Phosphoramidite Coupling to Oligonucleotides Bearing Unprotected Internucleosidic Phosphate Moieties

Andrei P. Guzaev* and Muthiah Manoharan

Department of Medicinal Chemistry, Isis Pharmaceuticals, 2292 Faraday Avenue, Carlsbad, California 92008

aguzaev@isisph.com

Received November 8, 2000

The coupling of 2-cyanoethyl thymidine phosphoramidite to solid-support-bound, phosphate-unprotected oligothymidylates and their phosphorothioate analogues was studied. The yield of the coupling reaction depended on the pK_{BH^+} values of protonated nitrogen bases that served as counterions to the phosphodiester functions of oligonucleotides. To maximize the coupling efficiency, the oligonucleotides were detritylated and washed with a mixture of 0.1 M DMAP and 0.1 M 1*H*-tetrazole, which resulted in a 98+% coupling efficiency. The utility of the results was demonstrated in the preparation of oligonucleotides with a mixed backbone that required the successive use of *H*-phosphonate and phosphoramidite methods of synthesis. Using this approach, 20-mer antisense oligonucleotides containing 2′-*O*-(2-methoxyethyl) ribonucleoside residues and phosphorothioate and phosphoramidate internucleosidic linkages were synthesized in high yield.

Introduction

The preparation of synthetic oligodeoxynucleotides is currently a well-established procedure that is carried out automatically on solid phase using either phosphoramidite[1] or *H*-phosphonate chemistry.[2] In both approaches, the synthesis is implemented by stepwise coupling of monomeric nucleoside building blocks to the 5′-terminus of the oligonucleotide chain to be elongated. In the phosphoramidite elongation cycle, the newly formed phosphite triester moiety is subsequently oxidized to give the phosphate triester or sulfurized to the thionophosphate triester. These moieties remain protected until the completion of the synthesis. The phosphate protecting group, which is most often the 2-cyanoethyl group,[3] is then removed under basic conditions. Thus, the 2-cyanoethyl or other phosphate protecting groups are not removed until the final deprotection of an oligonucleotide.

However, it has been reported that *o*-methylbenzyl protection can be removed by treatment with a solution of iodine during the normal oxidation protocol.[4] Thymidine *o*-methylbenzyl phosphoramidite was shown to couple to the 5′-hydroxy group of phosphate-unprotected, solid-support-bound oligodeoxyribonucleotides to afford eicosathymidylate with an average stepwise yield of 96%. This valuable observation is very important for the preparation of various modified oligonucleotides. Surprisingly, no further development of this initial discovery has been exploited. The coupling reaction of the most common 2-cyanoethyl phosphoramidites toward the 5′-hydroxy group of the oligonucleotides containing phosphodiester (PO) functions has rarely been used[5] and has never been studied on a systematic basis. More importantly, no information has been reported on phosphoramidite coupling in the presence of thionophosphodiester (PS) functions. In this communication, we report the coupling of 2-cyanoethyl phosphoramidites to solid-support-bound oligonucleotides unprotected at the PO and PS moieties.

We found that the coupling yields in this case are markedly lower than those with the standard, protected oligonucleotides. However, the efficiency of the synthesis could be improved by neutralizing the PO or PS residues with 1*H*-tetrazolides of tertiary amines. Under optimized conditions, the efficiency of the coupling reaction was restored to the commonly accepted levels for both PO and PS oligonucleotides. The practical utility of the methodology developed here has been demonstrated in the preparation of oligonucleotides with mixed backbones that required the successive use of the *H*-phosphonate and phosphoramidite approaches. To this end, 20-mer antisense oligonucleotides containing PS and phosphoramidate internucleosidic linkages and 2′-*O*-(2-methoxyethyl) ribonucleoside wings were synthesized in high yield.

Results and Discussion

Coupling of Phosphoramidite 2 to Unprotected Hexathymidylates Studied by ^{31}P NMR on Solid Support. It has been previously postulated that an activated phosphoramidite might react with an internucleosidic phosphate diester moiety to form a mixed anhydride, which could be cleaved in the presence of excess 1*H*-tetrazole to regenerate deoxyribonucleoside phosphorotetrazolide intermediates.[4] Indirectly, this has

* Tel: (760) 603 2445. Fax: (760) 929 0036.

(1) (a) Caruthers, M. H. *Acc. Chem. Res.* **1991**, *24*, 278−284. (b) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223−2311.

(2) (a) Hall, R. H.; Todd, A.; Webb, R. F. *J. Chem. Soc.* **1957**, 3291−3296. (b) Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. *Chem. Scr.* **1985**, *25*, 280−282. (c) Froehler, B. C.; Matteucci, M. D. *Tetrahedron Lett.* **1986**, *27*, 469−472.

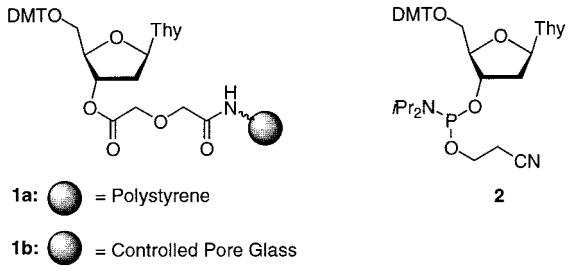
(3) (a) Sinha, N. D.; Biernat, J.; Köster, H. *Tetrahedron Lett.* **1983**, *24*, 5843−5846. (b) Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucleic Acids Res.* **1984**, *12*, 4539−4557.

(4) Caruthers, M. H. Kierzek, R.; Tang, J. Y. In *Biophosphates and their Analogues−Synthesis, Structure, Metabolism and Activity*; Bruzik, K. S., Stec, W. J., Eds.; Elsevier: Amsterdam, 1987; pp 3−21.

