
Recent developments in the chemical synthesis of polynucleotides

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INTRODUCTION

The application of chemically synthesized polynucleotides with defined sequence has played an essential role in the elucidation of the mechanism of many biological processes. In the sixties the use of synthetic oligonucleotides was invaluable for deciphering the Genetic Code.¹ Later the chemical synthesis of gene fragments of yeast alanine tRNA and E.coli suppressor tyrosine tRNA was accomplished. The DNA fragments coding for the tyrosine tRNA were linked with the aid of DNA ligase to form the first chemically synthesized active gene.^{2,3} These syntheses were carried out by the phosphodiester method (the immediate product of the condensation step is a phosphodiester). In the early seventies a reinvestigation of the first method of dinucleoside phosphate synthesis⁴ renewed interest in the phosphotriester approach (the immediate product of the condensation is a phosphotriester), which has now become a major route to oligodeoxynucleotide synthesis. These methods led to the first synthesis of gene fragments for peptide hormones.⁵⁻⁷ At present these methods are being perfected and the first automated DNA synthesis machines are commercially available.⁸ Although these methods still remain to be refined, simple methods for the synthesis of short deoxyribo-oligonucleotides are now in great demand due to the recent advances in recombinant DNA technology. The application of such compounds to site-specific, directed *in vitro* mutagenesis of cloned genes,^{9,10} the isolation of genes by probing clone banks with chemically synthesized oligonucleotides, and the synthesis of custom-tailored genes has already led to spectacular results in molecular biology.¹¹⁻¹³

Progress in the chemical synthesis of ribo-oligonucleotides has been slower.¹⁴ However, synthetic methods can now readily provide reasonable quantities of shorter oligonucleotides, which can then be joined by RNA ligase using well established techniques.¹⁵ The combination of these

chemical and enzymatic methods provided the basis for the recent total synthesis of tRNA molecules.^{16,17}

This article will discuss the present state of polynucleotide synthesis in liquid phase or on solid supports and review the most commonly used protecting groups and condensing reagents. For a more detailed treatment of many facets of polynucleotide synthesis see recent reviews¹⁴ or manuals.^{18,19}

1. Protecting groups for heterocyclic amino, ribose hydroxyl and phosphate functions

The chemical synthesis of polynucleotides involves formation of phosphodiester internucleotide linkages, mainly by activation of phosphomonoesters (in the phosphodiester method) or phosphodiesters (in the phosphotriester method) as shown in Fig. 1. Functional groups, except the ones involved in condensation, must be blocked with protecting groups which can be easily removed at the end of the synthesis. Heterocyclic amino groups are protected mostly with acyl groups according to their stabilities^{14e,18} and the 3'-hydroxyl groups in deoxynucleosides are usually protected with a benzoyl group. Some new compounds (e. g., t-butyl-di-phenylsilyl) have been introduced as lipophilic protecting groups for the deoxyribonucleoside 3'-hydroxyl.^{20a}

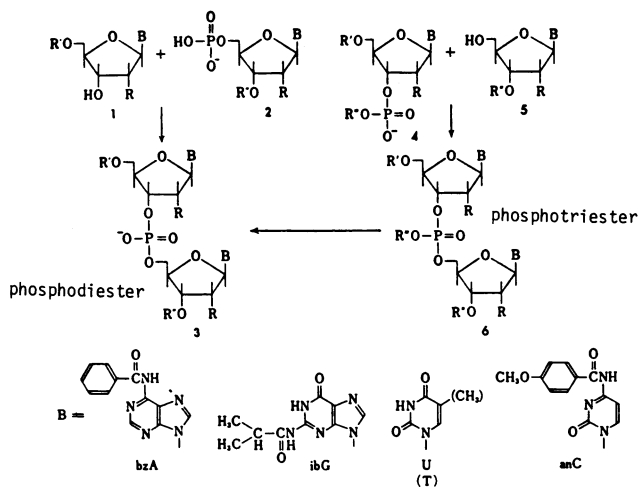


Figure 1. Phosphodiester- and Phosphotriester approach to the synthesis of the internucleotide linkage.

