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# Bioconjugatable Azo-Based Dark-Quencher Dyes: Synthesis and Application to Protease-Activatable Far-Red Fluorescent Probes

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**Abstract:** We describe the efficient synthesis and one-step derivatization of novel, nonfluorescent azo dyes based on the Black Hole Quencher-3 (BHQ-3) scaffold. These dyes were equipped with various reactive and/or bioconjugatable groups (azido, α-iodoacetyl, ketone, terminal alkyne, vicinal diol). The azido derivative was found to be highly reactive in the context of copper-catalyzed azide–alkyne cycloaddition (CuAAC) reactions and allowed easy synthetic access to the first water-soluble (sulfonated derivative) and aldehyde-modified BHQ-3 dyes, the direct preparation of which failed by means of conventional azo-coupling reactions. The aldehyde- and α-iodoacetyl-containing fluorescence quenchers were readily conjugated to aminooxy- and cysteine-containing peptides by the formation of a stable oxime or thioether linkage, respectively. Further fluorescent labeling of the resultant peptide conjugates with red- or far-red-emitting rhodamine or cyanine dyes through sequential and/or one-pot bioconjugations, led to novel Förster resonance energy transfer (FRET) based probes suitable for the in vivo detection and imaging of urokinase plasminogen activator, a key protease in cancer invasion and metastasis.

**Keywords:** azo compounds · click chemistry · dark quencher · dyes · FRET · water solubility

## Introduction

Nonfluorescent quenching dyes (Figure 1) are now regarded as key components in the design of smart optical bioprobes, especially those that rely on Förster resonance energy transfer (FRET), currently used in protease activity assays, nucleic acid hybridization, and real-time polymerase chain reactions (PCRs).[1] The principal advantage that these molecules offer over their fluorescent counterparts is the elimination of background fluorescence that originates from direct acceptor excitation or re-emission.[2] In this context, Black Hole Quencher (BHQ) dyes, a new generation of "true" dark quenchers with no native emission due to their polyaromatic azo backbone, have recently been developed and are now commercially available from Biosearch Technologies.[3] The non-emissive excitation of BHQ dyes can be related to photochemical isomerization of their azo bridge, either through a rotation mechanism around the N=N double bond or an inversion mechanism, in which a planar variation of one of the C-N-N angles can exist in the excited state.[4] This three-member family of quenchers (BHQ-1 to BHQ-3) covers the electromagnetic spectrum from $\lambda = 480$ nm to the near-infrared (NIR) region and provides excellent spectral overlap across the entire range of commonly used reporter fluorescent dyes. Unlike the dark quenchers derived from cyanine dyes (e.g., CyDyeQ from GE Healthcare or IRDye QC-1 from LI-COR Biosciences, Figure 1),[5] the native fluorescence emission of which is completely switched off by the incorporation of certain electron-donating (e.g., dialkylanilino) and/or -withdrawing (e.g., nitro) groups that favor either intramolecular charge transfer (ICT) or photoinduced electron transfer (PeT), BHQ dyes exhibit a greater (photo)chemical stability. Relative to other fully stable non-emissive dyes from an alternative trademarked family (QSY dyes from Invitrogen Molecular Probes),[6,7] the synthetic access to these azo-based dyes is more convenient and requires a limited number of steps.[8] Thus, their growing popularity is illustrated by the myriad of fluorescence-quenched probes reported during the past decade for use in challenging bioanalytical applications.[9] However, these nonfluorescent azo-based dyes are only currently available as carboxylic acid derivatives, which limits their conjugation to amine-containing biomolecules/biopolymers through the widely used *N*-hydroxysuccinimidyl (NHS) active ester chemistry. Phosphoramidite synthons derived from hydroxyl-containing

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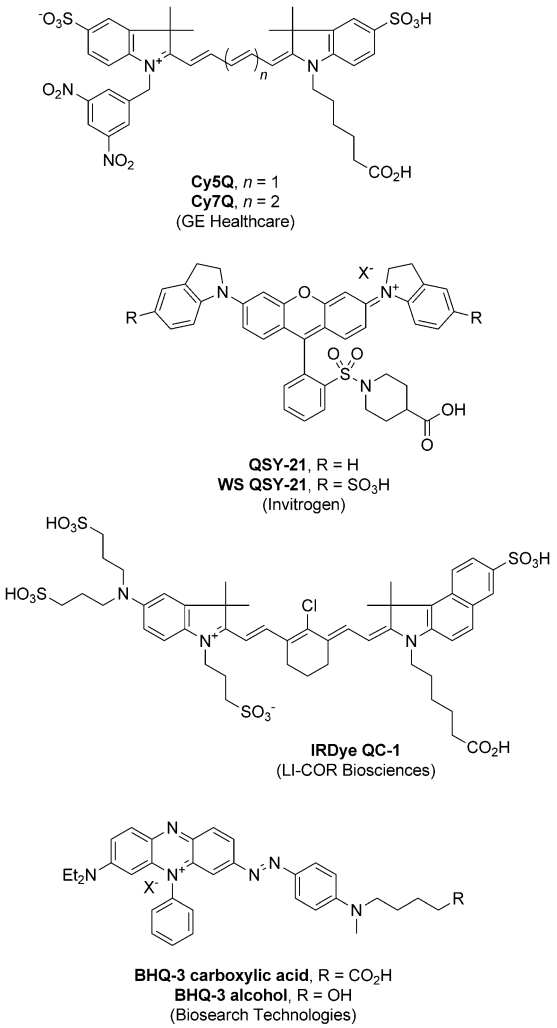



Figure 1. Structures of commercially available nonfluorescent, broad-range quencher dyes. All compounds are available as NHS esters except BHQ-3 alcohol, which is converted into a phosphoramidite building block suitable for the labeling of oligonucleotides by SPS.

BHQ dyes have also been developed but their use is restricted to the solid-phase synthesis (SPS) of oligonucleotide–quencher conjugates. To expand the scope of BHQ dyes in biolabeling applications we have revisited the chemistry of these azo dyes to design a highly convergent and efficient synthetic pathway with the aim to easily introduce a wide range of reactive and/or bioconjugatable groups (azido, α-iodoacetyl, ketone, terminal alkyne, vicinal diol) onto their aniline unit. This novel methodology was applied to BHQ-3 and five different quenchers were obtained. Furthermore, the implementation of copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction (click reaction)[10] with a versatile azido-modified BHQ-3 and unusual sulfonated or carbonylated terminal alkynes was also explored to produce the first fully stable water-soluble and benzaldehyde-functionalized dark quenchers. As illustrative examples of the utility of these novel bioconjugatable BHQ-3 derivatives, we have prepared enzyme-activatable red or far-red fluorogenic

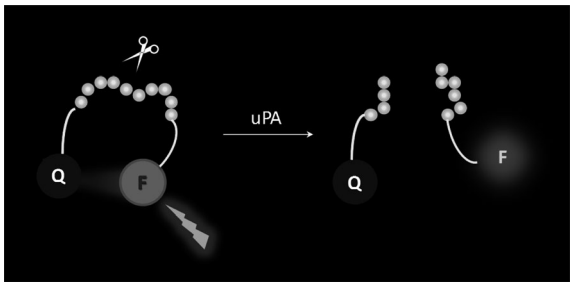



Figure 2. The principle of FRET-based uPA-sensitive probes studied in this work.

probes suitable for use in the detection of cell-secreted urokinase activity, reliant on the FRET phenomenon (Figure 2).[11] Urokinase plasminogen activator (uPA) is a serine protease that plays an important role in tumor-associated proteolysis and peptide-based far-red fluorescent molecular probes are valuable tools for the diagnosis of cancer by in vivo biomedical imaging.[12] High-yield grafting of the FRET donor (red- or far-red fluorophores sulforhodamine (SR) 101 or sulfonated cyanine dyes Cy 5.0 and Cy 5.5)[2] and quencher acceptor onto selected uPA peptidyl substrate was achieved, either in a three-step manner (with temporary cysteine protection) or in a sequential one-pot approach.

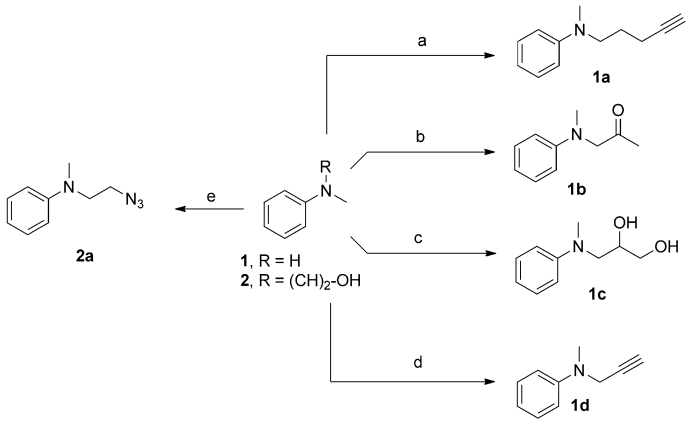
## Results and Discussion

**General considerations for synthesis of the BHQ-3 core**: The synthetic strategy currently employed to prepare the aryldiazo-N-arylphenazonium quencher moieties relies on an azo-coupling reaction between a 3-amino-7-(dialkylamino)-5-aryl-phenazonium salt (commercially available Methylene Violet 3RAX for BHQ-3) and an electron-rich N,N-dialkyl-substituted aniline with one of the two alkyl chains terminated with a carboxylic acid for subsequent bioconjugation by formation of a carboxamide linkage.[3,13] This $S_EAr$ reaction does not work well if the reaction mixture is too acidic; under the standard conditions required for generation of a nitrosonium ion $NO^+$ (sodium nitrite and a strong mineral acid, such as HCl or $H_2SO_4$), protonation of the activating N,N-dialkylamino group of the aniline reaction partner takes place. With these latter diazotization conditions, it is essential to adjust the pH of the reaction mixture to pH 6 before addition of the aniline partner and the isolated yields rarely exceed 20%. Therefore, the use of bench-stable, commercially available nitrosating agent, nitrosonium tetrafluoroborate, in a polar aprotic solvent is preferred to the standard conditions.[14] Surprisingly, such a diazo condensation has been carried out with only a limited number of asymmetric N,N-substituted anilines and, at present, only BHQ-3 derivatives functionalized either with a primary alcohol (subsequently converted into a phosphoramidite synthon suitable for coupling to synthetic DNA fragments) or a carboxylic acid are available. In this context, and with the objective to expand the conjugation chemistry of BHQ-3 dyes,

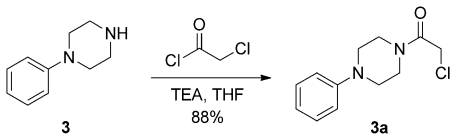
  

we decided to revisit this milder azo-coupling reaction with more sophisticated aniline substrates.

**Synthesis of functionalized unsymmetrical tertiary anilines**: To retain the beneficial electron-donating mesomeric effect of the N,N-dialkyl amino groups on the efficiency of the azo condensation with aromatic partners, tertiary anilines derived from *N*-methylaniline (**1**) or 2-*N*-methylanilinoethanol (**2**) were selected for the study. The introduction of alkyne-, azido-, vicinal diol-, and ketone-terminated alkyl chain substituents was achieved through nucleophilic substitution reactions (Scheme 1). Standard and previously reported conditions were used to readily achieve N-alkylations to give anilines **1a**, **1c**, **1d**, and **2a** in good yields. Effective ring-opening of glycidol to provide aminopropane-1,2-diol **1c** required microwave activation. Preparation of α-aminomethyl methyl ketone **1b** involved a further mild Swern oxidation step of the intermediate aminopropan-2-ol derivative. The N-acylation of *N*-methylaniline dramatically reduces the reactivity of this latter aromatic partner in azo-coupling reactions, therefore the amidification of **1** with an acyl halide derivative of α-chloroacetic acid or iodoacetic anhydride was not selected as the synthetic route to the required α-haloacetyl-functionalized aniline. A different approach based on the N-acylation of *N*-phenylpiperazine (**3**) with α-chloroacetyl chloride led to the bicylic aniline **3a** (Scheme 2) suitable for the synthesis of the α-iodoacetyl BHQ-3 derivative through the diazo-coupling method. The easy availability of these six functionalized tertiary anilines in large amounts has enabled us to consider a short methodological study designed to optimize the preparation of the diazo-dye framework found in both commercial BHQ-3 and the quenchers claimed in the present work.



