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Post-Synthetic Modification of DNA by Inverse-Electron-Demand Diels−Alder Reaction

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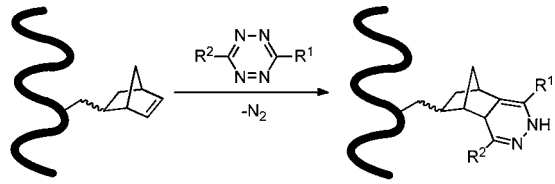
Methods for labeling biomolecules with fluorescent dyes and affinity tags have become indispensable tools in the modern life sciences. Those conjugation strategies that allow site-specific post-synthetic coupling of complex molecules under mild conditions are particularly sought.[1] While for many years *N*-hydroxysuccinimide (NHS) ester chemistry dominated both protein and nucleic acid functionalization,[2] cycloaddition reactions have recently gained considerable importance.[3−5]

Our groups have a long-standing interest in cycloadditions as tools for labeling and modifying biomolecules.[5,6] We and others recently set out to establish inverse-electron-demand Diels−Alder reactions as tools for the covalent and irreversible derivatization of various molecules, such as peptides and small-molecule drugs.[7,8] Herein we report the inverse-electron-demand Diels−Alder reaction[9] as a bioorthogonal reaction for the selective and efficient modification of oligonucleotides. Norbornene moieties are introduced as dienophiles into oligonucleotides during solid-phase synthesis, and the deprotected oligomers are then conjugated with water-stable tetrazine dienes (Scheme 1).

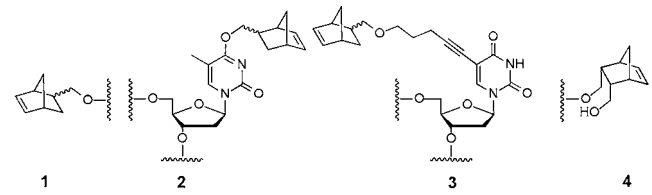
Scheme 1



R_1/R_2 = Aryl, heteroaryl

To allow the site-specific incorporation of dienophiles at both termini and at internal positions with and without influence on standard base pairing, building blocks **1**, **2**, **3**,[10] and **4** were synthesized from bicyclo[2.2.1]hept-2-ene derivatives as phosphoramidites and solid support, respectively (see the Supporting Information). All of the building blocks were incorporated into oligonucleotides (with typical coupling yields of ≥97%), and tetrazines **5**−**7**[7] were synthesized for fluorescence labeling and affinity tagging (Scheme 2). Diels−Alder reactions were initially investigated on hexa- and heptanucleotides ODN**1**_**1**−**1**_**4** modified either terminally or internally. First, the 5′-modified oligonucleotide ODN**1**_**1** was treated with 1 equiv of tetrazine **5** in aqueous solution. After 60 min of reaction time, the HPLC trace indicated high conversion (Table 1, entry 1), and the MALDI-TOF mass spectra of the collected peak fractions were in agreement with the calculated mass for the Diels−Alder product. Diels−Alder reactions of internally modified ODN**1**_**2** and 3′-modifed ODN**1**_**4** with either **5** or dansyltetrazine **6** also gave the cycloaddition products (Table 1, entries 2−5) in up to 96% yield for a 1:1 stoichiometry.

Scheme 2

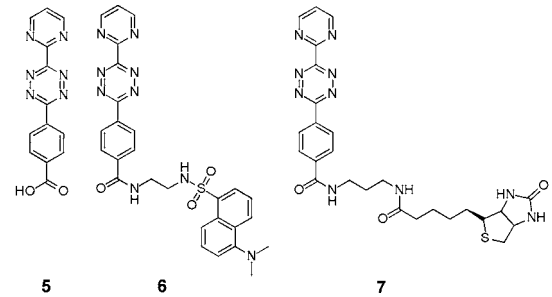


1 2 3 4

ODN1_1 5'-**1**TGCTCA-3'
ODN1_2 5'- TGC**2**TCA-3'
ODN1_4 5'- TGCTCA**4**-3'

