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REVIEWS

The Organic Chemistry Underlying DNA Synthesis

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The procedures used today for oligo-DNA synthesis are reviewed. The main applications of oligonucleotides in biological studies, the protecting group chemistry, the coupling procedures, and the methods for isolating and characterizing products are described.

Contents. Introduction. Uses of DNA Oligomers. Established methods of synthesis and general strategies. Types of solid supports. Protection of the nucleic bases. *Thymidine. Deoxycytidine. Deoxyadenosine. Deoxyguanosine.* Protection of the deoxyribose. Phosphorylation and phosphitylation. The coupling step. The final deprotection step. Purification methods in DNA synthesis. Characterization of oligonucleotides. Conclusion. References.

INTRODUCTION

The chemical synthesis of DNA sequences has become an unvaluable tool in genetic engineering (*1*). Even if several manufacturers offer now a custom synthesis of defined sequence oligodeoxyribonucleotides, it is still more economical for long run researches to have the facility in one's own laboratory.

For the molecular geneticist, this facility may just be the blind use of an automatic DNA synthesizer. At this level, most people are usually not concerned at all by the detailed chemistry of the synthesis. As a consequence, leading journals such as "Nucleic Acids Research" stipulate in their instructions to authors that "manuscripts describing . . . routine synthesis of oligonucleotides will not be published." Such a lack of interest for an area of sophisticated fine chemistry is made possible by the fact that the user is not much concerned with yields and purity along the synthesis because usually less than 1 o.d. of the oligomer is enough and HPLC techniques allow the isolation of the correct oligomer from an intricate crude mixture.

The point is however that the synthesis of oligodeoxyribonucleotides was made possible by the labor of excellent chemists who worked out and still improve methods of protection and coupling. Besides, it is still a challenge to synthesize large amounts of oligomers for NMR or X-ray studies.

This is why it seemed to us of interest to collect in a review the information on the detailed process of constructing DNA sequences. Progress in this area will

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also allow to improve the much more delicate RNA synthesis, a topic not covered here.

USES OF DNA OLIGOMERS

Before describing in details the various aspects of the synthesis, it is probably useful to briefly quote the main uses of oligodeoxyribonucleotides.

Hybridization Probes (Less Than 1 o.d. Necessary)

The routine synthesis of DNA on a solid support yields oligomers with free 3′ and 5′ alcohol functions. The 5′-end can be enzymatically phosphorylated (T4 polynucleotide kinase) to give a ^{32}P-labeled compound. A radioactive probe of typically 12–15 nucleotides long selectively hybridizes to the complementary sequence of single-stranded DNA or RNA. For such lengths, one mismatch significantly destabilizes the duplex. A ladder of bands of mRNA obtained on a gel by electrophoresis can be transferred to a membrane (e.g., nitrocellulose) and allowed to equilibrate with the probe. After washings under controlled conditions, an autoradiography will reveal the band that retained the probe (Northern blot (*2–5*)). The technique is also applied to DNA fragments resolved by gel electrophoresis (Southern blot (*6*)).

Colonies of cells on an agar plate can be partially transferred by contact to a membrane where the cells are lysed, their DNA denatured and adsorbed on the support. The membrane is treated by the hybridization probe. The autoradiography reveals the colonies featuring a given sequence in their genome (colony hybridization, Grunstein–Hogness procedure (*7, 8*).

The use of hybridization probes complementary to sequences of the genome where a defect is suspected allows to detect the mutation. It may be a useful diagnostic for hereditary diseases (*1*).

Instead of being labeled with ^{32}P, the oligonucleotide may be coupled to biotin, a small molecule forming an extraordinary stable complex with a protein called avidin. The ternary complex DNA (or RNA)–probe–avidin is detected by a sensitive chromogenic reaction (*9–11*).

Primers (Less Than 1 o.d. Necessary)

Synthetic oligomers hybridized to a complementary portion of a long single-stranded DNA or RNA molecule serve as primers for DNA polymerase (Klenow fragment) in the case of a DNA template, or reverse transcriptase in the case of a RNA template. These enzymes extend the duplex starting from the 3′-end of the primer. As hybridization is selective, one knows exactly from which point of the single stranded template the copying process starts. The selective priming allows to sequence either DNA (*12*) or RNA (*13*) templates; it also allows to obtain from very complicated mixtures of mRNA a small collection of DNA copies (cDNA) upstream to the primer (*14–16*).

Synthetic oligodeoxyribonucleotides may be used as site-specific mutagens (*17–*

19). The general strategy involves the use of a synthetic oligonucleotide carrying the desired mutation (point substitution of the heterocyclic base, deletion or insertion) as a primer to direct the DNA synthesis on a single-stranded circular DNA. The oligonucleotide is thus incorporated into a second (mutated) DNA strand. After ligation, transformation of this closed circular heteroduplex DNA into *Escherichia coli* cells, followed by *in vivo* DNA replication, resolves this heteroduplex into mutant and original, wild type gene. The mutant can be easily identified against a background of nonmutant DNAs by a hybridization probe.

Mixed Probes, Primers, and Related Problems

It is difficult to devise a convenient probe or primer when the sole available information is the sequence of a protein. Indeed, because of the degeneracy of the genetic code, numerous nucleic acid sequences exist for a unique peptide. Usually, a section of the protein sequence where the degeneracy is minimum is chosen and the corresponding collection of probes is synthesized; these are then used as a mixture in hybridization experiments (*1, 20–23*). An alternative is to construct with the most probable codons a unique but longer probe (*1, 24*).

A few teams try to solve the problem by replacing the ambiguous nucleotides by an artificial one able to be paired with any partner without destabilizing too much the whole duplex (*25–27*).

Gene Synthesis (Less Than 1 o.d. Necessary)

Several genes were recently constructed step by step by chemical synthesis of short duplexes followed by their enzymatic ligation (*28–30*). However, the total synthesis of a long gene is, for the moment, not competitive with its isolation from cells. The chemical synthesis of small DNA duplexes incorporating, e.g., sequences cleaved by restriction enzymes (linkers, polylinkers) is of much help for the molecular geneticist (*31, 32*).

Physical Chemistry and Chemical Creativity (10–50 mg or More Necessary)

Short duplexes may be crystallized and analyzed by X-ray diffraction (*33*) or NMR spectroscopy. They serve as models to study gene structure (*34*), intercalation of drugs (*35*), metal complexation, chemical modification, and so on. Completely new molecules may also be created. For example, the conjugate of a Fe^{II}–EDTA complex with an oligonucleotide regioselectively cleaves single-stranded DNAs (*36, 37*).

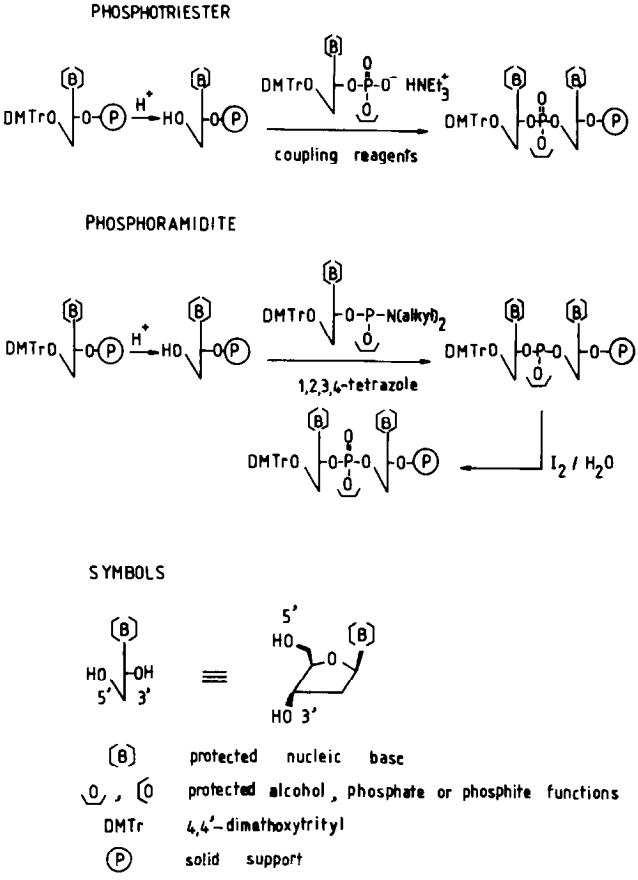
ESTABLISHED METHODS OF SYNTHESIS AND GENERAL STRATEGIES

Phosphotriester and Phosphoramidite Methods

Two methods are very popular nowadays. The so-called "phosphotriester method" was revitalized (after the pioneering work of Michelson and Todd (*38*))

by Letsinger (*39*), Reese (*40, 41*), and Eckstein (*42, 43*) and optimized by Narang ("modified phosphotriester method" (*44, 45*)), Gait (*46*), and Efimov (*47*). The so-called "phosphite triester method" was introduced by Letsinger (*48*) and modified to a more tractable form called "phosphoramidite method" by Caruthers's team (*49*). Both types of synthesis are routinely performed on a solid support. The phosphoramidite method is characterized by a more rapid and more complete coupling as compared to the phosphotriester one. The phosphite triester and phosphoramidite methods are on the way to be patented (*163, 457, 460*).

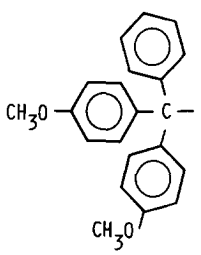
The repetitive steps of the phosphotriester and phosphoramidite types of synthesis are shown in Scheme 1. The chain grows by nucleophilic attack of the 5′-OH of the immobilized oligomer on the activated 3′-phosphate or phosphoramidite function of a soluble 5′-protected nucleotide building block. This one may be a mono- (in both methods), di- or trinucleotide (up to now, mainly in the phosphotriester method (*461*)). Other important steps are the acid deprotection of the 5′-*O*-(4,4′-dimethoxytrityl) group (DMTr) and, in the phosphoramidite method, the oxidation of the phosphite triester to the phosphate triester.



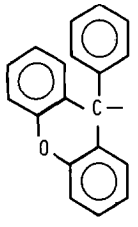
SCHEME 1. Main repetitive steps in the routine synthesis of an oligodeoxyribonucleotide on a solid support.

Alternative Strategies on a Polymeric Support

The 3′ to 5′ direction of synthesis, as well as the use of 3′-phosphorylated and 5′-protected soluble building blocks, is only one of the four theoretical possibilities (i.e., A in Scheme 2). This choice is tightly bound to the use of DMTr or Pixyl as ideal protecting groups for the 5′-OH function:



DMTr



Pixyl

These groups are specifically introduced on the primary alcohol function of a nucleoside, their cleavage is fast, quantitative, and can be monitored visually (strong color of the corresponding carbocations).

Approaches of type D, making use of the β-cyanoethyl phosphate protecting group, have been described (*50–52*). The treatment with a base cleaves this group by β-elimination. A careful choice of the base is necessary because it may be harmful to the triester functions of the growing chain and to the protections of the aglycone residues. Also, the linkage with the solid support has to be base-resistant.

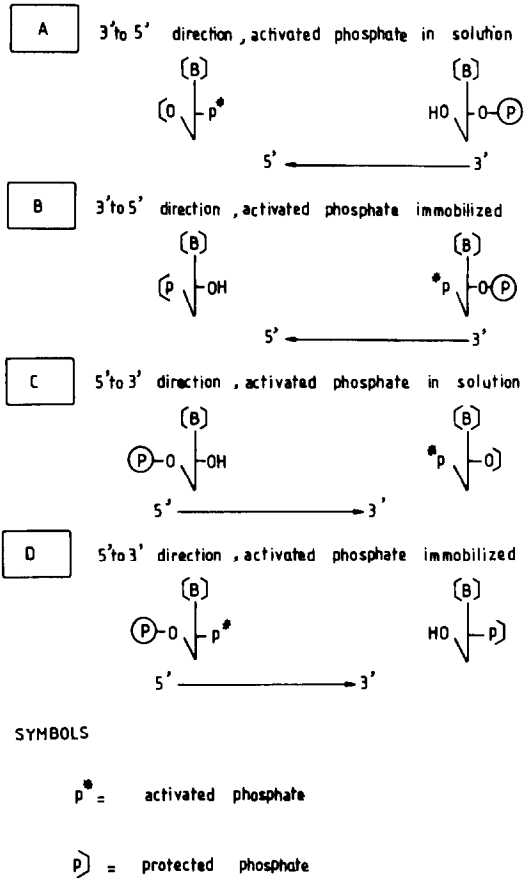
As compared to options A or D, methods B and C have the drawback to use a less reactive secondary alcohol function as a nucleophile for coupling. A strategy of type C was initially used with the phosphodiester method developed by Khorana (*53–56*). The 3′-OH protecting group was simply acetyl. Approach C is also feasible when using the phosphoramidite method (*456*).

Synthesis in Solution

Recent examples are rare. The key of the synthesis is here block coupling. Besides the obsolete phosphodiester method, the phosphotriester method was practiced up to now. The two main strategies are featured in Scheme 3. Catlin and Cramer's approach is usually preferred (*57–60*).

TYPES OF SOLID SUPPORTS

Four major types of supports are in common use. The nature of the linkage between a nucleoside and the polymeric backbones, as well as the usual amount of the first nucleoside that can be bound per gram of phase, are reported in Scheme 4. The first 5′-protected nucleoside is usually functionalized at the 3′-OH by succinic anhydride, and then coupled with the solid support.



SCHEME 2. Four theoretical possibilities of synthesizing an oligonucleotide chain on a polymeric support.

Styrene–Divinylbenzene Copolymer

This well-known support, used with limited success in the phosphodiester type of synthesis, proved to be very convenient for the phosphotriester strategy (*65*). The 1% crosslinked resin appeared more convenient than the classical 2% crosslinked Merrifield resin (*66*).

