Diels−Alder Bioconjugation of Diene-Modified Oligonucleotides

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In an effort to offer complementary technology for covalent biomolecule modification (bioconjugation), we have developed a method that exploits the aqueous acceleration of Diels−Alder reactions for this purpose. Three different diene phosphoramidite reagents have been synthesized that enable diene modification of synthetic oligonucleotides prepared by the phosphoramidite method. Clean and efficient Diels−Alder cycloaddition of these diene oligonucleotides with maleimide dieneophiles was carried out, and the labeled oligonucleotide bioconjugates were characterized by HPLC and electrospray mass spectrometry. Dieneophile stoichiometry, temperature, and pH are all parameters that were shown to influence the efficiency of the process.

Introduction

The capacity of oligonucleotides to assemble into complementary duplex structures with target nucleic acid sequences via the formation of Watson−Crick base pairs has led to a rich variety of technologies exploiting this property and, in conjunction with dramatic advances in information technologies, has contributed to unprecedented progress in the biotechnological arena.[1] In a general sense, covalently attached reporter groups on oligonucleotide probes enable quantitative information to be derived about the presence of a given nucleic acid target sequence. In applications related to nucleic acid sequencing, differential gene expression measurement, and genotyping, oligonucleotides covalently modified with one or more reporter groups (including haptens such as biotin[2] or organic fluorophores and/or fluorescence quenchers such as fluorescein and Cy3)[3] enable the derivation of genetic information in an ever more comprehensive and operationally streamlined fashion.

Additionally, covalent attachment of poly(ethylene glycol), peptides, or lipid modifiers to oligonucleotide based active pharmaceutical ingredients has led to improved activity and pharmacokinetic profiles for examples from this class of clinical candidates.[4] Oligonucleotide bioconjugates containing halide radioisotopes have been employed in pharmacological and imaging studies.[5]

Commonly employed methods for oligonucleotide bioconjugate construction include the introduction of the chemical modifier during solid-phase synthesis via the phosphoramidite method or by post-solid-phase synthesis 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 molecular label incompatibility with synthesis conditions as well as a relatively limited selection of readily available phosphoramidite reagents corresponding to the desired conjugate moieties. Post-solid-phase synthesis modification is one of the more commonplace methods for the derivatization of oligonucleotides, and condensation of 5′- or 3′-primary alkylamine modified oligonucleotides with electrophilic derivatives of the molecular labels has seen widespread use.[6] Advantages of amino oligonucleotide bioconjugation techniques include ready availability of these derivatized oligomers and amine reactive labels of interest; however, these methods suffer from competing reagent hydrolysis and potential cross reactivity with oligonucleotide functionality. Other frequently employed oligonucleotide bioconjugation methods employ the coupling of an alkyl mercaptan derivatized oligonucleotide with an α-haloacetyl,[7] a maleimide,[8] or an activated disulfide, such as a pyridyl disulfide.[9] Michael addition of mercaptan oligonucleotides to maleimides are particularly facile; however, the necessity of

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mercaptan oligonucleotide disulfide reduction directly prior to bioconjugation adds undesirable operational complexity to all these related methods.

While amine- and mercaptan-based techniques will always be mainstream methods for oligonucleotide conjugate formation, they are not without their problems, and complementary techniques would serve a valuable purpose in the field as well. In a particularly promising recent account, Greenberg has described a conceptually distinct variation on postsynthesis oligonucleotide bioconjugate formation in which a reactive 2′-amino uridine nucleoside monomer (introduced during automated solid phase synthesis) is selectively deprotected and condensed with electrophilic labels prior to oligonucleotide deprotection and cleavage from synthesis support.[10] Similarly, Grinstaff describes Pd(0)-catalyzed covalent modification of support-bound, 5-halouridine-containing oligonucleotides.[11]

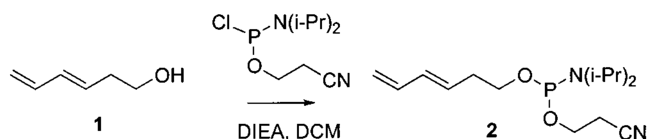
The Diels–Alder [4 + 2] cycloaddition between a diene and a dienophile remains among the more useful carbon–carbon bond-forming reactions available to synthetic organic chemists.[12] Breslow's discovery that aqueous solvents accelerate the Diels–Alder reaction unveiled further opportunities for exploitation of the methodology in synthetic and physical organic chemistry applications.[13–15] While the basis of the acceleration in water remains the subject of much study, we recognized the opportunity this phenomenon represented in the biotechnological arena. Specifically, the highly selective reaction between a diene and a dienophile could be exploited in covalent biomolecule modifications. The first example of the application of the Diels–Alder reaction in a nucleic acid context came from the work of Eaton and Tarasow.[16] This group identified RNAs capable of catalyzing a Diels–Alder reaction by in vitro selection from a library of modified RNAs. Seelig and Jäsche have recently published a similar example of an RNA-catalyzed Diels–Alder reaction.[17]

Initial results in our laboratory[18] have shown that the Diels–Alder reaction is indeed amenable to oligonucleotide bioconjugation.[19] The efficiency of the method compares very favorably with established techniques for oligonucleotide bioconjugation. We report herein the first full account of this complementary technique for oligonucleotide bioconjugation.

