SYNTHESIS OF N-[^{35}S]-SULPHO-2-AMINO TRICARBALLYLATE

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SUMMARY

The synthesis of [^{35}S]-labelled N-sulpho-2-amino tricarballylate (SAT) and methods for its purification and analysis are described. In the first stage of the two-step synthesis, chloro-[^{35}S]-sulphonic acid is reacted with pyridine to yield pyridine-[^{35}S]-sulphur trioxide. This agent is then utilized to sulphonate 2-amino tricarballylic acid yielding the ultimate product [^{35}S]-SAT, which was required for pharmacokinetic studies.

Key Words: N-sulpho-2-amino tricarballylate, [^{35}S]-label, sulphonation, sulphamate, phosphocitrate analogue, calcification inhibitor

INTRODUCTION

In recent years, a variety of both natural and synthetic compounds capable of inhibiting calcification *in vitro* have been investigated as therapeutic agents for the prevention and treatment of kidney stone disease. However, the majority of inhibitors tested to date have proven unsuitable as they are either a) destroyed rapidly in the body, b) unsuitable for oral administration due to poor absorption, c) insufficiently potent, or d) toxic when administered over long periods. We have recently described the synthesis of a new compound, N-sulpho-2-amino tricarballylate (SAT), a sulphamate analogue of the naturally occurring inhibitor phosphocitrate (1). SAT has proven to be a potent inhibitor of calcification *in vitro* although not as good as phosphocitrate, nevertheless preliminary studies have indicated that the bonds of SAT are much more resistant to enzymic degradation than the labile phosphate ester bond of phosphocitrate (2). To study the stability and pharmacokinetics of SAT *in vivo*

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and therefore evaluate its true therapeutic potential, the incorporation of a radiolabel into the molecule was clearly a necessity.

The method proposed for this synthesis was based on existing methods for preparing non-radiolabelled pyridine-sulphur trioxide (3) and SAT from this latter agent (1). These methods were scaled down and modified to achieve maximum utilization of the radiolabel. Hence, chloro-[^{35}S]-sulphonic acid was reacted with pyridine to yield pyridine-[^{35}S]-sulphur trioxide, which in turn was coupled with 2-amino tricarballylate to give [^{35}S]-SAT (Fig. 1).

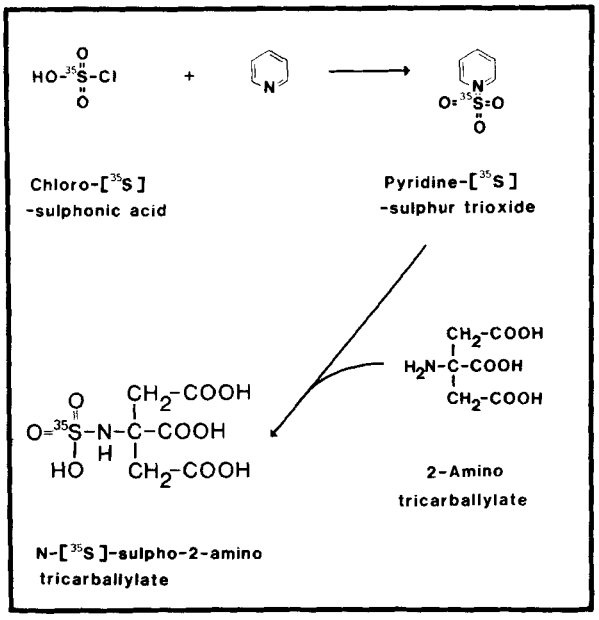

Fig. 1 Synthetic route for the preparation of N-[^{35}S] -sulpho-2-amino tricarballylate

MATERIALS

Chloro-[^{35}S]-sulphonic acid and [^{35}S]-sodium sulphate were obtained from Amersham, Buckinghamshire, England. The resins AG 2-X8 (Cl^-; 100-200 mesh) and AG 50W-X8 (H^+; 200-400 mesh) were products of the Bio-Rad Laboratories, Richmond, California, U.S.A. The AG 2-X8 resin was converted to the bicarbonate

form before use. All chemicals were reagent grade and obtained from commercial sources. The scintillation fluid comprised of a mixture of 0.6% 2,5 diphenyloxazole in toluene and cellusolve (10:6 v/v).

