Solid-Phase Supports for Oligonucleotide Synthesis

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ABSTRACT

This unit attempts to provide a reasonably complete inventory of over 280 solid supports available to oligonucleotide chemists for preparation of natural and 3′-modified oligonucleotides. Emphasis is placed on non-nucleosidic solid supports. The relationship between the structural features of linkers and their behavior in oligonucleotide synthesis and deprotection is discussed wherever the relevant observations are available. *Curr. Protoc. Nucleic Acid Chem.* 53:3.1.1-3.1.60. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

In comparison with chemistry in general, synthetic chemistry of nucleic acids is a very young area of research. However, even on this short scale, the chemistry of linkers for oligonucleotide synthesis is a recent development. Indeed, data presented in Figure 3.1.1 demonstrate that this subject hardly existed before 1983, with the more concerted research effort starting around 1989. Events occur in their historic order for a reason. One possible hypothesis as to why the chemistry of solid supports evolved as it has is offered below.

Before the invention of the phosphoramidite method, the synthesis of unmodified oligonucleotides was difficult enough to make derivatized oligonucleotides an unaffordable luxury. After the invention of phosphoramidite chemistry (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981; Caruthers, 1985), it took several years before the novel chemistry transformed into a well established routine method. A detailed account of the research conducted over the first 10 to 12 years of phosphoramidite chemistry can be found in an excellent review by Beaucage and Iyer (1992). At that time, terminal modifications were introduced almost exclusively at the 5′-terminus of oligonucleotides with the use of the respective specialty phosphoramidite building blocks (Beaucage and Iyer, 1993).

In the meantime, the revolutionary methods of sequencing invented in 1977 (Maxam and Gilbert, 1977; Sanger et al., 1977) led to accumulation of a large amount of information on sequences of genomic DNA. This, in turn, had two consequences. First, it armed the antisense method of mRNA silencing invented in 1978 (Zamecnik and Stephenson, 1978; Zamecnik, 1996) with numerous new targets for antisense oligonucleotides. Antisense research required oligonucleotides protected from degradation by exonucleases and equipped with enhancers of cellular internalization. In fact, the very first antisense oligonucleotides were capped at their 3′- and 5′-terminal hydroxy groups by reaction with phenylisocyanate (Zamecnik and Stephenson, 1978). In late 1980s, Paul Zamecnik and his group demonstrated an inhibition of HIV by synthetic oligonucleotides (Zamecnik et al., 1986; Goodchild et al., 1988; Agrawal et al., 1988; Sarin et al., 1988). Soon after, it was confirmed that various 3′-terminal modifications retarded the degradation of synthetic oligonucleotides to a substantial degree (Zamecnik et al., 1986; Stein et al., 1988; Tidd and Warenius, 1989; Orson et al., 1991; Saison-Behmoaras et al., 1991; Shaw et al., 1991; Zendegui et al., 1992). In this regard, solid supports **94** (Fig. 3.1.11; Asseline and Thuong, 1990) and **111a** (Fig. 3.1.13; Nelson et al., 1989) played their catalytic role in promoting the idea of terminal protection of oligonucleotides for in vitro and in vivo applications.

Second, the same pool of genetic sequence information enabled a rapid development of hybridization diagnostics. Again, as imperfect as it was, the solid support **111a** (Nelson et al., 1989) was instrumental in demonstrating the

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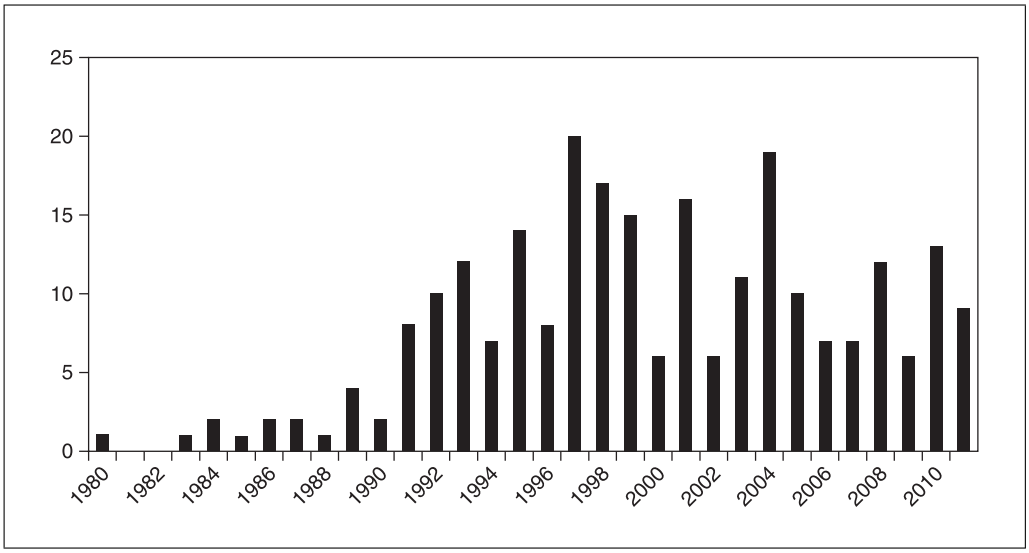

Figure 3.1.1 Annual number of articles on novel non-nucleosidic solid supports published in peer-reviewed journals.

advantages of oligonucleotides possessing two different labels at their 3′- and 5′-termini (in this case, 5′-[^{32}P]phosphate and 3′-biotin) for detection of a single-base-pair mutation.

From this point, the wheels were set in motion. Further motivation for the development of functionalized and labeled solid supports was drawn from the discovery of molecular beacons, oligonucleotides conjugated at their 5′- and 3′-termini to a fluorophore and the matching fluorescence quencher (Tyagi and Kramer, 1996; Wu et al., 2011). Manufacturing of antisense oligonucleotides on large scale and improvements in high-throughput synthesis on small scale created a demand for universal solid supports, which has been met so successfully that nucleosidic solid supports became nearly phased out from production settings. Recent success of conjugation by azide-alkyne 1,3-dipolar cycloaddition ("click" chemistry) led to a surprising observation that a solid-support-bound azido group was not reduced by phosphoramidite building blocks and hence linkers containing it could be conveniently attached to the 3′-terminus of oligonucleotides using the solid support **130** (Pourceau et al., 2009) to be conjugated post-synthetically to pendants containing triple bonds on solid phase or in solution. Those are just a few examples of successful and timely responses of solid-phase chemistry to the emerging challenges.

The previous version of this *Current Protocols* unit contained an excellent review on all aspects of the chemistry of solid supports for oligonucleotide synthesis (Pon, 2000; archived at *http://onlinelibrary.wiley.com/doi/10.1002/0471142700.nc0301s00/full*). To complement and expand that work, the present unit is focused on linkers used in the construction of the solid supports in an attempt to provide a reasonably complete inventory of the large toolbox of solid supports available to oligonucleotide chemists. An emphasis has been made on non-nucleosidic solid supports, although some nucleosidic solid supports specifically designed to introduce the 3′-terminal modifications are also reviewed. The relationship between the structural features of linkers and their behavior in oligonucleotide synthesis and deprotection is discussed wherever the relevant observations are available.

The useful features of over 75 pendant groups and their practical applications are so diverse that they were only mentioned very briefly or, in many instances, were not mentioned at all. Figures 3.1.2 to 3.1.19 present over 280 solid supports reported to date, primarily in peer-reviewed journals. In order to present a full account of linkers and pendant groups, yet to conserve space occupied by figures, some sacrifices had to be made. Wherever a linker is derivatized with a pendant group, the pendant is encoded in figures as R^n rather than as a chemical structure. The relationship between identifiers *n* and chemical structures of pendant groups is set in Figures 3.1.20 to 3.1.23 (see the appendix at end of unit), and in the relevant text. Table 3.1.1 (see the appendix at end of unit) will help readers to locate solid supports derivatized with a pendant or a common protecting group of interest. For instance, tetramethylrhodamine

(TAMRA) is encoded as R^{64} in Figure 3.1.23. Referring to Table 3.1.1, R^{64} is found in solid supports **120g, 120h, 122c**, and **122d**.

SOLID SUPPORT LINKERS FOR OLIGONUCLEOTIDE SYNTHESIS

In this unit, the chemical entity attached to a solid phase for the purpose of conducting oligonucleotide synthesis is divided in two parts serving different functions (Fig. 3.1.2).

The *linker* is a moiety bearing, most often, a hydroxy group to which the first phosphoramidite building block is coupled. In the traditional synthesis of natural oligonucleotides, the linker is a nucleoside that, in the process of oligonucleotide chain assembly, becomes the 3′-terminal nucleoside residue of the product. However, many non-nucleosidic linkers that perform functions other than hybridization with a complementary base have found use in oligonucleotide synthesis. They serve for the introduction of non-nucleosidic 3′-terminal moieties into oligonucleotides and, in the process of final deprotection, may remain unchanged, partially degraded, or

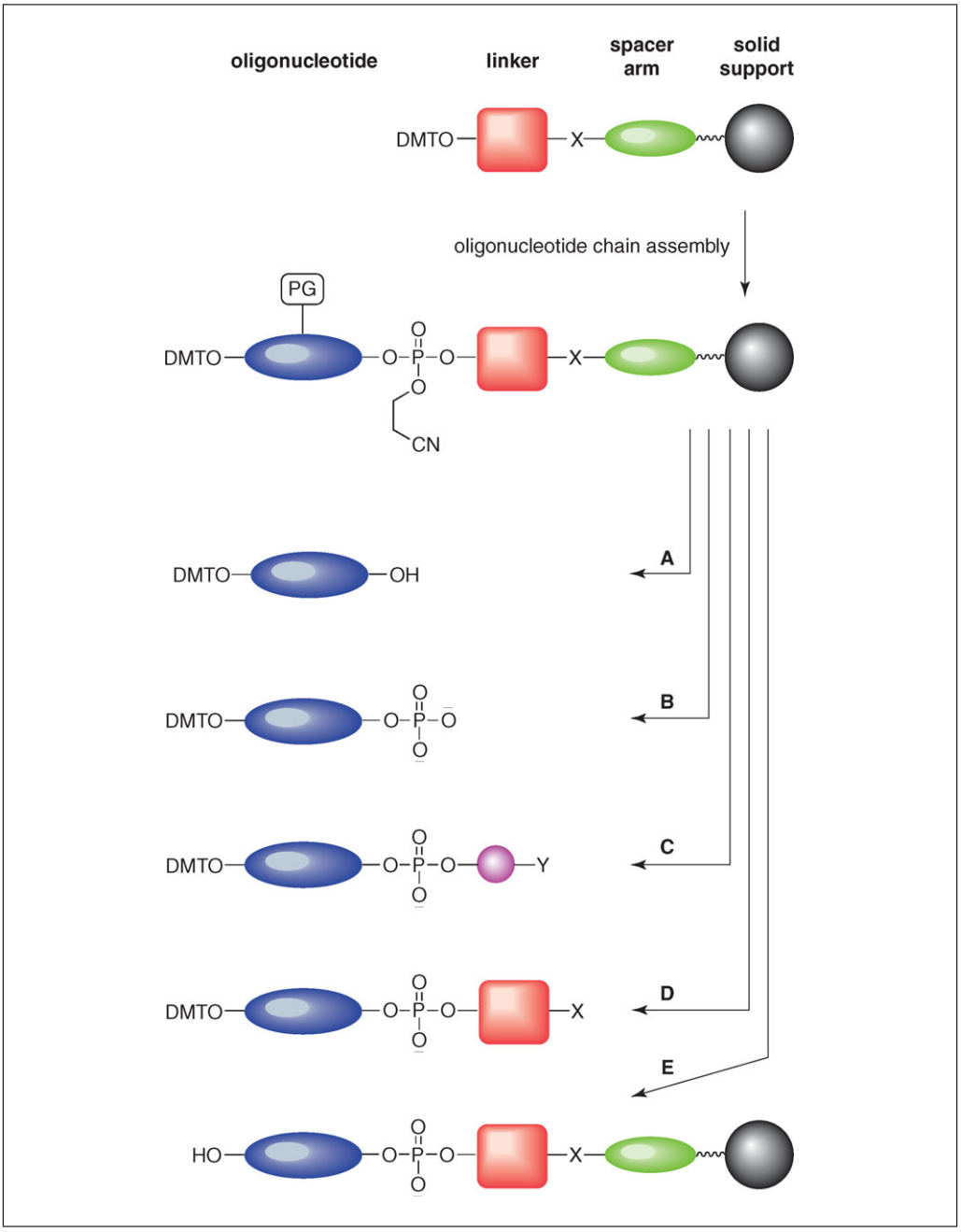

Figure 3.1.2 Schematic presentation of the release of synthetic oligonucleotides from solid supports classified by the type of 3′-modification to be retained in oligonucleotide products.

completely cleaved from the oligonucleotides as in the case of linkers for 3′-terminal phosphorylation. One type of linker, universal linker, is cleaved from the oligonucleotide by removing a portion of the assembled oligonucleotide itself (the 3′-terminal phosphate).

The *spacer arm* is a moiety that attaches a linker to the surface of solid supports. Most often, although not always, the surface of the solid support is pre-derivatized to bear either an amino or hydroxy group to accommodate the spacer arm. Respectively, one terminus of the spacer arm often bears a carboxylic function that can be conveniently coupled to the solid phase. The other terminus is designed for the attachment of the linker via a functional group X (Fig. 3.1.2). Often, the oligonucleotides are released together with the linker moiety by the cleavage of X, which requires this functional group to be cleavable under the conditions of oligonucleotide deprotection.

Although a linker and a spacer arm may be combined in the same chemical structure, most often this is not the case, and both moieties have to complement each other in the interest of a particular application. This requires a careful consideration of their chemical design. First, both the linker and the spacer should be compatible with all the conditions of oligonucleotide synthesis. The spacer arm should allow an efficient attachment of the linker to the support. Furthermore, the linker-spacer tandem must be designed to accommodate specific strategies of the release, which may be either compatible with or orthogonal to the strategies for the deprotection of nucleic bases and internucleosidic phosphates. The many different types of possible linker-spacer combinations can be formally divided into five groups, shown in Fig. 3.1.2:

A. Universal solid supports release oligonucleotides with an unprotected 3′-hydroxy group at their 3′-terminus. The phosphate group that coupled the 3′-terminal nucleoside residue to the solid support is cleaved from oligonucleotides and remains with the universal linker.

B. Solid supports for 3′-phosphorylation, a specific type of a more general group of solid supports referred to as traceless solid supports. As seen from the figure, only the linker and the spacer are removed, while the 3′-phosphate group remains attached to the oligonucleotide.

C. Convertible solid supports. While the release and the deprotection occur, the linker undergoes chemical reactions other than deprotection.

D. Conventional solid supports where the linker becomes a part of the oligonucleotide and does not undergo any chemical reactions other than deprotection. Nucleosidic solid supports are the best known example of this type of the supports.

E. Stable solid supports. In this case, the oligonucleotide only undergoes deprotection but remains permanently attached to the solid phase. Stable attachment of oligonucleotides to solid phases is not discussed in this article.

In all scenarios except for E, the events of nucleic base and phosphate deprotection, release from the solid phase, and, in cases A to C, conversion of the linker, may kinetically occur in a parallel fashion or sequentially in every possible order. For instance, in Scenario B, 3′-phosphorylated oligonucleotides may be formed by degradation of the linker still attached to the solid phase, as in the case of solid support **34** (Efimov et al., 1983). In other cases, as with solid support **40b**, the oligonucleotide has to be first released into the solution together with the linker, which makes possible the subsequent degradation of the linker in solution (Guzaev and Manoharan, 2001a). We do not feel it is necessary or important for further reading of this unit to list all 24 possible combinations of the events. However, we would like to emphasize that being mindful of the order of the events is important for the adoption of existing applications and the development of novel ones. The following sections discuss practical implementations of Scenarios A to D focusing on the behavior and practical applications of linkers.

Scenario A: Universal Linkers

Oligonucleotides with a free 3′-hydroxy group essential for the enzymatic extension are the ones used most frequently in life sciences. Until the late 1990s, the routine synthesis of these oligonucleotides has almost exclusively been carried out on nucleosidic solid supports containing 3′-terminal nucleosides attached via a readily cleavable ester linkage. An alternative approach uses universal solid supports. In this case, the 3′-terminal nucleoside residue is coupled to the universal support as a phosphoramidite building block in the first cycle of oligonucleotide synthesis. The oligonucleotide chain assembly then continues until completion, and the support-bound material is deprotected. It is important that, over the course of the final deprotection, the phosphate bridge formed between the universal linker and the 3′-terminal nucleoside be cleaved in such a manner that it remains with

the universal linker. The net result of the deprotection is the oligonucleotide with the unprotected 3′-terminal hydroxy group identical to that prepared on a nucleosidic solid support.

The indisputable convenience of the nucleosidic solid supports consists in a simple design and ease of the release of the target oligonucleotides. One limitation of this approach is that a minimum of four solid supports is required for the preparation of unmodified oligodeoxynucleotides. As the number of unnatural nucleosides used in oligonucleotides grows, so does the inventory of the respective solid supports for incorporation of these nucleoside residues at the 3′-terminus.

The decisive factor for the industry to switch to universal solid supports was the development of high-throughput DNA synthesizers driven by the growing demand for synthetic oligonucleotides. In these instruments, the solid support is packed in the wells of multiwell plates so that, depending on the format of the plate, 96 or 384 oligonucleotides are assembled simultaneously. When four nucleosidic solid supports were used in a multiwell plate format, each plate had to have its unique configuration of packing. The custom packing of plates with nucleosidic solid supports proved to be a time-consuming and an error-prone operation that could not be performed in advance. In contrast, the use of a universal solid support allowed uniform packing of plates with a single solid support well in advance.

The general requirements for a universal linker are simple. To gain wide acceptance, the universal solid support has to use a conventional phosphoramidite building block as the first monomeric unit and utilize conventional conditions and protocols of oligonucleotide synthesis. The post-synthetic conversion of the linker has to occur according to the route A in Fig. 3.1.2 via the dephosphorylation of the 3′-terminal nucleotide residue. Preferably, the dephosphorylation is carried out under the conditions used for deprotection of nucleic bases and inter-nucleosidic phosphate residues. Finally, the dephosphorylation should result in an oligonucleotide product at least as pure as the one obtained from syntheses on nucleosidic solid supports. As of today, it certainly appears that solid supports that do not comply with these requirements are only of academic interest (see, e.g., Ferreira et al., 2005).

Under nonenzymatic conditions sufficiently mild to maintain the integrity of oligonucleotides, the event of 3′-dephosphorylation only occurs via intramolecular reactions. The phosphate moiety is transferred from the 3′-hydroxy group of an oligonucleotide to a proximate functional group of a linker. To date, the only chemical design in which all these requirements are put in practice is a general structure of an alcohol with an additional, most often vicinal, functional group. The hydroxy group is used for the attachment of the phosphoramidite corresponding to the 3′-terminal nucleoside residue. The functional group has to be rendered unreactive for oligonucleotide chain assembly, but become unprotected for performing the function of phosphate transfer.

In one approach, the functional group was a primary amino group in a β-position to the hydroxy group, as shown in solid supports **16a** (Lyttle et al., 1996) and **16b** (Andersen, E. et al., 2003) in Figure 3.1.4. In the second, more developed design, the 3′-dephosphorylation occurs by intramolecular transesterification of the phosphate with the vicinal hydroxy group protected during the synthesis with an acyl protection. The processes occurring during the 3′-dephosphorylation of oligonucleotides bound to universal linkers are depicted in Figure 3.1.3. Under basic conditions, the solid-support-bound oligonucleotide **1** may lose the acyl protection forming **3** (route A). The intermediate **3** may be deprotected at the phosphotriester moiety to form a phosphodiester **4**. Alternatively, **3** rapidly undergoes 3′-dephosphorylation via a negatively charged transition state **5** leading to the desired product, an oligonucleotide **8** with a free 3′-terminal hydroxy group. The second possible reaction path, route B, begins with decyanoethylation of the phosphotriester residue in **1** to give **2** followed by the removal of the acyl group and the accumulation of **4**. The completely deprotected intermediate **4** is also capable of 3′-dephosphorylation via a transition state **6** to form the same desired product **8** plus 1,3,2-dioxaphospholane **9**.

Examination of the structures of the transition states **5** and **6** reveals that **6** is energetically substantially more demanding because it bears a double negative charge. Indeed, comparison of the rates of dealkylation of ribonucleoside 2′- and 3′-mono- and dialkylphosphates at pH 8 shows that the triesters are hydrolyzed more rapidly by over 2 orders of magnitude (Oivanen et al., 1991; Kosonen and Lönnberg, 1995). Thus, universal linkers operating as required by route A should a priori provide a more rapid kinetics of the 3′-dephosphorylation. That, however, would require removal of the acyl protection

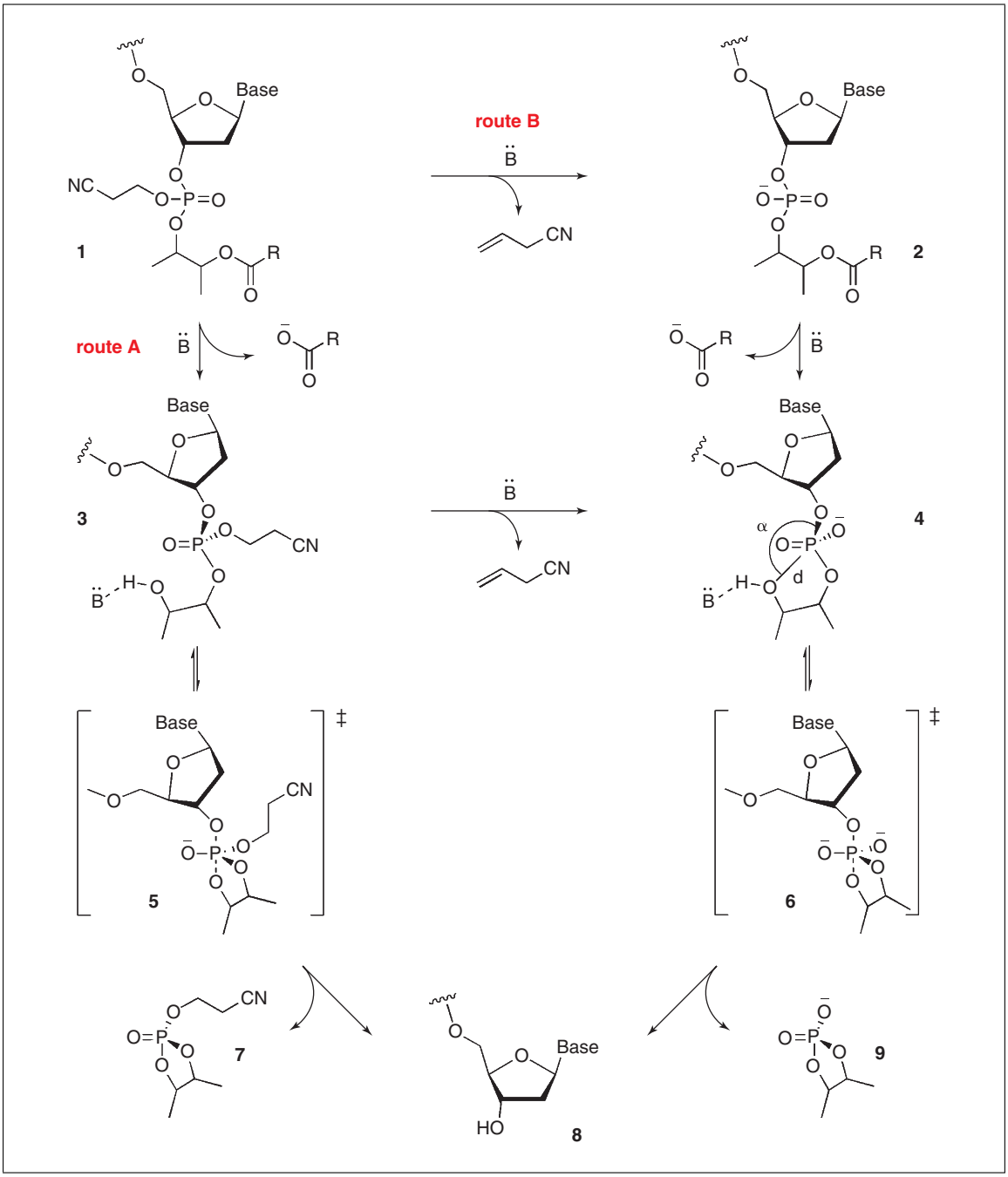

Figure 3.1.3 Mechanism of 3′-dephosphorylation of oligonucleotides assembled on universal solid supports.

in the linker to proceed at least 7 to 10 times more rapidly than the deprotection of the phosphate by β-elimination of 2-cyanoethyl group, which sets very narrow boundaries upon the choice of acyl protecting groups and the conditions for their removal. In turn, route B allows a wide choice of the OH-protecting groups in the linker. However, fine tuning of the structure of the linker itself is required to afford a reasonable rate of 3′-dephosphorylation in the negatively charged phosphodiester **4**. Both approaches currently find their use in oligonucleotide synthesis.

