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Fate of thiazolidine ring during fragmentation of penicillin by exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61

LIKE various β-lactamases, acylases and esterases[1], the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61 degrades benzylpenicillin and other β-lactam antibiotics[2-4]. The R61 enzyme, however, markedly differs from the other penicillin-degrading enzymes in causing fragmentation of the penicillin nucleus. By using 8-^{14}C-benzylpenicillin (benzyl labelled) as substrate, one of the fragments produced was shown to be ^{14}C-phenylacetylglycine[5]. The reaction with the R61 enzyme is also peculiar in that it is a slow process. This is because of the long half life of the stoichiometric complex transitorily formed between the antibiotic and the enzyme. Thus, for example, the value of the half life for the complex formed with benzylpenicillin is 80 min in 10 mM phosphate buffer (*p*H 7.0) and at 37 °C. As breakdown of the complex proceeds, however, phenylacetylglycine (when benzylpenicillin is used as substrate) is released and the enzyme concomitantly recovers its ability to bind penicillin. We have now characterised the fragment (hereby designated as the Y product) arising from the thiazolidine ring of penicillin as a result of the fragmentation of the antibiotic molecule by the R61 enzyme.

The large amount (about 20 μmol) of ^{14}C-phenylacetylglycine previously produced from 8-^{14}C-benzylpenicillin through a series of enzyme inactivation and reactivation (total time for the operation, 100 h) could be purified by filtration of the reaction mixture on a column of Sephadex G-25 on the basis that phenylacetylglycine was eluted at a volume slightly larger than the salt volume[5]. Examination of the fractions containing ^{14}C-phenylacetylglycine by NMR spectrometry failed to reveal any lines that could be attributed to methyl groups. In marked contrast, the NMR spectrum in D_2O of the non-radioactive

