Modified Nucleobases

International Edition: DOI: 10.1002/anie.201508893
German Edition: DOI: 10.1002/ange.201508893

Fluorogenic Labeling of 5-Formylpyrimidine Nucleotides in DNA and RNA

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Abstract: *5-Formylcytosine (5fC) and 5-formyluracil (5fU) are natural nucleobase modifications that are generated by oxidative modification of 5-methylcytosine and thymine (or 5-methyluracil). Herein, we describe chemoselective labeling of 5-formylpyrimidine nucleotides in DNA and RNA by fluorogenic aldol-type condensation reactions with 2,3,3-trimethylindole derivatives. Mild and specific reaction conditions were developed for 5fU and 5fC to produce hemicyanine-like chromophores with distinct photophysical properties. Residue-specific detection was established by fluorescence readout as well as primer-extension assays. The reactions were optimized on DNA oligonucleotides and were equally suitable for the modification of 5fU- and 5fC-modified RNA. This direct labeling approach of 5-formylpyrimidines is expected to help in elucidating the occurrence, enzymatic transformations, and functional roles of these epigenetic/epitranscriptomic nucleobase modifications in DNA and RNA.*

Nucleobase modifications in DNA and RNA are associated with fundamental biological processes and span a diverse range of chemical functionalities.[1] Modification-specific chemical reactions are required to elucidate the occurrence and roles of naturally modified nucleotides.[2] Formyl modifications at the 5-position of pyrimidine nucleobases have recently become the focus of much interest in the field of nucleic acid chemical biology. In DNA, 5-formylcytosine (5fC) is an epigenetic mark that is produced by successive oxidation of 5-methylcytosine (5mC) by TET-family dioxygenases.[3] 5fC is hypothesized to be an intermediate in active demethylation of 5mC, as well as to possess regulatory roles in cellular development.[4] In RNA, 5fC is a posttranscriptional modification in mitochondrial tRNAs that is known to modulate codon–anticodon interactions, but the exact biosynthetic pathway of 5fC installation in RNA remains to be elucidated.[5] In addition to oxidative modification of 5mC, the methyl group of thymine is also susceptible to oxidation, although less prominently by the action of enzymes, which have been shown to produce 5-hydroxymethyluracil (5hmU).[6] Instead, reactive oxygen species (ROS) are known to yield mutagenic 5-formyluracil (5fU) in DNA.[7] While 5-methyluracil (5mU) is a known modification of tRNA and rRNA, the oxidized products 5hmU and 5fU have not yet been described as natural modifications in RNA. However, based on recent reports on the enzymatic oxidation of 5mC in RNA to 5hmC,[8] it can be expected that 5hmU and 5fU in RNA are waiting to be discovered. Synthetic 5fU has been used in several nucleic acid studies associated with oxidative damage,[9] viral defense,[10] and affinity labeling.[11]

Aldehyde groups have been the abiding choice for refashioning nucleic acids with spectroscopic labels or affinity probes.[12] Amine, hydrazide, and aminoxyl derivatives have been used to attach fluorescent probes[13] or small molecule tags such as biotin to 5fC-modified DNA,[3a,4b,14] and most recently also to 5fU-modified DNA,[15] for detection, enrichment and sequencing. Formation of benzimidazole, benzoxazole, or benzothiazol heterocycles upon reaction of 5fU with 1,2-phenylendiamine or *o*-amino(thio)phenol derivatives generates fluorescent or spin-labeled nucleosides.[16] The synthesis of a benzothiazol chromophore was possible on DNA, but similar reactions were unsuccessful with 5fC.[16a] Alternative typical organic reactions of aldehydes should therefore be considered for labeling these biologically important nucleoside modifications.[17]

In this work, we explored an aldol-type condensation of 5fU and 5fC in synthetic DNA and RNA oligonucleotides with 2,3,3-trimethylindole derivatives to generate hemicyanine (Hcy)-like chromophores (Figure 1). Based on the inherently distinct electronic properties of cytosine and uracil nucleobases, we proposed to create two fluorescent nucleotides with different photophysical characteristics, with potential for the residue-specific detection of 5-formylpyrimidines in DNA and RNA. In addition, new environmentally sensitive fluorescent nucleotides may be generated that could serve as probes for nucleic acid folding and ligand or protein binding.

