Stepwise Regeneration and Recovery of Deoxyribonucleoside Phosphoramidite Monomers during Solid-Phase Oligonucleotide Synthesis

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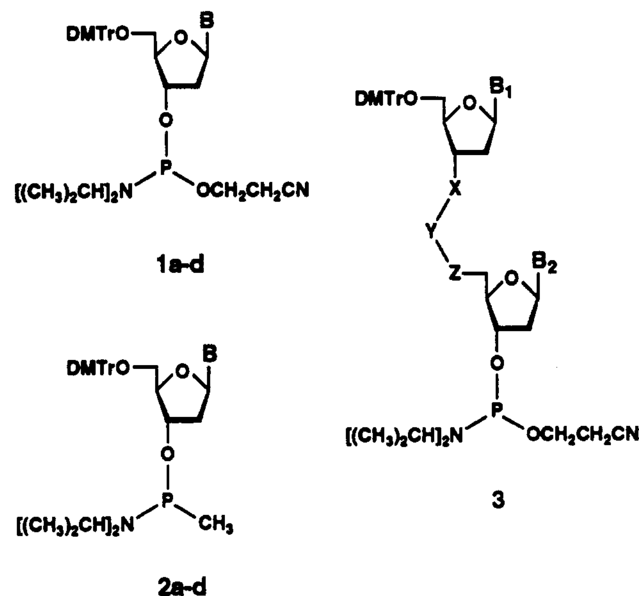
Summary: Deoxyribonucleoside phosphoramidites **1a-d** have been regenerated, in a stepwise manner, during solid-phase oligonucleotide synthesis and have been efficiently reutilized in the solid-phase synthesis of polydeoxyribonucleotides. The activator 1*H*-tetrazole has also been recovered and recycled.

In recent years, the application of synthetic oligonucleotides and their analogues to the regulation of gene expression has attracted considerable attention.[2] For example, oligodeoxyribonucleoside phosphorothioates and methylphosphonates have been employed extensively for such applications.[3] These oligonucleotide analogues are efficiently synthesized by the phosphoramidite approach[4] on solid supports *via* the deoxyribonucleoside phosphoramidites **1a-d** and the phosphonamidites **2a-d**, respectively.



1a = 2a, B= thymin-1-yl
1b, B= N^4-benzoylcytosin-1-yl
2b, B= N^4-isobutyrylcytosin-1-yl
1c = 2c, B= N^6-benzoyladenin-9-yl
1d = 2d, B= N^2-isobutyrylguanin-9-yl

DMTr = di-(*p*-anisyl)phenylmethyl
X,Y, Z = atom or functional group of atoms
B_1, B_2 = protected nucleobase

The solid-phase synthesis of oligonucleotides is inherently wasteful in that a large excess of nucleosidic phosphoramidite synthons (up to 10-fold the theoretical amount) is necessary for optimum coupling efficiency. Given that large-scale production of oligonucleotide analogues is required for the clinical evaluation of these potential therapeutic agents, the waste of valuable nucleoside phosphoramidites can assume gigantic proportions and, thus, can become a serious economic problem. In addition, dinucleoside phosphoramidites structurally related to **3**, along with nucleoside phosphoramidites having either modified nucleobase or carbohydrate entities, have increasingly been incorporated into oligonucleotide analogues in an effort to modulate their base pairing properties, lipophilicity, and nuclease resistance.[5] Because of the synthetic complexity of these phosphoramidite derivatives relative to that of **1a-d**, the waste of such building blocks, during oligonucleotide synthesis, constitutes a considerable loss in terms of time and labor. It must also be noted that the coupling efficiency of a number of modified nucleoside phosphoramidites is lower than that of **1a-d**,[5a] and this further aggravates the economics of large-scale synthesis of oligonucleotide analogues. Consequently, the development of a simple procedure for the efficient regeneration of these phosphoramidites during solid-phase oligonucleotide syntheses would be desirable to minimize the loss of expensive phosphoramidite synthons. To the best of our knowledge, the regeneration of deoxyribonucleoside phosphoramidite derivatives during oligonucleotide synthesis and their reutilization have not

