In situ generation of methylphosphonamidites for synthesis of oligonucleotide methylphosphonates

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5′-DMT-nucleoside methylphosphonamidites are generated *in situ* by selective activation of methylbis-(pyrrolidino)phosphine 1, and used effectively in the automated synthesis of oligonucleotide methylphosphonates.

Methylphosphonates or the corresponding mixed backbone oligonucleotides, which contain non-ionic internucleotide linkages, have been successfully used in gene expression studies and have demonstrated their potential for development as antisense therapeutics.[1] Oligonucleotide methylphosphonates are generally prepared using methylphosphonamidite synthons. Since the methylphosphonate backbone is susceptible to hydrolysis by aqueous basic solutions, some modified methods have been developed to improve their synthesis.[2–4] However, a major limiting factor for cost efficient synthesis of oligonucleotide methylphosphonates is the time and cost required to synthesize and purify nucleoside methylphosphonamidites. One potential approach in solving this problem is to generate the methylphosphonamidite *in situ.* Although several laboratories have studied the *in situ* methodology for the syntheses of phosphodiester, phosphorothioate and radiolabelled oligonucleotides,[5–10] to the best of our knowledge none have used *in situ* methods for the synthesis of oligonucleotide methylphosphonates.

In an effort to develop oligonucleotide methylphosphonate analogues as antisense therapeutics, we have investigated the *in situ* approach in order to achieve an economical synthetic process. A series of methylphosphitylating reagents were prepared and examined.[11] We report herein our preliminary results for *in situ* generation of nucleoside methylphosphonamidites using a methylphosphordiamidite, methyl-bis-(pyrrolidino)phosphine[12]† **1**, for oligonucleotide methylphosphonates synthesis.

The *in situ* generation of nucleoside methylphosphonamidites was first studied using ^{31}P NMR spectroscopy. The reactions were carried out at room temperature using **1** and 5′-DMT-nucleosides (dA^{Bz}, dC^{Bz}, dG^{iBu} and T) in the presence of tetrazole. The results showed that although **1** was activated by 1 equiv. of tetrazole to react with 5′-DMT-nucleosides immediately, the reaction did not give the desired products (the corresponding 5′-DMT-nucleoside methylphosphonamidites) selectively under these conditions. The major side products were identified as 3′-3′ dimers.‡ To avoid this problem, a series of different reaction conditions were examined. The results show that the corresponding 5′-DMT-nucleoside methylphosphonamidites were formed exclusively by selective activation of **1** using 0.2–0.3 equiv. of tetrazole (Scheme 1). Typically, 0.10 ml (0.045 mmol) of a 0.45 M tetrazole solution in MeCN was added to a solution of 5′-DMT-thymidine (81.7 mg, 0.15 mmol) and **1** (28 mg, 0.15 mmol) in $CDCl_3$ (1.0 ml). After stirring for 10 min at room temperature, the solution was transferred into an NMR tube and examined by ^{31}P NMR spectroscopy. The NMR results indicated that the corresponding 5′-DMT-thymidine methylphosphonamidite **2-T** was formed in a ratio of 97 : 3 as compared to the 3′-3′ dimer. Similar results were obtained for the other 5′-DMT-nucleosides (dA^{Bz}, dC^{Bz} and dG^{iBu}).

B = dA^{Bz}, dC^{Bz}, dG^{ibu} and T
DMT = 4,4′-dimethoxytrityl

Scheme 1

To further evaluate this approach, we prepared *in situ* a 0.1 M solution of 5′-DMT-thymidine methyl(pyrrolidino)phosphonamidite **2-T** on a 5 mmol scale.§ To make certain that 5’-DMT- thymidine was exclusively transformed into **2-T**, the *in situ* amidite solution was examined using ^{31}P NMR spectroscopy. Since 5′-DMT-thymidine can not be detected in ^{31}P NMR spectroscopy, an internal standard had to be used to determine the amount (or concentration) of **2-T** generated. In this way, 0.5 ml of a 0.1 M solution of 5′-DMT-thymidine methyl(diisopropylamino)phosphonamidite **3-T** (PerSeptive Biosystems, Framingham, MA) in $CDCl_3$ was mixed with 0.5 ml of an *in situ* generated solution of **2-T**, and examined by ^{31}P NMR spectroscopy (Fig. 1).

Fig. 1 ^{31}P NMR analysis of the methylphosphonamidite **2-T** solution generated *in situ*

As Fig. 1 shows, integration for compounds **2-T** (δ 126) and **3-T** (δ 119) was the same, and the 3′-3′-dimer (δ 177) was formed in less than 3% yield. The NMR results indicated that 5′-DMT-thymidine was almost quantitatively transformed into **2-T**, and a 0.1 M solution of **2-T** was prepared *in situ.*

