Tetrahedron Letters, Vol.32, No.14, pp 1715-1718, 1991
Printed in Great Britain

0040-4039/91 $3.00 + .00
Pergamon Press plc

A LONG CHAIN BIOTIN PHOSPHORAMIDITE REAGENT FOR THE AUTOMATED SYNTHESIS OF 5'-BIOTINYLATED OLIGONUCLEOTIDES

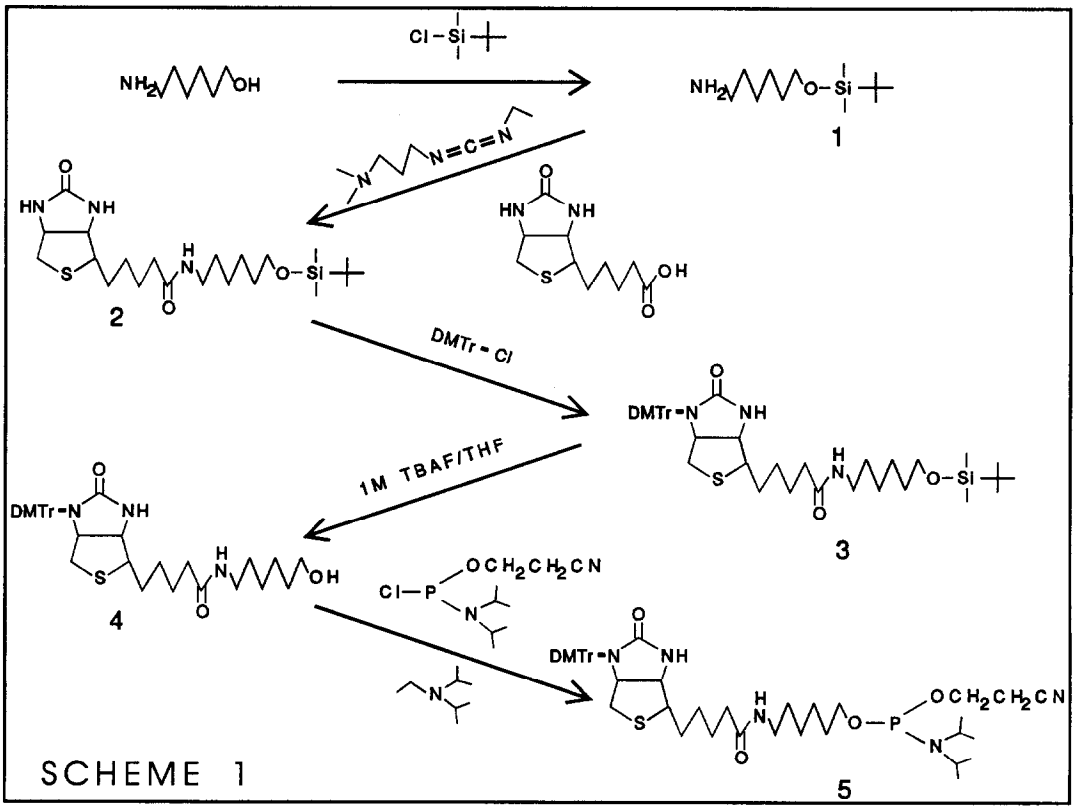
Richard T. Pon
Department of Medical Biochemistry, The University of Calgary, Calgary, Alberta, Canada, T2N 4N1

Abstract: An N-dimethoxytritylated biotin group coupled to a 6-aminohexanol linker was converted into a phosphoramidite to yield a biotinylating reagent which can be used in automated solid-phase synthesis to produce 5'-biotinylated oligonucleotides in high yields.

The use of labelled oligonucleotide sequences as probes for other nucleotide sequences is an extremely valuable technique in biology and medicine. However, isotopically labelled sequences are not always practical and a variety of methods have been developed for labelling oligonucleotides with biotin reporter groups[1], since these can be detected using non-radioactive reagents with high sensitivity. Chemical addition of biotin is usually performed by a two step procedure involving synthesis of an oligonucleotide modified with a primary amino group followed by coupling, in aqueous solution, to a biotin N-hydroxysuccinimide ester[2,3]. Recently, several groups have prepared biotin containing phosphoramidite reagents which incorporated biotin as part of the solid-phase synthesis of the oligonucleotides. These reagents generally biotinylated the 5'-terminus[4,5,6] although two N^4-biotinylated deoxycytidine reagents[7,8] could introduce biotin at any position. However, these latter reagents were unsuitable to us because of the lengthy and expensive synthetic procedures required to make them. The 5'-biotinylating reagents were also considered unsatisfactory because they had either an inadequately sized linker arm[4,6] or because they were insoluble in the acetonitrile[5].

