

Nucleobase Protection of Deoxyribo- and Ribonucleosides

UNIT 2.1

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Oligonucleotides carrying a variety of chemical modifications including conjugates are finding increasing applications in therapeutics, diagnostics, functional genomics, proteomics, and as research tools in chemical and molecular biology. The successful synthesis of oligonucleotides primarily depends on the use of appropriately protected nucleoside building blocks including the exocyclic amino groups of the nucleobases, the hydroxyl groups at the 2', 3', and 5'-positions of the sugar moieties, and the internucleotide phospho-linkage. This unit is a thoroughly revised update of the previously published version and describes the recent development of various protecting groups that facilitate reliable oligonucleotide synthesis. In addition, various protecting groups for the imide/lactam function of thymine/uracil and guanine, respectively, are described to prevent irreversible nucleobase modifications that may occur in the presence of reagents used in oligonucleotide synthesis. © 2017 by John Wiley & Sons, Inc.

Keywords: carbamates • cyanoethylation • depurination • nucleobases • *N*-acylation • photolytic cleavage • silyl protection

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SALIENT FEATURES OF OLIGONUCLEOTIDE SYNTHESIS

DNA and RNA are biopolymers comprised of nucleotide units. The synthesis of DNA and RNA as segments consisting of several nucleotides each, referred to as oligonucleotides, is quite a challenging endeavor, requiring the rapid and quantitative coupling of nucleosidic units to form internucleotidic linkages. In order to achieve high efficiency in the coupling reactions, it is necessary to direct the reaction to the desired site in the nucleoside. This is achieved by using appropriate protecting group strategies.

To date, five methods of oligonucleotide synthesis have been reported, all of which use protected nucleosides: (1) The *H*-phosphonate approach (Froehler, Ng, & Matteucci, 1986; Hall, Todd, & Webb, 1957; Michelson & Todd, 1955, and references therein; Garegg et al., 1986, and references therein; UNIT 3.4); (2) the phosphodiester approach, pioneered by Khorana and co-workers (Gilham &

Khorana, 1958; Khorana, 1979); (3) the phosphotriester approach, which heralded a new era in the nucleic acid synthesis by enabling more rapid synthesis and purification (Letsinger & Ogilvie, 1969, and references therein; Reese, 1978); (4) the phosphite strategy (Letsinger & Lunsford, 1976, and references therein; Matteucci & Caruthers, 1980); and (5) the phosphoramidite approach, presently the most popular method for oligonucleotide synthesis (Beaucage & Caruthers, 1981; UNIT 3.3). Over the years, improvements in coupling chemistries, along with advances in methods of solid-phase assembly, have revolutionized the art of oligonucleotide synthesis (Beaucage & Iyer, 1992, 1993a).

In the synthesis of oligonucleotides, persistent as well as transient protection of amino, imido, and hydroxy groups of nucleosides are employed. Transient protecting groups are used to temporarily block one of the hydroxy groups. For example, the 4,4'-dimethoxy trityl

Protection of Nucleosides for Oligonucleotide Synthesis

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group (DMTr) is used for transient protection of the 5' hydroxyl of a nucleoside (UNIT 2.3). The transient protecting group is removed at the beginning of each coupling cycle. This enables the coupling reaction to be directed to the desired hydroxy group during the synthesis cycle. On the other hand, persistent protecting groups are those that remain on the nucleoside throughout chain assembly and are only removed at the end of synthesis. For example, the exocyclic amino groups of nucleobases, the 2'-hydroxy groups of sugars (UNIT 2.2), and the internucleotidic phosphodiester functions, are protected using persistent protecting groups.

The present commentary addresses issues related to the persistent protection of nucleobases. The commentary is not a comprehensive review of published literature but is intended to highlight the salient features of nucleobase protection. In addition to this unit, readers should refer to reviews (Beaucage & Iyer, 1990, 1992, 1993a; Iyer & Beaucage, 1999) and primary literature for more comprehensive citations.

GENERAL ASPECTS OF NUCLEOBASE PROTECTION

The choice of nucleobase protecting groups and deprotection protocols is of paramount importance in the oligonucleotide synthesis and is dictated by many considerations. The protecting groups for nucleosides should be designed on the basis of several criteria, which have been summarized by Reese (1978). (1) It should be possible to introduce the protecting group using a stable reagent that is readily obtainable. (2) The group should be achiral. (3) The nuclear magnetic resonance (NMR) spectrum of the protected nucleoside should be simple to interpret. (4) The protecting group should be readily installed on the nucleoside. (5) The group should enhance the solubility of the nucleoside in organic solvents so that it can be adapted for coupling reactions. (6) The group should be stable to reagents employed in oligonucleotide synthesis. (7) The group should not cause other structural changes in the nucleoside during its installation or removal, or during oligonucleotide assembly.

A number of nucleobase protecting groups have been reported. Improved procedures for their installation and removal have also been developed (Somoza, 2008). Nevertheless, unforeseen problems have been reported when certain protecting groups are employed in oligonucleotide synthesis. For example, *N*-acylated purine nucleosides, particularly *N*-

acylated deoxyadenosine, are more prone to depurination than their unprotected counterparts, making the growing oligonucleotide chain susceptible to chain cleavage during the synthesis cycle. In fact, each protected purine and pyrimidine nucleobase exhibits different patterns of reactivity compared to the unprotected nucleoside. Certain protecting groups are also sensitive to reagents used in oligonucleotide synthesis, resulting either in their premature removal or in the production of base-modified products (reviewed by Beaucage & Iyer, 1992).

The following sections highlight the structural basis for the nucleophilicity and reactivity of nucleosides. An overview of the strategies employed for nucleobase protection is also provided.

NUCLEOSIDE TAUTOMERISM AND pK_a VALUES

The nucleophilicity of nucleobases (Fig. 2.1.1) is dictated by the pK_a of the amino and amido functions and their tautomeric forms. Table 2.1.1 lists the pK_a values of nucleobases. The amide-like nitrogens (N^3 of uridine and N^1 of guanosine) are acidic in character, whereas the ring nitrogens are basic. Therefore, at strongly alkaline pH, the proton at N^3 of uridine and thymidine and that at N^1 of guanosine are removed. Under acidic conditions (at pH ~ 3), the sites of protonation are N^1 of adenosine and N^3 of cytidine. At more acidic pH, the N^7 of guanosine and adenosine and the O^4 of uridine are protonated. Thus, all the bases remain mostly uncharged in the physiological range of pH 5 to 9 (Saenger, 1984).

It is noteworthy that each of the nucleosides A, C, and G becomes protonated at one of the ring nitrogens rather than on the exocyclic amino group. Thus, the electron pair of the amino group can delocalize into the heteroaromatic ring. Indeed the C–NH₂ bonds of A, C, and G are ~ 1.34 Å long and have 40% to 50% double-bond character (Saenger, 1984). The charge on a nucleobase, its tautomeric structures, and its ability to form and accept hydrogen bonds are also determined by its pK_a values as well as by the pH of the medium. At physiological pH, the major nucleobases exist almost exclusively in the amino and keto tautomeric forms. However, under appropriate pH conditions, the tautomeric equilibrium could shift to where both imino and enol forms could exist (Saenger, 1984). In turn, these factors significantly affect the reactivity of the nucleosides.

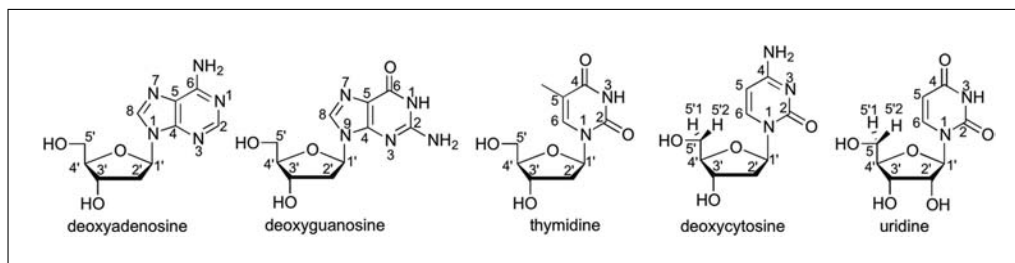


Figure 2.1.1 Structures and numbering of nucleosides.

Table 2.1.1 pK_a Values of Nucleoside Nucleobases

Nucleobase (site of protonation/deprotonation) ^a	pK_a
Thymidine (N^3)	9.93
Guanosine (N^1)	9.42
Uridine (N^3)	9.38
Cytidine (N^3)	4.17
Adenosine (N^1)	3.52

^aFor numbering of nucleoside positions, see Figure 2.1.1. For additional pK_a values, see Dunn & Hall (1975) and Clauwaert & Stockx (1986).

REACTIVITY OF NUCLEOSIDES

Nucleosides participate in electrophilic and nucleophilic substitution reactions as well as addition reactions (Shabarova & Bogdanov, 1994). Quite clearly, protecting groups and the protocols for their installation and removal should be designed to avoid various side reactions.

Nucleobases undergo substitution reactions with electrophilic reagents. For example, both *N*- and *O*-alkylation of the imide and lactam groups occur with alkylating agents. The N^7 position of purines is also a potential site for electrophilic attack. Because of these competing reactions, simple alkylation of exocyclic amino function is not a viable protection strategy for nucleobases. On the other hand, it is possible to chemoselectively acylate the exocyclic amino group. Thus, acyl-type protecting groups are widely used for the protection of the exocyclic amino groups of nucleosides.

The imide/lactam NH of thymidine, uridine (pK_a , 9.38), and guanosine (pK_a , 9.42) is weakly acidic and can deprotonate under basic conditions. The resulting nucleophilic anion can react with a variety of reagents such as activated phosphates, dicyclohexylcarbodiimide (DCC), mesitylene sulfonyl chloride, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), acid chlorides, phosphitylating reagents, and electrophilic

reagents that are employed during coupling reactions. These side reactions result in nucleobase-derived *N*- and *O*-products.

Nucleosides also react with a variety of nucleophilic reagents. For example, cytosine reacts with hydroxylamine at neutral and acidic pH to give the corresponding hydroxylamine derivative. It reacts with hydrazine at neutral pH to give the corresponding hydrazide. Indeed, nucleophilic substitution reaction of the exocyclic amino groups of cytosine proceeds rapidly in the presence of alkali and amines.

Under oxidative conditions, certain nucleobases (for example, adenine and cytosine) can form *N*-oxides. In addition, the C8 position of guanosine is vulnerable to hydrolytic attack under either strongly acidic or strongly alkaline conditions. The 5,6 double bond of pyrimidine nucleosides also reacts with halogens and halohydrins to give the corresponding addition products (Shabarova & Bogdanov, 1994). Selected examples of the side reactions that occur during oligonucleotide synthesis are given below.

Tri-*O*-acetyluridine (**1**) reacts with MSNT to produce the triazolo derivative **2** (Fig. 2.1.2; Reese & Ubasawa, 1980). During deprotection with ammonium hydroxide, **2** gives cytidine (**3**). Interestingly, during deprotection with either the tetramethylguanidinium salt of *syn*-4-nitrobenzaldoxime or tetrabutylammonium fluoride (TBAF), **2** reverts to uridine (den Hartog, Willie, Scheublin, & van Boom, 1982).

The triazolo derivative **2** is also known to react with other nucleophiles. For example, in the presence of methanol and 1,8-diazabicyclo[5.4.0]undecene-7 (DBU), **2** gives the corresponding 4-OMe derivative (Li, Reese, & Swann, 1987).

1 reacts with phosphorylating reagents at the uridine O^4 and undergoes 4-triazolization in the presence of the phosphorylating reagent, 2- or 4-chlorophenyl phosphorodiol-1,2,4-triazolide. The triazolo derivative is converted to the fluorescent pyridinium salt **4** in the presence of pyridine (Divakar & Reese, 1982; Huynh-Dinh, Langlois d'Estaintot,

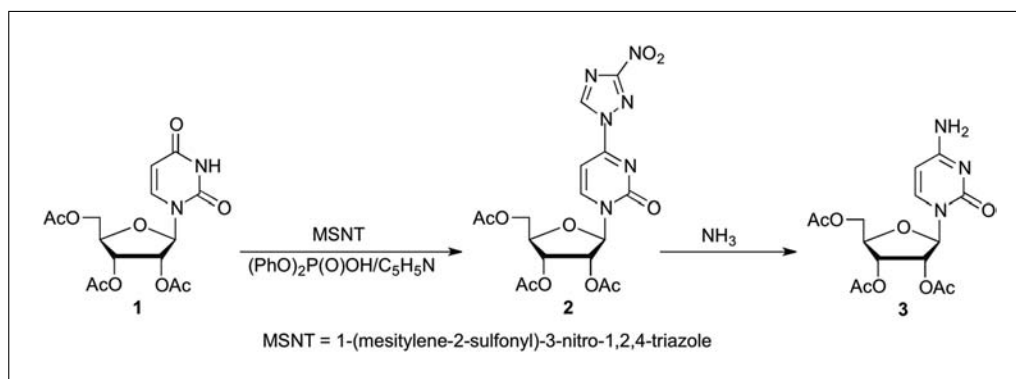


Figure 2.1.2 Conversion of tri-*O*-acetyluridine to cytidine via a nitrotriazolide intermediate. Reprinted from Iyer & Beaucage (1999) with permission from Elsevier Science Publishing.

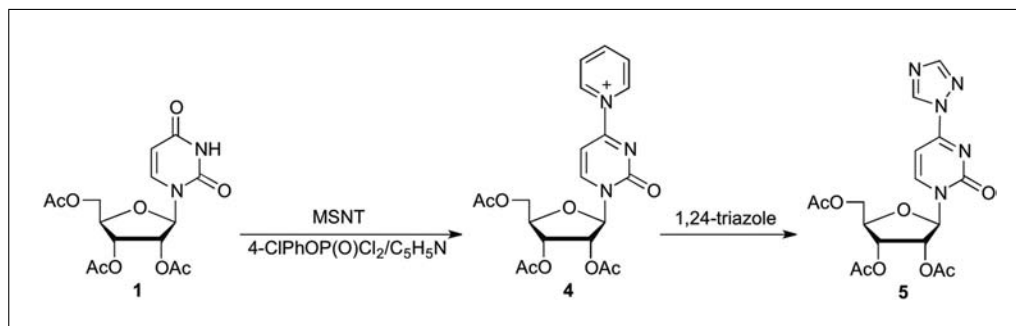


Figure 2.1.3 Formation of the triazolo derivative **5** via the pyridinium intermediate **4**. Reprinted from Iyer & Beaucage (1999) with permission from Elsevier Science Publishing.

Allard, & Igolen, 1985; Sung, 1982). Further reaction with triazole gives **5** (Fig. 2.1.3).

The purine nucleosides also react with electrophilic reagents. For example, guanine nucleosides react with MSNT to form the corresponding triazolo derivatives. Reaction of the *N*²-protected guanosine nucleoside **6** with mesitylene sulfonyl chloride gives the crystalline *O*⁶-sulfonated derivative **7** (Fig. 2.1.4; Bridson, Markiewicz, & Reese, 1977; Francois, Hamoir, Sonveaux, Vermeersch, & Ma, 1985). In the presence of pyridine, **7** is converted to the corresponding C6 pyridinium compound.

The side reactions that are predominant in phosphodiester and phosphotriester chemistry have been summarized (Reese and Ubasawa, 1980). These side reactions are of concern when the *H*-phosphonate method is employed in oligonucleotide synthesis in conjunction with phosphorochloridates as coupling reagents.

The imide and lactam functions of nucleosides also react with phosphitylating reagents. For example, the 2'-deoxyguanosine derivative **8** (Fig. 2.1.5) reacts with methyl phosphoramidites to give the *O*⁶-phosphitylated product **9** (Pon, Damha, & Ogilvie, 1985a; Nielsen, Dahl, Remaud, & Chattopadhyaya,

1987). During oxidation of **9** with iodine, *O*- to *N*-phosphoryl migration occurs (**9** → **10**; Pon, Damha, & Ogilvie, 1985b). Once formed, **11** induces depurination resulting in chain-cleaved products upon treatment with ammonium hydroxide during the final step of deprotection.

It is pertinent to mention that base modifications can also occur during the deprotection step following the assembly of the oligonucleotide. For example, thymine residues are significantly modified as *N*³-methylthymine during the deprotection of oligonucleoside methyl phosphotriesters with aqueous ammonium hydroxide (Urdea, Ku, Horn, Gee, & Warner, 1986). However, when deprotection of the methyl phosphate backbone of oligonucleotides is accomplished by treatment with thiophenol or 2-mercaptobenzothiazole, followed by aqueous ammonium hydroxide to remove nucleobase protecting groups, the formation of *N*³-methylthymine is considerably reduced (Andrus & Beaucage, 1988; McBride, Eadie, Efcavitch, & Andrus, 1987).

