

EN ROUTE TO FLUOROPHORES BASED ON OXYGEN HETEROCYCLES

Abstract

Small molecule fluorophores are effective tools for visualising biological events and they represent an important aspect of chemical biology. Since the first organic fluorophore, quinine, was discovered in 1845, efforts have been made to modify fluorescent substances using theoretical and synthetic approaches. The capability to use contemporary organic chemistry techniques to tailor chemical structures and logically tweak the photophysical properties and functioning of the fluorophore is a benefit of synthetic dyes. Although a wide array of fluorophores has been known over the years, most of these compounds are built on small 'core' dyes. Such dyes with various chemical functionalisations allow the accurate probing of several biochemical systems. In recent times, especially in the field of chemical biology, the technologies based on fluorescence have been found significant and it demands the basic understanding of the primary categories of small-molecule fluorophores. In this book chapter, we focus on the photophysical characteristics of a few fluorophores based on oxygen heterocycles and show both traditional and modern instances of how utility has been developed atop these foundations.

Keywords: Fluorophores; Coumarin; Benzofuran; Naphthofuran; Fluorescein; Biarsenical; Rhodamine; Oxygen Heterocycles

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I. INTRODUCTION

Fluorophores absorb photons of a certain wavelength and, in a matter of nanoseconds, release a photon with a longer wavelength [1-3]. The biology and material sciences have recently made extensive use of such luminous materials. These compounds find significant applications as light-emitting diodes. Besides that, high throughput screening, proteomics and in vivo imaging are just a few of the bioanalytical uses for fluorescently labelled heterocyclic compounds. The importance of such compounds increases when these substances show a range of medicinal effects, including analgesic, antifungal, anticancer, antimalarial, and anti-inflammatory actions [1-5].

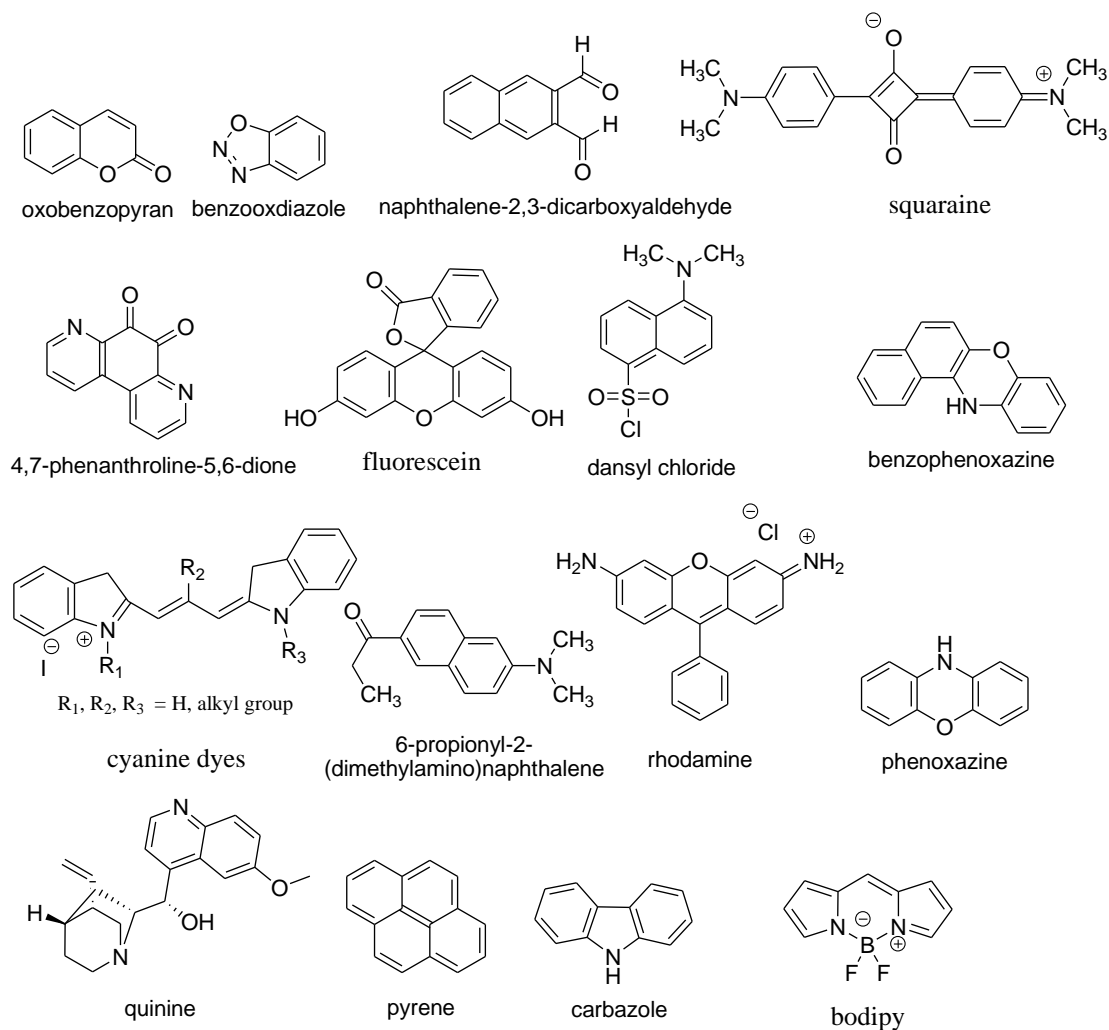
The past three and half decades have witnessed the applications of fluorescence in biological sciences. Over the years, steady-state fluorescence spectroscopic techniques have found significant applications in biochemistry and biophysics. However, time-resolved fluorescence spectroscopic techniques are now regarded as being primarily researched tools to understand the finer aspects of molecular behaviour in various environments. Since fluorescence detection is sensitive, most biochemical measurements no longer require expensive and challenging handling of radioactive tracers. Apart from biotechnology, genetic analysis and DNA sequencing, nowadays flow cytometry and forensics use the applications of fluorescence considerably. The saying 'Seeing is believing' applies perfectly to fluorescent imaging probes. Fluorescence imaging can sometimes identify single molecules and disclose the location and measurements of intracellular molecules [4-8].

Small-molecule fluorophores change their fluorescence emission in accordance with a change in their immediate environment. These are also responsive to chemical reactions and during binding with other biomolecules. Gradually, these fluorophores have evolved into strong tools for exploiting light to boost cell biology research, identify novel medications, detect environmental toxins, and improve cancer detection. The development of new drugs, cellular imaging, environmental research, and several medicinal applications all make extensive use of them today. These applications correspond to the growth of the research community working on fluorescent probe, which began as a small group in the latter part of the twentieth century and since then has grown to include over than 150 research groups globally. The entry of daring scientists from numerous other domains was necessary for this expansion [5-8].

Sensitive analytical methods are required in various branches of modern technology and this is especially true in the fields of cellular biology, environmental research, and medicine, where there have been numerous recent advances in reagents and techniques. Contrary to conventional dyes having good absorbance or labelling using radioactive units, fluorescent labelling is considered as in-depth as well as sensitive methodology for examining biomolecules. Biomolecules, such as NADH, amino acids having aromatic side chain, flavins etc. are examples of intrinsic fluorophores. However, labelling with fluorescent dyes improves sensitivity and visibility [9,10]. This field is made more promising owing to the substantial advancements in diverse fluorescence techniques and definitely accessibility of fluorophores across a wide spectrum of wavelengths. DNA, proteins, peptides and amino acids are the principal macromolecules that can be linked to fluorescent dyes either via covalent or noncovalent bonds. Organic synthesis has provided suitable platform to tweak the

fluorescent dyes for increasing the solubility in water, prolonged conjugation, quantum yields and photo stability.

Despite the fact that there are many fluorophores already in use, including coumarins [11,12], fluoresceins [13,14], cyanines [15,16], ellipticines [17,18], oxazines [19,20], acridines [21,22], pyrenes [23,24], carbazoles [25-38], quinines [39,40], bodipy dyes [41,42], etc., the pursuit for contemporary ones is still ongoing since they are essential to comprehend several biological processes.



Scheme 1: A Few Reputable Fluorescent Dyes Used for Labelling Study

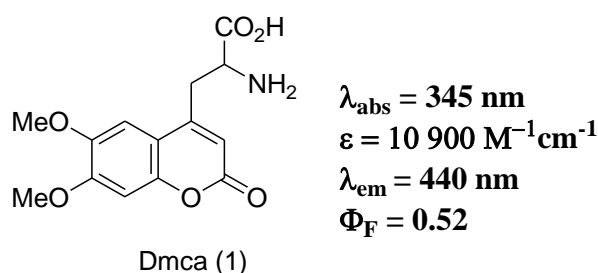
Scheme 1 portrays the fluorescent dyes which can be used for labelling ranging from near ultraviolet to having a wavelength around 500 nm. The examples include dansyl chloride, oxobenzopyrans, benzooxiazoles and naphthalene-2,3-dicarboxyaldehyde. However, when considered from 500 nm to the near-infrared spectrum, the fluorescent dye classes those are most frequently employed for bio-labelling are bodipy, fluorescein, squarines, rhodamine and cyanines (Scheme 1). Fluorophores based on coumarins, oligothiophenes, and benzooxiazoles have been reported for tagging biomolecules in the range till 500 nm. Below 500 nm emission range, fluorophores based on naphthalene skeleton

are also employed for bimolecular labelling. Fluorescein, rhodamine and BODIPY based fluorescent dyes are the best options for bimolecular labelling ranging from 500 nm to 700 nm, where issues with autofluorescence from biomolecules are negligible (Scheme 1). The abovementioned dyes possess high quantum yield and molar extinction coefficient [4]. Even, the designing of their water-soluble derivatives is likewise straightforward. This is what attracts the scientific community to use them as labelling dyes.

Near-infrared (NIR) fluorescent labelling dyes include squaraines and cyanine derivatives. The majority of applications for these NIR dyes involve DNA and gene sequencing. Recent advances also necessitate organic fluorophores with specialised photophysical, chemical, and electrochemical characteristics. To meet this rising need, synthetic efforts usually rely on the adaptability of heterocycle synthesis. Numerous fluorophores based on nitrogen and sulphur heterocycles have garnered popularity over the years. However, this chapter aims to portray several key ideas about the fluorophores based on oxygen heterocycles. Hopefully, by demystifying the subject, it would encourage more development in this area of research. This is not meant to be an exhaustive review. Recent literature has been used to choose illustrative cases.

