Clickable fluorophores for biological labeling—with or without copper†

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The synthesis of a set of new clickable fluorophores that virtually cover the whole visible spectrum reaching the near infra-red regime is presented herein. Besides dyes that are capable of participating in classical copper catalyzed 1,3-dipolar cycloaddition reactions with the counterparting function we have also prepared dyes containing a cyclooctyne moiety, an alkyne derivative that enables copper free clicking to azides. The suitability of these dyes for fluorescent labeling of biomolecules is presented by examples on model frameworks representing major biopolymer building blocks. The versatility of these dyes is presented in cell labeling experiments as well as by labeling the azide modified surface glycans of CHO-cells either by copper catalyzed or copper-free click reaction. These dyes are expected to have a large variety of applications in (bio)orthogonal labeling schemes both *in vivo* and *in vitro*.

Introduction

The *in vitro* and *in vivo* labeling of biomolecules by means of fluorescent tags represents an important tool in order to study complex biological processes often *via* imaging. The importance of fluorescent tags is due to the high sensitivity, the excellent spatial and temporal resolution and their potential for multi-channel imaging. The introduction of reporter tags rely on their selective and efficient reaction under physiological conditions with functional groups available at the biomolecule of interest. Amongst the known ligation reactions, so-called bioorthogonal chemical reporters have drawn much interest lately. The criteria for bioorthogonal reporters state that these reporter tags should be "non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes".[1a] In the bioorthogonal ligation toolkit the most valuable ones are: (a) the Staudinger ligation[1]; (b) the so-called click reaction represented by a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[2a,b]; (c) photoinduced dipolar cycloadditions of tetrazoles and alkenes[2c,d]; and (d) the inverse electron demand Diels–Alder reaction of tetrazines and strained alkenes.[2e,f] A recent review lists all these reactions in more details.[2g] Among these reaction types the introduction of fluorophore tags is synthetically most concise by a click reaction between azides and alkynes. The extreme rareness of azide and alkyne functions in biological systems further increases the importance of tagging by means of a copper catalyzed azide-alkyne cycloaddition. This reaction has been shown to be quite versatile in terms of biological applications.[3] CuAAC also finds widespread applications in high-throughput screening of libraries.[4] In cases where copper toxicity can be an obstacle in the ligation process, a strain-promoted cycloaddition between cyclooctynes and azides can be used as an alternative as proposed by the Bertozzi and Boons groups.[5,6] Very recently, we have presented our results on multiple labeling of biomolecules using a combination of copper free and copper mediated click chemistries in a sequential manner.[7]

Although the extreme sensitivity and the relatively easy detection modes of fluorescence make these techniques very appealing there has been little effort to develop clickable dyes for all colors present in the visible-NIR regime.[8] This had initiated our work presented herein to design clickable fluorophores that meet the criteria of bioorthogonality.

Results and discussion

The core part of the fluorescent tags **1–9** (Fig. 1) was selected on the basis of their emission bands *i.e.* to have a full coverage of the visible spectrum with edges at the near- infrared (NIR) region (Table 1). The incorporation of the clickable azido- or alkyne-functions was achieved by using appropriate halo-alkyl or propargyl amine derivatives. In the case of haloalkyl amine incorporation the halogenes (Br or I) were subsequently converted into the appropriate azido derivatives.

Once the syntheses of these novel dyes were accomplished we tested their applicability by ligating them to model frameworks.

Table 1 Photophysical properties of clickable fluorophores[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
$\lambda_{max(abs)}$	330	450	520	480	505	625	550	651	664
$\lambda_{max(em)}$	460	540	535	600	630	675	620	673	718
ε^b	0.34	0.68	6.3	4.7	5.7	1.8	7.0	7.3	10.0

[a] In methanol [b] $\times 10^4$ M^{-1} cm^{-1}.

