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This paper is dedicated to Professr Maciej Wiewiórowski
Review

Some aspects of oligoribonucleotides synthesis *via* the H-phosphonate approach°

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This review gives a short account of selected aspects of oligoribonucleotide synthesis *via* the H-phosphonate method. It includes: (i) recent methods for the preparation of suitably protected ribonucleoside 3'-H-phosphonates (the phosphonylation step), (ii) some chemical and stereochemical features of the formation of H-phosphonate internucleosidic linkages, and (iii) stereoselective synthesis of oligoribonucleoside phosphorothioates using chemo-enzymatic approach.

RNAs are a highly versatile class of molecules with a diverse and broad range of biological activities [1]. Although their main role is usually connected with a translation process in which they participate as messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), these molecules also play other important roles, as e.g. storing genetic information (e.g. in RNA viruses), participating in processing of mRNA precursors (as small nuclear RNAs, snRNA), or facilitating transport of proteins through phos-

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Abbreviations: t-BDMS, t-butyldimethylsilyl group; o-ClBz, *o*-chlorobenzoyl; Ctmp, 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl; o-NBn, *o*-nitrobenzyl group; rRNA, ribosomal RNA; THP, tetrahydropyranyl.

pholipid membranes (as 7S RNA in a complex with a protein). In addition, RNAs can exhibit enzymatic activity, either *per se* (e.g. in self-splicing) [2] or in combination with protein (e.g. in ribonuclease P) [3], or to act as small-molecule receptors [4, 5], that specifically bind to a target molecule.

This functional diversity of RNA caused high demand for synthetic oligoribonucleotides with defined sequences to be used as tools in a variety of chemical, biochemical, and biophysical investigations directed towards better understanding of physicochemical basis of the interaction of RNA with different types of substrates [5–8].

RNA fragments are exceedingly complex molecules from the point of view of their susceptibility to internucleotide bond cleavage [9, 10]. They undergo fast hydrolytic degradation under basic conditions and the 3′→2′ migration of the phosphoryl group under acidic ones [11, 12]. The latter process is always accompanied by a partial degradation of an oligonucleotidic chain. Intramolecular catalysis is apparently not confined to the participation of the neighbouring 2′-OH group, but may involve also some distant parts of the molecule, that labilise internucleotide linkages [13]. These potential problems in handling occur irrespective of the origin of RNA fragments (isolated from natural sources or synthesized chemically or enzymatically).

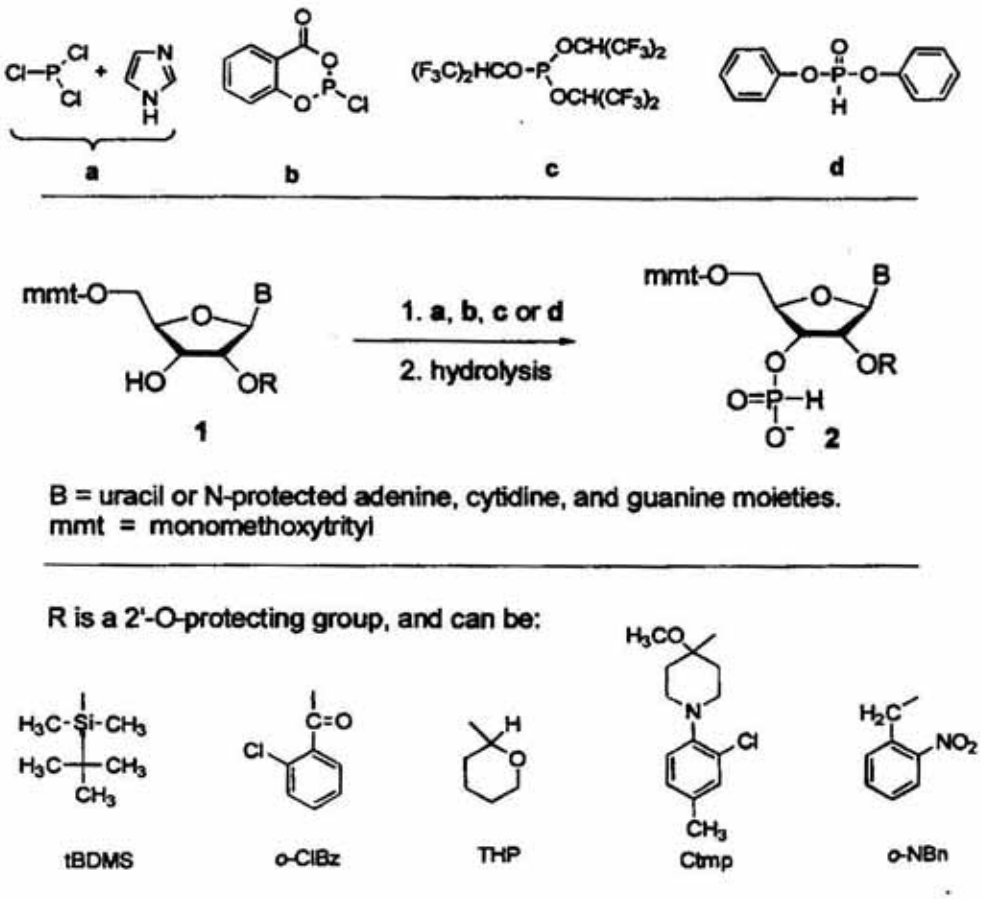
The development of synthetic methods for the preparation of oligo*ribo*nucleotides has always been dragging behind that for oligo*deoxy*ribonucleotides, largely due to the added synthetic complications caused by the presence of the vicinal 2′-hydroxyl group in these compounds. RNA fragments can, in principle, be prepared by the phosphodiester [14], phosphotriester [15, 16], phosphoramidite [17, 18], and H-phosphonate methodology [19, 20], but the effectiveness of these approaches varies. Irrespective of the method used, the choice of the 2′-OH protecting group is of prime importance from the synthetic point of view [21]. The chemical nature of this group often affects the efficiency and rate of the formation of an internucleotide bond, may suppress or stimulate the occurrence of side reactions during condensation and during final deprotection, and finally, its choice usually determines a synthetic strategy in terms of other protecting groups to be used.