(5) Braich, R. S.; Damha, M. J. *Bioconjugate Chem.* **1997**, *8*, 370−377.

10.1021/jo001591e CCC: $20.00
Published on Web 02/10/2001

been confirmed by the fact that the dimethoxytrityl responses measured after each elongation cycle were consistent with addition of only 1 equiv of phosphoramidite. No similar information was available for PS oligonucleotides. Considering the intrinsic differences in reactivity between the PO and PS diester groups, this information is of crucial importance. Therefore, the reactivity of the solid-support-bound oligonucleotides **4a** and **5a** with the phosphoramidite **2** was first studied.



To obtain **4a**, its 2-cyanoethyl-protected precursor **3a** was synthesized on a 40 μmol scale on a high-loaded polystyrene support **1a**[6] using phosphoramidite chemistry (Scheme 1). The solid-support-bound **3a** was treated with 1 M piperidine in anhydrous MeCN. The progress of the deprotection was monitored by ^{31}P NMR in gel phase without spinning.[7] After 1.5 h at room temperature, the peak at 67.8 ppm was replaced by a peak at 57.3 ppm, which reflected the conversion of the thionophosphate triester **3a** to the corresponding diester **4a** in more than 98% yield. At the same time, the diglycolyl linker[6] that anchored the oligonucleotide to the solid support was not cleaved to any appreciable extent. On completion of deprotection, **4a** was washed with excess MeCN and detritylated to give **5a**.

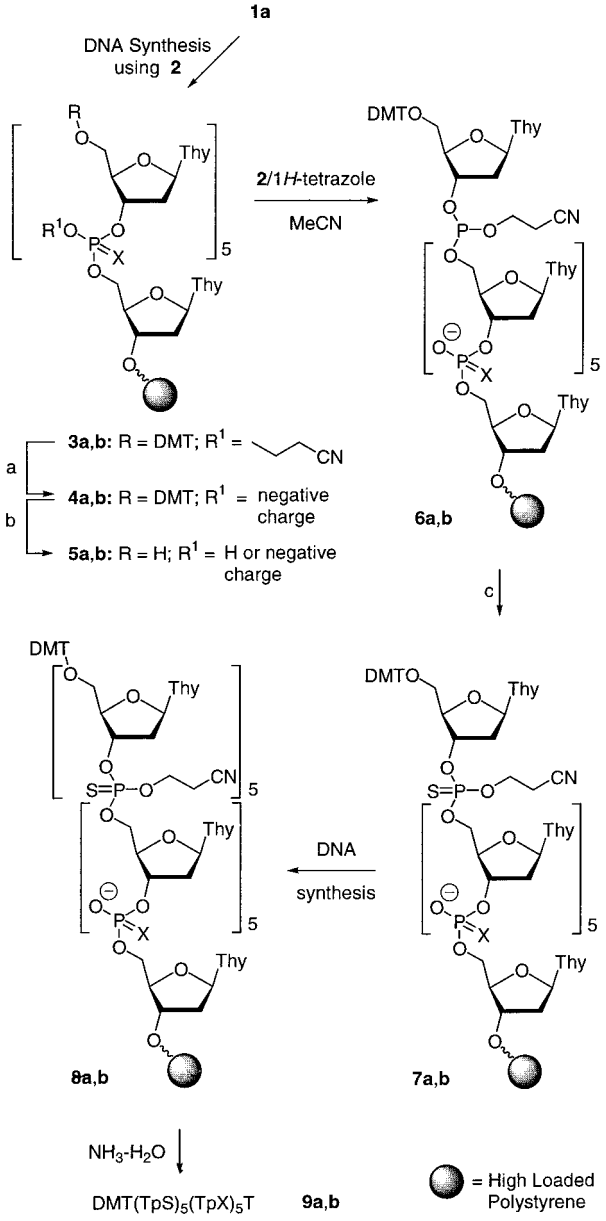
The solid supports **4a** and **5a** were treated with **2** (0.1 M in MeCN) in the presence of 1*H*-tetrazole for 10 min on a DNA synthesizer followed by excessive washing with MeCN. The ^{31}P NMR spectrum of the product obtained from **4a** revealed only the peak of the starting material. For **5a**, two peaks at 140.6 and 57.5 ppm in a ratio of 1:5.06 were observed, which agreed with the formation of **6a** in ca. 99% yield. When sulfurized with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide,[8] **6a** gave **7a** (peaks at 67.9 and 57.5 ppm) in quantitative yield.

In a similar manner, **4a** and **5a** were treated with the standard capping mixture (Ac_2O/*N*-methylimidazole/pyridine/THF) for 30 min in a NMR tube. No apparent changes in the ^{31}P NMR spectra of both compounds were observed.

When four additional coupling cycles were carried out with **7a**, solid-support-bound **8a** was obtained. In the ^{31}P NMR spectrum of **8a**, the peaks of the protected and the deprotected PS moieties were displayed at 67.8 and 56.8 ppm, respectively, in a 50:50 ratio. Additionally, a minor peak of desulfurized phosphates was observed at −1.5 ppm and accounted for ca. 2% of the total integration area.

Analogous observations were made when oligonucleotides **3b**−**8b** were synthesized. These experiments

Scheme 1[a]



[a] (a) 1 M piperidine/MeCN; (b) Cl_2HCCO_2H (3% in CH_2Cl_2); (c) 3*H*-1,2-benzodithiol-3-one 1,1-dioxide/MeCN. **3a**−**9a**: X = S. **3b**−**9b**: X = O.

