En Route to Fluorophores Based on Oxygen Heterocycles

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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. Despite the fact that many compounds are known, the ensemble of fluorescence probes is built on a small number of modular ‘core’ dyes. The development of these dyes with various chemical moieties allows for the accurate probing of biochemical and biological systems. Understanding the primary categories of small-molecule fluorophores is essential, given the significance of fluorescence-based technologies in chemical biology. Here, we look at the chemical and photophysical characteristics of popular 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

Contents

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. Light-emitting diodes, diagnostics, in vivo imaging and high throughput screening and are just a few of the bioanalytical uses for fluorescently labelled heterocyclic compounds. These substances have 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. Both the steady-state and time-resolved fluorescence spectroscopic techniques are now regarded as being primarily researched tools in biochemistry and biophysics. Since fluorescence detection is sensitive, most biochemical measurements no longer require the expensive and challenging handling of radioactive tracers. Nowadays, flow cytometry, genetic analysis, medical diagnostics, DNA sequencing, forensics, and biotechnology are just a few fields that use fluorescence significantly. Fluorescence has seen a sharp increase in use for imaging cells and molecules. 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 fluorescent probes are molecules whose fluorescence emission changes in response to a binding event, chemical reaction, or change in their immediate environment. Gradually, they 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

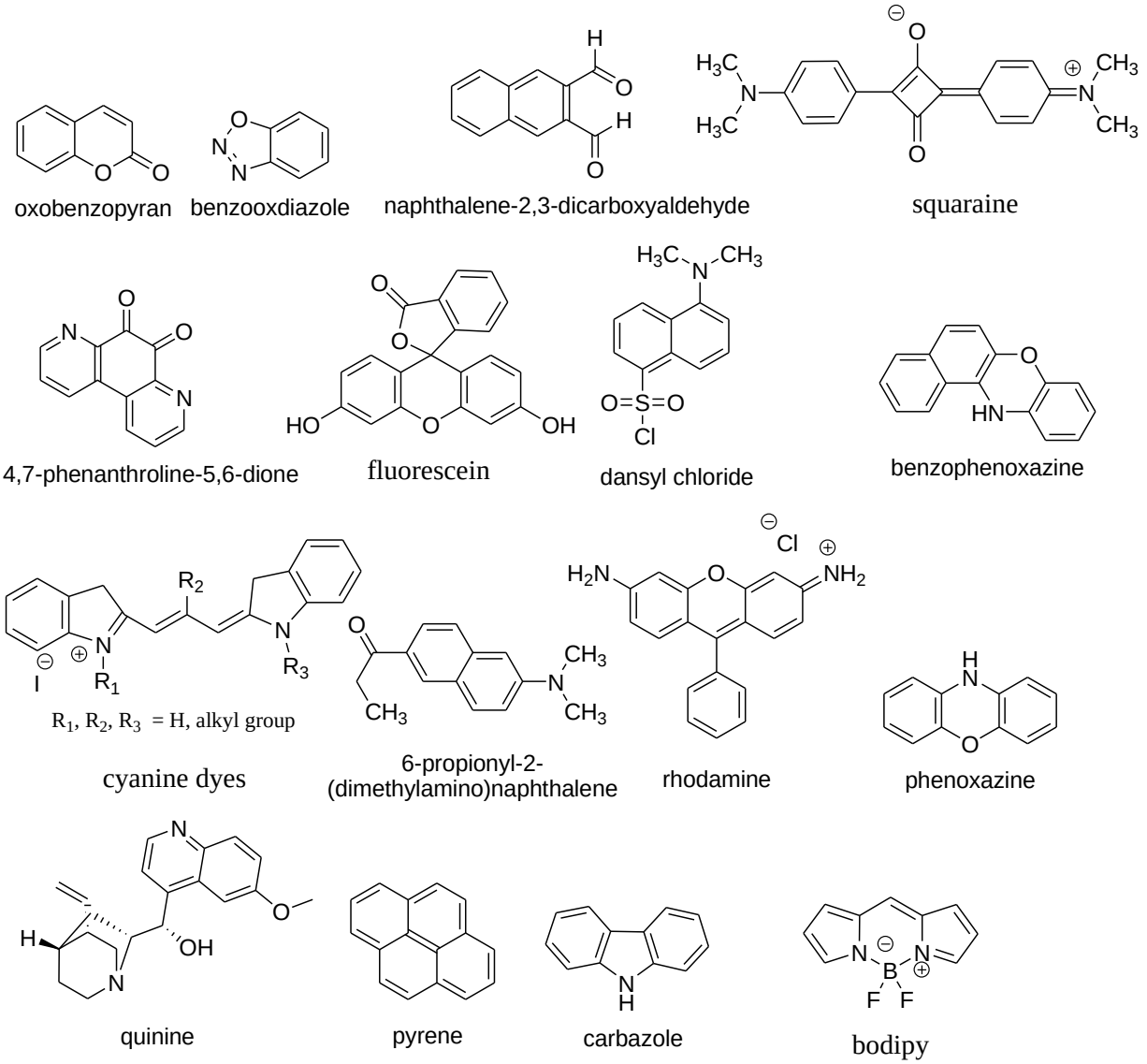
began as a small group in the late 20th century and has now grown to include more than 150 research groups globally. The entry of daring scientists from numerous other domains was necessary for this expansion [5-8].

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 absorbance-based dye or radioactive labelling, fluorescent labelling is one of the most in-depth and sensitive bioanalytical methods for examining biomolecules. Although some biomolecules, such as aromatic amino acids, NADH, and flavins, are intrinsic fluorophores, labelling with organic fluorescent dyes improves sensitivity and visibility [9,10]. This field is made more promising by the availability of fluorophores in a wide spectrum of wavelengths and substantial advancements in fluorescence tools. Fluorescent dyes can be linked to biomolecules via covalent or noncovalent bonds. Aside from DNA, proteins, peptides, and amino acids are the principal macromolecules that can be seen. Organic synthetic approaches are used to fine-tune fluorescent dyes for water solubility, photo stability, high quantum yields, prolonged conjugation, and integration of a variety of reactive functional groups based on the analyte.

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 quest for newer ones is still ongoing because they are crucial for understanding biological processes.

