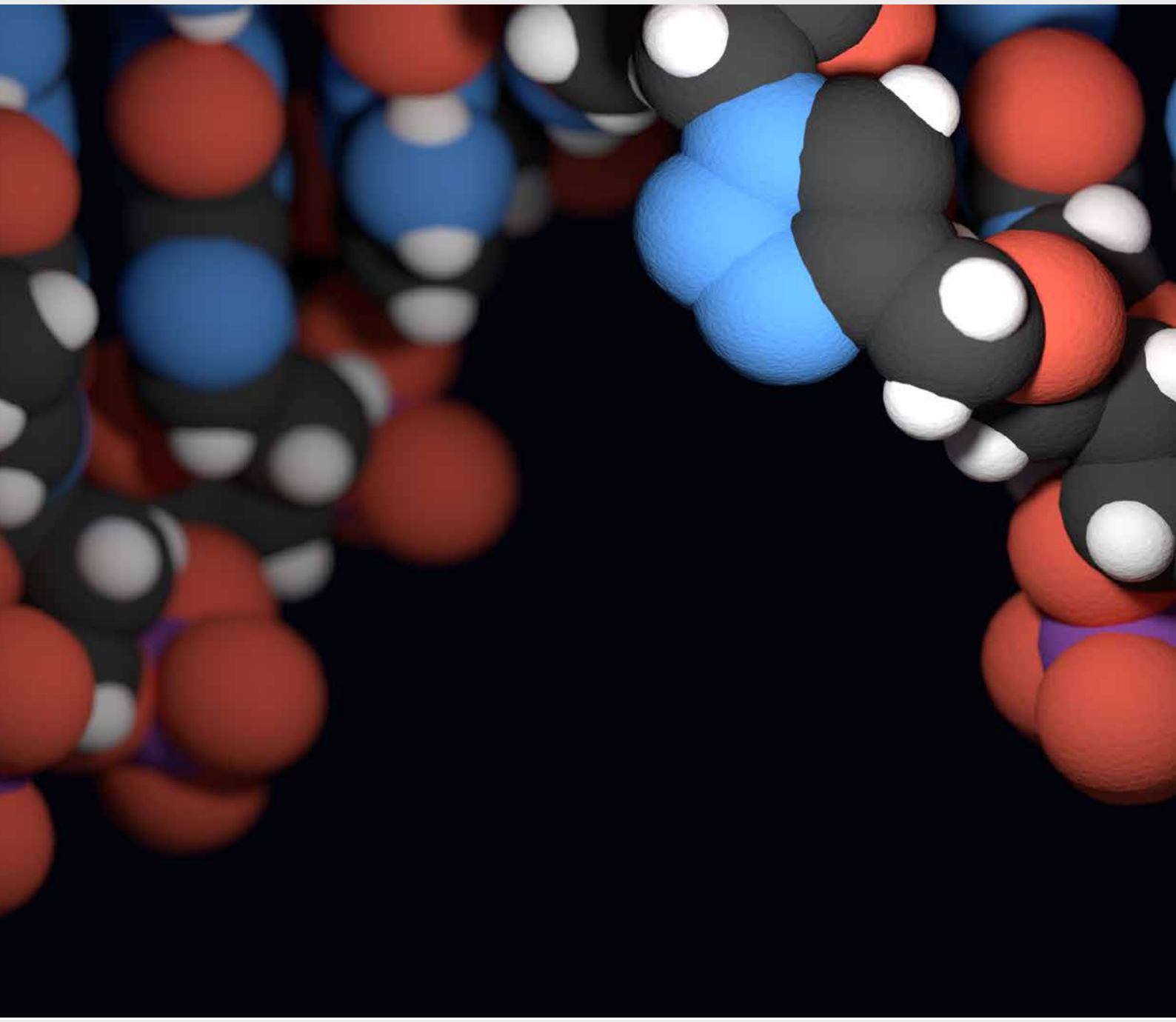
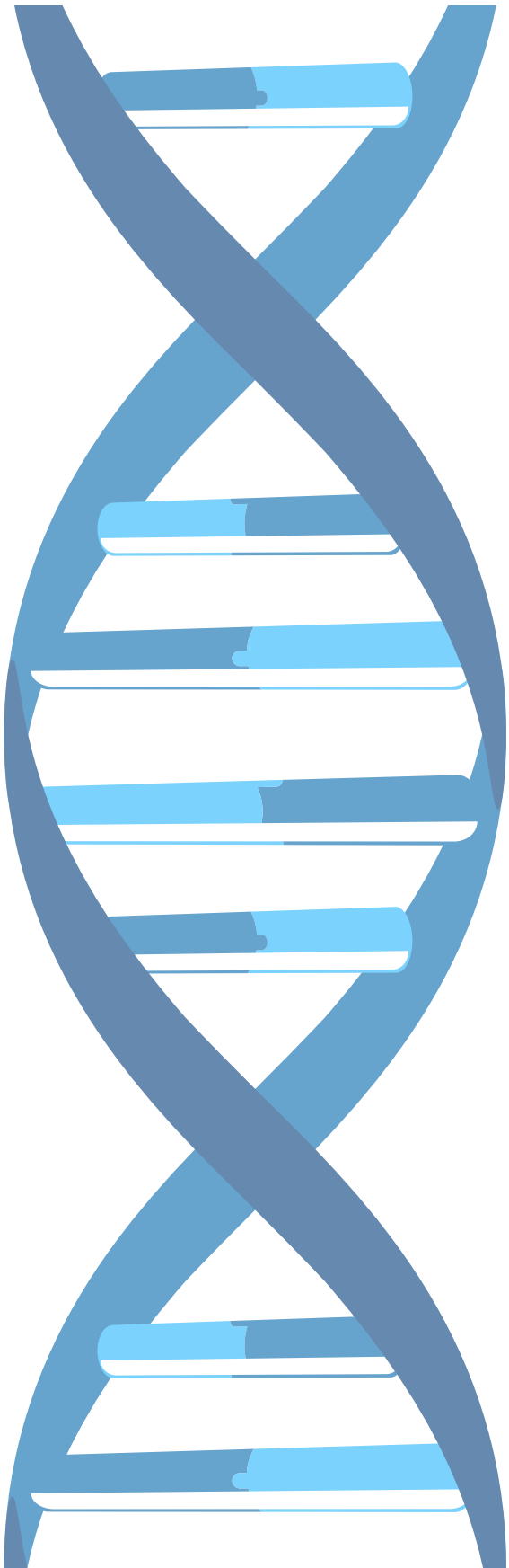
Solid Phase Oligonucleotide Synthesis



Biotage®

Solid Phase Oligonucleotide Synthesis



Introduction

Millions of oligonucleotides are synthesized every year for use in laboratories around the world. For most applications, very small quantities of DNA are required, and oligonucleotide synthesis is performed mainly on the 40 nmol scale or lower. This provides ample quantities for most biochemical and biological experiments. Much larger amounts of DNA (10 µmol or more) can be prepared for use in biophysical studies (NMR and X-ray crystallography) and in the extreme, solid-phase methods have been developed to allow the synthesis of multi-kilogram quantities of oligonucleotides for use as drug molecules (e.g. antisense oligonucleotides). For all these purposes, oligonucleotides are manufactured almost exclusively using automated solid-phase methods.

