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1 Article

2 Fluorophore-assisted click chemistry through 3 copper(I) complexation

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11 **Abstract:** The copper-catalyzed alkyne-azide cycloaddition (CuAAC) is one of the most powerful
12 chemical strategies for selective fluorescent labeling of biomolecules *in vitro* or in biological systems.
13 In order to accelerate the ligation process and ensure efficient formation of conjugates under diluted
14 conditions, external copper(I) ligands or sophisticated copper(I) chelating azides are used. This
15 latter strategy, however, increases the bulkiness of the triazole linkage, thus perturbing the
16 biological function or dynamic behavior of the conjugates. In a proof-of-concept study, we
17 investigated the use of an extremely compact fluorophore-based copper(I) chelating azide in order
18 to accelerate the CuAAC with concomitant fluorescence labeling; In our strategy, the fluorophore is
19 able to complex copper(I) species while retaining its photophysical properties. It is believed that this
20 unprecedented approach which was applied for the labeling of a short peptide molecule and the
21 fluorescent labeling of live cells, could be extended to other families of nitrogen-based fluorophores
22 in order to tune both the reaction rate and photophysical characteristics.

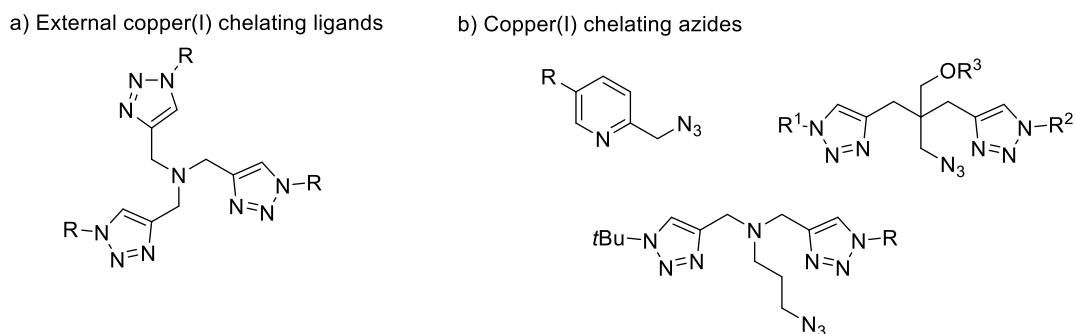
23 **Keywords:** click chemistry, CuAAC, chelating fluorophore, chelating azide, fluorescent labeling,
24 azaphthalimide, Kondrat'eva ligation

25 1. Introduction

26 The copper-catalyzed alkyne-azide cycloaddition (CuAAC) also known as click chemistry,[1]
27 has attracted tremendous interest in recent years for the site-specific *in vitro* modification of
28 biomolecules such as proteins, glycans, lipids or nucleic acids, as well as for the bioorthogonal
29 fluorescent labeling of cell extracts or living systems.[2-4] Prominent applications in this latter area
30 include among others the understanding of diverse biological processes,[5-6] the development of
31 detection tools,[7] and the real-time live-cell imaging and therapy[8].[9-12] In contrast to other
32 successful bioorthogonal chemistries such as strain promoted alkyne azide cycloaddition
33 (SPAAC)[13] and tetrazine-based inverse electron demand Diels–Alder ligation (IEDDA),[14,15]
34 CuAAC ligation has been widely adopted due to the small size and inertness of the alkyne and azide
35 handles that can be incorporated into biomolecules by using the genetic code expansion, or the
36 cellular metabolic machinery,[16] and to the fact that small triazole adducts also impose a minimal
37 perturbation of resulting conjugates.

38 Reaction of alkynes with azides generally involves the *in situ* formation of copper(I) catalyst
39 from a copper(II) source (*e.g.* CuSO₄) in the presence of reducing agent, sodium ascorbate. Although
40 coordinating ligands are not strictly required for CuAAC,[5] their combine use has shown to
41 significantly accelerate the alkyne-azide cycloaddition which thus ensures efficient bioconjugation
42 under diluted conditions imposed by biological systems, while preventing both the deactivation of
43 the copper(I) catalyst by biomolecules, and copper-mediated oxidative damages.
44 Tris(triazolylmethyl)amine derivatives such as TBTA or its water-soluble analogues THPTA, and
45 BTAA constitute an important family of such accelerating ligands (Figure 1a).[17-19]

46

47 **Figure 1.** Strategies of copper ligands

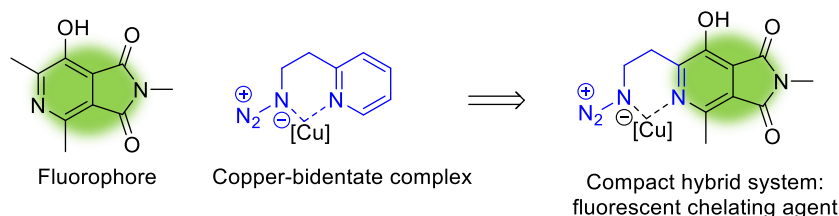
48

49

50 However, ligands, copper sources, and fluorescent bioorthogonal handle are generally required
 51 in excess amounts relatively to the chemical reporter group for the bioconjugate reaction to proceed
 52 accordingly with high yields.[20] These ligands are mostly used for cell-surface labeling, or require
 53 covalent attachment to cell penetrating peptides to facilitate their cellular uptake.[21] To circumvent
 54 these limitations, another strategy consists in using azides equipped with a chelating moiety which
 55 are capable of complexing the copper(I) species and thus accelerate the reaction in the absence of
 56 external ligands by both facilitating the formation of the metallacycle intermediate and increasing the
 57 electrophilicity of the azide function.[22-24] Highly performant azide chelating systems were
 58 designed in this context (Figure 1b).[25,26] Nevertheless, these chelating azides are constituted of
 59 polycyclic ligands with aromatic characters, and when linked to bulky and rigid fluorophores, water-
 60 solubilizing groups are required to counterbalance their overall hydrophobicity. As a consequence,
 61 subsequent biological, physico-chemical properties of the corresponding conjugates, in particular for
 62 short labeled biomolecules or pharmacophores, may be dramatically altered, which questions the
 63 benefit of using small alkyne, azide handles in CuAAC, with respect to other biorthogonal
 64 reactions.[27]

64

65 Following these considerations, in order to perform bioconjugate reaction with alkyne modified
 66 biomolecules, we want to report the unprecedented use of an extremely compact fluorophore-based
 67 ligand capable of complexing copper(I) species while retaining its photophysical characteristics
 68 useful for bioimaging applications. We previously reported the Kondrat'eva ligation based on a one-
 69 pot Diels-Alder/aromatization of 5-alkoxyazole with maleimide to furnish the corresponding
 70 azaphthalimide fluorophore, which has found different applications in chemical biology.[28-29]
 71 Interestingly, this small bicyclic azaphthalimide dye displays relatively high excitation and emission
 72 wavelengths (ex. 420 nm, em. 520 nm) suitable for live-cell imaging. Herein, we wish to combine both
 73 the fluorescent properties of the azaphthalimide dye and its underestimated copper-binding ability
 to design a readily available and extremely compact fluorescent copper-chelating azide (Figure 2).

