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Synthesis and Photophysical Properties of Conjugates of Green Fluorescent Protein (GFP) Chromophore and 2'-Deoxy-Uridine Developed as Labelled Building Blocks for Oligonucleotide Probes

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Abstract: Fluorescent probes with high signal/background ratio are needed for hybridization assays. Hence, the chromophore of green fluorescent protein (GFP), 4-hydroxybenzylidene-imidazolinone (HBI), was targeted as a potential fluorescent intercalator. For producing a building block for fluorescent oligonucleotide probes, 2'-deoxyuridine (dU) was conjugated with HBI via a flexible carbon-spacer. dU^{HBI} conjugates highly fluoresce in glycerol (λ_{em} 460 nm, Φ 0.31), and are 109-fold more emissive than in methanol, implying the potential of dU^{HBI}-labeled oligonucleotides as probes for hybridization assays.

Key words: Intercalator, GFP, 2'-deoxyuridine intercalator conjugates.

1. Introduction

Various fluorescent dyes, which are polyaromatic molecules capable of forming a planar structure, can function as intercalators, namely, fit in between base pairs of double-stranded DNA (dsDNA). Hence, these dyes can serve as an analytical signal to determine hybridization via the tracking of changes in fluorescence. Ethidium bromide, **1**, (Fig. 1) is one of the best known intercalators used for the detection of dsDNA though it has relatively low fluorescence enhancement upon binding and relatively low molar absorptivity [1]. Thiazole orange (TO, **2**) is another common intercalator. It has a non-planar chromophore composed of a benzothiazole derivative and a quinolinium ring, linked via a methine bridge [2]. Thiazole orange is used as an intercalative transduction agent in nucleic acid hybridization assays. In its free form in aqueous solution, it is a non-planar chromophore which has low fluorescence (Φ 2×10^{-4}), and upon binding, it provides dramatically enhanced fluorescence due to interruption of rotation about the methine bridge (Φ 0.1-0.4) [3]. Thus, TO provides ca. 3,000-fold enhancement of fluorescence upon binding dsDNA vs. 20-25-fold for ethidium bromide [4]. The positive charge of both **1** and **2** impacts not only their quantum yield of fluorescence but also increases association with the negatively charged dsDNA [3].

Green fluorescent protein (GFP), **3**, (Fig. 2) is known for its exceptional fluorescence (λ_{em} 504 nm; Φ 0.79; brightness 23,000 $M^{-1}\cdot cm^{-1}$) [5]. The fluorescence of GFP is leveraged as a powerful tool for numerous biochemical and biological applications [6]. 4-Hydroxy-benzylidene imidazolinone (HBI) may be considered as a potential fluorescent intercalator. Synthetic HBI analogue **4** (Fig. 2), in methanol is non-fluorescent, as is denatured GFP [7]. The source of fluorescence stems from planarity of the two parts of the π-conjugated system in HBI—the phenol and imidazolinone groups—interconnected by a methine bridge. Due to the conformational flexibility, the fluorescence of the isolated GFP chromophore in solution

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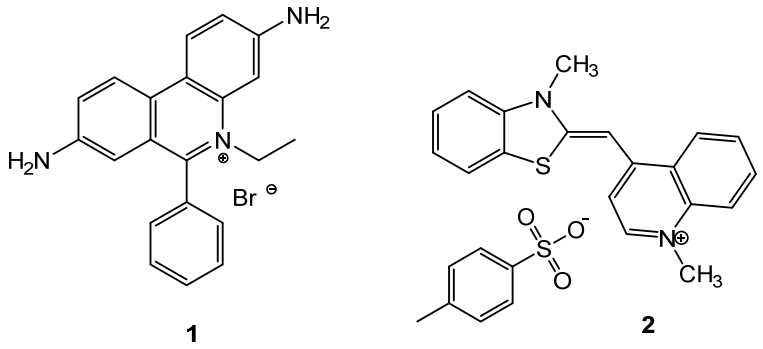


Fig. 1 Common dsDNA intercalators.

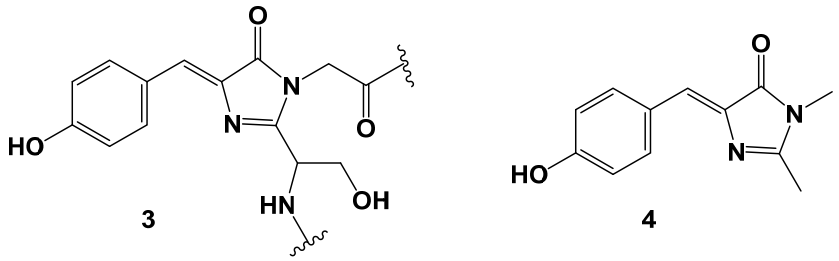


Fig. 2 Structure of the fluorophore of GFP and an analogue of GFP-chromophore.

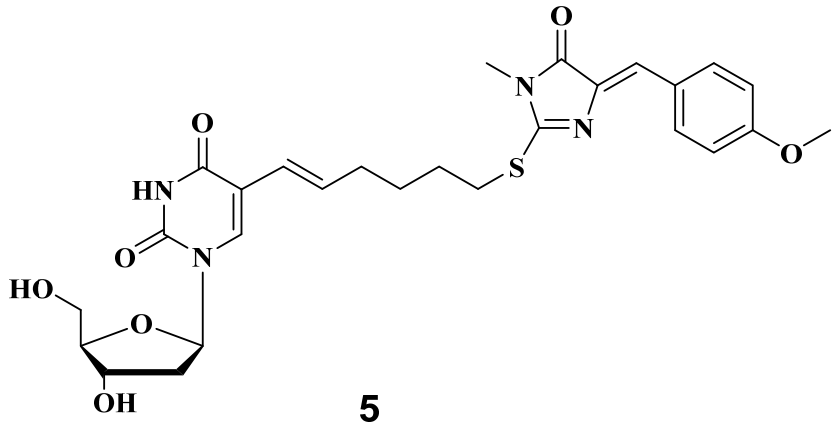


Fig. 3 HBI-modified 2'-deoxy-uridine nucleotide, dU^{HBI}, 3.

is quenched by radiationless internal conversion. An extensive network of interactions within the protein forces a planar structure of the GFP-chromophore, thus imposing conformational fixation and leaving fluorescence as the major pathway available to dissipate the energy of the excited state fluorophore [8].

