Journal Name

Dynamic Article Links ►

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Easy introduction of maleimides at different positions of oligonucleotide chains for conjugation purposes

Albert Sánchez, Enrique Pedroso and Anna Grandas*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

[2,5-dimethylfuran]-protected maleimides were placed at both internal positions and the 3'-end of oligonucleotides making use of solid-phase synthesis procedures. A new phosphoramidite derivative and a new solid support incorporating the protected maleimide moiety were prepared for this purpose. In all cases maleimide deprotection (retro-Diels–Alder reaction) followed by reaction with thiol-containing compounds afforded the target conjugate.

Introduction

Derivatization of oligonucleotides by covalent attachment of a non-oligonucleotide moiety, usually referred to as conjugation, is a common alternative to improve oligonucleotides performance in biological assays and suitability for therapeutic applications (1). 3'-Modification is known to impart protection to the most ubiquitous nucleases, namely 3'-exonucleases (2), and conjugation often improves oligonucleotides' cell permeation properties.

In general there is a rationale on the decision as to which moiety is to be linked to the oligonucleotide chain, but the linking site and the linkage type are most often dictated by the chemically available tools (resins or phosphoramidite derivatives commercially available, derivatives synthesized at the laboratory carrying out the study, etc.). Even though changes in the constitution of the conjugate will likely have an influence on the outcome of biological assays, there is not much information available on this type of structure-activity correlation.

Indeed, there are reports showing the outcome of such changes. It has been described, for instance, that the immune stimulating properties of conjugates in which a 28-mer β-amyloid peptide was attached to an oligonucleotide varied depending on whether the peptide was linked to either the 5'- or the 3'-end (3). It has also been reported that the stability of thiol-maleimide adducts may vary depending on the "external" environment, such as the presence of inorganic anions (4) or reducing agents (thiols) (5), but also depending on the structure of the conjugate itself (6). In a recent piece of work (6), Junutula and co-workers have shown that the stability and activity of antibody-drug conjugates varies depending on whether the conjugation site is placed in a highly solvent accessible and positively charged local environment, or in a partially accessible and neutral local environment.

In a different context, but also showing the importance of conjugates' constitution, an interesting example is the use of DNA as a ruler (7), which shows that the covalent attachment of peptides to different positions of the chain can provide information on the spatial distribution of protein binding sites (7, see references therein for other examples of applications of complex molecular structures).

Oligonucleotide conjugates can be assembled making use of solid-phase technologies when suitably derivatized building blocks of solid supports are available (8). Convergent solution synthesis is the other alternative. In this case the two moieties to be linked must be derivatized with functional groups ideally reacting regio- and chemoselectively, exploiting the so-called click chemistry (9). The par excellence click reaction is the Cu(I)-catalyzed azide-alkyne cycloaddition, but well-performing click reactions can exploit other functional groups as well. Maleimides are examples of useful functional units, since they can be involved in two different click reactions, the Michael-type reaction with thiols and the Diels-Alder cycloaddition.

For many years the most common alternative for the derivatization of oligonucleotides with a maleimide has been the reaction, in solution, of bifunctional compounds incorporating a carboxylic acid and a maleimide with the amine group of amino-derivatized oligonucleotides (10). The problem with this methodology is that amide formation does not always take place in high yield. Yet, attachment of the maleimide-containing bifunctional compound to amino-derivatized resin-linked oligonucleotides is not an alternative, because the ammonia treatment that removes oligonucleotide protecting groups degrades the maleimide (11).

Solid-phase assembly is only possible provided that the maleimide is protected and remains stable to the ammonia deprotection treatment. We have recently described that maleimide building blocks fulfilling this requirement can be obtained by reaction with 2,5-dimethylfuran, followed by removal of the ammonia-labile *endo* adduct (12). Bifunctional compounds containing a protected maleimide (*exo* adduct) and either phosphoramidite (**1**) or carboxyl (**2**) groups (Figure 1) can be used for the on-resin assembly of maleimido-oligonucleotides. Compound **1** has two advantages over **2**. On the one hand, that it can be incorporated after elongation of the oligonucleotide chain

using the same chemistry as for all nucleosides. On the other, it is not necessary to assemble amine-derivatized resin-linked oligonucleotides to which **2** must be coupled by forming an amide bond. Even though this type of reactions takes place on a solid matrix, which allows large excesses of the activated carboxyl-containing species to be added, coupling yields have been described as poorly reproducible and not always fully satisfactory (13).

