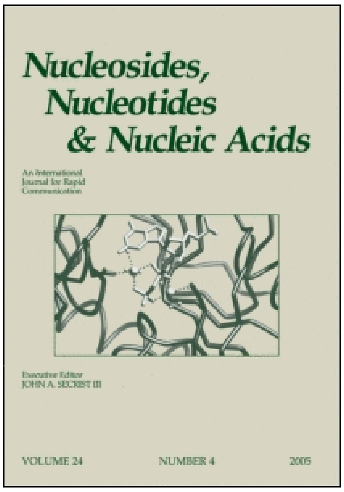

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Some Aspects of the Chemical Synthesis of Oligoribonucleotides

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SOME ASPECTS OF THE CHEMICAL SYNTHESIS OF OLIGORIBONUCLEOTIDES

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Summary. The present position regarding the protection of the 2'- and 5'-hydroxy functions in the chemical synthesis of oligoribonucleotides is discussed.

Introduction

In the past few years, remarkable progress has been made in the chemical synthesis of oligo- and poly-deoxyribonucleotides of defined sequence[1,2]. Advances in this area have been stimulated by the considerable need for synthetic oligodeoxyribonucleotides in biology - particularly in recombinant deoxyribonucleic acid (DNA) research - and has been made possible only by the development of the phosphotriester[3] and phosphite triester[2] approaches to oligonucleotide synthesis. Progress in the chemical synthesis of oligoribonucleotides has been less rapid. This is no doubt due partly to the fact that the requirements in biological research, at the present time, are mainly for synthetic oligodeoxyribo- rather than for oligoribo-nucleotides; however, it is due also to the increased complexity resulting from the need to protect the 2'-hydroxy functions throughout the synthesis of oligoribonucleotides. We have, for a considerable time, been interested in the particular problems associated with oligoribonucleotide synthesis and, in recent years[4-6], have undertaken studies directed towards the chemical synthesis of yeast alanine transfer ribonucleic acid ($tRNA^{Ala}$). The latter polyribonucleotide is composed of 76 nucleotide residues and, like other tRNA molecules, contains a number of modified ribonucleosides in addition to adenosine, cytidine, guanosine and uridine. In the past year, we have been particularly concerned with the synthesis of nearly one-half (from nucleoside 40

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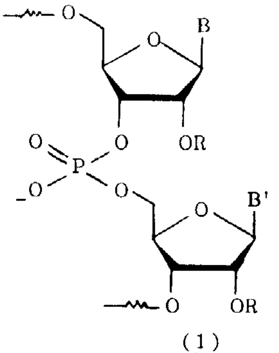
to nucleoside 76) of the yeast $tRNA^{Ala}$ molecule. In the course of these studies, it has been necessary to consider in greater detail which protecting groups should be used in oligoribonucleotide synthesis. The present article is devoted to a discussion of the factors involved in the choice of some of these protecting groups.

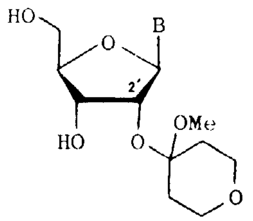
Protection of 2'-Hydroxy Functions

Probably the most crucial decision which has to be made in the synthesis of oligoribonucleotides (e.g. 1; R=H) is the choice of the protecting group (R) for the 2'-hydroxy functions. This protecting group has to remain intact until the final unblocking step at the end of the synthesis, and it must then be removable under conditions which are mild enough to prevent subsequent attack of the released 2'-hydroxy functions on vicinal phosphodiester groups with consequent cleavage or migration of the internucleotide linkages.

