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Fluorescent Unnatural Amino Acids: Introduction and Their Potential Applications

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Fluorescence spectroscopy is the simplest and most frequently used technique for probing complex biological events. Fluorescently labeled amino acids can show variety of properties, like environment sensitivity, chelation-enhanced fluorescence etc., which has provoked researchers to observe biological processes such as protein conformational changes, protein localization, enzyme activities and binding events. This review describes the design and applications of fluorescent unnatural amino acids. The capacity to incorporate fluorescent amino acids site-selectively into a protein or peptide gives the benefit of closely retaining the built-in function and structure of that protein.

1. Introduction

Amino acids are the building blocks for protein construction. They also play a crucial role as intermediates in metabolism.[1] Mainly 20 amino acids are found in protein structure, which differs in the side chain units.[2] The arrangement of those amino acids is according to the arrangement of the bases in the gene that encodes the protein. The biological properties of a specific protein are determined by the chemical properties of the amino acids in that protein.

Even though natural amino acids can serve the necessary purpose in our body system, but unnatural/chemically synthesized amino acids are also needed to serve many purposes. Unnatural amino acids can be used as building blocks, conformational constrains, pharmacologically active products and molecular scaffolds. These also signify a vast array of various structural elements for the growth of many peptidic and non-peptidic compounds. They are also broadly used as chiral building blocks owing to their structural diversity and functional flexibility.

1.1. Naturally Occurring Fluorescent Amino Acids

Out of 20 naturally occurring amino acids, phenylalanine (1), tyrosine (2), and tryptophan (3) are considered to be fluorescent amino acids due to their aromatic side chains. They possess promising spectroscopic features and therefore considered as "built-in" fluorescent probes (Figure 1 and Table 1). Out of these three, the inherent photophysical property of tryptophan is favorable towards its use as a natural fluorescent probe.

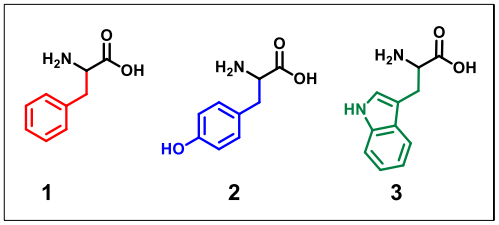

Figure 1: Naturally occurring fluorescent amino acids.

Table 1: Spectroscopic Properties of Emissive Native Amino Acids[4]

Compound no.	Name	λ_{max} (ε)	λ_{em}	φ	τ
1	**Phe**	258 (0.20)	282	0.024	
2	**Tyr**	275 (1.41)	310	0.14	3.3-3.8
3	**Trp**	279 (5.58)	365	0.01-0.4	

λ (Wavelength), ε (molar absorption coefficient), and τ (fluorescence lifetime) are in nm, LM^{-1} cm^{-1}, and ns, respectively.[4] φ signifies quantum yield.

1.2. Need for Unnatural Fluorescent Amino Acids

Naturally occurring fluorescent amino acids can serve the purpose of a fluorescent probe to monitor some biological events, but there is a need for unnatural amino acids also. The visualization of intracellular events and monitoring of intramolecular interactions inside a cell (e.g. explaining protein structures, functions, and dynamics; receptor-ligand binding;

enzyme activity; proteomics; protein sensing) can be done with the help of highly sensitive fluorescence probes.[5] Many of these aspects have already been studied using tryptophan, but all research needs cannot be fulfilled by only tryptophan or extrinsic fluorescent probes.[6,7] To overcome the deficiencies of tryptophan, microenvironment sensitive fluorescent unnatural amino acids could play an important role. Therefore, the synthesis and site-specific incorporation of fluorescent unnatural amino acids into a protein/peptide[8], the use of these amino acids to substitute natural aromatic amino acids in a built-in peptide/protein and the study of their outcome on secondary structure/conformation are also striking research goals.[9] But incorporation of unnatural amino acids into proteins can disturb protein folding and hence can disturb its function. For example, replacement of three tryptophan residues by 4-aminotryptophan causes destabilization of protein function.[10] Thus, design of spectroscopically valuable modified amino acids is a challenging task for those chemists interested in designing fluorescently modified unnatural amino acids.