Experimental Section

General Methods. Derivatized controlled pore glass (CPG) long-chain alkylamine-T solid support was obtained from Prime Synthesis. Deoxynucleoside phosphoramidites, solvents, and standard reagents for oligonucleotide synthesis were obtained from Proligo, Glen Research, Cruachem, Aldrich, and Burdick and Jackson. Biotin maleimide (biotin-BMCC; **6b**), fluorescein maleimide **6c**, and 1,6-bismaleimidohexane **8** were obtained from Pierce. *N*-Ethylmaleimide (**6a**) and coumarin maleimide **6d** were obtained from Aldrich. The 5K and 20K PEG maleimides, **6e** and **6f**, respectively, were obtained from Shearwater Polymers, Inc., and biotin maleimide **26** was purchased from Molecular Biosciences. NMR spectra were measured at 300 MHz. Electrospray mass spectral data were recorded in the negative ion mode or from M-Scan. At least three charge states were used to determine the reported molecular weights. High-resolution FAB mass spectroscopy data was obtained from the University of California, Berkeley, mass spectroscopy laboratory.

Scheme 1



The synthesis of all dienes, diene phosphoramidites and oligonucleotides (shown in Schemes 1, 2, 5, and 6) are described in the Supporting Information.

Preparation of a Stock Solution of Acyclic Hexadiene-Modified Oligonucleotide 5. Lyophilized 5′-diene oligonucleotide**5** was dissolved in 1 mL of 25 mM phosphate buffer (pH = 6.8) to give an oligonucleotide concentration of 2000 OD_{260nm}/mL (approximately 54 mg/mL or 6 mM). The oligonucleotide concentration was determined by measuring the absorbance at 260 nm of the oligonucleotide solution and converting to concentration using an extinction coefficient of 1 OD_{260nm} = 27ug/mL. The extinction coefficient was calculated using the method published by Breslauer[20] and using the values from Sugimoto.[21]

General Procedure: Formation of Diels–Alder Bioconjugates 7a–f. Generally, 2 equiv of maleimides **6a–f** were added to aliquots of the stock solution of **5**, although for reagent solubility reasons, as much as 10–12 equiv was sometimes used. Samples of the reaction mixture were withdrawn over time and analyzed by analytical anion-exchange HPLC on a 4 × 250 mm Dionex Nucleopak PA-100 strong anion-exchange column heated to 80 °C using the following method: a linear gradient of 36% buffer B to 65% buffer B over 30 min (buffer A: 25 mM Tris (pH = 7.5), 1 mM EDTA, 10% acetonitrile; buffer B: buffer A + 1 M NaCl). The column flow rate was 1 mL/min, and the chromatogram components were observed by UV detection at 260 nm. After the reactions appeared complete, the product was isolated by anion-exchange chromatography, using a 9 × 250 mm Dionex Nucleopak PA-100 column with a flow rate of 5 mL/min employing the same gradient described above. The purified conjugates were converted to the triethylammonium salt forms in order to obtain good MS data. The products were loaded onto a 4.6 × 250 mm PRP-1 column and washed with five column volumes of 25 mM triethylammonium carbonate followed by two column volumes of water. The conjugates were then eluted from the column in 50% acetonitrile/water and lyophilized. Anion-exchange HPLC and electrospray mass spectrometry were performed on the final lyophilized conjugates.

Synthesis of *N*-Ethyl Maleimide Oligonucleotide Conjugate 7a. Compound **7a** was prepared from 200 μL of the stock solution of **5** and 10 μL (2 equiv) of a stock solution of **6a** (prepared by dissolving 6.4 mg of **6a** in a solution of 150 μL of water and 50 μL of acetonitrile) according to the general procedure. After 2 h at 35 °C, the reaction was complete. The product was isolated as described above, and anion-exchange

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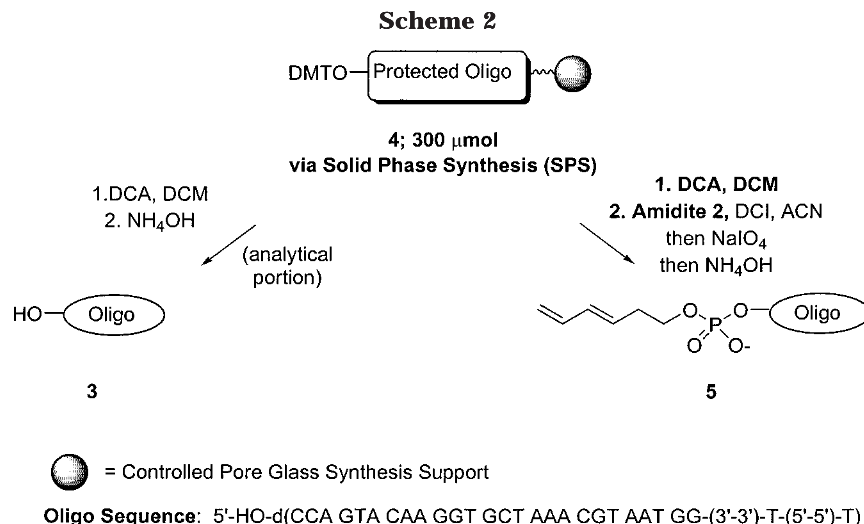
Scheme 2



HPLC analysis revealed a purity of 98%. Analysis by electrospray mass spectrometry gave a molecular weight of 8922.2 ± 0.5 (calcd 8922.7).

