Tetrahedron. Vol. 31, pp. 1517 to 1519. Pergamon Press 1975. Printed in Great Britain

POLY(3,5-DIETHYLSTYRENE) SULFONYL CHLORIDE: A NEW REAGENT FOR INTERNUCLEOTIDE BOND SYNTHESIS[1]

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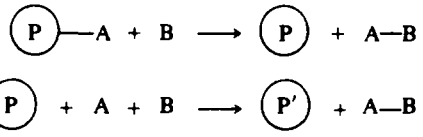
(*Received in the UK 25 November 1974; Accepted for publication 12 February 1975*)

Abstract—Insoluble crosslinked poly(3,5-diethylstyrene) sulfonyl chloride was prepared by bead copolymerization of 3,5-diethylstyrene and 5% divinylbenzene followed by chlrofosulfonation of the polymer. The insoluble polymeric sulfonyl chloride thus obtained was used for synthesizing internucleotide bonds. Dinucleoside phosphates were obtained in pure form and in high yields by relatively simple isolation and purification procedures such as extractions and precipitations, showing the polymer to be a convenient condensing agent for oligonucleotide synthesis.

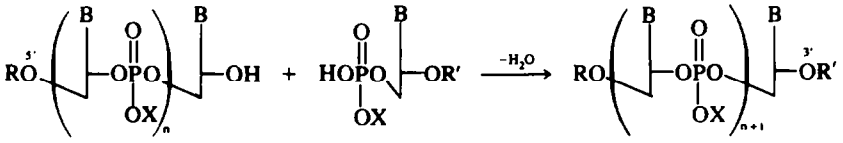
Chemically reactive insoluble polymeric compounds were recently used for organic synthesis.[2] In general such a polymeric reagent was allowed to react with a low-molecular weight substrate in a suitable medium in one of the following ways:



In the first reaction, A is bound via an active bond to the polymer P and is transferred to B. This approach has been used successfully in peptide synthesis where A was an acyl residue bound to an insoluble leaving group such as poly(4-hydroxy - 3 - nitrostyrene) and B was an amine.[3]

In the second reaction, P serves as a polymeric agent for the condensation of A with B, and several polymeric carbodiimides were prepared and used in this manner for peptide synthesis.[4]

The synthesis of oligonucleotide chains is usually affected by condensing two suitably protected nucleotides according to the following scheme:



n = 0, 1, 2, 3, ...
R,R′ = blocking groups
B = base residues such as thymine, adenine, etc.
X = Hydrogen or phosphate protecting group.

The condensing agents most frequently used are arylsulfonyl chlorides dissolved in pyridine[5] according to the following equation:

$$ROPO_3H^- + R'OH + ArSO_2Cl + C_5H_5N \rightarrow ROPO_2^-OR' + ArSO_3H + C_5H_5N \cdot HCl.$$

Although the mechanism of this reaction has not yet been completely understood, the first stage of the synthesis is probably the formation of a mixed sulfonate-phosphate anhydride. Nevertheless, the reaction forms considerable amounts of undesired side products, and sophisticated separation methods must be applied to isolate the end product. Firstly, direct nucleophilic attack by alcohol on the sulfonyl chloride leads to the formation of undesired sulfonate esters. This problem could be partially solved by using sterically hindered aromatic sulfonyl chlorides such as mesitylenesulfonyl chloride (MS) and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS).[5] Secondly, the high sensitivity of a sulfonyl chloride to hydrolysis has required the use of excess condensing agent. This causes the formation of large amounts of sulfonic acid which presents difficulties at the isolation stage since the solubility characteristics of sulfonic acids are similar to those of oligonucleotides. Finally, pyridinium 2,4,6-triisopropylbenzenesulfonate has detergent properties which cause stable emulsions to form during extraction with organic solvents.

It seems to us that a polymeric arylsulfonyl chloride could offer solutions to the above mentioned problems: excess polymeric reagent could be used and then removed at the end of the synthesis by filtration. Moreover, the sulfonate esters formed would be bound to the polymer and thus easily removed together with the polymer.

RESULTS AND DISCUSSION

Mono-α-bromination of 1,3,5-triethylbenzene followed by dehydrobromination with diisopropylethylamine gave 3,5-diethylstyrene in good overall yield (diisopropylethylamine was chosen as the base rather than pyridine,[1] which tends to quaternize). 3,5-Diethylstyrene was copolymerized with 5% divinylbenzene in aqueous medium to yield an insoluble polymer in bead form with an average size of 200 mesh. Treatment of the polymer with chlorosulfonic acid in chloroform gave a totally chlorosulfonated polymer (containing 3·7 mmol Cl per g) having a low content of sulfone bridges, as was indicated by its sulfur-to-chlorine ratio. The chlorosulfonated polymer swelled in common organic solvents such as benzene, chloroform, dioxan and pyridine, but not in

ether or hexane. The polymer is slightly sensitive to moisture and can be stored indefinitely in a tightly closed vessel. It is mechanically stable and completely insoluble in organic solvents or aqueous solutions, and can be readily filtered and washed with these solvents.

