SYNTHESIS OF (β-METHYL-^{3}H)-BENZYLPENICILLIN AND (β-METHYL-^{3}H)-6-AMINOPENICILLANIC ACID

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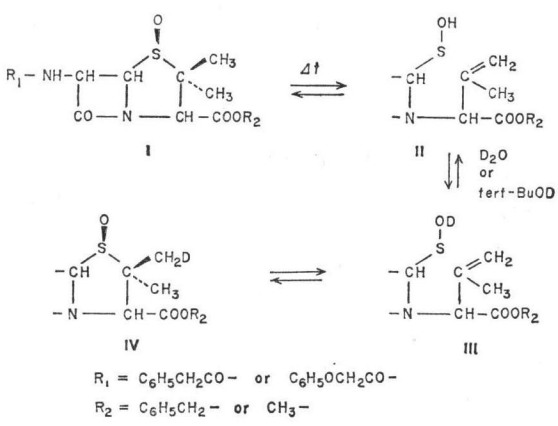
The synthesis of benzylpenicillin and 6-aminopenicillanic acid, labeled with tritium in the β-methyl group, is described. Benzylpenicillin S-sulfoxide benzyl ester is refluxed in benzene and tritiated water and is successively debenzylated and deoxygenated to (β-methyl-^{3}H)-benzylpenicillin. Removal of the side chain with a bacterial acylase gives (β-methyl-^{3}H)-6-aminopenicillanic acid.

Labeled penicillins are of interest in several fields of research, *e.g.* biosynthesis of the antibiotic, elucidation of its mode of action, and in pharmacological investigations. Only benzylpenicillin, labeled with (1-^{14}C)-phenylacetic acid, is commercially available; semisynthetic penicillins require the synthesis of the labeled side-chain for each new compound. Therefore, a better route to labeled penicillins is the synthesis of labeled 6-aminopenicillanic acid (6-APA), the common intermediate of all penicillins, to which different side chains can be attached.

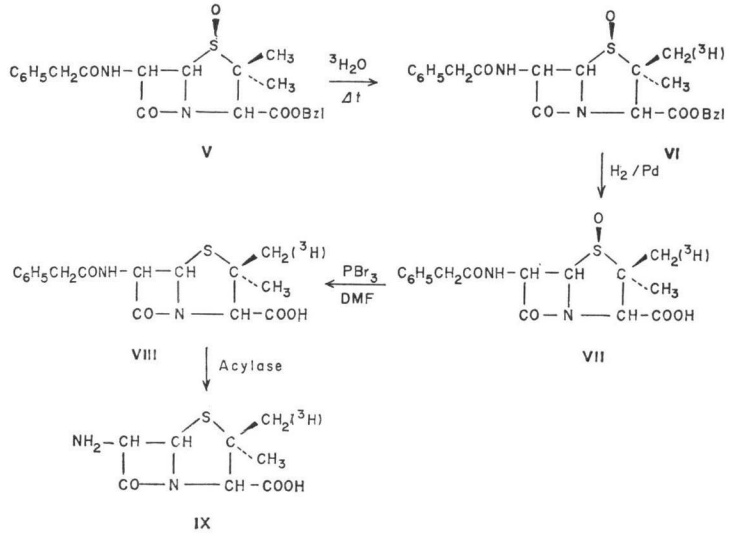
There are several possible methods for the preparation of labeled benzylpenicillin; from this compound, 6-APA can be obtained by the action of a bacterial acylase. Addition of (^{35}S)-sulfate to cultures of *Penicillium chrysogenum*, supplemented with phenylacetic acid, gives (^{35}S)-benzylpenicillin of high specific activity in 20~30 % yield[1,2]; however, the use of this isotope is restricted by its short half-life. Incorporation of labeled cystine or valine, the biochemical precursors of the penam ring system, gives (^{3}H) or (^{14}C)-labeled penicillin; the high cost of the amino acids and the low radiochemical yield limits their use to the preparation of small amounts of penicillin of low specific activity.

The penicillin sulfoxide-sulfenic acid equilibrium[3,4] has been used for the incorporation of deuterium into penicillin derivatives[5,6,7]. A penicillin S-sulfoxide ester (**I**) was heated in benzene with an excess of deuterated water or *tert*-butyl alcohol; the isotope was introduced specifically into the β-methyl group (**IV**) on carbon 2 through a sulfenic acid intermediate (**II** and **III**).



We used this reaction for the synthesis of tritiated benzylpenicillin and 6-APA. Benzylpenicillin S-sulfoxide benzyl ester (**V**)

was refluxed with benzene and tritiated water for 24 hours; the resulting (β-methyl-^{3}H)-S-sulfoxide ester (**VI**) was recrystallized from methanol. Hydrogenolysis in the presence of Pd/C gave (β-methyl-^{3}H)-benzylpenicillin S-sulfoxide (**VII**), which was deoxygenated with PBr_3/DMF to (β-methyl-^{3}H)-benzylpenicillin (**VIII**). (β-Methyl-^{3}H)-6-APA (IX) was obtained by hydrolysis with *Escherichia coli* NCIB 8743 acylase[8].



