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A NEW ACHIRAL REAGENT FOR THE INCORPORATION OF MULTIPLE AMINO GROUPS INTO OLIGONUCLEOTIDES

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Abstract. The synthesis of a new functionalized achiral linker reagent (**10**) for the incorporation of multiple primary amino groups into oligonucleotides is described. The linker reagent is compatible with conventional DNA-synthesis following the phosphoramidite methodology, and the linker can be incorporated in good yield.

Specific labelling of DNA-oligomers is of major importance in the construction of DNA-probes.[1] The hazardous nature associated with the traditional radio-isotopic labelling has prompted the search for alternative non-radioactive labelling methods. Particular attention is paid to photo-detectable fluorophores,[2] or other reporter molecules such as biotin.[3] Therefore, effort has been focused on routes to synthesise oligonucleotides, furnished with amino or thiol groups, which can function as handles for incorporation of the desired reporter molecule.[4]

Several methods have been developed for the synthesis of oligonucleotides containing primary aliphatic amino groups. The variety of methods currently available ranges from enzymatic or chemical post modification of fully deprotected oligonucleotides,[5] to the use of reagents compatible with the phosphoramidite based solid phase oligomerization technique.[6]

The use of phosphoramidite reagents probably offers the easiest route to amino modified oligonucleotides, as no operation is needed apart from the standard synthesis protocol. Simple amino alkanols of the general form NH_2-$(CH_2)_n$-OH, after protection of the primary amine and conversion of the hydroxyl group into the phosphoramidite group, have been widely used in this respect.[7] However, having only one hydroxyl group, chain elongation is not possible, and these linkers therefore can only be used for the incorporation of a single 5'-terminal amino group.

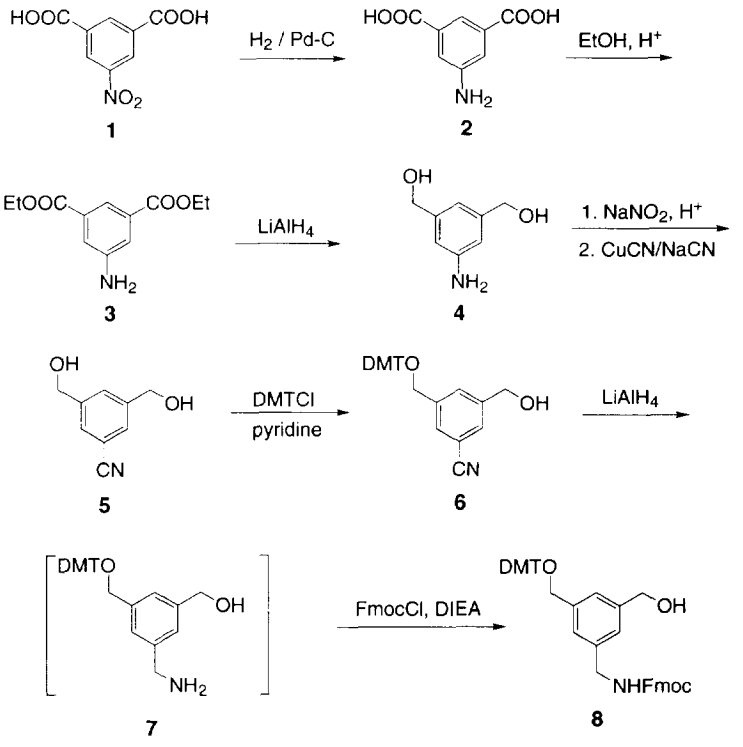
Recently, P. Nelson *et al.* [8] have reported the synthesis of 2-(4-aminobutyl)-1,3-propandiol, which in few steps can be converted into a linker compatible with the phosphoramidite method. Incorporation of amino groups in both the 3'- and 5'-end of the oligonucleotide has been demonstrated.

However, the reagent is produced as a racemic mixture of the 2*R* and 2*S*-form, thereby leading to 2^n diastereomers of the modified oligonucleotide upon incorporation of the reagent n times. Since purification and characterisation of such a mixture of diasteriomers can be difficult, there is a need for an achiral linker which allow multiple incorporation.

In this communication we wish to report our results on the synthesis of a simple achiral linker, compatible with standard phosphoramidite oligonucleotide synthesis.

The synthesis of the key intermediate **8** is shown in **Scheme 1**. Commercially available 5-nitroisophthalic acid was reduced to the corresponding anilino derivate **2**, using catalytic hydrogenation. Conventional esterification with ethanol gave the diester **3**, which upon reduction with $LiAlH_4$ gave 3,5-bis(hydroxymethyl)aniline (**4**) in 65-70 % yield from **1**.

