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DEVELOPMENT OF AN EFFICIENT OLIGONUCLEOTIDE DERIVATIZATION PROTOCOL

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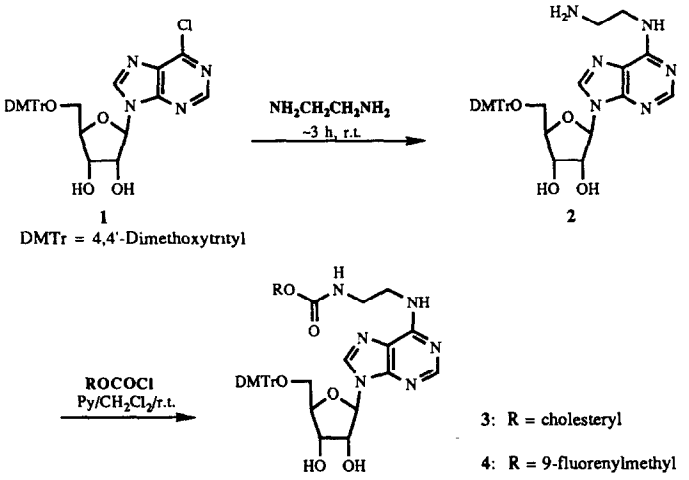
Abstract: An efficient procedure is presented for the introduction of a 6-*N*-(2-cholesteroloxycarboaminoethyl)- or 6-*N*-[2-(9-fluorenylmethoxycarboamino)ethyl] moieties into adenosine, which is compatible with solid phase incorporation into antisense oligodeoxyribonucleotides and has the further advantage of lack of diastereomer formation.

Despite all the advances in the synthesis of DNA analogues which have superior membrane permeability and cellular degradation characteristics compared to their native counterparts no single analogue has found universal acceptance as an antisense agent.[1,2] The modification of oligonucleotides to provide more potent antisense agents is pertinent to the successful therapeutic application of antisense DNA technology.[3] We herein describe a versatile method for the modification of oligonucleotides which is compatible with the high efficiency of solid phase synthesis. All modification steps occur prior to the solid phase synthesis of the oligonucleotide which still proceeds in very high yields. The method is demonstrated by the tethering of cholesterol to a 21 mer targeted against a serine protease implicated in the killer activity of T cells.[4,5] The lipophilicity of cholesterol transiently anchors the oligonucleotide to the cell membrane and may enhance the facility of cellular uptake and stabilize complexes formed with cellular nucleic acids.[6,7] The tethering procedure is compatible with the high pH necessary for removal of protecting groups.

The synthesis of 6-*N*-(2-cholesteroloxycarboaminoethyl)-5'-dimethoxytrityladenosine begins with commercially available 6-chloropurine riboside and is outlined in Scheme 1.



Scheme 1

6-Chloropurine 5'-dimethoxytritylriboside (1)

The riboside is tritylated at the 5'-position with 4,4'-dimethoxytrityl chloride in pyridine. To a solution of 6-chloropurine riboside (1.43 g, 5.0 mmol) in pyridine (25 mL), was added 4,4'-dimethoxytrityl chloride (2.04 g, 6.0 mmol) in portions over a 2 h period of time. The reaction mixture was stirred at room temperature overnight, and then concentrated to 5 mL *in vacuo*. The residue was diluted with chloroform (40 mL) and washed with 10% aqueous sodium bicarbonate solution (50 mL) and water (50 mL). The organic solution was dried over anhydrous sodium sulfate, filtered and concentrated to give an oil, which was subjected to flash chromatography on silica gel (2 × 30 cm column). Elution with methanol:chloroform (1:20) gave rise to a yellow foam (**1**) m.p. 98-100° (2.75 g, 4.67 mmol, 93% yield) which was characterized by spectroscopic and analytical methods.[8]

6-*N*-(2-Aminoethyl)-5'-(4,4'-dimethoxytrityl)adenosine (2)

Displacement of the chloro group then proceeds in neat ethylenediamine following a literature procedure for substitution of the 6-chloro position of adenosine with amines[9] at room temperature leaving a free primary amino group pendant to N6 of adenosine.

