PREPARATION OF 6-EPI-AMPICILLIN AND OF 6-EPI-α-HYDROXYBENZYLPENICILLIN

E. ROETS, A. VLIETINCK* and H. VANDERHAEGHE

Pharmaceutical Institute,
University of Leuven, B–3000 Leuven, Belgium

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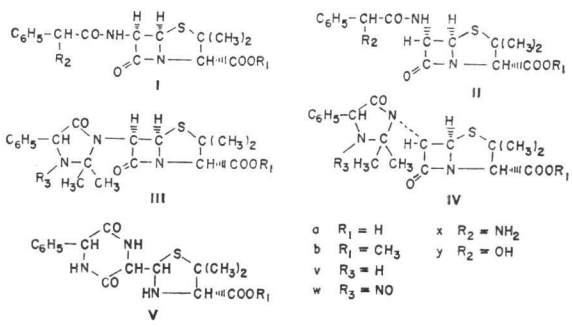
The preparation 6-epi-ampicillin by hydrolysis of 6-epihetacillin is described. During this conversion, the formation of a diketopiperazine was also observed. The best yield was obtained at pH 7.0 and room temperature for 3~7 hours. The lowest yield of 6-epi-ampicillin and the highest formation of the diketopiperazine occurred in pyridine - acetic acid - water. Treatment of ampicillin (with D-aminophenylacetyl side chain) with nitrous acid gave α-hydroxybenzylpenicillin with about 66% of L- and 34% mandelyl side chain. Reaction 6-epi-ampicillin gave 6-epi-α-hydroxybenzylpenicillin with practically the same ratio of L- and D-isomers.

Penicillin acylase of *Escherichia coli* not only hydrolyses benzylpenicillin but also some related compounds[1]. Because this enzyme preferentially cleaves L-phenylacetylamino acids, it has been stated that only compounds with an L-configuration can serve as substrates[2]. As 6-epi-benzylpenicillin, with a D-configuration at C–6, was available from previous work[3,4] we were interested in the behaviour of acylase towards this penicillin. We also wanted to examine related compounds like 6-epi-ampicillin and 6-epi-α-hydroxybenzylpenicillin.

Epimerisation of penicillins at C–6 was first described for methyl phthalimidopenicillanate[5] and hetacillin[6]. The epimerisation of other penicillin derivatives has been observed[3,4,7]. In solution hetacillin (**III av**) is in equilibrium with ampicillin (**Iax**)[8,9] but at a pH above 9.5, the formation of epi-hetacillin (**IVav**) also occurs[10,11]. Treatment of hetacillin with NaOH at pH 11.5 gave 6-epi-hetacillin in 82% yield[6]. We could obtain a somewhat better yield (96%) when the epimerisation was performed with 1, 5-diazabicyclo [4.3.0] non-5-ene (DBN) on silylated hetacillin. The epimerisation of hetacillin methylester (**IIIbv**) also was faster and gave a better yield when DBN was used instead of triethylamine[6].



The N-2-nitro-4-methylcarboxyl phenyl derivative of 6-epi-ampicillin was an intermediate in the transformation of 6-epi-hetacillin into 6-epi-aminopenicillanic acid but it was not isolated[12]. The hydrolysis of 6-epi-hetacillin (**IV av**) into 6-epi-ampicillin (**II ax**) in aqueous solution has been mentioned, but the product was only described as the benzyl ester[13]. When we repeated this reaction, we found that a secondary product was formed, in varying amounts. It was characterised[14] as the diketopiperazine **Va**. The formation of a diketopiperazine occurs only to a small extent with ampicillin, but it is an

* Present address: Pharmaceutical Institute, Universitaire Instelling Antwerpen.

important degradation reaction for cephalosporins having an aminogroup in the side chain[15,16].

The transformation of 6-epi-hetacillin (**IV av**) into 6-epi-ampicillin (**II ax**) was studied under different conditions. After storage for 1~8 days in methanol-acetic acid-water only 30% of product could be isolated, which consisted of 30~45% of diketopiperazine (**V a**) and 45~35% of 6-epi-ampicillin (**II ax**). Keeping epi-hetacillin in pyridine-acetic acid-water gave only the diketopiperazine (**V a**) in high yield. The best conversion was obtained in water at pH 7.0 and at room temperature for 3~7 hours. Some 65% of product could be isolated, and it was a mixture of 17~22% of diketopiperazine and 52~58% of 6-epi-ampicillin.

When ampicillin (**I ax**) was treated with nitrous acid, it was transformed into α-hydroxybenzylpenicillin (**I ay**). During this reaction partial racemisation and inversion of the configuration occurred in the side chain. The D-phenylglycyl part was transformed into a mixture of about 66% of L- and about 34% D-mandelyl side chain. This was deduced from the gas-liquid chromatogram of the trimethylsilyl derivative[17] of α-hydroxybenzylpenicillin and from the optical rotation of mandelic acid, obtained by hydrolysis of the penicillin. The same modification of the configuration of the side chain occurred during the transformation of 6-epi-ampicillin (**II ax**) into 6-epi-α-hydroxybenzylpenicillin (**II ay**).

