Synthesis of Oligodeoxyribo- and Oligoribonucleotides According to the *H*-Phosphonate Method

This protocol outlines a general procedure for the preparation of oligodeoxyribo- and oligoribonucleotides using *H*-phosphonate monomers. It is followed by an in-depth discussion of the advantages of the *H*-phosphonate approach as well as its underlying chemistry (see Commentary). The preparation of the *H*-phosphonate monomers can be achieved by a variety of methods; these are presented in UNIT 2.6.

Oligonucleotide synthesis employing *H*-phosphonates is considerably simpler than synthesis using the phosphotriester or phosphoramidite procedures. The elongation cycle includes only two chemical steps: deprotection of the terminal 5′-OH function of the support-bound oligonucleotide, and coupling of the 5′-OH with a nucleoside 3′-*H*-phosphonate in the presence of a condensing agent (Fig. 3.4.1). After completion of the desired number of elongation cycles (i.e., assembly of the oligomeric chain), a single oxidation cycle is performed to convert the internucleoside *H*-phosphonate functions to phosphodiesters (or some analog, such as phosphorothioates). Finally, the linkage between the oligomer and the support is cleaved under ammonolytic conditions, which is also the final deprotection step for oligodeoxyribonucleotide and oligoribonucleotide synthesis with 2′-*O*-2-chlorobenzoyl groups (also see UNIT 2.6). Purification by standard methods is then carried out to isolate the oligonucleotides.

The synthetic protocol described below was optimized for oligoribonucleotide synthesis with 5′-*O*-MMTr and 2′-*O*-TBDMS protection on a modified Gene Assembler (Pharmacia) with a polystyrene support. It also gives good results with 2′-*O*-alkyl RNA and fairly good results for oligodeoxyribonucleotide synthesis. Controlled-pore glass (CPG) can be used, but has proven to be less reliable in the authors' experience.

The efficiency of each elongation step in solid-phase oligonucleotide synthesis is usually high, but technical aspects of the procedure may have to be adjusted for each particular machine. The most important of these are probably (1) the time of *H*-phosphonate preactivation before it reaches the solid support, (2) the concentration of the condensing agent, and (3) the proportion of pyridine in the solvent mixture. Some recently introduced condensing agents—e.g., bis(pentafluorophenyl) carbonate (Efimov et al., 1993) and carbonium- and phosphonium-based condensing agents (Wada et al., 1997)—seem to make the condensation less sensitive to these factors.

The reaction conditions for the removal of the acid-labile 5′-*O*-MMTr or 5′-*O*-DMTr groups (usually 1% to 2% of various haloacetic acids in an anhydrous chlorinated solvent) have been shown not to affect the integrity of the *H*-phosphonate linkages within a relevant time (Stawinski et al., 1988). The most important factors affecting the condensation and oxidation steps are discussed later (see Commentary).

Materials

    1,2-Dichloroethane (DCE; BDH) over 4-Å molecular sieves
    Trifluoroacetic acid (TFA; Fluka)
    Dichloroacetic acid (DCA; Lancaster, 99%), distilled
    Acetonitrile (MeCN; Lab-Scan) over 3-Å molecular sieves

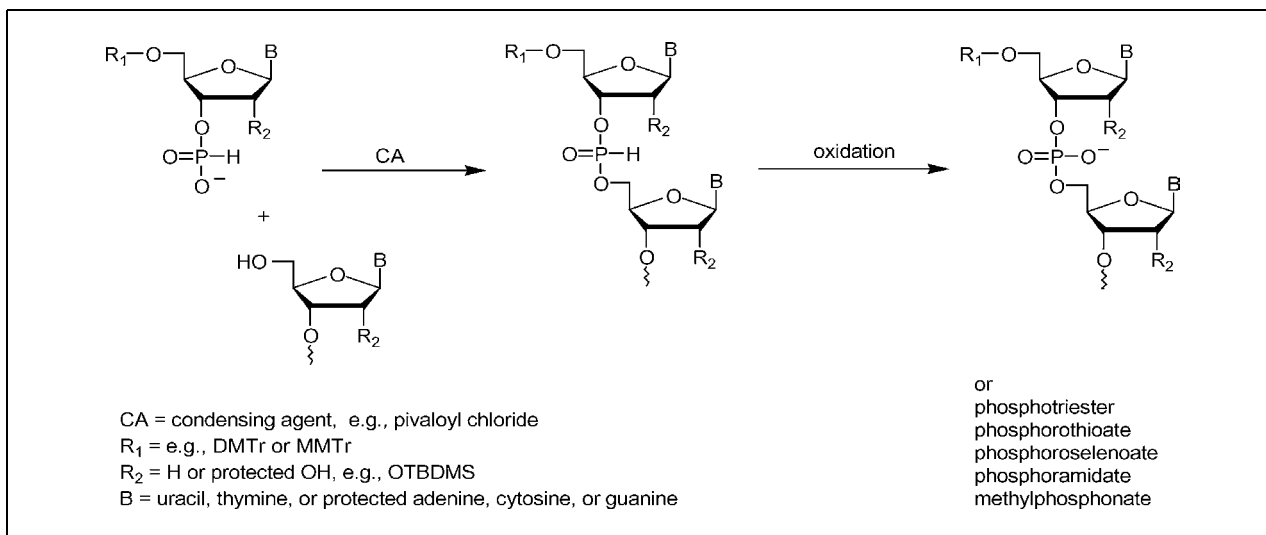**Contributed by Roger Strömberg and Jacek Stawinski**

Figure 3.4.1 Condensation of protected nucleoside *H*-phosphonate monoester with a nucleoside and conversion to the dinucleoside phosphate or backbone-modified analog.

Pyridine (Py; Lab-Scan, Anhydroscan) over 4-Å molecular sieves

Protected nucleoside 3′-*H*-phosphonate building blocks (triethylammonium salts; see Commentary and *UNIT 2.6*)

Pivaloyl chloride (Pv-Cl; Acros Organics, 99%), freshly distilled

Polystyrene support (PE Applied Biosystems) loaded at 20 to 30 μmol/g with a 5′-*O*-(4,4′-dimethoxytrityl) (DMTr)– or 5′-*O*-(4-monomethoxytrityl) (MMTr)–protected nucleoside succinate (or equivalent nucleoside-loaded solid support)

I_2

Diethyl ether

Concentrated (28% to 32%) aqueous ammonium hydroxide (NH_4OH) or 3:1 (v/v) concentrated NH_4OH/ethanol

Triethylamine trihydrofluoride (for RNA synthesis with 2′-*O*-TBDMS protection)

n-Butanol

20 mM sodium acetate buffer, pH 6.5, containing 30% and 10% MeCN

$LiClO_4$

0.1 M triethylammonium acetate (TEAA) buffer, pH 6.5

Automated oligonucleotide synthesizer (Gene Assembler, Pharmacia)

5-mL syringes with Luer lock

1.5-mL cryovials with screw caps

Glass sintered funnel of coarse porosity

Speedvac evaporator and a vacuum pump

C18 cartridge (Waters Sep-Pac)

Syringe filters (Millex-GV13 filter, 0.22-μm, 13 mm) and disposable syringes

4 × 250–mm Dionex NucleoPac PA-100 column

Lyophilizer

4.6 × 150–mm Supelcosil LC-18 (3 μm) column

Additional reagents and equipment for automated oligonucleotide synthesis (*APPENDIX 3C*) and purification by ion-exchange and reversed-phase HPLC (*UNIT 10.5*)

**Synthesis of
Oligonucleotides
According to the
H-Phosphonate
Method**

3.4.2

Table 3.4.1 Stepwise Oligonucleotide Synthesis Program

Step	Reagent[a]	Time (min)	Flow rate (mL/min)
Wash	DCE	2.0	2
Detritylation	*For 5'-O-MMTr-RNA or 2'-O-alkyl-RNA:*		
	1% TFA/DCE	1.0	2
	For 5'-O-DMTr-DNA:		
	3.5% DCA/DCE	2.0	2
	For mixed 5'-O-MMTr-RNA/2'-O-alkyl-RNA and DNA:		
	3.5% DCA/DCE	2.5	2
Wash	DCE	2.0	2
	MeCN	1.0	2
	3:1 (v/v) MeCN/Py	1.0	2
Coupling	50 mM phosphonate	0.1	1
	225 mM Pv-Cl	0.1	1
	50 mM phosphonate	0.1	1
	225 mM Pv-Cl	0.1	1
	50 mM phosphonate	0.1	1
	Pump forward	0.4	1
	Pump reverse	1.0	0.5
Wash	3:1 (v/v) MeCN/Py	1.0	2
	MeCN	1.0	2
Total time per cycle		10.9–12.4	

[a]Abbreviations: DCA, dichloroacetic acid; DCE, 1,2-dichloroethane; DMTr, 4,4'-dimethoxytrityl; MeCN, acetonitrile; MMTr, 4-methoxytrityl; Pv-Cl, pivaloyl chloride; Py, pyridine; TFA, trifluoroacetic acid.

