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A new environment-sensitive fluorescent amino acid for Fmoc-based solid phase peptide synthesis †

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A new 4-(*N*,*N*-dimethylamino) phthalimide-based environment-sensitive fluorescent building block for solid phase peptide synthesis 3, has been synthesized and incorporated into peptides. Peptides incorporating this residue show great potential for biological applications in sensing protein/protein interactions.

Fluorescent sensors have become increasingly important in the study of biological processes. Of particular interest are those incorporating environment-sensitive fluorophores, which manifest modified emission properties in response to changes in the surrounding environment. For example, the amino acid DANA/ALADAN **1**, (Fig. 1) which incorporates the 6-(dimethylamino) fluorophore, has been useful for monitoring the phosphorylation-dependent binding of peptides to proteins,[1] and for investigating protein–protein interactions.[2]

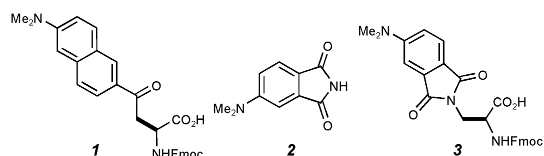


Fig. 1 Structures of DANA/ALADAN **1**, DMAP **2**, and DAPA **3**.

The 4-(*N*,*N*-dimethylamino)-phthalimide system **2** (4-DMAP) has been recognized as an extraordinarily environment-sensitive fluorophore that responds to changes in polarity and viscosity often found in organized media. 4-DMAP exhibits a 70-fold increase in the quantum yield and fluorescence emission spectral shifts as large as 100 nm on changing the solvent from water to 1,4-dioxane.[3] 4-DMAP has been extensively used in studies of micelles, solvent mixtures, and simple chemical systems.[4a,b] 4-DMAP also has convenient excitation and maximum emission wavelengths for biological applications. In addition to these photophysical properties, 4-DMAP has a size comparable to that of the natural amino acids tyrosine or tryptophan, which would render the residue less disruptive to specific native interactions than current environment-sensitive fluorophores (Fig. 2).

The application of 4-DMAP based sensors to the study of complex biological processes has been limited so far by the lack of simple chemical methods for the specific introduction of the probe into relevant biological systems.[5] Herein we present the synthesis of a 4-DMAP amino acid derivative **3** (DAPA) suitable for the incorporation of this fluorophore into peptides using standard Fmoc solid phase peptide synthesis.

The initial synthetic plan involved access to the key intermediate 4-(*N*,*N*-dimethylamino)-phthalic anhydride **6** (Scheme 1). The first step in the original scheme involved methylation of 4-aminophthalimide using dimethyl sulfate based on previously reported conditions.[4a] However, the

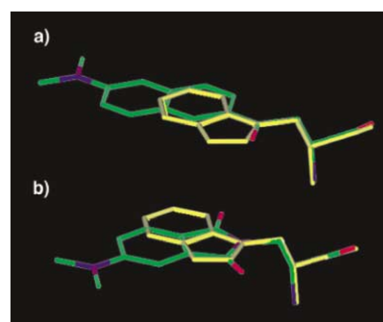
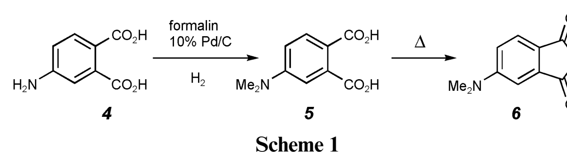


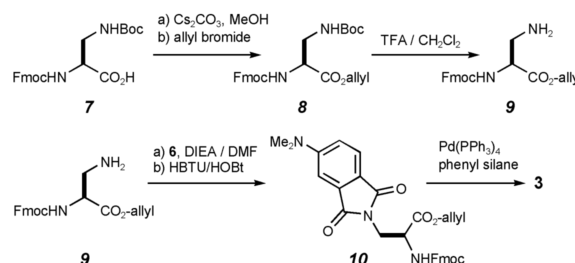
Fig. 2 Superposition of tryptophan with a) DANA/ALADAN **1**, and b) DAPA **3**, showing the similar sizes of **3** and tryptophan.

reaction proved irreproducible, affording the desired product only in low yield, which was attributed to the low nucleophilicity of the aromatic amine.[6] In contrast, reductive amination of 4-aminophthalic acid, **4**, with formalin and hydrogen in the presence of Pd/C afforded the desired 4-(*N*,*N*-dimethylamino)-phthalic acid **5** in excellent yield.[6] Sublimation of the diacid **5** under reduced pressure afforded the desired anhydride **6** also in excellent yield.



Scheme 1

Commercially available Fmoc-Dap(Boc)-OH, **7**, was protected[7] as the corresponding allyl ester **8** (Scheme 2). Deprotection of **8** with TFA in dichloromethane afforded the corresponding free amine **9** that was coupled, without further purification, to the 4-DMAP anhydride **6**. The initial reaction of the amine **9** with the anhydride **6** proceeded smoothly, but reaction of the resulting amide with the free acid for ring closure and formation of the phthalimide derivative was not possible using standard reported protocols. Additionally, heating or refluxing in piperidine or toluene resulted in partial or complete deprotection of the Fmoc protecting group and could also compromise the stereochemical integrity of the amino acid. Ultimately, activation of the acid intermediate using a HBTU/HOBt mixture resulted in the formation of the



Scheme 2

† Electronic supplementary information (ESI) available: Experimental details. See http://www.rsc.org/suppdata/ob/b4/b408001g/

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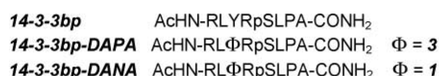
desired product **10** under mild conditions at room temperature. Final allyl deprotection of **10** gave the desired building block **3** in good yield (see supplementary information for experimental details). †

Upon completion of the building block synthesis, the incorporation of **3** into a peptide using standard Fmoc solid-phase peptide synthesis methods (SPPS) was evaluated. These peptides were used to assess the potential applicability of DAPA-based peptidic sensors in monitoring biological interactions.

