õSynthesis of lipid-oligonucleotide conjugates for RNA interference studiesö Grijalvo, S., Ocampo, S. M., Perales, J. C., Eritja, R. Chem. Biodivers., 8(2), 287-299 (2011). doi: 10.1002/cbdv.201000274

Synthesis of lipid-oligonucleotide conjugates for RNA interference studies.

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ABSTRACT

The synthesis of RNA molecules carrying lipids at their 3' termini and 5' termini is reported. These conjugates were fully characterized by MALDI-TOF mass spectrometry and HPLC chromatography. The ability of these conjugates to silence gene expression was evaluated in the inhibition of the tumor necrosis factor. All the lipid-siRNA derivatives were compatible with RNA interference machinery if transfected with oligofectamine. In the absence of a transfection agent some lipid-siRNA derivatives can exert a slight reduction of gene expression.

Introduction.- Many years ago the use of modified nucleic acids to inhibit gene expression by blocking translation and transcription or by stimulating the degradation of a particular messenger RNA (mRNA) became a promising approach in therapy. This process uses antisense technology [1-3] and, more recently, RNA interference (RNAi) [4]. Although both strategies inhibit the same stage of translation of a gene to a protein, their mechanisms are different. In the case of antisense technology, synthetic oligonucleotides are designed to selectively bind to the complementary mRNA transcript of a targeted gene thereby inhibiting mRNA translation to the corresponding protein [5]. In contrast the RNAi approach uses short double-stranded RNA (dsRNA) molecules to switch genes off. Components of the RNAi machinery specifically recognize the short interfering duplex RNAs (siRNA) and incorporate the guide RNA strand into a protein complex named the RNA-induced silencing complex (RISC). The

complex formed by the guide RNA strand and RISC catalyzes the degradation of a specific mRNA, thereby repressing the synthesis of a specific protein [6-7]. However, the use of oligonucleotides in both therapies is inefficient, since they are rapidly degraded by exonucleases and endonucleases in physiological conditions, and also because of their poor cellular uptake, which is primarily due to their relatively large molecular weight and their polyanionic nature. The lack of solutions to these two problems (especially in the case of cellular uptake), have caused a bottleneck in the development of nucleic acids in potential therapeutics [8].

In order to enhance the stability of oligonucleotides to nuclease degradation and increase cellular uptake a major research effort is being made to design and synthesize modified nucleic acids with similar hybridization properties but improved biological properties [9-11]. The synthesis of modified nucleic acids such as phosphorothioate [12], methyl phosphonate [13] and phosphoramidate oligonucleotides [14] locked nucleic acids [15] or peptide nucleic acids [16] has efficiently improved the stability of oligonucleotides in serum. On the issue of delivery progress has been made in the development of new non-viral carriers such as polymers [17], lipids [18], cell-penetrating peptides [19] or nanoparticles [20] for oligonucleotide delivery. However, despite considerable research, efficiency and specificity results are still not satisfactory in many cases. To date, most of the strategies for improving cellular uptake use carriers that can be covalently linked to ODNs using the post-synthetic conjugation [21-24] method or through the preparation of complexes (lipoplexes, polyplexes) [25-28]. The formation of these complexes is due to the existence of electronic interactions between polyanionic nucleic acids and the positively charged nature of the designed carrier. In particular, the conjugation of lipids to siRNA, such as cholesterol and fatty acids, produces molecules with improved inhibitory properties [29-32].

As part of our ongoing interest in gene-silencing methods [33-34] and in finding new carriers to improve inhibition and cellular uptake, we now propose a simple strategy in which several lipid tails are covalently linked to oligonucleotides at their 3' end (using controlled pore glass (CPG) supports) or at 5' end (phosphoramidite chemistry) (Figure 1). The target lipid molecules are based on the lipids that are used to formulate the stable nucleic acid-lipid particles (SNALP) [35-36] for the systemic administration of siRNA [37-38]. We studied the silencing properties of the conjugates carrying the lipid modification in the passenger strand of the siRNA. Our target for RNAi studies is the tumor necrosis factor (TNF- α) gene. The protein is involved in several biological processes such as apoptosis, inflammation and immunity and it has been implicated in the pathogenesis of many human diseases. The inhibition of this cytokine is of particular relevance for the development of anti-inflammatory drugs [24].

Figure 1

Results and Discussion.- 1. Synthesis of lipid derivatives. We considered the use of a glycerol backbone based structure to be a suitable scaffold for introducing our chemical modifications, covalently linked to oligonucleotides at their 3' end. This strategy was successfully used to introduce the 1-O-hexyldecylglycerol unit into antisense DNA [39]. The synthesis is outlined in Scheme 1. To prepare oligonucleotides carrying lipids C₁₄ and C₁₈ at the 3' position, controlled pore glass (CPG) supports were functionalized by reaction of the suitable trityl-lipid derivatives **4** or **9** with amino-functionalized CPG prior to DNA/RNA synthesis. To synthesize **4**, the alkylation reaction between commercially available solketal **1** and 1-bromotetradecane with sodium hydride (60%) in the presence of toluene was carried out to yield the respective alkylated compound **2**. Acetonide hydrolysis (*p*TsOH, MeOH) yielded the expected diol

3, which was selectively protected using the 4-monomethoxy trityl (MMTr) group. This reaction provided the desired trityl-protected alcohol **4** at a high yield. We used a similar synthetic route for the synthesis of trityl alcohol **9**. Mesylate **6** was used in the alkylation reaction instead of a bromo derivative. Then, compounds **7**, **8** and **9** were prepared in good yields and were fully characterized. Finally, with the two synthesized trityl compounds **4** and **9** on hand, we coupled them to CPG supports using the succinyl linker described in the literature [40-41]. CPG functionalization yielded glass beads that were loaded with our proposed lipids containing **5** (15 $\mu\text{mol/g}$) and **10** (16 $\mu\text{mol/g}$), respectively. To introduce lipids C_{28} and C_{14}N at the 5' position, we used the phosphoramidite approach (Scheme 2). For the synthesis of lipid phosphoramidite **15**; the selective protection of commercially available primary alcohol **11** with trityl chloride was carried out according to the literature [42]. Then, protected alcohol **12** was subjected to an alkylation reaction using 1-bromotetradecane in the presence of THF, which resulted in alkylated-trityl derivative **13** at a high yield. Subsequently, trityl deprotection under acid conditions (DCM:TFA 20%) easily produced alcohol **14** which was transformed into the desired phosphoramidite **15**. This was then directly used for the synthesis of oligonucleotides without further purification.

