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Synthesis and spectroscopic properties of fluorescent 5-benzimidazolyl-2′-deoxyuridines 5-fdU probes obtained from *o*-phenylenediamine derivatives†

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Fluorescent nucleosides (dU^{bmz}) with desirable fluorescence quantum yield (Φ) are synthesized from almost non-fluorescent 5-fdU and *o*-phenylenediamine derivatives. The fluorescence of these nucleosides is quite sensitive to pH and organic solvents. 4-Methoxybenzene-1,2-diamine was used for the detection of 5-fdU among natural nucleosides.

High-fidelity DNA replication is essential to maintain the genetic integrity of living cells. The mutation of bases tends to be associated with serious diseases, such as cancer.[1] In the past few years, numerous unnatural bases with diverse chemical biology functions have been reported. These unnatural bases minimally perturb DNA replication and transcription.[2] Naturally, identifying and detecting new bases with novel biological functions are becoming great challenges. Among these new bases is 5-formyl-2′-deoxyuridine (5-fdU), an oxidized thymidine lesion formed by reactive oxygen species resulting from UV light,[3] and the formation of this lesion may cause aging and/or carcinogenesis.[4] Thus, the convenient detection of 5-fdU is imperative. Several approaches for the detection of 5-fdU have been developed.[5] Until now, only one fluorescence-based approach for detecting 5-fdU has been reported,[6] but the reagent used for 5-fdU detection in this method is difficult to synthesize and is unstable. In addition, fluorescent nucleosides are playing an increasingly important role in the study of the biological functions of nucleic acids, including the monitoring of real-time biochemical events such as drug binding,[7] RNA folding and cleavage,[8] and RNA–protein interactions.[9] Recently, a complicated microwave-assisted synthesis of 5-benzimidazolyl-2′-deoxyuridines with antibacterial activity was reported.[10] In that work, both the microwaves and the protected hydroxyl hamper the detection of 5-fdU. Therefore, we synthesized series of fluorescent 2′-deoxyuridine derivatives without protecting hydroxyl groups in one step, and we used this reaction to distinguish 5-fdU from natural bases. Herein, we report the synthesis of a series of highly fluorescent 5-benzimidazolyl-2′-deoxyuridines (dU^{bmz}, Table 1) that were readily synthesized based on *o*-phenylenediamine derivatives from a commercial source. The *o*-phenylenediamine derivatives (**2a–j**) exhibit no fluorescence before the reaction with the target nucleoside, 5-fdU. After the reaction of **2a–j** with 5-fdU, the formyl group at the 5-position of 5-fdU is converted into a benzimidazole ring, which is directly conjugated to the uracil group to greatly improve the fluorescence (Scheme 1a).

First, we synthesized dU^{bmz} in one step (Scheme 1).[6] 5-Formyl-2′-deoxyuridine (5-fdU, **1**) was mixed with *o*-phenylenediamine derivatives (**2**) in DMF, and hydrogen peroxide was added as an oxidant to oxidize the C–N bond to a C=N double bond. The reaction occurred in the presence of $Sc(OTf)_3$ and gave 5-benzimidazolyl-2′-deoxyuridines (dU^{bmz}, **3**) with satisfactory yields, as shown in Table 1.

To explore the electronic effects of substituents on the *o*-phenylenediamine ring on the spectroscopic properties of the products (**3a–j**), the UV absorbance and fluorescence of dU^{bmz} were investigated. The UV absorbance and fluorescence spectra show that compounds **3a–f**, which were synthesized with *o*-phenylenediamine derivatives containing electron-donating groups, exhibit significantly higher fluorescence levels than compounds **3g–i**, which have much weaker fluorescence (see ESI†). The reason for this difference is likely that the structures of compounds **3a–j** include conjugated systems that undergo an intramolecular charge transfer process. The uracil moiety of compounds **3a–j** is an electron-withdrawing structure. When there are electron-donating groups on the *o*-phenylenediamine moiety, dU^{bmz} exhibits high fluorescence emission. In contrast, **3g–i**, which contain electron-withdrawing groups on the *o*-phenylenediamine moiety, exhibit no fluorescence emission.

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‡These two authors contributed equally to this work.

