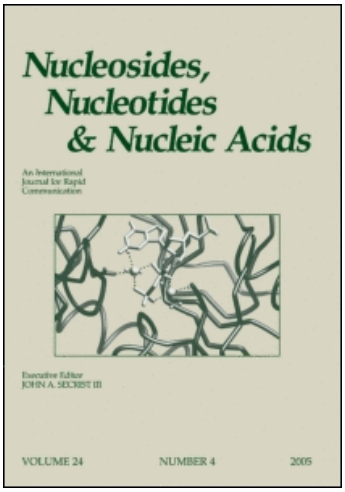

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Express Protocol for Functionalization of Polymer Supports for Oligonucleotide Synthesis

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EXPRESS PROTOCOL FOR FUNCTIONALIZATION OF POLYMER SUPPORTS FOR OLIGONUCLEOTIDE SYNTHESIS

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SUMMARY : A rapid and general one-pot method is described for the attachment of the leader nucleoside onto the polymer supports, suitable for polymer supported oligonucleotide synthesis following oxidation- reduction condensation. The method can also be used for support functionalisation in fully automated DNA synthesizer prior to oligonucleotide synthesis.

The introduction of stable synthons, improved polymer supports and the commercial availability of automated DNA synthesizers have simplified oligonucleotide synthesis[1,2] to such an extent that even a non-chemist can synthesize these molecules without much difficulty. However, there have been no parallel developments in the area of support functionalization chemistry. Only recently, some attempts have been made to simplify this process. Most of the methodologies used for the derivatization of supports are time consuming and involve the use of moisture sensitive and expensive reagents and therefore, require scrupulously anhydrous conditions[3-7].

In this communication, we describe a simple one pot method based on the use of a oxidation-reduction condensing agent (TPP-DTNP)[8] for anchoring nucleoside-3'-O-succinates onto amino group bearing polymer supports. The method is very fast (30 min) and gives fully functionalized polymer supports with

excellent nucleoside loadings. Though the method involves the use of nucleoside-3'-O-succinates, the preparation of these intermediates is no longer a limiting step. An expeditious method for the preparation of these intermediates has been reported[9] by our group. Therefore, it does not make much difference in the time of functionalization procedure. The supports with moderate nucleoside loadings (27-30 μmol/g LCAA-CPG), suitable for most automated oligonucleotide synthesis, have been obtained in just 2 min making the protocol suitable for support functionalisation in fully automated DNA synthesizer prior to oligonucleotide synthesis. The effect of various parameters, viz., solvent, oxidizing agent and base on the rate of functionalization has been studied in detail.

RESULTS AND DISCUSSION

In an attempt to simplify the process of functionalization, three parameters are considered of importance, (a) the method should be economical, rapid and should not involve multistep reactions and expensive reagents, (b) the procedure should avoid anhydrous conditions, and (c) the method should be applicable to a variety of polymer supports and amenable for large-scale preparations and automation.

The present communication outlines the application of oxidation-reduction condensation for functionalization of a variety of polymer supports. The method involves the use of an oxidation-reduction based coupling reagent (TPP-DTNP) to anchor leader nucleoside onto the amino group containing supports. In fact, the reaction mechanism involves the formation of an activated species, aryl-thiotriphenylphosphonium salt, in the first step, which subsequently reacts with the carboxylated nucleoside to give activated acyloxytriphenylphosphonium salt (Figure 1). In the final step, amino groups on the polymer support react very fast with the phosphonium salt to generate the fully functionalized supports with excellent nucleoside loadings (45-49 μmol/g of PS) (Table 1).

Effect of various parameters on rate of functionalization of polymer supports

The effect of various parameters (Fig. 2), viz., base or acylating catalyst, solvent and oxidizing agent on the rate of functionalization of polymer supports with 2'-deoxyribo-and ribonucleoside-3'-O-succinates was studied in detail.