An obvious practical conclusion from the mechanism of the alkaline hydrolysis of RNA (Jarvinen et al., 1991) is the use of ribonucleosides as universal linkers. Since early 1980s (Crea and Horn, 1980; Van der Marel et al., 1982; Cosstick and Eckstein, 1985), multiple reports describe attachment of ribonucleosides to solid phases in an inverted orientation (via N4 of cytidine or 5′-hydroxy group of uridine) to result in solid supports similar to **10** (Fig. 3.1.4, Schwartz et al., 1995). Oligonucleotide synthesis is then performed by the coupling of nucleoside

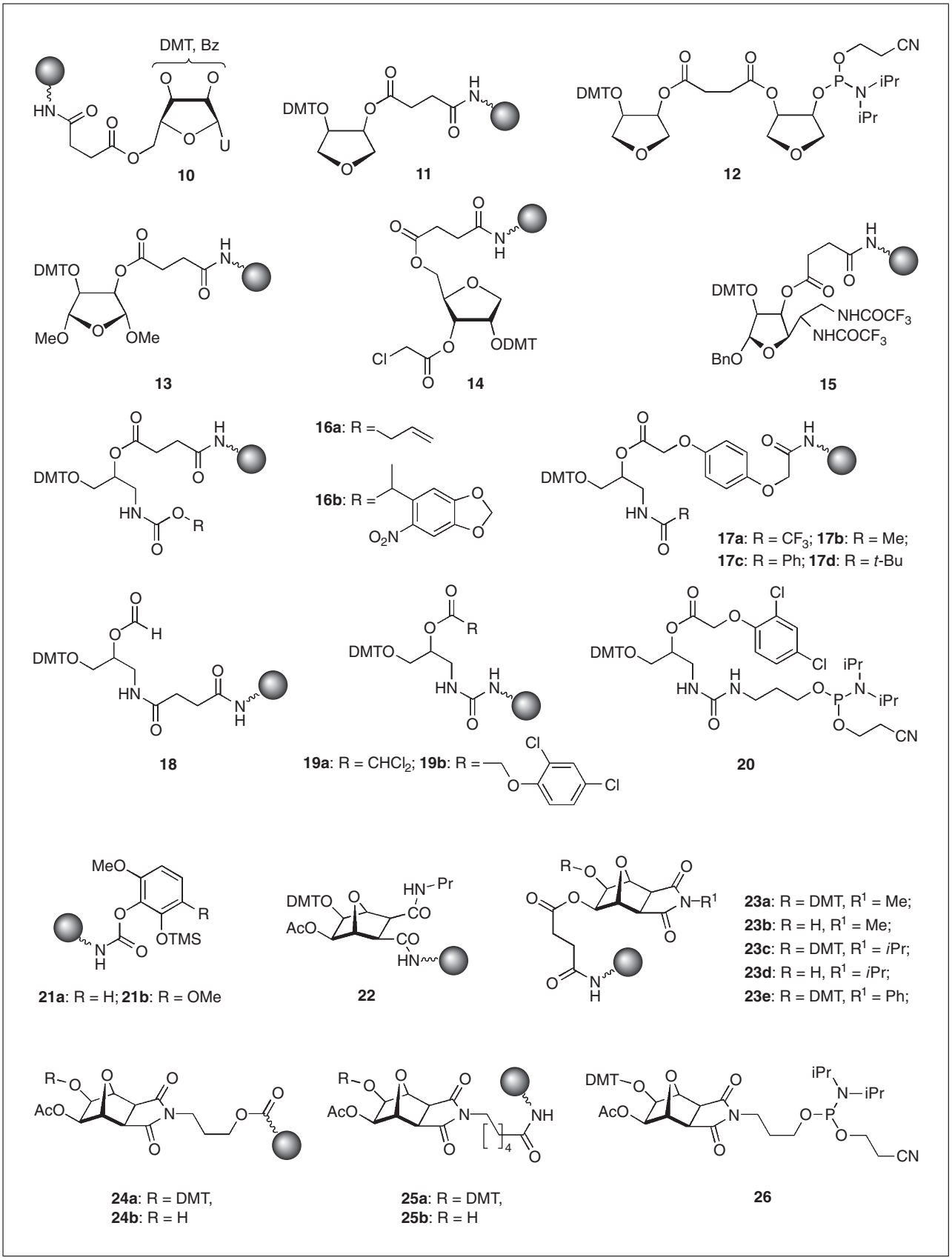

Figure 3.1.4 Structures of universal solid supports **10** to **19** and **21** to **25** and universal phosphoramidites **20** and **26.**

3′-phosphoramidites to one of the 2′ (or 3′) hydroxyl positions of the uridine linker in the normal manner, while the other hydroxy group remains acylated during the course of the synthesis. 3′-Dephosphorylation of the assembled oligonucleotide proceeds primarily according to route B (Fig. 3.1.3), with the hydrolysis of the phosphodiester linkage being the rate-limiting step. Complete deblocking can be performed by extended heating with ammonium hydroxide, although the rate of 3′-dephosphorylation depends on the nature of the 2′-deoxynucleoside adjacent to the universal linker in the following order: dA, dG > T > dC (deBear et al., 1987). Therefore, deprotection with ammonium hydroxide under conditions ranging from 16 hr at 50°C to 24 hr at 65°C were first proposed, and a subsequent paper has used 48 hr at 65°C (Schwartz et al., 1995). To facilitate 3′-dephosphorylation, alternative deprotection conditions were suggested. For instance, the use of 2 N NaOH made it possible to complete dephosphorylation in 10 min at 60°C (Pochet et al., 1987). However, the use of alkali hydroxides is generally not recommended because of possible deamination of cytosine bases. Additional treatment with lead acetate (18 hr, neutral pH, 37°C) carried out upon completion of the standard deprotection with ammonium hydroxide (Gough et al., 1983) did not find wide acceptance either. In order to use the supports in specific applications, minimal modifications to the structure of the linker were carried out. In one study, a ribonucleotide was attached to the solid phase through the N^4-position of a cytosine base (Pochet et al., 1987). In another study, a reversed uridine phosphoramidite reagent was used to convert PEG-polystyrene beads into universal supports (Lyttle, 1999). Additionally, a solid support derived from inosine was suggested (Kumarev, 2001).

Because a nucleic base is not essential for a universal linker to perform its function, the structure of a ribonucleoside has been reduced to a simpler design of 1,4-anhydroerythritol (Hardy et al., 1994). The developed phosphoramidite **(12)** allowed the synthesis of two oligonucleotide sequences in a consecutive manner. Cleavage with AH (concentrated aqueous ammonium hydroxide; 16 hr at 80°C) or AMA (mixture of concentrated aqueous ammonium hydroxide and 40% aqueous methylamine, 1:1 v/v; Reddy et al., 1994) for 16 hr at 60°C was recommended. Coupling of 1,4-anhydroerythritol linker to controlled pore glass (CPG) through a succinic spacer arm gave the solid support **11** (Nelson et al., 1997a,b). 3′-Dephosphorylation of oligonucleotides assembled on **11** was performed using either 0.5 M LiCl/AH (16 hr at 55°C) or 0.23 M triethylamine/0.5 M LiCl/AH (1 hr at 80°C) as cleavage agents. Various sets of equally inconvenient conditions, including aqueous 0.5 M NaCl plus 0.2 M NaOH for 30 min at room temperature, NaOH/MeOH under microwave radiation for 4 min (Kumar and Gupta, 1999); AH plus 1 M spermine plus 0.5 M LiCl at 70°C for 40 min or at 80°C for 10 min (Kumar et al., 2002); 1 M spermine plus 0.5 M LiCl at 60°C for 8 hr (Kumar et al., 2004); 0.05 M LiOH in 3.5 M triethylamine at 75°C for 60 min or 1 M spermine plus 0.5 M $ZnCl_2$ in AH at 60°C for 1 hr (Kumar and Gupta, 2003), were later suggested for similar solid supports containing a 1,4-anhydroerythritol linker.

The structure of the 1,4-anhydroerythritol linker was further improved by the introduction of two methoxy groups in the positions adjacent to the bridging oxygen (Scott et al., 1994). In comparison with **11**, the solid support **13** allowed the dephosphorylation to proceed about twice as rapidly. On the other hand, head-to-head comparison of **13** with inverted uridine solid support **10** did not reveal any advantages of **13** (Azhayev et al., 2004, 2005). The following conditions were recommended (Glen Research, 2008): ammonium hydroxide (80°C, 8 hr), AMA (55°C, 17 hr or 80°C, 3 hr), or 40% aqueous methylamine (55°C, 8 hr).

In an attempt to improve the kinetics of dephosphorylation, a solid support **14** was designed (Scheuer-Larsen et al., 1997). In **14**, the hydroxyl group adjacent to the phosphate linkage was protected with a base-labile chloroacetyl group. More rapid removal of the chloroacetyl protection allowed a larger fraction of the oligonucleotide to react according to route A (Fig. 3.1.3). Complete dephosphorylation would still require approximately the same time as with support **11**. However, a respectable ratio of dephosphorylated species to those still containing the 3′-terminal linker was reached more rapidly (ammonium hydroxide for either 12 hr at 55°C or 72 hr at 22°C).

The observation that various diamines promote the hydrolysis of RNA (Komiyama and Yoshinari, 1997) led to the development of a solid support **15** featuring 1,2-diamine fragment in its structure (Azhayev, 1999). Under the conditions of conventional

deprotection (ammonium hydroxide, 53°C for 27 hr or 80°C for 7 hr; ammonium hydroxide/40% aqueous methylamine, 53°C for 17 hr or 80°C for 2 hr), the solid support **15** somewhat outperformed the support **13**. However, the most interesting feature of **15** was observed on oligonucleotides partially deprotected to the extent that all protecting groups except for the 3′-linker were removed. When this material was heated in water under neutral, buffer-free conditions, the dephosphorylation proceeded nearly to completion in 24 hr at 80°C. Replacement of the phosphate linkage between the 3′-terminal nucleoside residue and the universal linker with a phosphorothioate linkage further facilitated the reaction to afford the 3′-deprotected oligonucleotide in 1 hr at 80°C.

A propensity of an acyclic 3-amino-1,2-propanediol linker (**111a**, Fig. 3.1.13; Nelson et al., 1989) to generate heterogenic pools of oligonucleotides has been reported in a number of communications (Reed et al., 1991; Petrie et al., 1992; Thaden and Miller, 1993; Vu et al., 1995). It was also noticed that the attachment of a nucleoside to the hydroxy group of the solid support via a methylphosphonate linkage, followed by treatment with 1,2-ethylenediamine, generated 3′-dephosphorylated nucleoside. Although under the conditions used both amino and hydroxy groups of the linker were deprotected rapidly, the event of dephosphorylation was attributed to the transesterification of the methylphosphonate diester by the hydroxy group (Thaden and Miller, 1993).

In a later study, a solid support **16a** based on 3-amino-1,2-propanediol, where the primary amino function was protected with *N*-allyloxycarbonyl group, was introduced (Lyttle et al., 1996). Upon completion of oligonucleotide synthesis, the *N*-allyloxycarbonyl protection was selectively removed by treating the support-bound material with tetrakis(triphenylphosphine) palladium(0), triphenylphosphine, and ammonium acetate hydrate in THF at 50°C for 10 min. The subsequent treatment of the solid support with 0.12% ammonium hydroxide in 0.1 M triethylammonium acetate, pH 10, for 2 hr at room temperature released the 3′-dephosphorylated oligonucleotide from the solid support. Addition of 0.5 M LiCl reduced the length of the reaction to 30 min. Finally, the base-protecting groups were removed with ammonium hydroxide for 5 hr at 55°C, which completed the deprotection procedure. Based on the fact that the deprotection of the amino group was carried out selectively, and that the further hydrolysis of the phosphate triester occurred under nearly neutral conditions, the authors explained the dephosphorylation by the attack of the amino group on P(V) of the phosphate triester, which agreed with the similar observations made later regarding the support **15** (Azhayev, 1999). It has to be noted, however, that no special effort has been made to remove Pd species from the solid support by means other than washing with acetonitrile, and traces of this metal could potentially act as a catalyst for dephosphorylation.

A conceptually similar approach to carry out the 3′-dephosphorylation under neutral conditions used a solid support **16b** where the primary amino group of 3-amino-1,2-propanediol bore a photolabile protection (Anderson, E., et al., 2003). Under the optimized conditions, the solid phase was irradiated with UV light at 365 nm for 3 hr to afford the yield of 42% for a 19-mer oligonucleotide.

The performance of 3-amino-1,2-propanediol as a universal linker was further advanced in solid supports **17** and **18** (Fig. 3.1.4; Azhayev and Antopolsky, 2001). To study the efficiency of 3′-dephosphorylation, oligonucleotides **28** to **28f** were synthesized on solid supports **18** and **27a** to **27f** and treated with AH or with 2 M ammonia in MeOH (Fig. 3.1.5). The data obtained suggested that only the fraction of **28** to **28f** where the *O*-acyl protection was cleaved first was 3′-dephosphorylated (route A in Fig. 3.1.5). The phosphotriester **29** that was formed underwent a rapid 3′-dephosphorylation to give oligonucleotide **31** with a free 3′-hydroxy group. However, if the 2-cyanoethyl protection was removed from **28** to **28f** prior to the *O*-acyl group, the formed phosphodiesters **30** to **30f** were hypothetically converted to **33** to **33f**, which did not undergo any dephosphorylation (Azhayev and Antopolsky, 2001). In other words, the dephosphorylation only occurred according to route A in Figure 3.1.5, while route B was completely nonoperational. At the same time, the nature of the *N*-acyl substituent in the solid supports **17a** to **17d** was less important for the outcome of the dephosphorylation. Variation of the *O*-acyl protection in **18** and **27a** to **27f** revealed that the formyl group (solid support **18**) offered a better yield of oligonucleotides. Under the suggested conditions of dephosphorylation (2 M ammonia in anhydrous MeOH, 20°C, 1 hr), 76% of hexathymidylate was recovered from the solid support, while the standard treatment with AH resulted in 42% yield. Solid supports

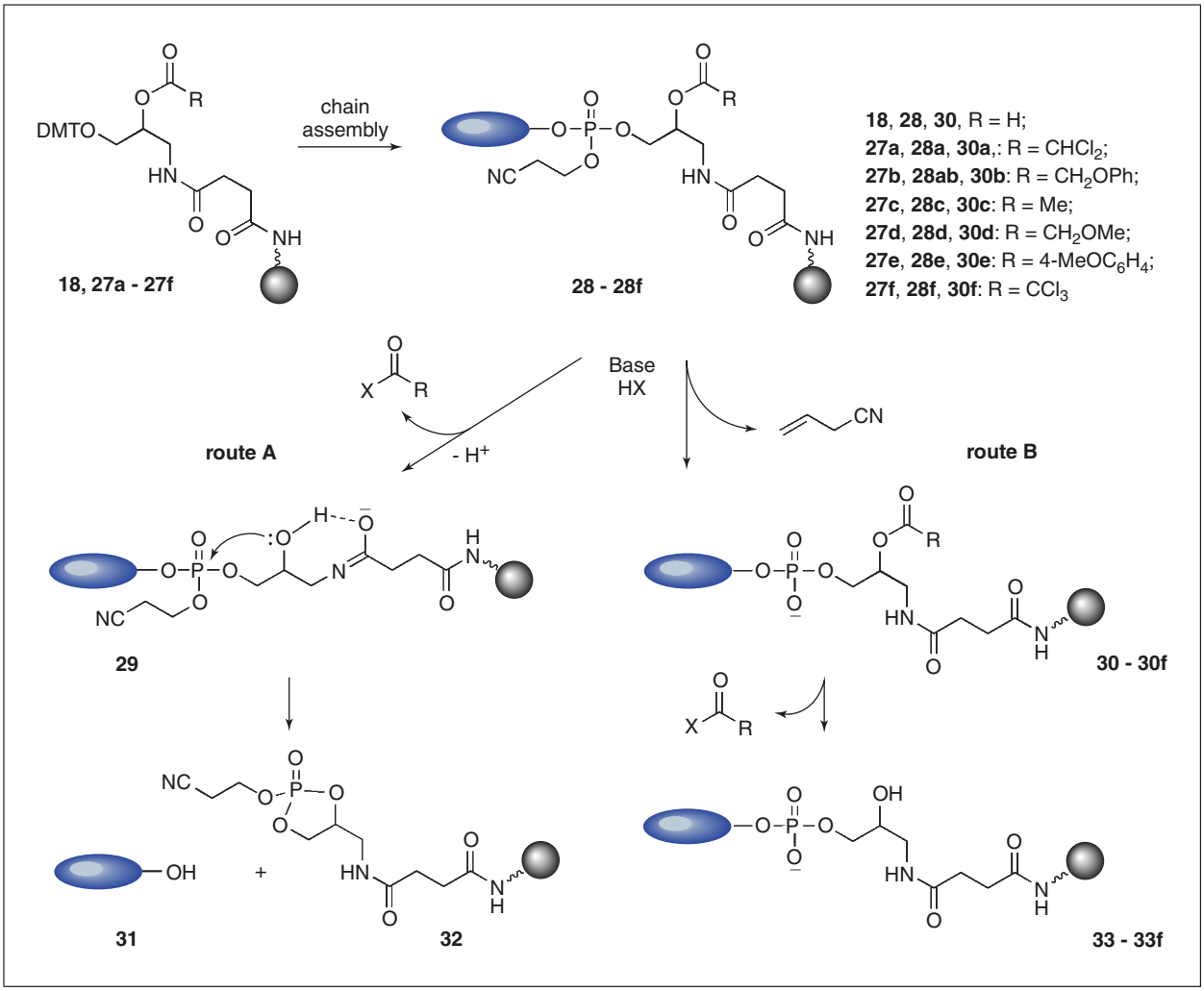

Figure 3.1.5 Processes occurring during the deprotection of oligonucleotides assembled on universal solid supports **18** and **27a** to **27f.**

27a and **27f** afforded yields very similar to those obtained from **18**, while **27c** bearing acetyl protection gave only 4% yield. It was also found later that the low stability of the formyl protecting group did not provide a reasonable shelf life (Yagodkin and Azhayev, 2009).

In a refined version, universal solid support **19a** featured a ureido linkage to the solid phase and dichloroacetyl or 2,4-dichlorophenoxyacetyl protection of the hydroxy group, **19a** and **19b**, respectively (Fig. 3.1.4; Yagodkin et al., 2011). Oligonucleotides synthesized on a more efficient dichloroacetyl version (**19a**) are released in yields ranging from 100% to 105% as compared to nucleosidic solid supports (2 M ammonia in MeOH, 20°C, 1 hr followed by the standard treatment with ammonium hydroxide). The solid support **19a** has a shelf life of 1 year at 4°C and is currently marketed by Metkinen Oy (*http://www.metkinen.fi*) and Glen Research (*http://www.glenresearch.com*). Various versions of these solid supports were used for the preparation of sugar- (Willams et al., 2009) and base-modified oligonucleotides (Seio et al., 2009, 2012). The same core structure was also used for preparation of a universal phosphoramidite building block **20** that allows one to convert any solid support possessing a hydroxy group to the universal support (Yagodkin and Azhayev, 2009).

The major disadvantage of the solid supports **17** to **19** described above is the dependence of the dephosphorylation on route A (Fig. 3.1.3), which sets a very narrow range of deprotection conditions and, consequently, a complex deprotection procedure involving at least two steps. In this respect, a universal solid support that allows the 3′-dephosphorylation to proceed under standard conditions of deprotection is more desirable. To date, only two linkers are capable of meeting this requirement.

One approach is implemented in solid support **21** derived from catechol (Anderson, K.M., et al., 2007). Of the two supports tested, version **21b** where R = OMe proved more efficient. In **21a** and **21b**, the 4,4′-dimethoxytrityl (DMT) protecting group commonly used in oligonucleotide chemistry was found to be too labile, and was replaced with trimethylsilyl (TMS) protection removable in the initial detritylation step. As seen from the structure, the release of oligonucleotides from the solid phase occurs via the hydrolysis of the carbamate moiety and requires, at room temperature, 50 and 20 min in AH or AMA, respectively. The 3′-dephosphorylation continues to proceed in solution according to path B (Fig. 3.1.3). When AH was used, the complete dephosphorylation of nonathymidylate was determined as 9, 6, and 2 hr at 55°, 65°, and 80°C, respectively. When AMA was used as a deprotection medium, the same oligonucleotide was 3′-dephosphorylated in 5, 2, and <1 hr at 55°, 65°, and 80°C, respectively. The support **21b** was also successfully used for RNA synthesis with 2′-*O*-*t*-butyldimethylsilyl- (2′-*O*-TBDMS-) and 2′-*O*-TOM-protected phosphoramidite building blocks. The deprotection was carried out by a mixture of aqueous ammonium hydroxide and ethanol (3:1) for 10 hr at 55°C. Frits embedding **21b** are marketed by CTGen, Inc. (*http://ctgen.com*).

More thoroughly studied and widely accepted in the industry are solid supports of 5,6-dihydroxy-7-oxabicyclo[2.2.1]heptane family **22** to **25** (Fig. 3.1.4). The solid support **22** featuring a chemically stable attachment to the solid phase material via an amide linkage was first synthesized and tested in oligonucleotide synthesis. The release of oligodeoxyribonucleotides, 2′-*O*-methyl, 2′-*O*-(2-methoxyethyl) oligoribonucleotides, and their phosphorothioate analogs by AH from **22** was studied in detail (Guzaev and Manoharan, 2003). The results showed that only 3′-dephosphorylated oligonucleotides were selectively released. 3′-Dephosphorylation proceeded primarily via a phosphodiester intermediate **2** (route B, Fig 3.1.3), although ~10% of the total amount was released via route A. For all sugar and phosphate backbone modifications studied, the rate of the release depended on the nature of the nucleic base in the 3′-terminal nucleoside residue in the order A ≥ C> T > G, with G being the slowest. Oligonucleotides attached to the linker via a phosphorothioate moiety were released 1.2 to 1.7 times more slowly than their phosphate counterparts. 2′-*O*-Modification in the 3′-terminal nucleoside facilitated the release by a factor of 1.5 to 2.3. In the worst case scenario (3′-terminal dG), 95% of the solid support-bound oligonucleotide material was released at 27°C in 4.5 and 7.5 hr for PO and PS backbones, respectively. The study demonstrated full compatibility of **22** with the conditions of oligonucleotide synthesis and deprotection, with the rate of 3′-dephosphorylation being well in the timeframe of the removal of base protecting groups. Direct comparison with a uridine-based universal solid support similar to **10** (Lyttle et al., 1999) showed that the release from **22** was more rapid by a factor of 12.9. An acceleration of the 3′-dephosphorylation was attributed to a locked *syn*-periplanar conformation of the two vicinal C-O bonds in the linker.

The encouraging results obtained from **22** motivated the design and synthesis of a set of solid supports **23** - **25** whose preparation on large scale was more practical (Guzaev and Manoharan, 2004). Linkers in solid supports **23** and **24** are attached to the solid phase via a readily cleavable ester function and are recommended for small scale applications where the rate of the release from the solid phase is critical for overall turnaround time. In contrast, linkers in **25a** and **25b** are attached via a hydrolytically stable amide group. These supports only release 3′-dephosphorylated oligonucleotides, and hence are recommended for large-scale oligonucleotide synthesis. It was found that all the compounds tested served as very efficient universal linkers, although their kinetics of 3′-dephosphorylation was slightly slower than that of **22**. A common feature shared by all DMT-protected universal supports of this family is that the initial removal of the DMT protecting group from **22**, **23a, 23c, 23e, 24a**, and **25a** is 4 to 5 times more slow than that from a solid support-bound 5′-*O*-DMT-thymidine. Users had to maintain and apply two different detritylation protocols for the initial detritylation and for all subsequent cycles of synthesis. It was however found that the shelf lives of unprotected solid supports **23b, 23d, 24b**, and **25b** exceeded 2 years at room temperature, and that conducting another detritylation subroutine on already detritylated supports **23b, 23d, 24b**, and **25b** did not negatively affect the following oligonucleotide synthesis. It is therefore advisable to use the unprotected versions of universal solid supports of this family.

It was also observed that treatment of the linker in **23e** with concentrated ammonium

hydroxide resulted in the release of aniline, a poorly volatile compound that could only be removed by chromatographic purification (Ravikumar et al., 2008). All other solid supports in this series were lacking an *N*-Ph fragment and hence did not suffer from this problem.

Because the event of dephosphorylation occurs according to routes A and B (Fig. 3.1.3), the solid supports **22** to **25** are very tolerant regarding choice of deprotection conditions. Besides conventional treatment with ammonium hydroxide at 60° to 65°C for 8 hr, suggested originally, a number of other deprotection conditions were recommended: AH for 2 hr at 80°C; AMA for 30 min at 80°C min, 65°C for 60 min, and 2 hr at 55°C; gaseous methylamine at 30 psi, for 30 min at 65°C (Glen Research, 2008); 0.05 M K_2CO_3 in anhydrous MeOH, for 17 hr at room temperature; and 25% aqueous *t*-butylamine (v/v), for 4 hr at 60°C (see Glen Research #1 in Internet Resources). For deprotection of oligoribonucleotides and oligonucleotides containing 2′-F-derivatized nucleoside residues, AH/EtOH (3:1 v/v) for 6 hr at 55°C was recommended (Prakash et al., 2005). Deprotection of oligonucleotides assembled on **23b** and **23e** using gaseous ammonia as a deprotection agent was studied in detail (Jensen et al., 2010). For samples wetted with water prior to the treatment with ammonia, the optimal conditions for the 3′-dephosphorylation were 80°C for 120 min or 90°C for 60 min at an ammonia gas pressure of 10 psi (0.69 Bar).

The solid supports **22** and **23e** were further tested in oligonucleotide synthesis on a medium and large scale. Oligodeoxyribonucleotide phosphorothioates and their 2′-*O*-methoxyethyl analogs were synthesized starting from the support **22** on 160 μmol scale (Kumar et al., 2006). The standard deprotection protocol, consisting of removal of 2-cyanoethyl phosphate protecting groups with a mixture of triethylamine:acetonitrile (1:1, v/v) at room temperature for 2 hr followed by release and deprotection with AH for 12 hr at 55°C was implemented. Isolated yields, contents on (*n-1*)-shortmers, and PS/PO ratios of chimeric oligonucleotide phosphorothioates containing 2′-deoxy- and 2′-*O*-methoxyethyl nucleoside residues were identical to those obtained from the syntheses starting from nucleosidic solid supports.

In another report, the solid support **23e** was tested on scales ranging from 0.2 μmol to 700 mmol, with the amount of oligonucleotide product in excess of 2 kg isolated from a single run (Ravikumar et al., 2008). The study confirmed that the high yields of the target oligonucleotides were obtained regardless of the solid support material used (CPG and polystyrenes HL30, PS200, NittoPhase, and OligoPrep) and loading of up to 325 μmol/g. Two additional benefits of using universal solid supports in large-scale oligonucleotide synthesis were recorded. First, the 3′-terminal dA residue suffers from depurination more severely when syntheses are conducted on nucleosidic rather than on universal solid supports. Second, solid supports loaded with nucleosides containing adenine and cytosine bases suffer from partial debenzoylation during their storage. Their use results in the contamination of the products with branched oligonucleotides termed as (2*n*-1) impurities. These impurities are not formed when universal solid supports are used.

The study also confirmed the concomitant formation of aniline during the deprotection of oligonucleotides assembled on the support **23e**.

The solid supports of this family have successfully been applied in the synthesis of natural oligoribonucleotides and their 2′-F-analogs (Prakash et al., 2005, 2009; Tedebark et al., 2011, Lima et al., 2012), LNA oligonucleotides (Ravikumar et al., 2008), oligonucleotides containing 2′,4′-(*N*-methoxy)aminomethylene-, 2′,4′-aminooxymethylene-, and 2′-*O*,4′-*C*-aminomethylene-bridged nucleoside analogs (Prakash et al., 2010), and oxepane and 2′-enopyranose nucleoside analogs (Sabatino and Damha, 2007).

Solid supports **23a** - **23d**, **24**, and **25** and phosphoramidite **26** are marketed by AM Chemicals LLC (*http://www.amchemicals.com/*). Glen Research is marketing **23a** and **23e**. ChemGenes, GE Life Sciences, and Kinovate Life Sciences (*http://www.kinovate.com/*) are marketing CPG and polystyrene versions of **23e**.

Scenario B: Linkers for 3′-Phosphorylation

Oligonucleotide 3′-phosphates and 3′-phosphorothioates find a widespread use in research. They are used as substrates for enzymatic and chemical ligation reactions. A variety of reporter groups and other modifiers can be attached by alkylation of sulfur in the 3′-phosphorothioate moiety. The ability of a phosphodiester and, particularly, of a phosphotriester moiety to serve as a leaving group in reactions of β-elimination and nucleophile substitution at a tetragonal carbon

atom prompted the development of a number of solid supports. As will be seen from the following pages, introduction of this modification is a well developed subject where researchers have a relatively extensive set of tools to their disposal.

Release of 3′-phosphate by elimination

All solid supports of this group are presented in Figure 3.1.6. Since the early years of oligonucleotide synthesis on solid phase, the hydroxyethylsulfonyl group was used in several solid supports as an anchor for 3′-phosphorylated or 3′-thiophosphorylated oligonucleotides.

A solid support **34** is shown with the first nucleotide residue attached. Upon treatment with triethylamine in dioxane for 2 to 3 hr at 20°C, **34** underwent the β-elimination to release oligonucleotides protected with 4-chlorophenyl group (Efimov et al., 1983).

Depending on the deprotection conditions, a similar solid support **35** permitted the release of phosphate-protected or deprotected oligonucleotides (Felder et al., 1984; Schwyzer et al., 1984). Upon assembling of oligonucleotides protected at the phosphate moieties with 4-chlorophenyl group, the protected oligonucleotides could be released by oxidizing the sulfide group of the linker with 0.4 M sodium periodate in water followed by treatment with excess tetramethylguanidine (TMG) in pyridine. Alternatively, 4-chlorophenyl protection was removed first with TMG/*o*-nitrobenzaldoxime. Upon oxidation to sulfoxide as described above, followed by β-elimination with 2 M sodium methoxide in MeOH/pyridine (1:1) for 16 hr, an oligonucleotide 3′-phosphate was released.

