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A New Method for the Preparation of Modified Oligonucleotides

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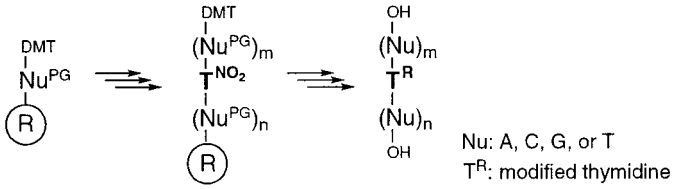
ABSTRACT



***N*-Nitrothymidine can be transformed into a phosphoramidite building block suitable for oligonucleotide synthesis using the standard phosphite triester solid-phase approach. The *N*-nitrothymidine residues remain stable during the elongation cycles and react smoothly with primary amines, furnishing oligonucleotides containing *N*3-modified thymidines. A number of *N*3-substituted oligonucleotides have been prepared using this methodology, some of them incorporating aminoalkyl or hydroxyalkyl groups.**

Progress in solid-phase synthesis methodology has provided efficient tools for the preparation of either unmodified or modified oligonucleotides.[1] The synthesis of DNA oligomers containing modified nucleobases, in particular, may be carried out using two different alternatives (Scheme 1). The first strategy for incorporating modified units requires the synthesis of a specific phosphoramidite building block for each of the nonnatural bases to be included. In this case, depending on the functional groups present in these units, a special protection scheme must be developed to guarantee their chemical compatibility with the protocols used during the solid-phase assembly of the DNA chain. In the second strategy, the oligonucleotide chain is elongated using a building block that is a precursor of the target nucleoside, a so-called "convertible nucleoside".[2] This precursor may be transformed into a range of differently modified nucleosides in the final steps of the synthesis (see Scheme 1).[3]

Recently, it was reported[4] that the substitution of the imidic hydrogen by a nitro group strongly activates the C4-carbonyl group of uridines and thymidines. As shown in Scheme 2, such activated nucleobases can then be easily modified by treatment with primary amines to provide a variety of *N*3-modified nucleosides through a ring-opening−ring-closing

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(1) *Oligonucleotides and Analogues. A Practical Approach;* Eckstein, F., Ed.; IRL Press: Oxford, 1991.

(2) See, for instance: (a) MacMillan, A. M.; Verdine, G. L. *J. Org. Chem.* **1990**, *55*, 5931−5933. (b) Allerson, C. R.; Chen, S. L.; Verdine, G. L. *J. Am. Chem. Soc.* **1997**, *119*, 7423−7433, and references therein. (c) Xu, Y.-Z.; Zheng, Q.; Swann, P. F. *Tetrahedron* **1992**, *48*, 1729−1740. (d) Xu, Y.-Z. *Tetrahedron* **1996**, *52*, 10737−10750, and references therein. (d) Harris, C. M.; Zhou, L.; Strand, E. A.; Harris, T. M. *J. Am. Chem. Soc.* **1991**, *113*, 4328−4329.

(3) For recent examples related to the "convertible nucleoside" strategy, see: (a) Hwang, J.-T.; Greenberg, M. M. *J. Org. Chem.* **2001**, *66*, 363−369. (b) Noll, D. M.; Noronha, A. M.; Miller, P. S. *J. Am. Chem. Soc.* **2001**, *123*, 3405−3411. (c) Banuls, V.; Escudier, J.-M.; Zedde, C.; Claparols, C.; Donnadieu, B.; Plaisancie, H. *Eur. J. Org. Chem.* **2001**, 4693−4700. (d) Robles, J.; Grandas, A.; Pedroso, E. *Tetrahedron* **2001**, *57*, 179−194. (e) Komatsu, Y.; Kumagai, I.; Ohtsuka, E. *Nucleic Acids Res.* **1999**, *27*, 4314−4323. (f) Kohgo, S.; Shinozuka, K.; Ozaki, H.; Sawai, H. *Tetrahedron Lett.* **1998**, *39*, 4067−4070. (g) Erlanson, D. A.; Wolfe, S. A.; Chen, L.; Verdine, G. L. *Tetrahedron* **1997**, *53*, 12041−12056. (h) Haginoya, N.; Ono, A.; Nomura, Y.; Ueno, Y.; Matsuda, A. *Bioconjugate Chem.* **1997**, *8*, 271−280.

