

Diene-modified nucleotides for the Diels–Alder-mediated functional tagging of DNA

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Received July 23, 2008; Revised December 16, 2008; Accepted December 18, 2008

ABSTRACT

We explore the potential of the Diels–Alder cycloaddition for the functional tagging of DNA strands. A deoxyuridine triphosphate derivative carrying a diene at position 5 of the pyrimidine base was synthesized using a two-step procedure. The derivative was efficiently accepted as substrate in enzymatic polymerization assays. Diene carrying strands underwent successful cycloaddition with maleimide-terminated fluorescence dyes and a polymeric reagent. Furthermore, a nucleotide carrying a peptide via a Diels–Alder cyclohexene linkage was prepared and sequence-specifically incorporated into DNA. The Diels–Alder reaction presents a number of positive attributes such as good chemoselectivity, water compatibility, high-yield under mild conditions and no additional reagents apart from a diene and a dienophile. Furthermore, suitable dienophiles are commercially available in the form of maleimide-derivatives of fluorescent dyes and bioaffinity tags. Based on these advantages, diene- and cyclohexene-based nucleotide triphosphates are expected to find wider use in the area of nucleic acid chemistry.

INTRODUCTION

The labeling of DNA and RNA strands with nucleotide triphosphate derivatives is an important topic in nucleic acids chemistry. Nucleotides carrying fluorophores, radio-nuclides, biotin, digoxigenin or other labels are enzymatically incorporated into nucleic acids in order to facilitate their detection and isolation, or to enhance their functional properties in cell biological experiments (1). The functional tagging of DNA can proceed either via the pre-modification approach using completely modified nucleotides for enzymatic incorporation, or via the post-modification route in which a nucleotide precursor is incorporated and afterwards linked to the final tag.

Based on its influence on the enzymatic incorporation, the chemical design of nucleotide derivatives is of great importance. Many nucleotide derivatives carry artificial labels at position 5 of the pyrimidine bases or at position 7 of deazapurin analogues because enzymes tolerate non-biogenic substitutions at these sites (2). In the post-modification approach, the exogenous linker also has to meet additional criteria with regard to linker length and terminal functional group (2). Only a limited number of coupling chemistries are compatible with the post-modification approach. One strategy uses a thiol group or sulfhydryl-reactive groups such as iodoacetamide, maleimide or pyridyl-disulfide (3). Another route employs the Huisgen 1,3-dipolar ‘Click’ cycloaddition, where an alkyne is joined with an azide to produce a triazole (4). Alkyne-modified nucleotides have been successfully incorporated into DNA strands and coupled to azide-carrying tags (5) to facilitate *in vitro* (6) and *in vivo* detection (7).

The Diels–Alder reaction is another route which offers the prospect of quickly and reliably creating a linkage between a base and a tag. The Diels–Alder reaction is a cycloaddition between a conjugated diene and a dienophile to form a cyclohexene structure (8). Its high specificity and high yield under aqueous and mild conditions (9) have made the reaction popular in the wider area of bio-conjugate chemistry (10). In the field of nucleic acid chemistry, the Diels–Alder approach has been employed in the context of solid-phase oligonucleotide synthesis using phosphoramidite chemistry. For example, Hill *et al.* (11) and Graham *et al.* (12) synthesized diene phosphoramidite reagents that enabled the Diels–Alder-mediated labeling with fluorophores (11,12), biotin and organic polymers (11) at the 5'-end (11) or at an internal position (12) of synthetic oligonucleotides. 5'-diene-modified oligonucleotides were also utilized to generate peptide–oligonucleotide conjugates (13). The Diels–Alder chemistry has, however, so far not been extended to nucleotide triphosphates.

Here we explored the potential of Diels–Alder chemistry for the labeling of DNA strands by synthesizing deoxyuridine derivative **3** (Figure 1A) which carries a terminal diene at position 5 of the pyrimidine ring. The labeling

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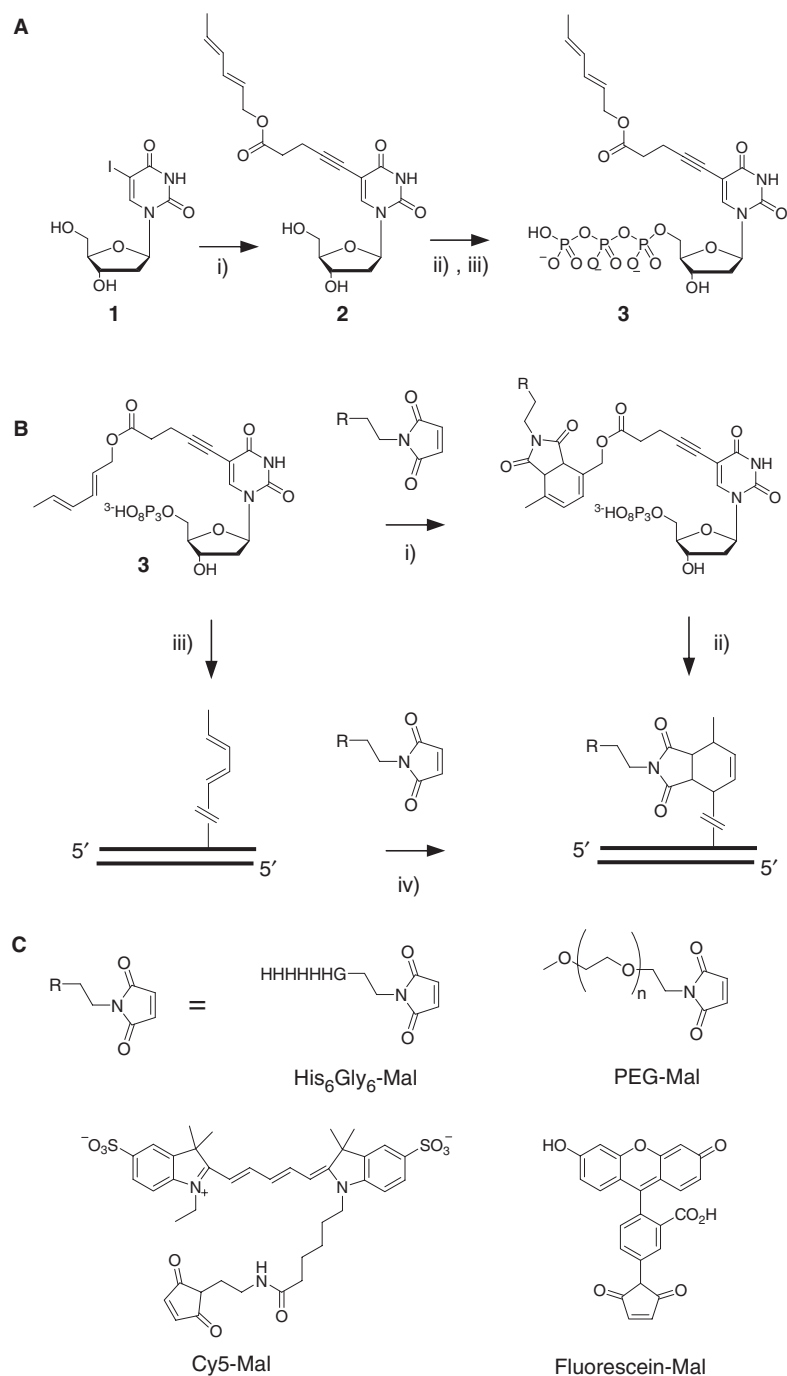


