RESOLUTION OF S-BENZYL-DL-PENICILLAMINE WITH PENICILLIN AMIDOHYDROLASE*

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Racemic N-phenylacetyl-S-benzylpenicillamine was resolved into optical isomers by action of penicillin amidohydrolase (E.C. 3.5.1.11.) in high yield and optical purity.

Optical resolution of S-benzyl-DL-penicillamine (β,β-dimethyl-S-benzylcysteine) has been achieved by crystallization of its salts with alcaloids[1-3]. The only one successfull enzymatic resolution consisted in cleavage of S-benzyl-DL-penicillamine amide with hog kidney amidase[4]. All other attempted resolutions with papain[5,6], ficin[5], L-amino acid oxidase or D-amino acid oxidase[4] failed. The treatment of N-acetyl-S-benzyl-(or S-methyl)-DL-penicillamine with acylase *I* was without success[4], the attempts to resolve racemic S-benzylpenicillamine by action of acylase *I* were also unsuccessful although various acyl groups such as formyl, chloroacetyl, or phenylacetyl were tried.

Penicillin G is transformed[7] into 6-aminopenicillanic acid by action of penicillin amidohydrolase (E.C.3.5.1.11.) from *Escherichia coli* cells. Removal of the phenylacetyl residue from some L-amino acids was described whereas acyl-D-amino acids were practically not hydrolyzed with this enzyme[8,9]. Resolution of some derivatives of amino acids, containing nucleobases, was reported[10] and the enzyme was employed also in correlation studies[11,12].

In the reaction of penicillin amidohydrolase with N-phenylacetyl-S-benzyl-DL-penicillamine** the phenylacetyl group is stereospecifically removed from the L-form and both enantiomers can be isolated in good yield and high optical purity. The use of the N-phenylacetyl group is essential because neither benzoyl nor chloroacetyl groups are cleaved off, although removal of the chloroacetyl group from other L-amino acids (phenylalanine, alanine, leucine) has been reported[8].

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** Part of the results was presented in the form of a preliminary communication[13].

The racemate was resolved also with enzyme, covalently bound to *B. megatherium* cells[14] or with immobilized *E. coli* cells of retained penicillin amidohydrolase activity[15] which enabled the recovery and re-use of the enzyme.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. The analytical samples were dried at room temperature and 150 Pa for 24 h. Thin layer chromatography was performed on silica gel coated plates (Silufol, Kavalier, Czechoslovakia). Solvent systems: S1 2-butanol–98% formic acid–water (75 : 13·5 : 11·5), S2 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5), S3 1-butanol–acetic acid–water (4 : 1 : 1), S4 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6). Spots were detected with ninhydrin or by the chlorination method. High performance liquid chromatography (HPLC) was carried out using a Separon SI C-18 column (Laboratorní přístroje, Prague) (15 × 0·6 cm) filled with spherical silica gel particles (6 μm diameter) with chemically bonded octadecyl chains. A 3 : 7 mixture of phosphate buffer (pH 4·4) and methanol was used as the mobile phase; detection by UV absorption at 230 nm. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter. Paper electrophoresis was performed in a moist chamber in 1M acetic acid (pH 2·4) or in a pyridine–acetate buffer (pH 5·7) on a Whatman No 3MM paper; 20 V/cm, 60 min.

Enzyme Preparations

Acylase *I* was isolated from hog kidneys[16]. Penicillin amidohydrolase (E.C. 3.5.1.11.) was obtained from *E. coli* cells[14]; 1 mg of this preparation liberated 8·5 μmol of 6-aminopenicillanic acid in 1 min. The activity was determined according to ref.[17]. As insoluble forms of the enzyme we used cells, immobilized by cross-linking, with penicillin amidohydrolase activity[15] (activity: 1 g of the moist material liberated 77 μmol of 6-aminopenicillanic acid in 1 min; particle size 100—800 μm, average 380 μm), or the enzyme bound to *B. megatherium* cells[14] (activity: 140 μmol of liberated 6-aminopenicillanic acid from 1 g of moist material in 1 min; particle size 200—650 μm).

S-Benzyl-DL-penicillamine was prepared according to ref.[1], m.p. 202—204°C; reported[1] m.p. 202°C and m.p. 201—202°C (ref.[18]). N-Benzoyl-S-benzyl-DL-penicillamine was synthesized according to ref.[18], m.p. 165—167°C, reported[18] m.p. 168—169°C and 156—159°C (ref.[1]).