Control Experiment: Reaction of 6a with 3. A mixture of **3** and **6a** under the conditions described above showed no change by HPLC over time.

Effect of Acetonitrile on the Formation of 7a. A 100 μL portion of the stock solution of **5** was placed into each of two 1 mL microcentrifuge tubes. To the first tube was added 100 μL of 25 mM phosphate buffer (pH = 6.8) and to the second tube was added 100 μL of acetonitrile. A stock solution of **6a** was prepared by dissolving 46.25 mg of *N*-ethylmaleimide in 1 mL of acetonitrile. To each of these two mixtures was added 10 μL of the *N*-ethylmaleimide stock solution (10 equivalents of **6a**). Both reactions were incubated at 25 °C and monitored by analytical anion-exchange HPLC. After 15 min, the reaction without acetonitrile was 50% complete while the reaction with acetonitrile present took 240 min to reach 50% completion.

Synthesis of Biotinylated Oligonucleotide 7b. Compound **7b** was prepared from 200 μL of the stock solution of **5** and 1.5 mg (approximately 2 equiv) of **6b** according to the general procedure. After 18 h at 35 °C, the reaction appeared complete. This product was isolated as described above, and anion-exchange HPLC analysis showed a purity of 97%. Analysis by electrospray mass spectrometry gave a molecular weight of 9333.4 ± 0.9 (calcd 9331.3).

Effect of Temperature on the Formation of 7b. Two 1 mL microcentrifuge tubes were each charged with 200 μL of the stock solution of **5**. To each was added 8 mg (0.015 mmol, approximately 10 equiv) of **6b**. The first tube was incubated at 35 °C and the second at 60 °C. Both reactions were followed by anion-exchange HPLC using the method described above. The reaction run at 35 °C took 90 min to reach 50% completion. The reaction at 60 °C took only 15 min to reach the 50% completion mark and was complete after 2 h.

Synthesis of Fluorescein-Labeled Oligonucleotide 7c. Compound **7c** was prepared from 200 μL of the stock solution of **5** and 3.8 mg (approximately 12 equiv) of fluorescein maleimide **6c** according to the general procedure. Most of the fluorescein did not appear to dissolve, although the reaction mixture did take on the expected yellow fluorescein color. The reaction mixture was vortexed at room temperature and, after 18 h, appeared complete. The product was isolated as described above and revealed to be 98% pure by anion-exchange HPLC. Electrospray mass spectrometry gave a molecular weight of 9224.6 ± 0.9 (calcd 9224.1).

Synthesis of Coumarin-Labeled Oligonucleotide 7d. Compound **7d** was prepared from 230 μL of the stock solution of **5** and 10 μL (ca. 1.2 equiv) of a stock solution of **6d** (prepared by dissolving 5.0 mg of **6d** in 100 μL of dimethyl formamide) according to the general procedure. After approximately 20 h at 35 °C, the reaction appeared complete. The product was isolated as described above and revealed to be 96% pure by anion-exchange HPLC. Electrospray mass spectrometry gave a molecular weight of 9096.9 ± 0.3 (calcd 9095.9).

Synthesis of 5K PEG Oligonucleotide Conjugate 7e. Compound **7e** was prepared from 200 μL of the stock solution of **5** and 11 mg (approximately 2 equiv) of 5K PEG maleimide **6e** according to the general procedure. After 20 h at 25 °C, the reaction appeared complete. The product was isolated as described above and showed a purity of 95% by anion-exchange HPLC analysis. Electrospray mass spectrometry gave a molecular weight centered at 13795.8 ±0.8 ($n = 110$) ± 44 (calcd 13797.6 ($n = 110$)).

Effect of Temperature on the Formation of 7e. Under the same conditions described above at 55 °C, the formation of **7e** was complete within 3 h.

