Solid-phase synthesis of base-sensitive oligonucleotides

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Dedicated to Professor Harri Lönnberg on occasion of his 60th birthday

Abstract
Oligonucleotides bearing N-acylated and O-alkylated nucleobases, biodegradable phosphate protections, cap-structures, internucleotidic H-phosphonates, methylphosphates, O-methylphosphorothioates and phosphotriesters are sensitive to nucleophiles, nucleopeptides are prone to β-elimination and some of the dihydropyrimidines undergo retro condensation. None of them withstand the standard ammonolytic deprotection, but an alternative synthetic procedure should be adopted. In this review, orthogonal protecting group schemes and the N-unprotected methods for the solid-phase synthesis of these important oligonucleotides are presented.

Keywords: Base-sensitive oligonucleotides, orthogonal protections, N-unprotected methods

Contents

1. Introduction

In the standard automated oligonucleotide synthesis benzoyl (Bz, for cytidine and adenosine) and isobutyryl (*i*Bu, for guanosine) are used to protect nucleobases, cyanoethyl (CE) to protect internucleotidic phosphates (in the phosphoramidite method) and the succinyl linker to immobilize the growing chain to a solid support. Strong alkaline conditions are required to expose nucleobases, which excludes the possibility for the preparation of base-sensitive oligonucleotides. Three potential choices are described in Figure 1 to sidestep this limitation. The most apparent one is *Strategy A*, in which orthogonal nucleobase protections and an orthogonal linker are adopted. After the chain assembly and incorporation of the base labile conjugate group, the protections are orthogonally removed either on a solid support (*Strategy A1*) or, alternatively, concomitant release occurs (*Strategy A2*). The former choice is usually preferred to the latter, since the cleavage reagents and the cleavage products of the protections may be washed away. In the *Strategy B* the oligonucleotide chain is assembled without nucleobase protections, and potential problems related to the deprotection step are omitted. The N-unprotected phosphoramidite method still requires internucleotidic phosphate protections, but the N-unprotected H-phosphonate method may be carried out completely without protecting groups. The only limiting step is cleavage of the linker. If chemoselective conjugation to an unprotected oligonucleotide is possible, *Strategy C* may be adopted. The terminal unit is anchored to a solid support via a base-stable but mildly cleavable linker. The oligonucleotide is assembled using standard building blocks and then the nucleobase (Bz, *i*Bu) and phosphate (CE) protections are removed by ammonolysis. The base-labile modification is selectively introduced to the fully deprotected but still solid-supported oligonucleotide (the most apparent site is 5'-OH group). Mild cleavage of the linker releases the desired modified oligonucleotide. Examples of the *Strategies A, B* and *C* are described more detailed in Chapters 3, 4 and 5, respectively.

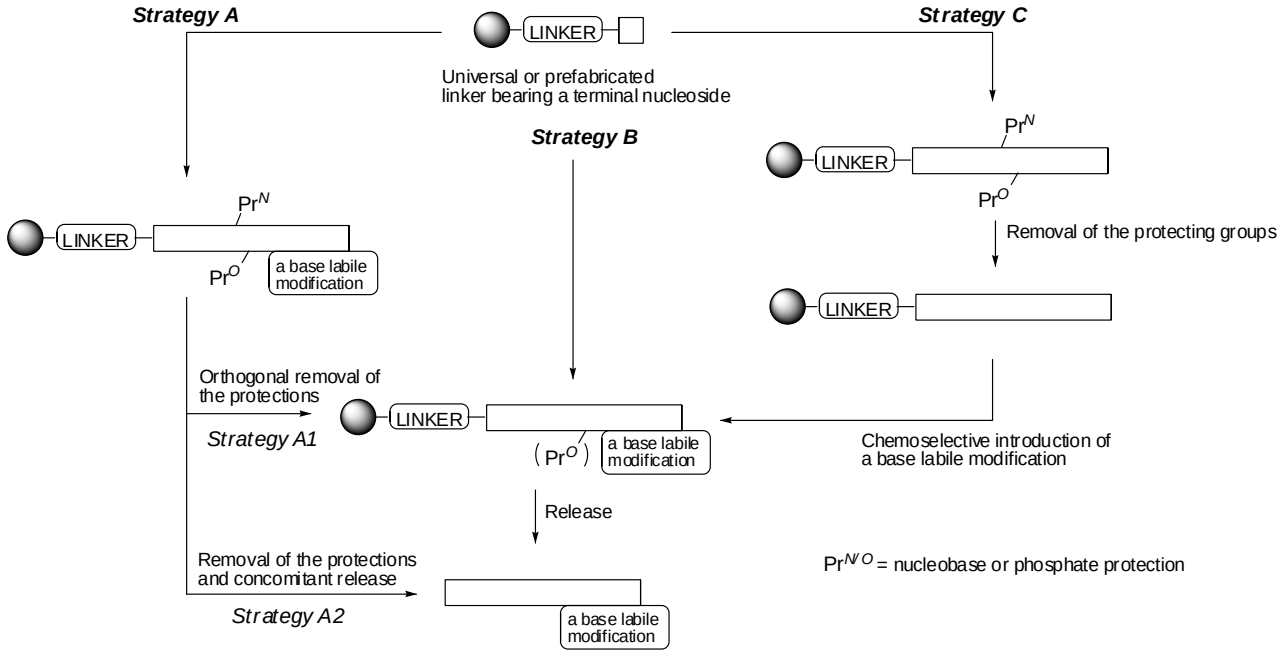


Figure 1. Strategies for the synthesis of base-sensitive oligonucleotides.

2. Orthogonal Linkers in Automated DNA Synthesis

Regardless of the adopted strategy (A, B or C), the cleavage of the linker should be mild enough to remain the base sensitive modification intact. Responding to this minimal requirement, several orthogonal linkers which may be cleaved in mild basic or acidic or completely neutral conditions are nowadays available. Sometimes the modifications are, however, stable enough upon a short exposure to mild protic alkaline treatment (hydrolysis, alcoholysis aminolysis etc.), for which the standard succinyl linker may essentially be applied (cf. Figure 9, below), but more base labile oxalyl (**1**, Alul et al., 1991),[1] malonic acid (**2**, Guzaev and Lönnberg, 1997),[2] diglycolic acid (**3**, Mullah et al., 1998)[3] or hydroquinone-*O,O'*-diacetic acid (**4**, Pon and Yu, 1997)[4] arms are primarily recommended. Solid supported nucleosides anchored *via* hydroquinone-*O,O'*-diacetic acid (Q-linker arm) are commercially available. The cleavage may be performed using *e.g.* only 2 to 3 min treatment with conc. ammonia, 50 mM K_2CO_3 in anhydrous methanol[5] (1 min at rt) or Et_3N•3HF (neat, 5 min at rt). The fluoride ion promoted cleavage is noteworthy, since it may impugn the motive of the silyl-linkers (cf. **11-14** in Figure 4).

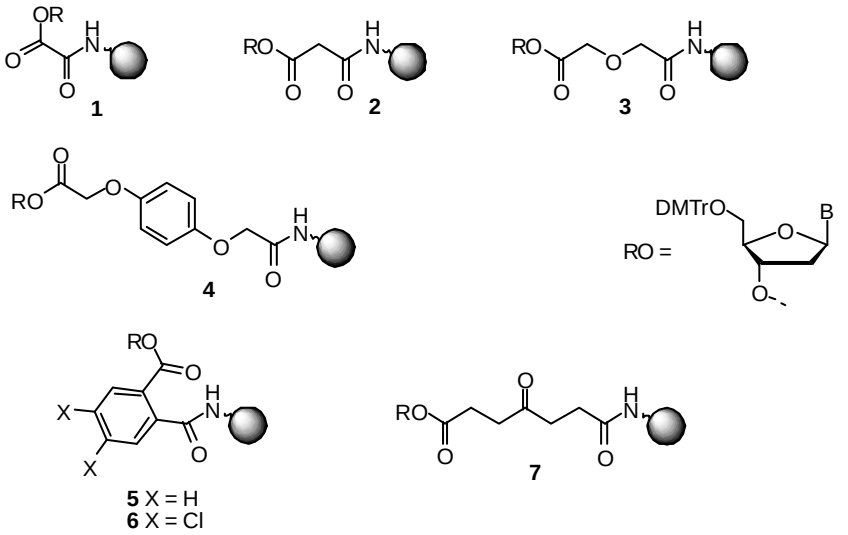


Figure 2. Dioic acid linkers which are cleaved in mild conditions.

The other mildly cleavable dioic acid arms are phthaloyl (**5**, Brown et al., 1989, **6**, Wada et al. 1998)[6,7] and recently described 4-oxoheptanedioic acid (**7**, Leisvuori et al., 2008)[8] linkers. As a contrast to **1-4**, a clear mechanistic difference is involved in **5-7**. In the phthaloyl linkers (**5** and **6**), intramolecular nucleophilic attack of the neighboring deprotonated amide group (*i.e.* imide formation) takes place in the presence of strong organic base, and hence nucleophilic conditions may be avoided. For example, 10% 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) in acetonitrile (5 min at rt) may be used for the cleavage of **6**.[7,9] It should be noted that the standard succinyl linker undergoes the same reaction, albeit the reaction is slow. The 4-oxoheptanedioic acid linker (**7**), bearing the 4-oxobutanoate structural motif, may be cleaved with a treatment of hydrazinium acetate. The similar cyclative reaction, which occurs in the levulinoyl deprotection, takes place.

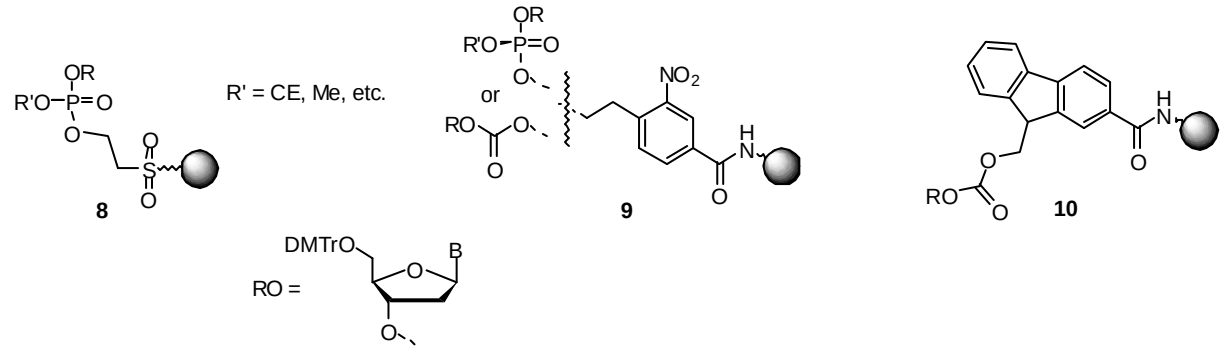


Figure 3. Linkers which may be cleaved by β-elimination.