74 **Figure 2.** Fluorophore-based copper(I) ligand for CuAAC-mediated fluorescent labeling.75 **2. Materials and Methods**76 **2.1. General information**

77 All chemicals were used as received from commercial sources without further purification.
 78 Solvents, unless otherwise stated, were purchased in reagent grade or HPLC grade and used as
 79 received, except tetrahydrofuran which was freshly distilled over sodium prior to use. PBS (pH 7.4,

0.1 M) and aq. mobile phases for HPLC were prepared with water purified by means of a MilliQ system (purified to 18.2 MΩ cm). All reactions were monitored by thin layer chromatography (TLC) and/or RP-HPLC. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. Visualization of spots was performed under a UV lamp at $\lambda = 254$ or 365 nm, and/or staining with a KMnO_4 solution/ $\text{K}_2\text{CO}_3 + 5\%$ NaOH, and developed with heat. Flash column chromatography purifications were performed manually on silica gel (40–63 μM) under pressurized air flow.

Instruments and Methods. HPLC system A: RP-HPLC analyses were performed with a Thermo Fischer Ultimate 3000 RS instrument, equipped with a diode array detector (DAD-3000RS). The temperature of the column compartment was fixed at 25 °C. A Thermo Hypersyl GOLD® C18 column (1.9 μm , 2.1 × 50 mm) was used with a binary solvent system composed of MeCN and 0.1% aq. formic acid (aq. FA, pH 2) as eluents (linear gradient from 5 to 100% MeCN over 10 min) at a flow rate of 0.600 mL/min. System B: Semi-preparative RP-HPLC were performed with an Interchim puriFlash® 4250 instrument equipped with a diode array detector, and a Thermo Hypersyl GOLD® C18 column (5 μm , 30.0 × 250 mm) with MeCN and water as eluents (linear gradient 10–65% MeCN for 50 min) at a flow rate of 40 mL/min. System C: Semi-preparative RP-HPLC were performed with an Interchim puriFlash® 4250 instrument equipped with a diode array detector, and a Thermo Synchronis® C18 column (5 μm , 21.2 × 250 mm) with MeCN and 0.1% aq. FA as eluents (linear gradient 5–95% MeCN for 35 min) at a flow rate of 15 mL/min. System D: Semi-preparative RP-HPLC were performed with an Interchim puriFlash® 4250 instrument equipped with a diode array detector, and a Thermo Hypersyl GOLD® C18 column (5 μm , 20.0 × 250 mm) with MeCN and water as eluents (linear gradient 25–70% MeCN for 25 min) at a flow rate of 40 mL/min. System E: Semi-preparative RP-HPLC were performed with a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV-visible 2000 detector, and a Hypersyl GOLD® C18 column (5 μm , 20.0 × 250 mm) with MeCN and 0.1% aq. FA as eluents (linear gradient 10–90% MeCN for 30 min) at a flow rate of 10 mL/min.

High Resolution Mass spectrometry (HRMS) were obtained by using a Waters Micromass LCT Premier XE® equipped with an orthogonal acceleration time-of-flight (oa-TOF) and an electrospray source in positive mode.

^1H , ^{13}C and NMR spectra were recorded on Bruker 300 machine operating at ambient temperature. The solvent resonance was used as the internal standard for ^1H -NMR (chloroform- d_3 at 7.26 ppm; DMSO- d_6 at 2.50 ppm; MeOD- d_4 at 3.31 ppm) and ^{13}C -NMR (chloroform- d_3 at 77.0 ppm; DMSO- d_6 at 29.8 ppm; MeOD- d_4 at 49.0 ppm). Chemical shift (δ) were quoted in parts per million (ppm). Coupling constants (J) were quoted in Hertz (Hz). The following abbreviations were used to give the multiplicity of the NMR signals: s: singlet, bs: broad singlet, d: doublet, t: triplet, dd: doublet of doublet...

Fluorescence spectroscopic studies (emission/excitation spectra and time course for kinetics monitoring) were performed on a Varian Cary Eclipse® spectrophotometer using a quartz fluorescence cell (Hellma, 104F-QS, 10 × 4 mm, lightpath: 10 mm, chamber volume 1.4 mL) and excitation/emission spectra were recorded at 20 °C. UV-visible absorption spectra were obtained on a Varian Cary 60 UV-Vis® (Varian, standard cell, 10 × 10 mm, chamber volume 3.5 mL) at 20 °C.

Fluorescence quantum yields were measured on a ORIBA Fluorolog 3 spectrophotometer with a quartz fluorescence cell (Hellma, 104F-QS, lightpath: 10 × 10 mm, chamber volume: 3.5 mL, excitation and emission slit: 2 nm) at 25 °C by a relative method using Lucifer Yellow ($\Phi_{\text{F}} = 21\%$ in Water, 430 nm)[30] as a standard. The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_{\text{F}}(\text{X}) = (A_{\text{s}}/A_{\text{x}}) (F_{\text{x}}/F_{\text{s}}) (n_{\text{x}}/n_{\text{s}})^2 \Phi_{\text{F}}(\text{S})$$

where A is the absorbance (in the range of 0.01–0.1 a.u.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively. The following refractive index values were used: 1.337 for PBS 0.1 M pH 7.4.

132 2.2. Chemical synthesis

133 Compounds **6**,^[25] **7**,^[31], **10**^[25] and **14**^[32] were prepared as described previously.

134

135 *Ethyl 2-(5-ethoxy-2-methyloxazol-4-yl)acetate* **2**. To a suspension of P₂O₅ (1.85 g, 13 mmol, 3 eq),
136 CaO (1.25 g, 22 mmol, 5 eq) and Celite® (400 mg) in dry chloroform (40 mL) stirred vigorously at room
137 temperature, was added diethyl *N*-acetylaspartate (1 g, 4.32 mmol) dissolved in dry CHCl₃ (5 mL).
138 The mixture was brought to reflux for 3 h, then P₂O₅, CaO and Celite® were further added in the
139 aforementioned amounts, and the reaction mixture was refluxed for further 3 h. The mixture was
140 cooled to 0 °C and a solution of saturated aq NaHCO₃ (100 ml) was added slowly with stirring for
141 30 min. Water (20 ml) was added and the solution was extracted with DCM (3 × 100 ml). The organic
142 phases were combined and then washed with a solution of saturated aq NaCl (150 mL), dried over
143 MgSO₄ and filtered through Celite®. The solvents were evaporated to dryness and the crude product
144 was purified by chromatography on silica gel in an AcOEt / Cyclohexane elution system (1:3 v/v) to
145 (1:1 v/v), the desired product was obtained as a yellow oil (620 mg, 2.90 mmol, 67%). The ¹H RMN
146 analysis is in agreement with the one described in the literature^[33] : ¹H RMN (300MHz, CDCl₃) δ =
147 4.17 (q, *J* = 7.2 Hz, 2 H), 4.14 (q, *J* = 7.2 Hz, 2 H), 3.41 (s, 2 H), 2.34 (s, 3 H), 1.36 (t, *J* = 7.2 Hz, 3 H), 1.28
148 (t, *J* = 7.2 Hz, 3 H).

