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Synthesis of Phospholipid-Oligodeoxyribonucleotide Conjugates

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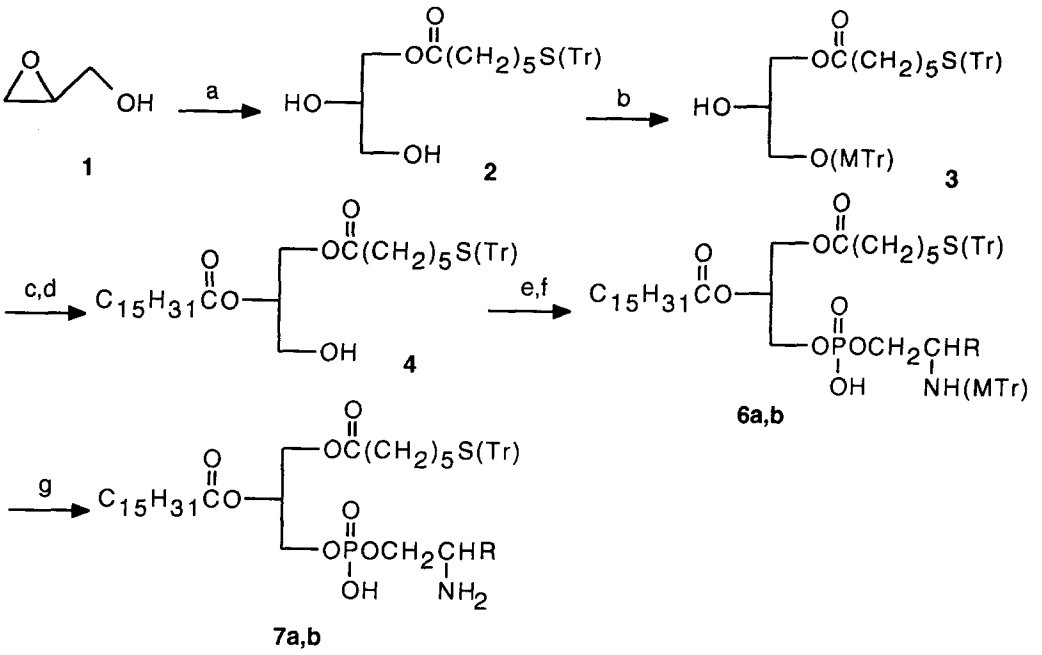
Abstract: Chemical synthesis of modified phospholipids and their attachment to oligodeoxyribonucleotides via dithiolinker is described.

Oligodeoxyribonucleotides bearing lipophilic groups such as cholesterol[1], fatty radicals[2] or phospholipids[3] have been shown more easily taken up by living cells and they are more active in blocking of viral replication in cell cultures as compared to non-derivatized oligonucleotides. These derivatives have been found to accumulate on the cell membrane and to penetrate into the cells via endosomal vesicles. In order to investigate another possibility of the transport of antisense oligomers into cell compartments such as a transport via a flip-flop mechanism, we proposed to couple oligonucleotides with phospholipids having near-natural structure. It is known for some natural phospholipids, e.g. phosphatidylethanolamine and phosphatidylserine, that their active transport from the outer to inner surfrace of the membrane bilayer is performed by a specialized enzyme called aminophospholipid translocase[4]. In this work, we describe the synthesis of modified phospholipids and their attachment to oligonucleotides via dithiolinker that could be cleaved in biological media.