Fluorescent dyes used for labelling from near ultraviolet to 500 nm wavelength range are oxobenzopyrans, benzooxdiazoles, dansyl chloride, naphthalene-2,3-dicarboxyaldehyde (Scheme 1). From 500 nm to the near-infrared spectrum, the fluorescent dye classes that are most frequently employed for bio-labelling are fluorescein, rhodamine, bodipy, suqarines, and

cyanines (Scheme 1). Fluorophores based on coumarins, oligothiophenes, phanquinones, and benzooxadiazoles have been described for tagging biomolecules in the emission range up to 500 nm. In the emission region below 500 nm, naphthalene-based fluorophores are also employed for bimolecular labelling. Fluorescein, rhodamine, and BODIPY fluorescent dyes are the best options for bimolecular labelling in the wavelength range 500-700 nm, where issues with autofluorescence from biomolecules are negligible (Scheme 1). The dyes mentioned above have high molar extinction coefficients and quantum yields [4].

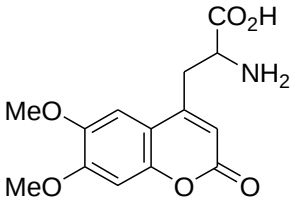


Scheme 1: A few reputable fluorescent dyes used for labelling study.

The synthesis of their water soluble derivatives is also simple. The researchers are drawn to employ them as labelling dyes because of this. Near-infrared (NIR) fluorescent labelling dyes include squaraines and cynine 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 groups of reagents for fluorescence derivatization is 3-oxo-3H-benzopyrans, also known as coumarins [11,12]. Many fluorophores of this sort are reported in the literature, and an unusually large number of articles allude to carboxylic acid compound determination employing such labels. Due to their wide spectrum range, strong emission quantum yields, photostability and good solubility in a variety of solvents, modified 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 a high fluorescence quantum yield (Φ_F = 0.52 and ε = 10,900 M^{-1} cm^{-1}, respectively), which enable the detection of the labelled peptides on a picomolar scale with a sensitivity comparable to radiolabeling.

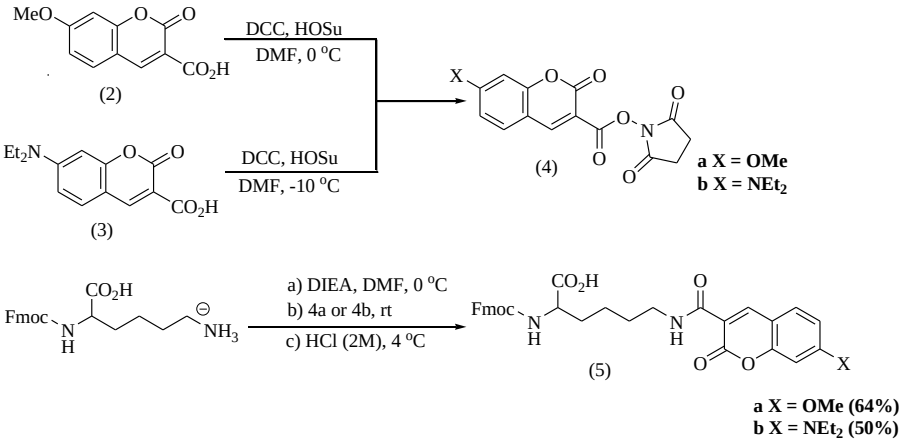


λ_{abs} = 345 nm
ε = 10 900 $M^{-1}cm^{-1}$
λ_{em} = 440 nm
Φ_F = 0.52

Dmca (1)

Scheme 2: Representative structure of Dmca (1)

Berthelot et al. synthesised two new oxobenzopyran-labeled lysines. Carboxylic acids (2) and (3) were activated via the *N*-hydroxysuccinimide ester and coupled with Fmoc-Lys-OH using familiar techniques (Scheme 3) [45-48].

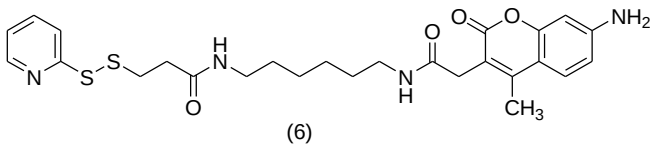


Scheme 3: Synthesis of two new oxobenzopyran-labeled lysines.

Compounds 5a and 5b have absorption and emission maxima (abs/em) at wavelengths of 350/404 and 431/480 nm, respectively. The lysine residue was linked to heterocycles (2) and (3), which caused red shifts in the absorption values of 15 and 24 nm, respectively. The labelled lysine derivatives 5a and 5b was proposed by the authors as a fluorescent building block for 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 thiol side chain of cysteine residues in proteins and peptides, a process known as S-nitrosylation. Certainly, an essential switch in controlling protein functions is protein S-nitrosylation, which is fundamentally the alteration of a cysteine thiol group in a protein by a nitrosyl [NO] group. Several proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [51,52], protein disulfide isomerase (PDI) [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-labeled cysteines [56]. In the labelling phase, AMCA-HPDP (6) (Scheme 4) was utilised. The S-nitrosylated proteins could then be easily identified as vivid blue bands under ultraviolet light when the labelled proteins were subjected to non-reducing SDS-PAGE analysis. The method offered a precise identification of nitrosocysteines with the AMCA adduct as the label when paired with liquid chromatography- tandem mass spectrometry (LC-MS/MS).

