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Blue Fluorescent Amino Acids as In Vivo Building Blocks for Proteins

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In vivo expression of colored proteins without post-translational modification or chemical functionalization is highly desired for protein studies and cell biology. Cell-permeable tryptophan analogues, such as azatryptophans, have proved to be almost ideal isosteric substitutes for natural tryptophan in cellular proteins. Their unique spectral features, such as markedly red-shifted fluorescence, are transmitted into protein structures upon incorporation. Among the azaindoles under study (2-, 4-, 5-, 6-, and 7-azaindole) 4-azaindole has exhibited the largest Stokes shift (~130 nm) in steady-state fluorescence measurements. It is also highly biocompatible and as 4-azatryptophan it can be translated into target protein sequences. However, its quantum yield and fluorescence intensity are still significantly lower when compared with natural indole/tryptophan. Since azatryptophans are hydrophilic, their presence in the hydrophobic core of proteins could be harmful. In order to overcome these limitations we have performed nitrogen methylation of azaindoles and generated mono- and dimethylated azaindoles. Some of these methyl derivatives retain the pronounced red shift present in the parent 4-azaindole, but with much higher fluorescence intensity (reaching the level of indole/tryptophan). Therefore, the blue fluorescence of azaindole-containing proteins could be further enhanced by the use of methylated analogues. Further substitution of any azaindole ring with either endo- or exocyclic nitrogen will not yield a spectral fluorescence maximum shift beyond 450 nm under steady-state conditions in the physiological milieu. However, green fluorescence is a special feature of tautomeric species of azaindoles in various nonaqueous solvents. Thus, the design or evolution of the protein interior combined with the incorporation of these azaindoles might lead to the generation of specific chromophore microenvironments that facilitate tautomeric or protonated/deprotoned states associated with green fluorescence.

Introduction

Accurate assessment of protein localization is crucial for complete understanding of its in vivo function. Most native chromophore-free proteins are colorless, which prevents studies of their location and dynamics deep within living tissues by using standard fluorescence microscopy. Genetically encoded fluorescent tags, such as fluorescent proteins (FPs), are widely used as visualization tools for such purposes.[1] The green fluorescent protein (GFP) from *Aquorea victoria* and other FPs recently became the most popular protein localization tools.[2] They proved to be useful markers to monitor and study protein turnover, transport, and molecular interactions by using techniques such as FRET, fluorescence lifetime imaging, bimolecular fluorescence complementation, fluorescence recovery after photobleaching or photoactivation.[3]

However, the principal disadvantage of these genetically encoded tags is that their fusion with target proteins might generate experimental artifacts.[4] For example, fusion constructs might alter the location or introduce oligomer formation of the native protein.[5] Therefore, complementary approaches are often necessary in order to gain a more accurate picture of particular in vivo protein functionalities. On the other hand, recently developed bioorthogonal transformations require the presence of a functional group in the protein and an addition of exogenous synthetic probes.[6] In this way, such ligation rather mimics a post-translational modification. Even the use of fluorophores like coumarines or FlAsH, although cell-permeable, is restricted since it requires the tailoring of the target sequences prior to standard labeling[7] or coupled enzymatic reactions.[8] In order to overcome these limitations, our long-term goal is to develop procedures for the direct cotranslational generation of colored proteins in living cells without the need for further post-translational modification or chemical functionalization by externally added reagents. In attempts to visualize natural chromophore-free proteins and even portions of the whole proteome it is highly desirable to generate color without structural and functional perturbations of target proteins.[9] As shown in Scheme 1 and Figure 1 tryptophan analogues, such as 4-azatryptophan (4AW), are very promising candidates for such minimal chromophores that can be used in imaging studies. Indeed, they are cell permeable and have proved to be almost ideal isosteric substitutes for natural tryptophan in cellular proteins. Their unique spectral features, such

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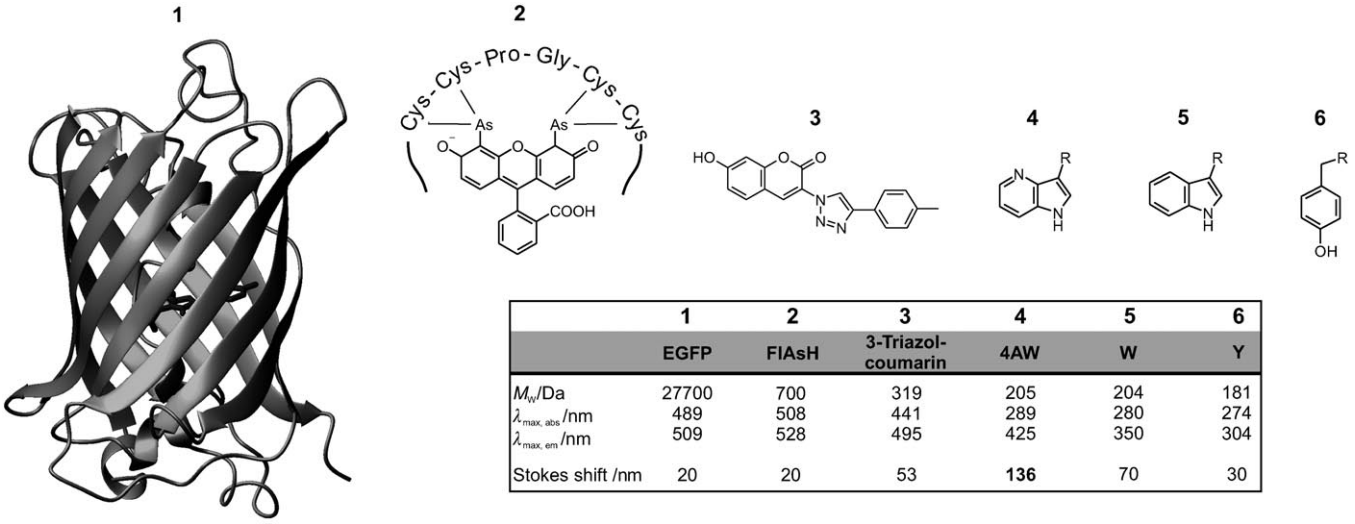


	1	2	3	4	5	6
	EGFP	FlAsH	3-Triazol-coumarin	4AW	W	Y
M_w/Da	27700	700	319	205	204	181
$\lambda_{max,\ abs}$/nm	489	508	441	289	280	274
$\lambda_{max,\ em}$/nm	509	528	495	425	350	304
Stokes shift /nm	20	20	53	**136**	70	30

Scheme 1. Comparison of fluorescent probes and spectral properties. Commonly used imaging tags and fluorescent probes that are relevant for protein studies. The illustration should approximate the real size differences of the chemical components. From the biggest to the smallest compound, **1**: member of the GFP family, EGFP; **2**: FlAsH (4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein)); **3**: representative of the coumarin family, 3-triazol-coumarin; **4**: 4-azatryptophan; **5**: tryptophan; and **6**: tyrosine. All GFP derived fluorescent proteins as well as FlAsH, require an engineering of the target amino acid sequence. Moreover, GFPs are relatively large in size, which can cause unpredictable perturbations within the studied system. The FlAsH method is based on the subsequent addition of arsenic compounds, which are toxic to many cells. In contrast, 4-azatryptophan is structurally very similar to W, that is, represents only a CH→N substitution. Furthermore, it comprises the largest Stokes shift of all depicted imaging probes. The details of its spectroscopic properties are provided in ref. [10]; R denotes $H_2CCH(NH_2)COOH$.