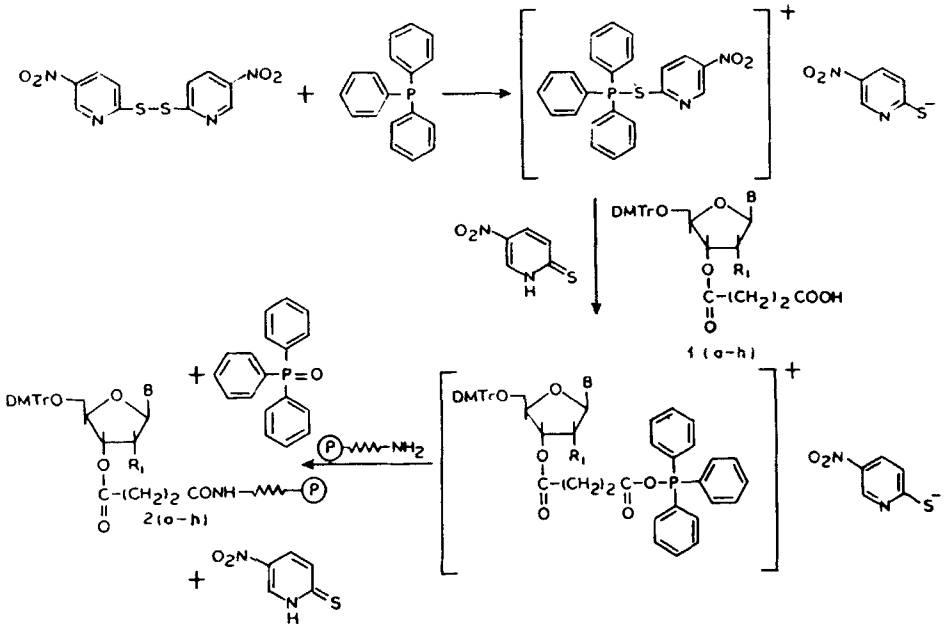

Fig.1 : Reaction mechanism involved in the reaction of TPP/DTNP with appropriately protected nucleoside-3'-O-succinate and amino group containing support

TABLE-1

Appropriately protected nucleoside		Loading in μmol nucleoside/g support		
		LCAA-CPG (50.02)*	AP-CPG (85.4)*	Fractosil (72.3)*
2a)	DMTdT	48.5	80.5	66.8
2b)	DMTdA(bz)	45.2	79.2	65.1
2c)	DMTdC(bz)	49.2	78.5	64.8
2d)	DMTdG(ibu)	47.9	79.6	66.3
2e)	DMTU-2'-TBDMS	45.5	69.5	58.6
2f)	DMTrA(bz)-2'-TBDMS	43.4	71.9	57.8
2g)	DMTrC(bz)-2'-TBDMS	42.2	72.5	59.3
2h)	DMTrG(ibu)-2'-TBDMS	42.5	71.5	58.4

* : amino group loading on the polymer supports

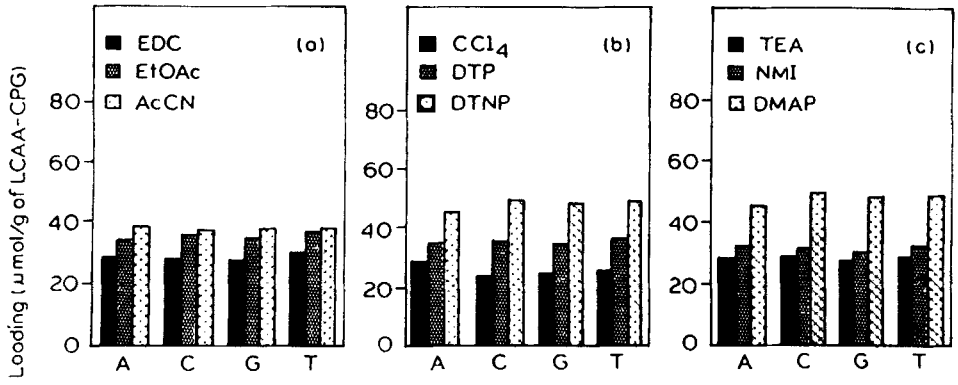

Fig.2 : The relative nucleoside loadings determined (a) in different solvents, using DTNP (oxidizing agent), TEA (base) and Rt = 2h; (b) using different oxidizing agents, DMAP (base) (2 eq. used in case of CCl_4), acetonitrile (solvent) and Rt = 30 min; (c) using different bases, DTNP (oxidizing agent), acetonitrile (solvent) and Rt = 30 min. A = DMTdA(bz), C = DMTdC(bz), G = DMTdG(ibu) and T = DMTdT

It was observed that the choice of solvent has a significant effect on the rate of functionalization of polymer supports by oxidation-reduction condensation. Acetonitrile was found to be the most appropriate solvent for this reaction. However, a minimum quantity of dichloroethane (25%) was employed in the reaction because of the poor solubility of DTNP in acetonitrile. Out of the three oxidizing reagents used,viz., DTNP, DTP and CCl_4, DTNP was found to be much superior. A tertiary base is usually recommended for this type of reactions. DMAP was found to be the most effective amongst the three bases used, viz., TEA, NMI and DMAP.

