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# Developments in the chemical synthesis of naturally occurring DNA and RNA sequences with normal and unusual linkages

Kelvin K. Ogilvie, Masad J. Damha, Nassim Usman and Richard T. Pon

Chemistry Department, McGill University, Montreal, Canada H3A 2K6

Abstract: A general method for the chemical synthesis of long oligoribonucleotide chains has been developed. The key features of the method are the use of alkylsilyl protecting groups for the 2'-position and the "phosphite" coupling procedure for internucleotide bond formation. The procedure has been adapted to an automated process using controlled pore glass supports. The successful synthesis of a 43-mer similar in sequence to the 3'-terminus of the f-met tRNA from E. coli is described. The method has been adapted to the synthesis of novel sequences including the branched trimer at the juncture point in the splicing of eucaryotic messenger RNA.

## INTRODUCTION

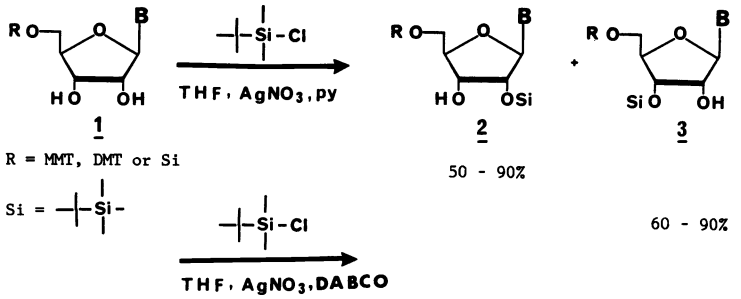
The development of methods for the chemical synthesis of long chains of RNA has been much slower than for DNA chains. The major reason for this has been the difficulty in developing a suitable protecting group combination for ribonucleosides. The presence of the 2'-hydroxyl group in the ribose series has complicated the protection of the hydroxyl groups. The group used to protect the 2'-position has to be chosen with additional care since it must ultimately be removed without leading to cleavage of the internucleotide linkage. Finally, once all protecting groups are removed from the product of synthesis, great care must be exercised in handling the free nucleotide. RNA chains are susceptible to chemical and enzymatic hydrolysis. Ribonucleases seem to be unusually abundant in secretions and in airborne organisms.

The current state of activity in RNA synthesis bears many similarities to that of DNA synthesis a decade ago. At that time the excitement over DNA was growing but biochemists and microbiologists were almost unanimously agreed that chemical synthesis would not be of particular use to them. That attitude has changed dramatically and nearly all gene syntheses make extensive or complete use of sequences prepared on "gene machines". The ready availability of chemically synthesized DNA sequences has been the key to the explosive rate of developments in understanding cellular mechanisms involving DNA. It appears that we are on the threshold of the same situation in RNA. In addition to their key roles in protein synthesis (e.g. mRNA, tRNA, rRNA and lariat processing) RNA molecules have recently been found to have enzyme-like catalytic activity and are the sole structural components of viroids. The complete understanding of their function at the molecular level awaits the recognition by biochemists and molecular biologists that chemistry can now provide the sequences they desire. We are on the verge of the RNA decade.

## PROTECTING GROUPS

Several research groups made important contributions to the development of protecting groups for ribonucleosides. The Khorana (1) and Reese (2) groups did extensive early work in this area. The key in much of this work was the use of tetrahydropyranyl or acyl (aroyl) groups on the 2'-hydroxyl group. These teams successfully prepared ribonucleotides as have Letsinger (3), Van Boom (4), Neilson (5) and others (6). Benzyl groups (7) have also been used to protect the 2'-position during the chemical synthesis of ribonucleotides.

Several years ago we began to explore the use of silyl groups for the protection of sugar hydroxyls in ribonucleosides (8). We found the existing routes to protected ribonucleosides to have far too many steps to be practical as general procedures. Fortunately, we found that the silyl groups could be directly introduced in good yields and with high selectivity. Treatment of a ribonucleoside 1 with silyl chlorides in the presence of silver salts leads to 50-90% yields of 2'-protected products 2 (9). Generally, the 2'-isomers are readily separated on chromatography from the 3'-isomers.



