Synthesis and application of bioorthogonal probes suitable for two-point binding labeling schemes

Theses of the PhD dissertation

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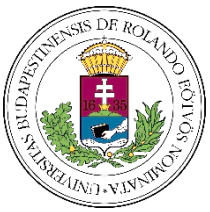

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Introduction

Chemical biology[1] is a discipline spanning in the fields of chemistry and biology. Being one of the most important interfacial sciences developed in the past decades, chemical biology relies on not only the toolbox of classical organic chemistry but also the state-of-the-art results of biotechnology and bioinformatics.

Fluorescence is one of the key elements of chemical biology. The combination of bioorthogonal ligation with fluorescence-based detection resulted in the emergence of a powerful labeling technique that enables the monitoring of biopolymers or cellular events.[2] Bioorthogonal chemistry allows the covalent modification of biomolecules through biocompatible, non-natural cognate functions (chemical reporters), while fluorescent labeling ensures good temporal and spatial resolutions with outstanding sensitivity.

Since the extant collection of fluorescent probes is built on a small set of well-known scaffolds, in the field of fluorophores the challenge lies not in developing new scaffolds but in enhancing the efficiency of labeling with fine-tuned dyes.[3] For example, labeling efficiency could be facilitated by minimizing the background fluorescence, a limiting factor for fluorescence detection. The source of background fluorescence is the presence of unreacted or non-specifically reacted probes, and it can be avoided by the application of so-called fluorogenic dyes. These labels have the unique feature of having low or zero emission and becoming fluorescent only upon reaction.

Another advantageous feature is selective and site-specific labeling, this can be adequately addressed with the use of self-labeling peptide tags, for example, tetracysteine motifs. These motifs can be selectively targeted with biarsenicals such as FlAsH, ReAsH or AsCy3.[4-5] These membrane permeable fluorogenic dyes have found broad applications. However, the considerable background labeling as a result of the interaction with competing non-specific thiols and the necessity of reducing agents to ensure the presence of reactive SH-groups limit the full potential that lies in the presumably remarkable sensitivity with these biarsenicals.

Research aim

My PhD research aimed at the development of fluorescent labeling schemes that combine the robustness of bioorthogonal chemistry with the two-point binding selectivity of biarsenicals.

The core concept was to modify various fluorescent scaffolds with two azide moieties. The presence of the azide groups plays a dual role:

- since dyes containing a single azide moiety are proved to possess good fluorogenic behaviour, we presumed that the instalment of two azide moieties results in more efficient fluorogenicity or even completely quenched fluorescence;
- these bisazides can participate in two strain-promoted azide-alkyne cycloaddition (SPAAC) with short peptide sequences that are modified with two cyclooctyne motifs as possible self-labeling tags;
- considering that one azide group still significantly lowers the fluorescence, bisazide dyes can only reach their maxima in fluorescence intensity if both azide groups participated in a SPAAC, thus assuring high selectivity through two-point binding

Consequently, the research plan included:

