Protection of 2′-Hydroxy Functions of Ribonucleosides

The methods used to protect 2′-hydroxy functions of ribonucleosides have recently been reviewed (Beaucage and Iyer, 1992; Sonveaux, 1994; Beaucage and Caruthers, 1996). In addition, there have been earlier brief reviews (Ohtsuka and Iwai, 1987; Reese, 1989). The main purpose of this article is to discuss 2′-protection in the context of effective oligoribonucleotide synthesis. For this reason, emphasis will be placed on what are now, or are likely to become, the 2′-protecting groups of choice in the synthesis of oligo- and poly-ribonucleotides (RNA sequences). As a result, only some of the protecting groups that have been suggested for this purpose are considered in detail here, and some interesting chemistry has necessarily been omitted.

CONSIDERATIONS FOR 2′-PROTECTING GROUPS IN OLIGORIBONUCLEOTIDE SYNTHESIS

There are three main general criteria that all protecting groups should fulfill (Reese, 1978). (1) They should be easy to introduce and, as part of this criterion, the reagents involved in their introduction should be readily accessible. (2) They should be stable and remain intact until it is appropriate to remove them. (3) They should be removable at the appropriate time using conditions under which the desired product is completely stable. In the case of chiral substrates such as ribonucleosides, achiral protecting groups are desirable for analytical (e.g., NMR, TLC, and HPLC) purposes. In the case of all substrates, it is desirable that the introduction of protecting groups should not result in unduly complex NMR spectra.

The successful chemical synthesis of polynucleotides (including RNA sequences) depends on the choice of suitable protecting groups and effective phosphorylation procedures. Arguably the most crucial single decision that has to be made in oligoribonucleotide synthesis is the choice of the protecting group (R; see **S.1**) for the 2′-hydroxy functions (Reese, 1978). This protecting group must remain intact until the very last step of the synthesis (Fig. 2.2.1), and must then be removable under conditions that are mild enough to avoid subsequent attack of the released 2′-hydroxy functions (see **S.2**) on the vicinal phosphodiester internucleotide linkages, thereby leading to their cleavage or migration.

Protecting groups are often removed hydrolytically under either basic or acidic conditions. Cleavage of interribonucleotide linkages can occur under relatively mild basic conditions (Järvinen et al., 1991; Kuusela and Lönnberg, 1994). This process, illustrated in Figure 2.2.2A, essentially involves an ester exchange reaction between the 2′-hydroxy function of the 3′-linked nucleoside residue and the 5′-hydroxy function of the 5′-linked nucleoside residue, leading to a (2′,3′)-cyclic phosphate (**S.3**). This intermediate then undergoes further base-catalyzed hydrolysis to give a mixture of isomeric 2′- and 3′-phosphates (**S.5** and **S.6**, respectively). Under acidic conditions (Fig. 2.2.2B), internucleotide cleavage and migration can both occur (Griffin et al., 1968). These processes are both believed to proceed via a phosphorane intermediate (**S.7**; Järvinen et al., 1991). If the P-O(2′) bond is then cleaved, starting material **S.2** is regenerated. If the P-O(3′) bond is cleaved, the isomeric product **S.8**

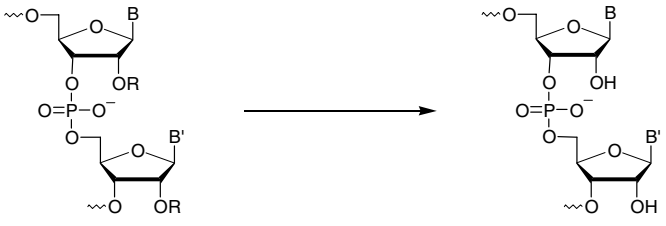

Figure 2.2.1 Scheme showing protected 2′-hydroxy functions. B and B′ are bases.

Contributed by Colin B. Reese
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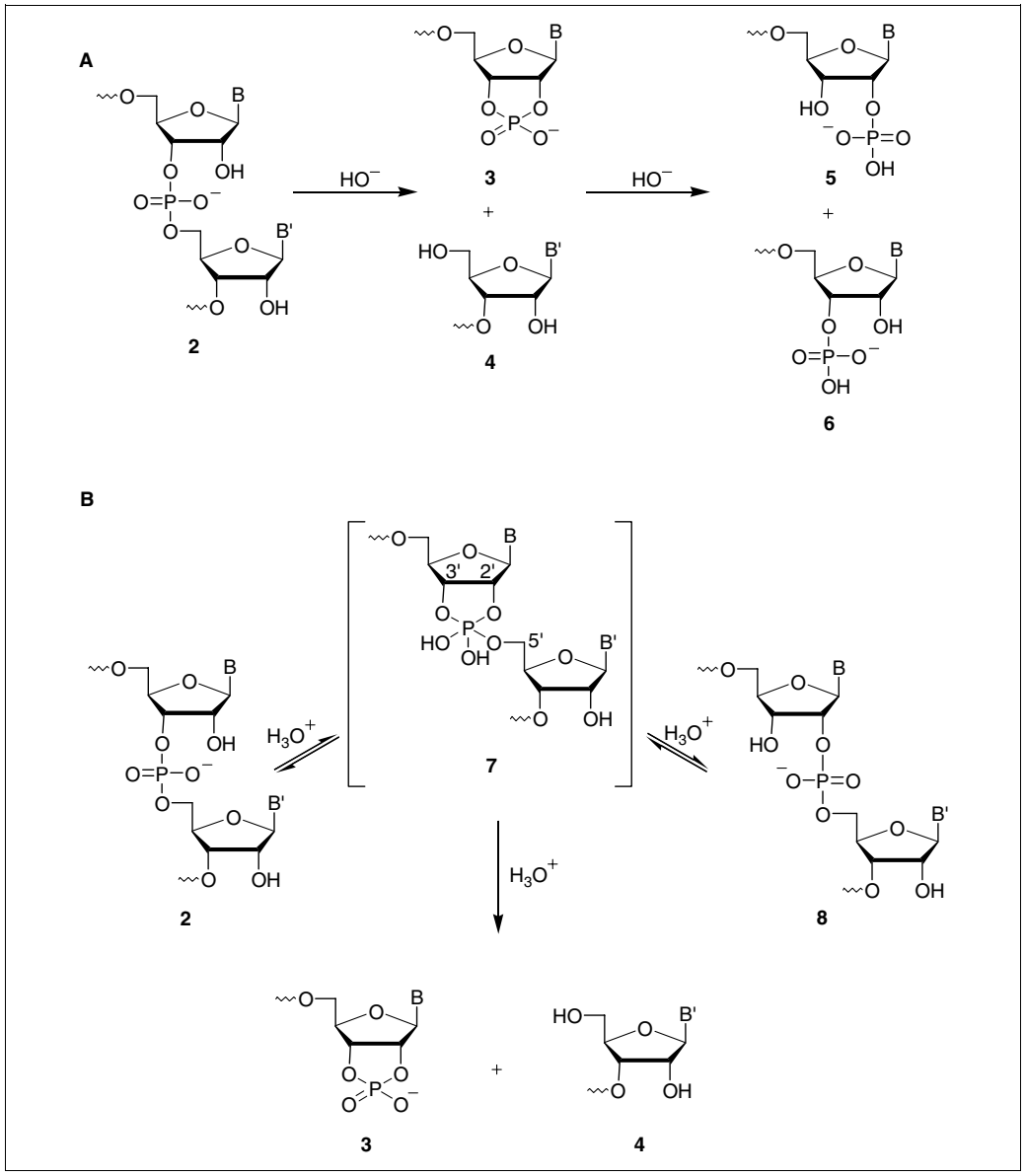


Figure 2.2.2 Scheme showing cleavage of interribonucleotide linkages under (**A**) basic and (**B**) acidic conditions. Although only shown in panel A, the hydrolysis of **S.3** can yield either the 2′-phosphate (**S.5**) or the 3′-phosphate (**S.6**) under either basic or acidic conditions.

with the migrated internucleotide linkage is obtained. Finally, if the P-O(5′) bond is cleaved, the (2′,3′)-cyclic phosphate **S.3** is obtained. The cyclic phosphate **S.3** undergoes further hydrolysis to give an isomeric mixture of the corresponding 2′- and 3′-phosphates (**S.5** and **S.6**, respectively) under acidic as well as under basic conditions.

The significance of these reactions in the context of oligo- and poly-ribonucleotide synthesis will be considered later. However, it is clearly of crucial importance that the 2′-protecting group should strictly satisfy the above criteria (1) and (2). As will become apparent, this is a very demanding requirement, as the 2′-protecting group must also be fully compatible with the groups that are used to protect the 5′-terminal hydroxy function, the base residues, and the internucleotide linkages. It is therefore appropriate to consider these other protecting group requirements at the outset.

Protection of the 5′-Terminal Hydroxy Function

Figure 2.2.3 illustrates a number of groups (R′ in **S.9**) used to protect the 5′-terminal hydroxy function. Although a good deal of work has been carried out on the synthesis of oligoribonucleotides in solution, most of the recent studies in this area have been concerned with

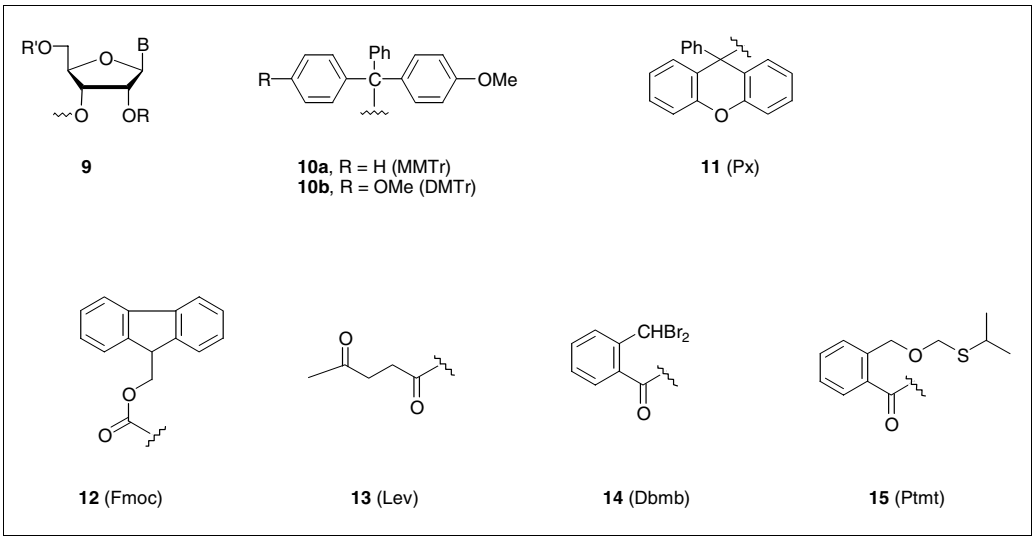


Figure 2.2.3 Several protecting groups for 5′-terminal hydroxy functions.

solid-phase synthesis. The 5′-terminal protecting group (R′) that has been used most widely for this purpose is the (di-*p*-anisyl)phenylmethyl group (also known as 4,4′-dimethoxytrityl or DMTr; **S.10b**; Schaller et al., 1963; *UNIT 2.3*). The 9-phenylxanthen-9-yl (pixyl or Px, **S.11**) group has very similar properties to the DMTr group and is equally suitable (Chattopadhyaya and Reese, 1978).

The somewhat less labile *p*-anisyl(diphenyl)methyl group (4-monomethoxytrityl or MMTr; **S.10a**; Schaller et al., 1963) has also been used, but its greater stability to acid makes it generally less suitable. The great advantage of the DMTr (**S.10b**) and Px (**S.11**) protecting groups in solid-phase synthesis, and perhaps also in solution-phase synthesis, is that they can be rapidly and quantitatively removed by treatment with acids, such as di- and tri-chloroacetic acids, in anhydrous dichloromethane solution (Sproat and Gait, 1984). A further advantage shared by all three of these protecting groups is that with acid treatment they give rise to colored carbocations that can easily be assayed spectrophotometrically. This permits coupling efficiencies to be monitored. Clearly, if one of these three protecting groups is used in oligoribonucleotide synthesis, the 2′-protecting group (R in formula **S.9**) must be completely stable under the acidic conditions required for 5′-deprotection.