Prepare reagents

1. Charge the synthesizer with solvents and detritylation solution needed for the steps shown in Table 3.4.1.

   These can usually be kept on the synthesizer until they are consumed.

2. Dissolve the protected nucleoside 3'-*H*-phosphonates (triethylammonium salts) in pyridine and evaporate the solvent (two times) under reduced pressure in a rotary evaporator. Use 15 μmol/coupling plus 15 μmol extra for margins and priming of solutions.

3. Dissolve the residue in 3:1 (v/v) MeCN/pyridine to a concentration of 50 mM and transfer to appropriate vessels for attachment to the synthesizer.

4. Prepare a solution of 225 mM pivaloyl chloride in 3:1 (v/v) MeCN/pyridine directly in the vessel that will be used for synthesis. Prepare 0.2 mL/coupling plus 1 to 2 mL extra for margins. Attach the vessel to the synthesizer.

5. Prime the solutions and connect a column/cartridge filled with 0.2 to 1 μmol nucleoside-loaded support.

6. Immediately before the final oxidation step, prepare an iodine solution by dissolving 0.4 g I_2 in 20 mL of 98:2 (v/v) pyridine/water.

   The solution should be prepared no more than 30 min before use.

Synthesis of Unmodified Oligonucleotides

3.4.3

Table 3.4.2 Final Oxidation Program

Step	Reagent[a]	Time (min)	Flow rate (mL/min)
Wash	DCE	2	2
Detritylation	1% TFA/DCE	1	2
Wash	DCE	2	2
	MeCN	1	2
Oxidation	2% I_2 in 98:2 (v/v) Py/H_2O	30	0.5
Wash	MeCN	10	2
Total time		46	

[a]Abbreviations: DCE, 1,2-dichloroethane; MeCN, acetonitrile; Py, pyridine; TFA, trifluoroacetic acid.

Synthesize oligonucleotide

7. Perform automated oligonucleotide synthesis (*APPENDIX 3C*) using the synthesis cycle shown in Table 3.4.1, with a final oxidation step as shown in Table 3.4.2. In the coupling step, program the synthesis so that the *H*-phosphonate and pivaloyl chloride are taken up in alternating 100-μL portions.

   The volume of the tubing between the valve and the column (including the pump hose of the peristaltic pump, through which the reagents flow) is 0.4 mL, which means that the front of the condensation mixture reaches the column when the last segment is taken up from the reagent bottles. The segments are then passed through the column (0.4 min at 1 mL/min), and then the flow is lowered and the direction reversed (1 min at 0.5 mL/min), giving a total condensation cycle time of 1.9 min, with an effective condensation time of ~1.5 min. The effective time of preactivation for the nucleoside H-phosphonate when it first reaches the column is ~0.3 min, for a total of 1.8 min at the end of condensation.

Purify oligonucleotide

8. Remove the cartridge/column from the synthesizer and wash the support with ~10 mL diethyl ether using a 5-mL syringe with a Luer fitting.

9. Air dry the support using the above 5-mL syringe with a Luer fitting by pushing air through the column a few times.

10. Remove the support from the cartridge and transfer it to a 1.5-mL cryovial (with screw cap).

11. Carry out ammonolysis by adding 3:1 (v/v) concentrated NH_4OH/ethanol (~1 mL for up to a 1-μmol scale synthesis) and incubating 8 to 16 hr at 20° to 25°C (8-hr incubations for sequences up to 25-mers; 16-hr incubations for longer sequences, e.g., 50- to 60-mers).

    The above conditions are for RNA oligomers with N^2-phenoxyacetyl guanosine protection, N^6-butyryl adenosine protection, and N^4-propionyl cytidine protection, which are recommended to avoid cleavage of TBDMS groups (and subsequent cleavage of RNA) upon ammonolysis at elevated temperatures (Stawinski et al., 1988), which is required for more stable base protection. Other reagents and conditions may be needed for other protecting groups. For DNA or for 2'-O-alkyl-RNA, concentrated NH_4OH should be used alone for ~20 hr at 55° to 60°C.

12. Filter off the support using a glass sintered funnel of medium porosity.

13. Wash support with 1 mL of 3:1 (v/v) concentrated NH_4OH/ethanol (or concentrated NH_4OH alone) and combine the filtrates.

14. Concentrate the oligonucleotide using a Speedvac evaporator and a vacuum pump.

15. For RNA synthesis with 2′-O-TBDMS protection, perform deprotection step with triethylamine trihydrofluoride (Westman and Strömberg, 1994) using the following steps:

    a. Dissolve the residue in 0.3 mL neat triethylamine trihydrofluoride and incubate 14 to 16 hr at room temperature.

    b. Add 30 μL water and 1 mL *n*-butanol, and incubate 1 hr at –20°C.

    c. Centrifuge and remove the liquid supernatant. Dissolve the pellet in HPLC buffer (see step 16).

16. Purify by ion-exchange and reversed-phase HPLC (*UNIT 10.5*).

    a. Dissolve the deprotected oligoribonucleotides in 0.5 mL of 20 mM sodium acetate buffer, pH 6.5, containing 30% MeCN and filter through a disposable C18 cartridge.

    b. Wash the cartridge with 1 mL of buffer, then combine fractions and filter through a disposable syringe attached to a 0.22-μm filter before HPLC purification.

    c. Purify by anion-exchange HPLC on a 4×250–mm Dionex NucleoPac PA-100 column using a linear gradient of $LiClO_4$ in 20 mM sodium acetate buffer, pH 6.5, containing 10% MeCN at a flow rate of 1 mL/min. For analysis, inject 0.2 OD_{260} units; for purification, inject 15 to 30 OD_{260} units of crude oligoribonucleotides.

    d. Lyophilize the collected fractions, dissolve in 1 mL of 0.1 M triethylammonium acetate (TEAA) buffer, pH 6.5, and filter through a disposable syringe attached to a 0.22-μm filter.

    e. Further purify by reversed-phase HPLC on a 4.6×150–mm Supelcosil LC-18 column using a linear gradient of MeCN in 0.1 M TEAA buffer, pH 6.5, at a flow rate of 1 mL/min.

    f. Collect the fractions containing the product, lyophilize, dissolve in 1 mL water, and lyophilize again.

COMMENTARY

Background Information

General information and synthetic strategies

Although the most common method today for synthesis of oligonucleotides and their analogs is the phosphoramidite approach (Beaucage and Iyer, 1993, see also *UNIT 3.3*), the newer *H*-phosphonate methodology can often be a preferred alternative (Garegg et al., 1985, 1986a,b,c; Froehler and Matteucci, 1986; Froehler et al., 1986). The use of *H*-phosphonates in nucleotide synthesis was pioneered by Sir Todd's group in Cambridge, UK, who in 1952 demonstrated the formation of *H*-phosphonate diesters in a condensation reaction of *H*-phosphonate monoesters with a protected nucleoside, promoted by diphenyl phosphorochloridate (Corby et al., 1952; Hall et al., 1957). This chemical principle was, however, not explored further; it was rediscovered three decades later (Garegg et al., 1985, 1986c) and explored for oligonu-cleotide synthesis (Froehler and Matteucci, 1986; Froehler et al., 1986; Garegg et al., 1986a,b,c, 1987a).