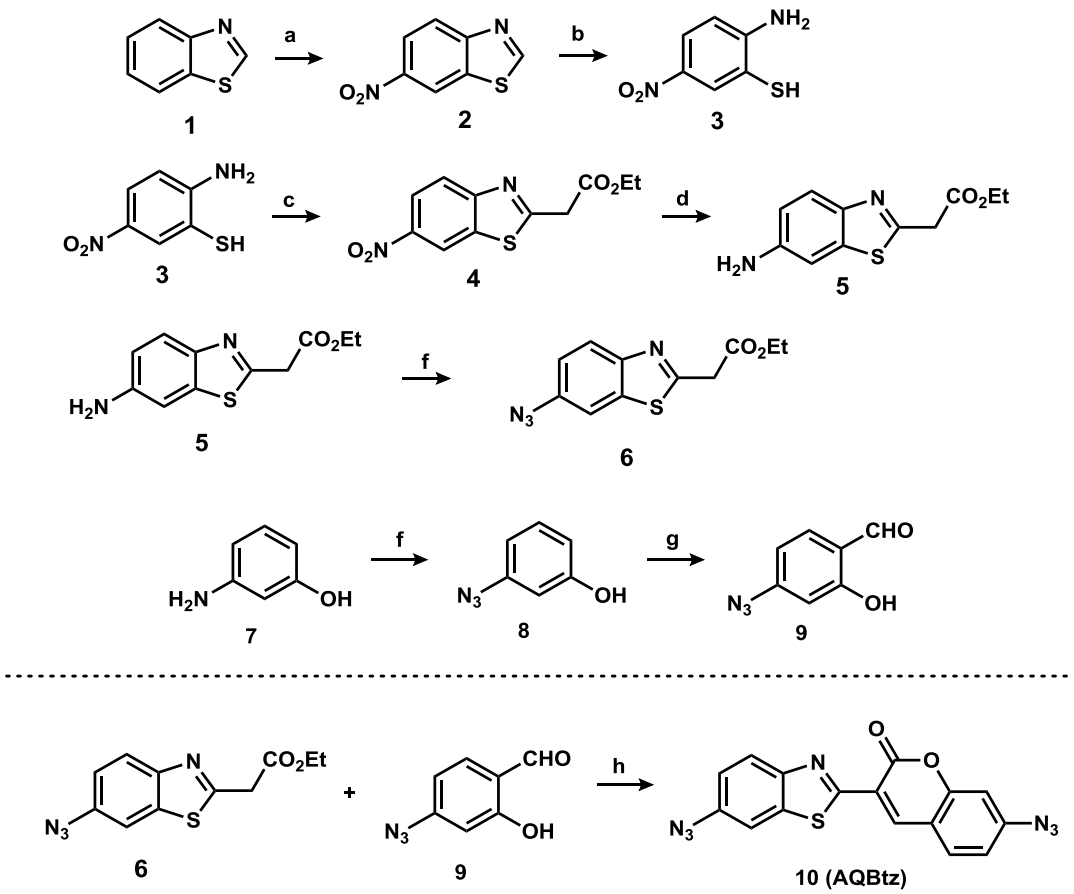
1. Synthesis of bisazide dyes with various scaffolds and measuring their main photophysical parameters
2. testing the concept and biological applicability of bioorthogonal two-point tagging labeling schemes

Results

The main results and conclusions can be summarized as follows.

1. The synthesis of AQBtz

To reach our aim we successfully synthesized benzothyazolyl-coumarine derivative AQBtz. (Scheme 1).



Scheme 1. Synthesis of probe **AQBtz** . (*a*) HNO_3/H_2SO_4, 0 °C, 2 h, 60 %; (*b*) $N_2H_4.H_2O$, MeOH, rt, 12 h, 96 %; (*c*) ethyl-cyanoacetate, 130 °C, N_2, 24 h, 41 %; (*d*) Fe/NH_4Cl, EtOH/H_2O, 60 °C, 3 h, 66 %; (*e*) 1. ^{t}BuONO, MeCN, 0 °C, 30 min; 2. TMS-N_3, N_2, rt, dark, ~77%; (*f*) 1. $NaNO_2$, HCl, 0-5 °C; 2. NaN_3, H_2O, 24 h, rt, dark, 87 %; (*g*) $(CH_2O)_n$, Et_3N, $MgCl_2$, MeCN, Ar, 70 °C, 12 h, dark, 50-80 %; (*h*) piperidine, EtOH, 55 °C, dark, 60 %.

2. AQBtz: fluorogenic properties and two-point binding experiments

After the successful synthesis of AQBtz we tested its main photophysical properties and stated that it shows practically no emission, proving the presumption about the remarkable quenching effect that lies with two azide moieties on the same fluorescent core to be correct.

We tested the fluorogenic properties of AQBtz by studying its reaction with cycloctyne BCN, a process that resulted in a 140-fold increase in fluorescence intensity compared to a control sample that contained no BCN but was stirred for 3 hours under the same conditions.

In order to test the two-point binding of AQBtz to bis-cyclooctynylated peptide targets, we have designed short peptide tags bearing two BCN units at different distances (synthesized by *Dr*.

Gábor Mező). The reaction of AQBtz with these model peptides resulted in cyclic, fluorescent conjugates (Figure 1.). We demonstrated that the fluorescence enhancement values, depending on the sequence of the peptide tag, were 130, 27 or 89.