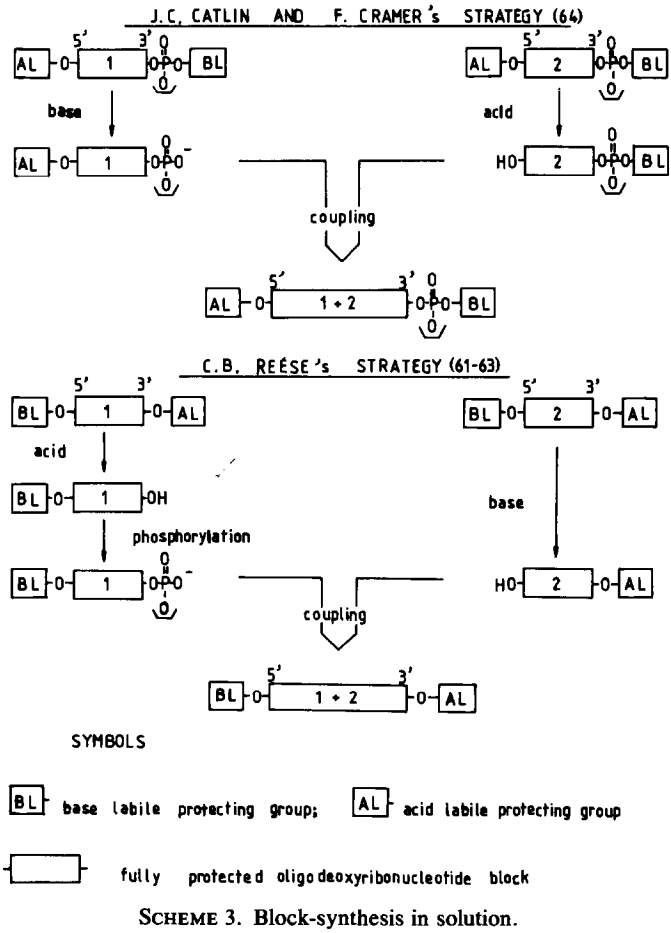
Composite Support Wolfchem SR 107

It is made of macroporous kieselguhr in which the copolymer of *N,N*-dimethylacrylamid, *N,N'*-bisacryloylethylenediamine, and *N*-acryloylsarcosine methyl ester was prepared *in situ* (*67*). This phase is sold by, e.g., Omnifit and can be used in a semiautomated phosphotriester-type synthesis. A copolymer of this nature was first introduced by Gait in the context of Khorana's phosphodiester strategy (*54*) and subsequently optimized. It must be stressed that the only convenient solvent for coupling is in this case pyridine (*68*) and that zinc bromide

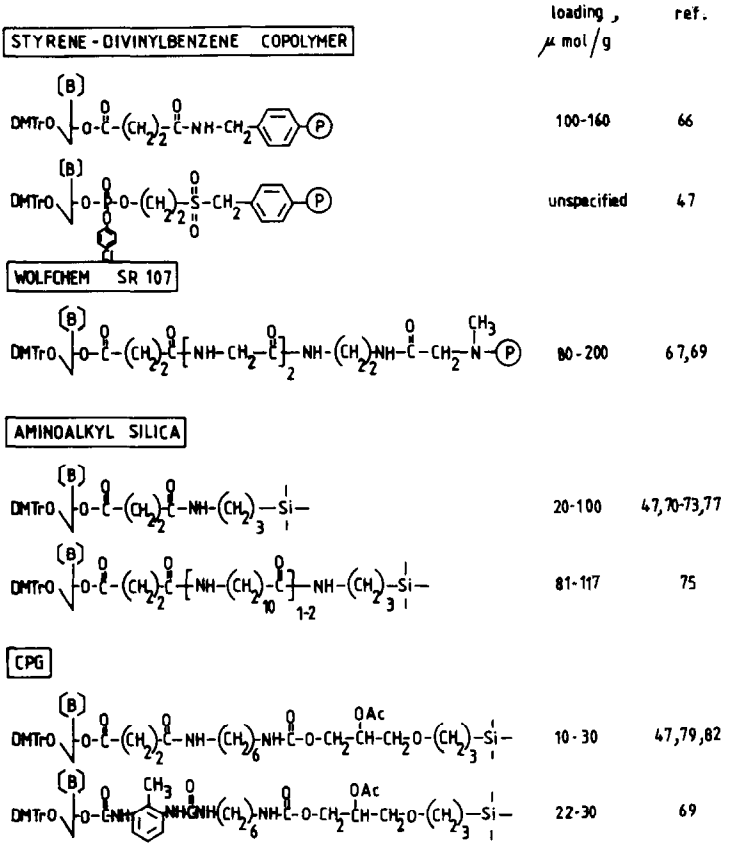
cannot be used for detritylation because it is deactivated by the amide functions of the backbone (*66*). Similarly, dichloroacetic acid is not convenient for detritylation of this resin (*173*).

Aminoalkylsilica

This support gained popularity as soon as the phosphite triester process was applied to solid-phase synthesis (*70–73*). The method of introduction of the amino functions on a HPLC-grade silica was described by Chow *et al.* (*74*) and is of common use (*47, 75–78*). The addition of a long hydrophobic spacer between the amino function and the oligonucleotide graft somewhat enhances the yields of the first coupling in the phosphotriester approach (*75*). The silica functionalized by each of the four monodeoxyribonucleosides is now commercially available (e.g., American Bionuclear, Applied Biosystem).



SCHEME 3. Block-synthesis in solution.



SCHEME 4. Solid-supported synthesis: nature of the linkages between the first nucleoside and the four main polymeric supports used in DNA synthesis, and range of loading.

Corning Controlled Pore Glass (CPG)

CPG, commercialized by Pierce, is probably the most versatile support: it has excellent mechanical and chemical properties and may be used with any solvent and coupling method (*47, 78–83*).

Other Polymeric Supports

Many soluble or insoluble polymeric supports were tested but did not gain, up to now, a large popularity: cellulose (*84, 85*), crosslinked polyacrylmorpholide (Enzacryl Gel K2) (*65, 86–89*), polyethylene glycol (*90*), polystyrene–Teflon (*50, 91–93*) and so on.

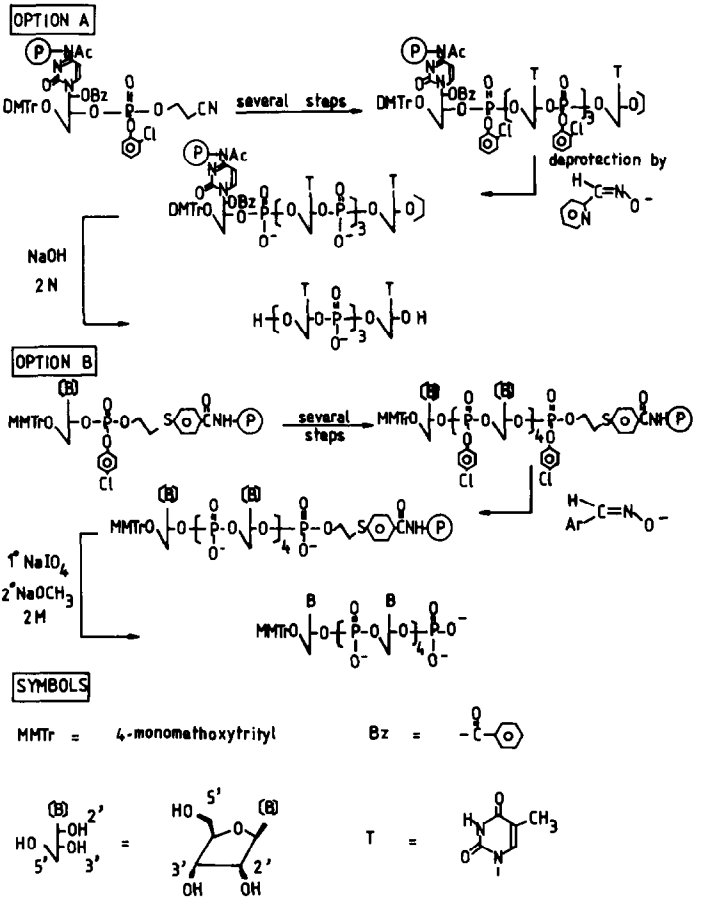
When a large number of different oligomers is required, the "segmented technique" may be useful. In this technique, cellulose disks are used as supports. Syntheses are normally carried out in parallel in four columns. Each addition of a nucleotide is carried out after the disks are sorted into four sets. The disk sets are then placed in the appropriate columns to receive dC, dT, dA, or dG protected monomers, respectively (*94–95, 463*).

Safety-Catch Spacers

It could be interesting to have a link between the polymeric backbone and the oligonucleotide that could survive the drastic conditions necessary to fully deprotect the synthesized oligomer. With this type of link, one could easily remove by simple washings the reagents and cleaved fragments produced by the final deprotection of the sequence. It should also be possible to directly construct, on, e.g., CPG, immobilized hybridization probes of moderate purity. Chemically resistant spacers, that are nevertheless cleavable in very specific conditions, have thus been proposed. Examples are illustrated in Scheme 5.

In option A (*78*), use is made of a 3′-5′ ribo-deoxyribonucleotide linkage that is cleaved in basic medium by anchimeric assistance of the 2′-OH of the ribose. Premature cleavage is avoided by a proper choice of the 2′-OH protecting group.

In option B (*96, 97*), the key function is a 2-phosphothioethyl group that is cleaved by β-elimination after oxidation of the thioether to the sulfone or the sulfoxide. When the chain is simply oligo-dT, *N*-chlorosuccinimide may be used



SCHEME 5. Examples of synthesis on a polymeric support using safety-catch spacers.

as an oxidizing agent (*90, 98*), but it is not recommended for other sequences (*96*). In this instance, $NaIO_4$/dioxane/water is preferred.

PROTECTION OF THE NUCLEIC BASES

The various steps of the construction of a DNA sequence are now presented in a chronological fashion, as they appear in the lab. One starts with the four simple nucleosides. The aglycone residues are first protected; then, one of the sugar alcohol functions. The protected nucleosides are eventually transformed into nucleotide building blocks. The coupling and other repetitive steps are then discussed, followed by the final deprotection, purifications, and control of the synthesized oligomers.

Thymidine

The thymine residue is usually not protected in DNA synthesis. However, thymidine features an acidic N—H bond (pK_a = 9.79) (*99*), so that a certain amount of the very nucleophilic anion may be present in basic medium. Accordingly, thymidine reacts with the water-soluble carbodiimide at pH 8–8.5 in water (*100, 101*) and with dicyclohexylcarbodiimide in anhydrous pyridine (*102*), to give compounds of type **a** (Scheme 6). With basic catalysts (pyridine, *N*-methylimidazole), the thymine residue also reacts to some extent with phosphorylating (*103–107*) and coupling reagents (*108*). It can also be phosphitylated (*109*) and *O*-silylated (*110*). The free base itself, thymine, can be 1,3-dibenzoylated by benzoyl chloride in acetonitrile/pyridine (*111*). Some of the characterized side products derived from thymidine under conditions of DNA synthesis are shown in Scheme 6. In a typical synthesis, the actual amount of side products may be small, because the reaction times are short. Moreover, most of the side products may revert to thymidine at the deprotection step (e.g., oximate treatment) (*104, 108*).

Several protecting groups have been devised for the more sensitive uridine (*112–116*), and some of them were also proposed for thymidine (*112, 113, 117*). The protection strategies are summarized in Scheme 6. Final deprotection can be effected by oximate treatment for the phenyl group, or by β-elimination with 1,5-diazabicyclo-[5,4,0]undecene-5 (DBU) for the *p*-nitrophenylethyl protection.

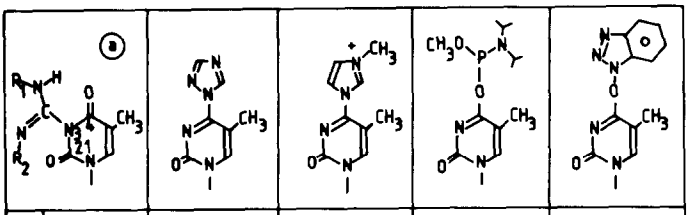
Deoxycytidine

Among the four nucleosides, deoxycytidine is the most basic (pK_a = 4.25) (*99*) and the most nucleophilic. For example, the cytosine residue of 5′-t-butyldimethylsilyldeoxycytidine is selectively acylated by benzoic anhydride in pyridine at room temperature, whereas 5′-t-butyldimethylsilyldeoxy-adenosine and -guanosine are 3′-*O* acylated under the same conditions (*118*). It was thus soon recognized that *N*-protection is unavoidable (*119, 120*).

Nature of the protecting groups. Numerous protecting groups have been proposed for deoxycytidine. Unusual ones are simply illustrated in Scheme 7.