Sensitivity to β-elimination may be utilized in sulfonylethyl-based (**8**, Schwyzer et al., 1984)[10,11,12,13], 2-(*o*-nitrophenyl)ethyloxycarbonyl (**9,** Eritja et al., 1991)[14] and 9-

fluorenylmethyloxycarbonyl (Fmoc, **10,** Avino et al. 1996)[15] linkers (Figure 3). Like phthaloyl linkers (**5** and **6**), these may be cleaved in basic but nonnuclephilic conditions (e.g. treatments with a mixture of DBU or Et_3N, cf. Chapter 3.3.). Linkers **8-9** bear also possibility to universality (*i.e.* the terminal nucleoside may be added as part of the automated synthesis).[11,13,14]

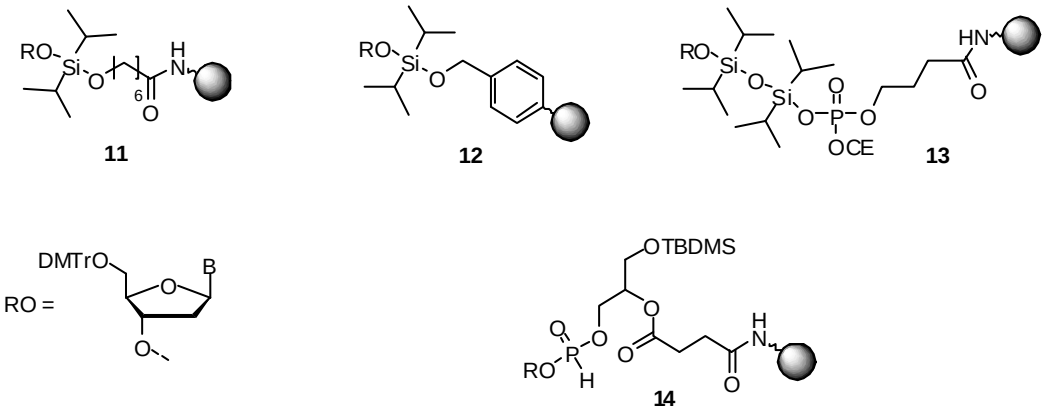


Figure 4. Silyl linkers.

Mild fluoride ion promoted cleavage and under neutral pH may be obtained with silyl linker arms (Routledge, et al., 1995) (Figure 4).[16-19] A typical cleavage procedure is treatment with a mixture of acetic acid buffered tetrabutylammonium fluoride (*e.g.* 1.0 M TBAF•H_2O with 1.0 M AcOH in THF, 1h at rt) or Et_3N•3HF (cf. Chapter 3.4. and linkers **4** and **23**). **14** (Ferreira et al., 2005) is a universal linker with a requirement that the terminal unit should be anchored as an *H*-phosphonate bond (cf. **23**). Desilylation exposes the hydroxyl group, which leads to a cyclative cleavage.[20]

Photolysis offers perhaps the mildest possible method for release. Although shortcomings, *e.g.* unexpected long irradiation time or thymine-thymine photodimerization (cf, chapter 3.6) may sometimes be involved, a photolabile linker (Greenberg, 1993) has most frequently been adopted for the synthesis of the base-sensitive oligonucleotides (Figure 5).[21-25] As a contrast to **15-17**, **18** (Dell'Aquila et al., 1997) is a universal linker and it may be used for the synthesis of 3'-phosphorylated oligonucleotides.

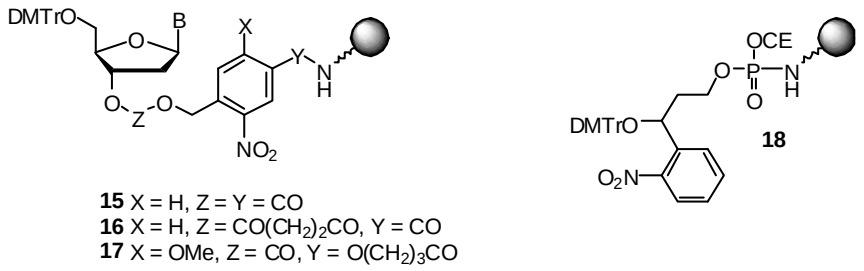


Figure 5. Photolabile linkers.

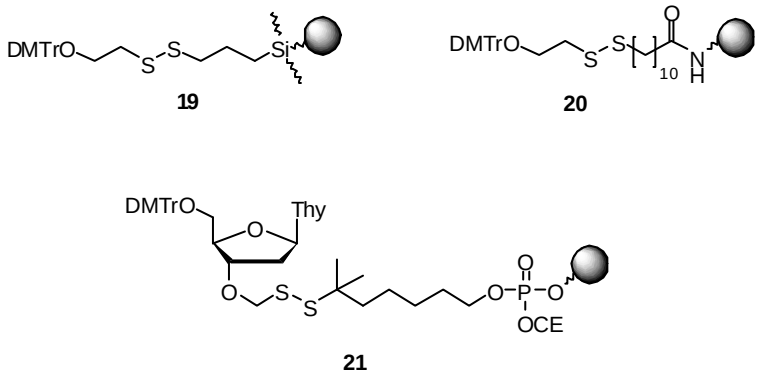


Figure 6. Disulfide linkers.

Base stable disulfide linkers (Kumar et al., 1991) offer possibility to on-resin removal of *N*-acyl nucleobase protections, but the cleavage may be performed in mild conditions using reductive treatments with dithiotreitol (DTT) or tris-2-carboxyethyl phosphine (TCEP) (Figure 6).[26,27] Thus they may be applied for the *Strategy C*, in which a base sensitive group is chemoselectively coupled to unprotected but still solid-supported oligonucleotides. **19** and **20** are universal linkers, in which cleavage occurs *via* episulfide formation to yield 3'-phosphorylated oligonucleotides. Recently an alternative dithiomethyl linker **21**, suitable for the synthesis of 3'-OH terminal oligonucleotides, has been reported.[28]

The universal allyl linker **22**[29] is also stable to the ammonolytic deprotection, but the cleavage may be performed in neutral conditions in presence of a Pd^0-catalyst (cf. chapter 3.5.). 3'-OH terminal oligonucleotides may be obtained with **23**. After Alloc deprotection, the reagents may be washed away. Intramolecular nucleophilic attack by the exposed amino group to a phosphorus atom releases the desired oligonucleotide in mild basic conditions (cf. **14** and the other aminopropanediol linkers).[30]

Figure 7. Universal Pd^0 labile linkers.

Phosphoramidate linkage **24** deserves special attention, since its simplicity. It may be cleaved by fluoride ion (Et_3N•3HF-THF, 1:1, *v/v*, 4h at rt) acting like a silyl linker (**11-14**), [65] but it is stable to ammonolysis if the linkage is able to leave one of the two *O*-alkyl groups (*e.g.* cyanoethyl group). After dealkylation, the linkage (**25**) becomes labile to acid (AcOH).[31] These both behaviours have been utilized for the synthesis of base sensitive oligonucleotides (Gryaznov and Letsinger, 1992, Guerlavais-Dagland et al., 2003, Chapter 3.4 and Chapter 5).

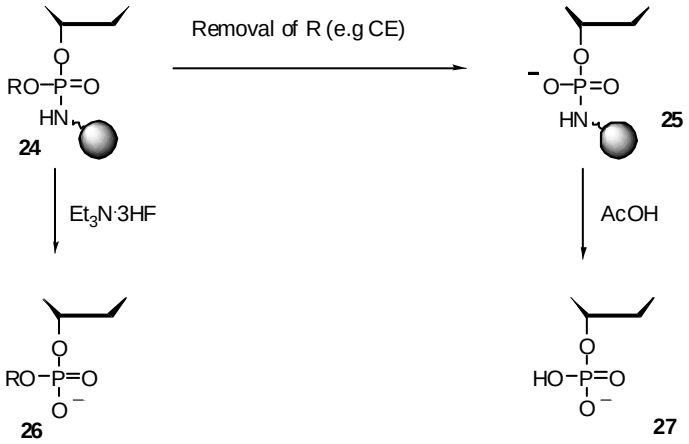


Figure 8. Cleavage of the phosphoramidate linkages.

3. DNA Synthesis Using Orthogonal Protection (Strategy A)

For the synthesis of base-sensitive oligonucleotides, the standard oligonucleotide protections (Bz, *i*Bu, CE) are frequently replaced with protections, which may be selectively removed in the presence of a fragile conjugate group. However, adopting of any of the orthogonal protecting group schemes is not necessarily straightforward. Synthesis of the non-standard building blocks may require effort and their coupling efficiency is not always as good as that of the standard ones. Retarded deprotection with longer oligonucleotides and contamination of the product caused by the deprotection step are also sometimes encountered. Furthermore, omitting of the standard acetic anhydride capping step, the self-evident manipulation in the N-unprotected methods (Chapter 4), should be considered with the orthogonal protecting group schemes, in which the transamidation may take place. Formation of unexpected N-acetylated oligonucleotides has been reported at least with the phenoxyacetyl (PAC, Chapter 3.1), [32,33] allyloxycarbonyl (Alloc, Chapter 3.5)[34] and 2,2'-bis(2-nitrophenyl)ethoxycarbonyl (diNPEOC, Chapter 3.6)[35] protected nucleosides. Potential mechanism for the transamidation of protected guanosine is described in Figure 9 (Zhu et al. 2001).[32] Protected amino group (**28**) is further acylated upon the repetitive capping steps (**29**). In the deprotection conditions, the orthogonal protection is cleaved but the acetyl cap remains intact (**30**). To overcome this shortcoming, the acetic anhydride capping step may be omitted but also alternative capping reagents may be considered. More hindered pivaloylanhydride[32] and diethyl *N,N*-diisopropyl phosphoramidite[34] has been used for this purpose.

