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A new bioorthogonal cross-linker with alkyne and hydrazide end groups for chemoselective ligation. Application to antibody labelling

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ABSTRACT

We describe here the synthesis of the first bioorthogonal cross-linking reagent based on aminocaproic acid core with a hydrazide function at one end to react with glycoproteins and an alkyne group at the other end for Cu(I)-catalyzed click chemistry to azide-derivatized probes. As an application, this cross-linker was used to orthogonally conjugate profluorescent 3-azido-7-hydroxycoumarin to immunoglobulin G (IgG). An immunoassay showed that IgG was mostly not affected by the Cu(I)-catalyzed click chemistry conditions. Successful conjugations and retained immunoreactivity demonstrate the potential of this new bioorthogonal cross-linker in chemoselective ligation.

1. Introduction

Biomolecules labelling is an important area of research with applications in the understanding of biological processes, the development of diagnostic tools[1] and new approaches for treatment of diseases.[2] Classical chemical modification of proteins relies on functional groups carried by amino acids side chains with lysine and cysteine being the most widely modified residues.[3] To covalently connect a probe or a biomolecule to a protein, the best strategy is to use a cross-linker that moves away the two molecules from each other, especially in the case of macromolecules, to decrease steric hindrance. Many bifunctional linkers, of the homobifunctional or heterobifunctional types (Fig. 1), are commercially available for this purpose. Most of these cross-linkers are designed to react with amino or sulfhydryl groups and are based on maleimide, *N*-hydroxysuccinimide (NHS) ester, pyridyl disulfide and α-haloacetyl reactive groups. The drawback of these reactive groups is their lack of selectivity and site-specificity since lysine residues are relatively abundant in proteins and cysteine residues are often involved in the structural integrity of the protein as disulfide bridges. Another possible side-reaction is protein homo-coupling. Although these cross-linkers still have widespread utility, recent developments in bioconjugation are directed towards alternative approaches exploiting chemoselective reactions between functional groups that are not naturally occurring in biomacromolecules and that only react with each other to ensure high selectivity and site-specificity. These reactions, that are termed bioorthogonal ligations, have found extensive applications in the selective derivatization of biological molecules during the past decade.[1,4–13]

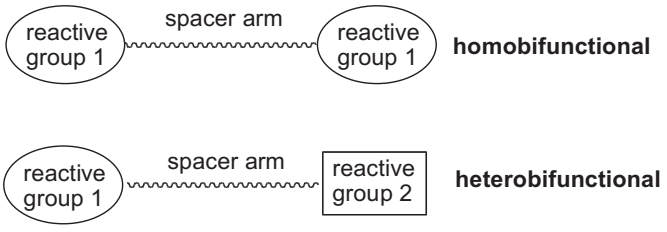

Fig. 1. Schematic design of homo and heterobifunctional cross-linkers.

One of the most successful approaches relies on the azide-alkyne [3+2] cycloaddition reaction, originally described by Huisgen.[14] This reaction leading to 1,2,3-triazole linkage, is the prototype of a set of selective reactions termed click chemistry reactions. It has enjoyed significant breakthroughs from Sharpless and Meldal groups who independently developed its copper(I)-catalyzed version (CuAAC),[15,16] and from Bertozzi group who developed its catalyst-free, strain-promoted (SPAAC) version.[17] The triazole ring is a highly stable linkage and has been described as an amide bond surrogate.[18,19] The growing interest for click chemistry reactions in

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the biological field is also attested by the recent commercial availability of azide or alkyne-tagged derivatives, such as biotin-based reagents, fluorescent dyes, nucleotides, nucleosides and non-canonical amino acids. Some spacers with azide or alkyne functionality at one end are also commercially available. Heterotrifunctional cross-linkers whose one of the reactive end groups is an azido group have been recently described for protein modification and immobilization[20] or to access sophisticated bioconjugates.[21]

We previously described the conjugation of decorated poly(amido) amine (PAMAM) dendrimer to immunoglobulin G (IgG) using a cross-linker derived from aminocaproic acid, with a hydrazide function in one end to react with oxidized IgG and form stable hydrazone, and a fluoro-nitroaromatic residue on the other end to react with nucleophiles, such as amines by aromatic nucleophilic substitution of the fluorine.[22] Furthermore the nitroaromatic moiety served as ^{1}H NMR and/or UV–vis probe.[23] As part of our current interest to explore new ways to site-selectively multi-label immunoglobulin G, we were interested in developing a new heterobifunctional and bioorthogonal linker to exploit the azide-alkyne [3+2] cycloaddition reaction to connect an azide-carrying probe to glycoproteins. In the literature, modification of intact IgG antibodies with alkyne or azide linkers for subsequent labelling by click reaction mostly relies on reagents carrying a *N*-succinimidyl ester function at the other end of the linker for ligation with primary amines of lysines residues.[24–27] Although a large number of lysine residues are located in the Fc region, favouring azide–alkyne functionalization of IgGs at their Fc region, these bifunctional linkers are not bioorthogonal.

Herein we describe the synthesis of a new bioorthogonal linker based on aminocaproic acid core with a hydrazide function at one end to react with sugar moieties of antibodies and an alkyne function at the other end for CuAAC reaction. As a proof of concept, this bioorthogonal linker was employed for the chemoselective ligation of a fluorescent dye to goat anti-rabbit secondary antibody (Fig. 2).

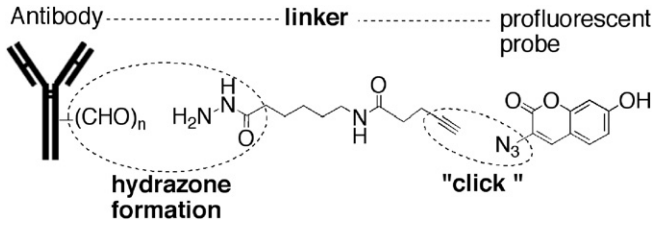


Fig. 2. Principle of the chemoselective ligation using the new bioorthogonal linker.

