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Gel-Phase ^{31}P-NMR. A New Analytical Tool to Evaluate Solid Phase Oligonucleotide Synthesis.

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Abstract: This paper shows Gel-phase ^{31}P-NMR spectra of synthetic intermediates obtained during solid-phase oligonucleotide synthesis on polystyrene for the first time. We have demonstrated the application of this technique using the phosphotriester, H-phosphonate and phosphite triester approaches. The use of Gel-phase ^{31}P-NMR for monitoring solid phase oligonucleotide synthesis is discussed.

The potential use of modified oligonucleotides as therapeutic agents has led to an increased interest in the large-scale synthesis of oligonucleotides and their analogues. One of the possible improvements in large-scale solid phase oligonucleotide synthesis would be to have a rapid, reliable and non-destructive analytical technique in order to monitor the synthesis. Also, almost all methodological studies in oligonucleotide synthesis have been carried out in solution, even though the reaction conditions in solid phase and solution syntheses are often quite different.

Sternlicht *et al.* [1] have reported on the application of conventional ^{13}C-NMR techniques for studying solvent-swollen cross-linked polymers. Subsequently gel-phase ^{19}F-NMR and ^{13}C-NMR have been successfully employed for the monitoring of solid phase peptide synthesis.[2-5] We reasoned that the gel phase NMR techniques could also be applied to monitor oligonucleotide synthesis taking advantage of several very attractive features of the ^{31}P nucleus. Firstly, different phosphorous derivatives are involved in the key steps of the synthetic cycle. Secondly, its NMR sensitivity is much higher than that of ^{13}C (383/1 considering the natural abundance of the nuclei). Finally, the signals of the polymer do not interfere with the observed ^{31}P-NMR resonances.

As solid support we have chosen a polystyrene-based resin which has long been used for the phosphotriester methodology [6] and also with the H-phosphonate methodology.[7] In addition we have recently shown that polystyrene is also compatible with the phosphite-triester approach,[8] which is by far the most effective and widely employed method for oligonucleotide synthesis.[9] Controlled pore glass (CPG),[10] the standard support used in oligonucleotide synthesis, is not suitable for this kind of study because it is rigid and it gives signals which are too broad to be observed.[11] Starting from DMT-G^{ib}-PS (where the nucleotide is attached to an aminomethylcopoly-(styrene-1%-divinylbenzene) resin through a succinyl linker) with a substitution degree of 0.17 mmol/g, we have prepared DMT-C^{bz}-p(Ar)-G^{ib}-PS (**1**) and DMT-T-p(Ar)-C^{bz}-p(Ar)-G^{ib}-PS (**2**) by the phosphotriester approach using a double coupling procedure.[12] The 81 MHz ^{31}P-NMR spectra in $CDCl_3$ of **1** and **2** are depicted in fig. 1.[13] The dimer **1** shows a single peak in the phosphate

triester region (the two diastereoisomeric products could not be distinguished) while the trimer **2** shows two very close signals, presumably corresponding to the two different phosphate groups.

As shown in fig. 1a the half-height width of the ^{31}P-NMR signal of **1** is quite small ($\Delta\nu_{1/2}$ = 26 Hz after 4200 transients).[14] The ^{31}P-NMR spin-lattice relaxation time (T_1) of resin **1** in $CDCl_3$ was measured by using the inversion recovery method. The observed value (1.006 ± 0.015 s) is similar to that measured for a solution of DMT-T-p(Ar)-G^{ib}-bz in the same solvent (0.969 ± 0.013 s). Considered together, line-width and T_1 values, provide a clear indication that the considerable degree of swelling of the dinucleotide-resin in $CDCl_3$ provides a highly flexible microenvironment for the phosphorus atoms and, therefore, of the reactive sites.[4]

In a similar way, we have prepared DMT-C^{bz}-p*-G^{ib}-PS (**3**) and DMT-T-p*-C^{bz}-p*-G^{ib}-PS (**4**) from DMT-G^{ib}-PS (0.27 mmol/g) by the H-phosphonate approach.[7] The results are similar to those obtained using the phosphotriester methodology: a single peak in the H-phosphonate region (δ 5.3 ppm) was observed for the dimer **3**, while **4** shows two very close signals (δ 6.6 and 5.9 ppm). After the oxidation of **4** the spectrum showed a broad signal (δ -3.7) as might be expected considering the high polarity of the molecule attached to the resin and the hydrophobicity of the solvent (Fig. 1c).

