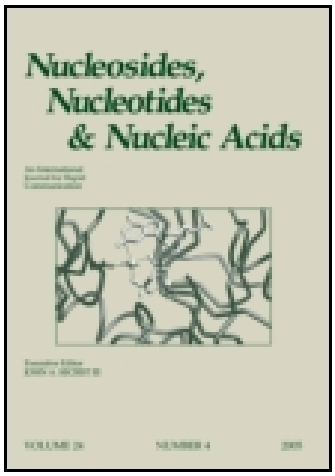

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COVALENT MODIFICATION AND SURFACE IMMOBILIZATION OF NUCLEIC ACIDS VIA THE DIELS-ALDER BIOCONJUGATION METHOD

Gregory M. Husar, Dominick J. Anziano, Michael Leuck, and David P. Sebesta*

Proligo, LLC, 2995 Wilderness Place, Suite 207, Boulder, Colorado 80301

ABSTRACT

The importance of chemically modified and surface immobilized nucleic acids has inspired the development of a wide variety of complementary techniques for covalent oligonucleotide preparation and immobilization. We are developing technology based on the use of a Diels-Alder reaction for accomplishing the covalent modification of oligonucleotides. Reported herein is preliminary progress toward the establishment of robust reagents for introducing the reactive functionality, as well as studies employing the BIACORE system to demonstrate surface immobilization by the method.

Covalently modified nucleic acids are serving as increasingly important tools in molecular biological, clinical and diagnostic applications (1). Commonly employed methods for constructing bioconjugates of oligonucleotides include the introduction of the chemical modifier via automated synthesis (most typically via specifically designed phosphoramidite reagents) or via post synthetic condensation with reactive handles introduced during automated synthesis. While the former approach involves fewer steps and in principle requires only a single purification, the preparation of labeled oligonucleotides by direct solid phase synthesis can be complicated by reagent incompatibility with synthesis conditions as well as a relatively limited selection of readily available specialty amidites.

*Corresponding author.

The second approach to oligonucleotide bioconjugates relies on introduction of nucleophilic reactive handles during solid phase synthesis (generally N-alkyl amines (2) or mercaptans (3–5)), followed by post-synthetic conjugation with electrophilic reactive labels. While post-synthetic conjugation affords more flexibility than relying on specialty amidite approaches, a number of complications can also afflict this method. In our hands, phosphoramidite reagents, such as the commonly employed MMT- or TFA-amino linkers, exhibit decreased stability and coupling efficiency relative to standard nucleoside phosphoramidites. We have consistently observed an increased lability for amidites derived from 1° vs 2° alcohols, along with decreased coupling performance during solid-phase synthesis. Additionally, in our hands, standard oligonucleotide deprotection conditions have led to detectable percentages of N-cyanoethylation of amino linker moieties, often complicating conjugate preparation by this method. Protected mercaptan reactive groups require an additional downstream processing step as well; specifically, the reduction of the dimeric disulfide construct which often results from exposure to air, consequently, amine condensation with electrophilic labeling reagents seems to have seen more use. With respect to post-synthetic conjugate formations from such functionally modified oligonucleotides, competing hydrolysis of labeling reagent, incomplete conversion or multiple labeling of the oligo can all lead to decreased yields and complicate isolation and purification of the desired conjugate products.

We report herein preliminary progress toward a complementary method for preparing covalently modified oligonucleotides by a post-solid phase synthesis method relying on a cycloaddition reaction as the covalent binding event (6). Specifically, the highly selective reaction between a diene and a dienophile (a Diels-Alder cycloaddition) has been exploited in covalent biomolecule modifications (7,8). Conceptually, the inertness and chemoselectivity of the Diels-Alder reactants offer advantages in bioconjugation applications. For example, competing hydrolysis of reactants under aqueous experimental conditions is less of a concern in a diene-dienophile condensation and, in fact, the Diels-Alder process is actually accelerated in aqueous solvents (9). Furthermore, due to the high selectivity dienes possess for reactions with dienophiles, the Diels-Alder cycloaddition constitutes a previously untapped dimension of chemical orthogonality for the covalent modification of nucleic acids and other biopolymers (10).