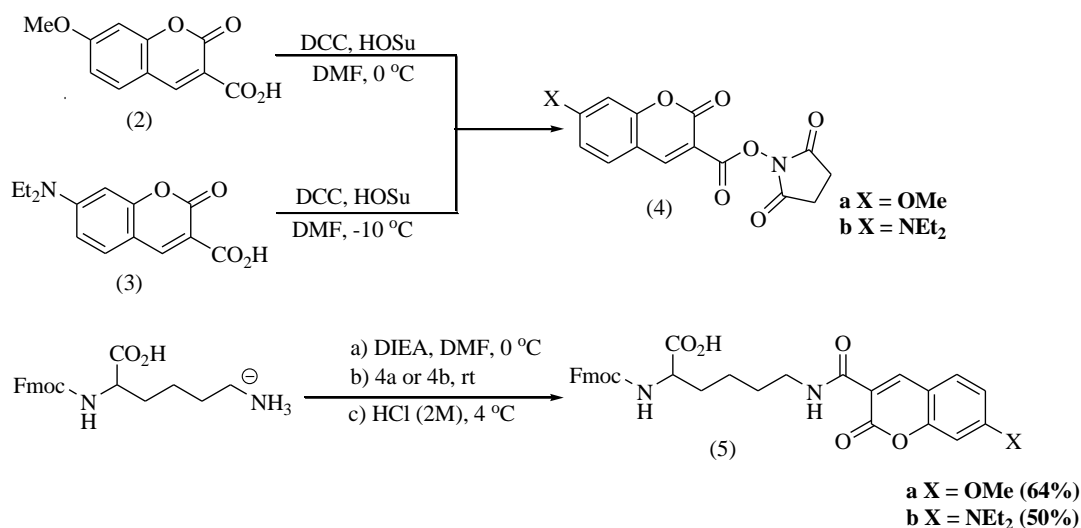
II. COUMARIN BASED FLUOROPHORES

One of the most effective and often used group of reagents for fluorescence derivatization is coumarin (3-oxo-3H-benzopyrans) [11,12]. A significant number of fluorophores of this type have been documented in scientific literature. Several articles allude to carboxylic acid based compound determination employing such labels. Owing to their wide spectrum range, high quantum yields, significant photostability and good solubility in a variety of solvents, fluorogenic amino acids containing oxobenzopyrans seem to be intriguing compounds. The maximal absorption and emission wavelengths of 2-amino-3-(6,7-dimethoxy-3-oxo-3H-benzopyran) propanoic acid (Dmca) (Scheme 2) are 345 and 440 nm, respectively [42-44]. These characteristics allow for the selective identification of Dmca-labeled peptides even in the presence of tryptophan residues. Additionally, Dmca has a large molar absorptivity (and $\epsilon = 10,900 \text{ M}^{-1} \text{ cm}^{-1}$) and a high fluorescence quantum yield ($\Phi_F = 0.52$), which enable the detection of the picomolar scale detection of the labelled peptides on a picomolar scale with a sensitivity comparable to radiolabeling.



Scheme 2: Representative Structure of Dmca (1)

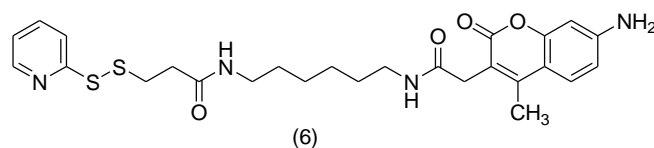
Berthelot et al. synthesised two new derivatives of lysine labelled by oxobenzopyran. Carboxylic acid derivatives (2, 3) were activated via the formation of N-hydroxysuccinimide ester and subsequently linked with Fmoc-Lys-OH (Scheme 3) [45-48].



Scheme 3: Synthesis of two New Lysine Derivatives labelled by Oxobenzopyran Unit

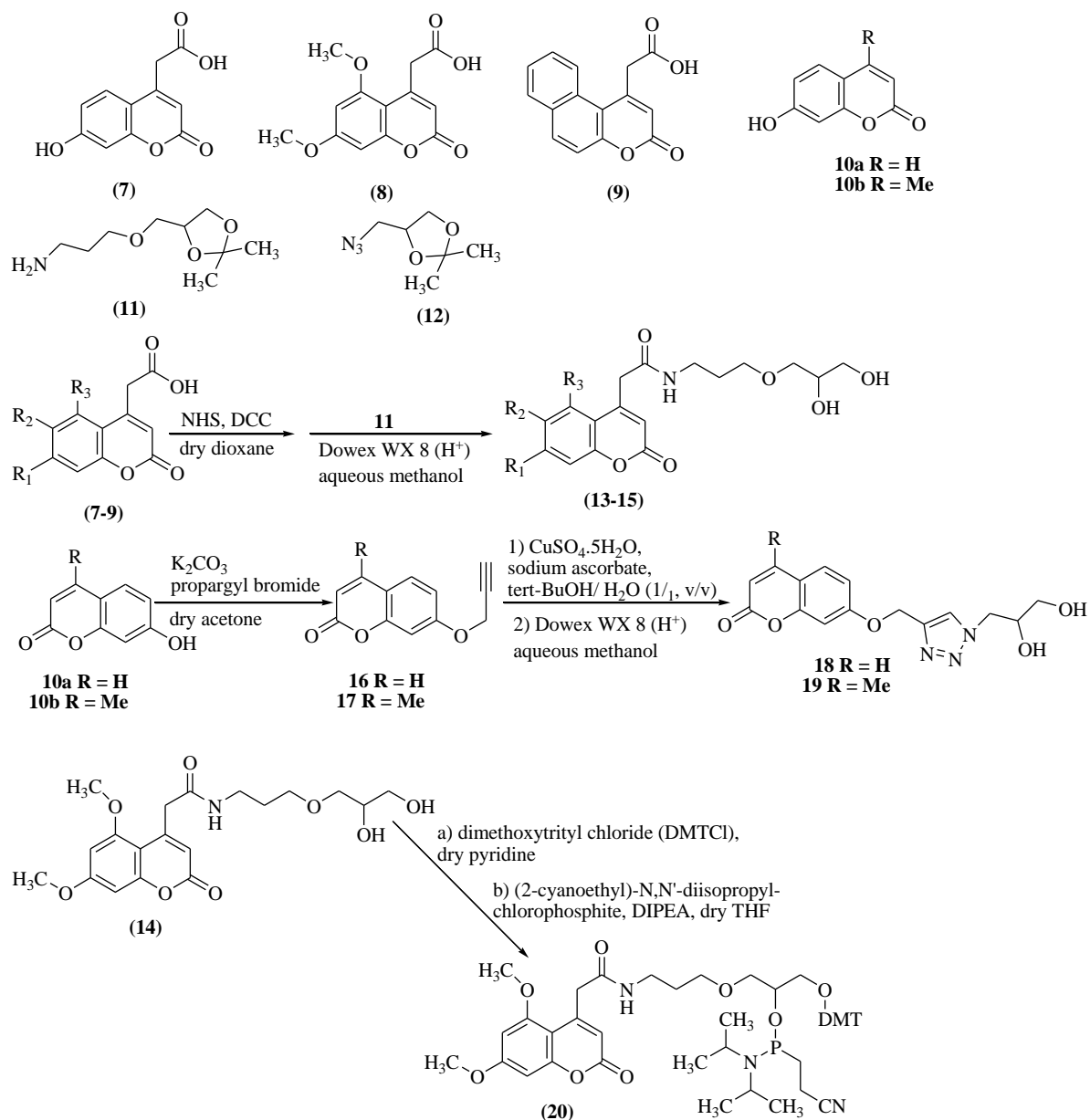
The absorption maxima of compounds (5a) and (5b) are ~350 nm and ~431 nm respectively whereas the emission maxima of 5a and 5b are ~404 nm and ~480 nm respectively. When the lysine residue was linked to heterocycles (2) and (3), a red shift was observed in the absorption maxima by 15 nm and 24 nm, respectively. The labelled lysine derivatives (5a) and (5b) was proposed by the authors as a fluorescent building block for the solid-phase peptide synthesis (SPPS) [49,50].

In the majority or all cell types and tissues, nitric oxide (NO) is created enzymatically. It has been demonstrated that certain significant effects of NO are mediated through the alteration of prosthetic metals in proteins, particularly haemoglobin iron. Later, it was discovered that a significant portion of NO's pervasive influence on cellular signal transduction is transmitted via the attachment of a NO group to the -SH side chain of cysteine residues present in proteins and peptides. This process is known as S-nitrosylation and certainly it plays as an essential switch in controlling protein functions. Several proteins, namely glyceraldehyde-3-phosphate dehydrogenase [51,52], protein disulfide isomerase [53], parkin [54] and β -actin [55] have been shown to be S-nitrosylated *in vitro* or *in vivo*. A fluorescence-based technique known as the AMCA switch method, developed by Han et al. in 2008, transforms S-nitrosylated cysteines into 3-(7-amino-4-methyl-2-oxo-2H-benzopyran) ethanoic acid (AMCA) fluorophore-labelled cysteines [56]. In the labelling phase, AMCA-HPDP (6) (Scheme 4) was utilised. S-nitrosylated protein units are easily identified as vivid blue bands under UV light source whereas the labelled proteins are subjected to non-reducing SDS-PAGE analysis. This method offered a precise identification of nitrosocysteines with the AMCA adduct when paired with liquid chromatography- tandem mass spectrometry (LC-MS/MS).



Scheme 4: Structure of AMCA-HPDP (6).

Ivana et al. described the development of fluorescent probes for site-specific insertion into oligonucleotides [57]. As sensitive fluorescent labels, oxobenzopyran derivatives (7-10) were employed, (11) and (12) were added as linkers to the basic oxobenzopyran skeleton to form the functionalized derivatives (13-15, 18, and 19) (Scheme 5). Commercially accessible hydroxyl derivatives of oxobenzopyran (10a) and (10b), as well as carboxylic acid containing heterocycles 7-9, were synthesised using the famous Pechman process [58].



