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H-Phosphonate oligonucleotides from phosphoramidite chemistry

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Abstract—A novel phosphoramidite methodology allowing the formation of *H*-phosphonate oligonucleotides was employed to introduce backbone modifications into oligonucleotides. Novel *N*,*N*-diisopropylamino-*para*-methoxybenzylphosphoramidites were prepared and used in a two-step synthesis cycle during the elongation process. The coupling step was directly followed by an acidic treatment performing concomitant deprotection and phosphite deprotection through an Arbuzov reaction to yield an *H*-phosphonate linkage.
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During the past years, the development of the antisense and antigene approaches using synthetic oligonucleotides interfering with gene expression led to the emergence of various chemical modifications to improve their nuclease resistance and their cellular uptake. Particularly, substantial attention was focused on modifications of the internucleoside linkages.

The phosphoramidite[1] and the *H*-phosphonate[2] chemical approaches, have appeared as the methods of choice to rapidly synthesize on solid support these phosphorus oligonucleotide analogues. Compared to the phosphoramidite chemistry where an oxidation of phosphite triester intermediates is performed after each coupling step, the *H*-phosphonate method appears more simple and versatile. Indeed, the oxidation of *H*-phosphonate diester linkages is carried out once at the end of the elongation process with the advantage that a common intermediate could give rise to oligonucleotides bearing various phosphorus linkages,[3] including phosphate-,[4] phosphorothioate-,[5] phosphoroselenoate-,[6] boranophosphate-,[7] and phosphoramidate diester[8,9] derivatives as well as chimeric oligonucleotides.[10]

However, the *H*-phosphonate method also has some disadvantages due to possible side reactions[11] mostly occurring during the coupling of a nucleoside *H*-phosphonate monoester with a free 5′-hydroxyl supported oligonucleotide using an acyl chloride (e.g., pivaloyl chloride[12]) as activator. If preactivation time of the *H*-phosphonate monoester with the acyl chloride is too long, bis P-acylation occurs resulting in low efficiency. O-Acylation of the 5′-hydroxyl reactant and P-acylation of the desired *H*-phosphonate diester are other sources of inefficiency of the *H*-phosphonate methodology. These are reasons why the more accessible and successful phosphoramidite approach remains the preferred method for the synthesis of oligonucleotides.

We decided to explore a phosphoramidite approach yielding oligonucleotides *H*-phosphonate diesters as versatile intermediates giving access to various phosphorus modifications.

Oxidation of a newly generated phosphite triester linkage is considered as an indispensable step in the elongation process of an oligonucleotide by the phosphoramidite approach. Attempts to postpone this oxidation at the end of the elongation process resulted in uncharacterized products.[13] Without oxidation, the phosphite triester linkages decompose under the acidic conditions, that is; trichloroacetic or dichloroacetic acid, used for the cleavage of the dimethoxytrityl group.

Regarding the literature concerning acidic hydrolysis of trialkylphosphites proceeding to dialkylphosphites (*H*-phosphonate diesters),[14] in the case of oligonucleotide phosphite degradation, the unshared electron pair of phosphorus atom of the phosphite is protonated to form a phosphonium salt (i.e., a protonated phosphite). Then, the nucleophilic attack of dichloroacetate ion on the phosphorus atom of the phosphonium ion gives rise to

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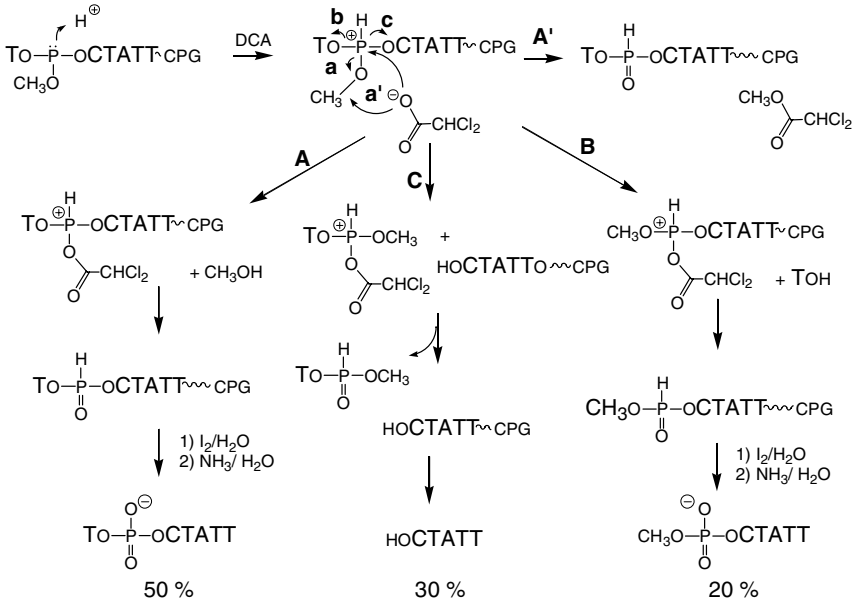
the elimination of one of the three alcohol substituents (Scheme 1, pathways A, B, and C) and to the corresponding unstable mixed anhydrides, which readily decompose to dialkyl *H*-phosphonates.

