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AN IMPROVED METHOD FOR SYNTHESIS OF BIOTIN PHOSPHORAMIDITES FOR SOLID PHASE BIOTINYLATION OF OLIGONUCLEOTIDES

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Abstract: Biotin (potassium salt) **2** was selectively and quantitatively N^1-tritylated to **3** in just 2 h in the presence of PEG-dimethyl ether. **3** was converted to its benzotriazolide active ester in 2 h at room temperature, which in the subsequent reaction in situ with aminoalkanol resulted in **4(a,b)**. The compounds **4(a,b)** were converted to the corresponding phosphoramidite reagents **5(a,b)** and were used for solid phase 5'-biotinylation of oligonucleotides in automated DNA synthesizer.

Non-radiolabelled oligonucleotides are used for several applications in modern biology. The use of biotin as a non-radiolabel reporter group for nucleic acids and proteins is now well established[1-8]. The detection of biotinylated molecules relies on the tight binding[9,10] of biotin to avidin and streptavidin. Though enzymatic methods are known for preparing biotinylated DNA, site specific biotinylation of oligonucleotides can only be achieved by chemical synthesis. The chemical labeling of oligonucleotides is generally achieved[11,12] by a two step procedure involving the synthesis of oligonucleotides modified with a suitable functional group ($-NH_2$ / -SH) followed by the reaction of suitable biotin derivative in aqueous solution.

Recently, single step solid phase biotinylation of oligonucleotides has been reported by several groups. In 1989, Alves et al.[13] and Cocuzza[14] reported independently two biotin phosphoramidite reagents for this purpose. The former method introduces short distance between biotin and the 5'-phosphate of the oligonucleotide and the reagent preparation involves multistep synthesis and purification scheme and also employs biotin methyl ester as a starting material, an expensive reagent. The main limitation with the latter one is that the reagent is insoluble in acetonitrile, the solvent most commonly used in automated DNA synthesizers. Moreover, the use of this reagent requires controlled deprotection conditions due to the lability of aromatic amide bond. In order to enhance the sensitivity in detection, a number of methods have been proposed for incorporating multiple biotinyl residues in synthetic oligonucleotides. Notably, the reagent 2'- deoxycytidine-3'-O-phosphoramidite carrying a duly protected biotin has been reported[15,16] for this purpose. However, the synthesis of this type of reagents

is not convenient. Moreover, the presence of nucleosidic component in these reagents limits their use. Though biotin reagents having non-nucleosidic component have also been reported[17,18], these suffer from the same problems as pointed out above for single biotin addition reagents.

Pon[19] described a new phosphoramidite reagent for solid phase biotinylation of oligonucleotides. This reagent takes care of all the limitations of the earlier two methods (single biotin adding reagents). However, the synthesis of his reagent involves multisteps (five steps) followed by an equal number of purification steps, which results in the loss of material and makes the synthesis scheme cumbersome and time consuming.

We wish to describe here a simple route to synthesize biotin phosphoramidite reagents for solid phase biotinylation of oligonucleotides. The synthesis protocol has been designed in such a way that it could retain all the advantages, viz., N^1- protection with DMTr group and variable alkyl chain arms etc. as offered by earlier described reagents[13,19] and involves minimum protection/deprotection strategy and purification steps. The protocol should also obviate the need to use biotin N-hydroxysuccinimide/p-nitrophenyl or biotin methyl ester, expensive reagents, as the starting material and coupling reagents like 1-(3-dimethylaminopropyl)- ethylcarbodiimide (DEC). In order to achieve this we have made use of polyethyleneglycol-dimethylether (PEG-DME) to solubilize biotin while carrying out N^1-tritylation of biotin.