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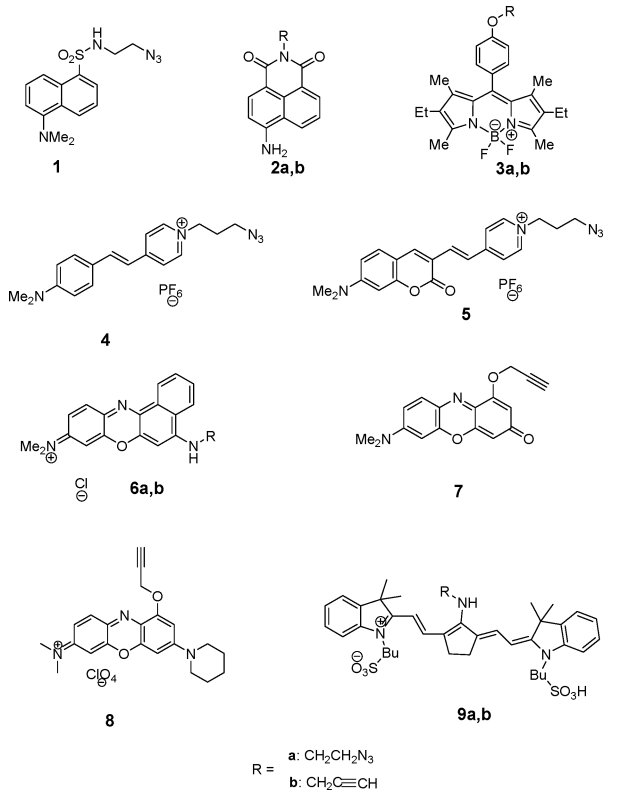


Fig. 1 Novel clickable fluorophores.

These model scaffolds **A**–**F** (Fig. 2) represent examples of major biological classes including sugars, amino acids, and nucleotides.

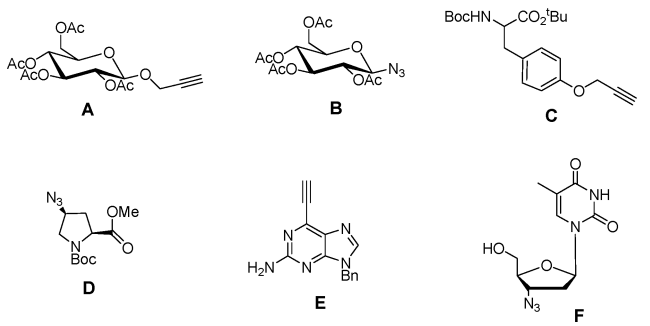


Fig. 2 Model frameworks for biological ligation.

Labeling experiments were carried out in an acetonitrile-water (50 v/v%) mixture in the presence of 10% CuI (10^{-3} M stock solution in DMSO and 10% triethylamine. In each case t.l.c. analyses revealed complete consumption of the starting materials. Purification of the products was carried out either by simple filtration of the precipitated products or by column chromatography. The fluorescently labeled biological building blocks were obtained in good to excellent yields providing further proof for the efficiency of CuAAC reaction in ligation processes (Table 2). As expected, the spectral properties of the dyes did not (or only slightly) changed upon conjugation.

Although fluorophores in the spectral region between the far red to the NIR region are particularly suitable for biological (both *in vitro* and *in vivo*) labeling as they are not interfered with by biological background luminescence, little effort has been made

Table 2 Isolated yields of representative ligation experiments[a]

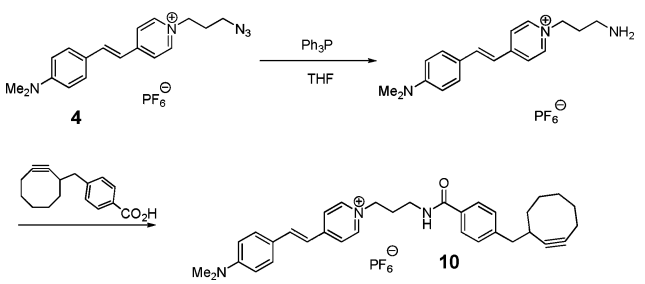
Conjugate	**1A**	**1C**	**2aA**	**3bD**	**4A**	**4C**	**5C**	**6aA**	**6aC**	**7B**	**8B**	**1E**	**11F**
Yield [%]	72	99	75	64	98	83	60	99	42	45	53	80	83