In confirmation of these pathways, we synthesized on solid support a short oligonucleotide d(TCTATT) with standard cyanoethyl-protected phosphoramidites for the first four coupling steps and a regular incorporation cycle (coupling, capping, iodine/water oxidation, detritylation). The last coupling step was performed with a methyl phosphoramidite followed by the detritylation step with dichloroacetic acid and then by the iodine–water oxidation. After regular ammonia deprotection, reverse-phase HPLC (Fig. 1A) showed three main compounds from which one corresponded to the full-length phosphodiester hexamer d(TCTATT) (t_R 21.5 min, m/z calcd 1756.2, 50% of the mixture). Following the mechanism described above, this compound should result from methanol cleavage (Scheme 1, pathway A) during acidic hydrolysis of the phosphite followed by oxidation of the resulting *H*-phosphonate diester. The two other compounds, the pentamers 5′-methyl phosphodiester d(CTATT) (t_R 19.4 min, m/z calcd 1546.0) and 5′-OH d(CTATT) (t_R 20.0 min, m/z calcd 1452.0) resulted from internucleoside bond cleavages (Scheme 1, pathways B and C, 20% and 30%, respectively). The identity of these three compounds was determined by MALDI-TOF mass spectrometry. The ratio between the three compounds in the mixture could be explained by the difference of the pK_a of the leaving alcohols.

However, another pathway where the dichloroacetate ion preferentially attacks the methyl group (Scheme 1, pathway A′), as observed during iodine/water oxidation of methylphosphite triesters (S_N2 mechanism)[15] might take place. Following this pathway, the acidic hydrolysis of the methylphosphite triester linkage gives rise to an *H*-phosphonate linkage. Interestingly, this Arbuzov-type reaction might explain why the full-length oligonucleotide is the main compound of the mixture.



Scheme 1. Acidic degradation of a methylphosphite triester linkage in the oligonucleotide model d(TCTATT).

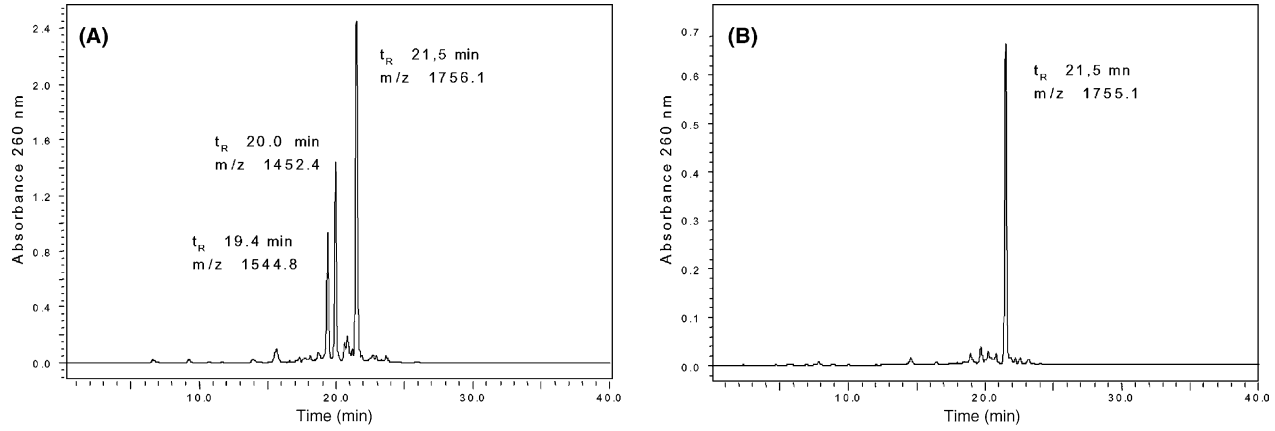


Figure 1. Reverse-phase HPLC chromatograms of the crudes resulting from the synthesis of d(TCTATT), (A) using dT methylphosphoramidite for the last coupling, (B) using dT *para*-methoxybenzylphosphoramidite for the last coupling.