RESULTS AND DISCUSSION

I. Reagents for Post-Synthetic Labeling of Oligonucleotides

An objective of our work is the direct comparison of the Diels-Alder bioconjugation method with other established techniques for biomolecule covalent modification with respect to operational simplicity and efficiency of conjugate preparation. Furthermore, comparable bioconjugate performance in applications is essential.

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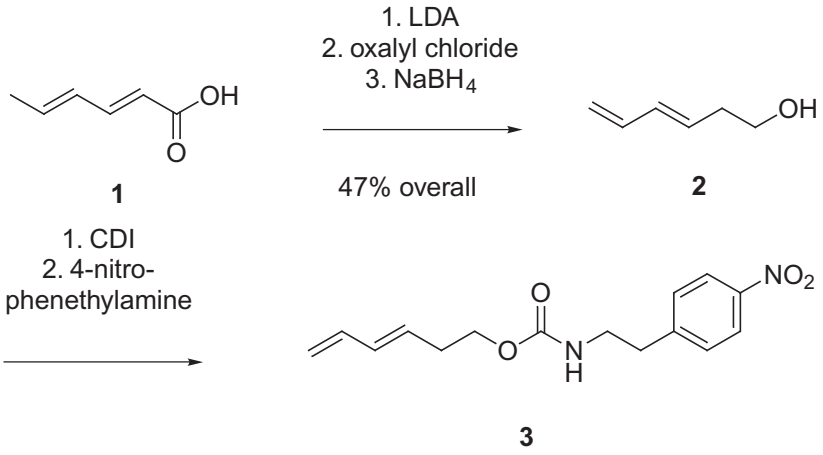
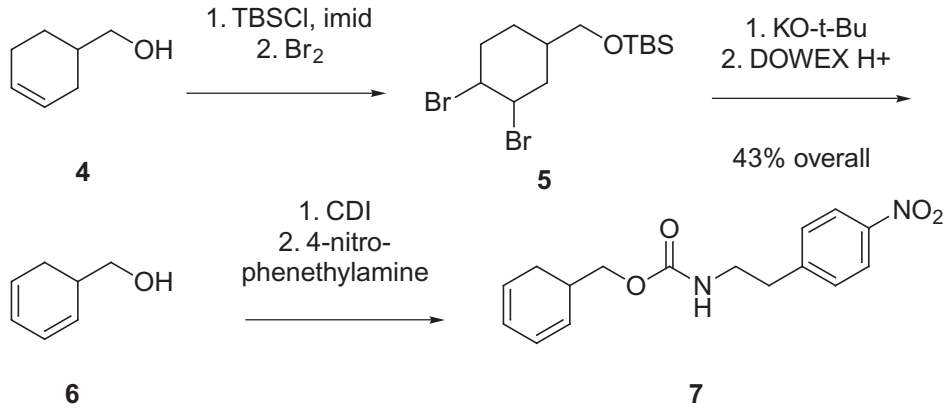


Scheme 1.

Initial efforts focussed on the design and synthesis of a reagent which could ultimately be employed in high throughput solid-phase oligonucleotide synthesis to reliably introduce the Diels-Alder reactive functionality. The decision at this stage to incorporate the diene (vs the dienophile) via solid phase synthesis was driven by two considerations: (1) the anticipated relative stability of unsubstituted 1,3-dienes toward the variety of reaction conditions encountered during oligo synthesis and (2) the abundance of relevant and structurally diverse dieneophile reactants available commercially as maleimide derivatives. A further design consideration was the incorporation of a 2° alcohol amidite precursor to avoid the previously mentioned shortcomings of primary alcohol derived amidites. Schemes 1 and 2 summarize the synthesis of two 1,3-diene core structures employed in the current studies (11).



Scheme 2.