The synthesis of biotin phosphoramidite reagents **5(a,b)** was carried out as shown in Fig.1. The synthesis of the reagents involves the conversion of biotin **1** to its potassium salt **2** by the reaction of

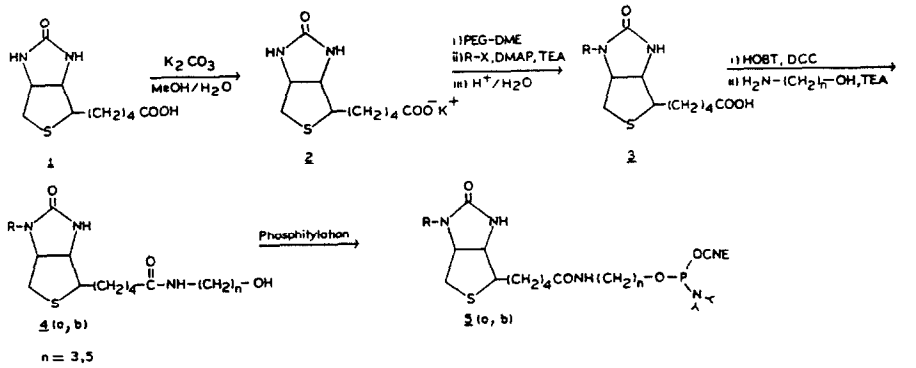

Fig. 1 Preparation of biotinylation reagent. R-X = 4,4'-dimethoxytrityl chloride, PEG-DME = polyethylene glycol dimethyl ether, DMAP = 4-dimethylaminopyridine, TEA = triethylamine, DCC = dicyclohexylcarbodiimide and HOBT = 1-hydroxybenzotriazole.

potassium carbonate, which was solubilized in pyridine by the addition of polyethyleneglycol-dimethylether (PEG-DME) and tritylated with 4,4'-dimethoxytrityl chloride to yield N^1-tritylated compound **3** in just 2 h in almost quantitative yields. Tritylation occurred selectively at the N^1-position, in full agreement with the results reported by Alves et al.[13] and Pon[19]. PEG-DME

protection at carboxyl group in 3 was removed during aq. work-up using 10% citric acid. The carboxyl group in compound 3 was then activated by N-hydroxybenzotriazole and dicyclohexylcarbodiimide (DCC) and reacted with an appropriate aminoalkanol in situ to obtain compounds **4(a,b)** in almost quantitative yield in just 2 h. Finally, the compounds **4(a,b)** were phosphitylated using 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite to yield the desired biotin phosphoramidite reagents **5(a,b)**.

The compounds **5(a,b)** have good solubility in acetonitrile and 0.1M solutions of these reagents were used in solid phase biotinylation of oligonucleotides in Pharmacia LKB Gene Assembler Plus. In order to test the utility of these reagents a number of 5'-biotinylated oligonucleotides were synthesized. In a typical experiment two oligonucleotides, viz., d(ATC TTC ATT G) and d(TCC ATC GAA A) were synthesized on 0.2 μmol scale using normal phosphoramidites. The last cycle was performed with biotin reagents with the "Tr-on" synthesis option in the similar way the normal nucleoside phosphoramidites are used except that the extended coupling time (600 sec).

The deprotection of biotinylated oligonucleotides was carried out by treatment with aq. ammonia (29%) at 55°C for 16 h (normal deprotection conditions for oligos). This one step process allows the removal of classical protecting groups (2- cyanoethyl from phosphates and acyl from nucleic bases). The N^1-DMTr-biotinylated oligonucleotides were purified on reverse-phase HPLC. The DMTr