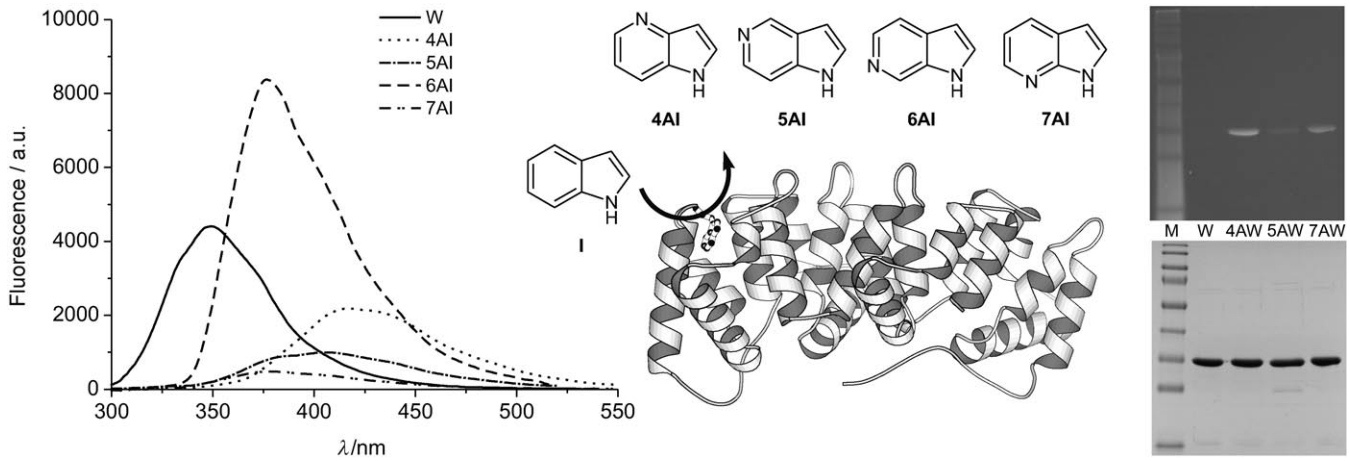


Figure 1. Molecular structure and fluorescence of free and protein incorporated azaindoles/azatryptophans. Left-hand panel: comparison of the fluorescence emission profiles of indole and azaindoldes. Middle panel: chemical structures of indole and azaindoles, which are the chromophores of the related tryptophans. Ribbon plot of human annexin A5 (side view) with W187 buried in the hydrophobic pocket at the convex side of the molecule. Right-hand panel: SDS-PAGE profiles of purified annexin A5 and its variants. Proteins were expressed either in the presence of tryptophan, 4-azatryptophan (4AW), 5-azatryptophan (5AW) or 7-azatryptophan (7AW). The upper part shows the gel upon UV exposure and the lower part after subsequent Coomassie blue staining. Note that simple UV irradiation reveals protein autofluorescence; M: molecular weight marker.

as markedly red-shifted fluorescence, charge conductivity and pH sensitivity, are transmitted into the protein structure upon incorporation.[10]

In this context, the major challenge for the synthesis chemistry of indole combined with protein engineering would be to identify possible indole-based chromophores suitable for serving as imaging tools. They should feature a significant shift in fluorescence towards the red spectral region combined with improved quantum yields. In an ideal case, such chromophores would be biocompatible and well incorporated into the target protein(s) by the endogenous translational apparatus. Their chemical structures in the context of an engineered or evolved surrounding protein matrix endow the resulting biologically active structures with unique spectral properties. Beside spectroscopy, other features gained in this way should be generally useful for biologically inspired research, innovations and applications.

Tryptophan As a Target for the Engineering of Protein Fluorescence

Tryptophan residues play crucial roles in protein stability and folding and numerous cellular functions, such as aromatic interactions with DNA bases. Since W is the main source of fluorescence and absorbance in proteins it was recognized very early to be a useful intrinsic probe for structure and function studies of proteins and enzymes. For example, the fluorescence maximum of most globular proteins is changed from 330 to 350 nm when the protein is denatured by urea.[11] This takes place upon solvent exposure of W residues, which are normally buried in the protein interior. In the latter case their maxima is close to 330 nm whereas the exposed residue maxima is near 350 nm. However, these spectral features are not suited for the majority of current imaging methods. The replacement of W residues by other canonical aromatic amino acids (phenylalanine, tyrosine, histidine) does not improve the protein's spectral features and might even perturb its structure or function due to differences in shape, size and even charge between W and these amino acids.

On the other hand, W's regular appearance and its low natural abundance make it an almost irreplaceable reporter and a very attractive target for engineering in protein science. The expanded genetic code opens up possibilities for a rational manipulation of W fluorescence. It exploits the diverse and rich chemistry of indole that offers numerous analogues/surrogates with predefined spectral properties.[12] The dominance of W in spectral properties of proteins is of great advantage in identifying introduced modifications since they are easily observable. In other words, spectroscopic properties delivered by chromophoric amino acids often emerge as intrinsic features of substituted target proteins. In this way, several isosteric tryptophan analogues, such as hydroxyl-, methyl-, amino- and fluorotryptophans, have been incorporated into the sequences of model proteins endowing them with enhanced or even novel spectral features.[13–15]

Even before the genetic code was deciphered Pardee et al. as well as Brawerman and Yčas reported experiments on the incorporation of 7-azatryptophan (7AW) and 2-azatryptophan (2AW) into the proteome of *Escherichia coli* and its phage.[16–17] A decade later Schlesinger reported an alkaline phosphatase to be the first enzyme fully substituted with azatryptophans in *E. coli* cells.[18] Szabo, Ross and their co-workers further extended this repertoire by introducing 5-hydroxytryptophan as a useful intrinsic fluorescent probe.[14–15] Its excitation is ~20 nm red shifted compared to that of W; this enables selective excitation in the range of 315 to 320 nm.[14] On the other hand, 4-hydroxytryptophan exhibited a blue-shifted absorbance and fluorescence.[19]

One of the most recent advances represents the incorporation of aminotryptophan (AmW) analogues that offer the possibility to design protein-based sensors[19] as well as novel classes of autofluorescent proteins.[20] Incorporation of 4-aminotryptophan (4AmW) into cyan fluorescence protein (CFP) substantially shifts its fluorescence emission to longer wavelengths and yields a gold fluorescence protein (GdFP). GdFP features a unique principle of photophysics that causes a Stokes shift of about 100 nm.[20] Similarly, incorporation of *para*-aminophenylalanine (pAmF) into GFP yields a Stokes shift of almost 70 nm.[21–22] Thus, pioneering works on generation of novel GFP protein classes, offer unprecedented opportunities to understand and manipulate the relationship between protein structure and its spectroscopic function. These efforts will certainly find more recognition and applications in future research.