Scheme 5: Synthesis of Fluorescent Probes Based on Oxobenzopyran Skeleton 13-15, 18 and 19.

In methanol, the optical characteristics of compounds 7-10, 13-15, 18, and 19 were examined (Table 1). Such conjugates (13-15) and (18-19) displayed emission maxima between 381 nm and 426 nm. Their fluorescence quantum yields varied from 0.02 to 0.30. Compound (14) was chosen for the production of a benzopyranylphosphoramidite linker (20)

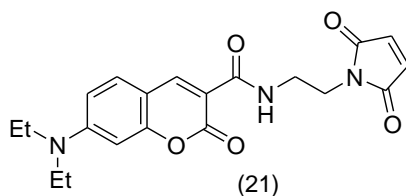
appropriate for direct insertion into oligonucleotides due to its superior fluorescence characteristics compared to the other members.

Table 1: Photophysical Possessions of a Few Fluorophores Related to Scheme 5

Compound	λ_{abs}	ϵ	λ_{em}	Φ_{F}	Compound	λ_{abs}	ϵ	λ_{em}	Φ_{F}
7	326 nm	12,487 ($\text{M}^{-1}\text{cm}^{-1}$)	392 nm	0.21	8	323 nm	3,032 ($\text{M}^{-1}\text{cm}^{-1}$)	418	0.47
9	318/ 349 nm	7,444 ($\text{M}^{-1}\text{cm}^{-1}$)	417 nm	0.10	10a	325 nm	14,509 ($\text{M}^{-1}\text{cm}^{-1}$)	392	0.08
10b	322 nm	15,552 ($\text{M}^{-1}\text{cm}^{-1}$)	387 nm	0.15	13	326 nm	11,883 ($\text{M}^{-1}\text{cm}^{-1}$)	396	0.24
14	323 nm	11,291 ($\text{M}^{-1}\text{cm}^{-1}$)	426 nm	0.30	15	319/350 nm	8,195 ($\text{M}^{-1}\text{cm}^{-1}$)	417	0.10
18	320 nm	11,301 ($\text{M}^{-1}\text{cm}^{-1}$)	387 nm	0.02	19	319 nm	20,338 ($\text{M}^{-1}\text{cm}^{-1}$)	381	0.05

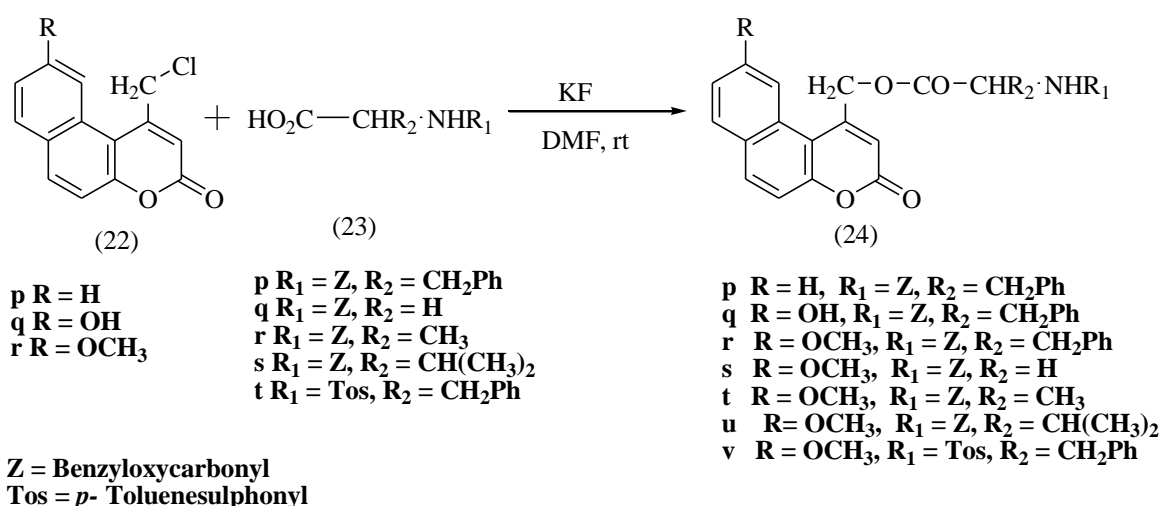
These oxobenzopyran heterocycles are significant in the derivatization of amino acids, peptides and nucleic acids, in addition to their application as fluorescent based probes in the investigation of enzymes [59,60].

Phosphate-binding protein (PBP) was labelled by Shutes et al. with fluorophore, MDCC (21). The research group used it to measure GTPase activity (Scheme 6). The MDCC-PBP probe makes it simple to examine the hydrolysis of GTP by GTPases in vitro and in real time [61].



Scheme 6: Representative Structure of MDCC (21)

Functionalized oxobenzo[f]benzopyrans (22p-r) were recently used to efficiently prepare various fluorescent *R*-amino acid derivatives (24p-v) (Scheme 7) [62]. The fluorescent conjugates that were produced (24p-v) exhibited high Stokes' shifts (66-131 nm). The λ_{abs} values were found between 345 nm and 360 nm whereas λ_{em} values were between 411 nm and 478 nm (Table 2).



Scheme 7: Synthetic Designing of Conjugates (24p-v)

Table 2: Photophysical Possessions Of Compounds (22p-r) and (24p-v) in Ethanol

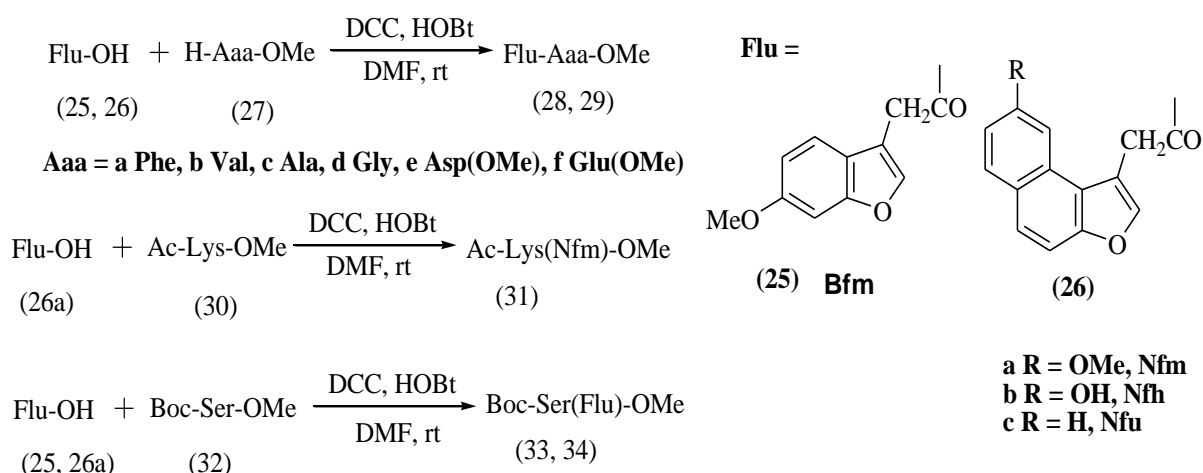
Compound	λ_{abs}	ϵ	λ_{em}	Φ_{F}	$\Delta\lambda$	Compound	λ_{abs}	ϵ	λ_{em}	Φ_{F}	$\Delta\lambda$
22p	352	11, 449 M ⁻¹ cm ⁻¹	418 (nm)	0.08	66 (nm)	24r	347 (nm)	12, 075 M ⁻¹ cm ⁻¹	478 (nm)	0.59	131 (nm)
22q	361 (nm)	12, 190 M ⁻¹ cm ⁻¹	462 (nm)	0.02	101 (nm)	24s	347 (nm)	11, 436 M ⁻¹ cm ⁻¹	471 (nm)	0.70	124 (nm)
22r	354 (nm)	12, 826 M ⁻¹ cm ⁻¹	472 (nm)	0.03	118 (nm)	24t	348 (nm)	11, 640 M ⁻¹ cm ⁻¹	477 (nm)	0.66	129 (nm)
24p	345 (nm)	14, 125 M ⁻¹ cm ⁻¹	411 (nm)	0.42	66 (nm)	24u	348 (nm)	11, 830 M ⁻¹ cm ⁻¹	478 (nm)	0.58	130 (nm)
24q	360 (nm)	10, 174 M ⁻¹ cm ⁻¹	456 (nm)	0.13	96 (nm)	24v	347 (nm)	12, 883 M ⁻¹ cm ⁻¹	475 (nm)	0.53	128 (nm)

Modest Φ_{F} values (0.02-0.08) were observed for (22p-r), which sharply rose during their reaction with amino acids (Φ_{F} = 0.13-0.70 for 24p-v). It was thus hypothesised that (22p-r) were prospective candidates for the fluorescent labelling of peptides and other biomolecules based on their photophysical possessions.

III. BENZOFURAN AND NAPHTHOFURAN BASED FLUOROPHORES

Similar polycyclic oxygen heterocycles that are referred to as fluorescent markers of biomolecules include benzofurans and naphthofurans. Piloto et al. derivatized α -amino acids (27, 30, and 32) in the amine function of the main chain of 27 or in the lateral chains in case of lysine (30) as well as in the hydroxyl group of the lateral chain of serine (32) using carboxylic benzofuran (25) and naphthofuran derivatives (26 a-c) (Scheme 8) [62-64].

After considering the photophysical data of the fluorescent amino acid residues (28a,b, 29a-h, 31, 33, and 34), it is evident from Table 3 that the 8-methoxy-naphtho[2,1-b]furan-1-yl ethanoic acid (26a) was found to be the most suitable derivative for labelling purposes. The absorption maxima of the amino acid-naphthofuran conjugates (29a-h, 31, and 34) was found to be around 298 nm whereas the emission maxima was ranging from ~325 nm to ~350 nm. The fluorescence quantum yields ranged between 0.10 and 0.44.