The method consists of condensing a protected nucleoside *H*-phosphonate monoester (*UNIT 2.6*) with a nucleoside in the presence of a coupling agent to produce the corresponding dinucleoside *H*-phosphonate diester. This, under various experimental conditions, can be converted to the dinucleoside phosphate or to a variety of backbone-modified analogs, e.g., phosphorothioates, phosphoramidates, etc. (Fig. 3.4.1).

The condensation step of the elongation cycles (i.e., the formation of an internucleoside *H*-phosphonate linkage between a nucleoside 3′-*H*-phosphonate monoester and the support-bound 5′-hydroxylic component) is usually carried out in pyridine-acetonitrile mixtures. Out of the various condensing agents initially tested, pivaloyl chloride (Pv-Cl) gave the best results in automated solid-support synthesis

of oligonucleotides, and it is still the most frequently used reagent. The reaction in pyridine or acetonitrile-pyridine mixtures using 2 to 5 equiv of Pv-Cl is usually fast and goes to completion in <1 min. Nowadays, an array of other condensing agents (see below) is available; these reagents can, in some instances, be superior to Pv-Cl.

The *H*-phosphonate methodology became commercially available (for use in automated synthesizers from Applied Biosystems and Biosearch) soon after the initial reports on oligonucleotide synthesis using this approach were published. The research group at Applied Biosystems also introduced 1-adamantanecarbonyl chloride as an alternative to Pv-Cl (Andrus et al., 1988). Although a capping step during oligonucleotide synthesis via *H*-phosphonate intermediates initially seemed to be superfluous, its incorporation into the synthetic protocol was shown to potentially improve the overall performance of the method. The procedure simply consists of an additional condensation step using isopropyl *H*-phosphonate after each nucleoside *H*-phosphonate coupling (Andrus et al., 1988). The use of 2-cyanoethyl *H*-phosphonate for capping was also reported, although this reagent has the disadvantage of being less accessible. The capping procedure routinely used in the phosphoramidite approach (acylation with acetic anhydride in the presence of nucleophilic catalysts more powerful than pyridine) is not compatible with *H*-phosphonate chemistry, owing to the occurrence of *P*-acylation (see below).

Since the introduction of the *H*-phosphonate methodology for synthesis of oligonucleotides, a number of improvements have been introduced. These involve new condensing agents and new reaction conditions. Oligodeoxynucleotide synthesis via *H*-phosphonates has been scaled up to 10 to 14 µmol using only a few equivalents of monomeric building blocks relative to a support-bound oligomer per condensation (Gaffney and Jones, 1988). The *H*-phosphonate approach was used in a cartridge-based procedure for simultaneous synthesis of multiple oligodeoxynucleotides (Seliger and Rösch, 1990). The *H*-phosphonate methodology has been used in the synthesis of a number of different oligonucleotide analogs, for example, phosphoramidates (Froehler, 1986) and phosphorothioates (Agrawal and Tang, 1990; Stein et al., 1990), including those with all-R_p-linkages (Almer et al., 1996). It has also been used in the synthesis of oligonu-

cleotides bearing modified heterocyclic bases (Ramzaeva et al., 1997; Seela and Wei, 1997). One particularly interesting feature of oligonucleotide synthesis when using the *H*-phosphonate methodology is that it can be performed without protection of the nucleobases (Kung and Jones, 1992; Wada et al., 1997; *UNIT 3.10*).

RNA synthesis is perhaps where the advantages of the *H*-phosphonate approach are most apparent. In the first report, the *t*-butyldimethylsilyl (TBDMS) group was used for protection of the 2′-OH and the protocol was similar to that described for DNA synthesis (Garegg et al., 1986a,b). A number of subsequent reports have differed in the choice of 2′-OH protection (see *UNITS 2.2* and *3.5*). These include the use of photolabile groups (e.g., *o*-nitrobenzyl; Tanaka et al., 1987), acid-sensitive groups (such as 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl; Ctmp; Sakatsume et al., 1989), and base-labile benzoyl derivatives (Rozners et al., 1988, 1990, 1992).

The *H*-phosphonate approach seems to be even more suited for RNA than for DNA synthesis. Potential problems such as double activation (i.e., the bis-acyl phosphite formation) and *P*-acylation are significantly suppressed when using ribonucleoside *H*-phosphonates (Stawinski et al., 1991b). The condensation reaction is virtually as fast as for DNA synthesis and at least as efficient, even with bulky 2′-*O*-protection. Synthesis of oligoribonucleotides of up to 50 to 60 nucleotide residues in length is readily achieved (Agrawal and Tang, 1990; Rozners et al., 1998), and the deprotection steps have been adjusted to more base-labile *N*-protection that gives better performance (Rozners et al., 1998).

The most common choice for 2′-OH protection in oligoribonucleotide synthesis is the TBDMS group (see *UNIT 3.5*). Studies on the stability of this group under the reaction conditions used for removal of *N*-acyl groups have been carried out (Stawinski et al., 1988; Wu and Ogilvie, 1990). A conclusion (relevant not only to the *H*-phosphonate approach) was that synthesis of longer RNA oligonucleotides with 2′-*O*-TBDMS protection would benefit from the use of more labile *N*-acyl-protecting groups for the heterocyclic base moieties. These were later duly introduced to the synthesis of oligoribonucleotides, both by the phosphoramidite approach (Wu and Ogilvie, 1988; Chaix et al., 1989) and the *H*-phosphonate method (Westman et al. 1993, 1994; Rozners et al., 1998).

Although Pv-Cl (Froehler et al., 1986; Garegg et al., 1986a) and perhaps adamantane carbonyl chloride (Andrus et al., 1988) are the most popular condensing agents used in solid-phase synthesis of oligonucleotides via the *H*-phosphonate approach, several others that may be considered for different applications have also been evaluated. These include various pivalic (Strömberg and Stawinski, 1987) or other carboxylic acid derivatives (Jäger et al., 1987), diphenyl phosphorochloridate (Hall et al., 1957; Garegg et al., 1985), 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane (Strömberg and Stawinski, 1987), bis(2-oxo-3-oxazolidinyl)phosphinic chloride (Strömberg and Stawinski, 1987), various dialkyl phosphorochloridates (Strömberg and Stawinski, 1987; Stawinski et al., 1988), arene sulfonic acid derivatives (Garegg et al., 1985; Jäger et al., 1987; Dan'kov et al., 1988), 1,3-dimethyl-2-chloro-imidazolium chloride (Sakatsume et al., 1989, 1990), bis(pentafluorophenyl)carbonate (Efimov et al., 1993), and a number of carbonium and phosphonium derivatives (Wada et al., 1997).