At first, we examined the use of the acid-labile tetrahydropyranyl group[7,8] for the protection of the 2'-hydroxy functions in oligoribonucleotide synthesis. As its use led[8] to the formation of two distinct diastereoisomeric 2'-protected building blocks for each ribonucleoside, we subsequently introduced the achiral 4-methoxytetrahydropyran-4-yl [methoxytetrahydropyranyl, as in 2'-O-methoxytetrahydropyranyladenosine (2a)] group[9] for this purpose. We have also examined the possibility of using ester groups (such as acetyl and benzoyl)[10] and benzyl[11] ether groups to protect 2'-hydroxy functions. The main disadvantages associated with the use of acyl protecting groups are (a) that it is sometimes difficult or indeed virtually impossible to obtain 2'-O-acyl ribonucleoside derivatives which are uncontaminated with the isomeric 3'-O-acyl derivatives, and (b) that base-catalyzed acyl migration (from O-2' to



(1)

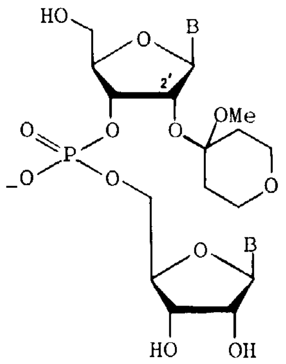


(2) a; B = adenin-9-yl
b; B = uracil-1-yl
c; B = cytosin-1-yl
d; B = guanin-9-yl

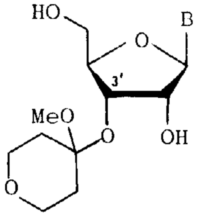
O-3' and *vice versa*) occurs[12] under very mild conditions. While benzyl ethers may readily be cleaved by catalytic hydrogenolysis, there is a possibility[11] that concomitant reduction of the pyrimidine base residues may occur. Perhaps the main reason, however, why the benzyl protecting group cannot be recommended is the uncertainty that 2'-O-benzylated oligomers would generally remain adsorbed on the catalyst surface until all the benzyl ether groups had been cleaved.

The t-butyldimethylsilyl (TBDMS)[13] and 2-nitrobenzyl (2-NB)[14] ether groups have also been used for the protection of 2'-hydroxy functions in oligoribonucleotide synthesis. It seems clear that care should be taken when the TBDMS protecting group is used for this purpose. While mixtures of 2'- and 3'-O-TBDMS derivatives of ribonucleosides seem to be more easily separable by adsorption chromatography than mixtures of corresponding 2'- and 3'-O-acyl derivatives, the TBDMS group is known[15], like common acyl groups[12], to migrate readily under mild conditions of basic catalysis. Thus, if the TBDMS group is used to protect 2'-hydroxy functions, there is a danger that the synthetic oligoribonucleotides will not contain exclusively 3'→5'-internucleotide linkages. Another feature of the TBDMS protecting group which needs clarification is whether the phosphodiester internucleotide linkages are completely stable under the conditions generally used (tetra-n-butylammonium fluoride in tetrahydrofuran) to remove the TBDMS protecting groups in the final unblocking step. The 2-NB protecting group, which may be removed photolytically by exposure to relatively long wavelength ultraviolet light[14], was introduced and used for several years by Ikehara and his coworkers. However, in their latest study on the synthesis of oligoribonucleotides, the latter workers have used[16] the acid-labile tetrahydrofuranyl instead of the 2-NB group to protect the 2'-hydroxy functions.

Recent studies[17] have confirmed the particular suitability of the methoxytetrahydropyranyl (MTHP) protecting group for the present purpose. In the first place, the rates of hydrolysis in 0.01*M*-hydrochloric acid at 22°C [Table] of all the common 2'-O-MTHP-ribonucleosides were determined. It can be seen [Table, entries nos. 1-4] that the rate of hydrolysis of 2'-O-methoxytetrahydropyranyluridine (2b) is approximately twice as fast as the rates of hydrolysis of the other 2'-O-MTHP ribonucleoside derivatives (2a, 2c and 2d). It is especially noteworthy that



