Published in final edited form as:

J Phys Chem B. 2017 August 03; 121(30): 7256–7266. doi:10.1021/acs.jpcb.7b03611.

PHOXI: A High Quantum Yield, Solvent-Sensitive Blue Fluorescent 5-Hydroxytryptophan Derivative Synthesized within Ten Minutes under Aqueous, Ambient Conditions

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Abstract

Multiple tryptophan (Trp) proteins are not amenable to fluorescence study because individual residue emission is not resolvable. Biosynthetic incorporation of an indole analogue such as 5-hydroxyindole has not provided sufficient spectroscopic resolution because of low quantum yield and small emission shift. Here, 5-hydroxyindole is used as the starting framework for building a blue emitting fluorophore of high quantum yield, 2-phenyl-6*H*-oxazolo[4,5-*e*]indole (PHOXI). This is a three reagent reaction completed in 10 min under ambient conditions in borate buffer at pH 8. Reaction conditions have been optimized using 5-hydroxyindole. Derivatization is demonstrated on tryptophanyl 5-hydroxytryptophan (5-HTP) and a stable β -hairpin “zipper” peptide with four tryptophan residues, TrpZip2, where Trp 4 has been replaced with 5-HTP, W4 $\rightarrow$ 5-HTP. Reaction optimization yields a PHOXI fluorophore that is essentially free of byproducts. Reaction specificity is demonstrated by the lack of reaction with *N*-acetyl-cysteine and amyloid β -40, a peptide containing all amino acids except tryptophan, proline, and cysteine and lacking 5-HTP. Fluorescence study of PHOXI-derivatized 5-hydroxyindole in different solvents reveals the sensitivity of PHOXI to solvent polarity with a remarkable 87 nm red-shift in water relative to cyclohexane while maintaining high quantum yield. Thus, PHOXI joins the ranks of solvatochromic fluorophores such as PRODAN. Surprisingly, DFT calculations reveal

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.7b03611. Preparation protocol for PHOXI, PHOXI ¹H NMR, mass spectra of tryptophanyl-5-hydroxytryptophan, and TrpZip2(5-HTP) before and after PHOXI derivatization; HPLC retention profile for *N*-acetylcysteine in the presence and absence of PHOXI reaction reagents; solvent parameter fits to lifetime, quantum yield, radiative and nonradiative decay rates for PHOXI in six solvents; fluorescence lifetime decay curves of PHOXI in six solvents; CD spectra of TrpZip2 peptide with Trp4 replaced by 5-HTP or PHOXI at three temperatures, and PHOXI photodegradation curves (PDF)

coplanarity of the oxazolo/indole extended ring system and the phenyl substituent for both the HOMO and LUMO orbitals. Despite the crowded environment of three additional Trps in TrpZip2, CD spectroscopy shows that the TrpZip2 β -hairpin structure is partially retained upon PHOXI incorporation. In an environment of smaller residues, PHOXI incorporation can be less disruptive of protein secondary structure, especially at molecular interfaces and other environments where there is typically less steric hindrance.

Graphical Abstract

INTRODUCTION

Fluorophores capable of site-specific incorporation into peptides and proteins are valuable tools for structural studies and imaging applications. Although macrofluorophores such as green fluorescent protein (GFP) can be easily introduced into biological systems during recombinant expression, their large size can often cause alteration of the structure or function of the system under study.^{1,2} Small molecule organic dyes circumvent this issue and can be designed to possess favorable photophysical properties that maximize their utility as biological probes, but they are not readily incorporated in site-specific positions.

Advances in organic synthesis and calculation methods have allowed for the development of a plethora of fluorophores with desirable photophysics. Among them, solvatochromic probes, usually designed to possess an electron donor and acceptor moiety flanking the aromatic system, have received particular attention because their fluorescence properties are highly sensitive to the surrounding environment, maximizing their utility in probing biological systems.^{3,4} Although many such probes have been developed, their use for investigation of protein structure, interactions, and dynamics is limited by current methods of site-specific incorporation into biomacromolecules.⁵ Incorporation of a solvatochromic probe into a specific site of a protein using a short and rigid linker maximizes the utility of any given fluorescence experiment. Current methods of incorporation that meet these criteria require synthesizing an amino acid derivative of the fluorophore and either introducing it into a peptide using solid phase peptide synthesis for subsequent introduction into a protein using expressed chemical ligation or by chemically acylating it onto an orthogonal tRNA for direct protein incorporation using nonsense suppression. Although the former method limits

the placement of the probe to ~40 amino acids from the C- or N-terminus of the target protein, the latter requires extensive chemical and biological manipulation and is not necessarily compatible for every fluorophore amino acid derivative and/or target protein of interest.

One of the most successful solvatochromic probes in the context of biological investigations, *N,N*-dimethylamino-6-propionyl-2-naphthylamine (PRODAN),⁶ and its derivatives (the DAN fluorophores) have been widely popular in studies of protein binding,^{7,8} structure,⁹ and membrane dynamics.¹⁰ Numerous structural analogues of PRODAN, which substitute its naphthalene core with other conjugated systems while keeping the electron donor/acceptor flanking groups, have been synthesized and possess even more desirable photophysical properties. For example, synthesis of the amino acid Aladan¹¹ (also known as DANA¹²), which bears the PRODAN side chain, has allowed for its introduction into peptides during solid phase peptide synthesis (SPPS) (via the corresponding Fmoc derivative) and into proteins by means of nonsense suppression. However, in most cases, the challenge of introducing these fluorophores into biological systems in a site-specific manner remains.

In our search for a fluorophore that is both a robust probe of the microenvironment and is capable of site-specific incorporation into biological molecules, our attention fell to 2-phenyl-6*H*-oxazolo[4,5-*e*]indole (PHOXI) produced by a fluorogenic oxidative coupling reaction between 5-hydroxytryptophan (5-HTP) and benzylamine in the presence of a mild oxidative agent in aqueous buffer (Scheme 1). This chemistry, first discovered in the 1980s during investigations of the drug 9-hydroxyellipticine, which contains 5-hydroxyindole within its structure,¹³ has been recently applied to fluorescence-based determination of 5-hydroxyindole containing analytes (serotonin, etc.) in increasingly complex biological samples^{14,15} and is touted to be selective for 5-hydroxyindoles within the biological milieu. Because there exist straightforward methods for incorporating 5-HTP into peptides via Fmoc SPPS¹⁶ and into proteins through selective pressure incorporation,¹⁷ we envision using this chemistry as a means of site-specifically labeling peptides and proteins. Furthermore, the few photophysical properties reported for PHOXI,¹⁸ namely, its high extinction coefficient ($1.54 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$),¹⁸ quantum yield (0.54),¹⁸ and emission in the visible region (461 nm) suggest this fluorophore has the essential features of a sensitive fluorescent tag.