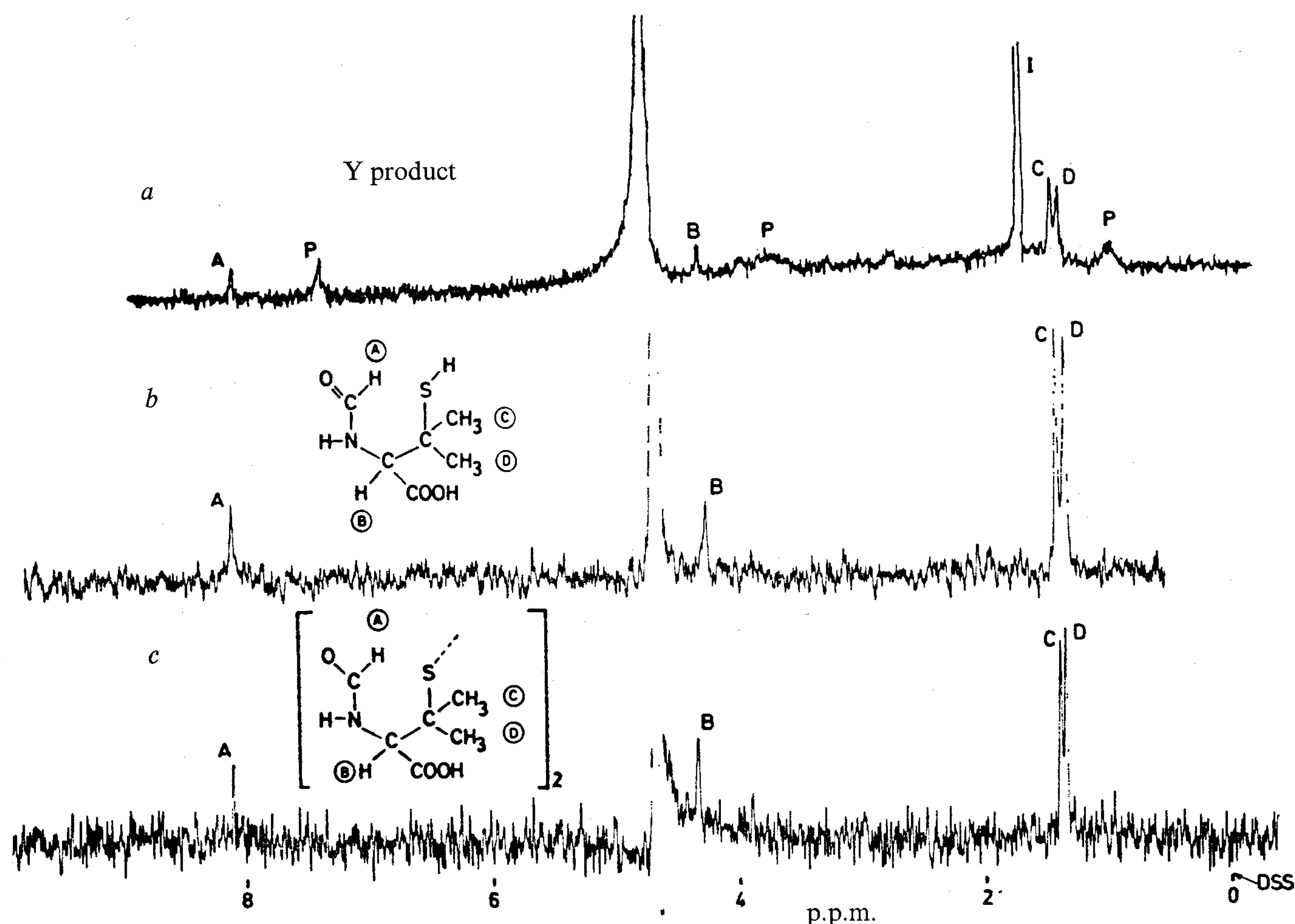

Fig. 1 NMR spectra in D_2O of Y product (*a*), *N*-formyl-D-penicillamine (*b*) and di-(*N*-formyl-D-penicillamine) disulphide (*c*). The spectra were obtained with a Varian XL100-15 spectrometer operated in the ^{2}D internal lock mode. The I line exhibited by the Y preparation (*p*H 7.0) was attributed to an impurity and the P lines were attributed to the presence of a small amount of penicilloic acid. The A, B, C and D lines, occurring in the ratios of 1 : 1 : 3 : 3, were attributed to the Y product. The 6 Hz distance between the methyl lines (C and D) excluded the presence of an intact thiazolidine ring (in which case the distance value should have been 38 Hz). The spectra of the Y product, *N*-formyl-D-penicillamine and di-(*N*-formyl-D-penicillamine)disulphide were virtually superimposable with respect to lines A (8.09, 8.11 and 8.09 p.p.m., respectively), B (4.31, 4.28 and 4.34 p.p.m., respectively), C (1.40, 1.38 and 1.36 p.p.m., respectively) and D (1.46, 1.45 and 1.41 p.p.m., respectively). The p.p.m. values are relative to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS). Further confirmation was obtained by extracting the Y product (after acidification) with ethylacetate. The extract was evaporated to dryness and the residue dissolved in $CDCl_3$ in the presence of dimethylsulphoxide. Proton B of the CH group located in the vicinity of the NH group gives a characteristic doublet (9 Hz splitting). The SH proton, however, could not be seen because of the presence of a large impurity peak in its resonance region and it was therefore impossible to decide whether the Y product was *N*-formyl-D-penicillamine or di-(*N*-formyl-D-penicillamine)disulphide. The *N*-formyl-D-penicillamine used as standard (spectrum *b*) was prepared by dissolving D-penicillamine HCl (550 mg) in 7 ml formic acid (95%) containing 200 mg of sodium formiate. After addition of 2 ml of acetic anhydride, the reaction proceeded for 1 h at room temperature. Melting point 146–148 °C; (α^{20}_D+63 (C = 1, pyridine)). The optical rotation is in agreement with the value published previously[6]. The di-(*N*-formyl-D-penicillamine)disulphide (spectrum *c*) was prepared from *N*-formyl-D-penicillamine by oxidation in air in the presence of FeCl[6].