A phosphoramidite reagent derived from sulfonyldiethanol was developed by Horn and Urdea (1986) for 5′-phosphorylation of oligonucleotides. This building block, after coupling to solid supports containing hydroxy (Nelson et al., 1997a) or amino groups (Gryaznov and Letsinger, 1993). The preparation of the respective solid support **36a** and a similar solid support **36b** was first described in detail only in 1995 (Efimov et al., 1995). The same linker was attached to the solid phase via a carbamate linkage (Koizumi et al., 1997), although the advantages of the obtained **36c** over **36b** remain unclear. The solid support **36b** has been marketed by Glen Research since mid-1990s. The recommended conditions for deprotection of 3′-terminal phosphate are: AH for 17 hr at room temperature, 4 hr at 55°C, or 0.05 M potassium carbonate in anhydrous MeOH for 8 hr at room temperature (Glen Research, 2011). Others, however, have found that heating for 2 days with AH at 60°C was necessary to complete the removal of the linker (Guzaev et al., 1998). Importantly, deprotection with AMA or initial decyanoethylation of oligonucleotides is not recommended (Glen Research, 2011).

Markiewicz and Wyrzykiewicz (1989) described preparation and properties of thio- and sulfonyl solid supports (**37**). Among the supports tested, that with X = O and Y = SO_2 provided the most rapid kinetics of β-elimination. In all cases, treatment with AH for 24 hr at 55°C was sufficient for complete deprotection of the 3′-terminal phosphate.

An *o*-nitrophenyl linker also served for generation of 3′-phosphorylated oligonucleotides using the solid support **38** (Eritja et al., 1991). Importantly, support **38** permitted the release of the desired oligonucleotides under non-aqueous conditions. To avoid the alkylation of nucleic bases with acrylonitrile, 2-cyanoethyl groups were removed first by 40% triethylamine in pyridine (three 1-hr treatments). The subsequent treatment with 0.5 M DBU in pyridine affected β-elimination in the linker and released the 3′-phosphorylated oligonucleotide. The standard deprotection with ammonium hydroxide was also a viable option for cleaving oligonucleotides from the solid support **38**.

In a recent report, a solid support **39** incorporating a structural motif of 2-cyanoethanol has been described (Paces et al., 2008). Expectedly, the support **39** readily released 3′-phosphorylated oligonucleotides under the standard conditions of oligonucleotide deprotection.

The deprotection chemistry of several solid supports operates on mechanisms that involve several steps including β-elimination. To initiate the release of a 3′-phosphate, linkers in solid supports **40** and **41** require the initial hydrolytic cleavage of an ester function connecting the linkers to the solid phase. Once the hydroxy group of the linker becomes unprotected, the linker undergoes a rapid retro-aldol reaction followed by β-elimination with the release of the 3′-(thio)phosphorylated oligonucleotides and, hypothetically, derivatives of methylenemalonic acid **43**. Under aqueous conditions, the rate-limiting step in the cleavage of **40a** and **40b** is the hydrolysis of the ester

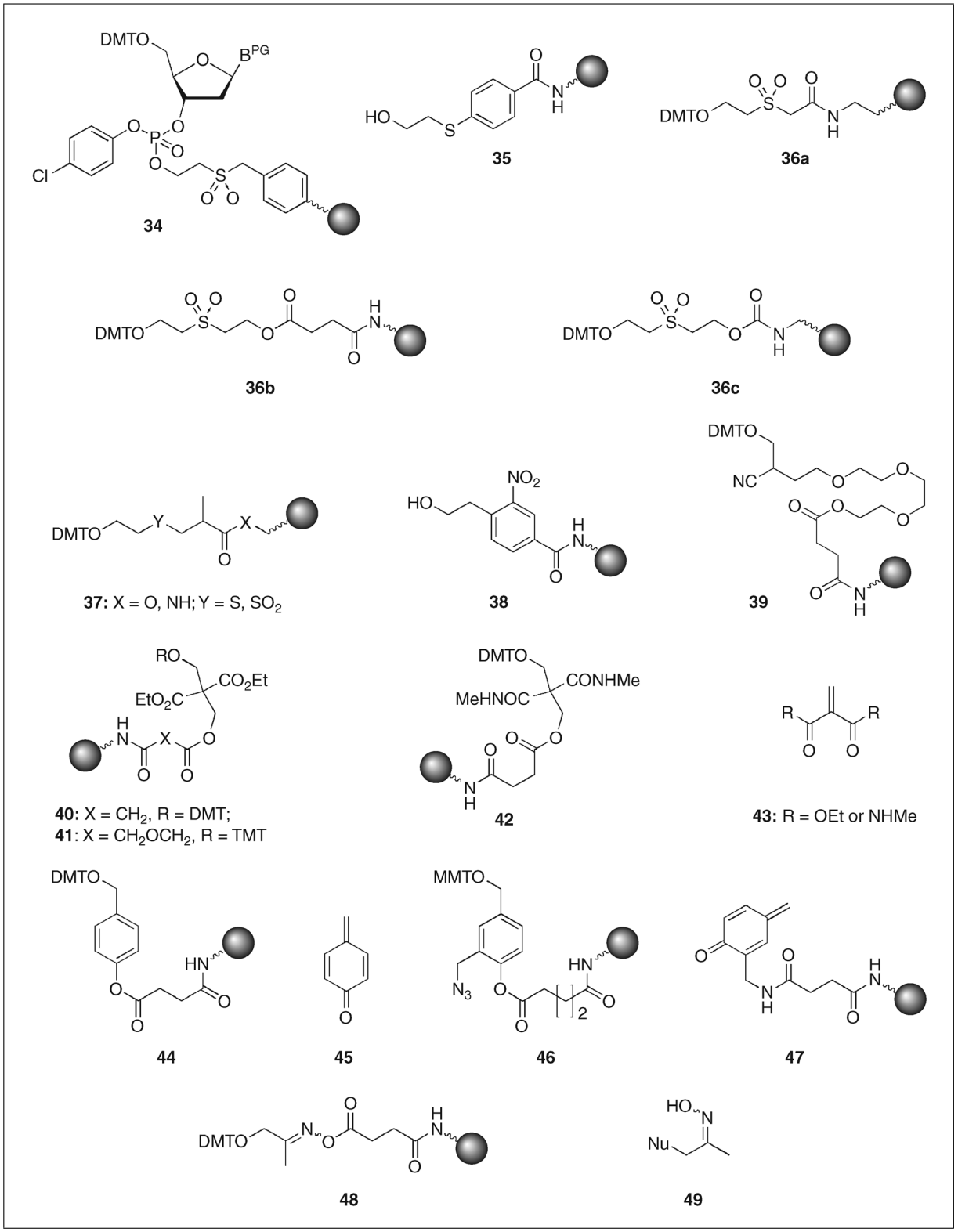

Figure 3.1.6 Structures of solid supports **34** to **42**, **44**, **46**, and **48** for the 3′-phosphorylation of synthetic oligonucleotides using reactions of β-elimination in the linker. Also shown are hypothetic structures of byproducts **43**, **45**, **47**, and **49**, formed by the solid supports **40** to **42**, **44**, **46**, and **48**, respectively.

bond, which was facilitated by using relatively labile malonyl spaces in **40a** (Guzaev and Lönnberg, 1997) or diglycolyl spacer in **40b** (Guzaev and Manoharan, 2001a). In these cases, treatment with aqueous ammonium hydroxide for 20 min at room temperature is sufficient to release the oligonucleotides from the solid phase and deprotect the 3′-phosphate. In 0.05 M K_2CO_3 in anhydrous methanol, β-elimination becomes a rate-limiting step and requires 180 min at room temperature (Guzaev and Lönnberg, 1997). Placement of two ester functions in close proximity to protecting groups of the trityl family makes the latter more stable under the acidic conditions. Accordingly, the removal of DMT group from the solid support **40a** required treatment with TFA (2% in CH_2Cl_2, 40 s) or with DCA (3% in CH_2Cl_2, 5 min). The use of a more labile 4,4′,4"-trimethoxytrityl (TMT) protection in **40b** permits the detritylation to proceed under the standard conditions (DCA, 3% in CH_2Cl_2, 45 sec). The solid support **41** (Guzaev et al., 1999) is commercially available from Glen Research and can be detritylated in a standard manner. However, the presence of two *N*-methylamido groups in place of ethyl ester functions somewhat retards the β-elimination so that the deprotection of the 3′-phosphate requires treatment with AH for 2 hr at 55°C or with AMA for 10 min at 65°C (see Glen Research #2 in Internet Resources).

Other elimination reactions have also been used to release 3′-phosphorylated oligonucleotides from solid supports. 4-Hydroxymethylphenol is useful as a phosphate-protecting group removable under very mild conditions. Attachment of this compound to solid phase gave a solid support (**44**) suitable for preparation of oligonucleotide phosphorothioates bearing 3′-terminal phosphorothioate group (Roland et al., 2001). Deprotection of solid support-bound material with AH overnight at 55°C was sufficient for the removal of the linker, with the postulated concomitant formation of 4-methylene-2,5-cyclohexadienone **45**. Cleavage of benzyl phosphates by treatment with iodine is well known. It remains therefore advisable to use *t*-Bu hydroperoxide for the synthesis of oligonucleotides with phosphate backbone on solid support **44**.

The same mechanism was used in a solid support **46**, where a base-labile TpTp dimer with methyl-protected phosphate groups was released under the neutral conditions of Staudinger reaction (Murata and Wada, 2006). Treatment of a support-bound material with 0.2 M methyldiphenylphosphine in aqueous dioxane reduced the azido group. The following *O*→*N*-acyl migration released the phenolic hydroxy group, which triggered elimination of the 3′-phosphorylated dimer with the formation of the solid support-bound 4-methylene-2,5-cyclohexadienone (**47**). The use of mercaptoethanol as a scavenger of **47** improved the outcome of the release.

Yet another example is presented by an oxime-based solid support **48** (Langenegger et al., 2003). The support **48** was compatible with the conditions of oligonucleotide synthesis, and released 3′-phosphorylated oligodeoxy- and oligoribonucleotides upon the standard deprotection with AH for 16 hr at 55°C or AMA for 30 min at 65°C. Based on literature examples, the cleavage of the linker was believed to proceed via elimination of nitroso-ene, which was expected to be rapidly trapped with ammonia or hydroxide-ion forming **49**.

Release of 3′-phosphate by intramolecular nucleophilic substitution

A large group of solid supports shown in Figure 3.1.7 release 3′-phosphorylated and 3′-thiophosphorylated oligonucleotides by nucleophilic substitution of the (thio)phosphate moiety by a neighboring nucleophilic group. The solid supports **50** to **52** incorporating a disulfide bridge have been described in several examples. The linkers in supports **50** to **52** withstand the conditions of oligonucleotide synthesis on small scale fairly well, although their use on scales exceeding 10 μmol results in lower yields. Upon the completion of oligonucleotide chain assembly, the disulfide bond is reduced using a solution of dithiothreitol (DTT), which releases an oligonucleotide bearing a mercaptoethyl phosphate at its 3′-terminus. Exposure of this material to weak basic conditions ionizes the mercapto function. The subsequent intramolecular nucleophilic attack of sulfide anion on the carbon in P-O-CH_2 fragment results in the formation of thiirane and a 3′-(thio)phosphorylated oligonucleotide.

The release of the 3′-phosphate may be successfully combined with the removal of nucleic base- and phosphate-protecting groups. Thus, oligonucleotides assembled on the support **50** were treated with 0.1 M DTT and 0.4 M NaOH in 20% aqueous MeCN or 0.1 M DTT in AH to release 3′-(thio)phosphorylated oligonucleotides to solutions in the course

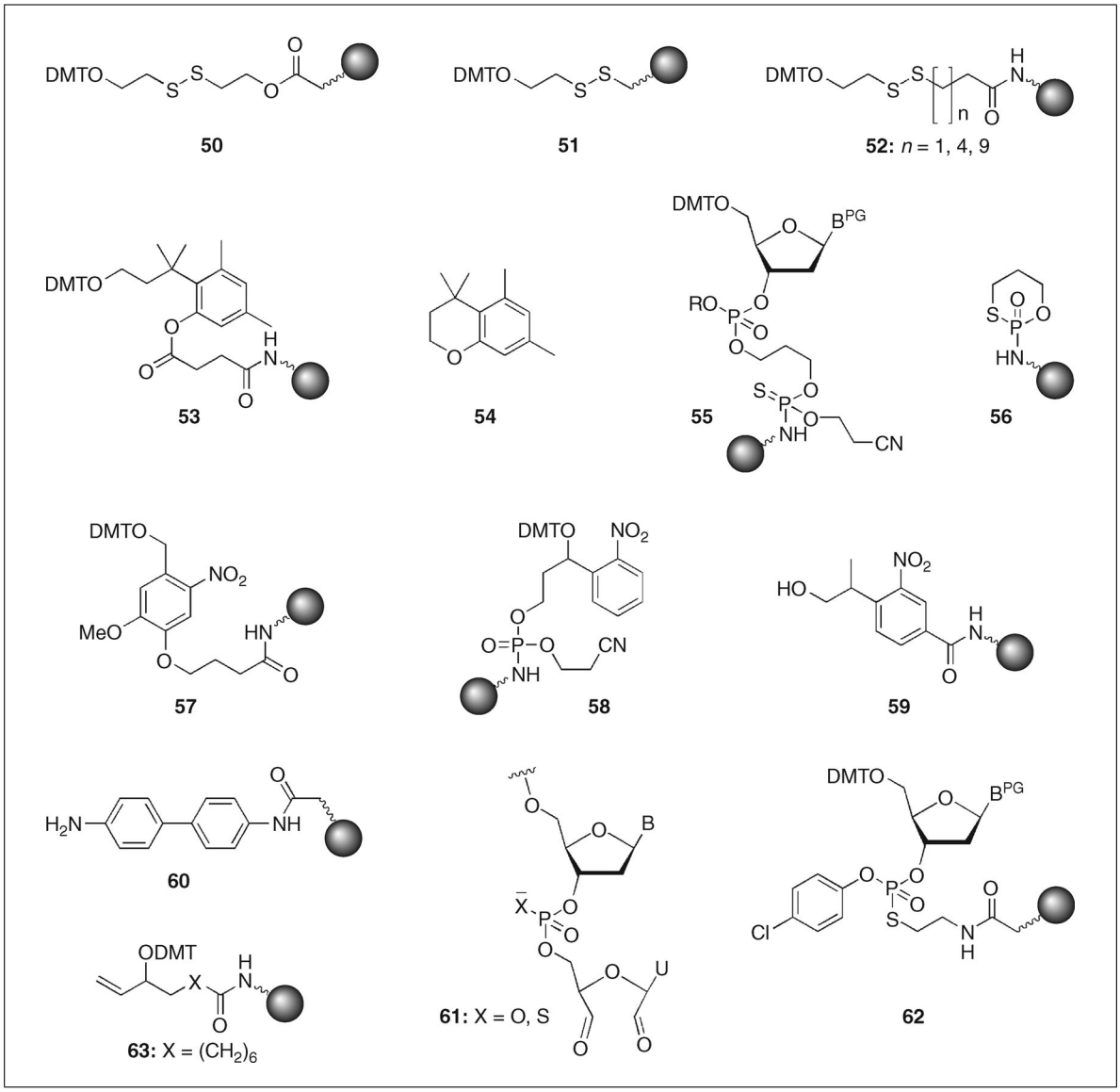

Figure 3.1.7 Structures of solid supports for the 3′-phosphorylation of synthetic oligonucleotides using reactions of intramolecular nucleophilic substitution (**50** to **53**), photolytic deprotection (**57** to **59**), cleavage of phosphoramidite bond (**60**), reactions of oxidation (**62**), and allylic rearrangement (**63**). Also shown are hypothetic structures of byproducts **54** and **56** formed by the solid supports **53** and **55**, respectively. Structure **61** is an intermediate formed from oligonucleotides assembled on solid support **75** (B = U) by oxidation with $NaIO_4$.

of the standard deprotection with ammonium hydroxide for 16 hr at room temperature (Asseline and Thuong, 1989; Asseline et al., 1992).

In contrast to **50**, the solid supports **51** (Kumar et al., 1991) and **52** (Salo et al., 1998) feature a hydrolytically stable connection of the disulfide linker to the surface of a solid phase. When deprotected under conditions similar to those described above for **50**, these solid supports also release the 3′-phosphorylated oligonucleotides. Additionally, solid supports **51** and **52** allow one to carry out the deprotection of nucleic bases and internucleosidic phosphates plus an optional labeling of the 5′-terminal amino groups with fluorescent reporters, while the majority of oligonucleotide material remains attached to the solid phase. During the deprotection of oligonucleotides assembled on **52** [X = $-(CH_2)_9-$] with AH, less than 15% of the solid support-bound oligonucleotide material was lost to the solution. After completing all steps above, the target oligonucleotide was selectively released and deprotected at the 3′-phosphate by treatment with 0.5 M aqueous DTT at pH 8.4 (Salo et al., 1998).

The idea of intramolecular nucleophilic attack was also used in solid support **53** (Cheruvallath et al., 2003). In this case,

oligonucleotides are first released into the solution via the hydrolysis of the ester bond. The deprotected phenolic hydroxy group could then attack the carbon in P-O-CH_2 fragment to form 3′-phosphorylated oligonucleotide, with 4,4,5,7-tetramethyl chroman **54** being an expected by-product. The fully deprotected material is obtained by treatment with AH for 16 hr at 55°C.

The nucleophilic properties of a negatively charged sulfur in phosphorothioate diesters was used in solid support **55**, which is shown with the first nucleotide residue attached (R is a substituted alkyl or 5′-*O*-nucleoside residue rather than 2-cyanoethyl group). Treatment of oligonucleotides assembled on **55** with gaseous ammonia for 16 hr at 10 bar and 25°C removed all protecting groups. In particular, removal of 2-cyanoethyl group in the phosphorothioamidate fragment created a negatively charged thiolate ion, which set the stage for intramolecular cyclization in the linker. However, in the absence of a polar solvent, the oligonucleotide remained attached to the solid phase. The following heating in aqueous 1× PBS, pH 7.2, for 2 hr at 90°C facilitated the nucleophilic attack of thiolate to release the oligonucleotide plus, hypothetically, 2-oxo-[1,3,2]-oxathiaphosphorinan (**56**; Grajkowski et al., 2008). The solid support was elegantly used in the synthesis of various oligonucleotide conjugates at the 5′- and 3′-termini, including circular oligonucleotides. It should be also noted that the solid support **53** was only tested for the release of phosphodiester groups, while it remains unclear whether a negatively charged phosphomonoester can be released in the same manner.

Release of 3′-phosphate by photolytic reactions

Photolysis was expected to offer orthogonal and mild conditions for the release of oligonucleotide that did not use any strong bases or even aqueous solutions. Photolabile support **57** (Fig. 3.1.7) with a linker arm derived from *o*-nitrobenzyl groups was used to release oligonucleotides 3′-phosphate (McMinn et al., 1998). The release of 3′-phosphorylated oligonucleotides was carried out by illuminating the support-bound material for 2 hr at 365 nm to yield 44% to 66% of the target compounds. The photolysis, however, caused small amounts (<3%) of thymine-thymine photodimers, and alkaline or other conditions still needed to be employed to remove base-protecting groups. In addition, the method did not permit the use of *N*-benzoyl-protected dA and dC nucleosides.

A photolabile solid support **58** was tested for preparation of a 3′-phosphorylated methyl phosphotriester analog of dodecathymidylate (Dell'Aquila et al., 1997). The release of oligonucleotides from **58** was carried out by illumination of the solid support-bound material with UV light from a high-pressure Hg-lamp for 15 min at 20°C to result in ∼50% yield for the oligonucleotide. The solid support **58** was also used for the synthesis of 3′-phosphorylated oligothymidylates bearing base-labile *S*-acylthioethyl phosphate protecting groups (Tosquellas et al., 1998a,b) and, using photolabile base-protecting groups, also mixed-base oligonucleotides (Alvarez et al., 1999). Unfortunately, none of these reports allows one to infer isolated yields of oligonucleotides obtained.

In a recent report, solid support **59** was used for preparation of oligonucleotides attached to the hydroxy group of the linker via an internucleosidic phosphate group (Johnsson et al., 2011). Oligonucleotides were released by irradiation for 20 min at 350 mn in MeCN with 1% DIPEA, yielding, after additional deprotection steps, 31% of crude oligonucleotide.

Release of 3′-phosphate by cleavage of phosphoramidate linkage

Cleavage of phosphoramidate linkages was used for the introduction of the 3′-terminal phosphates by two different methods. Anilidate protection of phosphorus removable by alkyl nitrites was extensively used in solution-phase oligonucleotide synthesis by the phosphotriester method (Ohtsuka et al., 1979). This strategy has been applied to preparation of oligonucleotide 3′-phosphates on a solid support **60**, where benzidine was attached to a solid phase material (Fig. 3.1.7; Markiewicz and Wyrzykiewicz, 1989). Upon the completion of chain assembly, 3′-phosphorylated product was released by treatment with a mixture of pyridine, acetic acid, and isoamyl nitrite (1:1:1 v/v/v) for 12 hr at room temperature, followed by the conventional deprotection of the released material with aqueous ammonium hydroxide.

Perhaps the simplest reported method of introducing the 3′-phosphate group used underivatized aminopropyl CPG. The 3′-terminal phosphoramidite was coupled to the amino group of an aminoalkylated solid support (aminopropyl or LCAA CPG) to form a phosphoramidite linkage. After the

oligonucleotide was assembled, the support-bound material was deprotected with AH, which left the oligonucleotide attached to the solid phase. The following treatment with 80% aqueous acetic acid for 4 hr at room temperature released the desired oligonucleotide 3′-phosphate into solution (Gryaznov and Letsinger, 1992). The effect of a rather long treatment of oligonucleotide material with acid on the extent of depurination of oligodeoxynucleotides has not been studied. However, the method was used for preparation of G-cap analogs of short 2′-*O*-Me RNA, where treatment with 80% aqueous acetic acid for 24 hr was permissible (Kadokura et al., 2001).

Release of 3′-phosphate by oxidation

Various oxidative methods and solid supports have been employed for generation of the 3′-terminal phosphate or phosphorothioate group. Thus, placement of a ribonucleotide unit at the 3′-terminus of an oligonucleotide followed by an oxidation of the vicinal diol with sodium periodate gives a dialdehyde intermediate **61** derived from the 3′-terminal uridine (Fig. 3.1.7). Under weakly basic conditions (pH 9.5), **61** undergoes β-elimination to generate the 3′-phosphomonoester group and to cleave the 3′-terminal uridine residue from the oligonucleotide. The method has been successfully used in nucleoside and RNA chemistry since mid-1950s (Dixon and Lipkin, 1954; Whitfeld, 1954) to be adapted later to oligonucleotide synthesis (Kathawala and Cramer, 1967, 1968; Nadeau et al., 1984; Krynetskaya et al., 1986; Bower et al., 1987). A deprotected oligonucleotide 3′-terminated with uridine, whether assembled on a solid support or synthesized in solution, may be converted, by the periodate oxidation described above, to the respective 3′-phosphorylated oligonucleotide that is shorter by the terminal uridine residue. Furthermore, this procedure has been well described by Alefelder et al. (1998). Provided that phosphoramidite coupling to the uridine solid support was followed by sulfurization, the same procedure can be applied to the synthesis of oligonucleotide 3′-phosphorothioates. However, over-oxidation of the phosphorothioate may result in ∼15% contamination of the 3′-phosphorothioate with the respective 3′-terminal phosphate (Alefelder and Eckstein, 1998).

Another method based on oxidation uses a mercaptoalkyl-derivatized solid support. An *N*-succinyl cystamine solid support (**62**) is shown in Figure 3.1.7 with the first 4-chlorophenyl-protected nucleotide attached (Tanaka et al., 1989). On completion of the oligonucleotide synthesis, the phosphate protection was removed using *syn*-pyridine-2-aldoxime and TMG. Next, the treatment with ammonia for 24 hr at 55°C removed the base-protecting groups while the oligonucleotide was left attached to the solid phase. After the resin was treated with 75 mM I_2 in pyridine/water (3:1) for 4 hr, the oligonucleotide 3′-phosphate was released in a high yield.

In a similar method, oligonucleotides were assembled on a mercaptoalkylated solid support of undisclosed structure (Kumar et al., 1997). Support-bound oligonucleotides were deprotection with AH, and the deprotecting agent was removed by washing the solid phase with methanol. The support bound material was then treated with 50 mM $AgNO_3$ for 15 min followed by addition of a double volume of 100 mM DTT. This released the desired oligonucleotide 3′-phosphates in unspecified yield. Reportedly, the method was also applicable to preparation of oligonucleotide 3′-phosphorothioates (Kumar et al., 1997).

Release of 3′-phosphate by Pd-catalyzed allylic rearrangement

Pd^0-Catalyzed deprotection of allyl carbamates and allyl phosphates has been widely used in organic synthesis. In oligonucleotide synthesis, the use of phosphoramidite building blocks protected with allyloxycarbonyl (Alloc) groups at nucleic bases and allyl group at phosphite moiety allowed deprotection of oligonucleotides at nearly neutral conditions (Hayakawa et al., 1990). The complementary solid support, **63**, derived from allyl alcohol has been reported to release oligonucleotides under orthogonal conditions (Fig. 3.1.7; Zhang and Jones, 1996). In testing solid support **63**, the nucleic base and phosphate deprotection steps with AH (3:1 mixture with EtOH) and 2′-*O*-desilylation with TEA/3HF were carried on solid support. The completely deprotected oligoribonucleotide was then released under almost neutral conditions using 0.5 equivalent of tetrakis(triphenylphosphine)palladium and triphenylphosphine in a mixture of *n*-butylammonium formate, pyridine, and THF for 4 hr at 60°C. Purified 8 to 13-mer oligoribonucleotides were isolated in 5% to 13% yield.

Scenario C: Convertible Linkers

Convertible linkers are linkers that, during the course of post-synthetic release from the

solid support, undergo reactions other than deprotection to attain functions not present in the original linker. Reactions of a functional group with a labeling reagent are considered standard labeling reactions and are not included in this chapter.

Conversion of linkers by nucleophilic substitution

A larger group of linkers operates on nucleophilic substitution at ester or thioester function and is presented in Figure 3.1.8. The first reported was a nucleosidic solid support (**63**) where thymidine was derivatized with 3′-*O*-(3-carboxypropyl)oxymethyl group and attached to a hydroxyalkylated solid phase (Hovinen et al., 1993a). Oligonucleotides assembled on **63** were released as 3′-carboxy derivatives by treatment with 0.1 M NaOH.

Alternatively, they were debenzoylated with 0.5 M hydrazinium acetate in pyridine and then were treated with 50% 1,3-propanediamine in EtOH to give analogs terminated with amino group. A more reactive and readily available solid support **66a** was reported later (Hovinen et al., 1993b, 1994). With this solid support, conversion to carboxamido-terminated oligonucleotides proceeded in 98% yield in the course of the standard deprotection with ammonium hydroxide. Treatment with 0.1 M aqueous NaOH (Hovinen et al., 1993b, 1994) or 33% aqueous triethylamine (Hausch and Jäschke, 1998, 2001) resulted a clean conversion to the respective carboxy-terminated analogs. However, the solid support **66a** was still of low reactivity towards amines: 50% solutions of amines in *i*-PrOH or those with the addition of 0.5 M DBU were required to convert solid support-bound oligonucleotides to the amino-terminated analogs in 67% to 88% yield. The use of amines in high concentrations, in turn, required the initial removal of benzoyl protection from dC^{bz} residues in the solid support-bound oligonucleotides by treatment with hydrazinium acetate.