N-Chloroacetyl-S-benzyl-DL-penicillamine

Chloroacetyl chloride (1·2 ml) and 4M-NaOH (4·2 ml) were added at 0°C to a stirred solution of S-benzyl-DL-penicillamine (2·4 g) in 1·5M-NaOH (11 ml) in the course of 45 min. After stirring for 1 h at room temperature, the mixture was acidified with hydrochloric acid (pH 2), and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, taken down, the residue dissolved in aqueous methanol and filtered through a column of Dowex 50. The eluates were concentrated to a small volume, cooled to 0°C and the separated crystals collected on filter and washed with water, affording 0·6 g (18%) of the product, m.p. 84—86°C (reported[1] m.p. 171—173°C). For $C_{14}H_{18}ClNO_3S.H_2O$ (333·8) calculated: 50·37% C, 5·43% H, 4·20% N, 10·62% Cl; found: 50·69% C, 5·50% H, 4·38% N, 10·19% Cl.

N-Formyl-S-benzyl-DL-penicillamine

A solution of S-benzyl-DL-penicillamine (2·4 g) in formic acid (5 ml) was heated to 100°C for 6 h. After cooling to 0°C the separated crystals were filtered and washed with formic acid and water, m.p. 155—158°C; yield 1·8 g (67%). The same melting point was reported[1] for the compound prepared by treatment with formic acid in acetic anhydride.

N-Phenylacetyl-S-benzyl-DL-penicillamine

Phenylacetyl chloride (1·4 ml) and 1M-NaOH (9 ml) were added at 0°C to stirred solution of S-benzyl-DL-penicillamine (2·4 g) in 1M-NaOH (11 ml) in the course of 45 min. After further stirring and cooling for 2 h, the mixture was acidified to pH 2, and the crystalline portion was filtered and washed with water, affording 2·2 g (61%) of the product, m.p. 98—99°C. For $C_{20}H_{23}NO_3S$ (357·5) calculated: 67·20% C, 6·48% H, 3·92% N; found: 67·20% C, 6·51% H, 3·99% N. Reported[1] m.p. 66—68°C.

Attempted Cleavage of N-Acyl Derivatives of S-Benzyl-DL-penicillamine with Acylase *I*

N-Formyl-S-benzyl-DL-penicillamine (100 mg) was incubated at 37°C with the acylase (30 mg) in 0·01M phosphate buffer, pH 7·8 (10 ml). After 4 h the mixture was analyzed by means of thin layer chromatography (S1): no spot corresponding to S-benzylpenicillamine was detected.

N-Chloroacetyl-S-benzyl-DL-penicillamine (60 mg) in water (3·6 ml) and 0·01M phosphate buffer, pH 7·5 (1·4 ml), was incubated with the acylase (20 mg) at 37°C for 12 h. Thin layer chromatography (S1) found no free S-benzylpenicillamine in the reaction mixture.

N-Phenylacetyl-S-benzyl-DL-penicillamine (50 mg) was incubated with the acylase (10 mg) in 0·1M phosphate buffer, pH 7·5 (3 ml), for 4 h. The mixture was analyzed as described above, with a negative result.

Attempted Cleavage of N-Benzoyl- and N-Chloroacetyl-S-benzyl-DL-penicillamine with Penicillin Amidohydrolase

The substrates (10 mg) were incubated at 37°C with the enzyme (5 mg) in a 0·2M phosphate buffer, pH 7·8 (0·3 ml). After 4 h the mixture was analyzed by means of HPLC; no peak corresponding to the free S-benzylpenicillamine was found.

S-Benzyl-L-penicillamine

A solution of N-phenylacetyl-S-benzyl-DL-penicillamine (20 g) in a 0·2M phosphate buffer, pH 7·6 (700 ml), and water (800 ml) was mixed with a solution of the enzyme, prepared by dissolution of penicillin amidohydrolase (0·5 g) in a 0·05M phosphate buffer, pH 7·2 (20 ml), and removal of a small insoluble portion by centrifugation. The mixture was incubated for 23 h at 40°C, its pH being maintained at 6·85. The mixture was centrifuged and the supernatant liquid acidified with 1M-HCl (pH 1·9) and cooled to 0°C. The separated crystals were filtered and washed with water; yield 10·5 g of crude N-phenylacetyl-S-benzyl-D-penicillamine.

