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Universal Dark Quencher Based on "Clicked" Spectrally Distinct Azo Dyes

Arnaud Chevalier,[†] Julie Hardouin,[‡] Pierre-Yves Renard,*[,†] and Anthony Romieu*[,†,§,∥]

Normandie Université, COBRA UMR 6014 & FR 3038, UNIV Rouen, INSA Rouen, CNRS, IRCOF, 1 Rue Tesnières, 76821 Mont-Saint-Aignan Cedex, France, Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005 Paris, France, and Laboratory PBS, UMR 6270 & FR 3038, Bât. Dulong, 76821 Mont-Saint-Aignan Cedex, France

pierre-yves.renard@univ-rouen.fr; anthony.romieu@u-bourgone.fr

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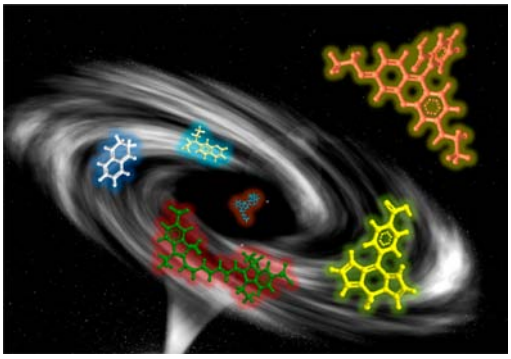
ABSTRACT



The first synthesis of an heterotrifunctional molecular scaffold derived from the popular DABCYL azo dye quencher has been achieved. The sequential derviatization of this trivalent azobenzene derivative with two other nonfluorescent azo dyes (Black Hole Quencher BHQ-1 and BHQ-3) and through effective reactions from the "bioconjugation chemistry" repertoire has led to an universal dark quencher (UDQ). This "clicked" poly azo dye is able to turn off an array of fluorophores covering the UV/NIR (300−750 nm) spectral range.

Over the past two decades, tremendous developments in the field of biomedical imaging have been achieved and have resulted in the transformation of anatomical imaging to molecular-specific imaging.[1]

Among the different functional imaging modalities now commonly used, those involving sensitive and quantitative detection of transmitted photons (i.e., bioluminescence and fluorescence optical imaging) are considered to be powerful tools to investigate mechanisms of disease and accelerate drug development in preclinical models. The successful implementation of fluorescence intensity imaging requires the use of a fluorescent probe capable of accurately reporting the location of its targeted bioanalyte. Optical imaging contrast agents that exhibit significant changes in their spectroscopic properties (i.e., fluorescence intensity or excitation/emission maximum wavelengths modifications) upon interaction with the intended target are preferred to obtain the best signal-to-background ratio.[2] A common approach to develop such "activatable" probes relies on the implementation of the Förster resonance energy transfer (FRET) mechanism through the attachment of a complementary fluorophore (donor) and quencher (acceptor) pair at either side of a cleavable substrate chosen to react selectively with the bioanalyte.[3] The efficacy of energy transfer is dependent on the spectral overlap between the donor emission and acceptor absorption, the distance between them, and their relative orientation. Consequently, a variety of nonfluorescent chromophores

[†] Laboratory COBRA, UMR 6014.
[‡] Laboratory PBS, UMR 6270.
[§] IUF.
[∥] Université de Bourgogne - UFR Sciences et Techniques, Faculté des Sciences Mirande, ICMUB - UMR CNRS 6302, Equipe "P2DA" - 9, avenue Alain Savary, BP 47870, 21078 DIJON Cedex, France.

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have been developed to offer specific quenchers for different emission ranges either in the UV−vis region or into the NIR spectrum. Among the many different structures reported in the literature and/or commercially available, the most popular are undoubtedly (1) cyanine dyes whose native fluorescence is abolished by intramolecular charge transfer (ICT) or photoinduced electron transfer (PeT) through the incorporation of electron-donating and/or -withdrawing groups (typically *N*,*N*-dialkylamino or nitro groups) within their core structures (e.g., IRDye QC-1 from LI-COR Biosciences),[4] (2) azo dyes[5] whose nonemissive features are 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 (e.g., BHQ dyes from Biosearch Technologies),[6] and (3) *N*,*N*′-diarylrhodamine derivatives developed by Invitrogen Molecular Probes and known as the trademark QSY.[7] However, as displayed in Figure 1, no single compound belonging to these dye classes is able to quench all fluorophores emitting in the broad UV/NIR spectral range.

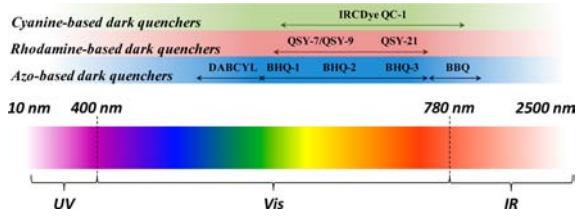

Figure 1. Wavelength range of commonly used commercially available dark quenchers.