Advantages of solid-phase synthesis

Solid-phase synthesis is widely used in peptide synthesis, oligonucleotide synthesis, oligosaccharide synthesis and combinatorial chemistry. Solid-phase chemical synthesis was invented in the 1960s by Bruce Merrifield, and was of such importance that he was awarded the Nobel Prize for Chemistry in 1984.

Solid-phase synthesis is carried out on a solid support held between filters, in columns that enable all reagents and solvents to pass through freely. Solid-phase synthesis has a number of advantages over solution synthesis:

- large excesses of solution-phase reagents can be used to drive reactions quickly to completion
- impurities and excess reagents are washed away and no purification is required after each step
- the process is amenable to automation on computer-controlled solid-phase synthesizers.

Solid supports

Solid supports (also called resins) are the insoluble particles, typically 50-200 µm in diameter, to which the oligonucleotide is bound during synthesis. Many types of solid support have been used, but controlled pore glass (CPG) and polystyrene have proved to be the most useful.

Controlled-pore glass (CPG)

Controlled-pore glass is rigid and non-swelling with deep pores in which oligonucleotide synthesis takes place. Glass supports with 500 Å (50 nm) pores are mechanically robust and are used routinely in the synthesis of short oligonucleotides. However, synthesis yields fall off dramatically when oligonucleotides more than 40 bases in length are prepared on resins of 500 Å pore size. This is because the growing oligonucleotide blocks the pores and reduces diffusion of the reagents through the matrix. Although large-pore resins are more fragile, 1000 Å CPG resin has proved to be satisfactory for the synthesis of oligonucleotides up to 100 bases in length, and 2000 Å supports can be used for longer oligonucleotides.

Polystyrene (PS)

Highly cross-linked polystyrene beads have the advantage of good moisture exclusion properties and they allow very efficient oligonucleotide synthesis, particularly on small scale (e.g. 40 nmol).

Solid supports for conventional oligonucleotide synthesis are typically manufactured with a loading of 20-30 µmol of nucleoside per gram of resin. Oligonucleotide synthesis at higher loadings becomes less efficinet owing to steric hindrance between adjacent DNA chains attached to the resin; however, polystyrene supports with loadings of up to 350 µmol / g are used in some applications, particularly for short oligonucleotides, and enable the synthesis of large quantities of oligonucleotides.

The Phosphoramidite method

Several methods of solution-phase oligonucleotide synthesis have been devised over the years, from Michelson and Todd's early experiments on the H-phosphonate and phosphotriester methods, and Khorana's phosphodiester approach, in the 1950s, to reinvestigation of the phosphotriester method and development of the phosphite triester method in the 1960 and 1970s. Each of these methods has its problems; the phosphoramidite method, pioneered by Marvin Caruthers in the early 1980s, and enhanced by the application of solid-phase technology and automation, is now firmly established as the method of choice.

Phosphoramidite oligo synthesis proceeds in the 3'- to 5'-direction (opposite to the 5'- to 3'-direction of DNA biosynthesis in DNA replication). One nucleotide is added per synthesis cycle. The phosphoramidite DNA synthesis cycle consists of a series of steps outlined in Figure 1.

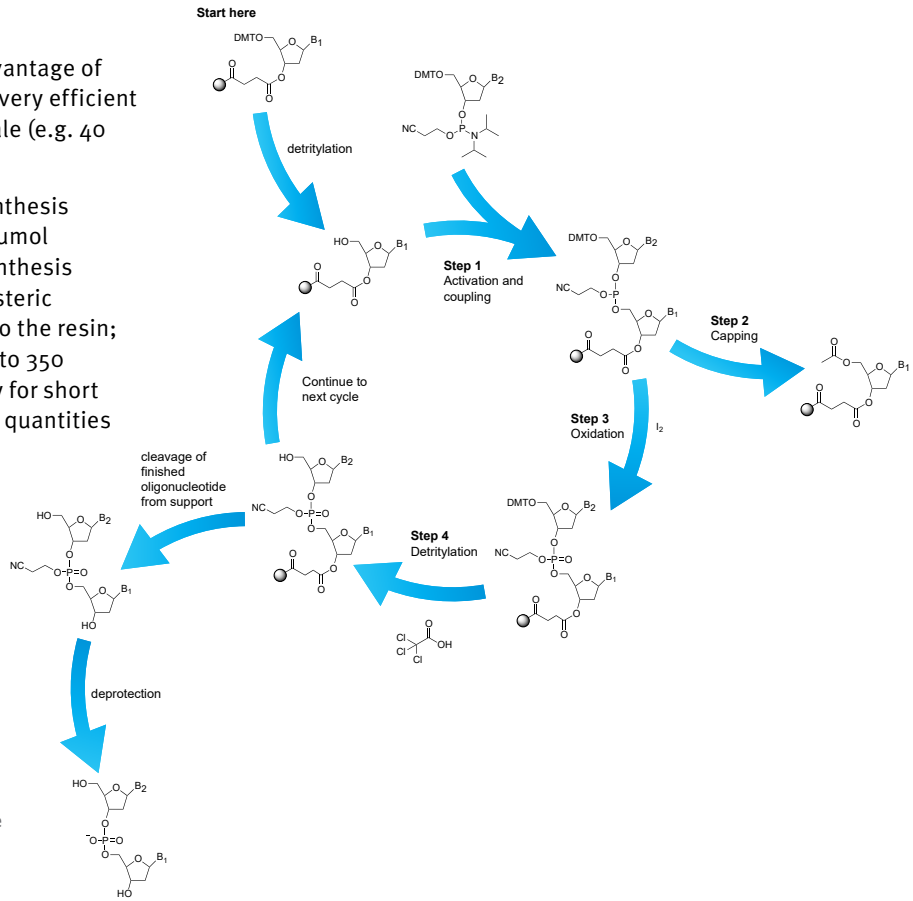


Figure 1. The phosphoramidite oligonucleotide synthesis cycle

Table 1. Phosphoramidite-oligonucleotide-synthesis-cycle: Typical timings used for the synthesis of DNA oligonucleotides in the phosphoramidite cycle.