Recently, this fluorogenic reaction was successfully used to introduce PHOXI into 5-HTP-containing peptides.¹⁸ Although this report sets an important precedent for the viability of this reaction to introduce the PHOXI fluorophore into peptides, the authors were employing this reaction to prepare cyclic peptides and thus did not investigate its photophysical properties. Furthermore, because the 5-HTP peptides were prepared using *in vitro* translation, their quantities were minute (sub- μM concentration in $\sim 10 \mu\text{L}$), only allowing for determination of reaction products by mass spectrometry without chromatographic separation. Although the authors claim, based on mass spectrometric evidence, that only the desired fluorophore-bearing products are formed, the proposed mechanism for this reaction¹⁹ suggests that unwanted products can arise from attack of intermediate **2** by nucleophiles other than the intended benzylamine. The susceptibility of **2** to attack even by weak nucleophiles is further illustrated by the myriad of oligomeric products produced upon oxidation of 5-hydroxyindole in the absence of benzylamine.²⁰ Finally, a recent study

exploring the analogous reaction of 5-hydroxyindole-2-acetic acid with benzylamine reported ~30% yields of the fluorescent adduct.²¹

Herein, we further probe the utility of the PHOXI derivatization reaction using model peptides. Using SPPS to produce 5-HTP peptides in greater quantities, we evaluate the derivatization reaction in greater concentrations ranges, such as those required for preparing sufficient amounts of labeled biomolecules for structural investigations. Working with greater quantities of 5-HTP peptides allowed us to perform chromatographic separation (RP-HPLC) to further interrogate the reaction products and optimize the derivatization conditions. Consistent with the reaction mechanism, we observed numerous nonfluorescent byproducts during 5-HTP peptide derivatization. Although some of these byproducts are unavoidable, we show how optimizing the derivatization conditions can minimize their formation. Furthermore, we show that PHOXI-labeled peptide derivatives are easily visualized and isolated by RP-HPLC with UV-vis detection.

We also attempted to create more structurally conservative analogues of PHOXI guided by the fact that 6-hydroxyindole-2-acetic acid has been shown to react with amines of the general structure $R-CH_2-NH_2$ to produce various oxazoles. In PHOXI, R is the phenyl ring, but we were interested in analogues of PHOXI, where R is a hydrogen, to minimize the size of this fluorophore and thus the structural perturbation caused by its introduction into peptides and proteins. There are precedents for using methylamine²² or ethanolamine¹⁹ as nucleophiles, albeit in organic media, to produce these unsubstituted PHOXI analogues. In contrast to benzylamine, our attempts to use these reagents to label peptides in a biologically compatible manner were not met with success (data not shown). Although methylamine derivatization did not produce any fluorescent products (contrary to a patent claim²²), the reaction with ethanolamine produced three fluorescent products, two of which were difficult to separate by RP-HPLC. The formation of these three fluorescent adducts is consistent with previous reports,¹³ but because of the difficulty in resolving them, derivatization with ethanolamine was not pursued further.

Finally, we further probed the structure and photophysics of PHOXI and describe the solvatochromism of this fluorophore for the first time. Our findings suggest that sensitivity of PHOXI fluorescence emission to the polarity of the environment is comparable to that of the DAN fluorophores. Although fluorescence emission of PHOXI is only slightly less sensitive to the polarity of the medium than PRODAN, multiparametric analysis using the Catalan 3P model^{23–25} shows that PHOXI's solvatochromism is more clearly dependent on a single parameter, general solvent polarity, with half the sensitivity to solvent acidity than that of PRODAN. Additionally, the quantum yields of PHOXI remained above 0.5 in all of the solvents studied. This is a considerable improvement in comparison to PRODAN, which displays very low quantum yields in extremely polar (water) and nonpolar (hexane) solvents. PHOXI's solvatochromism and straightforward photophysics along with its inherent capability to site-specifically incorporate into peptides via derivatization of 5-HTP make it a very promising fluorescent reporter tag.

MATERIALS AND METHODS

Materials

NH₂-Trp-5-HTP-OH (98.3% purity) and human A β -40 peptide (DAEFRHDSGYEVHHQKLVFFAEDVGSNK-GAIIGLMVGGVV, purity 98%) were custom synthesized by Lifetein (Hillsborough, NJ); 5-HTP-TrpZip2 (SWT-5-HTP-ENGKWTWK, 99% purity) was custom synthesized by Peptide 2.0 (Chantilly, VA). *N*-Acetylcysteine was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The purities of all peptides were confirmed by HPLC prior to use. Benzylamine (99%) was purchased from Acros Organics (Geel, Belgium) and distilled before use. 5-Hydroxyindole (97%) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and purified by sublimation prior to use.

Preparation of 2-Phenyl-6*H*-oxazolo[4,5-*e*] indole (PHOXI)

2-Phenyl-6*H*-oxazolo[4,5-*e*]indole was prepared from 5-hydroxyindole and benzylamine by adapting a procedure described by Boger et al.¹⁹ The procedure is further described in the Supporting Information. PHOXI ¹H NMR data confirm the structure (see Supporting Information).

PHOXI Derivatization of Peptides under Biological Conditions

For optimization of derivatization conditions, 10–100 μ M of 5-HTP-containing or control peptide was dissolved in 0.5 M borate buffer, pH 8.0 in the presence or absence of 0.5 M benzylamine in an HPLC microvial. The reactions were initiated by addition of K₃[Fe(CN)₆] to a final concentration of 2 mM. After 10 min, the reactions were stopped, and the products were analyzed by direct injection onto a Series 200 HPLC system (PerkinElmer, Waltham, MA) equipped with an XBridge C18 4.5 $\times$ 150 mm column (Waters Corporation, Milford, MA). The products were separated by eluting with a linear gradient of Buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile) at a flow rate of 1 mL/min as follows: 5 min 100% buffer A, 2 min increasing to 20% buffer B, 20 min increasing to 40% buffer B. The products and unreacted starting materials were visualized by a UV–vis flow-cell detector at 220 nm (peptide amide bonds), 280 nm (tryptophan and/or 5-HTP) and 326 nm (PHOXI). Product peaks with appreciable absorption at 326 nm were manually collected, lyophilized, and subjected to mass spectrometry to confirm the identity of PHOXI-derivatized products. Lyophilized NH₂-Trp-5-HTP-OH derivatization products were dissolved in 50% acetonitrile containing 0.1% formic acid and analyzed by direct infusion onto an Agilent 6220 ESI-TOF mass spectrometer. TrpZip2 derivatization products were analyzed using a MALDI-LTQ XL orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA) using α -cyano-4-hydroxycinnamic acid as matrix.