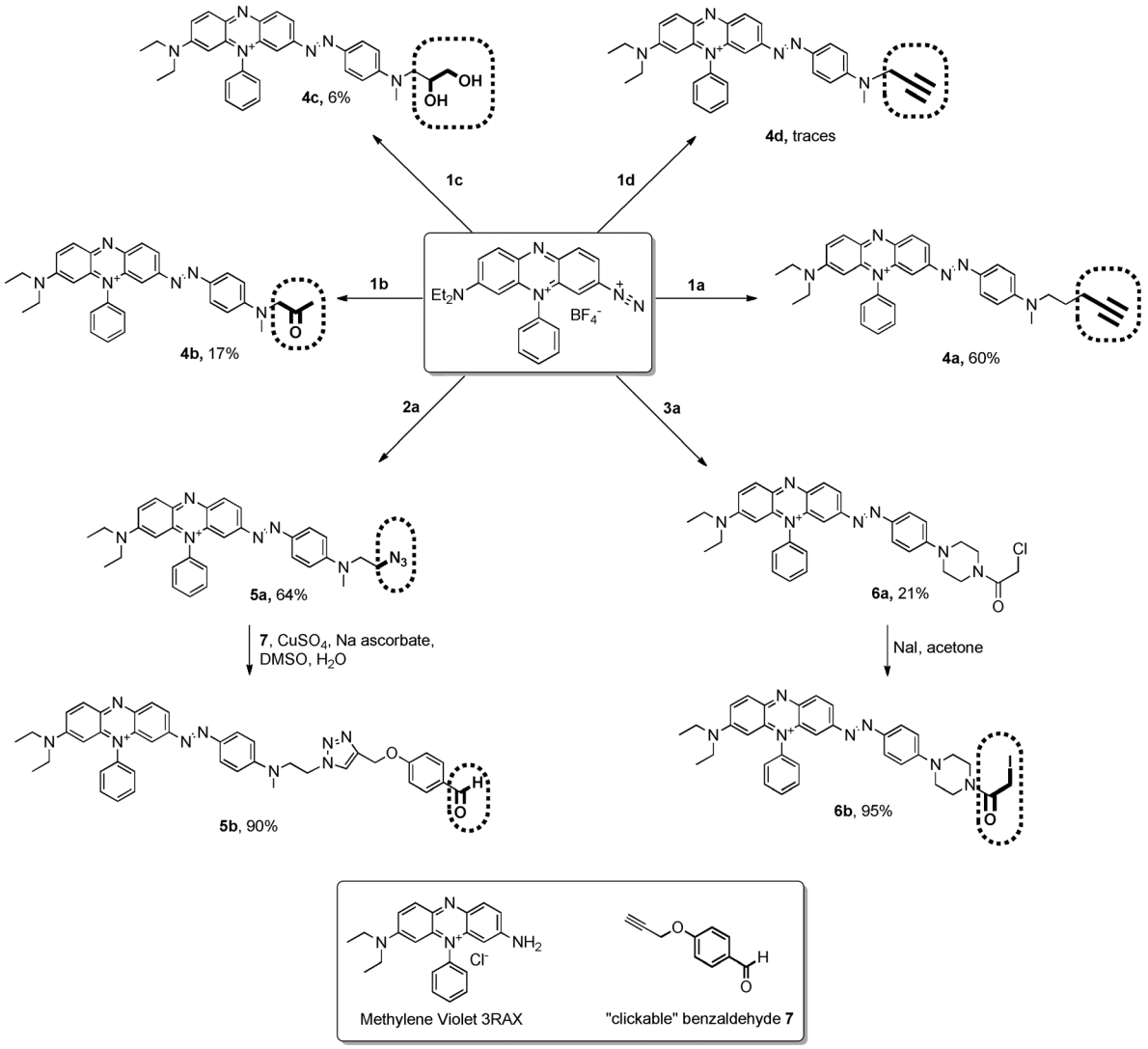

Scheme 1. Synthesis of functionalized unsymmetrical tertiary anilines. a) MsO–$(CH_2)_3$–CCH, $K_2CO_3$, neat, 100 °C, 2 h, 44 %; b) i) propylene oxide, EtOH, microwave (MW), 120 °C, 20 min, ii) oxalyl chloride, DMSO, triethylamine (TEA), $CH_2Cl_2$, −78 °C, 2 h, 71 % (over two steps); c) Glycidol, EtOH, MW, 120 °C, 20 min, 81 %; d) Propargyl bromide, $K_2CO_3$, [18]crown-6, toluene, 12 h, 75 %; e) i) Mesyl chloride, TEA, $CH_2Cl_2$, 4 °C–RT, 1 h, ii) $NaN_3$, DMF, 40 °C, overnight, 64 %.




Scheme 2. Synthesis of tertiary aniline **3a** for use in azo-coupling reactions.

**Synthesis of the functionalized BHQ-3 dyes**: First, we studied the influence of the reaction solvent to find an alternative to sulfolane, which was initially used by Ewing et al. for azo coupling with Methylene Violet 3RAX (Scheme 3).[13] The very high boiling point of sulfolane (285 °C/760 mmHg) makes its complete removal impossible (even with an effective vacuum system or by freeze drying). Moreover, its polar character makes column chromatography or HPLC purification (normal phase for BHQ-3 and reversed phase for **4a–d**, **5a**, **5b**, and **6b**) difficult and negatively affects the separation quality. Acetonitrile was found to be the best alternative because its polarity was high enough to get a homogeneous azo-coupling reaction mixture. Furthermore, it is one of the most popular solvents used in combination with an aqueous mobile phase for RP-HPLC analyses and purifications. Concerning the reagent ratio, $NOBF_4$ was used in stoichiometric amounts relative to Methylene Violet 3RAX for the initial diazotization step. Under these conditions, all targeted BHQ-3 derivatives were obtained, except dye **4d** (capped with the shorter-chain terminal alkyne), which was recovered in only trace amounts from the crude reaction mixture after RP-HPLC purification. The lack of reactivity of propargyl aniline **1d** relative to the pentyne analogue **1a** may be explained by the electron-withdrawing inductive effect of its carbon–carbon triple bond. A Finkelstein halogen-exchange reaction was performed on α-chloroacetyl derivative **6a** after the azo-bridge formation (near-quantitative conversion of **6a** into **6b** was achieved) because we suspected that the greater reactivity of the C–I bond could be troublesome in the condensation step. RP-HPLC purification with an aqueous solution of trifluoroacetic acid (TFA, 0.1 %) and $CH_3CN$ as the eluents provided the purified functionalized azo dyes **4a–c**, **5a**, and **6b** as TFA salts. The moderate isolated yields for compounds **4a** (60 %), **5a** (64 %), and **6b** (20 % over two steps) were explained by losses that occurred during the chromatographic purification and freeze-drying steps, which are unavoidable on the small scale used [Methylene Violet 3RAX (50 mg)], although the conversions were found to be almost quantitative. For ketone and vicinal diol derivatives **4b** and **4c**, the limited chemical stability of these functionalized quenchers explains the poor yields obtained. The structure of each BHQ-3 derivative was unambiguously confirmed by detailed measurements that included ESI-MS and NMR spectroscopic analyses. There is a growing need for fluorescence quenchers that bear a reactive handle suitable for bioconjugation through powerful and versatile ligation techniques, namely hydrazone and oxime ligations,[15] therefore it is necessary to find

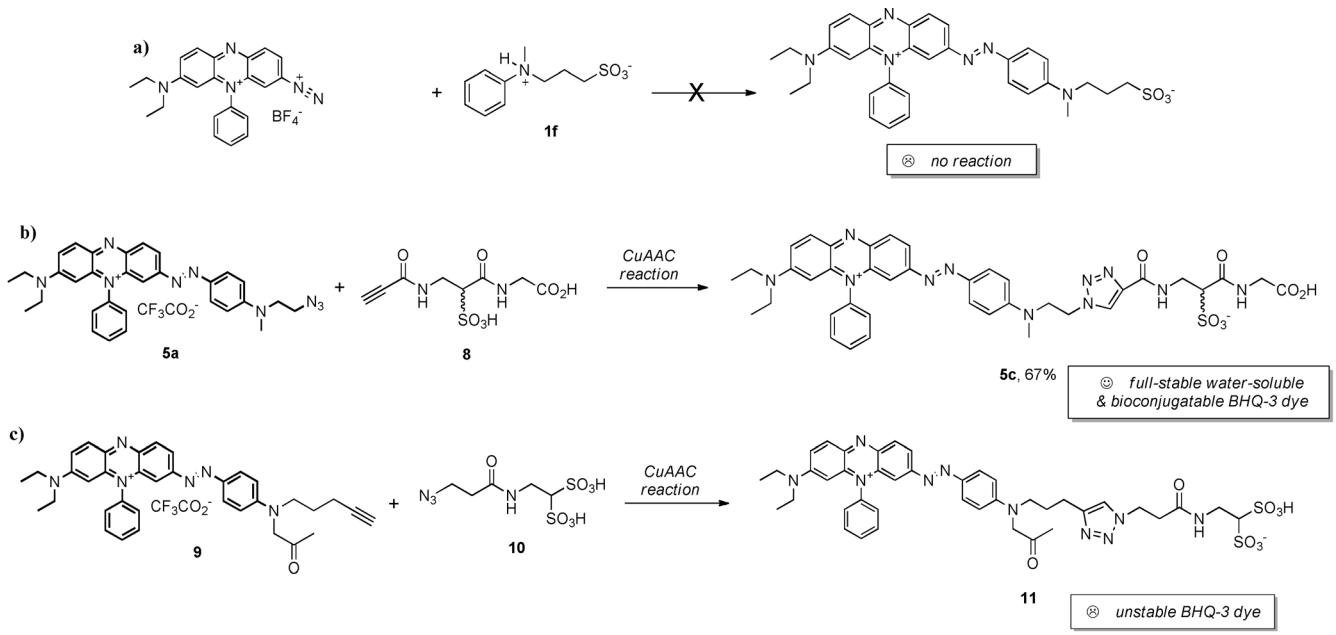
  




Scheme 3. Synthesis of functionalized BHQ-3 dyes (as their TFA salts). Reagents and conditions for the azo-coupling reaction: i) Methylene Violet 3RAX, $NOBF_4$, $CH_3CN$, 0 °C, 15 min, ii) Aniline **1a**–**d**, **2a**, or **3a**, $CH_3CN$, 0 °C, 1 h, RP-HPLC purification.

an alternative synthetic route to more-stable carbonyl-containing BHQ-3 derivatives in good yields. In this context, we considered modification of the azido derivative **5a** to help achieve this goal. Indeed, the specific chemical derivatization of the reactive group of this "convertible" BHQ-3 with a terminal alkyne functionalized with an aldehyde/ketone moiety may be easily performed by using the popular CuAAC reaction. The practical implementation of this "post-synthetic" carbonylation procedure involved the use of *para*-(propargyloxy)benzaldehyde (**7**) as the terminal alkyne and standard CuAAC reaction conditions ($CuSO_4$ and sodium ascorbate as the catalytic system). A mixture of DMSO/$H_2O$ (1:1) was used as the solvent and complete conversion into the fully stable benzaldehyde derivative **5b** was observed within 2 h. Furthermore, no diazo-bridge reduction mediated by sodium ascorbate was detected in the crude reaction mixture. The remarkable chemical inertness of azo dyes towards the click catalytic system is in agreement with previous observations by the Wagner group for 4-{[4-(dimethylamino)phenyl]azo}benzoic acid (DABCYL) analogues.[16] The clicked azo-based dark-quencher dye **5b** was isolated by RP-HPLC in a very good yield (90%) and spectroscopic data were in agreement with the structure assigned. In light of this successful functionalization of the BHQ-3 scaffold by click chemistry, we next investigated improvement of the water-solubility of this hydrophobic azo dye through a similar derivatization approach. Indeed, to the best of our knowledge, no water-soluble BHQ-3 analogue has been reported to date, even though it would be a valuable synthetic tool to ensure some of the physicochemical properties of smart optical bioprobes for challenging biological applications. One hydrophilic long-wavelength-absorbing QSY 21 disulfonate (Figure 1), synthesized from indoline-5-sulfonic acid, has recently been described.[7] Conjugation of BHQ dyes to synthetic DNA fragments is an effective way to counterbalance their native hydrophobic




Scheme 4. Synthesis of water-soluble bioconjugatable BHQ-3 dyes **5c** and **11** (as their TFA salts). Reagents and conditions for the CuAAC reaction: $CuSO_4$, sodium ascorbate, 1:1 DMSO/$H_2O$, Ar atmosphere, RT, 2 h; RP-HPLC purification. The isolated yield of **11** was not determined due to the degradation of the dye during purification and storage.

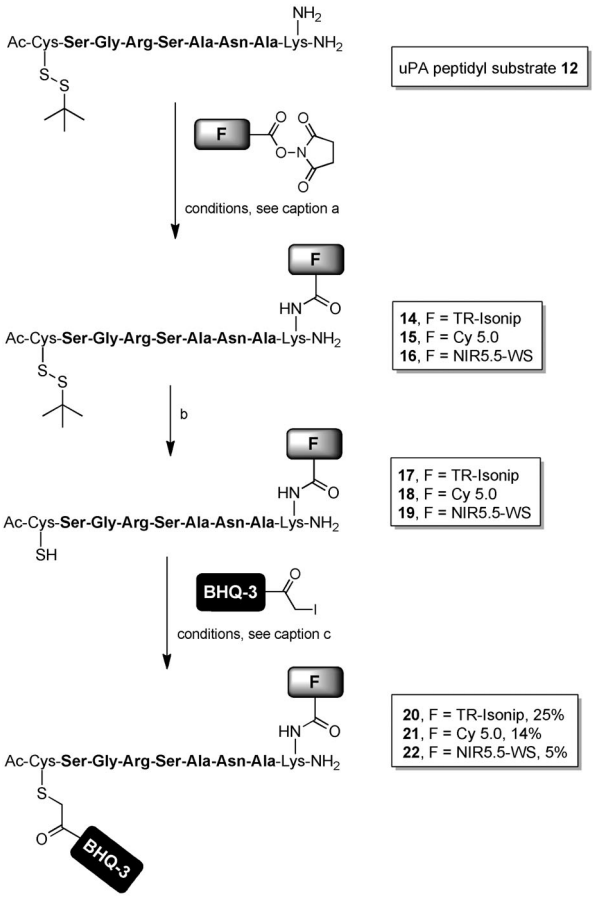
character, but such a water-solubilization strategy is difficult to apply and/or not relevant for non-nucleic acid based biomolecular architectures (e.g., FRET-quenched peptides). Initially, we performed a standard azo-coupling reaction between Methylene Violet 3RAX and *N*-methyl-*N*-sulfopropyl aniline (**1f**), readily obtained by $S_N2$ alkyaltion of **1** with 1,3-propanesultone. However, the reaction did not work (Scheme 4a) and the starting materials remained unchanged, even after prolonged reaction times. We assumed that sulfonated aniline **1f** exists mainly in its zwitterionic form, unreactive in the $S_EAr$ reaction due to the lack of any electron-donating mesomeric effect from a *para* substituent. To circumvent this issue, and by analogy with our previous work on the sulfonation of various fluorescent organic dyes,[17] we have explored the CuAAC reaction between azido quencher **5a** and sulfonated terminal alkyne **8** (Scheme 4b). The reaction was performed under the same conditions used for the click reaction of benzaldehyde derivative **7** and the targeted monosulfonated BHQ-3 derivative **5c** was obtained in a satisfying 67% yield. This monosulfonated azo dye was found to be soluble in water and related aqueous buffers in the concentration range suitable for biolabeling applications (1.0 μM–1.0 mM). Interestingly, the grafting of alkyne **8** also enables the introduction of a terminal carboxylic acid group suitable for bioconjugation through NHS chemistry. In addition to standard spectroscopic characterizations, we have also determined the log *P* value of **5c** by using a HPLC method recently developed by us for a series of water-soluble boron-dipyrromethene (BODIPY) dyes.[18] This is a relevant parameter for assessment of the propensity of a BHQ-3 quencher to form intramolecular dimers (H- or J-aggregates) with a selected reporter fluorescent dye (i.e. dye–quencher ground-state complex), which results in fluorescence quenching of the fluorogenic probe through a static mechanism.[19] Indeed, Ogawa et al. have recently shown that there is a relationship between dye/fluorophore lipophilicity and the degree of H-aggregate formation in aqueous solution.[20] Thus, a value of $\log P = 2.0$ was found by correlation of retention time to reference substances with known log *P* values. Motivated by this success, we decided to expand the click sulfonation strategy to the water solubilization of keto-alkyne-functionalized BHQ-3 **9** (Scheme 4c).[21] Indeed, the availability of two distinct reactive functional groups on the aniline moiety of **9** allows both the grafting of a nonbioconjugatable but more hydrophilic disulfonated linker such as **10** (see the Supporting Information for synthesis) and bioconjugation through oxime ligation. CuAAC reaction between **9** and **10** worked well under standard conditions but unfortunately the resultant azo dye **11** was found to exhibit poor chemical stability, even when stored at either 4 or −20 °C.