Numerous other 5′-protecting groups have been suggested (Sonveaux, 1994; *UNIT 2.3*), some of which are removable under mildly basic or virtually neutral conditions. Protecting groups in this latter category include 9-fluorenylmethoxycarbonyl (Fmoc; **S.12**; Pathak and Chattopadhyaya, 1985), levulinyl (Lev; **S.13**; van Boom and Burgers, 1976), 2-(dibromomethyl)benzoyl (Dbmb; **S.14**; Chattopadhyaya et al., 1979), and 2-(isopropylthiomethoxymethyl)benzoyl (Ptmt; **S.15**; Brown et al., 1989a). None of these protecting groups has found widespread use in the solid-phase synthesis of RNA sequences, but some have proved to be useful in solution-phase synthesis.

Protection of Base Residues

The protection of base residues is illustrated in Figure 2.2.4. In the solid-phase synthesis of RNA sequences (Rao et al., 1993), adenine, cytosine, and guanine residues are generally protected by N-acylation (as in **S.16**, **S.18**, and **S.19**, respectively), while uracil residues are left unprotected (as in **S.23**; *UNIT 2.1*). The *N*-acyl protecting groups are usually removed by ammonolysis in the step before the removal of the 2′-protecting groups. As RNA can undergo internucleotide cleavage (Fig. 2.2.2A) under ammonolytic conditions, the base-protecting groups must be removable using conditions under which the 2′-protecting groups are completely stable. Thus, the choice of an *N*-acyl protecting group for a particular base residue is, to some extent, dependent on the 2′-protecting group used.

The dimethylaminomethylene protecting group, which is also removable under ammonolytic conditions, has been recommended for the protection of adenine and guanine residues (as in **S.17** and **S.20**, respectively; Vinayak et al., 1992). Particularly in the solution-phase synthesis of RNA sequences, it may be desir-

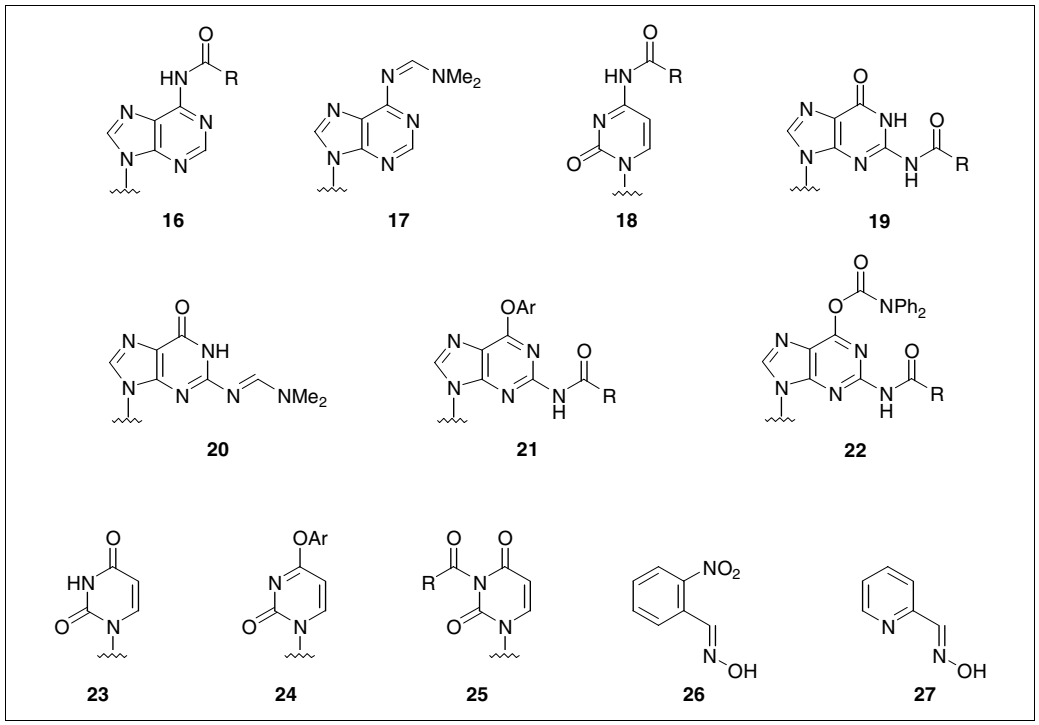


Figure 2.2.4 Several protecting groups for base residues (A: **S.16** and **S.17**; C: **S.18**; G: **S.19** to **S.22**; U: **S.23** to **S.25**). **S.26** and **S.27** are used in oximate treatment for the removal of aryl (Ar) groups.

able to protect guanine residues on O6 as well as on N2 (as in **S.21** and **S.22**). Aryl protecting groups are particularly suitable for this purpose (as in **S.21**; Ar = 2-nitrophenyl, 3-chlorophenyl, and 3,5-dichlorophenyl; Jones et al., 1981; Reese and Skone, 1984; Brown et al., 1989a); they may readily be removed by treatment with the N^1,N^1,N^3,N^3-tetramethylguanidinium salt of (*E*)-2-nitrobenzaldoxime **S.26** or of (*E*)-pyridine-2-carboxaldoxime **S.27** (oximate treatment; Reese and Zard, 1981) before the ammonolytic removal of the *N*-acyl protecting groups. The *N*,*N*-diphenylcarbamoyl group (as in **S.22**; Kamimura et al., 1984) is removable by ammonolysis (*UNIT 2.1*).

In the solution-phase synthesis of RNA sequences, it may also be desirable to protect uracil residues on O4 with an aryl group (as in **S.24**; Ar = 2,4-dimethylphenyl; Jones et al., 1981) or on N3 with an acyl group (as in **S.25**; R = 4-MeO·C_6H_4; Kamimura et al., 1984). O4-Aryl and N3-acyl protecting groups may be removed from uracil residues by oximate treatment and by ammonolysis, respectively (*UNIT 2.1*). It should be noted that the ammonolytic and oxime treatment conditions are both basic.

Protection of Internucleotide Linkages

Virtually all of the groups commonly used to protect the internucleotide linkages in both solid- and solution-phase oligo- and poly-ribonucleotide synthesis are removed under basic conditions (Fig. 2.2.5). The 2-cyanoethyl group (as in **S.28**; Sinha et al., 1983) is by far

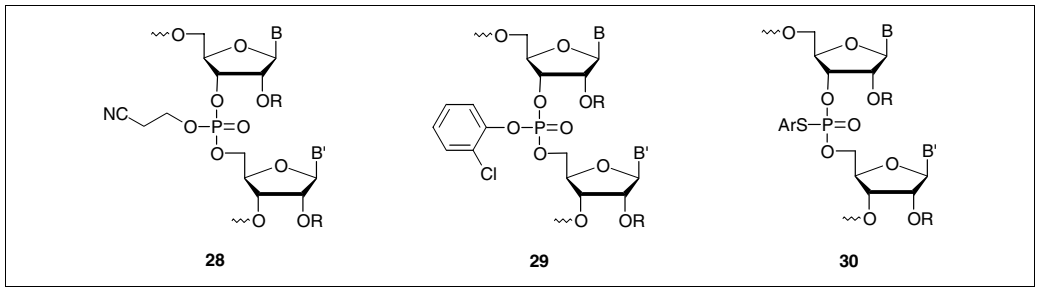


Figure 2.2.5 Several protecting groups for internucleotide linkages. Ar is phenyl or another aryl group.

the most commonly used protecting group for the internucleotide linkages in the solid-phase synthesis of RNA sequences, and the 2-chlorophenyl group (as in **S.29**; Reese, 1970) has been widely used for this purpose in solution-phase synthesis. Another approach to the synthesis of oligoribonucleotides was pioneered by Hata and co-workers (Honda et al., 1984) and involves intermediate *S*-aryl phosphorothioates (**S.30**, Ar = Ph). 2-Cyanoethyl protecting groups are usually removed at the same time as *N*-acyl base-protecting groups by treatment with ammonia (Sinha et al., 1983). 2-Chlorophenyl-protected oligo- and poly-ribonucleotides are best unblocked by treatment with the conjugate base of (*E*)-2-nitrobenzaldoxime **S.26** or (*E*)-pyridine-2-carboxaldoxime **S.27** (Reese et al., 1978; Reese and Zard, 1981). *S*-Aryl phosphorothioates (**S.30**), which are masked phosphodiesters, may also be unblocked by oximate treatment (Kamimura et al., 1984). In order to avoid internucleotide cleavage (Fig. 2.2.2A), it is necessary that the 2′-protecting group (R in **S.28**, **S.29**, and **S.30**) should be completely stable under the basic conditions used in the unblocking of the internucleotide linkages.

PROTECTION OF THE 2′-HYDROXY FUNCTION IN OLIGORIBONUCLEOTIDE SYNTHESIS

It is clear from the above discussion that the requirements for a 2′-protecting group in oligo- and polyribonucleotide synthesis are very demanding indeed. With regard to solid-phase synthesis, in addition to meeting the above general criteria for protecting groups, it is crucially important that 2′-protecting groups be stable to repeated exposure to the acidic conditions required to remove the 5′-terminal DMTr protecting group **S.10b**. The 2′-protecting groups must also be stable under the basic conditions (i.e., concentrated aqueous ammonia and oximate ions) required to unblock the base residues and the internucleotide linkages. It is further desirable that 2′-protecting groups not be excessively bulky and thereby impede the coupling process. For the successful removal of the 2′-protecting groups, it must always be borne in mind that RNA is a very sensitive material that is unstable under both acidic and basic conditions and in the presence of various hydrolytic enzymes. It is therefore desirable that manipulation should be kept to a minimum in the isolation of fully unblocked RNA.

Ether Protecting Groups

The protection of the 2′-hydroxy functions as readily cleavable ether groups would appear at first sight to be an attractive proposition. Indeed, the possibility of using the benzyl protecting group was first examined over 30 years ago (Griffin et al., 1966). Uridine was converted into its 2′-*O*-benzyl derivative **S.31**, which was then successfully converted via the protected dinucleoside phosphate **S.32** into uridylyl-(3′→5′)-uridine (**S.33**; Fig. 2.2.6). The benzyl protecting group, which is stable both to acid- and base-catalyzed hydrolysis, was removed by catalytic hydrogenolysis in the presence of palladized charcoal. However, it was subsequently reported that concomitant hydrogenation of the uracil 5,6-double bond can occur (Reitz and Pfleiderer, 1975). There is also a danger that the total removal of all of the 2′-protecting groups of per-2′-*O*-benzylated RNA sequences may not always be possible.

The 2-nitrobenzyl group (as in **S.34**; Fig. 2.2.7), which was introduced by Ikehara and

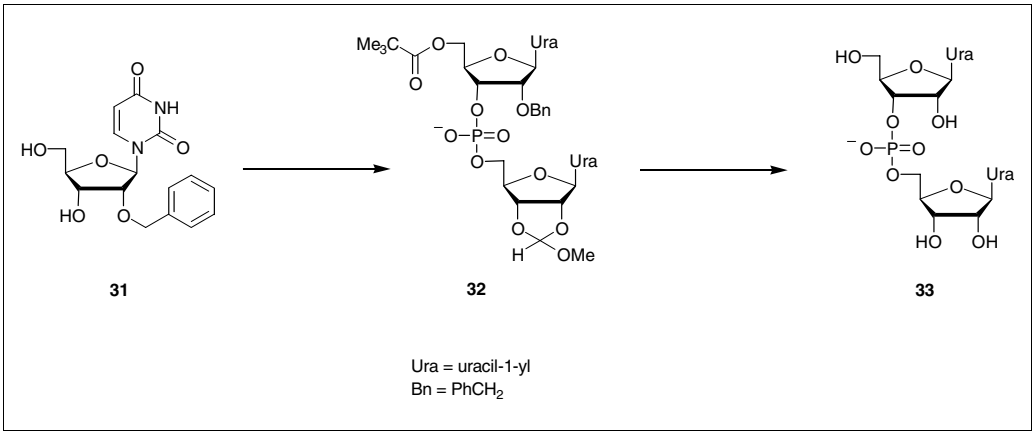
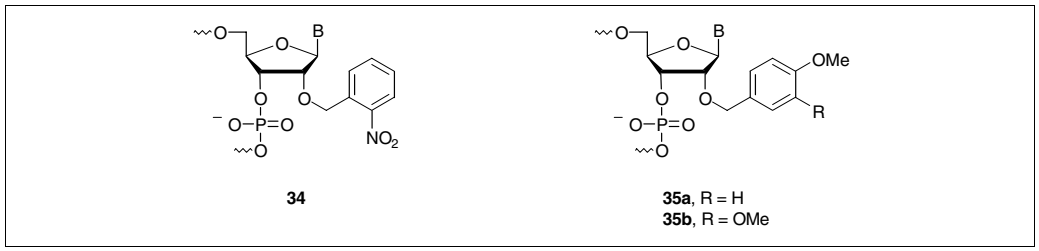


Figure 2.2.6 Scheme showing the preparation of uridylyl-(3′→5′)-uridine from 2′-*O*-benzyl (Bn)-uridine.



