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CONJUGATION OF OLIGONUCLEOTIDES VIA AN ELECTROPHILIC TETHER: *N*-CHLOROACETAMIDOHEXYL PHOSPHORAMIDITE REAGENT

Andrei Guzaev* and Muthiah Manoharan

Department of Medicinal Chemistry, Isis Pharmaceuticals, 2292 Faraday Ave., Carlsbad, CA 92008

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Abstract: A novel method for the preparation of oligonucleotides conjugated with nucleophilic ligands is described. A new phosphoramidite building block derived from *N*-chloroacetyl-6-aminohexanol is attached at the 5'-terminus on the last step of oligonucleotide synthesis. Postsynthetic treatment of support-bound modified oligonucleotide with a variety of amines and mercaptans affords conjugates in high yield.

Synthetic oligonucleotides having 5'-terminal functional or reporter groups are employed in a number of bioanalytical and antisense applications.[1,2] Appropriately, a number of procedures and reagents that allow their preparation have been described.[1-3] Among them, conjugation of electrophiles to oligonucleotides via tethers carrying nucleophilic groups is well known. For the opposite process of conjugating nucleophiles to electrophilic sites, the available methods to generate these sites in oligonucleotides are limited. These include periodate oxidation of a terminal ribose moiety[3] or the use of an internal abasic site within the oligonucleotide sequence.[4] Heterobifunctional reagents that bear a phosphoramidite moiety along with an electrophilic functional group that is reactive in orthogonal conditions are of particular interest. Previously, a method that utilizes the reactivity of (thio)ester group towards alkylamines has been reported.[5] On completion of chain elongation, the modified oligonucleotide could be converted into a variety of conjugates by treatment with linkers of different length and substitution that bear a primary amino group. The oligonucleotides derivatized with bifunctional reagents showed remarkable reactivity towards 0.5 to 1 M primary amines.[5] Thus the method allowed one to to generate a variety of conjugates starting from single modified oligonucleotide and using a relatively general protocol.

Several ligands that bear either NH_2 or SH function can be conjugated to oligonucleotides to improve their potential as antisense or diagnostic agents. These include lipids, long chain alkyl groups, polyamines, or peptides. Therefore, universal methods for preparation of oligonucleotide conjugates with the aid of electrophilic phosphoramidite building blocks are of particular interest. A haloacetyl linker that is reactive towards a variety of nucleophiles has been introduced previously into oligonucleotides by postsynthetic

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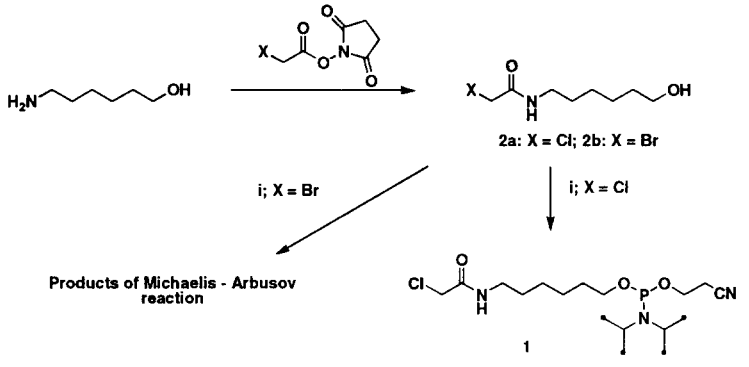
reaction in solution.[6] In this communication we report the preparation of a phosphoramidite building block **1** that enables conjugation of ligands bearing either primary amino or thiol groups to synthetic oligonucleotides.

Synthesis of phosphoramidite reagent **1** is presented in Scheme 1. First, 6-aminohexanol was reacted



Scheme 1. (i) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite/1 *H*-tetrazole/MeCN.

with succinimido chloro- or bromoacetate to give **2a** and **2b**. Compound **2a** was successfully converted into **1** by a routine procedure, isolated as a colorless oil by aqueous workup and precipitation, and characterized by ^{31}P NMR. In contrast, attempted conversion of bromoacetamido derivative **2b** into corresponding phosphoramidite resulted in products of Michaelis–Arbusov reaction as judged by ^{31}P NMR.

A similar but less pronounced problem was encountered for phosphoramidite **1**, which possesses both – electrophilic and nucleophilic centers. When stability of **1** (0.15 M in anhydrous MeCN) was monitored at room temperature by ^{31}P NMR, it was found that phosphoramidite moiety was transformed into species bearing tetracoordinated phosphorus within 5 to 6 days. Therefore, solutions of **1** in MeCN should be used immediately after preparation. They may, however, be stored at –20 °C for two weeks without substantial loss of activity.

