A, 0.23 for B and 0.666 for C.

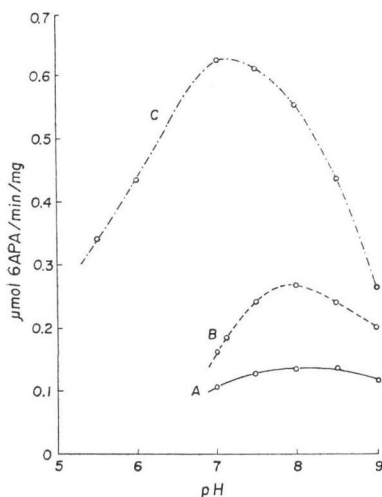
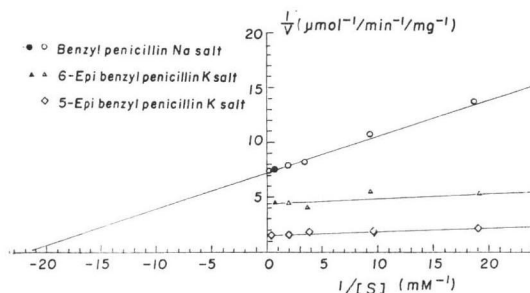


Fig. 2. LINEWEAVER-BURK plots for different penicillins.

Open circle, triangles and rhombs: pH-Stat method.
Closed circles and triangles: colorimetric method.



able to use a more or less purified preparation rather than a cell suspension, because in the latter case the pH-Stat method is perturbed by the release of acidic substances by the cells³.

Substrate Specificity

The V_{max} values of different penicillin and cephem derivatives were determined in the same way and are given in Table 2. For the phenylacetyl amino acids the V_{init} (initial rate of hydrolysis) are given. The (S) sulfoxide and the sulfone of benzylpenicillin were not hydrolysed by the *E. coli* enzyme preparation.

Table 1. Rate of deacylation of benzylpenicillin

[S] mmol/liter	1/[S]	V μmol/min/ mg	1/V
5.23	0.19	0.134	7.46
0.573	1.75	0.130	7.69
0.287	3.48	0.122	8.20
0.106	9.43	0.092	10.87
0.053	18.87	0.074	13.51

The appropriate quantity of sodium benzylpenicillin was dissolved in 40 ml of water containing 2.5 mg (0.325 u) of acylase. The rate was determined at pH 7.5 and 37°C.

Table 2. Rate of deacylation of different substrates at 37°C and pH 7.5

	V _{init} (μmol/min/mg acylase)	V _{max} (μmol/min/mg acylase)	
		L isomer	D isomer
I C ₆ H ₅ CH ₂ CONH - H		0.126	
II C ₆ H ₅ CH ₂ CONH - CH ₂ COOH		0.386	
III C ₆ H ₅ CH ₂ CONH - ⁺ CHCH ₃ COOH	0.60		0.017
IV C ₆ H ₅ CH ₂ CONH - ⁺ CH-CH ₂ CH ₃ COOH	0.224		0.003
V C ₆ H ₅ CH ₂ CONH - ⁺ CH-CH(CH ₃) ₂ COOH	0.022		0.000
VI C ₆ H ₅ CH ₂ CONH - ⁺ CH-CH ₂ -CH(CH ₃) ₂ CO-N-C-COOH H	0.784		0.007
VII C ₆ H ₅ CH ₂ CONH - ⁺ CH-C-SCH ₃ CO-N-C-C(CH ₃) ₂ COOH	0.217		0.011
VIII C ₆ H ₅ CH ₂ CONH - ⁺ CH-C-S CO-N-C-C(CH ₃) ₂ H	0.139		0.23
IX (D) C ₆ H ₅ -CH-CONH- NH ₂	0.068		0.085
X** (DL) C ₆ H ₅ -CH-CONH- OH	0.071		0.107
XI C ₆ H ₅ CH ₂ CONH - ⁺ CH-C-S CO-N-C-CH ₂ C-CH ₃ COOH	0.148		0.182

* Indicates the asymmetric carbon referred to with L- and D-isomer.

** Compounds X were a mixture of about 1 part D and 2 parts L isomer in the side chain.

Discussion

We have confirmed that benzylpenicillins with an hydroxy- or an amino group in α -position of the side chain are hydrolysed by *E. coli* acylase. The relative rates of deacylation (compounds VIII, IX and X of Table 2) are similar to those observed by COLE⁸⁾ using *E. coli* cell suspensions.

It has been stated²⁷⁾ that *E. coli* only hydrolysed phenylacetyl derivatives of L-amino acids. This was based on the study of phenylacetyl derivatives of α -aminophenylacetic acid¹⁵⁾, alanine, α -aminobutyric acid and valine¹³⁾. The derivatives of the D-isomers were not hydrolysed^{13,15)} although it was mentioned in another paper from the laboratory of ROMEO¹⁴⁾ that phenylacetyl-D-alanine was hydrolysed. Our results confirm that the hydrolysis of the L-isomers is definitely more rapid than that of the D-isomers, although it cannot be stated that these latter are not cleaved. Our data also indicate that the nature of the amino acid (compounds II~V of Table 2) has a marked influence upon the rate of the reaction.

The preferential hydrolysis of the L-derivative also exists when the amino acid is part of a ring as in the β -lactams VI and VII. It should be noted that the same stereospecificity of *E. coli* acylase was observed for the phenylacetyl derivatives of the alcohol and nitrile analogs of some amino acids¹⁴⁾.

When the β -lactam is incorporated in a penam or a cephem ring system, the enantiometric preference of the enzyme changes. The 6- or 7-epimers (with the D-configuration) are hydrolysed somewhat faster than the isomers with normal (L-configuration) configuration (compounds VIII to XI). The absence of deacylation of benzylpenicillin sulfoxide and sulfone also demonstrates the influence of this part of the molecule. 5-Epibenzylpenicillin, on the other hand, is hydrolysed quite readily (Fig. 1), despite a profound modification of the configuration of the penam nucleus.

The substrate specificity of few acylases has been studied. Hog kidney acylase hydrolyses acetyl, chloroacetyl and propionyl amino acids but not benzoyl derivatives^{36,37)}. The derivatives of the L-amino acids are cleaved preferentially, although after long incubation the acyl-D-amino acids are also hydrolysed to a small extent.³⁷⁾ It should be noted that there is a marked difference in the rate of hydrolysis of the acyl derivatives of different amino acids.³⁸⁾

Extraction of the mycelium of *Fusarium semitectum* with water yields an enzyme which cleaved phenoxyacetyl amino acids but not phenoxy methylpenicillin.³⁹⁾ Treatment of the extracted mycelium with 0.2 M NaCl gave an another preparation, which was purified and which hydrolysed phenoxy methylpenicillin but not acyl amino acids.⁴⁰⁾ There is no indication of the presence of two acylase enzymes in *E. coli*, although it cannot be excluded.

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