material obtained from the salt-containing fractions exhibited lines characteristic of methyl groups. The spectrum indicated that the thiazolidine ring had been opened and suggested that the split product was either *N*-formyl-D-penicillamine or di-(*N*-formyl-D-penicillamine)disulphide (Fig. 1). Direct chromatography of a sample of this fraction on Aminex A5 ion-exchange resin yielded only very minor ninhydrin-positive peaks. Mild acid hydrolysis (1 h in 1 N HCl at 60 °C, that is, in conditions which are known to release the formyl group from di-(*N*-formyl-D-penicillamine)disulphide[6]) produced one important ninhydrin-positive compound which had the same behaviour on the Aminex column as di-(D-penicillamine)-disulphide. Finally, mild acid hydrolysis followed by performic oxidation yielded a highly acidic ninhydrin-positive product which, on the same Aminex column, was indistinguishable from the product obtained by performic oxidation of D-penicillamine.

In order to characterise the Y product further and to study the kinetics of its release, various radioactive penicillins with the radioactive label in the thiazolidine portion of the molecule were prepared. ^{3}H-Benzylpenicillin (labelled in the β-methyl group, 1.7 mCi mmol^{-1}), 5-^{14}C-benzylpenicillin (0.07 mCi mmol^{-1}) and 5-^{14}C phenoxymethylpenicillin (that is, penicillin V; 0.08 mCi mmol^{-1}). As determined by the bioassay and iodometric assay, the purity of the compounds was higher than 98%. Thin-layer chromatography showed, besides the penicillin spot, a small amount of penicilloic acid (±2%) but no other radioactive impurities.

In a first series of experiments, maximal amounts of ^{3}H-Y and ^{14}C-Y products were prepared from ^{3}H-benzylpenicillin and 5-^{14}C-benzylpenicillin, respectively. In these assays, the R61 enzyme was maintained saturated by successive additions of radioactive penicillins to the reaction mixtures. The total period of time for the whole operations was 24 h and a total amount of 0.5 μmol of each radioactive benzylpenicillin was converted into degradation products. The accumulated ^{3}H-Y and ^{14}C-Y products were partially purified by filtration on Sephadex G-25. The following observations were made. (1) Both radioactive Y products had the same *Rf* values by thin-layer chromatography and with the two solvents used, these *Rf* values were identical to those of di-(*N*-formyl-D-penicillamine)-disulphide (Table 1). (2) At least 95% of the radioactive labels originally engaged in the reactions, cochromatographed on the Aminex A5 column exactly with exogenously added, non-radioactive di-(*N*-formyl-D-penicillamine)disulphide (as revealed by ninhydrin after acid hydrolysis of a sample of the fractions obtained; Fig. 2*a*). (3) Treatment of the ^{3}H-Y product with 1 N HCl for 60 min at 60 °C produced (95% yield) a radioactive compound which on the Aminex column cochromatographed exactly with exogenously added, non-radioactive di(D-penicillamine)disulphide (as revealed directly with ninhydrin; Fig. 2*b*). (4) HCl Treatment of the ^{14}C-Y product in the same conditions as above yielded a non-radioactive compound which also behaved on the Aminex column as di(D-penicillamine)disulphide (and was directly revealed with ninhydrin). Since the ^{14}C-Y product was formed by action of the R61 enzyme on 5-^{14}C-benzylpenicillin and since treatment of the ^{14}C-Y product under conditions known to release the formyl group from di-(*N*-formyl-D-penicillamine)disulphide actually resulted in the release of the radioactive label, it seems clear that the formyl group of di-(*N*-formyl-D-penicillamine)-disulphide arises from C5 of the penicillin nucleus. (5) Thin-layer chromatography in solvent B (for composition, see Table 1) of the hydrolysed ^{3}H-Y and ^{14}C-Y products revealed the presence of a radioactive ninhydrin-positive spot and of a non-radioactive ninhydrin-positive spot, respectively, exhibiting the same *Rf* value (0.27) as di-(D-penicillamine)disulphide. (6) Finally, performic oxidation of the HCl-treated ^{3}H-Y product yielded a very acidic ^{3}H compound (90% yield) which, on the Aminex column, cochromatographed exactly with exogenously added penicillamine previously oxidised with performic acid.