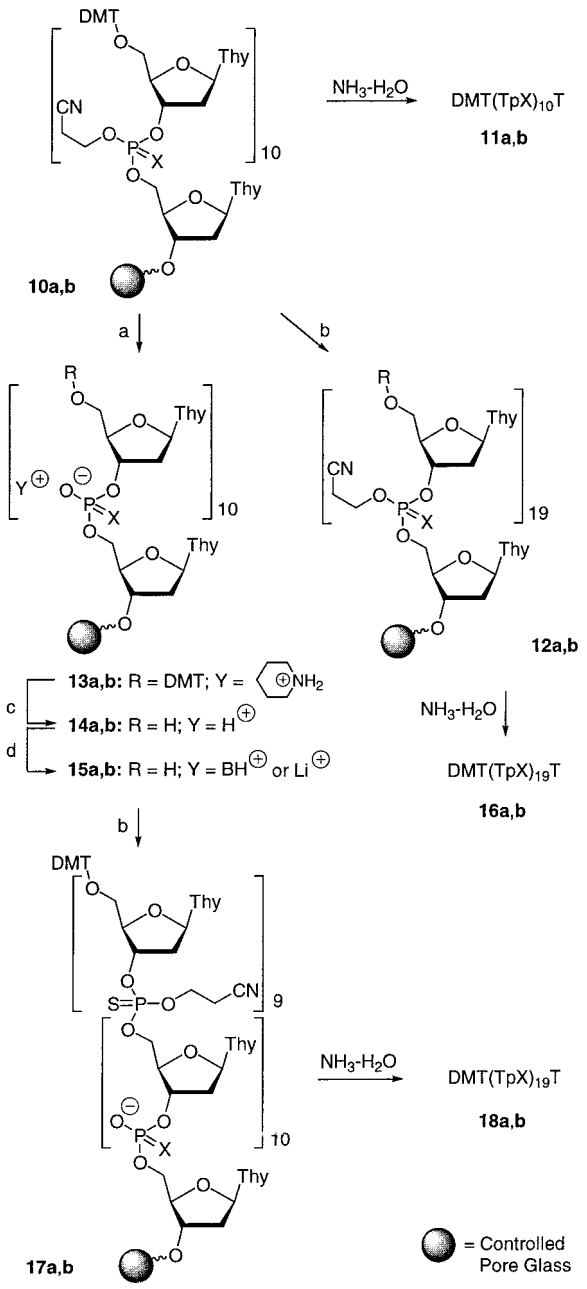
confirmed the hypothesis[4] that no stable products are formed between phosphodiester groups and the nucleoside phosphoramidite **2** or Ac_2O in the presence of 1*H*-tetrazole and *N*-methylimidazole, respectively. They also demonstrated that the hypothesis holds true for thionophosphate diesters.

The solid-support-bound compounds **8a** and **8b** were treated with concentrated aqueous ammonium hydroxide to give **9a** and **9b**, respectively. The reverse phase HPLC analysis of the crude products suggested an average coupling efficiency that did not exceed 94−95%. In addition, during the synthesis of **8a** and **8b** we observed some detritylation during the coupling step.

Possible explanations for the low coupling efficiency may be posited using compounds **13**−**15** in Scheme 2 as illustrations. After the phosphate-unprotected oligonucleotide **13** is detritylated on a DNA synthesizer by treatment with dichloroacetic acid (pK_a 1.30 in water[9]), the

(6) Pon, R. T.; Yu, S. *Nucleic Acids Res.* **1997**, *25*, 3629−3635.
(7) For CPG-bound oligonucleotides, magic angle spinning NMR may be used as described in Macdonald, P. M.; Damha, M. J.; Ganeshan, K.; Braich, R.; Zabarylo, S. V. *Nucleic Acids Res.* **1996**, *24*, 2868−2876.
(8) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699.

Scheme 2[a]



[a] (a) 1 M piperidine/MeCN; (b) DNA synthesis using, after detritylation, additional wash with 0.1 M base/MeCN, 0.1 M base + 0.1 M 1*H*-tetrazole, or 0.1 M $LiClO_4$/MeCN; (c) Cl_2HCCO_2H (3% in CH_2Cl_2); (d) 0.1 M base/MeCN, 0.1 M base + 0.1 M 1*H*-tetrazole, or 0.1 M $LiClO_4$/MeCN. **10a**−**18a**: X = S. **10b**−**18b**: X = O.

phosphodiester backbone may be at least partially converted to an acid **14** (Scheme 2). After washing with MeCN, **14** is treated with a phosphoramidite building block and 1*H*-tetrazole. In the standard protocols for DNA synthesis, phosphoramidite building blocks are used, depending on the synthetic scale, in a 2- to 10-fold excess over the 5′-hydroxy groups of the support-bound oligonucleotide. Thus, for the 11-mer oligonucleotide **14**, the concentration of the support-bound dinucleoside phosphoric acid in the reaction volume may exceed or at least be comparable to the concentration of the phosphoramidite. This may lead to the unwanted partial detritylation and inactivation of the monomer and result in lower coupling yields. Consequently, we hypothesized that both issues might be addressed if, prior to the coupling step, **14** was neutralized, i.e., converted to a salt **15** using a base or a salt of the base. Apparently, the counterion, BH^+ or M^+, should be inert toward nucleoside phosphoramidites.