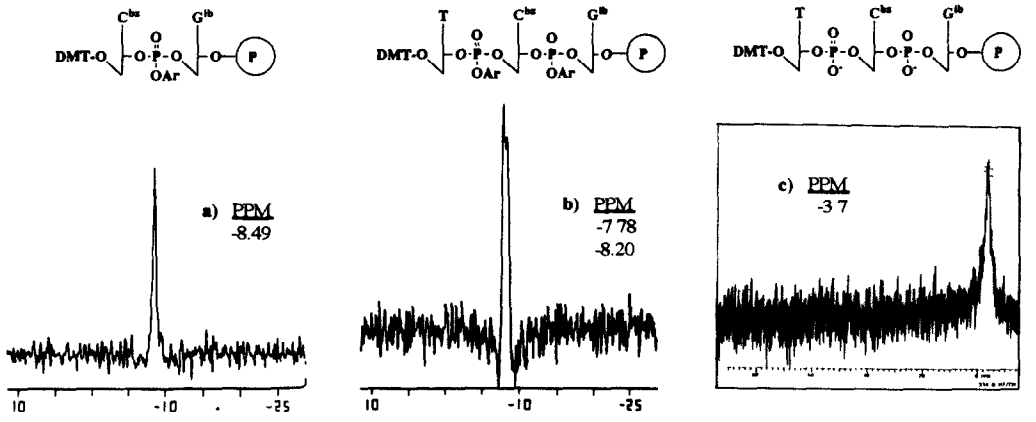

Figure 1: ^{31}P-NMR spectra of: a) DMT-C^{bz}-p(Ar)-G^{ib}-PS (**1**) in $CDCl_3$. Transients: 850. b) DMT-T-p(Ar)-C^{bz}-p(Ar)-G^{ib}-PS (**2**). Transients: 1656. Acquisition time: 0.8 s. Line broadening: 12 Hz. Pulse width: 30 μs.

c) DMT-T-p-C^{bz}-p-G^{ib}-PS (using H-Phosphonate method). Transients 980. Acquisition time: 0.842 s. Line broadening: 5 Hz. Pulse width: 17 μs.

The same oligonucleotide sequence was also synthesized using the phosphite-triester approach adapted to polystyrene [8] using O-methylphosphoramidites. Starting from DMT-G^{ib}-PS (0.27 mmol/g), 50 mg of the different stages of the synthesis of DMT-T-p(Me)-C^{bz}-p(Me)-G^{ib}-PS (**8**): that is to say of DMT-C^{bz}-p^{III}(Me)-G^{ib}-PS (**5**), DMT-C^{bz}-p(Me)-G^{ib}-PS (**6**), DMT-T-p^{III}(Me)-C^{bz}-p(Me)-G^{ib}-PS (**7**) and also **8**, were taken and the corresponding ^{31}P-NMR spectra were recorded.

The spectrum of **5** clearly showed two signals corresponding to the different diastereoisomeric phosphites attached to the resin (Fig. 2a). Oxidation of **5** to **6** went to completion and it was still possible to distinguish between the two diastereoisomeric phosphates (Fig. 2b). The second nucleotide coupling gave, at first, a product (**7**) where the signals corresponding to the phosphate and phosphite triesters could be seen (Fig. 2c).[15] Moreover, the 5'-terminal phosphite triester signal was also easily detected and clearly distinguishable from the eight phosphate triester resonances in the gel-phase spectrum of a decanucleotide-resin (results not shown), demonstrating the effectiveness of the method. After oxidation of **7**, the spectrum of **8** again showed two signals in the region of the phosphate triesters (Fig. 2d). No other signals were observed. In particular, we could not detect any extra signal from the described side reactions on guanine using both phosphotriester [16] and phosphite triester [17,18] methods. This may indicate that the extent of these side reactions under normal coupling conditions is too low to be observed by ^{31}P-NMR.