In a second series of experiments, a half life of 90 min for the ^{3}H-benzylpenicillin–R61 complex (in 10 mM phosphate

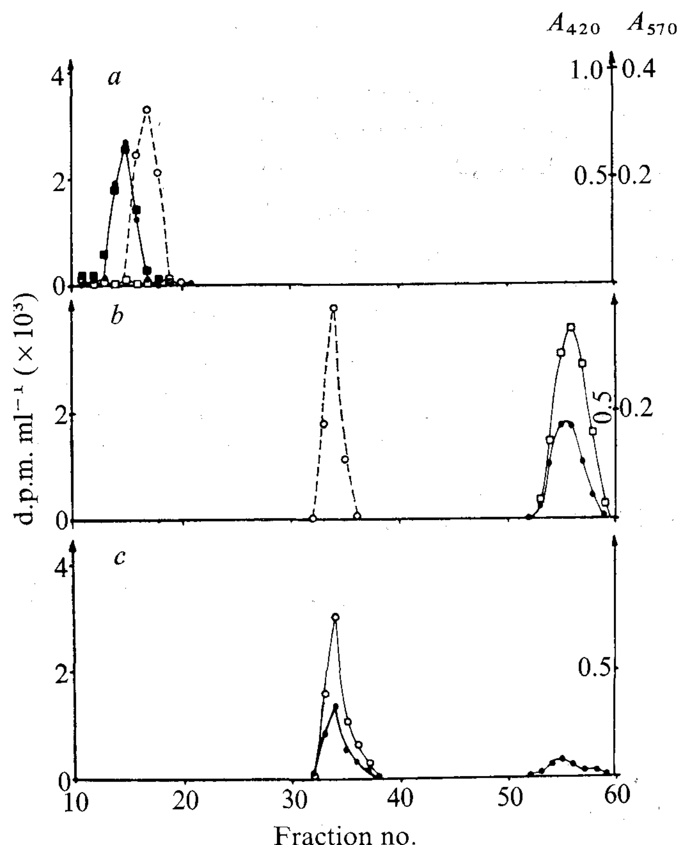

Fig. 2 Identification by chromatography on Aminex A5 of the Y products released from benzylpenicillin after interaction with the R61 enzyme under prolonged (*a* and *b*) and short (*c*) incubation times. The column (9×540 mm) was equilibrated against 0.2 M sodium citrate–HCl buffer (*p*H 3.17). Elution was performed with the same buffer at a flow rate of 80 ml h^{-1} (volume of the fractions: 3.2 ml). *a*, A mixture containing 10 nCi of ^{3}H-Y product and 200 μg of each *N*-formyl-D-penicillamine and di-(*N*-formyl-D-penicillamine)disulphide was deposited. Radioactivity (●) was measured on 1-ml samples. The *p*H of 0.4-ml samples was adjusted to 7.5–8.0 by addition of 40 μl of 2 M Tris base and the free sulphydryl groups (○) were estimated by adding 20 μl of 50 mM Tris-HCl buffer (*p*H 8.0) containing 40 μg of 5,5′-dithio-*bis*(2-nitrobenzoic acid) (A_{420}). The free amino groups were estimated by reaction with ninhydrin (A_{570}) before (□) and after (■) a 60 min incubation at 60 °C in 1 N HCl. *b*, A mixture containing 10 nCi of ^{3}H-Y product and 400 μg of di-(*N*-formyl-D-penicillamine)disulphide was incubated at 60 °C for 60 min in 1 N HCl. The *p*H was then adjusted to 2.2, 200 μg of D-penicillamine were added and the mixture was deposited on the column. Radioactivity (●) was measured on 1-ml samples. Free sulphydryl groups (○) and free amino groups (□) were estimated on 0.4-ml and 0.5-ml samples, respectively, as in *a*. *c*, Chromatography of the HCl-hydrolysed tritiated compound prepared by a 2-h incubation of ^{3}H-benzylpenicillin with the R61 enzyme (for conditions, see text). The radioactivity (●) was measured on 1-ml samples. Free sulphhldryl groups (○) were estimated as above. The A_{570} value after colour development of D-penicillamine with ninhydrin is about 7% of that obtained with equivalent amount of D-penicillamine disulphide.