However, the availability of an universal dark quencher (UDQ) that is effective over this ultrawide wavelength range should simplify the construction of multicolored families of fluorogenic substrates for multiplexed bioanalyses and high-throughput imaging assays. In this context, the Kool group has recently reported an elegant molecular approach to broaden the absorption spectrum (and thus quenching efficiency) of popular azo dyes, namely DABCYL and BHQ-2.[8] The resulting multipath quenchers (MPQ) have been designed to have multiple donor or acceptor groups in their structure, allowing for a multiplicity of conjugation pathways of varied length. The lead compound, namely MPQ6, possesses a valuable spectrum width (full-width half-maximum, $\Delta\lambda_{1/2\ \max}$) of 270 nm, ranging from 460 to 730 nm, but its ability to quench UV fluorophores was not investigated. A structurally simpler quencher structure derived from an anthraquinone scaffold (acide blue 40) was also identified and found to be effective in almost the same visible/NIR spectral range.[9] These two independent studies support the fact that it is virtually impossible to design an UDQ only through chemical modifications of a single quencher structure. Thus, an alternative approach based on the covalent association of several different nonfluorescent dyes through high-yielding conjugation reactions, deserves to be explored.

We report here the practical implementation of this new promising strategy by using DABCYL, BHQ-1, and BHQ-3 as the three azo dye components of the targeted UDQ **1** (see Scheme 3). Its effective quenching range is assessed in the context of disulfide-based FRET pairs involving a variety of fluorophores that range from UV-A (naphthalene) to NIR emission (sulfoindocyanine dye Cy 7.0).

The key issue for the use of this multiple azo dyes assembly methodology for the synthesis of **1** is the availability of an heterotrifunctional quencher core. Since such a diazo compound has never been described in the literature, we started from scratch, designing a DABCYL analogue equipped with easily derivatizable terminal alkyne and thiol moieties for the grafting of BHQ-1 and BHQ-3 dyes through copper-mediated azide−alkyne 1,3-dipolar cycloaddition (CuAAC) and S_N2 thio-alkylation reactions, respectively.[10] The third reactive group, namely carboxylic acid, is required for (bio)conjugation purposes involving the UDQ **1**. Our choice for a relatively cheap benzenic building block as starting material resulted in the use of 3-amino-5-nitrobenzoic acid because we thought that the presence of a nitro group within the resulting DABCYL-like core structure should enhance its "native" quenching ability linked to the photoinduced motions of N=N double bond through the PeT process.

As depicted in Scheme 1, a practical seven-step synthetic route to the key heterotrifunctional DABCYL analogue **6** was developed. First, alkylation of *N*-phenyldiethanolamine with propargyl bromide under phase-transfer conditions has enabled us to differentiate its two identical primary alcohols. Purification by flash column chromatography led to the monoterminal alkyne **2** in a good 72% yield. The remaining hydroxyl group of **2** was then readily converted into a primary amine using a sequence of mesylation, azidation, and Staudinger reduction to give the unsymmetrical *N*,*N*-disubstituted aniline **3**. Thereafter, amidification of **3** with 3,3′-dithiodipropanoic acid (DTDP) was achieved with BOP/DIEA in dry CH_3CN to provide the dianiline disulfide derivative **4**. We found that the disulfide bridge acts as a valuable protecting group for the thiol moiety, especially to avoid undesired side

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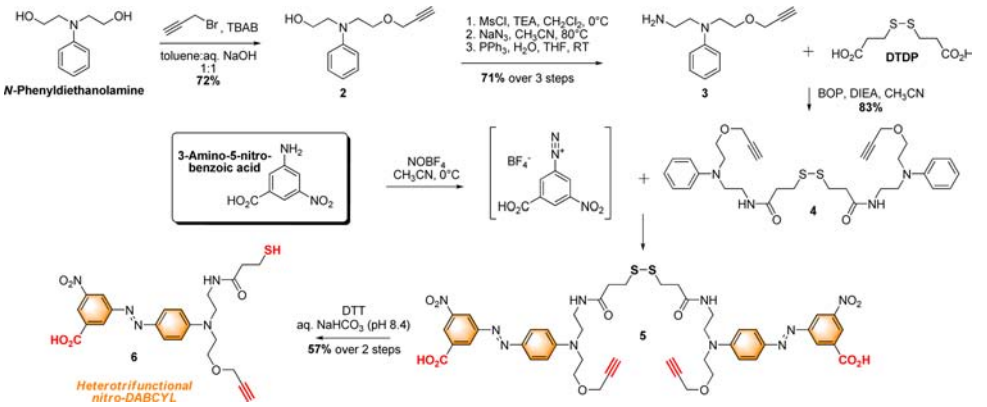
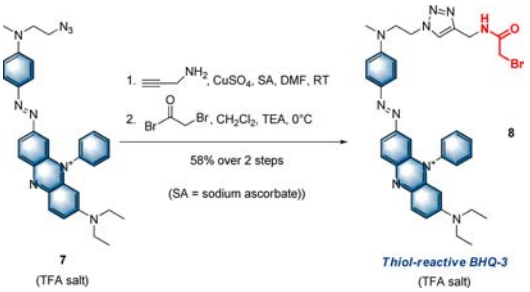
Scheme 1. Synthesis of Heterotrifunctional Nitro-DABCYL Dye (TFA Salt) **6**