Scheme 1

Lipophosphoramidite **19** was synthesized by a double alkylation reaction of commercially available protected triol **16** and 1-bromotetradecane, according to the literature [36]. The removal of the allyl group of compound **17** with $\text{Pd}[(\text{PhP})_3]_4$ in the presence of THF yielded the desired alcohol **18**, which was subsequently transformed into its phosphoramidite derivative **19**. As in the previous case, phosphoramidite **19** was used for DNA / RNA synthesis without further purification.

Scheme 2

2. *Synthesis of oligodeoxynucleotide-lipid conjugates.* Solid supports and phosphoramidites that have been previously described were used for the preparation of oligonucleotides carrying lipids at the 3' or 5' ends. We selected the self-complementary oligodeoxynucleotide sequence 5'-d(CGCGAATTCGCG)-3' known as the *Dickerson-Drew* dodecamer, as a model compound. Oligonucleotide sequences were assembled on a DNA synthesizer using standard protocols. After the assembly of the desired DNA sequence, supports were treated with concentrated ammonia at 55°C for a minimum of 6 hrs to yield oligonucleotides **20-23** carrying lipid molecules at the 3' or 5' ends. In all cases a major peak was obtained that had the expected molecular weight as determined by MALDI-TOF mass analysis (experimental section). This indicates that the oligonucleotide-lipid conjugates are stable to oligonucleotide synthesis conditions.

2. *Lipid-siRNA conjugates.* Next the synthesis of lipid conjugates of RNA was studied. We selected a previously described siRNA duplex to inhibit the expression of the TNF- α gene [43]. These siRNA are being studied as potential anti-inflammatory drugs. RNA conjugates of passenger strands carrying lipids at the 3' and 5' end were prepared (Table 1). A siRNA duplex carrying a cholesterol molecule at the 3' end of the passenger strand was also prepared for comparison purposes. Finally, RNA conjugates of the guide strand carrying lipids C₁₄ and C₁₈ at the 3' end of the guide strand were prepared (Table 1). Syntheses were carried out using standard RNA synthesis protocols and RNA monomers carrying the *tert*-butyldimethylsilyl (TBDMS) group in the 2' position. RNA conjugates carrying lipids at the 3' position were purified using *DMT-on* protocols whereas RNA conjugates carrying lipids at the 5' position were purified

according to *DMT-off* protocols. All modified conjugates were purified by semipreparative HPLC chromatography. In all cases we obtained a major peak that was isolated and characterized by MALDI- TOF mass spectrometry (see Table 1). All conjugates were annealed with equimolar amounts of the unmodified guide strands and purified from EtOH precipitation (Table 1).

Table 1

We examined the effect of the lipids on siRNA duplex stability. The thermal stability of the modified and unmodified duplexes was measured in 100 mM potassium acetate, 2 mM magnesium acetate, and 30 mM HEPES-KOH at pH 7.4 (Table 2). The melting curves of the duplexes were virtually identical (83.9°C, 83.6°C, 84.3°C; for siRNA-3, siRNA-5 and siRNA-6, respectively) compared to the siRNA-1 wild type (83.5°C), except for siRNA-4, which stabilized slightly when hybridized to its complement (86.9°C, with $T_m = 3.4$ °C). This was probably due to the presence of 2' OMe groups in the pyrimidines of the RNA molecule [44] (Table 2). These results indicate that the presence of lipid groups at the 3 and 5' position of the siRNA molecule does not affect their ability to form stable duplexes.

Figure 2

Next, we studied the TNF- inhibitory properties of the lipid-modified siRNA duplexes [40] using two different series of experiments. In the first case, siRNA transfection was carried out using commercially available cationic lipid (oligofectamine). To this end, HeLa cells were first transfected with plasmid expressing murine TNF- using lipofectin. One hour later, unmodified siRNA-1 or lipid-modified siRNA conjugates in the passenger strand (siRNA-2, siRNA-3, siRNA-4, siRNA-5, siRNA-6 and siRNA-7, respectively at 50 nM) as well as the scrambled sequence Scr were co-transfected using Oligofectamine. Cells were analyzed using an enzyme-linked

immunoabsorbent assay (ELISA) after twenty four hours of transfection. Inhibition results are depicted on Figure 2. Unmodified and modified siRNA conjugates at the 3' position (siRNA-2, siRNA-3, siRNA-4 and siRNA-5) showed 90-95% inhibition of the TNF- α production compared to the scrambled control Scr duplex. The inhibition results for the modified siRNA conjugates at the 5' position were somewhat lower (80% and 60% for siRNA-6 and siRNA-7, respectively). Although we obtained better inhibition results for modified siRNA conjugates at the 3' position, in general, the introduction of the lipid modifications did not disrupt the RNAi machinery. After verifying the functionality of our modifications, we evaluated whether the presence of the lipid modifications in siRNA duplexes could improve the cellular uptake process. After transfecting HeLa cells with the murine TNF- α plasmid, the cells were treated with unmodified and modified siRNA conjugates (siRNA-1, siRNA-2, siRNA-3, siRNA-4, siRNA-5 and siRNA-6 at 100 nM) in the absence of a transfection agent (Figure 3). The inhibition activity of TNF- α is much lower in this case, due to the absence of Oligofectamine. However, some chemically modified siRNAs silenced the TNF- α gene expression with promising inhibitory results (19%, 17% and 38% for siRNA-5, siRNA-6 and siRNA-7, respectively) compared to the scrambled siRNA duplex (siRNA 8). Surprisingly, the most active siRNA without a transfecting agent is the less active siRNA with oligofectamine. These results indicate that the most interesting siRNA derivative prepared in this work was the siRNA-7, which carries the C₂₈ lipid at the 5' end.

