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Click Chemistry

A Double-Clicking Bis-Azide Fluorogenic Dye for Bioorthogonal Self-Labeling Peptide Tags

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Abstract: Herein, we give the very first example for the development of a fluorogenic molecular probe that combines the two-point binding specificity of biarsenical-based dyes with the robustness of bioorthogonal click-chemistry. This proof-of-principle study reports on the synthesis and fluorogenic characterization of a new, double-quenched, bis-azide fluorogenic probe suitable for bioorthogonal two-point tagging of small peptide tags by double strain-promoted azide–alkyne cycloaddition. The presented probe exhibits remarkable increase in fluorescence intensity when reacted with bis-cyclooctynylated peptide sequences, which could also serve as possible self-labeling small peptide tag motifs.

Introduction

With the latest advancements in the field of fluorescent microscopy, an increasing number of efforts are made to develop bright, photostable, nontoxic fluorescent dyes that can bind to particular targets with high specificity.[1,2] Additional features such as fluorogenicity or environment sensitivity may further enhance efficiency of the labeling by reducing background signals.[3] To monitor biomolecular interactions, especially protein structures with high spatio-temporal resolution, site-specific labeling of targets with minimal perturbation of their original function should be achieved. Selective and site-specific labeling can be adequately addressed with the use of fluorescent proteins (FPs), self-labeling enzymes, self-labeling tags, or enzyme-mediated peptide labeling schemes.[4] However, FPs have low photostability and similarly to self-labeling enzymes (e.g., Halo-tag, SNAP-tag) in many cases they substantially perturb the function of the protein of interest as their size is often comparable.[4] Self-labeling tags and enzyme-mediated peptide labeling techniques enable the use of small synthetic dyes with improved photophysical properties. Self-labeling tags such as tetracysteine (Cys_4) motifs can be targeted with high selectivity with biarsenicals such as FlAsH, ReAsH, or AsCy3 probes.[5] These membrane permeable fluorogenic dyes have found broad applications, for example, to interrogate protein binding,[6] targeted protein inactivation,[7] or in live cell imaging.[8] 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 limits the full potential that lies in the presumably remarkable sensitivity with these biarsenicals.[9] Furthermore, all the aforementioned examples are restricted mostly to the N- or C-terminal labeling of the protein of interest with very few examples for internal protein labeling.[10]

The parallel evolution of genetic code expansion and bioorthogonal chemistry in the last decade resulted in the emergence of a powerful labeling technique that enables in vitro and in vivo labeling of proteins virtually at any location.[11] With their low background and site-specific reactivity, bioorthogonally applicable fluorogenic dyes allow for highly sensitive imaging of proteins without the need for exhaustive washing cycles.

Over the past two years we have introduced a series of azide-quenched fluorogenic dyes and demonstrated their use in bioorthogonal labeling schemes. In this styryl-type of fluorogenic framework the azide plays a dual role: it quenches the fluorescence of the aromatic core and serves as a bioorthogonal handle at the same time. Upon conjugation with alkyne-modified targets, an 8–55 fold fluorescence intensity change

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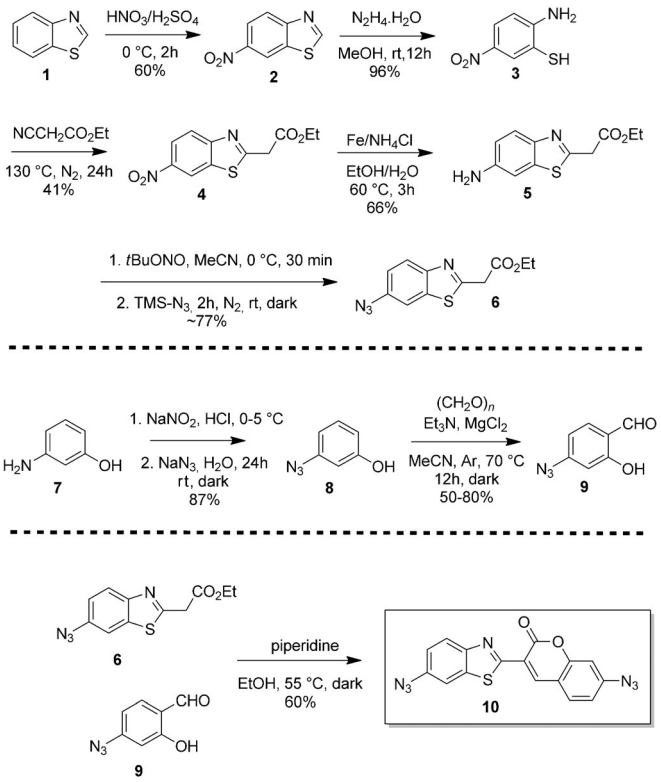
could be observed in phosphate buffered saline medium.[12] As a continuation of this work we set forth a study that aimed at developing double-functionalized bis-azide fluorogenic probes. We presumed that the quenching effect and binding specificity can be attenuated simultaneously with the installation of two azide moieties onto fluorescent frameworks. Such systems could combine the inherently high specificity of biarsenicals due to the two-point binding with the excellent fluorogenicity and robust bioorthogonality of azide-quenched structures. Moreover, they would be ideal candidates suitable for internal protein labeling with appropriately manipulated double-cyclooctynylated targets. In this proof-of-principle study we present the synthesis of a new bis-azide, bis-quenched probe for the very first time. We have also synthesized its monoazide and mono-clicked congeners to track down the contribution of the individual azide functions to the overall fluorogenicity. We also identified a bis-cyclooctynylated peptide scaffold that can be used as self-labeling tag in future applications.