We have recently shown that incorporation of an HBI-2'-deoxyuridine (dU) conjugate, dU^{HBI}, **5** (Fig. 3), into an oligonucleotide probe targeting HER2 mRNA (a breast cancer marker), allowed the sensitive detection of the target based on an 11-fold enhanced fluorescence of the probe. Fluorescence enhancement was due to hybridization of the ss-2'-OMe-RNA-dU^{HBI} probe with HER2 mRNA in total RNA extract from cancerous cells, and subsequent intercalation of the HBI moiety [9]. The length of the spacer that connects to HBI and 2'-deoxyuridine was not investigated. Hence, here, we prepare two new derivatives, with spacer lengths 4 and 7 atoms in order to evaluate the effect of spacer length on the fluorescent signal.

To address the demand for specific, simple, sensitive,

and cost-effective nucleic acid detection technologies, we targeted here the development of two dU^{HBI} conjugates for producing a building block for fluorescent oligonucleotide probes for the fluorescent detection of mRNA/microRNA targets.

Here, we report on the conjugation of the HBI-chromophore to dU via a flexible 4- and 7-atom carbon spacer to form dU^{HBI} analogues **6** and **7**. Next, we report on the proof of the potential of dU^{HBI} conjugates for hybridization assays.

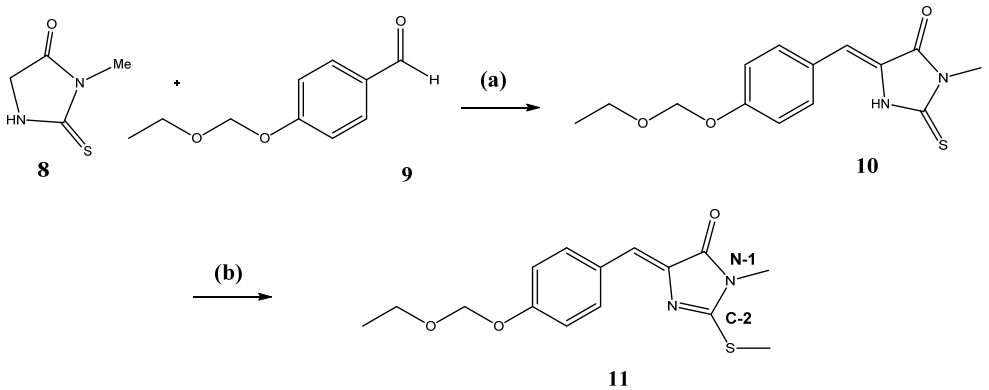
Compounds **6** and **7**, can later be incorporated in oligonucleotides. By covalently connecting the potential fluorescent intercalator moiety via a bound spacer to the nucleoside we eliminate the need for a positively charged fluorophore which will direct the latter in between the bases of the nucleic acid duplex. It is rather the spacer that directs the intercalation of the fluorophore. A high signal/background ratio is expected upon intercalation since the GFP-fluorophore is on one hand prone to efficient internal conversion in an aqueous medium, and on the other hand, highly emits light in rigid environment (e.g. when intercalated).

2. Results and Discussion

To simplify the proof of the above concept, we selected 2'-dU, as the nucleoside for conjugation with HBI-chromophore, via a flexible spacer. Next, we evaluated the photophysical properties of the conjugate in non- and highly-viscous solvents. Specifically, dU was selected due to its relatively easy application to various synthetic reactions; the dU nucleobase requires no protective groups and has a reactive C5 position suitable for chemical elaboration. Furthermore, natural H-bonding base-pairing and standard solution conformation of dU are not disturbed due to C5-substituents [10].

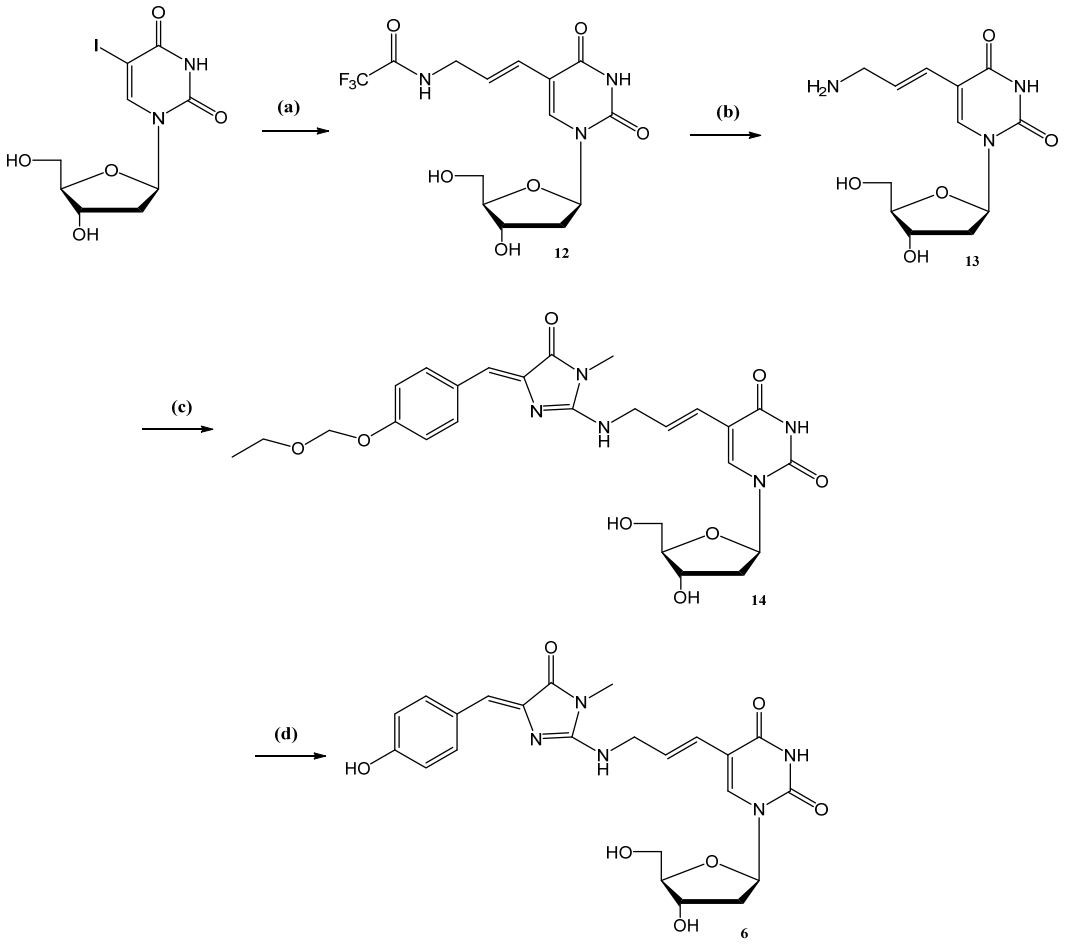
Specifically, the scaffold of the (5-(4-hydroxybenzylidene)-3-methyl-2-thioxoimidazolidin-4-one) chromophore, **11**, was synthesized in two steps starting from 3-methyl-2-thioxoimidazolidin-4-one, **8** [11]. The latter was treated with ethyl methyl ether-protected 4-hydroxybenzaldehyde (EOM), **9** [12] to give **10** by aldol condensation (82% yield) [13], followed by methylation with methyl iodide to afford the scaffold of the HBI chromophore **11** in 93% yield (Scheme 1) [14].