In this review, we discuss about the various unnatural fluorescent amino acids and their photophysical counterparts in particular. The fluorescent amino acid modifications can be classified into different categories depending on the type of modification.

2. Side-Chain-Modification of Amino Acids

2.1. Tryptophan, Tyrosine and Phenyl Alanine Mimic Fluorescent Unnatural Amino Acids:

Natural amino acids enable the biophysical study without any chemical modification in proteins. Unnatural amino acids (UAAs) were used due to their specific spectroscopic properties. The potential perturbation/destabilization due to the presence of unnatural amino acids in proteins can be minimized by mimicking natural fluorescent amino acids like tryptophan, tyrosine and phenyl alanine. Some example of natural fluorescent amino acids based UAAs are discussed below. Several unnatural amino acids mimicking tryptophan, tyrosine and phenyl alanine have therefore been developed and incorporated into proteins. Moroder and coworkers have developed and incorporated blue-colored fluorescent β-(1-azulenyl)-L-alanine (azuAla, **5**) from azulene (**4**) as tryptophan-mimetic fluorescent UAA. The planar aromatic azulene moiety mimics the tryptophan side chain to some extent, and the spectral assets of the azulene moiety provides UAAs of potential value as UV and fluorescence probes in synthetic peptides.[11]

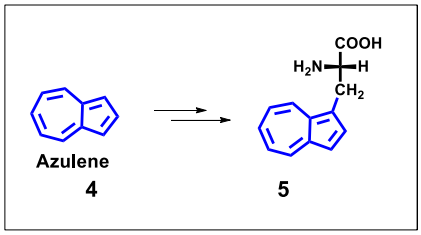

Figure 2: Azulene based fluorescent amino acid.

5-Hydroxytryptophan (5OHTrp, **6**)[12] and 7-azatryptophan (7azaTrp, **7**)[12,13] containing unnatural amino acid have also been synthesized and their optical properties have been explored. It was observed that both 5OHTrp (**6**) and 7azaTrp (**7**) showed a 20 nm red shift of their absorption maxima compared with tryptophan, which facilitates a particular excitation in the presence of other tryptophan in a protein.[14] Benzofuranylalanine (BfAla, **8**)[15] and benzothiophenyl (BtAla, **9**)[16] are two other examples of tryptophan mimics. These amino acids only vary from tryptophan in their ring heteroatoms.[17]

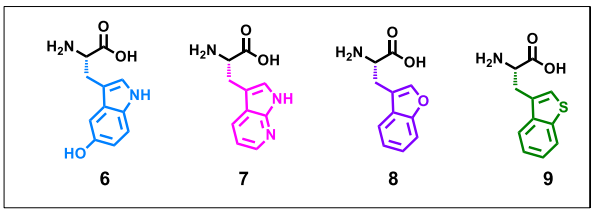

Figure 3: Tryptophan mimetic fluorescent unnatural amino acids.

In recent years, Hecht and co-workers have reported the synthesis and development of azaindole tryptophan analogue to exceed the limitations of 4-azatryptophan (**10, Figure 4**). The synthesized azaindole tryptophan analogue containing UAA (11, **Figure 4**) is more hydrophobic and having larger Stokes shifts (~150−160 nm) than that of tryptophan. They have also incorporated the 1H-pyrrolo[3,2-c]isoquinoline molecule into dihydrofolate reductase (DHFR) protein with minimal disruption of function. Their study revealed that the synthesized UAA containing tryptophan analogue could be selectively monitored in the presence of five Trp residues in that DHFR.[18]

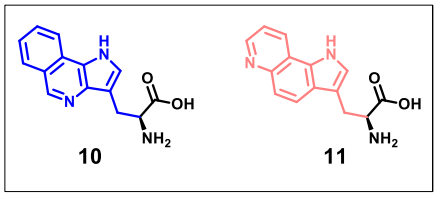

Figure 4: Structures of azaindole tryptophan derivative of unnatural amino acids**.**

In 2015, the same group have synthesized cyanotryptophans (12-14, **Figure 5**) and incorporated these molecules into dihydrofolate reductase (DHFR) protein at two different positions. The 6-CNTrp (**12, Figure 5**) was found to create an efficient Förster resonance energy transfer (FRET) pair with L-(7-hydroxycoumarin-4-yl)ethylglycine (HCO) at position 17 and when it (**12**, 6-CNTrp) was incorporated into a fragment of polymerase I, was found to transfer energy to DNA substrates with an acceptor fluorophore (**15, Figure 5**). Thus, they have established that a UAA can be utilized as a FRET partner for studying protein−nucleic acid interactions.[19]