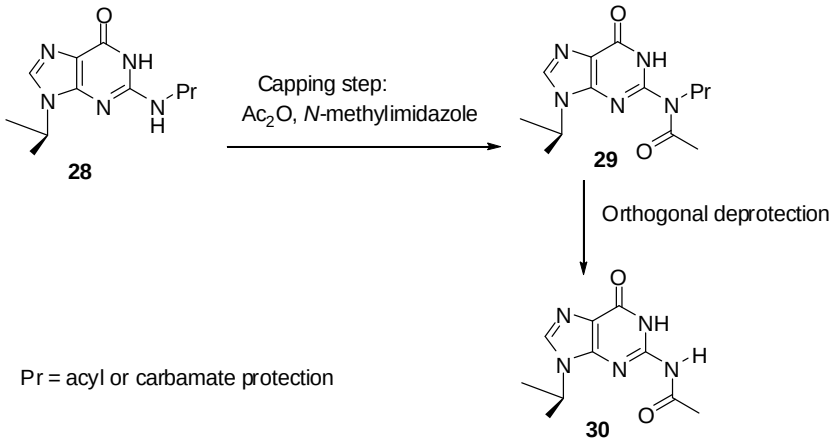


Figure 9. Potential transamidation upon the acetic anhydride capping step (Zhu et al. 2001).[32]

3.1 Protecting groups removable with mild alkaline conditions

In principle, the exocyclic amino groups may be selectively exposed by a base-catalyzed treatment in the presence of a base-sensitive modification. However, a real generality of any such a protecting group strategy should be impugned, since the deprotection time is always dependent on the number of protecting groups and nature of the heterocyclic bases. This is a contrast to the linkers (cf. **1-4**), in which cleavage time of a single bond is nearly constant. However, the 'fast-deprotecting' phosphoramidites [*i.e.* PAC (Köster at al. 1981, Schulhof et al. 1987)[36,37] protected adenosine and guanosine and isobutyryl or acetyl protected cytidine] have successfully been used for the synthesis of oligonucleotides bearing fragile O^4-alkylthymidine,[38] O^6-ethyl-2'-deoxyguanosine,[32] 2'-deoxyribosylurea,[39] 5,6-dihydrouridine,[40] (5*R*)-5,6-dihydro-5-hydroxythymidine[41] and 8-vinyl-2'-deoxyadenosine[42] units. Synthesis of (5*R*)-5,6-dihydro-5-hydroxythymidine bearing oligonucleotide (**37**) using the 'fast-deprotecting' phosphoramidites (**32-34**) is described in Figure 10. After standard chain assembly, deprotection and concomitant release from the succinyl arm is carried out by 50mM K_2CO_3 in anhydrous methanol (for 3h at 25 °C). To avoid potential unnecessary degradation of (5*R*)-5,6-dihydro-5-hydroxythymidine, the cleavage reaction is neutralized and quenched by adding of 2 molar equivalents of acetic acid.

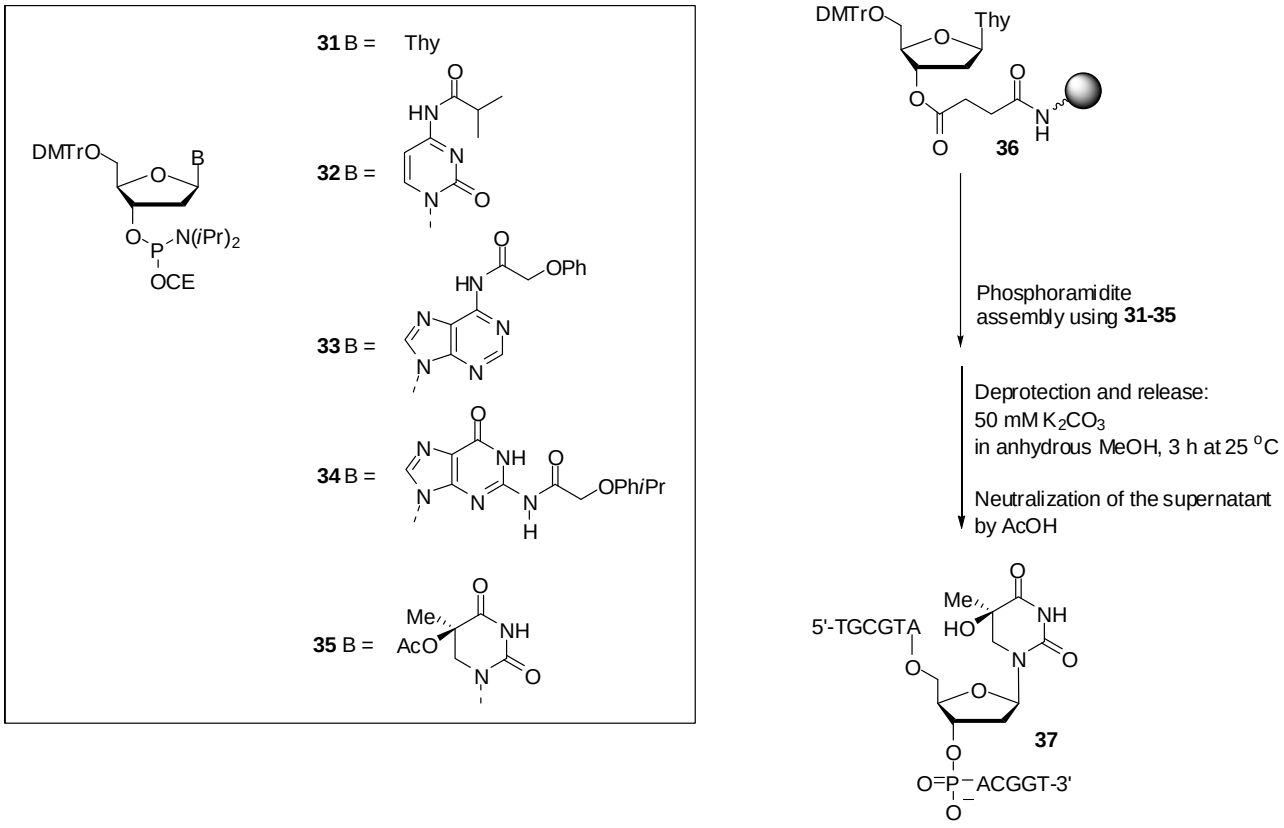


Figure 10. Synthesis of fragile oligonucleotide (**37**) bearing (5*R*)-5,6-dihydro-5-hydroxythymidine unit (cf. **35**). The 'fast-deprotecting' phosphoramidites (**32-34**) are used (Sambandam and Greenberg, 1999).[41]

3.2 Pentenoyl protected nucleosides

N-Pent-4-enoyl (PNT) protected nucleosides have gained unexpected little popularity in the preparation of base sensitive oligonucleotides (Iyer et al., 1996).[43,44] The protecting group precursor, *i.e.* pentenoylanhydride, is a cheap reagent, syntheses of the building blocks (**38-43**, Figure 11) are simple, coupling efficiency of the building blocks is better than that of the standard building blocks and the deprotection may be carried out by 50 mM K_2CO_3 in anhydrous methanol or by neutral treatments with I_2/THF/H_2O or NIS/THF/H_2O. Iodine is involved in the cleavage and hence difference for the standard phosphoramidite coupling procedure is the oxidation step, for which *t*BuOOH should be used. In the *H*-phosphonate strategy one-pot oxidation and deprotection procedure may be applied. Unfortunately the neutral iodine-promoted cleavage is limited only to short oligonucleotides and for the longer ones the K_2CO_3/MeOH treatment is required. The PNT protection has been applied for the synthesis of oligonucleotides bearing internucleotidic methylphosphate, *O*-methylphosphorothioate and 4-(2,6-dimethylbenzoyloxy)benzylphosphate groups. The latest is a prooligonucleotide, which bears the biodegradable phenolic ester group.

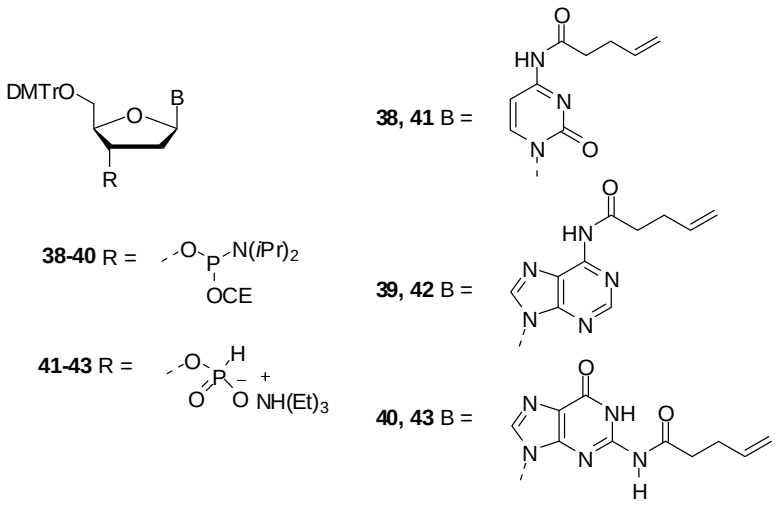


Figure 11. Pentenoyl (PNT) protected nucleoside building blocks (**38-43**).

3.3 Protecting groups removable with β-elimination

Some fragile molecules are not stable under mild protic alkaline conditions (required e.g. deprotections above), but they withstand basic media as long as exposure to potential nucleophiles may be eliminated. Nucleobase protections, such as *p*-nitrophenylethyloxycarbonyl (NPEOC),[53] 9-fluorenyloxycarbonyl (Fmoc),[45] arenesulfonylethoxycarbonyl[46,47] and cyanoethyloxycarbonyl groups[48] may undergo β-elimination in the presence of a non-nucleophilic brønsted base, and hence they are compatible with these base sensitive molecules. In general this compatibility is, however, encountered in the selective deprotection of CE groups to expose internucleotidic phosphates. For example, synthesis of oligothymidylates bearing base sensitive moieties [dihydropyrimidines[49] and biodegradable S-acyl-2-thioethyl[50] and 3-(pivaloyloxy)propyl[51] groups] are usually described prior to that of the corresponding heterooligonucleotides. Compatibility with the nucleobase protection chemistry is omitted, but even in that case the CE groups (in the standard phosphoramidite method) have to be removed, for which non-nucleophilic treatments with DBU (*e.g.* 10% solution in acetonitrile for 1 min)[52] or triethylamine (40% solution in pyridine for 3h)[53] are used. Stability of the linker upon these treatments is desirable, since otherwise the product is liberated into a strong basic environment, in which the resulted acrylonitrile may react with nucleobases.[53] On a solid phase the undesired cyanoethylation is not favoured, since resins may be washed with large volumes of DBU/Et_3N-mixtures, in which the concentration of acrylonitrile may be kept low.

