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SEPARATION OF DEGRADATION PRODUCTS OF DOUBLE-LABELED BENZYLPENICILLIN ON A CATION-EXCHANGE COLUMN

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SUMMARY

By using a cation-exchange resin and eluting with a lithium citrate buffer gradient, good separations were obtained for several degradation products of double-labeled benzylpenicillin.

INTRODUCTION

During ion-exchange chromatography of mycelial extracts and culture filtrates of *Penicillium chrysogenum*, grown in the presence of [^{35}S]sulphate, a number of unidentified labeled compounds were detected[1]. The fermentation was supplemented with phenylacetic acid to produce benzylpenicillin (penicillin G), and, before analysis, all samples were deproteinised with sulphosalicylic acid at pH 2; since benzylpenicillin is unstable in acid solution, some of the unknown compounds could be degradation products of the antibiotic. These degradation products are also of interest in other fields of research on penicillin, *e.g.*, in studies on their possible role in causing penicillin allergy[2], and in studies on the action of various microbial penicillin-degrading enzymes (β-lactamases[3], acylases[3] and DD-carboxypeptidase-transpeptidases[4,5]).

Thin-layer chromatography (TLC) has been used extensively in resolving benzylpenicillin from its decomposition products[6,7]; however, adequate separation of benzylpenicillin, benzylpenicilloic acid and benzylpenilloic acid is difficult to achieve. Blaha *et al.*[8] described the resolution of benzylpenicillin from five of its degradation products on an anion-exchange resin, with a citric acid–disodium phosphate buffer of pH 3.8 as mobile phase. Here, we report the separation of a number of decomposition products of benzylpenicillin on a cation-exchange column, with a lithium citrate buffer gradient as eluent, under conditions used for amino acid analysis. The use of benzylpenicillin labeled with carbon-14 in the side chain and with tritium in the nucleus permits the easy detection, identification and determination of several single-

or double-labeled derivatives of the antibiotic. Some derivatives of labeled phenoxymethylpenicillin (penicillin V) have also been tested.

EXPERIMENTAL

Materials

[^{14}C]Benzylpenicillin (labeled on the carbonyl group of the phenylacetyl side chain), [1-^{14}C] bromoacetic acid and L-[3,3-$^{3}H_2$]serine were products from The Radiochemical Centre (Amersham, Great Britain). [β-Methyl-^{3}H]benzylpenicillin was prepared by refluxing the penicillin G S-sulphoxide benzyl ester in benzene and tritiated water, followed by debenzylation and deoxygenation[9]. Degradation products of single- or double-labeled benzylpenicillin were obtained as described under Results and Discussion. [^{14}C]Phenylacetylglycine and [^{3}H]N-formyl-D-penicillamine were prepared by the action of *Streptomyces* R 61 on [^{14}C]benzylpenicillin[4] and [^{3}H]benzylpenicillin[5], respectively.

[1-^{14}C]Phenoxyacetic acid was synthesized[10] by refluxing an alkaline solution of phenol and [1-^{14}C]bromoacetic acid (yield 70%; specific activity 80 μCi/mmole); [^{14}C]phenoxymethylpenicillin was prepared[11] (with a yield of 90%) by condensing [1-^{14}C]phenoxyacetyl chloride with 6-aminopenicillanic acid. The phenylacetyl and phenoxyacetyl derivatives of L-[3,3-$^{3}H_2$]serine were obtained by Schotten–Baumann acylation in yields of 74 and 85%, respectively.