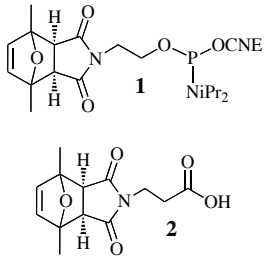


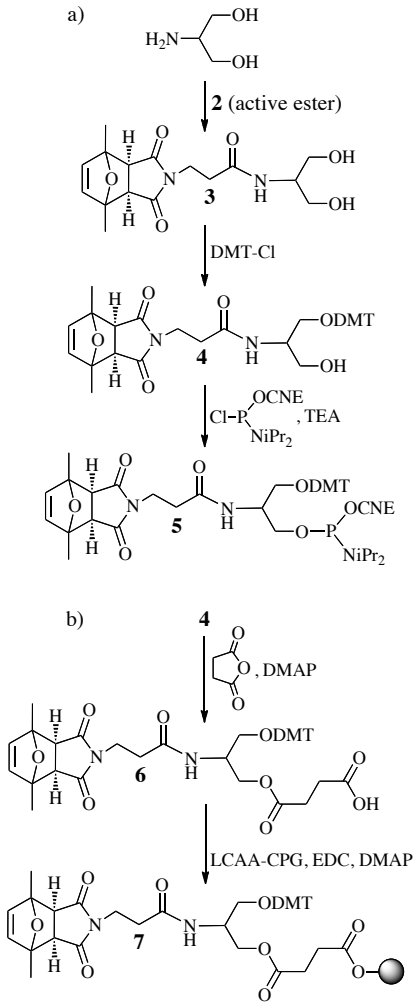
Figure 1. Structures of the [protected maleimido]-containing derivatives previously used in the synthesis of maleimido-oligonucleotides.

Incorporation of a protected maleimido unit after oligonucleotide elongation using standard nucleoside-3'-phosphoramidite derivatives, followed by deprotection with ammonia and heating to provide the free maleimide (retro-Diels–Alder reaction), affords 5'-maleimido-oligonucleotides. Chain elongation with nucleoside-5'-phosphoramidites might allow 3'-maleimido-oligonucleotides to be assembled, but these derivatives are much more expensive than standard nucleoside-3'-phosphoramidite. Since, as described above, it is important to have access to conjugates with different structures, we decided to prepare a new phosphoramidite building block allowing the maleimide unit to be placed at internal positions of the oligonucleotide sequence (in fact at any position of the chain), and a new solid matrix incorporating the protected maleimide moiety that is suitable for the synthesis of 3'-maleimido-oligonucleotides making use of 3'-phosphoramidite derivatives. In this manuscript we describe their synthesis and their use in the preparation of maleimido-oligonucleotides. These maleimido-oligonucleotides were reacted with thiols to assess the functionality of the maleimides.

Results and discussion

The synthesis of building block **5** and solid support **7** is summarized in Scheme 1. Both incorporate a DMT-protected hydroxyl to allow for further elongation of the chain. The two compounds **5** and **7** derive from common precursor **4**, which has the advantage that preparation of **4** provides a stock of a product from which either **5** or **7** can be obtained in one or two steps.

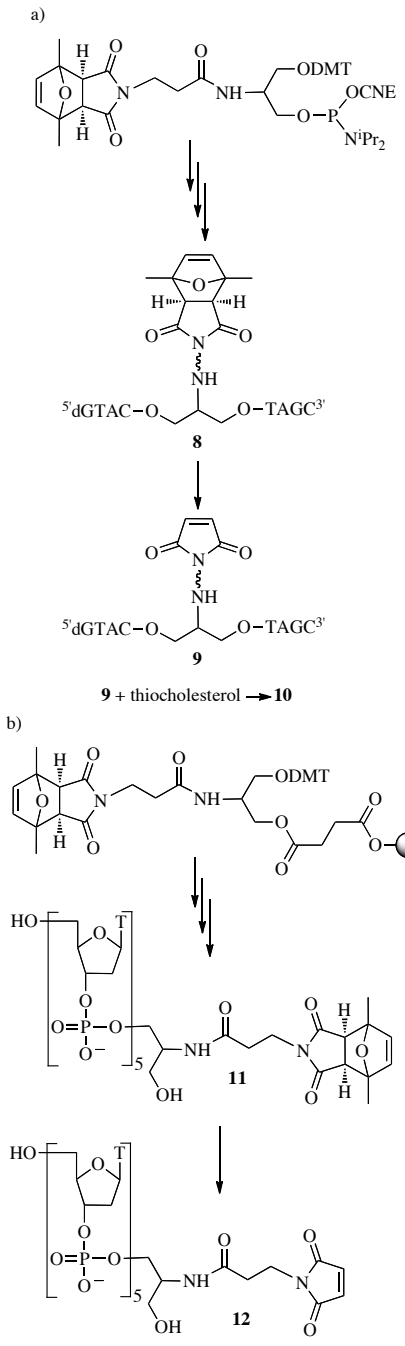
For the synthesis of **4**, the amino group of L-serinol, more nucleophilic than the hydroxyl groups, was derivatized first. Reaction between **2** (*exo* adduct), a carbodiimide and pentafluorophenol provided the pentafluorophenyl ester of **2**, and this active ester reacted with L-serinol to form the amide with no need to protect the hydroxyl groups. Then, one of the hydroxyl groups of the resulting compound (**3**) was protected by reaction with DMT-Cl. Use of a smaller amount of DMT-Cl (0.9 equiv) with respect to **3** facilitated formation and isolation of **4** as the main product. Phosphitylation of **4** afforded phosphoramidite **5**, while reaction of **4** with succinic anhydride and incorporation of the resulting **6** onto LCAA-CPG provided support **7**.