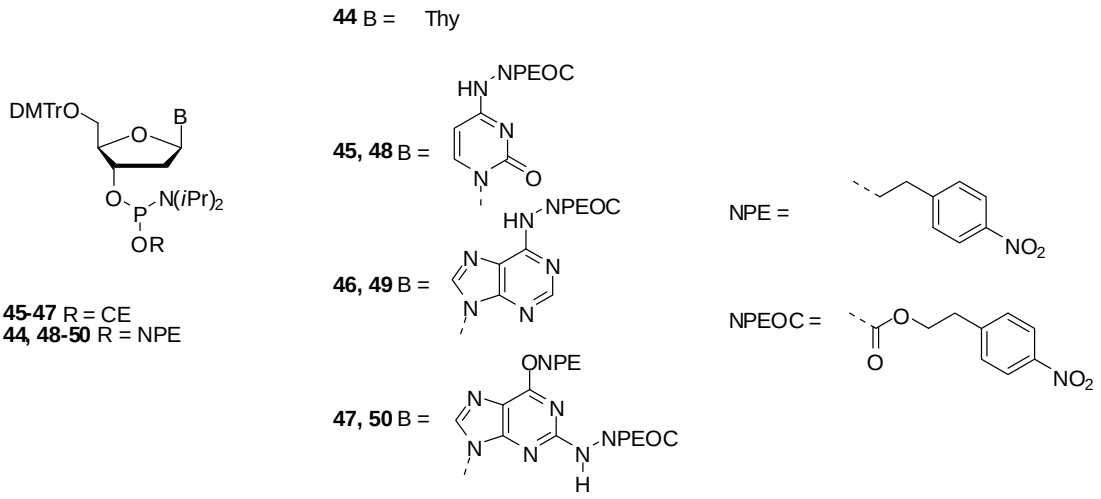


Figure 12. p-nitrophenylethyl (NPEOC/NPE) protected nucleoside phosphoramidites.

Oligonucleotides [d(GCT^{Pr}AGC) and d(GCT^{Bu}AGC)] bearing ammonia sensitive *O*-4-propyl (T^{Pr}) and *O*-4-butylthymidines (T^{Bu}) have been synthesized on a solid phase using *p*-nitrophenylethyl (NPE/NPEOC) nucleobase protections (**45-47,** Figure 12).[54-56] The oligonucleotides are assembled on *o*-nitrophenylcarbonate derivatized resin (**9**) and then two-step deprotection procedure is carried out. The cyanoethyl groups of the phosphates are first selectively removed by a mixture of 40% triethylamine in pyridine and then the resins are subjected to a mixture of 0.5 M DBU in pyridine, which cleaves the *p*-nitrophenylethyl protections and concomitantly releases the target oligonucleotides. As a contrast to acrylonitrile, the released *p*-nitrostyrene is not so strong alkylation agent, but the clear limitation of this procedure is the linker choice, since the product is released to a strong basic media.

More recently cyanoethyloxycarbonyl protections have been applied for the synthesis of oligonucleotides bearing (5*R*)-5,6-dihydro-5-(1-oxo-2-phenylethyl)thymidine unit (cf. **55** in Figure 13).[57] This dihydropyrimidine is prone to a retrocondensation reaction in alkaline condition (*e.g.* 50 mM K_2CO_3 in anhydrous MeOH and 0.5 M DBU in CH_3CN used to cleave the Pac and NPEOC protections). The standard phosphoramidite coupling procedure is used for the chain assembly, applying a temporal 5'-silyl protection. Demethylation of the phosphotriesters is accomplished with a treatment of disodium-2-carbamoyl-2-cyanoethylene-1,1,-dithiolate trihydrate (dccdt) (**57**), the cyanoethyl carbamates are removed by a mixture of triethylamine in DMF (1:1, *v/v*, 16 h at 55°C) (**58**) and then the fully deprotected oligonucleotide is released by photolysis. It may be notable that the unsubstituted cyanoethyloxycarbonyl group may be used for the guanine base (**54**), but the more base labile α,α-dimethylated derivative is required for adenine (**53**) and cytosine (**52**).

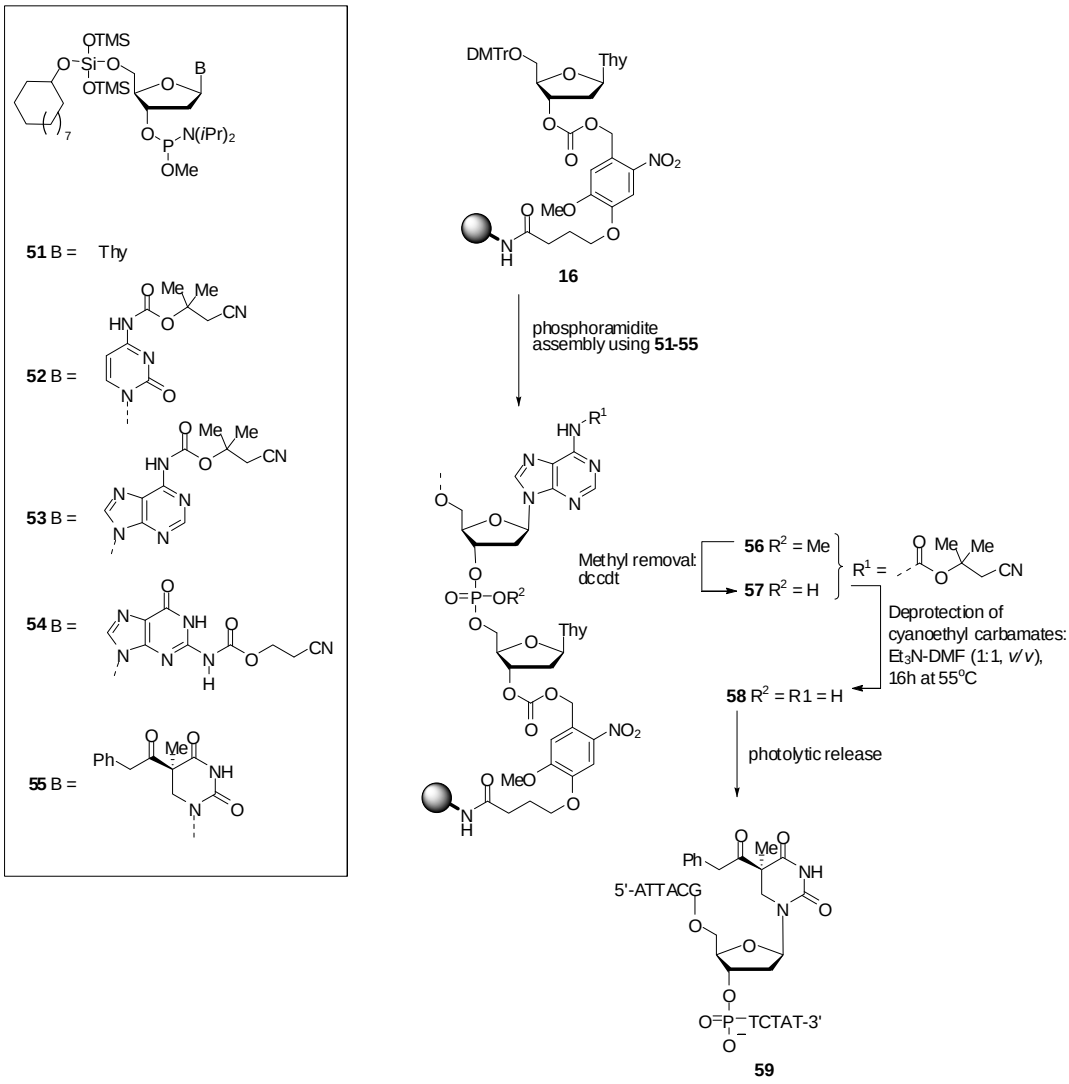


Figure 13. Synthesis of oligonucleotides bearing a fragile dihydropyrimidine unit (**55**) using cyanoethyloxycarbonyl protections (Chen et al., 2000).[57]

3.4 Silyl-based nucleobase protections

For many synthetic purposes, the silyl-based protecting groups are sufficiently stable in acidic and basic conditions (like in the automated DNA synthesis), but they may be rapidly cleaved by a mild treatment, in which the high affinity of a fluoride ion for silicon is utilized. This orthogonality may be applied for the synthesis of base sensitive molecules, albeit the common F^--source, TBAF•H_2O in THF as such, is strong enough *e.g.* to hydrolyse esters. In addition to 2'-*O*-*tert*-butyldimethylsilyl (TBDMS) and 2'-*O*-triisopropylsilyloxymethyl (TOM) protections of ribonucleosides, *O*-diphenylsilylethyl (DPSE)[58,59], *O*-trimethylsilylethyl (TSE)[60,61] *N*-[(*tert*-butyl)(diphenyl)silyloxymethyl]benzoyl (SiOMB)[62-65] and *N*-trimethylsilylethyloxycarbonyl (Teoc)[66] as alternative means for protecting phosphate groups and nucleobases have been

described. A nucleopeptide [H-Phe-Tyr-(pATAT)-NH_2] has been synthesized applying the SiOMB nucleobase protection for adenine,[63] but general applicability of the strategy has extensively demonstrated by the synthesis of *S*-acetyl thioethyl (MeSATE) prooligonucleotides (Figure 14. Guerlavais-Dagland et al., 2003).[65] The 3'-terminal nucleoside is anchored to a universal solid support *via* a phosphoramidite linkage (**65**), and the oligonucleotide is assembled by phosphoramidite coupling chemistry using TSE (**61-63**) and meSATE (**60**, **64**) phosphoramidites of SiOMB protected nucleosides. Removal of the silyl protections and concomitant cleavage of the phosphoroamidite linkage is carried out by a mild treatment with Et_3N•HF in THF (1:1, *v/v*, 4h at rt). 50 mM triethylammonium acetate buffer is then added to the cleavage mixture (for additional 36 h), which is required to release the remaining hydroxymethylbenzoyl group of guanosine base. It may be noteworthy that applicability of DPSE phosphate protection has also been evaluated for the synthesis, but it can not be removed by the Et_3N•HF-treatment.

Lewis acid promoted deprotection may be used for the Teoc group, which is poorly removable by fluoride reagents. A MeSATE phosphotriester analogue of d(5'-GCATTAGCTA-3') has been assembled on a photolabile linker using TSE and meSATE phosphoramidites of Teoc protected nucleosides.[67] After chain assembly, the resin is treated with a saturated mixture of $ZnBr_2$ in niromethane-isopropanol (7 h) to remove the Teoc and TSE protections, washed with 1 M EDTA solution to scavenge the Zn^{2+} cations and then irradiated by UV-light to release the desired prooligonucleotide.

