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Maleimide-Dimethylfuran *exo* Adducts: Effective Maleimide Protection in the Synthesis of Oligonucleotide Conjugates

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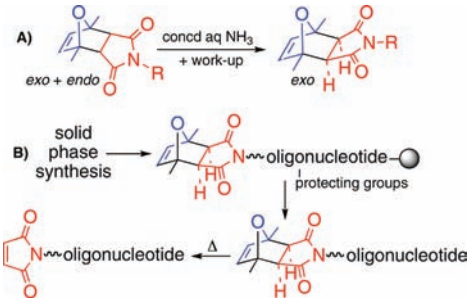
ABSTRACT



The reaction of maleimide-containing compounds with 2,5-dimethylfuran gives a mixture of *exo* and *endo* isomers from which the *exo* cycloadduct can be easily isolated taking advantage of its stability in concentrated aqueous ammonia. Bifunctional compounds incorporating a dimethylfuran-protected maleimide (*exo* adduct) have been attached to resin-linked oligonucleotide chains. Removal of protecting groups masking oligonucleotide functionalities followed by retro-Diels–Alder maleimide deprotection affords maleimido-oligonucleotides suitable for conjugation, as assessed by their reaction with different thiols.

Bio- and nanotechnological applications of oligonucleotides commonly require the use of analogs.[1] Modifications in the sugar–phosphate backbone or in the nucleobases generally improve stability in biological fluids, but to follow the fate of oligonucleotides or to enhance cell uptake, nucleic acid chains have to be attached to nonoligonucleotide molecules. The resulting hybrid compounds are referred to as conjugates.

Oligonucleotide conjugates can be assembled on a solid matrix or in solution.[2] Solution conjugation chemistry has made extensive use of the maleimide group. With very few exceptions,[3] maleimido-oligonucleotides have been prepared by forming an amide bond between amino-derivatized oligonucleotides and bifunctional compounds containing the maleimide moiety and a carboxyl group (or the corresponding activated ester) in solution.[4] Coupling of the maleimide to resin-linked oligonucleotides would be a more straightforward alternative. Yet, the instability of maleimides to oligonucleotide deprotection conditions (reaction with ammonia) renders this alternative impractical.[5]

We describe here a new, straightforward procedure to prepare maleimido-oligonucleotides based on the use of the *exo* maleimide-2,5-dimethylfuran cycloadduct, which

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is stable to concd aq ammonia. Suitably derivatized protected maleimide derivatives are attached to resin-linked oligonucleotides, affording [protected maleimido]-oligonucleotides after standard deprotection treatments. Then, maleimide deprotection (retro-Diels–Alder reaction) affords the target maleimido-oligonucleotides, which can be conjugated with thiol-derivatized small molecules or biomolecules.

Maleimides have commonly been protected by reaction with furan,[6] and the resulting succinimides are reported to be less labile to nucleophiles than maleimides.[7]

To identify a convenient protecting group, we compared three protected maleimide derivatives, namely those obtained by reaction of 3-maleimidopropanoic acid with furan, 2-methylfuran, or 2,5-dimethylfuran (**1**, **2** and **3**, respectively; see Figure 1). We found that the dimethylfuran-

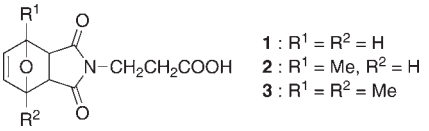


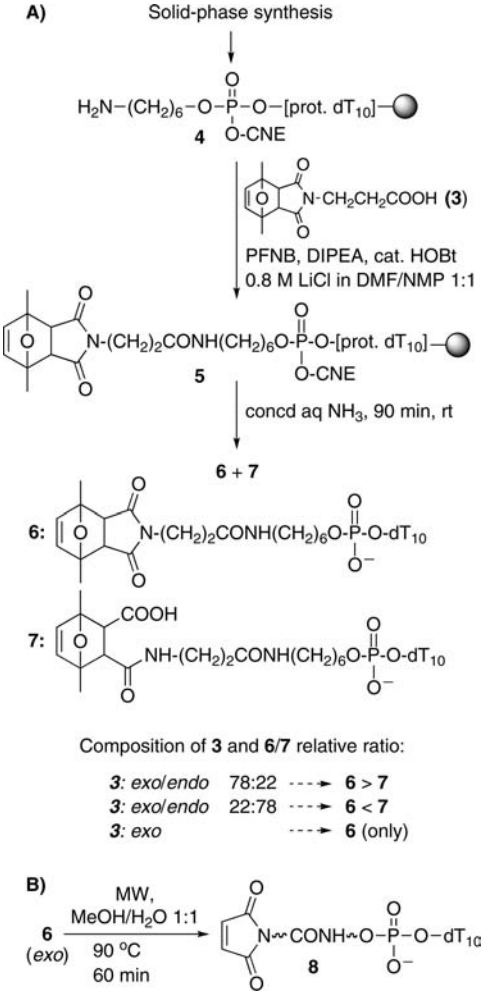
Figure 1. Furan-protected 3-maleimidopropanoic acid derivatives.

protected maleimide **3** was the best suited for our purpose (see Supporting Information).