Operation	Reagent/solvent	Time
Wash	acetonitrile	30 s
Detritylate	3% trichloroacetic acid in dichloromethane	50 s
Monitor trityl	-	-
Wash	acetonitrile	30 s
Flush	argon	10 s
Couple	0.1 M phosphoramidite monomer and 0.5 M tetrazole in acetonitrile	30 s
Wash	acetonitrile	30 s
Flush	argon	10 s
Cap	acetic anhydride/pyridine/THF 1/1/8 and 17.6% w/v N-methyl imidazole in acetonitrile	30 s
Wash	acetonitrile	30 s
Flush	argon	10 s
Oxidize	0.015 M iodine in water/pyridine/THF 2/20/78	45 s
Wash	acetonitrile	30 s
Flush	argon	10 s

Detritylation of the support-bound 3'-nucleoside

At the beginning of oligonucleotide synthesis the first protected nucleoside is pre-attached to the resin and the operator selects an A, G, C or T synthesis column depending on the nucleoside at the 3'-end of the desired oligonucleotide. The support-bound nucleoside has a 5'-DMT protecting group (DMT = 4,4'-dimethoxytrityl), the role of which is to prevent polymerization during resin functionalization, and this protecting group must be removed (detritylation) from the support-bound nucleoside before oligonucleotide synthesis can proceed. The mechanism of detritylation is shown in Figure 2.

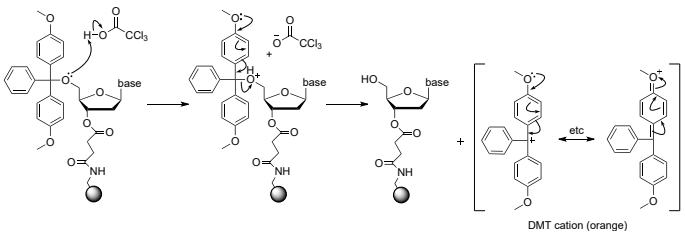


Figure 2. Phosphoramidite nucleoside detritylationMechanism of acid-catalyzed detritylation of a DMT-protected nucleoside phosphoramidite.

Activation and Coupling (Step 1)

Following detritylation, the support-bound nucleoside is ready to react with the next base, which is added in the form of a nucleoside phosphoramidite monomer. A large excess of the appropriate nucleoside phosphoramidite is mixed with an activator (tetrazole or a derivative), both of which are dissolved in acetonitrile (a good solvent for nucleophilic displacement reactions). The diisopropylamino group of the nucleoside phosphoramidite is protonated by the activator, and is thereby converted to a good leaving group. It is rapidly displaced by attack of the 5'-hydroxyl group of the support-bound nucleoside on its neighbouring phosphorus atom, and a new phosphorus-oxygen bond is formed, creating a support-bound phosphite triester (Figure 3).

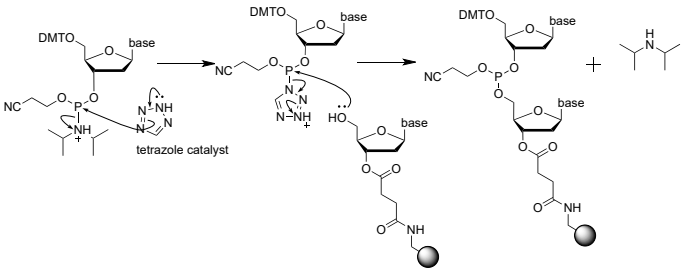


Figure 3. Phosphoramidite couplingMechanism of the phosphoramidite coupling reaction.

Nucleoside phosphoramidites are reasonably stable in an inert atmosphere and can be prepared in large quantities, shipped around the world and stored as dry solids for several months prior to use. Only upon protonation do nucleoside phosphoramidites become reactive.

Capping (Step 2)

It is not unreasonable to expect a yield of 99.5% during each coupling step, but even with the most efficient chemistry and the purest reagents it is not possible to achieve 100% reaction of the support-bound nucleoside with the incoming phosphoramidite. This means that there will be a few unreacted 5'-hydroxyl groups on the resin-bound nucleotide; if left unchecked, these 5'-hydroxyl groups would be available to partake in the next coupling step, reacting with the incoming phosphoramidite. The resulting oligonucleotide would lack one base, and would correspond to a deletion mutation of the desired oligo (Figure 4). If such deletion mutations were left unchecked they would accumulate with each successive cycle, and the final product would be a complex mixture of oligonucleotides, most of which would carry incorrect genetic information, and which would be difficult to purify. This would ruin any subsequent biochemical experiment.

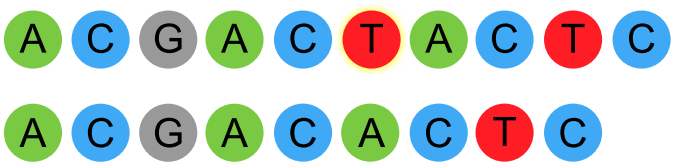


Figure 4. Deletion mutationSequence of the correct oligonucleotide (top); and a failure sequence (bottom) containing a deletion mutation, corresponding to the deletion of the thymine base at position 6.

Deletion mutations are avoided by introducing a "capping" step after the coupling reaction, to block the unreacted 5'-hydroxyl groups. Two capping solutions are used on the synthesizer: acetic anhydride and N-methylimidazole (NMI). These two reagents (dissolved in tetrahydrofuran with the addition of a small quantity of pyridine) are mixed on the DNA synthesizer prior to delivery to the synthesis column. The electrophilic mixture rapidly acetylates alcohols, and the pyridine ensures that the pH remains basic to prevent detritylation of the nucleoside phosphoramidite by the acetic acid formed by reaction of acetic anhydride with NMI (Figure 5). Acetylation of the 5'-hydroxyl groups renders them inert to subsequent reactions.