The feasibility of the proposed labeling reaction was established by exploring the condensation of 5-formyl-2′-deoxyuridine with the sulfonylindole **1a**, which yielded the new hemicyanine nucleoside Hcy1dU (Figure 2 and Figure S1 in the Supporting Information). Absorbance and emission properties of Hcy1dU were investigated in aqueous buffered solutions and both showed increasing intensity with increasing pH (Figure 2b). Fluorescence emission maxima in methanol and DMSO were slightly blue-shifted but of comparable intensity to those measured in buffered aqueous solutions,

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201508893.

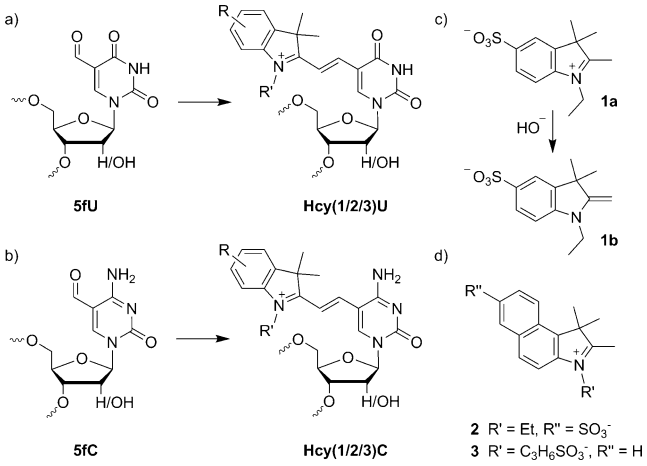

Figure 1. a) 5-formyluridine (5fU) and b) 5-formylcytidine (5fC) (oligo)-nucleotides and their conversion into hemicyanine(Hcy)-modified oligonucleotides using c) *N*-ethyl-2,3,3-trimethylindoleninium 5-sulfonate (**1**) or d) benzo(e)indoleninium reagents **2** and **3**.

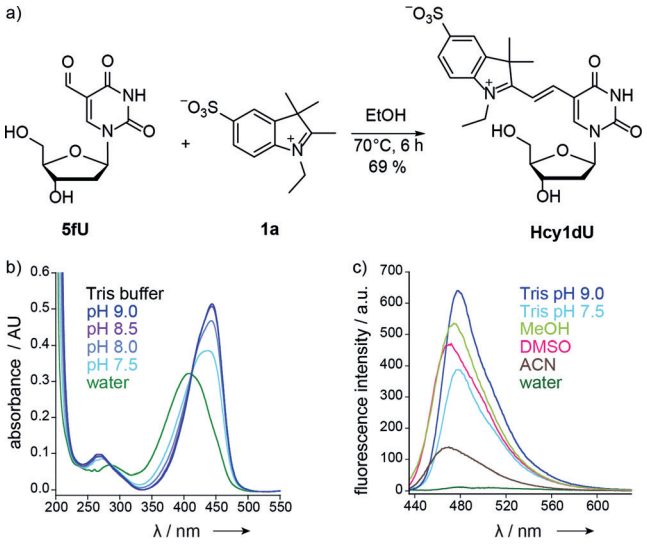

Figure 2. a) Synthesis of the Hcy1dU nucleoside. b) UV/Vis absorbance spectra of 10 μM Hcy1dU in aqueous solutions. c) Fluorescence emission spectra of Hcy1dU in various solvents (0.25 μM, excitation at $\lambda = 418$ nm).

while the emission intensity was considerably reduced in acetonitrile and in desalted aqueous solution (Figure 2c).