The transformation of α-hydroxybenzylpenicillin (**I ay**) into its 6-epimer by trimethylsilylation and treatment with DBN[4] was attempted. Only extensive decomposition was observed.

We observed the β-lactam peak in the infrared spectrum at 1795 cm^{-1} for hetacillin and at 1770 cm^{-1} for the 6-epimer. The converse was published for these products[6], but these data are incorrect because they were inverted during the writing of the article[18]. The β-lactam peak of the 6-epimer of ampicillin and of α-hydroxybenzylpenicillin also occurs at a lower wave number than those of the products with normal C–6 configuration. As the same observation was made for the 6-epimers of benzyl- and phenoxy-methylpenicillin[3,4], we believe that this shift is a general rule. It should be noted that a peak of the imidazolone part (called γ-lactam in the experimental part) is present in the spectra of the hetacillins.

Experimental

The melting points were determined with a Büchi-Tottoli apparatus and are corrected. The optical rotation was measured at room temperature on a Zeiss-Winkel polarimeter. For tlc we used Merck precoated silica gel F–254 plates, in benzene-acetone (80: 20) for esters and in acetone-acetic acid (95: 5) for free acids and their salts. Spots were located by U. V. illumination and exposure to iodine vapour. Column chromatography was performed over silica gel (Merck 0.05~0.2 mm).

IR-spectra were run on a Perkin-Elmer 257 spectrometer for KBr discs. Mass spectra were recorded on a single focusing AEI–MS 12 apparatus, operating at 8 kV accelerating voltage, 100 μA trapcurrent and 70 eV ionization energy. N. m. r. were taken on a Varian A 60 or XL 100 spectrometer with tetramethylsilane (TMS), hexamethyldisiloxane (HMDS) or sodium 2, 2-dimethyl-2-sila-pentane-5-sulphonate (DSSA) as internal standard. The first-order coupling constants are the measured peak spacings. GLC analysis was performed with a Pye Series 104 gas chromatograph equipped with hydrogen-flame ionization detector. A 4-mm i. d. × 150 mm glass U-tube column packed with 3% OV–17 on Gas-Chrom Q (100~120 mesh) (Applied Science Laboratories) was used. Before using the polar groups of the column were silylated with a 5% solution of BSA in dry acetone. The operating conditions were a gas flow rate of 60 ml min^{-1} for nitrogen with air and hydrogen calibrated for optimum sensitivity, a column oven temperature of 230°C and injector and detector temperature of 270°C. The purity of the 6-epi-penicillin was determined by iodometric assay according to ALICINO[19] and was expressed in percentage. The biotape method described by COLE[20] was used for microbiological assay.

For the combined gas chromatography-mass spectrometry, a 1: 2 stream splitter divided the outlet of the column between the flame ionization detector and the mass spectrometer. A membrane separator Varian (Type V 5620) allowed the eluted substances to flow into the ion source. The temperature of the separator and ion source was maintained 30~40°C above the column temperature.

Hetacillin (III av)

Hetacillin was prepared from ampicillin anhydrate according to HARDCASTLE *et al.*[21] in 62% yield, m.p. 181~183°C (decomp.), $[\alpha]_D^{20}$ +343° (*c* 0.5, pyridine), Rf 0.60, ν_{max} 3255 (NH), 1797 (β-lactam), 1725 (γ-lactam), 1705 (sh, COOH) cm^{-1}, δ($CDCl_3$-$DMSO_{d6}$ 3: 1, TMS), 1.46 (s, CH_3–C–S), 1.51 (s, CH_3–C–S), 1.54 (s, CH_3–C–N), 1.66 (s, CH_3–C–N), 4.38 (s, 3–H), 4.62 (s, C_5H_5–CH), 4.83 (d, J=4Hz, 5–H), 5.51 (d, J=4 Hz, 6–H), 7.19 to 7.71 (m, phenyl). HARDCASTLE *et al.*[21] found a m.p. of 183~184°C (decomp.) and $[\alpha]_D^{20}$ +366° (*c* 1, pyridine), while JOHNSON *et al.*[6] gave a $[\alpha]_D^{20}$ +343° for the same product.

