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DEOXYRIBONUCLEOSIDE 3'-PHOSPHORDIAMIDITES AS SUBSTRATES FOR SOLID SUPPORTED SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES AND THEIR PHOSPHOROTHIOATE AND DNA-TRIESTER ANALOGUES

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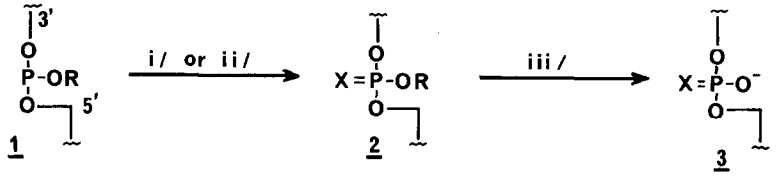
Abstract

5'-O-Dimethoxytrityl-base protected-nucleoside 3'-O-phosphordimorpholidites can be successfully used for the synthesis of oligodeoxyribonucleotides and their phosphorothioate or "triester" analogues.

All so far reported modifications of phosphite approach to the synthesis of oligodeoxyribonucleotides and oligoribonucleotides [1], originally designed by Letsinger [2], are distinguished by the intermediacy of phosphite type species (1). Their oxidation (sulphuration) leads to trialkyl phosphates (2, X=O) or trialkyl phosphorothioates (2, X=S), which are stable under conditions of further chain-extension [3]:



1 2 3

i/ X=O: I_2/H_2O/Lutidine/THF

ii/ X=S: S_8/Lutidine or S_8/CS_2 or S_8/Pyridine or KSCN

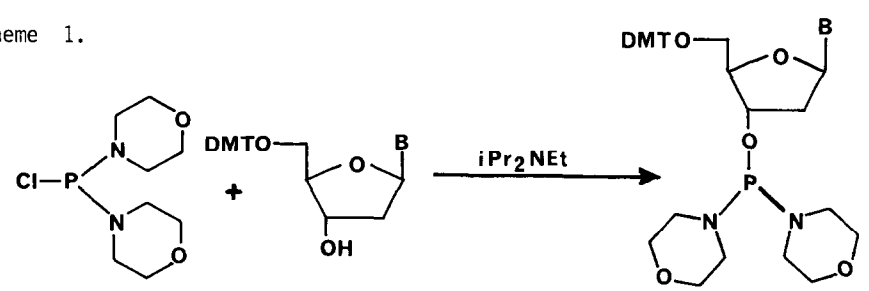
X=Se, KSeCN

iii/ OH^- or PhSH/Dioxane/Et_3N or NH_3aq.

Depending on the nature of -OR group attached to internucleotide phosphorus atom, phosphate deprotection involves hydrolysis with an attack of OH^- on phosphorus, attack of PhS^- on ester carbon atom, or β-elimination process [4].

In this communication we wish to present our modification of the synthesis of oligodeoxyribonucleotides, using 5'-O-dimethoxytrityl-base protected-nucleoside 3'-O-phosphordimorpholidites (6), available from the reaction of appropriately protected nucleoside (5a-d) with chlorodimorpholinophosphine (4) [5,6] (See Scheme 1).

Scheme 1.

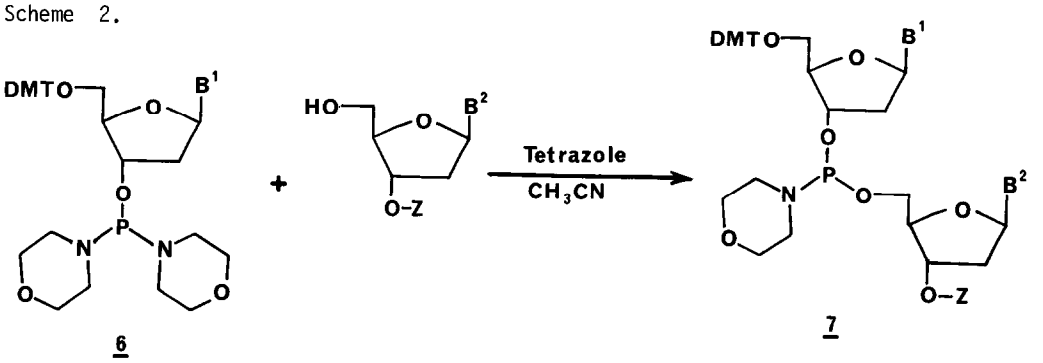


4 5 6

			Yield of 6 (%)	^{31}P-NMR, δ of 6 (ppm)(CD_3CN)
5,6	B = a	A^{NBz}	96	131.21
	b	G^{iBu}	70	131.46
	c	T	98	131.10
	d	C^{NBz}	77	131.63