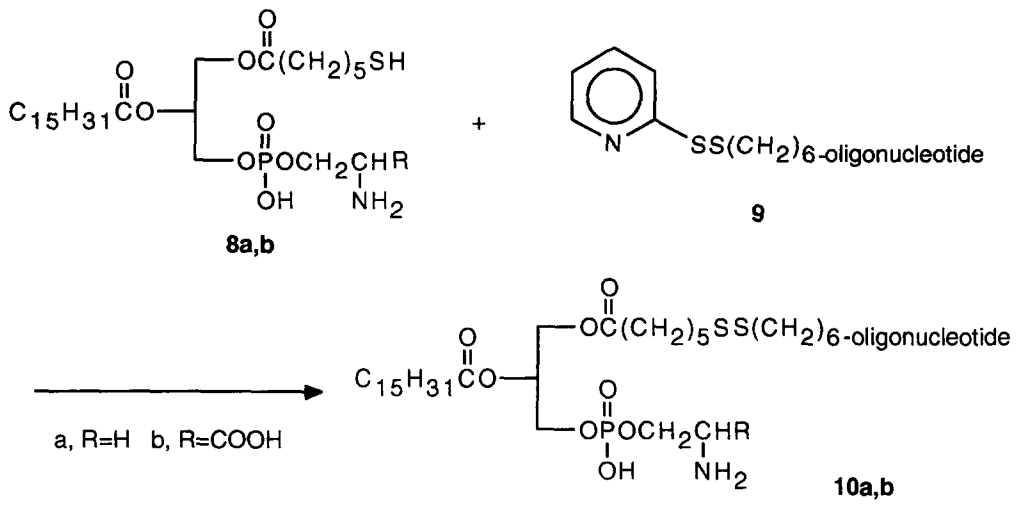
We started from (S)-(-)-glycidol which reacted with S-tritylthiocaproic acid **1**[5] in the presence of titanium(IV) isopropoxide[6]. The opening of epoxy-ring occurred smoothly in dichloromethane (4 h, 0°C) and 1-(S-tritylthiocaproyl)-sn-glycerol **2**[7] was obtained after purification on silicagel column (eluent: dichloromethane-pentane, 1:1) with a yield of 78%. In a second step we blocked the hydroxyl group with t-butyldimethylsilyl chloride in the presence of imidazole and acylated the remaining hydroxyl group with palmitoyl chloride in pyridine. However, the described procedure[6] where N-bromosuccinimide was used for deprotection of 1,2-O-diacyl-3-t-butyldimethylsilyl-sn-glycerol gave a low yield of 1,2-O-diacyl-sn-glycerol **4**. For this reason we choose monomethoxytrityl protecting group which selectively blocked the C(3) position of the compound **2**



a. (Tr)S$(CH_2)_5$COOH, Ti(OiPr)$_4$ b. MTrCl, Py c. $C_{15}H_{31}$COCl, Py d. TFA
e. (MTr)NHCH(R)CH_2OP(O)(H)O$^-$ Et_3NH^+ **5a,b** f. J_2, THF/H_2O g. TFA
R = H (a), R = COOMe (b) Tr = trityl, MTr = monomethoxytrityl

and after acylation could be cleaved by 2% trifluoroacetic acid in dichloromethane. The resulting 1-(S-tritylthiocaproyl)-2-palmitoyl-sn-glycerol **4**[8] was purified by the chromatography on silicagel (dichloromethane-pentane, 1:2) with a yield of 55%.

The synthesis of modified phospholipids was performed via H-phosphonates of N-protected ethanolamine and L-serine methyl ester obtained from the reaction of ethanolamine and L-serine methyl ester with monomethoxytrityl chloride in the presence of triethylamine. After the phosphorylation of the products with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one[9] in dioxane (1.5-excess, 1 h, r.t.) followed by the treatment with triethylammonium bicarbonate buffer, pH 8, the resulting Et_3N-salts of the two H-phosphonates **5a,b**[10] were purified on silicagel column with dichloromethane-methanol, 9:1, as eluent. The compound **4** reacted with H-phosphonates **5a,b** (3 equiv.) in the presence of adamantoyl chloride (12 equiv.)[11] in pyridine-acetonitrile, 1:1 (10 min, r.t.). After oxidation with carbon tetrachloride in aqueous pyridine (30 min, r.t.) and purification of the products by silicagel flash chromatography (eluent: dichloromethane-methanol, 9:1), N-monomethoxytrityl-ethanolamine- and L-serine methyl ester-containing phospholipids **6a,b** were obtained with yields of 90 and 78%, respectively. Removing of protecting groups by 2% TFA in dichloromethane and 0.15 M