Figure 1. Synthesis of diene-modified deoxyuridine derivative **3**, and its use for the functional tagging of DNA via Diels–Alder chemistry. (A) Two-step synthesis of **3** from 5-iodo-2'-deoxyuridine **1**. (i) CuI, Et₃N, Pd(Ph₃P)₄, pent-4-ynoic acid hexa-2,4-dienyl ester; (ii) POCl₃; (iii) (nBu₃NH⁺)₄.P₂O₇⁴⁻. (B) The pre-modification approach comprising (i) the Diels–Alder-mediated tagging of nucleotide **3** with a maleimide dienophile and (ii) the enzymatic incorporation of the cyclohexene product into DNA. The post-modification approach including (iii) enzymatic incorporation of **3** and (iv) Diels–Alder cycloaddition between the diene-tagged DNA and maleimide dienophiles. (C) The maleimide derivatives of peptide His₆Gly₁, and poly(ethylene glycol), Cy5, and fluorescein were used for the pre- and post-modification approach, respectively.

with nucleotide **3** was tested for the pre- as well as the post-modification approach. For pre-modification, diene-tagged nucleotide **3** was first reacted via Diels–Alder with a maleimide dienophile (Figure 1B, i) to produce a fully modified nucleotide carrying the cyclohexene linkage. In the second step (Figure 1B, ii), the cyclohexene

nucleotide was enzymatically incorporated into DNA. For the post-modification approach, nucleotide **3** was first applied (Figure 1B, iii) to enzymatically furnish DNA strands with diene groups. Diels–Alder reaction (Figure 1B, iv) with a maleimide dienophile yielded DNA carrying tags via a cyclohexene linkage.

Pre-modification was conducted with peptide His₆Gly₁-Mal carrying a terminal maleimide group (Figure 1C). The post-modification was tested with maleimide derivatives of poly(ethylene glycol) (PEG-Mal), and fluorophores Cy5 (Cy5-Mal) and fluorescein (Flu-Mal) (Figure 1C). Our results indicate that diene- and cyclohexene-derivatized nucleotides are sequence-specifically incorporated and allow for the functional tagging of DNA strands under mild conditions without additional catalysts.

MATERIALS AND METHODS

General

Trimethylphosphate was dried under a 3Å-molecular sieve prior to use. 5-Iodo-2'-deoxyuridine was stored in a desiccator several days before use. Anhydrous tributyl ammonium pyrophosphate solution was prepared following a published procedure (14). All other chemicals and solvents were of commercial quality and used as received. Deep Vent_R[®] (exo⁻) polymerase was purchased from New England Biolabs (NEB). DNA oligos, fluorescein-5-maleimide (Flu-Mal) and Cy5-Mal were ordered from Integrated DNA Technologies, Cambridge Bioscience, and GE Healthcare, respectively. Preparative ion exchange chromatography was performed on a Sephadex A-25 DEAE (120 ml) column or a Resource Q (1 ml) column on an Äkta Purifier system. Analytical HPLC was performed on a Varian Pro Star system equipped with a dual wavelength 325 detector and a Gemini C18 column (250 × 4.6 mm²). Preparative HPLC was performed on the same system using a Polaris C18 column (100 × 21.2 mm², 5 μm beads). Mass spectrometric analysis was performed on a Waters Acquity Ultra Performance LC/MS system equipped with an Acquity UPLC BEH C18 column (50 × 2.1 mm², 1.7 μm beads). NMR spectra were recorded at 293K on a Bruker AVANCE500 spectrometer (for ¹H- and ¹³C-NMR) and on a Bruker AMX300 spectrometer (for ³¹P-NMR). Coupling constants are reported in Hz. Unless described otherwise, oligonucleotides and DNA strands were analyzed via denaturing polyacrylamide gel electrophoresis (PAGE) using gels prepared using 18% acrylamide in *tris*-borate buffer containing 7M urea and 18% formamide, and visualized using the Stains-All stains.

Preparation of 5-(pent-4-ynoic acid hexa-2,4-dienyl ester)-2'-deoxyuridine, 2

For the Sonogashira coupling (15), 5-iodo-2'-deoxyuridine, **1** (395 mg, 1.05 mmol), palladium tetrakis(triphenylphosphine) (55 mg, 48 μmol) and copper iodide (28 mg, 121 μmol) were placed in a dried flask flushed with argon. Anhydrous DMF (5.0 ml) was added via syringe, followed by triethylamine (0.4 ml, 2.9 mmol) and a solution of sorbyl-4-pentynoate (1.0 g, 6.02 mmol) in anhydrous DMF (5 ml). After 22 h stirring at room temperature, the solvent was removed under reduced pressure. The resulting brown oil was purified by column chromatography using a gradient from 3% to 5% MeOH in DCM, yielding 158 mg of white powder (39%). ¹H-NMR (DMSO, 500 MHz): δ 1.71 (d, J 6.6,

3H), 2.09 (dd, J 6.6, 4.7, 2H), 2.53–2.63 (m, 4H), 3.54 (ddd, J 11.8, 5.0, 3.8, 1H), 3.59 (ddd, J 11.8, 5.0, 3.6, 1H), 3.78 (dd, J 6.6, 3.5, 1H), 4.19–4.23 (m, 1H), 4.56 (d, J 6.3, 1H), 5.07 (t, J 4.9, 1H), 5.22 (d, J 4.1, 1H), 5.62 (dt, J 15.1, 6.6, 1H), 5.72 (dq, 15.1, 6.6, 1H), 6.05 (ddd, J 15.1, 10.4, 1.6, 1H), 6.09 (t, J 6.6, 1H), 6.25 (dd, J 10.4, 15.1, 1H), 8.10 (s, 1H), 11.55 (s, 1H). ¹³C-NMR (DMSO, 126 MHz): 171.1, 161.6, 149.4, 143.0, 134.0, 130.6, 130.5, 124.2, 98.6, 91.5, 87.5, 84.6, 73.1, 70.1, 64.3, 60.9, 39.7, 32.8, 17.8, 14.8. ESI-MS(pos): 405 [M + H]⁺, HRMS 403.1519, calculated mass for C₂₀H₂₃N₂O₇: 403.1505. UV-vis λ_{max} (water): 228 nm, 291 nm (calc. ε₂₉₁: 10 600 l mol⁻¹cm⁻¹) (see also Supplementary Data S1).