In this review we will confine our considerations to the synthesis of oligoribonucleotides *via* the H-phosphonate approach and discuss two selected aspects of this methodology, namely, (i) the preparation of the appropriate building blocks (the phosphonylation step) and (ii) some characteristic features connected with the formation and further transformations of H-phosphonate internucleosidic linkages.

PREPARATION OF STARTING NUCLEOTIDIC UNITS, RIBONUCLEOSIDE 3′-H-PHOSPHONATES

Since no consensus has yet emerged as to the best 2′-OH protecting group, various building blocks have been proposed for oligoribonucleotide synthesis *via* the H-phosphonate approach [22]. These include derivatives of type **2** bearing in the 2′-position a fluoride-labile silyl group (usually the t-butyldimethylsilyl group, tBDMS) [19], acid-sensitive acetal groups [e.g. tetrahydropyranyl, (THP) [23], 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp)] [24], base-labile benzoyl derivatives [e.g. *o*-chlorobenzoyl (o-ClBz)] [25], and a photolabile *o*-nitrobenzyl group (o-NBn) [26]. In this paper we will only discuss a synthetic step which is specific for the H-phosphonate methodology, namely, the phosphitylation or phosphonylation of suitably protected nucleosidic precursors **1** to produce ribonucleoside 3′-H-phosphonates **2** as starting materials for the oligonucleotide synthesis (Scheme 1).

Although the phosphitylating/phosphonylating reagents **a** [19], **b** [27], **c** [24], and **d** [28] (Scheme 1) are rather universal, ribonu-



Scheme 1.