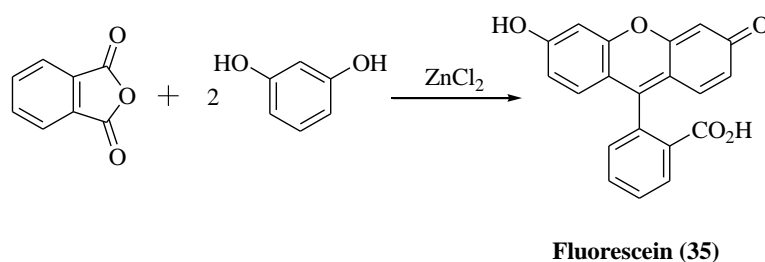
Scheme 8: Synthetic labelling of α -amino Acid Derivatives

Table 3: Photophysical Possessions of Compounds 25, 26a-c, 28a,b, 29a-h, 31, 33 and 34 in Ethanol

Compound	λ_{abs}	λ_{em}	Φ_{F}	$\Delta\lambda$	Compound	λ_{abs}	λ_{em} (nm)	Φ_{F}	$\Delta\lambda$ (nm)
Bfm-OH (25)	285 (nm)	315 (nm)	0.020	30 (nm)	Nfu-Val-OMe (29d)	292 (nm)	325 (nm)	0.13	33
Nfm-OH (26a)	298 (nm)	349 (nm)	0.020	51 (nm)	Nfm-Ala-OMe (29e)	298 (nm)	349 (nm)	0.24	49
Nfh-OH (26b)	301 (nm)	349 (nm)	0.062	48 (nm)	Nfm-Gly-OMe (29f)	297 (nm)	343 (nm)	0.24	46
Nfu-OH (26c)	293 (nm)	340 (nm)	0.076	47 (nm)	Nfm-Asp(OMe)-OMe (29g)	298 (nm)	346 (nm)	0.14	48
Bfm-Phe-OMe (28a)	288 (nm)	315 (nm)	0.064	27 (nm)	Nfm-Glu(OMe)-OMe (29h)	298 (nm)	347 (nm)	0.14	49
Bfm-Val-OMe (28b)	288 (nm)	315 (nm)	0.070	27 (nm)	Ac-Lys(Nfm)-OMe (31)	297 (nm)	347 (nm)	0.44	50
Nfm-Phe-OMe (29a)	298 (nm)	349 (nm)	0.32	52 (nm)	Boc-Ser(Bfm)-OMe (33)	287 (nm)	314 (nm)	0.06 4	27
Nfm-Val-OMe (29b)	298 (nm)	346 (nm)	0.37	49 (nm)	Boc-Ser(Nfm)-OMe (34)	298 (nm)	349 (nm)	0.13	51
Nfh-Val-OMe (29c)	300 (nm)	350 (nm)	0.10	50 (nm)					

IV. FLUORESCCEIN DYE BASED FLUOROPHORES

Fluorescein dye (35) is arguably the most widely used fluorescent probe in recent times [65,66]. Usually, Friedel-Crafts reaction can be used to generate such compounds using phthalic anhydride and resorcinol (1,3-dihydroxybenzene) in the presence of lewis acid catalyst, zinc chloride (Scheme 9). As an alternative, the catalyst might be methanesulfonic acid [67-69]. In aqueous medium, the λ_{abs} of 35 is found to be around 490 nm whereas the λ_{em} is around 512 nm.

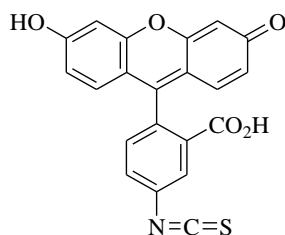


Scheme 9: Synthetic Strategy of Fluorescein (35)

Fluorescein is considered to be one of the most popularly used labels in the context of biological applications [4]. The combination of its extremely high molar absorptivity, good solubility in water, strong photostability and substantial fluorescence quantum yield makes it a particularly practical and sensitive fluorescent label along with a significant application in confocal laser-scanning microscopy as well as in flow cytometry [67,68]. Additionally, fluorescein has the advantage of having an excitation maximum around 494 nm, which is near to the argon laser's 488 nm spectral line. Although there are several fluorescent probes available for protein labelling but the amine-reactive probe produced from fluorescein is the commonly employed one. Fluorescein is commercially available in a variety of derivatives, including fluorescein succinimidyl ester and fluorescein isothiocyanate, which may bind to amino acids and large molecules covalently. The labelled compounds may be recognised with great sensitivity, which is useful in applications such as capillary electrophoresis. It is worthwhile to mention that Fluorescein's emission spectrum overlaps significantly with the absorption spectra of a related fluorescent dye, tetramethyl rhodamine. This makes the pair ideal for energy transfer experiments to estimate distances between the labelled macromolecules. Additionally, fluorescein-based protein conjugates are not highly vulnerable to precipitation, making it possible to acquire them at high levels of purity. Fluorescein based fluorophores and their conjugates show a few drawbacks, including (i) a fairly high rate of photobleaching; (ii) fluorescence dependence on pH; (iii) a fairly broad spectrum of fluorescence emission, restricting their efficacy in multicolor applications; and (iv) a proclivity to self-quench when conjugated to biopolymers, especially at high degrees of substitution. Quantitative analysis with fluorescein is challenging because to its photobleaching and pH sensitivity. Fluorescein can take on cationic, neutral, anionic, and dianionic forms in aqueous solution, which affects its fluorescence and absorption properties, depending on the pH [69-74]. In cases like fluorescence in situ hybridization and DNA sequencing where ultrasensitive detection is required, photobleaching lowers the sensitivity. These limitations have led to the development of fluorescein derivatives with improved characteristics.

Fluorescein labelling of peptides and proteins is typically accomplished using commercially available chemicals via the amino group of the lateral chain of lysine or thiol group of cysteine residues. In the particular labelling of NH_2 group, succinimidyl fluorescein derivative and during the labelling of SH, maleimidyl fluorescein derivatives are utilised [75,76]. Fluorescein isothiocyanate (FITC), however, is the widely utilised fluorophore in protein conjugation (36) (Scheme 10) [77,78]. Utilising soluble fluorescein isothiocyanate (FITC)-labeled casein, Twining described a straightforward, affordable, and sensitive protease assay [79]. The fluorescein thiocarbonyl derivative was formed by reacting casein with FITC. Trypsin, chymotrypsin, elastase, subtilisin, and thermolysin likewise cleaved this

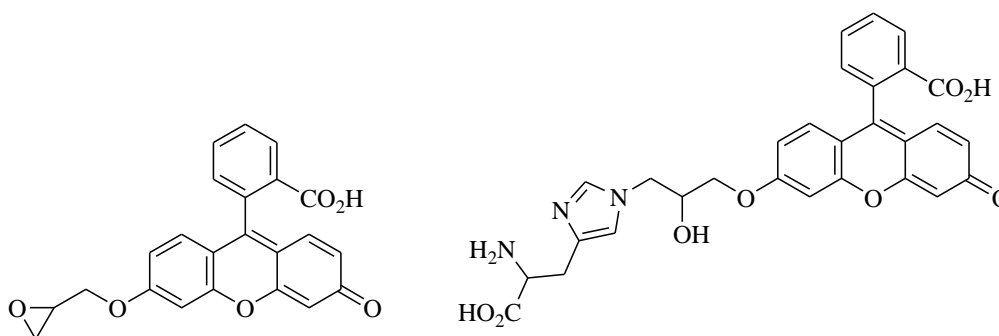
substrate in a linear time-dependent fashion. This assay can quantify enzymes in the nanogram and subnanogram ranges. Fluorescein isothiocyanate, an amine-reactive probe, was used in the research by Kila'r and Konecsni in order to label iron-free human serum transferrin, that was analysed employing varied dye-protein ratios [80]. Capillary electrophoresis was performed after the degree of labelling. A monomeric serum glycoprotein, transferrin binds two ferric ions and transports them to vertebrate cells via receptor-mediated endocytosis. This study helps to track the labelling of transferrin with FITC because the labelled protein can be employed as a receptor mediated endocytosis marker.



Fluorescein isothiocyanate (36)

Scheme 10: Representative Structure of Fluorescein Isothiocyanate (FITC) (36)

Li et al. reported the fabrication of another novel fluorescent probe, 3-epoxypropoxy fluorescein (EPF, 37), as well as its characteristics for histidine labelling (Scheme 11) [81]. The probe featured a fluorescein based unit with a long wavelength response along with an active epoxy labelling group. In alkaline environments, EPF interacts primarily with histidine in a selective fashion rather than other amino acids, causing a substantial increment in fluorescence intensity thereby permitting the detection of histidine. This type of increase in fluorescence resembles that of fluorescein diaion with increasing basicity of the neighbouring environment, implying that the addition reaction of histidine with the epoxy group provides the fluorophore with a basic molecular environment. The excitation maximum of EPF (37) is around 485 nm whereas its emission maximum is around 513 nm. These values are extremely similar to the values of its parent fluorescein. However, it also demonstrated that there is a lowering of quantum yield ($\Phi_F = 0.27$) compared to that of fluorescein ($\Phi_F = 0.95$).



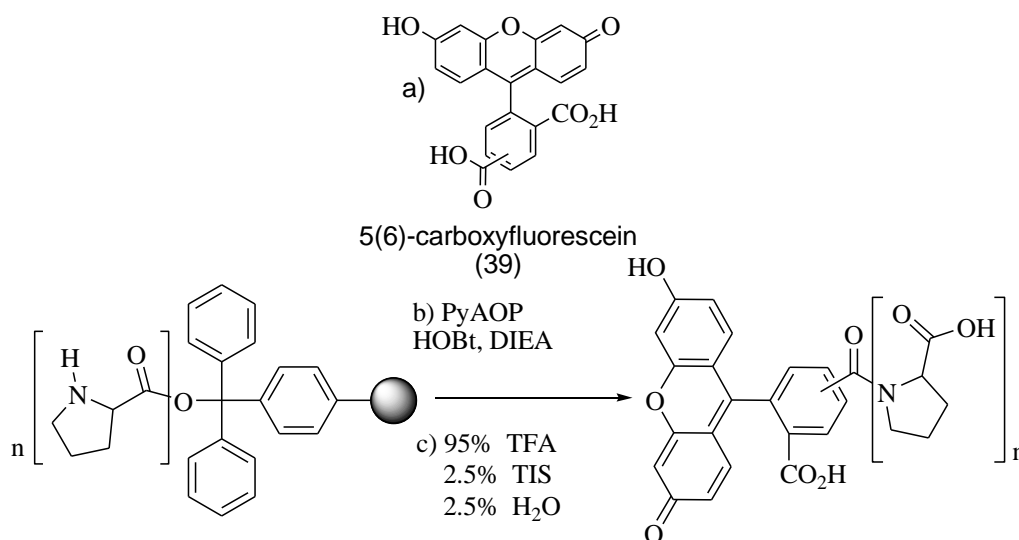
3-Epoxypropoxy fluorescein (EPF, 37)

fluorescent histidine derivative of EPF (38)