Figure 3

The inhibitory properties of siRNA duplexes carrying lipids at the guide strand (siRNA-9, siRNA-10) were also analyzed (Figure 4). Lipid-modified (siRNA-9, siRNA-10) and unmodified siRNA (siRNA-1) against TNF- α produced an 80-90%

inhibition of the production of TNF- α compared with the scrambled control siRNA duplex (siRNA 8). These results indicate that the introduction of the lipid at the 3'-end of either the guide or the passenger strand of an RNA duplex does not affect the inhibitory properties of the resulting siRNA duplex in HeLa cells.

Figure 4

Conclusions.

In summary, we have reported an efficient strategy for introducing novel lipid moieties at the 3' and 5' position of DNA and RNA molecules through covalent bonds that are stable to oligonucleotide synthesis conditions. The effect of these modifications on the cellular activity does not interfere with the RNAi machinery. In the presence of a transfection agent, we observed better RNAi inhibitory properties with siRNA conjugates carrying lipids at the 3' termini. However, in the absence of transfection agent, we observed better cellular uptake when the lipid C₂₈ was attached at the 5' position of the passenger strand. These promising results will be followed up with more detailed cellular uptake studies using these conjugates as well as the use of non-viral carriers to improve gene delivery.

Acknowledgement. This study was supported by the *Spanish Ministry of Education* (grant BFU2007-63287 and CTQ2010-20541), the *Generalitat de Catalunya* (2009/SGR/208) and CIBER-BBN (VI National R&D&i Plan 2008-2011, *Iniciativa Ingenio 2010, Consolider Program, CIBER Actions, Instituto de Salud Carlos III* with assistance from the *European Regional Development Fund*.

Experimental Part

Materials and Methods. All reactions were carried out under argon positive pressure in anhydrous solvents. Commercially available reagents and anhydrous solvents were used without further purification. Solvents were distilled prior to use and dried using standard methods. Analytical samples were homogeneous, as confirmed by TLC, and yielded spectroscopic results that were consistent with the structures assigned. Chemical shifts are reported in parts per million (ppm) relative to the singlet at $\delta = 7.24$ ppm of CHCl_3 for ^1H NMR and to the centre line of the triplet at $\delta = 77.0$ ppm of CDCl_3 for ^{13}C NMR. IR spectra were measured on film and were recorded with a BOMEM MB-120. Thin-layer chromatography (TLC) was performed on silica gel (Alugram Sil G/UV). MALDI mass spectrometry was recorded on a Fisons VG Tofspec spectrometer and ESI mass spectra on a Fisons VG Platform II spectrometer. Oligonucleotide sequences were prepared using solid phase methodology on an Applied Biosystems Model 3400 DNA synthesizer. Oligoribonucleotides were purified by DMT-on protocols using reversed-phase HPLC.

HPLC conditions. Conditions for semipreparative HPLC were as follows. HPLC solutions were solvent A: 5% acetonitrile (ACN) in 100 mM triethylammonium acetate (TEAA), pH 6.5; and solvent B: 70 % ACN in 100 mM triethylammonium acetate (pH 6.5). Column: PRP-1 (Hamilton) 250 x 10 mm. A flow rate 3 ml/min linear gradient from 15 to 100% in B (DMT-on) and 0 to 50% in B (DMT-off) was used with UV detection at 260 nm.

General procedure for the alkylation reaction. Solketal **1** (100 mg, 0.757 mmol) and 60% sodium hydride (NaH, 60.4 mg, 1.51 mmol, 2.0 eq) were stirred in toluene (3.0 mL) under argon for 20 minutes. The corresponding alkyl bromide C_{14} (420 mg, 1.51 mmol, 2.0 eq) or mesylate C_{18} (520 mg, 1.51 mmol) was dissolved in toluene (2.0 mL)

and added dropwise. The reaction was refluxed under argon overnight. It was then diluted with more toluene (10 mL) and the reaction mixture was cooled in an ice bath while quenched via the slow addition of cold water. The mixture was washed with water (5 mL) and brine (5 mL), using ethanol to aid phase separation if necessary. The organic phase was dried over anhydrous sodium sulphate and evaporated. The crude product was purified by flash chromatography (Ch:AcOEt 0% to 3%).

2,2-dimethyl-4-(tetradecyloxymethyl)-1,3-dioxolane, **2**. Yield: 78%; IR (film) 2925, 2854, 1462, 1379, 1370, 1117.4 cm^{-1} ; ^1H -NMR (400 MHz, CDCl_3) 4.26 (q, $J = 6.0$ Hz, 1H), 4.05 (dd, $J = 8.2$ Hz, 6.4 Hz; 1H), 3.73 (dd, $J = 8.2$ Hz, 6.4 Hz; 1H), 3.51 (m, 2H), 3.43 (m, 2H), 1.60 (m, 4H), 1.42 (s, 3H), 1.36 (s, 3H), 1.25 (m, 20H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C -NMR (400 MHz, CDCl_3) 109.6, 74.9, 72.1, 72.0, 67.1, 32.1, 30.0, 29.9, 29.8, 29.8, 29.8, 29.8, 29.7, 29.6, 29.5, 26.9, 26.2, 25.6, 22.9, 14.3; HR ESI MS: m/z calculated for $\text{C}_{20}\text{H}_{40}\text{NaO}_3$ ($\text{M}^+ + 23$), 351.2870, found m/z 351.2862; calculated for $\text{C}_{40}\text{H}_{80}\text{NaO}_6$ ($2\text{M}^+ + 23$), 679.5847, found m/z 679.5835.

