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NUCLEOSIDE H-PHOSPHONATES. IV. AUTOMATED SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES BY THE HYDROGENPHOSPHONATE APPROACH

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Abstract
A rapid and efficient synthesis of oligoribonucleotides on solid support is described via coupling of 5'-O-dimethoxytrityl-2'-O-t-butyldimethylsilyl ribonucleoside 3'-H-phosphonates II to the polymer bound nucleoside in the presence of pivaloyl chloride as coupling agent.

The emergence of RNA technology[1] and unusual properties of RNA molecules found during recent studies on the mechanism of self-splicing of messenger RNA precursors[2], as well as the known role of transfer RNAs in the translation of the genetic code[3], has rapidly increased the interest in RNA's chemistry, biochemistry and molecular biology.

In order to obtain deeper insight into the chemistry of RNA fragments and to provide the molecular biologist with a valuble research tool, a facile and rapid procedure for the synthesis of oligoribonucleotides has become an important synthetic goal.

In contrast to the fast progress in DNA synthesis, the development of chemical methods for the synthesis of RNA fragments has been rather slow, due to the problems with suitable protection of the 2'-hydroxyl function of ribonucleosides and because of lower efficiency of the coupling reaction.

Despite these difficulties, several oligoribonucleotides, which correspond to portions of tRNA molecules has been synthesized using the phosphorotriester approach[4]. Recently successful syntheses of oligoribonucleotides up to 19 nucleotidic units, using the chlorophosphite[5] and the phosphoroamidite approach[6], have also been reported. However, even using the rather reactive P(III) intermediates, the coupling reaction was considerably slower than in the deoxy series, and required recycling for 24 min[5] and 15 min[6] respectively during the condensation step. In addition, the relatively stable nucleoside morpholinophosphoroamidites, successfully used in oligodeoxyribonucleotide synthesis, were found to be difficult to activate to achieve efficient coupling in the ribo series[6].

In this communication, we would like to describe a new method for oligoribonucleotide synthesis, which consists of reaction of ribonucleoside 3'-H-phosphonates II (triethylammonium salts) with polymer bound nucleoside in the presence of pivaloyl chloride. After assembly of the

oligoribonucleotidic chain, having all internucleotidio bonds in the H-phosphonate form, the desired oligomers with phosphorodiester internucleotidic linkages were formed in a single oxidation step using 2% iodine in aq. pyridine, followed by deprotection.

The important step in the design of oligoribonucleotide synthesis, is the proper choice of the 2'-hydroxy protecting group for ribonucleosides. Our preliminary experiments, which have recently been confirmed by other laboratories[7], have shown, that it is rather difficult to obtain the required high selectivity of deprotection of the dimethoxytrityl group (DMT) from the 5' position in the presence of the tetrahydropyranyl group in the 2' position. Thus, we decided to use the t-butyldimethylsilyl group for protection of the 2'-hydroxy function in ribonucleotides, which is compatible with the DMT group in the 5' position.

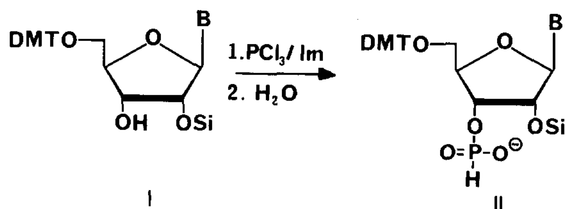
Starting materials, i. e. the suitably protected ribonucleoside 3'- H-phosphonates IIa-d were synthesized from the protected nucleosides Ia-d[8] in 75 - 90% yield using the PCl_3-imidazole procedure as described previously for deoxyribonucleosides[9].

The synthetic cycle, which was performed by an automated DNA synthesizer Nucsyn II (Kabi-Gen) is shown in Table 1.

Table 1. Protocol for machine-assisted oligonucleotide synthesis

Steps	Reagents and solvents (flow rate 3 ml per min)	Time
Elongation cycle		
1. detritylation	2.5% dichloroacetic acid in CH_2Cl_2	2 min
2. wash	acetonitrile	1 min
3. wash	acetonitrile-pyridine (1 : 1)	1 min
4. condensation	monomer IIa-d (40 mM solution) and pivaloyl chloride (200 mM solution) in acetonitrile -pyridine (1 : 1) were passed through the column in alternating mode in the form of 9 segments (3 sec each)	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	2.5% dichloroacetic acid in CH_2Cl_2	2 min
4. wash	acetonitrile	3 min

This cycle contains a 54 sec coupling step of ribonucleoside 3'- H-phosphonates IIa-d with a nucleoside (deoxy or ribo) bound to Controlled Pore Glass (CPG 500, aminopropyl arm, derivatized with 3'-succinylnucleoside). This was sufficient to produce the consistently high coupling yield (97-99%).