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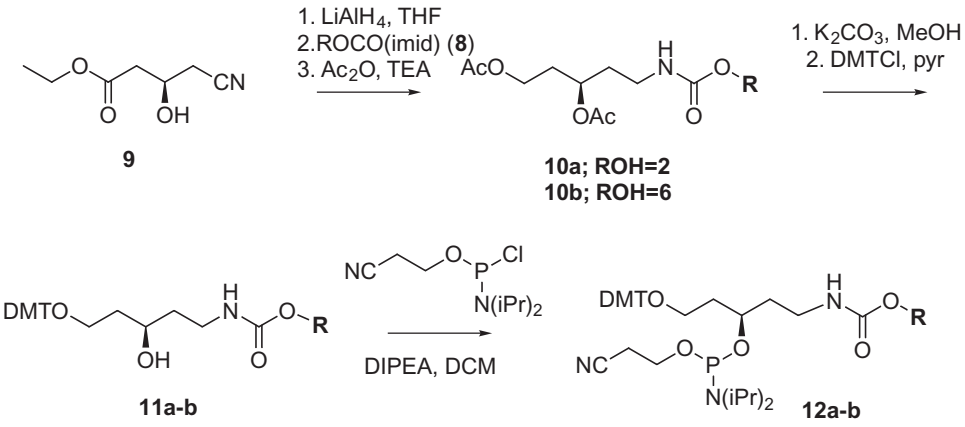

Scheme 3.

Acyclic hexadiene **2** and hydroxymethylcyclohexadiene **6** were converted to their respective 4-nitrophenethyl carbamates **3** and **7**. We wanted to confirm the stability of the diene functionality under various reaction conditions commonly employed during automated solid phase oligonucleotide synthesis and deprotection and the nitrophenyl moiety provided an inert chromaphore for UV detection. With the exception of 1.0 M TBAF, the diene functionality proved completely stable to all of the reaction conditions studied (12).

The next task was the conversion of diene alcohols **2** and **6** into suitable phosphoramidite reagents. Previously, acyclic diene alcohol **2** was directly phosphitylated and this primary amidite was employed to 5′-end label a synthetic oligonucleotide (7). Our interest was in designing a functional amidite which had a DMT group (for in-process analysis of the coupling step), a 2°-amidite and was extendable, enabling incorporation at any position within an oligonucleotide chain. Scheme 3 shows representative syntheses which afford diene amidites **12a** and **12b**. Preliminary solid phase oligonucleotide syntheses with these reagents are underway and evaluation of Diels-Alder bioconjugations and surface immobilizations with these diene oligo substrates will be reported in due course.

II. Surface Immobilization of Diene Modified Oligonucleotides

The BIACORE system offers the opportunity for the evaluation of surface phenomena and we designed a set of experiments which would confirm the application of the Diels-Alder bioconjugation as a new technique for surface immobilization (10,13). While the BIACORE system has been a very powerful tool for studying surface bound, non-covalent biomolecular associations (14), we were curious to determine whether or not it could be exploited for the optimization of key reaction parameters (e.g.; diene structure, linker structure, temperature, etc.)

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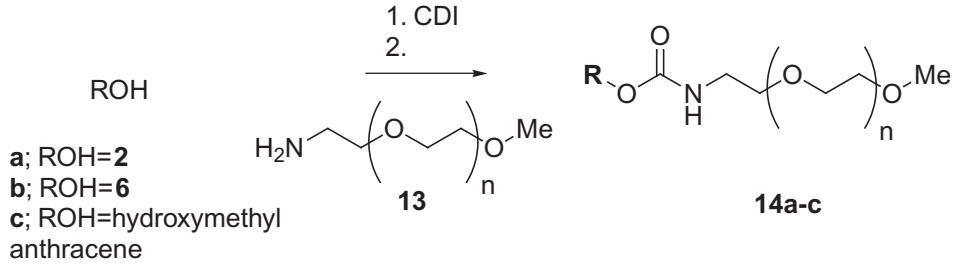
involved in covalent surface immobilization of biomolecules. A useful feature of the SPR system is the lack of a requirement for a reporter group of any kind on the molecule being immobilized. It is simply a change in mass density at the liquid/ solid interface which affects the signal. Due to the ease of preparation (and in some cases, commercial availability) of poly(ethylene glycol) (PEG) derivatives, we performed some of our initial experimentation with examples from this class of polymers.

