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Photophysics and Biological Applications of the Environment-Sensitive Fluorophore 6-*N*,*N*-Dimethylamino-2,3-naphthalimide

M. Eugenio Vázquez, Juan B. Blanco,† and B. Imperiali*

Contribution from the Department of Chemistry and Department of Biology, Massachusetts Institute of Technology, 77 Mass Avenue, Cambridge, Massachusetts 02139

Received August 23, 2004; E-mail: imper@mit.edu

Abstract: We have synthesized a new environment-sensitive fluorophore, 6-*N*,*N*-dimethylamino-2,3-naphthalimide (6DMN). This chromophore exhibits valuable fluorescent properties as a biological probe with emission in the 500−600 nm range and a marked response to changes in the environment polarity. The 6DMN fluorescence is red-shifted in polar protic environments, with the maximum emission intensity shifting more than 100 nm from 491 nm in toluene to 592 nm in water. Additionally, the fluorescence quantum yield decreases more than 100-fold from chloroform ($\Phi = 0.225$) to water ($\Phi = 0.002$). The scope and applications of the 6DMN probe are expanded with the synthesis of an Fmoc-protected amino acid derivative (**5**), which contains the fluorophore. This unnatural amino acid has been introduced into several peptides, demonstrating that it can be manipulated under standard solid-phase peptide synthesis conditions. Peptides incorporating the new residue can be implemented for monitoring protein−protein interactions as exemplified in studies with Src homology 2 (SH2) phosphotyrosine binding domains. The designed peptides exhibit a significant increase in the quantum yield of the long wavelength fluorescence emission band (596 nm) upon binding to selected SH2 domains (e.g., Crk SH2, Abl SH2, and PI3K SH2). The peptides can be used as ratiometric sensors, since the short wavelength band (460 nm) was found almost invariable throughout the titrations.

Introduction

Fluorescence spectroscopy has become one of the most valuable tools for the development of new probes for biochemical research,[1] being extensively used for monitoring ions,[2] small molecules,[3] and biological processes,[4] such as protein folding,[5] protein−protein interactions,[6] and phosphorylation events.[7] While many fluorescence applications rely on the use of intrinsic fluorophores, the development of new extrinsic fluorophores remains an essential element for the design of new fluorescent probes.

Environment-sensitive fluorophores are a special class of chromophores that have spectroscopic behavior that is dependent on the physicochemical properties of the surrounding environment.[8] Particularly useful are the solvatochromic fluorophores that display sensitivity to the polarity of the local environment, such as 2-propionyl-6-dimethylaminonaphthalene (PRODAN),[9] 4-dimethylamino phthalimide (4-DMAP),[10] and 4-amino-1,8-naphthalimide derivatives[11] (Chart 1). These molecules generally exhibit a low quantum yield in aqueous solution, but become highly fluorescent in nonpolar solvents or when bound to hydrophobic sites in proteins or membranes.

Introduced more than 20 years ago,[9a] PRODAN and derivatives still constitute the most widely used environment-sensitive fluorophores, regardless of certain limitations mainly resulting from the relatively intense fluorescence even in aqueous

† Departamento de Química Orgánica y Unidad Asociada al CSIC. Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain.

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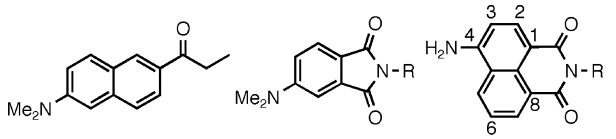
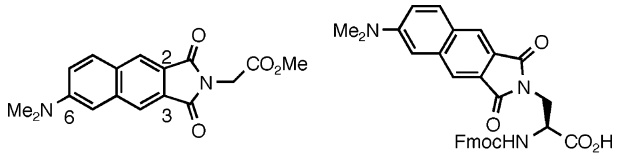
environments. Herein we present the synthesis and preliminary photophysical characterization of a new environment-sensitive fluorophore 6-*N*,*N*-dimethylamino-2,3-naphthalimide (6DMN), together with its application for the development of new probes for biological interactions using a new amino acid residue for Fmoc-based solid-phase peptide synthesis (Fmoc-Dap(6DMN)-OH, **5** (Chart 2)). The 6DMN fluorophore combines some of the advantageous fluorescence properties of PRODAN with the extreme sensitivity to the local polarity exhibited by the 4-aminophthalimide family of environment-sensitive fluorophores.[10] The 6DMN fluorophore may also find important technological applications analogous to those of 4-amino-1,8-naphthalimides in new materials such as liquid crystal displays[12] or light-emitting diodes.[13]

Chart 1. Examples of Environment-Sensitive Fluorophores PRODAN (**1**), 4-DMAP (**2**), and 4-Amino-1,8-naphthalimide (**3**)