2,2-dimethyl-4-(((10Z,13Z)-octadeca-10,13-dienyloxy)methyl)-1,3-dioxolane, **7**. Yield: 77%; IR (film) 3008, 2986, 2926, 2856, 1457, 1379, 1369, 1214, 1120, 847 cm^{-1} ; ^1H -NMR (400 MHz, CDCl_3) 5.34 (m, 4H), 4.25 (q, $J = 5.8$ Hz, 1H), 4.04 (dd, $J = 14.6$ Hz, 8.2 Hz, 1H), 3.71 (dd, $J = 14.6$ Hz, 8.2 Hz, 1H), 3.45 (m, 4H), 2.76 (t, $J = 5.8$ Hz, 2H), 2.00 (m, 4H), 1.56 (m, 2H), 1.41 (s, 3H), 1.35 (s, 3H), 1.29 (m, 20H), 0.88 (t, $J = 6.8$ Hz, 3H); ^{13}C -NMR (400 MHz, CDCl_3) 132.1, 128.9, 110.0, 72.7, 72.1, 70.5, 64.5, 32.1, 30.0, 29.9, 29.8, 29.8, 29.8, 29.6, 29.5, 26.3, 22.9, 14.3; HR ESI MS: m/z calculated for $\text{C}_{24}\text{H}_{44}\text{NaO}_3$ ($\text{M}^+ + 23$), 403.3183, found m/z 403.3176.

General procedure for the deprotection reaction. A solution of alkylated compound **2** (1.0 eq) and alkylated compound **7** (1.0 eq) along with *p*-toluenesulphonic acid monohydrate (0.5 eq) in methanol (5.0 mL) was stirred at room temperature overnight.

The solvent was evaporated and the residue was purified by flash chromatography, eluted with DCM:MeOH 0% to 2%.

3-(tetradecyloxy)propane-1,2-diol, **3**. Yield: 72%; IR (film) 2980, 2850, 2390, 2387 cm^{-1} ; ^1H -NMR (400 MHz, CDCl_3) 3.87 (m, 1H), 3.74 (dd, $J = 11.4$ Hz, 3.9 Hz, 1H), 3.67 (dd, $J = 11.4$ Hz, 5.0 Hz, 1H), 3.54 (m, 2H), 3.48 (m, 2H), 1.60 (m, 4H), 1.27 (m, 20H), 0.89 (t, $J = 6.8$ Hz, 3H); ^{13}C -NMR (400 MHz, CDCl_3) 72.8, 72.0, 70.5, 64.5, 32.1, 30.0, 29.9, 29.8, 29.8, 29.8, 29.6, 29.5, 26.3, 22.9, 14.3; HR ESI MS: m/z calculated for $\text{C}_{17}\text{H}_{36}\text{Na O}_3$ ($\text{M}^+ + 23$), 311.2557, found m/z 311.2555.

3-((10Z,13Z)-octadeca-10,13-dienyloxy)propane-1,2-diol, **8**. Yield: 70 %; IR (film) 3105, 2990, 2365, 1530, 1329 cm^{-1} ; ^1H -NMR (400 MHz, CDCl_3) 5.33 (m, 4H), 3.83 (m, 1H), 3.64 (m, 2H), 3.47 (m, 4H), 2.74 (m, 2H), 2.01 (m, 4H), 1.55 (m, 2H), 1.27 (m, 18H), 0.86 (t, $J = 6.8$ Hz, 3H); ^{13}C -NMR (400 MHz, CDCl_3) 130.4, 130.3, 130.2, 130.2, 128.2, 128.1, 128.1, 128.0, 72.6, 72.5, 72.0, 72.0, 70.8, 70.7, 64.4, 64.3, 31.8, 31.7, 29.9, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 27.4, 26.3, 26.2, 25.8, 25.8, 22.8, 22.7, 14.3; HR ESI MS: m/z calculated for $\text{C}_{21}\text{H}_{40}\text{Na O}_3$ ($\text{M}^+ + 23$), 363.2698, found m/z 363.2697.

General procedure for protection with MMTr. Diols **3** or **8** (1.0 eq) were co-evaporated twice with pyridine and dissolved again in pyridine (2 mL). DMAP (0.1 eq) and MMTr-Cl (1.5 eq) were added at room temperature and the corresponding mixtures were stirred at 40°C overnight. The reaction was quenched by addition of MeOH (5 mL). The crudes were concentrated *in vacuo* and the residues were purified by flash chromatography (DCM:MeOH:TEA 98:1:1).