2. Results and discussion

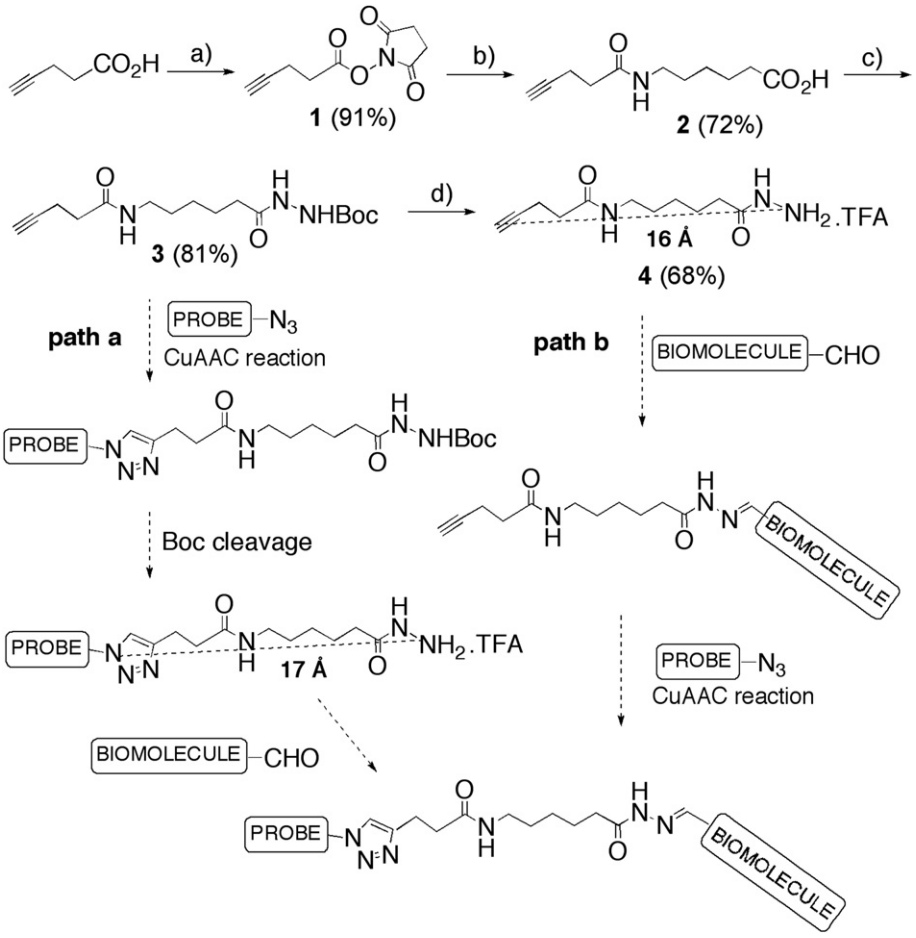
2.1. Synthesis of the bioorthogonal cross-linker 4

The synthetic route leading to the bioorthogonal linker **4** is presented in Scheme 1. Commercial pentynoic acid was reacted with TSTU in DMF for 3 h at room temperature to afford *N*-succinimidyl ester **1** in 91% yield after chromatography. Compound **1** has been previously obtained by reaction of pentynoic acid and *N*-hydroxysuccinimide mediated by EDAC and DMAP or DCC with longer reaction times and lower yields.[28,29] Purification of the active ester intermediate **1** is recommended because the TLC of the crude activation step displayed byproducts with polarities close to that of compound **2** that did not allow a clean purification of the compound of interest. Compound **1** was then reacted with aminocaproic acid in aqueous $NaHCO_3$/dioxane for 5 h to afford compound **2** in 72% yield. The carboxylic acid function of the aminocaproic moiety was then activated with TSTU and allowed to react overnight with *tert*-butyl carbazate in DMF/dioxane/H_2O to yield the protected hydrazide **3** in 81% yield. Removal of the Boc protecting group was classically carried out with trifluoroacetic acid in chloroform. The hydrazide function is highly reactive towards aldehydes and ketones and some organic solvents contain traces of these compounds. Consequently, the crude trifluoroacetate salt was triturated in ice-cooled chloroform, to afford the hydrazide linker **4** in 68% yield. Depending on the way the bioconjugation is envisaged, i.e., performing the CuAAC reaction before the hydrazone formation or the other way round, the protected hydrazide **3** as well as the free hydrazide **4** can provide starting points for the following chemoselective ligation. Path **a**, the 1,3-dipolar cycloaddition is carried out with the protected compound **3** and Boc cleavage achieved in a second step before hydrazone formation. Path **b**, the sequence of reactions is reverse with the coupling between the hydrazide group of cross-linker **4** first and the aldehyde-containing biomolecule then the azide–alkyne [3+2] cycloaddition. Cross-linker **4** has an extended length of 16 Å estimated by the ChemDraw software, that increases to 17 Å after triazole formation (path **a**). This length is well suited for intermolecular conjugation.

