

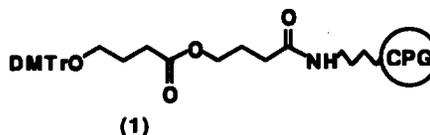
SYNTHESIS OF 3'-FUNCTIONALIZED OLIGONUCLEOTIDES ON A SINGLE SOLID SUPPORT

Jari Hovinen, Andrei Guzaev^{*}, Alex Azhayev, and Harri Lönnberg

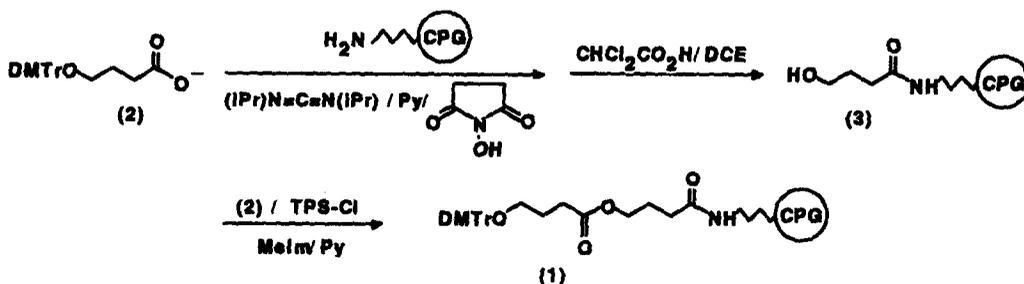
Department of Chemistry, University of Turku, FIN-20500 Turku, Finland

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 nucleases and hence they have found usage as antisense inhibitors.¹ 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 determinates 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.

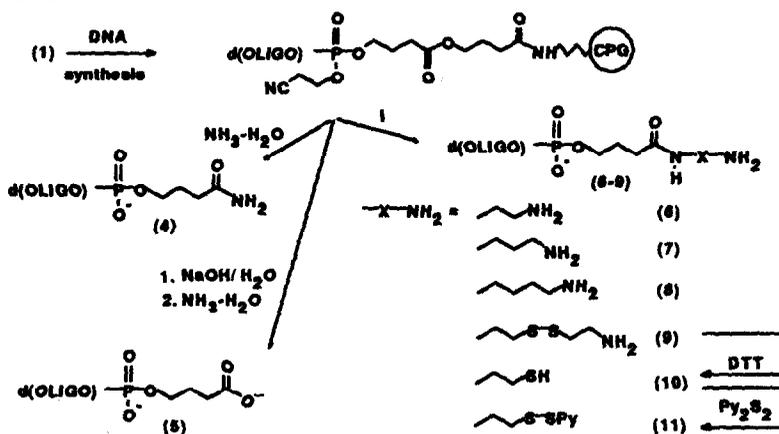


4-(4,4'-dimethoxytrityloxy)butyric acid¹⁰ (2) was immobilized on the long chain alkylamine controlled pore glass with the mixture of *N,N*-diisopropylcarbodiimide and *N*-hydroxysuccinimide (Scheme 1). After capping of the unreacted amino groups with a mixture of Ac_2O /pyridine/*N*-methylimidazole (1:5:1, 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.



Scheme 1. DMT = 4,4'-dimethoxytrityl; TPS = 2,4,6-trisopropylbenzenesulfonyl-; MeIm = N-methylimidazole.

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.



Scheme 2. DTT - 1,4-dithio-D,L-threitol; Py₂S₂ - 2,2'-dipyridyl disulfide; i: for the deprotection conditions see Table 1.

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

Product	Deprotection procedure
4	aq. NH ₃ 5 h at 55 °C
5	(i) 0.1 M aq. NaOH for 4 h. (ii) aq. NH ₃ , 5 h at 55 °C
6	(i) hydrazine acetate ^a (ii) 1,2-diaminoethane in <i>i</i> PrOH (1/1, v/v), 8 h at r.t.
7	(i) hydrazine acetate ^a (ii) 1,3-diaminopropane in <i>i</i> PrOH (1/1, v/v), 8 h at r.t.
8	(i) hydrazine acetate ^a (ii) 1,4-diaminobutane in <i>i</i> PrOH (1/1, v/v), 30 h at r.t.
9	(i) hydrazine acetate ^a (ii) cystamine in pyridine (1/1, v/v), for 3 days at 55 °C
10	9 overnight in aq. 1,4-dithio-D,L-threitol

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 N^4 -benzoyl protection of cytidine considerably enhances the rate of transamination.¹³ The oligonucleotides prepared were isolated by an ion exchange HPLC,¹⁴ purified on a RP column,¹⁵ and desalted by gel filtration.¹⁶ 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.

