Tetrahedron Letters,Vol.30,No.46,pp 6287-6290,1989
Printed in Great Britain
0040-4039/89 $3.00 + .00
Pergamon Press plc

A PHOSPHORAMIDITE REAGENT FOR AUTOMATED SOLID PHASE SYNTHESIS OF 5'-BIOTINYLATED OLIGONUCLEOTIDES

Anthony J. Cocuzza

Central Research and Development Department (Contribution Number 5218)
E. I. duPont de Nemours & Company, Inc.
Experimental Station
Wilmington, Delaware 19880-0328

Abstract: Biotin phosphoramidite **1** has been synthesized by a simple two-step procedure. With this reagent 5'-biotinylated oligonucleotides are easily prepared using an automated DNA synthesizer.

Labeled oligonucleotides have been used widely as hybridization probes for the detection of specific gene sequences including those associated with human genetic disease. While such probes are most commonly labeled by enzymatic incorporation of the isotope ^{32}P, they may also be labeled with biotin which is highly detectable by virtue of its tight binding to proteins avidin and streptavidin. Biotin is a safer and at times more convenient alternative to radioactive labels. Of particular recent interest are oligonucleotides to which biotin has been attached to the 5' hydroxyl group of the terminal nucleoside base[1,2]. In addition to hybridization probes, such 5'-biotinylated oligonucleotides have demonstrated application in ligase-mediated gene detection[3], direct dideoxy sequencing following PCR[4], and non-radioactive sequencing of DNA[5]. However, the use of 5'-biotinylated oligonucleotides in these and future applications is limited primarily by the lack of an efficient method for their preparation.

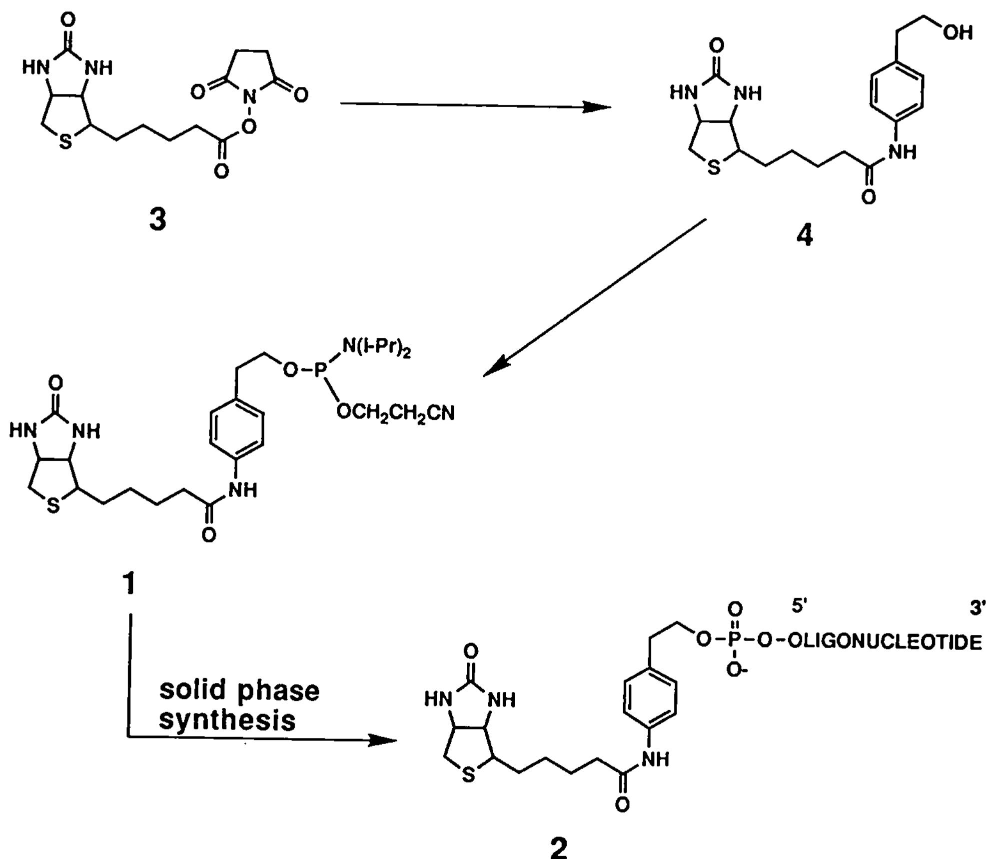
Typically, 5'-biotinylated oligonucleotides are prepared by the solid phase synthesis of oligonucleotides carrying a reactive aliphatic amine which is then