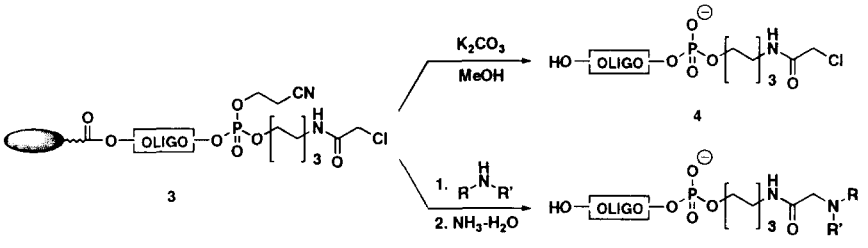
In order to examine the usefulness of phosphoramidite **1**, model oligothymidylates **3a** and **3b** were assembled on an ABI 380B DNA Synthesizer on a 1 to 10 μmol scale using routine protocols and common ancillary reagents. The solid support-bound **3a** and **3b** were first deprotected with either 0.05 M K_2CO_3 in MeOH or concentrated ammonium hydroxide to give appropriately chloroacetyl- or glycylaminohexyl derivatized oligonucleotides **4a** and **b** and **5a** and **b** as major products. Oligonucleotides **4,5 a,b** were characterized by their ESMS (Scheme 2 and Table 1).

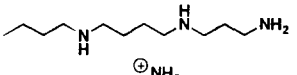
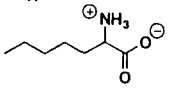
Conjugation with amines. Next, **3a** and **3b** were treated with a variety of amines followed by deprotection with concentrated ammonia, which converts any unreacted starting material into **5**. The excess reagent was removed either by neutralization with Dowex 50W×8 (PyH^+) or, for water-insoluble amines, by extraction with an organic solvent. The reaction mixtures were analyzed by reverse-phase HPLC. Peaks of target conjugates, **6–10**, and a peak of side product, **5**, were taken into the account for yield determination.

The results presented in Table 1 suggest that primary alkylamines in 0.25 to 1.0 M concentration are highly reactive towards the chloroacetamido group in **3**.

In contrast, secondary amines give only low conjugation yields (24% for **di-*n*-butylamine**). Consequently, spermine, in which both primary and secondary amino functions are present, reacts with **3b** to form exclusively linear conjugate **9b**.

To our knowledge, attachment of amino acids to solid support-bound oligonucleotides via amino group of the former has not been reported yet. In this method, lysine in the presence of DBU was able to form the conjugate **10** in moderate yield.



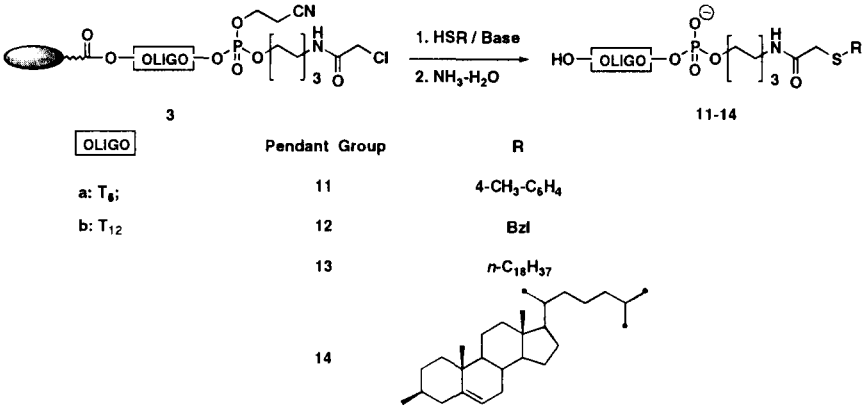
OLIGO	Pendant Group	R	R'
a: T_6;	5	H	H
b: T_{12}	6	H	*n*-Bu
	7	*n*-Bu	*n*-Bu
	8	H	*n*-$C_{12}H_{25}$
	9	H	
	10	H	

Scheme 2. Reactivity of chloroacetyl modified oligonucleotide **3a,b** towards K_2CO_3 and amines.