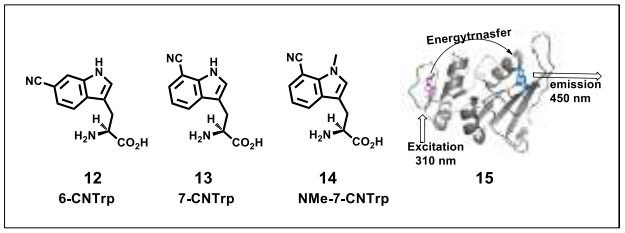

Figure 5: Incorporation of cyanotryptophan (12-14) analogues into dihydrofolate reductase (DHFR) protein.

Recently, Hecht synthesized four positional isomers of biphenyl-phenylalanine (18-21, **Figure 6**) and investigated their photophysical properties. All fluorophoric phenylalanine derivatives were then incorporated into multiple positions of *ec*DHFR. The modified *ec*DHFR with those amino acids was found to consume NADPH at a faster rate than normal. These outcomes indicated that biphenyl-phenylalanines can be introduced into DHFR with minimal impact on the structure and function of the enzyme.[20]

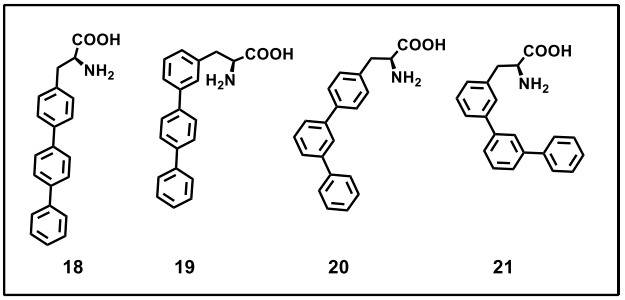

Figure 6: Structure of biphenyl-phenylalanines and coumaryl derivative of UAAs.

An approach to generate fluorescent UAAs to mimic the tyrosine analogue was developed by Meng-Lin Tsao and co-workers. They have genetically incorporated 2-naphthol analogue of tyrosine, 2-amino-3-(6-hydroxy-2-naphthyl)propanoic acid (NpOH), into proteins in *Escherichia coli*. They have also established that the 2-naphthol side chain in NpOH can serve as target for site specific protein modifications through selective azo coupling reaction under neutral pH using diazonium reagents (**16-17, Figure 7**).[21]

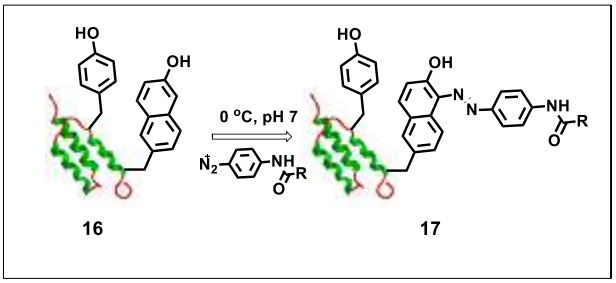

Figure 7: Incorporation of 2-naphthol analogue of tyrosine into *Escherichia coli* proteins.

Wang *et al*. have synthesised styryl conjugated tyrosine mimics with excellent optical properties (**23 a-f, 24 a'-g', Figure 8**). These fluorescent unnatural amino acids consist of stilbene and meta-phenylene vinylene units and covers the emission colour from blue to near IR region. They have also shown distinct red, green and blue (RGB) emission spectra simply by adjusting the pH of solution.[22]

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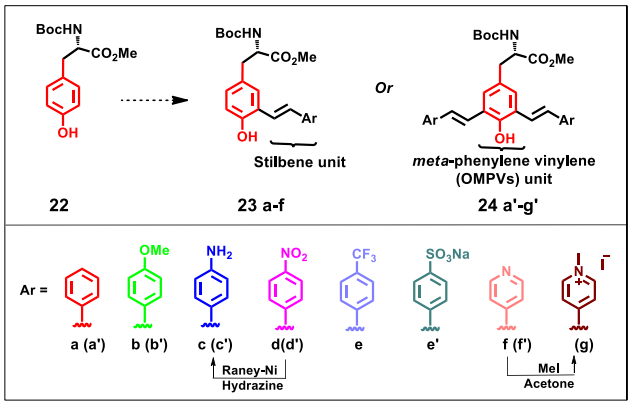

Figure 8: Tyrosine mimetic fluorescent unnatural amino acids by extending the π-conjugation of aromatic side chain.