To favor the removal of the protecting group avoiding internucleoside bond cleavage, we replaced then the methyl group with a *para*-methoxybenzyl group. Indeed, benzyl and substituted benzyl-protected nucleoside phosphoramidites have previously been applied to the synthesis of internucleoside phosphodiester analogues under diverse range of Arbuzov-type reaction conditions.[16,17] Considering Arbuzov-type reactions, it was reported by Nielsen and Caruthers that alkyl deoxynucleoside phosphites having alkyl groups, that is, tertiary alkyl or benzyl groups, which stabilize the S_N1 character of the second stage of the Arbuzov reaction react selectively with most electrophiles and eliminate only the appropriate protecting group.[18] For example, it was shown that aqueous iodine oxidation of 2-cyano-1,1-dimethylethyl deoxydinucleoside phosphite and *o*-methylbenzyl deoxydinucleoside phosphite,[16] where iodine acts as the electrophile and water as the nucleophile involved in the Arbuzov-type reaction, yield the corresponding phosphate diesters with selective loss of the protecting group.

Due to this observation, we prepared 5′-*O*-dimethoxytrityl 3′-*O*-*p*-methoxybenzyl phosphoramidites from the corresponding phosphine.[19] The hexamer d(TCTATT) was then synthesized with standard cyanoethyl-protected phosphoramidites for the first four incorporations. The coupling step of the last *p*-methoxybenzyl phosphoramidite was followed by the detritylation step and then by the iodine–water oxidation. After regular ammonia deprotection, the reverse-phase HPLC of the crude (Fig. 1B) showed more than 98% of the desired full-length oligonucleotide (t_R 21.5 min) without significant cleavage of the oligonucleotide.

To illustrate the feasibility of the method, several phosphorus-modified oligonucleotides were synthesized. The phosphodiester and phosphorothioate dT_6 were prepared on LCAA-CPG from the *p*-methoxybenzyl phosphoramidite of dT. A two-step synthesis cycle was used for the elongation of the oligonucleotide. The first step was the condensation of the phosphoramidite with a 5′-OH nucleoside, covalently linked to the solid support through a standard succinyl linker, in the presence of 1-*H*-tetrazole as activator. The second step was an acidic treatment with 2% dichloroacetic acid in CH_2Cl_2 performing both 5′-hydroxyl and phosphite deprotection. Hence, the oligonucleotide with *H*-phosphonate internucleoside linkages was obtained. This elongation process was followed by an oxidation step with I_2/H_2O in presence of Et_3N or by a treatment with 2.5% S_8 in a CS_2/pyr/Et_3N (1:1:0.1, v/v/v) mixture[20] to, respectively, yield the phosphodiester and the phosphorothioate dT_6. The cleavage of the ODN from the solid support was achieved with a standard aqueous ammonia treatment. The crudes were analyzed by reverse-phase HPLC and MALDI-TOF MS (negative mode). Both HPLC chromatogram and mass spectrum of the phosphodiester dT_6 showed the hexamer as the main product (t_R 19.6 min, m/z calculated 1762.2) contaminated with compounds of lower retention times (t_R 18.9 and 19.3 min) corresponding to shorter sequences dT_4 and dT_5 and with compounds of higher retention times (t_R 23.8, 24.1, 25.6 min, m/z calculated 1866.4) corresponding to an hexamer containing a benzylphosphonate linkage instead of a phosphodiester linkage. Similarly, the HPLC and the mass spectrum (Fig. 2) of the phosphorothioate dT_6 revealed the contamination of the desired oligonucleotide (t_R 15.5–17.6 min, m/z calcd 1839.9) with a compound of higher retention time and higher mass (t_R 22.0 min, m/z calcd 1930.6) corresponding to a benzylphosphonate-containing oligonucleotide. In the case of the phosphorothioate dT_6, the broadness of the HPLC peaks was due to the diastereoisomers partially resolved.

These unwanted alkylation reaction products might derive from a reaction of the highly reacting *p*-methoxybenzyl carbocation formed during the second step of the Arbuzov-type reaction (Scheme 2, pathway A) with another starting *p*-methoxybenzylphosphite molecule, in a true Arbuzov reaction (Scheme 2, pathway B), generating undesirable *p*-methoxybenzylphosphonate linkages.[21]

Our chemical approach was also applied to the synthesis of a cationic dimethylaminopropyl (DMAP) phosphoramidate oligonucleotide. These oligonucleotides have