Results and Discussion

To reach our aims we chose a benzothiazolyl-coumarin framework (Scheme 1). We presumed that the target probe could be assembled from appropriately substituted 2-benzothiazolyl acetate and similarly substituted salicylic aldehyde. Previous attempts to make 6-nitrobenzothiazolyl acetate by direct nitration of ethyl 2-(benzo[d]thiazol-2-yl)acetate have failed, therefore a different approach was necessary. Since other benzothiazoles can be nitrated regioselectively in good yields, we chose plain benzothiazole (**1**) as a starting material. As expected, standard nitration of **1** furnished the desired 6-nitro-substituted product (**2**) in good yield. Disassembling the thiazole ring of **2** with hydrazine-hydrate afforded 2-amino-5-nitrobenzenethiol (**3**) in excellent yield. Compound **3** was then treated with ethyl-cyanoacetate to afford **4** (41%, Scheme 1). Nitro-benzothiazole derivative (**4**) was then reduced to the corresponding amine (**5**), which was subjected to diazotization and treatment with TMS-N_3 to furnish ethyl 2-(6-azidobenzo[d]-thiazol-2-yl)acetate (**6**) (Scheme 1).



Scheme 1. Synthesis of probe **10**.

The other building block, 4-azido-2-hydroxybenzaldehyde (**9**) was obtained by diazotization and azide substitution of 3-aminophenol (**7**) to get 3-azidophenol (**8**), which was subsequently orthoformylated using anhydrous paraformaldehyde in the presence of anhydrous $MgCl_2$. Azido-building blocks **6** and **9** were then condensed in the presence of piperidine to afford target probe **10** in good yield (Scheme 1).

With the bis-azide probe in hand we have tested its main photophysical properties and fluorogenicity. Probe **10** showed excitation and emission maxima at around 410 nm and 493 nm, respectively. Next, we wished to determine the fluorogenic properties of **10**. We chose bicyclo[6.1.0]non-4-yn-9-yl-methanol (BCN)[13] as counterpart function. The rationale behind using BCN as reaction partner was that it can be routinely encoded genetically into proteins as its Lys-derivative through amber suppression technology.[13] To our delight, addition of two equivalents of BCN to a solution of **10** resulted in a 140-fold increase in fluorescence intensity in aqueous media. To get a better picture, we also tested two other cyclooctynes, COMBO[14a] and CyO[14b] (see the Supporting Information for structures) as reaction partners but found no significant alteration of the fluorogenic behavior compared to that of BCN.

Next we were curious about the fluorogenic performance of bis-azide **10** in the presence of bis-cyclooctynylated peptide targets as possible self-labeling tags. To this end, we have designed short peptide tags bearing two BCN units at different distances. For the design of these peptides, we used core sequences of Cys_4 tags previously tested successfully for FlAsH and AsCy3 probes. In these peptides, the N- and C-terminal Cys residues were replaced by Lys(ε-*N*-BCN) building blocks at the N- and C-termini, respectively (Figure 1).[5]

When added to probe **10**, peptides (BCN)KPGK(BCN) and (BCN)KPGAK(BCN), however, induced only a fraction of the fluorescence enhancement observed with plain BCN. Gratifyingly, with peptide (BCN)KAEAAK(BCN), the fluorescence intensity increase was quite comparable with the maximum value, and a 130-fold enhancement was detected (Figure 2).

To get a better insight into the fluorescence performance of these conjugates, their structures were studied with theoretical methods. First, a molecular mechanical (MM) conformation analysis was carried out for each species using the MMFF[15] force field and the Marvin[16] program. Then, for the most stable conformers, lying at most at 40 kJ mol^{-1} above the lowest energy one, geometry optimizations were performed at the AM1 (Austin model 1) level[17] with the Gaussian 09 package.[18] Finally, accurate conformational energies were obtained

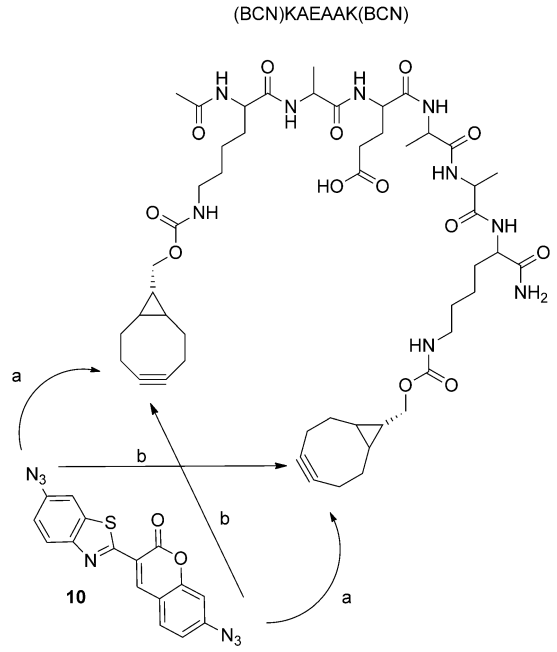


Figure 1. Representative example for the formation of cyclic peptide-**10** conjugates. Possible reactions between probe **10** and target peptide (BCN)KAEAAK(BCN). Note that in either case a cyclic bis-clicked product is formed.

