PREPARATION OF PENICILLIN N AND ISOPENICILLIN N

H. VANDERHAEGHE, A. VLIETINCK, M. CLAESEN* and G. PARMENTIER

Rega Institute, University of Leuven, Belgium

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Penicillin N and isopenicillin N (D- and L-4-carboxy-4-amino-*n*-butyl-penicillin) were synthetized from 6-aminopenicillanic acid (6-APA) by two different methods.

In the first synthesis the mixed anhydrides of the two enantiomers of the 1-benzylester of 2-carbobenzyloxyaminoadipic acid were prepared and by condensation with 6-APA, the L- and D-isomers of 4-benzyloxycarbonyl-4-carbobenzyloxy-amino-*n*-butylpenicillin were obtained. By hydrogenation of these two epimers in the presence of Pd/C, isopenicillin N and penicillin N were obtained. This hydrogenolysis was incomplete, but by chromatography on a column of Sephadex G10, it was possible to purify these penicillins and to remove 4-carboxy-4-carbobenzyl-oxyamino-*n*-butylpenicillin.

In the second synthesis, the 1-benzylesters of L- and D-2-azidoadipic acid were transformed into the acid chloride, and by reaction with 6-APA, the two epimers of 4-benzyloxycarbonyl-4-azido-*n*-butylpenicillin were prepared. By hydrogenation of these compounds in the presence of Pd/C, isopenicillin N and penicillin N were obtained. Both penicillins were also purified by chromatography on Sephadex G10.

Penicillin N (formerly called cephalosporin N) is produced together with cephalosporin C and cephalosporin P by *Cephalosporium acremonium*.[1] Penicillin N (**VIb**) has D-2-aminoadipic acid as side chain.[2] Another antibiotic, synnematin B, produced by *Cephalosporium salmosynnematum*[3] was shown to be identical with penicillin N.[4] Synnematin B has been used for the treatment of typhoid fever in man.[5,6]

Isopenicillin N (**VIa**), which has L-2-aminoadipic acid as side chain, has been isolated from the mycelium of *Penicillium chrysogenum*.[7~9] This product is of interest in the biosynthesis of penicillins, because of its possible transformation into other penicillins by an acyl transferase enzyme.[10,11] It has also been postulated that isopenicillin N could be cleaved by penicillin acylase,[11] whereas penicillin N is not attacked by this enzyme.[12] To study a possible cleavage of isopenicillin N by penicillin acylase, it was necessary to prepare this penicillin.

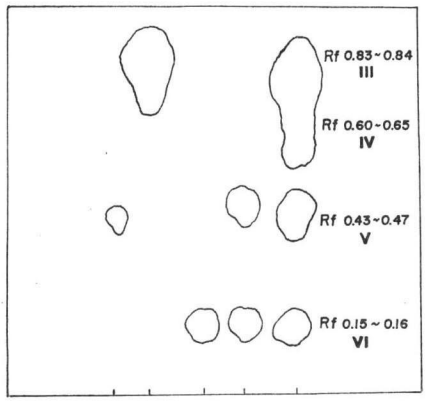
Isopenicillin N can be obtained only by a very laborious extraction from the mycelium of *Penicillium chrysogenum*,[7] whereas penicillin N is available in only very limited quantities under the form of synnematin B (Salmotin, Abbott). For this reason, it was decided to synthetize both penicillin N and isopenicillin N from 6-aminopenicillanic acid. As these penicillins are unstable in acid and alkaline media, only derivatives of 2-aminoadipic acid with protective groups which could be removed by hydrogenolysis were used in this synthesis.

In the first method the 1-benzylester of 2-carbobenzyloxyaminoadipic acid (**I**) obtained during a previous study[13] was transformed into a mixed anhydride (**II**) and condensed with 6-aminopenicillanic acid (6-APA). This synthesis was performed with both the L- and the D-isomers of **I**, and good yields of the two epimers of 4-benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin (**III**) were obtained.

* Present address: Ecole de Pharmacie, Université de Louvain, Belgique.

