ORGANIC LETTERS

2003
Vol. 5, No. 3
341−344

Novel Method for the Immobilization of Nucleotides

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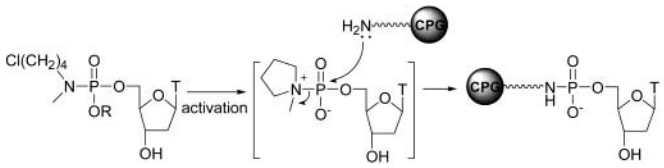
Received November 27, 2002

ABSTRACT



A novel method for the immobilization of nucleotides has been developed. The strategy employs a highly reactive pyrrolidinium phosphoramidate zwitterion intermediate that undergoes nucleophilic attack by long-chain alkylamine-controlled pore glass (LCAA-CPG) to generate an immobilized nucleotide. Quantification of nucleotide loading was accomplished by acidic hydrolysis of the P–N bond and subsequent HPLC analysis of TMP in the presence of an internal standard. Typical nucleotide loadings of 51–59 μmol/g of support were observed.

Numerous methods exist for the attachment of nucleotides to solid supports; however, the extension of these methods to the immobilization of modified nucleotides has, in many cases, demanded alteration of standard solid-phase coupling and cleavage conditions.[1] Recently, we reported a new method for the preparation of nucleoside diphosphates that employs a highly electrophilic zwitterionic phosphoramidate intermediate (**3**) as the phosphorylating reagent (Scheme 1).[2] The reactivity of the zwitterionic intermediate toward nucleophiles under both aqueous and anhydrous conditions has been investigated[2,3] and has inspired the extension of this method to the immobilization of nucleotides and other phosphates. The approach relies upon the assumption that a primary amine attached to controlled pore glass (CPG) will undergo nucleophilic attack at phosphorus of zwitterionic intermediate **3** following activation of the phosphoramidate ester (**1**). Cleavage of the nucleotide from the solid support can be accomplished via acid-catalyzed hydrolysis of the P−N bond, a potentially desirable alternative to the standard base-catalyzed cleavage conditions employed in the preparation of oligonucleotides (concentrated NH_4OH/CH_3NH_2) using automated DNA synthesis.[4] Furthermore, this method may provide an attractive means for the preparation of unique DNA analogues such as DNA−peptide conjugates that might otherwise require careful consideration for timing of deprotection of multiple base-sensitive groups and base-catalyzed cleavage from the support.[5] The synthesis of biopolymers using photolytic cleavage[1c] has provided an additional method for the production of such molecules under neutral conditions; however, deprotection via photolysis, although optimized for the cleavage of oligonucleotides bearing standard bases and protecting groups, still poses potential problems that arise from UV-induced damage to modified biopolymers. Our efforts to develop a novel method for the attachment of nucleotides to solid supports have resulted in generation of CPG derivatized with a thymidine phosphoramidate. We report herein the coupling of a chlorobutyl

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(1) (a) Lyttle, M. H.; Hudson, D.; Cook, R. M. *Nucleosides Nucleotides* **1999**, *8*, 1809−1824. (b) Lyttle, M. H.; Dick, D. J.; Hudson, D.; Cook, R. M. *Nucleic Acids Res.* **1996**, *24*, 2793−2798. (c) Venkatesan, H.; Greenberg, M. *J. Org. Chem.* **1996**, *61*, 525−529. (d) Eritja, R.; Robles, J.; Fernandez-Forner, D.; Alberico, F.; Giralt, E.; Pedroso, E. *Tetrahedron Lett.* **1991**, *32*, 1511−1514.

(2) Freel Meyers, C. L.; Borch, R. F. *Org. Lett.* **2001**, *3*, 3765−3768.

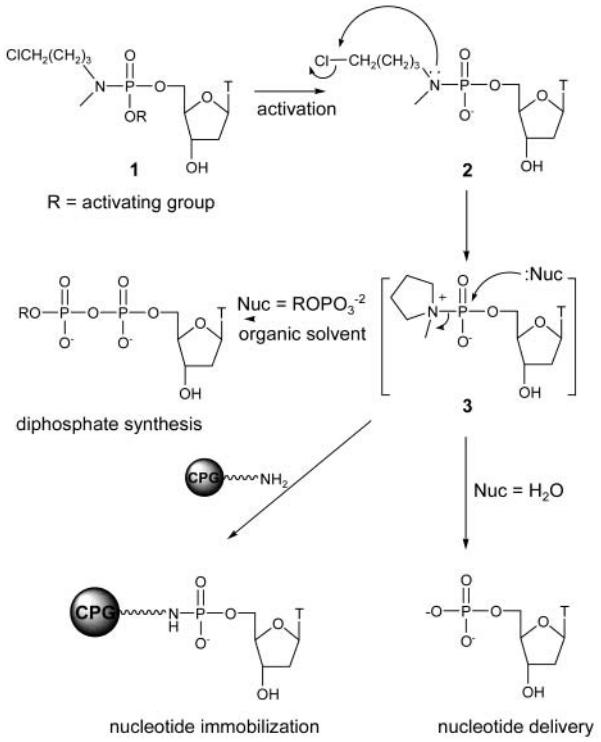
(3) (a) Freel Meyers, C. L.; Borch, R. F. *J. Med. Chem.* **2000**, *43*, 4319−4327. (b) Tobias, S. C.; Borch, R. F. *J. Med. Chem.* **2001**. *44*, 4475−4480.

