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Synthesis and Deprotection of Oligonucleotides under Aprotic Conditions

Tongqian Chen,† Jiasheng Fu,† and Marc M. Greenberg*

Department of Chemistry, Colorado State University, Ft. Collins, Colorado 80523

mgreenbe@lamar.colostate.edu

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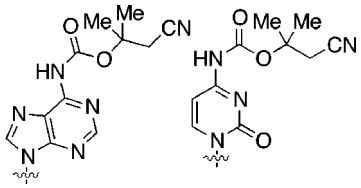
ABSTRACT



Oligonucleotides containing an alkali-labile nucleotide are synthesized and deprotected using a synthetic method that eliminates the use of Brønsted acid and base. The development of a new family of exocyclic amine-protecting groups is an integral component of this approach.

Modified oligonucleotides containing labile nucleotides are useful for elucidating mechanistic issues and as diagnostic tools.[1] Synthesizing some of these biopolymers poses significant challenges due to the routine use of protic acid during synthesis and protic base during deprotection.[2] We wish to report the development and application of a method for synthesizing oligonucleotides that accommodates the requirements of an alkali-labile nucleotide. The method eliminates the use of Brønsted acid and base by utilizing a new family of exocyclic amine protecting groups (described below), in conjunction with published chemistry that employs silicon and methyl groups to protect the 5′-hydroxyl and phosphate diester functional groups, respectively.[3]

Although methods are available that utilize monomers (most often phosphoramidites) with unprotected nitrogens, reagents for solid-phase oligonucleotide synthesis typically contain protecting groups for the exocyclic amines.[4] Solid-phase oligonucleotide synthesis also requires facile deprotection of the 5′-terminal hydroxyl in the growing chain and protection of the phosphate diesters, as well as a linkage between the 3′-terminus and the support that can be severed under mild conditions. Phosphate diesters are most commonly protected as their β-cyanoethyl or methyl triesters. These groups can be removed under aprotic conditions. Recently, silicon-protecting groups were introduced as a means of protecting the 5′-hydroxyl.[3] The exocyclic amines of nucleobases pose the greatest challenge for protecting groups in solid-phase synthesis. A variety of protecting groups, which are cleaved under mild protic and/or aprotic alkaline conditions, are available.[1a,b] The exocyclic amines can also be revealed using Pd (0) labile or photoactive protecting groups.[5] Each of these methods has limitations. Ideally, protecting groups that can be cleaved using reagents and methods that are readily available and easy to use are most desirable. Some molecules (e.g., **1**) are unstable to the

† These authors contributed equally to this work.

(1) (a) Iyer, R. P.; Beaucage, S. I. In *Comprehensive Natural Products Chemistry, Vol. 7*; Kool, E. T., Ed.; Elsevier: Amsterdam, 1999; 105. (b) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123. (c) Greenberg, M. M. *Chem. Res. Toxicol.* **1998**, *11*, 1235.

(2) a) Greenberg, M. M.; Barvian, M. R.; Cook, G. P.; Goodman, B. K.; Matray, T. J.; Tronche, C.; Venkatesan, H. *J. Am. Chem. Soc.* **1997**, *119*, 1828. (b) Greenberg, M. M.; Matray, T. J. *Biochemistry* **1997**, *36*, 14071. (c) Sambandam, A.; Greenberg, M. M. *Nucleic Acids Research* **1999**, *27*, 3597.

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(5) (a) Hayakawa, Y.; Wakabayashi, S.; Kato, H.; Noyori, R. *J. Am. Chem. Soc.* **1990**, *112*, 1691. (b) Stutz, A.; Pitsch, S. *Synlett* **1999**, 930. (c) Hayes, J. A.; Brunden, M. J.; Gilham, P. T.; Gough, G. R. *Tetrahedron Lett.* **1985**, *26*, 2407.