(3) a; B = adenin-9-yl
b; B = uracil-1-yl



(4) a; B = adenin-9-yl
b; B = uracil-1-yl

the presence of a vicinal 3'→5'-phosphodiester internucleotide linkage appears to facilitate the hydrolysis of a 2'-O-MTHP protecting group. Thus (3a) undergoes acid-catalyzed hydrolysis *ca.* 1.7 times as fast as (2a) [Table, entries nos. 5 and 1], and (3b) undergoes hydrolysis at a rate more than 3 times faster than that of (2b) [entries nos. 6 and 2]. Fortunately, the acid-catalyzed hydrolysis of MTHP groups in the final unblocking step of oligoribonucleotide synthesis will necessarily always be assisted in this way, and it can be estimated[17] that under the standard unblocking conditions [0.01*M* - hydrochloric acid (pH 2), room temperature], the MTHP protecting groups will be *ca.* 99.9% removed from a partially-protected oligoribonucleotide within 4 hr. Finally, as can be seen from the Table [compare entries nos. 1 and 7, and entries nos. 2 and 8], 3'-O-MTHP are several times more labile to acid-catalyzed hydrolysis than 2'-O-MTHP protecting groups. This observation is of importance in connection with the synthesis[18] of 5'-O-triphosphoryladenylyl-(2'→5')-adenylyl-(2'→5')-adenosine [2-5A] and its analogues.

Unprotected oligo- and poly-ribonucleotides are susceptible both to acid and base-catalyzed hydrolysis; they also readily undergo digestion in the presence of hydrolytic enzymes such as ribonuclease A. At the end of a synthesis, it is a wise precaution therefore to isolate oligoribonucleotides with their 2'-protecting groups still intact, and to store these products in this partially-protected form. Should the oligoribonucleotides be required, say, for a biological investigation, the 2'-hydroxy functions can then be unblocked. It is clearly essential (see above) that the 2'-protecting groups should be removable under conditions

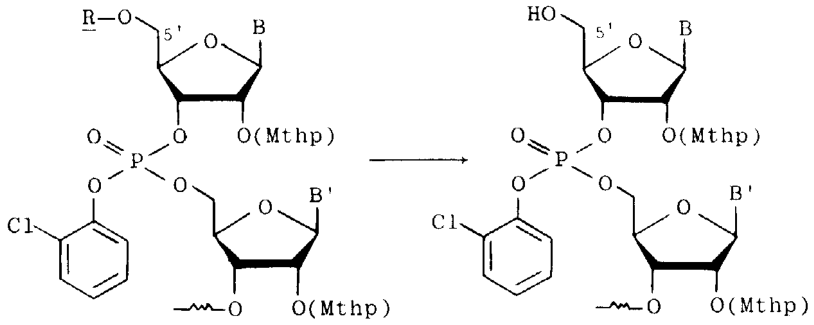
TABLE. Removal of 2'- and 3'-O-Methoxytetrahydropyranyl Protecting Groups

Entry No.	Substrate	$t_{\frac{1}{2}}$(min)	$t_{0.99}$(min)[a]
1	(2a)	34	226
2	(2b)	18.7	124
3	(2c)	40	266
4	(2d)	35	233
5	(3a)	19.9	132
6	(3b)	6.1	41
7	(4a)	7.4	49
8	(4b)	6.3	44

[a] $t_{0.99}$ represents the time required for 99% removal of the MTHP group.

under which fully-unblocked oligoribonucleotides are completely stable. It is well established[8] that aqueous acid can promote both the cleavage and migration (from 3'→5' to 2'→5', and *vice versa*) of inter-ribonucleotide linkages. A number of years ago, we reported[8] that, under the conditions of acid hydrolysis required to remove tetrahydropyranyl groups (let alone the more labile methoxytetrahydropyranyl[9] groups) from the 2'-hydroxy functions of oligoribonucleotides, cleavage and migration of the internucleotide linkages occur only to a negligible extent. These conclusions have recently been challenged[19]. However, we have very carefully re-investigated this matter[17], and have completely confirmed our original findings[8].