In a more detailed study, solid supports **66b** and **66c** comprising one or two residues of glycolic acid were studied (Hovinen et al., 1994; Guzaev et al., 1995). The higher acidity of carboxylic and α-hydroxy groups of glycolic acid led to the greater reactivity of the ester function in both solid supports, particularly in **66c**. Indeed, only 10% to 20% solutions of α,ω-diamines were required for the successful conversion of the support-bound oligonucleotides to the target compounds in 88% to 91% yield. The lower concentrations of the amines plus the use of aqueous solutions reduced the extent of transamination of dC^{bz} residues to less than 1%, and hence the initial removal of benzoyl groups was no longer required. However, due to the intrinsic relatively low N/O selectivity of ester function towards nucleophilic attack, amino-terminated oligonucleotides were contaminated with 4% to 9% of those bearing terminal carboxy function. A 4-methoxytrityl (MMT) version of the solid support **66c** has been reported later and was used for preparation of oligonucleotides derivatized with 3′-hydrazido group by reaction with 24% hydrazine for 18 hr at 4°C (Achilles and von Kiedrowski, 2005).

The method was substantially improved by taking advantage of the fact that thioesters were much more susceptible to the attack of nitrogen nucleophiles than esters while their rates of base-catalyzed hydrolysis were very similar. Accordingly, oligonucleotides assembled on the solid support **68** were readily converted to product **69**, ammonolyzed to primary amide with AH, converted to acylhydrazides by action of hydrazine hydrate in a mixture of pyridine and acetic acid (5:1), and aminolyzed with 1 M aqueous 1,4-butanediamine in 30 min. With less reactive amines the reaction proceeded more slowly: with 0.2 M aqueous 1-(3-aminopropyl) imidazole (Hovinen et al., 1995), 0.2 M aqueous histamine (Hovinen et al., 1995; Ma and Taylor, 2000, 2001), or 0.5 M aqueous cystamine (Azhayeva et al., 1995a,b; Hakala and Lönnberg, 1997) the reaction required 6 hr. The attachment of aminoalkyl-tethered aza-crown derivatives required overnight treatment with a 0.5 M aqueous solution of the reagent (Niittymäki et al., 2004). An ability to introduce cystamine at the terminus of oligonucleotides proved to be particularly convenient because it provided an expeditious access to 3′-mercaptoalkylated oligonucleotides, useful intermediates in post-synthetic cyclization of oligonucleotides (Azhayeva et al., 1995a,b) and their immobilization on polymeric particles (Hakala and Lönnberg, 1997). Using the solid support **68**, oligonucleotide-3′-carboxylates may be generated by action of 0.05 M aqueous or methanolic K_2CO_3. Very similar convertible, thioester-based solid supports were successfully used in peptide synthesis later (Vlattas et al., 1997).

The reactivity of ester function in solid support **70** and related, more complex supports towards amines was used to generate methylamides of oligonucleotide-cholesterol conjugates **71** (Chaltin et al., 2005).

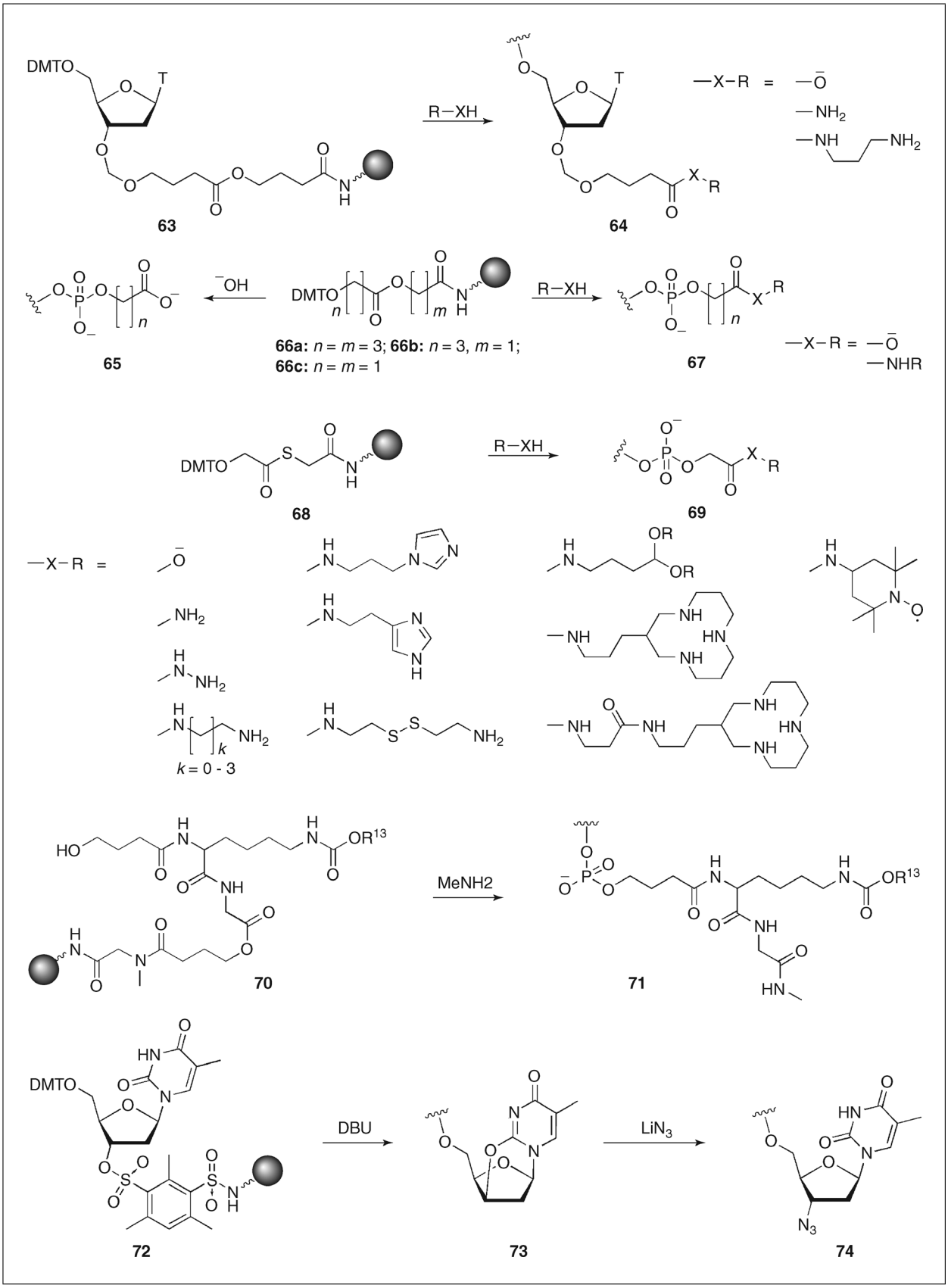

Figure 3.1.8 Structures of solid supports **63**, **66**, **68**, **70**, and **72** convertible under the conditions of nucleophilic substitution, and examples of the 3′-modifiers introduced into synthetic oligonucleotides.

Nucleophilic substitution at sp^3-hybridized carbon was used in solid support **72** (Shchepinov and Stetsenko, 1997). In this case, oligonucleotides comprising dC- and T residues assembled on **72** were treated with a mixture of DBU and DMAA at 70°C for 1 hr. Under these conditions, mesytylenesulfonyl group was substituted with C^2-O of thymidine to form a derivative of anhydrothymidine **73**. The following treatment with LiN_3/DMAA at 100°C for 1.5 hr gave oligonucleotides terminated at its 3′-terminus with 3′-deoxy-3′-azidothymidine **74** in 45% yield.

Conversion of linkers by oxidation of vicinal diols

A large group of convertible linkers presented in Figure 3.1.9 make use of oxidative cleavage of vicinal diols yielding aldehyde groups. Linkers comprising protected vicinal diols are readily available, and their use in oligonucleotide synthesis is well described. The aldehyde groups formed are further reacted with amines, optionally under conditions of reductive amination, or with hydroxylamines, acylhydrazides, or thiosemicarbazides, to form Schiff bases (amines if the reductive conditions were used), oximes, acylhydrazones, or thiosemicarbazones, respectively.

The oldest and the best tested is the solid support **75**, whose primary function is providing the 3′-terminal ribonucleotide unit in conventional oligoribonucleotide synthesis. However, this method of terminal modification has been developed well before the inception of oligonucleotide synthesis on solid phase. As early as in 1960, the formation of hydrazine and semicarbazone was used for labeling of tRNA from yeast, respectively, with 2,4-dinitrophenyl hydrazine (Monier et al., 1960) and 2-hydroxy-3-naphthoic acid hydrazide (Zamecnik et al., 1960). Periodate oxidation was applied later to synthetic oligonucleotides (Kathawala and Cramer, 1967,1968; Nadeau et al., 1984; Krynetskaya et al., 1986), and so was conjugation with various pendants. Because both periodate oxidation and the conjugation reaction proceed in solution, they will be discussed regardless of whether the starting material was isolated RNA or synthetic oligonucleotides terminated with 3′-ribonucleoside units.

We have already discussed above the use of 3′-terminal ribonucleosides for generation of oligonucleotide 3′-phosphates by oxidative cleavage of **75** with basic aqueous $NaIO_4$ via intermediary formation of dialdehyde (**61**; Fig. 3.1.9). In the present case, the oxidation with $NaIO_4$ was conducted under neutral or slightly acidic conditions where the dialdehyde **61** was a relatively stable intermediate. The following conditions were reported for preparation of dialdehyde **61**: 0.1 M $NaIO_4$ in 0.05 M NaOAc, pH 5.0, for 1 hr at room temperature in the dark (Zamecnik et al., 1960); 5 mM $NaIO_4$ (~100-fold excess) in 50 mM NaOAc, pH 5.0 for 1 hr at 4°C in the dark (Agrawal, 1994); 0.1 M $NaIO_4$ for 15 min at room temperature (Timofeev et al., 1996); 50 mM $NaIO_4$ for 10 min at room temperature (Guschin et al., 1997); 25 mM $NaIO_4$ (2-fold excess) in 50 mM sodium borate buffer, pH 8.6, for 10 min at room temperature in the dark followed by quenching with excess glycerol for 10 min (Alefelder et al., 1998).

Although the oxidation yields two aldehyde groups, each of which can be theoretically used for the attachment of a pendant group, the conjugation of **61** yields only 1:1 adducts regardless of the nature of the nitrogen nucleophiles. Product distribution and kinetics of the conjugation of **61** (B = G) with acylhydrazides has been studied by Achilles and von Kiedrowski (2005). At pH 6.1, the reaction of **61** with acylhydrazide was too rapid to be monitored by HPLC. At pH 7.5, the reaction became sufficiently slow to observe a smooth formation of the conjugate **76a**, with yields reaching their maximum of about 80% in 12 to 20 hr at 30°C. It was also observed that **76a** was formed as a mixture of at least three diastereomers and that the starting acylhydrazine was consumed, to a minor extent, in a side reaction with **76a** to form oligomeric products.

A variety of terminal modifiers attached at the 3′-terminus of RNA and oligonucleotides are listed below in accordance with the nature of the nitrogen nucleophiles reacted with the dialdehyde **61**:

Acyl hydazides: 2-hydroxy-3-naphthoic acid hydrazide (Zamecnik et al., 1960), proflavinyl acetic acid hydrazide (Beardsley and Cantor, 1970), carbohydrazide, proflavine monosuccinic acid hydrazide, anthracene-9-carbohydrazide, and 1-pyrenylbutyric acid hydrazide (Reines and Cantor, 1974). Reaction with carbohydrazide formed an intermediate that was subsequently conjugated with a variety of pendants containing aldehyde groups (Reines and Cantor, 1974). Oligonucleotides **61** were also immobilized on polyacrylamide gel containing acylhydrazide groups (Khrapko et al., 1991; Timofeev et al., 1996; Guschin et al., 1997).

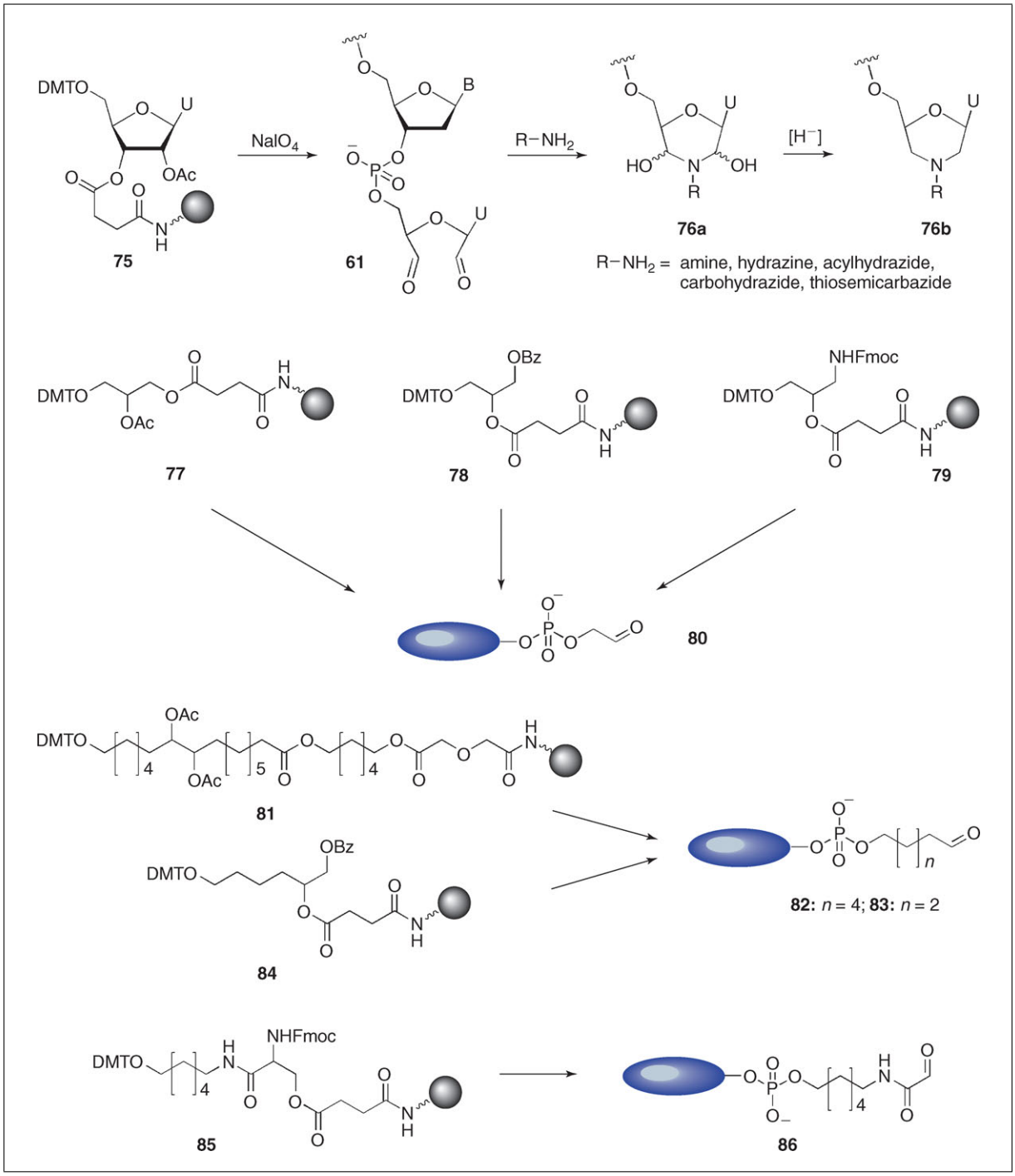

Figure 3.1.9 Structures of solid supports **75**, **77** to **79**, **81**, **84**, and **85** convertible under the conditions of oxidation and examples of the 3′-modifiers introduced into synthetic oligonucleotides.

Thiosemicarbazides: fluorescein thiosemicarbazide (Epe et al., 1982).

Hydrazines: 2,4-dinitrophenylhydrazine (Monier et al., 1960) and 9-hydrazino acridine (Beardsley and Cantor, 1970).

Primary amines: acriflavine (Churchich, 1963; Millar and Steiner, 1966; Beardsley and Cantor, 1970), 1,5-diaminopentane (Broker et al., 1978), 1,6-diaminohexane (Agrawal, 1994), and poly-L-lysine (Bayard et al., 1986; Lemaitre et al., 1987). Oligonucleotides **61** were also immobilized on the surface of polyacrylamide gel containing primary amino groups (Pevzner et al., 1991; Timofeev et al., 1996). The subsequent reduction led to the stable derivatives of 3′-morpholino nucleoside analogs **76b** (Khrapko et al., 1991; Agrawal, 1994; Timofeev et al., 1996). The latter step was conducted using $NaBH_4$ (Lemaitre et al., 1987; Agrawal, 1994) or

pyridine-borane (Timofeev et al., 1996) as reducing agents.

Non-nucleosidic structures have also been extensively used for introduction of a vicinal diol moiety and for generation of an aldehydo group at the 3′-terminus of oligonucleotides. Upon the completion of oligonucleotide chain assembly and deprotection, isomeric solid supports **77** (Urata and Akagi, 1993) and **78** (Achilles and von Kiedrowski, 2005; Chillemi et al., 2006) derived from glycerol gave identical 3′-glycerophosphate derivatives of oligonucleotides. Solid supports **81** (Skrzypczynski and Wayland, 2003) and **84** (Achilles and von Kiedrowski, 2005) served the same function of generating a 3′-terminal vicinal diol. They differ from **77** and **78** in that longer spacers were introduced between the 3′-terminal phosphate and the diol fragment.

Aldehyde **80** was prepared from the 3′-glycerophosphate oligonucleotides under the following conditions: 5 mM $NaIO_4$ for 1.5 hr at 0°C followed by quenching the excess periodate with L-methionine (Urata and Akagi, 1993); 20-fold excess of $NaIO_4$ for 1 hr at room temperature (90% isolated yield, Villien et al., 2004); 50-fold excess of $NaIO_4$ for 1 hr (Edupuganti et al., 2003, 2004a,b); 1.4-fold excess of $NaIO_4$ for 75 min at room temperature followed by 20-fold excess of $Na_2S_2O_3$ (Achilles and von Kiedrowski, 2005); and 90 mM $NaIO_4$ (165-fold excess) for 10 min at room temperature (Meyer et al., 2010). To obtain aldehyde **82**, the respective diol was reacted with 10 mM $NaIO_4$ (10-fold excess) for 30 min at pH 7.6 and room temperature in the dark (Skrzypczynski and Wayland, 2003). Aldehyde **83** was prepared using 1.4-fold excess of $NaIO_4$ for 75 min at room temperature followed by 20-fold excess of $Na_2S_2O_3$ (Achilles and von Kiedrowski, 2005).

Solid supports **79** (Forget et al., 2001) and **85** (Spinelli et al., 2007) lead, upon conducting oligonucleotide synthesis and basic deprotection, to oligonucleotides derivatized at the 3′-terminus with 1,2-aminoalcohols. These could also be readily oxidized to the respective aldehydes **80** and **86** using excess $NaIO_4$. The solid support **79**, however, proved unreliable as its use led to poorly reproducible results at the steps of oligonucleotide synthesis and deprotection (Villien et al., 2004; Singh et al., 2005). In view of the discussion for solid supports **16** to **20** (see above), this conclusion is not very surprising.

Collectively, the results compiled above demonstrate that the oxidation with $NaIO_4$ is fairly robust; it does not result in any appreciable degradation of nucleosidic residues, nor does the yield of aldehydes suffer from minor variations in the concentration of $NaIO_4$, the time, and the temperature. Conveniently, 3′-phosphoglycaldehydes **80** were stable in solution for at least 1 week (Urata and Akagi, 1993). Glyoxylic aldehyde **86** was stable for several months at −20°C (Spinelli et al., 2007).

Aldehydes **80**, obtained with the aid of solid support **77** to **79** were primarily used for conjugation with peptides via oxime bond formation for preparation of cyclic oligonucleotides and conjugation with peptides and derivatives of fluorescein, lactose, and mannose (Forget et al., 2001; Edupuganti et al., 2003, 2004a,b; Villien et al., 2004; Meyer et al., 2010). The stability of the oxime conjugates from **80** and **86** was limited and dependent on the storage medium (Spinelli et al., 2007). Thus, oximes obtained from 3′-phosphoglycaldehyde **80** and hydroxylamine-derivatized RGD peptide were labile under mild acidic conditions (>50% hydrolysis in 120 hr at pH 4.0) and were stable at pH 9.0 (<6% hydrolysis in 120 hr). In contrast, oximes from glyoxylic aldehyde **86** and the same peptide were relatively stable in acidic media (less than 3% hydrolysis in 120 hr at pH 4.0) and were hydrolyzed under basic conditions (36% hydrolysis in 120 hr at pH 9.0).

Several other applications of oligonucleotides **80** and **82** have been reported. Reductive amination of oligonucleotide **82** synthesized on **81** allowed the preparation of conjugate with bis(3-aminopropyl)-terminated polytetrahydrofuran (Skrzypczynski and Wayland, 2003). Aldehyde **80** smoothly reacted with RGD peptide containing cysteine at its *N*-terminus at pH 5 in 5 hr (Villien et al., 2004). The thiazoline cycle thus formed was hydrolytically unstable and quantitatively decomposed to the starting materials under neutral conditions in 24 hr. The 3′-phosphoglycaldehyde **80** was also reduced to the corresponding alcohol by $NaBH_4$ or oxidized to 3′-phosphoglycolate with $NaClO_2$ (Urata and Akagi, 1993).

The optimistic picture of a problem-free conjugation of aldehydes derived from solid supports **77** to **79** and **84** presented above was questioned more recently by Achilles and von Kiedrowski, who studied product distribution and kinetics of conjugation of 3′-aldehydo oligonucleotides **80** and **83** with acylhydrazides at pH 6.1 and 30°C (Achilles and von Kiedrowski, 2005). In contrast to **61**, 3′-phosphoglycaldehyde **80** formed, in

addition to the target conjugate, two main side products. Apparently, **80** suffered from the oxidative loss of glyoxal to form the respective 3′-phosphorylated oligonucleotide. Glyoxal was believed to react further with nucleic bases containing amino groups. Under the same conditions, the aldehyde **82** formed only a small percentage of the desired conjugate, while the main material was consumed in a side reaction, which proceeded independently of the presence of the acylhydrazide. Finally, the study questioned the usefulness of acylhydrazides as conjugation reagents, as they evidently formed polymeric side products with oligonucleotides.

Scenario D: Conventional Linkers

Conventional linkers are defined as linkers that, in the process of deprotection, do not undergo any chemical conversions other than deprotection of functional groups. Because of a broad range of applications and hence the widest structural diversity, conventional linkers constitute the largest group of linkers.

Most protected nucleosides attached to solid supports formally belong to this group, too. Nucleosides are attached to solid phases via the same spacer arms as all other linkers. The stability and the conditions of the cleavage of spacer arms remain relatively independent of whether the arm is used to attach a nucleoside or any other linker with a secondary hydroxy group. Protection strategy and stability of solid support-bound nucleosides under the conditions of oligonucleotide synthesis and deprotection are very important questions. However, the properties of nucleosides attached to solid phases do not substantially differ from those of nucleoside residues in any other position of oligonucleotide chain. In turn, protection strategies for phosphoramidite building blocks have been covered by a number of excellent reviews (Beaucage and Iyer, 1992; Sonveaux, 1994; Iyer, 1999; Bellon and Wincott, 2000; Hayakawa, 2001; Hayakawa et al., 2001; Scaringe, 2001). Therefore, nucleosidic linkers are excluded from the scope of this unit.

Linear linkers

Linear linkers are the most reliable linkers in oligonucleotide synthesis for more than one reason. First, they often bear only one functional group protected by the attachment to the solid phase. Second, the protected functional group can be readily separated from the nearest phosphotriester or phosphodiester moiety by a number of atoms sufficiently large to ensure the absence of intramolecular reactions associated with these two functions. The simple and straightforward design of linear linkers results in 3′-derivatized crude oligonucleotides as pure or almost as pure as their underivatized counterparts. However, oligonucleotides carrying an application-oriented 3′-pendant group are required more often than those with a 3′-function. When linear linkers are used, the pendant has to be introduced in an additional post-synthetic reaction in solution phase. The fact that the post-synthetic labeling is more laborious and more time consuming than the synthesis and deprotection of the parent 3′-functionalized oligonucleotide constitutes the greatest disadvantage of linear linkers.

Linear solid supports for 3′-hydroxyalkylation

3′-Hydroxyalkyl modification is, perhaps, the simplest one to introduce. Indeed, while the technique of attaching α,ω-alkanediols to solid phases is identical to that for 2′-deoxynucleosides, the former enjoy the structural simplicity rarely seen in oligonucleotide chemistry. A number of diols bearing a DMT protecting group at one terminus and succinate arm at the other were attached to solid phases to give the respective solid supports shown in Figure 3.1.10: 1,6-hexanediol, **87a**, (Hinrichsen et al., 1992; Walsh et al., 1997), 1,3-butanediol, **87b**, (De Vos et al., 1994), ethyleneglycol, **88a**, ($n = 1$; Koizumi, 1997) and its linear oligomers of various lengths, **88b** to **88d** ($n = 6$ to 12, 16 to 32, and 70 to 110, respectively; Jäschke et al., 1993), **88e** to **88g** ($n = 5$ to 14, 13 to 32, and 40 to 120, respectively; Jäschke et al., 1994), and **88h** ($n = 6$; Koizumi et al., 1997).

In a different design, 1,3-propanediol (Lyttle et al., 1997) or a mono-DMT-protected 1,12-dodecanediol (Laing et al., 2010) were attached via one of the carboxylic groups of trimellitic acid to give solid supports **89a** and **b**, respectively. In both cases, one of the carboxylic functions of trimellitic spacer remained unprotected, which was, perhaps, less than an ideal design.