Table 1 Structure of *o*-phenylenediamine derivatives and dU^{bmz} with yield

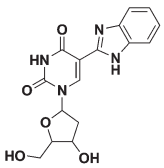
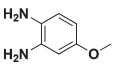
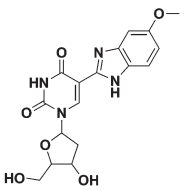
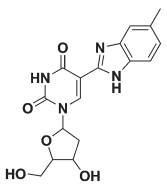
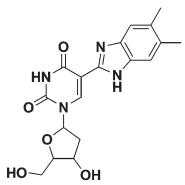
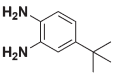
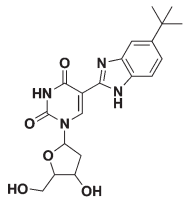
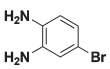
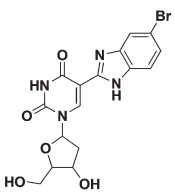
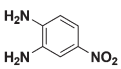
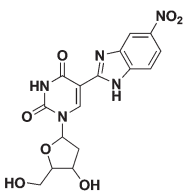
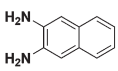
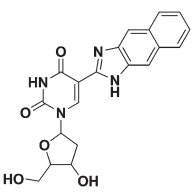
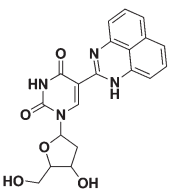
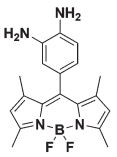
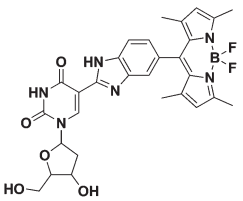
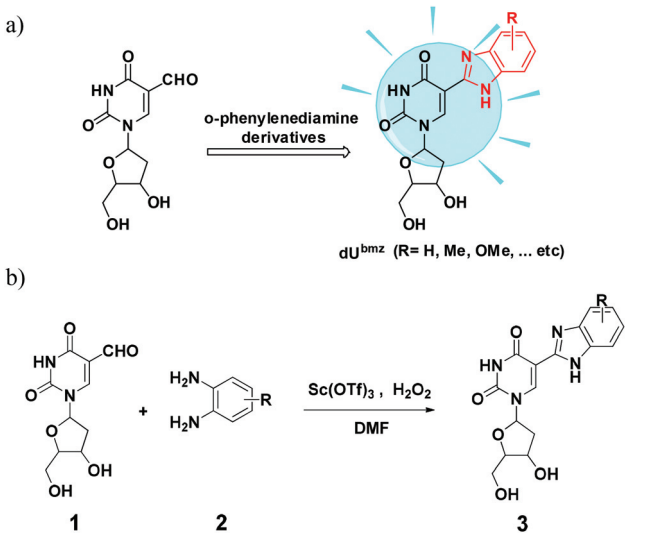
Substrate	Product	Yield
2a 	**3a** 	83%
2b 	**3b** 	77%
2c 	**3c** 	93%
2d 	**3d** 	87%
2e 	**3e** 	84%
2f 	**3f** 	83%
2g 	**3g** 	92%
2h 	**3h** 	80%
2i 	**3i** 	79%

Table 1 *(Contd.)*

Substrate	Product	Yield
2j 	**3j** 	77%



Scheme 1 (a) The diagram for production of fluorescent dU^{bmz}. (b) Synthesis of dU^{bmz}.

Table 2 Photoproperties of **3a–3f** and **1** in 100 mM HCl (aq)

	R	λ_{max}^{abs} [nm]	λ_{max}^{flu} [nm]	Stokes shift [nm]	Φ
3a	H	320	405	85	0.039
3b	OMe	335	475	140	0.023
3c	Me	324	426	102	0.065
3d	2Me	330	448	118	0.088
3e	*t*-Bu	325	423	98	0.070
3f	Br	329	405	76	0.016
1		260	308	48	0.00003

Table 2 shows that the Stokes shift of the fluorescent nucleosides becomes longer with the greater electron-donating ability of R. This result is consistent with the character of the intramolecular charge transfer effect. Among dU^{bmz}, the structure with the methoxy group (R = MeO) has the longest Stokes shift. In Table 2, we present the main optical properties of compounds **3a–3f**, including the maximum excitation wavelength (λ_{max}^{abs}), the maximum fluorescence emission wavelength (λ_{max}^{flu}), the Stokes shift, and the fluorescence quantum yield (Φ) in 100 mM aqueous hydrogen chloride (HCl) solution. Compared with that of 5-fdU (Φ = 0.00003), the fluorescence quantum yields (Φ) of these fluorescent nucleosides (**3a–3f**)

are excellent. All of the fluorescence quantum yields were determined using quinine sulfate (0.56) in 100 mM H_2SO_4 as a standard.