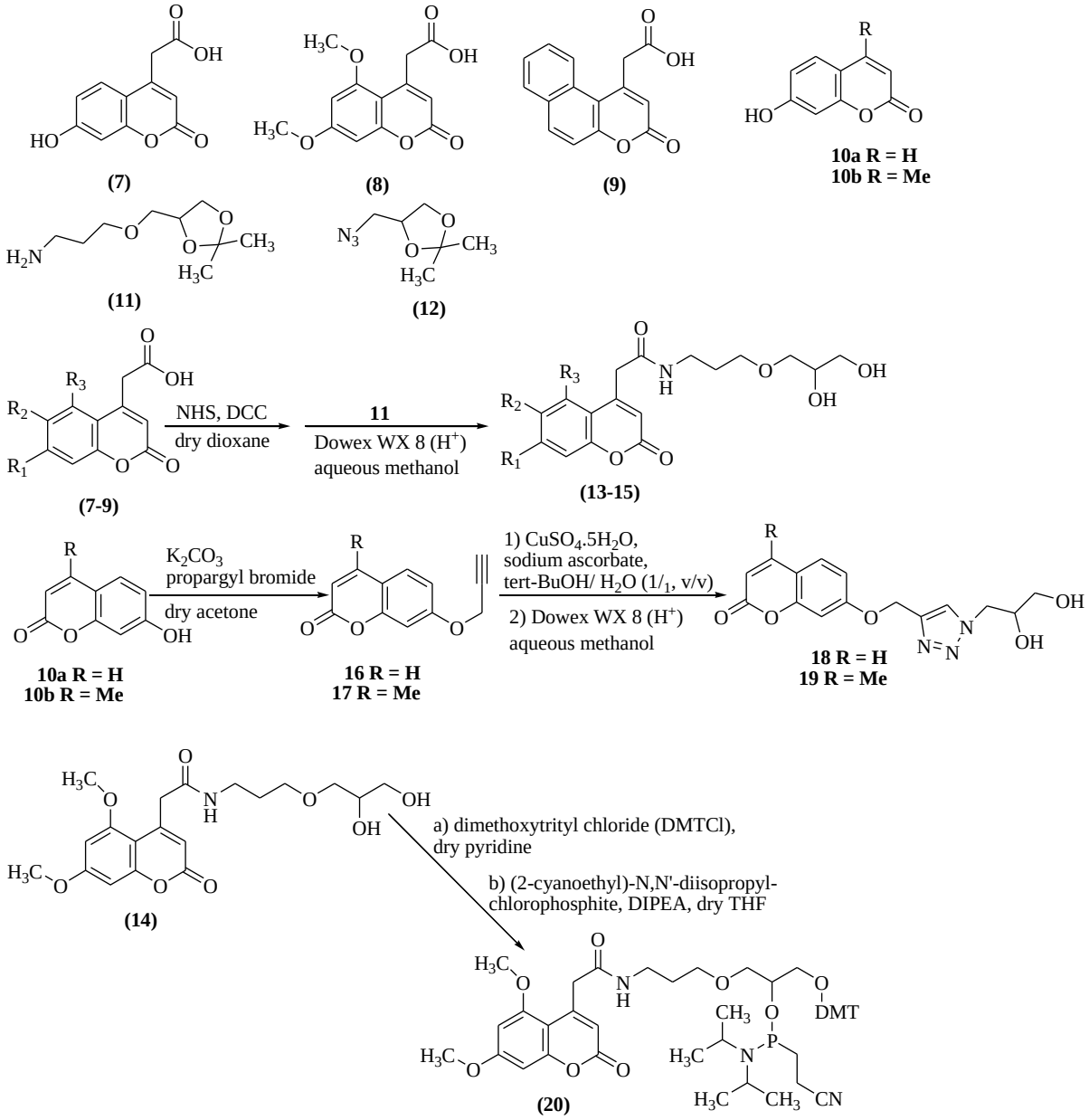


(6)

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

Ivana and Pavol described the development of novel fluorescent probes for site-specific insertion into oligonucleotides [57]. As sensitive fluorescent labels, oxobenzopyran derivatives (7-10) were employed, and two linkers (11 and 12) were added to the basic oxobenzopyran skeleton to form the functionalized derivatives (13-15, 18, and 19) (Scheme 5). Commercially

accessible hydroxy oxobenzopyran derivatives 10a and 10b, as well as carboxylic acid heterocycles 7-9, were synthesised using the famous Pechman process [58].



Scheme 5: Synthesis of oxobenzopyran-based fluoroscent probes 13-15, 18 and 19.

In methanol, the optical characteristics of compounds 7-10, 13-15, 18, and 19 were examined (Table 1). Conjugates 13-15 and 18-19 displayed emission peaks between 381 and 426 nm, with fluorescence quantum yields ranging from 0.02-0.30. Compound 14 was chosen for the

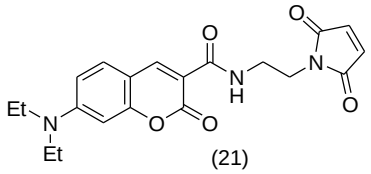
production of a benzopyranylphosphoramidite linker 20 appropriate for direct insertion into oligonucleotides due to its superior fluorescence characteristics.

Table 1: Photophysical possessions of a few fluorophores related to Scheme 5.

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

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

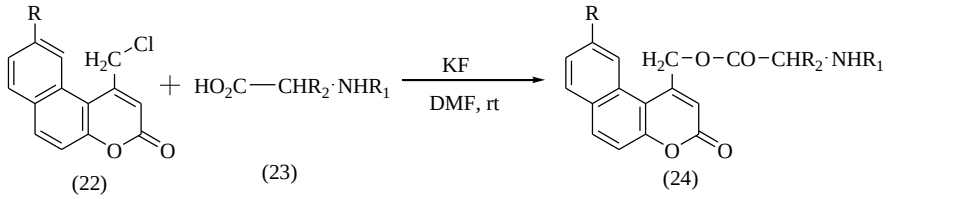
Shutes et al. described labelling phosphate-binding protein (PBP) with fluorophore, MDCC (21) and used it to measure GTPase activity (Scheme 6). The MDCCPBP probe makes it simple to examine GTP hydrolysis by GTPases in vitro and in real time [61].



(21)

Scheme 6: Representative structure of MDCC (21).

Recently, functionalized oxobenzo[f]benzopyrans 22a–c were successfully employed to prepare a number of fluorescent *R*–amino acid derivatives (24a–g) (Scheme 7) [62]. The resulting fluorescent conjugates (24a-g) exhibited strong Stokes' shifts (66-131 nm), with λ_{abs} values between 345 and 360 nm and λ_{em} values between 411 and 478 nm (Table 2).



a R = H
b R = OH
c R = OCH_3

a R_1 = Z, R_2 = CH_2Ph
b R_1 = Z, R_2 = H
c R_1 = Z, R_2 = CH_3
d R_1 = Z, R_2 = $CH(CH_3)_2$
e R_1 = Tos, R_2 = CH_2Ph

a R = H, R_1 = Z, R_2 = CH_2Ph
b R = OH, R_1 = Z, R_2 = CH_2Ph
c R = OCH_3, R_1 = Z, R_2 = CH_2Ph
d R = OCH_3, R_1 = Z, R_2 = H
e R = OCH_3, R_1 = Z, R_2 = CH_3
f R= OCH_3, R_1 = Z, R_2 = $CH(CH_3)_2$
g R = OCH_3, R_1 = Tos, R_2 = CH_2Ph

Z = Benzyloxycarbonyl
Tos = *p*- Toluenesulphonyl

Scheme 7: Synthesis of Fluorescent Conjugates 24a-g.

