chemistry proceedings

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Proceeding Paper

Straightforward Synthesis of BHQ-3 Amine: An Azo Dark-Quencher for FRET-Based Protease Activity Assays †

Cátia D. F. Martins [1,2], Maria Manuela M. Raposo [1] and Susana P. G. Costa [1,*]

[1] Centre of Chemistry, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; catiadf_martins@hotmail.com (C.D.F.M.); mfox@quimica.uminho.pt (M.M.M.R.)
[2] Advanced (Magnetic) Theranostic Nanostructures Lab, International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-330 Braga, Portugal
* Correspondence: spc@quimica.uminho.pt
† Presented at The 28th International Electronic Conference on Synthetic Organic Chemistry (ECSOC 2024), 15–30 November 2024; Available online: https://sciforum.net/event/ecsoc-28.

Abstract: A Black Hole Quencher-3 (BHQ-3) derivative was synthesized through an azo-coupling reaction between Methylene Violet 3RAX and a tertiary aniline functionalized with a pendant primary amine, allowing subsequent peptide conjugation. The synthesized compounds were characterized using NMR, UV-Vis absorption, fluorescence spectroscopy, and mass spectrometry. The spectral properties of a Cy5/BHQ-3 amine pair were investigated through titration experiments in PBS (pH 7.4). The results confirmed Förster Resonance Energy Transfer (FRET), along with additional dynamic quenching, as evidenced by Stern-Volmer analysis. The Stern-Volmer constant (K_{SV}) was determined to be 1.40×10^5 M^{-1}. These findings confirm the potential of this system for use in molecular probes and bioimaging applications.

Keywords: azo dyes; BHQ-3; FRET; proteases; quenching

Citation: Martins, C.D.F.; Raposo, M.M.M.; Costa, S.P.G. Straightforward Synthesis of BHQ-3 Amine: An Azo Dark-Quencher for FRET-Based Protease Activity Assays. *Chem. Proc.* **2024**, *6*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s): Name

Published: 15 November 2024

1. Introduction

Non-fluorescent azo dyes are extensively employed in advanced optical bioprobes, particularly those utilizing the Förster resonance energy transfer (FRET) mechanism [1]. These probes find widespread application in protease activity assays, nucleic acid hybridization, and real-time PCRs, effectively quenching the fluorescence of energy donors [2]. The azo (-N=N-) group in these dyes is primarily responsible for their non-fluorescent nature, while their robust photochemical stability makes them ideal fluorescence quenchers for FRET applications [3].

Among azo-based quenchers, Black Hole Quenchers (BHQs), especially BHQ-3, have gained significant attention due to their broad absorption spectrum, which extends into the near-infrared (NIR) region [4,5]. This wide spectral range allows BHQ-3 to effectively quench a variety of fluorophores, including FAM, FPR-675, Cy5, and IRDye 800CW [2].

In particular, pentamethine cyanine (Cy5) derivatives, which possess far-red excitation and emission wavelengths (650–670 nm), are particularly well-suited for use with BHQ-3 as a quencher due to the strong spectral overlap between Cy5 emission and BHQ-3 absorption [6]. This overlap is crucial for achieving efficient FRET, significantly boosting the sensitivity of systems like enzyme detection assays [7]. Consequently, this FRET pair has been widely exploited in various protease activity assays, where the cleavage of a peptide or protein substrate induces changes in the fluorescence signal due to FRET, providing a sensitive readout of enzymatic activity [8]. Additionally, the far-red emission of Cy5 derivatives is well-suited for deep tissue bioimaging, where longer wavelengths improve tissue penetration and reduce light scattering [9,10]. The general structures of BHQ-3 and Cy5 derivatives are depicted in Figure 1.