Coupling of Phosphoramidite 2 to Oligonucleotides with Neutralized Phosphodiester Groups. To study the effect of the counterion on the efficiency of the phosphoramidite coupling, model experiments were carried out as presented in Scheme 2. Starting from **1b**, protected 3′-segments **10a** and **10b** were first synthesized on a 30 μmol scale in a standard manner. Small aliquots of **10a** and **10b** (0.5 μmol) were conventionally deprotected to give **11a** and **11b**, which were characterized by ESMS and HPLC. Approximately one-half of **10a** and **10b** was converted to the unprotected 3′-segments **13a** and **13b** as described for compound **8a**. With these as starting material, further chain elongation was carried out on a 1 μmol scale until protected 9-mer 5′-segments were assembled to give **17a** and **17b** and **18a** and **18b**. For comparison, control samples of the same sequence, **12a** and **12b** and **16a** and **16b**, were synthesized from **10a** and **10b** on a parallel column using the identical scale, protocols, and conditions. In comparison with the experimental design for compound **8**, this offered two distinct advantages. First, a longer 11-mer 3′-segment allowed one to observe a more pronounced negative effect of the unprotected phosphate backbone. Second, at a high coupling efficiency, assembling a protected 9-mer 5′-segment resulted in greater differences in yields between the experimental and the control samples, **16** and **18**, respectively. This led to a more accurate determination of the stepwise yields for the 5′-segment of **16** and **18** and more reliable results regarding the coupling efficiency.

The standard protocol for the DNA synthesis on a 1 μmol scale was modified in two aspects. First, delivery and wait times on the capping step were each extended to 45 s. This reduced dramatically the abundance of DMT-positive (n − 1)-mer and shorter oligonucleotides and thus simplified the calculation of yields for the full-length oligonucleotides. Second, the standard detritylation subroutine was followed by a modified washing protocol. To convert the detritylated, support-bound oligonucleotides to the required salt, a solution of a neutralizer, i.e., an organic base or a salt was delivered to the columns for 45 s. Then, in accordance with the standard protocol, the columns were washed with MeCN, and the coupling subroutine was carried out.

For neutralization of the PS and PO backbones, a number of amines, i.e., pyridine (Py), 2,6-lutidine (Lut), 2,4,6-collidine (Col), *N*-methylmorpholine (NMM), *N*,*N*-diisopropylethylamine (DIPEA), and triethylamine (TEA) were used as 0.1 M solutions in MeCN. The pK_{BH^+} (MeCN) values of the conjugated acids of these amines covered a wide range from 12 to 18.5, so that a possible dependence of the acidity of a protonated amine on the coupling efficiency could be revealed.[10]

On completion of the synthesis, **12a** and **12b** and **17a** and **17b** were treated with concentrated ammonium hydroxide for 30 min to give crude **16a** and **16b** and **18a** and **18b**, respectively. These were analyzed by reverse phase HPLC. Average stepwise coupling yields and total

(9) Gould, E. S. *Mechanism and Structure in Organic Chemistry*; Henry Holt and Company: New York, 1960; p 201.

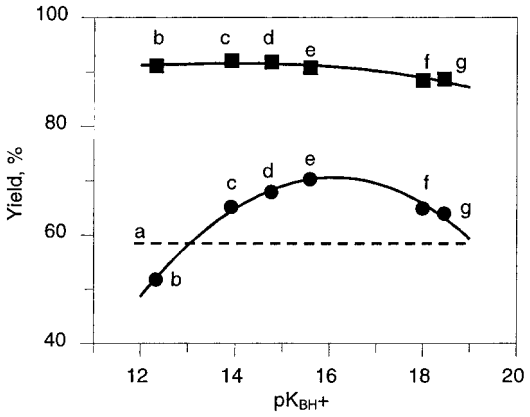


Figure 1. Total yields of the 5′-segments of **16a** (■) and **18a** (●) as a function of pK_{BH^+} (MeCN) values of protonated nitrogen bases. (a) Standard cycle (shown as a horizontal grid); (b) Py; (c) Lut; (d) Col; (e) NMM; (f) DIPEA; (g) TEA.

yields for the 5′-segments of the full-length oligonucleotides **16a** and **16b** and **18a** and **18b** were next calculated. The data obtained are presented in Figures 1 (**16a** and **18a**) and 3 (**16b** and **18b**). The total yields of the 5′-segments of the oligonucleotides are plotted against the pK_{BH^+} (MeCN) values of the protonated bases that served as counterions for the PS or PO residues. Horizontal grids in Figures 1−3 represent the yields of **18a** and **18b** obtained using the standard cycle.

As seen from Figures 1 and 3, the yields of **16a** and **16b** were not influenced by the nature of the neutralizer except when DIPEA and TEA free bases were used for the preparation of **16a** (Figure 1). In agreement with the preliminary results for **9a** and **9b**, the preparation of **18a** and **18b** using the standard cycle resulted in significantly lower yields of the products. The stepwise yield of **18b** (96.4%) correlated perfectly with the reported value of 96%.[4] In contrast, when tertiary amines were used as neutralizers, improved yields of **18a** and **18b** were obtained. This effect depended on the pK_{BH^+} value of the counterion. As seen in Figure 1, for the free amines, the yields of **18a** reached their maximum around pK_{BH^+} value of protonated NMM. With more basic amines, DIPEA and TEA, lower yields of both **16a** and **18a** were obtained. We speculated that amines as strong as DIPEA and TEA might cause a partial decyanoethylation in the course of the neutralization step, which generated additional unprotected phosphates and thus decreased the efficiency of the synthesis for both **16a** and **18a**.

