SYNTHESIS OF 3'-FUNCTIONALIZED OLIGONUCLEOTIDES ON A SINGLE SOLID SUPPORT

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Abstract: Oligodeoxyribonucleotides tethered with an amino-, carboxy-, amidocarbonyl-, or mercaptoalkyl spacer arm at 3'-terminus were obtained by assembling the chains on a modified aminoalkyl-CPG (1) and releasing them with appropriate reagents.

Oligonucleotides that bear a functional group tethered to their 3'-terminus are useful in preparation of labeled probes for diagnostic purposes. Furthermore, 3'-modification of oligonucleotides significantly increases their resistance to the action of nuclease and hence they have found usage as antisense inhibitors1 Synthesis of these compounds proceeds by preparation of a modified solid support. Some methods have been reported2-4 that enable synthesis of various 3'-functionalized oligonucleotides on a single polymer support. They either include the preparation of oligonucleotide 3'-phosphates2 or 3'-thiophosphates,3 followed by post-synthetic acylation2 or alkylation3 of the deprotected and isolated material, or they are based on a machine assisted synthesis of peptide - oligonucleotide conjugates that allow the introduction of several amino groups at the 3'-terminus of the DNA sequence.4 Other methods for the synthesis of 3'-tethered oligonucleotides are also available, but the structure of the solid support employed strictly determines the nature of the functional group and the length of the spacer arm.1,5-8 We have recently reported on preparation of a solid support containing a 3'-derivatized thymidine unit that enables a versatile functionalization of the 3'-terminus.9 We now describe the preparation of a more generally applicable solid support (1) and the synthesis of oligonucleotides bearing carboxy-, amino-, amidocarbonyl-, or mercaptoalkyl spacer arm at their 3'-end. The functional group is introduced (mercapto in the form of disulfide) during the deprotection and removal of the oligonucleotide from the solid support, without further chemical transformations or additional purification procedures.

4-(4,4'-dimethoxytrityloxy)butyric acid10 (2) was immobilized on the long chain alkylamine controlled pore glass with the mixture of N,N-diisopropylcarbodiimide and N-hydroxysuccinimide (Scheme 1). After capping of the unreacted amino groups with a mixture of Ac₂O/pyridine/N-methylimidazole (1:5:1, v/v) and detritylation, solid support, 3, was acylated with 2 by a mixed anhydride method11 by using N-methylimidazole as a catalyst. The unreacted hydroxyl functions were capped as described above. After standard work up,12 dimethoxytrityl cation assay showed a loading of 22 μmol of DMTr-groups per gram of 1.
The sequences d(GCCGTGGAGTCGTT) bearing various 3'-functionalities were synthesized on the solid support 1 (Scheme 2). The oligonucleotides were assembled on an Applied Biosystems 392 DNA Synthesizer (0.2 μmol of 1) by using phosphoramidite chemistry and the recommended protocols (DMTr-Off synthesis). No differences in coupling efficiency were detected between 1 and the commercial nucleoside derivatized columns.

Table 1. Deprotection procedures to obtain the 3'-modified oligonucleotides (4-10).

<table>
<thead>
<tr>
<th>Product</th>
<th>Deprotection procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>aq. NH₃ 5 h at 55 °C</td>
</tr>
<tr>
<td>5</td>
<td>(i) 0.1 M aq. NaOH for 4 h. (ii) aq. NH₃, 5 h at 55 °C</td>
</tr>
<tr>
<td>6</td>
<td>(i) hydrazine acetate® (ii) 1,2-diaminoethane in iPrOH (1/1, v/v), 8 h at r.t.</td>
</tr>
<tr>
<td>7</td>
<td>(i) hydrazine acetate® (ii) 1,3-diaminopropane in iPrOH (1/1, v/v), 8 h at r.t.</td>
</tr>
<tr>
<td>8</td>
<td>(i) hydrazine acetate® (ii) 1,4-diaminobutane in iPrOH (1/1, v/v), 30 h at r.t.</td>
</tr>
<tr>
<td>9</td>
<td>(i) hydrazine acetate® (ii) cystamine in pyridine (1/1, v/v), for 3 days at 55 °C</td>
</tr>
<tr>
<td>10</td>
<td>9 overnight in aq. 1,4-dithio-D,L-threitol</td>
</tr>
</tbody>
</table>

a. 0.5 M hydrazine hydrate in pyridine : acetic acid (4:1, v/v), 24 - 30 h at room temperature.¹³
After the chain assembly was completed, the solid supports were treated with appropriate reagents, as described in Table 1. To get the 3'-aminoalkylated oligonucleotides (6-9) it is essential to deprotect cytosine (and adenine) residues with hydrazinium acetate before treatment with appropriate α,ω-diaminoalkane or cystamine, because N₄-benzoyl protection of cytidine considerably enhances the rate of transamination. The oligonucleotides prepared were isolated by an ion exchange HPLC, purified on a RP column, and desalted by gel filtration. HPLC retention times are given in Table 2. Chromatographical profiles of 4, 5, 7, 9 and 10 are shown in Fig. 1 as illustrative examples.