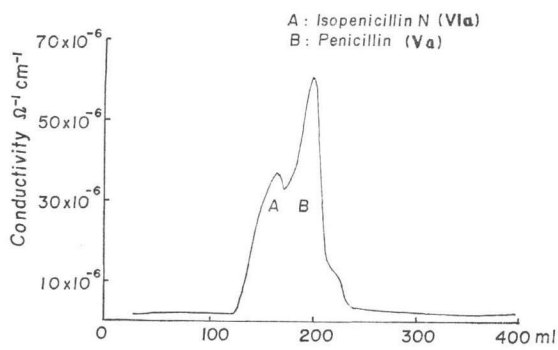
After hydrogenation of **III** in the presence of Pd/C, paper chromatography of the reaction mixture and revelation by bioautography indicated the presence of three penicillins, **IV**, **V**, and **VI**, besides the starting product **III** (Fig. 1). A further hydrogenation of the reaction mixture after removal of the catalyst gave a solution containing only two components. For these products the structures **V** and **VI** were inferred because previous studies had shown that a benzylester could be cleaved easily by hydrogenolysis, *e.g.*, a good yield of 4-carboxy-*n*-butylpenicillin could be obtained by this method from 4-benzyloxycarbonyl-*n*-butylpenicillin.[14] Identification of **V** as 4-carboxy-4-carbobenzyloxyamino-*n*-butylpenicillin was made by comparison with a sample which had been prepared by reaction of 4-carboxy-4-carbobenzyloxyaminovalerylazide (**VII**) with 6-aminopenicillanic acid.[15] The penicillin **VI**, which was the most polar component in our chromatography system, was shown by comparison with an authentic sample of penicillin N (synnematin B) to be 4-carboxy-4-amino-*n*-butylpenicillin. The other penicillin which is formed in small amounts during the first hydrogenation must have structure **IV**.

Fig. 1. Chromatogram on Whatman No. 1 paper with *n*-BuOH-pyridine-water, 1:1:1, of the mixture obtained after one hydrogenolysis of **IIIa** or **IIIb**, together with reference products. Detection by bioautography on agar plates seeded with *Staphylococcus aureus*.



To remove **V** from the reaction mixture containing **V** and **VI**, two more hydrogenations were necessary. It is well known that it is very difficult to split off an N-benzyloxycarbonyl group by catalytic reduction if the molecule contains a sulfur atom.[16] These successive hydrogenations involved a great loss of product due to adsorption on the catalyst and formation of decomposition products. This problem could be solved when we found it possible to separate the mixture of **V** and **VI**, obtained after two hydrogenations, on a column of Sephadex G10 (Fig. 2). By this method it was possible to obtain isopenicillin N (**VIa**) and penicillin N (**VIb**) with a purity of respectively 76 % and 68 %, in the iodometric assay, which is specific for the β-lactam-thiazolidine structure.[17,18]

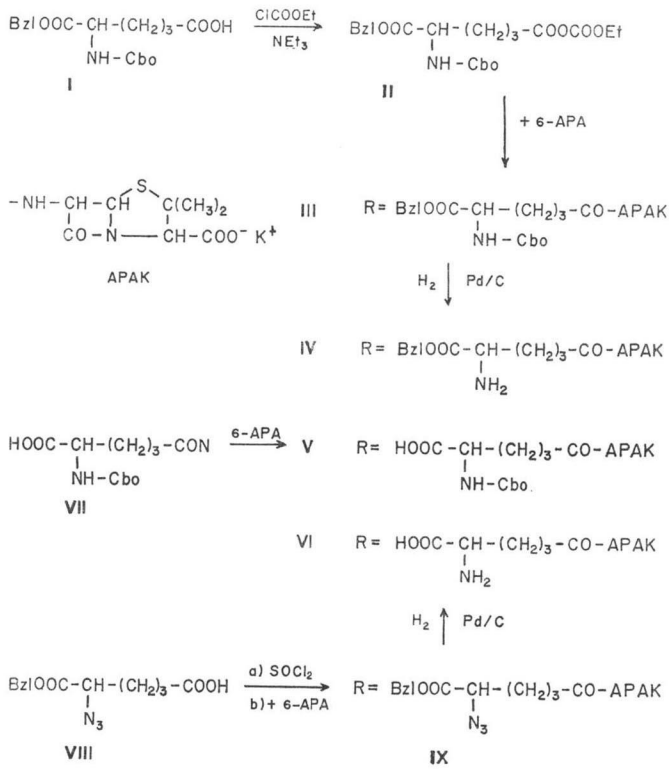
Fig. 2. Purification of isopenicillin N on a Sephadex G10 column (Inside diameter 3.2 cm, length 75 cm). The second peak (salt) is not shown.