$\lambda_{\text{ex}} = 485\text{nm}$, $\lambda_{\text{em}} = 513\text{nm}$
 (aqueous solution pH 12.0 NaOH)

Scheme 11: Representative Structures of 3-Epoxypropoxy Fluorescein (EPF, 37) and Structure of the Probable Histidine Fluorescent Derivative of EPF (38)

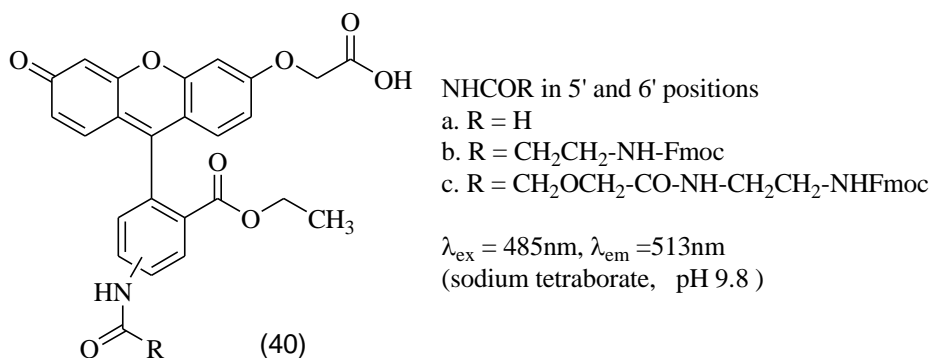
Owing to its high quantum yield and high absorption in the visible spectrum, fluorescein is the most widely used dye in a wide range of technological applications. Fluorescein derivatives, such as 5(6)-carboxyfluorescein (39), are particularly sensitive in an acidic or near-neutral pH range [82]. Fernández-Carneado et al. investigated the solid-phase fluorescent labelling of proline peptides with 5(6)-carboxyfluorescein (39) in the N terminals of hexaproline P6, dodecaproline P12, and 18-proline P18 (Scheme 12) [83]. The researchers pointed out that the disclosed technique might be used in other domains as well. Examples include solid-phase combinatorial synthesis of natural product-like compounds, polyamides and PNAs, where fluorescent labelling for cell biology is required.



Scheme 12: Conditions for the 5(6)-Carboxyfluoresceination Solid-Phase Reaction

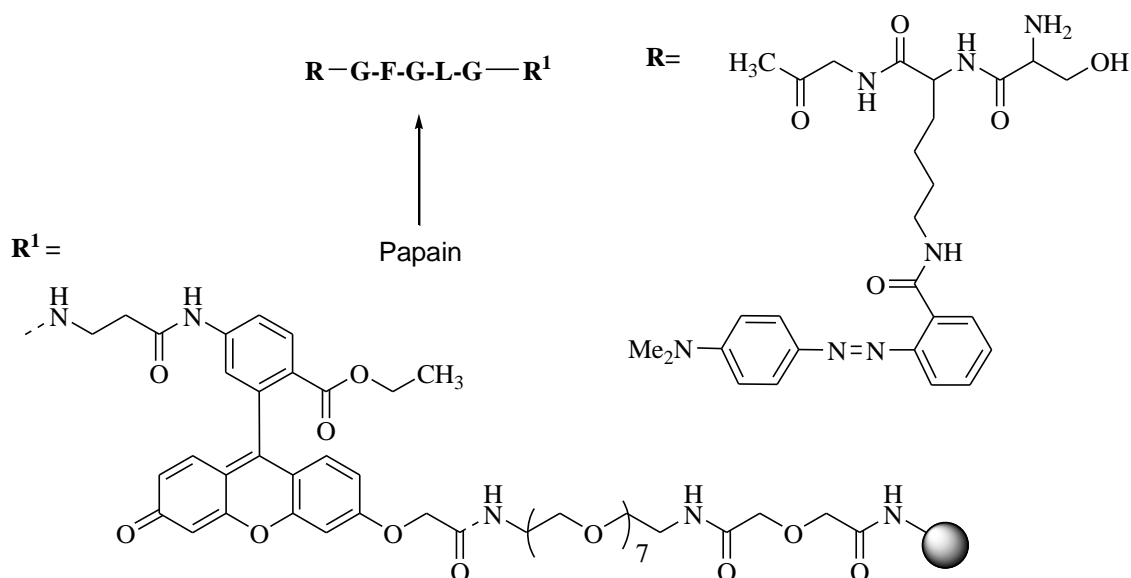
Recently, two sets of fluorescein-labelled peptides comprising O-mannosylated serine units integrated into peptide scaffolds with varying numbers of alanines connected to an NR-amino group or a side chain N-amino group of lysine were prepared using the 5(6)-carboxyfluorescein (39) [82,84]. The capacity of these O-mannosylated peptides to bind to mannose receptors or human APC subsets was supposed to be investigated *in vitro*. The two most significant issues that restrict the use of fluoresceins in bioanalysis are their low photostability and pH dependent fluorescence, as was already stated. Therefore, the goal of numerous investigations that have been published in literature has been the development of further heterocyclic compounds with superior photochemical capabilities.

A few novel fluorescent amino acids had been characterised in terms of their economical synthesis, photophysical characterisation and bioanalytical uses. In comparison to analogues based on fluorescein (35), these compounds (40a-c, Scheme 13) produced from commercial fluorophores have much higher photostability and pH-independent quantum yields. Compounds (40a-c) show two absorbance maxima centred on 456 nm and 481 nm. The fluorescence emission spectra of these compounds (λ_{em} 520 nm) are slightly red shifted in comparison to fluorescein though the fluorescence quantum yields for 40 ($\Phi_F = 0.19$) are considerably lower than those reported for fluorescein dianion ($\Phi_F = 0.92-0.93$). This is in accordance with those reports published for 3-O-alkyl ethers ($\Phi_F = 0.13-0.31$) [85-87].



Scheme 13: Representative Structures of a Few Fmoc-Protected Fluorescent Amino Acids

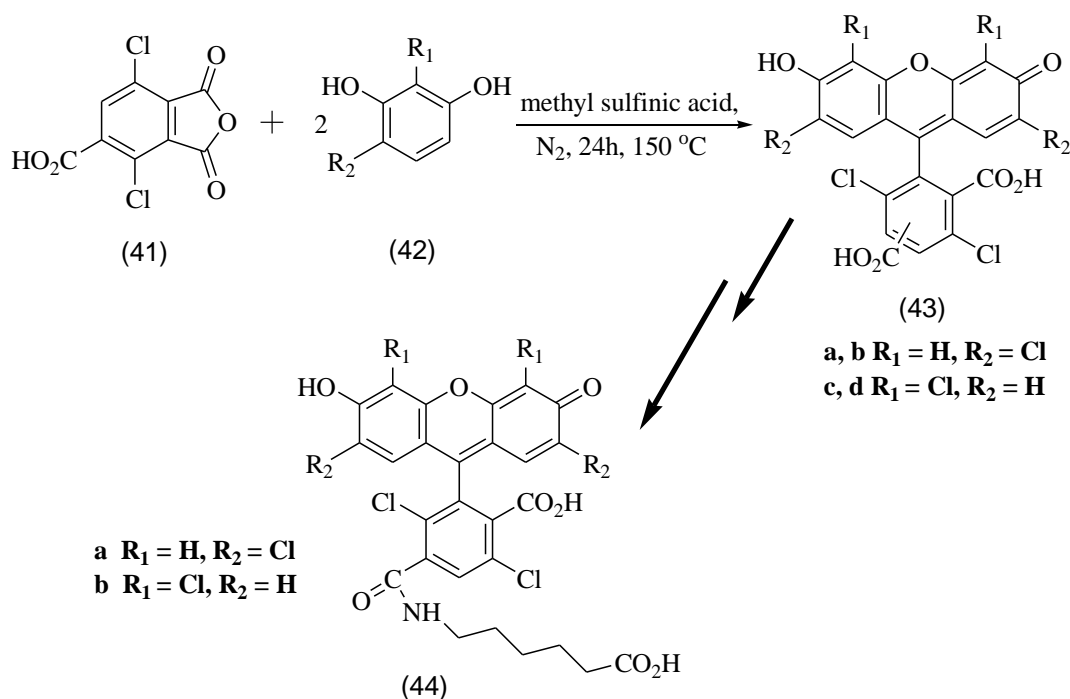
The broadening of the emission spectra and a drop in quantum yield are most likely to be caused by changes in the D_{2h} molecular symmetry of the xanthene unit present in these compounds, as well as vibrational modes and an enhanced rate of nonradiative internal conversion from excited state to ground state. Loss of D_{2h} symmetry in case of fluorescein monoanion causes a similar broadening of its emission spectrum as well as a comparable drop in its fluorescence quantum yield ($\Phi_F = 0.25 - 0.37$). When compared to 40a, the fluorescence quantum yields of Fmoc-protected amino acids (40b,c) ($\Phi_F = 0.04-0.12$) are found to be prominently lower [81]. This can be the outcome of the Fmoc chromophore-induced fluorescence quenching. In reality, Fmoc-deprotection increases the fluorescence of the compounds (40b,40c) significantly. For compounds (40a-c), the quantum yields and fluorescence maxima are not pH dependent. After being incubated in an aqueous pH 9.8 buffer for a number of hours, fluorophores 40a show no discernible modifications. These fluorophores' pH independence is extremely useful in applications that call for measuring and comparing fluorescence intensity across various conditions. An effective designing of a new type of fluorescent amino acids was proposed by Burchak et al. [88]. The Fmoc-protected dyes can be made from aminofluoresceins and from other affordable commercially available precursors in a four-step process with an overall yield of 30%. The dyes display fluorescence that is pH-independent and favourable for biological applications. These are significantly more photostable than fluorescein. The fluorescent amino acids that have been protected by Fmoc are prepared for usage in solid phase peptide synthesis. They built a fluorogenic substrate for cysteine protease papain to illustrate the applicability of the synthesised fluorophores for the solid phase synthesis of fluorogenic substrates. Fluorescein amino acid and Methyl red (MR) are the chromophores which are separated by the peptide sequence GGFGGLG in the substrate (Scheme 14). This sequence has been regarded as a considerably good papain substrate because of its ability to cleave at the G-L link. Phenylalanine unit at the P2 position and the leucine unit at the P10 location influence papain specificity. The MR dye is commonly employed in the construction of self-quenched oligonucleotide probes for real-time PCR since it has been proven to efficiently quench the fluorescence of fluorescein derivatives. Fmoc-chemistry was used to create the substrate on TentaGel resin [88].