In this communication, we would like to describe a phosphoramidite reagent, *[1-N-(4,4'-dimethoxytrityl)-biotinyl-6-aminohexyl]-2-cyanoethyl-N,N,-diisopropylaminophosphoramidite,* **5**, which contains a 6-aminohexyl linker arm. A long linker arm is required for maximum sensitivity[9], since the biotin group must be accessible to the large proteins used in biotin detection systems. This reagent also has a dimethoxytrityl group to increase solubility and provide compatibility with existing methods of oligonucleotide synthesis and purification. Thus standard methods based on released trityl colour can be used for monitoring coupling yields and trityl dependent reversed-phase HPLC or solid-phase extraction methods can be used for purification.

The synthesis of the biotin phosphoramidite, **5**, (Scheme 1) required only five steps and was designed to avoid the use of the expensive biotin N-hydroxysuccinimide reagents commonly used[2,3,5,6,9]. In this procedure, 6-aminohexanol, was protected by silylation with t-butyldimethylsilyl chloride to yield **1**. This product was then coupled to biotin using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in anhydrous pyridine to yield biotinyl-6-amino-1-t-butyldimethylsilylhexan-1-ol, **2**. Compound **2** was then tritylated with dimethoxytrityl chloride to yield the N^1-tritylated compound **3**. Tritylation occurred only at the N^1-position, in agreement with the results reported by Alves *et. al*[4]. Compound **3** was then desilylated by treatment with tetrabutylammonium fluoride (TBAF) in THF. Finally, compound **4** was phosphitylated using 2-cyanoethyl-N,N,-diisopropylchlorophosphoramidite to yield [1-N-(4,4'-dimethoxytrityl)-biotinyl-6-aminohexyl]



-2-cyanoethyl-N,N,-diisopropylaminophosphoramidite, **5**.

Compound **5** was readily soluble in acetonitrile and 0.1 M solutions were used on an Applied Biosystems 381A DNA synthesizer. 5'-Biotinylated sequences were prepared using our standard synthesis cycles with the "Tr-On" synthesis option. The N-DMT group of the biotin could be removed, while still on the synthesizer, by manually performing an extended length detritylation step (5% dichloroacetic acid/dichloroethane, 180 s). Alternatively, the tritylated sequence may be purified by reversed-phase HPLC[10]. The extended detritylation required because of the increased stability of an N-DMT group, was the only modification in the synthesis procedure neccessary.

The coupling efficiency of the biotinylation reagent was determined by trityl colour analysis and found to be between 85-95%. This was less than the 98-99% obtained for nucleotide additions but was still considered satisfactory. Higher biotinylation yields may be possible by either extending the duration of the biotin phosphoramidite coupling reaction or by increasing the phosphoramidite concentration, as has been the case with previously reported biotin phosphoramidite reagents[4,6-8]. However, the benefit of increased yields was not considered sufficient justification for the added cost of higher concentrations or the inconvenience of special coupling cycles. The biotinylated oligonucleotide was easily distinquished from the non-biotinylated products by a marked retardation of electrophoretic[3] and chromatographic[11] mobility.

It was essential that the aminohexyl linker joining the biotin to the oligonucleotide be resistant to the deprotection conditions used (28% NH_4OH, 16 h, 50°). To verify this a biotinylated octathymidylic acid sequence was prepared, cleaved from the support with 28% NH_4OH and aliquots were sealed into screw capped glass tubes. These were heated at 50° for periods of either 2, 4, 8, 16, or 24 hours before being evaporated to dryness. Gel electrophoresis (Figure 1) of the samples showed that there was no significant loss of biotin, even when treated for 24 hours at 50°.

The sensitivity of the 5'-biotinylated sequences as DNA probes was examined by making a 26-base long oligonucleotide complementary to a portion of a C-DNA HIV sequence. For comparison, the same sequence was made with a 5'-amino terminus and biotinylated with D-biotinyl-aminocaproic acid N-hydroxysuccinimide ester[3]. This two step procedure gave only a 10-20% yield of biotinylation versus the 93% yield obtained with compound **5**. The sensitivity of both of these probes, in a dot-blot hybridization[12], was similar with 0.4 femtomoles of DNA being detectable.

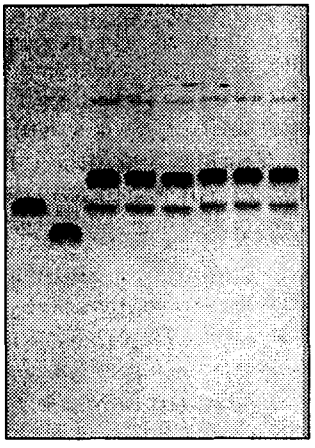


Figure 1. The effect of ammonium hydroxide hydrolysis (50°) on the biotin ligand. Lane 1: nonbiotinylated $(Tp)_7T$; Lane 2: 5'-phosphorylated sequence $p(Tp)_7T$; Lanes 3 to 8: Biotinylated $(Tp)_7T$ with 0, 2, 4, 8, 16, and 24 hours treatment with 50° NH_4OH.