As the main problem of the transformation of **III** into **VI** was the removal of the N-carbobenzyloxy group, a second synthesis using the 1-benzylester of 2-azidoadipic acid[19] was attempted. Previous studies had shown that an azido group in a penicillin could be transformed readily by hydrogenation into an amino

group.[20,21] The 1-benzylesters of L- and of D-2-azidoadipic acid (**VIIIa** and **VIIIb**) were transformed into the acid chloride, and condensation with 6-aminopenicillanic acid gave good yields of both epimers of 4-benzyloxycarbonyl-4-azido-*n*-butylpenicillin (**IXa** and **IXb**). Paper chromatography of this penicillin in the system *tert*-butanol - pyridine - water indicated the presence of another penicillin which could not be completely removed by recrystallization. This impurity was also present when **IX** was prepared by the mixed anhydride method. The conversion of **IX** to **VI** by catalytic hydrogenation proceeded more readily than with **III**. Paper chromatography of **VI** prepared by this method indicated the presence of a small amount of another penicillin, which originated from the impurity present in **IX**. It was again possible to obtain homogeneous **VIa** (isopenicillin N) and **VIb** (penicillin N) by passage through a column of Sephadex G10.

As has been noted by other authors,[7,8] isopenicillin N and penicillin N could not be distinguished by chromatography in different solvent systems. The differentiation between penicillin N and isopenicillin N was also impossible on the basis of the optical rotation, because both products had practically the same $[\alpha]_D$. This may be due in part to the fact that both penicillins contained a rather large amount (10~20 %) of decomposition products like penicilloic acid. For this reason it was decided to examine the enzymatic determination of the configuration of 2-aminoadipic acid present in the hydrolysate of these penicillins. This could be performed only with L-amino acid oxidase, because D-2-aminoadipic acid is inert to hog kidney D-amino acid oxidase.[22] The presence of L-2-aminoadipic acid in isopenicillin N was demonstrated by



a) (e.g. IIIa, IVa) refers to L-isomer.
b) (e.g. IIIb, IVb) refers to D-isomer.

treatment of the paper chromatogram of the hydrolysate with *Crotalus adamanteus* L-amino acid oxidase.

The difficulties experienced in the purification of penicillin N and isopenicillin N are similar in both methods of preparation. The synthesis using the benzylester of 2-carbobenzyloxyamino-adipic acid (**I**) must be preferred because the separation of the enantiomers of 2-azidoadipic acid is difficult and gives a low yield.[19] The preparation of **I** involves less difficulty because the separation of the enantiomers of 2-aminoadipic acid by the enzymatic method has become more practical since the recent improvement in the synthesis of 2-chloroacetylaminoadipic acid.[13,23]

Examination of the action of penicillin acylase on penicillin N and isopenicillin N demonstrated that both penicillins were resistant to this enzyme isolated from *Fusarium avenaceum* and from *Penicillium chrysogenum*.[24]

There was no significant difference between the antibacterial properties of the two penicillins (Table 1).