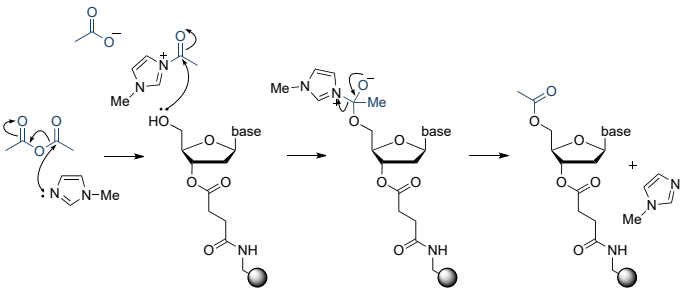


Figure 5. Phosphoramidite cappingMechanism of the capping step in phosphoramidite oligonucleotide synthesis.

Oxidation (Step 3)

The phosphite-triester (P(III)) formed in the coupling step is unstable to acid and must be converted to a stable (P(v)) species prior to the next TCA detritylation step. This is achieved by iodine oxidation in the presence of water and pyridine (Figure 6). The resultant phosphotriester is effectively a DNA backbone protected with a 2-cyanoethyl group. The cyanoethyl group prevents undesirable reactions at phosphorus during subsequent synthesis cycles.

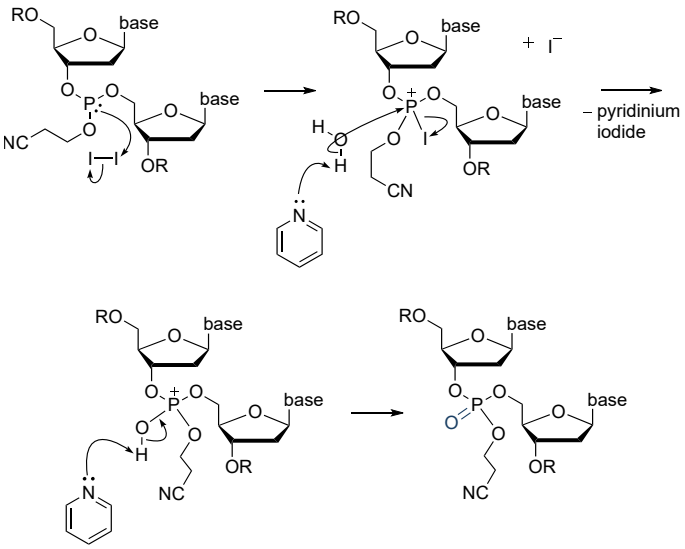


Figure 6. Phosphoramidite oxidationMechanism of the oxidation step in phosphoramidite oligonucleotide synthesis.

On some DNA synthesizers there is a second capping step after iodine oxidation. The purpose of this is to dry the resin, as residual water from the oxidation mixture can persist and inhibit the next coupling reaction. The excess water reacts with the acylating agent to form acetic acid which is washed away in the THF/pyridine solvent mixture.

Detritylation (Step 4)

After phosphoramidite coupling, capping and oxidation, the DMT protecting group at the 5'-end of the resin-bound DNA chain must be removed so that the primary hydroxyl group can react with the next nucleotide phosphoramidite. Deprotection with trichloroacetic acid in dichloromethane is rapid and quantitative. An orange colour is produced by cleaved DMT carbocation, which absorbs in the visible region at 495 nm. The intensity of this absorbance is used to determine the coupling efficiency. Most commercially available DNA synthesizers have hardware to measure and record the trityl yield for each cycle so that the efficiency of synthesis can be monitored in real time.

The cycle is repeated, once for each base, to produce the required oligonucleotide.

Effect of coupling efficiency on yield

The importance of a high average stepwise yield (coupling efficiency), and its effect on the overall yield of oligonucleotide synthesis, is illustrated below.

Length (bases)	90%	95%	97%	98.5%	99.5%
10	38.7%	63.0%	76.0%	87.3%	95.6%
20	13.5%	37.7%	56.1%	75.0%	90.9%
50	0.6%	8.1%	22.5%	47.7%	78.2%
100	< 0.01%	0.6%	4.9%	22.4%	60.9%
150	< 0.01%	0.05%	1.1%	10.5%	47.4%
200	< 0.01%	< 0.01%	0.2%	4.9%	36.9%

The cumulative effect of a series of poor couplings is two-fold, resulting in

- a poor overall yield of the desired oligonucleotide
- a product that is extremely difficult to purify.

It is clear from the table that very high stepwise yields are necessary to synthesize oligonucleotides over 100 bases in length. In practise 98.5% is readily attainable and average stepwise yields of 99.5% or higher can be achieved provided that all reagents are pure. Carefully dried anhydrous solvents must be used as the phosphoramidite coupling reaction is very sensitive to moisture.

Cleavage from the solid support

The linker is the chemical entity that attaches the 3'-end of the oligonucleotide to the solid support. It must be stable to all the reagents used in solid-phase oligonucleotide assembly, but cleavable under specific conditions at the end of the synthesis. The linker used most frequently in oligonucleotide synthesis is the succinyl linker. This is readily cleaved by treatment with concentrated ammonium hydroxide at room temperature for one hour (Figure 7).

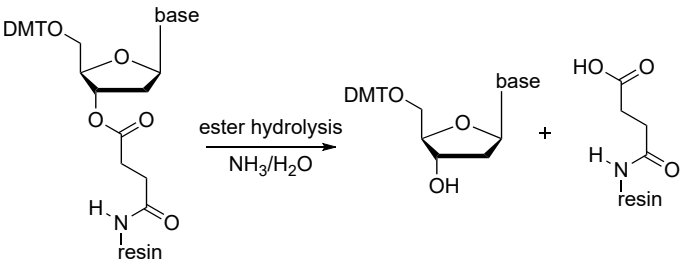


Figure 7. Oligonucleotide resin cleavageMechanism of cleavage of an oligonucleotide from its solid support, using ammonium hydroxide.

The cleavage reaction is carried out automatically on some synthesizers, and the ammoniacal solution containing the oligonuleotide is delivered to a glass vial. Alternatively, the cleavage can be carried out manually by taking the column off the synthesizer and washing it with syringes containing ammonium hydroxide.

Oligonucleotide deprotection

The oligonucleotide, now dissolved in concentrated aqueous ammonia, is heated to remove the protecting groups from the heterocyclic bases and phosphodiester backbone (Figure 8). The aqueous solution is then removed by evaporation and the oligonucleotide is ready for purification.

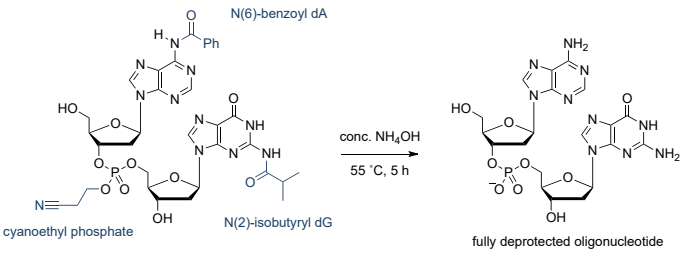


Figure 8. Oligonucleotide deprotectionMechanism of deprotection of an oligonucleotide synthesized using the phosphoramidite method.

