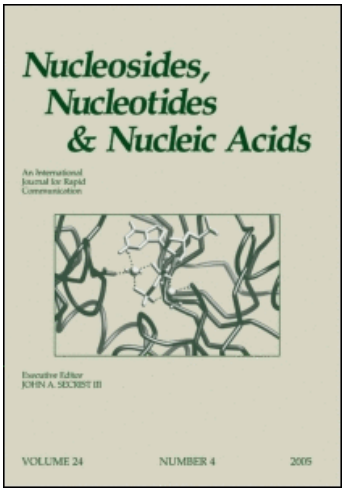

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Kumar, Pradeep , Sharma, A. K. and Gupta, K. C.(1996) 'Base-Labile Group Protected Biotinphosphoramidite Reagents for Solid Phase Biotinylation of Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 15: 7, 1263 — 1273
To link to this Article: DOI: 10.1080/07328319608002428
URL: http://dx.doi.org/10.1080/07328319608002428

NUCLEOSIDES & NUCLEOTIDES, 15(7&8), 1263-1273 (1996)

BASE-LABILE GROUP PROTECTED BIOTINPHOSPHORAMIDITE REAGENTS FOR SOLID PHASE BIOTINYLATION OF OLIGONUCLEOTIDES

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ABSTRACT

A general and rapid method is described for the synthesis of N^1-base labile group protected biotinphosphoramidite reagents, useful for the synthesis of biotinylated oligonucleotides in automated DNA synthesizer.

INTRODUCTION

Non-radiolabelled oligonucleotides find important applications in modern molecular biology, medical diagnostics and DNA sequence analysis. Out of several reporter groups employed for this purpose, biotin is preferred[1-4] and used extensively because of its ease in detection due to its tight binding to avidin and streptavidin.[5]

Though enzymatic labelling of DNA or RNA with biotin and other reporter groups is well known, chemical methods are advantageous for the preparation of well defined or site specific biotinylated probes. Chemical labelling[6,7] of synthetic oligonucleotides is usually carried out by a two step procedure involving the solid phase synthesis of oligonucleotides carrying a reactive functional group (-NH_2/-SH) which is then derivatized using a suitable reagent of biotin in aqueous solution.

Recently, single step solid phase biotinylation has been reported from several research groups. In this approach biotin is modified and converted to a suitable phosphoramidite reagent, which is then used in analogous way to normal nucleoside phosphoramidites. Alves et al.[8] and Cocuzza[9] reported two such reagents where in the former one they employed 4,4'-dimethoxytrityl (DMT) for N^1-protection to provide lipophilicity to biotin reagent while in the case of second one p-aminophenethyl spacer group was used for the same purpose. However, the former method introduces short distance between biotin and the 5'-phosphate of oligonucleotide. Furthermore, the reagent preparation itself involves multisteps synthesis and purification scheme, and also employs biotin methyl ester as an expensive starting material. The reagent reported by Cocuzza[9] is insoluble in acetonitrile, the most commonly used solvent in DNA synthesizer and also requires controlled deprotection conditions as the aromatic amide bond is labile under deprotection conditions (aq. ammonia treatment at 55°C).

A number of reagents have also been reported for the incorporation of multiple biotinyl residues in synthetic oligonucleotides for greater sensitivity in detection. In particular, 4-N-(6-N-biotinylaminohexyl)-2'-O-deoxycytidine or -5-methyl-2'-deoxycytidine-3'-O-phosphoramidite[13] to generate biotinylated nucleoside tails on 5'-end of synthetic oligonucleotides. However, these reagents introduce undesirable nucleosidic material to the oligomers and also involve tediousprotocols to synthesize them. Biotin reagents having non- nucleosidic linker have also been reported for the introduction of multiple biotinyl residues to synthetic oligonucleotides. In particular, a reagent 1-O-(4,4'-dimethoxytrityl)-3-O-(N-biotinyl-3-aminopropyl)-glyceryl-2-O-(N,N-diisopropylamino) (2-cyanoethyl)-phosphoramidite is now commercially available.