The biotin phosphoramidite described can be economically synthesized and it provides a faster, easier, and less expensive method for making biotinylated oligonucleotides than the previous two step procedure[2,3]. It should therefore be quite useful to those laboratories which require non-isotopically labelled oligonucleotide probes.

Experimental

1, Preparation of 6-Amino-t-butyldimethylsilylhexan-1-ol, **1**.

6-Amino-1-hexanol (10 g, 85.3 mmol) was coevaporated to dryness with pyridine (3x), redissolved in pyridine (75 ml) and t-butyldimethylsilyl chloride (15.4 g, 102 mmol) was added. After stirring overnight, the reaction was concentrated by evaporation, diluted in $CHCl_3$, neutralized with aqueous $NaHCO_3$ and purified on a silica gel column by elution with triethylamine/MeOH/$CHCl_3$ 2.5:10:87.5. Yield: 17 g (86%). M/S M(231); ^1H NMR (200MHz, $CDCl_3$): δ0.04 (6H, s, CH_3Si); δ0.89 (9H, s, tBuSi); δ1.28-1.53 (8H, m, $4CH_2$); δ1.56 (2H, s, NH_2); δ2.69 (2H, t, CH_2-N); δ3.60 (2H, t, CH_2-O).

2, Preparation of Biotinyl-6-amino-1-t-butyldimethylsilylhexan-1-ol, **2**.

Biotin (12.2 g, 50 mmol) and **1** (50 mmol, 11.6 g) were coevaporated to dryness with pyridine (3x) and then resuspended in more pyridine (250 ml). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (60 mmol, 11.5 g) was added and the reaction was stirred (48 h). TLC (20% MeOH/$CHCl_3$) showed complete disappearance of the starting amine (negative ninhydrin test) and formation of **2** (Rf 0.68, stained yellow with I_2). The pyridine was removed by evaporation and the residue was re-dissolved in 10% MeOH/$CHCl_3$ (250 ml) and purified by elution on a silica gel column with a 5-10% MeOH/$CHCl_3$ gradient. Yield: 18.6 g (81%). M/S M (457); ^1H NMR (200MHz, $CDCl_3$): δ0.04 (6H, s, CH_3Si); δ0.89 (9H, s, tBuSi); δ1.25-1.8 (14H, m, $7CH_2$); δ2.20 (2H, t, CH_2-N); δ2.68-3.00 (2H, m, CH_2CON); δ3.1-3.30 (3H, m, CHS); δ3.59 (2H, t, CH_2-O); δ4.32 (1H, m, CHN); δ4.49 (1H, m, CHN); δ5.46 (1H, bs, NH); δ5.94 (1H, bs, NH); δ6.36 (1H, bs, NH).

3, Preparation of 1-N-(4,4'-Dimethoxytrityl)-biotinyl-6-amino-1-t-butyldimethylsilylhexan-1-ol, **3**.

Compound **2** (20 mmol, 9.15 g) was coevaporated to dryness with pyridine (3x) and redissolved in more pyridine (50 ml). Dimethoxytrityl chloride (22 mmol, 7.45 g) and 4-dimethylaminopyridine (1 mmol, 122 mg) were then added. After 24 h, TLC (10% MeOH/$CHCl_3$) showed incomplete reaction and more dimethoxytrityl chloride (2 mmol) was

added. After another 16 h, the reaction was complete, and the solution was concentrated to 1/3 volume, redissolved in $CHCl_3$ and neutralized with aqueous $NaHCO_3$. The product was then purified by elution on a silica gel column with a 0-5% MeOH/$CHCl_3$ gradient. Yield: 13.4 g (88%). TLC (5% MeOH/$CHCl_3$) : Rf= 0.36. ^1H NMR (200MHz, $CDCl_3$): δ0.047 (6H, s, CH_3Si); δ0.89 (9H, s, tBuSi); δ1.25-1.75 (14H, m, $7CH_2$); δ2.04 (2H, t, CH_2-N); δ2.2-2.53 (2H, m, CH_2CON); δ3.02-3.30 (3H, m, CHS); δ3.60 (2H, t, CH_2-O); δ3.80 (6H,s, $2CH_3O$); δ4.32 (2H, m, 2CHN); δ5.21 (1H, bs, NH); δ5.55 (1H, bs, NH); δ6.82 (4H, δ, ArH); δ7.25 (9H,m, ArH).

4, Preparation of 1-N-(4,4'-Dimethoxytrityl)-biotinyl-6-amino-hexan-1-ol 4.

