[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

The Anhydride of Benzylpenicillin[1]

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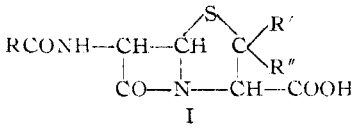
Previous studies in this and other Laboratories have shown that the replacement of the methyl groups R′ and R″ and the alkyl group R of the penicillin molecule, as illustrated on the basis of the β-lactam formula I, with various groups may result in the formation of compounds which still possess antibiotic activity.[2] Moreover, the sulfur of the thiazolidine ring may be oxidized to a sulfone without completely destroying the antibiotic activity.[3]



I

However, experiments in this Laboratory have demonstrated that compounds in which the 4-membered β-lactam ring has been replaced by a 5-membered γ-lactam ring do not possess antibiotic activity.[4]

It was of interest to extend the study of the relationship of structure to antibiotic activity to derivatives of the free carboxyl group of penicillin. Several neutral esters of the free carboxyl group of penicillin had already been prepared in other laboratories by the action on penicillin of diazomethane or related compounds.[5] These compounds, although possessing antibiotic activity *in vivo* in mice[5b,c] and in rats,[6] were relatively inactive *in vitro*[5] or *in vivo* when tested in monkeys, dogs and rabbits.[6]

Since there is a limit to the type of derivatives of the carboxyl group that may be prepared through the use of diazo compounds, other methods were sought for the substitution of the penicillin carboxyl group. The preparation of an acid anhydride of benzylpenicillin would presumably make possible the synthesis of a large number of penicillin derivatives not readily amenable to synthesis by the diazo reaction. However, preparation of the anhydride under standard conditions was not considered to be feasible, due to the lability of the penicillin molecule to acids.[7] Therefore, attention was focused on the development of modified procedures for the preparation of acid anhydrides.

In experiments with model compounds it was found that treatment of a chloroform solution of *p*-nitrobenzoic acid with thionyl chloride in the presence of a slight excess of pyridine resulted in the formation of a good yield of the acid anhydride. Barnett and Cook had already reported the preparation of anhydrides by the action of thionyl chloride on suspensions of organic acids in pyridine.[8] However, they did not obtain the anhydride of *p*-nitrobenzoic acid, the compound that was used as a model in our procedure.

Conditions were developed by which benzylpenicillin (or its triethylammonium salt) in chloroform solution containing pyridine was converted to the anhydride in 71% yield by the action of thionyl chloride. The product was obtained as an amorphous solid, which, however, gave carbon and hydrogen analyses in agreement with the anhydride structure. The product was further characterized by its chemical reactions. Thus, treatment with buffer at *p*H 6.0 converted it to benzylpenicillin; and treatment with methanol resulted in the formation of both benzylpenicillin and the methyl ester of benzylpenicillin. The latter compound was identical with the product prepared by the action of diazomethane on benzylpenicillin.[5a]

It was of particular interest to prepare a derivative of benzylpenicillin in which the acidic carboxyl group was converted to a basic group, as such a compound might be an effective antibiotic for organisms which are resistant to benzylpenicillin. Accordingly, the anhydride of benzylpenicillin was allowed to react with β-dimethylaminoethanol, and the β-dimethylaminoethyl ester was isolated as its crystalline hydrochloride. When this compound was dissolved in 1% phosphate buffer at *p*H 6.0 and assayed against *Bacillus subtilis* using the sodium salt of benzylpenicillin (1667 units/mg.) as the standard, it possessed about 1000 units/mg. of antibiotic activity. However, an investigation of the stability of this compound in 0.1 *M* phosphate buffer at *p*H 7.3 showed that it was rapidly hydrolyzed to benzylpenicillin. Therefore, it appeared that the *in vitro* activity of the β-dimethylaminoethyl ester was probably due to its reversion to benzylpenicillin during the assay interval.

It should be pointed out that the rapid hydrolysis of the β-dimethylaminoethyl ester under conditions in which benzylpenicillin is quite stable

(1) This work was supported in part by research grants from the Rockefeller Foundation and the National Institute of Health.