Scheme 1. Synthesis of [protected maleimido]-derivatized building block **5** (a) and solid support **7** (b).

Derivatives **5** and **7** were used in the synthesis of two maleimido-oligonucleotides, **9** and **12** (Scheme 2). In both cases chain elongation proceeded smoothly, showing that the two derivatives behaved as standard building blocks. The [protected maleimido]-oligonucleotides (**8** and **11**) obtained after reaction with ammonia at room temperature were purified by HPLC (Figure 2) and characterized by mass spectrometry.

Maleimide deprotection was carried out using the method that had performed better in our previous work (12, 14), namely heating a suspension of the oligonucleotide in anh. toluene at 90 ^{o}C for 4 h. Maleimide deprotection yields were above 90 %, as assessed by HPLC analysis of the crudes (Figure 3).



Scheme 2. Synthesis of [protected maleimido]-oligonucleotides, maleimido-oligonucleotides and conjugates.

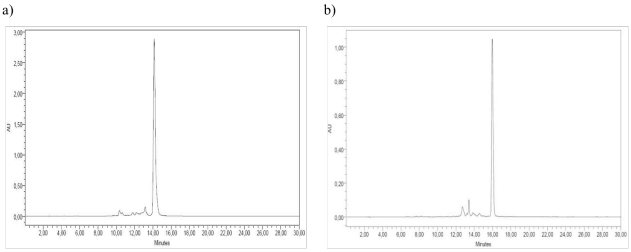


Figure 2. Crude $^{5'}$dGTAC-[protected maleimido monomer]-TAGC **8** (a) and dT_5-$^{3'}$[protected maleimide] **11** (b).

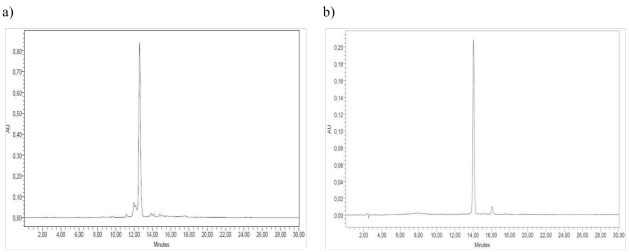


Figure 3. Crude $^{5'}$dGTAC-[maleimido monomer]-TAGC **9** (a) and dT_5-$^{3'}$[maleimide] **12** (b).

Finally, it was verified that reversal of the Diels-Alder dimethylfuran-maleimide cycloadduct afforded oligonucleotides with appending fully reactive maleimides. For this purpose, maleimido-oligonucleotides **9** and **12** were reacted with thiol-containing compounds, as shown in Scheme 2. Michael-type thiol-maleimide reactions yielded the target conjugates, as confirmed by mass spectrometric analysis of the product corresponding to the main peak of the HPLC trace of the crude (Figure 4). The maleimide moiety linked to the 3'-end of the oligonucleotide thoroughly reacted with two primary thiols, those of biotin-SH and glutathione, to yield conjugates **13** and **14**, respectively (Figure 4b,c). Likewise, reaction between the maleimide placed at an internal position and the secondary thiol of thiocholesterol, somehow more demanding because the reaction groups were more hindered, cleanly furnished conjugate **10** (Figure 4a).

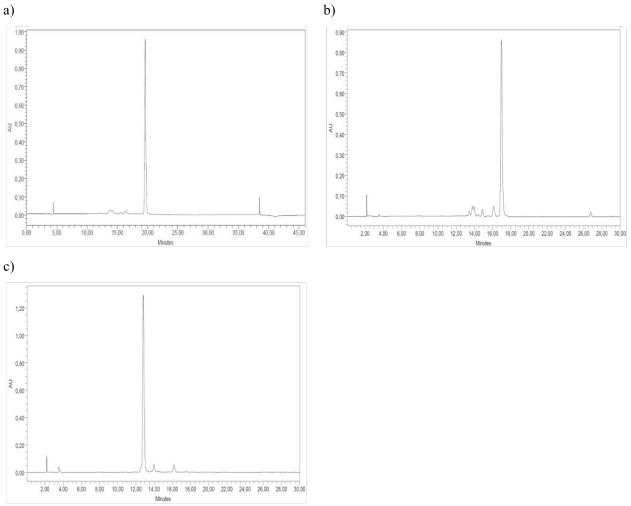


Figure 4. Crude conjugates **10** (a), **13** (b) and **14** (c).