To validate these two paths, a fluorescent probe was chosen to label goat anti-rabbit IgG as a model for bioconjugation using this new bioorthogonal cross-linker. This choice was motivated by the relevance of fluorophore-antibody conjugates in the field of immunoassays.[30] Interestingly, a series of non-fluorescent 3-azido coumarins have been described for use in CuAAC chemoselective ligation to yield coumarin-triazole. Among these, the 7-hydroxy derivative **5** (Fig. 3) displayed strong relative fluorescent intensity after the triazole linkage formation,[31] and was used as a probe for effective click reaction with alkynes,[32–35] and to monitor the progress of the CuAAC by measuring the increase in fluorescence intensity.[36–38]

2.2. Preparation of fluorophore-IgG conjugate following path a of Scheme 1

3-Azido-7-hydroxycoumarin **5**, prepared according to published procedure,[31] was reacted with the protected linker **3** using the $CuSO_4$-ascorbate catalytic system in ethanol/water (1:1)[31] for 24 h to provide the protected coumarin-triazole-linker **6** in 75% yield (Scheme 2). Removal of the Boc protecting group was then carried out with trifluoroacetic acid in chloroform to afford the free hydrazide **7** as the trifluoroacetate salt in 78% yield. Fluorescence analysis with variable excitation wavelength was performed to find out the optimum wavelength of excitation and emission to use with compound **7** and showed that a maximum of fluorescence at 476 nm was reached upon excitation at 390 nm in PBS pH 7.4 (Fig. 4a). Conversely, azido-coumarin **5** showed very weak fluorescence when excited at this wavelength (Fig. 4b). The last step was the orthogonal ligation between aldehyde groups of goat anti-rabbit IgG generated by mild oxidation of the carbohydrate moieties with sodium periodate[22,39,40] and a large excess of coumarin-triazole-hydrazide **7** (30 equiv) in acetate buffer pH 5.5. After reductive amination with ethanolamine and $NaBH_3CN$ to block the remaining free aldehyde functions, the immunoconjugate **8a** was purified by extensive dialysis in PBS pH 7.4, ultrafiltration and size exclusion chromatography. The conjugation ratio, defined as the average number of coumarins conjugated per IgG, was estimated to be 2.4. It was evaluated by UV–vis spectroscopy at 280 and 390 nm, which are the maximal absorbance wavelengths (λ_{max}) of IgG and coumarin-triazole derivative, respectively. In the chosen reaction conditions employed for IgG oxidation (10 mM $NaIO_4$, pH 3.8, 1 h), an average number of four labelling sites are expected to be generated according to Wolfe et al.[40] Conjugate **8a** showed strong fluorescence emission at 476 nm upon excitation at 390 nm in PBS



Scheme 1. (a) TSTU, DMF, DIPEA, rt 2 h; (b) aminocaproic acid, $NaHCO_3$, dioxane/H_2O (1:1, v/v), rt 5 h; (c) TSTU, DMF/dioxane (2:1, v/v), DIPEA, rt, 1 h then $H_2NNHBoc$, H_2O, DIPEA, overnight, rt; (d) TFA/$CHCl_3$ (1:1, v/v), rt, 1.5 h.

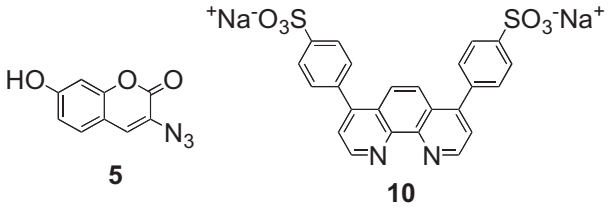
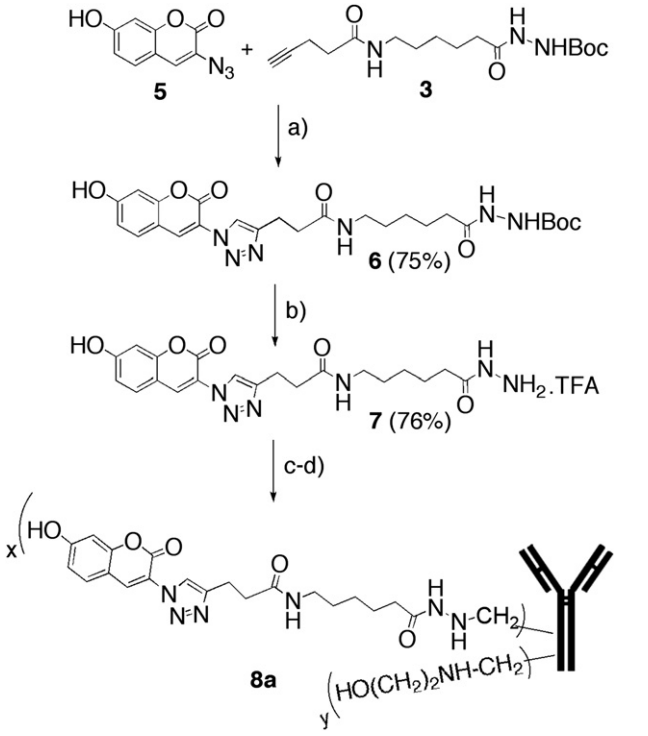


Fig. 3. Profluorescent 3-azido-7-hydroxycoumarin **5** and copper-binding ligand 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid disodium salt **10**.

pH 7.4 whereas excitation of commercial goat anti-rabbit IgG at 390 nm did not result in any fluorescence emission (Fig. 4c).

2.3. Preparation of fluorophore-IgG conjugate following path b of Scheme 1

In path **b**, goat anti-rabbit IgG was first oxidized with $NaIO_4$ to generate aldehyde groups and allowed to react with the deprotected bioorthogonal hydrazide–alkyne cross-linker **4** in acetate buffer pH 5.5 (Scheme 3). Subsequent treatments with ethanolamine and $NaBH_3CN$ as previously described (vide supra), followed by extensive dialysis and ultrafiltration, afforded IgG bearing the alkyne arm **9**. The reactive handle was further modified by the CuAAC reaction with the profluorescent 3-azido coumarin **5**. However, copper and ascorbate byproducts have been described to have potential side effects on biological molecules, such as aggregation or denaturation.[25,37,38,41] A way to circumvent these



Scheme 2. (a) $CuSO_4$, ascorbate, EtOH/H_2O (1:1), rt 48 h; (b) TFA/$CHCl_3$ (1:1, v/v), rt 2.5 h; (c) IgG/CHO, 0.1 M AcONa pH 5.5, rt 18 h; (d) ethanolamine·HCl, rt 2 h then $NaBH_3CN$, rt 2 h.

