Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 13, pp. 1639-1644, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00278-3

NOVEL NON-NUCLEOSIDIC PHOSPHORAMIDITES FOR OLIGONUCLEOTIDE MODIFICATION AND LABELING

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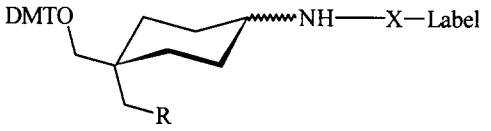
Abstract: A series of novel labeled phosphoramidites and CPG supports **1a–f** with a new non-nucleosidic backbone based upon cyclohexyl-4-amino-1,1-dimethanol have been synthesized. These reagents have been used to label oligonucleotides with biotin and fluorescein at the 5′-, the 3′-, and internal positions.

Oligodeoxynucleotides bearing suitable reporter groups have proven to be potent tools in molecular biology, finding applications in diagnostics, sequencing, and molecular studies of nucleic acids.[1,2] Various nucleosidic as well as non-nucleosidic phosphoramidites have been successfully incorporated into oligomers to obtain the desired functionalities. The non-nucleosidic tails used for labeling of oligonucleotides include both branched and linear structures. Branched dendrimeric structures have, for example, been assembled on the 5′-terminus of the oligonucleotide by using bis(4,4′-dimethoxytrityl)alkanetriol phosphoramidite as a building block.[3–5] Linear tails have also, in turn, been prepared by using non-nucleosidic phosphoramidites derived from

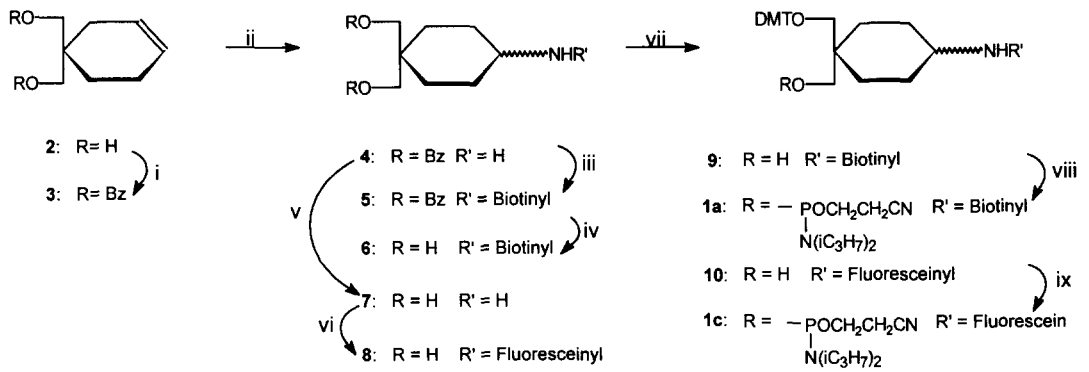


Compound	X	Label	R
1a	-	Biotinyl	-O-P-$OCH_2CH_2CN(N\text{-}^iPr_2)$
1b	$-CO(CH_2)_5NH-$	N-(4-^{t}Bu-Bz)-Biotinyl	-O-P-$OCH_2CH_2CN(N\text{-}^iPr_2)$
1c	-CO-	Fluoresceinyl	-O-P-$OCH_2CH_2CN(N\text{-}^iPr_2)$
1d	-CO-	$-(CH_2)_5NHCOCF_3$	-O-P-$OCH_2CH_2CN(N\text{-}^iPr_2)$
1e	-	Biotinyl	$-OCO(CH_2)_2CONH$-CPG
1f	-	Fluoresceinyl	$-OCO(CH_2)_2CONH$-CPG

substituted diols that carry either a protected amino function or a reporter group.[5–16] Among them, derivatives of 3-amino-1,2-propanediol, glycerol and 2-(4-aminobutyl)-1,3-propanediol have been used extensively. In order to improve the efficiency of synthesis, as well as to mimic the stereochemical properties of the natural polynucleotide backbone, 1,3-diols have drawn more interest as compared to 1,2-diols. In this paper, we report a series of phosphoramidites **1a–d** based upon a unique cyclohexyl-4-amino-1,1-dimethanol backbone.[17] We envisioned that in such a stereochemically defined structure, steric hindrance would be minimal and thus lead to high coupling efficiency during oligonucleotide synthesis. Further, reporter groups such as biotin or fluorescein would be kept away from the oligonucleotide chain by the rigid cyclohexyl ring resulting in higher hybridization efficiency. In addition, the present backbone will retain the natural three carbon internucleotide phosphate distance found in DNA and RNA.

