Synthesis of Tritium-Labelled Isopenicillin N, Penicillin N and 6-Aminopenicillanic Acid

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1. Phenoxymethylpenicillin sulphoxide 4-methoxybenzyl ester was labelled with ^3H in its 2- β -methyl group. Its specific radioactivity was 362 mCi/mmol. 2. Removal of the side chain of this compound yielded the corresponding ester of 6-aminopenicillanic acid sulphoxide and coupling of the latter with the appropriate protected α -amino adipic acid gave 4-methoxybenzyloxycarbonylisopenicillin N sulphoxide di-4-methoxybenzyl ester or the corresponding derivative of penicillin N. 3. Removal of the protective groups by hydrogenolysis and reduction of the sulphoxide group yielded ^3H -labelled isopenicillin N or penicillin N. 4. ^3H -labelled phenoxymethylpenicillin sulphoxide was obtained by hydrogenolysis from its 4-methoxybenzyl ester. Reduction of its sulphoxide group and subsequent removal of the side chain gave ^3H -labelled 6-aminopenicillanic acid.

Penicillin N [compound I; $\text{RCO} = \delta$ -(D- α -aminoadipyl)], isopenicillin N [compound I; $\text{RCO} = \delta$ -(L- α -aminoadipyl)] and 6-aminopenicillanic acid (compound II) have all been considered to be possible precursors of benzylpenicillin (compound I; $\text{R} = \text{C}_6\text{H}_5\text{CH}_2$) and of other penicillins with non-polar side chains produced by *Penicillium chrysogenum*. Isotopically labelled penicillin N, isopenicillin N and 6-aminopenicillanic acid were required for studies of their behaviour in a cell-free system from *P. chrysogenum*. The present paper describes syntheses of these compounds labelled with ^3H in the β -methyl group of the thiazolidine ring.

The synthesis of δ -(D- α -aminoadipyl)glycine (a degradation product of penicillin N) by the azide coupling procedure was described by Abraham &

Newton (1954). The use of this procedure for the synthesis of a mixture of penicillin N and isopenicillin N from benzyloxycarbonyl-DL- α -amino adipic acid δ -azide and 6-aminopenicillanic acid was reported briefly by Doyle *et al.* (1962). More recently, Vanderhaeghe *et al.* (1974) described the synthesis of penicillin N and isopenicillin N by two methods. One involved the coupling of the α -benzyl ester of *N*-benzyloxycarbonyl- α -amino adipic acid with 6-aminopenicillanic acid by a mixed-anhydride procedure and the other the coupling of the benzyl ester of 2-azido adipic acid via its acid chloride.

In the first method studied during the present work for the synthesis of isopenicillin N, *N*-benzyloxycarbonyl-L- α -aminoadipyl δ -azide was coupled with the triethylamine salt of 6-aminopenicillanic acid in a mixture of chloroform and dichloromethane. A product was obtained (in 18% yield) which showed activity against *Staphylococcus aureus* and migrated to the same position as authentic benzyloxycarbonylpenicillin N on paper electrophoresis at pH 4.5. Hydrogenolysis of the *N*-benzyloxycarbonyl derivative of isopenicillin N gave isopenicillin N mixed with unchanged starting material. The potassium salt of isopenicillin N was isolated by chromatography in 70% (v/v) propan-2-ol on a column (25 cm $\times$ 1 cm) of Sephadex G-25 much as described by Smith *et al.* (1967). The product was 70% pure by hydroxamic acid assay (Boxer & Everett, 1949). It showed about 25% of the activity of penicillin N against *Staph. aureus* (Oxford strain) but only about 3% of the activity of the latter against a strain of *Salmonella typhi*. In its relative activity against these organisms and its R_F value on paper chromatography

in butan-1-ol-acetic acid-water (4:1:5, by vol.) it appeared to be identical with a sample of synthetic isopenicillin N (approximately 75% pure by hydroxamic acid assay) which was provided by Professor Vanderhaeghe (Rega Institute, Leuven, Belgium).

This route to isopenicillin N was investigated because of its relative simplicity and the ready availability of the starting materials. However, the

overall yield of product was low (4.6% based on 6-aminopenicillanic acid). For the synthesis of isopenicillin N and penicillin N labelled with ^3H in the ring system it was desirable to explore a different procedure and one which would also yield ^3H -labelled 6-aminopenicillanic acid. The sequence of reactions used is summarized in Scheme 1.

The method used for the introduction of ^3H into

Scheme 1. Stages in the synthesis of isopenicillin N, penicillin N and 6-aminopenicillanic acid ^3H -labelled (as indicated by the asterisk) in the 2- β -methyl group

the penicillin ring system was based on Cooper's (1970) finding that when phenoxymethylpenicillin *S*-sulphoxide methyl ester was refluxed in benzene containing an excess of $^2\text{H}_2\text{O}$, an average of one ^2H atom was incorporated specifically into its β -methyl group. The sulphoxide was assumed to be in thermal equilibrium with the corresponding sulphenic acid. In the present case the 4-methoxybenzyl ester of the penicillin sulphoxide was used, because this group is both acid-labile and removable by hydrogenolysis.

Phenoxymethylpenicillin 4-methoxybenzyl ester was prepared by reaction of the penicillin potassium salt with 4-methoxybenzyl chloride in dimethylformamide, and was oxidized to the sulphoxide with 3-chloroperbenzoic acid at 0°C in chloroform (cf. Kaiser *et al.*, 1970). The product (Scheme 1, compound III; $\text{R} = 4\text{-methoxybenzyl}$) was isolated in 76% overall yield after recrystallization from chloroform and light petroleum (b.p. $40\text{--}60^\circ\text{C}$). Its i.r. and p.m.r. (proton-magnetic-resonance) spectra were identical with those reported for a sample of the same compound which was synthesized by Chauvette *et al.* (1971) during the course of the present work.