[a] In each case t.l.c. analysis showed complete consumption of starting materials.

to develop such (bio)orthogonal labels in this spectral regime. Nile Blue (**6**), oxazine (**8**) and the cyanine dye (**9**) derivatives are labels that are compatible with the 635-nm diode laser which is widely used in fluorescence instrumentation such as cell sorters and imagers.

Clickable dyes **4** and **5** are noteworthy for their large Stokes-shifts (120 and 125 nm, respectively). Dyes of this kind are exceptionally useful in Förster or fluorescence resonance electron transfer (FRET) applications as they do not interfere with the spectral bands of the second fluorophore, a common problem in FRET technology.

The introduction of strained cyclooctynes in azide-alkyne click reactions made it possible to overcome the limitations of the copper catalyzed process (*e.g.* copper toxicity). To extend our studies to the copper free version of click tagging we have designed and synthesized compounds that incorporate cyclooctyne functionality. Due to its relatively long wavelength emission and synthetic simplicity we have chosen the framework of **4** as a starting azide. Phosphine reduction of **4** (Staudinger-reduction)[9] furnished the appropriate amine which subsequently could easily be converted into a cyclooctyne derivative, **10**, using a cyclooctyne-carboxylic acid derivative (Scheme 1). Later, the cyclooctyne derivative (**11**) of compound **1** was also synthesized in a similar manner and its versatility was demonstrated by labeling the HIV drug **F**.[10,11]



Scheme 1 Synthesis of strained alkyne containing dye **10**.

Azido sugars have frequently been used for click-labeling of surface glycoproteines, recently.[8] We have adapted this system to demonstrate the ability of our dyes to undergo bioorthogonal labeling reactions, efficiently. Prior to labeling, Chinese hamster ovary (CHO) cells were incubated with **9b** and **10** to test the possible cytostatic effects of these dyes. Experimental results have shown that neither dye is toxic up to 50 μM concentration (see ESI†). Subsequently, CHO cells were treated with azidoacetylmannosamine (ManNAz) for 3 days.

The resulting cells bearing azido sialic acid on their surface glycoproteins were then fixed and treated with **9b** or **10**. In case of **9b**, $CuSO_4$ with sodium-ascorbate and tris-(benzyltriazolylmethyl)amine (TBTA)[12] were used to facilitate the reaction, whereas labeling with **10** did not require any further auxiliary reagents.

Results have shown that after 20 min reaction, both labels had efficiently tagged the modified cells, whilst cells not treated with ManNAz have shown only low intensity fluorescence as a result of non-specific binding of the dyes (Fig. 3, A–D).

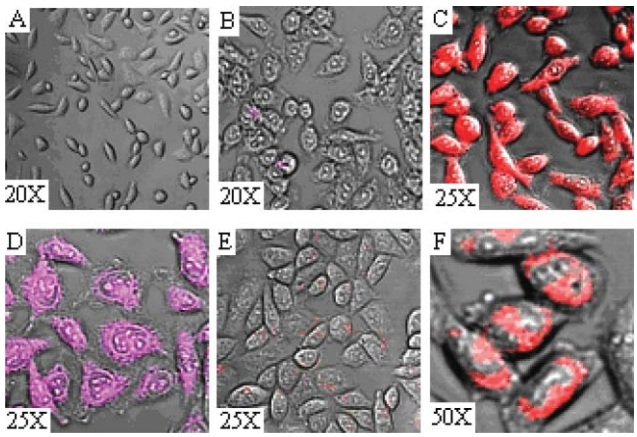


Fig. 3 Confocal luminescence images of fixed CHO cells treated with (A) PBS buffer, (B) **9b**/$CuSO_4$/ascorbate/TBTA, (C) ManNAz and **10**, (D) ManNAz and **9b**/$CuSO_4$/ascorbate/TBTA and native labeled CHO cells treated with (E) **10** and (F) ManNAz and **10**.