Hetacillin Methyl Ester (III bv)

Esterification of hetacillin with CH_2N_2 and crystallization from ether-*n*-pentane gave hetacillin methyl ester in 77% yield, m. p. 99.5~100°C (decomp.), $[\alpha]_D^{20}$ +321° (*c* 0.5, pyridine), Rf 0.10, *m/e* 403, ν_{max} 3365 (NH), 1782 (β-lactam), 1750, 1210 (ester), 1705 (γ-lactam) cm^{-1}, δ ($CDCl_3$, TMS), 1.48 (s, CH_3–C–S), 1.52 (s, CH_3–C–S), 1.70 (s, $2CH_3$–C–N), 2.23 (s, NH), 3.74 (s, OCH_3), 4.54 (s, 3–H), 4.66 (s, C_6H_5–CH), 4.75 (d, J=4 Hz, 5–H), 5.57 (d, J=4 Hz, 6–H), 7.16 to 7.60 (m, phenyl). HARDCASTLE *et al.*[21] found a m.p. of 101.5~102°C (decomp.).

6-Epi-hetacillin (IV av)

(a) Epimerization in aqueous solution. Hetacillin (95.2 g, 245 mmoles) was dissolved in 750 ml of 0.24 M NaOH and stirred for 30 minutes at room temperature, while the pH was kept at 11.5. The yellow solution was filtered and acidified with 4 N HCl (52 ml) to pH 2.0. After storage at 5°C the 6-epi-hetacillin was filtered off, washed twice with ice-water and twice with cold dry acetone. After drying over P_2O_5 *in vacuo* 78.0 g (82%) 6-epi-hetacillin was obtained, m.p. 162~164°C (decomp.), $[\alpha]_D^{20}$ +231° (*c* 1, pyridine), Rf 0.70, ν_{max} 3590, 3510 (water, OH), 2900 to 2300 (COOH, dimer), 1770 (β-lactam), 1740, (γ-lactam), 1710 (sh, COOH), 772, 698 (phenyl) cm^{-1}, δ ($CDCl_3$–$DMSO_{d6}$ 3: 1, TMS), 1.46 (s, CH_3–C–S), 1.52 (s, CH_3–C–S), 1.52 (s, CH_3–C–N), 1.59 (s, CH_3–C–N), 4.41 (s, 3-H), 4.49 (d, J=1.8 Hz, 6–H), 4.66 (s, C_6H_5–CH), 5.38 (d, J=1.8 Hz, 5–H), 7.17 to 7.60 (m, phenyl).

The biological activity against *Micrococcus pyogenes* var. *aureus* ATCC 6538 P as compared with ampicillin was 1.14% (biotape method). Therefore 6-epi-hetacillin was suspended in 700 ml methanol and stirred vigorously for 30 minutes. The undissolved product was filtered off, washed twice with acetone and dried over P_2O_5 *in vacuo* yielding 66.7 g (70%) of pure 6-epi-hetacillin, m.p. 162~164°C (decomp.), $[\alpha]_D^{20}$ +230° (*c* 1, pyridine). Biological activity as compared with ampicillin was 0.37% (biotape method). A m.p. of 164~165°C (decomp.) and $[\alpha]_D^{20}$ +232° (*c* 1, pyridine) was reported by JOHNSON *et al.*[6]

(b) Epimerisation with BSA and DBN.

Hetacillin (1.17 g, 3 mmoles) was suspended in 15 ml dry methylene chloride and chilled to 0°C. BSA (0.75 ml, 3 mmoles) was added and the suspension was stirred for 15 minutes. The hetacillin dissolved and the solution became yellow. DBN (0.36 ml, 3 mmoles) was added at 0°C and stirring was continued for another 15 minutes at 0°C. The solution was poured into a mixture of 15 ml ice-water and 3 ml 2 N HCl. The pH of the slurry was brought at 2.3 by addition of 2 N HCl. The precipitated 6-epi-hetacillin was collected, washed two times with cold water, twice with dry acetone and finally with dry ether. After drying *in vacuo* the yield was 1.12 g (96%).

The product was identical in all respects with the product described under (a). There was no penicillin with natural configuration present as assayed by the penicillinase method[4].

6-Epi-hetacillin Methyl Ester (IV bv)

(a) Methylation of 6-epi-hetacillin.

Esterification of 6-epi-hetacillin with CH_2N_2 and crystallisation from ether gave 6-epi-hetacillin methyl ester in 79% yield, m. p. 158~159°C, $[\alpha]_D^{20}$ +202° (*c* 0.5, pyridine), *m/e* 403, Rf 0.29, ν_{max} 3335 (NH), 1775 (β-lactam), 1742 (γ-lactam and ester), 1210 (ester), 1685 cm^{-1}, δ ($CDCl_3$, TMS), 1.46 (s, CH_3–C–S), 1.52 (s, CH_3–C–N), 1.60 (s, CH_3–C–N), 2.23 (s, NH), 3.72 (s, OCH_3), 4.56 (s, 3–H), 4.62 (d,

J=1.5 Hz, 6–H), 4.65 (s, C_6H_5–CH), 5.44 (d, J=1.5 Hz, 5–H), 7.19 to 7.60 (m, phenyl).

(b) Epimerisation with DBN in CH_2Cl_2.