On the basis of the results of several exploratory experiments ^3H was incorporated into 20 mmol of phenoxymethylpenicillin sulphoxide ester at The Radiochemical Centre, Amersham, Bucks., U.K., the reaction being carried out for 24 h at 100°C in dioxan containing 50 Ci (27.8 mmol) of $^3\text{H}_2\text{O}$. After the removal of labile ^3H in the amide side chain, the ^3H -labelled product (compound IV, $\text{R} = 4\text{-methoxybenzyl}$) was recovered in 95% yield on precipitation from dimethylformamide by the addition of water. Its specific radioactivity was 362 mCi/mmol. Degradation of a sample of the product by the procedure of Dougall *et al.* (1957) yielded DL- β -hydroxyvaline with a specific radioactivity of 369 mCi/mmol. Hence all the ^3H in compound (IV) was present in the methyl groups of the C_5 fragment of the thiazolidine ring. The specific radioactivity was 33% of that to be expected if there had been complete equilibration between the ^3H of the $^3\text{H}_2\text{O}$ water and the amide group of the side chain and all three H atoms of the penicillin β -methyl group. This ^3H -labelled compound was used for the preparation of labelled isopenicillin N, penicillin N and 6-aminopenicillanic acid. It was envisaged that the sulphoxide would function as a protective group for the relatively labile sulphur of the thiazolidine ring at some stages in these syntheses.

Removal of the phenoxacetyl side chain from compound (IV) was carried out by treatment with PCl_5 and then with butan-1-ol under conditions similar to those used with phenoxymethylpenicillin dimethylsilyl ester by Weissenburger & van der Hoeven (1970). The 4-methoxybenzyl ester of 6-aminopenicillanic acid sulphoxide (compound V) was isolated as its crystalline 4-toluenesulphonate in 26% yield. Its specific radioactivity was 373 mCi/mmol.

For the synthesis of the δ -(L- α -aminoadipyl) derivative of compound (V), the amino and α -carboxyl groups of L- α -aminoadipic acid were protected by the 4-methoxybenzyloxycarbonyl and 4-methoxybenzyl groups respectively. N-4-Methoxybenzyloxycarbonyl-L- α -aminoadipic acid was prepared in 52% yield by reaction of 4-methoxybenzyloxycarbonyl azide with the amino acid, the procedure being a modification of that described by Weygand & Hunger (1962). Selective esterification of its α -carboxyl group was carried out by reaction with 4-methoxybenzyl chloride in the presence of one equivalent of triethylamine, the method being based on that of Nefkens & Nivard (1964) for selective esterification of N-protected glutamic acid. The crystalline dicyclohexylamine salt of the pure α -ester was isolated in 36% yield, based on the N-protected amino acid. The assignment of the α -ester structure was verified by its conversion into δ -(L- α -aminoadipyl)-glycine (Abraham & Newton, 1954) after coupling with glycine ethyl ester. The 4-methoxybenzyloxycarbonyl derivative of D- α -aminoadipic acid α -ester was prepared similarly.

Free 4-methoxybenzyloxycarbonyl-L- α -aminoadipic acid α -4-methoxybenzyl ester was liberated from its dicyclohexylamine salt and obtained as a clear gum, which slowly crystallized by evaporation of its solution in ether. The preparation of L-alanyl- α -aminoadipic acid from this compound and its analysis by the method of Manning & Moore (1968) indicated that the degree of racemization of the α -aminoadipic acid residue was less than 0.6%.

Coupling of the protected L- α -aminoadipic acid (free acid) with the ^3H -labelled ester of 6-aminopenicillanic acid sulphoxide (compound V) was carried out on a 0.1 mmol scale by the dicyclohexylcarbodi-imide-hydroxybenzotriazole procedure (Konig & Geiger, 1970). Unlike the coupling of the protected α -aminoadipic acid with glycine ethyl ester, which was complete within 10 min under similar conditions, the coupling with compound (V) required more than 2 h at room temperature (20°C) and was brought to completion by stirring the mixture overnight. This slow coupling of compound (V) presumably reflects the relatively weak nucleophilic character of its amino group.

The fully protected isopenicillin N sulphoxide (compound VI; $\text{R} = 4\text{-methoxybenzyl}$) was purified by chromatography in methyl acetate on a column of silica gel. Fractions of the eluate were analysed by t.l.c. in methyl acetate, the product, with an R_F of 0.6, being located with ninhydrin-trifluoroacetic acid and with Cl_2 /starch-KI. Appropriate fractions were pooled to give a product, in 84% yield, with a specific radioactivity of 341 mCi/mmol. This product showed satisfactory p.m.r. and i.r. spectra, but elementary analysis suggested that it was contamin-

ated with about 9% of silica gel. A subsequent preparation, which was chromatographed twice on silica gel, showed a specific radioactivity of 359 mCi/mmol.

The three protective groups were removed from compound (VI) (0.05 mmol; 18 mCi) by hydrogenolysis in the presence of three times its weight of 10% Pd-charcoal catalyst. T.l.c. in butan-1-ol-acetic acid-water (4:1:4, by vol., upper phase) showed that the fully protected sulfoxide rapidly disappeared and was replaced by several compounds with lower R_F values which were presumably partially deprotected derivatives. Spots due to these compounds then declined in intensity. After 18 h the major component of the final mixture was ninhydrin-positive, showed a lower R_F value than those formed earlier, and had a mobility similar to that of penicillin N when subjected to electrophoresis on paper at pH 4.5 and 6.5. This compound was isolated as an amorphous triethylamine salt (5 mg; yield 21% by weight and 26% by radioactivity), with properties expected of isopenicillin N sulfoxide (compound VII), after chromatography on Dowex 1X8 in 0.5M-acetic acid. When chromatographed on paper in butan-1-ol-acetic acid-water (4:1:5, by vol.) at 4°C it gave a single spot with an R_F value (0.12) lower than that of penicillin N (R_F 0.35). Unlike penicillin N, it gave no zone of inhibition against *Staph. aureus* by bioautography after paper electrophoresis in 5 μ g quantities. The sulfoxide of cephalosporin C was previously found to show a lower R_F value than cephalosporin C itself in butan-1-ol-acetic acid-water and to have only about 10% of the activity of the latter against *Staph. aureus* (Abraham & Newton, 1961).