(2) (a) du Vigneaud, Wood and Wright, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1948, Ch. XXIII; (b) Boon, Carrington, Wintersteiner and MacCorquodale, *ibid.*, Ch. V; (c) Behrens, *ibid.*, Ch. XIX; (d) Committee on Medical Research, OSRD, Washington, and the Medical Research Council, London, *Science*, **102**, 627 (1945); (e) du Vigneaud, Carpenter, Holley, Livermore and Rachele, *ibid.*, **104**, 431 (1946).

(3) Peck and Folkers, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1948, Ch. VII, p. 156.

(4) du Vigneaud and Carpenter, *ibid.*, Ch. XXVII.

(5) (a) Wintersteiner, *ibid.*, Ch. V, p. 93; (b) Meyer, Hobby and Chaffee, *Science*, **97**, 205 (1943); (c) Meyer, Hobby and Dawson, *Proc. Soc. Exp. Biol. and Med.*, **53**, 100 (1943); (d) Hickey, *Science*, **101**, 462 (1945).

(6) Richardson, Walker, Miller and Hanson, *Proc. Soc. Exp. Biol. and Med.*, **60**, 272 (1945).

(7) Hunter, Hinman and Carter, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1948, Ch. XXIV.

(8) Barnett and Cook, *Chem. News*, **129**, 191 (1924).

might be used to advantage in various approaches to the synthesis of penicillin. By the use of this ester the penicillamine carboxyl might be covered during various procedures with a group that could be readily removed in the final product. Since both the anhydride and the β-dimethylaminoethyl ester of benzylpenicillin can yield benzylpenicillin under physiological conditions, they might possibly prove to be useful therapeutic agents.

Experimental[9]

Materials.—It is essential that the chloroform be free of alcohol. The chloroform (USP) was purified by shaking it for five minutes with 3 portions (10% of the volume of the chloroform) of concentrated sulfuric acid. The chloroform layer was then shaken in order with equal volumes of water, 2 *N* sodium hydroxide, water (twice) and saturated sodium chloride solution. It was then dried over phosphoric anhydride and distilled. The purified chloroform could be stored at 5° for as long as four days before being used in the reaction.

The pyridine was dried for one month over barium oxide, distilled from barium oxide and stored over barium oxide.[10] Purified thionyl chloride was used.[11] Crystalline sodium penicillin G which was at least 90% benzylpenicillin was used.[12]

Anhydrides of *p*-Nitrobenzoic Acid and Cinnamic Acid.—The acid (0.1 mole) was dissolved in 50 cc. of chloroform at 25° by the addition of 3.6 cc. (0.045 mole) of pyridine. Thionyl chloride (0.40 cc., 0.0055 mole) was added. After five minutes the reaction mixture was diluted with 40 cc. of chloroform and the resulting solution was shaken successively with two 30-cc. portions of 1 *N* sodium bicarbonate, with two 40-cc. portions of 1 *N* hydrochloric acid and with saturated sodium chloride solution, and then was dried over anhydrous sodium sulfate. The chloroform was removed *in vacuo* to leave the anhydrides as crystalline products.

The yield of *p*-nitrobenzoic acid anhydride was 1.30 g. (82%). After 1 crystallization from 35 cc. of toluene, 0.915 g., m. p. 189–191°,[13] remained.

The yield of cinnamic acid anhydride was 1.10 g. (79%), m. p. 132–134°. The compound was recrystallized from 12 cc. of carbon tetrachloride to give 0.965 g., m. p. 135–136°.[14]

Benzylpenicillin Anhydride.—The sodium salt of benzylpenicillin (643 mg., 1.8 millimoles, 1,072,000 units) was dissolved in 100 cc. of water. Ether (250 cc.) was added, followed by 50 cc. of 2 *M* phosphate buffer at *p*H 1.6, and the resulting mixture was shaken. The ether layer was separated, and the aqueous layer was shaken with another portion of ether (75 cc.). The combined ether layers were stored at −70° for two hours and then filtered. The ether was evaporated *in vacuo* to yield a white fluffy residue.