Following our research focus on developing nanoconstructed FRET-labeled peptide probes for real-time monitoring of protease activity in therapeutic and medical imaging applications [11–13], we present a straightforward synthesis of a BHQ-3 derivative. This derivative is designed for subsequent conjugation to specific peptides, enabling its use in FRET-based protease activity assays. Additionally, we investigated the quenching mechanism of the Cy5/BHQ-3 FRET pair through comprehensive photometric and fluorimetric studies, which confirmed the efficiency of this system.

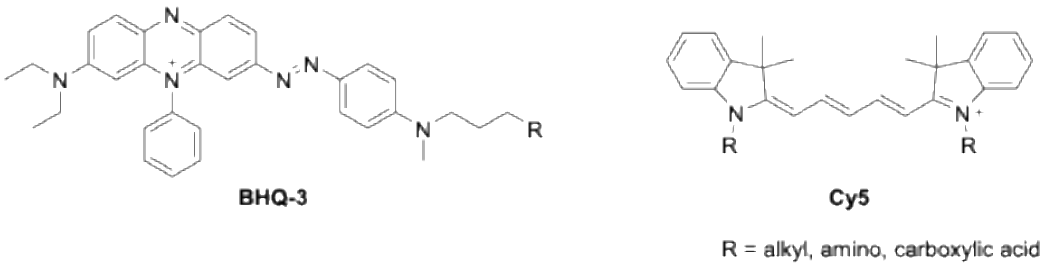


Figure 1. General structures of BHQ-3 and Cy5 derivatives.

2. Experimental Section

2.1. General Synthesis

TLC analyses were performed on 0.25 mm thick precoated silica plates with fluorescent indicator F_{254} (Merck KGaA, Darmstadt, Germany) and visualized under UV light at 254 and 365 nm. Silica gel (particle size 0.035–0.070 mm, 60 Å; Acros Organics, Geel, Belgium) was used for column chromatography. NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer, operating at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C, using the solvent peak as an internal reference at 25 °C in MeOH-d_4. Assignments were supported by two-dimensional heteronuclear correlation techniques. ESI-MS analyses were carried out on a Thermo Scientific LxQ Series linear ion trap equipped with an electrospray ionisation source. All reagents were purchased from Acros Organics and Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Cy5 NHS ester was purchased from Lumiprobe Corporation (Baltimore, MD, USA).

2.2. Synthesis

2.2.1. Synthesis of N′-Methyl-N′-phenylpropane-1,3-diamine (1)

N-Methylaniline (500 mg, 4.66 mmol, 1 equiv) and 3-bromopropan-1-amine hydrobromide (1.22 g, 5.59 mmol, 1.2 equiv) were mixed in absolute ethanol (4 mL) and heated under reflux. The progress of the reaction was monitored by TLC using mixtures of dichloromethane/methanol as the mobile phase. After 12 h, the solvent was removed under reduced pressure, and the crude residue was purified via silica gel column chromatography, employing mixtures of dichloromethane and methanol of increasing polarity as eluent. Compound **1** (Figure 2) was obtained as a beige solid (542 mg, 71%).

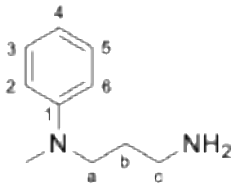


Figure 2. Structure of tertiary aniline **1**.

^{1}H NMR (400 MHz, MeOH-d_4): δ = 1.98 (2H, br s, CH_2-b), 3.05 (2H, t, J = 8.0 Hz, CH_2-c), 3.34 (3H, s, NCH_3), 3.43 (2H, t, J = 8 Hz, CH_2-a), 7.57 (1H, t, J = 7.6 Hz, H-4), 7.65 (2H, dt, J = 1.6 Hz and 6.8 Hz, H-2 and H-6), 7.76 (2H, d, J = 8.0 Hz, H-3 and H-5) ppm.

^{13}C NMR (100.6 MHz, MeOH-d_4): δ = 24.67 (CH_2-b), 37.75 (CH_2-c), 46.25 (NCH_3), 56.80 (CH_2-a), 122.17 (C-3, C-5), 131.13 (C-4), 131.89 (C-2, C-6), 141.97 (C-1) ppm.