Deprotection of heterocyclic bases

The exocyclic primary amino groups on the heterocyclic bases (A, C, and G) are nucleophilic and must therefore be protected during oligonucleotide synthesis. The protecting groups are removed quantitatively by treatment with concentrated ammonium hydroxide at 55 ºC for 5 hours in the final deprotection step. The most commonly used protecting groups for the heterocyclic bases are shown in Figure 9.

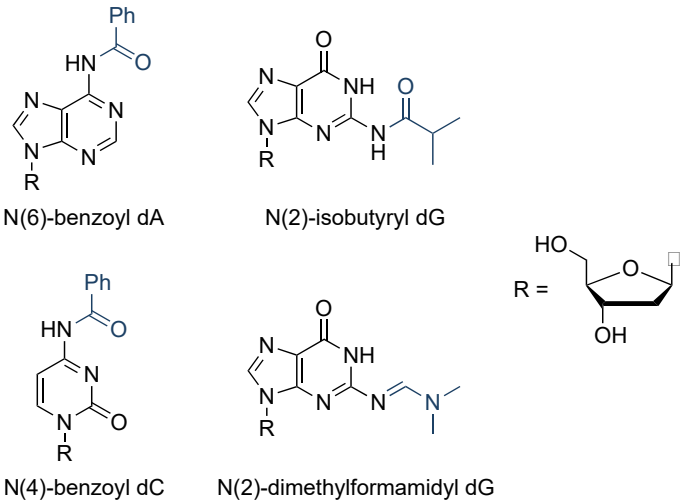


Figure 9. DNA base protecting groupsStructures of protecting groups commonly employed for the protection of adenine, cytosine and guanine bases during phosphoramidite DNA oligonucleotide synthesis.

The benzoyl groups on A and C are cleaved quickly in ammonium hydroxide but the isobutyryl protecting group on guanine is much more resistant to hydrolysis, and the rate determining step in oligonucleotide deprotection is cleavage of the isobutyryl group from guanine bases. In the case of certain chemically modified oligonucleotides, heating in ammonia can lead to degradation, so a more labile guanine protecting group is required in these cases. The most popular of the labile guanine protecting groups is dimethylformamidine (dmf dG), which allows oligonucleotide deprotection to be carried out under much milder conditions (conc. ammonium hydroxide at 55 ºC for 1 hour). A different set of "ultramild" protected monomers must be used in the synthesis of modified oligonucleotides with chemical groups that are extremely sensitive to aqueous ammonia. The most popular of these are shown in Figure 10.

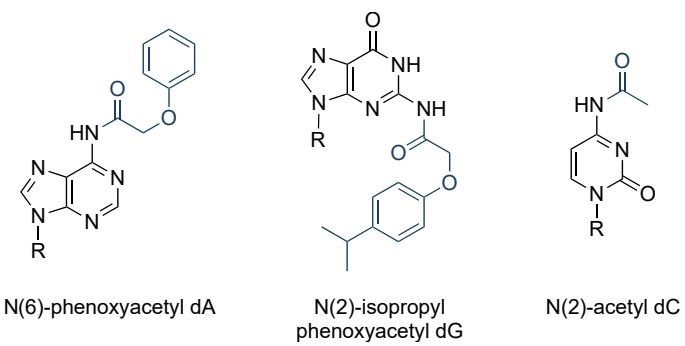


Figure 10. Ultramild protecting groupsStructures of heterocyclic base protecting groups designed for removal under "ultramild" conditions after phosphoramidite DNA oligonucleotide synthesis.

Ultramild protecting groups can be removed using a methanolic solution of potassium carbonate, or a mixture of 33% aqueous ammonia and 40% aqueous methylamine, at room temperature.

The reason for using acetyl dC as a protecting group is to avoid the transamidation side reaction that occurs with benzoyl dC and methylamine (Figure 11). The transamidation reaction does not occur with acetyl dC owing to the very rapid hydrolysis of the acetyl group.

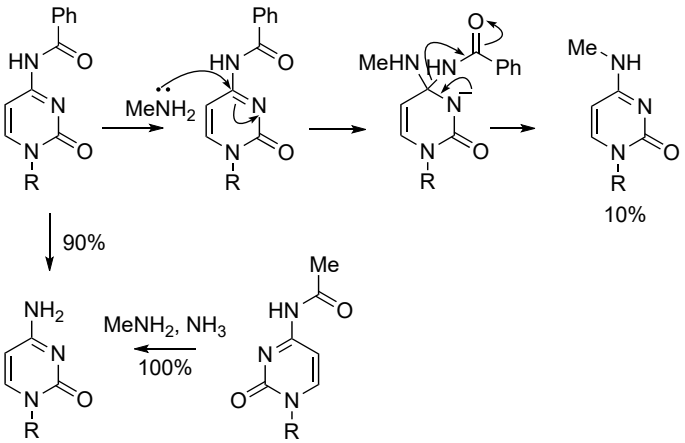


Figure 11. Cytosine deprotection side reactionMechanism of the side reaction that occurs in the deprotection of cytosine with methylamine and ammonium hydroxide.

Deprotection of the phosphodiester backbone

The phosphate groups are protected as 2-cyanoethyl phosphotriesters throughout oligo synthesis, and must be deprotected once synthesis is complete. The cyanoethyl groups are removed quickly in concentrated ammonium hydroxide, owing to the highly acidic nature of the hydrogens on the carbon atom adjacent to the electron-withdrawing cyano-group. The mechanism is as β-elimination (Figure 12).

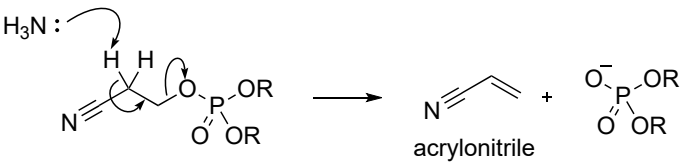


Figure 12. Cyanoethyl phosphodiester deprotectionMechanism of deprotection of the cyanoethyl protecting group employed to protect phosphodiester groups in phosphoramdite oligonucleotide synthesis.