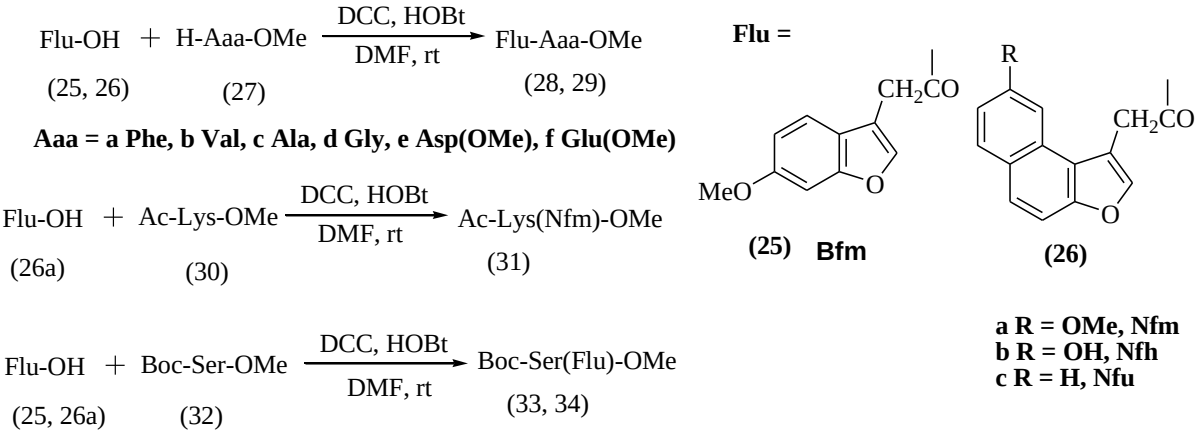
Table 2: Photophysical possessions of compounds 22a-c and 24a-g in ethanol.

Compound	**λ_{abs}, nm (ε, M^{-1} cm^{-1})**	**λ_{em} (nm)**	**Φ_F**	**$\Delta\lambda$ (nm)**	**Compound**	**λ_{abs}, nm (ε, M^{-1} cm^{-1})**	**λ_{em} (nm)**	**Φ_F**	**$\Delta\lambda$ (nm)**
22a	352 (11, 449)	418	0.08	66	24c	347 (12, 075)	478	0.59	131
22b	361 (12, 190)	462	0.02	101	24d	347 (11, 436)	471	0.70	124
22c	354 (12, 826)	472	0.03	118	24e	348 (11, 640)	477	0.66	129
24a	345 (14, 125)	411	0.42	66	24f	348 (11, 830)	478	0.58	130
24b	360 (10, 174)	456	0.13	96	24g	347 (12, 883)	475	0.53	128

Heterocycles 22a–c had modest Φ_F values (0.02-0.08), which sharply rose in response to the amino acids (Φ_F = 0.13-0.70, 24a–g). The heterocycles 22a-c were proposed as prospective candidates for the fluorescent labelling of peptides and other biomolecules on the basis of their photophysical possessions.

III. Benzofuran and naphthofuran based fluorophores

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



Scheme 8: Synthetic labelling of amino acid derivatives.

Table 3 clearly indicates that the 8-methoxy-naphto[2,1-b]furan-1-yl ethanoic acid (26a) was discovered to be the most suited derivative for labelling purposes after taking into account the photophysical data of the fluorescent amino acid residues (28a,b, 29a-h, 31, 33 and 34). The absorption and emission maxima of the amino acid-naphthofuran conjugates (29a-h, 31, and 34) were around 298 and 325-350 nm, respectively; the fluorescence quantum yields ranged between 0.10 and 0.44.

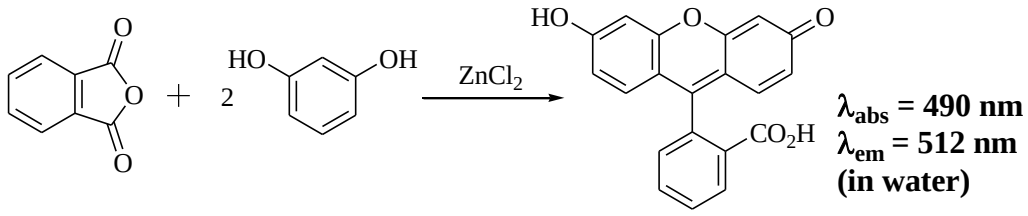
Table 3: Photophysical possessions of compounds 25, 26a-c, 28a,b, 29a-h, 31, 33 and 34 in ethanol

Compound		**λ_{abs} (nm)**	**λ_{em} (nm)**	**Φ_F**	**λ (nm)**	**Compound**		**λ_{abs} (nm)**	**λ_{em} (nm)**	**Φ_F**	**λ (nm)**
25	Bfm-OH	285	315	0.020	30	29d	Nfu-Val-OMe	292	325	0.13	33
26a	Nfm-OH	298	349	020	51	29e	Nfm-Ala-OMe	298	349	0.24	49
26b	Nfh-OH	301	349	0.062	48	29f	Nfm-Gly-OMe	297	343	0.24	46
26c	Nfu-OH	293	340	0.076	47	29g	Nfm-	298	346	0.14	48

							Asp(OMe)-OMe				
28a	Bfm-Phe-OMe	288	315	0.064	27	29h	Nfm-Glu(OMe)-OMe	298	347	0.14	49
28b	Bfm-Val-OMe	288	315	0.070	27	31	Ac-Lys(Nfm)-OMe	297	347	0.44	50
29a	Nfm-Phe-OMe	298	349	0.32	52	33	Boc-Ser(Bfm)-OMe	287	314	0.064	27
29b	Nfm-Val-OMe	298	346	0.37	49	34	Boc-Ser(Nfm)-OMe	298	349	0.13	51
29c	Nfh-Val-OMe	300	350	0.10	50						

IV. Fluorescein 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 it from phthalic anhydride and 1,3-dihydroxybenzene (resorcinol) in the presence of zinc chloride (Scheme 9). As an alternative, the catalyst might be methanesulfonic acid [67-69].