The toxic effects of Cu(I) during the labeling process had been demonstrated earlier[5b] and we also observed significant cell death when we attempted to label live cells with **9b** in the presence of a copper catalyst. On the other hand, as expected, the cyclooctyne bearing label (**10**) was found to be quite useful in live-cell tagging and efficient labeling was observed after 1 hour reaction time (Fig. 3, E–F).

Experimental

General

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka) and used without further purification. Analytical thin-layer chromatography (TLC) was performed on Polygram SIL G/UV 254 pre-coated plastic TLC plates with 0.25 mm silica gel from Macherey-Nagel + Co. Silica gel column chromatography was carried out with Flash silica gel (0.040–0.063 mm) from Merck. The NMR spectra were recorded on a Bruker DRX-250, Bruker DRX-300 or Varian Inova 600 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), quint (quintuplet), m (multiplet), dd (doublet of a doublet).

Syntheses of selected clickable labels‡

Synthesis of 1. Dansyl chloride (2.7 g, 10 mmol) and bromoethylamine hydrobromide (2.05 g, 10 mmol) was stirred at r.t. in 50 mL CH_2Cl_2 for 3 hours in the presence of Et_3N (2.79 mL, 20 mmol). The solvent was evaporated and the residue was dissolved in MeCN (50 mL). NaN_3 (1.6 g, 25 mmol) was added and the mixture was refluxed overnight. After cooling to r.t. the solvent was removed and the product was purified on silica using hexane/EtOAc 1:1 as eluent to afford the product as a greenish-yellow oil (2.26 g, 71%). ^1H ($CDCl_3$): δ = 2.89 (6H, s); 3.01–3.07 (2H, m); 3.27 (2H, t, J = 5.8 Hz); 5.08 (1H, t, J = 5.8 Hz); 7.20 (1H, d, J = 7.3 Hz); 7.52 (1H, dd, J = 7.3 Hz, J = 8.8 Hz); 7.59 (1H, dd, J = 7.3 Hz, J = 8.8 Hz); 8.24–8.28 (2H, m); 8.56 (1H, d, J = 8.8 Hz). ^{13}C ($CDCl_3$) δ = 42.2; 45.2; 50.7; 115.2; 118.4; 123.0; 128.5; 129.3; 129.7; 130.5; 144.4; 151.9; 171.1. IR: ν(neat) = 3290, 2940, 2832, 2787, 2099, 1572, 1309, 1140 cm^{-1}. HRMS (ESI) $[M + H]^+$ calcd. for $C_{14}H_{18}N_5O_2S$: 320,1181; found: 320,1178.

Synthesis of 2a. A mixture of 4-amino-1,8-naphthalic anhydride (0.91 g, 4 mmol) and ethanolamine (0.543 g, 8 mmol) were heated at 80 °C overnight in 10 mL DMF. After cooling, the solution was poured into 200 mL of ice-cold water. The precipitate was filtered, washed with water and acetone and dried *in vacuo.* The obtained product was then dissolved in DMF (10 mL), NaN_3 (756 mg, 11.4 mmol) was added to it and the reaction mixture was stirred at 80 °C for 24 h. The solvent was then removed *in vacuo* and the product was extracted with ethyl acetate, dried over Na_2SO_4, and purified by flash chromatography ($CHCl_3$). Yield: 825 mg, 69%. m.p. 214–216 °C. ^1H ($CDCl_3$) δ = 3.57 (2H, t, J = 6.2 Hz); 4.25 (2H, t, J = 6.0 Hz); 6.85 (1H, d, J = 8.4 Hz); 7.64 (1H, t, J = 7.7 Hz); 8.19 (1H, d, J = 8.4 Hz); 8.43 (1H, d, J = 7.4 Hz); 8.62 (1H, d, J = 7.7 Hz). Anal. Calcd. for $C_{14}H_{11}N_5O_2$: C: 59.87; H: 4.54; N: 23.85. Found: C: 59.96; H: 4.51; N: 23.74.