A solution of hetacillin methyl ester (403.4 mg, 1 mmole) in 5 ml dry CH_2Cl_2 was chilled to 0°C and treated with a solution of DBN (0.12 ml, 1 mmole) in 1 ml dry CH_2Cl_2. The progress of the reaction was followed by tlc. Within 2 minutes the equilibrium was reached.

After 15 minutes, the solution was diluted with 25 ml CH_2Cl_2 and poured into a mixture of 20 ml ice-water and 1 ml 1 N AcOH. The organic layer was separated and the water layer was extracted two times with 10 ml CH_2Cl_2. The combined organic layer was washed two times with 20 ml ice-water, dried (Na_2SO_4) and evaporated to dryness. After triturating with 20 ml dry ether, 315 mg (78%) 6-epi-hetacillin methyl ester crystallised, m.p. 157~158°C, $[\alpha]_D^{20}$ +199° (*c* 0.5, pyridine). Spectral data were in agreement with those described in the previous section.

(c) Epimerisation with triethylamine in CH_2Cl_2.

A solution of hetacillin methyl ester (403.4 mg, 1 mmole) in 4 ml dry CH_2Cl_2 was chilled to 0°C and treated with triethylamine (0.7 ml, 5 mmoles). The progress of the reaction was followed by tlc. After 120 hours storage at 0°C, the equilibrium was not yet reached. After 14 days the coloured reaction mixture was diluted with 20 ml CH_2Cl_2, washed three times with ice-water, dried (Na_2SO_4) and evaporated to a yellow oil, which was crystallised from CH_2Cl_2 - *n*-pentane. Several recrystallisations were necessary to obtain 150.0 mg (37%) 6-epi-hetacillin methyl ester, m.p. 155~157°C. A m.p. of 156~158°C and $[\alpha]_D^{20}$ +202° (*c* 1, pyridine) was reported by JOHNSON *et al.*[6]

6-Epi-ampicillin (**II ax**)

A suspension of 6-epi-hetacillin (35.0 g, 90 mmoles) in 700 ml water at 32°C was adjusted to pH 7.0 by dropwise addition of a saturated barium hydroxide solution over a period of 30 minutes. The solution was diluted to 875 ml with water, kept for 3 hours at 32°C and brought to pH 7.0 by addition of 2 N H_2SO_4 solution. The solution was chilled to 0°C and acidified with 2 N H_2SO_2 to pH 2.0 under vigorous stirring. The precipitate ($BaSO_4$) was washed twice with ice-water and discarded. The filtrate was again adjusted to pH 4.5 with saturated barium hydroxide solution, concentrated *in vacuo* below 30°C to 50 ml and filtered. The filtrate was freeze-dried yielding 27.0 g (86%) 6-epi-ampicillin, m.p. 189~192°C (decomp.) $[\alpha]_D^{20}$ +134° (*c* 0.5, water), Rf 0.15, ν_{max} 3060~2700, 2610 (NH_3^+), 1765 (β-lactam), 1670 (amide), 1640~1510 (COO^-, NH_3^+), 1392 (COO^-) cm^{-1}, δ (NaOD–D_2O, DSSA), 1.47 (s, CH_3), 1.53 (s, CH_3), 4.27 (s, 3–H), 5.10 (s, C_6H_5–CH), 5.21 and 5.23 (AB, J=1.5 Hz, 5–H and 6–H), 7.45 (s, phenyl). The purity as assayed by the iodometric method was 74.9%. From the blank the presence of 15.4% of the diketopiperazine (**V**) was deduced. Loss on drying (3 hours at 60°C *in vacuo* over P_2O_5) was 5.68%. There was no penicillin present with natural configuration as assayed by the penicillinase method[4]. In the interpretation of the NMR spectrum, the peaks corresponding to **V** were eliminated.

A similar preparation from 50.0 g of 6-epi-hetacillin but using NaOH as base and HCl as acid afforded 41 g of 6-epi-ampicillin (yield 62%, taking into account 9.6 g of starting product which could be recovered). The purity was 58.3% and 17.3% of **V** was present. This product contained a much larger quantity of salt than the epi-ampicillin prepared using Ba $(OH)_2$ and H_2SO_4.

2-(6′-Phenylpiperazin-2′, 5′-dion-3′yl) 5, 5-dimethylthiazolidine-4-carboxylic acid (**V a**).

(a) From neutral aqueous solution.