Fluorescein (35)

Scheme 9: Representative synthesis of fluorescein (35).

This polycyclic fluorophore exhibits absorption and fluorescence maxima in the visible area of the electromagnetic spectra (λ_{abs} 490 nm and λ_{em} 512 nm, in water) and this is considered as one of the most popularly used labels in 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

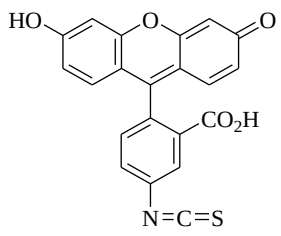
fluorescent label along with a significant application in confocal laser-scanning microscopy and flow cytometry [67,68]. Fluorescein also has the benefit of an excitation maximum at 494 nm, which is close to the 488 nm spectral line of the argon laser.

Despite the fact that there are several commercially available fluorescent probes for labelling proteins through either covalent or noncovalent interactions, the most popular and commonly employed fluorophore for this purpose is an amine-reactive probe produced from fluorescein. 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. Fluorescein's emission spectrum overlaps significantly with the absorption spectra of tetramethyl rhodamine, a related fluorescent dye, making this pair ideal for energy transfer experiments to estimate distances within and between labelled macromolecules. Additionally, fluorescein-based protein conjugates are not highly vulnerable to precipitation, making it possible to acquire them at high purity levels. Fluorophores made from fluorescein and their macromolecular conjugates do, however, have some drawbacks, including (i) a fairly high rate of photobleaching; (ii) fluorescence dependence on pH; (iii) a relatively broad fluorescence emission spectrum, which limits their efficacy in multicolor applications; and (iv) a tendency to self-quench on conjugation to biopolymers, particularly at high degrees of substitution. Quantitative analysis with fluorescein is challenging because to its photobleaching and pH sensitivity. Fluorescein exists in cationic, neutral, anionic, and dianionic forms in aqueous solution rendering its absorption and fluorescence characteristics pH dependant [69-74].

For applications requiring ultrasensitive detection, such as DNA sequencing, fluorescence in situ hybridization, and the localization of low-abundance receptors, in particular,

photobleaching lowers sensitivity, which is undesirable. Due to these restrictions, fluorescein derivatives with better stability qualities have been developed.

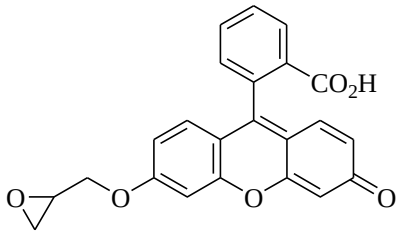
Fluorescein labelling of peptides and proteins is typically accomplished using commercially available chemicals via the amino or thiol groups of the lateral chain of lysine or cysteine residues. In the particular labelling of NH_2 and SH, Succinimidyl and maleidimyl fluorescein derivatives are utilised, respectively [75,76]. Fluorescein isothiocyanate (FITC), however, is the most 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 thiocarbamoyl derivative was formed by reacting casein with FITC. Trypsin, chymotrypsin, elastase, subtilisin, and thermolysin likewise cleave 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 electophoresis was performed after the degree of labelling. A monomeric serum glycoprotein called transferrin binds two ferric ions and transports them to vertebrate cells via receptor-mediated endocytosis. The goal of this study was to track the labelling of transferrin with FITC because the labelled protein can be employed as a receptor mediated endocytosis marker.



Fluorescein isothiocyanate (36)

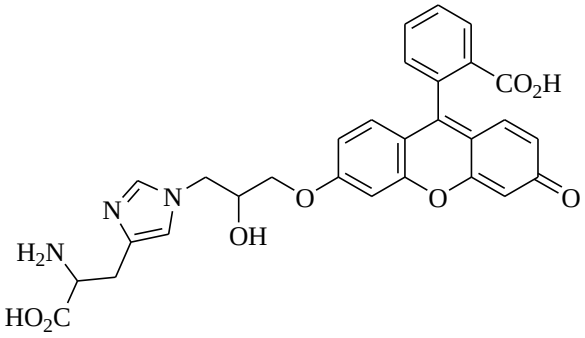
Scheme 10: Representative structure of fluorescein isothiocyanate (FITC) (36).

Li et al. reported the fabrication of a novel fluorescent probe, 3-epoxypropoxy fluorescein (EPF, 37), as well as its characteristics for histidine labelling (Scheme 11) [81]. The probe featured a fluorescein fluorophore with a long wavelength response and an active epoxy labelling group. In alkaline environments, EPF interacted primarily with histidine rather than other amino acids, causing a substantial increase in fluorescence intensity and so permitting the detection of histidine. This type of increase in fluorescence resembled that of fluorescein diaion with increasing media basicity, implying that the addition reaction of histidine with the epoxy group provides the fluorophore moiety with a basic molecular environment. The EPF (37) displayed an excitation maximum at 485 nm and an emission maximum at 513 nm, which are extremely similar to the values of its parent fluorescein, however it also demonstrated a significantly lower quantum yield (0.27) compared to the quantum yield of fluorescein (0.95).



3-Epoxypropoxy fluorescein (EPF, 37)

λ_{ex} = 485nm, λ_{em} =513nm
(aqueous solution pH 12.0 NaOH)