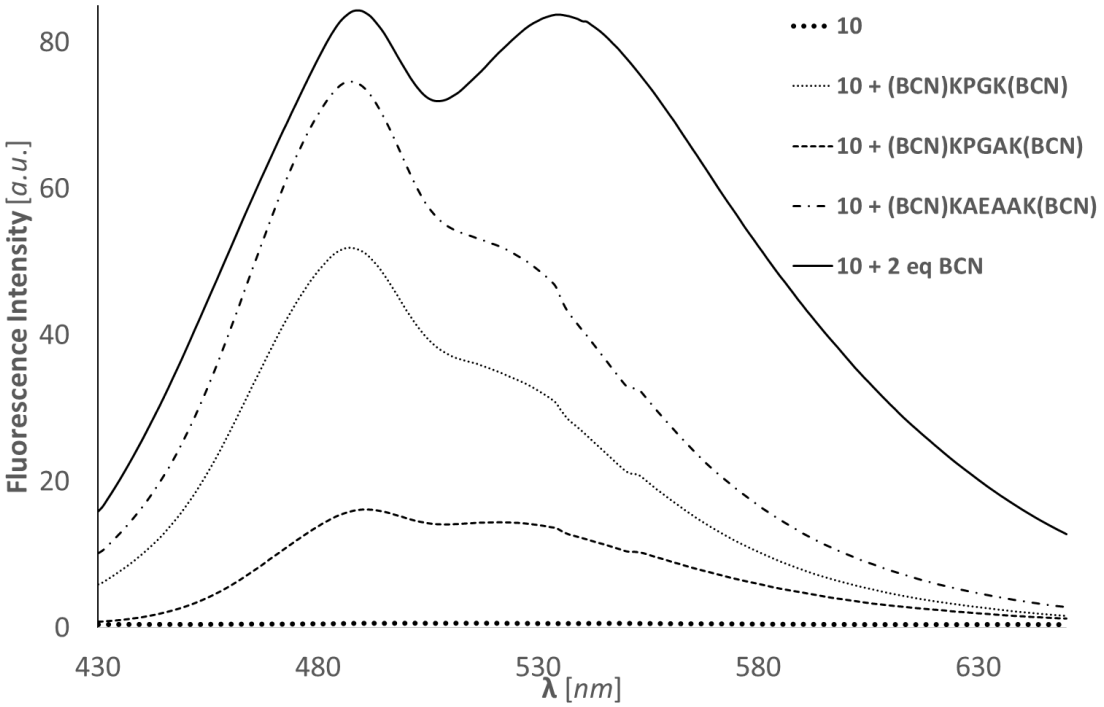


Figure 1. Fluorescent spectra of AQBz conjugates. (λ_{exc} = 410 nm, c = 0.25 μM in PBS, pH= 7.4).

These findings were supported by theoretical investigations to reveal that the degrees of rotation about the C-C bond between the benzothiazole and coumarin cores are different for the various probe-peptide cyclic conjugates. The deviation from the optimal 180° of the dihedral angle formed by the two rings explains the different fluorogenic performances as this gives rise to non-planar arrangement of the two units, leading to less fluorescent species. To quantify the average deviation from the planarity for the various conjugates we considered the conformers which are available at room-temperature and calculated their average dihedral angles (*Dr. Mihály Kállay*). The calculated angles correlate well with the observed fluorescence intensities, as listed in Table 1.

conjugate	dihedral angles for a and b structures (°)[a]	fluorescence enhancement[b]
10-BCN_2	2	140
10-(BCN)KPGK(BCN)	31 and 73	89
10-(BCN)KPGAK(BCN)	59 and 58	27
10-(BCN)KAEAAK(BCN)	**8 and 45**	**130**

Table 1. Dihedral angles and fluorescence enhancement values of conjugates

We were curious how the individual azide groups contribute to the quenching effect, thus we synthesized monoazide-monotriazole congeners **11** and **12**. We found that these monotriazoles had initial, intrinsic fluorescence, giving rise to moderate fluorescence enhancement values following reaction with 1 eq. of BCN (11 and 11, respectively). Similarly, the previously best performing (BCN)KAEAAK(BCN) sequence also resulted only modest enhancements in fluorescence intensities (4 and 7 for **11** and **12**, respectively). As a further attempt to understand the role of the respective azide groups we devised compounds **13** and **14** to elaborate their role in the total fluorogenicity (*Anna Eszter Fodor*, BSc Thesis). We found that monoazides **13** and **14** showed similarly moderate, 5.5 and 5-fold enhancements, respectively, thus we stated that in the complete quenching effect the presence of both azide groups is necessary as their effect is multiplicative.

