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Tuning the optical properties of BODIPY dye through Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) reaction

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Borondipyrromethenes (BODIPY) are a class of fluorescent dyes whose fluorescence quantum yields are generally high and independent of the solvent. In this paper, we report the synthesis of a new type of BODIPY compound that carries an azido group on the 3-position of the pyrrole core. The azido group quenches the fluorescence of the dye due to its weak electron-donating effect. The fluorescence of the BODIPY dye can be switched on after reacting with alkynes via a Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) reaction. We further demonstrate that this azido-BODIPY compound can be used in the cell imaging applications.

CuAAC reaction, fluorogenic, BODIPY dye, cell imaging, fluorescent probe

1 Introduction

It has been found that Cu(I) catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) reaction [1, 2], a prototypical click chemistry, could activate the fluorescence of some pro-fluorophores with either azido or alkynyl groups by the formation of triazole rings [3–6]. This fluorogenic process has shown widespread applications, especially in the emerging field of cell biology and functional proteomics [7], due to the high reaction efficiency of the CuAAC reaction under mild reaction conditions and the distinct fluorescence properties of the products [8]. So far only a few types of fluorophores, including coumarin, anthracene, and naphthalimide, have been explored [8], and all of them can only be irradiated by UV light. In biological systems, irradiation at UV range may cause high background noise and cell damage. Therefore, fluorophores with longer excitation and emission wavelengths, which can be activated *in situ*, are highly demanded to develop.

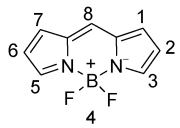

Figure 1 Structure of the BODIPY core.

BODIPY (borondipyrromethene) dyes are a type of interesting fluorophores which have numerous advantages including great chemical and photo stability, relatively high absorption coefficients, high quantum yields (ca. ϕf = 0.5–0.8), visible excitation wavelength (ca. 500 nm), narrow emission bands and high emission intensity. They have been used as laser dyes [9–20], markers [21, 22], sensors [23–28] in modern chemistry and biology [29]. It is well known that the functionalities on 3-, 4-, 5- and 8-positions of the BODIPY core (Figure 1) strongly impact its optical properties [30–33], which led to many efforts to design BODIPY-based sensor molecules [34–39]. In particular, an internal charge transfer (ICT) process has often been employed by introducing an electron donating amino group at the 3-position of the pyrrole ring of the BODIPY. For example, it has been shown that mono- or di-substitution of

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3,5-dichloro BODIPY caused the products to have quite different absorption and emission spectra [32, 40].

Based on our previous study, we postulated that when an azido group is introduced to the 3-position of the BODIPY core, the electron donating effect of the α-nitrogen of the azido group will quench the fluorescence via an ICT process. Upon CuAAC reaction with a terminal alkyne, the formation of triazole ring will lower the electron donating ability of this nitrogen, release the fluorescence quenching, and lead to a fluorogenic phenomenon [3].

2 Experimental

2.1 General experimental section

Chemical reagents and solvents were purchased from VWR Scientific, and were used without further purification. ^{1}H NMR spectra were recorded on Varian 300 NMR spectrometer and the chemical shifts were reported relative to TMS. ^{13}C NMR spectra were recorded either on Varian 300 NMR or Varian 400 NMR spectrometer. Analytical thin-layer chromatography (TLC) was performed on silica 60F-254 plates. Flash column chromatography was carried out on silica gel. The UV-vis absorption spectra were measured on Agilent 8453 spectrometer. Emission spectra were measured on Varian Cary Eclipse fluorescence spectrophotometer. Fluorescent microcopy was performed on Olympus IX 86. RPMI 1640 without folic acid and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). KB cells and Human Mesenchymal Stem Cell (HMSCs) were purchased from ATCC.