The reactivity of nucleosides and the attendant risk of base modification suggest an apparent need to protect imide and lactam groups of nucleosides in oligonucleotide synthesis. It is expected that the use of protecting groups at

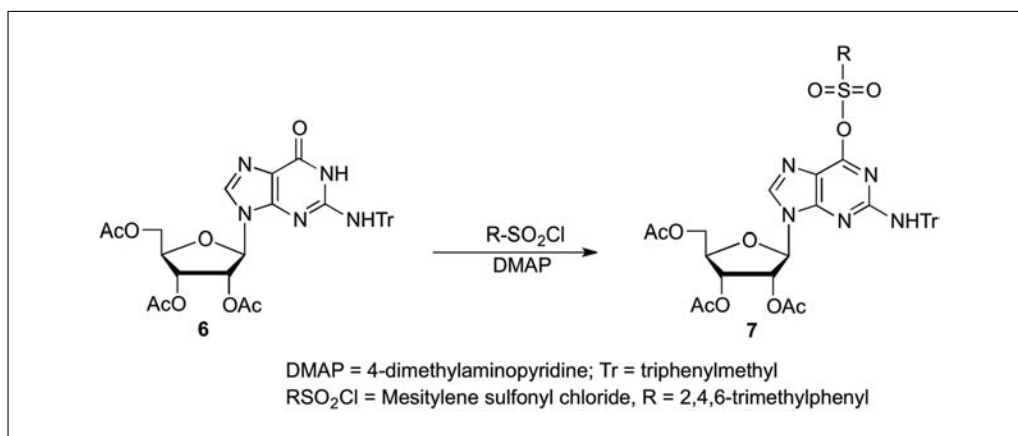


Figure 2.1.4 Formation of an *O*⁶-sulfonated derivative of guanosine. Reprinted from Iyer & Beaucage (1999) with permission from Elsevier Science Publishing.

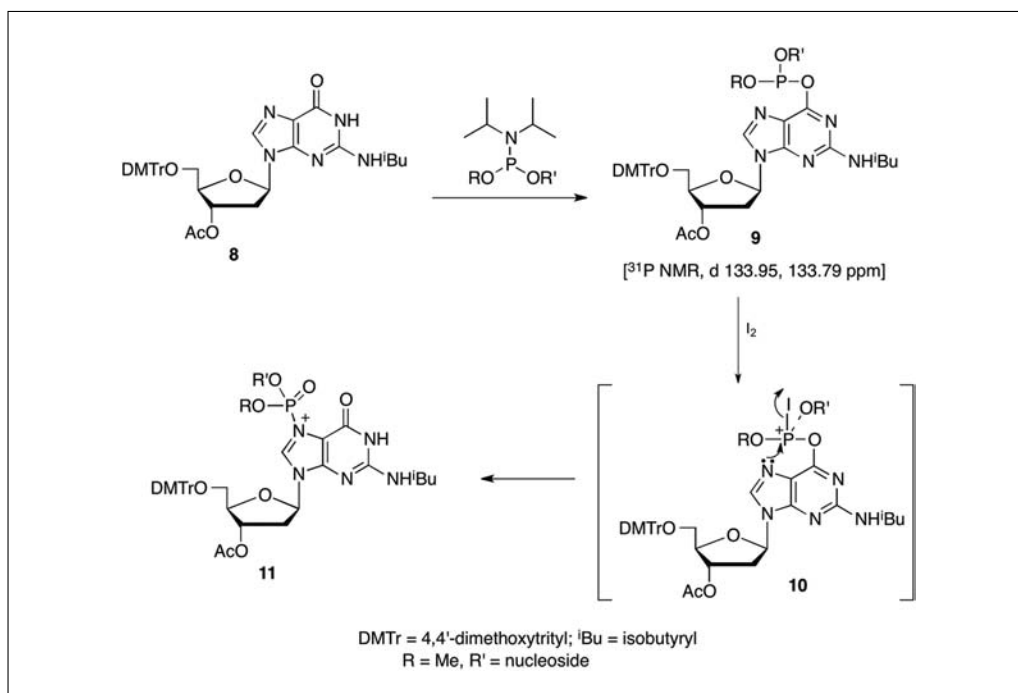


Figure 2.1.5 Reaction of an *N*²-isobutryl-2'-deoxyguanosine derivative with phosphitylating reagents.

the imide/lactam function of nucleosides can potentially avert the side reactions. This has been borne out by success in the synthesis of a number of polynucleotides (Reese, 1978).

PROTECTION OF IMIDE AND LACTAM FUNCTIONS

A number of groups have been proposed for the protection of thymine and uracil at *N*³ and *O*⁴ and of guanine at *O*⁶. A representative list of these groups is shown in Figure 2.1.6. For the benefit of the readers, references that include experimental details are cited.

The imide- and lactam-protecting groups can be installed in two steps: sulfonylation or triazolation, followed by displacement of

the resulting *O*-sulfonate or triazolidine by the nucleophilic protecting group. The protecting groups can also be installed directly—for example, via Mitsunobu alkylation (UNIT 1.2) or via the transient protection approach. The preparation of these protected nucleosides appears to be straightforward (Gaffney & Jones, 1982; Jones, Reese, Sibanda, & Ubasawa, 1981; Kamaike, Hasegawa, & Ishido, 1988; Nyilas, Zhou, Welch, & Chattopadhyaya, 1987; Trichtinger, Charubala, & Pfeleiderer, 1983).

However, it is still a matter of debate whether imide and lactam protection is necessary in oligonucleotide synthesis. There are a number of factors to be considered. (1)

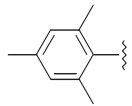
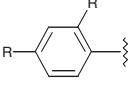
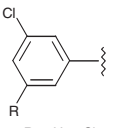
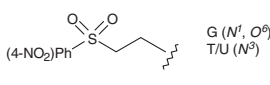
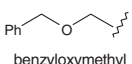
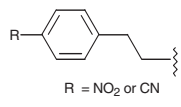
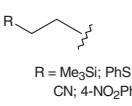
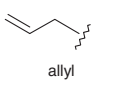
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 2,4,6-trimethylphenyl	U (O^4)	<i>syn</i> -4-Nitrobenzaldoxime/ tetramethylguanidine	Nyilas et al., 1988 MacMillan & Verdine, 1991
 2-nitrophenyl	G (O^6) U (O^4)	<i>syn</i> -4-Nitrobenzaldoxime/ tetramethylguanidine	Jones et al., 1981 Zhou & Chattopadhyaya, 1986 Zhou et al., 1986 Reese & Skone, 1984
 R = H or Me	T (O^4) U (O^4)	<i>syn</i> -4-Nitrobenzaldoxime/ tetramethylguanidine	Jones et al., 1981 Reese & Skone, 1984 Scalfi-Happ et al., 1987 Rao et al., 1987
 R = H or Cl	G (O^6)	<i>syn</i> -4-Nitrobenzaldoxime/ tetramethylguanidine	Reese & Skone, 1984 Rao et al., 1987 Hagen & Chládek, 1989
 2-(4-nitrophenylsulfonyl)ethyl	G (N^1 , O^6) T/U (N^3)	NH ₄ OH, heat	Engels & Mag, 1982
 benzyloxymethyl	T/U (N^3)	10% Pd/H ₂	Krecmerová et al., 1990
 R = NO ₂ or CN	G (O^6) T (O^4)	Pyridine-2-aldoxime/ tetramethylguanidine 0.5 M DBU/pyr	Pfister et al., 1988 Van Aerschot et al., 1988
 R = Me ₃ Si; PhS; CN; 4-NO ₂ PhS	G (O^6)	R=Me ₃ Si: <i>n</i> -Bu ₄ NF/THF R=PhS: 4-NO ₂ PhS: NaIO ₄ / NH ₄ OH, heat; R=CN; DBU, or NH ₄ OH	Hagen & Chládek, 1989 Gaffney & Jones, 1982 Engels & Mag, 1982
 allyl	G (O^6) T (O^4)	Pd(PPh ₃) ₄ /PPh ₃ , <i>n</i> -BuNH ₃ ⁺ HCO ₂ ⁻	Hayakawa et al., 1993

Figure 2.1.6 (*image continues on next page*) Protecting groups for the imide/lactam functions of guanine, uracil, and thymine.

As noted before, when using the phosphotriester method in oligonucleotide synthesis, O^6 -modified guanine and O^4 -modified uracil revert to guanine and uracil residues upon “oximate” treatment that follows the chain assembly. (2) When the phosphoramidite method is used, O^6 -phosphitylated deoxyguanosine reverts to deoxyguanosine on contact with water or acetate ions (Mag & Engels, 1988). Thus, during the synthesis cycle, if capping is performed (using acetic anhydride) after coupling, any O^6 -modified deoxyguanosine can potentially revert back to deoxyguanosine. (3) Base mod-

ifications generated during oligonucleotide chain assembly depend on the reagents employed and the contact time. Solid-phase oligonucleotide synthesis uses an automated pulsed delivery of reagents, resulting in shorter reaction times compared with solution-phase synthesis. Consequently, side reactions during oligonucleotide chain assembly appear to be minimal.

In recent reports, the N^3 -position of the pyrimidine ring was temporarily blocked with a 2-(phenylthio)ethyl group via a two-step strategy that enabled the synthesis of sugar-modified thymidine derivatives (D’Onofrio,

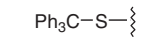
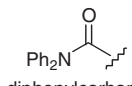
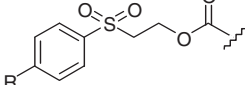
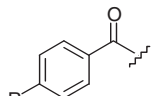
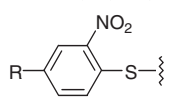
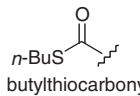
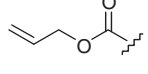
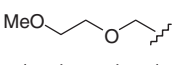
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 triphenylmethyl sulfenyl	U (<i>N</i> ³)	0.1 M I ₂ /THF/collidine/H ₂ O	Takaku et al., 1988
 diphenylcarbamoyl	G (<i>O</i> ⁶)	NH ₄ OH	Kamimura et al., 1983b
 R = H; Me	U (<i>N</i> ³)	DBU/morpholine/pyr	Nyilas et al., 1988
 R = H; Me; OMe; Cl	U (<i>N</i> ³ , <i>O</i> ⁴)	NH ₄ OH	Sekine, 1989 Kamimura et al., 1983a Kamaike et al., 1988
 R = H; Me; NO ₂	U (<i>N</i> ³)	Pyrr/H ₂ O or 0.2 M <i>n</i> -Bu ₄ NF/ THF	Sekine, 1989 Welch et al., 1985
 butylthiocarbonyl	G (<i>N</i> ² , <i>O</i> ⁶); U (<i>N</i> ³)	NH ₄ OH/MeOH	Fujii et al., 1987
 allyloxycarbonyl	U (<i>N</i> ³ , <i>O</i> ⁴)	Pd[PPh ₃] ₄ /PPh ₃ , <i>n</i> -BuNH ₂ /THF	Hayakawa et al., 1986
 (methoxyethoxy)methyl	U (<i>N</i> ³)	NH ₄ OH, heat, or Ph ₃ C ⁺ BF ₄ ⁻ /MeCN/H ₂ O	Ito et al., 1986

Figure 2.1.6 (image continued from previous page)

Napoli, Fabio, & Montesarchio, 2006). The 2-(phenylthio)ethyl residue was regiospecifically inserted at the *N*³-position of the pyrimidine by Mitsunobu reaction using 2-(phenylthio)ethanol and was found to be stable under strongly basic conditions, thereby allowing selective *O*-alkylation of the ribose moieties. In addition, the protection of the nucleobase also avoided undesired *N*- or *O*-alkylations. After oxidation of the thioether to sulfone, the thymine protecting group was removed (via a β -elimination) under the same basic conditions used in the final deprotection and cleavage of oligonucleotides from the support during solid-phase oligonucleotide synthesis.

Thus, in principle, appropriate adjustment of synthesis protocols may obviate the necessity for imide and lactam protection. Nevertheless, β -cyanoethyl protection is generally recommended for protection of guanosine at *O*⁶ or uridine at *O*⁴, and anisoyl for uridine at *N*³.

PROTECTION OF EXOCYCLIC AMINO GROUPS

N-Acyl Protection

On the basis of a number of criteria for protecting groups, as outlined previously, *N*-acyl-type protection for the exocyclic amino groups has emerged as a logical choice and continues to be employed even after many decades of advances in oligonucleotide synthesis. Figure 2.1.7 shows selected examples of *N*-acyl protecting groups and the reagents used to affect their deprotection.

The *N*-acyl groups are introduced into nucleosides by a number of procedures. (1) They can be installed by peracylation of the nucleoside followed by chemoselective *O*-deacylation (Büchi, & Khorana, 1972; Rigoli, Østergaard, Canady, Guenther, & Hrdlicka, 2009; Schaller, Weimann, Lerch, & Khorana, 1963). (2) The “transient” protection approach (Ti, Gaffney, & Jones, 1982) has

Protection of Nucleosides for Oligonucleotide Synthesis

2.1.7

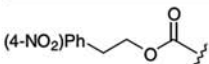
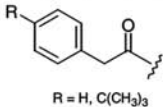
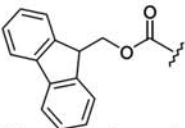
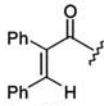
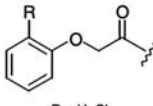
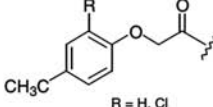
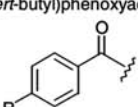
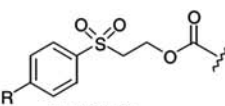
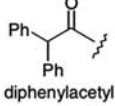
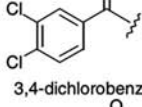
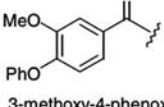
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 4-nitrophenylethoxycarbonyl	G (N ³); A (N ⁶); C (N ⁴)	DBU or DBU/pyr	Himmelsbach et al., 1984 Pfleiderer et al., 1985 Pflister & Pfeiderer, 1989 Pfleiderer, 2007 Weiler & Hoheisel, 1996
 R = H, C(CH ₃) ₃	G (N ³)	5 M NH ₃ / MeOH	Jones et al., 1981 Rao et al., 1987 Köster et al., 1981 Balgobin et al., 1981
 9-fluorenylmethoxycarbonyl	G (N ³); A (N ⁶); C (N ⁴)	NH ₄ OH/pyr Et ₃ N/pyr	Scalfi-Happ et al., 1987 Hagen & Chládek, 1989 Heikkilä & Chattopadhyaya, 1983
 α-phenylcinnamoyl	G (N ³); A (N ⁶); C (N ⁴)	NH ₄ OH, heat	Nagaich & Misra, 1989
 R = H, Cl	G (N ³); A (N ⁶)	NH ₄ OH	Chaix et al., 1989 Schulhof et al., 1987 Singh & Misra, 1988
 R = H, Cl 4-(tert-butyl)phenoxyacetyl	G (N ³); A (N ⁶); C (N ⁴)	0.2 M NaOH/MeOH or NH ₄ OH or NH ₃ gas	Köster et al., 1981 Sinha et al., 1993 Boal et al., 1996
 R = H, OMe, Cl, NO ₂ , NMe ₂ , CMe ₃	G (N ³); A (N ⁶); C (N ⁴)	NH ₄ OH	Brown et al., 1989 Rao et al., 1987 Tanimura et al., 1988 Schaller et al., 1963 Balgobin et al., 1981
 R = H; Cl; NO ₂	G (N ³); A (N ⁶); C (N ⁴)	Tetramethylguanidine/morpholine/pyr; DBU/morpholine/pyr; NH ₄ OH/morpholine/pyr.	Nyilas et al., 1988
 diphenylacetyl	G (N ³)	NH ₄ OH, heat	Marugg et al., 1984
 3,4-dichlorobenzoyl	G (N ³)	0.2 M NaOH/MeOH	Köster et al., 1981
 3-methoxy-4-phenoxybenzoyl	G (N ³); A (N ⁶); C (N ⁴)	NH ₄ OH, heat	Mishra & Misra, 1986

Figure 2.1.7 (image continues on next page) Protecting groups for the exocyclic amino function of nucleobases.

been widely used. In this procedure, the nucleoside is persilylated using a silylating agent, and the acyl function is installed on the amino group using the corresponding acid chloride or acid anhydride. (3)

Some acyl functions such as benzoyl, α-phenylcinnamoyl, phthaloyl, and naphthaloyl are directly incorporated on the nucleobase using the corresponding acid chloride or anhydride (Beier & Pfeiderer, 1997, 1999; Bhat

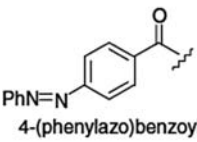
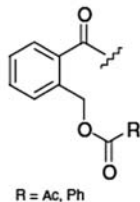
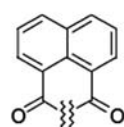
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 4-(phenylazo)benzoyl	C (<i>N</i> ⁶)	0.2 M NaOH/MeOH (1:1)	Köster et al., 1981
 R = Ac, Ph	G (<i>N</i> ⁶); A (<i>N</i> ⁶); C (<i>N</i> ⁶)	0.05 M K ₂ CO ₃ /MeOH; NH ₄ OH, RT	Kuijpers et al., 1990
 1,8-naphthaloyl	G (<i>N</i> ⁶); A (<i>N</i> ⁶); C (<i>N</i> ⁶)	NH ₄ OH, heat	Dikshit et al., 1988
RT, room temperature; pyr, pyridine.			

Figure 2.1.7 (image continued from previous page)

et al., 1989; Karpeisky, Sweedler, Haeblerli, Read, Jarvis, & Beigelman, 2002; Watanabe & Fox, 1966). (4) *N*-Lavulinyll derivatives of cytidine, adenosine, and guanosine were also prepared (Ogilvie, Nemer, Hakimelahi, Proba, & Lucas, 1982). Zhu, Williams, & Scott (2003) reported an improved synthesis of *N*-benzoylated nucleosides by using chlorotrimethylsilane (TMSCl) and benzoyl chloride in near equimolar quantities; the major advantage of this method is the easier work-up and higher yields.