We decided to analyze the optical properties of dU^{bmz} in 100 mM aqueous hydrogen chloride (HCl) solution because the fluorescence of these compounds is sensitive to pH (see Fig. S45–S49†). When the pH of the buffer is lower than 7, the fluorescence intensity of dU^{bmz} would increase quickly with decreasing pH. However, if the pH is neutral or basic, the fluorescence intensity would remain relatively low. Under acidic conditions, abundant hydrogen ions may facilitate the formation of the imidazole dication due to the protonation of both nitrogen atoms. A hydrogen bond is formed spontaneously between the hydrogen atom of the secondary amine in the benzimidazole and the oxygen atom on C4 of deoxyuridine. This hydrogen bond keeps the benzimidazole ring and the uracil base in the same plane.[11] This behavior is the proposed reason for the change in fluorescence at an acidic pH.

Compound **2j** has been used as a fluorescent probe for nitric oxide (NO).[12] This compound is almost non-fluorescent before combination with NO because of its sufficiently high HOMO energy level. However, the triazolobenzene produced after the reaction with NO has a lower HOMO energy level, and this compound is highly fluorescent. Analogously, **3j**, which contains a benzimidazolyl structure synthesized from 5-fdU and **2j**, should be highly fluorescent. As shown in Fig. S50,† at approximately 503 nm there is a peak at which there is a much higher fluorescence intensity for **3j** than for **2j**. **3j** and **2j** were even different colors in water exposed to UV light, and these differences were visible with the naked eye. Thus, probe **2j** can also be used to detect 5-fdU using fluorescence.

In addition to water or buffer as a solvent, we also tested the fluorescence and UV absorbance of dU^{bmz} in different types of organic solvents. The solvent-related properties of nucleosides **3a–3f** are summarized in Fig. 1. As expected, the unique structure had an impact on the photophysics of **3a–3f** in organic solvents. **3a–3f** in 100 mM HCl (aq) have high fluorescence quantum yields (Φ). The behavior in water is similar to that in dimethylsulfoxide (DMSO). The fluorescence of dU^{bmz} in organic solvents is lower than that in 100 mM HCl (aq), most likely because the formation of the imidazole dication under acid conditions could hold the benzimidazole ring and the uracil in the same plane *via* hydrogen bonding. Therefore, the fluorescence of dU^{bmz} in 100 mM HCl (aq) is higher than that in organic solvents, in which there is only one proton on the benzimidazole ring. Somewhat surprisingly, all compounds exhibit almost identical fluorescence quantum yields (Φ) in 1,4-dioxane. Due to the distinct solubilities of these compounds in organic solvents with diverse polarities, the fluorescence quantum yields (Φ) of dU^{bmz} in acetone, methanol, ethanol, and acetonitrile are quite low. Although it was very low, the fluorescence quantum yield (Φ) of dU^{bmz} containing bromide (R = Br) is greater in DMSO than in aqueous solution and is significantly greater than that of compounds with other substituent groups in organic solvents (acetone, methanol, ethanol, and acetonitrile, see ESI†). The assay results indicate that the fluorescence quantum yields (Φ) of **3a–3f** display moderate sensitivity to different solvents. Further experiments are required to elucidate the influence of the solvent.

Because of the excellent fluorescence properties of dU^{bmz}, this series of compounds such as **3a–3e** (pK_a = 4.4, 4.9, 4.8, 4.3, 4.5, see Fig. S56†) can be used in fluorescence detection, fluorescence labeling, cell imaging, and other applications.[13] The water solubility of these nucleoside analogs is significant for research in the biology field. Therefore, dU^{bmz}, which have fluorescence intensities in H_2O, especially under the acidic conditions that are crucial to organisms, could be used to exploit this advantage in biological detection. Above all, we have demonstrated that it is possible to use *o*-phenylenediamine derivatives to detect 5-fdU (Fig. 2).