Functionalisation of polymer supports in automated DNA synthesizer

Due to fast reaction kinetics (Fig. 3), functionalisation of polymer supports was tried in automated DNA synthesizer keeping the coupling time of 2 min in the Pharmacia. LKB Gene Assembler Plus. Nucleoside loadings were determined by analyzing a small portion of the supports and were found to be in the range of 27-30 μmol/g LCAA-CPG. The derivatized supports, after capping, were directly used for oligomer synthesis. The method could be of interest for the functionalization of supports for rare or unusual nucleosides.

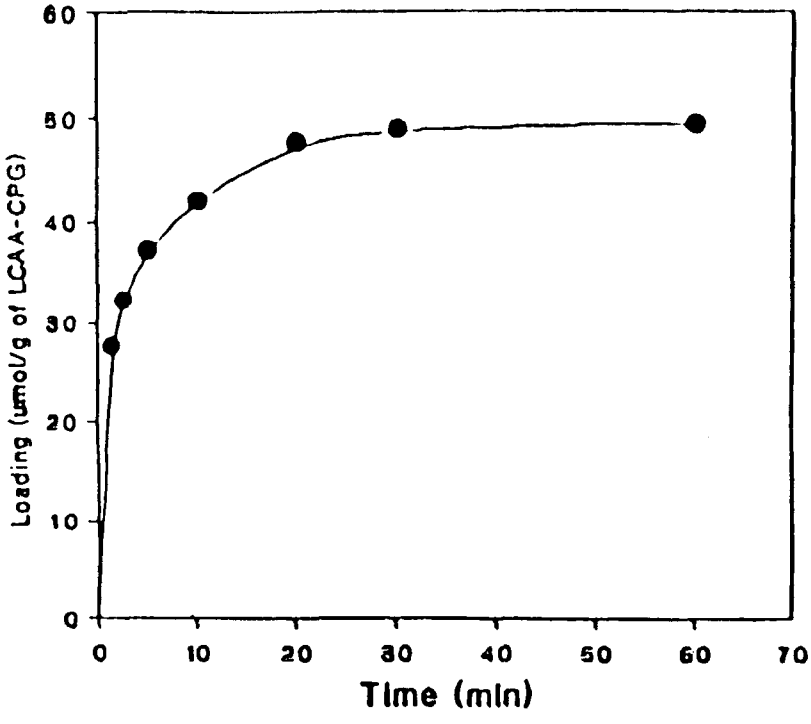

Fig. 3 : Time course of the reaction of DMT-dC(bz)-3'-O-succinate with LCAA-CPG

Oligonucleotide Synthesis

A number of oligonucleotides of different chain length were synthesized in order to test the efficiency of the polymer supports. Identical oligomers were synthesized on supports prepared by the standard procedure[10]. The coupling efficiency based on the released DMTr cation exceeded 98% and was found to be comparable to that of oligomers synthesized using standard supports. The synthesized oligomers were found to be identical in respect of their retention time, gradient concentration (FPLC and HPLC) and isolable yields (determined by measuring absorbance at 260nm) to those using standard supports.

Study the effect of reagents on nucleic base modification during support functionalization

The effect of reagents on nucleic base modification during support functionalization was carried out as described earlier[9]. A small quantity (100 mg)