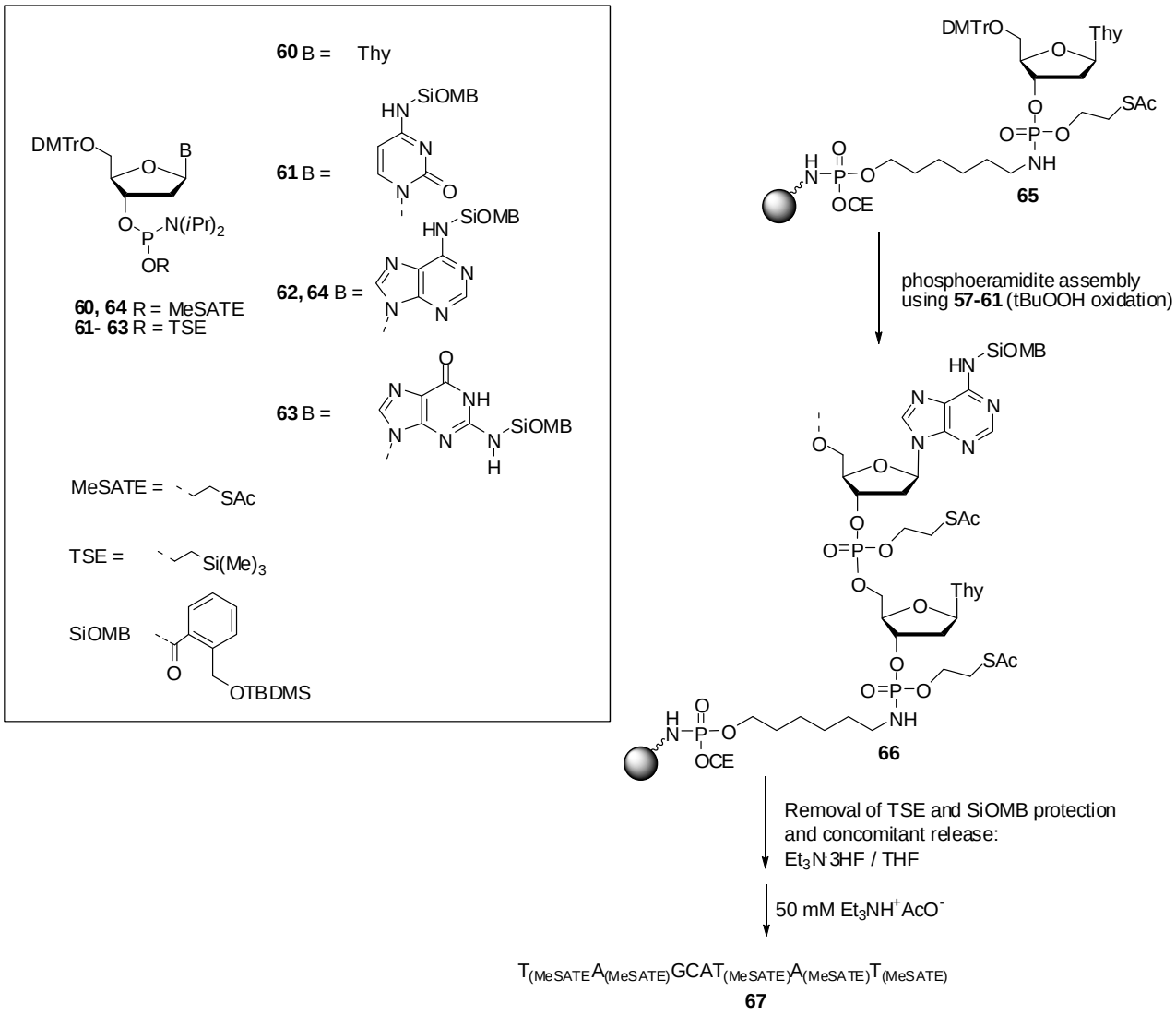


Figure 14. Synthesis of a MeSATE prooligonucleotide using fluoride labile (TSE, SiOMB) protections (Guerlavais-Dagland et al. 2003).[65]

3.5 Allyl protecting groups in the oligonucleotide synthesis

Since the allyl protecting group strategy for the solid phase oligonucleotide synthesis has been introduced (Hayakawa et al. 1990),[68,69] its applicability for the synthesis of base sensitive oligonucleotides was rapidly realized. Advantages of the strategy are orthogonality of the allyl group for a wide range of functional groups and efficiency of the deprotection, for which a neutral Pd^0-catalyzed treatment may be used. The original deprotection conditions involves treatment with a mixture of $Pd_2(dba)_3 \bullet CHCl_3$ (catalyst), triphenylphosphane (regenerator of Pd^0) and a large excess of formic acid and butyl amine (1:1, *v/v*, allyl scavenger) in THF (0.5-1 h at 50 °C). The solid supported oligonucleotide is then washed with a mixture of *N*,*N*-diethyldithiocarbamate (ddtc) to remove the residual palladium. More detailed studies have

shown that contamination of oligonucleotides by traces of Pd may sometimes be a problem regardless of extensive washing.[34] In spite of this occasional shortcoming, a number of base sensitive oligonucleotides (see Figure 15) has successfully been prepared applying the allyl protecting group strategy. *O*-allyl (All), *N*-allyloxycarbonyl (Alloc) protected nucleoside building blocks (**68**-**79**) are described in Figure 15.

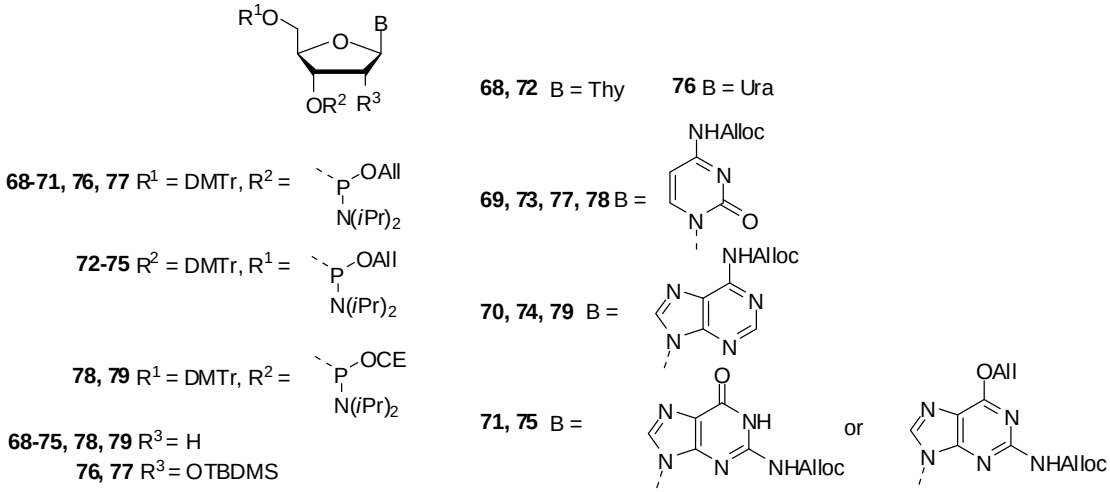


Figure 15. Nucleoside building blocks used in the allyl protecting group strategy.

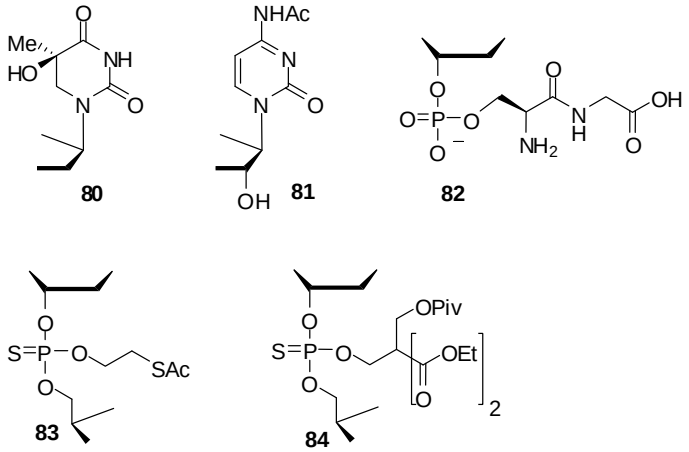


Figure 16. Base sensitive moieties incorporated to hetero oligonucleotides using the allyl protecting group strategy.

An oligonucleotide bearing a base-labile (5*R*)-5,6-dihydro-5-hydroxythymine has been synthesized using the allyl protecting groups on a photolabile *o*-nitrobenzyl support (Figure 17).[70,71] It may be worth of noting that instead of the allylcarbamate and allylester protection of **68**-**71** the modified thymine building block (**85**) bears two allylcarbonate protections, which also

withstand upon the chain elongation. Pd^0-catalyzed allyl deprotection and the photolytic release from **16** result the desired base sensitive oligonucleotide (**86**).

N-acetyl cytidine containing RNA [5'-$UC^{Ac}CC^{Ac}(UC^{Ac})_3UCUC^{Ac}CC^{Ac}UC^{Ac}$, ($C^{Ac}$ = N^4-acetyl cytosine, cf. **81**)] has been synthesized employing **76**, **77**, allyl phosphoramidite of 2'-*O*-TBDMS protected N^4-Ac-cytidine and photolabile support **17**.[72] After allyl deprotection and a photolytic release, the 2'-*O*-TBDMS protections are removed in solution by a mixture of TBAF, which results the fully deprotected oligoribonucleotide.

Allyl protected 5'-phosphoramidites (**71-75**) have been used for the synthesis of an oligonucleotide-3'-dipeptide conjugate.[73] The 5'→3'-elongation affords the oligonucleotide chain and then the 3'-hydroxyl is phosphitylated with a dipeptide phosphoramidite. A phosphodiester is obtained (cf. **82**) which is prone to β-elimination. The Alloc protections are removed and a brief exposure to conc. ammonia releases the conjugate from the standard succinyl support in a good yield.

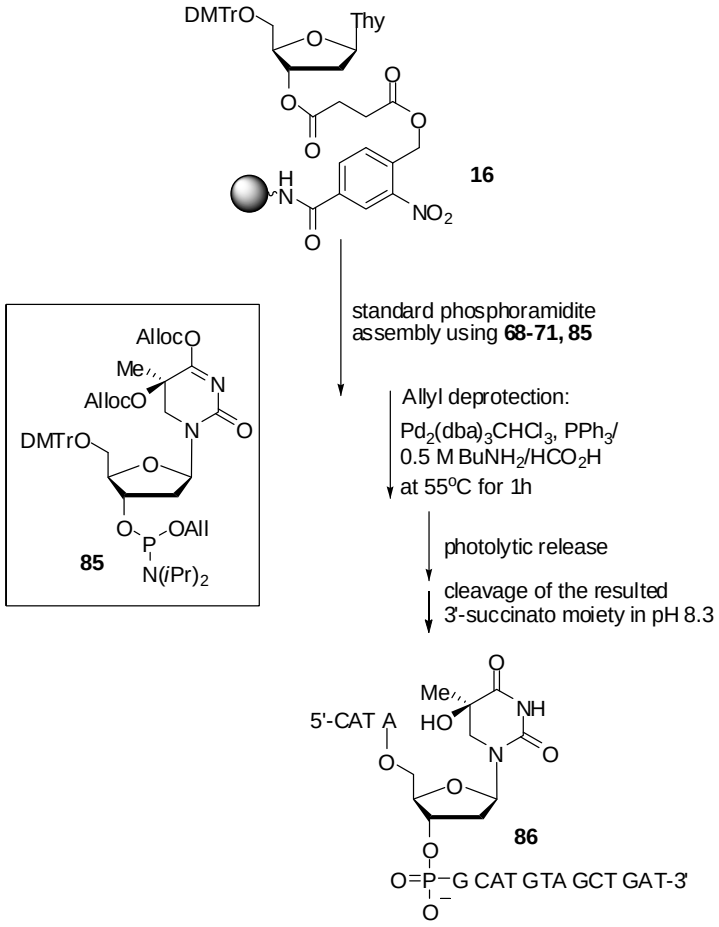


Figure 17. Incorporation of a base-labile (5*R*)-5,6-dihydro-5-hydroxythymine residue to an oligonucleotide sequence (Matray and Greenberg, 1994).[70]

Allyl deprotection and the ddtc washing step have been optimized for the synthesis of the MeSATE-prooligonucleotides (cf. **83**).[34] Optimized milder procedure is required, since butylamine as an allyl scavenger and the ddtc washing (pH = 9.7) step of the original protocol are able to cleave thioesters. A dodecanucleotide d(ACACCCAATTCT), containing 6 internucleotidic MeSATE phosphotriesters is assembled on a photolabile support using the Alloc as a nucleobase protection. A mixture of $Pd_2(dba)_3$•$CHCl_3$ (2.5 equiv/allyl group), PPh_3 (25 equiv/allyl group), dimedone as an allyl scavenger (250 equiv/allyl group, 0.6 M final concentration) in THF is used for the allyl deprotection and the resin is washed with a buffered (pH = 6.7) ddtc solution to remove the residual palladium. Using these conditions the desired prooligonucleotide may be obtained without degradation.