Scheme 1



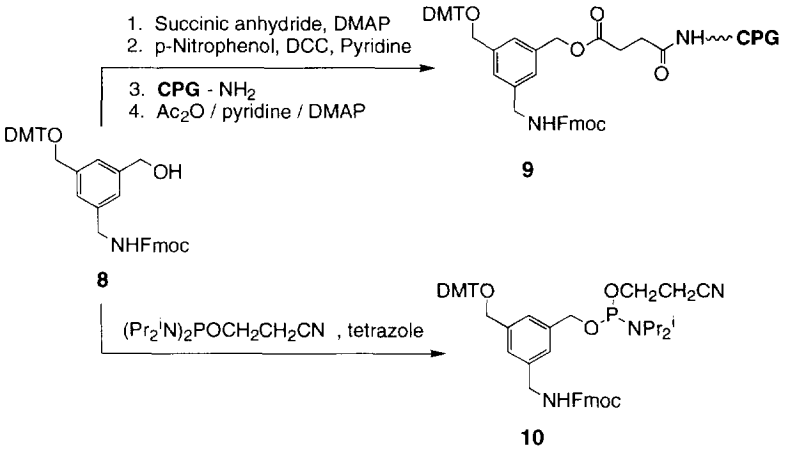
Diazotation of the anilino group and subsequent reaction with CuCN in aqueous solution furnished the nitrile **5** in 60-65 % yield.[9]

Attempts to reduce the cyano group in **5** to the aminomethyl group using various metal hydrides and solvent systems failed - probably due to the initial formation of insoluble metal alkoxides. Catalytic hydrogenation over either Raney Nickel or palladium on charcoal resulted in selective hydrogenolysis of one of the two hydroxymethyl groups.[10] Reduction was finally achieved with $LiAlH_4$ after prior protection of one of the two hydroxymethyl groups with a DMT group. This group assured good solubility during the reduction, and the benzylic amine **7** was obtained and Fmoc protected to give **8** [11] in 68 % overall yield from **6**.

The key intermediate **8** was then subjected to the transformations shown in **Scheme 2**. Derivatisation of CPG followed a standard procedure:[12] **8** was treated with succinic anhydride, and the resulting acid converted to the p-nitrophenyl ester. The ester was reacted with LCA-CPG, and after capping with acetic anhydride, the modified CPG **9** was obtained.[13]

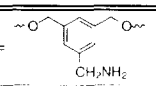
Treatment of **8** with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite and tetrazole,[14] followed by standard aqueous extraction and silica gel column chromatography afforded the stable phosphoramidite **10** as a fine powder in 66 % yield.[15]

Scheme 2



The reagent **9** and **10** were used to incorporate 5-(aminomethyl)-1,3-benzenedimethanol into various oligodeoxyribonucleotides (**Table**). Standard solid support phosphoramidite chemistry was used, apart from the oxidation step, where iodine/water was exchanged with *tert*-butyl hydroperoxide,[16] since the benzylic phosphate esters were not compatible with iodine.[17] The isolated yield of modified oligonucleotides were in the range 50-75 % after ethanol precipitation, calculated from optical density measurements.

Table

Sequence **X** = 	Reagent	Coupling efficiency **X**[a]	Coupling efficiency average[b]
5' - **X** - GTA GAT CAC T - 3'	10	95 %	97.5
5' - GTA GAT CAC T - **X** - 3'	9	--------	99.5
5' - **X** TGT ACG TCA CAA CTA **X** - 3'	9	--------	
	10	98 %	99.2
5' - **X** - CAT GAT CTG ACA GAG GGA ACC CAG T - 3'	10	90 %	99.2
5' - CAT GAT CTG ACA GAG GGA ACC CAG T - **X** - 3'	9	--------	99.3
5' - **XXX** - GTA GAT CAC T - 3'	10	95-99 %	98.8
5'- GTA GAT CAC T**XX** A GT GAT CTA C -3'	10	97-99 %	99.2

a) Coupling efficiency for reagent **10** (determined from the DMT-cation release).
b) Average coupling efficiency for the sequence indicated, including X.

The sequence 5' - **X** - GTA GAT CAC T - 3' was chosen for initial labelling studies. Labelling of this sequence with fluorescein isothiocyanate proceeded smoothly,[18] with completion within 2 hours according to HPLC. Multiple labelling of 5' - **XXX** - GTA GAT CAC T - 3' has also been realised, although after 16 h, the product was a mixture of mono-, bis-, and tris- fluorescein labelled oligomer.