HBI analogue, **11**, was coupled to dU via a spacer of 4 atoms (Scheme 2). Specifically, 5-iodo-2'-deoxyuridine was treated with N-allyltrifluoroacetamide under Heck reaction conditions in 0.1 M NaOAc (pH 5.2) to give **12** in 72% yield [15]. Next, the trifluoroacetamide protecting group in **12**, was removed by hydrolysis in ammonium hydroxide to give **13** in a quantitative yield [15]. 5-(3″-Aminoallyl)-2'-deoxyuridine, **13**, was coupled with **11** in ethanol at 70 °C for 6 days to give **14** in 76% yield [16]. Finally, the phenol EOM-protecting group was removed with trifluoroacetic acid to give **6** in a quantitative yield. HBI-analogue **11** was coupled to dU also via a spacer of 7 atoms (Scheme 2).



8 **9** **10** **11**

Scheme 1 Reaction conditions: (a) piperidine, ethanol, 82%; (b) methyl iodide, acetonitrile, 7 h, 93%.



Scheme 2 Reaction conditions: (a) N-allyltrifluoroacetamide, NaOAc buffer (0.1 M, pH = 5.2), $Na_2[PdCl_4]$, DMF, 72% yield; (b) 28% ammonium hydroxide, 100%; (c) 11, ethanol, 6 days, 76% yield; (d) TFA, DCM, rt, 100%.

Specifically, we coupled a 6-atom spacer at the dU C5-position by Suzuki reaction of 5-iodo-2'-deoxyuridine with (E)-6-chloro-1-hexenylboronic acid pinacol ester to get **15** at 83% yield [17], followed by S_N2 reaction with sodium azide to obtain **16**. The latter, without purification, was reacted with triphenylphosphine to give **17** at 79% yield [18]. Compound **17** was treated with HBI analogue, **11**, in ethanol at 70 °C for 5 days to get **16** at 82% yield. Finally, the phenol EOM-protecting group was removed with trifluoroacetic acid to give **7** in a quantitative yield.

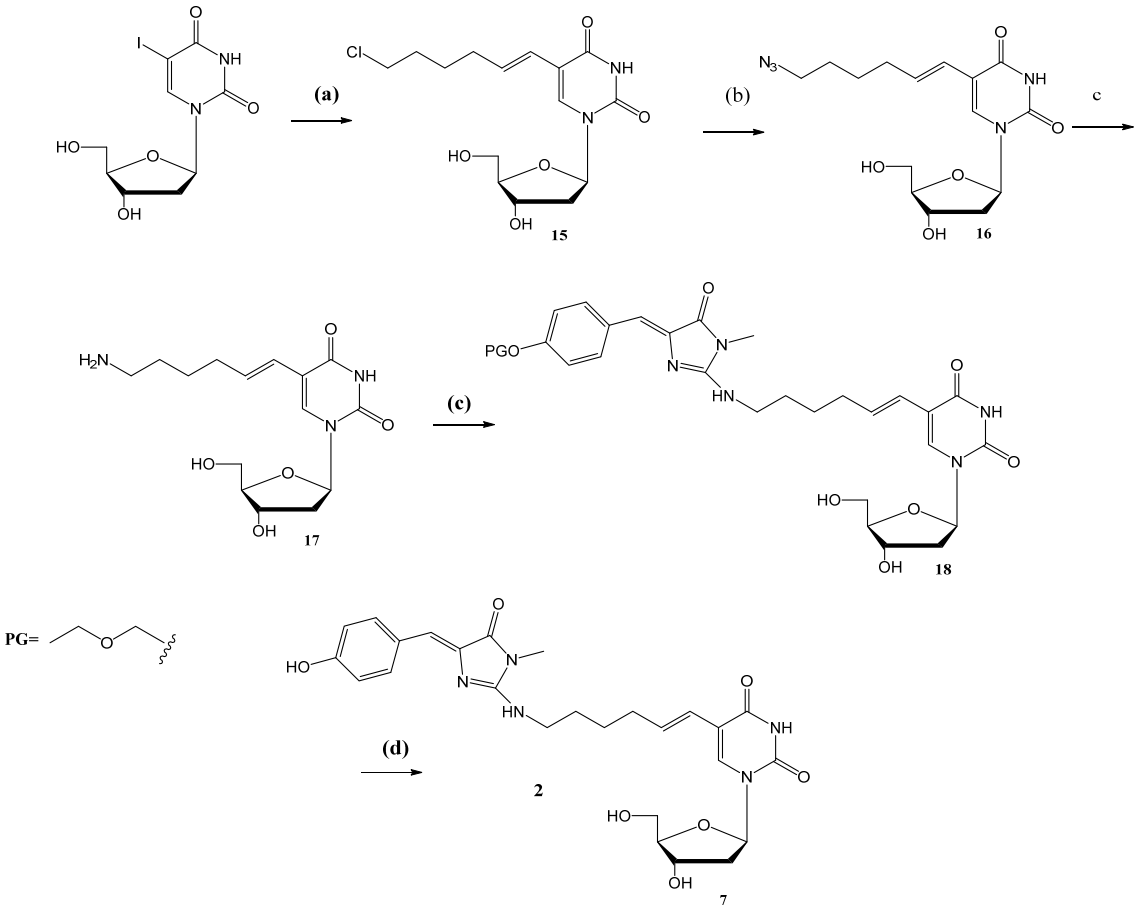
The photophysical properties of compounds **6** and **7** were evaluated in various solvents at a range of viscosities which was reported in details in previous study [9]. UV spectra of compounds **6** and **7** in all solvents exhibited λ_{abs} 386 nm and ϵ 20,600 $M^{-1}\cdot cm^{-1}$. Ultraviolet–visible (UV-vis) spectra of compounds **6** and **7** were also measured at different pH values: 1.4, 7.5, and 12. The maximum absorbance wavelength was pH-dependent, λ_{abs} 369 nm, pH 1.4 (positively charged HBI-moiety); 386 nm, pH 7.5; and 416 nm, pH 12 (negatively charged HBI-moiety).