In order to overcome these limitations of earlier reagents[8,9], Pon has recently described an N^1-protected biotin reagent with 6-aminohexyl linker arm at carboxyl terminal. The use of DMT group for N^1-protection imparts good solubility to the reagent and long linker arm was believed to help in easy accessibility of biotin to large proteins used in detection system. However, the multisteps (five steps) synthesis and purification have been the main limitations of this protocol. These limitations have been taken care of by the use of a phase transfer catalyst (PTC) in a recent publication[15] from this laboratory. However, the use of DMT (acid labile group) for N^1- protection requires an additional deprotection step for its removal from HPLC purified sequence and the synthesis protocol is still very much time consuming. In order to eliminate these problems,

we have further simplified and developed a rapid and general method for the synthesis of biotinphosphoramidite reagents having base labile N^1-protection. The reagents were successfully used for the synthesis of a number of biotinylated oligonucleotides in automated DNA synthesizer.

RESULTS AND DISCUSSION

In the present communication we have tried to develop a general and rapid protocol for the synthesis of biotin reagents, useful for solid phase biotinylation of oligonucleotides. While designing the strategy for the synthesis of biotin reagents, we were guided by two important considerations. The first is that the reagent should have a lipophilic N^1-protecting group and like DMT it should not require an additional deprotection step from HPLC purified oligonucleotides. The second consideration is that the synthesis protocol should involve minimum protection/deprotection and purification steps, inexpensive starting material and the reagent should be suitable for use in automated DNA synthesizer in an analogous manner to normal nucleoside- phosphoramidites and directly lead to biotinylated oligonucleotides upon final deprotection.

In order to satisfy these criteria, we have made use of a well known oxidation-reduction condensation[16] in coupling aminoalkanol (propyl or pentyl in the proposed study) to the carboxyl terminal of biotin using triphenylphosphine-carbontetrachloride (TPP/CCl_4) as a condensing reagent in just 30 min. The substitution at carboxyl terminal of biotin provided enough lipophilicity (solubility) and enables it to undergo further modification (N^1-acylation) in pyridine (Fig. 1). Biotinyl-n-aminoalkanol **2(a,b)** were precipitated by ether and directly subjected to N^1-modification using an appropriate acylating reagent to obtain 1-N-acyl-biotinylaminoalkanol **3a(i-iv)** and **3b(i-iv)** in 2h at room temperature in 80-85 % yield after column chromatography. These were subsequently converted to the corresponding phosphoramidites **4a(i-iv)** and **4b(i-iv)**.

The reagents **4a(i-iv)** and **4b(i-iv)** were found to possess good solubility in acetonitrile, the most commonly used solvent in DNA synthesizer. To demonstrate the utility of these reagents in solid phase biotinylation of oligonucleotides, a number of oligonucleotides were assembled on Pharmacia Gene Assembler Plus at 0.2 μmol scale following manufacturer's recommendations.[17] The last coupling was performed with one of these reagents (using 0.2M solution in acetonitrile) in an analogous manner the normal nucleoside-phosphoramidites are used except the extended coupling time (300 sec).