To label the 5fU modification in DNA, analogous condensation reactions with **1a** were examined under various conditions with 5fU-containing DNA oligonucleotides D1–D5, which were prepared by solid-phase synthesis[18] (Table 1 and Figure S2). Analyses of labeling reactions by RP-HPLC (monitored at $\lambda = 260$ and 445 nm) and ESI-MS revealed efficient formation of the Hcy1U-labled DNA, showing approximately 80% conversion after incubation at 45°C for 6 h at pH 6.0 (Figure 3a). Analysis of the labeling reaction by anion exchange HPLC under denaturing conditions (pH 8, 6 M urea, 80°C) showed only approximately 40% conversion to new products, of which only a tiny fraction was detectable

Table 1: 5fU- and 5fC-modified oligonucleotides.

Name	5′-sequence-3′[a]
D1	CTCTTGA**G(fU)G**TTATG
D2	CTCTTGA**A(fU)A**TTATG
D3	CTCTTGA**C(fU)C**TTATG
D4	CTCTTGA**T(fU)T**TTATG
D5	GACTCA**A(fU)A**GCCGTG
D6	CTCTTGA**G(fC)G**TTATG
D7	CTCTTGA**A(fC)A**TTATG
D8	CTCTTGA**C(fC)C**TTATG
D9	CTCTTGA**T(fC)T**TTATG
D10	CATA**G(fC)G**CTCAAGAGAAATCTCGATGG
D11	C**G(fC)G**GAGCTCGCTTGTCG
R1	r(GGAAGAG**A(fU)G**GCGACGG)
R2	r(GGAAGAG**A(fC)G**GUGAUGG)

[a] Primer binding site is underlined.

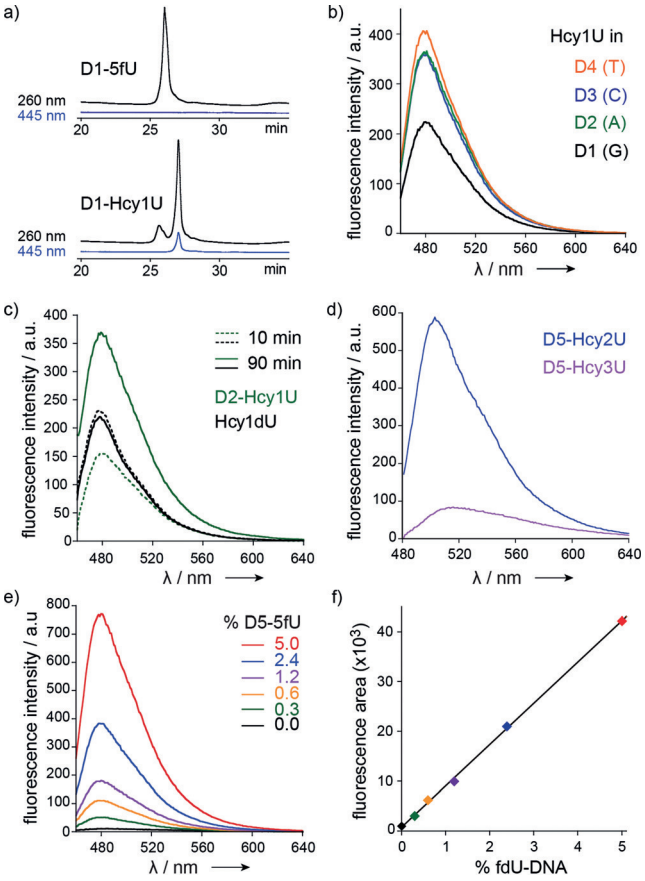

Figure 3. a) RP-HPLC trace at $\lambda = 260$ nm (black) and 445 nm (blue) of 5fU-modified DNA (D1) and D1-Hcy1U generated by reaction with **1a** at pH 6.0, 45°C, 6 h. b–d) Fluorescence emission spectra of HcyU-labeled DNAs D1–D5. $c = 0.25$ μM in 10 mM Tris, pH 9.0, 150 mM NaCl, 25°C, excitation at $\lambda = 445$ nm (Hcy1U) anf 465 nm (Hcy2U and Hcy3U). e) Fluorescence intensity dependence on 5fU content in DNA. f) Analysis of data shown in (e).