In the early days of phosphoramidite oligonucleotide synthesis, the phosphate groups were protected as methyl triesters, and it was necessary to use thiophenol in their deprotection (Figure 13). Thiophenol is a foul-smelling, toxic liquid; and the development of β-cyanoethyl phosphoramidites by Koester was a particularly popular advance.

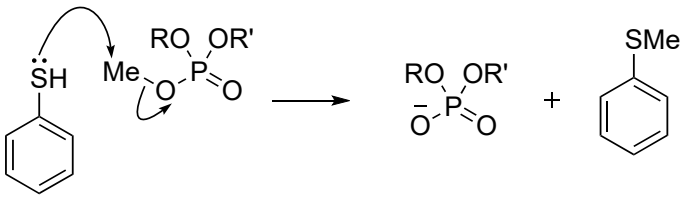


Figure 13. Methyl phosphodiester deprotectionMechanism of removal of the methyl group, used to protect phosphodiester groups in the early days of phosphoramidite oligonucleotide synthesis, using thiophenol.

Formation of adducts

Acrylonitrile, a by-product of phosphodiester deprotection (Figure 12), is a Michael acceptor. Under the strong basic conditions used in oligonucleotide deprotection, 2-cyanoethyl adducts can form with the hetereocyclic bases, particularly thymine (Figure 14).

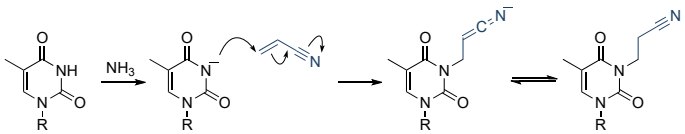


Figure 14. Formation of cyanoethyl adductsMechanism of reaction of thymine with acrylonitrile under strongly basic conditions, to form a 2-cyanoethyl adduct. These adducts often form adventitiously during phosphoramidite oligonucleotide synthesis.

If these cyanoethyl adducts are a problem, the resin cleavage and phosphoramidite backbone deprotection steps can be reversed. If the support-bound oligonucleotide is treated with a solution of a weak base in an organic solvent (e.g. 10% diethylamine in acetontrile, or 1:1 triethylamine/acetonitrile), the cyanoethyl protecting groups are removed from the phosphate backbone, but the oligo remains bound to the support (Figure 15).

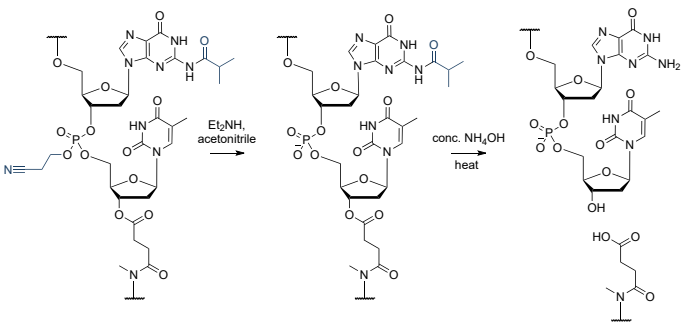


Figure 15. Deprotection of the phosphoramidite backbone of a solid support-bound oligonucleotideAfter deprotection of the cyanoethyl protecting group, the oligonucleotide is cleaved from the solid support. This prevents formation of cyanoethyl adducts.

Synthesis of nucleoside phosphoramidite monomers

The A, G, C and T phosphoramidites used in oligonucleotide synthesis are prepared on large scale from the free nucleosides which are obtained from natural sources. Using these precursors averts the need to build the required chirality into the molecules - not a trivial task, for deoxynucleosides contain three stereogenic centres.

Protection of nucleosides

Synthesis of the four monomers starts with protection of the amines (actually amidines and guanidines) of the heterocyclic bases as shown below for deoxyadenosine (Figure 16).

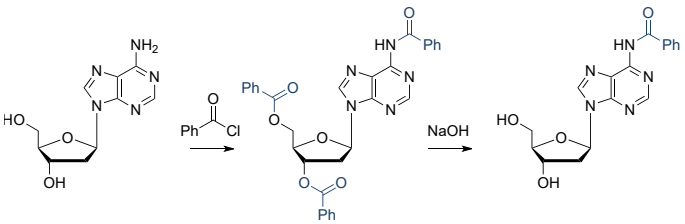


Figure 16. Benzoyl protection of adenineMechanism of protection of the adenine base for use in phosphoramidite oligonucleotide synthesis.

No protection is not necessary for thymine. The protected cytosine and guanine bases are shown in Figure 17.

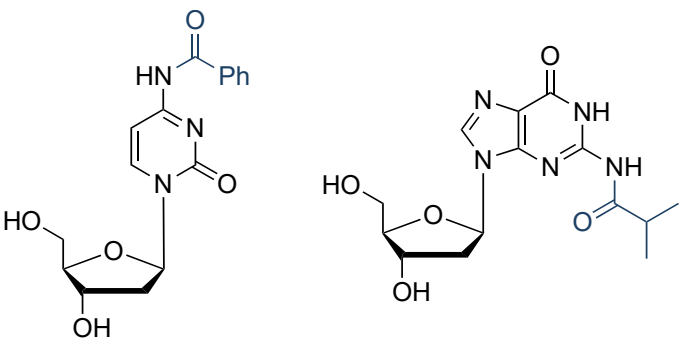


Figure 17. Protected cytosine and guanineStructures of protected cytosine and guanine heterocyclic bases for use in phosphoramidite oligonucleotide synthesis.

Protected dA, dC and dG can also be prepared by transient protection of the alcohol functions followed by benzoylation of the amino groups (Figure 18). The advantage of this method is that the silylation and benzoylation can be carried out in a single reaction vessel without isolation or purification of the intermediate silylated nucleoside ("one pot" synthesis).

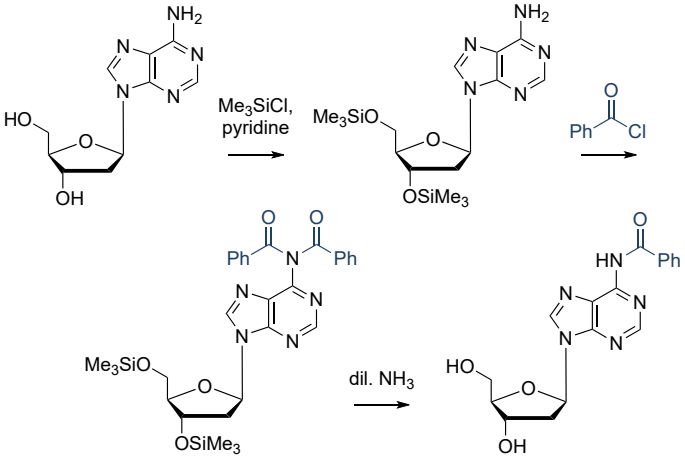


Figure 18. Transient protection of adenineMechanism of protection of the adenine base using the transient protection method.