At this point, we hypothesized that the use of a free base was not mandatory for an efficient cation exchange between the solid-support-bound PS or PO moieties and the solution of a neutralizing agent. To test this hypothesis, solutions of DBU[10a] in MeCN were mixed with different concentrations of 1*H*-tetrazole (Tet) or with AcOH. As an example of inorganic salts, $LiClO_4$, which is readily soluble in MeCN, was tested. These agents

(10) For pK_{BH^+} values in MeCN, see: (a) Py, 12.33; Lut, 13.92; Col, 14.77; DMAP, 17.74; DBU, 24.13 (Kaljurand, I.; Bodima, T.; Leito, I.; Koppel, I. A.; Schwesinger, R. *J. Org. Chem.* **2000**, *65*, 6202−6208). (b) NMM, 15.59 (Izutsu, K. *Acid−Base Dissociation Constants in Dipolar Aprotic Solvents*; Blackwell Scientific: Oxford, 1990; p 166). (c) DIPEA, 18.00 (estimated). To the best of our knowledge, the experimental value for DIPEA has not been reported. (d) TEA, 18.46 (Coetzee, J. F.; Padmanabhan, G. R. *J. Am. Chem. Soc.* **1965**, *87*, 5005−5010). (e) TMG, 23.3 (Schwesinger, R. *Nachr. Chem., Technol. Lab.* **1990**, *38*, 1214−26).

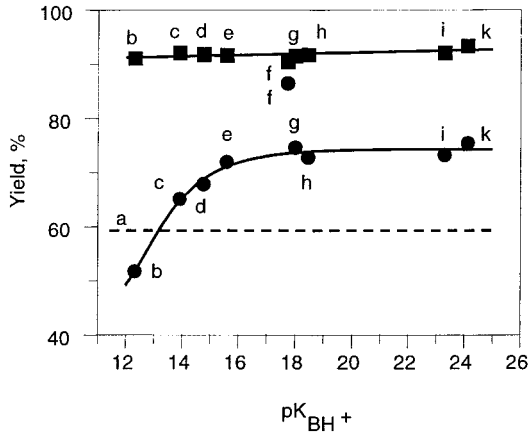


Figure 2. Total yields of the 5′-segments of **16a** (■) and **18a** (●) as a function of pK_{BH^+} (MeCN) values of protonated nitrogen bases. (a) Standard cycle (shown as a horizontal grid); (b) Py; (c) Lut; (d) Col; (e) NMM−Tet; (f) DMAP−Tet; (g) DIPEA−Tet; (h) TEA−Tet; (i) TBD−Tet; (k) DBU−Tet.

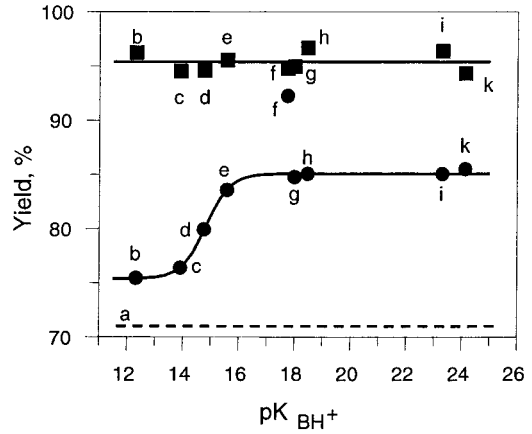


Figure 3. Total yields of the 5′-segments of **16b** (■) and **18b** (●) as a function of pK_{BH^+} (MeCN) values of protonated nitrogen bases. (a) Standard cycle (shown as a horizontal grid); (b) Py; (c) Lut; (d) Col; (e) NMM−Tet; (f) DMAP−Tet; (g) DIPEA−Tet; (h) TEA−Tet; (i) TBD−Tet; (k) DBU−Tet.

were used as neutralizers in the synthesis of **16a** and **18a** as described above for the free amines. The highest yields of **16a** and **18a** were obtained with a mixture of 0.1 M DBU and 0.1 M Tet (93.3 and 75.4%, respectively). Very similar results were obtained for both oligonucleotides when a slight excess (0.11 M) of Tet was present. In contrast, the yields of **16a** and **18a** were dramatically lower (by 29% and 11%, respectively) when excess DBU was used (0.10 M DBU + 0.09 M Tet). Similarly, using an equimolecular mixture of DBU and glacial AcOH or a solution of $LiClO_4$ reduced the yield of **16a** by more than 20%.

On the basis of this observation, a number of strong aliphatic amines, i.e., NMM, 4-(dimethylamino)pyridine (DMAP),[10a] DIPEA, TEA, and *N*,*N*,*N*′,*N*′-tetramethylguanidine (TMG),[10e] were tested as equimolecular mixtures with Tet. At 0.1 M concentration, all salts with Tet were readily soluble in dry MeCN and thus could be used safely on a DNA synthesizer. As seen in Figures 2 and 3, the use of these agents had no adverse effect on the synthesis of **16a** and **16b**. Moreover, the yields of **18a** and **18b** obtained with 1*H*-tetrazolides of NMM, DIPEA, and TEA were higher than with the corresponding free amines. The mixture of DMAP and Tet represented a

special case, and the results obtained are discussed separately (vide infra). With other neutralizing agents, the yields of **18a** and **18b** reached a plateau around the pK_{BH^+} value of protonated DIPEA and then remained independent of the acidity of the protonated amine.

Interestingly, the pattern of the experimental curves was characteristic for the titration, which is most apparent for **18b** (Figure 3). Indeed, the datapoints in Figure 3 were best fitted using the Henderson−Hasselbach equation,[11] which, in the present case, was transformed to

$$pK_{BH^+} = (14.83 \pm 0.05) + \lg\left(\frac{Y - Y_{min}}{Y_{max} - Y}\right)$$

where

$$\frac{Y - Y_{min}}{Y_{max} - Y} = \frac{[A^-]}{[HA]}$$

One may expect that, at low pK_{BH^+} of a neutralizing base, the internucleosidic moiety may present itself as the *O*,*O*′-dinucleoside phosphoric acid (HA). At high pK_{BH^+}, it is mostly ionized to form the corresponding phosphate anion (A^-). It can be seen from Figure 3 that both HA and A^- display a negative effect on the yield of **18b** (*Y*, %). However, the effect of A^- is less pronounced, which is reflected in a higher yield of **18b** ($Y_{max} = 85.1\%$) at high pK_{BH^+}. In contrast, the species HA that are dominant at low pK_{BH^+} reduce the yield of **18b** more substantially ($Y_{min} = 75.4\%$).