The synthesis of several dinucleoside phosphates using poly(3,5 diethylstyrene) sulfonyl chloride as condensing agent was examined. Thus, using anhydrous pyridine 5'-O-tritylthymidine was condensed with pyridinium 3'-O-acetylthymidine-5' phosphate using the polymer as a condensing agent (molar ratio 1:2:4). The colorless reaction mixture was kept overnight at room temperature. Water was then added and the polymer was removed by filtration. The dinucleoside phosphate was extracted with chloroform and precipitated with ether as its pyridinium salt. The product was obtained as a white amorphous powder and was shown to be homogeneous by thin layer chromatography. The yield was 90% based on the mononucleoside. Removal of protecting groups and enzymatic degradation gave thymidilic acid (pT) and thymidine (T) in a 1:1 ratio.

Similar results were obtained when three other dinucleoside phosphates were prepared by this method.

The polymeric sulfonyl chloride method was compared with that using the usual reagent 2,4,6-triisopropylbenzenesulfonyl chloride (TPS). The yields of dinulceoside phosphate prepared by the two reagents are similar, but isolation of the desired product prepared with the polymeric reagent is simpler due to the absence of sulfonate esters and sulfonic acid from the solution. Moreover, the polymeric reagent technique avoids the formation of stable emulsions during extraction with organic solvents. Furthermore, in the TPS synthesis, thin layer chromatography showed the final product to be contaminated with unidentified coloured impurities and with 2,4,6-trisopropylbenzenesulfonic acid which was developed by spraying with thymol blue. On the other hand, using the polymeric reagent, the dinucleoside phosphates were easily obtained in the pure state. These results show that poly(3,5-diethylstyrene) sulfonyl chloride is a convenient condensing agent for nucleotide synthesis.

The polymer was also a useful tool in investigating the mechanism of phosphorylation using aromatic sulfonyl chlorides. It was postulated that the sulfonyl chloride initially reacts with the phosphate ester to give a mixed sulfonate-phosphate anhydride. The mixed anhydride reacts with a second phosphate to give a symmetrical pyrophosphate. By repeating these steps, a triester of cyclic trimetaphosphoric acid is obtained. This triester is a slow phosphorylating agent, but the presence of catalytic amounts of aromatic sulfonyl chloride appears to considerably enhance the rate of phosphorylation.[6] Our findings support this theory. The insoluble polymeric sulfonyl chloride was allowed to react with a mononucleotide and filtered. Analysis of the polymer gave negative phosphate content, i.e. no mixed anhydride of the type polyaromatic-sulfonate-phosphate could be isolated. On the other hand, the filtrate, when reacted with a mononucleoside, gave a rather low yield (10%) of a dinucleoside phosphate. This indicates that the formation of pyrophosphates and probably a trimetaphosphate is a fast step, and it proves that excess of arylsulfonyl chloride is essential for efficient condensation.

Similar results were obtained when the phosphotriester approach[7] was investigated. Thus when a phosphodiester such as dibenzyl phosphate was allowed to react with the polymeric reagent, tetrabenzyl pyrophosphate was obtained in solution, and no phosphorous-containing residue was attached to the polymer. However, 5'-O-tritylthymidine-3' β-cyanoethyl phosphate was successfully condensed with thymidine by use of the polymeric sulfonyl chloride.[1]

These results may seem to be in contradiction to those obtained by Letsinger,[8] who was able to activate successfully a polymeric-bound phosphate by mesitylenesulfonyl chloride (MS) and to remove excess MS prior to addition of the nucleoside. However, in this case the formation of pyrophosphate was impossible, due to the mutual isolation of the phosphate groups through binding to the polymer. Therefore the reactive mixed sulfonate-phosphate anhydride was probably obtained on the polymeric support and could serve as a good phosphorylating agent.