Next, we measured the quantum yield of nucleosides **6** and **7** in various solvents and at a range of glycerol: methanol ratios, representing a range of viscosities (from 0.54 to 934 cp). The fluorescence of compounds **6** and **7** is viscosity-dependent and increases in viscous solvents; for example, compounds **6** and **7** barely fluoresce in methanol (Φ 2.84 × 10^{-3}); however, in glycerol, which is ca. 3,000 times more viscous than methanol, **6** and **7** were 109-fold more emissive (quantum yield 0.31, at 460 nm). Notably, in 1,3-butanediol and in glycerol: methanol (7:3) mixture,

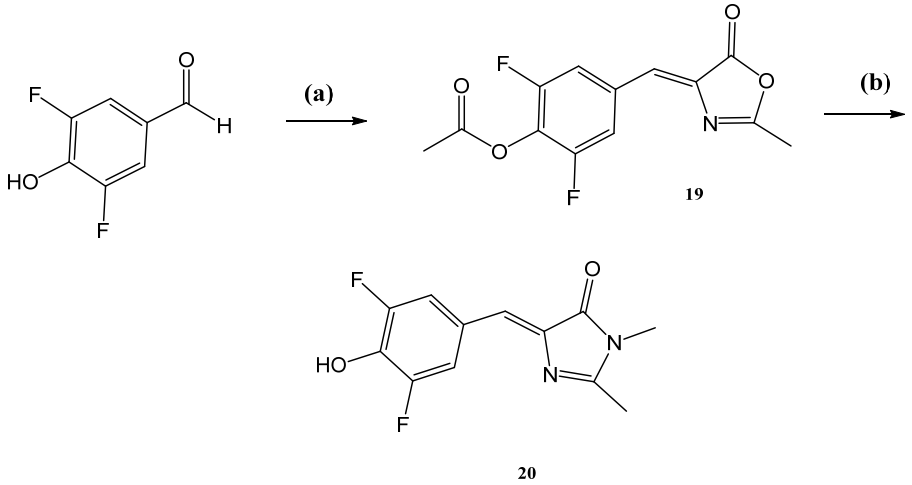
which shares the same viscosity, Φ of **6** and **7** is 0.019, and 0.013, respectively, thus implying that viscosity is a major parameter affecting the emission of HBI analogues. This finding supports our hypothesis that rigid medium limits the free-rotation around the methine-bridge of the HBI analog, thus resulting in a fluorescent signal, and hence, high fluorescence is expected upon intercalation of the HBI-moiety.

As mentioned above, the maximum absorbance wavelength of **6** and **7** was pH-dependent and red-shifted from 369 nm at pH 1.4, to 416 nm at pH 12, indicating the effect of the delocalization of the phenolate anion over the HBI chromophore. The 1,000-fold higher acidity of 2,6-difluoro-phenol than that of phenol (pK_a values of phenol and 2,6-difluoro-phenol are 9.99 and 6.88, respectively) [19], implied on a means for improving the photophysical properties of analogues **6** and **7**. Therefore, we synthesized 3,5-difluoro-4-hydroxy-benzylidene-imidazolinone (DFHBI), **20** (Scheme 3) [20]. DFHBI was obtained in two steps starting with 3,5-difluoro-4-hydroxy-benzaldehyde treated with N-acetylglycine and sodium acetate in acetic anhydride, to give **19**, followed by treatment with methylamine solution and K_2CO_3 in ethanol to give **20** in 73% yield.

The photophysical properties of **20** were evaluated in several solvents at a range of viscosities. UV spectra of **20** in these solvents exhibited λ_{abs} 418 nm and ϵ 25,700 $M^{-1}\cdot cm^{-1}$, showing 32 nm red-shift of λ_{abs}, and 5,100 $M^{-1}\cdot cm^{-1}$ increase of the molar extinction coefficient as compared to the parent compound, HBI [21]. UV-vis absorbance of **20** was also measured at different pH values: 1.4, 7.5, and 12. The maximum



Scheme 3 Reaction conditions: (a) (E)-6-chloro-1-hexenylboronic acid pinacol ester, Na_2CO_3 (3 eq), TPPTS (0.25 eq), $Pd(OAC)_2$ (0.05 eq), CH_3CN:H_2O 80 °C, 83%; (b) NaN_3, $AcNMe_2$; (c) PPh_3, Et_2O, 0 °C, 79%; (d) 9, ethanol, 5 days, 82%; (e) TFA, DCM, rt, 100%.