1-((4-methoxyphenyl)diphenylmethoxy)-3-(tetradecyloxy)propan-2-ol, **4**. Yield: 80%; IR (film) 3200, 1630, 1521, 1232, 990 cm^{-1} ; ^1H -NMR (400 MHz, CDCl_3) 7.37 (m, 2H), 7.23 (m, 5H), 7.19 (m, 5H), 6.77 (m, 1H), 6.74 (m, 1H), 3.86 (m, 1H), 3.73 (s, 3H), 3.46

(dd, $J = 9.7$ Hz, 4.2 Hz, 1H), 3.39 (dd, $J = 9.7$ Hz, 6.4 Hz, 1H), 3.35 (m, 2H), 3.11 (m, 2H), 2.34 (broad d, 1H), 1.47 (m, 2H), 1.19 (m, 22H), 0.81 (t, $J = 6.6$ Hz, 3H); ^{13}C -NMR (400 MHz, CDCl_3) 158.9, 158.7, 150.0, 147.3, 144.6, 135.7, 130.5, 129.4, 128.7, 128.6, 128.1, 128.0, 128.0, 127.3, 127.1, 123.9, 113.4, 113.3, 86.5, 81.9, 72.2, 71.8, 70.1, 64.7, 55.4, 55.4, 32.1, 30.0, 29.9, 29.8, 29.8, 29.8, 29.7, 29.5, 26.3, 22.9, 14.3; HR ESI MS: m/z calculated for $\text{C}_{38}\text{H}_{54}\text{NaO}_5$ ($\text{M}^+ + 23$), 613.7623, found m/z 613.7622.

1-((4-methoxyphenyl)diphenylmethoxy)-3-((10Z,13Z)-octadeca-10,13-dienyloxy)propan-2-ol, **9**. Yield: 67%; IR (film) 3300, 2980, 2732, 1600, 1475, 1200, 995 cm^{-1} ; ^1H -NMR (400 MHz, CDCl_3) 7.57 (m, 1H), 7.36 (m, 2H), 7.20 (m, 7H), 7.10 (d, $J = 8.8$ Hz, 2H), 6.75 (d, $J = 8.8$ Hz, 2H), 5.29 (m, 4H), 3.87 (m, 1H), 3.70 (s, 3H), 3.45 (dd, $J = 9.7$ Hz, 4.2 Hz, 1H), 3.37 (m, 1H), 3.10 (m, 2H), 3.01 (m, 2H), 2.69 (t, $J = 6.5$ Hz, 2H), 2.42 (broad d, 1H), 1.96 (m, 2H), 1.46 (m, 2H), 1.20 (m, 18H), 0.80 (t, $J = 6.7$ Hz, 3H); ^{13}C -NMR (400 MHz, CDCl_3) 158.8, 149.9, 147.4, 144.6, 139.5, 136.1, 130.5, 129.4, 128.6, 128.1, 128.0, 128.0, 127.3, 127.1, 123.9, 113.4, 86.5, 81.9, 72.3, 71.8, 70.1, 64.7, 55.4, 31.7, 29.9, 29.9, 29.8, 29.7, 29.6, 29.5, 27.5, 27.4, 26.3, 25.8, 22.8, 14.3; HR ESI MS: m/z calculated for $\text{C}_{42}\text{H}_{58}\text{NaO}_5$ ($\text{M}^+ + 23$), 665.8249, found m/z 665.8250.

General procedure for functionalization of CPG solid supports, 5 and 10. MMTr-protected alcohols **4** or **9** along with DMAP (1.5 eq) and succinic anhydride (1.5 eq) were dissolved in dichloromethane (1 mL). Reactions were stirred overnight at room temperature. Dichloromethane was added (5 mL) and the organic layers were then washed with a 0.1M Na_2HPO_4 solution. The resulting organic layers were dried on sodium sulphate and the solvent was evaporated. The residues were used in the next step without further purification. 2,2-Dithio-bis-(5-nitropyridine) (0.1 mmol) dissolved in 400 μL of acetonitrile was mixed with a solution of the appropriate MMTr-protected

compounds (0.1 mmol) and DMAP (0.1 mmol) in acetonitrile (500 L). A solution of TPP (0.1) dissolved in acetonitrile (200 L) was added to the respective clear solutions at room temperature. The mixtures were then vortexed for a few seconds and added to a syringe containing LCAA-CPG (500 mg, 0.05 mmol amino groups). They were allowed to react for 2 hours at room temperature. The reactions were arrested by adding methanol (500 L). The supports were then washed with methanol (3 x 10 ml), acetonitrile (3 x 10 mL) and diethyl ether (3 x 10 mL). The solid supports were dried *in vacuo* and then subjected to capping of the residual amino functionalities with a mixture of acetic anhydride/pyridine/tetrahydrofuran (500 L) and 1-methylimidazole in tetrahydrofuran (500 L). After 30 minutes, the resins were washed with methanol (3 x 10 mL), acetonitrile (3 x 10 mL) and diethyl ether (3 x 10 mL). The solid supports **5** and **10** were dried first in the air and then under high vacuum. Loading (**5a**) (mol/g) = 15; loading (**10**) (mol/g) = 16.

N,N-dimethyl-2-(tetradecyloxy)-3-(trityloxy)propan-1-amine, **13**. Protected alcohol **12** (300 mg, 0.830 mmol) and 60 % NaH (133 mg, 3.32, 4.0 eq) were suspended in anhydrous THF (4.5 mL) and stirred for 15 min at room temperature. Alkyl bromide (575 mg, 2.1 mmol, 2.0 eq) was added dropwise. The reaction was heated to reflux overnight. It was then cooled at 0°C and water (1.5 mL) was added carefully. The solvent was evaporated and the residue was dissolved in dichloromethane (10 mL), washing the organic layer with water (2 x 20 mL) and brine (2x 20 mL). The organic layer was dried on sodium sulphate and the solvent was evaporated. Crude was purified by flash chromatography (Ch:TEA 99:1 → Ch:AcOEt:TEA 98:1:1).