(4) (a) Ariza, X.; Bou, V.; Vilarrasa, J.; Tereshko, V.; Campos, J. L. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2454−2455. (b) Ariza, X., Bou; V.; Vilarrasa, J. *J. Am. Chem. Soc.* **1995**, *117*, 3665−3673. (c) Ariza, X.; Farràs, J.; Serra, C.; Vilarrasa, J. *J. Org. Chem.* **1997**, *62*, 1547−1549. (d) Serra, C.; Aragonès, C.; Bessa, J.; Farràs, J.; Vilarrasa, J. *Tetrahedron Lett.* **1998**, *39*, 7575−7578. (e) Serra, C.; Farràs, J.; Vilarrasa, J. *Tetrahedron Lett.* **1999**, *40*, 9111−9113. (f) Ariza, X.; Vilarrasa, J. *J. Org. Chem.* **2000**, *65*, 2827−2829.

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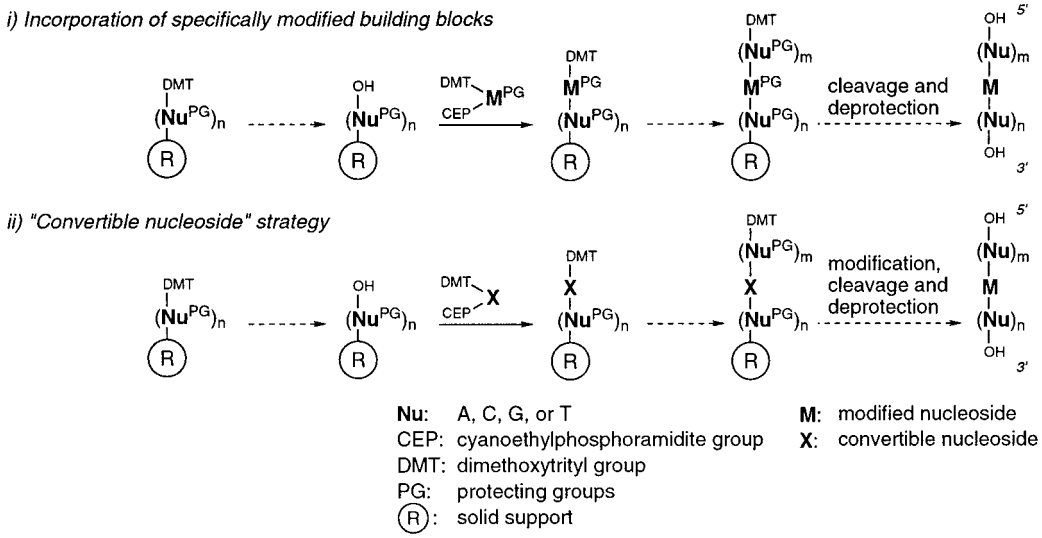
Scheme 1. Strategies for the Solid-Phase Synthesis of Modified Oligonucleotides Using the Phosphite Triester Methodology

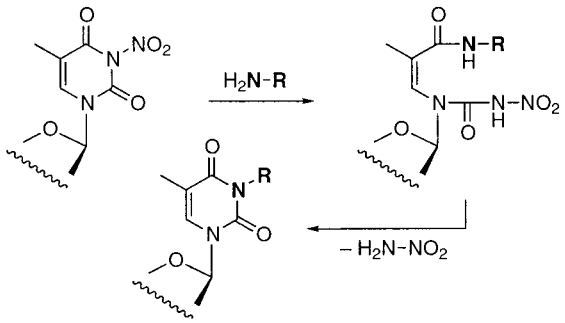


mechanism.[4a,b] In this Letter we describe the preparation of oligodeoxynucleotides with thymines modified at the *N*3 position, making use of the 3′-[(*β*-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite of 5′-*O*-(4,4′-dimethoxytrityl)-*N*-nitrothymidine (**4**, DMT-T^{NO_2}-CEP) as the key building block (Scheme 3). Reaction of *N*-nitrothymidine-containing oligonucleotide−resins with primary amines yields oligonucleotides with *N*3-modified thymines at the positions in which thymidine had been replaced by 3-nitrothymidine.

The strategy proposed in the present work may give access to a variety of modified oligomers using a single building block. To the best of our knowledge, this is the first example of a "convertible nucleoside" that allows the *N*3 position of the pyrimidine moiety to be manipulated.