To protect the primary 5'-hydroxyl of ribo, the acid labile mono- or dimethoxytrityl group is used most commonly.^{18,19,20b} Other protecting groups which can be removed under different conditions have also been investigated. Levuliny,²¹ o-dibromomethylbenzoyl²² and trityloxyacetyl²³ groups are removed by treatment with hydrazine, silver perchlorate and dilute ammonia, respectively. These groups and the conditions for their removal are summarized in Fig. 2 and Table I.

The 2'-hydroxyl group of ribonucleosides needs to be protected with stable groups which can be removed at the final deprotection step, especially in the phosphotriester synthesis. Ketals, such as tetrahydropyranyl^{20b} or methoxytetrahydropyranyl,^{14d} are used as acid removable protecting groups. 2'-O-(o-Nitrobenzyl) derivatives of ribonucleosides²⁴ and t-butyl dimethylsilyl nucleosides^{25a,b} have also been described. The tetraisopropyl disiloxo group^{25c} was found useful in protecting the 3'- and 5'-hydroxyl groups at the same time. The structures of some protecting groups for the secondary hydroxyl groups are shown in Figure 3.

For phosphate protection a variety of reagents have been developed. The β -cyanoethyl^{20c} group has mostly been used to protect phosphomono and phosphodiester. Lipophilic derivatives of 2-thioethyl ester^{1b} and aromatic amidates of phosphomonoesters have also been described.^{14e} The phosphotriester approach requires more stable protecting groups for phosphates; o-chloro- or p-chlorophenyl groups were found to possess sufficient stability^{14d}. The o-chlorophenyl group can be removed by treatment with oxamate²⁶ faster than the p-chloro derivative. This is important for the

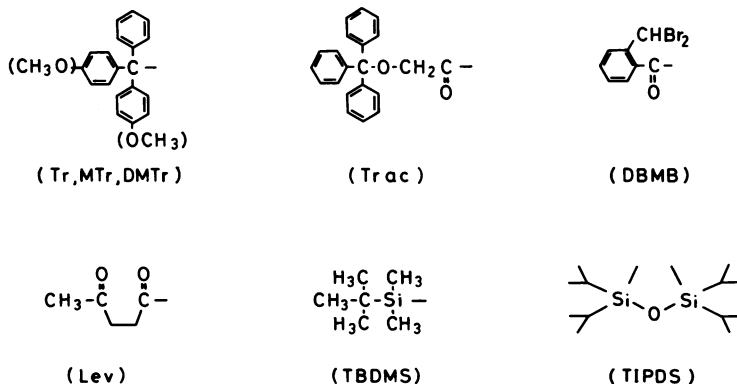


Figure 2. The structures of commonly used protecting groups for the 5'-hydroxyl group.

Table I
Commonly Used Protecting Groups and Conditions for their Removal

Groups to be protected	Protecting group	Removal conditions	References
-NH ₂	Acyl	conc. NH ₄ OH	18
5'-OH	Di- or Mono-methoxytrityl	Benzenesulfonic acid, Acetic acid or ZnBr ₂	18, 19, 20b 72
	Trityloxyacetyl	dilute NH ₄ OH	23
	Levulinyl	NH ₂ NH ₂	21
	o-Bromomethyl-benzoyl	AgClO ₄	22
	3'-OH	Benzoyl	conc. NH ₄ OH
2'-OH	Ketal	dilute HCl	14d, 20b
	o-Nitrobenzyl	UV-light	24
	t-Butyldimethylsilyl	Bu ₄ N ⁺ F ⁻	25
Phosphoryl	β-Cyanoethyl	Alkali, Et ₃ N	18, 19, 20c
	β, β, β-Trichloroethyl	Zn-TPSOH	21
	p- or o-Chlorophenyl	Tetramethylguanidinium pyrdine aldoximate	26
	Anilido or Anisido	Isoamyl nitrite	38, 41

complete removal of protecting groups from large oligonucleotides. As discussed later, the triester block condensation method requires two kinds of phosphate protecting groups which can be removed selectively. A summary of these groups as well as the basic means of their removal is given in Table I.