Experimental

Tritiated water (1 Ci/ml) was obtained from the Nationaal Instituut voor Radioelementen, I.R.E., Fleurus, Belgium.

E. coli NCIB 8743 was fermented in 300-ml Erlenmeyer flasks on a gyrotory shaker (240 rpm) at 27°C in 100 ml of the medium of SIKYTA and SLEZAK[9], supplemented with 0.1 % ammonium sulfate and 0.1 % phenylacetic acid, for 27 hours; 1 ml of 10 % potassium phenylacetate was added after 15, 18 and 21 hours. The bacterial suspension (5 liters) was centrifuged, washed with water and stored at 4°C in 75 ml of water.

Radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3390, with absolute activity analyzer, model 544. Standardization was performed with (^{3}H)-*n*-hexadecane. All samples were combusted with cellulose in a Packard sample oxidizer, model 306; for aqueous samples, 0.2 ml of Combustaid (Packard) for 0.5 ml of water was added.

For t.l.c., Merck precoated silica gel F-254 plates were used with the following solvent systems: benzene-acetone (80:20) for the ester, and acetone-acetic acid (95:5) for free acids and their salts. Spots were located by U.V. illumination and exposure to iodine vapour. Scanning was performed with a Packard radiochromatogram scanner, model 7200.

I.r. spectra (KBr-discs) were run on a Perkin-Elmer 257 spectrometer. N.m.r. spectra (D_2O) were taken on a Hitachi Perkin-Elmer spectrometer, model R-24, with sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSSA) as internal standard.

The purity of penicillin and 6-APA was determined by iodometric titration, according to ALICINO[10].

(β-Methyl-^{3}H)-benzylpenicillin S-sulfoxide benzyl ester (**VI**)

Tritiation was performed in a 250 ml flask with Liebig-cooler, put at an angle of 120°, and equipped with a side-tube which acted as a Dean and Stark water trap when operated

downwards. Two preparations were carried out. Benzylpenicillin S-sulfoxide benzyl ester (6.60 g, 15 mmol; dried in vacuum over P_2O_5)[11] was refluxed with 75 ml of sodium dried benzene and 0.5 ml of ^3H_2O (500 mCi) for 24 hours. After removal of the tritiated water by azeotropic distillation, the solution was evaporated to dryness. Labile tritium was exchanged with 50 ml of methanol-dichloromethane 4:1 for 3 periods of 3 hours. The product was crystallized from 40 ml of methanol. Yield 4.98 g; specific activity 1977 μCi/mmol. A second fraction (0.24 g) was obtained by concentration of the mother liquor and recrystallization from methanol-ether.

For the second preparation, the recovered ^3H_2O was refluxed with 3.30 g (7.5 mmol) of benzylpenicillin S-sulfoxide benzyl ester and 40 ml of benzene. Yield 2.60 g; specific activity 1641 μCi/mmol. Second fraction 0.13 g.

The first fractions of both preparations were combined and recrystallized from 75 ml of methanol; yield 7.10 g; specific activity 1838 μCi/mmol. The second fractions were mixed with the mother liquor and recrystallized twice from methanol; yield 0.495 g; specific activity 1833 μCi/mmol. Both products gave one spot on t.l.c. and were pure on scanning; Rf=0.52.

Total chemical yield 7.595 g=76.7 %; radiochemical yield 31.68 mCi=6.33 %.

(β-Methyl-^{3}H)-benzylpenicillin S-sulfoxide (**VII**)

A solution of 3.75 g of **VI** (8.5 mmol, 15.62 mCi) in 250 ml of ethyl acetate was hydrogenated over 10 % Pd-C (4.2 g) for 4 hours at room temperature and at a pressure of 3.5 kg cm^{-1}. The catalyst was filtered off and washed with ethyl acetate (6×75 ml). The combined filtrates were concentrated to 5 ml and the sulfoxide was crystallized by the addition of 25 ml of ether. Yield 2.11 g=70.8 %. Radiochemical yield 10.66 mCi=68.2 %. The presence of some active carbon from the catalyst lowered the specific activity to 1772 μCi/mmol. Scanning of a chromatogram showed that 98 % of the radioactivity was present in the sulfoxide (Rf=0.48), 1 % at the starting point and 1 % in the benzyl ester; both impurities were easily removed in the following step.