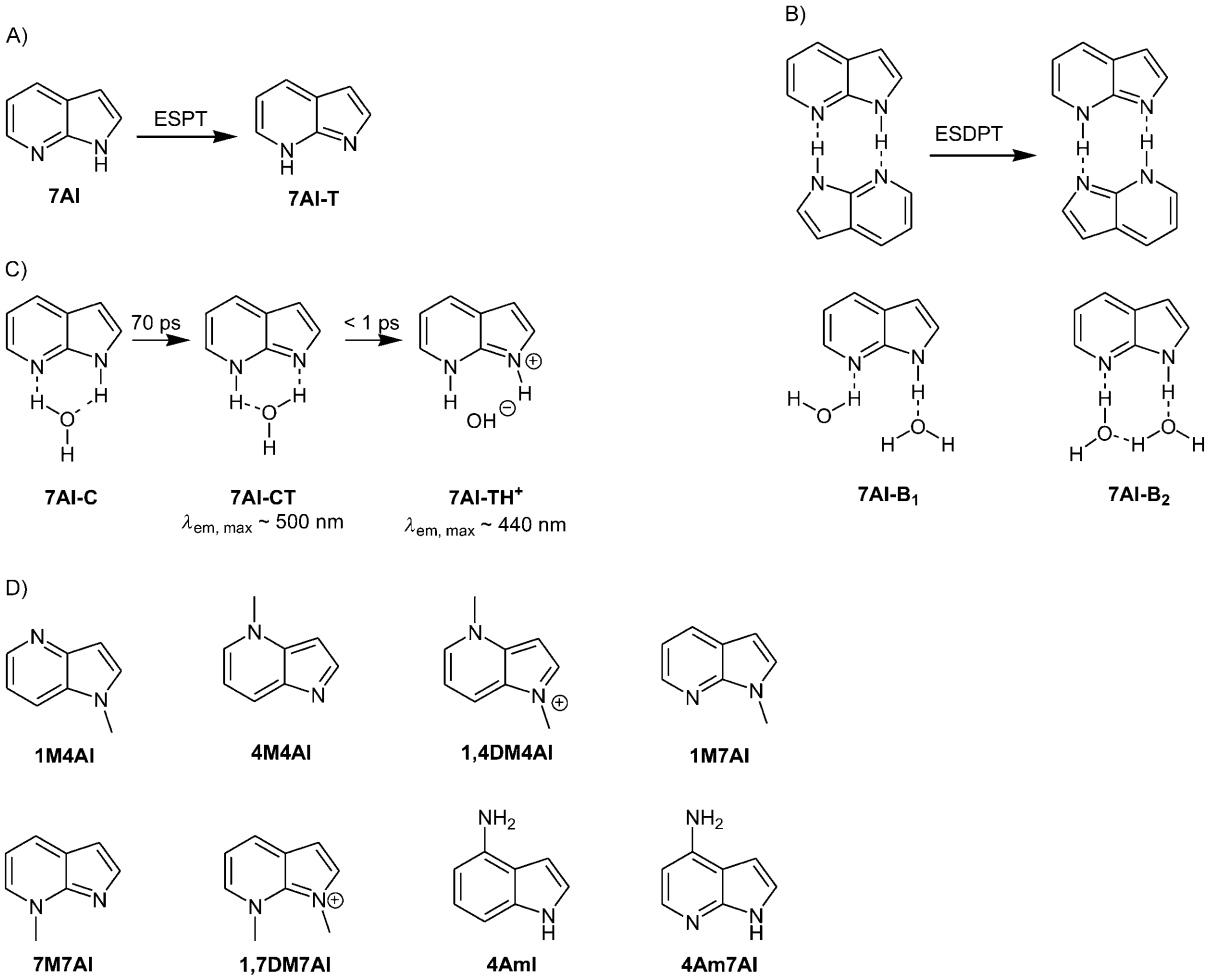
Photophysics of 7-Azatryptophan and Its Methylated Derivatives

During the first decades of studies with azatryptophan (AW) the photophysics of 7-azatryptophan (7AW) and its chromophoric side chain 7-azaindole (7AI) were in the focus of various researches. This included parallel studies of their properties in solution as well as studies of their spectral properties in protein structures. Recently, this research was extended to other azaindole isomers, such as 4-azaindole (4AI), 5-azaindole (5AI) and 6-azaindole (6AI). Before testing their suitability for protein engineering it is essential to understand the basic principles behind their unique photophysics. All these photophysical studies were mainly performed with indole and azaindoles to avoid complications by the charged (zwitterionic) nature of the related amino acid. For that reason we also focused on the azaindoles and their derivatives rather than related amino acids. Nonetheless, due to the better solubility in water we used W as a reference sample in all measurements, since its differences to free indole are well documented (e.g., red shift of 10 nm in fluorescence without changes in intensity).

Generally, in the structure of all azaindoles two tautomers can be evidenced. For 7-azaindole these tautomers are labeled 7AI and 7AI-T (Scheme 2 A). In the ground electronic state the 7AI form is energetically favored and is the only thermally accessible form.[23] Conversely, in the excited state the tautomeric form 7AI-T is more stable.[23] This phenomenon is known as excited state proton transfer (ESPT) and leads to a more stable tautomer after light exposure. The same holds true for the other azaindoles.[24]

One of the most striking features of 7AI is its extreme sensitivity to the nature of the solvent microenvironment.[25] For example, in nonpolar solvents dimerization of 7AI takes place and besides the usual violet emission maximum ($\lambda_{max\,em}$ ~ 390 nm) a second peak in the green spectral region ($\lambda_{max\,em}$ ~ 500 nm) emerges.[26] The appearance of the second peak is attributed to the formation of a 7AI dimer and cooperative double-proton transfer in the excited state (ESDPT). This yields the tautomeric form (7AI-T) for both dimer molecules (Scheme 2 B).[25–27]

In linear alcohols ESDPT also occurs.[25,27] The formation of a 7AI-C-like cyclic intermediate (Scheme 2 C) facilitates the interactions of 7AI with the solvent molecules, and again results in the more stable tautomeric form.[27] However, in water only a single and smooth fluorescent band is detectable for 7AI with a maximum at 386 nm.[27] This observation can be explained by taking into account that most of the solute molecules are