Preparation of 5-(pent-4-ynoic acid hexa-2,4-dienyl ester)-2'-deoxyuridine-5'-triphosphate triethylammonium salt, 3

Following an adapted procedure (16), nucleoside **2** (83 mg, 0.22 mmol) and 1,8-bis(dimethylamino)naphthalene (proton sponge) (71 mg, 0.33 mmol) were placed in a dried two-neck flask and dissolved in trimethylphosphate (2.0 ml) under argon. The reaction mixture was cooled to -20°C, and POCl₃ (30 μl, 0.32 mmol) was added via syringe. After 20 min stirring, a suspension of tributylammonium pyrophosphate (14) in anhydrous DMF (0.5 M, 2 ml) and tributylamine (0.16 ml) was added. After 45 min, the mixture was hydrolyzed with a solution of triethylammonium bicarbonate (TEAB) (0.1 M, 20 ml) and allowed to warm up to room temperature. Volatile solvents were removed under reduced pressure, and the residue was purified by ion exchange chromatography (Sephadex A-25 DEAE) at a flow rate of 10 ml/min using the step gradient: 0.1 M TEAB pH 7. 5 for 120 ml, 0.1 M to 0.45 M in 120 ml, 0.45 M to 0.8 M in 600 ml, followed by 0.8 M for 200 ml. The fractions were concentrated and further purified by preparative HPLC at a flow rate of 10 ml/min using a gradient from 20% MeCN/0.1 M TEAB, pH 7.0 to 45% MeCN/0.1 M TEAB for 7 min, followed by 45% MeCN/0.1 M TEAB for 3 min. ¹H-NMR (D₂O, 500 MHz): δ 1.19 (t, J 7.3, 27H), 1.61 (d, J 6.6, 3H), 2.26 (dd, J 14.0, 6.6, 2H), 2.31 (ddd, J 14.0, 6.3, 3.9, 1H), 2.58–2.62 (m, 2H); 2.63–2.67 (m, 2H), 3.10 (q, J 7.3, 18H), 4.07–4.15 (m, 3H), 4.51–4.55 (m, 1H), 4.58 (d, J 6.3, 2H), 4.58 (dt, J 15.1, 6.6, 1H), 5.68 (dq, J 15.1, 6.6, 1H), 5.95 (ddd, J 15.1, 10.4, 1.6, 1H), 6.17 (t, J 6.6, 1H), 6.20 (dd, J 15.1, 10.4, 1H), 7.92 (s, 1H). ¹³C-NMR (D₂O, 126 MHz): δ 175.0, 164.8, 150.8, 144.2, 135.3, 132.5, 130.5, 123.7, 100.3, 94.1, 85.9, 85.8, 72.2, 70.9, 66.0, 46.9, 38.8, 33.4, 17.7, 15.5, 8.5. ³¹P-NMR (D₂O, 500 MHz): δ -21.69 (t, J 21.1, 1P), -10.17 (d, J 21.1, 1P), -8.75 (d, J 21.1, 1P). ESI-MS (neg): 643, HRMS: 643.0488, calculated mass for C₂₀H₂₆N₂O₁₆P₃: 643.0495. UV-vis λ_{max} (water): 228 nm, 291 nm (see also Supplementary Data S2).

Peptide synthesis

The maleimide-peptide conjugate HOOC-(His)₆Gly-Mal was synthesized using standard Fmoc solid-phase peptide synthesis (SPPS) on a Syro automated system using

chloro-trityl resin pre-loaded with His and HBTU/DIPEA coupling chemistry following an adapted procedure (17) without the thiol scavenger DTT in the cleavage mixture. Maleimide- β -alanine was added as the last amino acid. The peptide was analyzed by LC/MS. ESI-MS(pos): 525.5 $[M + 2H]^{2+}$, 1049.5 $[M + H]^+$ (see also Supplementary Data S3c), HRMS: 1049.4211, calculated mass for $C_{45}H_{53}N_{20}O_{11}$: 1049.4203.

Preparation of a peptide conjugate of 5-(pent-4-ynoic acid hexa-2,4-dienyl ester)-2'-deoxyuridine-5'-triphosphate triethylammonium salt, 4

A solution of 50 mM of HOOC-(His)₆Gly-Mal was prepared by dissolving lyophilized peptide in 500 mM Na₂HPO₄ buffer (final pH 6.1). Aliquots (300 or 72 μ l) were added to a solution of triphosphate nucleotide **3** (30 mM, 100 μ l), and the reaction mixtures were shaken at 20°C for 1.5 and 7 h, respectively. LC-MS and ion exchange chromatography analysis indicated full conversion of the starting material for both reactions. The product was purified by ion exchange chromatography by using a Resource Q column at a flow rate of 2.5 ml/min using 0.1 M TEAB pH 7.5 for 5 ml followed by a gradient of 0.1 M to 0.45 M TEAB over 10 ml. The fractions containing products were lyophilized. ESI-MS(neg): 845.6 $[M - 2H]^{2-}$, 1692.3 $[M - H]^-$; HRMS: 1693.4810, calculated mass for $C_{65}H_{80}N_{22}O_{27}P_3$: 1693.4776. UV-vis λ_{max} (water): 291 nm (see also Supplementary Data S3a, b, d-f).