(β-Methyl-^{3}H)-benzylpenicillin (**VIII**)

A solution of 2.015 g of **VII** (5.75 mmol, 10.19 mCi) in 50 ml of anhydrous DMF was cooled to −20°C and treated with PBr_3 (4.3 ml) for 30 seconds. The reaction mixture was poured into an ice-cold suspension of 20 g of $NaHCO_3$ in 175 ml of water. The solution was covered with 150 ml of ethyl acetate and acidified to pH 2.2 at 0~2°C with 20 % H_3PO_4. After separation, the aqueous layer was extracted once more with 75 ml of ethyl acetate. The combined organic layer was washed with 10 ml of ice-water and with 10 ml of a saturated solution of NaCl, shaken with anhydrous Na_2SO_4 and filtered. After addition of 3 ml of 2 M potassium 2-ethylhexanoate in acetone, the solution was concentrated under vacuum to 25 ml; the potassium salt of benzylpenicillin crystallized after mixing with 300 ml of acetone. Yield 1.38 g =64.4 %. Radiochemical yield 6.40 mCi=62.8 %. Specific activity 1728 μCi/mmol. Iodometric assay showed that the product contained 95.0 % penicillin and 0.3 % penicilloic acid; when corrected for purity, the specific activity of the penicillin was 1813 μCi/mmol (=98.6 % of the benzyl ester). Scanning of a t.l.c. resulted in 99.4 % penicillin (Rf=0.57) and 0.6 % penicillinoic acid (Rf=0). I.r. γ_{max} 3370, 1672, 1495 (amide), 1780 (β-lactam), 1615 (COO^-), 760, 698 (phenyl) cm^{-1}. N.m.r. δ 1.46 (s, CH_3), 1.53 (s, CH_3), 3.57 (s, CH_2-CO), 4.11 (s, 3-H), 5.32 (d, J 4Hz, 5-H), 5.39 (d, J 4Hz, 6-H), 7.29 (s, phenyl).

(β-Methyl-^{3}H)-6-aminopenicillanic acid (**IX**)

Twenty-five ml of the washed aqueous suspension of *E. coli* cells was adjusted to pH 7.5 and diluted with a solution of 1.30 g of **VIII** (3.5 mmol; 6.05 mCi) in 175 ml of water. The mixture was stirred at 37°C while the pH was kept at 7.5 with 1 N NaOH (pH-stat); after 2.5 hours, 3.5 ml of base was consumed. After removing the bacterial cells by centrifugation, the supernatant was cooled to 0~2°C and acidified to pH 2.2 with 2 N HCl; phenylacetic acid was extracted with two 80-ml portions of cold ether. The aqueous phase was brought to pH 4.1 with 1 N NH_3 and concentrated under vacuum at 25°C to a volume of 3 ml; the (β-methyl-^{3}H)-6-APA was filtered and washed with cold water and acetone; yield 632 mg. Iodometric titration

resulted in 83.5 % 6-APA and 3.6 % of the penicillinoic acid; when corrected for purity, the yield was 73.4 %. Radiochemical yield: 4.68 mCi=77.3 %.

Part of the 6-APA obtained (575 mg, 4.26 mCi) was recrystallized by addition of 2 N NaOH to pH 7.5 and precipitation at pH 4.1 with 2 N HCl. Yield 455 mg, 3.70 mCi; specific activity 1,761 μCi/mmol (=95.8 % of the benzyl ester). Iodometric assay showed 90.0 % 6-APA and 5.7 % 6-APA-penicillinoic acid; the specific activity of the pure product was 1840 μCi/mmol. I.r. γ_{max} 3100~2700, 2150, 2070, 1530 (NH_3^+), 1770 (β-lactam), 1625, 1415 (COO^-) cm^{-1}. N.m.r. (+NaOD) δ 1.51 (s, CH_3), 1.61 (s, CH_3), 4.20 (s, 3-H), 4.64 (d, J 4 Hz, 6-H), 5.55 (d, J 4 Hz, 5-H).

Discussion

Preliminary experiments with deuterated water showed that a refluxing period of 24 hours is optimum for incorporation of isotope into the β-methyl group of the penicillin sulfoxide benzyl ester. Although some yellow color develops during heating, the product is reasonably stable under these conditions and yields of 75~80 % are obtained. Two consecutive reactions with 0.5 ml of ^3H_2O (18 mCi/mmol) gave tritiated sulfoxide esters with specific activities of 11 and 9 % of the starting material. Higher specific activities, if needed, can be obtained by using a larger excess of ^3H_2O of 5~10 Ci/ml. Labile tritium on the amide group should be completely removed by two or more equilibrations with methanol; since the ester crystallizes in this solvent, dichloromethane was added to keep a homogeneous solution. Care should be taken to trap the tritiated methanol completely during evaporation, or radioactive contamination will result.

Although the sulfoxide does not poison the Pd-catalyst during debenzylation, large differences in activity of several commercial catalysts were noted. Therefore, all catalysts used should first be tested on the non-labeled product.

Deoxygenation of the sulfoxide can easily be performed with PBr_3 in DMF at $-20°C$; since benzylpenicillin is acid-labile, yields in this step are mainly dependent on the quick extraction and neutralisation of the penicillin.

Starting from the tritiated penicillin sulfoxide ester, (β-methyl-^{3}H)-6-APA can be obtained in overall yields of 35~40 %.

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