1 **2** **3**

Chart 2. 6-Dimethylamino-2,3-naphthalimide 6DMN Derivatives Studied: Model Compound **4** (6DMN-GlyOMe), Amino Acid Building Block **5** (Fmoc-Dap(6DMN)-OH)



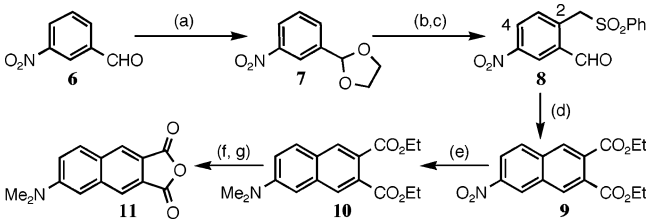
4 **5**

Previously, we reported that solvatochromic amino acids could be used to probe phosphorylation-dependent peptide−protein interactions.[14] In the previous study, the DANA amino acid (which included the PRODAN fluorophore) was used to signal binding by a phosphoserine peptide to 14−3−3, which is a protein involved in cell cycle control.[15] Another common phosphopeptide binding motif in nature is the Src homology 2 (SH2) domain. SH2 domains are small protein modules (approximately 100 amino acids) that are involved in tyrosine kinase signaling networks.[16] SH2 domains specifically recognize short phosphotyrosine-containing peptide sequences in target proteins through interactions with the phosphotyrosine side chain and a short sequence of *C*-terminal residues (three to five amino acids).[17] The specificity of the interaction of the peptide for any of the different SH2 variants is largely determined by the nature of those residues adjacent to the phosphotyrosine.[17b,18] Therefore, SH2 domains facilitate phosphorylation-dependent protein−protein interactions that result in signal propagation within the cell.[19] Examination of the structures of phosphopeptides bound to SH2 domains reveals that the interaction of the phosphopeptides with the SH2 domains takes place on the surface of the protein, and the phosphopeptides remain largely exposed to solvent even when bound to the proteins.[20] Thus, initial experiments using DANA as fluorescent reporter did not reveal a significant increase in fluorescence upon binding. Development of fluorescent sensors for specific SH2 domains would allow for a deeper understanding of their function within complex cellular networks and aid in the screening of small-molecule inhibitors that could be used to modulate SH2 activity and, ultimately, serve as pharmaceutical agents.[21]

Results and Discussion

Synthesis of 6DMN Fluorophore and the Amino Acid Building Block (5). Synthesis of the 6-*N*,*N*-dimethylamino-2,3-naphthalene derivatives **4** and **5** required access to the key anhydride intermediate **11** (Scheme 1). The first step involved protection of the commercially available 2-nitrobenzaldehyde, **6**, by refluxing in toluene with acid catalysis and continuous removal of water using a Dean−Stark apparatus.[22] The resultant nitroarene **7** was then coupled with chloromethyl phenyl sulfone via a vicarious nucleophilic substitution.[23] The reaction proceeded in acceptable yield, affording the mixture of isomers at positions 2 and 4, in a 1:1 ratio. The desired 2-(2-phenylsufonylmethyl-5-nitrophenyl)-1,3-dioxolane isomer was separated by silica gel chromatography and, after deprotection of the aldehyde, afforded intermediate **8** in good yield. The naphthalene moiety was then assembled by reaction of **8** in a multistep process. Addition of the nucleophilic sulfone anion to the electrophilic diethyl maleate, followed by addition of the resulting anion to the electrophilic aldehyde and subsequent elimination of phenylsulfinic acid and water, gave the desired

Scheme 1. Synthesis of 6-*N*,*N*-Dimethylamino Naphthalic Anhydride Intermediate **11**[a]



[a] (a) $HOCH_2CH_2OH$, *p*-TsOH, toluene, reflux, overnight, 100%; (b) $ClCH_2SO_2Ph$, KOH, DMSO, 23 °C, overnight, 69%; (c) AcOH/water 4:1, reflux, 5 h, 100%; (d) diethyl maleate, 18-crown-6, K_2CO_3, CH_3CN, 23 °C then reflux, 3 h, 74%; (e) formalin, 10% Pd/C, H_2 (1 atm) room temperature, 4 h, 84%; (f) KOH aq 40%, reflux, 4h; (g) △, vacuum, 4 h, 77%.

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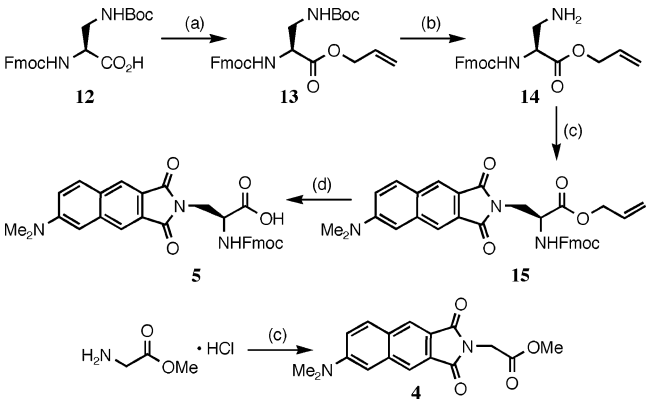
naphthalene derivative **9**.[24] Reductive amination of intermediate **9** with formalin and hydrogen in the presence of Pd/C afforded the desired diethyl 6-*N*,*N*-dimethylamino-2,3-naphthalene dicarboxylate **10** in excellent yield. Treatment of **10** with aqueous potassium hydroxide yielded the corresponding diacid, which was dehydrated by sublimation under reduced pressure to afford the desired anhydride **11**.