In conclusion, a new achiral linker reagent (**10**) and a modified CPG-support (**9**), both for the incorporation of primary amino groups, has been synthesised. The linker can be incorporated into the oligonucleotide at any position using conventional phosphoramidite chemistry. Apart from the necessity of using *tert*-butyl hydroperoxide in the oxidation step, the linker is fully compatible with the standard synthesis protocol for automated DNA-synthesis, and has been used with success in repetitive coupling cycles. The linker can be incorporated in good overall yield, and provide access to oligo-nucleotides with easily labelled and highly reactive amino groups. Thus, besides being achiral, the linker offers a good alternative to the already existing reagents of this type.

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References and Notes:

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8. Nelson, P. S.; Kent, M.; Muthini, S. *Nucleic Acids Res.* **1992**, 20, 6253-6259.
9. The water soluble nitrile **5** was isolated by continuos extraction with dichloromethane.
10. This selective hydrogenolysis appear to be general for bis(hydroxymethyl) aromatic compounds. For experimental details, see Behrens, C.; Egholm, M.; Buchardt, O. *Synthesis* **1992**, 12, 1235.
11. Mp= 68-72°. ^{1}H-NMR (250 MHz, DMSO-d_6): δ 3.80 (s, 6H (MeO-)); 4.10 (s, 2H ($C\underline{H}_2$ODMT)); 4.27 (d,2H ($C\underline{H}_2$OH)); 4.36 (d, 2H (OC$\underline{H}_2$CH)); 4.39 (t, 1H (OCH$_2$C$\underline{H}$)); 4.56 (d, 2H ($C\underline{H}_2$NHFmoc)); 5.26 (t,1H (CH$_2$N$\underline{H}$Fmoc)); 6.96-7.97 (m, 24H (Aromatic)). ^{13}C-NMR (250 MHz, DMSO-d_6): δ 43.96; 46.79; 55.16; 63.00; 65.22; 65.62; 113.41; 120.24; 123.58; 124.27; 125.28; 126.87; 127.16; 127.73; 128.05; 129.80; 135.81; 158.25 (only the strong signals are reported).
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15. Ethyl acetate / dichloromethane /triethylamine (50:45:5) was used as eluent on a silica gel 60 - column packed in the same solvent. The foam obtained after column purification was dissolved in a minimum of ethyl acetate, and precipitated from hexane to give the analytical pure material. The phosphoramidite is stable for at least 2 years when stored dry at -18°C. ^{31}P-NMR (90 MHz, $CDCl_3$): δ 148.5 ppm. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.19 (t, 12H ($(C\underline{H}_3)_2CH)_2N$); 2.57 (t, 2H ($CH_2C\underline{H}_2CN$)); 3.63 (dt, 2H ($C\underline{H}_2CH_2CN$)); 3.78 (s, 6H (MeO-)); 3.82 (m, 2H ($(CH_3)_2C\underline{H})_2N$); 4.18 (s, 2H ($C\underline{H}_2$ODMT)); 4.22 (t, 1H ($OCH_2C\underline{H}$)); 4.39 (d, 2H ($C\underline{H}_2$NHFmoc)); 4.43 (d, 2H ($OC\underline{H}_2CH$)); 4.71 (ddd, 2H ($C\underline{H}_2$OP)); 5.15 (t, 1H ($CH_2N\underline{H}$Fmoc)); 6.82-7.75 (m, 24H (Aromatic)). ^{13}C-NMR (400 MHz, $CDCl_3$) δ 20.34; 20.41; 24.60; 24.63; 24.67; 24.70; 43.16; 43.28; 47.28; 55.20; 58.41; 58.60; 65.24; 65.41; 66.80; 113.13; 119.91; 124.81; 125.03; 125.27; 126.75; 127.01; 127.63; 127.81; 128.16; 130.05; 136.19; 140.02; 158.47 (only the strong signals are reported). Elemental analysis, found: C, 72.65; H, 6.45; N, 4.70. $C_{54}H_{58}N_3O_7P$ requires: C, 72.71; H, 6.55; N, 4.71.

16. Biosearch 8750 DNA-syntheziser, 0.2 or 1 μmol scale. Standard synthesis cycles and cyanoethyl phosphoramidites were used, but with *tert*-butyl hydroperoxide (1.1 M in acetone/CH_2Cl_2 1:1, 3 min) as the oxidation reagent. For experimental details see Marugg, J.E.; Nielsen, J.; Dahl, O.; Burik, A.; van der Marel, G.A.; van Boom, J.H.; *Recl. Trav. Chim. Pays-Bas.* **1987**, 106, 72-76.

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18. The oligonucleotide was dissolved in a 0.1 N aqueous Na_2HPO_4 - solution, and a DMF solution of fluorescein isothiocyanate (ca. 100 fold excess) containing triethyl amine was added. After 2 h the product was purified by size exclusion chromatography using a NAP-10 -column (Pharmacia).

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