The residue, consisting of the free acid of benzylpenicillin (or an equivalent amount of the triethylammonium salt of benzylpenicillin) was dissolved in 30 cc. of purified chloroform. To this solution was added 5.4 cc. of a freshly prepared solution of pyridine (0.86 cc., 11 millimoles) and thionyl chloride (0.0972 cc., 1.35 millimoles) in chloroform (4.44 cc.). After the solution had been allowed to stand for five minutes at room temperature, it was diluted with 145 cc. of chloroform. An aliquot which was removed for assay[15] indicated the presence of 980,000 units of antibiotic activity. The chloroform solution was shaken with a 200-cc. and a 50-cc. portion of 2 *M* phosphate buffer at *p*H 1.6. Then it was shaken with two 150-cc. portions of 2 *M* phosphate buffer at *p*H 7.0 in order to remove any benzylpenicillin. The *p*H 7.0 buffer layer contained 104,000 units of unreacted or regenerated benzylpenicillin. The chloroform layer contained 870,000 units.

The water and clinging buffer were frozen out of the chloroform by short storage of this solution at −70°. The mixture was filtered while cold, and the filtrate was concentrated *in vacuo* in the absence of air to a volume of 15 cc. The concentrated solution was added dropwise with vigorous shaking to 200 cc. of hexane. A white flocculent precipitate formed. The precipitate was collected and while still moist with hexane was placed in a vacuum desiccator to dry under suction. The anhydride of benzylpenicillin weighed 415 mg. (71%); $[\alpha]^{22}D$ + 175° (*c*, 1.0 in chloroform).

Anal. Calcd. for $C_{32}H_{34}O_7N_4S_2$: C, 59.1; H, 5.27; N, 8.61; S, 9.85. Found: C, 58.8; H, 5.60; N, 8.52; S, 9.88.

The antibiotic activity obtained by treating the anhydride with buffer at *p*H 6.0 varied from 1660 to 1820 units/mg. in various experiments. The calculated activity, assuming complete conversion to benzylpenicillin, would be 1830 units/mg.

Reaction of the Anhydride of Benzylpenicillin with Buffer at *p*H 6.0.—The anhydride (50 mg., 0.0768 millimole) was dissolved in 10 cc. of acetone. The solution was diluted with 90 cc. of 1% phosphate buffer at *p*H 6.0. The solution became quite cloudy. However, no precipitate formed, and the cloudiness rapidly diminished in intensity. After the solution had been allowed to stand for one and one-quarter hours, it contained 90,000 units of penicillin activity. The solution was acidified by the addition of 30 cc. of 2 *M* phosphate buffer at *p*H 1.6, and the benzylpenicillin was extracted into a 100-cc. and a 50-cc. portion of ether. The combined ether layers contained 83,000 units. The ether solution was stored at −70° for one and one-half hours and then was filtered. The filtrate was concentrated to dryness *in vacuo* in the absence of air. The residue was dissolved in 10 cc. of commercial absolute ether, and the solution was filtered through cotton to remove a small amount of insoluble material. One cubic centimeter of an ethereal solution of triethylamine (1%) was added. Immediately the triethylammonium salt of benzylpenicillin separated in crystalline form. After the mixture had been allowed to stand for twelve hours at 5°, the crystals (50 mg. (75%)) were collected. After 1 recrystallization from 2 cc. of acetone, 36 mg. of compound was obtained, micro m. p. 137–145° (dec.) when heated at 10°/min. at the melting point[16]; $[\alpha]^{23}D$ +247° (*c*, 0.23 in 1% phosphate buffer at *p*H 6.0).[16] The melting point was not depressed upon admixture with authentic triethylammonium salt of benzylpenicillin.

Reaction of the Anhydride of Benzylpenicillin with Methanol.—Methanol (0.5 cc.) and pyridine (0.1 cc.) were added to a solution of 130 mg. (0.2 millimole, 238,000 units by assay) of the anhydride of benzylpenicillin in 50

(9) All melting points are corrected and are capillary melting points unless otherwise specified.

(10) Zerewitinoff, *Ber.*, **47**, 2417 (1914).

