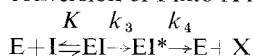


Fragmentation of benzylpenicillin after interaction with the exocellular DD-carboxypeptidase-transpeptidases of *Streptomyces* R61 and R39

THE killing target of penicillin in bacteria is a membrane-bound transpeptidase which catalyses peptide cross linking during wall peptidoglycan synthesis^{1,2}. *Streptomyces* R61 and R39 excrete during growth DD-carboxypeptidase-transpeptidase enzymes^{3,4} which seem to be soluble forms of the corresponding membrane-bound transpeptidases⁵. The exocellular enzymes (E) convert penicillin (I) in to a chemically altered and biologically inactive compound (X)^{6,7}. Kinetically, the simplest mechanism⁵ for the conversion of I into X is



The first step, a rapid equilibrium process, leads to the formation of an equimolar and inactive enzyme-antibiotic complex EI. This complex isomerises into a modified complex EI* which, in turn, undergoes irreversible breakdown. If the experiment is carried out in conditions in which the enzyme is stable, the enzyme is reactivated and recovers its initial penicillin sensitivity. The breakdown of complex EI* is a slow process. At 37 °C and in 10 mM Na phosphate buffer, pH 7.0 (in which conditions the R61 enzyme is stable) the half life of the R61 enzyme-benzylpenicillin EI* complex is 80 min (ref. 7). At 37 °C and in 0.1 M Tris-HCl buffer, pH 7.7, containing 0.1 M NaCl and 0.05 M MgCl₂ (in which conditions the R39 enzyme is stable), the half life of the R39 enzyme-benzylpenicillin is 4,250 min (ref. 6). As the enzyme is reactivated during the process, no enzyme is irretrievably lost on reaction with penicillin and after several cycles of inactivation and reactivation, both the enzyme and the accumulated X

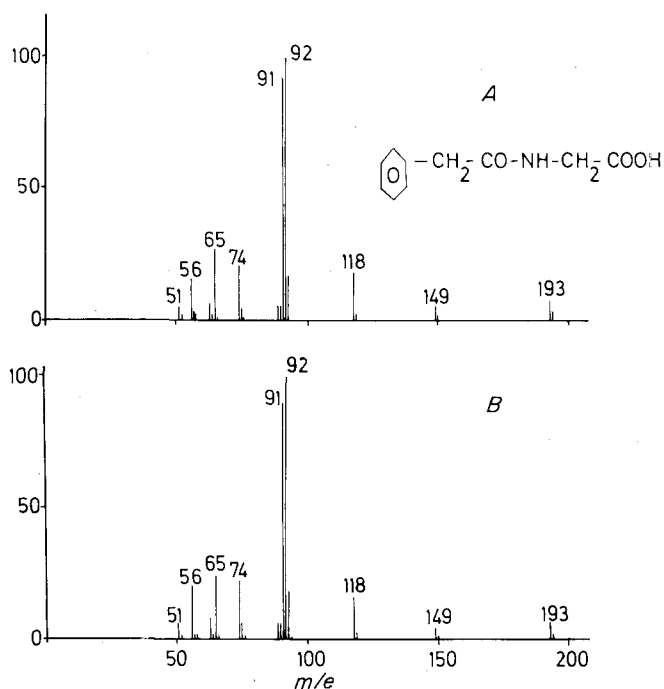


Fig. 1 Mass spectra of authentic phenylacetyl glycine (A, molecular weight = 193) and X compound (B). ¹⁴C-X (0.5 μeq) was dissolved in 100 μl of 6 N HCl and extracted, at room temperature, with 300 μl of ethyl acetate (yield of the extraction 60% in terms of d.p.m.). Mass spectrography was carried out on a sample of the extract containing 5 neq of ¹⁴C-X and compared with that given by 1 μg of phenylacetyl glycine. Spectra were recorded at 70 eV with an ionisation current of 60 μA. The sample was directly introduced (193:molecular ion M⁺).

compound can be reisolated. This technique was applied to ¹⁴C-benzylpenicillin (benzyl labelled) and the ¹⁴C-X compound produced was isolated.

A solution of R61 enzyme (0.9 μmol; molecular weight 38,000) in 20 ml of phosphate buffer was supplemented at 37 °C with 0.72 μmol of ¹⁴C-benzylpenicillin (0.7 mCi mmol⁻¹). Every 30 min, an additional 0.144 μmol of ¹⁴C-benzylpenicillin was added until a total amount of 29 μmol of antibiotic was used. Filtration on Sephadex G-25 and elimination of phosphate by addition of acetone at 0 °C yielded 19 μeq of purified ¹⁴C-X. Similarly, a solution of R39 enzyme (11.7 nmol; molecular weight 53,000) in 100 μl of Tris-NaCl-MgCl₂ buffer was supplemented with 14.5 nmol of ¹⁴C-benzylpenicillin (25 mCi mmol⁻¹) and the mixture was incubated for 51 h at 37 °C. Filtration on Sephadex G-25 yielded 4 neq of ¹⁴C-X.