149

150 *2-(5-Ethoxy-2-methyloxazol-4-yl)ethan-1-ol* **3**. To a suspension of LiAlH₄ (810 mg, 21.30 mmol, 2.5
151 eq) in anhydrous THF (120 mL) cooled at 0 °C, the oxazole ethyl 2-(5-ethoxy-2-methyloxazol-4-
152 yl)acetate **2** (1.8 g, 8.53 mmol) in dry THF (20 mL) was added dropwise. After 15 min at 0 °C, the
153 solution was allowed to warm at room temperature and further stirred for 3 h. Then, the mixture was
154 cooled to 0 °C and water (5 mL), a solution of aq NaOH (5 M, 5 mL), and water (15 mL) were added
155 successively. The mixture was stirred for 15 min, then MgSO₄ was added. The resulting cake was
156 filtered through Celite®, washed with ethyl acetate and the filtrate was evaporated to dryness. The
157 crude reaction mixture was then purified quickly by chromatography on a short plug of silica gel
158 (AcOEt) and the desired product was obtained as yellow oil (650 mg, 3.80 mmol, 46%). ¹H RMN
159 (300MHz, CDCl₃) δ = 4.09 (q, *J* = 7.2 Hz, 2 H), 3.77 (t, *J* = 6 Hz, 2 H), 2.56 (t, *J* = 6 Hz, 2 H), 2.26 (s, 3 H),
160 1.30 (t, *J* = 7.2 Hz, 3 H). ¹³C RMN (75MHz, CDCl₃) δ = 154.1, 152.6, 114.2, 70.5, 61.5, 27.6, 15.0, 14.2. IR
161 (neat, cm⁻¹) : 3347, 2930, 1671, 1583, 1379, 1264, 1237, 1018, 1049, 603, 473. HRMS (ESI+) : Calc. for
162 C₈H₁₄NO₃ [M+H]⁺ : 172.0974, found : 172.0972.

163

164 *2-(5-Ethoxy-2-methyloxazol-4-yl)ethyl methanesulfonate* **4**. To the alcohol 2- (5-ethoxy-2-
165 methyloxazol-4-yl) ethan-1-ol **3** (300 mg, 1.75 mmol) in dry THF (30 mL) at 0 °C, triethylamine (500
166 μL, 3.68 mmol, 2.1 eq) and mesyl chloride (270 μL, 3.50 mmol, 2 eq) were successively added dropwise
167 and the resulting mixture was stirred at room temperature for 3 h. Water (30 mL) was added to the
168 solution, and the mixture was further stirred for 15 min. The aqueous phase was extracted with
169 AcOEt (3 × 20 mL), the organic phases were combined and then washed with a solution of saturated
170 aq NaCl (30 mL), dried over MgSO₄ and filtered through Celite®. The solvent was evaporated to
171 dryness and the crude product was purified by chromatography on silica gel (AcOEt), the desired
172 product was obtained as a yellow oil (430 mg, 1.72 mmol, 98%). ¹H RMN (300MHz, CDCl₃) δ = 4.40
173 (t, *J* = 6.9 Hz, 2 H), 4.13 (q, *J* = 7.2 Hz, 2 H), 2.96 (s, 3 H), 2.80 (t, *J* = 6.9 Hz, 2 H), 2.30 (s, 3 H), 1.35 (t, *J*
174 = 7.2 Hz, 3 H). RMN ¹³C (75MHz, CDCl₃) δ = 154.9, 152.8, 111.0, 70.6, 68.1, 37.4, 25.1, 15.0, 14.2. IR
175 (neat, cm⁻¹) : 2983, 2936, 1673, 1444, 1349, 1267, 1169, 1018, 951, 902, 800, 526. HRMS (ESI+) : calc. for
176 C₉H₁₅NO₃S [M+H]⁺ : 250.0749 ; found : 250.0753.

177

178 *4-(2-Azidoethyl)-5-ethoxy-2-methyloxazole* **5**. To a solution of oxazole 2- (5-ethoxy-2-methyloxazol-
179 4-yl) ethyl methanesulfonate **4** (400 mg, 1.60 mmol) in dry DMF (8 mL), sodium azide (620 mg, 9.60
180 mmol, 6 eq) was added. After 15 min stirring at room temperature, the solution was heated at 50 °C
181 for 8 h. Then, the mixture was cooled to RT and water (10 mL) was added. The aqueous phase was
182 extracted with AcOEt (2 × 30 mL) and the combined organic phases were washed with a solution of
183 saturated aq NaCl (15 mL), dried over MgSO₄, filtered through Celite® and then the solvent was

184 evaporated to dryness. The crude reaction mixture was then purified by chromatography on silica
185 gel in a AcOEt / Cyclohexane elution system (1:5 v/v) to (1:1 v/v), the desired product was obtained
186 as yellow oil (240 mg, 1.22 mmol, 76%). ¹H RMN (300MHz, CDCl₃) δ = 4.15 (q, J = 7.2 Hz, 2 H), 3.5 (t,
187 J = 6.9 Hz, 2 H), 2.63 (t, J = 6.9 Hz, 2 H), 2.32 (s, 3H), 1.37 (t, J = 7.2 Hz, 3 H). ¹³C RMN (75MHz, CDCl₃)
188 δ = 154.56, 152.60, 112.74, 70.39, 49.83, 24.75, 15.01, 14.28. IR (neat, cm⁻¹) : 2979, 2930, 2094 (N3), 1671,
189 1587, 1379, 1265, 1229, 1171, 1020, 952, 644. HRMS (ESI+) : Calc. for C₈H₁₂N₄O₂ [M+H]⁺ : 197.1039 ;
190 found : 197.1042.