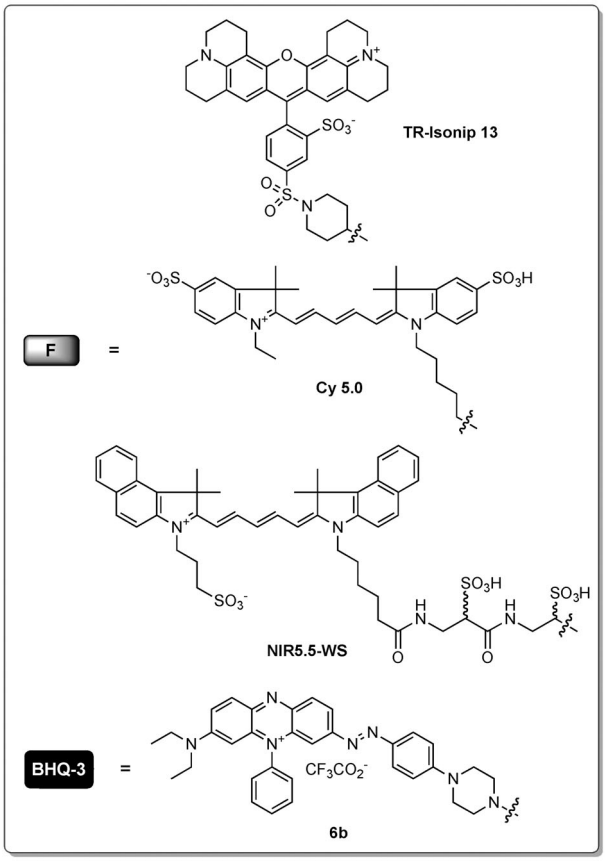
In summary, the azo-condensation reaction between Methylene Violet 3RAX and functionalized tertiary anilines, performed under mild conditions, has led to a wide range of bioconjugatable BHQ-3 dyes not reported to date. Because no changes in the BHQ-3 chromophore were made by this synthetic approach, the photophysical properties of these quenchers were assumed to be the same as the parent BHQ-3 terminated with a carboxylic acid ($\lambda_{max}$ ($CH_3CN$) = 653 nm, extinction coefficient ($\varepsilon$) = 17400 $mol^{-1}dm^3cm^{-1}$; $\lambda_{max}$ [phosphate buffered saline (PBS)] = 606 nm, $\varepsilon$ = 14900 $mol^{-1}dm^3cm^{-1}$).[22] The azido derivative **5a** can be easily conjugated to phosphine- or alkyne-modified (bio)-molecules through Staudinger ligation or CuAAC reaction,

respectively. The benzaldehyde/BHQ-3 hybrid **5b** is suitable for hydrazone and oxime ligations, whereas α-iodoacetyl quencher **6b** is expected to readily react with (bio)molecules that contain free-thiol groups. Finally, the BHQ-3 diol **4c** is able to strongly bind boronate-containing (bio)polymers (diol–boronate bioconjugation), access to which is made easier by the recent availability of valuable boronate (bio)-molecules such as *para*-boronophenylalanine.[23] In addition to increasing the number of different bioorthogonal handles onto the BHQ-3 scaffold, the present synthetic strategy is particularly well-suited for the provision of "click-convertible" azo dyes, the water solubility of which is dramatically improved through a mild and efficient sulfonation reaction. To demonstrate the broad utility of these functionalized azo-based dark quenchers in bioconjugation chemistry, the aldehyde- and α-iodoacetyl-modified BHQ-3 dyes **5b** and **6b** were coupled to peptides with a single aminooxy or sulfhydryl reactive group, respectively, to prepare novel, internally quenched uPA-sensitive (far-)red fluorescent probes.

**Synthesis of (far-)red-emitting dye/BHQ-3 fluorogenic probes through sequential dual-peptide labeling**: uPA is a serine protease that plays a critical role in malignancies and its overexpression has been linked to poor clinical prognosis, particularly in breast cancer.[24] Thus, noninvasive imaging of uPA overexpression would have significant potential to improve novel cancer therapies. Some uPA-sensitive far-red fluorescence imaging agents have been already reported but all were designed so that several molecules of the same far-red emitting fluorophore (mainly a cyanine dye—Cy 5.5, Cy 7.0—or related photostable analogues) were assembled in complex protease-cleavable polymeric-peptidyl hybrid architectures to ensure close proximity within two fluorophores, which resulted in efficient fluorescence quenching (static quenching through the formation of H-dimers).[11,12] Heptapeptide H–Ser–Gly–Arg–Ser–Ala–Asn–Ala–OH has been reported to be a highly potent substrate for uPA (cleavage site between the arginine and serine residues), therefore we decided to label this peptide with a FRET pair that consisted of a (far-)red emitting dye (SR101, Cy 5.0, or Cy 5.5 fluorescent core) and a BHQ-3 to obtain structurally more-simple uPA-cleavable, activatable probes. To achieve chemoselective labeling reactions, cysteine and lysine residues were incorporated at the N- and C-terminus, respectively, as handles for α-iodoacetyl BHQ-3 **6b** and amine-reactive fluorophore introduction (Scheme 5). Peptide Ac–Cys(S*t*Bu)–Ser–Gly–Arg–Ser–Ala–Asn–Ala–Lys–$NH_2$ (**12**) was readily obtained by standard solid-phase peptide synthesis techniques, was used. First, acylation of the ε-amino group of **12** with the NHS active ester of the selected fluorophore (**13**, Cy 5.0, or NIR5.5-WS) in dry *N*-methylpyrrol-

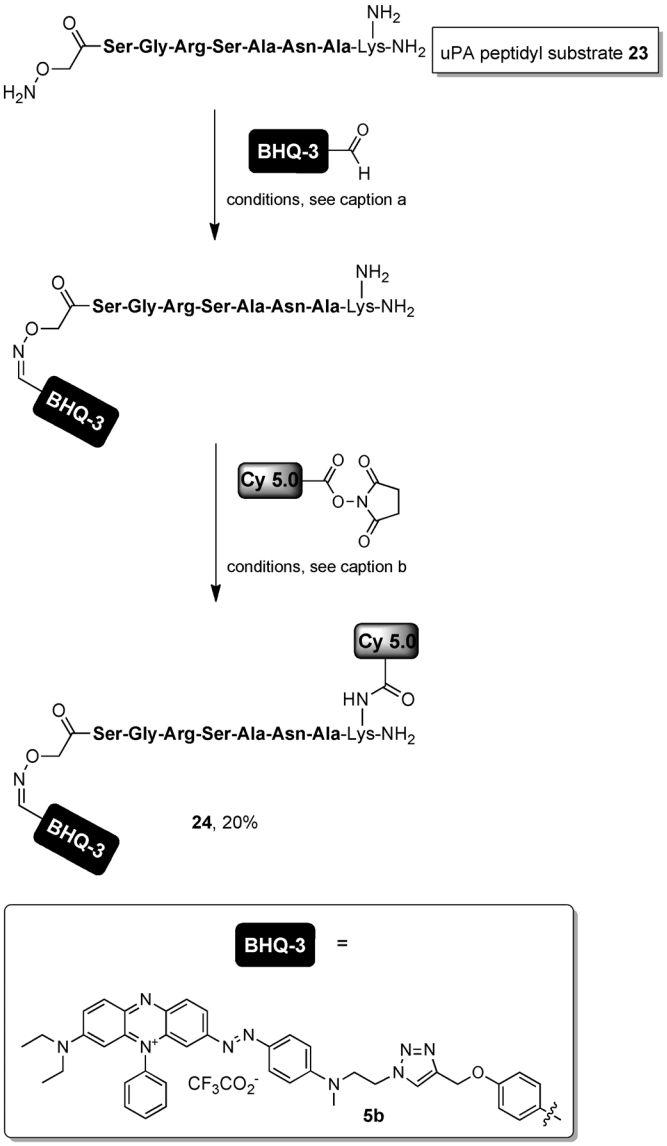



Scheme 5. Synthesis of uPA-activatable far-red or NIR fluorescent probes **20**–**22** by a sequential three-step bioconjugation procedure. Reagents and conditions: a) Fluorophore NHS ester (0.85 equiv), DIEA, NMP, RT; RP-HPLC purification. b) DTT, $NaHCO_3$ (aq) [0.1 M, pH 8.5], RT, 1 h; RP-HPLC purification. c) **6b** (1.5 equiv), $NaHCO_3$ (aq) [0.1 M], RT, 2 h; RP-HPLC purification.

idone (NMP) and in the presence of *N*,*N*-diisopropylethylamine (DIEA) was achieved to give **14**–**16**. The unusual SR101 derivative, TR-Isonip (**13**), which displays $\lambda_{max}$ (PBS) = 608 nm and relative quantum yield ($\Phi_F$) [PBS] = 45%, bears the tertiary sulfonamide moiety of isonipecotic acid at the 2′ position instead of a sulfonic acid group, and made the conjugation step easier due to the greater stability of the NHS active ester relative to the sulfonyl chloride moiety found in commercially available Texas Red (TR).[25] Furthermore, this is the most common way to avoid the undesired ring-chain tautomerism of TR–amine conjugates, which leads to formation of the colorless and nonfluorescent spirosultam.[26] In addition to this red-emitting xanthene, cyanine dyes Cy 5.0 and NIR5.5-WS (already reported in the literature with $\lambda_{max}$ = 665 and 700 nm and $\Phi_F$ (PBS) = 20 and 7%, respectively)[27] have been used as far-red and NIR fluorophores. The removal of the S*t*Bu protecting group from the cysteine residue was achieved by treatment with an excess of dithiothreitol (DTT) in aqueous sodium bicarbonate buffer (pH 8.5) to affoed **17**–**19**. Finally, iodoacetamido-activated BHQ-3 **6b** was readily attached to the cysteine residue of the fluorescent peptide in a mixture of aqueous sodium bicarbonate buffer (pH 8.5) and $CH_3CN$. The uPA fluorogenic substrates **20**–**22** were obtained after purification by RP-HPLC (overall yields 5–25%, purity > 95%). Their structures were unambiguously confirmed by ESI-MS analysis. To avoid the time-consuming isolation steps required after each synthetic step of this dual-labeling scheme and to improve the overall yield, a consecutive combination of two orthogonal chemoselective reactions (oxime ligation and Schotten–Baumann amidification) in a sequential one-pot synthesis[28] from benzaldehyde BHQ-3 **5b** was next considered (Scheme 6). Thus, the octapeptide H–Ser–Gly–Arg–Ser–Ala–Asn–Ala–Lys–$NH_2$ was N-acylated with the NHS active ester of a previously reported N-protected derivative of aminooxyacetic acid (Aoaa). The acid-labile 1-ethoxyethylidene group (Eei) was preferred to the standard di-*tert*-butoxycarbonyl protection because it has been recently shown that this oxime is more adequate for stepwise SPPS of Aoaa-containing peptides, especially in preventing the N-overacylation side reactions frequently encountered during such syntheses.[29] After TFA-mediated deprotection and RP-HPLC purification, super-nucleophilic peptide **23** was reacted with benzaldehyde-modified quencher **5b** in aqueous sodium acetate buffer (pH 4.4). Under these mildly acidic conditions, the free ε-amino group of lysine is protonated and unreactive toward the quencher.[30] The complete consumption of **5b** and **23** was checked by RP-HPLC, then the pH of the reaction mixture was adjusted to pH 8 by addition of aqueous sodium bicarbonate buffer. Subsequently, the NHS active ester of Cy 5.0 was added and its aminolysis by the lysine residue was performed at 4°C. The resulting uPA fluorogenic substrate **24** was isolated in pure form by RP-HPLC (overall yield = 20%, purity > 95%). These results establish compounds **5b** and **6b** as useful new fluorescence quenchers for oxime and thioalkylation ($S_N2$ reaction or Michael addition) orthogonal bioconjugation.




Scheme 6. Synthesis of uPA-activatable far-red fluorescent probe **24** by a one-pot, two-step bioconjugation procedure. Reagents and conditions: a) **5b** (1 equiv), NaOAc (aq) [0.1 M, pH 4.4], $CH_3CN$, RT; then pH adjusted to ≈pH 8.0 with $NaHCO_3$ (aq). b) Cy 5.0 NHS ester (1 equiv), NMP, 4°C, 2 h; RP-HPLC purification.

**In vitro activation of the uPA-sensitive fluorescence-quenched probes**: The results from the fluorogenic cleavage assay with commercial uPA (from human urine) are summarized in Table 1 and Figures 3 and 4. For example, almost complete quenching (calculated efficiency = 98.8%, Table 1, entry 2) was found for **21** until it was cleaved by uPA, which caused an 83-fold increase in fluorescence at $\lambda_{em}$ = 663 nm over time (a plateau was reached within 25 min). As expected, no significant fluorescence signal changes were observed in the absence of protease (Figure 3, inset). Similar behaviors were observed for the BHQ-3-based quenched probes **20** and **22** labeled with dyes **13** and NIR5.5-WS, respectively (Figure 3). Because their fluorescence emission is centered

Table 1. Spectral properties of fluorogenic probes **20**–**22** and **24** before and after cleavage with uPA.

| Entry | Probe | $\lambda_{abs}$ before [nm][a] | $\lambda_{em}$ after [nm][a] | Fluorescence emission intensity[b] | Quenching efficiency [%][c] |
|---|---|---|---|---|---|
| 1 | **20** | 590 | 609 | 22/2087 | 98.9 |
| 2 | **21** | 600 | 663 | 43/3588 | 98.8 |
| 3 | **22** | 620, 694 | 700 | 35/1159 | 97.0 |
| 4 | **24** | 607 | 665 | 112/2042 | 95.0 |

[a] UV-Vis absorption and fluorescence spectra of each probe were recorded before and after uPA digestion at 25 °C (absorption) or 37.5 °C (emission) either in PBS (for **20**, **21**, and **24**) or in PBS+2% DMSO (for **22**). [b] Area under the emission curve before/after uPA digestion. [c] Quenching efficiency was calculated based on the equation: $100\times[1-$(fluorescence emission intensity of the probe)/(fluorescence emission intensity of the probe after complete digestion with uPA)$]$.