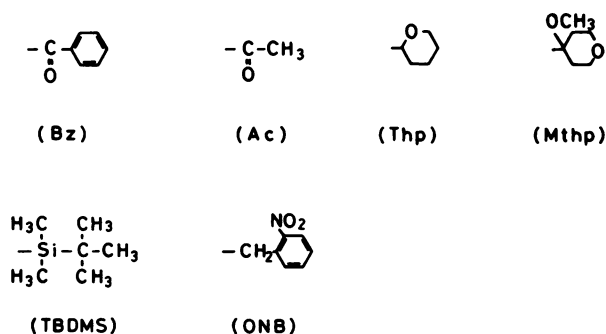


Figure 3. The structures of some protecting groups for the secondary hydroxyl groups.

2. Phosphorylating and condensing reagents

Phosphomonoesters can be activated with dicyclohexylcarbodiimide (DCC). However, DCC acts slowly and is suitable only for short oligonucleotides. Faster activation is achieved by arenesulfonyl chlorides, such as mesitylenesulfonyl chloride (MS) or triisopropylbenzenesulfonyl chloride (TPS).¹ In addition, these condensing reagents slowly activate phosphodiesters. This is a drawback in the phosphodiester approach, since internucleotide phosphates are esterified during condensation reaction which leads to side products. Arenesulfonyl chlorides give also some side products in the phosphotriester synthesis. The introduction of arenesulfonyl 1,2,4-triazolide²⁷ improved the phosphotriester yield by reducing sulfonylation and formation of colored impurities. Establishment of more active tetrazolide and 3-nitrotriazolide derivatives (e.g. MSTe²⁸, TPSTe²⁸ and MSNT²⁶ etc.) increased the rate of activation of phosphodiesters greatly. The structures of some condensing reagents are shown in Fig. 4. Mesitylene- and triisopropylbenzenesulfonyl 5-(pyridin-2-yl)tetrazolides were found to form stereospecific phosphotriesters when *o*-chlorophenyl esters of deoxynucleoside 3'-phosphates were condensed with 5'-unprotected deoxynucleosides²⁹. This should facilitate the isolation of condensation products by silica gel chromatography, an advantage in product purification.

Although the mechanism of activation by MST is not clarified, phosphotriazolides are presumably active intermediates. As shown in Figure

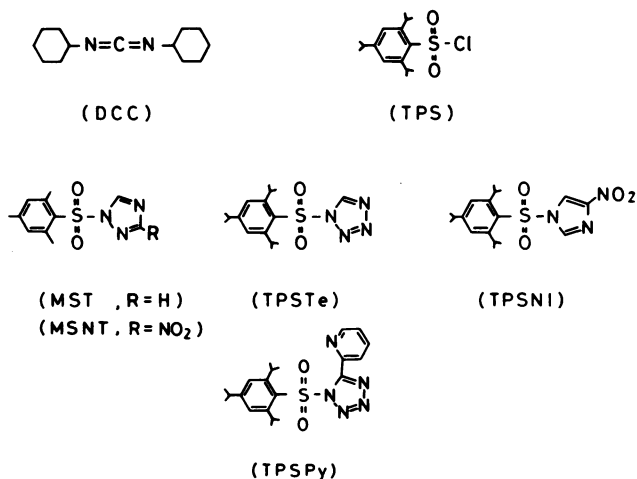


Figure 4. The structures of some condensing reagents.

chlorides have also been used simultaneously in coupling reactions of thymidine phenylthiophosphate.^{32b}

3. Deoxyoligoribonucleotide synthesis in solution

The new methods for protection and activation of nucleotides have reduced the time required for polynucleotide synthesis both in the phosphodiester and phosphotriester approach. Two tyrosine suppressor tRNA genes with altered promoter sequences (ca. 200 nucleotides long) were synthesized³³ by joining of oligonucleotides with approximate chain length of 12, which were prepared by the previously described phosphodiester methods.^{2,3,18} New separation and purification procedures of the products by high-performance liquid chromatography on alkylated silica gel³⁴ also contributed to shortening the time required for obtaining a large number of oligonucleotides.

