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NUCLEOSIDE H-PHOSPHONATES. III. CHEMICAL SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES BY THE HYDROGENPHOSPHONATE APPROACH

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Abstract

A rapid synthesis of oligodeoxyribonucleotides on solid support is described via coupling of the deoxyribonucleoside 3'- H-phosphonates in the presence of various condensing reagents.

The formation of internucleotidic bonds via phosphorotriester[1] and phosphite triester[2] intermediates are two well established methods for the chemical synthesis of oligonucleotides.

Because of the higher reactivity of P(III) intermediates, the phosphite triester method and the phosphoroamidite approach in particular, has attracted considerable attention during the last few years. Several different nucleoside phosphoroamidites have been proposed as starting materials for oligonucleotide synthesis, but the choice is still a compromise between stability and reactivity[3]. Since formation of the internucleotidic bonds in that approach is brought about via protonation of the starting material rather than activation by condensing reagents, as in the phosphorotriester method, rigourously anhydrous conditions are required during the condensation, which often restricts the phosphoroamidite method to skilled experimentalists.

Searching for a method of oligonucleotide synthesis which would combine the advantages of both the phosphorotriester and the phosphite triester methods, as well as those of the phosphorodiester method (e.g. the lack of protecting group on the phosphorus center), we turned our attention into nucleoside H-phosphonates[4]. These phosphite monoesters, which exist predominantly in the H-phosphonate form, theoretically should fulfill most of the requirements for a good starting material in oligonucleotide synthesis.

Recently we have reported on an efficient method for the synthesis of nucleoside H-phosphonate diesters[5] which involves reaction of the nucleoside H-phosphonate monoesters with a suitably protected nucleoside in the presence of condensing reagents. It was found that the coupling reaction using H-phosphonate monoesters in the presence of condensing reagents, is at least as fast (and probably faster, see below) as in the phosphoroamidite approach, and H-phos-

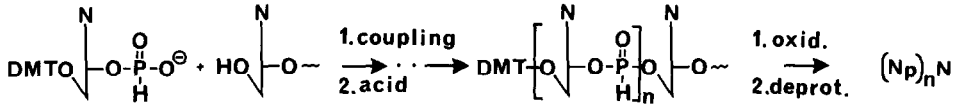
phonate diesters do not undergo further activation by coupling reagents[5,7]. In that last respect H-phosphonate diesters resemble phosphorotriesters. On the other hand, since the internucleotidic bond is in the H-phosphonate form, only one step, namely the oxidation, is required to convert it into the desired phosphorodiester bond. That can be conveniently done under mild conditions at the end ofoligomer synthesis by treatment with 2% I_2 in pyridine-water (98:2)[6]. This was found to be superior to other oxidation procedures investigated by us[6] or others[10]. ^{31}P NMR studies on the H-phosphonate diester formation[7], allowed us to design the proper reaction conditions (see below) for the synthesis of oligonucleotides on solid support.

Table 1. Protocol for machine-assisted oligonucleotide synthesis

Steps	Reagents and solvents (flow rate 3 ml per min)	time
Elongation cycle		
1. detritylation	2% dichloroacetic acid in CH_2Cl_2	2 min
2. wash	acetonitrile	1 min.
3. wash	acetonitrile-pyridine (1 : 1)	1 min.
4. condensation	monomer (20-30 eqv.,20 mM solution) and coupling agent (100-150 eqv.,100 mM) in acetonitrile-pyridine (1 : 1) were passed through the column in alternating mode in the form of 6-9 segments (3 sec each)	48-54 sec.
5. wash	acetonitrile-pyridine (1 : 1)	0.5 min.
6. wash	acetonitrile	1.5 min.
End cycle		
1. oxidation	2% I_2 in pyridine-water (98:2)	10 min.
2. wash	acetonitrile	2 min.
3. detritylation	2% dichloroacetic acid in CH_2Cl_2	2 min.
4. wash	acetonitrile	3 min.

Starting materials, i.e. the suitably protected deoxyribonucleoside 3'- H-phosphonates were synthesized from the corresponding 5'-O-dimethoxytritylated nucleosides (T, dA^{bz}, dC^{bz}, dG^{isb}) in 75 - 90% yield using the PCl_3-imidazole procedure as reported previously for 5'-O-dimethoxytritylthymidine 3'- H-phosphonate.[5] The syntheses of oligodeoxyribonucleotides were performed with an automatic DNA synthetizer Nucsyn II (Kabi-Gen) using a glass column type reactor charged with Controlled Pore Glass (CPG 500, aminopropyl arm, derivatized with 3'-succinyl deoxyribonucleosides) or with silica gel(Fractosil 1000) bound nucleoside (ca 20 µmoles per gram of support). Because of our previous findings about the mechanism of H-phosphonate diester

formation[7] we tried to avoid mixing the H-phosphonates with coupling agent before reaction. Thus both reagents were passed through the column in alternating mode of 6-9 segments each. When the reactor was filled to ca 50-80% of its volume with the support, the remaining volume served as a mixing chamber for the nucleoside 3'- H-phosphonate and coupling agent.