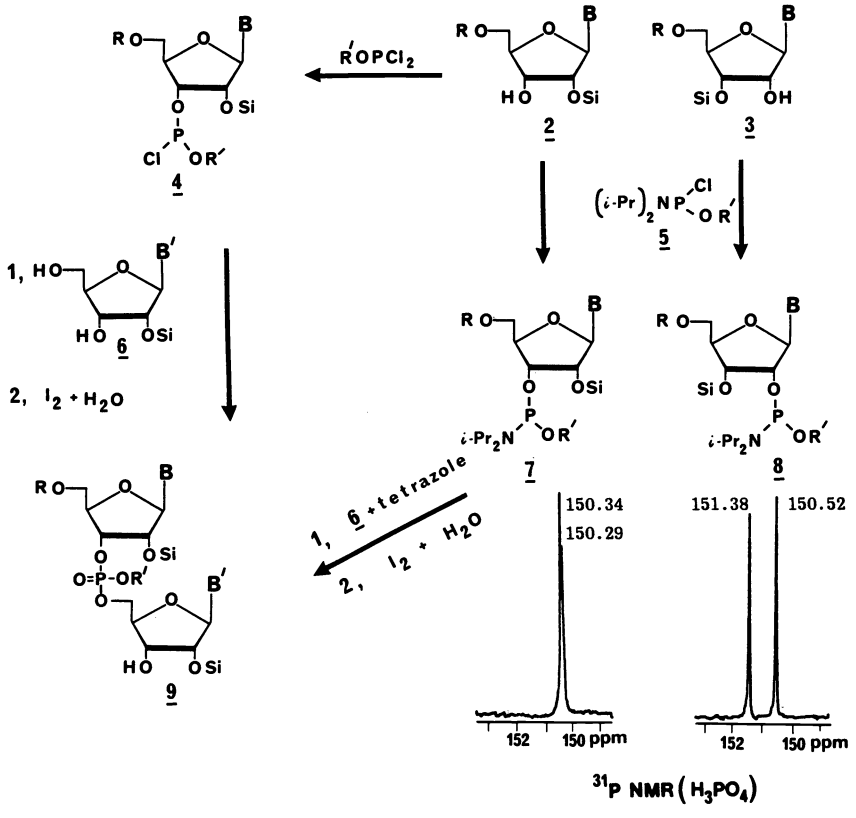

1
R = MMT, DMT or Si
Si = —|—Si—
2
3

Conditions were also developed that permit selective silylation at the 3'-position (9). Thus, compounds 3 can be obtained in 70-90% by using DABCO as a catalyst (for a review see ref. 10). Migration of silyl groups can occur under some conditions, particularly those involving basic solutions and protic solvents (8, 11). Contrary to some suggestions (12), however, the silyl protecting groups that we use are completely stable to the conditions commonly employed in both traditional- and phosphite-triester procedures (11, 13).

It is important to note that silyl chlorides are quite hygroscopic. Thus, for very small scale reactions or when using older reagents, larger than reported excesses of silylating agent may be required. The silver nitrate should be finely ground. Tetrahydrofuran is dried over activated molecular sieves (4Å) and distilled from sodium (and benzophenone). We generally use the t-butyldimethylsilyl (TBDMS) protection for A, C and U and the triisopropylsilyl (11, TIPS) group for guanosine. Base rings in A, C and G are protected at the amino groups with the benzoyl group introduced through the transient protection route. An additional problem can exist at the $O^6$-position of guanosine and this is dealt with below.