We began by converting all four flow cells of the commercially available carboxymethyl dextran coated gold chip (BIACORE CM5 chip) into a maleimide (i.e.; dienophile) modified surface via sequential addition of EDC-NHS, ethylene diamine and the bifunctional reagent sulfo-EMCS (15). A control experiment confirming the presence of surface bound maleimide was conducted by subjecting the first flow cell to a 1.5 mM solution of methoxy poly(ethylene glycol) mercaptan (MeO-PEG-SH; MW = 5000). At 25°C, a change in the relative baseline response signal of roughly 2000 response units (RU) was observed after 5 min exposure at 2 μL/min flow rate. For the purposes of studying covalent surface immobilization chemistry, it is the magnitude of this change, and the rate of the change, in the baseline response signal that are the key pieces of information. No additional increase in the baseline response was observed upon continued exposure at this temperature to the mercaptan PEG.

The second dieneophile flow cell was subjected to PEG cyclohexadiene substrate **14b** (Scheme 4) for 10 min under otherwise identical conditions, however, no appreciable change in the baseline response signal was observed after that length of time. In order to realize the same roughly 2000 RU increase in the baseline response observed upon exposure to the solution of the thiol substrate, approximately 18 h was required, indicating the Michael addition of the thiol group to the maleimide to be much faster than the diene cycloaddition. Table 1 shows the results of a series of experiments conducted at 25°C and 37°C with PEG dienes **14a–c**, along with some control PEG mercaptan data. As can be seen, higher degrees of surface functionalization occur at the higher temperature, and the degree of the increase is consistent from diene to diene. It is interesting, however, that no appreciable difference in the degree of surface substitution was observed when changing from the acyclic diene



Scheme 4.

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Table 1.

Entry	Substrate[a]	RU 25°C (time)[b]	RU 37°C (time)[b]
1	MeO-PEG-SH (1.5 mM)	1727 (5 min)	3346 (5 min)
2	**14a** (1.5 mM)	247 (3 h)	629 (3 h)
3	**14b** (1.5 mM)	211 (3 h)	580 (3 h)
4	**14c** (1.5 mM)	267 (3 h)	658 (3 h)

[a]All flow rates were 2 μL/min; all substrates were solutions in 100 μM phosphate buffer (pH=6.5); [b]All RU represent relative increases in the CM5 maleimide flow cell baseline response after adequate elution of running buffer (100 μM phosphate, pH=6.5).

moiety to the cyclic diene examples. Based on Breslow's original data on aqueous acceleration of Diels-Alder reactions (9), we expected to see some difference in going from the linear acyclic substrate **14a** to cyclic diene **14b**, and particularly expected anthracene derivative **14c** to display accelerated reaction rates. It is conceivable that exposure of the diene functionality from the solution phase structure of the PEG substrates is rate limiting, thus complicating the kinetic analysis of the surface derivatization. Further investigations on the influence of diene structure in Diels-Alder bioconjugation applications are warranted and are currently underway.

The immobilization of oligonucleotide substrates were addressed next. The results of a variety of experiments are summarized in Table 2. Based on our experience in the PEG series, a thiol substrate immobilization was carried out as a standard for comparison. Initially, 1.5 mM solutions of thiol oligo (16) (Entry 1) and cyclohexadiene oligo **16** (Entry 2; for preparation of **16**, see Scheme 5) (17) were used, however, some later experiments were performed with more dilute solutions (0.5 mM; Entries 4, 5). It is noteworthy that the increase in the relative response observed in going from 25°C to 37°C was more dramatic in the oligo diene series (4.4 fold increase at 37°C) than in the PEG diene case (average 2.5 fold increase).