(11) Fieser, "Experiments in Organic Chemistry," D. C. Heath and Company, New York, N. Y., 1935, p. 339.

(12) The crystalline sodium penicillin G was supplied by the Antibiotics Study Section, Research Grants Division, National Institute of Health.

(13) Thiele, *Ann.*, **314**, 296 (1901).

(14) Wedekind, *Ber.*, **34**, 2070 (1901).

(15) In order to assay material containing the anhydride, the anhydride was converted in a nearly quantitative yield to the sodium salt of benzylpenicillin by the following procedure. If the anhydride was in a water-immiscible organic solvent, the solvent was first removed *in vacuo*. The residue was then dissolved in a sufficient amount of purified acetone (Shipsey and Werner, *J. Chem. Soc.*, **103**, 1255 (1913)) to give a concentration of less than 1 mg./cc. The acetone solution was immediately diluted with at least 9 volumes of 1% phosphate buffer at *p*H 6.0. This solution and/or dilutions of it in phosphate buffer were assayed against *Bacillus subtilis* ATCC 6051 by a method similar to that of Vincent and Vincent (*Proc. Soc. Exp. Biol. and Med.*, **55**, 162 (1944)). Crystalline sodium benzyl penicillin was used as a standard.

(16) du Vigneaud, Carpenter, Holley, Livermore and Rachele, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1948, Ch. XXVIII.

cc. of chloroform. After the solution had been allowed to stand for one hour at room temperature, it contained 120,000 units of activity. The chloroform solution was washed with 30 cc. of 2 *M* phosphate buffer at *p*H 1.6, and then the benzylpenicillin which was generated in the reaction of the anhydride with methanol was extracted from the chloroform into two 50-cc. portions of 2 *M* phosphate buffer at *p*H 7.8. The extracted chloroform layer which contained 1,500 units was used for the isolation of the methyl ester of benzylpenicillin.

The *p*H 7.8 buffer layer was acidified, and the benzylpenicillin was extracted into ether. From the ether solution, which contained 74,000 units, there was obtained 37 mg. (42%) of the triethylammonium salt of benzylpenicillin by a procedure similar to that described above. After one recrystallization from acetone, the material had a micro melting point of 137–147° (dec.), $[\alpha]^{23}D$ +245° (*c*, 0.53 in 1% phosphate buffer at *p*H 6.0).[16] The melting point was not depressed on admixture with authentic triethylammonium salt of benzylpenicillin.

The chloroform solution containing the methyl ester was dried over anhydrous sodium sulfate and then was concentrated to dryness. The residue was dissolved in 2 cc. of carbon tetrachloride, and hexane was added to opalescence. When the solution was seeded with a trace of the methyl ester of benzylpenicillin and the mixture was allowed to stand at 5°, an oil formed which gradually crystallized. The crude product weighed 53 mg. (76%), micro m. p., 86–92°. Considerable difficulty was experienced in obtaining this material in pure form. It was recrystallized from heptane; this product (38 mg.) was washed with cold carbon tetrachloride and then was recrystallized from a mixture of carbon tetrachloride and hexane. The resulting product (11 mg.) was recrystallized a final time from 3.5 cc. of heptane to give 5 mg. of pure methyl ester of benzylpenicillin, micro m. p. 95–98°, $[\alpha]^{21}D$ + 301° (*c*, 0.25 in methanol).[5a] The melting point was not lowered on admixture with authentic methyl ester of benzylpenicillin, prepared by the action of diazomethane on benzylpenicillin.[5a]