(4) (a) Boal, J. H.; Wilk, A.; Harindranath, N.; Max, E. E.; Kempe, T.; Beaucage, S. L. *Nucl. Acids Res.* **1996**, *24*, 3115−3117. (b) Reddy, M. P.; Michael, M. A.; Farooqui, F.; Girgis, S. *Tetrahedron Lett.* **1994**, *35*, 5771−4314.

(5) Basu, S. and Wickstrom, E. *Tetrahedron Lett.* **1995**, *36*, 4943−4946.

10.1021/ol0273607 CCC: $25.00
Published on Web 01/15/2003

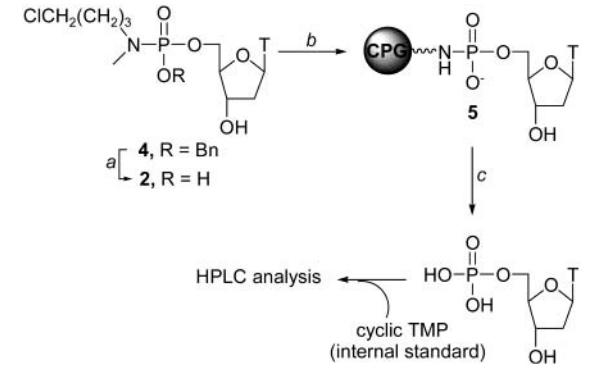
Scheme 1



thymidine phosphoramidate to CPG using various phosphoramidate ester activation conditions and subsequent nucleotide cleavage from CPG using optimized aqueous acidic conditions.

CPG coupling reactions were carried out using chlorobutyl phosphoramidic acid **2** (ca. 120-fold excess, Scheme 2) and

Scheme 2[a]



[a] Reaction conditions: (a) H_2, Pd/C, THF; (b) TEA, LCAA-CPG, CH_3CN/H_2O, rt, overnight; (c) 50:50 formic acid/water, rt, 8 h.

long-chain alkylamino-controlled pore glass (LCAA-CPG). Chlorobutyl phosphoramidate ester **4** is a stable precursor to the reactive phosphorylating agent **2** and was used previously in our laboratory for the preparation of TDP-D-glucose and TDP-L-rhamnose.[2] Activation of benzyl ester **4** to phosphoramidic acid **2** was accomplished via hydrogenolysis. The resulting crude hydrogenolysis mixture was then added to LCAA-CPG, and the suspension was tumbled overnight in the presence of excess triethylamine. The supernatant was removed following centrifugation, and the solid support was washed several times with water and acetonitrile to remove residual phosphoramidate and phosphate byproducts.

To assess the loading of immobilized phosphoramidate on the solid support, thymidine 5′-monophosphate (TMP) was cleaved using acid-catalyzed hydrolysis and quantified by HPLC analysis. Briefly, CPG derivatized with thymidine phosphoramidate (**5**) was tumbled in the presence of aqueous acid. Cyclic TMP (internal standard) was added to the suspension following acidic cleavage, and the supernatant containing TMP and cyclic TMP was removed following centrifugation and added to ammonium hydroxide. The resulting mixture was immediately analyzed by HPLC (98:2 0.1% TFA in H_2O/CH_3CN; TMP $R_t = 7.9$ min, cyclic TMP $R_t = 10.4$ min), and the concentration of TMP was determined from the ratio cyclic TMP:TMP calculated from HPLC peak areas. Acidic hydrolysis and HPLC analysis of the TMP/cyclic TMP mixture was repeated until no additional TMP was cleaved from the solid support.

The cleavage of TMP from the solid support was studied using a variety of aqueous acidic conditions. The cleavage reaction times and TMP loading are presented in Table 1.

Table 1. Aqueous Acidic Cleavage of TMP from Derivatized CPG

entry	reaction conditions[a]	reaction time[b]	loading (μmol/g of support)[c]
1	25% v/v $AcOH/H_2O$, rt	5 days	51−53
2	25% v/v Cl_2CHCO_2H/H_2O, rt	<3 h	56−59
3	50% v/v formic acid/H_2O, rt	8 h	53−55

[a] TMP was stable under all conditions tested. [b] Reaction times were determined on the basis of the total time taken to cleave all TMP from the support. A procedure for the determination of the cleavage rate is described in Supporting Information. [c] Minimum of two CPG coupling reactions were analyzed in each case.