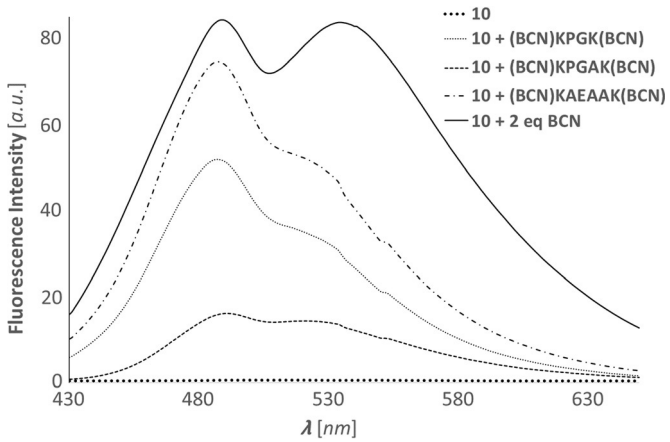


Figure 2. Fluorescent spectra of probe **10** conjugates. (λ_{exc} = 410 nm, c = 0.25 μM in PBS, pH 7.4).

invoking the local direct random phase approximation (dRPA)[19] and the aug-cc-pVDZ basis set[20] using the MRCC suite of quantum chemistry programs.[21] These studies revealed 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 that are available at room-temperature and calculated their average dihedral angles weighting by the corresponding Boltzmann factors; the results are presented in Table S4 (see the Supporting Information for further details and optimized structures).

Please note that probe **10** can bind to the aforementioned peptides in two ways (a and b), but even if we consider a 50:50 chance for the two binding modes, the calculated dihedral angles correlate well with the observed fluorescence intensities (Table 1).

Table 1. Dihedral angles and fluorescence enhancement values of conjugates.

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

[a] The two dihedral angles refer to the two binding modes of **10** to peptides [b] In PBS, pH 7.4

We also ran HPLC MS and MSMS measurements to identify the molecular weight and the possible structure of these double-clicked cyclic conjugates. In each case one sole mass was observed, indicating the formation of the bis-clicked products (a and b). Notably, no indication for the formation of linear or polymeric products was observed, which suggests preference for an intramolecular second click-reaction. Singly and doubly charged ions were observed in the LC-MS spectrum. The molecular weights calculated from these data were in good agreement with the expectations. Both the singly and doubly charged ions were selected as precursor ions in the MSMS measurements. The spectra of each sample gave similar fragment ions with a common base peak of 570 Da, and some characteristic fragment ions up 812 Da. The amino acid part does not play any role in the main fragmentation pattern. This can be explained only with the occurrence of two simultaneous SPAAC between dye **10** and the bis-cyclooctynylated peptides, resulting in single products with cyclic structures.

Finally, we were curious how the individual azide groups contribute to the quenching effect. To this end, we tried to generate the monoclicked product. However, even in very dilute solutions or with half equivalents of BCN we could only observe the formation of the bis-clicked product. This important observation clearly suggests that mono-clicked species are more reactive than the bis-azide, and thus a high driving force towards the formation of the double-clicked, cyclic product in case of peptides. Since the monotriazoles cannot be synthesized this way, we devised a synthetic route (the Supporting Information, Scheme S1) that allows the isolation of both monotriazole-monoazide, **11** and **12** (Figure 3). We found that these monotriazoles had initial, intrinsic fluorescence, giving rise to moderate fluorescence enhancement values following reaction with 1 equiv of BCN (11 and 11, for **11** and **12**, respectively, Table 2). Similarly, the previously best performing

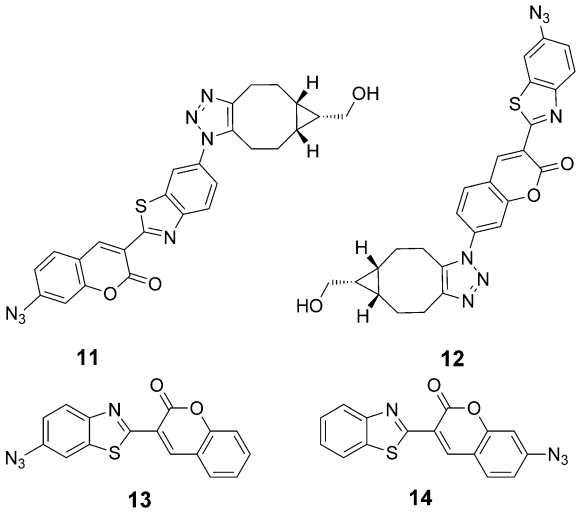


Figure 3. Compounds **11**–**14**.