The phosphotriester approach has been greatly improved with the introduction of the more reactive condensing reagents (see above). The other major improvement in the phosphotriester synthesis has been the use of new purification procedures. Separation of triesterified phosphate intermediates can be performed by chromatography on silica gel. Short column chromatography on fine particles of silica gel increased resolution of protected oligonucleotides^{14d} and reversed-phase chromatography is also used to purify protected oligonucleotides.³⁰ The latter method can resolve impurities which can not be removed by silica gel chromatography. Thin layer chromatography on alkylated silica gels has been used to monitor the extent of condensation reactions as well as the purity of the final products.²⁵ Unlike silica gel this material will also resolve compounds with phosphate dissociations. Phosphotriester intermediates with a terminal phosphodiester group are now purified by reversed-phase chromatography on C-8 or C-18 silica gels. Purification of oligonucleotide intermediates is essential for polynucleotide synthesis which involves several condensation steps. Compared to solid phase synthesis, solution phase synthesis has some advantages in purifying intermediates; in addition it allows product preparation on a large scale. This was shown in the recent synthesis of a few deoxyribopolynucleotides with defined base sequence which were used for X-ray³⁰ and NMR³¹ investigations. These studies required products of high X purity in large quantity.

Figure 5 shows methods for obtaining nucleotide intermediates in the triester synthesis. Two types of protected oligonucleotides are required

for the block condensation strategy: oligonucleotides with or without a 3'-terminal phosphodiester group. The 3'-terminal dimer (9, R'=Bz) can be synthesized by treating 3'-O-benzoyl-N-protected deoxynucleosides (8) either with the phosphotriazolide (3) or with phosphodiester (4) plus a condensing reagent. The 5'-dimethoxytrityl group is removed for elongation in the 5'-direction. The other type of oligonucleotide (e.g. 9, R'= phosphoryl) can be obtained by condensing nucleotide (7) instead of 8. Nucleotides (9) are converted to the 3'-diesterified block by removing one of the protecting groups on the phosphate without damaging other protecting groups. For this purpose a combination of chlorophenyl (Ar) and β -cyanoethyl (R) is used frequently.^{30,37} Trichloroethyl,^{21c,35a} anilido,³⁸⁻⁴⁰ or anisido⁴¹ are also used instead of β -cyanoethyl as more stable protecting groups; these are supposed to be advantageous in the isolation or purification of intermediates. Figure 6 shows two examples of oligonucleotide synthesis by the triester approach involving the β -cyanoethyl group (a) or the phosphoroanilidate approach (b). Figure 6a depicts the synthesis of a fragment used in the synthesis of the insulin gene⁵. The dodecanucleotide in Figure 6b corresponds to a part of the DNA sequence of the bacteriorhodopsin gene.³⁹

A simplified procedure to prepare dinucleoside phosphates, which involves condensation of 3',5-unprotected deoxynucleosides is also available,^{42,43} 3'-3'-linked side products from this reaction could be separated by thin layer chromatography on silica gel.⁴³

An alternate triester method based on phosphite intermediates dates back a long time⁴ and was worked out by Letsinger and his colleagues.⁴⁴ The phosphite coupling method using *o*-chlorophenyl phosphorodichloridate or trichloroethyl phosphorodichloridate³⁹ has allowed the synthesis of oligomers containing internucleotide phosphite linkages.⁴⁰ The use of methyl phosphorodichloridate and the removal of the methyl group after oxidation to triesters has also been reported⁴¹. These procedures were successfully applied to the solid phase synthesis of deoxy-oligonucleotides (see below).