Scheme 2. Schematic representation of 4- and 7-azaindoles with related tautomeric structures as well as the methylated derivatives. Methylation can be used as a chemical tool to arrest one particular tautomeric state in order to study its optical properties. A) Upon excited state proton transfer (ESPT) of 7-azaindole (7AI) its tautomer (7AI-T) is formed.[23] B) Excited state double proton transfer (ESDPT) in apolar solvents enables tautomerization after dimerization.[26] C) Compound 7AI and its interactions with water. Note that the 7AI-CT state has a characteristic fluorescence in the green region whereas the protonated form (7AI-TH$^+$) exhibits a blue fluorescence. On the right the blocked species (7AI-B$_1$ and 7AI-B$_2$) dominating in bulk water are depicted. The B$_1$ represents hydrogen bonding by two independent solvent molecules whereas B$_2$ resembles a cyclic hydrogen bonding system. In polar solvents (e.g., ethanol) the 7AI-CT state can be observed.[25] D) Chemical structures of 1-methyl-4-azaindole (4M4AI), 4-methyl-azaindole (4M4AI), 1,4-dimethyl-4-azaindolium iodide (1,4DAI), 1-methyl-7-azaindole (1M7AI), 7-methyl-7azaindole (7M7AI), 1,7-dimethyl-7-azaindolium iodide (1,7M7AI; iodide as counter ion is excluded from the formulas in case of the dimethylated derivatives), 4-aminoindole (4AmI) and 4-amino-7-azaindole (4Am7AI). For a detailed discussion see the text.

inappropriately solvated. Only a small portion of molecules are able to form the cyclic hydrogen-bonded complex 7AI-C with subsequent ESDPT and formation of 7AI-CT ($\lambda_{max\,em}$ ~ 500 nm, Scheme 2C). Unfortunately, the latter species will be rapidly protonated to 7AI-TH$^+$ (Scheme 2C). This cationic species has a fluorescence emission maximum of approximately 440 nm.[27]

The majority of 7AI molecules in bulk water establish hydrogen bonds with two different water molecules due to the high geometry of the water molecule itself as well as to the highly ordered water network in bulk solution.[9] This leads to so-called blocked species as depicted in Scheme 2C (7AI-B$_1$ and 7AI-B$_2$). There is no rapid conversion of the blocked species to one of the others on the time scale of their fluorescence lifetimes.[27] Thus, in aqueous solvents, 7AW fluorescence is strongly quenched, which limits its general use as a fluorophore.[28]

In order to arrest the tautomeric form in a stable state in water, selective methylation of either the pyrrole (N^1) or pyridine nitrogen (N^7) was performed.[23,27,29] The attachment of a methyl group to N^7 ended in the formation of 7-methyl-7-azaindole (7M7AI, Scheme 2D). Compound 7M7AI exhibits a fluorescence emission maximum at 442 nm, whereas at pH values above 10 the emission maximum is shifted to 510 nm.[27] Thus, high pH values facilitate deprotonation resulting in the green fluorescence of 7M7AI (λ_{max} = 510 nm). Unfortunately, 7M7AI is very unstable[23,29] and unattractive for spectroscopic research. Namely, it has only a very low fluorescence intensity with negligible quantum yields (50-times lower when compared with 7AI).[27]

Conversely, the methylation of N^1 leading to 1M7AI (Scheme 2D) suppresses the major nonradiative pathways in the excited state determined by the N^1 proton and its

interactions with the solvent.[25,27] Although this is reflected in a relatively long fluorescence lifetime the red shift is rather moderate ($\lambda_{max\,em} = 386$ nm, Figure 2). In the literature there are also reports about slightly higher values for the emission maximum (396–405 nm).[25,27] However, it is reasonable to conclude that 1M7AI reflects the blocked species of 7AI (Scheme 2C and D). Nevertheless, because of its improved fluorescence intensity and quantum yield it is a suitable optical probe. First reports of its use in a biological context by, for example, incorporation of 1-methyl-7-azatryptophan into small peptides are already published.[25]

Interestingly, simultaneous methylation of both N^1 and N^7 gives 1,7-dimethyl-7-azaindolium iodide (1,7M7AI, Scheme 2D; see the Experimental Section) a species that emits blue light at approximately 450 nm. However, fluorescence intensity is even lower than that of 7AI (Figure 2). Thus, the properties of 1M7AI (high fluorescence intensity and quantum yield) and 7M7AI (pronounced red shift) could not be combined in a way that desirable spectral features are displayed. In addition, the charged nature of this molecule could cause difficulties for its possible biological applications. For example, this could be a major obstacle for efficient cellular uptake through transporter systems as well as for recognition and tRNA charging reactions.

4-, 5- and 6-Azatryptophan

Only recently photophysical studies of 4AI, 5AI and 6AI have also been reported.[24,30] Both, experiments and theoretical studies show unambiguously that the stability of the N^1H tautomers and the N^nH tautomers ($n = 4, 5, 6$) are similar to that of 7AI. However, the mechanism of tautomerization of 4AI, 5AI and 6AI differs from that of 7AI due to the geometric arrangement of the pyridine and pyrrole nitrogens of the respective azaindole systems.[24] Here, excitation is thought to be subsequently followed by N^n ($n = 4, 5, 6$) abstracting a proton of a solvent molecule. Only after that the deprotonation of the N^1 takes place.

The absorbance of 6AI is red shifted to 320 nm (325 nm for the corresponding tryptophan derivative 6AW).[30] At physiological pH this chromophore is protonated ($\lambda_{max\,em} = 380$ nm) since its pK_a value is 8 whereas above pH 10 fluorescence can