MS (ESI, %): *m*/*z* 165 ([M+H]$^+$, 100).

2.2.2. Synthesis of Black Hole Quencher Amine (BHQ-3, 3)

Methylene Violet 3RAX (100 mg, 0.264 mmol, 1 equiv) was dissolved in dry acetonitrile (2 mL), and the solution was stirred at 0 °C for 15 min. Subsequently, solid $NOBF_4$ (33.9 mg, 0.290 mmol, 1.1 equiv) was added, and the reaction mixture was mixed at 0 °C for 15 min. Following this, *N*,*N*-substituted aniline **1** (52 mg, 0.317 mmol, 1.2 equiv) was dissolved in dry acetonitrile (1 mL) and added dropwise to the diazonium salt intermediate **2**. The reaction mixture was maintained at 0 °C for 2 h, and the progress of the reaction was monitored by TLC using a mixture of dichloromethane and methanol as eluent. The solvent was then evaporated under reduced pressure, and the crude residue was purified by flash column chromatography on silica gel, employing a gradient of dichloromethane and methanol with increasing polarity. The purified BHQ-3 amine **3** (Figure 3), was obtained as a dark blue solid (54 mg, 39% yield).

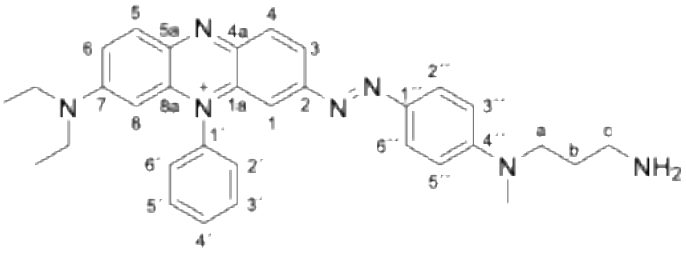


Figure 3. Structure of BHQ-3 amine **3**.

^{1}H NMR (400 MHz, MeOH-d_4): δ = 1.13 (3H, br s, $N(CH_2CH_3)_2$, 1.36 (3H, br s, $N(CH_2CH_3)_2$, 2.08 (2H, qui, *J*= 8.0 Hz, CH_2-b), 3.07 (2H, t, *J* = 7.6 Hz, CH_2-c), 3.34 (3H, s, NCH_3), 3.47 (2H, br s, $N(CH_2CH_3)_2$), 3.78 (2H, t, *J* = 7.6 Hz, CH_2-a), 3.86 (2H, br s, $N(CH_2CH_3)_2$), 5.91 (1H, d, *J* = 2,4 Hz, H-8), 7.08 (2H, d, *J* = 9.2 Hz, H-3″ and H-5″), 7.40 (1H, d, *J* = 2.0 Hz, H-1), 7.70 (2H, dd, *J* = 1.2 and 7.2 Hz, H-3′ and H-5′), 7.91(2H, d, *J* = 9.6 Hz, H-2″ and H-6″), 7.93–7.98 (4H, m, H-4′, H-6, H-2′ and H-6′), 8.18 (1H, d, *J* = 10 Hz, H-5), 8.26 (1H, dd, *J* = 1.6 and 8.8 Hz, H-3), 8.41 (1H, d, *J* = 8.8 Hz, H-4) ppm.

^{13}C NMR (100.6 MHz, MeOH-d_4): δ = 11.64 ($N(CH_2\underline{C}H_3)_2$), 26.53 ($CH_2$-b), 30.41 ($N(CH_2\underline{C}H_3)_2$), 38.30 ($CH_2$-c), 39.89 ($NCH_3$), 47.96 ($N(\underline{C}H_2CH_3)_2$), 51.25 ($CH_2$-a), 93.34 (C-8), 110.07 (C-1), 115.16 (C-3″ and C-5″), 120.84 (C-3), 125.19 (C-6), 128.98 (C-3′ and C-5′), 130.13 (C-2″ and C-6″), 132.88 (C-4′), 132.95 (C-2′ and C-6′), 134.15 (C-4), 135.35 (C-1a), 135.92 (C-5), 137.42 (C-1′), 139.54 (C-5a or C-8a), 140.39 (C-4a), 143.90 (C1″), 145.55 (C-5a or C-8a), 155.06 (C-2), 156.97 (C-4″ and C-7) ppm.

