Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 2, pp. 147-152, 1996

0960-894X/96 $15.00 + 0.00

0960-894X(95)00579-X

NEW NON NUCLEOSIDIC PHOSPHORAMIDITE REAGENT FOR SOLID PHASE SYNTHESIS OF BIOTINYLATED OLIGONUCLEOTIDES

Philippe Neuner

IRBM P. ANGELETTI, via Pontina Km 30.600, 00040 Pomezia, Rome, Italy

ABSTRACT : The synthesis of a 4-N-(6-aminohexanoyl)-4-amino-1,3-butanediol backbone functionalized with a protected biotin moiety is described. The CE phosphoramidite building block was used to incorporate the reporter group into a synthetic probe during solid phase synthesis. The ability of the modified oligomers to form DNA duplex structures with their target sequences and the stability of these structures was measured. Finally, the addition of an extra dT at the 5'-end allowed an efficient 5'-end ^{32}P labelling of the biotinylated oligomers.

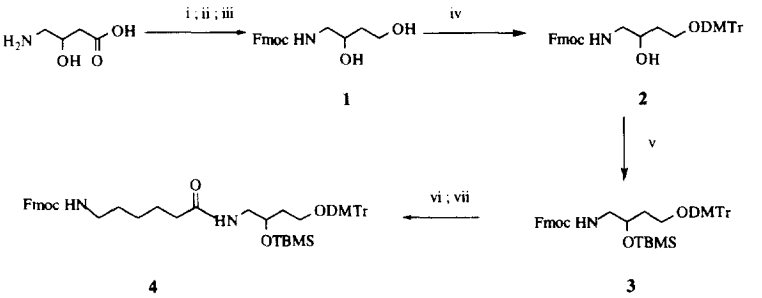
Introduction

The introduction of reporter groups into oligonucleotides during solid phase synthesis has attracted considerable attention over the last years. Non-radioactive oligonucleotides labelled with biotin have found applications as hybridization probes and diagnostic reagents for the detection of DNA and RNA, taking advantage of the strong affinity of streptavidin (Strp) for biotin.[1-4]

We report here a simple and efficient method to prepare a 4-N-(6 aminohexanoyl)-4-amino-1,3-butanediol backbone **4**. The backbone was derivatized with (+) biotin and the CE phosphoramidite building block **8** was used to incorporate the reporter group into oligonucleotides during solid phase synthesis. The accessibility of the biotinylated probe to streptavidin depends upon the flexibility of the linker arm and may be critical for optimal selection and nucleic acid-protein complex formation. For the present work, we chose a six carbon amino alkyl linker. The biotin moiety was tritylated at its 1-N position according to Pon[5] to increase the solubility of the phosphoramidite **8** in acetonitrile. We tested the ability of the modified probe to form a duplex DNA structure with its target sequence when the biotinylated reagent was introduced at various positions in a synthetic DNA fragment, and also measured the melting temperature (Tm) of the duplexes. All attempts at 5'-end ^{32}P kinasing the oligomer when the biotinyl backbone was directly introduced at the 5' end were unsuccessful[6], but this difficulty was overcome by the introduction of an extra dT residue at the 5'-end of the oligomer, immediately after the biotinyl moiety.