2.2 Synthesis of azido BODIPY 5 and sequential Cu-AAC reaction

2.2.1 Synthesis of azido BODIPY ***5***

To 30 mL acetonitrile solution of monochloride BODIPY **4** [41–43] (240 mg, 0.69 mmol), 3 equivalents of sodium azide (134 mg, 2.07 mmol) were added. The mixture was stirred in dark at room temperature for 3 h, monitored with TLC. The reaction was concentrated under vacuum and the resulting residue was purified by chromatography to get BODIPY **5** as a red solid (210 mg, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.79 (s, 1 H), 7.48 (d, J = 8.4 Hz, 2 H), 7.04 (d, J = 8.4 Hz, 2 H), 6.99 (d, J = 4.8 Hz, 1 H), 6.84 (d, J = 4.2 Hz, 1 H), 6.50 (m, 1 H), 6.35 (d, J = 4.2 Hz, 1 H), 3.91 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 161.8, 152.4, 144.0, 140.8, 134.0, 133.3, 132.9, 132.2, 128.8, 125.9, 117.4, 114.1, 109.9, 55.5; HRMS calculated for $(C_{16}H_{12}BF_2N_5O + Na)^+$: m/z 345.1607, found m/z 345.1613.

2.2.2 Typical experimental procedure for CuAAC reactions

Azido BODIPY **5** (17 mg, 0.05 mmol), 1-ethynyl-3-(trifluoromethyl)benzene (9 mg, 0.05 mmol) were suspended in a 7:3 mixture (*V*/*V*) of ethanol and water (8 mL). Copper sulfate (3 mg, 0.015 mmol), sodium ascorbate (6 mg, 0.03 mmol) and tris[(*N*-benzyltriazolyl)-methyl]amine ligand (8 mg, 0.015 mmol) were added. The mixture was then stirred at room temperature for 12–16 h, and monitored by TLC. EtOAc (20 mL × 2) were added to extract the product. The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the resulting residue was purified with flash chromatography on silica gel to get the triazole compound as a yellow solid (22 mg, 89%).

6: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.12 (s, 1 H), 7.94 (s, 1 H), 7.73–7.69 (m, 2 H), 7.57 (d, J = 8.1 Hz, 2 H), 7.45–7.39 (m, 2 H), 7.15 (s, 1 H), 7.17–7.01 (m, 4 H), 6.62 (s, 1 H), 3.92 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 146.1, 135.4, 130.5, 130.2, 129.6, 128.7, 127.8, 126.6, 125.9, 125.6, 125.5, 125.1, 124.4, 120.9, 55.5, 45.9. HRMS calculated for $C_{24}H_{17}BF_3N_5O$ (M^+): m/z 459.1593, found m/z 459.1587.

7: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.08 (s, 1 H), 7.91 (s, 1 H), 7.87 (d, J = 8.1 Hz, 2 H), 7.55 (d, J = 8.1 Hz, 2 H), 7.28 (d, J = 8.1 Hz, 2 H), 7.18 (d, J = 4.2 Hz, 1 H), 7.09–7.06 (m, 3 H), 6.99 (d, J = 2.1 Hz, 1 H), 6.63 (s, 1 H), 3.92 (s, 3 H), 2.65 (t, J = 7.2 Hz, 2 H), 1.67–1.63 (m, 2 H), 1.37–1.34 (m, 4 H), 0.92–0.82 (m, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 162.3, 148.4, 147.1, 143.6, 143.3, 139.3, 134.3, 133.4, 132.5, 132.4, 131.1, 128.9, 127.2, 126.1, 125.8, 120.3, 120.2, 120.1, 119.0, 114.1, 113.0, 55.6, 35.8, 33.2, 31.9, 22.6, 14.0. HRMS calculated for $C_{29}H_{28}BF_2N_5O$ (M^+): m/z 511.2469, found m/z 511.2483.