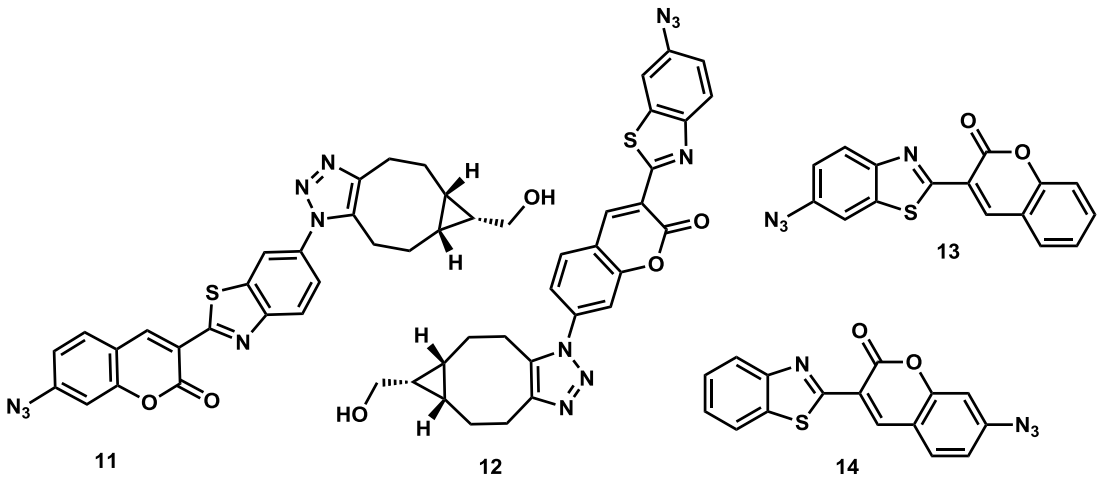


Figure 2. Compounds **11** – **14**.

3. Cyanine bisazides: synthesis

Since AQBtz was UV-excitable, thus not fully compatible with *in vivo* labeling schemes, in the second half of our research we set forth a study that aims the design, synthesis and fluorogenic

characterization of further bisazide dyes based on the cyanine scaffold. We synthesized five cyanine bisazides (**15** – **19,** Figure 3**,** syntheses: schemes 2-3**; 15** and **16** by *Dr. Attila Kormos*).

