Tetrahedron Letters, Vol. 33, No. 40, pp. 5869-5872, 1992
Printed in Great Britain

0040-4039/92 $5.00 + .00
Pergamon Press Ltd

H-Phosphonate DNA Synthesis Without Amino Protection

Pei-Pei Kung and Roger A. Jones*

Department of Chemistry
Rutgers, The State University of New Jersey
Piscataway, NJ 08855

Key Words: Oligonucleotide synthesis, H-phosphonate

Abstract: DNA synthesis by the H-phosphonate method does not require the use of amino protecting groups. The cytosine amino group, and the guanine O6/N1 positions, do react with the condensing agent to some extent, but these derivatives are cleaved under the standard aqueous ammonia conditions commonly used for deprotection and cleavage of the oligonucleotide from the solid support.

In early work on oligonucleotide synthesis using the diester approach, the cytosine amino group was found to be phosphorylated, when not protected, although neither the adenine nor guanine amino groups were.[1] This same pattern of reactivity was later found for the triester approach.[2] More recent experiments using the phosphoramidite method have found that both the adenine and cytosine amino groups react with activated phosphoramidites.[3] We now report that, in the H-phosphonate approach, there is no phosphonylation of the adenine, cytosine, or guanine amino groups. Instead, we find that cytosine and guanine, but not adenine, are subject to acylation by the pivaloyl or adamantoyl chlorides used as the condensing agent. In the case of guanine, the site of acylation is not the 2-amino group, but is rather the O6/N1 position. Moreover, because of the lability of these guanine adducts,[4] and the reactivity of the C4 position of cytosine nucleosides,[5] these modifications are readily reversible under the standard ammonia deprotection conditions used for DNA synthesis. Thus, DNA synthesis using the H-phosphonate method does not require amino protection.

The preparation of H-phosphonate monomers without amino protecting groups is complicated by the fact that each of the amino groups, and particularly the guanine amino group, is subject to reaction with dimethoxytrityl chloride.[6-9] Our approach (Figure 1) is first to prepare the *N*-dimethylaminomethylene derivatives **2** of the 2'-deoxynucleosides **1** by reaction with dimethylformamide dimethylacetal in methanol. Formation of these amidine derivatives is a much cleaner reaction than is acylation, and proceeds in near quantitative yield.[10-14] Although the cytosine and, to a lesser extent, the adenine dimethylaminomethylene derivatives are readily hydrolyzed, they can be prepared and used directly in the tritylation reaction, without purification, to give **3**.[15] Phosphonylation, again without purification, then gives the H-phosphonates **4**, which are purified by silica chromatography.[16] After this chromatography, the monomers have each lost the dimethylaminomethylene group to some extent; cytosine entirely, adenine largely, and guanine about half. Treatment of the adenine and guanine derivatives with aqueous ammonia then completes the amino deprotection to give **5**. We have found, moreover, that the dimethylaminomethylene group can be reintroduced at this stage, if desired, simply by treatment of these H-phosphonates with dimethylformamide dimethylacetal in methanol.

To evaluate the use of these amino-unprotected derivatives **5** in DNA synthesis we next carried out several short oligonucleotide syntheses. For comparison, we also carried out syntheses using the amidine derivatives **4**. After completion of the synthesis and oxidation, samples of the support-bound products were treated with aqueous ammonia and the ammonia solutions were examined by HPLC. After treatment under the

standard ammonolysis conditions, 65 °C for 4-12 h, or 2-3 d at room temperature, there was no detectable difference, by HPLC, between molecules made using derivatives **5** and those made using derivatives **4**. After treatment with concentrated ammonia at room temperature for about 18 h, as shown for the molecule d[DMT-TTCAGT] in Figure 2 (reversed-phase HPLC on a C-18 Nova-Pak column, using a gradient of 2 to 40 % acetonitrile to 0.1 M triethylammonium acetate in 5 min at 4 mL/min), the same single main product was obtained from the unprotected monomers **5** (left) and the amidine-protected monomers **4** (middle). The right panel in Figure 2 is a coinjection of these two mixtures. Examination of the ammonia solution at shorter times, however, revealed the presence of a more complicated mixture. Figure 3 shows chromatograms of the molecule d[DMT-TCAGT], prepared using adamantoyl chloride (AdCl) as the condensing agent, after treatment with concentrated aqueous ammonia for ~ 15 min (left), 1 h (middle), and overnight (right). A sample of this material, treated only briefly with aqueous ammonia, was purified using a 25 mm x 10 cm Nova-Pak C-18 column.