MS (ESI, %): *m*/*z* 518 ([M+H]$^+$, 100).

2.3. General UV/Vis and Fluorescence Studies

UV-visible absorption spectra were recorded using a Shimadzu UV/2501PC spectrophotometer. Fluorescence spectra were obtained with a Horiba FluoroMax-4 spectrofluorometer, using standard quartz cuvettes with a 1 cm optical path length. All measurements were carried out at room temperature. Stock solutions of BHQ-3 amine (**3**) and Cy5-NHS ester (100 μM) were prepared in phosphate-buffered saline (PBS, pH 7.4).

3. Results and Discussion

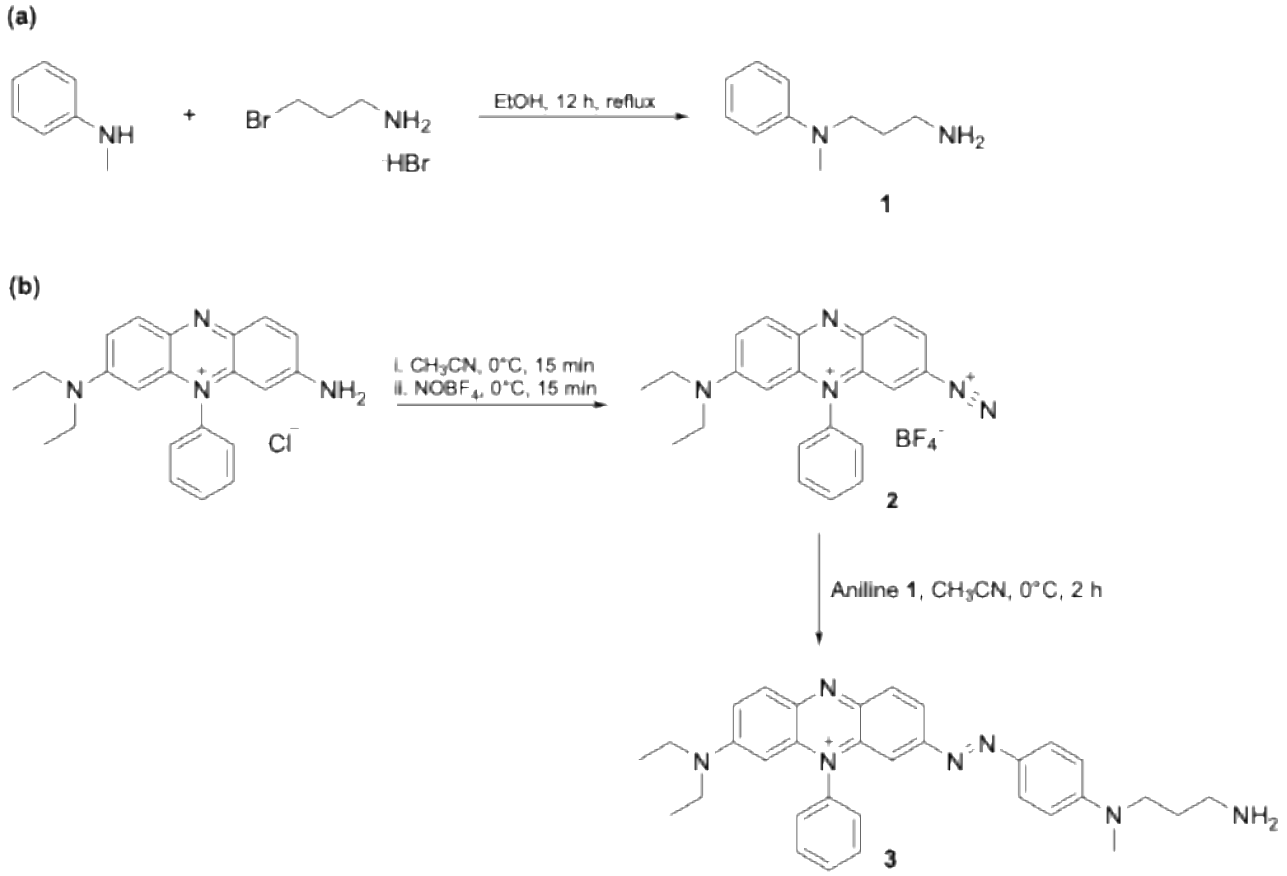
3.1. Synthesis of the Functionalized BHQ-3 Amine Dye