In the first experiments aimed toward obtaining a maleimido-oligonucleotide (**6**, Scheme 1A), different batches of **3** containing different ratios of *exo*/*endo* cycloadducts were coupled to the amino-oligonucleotide-resin **4**. HPLC analysis of the crudes obtained after ammonia treatment (Figure S1) showed that their composition varied depending on the batch of **3**. Since we found a good correlation between the amount of target **6** in the crude and that of *exo* adduct in **3**, we concluded that only the *exo* isomer was stable to ammonia and, thus, suitable for the preparation of maleimido-oligonucleotides. A side product resulting from hydrolysis of the succinimide (**7**) was formed from the *endo* adduct.

In none of the reaction conditions tested was it possible to obtain only the *exo* adduct of **3**, and separation by column chromatography appeared to be difficult and low yielding. Pure *exo* **3** could be obtained by exploiting the different stabilities to ammonia of the two cycloadducts, after an overnight treatment of the *exo*/*endo* mixture

Scheme 1. First Assays to Prepare Maleimido-dT$_{10}$



with ammonia at room temperature and subsequent workup.

Coupling of **3** (pure *exo*) to **4**,[8,9] followed by reaction with concd aq ammonia, afforded the target [protected maleimido]-dT$_{10}$ **6** (Figure S1c). Finally (Scheme 1B), microwave irradiation of a solution of **6** in MeOH/water (60 min, 90 °C) provided the desired maleimido-dT$_{10}$ **8** (75% maleimide deprotection yield, Figure S2).

With the aim of incorporating the protected maleimide using the same chemistry as that for standard nucleoside building blocks, we prepared the phosphoramidite of a protected maleimide derivative (**11**, Scheme 2).

N-(2-Hydroxyethyl)maleimide (**9**) was synthesized following described procedures with minor modifications.[10]

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(9) Abbreviations: ACN = acetonitrile, CNE = 2-cyanoethyl, CPG = controlled pore glass, DIPEA = *N*,*N*-diisopropylethylamine, HOBt = 1-hydroxybenzotriazole, Pac = phenoxyacetyl, iPrPac = (4-isopropylphenoxy)acetyl, PFNB = pentafluorophenyl 4-nitrobenzene-sulfonate, TBDMS = *tert*-butyldimethylsilyl.

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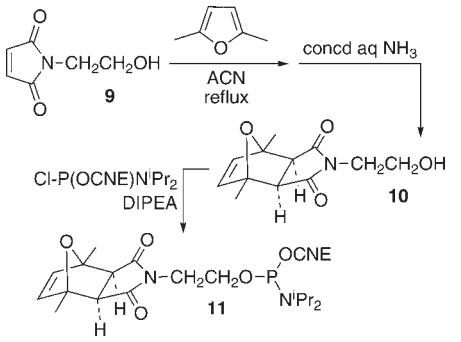
Reaction with 2,5-dimethylfuran protected the maleimide double bond, and treatment with ammonia removed the

Scheme 2. Preparation of [Protected Maleimido]-Phosphoramidite **11** (*exo* Adduct)



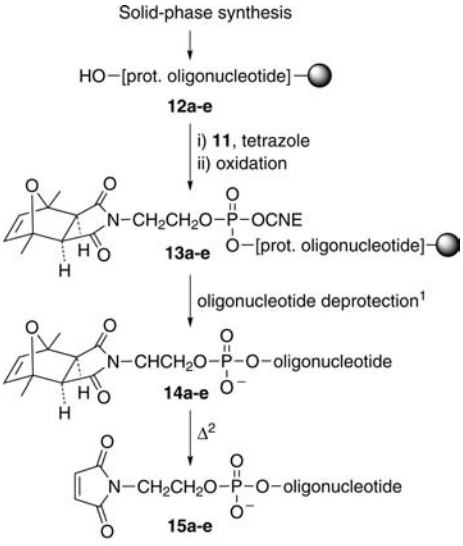
endo adduct. Finally, the hydroxyl group of **10** (pure *exo* isomer) was phosphitylated.