For the assembly of the amino acid, commercially available Fmoc-Dap(Boc)-OH (**12**) was allyl-protected (Scheme 2). The side-chain amine of the fully protected intermediate **13** was selectively deprotected by treatment with trifluoroacetic acid (TFA) to afford the free amine **14**, which was coupled without further purification to the naphthalic anhydride **11**. As expected from previous studies in the group,[25] the first coupling reaction of the free amine **14** with the anhydride proceeded smoothly. However, the ring closure reaction and formation of the corresponding 6DMN fluorophore took place only after activation of the free acid using a 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole (HBTU/HOBt) mixture. This procedure afforded the desired protected amino acid Fmoc-Dap(6DMN)-OAllyl (**15**) under mild conditions. Allyl deprotection of the fully protected building block **15** afforded the desired building block **5** in good yield. For the synthesis of the model compound **4**, glycine ester hydrochloride was reacted in a similar way with anhydride **11**.

Scheme 2. Coupling of Anhydride **11**, Synthesis of **5**[a]



[a] (a) 1. Cs_2CO_3, MeOH, 23 °C, 10 min, 2. Allyl bromide, 23 °C, 78%; (b) TFA, CH_2Cl_2, 0 °C, 30 min, 23 °C, 2h; (c) 1. **11**, DIEA, DMF, 23 °C, 15 min, 2. HBTU, HOBt, 23 °C, overnight, >95%; (d) $Pd(PPh_3)_4$, phenyl silane, CH_2Cl_2, 23 °C, 30 min, >95%.

Photophysics of 6DMN: UV Spectroscopy, Steady-State Emission Spectra, Quantum Yield, and Solvatochroism. The 6DMN fluorophore is closely related to the 4-amino-1,8-naphthalimides; however, as has been already established for unsubstituted aromatic dicarboximides, the photophysical properties of these systems have a strong dependence on the position of the dicarboximide group on the naphthalene system, and therefore the new system required detailed characterization.[26] The most significant feature in the UV spectrum is the intense absorption band at 378 nm. This band is not present in the unsubstituted 2,3-naphthalimide, while the other two bands at shorter wavelengths (282 and 237 nm) are qualitatively similar

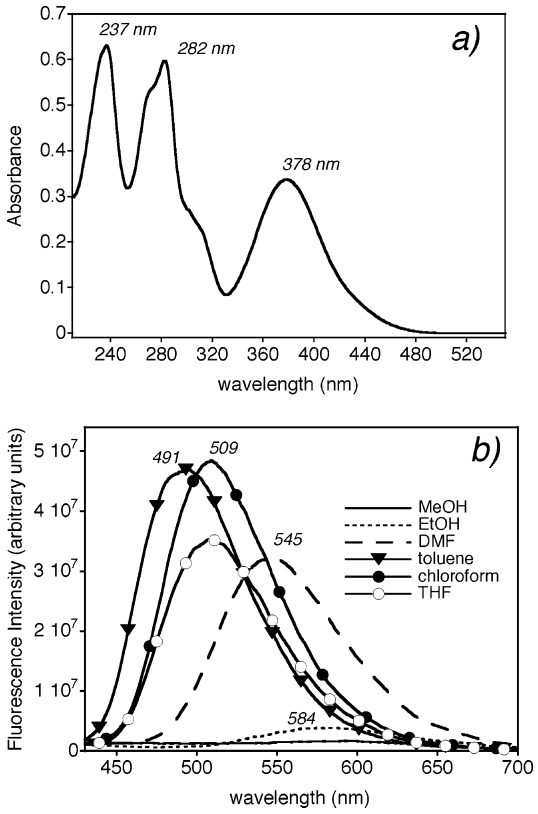


Figure 1. (a) UV absorption spectra of **4** (40 μM) in methanol. (b) Fluorescence emission spectra of **4** in different solvents. Labels show maximum emission wavelengths for selected solvents (toluene, chloroform, and methanol).

to those found in the unsubstituted parent compound.[26] The absorption spectrum was found to be relatively insensitive to changes in the polarity of the environment, with only a small red shift of the long wavelength absorption band from 372 nm in 1,4-dioxane to 388 nm in water (Figure 1a).