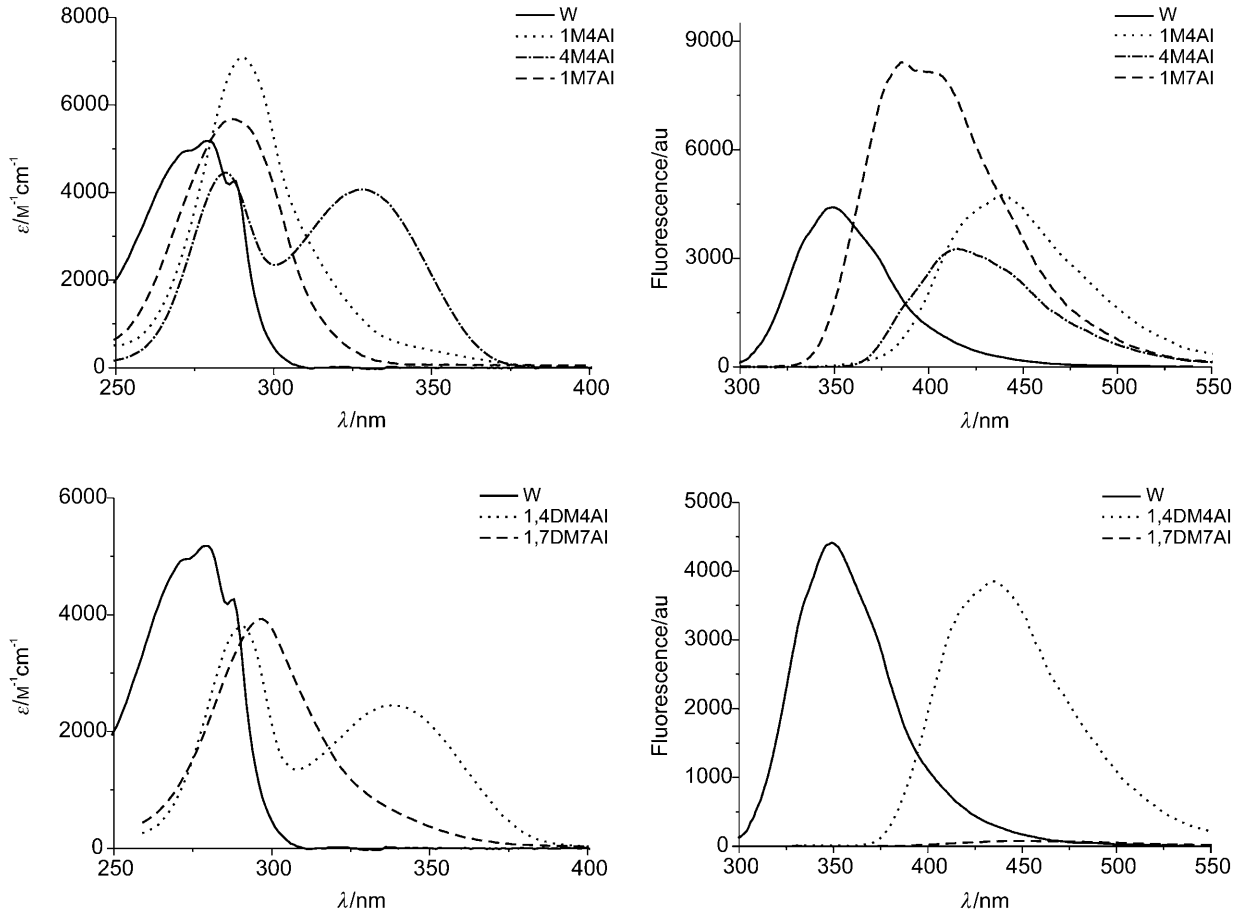


Figure 2. Absorption (left) and emission (right) profiles of tryptophan and related mono- and dimethylated 4- and 7-azaindoles. Upper graphs: the spectral properties of W and 1-methyl-4-azaindole (1M4AI), 4-methyl-4-azaindole (4M4AI) and 1-methyl-7-azaindole (1M7AI) are depicted. Note the additional absorption maxima for 4M4AI absent in W or 1M4AI. Furthermore, the emission profiles clearly show a more pronounced red shift and higher intensity for 4M4AI compared to 1M4AI. Lower graphs: the spectral properties of W and 1,4-dimethyl-4-azaindolium iodide (1,4M4AI) and 1,7-dimethyl-7-azaindolium iodide (1,7M7AI). Again, the absorption profile of the 4-azaindole derivative shows a second excitation maximum comparable to that of 4M4AI. The emission profile shows almost no detectable fluorescence for 1,7M7AI whereas 1,4M4AI exhibits a fluorescence profile similar to that of 4M4AI.

be detected in the blue spectral region ($\lambda_{max\,em} = 440$ nm). This is attributed to the excited-state tautomer N^6H.[30]

In comparison with the traditionally used 7AI, the 6AI chromophore has the advantage of a slight red shift in absorption (30 nm) and much higher fluorescence intensity. In protein science such an optical probe is especially interesting for studying protein–protein interactions in the presence of native tryptophan side chains. Its practical use was already shown by incorporation and subsequent spectral investigation of calmodulin.[30] It is worth to note, that this chromophore is less biocompatible than 7AI. For example, its translation into protein sequences is not efficient and difficult to reproduce.[10,30]

Similar to the above-mentioned examples, it was found that in case of 4AI and 5AI excited-state tautomerizaton occurs in the presence of strong bases (pH > 10). Under these basic conditions the chromophore emission maxima shift to 480 and 450 nm, respectively.[24] In neutral aqueous solution both 4AI and 5AI possess fluorescence emission maxima at 418 and 410 nm (Figure 1).[24] The pronounced red shift and the markedly higher fluorescence intensity of 4AI compared to 7AI and 5AI (Figure 1) makes it a powerful tool for protein fluorescence design. Recently, we could confirm these assumptions experimentally by converting the colorless protein annexin A5 into its blue fluorescent counterpart by W→4AW substitution.[10]

In order to investigate closely the tautomeric species of 4AI we and others[24] methylated 4AI both at N^1 and N^4. 1-Methyl-4-azaindole (1M4AI, Scheme 2D) has an absorption profile similar to that of the parent compound 4AI, but its fluorescence is red shifted by approximately 20 nm ($\lambda_{max} = 438$ nm). Noteworthy, the fluorescence intensity is almost doubled and reaches the level of I/W (Figures 1 and 3). Assuming a similar biocompatibility of 4AI and 1M4AI, the incorporation of 1M4AI into protein sequences could provide structures with a more intense blue fluorescence than already described for 4AI-containing proteins.[10]

In contrast, the regioisomer 4-methyl-4-azaindole (4M4AI, Scheme 2D) exhibits two absorbance maxima at 284 and 332 nm. Although, it has lower intensities compared to I/W (Figure 2) the second absorption shoulder is especially useful for selective excitation of related proteins in biological samples. Its fluorescence intensity as well as emission maximum ($\lambda_{max\,em} = 414$ nm, Figure 2) are comparable to 4AI (Figure 1). Expectedly, at high pH values 4M4AI's emission profile is red shifted to 480 nm.[24]

To the best of our knowledge there are no reports about chemical synthesis of dimethylated 4-azaindole. Therefore, we chemically synthesized 1,4-dimethyl-4-azaindole iodide (1,4M4AI; see the Experimental Section). As described above, dimethylation of 7AI almost extinguished its fluorescence (Figure 2). Conversely, the 1,4M4AI emission spectrum is almost identical with that of 1M4AI ($\lambda_{max\,em} = 435$ nm). Also, fluorescence intensities were not significantly changed compared to 1M4AI (Figure 2). Moreover, it features two absorption bands (290 and 338 nm) suitable for selective excitation of biological samples. In this way, 1,4M4AI as a chromophore with potential biological use combines two highly desired features: selective excitation wavelength (338 nm) and red shifted fluorescence ($\lambda_{max\,em} = 435$ nm, Stokes shift ~100 nm). However, due to the cationic nature of 1,4M4AI its biocompatibility should be investigated especially with respect to intracellular accumulation.