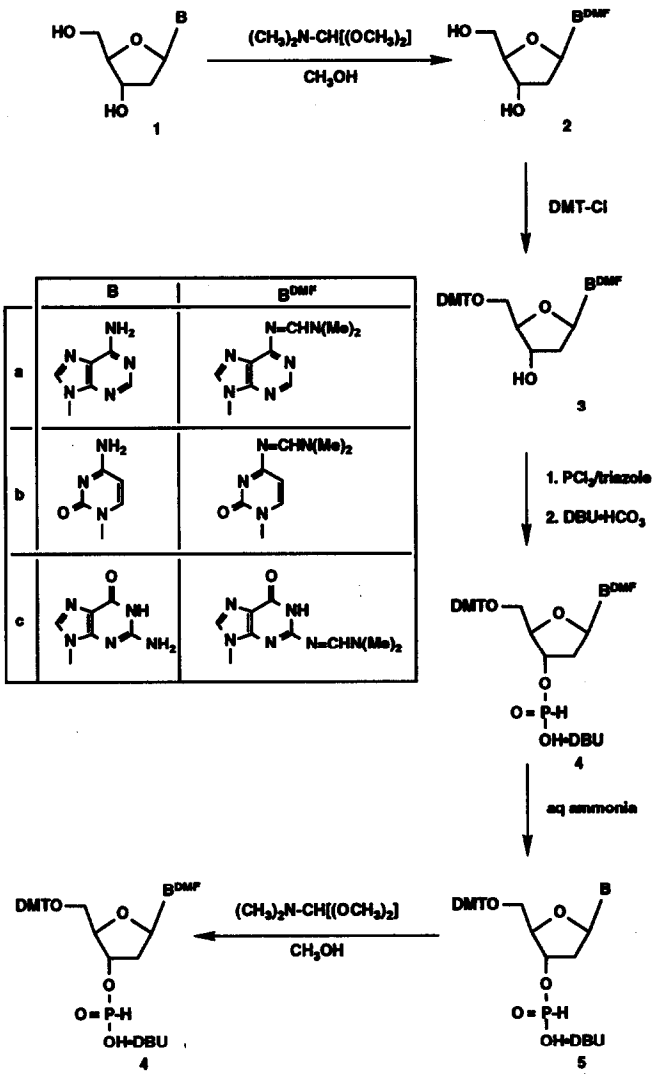
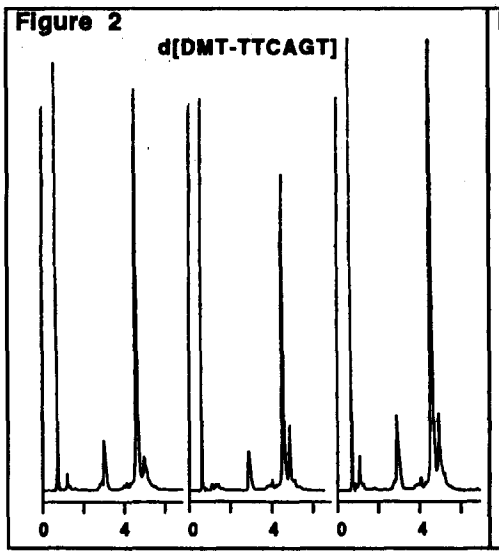


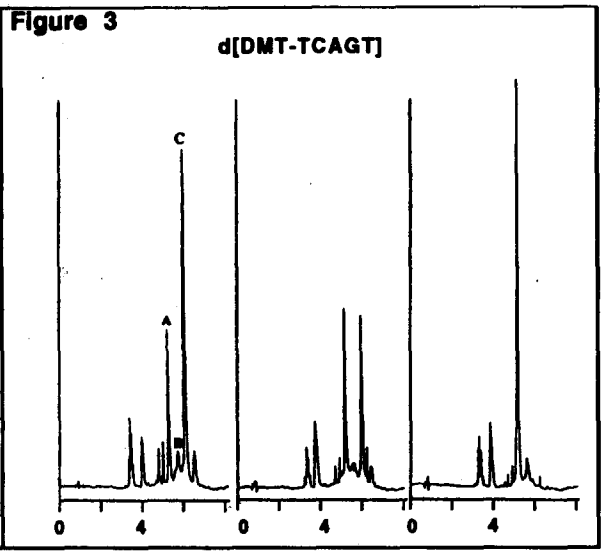
Figure 1

Fractions corresponding to peaks A, B, and C were then treated at pH 4.6 with a combination of nuclease P1 and calf alkaline phosphatase. Analysis of the resulting mixture by HPLC revealed that peak A contained the expected mixture of dC, dG, dT, and dA, while in peaks B and C, dG and dC, respectively, were modified. Similarly, synthesis of d[DMT-TCAGT] using pivaloyl chloride as the condensing agent, followed by brief ammonia treatment, gave a product mixture from which we were able to isolate fractions that, upon enzymatic degradation, showed modification of dG and dC. We have found that reaction of 5'-dimethoxytrityl-2'-deoxyguanosine, or the N^2-isobutyryl or dimethylaminomethylene compounds with either AdCl or pivaloyl chloride in pyridine gave the corresponding O^6/N^1-adamantoyl/pivaloyl-2'-deoxyguanosine derivatives, identified by NMR and UV. Further, reaction of 2'-deoxycytidine with either AdCl or pivaloyl chloride gave N^4-adamantoyl/pivaloyl-2'-deoxycytidine, which was identical, by HPLC, to the material obtained by enzymatic degradation.