A suspension of 6-epi-hetacillin (1.0 g, 2.56 mmoles) in 10 ml water was brought at pH 7.0 with 1 N NaOH solution. The solution obtained was held at room temperature for five days. The yellow solution which contained mainly the degradation product was brought to pH 3.2 with 0.5 N HCl solution under vigorous stirring. The precipitate was collected by filtration and washed several times with cold water. After drying *in vacuo* over P_2O_5, the yield was 470 mg (52%), m.p. 189~191°C (decomp.), $[\alpha]_D^{20}$ +222° (*c* 0.5, 0.05 N $NaHCO_3$), Rf 0.48, ν_{max} 3340~3300, 1660, 1450, 1330~1290 (amide), 3200 (amine), 1720~1700 (sh, COOH), 1130 (sec, amine) cm^{-1}, δ ($DMSO_{d6}$, HMDS), 1.14 (s, CH_3), 1.37 (s, CH_3), 3.62 (s, 4–H), 4.19 (m, 3′–H), 4.96 (m, 6′–H), 5.17 (d, J=4 Hz, 2–H), 7.22 to 7.68 (m, phenyl), 8.08 (d, J=2.5 Hz, 4′–H) and 8.53 (d, J=2 Hz, 1′–H).

On addition of D_2O the amide and amine protons disappear, the multiplets at 4.19 and 4.96 collapse to doublets with J 4 Hz and J 1 Hz respectively. The compound shows a spectrofluorimetric exci-

tation maximum at 363 nm and emission maximum at 453 nm (uncorrected). U. V. (1 N $NaHCO_3$) λ_{max} 304 nm (ε: 314), 2 N HCl λ_{max} 303.5 nm (ε: 496).

(b) From aqueous pyridine-acetic acid.

6-Epi-hetacillin (1.0 g, 2.56 mmoles) was dissolved in 25 ml of a mixture of pyridine - acetic acid - water, (20: 1: 80). The solution was kept for 8 days at 5°C. The mixture was acidified under vigorous stirring to pH 3.2 with 40% phosphoric acid solution. The precipitate was collected by filtration and washed several times with cold water. After drying *in vacuo* over P_2O_5, the yield was 644 mg (72%), m.p. 188~190°C (decomp.), $[\alpha]_D^{20}$ +222° (*c* 0.5, 0.05 N $NaHCO_3$). The spectral data were identical with those described under (a).

Electrometric titration gave a purity of about 95%. This product consumes 6 equivalents of iodine in the iodometric titration. This value was used for the correction of the blank of the iodometric assay of 6-epi-ampicillin.

Methyl 2-(6′-phenylpiperazin-2′, 5′-dion-3′yl) 5, 5-dimethyl-thiazolidine-4-carboxylate (**V b**).

A suspension of 2-(6′-phenylpiperazin-2′, 5′-dion-3′yl) 5, 5-dimethylthiazolidine-4-carboxylic acid (1.0 g, 2.86 mmoles) in 20 ml ether and 0.5 ml water was chilled to 0°C and treated with an etheral solution of diazomethane until the yellow colour persisted. The suspension was kept for 2 hours at 0°C, concentrated to near dryness and triturated with 20 ml dry ether. The gelatinous precipitate was collected, washed twice with ether and dried *in vacuo* over P_2O_5, yielding 812 mg (78%), m.p. 218~220°C (decomp.), $[\alpha]_D^{20}$ +162° (*c* 0.5, pyridine), *m/e* 363, Rf 0.98, ν_{max} 3320, 1680~1660, 1442, 1332 (amide), 3210 (amine), 1738, 1209 (ester) cm^{-1}, δ ($DMSO_{d6}$, HMDS), 1.11 (s, CH_3), 1.37 (s, CH_3), 3.64 (s, OCH_3), 3.73 (d, J=12 Hz, 4–H), 4.23 (m, 3′–H), 4.35 (dd, J=9 and 12 Hz, 3–H), 4.97 (m, 6′–H), 5.20 (dd, J= 4 and 9 Hz, 2–H), 7.23 to 7.65 (m, phenyl), 8.12 (d, J=2.5 Hz, 4′–H) and 8.55 (d, J=2 Hz, 1′–H).

On addition of D_2O the amide and amine protons disappear, the multiplets at 4.23 and 4.97 collapse to a double doublet with J=1 and 4 Hz and to a doublet with J=1 Hz respectively, and the double doublet at 5.20 collapses to a doublet with 4 Hz.

The ion at 377 in the mass spectrum indicated that the product contained a small amount of the N-methylated derivative.

α-Hydroxybenzylpenicillin (**I ay**)

Ampicillin anhydrate (20.0 g, 57.2 mmoles) was suspended in 400 ml ice-water and the pH was adjusted to 4.2 by addition of 5% phosphoric acid solution. A solution of sodium nitrite (5.0 g, 72.4 mmoles) in 70 ml ice-water was added under vigorous stirring over a period of 1 hour. The pH was kept at pH 4.2 by simultaneous addition of dilute phosphoric acid for 1 hour at 0°C and for 3 hours at room temperature.