Synthesis of 4. N-(3-azido-propyl)-4-methyl-pyridinium-iodide (0.213 g, 0.701 mmol) and 4-(dimethylamino)-benzaldehyde (0.104 g, 0.701 mmol) were stirred in EtOH (40 mL) at 60 °C for 10 hours. The solvent was evaporated and the product was purified on silica (MeCN, then 1% NH_4PF_6 in MeCN). To the combined fractions was added excess NH_4PF_6 then the solvent was removed *in vacuo.* The remainder of the solid was suspended in water and filtered, washed with several portions of water and dried *in vacuo.* Red solid (0.138 g, 43%). m.p. = 144–146 °C. IR: ν(neat) = 1529; 1585; 1645; 2099; 2919; 3096 cm^{-1}. ^1H-NMR (DMSO): δ = 2.16 (2H, t, J = 6.0 Hz); 3.02 (6H, s); 3.46 (3H, s); 4.49 (2H, t, J = 6.0 Hz); 6.78 (2H, d, J = 7.9 Hz); 7.17 (1H, d, J = 16.3 Hz); 7.60 (2H, d, J = 8.4 Hz); 7.92 (1H, d, J = 16.3 Hz); 8.05 (2H, d, J = 5.5 Hz); 8.75 (2H, d, J = 5.8 Hz). ^{13}C-NMR (DMSO) : δ = 29.8; 40.0; 48.0; 57.2; 112.3; 117.4; 122.7; 122.8; 130.6; 142.6; 143.9; 152.3; 154.2. HRMS (ESI) $[M]^+$ calcd. for $C_{18}H_{22}N_5^+$: 308.1870; found: 308.1866.

Synthesis of 5. 7-(Diethylamino)-2-oxo-2H-chromene-3-carbaldehyde (0.42 g, 1.71 mmol) and N-(3-azido-propyl)-4-methyl-pyridinium-iodide (0.55 g, 1.71 mmol) were refluxed for 3 hours in ethanol (20 mL) in the presence of 5 drops of piperidine. After cooling to room temperature the solvent was removed and the product was purified on a column (SiO_2) using acetonitrile as eluent. The product was dissolved in acetonitrile, and an excess of NH_4PF_6 was added. After solvent removal, the purple crystalline product was suspended in water and filtered. The solid was washed several times with water and dried *in vacuo* to give the title compound as a purple crystalline solid containing one molecule of water (0.4 g, 41%). M.p.: 200–201 °C. IR (neat) ν = 1416, 1503, 1570, 1712, 2097, 2978, 3069 cm^{-1}. ^1H NMR

‡ Synthetic procedures for the dyes not listed in the experimental section can be found in the ESI.

(DMSO-d_6)): δ = 1.14 (6H, t, J = 6.9 Hz); 2.18 (2H, quint. J = 13.4 Hz); 3.40 - 3.57 (6H, m); 4.52 (2H, t, J = 7.1 Hz); 6.58 (1H, d, J = 1.9 Hz); 6.78 (1H, dd, J = 1.9 Hz, J = 9.0 Hz); 7.53 (1H, d, J = 9.0 Hz); 7.66 and 7.82 (2H, AB, J = 16.1 Hz); 8.13 - 8.21 (3H, m); 8.85 (2H, d, J = 6.9 Hz). ^{13}C NMR (DMSO-d_6): δ = 12.3; 28.4; 44.3; 47.5; 57.2; 96.1; 110.0; 113.6; 122.5; 123.2; 130,7; 137.0; 144.0; 145.4; 151.9; 153.4; 156.3; 159.5. Anal. calcd. for $C_{23}H_{28}F_6N_5O_3P$; C: 48.68; H: 4.97; N: 12.34 Found: C: 48,58; H: 4.80; N: 12.45.

