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SOLID PHASE 5'-PHOSPHORYLATION OF OLIGONUCLEOTIDES

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Abstract: S-triphenylmethyl O-methoxymorpholinophosphinyl 2-mercaptoethanol can be used to prepare 5'phosphorylated oligonucleotides. Deblocking to 5'phosphates is with $AgNO_3$ or I_2 followed by mildly basic conditions.

The synthesis of oligonucleotides by current solid phase phosphoramidite methods (1,2) yields products containing a free 5'-hydroxyl group. For many applications, especially total gene synthesis, 5'-phosphorylated oligomers are required. These can be prepared enzymatically using polynucleotide kinase but addition of the 5'phosphate group as an integral part of the solid phase synthesis is far more efficient. This has recently been achieved using a nitrophenylethyl protected phosphoramidite (3). The relatively hydrophobic phosphodiester initially formed could be separated from failure sequences by reverse phase hplc and the nitrophenylethyl group then removed with strong base. Other methods include:

(1) a methoxytrityloxyethylaniline protected 5'-phosphate of thymidine (4); however, its preparation is labourious and an additional thymidine is is also added to the sequence;

(2) a dimethoxytrityloxyethylsulphonylethoxy protected phosphoramidite (5); however, the cleavage of this group with NH_3 prevents purification by reverse phase hplc.

The reagent S-triphenylmethyl O-methoxymorpholinophosphinyl 2-mercaptoethanol (I) (Fig.1) is an alternative to the above procedures. I is easy to prepare as a stable solid, can be used to prepare 5'-phosphorylated oligonucleotides in automated solid phase DNA synthesis and retains the advantage of reverse phase hplc purification (6).

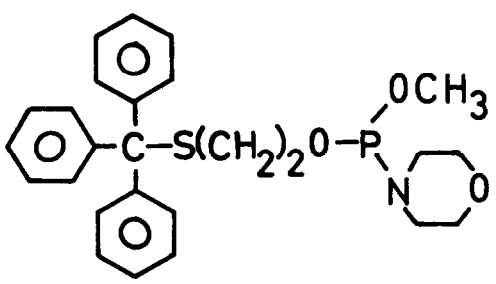

Fig.1. The structure of reagent I S-triphenylmethyl O-methoxymorpholinophosphinyl 2-mercaptoethanol.

I is prepared by the addition of 1 equivalent of methoxymorpholinochlorophosphite (2) (5mmol, 0.75ml) to S-triphenylmethyl 2-mercaptoethanol (7) (5mmol, 1.6g) dissolved in 25ml of dry and acid free CH_2Cl_2 containing diisopropylethylamine (10mmol, 1.9ml). The reaction is performed on ice with the exclusion of atmospheric moisture. It can be conveniently monitored by tlc on silica gel plates containing a fluorescent indicator (eluent petroleum ether (9), triethylamine (1); Rf of starting material 0.2, Rf of product 0.9) and is usually complete in 10 min. After warming to room temperature, 1ml of CH_3OH is added and after a further 5 min the mixture is extracted with 2 x 25ml 5% $NaHCO_3$ and 25ml saturated NaCl. The CH_2Cl_2 phase is dried over Na_2SO_4 and evaporated to about 2ml of a yellowish oil. This oil is dissolved in about 5ml of petroleum ether containing 10% triethylamine and purified by flash chromatography (8) on silica gel (column size 20 x 2.5cm) using the same solvent mixture as eluent. The fractions that contain product (detected by the above tlc system) are pooled and evaporated to about 1ml of a clear oil. This oil is dried in vacuo, over silica gel, and dissolved in about 3ml of dry diethylether containing 1% of triethylamine. After storage in a tightly sealed container, at -20°C for 3 days the product crystallises and can be obtained by filtration. Yields of 50% are typical and the product appears pure by tlc (above system) and ^{31}P NMR spectroscopy (δ = 144.1ppm relative to external 85% H_3PO_4).

The utility of I is illustrated in the preparation of d(pTGAGGATATCAGGT). This synthesis was performed using standard solid phase phosphoramidite techniques (1,2) with 75mg of controlled pore glass containing 1.3µmol of bound protected thymidine. 25µmol of incoming protected deoxynucleoside and 65µmol of tetrazole were used to prepare the desired sequence. At the end of this stage an extra round of synthesis is performed using 25µmol (12mg) of I together with 65µmol of tetrazole, i.e. I is used identically to normal protected deoxynucleoside phosphoramidites. After this coupling a capping and oxidation step are performed as normal but no attempt is made to remove the 5'-trityl group at this stage. Instead, after cleavage from the resin and removal of all the other protecting groups, the product is purified by reverse phase hplc. Fig.2A shows the hplc trace of this crude mixture - it comprises a peak eluting at 2 min. that contains failure sequences and a peak eluting at 11 min containing the product which still bears a hydrophobic trityl group. Thus the trityl group greatly aids in the purification of the oligomer and Fig.2B shows the hplc of the tritylated d(pTGAGGATATCAGGT) after hplc purification.