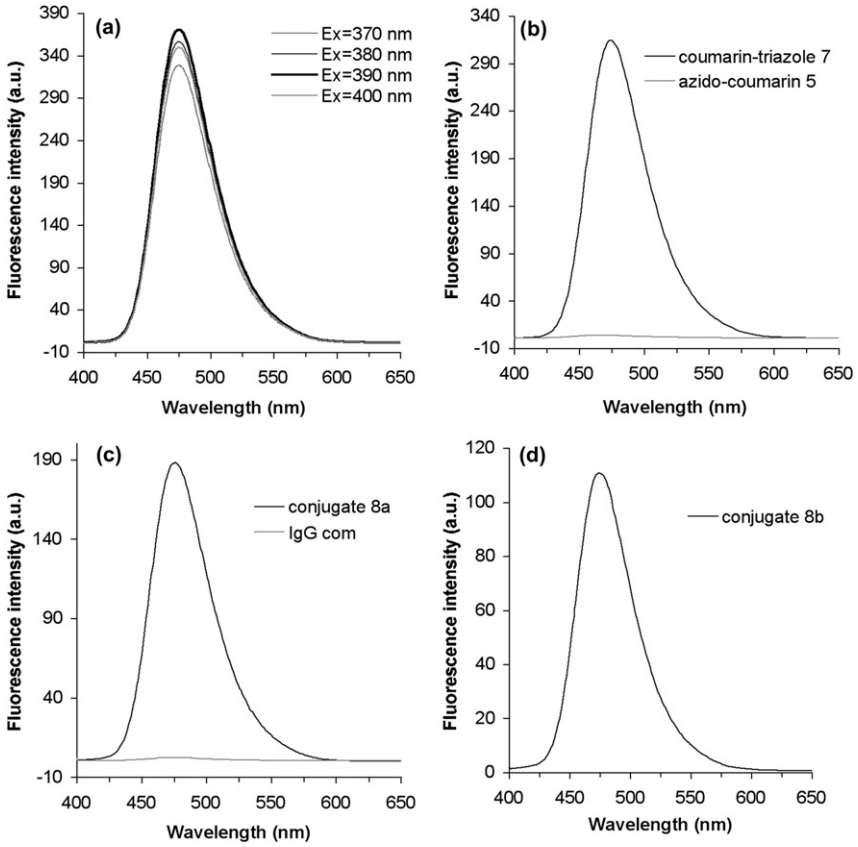
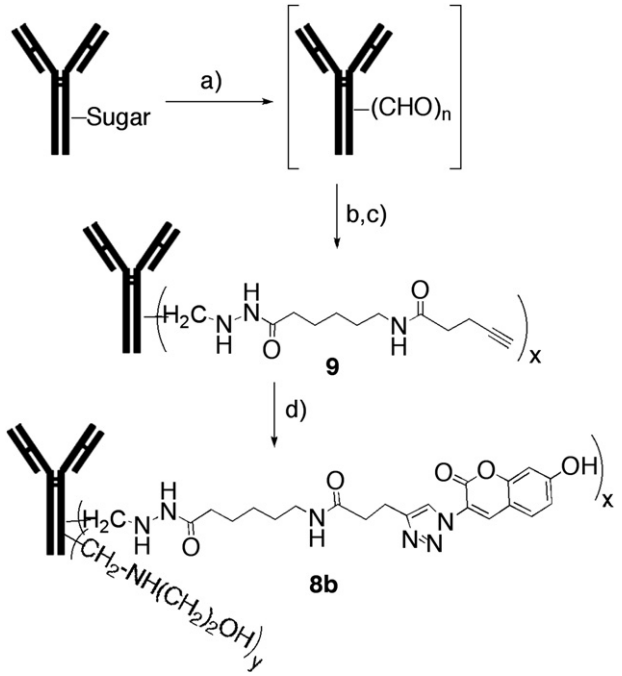


Fig. 4. (a) Superimposition of fluorescent emission spectra of coumarin-triazole **7** upon excitation at different wavelengths in PBS pH 7.4 at 20.0 °C. Fluorescent emission spectra (λ_{exc}=390 nm) recorded in PBS pH 7.4 at 20.0 °C of the following samples: (b) Coumarin-triazole **7** (black line) and azido-coumarin **5** (grey line) ([coumarin]=9.2 μM). (c) Conjugate **8a** ([coumarin]=9.2 μM, black line) and underivatized IgG (grey line). (d) Conjugate **8b** ([coumarin]=9.2 μM).



Scheme 3. (a) 10 mM $NaIO_4$, pH 3.7, rt 1 h; (b) **4**, 0.1 M AcONa pH 5.5, rt 18 h; (c) ethanolamine·HCl, rt 2 h then $NaBH_3CN$, rt 2 h; (d) **5**, $CuSO_4$, ascorbate, ligand **10**, 40 mM NaPB pH 7.8, 24 h.

drawbacks was the addition of a copper-binding ligand that kept the metal away from the protein and preserved the integrity of the biological molecule.[25,41] We chose the water-soluble sulfonated bathophenanthroline sodium salt **10** (Fig. 3) that was successfully added to the $CuSO_4$-ascorbate catalytic system for the ligation of proteins and virus scaffold.[41–43] As this complex was described as highly oxygen sensitive, the reaction was carried out under an inert atmosphere with degassed water and buffer. After purification by extensive dialysis in PBS pH 7.4, ultrafiltration and size exclusion chromatography, the resulting conjugate **8b** was analyzed by UV–vis spectroscopy and fluorescence. This time, the average number of coumarins conjugated per IgG was estimated to be 1.6 and a fluorescent emission signal was again observed at 476 nm upon excitation at 390 nm (Fig. 4d) confirming the formation of the triazole linkage and hence the covalent coupling to the alkyne handle of the IgG by click chemistry. However, a lower fluorescence intensity at 476 nm was observed for **8b** compared to **8a** although spectra were recorded in the same conditions of temperature, buffer and fluorophore concentration. This quenching can be attributed to different environmental factors around the fluorophores in the two conjugates.[44] Indeed, in path **a** (Scheme 1), the aldehyde–hydrazide ligation was performed with a bulkier azide derivative than in path **b**. Therefore, we can envisage that different aldehyde groups with different local environments may be involved in the conjugation.