Primer extension assay using **3** and **4**

Samples for DNA extension included 300 pmol of a primer with the sequence 5'-ATGGGACTAACTAATC TTTGCTTA-3' and 350 pmol of template with the sequence 5'-TATTCCATGTGATAAGCAAAGATTAG TTAGTCCCAT-3' dissolved in a polymerase extension mix. The mix comprised 2U Deep Vent_R[®] (exo⁻) (NEB), 1X Thermopol[™] buffer (NEB), and 40 μ M of natural dNTPs or dUTP derivatives **3** and **4**, as specified in Figures 3 and 4, respectively. The reaction mixtures were heated to 85°C for 1 min, incubated at 57°C for 10 min, and immediately chilled on ice. Due to the lack proofreading activity in Deep Vent_R[®] (exo⁻), primer extension times longer than 20 min were found to lead to mis-incorporation. The extension products (6–9 μ l) were denatured by addition of formamide (5 μ l) and subsequent brief heating to 95°C, followed by analysis using PAGE.

Diels–Alder reactions with diene-modified DNA

The diene-modified DNA samples (50–75 μ l) were purified using Qiagen nucleotide removal kits and eluted from the columns using 100 μ l of deionized water. The samples were concentrated to 2–4 μ l on a SpeedVac evaporator and then mixed with a solution of the maleimide bearing reagent.

Reaction with PEG–Mal (MW 5 kDa). Solutions of PEG–Mal of different concentrations (0.1, 1 and 10 mM) were prepared, and 10 μ l each were added to the concentrated diene-modified DNA samples which had been mixed with 500 mM Na₂HPO₄ buffer, pH 6.1 (5 μ l). The reaction mixtures were incubated at 20°C for 24 h,

diluted with 20 μ l of deionized H₂O and analyzed by denaturing PAGE. The extent of reaction was quantified by analyzing the intensities of the bands using image analysis software Scion Image (Scion Corporation) as described (18). The images were analyzed using Scion Image as described above.

Reaction with Cy5–Mal. The concentrated DNA samples were mixed with a solution of Cy5–Mal (5 mM, 4 μ l) in 500 mM Na₂HPO₄ buffer, pH 6.1. The reaction mixtures were then incubated at 37°C for 16 h, diluted with 30 μ l of deionized H₂O, and purified using Micro Bio-Spin P6 columns (Bio-Rad) to remove excess reagent. The samples were analyzed by denaturing PAGE and fluorescence scanning using an FLA-3000 Imager equipped with a 633 nm laser and a 675 nm filter, as well as staining with Stains-All.

Test on the hydrolytic stability of the ester linkage of **4**

A 28 bp DNA sample 5'-AATTTAAAGTGCAACCAAT GTGAGTCAU(His₆)-3' carrying a His₆-tag at the 3'-terminal uracil base was generated by template-directed linear amplification using primer 5'-AATTTAAAGTGC AACCAATGTGAGTCA-3', the corresponding template, and modified base **4**. The amplification product was subsequently purified by His₆ affinity chromatography (V. Borsenberger, N. Mitchell, S. Howorka, unpublished data). To determine the hydrolytic stability of the ester linkage, a solution the His₆-tagged DNA was diluted in 1X Thermopol buffer to a final concentration of 6 μ M and incubated at 95°C for 20 min. Aliquots were taken, kept on ice and analyzed by denaturing PAGE and Scion Image.

PCR amplification of diene-tagged DNA and Diels–Alder-mediated tagging with fluorescent dye Flu–Mal

DNA fragments with a length of 500 bp were obtained by PCR. The amplification reactions contained nucleotide mixes with all four natural nucleotides (200 μ M each) or a mix with dUTP derivative **3** at a ratio of 1:3 to dTTP. This mixture of dTTP and **3** was chosen as ratios higher than 1:1 led to the precipitation of the PCR product after Diels–Alder reaction with Flu–Mal, most likely due to the increased hydrophobicity of the DNA duplexes caused by multiple fluorescein tags. For PCR reaction, the nucleotide mixes were combined with 5 fmol of *Nde*I-linearized plasmid pT7*sbsB*His (18), 200 pmol each of forward and reverse primer with the sequence 5'-ATGCTAGTTATTG CTCAGCGGTGG-3' and 5'-CCAACAGGTGCAAATG TTTAG-3', respectively, 1U Deep Vent (exo⁻), 8 μ l of DMSO, and 1X Thermopol buffer yielding a total volume of 50 μ l. Thermocycling conditions comprised a denaturation step at 94°C for 2 min, 30 cycles of 95°C for 15 s, 57°C for 45 s and 72°C for 1 min, followed by an incubation step at 72°C for 10 min. PCR products were purified using the Qiagen PCR purification kit and eluted in 40 μ l of ultrapure water. The samples were mixed with a solution of 500 mM NaH₂PO₄, pH 6.1, 1 M NaCl (10 μ l) and 0.2 mg of Flu–Mal. The reaction mixtures were then concentrated to 4–6 μ l and incubated overnight

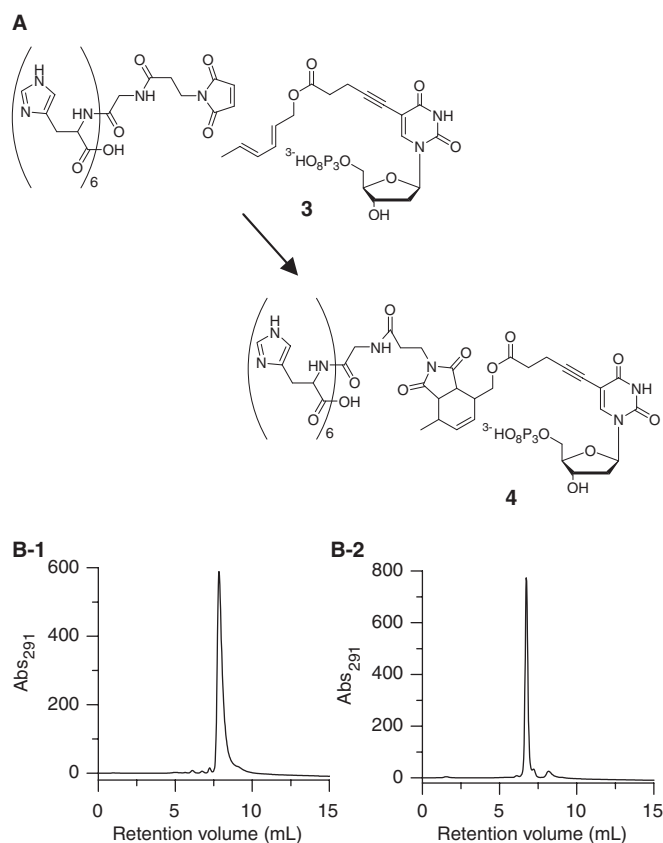


Figure 2. (A) Diels–Alder cycloaddition between diene nucleotide **3** and maleimide-tagged peptide HOOC-(His)₆Gly-Mal yielding nucleotide **4**. (B) Corresponding ion exchange chromatography traces (B-1) of starting material **3** and (B-2) of crude reaction mixture containing **4** after treatment with 1.2 equivalents of HOOC-(His)₆Gly-Mal for 7 h.

at 37°C. Afterwards, the samples were diluted in ultrapure water (30 µl), and excess dye was removed by purification using Micro Bio-Spin P30 columns (Bio-Rad). The reaction mixtures were analyzed by 1.5% agarose gel electrophoresis in TAE buffer, and visualized using fluorescence excitation at 303 nm, and ethidium bromide staining.