An interesting approach for preparation of oligonucleotides derivatized at their 3′-terminus with ethylene glycol, 1,6-hexanediol, or di-, tri-, tetra-, and hexaethyleneglycols was developed by Laurent and co-workers (Laurent et al., 2004; Laurent and Chaix, 2006). The diols were adsorbed on the surface by heating a native CPG (CPG with underivatized surface) with 3.7to 7.5 M solutions of diols in diglyme for 16 hr at 80°C. The diols were retained on

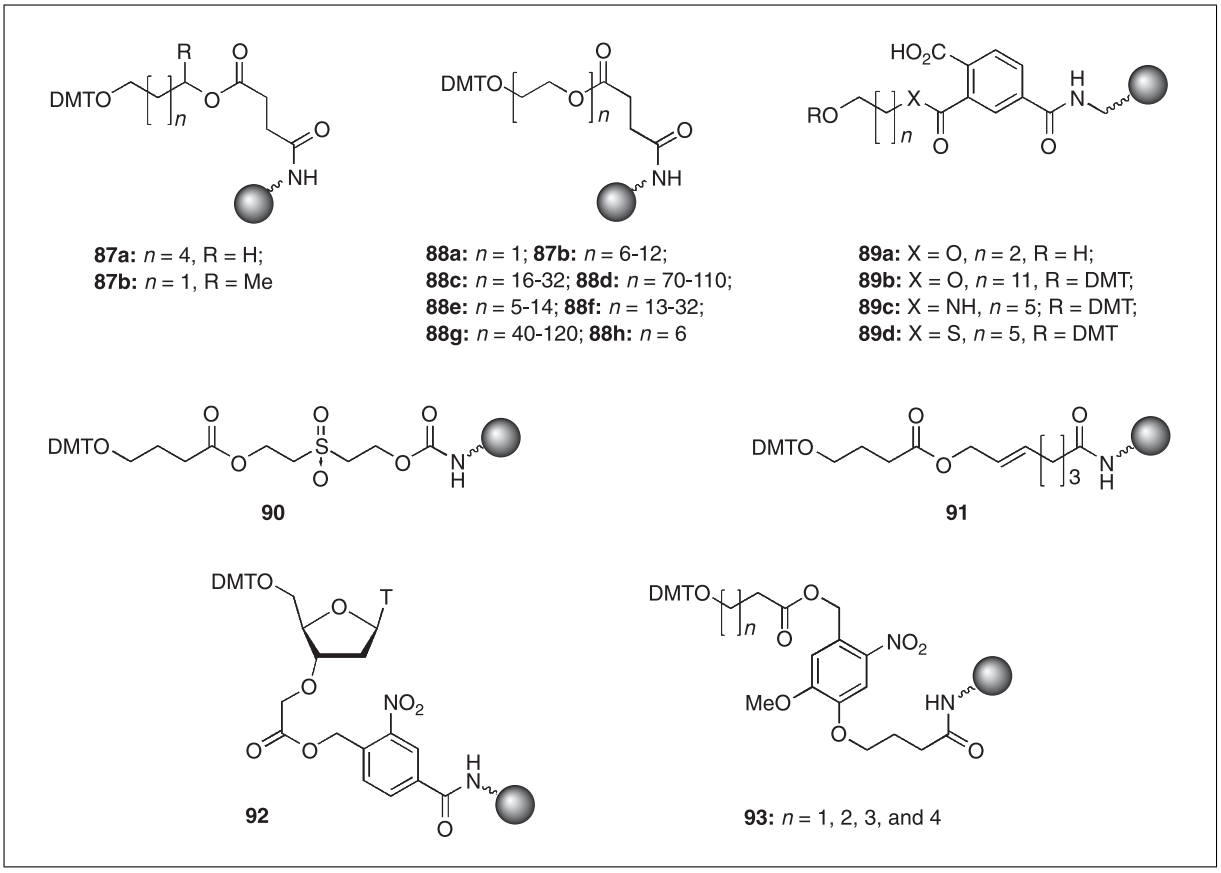

Figure 3.1.10 Structures of linear solid supports **87** to **89** for preparation of 3′-hydroxyalkylated oligonucleotides and solid supports **90** to **93** for preparation of 3′-carboxyalkylated oligonucleotides.

the surface by the forces of hydrogen bonding over the course of oligonucleotide synthesis, with one hydroxy group available for phosphoramidite coupling and the other protected by bonding to the surface. Expectedly, the loading with the diols depended on the specific surface area of CPG. For CPG500 having a specific surface area of 48.6 m^2/g, the loading of 38.8 μmol/g was determined, which was very similar to that obtained by conventional means.

With all solid support considered above, conventional deprotection of support-bound oligonucleotides with AH was sufficient for the release of 3′-(ω-hydroxyalkyl) oligonucleotides to solution.

Linear solid supports for 3′-carboxyalkylation

Convertible solid supports **63**, **66**, **68**, and **70** (Fig. 3.1.8), discussed earlier, form a variety of 3′-terminal modifications under nucleolytic deprotection conditions. Upon treatment with aqueous alkali, oligonucleotides assembled on these solid supports are released with 3′-(carboxyalkyl) modification (see above in "Scenario C: Convertible Linkers").

Several solid supports were designed specifically for the introduction of 3′-(carboxyalkyl) modification. Thus, oligonucleotides assembled on a solid support **90** (Fig. 3.1.10) were released with 0.2 M NaOH in 60% aqueous dioxane for 15 min at room temperature (Chakhmakhcheva et al., 1997; Efimov et al., 1998). Under anhydrous conditions, the release was performed using 0.5 M DBU in THF for 2 hr. In this case, the released oligonucleotides were additionally treated with AH for 16 hr at 60°C (Patnaik et al., 2012).

A solid support (**91**) was developed for releasing 3′-(carboxyalkyl) oligonucleotides under conditions orthogonal to the basic deprotection of nucleic bases and internucleosidic phosphates (Matray et al., 1997). Under the optimized conditions, oligonucleotides were released by action of $Pd_2(dba)_3 \cdot CHCl_3$ (4- to 8-fold excess over the support-bound material) and 1,2-*bis*(diphenylphosphino)ethane (2.5- to 5-fold over Pd complex) in 60 mM tetrabutylammonium formate buffer in 1 hr at 55°C. With a 2-fold excess of Pd(0) complex, the reaction time had to be extended to 2 hr (Greenberg et al., 1998). After completion of

deprotection, oligonucleotides were additionally treated with *N*,*N*-diethyldithiocarbamate to remove Pd bound to nucleic bases. The reported yields of oligonucleotides released under these conditions were as high as those obtained by alkaline hydrolysis. However, the conditions were not completely orthogonal because the cleavage of ester function was accompanied by the removal of 2-cyanoethyl groups from internucleosidic phosphate moieties.

In the early 1990s, several photolabile *o*-nitrobenzyl solid supports were introduced for oligonucleotide synthesis (Greenberg, 1993; Greenberg and Gilmore, 1994). In order to obtain fully protected, 3′-(carboxyalkyl)-derivatized oligonucleotides suitable for selective conjugation in solution, Greenberg (1995) developed two photolabile solid supports, **92** and **93**. The use of nucleosidic solid support **92** allowed the release of 12- to 20-mer oligonucleotides 3′-terminated with thymidine-3′-*O*-glycolate in 45% to 79% isolated yields after UV irradiation for 3 hr. A more readily available nitroveratroyl solid support **93** was also more efficient, so that the release occurred 3 to 6 times more rapidly than that from **92** to afford the fully base- and phosphate-protected oligonucleotides in 71% to 92% yields after UV irradiation at 365 to 400 nm in 90% aqueous MeCN for 0.5 to 1 hr (Yoo and Greenberg, 1995). The same group also found that dA^{ibu}- and dC^{ac}- protected oligonucleotides were more compatible with the photolytic conditions than the benzoyl-protected counterparts. In contrast to these results, the same group reported later that, under the identical conditions, the photolysis for 2 hr was required to obtain isolated yields in a range of 80% (Kahl and Greenberg, 1999).

Linear solid supports for 3′-aminoalkylation

3′-Aminoalkylated oligonucleotides do not require any activation for their conjugation with reactive derivatives of carboxylic acids, some of which are commercially available and others can be readily prepared. This feature renders 3′-aminoalkyl modifications more useful in preparation of various 3′-derivatized oligonucleotides than their 3′-carboxyalkyl counterparts. Therefore, a greater effort was focused on the development of reliable solid supports for introduction of 3′-aminoalkyl modification.

The 3′-aminoalkyl group can be conveniently introduced in a flexible manner using convertible solid supports **66** and **68** (see Fig. 3.1.8. and "Scenario C: Convertible Linkers").

A number of methods have been developed where an α,ω-aminoalcohol linker was directly attached, by its amino group, to solid supports via a spacer that simultaneously served as an amino protecting group.

In solid support **94**, *bis*(2-hydroxyethyl)disulfide spacer was used as an amino protecting group (Fig. 3.1.11; Asseline and Thuong, 1990; Asseline et al., 1992). The chemistry and the conditions of deprotection for **94** were very similar to those used for 3′-phosphorylation with the aid of **50** (Fig. 3.1.7). Upon the completion of oligonucleotide synthesis, the deprotection with AH was carried out in the presence of 0.1 M DTT that reduced the disulfide linkage. The spontaneous elimination of episulfide followed by the degradation of an intermediary carbamate anion resulted in deprotection of the 3′-terminal amino group.

In a solid support **95a**, the amino group was protected with phthalimido group by the attachment to solid phases via a trimellitic spacer (Fig. 3.1.11; Petrie et al., 1992; Gamper, 1993). Although the original publication did not disclose the details of deprotection procedure, a variety of deprotection conditions for similar solid supports **95b** and **95c** was reported later by others.

The release of the 3′-terminal amino group in oligonucleotides assembled on **95b** can be carried out in a mixture of AH and MeOH (5:3) in the presence of 2,2′-dipyridyldisulfide and phenol. These conditions were also employed for simultaneous deprotection of the 3′-terminal amino and a 5′-terminal mercapto functions and conversion of the latter to a 2′-pyridylthio-derivative (Aubert et al., 2000).

A diverse set of conditions was later developed at Glen Research Corp. for solid support **95c** (Glen Research, 2001, 2002). Thus, to release ~80% to 90% of the target oligonucleotide from the solid support, deprotection with AH for 24 hr at room temperature, 15 hr at 55°C, or 4 hr at 65°C was recommended. With a mixture of AH and EtOH (3:1 v/v), the reaction time should be extended to 48 hr at room temperature. When AMA was used, 10 min at 55°C was sufficient, while deprotection with 0.4 M NaOH in 80% aqueous MeOH required 15 hr at room temperature. However, another study found that only about a half of the oligonucleotide material was released from **95c** after treating with AH for 16 hr at 55°C (Leuck et al., 2004). Finally, deprotection with K_2CO_3 in MeOH was not recommended, as it

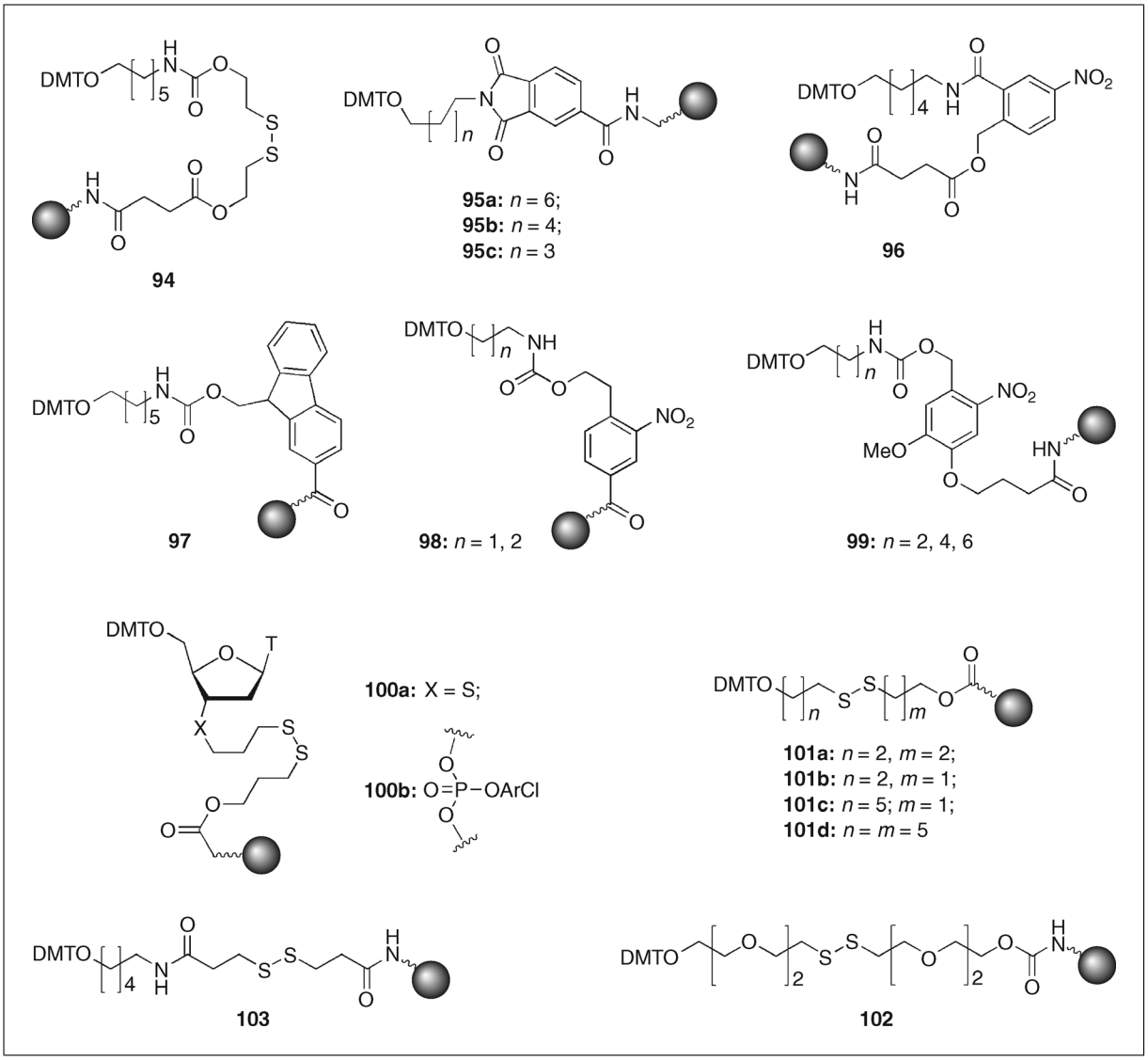

Figure 3.1.11 Structures of linear solid supports **94** to **99** for preparation of 3′-aminoalkylated oligonucleotides and solid supports **100** to **103** for preparation of 3′-mercaptoalkylated oligonucleotides.

results in the release of only about 15% of the 3′-aminoalkylated oligonucleotide (Glen Research, 2001, 2002).

A similar solid support **89c**, where *O*-DMT-protected 1,6-aminohexanol was attached to the trimellityl spacer via a carboxamide rather than an imide function, was also used as a precursor of 3′-aminohexyl oligonucleotides. Deprotection with AH for 18 to 24 hr at 55°C was reported in this instance (Lyttle et al., 1997).

A more reactive solid support **96** was obtained by attaching 1-*O*-DMT-6-aminohexanol to solid-phase material via a 2-(hydroxymethyl)-5(6)-nitrobenzoyl (HMNB) spacer (Leuck et al., 2004). [The authors repeatedly used 2-(hydroxymethyl)-6-nitrobenzoyl as a name of their linker although the structure of 2-(hydroxymethyl)-5-nitrobenzoyl linker as in **96** was invariably shown in their schemes.] At room temperature, oligonucleotides assembled on **96** were released from the solid phase with AH and AMA in 40 and 5 min, respectively. To complete the deprotection of the amino group, further treatment with AH (2 hr, 55°C or 24 hr, room temperature) or AMA (30 min, 65°C) was sufficient. This demonstrated that, in comparison with **95c**, the support **96** allowed a substantially more rapid deprotection of the terminal amino function. The formation of 6-nitrophthalide as a side product was postulated, although the latter was likely to be aminolyzed under the conditions of deprotection with AH or AMA (Suedkamp and Meise, 1985). A similar solid support lacking the nitro-group was proven to release the deprotected amino function much more slowly ,and required heating with AH for 6 hr at 55°C.

The Fmoc protecting group is widely used in solution-phase chemistry and in peptide synthesis on solid phase. An attempt to use a solid support **97** where 1-*O*-DMT-6-aminohexanol was attached via a derivatized Fmoc spacer for preparation of 3′-aminoalkylated oligonucleotides proved less successful (Aviñó et al., 1996). Upon the standard deprotection, crude oligonucleotides were of 50% to 60% purity and were contaminated with a major impurity (20% to 30%), with the pure material isolated in 7% yield. Similarly, starting from a solid support **98** based on NPEOC protecting group, target oligonucleotides were obtained in 12% to 16% and 9% to 14% isolated yields when deprotection was carried out in AH and 0.5 M DBU in pyridine, respectively (Aviñó et al., 1996).

In a photolytic approach, a solid support **99** has been extensively studied by Greenberg's group (McMinn and Greenberg, 1996, 1997, 1998, 1999; McMinn et al., 1997; Kahl et al., 1998). 3′-Aminoalkylated, phosphate-protected eicosathymidylate was released from **99** in yields ranging from 70% to 98% in 3 hr when the UV irradiation was carried out in a photo-reactor at 350 nm, or in 2 hr when a trans-illuminator was used (McMinn and Greenberg, 1996, 1997). A relatively minor concomitant formation of TT-photodimerized products was observed to a 2% extent. With mixed-base oligonucleotides, certain restrictions applied to the choice of base-protecting groups. Thus, while benzoyl group could not be used in the photolytic deprotection, phenoxyacetyl group was compatible with the conditions of UV irradiation. However, in the absence of basic deprotecting agents, the migration of phenoxyacetyl from bases to the terminal aliphatic amino group occurred. It was however found that isobutyryl protection of dA and dC nucleoside residues was compatible with photolytic release of oligonucleotides from the solid support **99** (McMinn and Greenberg, 1997).

Linear solid supports for 3′-mercaptoalkylation

Mercaptoalkylated oligonucleotides are readily conjugated to various pendants via the formation of a disulfide bond or, for oligonucleotides containing no internucleosidic phosphorothioate moieties, by alkylation with compounds prone to nucleophilic substitution. The formation of disulfides is particularly convenient due to its high selectivity and rapid kinetics of conjugation under conditions essentially harmless for oligonucleotides as well as for most conjugating groups.

As with all other functions, the 3′-mercaptoalkyl group can be introduced using convertible solid supports **66** and **68** (see Fig. 3.1.8 and "Scenario C: Convertible Linkers").

The first two solid supports **100a** and **b** for introduction of 3′-mercaptoalkyl groups contained a nucleoside moiety (Fig. 3.1.11; Zuckermann et al., 1987). Although less convenient for broader applications, this work demonstrated the usefulness of the mercapto function. The structure of the solid support **100b** was soon reduced to the more practical versions **101a** to **101** (**101a**: Guzaev, 1991; see Glen Research #3 in Internet Resources; **101b** and **101c**: Gupta et al., 1991; **101d**: see Glen Research #3 in Internet Resources). A conceptually similar solid support **102** was designed to introduce a longer and a more hydrophilic linker between an oligonucleotide and a 3′-modifier (Bonfils and Thuong, 1991; Asseline et al., 1992).

The deprotection of oligonucleotides assembled on solid supports **101** and **102** may be carried out in a single step using AH containing 0.05 M DTT to 0.1 M DTT for a period of time sufficient for the removal of nucleic base-protecting groups. Under these conditions, the disulfide bridge is reduced so that the crude product contains 3′-mercaptoalkyl groups.

Alternatively, the solid support-bound material may be released and deprotected without the addition of DTT. In this event, the crude product contains the disulfide bond. Upon isolation of partially protected product by HPLC, the disulfide bridge is reduced by 50 to 100 mM DTT at pH 8.3 to 8.5 for 30 min (see, for instance, Glen Research #3 in Internet Resources). More aggressive conditions or longer reaction times suggested by others are quite unnecessary. Under special circumstances (the presence of acridine reporters unstable to ammonium hydroxide), deprotection with 0.4 M NaOH was used followed by treatment with 0.1 M DTT (Asseline et al., 1992).

A linker containing the mercapto group can be attached to a solid phase via a thioester function as in a solid support **89d** (Lyttle et al., 1997). In this case, ammonolysis of solid support-bound oligonucleotides resulted in fully deprotected 3′-mercaptohexyl oligonucleotides. In order to prevent partial formation of symmetric disulfides, small quantities of DTT were added to deprotection mixtures.

In a slightly different design, a solid support **103** was built around 3,3′-dithiodipropionic acid to give, upon oligonucleotide synthesis and deprotection with AH plus 50 mM DTT for 16 hr at 55°C, 3′-mercaptoalkylated oligonucleotides (Gupta et al., 1990). This communication, however, does not reveal any information concerning the yields of target oligonucleotides.

Branched linkers

"Faster," the very first part of the Olympic motto "*Citius, Altius, Fortius*," is also a key concept for the survival of a commercial manufacturer of synthetic oligonucleotides. To maintain a high throughput of the synthesis of oligonucleotide conjugates, it has been most desirable to start the chain assembly from a solid support already containing the required pendant moiety and thus avoid the use of time-consuming procedures for post-synthetic labeling in solution. Design of linear solid supports that fulfill this requirement is difficult or, for many reporters having only one functional group, plainly impossible. The task became feasible with the use of branched linkers having at least three functional groups. Of these, two functions, often hydroxy groups, serve for the attachment of the linker to a solid support and as a starting point for oligonucleotide synthesis. The third function is located in the side chain and, in the course of oligonucleotide synthesis, is rendered unreactive by a protecting group, or carries a desired (often protected) pendant.

Side reactions of branched linkers

The seemingly facile task of attaching a trifunctional compound to a solid phase and using it in oligonucleotide synthesis is not necessarily so simple in practical implementation. In particular, the branched linkers frequently display a high concentration of functional groups in a limited space, which often results in side reactions occurring in the course of basic deprotection. A side reaction does not have to be particularly pronounced to render a linker unacceptable in the industrial environment. Some 20 years ago, an impurity of less than 5% might have gone unnoticed. Currently, however, acceptance criteria for synthetic oligonucleotides set by many pharmaceutical users and specifications set by many manufacturers require that no single impurity in excess of 1% be present in products of small-scale synthesis. This sets a high bar for the performance of all reagents including the linkers. Below, we mention a few examples of structural motifs that have a high likelihood of side reactions to occur.

Compounds derived from 1,2-diols are relatively readily available and hence have been widely used in preparation of branched linkers. One should, however, bear in mind that some 1,2-diols are used as universal linkers (see Fig. 3.1.4 and "Scenario A: Universal Linkers" above). Most other 1,2-diols, upon attachment to the 3′-terminus of an oligonucleotide, are impractical in that application. However, they are capable, to a minor extent, of dephosphorylation of the 3′-terminal nucleotide residue.

Of longer diols, linkers derived from 1,4-butanediols might also be less than an ideal choice. Indeed, the 4-hydroxybutyl group has been demonstrated to serve as a convenient protecting group for internucleosidic phosphate in oligonucleotide synthesis (Grajkowski et al., 2007). One may expect that, when placed at the 3′-terminus of an oligonucleotide as in hypothetical structure **104a**, the 1,4-butanediol linker might generate, along with the main product, a small percentage of the respective oligonucleotide 3′-phosphate **105** plus tetrahydrofuran (Fig. 3.1.12). Importantly, the cleavage of the linker from **104a** requires the protected phosphate and the unprotected hydroxy group. If the phosphate protection is removed first, the formation of **105** in the course of ammonolytic deprotection should not be observed. Analogously, a hypothetical compound **104b** is expected to generate an *S*-methyltetrahydrothiophenium salt (Cieślak et al., 2004).

Elimination of a linker to form a 3′-phosphorylated oligonucleotide is another concern. To avoid using structural motifs prone to β-elimination, refer to the respective part of "Scenario B: Linkers for 3′-Phosphorylation".

Often, a pendant group is connected to the side chain of a branched linker via an amide, a thioamide, or a carbamate group. Each of these groups contains two nucleophilic centers (nitrogen plus oxygen or sulfur in Y = S). If the distance between one of the nucleophilic atoms and the carbon directly attached to the phosphate group permits the formation of a five-membered ring as in **106**, the intramolecular nucleophilic attack occurs rapidly to give **105** plus the respective **107** to **110** as shown in Fig. 3.1.12 (Grajkowski et al., 2001, 2007, 2008; Guzaev and Manoharan, 2001a,b; Wilk et al., 2002; Ausín et al., 2010).

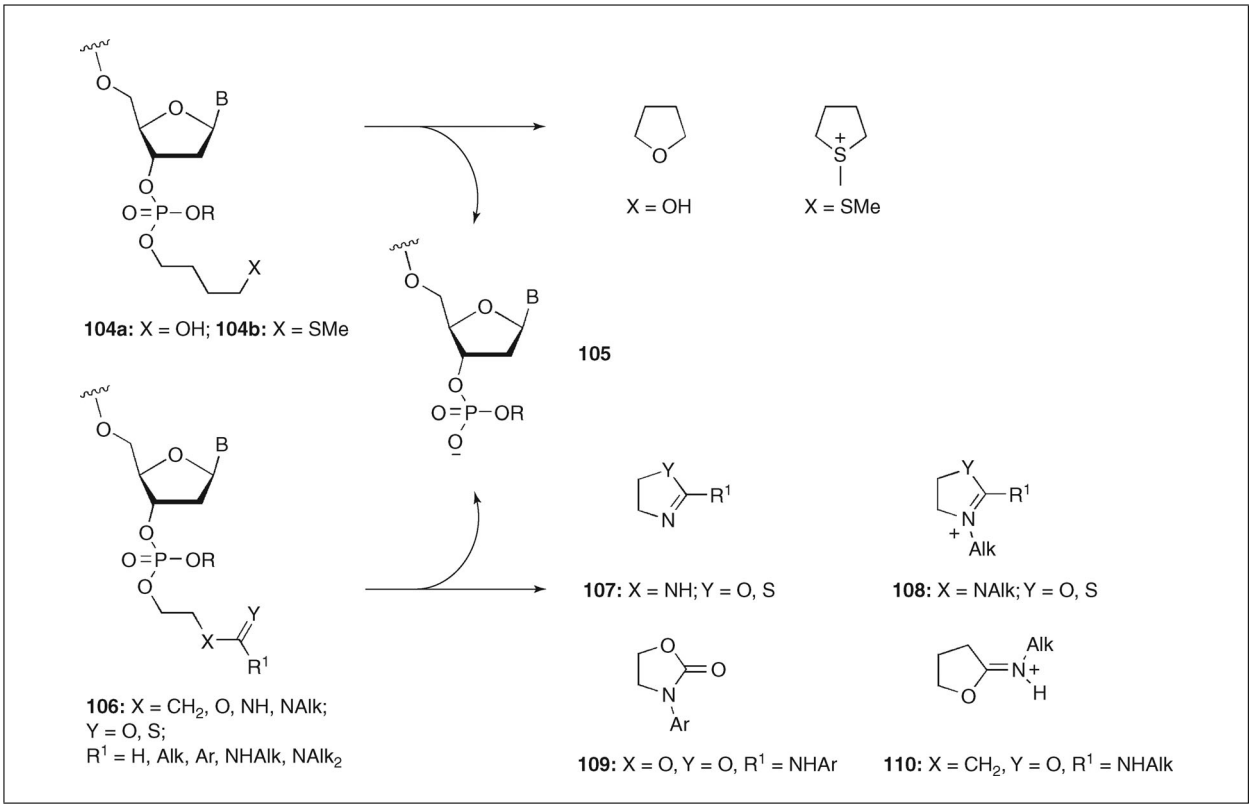

Figure 3.1.12 Examples of side reactions in 3′-derivatized oligonucleotides leading to the formation of oligonucleotide-3′-phosphate with the loss of the linker.

Importantly, the formation of **108** and **110** is an acid- and base-independent process and in selected cases (**108**, R = 4-MeOC$_6$H$_4$, Alk = *i*Pr) proceeds rapidly not only in water but also in MeCN (Guzaev and Manoharan, 2001b). It can therefore be concluded that a distance between amide, thioamide, or carbamate groups and the nearest phosphate should be observed carefully. Any structural motifs shown in **104a**, **104b**, and **106** should be avoided in construction of novel linkers.

Branched linkers derived from 1,2-diols

The first solid support containing a branched linker **111a** (Fig. 3.1.13) was derived from 3-amino-1,2-propanediol (aminoglycerol) where the amino group was protected with Fmoc (Nelson et al., 1989). The design of the linker suffered from several shortcomings. PAGE analysis of oligonucleotides synthesized on **111a** revealed the formation of several products (Reed et al., 1991; Petrie et al., 1992). In a detailed study, oligonucleotides and their methylphosphonate analogs were released from **111a** by ethylenediamine (Thaden and Miller, 1993). With oligonucleotides attached to **111a** via a methylphosphonate linkage, the linker behaved in a universal manner: the material released to the solution possessed no linker and no 3′-terminal methylphosphonate residue. When the linkage to the support was 2-cyanoethyl-protected phosphotriester, a heterogeneous oligonucleotide material was released in 40% to 60% yield. The authors suggested that *O*→*N* migration of the succinyl spacer occurred during the final deprotection, thus rendering a large portion of the material permanently attached to the solid phase.

