Semisynthetic Penicillins

VI.* The Use of the *o*-Nitrophenylsulphenyl Protecting Group in the Preparation of Aminopenicillins

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Zervas [1,2] recently introduced the *o*-nitrophenylsulphenyl (NPS) group into peptide chemistry for the protection of amino groups. NPS-Amino acids [3] can be coupled to amino acid esters under anhydrous conditions by several methods, *e.g. via* mixed anhydrides or with the aid of carbodiimides. The NPS-group can then be removed by treatment with acids in aqueous organic solvents or preferably with hydrogen chloride or bromide in dry organic solvents, such as ether and methanol.

We have explored the use of the NPS-group for the synthesis of aminopenicillins and found that NPS-amino acids can be coupled to 6-aminopenicillanic acid (6-APA) in aqueous solutions *via* the mixed ethoxyformic anhydrides. The resulting NPS-aminopenicillins can be separated from unreacted 6-APA by extraction with organic solvents at pH 2–3, and can be isolated from the organic solutions by extraction with potassium bicarbonate and freeze-drying the aqueous extract.

The sensitive β-lactam ring of the penicillins is rapidly split on treatment with strong acids under anhydrous conditions.[4,5] The penicillins are also inactivated in aqueous acid solutions, although the α-aminopenicillins have been found comparatively stable under such conditions.[6] When aliquots of α-(*o*-nitrophenylsulphenylamino)-benzylpenicillin, dissolved in 75 % dioxane, were acidified to different pH-values for 10 min, significant amounts of α-aminobenzylpenicillin were formed only at pH values below 2 (Fig. 1, curve 1). The yield of the aminopenicillin in solution rapidly increased with decreasing pH to a maximum at pH 0.2–0.5. At still lower pH values the yield fell off rather sharply, obviously due to inactivation of the penicillins, as only traces of the NPS-aminopenicillin were recovered. In experiments on a larger scale this inactivation will be important. It was thus desirable to find a method which could be used in less acidic media where the penicillins are more stable.

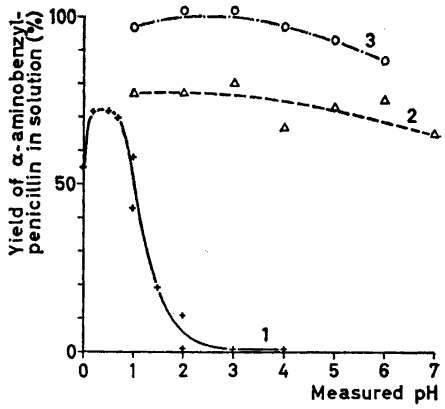

Fig. 1. Removal of the NPS-group from α-(*o*-nitrophenylsulphenylamino)-benzylpenicillin at different pH-values. Curve 1: with acid alone; curve 2: after addition of $Na_2S_2O_3$; curve 3: after addition of NaI.

An analytical method for sulphenamides, given by Foss,[7] involves splitting of the S–N bond by thiosulphate:

$$R_1SNR_2R_3 + S_2O_3^{2-} + 2H^+ \rightarrow R_1SS_2O_3^- + R_1R_2NH_2^+$$

We therefore investigated if the NPS-group could be removed under similar conditions.[8] Curve 2 (Fig. 1) shows the results from experiments performed as described above, but with the addition of two equivalent of sodium thiosulphate to the test samples. It can be seen that the thiosulphate at pH-values above 1 greatly enhances the formation of the aminopenicillin, although the yield in solution is only slightly better than that obtained with acid alone at pH 0.5. However, the relative pH-independence of the reaction in the presence of thiosulphate and the possibility to remove the NPS-group even in neutral solutions makes this method more favourable.

As it is probable that the removal of the NPS-group involves a nucleophilic attack on the sulphur atom of the sulphenamide group, we tried other nucleophilic agents for the reaction. Curve 3 (Fig. 1)

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shows the result obtained with sodium iodide. On addition of this reagent to the reaction solution, free iodine was immediately formed, as well as a yellow precipitate identified as *o,o'*-dinitrodiphenyl disulfide. The iodine was reduced by titration with thiosulphate during the experiment. The yields of the aminopenicillins are here even better than those obtained with thiosulphate.