Scheme 4 Reaction conditions: (a) N-acetylglycine, NaOAc, acetic anhydride, 7.5 h, 100 °C , 69% yield; (b) 2 eq. MeI, EtOH, 21 h, reflux, 73% yield.

absorbance wavelength was pH-dependent, λ_{abs} 395 nm, pH 1.4 (positively charged HBI-moiety); λ_{abs} 420 nm, pH 7.5; and λ_{abs} 470 nm, pH 12 (negatively charged HBI-moiety). Compound **20** in glycerol emitted at 485 nm, and was 135-fold more emissive (Φ 0.42) than in methanol.

These results support our notion that compounds **6** and **7** can be incorporated in an oligonucleotide and used for hybridization assays. Furthermore, the importance of the phenolate anion for obtaining a red shifted spectrum was observed at basic pH (12) where a red shift about 47 nm was observed vs. the signal at neutral pH (7.4). Indeed, when we substituted the HBI-phenol ring with electron withdrawing groups (F atoms) that stabilize the phenolate, longer absorption and emission wavelengths were measured.

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In conclusion, a route for the synthesis of novel dU^{HBI} conjugates (**6** and **7**) has been established through conjugating a 4- and 7-carbon-atoms boronic ester bearing a terminal amine to C5-position of 5-I-2'-deoxyuridine by Heck and Suzuki coupling reactions. Next, this terminal primary amine was used for S_N2 reaction for coupling with the HBI chromophore, **11**, at its C2-position by displacing MeSH. These dU^{HBI} conjugates upon introduction into the rigid environment of glycerol, vs. methanol, displayed dramatically enhanced quantum yield. Upon stabilization of the anion of HBI by two F atoms, as in model compound **20**, emission in glycerol is red-shifted (485 nm), and the quantum yield is further increased (Φ 0.42 in glycerol vs. 0.3 for **6**/**7**). Hence, we predict that analogues **6**/**7**, and in particular **20**, can be applied for hybridization assays which require large signal/background ratios. This proof of concept forms the basis for the future novel use of GFP-chromophore as an intercalator, thus potentially producing a universal platform for detection of genetic material. The application of dU^{HBI} conjugates as labelled building blocks of oligonucleotide probes for detection of mRNA and microRNA will be published in due-course.

3. Experimental

3.1 General

Reagents and solvents were purchased from

commercial sources and were used without further purification. All moisture sensitive reactions were carried out in flame-dried reaction flasks with rubber septa, and the reagents were introduced with a syringe. All reactants in moisture sensitive reactions were dried overnight in a vacuum oven. Progress of reactions was monitored by TLC (Thin-layer chromatography) on precoated Merck silica gel plates (60F-254). Visualization was accomplished by UV light. Medium pressure chromatography was carried out using automated flash purification system (Biotage SP1 separation system, Uppsala, Sweden). Compounds were characterized by nuclear magnetic resonance using Bruker, DPX-300 and DMX-600 spectrometers. ^{1}H NMR spectra were measured at 300, 400 and 600 MHz. Compounds were analyzed under electron spray ionization (ESI) conditions on a Q-TOF micro-instrument (Waters, UK). Modified oligonucleotides were synthesized by standard automated solid-phase method on an AKTA OligoPilot (GE healthcare), an ABI DNA/RNA synthesizer (Forster City, USA). MALDI-TOF mass spectra of oligonucleotides were measured with mass spectrometer in a negative ion mode with THAP matrix. Absorption spectra were measured on a UV-2401PC UV-VIS recording spectrophotometer (Shimadzu, Kyoto, Japan). Emission spectra were measured using Cary Eclipse Fluorescence Spectrophotometer. Absorption and fluorescence spectra were recorded in all the solvents.

*3.2 Fluorescence Measurements of **6, 7** and **20***

Samples were measured in a 10 mm quartz cell, with 710 V sensitivity and a 5 nm slit. Samples of 10 μM were measured in methanol, and were excited at λ = 380 nm.

3.3Quantum Yield Measurements

The quantum yield of **6**, **7**, and **20** was calculated from the observed absorbance and the integration of the fluorescence emission band. The fluorescence quantum yields of **6**, **7**, and **20** were determined relative to the quantum yield of quinine sulfate (0.58) in 0.1 M H_2SO_4 according to Eq. (1).

$$\phi_F = \phi_E I/I_R \times OD_R/OD \times \eta^2/\eta_R \qquad (1)$$

3.4 Synthesis

3.4.1 (Z)-5-(4-(Ethoxymethoxy)benzylidene)-3-methyl-2-thioxoimidazolidin-4-one (**10**)

A solution of 4-methylethyletherbenzaldehyde **9** (416 mg, 2.49 mmol, 1 eq), 3-methyl-2-thioxoimidazolidin-4-one **8**, (250 mg, 2.98 mmol, 1.2 eq), and piperidine (327 mg, 0.24 mmol, 0.1 eq) in ethanol (10 mL) was stirred under reflux for 24 h. After the reaction was completed, the mixture was cooled to ambient temperature and then poured into water and extracted with methylene chloride (50 mL). The resulting organic phase was washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude material was then purified by silica gel chromatography using Hexane: EtOAc (7:3) to give **10** in 72% yield (572 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (d, *J* = 8 Hz, 2H), 6.89 (d, *J* = 7 Hz, 2H), 6.62 (s, 1H), 6.02 (s, 2H), 3.59 (q, *J* = 4 Hz, 2H), 3.52 (s, 3H), 1.23 (t, *J* = 4 Hz, 3H) ppm. HRMS calcd for $C_{14}H_{16}N_2O_3S$ *m/z* 292.0769, found 292.0771.