The labelled isopenicillin N sulfoxide triethylamine salt (5 mg) was reduced to isopenicillin N by PBr_3 in dimethylformamide under conditions similar to those used for the reduction of a number of cephalosporin sulfoxides (Kaiser *et al.*, 1970; Wright *et al.*, 1971). The product was separated from sodium bromide and dimethylformamide by filtration through a column of Bio-Gel P2 and purified by chromatography in 70% (v/v) propan-2-ol on Sephadex G-25 under conditions similar to those used by Smith *et al.* (1967). The relative amounts of radioactive isopenicillin N in small samples of the eluate fractions were determined by paper chromatography in 70% (v/v) propan-2-ol and by electrophoresis at pH 4.5 and 6.5 of mixtures of the samples with unlabelled penicillin N. The penicillin N was located by a ninhydrin dip and the radioactivity in the same position was assumed to include all that due to isopenicillin N, since the two compounds were indistinguishable under these conditions (Flynn *et al.*, 1962; Cole & Batchelor, 1963). Plots of the resulting radioactivities against fraction numbers are shown in Fig. 1. They indicated that early fractions from the

column which contained isopenicillin N also contained another radioactive compound. Fractions 25-40 were pooled, the propan-2-ol was extracted with ether, and the aqueous phase freeze-dried to yield isopenicillin N (sodium salt) (compound VIII) containing a total radioactivity of 0.78 mCi. The yield, based on the sulfoxide, was 17%.

This product contained a major radioactive component which was indistinguishable from penicillin N in its R_F value on paper chromatography in 70% (v/v) propan-2-ol and in its mobility on electrophoresis at pH 4.5 and at 6.5 (Fig. 2). On paper chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.) at room temperature it underwent partial decomposition to give products of lower R_F values, and the appearance of ninhydrin-positive

Fig. 1. Analysis of radioactive isopenicillin N-containing fractions obtained after chromatography on Sephadex G-25 in 70% (v/v) propan-2-ol

The curves show the radioactivity found in the position occupied by penicillin N after a sample (5 μ l) of each fraction was subjected to (a) paper chromatography in 70% (v/v) propan-2-ol, (b) electrophoresis on paper at pH 4.5, and (c) electrophoresis on paper at pH 6.5 (for details, see the Experimental section).

Fig. 2. Profiles of radioactivity given by ^3H -labelled isopenicillin N (5 μg samples) on (a) electrophoresis on paper at pH 4.5, (b) electrophoresis on paper at pH 6.5 and (c) paper chromatography in 70% (v/v) propan-2-ol

In each system the peak of radioactivity coincided in position with that of unlabelled penicillin N detected with ninhydrin. Electrophoresis was at 70 V/cm for approx. 30 min. The arrows show the points of application of the samples.

spots on the paper was virtually identical with that observed with a parallel sample of unlabelled penicillin N. From the radioactive profile obtained after electrophoresis at pH 6.5 it was calculated that 63% of the total radioactivity of the preparation was due to isopenicillin N. Isopenicillin N penicilloate (with a mobility twice that of the penicillin) and other acidic compounds appeared to account for 33%, and material with no net change at pH 6.5 for 4%.

Bioautography after electrophoresis on paper at pH 4.5, with *Staph. aureus* (Oxford strain) as the test organism, showed that 20 μg of the preparation of isopenicillin N was required to produce a zone of inhibition similar in size to that produced by 5 μg of penicillin N. This was consistent with the finding that

unlabelled isopenicillin N, synthesized earlier by the azide-coupling method, showed an activity against *Staph. aureus* which was 25% of that of penicillin N.

A synthesis of penicillin N labelled with ^3H in its 2- β -methyl group was carried out by the procedure used for the synthesis of isopenicillin N, except that the labelled ester of 6-aminopenicillanic acid sulfoxide was coupled with protected D- α -amino-adipic acid instead of with the protected L isomer. The yields at all stages were similar, as was the purity of the final product as judged from its chromatographic and electrophoretic behaviour. However, in contrast with labelled isopenicillin N, the labelled penicillin N produced a zone of inhibition, on bioautography with *Staph. aureus* as the test organism, which was similar to that obtained from a sample of authentic non-labelled penicillin N producing a ninhydrin spot of similar intensity.

For the synthesis of 6-aminopenicillanic acid labelled with ^3H in its β -methyl group, phenoxymethylpenicillin sulfoxide 4-methoxybenzyl ester (3 mmol, 362 mCi/mmol, compound IV) was first converted into the corresponding acid (compound IX) by hydrogenolysis over Pd-charcoal in a solvent system similar to that used by Stelakatos & Argyropoulos (1970) in the removal of 4-methoxybenzyl ester groups. Phenoxymethylpenicillin sulfoxide, contaminated with two minor impurities, was obtained as a white crystalline solid (yield 61%). On t.l.c. it was indistinguishable from an authentic sample of the unlabelled compound prepared by the method of Morin *et al.* (1969).

The phenoxymethylpenicillin sulfoxide was reduced to phenoxymethylpenicillin (compound X) on a 1.64 mmol scale by treatment with PBr_3 in dimethylformamide. The product was converted into the potassium salt which was recrystallized from aq. acetone and obtained in about 22% yield. Removal of the side chain from 0.4 mmol of the penicillin by treatment with PCl_5 was carried out as described by Weissenburger & van der Hoeven (1970). ^3H -labelled 6-aminopenicillanic acid (compound XI) was obtained in 53% yield and with a radioactivity of 371 mCi/mmol. It was indistinguishable from authentic unlabelled 6-aminopenicillanic acid by m.p. and mixed m.p. and on t.l.c.