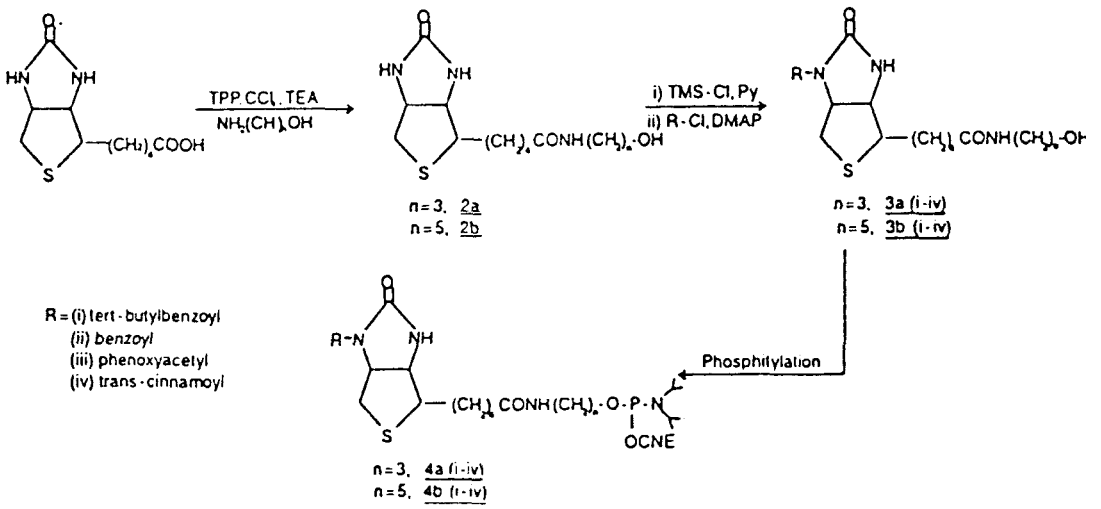

Fig. 1. Synthesis of biotinphosphoramidite reagents

Deprotection of biotinylated oligonucleotides was carried out with aq. ammonia (29%) at 55°C for 16h (usual conditions for the deprotection of oligomers) to remove various protecting groups (2–cyanoethyl from phosphates, acyl groups from nucleic bases and N^1 position of biotin). After usual workup and desalting (Sephadex G 25), the biotinylated oligomers were purified on reverse phase HPLC using C-18 column. The desired material with highest retention time was collected and after concentration, analyzed on C-18 column. Figures 2A and 2B show the HPLC profiles of crude biotinylated oligomers d(CTC TCT CT) and d(ATC TTC ATT G). It is clear from the elution pattern that the target biotinylated oligomers are quite stable under deprotection conditions, in full agreement with the deprotection conditions used by Alves et al.[8], Pon[10] and Kumar et al.[15] Figures 3A and 3B show the elution pattern of the biotinylated oligomers d (CTC TCT CT) and d (ATC TTC ATT G) on co-injecting with the corresponding non-biotinylated oligomers. The purified biotinylated oligomers were further characterized by dot-blot analysis and results are shown in Fig. 4.

EXPERIMENTAL

Thin layer chromatography (TLC) was performed on aluminium plates coated with silica gel $60F_{254}$ (Merck, Germany). Silica gel (120-200 mesh) was