Conclusions

In summary, a phosphoramidite derivative and a solid support incorporating 2,5-dimethylfuran-protected maleimide moieties were synthesized from a common precursor (**4**), obtained from L-serinol. Both were satisfactorily used in the preparation of maleimido-oligonucleotides that subsequently underwent addition of thiols, thus proving that maleimide deprotection affords reactive maleimides. This new building block and resin expand the repertoire of possibilities allowing the derivatization of oligonucleotides at different positions, and thus give access to differently decorated oligonucleotide chains.

Experimental

General

Nucleoside phosphoramidites (dA^{Pac}, dC^{Ac}, dG^{Dmf} and dT), CPG supports, and oligonucleotide synthesis reagents were from either Link Technologies, Glen Research Corporation or Applied Biosystems. [Protected maleimido]-propanoic acid (**2**, *exo* adduct) was prepared as previously described (12). Biotin-SH was prepared following described procedures (14). Glutathione and thiocholesterol were from Sigma-Aldrich. Acid-free DCM was obtained by filtration through basic alumina.

TLC was carried out on silica gel plates 60 F_{254} from Merck.

Samples were lyophilized in a FreezeMobile Virtis instrument.

The amount of free thiols in thiol-containing compounds was quantified by the Ellman test, as described in the Supporting Information of reference 12.

Oligonucleotide synthesis. Oligonucleotide chains were assembled in a 3400 ABI automatic synthesizer at the 1 μmol scale, using standard phosphoramidite synthesis cycles. Phosphoramidite **5** and solid support **7** were used as any standard reagent. After chain elongation, treatment with concd. aq. ammonia at room temperature (1 h in case of homo-dT oligomer **11**; 8 h when all the bases were present) removed protecting groups from the oligonucleotide. After filtration and washing, ammonia was evaporated under reduced pressure, and the sample lyophilized. Oligonucleotides were purified by reversed-phase HPLC, quantified by UV spectroscopy (λ = 260 nm), and characterized by mass spectrometry.

Maleimide deprotection by heating in toluene. The [protected maleimido]-containing oligonucleotide was dried by coevaporation with anh. toluene (3-4 ×), and a new batch of anh. toluene was added (the amount that would be required to obtain a 25 μM solution). The mixture was heated 90 ºC, toluene was removed under reduced pressure, and the crude was dissolved in water for HPLC analysis and mass spectrometric characterization. The crude was typically used at the subsequent conjugation step without any purification.

General procedure for Michael-type conjugation reactions. Aliquots of aqueous solutions containing the required amounts of maleimido-containing oligomer and the corresponding thiol-containing compound (5 to 10-fold molar excess) were mixed, and the mixture was diluted with 0.5 M triethylammonium acetate, pH = 7.8-7.9 (final concentration of oligomer: 50-150 μM). The mixture was stirred at room temperature under an Ar atmosphere. Reaction crudes were analyzed by HPLC. Conjugates were purified by HPLC and characterized by MALDI-TOF MS.

Synthesis of conjugate **10**: thiocholesterol (not soluble in water) was dissolved in THF, and the reaction was carried out in a 3:2 (v/v) 0.5 M triethylammonium acetate (pH = 7.8)/THF mixture.

HPLC. Reversed-phase HPLC analysis and purification was performed using analytical and semipreparative Waters or Shimadzu systems. Analysis and purification conditions were:

Oligonucleotide analysis conditions: Kromasil C18 column (10 μm, 100 Å, 250 × 4.0 mm) from Akzo Nobel; solvent A: 0.05 M triethylammonium acetate, solvent B: H_2O/ACN 1:1 (v/v), gradient from 5 to 60 % of B in 30 min, flow: 1 mL/min, detection wavelength: 254 nm.

Oligonucleotide purification conditions (semipreparative scale): Jupiter C18 column (10 μm, 300 Å, 250 × 10.0 mm) from Phenomenex, solvent A: 0.1 M triethylammonium acetate, solvent B: H_2O/ACN 1:1 (v/v), gradient from 5 to 60 % of B in 30 min, flow: 3 mL/min, detection wavelength: 260 nm.

Cholesterol-containing conjugate analysis and purification conditions: Kromasil C4 column (10 μm, 100 Å, 250 × 4.6 mm) from Teknokroma, solvent A: 0.05 M triethylammonium acetate (pH = 7.0), solvent B: acetonitrile, flow: 1 mL/min, detection wavelength: 254 nm. After injection of the samples, the column was eluted for 5 min with 10% of B, followed by gradient from 10 to 90 % of B in 25 min. Subsequently, the column was eluted for 10 min with 90% of B.