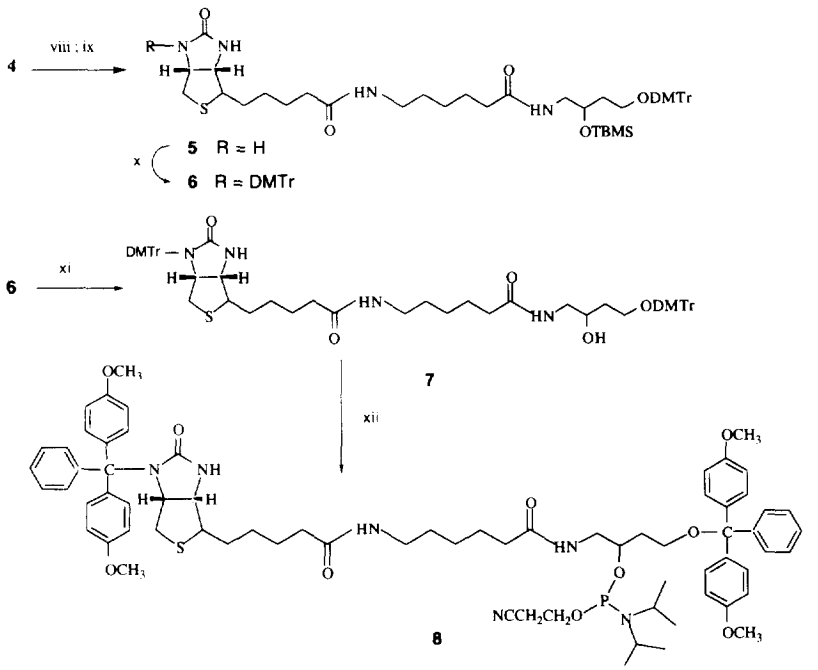
The commercially available (+/-) 4-amino-3-hydroxybutyric acid in dry methanol saturated with HCl was heated under reflux overnight, leaving after neutralization and evaporation of the solvent, the methyl ester as a colourless oil. The free amino group was protected with 9-fluorenylmethyl chloroformate (Fmoc) in 10% $NaHCO_3$/dioxane 1:1 overnight and subsequent reduction of the methyl ester with $NaBH_4$/LiCl in THF/MeOH afforded the 4-N-Fmoc-amino-1,3-butanediol **1**[7]. The primary alcohol **1** was selectively protected with 4,4'-dimethoxytrityl chloride (DMTr-Cl) in pyridine to afford **2** in 85% yield after FC using ethyl acetate/dichloromethane 1:9 (v/v) as eluent. The secondary alcohol in **2** was protected with tert-butyl-dimethylsilyl chloride (TBDMS-Cl) in dry DMF in the presence of 4 eq. of imidazole to leave the fully-protected **3** in 84% yield after purification[8]. The Fmoc blocking group was cleaved in **3** leaving the free amine which was directly coupled to the protected N6-Fmoc-aminohexanoic acid[9] using the mixed anhydride method[10], leaving the

fully-protected 1-O-DMTr-3-O-TBDMS-4-N-(N6-Fmoc-aminohexanoyl) 4-amino-1,3-butanediol **4** in 90% yield after isolation[11] (Scheme 1).



Scheme 1: Reagents i, MeOH/HCl; ii, 9-fluorenylmethyl chloroformate in 10% $NaHCO_3$/dioxane; iii, $NaBH_4$/LiCl in THF/MeOH; iv, 4,4'-dimethoxytrityl chloride in pyridine; v, tert-butyldimethylsilyl chloride, imidazole in DMF; vi, 20% piperidine in DMF 30 min.; vii, mixed anhydride (N-Fmoc ∑Ahx) at 0°C in THF.

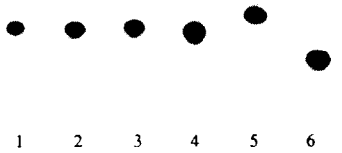
The intermediate **4** was converted to compound **5** by removing of the Fmoc protecting group and directly reacted with (+) biotin-hydroxysuccinimidyl ester[12] or alternatively with (+) biotin-2-nitrophenyl ester[13]. Compound **5** was then tritylated with dimethoxytrityl chloride to yield the N1-tritylated biotinyl moiety **6**. The TBDMS blocking group in **6** was cleaved off with 1M TBAF/THF treatment and **7**[14] was phosphorylated using 2-cyanoethyl-N,N, diisopropylchlorophosphoramidite to yield **8**[15] (Scheme 2).



Scheme 2: Reagents. viii, 20% piperidine in DMF 30 min.; ix, Biotin-NHS or Biotin-2-nitrophenol in DMF/TEA; x, 4,4'-dimethoxytrityl chloride, 4-dimethylaminopyridine in pyridine; xi, 1M TBAF/THF; xii, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, diisopropylethylamine in dichloroethane.