Scheme 14: Representative Structure of Papain Substrate (--- Shows Points of Attachment of Fluorophore)

In case of multicolour imaging, such as high content analysis of living cells, fluorescent probes having finer fluorescence bands have been found useful. Although fluorescein derivatives display broad fluorescence bands, selective substitution of fluoresceins by chlorine atoms have been found to be an efficient method of generating probes with smaller emission bands.

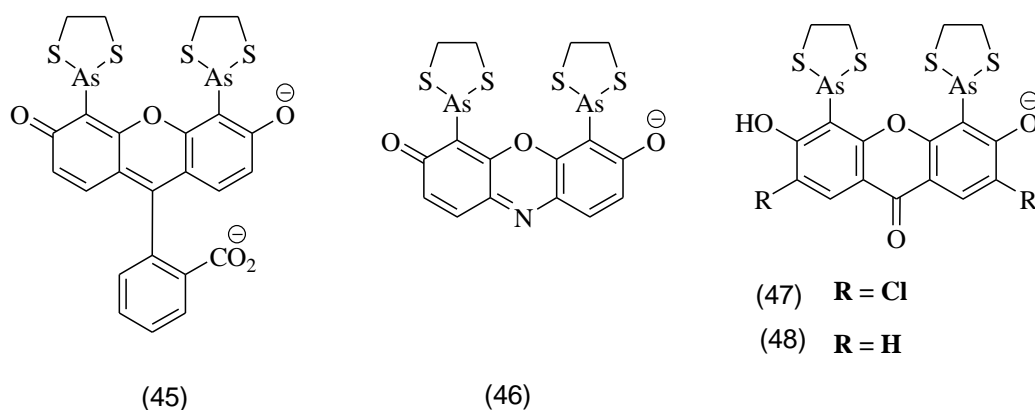
In 2008, Tian et al. reported the synthesis of two chlorinated fluoresceins namely, 4,7,2',7'-tetrachloro-6-(5-carboxypentyl)fluorescein (44a) and 4,7,4',5'-tetra-chloro-6-(5-carboxypentyl)fluorescein (44b) as fluorescent probes for labelling of protein (Scheme 15) [89]. As a spacer linker, these two fluoresceins include 6-aminohexanoic acid. Even at quite high degrees of labelling, the fluorescence possessions of protein conjugates generated from compounds 44a and 44b are not significantly diminished compared to those fluorescein molecules that do not contain this type of linker. Furthermore, compared to nonchlorinated fluoresceins, these fluorophores are found to be more photostable. Even, in the physiological pH range, these compounds are basically pH insensitive. Such spectral possessions make the new chlorinated fluoresceins appealing fluorophores for a wide range of biological applications.



Scheme 15: Synthetic Designing of Two Chlorinated Fluoresceins, 44a and 44b

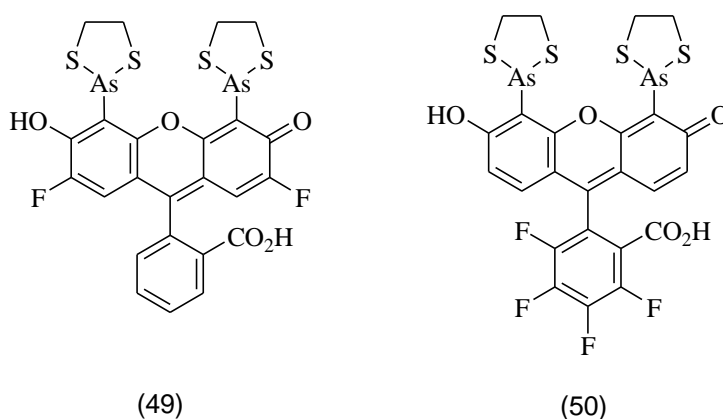
V. BIARSENICAL DYE BASED FLUOROPHORES

Griffin et al. pioneered the use of biarsenical dyes for the *in vivo* labelling of target peptides or proteins with tiny fluorescent dyes (Scheme 16) [90]. A fluorescent dye containing two arsenic moieties binds to a genetically encoded pattern of four cysteines in the sequence Cys-Cys-Xaa-Xaa-Cys-Cys with high affinity in this approach (where Xaa represents any amino acid other than cysteine) [91]. Two As(III) substituents in the cell permeable fluorescent dye FlAsH [4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein] (45) pair with the four cysteine thiol groups in the motif. As the dye binds to the motif, its fluorescence intensity rises.



Scheme 16: Representative Structures of Biarsenical Dyes 41-44

Though fluorescein derivatives were the original FAsH dyes but other cell-permeable biarsenical dyes, such as ReAsH (46) ($\lambda_{\text{ex}} = 593 \text{ nm}$ and $\lambda_{\text{em}} = 608 \text{ nm}$), a phenoxazine derivative, and CHoXAsH (47) ($\lambda_{\text{ex}} = 380 \text{ nm}$ and $\lambda_{\text{em}} = 430 \text{ nm}$), a xanthene derivative related to the FAsH compounds, are also available. It is worthwhile to mention that they have distinct optical properties (Scheme 16). In comparison to other conventional approaches, the binding and the membrane permeability capabilities of these biarsenical dyes offer substantial advantages for in-cells labelling. Spagnuolo et al. studied the interaction of biarsenical dyes with visible fluorescent proteins (VFPs) as the FRET donor-acceptor (DA) pairs [92]. The authors developed fluoro-substituted versions of fluorescein derivatives, F2FAsH (49) and F4FAsH (50) (Scheme 17), which were found to be significantly more effective in these areas than the original fluorescein derivative FAsH (45). This is especially due to the inadequate photostability as well as pH sensitivity of such fluorescein derivatives in the physiological range [93].



Scheme 17: Representative Structures of Fluorinated Fluorescein Biarsenical Dyes 45 and 46.

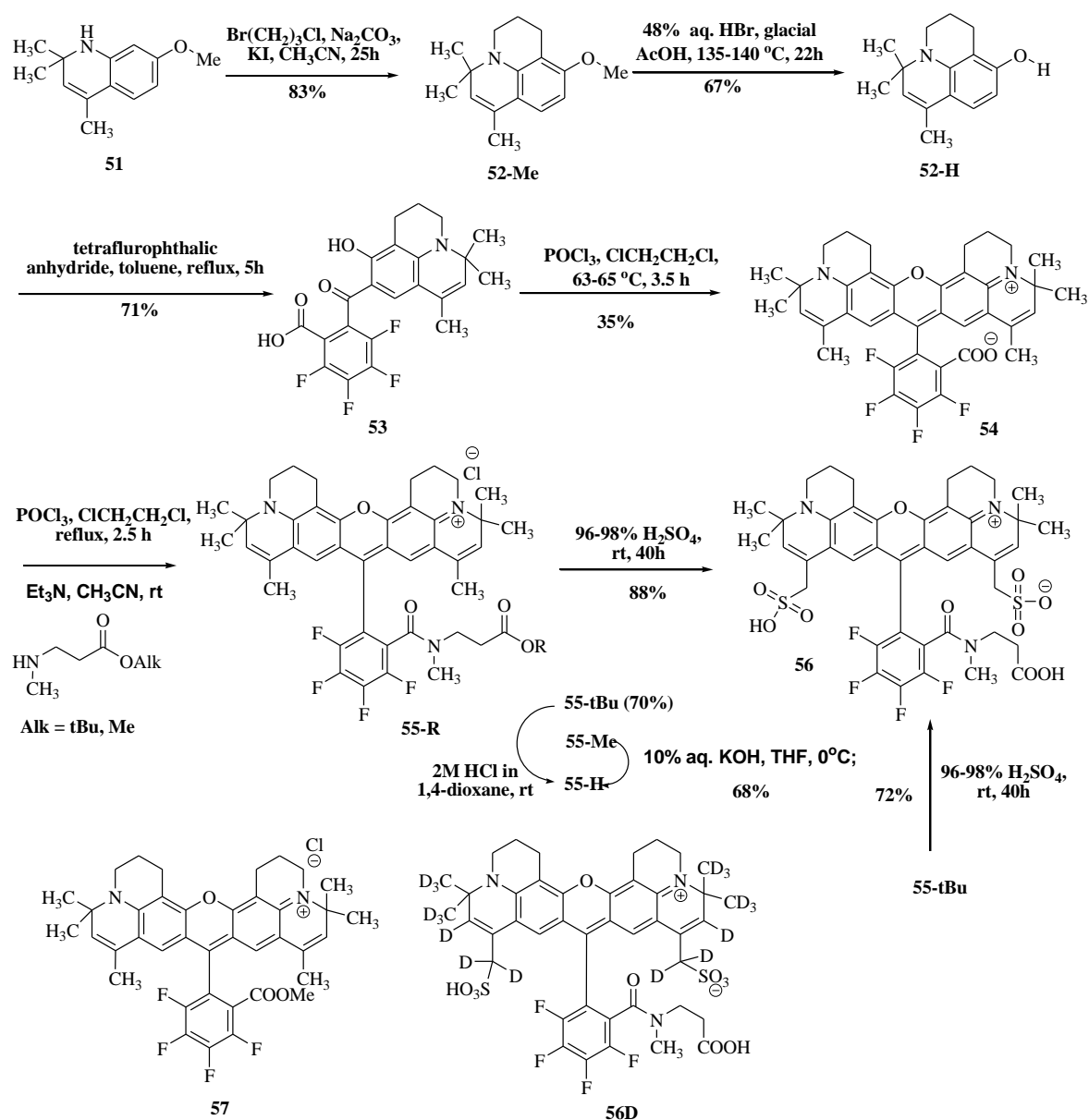
In comparison to FAsH (45), F2FAsH (49) possesses a comparatively higher absorbance along with a larger Stokes' shift and a higher fluorescence quantum yield. Even F2FAsH (49) shows a higher level of photostability and a lower pH dependence. By emitting F4FAsH (50) in the wavelength range between FAsH (45) and ReAsH (46), a new colour with exceptional luminosity is produced [93].