The synthetic methodology developed for the preparation of Black Hole Quencher-3 derivative (BHQ-3 amine, **3**) is based on an azo-coupling reaction between a 3-amino-7-

(dialkylamino)-5-aryl-phenazonium salt, commercially known as Methylene Violet 3RAX, and an *N*,*N*-substituted aniline (compound **1**) functionalized with a primary amine. This primary amine enables subsequent conjugation to the C-terminal of a specific peptide through amide bond formation.

The synthesis of tertiary aniline (compound **1**) was accomplished via alkylation of *N*-methylaniline with 3-bromopropylamine hydrobromide (Scheme 1a). The crude product was purified using silica gel chromatography, and its structure was confirmed through NMR spectroscopy and mass spectrometry. Methylene Violet 3RAX was then subjected to diazotization using nitrosonium tetrafluoroborate as the nitrosating agent, forming the corresponding diazonium cation (compound **2**). This intermediate was subsequently used in an electrophilic aromatic substitution (S_EAr) reaction with aniline **1**, yielding the BHQ-3 amine derivative (compound **3**) (Scheme 1b). The crude product was further purified by dry flash chromatography and fully characterized by NMR spectroscopy and mass spectrometry. This synthetic strategy resulted in a moderate yield of BHQ-3 amine at 39%.

It is noteworthy that the S_EAr reaction described above is significantly hindered under highly acidic conditions. The conventional generation of nitrosonium ions (NO^+) using sodium nitrite and strong acids (e.g., HCl or H_2SO_4) often leads to protonation of the *N*,*N*-dialkylamino group, requiring pH adjustment and potentially decreasing the reaction efficiency [4,14]. Thus, the use of nitrosonium tetrafluoroborate in a polar aprotic solvent is preferred over traditional methods for optimal reaction conditions and yields.



Scheme 1. Synthesis of BHQ3-amine **3**: (**a**) asymmetrical tertiary aniline **1**; (**b**) BHQ-3 amine **3**.

3.2. UV/Vis and Fluorescence Studies

The photophysical properties of Cy5-NHS and BHQ-3 amine (**3**) were initially examined in PBS (pH 7.4). As expected, BHQ-3 amine displayed a strong absorption band with a high molar absorptivity (log ε = 4.33) at 625 nm (Table 1), while Cy5-NHS exhibited a narrow fluorescence emission band centered at 659 nm. The spectral overlap between the

fluorescence emission of Cy5 and the absorption spectrum of BHQ-3 amine, as depicted in Figure 4a. This overlap is crucial for enabling non-radiative energy transfer between Cy5 (donor) and BHQ-3 (acceptor), since FRET relies on both spectral overlap and the proximity between donor and acceptor molecules (effective up to 10 nm) [2,7].

Table 1. UV-visible absorption and fluorescence data for Cy5 and BHQ-3 (5 μM) in PBS (pH 7.4).