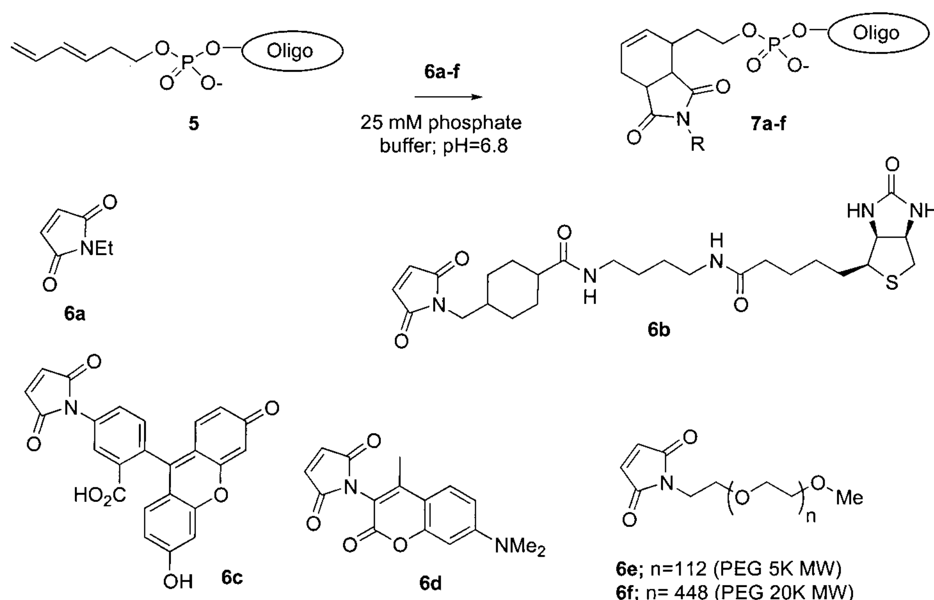
Synthesis of 20K PEG Oligonucleotide Conjugate 7f. Prepared from 200 μL of the stock solution of **5** and 50 mg (approximately 2 equiv) of 20K PEG **6f**. After 20 h at 35 °C, the reaction was complete. This material was not characterized further.

Formation of Oligonucleotide Dimer 9. Compound **9** was prepared from 200 μL of the stock solution of **5** and 22 μL (ca. 1/3 equiv) of a stock solution of **8** (prepared by dissolving 5 mg (0.02 mmol) of **8** in 1 mL of 1,4 dioxane) according to the general procedure. After 16 h at 25 °C, no further conversion of **5** was observed by anion-exchange HPLC analysis. An approximate yield of 75% was calculated on the basis of the area of the peak due to **5** versus the peak area of the oligonucleotide dimer **9** (no peak due to monoconjugated oligonucleotide was observed, although this species could have coeluted with **5**). The product was isolated as before, and anion-exchange analysis showed a purity of 98%. Electrospray mass spectrometry gave a molecular weight of 17873.1 ± 0.5 (calcd 17872.3).

Synthesis of Biotinylated Oligonucleotide 27 via Diels–Alder Bioconjugation of 24 and Biotin Maleimide 26. Acyclic diene modified oligonucleotide **24** (prepared as described in the Supporting Information; 27.5 OD) was dissolved in 1.0 mL of H_2O. This solution was aliquoted into five 1.5 mL microcentrifuge tubes in equal 200 μL volumes (each tube containing 5.5 OD_{260}, 40.3 nmol). All samples were dried under centrifugal conditions and reduced pressure at 85 °C.

One of the tubes containing oligonucleotide **24** (5.5 OD) was preheated to 40 °C for 1 min and then treated with 26.9 μL (10 equiv) of a stock solution of **26** (prepared from 3.94 mg of **26** in 0.500 mL of 100 mM phosphate buffer (pH = 6.3 or 5.5)). The solutions were prepared to afford a final diene oligonucleotide concentration of 1.5 mM. The solution was vortexed for 15 s, placed in a microcentrifuge and spun for 15 s, and then transferred to a vibrating heating block (40 or 70 °C). Aliquots (2 μL) were sampled at 15 min intervals. The 2 μL aliquots were diluted to 22 μL with 0.1 M NaOH solution, diluted to 200 μL with water, and analyzed by analytical RP HPLC. (Control experiments confirmed that this NaOH treatment

Scheme 3



effectively arrested the formation of the Diels–Alder adduct.) All reverse-phase analyses were performed on a 4.1 × 250 Hamilton PRP-1 column with a 10 μm particle size using the following methods: a linear gradient of 0% buffer B to 25% buffer B over 25 min, or 0% buffer B to 40% buffer B over 40 min (buffer A: 100 mM triethylammonium acetate (pH 7.0); buffer B: acetonitrile). The column flow rate was 1.5 mL/min.

At 40 °C, conversion to **27** was complete (product peak confirmed by LC/MS analysis (Mscan)) within 90 min at pH = 6.3 and within 30 min at pH = 5.5. At 70 °C, 80% conversion was observed within 20 min at pH = 6.3. Electrospray MS analysis of **27** showed a MW of 5554.1 (calcd 5555.0).

Synthesis of Biotinylated Oligonucleotide 28 via Diels–Alder Bioconjugation of 25 and Biotin Maleimide 26. Treatment of **25** under the conditions described above afforded biotinylated oligonucleotide **28**. Electrospray MS analysis of **28** showed a MW of 5566.1 (calcd 5567.0). At 40 °C, complete conversion to **28** was observed within 30 min at pH = 5.5 (product peak confirmed by LC/MS analysis (Mscan)), while at pH 6.3, 42% conversion was observed after 30 min. At 70 °C, 72% conversion was observed within 20 min at pH = 6.3 with complete conversion observed after 16 h (no time points between 20 min and 16 h were sampled).