4. Ribo-oligonucleotide synthesis in solution

In the past most notable achievements of ribo-oligonucleotide synthesis, such as the preparation of all possible triplets⁴⁷ or of many dinucleotides containing nucleoside analogs,⁴⁸ have been made by the phosphodiester strategy. However, much effort has been spent recently in developing a phosphotriester approach for ribo-polynucleotide synthesis. Present evidence points to advantages of this approach in the protection of the

internucleotide phosphate linkages during condensation.¹⁴ There are now several routes to the synthesis of oligoribonucleotides by the phosphotriester method in liquid phase. Protecting groups for the 5'-hydroxyl functions are selected in connection with the stability of other protecting groups. The protection of the 2'-hydroxyl group is an additional problem in

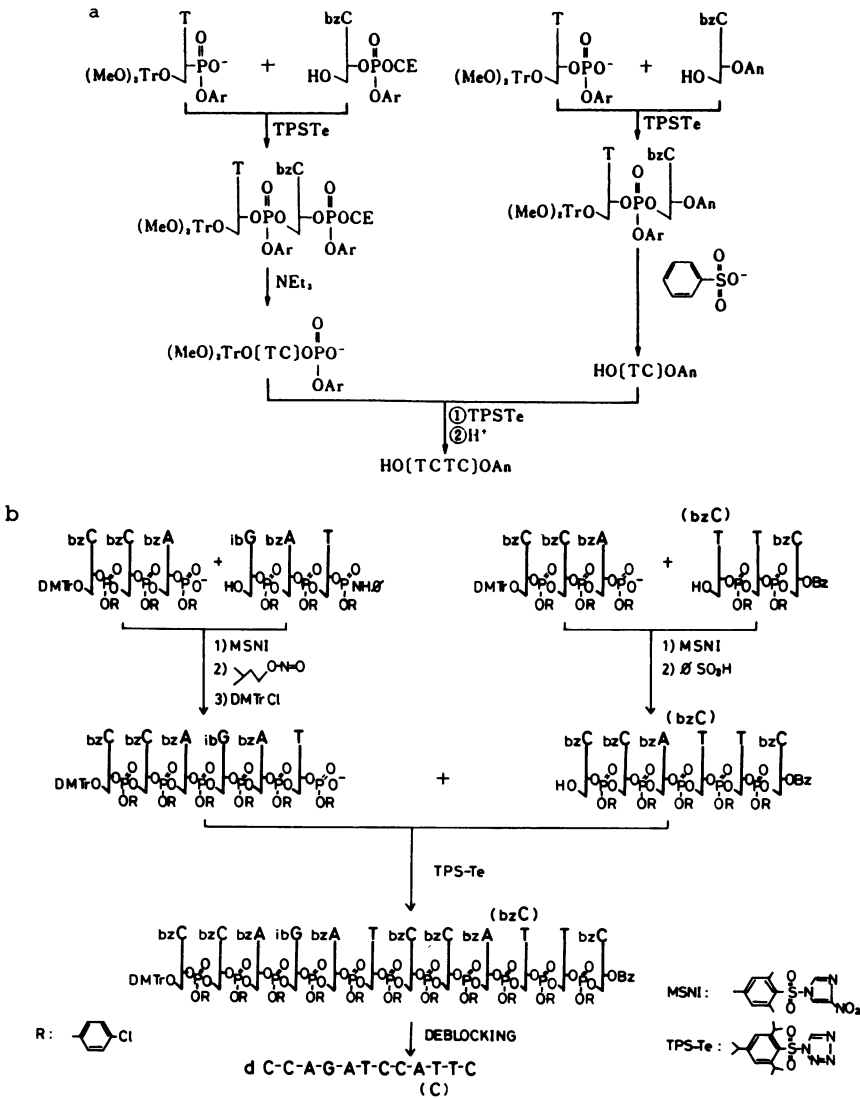


Figure 6. Two examples of deoxy-oligonucleotide synthesis by the phosphotriester method.