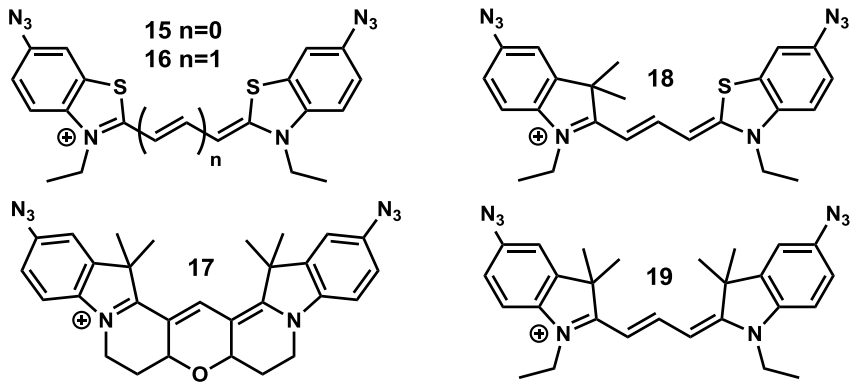
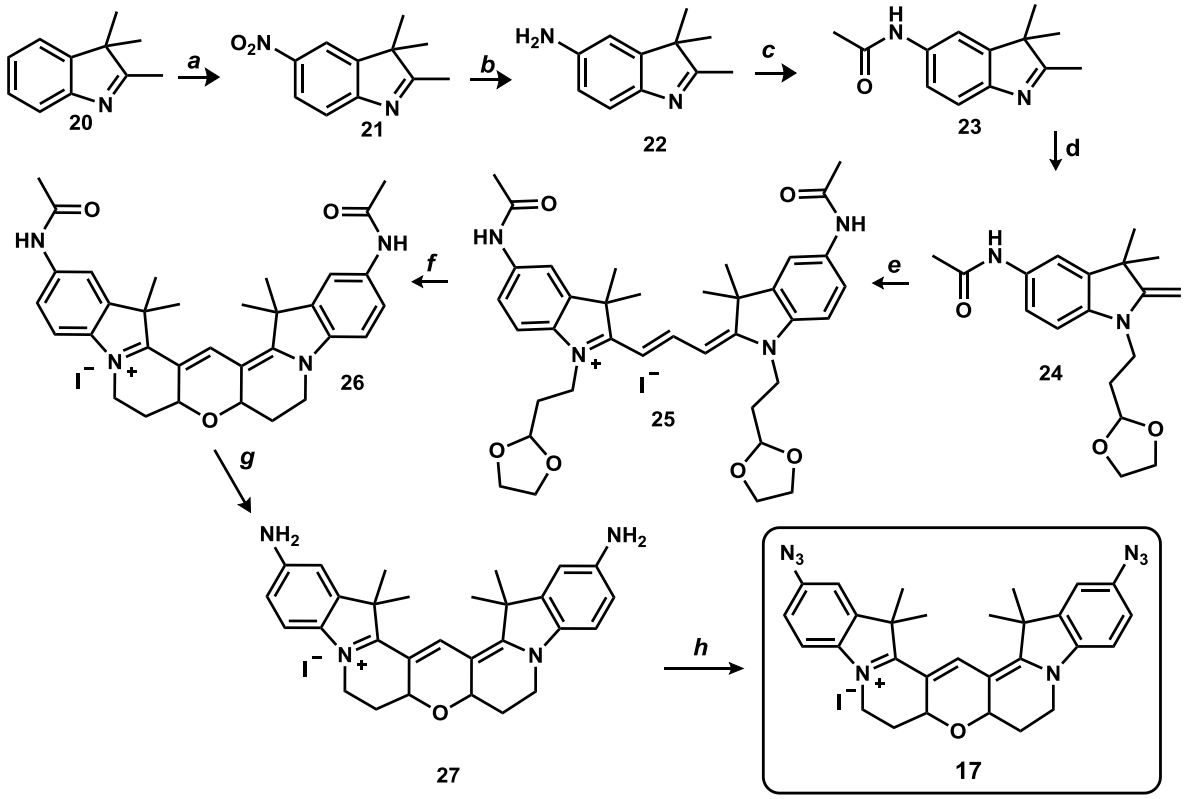
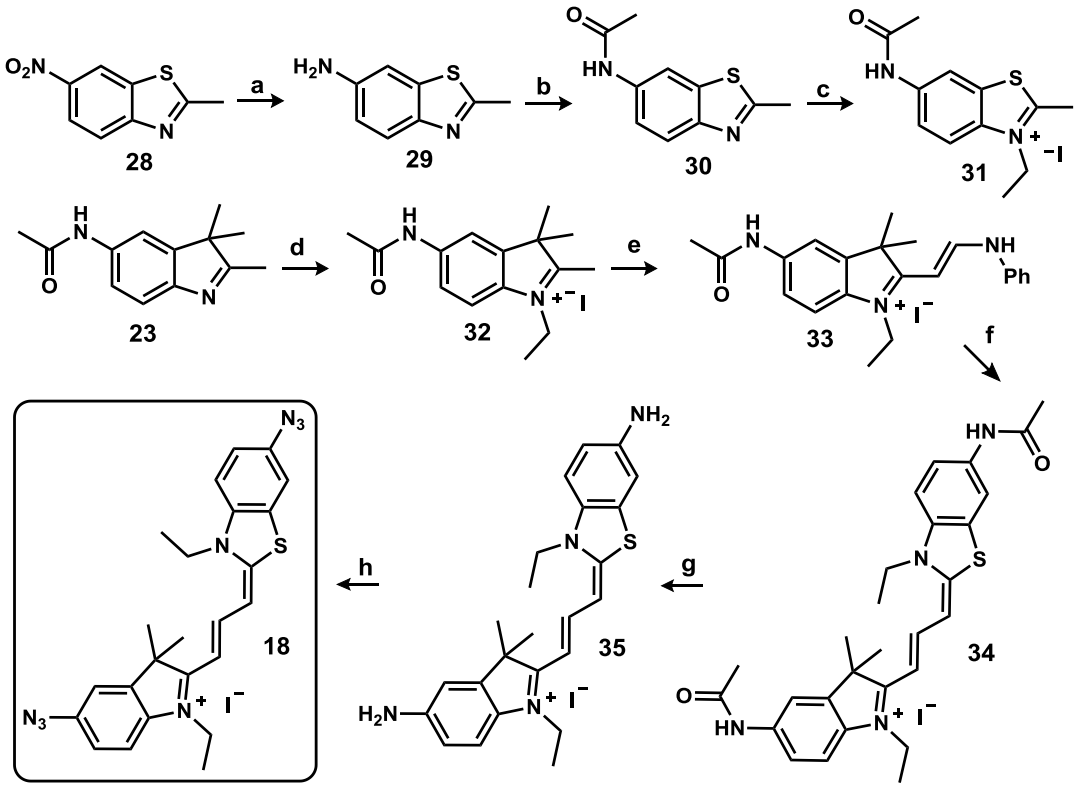