Hydrochloride of the β-Dimethylaminoethyl Ester of Benzylpenicillin.—To a solution of 325 mg. (0.5 millimole, 593,000 units by assay) of the anhydride of benzylpenicillin in 19 cc. of chloroform, there was added 1 cc. of a chloroform solution of β-dimethylaminoethanol (10%). After the solution had been allowed to stand for ten minutes at room temperature, it was diluted to 100 cc. with chloroform. An aliquot which was removed for assay indicated the presence of 467,000 units. The chloroform solution was shaken with three 100-cc. portions of 2*M* phosphate buffer at *p*H 7.0 in order to remove the benzylpenicillin liberated in the reaction. The combined buffer layers contained 312,000 units, while the extracted chloroform layer contained 157,000 units. The chloroform layer was dried over anhydrous sodium sulfate and then concentrated to dryness *in vacuo*. The residual brown oil was dissolved in 1.5 cc. of chloroform. The addition of 30 cc. of commercial absolute ether to the chloroform solution caused the formation of a small amount of precipitate which was removed by filtration through cotton. In the cold room at 5°, the filtrate was shaken with 3 cc. of water to which 0.1 *N* hydrochloric acid was added in small portions until the *p*H of the aqueous layer reached 3.0. This required about 3.5 cc. of 0.1 *N* hydrochloric acid. Immediately the aqueous layer was frozen and lyophilized. The resulting product was dissolved in a total of 4 cc. of acetone, the solution was filtered, and 2 cc. of commercial absolute ether was added to the filtrate. When the walls of the vessel were scratched, crystals started to separate. More ether was added in small portions until a total of 12 cc. had been added. The crude product weighed 150 mg. (68%), micro m. p. 105–135° (dec.). This material was recrystallized from 2 cc. of ethanol by the addition of 3 cc. of ether to give 75 mg. of product with a melting point of 136–138° (dec.). Another recrystallization from the same solvents gave 62 mg., m. p. 138–139° (dec.); $[\alpha]^{21}D$ +202° (*c*, 1.1, in ethanol).

Anal. Calcd. for $C_{20}H_{27}O_4N_3S \cdot HCl$: C, 54.3; H, 6.38; N, 9.50; Cl, 8.02. Found: C, 54.4; H, 6.88; N, 9.27; Cl, 7.94.

Stability of the Hydrochloride of the β-Dimethylaminoethyl Ester of Benzylpenicillin in 0.1 *M* Phosphate Buffer at *p*H 7.3.—The hydrochloride of the β-dimethylaminoethyl ester of benzylpenicillin (23.4 mg.) was dissolved in 20 cc. of 0.1 *M* phosphate buffer at *p*H 7.3 at 26°. An aliquot that was removed for assay indicated that the solution contained 23,400 units (1,000 units/mg.). After the solution had been allowed to stand for two hours, 25 cc. of 2 *M* phosphate buffer at *p*H 1.6 was added, and the benzylpenicillin was extracted into a 50-cc. and a 25-cc. portion of ether. An aliquot of the ether solution was removed for assay. The results indicated the presence of 22,700 units in the ether layer. The benzylpenicillin was isolated from this ether layer as its triethylammonium salt by a procedure similar to that described previously. The isolated salt weighed 6 mg. (26%), micro m. p. 133–141° (dec.). The melting point was not lowered on admixture with authentic triethylammonium salt of benzylpenicillin.

The hydrochloride of β-dimethylaminoethyl ester of benzylpenicillin (8.8 mg.) was dissolved in 8.8 cc. of 0.1 *M* phosphate buffer (*p*H 7.3) at 37°. Aliquots (0.5 cc.) were removed as soon as solution was complete (two minutes) and at various time intervals thereafter. Each aliquot was immediately added to 0.5 cc. of 10% phosphoric acid, and the resulting solution was shaken with 3 cc. of ether. The ether layers were dried over anhydrous sodium sulfate, a 2-cc. aliquot of each layer was evaporated to dryness *in vacuo*, the resulting residues were dissolved in buffer and these solutions were assayed.

The amounts of the total activity that were present in the solution as benzylpenicillin at the various time intervals were 10% at two minutes, 66% at fifteen minutes and 98% at thirty minutes.

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Summary

The anhydride of benzylpenicillin has been prepared by the use of a modified procedure for the synthesis of acid anhydrides. The methyl and β-dimethylaminoethyl esters of benzylpenicillin were prepared by reaction of the anhydride with the appropriate alcohols. The β-dimethylaminoethyl ester of benzylpenicillin rapidly hydrolyzes in aqueous solution at *p*H 7.3 to benzylpenicillin.

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