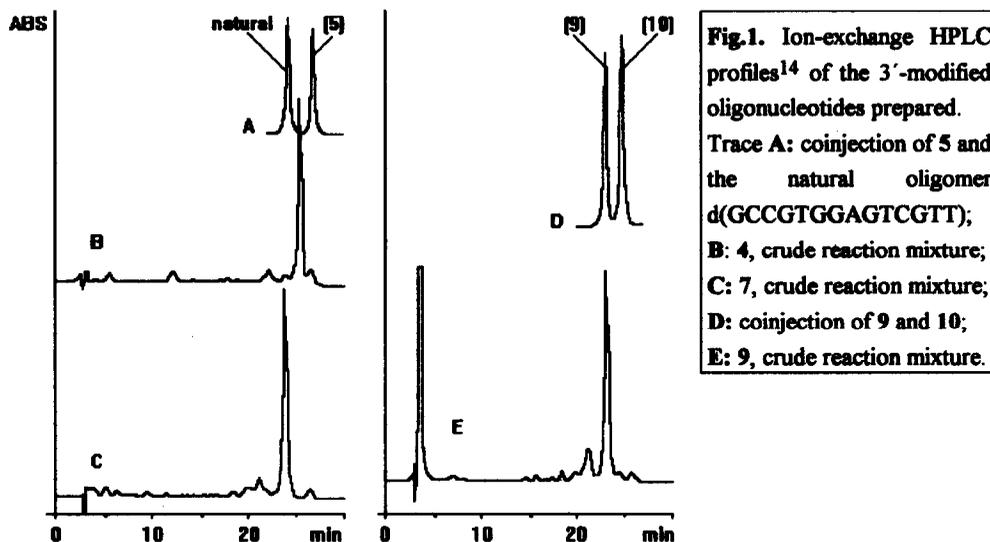


Fig.1. Ion-exchange HPLC profiles¹⁴ of the 3'-modified oligonucleotides prepared. Trace A: coinjection of 5 and the natural oligomer d(GCCGTGGAGTCGTT); B: 4, crude reaction mixture; C: 7, crude reaction mixture; D: coinjection of 9 and 10; E: 9, crude reaction mixture.

The oligonucleotides prepared were initially digested by phosphodiesterase II and then by alkaline phosphatase.¹⁷ The product distribution was analyzed by RP HPLC.¹⁸ 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).

	Relative retention time (min)								
	natural ^a	4	5	6	7	8	9	10	11
Ion exchange ¹⁴	24.5	+0.8	+2.5	-0.9	-1.1	-1.2	-1.2	+0.6	-
Rev. phase ¹⁵	16.4	-0.1	-0.4	0	0	-0.1	+0.5	+0.5	+3.9

a. Absolute retention time (min).

Acknowledgements. Financial support from the Research Council for Natural Sciences, the Academy of Finland, is gratefully acknowledged.