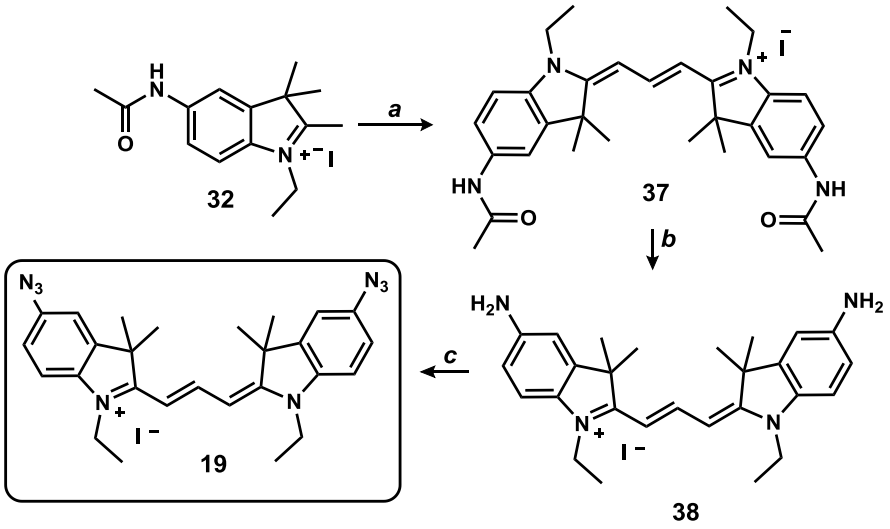
Figure 3. Cyanine bisazide dyes



Scheme 2. Synthesis of cyanine **3** (*a*) KNO_3, H_2SO_4, 1h, 0 ^{0}C, 92 % (*b*) $SnCl_2x2H_2O$, ccHCl, 1.5 h, Δ, 88 % (*c*) Ac_2O, MeCN, rt, 10 min, 92 % (*d*) 2-(2-Iodoethyl)-1,3-dioxolane, MeCN, 100 ^{0}C, 48 h, N_2, 33-80 %; (*e*) $(EtO)_3CH$, EtOH, Δ, 24 h, N_2, 54 % (*f*) DCM/H_2SO_4, rt, 30 min, 75-80 %; *(g)* BF_3*OEt_2, MeOH, 50 ^{0}C, 2 h, N_2, 60 %; *(h)* $NaNO_2$, HCl, 0°C, 30 min, then NaN_3, 0°C → rt, 1h, 55 %.



Scheme 3. Synthesis of dye 18 (*a*) $SnCl_2$, HCl, 1.5 h, Δ, 80 % (*b*) Ac_2O, MeCN, rt, 10 min, 96 % (*c*) EtI, 120 ^{0}C, 12 h, 59 % (*d*) EtI, MeCN, Δ, 24 h, 84 % (*e*) *N*, *N*-diphenyl-formamidine, $(EtO)_3CH$, EtOH, Δ, 24 h, 82 % (*f*) **31**, Ac_2O, piridine, Δ, 2 h, 67 %. (*g*) BF_3*OEt2, MeOH, 50 ^{0}C, 12 h, N_2, 78 %; (*h*) $NaNO_2$, HCl, 0°C, 30 min, then NaN_3, 0 °C → rt, 1h, 56 %.



Scheme 4. Synthesis of bisazide dye **5**. (*a*) $HC(OEt)_3$, pyridine, 12 h, Δ, 62 % (*b*) BF_3*OEt_2, MeOH, 50 ^{0}C, 12 h, N_2, 86 % (*c*) $NaNO_2$, HCl, 0 °C, 30 min, then NaN_3, 0 °C → rt, 1h, 64 %.

4. Cyanine bisazides: fluorogenic properties and two-point binding

When establishing the main photophysical properties of dyes **15-19**, we measured low intrinsic fluorescence, suggesting that the azide-mediated quenching works less efficiently with extended conjugation. We tested the fluorogenic properties of these cyanines by studying their reaction with BCN and measured fluorescence enhancement values between 7–29 (Table 2.)