Table 2.

Entry	Substrate[a]	RU 25°C (time)	RU 37°C (time)
1	Oligo-SH (1.5 mM)	682 (10 min)	–
2	Oligo-cyclic diene (**16**; 1.5 mM)	681 (10 h)	–
3	5K-PEG-SH (1.5 mM)	1814 (5 min)	3023 (5 min)
4	Oligo-SH (0.5 mM)	325 (5 min)	2618 (1 h)
5	Oligo-cyclic diene (**16**; 0.5 mM)	241 (3 h)	1071 (3 h)

[a]All flow rates were 2 μL/min; all substrates were solutions in 100 μM phosphate buffer (pH=6.5); [b]All RU represent relative increases in the CM5 maleimide flow cell baseline response after adequate elution of running buffer (100 μM phosphate, pH=6.5).

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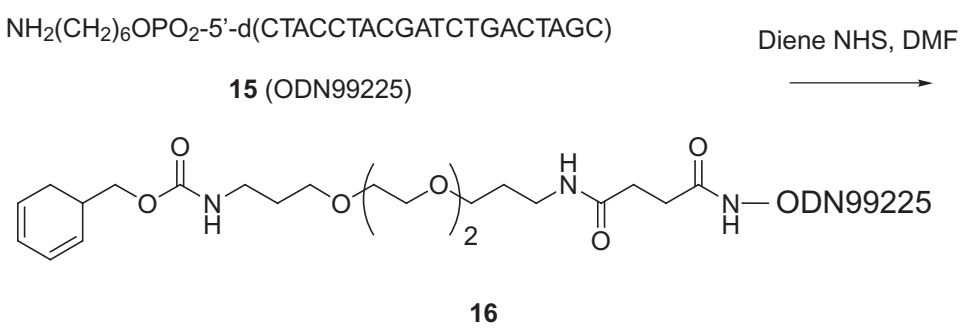

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Scheme 5.

CONCLUSION

We have prepared reagents for introducing Diels-Alder functionality to synthetic oligonucleotides. We have demonstrated the surface immobilization of PEG and oligonucleotide substrates by the Diels-Alder method and have begun a systematic optimization of the immobilization process employing the BIACORE system.

ACKNOWLEDGMENTS

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11. All new compounds were adequately characterized by an appropriate combination of ^{1}H, ^{13}C and ^{31}P NMR spectra, ES/MS and HPLC analysis for purity.
12. The 4-nitrophenethylamine-derived carbamate derivatives **3** and **7** displayed complete stability (as determined by RP HPLC analysis) upon 16 h exposure to each of the following reaction conditions: Cap A; Cap B; Cap A+ Cap B; 10% DCA/PhMe; 3% TCA/DCM (deblock); I_2/pyr/water (oxidizer); 0.45 M tetrazole/ACN; 0.5 M DCI/ACN; Conc. NH_4OH; $MeNH_2$ solution; N-methylimidazole solution; Ethyl thiotetrazole solution; t-butylhydroperoxide/PhMe; TEA-HF; phenylacetyl disulfide. Decomposition of both **3** and **7** was observed upon exposure to 1.0 M TBAF/THF for 16 h.
13. For examples of oligonucleotide immobilization by various methods onto BIACORE chips and gold surfaces, see: Brockman, J.M.; Frutos, A.G.; Corn, R.M. *J. Amer. Chem. Soc.* **1999**, *121*, 8044–8051 and references cited therein.
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16. The thiol oligo substrate was a 5′-alkyl mercaptan modified 6.6 kD oligodeoxynucleotide and was a generous gift from Dr. Brian Hicke of Gilead Sciences.
17. The diene NHS reagent used to prepare oligo diene **16** was prepared from **6** by the following sequence: (a) CDI; (b) $[NH_2(CH_2)_3O(CH_2)_2OCH_2]_2$; (c) succinic anhydride; (d) DCC/NHS.

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