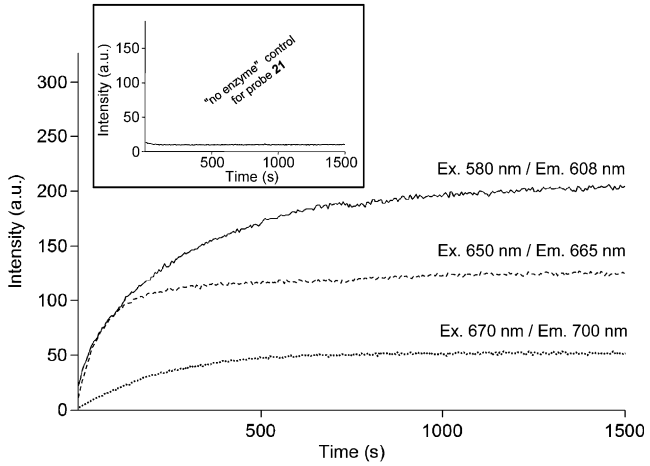



Figure 3. Fluorescence emission time course (kinetics mode) of probes **20** (——), **21** (-----), and **22** (•••••) with uPA from human urine (0.6 U, incubation time = 25 min) in PBS at 37.5 °C (probe concentration = 1.0 μM); inset: control without uPA for probe **20**; similar control results were achieved for probes **21** and **22**.

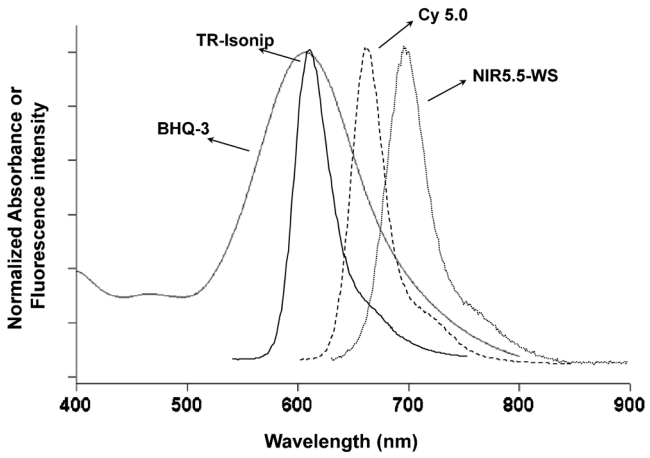



Figure 4. Normalized absorbance spectrum of BHQ-3 carboxylic acid (——) and the fluorescence emission spectra of **13** (——), Cy 5.0 (-----), NIR5.5-WS (•••••) in PBS at 25 °C.

at $\lambda_{em}=609$ and 700 nm, respectively, these results clearly show that our functionalized BHQ-3 derivatives are able to quench a wide range of fluorophores from the red to NIR spectral region. This is further supported by good overlap of the fluorescence emission spectra of selected cyanine and xanthene dyes and the absorption spectrum of BHQ-3 carboxylic acid (Figure 4). Furthermore, the magnitude differences in fluorescence intensity upon complete enzymatic cleavage between the three probes **20**–**22** are consistent with the differences in quantum yield of the released red- or NIR-emitting fluorophores. Finally, the same enzymatic activation was performed with the fluorogenic probe **24** (oxime linkage with BHQ-3 dye; Table 1, entry 4 and Figure 5). A

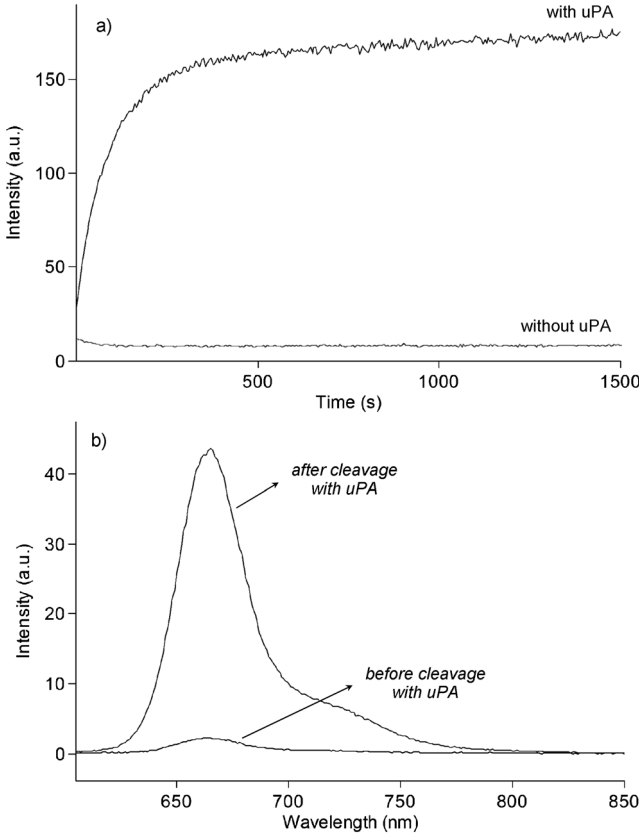



Figure 5. a) Fluorescence emission time course (kinetics mode, $\lambda_{ex}=$ 650 nm, $\lambda_{em}=665$ nm) of probe **24** with (0.6 U) and without uPA from human urine (incubation time 25 min) in PBS at 37.5 °C (probe concentration = 1.5 μM); b) Fluorescence emission spectrum (scan mode, $\lambda_{ex}=$ 595 nm) of probe **24** before and after incubation with uPA.

less-satisfying quenching efficiency was obtained (95%) that suggests a less-than-optimal proximity between the fluorophore and quencher required for efficient FRET and/or static quenching through the formation of an intramolecular ground-state complex (see above). Indeed, it seems unlikely that this partial dequenching is the result of a slow hydrolysis of the oxime bond of **24**, which is known to be very stable at physiological pH.[31]

## Conclusion

Five novel bioconjugatable dark quenchers based on the BHQ-3 scaffold were synthesized by an optimized diazo-coupling reaction. Various reactive and/or bioconjugatable groups (namely azido, α-iodoacetyl, ketone, terminal alkyne, vicinal diol) were easily introduced within the core structure of the azo dyes for the first time. Furthermore, azido-modified dark quencher **5a** proved to be a useful precursor for the preparation of the first water-soluble BHQ-3 **5c** and aldehyde-modified BHQ-3 **5b** through effective "post-synthetic" click reactions with unusual terminal alkynes. This study fills a significant gap in the field of tunable, functionalized azo dyes and complements a recent report from the Kool group related to the first hydrazine- and aminooxy-modified quenchers (DABCYL and BHQ-2 derivatives) designed for hydrazone and oxime orthogonal bioconjugation.[32] All these compounds are attractive chromophores for the design of the next generation of enzyme-activated probes based on either a single biopolymer (e.g., peptide or oligonucleotide) or a sophisticated nanoplatform, suitable for medical-imaging applications.[33] This was illustrated by the rapid and convenient synthetic access to uPA-activated fluorescent-imaging probes, of which the spectral properties and reactivity towards uPA open the way for their in vivo validation in the context of cancer diagnosis.

## Experimental Section

**General remarks**: TLC analyses were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. The spots were visualized by illumination with a UV lamp ($\lambda = 254$ nm) and/or staining with a solution of phosphomolybdic acid or $KMnO_4$. Flash column chromatography was performed on Geduran Si 60 silica gel (40–63 μm; 63–200 μm for SR101 derivatives) from Merck. All chemicals were used as received from commercial sources without further purification, unless otherwise stated. All solvents were dried by standard procedures (acetone: storage over 3 Å molecular sieves; $CH_2Cl_2$: distillation over $P_2O_5$; $CH_3CN$: distillation over $CaH_2$; DMSO: distillation over $CaH_2$). Anhydrous DMF was obtained from Carlo Erba-SdS or Fisher Scientific. Peptide-synthesis-grade DIEA, NMP, and piperidine were purchased from Iris Biotech GmbH. Triethylamine (TEA) was distilled over KOH and stored over BaO. Methylene Violet 3RAX and uPA from human urine (500 U mg$^{-1}$ protein, lyophilized form) were provided by Sigma–Aldrich. HPLC gradient-grade acetonitrile ($CH_3CN$) and methanol ($CH_3OH$) were obtained from VWR. PBS (phosphate (100 mm)+NaCl (150 mm), pH 7.5) and aqueous mobile phases for HPLC were prepared with water purified by means of a Milli-Q system (purified to 18.2 MΩ cm). Triethylammonium acetate (TEAA, 2.0 M) and triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled TEA and glacial acetic acid or $CO_2$ gas, respectively. Sulfonated terminal alkyne **8** was prepared by using a solid-phase method recently reported by us.[17] The N-protected derivative of aminooxyacetic acid, Eei-Aoaa-OH, and its NHS active ester were synthesized in accordance with published procedures.[17] Sulfoindocyanine dyes Cy 5.0 (also named Cy 5.29) and NIR5.5-WS (also named Cy 5.5) were prepared by literature procedures.[27]

**Instruments and methods**: A CEM Discover microwave (MW) unit was used for the reactions conducted under MW irradiation. The syntheses of peptides **12** and **23** were carried out on an Applied Biosystems 433A synthesizer by using standard 9-fluorenylmethoxycarbonyl (Fmoc)/*t*Bu chemistry.[34] $^1$H- and $^{13}$C NMR spectra were recorded on either a Bruker AC 200 or DPX 300 spectrometer, or with a Bruker AVANCE I 400 spectrometer (broad band fluorine observation BBFO) probe, 5 mm). Chemical shifts are expressed in parts per million (ppm) relative to the residual solvent signal.[35] Coupling constants ($J$) are expressed in Hz. IR spectra were recorded with a universal attenuated total reflectance (ATR) sampling accessory on a Perkin–Elmer FTIR Spectrum 100 spectrometer. Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a photodiode array (PDA) detector. Semi-preparative HPLC was performed on a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV-Vis 2000 detector. ESI mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus. UV-Vis spectra were obtained with a Varian Cary 50 scan spectrophotometer by using either a rectangular quartz cell (Varian, standard cell, Open Top, 10×10 mm, 3.5 mL) or a quartz micro cell (Hellma, 108.002-QS, light path: 10 mm, 500 μL). Fluorescence spectroscopic studies (emission/excitation spectra) were performed with a Varian Cary Eclipse spectrophotometer with a semi-micro quartz fluorescence cell (Hellma, 104F-QS, 10×4 mm, 1400 μL) for quantum yield determination or an ultra-micro quartz fluorescence cell (Hellma, 105.251-QS, light path: 3×3 mm, 45 μL) for uPA assays. Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (excitation and emission filters: auto, excitation and emission slit = 5 nm). The quantum yield of TR-Isonip (**13**) was measured at 25 °C by a relative method with SR101 as a standard ($\Phi_F(s) = 90\%$ in EtOH).[36,37] The following equation was used to determine the relative fluorescence quantum yield [$\Phi_F(x)$]:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

in which A is the absorbance (in the range 0.01–0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used for the measurements), and the subscripts S and X represent standard and unknown, respectively.

**HPLC separations**

*System A*: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 4.6×100 mm) with $CH_3CN$ and TFA (0.1% (aq), pH 2.2) as eluents [20% $CH_3CN$ (5 min), followed by a linear gradient from 20–100% $CH_3CN$ (45 min)] at a flow rate of 1.0 mL min$^{-1}$. Triple UV-Vis detection was achieved at $\lambda = 220$, 260, and 600 nm.

*System B*: Semi-preparative RP-HPLC (Varian Kromasil $C_{18}$ column, 10 μm, 21.2×250 mm) with $CH_3CN$ and TFA [0.1% (aq)] as eluents [0% $CH_3CN$ (5 min), followed by a gradient of 0–30% $CH_3CN$ (20 min), then 30–40% $CH_3CN$ (30 min), then 40–80% $CH_3CN$ (110 min)] at a flow rate of 20.0 mL min$^{-1}$. Double visible detection was achieved at $\lambda = 500$ and 700 nm.

*System C*: Semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 21.2×250 mm) with $CH_3CN$ and TFA [0.1% (aq)] as eluents [0% $CH_3CN$ (10 min), followed by a gradient of 0–15% $CH_3CN$ (30 min), then 15–30% $CH_3CN$ (60 min)] at a flow rate of 15.0 mL min$^{-1}$. Double UV detection was achieved at $\lambda = 220$ and 250 nm.

*System D*: As for system A, but with the following gradient: 0% $CH_3CN$ (2.5 min), followed by a linear gradient of 0–80% $CH_3CN$ (35 min) at a flow rate of 1.0 mL min$^{-1}$. Triple UV-Vis detection was achieved at $\lambda =$ 230, 254 and 666 nm.

*System E*: Semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 10.0×250 mm) with $CH_3CN$ and TFA [0.1% (aq)] as eluents [0% $CH_3CN$ (5 min), followed by a gradient of 0–20% $CH_3CN$ (15 min), then 20–30% $CH_3CN$ (25 min), then 30–80% $CH_3CN$ (125 min)] at a flow rate of 4.5 mL min$^{-1}$. Double visible detection was achieved at $\lambda = 560$ and 700 nm (for TR-Isonip derivatives), $\lambda = 620$ and 700 nm (for Cy 5.0 derivatives), or $\lambda = 640$ and 700 nm (for NIR5.5-WS derivatives).

*System F*: Semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 10.0×100 mm) with $CH_3CN$ and TFA [0.1% (aq)] as eluents [0% $CH_3CN$ (5 min), followed by a gradient of 0–20% $CH_3CN$ (15 min), then 20–30% $CH_3CN$ (25 min), then 30–80% $CH_3CN$ (125 min)] at a flow rate of 4.5 mL min$^{-1}$. Double visible detection was

achieved at $\lambda = 560$ and 700 nm (for TR-Isonip derivatives), $\lambda = 620$ and 700 nm (for Cy 5.0 derivatives), or $\lambda = 640$ and 700 nm (for NIR5.5-WS derivatives).