Reaction of corresponding 5'-O-dimethoxytrityl-base protected-nucleoside 3'-O-phosphordimorpholidite (6) with base protected-3'-O-methoxyacetyl nucleoside, performed in acetonitrile solution in the presence of tetrazole (5-fold molar excess), leads to 3'-(5'-O-dimethoxytrityl-base protected-nucleoside)-5'-(3'O-methoxyacetyl-base protected-nucleoside)-phosphormorpholidite (7) - this process is completed within 3 minutes (See Scheme 2).

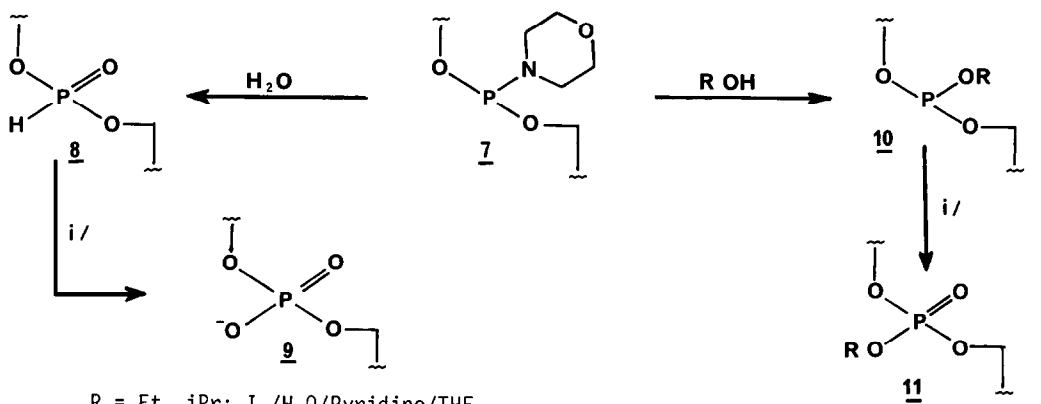
Scheme 2.



Z = Linker to solid support (LCA CPG, Applied Biosystems)

or Z = methoxyacetyl

As exemplified by dithymidyl(3',5')phosphormorpholidite (δ_{31P} 139.98 ppm, single line observed only), amidite P-N bond can be easily cleaved under mild acidic conditions [7] leading to 5'-O-(3'-O-methoxyacetyl)thymidyl 3'-O-(5'-O-dimethoxytrityl)thymidine H-phosphonate (8) (δ_{31P} 2.83 and 1.68 ppm, J_{P-H} 680 Hz), which subsequently was oxidized to give 5'-O-(3'-O-methoxyacetyl)thymidyl 3'-O-(5'-O-dimethoxytrityl)thymidine phosphate (9) (μBondapak C_{18} 0.78x30 cm column, HPLC assay). Alcoholysis [8] of 7 leads to dinucleoside O-alkyl phosphite 10 which was further oxidized, yielding corresponding dinucleotide O-alkyl ester 11 (See Scheme 3) [9].



R = Et, iPr; I_2/H_2O/Pyridine/THF

In order to estimate coupling efficiency of all four substrates 6a-d, we have synthesized, using chain elongation cycle shown in Scheme 4, the decamer 5' GGGAATTCCC 3' which contains Eco RI recognition sequence 5' GAATTC 3'.
Decamer GGGAATTCCC, obtained according to this methodology, was identical with genuine sample prepared *via* β-cyanoethyldiisopropyl phosphoramidite method [10], as proved by HPLC analysis, electrophoretic mobility on 20% polyacrylamide gel and digestion with Eco RI endonuclease leading to GGG and pAATTCCC fragments. Independently, this decamer was treated with snake-venom phosphodiesterase followed by alkaline phosphatase; the ratio of mononucleosides, estimated by HPLC analysis, was consistent with decamer sequence (2T:2A:3G:3C).
Coupling efficiency, estimated on the basis of trityl cation assay, was as follows:
A=96%, G=95%, T=89%, C=99%.