34 **35a**, R = H; **35b**, R = OMe

Figure 2.2.7 The 2-nitrobenzyl (**S.34**), 4-methoxybenzyl (**S.35a**), and 3,4-dimethoxybenzyl (**S.35b**) protecting groups.

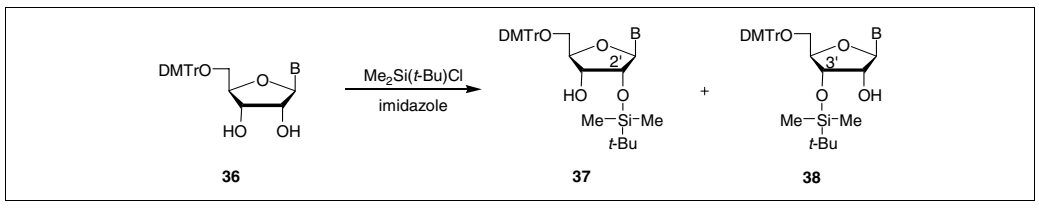
co-workers (Ohtsuka et al., 1978), is potentially a more useful 2′-protecting group. Like the benzyl group, it is stable both to acid- and base-catalyzed hydrolysis. However, it may be cleaved photochemically by irradiation with ultraviolet light ($\lambda > 280$ nm). It was later reported that photolytic cleavage of the 2-nitrobenzyl protecting group proceeds more efficiently in slightly acidic (pH 3.5) 0.1 mol dm^{-3} ammonium formate solution (Hayes et al., 1985). A serious drawback to the use of the 2-nitrobenzyl protecting group is that the photolytic cleavage reaction does not always proceed quantitatively (Ohtsuka and Iwai, 1987), especially in the unblocking of relatively high-molecular-weight RNA sequences.

Takaku and co-workers have used the 4-methoxybenzyl (as in **S.35a**; Takaku and Kamaike, 1982; Takaku et al., 1984) and 3,4-dimethoxybenzyl (**S.35b**; Takaku et al., 1986) groups to protect 2′-hydroxy functions in solution-phase oligoribonucleotide synthesis. The 4-methoxybenzyl protecting groups were removed from a hexaribonucleoside pentaphosphate (Takaku et al., 1984) by treatment for 3 hr at room temperature with a reagent prepared by adding triphenylmethyl tetrafluoroborate (~0.10 mmol/mL) to acetonitrile/water (4:1 v/v). However, Takaku et al. (1986) reported that incomplete unblocking and some cleavage of the glycosidic linkages can occur under these presumably rather acidic conditions. Some cleavage and migration of the internucleotide linkages might also be expected to occur. The 3,4-dimethoxybenzyl protecting group may be removed (Takaku et al., 1986) under somewhat milder conditions by treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in wet dichloromethane; this group would appear to be potentially more promising than the 4-methoxybenzyl group for the protection of the 2′-hydroxy functions in oligoribonucleotide synthesis.

The *tert*-Butyldimethylsilyl Protecting Group

The *tert*-butyldimethylsilyl group (TBDMS; as in **S.37** and **S.38**; Fig. 2.2.8) was originally suggested by Stork and Hudrlik (1968) for the protection of enols, and was first used by Corey and Venkateswarlu (1972) for the protection of alcoholic hydroxy functions. Ogilvie and co-workers (Ogilvie et al., 1974) then introduced it as a protecting group for the 2′-hydroxy functions of ribonucleoside building blocks. The TBDMS group is at present the most widely used 2′-protecting group in solid-phase oligoribonucleotide synthesis (Damha and Ogilvie, 1993). It meets some but by no means all of the above general requirements for protecting groups. It may be readily introduced (Usman et al., 1987), for example, by treating a 5′-*O*-DMTr-ribonucleoside derivative (**S.36**) with *tert*-butylchlorodimethylsilane and imidazole in *N*,*N*-dimethylformamide (DMF) solution (Fig.2.2.8).

Although the regiochemistry of the silylation reaction can be controlled to some extent (Hakimelahi et al., 1982), a mixture of 2′- and 3′-isomers (**S.37** and **S.38**, respectively) is in-



36 **37** **38**

Figure 2.2.8 Scheme showing introduction of the *tert*-butyldimethylsilyl (TBDMS) protecting group.

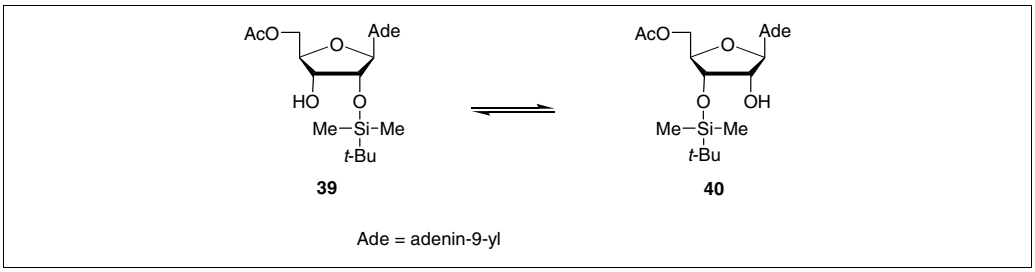


Figure 2.2.9 Scheme showing the interconversion of the 2′-*O*- (**S.39**) and 3′-*O*- (**S.40**) TBDMS adenosine derivatives.

variably obtained. Fortunately, such isomeric mixtures can usually be separated by chromatography on silica gel. However, great care has to be taken in the purification and isolation of the 2′-protected ribonucleoside building blocks (**S.37**), as the TBDMS group readily migrates from the 2′- to the 3′-hydroxy function and vice versa.

Interconversion between the adenosine derivatives **S.39** and **S.40** (Fig. 2.2.9) was found to be a base-catalyzed first-order equilibration reaction (Jones and Reese, 1979). Equilibration rates were observed to be the same in both directions, and the equilibrium constant was estimated to be 1.0. The half time ($t_{1/2}$) for equilibration in anhydrous pyridine solution at 36°C was 19 hr. The equilibration rate was increased by a factor of 3.0 when 0.1 mol equiv. (with respect to substrate) of benzylamine (pK_a 9.34) was added. Equilibration was faster still ($t_{1/2}$ = ~1 hr at 36°C) in methanol-d_4 solution without added base. When 0.1 mol equiv. of triethylamine (pK_a 10.87) was added to the methanol-d_4 solution at 20°C, equilibration was complete within ~5 min.

Precautions must be taken to avoid migration of the TBDMS protecting group during the purification and isolation of 2′-*O*-TBDMS-5′-*O*-DMTr-ribonucleoside derivatives (**S.37**) and during the course of their conversion into the required monomeric building blocks. Otherwise, the resulting synthetic RNA sequences will be contaminated with material containing (2′→5′)-internucleotide linkages. Thus, in the preparation of 3′-phosphoramidite building blocks (**S.41**; Fig. 2.2.10), it is advisable that the presence of a strong base such as diisopropylethylamine be avoided. Usman and coworkers (Scaringe et al., 1990) have recommended that a mixture of 2,4,6-collidine and 1-methylimidazole be used. Although the presence of contaminating isomeric 2′-phosphoramidites (**S.42**) can be detected above a certain level by ^{31}P NMR spectroscopy, these impurities cannot readily be removed.

It was recently reported that the oligoribonucleotide r[(Up)$_{20}$U], prepared by treating its per-2′-*O*-TBDMS derivative with tetra-*n*-butylammonium fluoride, contained an average of 1.3% (2′→5′)-internucleotide linkages (Morgan et. al., 1995). As acid was not used either during or after the unblocking process, a reasonable explanation for this observation is that the phosphoramidite **S.41** (B = uracil-1-yl) used in its synthesis was contaminated with 1.3% of its 2′-isomer (**S.42**; B = uracil-1-yl). Using an analytical procedure similar to that described by Morgan et al., it was later concluded that some commercially supplied 2′-*O*-TBDMS-protected ribonucleoside phosphoramidites (**S.41**) were contaminated with comparable amounts (i.e., >1%) of isomeric 2′-phosphoramidites (**S.42**; Reese et al., unpub. observ.). However, some other batches of commercially supplied material were estimated to contain smaller quantities of the corresponding

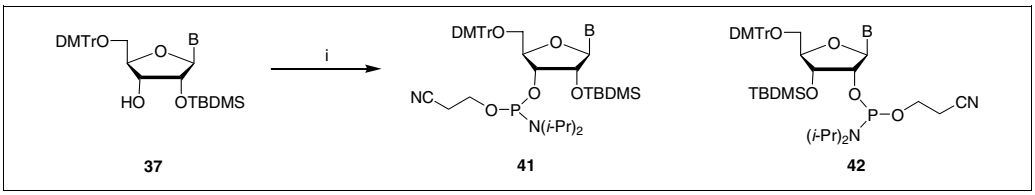


Figure 2.2.10 Scheme showing conversion of 2′-*O*-TBDMS-5′-*O*-DMTr-ribonucleoside into its corresponding 3′-phosphoramidite (**S.41**) and the structure of the possibly contaminating isomeric 2′-phosphoramidite (**S.42**). Reagents (i): $NCCH_2CH_2OPN(i\text{-}Pr)_2Cl$, base.

2′-isomer (**S.42**; Reese et al., unpub. observ.). It would therefore appear that migration of the TBDMS group can to a large extent be controlled if careful manufacturing protocols are observed.

The use of the TBDMS protecting group in the solid-phase synthesis of RNA sequences can lead to long coupling times and unsatisfactory coupling efficiencies, possibly due to the bulk of this protecting group. However, the use of 5-ethylthio-1*H*-tetrazole (**S.43b**; Fig. 2.2.11) instead of 1*H*-tetrazole (**S.43a**) as the phosphoramidite activator can result in shorter coupling times and higher quality products (Sproat et al., 1995; *UNIT 3.5*). One important advantage of the TBDMS protecting group is that it appears to be stable under the acidic conditions used to remove the 5′-terminal DMTr protecting group in solid-phase synthesis.

Problems have arisen in the unblocking of 2′-*O*-TBDMS-protected RNA sequences. The standard unblocking procedure used in the solid-phase synthesis of DNA sequences involves heating the fully loaded solid support with concentrated aqueous ammonia at 55°C overnight (Brown and Brown, 1991). These ammonolytic conditions lead to the release of the oligodeoxyribonucleotides from the solid support, the removal of the 2-cyanoethyl protecting groups from the internucleotide linkages, and the removal of *N*-acyl protecting groups from the base residues (in oligodeoxyribonucleotide synthesis, the adenine and cytosine residues are usually protected with benzoyl groups as in **S.16** and **S.18**, R = Ph, and the guanine residues are usually protected with isobutyryl groups as in **S.19**, R = Me_2CH). If these ammonolysis conditions are also used in the unblocking of 2′-*O*-TBDMS-protected oligoribonucleotides, appreciable loss of the 2′-*O*-TBDMS protecting groups and concomitant cleavage of the internucleotide linkages are likely to occur (Stawinski et al., 1988). This problem of premature removal of 2′-*O*-TBDMS protecting groups has been largely overcome by protecting the base residues with more labile acyl groups (Chaix et al., 1989), and by replacing concentrated aqueous ammonia with a more selective reagent such as 35% aqueous ammonia/ethanol (3:1 v/v) ammonia/ethanol (Mullah and Andrus, 1996), anhydrous ethanolic ammonia (Goodwin et al., 1994), or aqueous methylamine (Wincott et al., 1995). Téoule and co-workers (Chaix et al., 1989) have recommended that adenine and guanine residues be protected with phenoxyacetyl groups (as in **S.16** and **S.19**, R = CH_2OPh) and that cytosine residues be acetylated (as in **S.18**, R = Me). These workers found that the half times for removal of the latter protecting groups in aqueous ammonia/ethanol (1:1 v/v) at room temperature ranged from 10 to 15 min. It should therefore be possible completely to unblock base residues that are protected in this way without any significant loss of the 2′-*O*-TBDMS protecting groups and without internucleotide cleavage.