Results and Discussion

First-Generation Studies. Our initial intentions were to introduce a 5′-diene functional handle. The decision at this stage to incorporate the diene (vs the dienophile) via solid-phase synthesis was driven by the anticipated relative stability of unsubstituted 1,3-dienes toward the variety of reaction conditions encountered during oligonucleotide synthesis as well as the abundance of relevant and structurally diverse dieneophile reactants available commercially as maleimide derivatives. Our efforts began with the synthesis of acyclic diene **1**, which was prepared from ethyl sorbate as described by Choi and co-workers.[28] Direct phosphitylation of **1** was carried out to afford the simple diene phosphoramidite **2** (Scheme 1).

Diene-modified oligonucleotide synthesis began with the selection of the 28mer DNA fragment with the following sequence: 5′-d(CCA GTA CAA GGT GCT AAA CGT AAT GG-[3′-3′]-T-[5′-5′]-T). This oligodeoxynucleotide is a truncated sequence of one of many DNA aptamers identified by the SELEX process,[22,23] having a high binding affinity to L-selectin.[4b] The synthesis was conducted on a Milligen 8800 oligonucleotide synthesizer using standard solid-phase protocols at a 300 μmole scale.[24] After completion of the 5′ terminal solid-phase addition cycle (trityl-off), the diene phosphoramidite **2** was coupled (0.2 M acetonitrile solution, 4 equiv, 30 min; Scheme 2). Oxidation was carried out with 0.5 M $NaIO_4$ in water for 10 min, and then the excess oxidant was removed by washing with water followed by acetonitrile. The oligonucleotide was then cleaved from the solid support and deprotected under standard conditions using ammonium hydroxide. Anion-exchange analysis of the crude diene oligonucleotide **5** showed 45% full-length material, and this was purified by reversed-phase HPLC.[25] The product-containing fractions were pooled and converted into the sodium salt form on a PRP-1 column. The purified oligonucleotide was then lyophilized to a white powder that was ca. 85% pure by analytical anion-exchange HPLC. Mass spectrometric analysis of this material gave the expected mass.

For bioconjugation experiments, a stock solution of **5** was prepared by dissolving a sample in 25 mM phosphate, pH = 6.8, to give an approximate concentration of 6 mM oligonucleotide (approximately 54 mg/mL).

Scheme 3 shows the conversion of diene oligonucleotide **5** into a number of Diels–Alder bioconjugates. Treatment of an aliquot of the 6 mM stock solution of **5** with 2 equiv of *N*-ethyl maleimide (**6a**) at 35 °C resulted in complete transformation within 2 h to a new product with a slightly shorter retention time (as determined by HPLC

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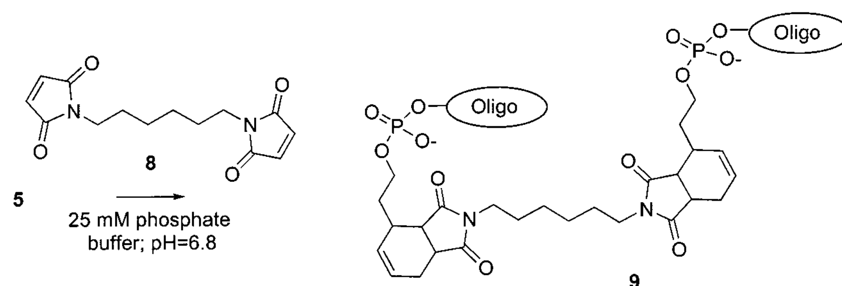
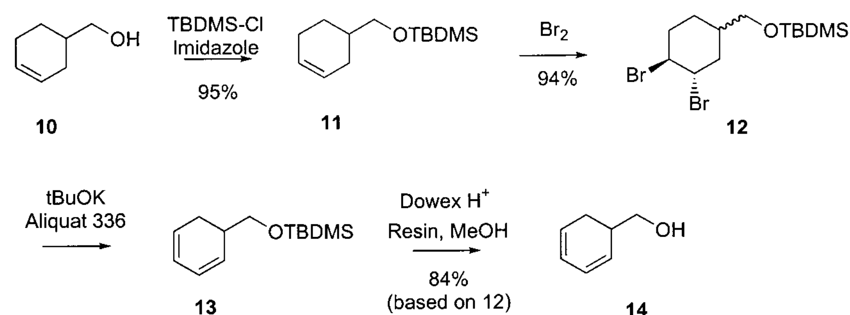
Scheme 4



Scheme 5



analysis). Isolation and mass spectrometric characterization of the product confirmed the formation of the expected Diels–Alder cycloadduct **7a**. As a control experiment, excess *N*-ethylmaleimide was added to oligonucleotide **3**, which lacks the hexadiene functionality. No conversion of **3** was observed under the reaction conditions.