Methods

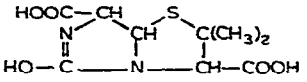
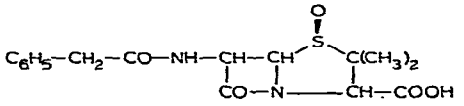
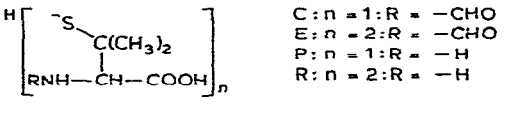
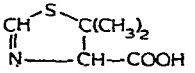
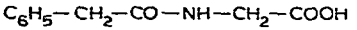
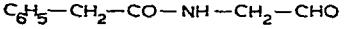
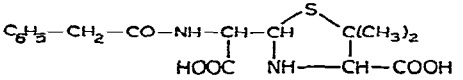
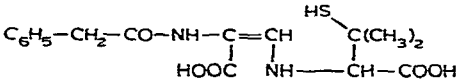
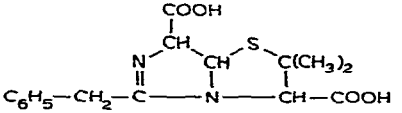
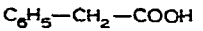
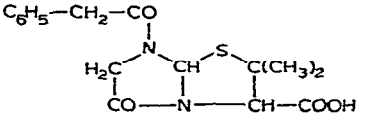
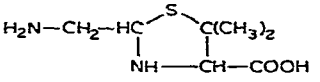
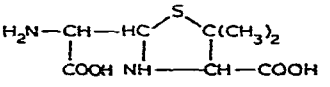
A Technicon amino acid analyzer was used, with a column (140 cm × 6 mm) of Chromobeads (type B; Li^+); the lithium buffer gradient and elution conditions were as described earlier[1,12]. All samples were buffered with 0.2 *M* lithium citrate of pH 2.2 before being applied to the column. Part of the effluent from the column was used for reaction with ninhydrin; the remainder was directed to a fraction collector, fractions of 1.6 or 2.1 ml being collected in Minivials at 3 or 4-min intervals. Counting was performed with 3 ml of Instagel (Packard Instrument Co., Downers Grove, Ill., U.S.A.) in a Packard Tri-Carb liquid scintillation spectrometer (model 3390) with an absolute activity analyzer (model 544). Standardisation was carried out with [^{3}H]- and [^{14}C]-*n*-hexadecane.

RESULTS AND DISCUSSION

The structures of the penicillin-degradation products studied are given in Table I; the elution pattern of these compounds is shown in Fig. 1.

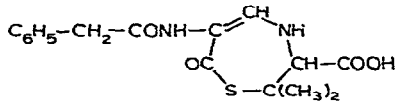
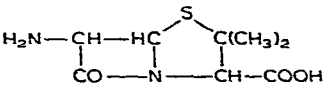
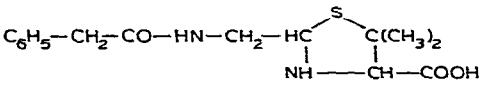
When intact [β-methyl-^{3}H] [^{14}C]benzylpenicillin was chromatographed on the cation-exchange column, two double-labeled peaks (H and J in Fig. 1) were obtained. The compounds responsible for these peaks were also present in culture filtrates of *Penicillium chrysogenum* containing [^{35}S]benzylpenicillin[1]. The compound giving peak H was the only product formed by alkaline degradation of the penicillin or by the action of penicillinase; it is therefore benzylpenicilloic acid. The compound giving peak J is the major product obtained after degrading the penicillin for two hours at 37° and pH 2.7; it is therefore benzylpenillic acid, the acid-catalyzed rearrangement product of benzylpenicillin. Under these conditions, two more peaks were formed;

TABLE I
STRUCTURES OF DEGRADATION PRODUCTS OF BENZYLPENICILLIN

Compound	*Peak (see Fig. 1)*	*Structure*
8-Hydroxypenillic acid	A	
Benzylpenicillin S-sulphoxide	B	
N-Formyl-D-penicillamine, reduced	C	
N-Formyl-D-penicillamine, oxidized	E	
D-Penicillamine, reduced	P	
D-Penicillamine, oxidized	R	
D-5,5-Dimethyl-2-thiazoline-4-carboxylic acid	D	
Phenylacetylglycine	F	
Benzylpenilloaldehyde	G	
Benzylpenicilloic acid	H	
Benzylpenamaldic acid	I	
Benzylpenillic acid	J	
Phenylacetic acid	K	
Benzylpenillonic acid	L	
2-Aminomethyl-5,5-dimethyl-thiazolidine-4-carboxylic acid	M	
Penicic acid	N	

(Continued on p. 96)

TABLE I (*continued*)

Compound	*Peak (see Fig. 1)*	*Structure*
Benzylthiazepine	O	
6-Aminopenicillanic acid	Q	
Benzylpenilloic acid	S	

besides benzylpenicilloic acid (peak H), a small double-labeled peak (I) was eluted after the former compound. This probably corresponds to benzylpenamaldic acid, as reported by Blaha *et al.*[8]. Since products H and I are closely related structurally, there is only a slight difference in their retention times; also, the tailing of peak I is typical of thiol compounds.