Compound	UV-vis		Fluorescence	
	λ_{max} (nm)	log ε	λ_{emi} (nm)	Stokes' Shift (nm)
Cy5-NHS	640	4.82	659	19
BHQ-3 amine (**3**)	625	4.33	-	-

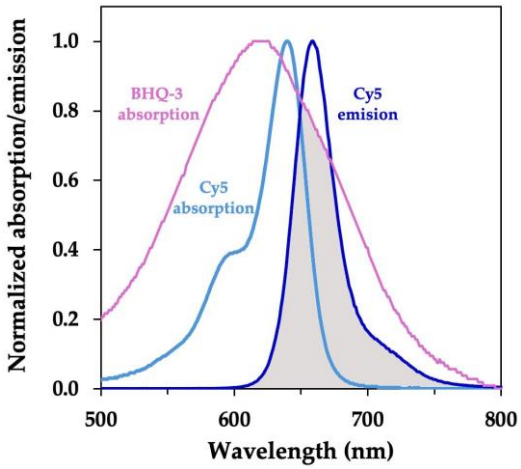


Figure 4. Normalized absorption and emission spectra of BHQ-3 amine (**3**) and Cy5 (5 μM) in PBS (pH 7.4). The shaded area highlights the spectral overlap between the emission spectrum of Cy5 and the absorption spectrum of BHQ-3 amine.

Incorporating the Cy5/BHQ-3 amine pair into peptides to develop fluorescent probes presents significant potential for enzyme detection. The peptide substrate can be designed so that enzymatic cleavage separates the Cy5 donor from the BHQ-3 quencher, thereby disrupting the FRET process and restoring Cy5 fluorescence. This "turn-on" fluorescence signal can be employed to detect specific enzyme activity, such as proteases, with high sensitivity and selectivity in aqueous environments. The strong quenching capacity of BHQ-3 minimizes background fluorescence, while the efficient FRET mechanism facilitates highly responsive and precise detection.

To further explore the quenching mechanism between Cy5 and BHQ-3 amine (**3**), titration experiments were conducted. Solutions were prepared with a fixed concentration of Cy5 (5 μM) and varying concentrations of BHQ-3 amine, ranging from 0 to 50 μM. The fluorescence spectra of these solutions were recorded, showing a progressive decrease in Cy5 fluorescence intensity as the concentration of BHQ-3 increased, confirming the efficiency of the FRET process (Figure 4a). The fluorescence emission maximum of Cy5 at 659 nm was gradually quenched without a detectable shift in wavelength.

However, this progressive quenching of Cy5 fluorescence suggests that dynamic quenching also contributes to the overall quenching mechanism [15,16]. Dynamic quenching occurs when collisions between the fluorophore and quencher reduce fluorescence intensity. This process can be quantitatively described by the Stern-Volmer equation [17]:

$$\frac{F_0}{F} = 1 + K_{sv}\,[Q] = 1 + K_q\tau_0\,[Q] \quad (1)$$

In this equation, F_0 and F represent the fluorescence intensities of Cy5 in the absence and presence of BHQ-3 amine, respectively, and $[Q]$ represents the concentration of the

quencher. The Stern-Volmer constant, K_{SV}, reflects the overall quenching efficiency, while K_q is the bimolecular dynamic quenching constant.

By fitting the experimental titration data to the Stern-Volmer equation, K_{SV} was determined to be 1.40×10^5 M^{-1}. The linearity of the Stern-Volmer plot (Figure 4b) confirms the presence of dynamic quenching. Considering the known fluorescence lifetime (τ_0) of Cy5 in the absence of a quencher (typically 1 ns) [18,19], the dynamic quenching constant K_q was calculated to be 1.40×10^{14} M^{-1} s^{-1}.

The results for both K_{SV} and K_q indicate that BHQ-3 amine is a highly effective quencher of Cy5 fluorescence. The notably high K_q value suggests a rapid quenching process, likely driven by a strong interaction between the quencher and fluorophore on the nanosecond timescale. These findings demonstrate the potential use of this quenching system in molecular probes and bioimaging applications, where rapid and efficient fluorescence quenching is essential.