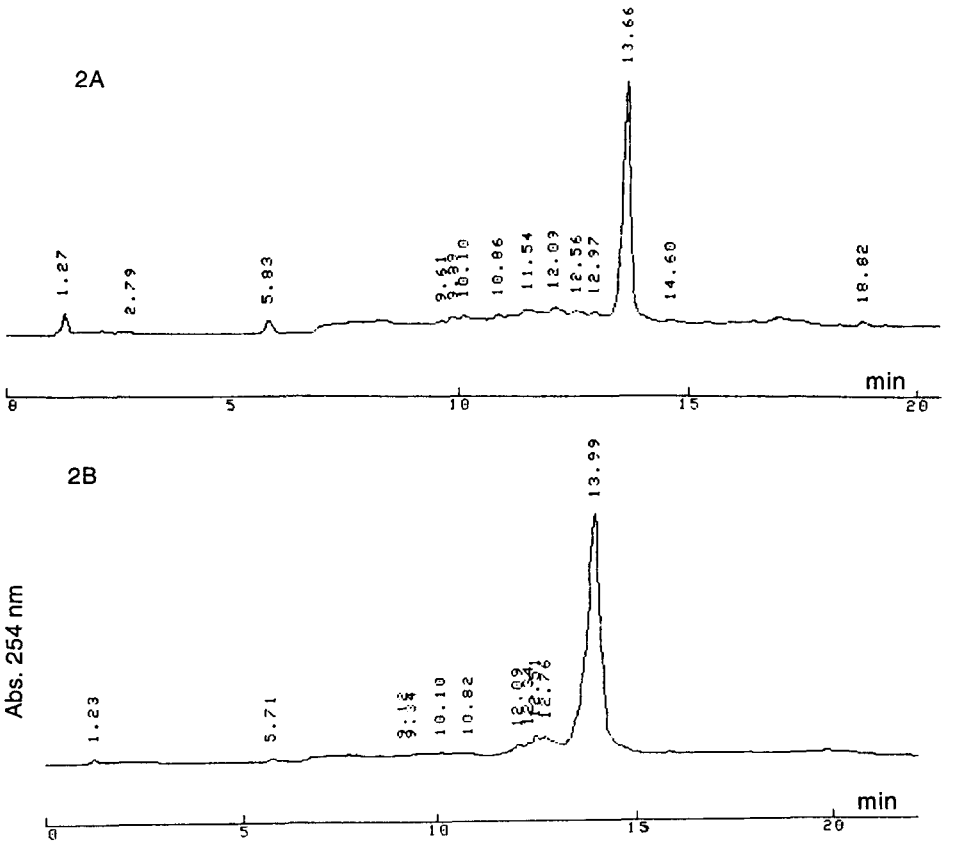

Fig. 2. Reverse phase HPLC profiles of crude (A) biotinylated d(CTC TCT CT) and (B) biotinylated d(ATC TTC ATT G). For HPLC conditions, see ref. 18.

used for column chromatography wherever required. All the solvents required for synthesis were procured from local suppliers and purified prior to use. Detection of biotinylated compounds on TLC was carried out by 4-dimethylaminocinnamaldehyde spray (Sigma, US). Nuclear magnetic resonance (NMR) spectra were recorded on 400 MHz NMR spectrometer (Bruker FT-400) using TMS as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and signals are quoted as s, singlet; d, doublet; t, triplet; m, multiplet; br, broad and Ar, aromatic. Jeol JMS D 300 mass spectrometer was used for obtaining mass spectra. Oligonucleotide synthesis was performed on Pharmacia LKB. Gene Assembler Plus. High performance liquid chromatography