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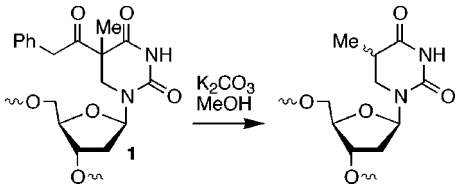
most mild alkaline conditions previously reported (Scheme 1). This dihydropyrimidine (**1**), which is of interest to our

Scheme 1



group for mechanistic studies, undergoes a retrocondensation reaction when subjected to the conditions used to cleave phenoxyacetyl protecting groups [anhydrous K_2CO_3 (0.05 M)/MeOH].[2c]

The ketone (**1**) also undergoes decomposition to an undefined set of products when exposed to DBU (0.5 M)/CH_3CN, which is used to cleave *p*-nitrophenylethyl carbamates.[6]

We reasoned that β-cyanoethyl carbamates would be amenable to mild, aprotic, alkaline cleavage conditions (e.g., triethylamine), which would also be compatible with molecules such as **1**.[7] Nucleosides **2a**, **3a**, and **4** (Figure 1) were

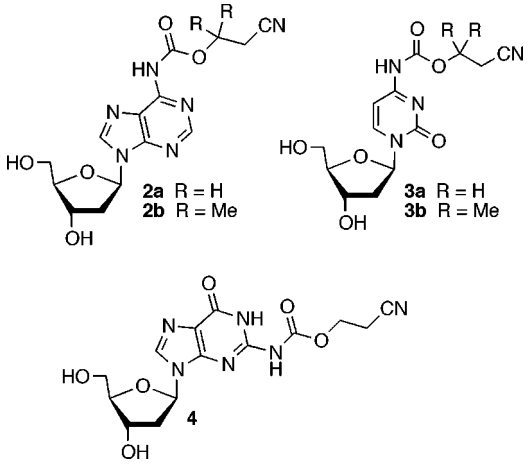


Figure 1. Acrylonitrile-derived carbamate protecting groups.

synthesized via conventional methods from the free nucleosides. Synthetic details will be reported at a later date. Treatment of these nucleosides with Et_3N/DMF (1:1 by volume) 55 °C resulted in their clean conversion to the respective deprotected nucleosides. However, with the exception of the deoxyguanosine derivative (**4**, Table 1), the

Table 1. Rates of β-Elimination from Nucleoside Carbamates Induced by Et_3N/DMF at 55 °C[a]

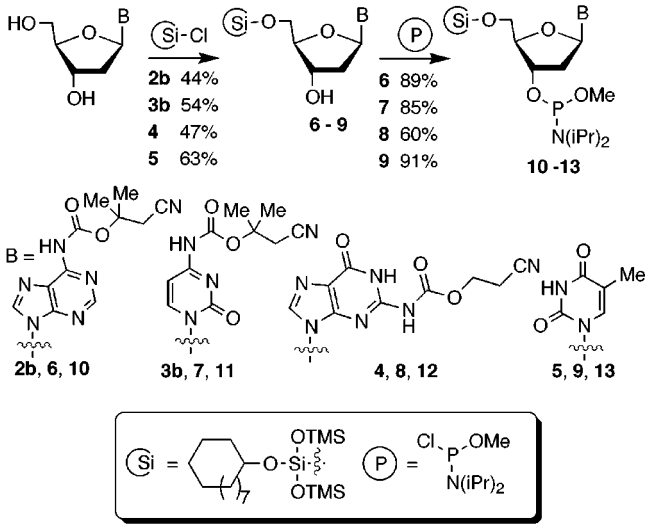
nucleoside	$10^4 \times k_{Elim}$ (s^{-1})[b]	$t_{1/2}$ (h)
2b	0.96 ± 0.06	2.0
3a		~12
3b	1.18 ± 0.07	1.6
4	1.32 ± 0.14	1.5

[a] Reaction rates were followed by measuring the disappearance of starting material as a function of time by C_{18}-HPLC. [b] Rate constants are expressed as an average of two or more separate experiments ± the standard deviation from this average.

rates for the reactions were too slow to be useful for oligonucleotide deprotection. Synthesis of the α,α-dimethyl substituted carbamates (**2b**, **3b**) provided substrates that were far more reactive under the above deprotection conditions (Table 1), and these were used to protect deoxycytidine and deoxyadenosine during solid-phase oligonucleotide synthesis.[8]

The free nucleosides (**2b**, **3b**, **4**, and **5**) were transformed into the respective 5′-silyl-methyl phosphoramidites (**10**−**13**) via sequential silylation and phosphitylation (Scheme 2).

Scheme 2



Selective silylation of 2′-deoxynucleosides required significant experimentation for each nucleoside, and details will be reported. The requisite phosphoramidite (**15**) of the 5*S*-diastereomer of the benzyl ketone was prepared in a like manner from 5*S*-**1**, which was in turn obtained from the bis-TBDMS ether of 5,6-dihydrothymidine (**14**, Scheme 3).[9]

Prior to carrying out solid-phase oligonucleotide synthesis, **2b**, **3b**, and **4** (or the appropriate 3′,5′-hydroxyl protected

(6) (a) Himmelsbach, F.; Schulz, B. S.; Trichtinger, T.; Charubala, R.; Pfleiderer, W. *Tetrahedron* **1984**, *40*, 59. (b) Stengele, K.-P.; Pfleiderer, W. *Tetrahedron Lett.* **1990**, *31*, 2549.