Yield: 77%; IR (film) 3059, 2924, 2853, 2818, 2766, 1490, 1449, 1074, 909 cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) 7.39 (m, 4H), 7.19 (m, 11H), 3.45 (m, 1H), 3.38 (m, 2H), 3.08 (m, 2H), 2.31 (s, 6H), 1.49 (m, 2H), 1.18 (m, 22H), 0.81 (t, *J* = 6.6 Hz, 3H); ¹³C-

NMR (400 MHz, CDCl₃) 144.4, 128.9, 127.9, 127.1, 86.7, 77.9, 70.7, 70.6, 64.9, 61.7, 60.2, 46.5, 45.7, 32.1, 30.4, 30.0, 29.9, 29.8, 29.6, 26.4, 22.9, 14.4; HR ESI MS: m/z calculated for C₃₈H₅₆NO₂ (M⁺ + H), 558.4306, found m/z 558.4298.

3-(dimethylamino)-2-(tetradecyloxy)propan-1-ol, **14**. Alkylated alcohol **13** (358 mg, 0.642 mmol) was dissolved in a mixture of 20 % TFA in DCM (2:8). The reaction was stirred for two hours at room temperature. The organic layer was carried to basic pH with 1M NaOH solution and was extracted three times with dichloromethane (20 mL). The organic layer was dried with sodium sulphate and the solvent was evaporated. The residue was purified by flash chromatography (DCM:MeOH:TEA 90:9:1).

Yield: 76%; IR (film) 3110, 2960, 2874, 1530, 1470, 1065, 864 cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) 3.79 (dd, J = 10.9 Hz, 4.1 Hz, 1H), 3.68 (m, 1H), 3.48 (m, 5H), 2.60 (m, 2H), 2.32 (s, 6H), 1.54 (m, 2H), 1.25 (m, 20H), 0.88 (t, J = 6.6 Hz, 3H); ¹³C-NMR (400 MHz, CDCl₃) 75.3, 70.1, 65.6, 62.9, 46.5, 32.1, 30.3, 29.9, 29.8, 29.8, 29.8, 29.8, 29.6, 29.5, 26.3, 22.9, 14.3; HR ESI MS: m/z calculated for C₁₉H₄₁NO₂ (M⁺ + H), 316.2143, found m/z 316.2142.

2,3-bis(tetradecyloxy)propan-1-ol, **18**. A mixture of protected alcohol **17** (100 mg, 0.191 mmol), *N, N*- dimethylbarbituric acid (80 mg, 0.514 mmol, 2.7 eq) and Pd[(PPh₃)₄] catalyst (12.3 mg, 0.011 mmol, 0.05 eq) was dissolved in anhydrous THF (2.5 mL) and heated at 90°C in a sealed-tube overnight. The solvent was evaporated and the residue was purified by flash chromatography (DCM:MeOH 0% to 1%) to yield a white solid.

Yield: 97%; ¹H-NMR (400 MHz, CDCl₃) 3.72 (m, 1H), 3.61 (m, 2H), 3.50 (m, 2H), 3.30 (m, 2H), 2.91 (m, 2H), 2.15 (m, 2H), 1.54 (m, 8H), 1.26 (m, 12H), 0.88 (t, J = 6.88 Hz, 6H).

General procedure for the synthesis of lipo-phosphoramidites 14 and 19.

Diisopropylethylamine (2 eq) was added dropwise at room temperature to a solution formed by alcohol **14**, or **18** (1 eq) in DCM (2 mL). Subsequently, DCM solutions were cooled at 0°C and -cyanoethyl-*N*, *N*-diisopropylaminophosphochloride (1.0 eq) was added. The resulting reactions were removed from the ice bath and allowed to warm to room temperature, with stirring after 2 hours. The reactions were diluted with more dichloromethane (10 mL) and extracted with 10 mL of cold 0.5 M sodium bicarbonate. The organic layers were dried with anhydrous sodium sulphate and evaporated to leave a clear colourless syrup. A vacuum pump was used to remove the last traces of solvent and diisopropylethylamine. The resulting products were used without further purification.

2-cyanoethyl 3-(dimethylamino)-2-(tetradecyloxy)propyl diisopropylphosphoramidite, 15. Yield: 69%; ¹H-NMR (400 MHz, CDCl₃) 4.18 (m, 2H), 3.88 (m, 1H), 3.56 (m, 6H), 2.76 (m, 2H), 2.63 (m, 2H), 2.26 (s, 6H), 1.56 (m, 2H), 1.25 (m, 22H), 1.19 (m, 12H), 0.88 (t, *J* = 6.5 Hz, 3H); ³¹P -NMR (300 MHz, CDCl₃) 150.1.

2,3-bis(tetradecyloxy)propyl 2-cyanoethyl diisopropylphosphoramidite, 19. Yield: 80%; ¹H-NMR (300 MHz, CDCl₃) 4.12 (m, 2H), 3.79 (m, 1H), 3.47 (m, 8H), 3.26 (m, 1H), 2.69 (m, 2H), 2.56 (m, 2H), 1.48 (m, 4H), 1.18 (m, 44H), 1.12 (d, *J* = 6.7 Hz, 6H), 1.10 (d, *J* = 6.7 Hz, 6H), 0.81 (t, *J* = 6.6 Hz, 6H); ³¹P -NMR (300 MHz, CDCl₃) 150.1.

Oligodeoxynucleotide synthesis. Oligodeoxynucleotide sequences were prepared using solid phase methodology. The syntheses were carried out on an Applied Biosystems Model 3400 DNA synthesizer using a 1 μmol scale. Oligonucleotides were purified by reversed-phase HPLC, as described above. Mass spectrometry of modified dodecamers: **20** [5'-d(CGCGAATTCGCG)-C₁₄-3'] found 3994, expected 3996; **21** [5'-d(CGCGAATTCGCG)-C₁₈-3']: found 4045, expected 4048; **22** [C₂₈-5'

d(CGCGAATTCGCG)-3': found 4196, expected 4192; **23** [C₁₄N-5'
d(CGCGAATTCGCG)-3': found 4028, expected 4025.