Following these considerations, the inflection point of the curve for **18b** in Figure 3 (14.83 ± 0.05) may correspond to an average pK_a value of protonated internucleosidic PO moieties under the conditions of phosphoramidite coupling. In general, diesters of thionophosphoric acid are more acidic than the corresponding derivatives of phosphoric acid. Indeed, comparison of the curves for **18a** and **18b** (Figures 2 and 3, respectively) shows that the former is shifted towards the more acidic p*K* values. Using the set of amines chosen for this work, the curve for **18a** was not observed in full detail and the inflection point could not be determined with confidence.

It has been reported recently that tertiary ammonium azolide salts provide a more efficient catalysis in alcoholysis of dialkyl tetrazolylphosphonite than the corresponding azoles or tertiary amines.[12] The catalytic effect of the salts correlated with the difference in the p*K* values of the acid and the base components, with the salts of stronger protolytes being more powerful catalysts. Therefore, it is also possible that the observed improvement in the yields of **18a** and **18b** arises, at least partially, from the catalytic effect of tertiary ammonium salts on the phosphoramidite coupling.

The effect of DMAP−Tet, of all the neutralizing agents tested, on the phosphoramidite coupling to phosphate-unprotected oligonucleotides is noteworthy. Indeed, the yields of **18a** and **18b** that were obtained using DMAP−Tet departed from the general trend and were markedly higher than might be expected from the pK_{BH^+} value of the protonated DMAP (Figures 2 and 3). The mean total yields of the 5′-segments of **18a** and **18b** were 86.4 ± 0.2% and 92.2 ± 0.3% ($n = 4$), or only 4% and 3% lower than the yields of **16a** and **16b**, respectively. These data corresponded to the average stepwise yield of 98.4% and 99.1% for the PS and PO coupling cycles.

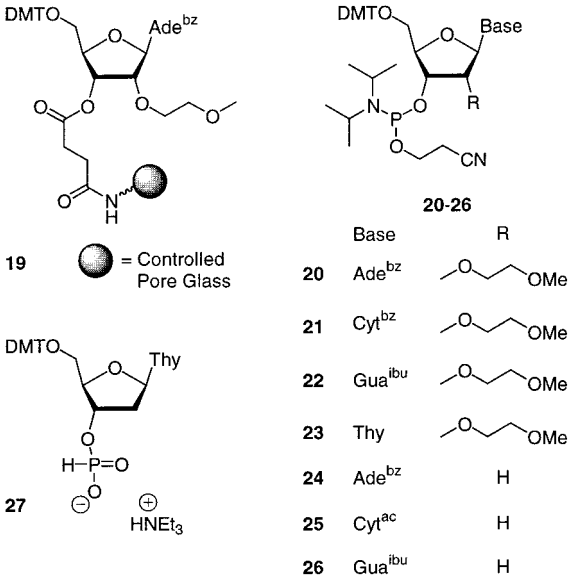
To explain the remarkable effect of the DMAP 1*H*-tetrazolide, one may consider the outstanding catalytic ability of DMAP in nucleophilic reactions. According to the original hypothesis, an activated phosphoramidite may form a mixed anhydride with an internucleosidic phosphate diester group. In the presence of excess 1*H*-tetrazole, this intermediate could be cleaved to regenerate the activated phosphoramidite, nucleoside phosphorotetrazolide.[4] As seen from the results of the ^{31}P NMR studies, the mixed anhydride is a short-lived intermediate that is not observed directly. However, by forming the mixed anhydride, the internucleosidic phosphates may efficiently compete with the 5′-hydroxy groups for the reactive species. Accordingly, the positive effect of DMAP may consist in catalyzing the reverse reaction, i.e., the regeneration of the nucleoside phosphorotetrazolide. Alternatively, DMAP may catalyze the coupling to the 5′-hydroxy groups in a manner similar to the reported observations concerning the synthesis of oligoribonucleotides.[13]

Summarizing the results of the model studies, one may conclude that the efficiency of the phosphoramidite coupling to phosphate-unprotected oligonucleotides is lower than with the standard, protected oligonucleotides. We demonstrated however that the efficiency of the synthesis can be improved by using 0.1 M DMAP 1*H*-tetrazolide in MeCN for the neutralization of the PS or PO backbone. Under optimized conditions, stepwise yields as high as 98+% were obtained.

Synthesis of Modified Oligonucleotides Using Phosphoramidite and H-Phosphonate Methods in Succession. The practical utility of phosphoramidite coupling to phosphate-unprotected oligonucleotides was demonstrated by preparing chimeric antisense oligonucleotides **32a** and **32b** against human MDM2 mRNA. As depicted in Scheme 3, compounds **32a** and **32b** comprised three segments consisting of PS and nucleoside-3′-phosphoramidate (PN) linkages. In addition, six 2′-*O*-(2-methoxyethyl) ribonucleoside (MOE) residues[14] were introduced at each terminus of **32a** and **32b**.