The amino group in the aminoglycerol linker was also protected with trifluoroacetyl (TFA) and with a phthaloyl group to give, respectively, solid supports **111b** and **111c** (Vu et al., 1995). The phthaloyl group was stable under the conditions of extended capping and detritylation, and was successfully removed by treatment with AMA for 5 to 10 min at 56°C. The yields of oligonucleotides synthesized on the solid support **111c** protected with phthaloyl group were not appreciably different from that of control, unmodified oligonucleotides. However, careful analysis of crude oligonucleotides synthesized on **111c** by PAGE showed the presence of the target oligonucleotides (75% to 77%) contaminated with its N-acetylated derivative (1% to 4.5%) and 3′-dephosphorylated species (7% to 12%). It was also demonstrated that TFA and, particularly, Fmoc-protected solid

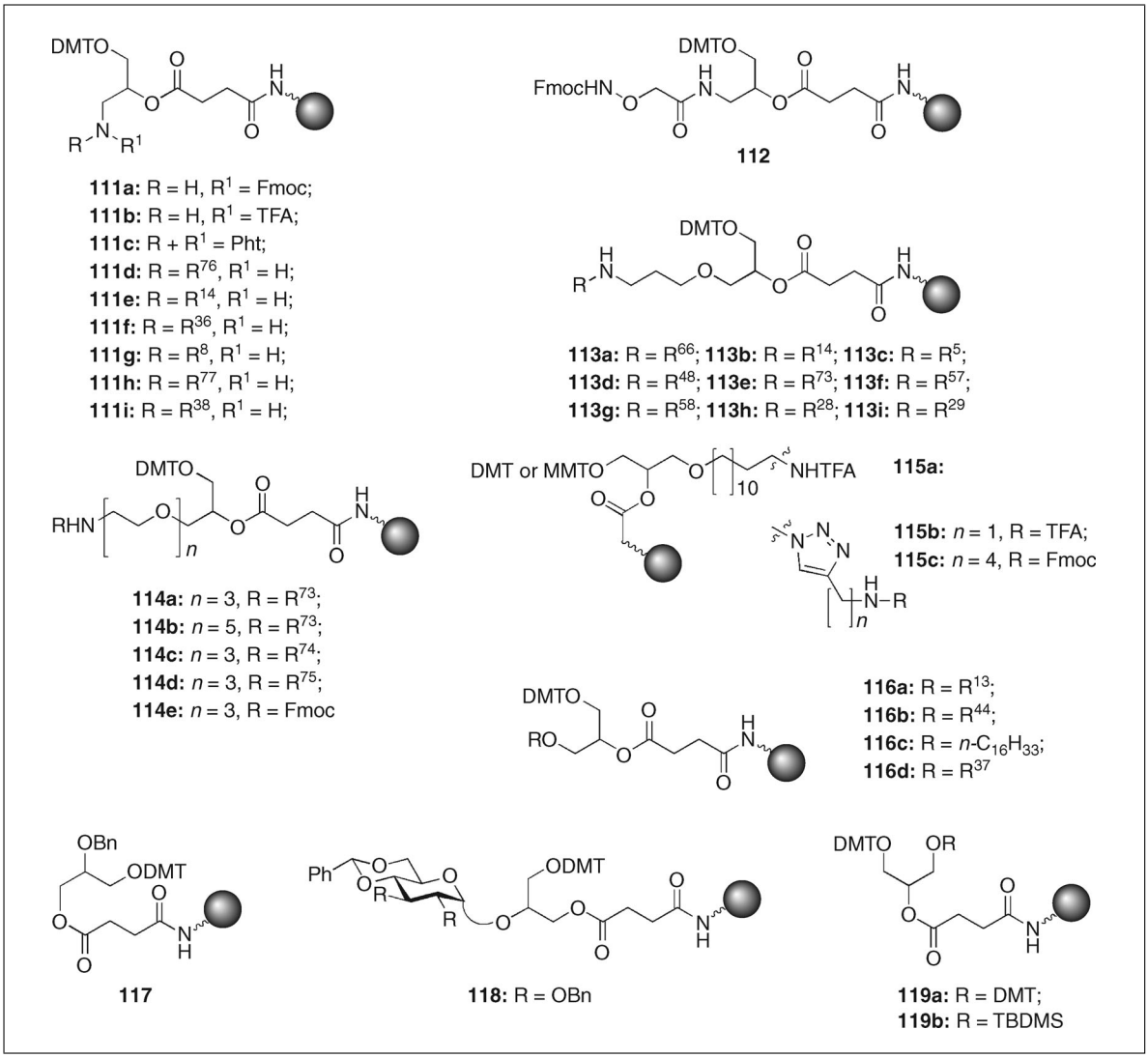

Figure 3.1.13 Structures of branched solid supports **111** to **119** derived from 1,2-diols for introduction of 3′-amino function and pendant groups. For universal solid supports excluded from this figure, see Figure 3.1.4.

supports **111a** and **111b**, respectively, cannot be used for the synthesis of oligonucleotides of acceptable quality.

Due to its inherent disadvantages, aminoglycerol linker has been used for preparation of solid supports and the respective oligonucleotides bearing hydrophobic pendants, e.g., in cases where a good separation of the target conjugates from side products could be achieved by reversed-phase HPLC. Along these lines, conjugates of oligonucleotides bearing a minor groove binder, R^{76}, and cholesterol, R^{14}, were prepared using solid support **111d** (Lukhtanov et al., 1995) and **111e** (Reed et al., 1995), respectively. However, intermediates in the synthesis of **111e** were not characterized, nor were the loading of **111e** or yields of oligonucleotide product determined. Oligonucleotides conjugated to a second-generation dendrimer, R^{36}, were synthesized on a solid support **111f** in typical isolated yields of 20% to 25% (Skobridis et al., 2005). The conjugates showed slightly higher binding affinity to the complementary oligoribonucleotides (+3°C), and their cellular uptake was greater than that of the unmodified counterparts by a factor of 10 (PO) to 15 (PS) (Skobridis et al., 2005). A thio-phosphoramidate oligonucleotide modified with palmitoyl group, R^8, was synthesized on solid support **111g** and was shown to enhance the potency of telomerase inhibition (Herbert et al., 2005). Hydrophobic nonaggregating azaphthalocyanine, R^{77}, an efficient dark quencher of fluorescence in a range of 300 to 740 nm, was conjugated to oligonucleotides using solid support **111h** (Kopecky et al., 2010). Similarly, conjugates of pyrenylbutyric acid, R^{38}, were prepared on solid support **111i** (Li et al., 2011).

Other uses of aminoglycerol are exemplified by a solid support **112** aimed at preparation of 3′-aminooxy-derivatized oligonucleotides. Surprisingly enough, the authors reported regular yields and high purity of crude oligonucleotides synthesized on **112** (Sethi et al., 2009).

In 1990, a protected phosphoramidite building block derived from 3-*O*-aminopropylglycerol has been used for preparation of multi-labeled oligonucleotides (Misiura et al., 1990). The respective solid supports **113a** to **113i**, where the distance between the amino group and the nearest hydroxy group was extended to seven single bonds, were substantially more reliable than **111**. A number of reporter groups including fluorescein, R^{66} (Theisen et al., 1992), cholesterol, R^{14} (Vu et al., 1993, 1994), adamantoyl, R^{5} (Habus et al., 1995), 9-(2-methoxy-6-chloroacridinyl), R^{48} (Asseline et al., 1996), dipyrido[3,2-*a*:2′,3′-*c*]phenazine-11-carbonyl, R^{73} (dppz; Ossipov et al., 1999), folic acid attached via α- or γ-carboxy group of L-glutamate fragment, R^{57} and R^{58}, respectively (Kazanova et al., 2007), dabsyl, R^{28} (Nakayama et al., 2008), and dabcyl, R^{29} (Matsumoto et al., 2010). In addition to the standard deprotection conditions using AH at 55°C, alternative conditions were used under special circumstances. Thus, oligonucleotides labeled with 2-methoxy-6-chloroacridine, R^{48}, were deprotected with 0.4 M NaOH in 50% aqueous MeOH for 3 hr (Asseline et al., 1996). Similarly, benzyl protection at α- or γ- ester functions of folic acid, R^{57} and R^{58}, was removed with 1 M NaOH followed by the standard treatment with AH at 55°C for 16 hr (Kazanova et al., 2007).

A relatively simple synthetic access to 1-*O*-aminoalkylated glycerols prompted preparation of solid supports **114a** to **114e** where a protected amino group or a reporter was separated from the backbone by long hydrophilic tri- and penta(ethyleneoxide) spacer arms. In **114a** and **114b**, the amino group in the side chain was acylated with dipyrido[3,2-*a*:2′,3′-*c*]phenazine-11-carboxylic acid, R^{73} (Ossipov et al., 1999). In the ensuing publications, the authors succeeded in preparation of **114c** and **114d** and their use for the synthesis of oligonucleotides labeled with ruthenium complexes [Ru(1,10-phenantroline)$_2$ (dipyrido[3,2-*a*:2′,3′-*c*]phenazine)]$^{2+}$, R^{74} (Ossipov et al., 2001), and [Ru(2,2′:6′,2"-terpyridine)(dipyrido[3,2-*a*:2′,3′-*c*]phenazine)Cl]$^{+}$, R^{75} (Ossipov et al., 2002), respectively. To keep the integrity of the complexes, oligonucleotides prepared on **114d** were deprotected with AH at room temperature for 17 hr. With **114a** to **114c**, the standard deprotection conditions were used. A Fmoc-protected solid support **114d** was also used for preparation of 3′-amino-derivatized oligonucleotides and their post-synthetic conversion to the desired complexes (Ossipov et al., 2002). More recently, similar solid supports **115a, 115b**, and **115c** where long alkyl chains were inserted between the TFA- and Fmoc-protected amino group and 1-*O*-position of glycerol were reported (Grijalvo et al., 2010).

A number of other oligonucleotide modifiers, primarily of hydrophobic nature, has been attached via alkylation of the primary hydroxy group in glycerol. The group led by T. Brown described solid supports **116a** and **116b** derivatized, respectively, with cholesterol, R^{13} (MacKellar et al., 1992) and, via its phenolic hydroxy group, with Vitamin E, R^{44} (Will and Brown, 1992). Interestingly, when a nucleoside phosphoramidite was attached to cholesterol-derivatized solid support **116a**, the phosphite triester formed could not be sulfurized with *N*,*N*,*N*′,*N*′-tetraethyl thiuramdisulfide, and its oxidation with the standard I_2 solution required 10 min. In contrast, oligonucleotide synthesis proceeded in a normal fashion when **116b** was used. Moreover, Vitamin E–derivatized oligonucleotides were stable in concentrated ammonium hydroxide for >24 hr at 55°C. Other reported examples featured solid supports **116c** and **116d** derivatized, respectively, with *n*-hexadecyl (Rait et al., 2000) and pyrenylmethyl, R^{37} (Christensen and Pedersen, 2002; Pasternak et al., 2008).

Solid supports **117** and **118**, where linkers were derived, respectively, from 2-*O*-benzylated or 2-*O*-glucosylated glycerols, have been used in preparation of cell wall components by phosphoramidite method (Veeneman et al., 1990; Hogendorf et al., 2011).

Solid supports **119a**, and **119b** (De Napoli et al., 1999a,b; Masuda et al., 2010; Putta et al., 2011) for the synthesis of fork-like oligonucleotides will be discussed in "Linkers for synthesis of branched oligonucleotides," below.

2-*O*-Acetyl and 1-*O*-benzoyl-protected glycerol solid supports **77** (Urata and Akagi, 1993) and **78** (Achilles and von Kiedrowski, 2005; Chillemi et al., 2006) shown in Fig. 3.1.9 were used for the attachment of glycerol to the 3′-terminus of oligonucleotides. Oligonucleotides obtained were used for

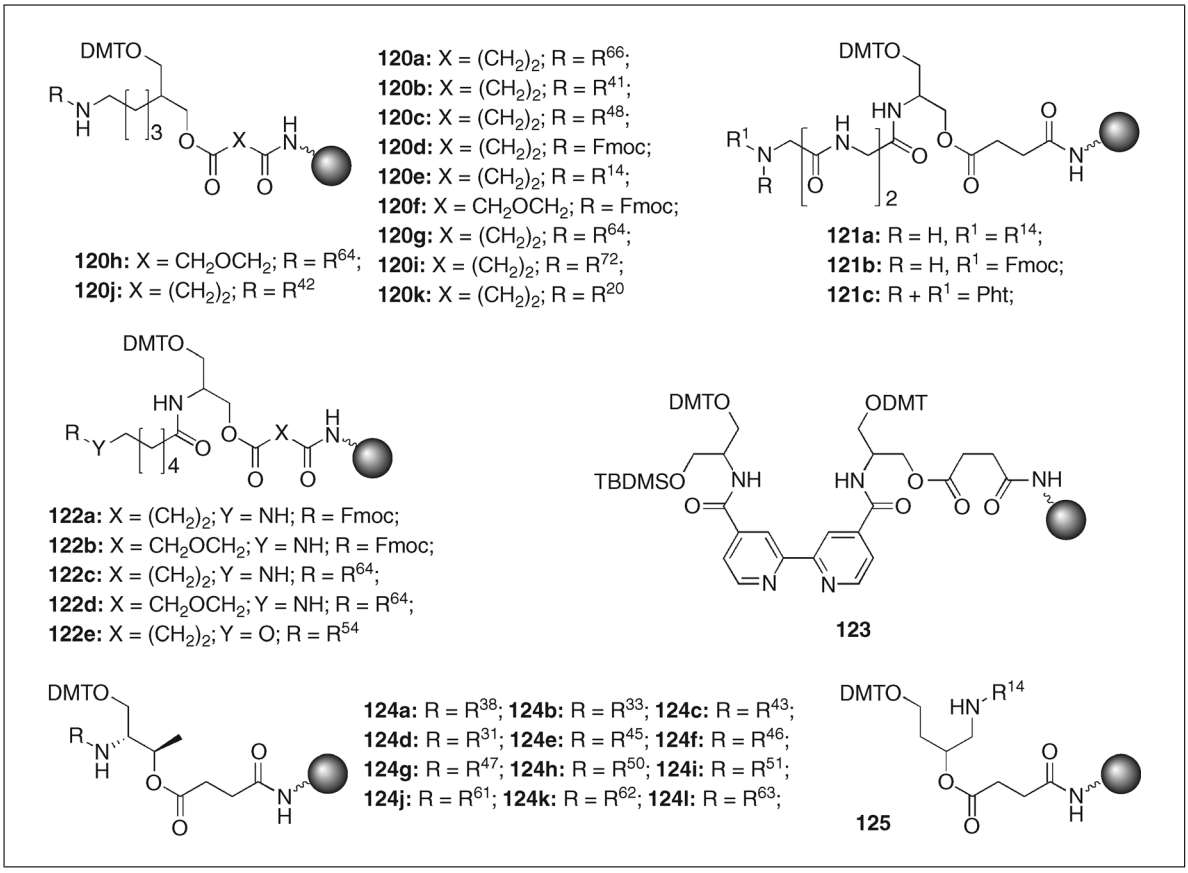

Figure 3.1.14 Structures of branched solid supports **120** to **125** derived from 1,3-propanediols with additional amino- or aminoalkyl group.

generation of 3′-aldehydo groups (Urata and Akagi, 1993; Achilles and von Kiedrowski, 2005) as described above in "Scenario C: Convertible Linkers" or were enzymatically converted to 3′-*O*-lysophosphatidyl oligonucleotides (Chillemi et al., 2006).

Branched linkers derived from amino- and aminoalkyl-1,3-propanediols

Linkers derived from 1,3-diols (Fig. 3.1.14) were introduced very shortly after it became apparent that a variety of problems was associated with the use of the solid support **111** in oligonucleotide synthesis. Among non-nucleosidic reagents, they represent one of the most preferred embodiments in oligonucleotide chemistry. It has been argued that, when inserted in the middle of an oligonucleotide chain, 1,3-diols serve as better mimics of the natural ribose unit and thus destabilize hybridization of the derivatized oligonucleotide to a lesser extent. Although this argument is not applicable to solid supports, the 1,3-diol-derived linkers are still more preferable over their 1,2-counterparts because they are essentially incapable of transesterification of the nearest phosphate residue, and thus they do not generate, to an appreciable extent, 3′-underivatized oligonucleotides as impurities.

Solid supports **120a** to **120d** derived from 2-(4-aminobutyl)-1,3-propanediol where the amino group was acylated with *bis*-pivaloyl fluorescein, R^{66}, or biotin, R^{41}, alkylated with acridine, R^{48} or protected with Fmoc, were the first tested among the supports of this group (Fig. 3.1.14; Nelson et al., 1992). The final deprotection step was carried out under the standard conditions except for acridine-labeled oligonucleotides that were deprotected with 0.4 M NaOH in 20% aqueous methanol.

The solid support **120b** was also used in an unconventional manner. 5′-*O*-3′-*O*-Bis-DMT- and 5′-*O*-Fmoc-3′-*O*-DMT-thymidines were alkylated at the N3 of the base moiety by detritylated **120b** where the pseudo-5′-hydroxy group was activated by action of Ph_3P/DEAD (Amato et al., 2004). The resulting branching solid support was used in preparation of 3′-3′-linked, acridine labeled pyrimidine oligonucleotides capable of triple-helix formation.

The Fmoc-protected version **120d** proved useful in preparation of

oligonucleotide-peptide conjugates (Basu and Wickstrom, 1995). Using *N*-Fmoc-protected building blocks, a peptide chain was first assembled starting from the primary amino group of **120d** in 70% yield. The oligonucleotide chain was next assembled using 2-(acetoxymethyl)benzoyl-protected dA and dG phosphoramidites and dC^{Ac} phosphoramidite. Upon completion of the synthesis, base-protecting groups were removed by treatment with 50 mM K_2CO_3 in anhydrous MeOH plus an additional treatment with aqueous ammonium hydroxide for 30 min at room temperature to give the desired conjugate in 45% yield.

In a similar fashion, 2-(4-aminobutyl)-1,3-propanediol was reacted with cholesterol chloroformate and converted to solid support **120e**, R^{14} (Reed et al., 1995). No complications with isolation of pure oligonucleotide product were reported.

To expand an array of reporter groups introducible at the 3′-terminus, the amino group in Fmoc-protected solid supports **120d** and **120f** were deblocked and acylated with tetramethylrhodamine (TAMRA, R^{64}) reporter (Mullah and Andrus, 1997; Mullah et al., 1998; Ota et al., 1998). The low stability of the dye in the presence of ammonia prompted authors to attach the linker via the conventional succinyl and more labile diglycolyl spacers to give solid supports **120g** and **120h**, respectively. Oligonucleotides were released from the solid support with a mixture of *t*-butylamine/methanol/water (1:1:2) at room temperature. With diglycolyl spacer, more than 98% of the support-bound material was released in 20 min, while the succinyl spacer released 27% and 50% of the material in 1 and 2 hr, respectively. Base-labile protecting groups were then removed by heating the solution of the released oligonucleotide at 65°C for 3 hr or at 85°C for 1 hr. The use of double-labeled oligonucleotides assembled on **120g** for detection of intermolecular interactions in structured nucleic acids has been thoroughly studied (Ota et al., 1998).

The scaffold of 2-(4-aminobutyl)-1,3-propanediol has also been used to introduce fluorescein with the aid of solid support **120i** where the protected chromophore was attached via thiourea linkage as shown in R^{72}. It has been, however, demonstrated that deprotection of the support-bound material with AH has to be carried out with a caution. While deprotection at room temperature resulted in the desired labeled oligonucleotides, treatment at 55°C overnight converted the thiourea linkage to the respective guanidine quantitatively (Dubey et al., 1998).

In addition to the biotinylated solid support **120b**, another version, **120j**, containing a protected biotin pendant, R^{42}, was introduced (Upadhya et al., 2005). This study identified a side reaction, an oxidation of the sulfide moiety of biotin to the respective sulfoxide during the course of oligonucleotide chain assembly on solid support **120j**. The oxidation was independent of the nature of the oxidizer used (standard I_2 reagent, 10-camphorsulfonyl oxaziridine, or *t*-butylhydroperoxide) and depended chiefly on the time of the exposure of the solid support to the oxidizer, which was proportional to the length of an oligonucleotide. The authors provided literature evidence of the fact that the oxidation was inconsequential for the affinity of biotin to avidin.

With an objective to target parenchymal liver cells, a solid support **120k** where 2-(4-aminobutyl)-1,3-propanediol was acylated with triantennary β-aminogalactosyl conjugate of cholic acid, R^{20}, was synthesized and used in preparation of 20-mer antisense oligonucleotides (Maier et al., 2003). Due to the steric bulk of the conjugate, the nature of the solid support material was of crucial importance for the success of the synthesis, with the best results obtained on macroporous polystyrene 1000 Å. Comparison of the performance of the solid support and the respective phosphoramidite showed that, with respect to the efficiency of the synthesis, the preferred site of conjugation was the 3′-terminus of oligonucleotides.

Serinol, a structural isomer of aminoglycerol, was used as a scaffold for the synthesis of solid supports **121** to **123**. In contrast to **120**, preparation of **121** to **123** involved only manipulations with protecting groups and acylation, the relatively routine and water-insensitive steps. In **121a** to **121c**, a triglycine linker was assembled as a side arm where the *N*-terminal amino group was either reacted with cholesteryl chloroformate to give **121a** (Vu et al., 1994) or protected with Fmoc or phthaloyl groups as in **121b** and **121c**, respectively (Vu et al., 1995). The carbamoyl group that attached cholesterol in **121a** was fairly stable under the conditions of conventional ammonolysis and was almost quantitatively cleaved by treatment with 0.4 M NaOH in 75% aqueous MeOH overnight. Oligonucleotides assembled on phthaloyl-protected solid support **121c** were

deprotected by treatment with AMA for 5 to 10 min at 56°C to give the desired oligonucleotides in an acceptable purity. In support of earlier findings, Fmoc protection in **121b** was particularly labile so that the main product of oligonucleotide synthesis was the respective N-acetylated derivative (Vu et al., 1995). In a similar manner, the amino group of serinol was acylated by 6-(Fmoc-amino)hexanol to obtain solid supports **122a** and **122b**. Fmoc group was then replaced with TAMRA dye, R^{64}, on solid phase, and the solid supports **122c** and **122d** were used in oligonucleotide synthesis (Mullah et al., 1998). Labeled oligonucleotides synthesized on **122c** and **122d** were deprotected as described above for **120f** and **120g**.

A solid support **122e**, allowed the introduction of psoralen, R^{54}, an intercalator and a UV-triggered cross-linking agent, at the 3′-terminus of oligonucleotides (Kurfürst et al., 1993). Deprotection of psoralen-tethered oligonucleotides was carried out at room temperature overnight using 0.4 M NaOH in 50% to 75% aqueous MeOH or AH.

In solid support **123**, two serinol linkers were attached to 2,2′-bipyridine 4,4′-dicarboxylic acid to give an unsymmetrically protected, fork-like solid support where bipyridine fragment was expected to coordinate transition metals (Galeone et al., 2001). Starting with the DMT-protected hydroxy groups, two 8-mer oligonucleotides linked at their 3′-termini were assembled simultaneously. The limited stability of the amide group connecting bipyridine and serinol moieties necessitated very mild deprotection conditions (25% aqueous ammonium hydroxide, 1.5 hr at room temperature), which also partially removed TBDMS protection from the linker. The remaining TBDMS group was removed by treatment with TBAF.

After the usefulness of serinol-based linkers has been established, the next logical step was to use L-threoninol for the same function. L-Threoninol is a more convenient starting material than serinol because of the high selectivity of the reaction with DMT-Cl at the primary hydroxy function. First introduced were phosphoramidite building blocks derived from L-threoninol (Asanuma et al., 2003), while a number of solid supports **124a** to **124l** derived from this scaffold were been reported several years later. The list of reporters used in **124a** to **124l** includes pyrene-1-butyric acid, R^{38}, anthraquinone-2-carboxylic acid, R^{33} (Ben Gaied et al., 2009), 4-methyl-trans-stilbazole, R^{43} (Kashida et al., 2011), and a number of intercalating and/or fluorescent carboxylic acids, R^{31}, R^{45} to R^{47}, R^{50}, R^{51}, and R^{61} to R^{63} (Aviñó et al., 2008, 2012). At this point, it remains unclear whether the supports of general structure **124** offer any advantages over serinol-based solid supports aside from the ease of preparation.

In yet another design, 4-amino-1,3-butanediol linker was obtained by reduction of 3-hydroxy-4-aminobutyric acid and converted to a solid support **125** featuring cholesterol pendant R^{14} (Reed et al., 1995).

Branched linkers derived from 1,3-propanediols with additional carboxylic acids

The choice of readily available 1,3-propanediols bearing a carboxylic group is more limited. One convenient approach uses readily available derivatives of *bis*(hydroxymethyl)malonic acid for construction of solid supports **126a** to **126c** (Fig. 3.1.15; Guzaev et al., 1996) and **126d** (Guzaev and Lönnberg, 1999). Among the advantages of linkers used in **126a** to **126d** is the lack of chirality and the fact that a single incorporation of the linker introduces two groups of interest. These were exemplified by Fmoc- or TFA-protected amino functions, dansyl, R^{30}, or octyl groups. These solid supports were constructed by attachment of the respective linkers to the solid support **40a** (Fig. 3.1.6) via a phosphotriester linkage. This design allowed a permanent protection of the pseudo-3′-terminal hydroxy group in oligonucleotide conjugates **126e** with a terminal phosphate function, a requirement dictated by the lability of esters and amides of *bis*(hydroxymethyl)malonic acid under basic conditions of deprotection. The solid support **126d** was used in the synthesis of oligonucleotide analogs bearing hydrophobic octyl groups at their 3′- and 5′-termini (Guzaev and Lönnberg, 1999). It was demonstrated that the ester functions in oligonucleotides synthesized on **126d** are efficiently protected against a nucleophilic attack by the two negative charges of the neighboring phosphodiester groups. This permitted the deprotection of oligonucleotide conjugates derived from **126d** with AH at room temperature for 2 days or for 1 hr at 65°C without a substantial loss of octyl groups (less than 5%).