The original method by Ti et al. (1982) for installation of protecting groups has also been further simplified by eliminating the use of ammonium hydroxide during work-up. Several approaches have been reported: (1) The exocyclic amino groups of cytidine and deoxycytidine were directly acylated using activated esters (Igolen & Morin, 1980), acid chlorides (Mishra & Misra, 1986, and references therein), or alkyl oxycarbonyl benzotriazoles (Himmelsbach, Schulz, Trichtinger, Charubala, & Pfeiderer, 1984). (2) Site-selective incorporation and removal of *N*-acyl protecting groups have also been achieved by enzymatic methods (Prasad & Wengel, 1996). (3) Selective acylation of nucleosides has also been carried out using *N*-acyltetrazole (Bhat, 1998) and carboxylic acids activated in situ with carbonyldiimidazole (CDI; Sinha, Davis, Schultze, & Updhyay, 1995). (4) *N*-Benzoyltetrazole has been developed as a

mild and selective reagent for the monobenzylation of the exocyclic amino groups in adenine and cytosine nucleobases (Bhat, & Sanghvi, 1997). (5) In a related report, 1-acetyl-4-nitro-benzotriazole readily acetylated the exocyclic amino group of cytidine (Reid, McHugh, Richie, & Graham, 2006); this one step-strategy was found to be advantageous for the large-scale production of *N*-acetylated nucleosides. (6) *N*-isopropoxy acetylated adenosine, guanine, and cytosine nucleosides were successfully prepared using isopropoxy-acetic anhydride and found to be stable under solid-phase nucleotide synthesis conditions. The *N*-isopropoxy acetyl group was removed much faster than the traditional benzoyl or isobutyryl group and completed within the cleavage of oligonucleotides from solid support (Uznanski, Grajkowski, & Wilk, 1989).

Use of polymer supported reagents

The exocyclic amino function of a purine nucleoside has been selectively protected using activating agents to effect *N*-acylation or *N*-benzylation at the exocyclic amino site. In this context, the use of polymer-bound coupling supports was found to be valuable. Thus, *N*⁶-benzoyldeoxyadenosine was prepared from adenosine in 81% yield via *N*-benzylation using polymer-supported cyclohexylcarbodiimide (Sanghvi, Misra, & Tripathi, 2002).

In addition to installing the *N*-acyl protecting groups via their carboxylic acid chloride or anhydride forms, the corresponding acids have also been employed. Thus, the carboxylic acid was first activated using DCC to form an active ester, which was then used for the *N*-acylation of purine nucleosides. Typically, the separation of dicyclohexylurea from the *N*-acylated product is difficult, which could be circumvented by the use of polymer-supported-carbodiimide (Tripathi, Misra, & Sanghvi, 2005).

Use of activated esters

A novel and cost-effective *N*-acylation procedure for the exocyclic amino function of ribonucleosides and deoxyribonucleosides was achieved by the reaction of the corresponding carboxylic acids activated as their respective *p*-nitrophenyl esters with the exocyclic amino group in the presence of DCC as the coupling agent in conjunction with dimethylaminopyridine (DMAP), 1-hydroxybenzotriazole (HOBt), or *N*-hydroxysuccinimide as catalysts (Mishra, Mishra, Diwedi, & Misra, 2006).

An efficient one-pot *N*-acylation of deoxy- and ribo-cytidine using carboxylic acids activated in situ with 2-chloro-4,6-dimethoxy-1,3,5-triazine **12** has also been reported (Rode, Son, & Hong, 2010). Diverse carboxylic acids efficiently reacted with pyrimidine and/or purine nucleobases in the presence of cyanuric chloride (2,4,6-trichloro-1,3,5-triazine **13**) and NaH/Et₃N in DMF/MeCN (1:1) affording a facile and selective one-pot *N*-acylation of nucleobases in good yields (Rad, Behrouz, Asrari, & Khalafi-Nezhad, 2014).

Use of microwave techniques for rapid installation and removal of protecting groups

Microwave radiation has been occasionally used for nucleobase protection. For example, *N*-acetylcytidine was prepared in quantitative yields in 40 to 60 sec by microwave-assisted synthesis (Nahar, 1997). Similarly, *N*-protected deoxyribonucleosides have also been prepared by microwave-assisted synthesis (Rao, Kumar, Chauhan, Garg, & Gupta, 2002). The exocyclic amino groups of adenine and cytosine were protected by reacting with benzyl chloroformate under microwave irradiation to provide the corresponding benzyloxy carbonyl nucleosides (Azzena, Dettori, Pisano, & Pittalis, 2007). An overview of various *N*-acylation and *N*-benzoylation reactions is shown in Figure 2.1.8.

Overall, most *N*-acyl protecting groups are stable in neutral or acidic medium and moderately stable at high pH (pH >13). However, many *N*-acyl protecting groups are readily removed by ammonolysis. This observation forms the basis for the widespread use of 28% NH₄OH as a deprotection reagent when *N*-acyl-protected nucleosides are employed in oligonucleotide synthesis.

A salient feature of *N*-acyl protecting groups is that their stability in alkaline pH can be modulated by the steric and electronic characteristics of specific acyl groups. A study comparing the stability of various *N*-acyl nucleosides in alkaline medium (0.2 N NaOH/MeOH) has been reported (Köster, Kulikowski, Liese, Heikens, & Kohli, 1981). Importantly, the stability of the acyl function towards alkaline hydrolysis is determined by the nature of the heterocyclic base. For example, the rate of deacylation of *N*-acyl derivatives of deoxycytidine is faster than that of deoxyadenosine or deoxyguanosine. The hydrolytic lability is also determined by inductive, resonance, and steric effects. For example, in a series of *N*-acyl nucleosides, *N*-benzoyl nucleosides are hydrolyzed sixteen times faster than *N*-(2,4-dimethyl)-benzoyl nucleosides, presumably because of steric effects. Similarly, *N*-(2,4-dimethoxy)-benzoyl nucleoside is hydrolyzed eight times faster than *N*-(4-dimethyl-amino)benzoyl nucleoside, perhaps due to a combination of inductive and resonance effects.

The choice of a particular *N*-acyl protecting group also depends on the type of coupling chemistry that is employed. For example, when phosphodiester and phosphotriester chemistries are used in oligonucleotide synthesis, it is necessary to select robust *N*-acyl protecting groups that can withstand the harsh reagents and conditions employed during synthesis. This requirement is met by the benzoyl group for adenine and cytosine, and the isobutyryl group for guanine. However, removal of these protecting groups requires prolonged heating (12 to 14 hr) with 28% NH₄OH at 55°C. In spite of this limitation, these protecting groups have remained popular even with the advent of automated solid-phase oligonucleotide synthesis using phosphoramidite and *H*-phosphonate chemistries.

Phenylacetyl Protection

The phenylacetyl group was introduced for the first time as a suitable protecting group for the *N*² amino group of deoxyguanosine for the synthesis of

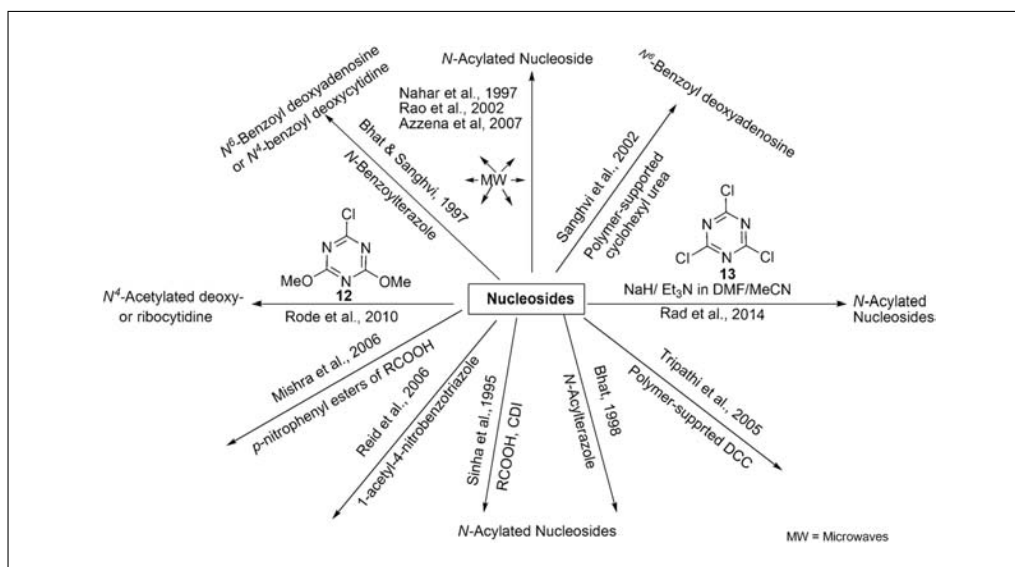


Figure 2.1.8 An overview of preparation of *N*-acylated nucleosides.

oligodeoxyribonucleotides (Kohli & Kumar, 1979). Later it was also used for the protection of the exocyclic amino group of adenine, guanine, and cytosine. The installation of the protecting group is achieved by treatment of the silylated nucleoside with phenylacetyl chloride (Dineva, Galunsky, Kasche, & Petkov, 1993; Waldmann, Heuser, & Reidel, 1994; Waldmann & Reidel, 1997; Waldmann et al., 1997; Fig. 2.1.7). Deprotection was carried out using concentrated ammonium hydroxide. Interestingly, the phenylacetyl group could be cleaved enzymatically from oligonucleotides in solution or on a solid support. An improved procedure for the protection of 2'-deoxyguanosine has been reported by pretreating phenylacetyl chloride with 1-hydroxybenzotriazole, and the resulting adduct was added to the tris[trimethylsilyl]ether derivative of 2'-deoxyguanosine (Benseler & McLaughlin, 1986). The phenylacetyl protecting group has been used for the *N*² position of *O*⁶-alkylguanine containing oligodeoxynucleosides (Smith, Xu, & Swann, 1990).

Fluoride-Labile Protection

Apart from the use of *N*-acyl-type protecting groups for nucleobases, fluoride-labile protecting groups represent another class of nucleobase protection. Various fluoride-labile protecting groups have been developed and successfully used for the synthesis of base-sensitive oligonucleotides and acylated RNA.

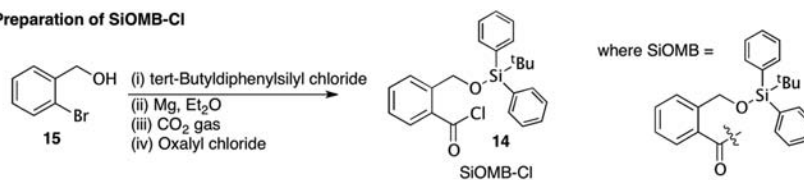
The exocyclic fluoride-labile amino protecting group 2-*tert*-butyldiphenylsiloxy-methyl benzoyl (SiOMB) was developed by Dreef-Tromp et al. (1990) by the use of

SiOMB-Cl **14**, which was prepared from **15** (Fig. 2.1.9). Nucleosides **16a** and **b**, and **c** were silylated and further reacted with **14** to afford SiOMB-protected nucleosides **17a**, **b**, and **c**, respectively, as shown in Figure 2.1.9. An alternative preparation of **14** has also been reported (Guerlavais, Meyer, & Morvan, 2002). Nucleosides **17a-c** were successfully utilized for the preparation of a short TCCT Me-S-acetyl thioethyl (SATE) prooligonucleotide following the removal of the SiOMB protecting group by mild conditions using TMSCl and water (Guerlavais, Meyer, Imbach, & Morvan, 2001).

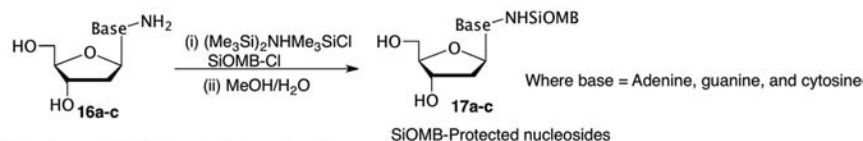
The preparation of the base-sensitive methyl SATE oligonucleotide prodrugs (prooligonucleotides) by combination of the SiOMB group and (trimethylsilyl)ethyl (TSE) groups was achieved using phosphoramidite chemistry on a photolabile solid support. Deprotection of the fluoride-labile nucleobase was achieved under milder conditions using Et₃N/HF (Guerlavais-Dagland, Meyer, Imbach, & Morvan, 2003).

Stutz, Höbartner, & Pitsch (2000) introduced novel 2'-*O*-triisopropylsiloxy-methyl (Tom) RNA phosphoramidites with fluoride-labile *N*-{[2-[(triisopropylsilyl)oxy]benzyl]oxy}carbonyl (tbc) **18a-c** and *N*-{[4-[(triisopropylsilyl)oxy]benzyl]oxy}carbonyl base protecting groups **19a-c** (Fig. 2.1.10). A solid support containing an immobilized *N*⁶-*t*-boc-protected adenosine with an orthogonal photolabile 2-*O*-[(*S*)-1-(2-nitrophenyl)ethoxy]methyl ((*S*)-npeom) protecting group was used for the synthesis of 3'/(2')-*O*-aminoacylated RNA sequences. Representative synthesis of tbc-protected

(i) Preparation of SiOMB-Cl



(ii) Protection of nucleobases with SiOMB group



(iii) Structures of SiOMB protected nucleosides

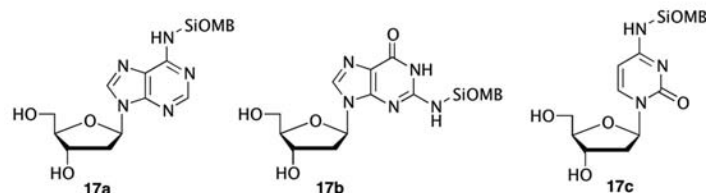
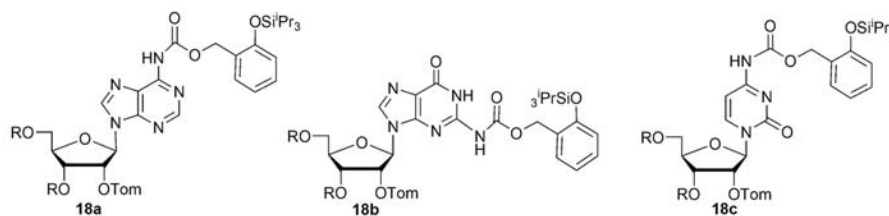


Figure 2.1.9 Nucleobase protection with 2-*tert*-butyldiphenylsiloxyethyl benzoyl (SiOMB) group.

*N*²-*t*boc protected nucleosides



*N*⁴-*t*boc protected nucleosides

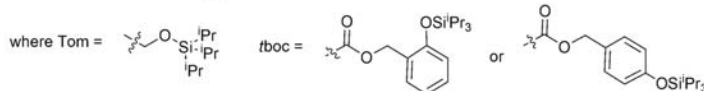
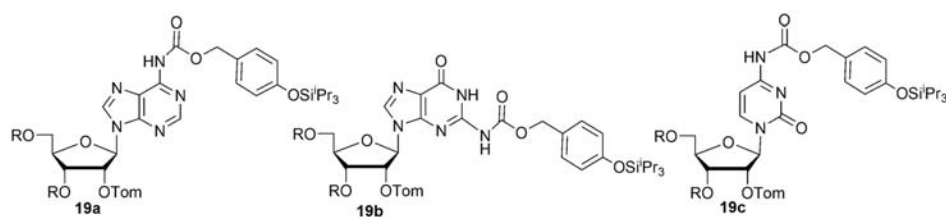


Figure 2.1.10 *t*boc-protected nucleosides.

adenine nucleoside **22** from nucleoside **20a** via a two-step reaction using reagent **21** is shown in Figure 2.1.11. Both *N*²-*t*boc and *N*⁴-*t*boc nucleobases were successfully deprotected in <2 min by buffered tetrabutyl ammonium fluoride. The mechanism of deprotection of *t*boc is shown in Figure 2.1.12.

Lavergne et al. (2011) used fluoride-labile *t*boc nucleobase protection at adenine and cytosine for the preparation of pro-RNA 2'-*O*

masked with biolabile pivaloylmethyl groups in an RNA interference assay. However, since the protection of *N*²-amino of guanosine could not be achieved using *t*boc, the DMTr group was used. DMTr protection of guanosine is rarely used.

Nucleosides were also protected using an *N*-(trimethylsilylethoxycarbonyl) (Teoc) group. Figure 2.1.13 shows the synthesis of trimethylsilylethyl (Tse)-protected adenine

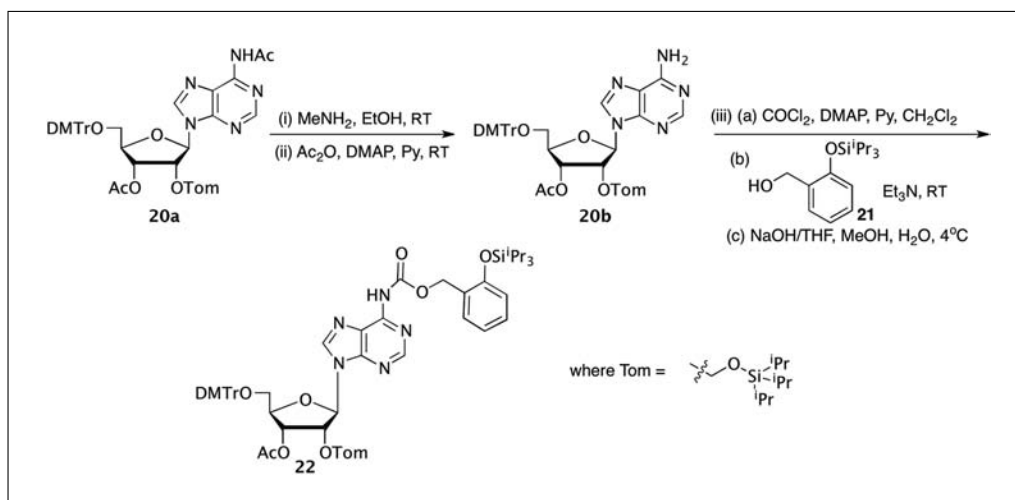


Figure 2.1.11 Synthesis of *t*boc-protected adenine nucleoside.