Starting from **19**, a solid-support-bound, protected oligonucleotide **28**, whose thionophosphate triester internucleosidic linkages (PPS) were protected with 2-cyanoethyl groups, was first synthesized by the phosphoramidite method using the commercially available building blocks **20** and **22**−**26**. The synthesis then proceeded with assembly of the segment 2 by the *H*-phosphonate method using **27** as a building block and pivaloyl chloride as the activator. The product **29** was converted to **30a** and **30b** by oxidative amidation of *H*-phosphonate linkages with a solution of a primary amine in CCl_4 as described previously.[15] Simultaneously, the 2-cyanoethyl protecting groups were removed to convert the PPS groups in segment 1 to the deprotected thionophosphate diester internucleosidic linkages, PS. In addition, treatment with primary amines partially removed the base protecting groups. These were restored by acylation with the standard capping reagent (Ac_2O/*N*-methylimidazole/Py/THF) for 2 h.

(11) See, for example: Atkins, P. W. *Physical Chemistry*, 3rd ed.; W. H. Freeman and Co.: New York, 1985; p 280.
(12) Nurminen, E. J.; Mattinen, J. K.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1999**, 2551−2556.
(13) Pon, R. T. *Tetrahedron Lett.* **1987**, *28*, 3643−3646.
(14) Martin, P. *Helv. Chim. Acta* **1995**, *78*, 486−504.
(15) Maier, M. A.; Guzaev, A. P.; Manoharan, M. *Org. Lett.* **1999**, *2*, 1819−1822.



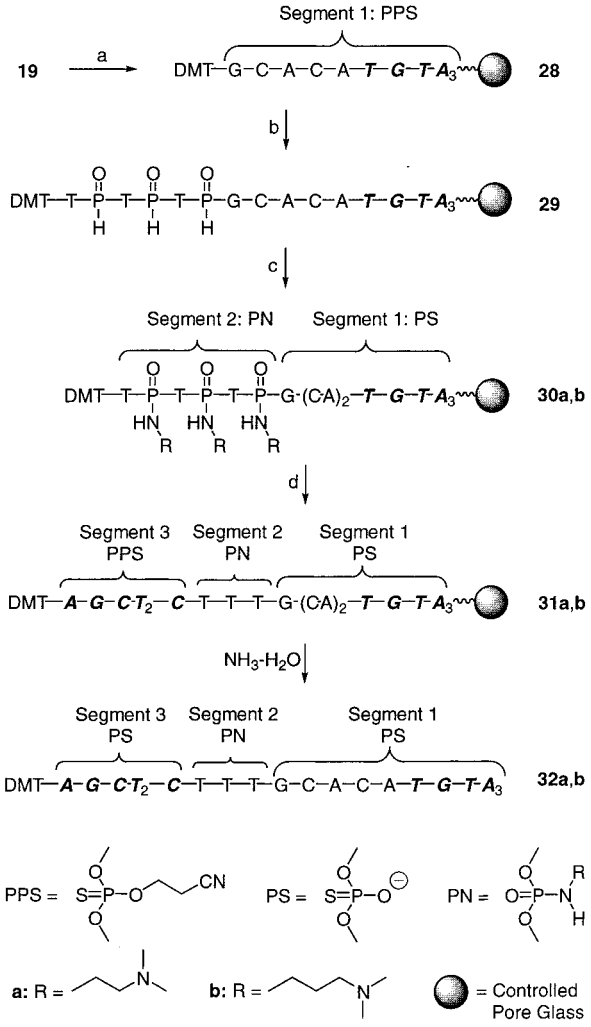
It has been previously reported that the chain elongation by the *H*-phosphonate approach can be carried out efficiently with solid-support-bound, phosphate-unprotected oligonucleotides.[16] Therefore, the synthesis of the oligonucleotide analogues of **32a** and **32b** could be completed using the *H*-phosphonate building blocks. However, the *H*-phosphonate counterparts of the building blocks **20**−**23** are not available commercially, and hence they have to be synthesized, purified, and characterized as described previously.[15] Similarly, many other nucleosidic and nonnucleosidic building blocks for the preparation of modified oligonucleotides are less commonly used and are commercially available only as 2-cyanoethyl phosphoramidites.

Using the procedure described here allows one to avoid the synthesis of unusual *H*-phosphonate building blocks. Starting from **30a** and **30b**, the synthesis was resumed by the phosphoramidite method with **20**−**23** as building blocks. The synthesis was carried out using the modified elongation cycle where detritylation was followed by washing with the neutralizer (0.1 M DMAP and 0.1 M Tet) as described above for **18a** and **18b**. The products **31a** and **31b** were deprotected with concentrated ammonium hydroxide in a conventional manner to give **32a** and **32b**. These were isolated by reverse phase HPLC in 33% and 36% yield, respectively, and characterized by ESMS. By comparison, when the standard cycle was used, the yield of **32a** was only 12%.

Conclusions

From the results obtained, one may conclude that the phosphoramidite coupling to phosphate-unprotected oligonucleotides does not result in formation of unwanted side products. Moreover, the coupling reaction may be carried out in 98+% yield, i.e., almost as efficiently as with the standard, phosphate protected oligonucleotides. To achieve this, the PS and PO residues were converted to their 4-(*N*,*N*-dimethylamino)pyridinium salts using an equimolecular mixture of DMAP and 1*H*-tetrazole prior to each coupling reaction. The method introduced here is useful for the preparation of oligonucleotides with mixed backbones that require the successive use of *H*-phosphonate and phosphoramidite approaches. It may also allow the efficient synthesis of oligonucleotides using very labile phosphate protecting groups that can be removed under automated synthesis conditions.[17]

Scheme 3[a]



[a] (a) Phosphoramidite synthesis using **20** and **22**−**26**; (b) *H*-phosphonate synthesis using **27** and pivaloyl chloride as an activator; (c) **i**: RNH_2 (5% in CCl_4), 3 h/rt; **ii**: Ac_2O/*N*-methylimidazole/Py/THF, 2 h/rt; (d) phosphoramidite synthesis using **20**−**23** and washing with 0.1 M DMAP + 0.1 M 1*H*-tetrazole in MeCN after detritylation. 2′-*O*-(2-Methoxyethyl) ribonucleoside residues are shown in bold italicized letters.