Liberation of the 3'-mercaptoalkyl group to give 10 was achieved by reducing 9 with 1,4-dithio-D,L-threitol. The presence of the mercapto function was additionally verified by allowing 10 to react with 2,2'-dipyridyl disulphide to give quantitatively 11.

The oligonucleotides prepared were initially digested by phosphodiesterase II and then by alkaline phosphatase. The product distribution was analyzed by RP HPLC. Along with the nucleosides expected, the enzymatic digestions gave in each case an additional product, which coeluted with authentic samples of appropriately derivatized thymidine-3'-(3-carboxypropyl)phosphates, synthesized via an independent route.

Table 2. HPLC retention times of 4-11 relative to d(GCCGTGGAGTCGTT).

<table>
<thead>
<tr>
<th>Relative retention time (min)</th>
<th>natural⁴</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion exchange⁴</td>
<td>24.5</td>
<td>+0.8</td>
<td>+2.5</td>
<td>-0.9</td>
<td>-1.1</td>
<td>-1.2</td>
<td>-1.2</td>
<td>+0.6</td>
<td>--</td>
</tr>
<tr>
<td>Rev. phase⁵</td>
<td>16.4</td>
<td>-0.1</td>
<td>-0.4</td>
<td>0</td>
<td>0</td>
<td>-0.1</td>
<td>+0.5</td>
<td>+0.5</td>
<td>+3.9</td>
</tr>
</tbody>
</table>

a. Absolute retention time (min).

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REFERENCES AND NOTES


10. 4-hydroxybutyric acid (as sodium salt; 5.0 mmol, 0.63 g) was dissolved in 50 mL of dry pyridine. DMTTr-chloride (1.1 eq, 2.05 g) was added and the mixture was stirred at room temperature for 4 h. The reaction was quenched by addition of ice. The mixture was concentrated and dissolved in EtOAc. After aqueous work up, the organic extract was concentrated, dissolved in methanolic water and passed through Dowex-50 resin (pyridinium form). Concentration in vacuo yielded 2.13 g (4.5 mmol, 90 %) of 2 as a pale yellow oil. $^1$H NMR (CDCl$_3$) 7.35 - 6.72 (13H DMTTr), 3.67 (6H, s, 2 x OCH$_3$); 2.99 (1H, t, OCH$_2$); 2.26 (2H t, CH$_2$COO$^-$); 1.80 (p, 2H, CH$_2$). Spectrum exhibited also the signals of pyridinium salt. 2 contained some DMTTr-alcohol as impurity. It was used for the next reaction without further purification.

11. 2 (0.5 mmol, 0.24 g) was dissolved in 2.5 mL of dry pyridine. 2,4,6- Triisopropylbenzenesulfonyl chloride (0.45 mmol, 136 mg; predissolved in 125 mL of pyridine) and N-Me (0.45 mmol, 36 mL) were added and the mixture was transferred to predried 3 (0.5 g). The mixture was shaken overnight at room temperature. Work up was performed as described in ref. 12.


14. Synchropak AX-300, 6.5 µm, 4.6x250 mm, SynChrom; flow rate 1.0 mL min$^{-1}$, $\lambda$ = 260 nm, A = 0.05 M KH$_2$PO$_4$, 50 % formamide, B = A + 0.6 M (NH$_4$)$_2$SO$_4$, from 10 to 80 % B in 30 min.

15. Nucleosil 300 SC18, 4.0x250 mm, Macherey-Nagel; flow rate 1.0 mL min$^{-1}$, $\lambda$ = 260 nm, A = 0.05 M NH$_4$OAc, B = 0.05 M NH$_4$OAc in 50 % MeCN, from 0 to 70 % B in 30 min.

16. TSKgel G2000SW, 7.5 x 300 mm, Tosoh Haas; flow rate 1.0 mL min$^{-1}$ of water.


18. Hypersil C18, 5 µm, 4.0 x 250 mm, Shandon; isocratic elution of 0.05 M NH$_4$OAc containing 5 % MeCN, flow rate 1.0 mL min$^{-1}$, $\lambda$ = 260 nm.

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