buffer (*p*H 7.0) and at 37 °C) was determined on the basis of the release of the radioactive label. In previous studies[3], an average half life of 80 min had been determined for the complex formed with the R61 enzyme. Within the limits of experimental errors therefore, recovery of enzyme activity and release of the two fragments arising from the benzylpenicillin molecule occur with the same rate constant (1.4×10^{-4} s^{-1}). This result together with the facts that after complete enzyme recovery, more than 90% of the original radioactive labels were recovered as ^{14}C-phenylacetylglycine and either ^{3}H- or ^{14}C-di-(*N*-formyl-D-penicillamine)disulphide (depending on the radioactive benzylpenicillins used), show clearly that phenylacetylglycine and

di-(*N*-formyl-D-penicillamine) are produced in stoichiometric amounts.

Since the Y product characterised as di-(*N*-formyl-D-penicillamine)disulphide was isolated after several cycles of enzyme inactivation and reactivation (that is, after prolonged incubation), we wondered whether it was actually the one initially released through breakdown of the penicillin molecule. In a third series of experiments, two samples, each containing 5 nmol of ^{3}H-benzylpenicillin and 6 nmol of R61 enzyme, were incubated for 2 h at 37 °C under nitrogen. Reaction of one of the samples with 5,5′-dithio-*bis*(2-nitrobenzoic acid) indicated free SH groups. Non-radioactive *N*-formyl-D-penicillamine (125 μg) was added to the second sample and the solution was treated with 1.2 N HCl (final concentration) for 16 h at 60 °C, that is in conditions where release of the formyl group from *N*-formyl-D-penicillamine was known to occur[6]. Chromatography on Aminex A5 revealed that 80% of the radioactive product present in the hydrolysate was penicillamine (Fig. 2*c*). About 20% of penicillamine disulphide was detected. Thus *N*-formyl-D-penicillamine was probably the primary product released and di-(*N*-formyl-D-penicillamine)disulphide was a product subsequently formed by oxidation. Surprisingly, however, by incubating 50 nmol of *N*-formyl-D-penicillamine for 16 h at 37 °C, in 100 μl of 10 mM phosphate buffer (*p*H 7.0), either as such, or in the presence of 0.5 nmol of heat-inactivated R61 enzyme (5 min at 100 °C), or in the presence of the same amount of active R61 enzyme, it was observed that 80–90% of the SH groups originally present remained titratable by dithio-*bis*-nitrobenzoic acid after incubation in the absence of enzyme or in the presence of inactivated enzyme but that 80% of these groups had disappeared after incubation in the presence of native enzyme. Thus, it is not impossible that the enzyme preparation somehow participated in the oxidation of the *N*-formyl-D-penicillamine initially released.