Parameters of the underlying chemistry

Activation of H-phosphonate monoesters

The formation of an internucleoside linkage by the *H*-phosphonate approach, using acyl chlorides, proceeds via a mixed phosphono-carboxylic anhydride that reacts with the nucleosidic component to produce an *H*-phosphonate diester (Garegg et al., 1987c; Fig. 3.4.2). The order in which the reactants are added in solution synthesis with near equimolar amounts of phosphonate and nucleoside was found to be important for the efficiency of *H*-phosphonate diester formation (Garegg et al., 1985, 1986c). The coupling reaction is virtually quantitative when the condensing
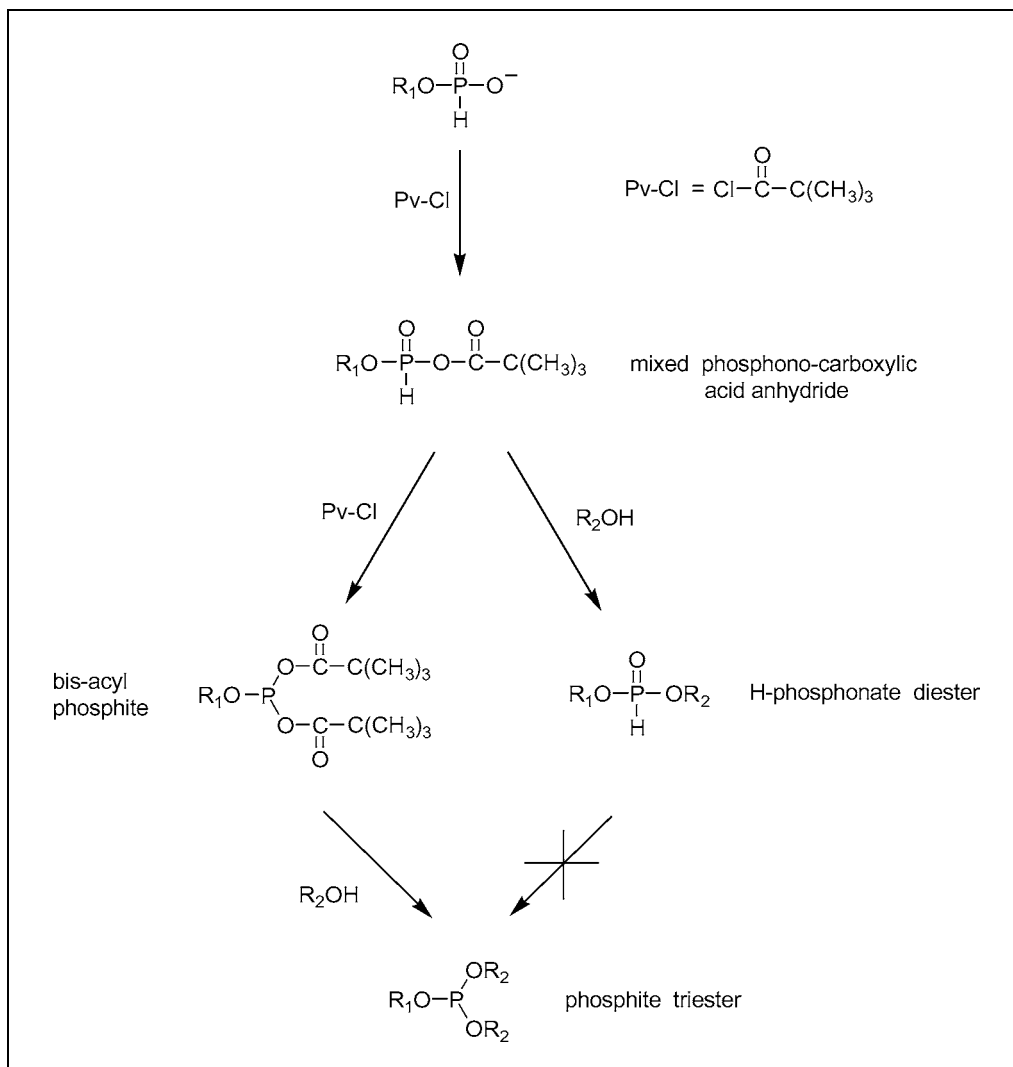

Figure 3.4.2   Activation of *H*-phosphonate monoesters. R$_1$ = protected nucleoside-3′-yl; R$_2$ = protected nucleoside-5′-yl.

agent is added to a solution containing both the alcohol and the *H*-phosphonate monoester. If, on the other hand, the *H*-phosphonate is preactivated with a condensing agent before the addition of an alcohol, the reaction is considerably less efficient. This phenomenon was investigated and traced back to the further activation of the initially formed intermediates in the absence of an alcohol (Garegg et al., 1987c,d,e). Irrespective of the condensing agent used, tricoordinated phosphite derivatives were formed; upon reaction with an alcohol, these species (the chemical nature of which depends on the coupling agent used) always gave various unwanted products in addition to the *H*-phosphonate diesters.

Preactivation of *H*-phosphonate monoesters with acyl chlorides produces bis-acyl phosphites (Garegg et al., 1987c). The consequence of such preactivation is that, in solution synthesis with near equimolar amounts of *H*-phosphonate and alcohol, formation of phosphite triesters is unavoidable (at least to some extent). It is quite clear that such species must also be formed to some extent when the condensing agent and *H*-phosphonate are mixed before entering the column in a synthesizer. No side products that could arise from this species have so far been detected in solid-phase synthesis products, but it has been shown that too long of a preactivation substantially slows down the coupling reaction, and the competing reaction of alcoholic functions with the condensing agent becomes more pronounced (Gaffney and Jones, 1988). It has also been shown that even if condensation with nucleoside bis-acyl phosphites gave the correct product on a solid support, the reaction of a bis-acyl phosphite with a nucleoside is substantially slower than that of the corresponding *H*-phosphonate which is postulated to proceed via the mixed phosphono-carboxylic anhydride (shown in Fig. 3.4.2; Garegg et al., 1987c; Efimov and Dubey, 1990).

Because an excess of *H*-phosphonate to alcohol is normally used in solid-phase synthesis, some formation of bis-acyl phosphite is tolerable. It is clear that the preactivation time should be minimized so that the mixture reaching the column has the highest ratio of mixed phosphono-carboxylic anhydride to bis-acyl phosphite as possible. Otherwise, the oligonucleotide synthesis will lose in efficiency. A preactivation time that is too long can probably not be compensated to the full extent by longer condensation time, because competing reactions become more prominent. Apart from minimizing preactivation time, it is

also possible to reduce or change the amount of condensing agent (which is usually used in excess) and control the reaction by varying solvent composition (see below). It is convenient to use enough condensing agent to achieve conditions that are not too moisture sensitive; but over a certain level, additional excess of acyl chloride increases the level of side reactions that may lower the coupling efficiency and overall yield.

Particularly noteworthy was the observation that *H*-phosphonate monoesters when activated in the absence of a base (like pyridine) produce only monoactivated species (Garegg et al., 1987c). Efimov and co-workers found that replacing pyridine by the slightly weaker base quinoline is sufficient to suppress the formation of bis-acyl phosphite and keep its amount <5% to 10% within the period of time necessary for a condensation (Efimov and Dubey, 1990; Efimov et al., 1990). This novel solvent mixture (acetonitrile-quinoline, 4:1 v/v) was used in the synthesis of oligodeoxynucleotides up to 39 units in length (Efimov and Dubey, 1990). Although the replacement of pyridine by quinoline in oligonucleotide synthesis via the *H*-phosphonate approach offers some advantages, the rate of condensation is usually lower and quinoline gives some side reactions with activated *H*-phosphonate monoesters (Stawinski et al., 1991a). The influence of base strength on preactivation of ribonucleoside *H*-phosphonates was examined, and substituted pyridines that were less basic than pyridine itself were found to suppress formation of tricoordinated species (Stawinski et al., 1991b). With these less basic pyridine derivatives, the side reactions observed with quinoline could not be detected. Steric factors are also likely to be important for double activation, because, for example, the formation of tricoordinated species is slower for 2′-*O*-TBDMS-protected ribonucleoside *H*-phosphonates than for the corresponding 2′-deoxyribo derivatives.

Recently, bis(pentafluorophenyl) carbonate (PFPC) was advocated as a condensing agent for *H*-phosphonate diesters formation (Efimov et al., 1993). The reagent may be as reactive as Pv-Cl in promoting condensations and compares favorably with Pv-Cl in regard to its reactivity toward heterocyclic bases and the 5′-hydroxyl function of a nucleosidic component (Efimov et al., 1993). Although the reagent produces double-activated species from *H*-phosphonate monoesters, these are as reactive as the initially formed monoactivated intermediate. Truncated sequences are claimed to be

significantly reduced compared to the protocol that uses Pv-Cl as a condensing agent. The utility of the reagent was demonstrated in the synthesis of several oligodeoxyribonucleotides (chain length 22 to 40) and oligonucleotide-phospholipid conjugates (Efimov et al., 1993).

Competing O-acylation

Direct reaction of a condensing agent with hydroxylic functions in competition with the desired coupling reaction is another potential source of inefficiency in oligonucleotide synthesis. The suggestion that capping is important when using the *H*-phosphonate approach for oligonucleotide synthesis points to a rather low extent of acylation during the condensation step (Andrus et al., 1988; Gaffney and Jones, 1988). Self-capping by the acyl chlorides used for condensation was estimated to account for only ~10% to 50% of nonphosphonylated hydroxyls (Gaffney and Jones, 1988), which represents 0.1% to 0.5% of an average coupling yield of 99%. When a four-fold excess of *H*-phosphonate to nucleoside and 5 equiv Pv-Cl were reacted for 1.5 min, however, the total amount of 5'-*O*-acylation was ~0.8% (Gaffney and Jones, 1988). The extent of *O*-acylation depends heavily on reaction conditions, such as ratios and concentrations of reagents, and is likely to be substantially lower when a larger excess of *H*-phosphonate is used in conjunction with fewer equivalents of Pv-Cl.