at $\lambda = 445$ nm (Figure S3). This result can be explained by reversible addition of urea to the Michael-acceptor moiety of Hcy1U and retro-aldol reaction. The urea addition product was identified by ESI-MS after PAGE purification. Upon

treatment with acetic acid, the desired Hcy1U-labeled DNAs were restored (Figure S4). For preparative labeling reactions, excess reagent **1a** was removed by precipitation, ultrafiltration, or size-exclusion chromatography, and the identity of Hcy1U-labeled DNAs D1–D5 was confirmed by ESI-MS (Table S2).

The fluorescence emission of Hcy1U-labeled DNA oligonucleotides was investigated and compared to that of the Hcy1dU nucleoside described above. The excitation and emission maxima were found to be $\lambda = 445$ nm and 480 nm, respectively. The emission intensity of Hcy1U-labeled DNA was also dependent on the pH of the solution (Figure S5). Almost 4-fold enhancement was observed upon increasing the pH from 8.0 to 9.0. Furthermore, the fluorescence emission intensity was influenced by the sequence context of the labeled nucleotide (Figure 3b). The lowest emission intensity was observed for D1, where Hcy1U is embedded between guanine nucleotides, which are known to partially quench fluorescence emission by photo-induced electron transfer. The highest emission intensity was observed for D4, which contained Hcy1U embedded between thymidine nucleotides. Interestingly, the fluorescence signal reached its maximum intensity only very slowly upon incubation at pH 9, with observed rate constants in the range of 0.05–0.1 min^{-1} (Figure S5). No time-dependent fluorescence increase was observed for the Hcy1dU mononucleoside (Figure 3c) in buffered solution at pH 9. The molecular explanation for the slow fluorescence increase is difficult to define and may involve a combination of factors related to local electrostatic or solvent-mediated effects. Covalent adduct formation with buffer components and degradation of the labeled DNA were ruled out by HPLC and MS analysis after incubation at pH 9 for several hours.

To tune the fluorescence properties of the labeled DNAs, analogous condensation reactions were performed with the extended benzo[*e*]indoleninium reagents **2** and **3**. The extended π-system resulted in the expected red-shift of the emission maximum in both cases (Figure 3d). The sulfonyl group on the aromatic core in Hcy2U lead to significantly higher fluorescence compared to the sulfonate at the N-alkyl substituent in Hcy3U. This result indicates that the substitution pattern substantially alters the brightness of the fluorophore, likely also by influencing stacking interactions with adjacent nucleotides.

Next, we demonstrated that the new fluorogenic reaction can be used to obtain quantitative information on the extent of 5fU present in a DNA sample. Several 5fU nucleotides were incorporated by primer-extension reactions using 5fdUTP. Upon labeling with **1a**, the emission intensity correlated with the number of 5fU modifications present in the DNA (Figure S6). To examine the sensitivity for 5fU detection, we generated a model calibration curve by spiking known amounts (0.3–5%) of 5fU-modified DNA into samples of the unmodified DNA analogue, followed by labeling with **1a**. A linear correlation was obtained between fluorescence intensity and % 5fU-DNA in the mixture (Figure 3e,f). The lowest concentration of labeled DNA measured in this experiment was 25 nM, which corresponds to 0.02% 5fU nucleotides in the DNA sample.