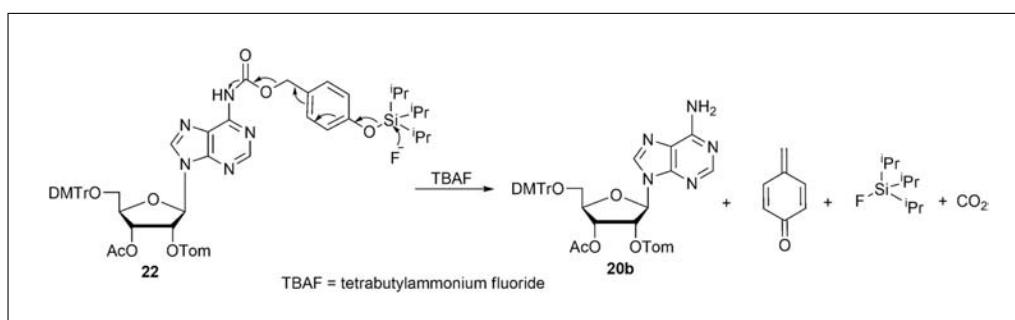


Figure 2.1.12 Mechanism of *t*boc deprotection.

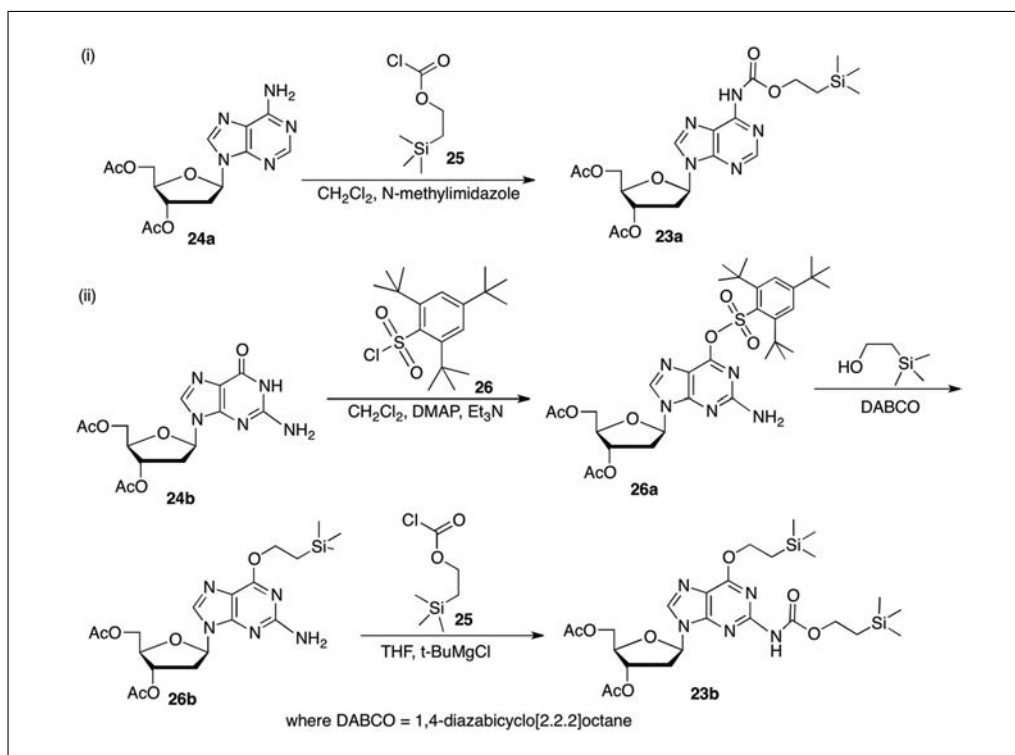


Figure 2.1.13 Nucleobase protection with Teoc and Tse groups.

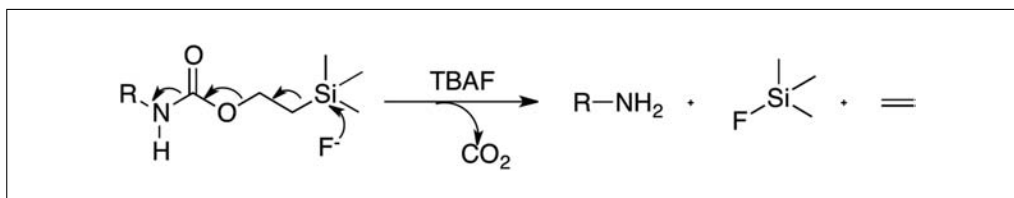


Figure 2.1.14 Mechanims of Teoc deprotection using TBAF.

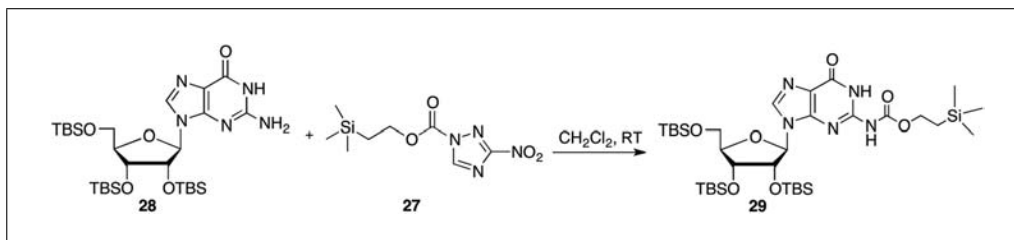


Figure 2.1.15 Teoc protection using 1-(2-trimethylsilyl)ethyl-3-nitro-1,2,4-triazole **27**.

and guanine nucleosides **23a** and **23b**, respectively, from acetyl protected adenine **24a** and intermediate guanine nucleosides **26b** prepared from **24b**. **24a** was reacted with trimethylsilylethyl chloroformate **25**, affording **23a**. In this case, the O^4 of guanine was also protected with the Tse group (Ferreira, Vasseur, & Morvan, 2004; Ferreira, & Morvan, 2005). Acetyl-protected guanine nucleoside **24b** was reacted with **26** to yield intermediate **26a**, which was further reacted with triethylsilylethanol and DABCO to form intermediate **26b**. In the final step, **26b** was reacted with **25** affording the desired Tse-protected guanine nucleoside **23b**.

The protected nucleosides were used in solid-phase oligonucleotide synthesis by using a disulfide linked with tris-2-carboxyethylphosphine and Tse phosphate protecting groups. The oligonucleotides were deprotected in a single step by treatment with ZnBr_2 in nitromethane/isopropanol under abasic conditions. Mechanism of Teoc deprotection using TBAF is shown in Figure 2.1.14. The fluoride-labile nucleobase protection strategy is detailed in a review by Virta (2009).

The silyl reagent, 1-(2-trimethylsilyl)ethyl-3-nitro-1,2,4-triazole **27** has also been used for the nucleobase protection of TBS-protected riboguanosine **28**, affording Teoc-protected riboguanosine **29** (Fig. 2.1.15; Shimizu & Sodeoka, 2007).

Caruthers and Roy (2015) recently reported the use of a sterically hindered di-*tert*-butylisobutylsilyl (BIBS) group (Liang, Hu, & Corey, 2011) for the protection the exocyclic nitrogen atoms of the nucleobases. The silyl-protecting group was found to be sufficiently sterically hindered and stable under standard oligonucleotide synthesis procedures.

An overview of the fluoride labile protecting groups and deprotection conditions is listed in Figure 2.1.16.

Cyano-ethyl and Cyano-ethoxy Carbonyl Protection

Manoharan et al. (1999) developed 2-(cyanoethoxycarbonyloxy)succinimide **30a**, a stable, crystalline, and convenient reagent for the protection of pendant alkylamines in oligonucleotides (Fig. 2.1.17). The 2-cyanoethyl (ce) group is the most common phosphate-protecting group in oligonucleotides; however, the ce and corresponding 2-(cyano-ethoxycarbonyl) (ceoc) groups were not utilized for nucleobase protection until 2000. Merk, Reiner, Kvasuk, & Pfeleiderer (2000) developed ceoc protection for the protection of the exocyclic amino group of adenine, guanine, and cytosine. The (2-cyanoethoxy)carbonylation reactions of nucleosides were carried out with either 2-cyanoethyl carbonochloride **30b** or 1-((2-cyano-ethoxy)carbonyl)-3-methyl-1 *H*-imidazolium chloride **30c** (Fig. 2.1.17), which were synthesized by the modified procedures of Merk et al. (2000) and Wiesler & Caruthers (1996), affording the ceoc-protected nucleosides **31a-c** (Fig. 2.1.18). In the case of guanine, the O^6 group was also protected with 4-nitrophenyl-ethyl (npe; Himmelsbach et al., 1984) or ce to prevent nucleobase anion formation and to avoid reduced deprotection kinetics of the N^2 -ceoc group. Deprotection was accomplished under non-protic conditions using the non-nucleophilic strong base DBU. The mechanism of deprotection follows a β -elimination pathway. Another advantage of this protection strategy is that the

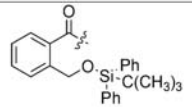
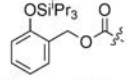
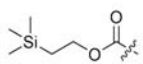
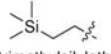
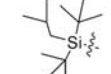
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 2-(tert-butyl(diphenylsiloxy)methyl)benzoyl (SiOMB)	G (<i>N</i> ²) A (<i>N</i> ⁹) C (<i>N</i> ⁴)	TBAF	Dreef-Tromp et al., 1990 Guerlavais et al., 2001 Guerlavais-Dagland et al., 2003
 2-triisopropylsilyloxybenzyl (tobc)	A (<i>N</i> ⁶) C (<i>N</i> ⁴)	1 M TBAF, 0.5 AcOH and THF, RT	Stutz et al., 2001 Pitsch, 2001 Lavergne et al., 2011
 4-triisopropylsilyloxybenzyl (tboc)	A (<i>N</i> ⁶) C (<i>N</i> ⁴)	1 M TBAF, 0.5 AcOH and THF, RT	Stutz et al., 2001 Pitsch, 2001
 N-(trimethylsilyloxy)carbamoyl (Teoc)	A (<i>N</i> ⁶) C (<i>N</i> ⁴) G (<i>N</i> ²)	ZnBr ₂ in nitromethane/ isopropanol (1:1)	Ferreira & Morvan, 2005 Shimizu & Sedeoka, 2007
 N-trimethylsilyl ethanol (Tse)	G (<i>O</i> ⁶)	ZnBr ₂ in nitromethane/ isopropanol (1:1)	Ferreira & Morvan, 2005
 di-tert-butylisobutylsilyl (BIBS)	A (<i>N</i> ⁶) C (<i>N</i> ⁴) G (<i>N</i> ²)	Et ₃ N/HF in dimethylformamide	Caruthers & Roy, 2015

Figure 2.1.16 Fluoride-labile protecting groups for the exocyclic amino function of nucleobases.

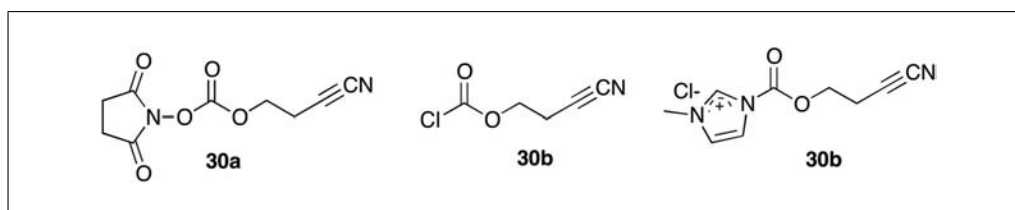


Figure 2.1.17 2-Cyanoethoxycarbonylating reagents.

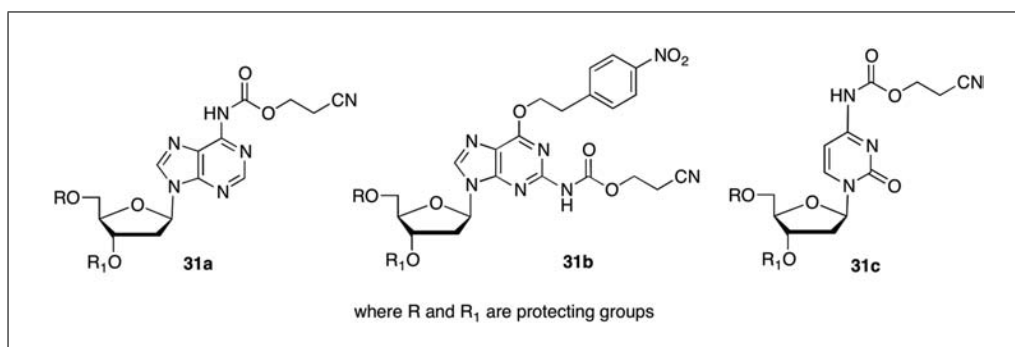


Figure 2.1.18 Structures of ceoc-protected nucleobases.

deprotection of oligonucleotide can be achieved in one step. The ceoc nucleobase protection was successfully used for the solid-phase synthesis of 2',3'-*O*-acetylated RNA oligonucleotides (Xu, Duffy, Chan, & Sutherland, 2014).

The exocyclic amino group of adenine and cytosine have also been protected using the 1,1-dimethyl-2-cyanoethoxy carbonyl

group (Chen, Fu, & Greenberg, 2000). Deprotection was carried out under aprotic, non-nucleophilic conditions, eliminating the use of Brønsted acid and base (Fig. 2.1.19).

Carbamate (or Benzyloxycarbonyl) Protection

The exocyclic amino groups of 2'-deoxyadenosine and 2'-deoxycytidine were

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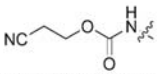
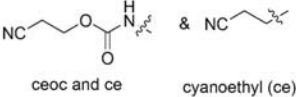
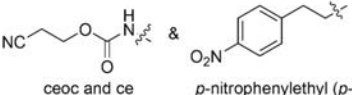
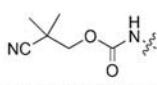
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 2-cyanoethoxycarbonyl (ceoc)	A (N^6) C (N^4)	DBU	Xu et al., 2014 Merk et al., 2000
 ceoc and ce cyanoethyl (ce)	G (N^2 -ceoc) and G (O^6 -ce)	DBU	Merk et al., 2000
 ceoc and ce <i>p</i> -nitrophenylethyl (<i>p</i> -npe)	G (N^2 -ceoc) and G (O^6 - <i>p</i> -npe)	DBU	Merk et al., 2000
 1,1-dimethyl-2-cyanoethoxycarbonyl	A (N^6) G (N^2) C (N^4)	Et ₃ N/DMF, 55°C Aprotic conditions	Chen et al., 2000

Figure 2.1.19 Cyanoethoxycarbonyl protecting groups.

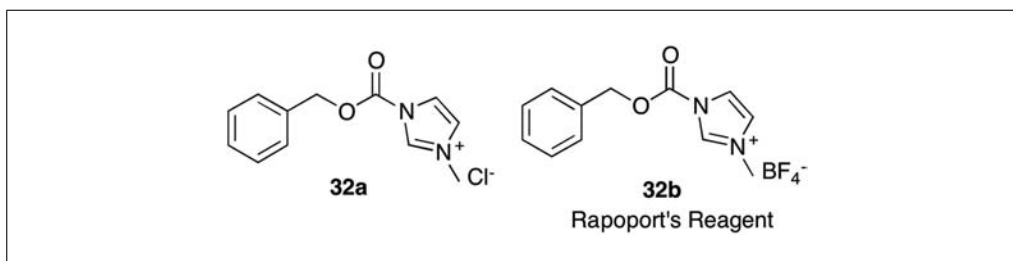


Figure 2.1.20 Benzyloxycarbonyl *N*-alkylimidazolium salts.

protected as benzyl carbamates utilizing benzyloxycarbonyl (Cbz) *N*-alkylimidazolium salts **32a** and **32b** (Fig. 2.1.20; Watkins, Kiely, & Rapoport, 1982). The oligonucleotides containing benzyl carbamates were deprotected by hydrogenation. Rapoport's reagent (1-(benzyloxycarbonyl)-3-methylimidazolium tetrafluoroborate) **32b** is the most widely utilized reagent for the protection of the *exo-N*⁶-group of purines with a carbobenzyloxy (Cbz) group. In the case of 2'-deoxyguanosine, the *O*⁶ oxygen was also protected as benzyl ether in addition to *N*-(benzyloxycarbonyl)carbamate.

In a recent report, more facile preparation of the carbamate-protected nucleoside was demonstrated by using various commercially available chloroformates. The *N*⁶-amino group of purine nucleosides **33** was efficiently protected with chloroformates **34** in the presence of *N*-methylimidazole (NMI), affording *N*⁶-carbamoyl adenosines **35** in good to excellent yields (Fig. 2.1.21; Cho, Coats, & Schinazi, 2012). The *N*⁶-Cbz-adenosine nu-

cleosides **36** were used for the preparation of the corresponding phosphoramidate pronucleotide **37** in excellent yield by reacting with phenyl phosphoryl chloride (Fig. 2.1.22; Cho, Amblard, Coats, & Schinazi, 2011, 2012).