2.4. Immunoreactivity

To investigate whether the CuAAC reaction affected the ability of the labelled antibody to recognize its antigen, fluorophore-IgG conjugates **8a** and **8b** were compared to underivatized IgG in an immunological assay to assess their relative affinity for rabbit IgG. To this end, a two-step immunoassay with a microplate coated-with rabbit IgG was set up. The first incubation step was performed by the addition of standard solutions of commercial

(underivatized) or coumarin-conjugated goat anti-rabbit IgG. Peroxidase-conjugated goat anti-rabbit IgG was incubated in a second step, and after addition of the enzyme substrate and colour development, immunoreactivities relative to the commercial IgG were calculated from the corresponding IC_{50} values (Fig. 5). Immunoconjugates **8a** and **8b** retained 80 and 55% of the immunoreactivity of the underivatized antibody, respectively. Firstly, this difference may be explained partly because immunoconjugate **8b**, obtained from path **b**, resulted from two consecutive reactions instead of one for **8a**. Secondly, antibody may be slightly sensitive to ascorbate byproducts known to potentially react with some amino acids side-chains leading to protein degradation, therefore affecting its concentration.[25,37,38,41] Nevertheless, in light of the immunoreactivity results obtained, we can assume that the $CuSO_4$-ligand bathophenanthroline-ascorbate catalytic system had only a slight effect on the coumarin-conjugated goat anti-rabbit IgG.

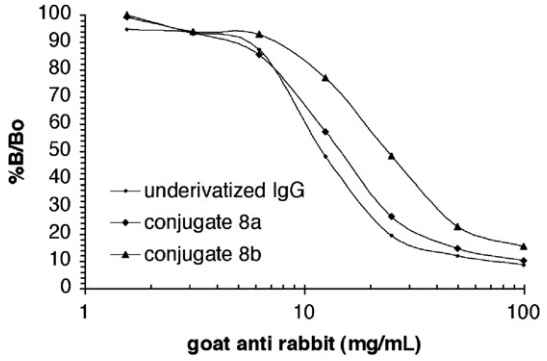


Fig. 5. Relative binding affinity of conjugates **8a** and **8b** compared to underivatized IgG on rabbit IgG-coated microplate.

3. Conclusion

In summary, we described the four-step synthesis of a new bioorthogonal cross-linker, derived from aminocaproic acid and carrying alkyne and hydrazide end groups, intended for conjugation between an aldehyde-containing molecule on the one hand and an azide derivative via CuAAC reaction on the other hand. The synthetic strategy is particularly attractive as it enables two paths for bioconjugation purposes depending on the stability of biological molecules to copper and sodium ascorbate. As a proof of concept, we studied the conjugation of an azido-profluorescent probe to immunoglobulin G. Oxidized IgG reacted with the fully deprotected cross-linker **4**, leading to site-selective addition of the 16 Å long functional handle with an alkyne end group. Subsequently, 3-azido-7-hydroxycoumarin was covalently linked via the CuAAC reaction. The occurrence of the cycloaddition was confirmed by the appearance of the fluorescence signal at 476 nm as a signature of the triazole ring. Labelling of goat anti-rabbit IgG was also undertaken following the other synthetic pathway where the CuAAC reaction was first performed to connect the probe of interest to the protected cross-linker **3**, followed by cleavage of the hydrazide protective group, and finally ligation of the hydrazide to oxidized IgG. Furthermore immunoreactivity studies showed that immunoglobulin G was mostly not affected by the conditions of the CuAAC reaction. For more sensitive biomolecules, the path involving the protected hydrazide that ends with the more friendly hydrazide–aldehyde coupling seems preferable. We have the feeling that this new cross-linker could be useful in the field of bioconjugation in conjunction with the increasingly popular copper(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction.

4. Experimental section

4.1. General

Pentynoic acid, *N*,*N*-diisopropylethylamine (DIPEA), *tert*-butyl carbazate, aminocaproic acid and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) were purchased from Sigma–Aldrich Co. Flash chromatography was performed on silica gel 60 (Merck, 40–63 mm). Dialysis was performed with Spectra/Por 4 membranes with a molecular weight cutoff of 12,000–14,000 Da (from Spectrum Laboratories, Inc.). Ultracentrifugation was performed using a Sorvall RC-5C plus (Thermo Scientific) superspeed centrifuge equipped with a SS-34 rotor using Centricon-30® centrifugal filter devices (30 kDa, Millipore). Size exclusion chromatographies were performed with HiTrap fast-desalting columns purchased from GE Healthcare. UV–vis spectra were recorded on a UV/mc^2 spectrometer (Safas, Monaco). NMR spectra were recorded on a BRUKER Avance 300 and a BRUKER Avance 400 spectrometers. Fluorescence spectra were recorded on a Jasco FP-6200 spectrofluorometer equipped with a temperature controller Jasco ETC-272T. Electrospray ionization (ESI) mass spectra were obtained on a API-3000 (Applied Biosystems, PE Sciex) coupled to an LC Agilent 1100 series. Chemical ionization (CI) mass spectra were obtained on a Focus GC/DSQ II (ThermoScientific).