RESULTS AND DISCUSSION

The chemical route for the synthesis of 5-(pent-4-ynoic acid hexa-2,4-dienyl ester)-2'-deoxyuridine-5'-triphosphate, **3** is illustrated in Figure 1A. 5-Iodo-2'-deoxyuridine, **1** was first subjected to Sonogashira coupling (Figure 1A, i) (6,15,19) with sorbyl-4-pentynoate to yield **2** as shown by HPLC, UV-vis spectrometry, ESI mass spectrometry, ¹H-NMR and ¹³C-NMR (Supplementary Data, S1). HPLC-purified nucleoside **2** was then reacted with POCl₃ and pyrophosphate (Figure 1A, ii) using the Yoshikawa procedure (20,21) to yield target nucleotide **3**. The purity of compound **3** was demonstrated by HPLC analysis (Supplementary Data, S2a). Further characterization by UV-vis spectrometry, ESI-MS, ¹H-NMR, ¹³C-NMR and ³¹P-NMR confirmed the chemical identity of **3** (Supplementary Data, S2b–f).

Nucleotide **3** was reacted with a maleimide-terminated peptide to test its ability to form Diels–Alder adduct **4**

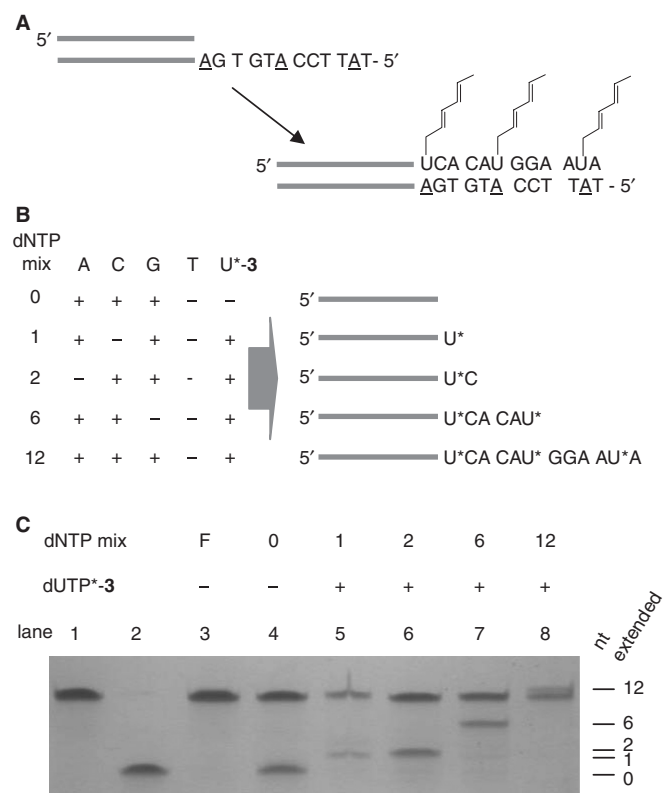


Figure 3. dUTP-diene **3** is specifically incorporated into DNA strands. (A) A duplex between the primer and the longer template serves as substrate for the primer extension assay. The template sequence directs the incorporation of up to three diene nucleotides **3** (abbreviated as U*-3 or dUTP*-3). (B) dNTP mixes of varying composition control the step-wise extension. (C) Denaturing PAGE analysis of the extension reactions. dNTP mixes 0, 1, 2, 6 and 12 are defined in (B). Mix F contains a full set of natural nucleotides. Lanes 1 and 2 are of template and primer, respectively.

(Figure 2A). Peptide His₆Gly₁-Mal was obtained via standard peptide synthesis. In order to avoid the hydrolysis of the maleimide group observed at pH values above 7.5 (22), the maleimide-tagged peptide was dissolved in a buffer at pH 6.1 (pH values between 3.8 and 6.1 were also used successfully in the Diels–Alder reaction between **3** and maleimide-derivatives). The Diels–Alder coupling was conducted at two different ratios of dienophile versus diene. With five equivalents of the maleimide dienophile, no diene-tagged nucleotide **3** could be detected via LC-MS after 90 min of incubation at 20°C (Supplementary Data S3a, S3b). Similarly, at 1.2 equivalents and a longer incubation of 7 h, the reaction was complete as demonstrated by ion exchange chromatography (Figure 2B). The quantitative conversion even at a small excess of dienophile, along with the lack of any detectable by-products are noticeable features of this reaction. Additional examination of nucleotide derivative **4** with UV-vis spectroscopy and ESI mass spectrometry confirmed its chemical identity (Supplementary Data S3d–f).

We explored whether the diene-modified nucleotide **3** can be sequence-specifically incorporated into DNA strands, by using a primer extension assay schematically illustrated in Figure 3A. A short DNA primer was first

hybridized to a longer strand yielding a non-hybridized section which served as template for the 3' extension of the primer. The sequence of the 12-nt long template overhang contained three adenines (Figure 3A, underlined) to probe the incorporation of up to three deoxyuridine derivatives **3**. The sequence of the template was designed to control the extension in a step-wise fashion by varying the composition of the dNTP mix (Figure 3B). In particular, key nucleotides required for extension beyond a given base position were omitted or included. For example, dNTP mix 0 contained dATP, dCTP, dGTP but neither dTTP nor dUTP***-3** (Figure 3B). Therefore, no polymerization should occur. By comparison, extension by 1 nt can be expected in dNTP mix 1 due to the presence of dUTP***-3** but the omission of dCTP (Figure 3B). The other mixes 2, 6 and 12 contained the nucleotide combinations required to achieve extension by 2, 6 and 12 nt, respectively (Figure 3B).