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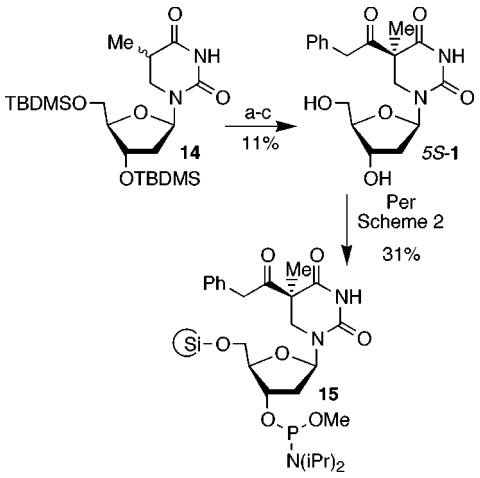
(8) Small amounts of a product ascribed to the nucleophilic addition of deoxyguanosine to acrylonitrile were observed during the deprotection of the monomer.

(9) Tallman, K. A. Ph.D. Dissertation, Colorado State University, Ft. Collins, CO, 2000.

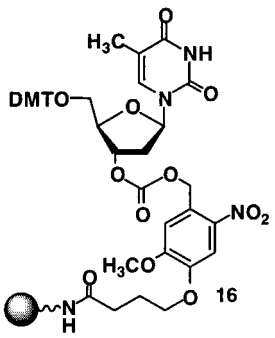
Scheme 3[a]



[a] Key: (a) sec-BuLi, DMPU, phenylacetyl chloride; (b) TFA, H_2O, THF; (c) $Et_3N{\cdot}3HF$, THF.

analogue) were tested separately for their stability to the desilylation (anhydrous $Et_3N{\cdot}3HF$, DMF), oxidation (*t*-BuOOH), and capping reagents. Exposure of the silyl protected β-cyano-α,α-dimethyl-ethyl of deoxyadenosine to acetic anhydride, 2,6-lutidine, and *N*-methylimidazole (standard capping reagents employed in oligonucleotide synthesis) resulted in a slow transformation into the respective *N*-acetyl derivative (65% in 15 h). Removal of *N*-methylimidazole from the reaction mixture eliminated this decomposition reaction.

Given that the substitution of the acetyl group for the carbamate was significantly slower than the capping procedure during automated synthesis, we attempted to synthesize an oligonucleotide (**17**, Figure 2) utilizing *N*-methylimidazole



5′-d(ATC CCT TAG TTC GAA CAT GCA ATT)
17

5′-d(ATT ACG (5S-1)TC TAT)
18

5′-d(TTT TCA ATT TAT (5S-1)CA TAT CAT ATT)
19

Figure 2. Solid-phase support and oligonucleotides prepared in this study.

during the capping process. Synthesis (1 μmol) was carried out on the photolabile linker previously reported by us on polystyrene support (**16**) using automated synthesis cycles similar to those reported by Scaringe in his paper introducing 2′-ACE technology for the synthesis of RNA.[3,10] The oligonucleotide was deprotected in three steps. First, demethylation of the phosphate triesters was accomplished using disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (0.2 M, 15 min, 25 °C).[3,11] The resin was washed following filtration. The washings were combined with the excess reagents, evaporated to dryness, and treated with concentrated aqueous ammonia. Following demethylation, the resin was treated with Et_3N in DMF (1:1 by volume, 16 h, 55 °C, eight or more half-lives established in studies on the monomers; see Table 1) in order to cleave the exocyclic amine-protecting groups. Again, the excess reagents and solvent obtained from washing of the resin were combined and evaporated to dryness. Finally, the fully deprotected, resin-bound oligonucleotide was cleaved from the solid-phase support via photolysis using a transilluminator (365 nm, 3 h).[10] Following collection of the solution and washing of the resin, this concentrated sample, as well as the washings from the previous two steps, was purified by denaturing polyacrylamide gel electrophoresis. Trace amounts of DNA were observed in the concentrated washings of the demethylation reaction, and none was detected in the respective washings obtained following cleavage of the exocyclic amines. The remainder of the oligonucleotide (**17**) was obtained from the sample subjected to all three deprotection steps, indicating that the carbonate linkage was stable to the exocyclic amine and phosphate-deprotection steps. Comparison of the yield of DNA obtained from photolysis to an equivalent sample, which was cleaved from the resin using concentrated aqueous ammonia, indicated that the photolysis proceeded in 67% yield. Analysis of the two samples of DNA by ESI-MS indicated that the photolytically cleaved material, which migrated as a single band in the gel, was a mixture of the desired product and modifications of this biopolymer containing between one and four acetyl groups (data not shown). The acetyl groups were assumed to result from the capping process, as observed above for the reaction of the carbamate protected monomers.