A number of other nucleophilic agents were tried for their efficiency of removing the NPS-group from penicillins. Addition of sodium azide, sodium fluoride, or sodium sulphide gave moderate yields of aminopenicillins, while good to excellent ones were obtained with sodium hydrogen sulphite, sodium dithionite and potassium thiocyanate. We have also found that other sulfenylamino groups can be split in the same way.[8] Analogous reactions have been described recently for the splitting of 2,4-dinitrophenylsulphenyl esters.[9]

Experimental. *α-(o-Nitrophenylsulphenylamino)-benzylpenicillin.* α-(*o*-Nitrophenylsulphenylamino)-phenylacetic acid was prepared by treating α-aminophenylacetic acid with *o*-nitrophenylsulphenylthiocyanate in 50 % dioxane at pH 7. It was isolated by extraction with ether at pH 3 and crystallized from carbon tetrachloride. M.p. 149–150° (decomp.) (Found: C 53.6; H 3.84; N 8.99; S 10.44. Equiv. wt. 305. Calc. for $C_{14}H_{12}N_2O_4S$: C 55.25; H 3.98; N 9.20; S 10.54. Equiv. wt. 304.34).

A mixture of the acid (6.1 g, 0.02 mole) and triethylamine (2 g, 0.02 mole) in 75 ml of dry dimethylformamide was treated dropwise under stirring at −10°C with ethyl chloroformate (2.2 g, 0.02 mole) dissolved in 5 ml of dry ether. After 20 min an ice-cooled solution of 6-aminopenicillanic acid (5.4 g, 0.025 mole) and triethylamine (2.8 g, 0.028 mole) in 50 ml of water was added rapidly and stirring was continued for 90 min without external cooling. The reaction mixture was diluted with water, washed with ether, acidified to pH 3 with dilute sulphuric acid and rapidly extracted with ether (3 × 100 ml). The combined ether extracts were washed with water and the penicillin was extracted by addition of N potassium bicarbonate till the aqueous phase was neutral. The aqueous extract was freeze-dried to give 7.4 g of the potassium salt of α-(*o*-nitrophenylsulphenylamino)-benzylpenicillin, with a purity of 84 % (hydroxylamine assay [10]).

Removal of the NPS-group. A stock solution of α-(*o*-nitrophenylsulphenylamino)-benzylpenicillin was prepared by dissolving 5.4 g (0.01 mole) of its potassium salt in 100.0 ml of 75 % dioxane. The NPS-penicillin used was prepared by treating α-aminobenzylpenicillin with *o*-nitrophenylsulphenyl chloride at pH 7. It was isolated by freeze-drying and contained about 5 % of α-aminobenzylpenicillin.

Aliquots of 10.0 ml of the stock solution were acidified with 6 N HCl to pH-values ranging from 0 to 7, the value being maintained for 10 min by addition of more acid, when necessary. The pH was then adjusted to 2 and the reaction mixture diluted with water and extracted thoroughly with ether. The aqueous phase obtained was neutralized, filtered, and assayed for α-aminobenzylpenicillin. In the experiments with thiosulphate and iodide, 2 ml of M $Na_2S_2O_3$ and 1 ml of 2 M NaI were added, respectively, before acidification. In the latter case, the iodine formed was titrated during the 10 min period with M $Na_2S_2O_3$.

For every stock solution a blank was made in which a sample was acidified directly to pH 2 and immediately extracted with ether. The NPS-aminopenicillin was extracted from the organic solution with M potassium bicarbonate and assayed. The acidic aqueous phase was neutralized and analyzed for aminopenicillin. The yields of α-aminobenzylpenicillin in solution, given in Fig. 1, are corrected for the amount present in the original solution. The penicillin assays were performed according to the hydroxylamine method;[10] pH was measured with a Radiometer type 22 pH meter, using a glass-calomel combination electrode GK 2021 B.