The novel backbone presented in this investigation is based upon readily available and inexpensive 3-cyclohexene-1,1-dimethanol **2**. The precursor was converted to dibenzoyl derivative **3** by reaction with benzoyl chloride in pyridine. Hydroboration of **3** by in situ generation of borane via the reaction of sodium borohydride with BF_3-Et_2O in diglyme, followed by reaction with hydroxylamine-O-sulfonic acid[18] resulted in the formation of desired compound **4** in 39% yield.[19] A small amount of corresponding 3- amino regioisomer was also formed and was easily separated by column chromatography. Though the formation of **4** proceeds in relatively low yields, it is easy to scale-up and useful quantities (>100 grams per batch) of **4** were readily obtained.

Scheme:



i. BzCl, pyridine; ii. BF_3Et_2O, $NaBH_4$, diglyme, H_2NSO_3H; iii. Biotin-NHSu, TEA, CH_2Cl_2; iv & v. CH_3ONa, CH_3OH; vi. Fluorescein-NHSu, TEA, DMF; vii. DMTCl, pyridine; viii. $CNCH_2CH_2O$-P(N-$iPr_2)_2$, tetrazole-DIPA salt; ix. $CNCH_2CH_2O$-P(Cl) (N-iPr_2), DIPEA.

The reaction of compound **4** with biotin-N-hydroxysuccinimide ester (biotin-NHSu) in the presence of triethylamine afforded the corresponding amide **5** in 90% purified yield. Removal of the benzoyl group from **5** followed by dimethoxytritylation resulted in the formation of compound **9**. It is likely that compound **9** and its derivatives exist as a mixture of geometric isomers. However, they were not resolved chromatographically and no attempt was made to assign their stereochemistry. The phosphitylation of compound **9** under standard conditions gave the biotin phosphoramidite **1a** in 75% yield.[20] Phosphoramidite **1a** when used as a 0.1 M solution in acetonitrile using standard 0.2 μmol scale DNA synthesis protocols, exhibits consistently high coupling efficiency (>98%).[21] Figure **1A** shows the capillary electrophoresis trace of a crude 17mer labeled at 5'- position by using biotin amidite **1a**. The desired full length oligo was 70% of the crude product mixture.[22] In order to facilitate the kinetics of biotin binding to streptavidin, we have also synthesized the long chain biotin phosphoramidite **1b**.

For fluorescein amidite, the benzoyl derivative **4** was initially deprotected with sodium methoxide in methanol to give compound **7**. Treatment of 5(6)-carboxyfluorescein with pivaloyl chloride gave the non-fluorescent lactone which was converted to its NHSu ester with dicyclohexylcarbodiimide and N-hydroxysuccinimide.[12] The coupling of NHSu ester with compound **7** in pyridine followed by dimethoxytritylation and phosphitylation gave the phosphoramidite derivative **1c**.[23] The fluorescein phosphoramidite **1c**, was used in the same manner as nucleoside phosphoramidites on an automated DNA synthesizer. However, a coupling time of 10 min was used for fluorescein incorporation which resulted in a coupling efficiency of > 97%.[24] This is a rather significant improvement over other non-nucleosidic fluorescein