Biscarbamate protection

tert-Butoxycarbonyl (*t*-Boc)-groups have rarely been used in nucleoside chemistry. Nevertheless, it is known that biscarbamate can be removed under mildly basic to neutral conditions and hence is suitable as an *N*-protecting group for nucleosides (Sikehi & Hultin, 2006, and references therein). A solvent-free reaction utilizing a simple low-energy ball mill apparatus was reported to convert the amino groups of nucleosides **38**, adenosine, 2-deoxyadenosine, cytidine, 2-deoxycytidine, guanosine, and 2-deoxyguanosine as well as some of their ribosyl *O*-protected derivatives, to the corresponding bis-*N*-Boc carbamates **39** (Sikehi, & Hultin, 2006). For guanosine compounds, the carbonyl group of the base moiety was also protected as its *O*-Boc enol carbonate

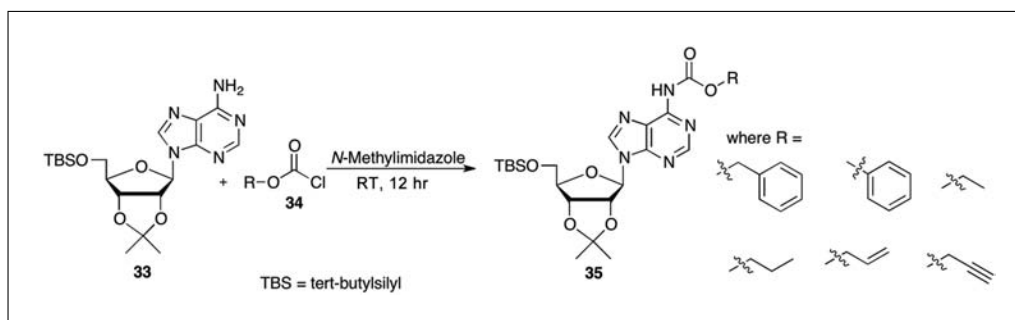


Figure 2.1.21 Carbamate protection of adenosine nucleoside.

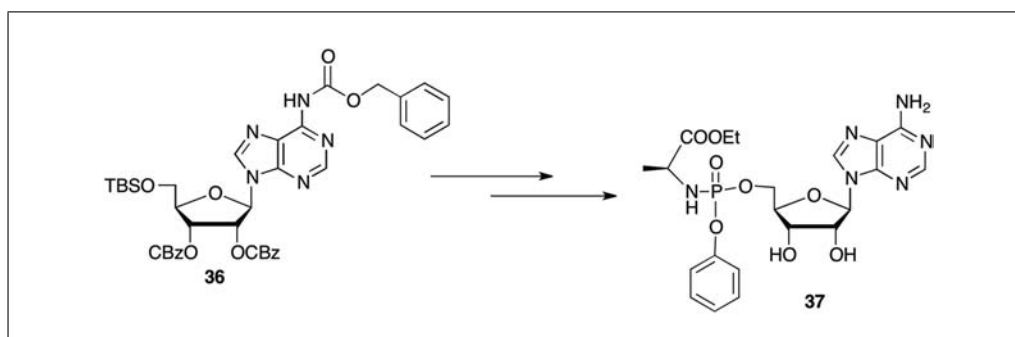


Figure 2.1.22 Preparation of phosphoramidate pronucleotides.

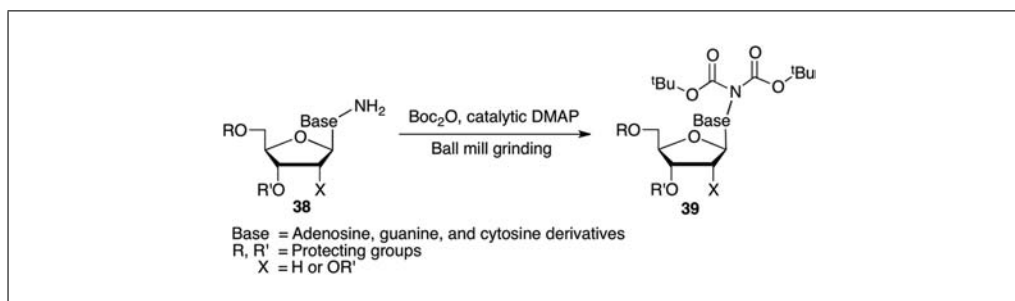


Figure 2.1.23 Di-*N*-Boc protection strategy of nucleosides.

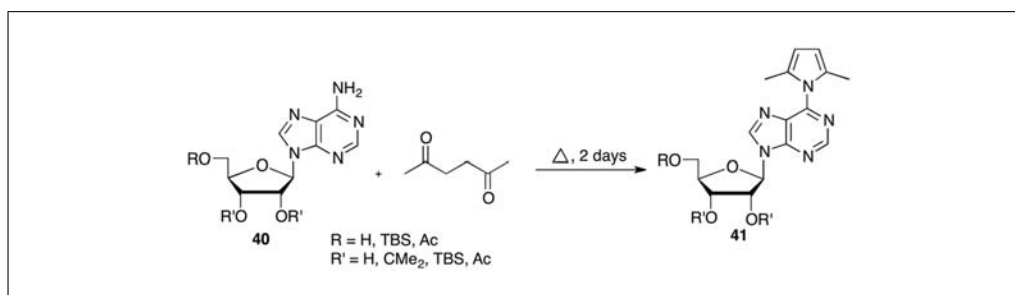


Figure 2.1.24 Exocyclic amino group protection with a 2,5-dimethylpyrrole moiety.

(Fig. 2.1.23). Transient *in situ* *O*-silylation facilitated the preparation of bis-*N*-Boc nucleosides from unprotected sugars. This strategy allows the protection of nucleosides with a base-stable and acid-labile group suitable for the preparation of base-sensitive oligonucleotides as well as for further manipulation.

However, the utility of this diBoc carbamate protection is yet to be explored in oligonucleotide synthesis.

Several other protecting groups developed in the nucleoside chemistry are described as

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follows. The amino groups of adenine and guanine nucleosides **40** were protected by heating the substrates in neat 2,5-hexanedione (Nowak & Robins, 2003). The resulting 2,5-dimethylpyrrole adducts **41** were found to be stable towards bases and were successfully hydrolyzed with trifluoroacetic acid/water to regenerate the amino function. A representative example with adenosine is demonstrated in Figure 2.1.24. Unfortunately, similar methodology could not be applied to cytidine nucleoside.

THE PROBLEM OF CYANO-ETHYLATION

The cyano-ethylation of a nucleobase is a common side reaction in oligonucleotide synthesis. In general, protected thymine is not used during oligonucleotide assembly. However, during the deprotection of oligonucleotides under basic conditions, acrylonitrile is formed during the removal of the cyanoethyl phosphate protecting group. Consequently, *N*³-cyanoethylation occurs via Michael addition at the *N*³ position of the thymine nucleobase with the liberated acrylonitrile. The formation of *N*³-cyanoethyl thymidine was efficiently suppressed by using nitromethane as a scavenger of acrylonitrile during oligo deprotection (Umemoto & Wada, 2005). A set of fully protected deoxynucleoside phosphoramidites **42a-d** completely eliminated the *N*-cyanoethylation (Fig. 2.1.25; Tsunoda, Kudo, Ohkubo, Seio, & Sekine, 2010). Fully protected adenine and cytosine nucleosides **42b** and **42d** were prepared by simply treating the substrate with phthaloyl chloride and Hünig's base in THF. Synthesis of fully protected

thymine cytosine and guanine nucleosides **42a**, **42d**, and **44** from **43a**, **42d**, and **44**, respectively, is shown in Figure 2.1.26.

The optimized synthesis of a number of 2'-*O*-Tom-containing RNA phosphoramidites **45a-g** (Fig. 2.1.27; Porcher & Pitsch, 2005) has been reported.

PROTECTION OF PURINE NUCLEOBASES: THE PROBLEM OF DEPURINATION

The development of suitable protecting groups for purine nucleobases has been an area of considerable interest because purine nucleosides rapidly depurinate under acidic conditions. The problem is compounded by the acid-labile DMTr group used for protection of the 5'-OH in solid-phase oligonucleotide synthesis. Prior to each coupling step in the synthesis cycle, the DMTr group is removed by exposure to a strong acid such as 2% dichloroacetic acid in dichloromethane. Consequently, the growing oligonucleotide chain is repeatedly exposed to strongly acidic conditions, potentially resulting in depurination and reduced yield of the desired "full-length" product.

The kinetics and mechanisms of nucleoside depurination have been investigated by several research groups (Oivanen, Lönnberg, Zhou, & Chattopadhyaya, 1987; Romero, Stein, Bull, & Cordes, 1978; Suzuki, Ohsumi, & Makino, 1994). The presumed mechanism for depurination of deoxyadenosine (Fig. 2.1.28) involves initial protonation of the nucleoside to produce the *N*¹-protonated form **46**, and then equilibration (prototropic shift) to the *N*⁷-protonated species **47** or **48**, followed

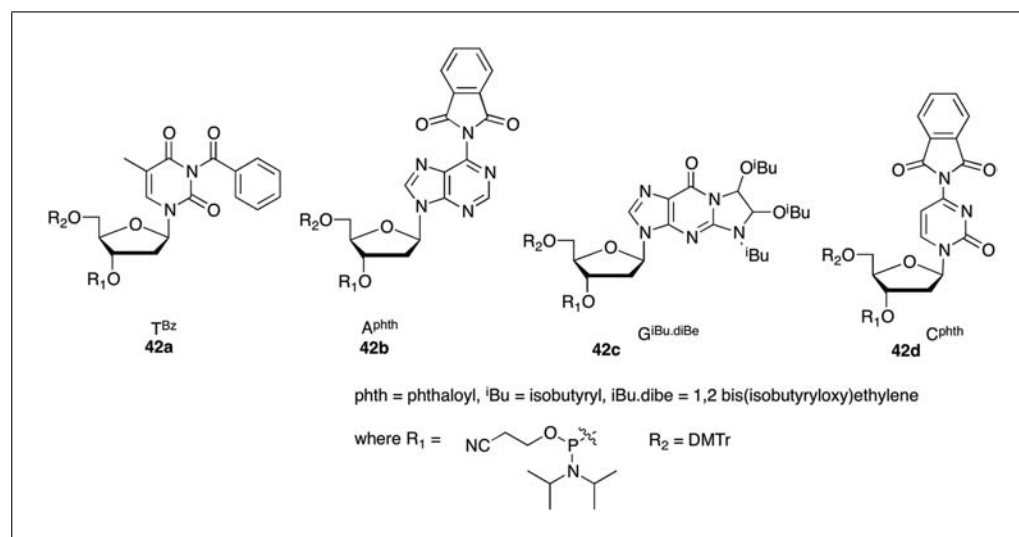


Figure 2.1.25 Structures of fully protected nucleobases to avoid cyanoethylation.

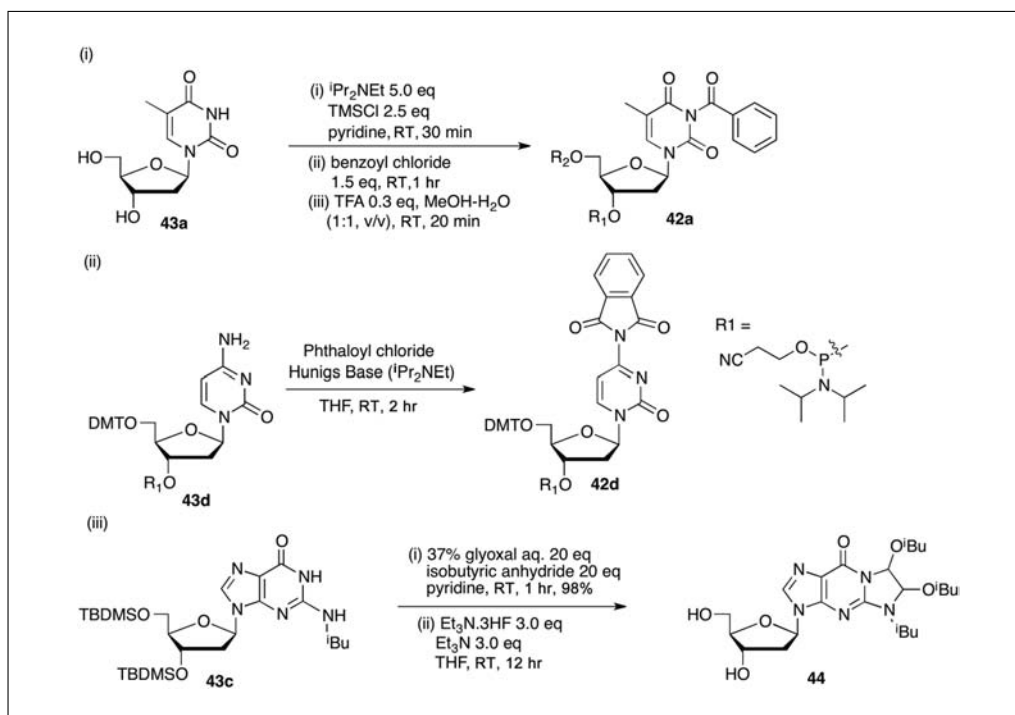


Figure 2.1.26 Synthesis of fully protected nucleobases.

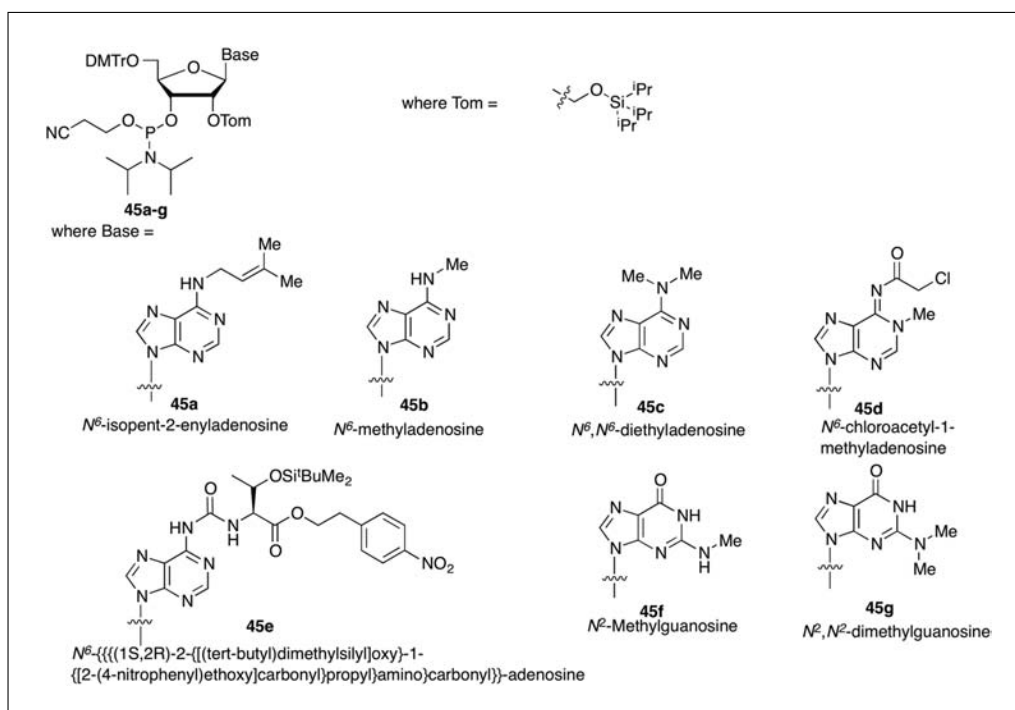


Figure 2.1.27 Miscellaneous nucleobase protecting groups for RNA synthesis.

by cleavage of the glycosidic bond to give **50** via the oxonium **49** (Zoltewicz & Clark, 1972; Zoltewicz, Clark, Sharpless, & Grahe, 1970). It is also conceivable that, at lower pH, depurination can occur via protonation of the purine nucleobase at both N^1 and N^7 . Presence of the 2'-OH has a significant effect

on the nucleoside's susceptibility to depurination. For example, guanosine and adenosine are more resistant to depurination compared with deoxyadenosine and deoxyguanosine. Deoxyadenosine itself depurinates 1200 times faster than adenosine (York, 1981).

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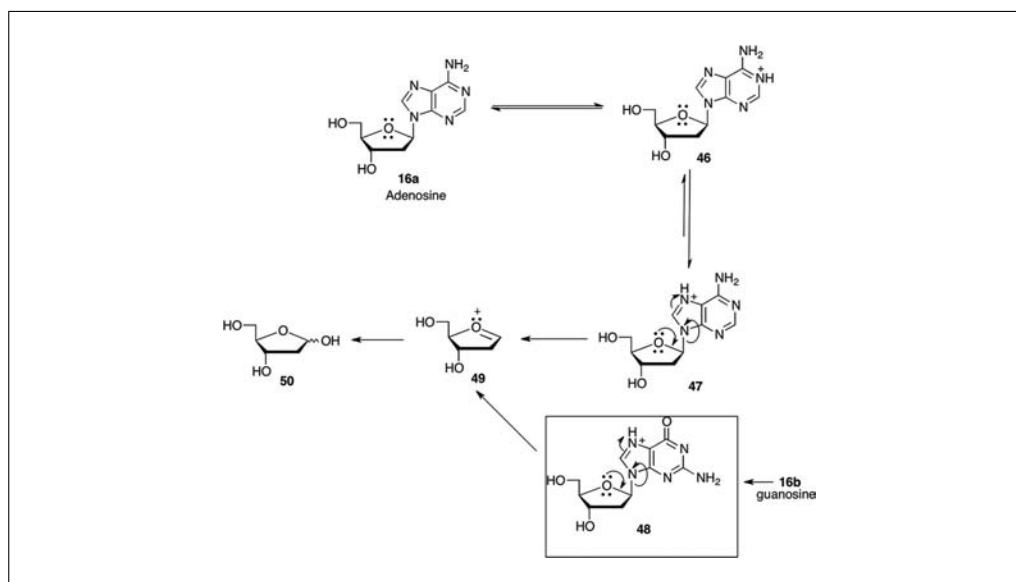


Figure 2.1.28 Schematic showing the proposed depurination mechanism for 2'-deoxyguanosine and 2'-deoxyadenosine catalyzed by protic acids. Modified from Iyer & Beaucage (1999) and reprinted with permission from Elsevier Science Publishing.