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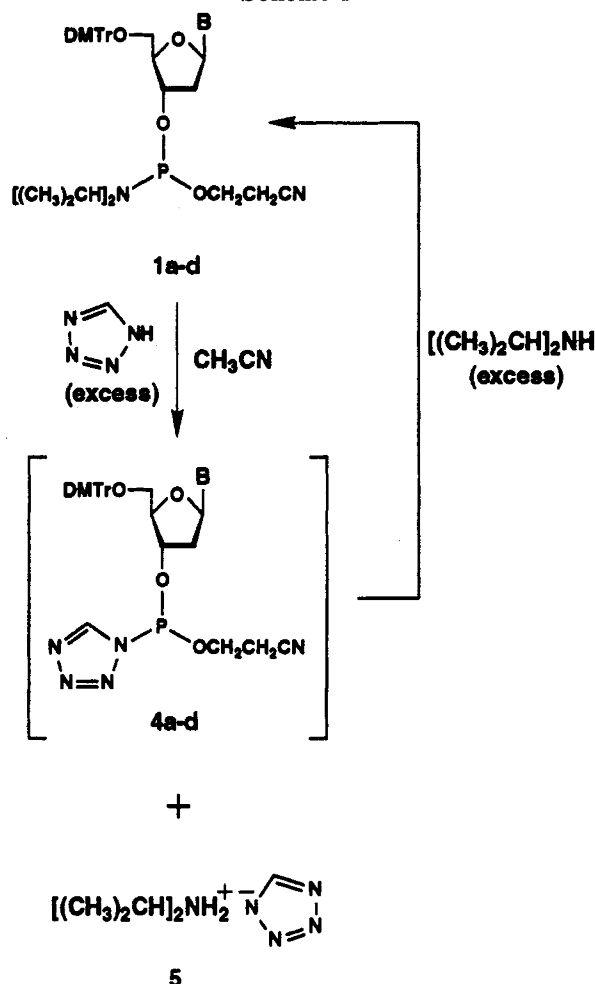
Scheme 1



as yet been reported.[6] Theoretically, the stepwise regeneration of phosphoramidites, for example, **1a–d**, would involve the individual collection of the unused portion of each activated monomer, from each coupling step, into reservoirs containing dry *N,N*-diisopropylamine. The simplicity of this concept is illustrated in Scheme 1.[7]

To test the feasibility of this approach, each of the deoxyribonucleoside phosphoramidites **1a–d**[8] were repeatedly coevaporated with anhydrous toluene and dried overnight under high vacuum. Each phosphoramidite was dissolved in dry acetonitrile[9] to a final concentration of 0.1 M, and an equal volume (5 mL) of 1*H*-tetrazole[10] was added. The reagents were briefly mixed and left at ambient temperature for 5 min under an argon atmosphere. Upon addition of 1 mL of *N,N*-diisopropylamine[11] by syringe to each solution of activated phosphoramidite, an immediate precipitate of the *N,N*-diisopropylammonium tetrazolide salt **5** was observed. The mixture was allowed to stand at ambient temperature for 24 h.[12] The tetrazolide salt was then filtered and washed with acetonitrile, and the combined filtrates were evaporated to dryness under reduced pressure. The material left was analyzed by ^{31}P-NMR spectroscopy[13] which indicated the formation of each of the deoxyribonucleoside phosphoramidites **1a–d** in yields greater than 80%. Further experimentation revealed that rigorous exclusion of moisture from the reagents and the solvents was necessary for the optimum regeneration of **1a–d**.

These encouraging results led us to attempt the stepwise recovery of deoxyribonucleoside phosphoramidites during solid-phase synthesis of an oligonucleotide on an automated DNA synthesizer. Each of the commercial phosphoramidites **1a–d** was dedicated to the synthesis of the corresponding decanucleotide homopolymer on a 10 μmol scale.[14] To maintain anhydrous conditions and experimental simplicity, activated 4-Å molecular sieves[15] were added to all the reagents and solvents required for solid-phase oligonucleotide synthesis except for the oxidant and the capping reagents.[16] At each coupling step, the excess activated phosphoramidites in acetonitrile were collected through a serum-capped and flame-dried glass bottle containing activated 4-Å molecular sieves (2 g) and 2.5 mL of dry *N,N*-diisopropylamine. Upon completion of the synthesis, the concentration of *N,N*-diisopropylamine in the collection bottle was *ca.* 10% relative to acetonitrile. The regenerated phosphoramidites were left at ambient temperature for a total time of 24 h[12] and were separated from the tetrazolide salt by filtration. The salt was thoroughly washed with acetonitrile. The filtrates were combined and evaporated to dryness under *vacuo*. The regenerated deoxyribonucleoside phosphoramidites **1a–d** were analyzed by ^{31}P-NMR spectroscopy, and the spectra are shown in Figure 1. The resonances corresponding to **1a–d** (δ 150–151 ppm) accounted for more than 85% of all the resonances. The resonances at δ 0–15 ppm probably arise from a relatively small amount (less than 15%) of hydrolysis side products. The recovered phosphoramidites were purified to homogeneity by silica gel chromatography and were isolated in *ca.* 55% yield based on the total