8: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.11 (s, 1 H), 7.64 (s, 1 H),7.51–7.46 (m, 2H), 7.22 (d, J = 3.6 Hz, 1 H), 7.03–7.00 (m, 3 H), 6.76–6.75 (m, 1 H), 6.45–6.43 (m, 1 H), 5.23 (s, 1 H), 5.13 (s, 1 H), 3.89 (s, 3 H), 3.26 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 167.2, 160.5, 151.0, 141.5, 137.4, 136.4, 133.8, 132.5, 131.0, 130.0, 125.8, 125.2, 116.9, 115.0, 113.0, 112.0, 54.5, 21.3.

9: ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.63 (s, 1 H), 7.88 (s, 1 H), 7.54 (d, J = 9.0 Hz, 2 H), 7.17–7.05 (m, 3 H), 6.98–6.97 (m, 1 H), 6.60–6.59 (m, 1 H), 3.91 (s, 3 H), 3.71 (t, J = 4.5 Hz, 2 H), 2.88 (t, J = 5.7 Hz, 2 H), 1.89–1.84 (m, 2 H), 1.75 - 1.67 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 162.2, 148.5, 147.3, 147.2, 143.2, 134.2, 133.3, 132.4, 131.1, 125.8, 122.3, 122.2, 122.1, 118.9, 114.2, 112.9, 62.6, 55.6, 32.2, 30.0, 25.3. HRMS calculated for $C_{22}H_{22}BF_2N_5O_2$ (M^+): m/z 437.1949, found m/z 437.1957.

10: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.17 (s, 1 H), 8.07 (d, J = 6.6 Hz, 2 H), 7.94 (s, 1 H), 7.74–7.65 (m, 4 H), 7.58 (d, J = 6.6 Hz, 2 H), 7.50–7.45 (m, 2 H), 7.40–7.35 (m, 1 H), 7.21 (d, J = 4.5 Hz, 1 H), 7.11–7.07 (m, 3 H), 7.02 (s, 1 H), 6.64 (s, 1 H), 3.94 (s, 3 H); HRMS calculated for $C_{30}H_{22}BF_2N_5O$ (M^+): m/z 517.2000, found m/z 517.2005.

11: ^{1}H NMR (300 MHz, DMSO): δ (ppm) 8.98 (s, 1 H), 7.90 (s, 1 H), 7.81 (d, J = 8.7 Hz, 2 H), 7.56 (d, J = 8.4 Hz,

2 H), 7.21 (d, J = 4.8 Hz, 1 H), 7.09–7.07 (m, 3 H), 6.97 (d, J = 3.9 Hz, 1 H), 6.75 (d, J = 8.7 Hz, 2 H), 6.61 (s, 1 H), 3.93 (s, 3 H), 3.44 (q, J = 6.6 Hz, 4 H), 1.19 (t, J = 6.6 Hz, 6 H). HRMS calculated for $C_{28}H_{27}BF_2N_6O$ (M^+): 512.2422, found m/z 512.2439.

12: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.17 (s, 1 H), 8.24 (s, 1 H), 8.15 (d, J = 5.7 Hz, 1 H), 7.95 (s, 1 H), 7.65–7.57 (m, 4 H), 7.18 (d, J = 3.6 Hz, 1 H), 7.10–7.07 (m, 3 H), 7.04 (s, 1 H), 6.65 (s, 1 H), 3.93 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 162.4, 147.6, 146.8, 146.3, 144.0, 134.5, 133.4, 132.5, 131.9, 131.7, 130.9, 130.7, 129.4, 125.7, 125.4, 125.2, 125.1, 123.0, 122.9, 122.7, 121. 2, 119.4, 114.3, 112.8, 55.6. HRMS calculated for $C_{26}H_{17}BF_5N_5O$ (M^+): m/z 509.1561, found m/z 509.1569.