Table 1. Antibacterial spectrum

	Minimum inhibitory concentration in μg/ml		
	Penicillin N	Isopenicillin N	Penicillin G
Staphylococcus aureus ATCC 6538P	3.5	3.5	0.03
Streptococcus pyogenes ATCC 8668	0.9	0.9	0.002
Streptococcus faecalis NTCC 8213	50	50	5
Corynebacterium xerosis 9755	3.5	3.5	0.1
Escherichia coli 9661	15	35	35
Proteus mirabilis	10	50	35

The purity of isopenicillin N, as estimated by iodometric assay, was 77 % and that of penicillin N 68 %.

Experimental

Melting points were determined in capillaries in a Büchi-Tottoli apparatus and are uncorrected. Infrared spectra were recorded with a Beckman spectrophotometer IR4 in KBr discs. Microanalyses were performed by Dr. A. BERNHARDT, Elbach über Engelskirchen, Germany. Mass spectra were recorded on an AEI MS9 apparatus with the direct insertion technique at 150°C. N.m.r. spectra were recorded on a Varian A60 or XL100 (for penicillin N and isopenicillin N) spectrometer with 2, 2-dimethyl-2-silapentane-5-sulfonate (DSSA) as internal standard. The penicillins were examined by descending paper chromatography. Amounts of 100 and 200 μg for penicillin N and isopenicillin N and 50 and 100 μg for the other penicillins were put on strips (1×40 cm) of Whatman No. 1 paper. Chromatography was performed with the system *n*-butanol-pyridine-water, 1:1:1, or *tert*-butanol-pyridine-water, 1:1:1. After chromatography, the strips were dried, put on agar plates seeded with *Staphylococcus aureus* ATCC 6538P, and incubated overnight at 37°C. For the examination of penicillin N and isopenicillin N the chromatograms were run in duplicate. One set of strips was dried under a hood and sprayed with 1 % sodium bicarbonate. After drying, the strips were treated with a 1 % solution of phenoxyacetyl chloride in acetone and dried before being put on the agar plates. Penicillin N and isopenicillin N (200 μg) were also chromatographed on Whatman No. 1 paper (45×25 cm) in the system *tert*-butanol-pyridine-water, 1:1:1, and revealed with ninhydrin reagent.

The purity of the penicillins was determined by iodometric assay and was based on the

assumption that 9 equivalents of iodine were consumed per mole of penicillin.[17,18] The estimated purity is probably too low because the amount of iodine consumed is between 8.5 and 9 equivalents. At any rate, the blanc values of the assay of penicillin N and isopenicillin N indicated that a certain amount of penicilloic or penilloic acid was present.

L-4-Benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin (**IIIa**)

L-2-Carbobenzyloxyaminoadipic acid 1-benzylester (**I**) (7.7 g, 20 mmoles), prepared from L-2-carbobenzyloxyaminoadipic acid[18] was dissolved in 30 ml of dioxane by moderate warming on a waterbath. This solution was diluted with 20 ml of acetone and cooled to 0~5°C. After addition of 2.8 ml (20 mmoles) of dry triethylamine, a solution of 1.90 ml (20 mmoles) of ethyl chloroformate in 50 ml of dry acetone was added with stirring for 10 minutes. The solution was further stirred for 15 minutes at 0~5°C and for another 20 minutes at room temperature.