Synthesis, deprotection and purification of the biotinylated oligonucleotides

Biotinylated oligonucleotides were synthesized on a 1 μmol scale on the Applied Biosystems® 380 B synthesizer using the "trityl on auto" ending procedure. The biotin CE-phosphoramidite **8** was carefully dried before use and then made up as a 0.15 M solution in anhydrous acetonitrile. The synthesis cycle was modified by increasing the coupling time to 300 secs for the biotin building block. Importantly, the coupling efficiency (96%) was measured by trityl cation released by the terminal nucleoside coupled to the modified biotin phosphoramidite. The oligonucleotides were cleaved from the support using 28% aqueous ammonia and the sealed vials containing the oligomers were kept for 16 h at 60°C. The crude trityl on oligomers were purified on Reverse Phase C_{18} HPLC and 5' trityl was cleaved in the standard manner[16]. Although the phosphoramidite **8** contains an asymmetrical carbon, the diastereoisomers were not resolved by HPLC or gel electrophoresis. An aliquot of each purified oligonucleotide was 5'-end ^{32}P labelled[17] and analyzed by 15% acrylamide/7M urea gel electrophoresis.



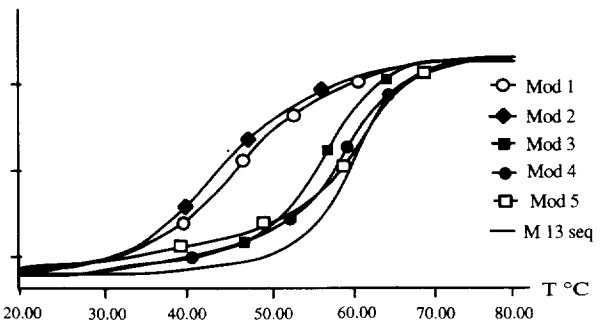
Lane 1	Mod 1	5' TCC CAG TC**X** CGA CGT 3'
Lane 2	Mod 2	5' TCC CAG T**X**A CGA CGT 3'
Lane 3	Mod 3	5' TCC CAG TCA CGA C**X**T 3'
Lane 4	Mod 4	5' T**X**C CAG TCA CGA CGT 3'
Lane 5	Mod 5	5' T**X** TCC CAG TCA CGA CGT 3'
Lane 6	M13 Seq	5' TCC CAG TCA CGA CGT 3'

Figure 1: Sequences of the oligonucleotides prepared with CE-phosphoramidite **8** (**X** designates the biotin moiety) and autoradiograph analysis of the HPLC purified 5'- ^{32}P end labelled oligomers.

Tm measurement

The melting temperature was determined spectrophotometrically at λmax=260 nm on a PERKIN ELMER® Cary instrument. About 0.5-0.7 A_{260} units of the duplex forming oligomers made of modified oligomer and complementary sequence (Compl. Seq) were dissolved in 1 ml of 10 mM tris-HCl, 100 mM KCl buffer pH 7.50.

Oligomers	Tm [°C]*
M13 seq	60.48
Mod 1	46.34
Mod 2	43.70
Mod 3	57.44
Mod 4	58.34
Mod 5	59.29

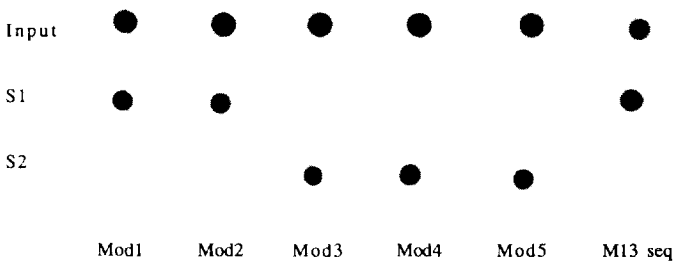


* Tm determined by calculation of the first derivative for each curve
Complementary sequence (Compl. Seq.) : 5' ACG TCG TGA CTG GGA 3'