In conclusion, the particular merits of the MTHP protecting group are (i) that it is achiral, (ii) that it does not migrate, (iii) that it is stable enough under phosphorylation conditions to remain intact throughout the assembly of fully-protected oligoribonucleotides, (iv) that its use leads to it being possible to isolate relatively stable partially-protected oligoribonucleotides (in which only the 2'-hydroxy functions and the terminal 2',3'-diol system are blocked) that can be stored until the fully-unblocked materials are required, and (v) that the final unblocking step (involving acidic hydrolysis) proceeds in homogeneous solution under mild conditions, and is virtually complete within a few hours without the occurrence of side reactions. It is, of course, likely that other protecting groups which are as suitable as, or indeed more suitable than the MTHP group will be developed in the future. However, it is not unlikely that some of these alternative protecting groups will also prove to be acid-labile.



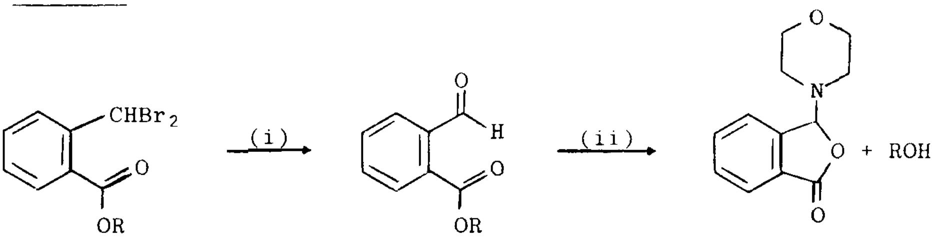
(5) a; R = 4-ClC$_6$H$_4$OCH$_2$CO
b; R = 2-(Br$_2$CH)C$_6$H$_4$CO

(6)

Protection of 5'-Hydroxy Functions

The situation with regard to the protection of the 5'-hydroxy functions in oligodeoxyribonucleotide synthesis by the phosphotriester approach is relatively straightforward. Whether the desired sequence is assembled in a stepwise manner or by block condensation reactions, the 5'-hydroxy functions may conveniently be protected by acid-labile protecting groups such as 4,4'-dimethoxytrityl[20] and 9-phenylxanthen-9-yl[21]. The only problem of significance which may then arise is that some depurination of protected adenine residues can occur[3] in the course of the acid-catalyzed unblocking step. The situation in the ribose series is more complicated. In order to extend an oligoribonucleotide sequence, it is necessary to remove the 5'-terminal protecting group (R) from a fully-blocked oligoribonucleotide (5), containing a number of acid-labile MTHP groups and base-labile phosphotriester functions, to give (6). It is clearly desirable that the protecting group (R) should, if possible, be removable under neutral conditions. In our first approach, we made R a particularly base-sensitive acyl group [e.g. *p*-chlorophenoxyacetyl[22], as in (5a)]. While it was possible to remove such a protecting group successfully[22] from dinucleoside phosphates and trinucleoside diphosphates, 5'-deacylation of longer oligoribonucleotides led to appreciable loss of material due to the concomitant hydrolysis of one or more of the phosphotriester linkages. Our first real success in the synthesis of oligoribonucleotides of relatively high molecular weight was achieved by using the 2-dibromomethylbenzoyl[23] [DBMB, as in (5b)] as the 5'-protecting group. We were then successful in synthesizing the 3'-terminal deca-

Scheme 1



(7) (8) (9)

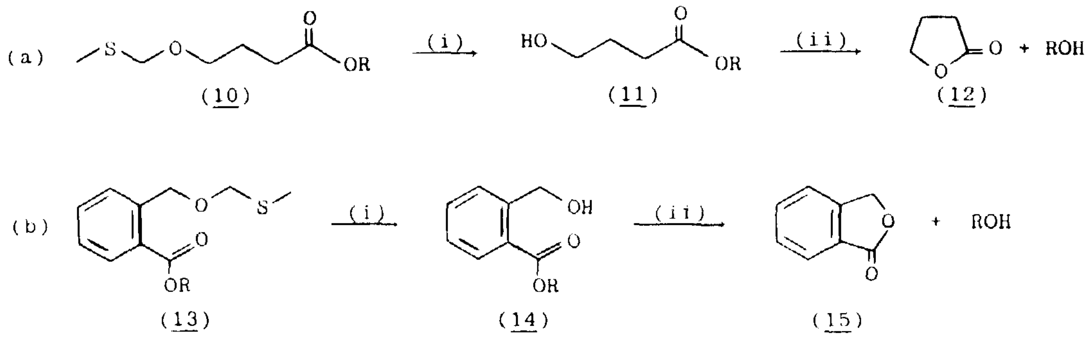
Reagents: (i) $AgClO_4$, 2,4,6-collidine/tetrahydrofuran-water (98:2 v/v); (ii) morpholine.