In contrast with benzylpenicillin, the sulphoxide derivative is more stable to acid; S-sulphoxide[β-methyl-^{3}H]benzylpenicillin is eluted as a single peak (B) with the first fractions of the column. The short retention time of this compound might be expected, as the sulphoxides of S-methylcysteine and methionine migrate much faster than the corresponding non-oxidised amino acids[12].

Degradation of [β-methyl-^{3}H] [^{14}C]benzylpenicillin with $HgCl_2$, as in the preparation of penicillamine, resulted in three single-labeled compounds (peaks G, P and R). Peaks P and R, which are ninhydrin-positive and tritium-labeled, have the same retention times as reduced and oxidized penicillamine, respectively[12]. Peak G contains carbon-14 and corresponds to the other half of the molecule, namely benzylpenilloaldehyde.

When [β-methyl-^{3}H] [^{14}C]benzylpenicilloic acid was heated for 2 h at 75° in the buffer solution of pH 2.2, the double-labeled peak H disappeared, and four compounds were obtained. Peak S is double-labeled and is much more retarded than benzylpenicilloic acid; since it is eluted before phenylalanine, its retention time conforms with that of a neutral amino acid with a weak basic group and an aromatic side chain. It can be identified as benzylpenilloic acid; this compound is indeed formed from benzylpenicilloic acid by thermal decarboxylation. Since the reaction mixture also contained single-labeled peaks G, P and R, part of the [β-methyl-^{3}H] [^{14}C]benzylpenilloic acid must have been further transformed into [^{14}C]benzylpenilloaldehyde and [^{3}H]penicillamine.

A number of minor degradation products of benzylpenicillin were also obtained after heating in acidic medium and were not identified; *e.g.*, one compound labeled with carbon-14 and two with tritium are eluted with the front. A double-labeled peak (O) is eluted before reduced penicillamine. Peak O was also formed when a solution (in dichloromethane) of the trimethylsilyl ester of labeled benzyl-