The primer extension assay was performed using Deep Vent_R[®] (exo⁻) polymerase. This enzyme belongs to the B-type polymerase family and has been shown to possess a tolerance for nucleotide analogues carrying exogenous tags at position 5 of pyrimidines (2). The reaction products were analyzed via gel electrophoresis to examine whether the experimental length of the extended primer matched the expected length (Figure 3C). Separate solutions of template strand of 36 nt length (Figure 3C, lane 1) and primer with 24 nt length (Figure 3C, lane 2) migrated at different heights. Upon polymerization with dNTP mix F containing a full set of natural nucleotides, the lower band disappeared (Figure 3C, lane 3) as the primer was successfully extended to a 36 nt DNA strand that co-migrated with the long template. dTTP, the natural analogue of dUTP***-3**, was specifically incorporated, because omitting the natural nucleotide completely blocked the extension from the 24 nt primer (Figure 3C, dNTP mix 0, lane 4). Polymerization in the presence of nucleotide mixes 1, 2, 6 and 12 containing dUTP***-3** resulted in bands with the expected extensions by 1, 2, 6 and 12 nt, respectively (Figure 3C, lanes 5 to 8). The difference in the migration heights was big for most of the DNA extension products with the exception for the step from 1 to 2 nt (Figure 3C, lanes 5 and 6).

Additional support for the successful enzymatic processing was obtained using peptide-tagged deoxyuridine derivative **4** (Figure 4A) which has a much bulkier substituent than nucleotide **3**. The extension reactions with **4** were carried out using the step-wise polymerization assay as described earlier. Similarly, the DNA extension products were analyzed by gel electrophoresis (Figure 4B). Polymerization in the presence of deoxyuridine derivative dUTP***-4** caused a primer band which was strongly up-shifted (Figure 4B, lane 6) compared to the non-extended primer (Figure 4B, lane 5). The large up-shift is in line with the presence of a bulky peptide tag. The incorporation of the second nucleotide dUTP***-4** also went also to completion as judged from the disappearance of the mono-tagged DNA band and the appearance of an up-shifted band of the double-tagged strand (Figure 4B, lane 8) which migrated on top of the template band. The incorporation of the second modified

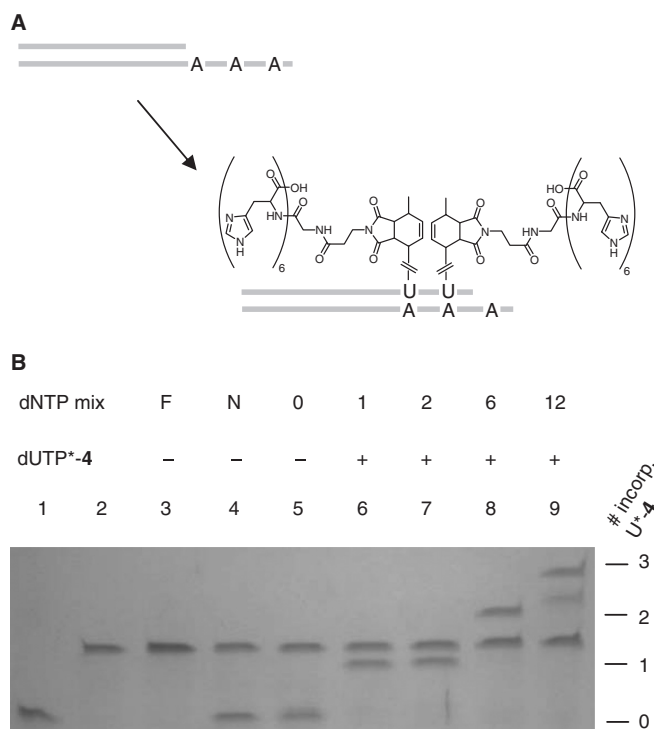


Figure 4. Hexahistidine-tagged nucleotide dUTP***-4** is specifically incorporated into DNA strands. (A) Schematic drawing of the primer extension. Only two of the three achieved incorporations are shown. (B) PAGE analysis of extension reactions. dNTP mixes of varying composition were used to control the step-wise extension. dNTP mixes F, 0, 1, 2, 6 and 12 are defined as in Figure 3B except that dUTP***-4** was used instead of dUTP***-3**. Polymerase mix N does not contain any nucleotides. Lanes 1 and 2 are primer and template, respectively.

nucleotide is noteworthy given that the two 2.4-nm long peptide tags are separated by only six bases corresponding to a distance of 1.5 nm. Steric clash may, however, be mitigated as the tags are likely positioned at opposite sides of the helical duplex. In support of this interpretation, incorporation of a third His₆-tagged nucleotide **4** did not go to completion (Figure 4B, lane 9). Complete incorporation was most likely hindered because the third and first tagged base were separated by an almost complete helical turn (10 bases) potentially leading to steric crowding between the two peptides positioned at the same side of the duplex.

After confirming that **3** can form Diels–Alder product **4**, we subjected diene-tagged DNA to Diels–Alder coupling using maleimide dienophiles. Diene-tagged DNA was first reacted with commercially available polymeric reagent PEG–Mal with a molecular weight of 5 kDa (Figure 5A). Due to its large hydrodynamic diameter of 4.2 nm (24) and the reduced accessibility of its maleimide group, this reagent is ideal to test whether the Diels–Alder chemistry can overcome hindering steric factors. Duplex DNA strands carrying one or two diene groups were prepared as described earlier, reacted with PEG–Mal at concentrations of 10, 1 and 0.1 mM, and analyzed using gel electrophoresis (Figure 5B). In comparison to the non-reacted DNA carrying one diene (Figure 5B, lane 1), treatment with 10 mM PEG–Mal led to the appearance of a highly

