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Internal labeling of oligonucleotide probes by Diels–Alder cycloaddition

Duncan Graham,* Antonio Grondin, Callum McHugh, Ljiljana Fruk and W. Ewen Smith

Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK

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Abstract—A new method of adding fluorescent labels to the middle of oligonucleotides is reported. Diels–Alder cycloaddition was used to add five fluorescent maleimides to an oligonucleotide containing a 2′-deoxyuridine modified at the 5-position with a spaced furan. This is a new approach to internal oligonucleotide chemistry that opens up a large range of possibilities for further conjugation.

Addition of external labels to oligonucleotides is necessary to satisfy the need of current DNA assays and allow the development of new approaches. The most common current approach for DNA analysis is the use of fluorescence spectroscopy.[1–3] Most fluorophores are added to the 5′ or 3′ terminus by a number of different methods. Internal labeling is more complex to achieve and a number of approaches have been employed. These include modification of the backbone, sugar or base. Addition of fluorophores to the base are most popular. Current approaches include the use of functional groups such as alkyl amines that react with activated dyes after oligonucleotide synthesis[4] or the use of a pre-labeled nucleoside phosphoramidite.[3] The post synthesis approach suffers from low yields and side reactions and the use of dye nucleoside phosphoramidites requires complex chemistry and expensive monomers. Brown et al.[5] recently reported the use of an Fmoc protected alkyl alcohol modified thymidine phosphoramidite that could be used to add commercially available dye phosphoramidites in high yield to the middle of an oligonucleotide. The aim of the research reported here was to produce a nucleoside phosphoramidite that could be used to add a label to the middle of an oligonucleotide after solid phase synthesis in a highly efficient manner.

We report the use of Diels–Alder cycloaddition for the addition of fluorescent dyes to a modified 2′-deoxyuridine residue after oligonucleotide synthesis. Hill et al.[6] recently reported the use of Diels–Alder cycloadditions as a method of adding fluorophores to the 5′-terminus of oligonucleotides. This demonstrated the advantages of using Diels–Alder reactions for conjugations to oligonucleotides, namely, a fast, quantitative reaction occurring in an aqueous buffer system. Additionally, as Diels–Alder reactions occur between a diene and a dienophile there is no need for use of selective protecting groups to prevent reaction with common nucleophiles and hence do not suffer from any side reactions. In this approach one phosphoramidite can be used to add any number of different dyes.

In this study we report the addition of a diene to the 5 position of 2′-deoxyuridine. The diene was chosen to be added to the nucleoside, as a number of commercially available dienophiles in the form of fluorescent maleimides can be used without further chemical modification. A range of dienes was considered for addition to a nucleoside and their reactions with a maleimide examined.[7] Furan was chosen due to the number of readily available derivatives, the speed and efficiency of the reaction with maleimides and stability to the conditions used for oligonucleotide synthesis.

The starting material for the synthesis was 5-iodo-2′-deoxyuridine which was initially protected on the 5′-hydroxyl with dimethoxytrityl **1**. A palladium catalyzed Sonogashira coupling was used to add an acetylenic furan derivative **2** prepared by the condensation of pentynoic acid and furfuryl amine. This protected furan 2′-deoxyuridine **3** was then phosphitylated using standard procedures to give the monomer **4** ready for solid phase oligonucleotide synthesis (Scheme 1).

