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APPENDIX

The preparation and properties of bromoacetylphosphonic acid

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Bromoacetylphosphonic acid, $\text{Br}-\text{CH}_2-\text{CO}-\text{PO}_3\text{H}_2$, was made by brominating dimethyl acetylphosphonate and de-esterifying with HBr. It proves to be a powerful alkylating agent, reacting rapidly with GSH, with a rate constant of about $6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6.

INTRODUCTION

Acetylphosphonic acid, $\text{CH}_3-\text{CO}-\text{PO}_3\text{H}_2$, can have biochemical uses as an analogue of pyruvate or of phosphate. Harrison, Perham and Slater (cited by Ambrose & Perham, 1976) found it to be an effective inhibitor of pyruvate dehydrogenase, with a K_i of $4 \mu\text{M}$, and Kluger & Pike (1977) reported that its monomethyl ester, which bears only a single charge at neutral pH, is an even better inhibitor, with a K_i of $0.05 \mu\text{M}$. It therefore seemed of interest to make an alkylating derivative of this compound, so we developed a synthesis of bromoacetylphosphonic acid. This molecule, $\text{Br}-\text{CH}_2-\text{CO}-\text{PO}_3\text{H}_2$, possesses two electrophilic centres, the activated bromomethyl group, expected to react preferentially with polarizable nucleophiles, and a carbonyl group, expected to be sensitive to basic nucleophiles (cf. Edwards, 1956). They are closer to the phosphono group than in the previously made bromoacetylphosphate, $\text{Br}-\text{CH}_2-\text{CO}-\text{O}-\text{PO}_3\text{H}_2$.

RESULTS

The bromoacetylphosphonic acid was synthesized by treating dimethyl acetylphosphonate, $\text{CH}_3-\text{CO}-\text{PO}(\text{O}-\text{Me})_2$, with Br_2 , and completing the de-esterification with HBr in acetic acid. It proves to react rapidly with GSH, at about $6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6.

EXPERIMENTAL

Synthesis

Dimethyl acetylphosphonate, $\text{CH}_3-\text{CO}-\text{PO}(\text{O}-\text{Me})_2$, was used as starting material, and was made by a modification of the method of Kabachnik & Rossiiskaya (1945), as follows. Acetyl chloride (20 ml, 0.28 mol) was cooled to 0°C . Trimethyl phosphite (30 ml, 0.25 mol) was added slowly with stirring and cooling, and the mixture was then heated to 80°C for 5 min to drive off unchanged acetyl chloride. The crude product (39.2 g, 38 g theoretical) was used for bromination without purification. To a portion (38 g, 0.25 mol if pure) a few drops of Br_2 were added and the mixture was warmed until decolorization started (about 50°C). The rest of the Br_2 (6.5 ml, 0.125 mol) was then added slowly with cooling to keep the temperature below 50°C . Less than the theoretical quantity of Br_2 was used to avoid

formation of dibromoacetylphosphonic acid. After this, 40 ml of 45% HBr in acetic acid was added, and the mixture was kept at about 20°C overnight [Cooke *et al.* (1953) had similarly de-esterified acetylphosphonic acid with anhydrous HBr]. The mixture was evaporated to dryness, and cooled with ice while it was dissolved in water and adjusted to pH 3.0 with cyclohexylamine. The solution was again evaporated to dryness, and traces of water were removed by re-evaporation from ethanol. The solid formed was washed by suspending and filtering off, five times with ethanol and five times with methanol, to remove cyclohexylamine salts of HBr, acetic acid and acetylphosphonic acid. A residue of 24.8 g remained (66% based on the Br_2 added, 34% on the trimethyl phosphite).

Characterization

On electrophoresis in 5% acetic acid/0.5% pyridine, pH 3.5, on Whatman 3MM paper cooled in white spirit, the product gave a single spot of 0.91 of the mobility of orthophosphate and 0.88 of the mobility of acetylphosphonic acid (with glucose as a standard of zero mobility) when stained for its carbonyl group by spraying with 5 mm-2,4-dinitrophenylhydrazine in 2 M-HCl, and for its ability to bind Fe^{3+} ions by spraying with FeCl_3 and sulphosalicylic acid solutions (the phosphate test of Wade & Morgan, 1953). [See Dixon & Sparkes (1974) for details of paper electrophoresis and staining.]

The crude product was stirred in methanol (50 ml per g) until dissolved; it crystallized on addition of diethyl ether. Elementary analysis gave C, 31.9; H, 5.7; N, 4.6%; $\text{C}_8\text{H}_{11}\text{BrNO}_4\text{P}$ requires C, 31.8; H, 5.7; N, 4.6%. Titration in water gives a pK of 5.2 and a molar mass of 299 g/mol (theoretical 302).

Preparation of the lutidine salt

Because of the difficulty of dissolving the cyclohexylamine salt, the salt with 2,6-lutidine was used for kinetic experiments. The cyclohexylamine salt was stirred with the sulphonic resin Zerolit 225 SRC13 (H^+ form) in water, and the suspension was filtered through a small bed of the same resin to obtain a solution of the free acid. This was adjusted to pH 3 with 2,6-lutidine, and was evaporated to dryness on a rotary evaporator. Ethanol was added and the solution again evaporated a few times until a

crystalline solid remained. Recrystallization from a solvent was not achieved.

Reaction with GSH

Solutions of 4 mM-bromoacetylphosphonic acid as its lutidine salt and of 0.2 mM-GSH were prepared, both adjusted to pH 6.0 with 2,6-lutidine. Reaction mixtures were made up from these solutions as follows: 0.1 mM-GSH in 2 mM- and 1 mM-bromoacetylphosphonate, and 0.05 mM-GSH in 0.5 mM-bromoacetylphosphonate. Samples (4 ml) were taken from these solutions at 1 min intervals, and assayed for thiol groups by addition to a mixture of 1 M-Tris/HCl buffer, pH 8.4, and 0.5 ml of 5 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), and measurement of absorbance at 410 nm. A plot of the logarithm of this absorbance against time gave psuedo-first-order rate constants of 0.59, 0.36 and 0.22 min⁻¹ respectively, giving a second-order constant of about 6 M⁻¹·s⁻¹. This is over 100 times the value calculated for iodoacetamide at pH 6 from

the p*K* of GSH and the value of 27 M⁻¹·s⁻¹ found by Halász & Polgár (1976) at high pH.

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