New Quencher Derivatives for Fluorescent Quenching Peptide Assays

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Doubly dye labeled peptides that utilize FRET quenching (Fig1) have many applications. A new class of efficient quencher molecules, termed Black Hole Quenchers™, have been introduced into peptides via phosphoramidite derivatives coupled to the hydroxylic functions of serine, tyrosine, or hydroxyproline residues. The spectral characteristics for the entire family (BHQ-1, BHQ-2, etc.) are matched to sets of fluorophores, providing high sensitivity and low background detection in a variety of fluorogenic assays.

Figure 1

FRET Quenching

1st Step: The dye molecule absorbs light and generates the dye excited state.

Dye —light absorption→ Dye*

2nd Step in the absence of quenching: Fluorescence!

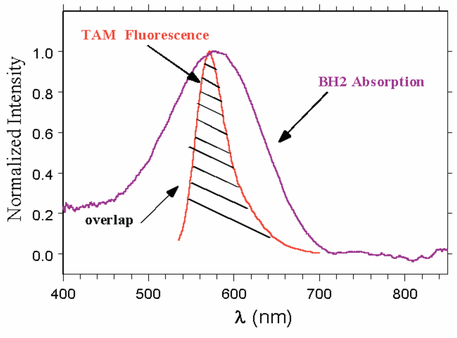
Dye —light emission→ Dye*

2nd Step when FRET occurs: The dye excited state TRANSFERS ENERGY to the quencher ground state generating the quencher excited state.

BHQ+Dye* —energy transfer→ Dye+BHQ*

3rd Step: The quencher then returns to the ground state.

BHQ* —radiationless decay→ BHQ



FRET Quenching efficiency depends on:

- $1/R^6$, where R is the distance between the donor and quencher.
- J, the overlap integral between donor emission and quencher absorption
- k, the orientation between the donor and quencher.

Materials and Methods

The following peptide was synthesized using FMOC synthesis chemistry:

Ser(Reporter)-Gly-Nle-Tyr-Gly-Arg-Gly-Ser(BHQ)

α-Chymotrypsin digestion site ↖ ↗ Trypsin digestion site

The quenchers were coupled to the hydroxyl groups on serine, tyrosine, and hydroxyproline on NovaGel resins using phosphoramidite chemistry. The solvent was CH_3CN and the activator used was ethyl thio-tetrazole (32 mg/mL). Couplings were done at 100 mg/ml for 0.5 h followed by 1 min oxidation. The reporter was coupled to the N-terminus in the same manner. Capping was not used in this synthesis. Four different peptides were synthesized with the following dye/quencher combinations: TAMRA/BHQ-1, ROX/BHQ-1, TAMRA/BHQ-2, and ROX/BHQ-2. Final products were HPLC purified on a Hamilton PRP-1 column (10 x 250 mm, 7 μm) using a linear gradient of 95:5 to 0:100 A:B over 20 min [A = 0.1% TFA (aq), B = 0.1% TFA in CH_3CN].

The lyophilized fractions from the HPLC purification were suspended in 1:2.5 CH_3CN/H_2O. This solution was diluted 1:40 in α-chymotrypsin digestion buffer (0.1 M NH_4HCO_3) and 5 μl (~0.5 U) of α-chymotrypsin solution (2 mg in 1 ml buffer) was added and allowed to react for 10 min before 2x serial dilutions were made out to 1:320 of the original solution. Fluorescence measurements were then taken for these solutions using a Molecular Devices SpectraMax Gemini fluorescence plate reader. Results are summarized in Figure 2.

Figure 2. Signal to noise ratios for BHQ Fret peptides.