## CONDENSATION METHODS

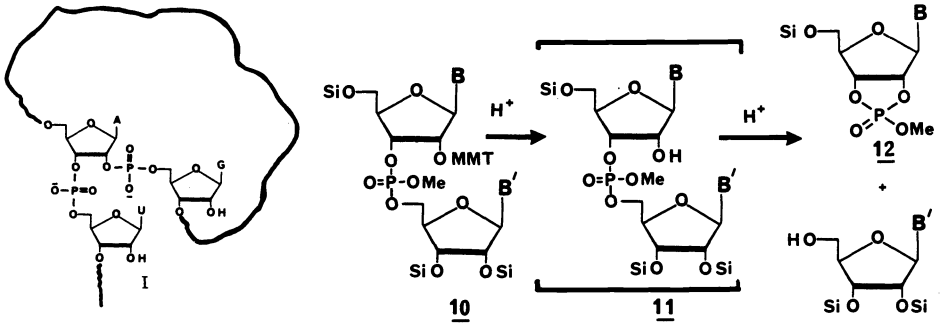
The phosphite condensation procedure introduced by Letsinger (14) dramatically altered the rate and efficiency of DNA synthesis. We immediately recognized the potential value of this method in ribonucleotide synthesis. Our first experiments showed us that the procedure was totally compatible with the silyl protecting groups, was fast, and was the cleanest condensation procedure we used (15). In addition, the problem of 3'-3' joining of monomers was greatly reduced relative to the deoxy series; the steric hindrance of 2'-protection was finally an advantage!



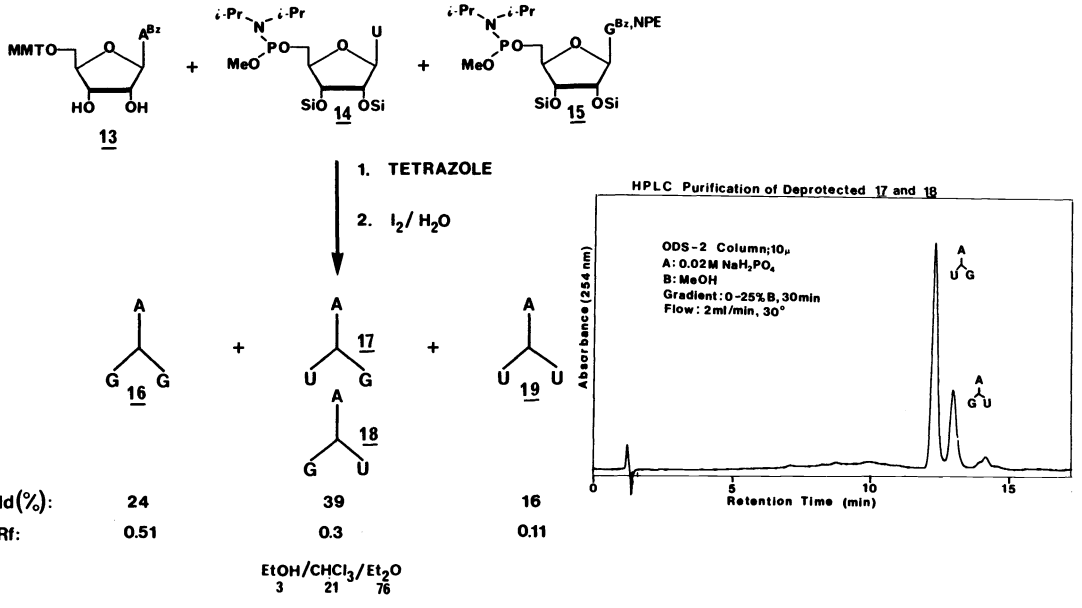
All our initial studies (15-18) were carried out using the chlorophosphite procedure. This involved generation of nucleoside 3'-chlorophosphites (4) just before use since they do not store well. These reagents are, however, ideal in the hands of skilled experimentalists and we prefer to use them when carrying out solution syntheses. Most people prefer to store reagents and for solid support syntheses this is clearly desirable. Thus, we have adapted the phosphoramidite reagents (19), initially introduced to DNA synthesis by Beaucage and Caruthers (20), to RNA synthesis. Either THF or acetonitrile can be employed as solvent. We generally use acetronitrile for solid support syntheses. Phosphoramidites must be activated by, for example, tetrazole which forms a reactive tetrazolide intermediate. Isomeric phosphoramidites 7 and 8 have been prepared and they are clearly distinguishable in the $^{31}P$ NMR (crude reaction mixture shown). This further supports the stability of the silyl groups under phosphorylation conditions.