Table 2. Fluorescence enhancement of compounds **11**–**14**

Compound	Fluorescence enhancement[a]	
	BCN	(BCN)KAEAAK(BCN)
11	11	4
12	11	7
13	–	5.5
14	–	5

[a] In PBS, pH 7.4, $c = 0.25$ μM.

(BCN)KAEAAK(BCN) sequence also resulted only modest enhancements in fluorescence intensities (4 and 7 for **11** and **12**, respectively, Table 2). This clearly indicates a two-step click reaction cascade resulting in de-quenched products.

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. We found that monoazides **13** and **14** showed similarly moderate, 5.5 and 5-fold enhancements, respectively. This is mainly due to the fact that like the aforementioned monotriazoles, these monoazides also exhibit more intense intrinsic fluorescence compared to that of bis-azide **10**.

Since we intend to apply bis-azide probes based on present study in physiological media in the future, it is important to elicit their stability. Azide-quenched dyes are known to be reduced by thiols, which in aqueous solutions leads to the formation of the corresponding, strongly fluorescent amines. Thus, the fluorescence intensity is a good indicator of any thiol-mediated side reactions if the conservation of fluorogenic properties is to be tested. Therefore we measured the changes in fluorescence for compound **10** in various reducing media. We found that the weak fluorescence intensity of dye **10** does not change substantially in reducing media such as excessive amounts of mercaptoethanol, cysteine, or glutathione (the Supporting Information, Table S3) especially in comparison to the intensive signal evolution of the dye upon SPAAC reaction with diverse strained alkyne reaction partners.

Conclusion

We have synthesized a new, double-quenched bis-azide fluorogenic probe that can participate in covalent, two-point binding bioorthogonal tagging schemes in combination with bis-cyclooctynylated sequences as possible self-labeling peptide tags. The probe showed excellent fluorescence enhancement when treated with a polar hexapeptide harboring two cyclooctyne motifs. This complementary peptide motif is simple and small (as few as six amino acids) for which the current preferred sequence is (BCN)KAEAAK(BCN). Studies revealed that such bis-azides participate in a two-step reaction cascade with peptides resulting in de-quenched cyclic products. Theoretical calculations underlined the importance of the conformation of the cyclic conjugates as the fluorescence of the final products was largely dependent on the dihedral angles between the two aromatic cores of probe **10**. Of the biscyclooctynylated peptide motifs screened, we have identified a polar hexapeptide sequence that allowed for nearly maximal fluorogenic performance. We also investigated the contribution of the individual azide moieties to the quenching by using monotriazole-monoazide and monoazide congeners. Such bis-azides based on this proof-of-principle study could offer highly specific and fluorogenic two-point binding labeling schemes with proteins bearing two genetically encoded cyclooctyne motifs. This work, together with the development of spectrally more advantageous bis-azide probes is currently being investigated in our laboratory and the results will be reported in due course.

Experimental Section

General

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma–Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals, Fluorochem) and used without further purification. All amino Fmoc-acid derivatives and Rink-Amide MBHA resin were purchased from Iris Biotech GmbH. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminum TLC plates from Merck. Column chromatography was carried out with silica gel (0.06–0.2 mm) from Zeochem. NMR spectra were recorded on a Varian Inova 500 MHz and Varian 600 MHz NMR spectrometers. 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), q (quartet), m (multiplet), dd (doublet of a doublet), td (triplet of a doublet), dt (doublet of a triplet), brs (broad singlet). Flash chromatography was performed on Teledyne Isco CombiFlash system. Analytical RP-HPLC-UV/Vis-MS experiments were performed on SHIMADZU LCMS-2020 system using a Gemini C18 column (100×2.00 mm I.D.) with 5 μm silica (110 pore size) as a stationary phase with a photodiode array UV/Vis (220–800 nm) and an ESI-MS detector. Linear gradient elution (0 min 0% B; 1.0 min 100% B; 3.5 min 100% B; 4.5 min 0% B; 5.0 min 0% B) with eluent A (2% NH_4HCO_3, 5% MeCN and 93% water) and eluent B (2% NH_4HCO_3 80% MeCN and

18% water) was used at a flow rate of 0.5 mL min^{-1} at 30 °C. The samples were dissolved in the mixture of water and MeCN (1:1 v/v).

Every synthetic procedure with the involvement of azide group was carried out under light-free conditions and the products were stored in low-actinic glassware at −4 °C.