Mass spectrometry. MALDI-TOF mass spectra were recorded on a 4800 *Plus* ABSciex instrument using reflector. Oligonucleotide and conjugates analysis conditions: 1:1 (v/v) 2,4,6-trihydroxyacetophenone/ammonium citrate (THAP/CA), negative mode. ESI (low and high resolution) mass spectra were obtained using an LC/MSD-TOF spectrometer from Agilent Technologies.

Synthesis of the [protected maleimido]-containing monomer 5 and solid support 7.

[Protected maleimido]-diol 3. 2,5-Dimethylfuran-protected 3-maleimidopropanoic acid (**2**, 300 mg, 1.13 mmol), DIPC (177 μL, 1.13 mmol) and pentafluorophenol (210 mg, 1.13 mmol) were dissolved in anh. DMF (*N,N*-dimethylformamide). After 30 min stirring, serinol (90 mg, 0.942 mmol) was added to the mixture containing the activated form of the carboxyl group, and left to react at room temperature under an Ar atmosphere for 12 h. The solvent was removed under reduced pressure, and the resulting crude was purified by silica gel column chromatography eluting with DCM (dichloromethane) and increasing amounts of MeOH (from 0 to 10 %). **3** was obtained as a white solid (318 mg, quantitative yield).

TLC (DCM/MeOH 4:1): Rf = 0.32; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.30 (s, 2H), 3.86-3.82 (m, 6H), 3.78 (td, *J* = 7.0, 3.8 Hz, 1H), 3.02 (t, *J* = 6.0 Hz, 2H), 2.84 (s, 2H), 2.57 (t, *J* = 6.0 Hz, 2H), 1.70 (s, 6H); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 174.8, 159.1, 140.9, 87.9, 64.4, 52.6, 52.5, 35.6, 34.5, 16.0 ppm; HRMS (ESI, positive mode): *m/z* 339.1539, calcd. for $C_{16}H_{23}N_2O_6$ $[M+H]^+$ 339.1551, *m/z* 361.1374, calcd. for $C_{16}H_{22}N_2O_6Na$ $[M+Na]^+$ 361.1370, *m/z* 699.2834, calcd. for $C_{32}H_{44}N_4O_{12}Na$ $[2M+Na]^+$ 699.2848.

DMT-*O*-[Protected maleimido]-diol 4. Compound **3** (318 mg, 0.94 mmol) and DMT-Cl (293 mg, 0.85 mmol) was dissolved in anh. pyridine (10 mL). After 15 h stirring at room temperature under an Ar atmosphere, pyridine was removed under reduced pressure. The resulting crude was purified by silica gel column chromatography eluting with hexanes/DCM 1:1, increasing amounts of DCM (until 100 %) and DCM/AcOEt mixtures (up to 7:3) always with triethylamine (4 %). The fractions containing the desired product were pooled, and after removing the solvent in vacuo were coevaporated with ACN to eliminate triethylamine traces, dissolved in acid-free DCM (25 mL) and washed with brine (3 × 25 mL). The organic phase was dried over anh. Na_2SO_4 and taken to dryness. A white solid (**4**, 410 mg, 75 % yield) was obtained.

TLC (DCM/MeOH 19:1): Rf = 0.33; ^{1}H NMR ($CDCl_3$, 400 MHz

$CDCl_3$): δ 7.39-7.19 (m, 9H), 6.83 (d, *J* = 8.9 Hz, 4H), 6.28 (s, 2H), 5.98 (d, *J* = 7.6 Hz, 1H), 4.03 (tt, *J* = 9.2, 4.7 Hz, 1H), 3.84 (dd, *J* = 13.8, 7.3 Hz, 2H), 3.79 (s, 6H), 3.70 (dd, *J* = 13.7, 7.0 Hz, 2H), 3.29 (qd, *J* = 9.7, 4.8 Hz, 2H), 2.78 (dd, *J* = 1.4 Hz, 2H), 2.47 (t, *J* = 6.6 Hz, 2H) 1.67 (s, 6H); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 174.8, 170.1, 158.7, 144.6, 141.0, 135.7, 130.1, 128.1, 127.1, 113.4, 87.8, 86.7, 63.6, 63.2, 55.4, 52.5, 51.7, 35.2, 34.2, 29.8, 16.0 ppm; HRMS (ESI, positive mode): *m/z* 663.2672, calcd. for $C_{37}H_{40}N_2O_8Na$ $[M+Na]^+$ 663.2677, *m/z* 1303.5430, calcd. for $C_{74}H_{80}N_4O_{16}Na$ $[2M+Na]^+$ 1303.5462.