Conjugation with mercaptans. In a similar fashion, support-bound **3a** and **3b** were reacted with aliphatic and aromatic mercaptans in the presence of a tertiary amine, either DBU or triethylamine (Scheme 3 and Table 1). Triethylamine was sufficient for conjugation with thiophenols. However, less acidic aliphatic thiols required a stronger base, DBU, to ensure ionization of the mercapto group and hence high nucleophilicity of the reagents.

Exposure of oligonucleotides to mercaptans under nonaqueous basic conditions does not result in any side reactions at nucleoside residues; neither is the oligonucleotide released from the solid support.[7] Consequently, the excess reagent was conveniently removed by washing. Finally, the solid support was treated with ammonia, and the reaction mixtures were analyzed by HPLC. As seen from Table 1, treatment with both aromatic and aliphatic mercaptans results in efficient formation of conjugates **11–14**.

Postsynthetic modification of oligonucleotides is often complicated by poor solubility of ligands at a concentration that enables favorable kinetics of the desired reaction and hence high coupling efficiency. In contrast, the conjugation of oligonucleotides **3a** and **3b** proceeds smoothly with 0.1 to 0.25 M mercaptans in chemically inert solvents. This allows efficient preparation of hydrophobic oligonucleotides **13** and **14** with octadecanethiol and thiocholesterol that are both poorly soluble and, in the latter case, sterically hindered.



Scheme 3. Use of mercaptans as pendant groups.

In summary, chloroacetamidohexyl phosphoramidite building block enables a simple and efficient preparation of oligonucleotides conjugated with a variety of primary amines or mercaptans.

Experimental

6-(Chloroacetylamino)hexanol (2a). A solution of *N,N'*-dicyclohexylcarbodiimide (8.24 g, 40 mmol) in THF (50 mL) was added dropwise to a mixture of chloroacetic acid (3.78 g, 40 mmol) and *N*-hydroxysuccinimide (6.33 g, 55 mmol) in THF (30 mL) under magnetic stirring at 0–4 °C. The stirring was maintained for 3 h in an ice bath and for 4 h at room temperature. The precipitate was filtered off and washed with THF (2 × 25 mL). Combined filtrates were evaporated in vacuo to one third of the initial volume (ca. 30 mL) and placed in an ice bath. A solution of 6-aminohexanol (4.69 g, 40 mmol) in THF (50 mL) was added dropwise under magnetic stirring. The reaction mixture was kept for 4 h in an ice bath, and stirring was continued overnight at room temperature. The solution was evaporated in vacuo; the residue was dissolved in water (50 mL) and filtered. The pH of filtrates was adjusted to 7.5-8 by adding 5% aq $NaHCO_3$, and the product was extracted with CH_2Cl_2 (5 × 30 mL). Combined extracts were dried over Na_2SO_4 and evaporated to a solid, which was re-crystallized from toluene to give **2a** (5.11 g, 66%) as white crystals, mp 65.5–66 °C. ^1H NMR ($CDCl_3$): 6.6 (1H, br s); 4.05 (2H, s, $ClCH_2$); 3.65 (2H, t, J = 6.2 Hz, OCH_2); 3.32 (2H, dt, J = 6.3 Hz, NCH_2); 1.6-1.2 (9H, m, 4×CH_2 and NH). ^{13}C NMR ($CDCl_3$): 165.85 (C=O); 62.65 ($ClCH_2$); 42.66 (OCH_2); 39.80 (NCH_2); 32.55, 29.31, 26.51, 25.34 (4×CH_2).

2-Cyanoethyl 6-(Chloroacetylamino)hexyl (N,N-diisopropyl)phosphoramidite (1). A solution of 1*H*-tetrazole (0.45 M in MeCN; 6.67 mL, 3.0 mmol) was added to a mixture of *N*-chloroacetyl-6-aminohexanol and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (1130 mg, 3.75 mmol). The mixture was magnetically stirred at room temperature for 1 h, and the reaction was found completed by ^{31}P NMR. The mixture was cooled to −10 °C and treated first with dry Et_3N (304 mg, 3.0 mmol; 25 % in MeCN) and then with aqueous $NaHCO_3$ (5%; 10 mL) and saturated NaCl (20 mL). The product was extracted with toluene (3 ×

40 mL) and dried over Na_2SO_4. The solvent was evaporated, the residue was dissolved in toluene (2 mL) and precipitated in hexane at –40 °C. Liquid precipitate was dried in vacuo to yield **1** (591 mg, 50%) as colorless oil, ^{31}P NMR (CD_3CN): 148.41.

Table 1. Preparation of Tethered Oligonucleotides by Postsynthetis Conversion of Chloroacetyl Linker.