191
192 *6-(2-Azidoethyl)-7-hydroxy-2,4-dimethyl-1H-pyrrolo[3,4-c]pyridine-1,3(2H)-dione* **1**. N-
193 methylmaleimide (47 mg, 0.43 mmol, 1.05 eq) was added to a solution of 4- (2-azidoethyl) -5-ethoxy-
194 2-methyloxazole **5** (80 mg, 0.41 mmol) in toluene (1.5 mL), was added. After stirring at room
195 temperature for 15 min and then at 70 ° C for 6 h, the mixture was concentrated under reduced
196 pressure. The residue was dissolved in CDCl₃ (1 mL) and then formic acid (10 μL, 0.265 mmol, 0.65
197 eq) was added. The mixture was allowed to stir at room temperature overnight. The reaction mixture
198 was concentrated under reduced pressure and the resulting residue was purified by semi-preparative
199 RP-HPLC according to system B to afford the desired product (43 mg, 0.16 mmol, 40%) as a yellow
200 solid. Melting point: 107.3 – 108.5 °C. ¹H RMN (300 MHz, MeOD) δ = 3.69 (t, J = 6.9 Hz, 2 H), 3.16 (t, J
201 = 6.9 Hz, 2 H), 3.06 (s, 3 H), 2.67 (s, 3 H) ppm. ¹³C RMN (75MHz, MeOD) δ = 169.51, 168.58, 157.58,
202 147.41, 146.43, 122.67, 122.15, 50.07, 32.95, 23.88, 19.77 ppm. IR (neat, cm⁻¹) : 3304, 2944, 2100 (N3),
203 1698, 1433, 1382, 1233, 1004, 756, 519. HRMS (API+) : Calc. for C₁₁H₁₂N₅O₃ [M+H]⁺ : 262.0940 ; found :
204 262.0946.

205
206 *7-Hydroxy-2,4-dimethyl-6-(2-(4-(2-oxo-2H-chromen-7-yl)-1H-1,2,3-triazol-1-yl)ethyl)-1H-pyrrolo[3,4-
207 c]pyridine-1,3(2H)-dione* **9**. To a solution of 6- (2-azidoethyl) -7-hydroxy-2,4-dimethyl-1H-pyrrolo [3,4-
208 c] pyridine-1,3 (2H) -dione **1** (13.55 mg, 0.052 mmol) in a solution of H₂O/t-BuOH (200 μL, 1:1, v/v),
209 were successively added the 7-ethynylcoumarin (8.65 mg, 0.052 mmol, 1 eq)[1] in DCM (100 μL),
210 sodium ascorbate (10.3 mg, 0.052 mmol, 1 eq), and copper sulfate pentahydrate CuSO₄.5H₂O (1.3 mg,
211 0.052 mmol, 1 eq). The reaction mixture was stirred at room temperature in the darkness for 4 h
212 (analytical RP-HPLC control, system A) and then the crude reaction mixture was concentrated under
213 reduced pressure. The resulting residue was triturated in Et₂O, then EtOH. The pellet recovered after
214 centrifugation was purified by preparative RP-HPLC according to system C, the desired product (5
215 mg, 0.12 mmol, 23%) was obtained as a yellow solid. Melting point: 179.9 – 181.2. °C. ¹H RMN (300
216 MHz, DMSO-d₆) δ = 8.72 (s, 1 H), 8.04 (d, J = 9.6 Hz, 1 H), 7.84 - 7.75 (m, 3 H), 6.46 (d, J = 9.6 Hz, 1 H),
217 4.85 (t, J = 6.9 Hz, 2 H), 3.49 (t, 2 H), 2.97 (s, 3 H), 2.59 (s, 3 H) ppm. ¹³C RMN (75MHz, DMSO-d₆) δ =
218 168.00, 166.46, 159.94, 155.49, 154.08, 145.72, 144.73, 143.92, 134.31, 129.12, 123.17, 121.17, 118.15,
219 115.76, 112.13, 47.49, 32.35, 23.56, 19.50 ppm. IR (neat, cm⁻¹) : 2924, 1706, 1681, 1621, 1430, 1223, 1000,
220 845, 754, 616, 590, 524, 456. HRMS (ESI+) : Calc. for C₂₂H₁₈N₅O₅ [M+H]⁺ : 432.1308 ; found : 432.1302.

221
222 *7-(1-Phenethyl-1H-1,2,3-triazol-4-yl)-2H-chromen-2-one* **11**. To a solution of 2-phenylethyl azide **7**
223 (89 mg, 0.52 mmol) in a solution of H₂O/t-BuOH (1.5 mL, 1:1, v/v) were successively added 7-ethynyl
224 coumarin (97 mg, 0.52 mmol, 1 eq) in DCM (300 μL), sodium ascorbate (104 mg, 0.52 mmol, 1 eq) and
225 copper sulfate pentahydrate CuSO₄.5H₂O (13 mg, 0.05 mmol, 0.1 eq). The reaction mixture was
226 allowed to stir at room temperature in the darkness for 12 h, then concentrated under reduced. The
227 obtained residue was dissolved in AcOEt (1 mL) and then purified by chromatography on silica gel
228 in a AcOEt / Cyclohexane elution system (3:1, v/v) to (3:0, v/v). The desired product (110 mg, 3.47
229 mmol, 66%) was obtained as a yellow solid. Melting point: 180 – 181.9 °C. ¹H RMN (300MHz, DMSO-
230 d₆) δ = 8.71 (s, 1 H), 8.04 (d, J = 9.3 Hz, 1 H), 7.79 (t, J = 7.8 Hz, 3 H), 7.25 (t, J = 6.5 Hz, 5 H), 6.46 (d, J =
231 9.3 Hz, 1 H), 4.68 (t, J = 6.9 Hz, 2 H), 3.23 (t, J = 6.9 Hz, 2 H) ppm. ¹³C RMN (75MHz, DMSO-d₆) δ =
232 159.9, 154.1, 144.8, 143.9, 137.5, 134.2, 129.1, 128.7, 128.4, 126.6, 122.8, 121.1, 118.1, 115.8, 112.0, 50.8,
233 35.5 ppm. IR (neat, cm⁻¹) : 3121, 2924, 1702, 1620, 1455, 1222, 1151, 939, 838, 754, 696, 614, 494. HRMS
234 (ESI+) : Calc. for C₁₉H₁₆N₃O₂ [M+H]⁺ : 318.1243 ; found : 318.1244.