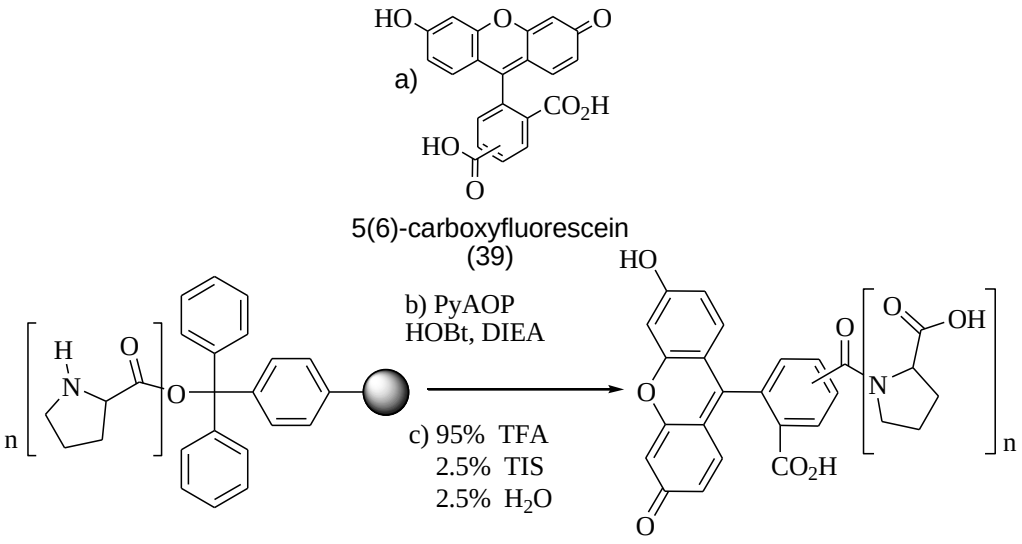


fluorescent histidine derivative of EPF (38)

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

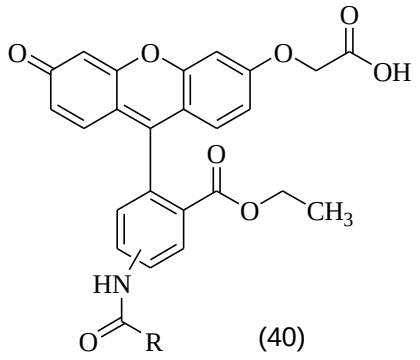
proline peptides with 5(6)-carboxyfluorescein (39) in the N terminals of hexaproline P6, dodecaproline P12, and 18-proline P18 (Scheme 12) [83]. The researchers pointed out that the disclosed technique might be used in other domains, such as solid-phase combinatorial synthesis of natural product-like compounds, polyamides, or PNAs, where fluorescent labelling for cell biology is needed.



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 showed two absorbance maxima around 456 nm and 481 nm. When compared to fluorescein (λ_{em} 512 nm), the fluorescence emission spectra of these compounds were widened and red shifted (λ_{em} 520 nm). The fluorescence quantum yields for 40 (Φ_F ca. 0.19) were much lower than those reported for fluorescein dianion (Φ_F ca. 0.92-0.93) and commensurate with those published for 3-*O*-alkyl ethers (Φ_F ca. 0.13-0.31) [85-87].



(40)

NHCOR in 5' and 6' positions
a. R = H
b. R = CH_2CH_2-NH-Fmoc
c. R = CH_2OCH_2-CO-NH-CH_2CH_2-NHFmoc

λ_{ex} = 485nm, λ_{em} =513nm
(sodium tetraborate, pH 9.8)

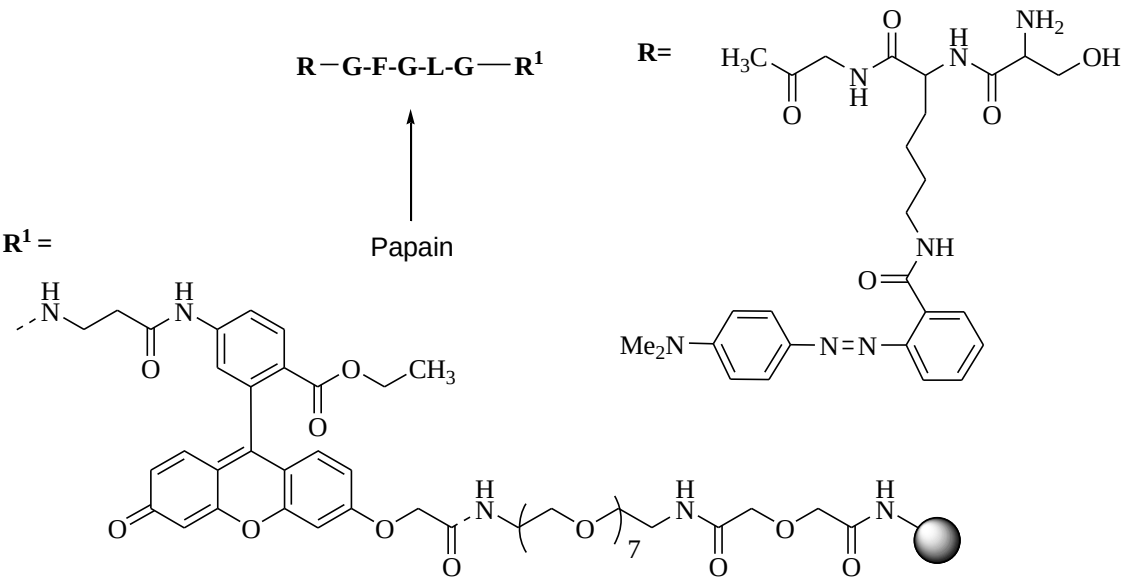
Scheme 13: Representative structures of a few Fmoc-protected fluorescent amino acids.

The broadening of the emission spectra and a decrease in quantum efficiency are most likely caused by changes in the D_{2h} molecular symmetry of the xanthene moiety present in these compounds, as well as vibrational modes and an enhanced rate of nonradiative internal conversion from excited to ground states. The loss of D_{2h} symmetry in the fluorescein monoanion causes a similar broadening of its emission spectrum as well as a comparable decline in its fluorescence quantum yield (Φ_F ca. 0.25 - 0.37). When compared to the dye 40a, the fluorescence yields of Fmoc-protected amino acids (40b,c) (Φ_F ca. 0.04-0.12) are noticeably lower [81]. This can be the outcome of the Fmoc chromophore-induced fluorescence quenching. In reality, Fmoc-deprotection increased the fluorescence of the compounds 40b,c

significantly. For compounds 40a-c, the quantum yields and fluorescence maxima were not pH dependent. After being incubated in an aqueous pH 9.8 buffer for a number of hours, fluorophores 40a showed no discernible modifications. These fluorophores' pH independence is extremely useful in applications that call for measuring and comparing fluorescence intensity across various conditions.