In the final unblocking step, the TBDMS protecting groups are removed from the 2′-hydroxy functions of the synthetic RNA sequences. Until recently, a solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran (Damha and Ogilvie, 1993) was almost always used for this unblocking process. However, it has been reported that the use of the latter reagent results in an inconvenient work-up procedure and can lead to incomplete unblocking (Sproat et al., 1995). More recently, it has been suggested that triethylamine trihydrofluoride (**S.44**; Fig. 2.2.11; Gasparutto et al., 1992; Westman and Strömberg, 1994) is a more suitable reagent for this purpose. Both the neat reagent **S.44**, which is slightly acidic as evidenced by the concomitant loss of 5′-*O*-DMTr protecting groups (Mullah and Andrus, 1996), and a solution of **S.44** and triethylamine in 1-methylpyrrolidone (Wincott et al., 1995) have been used.

There now seems to be little doubt that, if the above precautions are taken and the most suitable base-protecting groups and reagents are used, the TBDMS group may be used effectively for the protection of the 2′-hydroxy functions in the solid-phase synthesis of RNA sequences. 2′-*O*-TBDMS-ribonucleoside 3′-*H*-phosphonate building blocks (**S.45**; Fig. 2.2.12) have also been used successfully in the solid-phase synthesis of oligoribonucleotides (Rozners et al., 1994). It is reasonable to assume that the same precautions and considerations that apply to solid-phase synthesis based on phosphoramidite building blocks (**S.41**) should

Figure 2.2.11 Phosphoramidite activators 5-ethylthio-1*H*-tetrazole (**S.43b**) and 1*H*-tetrazole (**S.43a**), and the unblocking reagent triethylamine trihydrofluoride (**S.44**).

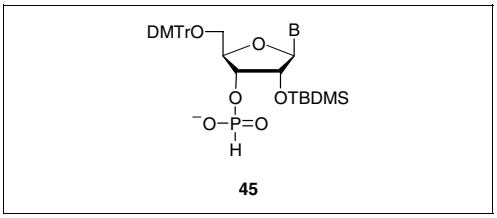


Figure 2.2.12 2′-*O*-TBDMS-ribonucleoside 3′-*H*-phosphonate.

be taken into account if good quality RNA sequences are to be obtained from the corresponding *H*-phosphonates (**S.45**).

Acetal Protecting Groups

In general, acetal groups have several distinct advantages over the TBDMS group as far as the protection of the 2′-hydroxy functions in oligoribonucleotide synthesis is concerned. First, acetal protecting groups can usually be placed regiospecifically on the 2′-hydroxy functions (see below) and, once in position, they cannot migrate. Secondly, they are completely stable under the basic conditions that normally obtain during the unblocking of internucleotide linkages and base residues. Thirdly, the 2′-protected RNA sequences obtained after the removal of the other protecting groups (Rao et al., 1993) can be purified under neutral or basic conditions without any danger of endonuclease-promoted digestion. However, there is one important drawback to the use of acetal protecting groups in that they are generally removed by acid-catalyzed hydrolysis. Unless the acidic conditions used are particularly mild, both cleavage and migration of the internucleotide linkages can occur (Griffin et al., 1968; Capaldi and Reese, 1994; Fig. 2.2.2B). While cleavage of internucleotide linkages is clearly highly undesirable, migration is a very much more serious matter as it is virtually impossible to free even a relatively low-molecular-weight RNA sequence from contaminating isomeric sequences containing one or more (2′→5′)-internucleotide linkages.

The tetrahydropyran-2-yl (Thp) group

In the 1960s, the use of the 2′-*O*-tetrahydropyran-2-yl protecting group (Thp) in oligoribonucleotide synthesis was examined (Smith et al., 1962; Smrt and Šorm, 1962; Griffin and Reese, 1964). Pure 2′-*O*-Thp derivatives of uridine and adenosine (**S.47a** and **S.47b**, respectively) were prepared according to the procedure indicated in Figure 2.2.13, and were converted into dinucleoside phosphates by the methods then available (Griffin and Reese, 1964; Griffin et al., 1968). Careful unblocking studies were carried out in 0.01 mol dm^{-3} hydrochloric acid (pH 2.0) at 24°C (Griffin et al., 1968), and the half time ($t_{1/2}$) for the conversion of 2′-*O*-Thp-UpU (**S.49**; Fig. 2.2.14) into completely unprotected uridylyl-(3′→5′)-uridine (UpU; **S.50**) was found to be 29 min. It can therefore be estimated that >99.9% removal of the Thp group would occur in <5 hr under these conditions.

It was also found that after UpU (**S.50**) had been allowed to stand in 0.01 mol dm^{-3} hydrochloric acid (pH 2.0) at 25°C for 216 hr, it underwent ~99% ribonuclease A–catalyzed digestion to uridine-3′-phosphate and uridine, thereby indicating that not more than 1% isomerization to uridylyl-(2′→5′)-uridine (**S.51**) had occurred (Griffin et al., 1968). Under these conditions (i.e., pH 2.0, 25°C, 216 hr), UpU also underwent ~0.5% hydrolytic cleavage (Fig. 2.2.2B). It can therefore be estimated that in the time required for >99.9% removal of the Thp protecting group from 2′-*O*-Thp-UpU, not more than 0.02% phosphoryl migration and 0.01% internucleotide cleavage would be expected to occur. Thus, it seemed reasonable to conclude from the data then available that the Thp group was suitable for the protection of the 2′-hydroxy functions in oligoribonucleotide synthesis.

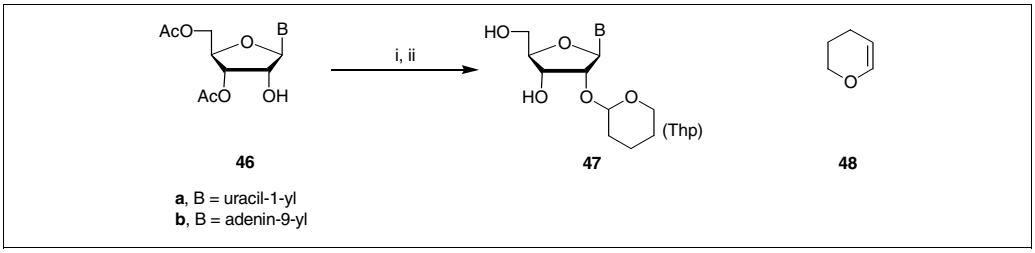


Figure 2.2.13 Scheme showing preparation of 2′-*O*-Thp derivatives of uridine and adenosine. Reagents: (i) 3,4-dihydro-2*H*-pyran (**S.48**), toluene-4-sulfonic acid (TsOH), dioxane; (ii) NaOMe, MeOH.

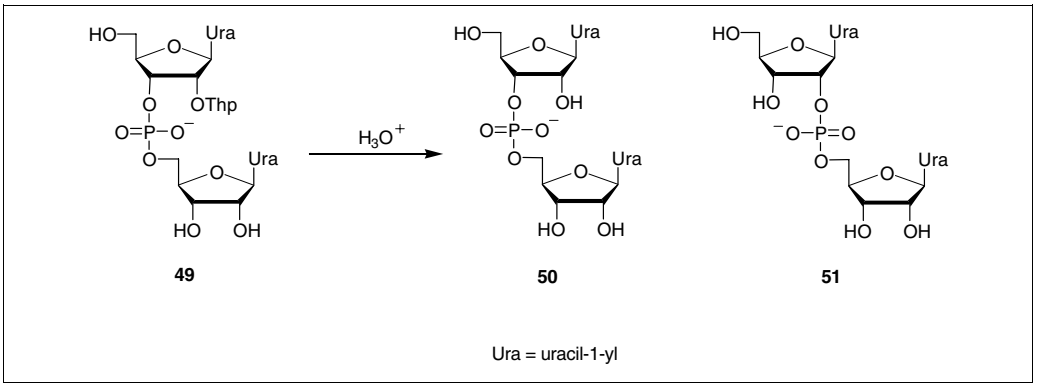


Figure 2.2.14 Scheme showing conversion of 2′-*O*-Thp-UpU into unprotected uridylyl-(3′→5′)-uridine (**S.50**) and the structure of its (2′→5′)-isomer (**S.51**).

A particular disadvantage of the Thp group is that it is chiral, and therefore its use in the protection of ribonucleoside derivatives and other chiral compounds leads to mixtures of diastereoisomers. Thus, two diastereoisomers each of 2′-*O*-Thp-uridine (**S.47a**) and 2′-*O*-Thp-adenosine (**S.47b**) were obtained (Fig. 2.2.13; Griffin et al., 1968). Although both pairs of diastereoisomers were easily separable and all four compounds were obtained as pure crystalline solids, this is clearly an undesirable complication.

The 4-methoxytetrahydropyran-4-yl (Mthp) group

A search for an achiral alternative to the Thp protecting group led to the introduction of the 4-methoxytetrahydropyran-4-yl group (Mthp; Reese et al., 1967; 1970). 2′-*O*-Mthp derivatives of ribonucleosides (**S.54**) were first prepared from 3′,5′-di-*O*-acyl-ribonucleosides (**S.53**; Fig. 2.2.15). However, they are more conveniently prepared from the corresponding 3′,5′-*O*-(1,1,3,3-tetraisopropyl- disiloxan-1,3-diyl) derivatives (**S.56**; Brown et al., 1989a). 2′-*O*-Mthp derivatives (**S.54**) are usually obtained as pure crystalline solids in satisfactory to good yields (Reese et al., 1970).

The half times for the hydrolysis of 2′-*O*-Mthp-uridine and 2′-*O*-Mthp-adenosine (**S.54**, B = uracil-1-yl and adenosine-9-yl, respectively) in 0.01 mol dm^{-3} hydrochloric acid at 22°C were found to be 18.7 and 34 min, respectively (Norman et al., 1984). It is interesting to note that the removal of the Mthp protecting group from 2′-*O*-Mthp-uridylyl-(3′→5′)-uridine (**S.57a**) and 2′-*O*-Mthp-adenylyl-(3′→5′)-adenosine (**S.57b**; Fig. 2.2.16) under the same conditions was found to proceed at significantly faster rates ($t_{1/2}$ = 6.1 and 19.9 min, respectively; Norman et al., 1984). The rate of removal of the Thp protecting group from 2′-*O*-Thp-UpU (**S.49**) is also faster than from 2′-*O*-Thp-uridine (**S.47a**; Griffin et al., 1968).

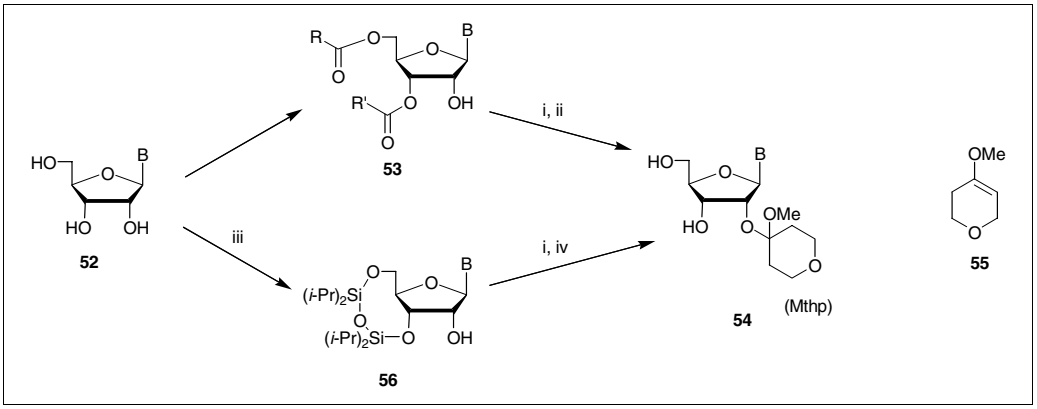


Figure 2.2.15 Scheme showing preparation of 2′-*O*-Mthp ribonucleoside derivatives (**S.54**) via 3′,5′-di-*O*-acyl-ribonucleosides (**S.53**) or 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl) derivatives (**S.56**). Reagents: (i) 4-methoxy-5,6-dihydro-2*H*-pyran (**S.55**), toluene-4-sulfonic acid (TsOH), dioxane; (ii) NH_3, MeOH; (iii) (*i*-Pr)$_2$Si(Cl)OSi(Cl)(*i*-Pr)$_2$, imidazole, MeCN; (iv) Et_4NF, MeCN.