235

236 *Alkyne-ISWLFVR-OH 12*. The 2-chlorotrityl chloride resin (1.0 g, 1.60 mmoles/g, 1.60 mmoles)
237 was swollen for 20 min in DCM (15 mL) under nitrogen bubbling. A solution of the amino acid Fmoc-
238 Arg(pbf)-OH (1.04 g, 1.60 mmoles, 2 eq) and DIPEA (1.64 mL, 9.60 mmoles, 6 eq) in DCM (10 mL)
239 was added on the resin, and agitated with nitrogen bubbling for 2.5 h at room temperature. The
240 resulting resin was then treated with MeOH (~ 5 mL) and agitated under nitrogen bubbling for 15
241 min. Then, the resin was washed successively by DCM/MeOH/DIPEA (15 mL, 17:2:1, v/v/v, repeated
242 3 times), DCM (3 × 15 mL), DMF (3 × 15 mL), DCM (3 × 15 mL) and then MeOH (3 × 15 mL). The resin
243 was dried under vacuum and the attachment level of the first residue was determined by UV/VIS
244 (55%, 0.88 mmoles).[34] Fmoc-Arg (pbf) -2-chlorotrityl resin (0.88 mmoles, 1 eq) was swollen for 20
245 min in DCM (15 mL) under nitrogen bubbling and then washed with DMF (3 × 15 mL). Deprotection
246 of the Fmoc group was performed with piperidine 20% in DMF (10 mL) for 1 min with nitrogen
247 bubbling. The resin was filtered and then deprotection was again performed for 10 min. After
248 washing with DMF (3 × 15 mL), subsequent Fmoc-amino acids were coupled in excess (2.64 mmol, 3
249 eq) on the deprotected amino-acid bearing resin with HBTU (2.95 eq) and DIPEA (6 eq) in DMF (~ 5
250 mL) for 1 h with nitrogen bubbling at room temperature. The coupling reactions were performed
251 twice with a filtration of the resin between them. The resin was washed with DMF (3 × 15 mL), and a
252 new cycle of deprotection / coupling was performed. The final H-peptide-resin was successively
253 washed with DMF (3 × 15mL), DCM (3 × 15 mL), MeOH (3 × 15 mL) and then was dried under
254 vacuum. The coupling of the 4-pentynoic acid (87 mg, 0.88 mmoles, 2 eq) was performed on half of
255 the N-term primary amine free ISWLFVR-resin (0.44 mmoles, 1 eq) with HBTU (326 mg, 0.86 mmol,
256 1.95 eq) and DIPEA (300 µL, 1.76 mmoles, 4 eq) in DMF (~ 5 mL) for 2 h at room temperature, under
257 nitrogen bubbling. The resulting peptide was cleaved from the resin with a solution of TFA/TIS/H₂O
258 (~ 10 mL, 95: 2.5: 2.5, v/v/v) for 2 h at room temperature. The cleavage solution was evaporated and
259 the peptide was precipitated with cold diethyl ether followed by washing by centrifugation 3 times
260 with diethyl ether (9000 rpm, 10 min). The resulting residue was solubilized in water and lyophilized
261 before a purification by semi-preparative RP-HPLC according to system D. The desired product was
262 obtained as a white solid (62 mg, 0.06 mmol, 14%). HRMS (ESI+) : Calc. for C₅₁H₇₄N₁₁O₁₀ [M+H]⁺ :
263 1000.5620 ; found : 1000.5628. HPLC System A (λ = 254 nm) : t_R = 3.66 min.
264

265 *Azaphthalimide-ISWLFVR-OH 13*. To a solution of the peptide-alkyne **12** in DMF (2 mg, 1.25 mL,
266 1.6 mM, 1 eq) was added a solution of azaphthalimide-azide **1** in DMF (2.04 mg, 1.25 mL, 3.2 mM, 2
267 eq). The mixture was then diluted with PBS buffer solution (2.50 mL, 0.1 M, pH = 7.4) and a solution
268 of copper sulfate pentahydrate in MilliQ water (1 mg, 2.50 mL, 1.6 mM, 2 eq) was added. The reaction
269 mixture was stirred at room temperature for 10 min, then a solution of sodium ascorbate in water
270 (19.81 mg, 2.50 mL, 40 mM, 50 eq) was added. The reaction was monitored by analytical RP-HPLC,
271 system A. The crude reaction mixture was then directly purified (without prior treatment) by
272 preparative RP-HPLC according to system E. The desired product (1.2 mg, 0.95 mmol, 38%) was
273 obtained as a yellow solid. HRMS (ESI+) : Calc. for C₆₂H₈₅N₁₆O₁₃ [M+H]⁺ : 1261.6482 ; found : 1261.6497.
274 HPLC System A (λ = 254 nm) : t_R = 3,63 min.
275

276 2.2. Live cells labeling

277 PC12 cells line, derived from a pheochromocytoma of the rat adrenal medulla, are cultured in a
278 humidified incubator at 37 °C in an atmosphere of 5 % CO₂. Cells were grown in Dulbecco's modified
279 Eagle's medium (DMEM) (Thermo Fisher Scientific, Illkirch, France) supplemented with 7 % heat-
280 inactivated fetal bovine serum (Sigma–Aldrich), 7 % horse serum (Lonza Bioscience, Walkersville,
281 MD, USA), 2.5 % HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (Thermo Fisher
282 Scientific), 1 % glutamine (Thermo Fisher Scientific), 100 units/mL penicillin and 100 µg mL⁻¹
283 streptomycin (Thermo Fisher Scientific).
284

285 PC12 cells were plated on glass bottom dishes (MatTek Corporation, MA, USA) for staining and
286 confocal microscopy observation (SP8). After medium removal and PBS rince, cells were incubated

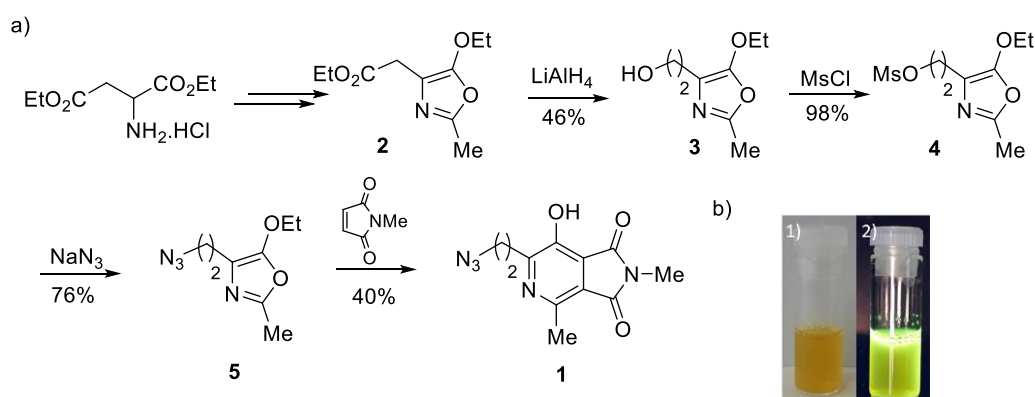
287 or not (“control” and “cocktail alone” conditions) for 30 min at 37°C in an atmosphere of 5 % CO₂
 288 with a 50 μM Alkyne 1-(4-pentyn-1-yl)-1H-pyrrole-2,5-dione **14** solution in PBS 0.1 M pH 7.4 (10 mL,
 289 containing 0.05% acetonitrile) prepared from a 5 mM alkyne **14** in acetonitrile/milliQ water 5:95 (1
 290 mL stock solution). “Cocktail” solution with azide **1** (50 mM), copper sulfate pentahydrate (50 mM),
 291 sodium ascorbate (1.25 mM), prepared 45 min before incubation (azide **1** stock solution 10 mM in
 292 DMSO/water 10:90 (500 mL) was added the copper sulfate pentahydrate stock solution 20 mM in
 293 water (250 mL). After 30 min of incubation at 20 °C, the sodium ascorbate stock solution 0.5 M in
 294 water (250 mL) was added, and the mixture was further incubated for 15 min at 20 °C). After 2 washes
 295 with PBS, cells were incubated during 30 min at 37 °C in an atmosphere of 5 % CO₂ with the “cocktail”
 296 solution implemented with 1 μM of the non-ionic surfactant polyol Pluronic F-127 (Thermo Fisher
 297 Scientific). Cells were washed one time with PBS before to be visualized under microscope.
 298