ODN**2** 5'- GGAGCTCAGCCTTCACTGC-3'
ODN**2**_**1** 5'-**1**GGAGCTCAGCCTTCACTGC-3'
ODN**2**_**2** 5'- GGAGC**2**CAGCCTTCACTGC-3'
ODN**2**_**3** 5'- GGAGC**3**CAGCCTTCACTGC-3'
ODN**2**_**1/3** 5'-**1**GGAGCTCAGCCTTCAC**3**GC-3'

ODN**3** 5'- GTGGATCCGACCGTGGTGCC-3'
ODN**3**_**1** 5'-**1**GTGGATCCGACCGTGGTGCC-3'



5 6 7

While short oligonucleotides allow straightforward analytical characterization, their utility in molecular biology is limited. Therefore, the 19-mers ODN**2**_**1**−**2**_**3** incorporating building blocks **1**−**3** were prepared (Scheme 2). Thermal denaturation analysis of duplexes involving these oligonucleotides revealed that modifica-

Table 1. Yields of Inverse-Electron-Demand Diels−Alder Reactions

entry	dienophile[a]	diene (equiv)	reaction time (h)	conversion (%)[b]
1	ODN**1**_**1**	**5** (1)	1	73
2	ODN**1**_**1**	**6** (1)	1	70
3	ODN**1**_**2**	**5** (1)	17	96
4	ODN**1**_**2**	**6** (1)	1	80
5	ODN**1**_**4**[c]	**5** (3)	17	94
6	ODN**2**_**1**	**6** (1)	1	78
7	ODN**2**_**2**	**6** (1)	1	80
8	ODN**2**_**3**	**6** (1)	0.2	50
9	ODN**2**_**3**	**6** (1)	1	80
10	ODN**2**_**3**	**6** (1)	3	90
11	ODN**2**_**1/3**	**6** (1)	1	65[d]
12	ODN**2**	**6** (2)	60	0
13	ODN**2**	**5** (10)	60	0

[a] Oligonucleotides were used at a concentration of 170 μM. [b] Determined by integration of the HPLC trace at 260 nm after Diels−Alder reaction. [c] 4,4′-Dimethoxytrityl (DMT)-on product. [d] Double Diels−Alder product; conversion was determined by denaturing polyacrylamide gel electrophoresis.

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tions **1** and **3** barely had an influence on duplex stability, while decreased stability due to the altered base-pairing pattern was observed for compound **2** (see the Supporting Information). For Diels−Alder reactions, ODN**2_3** was treated with equimolar amounts of **6**. After 12 min of reaction time at room temperature, HPLC and MALDI-TOF analysis indicated a positive reaction with a conversion that had already reached 50% (Table 1, entry 8). Higher conversion (up to 90%) was obtained by increasing the reaction time (Table 1, entries 9 and 10). The rate constant for this reaction was determined to be $20 \pm 2\ M^{-1}\ s^{-1}$. The cycloaddition products of the reactions of ODN**2_1** and **2_2** with **6** were also obtained in very good yields (Table 1). Control experiments using nonmodified ODN**2** and tetrazines **5** and **6** showed no reaction, even after 60 h of reaction time and using a 10-fold excess of tetrazine (Table 1, entries 12 and 13).

Post-synthetic modification procedures are most useful when they can be applied to large biomolecules that are not easily accessible by chemical synthesis. To investigate the utility of the method, we amplified a double-stranded 109-mer DNA by polymerase chain reaction (PCR) using dienophile-modified ODN**2_1** as the forward primer (see the Supporting Information). PCR yielded a clean product that was subsequently reacted with biotin−tetrazine **7** in a 1:1 stoichiometry. After reaction at room temperature, aliquots were withdrawn, mixed with the biotin-binding protein streptavidin, and loaded onto an agarose gel, where the bound protein caused a strong retardation of the DNA (Figure 1a). After 10 min, a faint product band was visible, and after 4 h, 30% of the DNA was found to be biotinylated. Increased product formation of up to 75% was obtained by using higher amounts of tetrazine (Figure 1c).