Compound **1** (203 mg, 0.345 mmol) was placed in 1,2-ethylenediamine (1.0 mL). The reaction mixture was stirred at room temperature for 3 h. When completed consumption of compound **1** was indicated by a TLC analysis, the reaction mixture was transferred to a separatory funnel containing ice-cold water (50 mL) and extracted with chloroform (2 × 20 mL). The extracts were combined, dried over anhydrous sodium sulfate, filtered, and concentrated to a yellow solid (**2**) m.p. 103-105° (170 mg, 0.278 mmol, 81% yield) which was characterized by spectroscopic and analytical methods.[10]

6-*N*-(2-Cholesteroloxycarboaminoethyl)-5'-dimethoxytrityladenosine (3)

Compound **2** was then dissolved in pyridine and the free amino group reacts with the chloroformate of cholesterol following a literature procedure for carbamate formation[6] in dichloromethane to give 6-*N*-(2-cholesteroloxycarboaminoethyl)-5'-dimethoxytrityladenosine in 84% yield.

To a solution of **2**[10] (90 mg, 0.147 mmol) in pyridine (1 mL), was added a solution of cholesteryl chloroformate (95% pure, 83 mg, 0.176 mmol) in dichloromethane (2 mL). After stirring at room temperature for 5 h, the reaction mixture was poured into a 10% aqueous sodium bicarbonate solution (20 mL) and extracted with dichloromethane (2 × 20 mL). The extracts were combined, dried over anhydrous sodium sulfate, filtered, and concentrated to a foam. The crude product was subjected to flash chromatography on silica gel. Elution with methanol:chloroform (1:20) gave rise to compound **3** (126 mg, 0.123 mmol, 84% yield), characterized by analytical and spectroscopic methods.[11]

The efficiency of this nucleotide derivatization procedure was further demonstrated by the preparation of 6-*N*-[2-(9-fluorenylmethoxycarbamino)ethyl]-5'-dimethoxytrityladenosine **4** in 87% yield from **2** under similar reaction conditions.[12]

6-*N*-[2-(9-Fluoroenylmethoxycarboamino)ethyl]-5'-dimethoxytrityladenosine (4)

To a solution of **2** (450 mg, 0.735 mmol) in pyridine (2 mL), was added a solution of 9-fluorenylmethyl chloroformate (229 mg, 0.882 mmol) in dichloromethane (2 mL). After stirring at room temperature for 11 h, the

reaction mixture was poured into a 10% aqueous sodium bicarbonate solution (30 mL) and extracted with dichloromethane (2 × 20 mL). The extracts were combined, dried over anhydrous sodium sulfate, filtered, and concentrated. The crude product obtained was purified by flash chromatography on silica gel. Elution with methanol:chloroform (1:20) gave a white solid (**4**) m.p. 108-110° (535 mg, 0.641 mmol, 87% yield), which was fully characterized by analytical and spectroscopic means.[11] All chemistry was done at room temperature and followed by standard aqueous workups. Intermediates and products were purified by flash chromatography on silica gel with methanol:chloroform mixtures and analyzed giving the proper ^{1}H-NMR, FAB-MS and IR spectra.

The free 2'- or 3'-hydroxyl groups of the ribose (**3**) were then covalently bound to controlled pore glass beads and used in solid phase DNA synthesis on an Applied Biosystems 390B DNA synthesizer.[13] There was no detectable loss in efficiency of the solid phase synthesis due to the attachment of the cholesterol group at N6.

A series of 18 mers with the modified adenosine added at the 3'-terminus were synthesized to target a serine protease implicated in mediating the killer activity of human T cells.[4,5] Preliminary studies indicate that these probes are effective in arresting killer activity at much lower concentrations than are their underivatized counterparts. Derivatized thiophosphates were also more efficient than underivatized thiophosphates. The efficiency of these probes is also being measured against cell proliferation by targeting the *c*-myc oncogene. Although cholesterol derivatized oligonucleotides have been reported previously[6,14,15] the approach outlined in this communication offers several advantages. The attached moiety has many degrees of freedom to rotate which may be advantageous to entering the cell and in stabilizing nucleic acid complexes in the cell. While other methodologies involve linking a primary amino group to a reactive reagent this is done after the solid phase synthesis and reduces the overall yield.[16] Further the aqueous solubility of oligonucleotides is often incompatible with the solubility of the moieties being attached. The presently described approach is free from all of these problems in addition to affording products free of diastereomer formation.