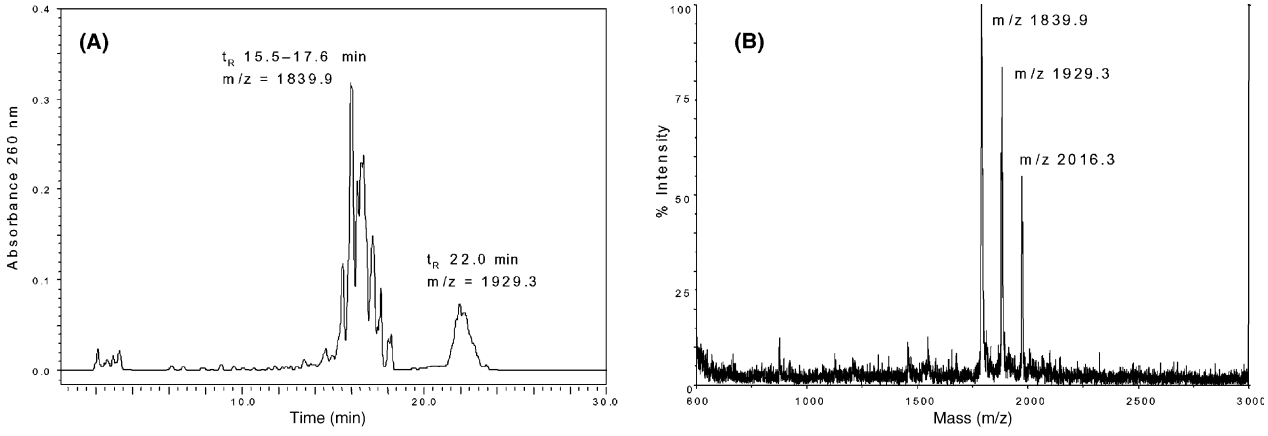
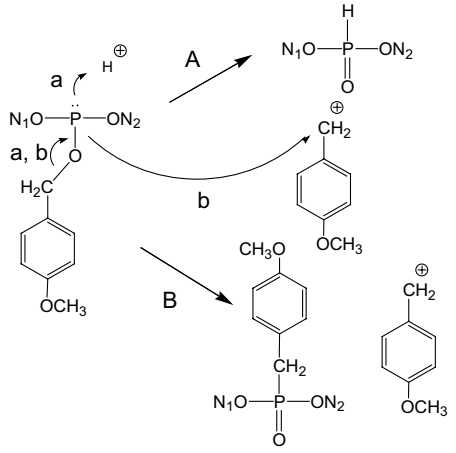


Figure 2. (A) Reverse-phase HPLC chromatogram and (B) MALDI-TOF mass spectrum of the crude resulting from the synthesis of the phosphorothioate dT_6.



Scheme 2. Arbuzov-type (a) and Arbuzov (b) reactions occurring toward the acidic treatment of a *p*-methoxybenzylphosphite triester giving rise to an *H*-phosphonate linkage (A) and to a benzylphosphonate linkage (B).

recently showed increasing interest.[22] Therefore, a PNHDMAP pentanucleotide d(TAGCT) was prepared on LCAA-CPG from *p*-methoxybenzyl phosphoramidites. The two-step synthesis cycle was followed with the oxidative amination of the *H*-phosphonate linkages in PNHDMAP links by CCl_4 in the presence of *N*-dimethylaminopropylamine. After deprotection and removal of the oligonucleotide from the solid support, the crude was analyzed by cationic exchange HPLC and MALDI-TOF MS (positive mode). Both HPLC chromatogram and mass spectrum showed a major compound corresponding to the desired PNHDMAP pentamer (t_R 11.2 min, m/z calcd 1815.7). Besides this pentamer, the mass spectrum showed other peaks, one of lower mass (m/z calcd 1737.5) resulting to an unwanted oxidation of one *H*-phosphonate linkage in a phosphodiester, and another one of higher mass (m/z calcd 1835.7) corresponding either to a sodium adduct or to the partial Arbuzov reaction on *p*-methoxybenzylphosphite triester intermediates yielding a benzylphosphonate linkage.

In conclusion, we reported here a new phosphoramidite method based on two-step synthesis cycle giving rise to *H*-phosphonate diester oligonucleotides allowing the preparation of various phosphate-modified oligonucleotide analogues. The acidic treatment required for the detritylation step allows conversion of the phosphite triester internucleosidic linkage in *H*-phosphonate linkage. Work is in progress to minimize side Arbuzov reaction.

Acknowledgements

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19. Synthesis of *p*-methoxybenzyloxy bis(diisopropyl-amino)-phosphane: Chlorobis(diisopropylamino)phosphane (3.5 g, 13 mmol) was added at 0 °C to a solution of *p*-methoxybenzyl alcohol (1.8 g, 13 mmol) and Et_3N (3.6 mL, 26 mmol) in dry Et_2O (50 mL). The resulting mixture was stirred at room temperature for 3 h, diluted with an Et_2O/Et_3N solution (9:1, v/v; 40 mL) and then filtered. The filtrate was concentrated to 2/3 and cyclohexane (40 mL) was added. The solution was concentrated once more. the crude was purified by silica gel chromatography. The desired phosphane was eluted with a cyclohexane/Et_3N solution (95:5, v/v). The appropriate fractions were combined, evaporated and co-evaporated with acetonitrile. Crystallization gave 4.2 g of phosphane (85% yield). R_f: 0.77 in toluene/Et_3N (9:1, v/v); mp: 54–56 °C; ^{31}P NMR ($CDCl_3$): δ 124.79 ppm.
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