METHODS

Preparation of Pyridine-[^{35}S]-Sulphur Trioxide

To a standard scintillation vial equipped with a magnetic stirrer bar was added 3.0 ml of anhydrous chloroform and 160 µl anhydrous pyridine. The vial was sealed with a rubber cap and cooled to 0° C by means of an ice-bath. Chloro-[^{35}S]-sulphonic acid (2 mCi/5 µl) was then diluted to 100 µl with non-radiolabelled chlorosulphonic acid, and drawn up into an airtight syringe fitted with teflon tubing. The rubber cap of the reaction vial was pierced, the tip of the teflon tubing introduced, and the syringe was clamped in an upright position above the level of the vial as depicted in Fig. 2.

The chloro-[^{35}S]-sulphonic acid was then added dropwise from the syringe over a 10 min period with stirring while the temperature was maintained at 0° C. At the end of this addition, the ampoule originally containing the chloro-[^{35}S]-sulphonic acid was rinsed with 100 µl of dry chloroform. This was again drawn up into the syringe, and the contents added to the reaction vial as before. The reaction mixture was allowed to stir for a further 5 min at 0° C before filtration under suction. The collected pyridine-[^{35}S]-sulphur trioxide was then washed with ice-cold anhydrous chloroform (2.0 ml), which had been used to rinse the reaction vial. The

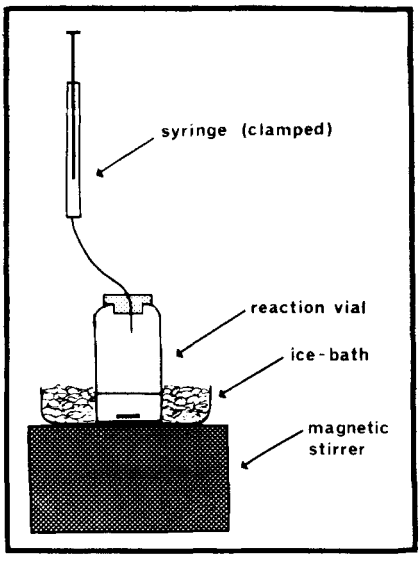

Fig. 2 Apparatus used for pyridine -[^{35}S]-sulphur trioxide synthesis

solid was dried thoroughly *in vacuo* over concentrated sulphuric acid in a desiccator for 2 h. The scintillation vial was retained for use in the next stage of the synthesis, and also dried in this fashion. The radiochemical yield of pyridine-[^{35}S]-sulphur trioxide was 27% (65 mg; sp. act. 8.3 μCi/mg).

Preparation of N-[^{35}S]-Sulpho-2-Amino Tricarballylate

To the scintillation vial used for the previous preparation was added 500 mg of 2-amino tricarballylic acid [prepared from diethyl acetone-dicarboxylate by the method of Dornow and Rombusch (4)] in 10 ml of water; the temperature was lowered to 10° C and the pH adjusted to 9.6 with 2 M NaOH. The pyridine-[^{35}S]-sulphur trioxide (65 mg) was added in 5 portions with continuous stirring over a 10 min period. The pH was maintained at 9.6 with 2 M NaOH using a pH-stat and the temperature held constant during this time. The mixture was then allowed to stir for a further 30 min with the pH still maintained at 9.6 while allowing the temperature to rise to 20° C. The resulting clear yellow solution was then passed through a 1.5 x 5.5 cm column of AG 50W-X8 (H^+) at 4° C to remove unreacted pyridine and 2-amino tricarballylate. After washing the column with a further 10 ml of water, the combined eluants were adjusted to pH 9.0 and loaded onto a 2.2 x 1.5 cm column of AG 2-X8 (HCO_3^-). After a preliminary wash with 250 ml of 0.35 M $NaHCO_3$ to remove [^{35}S]-sulphate, the stronger binding [^{35}S]-SAT was recovered by elution with 250 ml of 0.60 M $NaHCO_3$ collected in 5 ml fractions. The presence of [^{35}S]-sulphate and [^{35}S]-SAT was determined by measuring the radioactivity of aliquots (100 μl eluant/10 mls scintillant) taken from the 5 ml fractions collected (see elution profile, Fig. 3). Relevant tube contents containing [^{35}S]-SAT were pooled and decarbonated by stirring at 4° C for 10 min with an excess of AG 50W-X8 (H^+). The suspension was filtered, and the filtrate was adjusted to pH 7.0 with NaOH and lyophilized to give a white powder (25 mg; sp. act. 3 μCi/mg). The yield of radiochemical for this step was 15%, with the overall radiochemical yield for both steps being 4%.