Synthesis of 6a. This was obtained by condensation of 5-dimethylamino-2-nitrosophenol[13] hydrochloride with (3-azidopropyl)-1-naphthylamine[14] in 10 mL acidic ethanol. Specifically, to a cold solution (ice bath) of 5-dimethylamino-2-nitrosophenol hydrochloride (610 mg, 3 mmol) in ethanol (10 mL), (3-azidopropyl)-1-naphthylamine (658 mg, 2.91 mmol) and concentrated hydrochloride acid (0.25 mL) were added. The mixture was refluxed for about 9 hours and monitored by TLC (silica: chloroform and chloroform–methanol, 6:1). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (silica: chloroform–methanol, 5.5:0.5). N-[5-(3-Azidopropylamino)-9H-benzo[a]-phenoxazin-9-ylidene]-N-methyl-methanaminium chloride (**6a**) was obtained as a blue solid. Yield: 710 mg, 65%. m.p. 184–186 °C. ^{1}H NMR (CD_3Cl): δ = 2.03-2.18 (m, 2H,); 3.20 (s, 6H); 3.54 (t, J = 6.9 Hz, 2H); 3.84 (t, J = 6.9 Hz, 2H); 6.61 (d, J = 2.5 Hz, 1H), 6.72 (s, 1H); 6.97 (dd, J = 2.2 Hz, J = 2.2 Hz, 1H); 7.76 (s, 1H); 7.79 (s, 1H); 7.85 (m, 1H); 8.80 (t, J = 4.6 Hz, 1H); 9.40 (d, J = 8.0 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ = 27.1; 30.2; 42.6; 45.8; 99.0; 118.1; 120.6; 120.7; 121.3; 122.2; 122.6; 126.5; 126.9; 127.1; 129.3; 132.9; 147.0; 147.8; 148.6; 164.4. HRMS (PI-LSI) calcd. for $C_{21}H_{21}N_6O$ [M^+]: 373.1777; found: 373.1770.

Synthesis of 8. 5-(Piperidin-1-yl)benzene-1,3-diol[15] (0.5 g, 2.6 mmol) was refluxed with propargyl bromide (0.23 mL, 2.6 mmol) in THF for 2 days. 360 mg of K_2CO_3 was used as base. After cooling, the reaction was filtered, concentrated on a rotavapor, and then purified by column to get a colorless oil. Yield: 0.21 g, 35%. ^{1}H-NMR (DMSO-d6): δ = 1.80-1.62 (6H, m); 2.50 (1H, t, J = 2.5 Hz); 3.12-3.04 (4H, m); 4.53 (2H, d, J = 2.5 Hz); 5.23 (1H, t, J = 1.1 Hz); 5.83 (1H, t, J = 1.9 Hz); 5.96 (1H, t, J = 1.9 Hz); 6.01 (1H, t, J = 1.9 Hz). This product (0.2 g, 0.86 mmol) was further reacted with p-nitroso-N,N-dimethylaniline (0.19 g, 1.3 mmol) and 0.14 mL (1.3 mmol) of perchloric acid. The reaction was carried out in ethanol at 70 °C for 2 hours. The crude product was recrystallized in ethanol to yield dark blue crystals. Yield: 0.12 g, 30%. ^{1}-H-NMR (acetone-d6) δ = 1.78–1-87 (6H, m); 3.35 (1H, t, J = 2.5 Hz); 3.46 (6H, s); 3.97–4.05 (4H, m); 5.19 (2H, d, J = 2.5 Hz); 6.84 (1H, d, J = 2.7 Hz); 6.89 (1H, d, J = 2.5 Hz); 6.89 (1H, d, J = 2.5 Hz); 7.35 (1H, dd, J = 2.7 Hz, J = 9.6 Hz); 7.79 (1H, d, J = 9.6 Hz). IR (neat) ν = 3266, 2921, 2859, 1651, 1540, 1487, 1487 cm^{-1}. HRMS (PI-EI) calcd. for $C_{22}H_{24}N_3O_2^+$ [M^+]: 362.1863; found: 362.1877.