4.2. Synthetic procedures

*4.2.1. N-Succinimidyl-4-pentynoate (**1**).* DIPEA (426 μL, 2.45 mmol) and TSTU (0.675 g, 2.24 mmol) were added to a solution of pentynoic acid (0.2 g, 2.04 mmol) in DMF (3.5 mL) under an Ar atmosphere. The reaction mixture was allowed to stir for 3 h at room temperature. NaCl (saturated aqueous solution, 30 mL) and water (10 mL) were added and the aqueous solution was extracted with EtOAc (3×50 mL). The organic layers were combined, washed with 1 M HCl (120 mL) and dried over Na_2SO_4. The crude product was further purified by flash chromatography on silica gel (CH_2Cl_2/EtOAc, 1:1) to afford compound **1** as a white solid (0.36 g, 91%). Mp 70 °C; R_f (EtOAc/CH_2Cl_2, 1:1, v/v) 0.75; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.0 (t, 1H, J=2.6 Hz), 2.55 (m, 2H), 2.77 (s, 4H), 2.82 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.0, 25.5, 30.2, 70.0, 80.9, 167.0, 169.0; m/z (CI/CH_4) 195.86 ([M+H]$^+$); Anal. Calcd for $C_9H_9NO_4$: C, 55.39%; H, 4.65%; N, 7.18%. Found: C, 55.36%; H, 4.75%; N, 7.31%.

*4.2.2. 6-(Pent-4-ynamido)hexanoic acid (**2**).* Aminocaproic acid (0.25 g, 1.9 mmol) was dissolved in an aqueous solution of $NaHCO_3$ (0.16 g, 1.9 mmol, 2.5 mL). Compound **1** in dioxane (3 mL) and H_2O (0.5 mL) was added and the reaction mixture was allowed to stir for 5 h at room temperature. EtOAc (50 mL) and H_2O (30 mL) were added. The aqueous layer was acidified with 6 M HCl, and extracted with EtOAc (3×50 mL). The organic layers were combined and dried over Na_2SO_4. The crude product was further purified by flash chromatography on silica gel (EtOAc/CH_2Cl_2, 8:2) to afford compound **2** as a white foamy solid (0.29 g, 72%). Mp 69 °C; R_f (EtOAc/CH_2Cl_2, 8:2, v/v) 0.3; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (m, 2H), 1.48 (m, 2H), 1.58 (m, 2H), 1.96 (t, 1H, J=2.5 Hz), 2.27 (t, 2H, J=7.2 Hz), 2.35 (m, 2H), 2.44 (m, 2H), 3.2 (q, 2H, J=6.5 Hz), 6.35 (s, NH), 10.2 (br s, CO_2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.0, 24.2, 26.2, 29.0, 33.9, 35.2, 39.4, 69.5, 82.9, 171.8, 178.2; m/z (CI/NH_3) 212.19 [M+H]$^+$, 229.19 [M+NH_4]$^+$; Anal. Calcd for $C_{11}H_{17}NO_3$: C, 62.54%; H, 8.11%; N, 6.63%. Found: C, 62.45%; H, 8.22%; N, 6.53%.

*4.2.3. tert-Butyl 2-(6-(pent-4-ynamido)hexanoyl)hydrazinecarboxylate (**3**).* Compound **2** (0.25 g, 1.18 mmol) was dissolved in DMF (4 mL) and dioxane (1.5 mL). DIPEA (0.25 mL, 1.42 mmol) and TSTU (0.43 g, 1.42 mmol) were added. The solution was stirred for 1 h at

room temperature and *tert*-butylcarbazate (0.2 g, 1.47 mmol), DIPEA (0.2 mL, 1.18 mmol) and water (1.4 mL) were added. The reaction mixture was stirred at room temperature overnight, diluted in EtOAc (50 mL) and saturated aqueous $NaHCO_3$ (40 mL). The aqueous layer was extracted with EtOAc (3×50 mL), the combined organic layers were washed with water (100 mL) and dried over Na_2SO_4. The crude product was further purified by flash chromatography on silica gel (EtOAc) to afford compound **3** as a colourless oil that crystallized on the bench into a white solid (0.31 g, 81%). Mp 109 °C; R_f (EtOAc) 0.2; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (m, 2H), 1.45 (s, 9H), 1.49 (m, 2H), 1.66 (m, 2H), 1.99 (t, 1H, J=2.5 Hz), 2.23 (t, 2H, J=7.2 Hz), 2.38 (m, 2H), 2.48 (m, 2H), 3.19 (q, 2H, J=6.0 Hz), 6.16 (NH), 6.71 (NH), 7.98 (NH); ^{13}C NMR (75 MHz, $CDCl_3$) 14.9, 24.6, 25.9, 28.2, 28.8, 33.6, 35.2, 39.1, 69.3, 81.7, 83.1, 155.7, 171.3, 172.8; (CI/NH_3) m/z 326.26 $[M+H]^+$, 343.18 $[M+NH_4]^+$; Anal. Calcd for $C_{16}H_{27}N_3O_4$: C, 59.06%; H, 8.36%; N, 12.91%. Found: C, 58.85%; H, 8.27%; N, 12.66%.