Absorbance Measurements

Background-corrected absorption spectra were recorded on a Lambda 650 UV–vis spectrophotometer (PerkinElmer, Walnut Creek, CA) with a 2 nm band-pass, 1 cm path length, and 1 nm interval. Extinction coefficients of Trp (5.69×10^3 M⁻¹ cm⁻¹),²⁶ 5-HTP

$(4.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1})$,²⁷ and PHOXI $(1.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1})$ ¹⁸ were used to determine concentrations.

Steady-State Fluorescence Emission Measurements

Fluorescence emission spectra of PHOXI-derivatized peptides in water and of PHOXI in neat solvents were recorded on a Fluorolog 3 model FL-1000 (Horiba Jobin Yvon, Edison, NJ) fluorimeter. For PHOXI-derivatized peptides, emission spectra were recorded with an excitation wavelength of 280 nm (Trp) and 336 nm (PHOXI). For quantum yield measurements of PHOXI in neat solvents, the excitation wavelength used corresponded to the maximum absorbance of PHOXI in each solvent (332 nm for acetonitrile and 336 nm for 1,4-dioxane, methanol, toluene, and water), except for cyclohexane, where 322 nm had to be used to obtain a complete emission profile. For all fluorescence measurements, the step size was 1 nm, and the integration time was set to 0.1 s. The band pass was 2 nm and matched that of the absorption spectrophotometer. All concentrations were <0.01 mM to avoid inner filter effects.

Quantum Yield Determination

Aqueous quinine sulfate (QY = 0.54)²⁸ and harmaline (QY = 0.83)²⁹ in 0.1 M sulfuric acid were used as quantum yield standards. PHOXI quantum yields (22 ± 2 °C) in six neat solvents were determined by the method described by Williams et al.³⁰ Briefly, the quantum yield of the two quantum yield standards are first determined via reference against each other to ensure confidence in quantum yields measured for the unknown. The integrated fluorescence intensity of each fluorophore is determined from emission spectra at several fluorophore concentrations, all less than 0.01 mM, to avoid the inner filter effect. The integrated intensity is plotted as a function of the measured absorbance at each concentration. A linear fit is calculated, ensuring that the fit line runs through the origin. Obviously, the goodness-of-fit should be close to unity. The slope of the fit is the gradient, G , used in calculation of the quantum yield, QY:

$$\text{QY}_x = \text{QY}_s \left(\frac{G_x}{G_s} \right) \left(\frac{n_x^2}{n_s^2} \right) \quad (1)$$

where the subscript s signifies the quantities for the quantum yield standard, and x are the quantities for the fluorophore of unknown quantum yield. n is the refractive index for each solution: $n = 1.333$ for water, 1.334 for 0.1 M sulfuric acid, 1.497 for toluene, 1.427 for cyclohexane, 1.331 for methanol, 1.344 for acetonitrile, and 1.422 for 1,4-dioxane.³¹

Trp-PHOXI Förster Distance Calculation

The Förster distance in angstroms was calculated using the equation³²

$$R_0 = 0.2108 [k^2 \Phi_{\text{Trp}} n^{-4} J]^{1/6} \quad (2)$$

where R_0 is the critical Förster distance, k is the dipole orientation factor and was set to 2/3, Φ_{Trp} is the quantum yield of Trp in water (0.14),³³ n is the refractive index of the medium taken as 1.4 for biological macromolecules, and J is the overlap integral, defined as

$$J = \int_0^\infty F_{\text{Trp}}(\lambda) \varepsilon_{\text{PHOXI}}(\lambda) \lambda^4 d\lambda \quad (3)$$

where $F_{\text{Trp}}(\lambda)$ is the fluorescence quantum yield of tryptophan, and $\varepsilon_{\text{PHOXI}}(\lambda)$ is the absorption spectrum of PHOXI scaled to its extinction coefficient at 326 nm: $\varepsilon = 1.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.¹⁸

Time-Resolved Fluorescence Lifetime Decay Measurement

Fluorescence lifetime measurements ($22 \pm 2^\circ \text{C}$) of PHOXI in six neat solvents were carried out on a commercial instrument that employs a laser diode excitation source with a broad (1.47 ns) instrument response function (Horiba Jobin Yvon, Edison, NJ). Excitation at 340 nm (± 10 nm), 3 nm excitation and emission band-pass, was achieved with a NanoLED source (< 1 ns pulse). An experimental instrument response function, necessary for proper deconvolution, was collected from scattered light from an aqueous casein solution at 340 nm. Fluorescence decay was collected at the varied emission maxima for PHOXI as given in Table 1.

PHOXI Photodegradation Assays

A $0.33 \mu\text{M}$ solution of PHOXI in either toluene or water in a $50 \mu\text{L}$ quartz microcuvette was illuminated by a xenon lamp of a Fluorolog spectrofluorimeter at 336 nm for 5000 s. The lamp power at the cuvette was 8.5 mW, and the illumination area was 3 mm H $\times$ 2 mm W. Emission and excitation monochromator band pass were set to 8 nm. During illumination, the fluorescence at the maximum (459 nm for water, 390 nm for toluene) was recorded at 1 s intervals. The percentile fraction of residual fluorescence signal after 1 h of illumination was recorded.

Fluorescence Lifetime Decay Analysis

The lifetimes for decays collected on the diode instrument were determined by iterative convolution using the vendor-provided decay analysis software DAS6. Details of this method can be found in ref 34.

Circular Dichroism of PHOXI and 5-HTP-Containing TrpZip2 Peptide

A Chirascan CD spectrophotometer (Applied Photophysics, Surrey, UK) equipped with a sample temperature controller unit was used to obtain CD spectra of $25 \mu\text{M}$ aqueous solutions of TrpZip2 peptides bearing either 5-HTP or PHOXI in place of Trp 4. For each peptide, spectra were recorded at 25, 65, and 80°C .

Structure Calculations

Gaussian 09 software³¹ was used for *ab initio* quantum mechanical calculations. The ground-state structure was optimized using the B3LYP density functional theory method and

6-311G* basis set with an ultrafine grid. The polarizable continuum method with implicit solvents was used to allow for the solvent effects, and the structure was checked for imaginary frequencies to ensure that it was not in a transition state. Calculations were done on the BOB system at the CUNY High Performance Computing Center (College of Staten Island of the City University of New York, New York, NY). Chimera molecular visualization and analysis software v. 1.10.1 (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, CA) was used to calculate the oxazole-phenyl hydrogen bond distances of PHOXI and to create the image of the TrpZip2 hairpin shown in Figure 6.

RESULTS

Incorporation of PHOXI into Peptides under Biological Conditions for Fluorescence Enhancement

Two peptides, $\text{NH}_2\text{-Trp-5-HTP-OH}$ (WW_{OH}) and TrpZip2 substituted with 5-HTP in place of Trp in position 4 (SWT-5-HTP-ENGKWTWK or TrpZip2, $\text{Trp4} \rightarrow 5\text{-HTP}$) were used to evaluate the PHOXI derivatization reaction. Both peptides readily reacted with benzylamine in borate buffer pH 8.0 in the presence of 2 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ to produce fluorescent PHOXI adducts within 10 min.