## SYNTHESIS OF BRANCHED RNA

The excision of introns during the processing of messenger RNA has been receiving a great deal of attention. One of the mechanisms for the splicing of eucaryotic messenger RNA precursors involves the formation of circular RNA molecules (21-24) commonly referred to as "lariats". A unique feature of these structures is a branching trinucleotide that contains vicinal 2'-5' and 3'-5' phosphodiester linkages (I).




The synthesis of these trinucleotides clearly demonstrates the difficulties involved in RNA protecting groups. One would initially think that the synthesis of a molecule such as I would be straightforward. The coupling of a 2', 5'-protected adenosine to a 2', 3'-protected uracil would lead to a dinucleotide from which removal of the 2'-protecting group from the A unit and subsequent coupling to a suitably protected G unit would yield the desired trinucleotide. This route fails miserably when triester methods are employed for the formation of internucleotide linkages. Neither basic nor acidic conditions can be employed to remove a 2'-protecting group adjacent to a phosphotriester since cleavage of the triester occurs simultaneously with removal of the group from the 2'-position (the same situation holds for removal of a 3'-protecting group adjacent to a 2'-phosphotriester). The target compounds were prepared (25) in 39% yield by condensing 13 with 14 and 15. The protected isomers 17 and 18 could not be separated by chromatography at this stage. They were, however,



partially separated on cellulose analytical TLC plates and fully separated by HPLC after complete deprotection. It is of interest that these novel trinucleoside diphosphates are completely degraded by snake venom but are not affected by spleen phosphodiesterase. An alternate route to these molecules has also appeared (26).

## SYNTHESIS OF RIBONUCLEOTIDES

For most sequences, the synthesis of oligonucleotides on a solid support is the most efficient route. We have extensively investigated the synthesis of short and medium length chains via the solid support route (17-19, 27). Because the protected monomers for DNA sequences are easier to prepare we have used DNA sequences as models to test various conditions. It is important to note that in phosphite chemistry two slightly different cycles have been used to add each unit to the growing chain. The most widely used standard procedure is CYCLE A below (28). CYCLE B (29) has been used much less frequently.