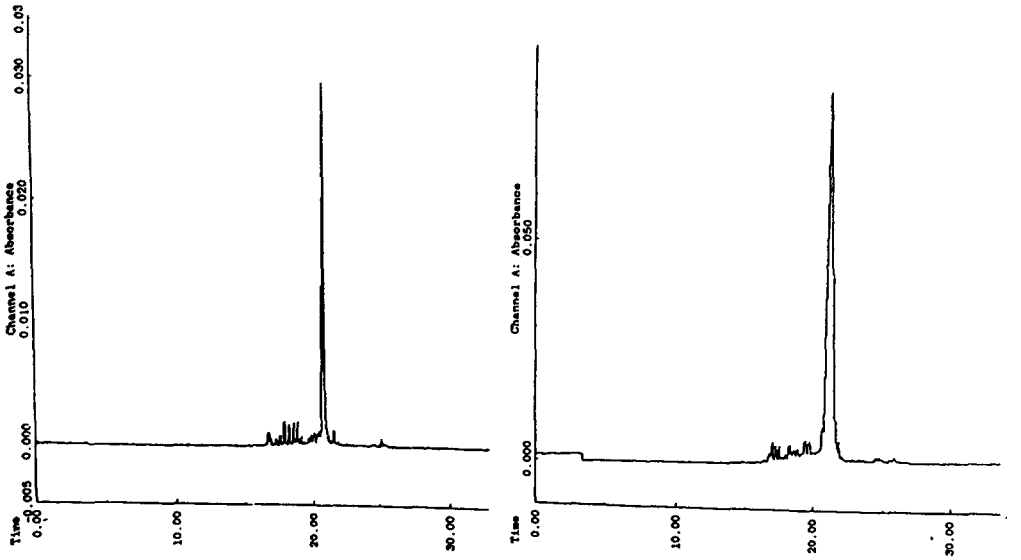


Figure 1: Microgel capillary electrophoretic traces of crude labeled oligonucleotides synthesized using biotin phosphoramidite (**1a**) and fluorescein phosphoramidite (**1c**). **Panel A**: biotinylated oligonucleotide 5'- B-GTA ATG CGA GCG CCA GT-3', where B = biotin. **Panel B**: fluoresceinated oligonucleotide 5'-F-TGG TCC GCT AAG ACA TTA GT-3', where F = fluorescein.

phosphoramidites that have been reported.[12] Figure **1B** shows the capillary electrophoresis trace of a 20mer labeled at 5′-position with **1c**. The full length oligo was 76% of the crude mixture.

For functionalization of oligonucleotides with primary amino group, we have also synthesized the amino phosphoramidite **1d**. This phosphoramidite is extremely stable and under normal conditions of automated synthesis, it consistently gave a coupling efficiency >98%. This compound can be used for the post-labeling of oligonucleotides with other reporter groups such as acridine and enzymes in addition to biotin and fluorescein.[25]

In order to label oligonucleotides with biotin and fluorescein at the 3′ end, biotin-CPG (500 Å) and fluorescein-CPG (500 Å) were synthesized by reaction of the corresponding succinate with LCCA-CPG.[26] Both biotin-CPG (loading 31.3 μmol/g) and fluorescein-CPG (loading 9.3 μmol/g) gave excellent coupling efficiency results (>99%) when 20 mers were synthesized using standard synthesis cycles.

Biotin phosphoramidite **1b** was tested as follows: the 30mer 5′-p-TTT TTT TTT TCG **B**AA TCA **B**GG TCA **B**AG CTG-3′ (**B** = biotin, p = phosphate) was synthesized with either **B** = **1b** or **B** = commercially available nucleosidic biotin-dC phosphoramidite. Both oligos were covalently bound to polystyrene (Covalink-NH_2) microtiter plates via 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide mediated coupling of the primary amino and phosphate groups. After washing off the unbound oligonucleotides, the biotin residues were detected by binding of streptavidin-alkaline phosphatase conjugate. Para-nitrophenyl phosphate was used as the substrate for the enzyme and the development of color was monitored at 405 nm. At concentrations of 5 fmol/μL and 10 fmol/μL, the observed signal intensity (OD 405 nm) was essentially identical for both the oligos tested. This indicates that the non-nucleosidic biotin phosphoramidite **1b** performs as well as nucleosidic biotin-dC phosphoramidite in functional assays. The ability of oligonucleotides synthesized using phosphoramidite **1b** to participate in hybridization assays was then tested and the results compared to those obtained by using commercially available nucleosidic biotin-dC phosphoramidite. The 56mer, Alu-011 with one internal biotin residue, is complementary to the template Alu-011A and has the sequence 5′-GGC CGG GCG CGG TGG CTC ACG CGT TAA **B**TT AAC GCT GTA ATC CCA GCA CTT TGG GA-3′. The template Alu-011A was immobilized on CovaLink polystyrene microtiter plates as described above. The biotinylated oligonucleotides (25 fmol/μL) were then hybridized with the template for 5 h at 42 °C. After washing off the excess probes, the bound biotinylated probes were detected by incubating with streptavidin-alkaline phosphatase conjugate. Under identical conditions, the **1b** labeled oligomers generated approximately a fivefold stronger signal than oligomers labeled with biotin-dC.