2.2. Coumarin Labelled Fluorescent Unnatural Amino Acids:

The coumarin derivatives are well known fluorescent probes due to their relatively high quantum yields, low bleaching property and moderate stability.[23] Furthermore the coumarin labelled amino acids can be synthesized from aspartic (Asp) and glutamic (Glu) acids by converting them into their corresponding β-ketoester that would further undergo condensation with phenols to give desired coumaryl amino acid. Pechmann condensation reaction is one of the easiest procedures for coumarin derivative synthesis.[24]

Garbay and coworkers have synthesized optically pure coumarin labelled amino acids from cheap chiral starting materials (**25-29**, **Figure 9**).[25] They also have studied cellular localization of coumaryl UAA labelled peptides by confocal fluorescence microscopy.

Katritzky and co-workers have demonstrated the pathway for the synthesis of coumarin-labeled amino acids and peptides by solid phase peptide synthesis (SPPS) reactions.[26] They have synthesized N^{α}- and N^{ε}-coumarin labeled Cbz- and Fmoc-L-lysines (**30-33, Figure 10**) in excellent yields using benzotriazole activated coupling reagents.

2.3. Dansyl Labelled Fluorescent Unnatural Amino Acids

The dansyl unit has been utilized as a fluorescent probe in the biomolecules including amino acids, due to its good spectroscopic qualities.[27]. Schultz and co-workers have synthesized dansyl labelled lysine modified UAAs and incorporated them site-specifically into proteins to know the

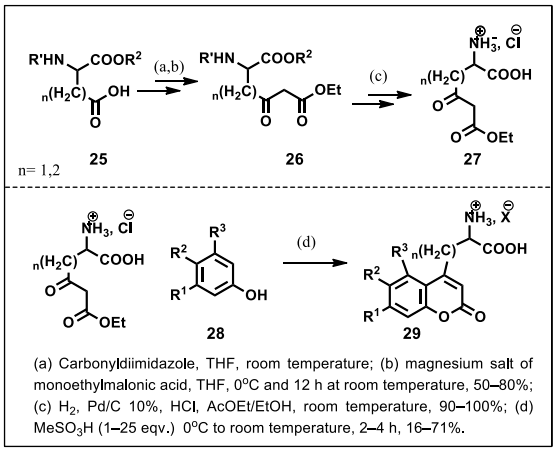

Figure 9: Synthesis of coumaryl based amino acids.

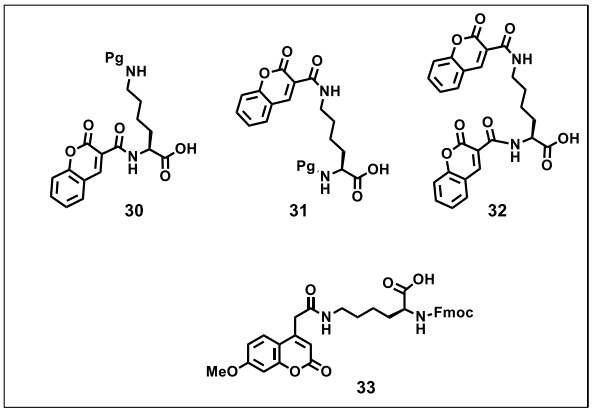

Figure 10: Example of coumarin-labelled lysine analogue.

structure and dynamics of it. Their study established that *E. coli* biosynthetic machinery can bear dansyl modified UAAs with slight similarity to native amino acids.[28,29]