The HPLC monitoring of reaction products observed during the PHOXI derivatization of $\text{NH}_2\text{-Trp-PHOXI-OH}$ under varying conditions is shown in Figure 1A. The fluorescence emission spectrum of $\text{NH}_2\text{-Trp-PHOXI-OH}$, showing characteristic PHOXI visible emission with $\lambda_{\text{max}} = 451$ nm and a marked increase in intensity ($2.2\times$) relative to that of $\text{NH}_2\text{-Trp-5-HTP-OH}$, is shown in Figure 2B, right. The PHOXI emission increase is due to the synergistic effects of a high extinction coefficient ($4.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ as measured here in 0.5 M borate pH 8.0) and quantum yield (0.54). Conducting the oxidation reaction in the absence of benzylamine (Figure 1A, lower left) produces a number of products. This is consistent with the highly reactive nature of *p*-quinoneiminemethide intermediate **2** (Scheme 1) that is formed upon oxidation of 5-HTP and with previous studies of oxidation of 5-hydroxyindole²⁰ showing a complex mixture of oligomeric products resulting from nucleophilic capture of intermediate **2** at the 4 position. However, in the presence of a large excess of benzylamine, the fluorescent PHOXI adduct is the major product of the reaction (Figure 1A, upper right). The concentration of the 5-HTP-bearing peptide had a profound effect on the number and amount of undesired byproducts produced during derivatization. Undesired byproducts are produced when the peptide concentration is increased from 10 to 100 μM (Figure 1A, lower right). These products appear at the same retention time as those formed during the oxidation of WW_{OH} in the absence of benzylamine. This finding suggests that at higher concentrations unwanted products result from capture of intermediate **2** by another molecule of peptide. We found that generally keeping the 5-HTP-bearing peptide at a concentration of 20 μM or below eliminates these byproducts.

These reaction conditions were applied in the derivatization of the peptide, TrpZip, $\text{W4} \rightarrow 5\text{-HTP}$. HPLC results monitoring this reaction are given in Figure 1B. The left plot in Figure 1B shows the elution time of TrpZip2 $\text{Trp4} \rightarrow 5\text{-HTP}$ in the absence of reactants. The right plot in Figure 1B shows the elution profile for reactants only (gray trace) and the completed

reaction (black trace) with the peak for the PHOXI-derivatized TrpZip2 peptide denoted as 5. We note the significantly reduced number of byproduct peaks in the HPLC for PHOXI TrpZip2. The fluorescence emission spectra for TrpZip2 Trp $\rightarrow$ 5-HTP and the PHOXI-derivatized peptide are shown in Figure 2C, right panel. Fluorescence spectra of these products are consistent with free PHOXI emission and excitation (Figure 2A), further corroborating their identity as PHOXI peptide adducts.

After separation of reaction products by HPLC, each peptide produced a major peak with high absorbance at 326 nm characteristic of PHOXI. These peaks were isolated, and their identities were confirmed by mass spectrometry as $\text{NH}_2\text{-Trp-PHOXI-OH}$ (calculated $[M + H]$ 508.19, observed $[M + H]$ 508.15) (see Figure S1) and $\text{SWT-PHOXI-ENGKWTWK}$, i.e., TrpZip2, Trp4 $\rightarrow$ 5-HTP (calculated $[M + H]$ 1726.79, observed $[M + H]$ 1726.92) (Figure S2).

To ensure that PHOXI derivatization conditions are selective for 5-HTP only, a control reaction was also conducted using peptide A β -40, which does not contain 5-HTP but has all canonical amino acids except for cysteine, tryptophan, and proline (Figure 3). This peptide was found to not be affected by the reaction conditions used for PHOXI derivatization. As the peptide sequence for TrpZip2, Trp4 $\rightarrow$ 5-HTP contains three additional Trps, the reaction of benzylamine with Trp is also eliminated as demonstrated by HPLC (Figure 1B) and mass spectroscopic analysis of the PHOXI-derivatized peptide. Lastly, a similar control reaction was conducted with *N*-acetylcysteine with a similar result, as shown in Figure S3. *N*-Acetylcysteine remained underivatized in the presence of the potassium ferricyanide and benzylamine reagents.

Spectroscopic Properties of PHOXI

Fluorescence Enhancement of PHOXI-Derivatized Peptides and Tryptophan-PHOXI FRET—Figure 2 shows the fluorescence emission and excitation spectra of 5-HTP (A), WW_{OH} (B), and TrpZip2, Trp4 $\rightarrow$ 5-HTP (C) before and after PHOXI derivatization. The excitation maxima of PHOXI analogues of 5-HTP, WW_{OH} , and TrpZip2, Trp4 $\rightarrow$ 5-HTP were found to be 336 nm for the first two species and 341 nm for the last. These wavelengths are outside of the Trp absorption envelope allowing for selective excitation of PHOXI in the presence of Trp. The excitation spectrum for 5-HTP in 0.5 M borate buffer, pH 8.0 (Figure 2A, left panel, gray line) is narrow, peaking at 316 nm. The normalized (50 $\times$) excitation spectrum for aqueous PHOXI is much broader and almost completely overlaps that of 5-HTP. It peaks at 332 nm with a small intensity at 280 nm, the wavelength used for Trp excitation. The emission spectra for 5-HTP and PHOXI are given in Figure 2A, right. The gray emission spectrum of 5-HTP (300 nm excitation) is narrow with an emission maximum at 346 nm. The breadth of the PHOXI excitation spectrum allows for excitation at 280 nm, a wavelength typically used to excite Trp emission, and at a wavelength outside of the 5-HTP excitation band, 336 nm. Emission spectra collected at these two excitation wavelengths prove useful for demonstrating FRET from Trp to PHOXI in WW_{OH} and TrpZip2, Trp4 $\rightarrow$ 5-HTP, as discussed below. The emission spectrum for PHOXI excited at 336 nm (Figure 2A, right, 17 $\times$, solid black line) consists of a single, broad band with a peak at 459 nm. The emission spectrum for PHOXI excited at 280 nm (Figure 2A, right, 100 $\times$,

dashed black line) is very weak, here being magnified 100×. The emission signal is at the level of noise in the emission region (300–375 nm) of 5-HTP and Trp.

Evidence of FRET from Trp residues to the PHOXI residue of WW_{OH} and TrpZip2, Trp4 → 5-HTP can be seen in the excitation and emission spectra of PHOXI-bearing peptides upon excitation at 280 nm (Figure 2B, C, right panels, dashed lines). Compared to the nil 280 nm-excited emission spectrum of PHOXI alone (Figure 2A, right panel, dashed line), these emission spectra show significant intensity at the PHOXI emission maximum of 459 nm, which can only have resulted from energy transfer from one or more Trp residues. We have computed the critical Forster distance, $R_0 = 23.5 \text{ \AA}$, for the PHOXI-Trp system. This value falls into the useful range for distance measurements in the context of biological macromolecules.