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8. For **1**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.71 (s, 1H, H2), 8.38 (s, 1H, H8), 7.4-7.1 (complex, 9H, ArH), 6.8-6.7 (d, 4H, ArH), 6.08 (d, 1H, H1'), 5.08 (m, 1H, H2'), 4.90 (m, 1H, H4'), 4.44 (dd, 1H, H3'),

4.49 (br s, 1H, OH), 3.76 (s, 6H, CH_3), 3.45 and 3.35 (dd, 2H, H5'), 3.20 (br s, 1H, OH); IR ($CHCl_3$) 3300 cm^{-1} (OH); MS M^+ 588.1770 (calcd. for $C_{31}H_{29}ClN_4O_6$: 588.1775).

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10. For **2**: ^{1}H-NMR (200 MHz, $CDCl_3$) δ 8.31 (s, 1H, H2), 8.03 (s, 1H, H8), 7.3-7.1 (complex, 9H, ArH), 6.8-6.7 (d, 4H, ArH), 6.36 (br s, 1H, NH), 5.92 (d, 1H, H1'), 4.78 (dd, 1H, H2'), 4.45 (m, 1H, H4'), 4.39 (dd, 1H, H3'), 3.75 (s, 6H, CH_3O), 3.8-3.7 (complex, 4H, NH_2 and $HNCH_2$), 3.41 and 3.22 (dd, 2H, H5'), 3.01 (t, 2H, CH_2NH_2), 2.36 (s, 1H, OH), 1.20 (s, 1H, OH); IR ($CHCl_3$) 3000-3400 cm^{-1} (OH and NH); MS (EI) m/z 310.1382 (M - DMTr + H)$^+$ (calcd. for $C_{12}H_{18}N_6O_4$: 310.1389); MS (FAB) M + Na^+, 634.99 (calcd. for $C_{33}H_{36}N_6O_6Na$: 635.25 and M+H^+, 613.00 (calcd. for $C_{33}H_{37}N_6O_6$: 613.28).
11. For **3**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.27 (s, 1H, H2), 7.95 (s, 1H, H8), 7.65 (br s, 1H, CONH), 7.4-7.1 (complex, ArH), 6.9-6.7 (d, 4H, ArH), 6.35 (br s, 1H, NH), 6.00 (d, 1H, H1'), 5.55 (br s, 1H, OH), 5.35 (br s, 1H, vinylic-H), 4.75 (t, 1H, H2'), 4.55-4.40 (complex, 3H, H3', H4' and cholesteryl 3-α-H), 3.75 (s, 6H, CH_3O-), 3.70 (m, 2H, NCH_2), 3.40 (m, 2H, $CONCH_2$), 3.35 and 3.20 (m, 2H, H5'), 2.5-0.6 (complex, 44H, including 1.0 (s), 0.95 (d), 0.85 (d) and 0.60 (s)); IR ($CHCl_3$) 3330 (OH) and 2950 cm^{-1} (C-H); MS (FAB) M + H^+ 1025.53 (calcd. for $C_{61}H_{81}N_6O_8$: 1025.61).
12. For **4**: ^{1}H-NMR δ 8.25 (s, 1H, H2), 7.90 (s, 1H, H8), 7.73 and 7.56 (d, 2H each, Form-ArH), 7.4-7.1 (complex, 13H, ArH), 6.75 (d, 4H, DMT-ArH), 6.65 (br s, 1H, NH), 6.33 (br s, 1H, NH), 6.02 (d, 1H, H1'), 4.73 (m, 1H, H2'), 4.40 (m, 1H, H4'), 4.35 (d, 2H, Form-CH_2), 4.25 (m, 1H, H3'), 4.17 (t, 1H, Form-CH), 3.70 (s, 6H, CH_3O), 3.70 and 3.45 (m, 2H each, CH_2N), 3.20 (m, 2H, H5'), 2.10 (br s, 1H, OH); IR ($CHCl_3$) 3315 (OH and NH), 1715 cm^{-1} (C=O); MS (FAB) M + Na^+ 857.02 (calcd. for $C_{48}H_{46}N_6NaO_8$: 857.32) and M + H^+ 835.12 (calcd. for $C_{48}H_{47}N_6O_8$: 835.34).
13. The procedures used for incorporation of the derivatized nucleoside into the 18 mer oligonucleotides followed our earlier published procedures in Gmeiner, W.H.; Pon, R.T.; Lown, J.W. *J Org Chem.* **1991**, *56*, 3602. Yields and efficiencies for the coupling reactions were comparable with those in that reference.
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