The mother liquors were applied on a column of Dowex 50 (H^+-form), the column was washed with water and the product eluted with 10% aqueous pyridine. Evaporation of the eluates afforded 4·8 g (72%) of S-benzyl-L-penicillamine, m.p. 192—194°C; $[\alpha_D]$ +93·3° (*c* 0·2, 1M-HCl). R_F 0·55 (S1), 0·18 (S2), 0·49 (S3), 0·63 (S4); $E^{Gly}_{5.7}$ 1·0, $E^{Gly}_{2.4}$ 0·28, k' 0·66 (for the N-phenylacetyl

derivative $k' = 1{\cdot}33$, for phenylacetic acid $k' = 0{\cdot}34$). For $C_{12}H_{17}NO_2S$ (239·4) calculated: 60·22% C, 7·16% H, 5·85% N; found: 60·65% C, 7·28% H, 5·86% N. The literature reports: $[\alpha]_D$ +90° (1M-NaOH) and m.p. 197—198°C (ref.[1]), $[\alpha]_D$ +91·3° (1M-HCl) (ref.[4]), and $[\alpha]_D$ +97° (*c* 1, 1M-HCl), m.p. 184—186°C (ref.[2]).

S-Benzyl-D-penicillamine

A part (2 g) of the crude N-phenylacetyl-S-benzyl-D-penicillamine, obtained in the above experiment, was dissolved in 0·25M-$NaHCO_3$ (60 ml), the solution was filtered and acidified with 1M-HCl (pH 2). The separated crystals were collected on filter and washed with water, affording 0·95 g (48%) of the compound, m.p. 125—127°C, $[\alpha]_D$ −7·5° (*c* 0·2, 1M-NaOH). For $C_{20}H_{23}NO_3S$ (357·5) calculated: 67·20% C, 6·48% H, 3·92% N; found: 66·75% C, 6·32% H, 4·09% N. Reported[1] m.p. 141—142°C and $[\alpha]_D$ −7·5°. In order to remove the phenylacetyl group a portion of the compound (0·5 g) was suspended in a mixture of acetic acid (28 ml), concentrated hydrochloric acid (8 ml) and water (15 ml). After reflux for 1 h, the mixture became homogeneous and the refluxing was continued for additional 3 h. During this time concentrated hydrochloric acid (8 ml) was added in portions and the mixture was refluxed for 2 h more. The solution was taken down, the residue dissolved in water (100 ml), washed with ether and applied on a column of Dowex 50 (H^+-form; 20 ml). The column was washed with water and the product eluted with 10% aqueous pyridine. Evaporation of the eluate afforded 0·24 g (72%) of the product, m.p. 191—192°C, $[\alpha]_D$ −91·1°. Its chromatographic and electrophoretic behaviour was the same as reported for the L-form.

Resolution with Immobilized Enzyme

a) *With immobilized* E. coli *cells*: Cells of penicillin amidohydrolase activity (15 g) were added to a solution of N-phenylacetyl-S-benzyl-DL-penicillamine (21·5 g) in a mixture of water (176 ml), 1M-NaOH (63 ml) and 0·2M Na-phosphate buffer, pH 7·5 (50 ml). The resulting suspension (pH 7·3) was stirred magnetically at 37°C and the reaction course was followed by HPLC. After incubation for 120 h, the cells were separated by filtration through a textile filter, washed with water and used in further incubation. The solution was worked up in the same manner as described in the above experiment with the soluble enzyme, yielding 3·1 g (43%) of S-benzyl-L-penicillamine, m.p. 190—192°C, $[\alpha]_D$ +96·4° (*c* 0·2, 1M-HCl). The procedure was repeated with the same fraction of cells, affording 3·4 g (47%) of the product, m.p. 191—193°C, $[\alpha]_D$ +97·6° (*c* 0·2, 1M-HCl).

b) *With immobilized enzyme*: A suspension of the immobilized enzyme (3·7 ml) was added to a solution of N-phenylacetyl-S-benzyl-DL-penicillamine (0·5 g) in a mixture of water (2·5 ml) and 1M-NaOH (1·5 ml) which was adjusted to pH 7 with 0·2M-NaH_2PO_4 (0·5 ml). The mixture was incubated at 37°C for 24 h, the immobilized enzyme removed by filtration and the solution worked up as described in the experiment with the free enzyme; yield 122 mg (73%) of S-benzyl-L-penicillamine, m.p. 187—190°C; $[\alpha]_D$ +93·2° (*c* 0·2, 1M-HCl).

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