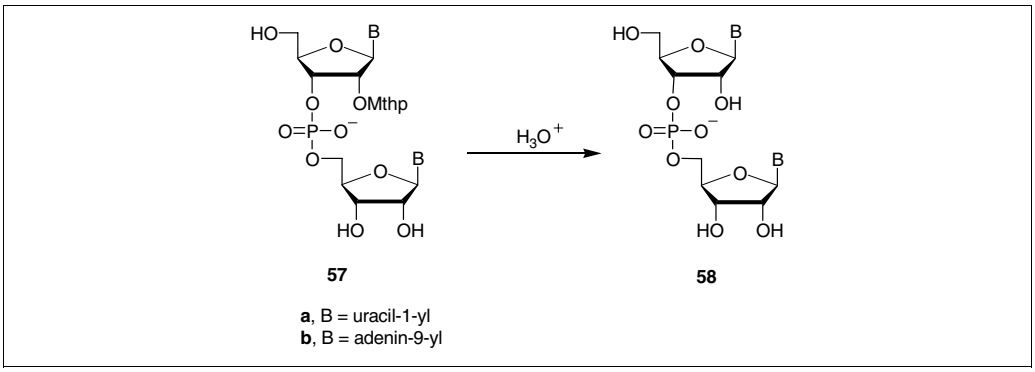


Figure 2.2.16 Scheme illustrating the removal of the Mthp protecting group.

The fact that the presence of a vicinal phosphodiester internucleotide linkage appears to facilitate the acid-catalyzed unblocking of a 2′-*O*-Mthp- or 2′-*O*-Thp-protected hydroxy function is clearly advantageous if migration and cleavage of the internucleotide linkages (Fig. 2.2.2B) in the final unblocking step of oligoribonucleotide synthesis are to be kept to a minimum. As well as being achiral, Mthp has an additional advantage over Thp in that it is more labile to acidic hydrolysis.

The Mthp and Thp protecting groups have been used in both solution- and solid-phase synthesis of RNA sequences. The Mthp group was introduced particularly for solution-phase synthesis, and it has been used successfully in the preparation of the 3′-terminal decamer, nonadecamer, and heptatriacontamer (37-mer) sequences (r[UpCpGpUpCpCpApCpCpA], r[ApUpUpCpCpGpGpApCpUpCpGpUpCpCpApCpCpA], and r[GpGpApGpApGpGpUpCpUpCpCp GpGpTpψpCpGpApUpUpCpCpGpGpApCpUpCpGpUpCpCpApCpCpA], respectively) of yeast alanine transfer RNA ($tRNA^{Ala}$; Jones et al., 1980, 1983; Brown et al., 1989a,b). This work has already been reviewed (Reese, 1989).

The tetrahydrofuran-2-yl (Thf) and 1,5-dimethoxycarbonyl-3-methoxypentan-3-yl (Mdmp) groups

The above approach to the solution-phase synthesis of RNA sequences was successful largely because treatment with acid was completely avoided until the final unblocking step. However, other workers (Ohtsuka et al., 1984) reported a solution-phase block synthesis of a tritriacontamer (33-mer) sequence of *E. coli* $tRNA_2^{Gly}$ using tetrahydrofuran-2-yl (Thf; **S.59**; Fig. 2.2.17) and DMTr groups for the protection of the 2′- and 5′-hydroxy functions, respectively. The 5′-terminal DMTr protecting groups were removed by treatment with zinc bromide in dry dichloromethane/isopropanol solution rather than with a protic acid. Although Thf is more labile than Thp (and probably also Mthp) to acid-catalyzed hydrolysis (Kruse et al., 1979), the latter combination of protecting groups was apparently effective.

There are a number of reports in the literature relating to solid-phase RNA synthesis in which the 2′-hydroxy functions are protected by Thp, Mthp, or Thf groups and the 5′-hydroxy functions are also protected with acid-labile groups (Tanaka et al., 1986; Kierzek et al., 1986; Iwai et al., 1987; Tanimura et al., 1989; Tanimura and Imada, 1990). Such acid-labile groups include DMTr (**S.10b**), 9-phenylxanthen-9-yl (**S.11**), and 9-(4-methoxyphenyl)xanthen-9-yl (**S.60**; Fig. 2.2.17; *UNIT 2.3*). Although some sequences appear to have been prepared successfully in this way, other reports suggest that this is an unsound strategy, particularly for the synthesis of comparatively high-molecular-weight RNA sequences (Reese and Skone, 1985; Christodoulou et al., 1986; Kierzek, 1994). Even when precautions are taken to maintain stringently anhydrous conditions, the repeated exposure of the growing protected oligoribonucleotide to di- or tri-chloroacetic acid in order to remove the 5′-protecting group in each synthetic cycle is likely to lead to some loss of such relatively labile 2′-

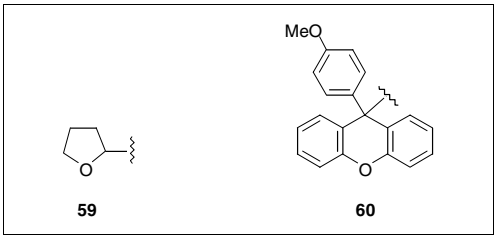


Figure 2.2.17 Tetrahydrofuran-2-yl (**S.59**) and 9-(4-methoxyphenyl)xanthen-9-yl (**S.60**) protecting groups.

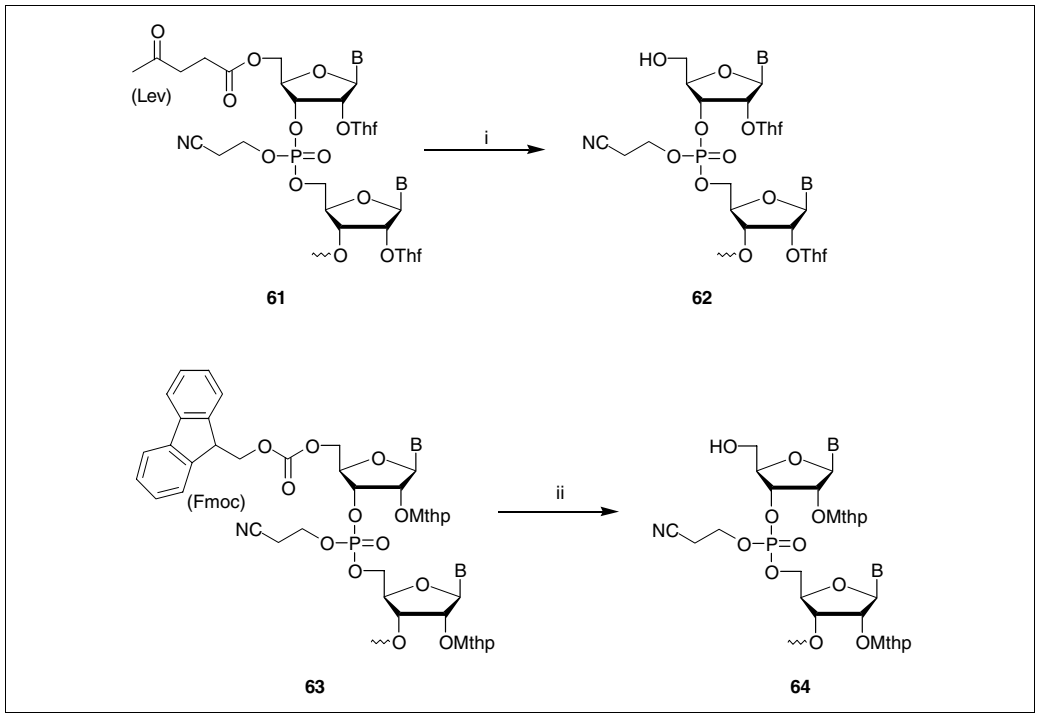


Figure 2.2.18 Scheme showing removal of levulinyl (top) and Fmoc (bottom) protecting groups. Reagents: (i) $N_2H_4 \cdot H_2O$, C_5H_5N, AcOH; (ii) 0.1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), MeCN.

protecting groups, resulting both in cleavage and migration of internucleotide linkages (Pathak and Chattopadhyaya, 1985; Reese and Skone, 1985; Kierzek, 1994). If Thp, Mthp, or Thf groups are to be used to protect the 2′-hydroxy functions, it would appear to be a better strategy in solid-phase synthesis to protect the 5′-terminal hydroxy function with a group that is readily removable under virtually neutral or mildly basic conditions. Thus, following van Boom's use of the levulinyl group (as in **S.61**; Fig. 2.2.18) for the protection of the 5′-hydroxy functions in solution-phase synthesis (den Hartog et al., 1981), other workers (Iwai and Ohtsuka, 1988) successfully used Lev in conjunction with Thf in solid-phase oligoribonucleotide synthesis. The Lev group was removed (Fig. 2.2.18) in the usual way (den Hartog et al., 1981) by treatment with hydrazine hydrate in pyridine/acetic acid solution. A number of RNA sequences, including a heneicosamer (21-mer), were prepared in this way. In another study (Lehmann et al., 1989), the Fmoc group (**S.12**) was used to protect the 5′-hydroxy functions in solid-phase oligoribonucleotide synthesis. These workers protected the 2′-hydroxy functions with Mthp groups (as in **S.63**) and removed the Fmoc group with 1,8- diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile solution (Fig. 2.2.18). As the authors (Lehmann et al., 1989) pointed out, it is very likely that some concomitant loss of the 2-cyanoethyl protecting groups from the partially protected oligoribonucleotides (**S.63**) occurs during DBU treatment. A nonadecamer and an icosamer (20-mer) RNA sequence were prepared successfully in this way.

Although the above approach using either 2′-*O*-Thf and 5′-*O*-Lev protection or 2′-*O*-Mthp and 5′-*O*-Fmoc protection (Fig. 2.2.18) was successful, it is much more convenient to use the acid-labile DMTr (**S.10b**) or Px (**S.11**) groups to protect the 5′-hydroxy functions in solid-phase RNA synthesis. The latter modified trityl groups can be rapidly and quantitatively removed under anhydrous conditions and the released carbocations can easily be assayed spectrophotometrically (Brown and Brown, 1991). For this reason, attempts have been made to develop somewhat more sophisticated acetal protecting groups that are stable under normal "detritylation" conditions and are also sufficiently labile to acidic hydrolysis in the final unblocking step for cleavage and migration of the internucleotide linkages (Fig. 2.2.2B) to be avoided. Chattopadhyaya and co-workers (Sandström et al., 1985) showed that the 1,5-dimethoxycarbonyl-3-methoxypentan-3-yl (Mdmp) group (as in **S.65**; Fig. 2.2.19), derived from dimethyl 4-ketopimelate, was

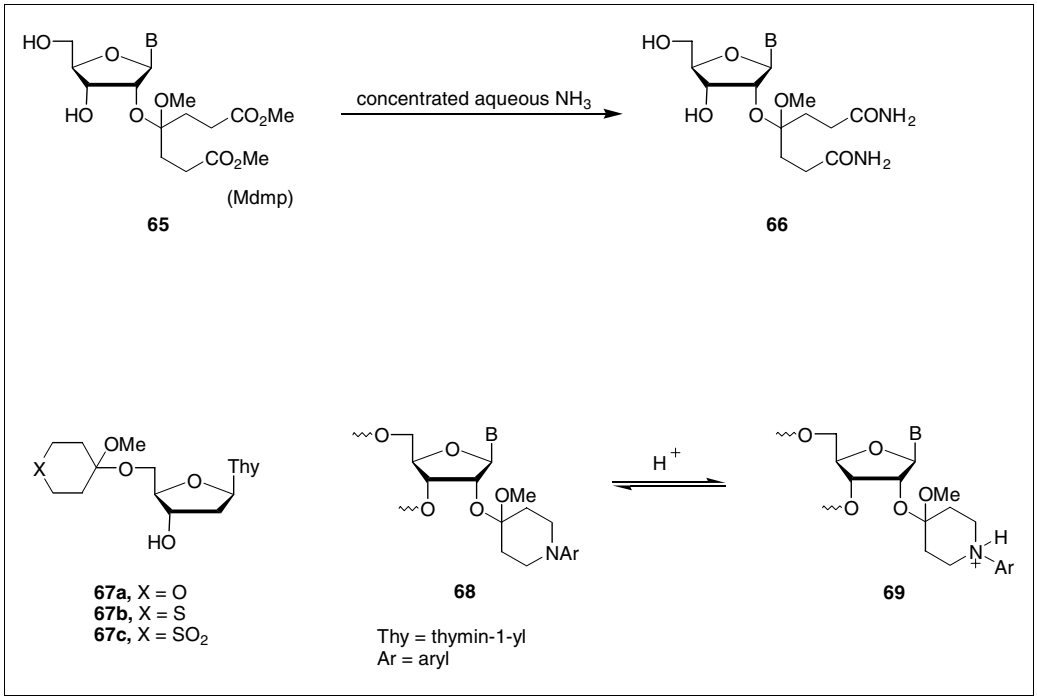


Figure 2.2.19 Acetal protecting groups labile to acidic hydrolysis.

converted under the standard ammonolytic unblocking conditions used in solid-phase synthesis into the corresponding bis-amide (**S.66**), which was seventeen times more labile to acidic hydrolysis than the bis-ester (**S.65**). However, the Mdmp protecting group itself is unlikely to find application in solid-phase synthesis of oligoribonucleotides as it undergoes hydrolysis in 4:1 (v/v) acetic acid/water solution even more rapidly than the Mthp group.