The ¹⁴C-X obtained with the R61 enzyme was identified as ¹⁴C-phenylacetyl glycine on the following grounds. (1) Acid hydrolysis with 6 N HCl at 120 °C yielded glycine (yield 100% with the amino acid analyser and 76% after transformation into dinitrophenylglycine) and ¹⁴C-phenylacetic acid (as revealed by thin-layer chromatography on Silica-Gel plates using two solvents; Table 1). (2) ¹⁴C-X and authentic phenylacetyl glycine exhibited the same R_f values by thin-layer chromatography on Silica-Gel plates using three solvents (Table 1). (3) A mixture containing a few neq of ¹⁴C-X and a 1,000-fold excess, on a molar basis, of non-radioactive phenylacetyl glycine was submitted to three successive crystallisations from ethylacetate. Crystals thus obtained exhibited constant specific radioactivities. (4) The mass spectrum of ¹⁴C-X was identical to that given by phenylacetyl glycine (Fig. 1). (5) Both ¹⁴C-X and phenylacetyl glycine, esterified with CH₃N₂, had the same retention time (6 min) by gas-liquid chromatography (at 200 °C, with H₂ and N₂ flow rates of 2 and 30 ml min⁻¹, respectively, and by using a capillary SP1000 column of length 20 m). Similarly, the ¹⁴C-X compound obtained with the R39 enzyme was also characterised as ¹⁴C-phenylacetyl glycine by cocrystallisation and cochromatography with authentic phenylacetyl glycine.

We conclude, therefore, that by interacting with benzylpenicillin, both R61 and R39 exocellular enzymes split the antibiotic molecule and that one of the fragments is phenylacetyl glycine. Evidently the methylene carbon atom of the glycine residue must arise from C₆ of the penicillin nucleus and, presumably, the C₇ is retained as the glycine carboxyl group (Fig. 2). The fate of the thiazolidine nucleus of the penicillin molecule has not yet been determined. Chemically, benzylpenicillin methyl ester can be degraded to methyl D-5,5-dimethyl-Δ³-thiazoline-4-carboxylate in trifluoroacetic acid. The N-phenylacetyl glycyl fragment was isolated by conversion to its N-benzylamide⁸. A possible mechanism for the action of the *Streptomyces* enzymes would be that the initial event is a rupture of the

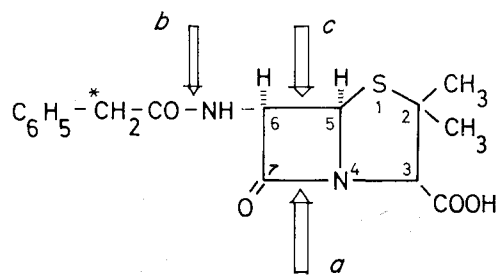


Fig. 2 ¹⁴C-benzylpenicillin. a, Site of action of penicillinase (reaction product benzylpenicilloic acid); b, site of action of amidase (reaction product 6-aminopenicillanic acid); c and a, possible sites of action of the DD-carboxypeptidase-transpeptidases of *Streptomyces* R61 and R39 (reaction product phenylacetyl glycine + unknown). *, [¹⁴C].

Table 1 R_f values of $^{14}\text{C-X}$ and phenylacetyl-glycine and of HCl-hydrolysed $^{14}\text{C-X}$ and phenylacetic acid, by cochromatography on Silica-Gel G thin-layer plates

Enzyme used for the preparation of $^{14}\text{C-X}$	Solvent	R_f values of			
		$^{14}\text{C-X}$	Added phenylacetyl-glycine	HCl-hydrolysed $^{14}\text{C-X}$	Added phenylacetic acid
R61	<i>a</i>	0.77	0.76	0.80	0.80
	<i>b</i>	0.71	0.70		
	<i>c</i>	0.40	0.40	0.56	0.55
R39	<i>a</i>	0.78	0.77		
	<i>b</i>	0.71	0.69		
	<i>c</i>	0.36	0.36		

Solvents: *a*, 1-butanol-H₂O-acetic acid-ethanol: 10:4:3:3 (v:v:v:v); *b*, water-1-butanol-acetic acid: 50:10:10 (v:v:v) upper phase; *c*, chloroform-methanol-acetic acid: 88:10:2 (v:v:v).

Note that the R_f values of benzylpenicilloic acid are 0.65 in solvent *a* and 0.55 in solvent *b*. Benzylpenicilloic acid is immobile in solvent *c*.

β -lactam amide bond (Fig. 2; arrow *a*) with attachment of C₇ to some active group in the enzyme. By a process of β elimination, in which functional groups of the enzyme could participate, the link between C₅ and C₆ would become a double bond and further degradation would result in removal of the thiazolidine moiety (Fig. 2; arrow *c*). Release of the phenylacetyl-glycine would then occur. There may be other possible mechanisms and the problem is being studied at present.

Whatever the mechanism, however, our studies point to the great importance of the nature of the substituents on C₆ of the penicillin molecule. Substitution at C₆ drastically reduces the activity of the β -lactam antibiotics in all cases tested; 6-methoxy-penicillin derivatives, however, remain better inhibitors than the corresponding 6-methyl derivatives⁹. These studies may also be related with the observations of Schmid and Plapp¹⁰ that binding of penicillin to *Proteus mirabilis* was inhibited by phenylacetyl-glycine and that in the presence of this compound, formation of sphaeroplasts was prevented. Experiments have shown that the extracted and partially purified membrane-bound transpeptidases from *Streptomyces* R61 and *S. rimosus*⁵ also perform fragmentation of benzylpenicillin with formation of phenylacetyl-glycine (M. Leyh-Bouille, J. Dusart and J. M. Ghuysen, unpublished). Moreover, penicillin is known to be degraded into a biologically inactive compound by interacting with the membrane-bound DD-carboxypeptidases of various *Bacillus* spp.¹¹. The fact that the membrane-bound targets of penicillin act as antibiotic-degrading compounds is important even if the overall process is slow. It leads to a better understanding of the phenomena of resistance which cannot be explained on the basis of production of either penicillinase or amidase (Fig. 2; arrows *a* and *b*, respectively). It may also be relevant to the necessity for the continuous supply of antibiotic during penicillin therapy.

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