3.4.2 (Z)-5-(4-(ethoxymethoxy)benzylidene)3-methyl-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one **(11)**

Methyl iodide (233.2 mg, 1.36 mmol) and (Z)-5-(4-(ethoxymethoxy)benzylidene)-3-methyl-2-thioxoimidazolidin-4-one **10** (400 mg, 1.36 mmol), were added to a mixture of $CHCl_3$ and CH_3CN (1:1), and solid potassium carbonate (378 mg, 1.36 mmol). The reaction mixture was stirred for 17 h at room temperature under argon atmosphere. The crude material was then purified by silica gel chromatography and eluted with hexane: EtOAc (6:4) to give **11** in 93% yield (383 mg). ^{1}H NMR ($CDCl_3$): δ 7.83 (s, H), 7.62 (d, *J* = 8 Hz, 2H), 6.88 (d, *J* = 7 Hz, 2H), 6.02 (s, 2H), 3.59 (q, *J* = 4 Hz, 2H), 3.52 (s, 3H), 2.57 (s, 3H), 1.23 (t, *J* = 3 Hz, 3H) ppm. HRMS calcd

for $C_{15}H_{18}N_2O_3S$ *m/z* 306.6933, found 306.7252.

3.4.3 5-(3″-Aminoallyl-*p*-methyl-ethyl-hydroxybenzylideneimidazolinone)-2'-deoxyuridine (**14**)

A solution of **11** (0.3 mmol) and **13** (6 mmol) in ethanol (3 mL) was heated at 100 ºC in a sealed tube for 6 days. The reaction mixture was cooled down to room temperature and concentrated under reduced pressure. The crude was then purified by silica gel column chromatography to obtain **14** in 76% yield. ^{1}H NMR ($CDCl_3$): δ 8.25 (s, 1H, H-6), 7.83 (s, H), 7.62 (d, *J* = 8 Hz, 2H), 6.88 (d, *J* = 7 Hz, 2H), 6.2 (d, *J* = 15.3 Hz, 1H), 6.02 (s, 2H), 5.99 (d, *J* = 15.3 Hz, *J* = 6.8 Hz, 1H), 5.85 (t, *J* = 7.7 Hz, 1H, H-1'), 4.37–4.40 (m, 1H, H-3'), 3.96–3.94 (m, 1H, H-4'), 3.59 (q, *J* = 4 Hz, 2H), 3.84 (d, *J* = 6.85, 2H), 3.52 (s, 3H), 2.29 (ddd, *J* = 13 Hz, *J* = 5 Hz, *J* = 2 Hz, 1H, H-5'), 1.99 (ddd, *J* = 13.2 Hz, *J* = 7 Hz, *J* = 5 Hz, 1H, H-5'), 1.94 (m, 2H, H2', H2"), 1.23 (t, *J* = 4Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 171, 162, 163, 158, 152, 150, 137, 132, 131, 130, 124, 122, 111, 107, 105, 94, 92, 90, 70, 63, 61, 41, 40, 35, 15 ppm. HRMS calcd for $C_{26}H_{31}N_5O_8$*m/z* 541.2173, found 542.2162.

3.4.4 5-(3″-Aminoallyl-p-hydroxybenzylidene imidazolinone)-2′-deoxyuridine (**6**)

A mixture of 5-(3″-aminoallyl-*p*-methyl-ethyl-hydroxybenzylideneimidazolinone)-2′-deoxyuridine, **14**, (120 mg, 0.22 mmol) and TFA (60 mg, 0.37 mmol), was dissolved in dry DCM at 0 °C, was stirred for 24 h at room temperature under argon atmosphere. The crude material was then purified by column chromatography using DCM: MeOH (8:2) as the eluent to give **6** in 100% yield (107 mg). ^{1}H NMR ($CDCl_3$): δ 8.25 (s, 1H, H-6), 7.83 (s, H), 7.62 (d, *J* = 8 Hz, 2H), 6.88 (d, *J* = 7 Hz, 2H), 6.2 (d, *J* = 15.3 Hz, 1H), 5.99 (d, *J* = 15.3 Hz, *J* = 6.8 Hz, 1H), 5.85 (t, *J* = 7.7 Hz, 1H, H-1′), 4.37-4.40 (m, 1H, H-3′), 3.96-3.94 (m, 1H, H-4′), 3.84 (d, *J* = 6.85, 2H), 3.52 (s, 3H), 2.29 (ddd, *J* = 13 Hz, *J* = 5 Hz, *J* = 2 Hz, 1H, H-5′), 1.99 (ddd, *J* = 13.2 Hz, *J* = 7 Hz, *J* = 5 Hz, 1H, H-5′), 1.94 (m, 2H, H2', H2") ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 171, 162, 163, 158, 152, 150, 137, 132, 131, 130, 124, 122, 111, 107, 105, 92, 90, 70, 63, 61, 41, 40 ppm. HRMS calcd for $C_{23}H_{25}N_5O_7$*m/z* 483.3863, found 484.6122.

3.4.5 5-(6-Chloro-1-hexene)-2'-deoxyuridine (**15**)

Water-acetonitrile (4:2 mL) was added through a septum to nitrogen-purged round-bottom flask 5-iodo-2'-deoxyuridine (730 mg, 204 mmol), trans vinyl-6-chloro-1-hexene boronic acid (1 g 408 mmol), $Pd(OAc)_2$ (14 mg, 0.05 mmol), TPPTS (93 mg, 0.15 mmol), and Na_2CO_3 (434.17 mg, 408 mmol). The mixture was stirred under reflux for 20 h at 100 °C, and the solvents were evaporated under reduced pressure. The crude material was then purified by column chromatography using DCM: MeOH (98:8) as the eluent to give **15** in 46% yield (315 mg). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.14 (brs, 1H, NH), 7.65 (s, 1H, H-6), 6.33 (d, *J* = 11 Hz, 1H, trans CH = CH), 6.25 (m, 1H, H-1′), 5.98 (d, *J* = 5 Hz, 1H, vinyl *cis*), 4.37-4.40 (m, 1H, H-3′), 3.96-3.94 (m, 1H, H-4′), 3.72 (t, *J* = 4 Hz, 2H, CH_2Cl) 2.29 (ddd, *J* = 13 Hz, *J* = 5 Hz, *J* = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH_2), 1.99 (ddd, *J* = 13.2 Hz, *J* = 7.7 Hz, *J* = 5.8 Hz, 1H, H-5′), 1.77 (m, 2H, H-1′), 1.29 (m, 2H, H-1′) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 163, 152, 150, 132, 130, 122, 105, 94, 90, 70, 61, 42, 41, 40, 27, 22 ppm. HRMS calcd for $C_{15}H_{21}N_2O_5Cl$ *m/z* 344.1139, found 345.1138.