The suspension turned into a yellow foam and was, after chilling to 0°C, acidified with dilute phosphoric acid to pH 2.5. A solution of 5 g ammonium sulfamate in 10 ml ice-water was added and the mixture was stirred rapidly for 15 minutes in order to destroy excess nitrous acid. The mixture was covered with 500 ml cold *n*-butyl acetate and acidified to pH 2.0. The organic layer was separated and the aqueous layer extracted three times with 200 ml *n*-butyl acetate. The combined organic layer was washed with two 200 ml portions ice-water, dried ($MgSO_4$) and decolourised with active carbon. The solution was filtered and the filtrate was treated with 20 ml 2 M potassium 2-ethylhexanoate in *n*-butyl acetate. Crystals soon appeared and the mixture was stored at 5°C for 16 hours. The crystals were collected by filtration, washed two times with 50 ml *n*-butyl acetate, twice with dry acetone and dried over P_2O_5 *in vacuo*, yielding 15.3 g (69%) α-hydroxybenzylpenicillin potassium salt, m. p. 204~205°C (decomp.), $[\alpha]_D^{20}$ +224° (*c* 0.5, H_2O), Rf 0.68, ν_{max} 3400~3340 (OH), 3300~3260, 1682, 1510, 1326 (amide), 1780 (β-lactam), 1598, 1395 (COO^-), 1058 (sec. OH) cm^{-1}, δ (D_2O, DSSA), 1.48 (s, CH_3), 1.58 (s, CH_3), 4.25 (s, 3–H), 5.21 (s, C_6H_5–CH), 5.45 (d, J=4 Hz, 5–H), 5.49 (d, J=4 Hz, 6–H), 7.36 (s, phenyl).

The purity assayed by the iodometric method gave 86.00%. The penicilloic acid content was 1.07%.

The product (3 mg) was dissolved in 1 ml of 1: 1 mixture of bis-(trimethylsilyl) acetamide (BSA) and trimethylchlorosilane (TMCS). After 30 minutes, the solution was injected on the column of the gas-chromatograph. The compound consisted of a mixture of 69% L-α-hydroxybenzylpenicillin (re-retention time 15.8 min.) and 31% D-α-hydroxybenzylpenicillin (retention time 18.0 min).

Unchanged ampicillin was recovered from the aqueous layer when the pH was adjusted to pH 4.5 with 4 N NaOH solution and concentrated under reduced pressure to 50 ml. The precipitate was collected, washed twice with 10 ml ice-water and 10 ml dry acetone yielding 4.02 g (20%) ampicillin.

COLE[20] reported an $[\alpha]_D^{20}+277°$ for the L-α-hydroxybenzyl-penicillin potassium with a purity of 92% and an $[\alpha]_D^{20}+168°$ for the D-α-hydroxybenzylpenicillin with the same purity.

Isolation of Mandelic Acid from α-Hydroxybenzylpenicillin.

A suspension of α-hydroxybenzylpenicillin potassium salt (2.0 g, 5.14 mmoles) in 20 ml 4 N HCl was refluxed for 1 hour. The product dissolved and the yellow solution was cooled and salted out with NaCl. The reaction mixture was extracted five times with 20 ml ether, dried (Na_2SO_4), evaporated *in vacuo* to dryness and crystallized from dry benzene to yield 639 mg (82%) of mandelic acid, m.p. 118°C, $[\alpha]_D^{20}+49.4°$ (*c* 0.5, EtOH). The rotation value indicated that the compound is a mixture of 66% L(+) and 34% D(−) mandelic acid since the pure D(−) and L(+) mandelic acid possess a value of −149.7° (EtOH) and +151.0° (EtOH) respectively[22].

α-Hydroxybenzylpenicillin Methyl Ester (**I by**).

A solution of α-hydroxybenzylpenicillin potassium salt (2.0 g, 5.14 mmoles) in 30 ml ice-water was covered with an equal volume ice-cold ether. While stirring the solution was acidified to pH 2.0 with 40% H_3PO_4. The organic layer was separated and the aqueous layer was extracted twice with 30 ml ice-cold ether. The combined ether layer was washed with ice-water and dried (Na_2SO_4). After filtration a solution of diazomethane in ether was added dropwise while stirring until a yellow colour persisted. The solution was concentrated under reduced pressure until the solvent was completely removed. The light-yellow oil weighed 1.356 g (72%), $[\alpha]_D^{20}+154°$ (*c* 1, acetone), Rf 0.24, *m/e* 364, ν_{max} 3400~3360 (polymeric OH), 1785 (β-lactam), 1743, 1212 (ester), 1676, 1510, 1300 (amide), 1060 (sec, OH) cm^{-1}, δ ($CDCl_3$, TMS), 1.42 (s, CH_3), 1.55 (s, CH_3), 3.68 (s, OCH_3), 4.04 (br, OH), 4.34 (s, 3–H), 4.96 (s, C_6H_5–CH), 5.34 (d, J=4 Hz, 5–H), 5.42 (dd, J=4 and 9 Hz, 6–H), 7.26 (s, phenyl), 7.42 (d, J=9 Hz, NH).

6-Epi-α-hydroxybenzylpenicillin (**II ay**).