Compound **3** (10 g, 13.2 mmol) was dissolved in 1M TBAF/THF solution (52 ml). After 16 h, TLC (10% MeOH/$CHCl_3$) showed complete desilylation. The solution was then concentrated, redissolved in $CHCl_3$, washed with H_2O (4x), and purified on a silica gel column using a 0-5% MeOH/$CHCl_3$ gradient. Yield: 8.7 g (80%). TLC (10% MeOH/$CHCl_3$): Rf= 0.47. ^1H NMR (200MHz, $CDCl_3$): δ1.25-1.75 (14H, m, $7CH_2$); δ2.05 (2H, t, CH_2-N); δ2.2-2.53 (2H, m, CH_2CON); δ3.02-3.30 (3H, m, CHS); δ3.61 (2H, t, CH_2-O); δ3.80 (6H,s, $2CH_3O$); δ4.35 (2H, m, 2CHN); δ5.21 (1H, bs, NH); δ5.69 (1H, bs, NH); δ6.82 (4H, δ, ArH); δ7.25 (9H,m, ArH).

5, Preparation of [1-N-(4,4'-dimethoxytrityl)-biotinyl-6-aminohexyl]-2-cyanoethyl-N,N,-diisopropyl-aminophosphoramidite 5.

Compound **4** (5.18 g, 8 mmol) was dissolved in diisopropylethylamine (5.5 ml, 32 mmol) and anhydrous $CHCl_3$ (25 ml) and then 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (1.8 ml, 8 mmol) was added. After stirring 1 h at room temperature, TLC (5% triethylamine/$CHCl_3$) showed complete conversion of compound **4** into compound **5**. Ethyl acetate (10 ml) was added and the reaction was washed with aqueous NaCl solution (3x) and H_2O (1x) before being purified on a silica gel column using first CH_2Cl_2/hexane/triethylamine 42:55:3 and then 5% triethylamine/$CHCl_3$. Yield: 3.6 g (81%). TLC (5% triethylamine/$CHCl_3$): Rf = 0.48. ^{31}P NMR ($CDCl_3$): 145.50 (s). ^1H NMR (200MHz, $CDCl_3$): δ1.20 (12H, m, $4CHCH_3$); δ1.25-1.9 (14H, m, $7CH_2$); δ2.07 (2H, t, CH_2-N); δ2.22-2.58 (2H, m, CH_2CON); δ2.64 (2H, t, CH_2CN); δ3.05-3.30 (3H, m, CHS); δ3.52-3.75 (4H, m, $2CH_2$-O); δ3.80 (6H,s, $2CH_3O$); δ4.36 (2H, m, 2CHN); δ5.13 (1H, bs, NH); δ5.61 (1H, bs, NH); δ6.82 (4H, δ, ArH); δ7.25 (9H,m, ArH). Analysis calculated for $C_{46}H_{64}N_5O_6PS$, M.W. 846.09: C, 65.30%, H, 7.62%, N, 8.28%. Found: C, 65.13%, H, 7.71%, N, 8.29%.

Acknowledgements: I would like to thank M. Inoue, Dept. of Pathology, University of Calgary for performing the biotin detection experiment and the Natural Sciences and Engineering and Research Council of Canada (NSERC) for financial support.

Bibliography

1, M. Wilchek and E. A. Bayer, Anal. Biochem. **171**, 1-32 (1988).
2, A. Chollet and E. H. Kawashima, Nucleic Acids Res. **13**, 1529-1541 (1985).
3, M. Bengtstrom, A. Jungell-Nortamo, and A.-C. Syvanen, Nucleosides & Nucleotides **9**, 123-127 (1990).
4, A. M. Alves, D. Holland, and M. D. Edge, Tetrahedron Letters **30**, 3089-3092 (1989).
5, A. J. Cocuzza, Tetrahedron Letters **30**, 6287-6290 (1989).
6, K. Misiura, I. Durrant, M. R. Evans, and M. J. Gait, Nucleics Acids Res. **18**, 4345-4354 (1990).
7, A. Roget, H. Bazin, R. Teoule, Nucleic Acids Res. **17**, 7643-7651 (1989).
8, U. Pieles, B. S. Sproat, G. M. Lamm, Nucleic Acids Res. **18**, 4355-4360 (1990).
9, A.H. Al-Halim and R. Hull, Nucleics Acids Res. **14**, 9965-9976 (1986).
10, M. W. Germann, R. T. Pon, and J. H. van de Sande, Anal. Biochem. **165**, 399-405 (1987).
11, K. Holmstrom and O. F. Rasmussen, Nucleic Acids Res. **18**, 4632 (1990).
12, M. Inoue, M. A. Duggan, D. I. Robertson, and V. Chang-Poon, J. Virological Methods **26**, 159-170 (1989).

(Received in USA 26 November 1990)