Syntheses of new compounds

2-Amino-5-nitrobenzenethiol (3): 6-Nitrobenzothiazole (3 g, 16.6 mmol) was suspended in absolute ethanol (30 mL) under nitrogen atmosphere. Hydrazine monohydrate (3.3 mL, 99.6 mmol) was added dropwise. During the addition the color of the suspension gradually turned red. The mixture was stirred for 12 h at room temperature then it was diluted with cold water (20 mL) followed by the slow addition of 2 M HCl (20 mL). The resulting orange suspension was extracted with dichloromethane (3×20 mL). The combined organics were dried ($MgSO_4$), filtered and concentrated to give compound **3** as orange solid in 96% that required no further purification. R_f = 0.40 (CH_2Cl_2/Methanol 20:1 v/v on neutral alumina TLC plates); M.p. = 83–85 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.31 (d, J = 2.3 Hz, 1H), 8.00 (dd, J = 8.9, 2.5 Hz, 1H), 6.70 (d, J = 8.9 Hz, 1H), 4.95 ppm (br s, 2H), 3.01 ppm (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 148.6, 137.7, 127.0, 121.0, 108.5, 106.0 ppm; LC-MS (ESI): m/z = 169 $[M-H]^-$.

Ethyl 2-(6-nitrobenzo[d]thiazol-2-yl) acetate (4): Finely powdered compound **3** (800 mg, 4.72 mmol) was triturated with ethyl-cyanoacetate (1.12 mL, 14.16 mmol) and the resulting orange gel was heated at 110 °C for 3 h under nitrogen atmosphere. The crude product was mixed with ethyl acetate, concentrated onto silica and purified by flash chromatography (hexanes/ethyl acetate 0 to 10% for 5 min, 10% for 10 min, 10 to 40% for 10 min). The title compound was obtained as yellow powder (41%). R_f = 0.65 (Hexanes/EtOAc 10:1 v/v); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.82 (d, J = 2.0 Hz, 1H), 8.35 (dd, J = 8.9, 2.1 Hz, 1H), 8.09 (d, J = 9.0 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 4.23 (s, 2H), 1.32 ppm (t, J = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 164.2, 162.9, 151.5, 131.6, 118.5, 116.6, 113.3, 57.6, 34.9, 9.3 ppm; LC-MS (ESI): t_{ret} = 2.18 min; m/z calcd: 267.04 $[M+H]^+$; found: 266.70; HRMS (ESI+, MeCN): m/z calcd for $C_{11}H_{10}N_2O_4S$: 267.0259 $[M+H]^+$; found: 267.0258.

Ethyl 2-(6-aminobenzo[d]thiazol-2-yl) acetate (5): Compound **4** (240 mg, 0.92 mmol), fine Fe powder (504 mmol, 9.02 mmol) and NH_4Cl (492 mmol, 9.2 mmol) was suspended in a mixture of ethanol (15 mL) and water (1 mL) and stirred for 1.5 h at 55 °C. The suspension was carefully treated with aqueous ammonia (25%, 2 mL), the inorganic precipitates were filtered off and the filtrate was extracted with diethyl ether (4×15 mL). The combined organics were dried ($MgSO_4$), concentrated onto silica and purified by flash chromatography (hexanes/ethyl acetate 0 to 70%) to yield compound **5** as red oil (66%). R_f = 0.42 (Hexanes/EtOAc 1:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.73 (d, J = 8.6, 1H), 7.05 (s, 1H), 6.78 (d, J = 7.2, 1H), 4.21 (q, J = 6.9, 2H), 4.06 (s, 2H), 3.81 (br s, 2H), 1.27 ppm (t, J = 7.0, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.8, 158.2, 146.3, 144.5, 137.5, 123.2, 115.6, 105.5, 61.7, 39.6, 13.9 ppm; LC-MS (ESI): t_{ret} = 2.20 min; m/z calcd: 237.07 $[M+H]^+$; found: 236.70; HRMS (ESI+, MeCN): m/z calcd for $C_{11}H_{12}N_2O_2SNa$: 259.0517 $[M+Na]^+$; found: 259.0512.

Ethyl 2-(6-azidobenzo[d]thiazol-2-yl)acetate (6): Compound **5** (50 mg, 0.211 mmol) was dissolved in anhydrous acetonitrile (5 mL) under nitrogen atmosphere and the resulting yellow solution was cooled to 0 °C, followed by the addition of *tert*-butyl nitrite (28 μL, 0.232 mmol). During the addition, the temperature was kept at 0 °C, while the color of the solution gradually turned deep red. After 15 min of stirring, azidotrimethylsilane (58 μL, 0.422 mmol) was added slowly and the mixture was stirred for 2 h at room temperature. Water (3 mL) was added to the reaction, followed by extraction with ethyl acetate. The combined organics were dried, concentrated onto silica and quickly purified on a short silica column (hexanes with 10% ethyl acetate) to yield the title compound as brown oil that readily decomposes upon light, heat or air. (77%). R_f = 0.50 (Hexanes/EtOAc 3:1 v/v); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.94 (d, J = 8.7 Hz, 1H), 7.49 (d, J = 1.7 Hz, 1H), 7.12 (dd, J = 8.7, 2.0 Hz, 1H), 4.25 (p, J = 7.0 Hz, 2H), 4.14 (s, 2H), 1.29 ppm (t, J = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.3, 162.5, 150.2, 137.6, 137.3, 123.7, 118.1, 111.2, 61.6, 39.7, 14.03 ppm; LC-MS (APCI): t_{ret} = 1.51 min; m/z calcd: 263.06 $[M+H]^+$; found: 262.65; HRMS (ESI+, MeCN): m/z calcd for $C_{11}H_{12}N_2O_2SNa$: 285.0422 $[M+Na]^+$; found: 285.0417.