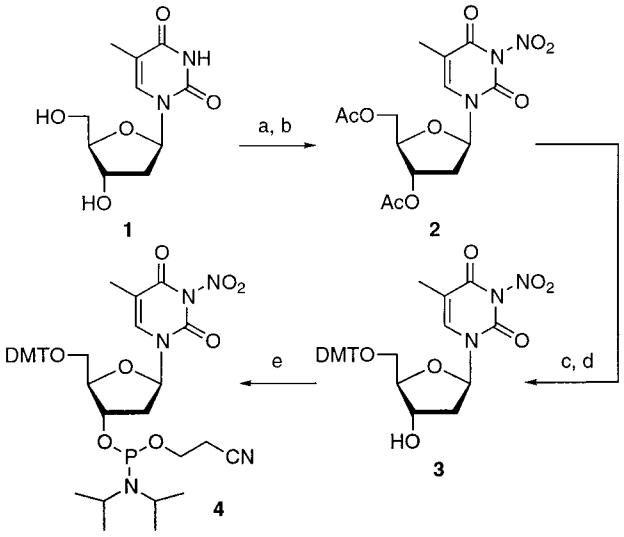
A preliminary series of experiments was carried out in order to establish the chemical compatibility of the *N*-nitro moiety with the solid-phase synthesis protocols. Thus, the protected *N*-nitrothymidine derivative **2** was chosen as model compound and was treated with all the reagents used for the oligonucleotide assembly.[5] None of those treatments produced any significant degradation of the *N*-nitronucleoside moiety.

Scheme 2. Reaction of *N*-Nitrothymidine Residues with Primary Amines



The *N*-nitronucleoside **2** was deacetylated, and the key phosphoramidite **4** was obtained in good yield (see Scheme 3) from the DMT-protected precursor **3** by treatment with (*β*-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of tetrazole. Compound **4** proved to be as stable as any of the other phosphoramidite derivatives and could be stored in the refrigerator for months without being degraded. Finally, the oligonucleotide−resins **5a**−**c**, bearing one or two T^{NO_2} residues, were prepared using the standard phosphite triester solid-phase methodology (see Table 1). As expected, phosphoramidite **4** could be easily incorporated

Scheme 3. Synthesis of DMT-T^{NO_2}-CEP (**4**)[a]



[a] (a) Ac_2O, py, rt (99%); (b) CF_3COONO_2 (3 equiv), CH_2Cl_2, 0 °C, 25 min (82%); (c) HCl/MeOH 0.17 M, rt, 24 h (88%); (d) DMT-Cl (1.2 equiv), DMAP (0.1 equiv), py, rt, 4 h (68%); (e) $[NC(CH_2)_2O]P(N^iPr_2)_2$ (1.2 equiv), tetrazole (0.5 equiv), ACN, rt, 1 h (83%).

Table 1. *N*-Nitrothymidine-Containing Oligonucleotide−Resins Prepared[a−c]

	oligonucleotide−resin
5a	T−T−T−T^{NO_2}−T−T−T−resin
5b	T−T^{NO_2}−T−T−T−T−T^{NO_2}−T−resin
5c	T−G^{iBu}−A^{Bz}−T^{NO_2}−C^{Bz}−A^{Bz}−T−resin

[a] TentaGel-NH_2 resin (Rapp Polymere GmbH) was used as the solid support. The first 5′-DMT-protected nucleoside was attached to the resin by means of a standard succinyl linker, and the initial substitution degree was in the range of 40−190 μmol/g. [b] Oligonucleotide elongation was carried out at 2−5 mmol scale following standard phosphite triester procedures: 0.1−0.15 M solutions of **4** or the commercially available (Glen Research Corp.) 3′-phosphoramidite derivatives (5′-DMT-Nu-P[O(CH_2)$_2$CN]N^{i}Pr$_2$, Nu = T^{NO_2}, T, A^{Bz}, C^{Bz}, G^{iBu}) in anhydrous ACN and 0.5−0.6 M solutions of tetrazole in anhydrous ACN were used for the coupling steps (15 min). A 0.1 M solution of ^{t}BuOOH in CH_2Cl_2 was used in the oxidation steps (1.5 min). All syntheses were performed in an Applied Biosystems 380B DNA synthesizer. [c] Average coupling yields, calculated from the UV absorbance at 498 nm of the DMT cation generated in the deprotection steps, ranged between 99.5% (**5a**) and 98.2% (**5c**).

into the corresponding oligomers using exactly the same conditions as those employed for other phosphoramidite derivatives, with no significant difference in the coupling yields. In addition, no evidence of degradation of the *N*-nitrothymidine moiety was found during the subsequent elongation cycles.

After assembly of the three oligonucleotide−resins **5a**−**c**, we turned our attention to their reaction with primary amines. As shown in Table 2, various oligonucleotide analogues were prepared. The modification reaction was straightforward and could be carried out efficiently under a variety of conditions. Either the neat amine, concentrated aqueous solutions, or dilute solutions of the amine in acetonitrile (ACN) afforded the target product, provided that the reaction times were long enough so as to allow for the completion of the reaction.