Meanwhile, 3.9 g (18.05 mmoles) of 6-aminopenicillanic acid was put into 6 ml of water. After the suspension was cooled to 0~5°C, 2.5 ml (18.05 mmoles) triethylamine was added with stirring at such a rate that the pH was always lower than 7.9. To this solution was added with stirring the cooled (0°C) solution of the mixed anhydride. After reaction for 20 minutes at 0~5°C, and for 60 minutes at room temperature, the mixture was diluted with 100 ml of water and the pH was adjusted to 8.1 by addition of N NaOH. The solution was washed with ether and the aqueous layer was acidified with 20% H_3PO_4 to pH 2.6 after cooling to 0°C and extracted with 150 ml of *n*-butyl acetate. After another extraction with butyl acetate, the organic layers were washed with water. The penicillin was removed from the organic phase by extraction with 0.5 N KOH until a pH of 6.5 was obtained. The aqueous solution was lyophilized, and 8.7 g (yield 80%) of the potassium salt of L-4-benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin (**IIIa**) was obtained, $[\alpha]_D^{26}$+160.8 (*c* 0.12, water), purity 95.3% (iodometric assay), ν max 1775 (β-lactam), 1730 (ester), 1670 and 1540 (amide), 1605 (COO^-) cm^{-1}; δ (D_2O, DSSA) 1.50 (s, CH_3), 1.60 (s, CH_3), 1.58~1.88 (m, β-CH_2 and γ-CH_2), 1.03~2.40 (m, α-CH_2), 3.85~4.20 (m, δ-CH), 4.26 (s, 3-H), 4.91 (s, br, $C_6H_5\underline{CH_2}OOC$ and $C_6H_5\underline{CH_2}OCO$), 5.50 (d, J 4 cps, 5-H), 5.57 (d, J 4 cps, 6-H), 7.12 (s, $\underline{C_6H_5}CH_2OOC$ and $\underline{C_6H_5}CH_2OCO$).

Analysis. Calculated for $C_{29}H_{32}N_3O_6SK$: N, 6.76. Found : N, 6.80.

Because of incomplete combustion of the potassium salt, no satisfactory results could be obtained for the carbon and hydrogen analysis. For this reason, 1.86 g (3 mmoles) of the potassium salt of **IIIa** was dissolved in 10 ml of water, and the cold solution was extracted with ether after acidification with N HCl. To the organic phase was added 543 mg (3 mmoles) of dicyclohexylamine dissolved in 10 ml of ether. The precipitate was recrystallized in benzene-cyclohexane. A quantity of 1.06 g of the dicyclohexylammonium salt of **IIIa**, mp. 72~75°C (dec.) was obtained.

Analysis. Calculated for $C_{41}H_{56}N_4O_6S$: C 64.37, H 7.38, N 7.34.
Found : C 64.52, H 7.55, N 7.62.

A sample of **IIIa** was extracted into ether, after acidification of the aqueous solution, and transformed into the methyl ester by reaction with diazomethane. The mass spectrum of the methyl ester ($C_{30}H_{35}N_3O_6S$: 597.76) showed the molecular ion peak at *m/e* 597.

D- and DL-4-Benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin

These penicillins were prepared by the method described for the L-epimer (**IIIa**). They were obtained in virtually the same yield and degree of purity, $[\alpha]_D^{26}$+181.5 (*c* 0.11, water) for the potassium salt of the penicillin with the D-side chain (**IIIb**) and +170.3 (*c* 0.23, water) for the potassium salt of the product with the DL-side chain. The carbonyl region of the i.r. spectrum and the n.m.r. spectrum had the same maxima as the L-isomer (**IIIa**).

As the potassium salt of **IIIb** gave only a satisfactory N analysis, the dicyclohexylammonium salt, mp. 72~74°C (dec.) was prepared from the potassium salt.

Analysis. Calculated for $C_{41}H_{56}N_4O_6S$: C 64.37, H 7.38, N 7.37.
Found : C 64.37, H 7.24, N 7.20.

D-4-Benzyloxycarbonyl-4-azido-*n*-butylpenicillin (**IXb**)

The 1-benzylester of D-2-azidoadipic acid (6.4 g, 23.1 mmoles) prepared from D-2-azidoadipic acid[19] was heated with 10 ml purified thionyl chloride and 1 drop of pyridine at 40°C for 2 hours. The excess reagent was removed by evaporation under reduced pressure, and the acid chloride was diluted with 10 ml of acetone. This solution was added with stirring to a cold (0°C) solution of 4.4 g (20.4 mmoles) 6-aminopenicillanic acid in 60 ml of M $KHCO_3$ and 50 ml of acetone. The mixture was stirred for 1 hour at room temperature, and some unreacted acid chloride was removed by extraction with ether. The aqueous layer was cooled and, after addition of 100 ml of butyl acetate, acidified with 20 % H_3PO_4 to pH 2.5. After another extraction of the aqueous layer with butyl acetate, the organic layer was washed with water, and the penicillin was reextracted with 0.5 N KOH until a pH of 6.5 was obtained.