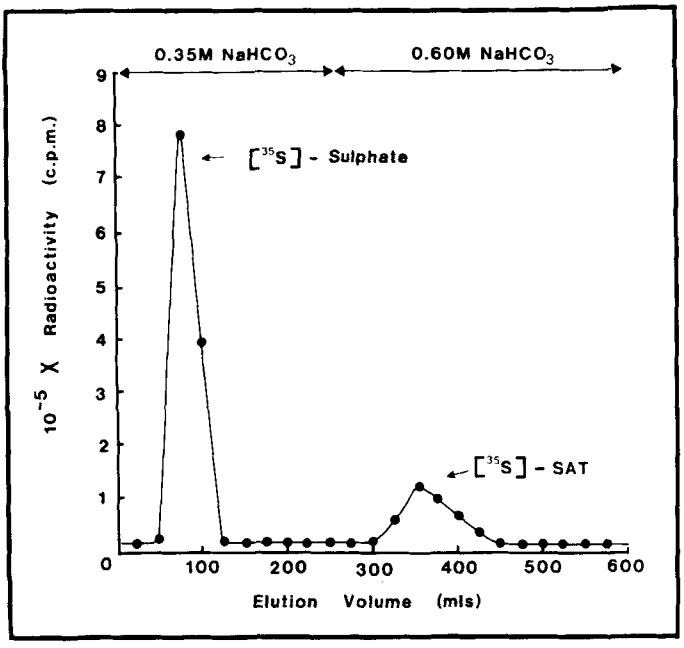

Fig. 3 Elution of [^{35}S]-SAT from AG 2X-8 resin

Analysis

Inorganic and N-hydrolyzable sulphate analyses (1), nitrogen determination (5) and high voltage paper electrophoresis were utilized to confirm the purity of [^{35}S]-SAT. Electrophoresis was performed by applying sample (1 µl; 1 mg/ml) of [^{35}S]-SAT and [^{35}S]-sulphate standard diluted to the same specific activity to Whatman No.3 MM paper and running at 2000 V for 1 h in an acid buffer [2.5% (v/v) acetic acid and 3% (v/v) formic acid]. The paper was dried and marked in a grid fashion before cutting into sections (3 cm wide and 1 cm long) incorporating the migration path of the compounds. These sections of paper were extracted with 2.0 ml of 1 M HCl, and the extracts were added to vials with 18.0 ml of scintillation fluid for counting.

RESULTS AND DISCUSSION

Radiolabelled [^{35}S]-SAT was successfully prepared from chloro-[^{35}S] -sulphonic acid with an overall radiochemical yield of 4%. The low yield was

largely attributable to the poor coupling of pyridine-[^{35}S]-sulphur trioxide with 2-amino tricarballylate (15%) a factor encountered with the synthesis of non-radiolabelled SAT (1) and presumably due to the steric hindrance surrounding the amino group of 2-amino tricarballylate. Nevertheless, the product was found to be of high purity as determined by sulphate analyses (free sulphate <0.1%; hydrolyzable N-sulphate 2.36 μmol/mg) and nitrogen analysis (2.41 μmol N/mg). This gave a sulphate to nitrogen ratio of 0.98 (theoretical value 1.00). Electrophoretic analysis (Fig. 4) confirmed these findings, showing the final product to be free of inorganic sulphate and any other detectable radiolabelled contaminants. In addition, the R_f of [^{35}S]-SAT in this system and its elution profile from AG 2-X8 resin was identical to non-radiolabelled SAT which had been fully characterized by physical ([^{1}H]-N.M.R., infra-red spectroscopy) as well as the abovementioned chemical techniques (1).

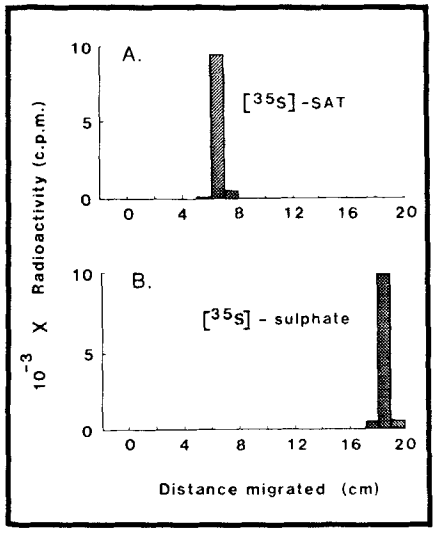

Fig. 4 Electrophoretic analysis of A) synthetic [^{35}S]-SAT, and B) standard [^{35}S]-sulphate

In summary, despite the low yield, N-[^{35}S]-sulpho-2-amino tricarballylate was produced in high purity by this simple and rapid two-step synthesis. The product obtained was of sufficient activity to support 20-30 pharmacokinetic experiments with laboratory rats, and these studies are currently being undertaken to evaluate the role that this new compound might have as a future drug in the treatment of kidney stone disease.

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