*System G*: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 4.6 × 100 mm) with $CH_3CN$ and TEAA (0.1 % (aq), 25 mM, pH 7.0) as eluents [0 % $CH_3CN$ (2.5 min), followed by a linear gradient of 0–80 % $CH_3CN$ (35 min)] at a flow rate of 1.0 mL min$^{-1}$. Triple UV-Vis detection was achieved at $\lambda = 230$, 254, and 666 nm.

*System H*: Semi-preparative RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 10.0 × 250 mm) with $CH_3CN$ and TEAB (50 mM (aq), pH 7.5) as eluents [0 % $CH_3CN$ (5 min), followed by a gradient of 0–20 % $CH_3CN$ (15 min), then 20–30 % $CH_3CN$ (25 min), then 30–80 % $CH_3CN$ (125 min)] at a flow rate of 4.5 mL min$^{-1}$. Double visible detection was achieved at $\lambda = 640$ and 700 nm.

*System I*: RP-HPLC (Thermo Hypersil GOLD $C_{18}$ column, 5 μm, 2.1 × 100 mm) with $CH_3CN$ and TFA (0.1 % (aq), pH 2.2) as eluents [0 % $CH_3CN$ (2.5 min), followed by a linear gradient of 0–80 % $CH_3CN$ (35 min)] at a flow rate of 0.25 mL min$^{-1}$. Triple UV-Vis detection was achieved at $\lambda = 230$, 254, and 666 nm.

**Compound 1a**: Compound **1** (434 mg, 4.0 mmol, 2 equiv), solid $K_2CO_3$ (560 mg, 4.0 mmol, 2 equiv), and mesylated 4-pentynol (300 mg, 2.0 mmol, 1 equiv) were heated neat at 100 °C for 2 h. The reaction mixture was dissolved in deioinized water and extracted with EtOAc (×2). The combined organic layers were evaporated under reduced pressure and the crude product was purified by flash chromatography on silica gel (cyclohexane/EtOAc 98:2) to give **1a** as a yellowish oil (154 mg, 44 %). $R_f = 0.77$ (cyclohexane/EtOAc 8:2); $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 1.81$ (quin, $^3J(H,H) = 6.8$ Hz, 2H), 2.00 (d, $^4J(H,H) = 0.75$ Hz, 1H), 2.25 (dt, $^3J(H,H) = 6.8$ Hz, $^4J(H,H) = 0.75$ Hz, 2H), 2.95 (s, 3H), 3.45 (t, $^3J(H,H) = 6.8$ Hz, 2H), 6.71 (m, 3H), 7.23 ppm (m, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 16.1$, 25.7, 38.6, 51.6, 69.0, 83.9, 112.3, 116.3, 129.3, 149.3 ppm; IR (neat): $\tilde{\nu} = 3297$ (CH, alkyne), 2939 (CH, Ar), 1598 (C=C, Ar), 1505 (C=C, Ar), 1367 cm$^{-1}$ (C–N); MS (ESI+): *m*/*z* (%): 174.1 $[M+H]^+$ (100); *m*/*z* calcd for $C_{12}H_{15}N$: 173.1.

**Compound 1b**[38]

*N-Alkylation*: Propylene oxide (404 mg, 7.0 mmol, 1.5 equiv) and absolute ethanol (0.5 mL) were added to **1** (500 mg, 4.6 mmol, 1 equiv). The reaction mixture was MW irradiated (300 W) at 120 °C for 20 min. The volatile compounds were removed under reduced pressure and the crude product (790 mg, 100 %) was used in the next step without further purification. $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 1.25$ (d, $^3J(H,H) = 6.2$ Hz, 2H), 2.17 (brs, 1H), 2.97 (s, 3H), 3.22 (d, $^3J(H,H) = 6.2$ Hz, 2H), 4.11 (m, 1H), 6.79 (m, 3H), 7.25 ppm (m, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 20.2$, 39.4, 61.8, 65.5, 113.4, 117.5, 129.2, 150.5 ppm; IR (neat): $\tilde{\nu} = 3366$ (OH), 2971, 2866, 1598, 1507, 1361, 1101, 1069 cm$^{-1}$; MS (ESI+): *m*/*z* (%): 166.1 $[M+H]^+$ (100); *m*/*z* calcd for $C_{10}H_{15}NO$: 165.1.

*Swern oxidation*: Dry DMSO (1 mL, 13.8 mmol, 4 equiv) was added to a solution of oxalyl chloride (710 μL, 8.3 mmol, 2.4 equiv) in dry $CH_2Cl_2$ (10 mL) at −78 °C and the mixture was stirred at −78 °C for 30 min. The crude secondary alcohol (570 mg, 3.45 mmol, 1 equiv) was added and the reaction mixture was stirred at −78 °C for 1 h before TEA (3.11 mL, 22.4 mmol, 6.5 equiv) was added. The mixture was stirred for 15 min then warmed to 0 °C. A saturated aqueous solution of $NaHCO_3$ (20 mL) was added and the immiscible layers were separated by decantation. The aqueous phase was extracted with $CH_2Cl_2$ (2 × 30 mL). The combined organic layers were washed with brine (50 mL) and deionized water (50 mL), then dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (cyclohexane/EtOAc 9:1) to give **1b** as a yellow oil (400 mg, 71 %). $R_f = 0.41$ (cyclohexane/EtOAc 8:2); $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 2.14$ (s, 3H), 3.07 (s, 3H), 4.01 (s, 2H), 6.62 (d, 2H), 7.26 ppm (m, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 27.2$, 39.7, 63.3, 112.1, 117.4, 129.5, 148.8, 208.7 ppm; IR (neat): $\tilde{\nu} = 2957$ (CH, Ar), 1727 (C=O), 1599, 1505, 1348 cm$^{-1}$ (C–N); MS (ESI+): *m*/*z* (%): 164.1 $[M+H]^+$ (100), *m*/*z* calcd for $C_{10}H_{13}NO$: 163.1.

**Compound 1c**:[39] Glycidol (510 mg, 6.9 mmol, 1.5 equiv) and absolute ethanol (0.5 mL) were added to **1** (494 mg, 4.6 mmol, 1 equiv). The reaction mixture was MW irradiated (300 W) at 120 °C for 20 min. The volatile compounds were removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (cyclohexane/EtOAc 6:4). Compound **1c** was obtained as a white solid (673 mg, 81 %). $R_f = 0.26$ (cyclohexane/EtOAc 1:2); $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 3.22$ (brs, 2H), 3.39 (s, 3H), 3.75 (m, 2H), 3.95 (m, 1H), 4.19 (dd, $^3J(H,H) = 4.9$ Hz, 1H), 4.45 (m, 1H), 7.23 (m, 3H), 7.69 ppm (t, $^3J(H,H) = 2.7$ Hz, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 39.5$, 56, 64.4, 69.7, 113.3, 117.6, 129.4, 150.1 ppm; IR (neat): $\tilde{\nu} = 3290$ (OH), 2880 (CH), 1598 (C=C, Ar), 1507 (C=C, Ar), 1376 cm$^{-1}$; MS (ESI+): *m*/*z* (%): 182.2 $[M+H]^+$ (100); *m*/*z* calcd for $C_{10}H_{15}NO_2$: 181.1.

**Compound 2a**: Compound **2** (311 mg, 2.0 mmol, 1 equiv) and dry TEA (343 μL, 2.5 mmol, 1.25 equiv) were dissolved in dry $CH_2Cl_2$ (5 mL). The resulting mixture was cooled to 4 °C and kept under an Ar atmosphere. Then, mesyl chloride (1.1 equiv) was added dropwise and the reaction mixture was stirred at RT for 1 h. The newly formed precipitate (triethylammonium chloride) was removed by filtration and the filtrate was diluted with $CH_2Cl_2$ (10 mL), washed with brine (2 × 10 mL) and deionized water (20 mL), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The resulting mesylate derivative was dissolved in dry DMF (15 mL) and $NaN_3$ (402 mg, 6.2 mmol, 3.1 equiv) was added. The reaction mixture was stirred at 40 °C overnight. DMF was removed under reduced pressure. The residue was dissolved in $Et_2O$ (20 mL) and the organic layer was washed with deionized water (2 × 20 mL), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (cyclohexane/EtOAc 95:5). Compound **2a** was recovered as a yellow oil (233 mg, 64 %). $R_f = 0.70$ (cyclohexane/EtOAc 8:2); $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 3.01$ (s, 3H); 3.45 (t, $^3J(H,H) = 6.0$ Hz, 2H), 3.55 (t, $^3J(H,H) = 6.0$ Hz, 2H), 6.75 (m, 3H), 7.26 ppm (m, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 39.0$, 48.9, 52.2, 112.4, 117.1, 129.5, 148.6 ppm; IR (neat): $\tilde{\nu} = 2890$ (CH, Ar), 2090 ($N_3$), 1598, 1504, 1360, 1344 cm$^{-1}$; MS (ESI+): *m*/*z* (%): 177.2 $[M+H]^+$ (100); *m*/*z* calcd for $C_9H_{12}N_4$: 176.1.

**Compound 3a**:[40] Compound **3** (500 mg, 3.1 mmol, 1 equiv) and TEA (868 μL, 6.2 mmol, 2 equiv) were dissolved in dry THF (10 mL) and the solution was kept under an Ar atmosphere at 0 °C. Chloroacetyl chloride (370 μL, 4.6 mmol, 1.5 equiv) was added dropwise and the reaction mixture was stirred at RT for 1 h. The reaction was quenched by addition of deionized water (20 mL). The layers were separated by decantation and the aqueous phase was extracted with EtOAc (2 × 20 mL). The combined organic layers were sequentially washed with brine then deionized water, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (cyclohexane/EtOAc 9:1). Compound **3a** was recovered as a yellowish oil (582 mg, 79 %). $R_f = 0.52$ (cyclohexane/EtOAc 8:2); $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 3.18$ (dd, $^3J(H,H) = 5.2$, 5.2 Hz, 2H), 3.23 (dd, $^3J(H,H) = 5.2$, 5.2 Hz, 2H), 3.67 (dd, $^3J(H,H) = 5.2$, 5.2 Hz, 2H), 3.79 (dd, $^3J(H,H) = 5.2$, 5.2 Hz, 2H), 4.13 (s, 2H), 6.95 (m, 3H), 7.3 ppm (dd, $^3J(H,H) = 7.2$ Hz, $^4J(H,H) = 1.8$ Hz, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 40.8$, 42.1, 46.2, 49.3, 49.7, 116.8, 120.8, 129.31, 150.7, 165.3 ppm; MS (ESI+): *m*/*z* (%): 239.27 $[M+H]^+$ (100), 241.27 $[M+H]^+$ (40); *m*/*z* calcd for $C_{12}H_{15}ClN_2O$: 238.09.

**Compound 7**:[41] *Para*-hydroxybenzaldehyde (200 mg, 1.7 mmol, 1 equiv) was dissolved in dry acetone (3 mL) and solid $K_2CO_3$ (456 mg, 3.3 mmol, 1.9 equiv) was added. The mixture was stirred under an Ar atmosphere and a solution of propargyl bromide in toluene (80 % *w*/*w*, 453 μL, 4.1 mmol, 2.4 equiv) was added. The reaction mixture was stirred overnight under an Ar atmosphere at 60 °C. The volatile compounds were removed under reduced pressure. The residue was dissolved in $CH_2Cl_2$ (20 mL), washed with deionized water (2 × 20 mL), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (cyclohexane/EtOAc 90:10) to give **7** as a yellow solid (246 mg, 94 %). $R_f = 0.42$ (cyclohexane/EtOAc 8:2); $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 2.57$ (t, $^4J(H,H) = 2.4$ Hz, 1H), 7.08 (dt, $^3J(H,H) = 8.7$ Hz, $^4J(H,H) = 2.7$ Hz, 2H), 7.85 (dt, $^3J(H,H) = 8.7$ Hz, $^2J(H,H) = 2.7$ Hz, 2H), 9.89 ppm (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 56.0$, 76.5, 77.7, 115.3, 130.7, 132.0, 162.5, 190.9 ppm; IR (neat): $\tilde{\nu} = 3212$ (CH, alkyne), 1679 (C=O), 1248,

1006 $cm^{-1}$; MS (ESI+): *m/z* (%): 161.0 $[M+H]^+$ (100); *m/z* calcd for $C_{10}H_8O_2$: 160.0.

**General procedure for the synthesis of functionalized BHQ-3 dyes**: Methylene Violet 3RAX (50 mg, 132.5 μmol, 1 equiv) was dissolved in dry $CH_3CN$ (1 mL) and the solution was stirred at 0 °C for 15 min. Solid $NOBF_4$ (17 mg, 145.5 μmol, 1.1 equiv) was added and the reaction mixture was stirred at 0 °C for 15 min. The functionalized tertiary aniline (1.2 equiv) was dissolved in dry $CH_3CN$ (0.5 mL) and this solution was slowly added to the preformed *N*-nitrosamine/diazonium salt intermediate. The reaction mixture was stirred at 0 °C for a further 1 h. Reaction completion was assessed by RP-HPLC (system A). $CH_3CN$ was removed under reduced pressure and the crude product was purified by semi-preparative RP-HPLC (system B). The product-containing fractions were lyophilized to give the TFA salt of the BHQ-3 derivative as a blue amorphous powder.