derivatized by an active ester of biotin[1]. Although a recent procedure has been decribed which allows the solid phase synthesis of biotinylated oligonucleotides[2], all of the methods currently available require specialized reagents as well as reaction and purification steps beyond those used in automated solid phase synthesis, the technique by which the vast majority of synthetic oligonucleotides are now prepared. This report describes a biotin phosphoramidite reagent **1** which can be directly used in automated oligonucleotide synthesizers to prepare 5'-biotinylated oligonucleotides. *This reagent makes biotin-labeled oligonucleotides as accessible as ordinary oligonucleotides.*

Reagent **1** has been used to prepare a number of oligonucleotides **2** in which biotin has been attached to the phosphate group of the terminal 5'-base through a *p*-aminophenethyl spacer group. The *p*-aminophenethyl group imparts excellent physical characteristics on phosphoramidite **1** making it easy to purify by precipitation and convenient to manipulate and store. The length of the *p*-aminophenethyl spacer group allows the biotinylated oligonucleotides **2** to bind efficiently to solid supported streptavidin, and its hydrophobicity makes it simple to analyze and purify these oligonucleotides by reverse-phase HPLC.

Phosphoramidite reagent **1** is easily prepared by a two-step procedure starting from commercially available biotin N-hydroxysuccinimide ester **3**. First, the intermediate *p*-aminophenethanol amide **4** is prepared by heating a DMF solution of **3** and *p*-aminophenethyl alcohol (1.15 eq) at 50° for 24 hr. After evaporation of the solvent, pure **4** is obtained by first boiling the reaction product for 15 min in refluxing ethanol, and then collecting the solid product (80% yield) by vacuum filtration of the cooled suspension. Phosphoramidite reagent **1** is prepared from **4** by a modification of the general method of Caruthers[6]. A solution of **4**, 2-cyanoethyl *N, N, N', N'*-tetraisopropylphosphorodiamidite[7] (1.2 eq), and diisopropylammonium tetrazolide (0.6 eq) in dry DMF is stirred 3 hr at room temperature. The reaction is first quenched by the addition of a small quantity of methanol, and then worked up by pouring the reaction mixture onto aqueous sodium bicarbonate and extracting with methylene chloride. The crude product is effectively purified by precipitation. A concentrated methylene chloride solution is added dropwise to a rapidly stirring 16-fold volume of hexane at 0°, and the precipitated product is collected (75% yield). Reagent **1** is obtained as a stable, dry powder which can be conveniently weighed out and manipulated in air[8].

We have now used **1** to prepare very conveniently a large number of 5'-biotinylated oligonucleotides on both a DuPont Coder™ 300 and an ABI 380A DNA Synthesizer. A vial containing a 0.1M solution of the reagent in 15% DMF/methylene chloride[9] is attached to the extra port on the synthesizer which is then programmed to use the reagent when attaching the terminal 5'-residue of an oligonucleotide. The instrument's standard synthesis cycle (tetrazole-catalyzed coupling, capping, and



oxidation) followed by removal of the nucleoside protecting groups affords directly high yields of biotinylated oligonucleotides[10]. For many applications these oligonucleotides may be used without purification. HPLC purification and identification of these modified oligonucleotides are greatly facilitated by their long retention times relative to oligonucleotide failure sequences which do not contain the relatively hydrophobic N-biotinyl *p*-aminophenethyl group[11]. We anticipate that users of automated DNA synthesizers will find it convenient to prepare 5'-biotinylated oligonucleotides using biotin phosphoramidite reagent **1**.[12]

Acknowledgements. The expert technical assistance of Dennis R. Chidester and Carol E. Burns are gratefully acknowledged.