The authors assert that the most important reason for the occurrence of competing 5'-*O*-acylation in machine-assisted synthesis is that the condensation is slowed down owing to formation of bis-acyl phosphites (see above). Acylation of 5'-OH functions has never been observed when the condensation reaction is carried out in solution without preactivation. Minimizing formation of the less reactive tricoordinated species (from preactivation) should result in higher coupling efficiency. Using fewer equivalents of condensing agent relative to *H*-phosphonate monoester is likely to be the most efficient way to decrease 5'-*O*-acylation, because it will affect the rate of both *O*-acylation and bis-acyl phosphite formation. Changes in solvent composition may also be beneficial (e.g., less pyridine).

P-acylation

Another side reaction that potentially can occur when using acyl chlorides as condensing agents in the *H*-phosphonate approach to oligonucleotide synthesis is the formation of acylphosphonates (Fig. 3.4.3). When the synthesis of an *H*-phosphonate diester is carried out with all reactants in solution under standard conditions (approximately equimolar amounts of *H*-phosphonate and nucleosidic components together with 2 to 3 equiv of coupling agent), the reaction can be quenched long before any *P*-acylation can be detected. In solid-support oligonucleotide synthesis, however, because of iterative condensation steps, reaction of *H*-phosphonate functions with the condensing agent could become a problem. Acylphosphonate formation was not detected when *H*-phosphonate diesters were exposed to 3 to 5 equiv of Pv-Cl in pyridine for 30 min (Regberg et al., 1988). In a study of *P*-acylation, however, a slow conversion of a di(deoxyribonucleoside) *H*-phosphonate to the corresponding pivaloylphosphonate went
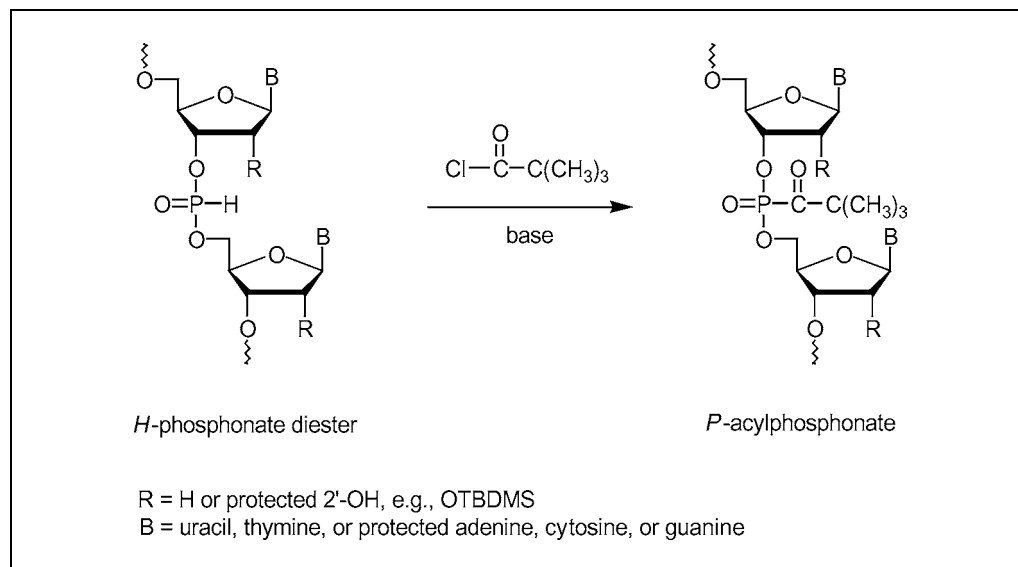

R = H or protected 2'-OH, e.g., OTBDMS
B = uracil, thymine, or protected adenine, cytosine, or guanine

Figure 3.4.3   *P*-acylation side reaction.

to completion in the presence of 5 equiv of Pv-Cl in pyridine-acetonitrile after 15 hr at 20°C (Kuyl-Yeheskiely et al., 1986).

The *P*-acylation during a condensation reaction should in principle be less pronounced, owing to the inhibitory effect of pyridinium ions that are formed (Strömberg, 1987). In a condensation mixture containing 5 equiv of Pv-Cl (in pyridine-acetonitrile 1:1 v/v), only ~15% of the produced *H*-phosphonate diester is pivaloylated in 14 hr (Strömberg, 1987). In the presence of bases stronger than pyridine (e.g., *N*-methylimidazole, triethylamine), the *P*-acylation is considerably faster (Strömberg, 1987). Note, however, that even if *P*-acylation occurred to some extent during oligonucleotide synthesis, this modification could not be easily carried through to the fully deprotected oligonucleotide. Oligonucleotidic chains bearing acylphosphonate modifications will be cleaved during final deprotection upon treatment with aqueous ammonia, and thus only a decrease in the yield of oligomers with the desired chain length is expected.

Reactions of protected ribonucleoside *H*-phosphonate diesters with Pv-Cl are considerably slower than those of deoxyribonucleoside derivatives (Stawinski et al., 1991b). The presence of a bulky 2′-*O*-TBDMS-protecting group vicinal to the *H*-phosphonate linkage in the ribonucleoside derivatives is likely to be one reason for the observed difference. In a typical condensation reaction (5 equiv of Pv-Cl in pyridine-acetonitrile; 1:1 v/v), only a few percent of the produced *H*-phosphonate diester is pivaloylated within 22 hr (Stawinski et al., 1991b), which corresponds to the time required for 880 standard condensations reactions. As a rough estimate, a 50-residue-long 2′-*O*-TBDMS-oligoribonucleoside *H*-phosphonate synthesized with 5 equiv of Pv-Cl and 50% pyridine-acetonitrile (1:1 v/v) gives truncated sequences (owing to *P*-acylation and subsequent cleavage) to a level of 0.1%; it is further estimated that decreasing the excess Pv-Cl to 3 equiv in pyridine-acetonitrile (1:3 v/v), would only produce ~0.025% *P*-acylation. For oligodeoxyribonucleotide synthesis, the corresponding levels should be roughly 0.9% and 0.2% to 0.3% for a coupling time of 1 min. This suggests that *P*-acylation could well be responsible for a substantial part of the observation that DNA synthesis with *H*-phosphonates, in contradistinction to oligoribonucleotide synthesis, is not quite as efficient as with phosphoramidites (when using 5 equiv of Pv-Cl in pyridine-acetonitrile, 1:1 v/v). It seems

clear that oligodeoxyribonucleotide synthesis would benefit even more than oligoribonucleotide synthesis from reducing the excess of coupling agent and pyridine (and possibly the basicity by using a substituted pyridine; see below).

In the earlier discussion on preactivation, some advantages connected with using a slightly weaker base than pyridine as a co-solvent during the condensation step were mentioned. Such conditions are also advantageous when considering *P*-acylation. The extent of this reaction is substantially decreased when quinoline or some substituted pyridines are used instead of pyridine (Stawinski et al., 1991b). In the case of deoxyribonucleoside *H*-phosphonate diesters, only traces of *P*-acylation can be detected (^{31}P NMR) after 22 hr when 5 equiv of Pv-Cl in quinoline-acetonitrile (1:3 v/v) is used. For ribonucleoside *H*-phosphonate diesters, this side reaction cannot be detected even after 22 hr under the same conditions (Stawinski et al., 1991b).

Other potential side reactions

Not many studies on the extent of base modification when using the *H*-phosphonate approach for oligonucleotide synthesis have been reported. Error rates in oligodeoxynucleotides that were produced using the *H*-phosphonate methodology have been estimated by sequencing (Vasser et al., 1990). The average number of substitutions was found to be 1/1783 bases. These are not necessarily caused by base modification during synthesis, but they at least indicate the maximum level of modification one may anticipate to be present in purified oligomers.