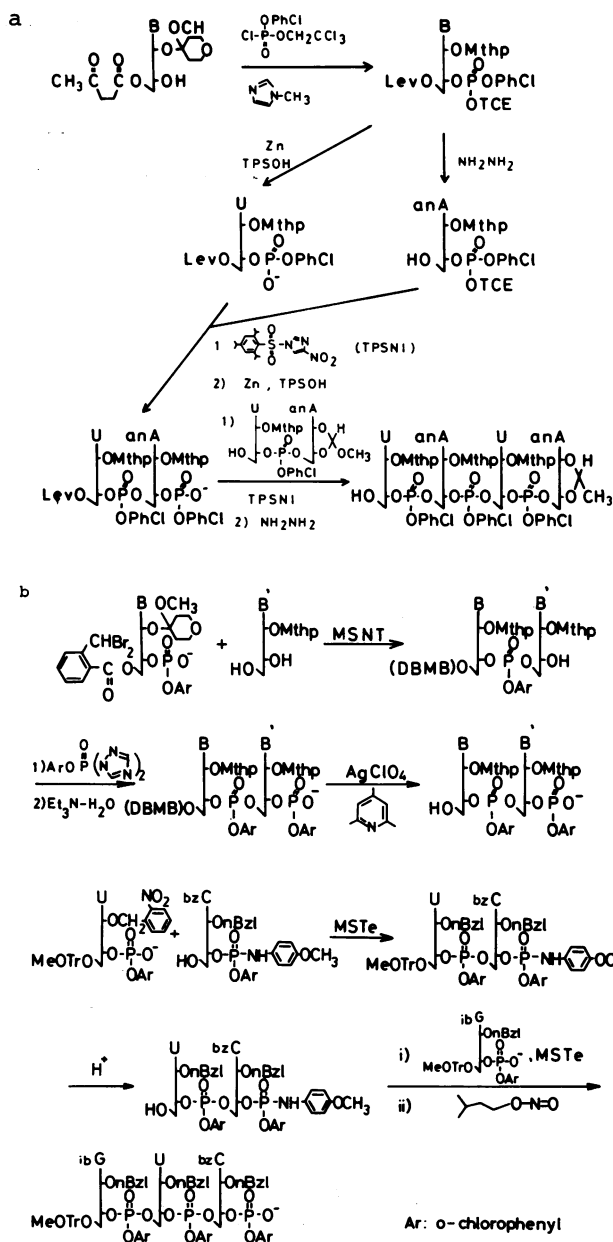


Figure 7. Three examples of ribo-oligonucleotide synthesis by the phosphotriester method.

ribo-oligonucleotide synthesis. Combinations of acid labile ketals for the 2'-hydroxyl group and alkaline labile acyl derivatives have been employed. Figure 7 shows three examples of the triester approach for the synthesis of ribo-oligonucleotides. A combination of 5'-O-levulinyl, 2'-O-methoxypropyl and *o*-chlorophenyl phosphate was used in the synthesis of a tetradecanucleotide (Fig. 7a).^{21a} *o*-Dibromomethylbenzoyl, which can be removed by treatment with silver cations, is introduced as shown in Fig.7b⁴⁹ A photolabile *o*-nitrobenzyl ether²⁴ has been used in combination with acid labile 5'-O-monomethoxytrityl and *p*-chlorophenyl phosphates (Fig.7c)⁵⁰ in the synthesis of oligonucleotides having the sequence of *E.coli* formylmethionine tRNA. These deca-,⁵¹ heptadeca-,⁵² and eicosanucleotides⁵³ are substrates for RNA ligase and allowed the construction of a tRNA.¹⁶ The some strategy of chemical synthesis of ribo-oligonucleotides and joining with RNA ligase allowed the synthesis of "mutant" tRNAs.⁵⁴

The phosphite coupling method⁴⁴ has been applied to the synthesis of a riboheptanucleotide using 2'-(*t*-butyldimethylsilyl)nucleosides,⁵⁵ and hexadecauridylic acid has been synthesized by block condensations.⁵⁶ A scheme for the preparation of a dimer by the phosphite method is shown in Figure 8.

5. Solid phase synthesis of polynucleotides

The strategy of polymer synthesis on an insoluble support has significant advantages over the solution method discussed above. The separation of the reaction intermediates from the starting compounds is much easier, consequently the speed of synthesis will be much faster. On the other hand, high yields in each condensation step or blocking of unreacted growing polynucleotide chains are required. Early attempts to synthesize