(6) The regeneration of deoxyribonucleoside *H*-phosphonate monomers from their activated congeners has, however, been reported. See: (a) Geiger, A.; Seliger, H.; Nehls, P. *Nucleosides Nucleotides* **1993**, *12*, 463–477. (b) Rosenberg, I.; Farras Soler, J.; Tocik, Z.; Ren, W.-Y.; Ciszewski, L. A.; Kois, P.; Pankiewicz, K. W.; Spassova, M.; Watanabe, K. A. *Nucleosides Nucleotides* **1993**, *12*, 381–401. (c) Seliger, H.; Rösch, R. *DNA Cell Biol.* **1990**, *9*, 691–696. (d) Gao, H.; Gaffney, B. L.; Jones, R. A. *Tetrahedron Lett.* **1991**, *32*, 5477–5480.

(7) The formation of the deoxyribonucleoside phosphorotetrazolides **4a–d** upon activation of **1a–d** with 1*H*-tetrazole has been proposed in the literature.[4b] See also: (a) Seliger, H.; Gupta, K. C.; Kotschi, U.; Spaney, T.; Zeh, D. *Chem. Scr.* **1986**, *26*, 561–567. (b) Seliger, H.; Gupta, K. C. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 685–687. (c) Seliger, H.; Gupta, K. C. *Nucleosides Nucleotides* **1985**, *4*, 249. (d) Berner, S.; Mühlegger, K.; Seliger, H. *Nucl. Acids Res.* **1989**, *17*, 853–864. (e) Berner, S.; Mühlegger, K.; Seliger, H. *Nucleosides Nucleotides* **1988**, *7*, 763–767. Our findings indicate that **4a–d** revert to the phosphoramidites **1a–d** in the presence of an excess of *N,N*-diisopropylamine.

(8) The deoxyribonucleoside phosphoramidites **1a–d** were purchased from Cruachem Inc. These were used in both regeneration and control experiments.

(9) Anhydrous acetonitrile was obtained from Applied Biosystems and used as received.

(10) A solution of *ca.* 0.45 M of 1*H*-tetrazole in acetonitrile was purchased from Applied Biosystems and used as is.

(11) *N,N*-Diisopropylamine obtained from Aldrich was refluxed over calcium hydride (Aldrich, –40 mesh) for at least 16 h prior to distillation and stored over activated 4-Å molecular sieves[15] in dry amber glass bottles.

(12) A period of 24 h was selected to simulate the time that may, actually, be required for large-scale (5–15 mmol) oligonucleotide syntheses.

(13) NMR spectra were recorded with a General Electric GN 300 NMR spectrometer operating at 7.05 T (300 MHz for ^{1}H). Proton-decoupled ^{31}P-NMR spectra were run in acetonitrile-d_3 with 85% phosphoric acid in deuterium oxide as an external reference.

(14) The regeneration of the phosphoramidites **1a–d** during solid-phase oligonucleotide synthesis has been performed on an Applied Biosystems Model 380B DNA synthesizer. A 10 μmol program has been modified to allow the recovery of **1a–d** and 1*H*-tetrazole in amounts sufficient for their reutilization. Details are provided in the supplementary material.

(15) Davison 4-Å molecular sieves (Fisher Scientific) were activated upon heating at 300 °C for 6 h and, then, allowed to cool in a dessicator under vacuum.

(16) The amounts of molecular sieves used are as follows: 30 g per bottle (4 L) of acetonitrile, 1 g per gram of deoxyribonucleoside phosphoramidite, 3 g per bottle (45 mL) of 1*H*-tetrazole, and 4 g per bottle (450 mL) of trichloroacetic acid. To ensure anhydrous conditions, the minimum contact time between reagents and sieves is 8 h.