The aqueous solution was lyophilized, and 8.4 g of product was obtained. This material was examined by paper chromatography in the system *tert*-butanol - pyridine - water, 1:1:1, and bioautography. It contained, besides the main product (Rf 0.75~0.80), a small amount of another biologically active product (Rf 0.52~0.53). The freeze-dried penicillin was taken up in acetone and a small amount (470 mg) of undissolved material was removed by centrifugation. The solution was evaporated at 25°C under reduced pressure, the residue was dissolved in water, and the solution was lyophilized to yield 7.1 g (68 %) of the potassium salt of D-4-benzyloxycarbonyl-4-azido-*n*-butylpenicillin (**IXb**), purity 94.3 % in the iodometric assay $[\alpha]_D^{25}+236$ (*c* 0.10, water), ν max 2160 (N_3—), 1775 (β-lactam), 1750 (ester), 1670 and 1540 (amide), 1610 (COO^-) cm^{-1}; δ (D_2O, DSSA), 1.50 (s, CH_3), 1.59 (s, CH_3), 1.60~1.90 (m, β-CH_2 and γ-CH_2), 1.04~1.40 (m, α-CH_2), 3.70~3.98 (m, δ-CH), 4.24 (s, 3-H), 5.07 (s, $C_6H_5CH_2OOC$), 5.49 (d, J 4 cps, 5-H), 5.54 (d, J 4 cps, 6-H), 7.24 (s, $C_6H_5CH_2OOC$). Paper chromatography of this material still revealed the presence of a trace of the component with Rf 0.51~0.53.

Analysis. Calculated for $C_{21}H_{24}N_5O_6SK$: C 49.10, H 4.71, N 13.64.
Found : C 49.03, H 4.79, N 13.46.

L-4-Benzyloxycarbonyl-4-azido-*n*-butylpenicillin (**IXa**)

This penicillin was obtained by the method described for the D-isomer. It also contained a small amount of another penicillin. The product showed a purity of 95.5 % in the iodometric assay, $[\alpha]_D^{24}+202$ (*c* 0.10, water). The i.r. and n.m.r. maxima given for **IXb** were also described for **IXa**.

A sample of **IXa** was extracted into ether, after acidification of the aqueous solution with 10 % phosphoric acid, and transformed into the methylester. The mass spectrum of this product ($C_{22}H_{27}N_5O_6S$: 489.6) gave the expected molecular ion peak at *m/e* 489.

DL-4-Carboxy-4-carbobenzyloxyamino-*n*-butylpenicillin (**V**)

DL-4-Carboxy-4-carbobenzyloxyaminovalerylhydrazide, prepared by the method of ABRAHAM and NEWTON,[25] was transformed into the azide (**VII**) and condensed with 6-aminopenicillanic acid according to the procedure of DOYLE *et al.*[15] The reaction product was extracted into ethyl acetate after acidification of the aqueous solution with 25 % phosphoric acid. The penicillin was reextracted from the organic phase with 0.5 N KOH until a pH of 7.0 was obtained, and the aqueous solution was concentrated at 30°C under reduced pressure and lyophilized. The DL-4-carboxy-4-carbobenzyloxyamino-*n*-butylpenicillin (**V**) had a purity of only 25 % according to the iodometric assay but was homogeneous on paper chromatography in the system *n*-butanol - pyridine - water, 1:1:1, and revelation by bioautography.

L-4-Carboxy-4-amino-*n*-butylpenicillin (Isopenicillin N)

Method A. A solution of 2.5 g of the potassium salt of L-4-benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin (**IIIa**) in 25 ml water was adjusted to pH 8.0 and was shaken under a pressure of 3 kg/cm² of hydrogen in the presence of 7 g of prehydrogenated and neutralized 10 % Pd/C. After 3 hours the catalyst was filtered off and washed with water and with an acetone-water, 1:1, mixture to remove the product adsorbed on the active carbon. The

filtrate was evaporated under reduced pressure at a temperature below 25°C to a volume of 25 ml.