REFERENCES AND NOTES

1. a) A. Chollet and E. H. Kawashima, *Nucleic Acids Res.* **1985**, *13*,1529. b) L. Wachter, J. A. Jablonski, and K. L. Ramachandran, *Nucleic Acids Res.* 1986, *14*, 7985. c) S. Agrawal, C. Christodoulou, and M. J. Gait, *Nucleic Acids Res.* **1986**,*14*, 6227. d) M. S. Urdea, B. D. Warner, J. A. Running, M. Stempien, J. Clyne, and T. Horn, *Nucleic Acids Res.* **1988**, *16*, 4937. e) A. F. Cook, E. Vuocolo, and C. L. Brakel, *Nucleic Acids Res.* **1985**,*16*, 4077. f) B. C. F. Chu and L. E. Orgel, *DNA* **1985**, *4*, 327.
2. T. Kempe, W. I. Sundquist, F. Chow and S. -L. Hu, *Nucleic Acids Res.* **1985**, *13*, 45.
3. U. Landegren, R. Kaiser, J. Sanders, and L. Hood, *Science* **1988**, *241*,1077.
4. L. G. Mitchell and C. R. Merril, *Anal. Biochem.* **1989**, *178*, 239.
5. P. Richterich, *Nucleic Acids Res.* **1989**, *17*, 2181.
6. A. D. Barone, J. -Y. Tang, and M. H. Caruthers, *Nucl. Acids Res.* **1984**, *12*, 4051.
7. J. Nielsen, J. E. Marugg, J. H. van Boom, J. Honnens, M. Taagaard, and O. Dahl, *J. Chem. Res. (S)* **1986**, 26.
8. 1H-NMR (CD_2Cl_2) : ∂ 8.30 (m, 1H, NH), 7.50 (d, J=7, 2H, ArH), 7.15 (d, J=7, 2H, ArH), 6.55 (d, 1H, NH), 5.52 (m, 1H, NH),4.47 (m, 1H, NCH), 4.30 (m, 1H, NCH), 3.5-3.9 (m, 6H, 2X CH_2O + 2X NC<u>H</u>(CH_3)$_2$), 3.14 (m, 1H, SCH), 2.80-2.95 (m, 3H, CH_2Ph + SCH), 2.65 (d, J=13, 1H, SCH), 2.57 (t, J=7, 2H, CH_2CN), 2.30 (t, J=7, 1H, CH_2CO), 1.75 (m, 4H, 2X CH2), 1.43 (m, 2H, CH_2), 1.15 (m, 12H, 2X CH(<u>CH</u>$_3$)$_2$). ^{1}H-decoupled ^{31}P-NMR (CD_2Cl_2): ∂ 148.96 (s).
9. Reagent **1** is not soluble in acetonitrile, the solvent most commonly used in automated DNA synthesizers. This mixture of DMF and methylene chloride provides optimum coupling efficiency. Both solvents should be dry and DMF should be free of amines.
10. Running the standard 55° ammonia deprotection reaction for the minimum time (4 - 6 hr) ensures that the linker amide bond will not be cleaved. That the biotin group of **2** remains intact throughout the synthesis cycle and deprotection steps was demonstrated by the quantitative removal of oligonucleotide **2** from aqueous solution by solid-supported streptavidin.
11. Retention times are consistently 8-10 minutes longer than the corresponding unbiotinylated oligonucleotides under standard HPLC conditions (C8 column, 5-15% acetonitrile in 0.1M aqueous TEAA, pH 7.0).
12. Since this paper was submitted, a report describing an alternative biotin phosphoramidite reagent has appeared: A. M. Alves, D. Holland, and M. D. Edge, *Tetrahedron Lett.* **1989**, *30*, 3089.

(Received in USA 27 July 1989)