* Corresponding author. E-mail: duncan.graham@strath.ac.uk

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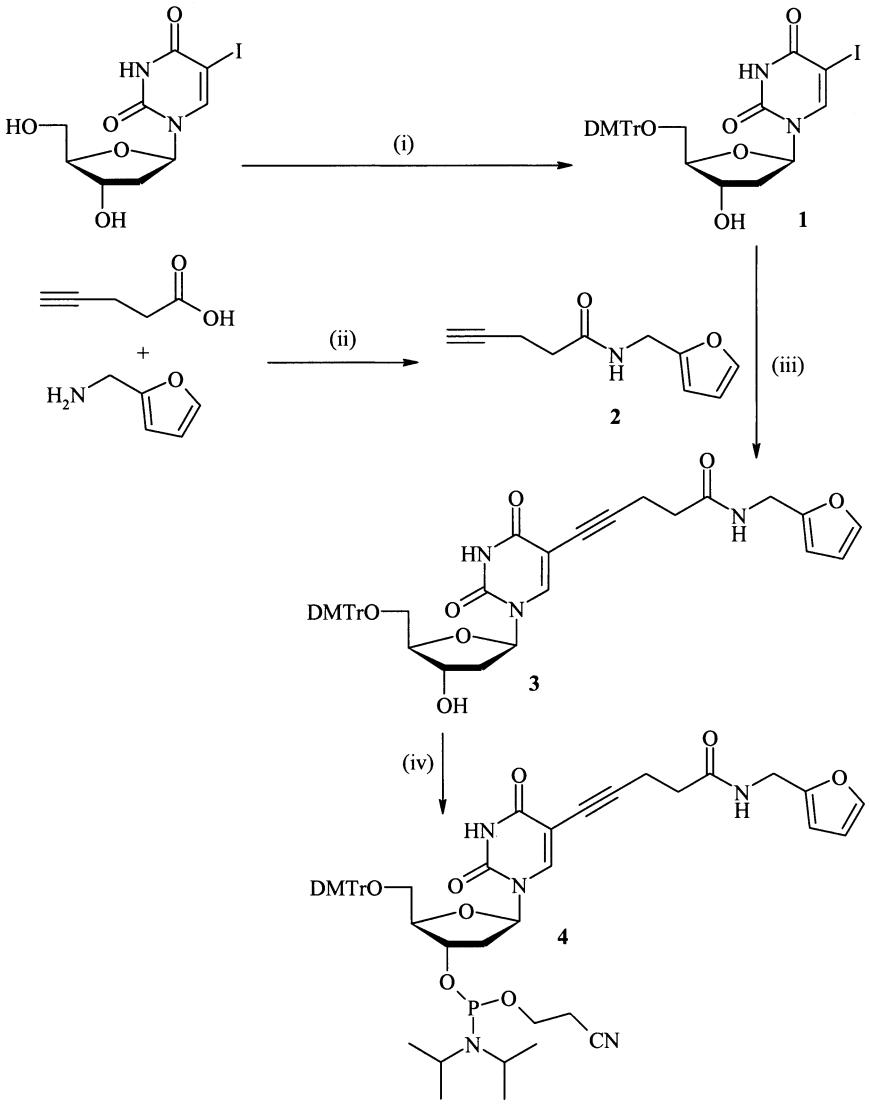

Scheme 1. (i) Pyridine, 4,4′-dimethoxytrityl chloride (1.2 equiv.), DMAP (0.1 equiv.), 80%; (ii) DMF, carbonyl diimidazole (1.1 equiv.), 40°C, 95%; (iii) DMF, triethylamine (5 equiv.), CuI (0.2 equiv.), **2** (1.5 equiv.), Pd $(PPh_3)_4$ (0.1 equiv.), 79%; (iv) THF, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphine (1.1 equiv.), DIPEA (3 equiv.), 98%.

The monomer was then used as a 50 mg/ml solution (THF/MeCN 1:1) on an Expedite 8909 oligonucleotide synthesizer with a 15-minute coupling cycle. The HPLC trace indicated excellent incorporation of the modified monomer (Fig. 1). A 20mer that has been used as an antisense oligonucleotide to inhibit MDR1 gene expres-