In analogy to the labeling of 5fU-DNA, we examined similar fluorogenic reactions on 5fC-modified oligonucleotides (Table S1 and Figure S2). The optimal labeling conditions developed for 5fU-DNA yielded only approximately 24% conversion of 5fC-DNA (Figure S7). Increasing the pH to 7.5 at 45 °C increased the yield of the new product to 65%. Upon incubation at 70 °C and pH 7.5, a second product was observed. Mass spectrometric analysis revealed that the first product was formed upon aldol addition of **1a**, but the condensation was not completed, that is, water was not yet eliminated. Based on ESI-MS data, the second product contained an additional molecule of **1a**. We hypothesize that a partially conjugated cyanine-like structure is formed on the nucleobase, although the structure of this extensive modification could not yet be unequivocally established. We thus focused on optimizing the reaction conditions to produce the designed Hcy1C-labeled DNA. A comprehensive screen of pH and buffer conditions with ^{32}P-labeled 5fC-DNA suggested that the desired product is preferentially formed at pH 7.5 (Figure S8). To further increase the nucleophilicity of the indole reagent, **1a** was treated with NaOH followed by neutralization with Dowex-H^+ to generate the enamine compound **1b** with an exocyclic double bond (which was confirmed by NMR spectroscopy). Using **1b** at pH 7.5 and 45 °C, the single addition product was obtained in 80% yield. Mass spectrometry of the isolated product revealed the non-dehydrated intermediate, which was further converted into the condensation product. The dehydration was successfully mediated by 3% acetic acid at 45 °C (Figure S9). The desired product was stable and could be purified by denaturing gel electrophoresis to homogeneity (Figure 4).

The optimized reaction conditions (i.e., using **1b** at pH 7.4 and 45 °C, followed by acid treatment) were used for preparative labeling of DNA oligonucleotides D6–D9, which contained 5fC between different adjacent nucleotides. The excitation and emission maxima of Hcy1C-labeled DNAs were significantly blue-shifted ($\lambda = 335$ and 400–415 nm, respectively) compared to the analogous Hcy1U-labeled DNAs, and the emission intensity was reduced. The Hcy1C label showed modest environmental sensitivity in single-stranded and double-helical environments (Figure 4c and Figure S10). In contrast to Hcy1U, no significant dependence of emission properties on pH (between pH 7 and 9) or incubation time was observed. The distinct excitation and emission wavelengths of Hcy1U and Hcy1C allowed discrimination of both modifications present in the same sample (Figure S11).

To further explore the potential of the aldehyde-specific labeling reaction beyond fluorescence readout, we examined the site-specific analysis of 5fC DNA in primer-extension assays. While 5-formyl modifications are usually bypassed by DNA polymerases,[19] the hemicyanine-nucleosides may act as a "roadblock" to abort primer extension and enable detection and quantification of underlying 5fC modifications. While unlabeled 5fC-DNA caused only minor pausing of Klenow DNA polymerase, a strong stop was detected when the labeled Hcy1C-DNA was used as template (Figure 4d and Figures S12, S13). In a proof-of-principle setup, we prepared samples with known 5fC content at a particular target