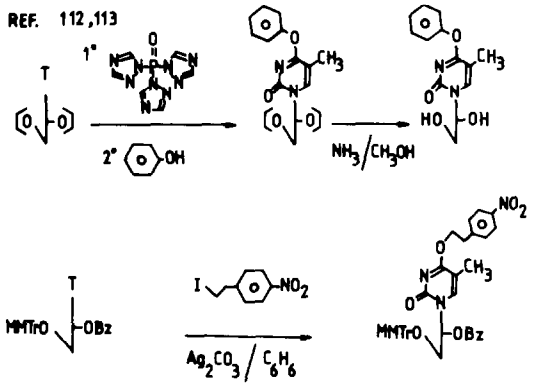
When, as usual, a treatment with concentrated ammonia is the final deprotec-

MODIFICATIONS OF THE THYMINE RESIDUE



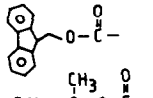
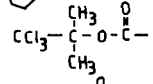
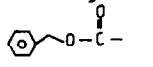
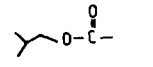
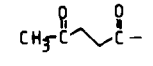
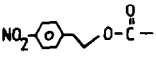
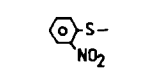
REF.	100	104, 107	106	109	103

PROTECTION OF THYMIDINE



REF. 117

SCHEME 6. Identified side products derived from thymidine under conditions of DNA synthesis, and proposed strategies of protection.

ref.	NH_2 protecting group	method of introduction (see scheme 8)	mode of cleavage
126		B	β - elimination
137		A	reductive elimination
135		A	hydrogenolysis
125		A	conc. NH_3
138		D	NH_2-NH_2
117		D	β - elimination
139		B	thiolysis

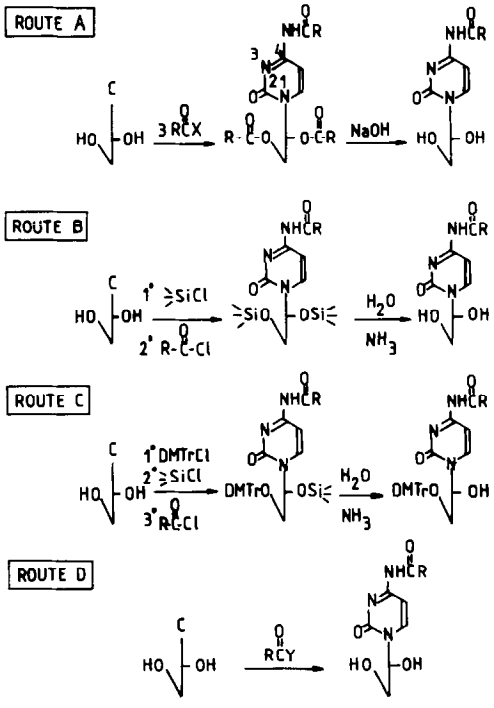
SCHEME 7. Protecting groups proposed for the cytosine residue, other than simply benzoyl or 4-methoxybenzoyl (discussed in the text).

tion step, a simple acyl protecting group is convenient. The acylation takes place at the exocyclic amino function (*121, 122*). The acetyl group is not recommended, being cleaved in weakly acidic medium (*123*) or even in hot ethanol (*121*). Benzoyl or 4-methoxybenzoyl groups are preferred, although removal of the benzoyl group by methanol (*124*) or hydrazine in pyridine–acetic acid (*125*) (a levulinyl-deprotection targeted reagent) was sometimes observed. The half-lives of acetyl, benzoyl, and 4-methoxybenzoyl protecting groups in 9 N ammonia are 1.5, 16, and 64 min, respectively (*123*). The *N*-benzoyl protecting group of deoxycytidine is completely cleaved in 6.5 h by a 1 : 1 (v/v) mixture of aqueous ammonia (5 M) and dioxane at room temperature (*126*). The 4-methoxybenzoyl group, being less susceptible to nucleophilic attack, was preferred in the diester approach where repeated chromatography in buffered aqueous media was necessary (*127*). The benzoyl group is now routinely used in modern synthetic approaches.

Methods of N-acylation of deoxycytidine. These methods are of four types, summarized in Scheme 8. The peracylation of the nucleoside, followed by selective saponification of the 3′ and 5′ ester functions (route A), was worked out by Khorana *et al.* (*120*). The secondary amide function is only slowly hydrolyzed in the strongly basic medium used, probably because it is in the anionic form.

Jones *et al.* (*128, 129*) optimized two methods based on transient silylation of the alcoholic functions of the sugar (routes B and C).

The most popular approach is to selectively *N*-acylate the nucleoside by a smooth acylating agent (route D). Many such reagents have been used in this



SCHEME 8. Strategies to synthesize *N*-4-protected deoxycytidine.

instance: activated esters (4-nitrophenylbenzoate or acetate (*130*), 2-(chloromethyl)-4-nitrophenylbenzoate (*131*), pentafluorophenyl benzoate (*132*), pentachlorophenylbenzoate (*46*)), acid anhydrides in an alcohol (*133, 134*), thioacetic acid (*121*), mixed anhydrides (*136*).

Deoxyadenosine

The protection of deoxyadenosine requires a special comment, because the chemical stability of the nucleoside is profoundly altered by the protection of the exocyclic amino function of the nucleic base. The discussion thus starts with an account of the encountered problems.

The depurination problem. Purine deoxyribonucleosides, nucleotides, and oligonucleotides can loose their purine residue in either strongly basic or moderately acidic conditions, leaving an unsubstituted sugar. This reaction is a nuisance because the growing chain is repeatedly submitted to acid de-dimethoxytritylation during the synthesis on a solid support.

The mechanism of acid depurination involves a rapid preequilibrium of protonation or diprotonation of the purine residue, followed by the rupture of the glycosidic linkage that is the rate-determining step (*140–145*). The unstable species are the *N*-7 protonated derivatives of deoxyguanosine and deoxyadenosine (Scheme 9).

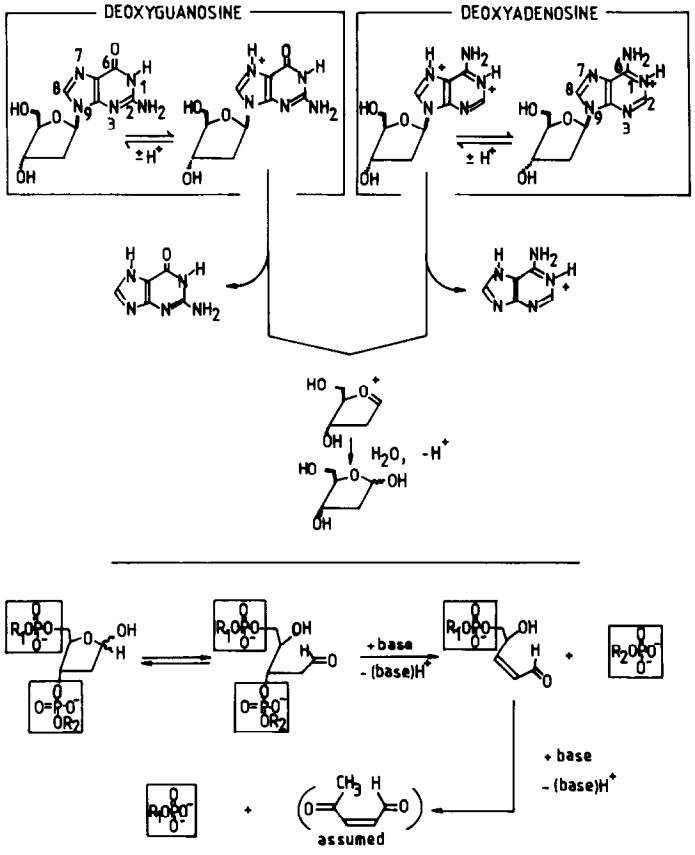
The case of deoxyadenosine is peculiar, because the site of first protonation is *N*-1 (*458*). Only a small concentration of the *N*-7 monoprotonated form may exist at equilibrium. Moreover, the *N*-1, *N*-7 diprotonated species is accessible only at low pH ($pK_{a_1} \cong -1.48$ (*146*), $pK_{a_2} \cong 3.63$ (*99*)). The acidic depurination is thus limited by the rather difficult access to a *N*-7 protonated form of deoxyadenosine. If, however, the adenine residue is *N*-6 acylated, the site of first protonation becomes *N*-7 (*147*). This quickly depurinating form is thus more readily accessible ($pK_a \cong 1.73$ (*148*)). The tendency to depurinate of *N*-6-benzoyl(dA) is even greater than that of *N*-2-i-butyryl(dG) (*149*).

A carbocation is generated at C-1′ in the rate-determining step of acid depurination. As a consequence, electron-withdrawing substituents on the 3′ and 5′ oxygens (like phosphate groups) reduce the tendency to depurinate. Deoxyadenosine in the middle of a sequence thus depurinates less easily than the nucleotide (*149*). That is why people usually avoid to start a sequence with *N*-benzoyl(dA) directly attached to a solid support with a 3′-succinate link (*88*).

When a partially depurinated oligomer is submitted to the strongly basic conditions of final deprotection, the chain is cleaved at the sites of depurination by double β-elimination (Scheme 9), generating truncated sequences (*150, 151*).

Smooth conditions of de-dimethoxytritylation were devised to allow the use of simple *N*-6-monoacylated (dA). However, this approach is still not fully satisfactory for sequences rich in dA.

In spite of the worsening of the depurination problem and the relatively weak reactivity of the exocyclic NH_2, it is still wise to protect this function in dA because it is susceptible to be attacked by trityl chloride (*148*) and phosphorylating agents (*120, 152, 153*). It seems, however, that the coupling itself, in the



SCHEME 9. Acidic depurination mechanism of purine nucleosides, and cleavage of the depurinated sequence in basic medium.

triester approach, could be realized without protection of the exocyclic amino function (*154, 155*). Also, phosphitylation at low temperature in Letsinger's approach can be performed without *N*-protection (vide infra).

N-6-Monoacylated derivatives. These derivatives are illustrated in Scheme 10. When deoxyadenosine is reacted with benzoyl chloride in neat pyridine, the *N*-6,*N*-6,*O*-3′,*O*-5′-tetrabenzoylated derivative is obtained (*120*). The fact that both *N*-actylations occur on *N*-6 was proved by Reese (*157*), Anzai *et al.* (*158*). The second *N*-acylation is very rapid in pyridine, probably because a certain amount of the ionized secondary amide function (—$N^{\ominus}$ COPhe) is present ($pK_a \cong 10.02$ (*148*)). The same type of peracylated products are formed with 4-methoxybenzoyl chloride (*120*), phenyloxycarbonyl (*159*), and *p*-nitrophenylethyloxycarbonyl chlorides (*117*). The *N*-monoacylated derivatives are obtained by selective *N*,*O*-deprotection in basic medium. This procedure is sometimes unpracticable because of considerable base-induced depurination (*120*). These compounds are also synthesized by use of the transient protection of the 3′- and 5′-OHs with trimethylsilyl chloride (*128, 160, 161*).

For the sake of simplicity, the *N*-6-benzoyl group is routinely used to protect dA, although it is not so satisfactory because it induces depurination. Some depurination of *N*-benzoyl(dA) was observed not only under acidic treatment, but also under the triester coupling conditions (*162*). In some instances, premature *N*-debenzoylation by methanolysis occurs (*124*).

N-6-Diacylated derivatives. Hata *et al.* proposed the phtaloyl protecting group. Deoxyadenosine protected in this way depurinates less easily than the *N*-6-monobenzoylated derivative (*75, 165*). The rationale is probably that electron-withdrawing substituents on the six-membered ring lower the pK_a of the nucleoside (*166*), rendering the quickly depurinating *N*-7 protonated form less accessible. Accordingly, the *N*-1-oxide of *N*-6-benoyl(dA) depurinates far less easily than *N*-6-benzoyl(dA) itself (*168*). The phtaloyl group was successfully used in DNA synthesis (*80, 81*) but Hata now prefers the succinylated derivative of dA (*169*).

Takaku *et al.* simply use *N*-6,*N*-6-dibenzoylated dA in order to circumvent the facile cleavage by nucleophiles of the imide ring, a drawback of the phtaloyl protection (*161*).

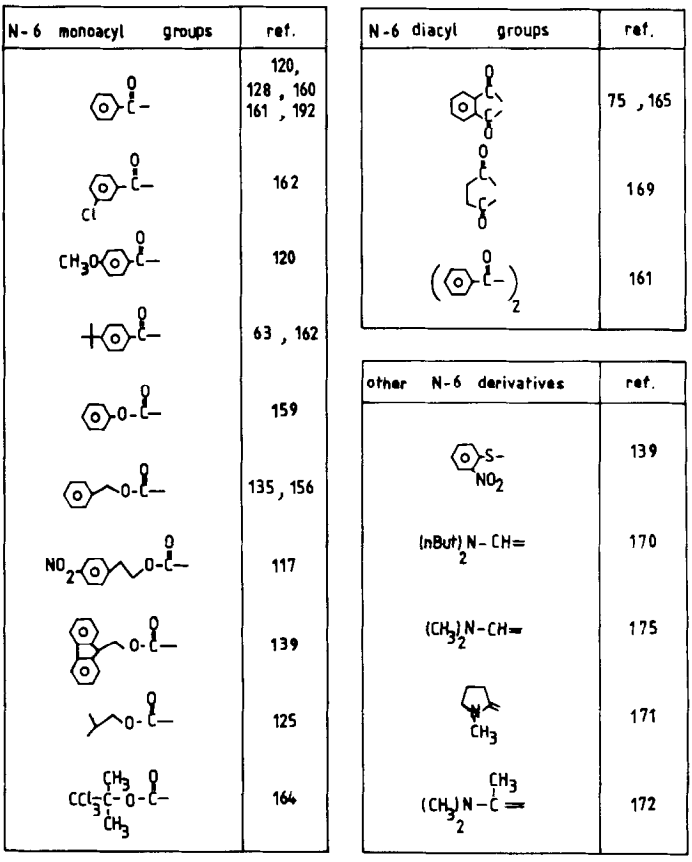
Other N-6 protections of deoxyadenosine. The tendency is now to protect the exocyclic amino function of dA by other methods than acylation, in order to avoid the *N*-1 to *N*-7 shift of protonation site due to protection. For the moment, the sterically crowded amidine protections (Scheme 10) are supposed to be ideal (*173*). Presumably, the first protonation of these compounds do not occur at *N*-7 (*170, 171*).