DMT-*O*-[Protected maleimido]-diol phosphoramidite 5. Compound **4** (225 mg, 0.35 mmol) was dried by coevaporation with anh. ACN (3 × 1 mL), and dissolved in anh. DCM (1 mL). Triethylamine (1 mL, 7.17 mmol) and (2-cyanoethoxy)chloro(diisopropylamino)-phosphine (83 μL, 0.37 mmol) was added, and the mixture was reacted at room temperature for 1 h under an Ar atmosphere. This was followed by addition of DCM (25 mL) and extraction with 5 % $NaHCO_3$ (2 × 25 mL) and brine (1 × 25 mL). The organic phase was dried over Na_2SO_4 and the solvent was removed under vacuum, adding ACN to prevent the amidite solution from being too rich in TEA. Phosphoramidite **5** was obtained as a white powder after lyophilization from anh. benzene (270 mg, 92 % yield; single spot by TLC). This solid was used for the assembly of maleimido-oligonucleotide **8** in spite of being slightly impurified with the phosphine hydrolysis product. Attempts to purify **5** by silica gel column chromatography were unsuccessful, because the collected fractions were less pure than the material obtained after the work-up.

TLC (DCM/MeOH 19:1): Rf = 0.56; ^{31}P NMR ($CDCl_3$, 121.42 MHz): δ 148.2, 148.0 ppm (plus one signal at 14.2 ppm); HRMS (ESI, positive mode): *m/z* 863.3774, calcd. for $C_{46}H_{57}N_4O_9PNa$ $[M+Na]^+$ 863.3755.

DMT-*O*-[Protected maleimido]-diol hemisuccinate 6. DMAP (3.8 mg, 0.031 mmol) and succinic anhydride (6.4 mg, 0.062 mmol) were added to a solution of alcohol **4** (40 mg, 0.062 mmol) in anh. pyridine (200 μL). The mixture was reacted at room temperature, under an Ar atmosphere, for 15 h, after which time TLC analysis showed that the starting material (**4**) had been completely consumed. After removing the solvent in vacuo, and coevaporation with ACN (3 × 1 mL), the resulting crude was dissolved in acid-free DCM (2 mL), and this solution was extracted with a 10 % aqueous citric acid solution (3 × 2 mL), water (1 × 2 mL), and brine (1 × 2 mL). The organic phase was dried over anh. Na_2SO_4, and solvent removal under reduced pressure afforded **6** as a whitish solid (45 mg, 98 % yield).

TLC (DCM/MeOH 19:1): Rf = 0.32; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.41 – 7.39 (m, 2H), 7.28 (dd, *J* = 8.9, 1.4 Hz, 6H), 7.19 (dd, *J* = 18.3, 8.1 Hz, 1H), 6.83 (dd, *J* = 8.9, 1.2 Hz, 4H), 6.29 (s, 2H), 6.13 (d, *J* = 8.2 Hz, 1H), 4.35 (s, 2H), 3.79 (s, 6H), 3.78 – 3.70 (m, 2H), 3.23 (dd, *J* = 9.1, 4.0 Hz, 1H), 3.10 (dd, *J* = 9.0, 6.8 Hz, 1H), 2.78 (s, 2H), 2.70 – 2.60 (m, 2H), 2.60 – 2.48 (m, 4H), 2.45 – 2.38 (m, 2H), 1.67 (d, *J* = 3.9 Hz, 6H); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 175.2, 174.6, 172.3, 169.5, 158.7, 144.7, 141.0, 135.9, 130.1, 129.3, 128.2, 128.0, 127.0, 113.3, 87.9, 86.4, 63.6, 62.0, 55.4, 52.6, 48.5, 35.4, 34.3, 29.8, 29.6, 29.1, 15.9 ppm; HRMS (ESI, positive mode): *m/z* 763.2833, calcd. for $C_{41}H_{44}N_2O_{11}Na$ $[M+Na]^+$ 763.2837, *m/z* 1503.5780, calcd. for $C_{82}H_{88}N_4O_{22}Na$ $[2M+Na]^+$ 1503.5782.

DMT-*O*-[Protected maleimido]-diol-succinyl-LCAA-CPG 7 (15). LCAA-CPG (100 mg, 71 μmol/g) was introduced in a 2 mL-polyethylene syringe fitted with a polypropylene disc, washed (DCM: 3 × 2 mL, 3 % TCA in DCM: 3 × 2 mL, 20 % TEA in DCM: 5 × 5 mL, and acid-free DCM: 5 × 5 mL), and thoroughly dried. Compound **6** (21 mg, 0.028 mmol), DMAP (2 mg, 0.014 mmol) and EDC (54 mg, 0.28 mmol) was dissolved in a 70:1 (v/v) mixture (710 μL) of anh. pyridine and anh. TEA, respectively, and the resulting solution poured into the syringe containing the LCAA-CPG. The mixture was gently stirred using a shaker under an Ar atmosphere for 3.5 h. The CPG beads were then filtered, washed (pyridine: 10 mL, MeOH: 20 mL, and acid-free DCM: 30 mL) and dried. Removal of the DMT group from an aliquot of DMT-*O*-[protected maleimido propanoyl]-*N*-succinylserinol-LCAA-CPG showed that the solid matrix (**7**, substitution degree = 31 μmol/g) was suitable for solid-phase oligonucleotide synthesis. Possible unreacted amines were capped by reaction with a 1:1 (v/v) mixture of the Cap A and Cap B reagents used at the capping step in solid-phase oligonucleotide synthesis (2 h, room temperature), after which time the glass beads were filtered, washed (MeOH: 20 mL, acid-free DCM: 30 mL) and dried.