The 1-Aryl-4-methoxypiperidin-4-yl (Ctmp and Fpmp) groups

Acetal hydrolysis is a second-order reaction; its rate, which is proportional to the concentrations both of substrate and hydrogen ions, is very sensitive to inductive effects (Kreevoy and Taft, 1955). Thus 5′-*O*-(4-methoxytetrahydrothiopyran-4-yl)-thymidine (**S.67b**; Fig. 2.2.19) was found to be ~5 times more labile to acidic hydrolysis than the corresponding Mthp derivative (**S.67a**) and was estimated to be >2000 times more labile than the corresponding sulfone (**S.67c**; van Boom et al., 1972). It seemed possible that, if the aryl substituent Ar were selected carefully, a 1-aryl-4-methoxypiperidin-4-yl protecting group (as in **S.68**) could be identified that would be almost fully protonated (as in **S.69**) under detritylation conditions (i.e., in dichloromethane containing, for instance, 2% to 3% trichloroacetic acid), but would be virtually unprotonated (as in **S.68**) under the milder conditions of acidic hydrolysis obtaining in the final unblocking step of oligoribonucleotide synthesis. Although it would, of course, depend on the aryl substituent, it seemed possible that the rate of hydrolysis of the unprotonated and protonated piperidinyl species might correspond approximately to those of the Mthp (**S.67a**) and sulfone (**S.67c**) derivatives, respectively. In the overall rate expression for the hydrolysis of a 1-aryl-4-methoxypiperidin-4-yl derivative, it is reasonable to assume that the component relating to the hydrolysis of the conjugate acid **S.69** is likely to be negligible in comparison with that relating to the unprotonated **S.68** and that, as a first approximation, it can be ignored. If this is the case, the observed rate of hydrolysis of the 1-aryl-4-methoxypiperidin-4-yl acetal system should be pH independent. For example, if the pH of the hydrolytic medium is lowered by one unit, the concentration of the unprotonated acetal **S.68** will decrease by an order of magnitude, and at the same time the rate of hydrolysis of the remaining unprotonated acetal will increase by an order of magnitude.

Despite the very limited synthetic methodology available at the outset, it was possible to prepare 2′-*O*-[1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl] (Ctmp) ribonucleoside derivatives (**S.71a**; Fig. 2.2.20A) and

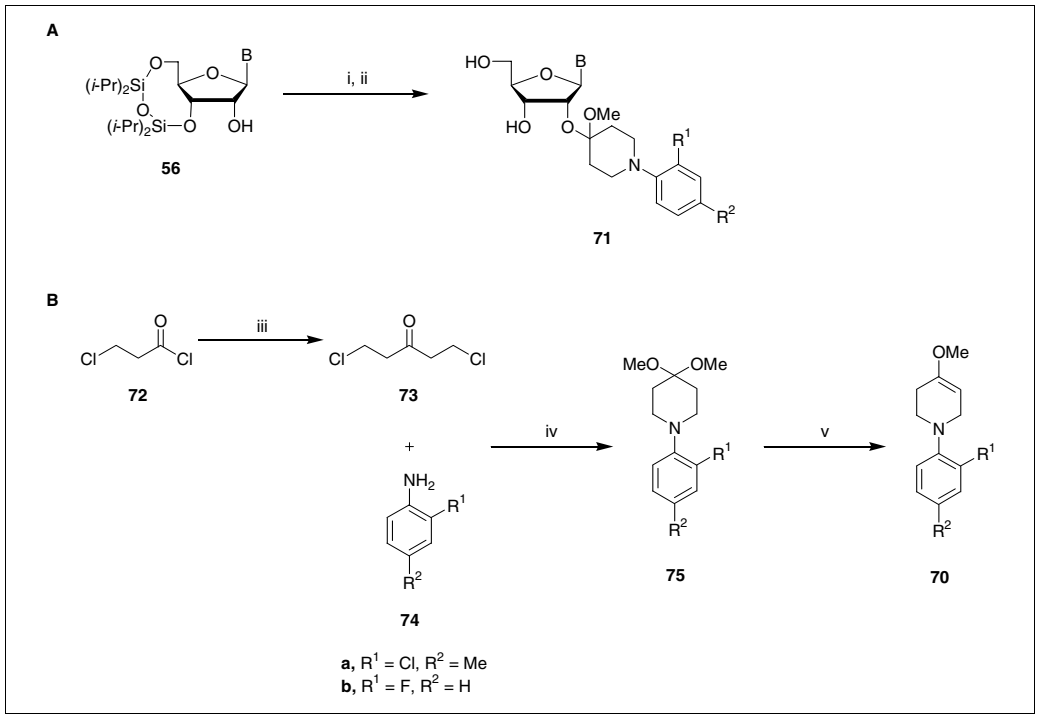


Figure 2.2.20 (**A**) Scheme showing preparation of Ctmp (**S.71a**) and Fpmp (**S.71a**) ribonucleoside derivatives. (**B**) Preparation of the 1-aryl-4-methoxy-1,2,5,6-tetrahydropyridines (**S.70**) required in (**A**). Reagents (i) **S.70**, CF_3CO_2H, CH_2Cl_2; (ii) Et_4NF, MeCN; (iii) ethylene, $AlCl_3$, CH_2Cl_2; (iv) toluene-4-sulfonic acid monohydrate ($TsOH{\cdot}H_2O$), MeOH, and reflux followed by $(MeO)_3CH$; (v) $(i\text{-}Pr)_2NEt$, $Et_2O \rightarrow BF_3$, CH_2Cl_2, 0°C.

show that the Ctmp protecting group had the desired properties (Reese et al., 1986). Thus it can be seen from Figure 2.2.21 that the rate of hydrolysis of 2′-*O*-Ctmp-uridine (**S.71a**, B = uracil-1-yl) at 30°C is only 1.75 times faster at pH 0.5 than it is at pH 2.5. At 25°C, 2′-*O*-Ctmp-uridine is ~40 times more *stable* than 2′-*O*-Mthp-uridine (**S.54**, B = uracil-1-yl) at pH 1.0 (Reese et al., 1986), but it is nearly 1.6 times more *labile* than 2′-*O*-Mthp-uridine at pH 3.0.

The first general criterion that all protecting groups should meet (see above) is that they should be easy to introduce, and an important part of this criterion is that the reagent required should be readily accessible. The Ctmp and related piperidine-derived protecting groups are easy to introduce, but until recently the preparation of the enol ether reagents (such as **S.70**) involved a number of steps. However, these 1-aryl-4-methoxy-1,2,5,6-tetrahydropyridine derivatives can now be readily prepared (Fig. 2.2.20B) in two steps and in good overall yields (Faja et al., 1997) from 1,5-dichloropentan-3-one (**S.73**; Owen and Reese, 1970) and the appropriate primary aromatic amine (**S.74**). The procedure for the preparation of 2′-*O*-(1-aryl-4-methoxypiperidin-4-yl) ribonucleoside derivatives (such as **S.71**; Fig.

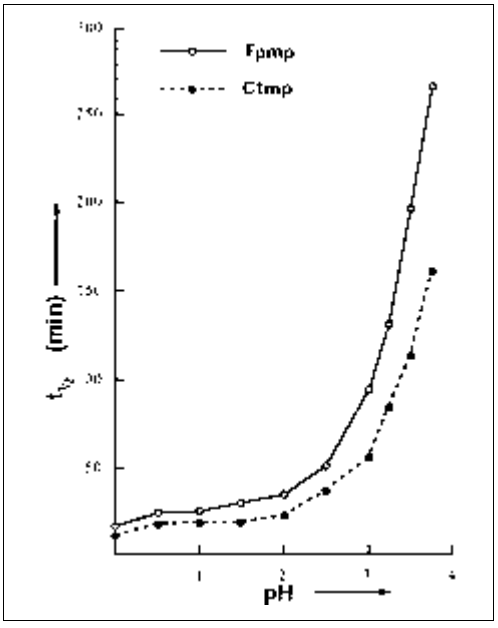


Figure 2.2.21 Dependence of half times ($t_{1/2}$) on pH for hydrolysis of 2′-*O*-Ctmp-uridine (**S.71a**) and 2′-*O*-Fpmp-uridine (**S.71b**) at 30°C.

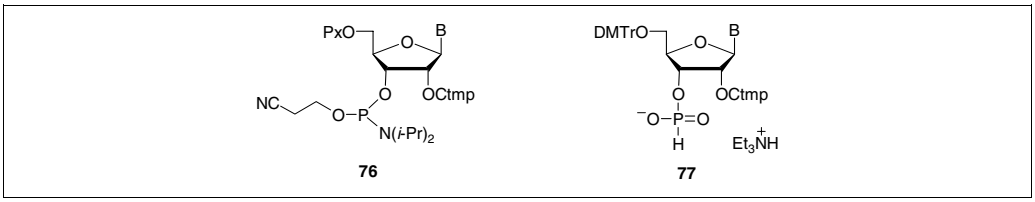


Figure 2.2.22 Ctmp-protected phosphoramidite (**S.76**) and *H*-phosphonate (**S.77**).

2.2.20A; Rao et al., 1987, 1993) is closely similar to that used in the preparation of the corresponding 2′-*O*-Mthp derivatives (**S.54**; Fig. 2.2.15), except that a much smaller excess of the enol ether reagent **S.70** is needed.

The 2′-*O*-Ctmp protecting group was used in conjunction with the 5′-*O*-Px protecting group (Rao et al., 1987) or the 5′-*O*-DMTr protecting group (Sakatsume et al., 1989) in the solid-phase synthesis of oligoribonucleotides. Phosphoramidite building blocks (**S.76**; Fig. 2.2.22) were successfully used in the preparation of the 3′-terminal nonadecamer sequence r[ApUpUpCpCpGpGpApCpUpCpGpUpCpCpApCpCpA] of yeast tRNA^{Ala} (Rao et al., 1987), and *H*-phosphonate building blocks (**S.77**) were used successfully in the preparation of the octadecamer sequence, r[ApGpUpApUpApApGpApGpGpApCpApUpApUpG] (Sakatsume et al., 1989). However, the required enol ether reagent (**S.70**) was difficult to prepare by the original procedure (Reese et al., 1986), and its preparation by the improved protocol (Fig. 2.2.20B; Faja et al., 1997) involves either the use of an expensive aromatic amine (**S.74a**) or an additional chlorination step.

It was later found that several other 1-aryl-4-methoxypiperidin-4-yl groups were also suitable for the protection of the 2′-hydroxy functions in solid-phase oligoribonucleotide synthesis. Among these is the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) protecting group (as in **S.71b**; Reese and Thompson, 1988). The Fpmp protecting group has two distinct advantages over the Ctmp group. First, the enol ether reagent **S.70b**, which is a low-melting solid, is readily prepared (Faja et al., 1997) from 2-fluoroaniline (**S.74b**), which is an inexpensive starting material. Secondly, the Fpmp group is somewhat more stable than the Ctmp group to acidic hydrolysis in the pH range of 0.5 to 1.0 (~1.4 times at 30°C; Fig. 2.2.21), and therefore the risk of concomitant 2′-unblocking in the detritylation steps is even smaller. However, removal of the 2′-*O*-Fpmp protecting group occurs more slowly than removal of the 2′-*O*-Ctmp group in the final unblocking step of oligoribonucleotide synthesis, and this can be disadvantageous (see below). It can be seen from Figure 2.2.21 that at 30°C the rate of hydrolysis of 2′-*O*-Fpmp-uridine (**S.71b**; B = uracil-1-yl) is only about twice as fast at pH 0.5 as at pH 2.5.