The TMP loading in each case is higher than typical nucleoside loading on CPG used for the automated synthesis of oligonucleotides (30−40 μmol/g CPG).[6] The shortest cleavage reaction time for the aqueous acidic conditions considered is 3 h in 1:3 dichloroacetic acid/water (entry 2). Treatment of the derivatized CPG with the less toxic 1:1 formic acid/water (entry 3) resulted in complete cleavage of TMP over a longer time period (8 h), but cleavage was more rapid under these conditions relative to cleavage carried out in 1:3 acetic acid/water (5 days, entry 1). For circumstances in which quantitative recovery of nucleotide from the solid support is not required, shorter cleavage times can be used. For example, cleavage is 61, 91, and 98% complete after

(6) *Current Protocols in Nucleids Acid Chemistry;* Harkins, E. W., Ed.; Wiley & Sons: New York; 2001, p 3.2.20.

1.5, 3, and 5 h, respectively, in formic acid/water. It should be noted that TMP is stable under all cleavage conditions tested. In addition, the stability of a model oligonucleotide (5′-TGACTGACTGACTGACTGAC-3′) was monitored in 1:3 formic acid/water; <5% of the oligonucleotide had decomposed after 3 h at room temperature.

Additional investigation of the CPG coupling reaction was carried out for the purpose of defining a broader set of reaction conditions that might be employed for the immobilization of oligonucleotides and other biomolecules. Thus, CPG coupling reactions were carried out under different aqueous/organic conditions and with varying ratios of chlorobutyl phosphoramidate **2** and solid support. The results are summarized in Table 2. Comparable loading of

Table 2. Variation of CPG Coupling Reaction Conditions

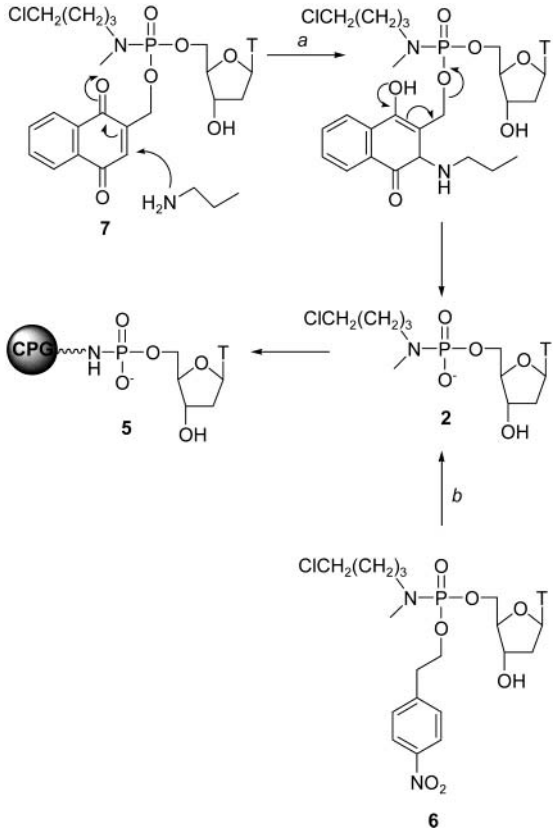
entry	ratio of **2**:CPG-NH_2[a]	reaction conditions	loading (μmol/g of support)[b]
1	120	TEA (3 equiv), CH_3CN, rt, overnight	54−57
2	120	TEA (3 equiv), 95:5 CH_3CN/H_2O, rt, overnight	54−55
3	120	TEA (3 equiv), 50:50 CH_3CN/H_2O, rt, overnight	49−53
4	50	TEA (3 equiv), 95:5 CH_3CN/H_2O, rt, overnight	44−48
5	5	TEA (3 equiv), 95:5 CH_3CN/H_2O, rt, overnight	40−42

[a] CPG-NH_2 loading was ~80 μmol/g of support (5 mg of support used in each case). [b] Minimum of two CPG coupling reactions were analyzed in each case.

TMP was observed from both partial aqueous and anhydrous coupling reactions (entries 1−3). Furthermore, TMP loading was reduced by only ~20% when the amount of phosphoramidate coupling reagent was decreased from a 120-fold excess to a 5-fold excess (entries 2, 4, and 5). The unexpected efficiency of nucleotide loading observed for CPG coupling reactions carried out in aqueous solvent suggests that this methodology for the immobilization of phosphates can be extended to the attachment of oligonucleotides and other biomolecules.

