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Azo-Sulforhodamine Dyes: A Novel Class of Broad Spectrum Dark Quenchers

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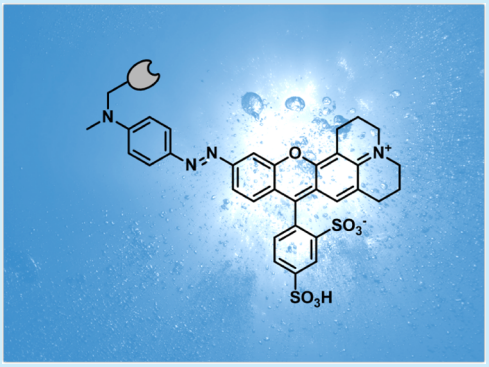
Supporting Information

ABSTRACT: A rapid access to a novel class of water-soluble dark quencher dyes was achieved using an azo-coupling reaction between a fluorescent primary arylamine derived from a sulforhodamine 101 scaffold and a tertiary aniline equipped with different bioconjugatable groups. The thus obtained nonfluorescent azo-sulforhodamine hybrids display a broad quenching range spanning the visible to NIR regions. This was demonstrated through the preparation and enzymatic activation of FRET-based fluorogenic substrates of urokinase.



During the past few years, biomedical imaging has undergone manifold and rapid developments which make it particularly suited for visualization of physiological structures, measurements of biological functions, and evaluation of cellular and molecular events without requiring invasive procedures.[1] Compared to other imaging modalities, optical imaging techniques (i.e., bioluminescence and fluorescence intensity imaging) that refer to both sensitive and quantitative detection of transmitted visible- or NIR-light photons are cost-effective and widely available and do not involve any form of ionization radiation.[2] The successful implementation of fluorescence intensity imaging generally involves the use of "activatable" probes (also known as "smart" imaging probes) which exhibit significant changes in their spectroscopic properties (i.e., a turn-on emission increase or a shift in excitation/emission profiles) upon interaction and/or subsequent reaction with the targeted bioanalyte (e.g., an enzyme, a biomolecule, or a biologically relevant anion or cation).[3] A turn-on response gives a bright signal against a dark background, which is the preferred way to achieve the high signal-to-noise responses and to maximize spatial resolution. Historically, the most popular and convenient approach to designing "smart" imaging probes is the one based 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 (mostly, an hydrolytic enzyme).[4] The performances of such dual labeled probes are closely linked to the efficacy of energy transfer and hence also to the spectral overlap between the donor emission and acceptor absorption, the distance between them, and their relative orientation. Research efforts devoted to the field of such "activatable" probes have promoted the chemistry of nonfluorescent FRET acceptors bearing a bioconjugatable handle and displaying a specific dynamic quenching range fully compatible with the selected fluorescent label (acting as FRET donor). Thus, various dark quenchers mainly belonging to the family of azo or cyanine dyes have been reported and/or commercialized. In general, azo dyes are nonfluorescent owing to ultrafast conformational change around the N=N bond after photoexcitation (e.g., BHQ dyes from Biosearch Technologies).[5] Alternatively, the native fluorescence of cyanine dyes is readily 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., CytoCy5S from GE Healthcare and IRDye QC-1 from LI-COR Biosciences).[6] All these compounds are commonly used in the design of FRET-based probes for *in vitro* detection or *in vivo* imaging of biologically relevant enzyme activities.[7] Despite these achievements, the quencher dyes do not necessarily have ideal physicochemical and spectral properties, and overall (photo)chemical stability (especially for the cyanine deriva-