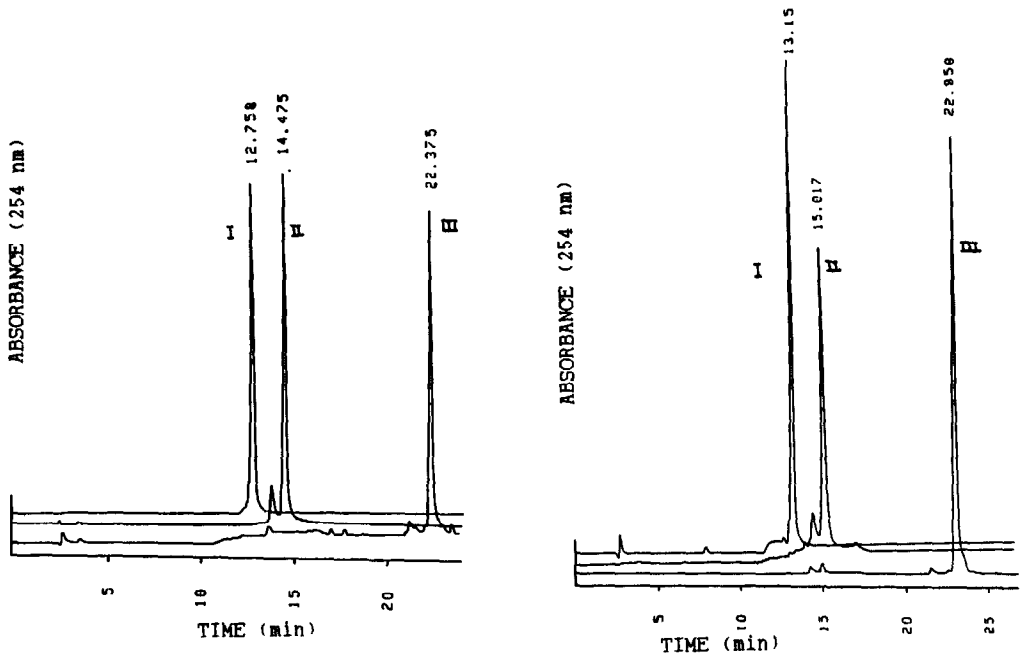

Fig.2 Reverse phase HPLC profiles of (a) d(ATC TTC ATT G) peak I, d(5'-biotin-NH-$(CH_2)_3$-PO_4-ATC TTC ATT G) peak II and d(5'-(N^1-DMTr)biotin-NH-$(CH_2)_3$-PO_4-ATC TTC ATT G) peak III; (b) d(TCC ATC GAA A) peak I, d(5'-biotin-NH-$(CH_2)_5$-PO_4-TCC ATC GAA A) peak II and d(5'-(N^1-DMTr)biotin-NH-$(CH_2)_5$-PO_4-TCC ATC GAA A) peak III. For conditions, see ref. 21.

group was removed following standard protocol[13,19] and the purity of the biotinylated oligonucleotides were again checked on HPLC (Fig. 2). The DMTr group from N^1-DMTr-biotinylated oligonucleotides can be removed in the machine, if desired, using extended detritylation step. The presence of biotin moiety in HPLC purified oligonucleotides was further confirmed by enzymatic assay using ELISA[20].

EXPERIMENTAL

Synthesis of 1-N-(4,4'-dimethoxytrityl)biotin 3

Biotin 1 (10 mmol) was reacted with potassium carbonate (5 mmol) in methanol : water (2:1). The resulting solution was concentrated to dryness, redissolved in dry pyridine (50 ml) and PEG-DME (10 mmol) was added. The suspension was stirred for 30 min prior to dryness with pyridine (3x). The mixture was finally taken in dry pyridine (10 ml), reacted with 4,4'-dimethoxytrityl chloride (30 mmol) in the presence of 4-dimethylaminopyridine (DMAP) (2.5 mmol) and triethylamine (10 mmol) at 70°C for 2 h. After stirring for 2 h, the reaction was found to be complete (tlc analysis). The reaction mixture was concentrated by evaporation, diluted with chloroform and subjected to extraction with 10 % cold citric acid solution and water, respectively. The organic phase was separated, dried over anhydrous sodium sulfate and concentrated to get oily mass in almost quantitative yield. The purity of the compound was checked on tlc and characterized by nmr, ir and mass spectrometry.

R_f = 0.17 (Chloroform : Methanol, 9 : 1)

^1H NMR ($CDCl_3$) δ = 1.25 (m, 6H, 3 x -CH_2-), 2.27 (m, 2H, -CH_2CO-), 3.0 (m, 1H, -CHS-), 3.25 (m, 2H, -CH_2S-), 3.8 (s, 6H, 2 x -OCH_3), 4.35 (m, 2H, 2 x -CHN-) and 6.82-7.3 (m, 13H, Ar-H).