Solvatochromism—To glean the utility of PHOXI as a spectroscopic probe capable of providing structural information, we synthesized it from 5-hydroxyindole and benzylamine and studied its spectroscopic properties in six different neat solvents spanning a range of polarities. The results are summarized in Table 1, and absorption and emission spectra are shown in Figure 4A, B.

PHOXI fluorescence emission maxima were found to be very sensitive to solvent, exhibiting red-shifted emission with increasing solvent polarity. PHOXI emission red-shifts by 87 nm in water relative to that of cyclohexane, suggesting that PHOXI fluorescence emission is highly sensitive to the environment. Vibrational structure is present in the emission spectra of PHOXI in the nonpolar solvents cyclohexane and toluene but not in more polar solvents (Figure 4B).

Because of the increase in dipole moment that accompanies electronic excitation in a solvatochromic molecule, considerable solvent reorganization is expected for solvents that are both hydrogen bond donors and acceptors, i.e., water and methanol. The energy of reorganization necessary upon excitation is responsible for the observed large Stokes shifts for PHOXI in these solvents (Table 1), the diminished radiative rate, and the concomitant lower quantum yield (0.54–0.55) relative to those for other solvents. At the same time, the excited state is stabilized by the solvent reorganization, resulting in a longer lifetime of 4.06 ns for methanol and 5.71 ns for water. For aprotic solvents like 1,4-dioxane and acetonitrile, less reorganizational energy is required upon PHOXI excitation because these are only hydrogen bond acceptors. Thus, the Stokes shift is not as great, the radiative rate is not as diminished as for water, and thus quantum yield is higher: 0.72 for acetonitrile and 0.78 for 1,2-dioxane. The excited state is still somewhat stabilized by noncovalent interactions, yielding fluorescence lifetimes of 3.29 ns for acetonitrile and 2.51 ns for 1,4-dioxane. The excited state is not stabilized in non-hydrogen bonding solvents like cyclohexane, so the fluorescence lifetime is only 1.69 ns. As little energy is required for solvent reorganization upon excitation, the Stokes shift is small, and quantum yield is high, certainly within the range for that obtained with acetonitrile given the $\pm 10\%$ uncertainty.

The observation of an inverse relationship between fluorescence quantum yield and lifetime with changes in solvent hydrogen bonding capability is not without precedent. In a study of

the xanthene dye, rhodamine 6G chloride cation, fluorescence lifetime increased while quantum yield decreased as the hydrocarbon chain in a series of alcohols decreased.³⁵ The trend culminates in water, where the longest lifetime of 4.08 ns was matched by the lowest quantum yield of 0.09 ± 0.02 . Because the radiative rate, k_r , is given by³⁵

$$k_r = \phi / \tau \quad (4)$$

where ϕ is the quantum yield and τ is the fluorescence lifetime, it follows that the nonradiative rate, k_{nr} , is given by

$$k_{nr} = (1 - \phi) / \tau \quad (5)$$

Because the distribution of excitation energy between k_r and k_{nr} changes with solvent, it follows that the ratio, ϕ / τ , can change with solvent as well. Thus, ϕ and τ are not necessarily directly proportional under all solvent conditions.

Although PHOXI absorption maxima did not vary significantly (spanning a range of only 10 nm), the quantum yields were found to vary but remained high (between 0.54 and 0.78, Table 1) in all solvents studied. Fluorescence lifetimes exhibited single exponential decay and varied between 1.69 ns (cyclohexane) and 5.71 ns (water), increasing with solvent polarity (see Figure S5).

To better understand the solvatochromic nature of PHOXI, we fit its solvent-associated spectral parameters to linear functions of a generalized solvent parameter, SP, using empirical solvent parameters described by Catalán et al.^{23–25} and supplying proportionality constants. A table of solvent parameters used in the analysis is given in Table S1. Figure 4C shows the dependence of PHOXI emission maxima on SP; an analogous fit of the emission of PRODAN in the same six solvents is included for comparison (PRODAN emission data used to construct the graph can be found in ref 32). For PHOXI, a good linear fit of SP to emission maximum ($R^2 = 0.98$) was achieved when

$$SP = 0.79SPP + 0.12SB + 0.09SA \quad (6)$$

where SPP is solvent polarity-polarizability, SB is solvent basicity, and SA is solvent acidity, whereas for PRODAN the best fit ($R^2 = 0.99$) is realized when

$$SP = 0.69SPP + 0.13SB + 0.18SA \quad (7)$$

Notably, almost all of the variance in PHOXI spectral shifts is explained by SPP with only minor contributions from acidity and basicity. In fact, a single parameter fit to SPP alone

gives $R^2 = 0.94$, suggesting that the dipolar nature of the PHOXI excited state is primarily responsible for the observed solvatochromism. In contrast, PRODAN fluorescence emission displays roughly twice the sensitivity to solvent acidity. For PHOXI, the minor contributions of acidity and basicity are not surprising because it contains both an acidic group (the indolic NH) and a basic group (the oxazole O or N).

Variation in fluorescence lifetimes was also found to be primarily affected by SPP ($R^2 = 0.99$), as shown in Figure S4. As ascertained from quantum yield and fluorescence lifetime data, radiative decay (Γ , $R^2 = 0.97$) decreases with increasing solvent polarity. Nonradiative decay (k_{nr} , $R^2 = 0.80$) and quantum yield (ϕ_F , $R^2 = 0.71$) are not as well modeled by the Catalán et al. generalized solvent parameter, SP (Figure S4).

The change between the ground and excited state dipole moments of PHOXI can be ascertained from its spectral shifts in solvents of differing polarities by means of the Lippert–Mataga equation³²

$$\gamma_{\text{Stokes}} = \frac{2}{hca^3} (\Delta\mu)^2 \Delta f + \text{constant} \quad (8)$$

where γ_{Stokes} is the Stokes shift expressed as energy, h is Planck's constant, c is the speed of light, a is the Onsager radius of the solvent cavity occupied by the fluorophore, μ is the difference in dipole moments in the ground and excited states, and f is the orientation polarizability function defined as

$$\Delta f = \frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (9)$$

where ϵ is dielectric constant and n is the refractive index of the solvent.

Because this equation does not take into account the contributions of specific solvent interactions, we included the SA term into a multiparameter fit along with f , mirroring the analysis of PRODAN solvatochromism by Catalán.³⁷ Regression analysis of PHOXI Stokes shifts affords

$$\gamma_{\text{Stokes}} = 15035(0.63\Delta f + 0.37\text{SA}) + 4205 \quad (10)$$

with $R^2 = 0.90$.