A prooligonucleotide d(CCTTACA), bearing a base labile 2,2-bis(ethoxycarbonyl) 3-(pivaloyloxy)propyl protection (**92**), has recently been synthesized on a 4-oxoheptanedioic acid linker **87** (Figure 18).[8] It may be worth of noting that cyanoethyl phosphoramidites of *N*-Alloc protected nucleosides (**78** and **79**) are used for the synthesis. After chain assembly, the Alloc protections may be removed first [$(PPh_3)_4Pd^0$ and $PhSiH_3$ in THF] and then the cyanoethyl groups are removed (1.5% DBU in acetonitrile). The desired prooligonucleotide is released by a mixture of 0.05 M hydrazinium acetate, which remains the 2,2-bis(ethoxycarbonyl) 3-(pivaloyloxy)propyl protection intact.

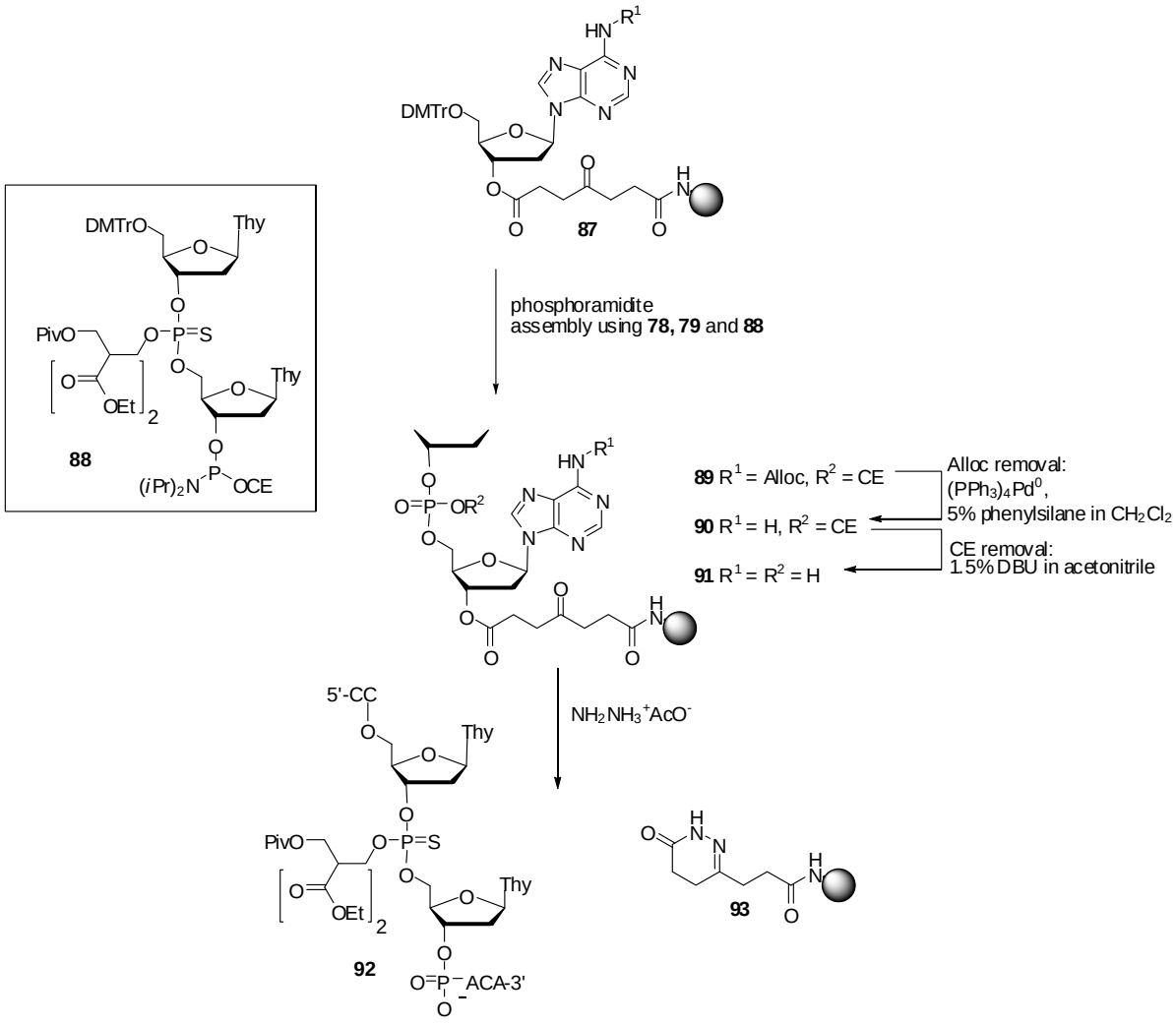


Figure 18. Synthesis of biodegradable 2,2-bis(ethoxycarbonyl) 3-(pivaloyloxy)propyl protected prooligonucleotide (Leisvuori et al., 2008).[8]

3.6 Photocleavable nucleobase protections

Photolabile linkers are used above in combination with other orthogonal protections. Advantage is the neutral cleavage, in which potential reagent contamination may be omitted. Applicability of the photolabile protections has also been demonstrated for the 5'-OH group (in photolithographic DNA synthesis),[74,75] for the 2'-OH group of ribonucleosides,[76] for the internucleotidic phosphates[77] and, finally, for the exocyclic amino groups of nucleobases,[35,78] when a base sensitive conjugate groups are incorporated. Deprotection is generally performed by irradiation at wavelengths > 300 nm to avoid damage of nucleic acids.[79] Potential side reactions are *e.g.* thymine-thymine photodimers[80] and adducts caused by reaction between the exposed amino groups and the photoproduct of the protecting group (nitrosoaldehyde if formed).[35] Formation of the latter side reaction may be eliminated by irradiating the protected oligonucleotide in slightly acidic conditions, which also increases photolysis rate. It may also be

noteworthy that the irradiation time is usually dependent on the concentration of the substrate.[35] In the first description involving photolabile nucleobase protections, *N*-nitrobenzyloxycarbonyl (NBOC) and 2'-*O*-nitrobenzyloxymethyl (NBOM) groups have been applied for the preparation of 3'-*O*-aminoacylated RNA sequences (cf. **101-104** in Figure 18, Stutz and Pitsch 1999).[78] Then a real applicability of the photolabile protecting group strategy has been studied more detailed by the synthesis of base sensitive *S*-pivaloyl thioethyl (*t*BuSATE) prooligonucleotides, in which compatibility of 6-nitroveratryloxycarbonyl (NVOC) and 2,2'-bis(2-nitrophenyl)ethoxycarbonyl (diNPEOC) protections have been compared.[35] Cleavage rate of the NVOC group was found to be too slow, in which complete photolytic deprotection occurs at the expense of side reactions. The retarded deprotection was overcome by the more photolabile diNPEOC group and it was successfully used for the synthesis of *t*BuSATE phosphotriester and phosphorothionotriester analogues of d(5'-ACACCCAATTCT)-3' on a photolabile support. After chain assembly, resins are subjected to an appropriate volume (10 mL / 1 μmol of the oligonucleotide) of mixture of AcOH, dioxane and water (5:57:38, *v/v/v*,) and irradiated by a high-pressure Hg lamp (filtered with a pyrex glass, for 40 min at 20 °C) to release the desired prooligonucleotides. Nucleoside building blocks (**94-100**) used in the syntheses are described in Figure 19.

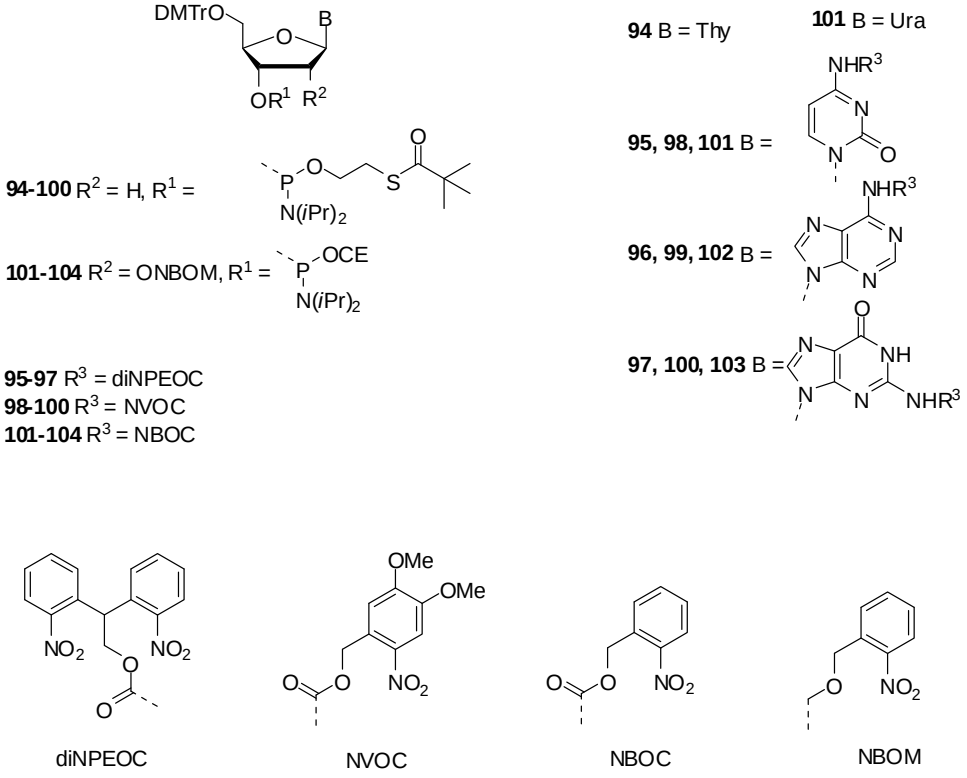


Figure 19. Nucleoside building blocks bearing photolabile protections.