In general, we have observed that 6DMN shows fluorescence properties parallel to those exhibited by the closely related fluorophore 4-DMAP, showing, however, a far higher emission intensity (Figure 1b). The maximum excitation wavelength of 6DMN is at 375 nm, and the fluorescence emission spectra are strongly dependent on the polarity of the environment: the maximum emission wavelength is red-shifted almost 100 nm from 491 nm in toluene to 589 nm in methanol, and the fluorescence quantum yield decreases more than 100 times from chloroform ($\Phi = 0.225$) to water ($\Phi = 0.002$).

In contrast to the behavior of the parent compounds (2,3-naphthalimides), which hardly show any fluorescence response to changes in the polarity of the solvents,[26] 6DMN shows outstanding solvatochromic fluorescence properties. The most remarkable effect is the quenching observed in protic solvents that results in a strong reduction of the fluorescence quantum yield and a significant red shift of the emission band to the vicinity of 590 nm. Polar aprotic environments (acetonitrile, *N*,*N*-dimethyl formamide, acetone, tetrahydrofuran) also induce an increase in the quantum yield, as well as a marked blue shift. Apolar aprotic solvents such as chloroform, dichloromethane, toluene, or 1,4-dioxane cause the largest blue shift and increase in the quantum yield relative to polar protic solvents. For example, in toluene the maximum emission intensity is at 491 nm, and in water it is at 592 nm (see Table 1).

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Table 1. Photophysical Properties of **4** in Different Solvents

solvent	absorbance maximum (nm)	emission maximum (nm)	quantum yield (Φ)	solvent type
water[a]	388	592	0.002	protic
methanol	382	589	0.012	
2-propanol	385	589	0.018	
ethanol	379	584	0.027	
acetonitrile	380	549	0.135	polar aprotic
DMF	380	545	0.155	
acetone	375	532	0.148	
tetrahydrofuran	373	510	0.147	
1,4-dioxane	372	498	0.220	
dichloromethane	379	517	0.210	apolar aprotic
chloroform	380	509	0.225	
toluene	373	491	0.208	

[a] Because of solubility limitations, the quantum yield in water was calculated using peptide **Abl-bp** (see Table 2).

It is known that the solvent sensitivity to polarity can be analyzed in terms of difference in the dipole moments in the ground and excited states, and it has been found that the most sensitive fluorophores are those with the largest changes in the dipole moment. This can be estimated from a Lippert−Mataga plot, which is essentially a plot of the Stokes shift of the fluorescence emission versus the solvent polarity.[27,28] The difference in the maximum absorption and emission wavelengths, expressed in wavenumbers ($\Delta\bar{\nu}$), is fitted to the following equation:

$$\bar{\nu}_A - \bar{\nu}_F = \Delta\bar{\nu} = \frac{2}{hca_0^3}\left(\frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right)(\mu_e - \mu_g)^2 = \frac{2\Delta f}{hca_0^3}\Delta\mu^2 \quad (1)$$

where $\mu_e - \mu_g$ is the difference between the dipole moments of the excited and the ground states, respectively ($\Delta\mu$), c is the velocity of light, h is Plank's constant, and a_0 is the radius of the Onsager cavity around the fluorophore. The parameters ϵ and n are the solvent dielectric constant and refraction index, respectively, which are grouped in the term Δf, known as orientation polarizability. The Onsager radius was calculated from the optimized structure obtained with a DFT minimization using the Gaussian program[29] (B3LYP functional using 6-31G-(d) orbital base). The Onsager radius (4.19 Å) was taken as half of the average distance between nitrogen of the amine donor and the two carbonyl oxygens, which corresponds to the longest distance across the molecule where charge separation can take place.[30] As can be seen in Figure 2a, the Stokes shift of a series of mixtures of 1,4-dioxane/acetonitrile changes linearly in response to the solvent polarity, which correlates with an increase in the dipole moment ($\Delta\mu$) of 5.5 D. This value is similar to reported values for other environment-sensitive fluorophores.[30,31] Lippert plots also give evidence of specific