3-Azidophenol (8): 3-Aminophenol (compound **7**, 2 g, 18.3 mmol) was dissolved in 2 M HCl (40 mL). The solution was cooled to 0 °C and aqueous solution of $NaNO_2$ (1.57 g, 22.8 mmol in 8 mL water) was slowly added. The mixture was stirred for 30 min followed by the addition of sodium azide (2.25 g, 34.5 mmol) in water (10 mL). After 3 h of stirring, the resulting brown solution was extracted with ethyl acetate (3×50 mL). The combined organic layers were washed with brine (20 mL). Removal of the solvents resulted in pure compound **8** as thick red oil. (87%). R_f = 0.70 (Hexanes/EtOAc 2:1 v/v); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.18 (t, J = 8.1 Hz, 1H), 6.66–6.56 (m, 2H), 6.50 ppm (t, J = 2.2 Hz, 1H); ^{13}C NMR (63 MHz, $CDCl_3$): δ = 157.0, 142.0, 131.2, 112.6, 111.9, 106.7 ppm; MS (APCI): m/z = 136 $[M+H]^+$.

4-Azido-2-hydroxybenzaldehyde (9): Compound **8** (1.4 g, 10 mmol), anhydrous $MgCl_2$ (1.86 g, 20 mmol) and paraformaldehyde (2.1 g, 70 mmol) were combined in an oven-dried round-bottom flask. The flask was charged with argon followed by the addition of dry MeCN (40 mL) and, finally, freshly distilled triethylamine (5.3 mL, 38 mmol). This mixture was vigorously stirred for 7 h at 70 °C. Subsequently, the resulting yellow suspension was cooled and aqueous 2 M HCl (75 mL) was added. After extraction with ethyl acetate (2×50 mL) the combined organics were washed with brine (30 mL), concentrated onto silica and purified (column chromatography, hexanes with 10% ethyl acetate) to yield compound **9** as pale-yellow solid with characteristic almond-like odor (75%). R_f = 0.45 (Hexanes/EtOAc 10:1 v/v); M.p. = 110 °C (decomposes); ^{1}H NMR (250 MHz, DMSO): δ = 11.03 (s, 1H), 10.13 (s, 1H), 7.66 (d, J = 8.4 Hz, 1H), 6.68 (dd, J = 8.3, 1.9 Hz, 1H), 6.63 ppm (d, J = 2.0 Hz, 1H); ^{13}C NMR (63 MHz, DMSO): δ = 190.3, 162.3, 147.5, 131.4, 120.1, 111.2, 107.0 ppm; MS (APCI): m/z = 164 $[M+H]^+$

7-Azido-3-(6-azidobenzo[d]thiazol-2-yl)-2*H*-chromen-2-one (10): Compound **6** (20 mg, 0.07 mmol) was dissolved in absolute ethanol and piperidine (2 μL) was added. The color of the solution turned red. Compound **9** (12 mg, 0.07 mmol) was added portion-wise and the resulting suspension was stirred for 1 hour at 50 °C. The precipitation was filtered, washed repeatedly with cold ethanol and dried in vacuo. Target dye **10** was obtained as bright-orange solid (60%). R_f = 0.60 (Hexanes/EtOAc 3:1 v/v); ^{1}H NMR (700 MHz, DMSO): δ = 9.18 (s, 1H), 8.10–8.04 (m, 2H), 8.01 (d, J = 1.8 Hz, 1H), 7.33 (d, J = 1.5 Hz, 1H), 7.30 (d, J = 2.0 Hz, 1H), 7.28 (d, J = 1.8 Hz, 1H), 7.24 (d, J = 1.6 Hz, 1H), 7.22 ppm (d, J = 2.0 Hz, 1H); ^{13}C NMR (101 MHz, DMSO): δ = 159.7, 159.3, 154.5, 149.6, 145.1, 141.4, 137.5, 136.8, 131.7, 123.6, 118.9, 117.8, 116.7, 116.0, 112.2, 106.6 ppm; LC-MS (ESI): t_{ret} = 3.36 min, m/z calcd: 362.05 $[M+H]^+$; found: 361.70; HRMS (ESI+, MeCN): m/z calcd for $C_{17}H_7N_7O_2SNa$: 384.0280 $[M+Na]^+$; found: 384.0283.