Starting from (*R*)-(+)-3-hydroxybutyrolactone and (*R*)-(−)-pantolactone, a set of linkers and the respective solid supports **127a** to **127g** derived from (*R*)-2,4-dihydroxybutyramide were synthesized. The

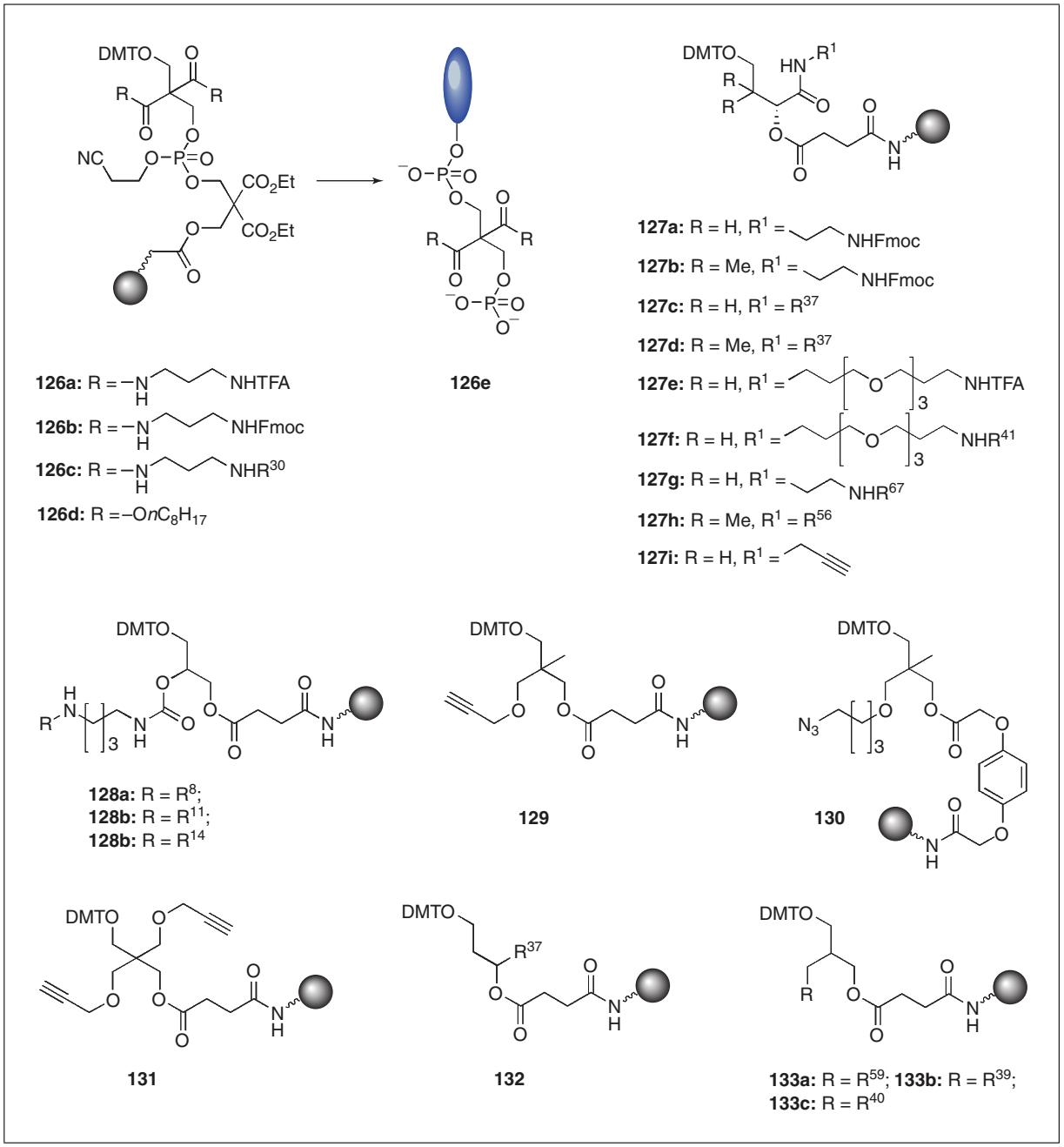

Figure 3.1.15 Structures of branched solid supports **126** and **127** derived from 1,3-propanediols with additional carboxylic group and solid supports **128** to **133** derived from other acyclic 1,3-propanediols.

strategy offered a convenient attachment of 1-pyrenylmethyl group, R^{37}, and of amino-terminated side chains of various lengths protected with Fmoc or TFA or labeled with biotin, R^{41}, and fluorescein, R^{67} (Dioubankova et al., 2002, 2006). When the respective phosphoramidite building blocks were attached at the 5′-terminus and the final deprotection was carried out DMT-Off, the non-nucleosidic moiety was eliminated (Dioubankova et al., 2006). Otherwise, no complications in use of the linkers were reported. Derivatization of the linker with terpyridine, R^{56}, and propargylamine resulted in solid supports **127h** (Freville et al., 2006) and **127i** (Ustinov et al., 2008), respectively. Oligonucleotides synthesized on **127i** were used in post-synthetic conjugation by Cu(I)-catalyzed azide-alkyne cycloaddition (Ustinov et al., 2008; Mercier et al., 2011). When this reaction was carried out at elevated temperatures in neutral solution, loss of the linker from the 3′-terminus to the extent of 30% to 40% was observed.

Branched linkers derived from other acyclic 1,3-propanediols

Yet another way to construct 1,3-diol-type scaffold with a side arm consisted in the attachment of 1,4-diaminobutane to the secondary hydroxy group of glycerol by a carbamate linkage as in solid supports **128** (Fig. 3.1.15; Ueno et al., 2008). The free amino group of the linker was converted to cholesteryloxycarbonyl derivative, R^{14}, by reaction with the respective chloroformate or acylated to amides of palmitoic acid, R^{8}, and oleic acid, R^{11}. The solid supports **128a** to **128c** thus obtained were used in preparation of sense and/or antisense strands of siRNA. In spite of mild conditions of deprotection (AH:EtOH, 3:1 v/v, 12 hr at room temperature), the target oligonucleotides were obtained in yields lower than usual regardless of the nature of the hydrophobic modifier. Less than 20 OD of the product was isolated for 9 out of 12 oligonucleotides synthesized on 1 μmol scale.

Solid supports **129** and **130** were derived from tris(hydroxymethyl)ethane where one of the three identical hydroxy groups was alkylated to introduce, respectively, propargyl (Pourceau et al., 2008) or 4-azidobutyl (Pourceau et al., 2009) side arms. Interestingly, the azido group in **130** did not suffer from Staudinger reaction with phosphoramidite building blocks during the course of oligonucleotide synthesis. Oligonucleotides assembled on **129** and **130** were used in post-synthetic conjugation by Cu(I)-catalyzed 1,3-dipolar azide-alkyne [3 + 2] cycloaddition.

Solid support **131** is similar in its function to **129**. However, **131** allowed simultaneous conjugation of two groups to the 3′-terminus of oligonucleotides (Lietard et al., 2008; Ryazantsev et al., 2012).

However, less often, a reporter group may be directly attached to the 1,3-propanediol backbone by a C—C bond. The documented examples describe synthesis and use of solid support **132** derived from 2-(1-pyrenylmethyl)-1,3-propanediol (Balakin et al., 1998; Korshun et al., 1999) and similar solid supports **133a** to **133c** with fluorescent dyes 2-[(4-phenylethynyl) phenyl]-benzoxazole, R^{59} (Malakhov et al., 2001), 9,10-*bis*(phenylethynyl)anthracene, R^{39}, and 1-(phenylethynyl)pyrene, R^{40} (Malakhov et al., 2004) attached to 1,3-propanediol backbone.

Branched linkers derived from cyclic 1,3-diols

Of all non-nucleosidic linkers, those derived from 2-deoxy-D-ribose where the side chain is attached to the anomeric carbon in the natural β-configuration are the closest mimics of 2′-deoxynucleosides. When introduced in the middle of an oligonucleotide chain, the sugar moiety facilitates the direction of the side arm toward the interior of the double or the triple helix. With certain types of the labeling groups (intercalators as in **136b** to **136e**, Fig. 3.1.16, or cross-linking agents), this property may offer an advantage. Among other advantages are stereochemical homogeneity of linkers and of oligonucleotides produced and their unparalleled stability under the basic conditions of oligonucleotide deprotection. The respective solid supports disclosed in literature, **134a** to **134d** (Smith et al., 1996), **135** and **136a** to **136e** (Ben Gaied et al., 2009), are shown in Figure 3.1.16.

The solid supports **137a** and **137b** (Fig. 3.1.16) were loaded with a cyclic linker selected for the ease of its synthetic preparation on large scale (Su et al., 1997). The linker was a mixture of two stereoisomers different by the orientation of the amino group acylated with biotin, R^{41}, or protected fluorescein, R^{66}. One advantage of **137a** and **137b** was that the amido group was facing away from the phosphodiester backbone of oligonucleotides, which prohibited its participation in any side reactions involving intramolecular nucleophilic substitution.

Oligonucleotides 3′-terminated with peracetylated 1-*O*-methyl-α-D-glucose and sucrose were prepared on solid supports **138** and **139**, respectively (Fig. 3.1.16; glucose: Adinolfi et al., 1998, 2001, 2002; sucrose: Adinolfi et al., 2004). Solid-phase synthesis on **138** or **139** with the use of the respective sugar-derived phosphoramidite building blocks bearing the phosphite moiety at the 4-*O* of glucose allowed multiple incorporation of sugar residues at both termini of 18- to 22-mer oligonucleotides, although yields of products were not disclosed (Adinolfi et al., 2002, 2004). In all cases listed in this paragraph, the final deprotection was carried out using AH at 55°C.

Solid support **140a** featured an orthogonally protected 1-*O*-methyl-α-D-glucosamine as a bifunctional linker and was designed for preparation of oligonucleotide-peptide conjugates (Di Fabio et al., 2001). The solid support was tested in the attachment of acetyl or leucine at the amino group of 1-*O*-methyl-α-D-glucosamine to give supports **140b** and **140c**. To these, dA^{bz} phosphoramidite was coupled at the 6-*O* of the sugar to form the desired conjugates in 95% and 93% yield, respectively.

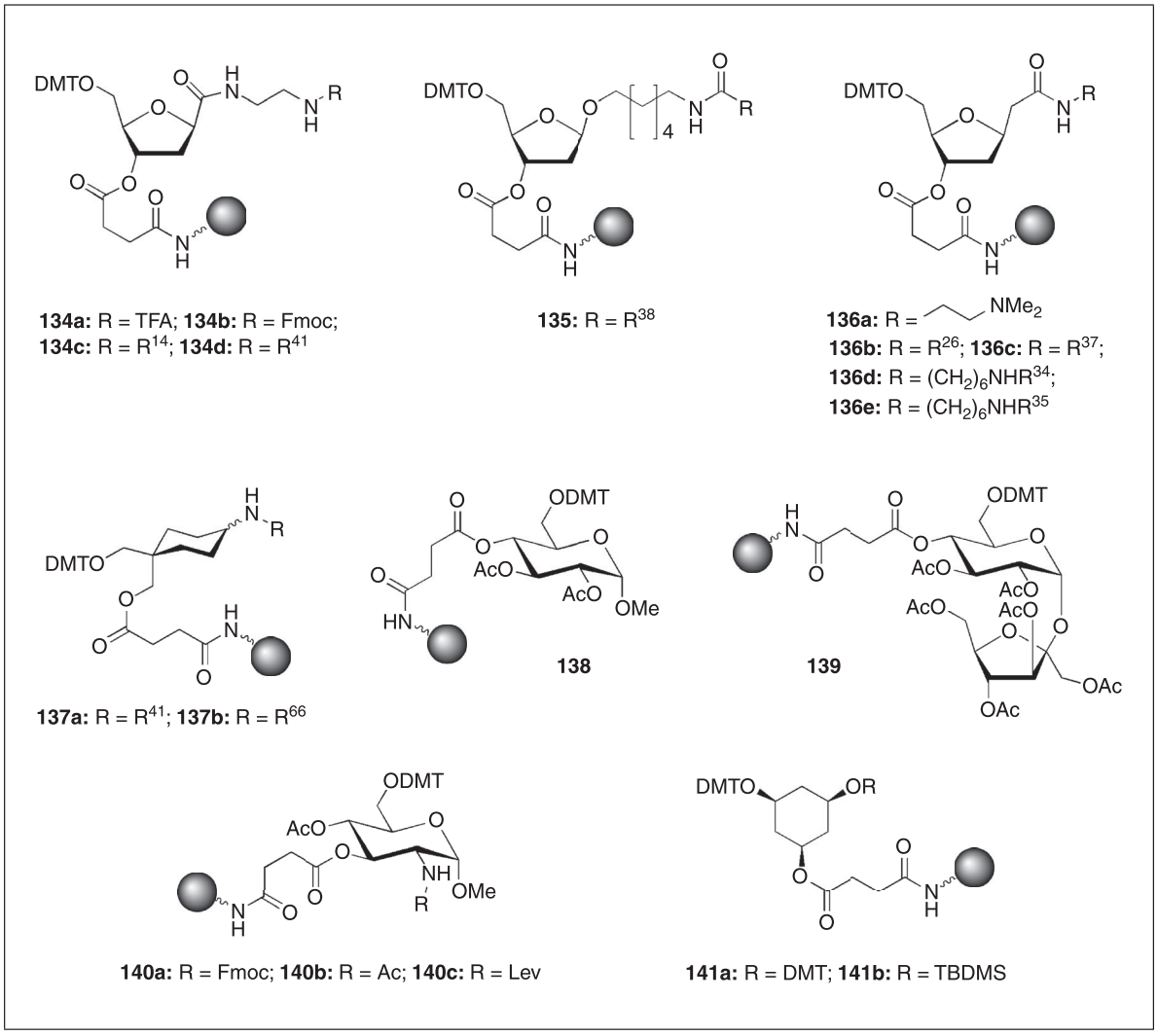

Figure 3.1.16 Structures of branched solid supports **134** to **141** derived from cyclic 1,3-diols.

Symmetrical bis-DMT-protected or asymmetrical DMT- and TBDMS-protected 1,3,5-cyclohexanetriol-derived solid supports **141a** and **141b** (De Napoli et al., 1999a,b) for the synthesis of fork-like oligonucleotides will be discussed in "Linkers for synthesis of multiantennnary oligonucleotides" below.

Branched linkers derived from cyclic 1,4-diols

Hydroxyprolinol proved to be a very stable and reliable linker where the most common side reactions of diol linkers bearing an additional amido group were not reported. The linker was first introduced in 1991 by solid supports **142a** to **142c** bearing cholesteryloxycarbonyl, R^{14}, 3-(9-acridinyl)propionyl, R^{52},- and 5-(9-acridinyl)pentenoyl groups, R^{53}, attached to the secondary amino group (Fig. 3.1.17; Reed et al., 1991). In a subsequent communication, an improved method of preparation of **142a** and **142c** was been reported (Gamper et al., 1993). Oligonucleotides prepared on solid supports **142a** to **142c** were deprotected with AH for 19 to 24 hr at 45°C to be isolated in 80% to 120% yield relative to the respective 3′-unmodified oligonucleotides. Soon after, the respective phosphoramidite building blocks were synthesized and successfully tested (Hébert et al., 1994). Later on, the selection was expanded by solid supports **142d** to **142h** bearing *O*-methyl lithocholamide, R^{15}, stigmasterol, R^{16}, stigmastanol, R^{17}, *O*-benzoyl estradiol, R^{18}, and 1-*O*-2-*O*-dihexadecylglycerol, R^{22}, respectively (Reed et al., 1995), as well as solid supports **142i** (dabsyl, R^{28}; Adamczyk et al., 2000), **142j** to **142l** (dyes of fluorescein family, R^{69}-R^{71}; Kutyavin et al., 2006), and **142m** bearing *N*-(5-hexynoyl) pendant, R^4, (Kvach et al., 2007).

Hydroxyprolinol solid supports **143a** to **143l** featuring a side arm extended by 6-aminohexanoic acid have also been widely

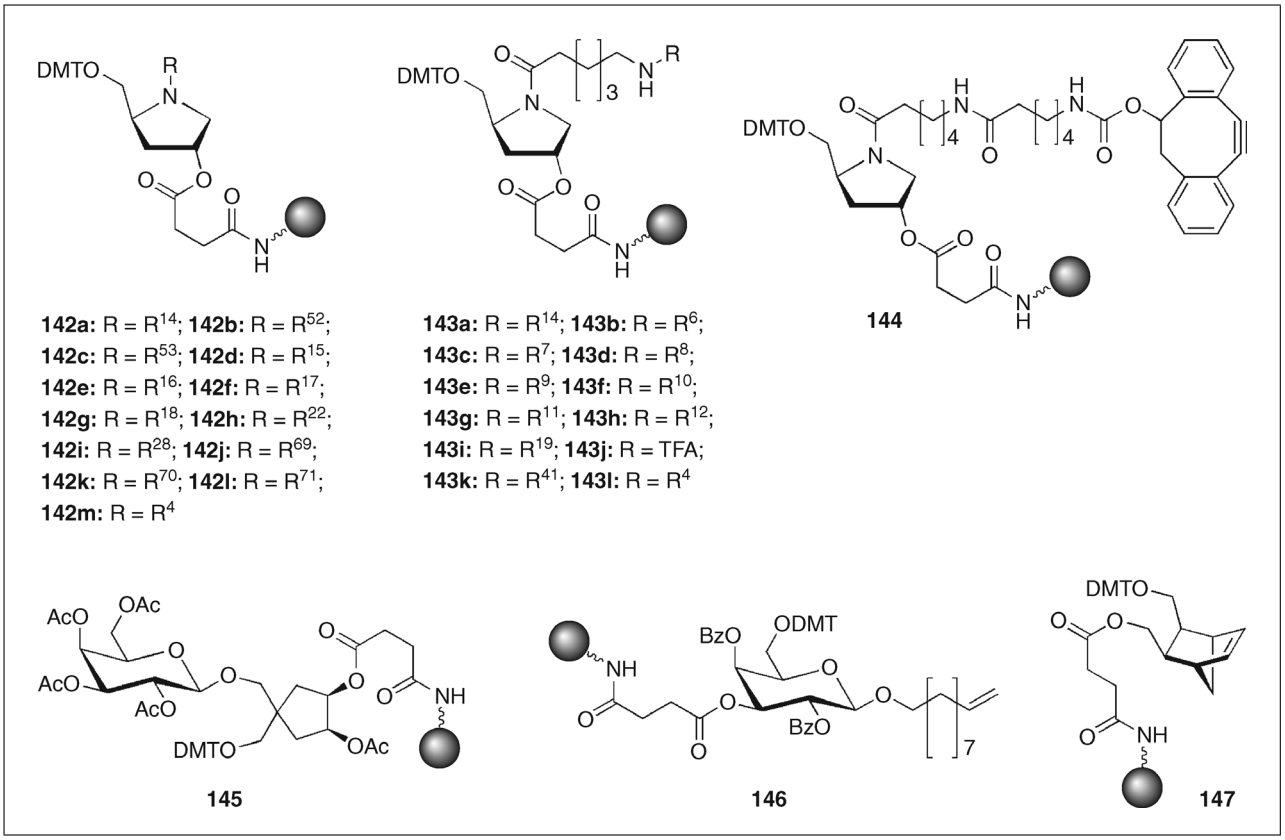

Figure 3.1.17 Structures of branched solid supports **142** to **147** derived from cyclic 1,4-diols.

used. In an extensive study, solid support **143a** bearing a cholesteryloxycarbonyl group, R^{14} (Soutschek et al., 2004; Wolfrum et al., 2007) and solid supports **143b** to **143j** acylated, respectively, with lauroyl, R^6, myristoyl, R^7, palmitoyl, R^8, stearoyl, R^9, docosanoyl, R^{10}, oleoyl, R^{11}, linoleyl, R^{12}, and (oleylcarbamoyl)lithocholyl group, R^{19}, were prepared (Wolfrum et al., 2007). These solid supports were used for preparation of 3′-derivatized siRNA, of which cholesteryl- and docosanoyl-bearing compounds demonstrated the strongest in vivo silencing of apoB mRNA due to a more efficient binding to HDL and LDL particles and the following cellular uptake in vivo (Wolfrum et al., 2007).

Using the same scaffold, solid supports **143j** and **143k**, where the amino group of the side chain was protected with TFA or acylated with biotin, R^{41}, were prepared (Kvach et al., 2007). Oligonucleotides synthesized on these supports were immobilized on Sepharose beads.

Oligonucleotides synthesized on solid supports **143l** and **144** derivatized with 5-hexynoyl group, R^4, and a cyclooctyne derivative, respectively, were used for post-synthetic conjugation using Cu(I)-catalyzed (**143l**) and copper-free strain-promoted (**144**) azide-alkyne 1,3-dipolar [3 + 2] cycloaddition (Jayaprakash et al., 2010).

A cyclic, β-D-galactosylated linker used in solid support **145** was designed to improve stability of oligonucleotide in serum (Ikeda et al., 2010). Indeed, oligonucleotides assembled on **145** were intact to ∼80% extent after incubation for 180 min. At the same time, siRNA containing the linker at the 3′-terminus of sense, antisense, or both strands maintained their silencing activity, although at a somewhat reduced level.

Another solid support **146** constructed around β-D-galactose was used in synthesis of phosphorylated tetra- and hexasaccharide fragments of the lipophosphoglycan from *Leishmania* using H-phosphonate method (Ross et al., 2000). An 1-*O*-dec-9-enyl aglycone moiety was introduced to assist further biochemical assays.

A bicyclo[2.2.1]hept-2-ene linker in solid support **147** served as a dienophile in post-synthetic conjugation reactions using inverse-electron-demand Diels-Alder cycloaddition (Schoch et al., 2010). Oligonucleotides assembled on a solid support **147** have been shown to react with dansyl or biotin-derivatized 1,2,4,5-tetrazines as heterodienes in up to 96% yield and 1:1 stoichiometry.

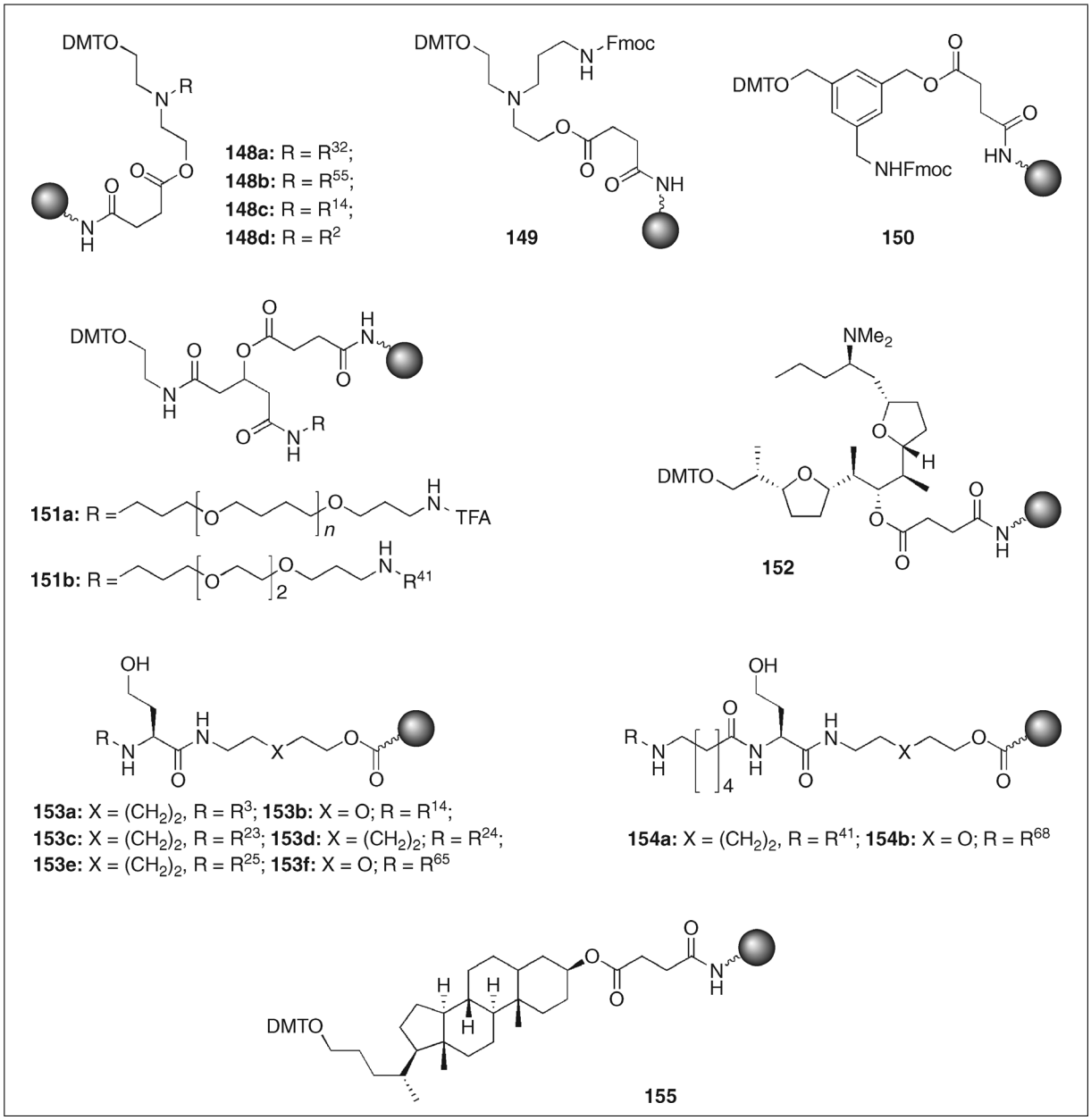

Figure 3.1.18 Structures of branched solid supports **148** to **155** derived from 1,5- and longer diols with additional amino group for introduction of 3′-amino function and pendant groups.

Branched linkers derived from 1,5- and longer diols

To date, two 1,5-diol-type linker designs are known in the literature. Lin and Matteucci (1991) used diethanolamine-based solid support **148a** for labeling of antisense oligonucleotides with antraquinone, R^{32} (Fig. 3.1.18). Importantly, attachment of labels or side arms to nitrogen of diethanolamine only generated achiral compounds. Diethanolamine scaffold was also used in solid supports **148b, 148c**, and **148d** for the attachment of 5-deazaflavin, R^{55} (Nakamura et al., 1993) cholesterol, R^{14} (Reed et al., 1995), and methacryloyl, R^2, group (Rubina et al., 2004), respectively. Another diethanolamine linker, now containing Fmoc-protected aminopropyl side arm was synthesized and converted to solid support **149** (Thaden and Miller, 1993).

The support **149** was used in the synthesis of methylphosphonate analogs of oligonucleotides. Due to the lability of methylphosphonate backbone in aqueous bases, the final deprotection was carried out in two steps using first 0.5 M hydrazine hydrate in pyridine-AcOH (4:1) and then with 1,2-diaminoethane in EtOH (1:1) for 6 hr at room temperature. Oligonucleotides

synthesized with the phosphotriester moiety between the 3′-terminal nucleoside and the linker cleanly gave the desired 3′-amino-derivatized products. Interestingly, when said linkage was methylphosphonate, a mixture of 3 products was formed, one of which was oligonucleotide 3′-methylphosphonate. Upon storage in aqueous solution for 16 hr at 37°C, the conversion of intermediates was complete to give oligonucleotide 3′-methylphosphonate as the only product. The observation was explained by nucleophilic attack of the tertiary nitrogen of the linker on the carbon linked to the methylphosphonate group, resulting in formation of an aziridinium intermediate and elimination of the oligonucleotide 3′-methylphosphonate.

The second 1,5-diol-type linker was implemented in solid support **150** (Behrens et al., 1995; Behrens and Dahl, 1999). As in the previous case of diethanolamine scaffold, the use of 5- substituted 1,3-*bis*(hydroxymethyl)benzene generated an achiral linker. A limitation of **150** was that, upon the attachment of the first phosphoramidite building block, a benzyl phosphotriester moiety unstable under the conditions of iodine oxidation is formed. This necessitated the use of *t*-butyl hydroperoxide as an oxidizer, which allowed preparation of 3′-derivatized oligonucleotides in an isolated yield of 50% to 75%.

Starting from 3-hydroxyglutaric anhydride, solid supports **151a** and **151b** containing 1,6-diol-type linkers were synthesized (Skrzypczynski and Wayland, 2004). A TFA-protected aliphatic amino group and biotin, R^{41}, were attached via long polytetrahydrofuran (average M_n *ca*. 1100) or triethyleneoxide side arms, respectively. All synthesized oligonucleotides were deprotected with AH for 17 hr at 55°C to give reaction mixtures of various complexities.

1,7-Diol-type solid supports **152** bearing the anionophoric product of alkaline hydrolysis of pamamycin, an antibiotic from *Streptomyces aurantiacus* with penetration enhancement potency, was used in the synthesis of a series of antisense oligodeoxynucleotides (Uhlmann et al., 1998).

Stereochemically defined 1,10- or 1,11-diols derived from homoserine were assembled on solid phase (Stetsenko and Gait, 2001) to give solid supports **153a** to **153f**. The pendants were attached, via formation of an amide bond, at the α-amino group of homoserine: 3-pentynoyl, R^3, cholesterol, R^{14}, *N*-Fmoc-S-StBu cysteine, R^{23}, trilysine protected with TFA at all ε-amino groups and with Fmoc at the *N*-terminus, R^{24}, 4-iodophenylacetyl, R^{25}, and diacetyl 6-carboxyfluorescein, R^{65}. Versions with an extended side chain **154a** and **154b** labeled with biotin, R^{41}, and fluorescein, R^{68}, were synthesized, too. Good to excellent yields of the desired oligonucleotides and their phosphorothioate analogs (47% to 93%) were reported.