299 Images (1024x1024 pixels) were acquired by using an upright fixed-stage Leica TCS SP8 confocal
 300 microscope (Leica Microsystems, Nanterre, France) equipped with diode laser at 405 nm to excite
 301 fluorophore and a conventional scanner at 400Hz. Using a 25× objective (NA 0.95, water immersion)
 302 fluorescence emission was detected through a hybrid detector (HyD) in photon counting mode with
 303 a specific band from 500 to 550 nm. Mosaic image (square of 5 by 5 images at 1024x1024 pixels) was
 304 performed to obtain a large view and a merging was realized after acquisition through module LAS
 305 X Navigator. For image acquisitions, focuses on cells were performed in bright-field mode before
 306 fluorescence acquisition. For the fluorescence quantification, all values are expressed as fluorescence
 307 intensity (A.U.) means of at least 100 cells +/-sem . Statistical analysis was performed using the
 308 GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA) and a one-way Analysis of
 309 Variance (ANOVA) with a Tukey-Kramer multiple comparisons tests

310 3. Results and Discussion

311 3.1. Synthesis of the fluorophore-based chelating azide

312 The fluorescent chelating agent **1** was prepared from commercial L-aspartic acid diethyl ester
 313 hydrochloride (Scheme 1). First, the reported oxazole ester **2** obtained in two steps,[33] was reduced
 314 into the corresponding alcohol derivative **3** in 46% isolated yield. The hydroxyl group was then
 315 converted into mesylate intermediate **4**, which was subsequently displaced with azide ion to afford
 316 compound **5** in 74% over two steps. Finally, the oxazole **5** underwent a [4+2]
 317 cycloaddition/aromatization process with *N*-methylmaleimide to furnish the corresponding
 318 azaphthalimide **1** in 40% isolated yield. Importantly, this compound proved to be relatively soluble
 319 in PBS pH 7.4 as the only solvent, up to 4 mM (Scheme 1a and b). Interestingly, LogS value of
 320 azaphthalimide **1** was found to be -1.58, which is in the range of those calculated for water-soluble
 321 tris(triazolylmethyl)amine THPTA and BTAA (Table S8).
 322



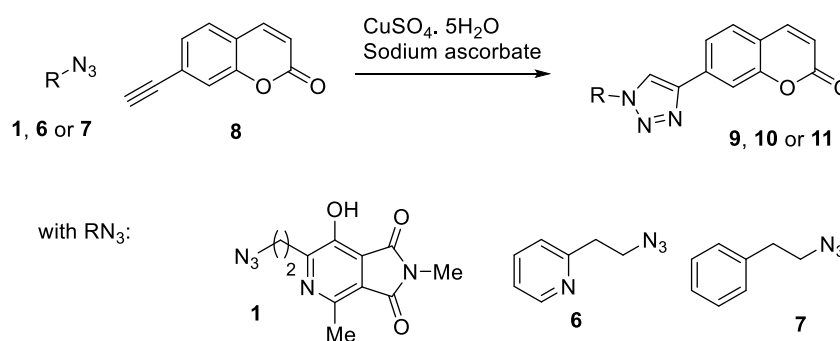
323

324 **Scheme 1.** a) Synthetic access to the azaphthalimide **1**. b) Photographs of **1** (1 mg/mL PBS 0.1 M pH
 325 7.4) taken under visible light (1) or under 365 nm light (2).

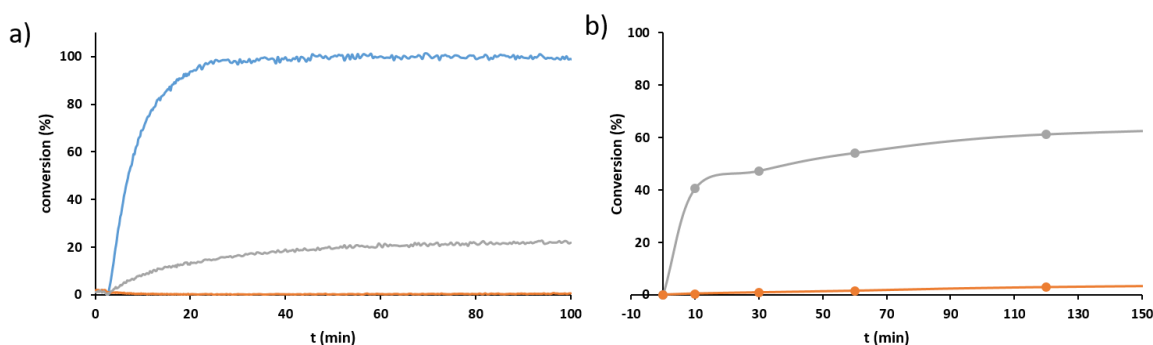
3.1. Comparative study with chelating and non-chelating azide

The azaphthalimide **1** in hand, its ability to accelerate 1,3-dipolar cycloadditions was compared to a reported effective but not fluorescent pyridine-based chelating azide, 2-(2-azidoethyl)pyridine **6**, [25] as well as a non-chelating derivative, 2-phenylethyl azide **7**. We chose these three azide-based reagents, since in all of them, the azide function is separated from the aromatic ring by a two carbon-atom alkyl chain length in order to form a 6-membered metallocycle system (for chelating azides **1** and **6** only). The fluorogenic 7-ethynyl coumarin **8** whose fluorescence increases upon reaction with azides,[25] was chosen as the click partner in order to monitor the click reactions progress by fluorescence spectroscopy. For compound **6** and **7**, an excitation and emission wavelength of 320 nm and 400 nm was selected, corresponding to the absorption and emission of the triazole-substituted coumarin (Figures S2-S3). With regards to azaphthalimide azide **1**, the coumarin and azaphthalimide scaffold display complementary photophysical properties to constitute a suitable FRET pair with an excitation at the coumarin wavelength ($\lambda_{\text{ex}} = 320 \text{ nm}$), and an emission at the azaphthalimide emission wavelength ($\lambda_{\text{em}} = 550 \text{ nm}$, Figure S1). For each triazole product, a calibration curve was performed to convert fluorescence intensity signal into product conversion (Figures S4-S6). Of note, the presence of copper(I) did not significantly modify the fluorescence emission of either triazole products **9-11**.

