phene. After refluxing for 20 min, 65.2 g (0.3 mole) of isopropyl *p*-toluenesulphonate in 300 ml dry ether were added at such a rate as to maintain gentle reflux. When the addition was complete the reaction mixture was refluxed for 2 h and then hydrolyzed by iced acid. The ether layer, after washing with water, was concentrated and then steam-distilled. The distillate was extracted with ether and the ether phase dried and distilled yielding 7 g (18 %) of a mixture containing 55 % 2-isopropylthiophene (A) and 45 % 3-isopropylthiophene (B) b.p. 151–156°, $n_D^{20} = 1.5054$. (Found: C 65.99; H 7.87; S 25.23. Calc. for $C_7H_{10}S$ (126.2) C 66.61; H 7.99; S 25.41). NMR-data: ($CCl_4$) $\tau_{CH_3}$ (A) = 8.71, $J_{CH_3-CH}$ = 7.0 c/s $\tau_{CH_3}$ (B) = 8.79, $J_{CH_3-CH}$ = 7.0 c/s

*2- and 3-Isopropyl-5-thiophenecarboxylic acid.* 50 ml of 1.1 N butyllithium were added to 7 g (0.055 mole) of isopropylthiophenes in 60 ml dry ether. After refluxing for 30 min the solution was poured on to dry carbon dioxide and 100 ml dry ether. The mixture was hydrolyzed with water and the water phase was acidified with dilute hydrochloric acid and extracted with ether. After removal of the ether 7 g (75 %) of the acids were obtained consisting of 54 % of 2-isopropyl-5-thiophenecarboxylic acid (C) and 46 % of 3-isopropyl-5-thiophenecarboxylic acid (D)

NMR-data: (acetone) $\tau_{H_3}$ (C) = 3.10, $\tau_{H_4}$ (C) = 2.37, $J_{34}$ (C) = 3.7 c/s, $\tau_{H_2}$ (D) = 2.60, $\tau_{H_4}$ (D) = 2.27, $J_{24}$ (D) = 1.7 c/s.

The NMR-spectra were recorded on an Varian Associates A-60 high resolution spectrometer.


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# Preparation of $^{35}S$-Labelled 6-Aminopenicillanic Acid

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In order to carry out certain pharmacological work with new penicillins prepared by reaction of 6-aminopenicillanic acid with different acid chlorides, it was desirable to have such compounds radioactively labelled in the heterocyclic moiety. From the technical as well as the economic point of view it seemed preferable to start with $^{35}S$-labelled benzylpenicillin, which could readily be split into radioactive 6-aminopenicillanic acid and phenylacetic acid by means of enzymatic hydrolysis [1-4].

The biosynthetic preparation of $^{35}S$-labelled benzylpenicillin has been previously described by Smith and Hockenhull [5] and by Perret [6]. They reported that 10–20 % of the $^{35}S$ added (as $Na_2{}^{35}SO_4$) was incorporated in the penicillin formed during the fermentation. In our experiments about 27 % of the radioactive sulphur added was present in the penicillin formed in the broth. About 60 % of this material could be isolated as crystalline potassium penicillin.

## *Experimental*

*Biosynthesis and isolation of $^{35}S$-labelled benzylpenicillin.*

*Organism. Penicillium chrysogenum* (Astra strain 1155), was grown on a sporulating medium of the following composition: 30 g crushed barley, 5 ml asparagine solution (0.1 % asparagine + 3 % glycerol in water) in a 250 ml conical flask.

*Seed.* The spores from one flask were suspended in 100 ml of sterile water. 2 ml of that suspension were used for seeding one 500 ml conical flask containing 100 ml of the following medium: Corn steep liquor 7.6 %, sucrose 2.0 %, $CaCO_3$ 1.0 %, and soy been oil 0.25 %. pH was 6.2. A suitable number of flasks seeded in that way were incubated at 27°C for 72 h on a rotary shaker (220 r.p.m., two inches circular stroke).

*Fermentation.* The fermentation was carried out in 22 conical flasks of 500 ml capacity, each containing 100 ml of the following medium: Peanut meal 1.5 %, cotton seed meal 1.5 %, lactose 7.5 %, $CaCO_3$ 1.0 %, $Na_2SO_4$ 0.4 %, soy been oil 0.25 %. To each flask was added, after sterilization, 0.4 ml of a sterile solution containing 15.9 mC $Na_2{}^{35}SO_4$ in water followed by 5 ml of the abovementioned 72-h seed culture. The flasks were incubated on a rotary shaker at 27°C for 7 days with additions of 1 ml 5 % phenylacetic acid twice a day, starting from the 24th hour of fermentation. The penicillin titre at the end of the fermentation was 2.8 mg/ml, estimated by the hydroxylamine assay method [7].

*Isolation.* The contents of the 22 flasks were combined, cooled and adjusted to pH 5.5. After stirring for about 30 min the mixture was filtered on a layer of Celite. To the filtered broth (about 2 l) was added 20 ml of an 8 % cetyltrimethylammonium bromide solution and 500 ml of methylisobutyl ketone, followed by 16 ml of 5 M sulphuric acid under vigorous stirring until the pH had dropped to 2.1 in the mixture. The resulting emulsion was broken up in a bucket centrifuge and the methylisobutyl ketone phase was dried with anhydrous sodium sulphate. To the clear solution, volume 375 ml, was added 20 ml of 50 % potassium acetate solution and the stirring was continued for about one hour during which time the potassium salt of benzylpenicillin crystallized. After cooling overnight the salt was filtered, washed with methylisobutyl ketone, butanol, and acetone and dried *in vacuo* at 40°C. Yield was 4.3 g of potassium benzylpenicillin, purity 74.5 % (estimated by the hydroxylamine assay method). The radioactivity (estimated by the liquid scintillation method) was 12.4 mC/g.

Paper chromatography using paper strips impregnated with phosphate buffer (pH 5.1) developed with wet ether showed that in addition to benzylpenicillin the product also contained small amounts of three other penicillins. The quotient $^{35}S/^{32}S$ expressed as mC $^{35}S$ per g $^{32}S$ was practically the same in the crystalline penicillin (144) as in the broth at the start of the fermentation (152).

*Enzymatic splitting of $^{35}S$-labelled benzylpenicillin.* To 2.0 g of the radioactive potassium benzylpenicillin, dissolved in 100 ml of water, an enzyme preparation was added, consisting of a paste obtained by centrifuging one liter of an *Escherichia coli* 20 h fermentation broth (Astra strain 1339). The temperature was maintained at 35°C and the pH at 7.8 by continuous addition of a 10 % sodium hydroxyde solution. The enzymatic reaction ceased after 4 h when about 95 % of the benzylpenicillin had been split as indicated by the sodium hydroxide consumption. After cooling and addition of Celite the mixture was filtered.

To the filtrate was added 50 ml of butyl acetate and the pH was adjusted to 2.9 by the addition of 1 N hydrochloric acid. The aqueous phase was separated and neutralized to pH 7.3 with a 10 % sodium hydroxyde solution. Celite was then added, the mixture filtered and the clear solution concentrated *in vacuo* at 20°C to about 10 ml volume. After cooling and acidifying the concentrated solution to pH 4.3, the 6-aminopenicillanic acid readily crystallized. After stirring for about one hour the substance was filtered, washed with water and acetone and finally dried *in vacuo* at 40°C. Yield was 0.69 g. Purity was 94.7 % estimated by the hydroxylamine assay method. Radioactivity 25 mC/g. The material was found to be chromatographically pure.

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