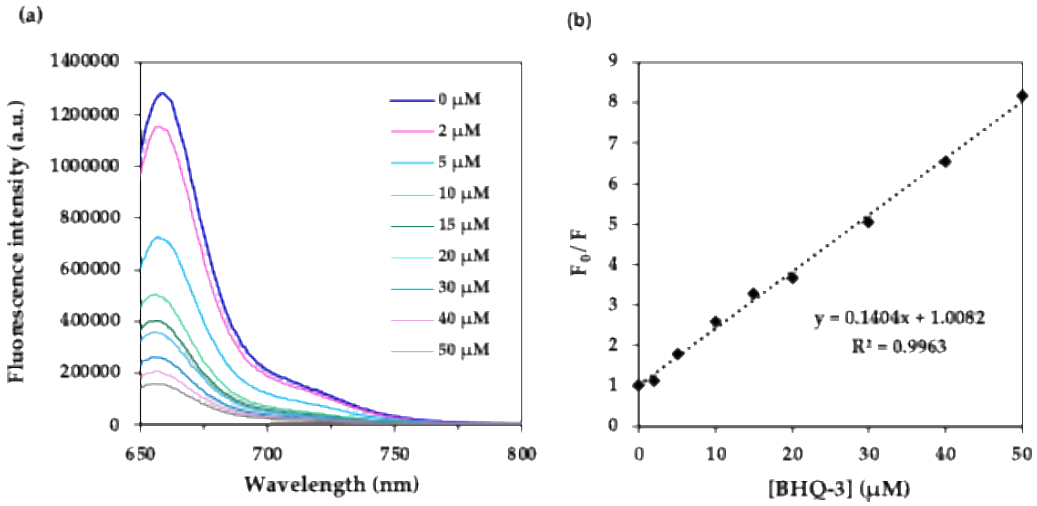


Figure 5. (**a**) Fluorescence spectra of a 5 µM Cy5 solution at varying concentrations of BHQ-3 amine (**3**). (**b**) Stern-Volmer plots illustrating the variation of F_0/F as a function of BHQ-3 concentration.

4. Conclusions

In conclusion, the Black Hole Quencher-3 (BHQ-3 amine, compound **3**) was synthesized via an optimized azo-coupling reaction between Methylene Violet 3RAX and a tertiary aniline functionalized with a primary amine. This synthetic route will enable the conjugation of compound **3** to peptides, making it a versatile tool for bioconjugation. The quenching mechanism of the Cy5/BHQ-3 amine pair was studied, confirming efficient FRET due to strong spectral overlap, along with dynamic quenching, as revealed by Stern-Volmer analysis. These results highlight the potential of this FRET pair for developing highly sensitive peptide-based fluorescent probes for enzyme detection in bioimaging and diagnostic applications.

Author Contributions: Conceptualization, C.D.F.M. and S.P.G.C.; methodology, C.D.F.M. and S.P.G.C.; validation, S.P.G.C. and M.M.M.R.; formal analysis, C.D.F.M. and S.P.G.C.; investigation, C.D.F.M.; resources, S.P.G.C. and M.M.M.R.; writing—original draft preparation, C.D.F.M.; writing—review and editing, C.D.F.M., M.M.M.R. and S.P.G.C.; supervision, S.P.G.C. and M.M.M.R.; project administration, S.P.G.C. and M.M.M.R.; funding acquisition, S.P.G.C. and M.M.M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação para a Ciência e Tecnologia—FCT (Portugal) through CQ-UM (UID/QUI/00686/2020), project PTDC/QUI-OUT/3143/2021 and a PhD grant to C. D. F. Martins (SFRH/BD/05277/2020). The NMR spectrometer Bruker Avance III 400 is part of the National NMR Network and was purchased within the framework of the National Program for

Scientific Re-equipment, contract REDE/1517/RMN/2005 with funds from POCI 2010 (FEDER) and FCT.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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