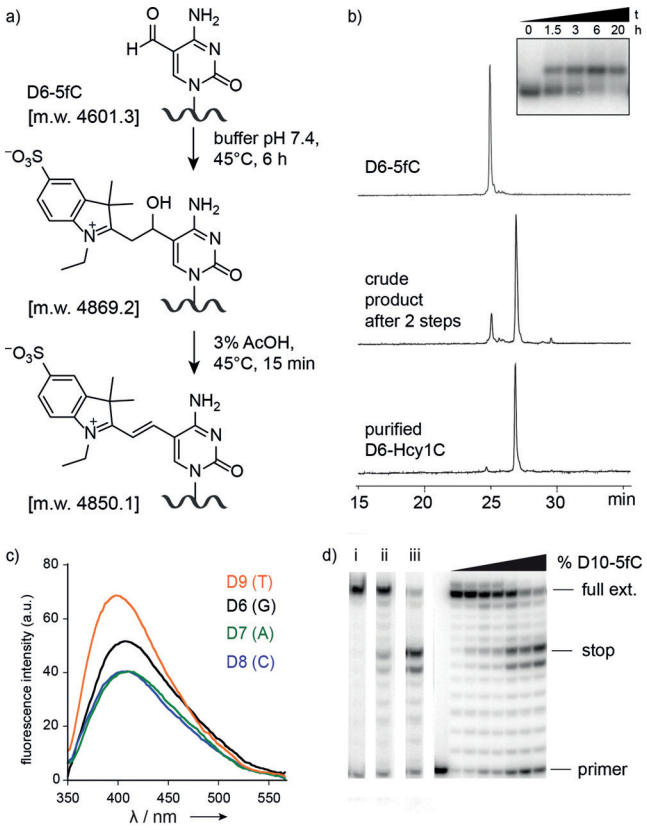

Figure 4. a) Labeling Scheme for 5fC-modified DNA. b) Anion-exchange HPLC traces (monitored at $\lambda = 260$ nm). Inset: PAGE analysis of reaction using 5′-^{32}P-labeled D6. m.w. = molecular weight. c) Fluorescence emission spectra of Hcy1C-labeled DNAs D6–D9; the nucleotides flanking the Hcy1C nucleoside are given in parentheses. 5 μM DNA in K-phosphate buffer, pH 7, excitation at $\lambda = 335$ nm. d) Primer-extension assay with Klenow *exo*- DNA polymerase. Templates: i) unmodified DNA; ii) D10-5fC; iii) D10-Hcy1C. Right-hand gel: increasing 5fC content in the template from 0–100%. Experimental details are given in the Supporting Information.

nucleotide, and correlated the extent of aborted product to the 5fC content (Figure 4d). These results demonstrate that the condensation reaction with indoleninium reagents can be used to examine the status of the 5fC modification at a particular position of the target DNA. This approach could be combined with established oxidative and reductive treatments of DNA to distinguish different oxidation states of 5mC modifications.[4b,14b] Furthermore, the Hcy-labeling reaction has the potential to be explored for genome-wide analysis of 5-formylpyrimidines.

5-Formylpyrimidines also play important roles in RNA biology, but methods for their detection and analysis in RNA lag behind those available for DNA. Herein, we applied the optimized conditions for labeling 5fU and 5fC in DNA to the analogously modified RNA oligonucleotides, which were prepared by in vitro transcription (Figure S2). The labeling reaction conditions were easily transferable and the products were characterized by HPLC and ESI-MS (Figure S14). Fluorescence properties for Hcy1U- and Hcy1C-labeled RNAs were comparable to the corresponding ones in DNA. These findings clearly open the way for the further exploration of 5fC and 5fU modifications in RNA.

In conclusion, we have demonstrated efficient labeling of naturally occurring 5fC and 5fU in nucleic acids by a new fluorogenic aldol-type condensation reaction under mild and biocompatible reaction conditions. The inherent properties of uracil and cytosine nucleobases were exploited for residue-specific labeling and detection by fluorescence readout. Interrogation of 5fC levels at a particular target site was demonstrated by primer-extension stop assays. Besides providing new insight into the distinct reactivity of 5fU and 5fC, the new labeling reaction will find direct applications in the detection and mapping of formyl-modified nucleotides in biological samples. In future studies, the new fluorescent labels may be used to monitor 5fC to 5fU deamination, and could potentially be tuned for metabolic labeling and optical mapping.

Acknowledgements

This work was supported by the Cluster of Excellence and DFG Research Center Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB) and the DFG Priority Program Chemical Biology of Native Nucleic Acid Modifications (SPP1784).

Keywords: DNA · epigenetics · fluorogenic probes · modified nucleobases · RNA

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1912–1916
Angew. Chem. **2016**, *128*, 1946–1950

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Received: September 22, 2015
Published online: December 17, 2015