Experimental

Materials

Phenoxymethylpenicillin (potassium salt) was from the Lilly Research Centre Ltd., Windlesham, Surrey, U.K. 6-Aminopenicillanic acid was from the Beecham Research Laboratories, Brockham Park, Surrey, U.K. Penicillin N was a laboratory sample and was purified by the method of Smith *et al.* (1967) to give a product with an activity of 99 u/mg. 4-Methoxybenzyl chloride was from Eastman-Kodak

Ltd., Rochester, N.Y., U.S.A., and 4-methoxybenzyl-oxy-carbonyl azide from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Pd-charcoal catalyst (10%) was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. 1-hydroxybenzotriazole was provided by Dr. G. T. Young (Dyson Perrins Laboratory, Oxford, U.K.). DL- α -Amino adipic acid was from Glaxo Laboratories Ltd., Slough, Bucks., U.K. L- and D- α -amino adipic acids were prepared from the DL compound by the method of Greenstein *et al.* (1953) as modified by Wood & Hartline (1971). L- α -Amino adipic acid was also prepared from L-glutamic acid by the method of Rudinger & Farkasova (1963). Other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of AnalaR grade where available.

Dimethylformamide was distilled *in vacuo* at 40°C and dried over molecular sieves. Dioxan, chloroform and dichloromethane were purified by passage through a column of activated alumina (Woelm, basic, grade 1) (Wohlleben, 1955, 1956). Light petroleum was of b.p. 40–60°C.

T.l.c.

T.l.c. was carried out on silica gel G from E Merck, Darmstadt, Germany. Compounds were detected by the Cl_2 /starch-KI method of Rydon & Smith (1952), or by a ninhydrin spray, or both. Compounds which were N-protected by the 4-methoxybenzyl-oxy-carbonyl group were sprayed lightly with trifluoroacetic acid after ninhydrin, before heating at 100°C.

Paper chromatography and electrophoresis

Chromatograms were run overnight on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol., upper phase) or in propan-2-ol-water (7:3, v/v) at 4°C. Electrophoresis was carried out in an apparatus similar to that of Katz *et al.* (1959). Compounds were usually detected by use of the cadmium-ninhydrin reagent of Heilmann *et al.* (1957), but penicillins were sometimes detected by bioautography with *Staphylococcus aureus* as the test organism (Hale *et al.*, 1961) and by spraying with 0.5M-NaOH followed by starch-iodine (Thomas, 1961).

Detection and counting of ^3H -labelled compounds

Counting was done in a Nuclear-Chicago Unilux 11A scintillation counter. For determinations of specific radioactivity, counts of more than 10000 were recorded. The efficiency of the counter was calculated by use of an *n*-[1,2- ^3H]hexadecane standard. For the detection and estimation of labelled compounds on paper after chromatography or electrophoresis, a strip of paper (4 cm wide) along the direction of travel of the compound was cut into 1 cm segments. The pieces of paper thus obtained were mixed with water-

miscible scintillation fluid (3 ml) and counted for 1 min.

Antibacterial activities

These were determined by the hole-plate method (Smith *et al.*, 1967).

N-Benzyloxycarbonylisopenicillin N

6-Aminopenicillanic acid (650 mg) was suspended in dichloromethane (6 ml), triethylamine (0.9 ml) was added and the mixture stirred for 30 min by which time most of the 6-aminopenicillanic acid was in solution. *N*-Benzyloxycarbonyl-L- α -amino adipic acid δ -hydrazide (930 mg, prepared as described by Abraham & Newton, 1954) was dissolved in water (22.5 ml) containing 11 M-HCl (1.2 ml). Chloroform (21 ml) was added and the mixture stirred at 0°C. A solution of NaNO_2 (330 mg) in water (2.7 ml) was added dropwise during 5 min and stirring continued for a further 5 min at 0°C. The chloroform solution of the azide was separated, washed twice with ice-cold water and dried briefly over anhydrous Na_2SO_4 . It was then added slowly to the cooled solution of 6-aminopenicillanic acid. The mixture was kept at 0°C for 30 min and then for 18 h at room temperature. Ice-cold water (30 ml) was then added, followed by 1 M-HCl (0.6 ml) to bring the pH to 6.6. The chloroform-dichloromethane layer was removed and washed once with ice-cold water (9 ml). The aqueous layers were combined, ethyl acetate (20 ml) was added and then 2 M-HCl dropwise with shaking until the pH fell to 2.0. The aqueous layer was extracted again with ethyl acetate (2 $\times$ 20 ml) at 0°C, and the extracts were combined and washed once with cold water (5 ml). The product was extracted from the ethyl acetate into water (15 ml) by addition of 1 M-KOH to adjust the pH to 6.4. The aqueous layer was freeze-dried, after removal of the ethyl acetate *in vacuo*, to give a white solid (290 mg). After electrophoresis on paper at pH 4.5, the main constituent of this product, as detected by the methods of Thomas (1961) and Rydon & Smith (1952), migrated to the same position as aspartic acid and showed activity on bioautography with *Staph. aureus* as the test organism. It was contaminated with a small amount of material which migrated to the same position as *N*-benzyloxycarbonyl- α -amino adipic acid.

Isopenicillin N

The preparation of *N*-benzyloxycarbonylisopenicillin N (potassium salt; 100 mg) was dissolved in water (2 ml) and 1 M-acetic acid added to bring the pH to 5.0. The solution was added to a suspension of Pd-charcoal catalyst (300 mg) in water (2 ml) previously saturated with H_2 . Hydrogenation was carried out for 10 min by bubbling a stream of H_2 through the mixture. The catalyst was removed by filtration and the filtrate freeze-dried. The product

(42mg) was analysed by paper electrophoresis at pH4.5, followed by bioautography on *Staph. aureus* and by paper chromatography in butan-1-ol-acetic acid-water (4:1:5, by vol.) at 4°C followed by detection with ninhydrin. Two biologically active compounds were detected. One (isopenicillin N) migrated to the same place as cephalosporin C at pH4.5. The other, which appeared to be unchanged starting material, migrated to the same place as aspartic acid. The two compounds were present in approximately equal amounts.