A solution of 6-epi-ampicillin (29.3 g, 62.8 mmoles, purity 74.9%, containing 15.4% diketopiperazine derivative and less than 5% 6-epi-hetacillin) in 600 ml ice-water was treated as described under α-hydroxybenzylpenicillin. The reaction mixture was covered with 500 ml cold ether and acidified to pH 2.1. The product which did not dissolve after shaking was filtered off and the aqueous layer was extracted twice with 500 ml cold ether. The combined organic layer was washed with two portions of 100 ml cold water, dried (Na_2SO_4) and decolourised with active charcoal. After addition of 200 ml ice-water, the pH raised to 6.5, under vigorous stirring, with 1 N KOH solution (36.4 ml). The aqueous layer was separated and the ether layer extracted once with 100 ml water. The combined aqueous layer was freed from traces ether and lyophilised. The yield was 14.45 g (59%) 6-epi-α-hydroxybenzylpenicillin potassium salt, m.p. 178~180°C (decomp.), $[\alpha]_D^{20}+177°$ (*c* 0.5, H_2O), Rf 0.70, ν_{max} 3600~3200 (OH), 1760 (β-lactam), 1680~1650, 1515, 1330~1300 (amide), 1605, 1390 (COO^-), 1062 (sec. OH) cm^{-1}, (D_2O, DSSA), 1.47 (s, CH_3), 1.55 (s, CH_3), 4.29 (s, 3–H), 4.88 (d, J=1.5 Hz, 6–H), 5.19 (s, C_6H_5–CH), 5.24 (d, J=1.5 Hz, 5–H), 7.41 (s, phenyl).

The purity assayed by the iodometric method was 72.1%. The penicilloic acid content was 2.9%. The product was very hygroscopic and loss on drying (60°C, *in vacuo* over P_2O_5) was 12.3%. Some 6-epi-N-nitrosohetacillin was present and could not be removed. The diketopiperazine, which was present in the starting product, reacted with nitrous acid, but it was not extracted into ether.

Isolation of Mandelic Acid from 6-Epi-α-hydroxybenzylpenicillin.

A suspension of 6-epi-α-hydroxybenzylpenicillin potassium salt (2.5 g, purity 48.8%, 3.14 mmoles) in 20 ml 4 N HCl was refluxed for 1 hour. The reaction mixture was worked up as described for the α-hydroxybenzylpenicillin potassium salt and yielded 400 mg (84%) mandelic acid, m.p. 118°C, $[\alpha]_D^{20}$ +49.5° (*c* 0.4, EtOH). The optical rotation indicated that mandelic acid is a mixture of 66% L(+) and 34% D(−) isomer.

6-Epi-α-hydroxybenzylpenicillin Methyl Ester (**II by**).

A solution of 6-epi-α-hydroxybenzylpenicillin potassium salt (0.5 g, 0.86 mmoles, purity 66.9%,

containing *ca.* 10% 6-epi-N-nitrosohetacillin potassium salt) was treated as described for α-hydroxybenzylpenicillin potassium salt. The yellow foam (418 mg) was chromatographed over 25 g silica gel using a gradient of benzene to benzene - ethylacetate (50: 50).

Fractions (20 ml) 74~80 yielded 27.0 mg (7.3%) of an oil which was identified as 6-epi-N-nitrosohetacillin methyl ester. Fractions (84~97) yielded 214.0 mg (68%) 6-epi-α-hydroxybenzylpenicillin methyl ester as a colourless oil, $[\alpha]_D^{20}+119°$ (*c* 1, acetone), Rf 0.18, *m/e* 364, ν_{max} 3340~3380 (OH), 1778 (β-lactam), 1745, 1212 (ester), 1672, 1510, 1305 (amide), 1060 (sec. OH) cm^{-1}, δ ($CDCl_3$, TMS), 1.42 (s, CH_3), 1.56 (s, CH_3), 3.68 (s, OCH_3), 4.06 (br, OH), 4.44 (s, 3-H), 5.06 (dd, J=1.6 and 8 Hz, 6-H), 5.11 (s, C_6H_5-CH), 5.15 (d, J=1.6 Hz, 5-H), 7.31 (s, phenyl), 7.63 (d, J=8 Hz, NH).

6-Epi-N-nitrosohetacillin Potassium Salt

To a suspension of 6-epi-hetacillin (4.0 g, 10.27 mmoles) in 120 ml ice-water at pH 3.5 was added under vigorous stirring at 0°~5°C and in a time interval of 30 minutes a solution of sodium nitrite (0.85 g, 12.32 mmoles) in 20 ml water. Meanwhile the suspension was kept at pH 3.5 by adding dropwise a solution of 10% phosphoric acid. Stirring was continued at the same pH for a further 1 hour 30 minutes at room temperature. The yellow suspension was then chilled to 0°C, acidified to pH 3.0 with dilute phosphoric acid, treated with a solution of 0.6 g ammonium sulfamate in 5 ml ice-water and stirred for 15 minutes.