Paper chromatography (Fig. 1) of this solution in the system *n*-butanol - pyridine - water, 1:1:1, and revelation by bioautography indicated the presence of four penicillins, *viz.*, **VI** (Rf 0.15~0.16), **V** (Rf 0.43~0.47), **IV** (Rf 0.60~0.65), and a small amount of starting material **III** (Rf 0.83~0.84).

This solution was hydrogenated again in the presence of 7 g 10 % Pd/C. After 1.5 hours the catalyst was filtered off and washed with water and with acetone - water. The filtrate was concentrated under reduced pressure to a volume of 25 ml, and by lyophilization 1.3 g of solid was obtained. This product contained components **V** and **VI**. Other experiments had shown that two more hydrogenations were necessary for complete removal of component **VI** but that these operations increased the amount of decomposition products in such a way that penicillin **VI** presented a low degree of purity in the iodometric assay. For this reason the mixture of **V** and **VI** obtained after two hydrogenations was purified on a Sephadex G10 column (Fig. 2). Amounts of 20~75 mg of product were put on the column, and elution was performed with sterile distilled water at 0°C. The components in the effluent were detected by recording the conductivity. Two peaks were observed : the first, containing penicillin; the second, containing salt.

The penicillin peak (which was a double peak when small amounts were put on the column) was collected in four fractions and examined by paper chromatography. In this way 1.1 g of material containing **V** and **VI** was purified, and the corresponding fractions obtained in each operation were combined, evaporated under reduced pressure, and lyophilized. The first part of the penicillin peak yielded 162 mg of **VIa**, which according to the iodometric assay had a purity of 54 % but contained a large amount of penicilloic acid. From the second and the third part of the peak, 377 mg of the potassium salt of **VIa** (isopenicillin N) with a purity of 76 % was obtained. The fourth part of the penicillin peak yielded 184 mg of material, which according to the paper chromatographic examination contained **VI** and some **V**.

Isopenicillin N with a purity of 77 % (iodometric assay) gave $[\alpha]_D^{23}+209$ (*c* 0.50, water), ν max. 1775 (β-lactam), 1670 and 1540 (amide), 1605 (COO^-) cm^{-1}, no ester band, δ (D_2O, DSSA), 1.54 (s, CH_3), 1.65 (s, CH_3), 1.66~1.98 (m, β-CH_2 and γ-CH_2), 2.42 (t, J 6 cps, α-CH_2), 3.74 (t, J 6 cps, δ-CH), 4.24 (s, 3-H), 5.47 (d, J 4 cps, 5-H), 5.55 (d, J 4 cps, 6-H).

Analysis. Calculated for $C_{14}H_{20}N_3O_6SK \cdot \frac{1}{2}H_2O$: C 41.36, H 5.70, N 10.34, S 7.89.
Found : C 41.17, H 5.52, N 10.39, S 7.85.

Method B. A solution of 2.5 g of the potassium salt of L-4-benzyloxycarbonyl-4-azido-*n*-butylpenicillin (**IXa**) in 15 ml water at pH 7.8 was hydrogenated at 3 kg/cm^2 in the presence of 1.25 g of 10 % Pd/C. After 2 hours the catalyst was filtered off and washed with water and with a water - acetone, 1:1, mixture. The filtrate was concentrated under reduced pressure to a volume of 20 ml. The solution was hydrogenated again in the presence of 1.25 g 10 % Pd/C for 1.5 hours. After filtration of the catalyst and washing with water and water - acetone, the solution was concentrated under reduced pressure at temperature below 30°C, and by lyophilization 1.5 g of product was obtained. Paper chromatography in the system *tert*-butanol - pyridine - water, 1:1:1, indicated the presence of a small amount of another penicillin (Rf 0.53~0.55) besides isopenicillin N (Rf 0.30). The second penicillin gave a clearly visible inhibition zone when the chromatogram was treated with 1 % sodium bicarbonate and 1 % phenoxyacetyl chloride before autoradiography.