Phosphoramidite **11** was coupled to a series of resin-linked, protected oligonucleotides (**12**) differing in length and composition (four 2′-deoxyoligoribonucleotides and an RNA sequence, Scheme 3). Since the protected maleimide moiety is not stable to the conditions required to remove standard nucleobase protecting groups, ultra mild protection was used (phenoxyacetyl, acetyl, and isopropylphenoxyacetyl for A, C, and G, respectively). It is worth mentioning here that, in addition to not being degraded by concd aq ammonia (rt), the protected maleimide is stable to 0.05 M K_2CO_3 in MeOH (4 h, rt) and to the 1:1 ammonia/methylamine (AMA) deprotection mixture (2 h, rt). Deprotection and cleavage of **13a**−**e** provided [protected maleimido]-oligonucleotides **14a**−**14e** (Scheme 3, Figure S3a−e), which have proved to be highly stable compounds.

Maleimide deprotection was first performed by microwave irradiation of 1:1 MeOH/water solutions of **14a**−**14e** (90 min, 90 °C). The extent of the retro-Diels−Alder reaction ranged between 92 and 97% (Figure S4a−e), and mass spectrometry confirmed the identity of **15a**−**15e**. Yet, we observed that these conditions occasionally afforded less homogeneous crudes.

Additional experiments were carried out with **14b** to find maleimide deprotection conditions yielding consistent, satisfactory results. Overnight heating of 1:1 MeOH/water or 3:1 acetonitrile/water solutions at 55 and 75 °C, respectively, afforded mixtures containing the target compound, unreacted starting material, and hydrolyzed maleimide (Figure S5). To prevent formation of hydrolysis-derived side products, different aliquots of **14b** were dried by coevaporation with toluene (3 ×), toluene was added to the resulting solid, and the mixture was heated at 90 °C for periods ranging between 1 and 5 h. The maleimide deprotection yield was found to be 65% after 1 h, 92% after 2 h, and 95% after 3 h. A heating time of 3 h afforded maleimido-oligonucleotide **15b** at a degree of homogeneity similar to that of the product obtained after microwave irradiation, as shown by HPLC (Figure S6a). Prolonged

Scheme 3. Maleimido-oligonucleotides: On-Resin Assembly and Deprotection

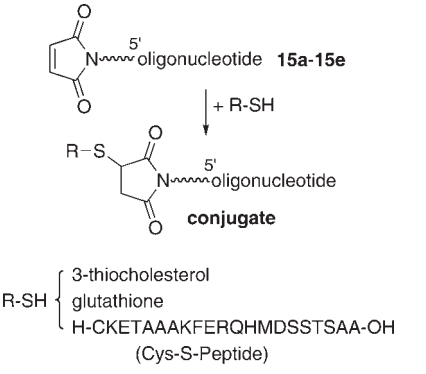


heating gave less homogeneous crudes. Maleimido-oligonucleotides **15c** and **15d** were also obtained using this deprotection procedure (Figure S6b,c).

Finally, to prove their aptness for conjugation, maleimido-oligonucleotides **15a**−**15e** were reacted with different thiols (Scheme 4), either lipophilic compounds such as thiocholesterol or water-soluble peptides (glutathione and the 21-mer H-Cys-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH (Cys-S-Peptide), which is the cysteine-derivatized S-peptide from ribonuclease S).[11]

Michael-type conjugation reactions were carried out using crude maleimido-oligonucleotides, which are not

Scheme 4. Conjugation Reactions



highly stable,[5] after the maleimide deprotection step. The small amounts of [protected maleimido]-oligonucleotide that may still be present in the crude do not react and can be easily separated from the conjugate in the subsequent purification step. Conjugates (Scheme 4) were obtained by reacting maleimido-oligonucleotides **15a**−**15e** with 5−10 equiv of thiol at a slightly basic pH (7.7), overnight at room temperature, in 0.5 M triethylammonium acetate (3:2 THF/triethylammonium acetate mixtures were used in reactions with thiocholesterol). Conjugation reaction crudes were analyzed by either HPLC or PAGE (denaturing conditions), using C4 rather than a C18 stationary phase in the case of cholesterol-oligonucleotide conjugates (Figures S7−S10).[12] Mass spectrometric characterization confirmed the identity of all conjugates.

In summary, ammonia treatment (room temperature) of the mixture formed upon reaction of maleimide-containing compounds with 2,5-dimethylfuran allowed the stable *exo* adducts to be easily isolated. Dimethylfuran-protected maleimide derivatives provided with the required functional group were used for the first time for the on-resin assembly of maleimido-oligonucleotides. [Protected maleimido]-oligonucleotides were obtained after removal of oligonucleotide protecting groups under standard conditions. Subsequent retro-Diels−Alder reaction deprotected the maleimide moiety in high yield, affording oligonucleotides with fully reactive appending maleimide groups.

Both reaction of maleimido-oligonucleotides with thiols[13] and Diels−Alder cycloadditions[14] may be useful to prepare oligonucleotide conjugates with improved pharmacokinetic properties. Maleimido-oligonucleotides may also find application for immobilization on different surfaces, as well as in the preparation of microarrays and nanosensors.[15]

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Supporting Information Available. Experimental procedures, compound characterization data and spectra, and HPLC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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