Similarly, oligonucleotide diene **5** was treated with 2 equiv of biotin maleimide **6b**. This homogeneous solution was warmed to 35 °C, and the course of the reaction was followed by anion-exchange HPLC. After approximately 18 h, the peak corresponding to **5** had disappeared and a new peak had grown in (95% yield by HPLC). This product was isolated by anion exchange chromatography and analyzed by electrospray mass spectrometry. The mass obtained agreed with the expected mass of the Diels–Alder adduct **7b**. Much faster formation of biotinylated oligonucleotide **7d** was accomplished upon treatment of **5** with 10 equiv **6b** at 60 °C. Under these conditions, complete conversion to **7b** was observed in 2 h.

The diene oligonucleotide **5** was treated with 12 equiv of fluorescein maleimide **6c** or 1.2 equivalents of coumarin maleimide **6d**. The reaction with the fluorescein maleimide was conducted at room temperature (approximately 25 °C), while the coumarin maleimide cycloaddition was run at 35 °C. The coumarin maleimide **6d** was readily soluble in the solvent system used (5% DMF in phosphate buffer); however, the fluorescein maleimide solubility was limited and most of this solid settled to the bottom of the reaction solution. Anion-exchange analysis of the reaction mixtures showed the diene oligonucleotide peak decreasing as a new peak grew in with time. Both reactions took approximately 20 h to go to completion, with HPLC yields of 95% and 93% for the coumarin maleimide and fluorescein maleimide reactions, respectively. Both products were isolated by anion exchange chromatography and, in each case, the isolated products gave the correct mass for the expected Diels–Alder cycloaddition products **7c** and **7d**. Both of the isolated products were colored due to the fluorescent nature of the fluorescein and coumarin moieties.

The ability to generate poly(ethylene glycol) bioconjugates by the Diels–Alder bioconjugation method was demonstrated. Treatment of the stock solution of **5** with two equivalents of PEG maleimide **6e** at 25 °C cleanly afforded the expected PEG–oligonucleotide conjugate **7e** after 20 h. At 55 °C, complete conversion to **7e** occurred in only 3 h. Similarly, the 20 K PEG–oligonucleotide conjugate **7f** was prepared (2 equiv **6f**, 35 °C; 20 h). The PEG–oligonucleotide conjugates were isolated by reversed-phase chromatography, and the molecular weights confirmed by mass spectrometry.[26,27]

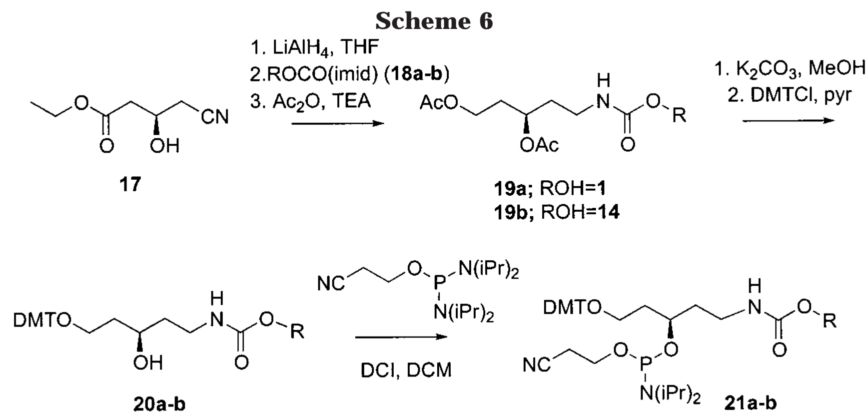
A particularly compelling application of the Diels–Alder conjugation methodology is in linking more than one oligonucleotide to single platform. A solution of **5** was treated with approximately 0.33 equiv of 1,6-bismaleimidohexane (**8**) at 25 °C (Scheme 4). After 16 h the dimer conjugate **9** had formed in approximately 80% yield (based on **8**). The dimer product was isolated by anion-exchange chromatography and the molecular weight confirmed by electrospray MS.

While we have not investigated the stereochemistry of Diels–Alder reactions within the context of the oligonucleotide conjugations, diene **1** forms the endo adduct with *N*-methyl maleimide in toluene[28] and in aqueous buffer.[29]