Accounting for the variance in Stokes shifts caused by SA in the above equation and substituting into the Lippert–Mataga equation gives

$$\Delta\mu = \sqrt{\frac{9402hca^3}{2}} \quad (11)$$

Assuming an Onsager cavity radius of 4.94 Å (taken as half the distance between C4 of the phenyl substituent and the indolic N, which are the furthest heavy atoms flanking the PHOXI conjugated system as ascertained from its geometry-optimized ground state structure), the transition dipole moment of PHOXI is 10.6 D.

Structural Properties of PHOXI—Surprisingly, repeated DFT calculations show that PHOXI adopts a planar conformation, presumably because greater stabilization is attained if the oxazole oxygen lone pair does not participate in the aromatic system while the phenyl substituent does. Figure 5 illustrates the calculated planar geometry of PHOXI showing its ground state dipole vector (A) as well as its HOMO (B) and LUMO (C) orbitals.

We probed the structural perturbation caused by PHOXI substitution by obtaining CD spectra of TrpZip2 bearing either 5-HTP or PHOXI at position 4; these results are shown in Figure S6. TrpZip2 is engineered to adopt a β -hairpin structure, stabilized by stacking interaction of two pairs of tryptophan residues.³⁸ The NMR structure showing these interactions is given in Figure 6. The NMR structure reveals that the four Trp residues are arranged in two pairs with orthogonal molecular planes: Trp 4 aromatic C(4)H points into the center of the Trp 9 pyrrole ring whereas Trp 11 C(4)H points into the center of the Trp 2 pyrrole ring. Additionally, the indole molecular planes of Trps 2 and 9 are antiparallel and at a relative angle of slightly less than 90°. Overall, the Trp indoles in TrpZip2 lie in a closely packed arch about one face of the β hairpin. Although Trp 4 is replaced by the bulkier PHOXI, the PHOXI derivative of TrpZip2 still retains some of its β -hairpin structure. As shown in Figure S6, the positive ellipticity at 230 nm of PHOXI-bearing TrpZip2 at 25 °C is approximately the same as that of 5-HTP-bearing TrpZip2 at 80 °C. Thus, despite the fact that PHOXI is surrounded by three copies of the bulkiest possible amino acid residue, some native β -hairpin structure is maintained. This provides for some optimism regarding preservation of secondary structure upon PHOXI incorporation in proteins, especially at molecular interfaces and other environments where there is typically less steric hindrance.

Photodegradation—Susceptibility of PHOXI to photodegradation was also tested by exposure to UV light and monitoring the decrease of its fluorescence intensity at the maximum over time. These results are shown in Figure S7. The experimental setup was identical to that used to probe photostability of PRODAN and its analogue FR0.³⁶ After 1 h, a solution of PHOXI in toluene had 72% of its fluorescence intensity remaining compared to 10% for PRODAN³⁶ and 76% for FR0.³⁶

DISCUSSION

Incorporation into Peptides

We have used two 5-HTP-containing model peptides to show that the PHOXI fluorophore can be introduced by a quick and selective oxidation reaction in aqueous buffer in the

presence of benzylamine. Although unintended reaction byproducts may be observed during derivatization of 5-HTP-bearing peptides, the A β 40 peptide, which is devoid of 5-HTP, is completely unaffected by the reaction conditions, attesting to the selectivity of this reaction. Although *N*-acetylcysteine in the presence of benzylamine here showed no product formation, Yamagishi et al. found side-product formation in the cyclization reaction of a 14 amino acid peptide bearing a Phe functionalized with benzylamine and 5-HTP (F_{bza}-R-T-R-N-A-W_{OH}-A-G-E-G-F-C-D).¹⁸

Because the byproducts present during the oxidation of 5-HTP peptides in the presence of benzylamine are the same as those formed during oxidation of 5-HTP peptides in its absence, we presume that these species are formed by capture of the proposed reaction intermediate **2** by nucleophiles other than the intended benzylamine.

Furthermore, we found that lowering the 5-HTP substrate concentration dramatically reduces the number and amount of these undesired products. This suggests that at higher substrate concentrations capture of intermediate **2** by nucleophilic moieties on another molecule of substrate leads to the formation of these products. At substrate concentrations below 20 μ M, the products formed by this intermolecular capture are eliminated.

Although the presence of these side products is less than desirable in the context of peptide labeling, their formation is consistent with the proposed reaction mechanism¹⁹ and is additionally documented in previous reports.^{20,21} However, even with this inherent limitation, we found that PHOXI-derivatized products are produced essentially free of undesired byproducts at low substrate concentrations and are easily visualized and isolated by RP-HPLC for every peptide tested. Overall, the oxidative coupling reaction of 5-HTP to produce PHOXI offers a fast and easy protocol for labeling of peptides: the reaction is assembled from just three components in aqueous buffer and is completed in 10 min or less. Furthermore, because PHOXI is assembled *in situ* using water-miscible benzylamine, it avoids the need for inclusion of harsh organic solvents during labeling reactions, an otherwise necessary step during labeling with many common organic fluorophores, which suffer from low water solubility.

Taken together, these findings suggest that PHOXI-labeled peptides are easily accessed through oxidation of 5-HTP peptides in the presence of benzylamine and subsequent RP-HPLC purification. The PHOXI fluorophore is introduced site-specifically at the 5-HTP site, and because it only modifies the indole side chain of 5-HTP, the PHOXI label is introduced in a conformationally restricted manner, being linked to the peptide backbone through a single methylene bridge. For a probe to be useful in characterization of the microenvironment, this short and rigid tether offers clear advantages over other common fluorophore attachment strategies (such as through side chain and terminal amine groups as well as cysteine thiol groups), which result in longer flexible tethers and greater degrees of freedom.

The PHOXI labeling strategy is applicable to a wide variety of biomolecules, primarily due to the ease of introduction of 5-HTP into peptides and proteins. For example, 5-HTP is easily incorporated as its Fmoc derivative during SPPS using standard protocols,¹⁶

incorporated into proteins in response to a Trp codon during recombinant expression in a Trp auxotrophic host,¹⁷ or introduced into a desired site by means of nonsense suppression. Furthermore, recent work utilizing PHOXI as a fluorescent linker for peptide immobilization suggests that an Fmoc-protected tryptophanyl PHOXI derivative can be introduced during SPPS as well.⁴⁰

Applying this chemistry to 5-HTP protein substrates is expected to present challenges associated with separation of the correctly PHOXI-labeled protein from possible undesired byproducts. Although RP-HPLC purification may prove sufficient for some protein substrates, inadequate resolution and unwanted protein denaturation in HPLC buffers are among the expected challenges. However, a workaround solution based on substrate flexibility of this fluorogenic coupling reaction can be envisioned. Several groups^{14,18} reported that benzylamines substituted at one or more of the phenyl positions still react with 5-HTP to form fluorescent adducts analogous to PHOXI. One of these reaction variants included the introduction of biotinyl benzylamine upon oxidation of a 5-HTP peptide substrate.¹⁸ Because unwanted byproducts arise from the capture of the reactive intermediates by nucleophiles other than the intended benzylamine, using benzylamines functionalized with non-nucleophilic affinity tags can facilitate the separation of correctly labeled protein products through affinity purification.