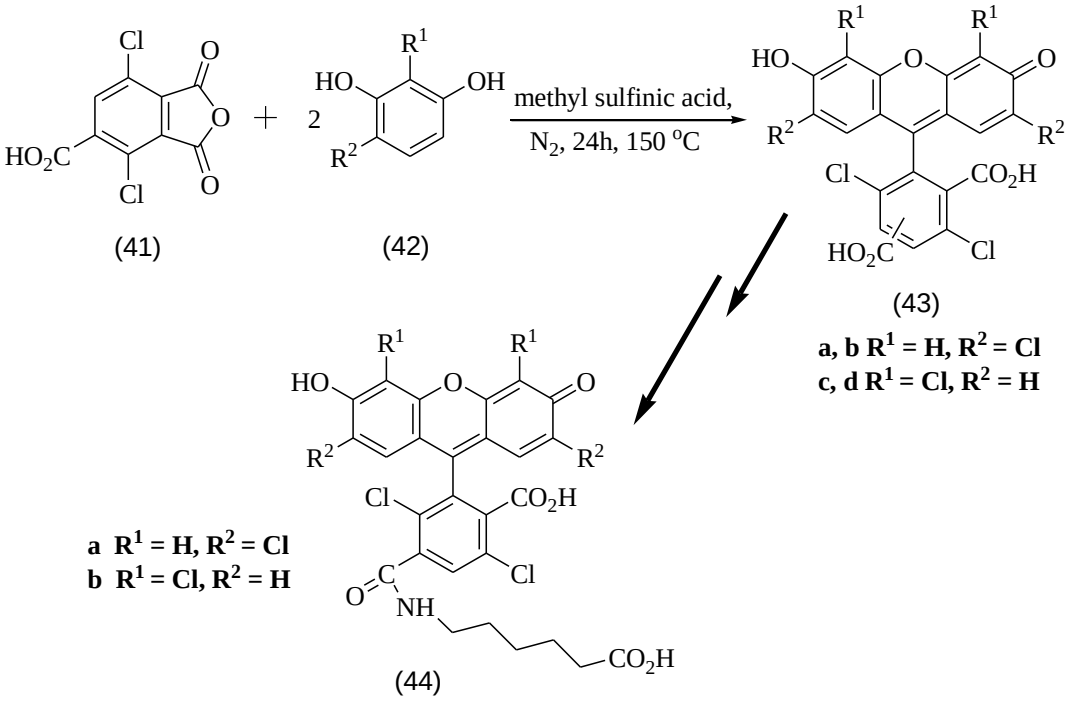
An effective synthesis of new type fluorescent amino acids was described by Burchak et al. [88]. The Fmoc-protected dyes can be made from aminofluoresceins and other affordable commercially available precursors in a four-step process with a 30% overall yield. The dyes display steady pH-independent fluorescence that is favourable for biological applications and are significantly more photostable than fluorescein. The fluorescent amino acids that have been Fmoc-protected 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) chromophore are separated by the peptide sequence GGFGLG in the substrate (Scheme 14). This sequence has been regarded as a good papain substrate because of the cleavage at the G-L link. The phenylalanine at the P2 position and the leucine 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 (20 lm beads) [88].



Scheme 14: Representative structure of fluorogenic papain substrate (- - - indicates points of fluorophore attachment).

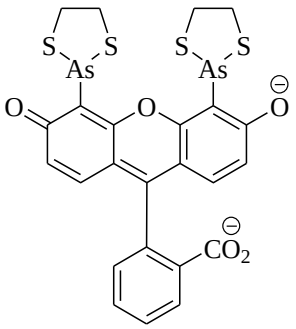
In case of multicolour imaging, such as high content analysis of living cells, fluorescent probes with narrower fluorescence bands are significantly useful. Although fluorescein derivatives have very broad fluorescence bands, selective substitution of fluoresceins by chlorine were found to be an efficient method of generating probes with smaller emission bands. Tian et al. reported in 2008 the synthesis of two novel chlorinated fluoresceins, 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 protein labelling (Scheme 15) [89]. As a spacer linker, these two fluoresceins include 6-aminohexanoic acid. Even at quite high degrees of labelling, the fluorescence of protein conjugates generated from compounds 44a and 44b are not significantly diminished compared to fluorescein molecules that do not contain this linker. Furthermore, these fluorophores are more photostable than nonchlorinated fluoresceins and, in the physiological pH range, are basically pH insensitive. These characteristics 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 [91]. Xaa represents any amino acid other than cysteine. 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.



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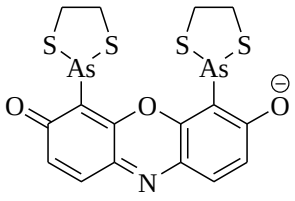

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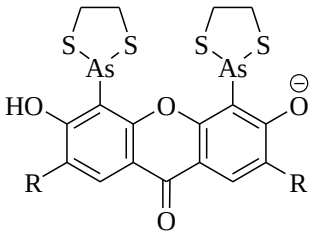
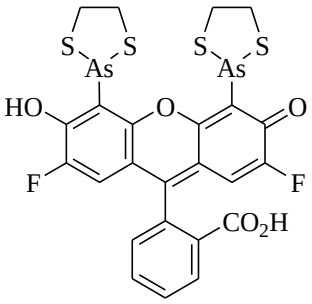


(47) **R = Cl**

(48) **R = H**

Scheme 16: Representative **s**tructures of biarsenical dyes 41-44.

Though fluorescein derivatives were the original FlAsH dyes but other cell-permeable biarsenical dyes, such as ReAsH (46) ($\lambda_{ex}/\lambda_{em}$ 593/608 nm), a phenoxazine derivative, and CHoXAsH (47) ($\lambda_{ex}/\lambda_{em}$ 380/430 nm), a xanthene derivative related to the FlAsH compounds, are also available. It is worthwhile to mention that they have distinct optical properties (Scheme 16). In comparison to other conventional approaches, the unique binding and membrane permeability capabilities of these biarsenical dyes offer significant 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, F2FlAsH (49) and F4FlAsH (50) (Scheme 17), which were found to be significantly more effective in these areas than the original fluorescein derivative FlAsH (45), due to the limited photostability and pH sensitivity of fluorescein derivatives in the physiological range [93].