7-Azido-3-(6-((5a*S*,6*R*,6a*R*)-6-(hydroxymethyl)-5,5a,6,6a,7,8 hexahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-1(4*H*)-yl)ben-

zo[d]thiazol-2-yl)-2*H*-chromen-2-one (11). (1*R*,8*S*,9*S*)-Bicyclo-[6.1.0]non-4-yn-9-ylmethanol (11 mg, 0.076 mmol) and compound **6** were combined in a round-bottom flask. Dry MeCN (3 mL) was added under and the resulting solution was stirred at 36 °C under nitrogen atmosphere. After 2 h of stirring, TLC analysis indicated that all of the starting materials have been consumed. The solvent was evaporated. The ethanolic solution of the residue (2 mL EtOH) was added dropwise to a stirring mixture of compound **9** (12 mg, 0.07 mmol) and piperidine (2 μL). After 20 min of stirring at reflux temperature, the precipitate was filtered, washed repeatedly with cold ethanol and dried in vacuum. Yellow powder 75%.R_f = 0.5 (Hexanes/EtOAc 1:3 v/v). ^{1}H NMR (500 MHz, DMSO): δ = 9.19 (1H, s), 8.34 (1H, s), 8.17 (1H, d, J = 8.4 Hz), 8.05 (1H, d, J = 8.2 Hz), 7.62 (2H, d, J = 8.0 Hz), 7.17 (1H, d, J = 7.9 Hz), 4.28 (2H, s), 3.48 (4H, s), 3.06 (2H, s), 2.89 (3H, dd, J = 31.9 Hz, 12.4), 2.73–2.56 (2H, m), 2.25–1.91 (3H, m), 1.60 (4H, d, J = 8.5 Hz), 1.07–0.93 (2H, m), 0.88 ppm (3H, s); ^{13}C NMR (126 MHz, DMSO): δ = 162.7, 159.7, 155.0, 152.5, 145.9, 144.6, 142.5, 137.0, 135.1, 133.6, 132.4, 125.1, 123.4, 120.4, 118.1, 117.2, 116.3, 109.1, 107.1, 57.8, 25.9, 23.7, 22.5, 21.4, 19.4, 18.9 ppm; LC-MS (ESI): t_{ret} = 1.79 min, *m/z* calcd: 512.14 $[M+H]^+$; found: 512.00; HRMS (ESI+, MeCN): *m/z* calcd for $C_{26}H_{21}N_7O_3SNa$: 534.1324 $[M+Na]^+$; found: 534.1319.

3-(6-Azidobenzo[d]thiazol-2-yl)-7-((5a*S*,6*R*,6a*R*)-6-(hydroxymethyl)-5,5a,6,6a,7,8-hexahydrocyclopropa[5,6]cycloocta[1,2-d][1,2,3]triazol-1(4*H*)-yl)-2H-chromen-2-one (12): (1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (20 mg, 0.133 mmol) and compound **9** (24 mg, 0,146 mmol) were combined in a round-bottom flask. Dry MeCN (3 mL) was added and the resulting solution was stirred at 36 °C under nitrogen atmosphere. After 2 h of stirring, TLC analysis showed that all of the starting materials have been consumed. The solvent was evaporated and the ethanolic solution of the residue (3 mL EtOH) was added dropwise to a stirring mixture of compound **6** (17 mg, 0.063 mmol) and piperidine (2 μL). After 20 min of stirring at reflux temperature, the precipitate was filtered, washed repeatedly with cold ethanol and dried in vacuum. Yellow powder, 71%. R_f = 0.5 (Hexanes/EtOAc 1:3 v/v).^{1}H NMR (500 MHz, DMSO): δ = 9.19 (1H, s), 8.34 (1H, s), 8.17 (1H, d, J = 8.4 Hz), 8.05 (1H, d, J = 8.2 Hz), 7.62 (2H, d, J = 8.0 Hz), 7.24 (1H, s), 7.17 (1H, d, J = 8.0 Hz), 4.28 (2H, s), 3.48 (4H, s), 3.06 (2H, s), 2.89 (3H, dd, J = 31.9 Hz, 12.4), 2.73–2.59 (2H, m), 2.08 (3H, d, J = 22.1 Hz), 1.61 (4H, s), 1.05–0.94 (2H, m), 0.88 ppm (3H, s);^{13}C NMR (126 MHz, DMSO): δ = 159.8, 159.5, 153.8, 150.0, 145.0, 139.9, 138.21, 135.20, 131.68, 122.77, 120.69, 119.89, 113.82, 112.71, 110.00, 57.80, 31.51, 25.80, 23.84, 23.24, 22.40, 21.84, 21.34, 20.82, 19.39, 18.70 ppm; LC-MS (ESI): t_{ret} = 1.85 min, *m/z* calcd: 512.14 $[M+H]^+$; found: 512.00; HRMS (ESI+, MeCN): *m/z* calcd for $C_{26}H_{21}N_7O_3SNa$: 534.1324 $[M+Na]^+$; found: 534.1319.