The impure isopenicillin N was also purified on a column of Sephadex G10, as described under Method A. The recording of the conductivity of effluent indicated that only a small amount of salt (second peak) was present. The first peak was also divided into four fractions. The purest isopenicillin N was obtained from the second and the third fractions : the fourth fraction contained isopenicillin N and the penicillin of unknown structure. From 770 mg of impure penicillin, 381 mg of the potassium salt of isopenicillin N with a purity of 77 %, was obtained, $[\alpha]_D^{25}+212$ (*c* 0.5, water), ν max. 1775 cm^{-1} (β-lactam CO); the azido and ester bands

were absent. Amide and carboxylate bands were present. This sample gave the same Rf value on paper chromatography in the system *n*-butanol - pyridine - water, 1:1:1, as did the isopenicillin N obtained by method A.

D-4-Carboxy-4-amino-*n*-butylpenicillin (Penicillin N)

Method A. Penicillin N was prepared by hydrogenation of 2.5 g of D-4-benzyloxycarbonyl-4-carbobenzyloxyamino-*n*-butylpenicillin (**IIIb**) in the presence of 7 g 10 % Pd/C, as described for isopenicillin N. A second hydrogenation yielded 900 mg of a product which by paper chromatography was shown to contain **Vb** and **VIb**. After chromatography on a column of Sephadex G10, penicillin N (**VIb**) free from **Vb**, could be obtained. Its purity was 67.8 % by iodometric assay.

Method B. Hydrogenation of 4 g of D-4-benzyloxycarbonyl-4-azido-*n*-butylpenicillin (**IXb**) in the presence of 2 g 10 % Pd/C gave, after filtration of the catalyst, evaporation, and lyophilization, 2.5 g of solid. Paper chromatography of this product revealed the presence of another penicillin besides penicillin N. This product was purified on a column of Sephadex G10 as described for isopenicillin N. Its purity was 68.4 % by iodometric assay, $[\alpha]_D^{23}+214$ (*c* 0.31, water).

These samples of penicillin N had the same Rf value on the paper chromatogram as penicillin N (Salmotin) obtained by fermentation. The i.r. and n.m.r. spectra and the Rf values in the two paper chromatography systems described under isopenicillin N were identical for penicillin N and isopenicillin N.

Determination of the configuration of 2-aminoadipic acid in penicillin N and isopenicillin N

Penicillin N and isopenicillin N (10 mg) were dissolved in 5.7 N HCl (2.5 ml) and heated in a sealed tube under nitrogen at 110°C for 20 hours. The contents of the tubes were evaporated and dried *in vacuo* over P_2O_5 and KOH pellets. The hydrolysates together with the reference amino acids L- and D-2-aminoadipic acid and D-penicillamine were chromatographed in duplicate on Whatman No. 1 paper in the system *n*-butanol - acetic acid - water, 4:1:5. After drying, one chromatogram was sprayed with ninhydrin solution; the other, with a solution containing 10 mg *Crotalus adamanteus* L-amino acid oxidase (Calbiochem) per ml of 0.2 M Tris buffer pH 7.2. This chromatogram was dried in the air and then sprayed with a 0.4 % 2, 4-dinitrophenylhydrazine solution in 2 N HCl. The keto acids formed from the L-amino acids appear as dark yellow spots on a yellow background.

The hydrolysate of penicillin N and isopenicillin N contained three ninhydrin-positive components *inter alia*, 2-aminoadipic acid and penicillamine. On the chromatogram of the hydrolysate of isopenicillin N, only one spot, whose Rf value corresponded to that of 2-aminoadipic acid, appeared after reaction with L-amino acid oxidase and 2, 4-dinitrophenylhydrazine. In the hydrolysate of penicillin N a weak spot, probably due to some racemisation of D-aminoadipic acid, was detected.

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