To evaluate their effectiveness in automated solid-phase DNA synthesis, a 16-mer of oligonucleotide methylphosphonate, 5′-d$T_sT_sT_sT_sT_sT_{Me}T_{Me}T_{Me}T_{Me}T_{Me}T_{Me}T_{Me}T_{Me}T_{Me}T_{Me}$T-3′ **16T**, was synthesized as a model to test the methylphosphonamidite generated *in situ*.¶ This oligomer consists of ten methylphosphonate linkages (PMe) at the 3′-end and five phosphorothioate linkages (PS) at the 5′-end. The coupling efficiency of *in situ* amidite **2-T** was studied by measuring

spectrophotometrically the dimethoxytrityl cation (λ 498 nm) released at each cycle and by analysing the final product. The synthesis was carried out using a 0.1 M solution of **2-T** prepared *in situ* (used in the first ten coupling cycles) followed by a commercial 5′-DMT-thymidine-2-cyanoethoxy(*N*,*N*-diisopropylamino)phosphoramidite **T**, (PerSeptive Biosystems). The commercial methylphosphonamidite **3-T** was also examined in the synthesis of **16T** as a comparison. The results showed that methylphosphonate linkages can be synthesized using **2-T** prepared *in situ* with an average stepwise yield of 98%. The coupling yield was the same as that of the commercial methylphosphonamidite **3-T**. The *in situ* amidite 2-T was also found to be stable for at least one week. The capillary gel electrophoresis (CE) result of **16T** synthesized using one week old *in situ* amidite solution was nearly identical to that of freshly prepared *in situ* amidite solution. The CE and ^{31}P NMR analysis results of the crude oligonucleotide **16T** are given in Figs. 2 and 3.

In summary, our studies show that the methylphosphordiamidite **1** can be selectively activated to react with deoxyribonucleosides, and chemoselectively generate the corresponding deoxyribonucleoside methylphosphonamidites *in situ*. The *in situ* methodology using **1** is easily adapted to the current phosphoramidite approach for the synthesis of oligonucleotide methylphosphonates. This method may find applications in large-scale syntheses of oligonucleotide methylphosphonates and in the synthesis of oligonucleotide methylphosphonate analogues containing modified nucleosides[13] as well as radioisotopically labelled nucleosides .

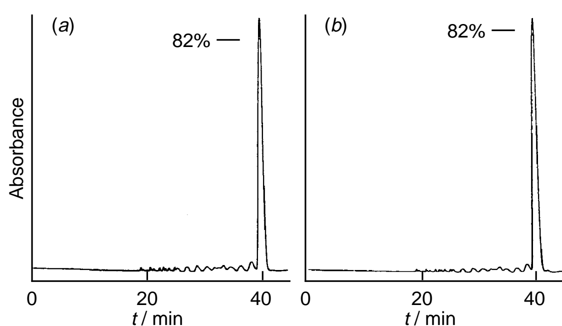

Fig. 2 Capillary gel electrophoresis analysis of the crude oligonucleotide **16T** synthesized using (*a*) the commercial methylphosphonamidite **3**-**T** and (*b*) the *in situ* methylphosphonamidite **2**-**T**

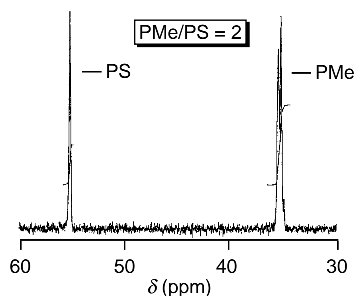

Fig. 3 ^{31}P NMR analysis of the crude oligonucleotide **16T** synthesized using the *in situ* methylphosphonamidite **2**-**T**

Footnotes

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† Compound **1** was prepared in 79% yield from methyldichlorophosphine and 1-(trimethylsilyl)pyrrolidine. ^{31}P NMR ($CDCl_3$): δ 64.1.

‡ A 3′-3′ dimer was also synthesized by the reaction of methyldichlorophosphine (1.0 equiv.), 5′-DMT-thymidine (2.5 equiv.) and triethylamine (2.5 equiv) in CH_2Cl_2. ^{31}P NMR ($CDCl_3$): δ 177.5.

§ To 5.0 mmol (2.72 g) of 5′-DMT-thymidine in THF (15.0 ml) was added a solution of **1** (0.93 g, 5.0 mmol) in MeCN (30.0 ml) at room temperature. The solution was stirred for 1 min. To the solution was added dropwise a 0.45 M solution of tetrazole (1.5 mmol, 3.3 ml) in MeCN. The mixture was stirred for 5 min, and then the thymidine methylphosphonamidite solution (0.1 M) was ready to use.

¶ The oligonucleotide methylphosphonate **16T** was synthesized on a 1 µmol scale using an automated synthesizer (Millipore 8909 Expedite™, Bedford, MA). For methylphosphonate linkages the synthesis was carried out using a modified protocol, in which capping was performed after the iodine oxidation step. An oxidizing agent of low water content (0.1 M I_2 in THF–lutidine– H_2O, 74.75 : 25 : 0.25) was used to minimize backbone hydrolysis.[2] Treatment (2 h) with 7 M ammonia in MeOH at 55 °C was carried out to cleave the oligomer from the support.[3] The mixture was filtered to remove the CPG. After the NH_3–MeOH solution was removed by lyophilisation (Speed Vac, Savant Instruments Inc., Farmingdale, NY), the remaining crude products were submitted for CE, RP-HPLC, NMR and MALDI-TOF analysis. MS (MALDI-TOF): calc. for **16T** (M + H) 4867. Found, 4868.

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