In summary, among all the above described azaindole derivatives 1M4AI seems to be the most promising chromophore for the engineering of protein fluorescence. Namely, it combines a very pronounced red shift in fluorescence emission (Stokes shift of 150 nm) and good fluorescence intensities.

Attributes and Perspectives of Indole-Based Chromophores As Possible Imagining Tools

In any chromophore structure, it is generally expected that an increase in the size of delocalized π system results in an increase in the excitation and emission wavelength.[31] The best documented example among natural FPs is DsRed from *Discosoma striata*. Here, the basic chromophore structure of GFP is further augmented by an extension of the π-bonding system by additional conjugation through the protein backbone (i.e., C^{α}-*N*-bond of neighboring residue).[32] Any attempt to use

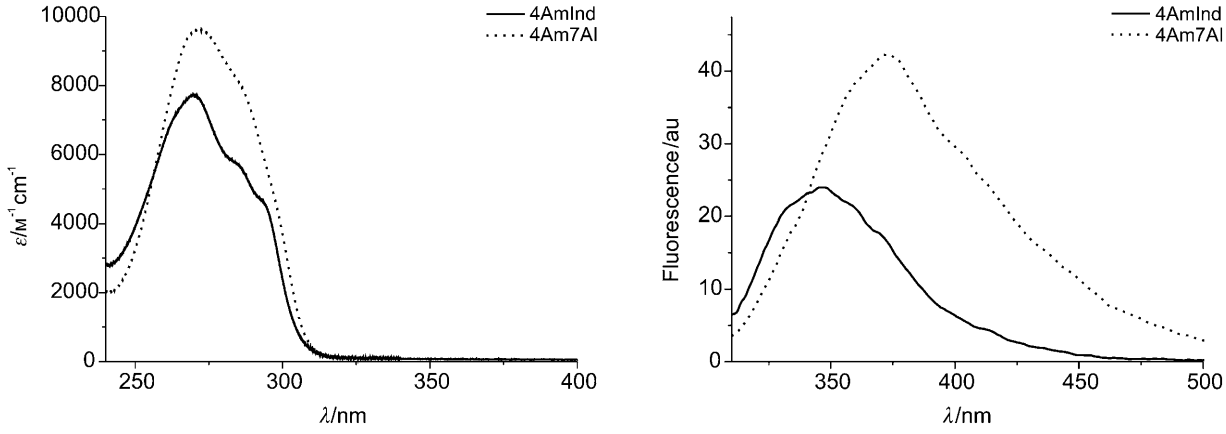


Figure 3. Absorption (left) and emission profiles (right) of 4-aminoindole (4AmI) and 4-amino-7-azaindole (4Am7AI). It is obvious that amination of 7AI provides two-fold increase in intensity and a considerable red shift (27 nm). In comparison with monomethylated counterparts this is a rather modest improvement. But 4Am7AI could be an especially interesting candidate for insertion into the chromophores of FPs, such as ECFP.

azaindole-based chromophores capable of providing detectable emission in blue or even green regions, should consider the possibilities offered by the chemistry of the indole ring. The goal is to identify chemical substitutions that lead to significant spectral red shifts, if possible without necessity for the expansion of the aromatic system with a third ring. Until now, the red shift of azaindoles under physiological conditions have never extended beyond 450 nm.

The substitution of endocyclic –CH groups of indole with nitrogen leads to changes in the photophysics of the whole aromatic system.[33] Furthermore, similar effects can be achieved by introducing electron-donating amino and hydroxy groups at different positions of the indole moiety. Both substitutions induce an intramolecular charge transfer that is sensitive to pH changes.[19] The DNA/RNA purine nucleotides adenine and guanine and their analogues (such as 6-aminopurine) harbor a combination of both endo- and exocyclic nitrogen substitutions. The lone electron pairs of these nitrogen atoms are responsible for unique features like charge transfer, the fluorescence dependence of the solvent and pH value.[19] However, additional endocyclic nitrogen atoms in the azaindole system do not contribute to the extension of the π-electron system with their lone electron pair. Accordingly, such substances tend to be more basic.

Similar to 7AI in apolar solutions, aminoindoles also possess dual fluorescence emission with one peak centered around 350 nm and the other at around 520 nm.[34] The second spectral peak is normally not visible in water for the following reason. Water as a solvent acts as proton donor in the S_o state while the intramolecular charge transfer from the amino group to the indole is most effective in the S_1 excited state. This also explains the decrease in the relative fluorescence intensity of aminoindoles when compared to the natural indole. In other words, interactions such as hydrogen bonding of water molecules to the aminoindole in the S_1 state cause enhancement of nonradiative decay processes and explain the absence of the second fluorescence band in aqueous buffered solutions. However, in particular water-free milieus, such as membrane bilayers or apolar protein interiors, these substances might be conceived as dual-fluorescence protein-based sensors.

In protein fluorescence design it might be of outstanding importance to generate water-free protein interiors (i.e., cavities) especially suited for the accommodation of amino- or azaindoles and their derivatives. Suitable arrangements of amino acid side chains in such microenvironments could establish interactions that are, for example, able to stabilize green emitting chromophore species. This concept is indeed conceivable, if one considers, for example, the fluorescence of GFP. The chromophore (4-(*p*-hydroxybenzilidene)imidazolid-5-one) of GFP is completely encoded in its amino acid sequence and autocatalytically formed by the post-translational reaction between the side chains of residues 65–67 with molecular oxygen as the only externally required component.[35] The unique absorbance and fluorescence properties of GFP are not an inherent property of the isolated chromophore, which is nonfluorescent in water.[36] Its fluorescence is possible only in the context of the properly folded protein.[37] In other words, the fluorescence of GFP is not an intrinsic property of the cyclyzed Ser-Tyr-Gly tripeptide since this sequence can be found in a number of other proteins. However, its cyclization is possible only in the context of the GFP structure. For this reason, neither is the Ser-Tyr-Gly tripeptide in the structure of proteins other than GFP cyclized nor is the tyrosine side chain oxidized. Thus, the green fluorescence of GFP is a reporter of the interactions between the chromophore and its surrounding protein matrix.