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tives). For instance, the issue of water solubility (and cell permability) of a quencher is not generally taken into account. Furthermore, the vast majority of these nonfluorescent chromophores display a strong spectral overlap only with a limited number of fluorophores emitting in a specific range of the UV−vis spectrum. With the aim of improving their quenching ability and at converting them into water-soluble derivatives with a wide range of bioconjugatable handles, some azo dyes belonging to the BHQ family have been the focus of several recent works from Kool et al. and our research group.[8,9] Conversely, very little attention has been focused toward dark quenchers based on a xanthene scaffold whereas they might take advantage of superior properties that possess fluoresceins and rhodamines: (1) high molar extinction coefficients, (2) high chemical stability especially under harsh conditions of pH and temperature, and (3) easy modulation of the quenching ability through the chemical modification of their aniline or phenol moiety. To the best of our knowledge, only three *N,N′*-diarylrhodamine derivatives developed by Invitrogen Molecular Probes (known as QSY 7, 9 and 21)[10] are currently available. In this context, it seems interesting to explore the feasibility of designing a novel class of water-soluble nonfluorescent long-wavelength rhodamines acting as broadly absorbing dark quenchers. Two independent publications have recently reported that the direct conjugation of an azo group to the conjugated system of a rhodamine fluorophore is an effective way to switch-off its fluorescence, which can be readily recovered upon bioreduction.[11,12] This feature has allowed the design of fluorescent chemodosimeters, readily effective for tumor hypoxia imaging through the detection of edogeneous reductases. Interestingly, this fluorescence suppression strategy was also applied to other aniline-based fluorophores such as 4-amino-*N*-butyl-1,8-naphthalimide to obtain an effective colorimetric and ratiometric probe for the detection of cyanide ions,[13] and 3,5,8-triaryl-2-amino BODIPY dyes[14] (Figure 1).

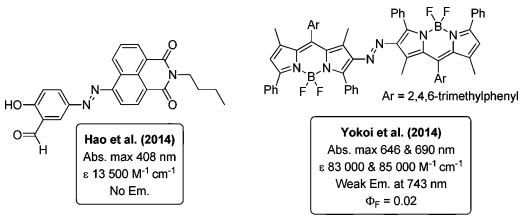


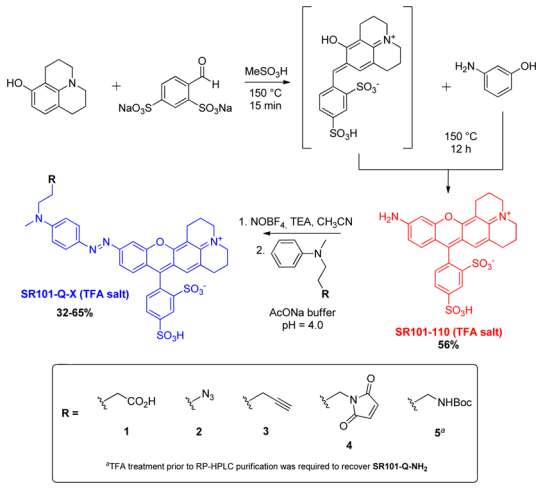
Figure 1. Nonfluorescent azo dyes derived from fluorescent anilines and recently reported in the literature.

Thus, the conversion of the single primary aniline moiety of an unsymmetrical rhodamine dye into π-extended azobenzene derivatives should enable novel dark quenchers with valuable properties to be rapidly obtained.

We report here the practical implementation of this synthetic strategy by using an unsymmetrical derivative of sulforhodamine 101 namely **SR101-110** and functionalized tertiary anilines to introduce five distinct bioconjugatable groups (alkyne, amino, azido, carboxylic acid and maleimide) onto the diaryl-azo scaffold. Indeed, **SR101-110** exhibits structural features (i.e., two sulfonate groups on the *meso* phenyl ring and a positively charged julolidine unit) that are valuable to keep within the core structure of a targeted quencher, aimed at improving water solubility and shifting absorption to the red region as compared to conventional BHQ dyes. The effective quenching range of these bioconjugatable azo-sulforhodamine hybrids is assessed in the context of a peptidyl-FRET substrate for a serine protease namely the urokinase plasminogen activator (uPA), known to play an important role in tumor-associated proteolysis,[15] involving a variety of fluorophores that range from visible blue light (7-*N,N*-diethylaminocoumarin, DEAC) to NIR emission (sulfoindocyanine dye Cy 7.0).