*4.2.4. N-(6-Hydrazinyl-6-oxohexyl)pent-4-ynamide TFA salt (**4**).* Hydrazide **3** (0.1 g, 0.3 mmol) was allowed to react with TFA (0.8 mL) in $CHCl_3$ (0.8 mL) for 1.5 h at room temperature. The mixture was concentrated under reduced pressure, $CHCl_3$ (5–10 mL) was added and the mixture was concentrated (five times) to yield a pasty residue. $CHCl_3$ (5 mL) was added and the mixture was left at 4 °C overnight. The resulting solid was triturated in cold $CHCl_3$, filtered off, rinsed with cold $CHCl_3$ and dried under vacuum to yield the bioorthogonal cross-linker TFA salt **4** as a beige solid (71 mg, 68%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.26 (m, 2H), 1.38 (m, 2H), 1.52 (m, 2H), 2.18 (t, 1H, J=7.5 Hz), 2.23 (m, 2H), 2.34 (m, 2H), 2.76 (t, 1H, J=2.5 Hz), 3.02 (q, 2H, J=6.4 Hz), 7.9 (br s, NH); ^{19}F NMR (282 MHz, DMSO-d_6) δ −73.73 (TFA salt); ^{13}C NMR (75 MHz, DMSO-d_6) δ 14.2, 24.3, 25.7, 28.7, 32.6, 34.1, 38.2, 71.2, 83.7, 170.0, 171.3; ESI-MS (ESI+) m/z 226.2 $[M+H]^+$, 248.4 $[M+Na]^+$, 451.4 $[2M+H]^+$, 473.5 $[2M+Na]^+$. Anal. Calcd for $C_{13}F_3H_{20}N_3O_4$: C, 46.02%; H, 5.94%; N, 12.38%. Found: C, 46.21%; H, 6.08%; N, 12.12%.

*4.2.5. CuAAC between tert-butyl 2-(6-(pent-4-ynamido)hexanoyl) hydrazinecarboxylate (**3**) and 3-azido-7-hydroxycoumarin (**5**).* To a mixture of alkyne spacer **3** (0.104 g, 0.32 mmol) and 3-azido-7-hydroxycoumarin **5** (65 mg, 0.32 mmol, prepared according to published procedure),[31] in EtOH/H_2O (7 mL, 50:50 v/v) was added a freshly prepared solution of sodium ascorbate (64 μL, 64 μmol, 1 M in H_2O) and $CuSO_4 \cdot 5H_2O$ (53.3 μL, 16 μmol, 7.5% in H_2O). The heterogeneous mixture was allowed to stir for 24 h at room temperature. EtOH was evaporated under vacuum and the residue was diluted in EtOAc (30 mL) and water (30 mL). The aqueous layer was extracted with EtOAc (3×40 mL), the combined organic layers were dried over Na_2SO_4. The crude product was further purified by flash chromatography on silica gel (EtOAc/MeOH, 92:8, v/v) to afford compound **6** as a pale yellow solid (0.127 g, 75%). Mp 114 °C; R_f (EtOAc/MeOH, 92:8, v/v) 0.3; ^{1}H NMR (300 MHz, CD_3OD) δ 1.35 (m, 2H), 1.44 (s, 9H), 1.46 (m, 2H), 1.63 (m, 2H), 2.20 (t, 2H, J=7.4 Hz), 2.6 (t, 2H, J=7.4 Hz), 3.06 (t, 2H, J=7.4 Hz), 3.17 (t, 2H, J=6.9 Hz), 6.76 (d, 1H, J=2.4 Hz), 6.85 (dd, 1H, J=8.5 and 2.2 Hz), 7.6 (d, 1H, J=8.7 Hz), 8.31 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 22.6, 26.1, 27.4, 28.6, 30.0, 34.6, 36.3, 40.2, 81.7, 103.4, 111.9, 115.6, 20.7, 124.3, 131.9, 136.8, 147.7, 156.4, 157.7, 158.1, 164.3, 174.4, 175.6; ESI-MS (ES^+) m/z 551.6 $[M+Na]^+$; Anal. Calcd for $C_{25}H_{32}N_6O_7$, 0.56 $CHCl_3$ (used to removed traces of EtOAc and MeOH by evaporation; whatever the solvent used, half equiv was recovered in the ^{1}H spectrum): C, 51.56%; H, 5.51%; N, 14.11%. Found: C, 51.31%; H, 5.52%; N, 13.85%.

*4.2.6. N-(6-Hydrazinyl-6-oxohexyl)-3-(1-(7-hydroxycoumarin-3-yl)-1H-1,2,3-triazol-4-yl) propanamide TFA salt (**7**).* Compound **6** (90 mg, 0.17 mmol) was allowed to react with TFA (1 mL) in $CHCl_3$ (1 mL) for 2.5 h at room temperature. The mixture was concentrated under reduced pressure, $CHCl_3$ (5–10 mL) was added and the mixture was concentrated (five times) to yield a pasty residue. $CHCl_3$ (5 mL) was added and the mixture was left at 4 °C overnight. The resulting solid was triturated in cold $CHCl_3$, filtered off, rinsed with cold $CHCl_3$ and dried under vacuum to yield the hydrazide **7** as a pale yellow solid (70 mg, 76%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.22 (m, 2H), 1.36 (m, 2H), 1.49 (m, 2H), 2.14 (t, 2H, J=7.0 Hz), 2.45 (t, 2H, J=7.5 Hz), 2.91 (t, 2H, J=7.5 Hz), 2.99 (q, 2H, J=5.9 Hz), 6.83 (d, 1H, J=1.9 Hz), 6.9 (dd, 1H, J=8.4 and 2.2 Hz), 7.72 (d, 1H, J=8.7 Hz), 7.89 (t, NH, J=5.3 Hz), 8.27 (s, 1H), 8.54 (s, 1H), 10,46 (br s, 1H), 10.99 (br s, 1H); ^{19}F NMR (282 MHz, DMSO-d_6) δ −73.71 (TFA salt); ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.2, 24.5, 25.9, 28.8, 32.8, 34.6, 38.3, 102.1, 110.3, 114.3, 119.4, 122.8, 130.9, 135.8, 146.3, 154.6, 156.3, 158.2 (q, J=31 Hz, C–CF_3), 162.4, 170.8, 171.7; ESI-MS (ES^+) m/z 429.4 $[M+H]^+$; HRMS (ES^+) m/z calculated for $C_{20}H_{25}N_6O_5$ $(M+H)^+$ 429.1886, observed 429.1875.