Deoxyguanosine

The exocyclic amino function of the guanine residue is neither very basic (the predominant site of protonation of the nucleoside is *N*-7, with $pK_a = 1.6$) (*99*), nor very nucleophilic. For example, guanosine and deoxyguanosine can be poly-*O*-acetylated by acetic anhydride without *N*-acylation (*120, 176, 177*). Accordingly, *N*-protection during the coupling step of the triester method is perhaps not so important (*154*). Direct alkylation of the unprotected deoxynucleoside by 4,4′-dimethoxytrityl chloride gives however a 5′-*O*,*N*-dialkylated product (*120, 128, 185*). The *N*-protection is thus necessary, at least at this stage of the synthesis.

The major site of unwanted reactions of the guanine residue is the enolizable lactame function of the six-membered ring. Indeed, the facile ionization of the hydrogen attached to *N*-1 ($pK_a = 9.33$) (*99*) gives a strongly nucleophilic amide anion. The oxygen on C-6 is thus sulfonylated, phosphorylated, phosphitylated, silylated, and even sometimes acylated in basic media (Scheme 11). Deoxyguanosine is also modified by carbodiimides at pH 8–8.5 (*100, 101*). In addition, the *N*-2-i-butyrylguanine residue of a growing chain may couple with an activated nucleotide, leading eventually to the formation of a furcated sequence (*187*). The reactivity of the guanine residue is thus similar, but far more pronounced, than that of the thymine residue.

N-2 protection. This protection is usually an acylation. The site of acetylation and benzoylation of the guanine residue was demonstrated to be *N*-2 by Reese and Saffhill (*188*). The acetyl group proved however to be too labile for the diester



SCHEME 10. Protecting groups for the adenine residue.

methodology (*189*) and the benzoyl group too difficult to remove at the very end of the synthesis (*190*). That is why Khorana and almost everybody after him chosen the i-butyryl group as a good compromise between the two extremes (*191*). More lipophilic groups were sometimes used to optimize the solubility properties of the protected sequences in the solution synthesis. The reported *N*-protecting groups of the guanine residue are gathered in Scheme 12.

The effect of the *N*-2-acylation on the acidic depurination rate of deoxyguanosine has not been studied in details, the problem being overshadowed by the faster depurination of protected dA (*41, 113, 149*).

Two methods are used to introduce an acyl group on dG: *O,N*-peracylation followed by selective saponification (*190, 192*) or transient protection of the oxygen atoms by trimethylsilyl chloride (*128*).

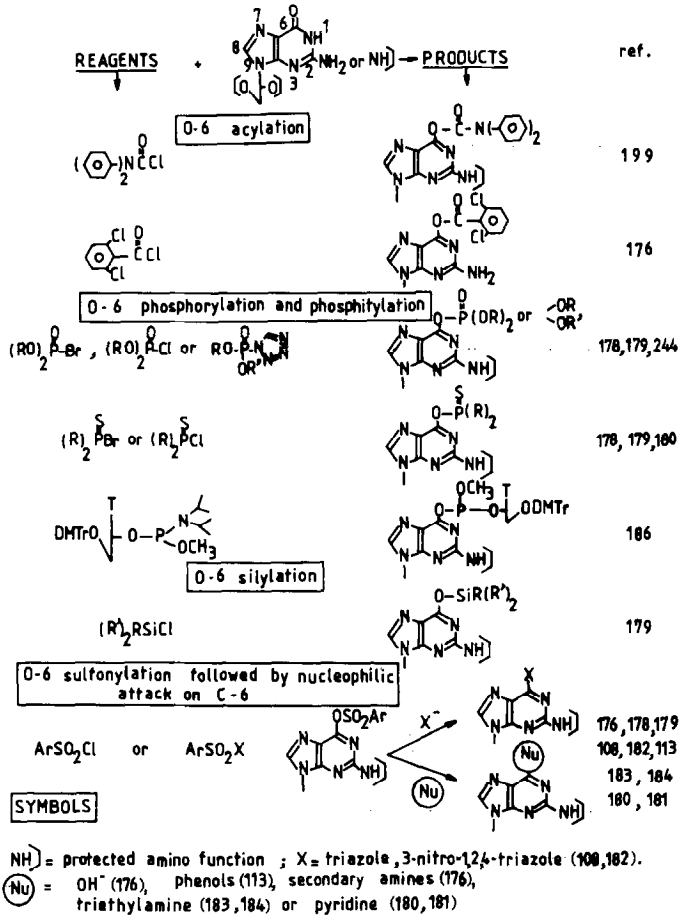
O-6 protection. The various proposed protecting groups are illustrated in Scheme 13. The best documented approach is that using the *p*-nitrophenylethyl group (*184, 195, 196, 117, 197, 198*). Two procedures exist to introduce it: sulfonylation of *O*-6, followed by triethylamine catalyzed displacement with *p*-nitrophenylethanol, or Mitsunobu's alkylation (*194*).

The *p*-nitrophenylethyl group is removed at the end of the synthesis by β-elimination using DBU in pyridine.

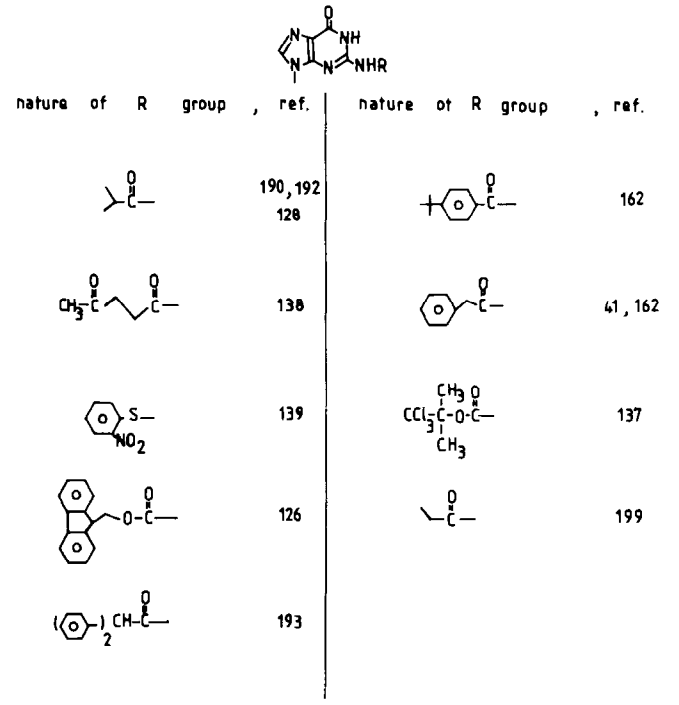
PROTECTION OF THE DEOXY-RIBOSE

Dimethoxytrityl and Pixyl Groups

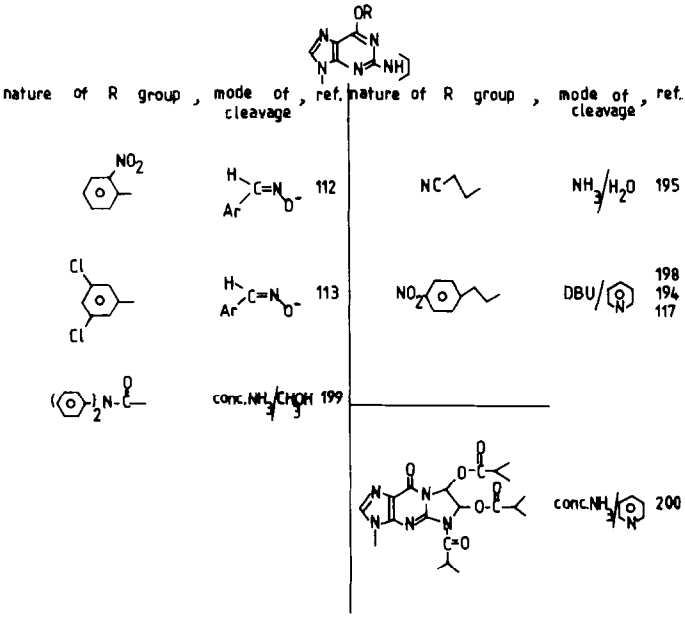
Properties. These acid-labile 5′-*O* protections are keystones of DNA synthesis, specially in the solid-phase method. They present major advantages:
—The 4,4′-dimethoxytrityl and pixyl chlorides are very selective alkylating agents for the primary 5′-OH.
—The protected building blocks are lipophilic, rendering solvent extraction and chromatography easier, even at the very end of the synthesis.
—The protected building blocks are specifically stained on TLC (with a spray of trichloroacetic acid/chloroform 10 : 90, v/v).



SCHEME 11. Reactions at *O*-6 for the guanine residue.



SCHEME 12. *N*-2 protecting groups for the guanine residue.



SCHEME 13. *O*-6 protecting groups for the guanine residue.

—The rate of acid deprotection is high and the deprotection is quantitative.
—The yield of deprotection can be monitored at each step of a synthesis on a polymeric support by spectrophotometry of the waste (*46, 120, 202*).
—An acid-labile group is probably the least poor choice in a solid supported synthesis, when frequently repeated deprotections are needed. Basic media and nucleophiles would be aggressive towards the succinate link with the support, the triester and the *N*-acyl functions of the growing sequence.

The 4,4'-dimethoxytrityl is the most frequently used group, probably because of the larger commercial availability of the corresponding chloride. The final reverse-phase HPLC of the 5'-end dimethoxytritylated oligomer is also easier than for the pixylated sequence (vide infra). The pixyl group has about the same acid-sensitivity as the dimethoxytrityl one, but provides more easily crystallized building blocks (*162, 203*).

The presence of the two *p*-methoxy substituents results from a trial and error optimization: roughly, each *p*-methoxy substituent enhances the rate of acid cleavage by a factor of 10 (*204*). The trityl group itself is far too stable (*120*) and the tri-*p*-methoxy too labile (*205*). Pyridine (1%) is usually added when DMTr-protected building blocks are chromatographed on silica (*206*). The chloroform used as a solvent or eluant must be devoid of hydrochloric acid (e.g., by passing over basic chromatographic alumina) (*202*). The references corresponding to the description of the synthesis of the major base and 5'-*O* protected nucleosides are given in Table 1.

Recurrent deprotection of DMTr or Px. The conditions of acid cleavage of the DMTr group, when performing a synthesis on a solid support, were optimized in order to be rapid, complete and devoid of depurination. The oldest method (i.e., acetic acid/water, 80 : 20, v/v) is restricted to the ultimate end-detritylation of the otherwise fully deprotected oligomer. At this stage, indeed, unprotected dA resi-

TABLE 1

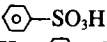
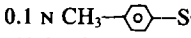
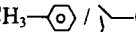
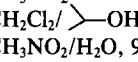
MAIN NUCLEOSIDE BUILDING BLOCKS AND REFERENCES TO PAPERS WHERE THEIR SYNTHESIS IS DESCRIBED

Protected nucleosides	References
5'-*O*(DMTr)dT	*45, 120, 201*
5'-*O*(DMTr)dC^{bz}	*46, 120, 128, 201*
5'-*O*(DMTr)dA^{bz}	*46, 120, 128, 201*
5'-*O*(DMTr)dA^{pht}	*165*
5'-*O*(DMTr)dA^{dibz}	*161*
5'-*O*(Px)dA^{dbam}	*194*
5'-*O*(DMTr)dG^{ib}	*46, 201*
5'-*O*(DMTr)dG^{ib,pnpe}	*181, 194, 198*

Note. bz = benzoyl; pht = phtaloyl; dibz = dibenzoyl; dbam = dibutylaminomethylene; ib = *N*-2-isobutyryl; pnpe = *O*-6-*p*-nitrophenylethyl; DMTr = 4,4'-dimethoxytrityl; Px = pixyl.

TABLE 2

REAGENTS FOR THE RECURRENT CLEAVAGE OF THE 4,4'-(Dimethoxy)-trityl Group

Reagents	Solvent or mixture of solvents (v/v)	References
	(a) Protic acids	
2% 	$CHCl_3/CH_3OH$, 7:3	*201, 207*
0.1 N 	CH_3CN	*70*
2% CF_3COOH		*210*
2% CF_3COOH	$CHCl_3/CH_3OH$, 9:1	*47*
1% CF_3COOH	$CHCl_3$	*165*
1% CF_3COOH	CH_2Cl_2	*169*
10% CCl_3COOH	$CHCl_3/CH_3OH$, 7:3 or 98:2	*149, 151*
10% CCl_3COOH	$CHCl_3$	*208*
3% CCl_3COOH	CH_3NO_2	*161*
5% $CHCl_2COOH$	CH_2Cl_2	*211*
3% $CHCl_2COOH$	CH_2Cl_2	*79, 209, 173*
	(b) Zinc bromide	
$ZnBr_2$, saturated	CH_3NO_2	*71*
$ZnBr_2$, 1 M	CH_2Cl_2/  , 85:15	*66, 214*
$ZnBr_2$, saturated	CH_3NO_2/H_2O, 99:1	*74, 76, 215, 216*
	(c) Other reagents	
$(C_2H_5)_2AlCl$	CH_2Cl_2	*218*
	CH_2Cl_2	*218*

dues in the sequence are far less sensitive to acids than *N*-6-benzoyl(dA) and moreover, it is easy to get rid of the excess acetic acid.

A strong organic acid under anhydrous conditions is frequently used for recurrent deprotection (as shown in Table 2). The duration of the acid treatment must be strictly controlled. The benzenesulfonic and related acids were soon abandoned because of extensive depurination of *N*-6-benzoyl(dA) (*151, 344*). It was also recognized that a strong carboxylic acid in a solvent mixture containing an alcohol generates traces of water due to some *in situ* esterification of the acid (*208*). Alcohols are thus to be avoided, in order to ensure reproducible conditions. A weak concentration of dichloroacetic acid in an aprotic solvent is the reagent of choice for detritylation because its action is rapid (~2 min) and depurination of *N*-6-benzoyl(dA) is kept to a minimum (*173, 79, 209*). These conditions however do not work well when using the WOLFCHEM SR107 resin, owing to the basic nature of this support. Treatment with 10% trichloroacetic acid is then necessary (*173*).