EXPERIMENTAL

Material and general methods. Chemically pure 1,3,5-triethylbenzene was commerically available (Aldrich Chemicals), but it was alternatively isolated from technical grade triethylbenzene (Eastman Kodak) according to literature.[9] NMR spectra were measured on a Varian A-60 spectrometer in CCl_4 solns, with TMS as internal reference. Pyridinium salts of d-pT-OAc and d-pC ^{Ac}-OAc were prepared according to literature[10] and were isolated by adding their conc pyridine solns to a large excess of anhyd ether. N-anisoyl-5'-O-monomethoxytrityldeoxycytidine was obtained from P-L biochemicals, Inc., Milwaukee, U.S.A. Analytical grade pyridine was dried and stored over calcium hydride. Condensations were carried out in a drybox under a N_2. TLC was carried out on silica gel plates (containing a fluorescence indicator) using chloroform-methanol (7:3 v/v) (system A), and on cellulose plates (also with fluoroescence indicator) using 2-propanol-conc ammonia-water (7:1:2 v/v) (system B). The TLC plates were obtained from Riedel de Haen, Hannover, W. Germany. Dinucleoside monophosphates were analyzed by enzymatic degradation according to the literature.[7]

α-Bromoethyl-3,5-diethylbenzene. To a soln of 1,3,5-triethylbenzene (49 g, 0·3 mol) in anhyd CCl_4 (500 ml) NBS (43 g, 0·24 mol) was added together with dibenzoyl peroxide (1 g). The mixture was stirred and refluxed with exclusion of moisture until all the NBS has reacted (about 2 hr). It was then cooled and the succinimide filtered off. The filtrate was washed with water (two 200 ml portions) and dried. CCl_4 was removed *in vacuo* and the residue was fractionated under high vacuum. α-Bromoethyl-3,5-diethylbenzene was collected at 106–108°/1·5–2 mm Hg, yield: 51 g (88%); NMR (CCl_4) δ 1·2 (6H, t, J = 8 Hz), 1·95 (3H, d, J = 7 Hz), 2·58 (4H, q, J = 8 Hz), 5·03 (1H, q, J = 7 Hz), 6·85 (1H, s), 6·95 (2H, s). (Found: C, 59·53; H, 7·25; Br, 33·0. Calcd. for $C_{12}H_{17}Br$ (241·04): C, 59·71; H, 7·11; Br, 33·18%).

3,5-Diethylstyrene. A mixture of α-bromoethyl-3,5-diethylbenzene (135·2 g, 0·56 mol), ethyldiisopropylamine (72·5 g, 0·56 mol) and t-butylcatechol (1 g) was heated on an oil bath at 140° for 2 hr. The temp was then raised to 160° and the mixture heated for 2 more hr. After cooling, the mixture was taken up in methylene chloride (500 ml) and washed with water (two 500 ml portions), 0·5 N HCl (two 500 ml portions), and water (500 ml). The organic layer was dried and the methylene chloride removed *in vacuo*. t-Butylcatechol (1 g) was added to the residue and fractionation in high vacuum gave 3,5-diethylstyrene (b.p. 50°/1 mm Hg), yield: 67 g (75%) (the styrene was immediately polymerized or stored at −20°); NMR (CCl_4) δ 1·2 (6H, t, J = 7·5 Hz), 2·55 (4H, q, J = 7·5 Hz), 5·02–6·68 (3H, m, olefinic), 6·82 (1H, s), 6·95 (2H, s). (Found: C, 89·57; H, 10·32. Calcd. for $C_{12}H_{16}$ (160·13): C, 89·93; H, 10·07%).

Copoly(3,5-diethylstyrene-divinylbenzene). Polyvinyl alcohol (1 g) was dissolved in 400 ml of distilled water. The soln was filtered into a 500 l Erlenmeyer flask equipped with a ground-glass, mechanical stirrer. 3,5-Diethylstyrene (19 g), freshly distilled divinylbenzene (2 g, 50%), and dibenzoyl peroxide (60 mg) were added without stirring. The flask was placed on a bath at 90°,

and the mechanical stirrer was tightly closed using a teflon sleeve over the ground-glass joint. The mixture was stirred overnight at 90°. If polymerization did not occur, more dibenzoyl peroxide (another 60 mg) was added and the stirring continued for a further 24 hr. The polymer beads then obtained were filtered over a piece of cloth, washed with excesses of water, MeOH, chloroform, and MeOH, and finally dried, yield: 19·5 g (97%). The polymer was purified by Soxhlet extraction with dioxan.

Poly(3,5-diethylstyrene)sulfonyl chloride. Poly(3,5-diethylstyrene) (10 g) was dried *in vacuo* at 100° over P_2O_5 and suspended in 60 ml abs, EtOH-free chloroform. The suspension was cooled to 0° and chlorosulfonic acid (20 ml) was added dropwise over 30 min with stirring and the exclusion of moisture. The mixture was brought to room temp and stirred for a further 2 hr. The polymer was then filtered and washed with abs chloroform and anhyd ether (caution: heat is evolved during this step causing the ether to boil). The product is dried *in vacuo* over P_2O_5 and KOH and is stored in the desiccator. (Found: S, 12·1; Cl, 13·2. Calcd. for $(C_{12}H_{15}SO_2Cl)_n$ (258·64): S, 12·4; Cl, 13·7%).