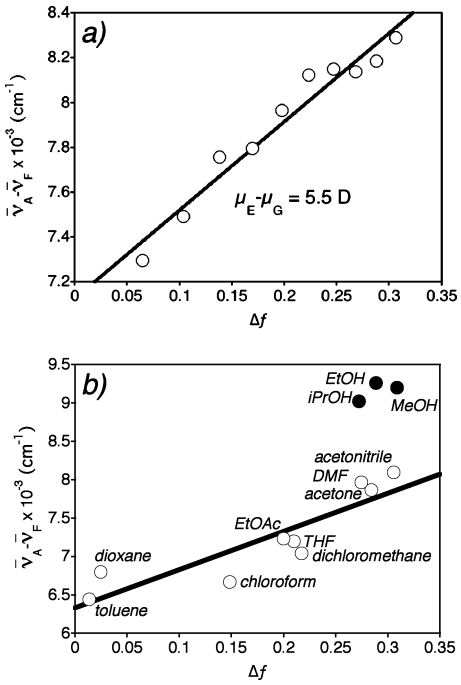

Figure 2. (a) Plot of the Stokes shift vs the solvent polarity measured as the orientation polarizability Δ×c4, derived from fluorescence spectra of **4** in a series of 1,4-dioxane/acetonitrile mixtures. Δ×c4 is obtained for each mixture using ϵ and n values calculated using the molar fractions as follows: $\epsilon = \chi_{acetonitrile} \times 38.8 + \chi_{diox} \times 2.218$; $n = \chi_{acetonitrile} \times 1.3442 + \chi_{diox} \times 1.4224$; $\chi_{acetonitrile} + \chi_{diox} = 1$. (b) Lippert plot of **4** in different solvents. The line corresponds to the best linear fit to the data, excluding the values obtained for protic solvents (methanol, 2-propanol and ethanol, ●).

solvent effects for protic solvents; as can be seen in Figure 2b, in most solvents the Stokes shift is proportional to the orientation polarizability. However, protic solvents induce a disproportionately large Stokes shift. This phenomenon has also been observed for other environment-sensitive fluorophores and has been explained as the result of emission from two different excited states: one with higher dipole moment that is stabilized in polar environments and another less polar excited state responsible for the emission in less polar media.[32]

SH2 Binding Peptides: Design and Synthesis. Upon completion of the building block synthesis, **5** was incorporated into peptides using standard Fmoc solid-phase peptide synthesis (SPPS). The peptide targets were designed for use in evaluating the potential applicability of 6DMN-based peptidic sensors in monitoring phosphorylation-dependent peptide−protein interactions in the context of SH2 domains. The phosphotyrosine peptide-binding SH2 domains were selected as targets for their high biological significance and the unique challenges presented for probe design.[19,33] SH2 domains interact with phosphorylated

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tyrosine-containing sequences at peptide−protein or protein−protein interfaces. With phosphotyrosine-dependent protein−protein interactions, the essential phosphoamino acid contacts are commonly complemented by other simultaneous protein−protein interactions that greatly enhance selectivity.[17,34] In general, SH2 domains are highly conserved, and the largest energetic contribution to their interaction with peptides or proteins comes from the contacts made with the phosphotyrosine. It is therefore challenging to achieve tight and selective binding to a specific SH2 domain with small molecules or peptides.[35] Additionally, analysis of the available structures of phosphopeptides bound to SH2 domains reveals that the peptides are docked onto the protein surface, rather than sequestered in a deep pocket, as is the case with the phosphoserine peptide-binding proteins such as 14−3−3.[36] The shallow surface for interaction exposes most of the peptide side chains to the bulk aqueous environment, thus making it more difficult to use polarity-based (environment-sensitive) sensors as probes for the interaction, since the polarity of the microenvironment surrounding the fluorophore does not necessarily change upon binding to the SH2 domain.

Peptides for SH2 binding were designed on the basis of previous studies with peptide libraries that identified the optimized amino acid sequence for binding to different members of the SH2 domain family.[37] The binding sequences with the highest scores from the library targeting Abl SH2 and Crk SH2 domains were incorporated into peptides, along with the reporter amino acid, **5**, inserted at the (+2) position relative to the phosphotyrosine residue[38] (Table 2).

Table 2. Peptide Sequences and Corresponding SH2 Domain Targeted[a]

peptide	target SH2	peptide sequence
Crk-bp	Crk	Ac-Glu-Dap(6DMN)-Gln-**pTyr-Asp-His-Pro**-Asn-Ile-($CONH_2$)
Crk-bp2	Crk	Ac-Glu-Dap(6DMN)-Gly-**pTyr-Asp-His-Pro**-Asn-Ile-($CONH_2$)
Abl-bp	Abl	Ac-Glu-Dap(6DMN)-Gly-**pTyr-Glu-Asn-Val**-Gln-Ser-($CONH_2$)
Abl-bp2	Abl	Ac-Glu-Dap(6DMN)-**pTyr-Glu-Asn-Val**-Gln-Ser-($CONH_2$)

[a] pTyr refers to phosphotyrosine. All peptides are capped as *N*-terminal acetyl and *C*-terminal amide derivatives. The SH2 recognition sequence is noted in bold.

Peptides including the fluorescent environment-sensitive amino acid were synthesized using standard Fmoc-SPPS, cleaved/deprotected from the solid support, and purified by reverse-phase high-performance liquid chromatography (HPLC). The new 6DMN side chain proved resistant to the standard mildly basic amino acid coupling conditions (0.12 M diisopropylethylamine), the Fmoc deprotection conditions (20% piperidine), and the acidic resin cleavage and deprotection cocktail (95% TFA).