Thus, the engineering of these FPs always includes two approaches: 1) covalent modification of the chromophore by classical site-directed mutagenesis (e.g., Ser65Thr), and 2) manipulation of electrostatic interactions between the chromophore and its surrounding protein matrix. For example, ECFP (enhanced cyan FP) was generated by random mutagenesis. This allowed for identification of the structure with a series of mutations in the neighborhood of the chromophore responsible for greatly improved quantum yield and fluorescence lifetime. On the other hand, in EGFP covalent modification of the chromophore (Ser→Thr) favors its anionic form ($\lambda_{max\,em}$ = 509 nm) responsible for strong green fluorescence especially attractive for numerous practical applications in molecular and cellular biology. The most recent examples include the conversion of a nonfluorescent chromoprotein into a fluorescent protein by semirandom mutagenesis.[38]

However, the basic advantage of GFP-like chromophores is that their excitation wavelengths are usually above 350 nm, whereas azaindole-based chromophores are usually excitable below this value. Future work should clarify whether it is possible to circumvent this problem by extensive modifications of azaindole chromophores (e.g., introduction of novel substituents like cyano, nitro, nitroso, imidazole, pyrrole, etc.).

From a general point of view, suitable protein microenvironments might also be able to stabilize particular tautomeric states of incorporated amino acid like chromophores. In other words, these microenvironments could lead to the stabilization of fluorescence species not assessable in bulk water. For the evolution of such protein environments classical protein engineering (i.e., site directed mutagenesis) and evolutionary methods (i.e., guided evolution) should be combined with an expanded genetic code. In addition, the newly evolved indole analogues could also be introduced to the chromophores of FPs to expand their spectral properties. In this context a suitable candidate for further improvement of the spectral properties could, for example, be 4-amino-7-azaindole (Figure 3). Taken together, all the mentioned azaindole derivatives could be solely used as chromophores or as part of post-translationally generated chromophores in FPs.

Methodological Issues

There are a few crucial methodological issues that should be accounted for in order to employ the methylated azaindoles for protein incorporation. Firstly, these chromophores should be chemically synthesized as amino acids capable of mimicking protein building blocks. Secondly, the biocompatibility of these compounds should be established. Thirdly, given

that cotranslational incorporation is possible, engineering of the protein target itself would be most probably necessary as discussed above. Finally, the influence of these chromophores on the protein structural integrity upon insertion into hydrophobic interior should be considered as well. For example, we found that both 4-aminoindole as well as 4-azaindole—as W analogues—deliver hydrophilicity into the apolar protein interior, which impairs folding cooperativity and stability by disruption of the interaction network within the core.[39] In future studies, the use of methylated azaindoles might be helpful to circumvent these problems.

For successful in vivo protein biosynthesis with noncanonical amino acids the following conditions must be fulfilled: 1) effective uptake/import of the noncanonical amino acid into the cell, 2) its intracellular metabolic and chemical stability and accumulation at levels sufficient for activation and tRNA aminoacylation, and 3) ribosomal translation into the nascent polypeptide chain in response to a sense or stop codon. To date, there are no studies about possible intracellular uptake and transport of methylated azatryptophans although there are plenty of data about carbon methylated tryptophan derivatives.[40] In spite of the fact that azaindoles/azatryptophans fulfill all the above listed criteria their methylated derivatives possess no protonated pyrrole nitrogen. As a consequence they are not substrates of Trp synthase (Trp metabolism) or for tryptophanyl-tRNA synthetase (Trp translation), that is, they are bioorthogonal in the cellular milieu. This was confirmed by studies of indole isosters in which the pyrrole nitrogen was replaced with other heteroatoms (e.g., sulfur or oxygen).[41] Therefore, incorporation of methylated azatryptophans would require the installation of novel aminoacylation pathways as well as codon reassignment in the genetic code. Interestingly, W analogues in which the pyrrole nitrogen is replaced with other heteroatoms are found to be substrates for engineered Phe tRNA synthetase.[42]

Currently existing approaches for in vivo incorporation of noncanonical amino acids into proteins are normally performed in bacterial hosts. Their transmission to eukaryotic cells is not a trivial task. The use of stop codon suppression is additionally limited by read-through efficiency (usually ~40%)[43] and permissiveness of the particular sequence positions (i.e., not all positions in the sequence are equally well suited for labeling). Last but not least, evolved enzymes for amino acid recognition and cognate tRNA have poor catalytic performance and selectivity.[43] The other approach, known as selective pressure incorporation (SPI)[43] relies on the relaxed substrate specificity of aminoacyl-tRNA synthetases as the crucial enzymes in the interpretation of the genetic code. Thereby, the activation and transfer onto cognate tRNAs of a variety of structurally and chemically similar substrate analogues is possible without the necessity for extensive host cell engineering. This straightforward in vivo approach is especially suited for multisite substitutions in a target protein sequence.

The biggest challenge will certainly be to enable broader in vivo use of these novel chromophores as a marker in cell biology. This is currently limited due to the necessity of special incorporation protocols. Fortunately, there is already solid data that support the proposal that such reprogrammed protein translation in mammalian cells is possible.[44] It is usually assisted by the supplementation of cells with plasmids encoding modified translation components (aminoacyl-tRNA synthetases, tRNAs, etc.) that are compatible with the endogenous translational apparatus of the host. In the coming years, we anticipate significant progress in the development of reprogrammed translation with an expanded genetic code for use in mammalian cells.

Synthetic Biology: from DNA Mimicry to Molecular Switches, Recognition, Novel Biomaterials and Base Pairs

The slightly basic indole moiety of the canonical amino acid W substituted by endocyclic nitrogen acquires stronger basicity. Nucleobases and amino acids are strictly separated building blocks in the synthesis of biopolymers, such as DNA/RNA on the one hand and polypeptides on the other hand. Current developments in the field of code engineering by introducing amino- and azaindole-based amino acids as protein building blocks can also be seen as a bridge between these two worlds. This will further assist novel research in biologically-inspired material science.

DNA nucleobases exhibit a combination of both exocyclic and endocyclic nitrogen atoms facilitating charge transfer and basicity. These features play a key role in biological recognition as well as in numerous intra- and intermolecular interactions. All these features could be transferred to the level of proteins by introduction of various nitrogen substituted indole analogues. Azaindoles are indeed similar to the nucleic acid purine bases since they contain pyridine nitrogen with a lone electron pair capable of hydrogen bonding and excited-state tautomerization. Subsequently, as protein building blocks, azaindole and its derivatives can decorate them with new functions and properties Nature has exclusively reserved for nucleic acids. This might be a crucial feature for the design of future protein-based optoelectronic data storage devices or molecular wires for information transduction.[45]

DNA mimicry is the property of some proteins to mimic DNA structures.[46] They normally interact with enzymes that bind DNA, such as restriction and repair enzymes, gyrase or nucleosomal and nucleotide-associated proteins (molecular switches). They also include repressor or activator proteins, which can either block the access of the polymerase to DNA or enhance enzyme binding to DNA. DNA mimicry might be further developed if specific, for example, purine-mimicking W analogues can be incorporated into proteins. Molecular recognition designed in this way opens up possibilities to develop novel generations of molecular switches for tight control and regulation of enzyme interactions with genomic DNA. In other words, novel synthetic biology of molecular recognition and molecular switching at the genome level might be established.

