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. dUHBI conjugates highly fluoresce in glycerol (λem 460 nm, Φ 0.31), and are 109-fold more emissive than in methanol, implying the potential of dUHBI-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 (λem 504 nm; Φ 0.79; brightness 23,000 M⁻¹·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}, 5.

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\textsuperscript{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\textsuperscript{HBI} analogues 6 and 7. Next, we report on the proof of the potential of dU\textsuperscript{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).

![Scheme 1](image)

Scheme 1  Reaction conditions: (a) piperidine, ethanol, 82%; (b) methyl iodide, acetonitrile, 7 h, 93%.
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$_{N}$2 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 $\lambda_{\text{abs}}$ 386 nm and $\epsilon$ 20,600 M$^{-1}$·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, $\lambda_{\text{abs}}$ 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 ($\Phi$ 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-butandiol and in glycerol: methanol (7:3) mixture,
which shares the same viscosity, $\Phi$ 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 (pKa 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$_2$CO$_3$ 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 $\lambda_{abs}$ 418 nm and $\varepsilon$ 25,700 M$^{-1}$·cm$^{-1}$, showing 32 nm red-shift of $\lambda_{abs}$, and 5,100 M$^{-1}$·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:](image)

(a) (E)-6-chloro-1-hexenylboronic acid pinacol ester, Na$_2$CO$_3$, (3 eq), TPPTS (0.25 eq), Pd(OAc)$_2$ (0.05 eq), CH$_3$CN:H$_2$O 80 °C, 83%; (b) NaN$_3$, AcNMe$_2$; (c) PPh$_3$, Et$_2$O, 0 °C, 79%; (d) 9, ethanol, 5 days, 82%; (e) TFA, DCM, rt, 100%.
absorbance wavelength was pH-dependent, $\lambda_{\text{abs}}$ 395 nm, pH 1.4 (positively charged HBI-moiety); $\lambda_{\text{abs}}$ 420 nm, pH 7.5; and $\lambda_{\text{abs}}$ 470 nm, pH 12 (negatively charged HBI-moiety). Compound 20 in glycerol emitted at 485 nm, and was 135-fold more emissive ($\Phi$ 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 dUHBI 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 $\text{S}_\text{N}2$ reaction for coupling with the HBI chromophore, 11, at its C2-position by displacing MeSH. These dUHBI$^\text{III}$ 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 ($\Phi$ 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 dUHBI$^\text{III}$ 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 $\lambda = 380$ nm.

3.3 Quantum 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$_2$SO$_4$ according to Eq. (1).

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

3.4 Synthesis

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

A solution of 4-methyllethyletherbenzaldehyde 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 at 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$_2$SO$_4$ 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$_3$): $\delta$ 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$_2$O$_3$S $m/z$ 292.0769, found 292.0771.

3.4.2 (Z)-5-(4-(ethoxymethoxy)benzylidene)-3-methyl-2-(methylthio)-3,5-dihydro-4H-imidazo[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$_3$CN (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$_3$): $\delta$ 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 C13H18N2O5S m/z 306.6933, found 306.7252.

3.4.3 5-(3′-Aminoallyl-p-methyl-ethyl-hydroxybenzylideneimidazolinone)-2′-deoxyuridine (14)

A solution of 11 (0.5 mmol) and 13 (6 mmol) in ethanol (3 mL) was heated at 100 °C 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 using DCM: MeOH (8:2) as the eluent to give 14 in 76% yield. 1H NMR (CDCl3): 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-5′), 1.99 (ddd, J = 13.2 Hz, J = 13 Hz, J = 7 Hz, J = 5 Hz, 1H, H-5), 1.94 (m, 2H, H2′, H2″), 1.23 (t, J = 4 Hz, 3H) ppm. 13C NMR (100 MHz, CDCl3): 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 C26H31N5O8 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). 1H NMR (CDCl3): 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-5′), 3.96-3.94 (m, 1H, H-4′), 3.59 (q, J = 4 Hz, 2H), 2.16 (m, 2H, CH = CH-CH2), 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. 13C NMR (100 MHz, CDCl3): δ 163, 152, 150, 132, 130, 122, 105, 94, 90, 70, 61, 42, 41, 40, 27, 22 ppm. HRMS calcd for C19H21N5O7 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 PPh3 (4.72 g, 18.0 mmol) and left to stir at 0 °C. After 3 h, H2O (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 Et2O (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 then washed with diethyl ether (5 × 10 mL), and the combined organic extracts were dried with Na2SO4, and carefully concentrated in vacuo. The
crude product was carried on to the next step without further purification. 1H-NMR (400 MHz, CDCl3): 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′), 3.59 (q, J = 4 Hz, 2H), 2.7 (t, J = 4 Hz, 2H, CH2NH2), 2.29 (ddd, J = 13 Hz, J = 5 Hz, J = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH2), 1.77 (m, 2H, H-1′), 1.29 (m, 2H, H-1′) ppm. 13C NMR (100 MHz, CDCl3): δ 171, 162, 163, 160, 158, 152, 150, 146, 139, 137, 132, 131, 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 C15H23N3O5 / 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 °C 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. 1H-NMR (400 MHz, CDCl3): 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, 1H), 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, CH2NH2), 2.29 (ddd, J = 13 Hz, J = 5 Hz, J = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH2), 1.99 (ddd, J = 13 Hz, J = 13 Hz, J = 7 Hz, 1H), 1.77 (m, 2H, H-1′), 1.29 (m, 2H, H-1′), 1.23 (t, J = 4 Hz, 3H) ppm. 13C NMR (100 MHz, CDCl3): δ 171, 162, 163, 160, 158, 152, 150, 146, 139, 137, 132, 131, 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 C29H37N5O8 / 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-hydroxybenzylidene 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). 1H-NMR (400 MHz, CDCl3): 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, CH2NH2), 2.29 (ddd, J = 13 Hz, J = 5 Hz, J = 2 Hz, 1H, H-5′), 2.16 (m, 2H, CH = CH-CH2), 1.99 (ddd, J = 13 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. 13C NMR (100 MHz, CDCl3): δ 171, 162, 163, 160, 158, 152, 150, 146, 139, 137, 132, 131, 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 C26H31N5O7 / 525.0493, found 526.1359.

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