Synthesis of 9b. IR-806 (Sigma-Aldrich) (0.1 g, 0.136 mmol), propargylamine (0.009 mg, 0.16 mmol) and triethylamine (0.016 mg, 0.16 mmol) in DMF (5 mL) were stirred in the dark for 24 hours. The solution was poured into methyl-*t*-butyl ether (MTBE) (50 mL) and the precipitated product was filtered and washed with several portions of MTBE (0.10 g, 97%). M.p.: 93–95 °C. IR (neat) ν = 3201, 2925, 1666, 1515, 1453 cm^{-1}. ^{1}H NMR (DMSO): δ = 1.64 (12H, s); 1.69–1.79 (8H, m); 2.50–2.52 (2H, m); 2.74 (3H, s); 3.34 (4H, s); 3.69–3.71 (1H, m); 3.92–4.00 (4H, m); 4.42–4.50 (2H, m); 5.67 (2H, d, J = 12.4 Hz); 7.06 (2H, t, J = 7.7 Hz); 7.18 (2H, d, J = 8.1 Hz); 7.28 (2H, t, J = 7.7 Hz); 7.45 (2H, d, J = 7.2 Hz); 7.92–7.93 (2H, m). ^{13}C NMR: δ = 22.5; 25.3; 25.9; 27.5; 28.1; 35.1; 40.0; 42.3; 47.1; 50.8; 76.9; 80.4; 96.8; 109.2; 121.9; 122.5; 126.0; 128.0; 139.9; 142.7; 163.8; 166.7. HRMS (ESI) calcd. for $C_{40}H_{48}N_3O_6S_2^-$ [$M]^-$: 730.2990, found: 730.2978.

Synthesis of 10. Compound **4** (50 mg, 0,11 mmol) and support bound triphenylphosphine (100 mg, 3 mmol/g) was stirred in dichloromethane-water (9/1 v/v%) for 24 hours, until all the starting materials were reduced. The reaction mixture was filtered through Celite and the filtrate was concentrated *in vacuo* (43 mg, 91%). This intermediate was used subsequently without further purification and characterization. The product of the previous reaction was dissolved in acetonitrile (10 mL) and HBtU (35 mg, 0.0936 mmol), HOBt·H_2O (16 mg, 0.103 mmol) and CyOCOOH (25 mg, 0.103 mmol) were added together with 36 μL of N,N-diisopropylethylamine. The solution was stirred for 14 hours, and then concentrated in vacuo. The product was purified on a column (SiO_2, acetonitrile) to give compound **10** as a red solid (35 mg, 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.38-1.41 (1H, m); 1.53–1.61 (1H, m); 1.71–1.83 (3H, m); 1.84–2.01 (3H, m); 2.06–2.14 (2H, m); 2.28 (2H, m); 2.52–2.61 (1H, m); 2.62–2.71 (2H, m); 3.03 (6H, s); 3.50 (2H, m); 4.38 (2H, m); 6.68 (2H, d, J = 7.9 Hz); 6.76 (1H, d, J = 15.8 Hz); 7.02 (1H, bs); 7.22 (2H, d, J = 6.6 Hz); 7.43–7.50 (3H, m); 7.64 (2H, d, J = 4.8 Hz); 7.70 (2H, d, J = 6.6 Hz); 8.36 (2H, d, J = 4.8 Hz). ^{13}C NMR: δ = 23.5; 31.0; 32.6; 33.3; 37.4; 39.1; 39.2; 42.7; 42.9; 44.4; 60.8; 97.5; 99.1; 114.9; 119.0; 125.2; 129.7; 131.8; 133.3; 133.9; 145.4; 145.6; 147.2; 154.8; 157.0; 170.9; 170.7. HRMS (ESI) calcd. for $C_{34}H_{40}N_3O^+$ [M^+]: 506.3171; found: 506.3176.