The most selective reagent for cleavage of the 5'-*O*-DMTr group without depurination is zinc bromide in a aprotic solvent, either in homogeneous (≤0.1 M in

nitromethane) (*149, 161, 212*) or heterogeneous conditions (dichloromethane) (*213*). A major drawback is that this reaction is too slow for a solid supported synthesis (*209*). The rate of deprotection and the solubility of zinc bromide are enhanced by the addition of various alcohols (isopropanol (*1, 66, 214*), methanol (*202*)) or even water (*76, 215, 216*). In this case however, zinc bromide can react with these solvents and generate oxacids of type $H_2[Zn(OR)_2Br_2]$ (*217*). As a consequence, the deprotection is perhaps more rapid (*205*), but also less selective (*212, 214*). The WOLFCHEM SR107 resin is incompatible too with zinc bromide.

The use of zinc bromide tends to disappear in the case of solid supported synthesis.

Other 5'-O and 3'-O Protecting Groups

They are illustrated in Scheme 14. Their use is only occasional. Some of them were devised for specific purposes: **1** serves as an anchor for two sequences, allowing purification at each step by gel permeation chromatography; lipophilic groups like **9** enhance the solubility of the building blocks for solution synthesis.

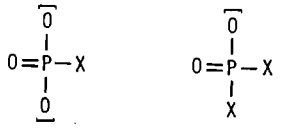
In Reese's strategy of solution synthesis (vide supra), there is a need for two orthogonal 5'-*O* and 3'-*O* protecting groups. Reese and Chattopadhyaya thus introduced a series of base-labile or safety-catch type protections (**9**, **11**, **12**, **15**) that can be used in conjunction with acid-labile groups.

PHOSPHORYLATION AND PHOSPHITYLATION

In common practice, the four 5'-*O* and base-protected nucleosides are phosphorylated or phosphitylated on the 3'-OH function, to generate the nucleotides in a form that can be purified, stored and/or commercialized. The *persistent phosphate protecting group* (i.e., the one remaining until the synthesis is completed) is introduced at this stage. This transformation of a nucleoside to a nucleotide is a preliminary preparation, rarely included in the repetitive steps performed on a solid support (*241*).

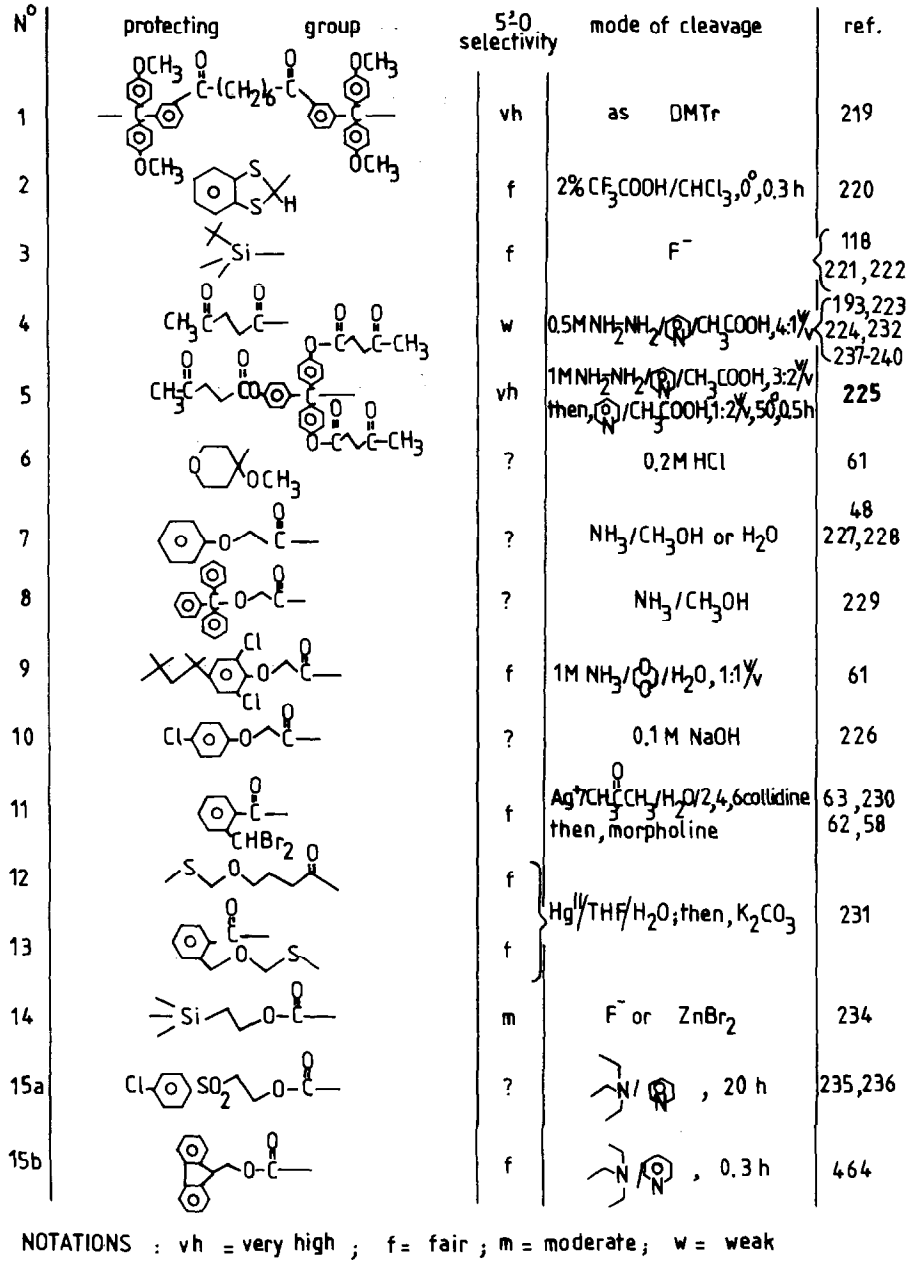
Phosphorylation

The phosphorylating agents may be monofunctional (**b**) or bifunctional (**c**):



(b) (c)

Compounds of type (**b**) carrying a persistent and a transient protecting group are discussed first.



SCHEME 14. 5'-*O* and 3'-*O* deoxyribose protecting groups used in DNA synthesis (other than DMTr and Px, discussed in the text).

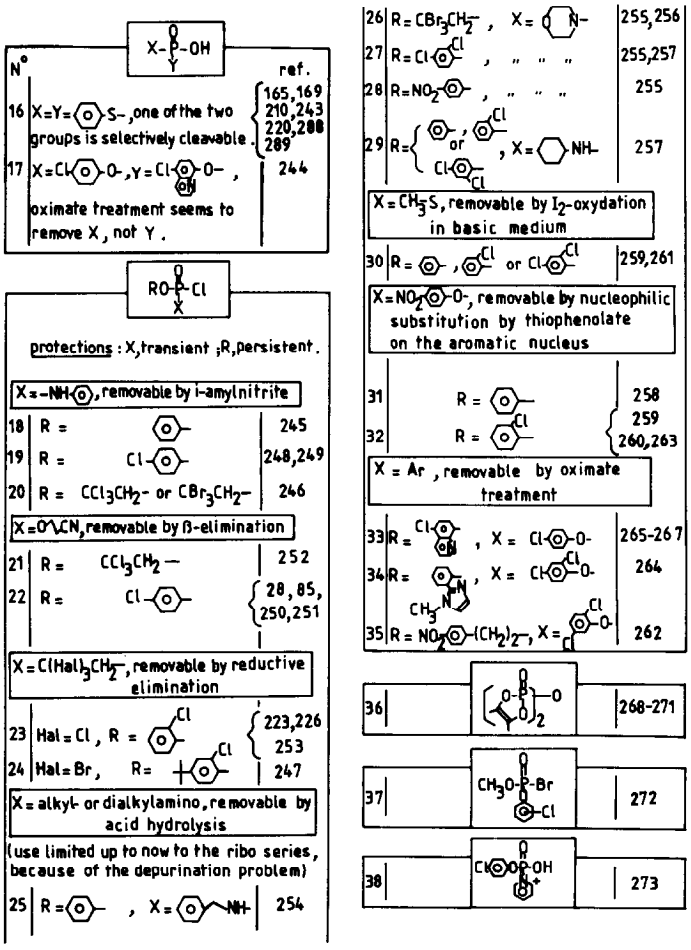
Monofunctional phosphorylating agents carrying a persistent and a transient protecting group. The successive steps of the synthesis of a sequence using this approach are:

—the phosphorylation, followed by an exhaustive purification of the fully protected nucleotide;

—the cleavage of the transient protecting group, followed by a minimal purification of the diester salt;
—the coupling of this building block with the growing sequence.

Phosphorylation itself may occasionally be a simple coupling with a diester ((**b**), X = OH) but, in general, a phosphochloridate ((**b**), X = Cl) or bromidate ((**b**), X = Br) is used. These reagents (**16–38**) are illustrated in Scheme 15. It must be recognized that, after Narang's optimization of bifunctional phosphorylating agents (vide infra), the approach described here tends to loose popularity. The main reason is probably that the necessary phosphorylating agents have to be synthesized in an extra step. The phosphochloridates **22**, **23**, and **33** are among the most frequently advocated.

The phosphorylation was formerly performed by action of the phosphochloridate alone on the nucleoside in pyridine, but this reaction is not clean (*250*) and



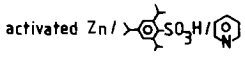
SCHEME 15. Monofunctional phosphorylating agents carrying a transient and a persistent protecting group.

the aglycone residue (*N*-4-acylcytosine, *N*-2-acylguanine) can be modified (*223, 249*). A mixture of the phosphochloridate and *N*-methylimidazole in acetonitrile, THF or dioxane gives better results (*85, 223, 265*). However, *N*-2-acyldeoxyguanosine is still phosphorylated on the aglycone residue under these conditions (*250*). *N*-Methylimidazole is not a strong base enough to prevent a partial loss of the dimethoxytrityl group (*266*). Finely ground molecular sieve (4 Å) is to be used as an acid-scavenger (*274*). Alternatively, the phosphochloridate can be transformed into a tetrazolide before the reaction with the nucleoside (*266*). The alcohol function of the nucleosides can also be activated by transformation to an alcoholate-type derivative before phosphorylation (*275, 276*).

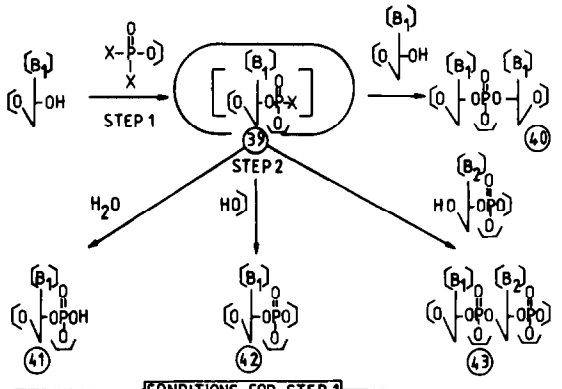
The next step after the phosphorylation is the cleavage of the transient protecting group. The conditions of deprotection are also indicated in Scheme 15. The β-cyanoethyl group (**21**, **22**) is popular because it is very easily removed by β-elimination with weak bases (triethylamine (*277*) or t-butylamine (*278, 279*)). The cleavage of the trihaloethyl group (**23**, **24**) is not so easy and required numerous optimizations as illustrated in Scheme 16. It is not recommended, except for very small oligomers. In some instances (e.g., **35**), an aryloxy group on phosphorus can be selectively removed by oximate (*285*).

Bifunctional phosphorylating agents carrying only the persistent protecting group. In this case, the primary product of phosphorylation **39** (Scheme 17) is not isolated as such, but rather reacted with either water, a nucleophile (e.g., $HO(CH_2)_2CN$) or a suitably protected nucleotide, as shown in the scheme. The main side-reaction to be avoided is of course the formation of the 3′-3′ symmetrical dimer (**40**).

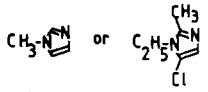
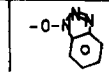
Monoesters of phosphoric acid were formerly condensed with the nucleoside using dicyclohexylcarbodiimide (*39, 152*) or an arylsulfonyl chloride (*61, 290, 291*) (method A). Arylsulfonyl chlorides alone as coupling agents were however aban-

REAGENT	ref.
activated Zn / 	223,280
Zn / $CH_3\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}CH_3$	281
Co(I) phtalocyanine	191
Na /  / HMPT (also cleaves the MMTr group)	228,48
Cu / Zn / $CH_3\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}CH_3$ / DMF	282
But_3P / DMF / Et_3N	211,283
electrochemical removal	284

SCHEME 16. Methods to cleave the trihaloethyl protecting groups.