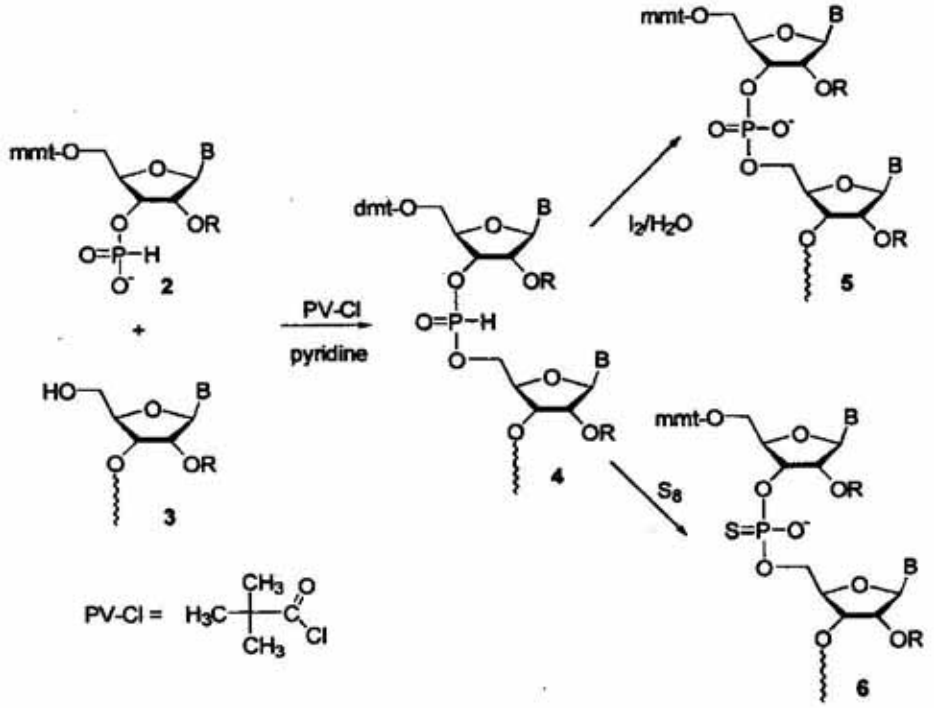
cleoside 3′-H-phosphonates **2** bearing different 2′-OH protecting groups, are usually prepared using different phosphonylating agents. Thus, for **2** with tBDMS group, PCl_3/azole (**a**) [19], or diphenyl H-phosphonate (**d**) [28] were used, for **2** with *o*-chlorobenzoyl group, reagent **a** [25], for **2** with 2′-OTPH group, reagent **a** or salicylchlorophosphite **b** [29], for **2** with *o*-nitrobenzyl group, reagent **a** [26], and for **2** with an acetal-type of 2′-OH protection (group Ctmp), phosphite **c** [24] was claimed to give superior results.

Since most of the proposed reagents give comparable yields of product **2** and the reaction conditions are usually mild, a decisive factor in choosing a particular phosphonylating reagent is usually its accessibility and the experimental simplicity of the procedure involved. From this point of view, cheap, commercially available, and easy to use diphenyl H-phosphonate **d** can be a reagent of choice for most applications. With this reagent, the phosphonylation of ribonucleosides **1** is usually complete within a few minutes, and after hydrolysis and aqueous work-up, ribonucleoside H-phosphonates **2** (R = tBDMS) can be obtained in a 75–90% yield after silica gel column chromatography [28]. Since the 2′,3′-migration of some protecting groups in **1** may occur during the course of the reaction [30–33], precaution should always be taken to check isomeric purity of the produced H-phosphonates **2**.

SYNTHESIS OF OLIGORIBONUCLEOTIDES *VIA* THE H-PHOSPHONATE METHOD

The H-phosphonate method for the synthesis of oligonucleotides [34–36] is based on the reaction of a suitably protected nucleoside 3′-

H-phosphonate (e.g. **2**) with a nucleosidic component (e.g. **3**) in the presence of a condensing agent, followed by oxidation of the produced H-phosphonate diesters **4** (Scheme 2). By changing the oxidation conditions, various heteroatoms (e.g. O, N, S, Se) can be introduced into the non-bridging position of the H-phosphonate diester bond to form oligonucleotides with a natural (e.g. phosphodiester **5**) or modified phosphorus centre (e.g. phosphorothioate **6**). The most commonly used condensing agent is pivaloyl chloride, although an array of other coupling agents have been developed for this purpose [20]. Generally recognized advantages of the H-phosphonate methodology are: (i) nucleoside H-phosphonates are stable and easy to handle solids, (ii) protection at the phosphorus center is not required, (iii) oxidation can be performed as one synthetic step after the assembly of an oligonucleotidic chain is complete, (iv) an easy access to oligonucleotide analogues by changes in the oxidation step.



Scheme 2.

For other abbreviations see Scheme 1.

There are two processes which potentially may lower the efficiency of synthesis of oligonucleotides *via* the H-phosphonate approach. These are (i) double activation of the starting material **2** by a condensing agent [37] and (ii) P-acylation of the produced H-phosphonate internucleotide bond [38, 39]. The first process generates tervalent nucleoside bisacyl phosphites, which are less reactive in the condensation reaction than the initially formed phosphono-carboxylic anhydrides [37]. Due to this, side reactions (e.g. acylation of a nucleosidic component) may become more pronounced. The second process generates P-acylphosphonates that are formed in the reaction of the produced H-phosphonate diesters with pivaloyl chloride. Acylphosphonates are resistant towards oxidation, but regenerate a H-phosphonate function during basic deprotection of oligonucleotides. Since H-phosphonate diesters are very labile under these conditions, they immediately break down leading to the scission of an oligonucleotidic bond.