**Quencher 4a**: Purification by semi-preparative RP-HPLC (system B), yield = 60%. HPLC (system A): retention time ($t_R$) = 25.9 min, purity = 99%; $^1$H NMR (300 MHz, [D$_4$]MeOH): $\delta$ = 1.08 (brt, 3H), 1.32 (brt, 3H), 1.84 (q, $^3J$(H,H) = 6.8 Hz, 2H), 2.28 (dt, $^4J$(H,H) = 2.6 Hz, $^3J$(H,H) = 6.8 Hz, 2H), 2.38 (t, $^4J$(H,H) = 2.6 Hz, 1H), 3.13 (s, 3H), 3.41 (brq, 2H), 3.62 (t, $^3J$(H,H) = 7.3 Hz, 2H), 3.81 (brq, 2H), 5.81 (d, $^4J$(H,H) = 2.5 Hz, 1H), 6.80 (d, $^3J$(H,H) = 9.3 Hz, 2H), 7.26 (d, $^4J$(H,H) = 1.8 Hz, 1H), 7.68 (m, 3H), 7.72 (s, 1H), 7.86 (dd, $^4J$(H,H) = 2.5 Hz, $^3J$(H,H) = 10.0 Hz, 1H), 7.12 (m, 2H), 8.28 ppm (d, $^3J$(H,H) = 9.0 Hz, 1H); $^{13}$C NMR (75 MHz, [D$_4$]MeOH): $\delta$ = 16.4, 26.7, 39.1, 47.9, 50.1, 52.2, 70.6, 84.1, 93.2, 112.0, 112.9, 121.8, 124.9, 127.9, 128.9, 132.9, 132.9, 133.8, 135.1, 135.8, 137.4, 139.3, 140.7, 145.0, 145.2, 154.9, 156.7, 157.8 ppm; IR (neat): $\tilde{\nu}$ = 2928, 1689, 1595, 1516, 1355, 1242, 1142, 1096 $cm^{-1}$; UV-Vis ($CH_3CN$): $\lambda_{max}$ = 658 nm; MS (ESI+): *m/z* (%): 527.33 $[M]^{+\bullet}$ (100); *m/z* calcd for $C_{34}H_{35}N_6^+$: 527.68.

**Quencher 4b**: Purification by semi-preparative RP-HPLC (system B), yield = 17%. HPLC (system A): $t_R$ = 24.7 min, purity = 93%; $^1$H NMR (200 MHz, $CD_3CN$): $\delta$ = 1.02 (brt, 3H), 1.27 (brt, 3H), 2.15 (s, 3H), 3.08 (s, 3H), 3.38 (brq, 2H), 3.69 (brq, 2H), 4.35 (s, 2H), 5.79 (d, $^4J$(H,H) = 2.5 Hz, 1H), 6.69 (d, $^3J$(H,H) = 9.2 Hz, 2H), 7.29 (d, $^4J$(H,H) = 1.8 Hz, 1H), 7.56 (m, 2H), 7.74 (d, $^3J$(H,H) = 9.2 Hz, 2H), 7.78 (m, 1H), 7.89 (m, 3H), 8.05 (d, $^3J$(H,H) = 9.8 Hz, 1H), 8.13 (dd, $^4J$(H,H) = 1.8 Hz, $^3J$(H,H) = 9.0 Hz, 1H), 8.30 ppm (d, $^3J$(H,H) = 9.0 Hz, 1H); too little of this compound was obtained to be well-characterized by $^{13}$C NMR spectroscopy; IR (neat): $\tilde{\nu}$ = 2916, 1725, 1686, 1631, 1589, 1516, 1467, 1349, 1242, 1136, 1090 $cm^{-1}$; UV-Vis ($CH_3CN$): $\lambda_{max}$ = 640 nm ($CH_3CN$); MS (ESI+): *m/z* (%): 517.33 $[M]^{+\bullet}$ (100); *m/z* calcd for $C_{32}H_{33}N_6O^+$: 517.64.

**Quencher 4c**: Purification by semi-preparative RP-HPLC (system B), yield = 6%. HPLC (system A): $t_R$ = 20.7 min, purity = 95%; $^1$H NMR (300 MHz, [D$_4$]MeOH): $\delta$ = 1.08 (brt, 3H), 1.30 (brt, 3H), 3.10 (s, 3H), 3.32 (brq, 2H), 3.47 (d, $^3J$(H,H) = 9.0 Hz, 2H), 3.62 (d, $^3J$(H,H) = 3.0 Hz, 1H), 3.67 (d, $^3J$(H,H) = 3.0 Hz, 2H), 3.72 (brq, 2H), 3.83 (m, 1H), 5.81 (d, $^4J$(H,H) = 2.5 Hz, 1H), 6.82 (d, $^3J$(H,H) = 9.3 Hz, 2H), 7.26 (d, $^4J$(H,H) = 1.8 Hz, 1H), 7.62 (m, 2H), 7.72 (d, $^3J$(H,H) = 9.3 Hz, 2H), 7.89 (m, 4H), 8.09 (d, $^3J$(H,H) = 10.0 Hz, 1H), 8.16 (dd, $^4J$(H,H) = 1.8 Hz, $^3J$(H,H) = 9.0 Hz, 1H), 8.30 ppm (d, $^3J$(H,H) = 9.0 Hz, 1H); too little of this compound was obtained to be well-characterized by $^{13}$C NMR spectroscopy; IR (neat): $\tilde{\nu}$ = 3244, 2916, 1680, 1586, 1510, 1467, 1349, 1300, 1239, 1178, 1121, 1066 $cm^{-1}$; UV-Vis ($CH_3CN$): $\lambda_{max}$ = 638 nm; MS (ESI+): *m/z* (%): 535.40 $[M]^{+\bullet}$ (100); *m/z* calcd for $C_{32}H_{35}N_6O_2^+$: 535.66.

**Quencher 4d**: Due to its poor chemical stability, this compound was only obtained in trace amounts and NMR characterization was not possible.

**Quencher 5a**: Purification by semi-preparative RP-HPLC (system B), yield = 64%. HPLC (system A): $t_R$ = 24.8 min, purity = 96%; $^1$H NMR (300 MHz, [D$_6$]DMSO): $\delta$ = 1.01 (brt, 3H), 1.26 (brt, 3H), 3.09 (s, 3H), 3.32 (brq, 2H), 3.51 (t, $^3J$(H,H) = 6.0 Hz, 2H), 3.64 (t, $^3J$(H,H) = 6.0 Hz, 2H), 3.73 (brq, 2H), 5.75 (d, $^4J$(H,H) = 2.5 Hz, 1H), 6.78 (d, $^3J$(H,H) = 9.0 Hz, 2H), 7.21 (d, $^4J$(H,H) = 1.8 Hz, 1H), 7.6 (m, 2H), 7.65 (d, $^3J$(H,H) = 9 Hz, 2H), 7.8 (dd, $^4J$(H,H) = 2.5 Hz, $^3J$(H,H) = 10.0 Hz, 1H), 7.89 (m, 3H), 8.04 (m, 2H), 8.22 ppm (d, $^3J$(H,H) = 9 Hz, 1H); $^{13}$C NMR (75 MHz, [D$_6$]DMSO): $\delta$ = 11.6, 14.0, 39.4, 52.4, 93.2, 111.8, 112.4, 113.1, 121.5, 125.1, 127.7, 128.9, 132.9, 133.0, 133.8, 134.9, 135.8, 137.3, 139.2, 140.6, 145.4, 154.6, 156.8, 157.5 ppm; IR (neat): $\tilde{\nu}$ = 2132, 2102, 1589, 1516, 1461, 1343, 1197, 1139, 1096 $cm^{-1}$; UV-Vis ($CH_3CN$): $\lambda_{max}$ = 636 nm; MS (ESI+): *m/z* (%): 530.20 $[M]^{+\bullet}$ (100); *m/z* calcd for $C_{31}H_{32}N_9^+$: 530.65.

**Quencher 5b**: Derivative **5a** (7 mg, 13.2 μmol, 1 equiv) and **7** (2.5 mg, 15.8 μmol, 1.2 equiv) were dissolved in 1:1 DMSO/$H_2O$ (3 mL). Aqueous solutions of sodium ascorbate (0.5 mg, 2.5 μmol, 0.19 equiv) and $CuSO_4$ pentahydrate (0.3 mg, 1.25 μmol, 0.09 equiv) were sequentially added and the reaction mixture was stirred at RT under an Ar atmosphere for 2 h. The reaction was monitored by RP-HPLC (system A). On completion, the mixture was diluted with an aqueous solution of TFA (0.1%, 4 mL) and purified by semi-preparative RP-HPLC (system B). After lyophilization, **5b** was obtained as a dark-blue amorphous powder (6.7 mg, 75%). HPLC (system A): $t_R$ = 26.1 min, purity = 95%; $^1$H NMR (300 MHz, [D$_6$]DMSO): $\delta$ = 1.00 (brt, 3H), 1.24 (brt, 3H), 2.90 (s, 3H), 3.36 (brd, 2H), 3.81 (brq, 2H), 3.99 (brt, 2H), 4.65 (brq, 2H), 5.23 (s, 2H), 5.71 (s, 1H), 6.71 (d, $^3J$(H,H) = 7.5 Hz, 2H), 7.1 (dd, $^4J$(H,H) = 2.2 Hz, $^3J$(H,H) = 8.7 Hz, 2H), 7.25 (m, 1H), 7.68 (d, $^3J$(H,H) = 7.2 Hz, 2H), 7.79 (m, 4H), 7.92 (m, 3H), 8.00 (d, $^3J$(H,H) = 9.9 Hz, 1H), 8.20 (m, 2H), 8.28 (d, $^4J$(H,H) = 2.4 Hz, 1H), 8.43 (dd, $^4J$(H,H) = 2.4 Hz, $^3J$(H,H) = 9.0 Hz, 1H), 9.83 ppm (s, 1H); $^{13}$C NMR (75 MHz, [D$_6$]DMSO): $\delta$ = 11.1, 13.7, 38.2, 46.5, 47.1, 51.5, 61.3, 91.5, 111.1, 111.8, 115.2, 118.6, 119.8, 124.6, 125.6, 126.4, 127.72, 129.8, 131.6, 131.8, 132.5, 133.9, 134.3, 135.7, 138.0, 139.0, 142.2, 143.3, 144.2, 152.9, 154.8, 155.5, 157.7, 158.2, 162.9, 191.4 ppm; IR (neat): $\tilde{\nu}$ = 1689, 1628, 1595, 1513, 1349, 1242, 1139, 1099 $cm^{-1}$; UV-Vis ($CH_3CN$): $\lambda_{max}$ = 640 nm; MS (ESI+): *m/z* (%): 690.20 $[M]^{+\bullet}$ (100); *m/z* calcd for $C_{41}H_{40}N_9O_2^+$: 690.81.

**Quencher 5c**: BHQ-3 derivative **5a** (10 mg, 18.5 μmol, 1 equiv) and **8** (6.25 mg, 22.5 μmol, 1.2 equiv) were dissolved in 1:1 DMSO/$H_2O$ (3 mL). Aqueous solutions of sodium ascorbate (0.7 mg, 3.7 μmol, 0.2 equiv) and $CuSO_4$ pentahydrate (0.1 mg, 1.85 μmol, 0.1 equiv) were sequentially added and the reaction mixture was stirred under an Ar atmosphere at RT for 2 h. The reaction was monitored by RP-HPLC (system A). On completion, the mixture was diluted with an aqueous solution of TFA (0.1%, 4 mL) and purified by semi-preparative RP-HPLC (system B). After lyophilization, **5c** was obtained as a dark-blue amorphous powder (10 mg, 67%). HPLC (system A): $t_R$ = 17.2 min, purity = 99%; $^1$H NMR (300 MHz, [D$_6$]DMSO): $\delta$ = 0.98 (brt, 3H), 1.23 (brt, 3H), 2.90 (s, 3H), 3.5–3.8 (m, 10H), 4.02 (brq, 2H), 4.66 (brq, 2H), 5.69 (m, 1H), 6.78(d, $^3J$(H,H) = 9.0 Hz, 2H), 7.23 (m, 1H), 7.70 (d, $^3J$(H,H) = 8.9 Hz, 2H), 7.76 (d, $^3J$(H,H) = 7.2 Hz, 2H), 7.95 (m, 3H), 7.98 (m, 1H), 8.18 (m, 3H), 8.25 (m, 1H), 8.40 (d, $^3J$(H,H) = 9.0 Hz), 8.61 ppm (s, 1H); $^{13}$C NMR (75 MHz, [D$_6$]DMSO): $\delta$ = 11.0, 13.6, 37.6, 41.1, 46.4, 47.2, 51.3, 63.7, 91.4, 111.1, 111.8, 119.8, 124.5, 126.3, 127.0, 127.6, 131.5, 132.4, 133.7, 137.2, 135.6, 137.8, 138.9, 142.6, 143.3, 144.0, 152.7, 154.6, 155.4, 158.9, 167.2, 170.9 ppm; IR (neat): $\tilde{\nu}$ = 3315, 2959, 1737, 1662, 1597, 1517, 1467, 1354, 1241, 1143, 828 $cm^{-1}$; UV-Vis (PBS): $\lambda_{max}$ = 610 nm; MS (ESI+): *m/z* (%): 404.67 $[M2H]^{2+}$, 808.27 $[M+H]^+$ (100); *m/z* calcd for $C_{39}H_{41}N_{11}O_7S$: 807.29.

**Quencher 6b**: Quencher **6a** was synthesized from Methylene Violet 3RAX and **3a** by the general procedure described above, and rapidly purified by triturating the crude with a mixture of $Et_2O$/MeOH (9:1). The recovered blue powder was dissolved in dry acetone (2 mL) and NaI (200 mg, 265 μmol, 10 equiv) was added. The reaction mixture was stirred at RT for 1 h. The reaction was monitored by RP-HPLC (system A). On completion, acetone was removed under reduced pressure and the crude product was purified by semi-preparative RP-HPLC (system B). After lyophilization, **6b** was obtained as a dark-blue amorphous powder (5 mg, 42% over two steps). HPLC (system A): $t_R$ = 25.1 min, purity = 95%; $^1$H NMR (300 MHz, [D$_6$]DMSO): $\delta$ = 0.98 (brt, 3H), 1.23 (brt, 3H), 3.36 (brq, 2H), 3.60 (m, 8H), 5.69 (d, $^4J$(H,H) = 2.2 Hz, 1H), 7.07 (d, $^3J$(H,H) = 9.2 Hz, 2H), 7.26 (d, $^4J$(H,H) = 1.9 Hz, 1H), 7.76 (m, 4H), 7.92 (m, 3H), 8.02 (dd, $^4J$(H,H) = 2.2 Hz, $^3J$(H,H) = 10.0 Hz, 1H), 8.19 (m, 2H), 8.43 ppm (d, $^3J$(H,H) = 9.2 Hz, 1H); $^{13}$C NMR (75 MHz, [D$_6$]DMSO): $\delta$ = 11.0, 13.7, 41.1, 45.6, 45.8, 46.5, 91.5, 111.6, 113.7, 119.7, 124.8, 126.3, 127.72, 131.63, 132.9, 133.8, 134.3, 135.7, 138.0, 139.0, 144.1, 144.5, 153.7, 154.8, 155.2, 157.7, 158.2, 166.7 ppm; IR (neat): $\tilde{\nu}$ = 1962, 1943, 1589, 1513, 1464, 1346, 1136, 1094 $cm^{-1}$; UV-Vis ($CH_3CN$): $\lambda_{max}$ =

612 nm; MS (ESI+): *m/z* (%): 684.13 $[M]^{+\bullet}$ (100); *m/z* calcd for $C_{34}H_{35}IN_7O^+$: 684.59.