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Reaction of Two Enzyme Fractions from *Streptomyces griseus* Protease with Diisopropylphosphorofluoridate

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Several proteolytic and other enzymes are inactivated by DFP * (see Ref. 1). All these enzymes have esterase activity, and are phosphorylated on a serine residue at the active site during inactivation. It has also been reported that rat-liver tissue contains many proteins that are phosphorylated on a serine residue by DFP (see Ref. 2). These proteins seem to have esterase activity.[3]

Since Pronase (Calbiochem, U.S.A.), a new commercial protease preparation obtained from *Streptomyces griseus*, also has esterase activity,[4] a preliminary experiment was made to ascertain whether this preparation would be phosphorylated on a serine residue by $DF^{32}P$. 10 mg of Pronase were dissolved in 0.5 ml of 0.1 M Tris-acetic acid buffer (pH 7.5) and incubated with 5×10^{-4} M $DF^{32}P$ (Radiochemical Centre, Amersham, England) for 10 min at 20°C. The enzyme was then precipitated by trichloroacetic acid at a final concentration of 10 % (w/v). The precipitate was washed twice with acetone and dried *in vacuo*. After acid hydrolysis, $Ser^{32}P$ was isolated from the hydrolysate, using the same methods as previously described.[5]

Hiramatsu and Ouchi [6] have shown by starch zone electrophoresis that Pronase contains four different fractions with proteolytic activity. The activity of one of these fractions was found to be inhibited by DFP. It was therefore of interest to see whether this fraction would have esterase activity and — in this event — could be phosphorylated on a serine residue by DFP. For this reason, Pronase was chromatographed on CM-cellulose, and the fractions containing esterase activity were used for an experiment with $DF^{32}P$.

300 mg of Pronase (Calbiochem, Lot no. 42834) in 15 ml of 0.01 M calcium acetate-acetic acid buffer (pH 5.0), was purified with respect to esterase activity (measured with N-benzoyl-L-arginine ethyl ester as substrate)[7] by adding solid ammonium sulphate until 0.7 saturation at 4°C. The precipitate was dissolved in 9 ml of the buffer, and dialyzed overnight against the same buffer. The enzyme solution was then chromatographed on a 2 × 6.5 cm CM-cellulose column, equilibrated with the aforementioned buffer. Elution was performed with a linear gradient of 0.01 M to 0.20 M calcium acetate-acetic acid buffer, pH 5.0. Two main protein peaks were eluted, one appearing with the front of the effluent and one at a buffer concentration of about 0.03 M. The latter peak contained all the esterase activity. The fractions of the second peak were pooled and, after adjustment of the pH to 7.4 with Tris, incubated at 20°C with $DF^{32}P$ (3500 counts/min/mμmole) at a final concentration of 10^{-3} M. After 2 h, when all the esterase activity had disappeared, the incubation mixture was filtered at 4°C through a Sephadex G 50 column with 0.001 M Tris acetate-acetic acid buffer, pH 5.0, to remove excess $DF^{32}P$.

About one-third of the labelled protein was used for isolation of $Ser^{32}P$ after acid hydrolysis, as described earlier.[5] The recovery of $Ser^{32}P$ was 28 %, calculated from the total radioactivity eluted from the Dowex 50 column. This value indicates that all the labelled phosphorus had been bond to serine in the protein.[5]

Half the total labelled enzyme solution was titrated to pH 8.6 with Tris, and then applied to a 1.2 × 22 cm TEAE-cellulose column equilibrated with 0.001 M Tris-acetic acid buffer, pH 8.6. The column was eluted with a linear gradient of the same buffer to 0.20 M Tris-acetic acid buffer, pH 8.6, also containing 0.1 M calcium acetate. Two labelled protein

* Abbreviations: DFP, diisopropylphosphorofluoridate. $DF^{32}P$, radioactive diisopropylphosphorofluoridate. SerP, phosphorylserine. $Ser^{32}P$, radioactive phosphorylserine.