The designed peptides were examined for changes in fluorescence upon binding to the respective SH2 domains. The SH2 domains (Crk SH2, Abl SH2, and C-terminal PI3K SH2) were expressed in bacteria as GST fusion proteins for purification purposes (GST-Crk SH2, GST-Abl SH2, and GST-PI3K SH2). It has been shown previously that the GST fusion does not affect the affinity of SH2 domains for phosphotyrosine-containing peptides.[39]

Binding to SH2 Domains: Influence of *N*-Terminal Backbone Flexibility on the Fluorescent Signal. Peptides **Crk-bp** and **Crk-bp2** were designed to evaluate the influence of conformational flexibility on the fluorescence emission upon SH2 binding. **Crk-bp** and **Crk-bp2** share the same *C*-terminal sequence recognized by Crk SH2 (pTyr-Asp-His-Pro), but they differ in the *N*-terminal amino acid residue between the phosphotyrosine and the fluorescent Dap(6DMN). **Crk-bp** contains a glutamine found in many Crk binding sites.[37] In **Crk-bp2**, the glutamine is substituted for a glycine. Introduction of glycine next to the reporting amino acid residue was proposed to allow greater flexibility and potentially enhance interactions between the protein and the 6DMN side chain, inducing an increased fluorescent signal upon binding to the target SH2. Such an interaction would be conformationally inaccessible with the α-substituted amino acids.

The fluorescence spectrum of the peptide **Crk-bp** shows two emission maxima: a band at 470 nm, similar to the fluorescence emission observed with model compound **4** in very hydrophobic solvents, and another band at 620 nm, similar to that observed for **4** in polar protic solvents such as methanol or water. Incubation of **Crk-bp** with the target SH2 domain, GST-Crk SH2, results in an approximately 5-fold enhancement of the long wavelength emission band, which is blue-shifted 55 nm from 620 to 565 nm (Figure 3a). Incubation of the glycine-containing peptide, **Crk-bp2**, with GST-Crk SH2 induces a similar blue shift on the long wave emission band, but a much larger increase in the emission intensity (11-fold, Figure 3b). Binding constants with GST-Crk SH2 are 4.8 ± 2 μM and 2.4 ± 1 μM, respectively. The larger fluorescence increase observed for **Crk-bp2** may be the result of the higher flexibility of the peptide that allows for better docking of the fluorescent side chain in a hydrophobic environment created by the protein. Incubation of **Crk-bp2** with other SH2 domains (GST-Abl SH2 or GST-PI3K SH2), to assess the selectivity of the peptides, induces only a modest increase in the fluorescence emission intensity of the long wavelength band (Figure 3c) and also shows reduced affinities for these other nontarget SH2 domains (binding constants 20 ± 2 μM for GST-Abl-SH2 and 40 ± 6 μM for GST-PI3K SH2).

A similar phenomenon in the fluorescence emission (increase of quantum yield of the long wavelength emission band) has been previously observed for the related fluorophore *p*-(*N*,*N*-diethylamino)benzoic acid in aqueous β-cyclodextrin solutions. This has been attributed to burial of the donor moiety (an amine) in the hydrophobic cyclodextrin cavity, with exposure of the acceptor group into bulk water.[40] The observed increase in fluorescence emission upon protein binding has been associated with a destabilization of the nonemissive twisted-intramolecular charge-transfer state as a direct consequence of the reduced polarity of the fluorophore environment,[41] although a more comprehensive study would be needed to obtain a definitive conclusion about the mechanism.

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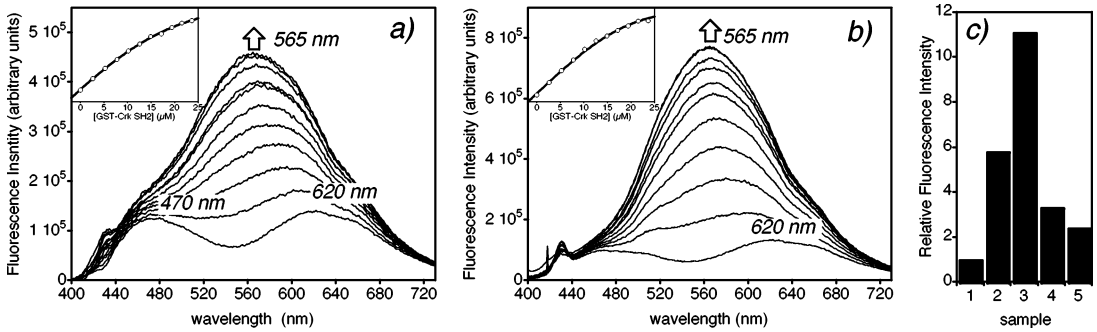

Figure 3. Fluorescence titrations of peptides (a) **Crk-bp** and (b) **Crk-bp2** (20 μM in PBS buffer, pH 7.5) with GST-Crk SH2. Insets show plots of the fluorescence emission intensity with the best fitting binding curves. Spectra were corrected for the dilution upon addition of the protein solution. (c) Relative fluorescence emission intensities at 565 nm for (1) peptides **Crk-bp** and **Crk-bp2** in buffer, (2) peptide **Crk-bp** saturated with GST-Crk SH2, (3) peptide **Crk-bp2** saturated with GST-Crk SH2, (4) peptide **Crk-bp2** saturated with GST-Abl SH2, and (5) peptide **Crk-bp2** saturated with GST-PI3K SH2.