3.4.6 5-(6-Amino-1-hexene)-2'-deoxyuridine (**17**)

5-(6-azido-1-hexene)-2'-deoxyuridine **16** was dissolved in anhydrous diethyl ether (10 mL), and the contents were cooled to 0 °C. The vessel was charged with PPh_3 (4.72 g, 18.0 mmol) and left to stir at 0 °C. After 3 h, H_2O (1 mL) was added and the reaction was stirred and allowed to warm to room temperature overnight. The reaction mixture was poured carefully into a separatory funnel containing 10% HCl, and the resulting mixture was extracted with Et_2O (3 × 10 mL). The combined organic extracts were discarded. The aqueous layer was treated with 6 M aqueous NaOH solution until the solution reached a pH of 10. The crude amine was extracted with diethyl ether (5 × 10 mL), and the combined organic extracts were dried with Na_2SO_4, and carefully concentrated in vacuo. The

crude product was carried on to the next step without further purification. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.14 (brs, 1H, NH), 7.65 (s, 1H, H-6), 6.33 (d, *J* = 11 Hz, 1H, trans CH = CH), 6.25 (m, 1H, H-1′), 5.98 (d, *J* = 5 Hz, 1H, vinyl *cis*), 4.37-4.40 (m, 1H, H-3′), 3.96-3.94 (m, 1H, H-4′), 2.7 (t, *J* = 4 Hz , 2H, CH_2NH_2), 2.29 (ddd, *J* = 13 Hz, *J* = 5 Hz, *J* = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH_2), 1.99 (ddd, *J* = 13.2 Hz, *J* = 7.7 Hz, *J* = 5.8 Hz, 1H, H-5′), 1.77 (m, 2H, H-1′), 1.29 (m, 2H, H-1′) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 163, 152, 150, 132, 130, 122, 105, 94, 90, 70, 61, 45, 42, 30, 27, ppm. HRMS calcd for $C_{15}H_{23}N_3O_5$ *m*/*z* 325.1093, found 326.1189.

3.4.7 5-(6-*p*-methyl-ethyl-hydroxybenzylidene imidazolinone-1-hexene)-2'-deoxyuridine (**18**)

A solution of **17** (0.3 mmol) and **11** (6 mmol) in ethanol (5 mL) was heated at 100 ºC in a sealed tube for 5 days. The reaction mixture was cooled down to room temperature and concentrated under reduced pressure. The crude was then purified by silica gel column chromatography to obtain **18** in 63% yield. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.25 (d, *J* = 8 Hz, 2H),7.65 (s, 1H, H-6), 6.89 (d, *J* = 7 Hz, 2H), 6.62 (s, 1H), 6.33 (d, *J* = 11 Hz, 1H, trans CH = CH), 6.25 (m, 1H, H-1′), 6.02 (s, 2H), 5.98 (d, *J* = 5 Hz, 1H, vinyl *cis*), 4.37-4.40 (m, 1H, H-3′), 3.96-3.94 (m, 1H, H-4′),, 3.59 (q, *J* = 4 Hz, 2H), 3.52 (s, 3H), 2.7 (t, *J* = 4 Hz , 2H, CH_2NH_2), 2.29 (ddd, *J* = 13 Hz, *J* = 5 Hz, *J* = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH_2), 1.99 (ddd, *J* = 13.2 Hz, *J* = 7.7 Hz, *J* = 5.8 Hz, 1H, H-5′), 1.77 (m, 2H, H-1′), 1.29 (m, 2H, H-1′), 1.23 (t, *J* = 4 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 171, 162, 163, 160, 158, 152, 150, 146, 139, 137, 132, 131, 130, 129, 128, 126, 124, 122, 114, 111, 107, 105, 94, 92, 90, 82, 70, 63, 61, 56, 41, 42, 40, 35, 30, 27 15 ppm. HRMS calcd for $C_{29}H_{37}N_5O_8$ *m*/*z* 583.1393, found 584.1389.

3.4.8 5-(6-*p*-Hydroxybenzylidene imidazolinone -1-hexene)-2'-deoxyuridine (**7**)

A mixture of 5-(6-*p*-methyl-ethyl-hydroxybenzy-lidene imidazolinone-1-hexene)-2'-deoxyuridine, **18**, (100 mg, 0.17 mmol) and TFA (55 mg, 0.35 mmol), was dissolved in dry DCM at 0 °C, was stirred for 24 h at room temperature under argon atmosphere. The crude material was then purified by column chromatography using DCM: MeOH (7:3) as the eluent to give **7** in 100% yield (90 mg). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.25 (d, *J* = 8 Hz, 2H),7.65 (s, 1H, H-6), 6.89 (d, *J* = 7 Hz, 2H), 6.62 (s, 1H), 6.33 (d, *J* = 11 Hz, 1H, trans CH = CH), 6.25 (m, 1H, H-1′), 5.98 (d, *J* = 5 Hz, 1H, vinyl *cis*), 4.37-4.40 (m, 1H, H-3′), 3.96-3.94 (m, 1H, H-4′), 3.52 (s, 3H), 2.7 (t, *J* = 4 Hz , 2H, CH_2NH_2), 2.29 (ddd, *J* = 13 Hz, *J* = 5 Hz, *J* = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH_2), 1.99 (ddd, *J* = 13.2 Hz, *J* = 7.7 Hz, *J* = 5.8 Hz, 1H, H-5′), 1.77 (m, 2H, H-1′), 1.29 (m, 2H, H-1′) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 171, 162, 163, 160, 158, 152, 150, 146, 139, 137, 132, 131, 130, 129, 128, 126, 124, 122, 114, 111, 107, 105, 92, 90, 82, 70, 63, 61, 56, 41, 42, 40, 35, 30 ppm. HRMS calcd for $C_{26}H_{31}N_5O_7$ *m*/*z* 525.0493, found 526.1359.