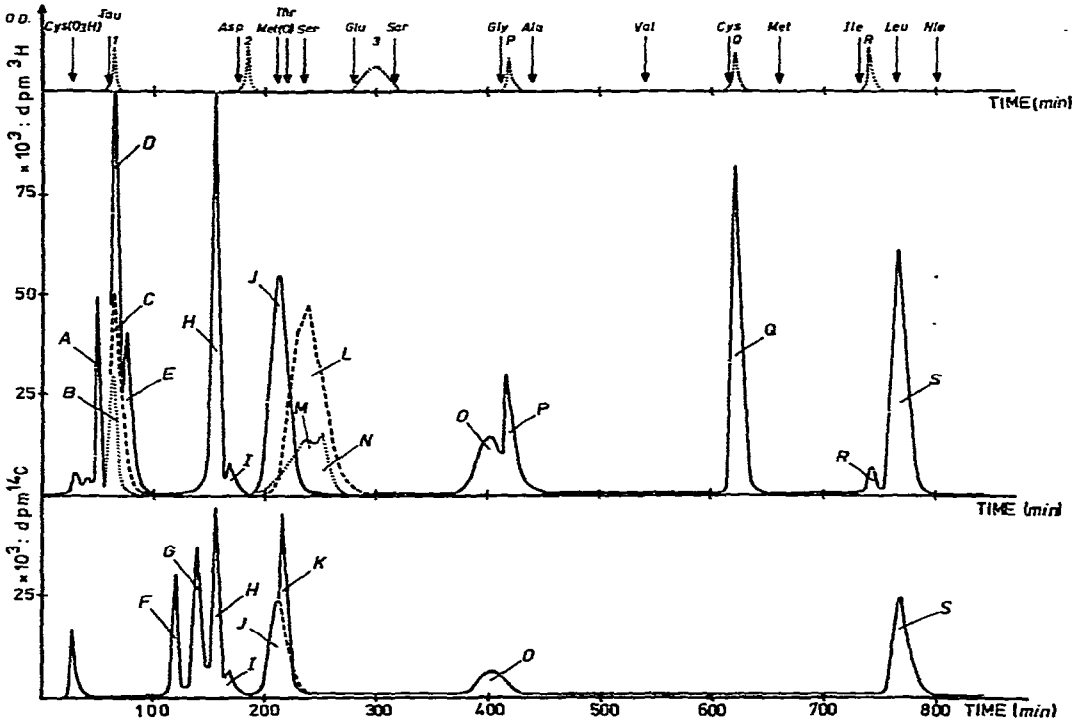


Fig. 1. Separation of degradation products of labeled benzylpenicillin on a cation-exchange column, with a lithium citrate buffer gradient as eluent. Peaks A–S refer to the compounds listed in Table I. Peaks: 1 = penicillaminic acid, 2 = S-carboxymethylpenicillamine, 3 = the mixed disulphide of D-penicillamine and N-formyl-D-penicillamine (these compounds were detected with ninhydrin). The elution positions of some common amino acids are indicated by arrows.

penicillin was stored for 2 days at room temperature in the presence of bis(trimethylsilyl)acetamide and triethylamine; this peak is probably due to benzylthiazepine[13].

When [β-methyl-^{3}H] [^{14}C]benzylpenicillin was treated with the acylase from *Escherichia coli* NCIB 8743, the molecule was split into [^{14}C]phenylacetic acid (peak K) and [^{3}H]6-aminopenicillanic acid (6-APA; peak Q), the latter being eluted after cystine. Degradation of [β-methyl-^{3}H]6-APA with penicillinase resulted in a broad double peak (M and N). This is probably caused by decarboxylation (during the run) of some of the penicic acid formed (peak N) to yield the slower moving D-2-aminomethyl-5,5-dimethylthiazolidine-4-carboxylic acid (peak M). When a solution of [β-methyl-^{3}H]6-APA in 2% sodium hydrogen carbonate solution was stored overnight, then acidified and extracted with ethyl acetate, 8-hydroxypenillic acid[14] (peak A) was obtained. The large difference in elution time between 8-hydroxy- and benzylpenillic acid (peaks A and J) clearly demonstrates the retarding effect of an aromatic group and the accelerating effect of a hydroxyl function.

The exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R 61 degrades double-labeled benzylpenicillin into [^{14}C]phenylacetylglycine (peak F, eluted before benzylpenilloaldehyde) and oxidized N-formyl-D-penicillamine[5] (peak E). When this compound was treated with dithiothreitol, the reduced N-formyl-D-

penicillamine (peak C) moves slightly ahead of the oxidized form. If desired, the elution position of the reduced N-formyl-D-penicillamine can be further altered by acid hydrolysis to form D-penicillamine or by atmospheric oxidation in the presence of D-penicillamine, which results in a mixture of oxidized penicillamine, oxidized N-formyl-D-penicillamine and the mixed disulphide of both products; this mixed disulphide is eluted between glutamic acid and sarcosine. Further, penicillamine itself can be transformed into penicillaminic acid by oxidation with performic acid, or into the S-carboxymethyl derivative by reaction with iodoacetic acid at pH 7; these compounds are eluted, respectively, after taurine and aspartic acid. If no tritium-labeled benzylpenicillin is available, penicillamine and its derivatives can also be detected with ninhydrin reagent.

Some degradations of [^{3}H]benzylpenicillin were also performed under anhydrous conditions. When [β-methyl-^{3}H]benzylpenicillin is refluxed in trifluoroacetic acid for 20 min, D-5,5-dimethyl-2-thiazoline-4-carboxylic acid (peak D) is formed[15,16]. In addition, Hammarström and Strominger recently reported the formation of this thiazoline from benzylpenicillin by the action of the D-alanine carboxypeptidase of *Bacillus stearothermophilus*[17]. The thiazoline has the same retention time as reduced N-formyl-D-penicillamine (peak C). However, since the thiazoline is very unstable in aqueous solutions at pH 2–3 and is rapidly transformed into N-formyl-D-penicillamine, the identical retention time for both compounds is probably caused by complete hydrolysis of the thiazoline during the run.

The methyl ester of [β-methyl-^{3}H]benzylpenillonic acid was prepared by refluxing the methyl ester of tritiated benzylpenicillin for 3 h in toluene containing a trace of iodine and was purified by preparative TLC. Mild alkaline hydrolysis gave [β-methyl-^{3}H]benzylpenillonic acid, which crystallized from diethyl ether[18]; this compound (peak L) is eluted after benzylpenillic acid and phenylacetic acid. The presence of some epimers of benzylpenillonic acid might explain the broad and irregular peak obtained.