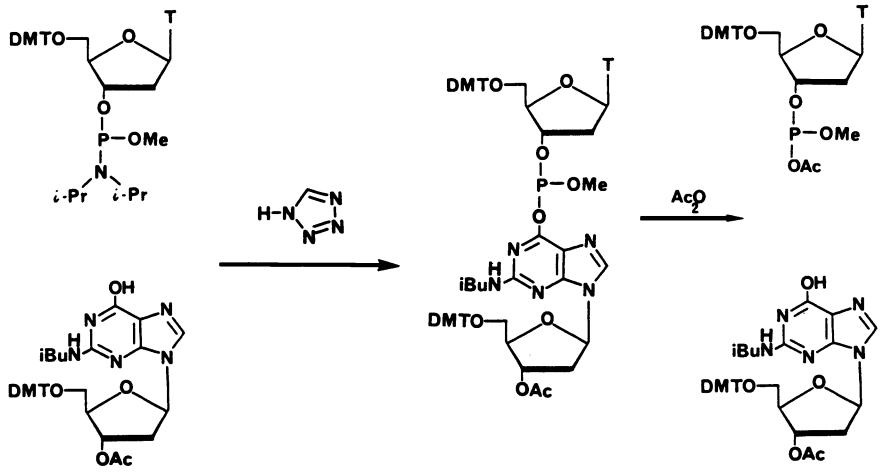
COMPLETE AUTOMATED SYNTHESIS CYCLE B
RNA SYNTHESIS

CYCLE A

| | | Step | Reagent* | Time (min) |
|---|---|---|---|---|
| COUPLING | | | | |
| OXIDATION | ($I_2$) | | | |
| CAPPING | | 1 | Acetonitrile | 0.67 |
| | | 2 | 0.25M $Ac_2O$/DMAP/Collidine/THF | 0.50 |
| | | 3 | Acetonitrile | 0.50 |
| | | 4 | 5% Trichloroacetic acid/Dichloroethane | 2.00 |
| | | 5 | Acetonitrile | 0.67 |
| | | 6 | 0.1M Nucleoside Phosphoramidite | |
| CYCLE B | | | + 0.5M Tetrazole/Acetonitrile | 0.25 |
| | | 7 | Recycle | 14.75 |
| COUPLING | | 8 | 0.25M $Ac_2O$/DMAP/Collidine/THF | 1.50 |
| CAPPING | | 9 | 0.1M $I_2$ THF/Pyridine/$H_2O$:7/2/1 | 0.50 |
| OXIDATION | ($I_2$) | | | |

*Flow rate + 5ml $min^{-1}$ Total Cycle Time + 21.45 min

We prepared a series of 24-unit homodeoxyribonucleotides using CYCLE A and 5'-DMT-N-acyl-3'-phosphoramidites (30, 31). Coupling yields as determined by trityl color measurements were over 93% in all cases. Fully deblocked oligonucleotides were obtained cleanly in all cases except for dG. In the dG case no identifyable nucleotide chain could be detected. However, when deoxyguanosine units bearing $O^6$-protection were used, a 24-unit chain was obtained in yields comparable to the other deoxynucleoside cases. We were able to clearly demonstrate through $^{31}P$ NMR that phosphoramidites can react with the $O^6$ of guanine units. We were also able to show that the problem with the synthesis only occurs when the reaction with phosphite is followed directly by iodine oxidation. We believe that the initial product of coupling at $O^6$ reacts with iodine leading ultimately to depurination. The initial $O^6$-phosphitylated product can be rapidly decomposed by nucleophiles such as water or acetate ions (32). This explains why no such problems are experienced when CYCLE B is used. In CYCLE B, capping (employing acetic anhydride) immediately follows coupling and preceeds iodine oxidation. In CYCLE A, oxidation follows coupling and preceeds capping. Thus, one must be careful with sequences involving large numbers of guanine residues. Either $O^6$-protection must be employed or CYCLE B must be used (32).



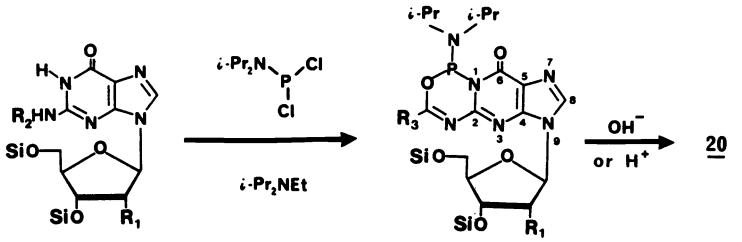
The same problems exist in the ribo area and the same precautions need to be employed. Homopolymers of 15 units were prepared from each of the common ribophosphoramidites (32). Very good yields were obtained from A, C and U units and from $O^6$-protected G units when using CYCLE A. However, no product was obtained from CYCLE A and $O^6$-unprotected G. Excellent yields were obtained when $O^6$-unprotected riboG was used with CYCLE B. Another possibility is the use of TAMA (33) as a catalyst in the coupling reaction. When this reagent is used no side reaction occurs even when $O^6$ is unprotected and CYCLE A is used.

We have also spent considerable time developing techniques that allow us to deprotect and handle oligoribonucleotide chains. The steps involved in deprotection are summarized in the scheme below.