In 2006, the same group synthesized 2-amino-3-(5-(dimethylamino)naphthalene-1-sulfonamide)propanoic acid (dansylalanine) (**34, Figure 11**) and reported the strategy for the selective incorporation of it into proteins at defined sites. The synthesized fluorescent amino acid was genetically encoded in *Saccharomyces cerevisiae* by utilizing an amber codon (**35-36, Figure 11**). The fluorophore, after incorporation in human superoxide dismutase can be utilized to observe the unfolding of the protein in presence of guanidinium chloride. This approach

can facilitate the biochemical and cellular studies of protein structure and function.[28]

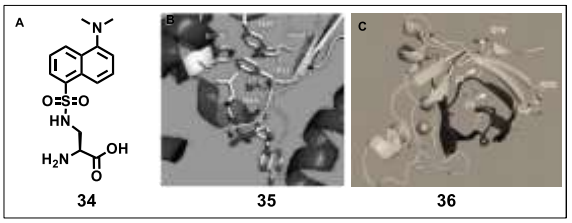

Figure 11: (A) Dansyl decorated unnatural amino acid and (B-C) its incorporation into protein.

Dansyl phenyl alanines have also been proposed but to the best of our knowledge, it has not been explored.[30] Here are two examples of dansyl modified phenyl alanines (**37-38, Figure 12**).

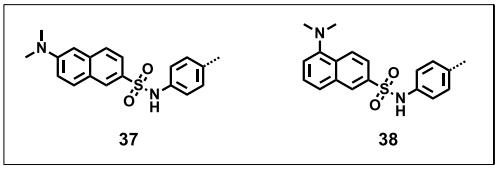

Figure 12: Phenyl alanine based dansyl decorated unnatural amino acids.

Imperiali *et al*. have synthesized a library of peptides with the amino acids having 4-DMAP, 6-DMN, BADAN,[31] dansyl and NBD groups (**39-45**, **Figure 13**). The UAAs based on the dansyl fluorophore has been subjugated for probing peptide–protein interactions and protein unfolding study.[32]

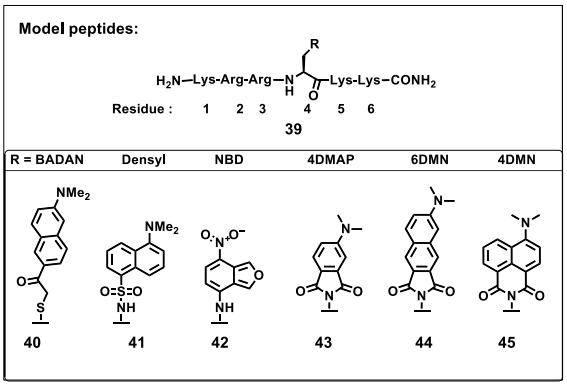

Figure 13: Solvatochromic amino acids incorporated model peptide series.

2.4. Diaminopropionic Acid Fluorescent Unnatural Amino Acids

N,N-dimethylamine derivative containing UAA is another example of amino acid modification through charge transfer group. Imperiali and co-workers have developed route for the synthesis of 4-(*N*,*N*-dimethylamino) phthalimide-based environment-sensitive fluorescent unnatural amino acid building block that was incorporated into peptides via Fmoc-based solid phase peptide synthesis protocol (**46-47**, **Figure 14**). This UAA labelled peptide serves the purpose of a very good probe for biological applications in sensing protein–protein/peptide–protein interactions.[33]

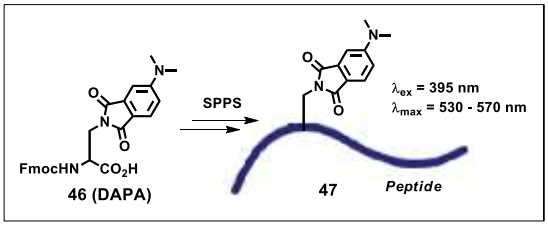

Figure 14: synthesis of 4-(*N*,*N*-dimethylamino) phthalimide-based environment-sensitive fluorescent unnatural amino acid and its incorporation into peptides.