Guanosine-containing dinucleoside *H*-phosphonates have been reported to react with some condensing agents (Regberg et al., 1988). The guanine residue reacts with Pv-Cl to produce a modification (most likely pivaloylation of the lactam function) that is reversed by treatment with ammonia for a much shorter time than that used for the deprotection of oligonucleotides. Because this reaction is also considerably slower than the condensation, protection of the heteroaromatic lactam system in guanine seems to be unnecessary for most applications when using *H*-phosphonate chemistry. It may, however, be advisable to analyze for base modifications if novel condensing reagents are being used.

Some concern has been expressed about the possibility of a partial loss of the 4,4′-dimethoxytrityl group from 5′-protected

H-phosphonates upon standing in vials on automated synthesizers (Froehler et al., 1986; Froehler and Matteucci, 1987). It was estimated that 5′-*O*-(4,4′-dimethoxytrityl)thymidine *H*-phosphonate (triethylammonium salt (TEAH⁺)) dissolved in pyridine-acetonitrile (1:1 v/v) loses 2% of its 5′-protection within 4 weeks (Froehler and Matteucci, 1987). Assuming a first-order reaction, this would translate to ∼0.05% detritylation per 1000 min. If fresh solutions are prepared before synthesis, this can be considered a measure of the extent to which the loss of 5′-protection could be for the last added nucleotide unit in the synthesis of a 100-mer (assuming a 10-min cycle time). The replacement of TEAH⁺ by 2,3,4,6,7,8,9,10-octahydropyrimido[1,2-a]azeponium (DBUH⁺) salts of nucleoside *H*-phosphonate monoesters was also claimed to decrease the amount of oligomers with a chain length of $n + 1$ (Froehler and Matteucci, 1987) owing to suppression of partial detritylation during the condensation step. It is, therefore, conceivable that the loss of the 5′-*O*-(4-monomethoxytrityl group) frequently used in RNA synthesis should probably be negligible under these conditions. Although the detritylation step has been shown to be completely compatible with *H*-phosphonate linkages (Stawinski et al., 1988), be aware that using acid-labile 2′-*O*-protecting groups in *H*-phosphonate-based oligoribonucleotide synthesis may cause problems (Rozners et al., 1994). These can be alleviated by using the fluoride-labile 2′-*O*-TBDMS or base-labile 2′-*O*-(2-chlorobenzoyl) groups. The former group can be recommended for the synthesis of longer oligoribonucleotides, whereas the latter can be selected for shorter fragments, owing to the simplicity and cost of preparation of starting materials.

The oxidation step

In the phosphoramidite approach, the oxidation step is carried out after each coupling reaction (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981; see also UNIT 3.3). It may be feasible to do so via the *H*-phosphonate method, because the condensation reaction can be accomplished in the presence of phosphodiester functions (Földesi et al., 1989; Gryaznov and Potapov, 1990). Given that *H*-phosphonate functions remain intact throughout the oligomeric chain assembly, the oxidation reaction can be carried out in a single step upon completion of the synthesis. This can be advantageous for the preparation of uniformly modified oligonucleotides (e.g., elemental sulfur can produce phosphorothioate oligomers in one step from *H*-phosphonate oligonucleotides).

The final oxidation step in the *H*-phosphonate method for oligonucleotide synthesis is usually performed using a solution of iodine in aqueous pyridine (Garegg et al., 1986a,b, 1987b) or with iodine-water in the presence of another base (triethylamine, *N*-methylimidazole; Froehler and Matteucci, 1986; Froehler et al., 1986). Completeness of the oxidation reaction is, of course, important for efficient synthesis of oligonucleotides. Any oligomeric chain containing an *H*-phosphonate function will be immediately cleaved in the subsequent ammonia treatment step (Hammond, 1962). Incomplete oxidation will produce shorter than expected oligomers, thus reducing the yield of the desired product. An oxidation time of 10 min with 2% iodine in pyridine-water (98:2) seems to be sufficient for solid-phase synthesis of 20-mers (Garegg et al., 1986a,b). Because the oxidation reaction needs to be carried out only once after chain assembly, one can extend the oxidation time (rather than using stronger bases) to ensure complete reaction within a marginal time loss. A longer oxidation time may still be advisable for longer oligonucleotides.

Competitive hydrolysis of *H*-phosphonate diesters during aqueous oxidation may be considered a potential problem. This has not yet been reported, but it may be justified to discuss this issue because *H*-phosphonate diesters are very unstable under aqueous basic conditions (Nylén, 1937; Westheimer et al., 1988). The rate constant (k_{OH}) for hydroxide-catalyzed oxidation with iodine was reported to be ∼1400 to 4300 times larger than that for hydrolysis of a series of dialkyl *H*-phosphonates (Nylén, 1937, 1938). These values have been used to estimate that an aqueous pyridine solution containing 2% iodine should give no more than ∼5 to 25 ppm *H*-phosphonate diester cleavage (Stawinski and Strömberg, 1993). This suggests that hydrolysis during oxidation is not a serious problem in oligonucleotide synthesis according to the *H*-phosphonate approach.

The rate of oxidation of nucleoside *H*-phosphonate diesters with iodine-pyridine-water can be increased by including a presilylation step (e.g., treatment with trimethylsilyl chloride) in the synthetic protocol (Hata and Sekine, 1974; Garegg et al., 1987b). This has, however, been used infrequently in oligonucleotide synthesis.

Synthesis of Unmodified Oligonucleotides

3.4.11

Critical Parameters

The detritylation step has been shown to be completely compatible with *H*-phosphonate linkages (Stawinski et al., 1988). However, using acid-labile 2′-*O*-protecting groups in *H*-phosphonate-based oligoribonucleotide synthesis may cause problems (Rozners et al., 1994). These problems can be alleviated by using the fluoride-labile 2′-*O*-TBDMS or base-labile 2′-*O*-2-chlorobenzoyl groups. The former group can be recommended for the synthesis of longer oligoribonucleotides, while the latter can be a choice for shorter fragments, due to the simplicity and cost of preparing the starting materials.

The preactivation time, i.e., contact of a condensing agent with nucleoside 3′-*H*-phosphonates before they reach the reaction chamber, should be minimized as much as possible, since an extended preactivation time adversely affects rate and efficiency of internucleotide bond formation.

The amount of condensing agent and proportion of pyridine in the solvent is also important. It is convenient to use enough condensing agent to obtain conditions that are not too moisture sensitive. Over a certain level, however, excess acyl chloride increases the extent of side reactions that may lower the coupling efficiency and overall yield.

The quality of the pivaloyl chloride is important for the outcome of the synthesis. Since the reagent decomposes with time, it should be distilled approximately once a month. The distilled material, divided into a number of smaller vials to avoid too frequent opening and closing of the same bottle, can be safely stored at −18° to −20°C.

The best results are obtained if the reagent solutions are prepared fresh just before the start of oligonucleotide synthesis. This is most beneficial for the pivaloyl chloride solution and the oxidation solution. The latter degrades with time (particularly when a base stronger than pyridine is used in the oxidation solution) and it is recommended that a fresh oxidation solution be used for each synthesis. A shorter oxidation time than that stated in the protocol can be used for shorter oligonucleotides, but since this is only carried out once, the time savings are not significant and a standard time of 30 min that appears to result in complete oxidation (with some margin for 50- to 60-mers) may be best.

Anticipated Results

Since 2′-protected ribonucleoside 3′-*H*-phosphonates are less susceptible to double activation with a condensing agent, and diribonucleoside *H*-phosphonate diesters are more resistant to *P*-acylation by acyl chlorides used as condensing agent, the *H*-phosphonate approach is particularly suited for the preparation of oligoribonucleotides. Because oxidation is carried out as a final synthetic step, the *H*-phosphonate approach can also be a method of choice for the preparation of uniformly modified oligonucleotides.