CONDITIONS FOR STEP 1

method	nature of X	solvent	co-reagent
A	OH	pyridine	dicyclohexylcarbodiimide, $ArSO_2Cl$, $ArSO_2Cl$/ tetrazole
B	Cl	" " "	none
C	Cl	acetonitrile, dioxane	
D		acetonitrile, dioxane, THF	none
E		dioxane, THF	none

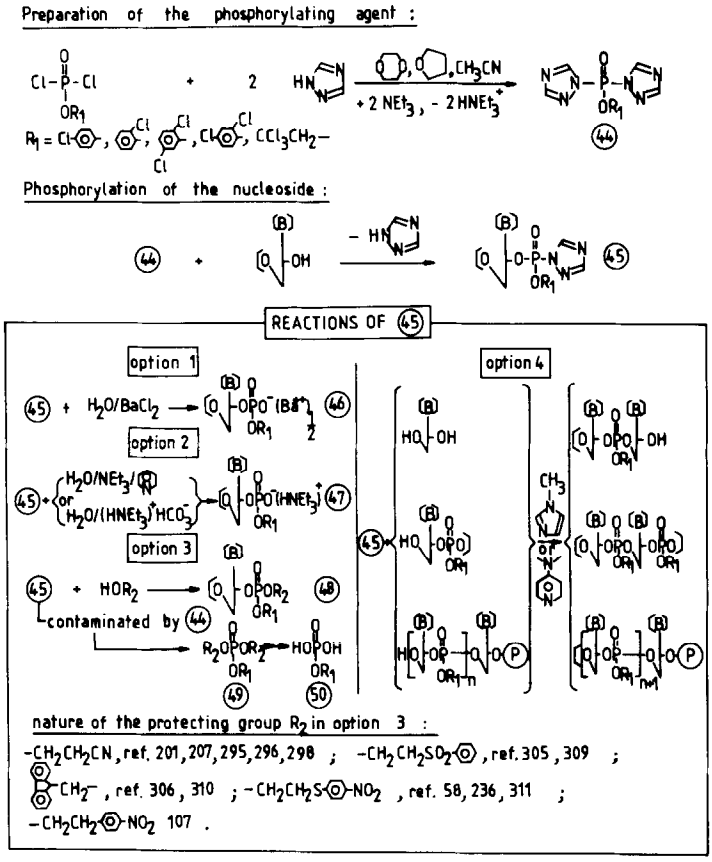
SCHEME 17. Principle of phosphorylation of a nucleoside by use of a bifunctional phosphorylating agent.

doned because the symmetrical dimer (**40**), pyrophosphates and modified *N*-2-acyldeoxyguanosine were side products. In the modern version, tetrazole is added as a coreagent (*292*).

Neat phosphochloridates in pyridine (method B) were also abandoned, because they are too reactive: the nucleoside is very rapidly consumed, but low yields of the correct product are obtained. 1-Methylimidazole as a coreagent (method C) is not satisfactory, but 5-chloro-1-ethyl-2-methylimidazole improved the yields (in the case of 5′-O-MMTr(dT)) (*106*).

A major progress was made when Narang *et al.* introduced method D (*293–296*). The bis-triazolide derivatives **44** (Scheme 18) respect the *N*-acylated aglycone residues when the reaction time is short (*103, 297*). Pyridine is avoided as a solvent (*298*). The bis-triazolide is prepared just before use and cannot be stored. There is no formation of symmetrical dimers **40** in this method (*303*). Details are given in Scheme 18.

Option 3 of Scheme 18 needs some comment (*201, 207, 295, 296*). As an excess of phosphorylating agent **44** is routinely used to drive the phosphorylation to



SCHEME 18. Narang's method of phosphorylation, using the bis-triazolide derivative **44**.

completion, a symmetrical triester **49** is always a side-product. Contamination of **48** by **49** is a nuisance because, after removal of the transient protecting group R_2, the resulting di-salt **50** competes with the nucleotide monosalt for coupling with a growing sequence. Chromatography on silanized silica can remove **49** from 5′-*O*-DMTr fully protected nucleotides **48** (*181*). Options 1 (*187, 299*) and 2 (*62, 63, 94, 95, 162*) circumvent this problem. In these cases, precipitation of the barium salt **46** or extraction of the nucleotide monosalt **47** by chloroform from an aqueous bicarbonate solution ensure the necessary separation from **50**. If needed, a transient protecting group may then be introduced by a classical coupling with an alcohol, to give compounds of type **48** (*300*).

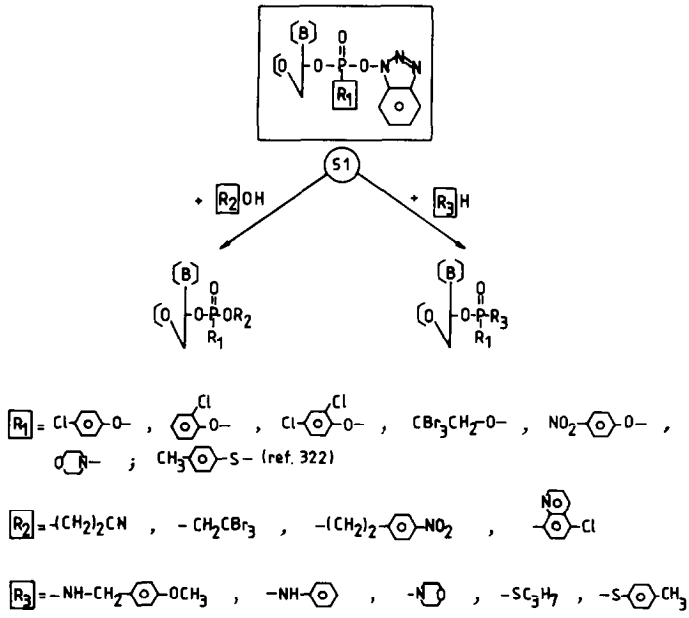
Option 4 of Scheme 18 (*276, 303–307, 298*) represents a rapid synthesis of dinucleotide mono- or diphosphates as well as an interesting way to elongate an immobilized sequence (*308*). Compound **45** indeed selectively reacts with the 5′-OH function of an unprotected deoxyribose residue (*276, 303, 304*). The interfering excess phosphorylating agent **44**, contaminating **45**, is sometimes selectively destroyed by addition of water/pyridine (*298*). The coreagents have to be used

with caution in option 4: 1-methylimidazole and especially 4-dimethylaminopyridine cleave the β-cyanoethyl protection (*298, 273*).

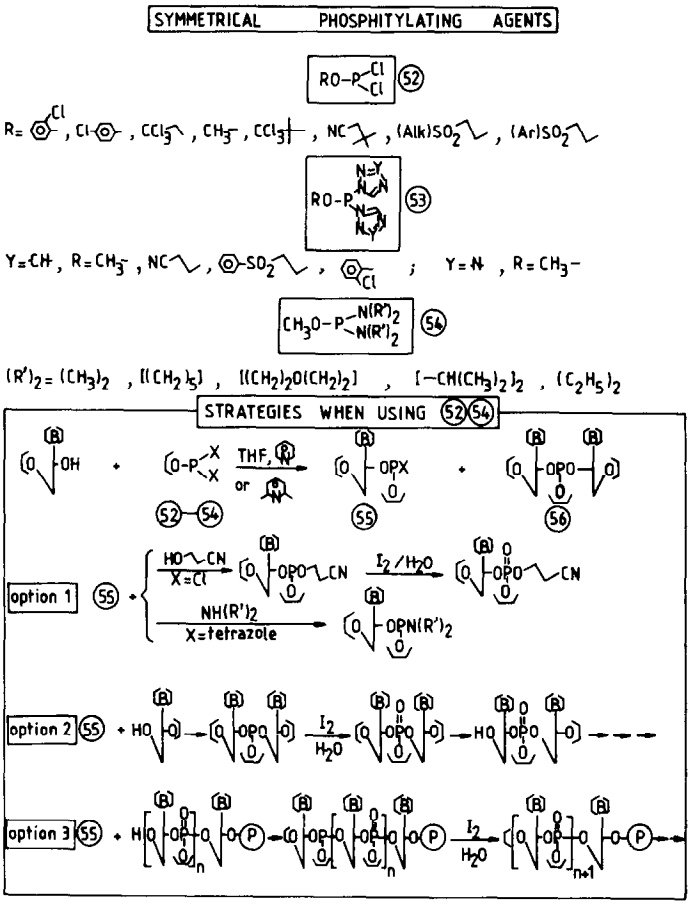
A variant of Narang's method was introduced by Van Boom who used a phosphotriester derivative from 1-hydroxybenzotriazole (method E, Scheme 17) instead of the bis-triazolide **44** (*312–317*). This phosphorylating reagent is more reactive: traces of the symmetrical 3'-3' dimer **40** are formed (*318*) and, if *N*-methylimidazole is present, thymidine as well as *N*-acylated deoxyguanosine are modified (*103*). When an excess of reagent is used in the phosphorylation step, this excess further reacts with the nucleophile introduced at the next stage. The nucleophile is, for example, a nucleoside with a free 5'-OH function: traces of the corresponding 5'-5' symmetrical dimer are thus observed (*319*). Just as in options 3 and 4 of Scheme 18, the intermediate **51**, illustrated in Scheme 19 (*320, 321*), may be used to obtain fully protected nucleotides (as shown in the scheme), dinucleotides (*193*), or to elongate an immoblized sequence (*314, 318*).

Phosphitylation

Letsinger's approach: symmetrical reagents. Phosphodichloridites of type **52** (Scheme 20) were first introduced in oligonucleotide chemistry by Letsinger (*48, 228, 323*) in 1975. These compounds react quickly at −78°C with the free 3'-OH function of a 5'-*O* protected nucleoside, in a mixture of THF and pyridine or 2,6-lutidine. Usually, a solution of the nucleotide is slowly added to a cold solution of an equimolar amount of phosphodichloridite. The quantity of pyridine must be adjusted to solubilize the nucleoside (*228*). It is in principle not necessary to have



SCHEME 19. Van Boom's approach corresponding to option 3 in Scheme 18.



SCHEME 20. Types and uses of symmetrical phosphitylating agents.

the aglycone residues protected, provided the temperature is limited to −78°C during the manipulations (*124, 228, 282*). *N*-Protection is however required for other reasons (solubility (*228*), selective dimethoxytritylation, capability to perform a capping with phenylisocyanate in an automated synthesis).

The main problem when using the phosphodichloridites **52** is the formation of large quantities of the symmetrical 3′—3′ dimer **56** (*283, 324*) as well as the contamination of the crude mixture by the corresponding amount of unreacted phosphodichloridite. This last contaminant strongly interferes with the subsequent steps (*211*) (Scheme 20, options 1–3). To limit these side-reactions, people chose voluminous protections (**52**, R = CCl_3—$C(CH_3)_2$— (*211, 283, 336*) or R = NC—CH_2—$C(CH_3)_2$— (*335*)) or moved to other leaving groups as in compounds **53** (*82, 336, 337*) and **54** (*339, 340, 341, 462*).

The uses of the symmetrical phosphitylating agents **52–54** are described in the second part of Scheme 20. The phosphitylated nucleosides **55** may react with an alcohol (*327*) or an amine (*328, 329*). Triester or phosphoramidite building blocks are so obtained (option 1). Compound **55** (X = Cl) gives, by reaction with a

suitably protected nucleoside, a dinucleotide monophosphate (option 2) (*48, 228*). Crude **55**, resulting from the action of **52** or **54** (activated by 4,5-dichloroimidazole), may also serve to elongate an immobilized sequence (*149, 72, 74, 70, 325*) (option 3). Phosphitylation of a growing sequence by **52**, **53**, or **54** was even included in the repetitive operations of a solid-supported synthesis (*242, 326, 462*).

The nature of the persistent phosphate protecting group R, featured in Scheme 20, was not varied as much as in the phosphotriester approach. The familiar chlorophenyl groups were not recommended, at least when using phosphodichloridites, because of low yields (*48*). The trichloroethyl group of the pioneering work (*228, 232, 323*) was soon superseded by the methyl group. Methyl is cleaved by nucleophilic attack on the carbon with thiolates (*202, 330, 345*), t-butylamine (*202, 324*), or even concentrated ammonia (*331, 332*). A drawback is that methyl esters of derivatives of phosphoric acid are methylating agents. As a consequence, some methylation of the aglycone residues was detected in the chromatographic traces of the synthesized sequences (*334*). There is a continuous search for a better persistent phosphate protecting group, useful for the phosphite method. Aryl- or alkylsulfonylethyl groups, cleaved by β-elimination, were recently proposed (*338*).

Caruther's approach: dissymetrical reagents. The use of phosphodichloridites had several drawbacks: the symmetrical 3′—3′ dimer was a major side product; the reaction had to be performed at low temperature in the strict absence of water and oxygen; the phosphitylated nucleosides could only be kept at low temperature (−20 to −70°C).

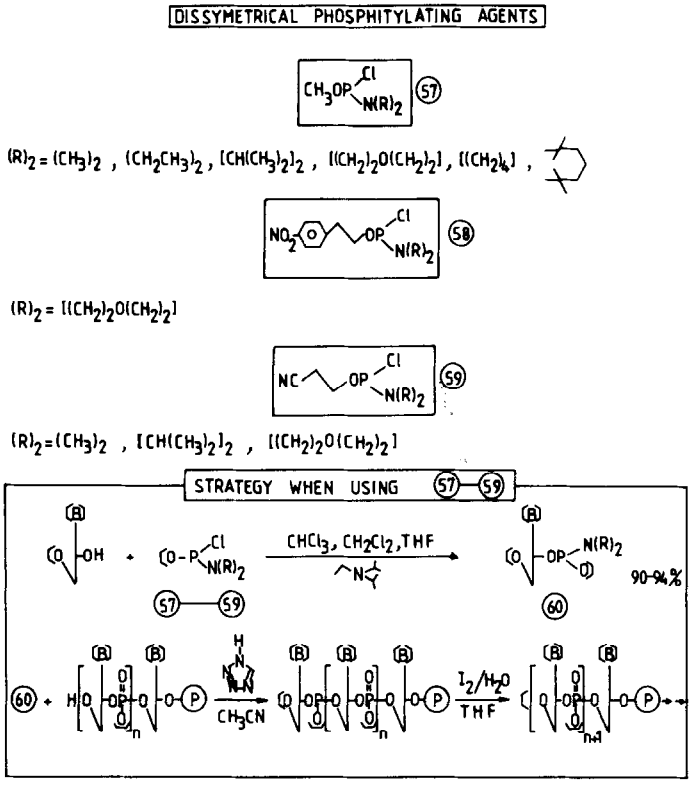
People thus looked for phosphitylating agents leading to more easily manipulatable nucleotide derivatives. Caruthers *et al.* introduced in 1980 (*342*) the dissymetrical phosphitylating agents **57** (Scheme 21), opening the way to *an extraordinary performant method of solid-supported oligo-DNA synthesis*. This one is illustrated in the second part of Scheme 21 (*49, 202, 205, 172, 344, 345, 215, 216, 76*).