Figure 2: Tm value and thermal dissociation curves of the duplex forming oligonucleotides

Binding of the biotinylated duplex to streptavidin

The ability of the duplex (modified oligomer/complementary sequence) to bind streptavidin was tested. The biotinylated oligomer was allowed to hybridize with its 5'-end ^{32}P-labelled complementary strand. The duplex DNA was incubated with streptavidin-coated magnetic beads and the non-hybridized labelled strand (S1) was collected. The immobilized double-stranded DNA was then denatured under alkaline conditions and the labelled strand (S2) was recovered. For each sample, equivalent amounts of S1 and S2 were applied on a Tlc plate and autoradiographed.



Input : Double stranded DNA before incubation with streptavidin-coated beads.
S1 : 2 µl of supernatant collected after 30 min incubation of the DNA duplex with Strp-coated beads.
S2 : 2 µl of supernatant collected after denaturation of the immobilized DNA duplex on streptavidin.

Figure 3: Strp-biotin complex analysis of the duplex formed by the modified oligomer and the 5'-^{32}P labelled complementary strand.

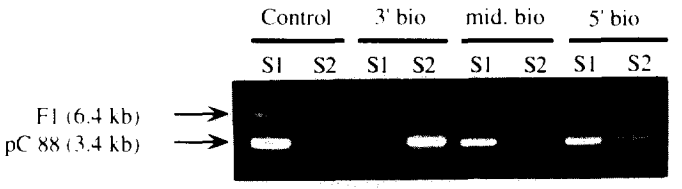
Isolation of ssDNA plasmids using the biotinyl complementary probe

Three biotinylated probes (3', 5' and internally labelled) complementary to the (+) strand of pC88 (3.4 kb) plasmid[18] and F1.IR1 (6.4 kb) genome were synthesized as described above. About 500 pmol of each biotinylated oligomer were incubated with 1 mg of streptavidin-coated magnetic beads (M-280 dynabeads®) in 100 µl of buffer (10 mM Tris-HCl, 1 M NaCl, 1mM EDTA) at room temperature for 1 h. The supernatant was removed and the particles were washed twice with 200 µl of 5 x SSC buffer to remove the excess of biotinylated oligomers. 3 µg of a mixture of ssDNA pC88 and F1.IR1 in 100 µl of 5 x SSC buffer were added to each of the immobilized biotinyl probe. The mixture was incubated at 95°C for 5 min and then gradually cooled to 43°C (1°C/min.). Annealing was performed for 6 h at 43°C under agitation. After hybridization, the supernatant S1 containing the non hybridized ssDNA was collected. The beads were washed twice with 200 µl of 5 x SSC buffer and re-suspended in 100 µl of H_2O. After heating to 80°C for 5 min, the supernatant S2 containing the eluted ssDNA was recovered. S1 and S2 were desalted and analyzed by electrophoresis on a standard 1.5% agarose gel containing ethidium bromide and photographed under UV illumination. 3 µg of a mixture of ssDNA pC88 and F1.IR1 incubated with 1 mg streptavidin-coated beads as described above in absence of any biotinylated probe was used as control. The biotinylated oligomers used in this experiment were:

3' Bio : 5' TTG CAG GGA GTC AAA GGC CGC T**X**T 3';

Mid bio: 5' TTG CAG GGA GTC **X**AA GGC CGC TTT 3'

5' Bio : 5' T**X**G CAG GGA GTC AAA GGC CGC TTT 3' (**X** designates the biotin moiety)



S1: Supernatant containing the non-hybridized pC88/F1.IR1 ssDNA templates with the respective biotinylated oligomers.
S2: Supernatant containing the eluted ssDNA fragments after denaturation.

Figure 4: Agarose gel analysis of S1 and S2 supernatants.