These processes which, in principle, are not observed in solution synthesis but may occur under solid phase conditions, can be attenuated by a proper choice of solvents (usually less basic solvents) or/and a suitable condensing agent [37, 40, 41].

In oligoribonucleotide synthesis, due to the presence of a bulky 2′-OH protecting group in vicinity of the H-phosphonate function, both double activation of H-phosphonate monoesters **2** and the P-acylation reaction of **4**, are heavily suppressed [42, 43]. This makes the H-phosphonate methodology particularly suited for the preparation of RNA fragments and their analogues [19, 44, 45]. There is also another feature, rather unique for the H-phosphonate methodology. Despite the presence of 2′-OH protecting group, which certainly may pose some steric hindrance for the incoming nucleosidic component, the condensation reaction in the ribo series is virtually as fast and efficient as that for DNA synthesis [19, 46].

A synthetic protocol for oligoribonucleotide synthesis *via* the H-phosphonate approach is usually simple [20] and involves two chemical steps in the elongation cycle: (i) deprotection of the 5′-OH position in the growing oligonucleotidic chain (usually, the removal of a monomethoxytrityl group under acidic conditions) and (ii) formation of the internucleotidic bond between a support-bounded oligonucleotide and activated nucleoside H-phosphonate monoesters. After completion of the desired number of elongation cycles, the oligonucleotide bearing all H-phosphonate internucleotide linkages is converted in one oxidation step into a compound containing natural internucleotide bonds (phosphodiesters, oxidation with iodine-water) [19] or to an oligonucleoside phosphorothioate, if sulfur is used as an oxidant [44, 47]. The produced oligonucleotide (or oligonucleotide analogue) is split from the support and the protecting groups are removed using standard procedures, which are practically the same for all methods for oligonucleotide synthesis.

STEREOCHEMICAL ASPECTS

Since the phosphorus center in H-phosphonate diesters (e.g. **4**, Scheme 2) is chiral, it gives rise to 2^n (n = number of internucleotide bonds) P-diastereomers. When ^{16}O is introduced to **4** during oxidation to produce phosphodiester **5**, chirality at the phosphorus center vanishes. In all other instances, chirality at the phosphorus center is preserved after oxidation, and a product (e.g. phosphorothioate **5**) is usually obtained as a mixture of 2^n diastereomers.

The following observations have been made concerning stereoselectivity in the formation of dinucleoside H-phosphonates. The two diastereomers, R_P and S_P, are formed in almost equal amounts when a condensation is carried out between a deoxynucleoside H-phosphonate and an alcohol (e.g. a nucleoside) [37]. However, with protected ribonucleoside 3′-H-phosphonates **2** (R= tBDMS) the formation of one diastereomer is always favored (about 80–85% of the diastereomer resonating at lower field in ^{31}P NMR) [47, 48]. This stereoselectivity is most likely due to steric influence from the 2′-O-protecting group and was found to be independent of the nature of the coupling agent used [49]. Since the stereoselectivity varied depending on the kind of nucleobase and reaction conditions used (especially the nature and concentration of a base/nucleophile catalyst is of crucial importance), it seems that there are several factors governing the preferential formation of one diastereomer [49, 50].

Since the number of P-diastereomers produced during the synthesis of even a medium size oligonucleotide of type **6** is enormous (e.g. for an oligonucleotide containing 20 internucleotidic bonds, over 10^6 different species can be formed), one cannot isolate a

stereochemically homogeneous species from such reaction mixtures. However, the observed stereoselectivity in the formation of the H-phosphonate bond during oligoribonucleotide synthesis, can be exploited e.g. for the preparation of short, uniformly modified oligoribonucleoside phosphorothioates, containing all R_P stereochemistry at phosphorus centers [50, 51]. The method consists of three stages. In the first one, an oligoribonucleotide of type **4** (R= tBDMS) with predominantly S_P-configured H-phosphonate linkages, is produced on solid phase. The second stage involves sulfurization (**4**→**6**), and because this is a stereospecific process [47], phosphorothioates enriched in R_P diastereomers are obtained. After splitting from the support and removing the protecting group, the crude reaction mixture is treated in the third stage with nuclease P1 from *Penicillium citrinum*, an enzyme that preferentially degrades S_P phosphorothioate linkages. The full length oligoribonucleotide fragment with uniform R_P stereochemistry is then separated from shorter fragments using the reversed phase HPLC technique. The practical limit of this method is set by the stereoselectivity of the H-phosphonate coupling (72–89%), which at present makes it possible to prepare all R_P-oligoribonucleoside phosphorothioate sequences containing up to 12 nucleotidic units [50, 51].

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