**TR-Isonip (13)**

*Disulfonyl dichloride*: SR101 (40 mg, 66 μmol, 1 equiv) was dissolved in dry $CH_2Cl_2$ (1 mL) and the solution was kept under an Ar atmosphere at 0 °C. Oxalyl chloride (30 μL, 330 μmol, 6 equiv) was added dropwise, followed by a drop of dry DMF. The reaction mixture was warmed to RT and stirred for 3 h. The volatile compounds were removed under reduced pressure to give the crude disulfonyl dichloride derivative, which was used in the next step without purification.

*Aminolysis*: The crude sulfonyl chloride was dissolved in dry $CH_2Cl_2$ (1 mL) and the solution was kept under an Ar atmosphere at 0 °C. Dry TEA (93 μL, 660 μmol, 10 equiv) and a solution of ethyl isonipecotate (12.4 mg, 79 μmol, 1.2 equiv) in dry $CH_2Cl_2$ (0.5 mL) were sequentially added dropwise. The reaction mixture was stirred at RT for 2 h. The reaction was quenched by dilution with an aqueous solution of TFA (0.1%) and the crude product was purified by semi-preparative RP-HPLC (system F). The product-containing fractions were lyophilized to give the TFA salt of the SR101 derivative as a bluish-red amorphous powder.

*Acid hydrolysis of ethyl ester*: The ethyl ester was dissolved in 1,4-dioxane (1.5 mL) and an aqueous solution of HCl (6.0 M, 1.5 mL) was added dropwise. The reaction mixture was stirred at RT for 10 h. The reaction was quenched by dilution with an aqueous solution of TFA (0.1%) and the crude product was purified by semi-preparative RP-HPLC (system F). After lyophilization, the TFA salt of **13** was obtained as a purple amorphous solid (19 mg, 40% over three steps). HPLC (system I): $t_R$ = 22.0 min, purity > 99%; $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta$ = 1.57 (qd, $^4J$(H,H) = 3.7 Hz, $^3J$(H,H) = 11 Hz), 1.84 (sext, $^3J$(H,H) = 5.8 Hz, 4H), 1.94 (m, 2H), 2.02 (m, 4H), 2.64 (m, 6H), 2.98 (tt, $^3J$(H,H) = 6.3, 9.3 Hz, 4H), 3.48 (m, 8H), 3.63 (m, 2H), 6.51 (s, 2H), 7.40 (d, $^3J$(H,H) = 7.9 Hz, 1H), 7.87 (dd, $^4J$(H,H) = 1.88 Hz, $^3J$(H,H) = 7.9 Hz, 1H), 8.28 ppm (d, $^4J$(H,H) = 1.88 Hz, 1H); $^{13}$C NMR (100 MHz, [D$_6$]DMSO): $\delta$ = 19.2, 19.3, 20.2, 26.9, 27.1, 45.3, 49.7, 50.2, 104.2, 112.6, 122.6, 126.4, 127.1, 127.3, 131.1, 134.4, 136.6, 148.2, 150.5, 151.3, 154.1, 175.1 ppm; UV-Vis (PBS): $\lambda_{max}$ ($\varepsilon$) = 595 nm (85000 mol dm$^3$ cm$^{-1}$); fluorescence (PBS): $\lambda_{em}$ = 609 nm ($\Phi_F$ = 45%), MS (ESI+): *m/z* (%): 718.33 $[M+H]^+$ (100), *m/z* calcd for $C_{37}H_{39}N_3O_8S_2$: 717.22.

**Solid-phase peptide synthesis**: The SPS of peptides **12** and **23** was performed by using standard Fmoc/*t*Bu chemistry and the Rink Amide methylbenzhydrylamine (MBHA) resin (Novabiochem, 100–200 mesh, loading 0.50 or 0.59 mmol g$^{-1}$) on a scale of 0.25 mmol. Coupling reactions were performed with commercially available Fmoc-protected amino acids from Iris Biotech or Novabiochem (4 equiv) and *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU, 4 equiv), 1-hydroxybenzotriazole (HOBt, 4 equiv), and DIEA (12 equiv) in peptide-grade DMF. For peptide **12**, final acetylation of the free N-terminal amino group was achieved by treatment with $Ac_2O$ (10 equiv), DIEA (10 equiv), and 4-dimethylaminopyridine (DMAP, 0.1 equiv) in NMP (2 mL) for 2 h. The reaction was monitored by the Kaiser ninhydrin test (negative after this treatment) and the resin was washed with NMP (3×10 mL), MeOH (3×10 mL), and $CH_2Cl_2$ (3×10 mL). For peptide **23**, acylation of the N-terminal amino group with the NHS active ester of Eei-Aoaa-OH (4 equiv) was achieved in NMP (1 mL) in the presence of DIEA (4 equiv) at RT for 5 h. The reaction was monitored by the Kaiser ninhydrin test (negative after this treatment) and the resin was washed with NMP (3×10 mL), MeOH (3×10 mL), and $CH_2Cl_2$ (3×10 mL).

*Deprotection*: A pre-cooled solution of TFA (6 mL) and triisopropylsilane (TIPS) in deionized water (2.5% v/v) was added to the resin (350 mg). After 3 h of stirring at RT the resin was filtered and washed with TFA. The filtrate was evaporated to dryness and cold $Et_2O$ was added to the oily residue. The resulting white precipitate was isolated by centrifugation, lyophilized, and purified by semi-preparative RP-HPLC. The product-containing fractions were lyophilized to give the TFA salt of the targeted peptide as a white amorphous powder.

**Peptide 12**: Purification by semi-preparative RP-HPLC (system C), yield = 54%. HPLC (system D): $t_R$ = 13.95 min, purity = 94%; MS (ESI+): *m/z* (%): 1022.33 $[M+H]^+$ (100); *m/z* calcd for $C_{39}H_{71}N_{15}O_{13}S_2$: 1021.48.

**Peptide 23**: Purification by semi-preparative RP-HPLC (system C), yield = 28%. MS (ESI+): *m/z* (%): 431.80 $[M+2H]^{2+}$ (25), 862.33 $[M+H]^+$ (100); *m/z* calcd for $C_{32}H_{59}N_{15}O_{13}$: 861.90.

**Peptide labeling**

*N-acylation of **12** with the NHS active ester of **13***: Compound **13** (4.0 mg, 5.6 μmol, 1 equiv) was dissolved in NMP (75 mL). (*O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TSTU; 1.85 mg, 6.1 μmol, 1.1 equiv) and DIEA (2.0 M in NMP, 16 μL, 32 μmol, 5 equiv) were sequentially added. The reaction mixture was periodically vortexed over a 1 h period. The reaction was monitored by RP-HPLC (system I). On completion, a solution of peptide **12** (9.5 mg, 6.7 μmol, 1.2 equiv) in NMP (75 μL) was added and the mixture was periodically vortexed until the fluorescent labeling was found to be complete (monitored by RP-HPLC). The mixture was diluted with an aqueous solution of TFA (0.1%) and the crude product was purified by semi-preparative RP-HPLC (system E). The product-containing fractions were lyophilized to give peptide **14** as a purple amorphous powder. HPLC (system I): $t_R$ = 20.7 min, purity = 98%; MS (ESI+): *m/z* (%): 861.20 $[M+2H]^{2+}$ (100), 1721.33 $[M+H]^+$, (70); MS (ESI−): *m/z* (%): 1833.13 $[M+TFA-H]^-$ (10), 1947.07 $[M+2TFA-H]^-$ (100); *m/z* calcd for $C_{77}H_{109}N_{17}O_{20}S_4$: 1720.69.

*N-acylation of **12** with the NHS active ester of Cy 5.0*: Cy 5.0 dye (5.0 mg, 7.6 μmol, 1 equiv) was dissolved in NMP (100 mL). TSTU (2.4 mg, 8 μmol, 1.05 equiv) and DIEA (2.0 m in NMP, 21 μL, 42 mmol, 5.5 equiv) were sequentially added. The reaction mixture was periodically vortexed over a 1 h period. The reaction was monitored by RP-HPLC (system D). A solution of peptide **12** (11.4 mg, 8.4 μmol, 1.1 equiv) in NMP (100 μL) of was added and the mixture was periodically vortexed until the fluorescent labeling was found to be complete (monitored by RP-HPLC). The mixture was diluted with an aqueous solution of TFA (0.1%) and the crude product was purified by semi-preparative RP-HPLC (system E). The product-containing fractions were lyophilized to give peptide **15** as a blue-purple amorphous powder. HPLC (system D): $t_R$ = 16.9 min, purity > 99%; MS (ESI+): *m/z* (%): 830.93 $[M+2H]^{2+}$ (100), 1661.20 $[M+H]^+$, (40); MS (ESI−): *m/z* (%): 1659.27 $[M-H]^-$ (5), 1772.60 $[M+TFA-H]^-$ (100); *m/z* calcd for $C_{72}H_{109}N_{17}O_{20}S_4$: 1659.69.

*N-acylation of **12** with the NHS active ester of NIR5.5-WS*: NIR 5.5-WS dye (5.0 mg, 5.05 μmol, 1 equiv) was dissolved in NMP (75 mL). TSTU (1.67 mg, 5.55 μmol, 1.1 equiv) and DIEA (2.0 m in NMP, 13 μL, 26 mmol, 5 equiv) were sequentially added. The reaction mixture was periodically vortexed over a 1 h period. The reaction was monitored by mass spectroscopy. A solution of peptide **12** (9.0 mg, 6.06 μmol, 1.2 equiv) in 75 μL of NMP was added and the mixture was periodically vortexed until the fluorescent labeling was found to be complete (monitored by RP-HPLC). The mixture was diluted with an aqueous solution of TFA (0.1%) and the crude product was purified by semi-preparative RP-HPLC (system E). The product-containing fractions were lyophilized to give peptide **16** as a blue-green amorphous powder. HPLC (system G): $t_R$ = 23.2 min, purity > 99%; MS (ESI+): *m/z* (%) 997.53 $[M+2H]^{2+}$ (85), 1995.40 $[M+H]^+$ (100); MS (ESI−): *m/z* (%): 996.73 $[M-2H]^{2-}$ (100); *m/z* calcd for $C_{88}H_{126}N_{18}O_{25}S_5$: 1995.77.

**General procedure for removal of the StBu protecting group**: Fluorescently labeled peptide was dissolved in an aqueous solution of $NaHCO_3$ (0.1 M, pH 8.5, 400 μL) and a solution of DTT (17.6 mg, 115 μmol, 14 equiv) in an aqueous solution of $NaHCO_3$ (0.1 M, 100 μL). The reaction mixture was protected from light (with aluminum foil) and stirred at RT for 1 h. The reaction was monitored by RP-HPLC (system E). The mixture was diluted with an aqueous solution of TFA (0.1%, 4 mL) and the crude product was purified by semi-preparative RP-HPLC. The product-containing fractions were lyophilized to give the free-sulfhydryl fluorescent peptide.

**Peptide 17**: Purification by semi-preparative RP-HPLC (system E), bluish-red amorphous powder. HPLC (system I): $t_R$ = 19.6 min, purity = 96%; MS (ESI+): *m/z* (%): 817.60 $[M+2H]^{2+}$ (100), 1634.47 $[M+H]^+$ (40); MS (ESI−): *m/z* (%): 1632.60 $[M-H]^-$ (5), 1745.33 $[M+TFA-H]^-$

(10), 1859.33 $[M+2TFA-H]^-$ (100), *m/z* calcd for $C_{72}H_{99}N_{17}O_{21}S_3$ 1633.64.

**Peptide 18**: Purification by semi-preparative RP-HPLC (system E), blue-purple amorphous powder. HPLC (system D): $t_R = 15.1$ min, purity = 94%; MS (ESI+): *m/z* (%): 787.40 $[M+2H]^{2+}$ (65), 1573.27 $[M+H]^+$, (100); MS (ESI−): *m/z* (%): 1570.47 $[M-H]^-$ (25), 1684.87 $[M+TFA-H]^-$ (100), 1819.67 $[M+Na+2TFA-2H]^-$ (25); *m/z* calcd for $C_{68}H_{101}N_{17}O_{20}S_3$: 1571.66.

**Peptide 19**: Purification by semi-preparative RP-HPLC (system E), blue-purple amorphous powder. HPLC (system D): $t_R = 22.0$ min, purity = 98%; MS (ESI+): *m/z* (%): 955.27 $[M+2H]^{2+}$ (100), 1908.40 $[M+H]^+$ (30); MS (ESI−): *m/z* (%): 1906.40 $[M-H]^-$ (100); *m/z* calcd for $C_{83}H_{117}N_{19}O_{25}S_4$: 1907.74.

**General procedure for the labeling of free-sulfhydryl peptides with α-iodoacetyl-modified BHQ-3 6b**: The free-thiol peptide (1 equiv) was dissolved in an aqueous solution of $NaHCO_3$ (0.1 M, pH 8.5, 200 μL) and **6b** (1.5 equiv) was added. The reaction mixture was protected from light (with aluminum foil) and stirred at RT for 2 h. The reaction was monitored by RP-HPLC. The mixture was diluted with an aqueous solution of TFA (0.1%, 4 mL) and the crude product was purified by semi-preparative RP-HPLC. The product-containing fractions were lyophilized to give the uPA-sensitive fluorescence-quenched probe. A stock solution of the fluorogenic uPA substrate was prepared in Milli-Q ultrapure water (except in the case of **19**, for which 9:1 water/DMSO was required for complete solubilization) and UV-Vis quantification was achieved upon complete hydrolysis with uPA protease by comparison of the fluorescence emission intensity at $\lambda_{max} = 609$, 663, or 700 nm with that of a solution of the free dye of known concentration (measured at $\lambda = 595$ nm, $\varepsilon = 85000\ mol^{-1}dm^3cm^{-1}$ for **13**; $\lambda = 650$ nm, $\varepsilon = 250000\ mol^{-1}dm^3cm^{-1}$ for Cy 5.0; $\lambda = 679$ nm, $\varepsilon = 197000\ mol^{-1}dm^3cm^{-1}$ for NIR5.5-WS).