4. DNA Synthesis without Amino Protection (Strategy B)

The orthogonal strategies in Chapter 3 entail introduction of the protecting groups to the nucleoside monomers and their removal at the final stage of the synthesis. These additional steps limit range of functional groups that can survive upon the synthesis. Additionally, quality of the crude product may be low, because of the contamination by the reagents used for the deprotection or by the cleavage products of the protecting groups. Ideally the chain assembly would to be performed without protections.[81,82] For the both commonly adopted chain elongation methods (the phosphoramidite and the H-phosphonate), N-unprotected variations (*i.e*. synthesis without exocyclic amino protections of adenine, guanine and cytosine) have been developed. Thanks to the tenacious pioneering work, nowadays these are respectable options, when base sensitive conjugate groups are aimed to be incorporated to an oligonucleotide synthesis.

4.1 The *N*-unprotected phosphoramidite method

In the pioneering N-unprotected phosphoramidite method, applicability of the standard phosphoramidite coupling procedure with the N-unprotected monomers has been demonstrated (Gryaznov and Letsinger 1991).[82] It was found that subjects for the undesired N-phosphitylation are adenine and cytosine, but the amino group of guanine is relatively resistant to phosphitylation. The phosphitylated amino groups, which themselves serve as phosphitylating agents, were cleaved after each coupling, but prior to oxidation to avoid formation of stable *N*-phosphates. For that additional step, a mixture of pyridinium hydrochloride and aniline was used as a phosphityl transfer reconverting the modified sites to the free amino groups. Extent of the undesired *N*-phosphitylation in this pioneering *N*-unprotected phosphoramidite method has been decreased later by using a mixture of pyridinium hydrochloride and imidazole as an activator.[83] The usual coupling chemistry (1*H*-tetrazole as an activator) has also recently been used for the synthesis of a base sensitive oligonucleotide, in which exocyclic amino group of guanine was remained unprotected upon the chain elongation.[84]

A good O-selectivity has been obtained by imidazolium triflate (IMT, **105**) promoted coupling together benzimidazolium triflate (BIT, **106**)-methanol washing step (Hayakawa and Kataoka 1998).[85] BIT is an efficient reagent, which promotes phosphityl transfer from an exocyclic amino group to methanol, but unfortunately it may also cause transesterification of the phosphite intermediate. Although this method has been reported to be successful for the synthesis of relatively long oligonucleotides, the transesterification has been proven to be a serious problem upon the chain elongation.[86]

The proton-block strategy (Sekine et al. 2003) involves protection of the nucleobases (dC, dA and dG: pK_a = 4.3, 3.8 and 2.5, in this order) simply by their unreactive protonated form by using promoters with low pK_a values.[86] Among these promoters, 5-nitrobenzimidazolium triflate (NBT, **107**, pKa = 2.76) has been used for the synthesis of a dodecamer d(CAGTCAGTCAGT)

nucleotide. In order to avoid potential detritylation caused by NBT in the coupling step, THF or a mixed solvent system MeCN-NMP (9:1, *v/v*) instead of MeCN should be used as a solvent. The more basic solvent system is more accessible to protonation, decreasing protonation to the 5'-ether site, and hence it suppresses detritylation. The method is, however, not generally recommended for the synthesis of longer than decamer nucleotides.

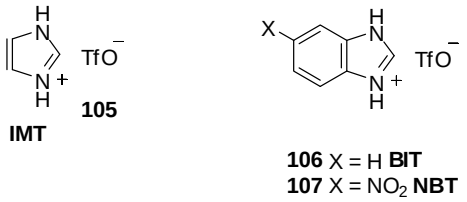


Figure 20

4.1.1 The phosphite triester method. A real *O*-selective condensation may be obtained by the phosphite triester method (Ohkubo et al. 2004, Figure 21).[52,87] The phosphoramidite monomer (**108**) reacts with the *N*-hydroxybenzotriazol (HOBt, **109**) derived promoters to form the active phosphite triester (**110**). *O*-selectivity of this reactive intermediate may be explained by the interactions of the frontier molecular orbitals (FMOs) between the phosphite intermediate and the nucleophiles (Figure 22).[88] The phosphite intermediate has a large HOMO on the phosphorus atom which interacts with the lone pair electrons. In contrast to a primary alcohol (5'-OH) bearing a large LUMO located on the hydrogen atom (A, Figure 22), there are no LUMOs in exocyclic amino groups (B, Figure 22). This FMO interaction raises the HOMO energy of the alcohol and decreases the LUMO energy of the phosphite intermediate (**110**) being basis of the favoured *O*-phosphitylation. It is worth noting, that the corresponding imidazolide intermediates, formed in the methods above, can not form similar interaction, since partial proton transfer from hydroxyl nucleophiles to a phosphorus atom is less favoured. It has also been reported that HOBt derivatives are able to participate to the phosphityl transfer from the undesired *N*-phosphitylation, which further increases *O*-selectivity (cf. postcondensation treatments with pyridinium hydrochloride-aniline and BIT-MeOH above).

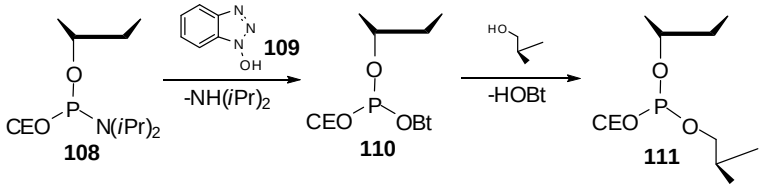
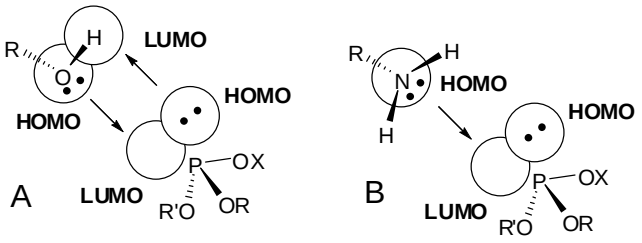


Figure 21. Coupling in the phosphite triester method (Ohkubo et al. 2004).[52]



R' = alkyl (the phosphite triester method) or H (the H-phosphonate method)
OX = an appropriate leaving group (e.g. OBt)

Figure 22. Difference in the interaction of frontier molecular orbitals between the active phosphite intermediate and hydroxyl- and aminonucleophiles (Wada et al., 1997).[88]

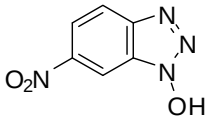
In order to combine a good *O*-selectivity with a high yield, several HOBt derivatives have been screened for the phosphite triester coupling.[87] HOBt itself gives an excellent *O*-selectivity and coupling yield, but its solubility in acetonitrile is low. This shortcoming may be overcome by using a mixed solvent of acetonitrile and NMP. Even a better coupling yield may be obtained with a more acidic promoter, 6-trifluoromethyl *N*-hydroxybenzotriazol (HO^{tf}Bt, **112**) (or mixture of IMT and HO^{tf}Bt), which is recommended to be used in automated synthesis of longer oligonucleotides {e.g. cytidine rich d[$C_5T_5(CT)_5$] has been synthesized.}. Reaction cycle of the method is described in Table 1.

Applicability of the phosphite triester method for the base-sensitive oligonucleotides has been demonstrated by the synthesis of an oligonucleotide d(GC^{Ac}ATCAGC^{Ac}C^{Ac}TCAT) having three 4-*N*-acetyldeoxycytidine residues.[87] Chain assembly is carried out on a silyl linker (cf. **12**) derivatized highly cross-linked polystyrene (HCP). Removal of the cyanoethyl groups [10% solution of DBU in acetonitrile, 1 min at 25 °C] and release from the support [1 M TBAF-AcOH (1:1, *v/v*) in THF] result the crude product oligonucleotide with an excellent purity.

Applicability of the phosphite triester method for the synthesis of aminoacylated oligo ribonucleotides has been demonstrated.[89] However, the required longer coupling time lowers the *O*-selectivity. The shortcoming may be resolved by a NBT (**107**) promoted post-condensation treatment, which increased the selectivity to more than 99% independently on the kind of protecting groups (TDMS, TOM) at the 2'-position.

Table 1. Reaction cycle of the phosphite triester method.

	operation	reagents	time / min
1	washing	CH_2Cl_2	
2	detritylation	3% DCA in CH_2Cl_2	1.5
3	washing	CH_2Cl_2-THF	
4	coupling	0.1 M *N*-unprotected amidite monomer / MeCN-NMP (15:1, *v*/*v*) + 0.2 M **112** / MeCN-NMP (15:1, *v*/*v*), twice	1.0 (2 x)
5	washing	MeCN	
6	oxidation	I_2 / pyridine-H_2O (9:1, *v*/*v*)	0.5
7	washing	MeCN	



112 $HO^{tf}Bt$

4.2 The N-unprotected H-Phosphonate method

The N-unprotected monomers may also be applied in the H-phosphonate strategy. In contrast to the N-unprotected phosphoramidite method, which still needs exposing of the phosphodiester groups, the H-phosphonate chain elongation may be performed completely without protecting groups. The main problem is not undesired phosphonylation of the exocyclic amino groups of adenine, cytosine or guanine, but acylation of nucleobases by condensing agents, when usual pivaloyl or adamantoyl chlorides are used. Subjects for the acylation are cytosine and guanine, but adenine in lesser extent, and in the case of guanine the site for acylation is the O^6/N^1 position, not the 2-amino group.[90] A base treatment, like standard ammonolysis, is required to release these acylated side products, and hence the N-unprotected H-phosphonate method with the commonly used condensing agents is not suitable for the synthesis of base-sensitive oligonucleotides.