The retention times of some derivatives of phenyl- and phenoxyacetic acid are listed in Table II. Although phenoxyacetic acid is eluted before phenylacetic acid, phenoxyacetylserine has a longer retention time than has phenylacetylserine. Also, the penicilloic and penilloic acids of phenoxymethylpenicillin are more retarded than the corresponding derivatives of benzylpenicillin.

TABLE II

RETENTION TIMES OF SOME DERIVATIVES OF PHENYL- AND PHENOXYACETIC ACID

Compound	*Retention time, min*
Phenylacetic acid	216
Phenoxyacetic acid	165
Phenylacetylserine	84
Phenoxyacetylserine	120
Benzylpenicilloic acid	156
Phenoxymethylpenicilloic acid	201
Benzylpenilloic acid	768
Phenoxymethylpenilloic acid	872

Since most of the degradation products of benzylpenicillin contain no basic functions, the resolution obtained on a cation-exchange resin is mainly caused by adsorption chromatography, *i.e.*, by interaction between the aromatic side chain and the resin matrix. Only 4 h are needed to separate peaks A to K; the elution times of these products can be influenced by changing the isopropyl alcohol content of the buffer (2% in the first chamber of the Autograd). Shorter retention times for the slower-moving compounds can be obtained by using a steeper gradient. Further studies are in progress to determine the role of other factors (*e.g.*, the concentration of sodium and the pH of the starting buffer) on the separation.

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REFERENCES

1 P. Adriaens, B. Meesschaert, W. Wuyts, H. Vanderhaeghe and H. Eyssen, *Antimicrob. Agents Chemother.*, 8 (1975) 638.
2 M. A. Schwartz, *J. Pharm. Sci.*, 58 (1969) 643.
3 E. P. Abraham, *Biosynthesis and Enzymic Hydrolysis of Penicillins and Cephalosporins*, University of Tokyo Press, Tokyo, 1974, p. 1.
4 J. M. Frère, J. M. Ghuysen, J. Degelaen, A. Loffet and H. R. Perkins, *Nature (London)*, 258 (1975) 168.
5 J. M. Frère, J. M. Ghuysen, H. Vanderhaeghe, P. Adriaens, J. Degelaen and J. De Graeve, *Nature (London)*, 260 (1976) 451.
6 E. J. Vandamme and J. P. Voets, *J. Chromatogr*, 71 (1972) 141.
7 P. E. Manni, R. A. Lipper, J. M. Blaha and S. L. Hem, *J. Chromatogr.*, 76 (1973) 512.
8 J. M. Blaha, A. M. Knevel and S. L. Hem, *J. Pharm. Sci.*, 64 (1975) 1384.
9 B. Meesschaert, P. Adriaens, H. Eyssen, E. Roets and H. Vanderhaeghe, *J. Antibiot.*, 29 (1976) 433.
10 C. M. Eaker, *Brit. Pat.* 682282 [*Chem. Abstr.*, 48 (1954) 2776].
11 G. Parker, *Brit. Pat.* 1047842 [*Chem. Abstr.*, 66 (1967) 28764].
12 P. Adriaens, B. Meesschaert, W. Wuyts, H. Vanderhaeghe and H. Eyssen, *J. Chromatogr.*, 140 (1977) 103.
13 A. Vlietinck, E. Roets, P. Claes, G. Janssen and H. Vanderhaeghe, *J. Chem. Soc., Perkin Trans. I*, 937 (1973).
14 D. A. Johnson and G. A. Hardcastle, Jr., *J. Amer. Chem. Soc.*, 83 (1961) 3534.
15 M. R. Bell, J. A. Carlson and R. Oesterlin, *J. Amer. Chem. Soc.*, 92 (1970) 2177.
16 M. R. Bell, J. A. Carlson and R. Oesterlin, *J. Org. Chem.*, 37 (1972) 2733.
17 S. Hammarström and J. L. Strominger, *J. Biol. Chem.*, 251 (1976) 7947.
18 R. L. Peck and K. Folkers, in H. T. Clarke, J. R. Johnson and R. Robinson (Editors), *The Chemistry of Penicillin*, Princeton Univ. Press, Princeton, N.J., 1949, p. 188.