Interestingly, *N*-acyl-protected purine nucleosides (particularly deoxyadenosine) are more prone to depurination than unprotected nucleosides. Among *N*-acyl-protected deoxyadenosines, protection at *N*⁶ with α -phenylcinnamoyl, naphthaloyl, 3-methoxy-4-phenoxybenzoyl, 9-fluorenylmethoxycarbonyl (Fmoc), and *tert*-butylphenoxyacetyl (*t*-PAC) groups (Fig. 2.1.7) provides greater resistance to depurination than with *N*⁶-benzoyl (reviewed in Beaucage and Iyer, 1992). It is believed that in the case of acyl-protected purine nucleosides under acidic conditions, the initial site of protonation is *N*⁷, rendering the protonated species more prone to glycosidic cleavage. Naturally, caution should be exercised in the synthesis of oligonucleotides whose sequence contains deoxyadenosines at the 3' terminus.

Bis-acylation has also been studied as a strategy to reduce depurination. Imide protecting groups **51** and **52**, as well as the diamide protecting group **53**, have been investigated (Fig. 2.1.29; Kume, Iwase, Sekine, & Hata, 1984; Kume, Sekine, & Hata, 1982). However, these oxidation groups were labile to aqueous pyridine, a solvent used in the oxidation step in solid-phase oligonucleotide synthesis. Thus, alternate oxidants have to be employed for the oxidation step. 2'-Deoxyadenosine derivatives **42a** and **42d** protected with the phthaloyl group suppressed depurination to a great extent compared to the traditional *N*-benzoyl protected deoxyadenosine analogs (Tsunoda et al., 2010).

Nucleosides protected with *O*-nitrophenylsulfonyl and tris(benzoyloxy)trityl groups **54** also appear to be more resistant to depurination (Honda et al., 1984, and references therein; Shimidzu & Letsinger, 1968). However, the derived nucleoside phosphoramidites couple less efficiently than the *N*-acyl-protected nucleoside phosphoramidites (Sekine, Masuda, & Hata, 1985).

Amidine Protecting Groups

Amidine-type protecting groups were introduced by Smrt & Sorm in 1967 (Fig. 2.1.30; Smrt & Sorm, 1967). Since *N*-acyl-protected purine nucleosides are sensitive to deacylation, alternate protecting groups have been investigated. Prominent among these are the amidine protecting groups, which are introduced using an exchange reaction with appropriate amidine acetals. Interestingly, *N*-amidine-protected nucleosides (Fig. 2.1.30) resist depurination 20-fold better than the corresponding *N*-benzoyl nucleosides (Caruthers, McBride, Bracco, & Dubendorff, 1985; Froehler & Matteucci, 1983; Holy & Zemlicka, 1969; Ohkubo et al., 2010; Smrt & Sorm, 1967, and references therein; Sproat, Iribarren, Guimil Garcia, & Beijer, 1991; Vongsutilers, Daft, Shaughnessy, & Gannett, 2009; Vu et al., 1990).

It is presumed that the protonation sites of amidine-protected nucleosides are *N*¹ and *N*⁶ instead of *N*¹ and *N*⁷, respectively, resulting in a slower rate of depurination. Indeed, amidine-protected nucleoside phosphoramidites are frequently employed in solid-phase

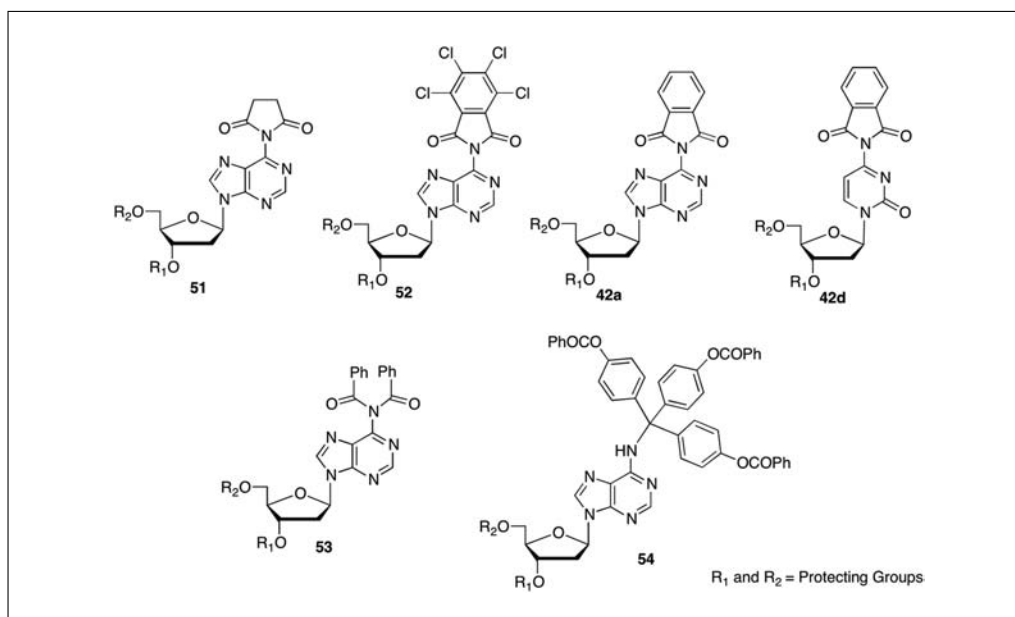


Figure 2.1.29 Examples of N^6 -protecting groups for 2'-deoxyadenosine derivatives that reduce depurination. Reprinted from Iyer & Beaucage (1999) with permission from Elsevier Science Publishing.

Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 (dimethylamino)methylene	G (N^3), A (N^6)	NH ₄ OH, heat	Smrt & Sorn, 1967 Vu et al., 1990 Sproat et al., 1991 Caruthers et al., 1985 McBride et al., 1986 Vinayak et al., 1992 Ohkubo et al., 2010 Vongsutlers et al., 2009
 (di- <i>n</i> -butylamino)methylene	G (N^3), A (N^6) C (N^4)	NH ₄ OH, heat; 0.5 M NH ₂ NH ₂ ·H ₂ O/pyr/AcOH	McBride et al., 1986 Froehler & Matteucci, 1983 Ohkubo et al., 2010
 (diisopropylamino)methylene	A (N^6)	NH ₄ OH/10% NH ₄ OAc/heat; 0.5 M NH ₂ NH ₂ ·H ₂ O/pyr/AcOH	Froehler & Matteucci, 1983
 1-(dimethylamino)ethylidene	G (N^3), A (N^6)	NH ₄ OH, heat	McBride et al., 1986
 (diisobutylamino)methylene	A (N^6)	NH ₄ OH/10% NH ₄ OAc/heat; 0.5 M NH ₂ NH ₂ ·H ₂ O/pyr/AcOH	Froehler & Matteucci, 1983
 <i>N</i> -methylpyrrolidin-2-ylidene	C (N^4)	NH ₄ OH, heat	McBride et al., 1986

Figure 2.1.30 Amidine protecting groups.

oligonucleotide synthesis. However, changes are required in the oxidation step to avoid nucleobase modifications (Mullah, Andrus, Zhao, & Jones, 1995). Deprotection of amidine-protected nucleosides were done

under basic conditions (Sproat et al., 1991; Vinayak, Anderson, McCollum, & Hampel, 1992; Vu et al., 1990).

Deprotection by treatment with aqueous ammonia is incompatible with the synthesis

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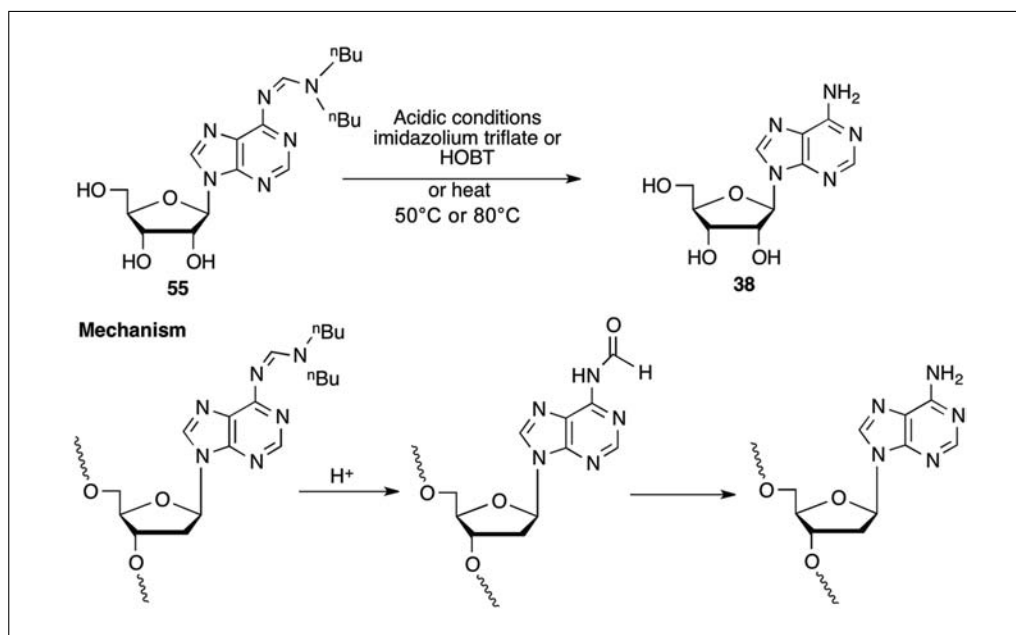


Figure 2.1.31 Deprotection of the dbf group under acidic or neutral conditions.

of base-labile oligonucleotides. Based on a report of formation of the formyl group from *N,N*-dimethylformamidine, Ohkubo et al. (2010) prepared oligonucleotides containing amidine-type protecting groups, *N,N*-dimethylformamidine (dmf), and *N,N*-dibutylformamidine (dbf). The resulting oligonucleotides **55** were rapidly deprotected under mild acidic conditions using imidazolium triflate or HOBT or simply by heating under neutral conditions (Fig. 2.1.31). Since the deprotection can be achieved under acidic conditions, this new deprotection strategy allows efficient preparation of RNA oligonucleotides or modified nucleotides bearing a base-labile group using a silyl-type linker.

Depurination is also affected by other factors such as the nature of the solid support (controlled-pore glass versus polystyrene; see UNIT 3.1), the composition of the de-blocking solution and deblocking time, the washing solvent, and the washing time that are employed in solid-phase synthesis of oligonucleotides (Paul & Royappa, 1996). Depurination is faster at terminal sites than at internal sites in an oligonucleotide chain (Suzuki et al., 1994). Interestingly, a solution of 15% dichloroacetic acid (DCA) in methylene chloride was ideal as a detritylating reagent that induced minimal depurination compared to the traditionally used 2% DCA/methylene chloride.

It is pertinent that with modern DNA synthesizers the pulsed delivery of reagents to the synthesis columns, in conjunction with optimized synthesis programs, results in short

contact times and has greatly minimized the depurination problem. Thus, *N*-acyl-protected nucleoside (benzoyl for dA and dC, and isobutyryl for dG) phosphoramidites and *H*-phosphonates can be used for the efficient synthesis of oligonucleotides.

Over the past few years, new applications of oligonucleotides in diagnostics and therapeutics have emerged, necessitating the expeditious synthesis of large numbers of oligonucleotides. In order to speed the synthesis process, more “labile” *N*-acyl protecting groups for nucleosides have been sought. As the simplest member of the family of *N*-acyl protecting groups, the *N*⁴-acetyl of cytosine has been used for “ultrafast” DNA synthesis using the phosphoramidite approach. Rapid deprotection rates were achieved using methylamine/ammonia (Reddy, Hanna, & Farooqui, 1994). However, this group is unsuitable for use in phosphodiester and phosphotriester chemistries.

The synthesis of oligonucleotide analogs carrying sensitive backbones requires protecting groups that (1) withstand the synthetic rigors of chain assembly and (2) can be removed chemoselectively under mild conditions. Nucleobases protected by phenoxyacetyl (Chaix, Molko, & Téoule, 1989; Singh & Misra, 1988; Sproat et al., 1991), and their derivatives such as *t*-PAC, show accelerated deacylation (Köster et al., 1981; Sinha et al., 1993) under mildly basic conditions. It is presumed that the inductive effect of the phenoxy group renders the amide carbonyl group more susceptible to nucleophilic attack, facilitating rapid

base-catalyzed hydrolysis. Thus, PAC- and *t*-PAC-phosphoramidites have been employed for rapid synthesis of oligonucleotides and certain analogs. As a rule, following oligonucleotide assembly on solid support, the PAC and *t*-PAC groups are removed under milder conditions (28% NH₄OH, room temperature; Sinha et al., 1993) or using gaseous amines under pressure (Boal et al., 1996). However, the use of *t*-PAC-protected nucleoside phosphoramidites results in *trans* acylation of the *t*-PAC protecting groups during the capping step when acetic anhydride is employed as a capping reagent. Thus, *tert*-butylphenoxyacetic anhydride should be used for the capping step in solid-phase oligonucleotide synthesis (Sinha et al., 1993).

The concept of neighboring-group participation has also been used to design acyl protecting groups and accelerate the deprotection step (Dreef-Tromp et al., 1990; Kuijpers, Huskens, & van Boeckel, 1990). For example, removal of the 2-(acetoxymethyl)benzoyl group from nucleosides under basic conditions is accelerated by intramolecular participation of the deacylated hydroxymethyl group.

Nucleobase protection for support-bound oligonucleotide synthesis

Solid-support-bound oligonucleotides are used for a variety of applications. This requires the use of special protecting groups that are stable in the conditions used for the removal of phosphate- and nucleobase-protecting groups. Allyloxycarbonyl protecting groups (Hayakawa, Kato, Uchiyama, Kajino, & Noyori, 1986; Hayakawa, Wakabayashi, Kato, & Noyori, 1990; Hyodo, & Hayakawa, 2006) have also been introduced and can be chemoselectively removed under neutral [using Pd(0)] or mildly basic conditions. However, the *p*-nitrophenylethyl (NPE) and (*p*-nitrophenyl)ethoxycarbonyl (NPEOC) groups (Pfleiderer et al., 1985; Pfleiderer, 2007; Pfister, Farkas, Charubala, & Pfleiderer, 1988; Schirmeister & Pfleiderer, 1987; Trichtinger et al., 1983) and the 2-dansylethoxycarbonyl group (Wagner & Pfleiderer, 1997) are selectively removed using DBU following a β -elimination nucleobase protection strategy. The NPE/NPEOC β -elimination nucleobase-protection strategy in conjunction with a succinyl linker has been used for the preparation of oligonucleotide arrays and high-quality primers (Weiler & Hoheisel, 1996) by solid-phase synthesis. Thus, support-bound oligonucleotides can

be prepared using building blocks that carry these protecting groups. The *N*-pent-4-enoyl (PNT) group has been introduced as a new acyl protecting group for situations where multiple deprotection protocols are used (Iyer et al., 1997, and references therein). PNT-protected nucleoside phosphoramidites can potentially be used for the rapid synthesis of oligonucleotides and oligonucleotide analogs, as well as for the preparation of support-bound oligonucleotides.

Several groups have reported nucleobase protection that may be particularly valuable in the synthesis of oligonucleotides intended for specific applications. Figure 2.1.7 shows a partial list of protecting groups. A complete list of such groups is covered in other reviews (Beaucage & Iyer, 1992; Iyer and Beaucage, 1999; Sonveaux, 1986). Nonetheless, it should be noted that many of these still need to be evaluated in routine oligonucleotide synthesis.

Thermolytic Protecting Groups

Inspired by the report of thermolabile phosphate protecting groups—4-methylthio-1-butyl and 3-(2-pyridyl)-1-propyl, developed by Beaucage and coworkers (Cieslak & Beaucage, 2003; Cieslak, Grajkowski, Livengood, & Beaucage, 2004)—Ohkubo et al. (2009) prepared DNA probes **56a–c** from nucleosides **16** incorporating thermolytic protecting groups, *N*-arylcabamoyl and *N*-(phenylsulphonyl)cabamoyl (psc), onto the amino groups of deoxycytidine, deoxyadenosine, and deoxyguanosine by reacting with corresponding isocyanates (Fig. 2.1.32). Deprotection was carried out by heating under neutral conditions, enabling a new system of molecular switching by simply heating and reprotection with isocyanates. Figure 2.1.32 also shows the deprotection mechanism under thermal conditions. A combination of thermolytic phosphate and amino protecting groups may lead to oligonucleotides that can be deprotected under non-basic conditions. *N*-quinoylecabamoylated nucleobases were deprotected under thermolytic conditions (Ohkubo, Kasuya, Miyata, Taguchi, & Sekine, 2007).