Dye	[a]I_{BCN} / $I_{bisazide}$	λ_{exc} [*nm*]	λ_{em} [*nm*]	[b]$\Phi_{bisazide}$	[b]Φ_{BCN}
15	14	410	487	0.00065	0.0073
16	7	560	575	0.0195	0.057
17	29	560	571	0.065	0.73
18	22	550	565	0.011	0.13
19	10	555	569	0.0125	0.082

[a]Calculated at the emission maxima of the product.
[b]Quantum yields relative to Coumarin 153 (in case of dye **1**) or to Rhodamine B (dyes **2**–**5**)
Table 2. Photophysical properties and fluorescence enhancement caused by double-click reactions with BCN of dyes **1-5**. (in PBS pH=7.4, c = 0.625 μM, at room temperature)

Two-point binding to bis-cyclooctynylated peptides ((BCN)KAEAAK(BCN) and Ac-K(BCN)AEAADAEAAK(BCN)-NH_2, **Peptide-2**) resulted in cyclic conjugates with fluorescence enhancement values between 11–39. To the best of our knowledge, these enhancement values are outstanding within fluorogenic cyanine dyes (Table 3, Figure 4).

Dye	[a]$I_{bis\text{-}clicked}$ / $I_{bisazide}$	λ_{exc} [*nm*]	λ_{em} [*nm*]	[b]$\Phi_{bis\text{-}clicked}$
15[c]	13	410	487	0.0065
15[d]	34	410	487	0.018
16[d]	12	560	577	0.16
17[d]	28	560	573	0.85
18[d]	39	550	565	0.21
19[d]	11	555	569	0.14

[a]in PBS pH=7.4, c = 0.625 μM, at room temperature, after 2 hours of stirring in the dark
[b]Quantum yields relative to Coumarin 153 (in case of dye **1**) or to Rhodamine B (dyes **2**–**5**)
[c]with **hexapeptide** (BCN)KAEAAK(BCN)
[d]with **Peptide-2**
Table 3. Photophysical properties and fluorescence enhancement caused by double-click reactions with biscyclooctynilated peptides.

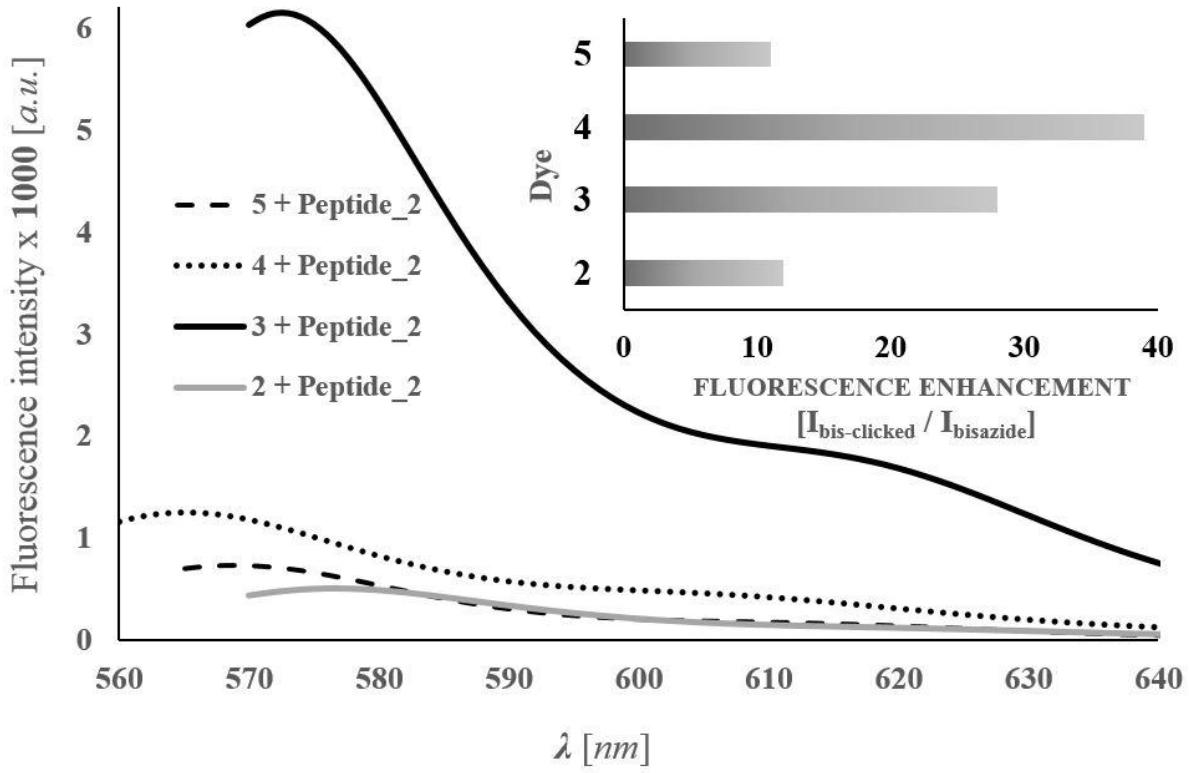


Figure 4. Comparative fluorescence enhancement of dyes **2-5** when reacted with bis-cyclooctynylated **Peptide-2**.