The precipitate formed after addition of more dilute phosphoric acid to pH 2.0 was dissolved in 75 ml cold *n*-butyl acetate.

The organic layer was separated and the aqueous layer extracted twice with 25 ml *n*-butyl acetate. The combined organic layer was washed with two 25 ml portions of ice-water, dried (Na_2SO_4) and decolourised with active charcoal. The solution was filtered and a solution of 2 M potassium 2-ethylhexanoate in *n*-butyl acetate was added.

The crystals which appear slowly were collected, washed twice with *n*-butyl acetate and finally twice with dry acetone. After drying *in vacuo* over P_2O_5 the yield was 2.95 g (63%), m.p. 187~189°C (decomp.), $[\alpha]_D^{20}+171°$ (*c* 0.5, H_2O), Rf 0.77, ν_{max} 3700~3300 (H_2O), 1768 (β-lactam), 1717 (γ-lactam), 1610, 1412 (COO^-), 758, 698 (phenyl) cm^{-1}, δ (D_2O, DSSA), 1.52 (s, CH_3-C-S), 1.61 (s, CH_3-C-S), 2.00 (s, CH_3-C-N), 2.12 (s, CH_3-C-N), 4.39 (s, 3-H), 4.87 (d, J=1.7 Hz, 6-H), 5.60 (d, J=1.7 Hz, 5-H), 5.77 (s, C_6H_5-CH), 7.41 (s, phenyl).

The product between the organic and aqueous layer was collected. After drying over P_2O_5 *in vacuo* it weighed 0.865 g (22%), m.p. 163~164°C (decomp.). From the infrared spectrum and chromatographic behaviour, there is evidence that the product is starting material.

6-Epi-N-nitrosohetacillin (**IV aw**)

6-Epi-N-nitrosohetacillin was prepared by acidifying a suspension of the potassium salt (0.4 g, 0.87 mmoles) in 10 ml ice-water with 40% H_3PO_4 to pH 2.0. The compound could not be extracted with an organic solvent and was therefore collected, washed twice with ice-water and dried thoroughly *in vacuo* over P_2O_5 yielding 270 mg (74%) 6-epi-N-nitrosohetacillin acid, m.p. 166~168°C (decomp.), $[\alpha]_D^{20}+230°$ (*c* 0.5, pyridine), ν_{max} 3590, 3505 (OH), 2900~2200 (COOH dimer), 1768 (β-lactam), 1740 (γ-lactam, COOH), 1400~1490 (*gem.* dimethyl), 770, 698 (phenyl) cm^{-1}.

6-Epi-N-nitrosohetacillin Methyl Ester (**IV bw**)

A suspension of 6-epi-N-nitrosohetacillin potassium salt (1.00 g, 2.19 mmoles) in 10 ml cold water was treated as described for α-hydroxybenzylpenicillin potassium salt.

The reaction mixture was evaporated to dryness, the white foam crystallized upon addition of 50 ml dry ether. The product was collected, washed with dry ether and dried *in vacuo* yielding 703 mg (74%) 6-epi-N-nitrosohetacillin methyl ester, m.p. 173~174°C (decomp.), $[\alpha]_D^{20}+174°$ (*c* 0.5, pyridine), Rf 0.54, *m/e* 432, ν_{max} 1785 (β-lactam), 1728 (ester, γ-lactam), 1220 (ester), 758, 700 (phenyl) cm^{-1}, δ ($CDCl_3$, TMS), 1.47 (s, CH_3-C-S), 1.62 (s, CH_3-C-S), 2.03 (s, CH_3-C-N), 2.08 (s, CH_3-C-N), 3.75 (s, OCH_3), 4.61 (d, J=1.6 Hz, 6-H), 4.62 (s, 3-H), 5.56 (d, J=1.6 Hz, 5-H), 5.56 (s, C_6H_5-CH), 7.32 (m, C_6H_5).

Gas-Liquid Chromatography

The penicillin (3.0 mg) was suspended in dry acetone (1 ml) and a mixture of bis-(trimethylsilyl) acetamide and trimethylchlorosilane 1: 1 (1 ml) was added. The product dissolved and was stored at

room temperature for 30 minutes. Usually 1 μl of this solution was injected. An authentic sample of D-α-hydroxybenzylpenicillin sodium salt monohydrate kindly supplied by M. COLE was used as reference. The chromatographic peaks were shown to be the trimethylester of the intact penicillin as could be deduced from the observation of the molecular ion using a gas chromatograph coupled with the mass spectrometer.

Trimethylsilylester of phenoxymethylpenicillin (Rt 1.00; 16.5 min), of benzylpenicillin (Rt 0.80), of hetacillin (Rt 2.25), of N-nitrosohetacillin (Rt 1.74), or L-α-hydroxybenzylpenicillin (Rt 0.96) and of D-α-hydroxybenzylpenicillin (Rt 1.09).

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