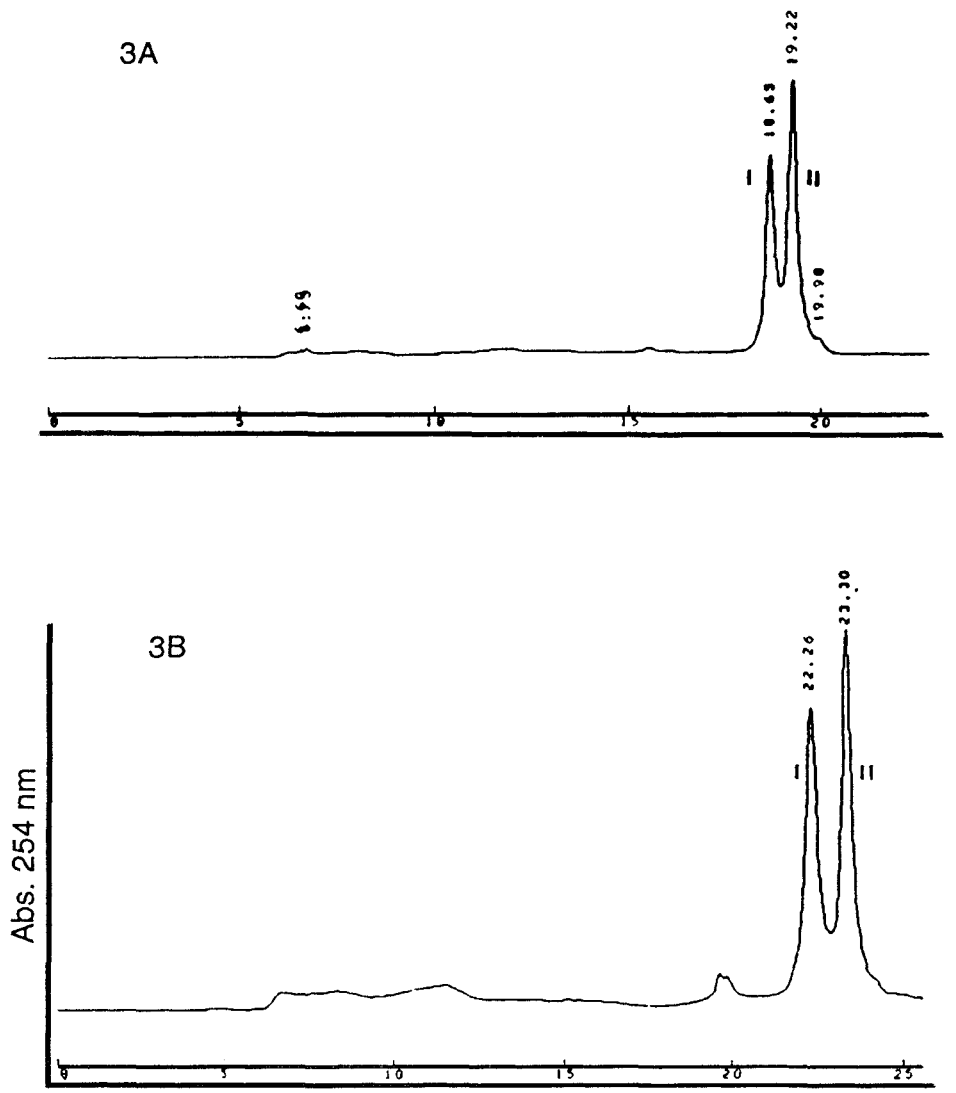

Fig. 3. Reverse phase HPLC profiles of (A) biotinylated d(CTC TCT CT) (peak II) coinjected with d(CTC TCT CT) (peak I) and (B) biotinylated d(ATC TTC ATT G) (peak II) coinjected with d(ATC TTC ATT G) (peak I). For HPLC conditions, see ref. 19.

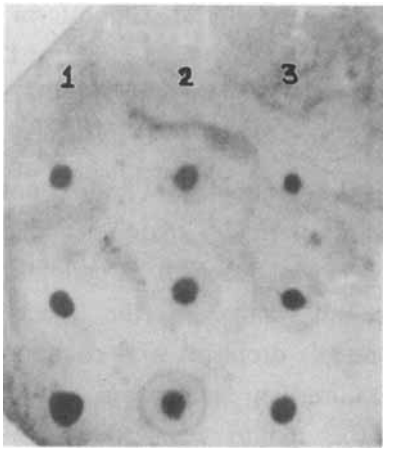

Fig. 4. Dot blot analysis of biotinylated d(ATC TTC ATT G), lane 1; d(CTC TCT CT), lane 2; standard DNA, lane 3.

(HPLC) was performed on a Shimadzu LC-4A fitted with a variable wavelength detector Shimadzu SPD-2AS (set at 254 nm). Lichrosphere RP C-18 column (Merck, Germany) was used for analysis and purification purposes.

Synthesis of biotinyl-n-aminoalkanol 2(a,b). To a suspension of biotin **1** (20 mmol) in dimethylformamide (80 ml) were added triethylamine (20 mmol) and triphenylphosphine (30 mmol). To the stirring mixture, carbontetrachloride (30 mmol) were added dropwise followed by addition of n-aminoalkanol (n = 3, 5) (30 mmol). The resulting mixture was vigorously stirred at room temperature. After completion of reaction (apprx. 30 min), water (0.5 ml) was added and the reaction mixture concentrated on a rotary evaporator to a semi-solid mass. It was dissolved in a minimum amount of methanol (15 ml) and the crude material was precipitated by addition of diethylether (200 ml). The solid was filtered and the process was repeated thrice in similar way to get the title compounds **2 (a,b)** in 85-90 % yield and were characterized by NMR and mass spectroscopy.

Biotinyl-3-aminopropanol (2a). Rf = 0.27 (Chloroform : Methanol :: 8 : 2). MS, m/z : 301 (M^+). ^1H NMR (DMSO)δ: 1.3-1.86 (m, 8H, 4 x -CH_2-), 2.2 (m, 2H, -CH_2CO-), 2.78 (m, 2H, -CH_2S-), 3.13 (m, 1H, -CHS-), 3.22 (m, 2H, -NCH_2-), 3.5 (t, 2H, -CH_2O-), 4.2 (m, 2H, 2 x -CHN-), 6.35 (m, 2H, 2 x -NH-).

Biotinyl-5-aminopentanol (2b). Rf = 0.28 (Chloroform : Methanol :: 8 : 2). MS, m/z : 329 (M^+). ^1H NMR (DMSO)δ: 1.28-1.78 (m, 12H, 6 x -CH_2-), 2.24 (m, 2H, -CH_2CO-), 2.72 (m, 2H, -CH_2S-), 3.15 (m, 1H, -CHS-), 3.25 (m, 2H, -NCH_2-), 3.53 (m, 2H, -CH_2O-), 4.33 (m, 2H, 2 x -CHN-), 6.39 (m, 2H, 2 x -NH-).