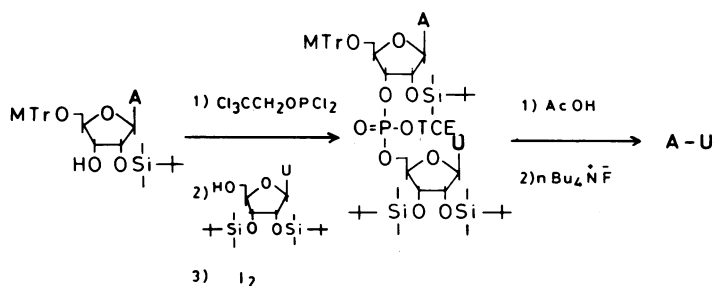


Figure 8. Ribo-oligonucleotide synthesis by the phosphite triester method.

polynucleotides on solid support matrices met with great difficulties. These involved adsorption of nucleotide intermediates on the support material and insufficient yields in condensation steps. This is summarized in earlier reviews.¹⁴ Reexamination of these methods showed that polyacrylamide, polyacrylmorpholide,⁵⁷ and polyacryldimethyl-amide⁵⁸ were satisfactory. These support materials were then successfully used in the triester approach to condense mono- or oligonucleotides^{31,59-65}

Polystyrene was found to be as good as polyacrylamide supports for deoxy-oligonucleotide synthesis except that it required hydrophobic conditions for removal of the product from the polymer.⁶⁶ Silica gel⁶⁷ was also used for the synthesis of deoxyoligonucleotides by the phosphite coupling method^{68,69} and by the triester method.⁷⁰ The phosphite method has been improved by introduction of stable N,N-dimethylphosphoramidite intermediates which can be prepared by treating N,5'-protected deoxynucleosides with chloro-N,N-dimethoxyaminomethoxyphosphine.⁷¹ The presentative schemes for solid phase synthesis by the phosphotriester and phosphoramidite methods are shown in Figure 9.

Removal of the 5'-dimethoxytrityl group in each step is one of problems in the synthesis of deoxyoligonucleotides on polymer supports, since the glycosidic linkage of N-benzoyldeoxyadenosine is very acid labile (especially when the 3'-position of the nucleoside is not phosphorylated) and detritylation of polynucleotides requires stronger acid conditions on the polymer. Therefore oligomers with a 3'-terminal deoxyadenosine have been avoided earlier in solid phase syntheses. However, the use of zinc bromide in the presence of isopropanol^{72a,b} or trichloroacetic acid^{72c} seem to resolve this problem.

It should be noted, that solid phase synthesis can be scaled down to produce very small amounts. For instance, as little as 14 μ moles of deoxy-heptadecamers on 50-60 mg of polymer support.⁶² This is convenient especially in the case of block condensations for larger oligonucleotides. The largest oligonucleotide reported by the solid phase approach is a 31 mer synthesized by trimer block condensation.⁶³ Purification by reversed phase chromatography was required before and after removal of the dimethoxytrityl protecting groups. This purification was based on the presence of the lipophilic trityl group in the desired product. Speed, the other feature of the solid phase synthesis was demonstrated by the rapid synthesis of gene fragments of α -interferon by block condensation of dinucleotides on polyacryldimethylamide resin support. These oligonucleotides ranging in size

from 14-15 were joined with DNA ligase to form a DNA duplex of 514 base pairs. This represents the largest DNA molecule synthesized chemically to date.⁶⁴ The phosphite approaches have so far utilized monomer units as intermediates. This is the simplest strategy which requires only four intermediates as starting materials. However, very pure starting materials and vigorously dry conditions are needed to maintain good yields and high product purity.