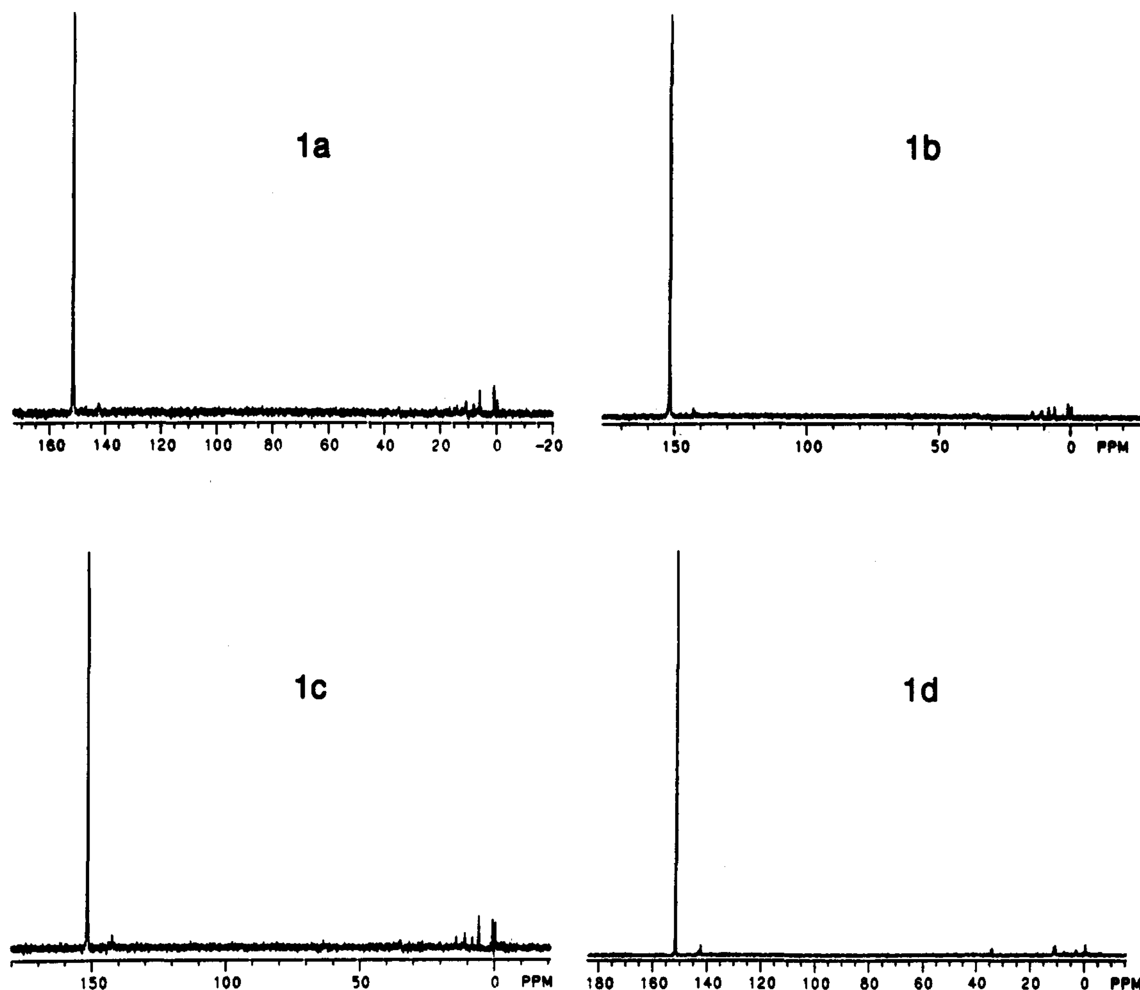


Figure 1. ^{31}P-NMR spectra of the deoxyribonucleoside phosphoramidites regenerated from activated **1a-d** during solid-phase oligonucleotide synthesis.

amount of phosphoramidites consumed during the synthesis.[17] The discrepancy between regenerated and isolated yields of **1a-d** may imply an inefficient elution of activated phosphoramidites from the solid support after each condensation step.