Discussion

Oligonucleotides containing at a pre-selected position the biotinyl phosphoramidite **8** instead of the nucleoside moiety can form a duplex with its complementary sequence. Substitution of the natural nucleoside with the biotinyl backbone at 5' or 3' end of a 15 mers probe, did not significantly alter the Tm values. By contrast, modification of a more central position drastically decreased the duplex stability, as mirrored by the lower Tm values[19]. The poor affinity of the internally biotinylated probes (mod 1 and mod 2) for their target sequence resulted in a low recovery of the ^{32}P labelled complementary strand (Figure 3). To confirm these results we synthesized a set of biotinylated 24 mers (5', 3' and internally) complementary to the (+) strand of a pC88 plasmid and F1.IR1 ssDNA genome. Increasing the length of the probe reduced the destabilization effect of the 1,3 butanediol moiety. All the biotinylated labelled probes successfully tagged the target sequence and in particular, the 3' biotinylated probe showed a higher efficiency in our experimental conditions. The length of the biotinylated probe and hybridization conditions are therefore essential factors to ensure an efficient capturing of the target template. Important to note is that circular ssDNA fragments as large as 6.4 kb can be efficiently tagged and recovered using this methodology. Finally, the 5'-end incorporation of the biotinylated backbone followed by the addition of an extra deoxythymidine nucleoside allowed an efficient 5'-end ^{32}P enzymatic labelling. We are currently using biotinylated oligonucleotides prepared with the biotinyl phosphoramidite **8** in PCR amplifications, solid phase DNA sequencing[20] and for isolation of specific genes in cDNA libraries[21].

Acknowledgments

We thank C. Prezzi, C. Fipaldini and C. Santini for valuable assistance and F. Felici and P. Monaci for stimulating discussions and critical reading. Special thanks to J. Clench for linguistic editing and M. Emili for photographic work.

References and notes

1. De Vos, M-J., Cravador, A., Lenders, J-P., Houard, S. and Bollen, A. **1990**, Nucleosides & Nucleotides, 9, 259-273.
2. Bengstrom, M., Harju, L. and Syvanen, A-C. **1991**, Nucleosides & Nucleotides, 10, 507-509.
3. Reyes-Engel, A. and Dieguez-Lucena, J., L. **1993**, Nucleic Acid Res., 21, 759-760.
4. Niemeyer, C., M., Sano, T., Smith, C., L. and Cantor, C., R. **1994**, Nucleic Acid Res., 22, 5530-5539.
5. Pon, R.T., **1991**, Tetrahedron Lett., 32, 1715-1718.
6. Fontanel, M-L., Bazin, H. and Teoule, R. **1993**, Analytical Biochemistry, 214, 338-340.
7. The crude N-Fmoc-4-amino-3-hydroxybutyric acid methyl ester was treated at room temperature for 12 hours with 3 eq of $NaBH_4$/LiCl mixture in MeOH/THF 1/1. The reaction mixture was diluted with dichloromethane and washed first with a 10% aqueous citric acid solution, then with a 1% aqueous sodium

bicarbonate solution. The organic layer was separated, dried over sodium sulfate and evaporated to dryness. The resulting oil was purified by flash chromatography (FC) using a gradient of 3-6% methanol in dichloromethane as eluent. The pure product was obtained in 73% yield. While this manuscript was in preparation, De Vos et al. reported the synthesis of 4-amino-1,3-butanediol through a different approach. De Vos, M.J., Van Elsen, A. and Bollen, A. **1994**, Nucleosides & Nucleotides, 13, 2245-2265.