The synthetic strategy currently used to prepare azo dye quenchers relies on the coupling reaction between a diazonium compound and an electron-rich tertiary aniline. The use of bench-stable, commercially available nitrosating agent, nitrosonium tetrafluoroborate ($NOBF_4$), in a polar aprotic solvent has recently emerged as valuable in performing this S_EAr reaction under mild conditions fully compatible with the moderate stability of functionalized anilines.[9,11] Thus, this diazo coupling methodology appears to be well suited to the conversion of a fluorescent aniline such as **SR101-110** into the corresponding azo-based quencher. Moreover, as shown in Scheme 1, **SR101-110** was readily obtained in good yield

Scheme 1. Synthesis of Azo-sulforhodamine Hybrids SR101-Q-X



through a concise synthetic route based on the sequential condensation of two different *meta*-aminophenols (i.e., 8-hydroxyjulolidine and 3-hydroxyaniline) with 4-formylbenzene-1,3-disulfonic acid in methanesulfonic acid heated at 150 °C.[16] After isolation by RP-HPLC purification and freeze-drying, the TFA salt of **SR101-110** was reacted with $NOBF_4$ in dry CH_3CN and in the presence of 1 equiv of TEA (aimed at deprotonating its primary aniline). Surprisingly, the newly formed diazonium salt intermediate was found to be unreactive toward tertiary aniline **1** in CH_3CN. The addition of acetate buffer (pH 4.0) helped to solve this lack of reactivity, and a good conversion into **SR101-Q-CO_2H** was obtained. Purification was achieved by semipreparative RP-HPLC to give **SR101-Q-CO_2H** in a good 52% isolated yield. Its structure was unambiguously confirmed by detailed measurements including NMR and ESI-HRMS analysis (see the Supporting Information (SI)).

To expand the scope of azo-sulforhodamine dyes in biolabeling applications involving popular reactions such as copper-catalyzed azide−alkyne cycloaddition (CuAAC), Staudinger ligation, or thiol-alkylation (Michael addition),[17] we have next explored the diazo coupling between **SR101-110** and four different *N,N*-dialkyl-substituted anilines terminated with azido, alkyne, maleimide, and amino groups, respectively.

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Anilines **2**, **3**, and **5** were prepared according to literature procedures, and maleimide-terminated derivative **4** was synthesized from *N*-methyl-*N*-phenylpropane-1,3-diamine (see the SI). The use of previously optimized conditions for the azo coupling has enabled us to readily obtain four additional bioconjugatable azo-sulforhodamine dyes (Scheme 1). These compounds were isolated in a pure form by semipreparative RP-HPLC and characterized by NMR and ESI-HRMS (see the SI). As illustrated in Figure 2 (and table portion in figure),

compound	Yield (%)[a]	λ_{max} (nm)	ε (M^{-1} cm^{-1})	$\Delta\lambda_{1/2\ max}$ (nm)
SR101-Q-CO$_2$H	52	592	39 000	510-671 (161)
SR101-Q-N$_3$	65	583	44 000	513-655 (142)
SR101-Q-CCH	54	590	37 000	516-667 (152)
SR101-Q-Mal	52	588	39 500	519-662 (153)
SR101-Q-NH$_2$	32	585	44 500	503-662 (159)