Reduction of lithocholic acid followed by the standard procedures gave an 1,12-diol-type solid support **155** that was used in preparation of antisense oligonucleotides (Reed et al., 1995).

Linkers for synthesis of multiantennary oligonucleotides

Linkers of this group were designed for preparation of two- and three-antennary oligonucleotides and are presented in Figure 3.1.19. When they were used in combination with the respective branching phosphoramidite building blocks, oligonucleotide dendrimers were synthesized (Shchepinov et al., 1997, 1999; Oliviero et al., 2004).

Solid supports **119a** (De Napoli et al., 1999a,b; Masuda et al., 2010; Putta et al., 2011), **119b** (De Napoli et al., 1999a,b), **141a** and **141b** (De Napoli et al., 1999a,b), **158a, 158b**, (Ueno et al., 2001), **158c** (Hoshika et al., 2003), **159**, **160** (Ueno et al., 2003), and **161** to **164** (Putta et al., 2010) were prepared in a conventional manner. In contrast, solid supports **156** and **157** were prepared by coupling of the respective phosphoramidite building blocks to T-CPG (Shchepinov et al., 1997, 1999) and solid support **36b** (Oliviero et al., 2004).

The uniform protection of hydroxy functions with DMT group in **119a, 141a**, **156, 157**, **158a** to **158c**, and **161** to **164**, only allowed assembly of oligonucleotides with identical branches. A number of two-antennary oligonucleotides synthesized starting from bis-DMT-protected solid supports **119a, 141a**, and **161** to **164** were tested for their immunemodulatory activity (Putta et al., 2010). Linkers in solid supports **119a**, **161**, and **162** were found to be more efficient for stimulation of immune response in the biological system studied.

In order to permit the assembly of two different oligonucleotide branches, the solid supports **119b, 141b**, **159**, and **160** were designed to bear two orthogonal protecting groups of their hydroxy functions. The supports **119b** and **141b** were protected with DMT and TBDMS groups. When these were used, the first

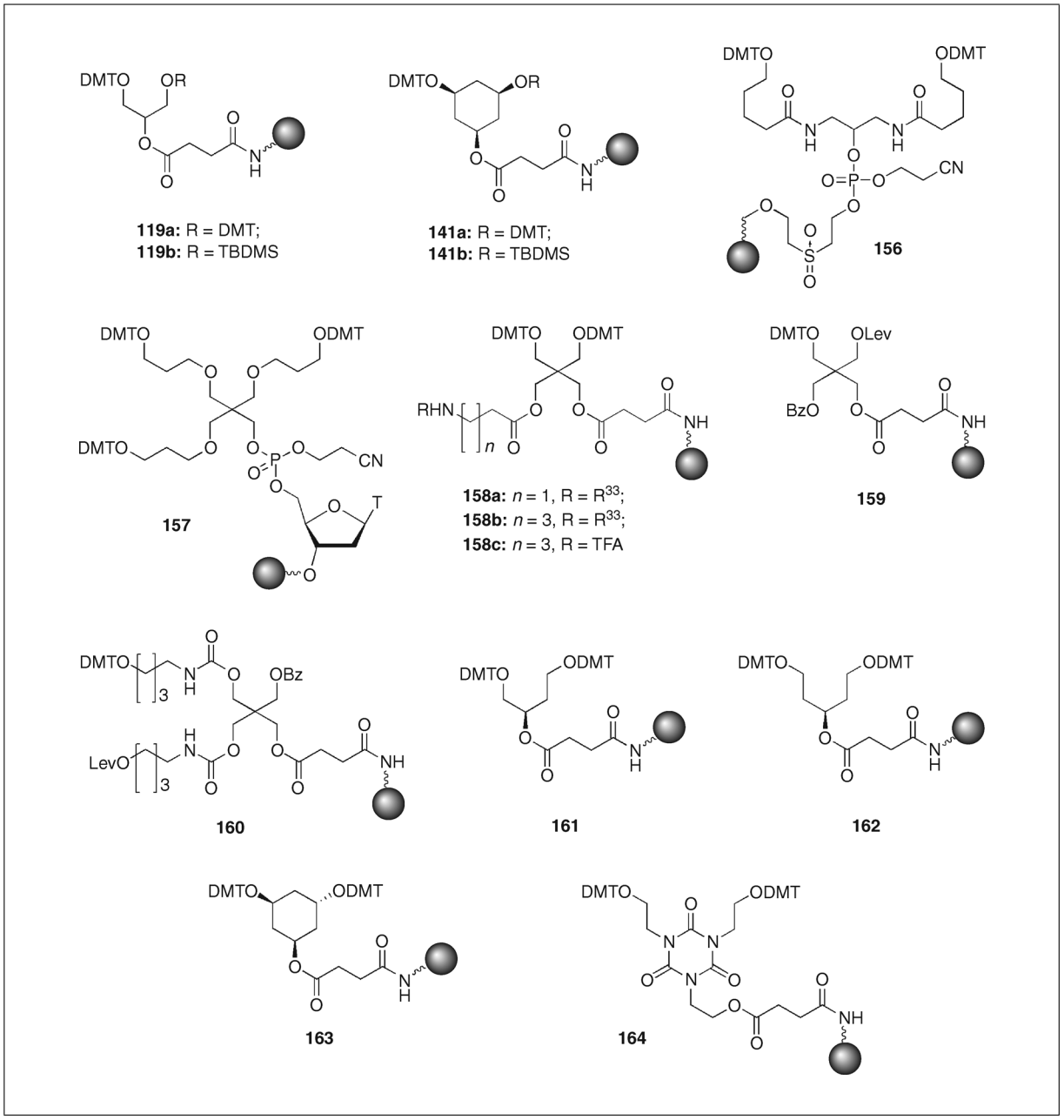

Figure 3.1.19 Structures of solid supports **156** to **164** for preparation of multiantennary oligonucleotides.

oligonucleotide arm was assembled starting with the DMT-protected hydroxy group (De Napoli et al., 1999a,b). TBDMS protection was then removed with $Et_3N{\cdot}3HF$ for 18 hr at room temperature, followed by the assembly of the second arm. The attempted removal of TBDMS protection with TBAF and TBAF/AcOH in THF or THF/water resulted in cleavage of succinoyl ester and premature release of oligonucleotide material.

In a more convenient manner, branched and circular oligonucleotides are prepared using a combination of orthogonal DMT and levulinyl groups (Azhayev et al., 1993; Guzaev et al., 1994; Azhayeva et al., 1995a,b). Following this strategy, solid supports **159** and **160** were prepared and used in the synthesis of unsymmetrically branched oligonucleotides (Ueno et al., 2003). Starting with the DMT-protected arm, the first branch was assembled and capped. The levulinyl group was removed by treatment with 0.5 M hydrazine hydrate in a mixture of pyridine and AcOH (4:1, 20 min), after which the synthesis of the second branch was carried out.

CONCLUSIONS

The chemistry of solid supports emerged as a response to challenges of life sciences and

pressure from end users of synthetic oligonucleotides. So far, these challenges were met within reasonable timeframes to the extent satisfying the contemporary standards of purity, convenience, and cost. In the competitive field of oligonucleotide synthesis, the standards grow higher very rapidly. It does not take much foresight to expect that further evolution of the chemistry of solid supports might include a more careful analysis of impurities generated by market-oriented linkers and a research effort towards the elimination of the respective side reactions. There is also little doubt that the rapidly expanding life sciences will create new demands for tailored oligonucleotides, which will be addressed, among other means, by development of novel linkers. We hope that this review provides sufficient information for an informed selection of appropriate linkers for user-specific applications and aids in designing novel solid supports.

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INTERNET RESOURCES

http://www.glenresearch.com/ProductFiles/20-5040.html

Glen Research #1.

http://www.glenresearch.com/Technical/TB_CPR_II.pdf

Glen Research #2: Chemical phosphorylation reagent II. Technical Bulletin.

http://www.glenresearch.com/Technical/Thiol-Modifier%20S-S-1.pdf

Glen Research. #3: Thiol-modifier S-S-phosphoramidite and supports.

APPENDIX

The relationship between identifiers *n* and chemical structures of pendant groups is set in Figures 3.1.20, 3.1.21, 3.11.22, and 3.1.23, and in the relevant text. Table 3.1.1 will help readers to locate solid supports derivatized with a pendant or a common protecting group of interest. For instance, tetramethylrhodamine (TAMRA) is encoded as R^{64} in Figure 3.1.23. Referring to Table 3.1.1, R^{64} is found in solid supports **120g, 120h, 122c**, and **122d**.

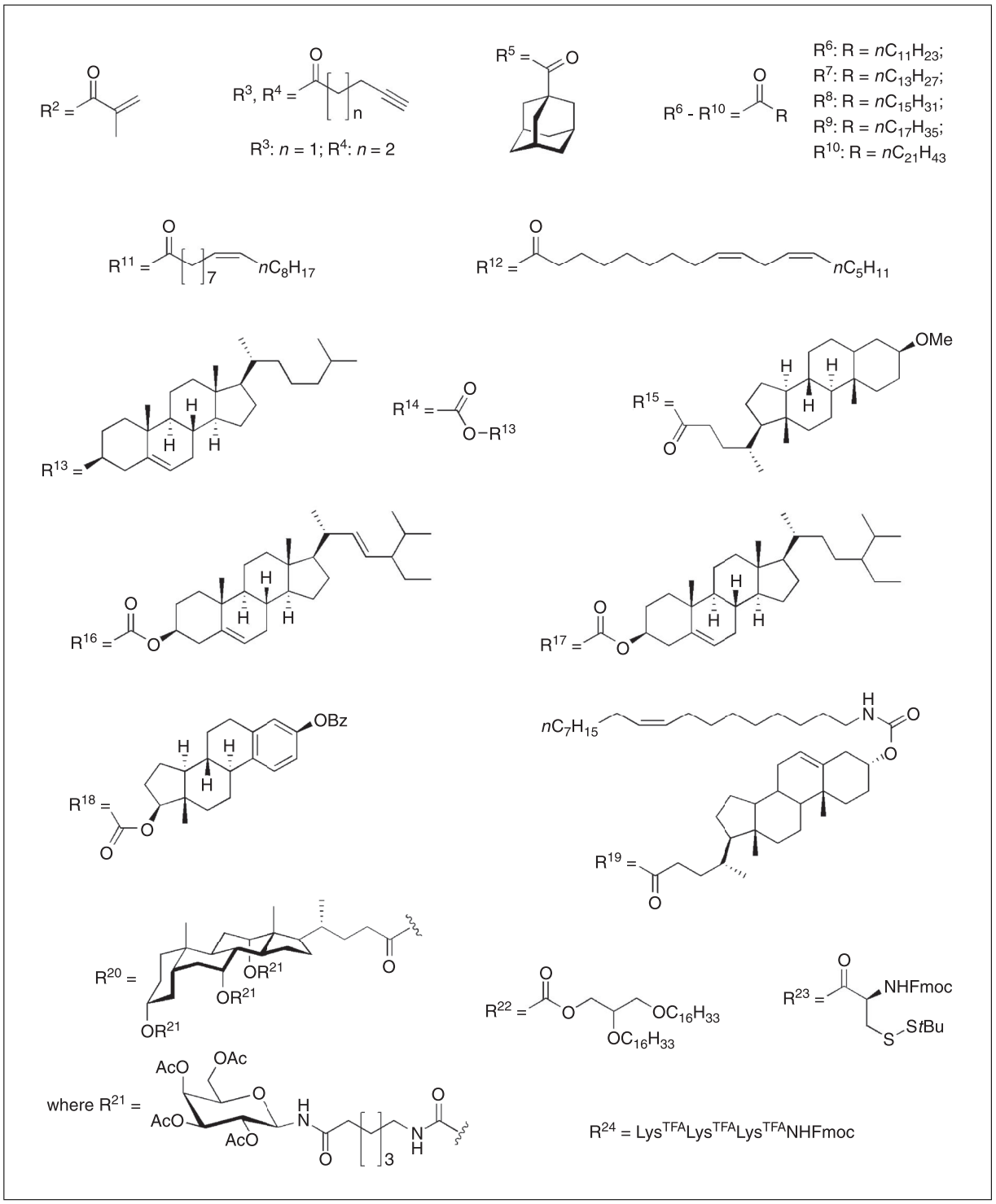

Figure 3.1.20 Structures of aliphatic, alicyclic, and saturated linear pendants R^2 to R^{24} occurring in solid supports in this unit (to locate specific supports, see Table 3.1.1).

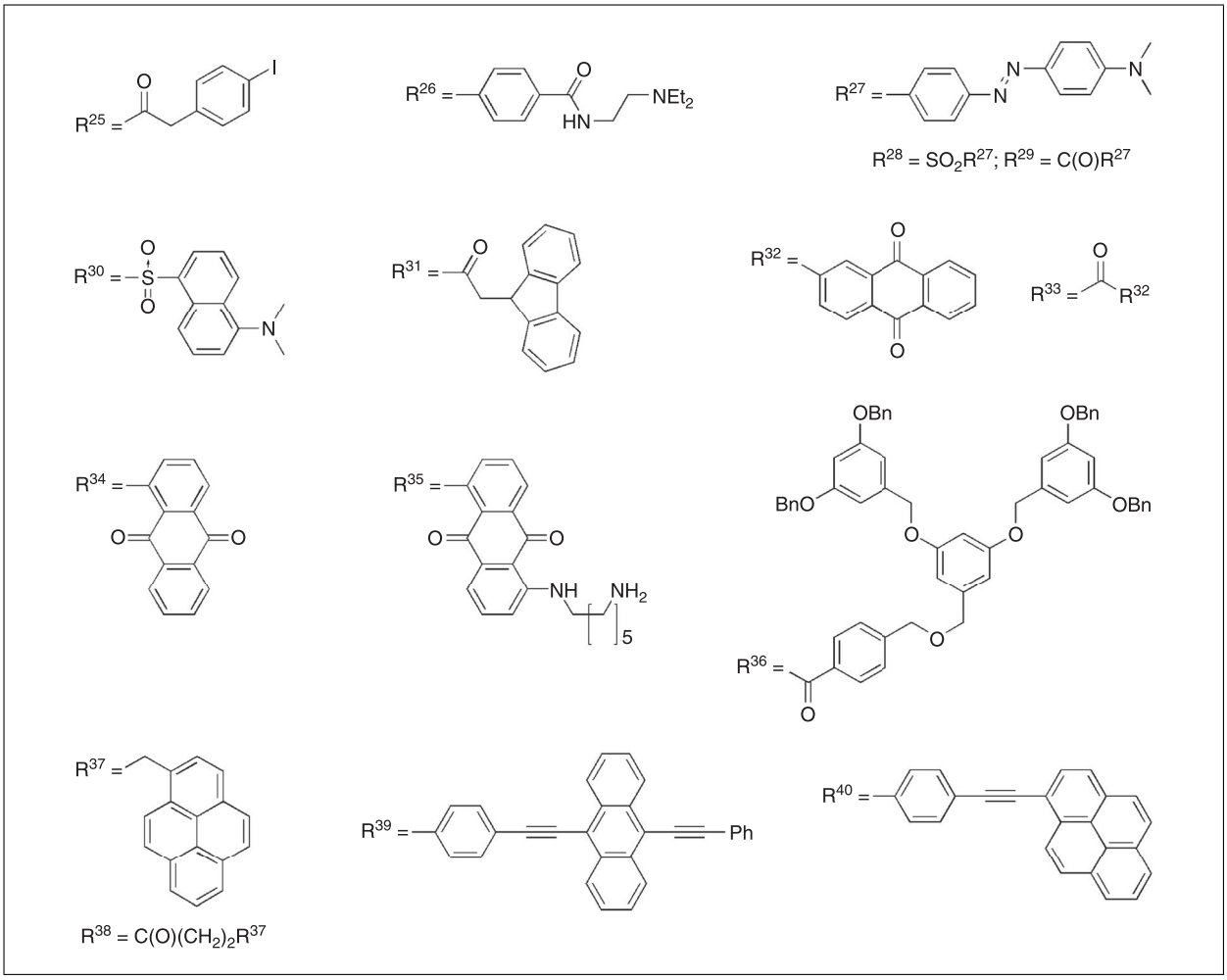

Figure 3.1.21 Structures of aromatic mono- and polycyclic pendants R^{25} to R^{40} (to locate specific supports, see Table 3.1.1).

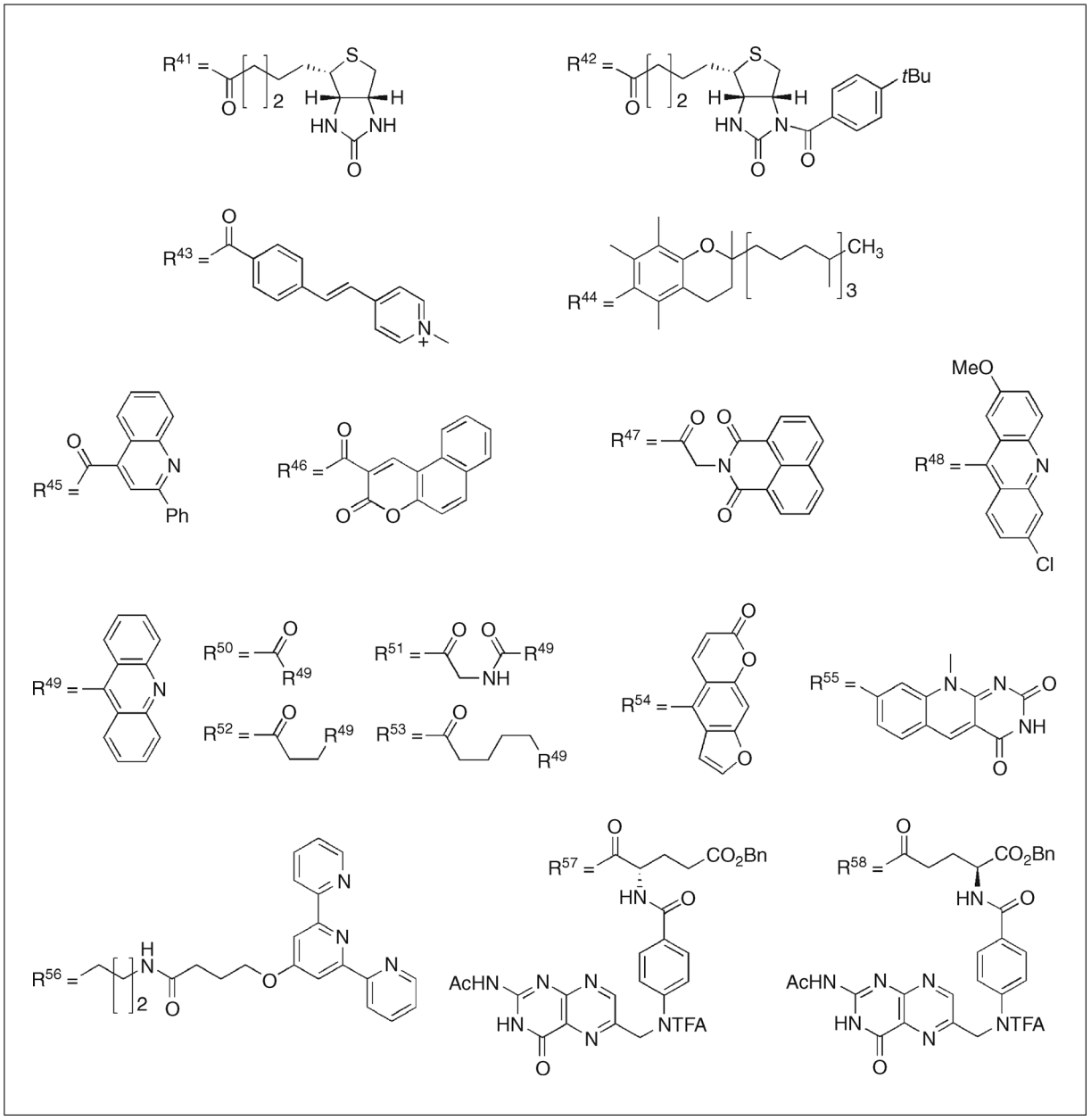

Figure 3.1.22 Structures of heterocyclic pendants R^{41} to R^{58} containing 1 to 3 cycles (to locate specific supports, see Table 3.1.1).

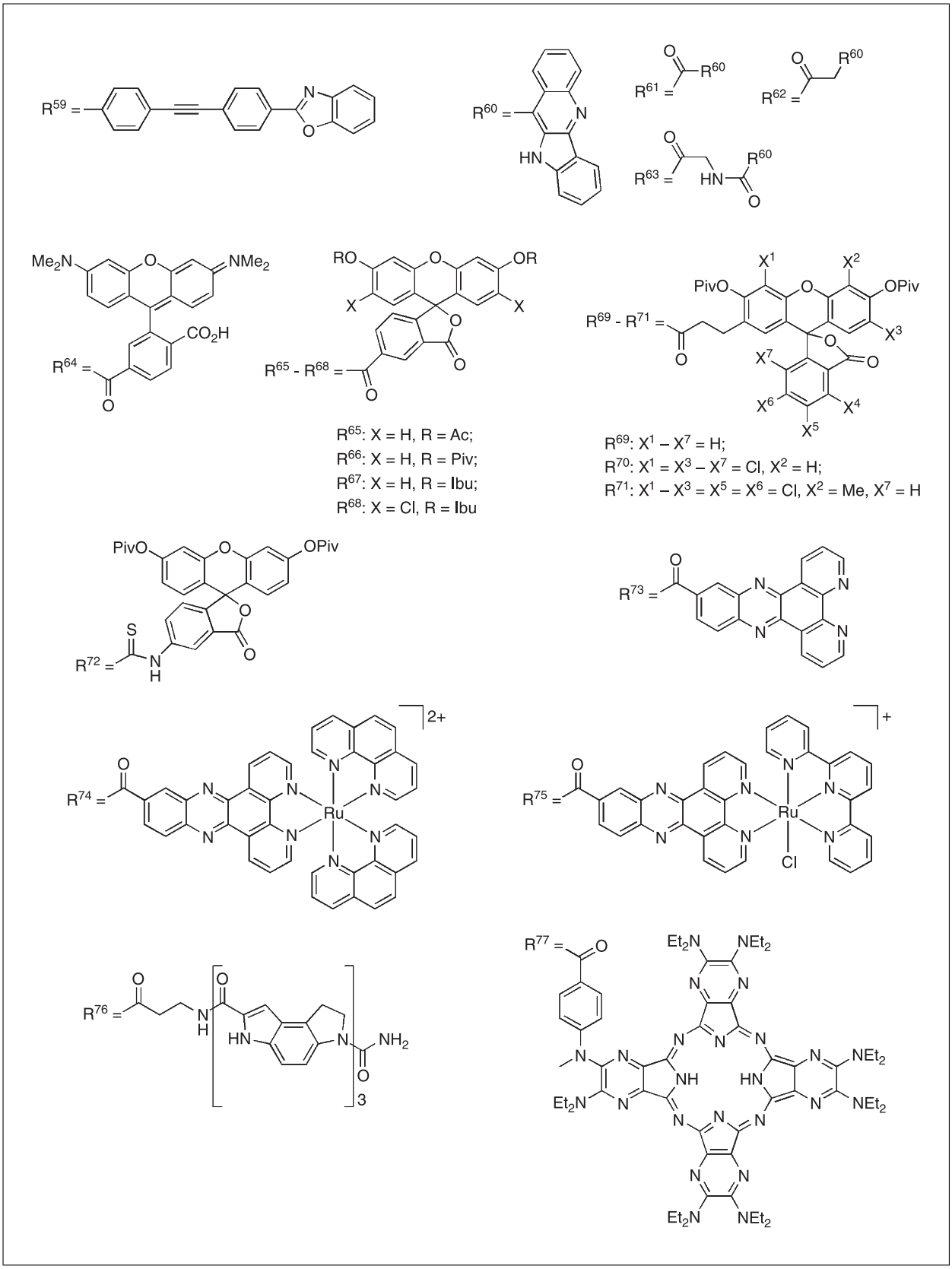

Figure 3.1.23 Structures of heterocyclic pendants R^{59} to R^{77} containing 4 or more cycles (to locate specific supports, see Table 3.1.1).

Table 3.1.1 Locator of Pendants and Common Protecting Groups Used in Solid Supports

Pendant	Solid support	Pendant	Solid support	Pendant	Solid support
R^1	nd	R^{34}	**136d**	R^{66}	**113a, 120a, 137b**
R^2	**148d**	R^{35}	**136e**	R^{67}	**127g**
R^3	**153a**	R^{36}	**111f**	R^{68}	**154b**
R^4	**142m, 143l**	R^{37}	**116d, 127c, 127d,**	R^{69}	**142j**
R^5	**113c**		**136c**	R^{70}	**142k**
R^6	**143b**	R^{38}	**111i, 124a, 135**	R^{71}	**142l**
R^7	**143c**	R^{39}	**133b**	R^{72}	**120i**
R^8	**111g, 128a, 143d**	R^{40}	**133c**	R^{73}	**113e, 114a, 114b**
R^9	**143e**	R^{41}	**120b, 127f, 134d,**	R^{74}	**114c**
R^{10}	**143f**		**137a, 143k, 151b,**	R^{75}	**114d**
R^{11}	**128b, 143g**		**154a**	R^{76}	**111d**
R^{12}	**143h**	R^{42}	**120j**	R^{77}	**111h**
R^{13}	**116a**	R^{43}	**124c**	Ac	**17b**, **22**, **24a, 24b, 25a**,
R^{14}	**111e, 113b, 120e,**	R^{44}	**116b**		**25b**, **26**, **27c**, **77, 81**,
	121a, **125**, **128c,**	R^{45}	**124e**		**138, 139**, **140a, 140b**,
	134c, 142a, 143a,	R^{46}	**124f**		**140c**, **145**
	148c, 153b	R^{47}	**124g**	Alloc	**16a**
R^{15}	**142d**	R^{48}	**113d, 120c**,	Bn	**15, 117, 118**
R^{16}	**142e**	R^{49}	Part of R^{50} - R^{53}	Bz	**10**, **17c**, **78, 84, 146**,
R^{17}	**142f**	R^{50}	**124h**		**159, 160**
R^{18}	**142g**	R^{51}	**124i**	Cl-acetyl	**14**
R^{19}	**143i**	R^{52}	**142b**	Cl_2-acetyl	**19a, 27a**
R^{20}	**120k**	R^{53}	**142c**	Cl_3-acetyl	**27f**
R^{21}	Part of R^{20}	R^{54}	**122e**	Formyl	**18**
R^{22}	**142h**	R^{55}	**148b**	Fmoc	**79, 85**, **111a, 114e**,
R^{23}	**153c**	R^{56}	**127h**		**115c, 120d, 120f**,
R^{24}	**153d**	R^{57}	**113f**		**121b, 122a, 122b**,
R^{25}	**153e**	R^{58}	**113g**		**126b, 127a, 127b**,
R^{26}	**136b**	R^{59}	**133a**		**134b**, **149, 150**,
R^{27}	Part of R^{28} and R^{29}	R^{60}	Part of R^{61}, R^{62}, & R^{63}	Lev	**159, 160**
R^{28}	**113h, 142i**	R^{61}	**124j**	Piv	**17d**
R^{29}	**113i**	R^{62}	**124k**	Pht	**111c, 121c**,
R^{30}	**126c**	R^{63}	**124l**	TBDMS	**119b, 141b**
R^{31}	**124d**	R^{64}	**120g, 120h, 122c,**	TFA	**15**, **17a, 111b, 115b**,
R^{32}	**148a**		**122d**		**126a, 127e, 134a**,
R^{33}	**124b, 158a, 158b**	R^{65}	**153f**		**151a, 158c**