Final deblocking involves initial cleavage of the sulphur-trityl bond. This cannot be achieved using acid under conditions mild enough to prevent oligonucleotide depurination. However, this bond is easily broken with $AgNO_3$ (9-11) or I_2 (12) (1 equivalent oligonucleotide + 25 equivalents $AgNO_3$ or I_2 in 50mM triethylammoniam acetate pH 6.5 - containing 50% CH_3OH in the I_2 case - for 10 min). The lability of the trityl-sulphur bond to I_2 here

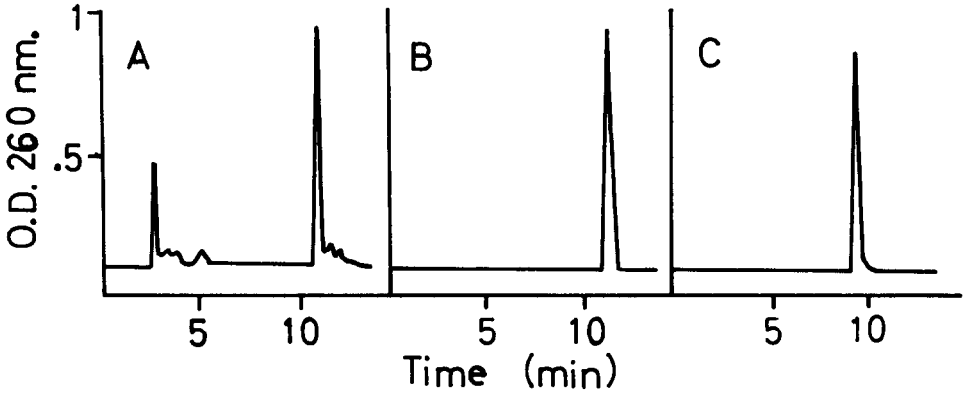

Fig.2. (A) Reverse phase hplc of crude $TrS(CH_2)_2OPO_3$-TGAGGATATCAGGT. (B) Reverse phase hplc of purified $TrS(CH_2)_2OPO_3$-TGAGGATATCAGGT. (C) Reverse phase hplc of purified d(pTGAGGATATCAGGT). An octadecylsilyl-silica column (25 x 0.45cm) was used with a 100mM triethyl ammonium acetate pH 6 buffer. For (A) and (B) a gradient of 20% to 70% CH_3CN in this buffer over 15 min was used whereas for (C) 5% to 45% CH_3CN over 15 min was used.

contrasts with its stability to I_2 during solid phase synthesis. Fig.3 shows that with $AgNO_3$ an intermediate oligonucleotide 5'-phosphate mercaptoethanol diester is the product (9-11) whereas with I_2 a disulphide dimer is produced (12). Deblocking is completed by the addition of 50 equivalents of dithiothreitol and raising the pH to 8.5 by $NaHCO_3$ addition. With $AgNO_3$ a silver dithiothreitol complex precipitates and is removed by centrifugation; with I_2 the dithiothreitol reduces both the excess I_2 and the disulphide dimer to monomer. In both cases, the mercaptide ion generated at pH 8.5 decomposes to the desired 5'-phosphate in 90 min. This decomposition probably involves ethylene sulphide formation (13). Deblocking by both methods is quantitative and the d(pTGAGGATATCAGGT) produced was purified by reverse phase hplc. Fig.2C shows the purity of the final product which was obtained in a yield of 15%. Its identify was confirmed by hydrolysis with snake venom phosphodiesterase (14). The mononucleotides dTMP; dGMP; dAMP and dCMP were produced in the expected ratio of 4; 5; 4; 1. No free nucleoside was produced indicative of 5-phosphorylation.

Thus I appears to be an ideal reagent for 5'-phosphorylation of oligonucleotides. Preliminary experiments with diisopropylaminocyanoethyl derivatives of 1 (15) suggest this is equally suitable. Furthermore, the specific and mild removal of the S-trityl mercaptoethanol group from phosphodiesters suggests that it may be generally useful in phosphate protection.

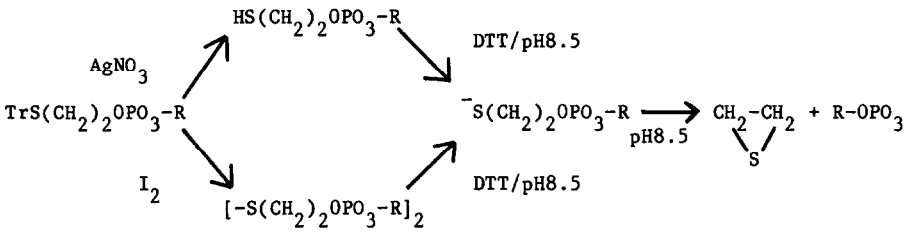

Fig.3. The mechanism of deblocking of $TrS(CH_2)_2OPO_3$-TGAGGATATCAGGT to d(pTGAGGATATCAGGT) with either $AgNO_3$ or I_2 followed by dithiothreitol and mildly basic conditions (R represents the oligonucleotide sequence and DTT is dithiothreitol). Negative charges on phosphate groups have been omitted for clarity.

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