The amino group of deoxycytidine is sufficiently reactive to be functionalized with active esters of benzoic acid which do not react with the hydroxyl functions. This affords a one-step synthesis of N(4)-benzoyl dC (Figure 19).

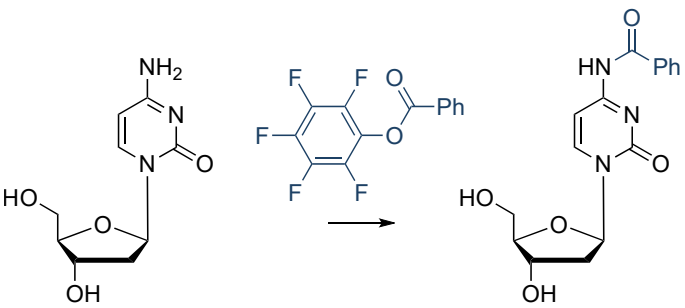


Figure 19. Protection of cytosine using pentafluorophenyl benzoateMechanism of protection of the cytosine nucleoside using the active ester method, with pentafluorophenyl benzoate.

Tritylation

The nucleosides (protected dA, dG and dC and unprotected T) are then tritylated selectively at the 5'-position using one equivalent of 4,4'-dimethoxytrityl chloride in pyridine at room temperature (Figure 20).

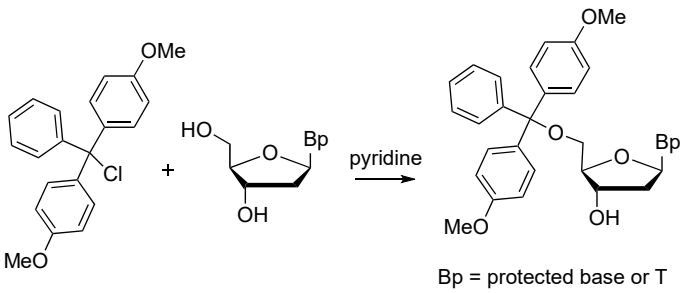


Figure 20. DMT nucleoside protectionMechanism of protection of the 5'-hydroxyl group with the 4,4'-dimethoxytrityl (DMT) group.

Phosphitylation

After purification, the DMT-nucleosides are phosphitylated at the 3'-position using 2-cyanoethyl diisopropylaminophosphorochloridite in the presence of the non-nucleophilic base diisopropylethylamine (DIPEA; Figure 21), to give phosphoramidite monomers. After silica gel column chromatography, precipitation into hexane, and filtration through a microfilter, the phosphoramidite monomers are ready to use in oligonucleotide synthesis.

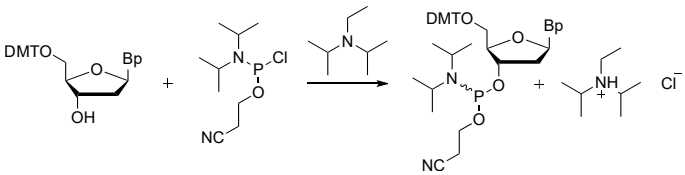


Figure 21. Nucleoside phosphitylationMechanism of phosphitylation of a DMT-nucleoside at the 3'-position.

The reagent for the above phosphitylation reaction is made by treating phosphorus trichloride with 2-cyanoethanol then with N,N-diisopropylamine in the presence of DIPEA (N,N-diisopropylethylamine, a non-nucleophilic base; Figure 22). Other secondary amines can be attached to the phosphorus but the nucleoside phosphoramidites of dimethylamine and diethylamine are rather unstable and bulkier amines react too slowly during the coupling step in oligonucleotide synthesis.

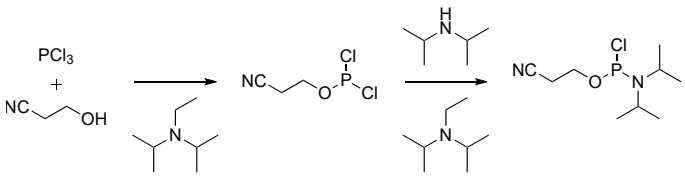


Figure 22. Synthesis of the phosphitylation reagentThe phosphitylating reagent, used to convert nucleosides to nucleoside phosphoramidites, is synthesized from phosphorus trichloride, 2-cyanoethanol and N,N-diisopropylamine, in the presence of DIPEA.

Because phosphorus is tetrahedral and the phophorus atom is a chiral centre, the phosphoramidite monomer produced in the phosphitylation reaction is a mixture of two diastereomers (Figure 23). These are distinct chemical compounds that give rise to two spots on TLC, two peaks in the HPLC spectrum (provided the resolution is sufficiently good) and two peaks in the 31P NMR spectrum.

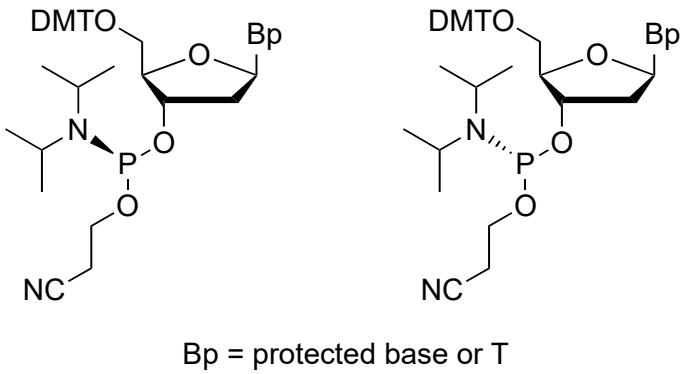


Figure 23. Phosphoramidite diastereomersStructures of the two diastereomers of the phosphoramidite monomer that result from the phosphitylation reaction.

Resin functionalization

Controlled pore glass and polystyrene resins are available commercially (with aminoalkyl groups attached) with loadings of 20—100 micromoles per gram. These resins are suitable for oligonucleotide synthesis provided that they have pores of 500 Å or more in diameter to allow the necessary reagents to diffuse, and the individual particles are more than 500 Å (50 nm) in diameter. Smaller particles will not permit rapid flow of solvents and reagents through the synthesis column and can block filters. The nucleoside attachment chemistry is shown in Figure 24. Four separate resins are needed for general oligonucleotide synthesis - one functionalized with each base (dA, dG, dC and dT).