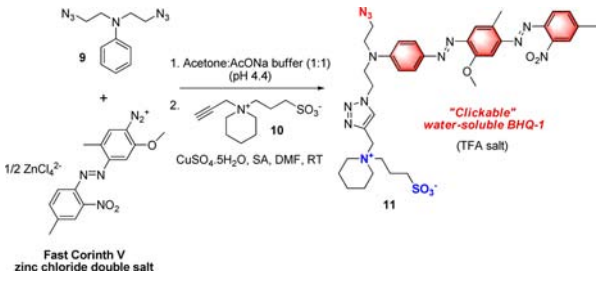
reactions (e.g., S_NAr) of such nucleophiles during the next azo coupling reaction involving the diazonium intermediate of 3-amino-5-nitrobenzoic acid. Furthermore, the chemical intertness of azo dyes toward dithiothreitol (DTT) recently highlighted by the Wagner group[11] has enabled us to consider the use of this mild reducing agent to easily convert the dimeric diazo compound **5** into the targeted heterotrifunctional nitro-DABCYL **6**. Purification was achieved by semipreparative RP-HPLC to give **6** in a satisfying overall yield (57% for the two last steps). Its structure was unambiguously confirmed by detailed measurements, including ESI-HRMS and NMR analyses (see the Supporting Information). Next, we focused on the preparation of functionalized BHQ-1 and BHQ-3 dyes that bear a reactive handle (azido and α-bromoacetyl group, respectively) suitable for their chemoselective conjugation to the nitro-DABCYL quencher **6**. The thiol-reactive BHQ-3 **8** was readily obtained from the known azido-modified derivative **7**[5] through sequential CuAAC reaction with propagylamine and subsequent alkylation with α-bromoacetyl bromide (Scheme 2). The synthesis of the "click-convertible" BHQ-1 **11** was achieved by azo coupling reaction between the commercially available Fast Corinth V diazonium salt and *N*,*N*-bis(2-azidoethyl)-aniline **9** (Scheme 3). After optimization of synthesis conditions, we found that the use of an equivolume mixture of acetone and acetate buffer (pH 4.4) is a suitable solvent to recover **11** in a good yield by simple filtration. To impart the water-solubility of this hydrophobic bis-azo dye, "postsynthetic derivatization" of only one azido group was achieved through CuAAC reaction with a terminal alkyne **10** carrying a sulfobetain moiety (see the Supporting Information for its synthesis). This feature is particularly useful to simplify the synthesis, purification, and final bioconjugation reactions of the final UDQ **1** that involve the use of water as (co)solvent.

The resulting "clicked" water-soluble BHQ-1 **11** was purified by semipreparative RP-HPLC (overall yield for

Scheme 2. Synthesis of Thiol-Reactive BHQ-3 **8**



Scheme 3. Synthesis of "Clickable" Water-Soluble BHQ-1 **11**



the two steps 27%). In order to obtain the targeted UDQ **1**, the reaction between nitro-DABCYL **6** and BHQ-3 derivative **8** was first achieved to avoid the oxidative dimerization of thiol group of **6** that may occur with the copper catalyst used in the "click" reaction with azido BHQ-1 **11** (Scheme 4). The resulting heterodimer DABCYL−BHQ-3 **12** was isolated by semipreparative RP-HPLC and finally subjected to a CuAAC reaction with **11** to give, after RP-HPLC purification, the desired universal dark quencher **1** in a moderate yet not optimized 18% overall yield (see the Supporting Information for ESI-HRMS and NMR characterizations). Interestingly, this multiazo quencher was found to be soluble in water and related aq. buffers over a concentration range (1.0 μM to 0.1 mM)