Experimental Section

Materials and Methods. Anhydrous MeCN (water content <0.001%) was purchased from Burdick and Jackson (Muskegon, MI). Standard phosphoramidites **2** and **24**−**26**, thymidine *H*-phosphonate **27**, and ancillary reagents for oligonucleotide synthesis were purchased from Glen Research (Virginia). 2′-*O*-(2-Methoxyethyl) ribonucleoside phosphoramidites **20**−**23** were obtained from Proligo Biochemie GmbH (Hamburg, Germany). All other reagents and dry solvents were purchased from Aldrich and used without further purification. Tentagel and controlled pore glass versions of the solid support **1** were synthesized as previously reported.[6]

^{31}P NMR spectra in gel phase were recorded at 80.950 MHz. The spectra were recorded using 10−15 mg of solid supports

(16) Gryaznov, S. M.; Potapov, V. K. *Tetrahedron Lett.* **1991**, *32*, 3715−3718.

(17) Guzaev, A. P.; Manoharan, M. *J. Am. Chem. Soc.* **2001**, *123*, 783−793.

3−**8** loaded at 208 μmol g^{-1} and CD_3CN as a liquid phase with the spinning switched off. With the phosphate-unprotected oligonucleotides **4**−**8**, the best resolution was obtained when an amine was added to the liquid phase (1 M piperidine for **4** or 5% Py for **5**−**8**).

HPLC Techniques. Crude **9a**, **11a**, **16a**, **18a**, and **32a** and **32b** were analyzed and isolated on a DeltaPak C18 column (15 μm; 300 Å; 3.8 mm × 300 mm) eluting with a linear gradient from 0% to 60% B in 40 min at a flow rate of 1.5 mL min^{-1}. Oligonucleotides **9b**, **11b**, **16b**, and **18b** were analyzed using a linear gradient from 0% to 60% B in 30 min; 0.1 M aqueous NH_4OAc and 80% aqueous MeCN were used as buffer A and buffer B, respectively.

Oligonucleotide Synthesis. The oligonucleotide synthesis was performed on an ABI 380B DNA synthesizer. The phosphoramidite synthesis was carried out either according to the manufacturer's recommendations (standard cycle) or by a modified procedure. Phosphoramidites **2** and **20**−**26** were used as 0.1 M solutions in dry MeCN. For the attachment of phosphoramidites **20**−**23**, the coupling time was extended to 10 min. For preparation of the PS oligonucleotides, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in MeCN) was used as a sulfur-transfer reagent.[8] For preparation of **16a** and **16b** and **18a** and **18b** by the modified cycle, the neutralizers, $LiClO_4$ or a tertiary amine (0.1 M in MeCN) or mixtures containing 0.1 M tertiary amine and 0.1 M 1*H*-tetrazole, were prepared. These were placed in positions 15 or 17 of the synthesizer (PS and PO cycles, respectively). The standard, 1 μmol protocol was modified in two respects. First, the delivery time for the capping reagents and the following wait time were extended to 45 s each. This reduced dramatically the presence of the 5′-DMT-protected 19-mer and shorter oligonucleotides and thus simplified the calculation of yields for the less efficient syntheses. Second, the standard detritylation subroutine was followed by a brief washing with MeCN and flushing with argon. To convert the support-bound oligonucleotide to the required salt, the solution of a neutralizer was next delivered to the columns for 45 s. Finally, the columns were washed with MeCN and flushed with argon prior to the coupling step, as in the standard protocol.

The *H*-phosphonate synthesis was carried out according to the manufacturers recommendations. Thymidine 3′-*H*-phosphonate **27** and the activator, pivaloyl chloride, were used as 0.05 M solutions in MeCN−Py (50:50) and 0.2 M solutions in MeCN−Py (95:5), respectively.

Solid-support-bound **8**, **10**, **12**, and **17a** and **17b** were deprotected with concentrated aqueous ammonium hydroxide for 30 min at room temperature. Compounds **31a** and **31b** were deprotected for 6 h at 55 °C. The products, 5′-DMT protected crude oligonucleotides **9a** and **9b**, **11a** and **11b**, **16a** and **16b**, **18a** and **18b**, and **32a** and **32b**, were analyzed by reverse phase HPLC and characterized by electron-spray LCMS. The modified oligonucleotides **32a** and **32b** were isolated and desalted by reverse phase HPLC.

Acknowledgment. The authors would like to thank Mr. Patrick D. Wheeler, Isis Pharmaceuticals, for his help in performing NMR experiments on solid support and Professor Harri Lönnberg, University of Turku, and Mr. Erkki Nurminen, Åbo Akademi University, Turku, Finland, for helpful discussions and for providing the references on the pK_{BH^+} values.

Supporting Information Available: ^{31}P NMR spectra for **3a**, **4a** and **4b**, **6a**−**8a**, and **8b**; table of the average stepwise yields and the total yields of **16a** and **16b** and **18a** and **18b** and algorithm for their calculation; ESMS data for **11a** and **11b**, **16a** and **16b**, **18a** and **18b**, and **32a** and **32b**; HPLC profiles of **9**, **16**, and **18a** and **18b** obtained under standard and optimal conditions; and HPLC profiles of crude **32a** and **32b**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO001591E