In order to synthesize these functionalized resins, the 5'-DMT nucleosides in Figure 20 are treated with succinic anhydride at room temperature in the presence of pyridine. Pyridine is a good solvent for acylation reactions and also prevents detritylation of the DMT ether by the acidic nucleoside succinate produced in the reaction. A large excess of each nucleoside succinate is then added to a batch of the amino-functionalized resin along with a diimide coupling agent and an acidic alcohol such as 4-nitrophenol which forms an active ester in which it acts as a good leaving group. This is followed by a capping step to block any unreacted amino groups which would otherwise cause problems in oligonucleotide synthesis (Figure 24).

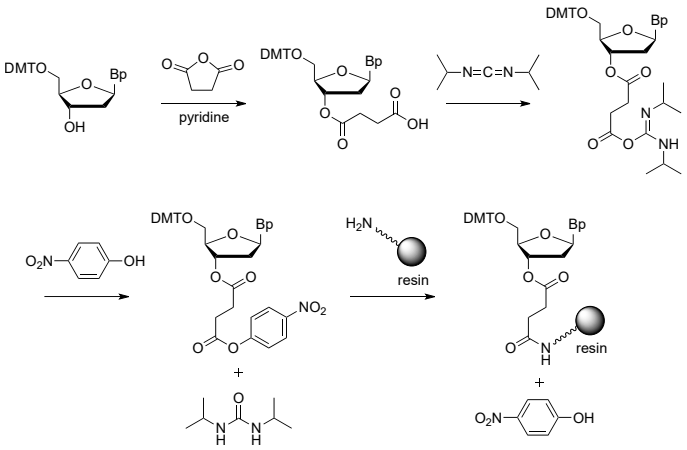


Figure 24. Resin functionalizationMechanism of functionalization of a resin by attaching a protected nucleoside phosphoramidite.

Calculation of resin loading

The loading of the nucleoside on the resin can be determined by trityl analysis. A small quantity of resin (1 mg) is treated with a strong acid (e.g. a 1:1 mixture of concentrated hydrochloric acid and ethanol) to cleave the DMT group. An aliquot of the resultant orange solution is taken and its absorbance at 495 nm is measured in a UV/visible spectrophotometer. This allows the amount of DMT cation to be determined (using the extinction coefficient of the DMT cation at 495 nm, E495 = 71 700 M−1 cm−1). The loading of the resin (per 1 mg of resin) is equal to this amount:

$$\text{Loading} = (E495 / A495) \times V \times (1/f)$$

where V is the volume of the cuvette and f is the fraction of the solution used in the absorbance measurement.

Oligonucleotide synthesis with universal supports

Standard solid-phase supports have the first base pre-attached to the resin. Universal supports do not have a base attached; instead, the first base is added in the first coupling step (Figure 25).

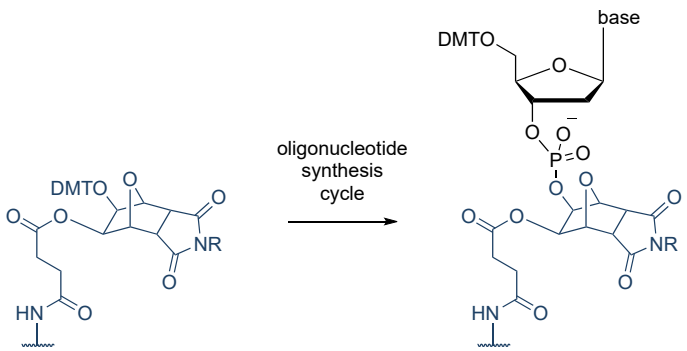


Figure 25. Synthesis of the first base of an oligonucleotide on a universal support.UnyLinker: R = Ph; UnySupport: R = Me.

The advantages of universal supports are obvious: a single type of column can be used for the synthesis of any oligonucleotide, whether it has A, C, G or T at the 3'-end; or, indeed, a modified (non-natural) base such as deoxyuridine, 5-methyldeoxycytosine or a fluorescent dye. With standard supports, separate synthesis columns must be used for each of the standard bases, and columns for modified bases may not be readily available.

The different chemistry of universal supports necessitates different conditions for cleavage of the oligonucleotide from the support, however, which can introduce problems.

Cleavage from universal supports

The cleavage of an oligonucleoide from a standard support involves ester hydrolysis, which occurs quickly in aqueous base at room temperature (e.g. 1 h in ammonia at room temperature). By contrast, cleavage from a universal support involves two steps: first, ester hydrolysis; then dephosphorylation (breaking of a P-O bond) (Figure 26). The first universal supports required treatment with a relatively strong base (e.g. methylamine) to effect dephosphorylation; even under these harsh conditions, however, incomplete cleavage was a frequent problem. Universal supports such as the UnyLinker/UnySupport have a strained structure in which neighbouring group participation (anchimeric assistance) speeds up the dephosphorylation reaction. Ammonia can then be used as the base for both resin cleavage and deprotection, as with standard supports. However, with the UnyLinker/UnySupport support, extended cleavage times must be used (e.g. 18 h at 55 ˚C).

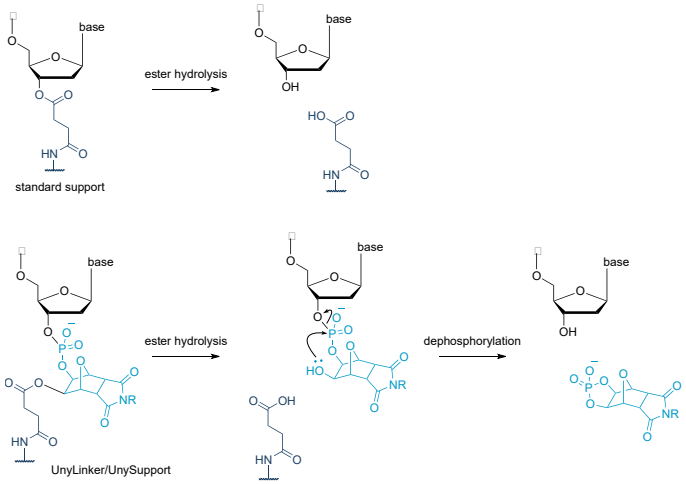


Figure 26. Cleavage of an oligonucleotide from a universal support.

The extended cleavage times required when using universal supports extend the time required for oligonucleotide synthesis, and also preclude the use of any reagents that are not stable to these conditions (such as "ultramild" reagents), and cannot be used in RNA synthesis.

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