NaOH in aqueous dioxane gave corresponding 1-(S-tritylcaproyl)-2-palmitoyl-3-phosphoethanolamine-sn-glycerol **7a** and 1-(S-tritylcaproyl)-2-palmitoyl-3-phospho-L-serine-sn-glycerol **7b** then purified by preparative TLC on silicagel[12].

To prepare the free thiol forms of these compounds we used the reaction with 0.02 M silver nitrate[13] in chloroform-ethanol, 1:1, followed by the treatment with dithiothreitol (DTT). The excess of DTT was removed by water extraction and the organic phases were concentrated *in vacuo*.

The phospholipid moieties were linked to oligonucleotide molecules via reaction of sulphur exchange starting from 5'-tritylthiol-linker bearing oligonucleotides[14]. These derivatives were converted to free thiols by the reaction with 0.5 M silver nitrate followed by DTT treatment and purified by ethanol precipitation. The thiol groups then were activated with 2,2'-dipyridyldisulphide and cetyltrimethylammonium salts of the oligonucleotide derivatives **9** were obtained. The coupling of activated oligomers **9** with thiol phospholipids **8a,b** was carried out in pyridine-chloroform, 1:2 (16 h, r.t.) and the final products **10a,b** were purified by reverse phase HPLC[15].

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7. ^{1}H NMR ($CDCl_3$) ppm 7.15-7.50 (m, 15H, Ar-H), 4.14 (m, 2H, 1-CH_2), 3.85 (m, 1H, 2-CH), 3.64 (m, 2H, 3-CH_2), 2.26 (t, 2H, $OCOCH_2$), 2.18 (t, 2H, S-CH_2), 1.38 (m, 6H, 3xCH_2). TLC: R_f 0.2 (dichloromethane-pentane, 1:1), yellow coloring in TFA vapour.
8. ^{1}H NMR ($CDCl_3$) ppm 7.10-7.60 (m, 15H, Ar-H), 5.05 (m, 1H, 2-CH), 4.25 (dd, 2H, 1-CH_2), 3.68 (d, 2H, 3-CH_2), 2.40 (2t, 4H, 2x$OCOCH_2$), 2.18 (t, 2H, S-CH_2), 1.38-1.66 (m, 6H, 3xCH_2), 1.30 (m, 26H, 13xCH_2), 0.85 (t, 3H, CH_3). TLC: R_f 0.5 (dichloromethane-pentane, 1:1), yellow coloring in TFA vapour.
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10. ^{31}P NMR (respectively to 85% H_3PO_4, $CDCl_3$) ppm 3.91 (J_{P-H} 5.09) **5a** and 4.32 (J_{P-H} 5.07) **5b**. TLC: R_f 0.3 **5a** and 0.25 **5b** ($CHCl_3$-MeOH, 9:1).
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12. ^{1}H NMR ($CDCl_3$) ppm 4.20 (m, 2H, alfa-CH_2), 3.59 (m, 2H, beta-CH_2) **6a** and 4.23 (m, 2H, alfa-CH_2), 3.88 (m, 1H, beta-CH) **6b**. TLC: R_f 0.35 **7a** and 0.3 **7b**, yellow coloring in TFA vapour.
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15. Column 4x250 mm, C18 (3um), buffer A: 5% acetonitrile - 0.1 M triethylammonium acetate buffer, pH 7.5 (TEAA), buffer B: 80% acetonitrile - 0.1 M TEAA. Gradient: 0-100% B/40 min. The conjugates **10a,b** were eluted at 45% B. Yields of isolated phospholipid-oligonucleotide conjugates were usually 55-70%.

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