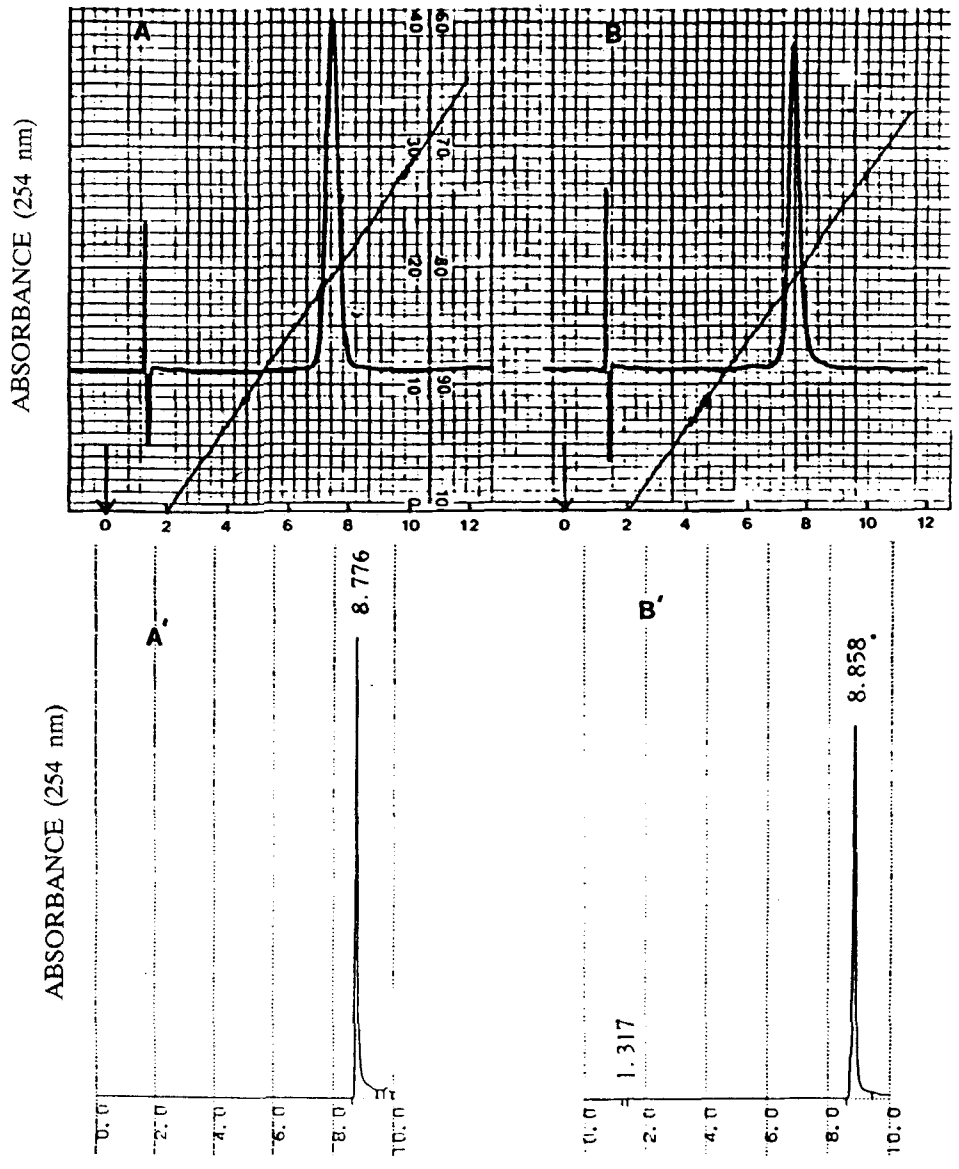

Fig.4 : Anion-exchange FPLC (A & B) and RP-HPLC (A' & B') analysis of d(GAA GTC GTA ACA AGG) synthesized on support **2d** and standard DMTdG(ibu) support. FPLC : Column : Mono Q HR 10/10, flow rate : 2 ml/min, gradient : 0% B for 2 min and 0-100% B in 35 min, buffer A : 0.5M NaCl containing 0.01M NaOH, pH 12 and buffer B : 1.0M NaCl containing 0.01M NaOH, pH 12. RP-HPLC : Column : Lichrosphere RP-18 (125 x 4 mm), flow rate 1 ml/min, gradient : 0-50% B in 25 min, buffer A : 0.1M ammonium acetate, pH 7.05 and solvent B : acetonitrile.

TABLE 2

Functionalization of LCAA-CPG with appropriately protected 2'-deoxy- and ribonucleoside-3'-O-succinates

	Methods					
	ORC	Kumar et al.[9]	Pon et al.[4]	Damha et al.[5]	Active ester[10]	Ogilvie et al.[11]
Functional groups on support	$-NH_2$	$-NH_2$	$-NH_2$	-COOH	$-NH_2$	$-NH_2$
Starting nucleosidic material	R-3'-O-suc.	R-3'-O-suc.	R-3'-O-suc.	R-3'-OH	R-3'-O-suc.	R-3'-O-suc.
Coupling time, h	0.5	6	24	16	30	72
Capping time, h	0.5	0.5	0.5	16	0.5	0.5
Loading (umol/g LCAA-CPG)	45-49	38-40	30-60	30-40	10-25	–
	*(42-45)**	*(38-40)*	–	*(30-40)*	–	*(18-20)*

*Values in parentheses are obtained for 2'-O-TBDMS-ribonucleoside-3'-O-succinates