Table 1 Thin-layer chromatography on silica gel (G) of radioactive Y product and of non-radioactive *N*-formyl-D-penicillamine and di-(*N*-formyl-D-penicillamine)disulphide

Compound	*Rf* values in solvents A	B
^{3}H- or ^{14}C-Y product	0	0.69
N-formyl-D-penicillamine	0.34	0.68
di-(*N*-formyl-D-penicillamine)disulphide	0	0.67

Solvent A, $CHCl_3/CH_3OH/CH_3COOH$: 88/10/2 (v/v/v).
Solvent B, 1-butanol/$H_2O/C_2H_5OH/CH_3COOH$: 10/4/3/3(v/v/v/v).
Both the peaks of radioactivity (detected by scanning the thin-layer plates in a radiochromatogram scanner) and the spots of di-(*N*-formyl-D-penicillamine)disulphide (as revealed by spraying the plates with an alcoholic solution of bromocresol green) were perfectly symmetrical.

Comparable results were obtained with 5-^{14}C-phenoxymethylpenicillin which was degraded into ^{14}C-*N*-formyl-D-penicillamine and non-radioactive phenoxyacetylglycine.

In summary, it seems that within the limits of experimental error, *N*α-(acyl)-glycine and *N*-formyl-D-penicillamine are released in stoichiometric amounts from the complex formed between penicillin and the exocellular R61 DD-carboxypeptidase-transpeptidase, and that release of these fragments and enzyme reactivation are concomitant events. If penicillin is benzylpenicillin, phenylacetylglycine is released. If penicillin is phenoxymethylpenicillin, phenoxyacetylglycine is released. Globally, the reaction consists in the addition of two H_2O molecules and results in the hydrolysis of the amide bond and in the rupture of both C5–C6 and C5–S linkages (Fig. 3). The nature of the acyl substituent on the antibiotic molecule is important; although it does not modify the pathway of the reaction, it obviously influences the half life of the complex formed between penicillin and the enzyme. The released *N*-formyl-D-penicillamine undergoes subsequent oxidation with formation of the disulphide derivative.

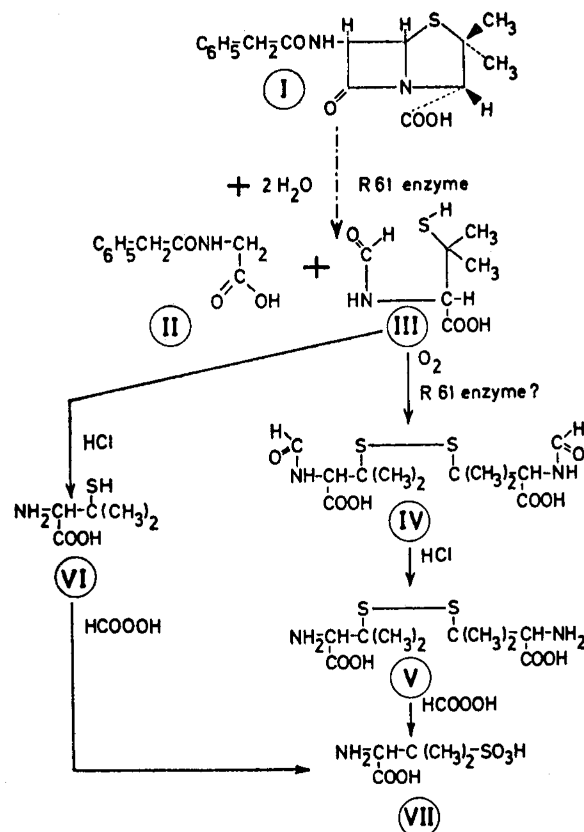

Fig. 3 Degradation of benzylpenicillin. I, benzylpenicillin; II, phenylacetylglycine; III, *N*-formyl-D-penicillamine; IV, di-(*N*-formyl-D-penicillamine)disulphide; V, D-penicillamine disulphide; VI, D-penicillamine; VII, dimethyl cysteic acid. Degradation of III into VI requires acid treatment (1 N HCl) during several hours at 60 °C. Degradation of IV into V can be achieved by the same acid treatment during 1 h.

Studies[7,8] with isolated membranes have shown that enzyme–penicillin complexes found in membranes do not always give rise to the products we have found with purified enzyme. Further work is required in order to unravel the exact mechanism of the reactions.

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