Fluorescence Enhancement and Solvatochromism

In addition to a reliable method for incorporation into specific sites of a biomolecule, our investigation of the photophysics of PHOXI revealed that it has many desirable characteristics of a sensitive fluorescent reporter tag and reporter of the peptide/protein microenvironment. The fluorescence emission spectrum of PHOXI lies in the visible region ($\lambda_{EM} = 459$ nm in water), and its high quantum yield (0.54) and absorption extinction coefficient contribute to its brightness. Consistent with the above properties, PHOXI peptide adducts display visible fluorescence emission and an approximately 5-fold increase in brightness relative to those of unmodified 5-HTP peptides.

PHOXI absorption also shows a significant overlap with the Trp emission envelope, allowing PHOXI to act as a FRET acceptor to Trp. The calculated critical Forster distance of 23.5 Å for this pair falls within the usable range (20–80 Å) for distance measurements for biological macromolecules. Because Trp is an intrinsic fluorophore, this furthers PHOXI's utility to provide structural information and enables it to be used in binding assays for Trp-containing binding partners. Also of significance here is the fact that PHOXI absorption and excitation wavelengths are outside of the Trp absorption envelope, allowing for selective excitation of PHOXI in the presence of Trp. As seen in Figure 2B, when H₂N-Trp-PHOXI-OH is irradiated with 336 nm light, only PHOXI emission is observed, whereas excitation at 280 nm yields both Trp and PHOXI emissions by way of FRET.

Keen sensitivity of PHOXI's fluorescence emission, lifetimes, and quantum yields to the polarity of the surrounding media allows it to be used as a sensitive reporter of the local microenvironment, greatly increasing the amount of structural, dynamics, and binding assay data that can be extracted from experiments using this probe. We found that PHOXI's spectral emission maximum red-shifts by 87 nm when the solvent is changed from the

nonpolar cyclohexane to water. Probes exhibiting this degree of solvatochromism are highly sought after, and many are rationally designed to impart this property.

A comparison of PHOXI's photophysical properties to other solvatochromic probes with absorption below 400 nm is shown in Table 2. It is noteworthy that none of these fluorophores have perfect photophysics for any given biological investigation, and none, except for PHOXI, have an intrinsic method of site-specific biosynthetic incorporation. The degree of PHOXI's solvatochromism is comparable to that of PRODAN, 6-*N,N*-dimethylamino-2,3-naphthylimide (6DMN), 4-(*N,N*-dimethylamino)-phthalimide (4DMP), and 5-(dimethylamino)-naphthalene-sulfonamide (dansyl) probes. The extinction coefficient of PHOXI is close to the average of the probes listed, and its high quantum yield in polar solvents makes it a great alternative to probes such as Fluoroprobe, 6DMN, and 4DMP, which exhibit low quantum yields in polar solvents.

Although PHOXI exhibits modestly lower bathochromic shifts with increasing solvent polarity relative to those with PRODAN, one of the most successful solvatochromic dyes in the realm of biological investigations, its sensitivity to specific solvent acidity effects is approximately half that of PRODAN. This more straightforward relationship will help to better ascertain the polarity of protein cavities. Although the amino acid analogue of PRODAN, Aladan, was recently reported to be an efficient reporter of protein electrostatics in the Shaker potassium channel and Kir2.1 proteins,¹¹ its sensitivity to H-bond donors complicates the attribution of such data to the polarity of protein cavities. In fact, Catalán has cautioned of attributing such spectral data to general polarity effects,³⁷ theorizing that the high polarity of the haem binding pocket in apomyoglobin as ascertained from PRODAN fluorescence⁴⁶ can also be attributable to specific interactions with pyrrole NH sites.

Furthermore, the molecular structure of PHOXI does not resemble the traditional scaffold of electron donor/acceptor moieties flanking an aromatic system that is the hallmark of other solvatochromic dyes in Table 2. This suggests that an alternative approach to the rational design of such probes is possible. Additionally, utilizing varying aryl amine nucleophiles can provide synthetic access to structural analogues of PHOXI with enhanced properties.

Structure

DFT calculations show that the oxazoloindole and phenyl rings of PHOXI remain coplanar in both HOMO and LUMO orbitals (Figure 5B, C). Hydrogen bonding between the oxazole nitrogen and oxygen and nearest neighbor aryl hydrogens (hydrogen bond distances = 2.56 and 2.55 Å, respectively) may stabilize such a conformation.⁴⁷ Coplanarity of the PHOXI rings can reduce structural perturbation.

CONCLUSIONS

The usefulness of Trp fluorescence for characterizing its immediate protein environment is lost when more than one appears in a sequence. For this problem to be overcome, Trp analogues have been biosynthetically swapped in, but the spectroscopic resolution provided is limited and often insufficient. Here, we have used a Trp analogue, 5-HTP, as the *in situ* building block for a larger chromophore, PHOXI, under mild, biologically compatible

reaction conditions. The reaction is complete in ~10 min, and given optimized reaction conditions, little if any side products may be formed. PHOXI exhibits high quantum yield in solvents of varying polarity, making it solvatochromic. PHOXI emission is significantly red-shifted relative to that of Trp, to 459 nm in water, providing for resolution of emission in a multi-Trp protein. Specificity of the reaction is demonstrated by lack of product formation with amyloid beta 40 peptide, the sequence of which contains all but three (Cys, Pro, Trp) native amino acids. Remarkably, DFT calculations have shown PHOXI to be planar. A TrpZip2 peptide construct where one out of four Trps (Trp4) was replaced by PHOXI partially maintained the native β -hairpin structure, demonstrating that even under sterically crowded conditions PHOXI can be inserted and cavity-dependent local structure can be retained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by National Institutes of Health Grant 5SC3GM105562. This research was supported, in part, by a grant of computer time from the City University of New York High Performance Computing Center under NSF Grants CNS-0855217, CNS-0958379, and ACI-1126113. Molecular analysis was performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institutes of Health Grant P41-GM103311). We would like to thank Dr. Ryan Murelli who provided facilities for PHOXI synthesis and purification.