First, azide **1**, **6** and **11** were reacted with coumarin **8**, and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($17.5 \mu\text{M}$ each), followed by the addition of sodium ascorbate (0.437 mM , 25 equiv.) in aqueous medium (Figure 3a).



344



345

Figure 3. Comparison of kinetics of CuAAC reactions of: a) 7-ethynyl coumarin ($17.5 \mu\text{M}$), with azide (**1** in grey, **6** in blue, and **7** in orange) in the presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($17.5 \mu\text{M}$) and sodium ascorbate ($437 \mu\text{M}$) in DMF:PBS pH 7.4:H₂O (1:1:2); b) 7-ethynyl coumarin ($175 \mu\text{M}$), with azide (**1** in grey, and **7** in orange) in the presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($175 \mu\text{M}$) and sodium ascorbate ($4.375 \mu\text{M}$) in DMF:PBS pH 7.4:H₂O (1:1:2).

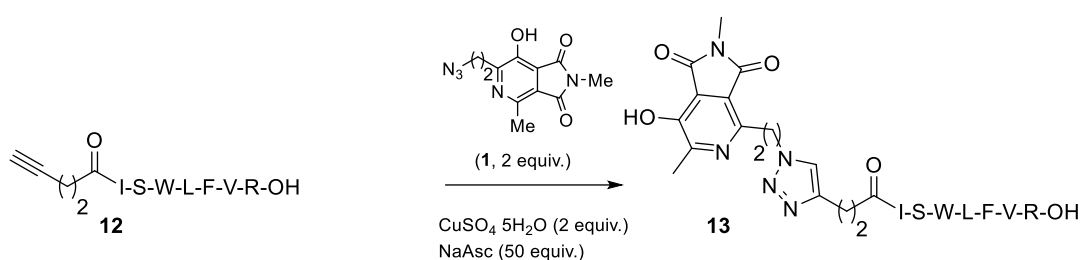
First, the 2-(2-azidoethyl)-pyridine **6** led to the rapid formation of the triazole product (Figure 3a, curve in blue), while on the other hand, the non-chelating azide **7** gave no detectable product, even after 100 min of reaction (curve in orange). Meanwhile, the azaphthalimide enables the formation of 20% of triazole. Although this conversion is significantly lower than that of **6**, this result showed that the azaphthalimide was able to accelerate the reaction presumably by acting as a bidentate ligand for CuAAC reactions. This chelating ability has been further validated with reactions carried

356

357 out at 175 μ M, and monitored by HPLC (Figure 2b, grey curve). In fact, a plateau at c.a. 60%
 358 conversion yield was quickly reached while almost no product was obtained with the non-chelating
 359 compound (orange curve). The reaction performed with the azaphthalimide was approximately
 360 100 times faster than the one achieved with the phenyl scaffold. Importantly, the presence of the
 361 copper complex did not impact the absorption and emission wavelengths of either fluorophores
 362 (Figure S1).

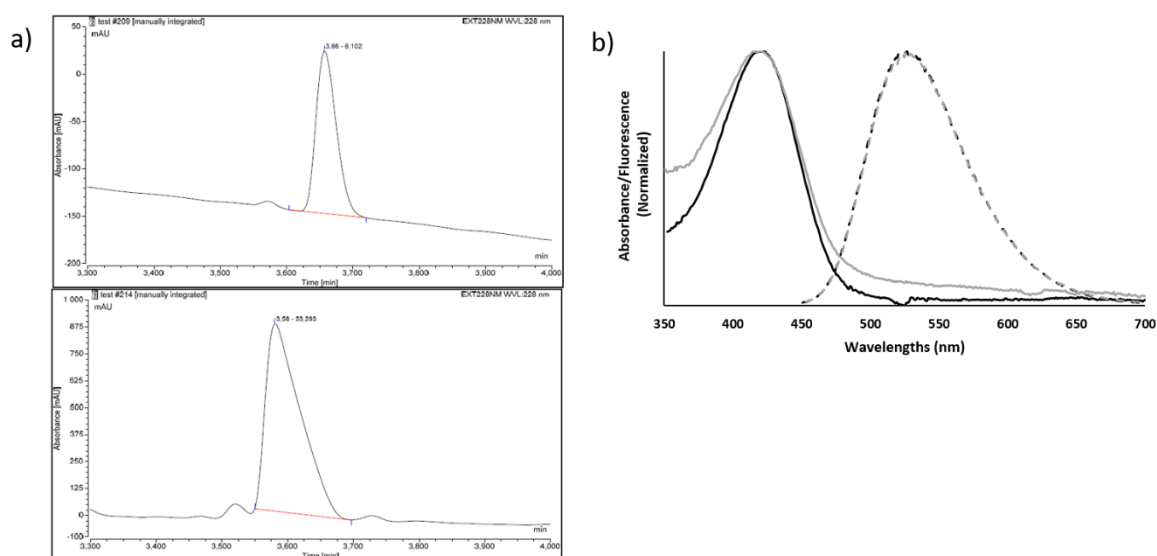
363 3.1. Applications

364 The potency of a small fluorophore-assisted click chemistry for minimal conjugate
 365 perturbation was illustrated through the fluorescent labeling of a heptapeptide-based short
 366 biomolecule (Scheme 2). The starting peptide was prepared according to standard solid-phase
 367 peptide synthesis protocols with the incorporation of the pentynoic acid at the N-terminus (see
 368 Materials and Methods).
 369



370 **Scheme 2.** Labeling of alkyne-oligopeptide **12** with azide **1**.