These experiments demonstrate that H-phosphonate DNA synthesis can be carried out without amino protecting groups. Although cytosine and guanine react with the acid chlorides commonly used as condensing

agents in this approach, no additional steps are needed during the synthesis or deprotection to cleave the resulting adducts. The amino-unprotected H-phosphonates can be prepared in high yield by using the dimethylaminomethylene group as a 'transient' protecting group. In addition, the labile cytosine dimethylaminomethylene derivative can be prepared from the H-phosphonate **5**, without requiring chromatography (where it would be cleaved),[13] and used in oligonucleotide synthesis to avoid acylation of this amino group by the condensing agents. This would be useful in cases where the standard ammonia conditions are too harsh.





The ability to use amino-unprotected monomers in DNA synthesis will be most useful for 2'-deoxyadenosine, because of the enhanced stability of the glycosidic bond relative to the commonly used *N*-benzoyl derivative. Further, preparation of amino-unprotected monomers for DNA synthesis proceeds in somewhat higher yield than does preparation of the *N*-acyl monomers, which is important for isotopically labeled or otherwise modified monomers. The effect, if any, on the coupling yields using these monomers was not apparent in these experiments. Other recent work, however, suggests that the 2'-deoxyadenosine coupling yields may be slightly better without amino protection, although many more examples will be needed to assess the generality of this still preliminary observation.[17]

References

1. Khorana, H. G.; Turner, A. F.; Vizsolyi, J. P. *J. Am. Chem. Soc.* **1961**, *83*, 686-698.
2. Narang, S. A.; Itakura, K.; Wightman, R. H. *Can. J. Chem.* **1972**, *50*, 769-770.
3. Gryaznov, S. M.; Letsinger, R. L. *J. Am. Chem. Soc.* **1991**, *113*, 5876-5877.
4. Daskalov, H. P.; Sekine, M.; Hata, T. *Tetrahedron Lett.* **1980**, *21*, 3899-3902.
5. Weber, H.; Khorana, H. G. *J. Mol. Biol.* **1972**, *72*, 219-249.
6. Smith, M.; Rammler, D. H.; Goldberg, I. H.; Khorana, H. G. *J. Am. Chem. Soc.* **1962**, *84*, 430-440.
7. Schaller, H.; Weimann, G.; Lerch, B.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 3821-3827.
8. Ralph, R. K.; Connors, W. J.; Schaller, H.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 1983-1988.
9. Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316-1319.