ABBREVIATIONS

CD	circular dichroism spectroscopy
DAN	6-dimethylamino-2-acyl-naphthalene
6DMN	6- <i>N,N</i> -dimethylamino-2,3-naphthylimide
4DMP	4-(<i>N,N</i> -dimethylamino)phthalimide
5-HTP	5-hydroxytryptophan
DFT	density functional theory
FR0	7-diethylamino-9,9-dimethyl-9 <i>H</i> -fluorene-2-carbaldehyde
GFP	green fluorescent protein
HOMO	highest occupied molecular orbital
LUMO	lowest unoccupied molecular orbital
PHOXI	2-phenyl-6 <i>H</i> -oxazolo[4,5- <i>e</i>]indole
PRODAN	<i>N,N</i> -dimethylamino-6-propionyl-2-naphthylamine
QY	quantum yield
RP-HPLC	reverse phase-high pressure liquid chromatography

SPPS	solid phase peptide synthesis
WW_{OH}	tryptophanyl 5-hydroxytryptophan

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Figure 1.

HPLC monitoring of the formation of PHOXI. All reactions are carried out in 250 mM borate buffer at pH 8.0 for 10 min. (A) Derivatization of $\text{NH}_2\text{-Trp-5-HTP-OH}$ in the presence and absence of reactants. (top left) $10\ \mu\text{M}$ $\text{NH}_2\text{-Trp-5-HTP-OH}$ only; (top right) $10\ \mu\text{M}$ $\text{NH}_2\text{-Trp-5-HTP-OH}$ with 2 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 0.5 M benzylamine; (bottom left) $10\ \mu\text{M}$ $\text{NH}_2\text{-Trp-5-HTP-OH}$ with 2 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ only; (bottom right) $100\ \mu\text{M}$ $\text{NH}_2\text{-Trp-5-HTP-OH}$ with 2 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 0.5 M benzylamine. Peaks are identified as $\text{NH}_2\text{-Trp-5-HTP-OH}$ (1), benzylamine (2), and $\text{NH}_2\text{-Trp-PHOXI-OH}$ (3). (gray trace) Monitoring absorbance at 320 nm; (black trace) monitoring absorbance at 280 nm. (B) Derivatization of $10\ \mu\text{M}$ Trp-Zip2 , $\text{Trp4} \rightarrow 5\text{HPT}$. (left panel) Peptide only; (right panel) reagents alone (gray trace) and reaction solution (black trace). Peaks are identified as benzylamine (2), peptide (4), and PHOXI-derivatized TrpZip2 (5).

Figure 2. (left panels) Fluorescence excitation and (right panels) emission spectra of 5-HTP and PHOXI and peptides derivatized with these chromophores. Spectra for 5-HTP-bearing species given by gray lines. Spectra for PHOXI-derivatized species given by black lines. Emission spectra for PHOXI-derivatized species were acquired at two excitation wavelengths: 280 nm (dashed black line) and 336 nm (solid black line). The emission monochromator was set to the emission maximum as shown in the right panels for collection of the excitation spectra given in the left panels. (A) Spectra for NH₂-5-HTP-OH (gray lines) and PHOXI (black lines). (B) Spectra for NH₂-Trp-5-HTP-OH (gray lines) and NH₂-Trp-PHOXI-OH (black lines). The emission intensity for NH₂-Trp-5-HTP-OH is scaled to 2.2× the original intensity. (C) Spectra for TrpZip2, Trp4 → 5-HTP (gray lines) and TrpZip2, Trp4 → PHOXI (black lines).

Figure 3.

PHOXI derivatization conditions do not affect residues other than 5-HTP: HPLC monitoring of PHOXI derivatization of A β -40. All reactions are carried out in 250 mM borate buffer at pH 8.0 for 10 min with the following additions: (A) derivatization reagents only: 2 mM K₃[Fe(CN)₆] and 0.5 M benzylamine, (B) 20 μ M A β -40 peptide only, (C) 20 μ M A β -40 peptide with 2 mM K₃[Fe(CN)₆] and 0.5 M benzylamine, and (D) Chromatogram C with A subtracted.

Figure 4. Spectroscopic properties of PHOXI in solvents of varying polarity: cyclohexane (solid black line), toluene (solid gray line), dioxane (dashed black line), acetonitrile (dashed gray line), methanol (dash-dotted black line), and water (dash-dotted gray line). (A) absorption, offset, (B) normalized fluorescence emission, (C) multiparameter fits of PHOXI and PRODAN (constructed from previously published emission data³⁶) fluorescence maxima to a global solvent parameter (SP) as defined in the Catalán 3P model.^{23–25}

Figure 5. DFT-calculated planar structure of PHOXI ground state showing the dipole vector (A), HOMO (B), and LUMO (C) orbitals.

Figure 6. NMR structure of the β -hairpin peptide, TrpZip2, with tryptophan residues specifically displayed (image created from PDB 1LE1³⁸ using Chimera software,³⁹ University of California at San Francisco).

Scheme 1. Fluorogenic Oxidative Coupling Reaction of 5-Hydroxyindole (1) and Benzylamine^a

^aInitial oxidation forms *p*-quinoneiminemethide (2), which is highly reactive to nucleophiles at position 4. Nucleophilic attack by benzylamine at this position, followed by oxidation to the imine, a 1,5-hydride shift, and ring closing yields (3) 2-phenyl-6*H*-oxazolo[4,5-*e*]indole (PHOXI).

Table 1

Spectroscopic Properties of PHOXI in Neat Solvents

solvent	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	ν_{Stokes} (cm^{-1})	ϕ_{r} ($\pm 10\%$)	lifetime (ns)
cyclohexane	331	331	372	3330	0.6	1.69
toluene	322	336	390	5415	0.69	1.91
1,4-dioxane	333	335	409	5580	0.78	2.51
acetonitrile	320	332	427	7831	0.72	3.29
methanol	325	336	437	7886	0.55	4.06
water	321	336	459	9366	0.54	5.71

Table 2
Comparison of PHOXI to Other Solvatochromic Fluorophores with Absorption <400 nm^a

dye	λ_{Abs} (nm)	ϵ_{max} (M ⁻¹ cm ⁻¹)	polar solvent (MeOH)		apolar solvent (toluene)		ν_{Fluo} (cm ⁻¹)	ref
			λ_{Fluo} (nm)	QY (%)	λ_{Fluo} (nm)	QY (%)		
Fluoroprobe	308 ^b	12000	695 ^c	<0.1 ^c	476	46	6620	41
FR0	396	43000	570	19	434	98	5500	36
dapoxyl derivatives	373	28000	584	39	457	86	4760	42
Prodan	361	18400	498	51	417	55	3900	6
6DMN	382	8000	589	1.2	491	21	3390	43
4DMP	396	6500	534	12 ^d	457 ^c	62 ^c	3160	44
dansyl derivatives	335	4600	526	49	471	81	2220	45
PHOXI	326	15400	437	55	390	69	2760	

^a λ_{Abs} , absorption maximum; ϵ_{max} , absorption coefficient; λ_{Fluo} , fluorescence emission maximum; QY, quantum yield.

Data in ^bcyclohexane, ^ctetrahydrofuran, ^d1,4-dioxane.