VI. RHODAMINE DYE BASED FLUOROPHORES

Rhodamine dyes, which are part of the xanthene dye family, are some of the first synthetic dyes used to dye fabrics. Many of their derivatives are very fluorescent, and they typically show high molar absorptivities in the visible region of the spectrum [13,16,65]. The substituents on the xanthene nucleus put a significant impact on the absorption and emission properties. Rhodamine based dyes find applications as laser dyes, photosensitizers as well as fluorescent markers in structural microscopic studies in addition to being used as colourants [94]. These are also used as fluorescent markers for labelling biologically active units including proteins, nucleic acids, lipids, carbohydrates, hormones etc.. This is especially owing to their high photostabilities, extinction coefficient, fluorescence quantum yields and low degree of triplet formation. Rhodamines are proven useful in the hands-on enactment of new physical principles, for example those that can assist in overcoming the diffraction limit

in (far-field) optical microscopy, in addition to their application in biological imaging and single-molecule-based spectroscopy. It is well known that STED, PALM, STORM and GSDIM allow optical resolution to be improved. For example, from a range of 200 nm to 350 nm to a range of 20 nm to 35 nm by switching between the dark and bright states of a fluorescent marker [95]. The rhodamine family, on the other hand, lacks water-soluble derivatives with high photostabilities and fluorescence quantum yields. Most fluorescent dyes which absorb in the far-red or even in the near-infrared optical range are preferred for a diverse biological applications. In this aspect, it is worthwhile to mention that, most of the derivatives from rhodamine family lack their absorbance and fluorescence band maxima in the far-red optical range. Excitation at wavelengths greater than 600 nm, in contrast to visible and especially ultraviolet, is essentially noninvasive and reduces the undesired background signal caused by cellular autofluorescence.

Red-emitting fluorescent markers are particularly useful in biological imaging because they reduce cellular autofluorescence and expand the scope of multicolor investigations. Kolmakov et al. engineered unique rhodamine dyes that can be excited by 630 nm laser light and emit roughly around 660 nm [95]. Such novel rhodamines are very photostable, with up to 80% fluorescence quantum yields, longer excited state lifetime (3.4 ns). The rates of intersystem-crossing are also comparatively low. They excel at both conventional and subdiffraction-resolution microscopy, for example STED, GSDIM. Even in various single-molecule experiments like FCS. The authors disclosed the synthesis of lipophilic (55,57) and hydrophilic (56) derivatives from the identical chromophoric scaffold. The addition of two sulfo groups increases water solubility and fluorescence quantum yield. Incorporation of reactive amino or thiol groups permits the dyes to be utilised as fluorescent markers in biology. Table 4 lists the spectroscopic possessions of the synthetically designed fluorescent dyes [95].

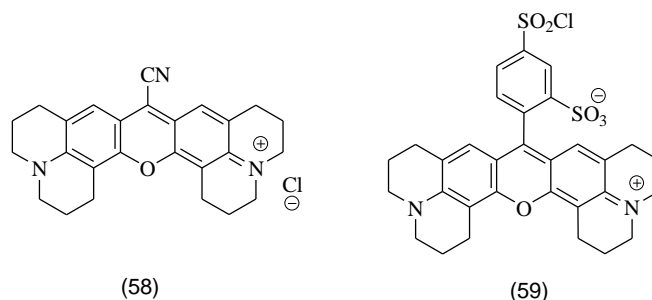


Scheme 18: Synthesis of Lipophilic (55,57) and Hydrophilic (56) Derivatives of Rhodamine

Table 4: Spectroscopic Possessions of the Synthetically Designed Fluorescent Dyes

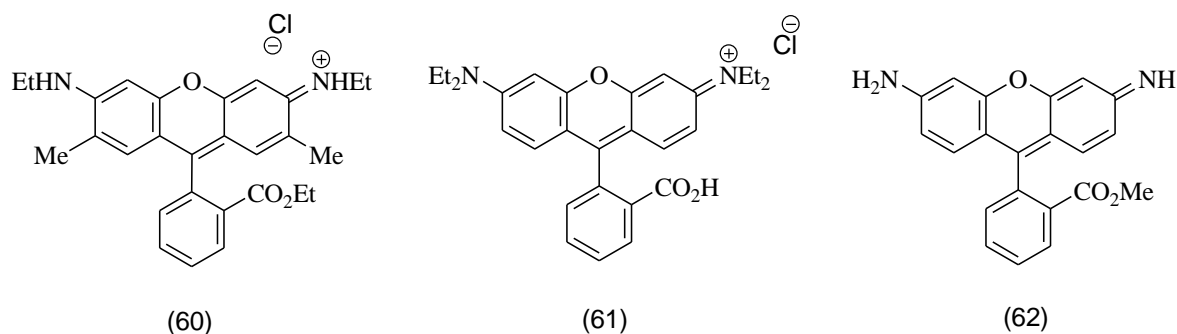
Compound	λ_{\max} (abs)	λ_{\max} (fl.)	Solvent	ϵ [$10^{-5} \text{M}^{-1} \text{cm}^{-1}$]	Φ_{fl} [%] in H ₂ O	τ_{fl}	$K_{\text{ISC}/T}$ [10^{-6}s^{-1}]
54	616(nm)	641(nm)	MeOH	-	63	-	-
57	632(nm)	655.5(nm)		-	-	-	-
55- <i>t</i> Bu	638(nm)	661(nm)		0.92	61	-	-
55-Me	638(nm)	661.5(nm)		0.66	62	-	-
55-H	638(nm)	661.5(nm)		0.73	53	3.6(ns)	-
56	637(nm)	660(nm)	H ₂ O	0.94	80	6.4(ns)	2.5/0.3
56D	637(nm)	660(nm)		0.92	78	6.4(ns)	2.5/0.3

Rhodamine 800 (58) and Texas Red (59) are two recent rhodamine compounds employed primarily in bioanalysis (Scheme 19) [96]. Horneffer et al. employed Texas Red as a fluorescent marker for localising proteins in MALDI preparations using confocal laser scanning microscopy [97]. This fluorophore's low pH dependence outperformed the possible fluorescence quenching in the acidic surroundings of standard MALDI matrices like 2,5- and 2,6-dihydroxybenzoic acids.

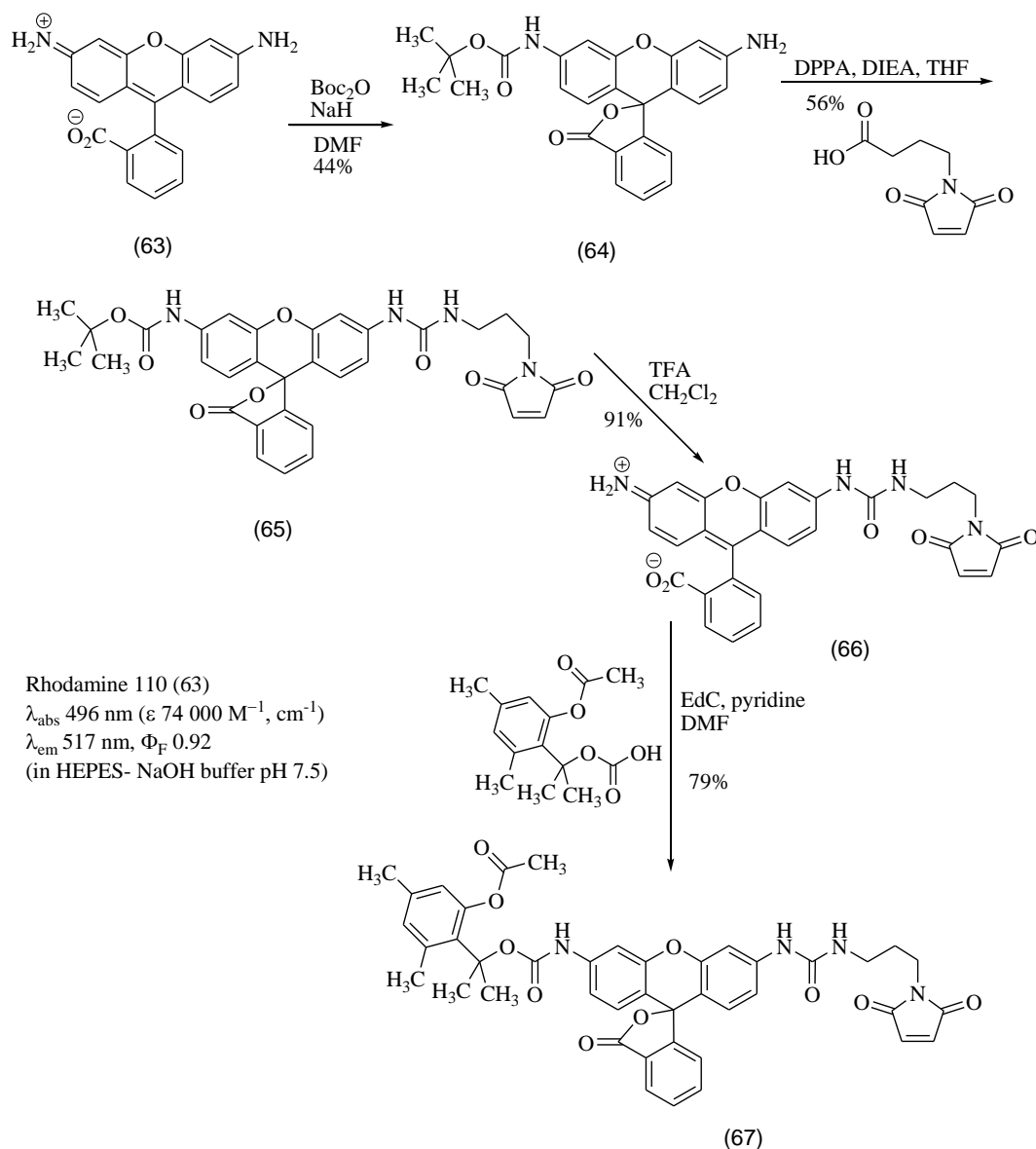


Scheme 19: Representative structures of Rhodamine 800 (54) and Texas Red (55)

Other xanthene based fluorophores used as fluorescent probes in biological research include Rhodamine 6G (60), Rhodamine 123 (61), and Rhodamine B (62) (Scheme 20). Rhodamine 110 (63) is also utilised in experiments related to enzymatic activity [98]. Lavis et al. reported the designing of a flexible 'latent' fluorophore 67 (Scheme 21), a Rhodamine derivative in which one of the nitrogen atoms is converted into urea and the other as a 'trimethyl lock' [99]. The first modification reduced rhodamine's half-fluorescence while enabling conjugation with the target. The second alteration allowed fluorescence to be completely unmasked by a single user-specified chemical transformation.