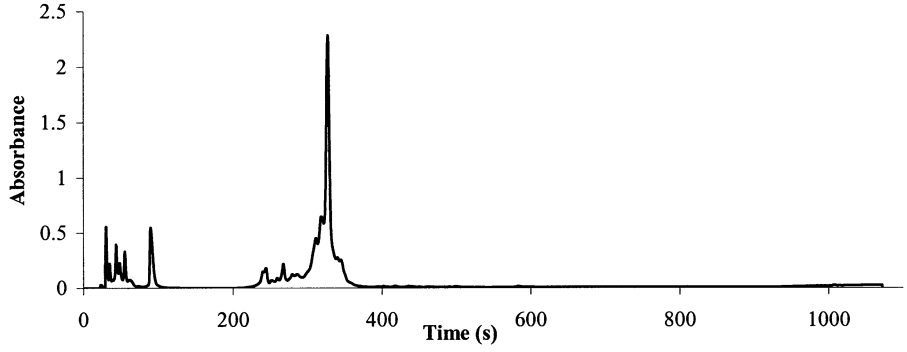

Figure 1. HPLC trace obtained for the crude modified 20mer after deprotection using a Chromolith reverse phase column running at 4 ml/min and a buffer system of A = 0.1 M ammonium acetate pH 7.0, B = MeCN. (CV = column volume 1.662 ml) 5 CV 5% B, 25 CV 5–30% B, 5 CV 30% B, 5 CV 30–100% B, 3 CV 100% B.

sion was synthesized.[8] The position marked with X represents the furan modified uridine.

5′CCT CGC GCX CCA GCC CTA CC

Diels–Alder cycloadditions were then attempted using this oligonucleotide and five fluorophore maleimides. The following dye maleimides were purchased from Molecular Probes and used without further manipulation, FAM, Texas red, TAMRA-5, TAMRA-6 and Alexa Fluor 532 (Fig. 2). The two isomers of TAMRA i.e. 5 and 6 were examined to see if the different isomers affected the cycloaddition. The oligonucleotide was dissolved in a phosphate buffer (25 mM, pH 5.5) and the fluorophore maleimides added in an acetonitrile buffer mixture that gave a final acetonitrile concentration of 30% v/v. The cycloadditions were left in the dark at 40°C for 3 h prior to analysis by reverse phase HPLC. The TAMRA dyes were not heated due to the thermal instability of the dye but were left to react for 4 h instead of 3 h. In each case 3 equiv. of dye maleimide were used to ensure complete reaction. The HPLC traces of the cycloadditions showed clear differences between the furan oligonucleotide peak and the cycloadduct peak indicating that the cycloadduct had formed in excellent yield. Fig. 3 shows the HPLC trace of the oligonucleotides produced by cycloaddition of three of the maleimides. All of the cycloadducts displayed a reduced retention time due to the increased polarity of the conjugated oligonucleotides. Only three are shown in Fig. 3 as the Alexa Fluor 532 and FAM conjugates had the same retention time as did the TAMRA 6 isomer and Texas Red conjugates.

The labeled oligonucleotides were then examined for fluorescence. The emission wavelengths observed for the oligonucleotides differed slightly from those quoted. This is presumed to be due to the use of an aqueous buffer as opposed to methanol apart from the value quoted for FAM,[9] which is in aqueous buffer and is identical (Table 1). Interestingly we found that the two

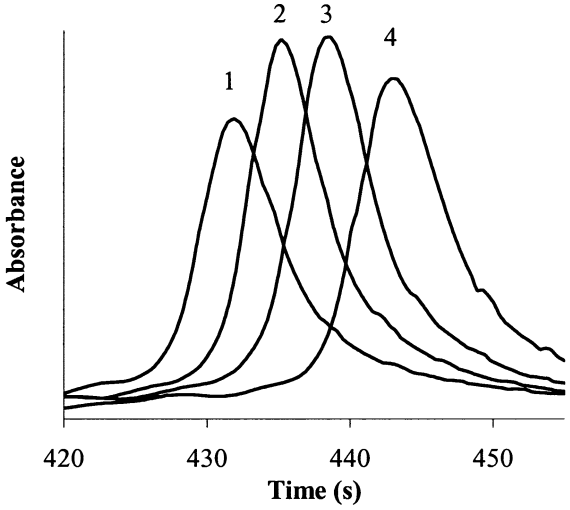

Figure 3. HPLC of the cycloadducts using a Chromolith reverse phase column at 4 ml/min and a buffer system of: A = 0.1 M ammonium acetate pH 7.0, B = MeCN. (CV = column volume 1.662 ml) 0–5 CV 5% B, 5–15 CV 5–10% B, 15–35 CV 10–25% B, 35–45 CV 25–100% B, 45–48 CV 100% B. Peak 1 = TAMRA 5, Peak 2 = Texas Red, Peak 3 = FAM, Peak 4 = furan oligo. (The peaks have been scaled for clarity.)