**Peptide 20**: Purification by semi-preparative RP-HPLC (system H), 25% (over three steps), dark-blue amorphous powder. HPLC (system I): $t_R = 27.0$ min, purity = 98%; MS (ESI+): *m/z* (%): 1095.27 $[M^+ + H]^{2+}$ (100), 730.73 $[M^+ + 2H]^{3+}$ (25); *m/z* calcd for $C_{106}H_{134}N_{25}O_{21}S_3^+$: 2188.93.

**Peptide 21**: Purification by semi-preparative RP-HPLC (system E), 14% (over three steps), dark-blue amorphous powder. HPLC (system H): $t_R = 20.2$ min, purity = 98%; MS (ESI+): *m/z* (%): 710.20 $[M+3H]^{3+}$ (100), 1064.93 $[M+2H]^{2+}$ (55); *m/z* calcd for $C_{102}H_{134}N_{24}O_{21}S_3$: 2126.93.

**Peptide 22**: Purification by semi-preparative RP-HPLC (system H), 5% (over three steps), dark-blue amorphous powder. HPLC (system E): $t_R = 25.2$ min, purity = 96%; MS (ESI+): *m/z* (%): 822.40 $[M+3H]^{3+}$ (100), 1233.33 $[M+2H]^{2+}$ (35); *m/z* calcd for $C_{117}H_{150}N_{26}O_{26}S_4$: 2463.01.

**One-pot, two-step peptide labeling (24)**: Aoaa-terminated peptide **23** (2.2 mg, 2.55 μmol, 1 equiv) was dissolved in aqueous NaOAc buffer (0.1 M, pH 4.4) and a solution of **5b** (1.7 mg, 2.50 μmol, 1 equiv) in $CH_3CN$ (5 μL) was added. The reaction mixture was periodically vortexed was monitored by RP-HPLC (system I). Upon complete consumption of **5b**, the pH was adjusted to approximately pH 8 by addition of aqueous $NaHCO_3$ buffer and the reaction mixture was cooled to 4 °C. The NHS active ester of Cy 5.0 (5 μmol, prepared as described above) was slowly added and the reaction mixture was kept at 4 °C for 2 h (periodically vortexed). The reaction was monitored by RP-HPLC (system I). Thereafter, the mixture was diluted with an aqueous solution of TFA (0.1%) and the crude product was purified by semi-preparative RP-HPLC (system E). After lyophilization, **24** was obtained as a dark-blue amorphous powder (1.8 mg, 20%). A stock solution of **24** was prepared in Milli-Q ultrapure water and UV-Vis quantification was achieved upon complete hydrolysis with uPA protease, by comparison of the fluorescence emission intensity at $\lambda_{max} = 665$ nm with that of a solution of free Cy 5.0 dye of known concentration (measured at $\lambda = 650$ nm, $\varepsilon = 250000\ mol^{-1}dm^3cm^{-1}$). HPLC (system I): $t_R = 18.8$ min, purity = 97%; MS (ESI+): *m/z* (%): 725.00 $[M+3H]^{3+}$ (100), 1087.07 $[M+2H]^{2+}$ (85); *m/z* calcd for $C_{106}H_{133}N_{25}O_{22}S_2$: 2171.95.

**General procedure for in vitro peptide cleavage by uPA**: A solution of fluorogenic peptide (1.0 μM) was prepared in PBS (45 μL) and transferred into an ultra-micro quartz fluorescence cell. A solution of uPA (25 μg) in buffer [100 μL; Tris-HCl (500 mM) < M + > NaCl (1.0 M) < M + > PEG 6000 (1%) + mannitol (2.0 M)] (5 μL, 0.6 U) was added and the mixture was incubated at 37.5 °C. After excitation at $\lambda = 580$ (for **20**), 650 (for **21** and **24**), or 670 nm (for **22**), fluorescence emission at $\lambda = 608$ (for **20**), 665 (for **21** and **24**), or 700 nm (for **22**) was monitored over time, with measurements recorded every 5 s.

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[1] a) R. T. Ranasinghe, T. Brown, *Chem. Commun.* **2005**, 5487–5502; b) R. P. Haugland, *The Handbook. A Guide to Fluorescent Probes and Labeling Technologies*, 11th ed., Invitrogen, Oregon, **2006**; c) C. R. Drake, D. C. Miller, *Curr. Org. Synth.* **2011**, *8*, 498–520.

[2] K. E. Sapsford, L. Berti, I. L. Medintz, *Angew. Chem.* **2006**, *118*, 4676–4704; *Angew. Chem. Int. Ed.* **2006**, *45*, 4562–4588.

[3] R. M. Cook, M. Lyttle, D. Dick, (Biosearch Technologies, Inc.), WO01/86001 A1, **2001**.

[4] F. d. S. Santos, R. R. Descalzo, P. F. B. Goncalves, E. V. Benvenutti, F. S. Rodembusch, *Phys. Chem. Chem. Phys.* **2012**, *14*, 10994–11001 and references cited therein.

[5] For cyanine-based broad-range quencher dyes, see: a) *GE Healthcare - Product Booklet*, *Vol.18–1146–98*, https://www.gelifesciences.-com/gehcls images/GELS/Related%20Content/Files/1314729545976/litdoc1314718114698 20110830214950.pdf; b) X. Peng, H. Chen, D. R. Draney, W. Volcheck, A. Schutz-Geschwender, D. M. Olive, *Anal. Biochem.* **2009**, *388*, 220–228; c) O. Redy, E. Kisin-Finfer, E. Sella, D. Shabat, *Org. Biomol. Chem.* **2012**, *10*, 710–715.

[6] R. P. Haugland, V. L. Singer, S. T. Yue, (Molecular Probes, Inc.), US6,399,392 B1, **2002**.

[7] Y. Kong, H. Yao, H. Ren, S. Subbian, S. L. G. Cirillo, J. C. Sacchettini, J. Rao, J. D. Cirillo, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 12239–12244.

[8] For other quenchers with broadened absorbance properties, see: a) W. Pham, R. Weissleder, C.-H. Tung, *Angew. Chem.* **2002**, *114*, 3811–3814; *Angew. Chem. Int. Ed.* **2002**, *41*, 3659–3662; b) W. Pham, R. Weissleder, C.-H. Tung, *Tetrahedron Lett.* **2003**, *44*, 3975–3978; c) K. Kopecky, V. Novakova, M. Miletin, R. Kucera, P. Zimcik, *Bioconjugate Chem.* **2010**, *21*, 1872–1879; d) P. Crisalli, E. T. Kool, *Bioconjugate Chem.* **2011**, *22*, 2345–2354; e) K. Kopecky, V. Novakova, M. Miletin, R. Kucera, P. Zimcik, *Tetrahedron* **2011**, *67*, 5956–5963; f) K. Kopecky, D. Satinsky, V. Novakova, M. Miletin, A. Svoboda, P. Zimcik, *Dyes Pigm.* **2011**, *91*, 112–119.

[9] For selected examples, see: a) P. Jothikumar, V. Hill, J. Narayanan, *BioTechniques* **2009**, *46*, 519–520, 524; b) K. Kiyose, K. Hanaoka, D. Oushiki, T. Nakamura, M. Kajimura, M. Suematsu, H. Nishimatsu, T. Yamane, T. Terai, Y. Hirata, T. Nagano, *J. Am. Chem. Soc.* **2010**, *132*, 15846–15848; c) A. V. Popov, T. M. Mawn, S. Kim, G. Zheng, E. J. Delikatny, *Bioconjugate Chem.* **2010**, *21*, 1724–1727; d) J. H. Ryu, A. Lee, S. Lee, C.-H. Ahn, J. W. Park, J. F. Leary, S. Park, K. Kim, I. C. Kwon, I.-C. Youn, K. Choi, *Bioconjugate Chem.* **2010**, *21*, 1378–1384; e) T. M. Mawn, A. V. Popov, N. J. Beardsley, K. Stefflova, M. Milkevitch, G. Zheng, E. J. Delikatny, *Bioconjugate*

  

*Chem.* **2011**, *22*, 2434–2443; f) X. Liu, R. Freeman, I. Willner, *Chem. Eur. J.* **2012**, *18*, 2207–2211.

[10] For a recent review about the CuAAC reaction, see: M. Meldal, C. W. Tornoe, *Chem. Rev.* **2008**, *108*, 2952–3015.

[11] For reviews about peptide-based FRET probes, see: a) C.-H. Tung, *Biopolymers* **2004**, *76*, 391–403; b) X. Chen, M. Sun, H. Ma, *Curr. Org. Chem.* **2006**, *10*, 477–489.

[12] a) B. Law, A. Curino, T. H. Bugge, R. Weissleder, C.-H. Tung, *Chem. Biol.* **2004**, *11*, 99–106; b) R. Malik, S. Qian, B. Law, *Anal. Biochem.* **2011**, *412*, 26–33.

[13] G. Ewing, K. Mullah, R. Graham, (Applera Corporation), WO03/019145 A2, **2003**.

[14] For a review about the synthesis of azobenzenes, see: F. Hamon, F. Djedaini-Pilard, F. Barbot, C. Len, *Tetrahedron* **2009**, *65*, 10105–10123.

[15] For comprehensive reviews about bioorthogonal/biocompatible reactions recently developed for bioconjugation, see: a) E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108–7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998; b) J. Kalia, R. T. Raines, *Curr. Org. Chem.* **2010**, *14*, 138–147.

[16] G. Leriche, "Etude de fonctions chimiques clivables en milieux biologiques et leurs applications en protéomique chimique et imagerie de fluorescence", Ph. D. Université de Strasbourg (Strasbourg, France), **2012**.

[17] C. Massif, S. Dautrey, A. Haefele, R. Ziessel, P.-Y. Renard, A. Romieu, *Org. Biomol. Chem.* **2012**, *10*, 4330–4336.

[18] A. Romieu, C. Massif, S. Rihn, P.-Y. Renard, G. Ulrich, R. Ziessel, *Org. Biomol. Chem.* **2012**, manuscript in preparation.

[19] M. K. Johansson, R. M. Cook, *Chem. Eur. J.* **2003**, *9*, 3466–3471.

[20] M. Ogawa, N. Kosaka, P. L. Choyke, H. Kobayashi, *ACS Chem. Biol.* **2009**, *4*, 535–546.

[21] See the Supporting Information for the synthesis of **9**.

[22] Determined by us because the extinction coefficient of the BHQ-3 dye has never been reported in the literature, in particular by Biosearch Technologies.

[23] E. Brustad, M. L. Bushey, J. W. Lee, D. Groff, W. Liu, P. G. Schultz, *Angew. Chem.* **2008**, *120*, 8344–8347; *Angew. Chem. Int. Ed.* **2008**, *47*, 8220–8223.

[24] J. D. A. Tyndall, M. J. Kelso, P. Clingan, M. Ranson, *Recent Pat. Anti-Cancer Drug Discovery* **2008**, *3*, 1–13.

[25] a) J. A. Titus, R. Haugland, S. O. Sharrow, D. M. Segal, *J. Immunol. Methods* **1982**, *50*, 193–204; b) C. Lefevre, H. C. Kang, R. P. Haugland, N. Malekzadeh, S. Arttamangkul, R. P. Haugland, *Bioconjugate Chem.* **1996**, *7*, 482–489.

[26] J. E. T. Corrie, C. T. Davis, J. F. Eccleston, *Bioconjugate Chem.* **2001**, *12*, 186–194.

[27] a) R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, A. S. Waggoner, *Bioconjugate Chem.* **1993**, *4*, 105–111; b) A. Romieu, D. Brossard, M. Hamon, H. Outaabout, C. Portal, P.-Y. Renard, *Bioconjugate Chem.* **2008**, *19*, 279–289.

[28] For examples of one-pot multi-step bioconjugation procedures, see: a) R. Mhidia, A. Vallin, N. Ollivier, A. Blanpain, G. Shi, R. Christiano, L. Johannes, O. Melnyk, *Bioconjugate Chem.* **2010**, *21*, 219–228; b) M. Galibert, O. Renaudet, P. Dumy, D. Boturyn, *Angew. Chem. Int. Ed.* **2011**, *50*, 1941–1944.

[29] S. Foillard, M. Ohsten Rasmussen, J. Razkin, D. Boturyn, P. Dumy, *J. Org. Chem.* **2008**, *73*, 983–991.

[30] J. Shao, J. P. Tam, *J. Am. Chem. Soc.* **1995**, *117*, 3893–3899.

[31] D. Forget, O. Renaudet, D. Boturyn, E. Defrancq, P. Dumy, *Tetrahedron Lett.* **2001**, *42*, 9171–9174.

[32] P. Crisalli, A. R. Hernandez, E. T. Kool, *Bioconjugate Chem.* **2012**, *23*, 1969–1980.

[33] a) S. Lee, J. H. Ryu, K. Park, A. Lee, S.-Y. Lee, I.-C. Youn, C.-H. Ahn, S. M. Yoon, S.-J. Myung, D. H. Moon, X. Chen, K. Choi, I. C. Kwon, K. Kim, *Nano Lett.* **2009**, *9*, 4412–4416; b) S. Lee, J. Xie, X. Chen, *Curr. Top. Med. Chem.* **2010**, *10*, 1135–1144.

[34] G. B. Fields, R. L. Noble, *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.

[35] G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg, *Organometallics* **2010**, *29*, 2176–2179.

[36] J. Olmsted, III, *J. Phys. Chem.* **1979**, *83*, 2581–2584.

[37] See: http://omlc.ogi.edu/spectra/PhotochemCAD/html/012.html.

[38] M. E. Jung, B. E. Love, *J. Chem. Soc. Chem. Commun.* **1987**, 1288–1290.

[39] S. A. Elfeky, W. Chen, J. S. Fossey, S. E. Flower, N. Masumoto, F. D'Hooge, L. Labarthe, T. D. James, C. Len, *Chem. Asian J.* **2010**, *5*, 581–588.

[40] a) D. A. Brown, P. S. Kharkar, A. K. Dutta, I. Parrington, M. E. A. Reith, *J. Med. Chem.* **2008**, *51*, 7806–7819; b) X. Zhang, X. Yang, S. Zhang, *Synth. Commun.* **2009**, *39*, 830–844.

[41] J.-B. Giguère, D. Thibeault, F. Cronier, J.-S. Marois, M. Auger, J.-F. Morin, *Tetrahedron Lett.* **2009**, *50*, 5497–5500.

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