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A NEW METHOD TO PREPARE 3'-MODIFIED OLIGONUCLEOTIDES

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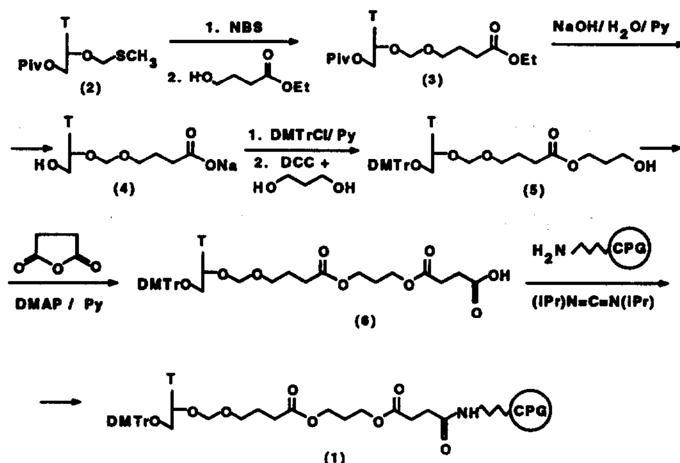
Abstract: A new method to prepare oligonucleotides bearing a carboxy- or aminoalkyl spacer arm at their 3'-terminus is described.

A wide range of chemical procedures have been developed to synthesise oligonucleotides bearing various functionalities at their 5'-terminus.[1] Synthesis of 3'-modified oligonucleotides is more laborious, because it involves preparation of a modified solid support. Only two methods have been reported[2,3] that enable synthesis of various 3′-functionalised oligonucleotides on a single polymer support. They include the preparation of oligonucleotide 3'-phosphates[2] or 3'-thiophosphates,[3] followed by post-synthetic acylation[2] or alkylation[3] of deprotected and isolated material. Some other methods enabling the preparation of 3'-tethered oligonucleotides are also available, but the length of the spacer arm and the nature of the functional group are strictly determined by the structure of the solid support employed.[4-7] Moreover, an elegant method for the preparation of oligonucleotides bearing several amino groups at their 3′-terminus has been established.[8] This approach is based on a machine assisted synthesis of oligopeptides. So far, no applicable solid support method to synthesise 3′-carboxyalkylated oligonucleotides is available. The only procedure described[4] is incompatible with standard procedures of base deprotection. We now report on a novel method to prepare 3'-amino- and 3′-carboxy-alkylated oligonucleotides using a single solid support (**1**, Scheme 1).

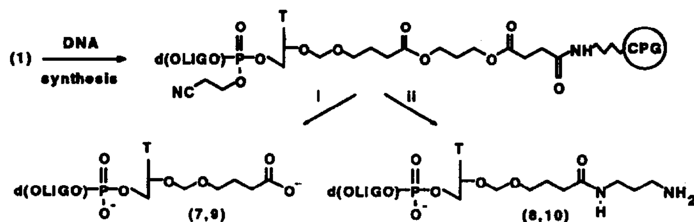
5'-*O*-Pivaloylthymidine[9] was first converted to 3'-*O*-methylthiomethyl-5'-*O*-pivaloylthymidine (**2**) as described previously for 5'-*O*-benzoylthymidine.[10] When **2** was treated with NBS in dry dichloroethane and then *in situ* with ethyl 4-hydroxybutyrate[11] in the presence of lutidine, **3** was formed in a high yield (87%). Deprotection with aqueous alkali and neutralisation with Dowex-50 resin (pyridinium form) gave **4**[12] almost quantitatively. Dimethoxytritylation of 5′-hydroxyl group of **4** followed by esterification with 1,3-propanediol in the presence of DCC gave **5**. The yield after purification on a silica gel column was 60 %.

Treatment of **5** with succinic anhydride[12] yielded the appropriate succinate (**6**),[13] which was immobilised on a long chain alkylamine CPG polymer support with N,N'-diisopropylcarbodiimide. Capping of the remaining amino groups by acetic anhydride / pyridine / N-methylimidazole (1:5:1, v/v) yielded a derivatised support (**1**). Dimethoxytrityl cation assay[14] showed loading of the spacer group to range from 7 to 9 μmol per gram of the solid support.



Scheme 1. NBS - N-bromosuccinimide; DMTr - 4,4'-dimethoxytrityl; CPG - controlled pore glass.

In order to demonstrate the usefulness of the support (1) in preparation of 3'-modified oligonucleotides, the model sequences d(T)$_5$-3′-$OCH_2O(CH_2)_3COOH$ (7) and d(T)$_5$-3′-$OCH_2O(CH_2)_3CONH(CH_2)_3NH_2$ (8) and the target sequences d(GC$_2$GTG$_2$AGTCGTT) bearing the same spacer arms at their 3′-end (9, 10) were synthesised (Scheme 2).



Scheme 2. i: 0.1M NaOH, then NH_3-H_2O at 55 °C; ii: hydrazine/acetic acid/pyridine, then 1,3-diaminopropane : EtOH (1:1, v/v).