Oligonucleotides and conjugates.

$^{5'}$dGTAC-[protected maleimido monomer]-TAGC 8 (87 % yield in the crude, as assessed by HPLC) was purified by HPLC at the semipreparative scale. Analytical HPLC: t_R = 14.2 min (Figure 2a); MALDI-TOF MS (negative mode): *m/z* 2807.1 $[M-H]^-$, M calcd. for $C_{94}H_{120}N_{32}O_{54}P_8$ 2808.6.

dT$_5$-$^{3'}$[protected maleimide] 11 (86 % yield in the crude, as assessed by HPLC) was purified by HPLC at the semipreparative scale. Analytical HPLC: t_R = 16.0 min (Figure 2b); MALDI-TOF MS (negative mode): *m/z* 1857.4 $[M-H]^-$, M calcd. for $C_{66}H_{87}N_{12}O_{41}P_5$ 1858.4.

dGTAC-[maleimido]-TAGC 9 was obtained after deprotection of $^{5'}$dGTAC-[protected maleimide]-TAGC **8** as described above (quantitative deprotection as assessed by HPLC of the crude, Figure 3a). Analytical HPLC (gradient from 5 to 60 % of B in 30 min): t_R = 12.6 min; MALDI-TOF MS (negative mode): *m/z* 2711.1 $[M-H]^-$, M calcd. for $C_{88}H_{112}N_{32}O_{53}P_8$ 2712.5. The crude was used without further purification in the conjugation reaction.

dT$_5$-$^{3'}$[maleimide] 12 was obtained after deprotection of dT$_5$-$^{3'}$[protected maleimide] **11** as described above (96 % deprotection yield as assessed by HPLC of the crude, Figure 3b). Analytical HPLC: t_R = 14.1 min; MALDI-TOF MS (negative mode): *m/z* 1761.4 $[M-H]^-$, M calcd. for $C_{60}H_{79}N_{12}O_{40}P_5$ 1762.3. The crude was used without further purification in the conjugation reactions.

Conjugate **10** (**9** + thiocholesterol). After stirring the conjugation reaction mixture overnight, the solvent was removed under vacuum, water was added and the insoluble residue was filtered (13 mm Syringe Filter 0.2 mm PTFE membrane). C18 HPLC analysis of the crude showed virtually complete disappearance of the peak of T$_5$-$^{3'}$maleimide (**9**); C4 HPLC trace of the crude is shown in Figure 4a, t_R = 19.6 min; MALDI-TOF MS (negative mode): *m/z* 3114.2 $[M-H]^-$, M calcd. for $C_{115}H_{158}N_{32}O_{53}P_8S$ 3114.8.

Conjugate 13 (**12** + biotin). Conjugation reaction time: 1 h. 95 %; conjugation yield (from the HPLC of the crude, Figure 4b): 95

%. Analytical HPLC: t_R = 17.0 min; MALDI-TOF MS (negative mode): *m/z* 2064.4 $[M-H]^-$, M calcd. for $C_{72}H_{100}N_{15}O_{42}P_5S_2$ 2065.4.

Conjugate 14 (**12** + glutathione). Conjugation reaction time: 1 h. 96 %; conjugation yield (from the HPLC of the crude, Figure 4c): 96 %. Analytical HPLC: t_R = 12.8 min; MALDI-TOF MS (negative mode): *m/z* 2068.3 $[M-H]^-$, M calcd. for $C_{70}H_{96}N_{15}O_{46}P_5S$ 2069.4.

Acknowledgments. This work was supported by funds from the Ministerio de Ciencia e Innovación (grant CTQ2010-21567-C02-01, and the project RNAREG, grant CSD2009-00080, funded under the programme CONSOLIDER INGENIO 2010), and the Generalitat de Catalunya (2009SGR-208). A. S. was a recipient fellow of the Generalitat de Catalunya.