4. Cyanine bisazides: Crosslinking of BCN-tagged proteins

We have also demonstrated that these bisazides are useful fluorogenic crosslinking platforms that are able to covalently attach monocyclooctynylated GFP with site-specifically incorporated BCN ($GFP^{Y39\rightarrow BCN}$). The protein was labeled with 2-fold excess of dyes **16**, **17** and **19**. Dyes **15** and **18** were excluded from this experiment due to the high tendency for non-specific sticking to proteins and insufficient solubility. In each case an intensely fluorescent band appeared in SDS PAGE gel analysis at a size double of GFP, whereas control experiment with TAMRA-azide showed only the formation of the monomeric labeled species (Figure 5). Interestingly, in case of dyes **16** and **18** the main product was the monolabeled GFP as suggested by the fluorescence intensities at the detection channel of the dyes. In case of dye **19**, however, mainly the crosslinked GFP was formed and the monolabeled species was only faintly fluorescent. This suggests that the bisazides, in particular dye **19** can indeed be used as fluorogenic crosslinkers (Figure 6). In case of wild-type GFP none of the samples showed fluorescence.

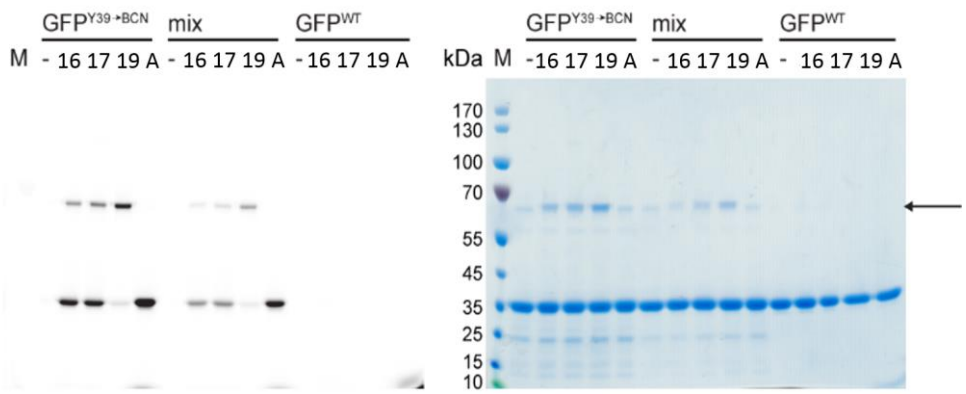


Figure 5. Fluorescent (left) and corresponding Coomassie stained SDS-PAGE (right) of $GFP^{Y39\rightarrow BCN}$, the mix (1:1 mixture of $GFP^{Y39\rightarrow BCN}$ and GFP^{WT}) and GFP^{WT} crosslinking reactions with compounds **16**, **17** and **19**. TAMRA-azide (**A**) was used as control. The first lane (-) for each sample shows the protein without dye. The black arrow indicates the GFP dimer on the gel.

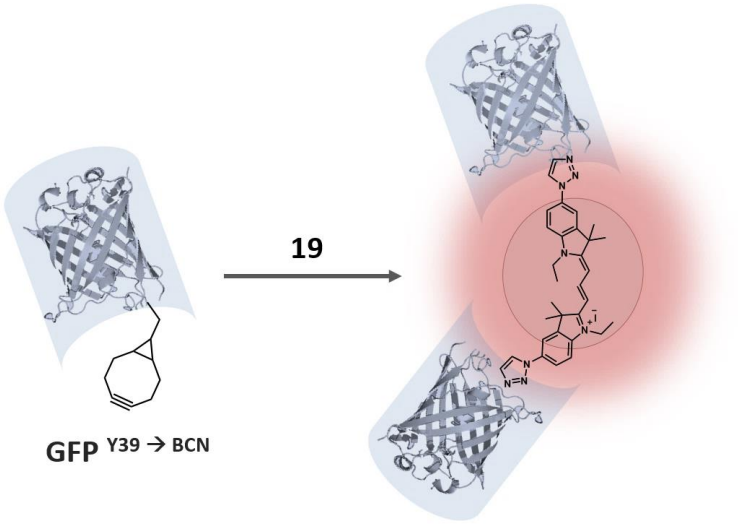


Figure 6. Dye 19 as effective, covalent fluorogenic crosslinker

Publications:

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