The crude isopenicillin N (42mg) was dissolved in 70% (v/v) propan-2-ol (1ml) and applied to a column (25 cm $\times$ 1 cm diam.) of Sephadex G-25 (fine) in 70% propan-2-ol. Elution was carried out at 4°C with 70% propan-2-ol, and fractions (2.5ml) were collected every 20min. Samples of the fractions (100 μ l) were analysed by the method of Thomas (1961), which determines their penicillin and penicilloate content by measurements of iodine uptake before and after hydrolysis with 1M-NaOH. Samples (4 $\times$ 5 μ l) of fractions were also spotted on to paper and sprayed with ninhydrin. Detection with ninhydrin and by the penicilloate assay indicated that isopenicillin N was present in fractions 50–75. These fractions were pooled and mixed thoroughly with ether (4 vol.). The aqueous layer was separated and the ether washed with water (5ml). The washings were added to the first aqueous phase, ether was removed in a rotary evaporator and the solution freeze-dried. The product (19mg) was a white powder, 70% pure by hydroxamic acid assay.

Introduction of ^3H into the 2- β -methyl group of phenoxymethylpenicillin sulphoxide 4-methoxybenzyl ester

Phenoxymethylpenicillin sulphoxide 4-methoxybenzyl ester (9.72g, 20mmol), dioxan (100ml) and $^3\text{H}_2\text{O}$ (0.5ml, 27.8mmol, 50Ci) were kept at 100°C for 24h in a flask fitted with a reflux condenser. The solution was then evaporated at room temperature to a colourless gum and labile ^3H removed by solution of the gum in a dimethylformamide-water (3:1, v/v) mixture and repeated evaporation *in vacuo* in a rotary evaporator. The product was obtained as an off-white solid (9.2g) by precipitation from dimethylformamide (100ml) with water (500ml). On t.l.c. in butan-1-ol-acetic acid-water (4:1:4, by vol., upper phase), or in ethyl acetate-benzene (3:2, v/v), it behaved as a single substance contaminated with only very small amounts of impurities. Its R_F value in ethyl acetate-benzene (0.33) was that of an authentic sample of the unlabelled compound. Its specific radioactivity was 362mCi/mmol.

4-Methoxybenzyloxycarbonyl-L- α -aminoadipic acid

L- α -Aminoadipic acid (0.322g, 2mmol) was dissolved in a mixture of 0.5M- Na_2CO_3 (3ml) and

0.5M- NaHCO_3 (7ml) and the pH of the solution adjusted to 9.5 with 2M-NaOH. 4-Methoxybenzyloxycarbonyl azide (0.515g, 2.5mmol) in dioxan (10ml) was added and the mixture stirred at room temperature for 4h, with the occasional addition of 2M-NaOH to maintain the pH at 9.5. The clear solution was extracted with ether (10ml). The pH of the aqueous phase was adjusted to 2.0 with 1M- H_2SO_4 and the solution stirred with ethyl acetate (10ml). After separation of the phases the aqueous layer was washed with ethyl acetate (5ml) and the combined extracts were dried and evaporated. The residual oil was triturated with light petroleum (5 $\times$ 10ml) and gave 0.55g (85%) of a white solid. This product was recrystallized from a mixture of ethyl acetate (6ml) and light petroleum (4.5ml) at -15°C to give 0.337g of 4-methoxybenzyloxycarbonyl-L- α -aminoadipic acid (m.p. 111–112.5°C) which appeared homogeneous on t.l.c. in butan-1-ol-acetic acid-water (4:1:4, by vol., top phase) and chloroform-methanol-acetic acid (90:5:5, by vol.) (Found: C, 55.5; H, 5.9; N, 4.4; $\text{C}_{15}\text{H}_{19}\text{NO}_7$ requires C, 55.4; H, 5.8; N, 4.3%).

Dicyclohexylamine salt of 4-methoxybenzyloxycarbonyl-L- α -aminoadipic acid α -4-methoxybenzyl ester

4-Methoxybenzyloxycarbonyl-L- α -aminoadipic acid (0.325g, 1mmol) was dissolved in dimethylformamide (0.5ml) containing 0.14ml (1mmol) of triethylamine. To the clear solution was added 4-methoxybenzyl chloride (0.17g, 1.1mmol) in dimethylformamide (0.5ml) and the mixture left for 24h at 37°C. Water (5ml) was added and the product extracted into ether (3 $\times$ 5ml). The ether was washed with McIlvaine's (1921) buffer (5 $\times$ 5ml), pH5.0, and then with water (5ml). The ethereal solution was dried and evaporated to a colourless gum which slowly crystallized to a white solid on standing at room temperature. T.l.c. in chloroform-methanol-acetic acid (90:5:5, by vol.) indicated that it contained the required product (R_F 0.66) mixed with a little δ -4-methoxybenzyl ester (R_F 0.63) and a little $\alpha\delta$ -di-4-methoxybenzyl ester (R_F 0.88). This material (0.33g, 0.8mmol) was dissolved in ether (10ml), and dicyclohexylamine (0.8mmol) in ether (4ml) was added. The resulting solution was stored at -15°C, when the dicyclohexylamine salt separated as a gum which crystallized on shaking. It was washed with ether and dried to give a product (0.273g, yield 44%) of m.p. 101–107°C. T.l.c. indicated that it was contaminated with only a trace of the $\alpha\delta$ -di-4-methoxybenzyl ester. Recrystallization of a sample (50mg) of the dicyclohexylamine salt of 4-methoxybenzyloxycarbonyl-L- α -aminoadipic acid α -4-methoxybenzyl ester from chloroform (0.2ml) and ether (5ml) at -15°C gave 41mg of dicyclohexylamine salt, m.p. 108–113°C (Found: C, 67.3; H, 8.3; N, 4.5; $\text{C}_{23}\text{H}_{27}\text{NO}_8$, $\text{C}_{12}\text{H}_{23}\text{N}$ requires C, 67.1; H, 8.0;

N, 4.5%). The corresponding derivative of D- α -aminoadipic acid (m.p. 109–112°C) was synthesized without crystallization of 4-methoxybenzyloxycarbonyl-D- α -aminoadipic acid and was obtained in 40% overall yield.