Departament de Química Orgànica and IBUB, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain. Fax: +3493339787; Tel: +34934021263; E-mail: anna.grandas@ub.edu

Abbreviations. ACN=acetonitrile, CNE=2-cyanoethyl, DCM=dichloromethane, DIPC=*N,N′*-diisopropylcarbodiimide, DMAP=*N,N*-dimethylaminopyridine, DMF=*N,N*-dimethylformamide, Dmf=dimethylaminomethylene (dimethylformamidine), DMT=4,4'-dimethoxytrityl, EDC=*N*-ethyl-*N′*-(3-dimethylaminopropyl)-carbodiimide·HCl, LCAA-CPG=long chain aminoalkyl controlled pore glass beads, Pac=phenoxyacetyl, TCA=trichloroacetic acid, TEA=triethylamine, THF=tetrahydrofuran.

References

1 *(a)* R. L. Juliano, X. Ming and O. Nakagawa, *Acc. Chem. Res.*, 2012, in press (DOI: 10.1021/ar2002123); (b) Y. Singh, P. Murat and E. Defrancq, *Chem. Soc. Rev.*, 2010, **39**, 2054-2070; *(c)* J. Kalia and R. T. Raines, *Curr. Org. Chem.*, 2010, **14**, 138-147.

2 C. D. Juby, C. D. Richardson and R. Brousseau, *Tetrahedron Lett.*, 1991, **32**, 879-882.

3 M. R. Putta, F.-G. Zhu, D. Wang, L. Bhagat, M. Dai, E. R. Kandimalla and S. Agrawal, *Bioconjugate Chem.*, 2010, **21**, 39-45.

4 J. Kalia and R. T. Raines, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 6286-6289.

5 A. D. Baldwin and K. L. Kiick, *Bioconjugate Chem.*, 2011, **22**, 1946-1953.

6 B.-Q. Shen et al., *Nat. Biotechnol.*, 2012, **30**, 184-189.

7 H. Eberhard, F. Diezmann and O. Seitz, *Angew. Chem. Int. Edit.*, 2011, **50**, 4146-4150.

8 H. Lönnberg, *Bioconjugate Chem.*, 2009, **20**, 1065-1094.

9 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem. Int. Edit.*, 2001, **40**, 2004-2021.

10 *(a)* A. Chollet, *Nucleosides & Nucleotides,* 1990, **9**, 957-966; ***(b)*** C.-H. Tung, M. J. Rudolph and S. Stein *Bioconjugate Chem*., 1991, **2**, 464-465; *(c)* Y. Uchiyama, H. Inoue, E. Ohtsuka, C. Nakai, S. Kanaya, Y. Ueno and M. Ikehara, *Bioconjugate Chem.*, 1994, **5**, 327-332; *(d)* J.-P. Bongartz, A.-M. Aubertin, P. G. Milhaud and B. Lebleu, *Nucleic Acids Res.*, 1994, **22**, 4681-4688; *(e)* J. G. Harrison and S. Balasubramanian, *Nucleic Acids Res.*, 1998, **26**, 3136-3145; *(f)* M. A. Zanta, P. Belguise-Valladier and J. P. Behr, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 91-96; *(g)* F. Kukolka and C. Niemeyer, *Org. Biomol. Chem.*, 2004, **2**, 2203-2206; *(h)* B. A. R. Williams and J. C. Chaput, in *Current Protocols in Nucleic Acid Chemistry*, ed. M. Egli, P. Herdewijn, A. Matsuda and Y. Sanghvi, John Wiley & Sons, USA, 2010, unit 4.41, pp. 4.41.1-4.41.20.

11 *(a)* J. D. Gregory, *J. Am. Chem. Soc.*, 1955, **77**, 3922-3923; *(b)* P. O. Tawney, R. H. Snyder, R. P. Conger, K. A. Leibbrand, C. H. Stitelerand and A. R. Williams, *J. Org. Chem.*, 1964, **26**, 15-21.

12 A. Sánchez, E. Pedroso and A. Grandas, *Org. Lett.*, 2011, **13**, 4364-4367.

13 *(a)* J. Casals, L. Debéthune, K. Alvarez, A. Risitano, K. R. Fox, A. Grandas and E. Pedroso, *Bioconjugate Chem.*, 2006, **17**, 1351-1359; *(b)* S. Zaramella, E. Yeheskiely and R. Stromberg, *J. Am. Chem. Soc.*, 2004, **126**, 14029-14035; *(c)* S. Peyrottes, B. Mestre, F. Burlina and M. J. Gait, *Tetrahedron*, 1998, **54**, 12513-12522; *(d)* C. N. Tetzlaff, I. Schwope, C. F. Bleczinski, J. A. Steinberg and C. Richert, *Tetrahedron Lett.*, 1998, **39**, 4215-4218.

14 A. Sánchez, E. Pedroso and A. Grandas, *Bioconjugate Chem.*, 2012, **23**, 300-307.

15 R. T. Pon, in *Current Protocols in Nucleic Acid Chemistry*, ed. S. L. Beaucage, D. E. Bergstrom, P. Herdewijn and A. Matsuda, John Wiley & Sons, Inc., USA, 2000, unit 3.2, pp. 3.2.1-3.2.23.