The 2′-*O*-Fpmp protecting group has been widely used in solid-phase oligoribonucleotide synthesis (Beijer et al., 1990; Rao et al., 1993; Capaldi and Reese, 1994; Pieles et al., 1994; Sproat et al., 1994; Rao and Macfarlane, 1995; McGregor et al., 1996). The 5′-*O*-Px-2′-*O*-Fpmp phosphoramidite building blocks **S.78** (Fig. 2.2.23) were used successfully in the synthesis of r[UpCpGpUpCpCpApCpCpA], r[ApUpUpCpCpGpGpApUpCpGpUpCpCpApCpCpA], and r[GpGpApGpApGpGpUpCpUpCpCpGpGpUpUpCpGpApUpUpCpCpGpGpApCpUpCpGpUpCpCpApCpCpA], the 3′-decamer, nonadecamer, and heptatriacontamer (37-mer) sequences, respectively, of unmodified yeast tRNA^{Ala} (Rao et al., 1993). Sproat and co-workers (Pieles et al., 1994) carried out the solid-phase synthesis of some modified oligoribonucleotides containing

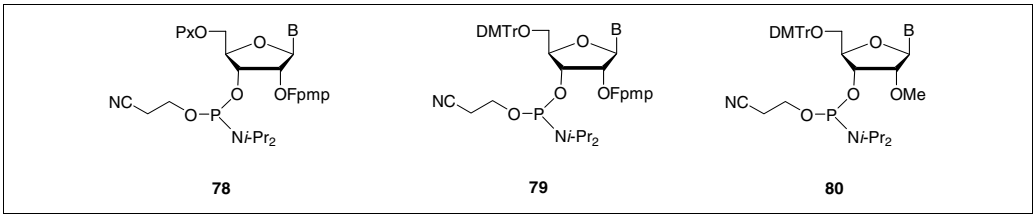
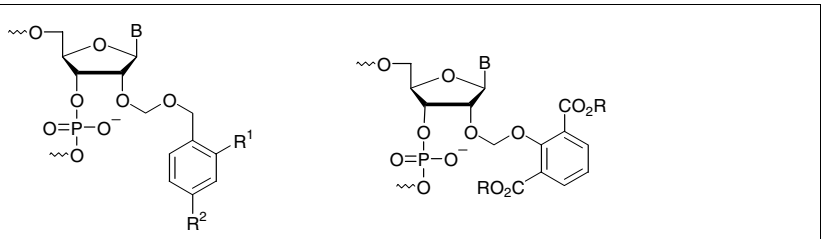


Figure 2.2.23 5′-*O*-Px-2′-*O*-Fpmp phosphoramidite (**S.78**), 5′-*O*-DMTr-2′-*O*-Fpmp phosphoramidite (**S.79**), and 5′-*O*-DMTr-2′-*O*-methyl-ribonucleoside phosphoramidite (**S.80**).



81a, $R^1 = NO_2$, $R^2 = H$
81b, $R^1 = H$, $R^2 = NO_2$

82a, R = Me
82b, R = H

Figure 2.2.24 Additional acetal protecting groups: (2-nitrobenzyloxy)methyl (**S.81a**), (4-nitrobenzyloxy)methyl (**S.81b**), (2,6-dimethoxycarbonyl)phenoxymethyl (**S.82a**), and (2,6-dicarboxy)phenoxymethyl (**S.82b**).

pseudouridine, 2′-*O*-methylpseudouridine, and some other 2′-*O*-methyl-ribonucleoside residues starting from the appropriate 5′-*O*-DMTr-2′-*O*-Fpmp and 5′-*O*-DMTr-2′-*O*-methyl-ribonucleoside phosphoramidites (**S.79** and **S.80**, respectively). In all of the early work (Rao et al., 1993), the 2′-*O*-Fpmp and 5′-terminal Px (or DMTr) protecting groups were removed by treatment with 0.01 mol dm^{-3} hydrochloric acid (pH ~2) at room temperature. However, it soon became clear that the susceptibility of the internucleotide linkages of oligoribonucleotides to acid-catalyzed cleavage and migration was sequence dependent, and that certain sequences were unstable at pH 2 and room temperature (Capaldi and Reese, 1994). Thus, despite the relative stability of uridylyl-(3′→5′)-uridine (**S.50**; Griffin et al., 1968) at pH 2 and room temperature, r[$(Up)_9$U] and r[$(Up)_{19}$U] both underwent virtually complete degradation in the course of the removal of the 2′-*O*-Fpmp protecting groups under the same conditions (Capaldi and Reese, 1994). However, when unblocking was carried out at room temperature above pH 3.0, virtually no internucleotide cleavage or migration could be detected (see Conclusions). Other workers subsequently reported that no cleavage or migration of the internucleotide linkages could be detected after r[$(Up)_{20}$U] had been allowed to stand at pH 3.25 in 0.5 M sodium acetate buffer solution at room temperature for 96 hr (Rao and Macfarlane, 1995), which is very much more than the time required to remove the 2′-*O*-Fpmp protecting groups. These workers went on to recommend that 2′-*O*-Fpmp protecting groups be removed at pH 3.25 and 30°C in 0.5 M sodium acetate buffer solution. They successfully unblocked RNA sequences containing up to 50 nucleoside residues under these conditions, and obtained oligoribonucleotides that were active as ribozymes and ribozyme substrates.

Other acetal groups

Three other interesting and potentially useful acetal groups have recently been suggested for the protection of the 2′-hydroxy functions in solid-phase oligoribonucleotide synthesis. Like the 2-nitrobenzyl group (as in **S.34**, see above), the (2-nitrobenzyloxy)methyl group (as in **S.81a**; Schwartz et al., 1992; Fig. 2.2.24) is removable photochemically; however, possibly for steric reasons, its use leads to faster and more efficient coupling reactions. The (2-nitrobenzyloxy)methyl protecting group has been used successfully in the solid-phase synthesis of a number of RNA sequences including a dodecamer, a hexadecamer, and a tritriacontamer (33-mer) sequence that are all components of ribozyme structures. The related (4-nitrobenzyloxy)methyl protecting group (as in **S.81b**; Gough et al., 1996), which has also been used successfully in solid-phase oligoribonucleotide synthesis, is removable by treatment with tetra-*n*-butylammonium fluoride in THF solution. Finally the (2,6-dimethoxycarbonyl)phenoxymethyl protecting group (as in **S.82a**; Rastogi and Usher, 1995), which has been used in the solid-phase synthesis of two dinucleoside phosphates, is extremely (over 100 times more than the Fpmp group) stable under standard detritylation conditions. After the assembly of the desired RNA sequences, the two methoxycarbonyl groups are saponified by treatment with aqueous sodium hydroxide, which also releases the product from the solid support and removes base-labile protecting groups. The resulting (2,6-dicarboxy)phenoxymethyl acetal system (as in **S.82b**) is estimated to be >1300 times more labile to acidic hydrolysis at pH 3.0 than the original (2,6-di-

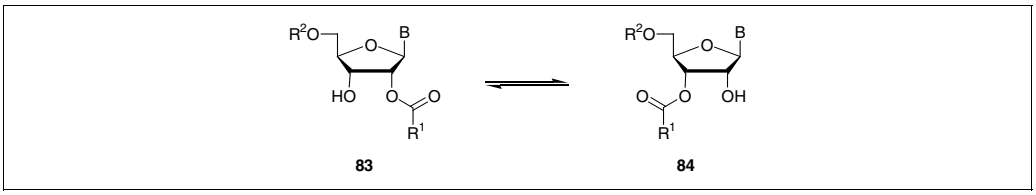


Figure 2.2.25 Scheme showing interconversion of isomeric 2′- and 3′-*O*-acyl-ribonucleoside derivatives.

methoxycarbonyl)phenoxymethyl acetal system; however, it is still ~2.3 times more stable at pH 3.0 than the Fpmp protecting group.

Ester Protecting Groups

It has been known for many years that isomeric 2′- and 3′-*O*-acyl-ribonucleoside derivatives (**S.83** and **S.84**, respectively; Fig. 2.2.25) interconvert under mildly basic conditions, and that the equilibrium mixture eventually obtained is generally somewhat richer in the 3′-isomer (Reese and Trentham, 1965). Unlike corresponding mixtures of 2′- and 3′-*O*-TBDMS derivatives (e.g., **S.39** and **S.40**; Fig. 2.2.9), it is usually very difficult or even impossible to separate isomeric mixtures of 2′- and 3′-esters (**S.83** and **S.84**) by standard chromatographic methods. Furthermore, acyl migration can occur during chromatography. For these reasons, 2′-*O*-acyl protecting groups have only very rarely been used in oligoribonucleotide synthesis. However, in an early study (Fromageot et al., 1968), N^2,$O^{2'}$,$O^{5'}$-tribenzoylguanosine (**S.85a**; Fig. 2.2.26) and N^4,$O^{2'}$,$O^{5'}$-triacetylcytidine (**S.85b**), two pure crystalline compounds, were both successfully coupled with 2′,3′-di-*O*-acetyluridine 5′-phosphate (**S.86**) by the now obsolete phosphodiester approach in solution to give, after deprotection, guanylyl-(3′→5′)-uridine (**S.87**; B = guanin-9-yl) and cytidylyl-(3′→5′)-uridine (**S.87**; B = cytosin-1-yl), respectively. Both of the latter dinucleoside phosphates were apparently free from their (2′→5′)-isomers.

Two later studies relating to the use of 2′-*O*-acyl protecting groups in solid-phase oligoribonucleotide synthesis are also of interest. In one study (Kempe et al., 1982), oligoribonucleotides and chimeric RNA:DNA sequences were prepared from 2′-*O*-benzoyl-protected phosphoramidites (**S.88**; Fig. 2.2.27). However, as these phosphoramidites (**S.88**) were contaminated with 1% to 3% of the isomeric 2′-phosphoramidites, the integrity of the internucleotide linkages in the target RNA sequences was to some extent compromised. The other study (Rozners et al., 1992) described the solid-phase synthesis of oligoribonucleotides from 2′-*O*-(2-chlorobenzoyl)-3′-*H*-phosphonate building blocks (**S.89**). This is a more promising approach for two reasons. First, it was possible to separate the isomeric

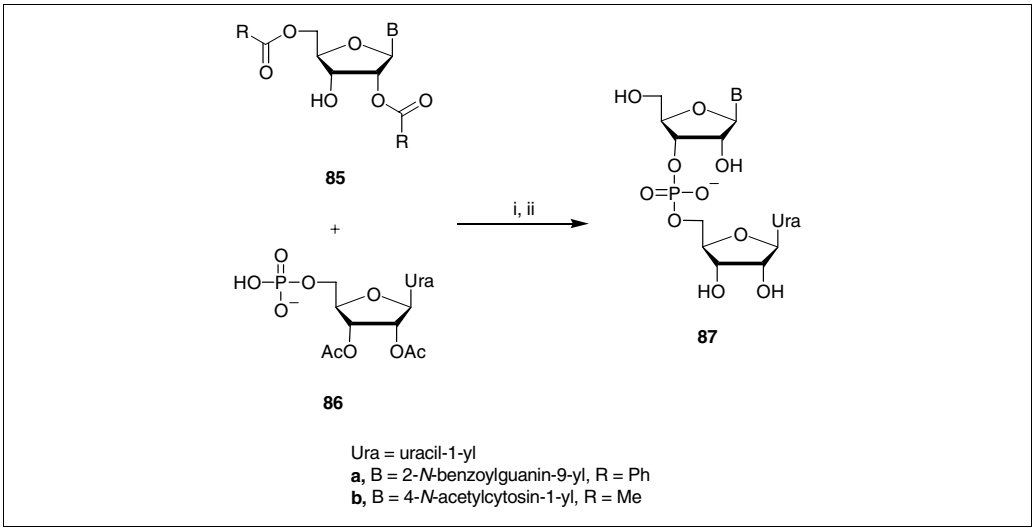


Figure 2.2.26 Scheme showing preparation of guanylyl-(3′→5′)-uridine and cytidylyl-(3′→5′)-uridine using a 2′-*O*-acyl protecting group. Reagents: (i) mesitylene-2-sulfonyl chloride, C_5H_5N; (ii) $MeNH_2$, EtOH, or NH_3, MeOH.