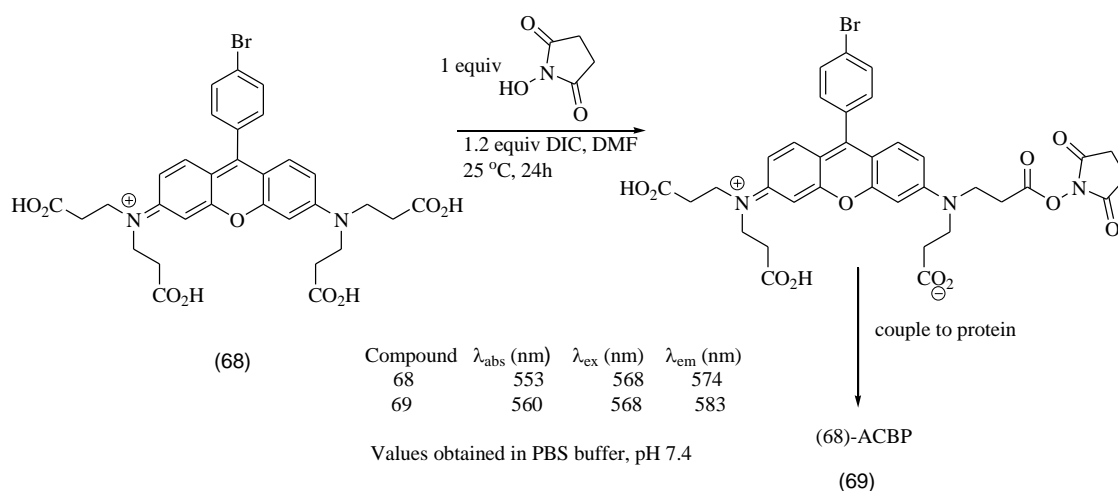


Scheme 20: Representative structures of Rhodamine 6G (60), Rhodamine 123 (61) and Rhodamine B (62)



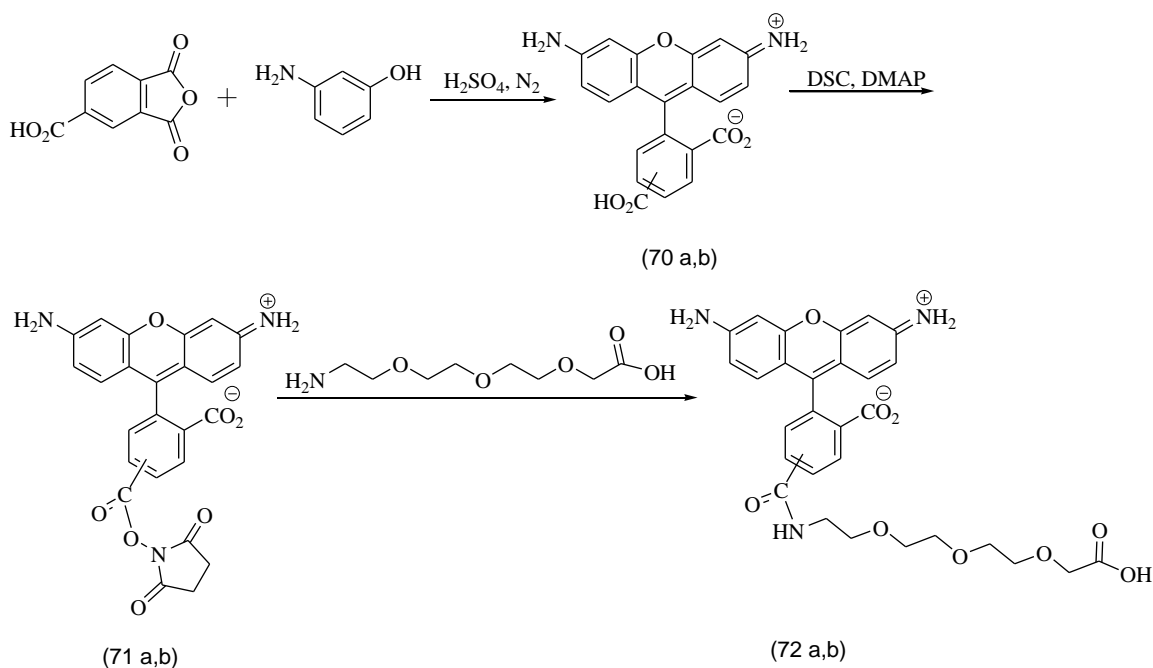
Scheme 21: Synthetic Route towards the Development of Fluorogenic Label, 67

Bandichhor et al. designed a novel fluorescent rhodamine derivative 68 in 2006 as well (Scheme 22) [100]. This probe differs from previous rhodamines with respect to the fact that it has four separate carboxylic acid functions to aid in protein conjugation and increase water solubility. Additionally, it has an aryl bromide activity that might be exploited in palladium-catalyzed processes (such as Suzuki and Sonogashira couplings) to connect additional molecules to the fluorescent dye. Dye 68 was linked to an ACBP (acyl-CoA binding protein) model protein. The features of this compound were investigated to ensure that the label did not considerably modify the protein's binding utility to its natural ligand *in vitro* and that its secondary structure was not dramatically altered. Other experiments demonstrated the new Chariot-peptide carrier system could import 68-ACBP (69) into live cells, where it accumulates, at least partially, in the nucleus. As a result, conjugate 69 can now be used in *in vivo* research.



Scheme 22: Fluorescent Labelling of the ACBP Protein using dye (68)

It is critical to determine the extent to which rhodamine-based fluorescent dyes influence the behaviour of target molecules when used as target molecules in biological processes. The homogeneity of the change in the target molecules is crucial if such a shift does in fact take place. The possible interaction between the rhodamine and the pharmacophore of melanocortin receptors was minimised by using a 6-aminohexanoic acid linker. Gao et al. studied the insertion of a linker molecule (11-amino-3,6,9-trioxaundecanoic acid) to 5- or 6-carboxy-rhodamine 70 (Scheme 23) in 2007 in order to reduce such potential steric issues [101]. The efficiency of this probe was tested by labelling goat-antimouse IgG and detecting alphasubunit in endothelial cells of a bovine pulmonary artery. Compounds 72a and 72b were found to be excellent green fluorescent probes, with strong fluorescence and good biocompatibility, and useful as markers for a range of proteins and other biopolymers.



Scheme 23: Synthesis of Rhodamine Derivatives 72a and 72b

VII. CONCLUSION AND FUTURE PERSPECTIVE

Over the past 30 years, the development and use of fluorescent probes based on oxygen heterocycles has advanced extraordinarily. Amongst such oxygen heterocycles, many fluorophores have been designed throughout the years and used in the imaging of biomolecules, cells, and organisms. The discovery of appropriate fluorescent labelling dyes based on oxygen heterocycles that play a crucial role in enabling the technologies has always been accompanied by the introduction of novel imaging platforms, such as in vivo NIR imaging, super-resolution imaging and multiphoton imaging. Despite the inherent advantages of the fluorophores discussed in this chapter, significant effort has to be given to enhance particular qualities or overcome some of their limitations. Furthermore, chemists can develop activatable fluorescent probes based on oxygen heterocycles since the fluorescence attributes of such organic compounds can be logically modulated via a variety of techniques.

Even though there are known organic fluorophore labels that emit electromagnetic radiation from the UV region to the NIR region, longer wavelength probes still have some limitations even though these are crucial for many biological applications. As a result, there is a dire need for novel fluorophores or derivatives of existing fluorophores with enhanced water solubility, where the excitation and emission maxima are located beyond approximately 600 nm and the fluorescence quantum yields are high. There is also an absolute need that these molecules include a functional group capable of effective covalent attachment to biomolecules.

VIII. ACKNOWLEDGEMENTS

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Abbreviations used in this chapter:

PNAs: peptide nucleic acids	Cys: cysteine	Gly: glycine
Glu: glutamic acid	τ : fluorescence lifetimes	Val: valine
BSA: bovine serum albumin	HOBt: 1-hydroxybenzotriazole	Phe:
phenylalanine		
PBS: phosphate-buffered saline	Boc ₂ O: di- <i>tert</i> -butyl pyrocarbonate	NIR: near-infrared
NHS: <i>N</i> -hydroxysuccinimide	DIC: diisopropylcarbodiimide	MeOH: methanol
Φ_F : fluorescent quantum yield	$\Delta\lambda$: Stokes' shift in nm	EDT:
ethanedithiol		

ϵ : molar absorptivity ($M^{-1} \text{ cm}^{-1}$)	HOSu: <i>N</i> -hydroxyssuccinimide	Pro: proline
SPPS: solid-phase peptide synthesis	HSA: human serum albumin	Lys: lysine
DIEA: diisopropylethylamine	LC: liquid chromatography	Ala: alanine
TIS: triisopropylsilane	DPPA: diphenylphosphoric azide	<i>t</i> Bu: <i>tert</i> -butyl

STORM: stochastic reconstruction microscopy

STED: stimulated emission depletion

PALM: photoactivation localization microscopy

GSDIM: ground-state depletion with individual molecular return

MALDI: matrix-assisted laser desorption/ionization

FRET: fluorescence resonance energy transfer

TRIS: tris(hydroxymethyl)aminomethane

λ_{abs} : wavelength of maximum absorption

λ_{em} : wavelength of maximum emission

λ_{ex} : wavelength of maximum excitation

$\Delta\nu$: Stokes' shift in cm^{-1}