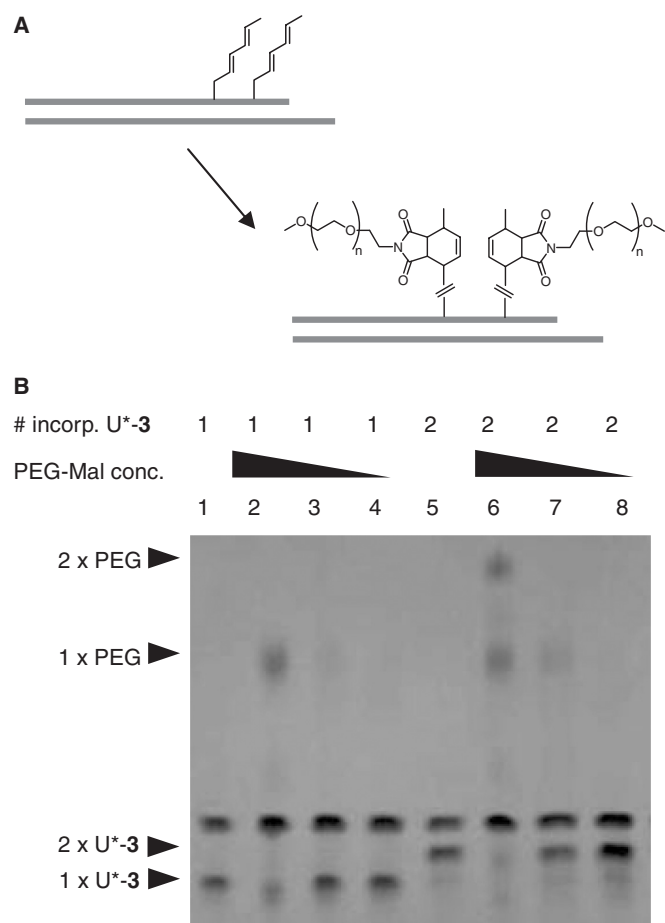


Figure 5. Diels–Alder reaction of single-diene and double-diene modified DNA with PEG–Mal. (A) Scheme of the cycloaddition reaction. (B) Denaturing PAGE analysis of reactions between single-diene (lanes 1–4) and double-diene modified DNA (lanes 5–8) and PEG–Mal solutions of three different concentrations, 10 mM (lanes 2 and 6), 1 mM (lanes 3 and 7) and 0.1 mM (lanes 4 and 8) incubated for 24 h at 22°C.

up-shifted band representing the PEG–DNA conjugate (Figure 5B, lane 2). The generation of the conjugate was accompanied by a corresponding decrease of gel band intensity of the unmodified DNA (Figure 5B, lane 2). Quantification of the intensities of the bands suggests that approximately 50% of DNA reacted with the polymeric reagent. Lower concentrations of PEG–Mal produced much weaker or no up-shifted bands (Figure 5B, lanes 3 and 4). DNA with two dienes (Figure 5B, lane 5) was also reacted with PEG–Mal. At the highest concentration, two up-shifted bands appeared, representing DNA strands with one and two attached PEG chains (Figure 5B, lane 6), while almost no diene-modified DNA band remained (Figure 5B, lane 6). At the two lower PEG–Mal concentrations, one (Figure 5B, lane 7) or no coupling occurred (Figure 5B, lane 8), respectively. The results confirm that Diels–Alder cycloaddition occurred despite the limited steric accessibility of the malimide group in the PEG chain. The coupling of two PEG chains with a hydrodynamic diameter of 4.2 nm is remarkable given that the two dienes in the DNA strands are only 1.5 nm apart.

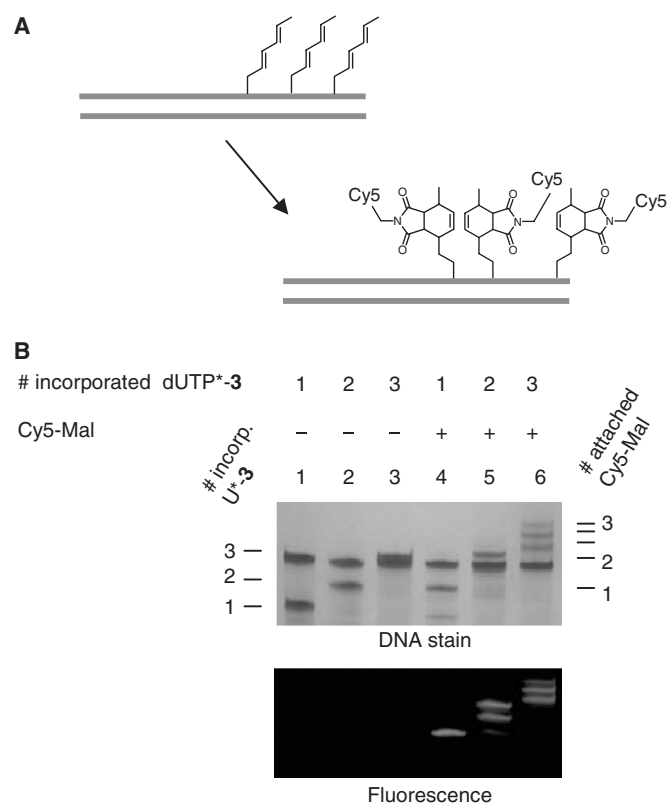


Figure 6. Diels–Alder cycloaddition of diene-modified DNA with Cy5–Mal. (A) Scheme of the cycloaddition reaction. (B) The samples were analyzed using denaturing PAGE, and bands were visualized using excitation at 633 nm and filtering at 675 nm (bottom) or by staining with Stains-All (top). The template strand from the primer extension mixture is present in all samples.

Functional tagging of diene-modified DNA was also conducted with maleimide-linked fluorophores Cy5 and fluorescein. The first dye, cyanine derivative Cy5–Mal, was reacted with DNA strands carrying up to three diene groups (Figure 6A), followed by PAGE analysis using fluorescence detection and DNA staining (Figure 6B). Prior to treatment with Cy5–Mal, DNA strands with one, two and three diene moieties exhibited the expected increasing migration heights as shown in Figure 6B (panel DNA-stain, lanes 1–3), respectively. Reaction with the fluorescent dienophile resulted in up-shifted bands (Figure 6B, lanes 4–6) in line with the formation of Diels–Alder products. For mono-diene DNA (Figure 6B, lane 4), the extent of cycloaddition was greater than 80% as determined from the intensities of the product and non-shifted unreacted DNA bands. A coupling yield of 80% was also obtained in the Diels–Alder-mediated tagging of mono-dienyl DNA with peptide His₆Gly1–Mal (data not shown). The successful cycloaddition with Cy5–Mal was also apparent upon fluorescence inspection. Reaction of diene-DNA with Cy5–Mal yielded a fluorescent band (Figure 6B, panel fluorescence, lane 4) which is not observed for the untreated sample (Figure 6B, panel fluorescence, lane 4). Furthermore, fluorescence analysis of double- and triple-diene DNA strands (Figure 6B, lanes 5 and 6) demonstrated that all dienes’

groups had been tagged with the fluorophore. The extent of coupling was not complete, and this was most likely due to steric hindrance caused by Cy5 molecules already coupled to DNA.