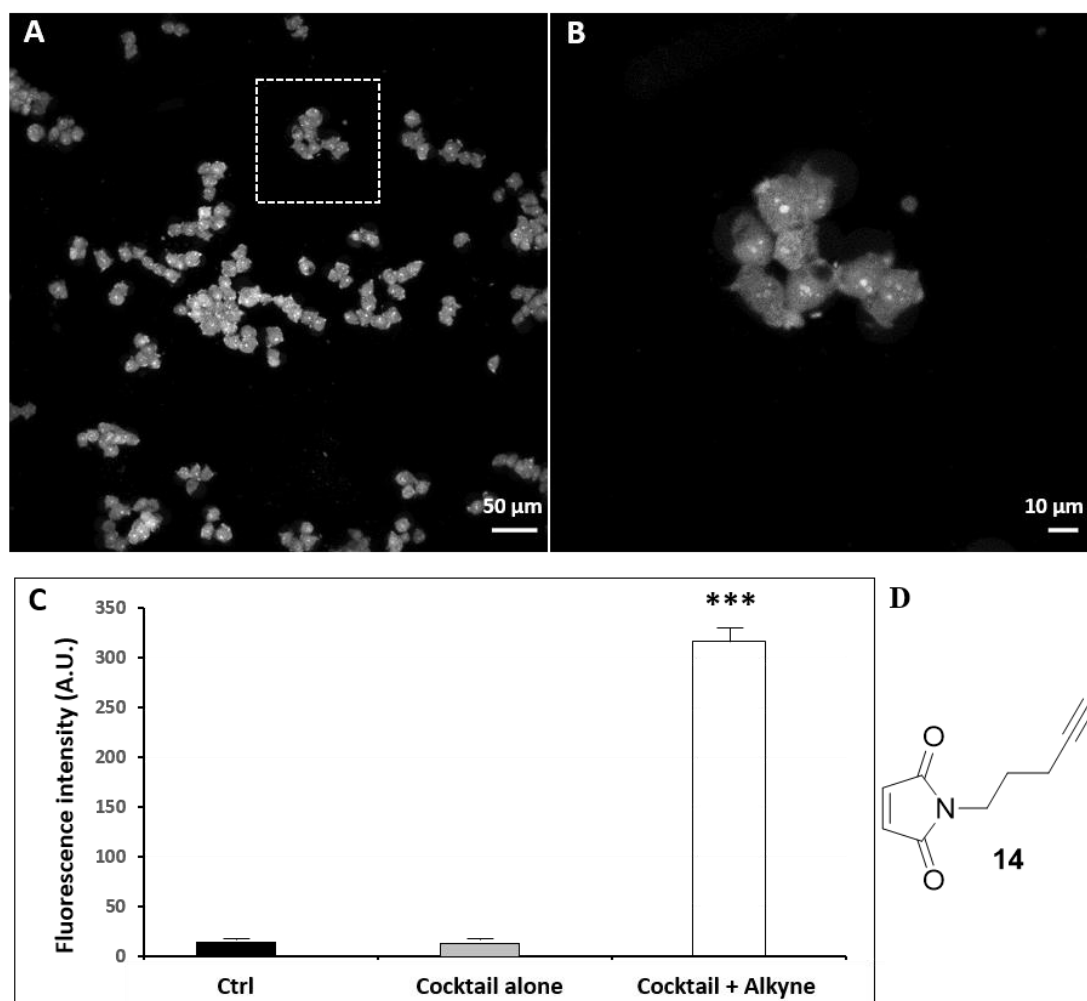
371 The reaction was monitored by HPLC and showed a clean and complete reaction within 30 min
 372 (Figure 3a). The isolated conjugate **13** obtained in 38% isolated yield after RP-HPLC and
 373 lyophilization was subject to photophysical measurements. Absorption and emission spectra
 374 recorded either in the presence or absence of copper(I) (generated *in situ* from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and
 375 sodium ascorbate), showed no change in position of peak maxima, which were found at $\lambda_{\text{abs}} \sim 416$ nm
 376 and $\lambda_{\text{em}} \sim 525$ nm, respectively (Figure 3b). Only a widening of the absorption peak was observed.
 377 Besides, quantum yield for the green fluorescence-labeled peptide was found to be around 8% in the
 378 presence and absence of *in situ* generated copper(I) determined in PBS pH 7.4 at 25 $^{\circ}\text{C}$. This value is
 379 in line with those reported for similar azaphthalimide derivatives.[29]
 380
 381



382
 383 **Figure 3.** Green-fluorescence labeling of peptide **12**. a) RP-HPLC chromatogram of peptide-alkyne
 384 **12** (top) and labeled peptide **13** after 30 min reaction (bottom). b) Normalized absorption (solid line)

385 and emission (dashed line) spectra of azaphthalimide-containing peptide **13** (in black) in the presence
 386 of *in situ* generated copper(I) (grey line) in PBS pH 7.4 at 20 °C.

387 After establishing that small conjugates could be readily labeled *in vitro* using an heptapeptide
 388 as model, then, the labeling of alkyne-modified live cells with azaphthalimide azide **1** was
 389 investigated. As a proof-of-concept study, the alkyne reporter group was first chemically
 390 incorporated into live cells by using a maleimide derivative **14** (50 µM in PBS for 30 min at 37 °C), a
 391 functional group known to react rapidly and covalently with biological thiols through a Michael
 392 addition process.[35] Cells were washed twice with PBS to remove unreacted alkyne-maleimide **14**
 393 in the supernatant, then a 50 µM solution of azaphthalimide-based chelating azide **1** in PBS pretreated
 394 with a 50 µM solution of *in situ* generated copper(I) (cocktail solution), was incubated for 15 min with
 395 cells at 37 °C, then rinsed with PBS. Subsequent confocal analysis microscopy revealed a strong
 396 fluorescence labeling of cells at 500-550 nm (Figure 4), highly statistically significant compared to
 397 control conditions (fluorescence poorly detectable in cells with only PBS incubation (ctrl) and
 398 “cocktail alone” when cells that were not incubated with the alkyne reporter group, thus
 399 demonstrating successful CuAAC mediated ligation of azaphthalimide azide and alkyne
 400 incorporated into cells, data not shown). Images revealed some discernible vesicles that could be
 401 identify as lipid droplets, in which the lipophilic alkyne reporter presumably accumulates (Figures
 402 4A,B, S7).[36]



403
 404 **Figure 4.** A, B. PC12 cells in « Cocktail+Alkyne » conditions, acquired under mozaic confocal image
 405 for cell population observation (A) and single image for cellular observation (B). C. Fluorescence
 406 intensity graph showing high significance (***) of the fluorescence intensity level after
 407 « Cocktail+Alkyne » incubation *versus* « cocktail alone » and PBS incubation (ctrl). D. Chemical
 408 structure of alkyne-maleimide **14**.

409 4. Conclusions

410 Herein, we disclosed an unprecedented “all-in-one” fluorophore-based chelating azide with an
411 exceptionally low molecular weight (260 Da), which proved useful for the fluorescent labeling of
412 small biomolecules, and biological systems. In this approach, the azaphthalimide played the role of
413 both the copper ligand and fluorophore in order to accelerate the CuAAC reaction with concomitant
414 installation of a fluorescent tag. Importantly, the fluorescence properties of the native fluorophore
415 were conserved upon its complexation to copper(I). This process advantageously does not require
416 neither an external ligand, a fluorophore, nor water solubilizing group which complicate the
417 synthesis and increase the risk of impacting the mobility of azides and properties of conjugated
418 molecules. This strategy could be extended to other families of nitrogen-containing fluorophores in
419 order to further improve the ligation rates or to red-shift the fluorescence emission.[37,38]

420 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, ¹H and ¹³C NMR
421 spectra for all new compounds; absorption, emission, and excitation spectra for compounds **9-11**; additional
422 information regarding live cells labeling.

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426 **Conflicts of Interest:** The authors declare no conflict of interest.

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