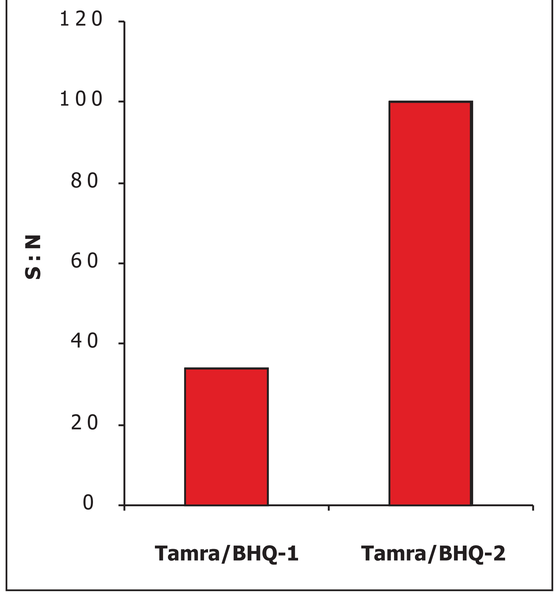
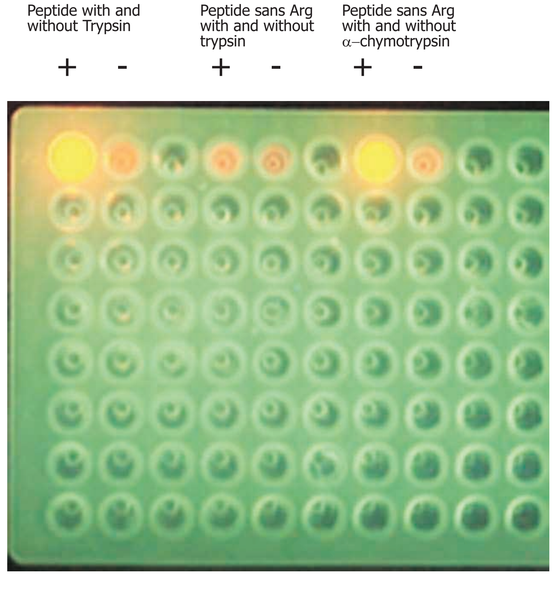


Figure 3. A by-product resulting from poor arginine coupling is shown not to be susceptible to cleavage by trypsin.



During the Fmoc deprotection step of the peptide synthesis, BHQ's attached to the hydroxyl functionalized resin were removed by β-elimination (Figure 4), the extent of this side reaction is shown in Figure 5.

Figure 4. Schematic representation of the loss of quencher moiety through a proposed β-elimination mechanism.

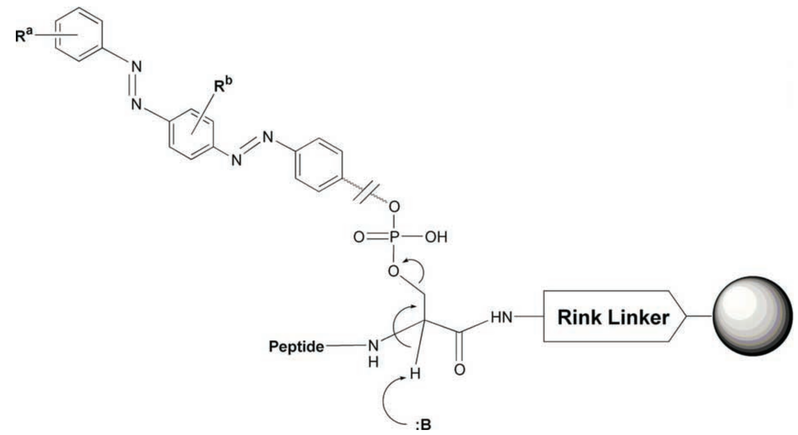
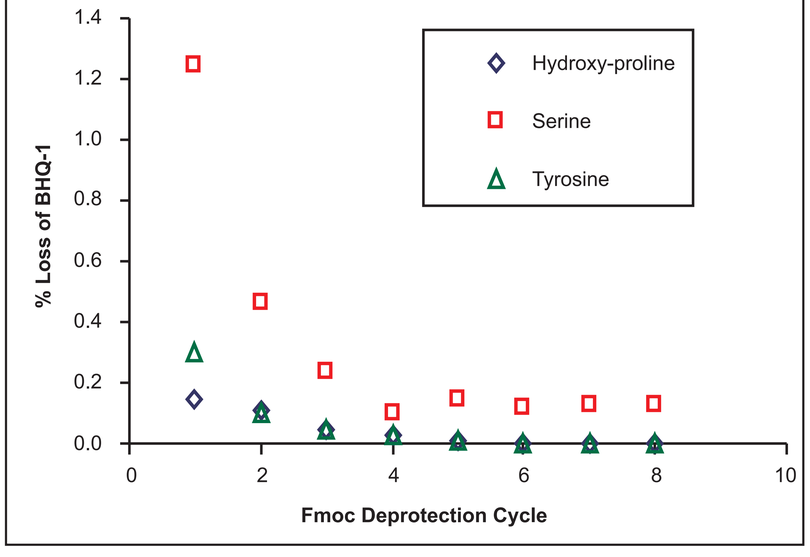


Figure 5. Extent of Elimination from BHQ Resins during Synthesis



Conclusions

- Fluorophore/BHQ FRET Peptides provide a sensitive detection method for enzyme cleavage assays.
- Hydroxyproline residues provide the most stable attachment of BHQ's via phosphoramidite chemistry.
- A new attachment method under development involves the use of H-Lys(BHQ)-OH, which is expected to simplify the procedure.