Synthesis of 1-N-acylbiotinyl-n-aminoalkanol 3a(i-iv) and 3b(i- iv). The compound **2(a,b)** (2 mmol) was dissolved in dry pyridine and co-evaporated twice on a rotary evaporator. Finally, it was dissolved in pyridine (15 ml) and the solution was chilled in an ice-salt bath. To the reaction mixture was added trimethylchlorosilane (4 mmol) dropwise with continuous stirring. After 30 min stirring at room temperature, an appropriate acyl chloride (2.5 mmol) and 4-dimethylaminopyridine (0.5 mmol) were added under external cooling. The reaction mixture was again allowed to stir at room temperature for additional 2h. The progress of the reaction was monitored on tlc. After completion of the reaction (approx. 2h), saturated aq. sodium hydrogen carbonate (1 ml) was added at 0°C with continuous stirring. The resulting mixture was concentrated under reduced pressure and then taken up in dichloromethane (50 ml). The organic phase was washed with saturated sodium bicarbonate (3 x 20 ml) and water (2 x 15 ml), respectively. It was then dried over anhydrous sodium sulfate and concentrated to syrupy mass. The title compounds **3a(i-iv)** and **3b(i-iv)** were purified by silica gel chromatography and fractions containing the pure compound were pooled together and concentrated in vacuo. The title compounds were obtained in 80-85 % yield and analyzed by NMR and MS.

1-N-tert-butylbenzoylbiotinyl-3-aminopropanol 3a(i). Rf = 0.25 (Chloroform : Methanol :: 9 : 1). MS, m/z : 449 (M^+-1). ^1H NMR ($CDCl_3$)δ: 1.16-1.8 (m, 17H, 4 x -CH_2-, 3 x -CH_3), 2.31 (m, 2H, -CH_2CO-), 3.02 (m, 2H, -CH_2S-), 3.2 (m, 1H, -CHS-), 3.31 (m, 2H, -NCH_2-), 3.45 (m, 2H, -CH_2O-), 4.23 (m, 1H, -CHN-), 5.22 (br, 1H, -CHN-), 6.59 (d, 1H, -NH-), 7.2-7.6 (m, 4H, Ar-H).

1-N-benzoylbiotinyl-3-aminopropanol 3a(ii). Rf = 0.23 (Chloroform : Methanol :: 9 : 1). MS, m/z : 406 (M^+). ^1H NMR ($CDCl_3$)δ: 1.23-1.92 (m, 8H, 4 x -CH_2-), 2.14 (m, 2H, -CH_2CO-), 2.67 (m, 2H, -CH_2S-), 3.03 (m, 1H, -CHS-), 3.24 (m, 2H, -NCH_2-), 3.5 (m, 2H, -CH_2O-), 4.17 (br, 1H, -CHN-), 5.15 (br, 1H, -CHN-), 7.3-7.5 (m, 5H, Ar-H).

1-N-phenoxyacetylbiotinyl-3-aminopropanol 3a(iii). Rf = 0.25 (Chloroform : Methanol :: 9 : 1). MS, m/z : 436 (M^+-1). ^1H NMR ($CDCl_3$)δ: 1.22-1.85 (m, 8H, 4 x -CH_2-), 2.18 (m, 2H, -CH_2CO-), 2.95 (m, 2H, -CH_2S-), 3.15 (m, 1H,

-CHS-), 3.3 (m, 2H, -NCH_2-), 3.52-3.7 (m, 4H, 2 x -CH_2O-), 4.24 (m, 1H, -CHN-), 5.21 (m, 1H, -CHN-), 6.8-7.4 (m, 5H, Ar-H).

1-N-trans-cinnamoylbiotinyl-3-aminopropanol 3a(iv). Rf = 0.27 (Chloroform : Methanol :: 9 : 1). MS, m/z : 432 (M^+). ^1H NMR ($CDCl_3$)δ: 1.2-1.95 (m, 8H, 4 x -CH_2-), 2.35 (m, 2H, -CH_2CO-), 2.8 (m, 2H, -CH_2S-), 3.08 (m, 1H, -CHS-), 3.25 (m, 2H, -NCH_2-), 3.52 (t, 2H, -CH_2O-), 4.23 (m, 1H, -CHN-), 5.21 (m, 1H, -CHN-), 6.6 (d, 1H, =CHCO-), 7.3-7.6 (m, 5H, Ar-H), 7.75 (d, 1H, PhCH=).