R-3'-O-suc. = Appropriately protected 2'-deoxynucleoside-3'-O-succinates

R-3'-OH = Appropriately protected 2'-deoxynucleosides

ORC = Oxidation-reduction condensation

of each of the derivatized supports and supports prepared by the standard procedure were subjected to aq. ammonia treatment in a sealed vial for 30 min at room temperature. After concentration, the released 5'-N-protected-2'-deoxynucleosides were extracted in acid free diethyl ether and the solvent removed. A portion of each of the cleaved nucleosides was subjected to UV analysis and the other portions were subjected to HPLC analysis. The released nucleosides were found to be identical in all respects (UV, HPLC retention time etc.) to the corresponding nucleosides released from the standard supports (data not shown), which clearly indicate that no modification of nucleic bases occurred during the course of functionalization of polymer supports. This was further confirmed by FPLC and HPLC analysis of an oligomer, d (GAA GTC GTA

ACA AGG), synthesized on derivatized as well as standard support under identical conditions. Both of the oligomer eluted with same retention time and gradient concentration (Fig. 4).

Comparison of the suggested method with the conventional methods

The data shown in Table 2 can be used to evaluate the suggested method for the functionalization of LCAA-CPG in respect of coupling and capping times as well as nucleoside loadings. This method not only gives better nucleoside loadings in much lesser time but also avoids anhydrous conditions and hence commercially available solvents can be employed.

EXPERIMENTAL

High Performance Liquid Chromatography (HPLC) was performed on a Shimadzu LC-4A system fitted with variable wavelength detector SPD-2AS (set at 254 nm) and C-R7A Chromatopac for recording and analysis. Analytical HPLC was carried out on Lichrosphere RP-100 (4 x 125 mm) column, Merck, Germany.

Anion-exchange chromatography was performed on a Pharmacia FPLC system consisting of a single path monitor UV-1. Mono Q HR 10/10 column (Pharmacia, Sweden) was used for analysis.

3-Aminopropylation of CPG and Fractosil 200 was carried out according to standard procedure[10] and the contents of the amino groups on the polymer supports were determined following the procedure[5].

Functionalization of polymer supports

A solution of an appropriately protected 2'-deoxyribo- or ribonucleoside-3'-O-succinate **1(a-h)** (0.2 mmol) and DMAP (0.2 mmol) in acetonitrile (1 ml) was mixed with a solution of DTNP (0.2 mmol) in acetonitrile: dichloroethane (1 ml, 0.75:0.25). To this mixture, a solution of TPP (0.2 mmol) dissolved in acetonitrile (0.5 ml) was added. After a brief agitation, an amino group bearing support (LCAA-CPG) (1 g, 0.1 mmol of amino groups) was added and the suspension was further agitated for 30 min. The support was then recovered on a sintered disc glass funnel and washed with acetonitrile, dichloromethane and diethyl ether (20 ml of each). After drying, the residual amino groups were capped following the standard procedure[12]. The washed support was dried and stored at 4°C.

Optimum time for functionalization of polymer supports

In order to optimize the conditions for functionalization of polymer supports, the above experiment was repeated with 2.5 g of LCAA-CPG (250 umol of amino groups). During agitation, small aliquots were withdrawn at the interval of 10 min and thoroughly washed on glass funnel. Loading of nucleoside on the withdrawn samples was determined spectrophotometrically[10].

Synthesis, deprotection and purification of oligonucleotides

The synthesis of d(CAA ATA AAT AAG CAT TTT), d(AGC CCG GTT TTC CAG AAC), d(GCT GTA CG), d(AAG CTA GTC CCA CCT ATT CC), d(AAA AA), d(TTT TT), d(CCC CC) and d(GAA GTC GTA ACA AGG) was carried out at 0.2 μmol scale on Pharmacia.LKB Gene Assembler Plus following the standard protocol[13]. The same sequences were synthesized on the standard supports under identical conditions.

The cleavage of oligonucleotides from the supports and the removal of protecting groups from exocyclic amino as well as internucleotidic phosphate groups were achieved following standard protocol[10]. The desalted oligomers obtained were analyzed on anion-exchange FPLC and reverse phase HPLC.

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Abbreviations used : MeCN, Acetonitrile; AP-CPG, 3-Aminopropyl- controlled pore glass; DCM, dichloromethane; DMAP, 4-dimethylaminopyridine; DTNP, 2,2'-Dithiobis(5-nitropyridine); DTP, 2,2'-dithiodipyridine; EDC, ethylene dichloride; EtOAc, ethyl acetate; NMI, N-methylimidazole; TEA, triethylamine; TPP, triphenylphosphine; PS, polymer support.

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