REFERENCES AND NOTES

- Gamber, H.B., Reed, M.W., Cox, T., Viroscio, J.S., Adams, A.D., Gall, A.A., Scholler, J.K., Meyer, Jr, R.B. *Nucleic Acids Res.*, **1993**, *21*, 145.
- Gottickh, M., Asseline, U., Thuong, N.T. *Tetrahedron Lett.*, **1990**, *31*, 6657.
- a) Asseline, U., Bonfils, E., Kurfürst, R., Chassignol, M., Roig, V., Thuong, N.T. *Tetrahedron*, **1992**, *48*, 1233; b) Thuong, N.T., Asseline, U.I., *Oligonucleotides and Analogues, A Practical Approach*; Eckstein, F. Ed.; IRL Press, Oxford, **1991**, 283.
- Haralambibis, J., Duncan, L., Angus, K., Tregear, G.W., *Nucleic Acids Res.*, **1990**, *18*, 493.
- Greenberg, M.M. *Tetrahedron Lett.*, **1993**, *34*, 251.
- For the procedures to introduce a 3'-aminoalkyl spacer, see: a) Nelson, P.S., Frye, R.A., Liu, E. *Nucleic Acids Res.*, **1989**, *17*, 7187; b) Asseline, U., Thuong, N.T. *Tetrahedron Lett.*, **1990**, *31*, 81; c) Nelson, P.S., Kent, M., Muthini, S. *Nucleic Acids Res.*, **1992**, *20*, 6253; d) Ref. 1; e) Gryaznov, S.M., Letsinger, R.L. *Tetrahedron Lett.*, **1993**, *34*, 1261; d) Nylas, A.; Agrawal, S.; Zamecnik, P. *Bioorg. Med. Chem. Lett.*, **1993**, *3*, 1371.
- For the procedures to introduce 3'-mercaptoalkyl spacer, see: a) Zuckermann, R., Corey, D., Schultz, P., *Nucleic Acids Res.*, **1987**, *15*, 5305; b) Gupta, K.C., Sharma, P., Sathayanarayana, S., Kumar, S. *Tetrahedron Lett.*, **1990**, *31*, 2471; c) Gupta, K.C., Sharma, P., Sathayanarayana, S., Kumar, S. *Nucleic Acids Res.*, **1991**, *19*, 3019; d) Guzaev, A.P., *Nucleic Acids Res. Symp. Ser.*, **1991**, *n24*, 236; e) Bonfils, E., Thuong, N.T. *Tetrahedron Lett.*, **1991**, *32*, 3053.
- For the procedures to introduce a 3'-hydroxyalkyl spacer, see: a) MacKellar, C., Graham, D., Will, D.W., Burgess, S., Brown, T. *Nucleic Acids Res.*, **1992**, *20*, 3411; b) Hinrichsen, R.D., Fraga, D., Reed, M.W. *Proc. Natl. Acad. Sci. USA*, **1992**, *89*, 8601; c) Jäschke, A., Furst, J.P., Cech, D., Erdman, V.A. *Tetrahedron Lett.*, **1993**, *34*, 301; d) Ref. 1.
- Hovinen, J., Guzaev, A.P., Azhayev, A. V., Lönnberg, H. *Tetrahedron Lett.* **1993**, *34*, 5163.
- 4-hydroxybutyric acid (as sodium salt; 5.0 mmol, 0.63 g) was dissolved in 50 mL of dry pyridine. DMTr-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. ¹H NMR (CDCl₃) 7.35 - 6.72 (13H, DMTr), 3.67 (6H, s, 2 x OCH₃); 2.99 (2H, t, OCH₃); 2.26 (2H, t, CH₂COO⁻); 1.80 (p, 2H, CH₂CH₂CH₂). Spectrum exhibited also the signals of pyridinium salt. **2** contained some DMTr-alcohol as impurity. It was used for the next reaction without further purification.
- 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 μL of pyridine) and *N*-methylimidazole (0.45 mmol, 36 μL) 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.
- Atkinson, T., Smith, M. in *Oligonucleotide Synthesis: A Practical Approach*; Gait, M.J. Ed; IRL Press: Oxford, **1984**, pp. 47-49.
- Miller, P.S., Cushman, C.D., Levis, J.T. *Oligonucleotides and Analogues: A Practical Approach*; Eckstein, F. Ed.; IRL Press: Oxford, **1991**, pp. 137 - 154.
- Synchropak AX-300, 6.5 μm, 4.6x250 mm, SynChrom; flow rate 1.0 mL min⁻¹, λ = 260 nm, A = 0.05 M KH₂PO₄, 50 % formamide, B = A + 0.6 M (NH₄)₂SO₄, from 10 to 80 % B in 30 min.
- Nucleosil 300 5C18, 4.0x250 mm, Macherey-Nagel; flow rate 1.0 mL min⁻¹, λ = 260 nm, A = 0.05 M NH₄OAc, B = 0.05 M NH₄OAc in 50 % MeCN, from 0 to 70 % B in 30 min.
- TSKgel G2000SW, 7.5 x 300 mm, Toso Haas; flow rate 1 mL min⁻¹ of water.
- USB Molecular Biology Reagents/Protocols, **1992**, pp. 86 - 87, and 131.
- Hypersil C18, 5 μm, 4.0 x 250 mm, Shandon; isocratic elution of 0.05 M NH₄OAc containing 5 % MeCN, flow rate 1.0 mL min⁻¹, λ = 260 nm.