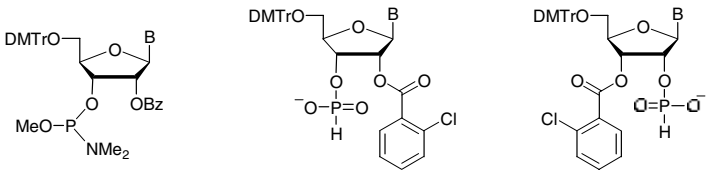


Figure 2.2.27 2′-*O*-Benzoyl-protected 3′-phosphoramidite (**S.88**), 2′-*O*-(2-chlorobenzoyl)-protected 3′-*H*-phosphonate (**S.89**), and the isomeric 2′-*H*-phosphonate (**S.90**).

2′- and 3′-*H*-phosphonates (**S.90** and **S.89**, respectively) by chromatography on silica gel, and thereby obtain isomerically pure building blocks (**S.89**). Secondly, after the desired RNA sequences had been assembled, the 2′-protecting groups could be removed by ammonolysis under conditions that were mild enough to avoid cleavage of the internucleotide linkages. A number of RNA sequences of moderate length were prepared successfully by this approach.

CONCLUSIONS

At present, the TBDMS group (as in **S.37**; Fig. 2.2.28) is the most widely used protecting group for 2′-hydroxy functions in solid-phase oligoribonucleotide synthesis. The Fpmp (as in **S.71b**; Fig. 2.2.28) group is also widely used. Both 2′-*O*-TBDMS- and 2′-*O*-Fpmp-protected phosphoramidites (**S.41** and **S.79**, respectively) are commercially available. So far, there is no report in the literature that constitutes a thorough and definitive comparison of these two protecting groups. However, it is worthwhile discussing how they meet the necessary criteria for protecting groups in oligoribonucleotide synthesis, and also whether improvements could be made by modifying them.

With regard to the introduction of these two protecting groups, the reagents required, namely *tert*-butylchlorodimethylsilane and 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine (**S.70b**; Fig. 2.2.28) are both readily available. However, as far as the introduction of these protecting groups is concerned, the Fpmp group has the edge over the TBDMS group inasmuch as it can be introduced regiospecifically (Fig. 2.2.20A) and cannot then migrate. As indicated above, great care has to be exercised in the preparation of TBDMS-protected building blocks (**S.41**) in order to avoid contamination with the isomeric 2′-phosphoramidites (**S.42**; Fig. 2.2.10), the presence of which will inevitably lead to (2′→5′)-internucleotide linkages in the final product.

In solid-phase synthesis involving phosphoramidite building blocks, it seems clear that coupling rates and efficiencies are generally lower when ribonucleoside rather than 2′-deoxyribonucleoside building blocks are used

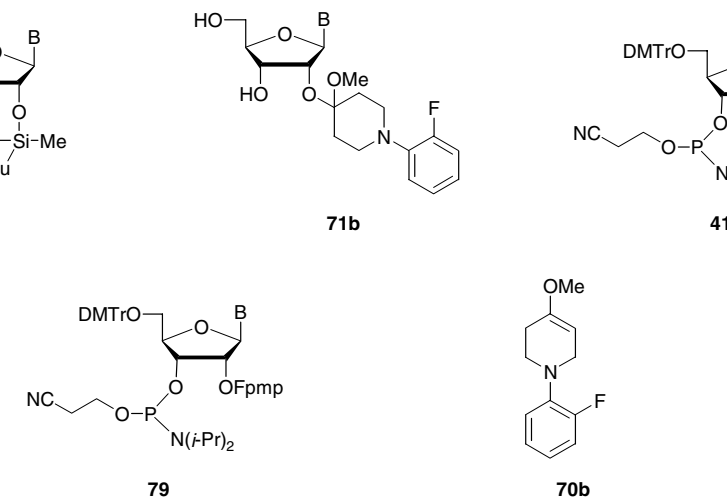


Figure 2.2.28 Structures relating to a discussion of the relative merits of the TBDMS and Fpmp protecting groups.

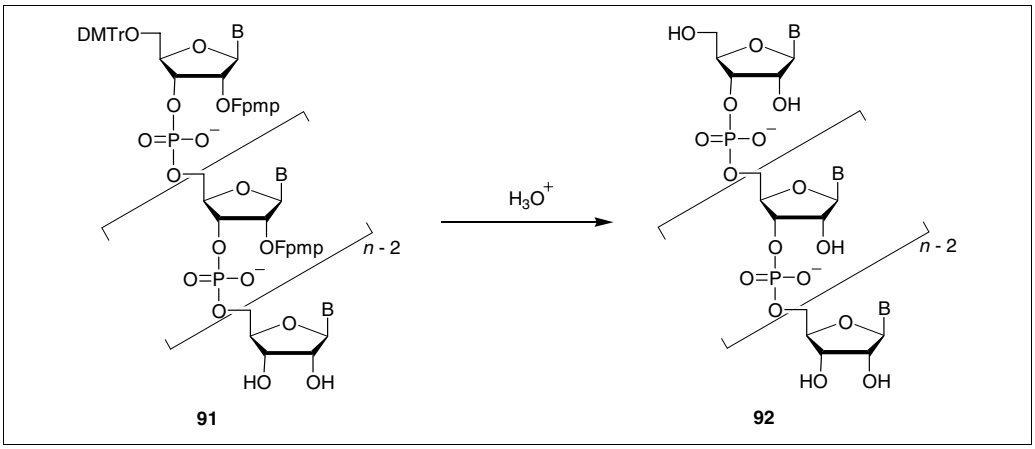


Figure 2.2.29 Unblocking of 2′-*O*-Fpmp-protected oligoribonucleotides under mild conditions of acidic hydrolysis.

(Hayakawa et al., 1996). It is not yet clear whether TBDMS-protected or Fpmp-protected phosphoramidites (**S.41** or **S.79**; Fig. 2.2.28) are the more hindered. Although TBDMS ethers (Kawahara et al., 1996) and Fpmp acetals are both susceptible to acid-catalyzed hydrolysis, the available evidence suggests that both groups remain intact under the anhydrous acidic conditions used during the detritylation steps. The Fpmp group has advantages over the TBDMS protecting group in the ammonolytic unblocking step at the end of the synthesis. First, in the Fpmp approach, adenine, cytosine, and guanine base residues are protected with relatively stable acyl groups (as in **S.16**, R = Me_3C, **S.18**, R = Ph, and **S.19**, R = $PhCH_2$, respectively; Fig. 2.2.4; Rao et al., 1993). However, it is advisable to use much more labile acyl protecting groups in the TBDMS approach (Chaix et al., 1989). More importantly, as the Fpmp protecting group is completely stable under the ammonolytic conditions, "Fpmp-on" RNA sequences (**S.91**; Fig. 2.2.29) are obtained (Rao et al., 1993). Such "Fpmp-on" oligoribonucleotides are stable to endonucleases and base, and may be conveniently purified and stored. On treatment with aqueous acid under very mild conditions (see below), they are readily converted into unprotected RNA sequences (**S.92**). "TBDMS-on" RNA sequences do not appear to have been purified and isolated in this way.

The one clear advantage that the TBDMS approach has over the Fpmp approach is that removal of the TBDMS protecting group in the final unblocking step does not normally involve acidic hydrolysis, and therefore cannot lead to migration of the internucleotide linkages. However, such migration in the Fpmp approach can be virtually eliminated by carefully controlling the unblocking conditions. Hecht and co-workers (Morgan et al., 1995) reported that when **S.91** (B = uracil-1-yl, n = 21; Fig.2.2.29) was unblocked in 0.5 mol dm^{-3} sodium acetate buffer, pH 3.25, at 25°C for 20 hr, analysis of the resulting r[$(Up)_{20}$U] (**S.92**; B = uracil-1-yl, n = 21) revealed that an average of 0.40% migration per internucleotide linkage had occurred. However, Reese et al. (unpub. observ.) have found that under somewhat milder unblocking conditions (0.5 mol dm^{-3} sodium acetate buffer, pH 4.0, at 35°C), unblocking of **S.91** (B = uracil-1-yl, n = 20) was complete after 9 hr and no migration of internucleotide linkages could be detected in the resulting r[$(Up)_{19}$U] (**S.92**; B = uracil-1-yl, n = 20). As has been suggested before (Capaldi and Reese, 1994), it cannot be concluded from the results obtained by Strömberg and co-workers (Rozners et al., 1994) in connection with the use of 2′-*O*-Ctmp-5′-*O*-DMTr-uridine 3′-*H*-phosphonate (**S.77**; B = uracil-1-yl; Fig. 2.2.22) and the corresponding Fpmp-protected *H*-phosphonate building block in the synthesis of r[$(Up)_{11}$U] and r[$(Up)_{11}$A] that Ctmp and Fpmp are unsuitable protecting groups for the 2′-hydroxy functions in the *H*-phosphonate approach to the solid-phase RNA synthesis. A much more likely explanation for Strömberg's observations is that r[$(Up)_{11}$U] and r[$(Up)_{11}$A], like r[$(Up)_9$U] and r[$(Up)_{19}$U] (Capaldi and Reese 1994), are particularly labile at pH 2.0 and room temperature.

Although it is clear that the solid-phase synthesis of relatively high-molecular-weight RNA sequences using TBDMS, Fpmp, or other groups to protect the 2′-hydroxy functions is now a feasible proposition, it is likely that even better protecting groups will be identified in the

future. The next generation of 2′-protecting groups could include modifications of TBDMS, Fpmp, and some of the other groups described above, and it could also include completely different groups. Any alternative silyl protecting group (**S.93**; Fig. 2.2.30) would need to be bulky to be sufficiently stable, and so far there is no evidence that any such group is likely to have superior properties to those of the TBDMS group itself. However, modification of the Fpmp group could well lead to improvements. A choice is already available between the Fpmp group, which has the advantage of being more stable at low pH (Fig. 2.2.21) during the detritylation steps, and the Ctmp group, which has the advantage of being more labile at high pH during the final unblocking step. It might well be possible, by a careful choice of R^1 and R^2, to identify a 1-aryl-4-alkoxypiperidin-4-yl protecting group (**S.94**; Fig. 2.2.30) that is as stable as (or perhaps even more stable than) the Fpmp group at low pH and as labile as (or perhaps even more labile than) the Ctmp group at high pH.

Most of the above discussion has been concerned with the small-scale synthesis of RNA sequences on a solid support. In the light of recent developments in the possible use of oligonucleotide analogs in chemotherapy, a demand has arisen for the development of methods for large-scale synthesis. This may well involve a shift from solid-phase to solution-phase methodology. While this need not necessarily affect the strategy of 2′-protection, the cost of the requisite monomeric building blocks is likely to become a matter of crucial importance. Therefore, particular emphasis will need to be laid on the first general criterion for protecting groups–that they should be easy to introduce and that the reagents involved should be readily accessible. It is not envisaged that this will present a problem for the Fpmp and most other related 1-aryl-4-alkoxypiperidin-4-yl protecting groups (**S.94**). Apart from the practical problems associated with the preparation of very large quantities of 2′-*O*-TBDMS-5′-*O*-DMTr-protected 3′-phosphoramidites (**S.41**) that are free from their 2′-isomers (**S.42**), there is no obvious reason why the TBDMS group should not also be used to protect 2′-hydroxy functions in the large-scale synthesis of RNA sequences in solution.

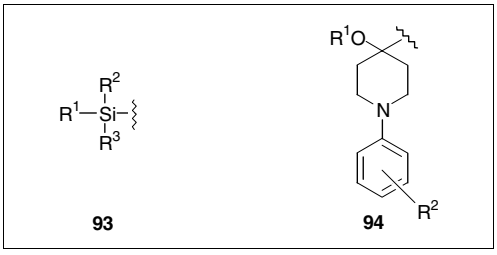


Figure 2.2.30 Substituted silyl and 1-aryl-4-alkoxypiperidin-4-yl protecting groups.

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The author would like to acknowledge the huge contributions that his co-workers have made over a period of more than 30 years to studies on the chemical synthesis of oligo- and poly-ribonucleotides, and especially to those studies relating to the problem of 2′-protection. Some of their names appear in the references below; they are all owed an enormous debt of gratitude.

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