Finally, alternative methods of phosphoramidate activation have been investigated to identify potential activation conditions for the immobilization of phosphoramidate biomolecules that are sensitive to hydrogenolysis conditions or that require aqueous activation conditions. The nitrophenylethyl moiety has been employed previously for the protection of phosphate groups[7] and can be removed via a base-catalyzed β-elimination reaction with DBU (Scheme 3). Use of the nitrophenylethyl substituent as an activating group for the liberation of the reactive phosphoramidate anion **2** circumvents the requirement for activation via hydrogenolysis and provides an alternative method for the immobilization of sensitive biomolecules. Thus, model phosphoramidate **6** was synthesized according to the procedure used for benzyl

Scheme 3[a]



[a] Reaction conditions: (a) LCAA-CPG, propylamine, TEA, CH_3CN, rt, overnight; (b) LCAA-CPG, DBU, CH_3CN, rt, 12−35 h.

phosphoramidate **4**. The activation of **6** (Scheme 3) in a mixture of acetonitrile and DBU (10 equiv) was carried out in the presence of LCAA-CPG. The TMP loading observed in this case (entry 2, Table 3) was somewhat lower than the TMP loading observed in CPG coupling reactions carried out following activation via hydrogenolysis but comparable to nucleotide loading on CPG used for automated DNA synthesis (30−40 μmol/g). Coupling reactions carried out in the presence of 1.1 equiv of DBU (entry 1, Table 3) afforded TMP loading similar to those obtained in reactions carried out in the presence of 10 equiv DBU. This method of phosphoramidate activation/immobilization is attractive because it precludes the need for multiple reaction vessels and is a viable alternative to activation via hydrogenolysis for the immobilization of phosphates.

The quinone activating group of phosphoramidate **7** (Scheme 3) was initially designed to undergo bioreductive activation for the intracellular delivery of alkylating agents[8] and more recently has been used for the intracellular delivery of nucleotides.[3] During the course of this work, model phosphoramidate **7** was found to undergo rapid activation via 1,4-addition of propylamine to the quinine moiety

(7) Ohtsuka, E.; Morioka, S.; Ikehara, M. *J. Am. Chem. Soc.* **1973**, *95*, 8437.

(8) Flader, C.; Liu, J.; Borch R. F. *J. Med. Chem.* **2000**, *43*, 3157−3167.

Table 3. Dependence of TMP Loading on Method of Phosphoramidate Activation

entry	starting phosphoramidate	reaction conditions	loading (μmol/g of support)[a]
1	**6**	DBU (1.1 equiv), CH_3CN, rt, overnight	29−33
2	**6**	DBU (10 equiv), CH_3CN, rt, overnight	25−27
3	**7**	$H_2N(CH_2)_2CH_3$ (1.05 equiv), TEA (2 equiv), CH_3CN, rt, overnight	33
4	**2**	TEA (3 equiv), CH_3CN, rt, overnight	53−55

[a] A minimum of two CPG coupling reactions was analyzed in each case.

followed by elimination of the phosphoramidate anion, a reaction that can be carried out under both aqueous and nonaqueous conditions. Thus, a CPG coupling reaction was carried out using phosphoramidate **7** in the presence of LCAA-CPG, propylamine and triethylamine (Table 3). In this case, a loading value comparable to that observed with the nitrophenethyl phosphoramidate **6** was observed.

In summary, a novel method for the attachment of nucleotides to solid supports has been developed. The strategy described here employs a highly reactive phosphorylating reagent used previously in this laboratory for the intracellular delivery of nucleotides and for the synthesis of sugar nucleoside diphosphates. Aqueous acidic conditions have been identified such that the intact nucleotide can be cleaved from the solid support prior to the determination of nucleotide loading by HPLC. The nucleotide cleavage conditions employed in this strategy may be advantageous under circumstances in which the immobilized phosphate is sensitive to a strong base. TMP loading of 51−59 μmol/g of support was observed in most cases, offering an increase over the typical nucleoside loading on CPG used for oligonucleotide synthesis (30−40 μmol/g of CPG).[5] Alternative activation methods were explored and afforded somewhat lower loading of nucleotide when compared to coupling reactions carried out following activation via hydrogenolysis. Finally, preliminary studies indicate that this strategy for the attachment of nucleotides to CPG may be extended to the attachment of oligonucleotides and other phosphate biomolecules. Efforts are underway to explore the scope of this methodology.

Acknowledgment. Financial support from the National Cancer Institute (CA34619) is gratefully acknowledged.

Supporting Information Available: Experimental procedures for phosphoramidate coupling of **2**, **6**, and **7** to LCAA-CPG, cleavage of nucleotide from CPG, and determination of nucleotide loading by HPLC. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0273607