1-N-tert-butylbenzoylbiotinyl-5-aminopentanol 3b(i). Rf = 0.27 (Chloroform : Methanol :: 9 : 1). MS, m/z : 477 (M^+-1). ^1H NMR ($CDCl_3$)δ: 1.2-1.75 (m, 21H, 6 x -CH_2-, 3 x -CH_3), 2.42 (t, 2H, -CH_2CO-), 3.05 (m, 2H, -CH_2S-), 3.22 (m, 1H, -CHS-), 3.44 (m, 2H, -NCH_2-), 3.58 (m, 2H, -CH_2O-), 4.24 (m, 1H, -CHN-), 5.23 (br, 1H, -CHN-), 7.3-7.65 (m, 4H, Ar-H).

1-N-benzoylbiotinyl-5-aminopentanol 3b(ii). Rf = 0.24 (Chloroform : Methanol :: 9 : 1). MS, m/z : 433 (M^+-1). ^1H NMR ($CDCl_3$)δ: 1.25-1.87 (m, 12H, 6 x -CH_2-), 2.18 (t, 2H, -CH_2CO-), 2.6 (m, 2H, -CH_2S-), 3.15 (m, 1H, -CHS-), 3.28 (m, 2H, -NCH_2-), 3.45 (m, 2H, -CH_2O-), 5.2 (br, 1H, -CHN-), 7.35-7.5 (m, 5H, Ar-H).

1-N-phenoxyacetylbiotinyl-5-aminopentanol 3b(iii). Rf = 0.26 (Chloroform : Methanol :: 9 : 1). MS, m/z : 464 (M^+-1). ^1H NMR ($CDCl_3$)δ: 1.15-1.7 (m, 12H, 6 x -CH_2-), 2.21 (m, 2H, -CH_2CO-), 2.9 (m, 2H, -CH_2S-), 3.14 (m, 1H, -CHS-), 3.2 (m, 2H, -NCH_2-), 3.36-3.7 (m, 4H, 2 x -CH_2O-), 4.25 (m, 1H, -CHN-), 5.23 (m, 1H, -CHN-), 6.71-7.35 (m, 5H, Ar-H).

1-N-trans-cinnamoylbiotinyl-5-aminopentanol 3b(iv). Rf = 0.30 (Chloroform : Methanol :: 9 : 1). MS, m/z : 459 (M^+-1). ^1H NMR ($CDCl_3$)δ: 1.25-1.8 (m, 12H, 6 x -CH_2-), 2.22 (m, 2H, -CH_2CO-), 2.75 (m, 2H, -CH_2S-), 3.1 (m, 1H, -CHS-), 3.25 (m, 2H, -NCH_2-), 3.65 (m, 2H, -CH_2O-), 4.23 (m, 1H, -CHN-), 5.05 (m, 1H, -CHN-), 6.42 (d, 1H, =CH-CO-), 7.2-7.7 (m, 5H, Ar-H), 7.8 (d, 1H, Ph- CH=).

Synthesis of (1-N-acylbiotinyl-n-aminoalkyl)-2-cyanoethyl-N,N- diisopropylamino-phosphoramidite 4a(i-iv) and 4b(i-iv). Compound **3a(i-iv)** or **3b(i-iv)** (1 mmol) was dissolved in dry dichloroethane (20 ml) and added diisopropylethylamine (2 mmol). The resulting solution was cooled in an ice-bath and 2-cyanoethyldiisopropylchlorophosphoramidite (1.2 mmol) was injected dropwise with continuous stirring. After stirring at room temperature for 40 min, the completion of reaction was monitored on t.l.c. and quenched by addition of anhydrous

methanol (200 µl). The reaction mixture was diluted with dichloroethane (25 ml) and washed with 10% aq. sodium bicarbonate (2 x 20 ml) and saturated sodium chloride solution (2 x 20 ml). The organic phase was dried over anhydrous sodium sulfate, filtered and concentrated. The resulting biotinphosphoramidite was purified on silica gel column using dichloromethane : ethyl acetate : triethylamine (4.5 : 4.5 : 0.4) as an eluent. The pure fractions were collected and concentrated under reduced pressure to obtain the desired compounds **4a(i-iv)** and **4b(i-iv)** in 85-90% yield.