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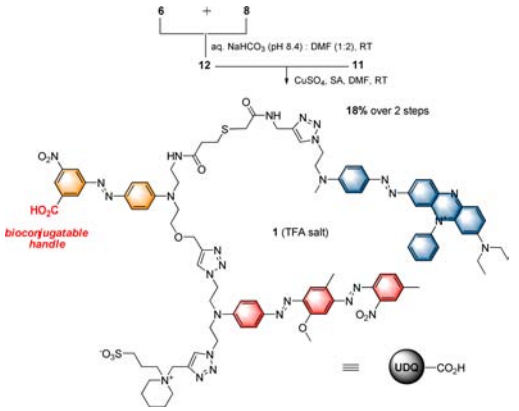
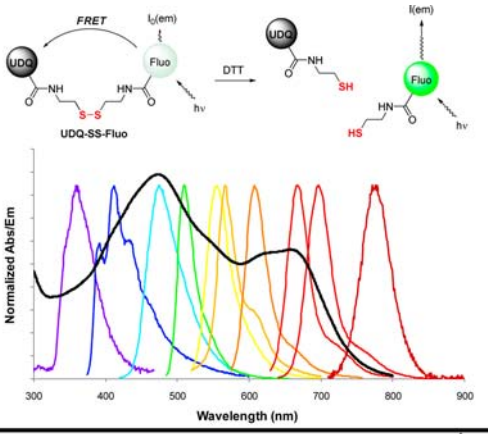
Scheme 4. Synthesis of UDQ **1**



full-compatible with bioanalytical applications involving FRET probes.

As illustrated in Figure 2, UDQ **1** exhibits a very broad absorption spectrum that spans from the UV to NIR region and is characterized by two maxima centered at 470 and 645 nm (ε 47400 and 29800 M^{-1} cm^{-1}) and an unprecedented $\Delta\lambda_{1/2\ max}$ of 460 nm. As expected, this nonfluorescent multiazo dye possesses a spectral absorption feature that is slightly different from the combined absorption spectra of its three parent azo dyes, namely nitro-DABCYL (λ_{max} = 450 nm), BHQ-1 (λ_{max} = 506 nm), and BHQ-3 (λ_{max} = 645 nm) (see the Supporting Information for the spectra). This supports the fact that such a building block approach is a valuable strategy to easily and accurately tune the effective operating wavelength range of dark quenchers. The quenching range of UDQ **1** was validated by measuring the quenching efficiency (QE) of 10 disulfide-based FRET probes ("UDQ-SS-Fluo") derived from cystamine and containing an array of organic fluorophores covering the near-UV and the entire visible spectrum up to the NIR (see Figure 2 and the Supporting Information for the chemical structures of selected fluorophores and synthesis of UDQ-SS-Fluo probes). QE was measured as the difference in fluorescence (area under the emission curves) of the FRET probe before and after reductive cleavage of its disulfide bridge with DTT, and expressed as a percentage (QE = 100 × [1 − I_o(em)/I(em)]). Data displayed in inserted table of Figure 2 show that UDQ **1** has excellent quenching efficiency (higher than 95%) for all selected fluorescent organic dyes, except for NIR-emitting cyanine dye Cy 7.0 (81%). This latter result may be ascribed to a nonoptimal spectral overlap of the emission spectrum of this cyanine fluorophore with the absorption spectrum of UDQ **1** and to the absence of static quenching,[12] which is provided through the formation of an intramolecular complex between the donor and acceptor



Fluo[a]	λ_{max}(Em)	QE[b]
Naphthalene	340 nm	97%
Anthracene	380, 400, 420 nm	98%
DEAC	450 nm	99%
BODIPY	509 nm	97%
R6G	560 nm	95%
Cy 3.0	565 nm	97%
SR101	610 nm	99%
Cy 5.0	670 nm	99%
Cy 5.5	695 nm	98%
Cy 7.0	770 nm	81%

[a]DEAC = 7-diethylaminocoumarine, R6G = rhodamine 6G and SR101 = sulforhodamine 101. [b]I_0(em) and I(em) were measured in PBS-CH_3CN (1:1) at 25 °C before and after treatment with DTT (100 equiv.).

Figure 2. Fluorescence quenching range of UDQ **1** determined in PBS−CH_3CN (1:1) ar 25 °C.

In summary, we have described the first universal dark quencher that possesses a valuable and unprecedented spectrum width of 460 nm ranging from 250 to 710 nm, suitable for turning off emission of a wide variety of fluorophores. This mutli-azo compound is water-soluble, and was obtained using a highly modular, convergent synthetic route based on the sequential derivatization of the nitro-DABCYL analogue **6** with (bio)conjugatable BHQ dyes. Interestingly, an heterotrifunctional molecular platform bearing a mild chemically cleavable azo linker (e.g., cleavage by treatment with sodium dithionite) such as **6** could be also a valuable synthetic tool for designing cleavable trifunctional tags currently used in chemical proteomics for tandem labeling of proteins and subsequent detection or enrichment.[13]

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Supporting Information Available. Synthetic procedures and analytical data reported herein.This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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(13) For a recent publication related to such cleavable trifunctional tags, see: Yang, Y.; Verhelst, S. H. *Chem. Commun.* **2013**, *49*, 5366–5368.