IR, (cm^{-1}) = 3150, 2850, 1685, 1595, 1495, 1430, 1235, 1170, 1015, 740 and 690

MS (+FAB), m/z 546 (M^+-1)

Synthesis of 1-N-(4,4'-dimethoxytrityl)biotinyl-n-aminoalkan-1-ol 4(a,b)

To a solution of dicyclohexylcarbodiimide (4.5 mmol) in dry tetrahydrofuran, 1-hydroxybenzotriazole (4.5 mmol) was added and stirred vigorously at room temperature for 15 min, then 3 (3 mmol) was added and the suspension stirred at room temperature for 2 h under anhydrous conditions. After 2 h, n-aminoalkanol (4.5 mmol) and triethylamine (4.5 mmol) were added simultaneously and the reaction mixture was stirred at room temperature for 2 h and then cooled to 0°C. The solid material which separated out was filtered off, the filtrate was concentrated by evaporation, the residue was taken in chloroform and subjected to extraction with 10 % cold citric acid solution and water, respectively. The organic phase was concentrated after drying over anhydrous sodium sulfate to obtain the title compounds in 90% yields after silica gel column chromatography. The compounds were characterized by their nmr and mass spectrometry.

(i) 1-N-(4,4'-Dimethoxytrityl)biotinyl-3-aminopropan-1-ol 4(a)

R_f = 0.33 (Chloroform : Methanol, 9 : 1)

^1H NMR ($CDCl_3$) δ = 1.5-1.7 (14H, m, 7 x -CH_2-), 2.29 (2H, t, -CH_2CO-), 2.9-3.3 (5H, m, -CHS-,

-CH_2S-, -CH_2O-), 3.6-3.8 (8H, m, -NCH_2-, -OCH_3), 4.3 (2H, m, -CHN-), 4.9 (1H, m, NH) and 6.8-7.2 (13H, m, Ar-H).

MS (+FAB), m/z 603 (M^+-1)

(ii) **1-N-(4,4'-Dimethoxytrityl)biotinyl-5-aminopentan-1-ol 4(b)**

R_f = 0.35 (Chloroform : Methanol, 9 : 1)

^1H NMR ($CDCl_3$) δ = 1.5-1.7 (18H, m, 9 x -CH_2-), 2.29 (2H, t, -CH_2CO-), 3.0-3.3 (5H, m, -CHS-, -CH_2S-, -CH_2O-), 3.6 (2H, t, -NCH_2-), 3.78 (6H, s, -OCH_3), 4.3 (2H, m, -CHN-), 4.9 (1H, m, NH) and 6.8-7.4 (13H, m, Ar-H).

MS (+FAB), m/z 631 (M^+-1)

Synthesis of [1-N(4,4'-dimethoxytrityl)biotinyl-n-aminoalkyl)]-2-cyanoethyl-N, N-diisopropylamino phosphoramidite 5(a,b)

To compound **4(a,b)** (2 mmol) dissolved in dry dichloroethane (15 ml) was added N-diisopropylethylamine (8 mmol). The reaction mixture was cooled in an ice bath and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (3 mmol) was added dropwise througha glass syringe over a period of 5 min with vigorous stirring. The reaction was allowed to stir at room temperature for 1 h and the progress of the reaction was checked on tlc. After completion of the reaction, dry methanol (0.5 ml) was added. The reaction mixture was diluted with dichloroethane (50 ml) and extracted twice with 10 % sodium carbonate solution followed by saturated sodium chloride. The organic phase was dried over anhydrous sodium sulfate. The sodium sulfate was filtered off and the organic phase concentrated on a rotary evaporator to yield a syrupy product, which was purified on a silica gel column using dichloroethane : ethyl acetate : triethylamine (45 : 45 : 10) as an eluent. The fractions containing the desired material were pooled together and concentrated to obtain the title compound **5(a,b)** in over 85% yield.

Further work is under progress to prepare a biotin reagent following the same chemistry for multiple biotinylation of oligonucleotides.

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21. HPLC conditions : column, Delta Pak C_4 300 Å (3.9 x 150 mm); gradient, 0-100% solvent B in 50 minutes at a flow rate of 0.5 ml/min. Solvent A = 0.1M Ammonium acetate, pH 7.2, solvent B = Acetonitrile. Auf 0.08.

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