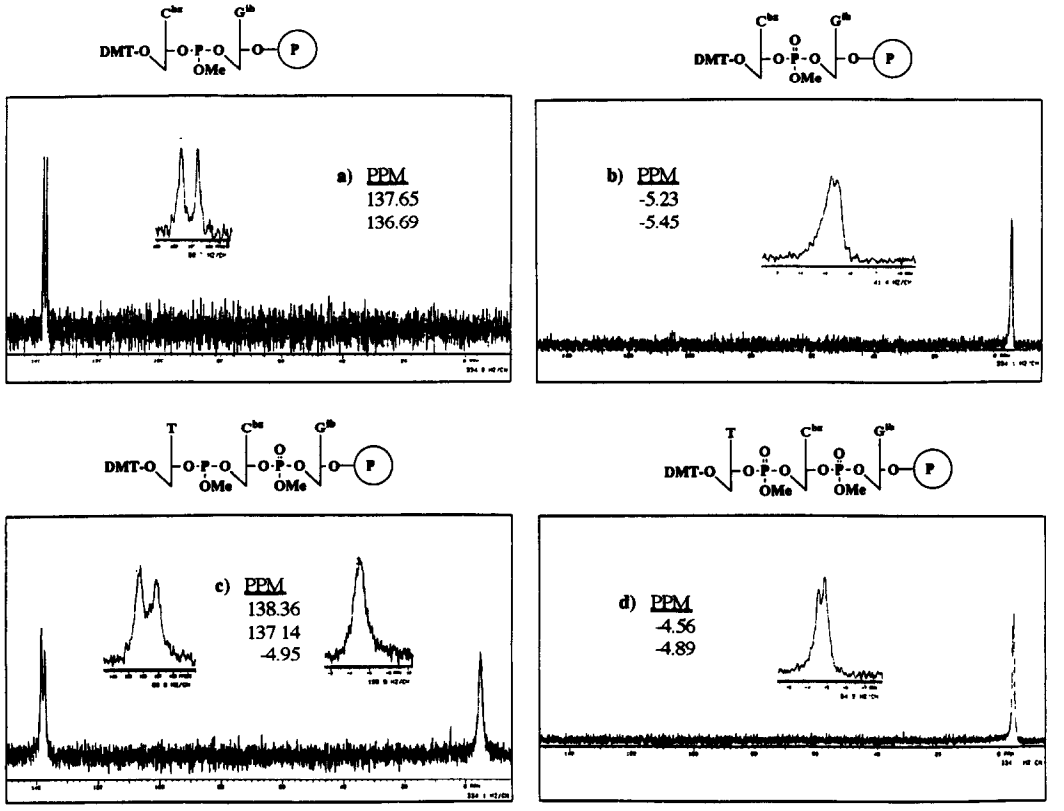

Figure 2: ^{31}P-NMR spectra of: a) DMT-C^{bz}-p^{III}(Me)-G^{ib}-PS (**5**) in $CDCl_3$. Transients: 980. b) DMT-C^{bz}-p(Me)-G^{ib}-PS (**6**) Transients: 2540. c) DMT-T-p^{III}(Me)-C^{bz}-p(Me)-G^{ib}-PS (**7**). Transients: 1856. d) DMT-T-p(Me)-C^{bz}-p(Me)-G^{ib}-PS (**8**). Transients: 1571.

Acquisition time: 0.842 s. Line broadening: 5 Hz. Pulse width: 17 μs.

It appears that ^{31}P-NMR is a useful technique for monitoring solid-phase oligonucleotide synthesis and is particularly interesting in methodological studies in order to ensure quantitative yields or to determine the extent of a side reaction on the resin [15,19]. The use of ^{31}P nuclei for NMR gives great selectivity in the analysis, especially considering the size of this kind of molecule, and takes advantage of the fact that the solid support is transparent to this technique. In addition, the great difference between the chemical shifts of P^{III} and P^{V} makes gel phase ^{31}P-NMR especially useful as a non-destructive method for monitoring the synthesis of oligonucleotides using the phosphite-triester approach, for example in large-scale synthesis. This technique might also be useful in other areas such as phosphorylation studies, synthesis of phosphopeptides, etc.

Abbreviations

DMT: 4,4'-dimethoxytrityl, C^{bz} :N-benzoyldeoxycytidine, T: thymidine, G^{ib}: N-isobutiryldeoxyguanosine, PS: polystyrene, p: phosphate triester, p^{*}: H-phosphonate diester, p^{III}: phosphite triester, Ar: 2-chlorophenyl, Me: methyl.

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12. The coupling steps employed with the phosphotriester method consists in a first treatment of the resin with the corresponding nucleotide (5 fold excess), 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) and N-methylimidazole in anhydrous pyridine during 15 minutes, and after washing with pyridine, another treatment with the nucleotide (3 fold excess) and MSNT in anhydrous pyridine.
13. The spectra were recorded on a Varian XL-200 spectrometer from resins swollen in $CDCl_3$ and made homogeneous by mechanical and ultrasonic stirring. The ^{2}H resonance of the solvent was used for the field-frequency lock. The amount of resin range from 30 to 100 mg, depending on each individual case. Either 5mm tubes or microsamples bulb were used.
14. After removal of the DMT protecting group the ^{31}P-NMR lines systematically broaden-up (results not shown).
15. A small and narrow signal in the region of H-phosphonate diesters was also detected, that could perhaps be explained by the presence of nucleoside H-phosphonate adsorbed to the resin during the synthetic cycle.
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18. The ^{31}P-NMR-spectrum of the polymeric phosphite triester **5** was carried out before and after addition of capping reagents (which are known to reverse guanosine phosphate adducts[16]) and no differences were observed between the spectra.
19. For instance, after the synthesis of the dimer **3** the ^{31}P-NMR spectrum showed a signal at 9.0 ppm (besides the expected H-phosphonate signal) that could be explained by the attachment of the activated H-phosphonate to non-capped amino groups on the resin. Using another batch of properly capped resin only the H-phosphonate signal was observed.