7-Azido-3-(benzo[d]thiazol-2-yl)-2H-chromen-2-one (13): Ethyl 1,3-benzothiazol-2-ylacetate (50 mg, 0.22 mmol) prepared according to literature[22] was dissolved in ethanol (3 mL) and catalytic amount of piperidine was added (3 μL). The color of the solution turned orange. Compound **9** (36 mg, 0.22 mmol) was added portionwise and the resulting suspension was stirred for 1 hour at 70 °C. A bright-orange precipitate was formed, which was filtered, washed with cold ethanol and dried in vacuo (57%). R_f = 0.8 (Hexanes/EtOAc 3:1 v/v); ^{1}H NMR (400 MHz, DMSO): δ = 9.24 (s, 1H), 8.19 (d, J = 7.9 Hz, 1H), 8.10 (dd, J = 11.3, 8.4 Hz, 2H), 7.59 (t, J = 7.6 Hz, 1H), 7.49 (t, J = 7.5 Hz, 1H), 7.35 (d, J = 1.5 Hz, 1H), 7.25 ppm (dd, J = 8.4, 1.9 Hz, 1H); ^{13}C NMR (101 MHz, DMSO): δ = 159.79, 159.26, 154.53, 151.93, 145.15, 141.61, 135.83, 133.22, 131.77, 126.67, 125.37, 122.43, 122.23, 117.94, 116.74, 115.99, 106.64 ppm; LC-MS (ESI): t_{ret} = 3.50 min, *m/z* calcd 321.04 $[M+H]^+$; found: 320.65; HRMS (ESI+, MeCN): *m/z* calcd for $C_{16}H_8N_4O_2SNa$: 343.0266 $[M+Na]^+$; found: 343.0265.

3-(6-Aminobenzo[d]thiazol-2-yl)-2H-chromen-2-one (14b): Compound **4** (300 mg, 1.12 mmol) was suspended in ethanol (10 mL) and piperidine (3 μL) was added. Distilled salicylaldehyde (120 μL, 1.12 mmol) was mixed in dropwise, and the resulting yellow suspension was stirred at reflux temperature for 1 hour followed by filtration. Dye precursor **14a** was obtained as yellow, highly insoluble precipitate which was filtered, washed repeatedly with diethyl ether and dried in vacuo (41%). Subsequently, finely powdered **14a** (80 mg, 0.24 mmol) was suspended in ethanol (20 mL) and $SnCl_2$ dihydrate (178 mg, 1.23 mmol) and *cc* HCl (1500 μL) were added. The resulting suspension was stirred at reflux temperature for 1.5 h. The cold reaction mixture was poured onto water and with slow, careful addition of NaOH (10%) the pH of the solution was adjusted between 6.5 and 7. The aqueous phase was washed with ethyl acetate (3×30 mL). The volatiles were evaporated and the crude product was purified (column chromatography, silica, CH_2Cl_2 with 1–10% methanol) to yield title compound **14b** as vivid red powder (32%). R_f = 0.35 (CH_2Cl_2/Methanol 20:1 v/v); ^{1}H NMR (500 MHz, DMSO): δ = 9.03 (s, 1H), 8.00 (d, J = 6.6 Hz, 1H), 7.73 (d, J = 8.7 Hz, 1H), 7.71–7.65 (m, 1H), 7.50 (d, J = 8.3 Hz, 1H), 7.46–7.39 (m, 1H), 7.12 (d, J = 1.8 Hz, 1H), 6.86 (dd, J = 8.7, 2.1 Hz, 1H), 5.60 ppm (s, 2H); ^{13}C NMR (126 MHz, DMSO): δ = 174.64, 159.81, 153.42, 147.88, 144.59, 139.97, 138.68, 133.26, 130.18, 125.55, 123.51, 120.45, 119.53, 116.57, 116.36, 103.50 ppm; LC-MS (ESI): t_{ret} = 2.20 min, *m/z* calcd: 295.05 $[M+H]^+$; found: 294.65; HRMS (ESI+, MeCN): *m/z* calcd for $C_{16}H_{10}N_2O_2SNa$: 317.0361 $[M+Na]^+$; found: 317.0357.

3-(6-Azidobenzo[d]thiazol-2-yl)-2H-chromen-2-one (14): Amine **14b** (30 mg, 0.10 mmol) was suspended in cold 6 M HCl (2 mL) and methanol was added until a clear solution was obtained. Aqueous $NaNO_2$ solution (8 mg, 0.11 mmol in 500 μL) was added dropwise while the temperature was maintained around 0 °C, followed by 30 min of stirring at 0 °C. NaN_3 (10 mg, 0.15 mmol) in 1 mL water was added. The ice bath was removed and after 3 h of stirring at room temperature the resulting precipitate was filtered, dissolved in ethyl acetate and washed with brine. Target monoazide **14** was obtained as yellow powder (34%); R_f = 0.8 (Hexanes/EtOAc 3:1 v/v). ^{1}H NMR (400 MHz, DMSO): δ = 9.21 (s, 1H), 8.09 (s, 1H), 8.07 (s, 1H), 8.02 (d, J = 2.2 Hz, 1H), 7.76 (t, J = 7.7 Hz, 1H), 7.62–7.41 (m, 1H), 7.30 ppm (dd, J = 8.7, 2.0 Hz, 1H); ^{13}C NMR (101 MHz, DMSO): δ = 159.58, 159.41, 153.32, 149.53, 141.80, 137.60, 136.96, 133.66, 130.22, 125.22, 123.66, 119.30, 118.92, 118.81, 116.24, 112.17 ppm; LC-MS (ESI): t_{ret} = 3.50 min, *m/z* calcd 321.04 $[M+H]^+$; found: 320.65; HRMS (ESI+, MeCN): *m/z* calcd for $C_{16}H_8N_4O_2SNa$: 343.0266 $[M+Na]^+$; found: 343.0265.

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