Another UAA containing 6-N,N-dimethylamino-2,3-naphthalimide (**49-51**, **Figure 15**) was synthesized by the same group, which is an environment sensitive good fluorescent probe. The chromophore shows significant emission properties in 500−600 nm range and this range changes with environment polarity (**48**, **Figure 15)**.[34]

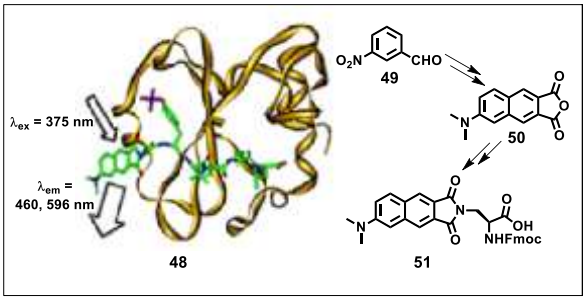

Figure 15: 6-N,N-dimethylamino-2,3-naphthalimide (6DMN)-based fluorescent unnatural amino acid synthesis and its incorporated optical properties.

They have further developed a 4-N,N-dimethylamine containing napthyl based fluorescent UAA probe (**52**, **Figure 16)**

which can serve as a bifunctional unit allowing the spatial release of a biologically active ligand and also make it possible to observe its binding to desired protein (**53**, **Figure 16)**. [35]

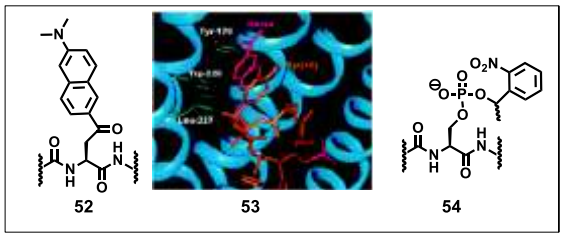

Figure 16: Substitution of Tyr(-2) for the enviromentally sensitive fluorescent amino acid DANA in the sequence RLYRpSLPA, that allows the monitoring of the binding process of protein.

All these charge transfer chromophores show valuable optical properties and their environment sensitivity makes them a useful fluorescent probe for incorporation into proteins and monitor peptide–protein interaction.

2.5. *Polyaromatic Hydrocarbon Labeled* Fluorescent Unnatural Amino Acids

Polyaromatic hydrocarbons are often highly emissive and when attached to an amino acid side chain give fluorescent building blocks. Many research groups have synthesized polyaromatic hydrocarbon containing UAAs and incorporated them into peptides or proteins.

Sisido *et al.* have synthesized polypeptides with α-helical secondary structure, carrying l-4-biphenylalanine and l-1-naphthylalanine units, separated by 11 amino acid units or 19.5 Å. They have investigated singlet energy transfer from biphenyl group to naphthyl group using steady-state fluorescence spectroscopy.[36] In the year 1999, they have synthesized various polyaromatic side chain containing UAAs and incorporated it site-specifically into proteins. Here are the examples of UAAs that were used in this study (**55-73**, **Figure 17)**.

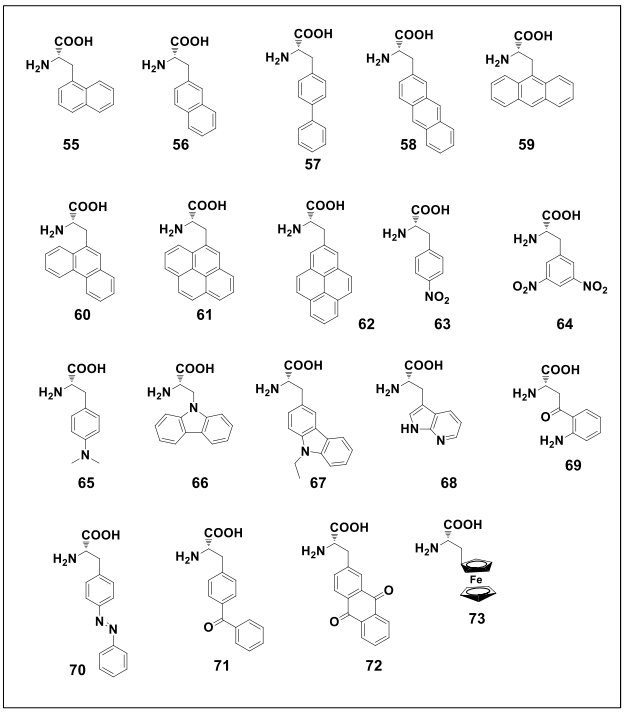

Figure 17: Examples of polyaromatic hydrocarbons containing fluorescent unnatural amino acids.