Oligoribonucleotides. The following RNA sequences were obtained from commercial sources (*Sigma-Proligo*, *Dharmacon*): passenger scrambled 5'-CAG UCG CGU UUG CGA CUG G-dT-dT-3' guide scrambled 5'-CCA GUC GCA AAC GCG ACU G-dT-dT-3' guide anti-TNF : 5'-GAG GCU GAG ACA UAG GCA C-dT-dT-3' and passenger anti-TNF : 5'-GUG CCU AUG UCU CAG CCU C-dT-dT-3' RNA monomers in capital letters, dT represents thymidine. The 3'-cholesterol passenger anti-TNF : strand 5'-GUG CCU AUG UCU CAG CCU C-dT-dT-3'-cholesterol was prepared using the cholesterol-tetraethyleneglycol (TEG)-3'-CPG support from commercial sources (*Glen Research*).

Oligoribonucleotides were prepared on a DNA synthesizer (*Applied Biosystems 3400*) using 2-cyanoethyl phosphoramidites. The following solutions were used: 0.4 M 1H-tetrazol in ACN (activation); 3% trichloroacetic acid in DCM (deprotection), acetic anhydride / pyridine / tetrahydrofuran (1: 1: 8) (capping A), 10 % *N*-methylimidazole in tetrahydrofuran (capping B), 0.01 M iodine in tetrahydrofuran / pyridine / water (7: 2: 1) (oxidation). Guanosine was protected with the dimethylaminomethylidene group, cytosine was protected with the acetyl group and adenosine with the benzoyl group. The 2'-OH protecting group for the RNA monomers was the *t*-butyldimethylsilyl (TBDMS) group. The average coupling yield was around 97-98% per step. Modified RNA strands were purified using DMT-on based protocols: RNAs were cleaved from the support with a mixture of concentrated ammonia and ethanol 3:1 at 55 °C for 60 minutes. The solvent was evaporated to dryness and a 1M solution of TBAF in THF was added. RNAs were incubated for 24 hours at room temperature. The deprotection reaction was quenched and modified RNA conjugates were desalted (Sephadex, NAP-10) and

purified by semi-preparative HPLC. DMTr groups were then deprotected (AcOH 80%, 30 min) and extracted them with ether. Finally, chemically modified RNA conjugates were desalted (Sephadex, NAP-5).

Melting experiments on siRNA duplexes. A 100 mM potassium acetate, 2 mM magnesium acetate and 30 mM HEPES-KOH solution at pH = 7.4 at a final concentration of approximately 0.25 OD for each strand was used for the melting experiments. The solutions were heated to 90°C and allowed to cool slowly to room temperature. Melting curves were recorded using a concentration of approximately 1 μ M for each strand in 1 mL of the tested solution in Teflon-stoppered quartz cuvettes of 1 cm optical path length. The melting temperatures were determined as the maxima of the first derivative of absorbance *versus* temperature. In all cases, the complexes displayed sharp, apparently two-state transitions. The data were analyzed by the denaturation curve-processing program.

Table 2

Annealing of the siRNA duplexes. Equimolar amounts of the corresponding passenger and guide strands were dissolved in a buffer containing 10 mM TRIS 50 mM NaCl (100 μ L) and annealed in a final volume of 100 μ L. The solutions of siRNAs were heated at 94°C for two minutes and allowed to cool until they reached room temperature. Then, 3M NaOAc pH = 5.2 were added (10 μ L) along with EtOH (96%) (275 μ L). Samples were stirred, centrifuged at 4°C (15 minutes, 12000 rpm) and precipitated at -20°C. Finally, the supernatant was removed and the respective pellets were carefully dried under argon.

Cell culture and siRNA conjugates treatments. PROTOCOL A (transfection of lipid-siRNA conjugates using Oligofectamine): HeLa cells were cultured under standard

conditions (37°C, 5% CO₂, Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 2 mM l-glutamine, supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL). All experiments were conducted at 40–60% confluence. HeLa cells were transfected with 250 ng of murine expressing TNF- α plasmid using lipofectin (*Invitrogen*), according to the manufacturer's instructions. One hour after transfection, m-TNF- α expressing HeLa cells were then transfected with unmodified siRNA-1 and modified (siRNA-2, siRNA-3, siRNA-4, siRNA-5, siRNA-6, siRNA-7, siRNA-9, or siRNA-10) and a scrambled sequence siRNA-8 at 50nM per well against TNF- α using oligofectamine (*Invitrogen*). The TNF- α concentration was determined from cell culture supernatant by an enzyme-linked immunoabsorbent assay kit (*Bender MedSystems*) following the manufacturer's instructions.

PROTOCOL B (transfection of lipid-siRNA conjugates in the absence of Oligofectamine): HeLa cells were co-transfected with murine expressing TNF- α plasmid as in the protocol A. One hour later, lipid-siRNA conjugates (siRNA-2, siRNA-3, siRNA-4, siRNA-5, siRNA-6 and siRNA-7) and a scrambled sequence siRNA-8 at 100nM were added. The TNF- α concentration was determined from cell culture supernatant by an enzyme-linked immunoabsorbent assay kit (*Bender MedSystems*), following the manufacturer's instructions.

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LEGENDS

Scheme 1. Synthesis of lipid-functionalized CPG solid supports **5** and **10** used for the preparation of DNA/RNA molecules carrying lipids at the 3' position. *Reagents and Conditions:* (a) NaH (60%), 1-Bromotetradecane or **6**, toluene, reflux, overnight, **2** (78%), **7** (77%); (b) *p*TsOH, MeOH, r.t., overnight, **3** (72%), **8** (70%); (c) MMTr-Cl, DMAP, pyridine, 40°C, overnight, **4** (80%), **9** (67%); (d) CPG derivatization.