With the reported protocol, one should be able to routinely synthesize oligoribonucleotides of up to ~50 to 60 residues with high yield when using 5′-MMTr and 2′-*O*-TBDMS protection (and N^4-propionyl for cytidine, N^6-butyryl for adenosine, and N^2-phenoxyacetyl for guanosine). For oligodeoxyribonucleotides, one could expect similar results. When using 2′-*O*-2-chlorobenzoyl protection, oligoribonucleotides of up to ~20- to 30-mers can be produced with fairly good yields. Average coupling yields per step are usually 98% to 99+% depending on the building blocks used.

Time Considerations

Using the protocol in this unit, the total time for the machine-assisted part of synthesis of a 20-mer and 50-mer RNA is 4 hr 13 min and 9 hr 40 min, respectively. The total time will depend upon protecting groups, linker to support, deprotection conditions, and purification procedures used.

The procedure should not be stopped before the oxidation step, because the *H*-phosphonate linkages are rather labile. One can take a break after oxidation. At that point, the oligonucleotide is more stable than at the corresponding stage in the phosphoramidite method, since phosphodiesters are obtained after this step. The support-bound oligonucleotide can be stored at –20°C until the ammonolysis step is carried out to release the oligonucleotide from the support and remove the *N*-acyl protection.

Literature Cited

Agrawal, S. and Tang, J.-Y. 1990. Efficient synthesis of oligoribonucleotide and its phosphorothioate analog using H-phosphonate approach. *Tetrahedron Lett.* 31:7541-7544.

Almer, H., Stawinski, J., and Strömberg, R. 1996. Solid support synthesis of all Rp-oligoribonucleoside phosphorothioates. *Nucl. Acids Res.* 24:3811-3820.

Andrus, A., Efcavitch, J.W., McBride, L.J., and Giusti, B. 1988. Novel activating and capping reagent for improved hydrogen-phosphonate DNA synthesis. *Tetrahedron Lett.* 29:861-864.

Beaucage, S.L. and Caruthers, M.H. 1981. Deoxynucleoside phosphoramidites—a new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.* 22:1859-1862.

Beaucage, S.L. and Iyer, R.P. 1993. The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron* 49:6123-6194.

Chaix, C., Molko, D., and Teoule, R. 1989. The use of labile base protecting groups in oligoribonucleotide synthesis. *Tetrahedron Lett.* 30:71-74.

Corby, N.S., Kenner, G.W., and Todd, A.R. 1952. Nucleotides. Part XVI. Ribonucleoside-5′ phosphites. A new method for the preparation of mixed secondary phosphites. *J. Chem. Soc.* 3669-3675.

Dan'kov, Y.V., Batchikova, N.V., Scaptsova, N.V., Besidsky, E.S., and Azhayev, A.V. 1988. H-Phosphonate solid-phase synthesis of oligodeoxyribonucleotides in syringe. *Bioorg. Khim.* 14:615-620.

Efimov, V.A. and Dubey, I.Y. 1990. Modification of the H-phosphonate oligonucleotide synthesis on polymer support. *Bioorg. Khim.* 16:211-218.

Efimov, V.A., Dubey, I.Y., and Chakhmakhcheva, O.G. 1990. NMR study and improvement of H-phosphonate oligonucleotide synthesis. *Nucleosides Nucleotides* 9:473-477.

Efimov, V.A., Kalinkina, A.L., and Chakhmakhcheva, O.G. 1993. Dipentafluorophenyl carbonate–a reagent for the synthesis of oligonucleotides and their conjugates. *Nucl. Acids Res.* 21:5337-5344.

Földesi, A., Balgobin, N., and Chattopadhyaya, J. 1989. Synthesis of a "branched" trinucleotide using the H-phosphonate chemistry. *Tetrahedron Lett.* 30:881-884.

Froehler, B.C. 1986. Deoxynucleoside H-phosphonate diester intermediates in the synthesis of internucleotide phosphate analogs. *Tetrahedron Lett.* 27:5575-5578.

Froehler, B.C. and Matteucci, M.D. 1986. Nucleoside H-phosphonates: Valuable intermediates in the synthesis of deoxyoligonucleotides. *Tetrahedron Lett.* 27:469-472.

Froehler, B.C. and Matteucci, M.D. 1987. The use of nucleoside H-phosphonates in the synthesis of deoxyoligonucleotides. *Nucleosides Nucleotides* 6:287-291.

Froehler, B.C., Ng, P.G., and Matteucci, M.D. 1986. Synthesis of DNA via deoxynucleoside H-phosphonate intermediates. *Nucl. Acids Res.* 14:5399-5407.

Gaffney, B.L. and Jones, R.A. 1988. Large-scale oligonucleotide synthesis by the H-phosphonate method. *Tetrahedron Lett.* 29:2619-2622.

Garegg, P.J., Regberg, T., Stawinski, J., and Strömberg, R. 1985. Formation of internucleotidic bond via phosphonate intermediates. *Chem. Scr.* 25:280-282.

Garegg, P.J., Lindh, I., Regberg, T., Stawinski, J., Strömberg, R., and Henrichson, C. 1986a. Nucleoside H-phosphonates. III. Chemical synthesis of oligodeoxyribonucleotides by the hydrogenphosphonate approach. *Tetrahedron Lett.* 27:4051-4054.

Garegg, P.J., Lindh, I., Regberg, T., Stawinski, J., Strömberg, R., and Henrichson, C. 1986b. Nucleoside H-phosphonates. IV. Automated solid phase synthesis of oligoribonucleotides by the hydrogenphosphonate approach. *Tetrahedron Lett.* 27:4055-4058.

Garegg, P.J., Regberg, T., Stawinski, J., and Strömberg, R. 1986c. Nucleoside hydrogenphosphonates in oligonucleotide synthesis. *Chem. Scr.* 26:59-62.

Garegg, P.J., Henrichson, C., Lindh, I., Regberg, T., Stawinski, J., and Strömberg, R. 1987a. Automated solid phase synthesis of DNA and RNA fragments by the hydrogenphosphonate approach. *In* Biophosphates and Their Analogs—Synthesis, Structure, Metabolism and Activity (K.S. Bruzik and W.J. Stec, eds.) pp. 93-98. Elsevier/North-Holland, Amsterdam.

Garegg, P.J., Regberg, T., Stawinski, J., and Strömberg, R. 1987b. Nucleoside H-phosphonates. VII. Studies on the oxidation of nucleoside hydrogenphosphonate esters. *J. Chem. Soc. Perkin Trans.* I:1269-1273.

Garegg, P.J., Regberg, T., Stawinski, J., and Strömberg, R. 1987c. Nucleosides H-phosphonates. V. The mechanism of hydrogenphosphonate diester formation using acyl chlorides as coupling agents in oligonucleotide synthesis by the hydrogenphosphonate approach. *Nucleosides Nucleotides* 6:655-662.

Garegg, P.J., Stawinski, J., et al. 1987d. Nucleoside H-phosphonates. VI. Reaction of nucleoside hydrogenphosphonates with arenesulfonyl chlorides. *J. Chem. Soc. Perkin Trans.* II:1209-1214.

Garegg, P.J., Stawinski, J., and Strömberg, R. 1987e. Nucleoside H-phosphonates. VIII. activation of hydrogenphosphonate monoesters by chlorophosphates and aryl sulfonyl derivatives. *J. Org. Chem.* 52:284-287.

Gryaznov, S.M. and Potapov, V.K. 1990. New approach in synthesis of natural and modified oligodeoxyribonucleotides by H-phosphonate method. *Bioorg. Khim.* 16:1419-1422.

Hall, R.H., Todd, A., and Webb, R.F. 1957. Nucleotides. Part XLI. Mixed anhydrides as intermediates in the synthesis of dinucleoside phosphates. *J. Chem. Soc.* 3291-3296.

Hammond, P.R. 1962. A simple preparation of alkyl ammonium phosphonates and some comments on the reaction. *J. Chem. Soc.* 2521-2522.

Hata, T. and Sekine, M. 1974. Silyl phosphites. I. The reaction of silyl phosphites with diphenyl disulphides. Synthesis of S-phenyl nucleoside phosphorothioates. *J. Am. Chem. Soc.* 96:7363-7364.

Jäger, A., Charubala, R., and Pfleiderer, W. 1987. Synthesis and characterization of deoxy and ribo H-phosphonate dimers. *Nucl. Acids Symp. Ser.* 18:197-200.