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References

[1] Minami, J., Yoshida, K., Soejima, T., Yaeshima, T., and Iwatsuki, K. 2010. "New Approach to Use Ethidium Bromide Monoazide as an Analytical Tool." *Journal of Applied Microbiology* 109: 900-9.

[2] Hwang, G. 2018. "Single-Labeled Oligonucleotides Showing Fluorescence Changes upon Hybridization with Target Nucleic Acids." *Molecules* 23: 124.

[3] Nygren, J., Svanvik, N., and Kubista, M. 1998. "The Interactions between the Fluorescent Dye Thiazole Orange and DNA." *Biopolymers: Original Research on Biomolecules* 46: 39-51.

[4] Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. 1992. "Simultaneous Amplification and Detection of Specific DNA Sequences." *Biotechnology* 10: 413-7.

[5] Xu, X., Yan, S., Hu, J., Guo, P., Wei, L., Weng, X., and Zhou, X. 2013. "One-Step to Get 5-Azidomethyl-2'-Deoxyuridine from 5-Hydroxymethyl-2'-Deoxyuridine and Detection of It through Click Reaction." *Tetrahedron* 69: 9870-4.

[6] Yang, L., Lin, G., Nelson, R. S., Jian, Y., Telser, J., and Li, L. 2012. "Mechanistic Studies of the Spore Photoproduct Lyase via a Single Cysteine Mutation." *Biochemistry* 51:

7173-88.

[7] Niwa, H., Inouye, S., Hirano, T., Matsuno, T., Kojima, S., Kubota, M., Ohashi, M., and Tsuji, F. I. 1996. "Chemical Nature of the Light Emitter of the *Aequorea* Green Fluorescent Protein." *Proceedings of the National Academy of Sciences* 93: 13617-22.

[8] Meech, S. R. 2009. "Excited State Reactions in Fluorescent Proteins." *Chemical Society Reviews* 38: 2922-34.

[9] Saady, A., Böttner, V., Meng, M., Varon, E., Shav-Tal, Y., Ducho, C., and Fischer, B. 2019. "An Oligonucleotide Probe Incorporating the Chromophore of Green Fluorescent Protein Is Useful for the Detection of Her-2 Mrna Breast Cancer Marker." *European Journal of Medicinal Chemistry* 173: 99-106.

[10] Kovaliov, M., Wachtel, C., Yavin, E., and Fischer, B. 2014. "Synthesis and Evaluation of a Photoresponsive Quencher for Fluorescent Hybridization Probes." *Organic & Biomolecular Chemistry* 12: 7844-58.

[11] Wenge, U., and Wagenknecht, H.-A. 2011. "Synthetic GFP Chromophore and Control of Excited-State Proton Transfer in DNA: An Alternative Concept for Fluorescent DNA Labels with Large Apparent Stokes' Shifts." *Synthesis 2011*: 502-8.

[12] Devon, F. 2017. "A Versatile Platform for Adding Functional Properties to Amyloid Fibrils." *Organic & Biomolecular Chemistry* 15: 8023-7.

[13] Nakamura, S., Ogasawara, S., Matuda, S., Saito, I., and Fujimoto, K. 2012. "Template Directed Reversible Photochemical Ligation of Oligodeoxynucleotides." *Molecules* 17: 163-78.

[14] Coulibaly, W. K., Paquin, L., Bénie, A., Bekro, Y.-A., Durieu, E., Meijer, L., and Bazureau, J. P. 2012. "Synthesis of N,N'-bis(5-arylidene-4-oxo-3,5-dihydro-4H-imidazol-2-yl)diamines Bearing Various Linkers and Biological Evaluation as Potential Inhibitors of Kinases." *European Journal of Medicinal Chemistry* 58: 581-90.

[15] Dey, S., and Sheppard, T. L. 2001. "*Ketone*-DNA: A Versatile Postsynthetic DNA Decoration Platform." *Organic Letters* 3: 3983-6.

[16] Debdab, M., Carreaux, F., Renault, S., Soundararajan, M., Fedorov, O., Filippakopoulos, P., Lozach, O., Babault, L., Tahtouh, T., Baratte, B., et al. 2011. "Leucettines, a Class of Potent Inhibitors of cdc2-Like Kinases and Dual Specificity, Tyrosine Phosphorylation Regulated Kinases Derived from the Marine Sponge Leucettamine B: Modulation of Alternative Pre-RNA Splicing." *Journal of Medicinal Chemistry* 54: 4172-86.

[17] Segal, M., Yavin, E., Kafri, P., Shav-Tal, Y., and Fischer, B. 2013. "Detection of mRNA of the Cyclin D1 Breast Cancer Marker by a Novel Duplex-DNA Probe." *Journal of Medicinal Chemistry* 56: 4860-9.

[18] Zilbershtein, L., Silberman, A., and Fischer, B. 2011. "8-(*p*-CF3-cinnamyl)-Modified Purine Nucleosides as Promising Fluorescent Probes." *Organic & Biomolecular Chemistry* 9: 7763-7773.

[19] Song, W., Strack, R. L., Svensen, N., and Jaffrey, S. R. 2014. "Plug-and-Play Fluorophores Extend the Spectral Properties of Spinach." *Journal of the American Chemical Society* 136: 1198-201.

[20] Han, K. Y., Leslie, B. J., Fei, J., Zhang, J., and Ha, T. 2013. "Understanding the Photophysics of the Spinach–DFHBI RNA Aptamer–Fluorogen Complex to Improve Live-Cell RNA Imaging." *Journal of the American Chemical Society* 135: 19033-8.

[21] Stepanenko, O. V., Verkhusha, V. V., Kuznetsova, I. M., Uversky, V. N., and Turoverov, K. K. 2008. "Fluorescent Proteins as Biomarkers and Biosensors: Throwing Color Lights on Molecular and Cellular Processes." *Current Protein and Peptide Science* 9: 338-69.