10. Zemlicka, J. *Coll. Czech. Chem. Comm.* **1963**, *28*, 1060-1062.
11. Zemlicka, J.; Holy, A. *Coll. Czech. Chem. Comm.* **1967**, *32*, 3159-3168.
12. Froehler, B. C.; Mateucci, M. D. *Nucleic Acids Res.* **1983**, *11*, 8031-8036.
13. McBride, L. J.; Kierzek, R.; Beaucage, S. L.; Caruthers, M. H. *J. Am. Chem. Soc.* **1986**, *108*, 2040-2048.
14. Vu, H.; McCollum, C.; Jacobson, K.; Theisen, P.; Vinayak, R.; Speiss, E.; Andrus, A. *Tetrahedron Lett.* **1990**, *31*, 7269-7272.
15. To a suspension of 3 mmol of the appropriate 2'-deoxynucleoside **1** in 20 mL of methanol, was added 1.2 mL (9 mmol) of dimethylformamide dimethylacetal. After stirring overnight at room temperature a clear solution was obtained. Evaporation gave a white residue which was pure by HPLC and had UV spectra identical to material prepared according to the literature.[11]
To the appropriate *N*-dimethylaminomethylene derivative **2**, dried three times by evaporation of pyridine and suspended in 50 mL of dry pyridine, was added 1.55 g (4.6 mmol) of 4,4'-dimethoxytrityl chloride. After stirring overnight under a nitrogen atmosphere the mixture was added to a cold 100 mL portion 5.4 M aqueous $NaHCO_3$. The mixture was then extracted with five 100 mL portions of a mixture of diethyl ether and methylene chloride (1/1; v/v). Concentration of the combined organic layers and purification of the residue by chromatography on silica gel using a gradient of 0 to 3 % methanol in methylene chloride containing 1 % pyridine gave pure **3**. Analytically pure samples of **3a**, **3b**, and **3c** were obtained in yields of 61 to 82 % by precipitation from methylene chloride/pentane:
3a (80 %): UV (CH_2Cl_2) max 235, 311 nm; min 260 nm. ^1H NMR (DMSO-d_6) δ (ppm) 8.88, (s, 1, H_8), 8.33 (s, 2, H_2 & N=CH), 7.21 (m, 11, NH_2 & Ar), 6.76 ("t", 4, J_{app}=9 Hz, Ar), 6.40 ("t", 1, J_{app}=6.3 Hz, $H_{1'}$), 5.37 (d, 1, J=4 Hz, 3'-OH), 4.47 (m, 1, $H_{3'}$), 3.97 (m, 1, $H_{4'}$), 3.70 & 3.69 (s & s, 3 & 3, OCH_3), 3.31 (m, 2, $H_{5',5''}$), 3.10 & 3.17 (s & s, 3 & 3, NMe_2), 2.89 & 2.32 (m & m, 1 & 1, $H_{2'}$ & $H_{2''}$). Anal. calcd. for $C_{34}H_{36}N_6O_5 \cdot 1/4\ H_2O \cdot 3/4\ C_5H_{12}$: C, H, N.
3b (61 %): UV (MeOH) max 232, 315 nm; min 222, 257 nm. ^1H NMR (DMSO-d_6) δ (ppm) 8.61 (s, 1, N=CH), 7.81 (d, 1, J=7.0 Hz, H_6), 7.31 (m, 9, Ar), 6.89 (m, 4, Ar), 6.15 ("t", 1, J_{app}=6.2 Hz, $H_{1'}$), 5.72 (d, 1, J=7 Hz, H_5), 5.30 (m, 1, 3'-OH), 4.26 (m, 1, $H_{3'}$), 3.91 (m, 1, $H_{4'}$), 3.73 (s, 6, two OCH_3), 3.22 (m, 2, $H_{5',5''}$), 3.16 & 3.02 (s & s, 3 & 3, NMe_2), 2.22 & 2.09 (m & m, 1 & 1, $H_{2'}$ & $H_{2''}$). Anal. calcd. for $C_{33}H_{36}N_4O_6 \cdot 1/2\ H_2O \cdot 1/4\ C_5H_{12}$: C, H, N.
3c (82 %): UV (CH_2Cl_2) max 275, 308 nm; min 257, 280 nm. ^1H NMR (DMSO-d_6) δ (ppm) 11.34 (br, 1, NH), 8.50 (s, 1, N=CH), 7.90, (s, 1, H_8), 7.25 (m, 9, Ar), 6.80 (m, 4, Ar), 6.27 ("t", 1, J_{app}=6.2 Hz, $H_{1'}$), 5.36 (m, 1, 3'-OH), 4.39 (m, 1, $H_{3'}$), 3.91 (m, 1, $H_{4'}$), 3.70 (s, 6, two OCH_3), 3.10 (m, 2, $H_{5',5''}$), 3.10 & 3.01 (s & s, 3 & 3, NMe_2), 2.66 & 2.30 (m & m, 1 & 1, $H_{2'}$ & $H_{2''}$). Anal. calcd. for $C_{34}H_{36}N_6O_6 \cdot 2/3\ H_2O \cdot 1/3\ C_5H_{12}$: C, H, N.
16. To a cold (ice-salt bath) solution of crude **3** in 90 mL of methylene chloride was added a cold solution of 1.35 mL (15 mmol) of PCl_3, 14.7 mL (133 mmol) of N-methylmorpholine, and 3.45 g (50 mmol) of 1,2,4-triazole in 120 mL of methylene chloride. After stirring for 1 h in the ice-salt bath and 40 min at room temperature, the mixture was poured into a 120 mL portion of 10 % pyridine in water. A 3 mL portion of 1.7 M DBU•HCO_3 was added, the layers separated, and the organic layer concentrated to a foam. The crude material was purified by chromatography on silica gel using a gradient of 0 to 20 % methanol in methylene chloride containing 1 % pyridine. The product fractions were concentrated to a foam containing a mixture of **4** and **5**. Treatment of this **4/5** mixture with aqueous ammonia gave the amino-unprotected derivatives **5** in overall yields (from **1**) of 72 to 90 %. Alternatively, treatment of the **4/5** mixture with dimethylformamide dimethylacetal in methanol for 4 h gave the derivatives **4** in overall yields (from **1**) of 66 to 75 %.
17. Gaffney, B. L.; Jones, R. A. unpublished results.

(Received in USA 19 May 1992; accepted 9 July 1992)