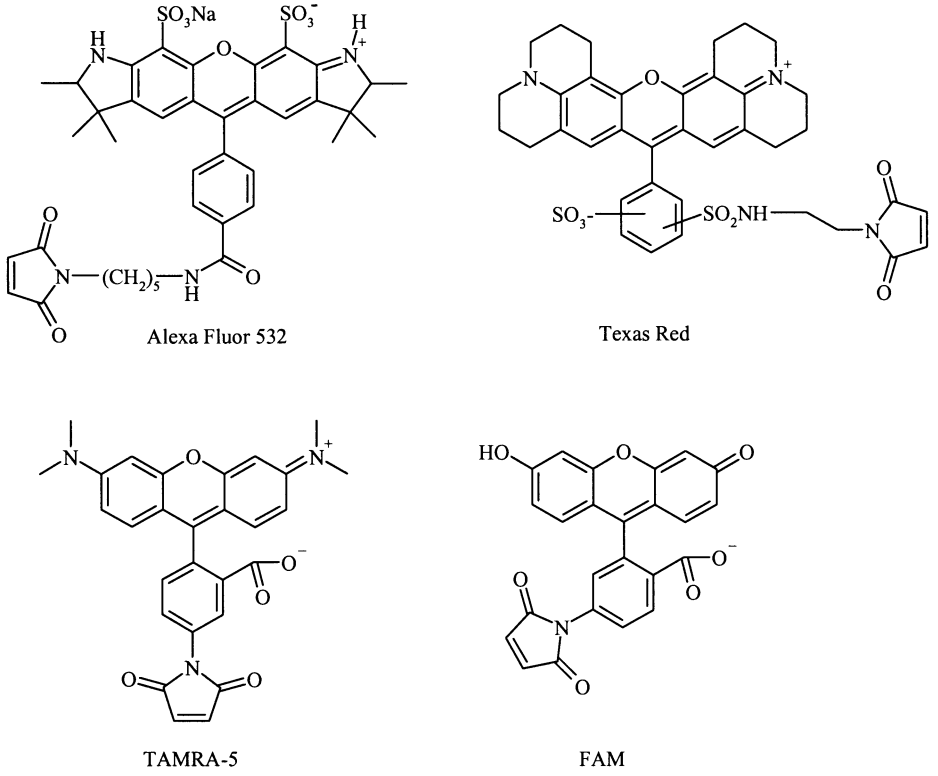

Figure 2. The fluorophore maleimides used in the cycloadditions.

Table 1. Comparison of the emission wavelengths of the labeled oligonucleotides with the literature values

Fluorophore	Emission (aq.) (nm)	Lit. value[9] (nm)
FAM	515	515 (aq.)
TAMRA-5	571	567 (MeOH)
TAMRA-6	558	567 (MeOH)
Alexa Fluor 532	533	552 (MeOH)
Texas Red	606	600 (MeOH)

TAMRA isomers gave emission wavelengths that differed by 13 nm where the literature values are identical. This proved that the oligonucleotides were fluorescent and that the cycloaddition does not interfere with the fluorescence emission.

In conclusion, this letter demonstrates a new method of internally labeling oligonucleotides that is fast, simple and high yielding. Different types of fluorophore were used to demonstrate the versatility of this approach and demonstrate the selectivity of the chemistry. Additionally the one modified phosphoramidite can be used with any fluorescent maleimide and is therefore a generic modification.

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