Preliminary results on the testing of fluorescein phosphoramidite **1c**, indicate that probes synthesized using **1c** allowed for the more sensitive detection of kappa immunoglobulin light chains on paraffin embedded tissue sections in in situ hybridization assays. Fluoresceinated probes synthesized using **1c** gave much stronger signal and less background relative to similar probes that had the fluorescein moiety introduced post-synthetically.[24]

To conclude, we report novel biotin, fluorescein and amino labeled phosphoramidites and CPG supports based upon the cyclohexyl backbone. These compounds can be used advantageously for the introduction of multiple reporter groups onto oligonucleotides in a cost effective and efficient manner. The natural 3-carbon atom internucleotide phosphate distance in DNA/RNA is retained in the above structures which will not affect the hybridization and annealing properties of the duplex. The reporter group is kept outside of the nucleotide chain by the rigid cyclohexane ring resulting in high coupling efficiency and oligonucleotide yields.

Acknowledgment: Technical assistance received from Deguang Yang and Zhong-Yi Lu is gratefully acknowledged. The authors wish to thank Kenneth A. Sikes for critical evaluation of the manuscript.

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19. Compound **4**: ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.9 (m, 4H, ortho ArH), 7.55 (m, 2H, para ArH),7.4 (m, 4H, meta ArH), 4.3 (s, 2H, -OCH_2), 4.1 (s, 2H, -OCH_2), 2.92 (bs, 2H, NH_2), 2.6 (m, 1H, -CH), 1.15–1.75 (m, 8H, 4X CH_2).

20. Compound **1a**: ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.4–6.65 (m, 13H, ArH), 6.1 (s, 1H, NH), 5.45 (d, 1H , NH), 5.35 (s, 1H, NH), 4.45–4.1 (m, 2H, -OCH_2), 3.75 (s, 6H, 2X OCH_3), 3.6–3.25 (m, 2H, -OCH_2), 2.8 (m, 2H, -CH_2S-), 2.7 (m, 1H, CH), 2.5 (m, 1H, CH), 2.2–1.4 (m, 24H, ring and chain CH_2), 1.3–0.9 (dd, 12H, 4X CH_3-CH); ^{31}P NMR (146 MHz, $CDCl_3$) 147.9, 148.4 ppm, M/S M^+ 888 (C_{48} H_{66} N_5 O_7PS).

21. Oligodeoxynucleotides were synthesized using a Eppendorf D–100 DNA Synthesizer utilizing standard β-cyanoethyl N,N-diisopropyl phosphoramidite monomers (dA^{Bz}, dG^{iBu}, and dC^{Bz}). Oligodeoxynucleotides were cleaved from the support and deprotected by heating at 55 °C overnight in concentrated aqueous ammonia solution. Fluoresceinated oligodeoxynucleotides were deprotected by heating at 60 °C for 5 h in concentrated aqueous ammonia solution.

22. The integrity of the label was confirmed by checking the MALDI-TOF mass spectrum of a 5′-biotin-$(T)_{19}$-T-3′ (biotin = **1a**) oligomer: M^+ calculated for $C_{218}H_{291}N_{43}O_{144}P_{20}S$ = 6466.12; found = 6479.08 utilizing a 2,4,6-trihydroxyacetophenone (THAP) matrix.

23. Compound **1c**: ^{1}H NMR (360 MHz, $CDCl_3$) δ 6.8–8.1 (m, 22H, ArH), 3.4–3.85 (m, 12H), 2.55–2.95 (m, 4H), 1.9–2.1(m, 6H), 1.6–1.85 (m, 5H), 1.15–1.45 (m, 4X CH_3, 4X CH_2), 1.2 (dd, 12H, 4X CH_3-CH); ^{31}P NMR (146 MHz, $CDCl_3$) 147.6, 147.1 ppm.

24. Preliminary results obtained from the use of phosphoramidite **1c** in in situ hybridization assays were presented at the Annual Symposium of National Society for Histotechnology held in Albuquerque, U.S.A., October 19-23, 1996, Abstract P10.

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(*Received in USA* 14 *January* 1997; *accepted* 22 *May* 1997)