Oligo Synthesis Without Nucleobase Protection

Because of the various issues associated with nucleobase protection, oligonucleotide synthesis has also been evaluated using building blocks bearing unprotected nucleobases. However, these efforts have met

Protection of Nucleosides for Oligonucleotide Synthesis

2.1.23

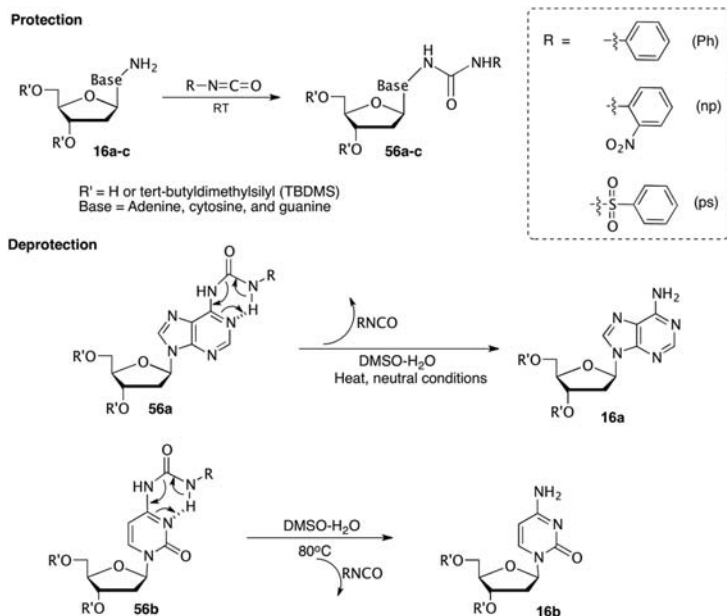


Figure 2.1.32 Thermolytic deoxyribonucleic acid protection and deprotection.

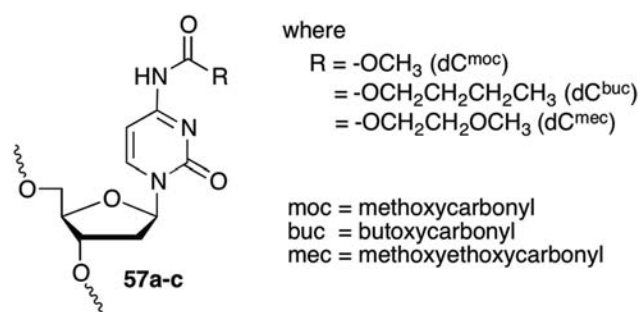


Figure 2.1.33 *N*-Acetyl-type protected dC: an interesting feature.

only limited success (Fourrey & Varenne, 1985; Gryaznov & Letsinger, 1991; Narang, Itakura, & Wightman, 1972; Uchiyama, Aso, & Noyori, 1993, and references therein). Nucleotide synthesis without nucleobase protection has been briefly reviewed (Hayakawa, Kawai, & Kataoka, 2001). DNA synthesis without base protection has been attempted by the phosphoramidite approach (Ohkubo, Seio, & Sekine, 2006; Sekine, 2004). However, this strategy has not found application in large-scale oligonucleotide synthesis.

***N*-Acetylated Nucleosides: An Interesting Feature**

N-Acetylated nucleosides have been extensively used in the oligonucleotide synthesis in commercial DNA synthesizers. Apart from that, *N*-acylated nucleosides such as 4-*N*-acetyldeoxycytidine and *N*-

threonylcarbonyladenine occur naturally in tRNA. Inspired by this, Wada, Kobori, Kawahara, & Sekine (1998) investigated the stability of oligodeoxyribonucleotides incorporating 4-*N*-acetyldeoxycytidine and found that the 4-*N*-acetyl group stabilizes the duplex with a complementary strand. In a different study, oligodeoxyribonucleosides **57a–c** containing 4-*N*-alkoxycarbonydeoxycytidine were synthesized, and their hybridization properties were evaluated (Fig. 2.1.33; Kobori, Miyata, Ushioda, Seio, & Sekine, 2002).

4-*N*-(*N*-arylcarbonyl)-dC derivatives **58a–c** were synthesized and incorporated into oligonucleotides (Miyata et al., 2004). The carbonyl group forms an intramolecular hydrogen bond with the cytosine ring nitrogen atom, potentially inhibiting the formation of Watson-Crick base pairing with the complementary guanine base. However, the

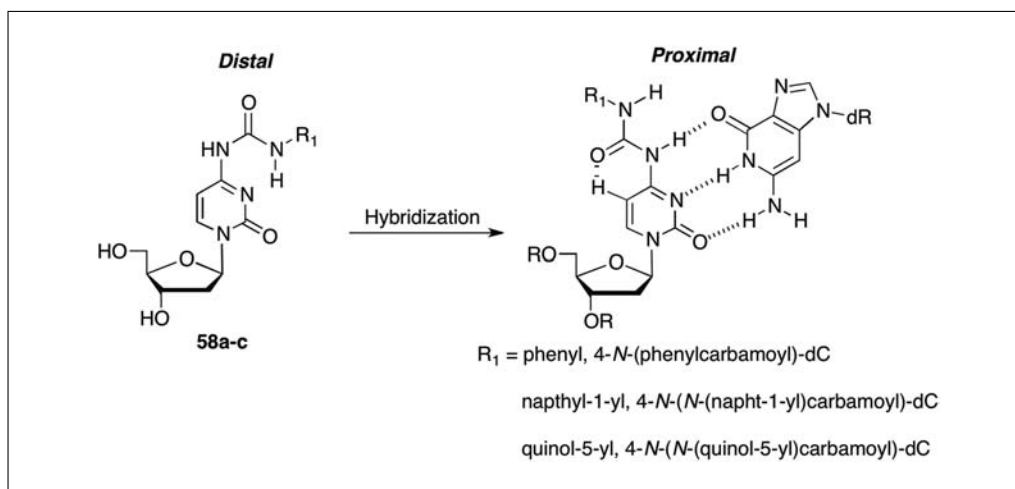


Figure 2.1.34 Watson-Crick base pairing of *N*-carbamoyl protected dC with dG.

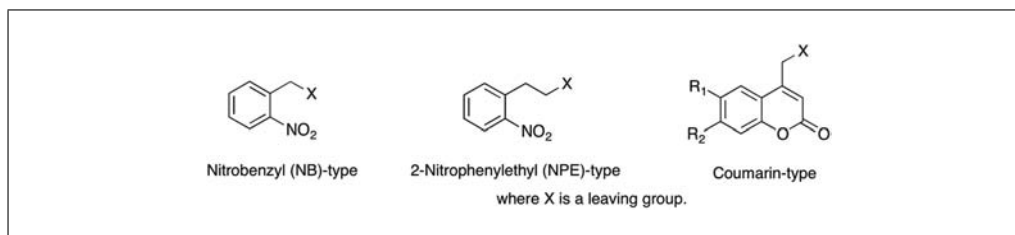


Figure 2.1.35 Types of photolabile nucleobase protection.

melting point temperatures and ^1H NMR analyses indicate that nucleosides change conformation from distal to proximal, allowing Watson-Crick base pairing (Fig. 2.1.34).

Based on the higher hybridization affinity of ssDNA containing 4-*N*-acetylcytosine compared to the unmodified oligonucleotides, a new strategy called the “Protected DNA Probes method” has been developed in which appropriately protected bases selectively bind to the complementary bases, but the base protecting groups are not removed at the end of synthesis (Ohkubo et al., 2008).

Photocleavable Protecting Groups

As mentioned above, *N*-acyl-type nucleobase protecting groups, the most common protecting group for oligonucleotides, are removed by bases such as aqueous NH_3 and aqueous or methanolic methylamine. Some of the synthetic oligonucleotides are not stable under such harsh basic treatment. In this context, mild base-labile protecting groups such as phenoxyacetyl have been developed and used in oligonucleotide synthesis. An alternate approach to circumvent this problem is the use of allyloxycarbonyl nucleobase protection, which can be removed using Pd^0 complexes.

One of the mildest deprotection strategies involves the use of photolytic removal of appropriate protecting groups. Photolabile protecting groups (Klan et al., 2013) are very useful for the synthesis of base-sensitive oligonucleotides. In this context, the deprotection can be accomplished by irradiation using light of an appropriate wavelength. Photolabile nucleobase protecting groups can be classified into three types, as shown in Figure 2.1.35.

The most common photolabile protecting group is the *o*-nitrobenzyl (NB) group, which is a carbamate-type nucleobase protecting group; the nucleobase protection is normally achieved by reacting nucleosides with [(2-nitrobenzyl)oxy]chloroformate in a biphasic mixture of aqueous Na_2CO_3 /dichloroethane at 25°C , affording the corresponding *N*-protected derivatives (Stutz & Pitsch, 1999). A representative example of photolabile protection of ribocytidine **59**, affording *o*-NB protected ribocytidine **60** is shown in Figure 2.1.36. Such photolabile nucleobase protecting groups have been used in the synthesis of 3'-aminoacylated RNA synthesis.

The proposed mechanism of deprotection of *o*-NB group under UV light is shown in Figure 2.1.37 (Barton, Chow, Cox, & Kirby, 1962).

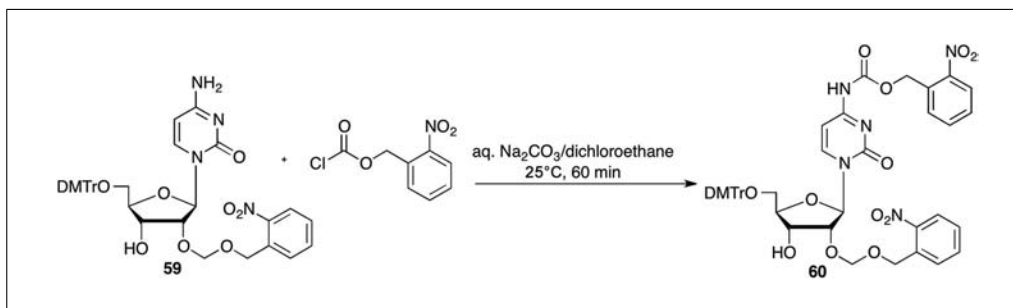


Figure 2.1.36 Photolabile nucleobase protection with *o*-nitrobenzyl group.

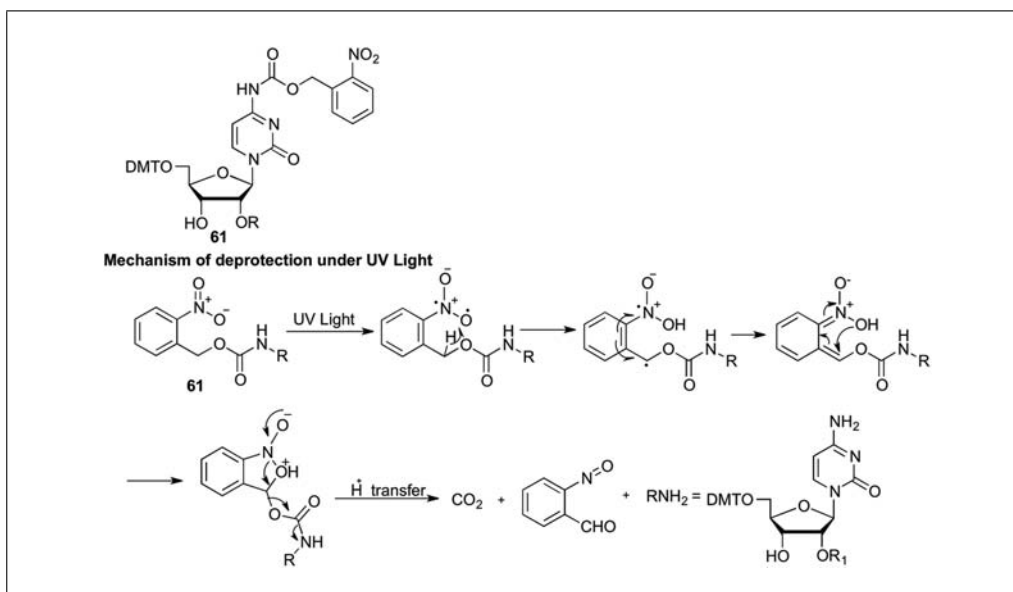


Figure 2.1.37 Mechanism of deprotection of *o*-nitrobenzyl group under UV light.

In order to improve photolytic deprotection with enhanced efficiency, several additional photoremovable groups have been developed. Figure 2.1.38 shows the *o*-NB protecting group together with other common photolabile protecting groups such as 1-(*o*-nitrophenyl)ethyl (NPE), 2-(*o*-nitrophenyl)propyl (NPP), 6-nitroveratryloxycarbonyl (NVOC), 2,2-bis(2-nitrophenyl)ethoxycarbonyl (diNPEOC), 4-*N*-[2-(2-nitrophenyl)propoxy]carbonyl (NPPOC), 1-(3-nitrodibenzofuran-1-yl)ethyl (NDBF), 6-nitropiperonyloxymethyl (NPOM), *p*-hydroxyphenacyl (pHP), 7-(diethylaminocoumarin-4-yl)methoxy carbonyl (DEACM), 6-bromo-7-hydroxycoumarinyl-4-yl-methyl (Bhcmoc), and 6-bromo-7-methoxycoumarinyl-4-yl-methyl (Bmcmoc). A combination of NPOM and NDBF caging group strategies has enabled two-photon caged phosphoramidite for DNA synthesis (Lusic, Uprety, & Deiters, 2010).

Photolabile nucleobase protection has been used to achieve exquisite control over biological processes through light regulation,

also referred to as photocaging. The concept was introduced independently by Engels (Engels & Reidys, 1978; Engels & Schlaeger, 1977) and Hofmann (Kaplan, Forbush, & Hoffman, 1978). Watson-Crick base pairing is blocked by caging groups, completely preventing oligonucleotide duplex formation. Duplex formation is restored when irradiated by light at a specific wavelength. Photolabile groups, when introduced at specific locations, render the molecule inactive; however, upon irradiation with light at a particular wavelength, the molecules can be activated (see detailed review by Liu & Deiters, 2014; Ruble, Yeldell, & Dmochowski, 2015; Shao & Xing, 2010; Tang et al, 2013). Caging groups can also be introduced at phosphate, 2'-OH, and nucleobases (Tang et al., 2013); however, nucleobase caging has been of particular interest.

Oligonucleotides containing NVOC protecting groups were used as the photocleavable protecting group at the N^3 position of thymidine, and duplex formation was achieved by photoradiation at 365 nm for 5 hr (Iwase, Kitani, Yamaoka, & Murakami, 2003).

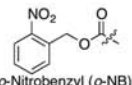
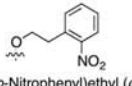
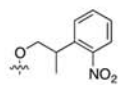
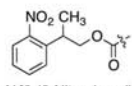
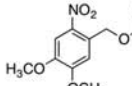
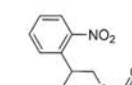
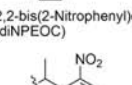
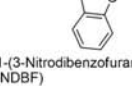
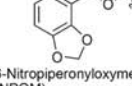
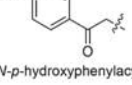
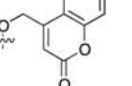
Protecting group	Nucleobase (protection site)	Deprotection conditions	References
 o-Nitrobenzyl (o-NB)	C(N ⁴) A(N ⁶) G(N ²)	Aq. sodium citrate buffer (pH 3.5)/t-BuOH Pyrex filtered light from mercury 250 W lamp or UV irradiation at 365 nm	Stutz & Pitsch, 1999 Seio et al., 2016
 (o-Nitrophenyl)ethyl (o-NPE)	N(O ⁴)	UV irradiation at 365 nm	Walbert et al., 2001 Schafer et al., 2011
 2-(o-Nitrophenyl)propyl (NPP)	T(O ⁴) G(O ⁶)	UV irradiation at 365 nm	Mikat & Heckel, 2007 Seio et al., 2016
 4-N-[2-(2-Nitrophenyl)-propoxy]carbonyl (NPPOC)	C(N ⁴)	UV irradiation at 290 nm	Furuta et al., 2007
 6-Nitroveratryloxycarbonyl (NVOC)	C(N ⁴) A(N ⁶) G(N ²) T(N ³)	UV irradiation at >300 nm	Alvarez et al., 1999 Iwase et al., 2003
 2,2-bis(2-Nitrophenyl)ethoxycarbonyl (diNPEOC)	A(N ⁶) G(N ²)	UV irradiation >300 nm	Alvarez et al., 1999
 1-(3-Nitrobenzofuran-1-yl)ethyl (NDBF)	C(N ⁴) A(N ⁶) T(O ⁴) T(N ³)	UV irradiation at 440 nm	Momotake et al., 2006 Schäfer et al., 2011 Rodrigues-Correia et al. 2013, 2014
 6-Nitropiperonyloxymethyl (NPOM)	T(N ³) G(N ²)	UV irradiation at 365 nm	Young et al., 2008, 2009 Lusic & Dieters, 2006 Lusic et al., 2007, 2008
 N-p-hydroxyphenylacetyl (N-PHP)	T(N ³) T(O ⁴)	UV irradiation at 330-350 nm UV irradiation at 313 nm	Givens et al., 2012 Rodrigues-Correia et al., 2013, 2014
 7-(Diethylaminocoumarin-4-yl) methyl (DEACM)	G(O ⁶) T(O ⁴) T(N ³)	UV irradiation at 400 nm	Menge et al., 2011. Rodrigues-Correia et al., 2013, 2014 Ohtsuki et al., 2016
 R = H, 6-Bromo-7-hydroxycoumarinyl-4-yl-methyl (Bhcmoc) R = Me, 6-Bromo-7-methoxycoumarinyl-4-yl-methyl (Bmcmoc)	C(N ⁴) A(N ⁶)	UV irradiation at 350 nm in aq. buffer at pH 7.2	Furuta et al., 2007 and references therein

Figure 2.1.38 Photolabile nucleobase protecting groups.