Scheme 2. Synthesis of lipophosphoramidites **15** and **19** to be introduced into DNA/RNA molecules at the 5' position. *Reagents and Conditions:* For lipophosphoramidite **15**: (a) Trityl chloride (reference 42); (b) NaH (60%), THF, reflux, overnight; 76%; (c) DCM:TFA 20%, r.t., 2 h, 76%; (d) NC(CH₂)₂OP(iPr₂N)Cl, DIEA, DCM, 0°C to r.t., 1h 30 min. For lipophosphoramidite **19**: (a) 1-bromotetradecane (reference 36); (b) Pd[(PPh₃)₄], *N, N*- dimethylbarbituric acid, THF, 90°C, sealed tube, overnight, 97%; (c) NC(CH₂)₂OP(iPr₂N)Cl, DIEA, DCM, 0°C to r.t., 1h 30 min

Figure 1. *Proposed lipid modifications to be introduced into DNA and RNA molecules. (a) Selected lipids were introduced at the 3' termini and (b) selected lipids were introduced at the 5' termini.*

Figure 2. *Plot of gene-specific silencing activities for unmodified (siRNA-1), modified (siRNA-2, siRNA-3, siRNA-4, siRNA-5, siRNA-6, siRNA-7 and siRNA-8) conjugates along with a scrambled sequence Scr (50 nM per well). Transfection of siRNA conjugates was carried out using Oligofectamine (Protocol A). Values are represented*

as the average $\pm ES$, $n = 3$ and are compared to a scrambled sequence. *** $p < 0.001$, ANOVA Test, Bonferrini post-test.

Figure 3. Plot of gene-specific silencing activities for unmodified (siRNA-1) and modified (siRNA-2, siRNA-3, siRNA-4, siRNA-5, siRNA-6 and siRNA-7) lipid-siRNA conjugates along with a scrambled sequence siRNA-8 at 100 nM without transfecting agent (Protocol B).

Figure 4. Plot of gene-specific silencing activities for unmodified (siRNA-1) and modified (siRNA-9 and siRNA-10) lipid-siRNA conjugates along with a scrambled siRNA-8 at 50 nM with transfecting agent (Protocol A).

Table 1. Modified and Unmodified RNA Sequences Used for the Preparation of the Corresponding siRNA Conjugates for RNA Interference Studies.

Compound number	Lipid ^a	Sequence (5'3') ^b	MS ^c	siRNA ^d
24	None P, wt	GUG CCU AUG UCU CAG CCU C-dT-dT	n. d.	siRNA-1
25	Chol ^e P	GUG CCU AUG UCU CAG CCU C-dT-dT-Chol	7319 / 7310	siRNA-2
26	C ₁₄ -3', P	GUG CCU AUG UCU CAG CCU C-dT-dT-C ₁₄	6909 / 6908	siRNA-3
27	C ₁₄ -3', P	GUG <u>CCU</u> AUG <u>UCU</u> <u>CAG</u> <u>CCU</u> C-dT-dT-C ₁₄	7013 [M+Na ⁺] / 6993	siRNA-4
28	C ₁₈ -3', P	GUG CCU AUG UCU CAG CCU C-dT-dT-C ₁₈	6964 / 6961	siRNA-5
29	C ₁₄ N-5', P	C ₁₄ N-GUG CCU AUG UCU CAG CCU C-dT-dT	6934 / 6937	siRNA-6
30	C ₂₈ N-5', P	C ₂₈ -GUG CCU AUG UCU CAG CCU C-dT-dT	7112 / 7106	siRNA-7
31	None P, scr	CAG UCG CGU UUG CGA CUG G-dT-dT	n. d.	siRNA-8
32	C ₁₄ -3', G	GAG GCU GAG ACA UAG GCA C-dT-dT-C ₁₄	7123 / 7120	siRNA-9
33	C ₁₈ -3', G	GAG GCU GAG ACA UAG GCA C-dT-dT-C ₁₈	7176 / 7174	siRNA- 10

^awt: wild type, P: passenger strand, G: guide strand, scr: scrambled, ^bRNA monomers are in uppercase letters, dT stands for thymidine. ^cMass spectra (found / expected). ^dsiRNA were prepared by hybridization with the corresponding guide strands (**24-30** with 5'-GAG GCU GAG ACA UAG GCA C-dT-dT-3' **31** with 5'-CCA GUC GCA AAC GCG ACU G-dT-dT-3' and **32** and **33** with 5'-GUG CCU AUG UCU CAG CCU C-dT-dT-3'). ^esiRNA conjugate with cholesterol at the 3'-termini was prepared for comparison purposes. ^fRNA with underline contains 2'-O-methyl-RNA monomers. n.d. not determined.

Table 2. Melting Temperatures (°C) of the siRNA Conjugates Prepared in this Study.^a

siRNA	Lipid	T _m (ΔT_m^b)
siRNA-1	None	83.5
siRNA-3	C ₁₄ -3ø	83.9 (0.4)
siRNA-4	C ₁₄ -3ø	86.9 (3.4)
siRNA-5	C ₁₈ -3ø	83.6 (0.1)
siRNA-6	C ₂₈ -5ø	84.3 (0.8)

^aConditions: 100 mM potassium acetate, 2 mM magnesium acetate and 30 mM HEPES-KOH solution at pH 7.4. ^b ΔT_m is the difference between the melting temperature of the lipid siRNA minus the melting temperature of the corresponding unmodified siRNA.

Scheme 1

Scheme 2

Figure 1

(a) General structure for DNA/RNA molecules at 3'-positions

(b) General structure for DNA/RNA molecules at 5'-positions

Figure 2

Figure 3

Figure 4

Graphical illustration for the table of contents