13: ^{1}H NMR (300 MHz, DMSO): δ (ppm) 8.98 (s, 1 H), 7.90 (s, 1 H), 7.81 (d, J = 8.7 Hz, 2 H), 7.55 (d, J = 8.7 Hz, 2 H), 7.20 (s, 1H), 7.10–7.07 (m, 3 H), 6.98 (s, 1 H), 6.70 (d, J = 8.7 Hz, 2 H), 6.60 (s, 1 H), 3.93 (s, 3 H), 3.23 (q, J = 7.2 Hz, 2 H), 1.30 (t, J = 7.2 Hz, 3 H). HRMS calculated for $C_{26}H_{23}BF_2N_6O$ (M^+): m/z 484.2109, found m/z 484.2119.

2.3 Cell labeling and detection by fluorescence microscopy

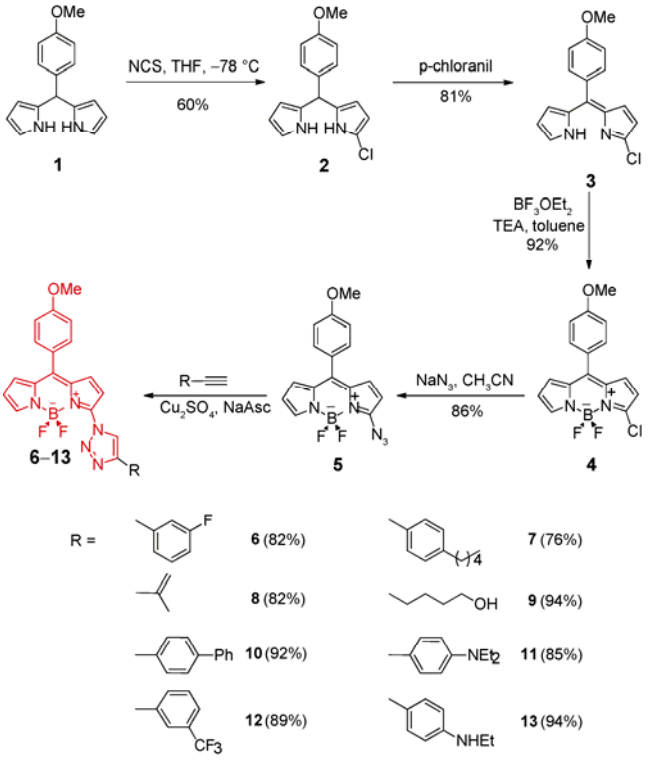
KB cells were cultured in RPMI 1640 medium without folic acid for 2 weeks prior to the experiment, adjusted to contain 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). HMSCs were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Both cells were maintained in humid 5% CO_2 atmosphere at 37 °C. Cells were plated into a 12-well plate at a density of 1 × 10^5 cells/well 24 h prior to the experiment. Then the medium was replaced with 1 mL of fresh medium containing 5 μg triazole folate compound **15** which was obtained by the congjuation of folate ligand **14** and BODIPY **5**. After 2 h, the cells were washed 3 times with PBS, and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The nucleus was labeled with DAPI for 10 min followed by several washes with PBS, and then visualized using a confocal microscope.

3 Results and discussion

As shown in Scheme 1, the monoazide derivatized BODIPY dyes were synthesized to test our hypothesis. The starting compound **1** was obtained according to the reported method by condensation of *p*-anisaldehyde with neat excess pyrrole under the catalysis of trifluoroacetic acid at room temperature [41]. It was purified by chromatography and recrystallization, and then chlorinated with one equivalent of *N*-chlorosuccinimide in tetrahydrofuran at 78 °C [42]. The monochlorinated compound **2** was oxidized with *p*-chloranil in dichloromethane to give **3,** which was then refluxed with triethylamine and BF_3-OEt_2 in toluene to afford the chloro-BODIPY precursor **4** [43]. The nucleophilic reaction was tested in several different conditions. The monochloro BODIPY **4** disappeared, and no desired azido compound was formed at room temperature in the most often used solvent such as DMSO and DMF. Finally with acetonitrile as the solvent, **4** was smoothly converted to the final azido BODIPY compound **5** in three hours at room temperature. The product was purified by choromatography, and confirmed by NMR, IR, and HRMS analysis. As we predicted, **5** showed very weak fluorescence under the irradiation of 520 nm while it had a strong absorption peak around this range.