For liberation of the free acid from the dicyclohexylamine salt, a sample of the latter (0.157 g, 0.25 mmol) was shaken with ether (25 ml) and cold McIlvaine's buffer (pH 3.0, 5 ml) until it had dissolved. The ether layer was washed with buffer (5 ml) and water (5 ml) and dried. On evaporation it gave 0.113 g of the free acid as a colourless gum.

Proof that this compound was an α -ester was obtained by coupling it with glycine ethyl ester by use of dicyclohexylcarbodi-imide under standard conditions (Bodanszky & Ondetti, 1966; König & Geiger, 1970). The coupling was carried out on a 0.1 mm scale in chloroform (4 ml). The ester groups were removed from the product by treatment with a mixture of pyridine (2 ml) and 2M-NaOH (2 ml) for 30 min at room temperature. The resulting acid was extracted into ethyl acetate at pH 2.5 and its *N*-4-methoxybenzyloxycarbonyl group removed by keeping it for 30 min in trifluoroacetic acid (1 ml) containing a few drops of anisole. The unprotected dipeptide was precipitated by ether. Its behaviour on paper electrophoresis at pH 4.5 was indistinguishable from that of an authentic sample of δ -(D- α -amino-adipyl)glycine prepared by Abraham & Newton (1954).

Degree of racemization

A sample (1 μ mol) of the *N*-4-methoxybenzyloxycarbonyl-L- α -aminoadipic acid 4-methoxybenzyl α -ester dicyclohexylamine salt was hydrolysed for 24 h in 6M-HCl at 105°C. The product was dissolved in 0.5 ml of borate buffer (0.43 M) and treated with 50 μ mol of the *N*-carboxyanhydride of L-alanine (Hunt & Du Vigneaud, 1938) as described by Manning & Moore (1968). The resulting mixture (0.75 ml) was adjusted to pH 1.0 and subjected to preparative electrophoresis on paper in a 30 cm-long band at pH 4.5 (70 V/cm) for 1 h. The dipeptide, which migrated about 4 cm towards the anode, was located by treatment of strips at each end of the band with ninhydrin. It was eluted with water (1 ml) and the eluate freeze-dried. The residue was dissolved in 1.5 ml of 0.2M-sodium citrate buffer, pH 2.2, containing 15 nmol each of methionine and norleucine. A sample (1.0 ml) of the solution was analysed in a Bical BC 100 L amino acid analyser with a high-sensitivity linearized colorimeter and a column (50 cm $\times$ 0.6 cm) of Aminex A5 resin. Elution was carried out with the series of buffers normally used in the analysis of protein hydrolysates. Similar analyses were carried out with L-alanyl-D- α -aminoadipic acid and with a mixture of the LL and LD isomers.

The LL dipeptide was eluted from the column after

norleucine and after the LD dipeptide. Methionine was eluted before the LD dipeptide.

³H-labelled 6-aminopenicillanic acid sulphoxide 4-methoxybenzyl ester 4-toluenesulphonate

Phenoxymethylpenicillin ester sulphoxide (0.778 g, 1.6 mmol, 362 mCi/mmol) was dissolved in redistilled dimethylaniline (0.52 ml, 4.10 mmol) and dichloromethane (2.5 ml) in a vessel carrying a drying tube of CaCl₂. The solution was cooled to between –60° and –65°C and 0.36 g (1.70 mmol) of AnalaR PCl₅ was added in one portion. The magnetically stirred mixture was allowed to warm to between –40°C and –45°C during 15 min and kept at this temperature for 2 h.

The mixture was then cooled to between –60° and –65°C and butan-1-ol (2.5 ml, 27.4 mmol) added dropwise with stirring over 15 min, the rate of addition being such that the temperature did not rise above –40°C. The yellow solution was stirred for a further 2 h between –40° and –45°C. Water (2.5 ml) was added, the mixture stirred vigorously for 5–10 s, and 5.0M-NH₃ (0.25 ml) then added in one portion. The final pH of the mixture, after stirring for 15 min, was 0.9.

Ethyl acetate (20 ml) and water (5 ml) were added to the stirred mixture and the pH was raised to 6.5 by addition of 2M-NaOH. The aqueous layer was separated and washed with ethyl acetate. The combined ethyl acetate solutions were dried and concentrated to 5 ml. 4-Toluenesulphonic acid (0.304 g, 1.6 mmol) in ethyl acetate (5 ml) was added and the mixture stored at –15°C overnight with occasional shaking. The 4-toluenesulphonate of 6-aminopenicillanic acid 4-methoxybenzyl ester sulphoxide appeared as white crystals which were collected, and washed with ethyl acetate and then with ether. This product (yield 0.22 g, 26%) had m.p. 152–155°C (decomp.) and behaved as a pure substance on t.l.c. in butan-1-ol-acetic acid-water (4:1:4, by vol., top phase) with *R_F* 0.48. Its specific radioactivity was 373 mCi/mmol.

The corresponding unlabelled product was prepared for analysis in the same manner. It had m.p. 152–155°C (decomp.) (Found: C, 52.70; H, 5.50; N, 5.14; S, 12.40; C₂₃H₂₈N₂O₈S₂ requires C, 52.67; H, 5.34; N, 5.34; S, 12.21%). P.m.r. spectrum: τ (Me₂SO perdeuterated), 2.3–3.3 (8H, complex, aromatic protons); 4.5–5.0 (4H, complex, H₅, H₆ and CO₂CH₂); 5.45 (1H, singlet, H₃), 6.25 (3H, singlet, OCH₃); 7.70 (3H, singlet, CH₃ of toluene sulphonate); 8.44 (3H, singlet, 2-CH₃); 8.89 (3H, singlet, 2-CH₃).