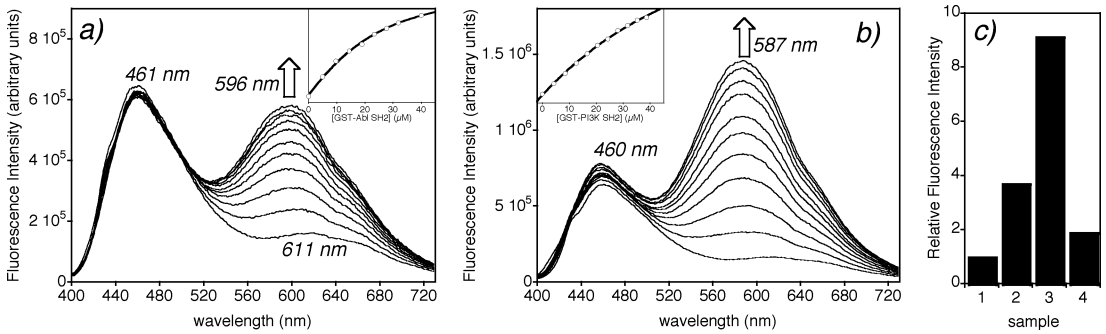

Figure 4. Fluorescence titrations of peptide **Abl-bp** (20 μM in PBS buffer, pH 7.5) with (a) GST-Abl SH2 or with (b) GST-PI3K SH2. Insets show plots of the fluorescence emission intensity at 599 nm, with the best fitting binding curves. Spectra were corrected for the dilution upon addition of the protein solution. (c) Relative fluorescence emission intensities at 590 nm for (1) peptide **Abl-bp**, (2) peptide **Abl-bp** saturated with GST-Abl SH2, (3) peptide **Abl-bp** saturated with GST-PI3K SH2, and (4) peptide **Abl-bp** saturated with GST-Crk SH2.

Binding to SH2 Domains: Selectivity of SH2 Binding. Peptide **Abl-bp** also showed dual fluorescence emission bands at 470 and 610 nm. When peptide **Abl-bp** was incubated with increasing amounts of GST-Crk SH2 there was no significant change in the fluorescence emission spectra (Figure 4c). However, there was a marked increase in the emission intensity of the long wavelength band induced by GST-Abl SH2 (almost 4-fold), and even more significantly by GST-PI3K SH2 (almost 10-fold), even though the affinity of **Abl-bp** for PI3K SH2 is lower than that for Abl SH2 (Figure 4). Binding constants for GST-Crk SH2, GST-Abl SH2, and GST-PI3K SH2 are 30 $\pm$ 8, 13 $\pm$ 2, and 42 $\pm$ 11 μM, respectively.

The larger fluorescence increase with GST-PI3K SH2 as compared to GST-Abl SH2 can be attributed to the different sets of specific interactions of each SH2 domain with the bound peptide. Although there is no structural information on Abl−peptide complexes, it has been proposed that there is an accessory interaction between an arginine side chain of the Abl SH2 domain with the backbone carbonyl of Gly (+1) and the side chain of residue (+2) of the bound peptide. The microenvironment of those peptide positions should be largely affected by the presence of the charged arginine in Abl SH2, which is likely more polar than the same position in PI3K SH2, for which no such interaction has been proposed.[42]

Finally, peptide **Abl-bp2**, with the fluorescent reporting amino acid directly adjacent to the phosphotyrosine, was synthesized to investigate if the interaction between the 6DMN side chain and the phosphotyrosine could be enhanced. The fluorescence emission spectra of this peptide show the dual fluorescence behavior found in **Abl-bp** and demonstrate higher selectivity for binding to Abl SH2 against any of the other SH2 domains. The binding constants for GST-Abl SH2, GST-Crk SH2, and GST-PI3K SH2 are 12 $\pm$ 3, 400 $\pm$ 93, and 100 $\pm$ 32 μM, respectively. However, the induced increase in emission intensity in the long wavelength band is clearly smaller than that for **Abl-bp** (Figure 5). Note that the **Abl-bp2** signal increase is higher with GST-Abl SH2 than GST-PI3K SH2.