The CuAAC reactions between BODIPY azide **5** and alkynes were first conducted in DMF/H_2O (4:1) following the common reaction conditions. However, the yield of the desired triazole products was very low while some unknown impurities were observed. Upon optimization, moderate to high yields of the CuAAC reaction was obtained when EtOH/H_2O (7:3) was used as solvent. Eight different alkynes with different electron density and structure diversity were selected to react with BODIPY azide **5**, and the yields of the designed triazole products were listed in Scheme 1.

The optical properties of the resulting triazole compounds were analyzed by UV-vis and fluorescence spectroscopy, which revealed that the CuAAC reaction could indeed trigger the fluorogenic process. The maximum absorption and emission wavelengths of triazole products in



Scheme 1 Synthesis of the azido-BODIPY dye and sequential CuAAC reactioin.

different solvent systems were summarized in Table 1. The results showed that alkynes with different structure and electron density could only slightly tune the maximum absorption and emission wavelength of the triazole products.

The fluorescence intensity comparison between the azido compound **5** and the triazole compounds were conducted in chloroform. For most products, a significant intensity increase was observed when comparing the fluorescence of the trazole products with the azido compound **5**. Generally, the electron withdrawing groups on the benzene ring (e.g. **6** and **12**) increased the fluorescence intensities of the triazole products, whereas the electron donating groups (e.g. **11** and **13**) significantly decreased the fluorescence signals as shown in Figure 2.

With all the results mentioned above, it clearly shows that the fluorescence of the azido compound **5** can be efficiently switched on through a CuAAC reaction. In addition to the preparation of the azido compound, its application in cancer cell imaging was also studied. The propargyl amine modified folate **14** was prepared according to reported method [44, 45], and then it was used as the targeting ligand to test whether the BODIPY azide **5** can be used in cell imaging study through *in situ* click reaction. In this study, KB cells were chosen as the targeting cells because they overexpress the folate receptors [46, 47] while human mesenchymal stem cells (HMSCs), which do not overexpress folate receptors, were used as a negative control. The cells were exposed to compound **14,** and then compound **5.** Unfortunately, a strong fluorescent signal was also detected from the negative control which only has compound **5**. We postulated that the problem could be resulted from the instability of the azido compound **5** under the experimental conditions.

In order to increase the stability of the BODIPY dye, the folate ligand **14** was conjugated to the compound **5** via CuAAC reaction to generate a stable triazole compound **15**, before it was used for the cell studies (Scheme 2). Following the aforementioned procedure, the KB cells and HMSCs were exposed to the mixture of the triazole compound **15** for 2 hours before the cells were washed and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Then the cellular uptake was examined with confocal microscope. As expected, the KB cells could efficiently uptake the triazole compound and show clear red fluorescence in cytoplasm when visualized under the Cy5 channel. As comparison, the very weak fluorescence signals of HMSCs after incubating with compound **15** is likely due to the lack of folate receptors (Figure 3). In both situations, no obvious cytotoxicity has been observed. The results demonstrated that our fluorogenic BODIPY dye can potentially be used in cell imaging studies.

Table 1 Optical properties of azide **5** and triazole products **6–13** in various solvents

Entry	Absorption (λ, nm)			Emission (λ, nm)		
	hexane	MeOH	$CHCl_3$	hexane	MeOH	$CHCl_3$
5	522	515	521	529	530	533
6	528	516	528	538	534	541
7	533	518	532	544	536	546
8	516	514	521	532	529	534
9	523	514	523	534	532	536
10	532	518	532	544	538	546
11	529	518	531	542	536	541
12	527	515	528	538	534	541
13	533	522	530	539	533	541