³H-labelled *N*-4-methoxybenzyloxycarbonyl isopenicillin *N* sulphoxide di-4-methoxybenzyl ester

6-Aminopenicillanic acid sulphoxide 4-methoxybenzyl ester was liberated from its 4-toluenesulphonate

by mixing the salt (52.4mg, 0.1mmol; 373 mCi/mmol) with triethylamine (0.015ml, 0.10mmol) in chloroform (2ml) to give a clear solution.

4-Methoxybenzyloxycarbonyl-L- α -amino adipic acid α -4-methoxybenzyl ester (from 62.6mg, 0.10 mmol, of the dicyclohexylamine salt), hydroxybenzotriazole (13.5mg, 0.10mmol) and dicyclohexylcarbodi-imide (22.7mg, 0.11 mmol) were mixed with chloroform (2ml) at 0°C, and the mixture was stirred at 0°C for 1h and then at room temperature for 1h. The solution of 6-aminopenicillanic acid sulphoxide ester in chloroform (2ml) was added and the mixture stirred overnight. T.l.c. in methyl acetate gave an intense spot due to the product (R_F 0.60) and four weak spots, one of which (R_F 0.24) corresponded to the original derivative of α -amino-adipic acid.

The reaction mixture (4ml) was applied directly to a column (2.2cm $\times$ 100cm) of silica gel (as for t.l.c.) equilibrated with methyl acetate. The mixture was washed on to the column with a mixture of chloroform (1ml) and methyl acetate (1ml) and elution then carried out with methyl acetate under a solvent head of 40cm. Fractions (about 2ml) were collected at 30min intervals and analysed by t.l.c. in methyl acetate. Fractions 121–135, containing the apparently pure product, were pooled and evaporated *in vacuo* to give 65.3mg (84%) of *N*-4-methoxybenzyloxycarbonylisopenicillin N sulphoxide di-4-methoxybenzyl ester as a white foam with specific radioactivity 341mCi/mmol. Elementary analysis indicated that it was contaminated with about 9% of silica gel (Found: C, 54.5; H, 5.3; N, 4.9; C₃₉H₄₅N₃O₁₂S requires C, 60.1; H, 5.8; N, 5.4%). I.r. spectrum: $\nu_{\max}$. (CHCl₃) 1800 (β -lactam C=O), 1750–1720 (ester carbonyls) cm⁻¹. P.m.r. spectrum: τ (C²HCl₃), 2.6–3.3 (13H, complex, 1H, doublet, J =10 Hz, CH₂CONH superimposed on 12H, complex, aromatic); 4.02 (1H, quartet, J =5 Hz and J =10 Hz, H₆); 4.57 (1H, crude doublet, J =9 Hz, side chain CO₂·NH·CH); 4.76, 4.90 (2H, AB multiplet, J =11 Hz, nucleus CO₂·CH₂); 4.94 (2H, singlet, side chain CO₂·CH₂); 5.01 (2H, singlet, side chain CO₂CH₂); 5.07 (1H doublet, J =5 Hz, H₅); 5.40 (1H, singlet, H₃); 5.70 (1H, crude triplet, side chain CO₂NHCH); 6.22 (9H, three singlets, three OCH₃); 7.6–8.6 [9H, 3H singlet 2 β -CH₃ superimposed on 6H complex side chain (CH₂)₃]; 8.97 (3H, singlet, 2 α -CH₃).

A second preparation of the fully protected isopenicillin N sulphoxide, carried out on a 0.3mmol scale, gave 0.14g of product with specific radioactivity 359mCi/mmol.

Isopenicillin N sulphoxide

³H-labelled *N*-4-methoxybenzyloxycarbonylisopenicillin N sulphoxide di-4-methoxybenzyl ester (39mg, 0.05mmol, 359mCi/mmol) was dissolved in a

mixture of tetrahydrofuran (2ml), methanol (2ml), and 0.05ml of aq. triethylamine acetate (0.15mmol), pH6.5. Water (approx. 2ml) was added until the mixture just failed to become cloudy; Pd-charcoal catalyst (117mg) was added to the solution and air removed from the mixture by a stream of N₂. Hydrogen [washed with alkaline permanganate and methanol-tetrahydrofuran-water (1:1:1, by vol.)] was then bubbled through the mixture and the progress of hydrogenolysis followed by analysis of samples by t.l.c. in butan-1-ol-acetic acid-water (4:1:4, by vol., upper phase). After 18h the mixture appeared to contain mainly isopenicillin N sulphoxide (R_F 0.04) with traces of partially protected products.

The catalyst was removed by filtration and washed with water (2 $\times$ 5ml) and the combined filtrates were applied directly to a column (2cm $\times$ 1cm diam.) of Dowex 1 X8 (acetate form). The column was washed with water (200ml) and the product (4.6mCi) eluted with 0.5M-acetic acid (80ml). The pH of the eluate was adjusted to 7.0 by addition of 2% (v/v) triethylamine and the resulting solution freeze-dried. The residue was dissolved in water (5ml) and samples (5 μ l) of the solution were analysed by paper electrophoresis at pH4.5 and 6.5, and by paper chromatography. The solution was then freeze-dried to give 5mg of the triethylamine salt of isopenicillin N sulphoxide as a pale-brown powder. The estimated yield was 21% by weight and 26% by radioactivity.