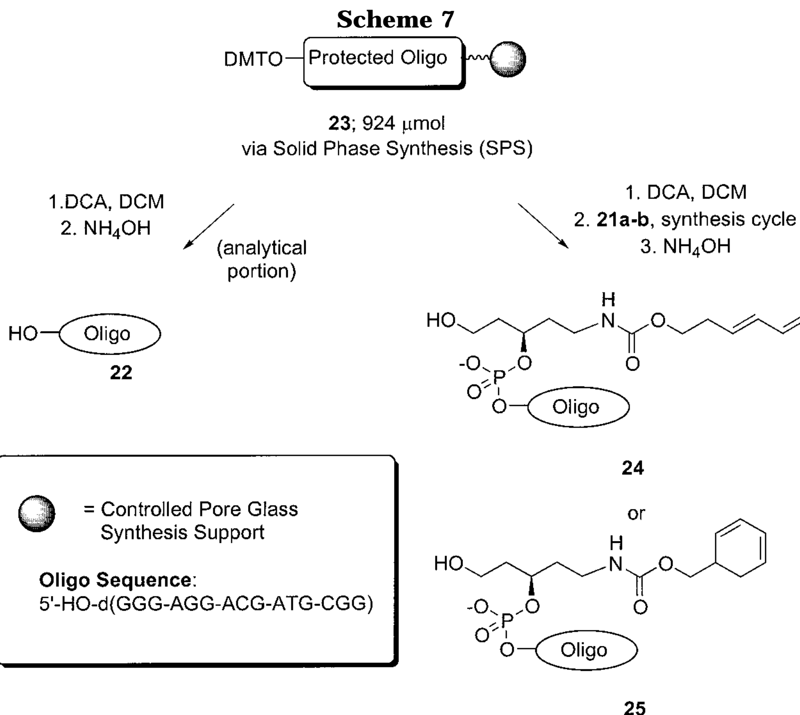
Second-Generation Studies. We were next interested in studying the influence of diene structure on conjugation rate and establishing a reagent suitable for the introduction of the diene functionality under high throughput automated oligonucleotide synthesis conditions. Cyclic 1,3-diene **14** was prepared as shown in Scheme 5. Hydroxymethyl cyclohexene **10** was silylated (TBSCl, imidazole; 95%), then treated with molecular bromine to form a diastereomeric mixture of dibromides **12**. Double elimination (potassium *tert*-butoxide, Aliquat 336) afforded silyl ether protected diene **13**. Failure to

(29) Hill, K. W.; Taunton-Rigby, J.; Tarasow. T. Unpublished results.

Scheme 6



Scheme 7



protect the hydroxyl group prior to elimination resulted in intramolecular bromide displacement and the formation of bicyclic ether products. Finally, acidic silyl ether cleavage and purification by distillation afforded hydroxymethyl cyclohexadiene substrate **14** in 75% overall yield.

Prior to proceeding further with diene oligonucleotide synthesis, we performed a series of control experiments establishing the compatibility of the diene functionality under the various reaction conditions encountered during automated solid-phase synthesis. To accomplish this, dienes **1** and **14** were transformed into nitrophenethylamine-derived carbamates **15** and **16** (Figure 1) by sequential addition of carbonyldiimidazole and 4-nitrophenethylamine in DMF. The 4-nitrophenethyl moiety provided a chemically inert chromophore for UV detection. These substrates were subjected to a number of reaction conditions and evaluated by HPLC after 16 h. The dienes both displayed complete stability to all conditions studied except 1.0 M tetrabutylammonium fluoride solution in THF. However, alternative desilylation conditions (TEA-HF) were suitable. Noteworthy is the stability of the dienes toward the standard oxidizing conditions (0.02 M I_2, pyr/water/THF).

Comfortable with the requisite stability of the diene functionality, we next designed and synthesized a versatile phosphoramidite reagent platform for diene incorporation during automated synthesis. Scheme 6 shows the synthesis of diene amidite reagents **21a** and **21b**. Chiral hydroxy nitrile **17** was reduced by treatment with $LiAlH_4$. Condensation of the unpurified amino diol with diene carbonyl imidazolides **18a** or **18b**, followed by peracetylation (for ease of purification and characterization) gave diacetate dienes **19a** and **19b**. Acetate cleavage (K_2CO_3/ MeOH), followed by selective protection of the primary hydroxyl gave phosphoramidite precursors **20a** and **20b**. Phosphitylation afforded the two diene amidites

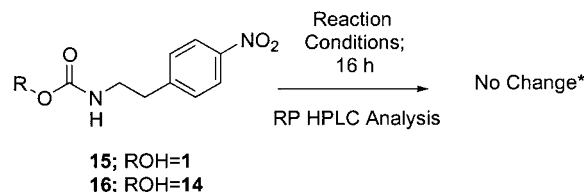
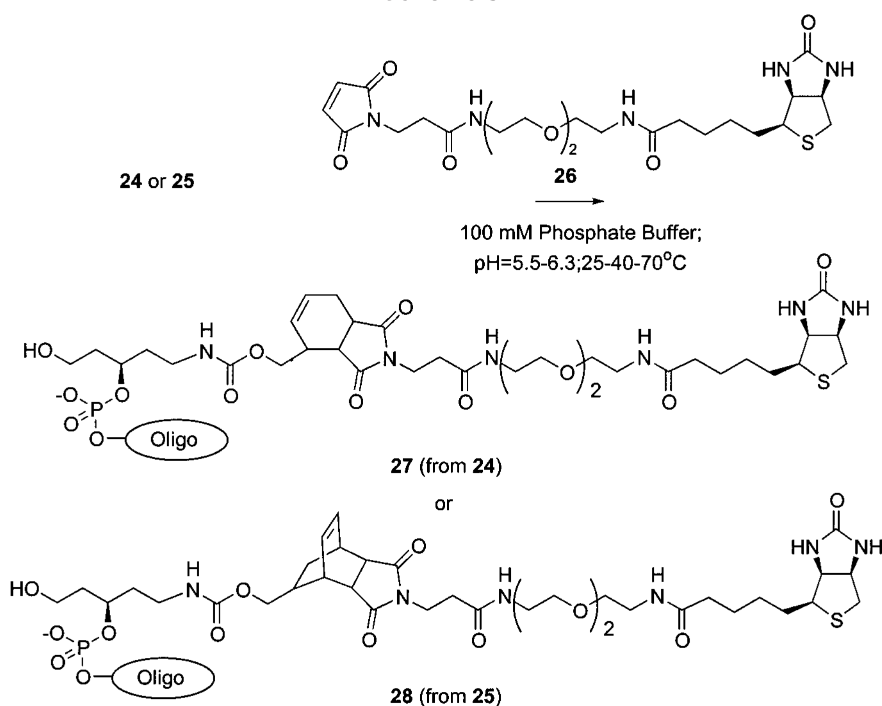