Protection of Nucleosides for Oligonucleotide Synthesis

2.1.27

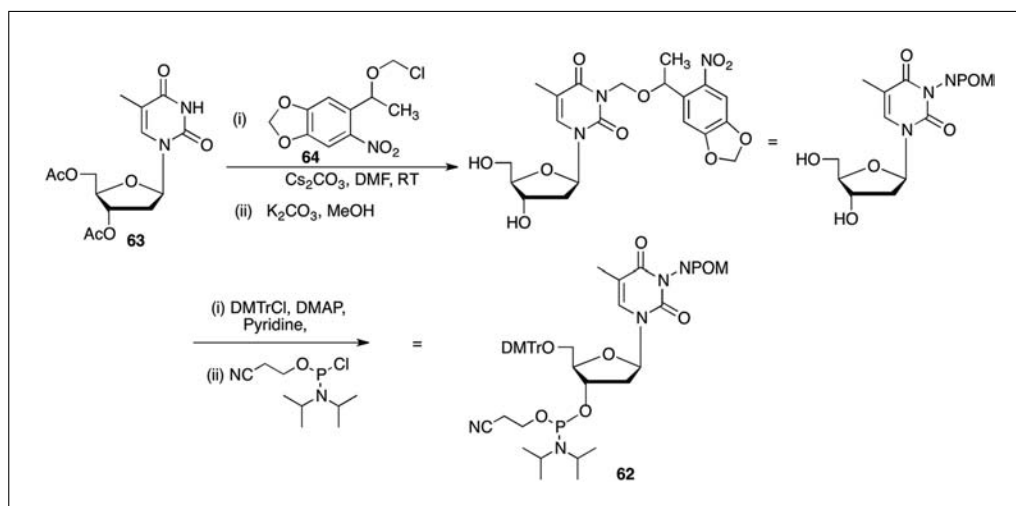


Figure 2.1.39 Synthesis of NPOM-caged thymidine phosphoramidite.

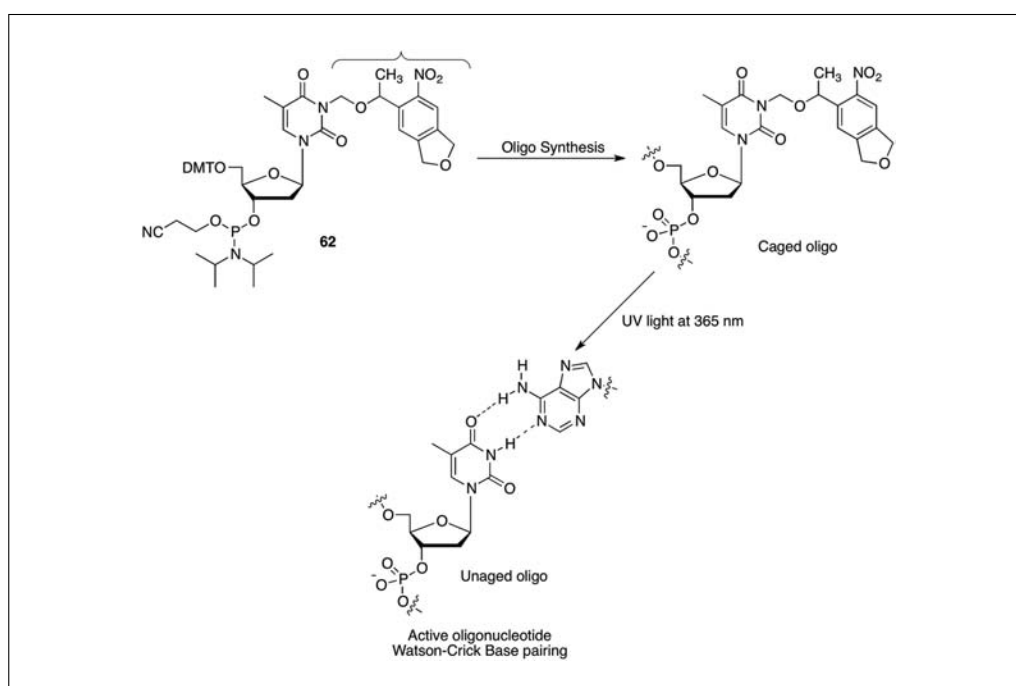


Figure 2.1.40 Nucleobase caging by NPOM protection.

NPOM-caged dT **62** introduced by the Deiters group is of particular interest. Figure 2.1.39 shows the synthesis of NPOM-caged thymidine phosphoramidite **62** (Lusic, Young, Lively, & Deiters, 2007) from nucleoside **63** using reagent **64**, and DNA incorporating NPOM-caged thymidine was prepared by standard phosphoramidite chemistry and irradiated at 365 nm to afford active oligo with Watson-Crick base pairing (Fig. 2.1.40).

NPOM-caged thymidine incorporated into DNA has also enabled light-controlled PCR (Young, Edwards, Lusic, Lively, & Deiters, 2008). NPOM-caged deoxyguanosine has

been reported to regulate peroxidase activity by photolysis (Lusic, Lively, & Deiters, 2008). The introduction of NPOM caging to 2'-OMe RNA has enabled photochemical OFF→ON and ON→OFF switching of their activity, thus allowing precise control of alternative splicing (Hemphill et al., 2015). Therefore, light can be used as a regulatory agent, allowing tight spatial and temporal control of splice switching in mammalian cells and animals.

Different levels of nucleobase caging have been developed for more complex photoregulation. The NDBF group is used as a photolabile “caging” group to temporarily mask the Watson-Crick interaction of both

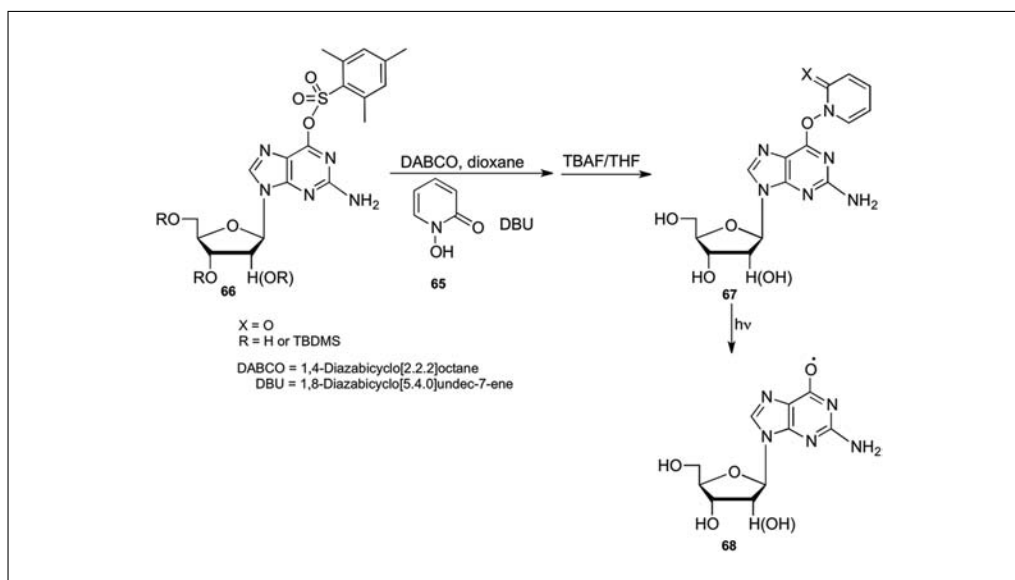


Figure 2.1.41 Scheme for the generation of guanine radical.

dA and dC, exhibiting improved masking capabilities resulting in 12 times faster deprotection rates than the NPE caging groups in these positions (Schäfer et al., 2011). Furthermore, NDBF groups can be selectively removed by irradiation at different wavelengths in the presence of NPE groups, thereby allowing for different (un)caging strategies of oligonucleotides beyond the usual irreversible triggering. dT-pHP can be selectively uncaged at 313 nm without forming undesired cyclic dimers (Rodrigues-Correia, Knapp-Bühle, Engels, & Heckel, 2014.). The O^6 of 2'-deoxyguanosine was protected with the DEACM group, and the corresponding phosphoramidite was incorporated into oligonucleotide. The photochemical deprotection of the resulting oligos was found to be 17 times faster than that of the NPP caging groups in the same position. Wavelength-selective deprotection of DEACM groups in the presence of NPP groups was up to 80 times faster (Menge & Heckel, 2011). DEACM, Bhcmoc, and Bmcmoc are coumarin-type photolabile nucleobase protecting groups with no stereocenters in contrast to some NPE-types protecting groups, thus avoiding chiral separation. Convertible nucleobase residues have been used for rapid access to nucleobase-caged RNA (Rodrigues-Correia, Stoess, Wilhelm, Berlin, & Heckel, 2013).

Photolabile guanine protection for generation of guanine radical

N-Hydroxypyridyl-2(1*H*)-one **65** was used as the photolabile modifier of guanine (e.g., **66**). Novel photolabile 6-[(1-oxido-2-pyridinyl)oxo]-6-deoxy- and 2',6-dideoxy-

guanosine derivatives **67** thus obtained were used for site specific, independent generation of the guanine radical ($G(-H)\bullet$) **68**, proceeding through the homolysis of the N–O bond (Fig. 2.1.41; Kaloudis et al., 2009). The generation of $G(-H)\bullet$ was confirmed through continuous photolysis product analysis and trapping studies, as well as laser flash photolysis experiments.

OTHER DEVELOPMENTS IN NUCLEOBASE PROTECTION

Current strategies for electrophilic solid-phase oligonucleotide synthesis, which include the use of linkers and nucleobase protection cleavable under mild basic and neutral conditions, are highly desirable. In a recent development, 1,3-dithian-2-yl-methoxycarbonyl (Dmoc) and the linker T-Dmoc-CPG were utilized for oligonucleotide-containing electrophilic linkages (Lin, 2013; Lin et al., 2016). Figure 2.1.42 shows the corresponding Dmoc-protected amidites **69a–c**. Dmoc protection of adenine nucleoside **70** was achieved by reacting the silylated nucleoside with reagent **71**, affording **72**, as shown in Figure 2.1.43. The resulting oligonucleotides were successfully deprotected under non-nucleophilic conditions using DBU in three steps. The mechanism of deprotection of Dmoc-protected nucleosides is shown in Figure 2.1.44. This strategy was successfully applied for the synthesis of base-sensitive and electrophilic oligonucleotides. However, the potential of this in standard oligonucleotide synthesis needs to be explored for large-scale oligo synthesis.

Protection of Nucleosides for Oligonucleotide Synthesis

2.1.29

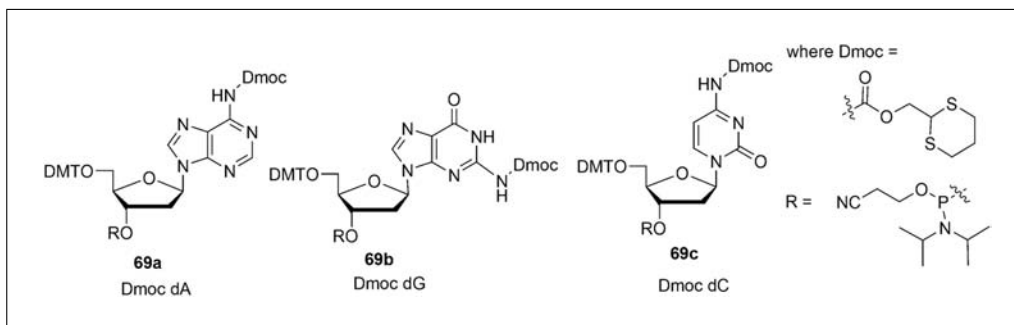


Figure 2.1.42 Dmoc-protected nucleosides for solid-phase oligo synthesis.

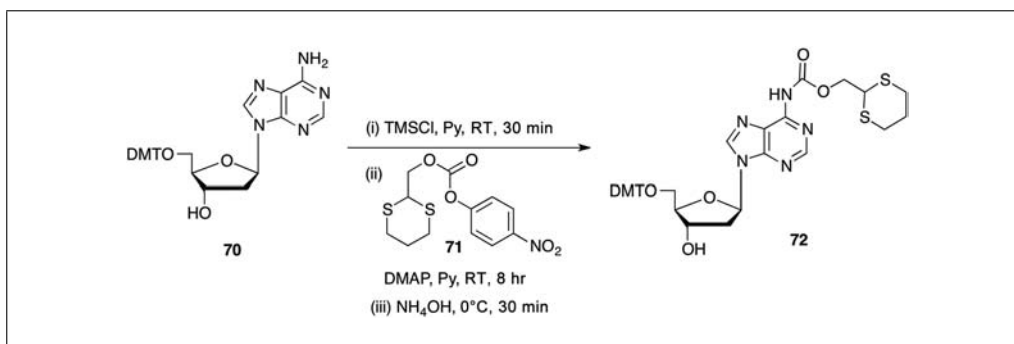


Figure 2.1.43 Synthesis of Dmoc-protected adenosine.

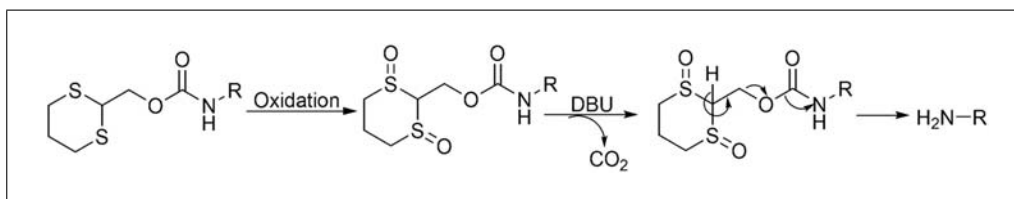


Figure 2.1.44 Mechanism of Dmoc deprotection.

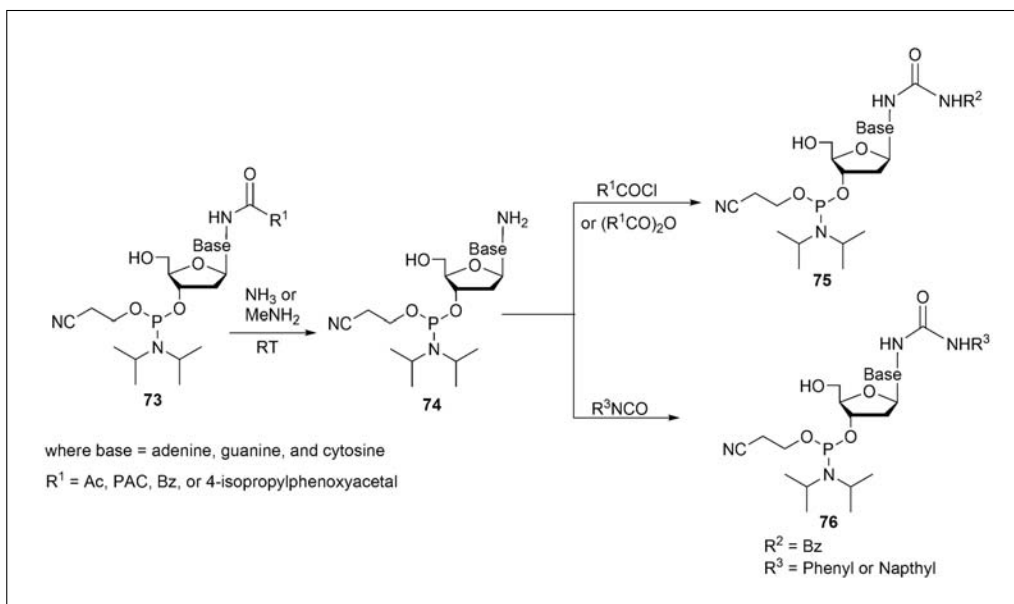


Figure 2.1.45 Conversion of *N*-protected deoxynucleoside phosphoramidites to other *N*-protected deoxynucleoside phosphoramidites.

In an interesting development, commercially available *N*-protected deoxynucleoside phosphoramidites **73** were treated with MeNH₂ or NH₃, affording the highly selective *N*-deacylated product **74**, which was transformed to other *N*-protected species, **75** and **76**, by facile *N*-acylation or *N*-carbamoylation with acylating or isocyanate reagents, respectively (Fig. 2.1.45; Ohkubo, et al., 2005). *N*-deacylated products **74** were successfully used in solid-supported oligonucleotide synthesis on highly crosslinked polystyrene beads using silyl type linker.

CONCLUSION

It is clear that a nucleobase protecting group should meet several criteria before it can be adapted for routine oligonucleotide synthesis. It is imperative, therefore, that the potential for side reactions be closely examined when designing new reagents, evaluating new protecting groups, and implementing modifications of established protocols during oligonucleotide synthesis, as well as during the manufacture of oligonucleotides and their analogs.

The development of nucleobase protecting groups and deprotection protocols has been crucial for the successful synthesis of oligonucleotides, functionalized oligonucleotides, oligonucleotide analogs, ribonucleotides, and phosphorylated biomolecules (reviewed in Beaucage & Iyer, 1992, 1993a,b,c). As in the past, oligonucleotides are expected to play a dominant role in fostering advances in functional genomics, proteomics, diagnostics, and therapeutics. Consequently, continued demand exists for simultaneous synthesis of large numbers of oligonucleotides in miniature formats, and for the synthesis and manufacture of novel analogs. We hope that the present commentary will serve as a framework for developing new protecting group strategies for oligonucleotide synthesis that could meet these challenges.

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