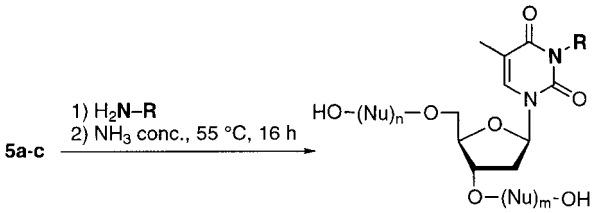
Treatment of the oligonucleotide−resins with the primary amines to some extent also cleaved the oligonucleotides from the resin and removed protecting groups. In any case, concentrated aqueous ammonia was added to the reaction mixture, and the reaction was left to proceed overnight at 55 °C, to ensure completeness of all of the deprotection processes (cleavage of the nucleoside−resin bond and removal of the phosphate and nucleobase protecting groups). This additional treatment was also found to reduce the amount of byproducts present in the final crude mixtures.

As summarized in Table 2, good results were obtained with all of the alkylamines, but reaction of resin **5c** with aniline led to a complex reaction mixture in which no modified nucleoside-containing product could be identified. This result indicates that aromatic amines are not nucleophilic enough to complete the reaction and establishes the lower limit to the scope of the proposed methodology.

It is worth noting that no remarkable differences were found in the reactions involving resins **5a** and **5b**, which

(5) (1) Tetrazole, ACN, 24 h, rt; (2) Ac_2O/2,6-lutidine, THF, 24 h, rt; (3) *N*-methylimidazole, THF, 24 h, rt; (4) I_2, H_2O/py/THF, 24 h, rt; (5) *t*-BuOOH 3 M, toluene, 24 h, rt.

Table 2. Modification of Resins **5a**−**c** with Primary Amines[a,b]



Modified oligomer

6: T-T-T-T^R-T-T-T

6a: R=Me, **6b**: R=Bn, **6c**: R=-(CH_2)$_3$$NH_2$

7: T-T^R-T-T-T-T-T-T^R-T

7: R=Me

8: T-G-A-T^R-C-A-T

8a: R=Bn, **8b**: R=-(CH_2)$_3$$NH_2$, **8c**: R=-($CH_2$)$_5$OH

resin	modification conditions	product
5a	$MeNH_2$ (33% in H_2O), 1 h, rt	**6a**
5a	$BnNH_2$ (neat), 1 h, rt	**6b**
5a	$H_2N(CH_2)_3NH_2$ (neat), 1 h, rt	**6c**
5b	$MeNH_2$ (33% in H_2O), 1 h, rt	**7**
5c	$BnNH_2$ (0.25 M in ACN), 4 days, rt	**8a**
5c	$H_2N(CH_2)_3NH_2$ (0.1 M in ACN), 24 h, rt	**8b**
5c	$HO(CH_2)_5NH_2$ (0.14 M in ACN), 48 h, rt	**8c**
5c	$PhNH_2$ (neat), 2 h, rt	

[a] In all cases, the analytical reverse-phase HPLC chromatograms of the crude products showed a single major peak (80−93%), which corresponded to the expected oligomers **6**−**8**, and very minor peaks associated with other nucleosidic byproducts. As expected, retention times of the modified oligonucleotides **6**−**8** (R ≠ H) were always higher than those of the corresponding unmodified ones (R = H). Reverse-phase HPLC analyses were performed on either Spherisorb (ODS-2, 25×0.4 cm, 5 μm) or Phenomenex (C18, 25×0.46 cm, 10 μm) columns using linear gradients of 0.05 M aqueous triethylammonium acetate and ACN/H_2O (1:1). [b] Identities of all of the major products were established either by electrospray or MALDI-TOF (2,4,6-trihydroxyacetophenone) mass spectrometry (negative mode) of the HPLC-purified material. Identities of **8a**−**c** were also established by enzymatic digestion of the HPLC-purified materials with snake venom phosphodiesterase and alkaline phosphatase. Samples of the modified nucleosides were used as standards.

carried one and two *N*-nitrothymidine residues, respectively. These results clearly point out that several modifications can be simultaneously introduced into oligonucleotide−resins bearing multiple *N*-nitrothymidine residues by means of a single final reaction with primary amines. Moreover, the modification reaction is compatible with the presence of additional free amine and hydroxyl groups in the modification reagent. Successful isolation of the target modified oligonucleotides **8a** and **8b**, after treatment of the oligonucleotide−resin **5c** with either 5-aminopentanol or 1,3-propanediamine (see Table 2), illustrates the fact that protection of the second functional group in the modification reagent is not necessary.

In summary, our results show that the phosphoramidite derivative **4** is a good building block for the synthesis of oligonucleotide−resins containing *N*-nitrothymidine residues. The *N*-nitrothymidine residues behave as "convertible nucleosides" that can be transformed into a range of *N*3-modified thymidines by reaction with primary alkylamines.

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Supporting Information Available: Enzymatic digestion results and NMR data of **2**, **3**, **4**, and *N*-nitrothymidine. This material is available free of charge via the Internet at http://pubs.asc.org.

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