Scheme 4.
Protocol for chain-elongation cycle

Detritylation	2% dichloroacetic acid/methylene chloride	1 min
Wash	acetonitrile	
Coupling	0.5M tetrazole in acetonitrile 0.2M phosphordiamidite 6 in acetonitrile	3 min
Wash	acetonitrile	
Hydrolysis	20% water in 0.5M solution of tetrazole in acetonitrile	5 min
Wash	acetonitrile	
End cycle:		
Oxidation	0.1M I_2 in H_2O/Lutidine/THF (1:2:2) [1]	10 min
Wash	acetonitrile	

As inspection of the protocol presented in Scheme 4 clearly demonstrates advantage of our approach over that commonly used in automated solid phase O-methyl or β-cyanoethyl phosphoramidite methodology [1], since oxidation is performed in one common step within the end cycle, before ammoniolytic cleavage from the solid support and base deprotection.

It should be pointed out that presented in this communication approach to the synthesis of oligodeoxyribonucleotides and their phosphotriester analogues, combines the advantages of classical phosphoramidite approach together with those offered by recently explored H-phosphonate method, developped by Stawiński et al. [11], and with recently published transformations described by Froehler [12]. Versatility seems to be the main advantage of our approach. Studies on the synthesis of phosphorothioylated oligonucleotides [13], their analogues containing phosphordithioate moiety(ies) and other backbone-modified DNA fragments are in progress

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5. N-Trimethylsilylmorpholine [14] (2-fold molar excess) was added to phosphorus trichloride with external cooling (approx. 20°C) and reaction mixture was left for 0.5 h. Product was distilled under reduced pressure and the fraction 112-114°C/0.05 mmHg was collected (yield 50%). As the product is crystalline, it was stored for handling convenience in methylene chloride solution.
6. Phosphitylation was performed in methylene chloride in the presence of diisopropylethylamine and monitored by means of TLC (C_6H_6-CH_2Cl_2-Et_3N = 3.5:6:0.5; Rf= 0.8 to 0.85). After 0.5 h, the reaction went to completion. Products 6a-d were isolated, as foam-like solids, by means of short column chromatography on 230-400 mesh silica gel (Merck) using C_6H_6-CH_2Cl_2-Et_3N (4:4:2) as eluting system.
7. Hydrolysis of 7, performed with 20% H_2O in 0.5M solution of tetrazole in acetonitrile (120 µl per column), does not affect 5'-DMT group and enables execution of trityl cation assay. However, this process can be performed in the presence of dichloroacetic acid (DCA) instead of tetrazole. Optimal conditions for simultaneous hydrolysis/detritylation step are under studies.
8. Alcoholysis was performed using anhydrous ethanol or isopropanol and resulting derivatives (10, R=Et, δ_{31P} 139.91 and 138.84 ppm; R=iPr, δ_{31P} 139.29 and 139.26 ppm) after oxidation were compared with genuine samples obtained in our earlier studies [10].
9. Reactions in solution were performed using 6c and 3'-O-methoxyacetyl thymidine in anhydrous acetonitrile in the presence of tetrazole, ^{31}P-NMR spectra were recorded on MSL 300 Bruker. The same reactions were repeated on solid support (5'-DMT-dT-LCA CPG Applied Biosystems). After cleavage from solid support, compounds 9 and 11 were compared with genuine samples, synthesized independently using standard methods [10].
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13. According to protocol presented in Scheme 4, the decamer $(Tp(S))_9T$ has been synthesized on 2 µmol scale using Fractosil 500 as solid support, loaded with DMT-dT at level 42 µmol/g. End cycle was modified using instead iodine oxidation, the addition of sulphur to all nine internucleotide H-phosphonate moieties (saturated solution of S_8 in diisopropylethylamine, column left for 8 h at room temperature).
Product $(Tp(S))_9T$, after cleavage from the support and removal of 5'-DMT group, did show in ^{31}P-NMR spectrum (1 mM EDTA in D_2O) the presence of multiplet centered at 57 ppm. Electrophoresis of the same product on 20% polyacrylamide gel did show the presence of major band characteristic for decamer, although the bands characteristic for shorter sequences were also present.
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