The dinucleotide d-Tr-TpT-OAc. An anhyd pyridine soln (4 ml) of pyridinium 3'-O-acetyldeoxythymidine-5' phosphate (880 mg, 2 mmol) and 5'-O-tritylthymidine (550 mg, 1 mmol) was treated with poly(3,5-diethylstyrene) sulfonyl chloride (1 g, 3·7 mmol). The mixture was stirred at room temp for 2 hr. Water (2 ml) was then added under cooling and the soln kept overnight at room temp. The polymer was then removed by filtration and washed with pyridine (4 ml). More water (6 ml) was added to the combined filtrate and washings. The soln was extracted with ether (four 50 ml portions) to remove unreacted tritylthymidine. Ethyldiisopropylamine (0·5 ml) was added to the aqueous phase and the dinucleotide was extracted into chloroform-n-butyl alcohol (9:1, two 20 ml portions). The combined organic phase washed with water (10 ml) and 1 N NaCl aq (10 ml), dried over Na_2SO_4 and evaporated *in vacuo* to dryness. The residue was coevaporated with pyridine (10 ml), dissolved in dry pyridine (3 ml), and precipitated by dropwise addition of the soln to an excess of dry ether, yield: 900 mg (90%, determined spectrophotometrically after removal of protecting groups). The product was pure on TLC (R_f = 0·30, system A). The protecting groups were removed according to literature.[11] TLC of the deprotected compound gave a single spot (R_f = 0·28, system B), identical to that of authentic TpT. The product was also subjected to enzymatic degradation by snake venom phosphodiesterase, it was hydrolyzed quantitatively to thymidine and thymidine-5' phosphate which were obtained in a molar ratio of 1·03:1 (T/pT).

The dinucleotide d-Tr-TpC^{Ac}-OAc. Pyridinium N,O$^{3'}$-diacetyldeoxycytidine-5' phosphate (700 mg, 1·6 mmol) and 5'-O-tritylthymidine (1·85 g, 3·2 mmol) were condensed in anhyd pyridine (6 ml) using poly(3,5-diethylstyrene) sulfonyl chloride (1·5 g, 5·5 mmol). The mixture was worked up as described for the dinucleotide d-Tr-TpT-OAc. Precipitation from pyridine-ether (as above) gave the fully protected dinucleotide as a white solid, yield: 1·05 g (75% spectrophotometrically). The product was pure on TLC (R_f = 0·65, system B). After removal of the protecting groups, TLC gave one spot (R_f = 0·25, system B). The unprotected dinucleotide was enzymatically digested to thymidine and deoxycytidine-5' phosphate. T/pC = 1·01.

The dinucleotide d-MMTr-C^{An}pC^{Ac}-OAc. Pyridinium N,O$^{3'}$-diacetyldeoxycytidine-3' phosphate (470 mg, 1 mmol) and N - anisoyl - 5' - O - monomethoxytrityldeoxycytidine (300 mg, 0·5 mmol) were condensed in anhyd pyridine (4 ml) using poly(3,5-diethylstyrene) sulfonyl chloride (1 g, 3·7 mmol). The mixture was worked up as described for the dinucleotide d-TrTpT-OAc except that the unreacted nucleoside was extracted with chloroform-ether (1:1, two 20 ml portions). The fully protected dinucleotide was obtained as before, yield: 390 mg (74% spectrophotometrically). TLC gave one spot (R_f = 0·67, system A). After removal of protecting groups, TLC gave one spot (R_f = 0·27, system B). The unprotected dinucleotide was enzymatically digested to deoxycytidine and deoxycytidine-5' phosphate. pC/C = 0·97.

The dinucleotide d-MMTr-C^{An}pT-OAc. Pyridinium 3'-O-acetylthymidine-5' phosphate (450 mg, 1 mmol) and N-anisoyl-5'-O-monomethoxytrityldeoxycytidine (300 mg, 0·5 mmol) were condensed as described for the dinucleotide d-MMTr-Cp^{An}C^{Ac}-OAc. The yield of fully protected dinucleotide was 425 mg (82% spectrophotometrically). After removal of protecting groups, TLC gave one spot (R_f = 0·3, system B). The unprotected dinucleotide was enzymatically digested to deoxycytidine and thymidine-5' phosphate. pT/C = 1·04.

Acknowledgement—This work has been supported by Grant No. AM 05098 from the National Institutes of Health.

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