³H-labelled isopenicillin N

A suspension of the triethylamine salt of isopenicillin N sulphoxide (5mg) in dimethylformamide (2ml) was cooled to 0°C. PBr₃ (25 μ l, 0.25mmol) was added and the mixture stirred for 60s at 0°C. The mixture was then cooled to -20°C and 0.5M-NaHCO₃ (2ml) rapidly added. The mixture was allowed to warm to room temperature and CO₂ removed *in vacuo* in a rotary evaporator. The resulting solution (pH7.0) was applied to a column (75cm $\times$ 1.5cm diam.) of Bio-Gel P2 resin equilibrated with water, and elution was carried out with water at a flow rate of 60ml/h, fractions being collected at 5min intervals. A sample (10 μ l) of each fraction was mixed with water-miscible scintillation fluid (3.0ml) and counted for radioactivity for 1min. Fractions 11–20 inclusive were pooled and freeze-dried to give 38mg of off-white solid.

This product was dissolved in water (2ml) and propan-2-ol added to the solution until its concentration was 70% (v/v). The turbid mixture was applied to a column (33cm $\times$ 1.2cm diam.) of Sephadex G-25 (fine) equilibrated with 70% (v/v) propan-2-ol at 4°C. Elution was carried at 4°C with 70% (v/v) propan-2-ol, fractions (5ml) being collected hourly. A sample (10 μ l) of each fraction was mixed with water-miscible scintillation fluid (3.0ml) and counted

for radioactivity for 1 min. Samples (5 μ l) of every other fraction were spotted on to paper, together with 5 μ g samples of penicillin N, and the mixtures analysed by paper electrophoresis at pH 4.5 and 6.5, and by paper chromatography in 70% (v/v) propan-2-ol. After detection with ninhydrin, spots corresponding to penicillin N were cut out, mixed with 3 ml of scintillation fluid, and counted for radioactivity. Fractions 25–40 inclusive were combined and the propan-2-ol was extracted with 4 vol. of ether. After removal of ether *in vacuo* from the aqueous phase, the latter was freeze-dried. The radioactivity of the product corresponded to 0.8 mg of isopenicillin N (sodium salt), with a specific radioactivity of 373 mCi/mmol.

³H-labelled phenoxymethylpenicillin sulphoxide

To a solution of phenoxymethylpenicillin sulphoxide 4-methoxybenzyl ester (1.46 g, 3 mmol, 362 mCi/mmol) in 60 ml of methanol–tetrahydrofuran (1:1, by vol.) containing two drops of water was added 2.92 g of 10% Pd-charcoal catalyst. Air was removed by a stream of N₂ washed with alkaline permanganate, water and methanol–tetrahydrofuran and the N₂ then replaced by H₂. Hydrogenolysis was continued overnight and the mixture then filtered and the catalyst washed with methanol (4 $\times$ 10 ml). The combined filtrates were evaporated to dryness to give a colourless gum which crystallized on standing. This was dissolved in 1 M-NaHCO₃ (30 ml) and shaken with ethyl acetate (2 $\times$ 15 ml). The pH of the aqueous phase was then adjusted to 2.3 with 1 M-HCl and the product extracted into ethyl acetate (60 ml). The extract was dried and evaporated to give a residue (0.67 g) which slowly crystallized.

The corresponding product from a similar experiment with unlabelled starting material was recrystallized from methanol (9 ml) and water (6 ml) at 0°C to give long colourless needles (yield 41%) which had m.p. 163.5–164.5°C and mixed m.p. with an authentic sample of phenoxymethylpenicillin sulphoxide prepared by the method of Morin *et al.* (1969), 163.5–164.5°C. The i.r. and p.m.r. spectra of the product were virtually identical with those of the authentic sample.

³H-labelled phenoxymethylpenicillin

Phenoxymethylpenicillin sulphoxide (0.67 g, 1.64 mmol) was dissolved in dry dimethylformamide (12 ml) and the solution cooled to –20°C. PBr₃ (1 ml, 10.2 mmol) was added in 5 s and the reaction mixture stirred for 60 s and then poured into an ice-cold suspension of Na₂HPO₄, 12H₂O (10 g, 27.6 mmol) in water (100 ml). The pH of the aqueous mixture (pH 2.6) was brought to 2.5 with a few drops of 1 M-HCl and the mixture kept at 0°C overnight. The white crystalline phenoxymethylpenicillin was filtered, washed with a little water and dried. It was

converted into the potassium salt by solution in a mixture of dioxan (20 ml) and water (10 ml) and addition of 0.2 M-KOH with stirring until the pH rose to 7.0. After removal of the solvent *in vacuo* the residue was crystallized from water (2 ml) and acetone (100 ml) at –15°C. The crystals were filtered, washed with acetone and dried (0.156 g). On t.l.c. in butan-1-ol–acetic acid–water (4:1:4, by vol., top phase) the product behaved as pure phenoxymethylpenicillin (potassium salt).

³H-labelled 6-aminopenicillanic acid

To a mixture of the radioactive phenoxymethylpenicillin (potassium salt) (0.156 g, 0.4 mmol), re-distilled dimethylaniline (0.13 ml, 1025 mmol), and dichloromethane (0.6 ml), was added dichlorodimethylsilane (0.04 ml, 0.325 mmol), at room temperature, in a vessel fitted with a CaCl₂ drying tube. The mixture was stirred for 30 min, then cooled to –50°C and PCl₅ (0.09 g, 0.425 mmol) added in one portion. The temperature was maintained at –40°C for 2 h and then decreased to –65°C. Butan-1-ol (0.6 ml, 7 mmol) was then added at such a rate that the temperature did not rise above –40°C. The mixture was stirred for 2 h and then poured into water (0.6 ml), and 4.5 M-NH₃ (0.25 ml) immediately added. After the pH had been adjusted to 4.1 by addition of solid (NH₄)₂CO₃ the mixture was kept at 0°C overnight. The crystalline product which separated was filtered and washed with 0.1 ml of aq. 50% (v/v) acetone and 0.1 ml of acetone. (Yield 45 mg, m.p. 202–204°C, mixed m.p. with authentic 6-aminopenicillanic acid 202–204°C, specific radioactivity 371 mCi/mmol.) It behaved as pure 6-aminopenicillanic acid (*R*_F 0.27) on t.l.c. in butan-1-ol–acetic acid–water (4:1:4, by vol., top phase).

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