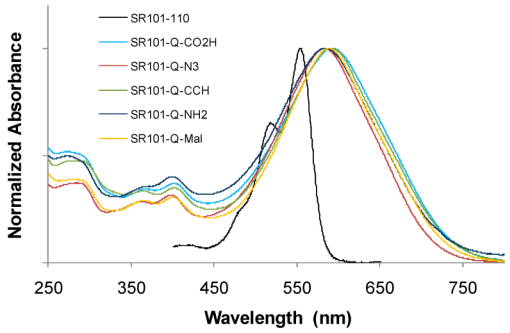


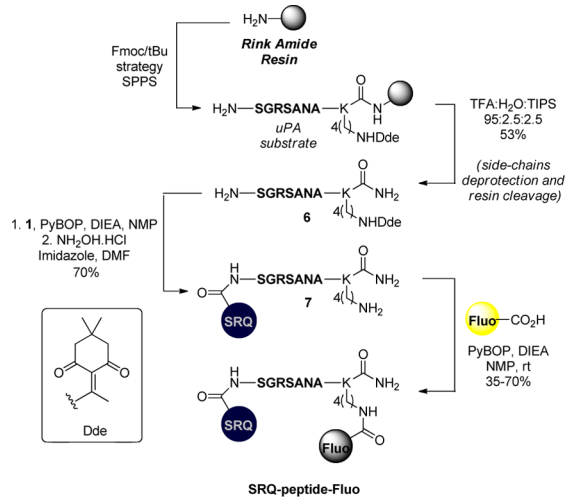
Figure 2. Absorption data of **SR101-Q-X** in PBS at 25 °C. [a] Isolated yields after purification by semipreparative RP-HPLC. Each quencher compound was recovered with a purity up to 99%.

SR101-Q-X exhibit a far broader absorption spectrum than that of parent fluorophore **SR101-110**, which covers the full visible spectrum, and was characterized by a maximum centered at 582−592 nm (according to the substitution pattern of aniline) and good $\Delta\lambda_{1/2\ max}$ values (142−161 nm).

As expected, sulfonate moieties onto the *meso*-phenyl ring make these azo-sulforhodamine hybrids significantly soluble in water and related aq buffers, over a concentration range (1.0 μM to 1.0 mM) full-compatible with bioanalytical applications involving FRET probes.

To assess the quenching range of the azo-sulforhodamine 101 scaffold and to confirm the bioconjugation ability of **SR101-Q-CO$_2$H**, we have chosen to prepare different uPA-sensitive fluorescence quenched probes ("SRQ-peptide-Fluo") using a wide range of fluorophores associated with this azo-based quencher. Heptapeptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-OH (also known as SGRSANA) has been reported to be a highly potent substrate of uPA (cleavage site between Arg and Ser residues);[15] therefore, we decided to label this peptide with a FRET pair composed of an organic fluorophore (emitting in the visible or NIR spectral region) and **SR101-Q-CO$_2$H**, abbreviated **SRQ** (Scheme 2). To achieve chemoselective labeling reactions through aminolysis of activated esters, a lysine residue whose ε-primary amine is masked with a trifluoroacetic acid (TFA)-stable protecting group (Dde) was incorporated at the *C*-terminus. Peptide H-Ser-Gly-Arg-Ser-Ala-Asn-Ala-Lys(Dde)-NH$_2$ **6** was obtained by standard solid-

Scheme 2. Synthesis of FRET-Based Fluorogenic uPA Substrates



phase peptide synthesis techniques (Fmoc/tBu strategy) and subsequent TFA deprotection. Acylation of its *N*-terminal α-amino group with HOBt active esters of **SR101-Q-CO$_2$H** (formed *in situ* with PyBOP/DIEA) in NMP/DIEA has led to the monolabeled peptide. The removal of the Dde protecting group from the lysine residue was achieved by treatment with an excess of hydroxylamine and imidazole to afford **7**.[18] These mild deprotection conditions were preferred to the standard use of hydrazine, since the sensitivity of some fluorophores (especially cyanine and rhodamine dyes) to such an α-nucleophile by either the reduction of ethylenic insaturations (mediated by diimide resulting from slow oxidation of hydrazine) or Michael-type addition to the xanthene core has already been observed.[19] Finally, amidification of the lysine side chain with the selected fluorophore carboxylic acid provided the uPA fluorogenic substrates. These dual-labeled peptides were purified by semipreparative RP-HPLC (overall yields 25−50%, purity >95%), and their structures were unambiguously confirmed by ESI-MS analyses (see the SI).