Reagents of type **57–59** rapidly react with the free 3′-OH function of a nucleoside (<15 min at room temperature). Only a small amount (~4%) of the symmetrical 3′-3′ dinucleotide is formed. Some other side-products may contaminate the mixture, but they seem not to interfere with the following steps of the synthesis (*202, 343, 346, 459*). The resulting phosphoramidites **60** are white powders more or less stable toward oxygen and atmospheric moisture, depending on the nature of R.

The amino function of **60** is transformed to an excellent leaving group by protonation with a weak acid (typically tetrazole). Under these conditions, the phosphoramidites **60** react very efficiently with the free 5′-OH function of an immobilized sequence (Scheme 21). Coupling yields are almost quantitative.

The persistent protecting group has not much varied: methyl (compound **57**) is by far the most common up to now. A methyl protection is however not ideal because of the undesired methylation of the base residues and the tedious deprotection by thiolates. The β-cyanoethyl derivative **59** is superseding **57** (*349, 456*). The *p*-nitrophenylethyl protection (compound **58**) was also advocated (*347*).

As the dimethylamino derivatives (**57** and **60**, R = CH_3) were too unstable (*76, 346*), the nature of the nitrogen substituents $(R)_2$ required an optimization. The



SCHEME 21. Types and uses of dissymetrical phosphitylating agents.

aim was to get easily manipulatable, but nevertheless sufficiently reactive, phosphoramidites **60**. The most useful compounds proved to be the diisopropylamino and morpholino derivatives (*209, 346, 348*). The latter (**60**, $(R)_2$ = morpholino) were even purified by chromatography on silica and characterized by elemental analysis.

THE COUPLING STEP

In the preceding chapter, numerous references were already made to internucleotidic couplings. In this chapter, the already encountered reactions are placed in their general context and discussed in depth.

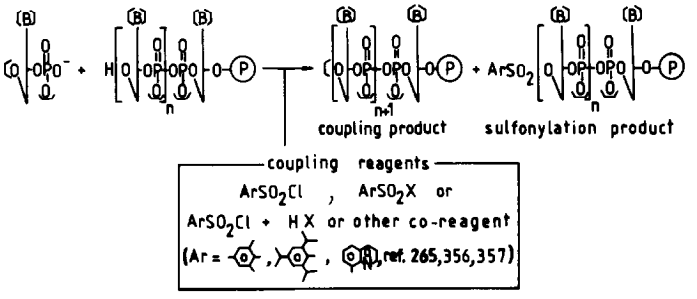
The Classical Phosphotriester Method

Principle. The coupling is illustrated in Scheme 22. An immobilized sequence, having a free 5'-OH function, is reacted with an excess of the triethylammonium (or barium) (*350*) salt of the appropriate nucleotide building block (mono-, di-, trimers). The salt in excess may sometimes be recovered (*350, 83*). *The key point of the reaction is to introduce on the phosphate function of the building block a*

good leaving group, that will be subsequently substituted by the immobilized 5'-OH function. For this purpose, coupling reagents with or without coreagents (e.g., tetrazole, *N*-methylimidazole) have been used.

Dicyclohexylcarbodiimide was not satisfactory as a coupling reagent in the phosphotriester approach, because it gave *N*-phosphorylureas (*351*). Arylsulfonyl chlorides and azolides are more convenient. However, the coupling reaction is then usually in competition with the sulfonylation of the free 5'-OH function (*352*) (Scheme 22). This competition is the major weakness of the method: it is yield-limiting. Sulfonylation-free coupling reagents like **71** and **72** have thus to draw the attention.

Neat arylsulfonyl chlorides (ArSO$_2$Cl) as coupling agents. Phenyl-, tolyl-, 2,4,6-trimethylphenyl-, 2,4,6-triisopropylphenyl- (*356*), and 8-quinoleyl sulfonyl chlorides (*357, 358*) were used in the pioneering work. The solvent was pyridine. Sterically crowded aryl substituents were preferred in order to reduce the undesir-

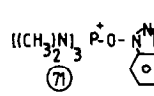


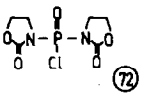
NATURE OF HX OR OTHER CO-REAGENT

N°	nature and date of first use		ref.	coupling solvent	coupling rate	N°	nature and date of first use		ref.	coupling solvent	coupling rate & remarks
61		1973	359	p	s	66		1979	62	p	r
62		1974	360	p	s	67		1975	106 366	an,dic,p,...	vr
63		1977	280	p	s	68		1982	273 366	an,dic,p,...	nr
64		1971	361	an	s	69		1981	363 364	p	stsp
65		1976	362	p	r	70		1985	365	dic, p, ..	vr

p= , an = CH_3CN , dic = CH_2Cl_2 ; s = slow, r = rapid, vr = very rapid ;
nr = not recommended , stsp = stereospecific coupling

SULFONYLATION-FREE COUPLING REAGENTS





SCHEME 22. The coupling step in the classical phosphotriester approach.

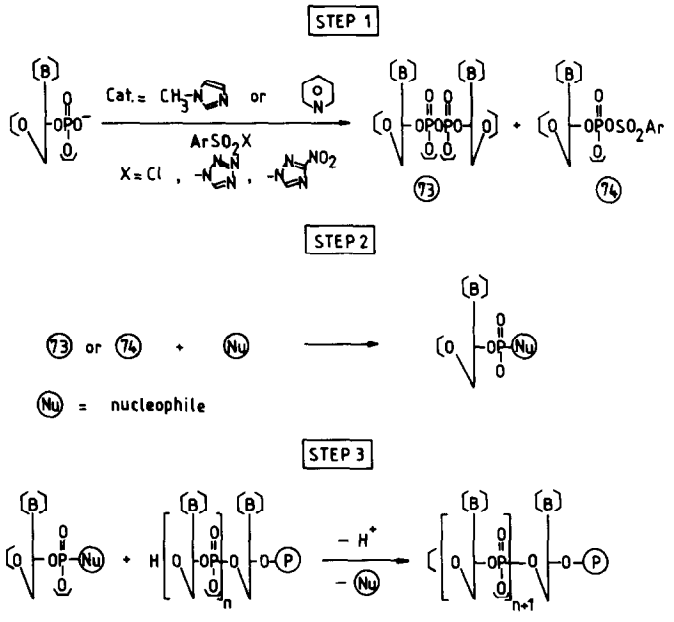
able sulfonylation (*355*). However, the 5′-OH sulfonylation remained a serious problem and people looked for more selective coupling agents.

Arylsulfonyl azolides (ArSO$_2$X) as coupling agents. Keeping large aryl groups, the nature of X in ArSO$_2$X was varied. Imidazolides, triazolides, and 4-nitroimidazolides (Scheme 22, H—X = **61–63**) gave too slow couplings (*370, 355*). The 1,2,3,4-tetrazolides (H—X = **65**) and 3-nitro-1,2,4-triazolides (H—X = **66**), used in pyridine, were, however, very satisfactory. The arylsulfonyltetrazolide must be prepared in a separate vessel just before coupling; the nitrotriazolide, on the contrary, is a stable, crystalline compound.

As will be shown later on, the role of X in the coupling reaction is not trivial: X$^-$ or H—X can be powerful catalysts. That is why an extra amount of HX may be added to ArSO$_2$X in the coupling mixture (*355, 367*).

Use of a mixture of an arylsulfonyl chloride and a coreagent. Very high rates of coupling are reached when the coupling agent is a mixture of an arylsulfonyl chloride (or nitrotriazolide) (*79*) and *N*-methylimidazole (*47, 273, 366*). Apparently, the excess *N*-methylimidazole **67** not only acts as a catalyst, but also as an acid-scavenger, because other solvents than pyridine are allowed (*365*). Deoxyguanosine must be protected on its enolizable lactame function, in order to remain unmodified under these conditions (*365*). Pyridine *N*-oxides are also good catalysts.

Mechanism. The mechanism of coupling of a phosphodiester salt with an alcohol in the presence of an arylsulfonyl chloride (*43*) or azolide was studied by ^{31}P NMR spectroscopy. The most probable reaction path is illustrated in Scheme 23.



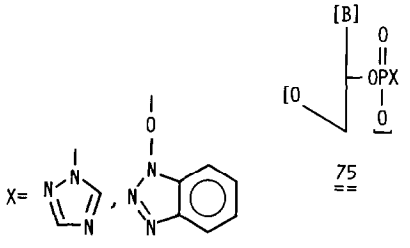
SCHEME 23. The mechanism of coupling in a classical phosphotriester approach.

On the action of the arylsulfonyl derivative, the nucleotide salt is very quickly transformed into a mixture of symmetrical 3',3'-pyrophosphate **73** and mixed anhydride **74** (*369–374*) (the ^{31}P chemical shifts of both species lie in the same range) (*371, 372*). This first step is catalyzed by pyridine or *N*-methylimidazole (*369*). Both intermediates **73** and **74** are sterically crowded. They are not prone to react with the 5'-OH function of the sequence to be elongated, and they accumulate in the solution.

Further reaction is however possible (Step 2), if the intermediates **73** and **74** react with a good and small nucleophile. This entering nucleophile must also be a good leaving group, in order to facilitate the next and final step. Azoles thus, after having decomposed **73** and **74** by nucleophilic attack (*367*), are substituted by the 5'-OH function, to give the elongated sequence (Step 3).

The Activated Ester or Amide Method

In this case, it is no more a nucleotide salt that is engaged in the coupling reaction, but a compound of type **75**:



These activated derivatives, discussed at length before, react quickly with the free 5'-OH function of an immobilized sequence, when *N*-methylimidazole is added (option 4 of Scheme 18). In the case of the hydroxybenzotriazole derivative, diisopropylethylamine is used as an acid-scavenger.

The Phosphite Method

In Letsinger's approach, the crude mixture of phosphorylation containing the phosphomonochloridite **55** (Scheme 20) is directly used for coupling at −78°C (20–30 min). *N*-Methylimidazole or tetrazole are sometimes added (*71, 211, 283*). As said before, the main problem here is to avoid any contamination of the crude coupling mixture by the phosphodichloridite **52** (Scheme 20). This method was however very often used, both in the ribo and deoxyribo series (*48, 72, 124, 149, 211, 228, 232, 233, 283, 323, 324, 331, 375–378*).

In Caruther's method, the isolated and purified nucleotide phosphoramidite **60** (Scheme 21) is coupled with the immobilized sequence at room temperature in acetonitrile, with 1,2,3,4-tetrazole as a coreagent. Caruther's method of coupling seems nowadays to be the best one with respect to rate and yield (*49, 202, 205, 344, 345, 346*). An elegant microtechnique using a simple syringe as coupling vessel has been described (*379*).

^{31}P NMR spectroscopy shows that the role of tetrazole is double (*346*): it proto-

nates the dialkylamino group of the phosphoramidite function and, next, it takes its place, generating a very reactive tetrazoylphosphoramidite **55** (Scheme 20, X = tetrazoyl).

The oxidation step after coupling (Scheme 21) deserves some comment. The popular reagent is iodine in wet THF, in the presence of 2,6-lutidine or collidine (30 min). The mechanism remains unknown (*380*). The use of water in this step is of course a nuisance: the support must then be treated with phenylisocyanate/pyridine or acetic anhydride for drying (these reagents also acylate the very few unreacted 5'-OH functions (*127*). Anhydrous oxidation conditions (*381*) are thus of interest.

The Alcoholate Method

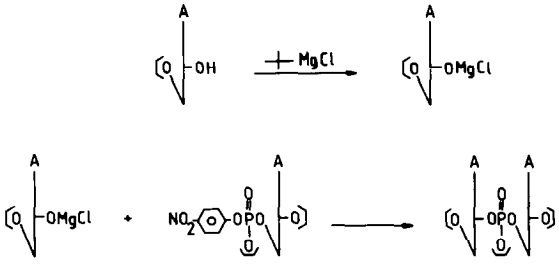
Almost all the current works on coupling are focused on the activation of the electrophilic partner. It is also possible to render the nucleophilic center more reactive. Dimers were synthesized by using this approach. The alcohol function of a nucleoside was reacted with a Grignard reagent (*382*) or potassium t-butylate (*383–385*), to generate a very reactive alcoholate function. This intermediate was then coupled with the activated ester of a nucleotide (Scheme 24). The method has severe limitations, due to the strongly basic conditions.

THE FINAL DEPROTECTION STEP

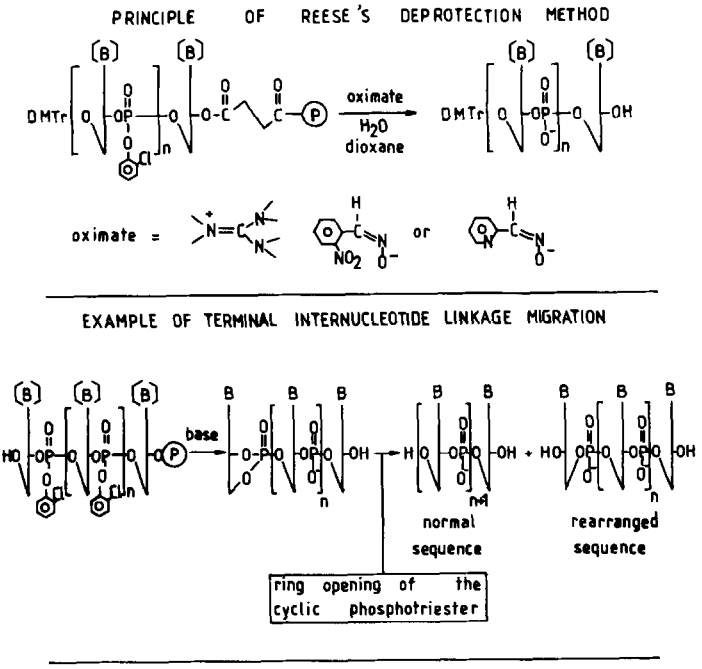
Principle

Particular deprotection methods were described when discussing the choice of nucleic base or phosphate protecting groups. We now summarize the techniques routinely used.