ribonucleoside nonaphosphate[4] and nonadecaribonucleoside octadecaphosphate[5,6] sequences of $tRNA^{Ala}$.

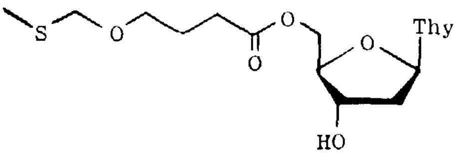
The DBMB group is an example of a 'protected' protecting group. Thus DBMB derivatives, such as (7), may be regarded as protected 2-formylbenzoate esters; when they are treated (Scheme 1) with silver perchlorate in slightly wet tetrahydrofuran or acetone, in the presence of 2,4,6-collidine at room temperature, the masked formyl group is 'unblocked' and 2-formylbenzoyl derivatives (8) are obtained. The latter intermediates (8) are extremely sensitive to hydroxide ions and certain other nucleophiles[24]; treatment of (8) with morpholine at room temperature rapidly leads to the release of unblocked alcohols (ROH). If DBMB derivatives are treated directly with alkali[23], they undergo hydrolysis at approximately the same rates as the corresponding acetate esters.

The removal of the 5'-terminal DBMB groups from fully-protected oligoribonucleotides (5b) [to give the partially-protected intermediates (6)] proceeds very satisfactorily in the case of relatively low molecular weight substrates. Thus, in the studies directed towards the synthesis of yeast $tRNA^{Ala}$, DBMB removal from the fully-protected 3'-terminal tetraribonucleoside triphosphate and heptaribonucleoside hexaphosphate was effected in 93 and 83% yields, respectively[6]. However, the removal of the DBMB group from the 3'-terminal decaribonucleoside nonaphosphate was effected less readily[6]. This indication that the removal of the DBMB group from high molecular weight fully-protected oligo- and polyribonucleotides was likely to be difficult, together with the observation[25] that the dibromomethyl side-chain reacted with toluene-*p*-thiolate ions under the conditions required[26] to remove the 2,4-dinitrobenzyl

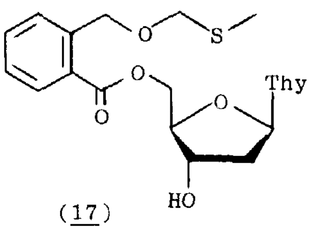
Scheme 2



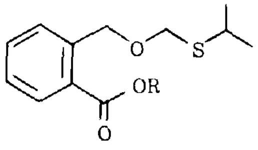
Reagents: (i) $Hg(ClO_4)_2$, 2,4,6-collidine/tetrahydrofuran-water (98:2 v/v); (ii) base



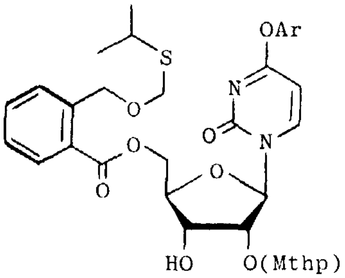
(16)



(17)



(18)



(19) Ar = 2,4-$Me_2C_6H_3$

protecting group from 3'-terminal phosphotriesters, prompted us to search for possible alternatives to the DBMB group.