Some research groups have synthesized 9-anthryl- alanine (**59**),[37,38] 9-phenanthryl-alanine (**60**),[38] anthraquinone based alanine (anthrAla)[39] and investigated their photophysical properties.

2.6. Other Fluorescent Unnatural Amino Acids

Wang *et al.* have reported polarity-sensitive fluorescent UAAs L-Anap, which was later incorporated genetically into proteins and its activities studied in mammalian cells (**Figure 18**).[40]

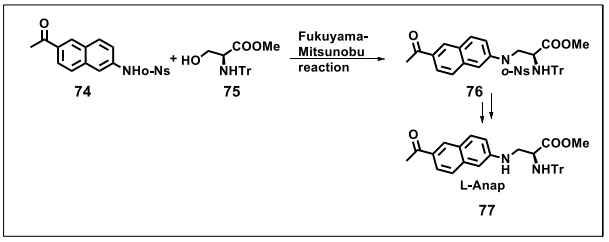

Figure 18: Polarity-sensitive fluorescent unnatural amino acids L-Anap.

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Sisido *et al*., synthesized multiply labelled fluorescent amino acids containing fluorescent peptides, based on their protein binding specificity. Thus, 8-mer peptides was synthesized by them and modified with several fluorescent UAAs (**78-83**, **Figure 19**). Before incubation, the peptides were mixed to anti-FLAG antibody and an EGFR. They were then recovered by gel filtration chromatography. The quantification of the binding peptides was carried out using a blend of fluorescence analysis with gel filtration method.[41]

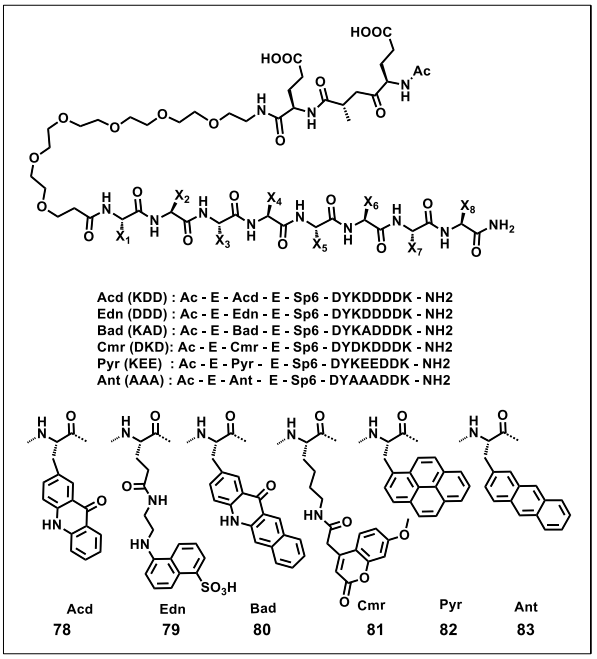

Figure 19: Structure of peptide modified with different fluorescent amino acids.

3. Applications

All the fluorescent UAAs discussed above are designed and synthesized for various applications. The applications are discussed below.[42]

a. **Structure, conformation and function**. One of the most important applications for designing UAAs is to determine the conformational behavior of proteins after incorporation of the modified amino acid and investigate the effect of amino acids on the natural protein function.

b. **Incorporation**. The fluorescent UAAs were synthesized for *in vitro* or *in vivo* incorporation of them into native proteins.

c. **Folding/unfolding**. Fluorescent UAAs, whose optical properties are dependent on solvent polarity, are responsible for unfolding (i.e., denaturation) of proteins.

d. **Electrostatics/polarity**. Electrostatics play a key role in defining the structure and function of proteins. Thus, fluorescent UAAs which are Coulombic interaction force sensitive can be used to study binding events of proteins.

e. **Photoswitch.** Photoswitchable fluorescent amino acids can be used to photocontrol a biological study.

4. Conclusion

The literature review describes a variety of fluorescent scaffolds with their optical properties and applications. The value of fluorescent amino acids in research helps to continue the progress in synthetic biological methods and sensitivity of imaging tools. Future utility of fluorescent UAAs will not only be better fluorophore scaffold designs and methods for multiple-fluorescent amino acid incorporation but also higher integration of imaging tools to see dynamic and delicate cellular functions in complex systems.[43]

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