8. We found it necessary to protect the secondary alcohol in **2** to avoid side reaction during the preparation of **4** using the mixed anhydride methodology.
9. The preparation of the Fmoc protected amino hexanoic acid is described by Haralambis J., Duncan L., Angus K. and Tregear G. W. **1990**, Nucleic Acid Res., 18, 493-499.
10. 1 eq. of N-methylmorpholine was added to a stirring solution of 1 eq. of 6-N-Fmoc-amino hexanoic acid in dry THF at -30°C under anhydrous conditions. After 15 min, 1 eq. of isobutyl chloroformate was added. The cloudy mixture was stirred at -30°C for 30 min, then at 0°C for 30 min. Tlc (2% MeOH/DCM) showed a new spot Rf 0.80 corresponding to the mixed anhydride. The stirring mixture was cooled to -30°C and the previously amino deprotected **3** in THF was added in one portion. Reaction was completed in 1.50 h.
11. **4** was purified by FC using petrol ether/ethyl acetate 3/1, then 2/1 and finally 1/1 as eluent. ^{1}H NMR ($CDCl_3$) δ: 0.09 (s, 6H, CH_3Si), 0.91 (s, 9H, TBDMS), 1.3-1.88 (m, 6H, CH_2), 2.22 (t, 2H), 3.25-3.35 (m, 6H), 3.86 (s, 6H OMe), 3.95 (t, 1H), 4.25 (m, 1H), 4.48 (d, 2H), 6.87-6.90 (dd, 4H), 7.31-7.86 (m, 17H ArH).Anal. Calc. for C_{52} H_{64} O_7 N_2 Si: C, 72.85; H, 7.52; N, 3.27. Found: C, 72.30; H, 7.85; N, 3.22.
12. Parameswaran, K.N. **1990**, Org. Prep. Proced. Int., 22, 119-121. The pure compound **5** was obtained in 75 % yield after flash chromatography (FC) on silica using a gradient of 6-10% methanol in dichloromethane. Rf : 0.30 in 10% methanol/dichloromethane.
13. Pieles, U., Sproat, B.S. and Lamm, G.M. **1990**, Nucleic Acids Res., 18, 4355-4360. Compound **5** was obtained in 80% yield after FC using the same eluent system described in reference 12.
14. Rf: 0.42 in 7% ethanol/dichloromethane/0.5% triethylamine. Yield 85%.^{1}H NMR ($CDCl_3$) δ: 1,30-1,75 (m, 12H, CH_2), 1.98-2.21 (m, 4H, CH_2CON), 2.21-2.54 (m, 3H, CHS), 3.00-3.46 (m, 6H), 3.80 (s, 12H, OMe), 3.95 (t, 1H), 4.20-4.45 (m, 2H, CHN), 6.80 (m, 8H ArH), 7.25-7.48 (m, 18H, ArH). Anal. Calc. for C_{62} H_{72} O_9 N_4 S: C, 70.99; H, 6.87; N, 5.34. Found: C, 69.51; H, 7.47; N, 5.55.
15. **8** was obtained as a white foam in 75% yield after FC using dichloromethane/ethyl acetate/0.5% triethylamine as eluent. ^{31}P NMR ($CDCl_3$) δ: 148.26, 148.15, 147.18, 147.00.
16. The purification was carried out using a gradient of buffer A (0.1M TEAA pH 7.50) and buffer B (30% buffer A/70% acetonitrile) at a flow rate of 2 ml/min. over 50 min. The purified tritylated oligomer was deblocked with 0.5 ml acetic acid/water (8/2), and after 30 min at room temperature the mixture was extracted with ether (3 × 0.5ml) and evaporated to dryness.
17. Maniatis, T., Fritsch, E.F., Sambrook, J. **1989**, Molecular Cloning; Cold Spring Harbor Laboratory, New York.
18. Felici F., Castagnoli L., Musacchio A., Jappelli R. and Cesareni G. **1991**, J. Mol. Biol., 222, 301-310.
19. Wilk, A., Koziolkiewicz, M., Grajkowski, A., Uznanski, B. and Stec, W.J. **1990**, Nucleic Acid Res., 18, 2065-2068.
20. Hultman, T., Bergh S., Moks T. and Uhlen M. **1991**, Biotechniques, 10, 84-93.
21. Li, W-B., Gruber C.E., Noon M.C., Polayes D., Schmidt B. and Jesse J. **1995**, Focus, 17, 35-39.

(*Received in Belgium* 26 *June* 1995; *accepted* 6 *December* 1995)