The regeneration of the free nucleic bases usually requires a treatment with concentrated ammonia at ca. 60°C for several hours. The point is to avoid the random cleavage of the deoxyribophosphate backbone under these severe conditions. It is therefore essential to remove *first* the persistent protecting groups of the phosphate *triester* functions with a specific reagent that will not cleave the sugar-phosphate links: the negatively charged phosphate *diester* functions so ob-



SCHEME 24. Activation of the nucleophilic partner for coupling.



SCHEME 25. Deprotection of the persistent phosphate protecting groups in a routine phosphotriester synthesis.

tained are indeed resistent to further nucleophilic attack. The final deprotection is thus a sequential operation that requires:

1. the selective deprotection of the persistent phosphate protecting group;
2. the release of the free aglycone residues. The point is here to get a *quantitative* deprotection (*88*).
3. the removal of the DMTr or Px group attached to the 5'-end of the sequence.

The sequence is usually cleaved from the polymeric support at either stage 1 or 2. However, resistent links are also available (vide supra).

Pfleiderer and Uhlmann (*107*) are working out a completely different strategy, where all the phosphate and base protections can be removed in one step by β-elimination.

Deprotection of the Phosphate Functions

When using the phosphotriester method. Aromatic groups (typically, the 2-chlorophenyl substituent) are almost always used to protect the phosphate functions of long sequences. Reese *et al.*'s reagents are of universal use for deprotection because of their high selectivity: the immobilized sequence is treated with the N^1,N^1,N^2,N^2-tetramethylguanidinium salt of *syn*-4-nitrobenzaldoximate, *syn*-2-nitrobenzaldoximate, or *syn*-pyridine-2-carboxaldoximate, in a mixture of dioxane and water (7 : 1, v/v) (Scheme 25) (*333, 386, 387*). These reagents also detach

the sequence from the support by cleaving the succinate linkage. On prolonged treatment, the acyl protection of the aglycone residues are cleaved too (*388*). *O*-Silyl groups do not withstand the oximate treatment (*389*).

During this deprotection, it is wise, if possible (e.g., at the end of a solution synthesis), to keep protected both OH-terminal function of the oligomer, because the migration of the terminal internucleotide linkages in basic media, although very limited in Reese's conditions (0.5%), is then completely avoided (Scheme 25) (*63, 390–392*).

When using the phosphoramidite approach. In this case, the persistent phosphate protecting group is usually methyl. It is cleaved by nucleophilic substitution on the carbon by thiolates (*202, 330, 345*), t-butylamine (*202, 324*), or even ammonia (*331*). As mentioned before (*332*), this last reagent is not recommended for long oligomers (20 nucleotides long (*345*)), because of chain cleavage and phosphoramidates formation (*333*). Thiolates also can induce some chain cleavage (*263*). This reagent does not cleave the succinate link with the solid support (*345, 394*). When the β-cyanoethyl groups is used as a phosphate protection, rapid β-elimination occurs during the ammonia treatment (*456*). As this last step is anyway necessary, the deprotection procedure is significantly simplified.

Deprotection of the Aglycone Residues

The oligomer is typically treated with concentrated aqueous ammonia for ca. 12 h, at 60°C in a sealed ampoule. The rates of removal of the *N*-acyl groups on cytosine, adenine, and guanine residues under these conditions are clearly dependent upon the nature of base residues. To give an example, the removal of *N*-benzoyl groups is complete at room temperature with a mixture of aqueous ammonia (5 M) and dioxane (1 : 1, v/v) in 6.5, 23.5, and 72.5 h from the corresponding 2′-deoxyribofuranosyl derivatives of cytosine, adenine, and guanine residues, respectively (*126*).

The treatment with hot ammonia also cleaves the chain at the depurinated sites (see Scheme 9) and removes the sequence from the support, if this has not yet been done.

Deprotection of the Terminal 5′-OH Function

The terminal 5′-O dimethoxytrityl or pixyl group withstands all the basic conditions described before. It may be removed at this stage by treatment with aqueous acetic acid (8 : 2, v/v) (*173*). Depurination seems not to be a problem now (*174*), because intrachain dA is more resistent to acids than *N*-6-benzoyl(dA).

The dimethoxytrityl group (and to a much lesser extend, the pixyl one (*173*)) has however the property to considerably retard the capped sequences in a reversed-phase chromatography. Its cleavage may thus be postponed: a considerable enrichment in the correct sequence is gained by isolating the dimethoxytritylated sequences from the rest of the mixture.

PURIFICATION METHODS IN DNA SYNTHESIS

Chromatographic Techniques

In a solution synthesis, the protected oligomers are purified by classical column chromatography on silica or on silanized silica (*395*). Sephadex LH-20 is also useful (*396*). The deprotected sequence obtained at the very end of a solid supported or solution synthesis may be purified by essentially two methods (*397*).

1-Anion exchange chromatography. The oldest method is low pressure anion exchange chromatography on columns of DEAE-cellulose (*61, 63, 398–400*), DEAE-Sephadex (*401*), QAE-Sephadex (*402, 403*), Dowex-I (*54*). Except with Sephadex, where the hydrodynamic volume of the oligomer may interfere, the main discriminating structural property is the net charge of the compound. The preferred eluants are a triethylammonium bicarbonate buffer (pH $\cong$ 7.5) (*61, 63, 399*) or a solution of sodium chloride in an acetate buffer (pH $\cong$ 4.7) (*404*). This type of chromatography may take several days (*127, 405*) and the resolving power goes down rapidly for large oligomers (*406, 407*). The resolution is enhanced by addition of 7 **M** urea, in order to prevent secondary interactions due to H-bonding (*404, 408*).

The modern version of these techniques (*439*) is high pressure anion exchange chromatography. Weak anion exchange columns (—NH_2, —NHR, or —NR_2 functionalities) were sometimes used (*89, 409–413*), but, in general, strong anion exchange columns are preferred (*414*). These columns are made up of either relatively large (30–50 μm), superficially porous, silica particles or of micro (10 μm) silica particles carrying chemically bonded quaternary ammonium cations (e.g., respectively the PERMAPHASE AAX column from du Pont (*87, 193, 28, 415, 416*) and the PARTISIL 10 SAX column from Whatman (*1, 67, 417, 419*)). The eluant is a mixture of a phosphate buffer (KH_2PO_4) and formamide (added to prevent hydrogen bonding (*420*)). A phosphate buffer is chosen for eluting because the phosphate anion competes favorably with the polydeoxyribophosphate for the cationic sites of the stationary phase. The resolving power is very good for up to 25-residues-long oligomers. *The longest sequence is always the most retained by such columns. This makes its identification rather easy.* The major drawback of the method is the short life of the columns (*423, 424*). A recent paper reports that the FPLC technique, using Mono Q prepacked columns (Pharmacia) is as good as the HPLC method (*425*).

2-Hydrophobic affinity chromatography. Sequences carrying one DMTr group are separable from uncapped oligomers by reversed phase HPLC, using a gradient of acetonitrile in a triethylammonium acetate buffer and various types of columns (μBONDAPAK C_{18} from Waters, ZORBAX ODS from du Pont, NUCLEOSIL C_{18} from Whatman, PRP-1 from Hamilton) (*1, 71, 419*). The retardation of end-capped sequences was already used long before in low pressure column chromatography (*127, 426–428*).

Fully deprotected oligonucleotides are also routinely purified by reversed-phase HPLC (*1, 71, 411, 429–432*). The resolving power is good for oligomers up to 15–20 units long (*419*). Low recovery due to irreversible adsorption into the column is

sometimes observed. A standard purification technique for fully deprotected oligomers (*420*) starts with an anion exchange HPLC. The slower moving peak is collected and further purified on a reversed phase column. The inverted order of operation is often impossible because the elution position of the correct sequence among truncated ones cannot be predicted in the hydrophobic affinity chromatography (*419, 421, 422*).

3-Other chromatographic methods. Various types of chromatography have been proposed: thin layer chromatography on avicel-cellulose (*433*), reversed-phase ion-pair chromatography (*424, 434–436*) (e.g., on LICHROSORB RP-8 or RP-18 columns from Merck), size-exclusion (*61, 422, 437*), and hydrophobic-anion exchange mixed-mode chromatography (*438*).

Polyacrylamide Gel Electrophoresis

This technique (PAGE) is routinely used to separate and recover one oligonucleotide from 50 to 100 o.d. of crude synthetic DNA (1,209). A denaturing 20% acrylamide gel containing 7 **M** urea gives the best results. The electrophoretic mobility of single-stranded DNA molecules of 20–1000 nucleotides in length is, under controlled conditions, a linear function of the log of the chain length, expressed by the number of nucleotides (*440*). The bands on the gel are visualized under UV light in a dark room, by lying the gel on TLC plates (covered with Saran wrap) containing a fluorescent indicator. The desired band may be sliced, crushed, and eluted with a buffer. The recovery from the gel is length-dependent, being of about 70% for a 20-mer (*57, 441, 442*).

CHARACTERIZATION OF OLIGONUCLEOTIDES

The two most useful methods are outlined below. Experimental details are given in Gait's recent book (*173*).

The "wandering spot" or "mobility shift" procedure. It is an excellent technique for sequencing DNA-oligomers up to 20 nucleotides long (*443–445, 454*). It was introduced by Ling in 1972 (*451*). The pure synthetic oligomer is enzymatically labeled by ^{32}P either at the 5′- or 3′-end, at choice. The 5′-end-labeled product is partially digested with snake venom phosphodiesterase to produce a mixture of sequences with parts of the original 3′-end lacking. Similarly, the 3′-end-labeled oligomer may be partially digested by spleen phosphodiesterase. The mixture of digestion is first fractionated by electrophoresis on a cellulose-acetate paper (50–85 cm long) at pH 2.8 (*446*) (at this pH, dpA and dpC are *N*-protonated, but not dpG and dpT). The "ladder" of spots is blotted to a thin layer plate of DEAE cellulose (*447*) and homochromatographied (*448*). On an autoradiography of the cellulose plate, each spot differs from the neighboring one by a vectorial shift (i.e., characterized by a length and an orientation). There is a typical vector corresponding to the depletion of either dpA, dpC, dpG, or dpT. A zigzagging path connecting the neighboring spots is drawn on the autoradiography. The shape of this line will tell the successive residues that have been removed by the

enzymatic reaction. The sequence is thus so determined. The method also allows to detect small amounts of impurities in the sample.

Maxam and Gilbert procedure. This well-known procedure was originally developed to sequence long polynucleotides (about 400 bases) (*442, 452*). Specific reagents cleave a 5′- or 3′-end-labeled oligomer by destroying either dA, dG, dC + dT, or dC. Four different and specific partial degradations of the sample are done in four different vials. The resulting families of truncated sequences are resolved by denaturing polyacrylamide gel electrophoresis. Comparison of the four "ladders" of spots on an autoradiography of the gel allows the sequence to be determined. The method of chemical cleavage was adapted to small oligomers (*453–455*). A disadvantage is that small amounts of impurities ($\leq$10%) in the synthetic product cannot be detected by this method.

CONCLUSION

This survey of the literature demonstrated the diversity of methods used in DNA synthesis. It is indeed useful to know in details what has already been attempted in the past, before going ahead. For practical purposes, however, the best approaches have to be pointed out. Short term perspectives have also to be delineated.

Nowadays, the best *small scale solid-supported DNA synthesis* is probably the phosphoramidite method, using CPG as a support and a β-cyanoethyl phosphate protecting group.

The phosphotriester method remains behind, essentially because of lower coupling yields. The use of dimeric or trimeric building blocks requires a costly preliminary synthetic effort. The development of sulfonylation-free coupling reagents will perhaps give a new impetus to this method. On the other hand, it is almost necessary to have a ^{31}P NMR facility, when using the phosphoramidite strategy, in order to check the purity of the sensitive phosphitylated building blocks.

The protection of the nucleic bases remains an important issue. Some early observations still hold: thymidine does not seriously require a protection and the *N*-benzoyl group is convenient for deoxycytidine. For the two other bases, major improvements were made. The O-6 protection of deoxyguanosine is a must. The amidine-type protections will perhaps put an end to the long search for a convenient N-protection for deoxyadenosine. Alternatively, the replacement of the ubiquitous 4,4′-dimethoxytrityl group by another group, cleavable in almost neutral conditions, will solve the problem of depurination.

The case by case optimization of the various persistent protecting groups leads to a situation where the final deprotection protocol is complicated. There is thus a real need for a more standardized protection strategy. A final deprotection based on general β-elimination (as proposed by Pfleiderer) is probably the most rational approach.

The purification of the deprotected sequence by anion exchange chromatogra-

phy is confronted with the problem of column longevity. The use of Mono Q FPLC columns will perhaps be a serious improvement.

Finally, it must be recognized that little effort has been concentrated on solution synthesis. This strategy is however the best one to obtain large amounts of short oligomers for spectroscopic, structural, or physicochemical studies. The phosphotriester block coupling is, until now, the only way to perform this type of synthesis.

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