Subsequently, synthesis of **18** was carried out omitting *N*-methylimidazole in the capping process. The three-step deprotection procedure was carried out as described above. Less efficient capping was evident in the purification gel, which revealed the presence of greater amounts of deletion products close in size to the full-length product. However, the product isolated from this gel was pure, as determined by ESI-MS analysis, and did not exhibit any evidence of *N*-acetylation (Figure 3). The suitability of the method was further demonstrated by the syntheses of **19**, which also was obtained as a single product (Figure 4). No evidence for nucleophilic trapping of acrylonitrile or the more hindered

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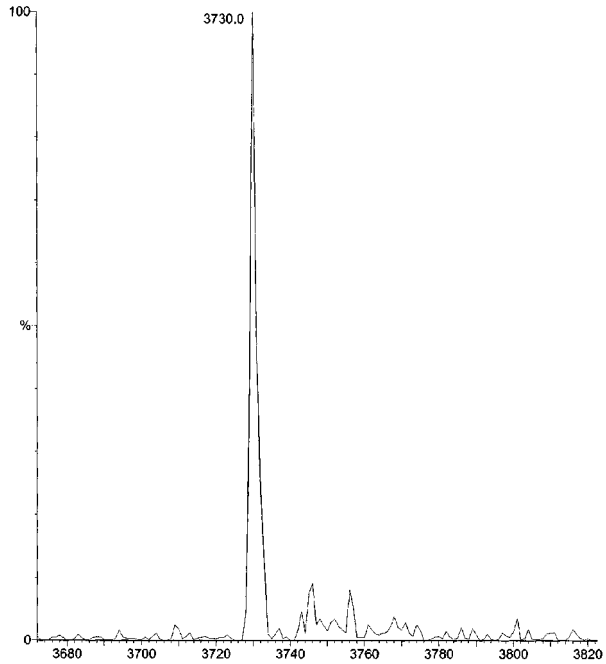

Figure 3. ESI-MS of **18**. Calculated *m*/*z* 3730.3.

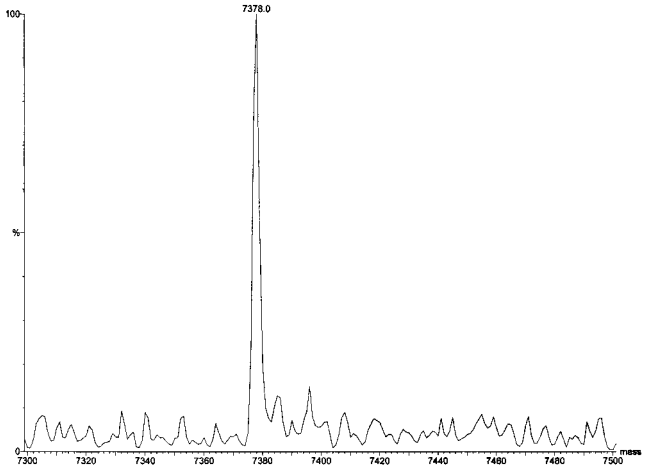

Figure 4. ESI-MS of **19**. Calculated *m*/*z* = 7376.7.

β,β-dimethyl analogue was observed.[8] Of special note is the lack of any evidence for the retrocondensation reaction noted above (Scheme 1) for **1**.

In summary, we have developed a method for synthesizing oligonucleotides using phosphoramidites that are deprotected under mild, anhydrous, aprotic conditions, using inexpensive, routinely available reagents (Et_3N). Most importantly, the method enables the synthesis of oligonucleotides containing a modified nucleotide that is labile to other mild alkaline deprotection conditions. Furthermore, the use of silyl-protecting groups for the primary hydroxyl group should also allow one to synthesize oligonucleotides containing modifications that are acid-labile and/or require an acid-labile-protecting group that is removed in the final step of the synthesis.

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