DMT- 4,4'-dimethoxytriphenylmethyl group

Si - t-butyldimethylsilyl group

Im - imidazole

Ia,IIa B=uracil

Ib,IIb B=N^4-benzoylcytosine

Ic,IIc B=N^6-benzoyladenine

Id,IId B=N^2-isobutyrylguanine

Figure 1. Synthesis of fully protected ribonucleoside 3'- H-phosphonates.

^{31}P NMR data of fully protected ribonucleoside 3'- H-phosphonate triethylammonium salts (in pyridine with 2% H_3PO_4 in D_2O as external standard).

Compound	Chemical shift (ppm)	$^1J_{PH}$(Hz)	$^3J_{PH}$(Hz)
IIa	1.2	604	10.1
IIb	1.9	617	10.3
IIc	2.6	621	9.7
IId	2.2	615	11.5

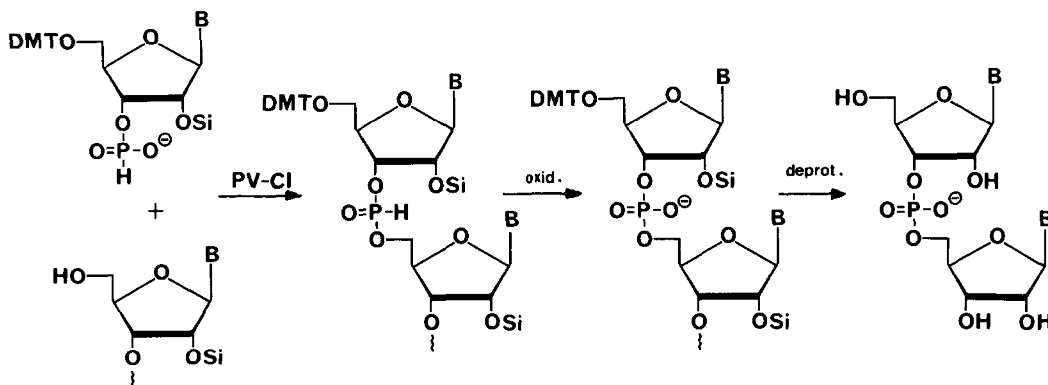


Figure 2. Formation of the internucleotidic phosphorodiester bond *via* the H-phosphonate approach.

In a typical experiment (conditions not optimized), 30 equiv. of IIa-d and 150 equiv. of pivaloyl chloride was used. When the assembly of the oligonucleotidic chain was completed, the

support-bound oligonucleotide H-phosphonate was oxidized during 10 min using 2% I_2 in aq. pyridine, deprotected with 2.5% dichloroacetic acid in CH_2Cl_2 and treated with ammonia (25%)-ethanol (3 : 1)[6] during 8 h. The polymer was removed by filtration and washed with ethanol. The combined filtrate and washings were evaporated and dissolved in 0.1 M TBAF in THF (0.5 ml, 2 h). The solution was concentrated, diluted with water (0.5 ml) and desalted on a Pharmacia PDT-10 column. The eluant was lyophilized and, after ^{32}P-labeling with polynucleotide kinase, the reaction mixtures were analysed on polyacrylamide gel electrophoresis (PAGE).

Using the above procedure, the following oligoribonucleotides have been synthesized: dodecamers $(Cp_{11})dT$, $(Up_{11})G$, rACAGUGUCCUAG and the 21-mer rAUGAAAUCGACAGUGUCCUAG.

All oligoribonucleotides appeared on PAGE as single spots but in the case of the 21-mer faint shadows from oligonucleotides with shorter chain length were observed. Synthesized oligonucleotides were characterized by sizing them against oligodeoxyribonucleotidic standards on PAGE and the nucleotide composition of each sequence was determined by enzymatic degradation with snake venom phosphodiesterase (SVPD), followed by HPLC analysis (Particil PXS 10 SAX column, isocratic elution with 0.05 M potassium phosphate buffer, pH 3.35)[10]. Complete digestion of pyrimidine oligomers with Pancreatic Ribonuclease confirmed the 3'-5' character of all phosphorodiester internucleotidic linkages.

These syntheses clearly demonstrate the potential utility of the H-phosphonate approach in chemical synthesis of RNA fragments. Starting materials, i. e. ribonucleoside 3'-H-phosphonates IIa-d are easy to prepare, purify and handle. The short and simplified elongation cycle and nearly quantitative yields open for the first time the real possibility of synthesis of longer RNA sequences such as transfer RNA. It is worth to stress, that the above presented H-phosphonate approach to oligonucleotide synthesis, is at present the only method which give good results both in the deoxy[11] and the ribo series.

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