These investigations led to the development[27] of the 4-(methylthiomethoxy)butyryl [MTMB; as in (10), Scheme 2(a)] and the 2-(methylthiomethoxymethyl)benzoyl [MTMT; as in (13), Scheme 2(b)] 'protected' protecting groups. While MTMB and particularly MTMT esters [as in (10) and (13), respectively] undergo ammonolysis more slowly than the corresponding acetates, the intermediate hydroxy esters [(11) and (14), respectively] obtained after treatment with mercury (II) perchlorate in the presence of 2,4,6-collidine in slightly wet tetrahydrofuran, are very base-labile indeed[27] (Scheme 2).

In the first instance, 5'-O-(MTMB)- and 5'-O-(MTMT)-thymidines [(16) and (17), respectively] were prepared by the regioselective acylation of thymidine with the respective acids [(10; R=H) and (13; R=H)] and appropriate activating agents[27]. The first step [Scheme 2(a)(i)] in the unblocking of 5'-O-(MTMB)-thymidine (16) was complete after 5 min at room temperature whereas, under the same conditions [0.05*M* substrate, 0.1*M*-$Hg(ClO_4)_2$, 0.25*M*-2,4,6-collidine], the first step [Scheme 2(b)(i)] in the unblocking of 5'-O-(MTMT)-thymidine (17) required[27] *ca.* 3 hr. On the other hand, the second step [Scheme 2(b)(ii)] in the unblocking of (17) [complete after 30 sec in 0.125*M*-K_2CO_3 at room temperature] was much faster than the second step [Scheme 2(a)(ii)] in the unblocking of (16).

These preliminary studies[27] suggested that neither the MTMB nor the MTMT group would be a suitable alternative to the DBMB group for the protection of the terminal 5'-hydroxy functions in oligoribonucleotide synthesis. The second step [Scheme 2(a)(ii)] in the unblocking of the MTMB group and the first step [Scheme 2(b)(i)] in the unblocking of the MTMT group both appeared to be too slow. It seemed possible that the latter step [Scheme 2(b)(i)] could be speeded up by replacing methylthiomethyl in the MTMT group by isopropylthiomethyl [as in the 2-(isopropylthiomethoxymethyl)benzoyl (PTMT) ester (18)]. The protected uridine building block (19) was isolated in 43% yield from the products of the reaction[28] between 2'-O-methoxytetrahydropyranyl-4-O-(2,4-dimethylphenyl)uridine, PTMT acid (18; R=H)[28] and N,N'-di-isopropylcarbodi-imide in acetonitrile solution. When (19) was treated[28] with mercury (II) perchlorate and 2,4,6-collidine in tetrahydrofuran-water (98:2 v/v) solution under the conditions described above for (16) and (17), the first step [corresponding to step (i) in Scheme 2(b)] was complete in 5 min. The second step in the 5'-deprotection of (19) corresponds to the second step [Scheme 2 (b)(ii)] in the unblocking of (17); it was most conveniently effected with 0.2*M*-triethylamine in tetrahydrofuran-water (2:1 v/v). Under the latter conditions, it was complete in 1 min at room temperature[28].

This result suggested that the PTMT might well prove to be complementary, and in some ways superior to the DBMB protecting group. Our most recent results have indeed confirmed this. Thus the 5'-O-PTMT group has been removed successfully[28] from the fully-protected 3'-terminal decaribonucleoside nonaphosphate sequence [UCGUCCACCA], and preliminary experiments[28] suggest that it can also be removed from the fully-protected nonadecaribonucleoside octadecaphosphate sequence [AUUCCGGACUCGUCCACCA] of yeast tRNA^{Ala}. The development of the PTMT protecting group may well prove to be of crucial importance in the synthesis of the target heptatriacontamer sequence of the tRNA molecule.

Acknowledgements

I should like to acknowledge the outstanding contributions which a number of my co-workers have made to our studies in the synthesis of oligo- and poly-nucleotides. Some of their names appear in the references below. I should also like to thank the Science and Engineering Research Council and the British Technology Group for recent support of this work.

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