Finally, azaindole-based substances can be especially useful for genetic code expansion by generation of noncanonical base pairs. Thereby, a third, and therefore noncanonical, base pair could be designed.[26] It is expected that this is stable in

duplex DNA and can be replicated by appropriate polymerases with high efficiency and selectivity. This would lead to a greatly expanded coding capacity of the genetic code.[47] These novel noncanonical pairings could be based on sterical complementarity or hydrophobic interactions or on hydrogen bonding through their tautomeric forms. For example, indole-like bases, such as 7-azaindole or benzotriazole, could be good promising substrates for incorporation into nucleic acids based on shape complementarity.[48,49]

Experimental Section

All chemicals were purchased from Sigma–Aldrich, unless otherwise stated; 4AI and 7AI were purchased from Molekula and Biosynth. 1-Methyl-7-azaindole and 1-methyl-4-azaindole were synthesized as described.[45,50] The protection of 4AI was performed as described.[51] For the deprotection the literature procedure was used.[52]

Syntheses

1-Methyl-7-azaindole (1M7AI): 7-Azaindole (472 mg, 4 mmol) and sodium hydride (192 mg, 4.8 mmol; 60% in oil) were cooled to 0 °C under a nitrogen atmosphere. Then anhydrous dimethylformamide (10 mL) was added and the mixture was stirred until the evolution of hydrogen was complete. After the dropwise addition of iodomethane (0.25 mL, 4 mmol) the reaction was allowed to warm to room temperature. After being stirred at this temperature for 3 h, the solvent was removed and the residue was purified by flash chromatography (PE:EE:DCM, 8:1.8:0.2) to give 494 mg (94%) of a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 08.34 (dd, J = 4.7, 1.5 Hz, 1H), 7.90 (dd, J = 7.8, 1.5 Hz, 1H), 7.18 (d, J = 3.5 Hz, 1H), 7.05 (dd, J = 7.8, 4.7 Hz, 1H), 6.45 (d, J = 3.5 Hz, 1H), 3.900 (s, 3H) ppm. The NMR data are in agreement with those reported in the literature.[50]

1-Methyl-4-azaindole (1M4AI): 4-Azaindole (354 mg, 3 mmol) and sodium hydride (144 mg, 3.6 mmol; 60% in oil) were cooled to 0 °C under a nitrogen atmosphere. Then dry dimethylformamide (10 mL) was added and the mixture was stirred until the evolution of hydrogen was complete. After the dropwise addition of iodomethane (0.19 mL, 3 mmol) the reaction was allowed to warm to room temperature. After being stirred at this temperature for 3 h, the solvent was removed and the residue was purified by flash chromatography (PE:EE, 1:1) to give 330 mg (83%) of a light brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.46 (dd, J = 4.6, 1.2 Hz, 1H), 7.62 (d, J = 8.2 Hz, 1H), 7.32 (d, J = 3.2 Hz, 1H), 7.12 (dd, J = 8.2, 4.6 Hz, 1H), 6.69 (dd, J = 3.2, 0.8 Hz, 1H), 3.81 (s, 3H) ppm.

tert-*Butyl-4-azaindole-1-carboxylate:* *N,N*-Dimethylamino-pyridine (49 mg, 0.4 mmol) and di-*tert*-butyl dicarbonate (1.05 g, 4.8 mmol) were added to a solution of 7-azaindole (472 mg, 4 mmol) in anhydrous acetonitrile (10 mL). After being stirred under nitrogen at room temperature for 3 h, the solvent was removed and the residue was purified by flash chromatography (PE:EE, 1:1) to give 874 mg (quant.) of a white solid; m.p. 69–71 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.34 (dd, J = 4.7, 1.3 Hz, 1H), 7.90 (d, J = 6.8 Hz, 1H), 7.83 (d, J = 3.7 Hz, 1H), 7.05 (dd, J = 8.3, 4.7 Hz, 1H), 6.78 (d, J = 3.7 Hz, 1H), 1.68 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 149.1, 148.8, 145.5, 128.9, 122.2, 118.7, 108.3, 84.5, 28.1 ppm. The NMR data are in agreement to those reported in the literature.[53]

4-Methyl-4-azaindole (4M4AI): Dimethylsulfate was added to a solution of *tert*-butyl-4-azaindole-1-carboxylate (775 mg, 3.55 mmol) in anhydrous toluene (10 mL). The mixture was stirred under nitrogen at reflux temperature for 3 h. After being cooled, the solvent was removed and the remaining white solid was dried in vacuo. The crude product was used for the next reaction without further purification. A mixture of ethyl acetate (25 mL) and concentrated hydrochloric acid (10 mL) were added to the methylated salt. After being stirred at room temperature for 18 h a concentrated aqueous solution of potassium hydroxide was added (pH 13) and the mixture was extracted with dichloromethane (4×100 mL). Drying with magnesium sulfate and evaporation of the solvent gave 450 mg (90%) of an orange-colored oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.28 (d, J = 1.8 Hz, 1H), 8.18 (d, J = 7.7 Hz, 1H), 7.57 (d, J = 6.2 Hz, 1H), 6.94 (dd, J = 7.7, 6.2 Hz, 1H), 6.44 (dd, J = 1.8, 0.9 Hz, 1H), 4.15 (s, 3H) ppm.

UV absorbance and fluorescence measurements: We acquired ε_M of indole and azaindoles by UV spectra measurement with the UV/Vis spectrometer lambda 19 (PerkinElmer and Life Sciences). Samples were measured in Tris-HCl buffer (100 mM, pH 7.5). Fluorescence spectra in a range of 300–500 nm were recorded with the luminescence spectrometer LS50B (PerkinElmer and Life Sciences) at 20 °C at the determined maximum absorbance wavelengths as follows: W (278 nm), 4AI (288 nm), 1M4AI (289 nm), 4M4AI (285 nm), 1,4M4AI (290 nm), 7AI (288 nm), 1M7AI (289 nm), 1,7M7AI (290 nm), 4AmI (270 nm), 4Am4AI (300 nm). Different excitation/emission slits were used for the different substances measured because the fluorescence emission of some derivatives could not be measured in a reasonable dilution at slit 2.5/2.5. Therefore, in case of 4AI, 7AI, 4AmI and 4Am4AI the slits were set to 5/5. By co-measuring with tryptophan as standard, measurements at different slits and concentrations were later normalized with respect to each other.

Excitation spectra were corrected automatically by the instrument for artifacts originating from the xenon lamp (energy output is wavelength and time dependent) and from the excitation monochromator (efficiency is wavelength dependent). Emission spectra were corrected with respect to artifacts originating from the emission monochromator (efficiency is wavelength dependent) and the sample multiplier (sensitivity is wavelength dependent). Correction files for emission spectra are yearly updated and provided by the manufacturer of the instrument (PerkinElmer and Life Sciences). All measurements were made with constant detector current.

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