The oligonucleotides were assembled on a Applied Biosystems 392 DNA Synthesizer (0.04 μmol of **1**). Phosphoramidite chemistry and recommended protocols (DMTr-Off synthesis) were used in all cases. No differences in coupling efficiency (>98% as determined from trityl responses) were detected between the modified CPG supports and commercial nucleoside-derivatised columns. After the chain assembly was completed, the solid supports were treated with appropriate reagents. To obtain **7** and **9** the supports were treated with 0.1 M NaOH. This cleaves the phosphate protections and releases the carboxylic acid

group at the 3′-terminus. For **9**, the partially deprotected oligomer was further treated with aqueous ammonia to cleave all remaining base protecting groups. To get the 3'-aminoalkylated oligonucleotide **10**, the support was treated with the mixture of hydrazine/AcOH/pyridine which deblocks A and C[15] (to avoid transamination of C in the next step), and with 1,3-diaminopropane to cleave the rest of the protecting groups and to introduce the desired amino function at 3′-end. For **8**, the treatment with 1,3-diaminopropane alone was enough to get the desired product. The deprotected material was analysed by ion exchange HPLC (Fig. 1). Compound **9** exhibited a longer and **10** a shorter retention time (t_R) than the corresponding unmodified sequence (Fig. 1, right). This in all likelihood results from the negative charge of the free carboxylate ion of **9**, which increases t_R approximately as an additional nucleotide. The amino group of **10** is, in turn, protonated in the eluant buffer (pH = 5.6), and this increases the mobility. The same effect is also seen on comparing the HPLC profiles of the modified pentamers **7** and **8** (Fig. 1, left). The oligonucleotides prepared were purified on a reversed phase column[16] and finally desalted by gel filtration.[17] The presence of the 3′-amino function was additionally verified by allowing **8** to react with phenyl isothiocyanate in a carbonate buffer.[18] The HPLC analysis[19] showed the disappearance of **8** and the appearance of a product with a longer t_R. The reaction was quantitative, and demonstrated the presence of the primary amino group at the 3′-terminus of **8**. Enzymatic digestion of derivatised oligomers gave the correct ratio of the expected products.

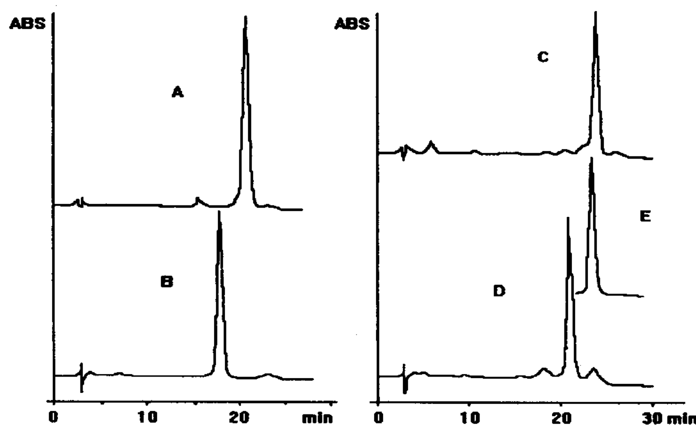


Fig. 1. Ion exchange HPLC profiles[21] of the crude oligonucleotides: left - **7** (A), **8** (B); right - **9** (C), **10** (D), and corresponding natural oligonucleotide (E).

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12. **3**: ^{1}H NMR (D_2O): 7.47 (1H, s, H-6); 6.10 (1H, dd, H-1′); 4.67 (2H, s, OCH_2O, partially overlapping with HDO signal); 4.27 (1H, m, H-3′); 3.97 (1H, m, H-4′); 3.68 (1H, dd, H-5′); 3.60 (1H, dd, H-5′′); 3.51 (2H, t, OCH_2); 2.33 (1H, ddd, H-2′′); 2.25 (3H, m, H-2′and t, CH_2COOH); 1.73 (5H, s, 5-CH_3 and p,$CH_2CH_2CH_2$).
13. **6**: purified on preparative TLC plates (CH_2Cl_2:MeOH:TEA, 89:8:3, v/v) as triethylammonium salt in 92 % yield. ^{1}H NMR ($CDCl_3$): 8.22 (1H, br, H-3); 7.67 (1H, s, H-6); 7.40 - 6.84 (13H, DMTr); 6.27 (1H, dd, H-1′); 4.69 (2H, s, OCH_2O); 4.37 (1H, m, H-3′); 4.16 (5H, m, H-4′and 2 x CH_2OCO); 3.76 (6H, s, 2 x OCH_3); 3.65 (1H, m, H-5′); 3.46 (2H, m,OCH_2); 3.31 (1H, m, H-5′′); 2.61 (4H, m, 2 x CH_2); 2.40 (1H, m, H-2′′); 2.25 (1H, m, H-2′) 1.97 (2H, p, CH_2); 1.90 (2H, p, CH_2); 1.43 (3H, s, 5-CH_3). Spectrum exhibited also signals of triethylammonium group (q 3.07 and t 1.33).
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16. RP-18 column (Nucleosil 300-5C18, 4.0 x 250 mm, Macherey-Nagel), flow rate 1.0 mL min^{-1}, λ = 270 nm A = 0.1 M NH_4OAc, B = A + 50 % MeCN (v/v); from 10 to 50 % B in 30 min.
17. TSKgel G2000SW (7.5 x 300 mm, Toso Haas) flow rate 1.0 mL min^{-1} of water.
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19. As in ref. 16, but from A to 50 % B in 30 min. t_R (**8**) 23.7 min; t_R (product) 28.4 min.
20. Applied Biosystems, *User Bulletin*, **1987**, *13*, p. A-4,
21. Ion exchange column (Synchropak AX-300, 6.5 μm, 4.6x250 mm), flow rate 1.0 mL min^{-1}, λ = 270 nm, A = 0.03 M KH_2PO_4, 50 % formamide, B = 0.03 M KH_2PO_4, 0.6 M $(NH_4)_2SO_4$, 50 % formamide. Left: from A to 20 % B in 25 min; Right: from 10 to 80 % B in 30 min.

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