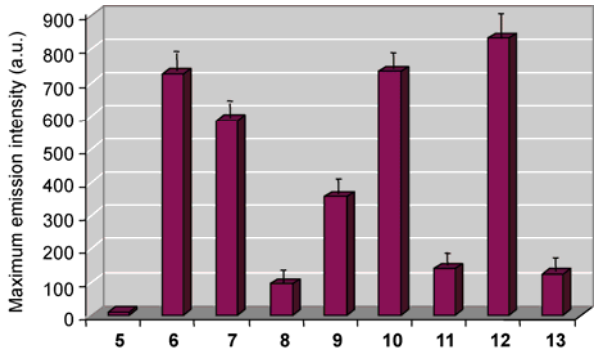
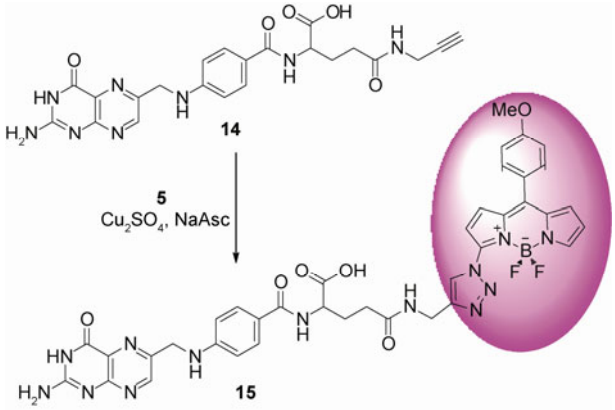


Figure 2 The comparison between the maximum emission intensity of BODIPY triazole products **6–13** and azide **5** in chloroform (optical density kept constant at 0.1 mM at their excitation wavelength).

4 Conclusions

In summary, we have synthesized a new type of BODIPY dye that the azido group on the 3-position of the pyrrole core could quench the fluorescence of the dye. The fluorescent emission could be significantly switched on after reacting with alkynes. It could be used to selectively target human KB cells when it was conjugated with the folate ligand, which demonstrated the potential applications of this dye in bioimaging. Further detailed study about the photophysical mechanism of switch on/off of fluorescence and



Scheme 2 Synthesis of BODIPY-folate conjugates.

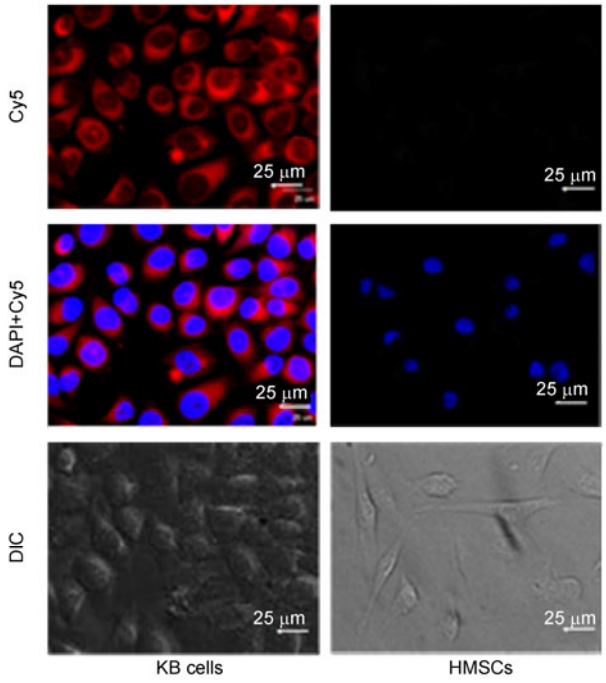


Figure 3 Confocal fluorescence images of KB cells (left panels) and HMSCs (right panels) labeled with folate-BODIPY conjugate **15**. The blue nuclei of cells were stained with 4',6-diamidino-2-phenylindole (DAPI). The cells were visualized using only Cy5 channel (Cy5), an overlay of DAPI and Cy5 channel (DAPI+Cy5), and differential interference contrast imaging (DIC). Scale bars are 25 μm.

the potential applications for *in situ* cell imaging are underway in our group.

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