To evaluate the efficiency of different coupling agents, we carried out syntheses of $(Tp)_{11}T$ using 20-40 molar excess of nucleoside H-phosphonate and 100-200 molar excess of different coupling agents: 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl), N,N-bis(2-oxo-3-oxazolidinyl)-phosphorodiamidic chloride[8] (OXP), diphenylchlorophosphate (DPCP) and pivaloyl chloride (PV-Cl). The coupling yields, estimated from the trityl assay, were in the range of 97-100% throughout the synthesis, but the samples differed on polyacrylamide gel electrophoresis (PAGE) after oxidation, deprotection (conc. ammonia, 55°C, 5-7 hr), and ^{32}P-labeling with polynucleotide kinase.

The crude reaction mixture from the synthesis with PV-Cl as a coupling agent revealed one spot of the expected $(Tp)_{11}T$ on PAGE. The picture was similar for reaction with OXP, but spots from shorter sequences were also visible. When TPS-Cl was used as coupling reagent, the dodecamer again was the main product, but spots from oligomers with n-1 and n-2 chain length, were rather strong, and practically no traces of shorter sequences could be detected. The reaction mixture from the DPCP synthesis, despite the fact that the yield in each step was high as judged from the trityl assay, showed several bands on PAGE, corresponding to oligomers with different chain length, and with $(Tp)_{11}T$ being a minor product.

Since the reactions with TPS-Cl, OXP and DPCP as coupling agents seem to require further investigations to find optimum conditions, all further syntheses were carried out with PV-Cl as a condensing reagent.

To obtain a rigorous test of our methodology, we carried out syntheses of oligomers with all four bases. Thus the following oligodeoxyribonucleotides have been synthesized: $d(Cp)_{11}T$, dCGGATCCG (8-mer), dACAGCGTAGAAA (12-mer), dGATCGACGTTCA (12-mer) and dCGTTTCAAAGATCGACGTTCA (21-mer). The crude reaction mixtures analysed on PAGE, after kination, showed the desired oligomer as one spot[9] with only faint shadows from shorter sequences. Some of the synthesized oligonucleotides are currently under biological studies.

It should be emphasized, that the various coupling agents investigated did not show any noticeable difference in terms of efficiency of coupling and the purity of products, when used for solution synthesis of dimers. Also the reaction mixtures from solid support syntheses carried out in a syringe, showed almost identical PAGE patterns. This indicates that for machine-assisted synthesis of oligonucleotides <u>via</u> the H-phosphonate approach, using coupling agents other than PV-Cl, technical changes are required.

Our studies on the activation of H-phosphonate monoesters have shown[7] that premixing of nucleoside H-phosphonates with PV-Cl produces nucleoside bis-acylphosphites, which can react with a nucleoside forming trinucleoside phosphites. We tried to establish whether this can influence the coupling yield (and purity of the final oligomer) during oligonucleotide synthesis on solid support. Thus, we carried out a synthesis of the hexamer $(Ap)_5A$ using the regular coupling procedure as described above and a procedure which involved premixing of H-phosphonate with pivaloyl chloride. After oxidation and deprotection, the product of the former synthesis consisted of the desired hexamer as one spot on PAGE, while the latter afforded the desired hexamer together with shorter oligomers. However, we do not consider these results as completely conclusive and more experiments are under the way.

The procedure presented above, which has not yet been optimized, clearly demonstrate the potential synthetic utility of nucleoside H-phosphonates in the chemical synthesis of oligonucleotides. Obvious advantages as e.g. stability of the starting materials, very fast and efficient coupling, no protection at the phosphorus center, together with a short and simplified elongation cycle (no capping step, only one oxidation step at the end of synthesis) and generally less rigourous requirements for anhydrous conditions (standard conditions as in the phosphotriester method), can make the approach via H-phosphonate intermediates a method of choice for the chemical synthesis of oligonucleotides.

Recently a similar approach to oligonucleotide synthesis has also been reported[10]

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