For the coupling of fluorescein derivative Flu-Mal, a diene-modified DNA duplex was generated via PCR. Prior to fluorescence tagging, the hydrolytic stability of the ester linkage in the deoxyuridine analogue (Figure 2A) was assessed by using typical PCR conditions such as high temperature and elevated pH. A probe strand was generated by enzymatically extending a 27-nt long DNA oligonucleotide by a single His₆-tagged nucleotide **4**. The band of the resulting conjugate migrated higher (Figure 7A, lane 2) than the non-modified primer (Figure 7A, lane 1) due to the presence of the large peptide moiety. The His₆-tagged DNA strand was then incubated in a commercial polymerase buffer (pH 8.8 at 25°C) for 20 min at 95°C to emulate the cumulative duration of multiple denaturing steps in a typical PCR amplification. Any ester hydrolysis would be expected to cleave the peptide tag from the DNA strand and result in the down-shift of the DNA band. Gel electrophoretic analysis of the heat-treated sample shows that the majority of DNA migrated in the top position (Figure 7A, lane 3) demonstrating that the ester linkage is stable towards hydrolysis. A barely visible down-shifted band indicates a minor extent of hydrolysis of less than 5% as determined using quantitative analysis of the gel band intensities. The results indicate that the nucleotide derivative is compatible with heat and pH conditions of the standard amplification method.

For the fluorescence-tagging with Flu-Mal, a PCR product carrying diene groups was generated. The PCR amplification was conducted using a complete nucleotide mix supplemented with dUTP***3** at a ratio of 1:3 to dTTP, and for control purposes, a nucleotide mix lacking **3**. As shown by gel electrophoretic analysis, the 500 bp PCR fragment was obtained using the natural and the **3**-supplemented mix (Figure 7B, lanes 1 and 2). When the PCR products were treated with Flu-Mal, diene-modified DNA gave rise to a fluorescent band (Figure 7B, lane 4); DNA lacking **3** did not react with the fluorophore (Figure 7B, lane 3). This strongly suggests that the labeling was due to the specific Diels–Alder coupling between multiple incorporated dienes **3** and dienophile Flu-Mal.

This report describes (i) the synthesis of a diene-modified nucleotide triphosphate and (ii) its successful application for tagging DNA strands via the Diels–Alder cycloaddition. The synthetic route to the diene-modified deoxyuridine derivative was simple and involved two steps. The applicability of the diene-nucleotide and the Diels–Alder-mediated labeling of DNA was demonstrated using the pre- and post-modification approach. In pre-modification, the conversion of **3** to Diels–Alder product **4** went to completion at a minor molar excess of 0.2 equivalents. In the post-modification route, the diene tag did not interfere with the polymerase, and the nucleotide was correctly and efficiently incorporated into DNA strands. As shown by the successful modification with maleimide-terminated fluorophores, the diene groups were accessible within the DNA but steric factors such as blockade by closely positioned tags influenced the

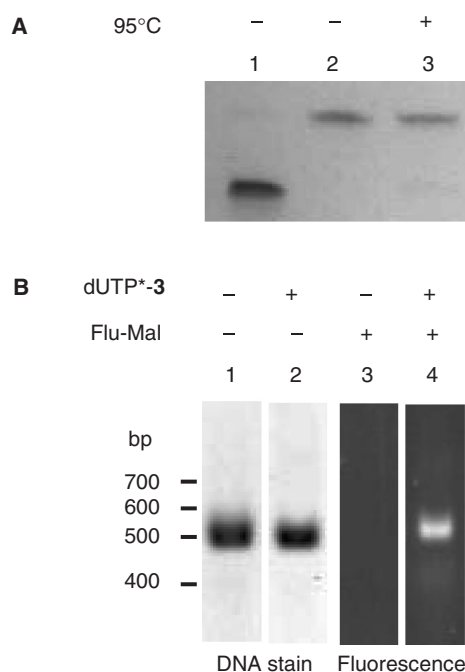


Figure 7. Thermal stability of the ester linkage in **4**, and application of **3** for the fluorescence tagging of diene-modified DNA generated by PCR. (A) The thermal stability of the ester linkage was tested on a 28-nt long ssDNA strand carrying the terminal His₆-tagged nucleotide **4**. The tagged strand was obtained by linear amplification of a 27-nt long primer. Non-extended primer (lane 1), and extended His₆-tagged DNA (lane 2) after incubation (lane 3) in 1X Thermopol buffer (NEB, pH 8.8 at 25°C) for 20 min at 95°C. (B) PCR products were obtained using dNTP mixes containing either dTTP (lanes 1 and 3) or a mix of dTTP and dUTP***3** (lanes 2 and 4), and were incubated with fluorescein derivative Flu-Mal (lanes 3 and 4) and analyzed using agarose gel electrophoresis followed by fluorescence detection (excitation 303 nm) or DNA staining using ethidium bromide.

extent of labeling. Reaction with a large PEG derivative confirmed that the post-modification Diels–Alder coupling can proceed despite sterically demanding conditions. The steric inhibition could be overcome by increasing the length of the linker between pyrimidine base and diene.

In conclusion, the pericyclic coupling chemistry features several advantages such water-solubility, high reaction yields, no detectable side-reactions and no requirement for additional catalysts. Furthermore, dienophile reagents are commercially available including maleimide derivatives of fluorophores or affinity tags which are usually sold for sulfhydryl-specific coupling schemes. The direct availability of maleimide reagents, along with the missing requirement for additional catalysts compares favorably to other water-soluble-specific coupling methodologies such as Click chemistry (5). For example, the versatile and powerful 1,3-dipolar cycloaddition uses azide or alkyne-derivatives which are not widely sold for dyes or affinity tags. In addition, the Click reaction requires catalysts such as Cu salts which can lose activity if not re-activated (7). Due to the favorable features of commercial compatibility and missing necessity for catalysts, diene-tagged nucleotides and the Diels–Alder reaction are expected to find wider use in the field of DNA bioconjugate chemistry with potential applications in

biosensing (17), *in situ* labeling, the generation of peptide-oligonucleotide conjugates, the immobilization of DNA strands on solid supports, and biochemical cross-linking studies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

ACKNOWLEDGEMENTS

We thank A.B. Tabor for use of a peptide synthesizer, and Mikiembo Kukwikila for acquiring and analyzing the ¹³C-NMR spectrum for compound 2.

FUNDING

UCL Chemistry, UCL Business PLC and the EPSRC (EP/D030005). Funding for open access charge: EPSRC.

Conflict of interest statement. None declared.

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