Oligo-nucleotide	Reagents / Conditions	Time, h	Yield, %	ES MS, MW		Retention time,
				Found	Calculated	min
4a	0.05 M K_2CO_3/MeOH	12	75	2017.1	2017.94	23.4[a]
4b	0.05 M K_2CO_3/MeOH	12	68	3842.2	3843.08	37.6[b]
5a	Conc. NH_3/H_2O	2	85	1999.3	1999.39	22.1[a]
5b	Conc. NH_3/H_2O	2	87	3824.3	3824.53	35.1[b]
6a	1 M *n*-$BuNH_2/H_2O$	6	90	2055.4	2055.54	23.7[a]
6b	1 M *n*-$BuNH_2$/EtOH	12	84	3880.1	3880.68	35.7[b]
7b	0.5 M $(n\text{-}Bu)_2NH$/EtOH	12	34	3936.0	3936.76	43.5[b]
8b	0.25 M *n*-$C_{12}H_{25}NH_2$/dioxane/55 °C	12	65	3992.2	3992.85	26.9[c]
9b	0.5 M Spermine/DMF	12	65	4010.1	4009.84	35.6[b]
10b	0.5 M L-lysine/0.25 M DBU/50% aq DMF/55 °C	12	35	3954.4	3953.7	32.3[b]
11a	0.1 M *p*-thiocresol/0.2 M Et_3N/dioxane	6	94	2106.3	2106.58	28.9[a]
11b	0.1 M *p*-thiocresol/0.5 M Et_3N/dioxane	6	86	3931.8	3931.72	42.8[b]
12b	0.1 M benzylmercaptane/0.5 M Et_3N/ dioxane	6	76	3917.69	3917.9	42.9[b]
12b	0.1 M benzylmercaptane/0.1 M DBU / dioxane	6	87	3917.75	3917.9	42.9[b]
13b	0.125 M *n*-$C_{18}H_{37}SH$/0.125 M DBU/ dioxane/55 °C	12	62	4094.5	4094.01	37.5[c]
14b	0.5 M thiocholesterol/0.25 M DBU/ dioxane/55 °C	12	74	4210.3	4210.24	39.2[c]

a. 0 to 60% B in 40 min; A = 0.1 M NH_4OAc; B = 0.1 M NH_4OAc in 50 % aq MeCN.
b. 0 to 25% B in 50 min; A = 0.1 M NH_4OAc; B = 80% aq MeCN.
c. 0 to 100% B in 50 min; A = 0.1 M NH_4OAc; B = 80% aq MeCN.

Oligonucleotide synthesis. Oligonucleotide synthesis was performed on an ABI 380B DNA Synthesizer using phosphoramidite chemistry, standard ancillary reagents and cycles. Phosphoramidite **1** (0.15 M in MeCN) was attached at the 5'-terminus on the last coupling step. Both the capping and detritylation subroutines are neither required nor recommended as part of the last synthetic cycle. The solid support-bound oligonucleotide was briefly dried in vacuo and subjected to the attachment of a pendant group.

HPLC Techniques. Crude oligonucleotides were analyzed on a Waters DeltaPak HPLC column (C18, 15 μm, 300 Å, 3.8 × 300 mm) using linear gradients shown in Table 1. Retention times for oligonucleotides **4-14** are presented in Table 1.

Attachment of pendant groups: treatment with amines. Solid support bound oligonucleotides **3a,b** were treated with amines (Scheme 2) using the conditions specified in the Table 1. The reaction mixture was diluted with concentrated ammonium hydroxide and left at room temperature for 2 h. The solution was collected and evaporated to dryness. The residue was dissolved in water (2 mL) and treated depending on the reagent employed for the derivatization. For **4**,**7**,**9**, and **10b** solutions were neutralized with Dowex 50wx8 (PyH^+), filtered, and analyzed by HPLC. For **8b**, the emulsion was extracted with CH_2Cl_2 (5 × 0.5 mL), filtered, and analyzed by HPLC. For **5a,b** and **6a,b** no special treatment was required. The solutions were filtered and subjected to HPLC.

Attachment of pendant groups: treatment with mercaptans. Solid support bound oligonucleotides **3a** and **b** were treated with mercaptans (Scheme 3) using the conditions specified in Table 1. The solid support was washed with dioxane (5 × 1 mL), concentrated ammonia was added, and the mixture was left for 2 h at room temperature. The solution was evaporated to dryness. The resulting pellet was dissolved in water (2 mL), filtered and analyzed by HPLC.

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