*4.2.7. Labelling of IgG following path **a** (conjugate **8a**).* $NaIO_4$ (0.1 M) in citric acid solution (10 mM, 24.7 μL) was added to a solution of goat anti-rabbit IgG (2 mg) in 0.01 M phosphate buffered saline pH 7.2 (142 μL) and the pH was adjusted to 3.7 with citric acid solution (10 mM, 80 μL). This solution was incubated for 1 h at room temperature in the dark then quickly purified on a 5 mL HiTrap fast-desalting column (50 mM AcONa+150 mM NaCl pH 5.0). Oxidized IgG solution was concentrated and transferred into 0.1 M AcONa pH 5.5 (350 μL) and allowed to react with hydrazide **7** (0.18 mg, 30 mol equiv) in DMSO (35 μL) at room temperature in the dark for 18 h. Ethanolamine·HCl (1 M in H_2O, pH 10.3, 60 μL) was added and the incubation was pursued for 2 h then 5 M $NaBH_3CN$ in 1 M NaOH (15 μL) was added and the incubation was continued for 2 h. The solution was dialyzed in PBS pH 7.4 at 4 °C for 48 h during which the buffer was changed every 12 h then transferred into a Centricon-30® centrifugal filter device and concentrated. The concentrate was reconstituted to 2 mL with PBS pH 7.4 and concentrated. This process was repeated twice, then the recovered solution was loaded on a HiTrap fast-desalting column and eluted with PBS pH 7.4. Fractions containing the labelled protein were concentrated using a Centricon-30® centrifugal filter device and analyzed by measuring absorbance at 280 and 390 nm. Absorbance measurement at 390 nm (ε=12,000 M^{-1} cm^{-1}) afforded the coumarin concentration and after subtraction of the contribution of the coumarin at 280 nm (ε=3300 M^{-1} cm^{-1}), the IgG concentration (A_{280}=1.4 for 1 mg/mL solution) was deduced. Fluorescence spectroscopy was performed in PBS pH 7.4 at 20.0 °C upon excitation at 390 nm.

*4.2.8. Labelling of IgG following path **b** (conjugate **8b**).* Goat anti-rabbit IgG (2 mg) was oxidized by $NaIO_4$ as described in path **a** and transferred into 0.1 M AcONa pH 5.5 (270 μL) and allowed to react with fully deprotected cross-linker **4** (0.09 mg, 30 mol equiv) in DMF (25 μL) at room temperature for 18 h. Ethanolamine·HCl (1 M in H_2O, pH 10.3, 60 μL) was added and the incubation was pursued for 2 h then 5 M $NaBH_3CN$ in 1 M NaOH (15 μL) was added and the incubation was continued for 2 h. The solution was dialyzed in PBS pH 7.4 at 4 °C for 4 days during which the buffer was changed every day then concentrated using a Centricon-30® centrifugal filter device and transferred into 40 mM NaPB pH 7.8. This solution containing conjugate **9** (500 μL) was put in a tube under an argon atmosphere with a gentle bubbling into the solution to remove oxygen from the buffer. This gentle bubbling was maintained throughout the reaction. 3-Azido-7-hydroxycoumarin **5** (54.13 μg, 0.266 μmol) was added then the following solutions prepared in degassed water: $CuSO_4$ (102.2 μg, 0.41 μmol, 100 μL) pre-mixed with bathophenanthroline sulfonated sodium salt (0.44 mg, 0.82 μmol, 100 μL) and sodium ascorbate (88 μg, 0.42 μmol, 100 μL).

The reaction mixture was incubated for 24 h at room temperature and dialyzed in PBS pH 7.4 at 4 °C for 48 h during which the buffer was changed every 12 h then concentrated using a Centricon-30® centrifugal filter device. The concentrate was reconstituted to 2 mL with PBS pH 7.4 and concentrated. This process was repeated twice, then the recovered solution was loaded on a HiTrap fast-desalting column and eluted with PBS pH 7.4. Fractions containing the labelled protein were concentrated using a Centricon-30® centrifugal filter device. The final solution was analyzed by measuring absorbance at 280 and 390 nm to determine the molar ratio of the probe to protein and by performing fluorescence spectroscopy upon excitation at 390 nm as explained for the conjugate **8a**.

4.3. Immunoreactivity of the antibody-fluorophore conjugates

Binding of antibody-fluorophore conjugates to rabbit IgG coated microplate was compared to the binding of unmodified goat anti-rabbit IgG in the following manner. A rabbit IgG solution (100 μL/well, 5 μg/mL in PBS pH 7.4) was pipetted in 96-well microtiter plate (Greiner, F-bottom) and left overnight at 4 °C. Nonspecific binding was further blocked by PBS+4% goat serum (200 μL/well). Goat anti-rabbit IgG or antibody-fluorophore conjugates **8a** or **8b**, dilutions ranging from 100 μg/mL to 0.39 μg/mL, were prepared in PBS pH 7.4 with 2% goat serum, applied to the wells in duplicate (100 μL/well) and incubated for 2 h at room temperature in the dark. Wells were washed four times with PBS+0.05% Tween 20, then goat anti-rabbit IgG-HRP conjugate diluted 1:8000 in PBS pH 7.4+2% goat serum was applied to the wells (100 μL/well) for 2 h at room temperature in the dark. Wells were washed as above, and a 0.7 mg/mL solution of *o*-phenylenediamine in citrate–phosphate buffer at pH 5.0+0.012% H_2O_2 (v/v) was applied to the wells (100 μL/well). The colour was developed for 10 min, and the enzymatic reaction was stopped by addition of 2.5 M H_2SO_4 (50 μL/well). The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad, Model 550).

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Supplementary data

HPLC-MS analysis and NMR spectra of compound **7** are provided. Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2012.09.062.

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