These achievements suggest that chemical-enzymatic synthesis of genes

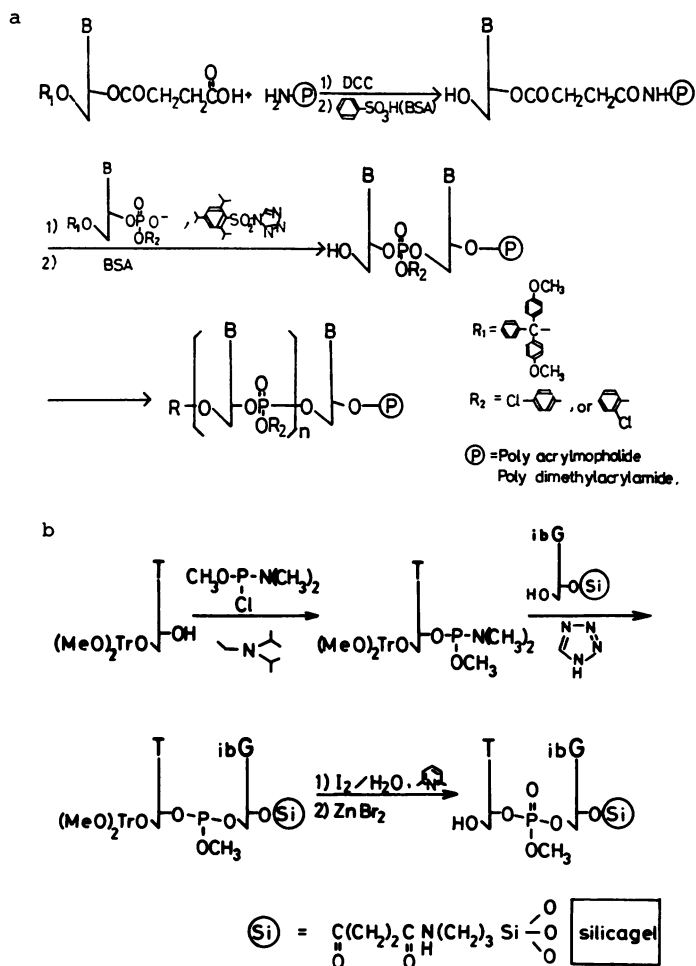


Figure 9. Two examples of deoxy-oligonucleotide synthesis on solid supports.

coding for relatively large proteins has become feasible. Automated and semiautomated synthesis carried out by machines can now perform many synthetic steps and economize man-power, but purification and characterization of the final product still requires time.

For ribopolynucleotide synthesis the polyacrylmorpholide support has been used to synthesize a heptanucleotide⁷³ and the phosphite coupling method combined with a silica gel support has allowed the synthesis of a hexanucleotide.⁷⁴ Since the solid phase approach requires a large excess of the incoming nucleotide unit to get a high yield in the condensation step, this strategy may not be so advantageous for ribopolynucleotide synthesis where the preparation of the protected intermediates needs more steps than are required for the corresponding deoxyribonucleotides.

CONCLUSION

The methods presently available for chemical polynucleotide synthesis can be divided into two categories, (i) synthesis in homogeneous solution and (ii) synthesis on solid supports. When only small amounts of polynucleotides are required (e.g., for biochemical studies) then the solid phase approach is the method of choice. However, when relatively large amounts of polynucleotides are desired (e.g., for physical studies of these compounds) then liquid phase synthesis is preferable, where the intermediates are purified after each condensation step.

The advantages of the solid support approach are the following. i) The scale of the synthesis can be reduced since the intermediates need not be isolated after each condensation step. Quantities of less than 1 μ mole are sufficient for many applications in molecular biology, e.g., to serve as substrates for DNA ligase,² as primers for reverse transcriptase,¹² as tools for in vitro mutagenesis¹⁰ or as probes for hybridization experiments.¹¹ ii) Most of the operations can be automated and soon commercially available machines may be successfully used. iii) The solid phase method is preferred in the synthesis of "mixed probes" containing multiple bases (corresponding to the third letter degeneracy of the Genetic Code) in the products. This is due to the fact that deoxyguanosine-containing oligomers are recovered in lower yield upon silica gel purification used in solution synthesis. iv) The time required for synthesis is substantially reduced because the purification process after each step of condensation is not needed. However, final purification and identification of the synthesized polynucleotides still remain time consuming tasks.

The introduction of reversed-phase chromatography for purifying the intermediates obtained in the homogeneous solution synthesis approach improved the quality of oligonucleotide products. It is now feasible to synthesize deoxy-oligonucleotides of chain length 20 in gram quantities by the phosphotriester method in solution.

The recent achievements in the chemical synthesis and modification of polynucleotides have set a new level of accomplishment and expectation in biochemistry, molecular biology and related areas. This, in turn, will bring about further advances in the field of nucleic acid chemistry. An exciting future lies ahead.

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