In summary, studies with the Crk-binding peptides show that it is feasible to design a good fluorescent probe for the Crk-SH2 domain. **Crk-bp2** binds to Crk-SH2 with an affinity of 2.4 μM and undergoes an 11-fold change in fluorescence emission intensity at 565 nm. Studies with the related **Crk-bp** peptide show that the flexible amino acid between the reporter amino acid and the phosphotyrosine in **Crk-bp2** is important for achieving the maximum fluorescent response with the Crk-SH2. Studies with the **Abl-bp** peptide, which is targeted at the Abl-SH2 domain, show good affinity (13 μM) for the targeted SH2 domain (Abl-SH2) together with a 4-fold signal increase. However, in this case the fluorescence increase with the alternative SH2 domain, PI3K-SH2, is larger, although the affinity for this domain is reduced. In this case, if the fluorescent amino acid is inserted directly adjacent to the phosphotyrosine, as in **Abl-bp2**, the fluorescent change is minimal and the affinity greatly reduced. These results suggest that some flexibility

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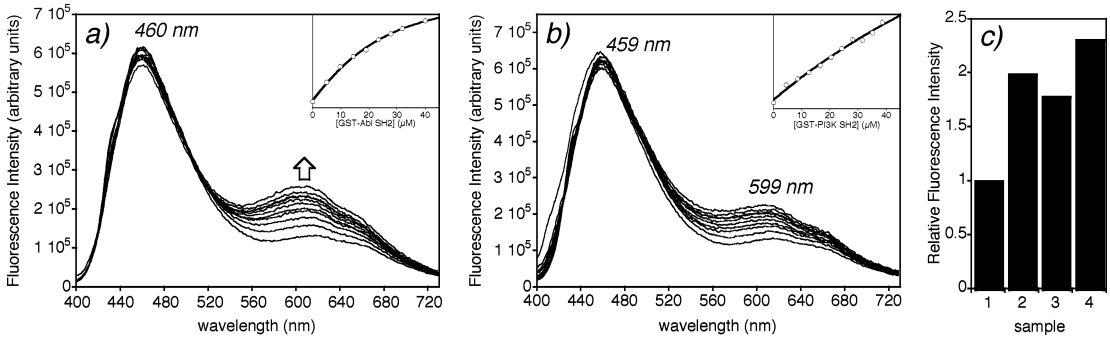

Figure 5. Fluorescence titrations of peptide **Abl-bp2** (20 μM in PBS buffer, pH 7.5) with (a) GST-Abl SH2 and with (b) GST-PI3K SH2. Insets show plots of the fluorescence emission intensity at 599 nm, with the best fitting binding curves. Spectra were corrected for the dilution upon addition of the protein solution. (c) Relative fluorescence emission intensities at 599 nm for (1) peptide **Abl-bp2**, (2) peptide **Abl-bp2** saturated with GST-Abl SH2, (3) peptide **Abl-bp2** saturated with GST-PI3K SH2, and (4) peptide **Abl-bp2** saturated with GST-Crk SH2.

between the signaling module and the phosphotyrosine is important to enable adoption of the conformation that simultaneously affords a fluorescence increase and accommodates good binding. In the case of the Abl-SH2 targeted peptides, we have observed large changes in the emission intensity with a nontarget SH2 domain (**Abl-bp** fluorescence increase with GST-PI3K SH2) with the current design. However, the modular nature of the probe, together with the ease with which further refinement of the peptide sequence can be processed through combinatorial methods, suggests that it should be possible to optimize the binding and reporting properties of each probe to effectively target specific SH2 domains. Thus, results comparable to those observed with **Crk-bp2** and the Crk-SH2 should be attainable. Taken together, these results show the potential applications of this new fluorophore for the development of fluorescent peptidic sensors for biological interactions. In the future, we also anticipate that larger fluorescence changes and higher specificities can be obtained if the 6DMN-based fluorescent amino acid is incorporated into full-length proteins via protein semi-synthesis or unnatural amino acid mutagenesis, since in this case shielding from water should be maximized in the protein−protein interface.

Conclusions

A new environment-sensitive fluorophore, 6DMN, has been synthesized. 6DMN displays excellent fluorescent properties for biochemical studies: long excitation (375 nm) and emission wavelengths (460−590 nm) and also a large quantum yield and Stokes shift. Additionally, a new amino acid building block containing this fluorescent group, Fmoc-Dap(6DMN)-OH (**5**), was synthesized to explore the application of 6DMN to probe biological interactions. This amino acid can be easily incorporated into peptides using standard Fmoc-SPPS methods and was used to synthesize peptides that selectively bind SH2 phosphotyrosine binding domains (Crk SH2 and Abl SH2). The results show that 6DMN fluorescence emission intensity significantly increases upon binding of the designed peptides to specific SH2 domains.

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Supporting Information Available: Experimental procedures and spectroscopic data for the synthesis of 6DMN-GlyOMe (**4**) and Fmoc-Dap(6DMN)-OH (**5**). Detailed peptide synthesis protocols. Quantum yield determination of **4**. GST fusion SH2 domains expression and purification. Fluorescence titration binding assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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