Synthesis of Unmodified Oligonucleotides

3.4.13

Kung, P.-P. and Jones, R.A. 1992. H-phosphonate DNA synthesis without amino protection. *Tetrahedron Lett.* 33:5869-5872.

Kuyl-Yeheskiely, E., Spierenberg, M., van den Elst, H., Tromp, M., van der Marel, G.A., and van Boom, J.H. 1986. Reaction of pivaloyl chloride with internucleosidic H-phosphonate diesters. *Recl. Trav. Chim. Pays-Bas* 105:505-506.

Matteucci, M.D. and Caruthers, M.H. 1981. Synthesis of deoxyoligonucleotides on a polymer support. *J. Am. Chem. Soc.* 103:3185-3191.

Nylén, P. 1937. Die Kinetik der Verseifung von Dialkylphosphiten. II. Allgemeine Säure- und Basekatalyse bei der Hydrolyse von Diäthylphosphit. *Svensk Kem. Tidskr.* 49:79-96.

Nylén, P. 1938. Die Säure- und Basekatalyse bei der Reaktion zwischen Dialkylphosphiten und Jod. *Z. Anorg. Allg. Chem.* 235:161-182.

Ramzaeva, N., Mittelbach, C., and Seela, F. 1997. 7-deazaguanine DNA: Oligonucleotides with hydrophobic or cationic side chains. *Helv. Chim. Acta* 80:1809-1822.

Regberg, T., Stawinski, J., and Strömberg, R. 1988. Nucleoside H-phosphonates. IX. Possible side-reactions during hydrogenphosphonate diester formation. *Nucleosides Nucleotides* 7:23-35.

Rozners, E., Kumpins, V., Rekis, A., and Bizdena, E. 1988. Solid phase synthesis of oligoribonucleotides by the H-phosphonate method using 2′-O-benzoyl protective group. *Bioorg. Khim.* 14:1580-1582.

Rozners, E., Rekis, A., Kumpins, V., and Bizdena, E. 1990. Synthesis of oligoribonucleotides by the H-phosphonate method using base-labile 2′-O-protecting groups. II. Some aspects of use of 2′-O-benzoyl and anisoyl protecting groups. *Bioorg. Khim.* 16:1531-1536.

Rozners, E., Renhofa, R., Petrova, M., Popelis, J., Kumpins, V., and Bizdena, E. 1992. Synthesis of oligoribonucleotides by the H-phosphonate approach using base labile 2′-O-protecting groups. 5. Recent progress in development of the method. *Nucleosides Nucleotides* 11:1579-1593.

Rozners, E., Westman, E., and Strömberg, R. 1994. Evaluation of 2′-hydroxyl protection in RNA-synthesis using the H-phosphonate approach. *Nucl. Acids Res.* 22:94-99.

Rozners, E., Westman, E., Stawinski, J., Garegg, P.J., Bizdena, E., and Strömberg, R. 1998. Oligoribonucleotide synthesis with H-phosphonates. An improved procedure with base labile N-acyl protection and two alternative 2′-O-protecting groups. Manuscript in preparation.

Sakatsume, O., Ohtsuka, M., Takaku, H., and Reese, C.B. 1989. Solid phase synthesis of oligoribonucleotides using the 1-[2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp) group for the protection of the 2′-hydroxy functions and the H-phosphonate approach. *Nucl. Acids Res.* 17:3689-3696.

Sakatsume, O., Yamane, H., Takaku, H., and Yamamoto, N. 1990. Use of new phospho-nylating and coupling agents in the synthesis of oligodeoxyribonucleotides via the H-phosphonate approach. *Nucl. Acids Res.* 18:3327-3331.

Seela, F. and Wei, C.F. 1997. 7-Deazaisoguanine quartets: Self-assembled oligonucleotides lacking the Hoogsteen motif. *Chem. Commun.* 1869-1870.

Seliger, H. and Rösch, R. 1990. Simultaneous synthesis of multiple oligonucleotides using nucleoside H-phosphonate intermediates. *DNA Cell Biol.* 9:691-696.

Stawinski, J. and Strömberg, R. 1993. H-phosphonates in oligonucleotide synthesis. *Trends Org. Chem.* 4:31-67.

Stawinski, J., Strömberg, R., Thelin, M., and Westman, E. 1988. Studies on the *t*-butyldimethylsilyl group as 2′-O-protection in oligoribonucleotide synthesis via the H-phosphonate approach. *Nucl. Acids Res.* 16:9285-9298.

Stawinski, J., Strömberg, R., and Westman, E. 1991a. Ribonucleoside H-phosphonates. Pyridine vs quinoline–influence on condensation rate. *Nucleosides Nucleotides* 10:519-520.

Stawinski, J., Strömberg, R., and Westman, E. 1991b. Studies on reaction conditions for ribonucleotide synthesis via the H-phosphonate approach. *Nucl. Acids Symp. Ser.* 24:228.

Stein, A., Iversen, P.L., Subasinghe, C., Cohen, J.S., Stec, W.J., and Zon, G. 1990. Preparation of ^{35}S-labelled polyphosphorothioate oligodeoxyribonucleotides by use of hydrogenphosphonate chemistry. *Anal. Biochem.* 188:11-16.

Strömberg, R. 1987. Nucleoside H-phosphonates. Chemical studies directed towards oligonucleotide synthesis. *Chem. Commun. Stockholm Univ.* 1:1-54.

Strömberg, R. and Stawinski, J. 1987. Evaluation of some new condensing reagents for hydrogenphosphonate diester formation. *Nucl. Acids Symp. Ser.* 18:185-188.

Tanaka, T., Tamatsukuri, S., and Ikehara, M. 1987. Solid phase synthesis of oligoribonucleotides using the o-nitrobenzyl group for 2′-hydroxyl protection and H-phosphonate chemistry. *Nucl. Acids Res.* 15:7235-7248.

Vasser, M., Ng, P.G., Jhurani, P., and Bischofberger, N. 1990. Error rates in oligodeoxynucleotides synthesized by the H-phosphonate method. *Nucl. Acids Res.* 18:3089.

Wada, T., Sato, Y., Honda, F., Kawahara, S., and Sekine, M. 1997. Chemical synthesis of oligodeoxyribonucleotides using N-unprotected H-phosphonate monomers and carbonium and phosphonium condensing reagents: O-Selective phosphonylation and condensation. *J. Am. Chem. Soc.* 119:12710-12721.

Westheimer, F.H., Huang, S., and Covitz, F. 1988. Rates and mechanisms of hydrolysis of esters of phosphorous acid. *J. Am. Chem. Soc.* 110:181-185.

Synthesis of
Oligonucleotides
According to the
H-Phosphonate
Method

3.4.14

Westman, E. and Strömberg, R. 1994. Removal of *t*-butyldimethylsilyl protection in RNA-synthesis. Triethylamine trihydrofluoride (TEA, 3HF) is a more reliable alternative to tetrabutylammonium fluoride (TBAF). *Nucl. Acids Res.* 22:2430-2431.

Westman, E., Stawinski, J., and Strömberg, R. 1993. RNA-synthesis using H-phosphonates. Synchronizing 2′-OH and N-protection. *Collect. Czech. Chem. Commun.* 58:236-237.

Westman, E., Sigurdsson, S., Stawinski, J., and Strömberg, R. 1994. Improving the H-phosphonate approach to oligoribonucleotide synthesis. *Nucl. Acids Symp. Ser.* 31:25.

Wu, T. and Ogilvie, K.K. 1988. N-Phenoxyacetylated guanosine and adenosine phosphoramidites in the solid phase synthesis of oligoribonucleotides: synthesis of a ribozyme sequence. *Tetrahedron Lett.* 29:4249-4251.

Wu, T. and Ogilvie, K.K. 1990. A study on the alkylsilyl groups in oligoribonucleotide synthesis. *J. Org. Chem.* 55:4717-4724.

Contributed by Roger Strömberg
Karolinska Institute
Stockholm, Sweden

Jacek Stawinski
Stockholm University
Stockholm, Sweden

**Synthesis of
Unmodified
Oligonucleotides**

3.4.15