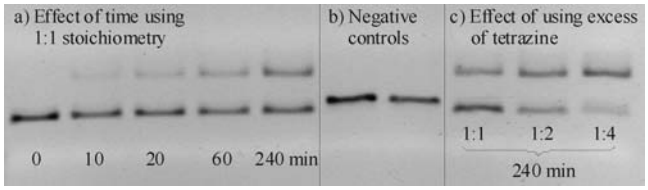

Figure 1. Gel-electrophoretic analysis of Diels−Alder reaction mixtures involving dienophile-modified double-stranded 109-mer PCR products and tetrazine **7** at room temperature (2% agarose gel, stained with ethidium bromide). Lower band: substrate DNA. Upper band: product DNA. The reaction mixture was treated with streptavidin before loading. The negative control was unmodified double-stranded DNA (same sequence) treated with either 2 (left) or 10 (right) equiv of **7** for 15 h at room temperature.

To extend the application of this approach, we furthermore synthesized ODN**2_1/3** carrying two dienophiles. Diels−Alder reaction for 1 h with 1 equiv of tetrazine **6** per dienophile gave 65% conversion to the double-labeled Diels−Alder product (Table 1, entry 11). Finally, double-stranded 109-mer DNA was amplified by PCR using unmodified or singly or doubly modified forward and reverse primers, yielding DNA with up to three norbornene moieties attached. After overnight Diels−Alder reaction with a 10-fold excess of **7** per dienophilic modification, conversions of >95% for the double-Diels−Alder product (Figure 2, lanes 4−5) and 75% for the triple-Diels−Alder product (Figure 2, lane 6) were obtained.

In conclusion, we have demonstrated the inverse-electron-demand Diels−Alder reaction as a highly efficient and chemoselective method for post-synthetic modification of DNA. The elimination of N_2 from the primary cycloaddition product prevents the reverse reaction. The method is suitable for small chemically synthesized oligonucleotides as well as for longer enzymatically amplified DNA strands for both single and multiple modifications.

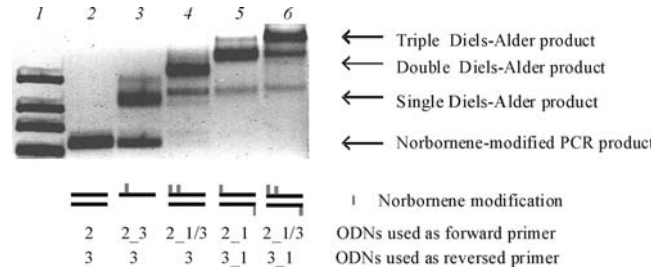

Figure 2. Multiple labeling of DNA by Diels−Alder reaction. A 2% agarose gel (stained with ethidium bromide) of reaction mixtures containing modified double-stranded 109-mer PCR products and tetrazine **7** at room temperature is shown. The tetrazine was used in 10-fold excess per dienophile, and the reaction mixture was treated with 1 equiv of streptavidin before loading. Lane 1: ultra-low-range DNA ladder. Lane 2: unmodified 109-mer. Lane 3: singly modified 109-mer. Lanes 4 and 5: doubly modified 109-mer. Lane 6: triply modified 109-mer.

In contrast to NHS-ester chemistry and copper-catalyzed azide−alkyne cycloaddition,[2,11] inverse Diels−Alder conjugation works efficiently at very low reactant concentrations and much lower excesses of labeling reagent, often even with equimolar amounts. These properties render the method attractive for conjugation of expensive and sensitive compounds. The reaction proceeds smoothly under mild conditions without the requirement of transition metals. This is particularly relevant for potential applications of this coupling chemistry in cells,[8c] where high concentrations of copper are not tolerated. In comparison with other recent bioconjugation methods,[12] the functional groups required for the inverse-electron-demand Diels−Alder reaction are relatively easy to synthesize and incorporate into oligonucleotides.

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Supporting Information Available: Full experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

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