Quenching Efficiency (QE) was measured as the difference in fluorescence (area under the emission curves) of the FRET probes before and after enzymatic digestion with uPA, and expressed as a percentage ($QE = 100 \times (1 - I_0(em)/I(em))$). Data displayed in the table portion of Figure 3 show that **SRQ** has an excellent quenching efficiency (>95%) for all selected fluorescent organic dyes, except for NIR-emitting cyanine dye Cy 7.0 (92.7%). Due to the lack of significant spectral overlap between emission curves of sulfoindocyanine dyes Cy 5.0, Cy 5.5, and Cy7.0 and absorption curve of **SRQ** (see Figure 3), static quenching (also known as contact quenching) wherein the fluorophore and the quencher form an intramolecular complex[20] is likely to occur in these three probes instead of conventional FRET quenching. This was supported by the shape of UV−vis absorption spectra which are not the simple sum of absorption curves of cyanine and SRQ dyes (see the SI).

In summary, we have developed an original approach to easily access a novel family of rhodamine-based quenchers that are structurally compact, water-soluble, and bioconjugatable. Contrary to commercially available QSY dyes whose synthesis is tedious and time-consuming, these nonfluorescent derivatives are readily obtained through a single-step protocol based on an azo coupling reaction involving a sulforhodamine as one of the

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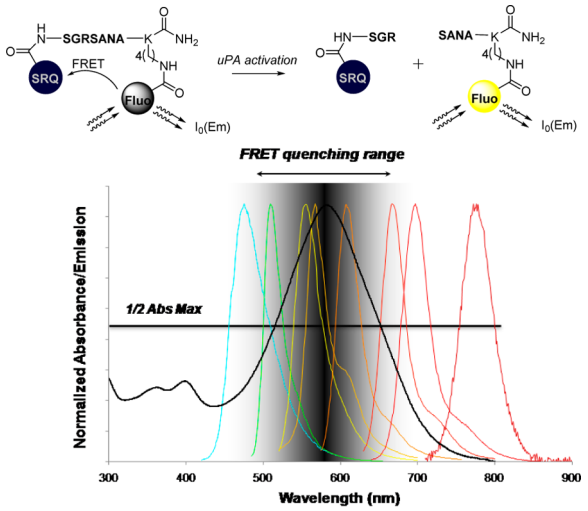

fluorophore[a]	λ_{max} (nm)	QE
DEAC	479 nm	95.8%
BODIPY	508 nm	97.8%
R6G	554 nm	97.2%
Cy3.0	563 nm	95.0%
TR-Isonip	610 nm	97.9%
Cy5.0	665 nm	98.9%
Cy5.5	693 nm	99.0%
Cy7.0	774 nm	92.7%

Figure 3. Fluorescence quenching range of **SR101-Q**. [a] DEAC = 7-*N*,*N*-diethylaminocoumarin, R6G = rhodamine 6G, and TRIsonip = Texas Red-isonipecotic acid; see SI for the chemical structures of fluorophores. [b] Fluorescence emission measurements were performed in PBS at 25 °C before and after enzymatic digestion with uPA.

two aniline partners. The potential utility of these unusual azo-sulforhodamine hybrids has been demonstrated by the fact that the **SR101-Q** scaffold is able to turn off emission of a wide variety of fluorophores, in the range 450−770 nm and through both static and dynamic quenching mechanisms. Furthermore, bis aryl-azo compounds such as **SR101-Q-CO_2H** or analogues derived from longer-wavelength rhodamines/rhodols are valuable scaffolds for designing "smart" optical bioprobes for tumor hypoxia imaging.[12,21]

■ ASSOCIATED CONTENT

Supporting Information

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

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Notes

The authors declare no competing financial interest.

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