In order to improve *O*-selectivity with high coupling efficiency, several benzotriazol-1-yloxy carbonium and phosphonium type condensing reagents have been extensively screened for the *H*-phosphonate coupling (Wada et al. 1997).[88] These reagents and the activated monomers (**114** and **115** in Figure 23) produced by them do not affect the amino groups of the nucleosides. Careful ^{31}P NMR studies have shown that the active intermediate for the condensation is the phosphonium or carbonium activated phosphite intermediate (**115**). *O*-selectivity may be explained by the differences in the interaction of the frontier molecular orbitals (FMOs) as discussed above (Figure 22). As a contrast to the phosphoramidite method, a hydroxyl bearing phosphite intermediate is formed (**115**), which favours formation of *H*-phosphonate diester (**116**) (Figure 23). Coupling rate has been found to be dependent on the carbonium/phosphonium core structure and the *N*-hydroxy benzotriazol leaving group. Fast coupling rate may be obtained with 2-(benzotriazol-1-yloxy)-1,1-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (BOMP, **117**). It bears good solubility properties and therefore it also suits well for automation. Employing BOMP with the *N*-unprotected *H*-phosphonate monomers dodecanucleotide [$d(CAGT)_3$] has been successfully assembled on an oxalate (cf. **1**) derivatized resin. Reaction cycle of the method is described in Table 2. It is worth noting that the oxidation step is also modified, allowing formation of the desired phosphodiester backbone in dry

conditions. Oxidation in aqueous condition may decrease yield of the desired oligomer, since the internucleotide *H*-phosphonate linkages are prone to hydrolysis.[91]

Synthesis of the base-sensitive oligonucleotides bearing the *H*-phosphonate backbone deserves special attention, since the free 3' or 5'-terminal hydroxyl group may react intramolecularly to the phosphorus atom under aqueous basic condition. A tetramer nucleotide d[Hexp(H)Cp(H)Ap(H)Tb(H)Hex] (Hex refers to 6-hydroxyhexyl group and p(H) to the *H*-phosphonate linkage) has been assembled on an oxalalate derivatized resin by the *N*-unprotected *H*-phosphonate method using BOMP activation.[92] The release from the support is performed by a mixture of propylamine in presence of a neutral silylating reagent, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide ($PrNH_2$-BSTFA-MeCN, 2:1:2, *v/v/v*, for 30 min at rt). After release, reagents are removed and the transiently protected product is desilylated by a neutral aqueous treatment.

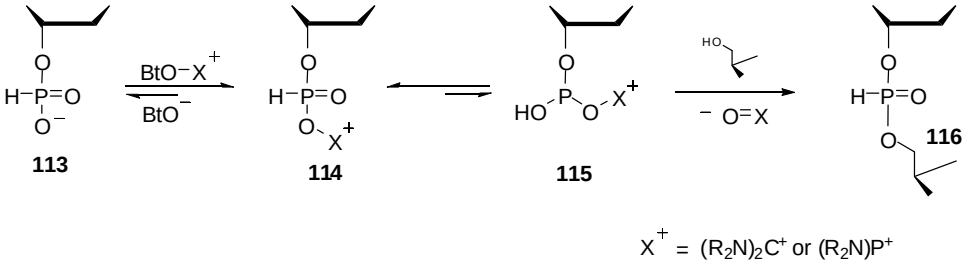
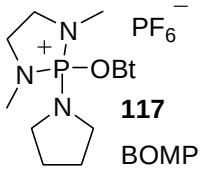


Figure 23. Mechanism of the H-phosphonate condensation by the carbonium and phosphonium condensing reagents (Wada et al. 1997).[88]

Table 2. Reaction cycle of the N-unprotected H-phosphonate method

	operation	reagents	time / min
1	washing	CH_2Cl_2	
2	detritylation	1% TFA / CH_2Cl_2	0.25
3	washing	CH_2Cl_2 + 1% quinoline / CH_2Cl_2	
4	coupling	0.05 M N-unprotected H-phosphonate monomer / pyridine + 0.2 M **117** / pyridine	2
5	washing	pyridine	
6	capping	0.05 M triethylammonium isopropyl phosphonate + 0.2M **117** / pyridine	2
7	washing	pyridine	
8	oxidation after completion of the chain assembly	0.2 M PNO and 0.5 BSA in CH_2Cl_2	10



5. Chemo Selective Coupling to an Unprotected Solid-supported Oligonucleotide (Strategy C)

In the N-unprotected methods below, the phosphodiester bond may be *O*-selectively introduced to the 5'-OH group in the presence of the exocyclic amino groups. When the goal is introduction of a base labile conjugate group to the 5'-end, even a more attractive alternative may be synthesis of the oligonucleotide by using standard nucleobase protections (or more base labile Pac-protections), their on-resin removal, and then chemoselective coupling of the base labile group. This last approach (*Strategy C*) requires linker which withstands upon ammonolysis, but is able to release the target oligonucleotide in mild conditions.

As an example of the *Strategy C*, a highly base labile N^2,N^2,7-trimethylguanosine-capped trinucleotide has been synthesized on HCP resin (Figure 24).[93] The phosphoramidate linkage (cf. Figure 8) between the first nucleoside and the resin is stable in ammonolysis, but becomes acid labile after elimination of the cyanoethyl group. The ribonucleotide (AUA) trimer is elongated by standard phosphoramidite chemistry which is followed by the terminal phosphorylation (**118**). The phosphate protections are removed by β-elimination using a mixture of DBU (**119**), the first pyrophosphorylation is carried out and then the oligonucleotide is subjected to ammonolysis to remove the nucleobase (Bz) protections (**120**). The final nucleoside may be chemoselectively introduced as a 5'-phosphoroimidazolide (**123**) resulting the fragile cap structure. At the final stage, release from the support by 80% acetic acid, deprotection by aqueous hydrogen chloride and dephosphorylation by alkaline phosphatase results the desired N^2,N^2,7-trimethylguanosine capped ribonucleotide trimer (**121**).

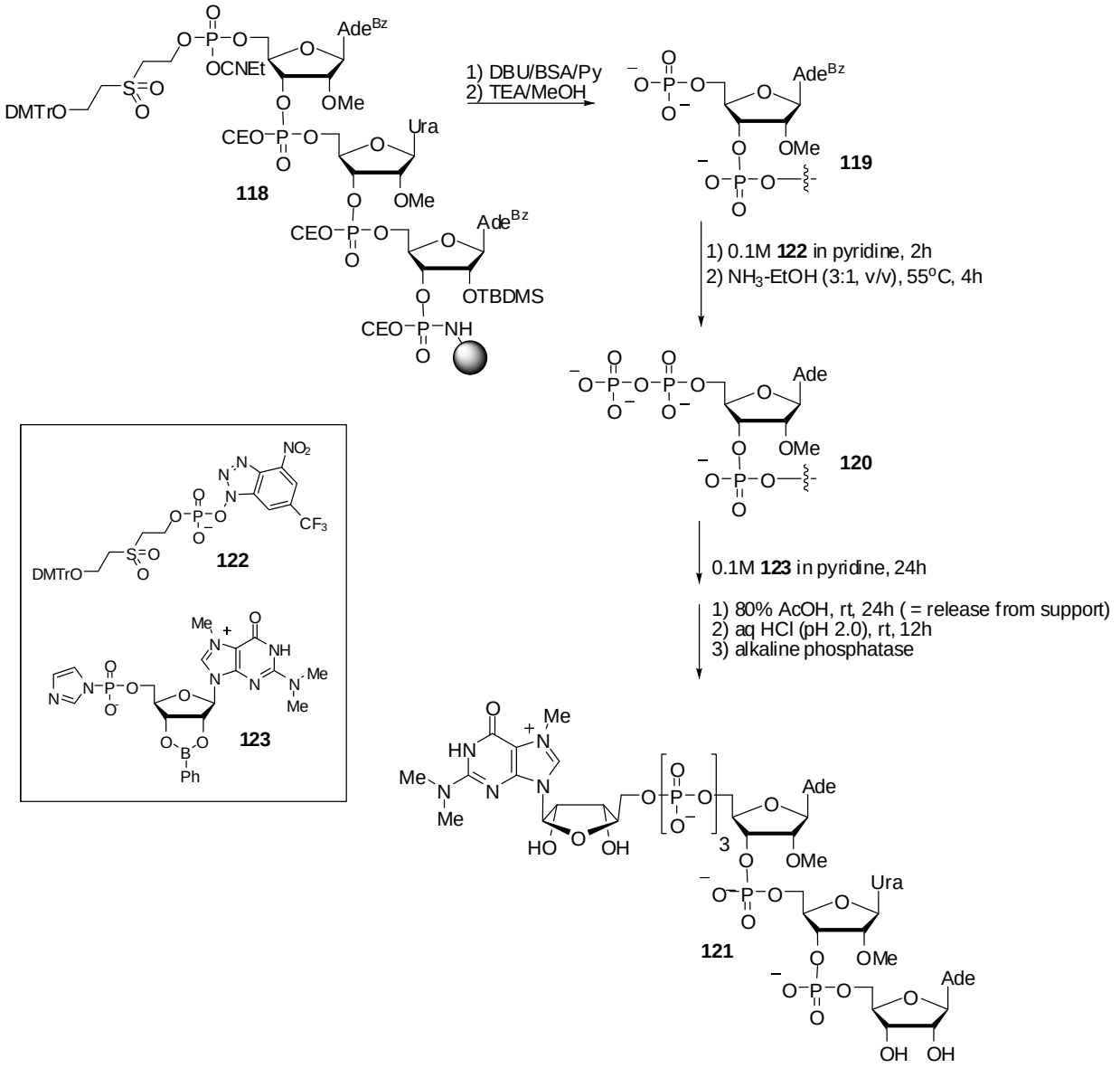


Figure 24. Synthesis of base labile N^2,N^2,7-trimethylguanosine-capped AUA (Kadokura et al. 2001).[93]

Almost similar approach has recently been used for the synthesis of 7-methylguanosine capped AUA.[94] Synthesis is carried out on a disulfide linker (**20**), which survives upon standard ammonolysis but may be reductively cleaved under mild condition. The trinucleotide is elongated, the 5'-OH is phosphorylated and the base protections are removed (conc. aq. NH_3, over night) on a support (TG) as above. The cap structure is chemoselectively introduced using unprotected 7-methylguanosine 5'-diphosphate imidazolide in presence of $ZnCl_2$, and the product is reductively released by a mixture of DTT, TEA and methanol. It is worth noting that the prolonged ammonolytic treatment which is required for the on-resin removal of the base

protections can not be performed on controlled pore glass (CPG). A base stable resin material, like tenta gel (TG) in the present case, is required.

6. Conclusions

It is hard to say, which one of the described orthogonal protecting group strategies is the best choice for the synthesis of base sensitive oligonucleotides. It is more likely that none of them can be preferred to others, but the adopted strategy should always be evaluated case-specifically. The most popular is the 'conventional' allyl protecting group scheme, which also facilitates synthesis of long oligonucleotides without notable problems involved in retarded deprotection.
The *N*-unprotected methods seem to be attractive choices nowadays. The *O*-selectivity upon the synthesis of the medium-size oligodeoxyribonucleotides is practically resolved and the *N*-unprotected monomers are readily available from the commercial building blocks.[95] However, problems may arise when the size of the oligonucleotide increases or the target is oligoribonucleotide.

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