Synthesis of 11. Compound **1** (0.133 g, 0.41 mmol) was mixed with support bound triphenylphospine (0.35 g, 3 mmol/g) in CH_2Cl_2 (1 mL). After being stirred at r.t. for 2 hours, water (0.5 mL) was added and the mixture was further stirred at r.t. for 16 h then filtered through Celite. The solid was washed thoroughly with CH_2Cl_2 and the filtrate was concentrated *in vacuo* to give 0.111 g of the desired product. The product (0.02 g, 0.068 mmol) was dissolved in acetonitrile (5 mL) and CyOCOOH (0.018 g, 0.075 mmol), HBTU (0.027 g, 0.682 mmol), HOBt (0.011 g, 0.075 mmol) and N,N-diisopropylethylamine (24 μL, 0.136 mmol) were added. The solution was stirred at r.t. for 24 h then concentrated. The product was purified on silica (eluent: hexane : EtOAc, 1:1 v/v%) to give compound **11** (30 mg, 86%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.29–1.41 (2H, m); 1.47–1.59 (1H, m); 1.62–1.94 (5H, m); 2.05–2.11 (2H, m); 2.54–2.67 (3H, m); 2.78 (6H, s); 3.03 (2H, dd, J = 6.0 Hz, J = 10.4 Hz); 3.38 (2H, dd, J = 6.0 Hz, J = 10.4 Hz); 5.95 (1H, t, J = 6.0 Hz); 6.81 (1H, t, J = 5.5 Hz); 7.04 (1H, d, J = 7.1 Hz); 7.08 (2H, d, J = 8.2 Hz); 7.34–7.43 (2H, m); 7.48 (2H, d, J = 8.4 Hz); 8.14 (1H, dd, J = 1.1 Hz, J = 7.3 Hz); 8.19 (1H, d, J = 8.7 Hz); 8.43 (1H, d, J = 8.5 Hz). ^{13}C NMR: δ = 22.6; 28.4; 29.9; 34.7; 36.5; 39.7; 40.1; 41.6; 43.0; 45.3; 94.8; 96.3; 115.2; 118.5; 123.1; 127.0; 128.5; 128.9; 129.4; 129.5; 129.8; 130.5, 131.5; 134.3; 144.1, 151.9; 168.0. HRMS (ESI) calcd. for $C_{30}H_{36}N_3O_3S$ [M + H]$^+$: 518.2477; found: 518.2471.

Preparation of fluorescent adducts

General procedure for the synthesis of adducts. Azido- or alkyne bearing fluorophores **1–11** (1 eq.) and modified building blocks **A**–**F** (1.1 eq.) were stirred in an acetonitrile-water (50 v/v%) mixture at r.t. in the presence of 10% CuI and triethylamine 20% for 16 hours. Products were purified either by simple filtration or column chromatography on silica. Characterization data of the fluorescent ligates can be found in the ESI.†

Fluorescent labeling of CHO cells

Chinese hamster ovary (CHO) cells were incubated for 2 days in a ManNAz containing medium. The cells bearing azido-sialic acid on their surface glycans were then fixed or directly treated with the appropriate labels and auxiliaries where needed. Labeled cells were then studied under a fluorescent confocal microscope. For a detailed experimental set up please refer to the ESI.†

Conclusions

In conclusion, the set of clickable fluorophores presented here is suitable for fluorescent labeling of biomolecules. These tagging molecules have moderate to high quantum yields (0.1 to 0.6), and this is best documented by the brilliant contrast of the microscopy images. These dyes are expected to have a large variety of applications in (bio)orthogonal labeling schemes both *in vivo* and *in vitro.* Besides the (cyclo)alkyne function, installation of additional functional groups *e.g.* maleimides, amines *etc.* can lead to further tools for tagging other desirable reactive groups.

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