The specific biological system used in the initial study includes the 14-3-3 proteins which are involved in phosphoserine-dependent signalling and are essential intermediates in cell cycle regulation.[8] These proteins are known to bind short phosphopeptides with high affinity and are therefore ideal systems to test the new fluorophore in a real biological interaction. The peptide sequence was selected on the basis of previously reported peptide library screening that identified the optimal sequence for 14-3-3 ζ isoform binding,[9] and the X-ray structure of 14-3-3 bound with the octapeptide **14-3-3bp**.[10] Previous work in our group demonstrated that placement of a fluorescent reporter within that sequence at pSer(-2) position induces a fluorescence change in DANA emission intensity (see Fig. 3).[11]



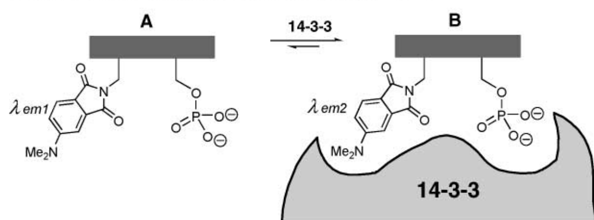


Fig. 3 Sequence of parent peptide **14-3-3bp** and modified fluorescent sensor **14-3-3bp-DAPA**. Binding of **14-3-3bp-DAPA** (A, λem1) induces changes in the emission intensity and the maximum emission wavelength (B, λem2).

Peptide **14-3-3bp-DAPA** containing the fluorescent environment-sensitive amino acid **3** was synthesized using standard Fmoc-based solid phase synthesis protocols, cleaved/deprotected from the solid support and purified by reverse phase HPLC. The new phthalimide side chain proved resistant to the standard mildly basic coupling conditions (0.12 M diisopropylethyl amine), Fmoc removal conditions (20% piperidine), and also resistant to the acidic treatment necessary for cleavage and deprotection of the peptides (95% TFA).

The spectral properties of the isolated peptide were found similar to those reported for 4-DMAP, with a UV absorption maximum at 421 nm, fluorescence excitation maximum at 395 nm and fluorescence emission dependent on the medium polarity (see supporting information). †

To test the utility of the fluorescent 4-DMAP amino acid side chain for monitoring binding of the designed peptide to 14-3-3, the fluorescent peptide was incubated with 14-3-3 ζ protein. Addition of the protein to a solution of **14-3-3bp-DAPA** induces a significant increase in the fluorescence emission intensity. This fluorescence increase is consistent with the anticipated binding of the reporter amino acid DAPA into a more hydrophobic environment and concurrent shielding from the surrounding water molecules (Fig. 4). Titration of **14-3-3bp-DAPA** with a 14-3-3 solution afforded a family of spectra that could be fit to a 1 : 1 binding model. The observed K_D is 4.6 ± 0.2 μM, which is in the same order of magnitude as previously reported values for similar peptides.[9]

The fluorescence emission intensity at 510 nm is 6-fold more intense when the peptide is complexed with 14-3-3 than in the absence of protein, and the maximum emission intensity is

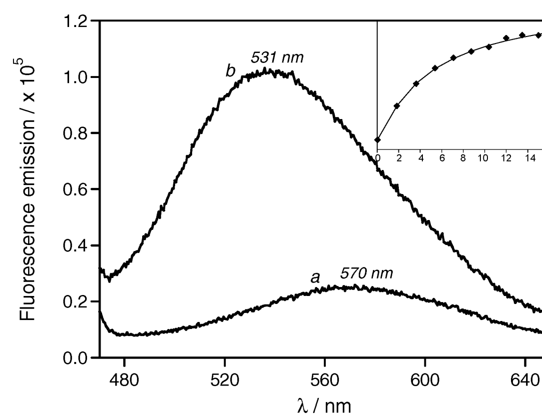


Fig. 4 Fluorescence emission of 1.3 μM **14-3-3bp-DAPA** (a) and 1.3 μM **14-3-3bp-DAPA** in presence of 16.5 μM 14-3-3 protein (b) showing the increase in fluorescence as well as the shift in the emission maximum. Insert of fluorescence titration. Observed $K_D = 4.6 \pm 0.2$ μM.

shifted about 40 nm from 570 to 531 nm. These changes are more significant than the reported change in emission intensity observed when using the peptide **14-3-3bp-DANA** containing DANA as the reporter side chain,[10] where only a four-fold increase in the fluorescence emission intensity and 20 nm blue shift from 522 to 501 nm is observed. This reflects the high sensitivity of the 4-DMAP side chain to changes in the surrounding environment.

In summary, a short and facile synthesis is outlined to access the new 4-DMAP-based environment-sensitive fluorescent Fmoc protected amino acid. This new building block can be successfully incorporated into peptides using Fmoc-based solid phase peptide synthesis without any modification of the standard protocols. These methods were used to synthesize a new fluorescent peptidic sensor for 14-3-3 proteins, demonstrating the potential of this new fluorescent probe to monitor protein/protein interactions.

Acknowledgements

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