The coupling efficiency of the recovered phosphoramidites **1a-d** was compared to that of control phosphoramidites *via* two independent syntheses of d(ATCCG-TAGCTAAGGTCATGC). In each synthesis, the stepwise coupling yields averaged 98%. Following complete deprotection, the crude oligomers were desalted by gel exclusion chromatography and were independently incubated with snake venom phosphodiesterase (SVP) and bacterial alkaline phosphatase (BAP) for 16 h at 37 °C.[18] Analysis of the hydrolysates by reversed-phase HPLC did not show detectable nucleobase modification.[19]

Because of the notorious sensitivity of N^4-benzoyl-2′-deoxycytidine derivatives to transamination with primary amines,[20] the stability of the protected nucleosides corresponding to **1a-d** toward *N,N*-diisopropylamine, under the conditions used for the regeneration of deoxyribonucleoside phosphoramidites, was investigated. According to TLC analysis, the formation of side products was negligible within 24 h. However, under forcing conditions involving a higher concentration of *N,N*-diisopropylamine (50% in acetonitrile), modification of the protected nucleosides corresponding to **1b** and **1d** occurred to some extent,[21] whereas nucleosides corresponding to **1a** and **1c** were inert.

The sensitivity of N^4-benzoylated deoxycytidine and N^2-isobutyrylated deoxyguanosine residues to *N,N*-diisopropylamine prompted us to apply recovered **1b** and **1d** to the solid-phase synthesis of d(CGCGGGCGCG-CGGGCGCCGG). This sequence should facilitate the detection of potential nucleobase modifications given its high GC content. After treatment of the crude, desalted, GC-rich oligomer with SVP and BAP, the analysis of the digests by reversed-phase HPLC did not show noticeable nucleobase modification.[19] These results demonstrate that nucleobase modification is negligible during the regenera-

(17) ^{31}P-NMR spectra of the regenerated and chromatographically purified phosphoramidites **1a-d** are displayed in the supplementary material.

(18) To 1 OD A_{260} of the oligomer was added 6 μL of 1 M Tris buffer (pH 9.0), 8 μL of 1 M magnesium chloride, 75 μL of water, 5 μL of snake venom phosphodiesterase (*Crotallus durissus*, 15 × 10^{-3} U), and 6 μL of bacterial alkaline phosphatase (7 × 10^{-1} U). The digestion reaction was incubated for 16 h at 37 °C. The enzymes were then heat-deactivated at 75 °C for 2 min.

(19) HPLC chromatograms of the enzymic hydrolysates are shown in supplementary material.

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(21) After 3 weeks at 20 °C, TLC showed two additional spots of similar intensity with higher and lower R_f values relative to the starting material. These side products account for no more than 10–20% of the starting material. Preliminary FAB-MS analysis of the fast-moving byproducts suggests isobutyrylation at O^6- or N^1- of N^2-isobutyrylguanine in one case and N^4-benzoylation of N^4-benzoylcytosine in the other. The complete characterization of these side products is in progress.

tion of the phosphoramidites **1a–d**, under the conditions described herein.

Interestingly, addition of stoichiometric amounts of trifluoroacetic acid to a dichloromethane solution of the salt **5**, isolated during the generation of **1a–d**, led to the precipitation of 1*H*-tetrazole in 65% yield. The regenerated 1*H*-tetrazole was sublimed (110 °C at 0.05 mmHg), dissolved in dry acetonitrile (0.45 M), and used in solid-phase oligonucleotide synthesis. The effectiveness of regenerated 1*H*-tetrazole was equivalent to that of the commercial activator according to trityl color determination.

In conclusion, we have delineated a simple, reliable, and efficient procedure for the regeneration and recovery of the deoxyribonucleoside phosphoramidites **1a–d** during solid-phase oligonucleotide synthesis. Although this method has not, as yet, been applied to other types of phosphoramidites, it seems reasonable to speculate that the procedure may be useful in the regeneration of a variety of modified nucleoside phosphoramidites including those structurally related to **3**. Deoxyribonucleoside phosphonamidites and ribonucleoside phosphoramidites could also, conceivably, be regenerated and recovered. This methodology should have a strong impact on the economic considerations stemming from large-scale syntheses of oligonucleotides and their analogues.

Supplementary Material Available: A modified synthetic program for the regeneration of **1a–d**, ^{31}P-NMR spectra of regenerated and chromatographically purified **1a–d**, the relative electrophoretic mobility of d(ATCCGTAGCTAAGGTCATGC) and d(CGCGGGCGCGCGGGCGCCGG) prepared from recovered and purified **1a–d**, and a protocol for the regeneration of 1*H*-tetrazole from **5** (10 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.