DEPROTECTION OF SYNTHETIC OLIGORIBONUCLEOTIDES

| Step | Reagent(s) and Conditions |
|---|---|
| 1. Removal of P-O-$CH_3$ | Thiophenol/$NEt_3$/Dioxane 1/2/2 R.T., 30 min |
| 2. Cleavage from Support | 15M $NH_4OH$/ETOH 3/1, 55°, 16 hr |
| 3. a) Desilylation | 1.0M TBAF/THF R.T. 2 hr |
| b) Desalting | Sephadex G-25F Size Exclusion Chromatography |
| 4. Purification | Polyacrylamide Gel Electrophoresis |
| 5. Desalting | Sephadex G-25F Size Exclusion Chromatography |

The methyl protecting groups on the phosphate linkages are removed by treatment with thiophenoxide ion at room temperature for 45 minutes. This is followed by cleavage of the sequence from the polymer with concommitant debenzoylation of the exocyclic amino protecting groups of adenosine, cytidine and guanosine using ammonium hydroxide/ethanol at 55° degrees for 16 hours. The 2'-O-silyl groups are removed using 1 M tetrabutylammonium fluoride/THF at room temperature for 2-4 hours and the completely deprotected oligonucleotide is then desalted directly using Sephadex size exclusion chromatography. The final purification is carried out using preparative polyacrylamide gel electrophoresis followed by a desalting step. The integrity of the purified sequences may be ascertained by either enzymatic degradation, followed by HPLC analysis of the products, or by RNA sequencing.




20 21 20

a, $R_1$ = OSi; $R_3$ = Ph; 96%.

b, $R_1$ = H ; $R_3$ = Ph; 93%.

c, $R_1$ = H ; $R_3$ = iPr; 67%.

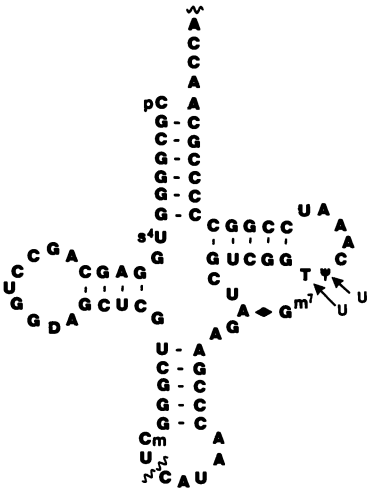
d, $R_1$ = H ; $R_3$ = $CH_3$; 60%.

It is critical that sterile equipment, reagents, and handling techniques be used in handling free oligoribonucleotides. For example all $H_2O$, Sephadex, silanized glassware and plasticware must be autoclaved in the presence of diethylpyrocarbonate (DEP). Large pieces of glassware may be sterilized by washing with a dilute aqueous solution of DEP and drying in an oven. During all steps of the deprotection, gloves must be worn to prevent enzymatic degradation by RNAases present on one's hands. If these procedures are rigorously adhered to, then the accidental degradation of the fully deprotected oligonucleotides may be completely avoided.

There is one final area in which caution must be exercised. The reagent that is used to prepare nucleotide phosphoramidites is generally chloro-N,N-diisopropylaminomethoxyphosphine or an equivalent. Dichloro-N, N-diisopropylaminophosphine may be obtained as an impurity during its preparation. This latter reagent will react with N-acylated guanine rings to produce a novel tricyclic derivative (21, 34). This new ring structure can be readily reconverted to the acylated guanine with dilute base or dilute acid.

We have attempted to extend our general procedures to the synthesis of long defined RNA sequences. Our target molecule has for some time been the fmet E. coli initiator tRNA sequence.

Once we were certain of our methods from the model studies with homosequences, we set about to test the limit of the method. The 43mer corresponding to the 3'-half of the tRNA sequence was prepared using the standard procedure. This molecule was obtained in an overall yield of 41% based on trityl color measurements. The chain was released from the polymer, deprotected and characterized by gel electrophoresis, sequence determination and total base analysis (35).



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