Oligonucleotide Assembly

A number of oligonucleotide sequences were assembled using a Pharmacia-LKB Gene Assembler Plus following manufacturer's recommendations with the 2-cyanoethyl phosphoramidite chemistry. The synthesis was performed at 0.2 µmol scale. For coupling with biotinphosphoramidite reagents **4a(i-iv)** and **4b(i-iv)**, 0.2M solution in absolute acetonitrile was used with extended coupling time (300 s).

Deprotection and purification of biotinylated oligonucleotides

The biotinylated oligonucleotides were subjected to concentrated ammonia treatment (29%) at 55 ^{o}C for 16h. This one step treatment cleaved the biotinylated oligonucleotides from the support and also removed the protecting groups (2-cyanoethyl from phosphates, acyl groups from nucleic bases and ureido ring of biotin). The ammoniacal solution was concentrated and desalted on Sephadex G25 column using 0.1M triethylammonium acetate as an eluent. The desalted sequences were subjected to HPLC purification on C-18 column and the desired fractions were collected, concentrated and analyzed on C-18 column.

ACKNOWLEDGEMENT

The financial support from the Department of Biotechnology, Govt. of India, New Delhi, is gratefully acknowledged. We are thankful to Dr. S.B. Katti, CDRI, Lucknow and Dr. Rakesh M. Vohra, IMT, Chandigarh for their help in obtaining NMR and MS spectra. We would also like to thank Dr. P.K. Swain for carrying out dot-blot analysis.

REFERENCES

1. Beaucage, S.L., Iyer, R.P., Tetrahedron, 1993, 49, 1625.
2. Agrawal, S., Protocols for Oligonucleotide Conjugates, Synthesis and

Analytical Techniques; Agrawal, S., Ed.; Humana Press: Totowa, NJ, 1994; vol 21.

3. Sinha, N.D., Striepeke, S., Oligonucleotides and Analogues, A Practical Approach; Eckstein, F., Ed; IRL Press: Oxford, 1991; pp. 185.
4. Kessler, C., Non-isotopic DNA Probe Techniques; Kricka, L.J., Ed.; Academic Press: San Diego, California, 1992; pp. 29.
5. Wilchek, M., Bayer, E.A., Methods in Enzymol., 1990, 184, 5.
6. Agrawal, S., Christodoulou, C., Gait, M.J., Nucl. Acids Res., 1986, 14, 6227.
7. Connolly, B.A., Nucl. Acids Res., 1987, 15, 3131.
8. Alves, A.M., Holland, D., Edge, M.D., Tet. Lett., 1989, 30, 3089.
9. Cocuzza, A.J., Tet. Lett., 1989, 30, 6287.
10. Pon, R.T., Tet. Lett., 1991, 32, 1715
11. Pieles, U., Sproat, B.S., Lamm, G.M., Nucl. Acids Res., 1990, 18, 4355.
12. Roget, A., Bazin, H., Teoule, R., Nucl. Acids Res., 1989, 17, 7643.
13. Nelson, P.S., Shermon-Gold, R., Leon, R., Nucl. Acids Res., 1989, 17, 7179.
14. Misiura, K., Durrant, I., Evans, M.R., Gait, M.J., Nucl. Acids Res., 1990, 18, 4345.
15. Kumar, P., Bhatia, D., Garg, B.S., Gupta, K.C., BioMed. Chem. Lett., 1994, 4, 1761.
16. Mukaiyama, T., Angew. Chem. Int. Ed. Engl., 1976, 15, 94.
17. Gene Assembler Plus Manual, Uppsala, Sweden.
18. HPLC conditions : column, Lichrosphere , gradient, 0-15% solvent B in 20 min at a flow rate of 1.0 ml/min. Solvent A = 0.1M ammonium acetate, pH 7.2, solvent B = acetonitrile.
19. HPLC conditions : column, Lichrosphere , gradient, 0-15% solvent B in 30 min at a flow rate of 1.0 ml/min. Solvent A = 0.1M ammonium acetate, pH 7.2, solvent B = acetonitrile.

Received December 5, 1995
Accepted February 16, 1996