Finally, 10 μM 4-methoxybenzene-1,2-diamine was added to aqueous solutions (10 mM CH_3COONH_4, pH = 4.5) containing 5-formyl-2′-deoxyuridine (5-fdU), 2′-deoxyguanosine (dG), 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), or 2′-deoxythymidine (dT) at a concentration of 100 μM, followed by the addition of dithiothreitol (DTT). Then, the fluorescence emission of the reaction mixture was tested at 400 nm–560 nm with an excitation wavelength of 370 nm after a 12 h incubation at

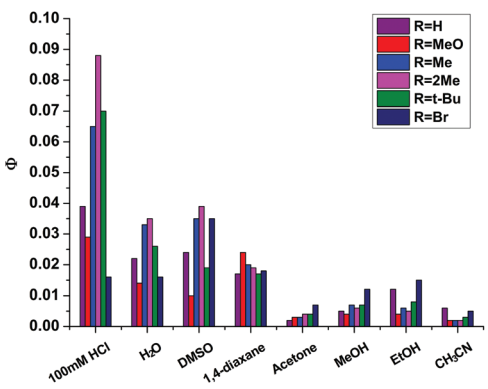


Fig. 1 Fluorescence quantum yield (Φ) of compounds **3a–3f** in several solvents (100 mM HCl (aq), H_2O, DMSO, 1,4-dioxane, acetone, MeOH, EtOH, and CH_3CN). Concentrations of compounds are 1 μM.

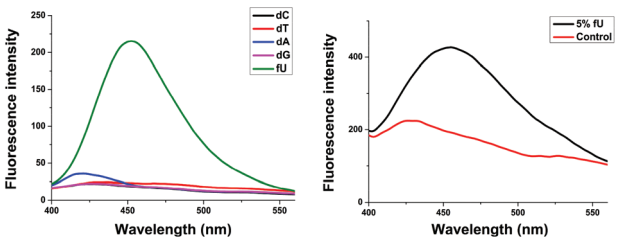


Fig. 2 (a) Fluorescence spectra of 10 μM 4-methoxybenzene-1,2-diamine toward 100 μM 5-fdU (green), dC (black), dT (red), dA (blue), dG (purple) in 10 mM CH_3COONH_4 buffer, pH = 4.5, with DTT 10 μM. (b) Fluorescence spectra of 10 μM 4-methoxybenzene-1,2-diamine toward total concentration of a 100 μM mixture of dA, dG, dT, dC, 5-fdU in 10 mM CH_3COONH_4 buffer, pH = 4.5, with 10 μM DTT, including 5% 5-fdU (black), control with only natural nucleosides (red).

37 °C (Fig. 2a). The results indicate that 4-methoxybenzene-1,2-diamine can selectively react with 5-fdU but not with the natural nucleosides. Additionally, we mixed 5-fdU into aqueous buffer (10 mM CH_3COONH_4, pH = 4.5) containing the four natural nucleosides to distinguish the mutated nucleoside 5-fdU from natural nucleosides. In Fig. 2b, relative to the control line, both an increasing fluorescence intensity and also an almost 30 nm red-shift of the maximum fluorescence emission peak can be observed. This phenomenon indicates that a 5% concentration of 5-fdU among normal bases can be easily detected by fluorescence. Furthermore, the acid reaction condition is helpful for improving the fluorescence intensity of the obtained dU^{bmz}. Thus, this method can be used to detect a small amount of 5-fdU in a mixture of nucleosides prepared by hydrolyzing oligonucleotides with S1 nuclease and alkaline phosphatase.[6] The commercially available compound 4-methoxybenzene-1,2-diamine can readily combine with 5-fdU containing a free hydroxyl to produce a highly fluorescent product in aqueous solution. In our future work, we may use *o*-phenylenediamine derivatives to detect 5-fdU in oligonucleotides and even in the genome.

In summary, we synthesized a series of fluorescent nucleosides containing a benzimidazole ring that have desirable fluorescence quantum yields (Φ), particularly in 100 mM HCl (aq) solution, using a very convenient method. The fluorescence of these nucleosides is sensitive to the pH and the presence of organic solvents. We also used 4-methoxybenzene-1,2-diamine, one of the starting materials, to detect 5-fdU among natural nucleosides. The fluorescent nucleosides synthesized, using the detection method explored in this paper, will be applied as fluorescent probes in the DNA and RNA mutation fluorescence detection field in the future.

Acknowledgements

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