Figure 1. Reaction conditions evaluated include the following: (a) A_2O, pyr, THF; (b) *N*-Me-imidazole, THF; (c) 3% TCA/ CH_2Cl_2; (d) 10% DCA, PhMe; (e) 0.02 M I2, pyr/water/THF; (f) *t*-Bu-OOH/PhMe; (g) 1.0 M TBAF/THF; (h) TEA-HF/ACN; (i) 0.45 M tetrazole/ACN; (j) 0.25 M 4,5-dicyanoimidazole; (k) NH_4OH, 25 °C; (l) Nh_4OH, 70 °C (4 h); (m) phenylacetyl disulfide. *Except 1.0 M TBAF/THF. Significant decomposition of **15** and **16** was onserved.

Scheme 8



21a and **21b** in reasonable overall yields. This amidite platform features the DMT protected alcohol, which facilitates colorimetric, in-process monitoring of the coupling step and enables extension beyond the diene functionality (vs exclusive 5′-end labeling).[30]

Incorporation of these reagents during automated synthesis was carried out next. First, the support-bound, fully protected oligodeoxynucleotide **23** was prepared. Oligonucleotide **23** is a 15mer of the following sequence: 5′-d(GGG-AGG-ACG-ATG-CGG). The synthesis was carried out on a controlled pore glass (CPG) synthesis support using standard conditions on a Milligen 8800 DNA synthesizer at a 935 μmol scale.[24] An analytical portion was detritylated and subjected to ammonium hydroxide deprotection. HPLC analysis of the resultant oligonucleotide **22** showed the crude synthesis to be 63% full-length material. Next, a number of 1 μmol portions of the support-bound, trityl-on crude oligonucleotide were separated and loaded into empty column casings for diene phosphoramidite coupling studies on an ABI 394 synthesizer. A coupling protocol employing 15 equiv of amidite **21a** or **21b** for two 15 min coupling steps enabled efficient incorporation of the acyclic and cyclic dienes (Scheme 7). Anion-exchange HPLC analysis of deprotected crude oligonucleotides revealed clean incorporation of the diene amidites; crude diene oligonucleotide purities ranging from 62−68% full-length material were observed. Electrospray MS analysis of the crude synthesis products revealed the presence of the expected diene molecular weights, and samples were purified to >80% purity by anion-exchange HPLC.

Our next objective was to determine the effect of the diene structure on the rate of Diels−Alder bioconjugation. A series of experiments was carried out in which **24** and **25** were each treated with either 2 or 10 equiv of biotin maleimide **26** (Scheme 8). These reactions were conducted at 25, 40 and 70 °C in phosphate buffers (pH = 5.5 or 6.3), and the progress of the reactions was monitored by HPLC analysis of aliquots sampled at regular intervals. Under equivalent reaction conditions (pH, temperature, concentration), the cyclic and linear diene modified oligonucleotides displayed comparable reaction rates. Diels−Alder bioconjugation reactions conducted at pH 5.5 were observed to be consistently faster than those at pH 6.3. For example, at 40 °C with 10 equiv of biotin maleimide **26**, diene oligonucleotide **24** (1.5 mM) was completely transformed into conjugate **27** within 30 min at pH 5.5, while 90 min were required at pH = 6.3.

Conclusion

In conclusion we have shown that the Diels−Alder reaction is a useful method of preparing oligonucleotide conjugates in aqueous solution under mild conditions. Reagents have been developed which facilitate ready incorporation of the Diels−Alder reactive functionality under standard solid-phase synthesis conditions. The method provides a complementary alternative to amine- and mercaptan-based oligonucleotide conjugations. Applications of the derived Diels−Alder bioconjugates and surface immobilization by the method will be described in due course.

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Supporting Information Available: Experimental procedures for synthesis and purification of all diene reagents and oligonucleotides. HPLC chromatograms for crude and purified oligonucleotides **5** and **7a**,**f**. Selected chromatograms for diene stability studies described in Figure 1 and formation of biotinylated oligonucleotide **28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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