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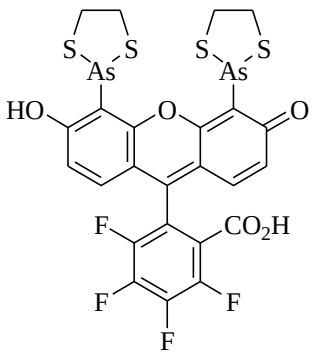


(50)

Scheme 17: Representative structures of fluorinated fluorescein biarsenical dyes 45 and 46.

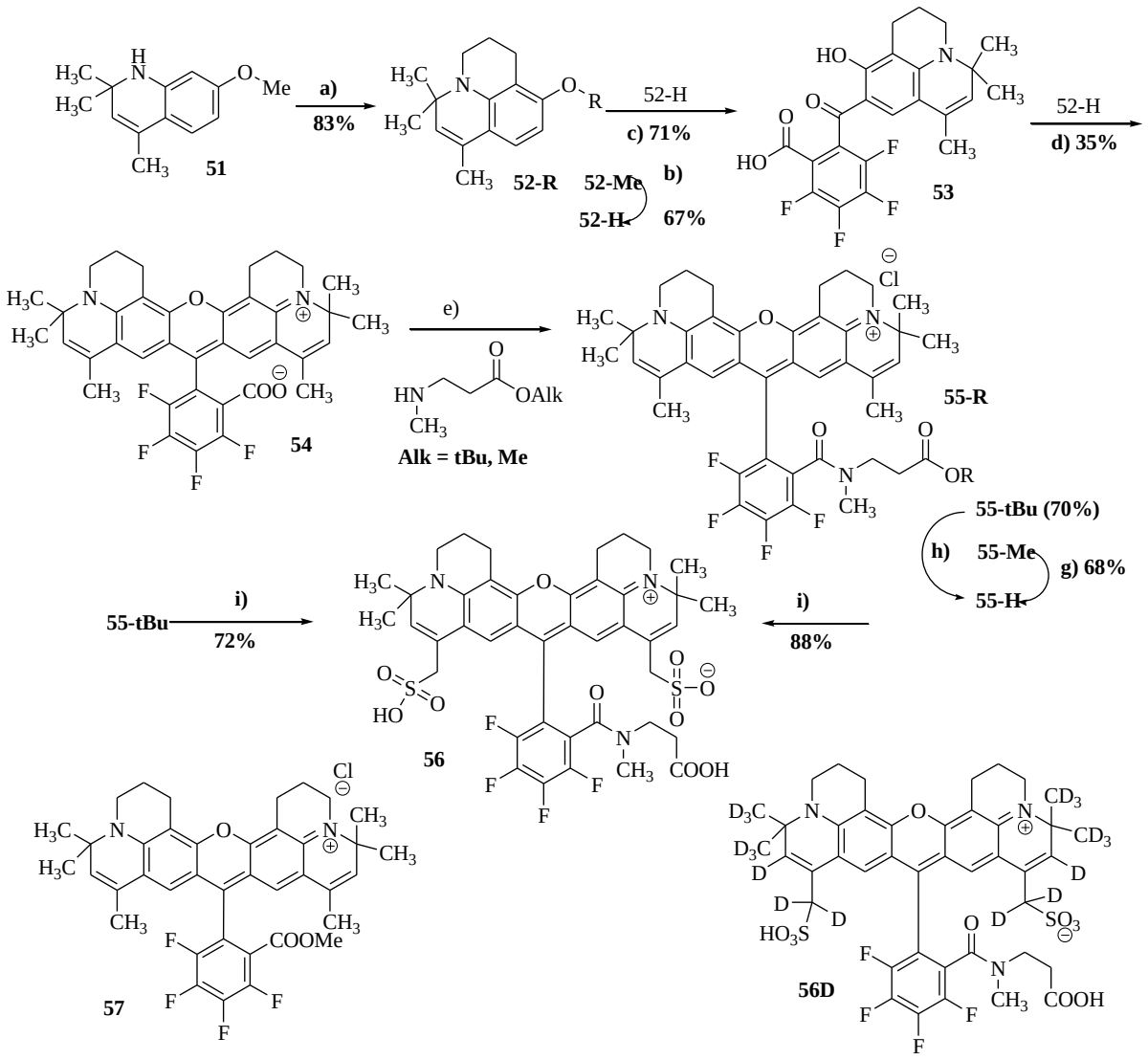
In comparison to FlAsH (45), F2FlAsH (49) has a higher absorbance, a larger Stokes' shift, a higher fluorescence quantum yield, a higher level of photostability, and a lower pH dependence. By emitting F4FlAsH (50) in the wavelength range between FlAsH (45) and ReAsH (46), a new colour with exceptional 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 have high molar absorptivities in the visible spectrum [13,16,65]. The xanthene nucleus substituents have a significant impact on the absorption and emission properties, and rhodamine dyes are used as laser dyes, photosensitizers, and fluorescent markers in structural microscopic studies in addition to being used as colourants [94]. Rhodamine dyes are used extensively as laser dyes and fluorescent markers for labelling proteins, nucleic acids, lipids, carbohydrates, toxins, hormones, and other biomolecules due to their strong photostabilities, high extinction, high fluorescence quantum yields, and low degree of triplet formation. Rhodamines proved useful in the practical implementation of some new physical principles, such as those that assisted in overcoming the diffraction limit in (far-field) optical microscopy, in addition to their application in biological imaging and single-molecule-based spectroscopy. STED (stimulated emission depletion), PALM (photoactivation localization microscopy), STORM (stochastic reconstruction microscopy) and GSDIM (ground-state depletion with individual molecular return) allowed optical resolution to be improved from about 200-350 nm to 20-35 nm by switching between the dark and bright states of a fluorescent marker [95]. The rhodamine family, on the other hand, lacks water-soluble compounds with high photostabilities and fluorescence quantum yields and absorbance and fluorescence band maxima in the far-red

optical range. Fluorescent dyes that absorb in the far-red or even near-infrared (IR) optical range are preferred for biological applications. 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) and comparatively low intersystem-crossing rates. They excel at both conventional and subdiffraction-resolution microscopy, such as STED and GSDIM, as well as single-molecule experiments like FCS. The authors disclosed the synthesis of lipophilic (55,57) and hydrophilic (56) derivatives from the same chromophore-containing scaffold. The addition of two sulfo groups results in high water solubility and a significant increase in fluorescence quantum yield. The addition 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].



a) $Br(CH_2)_3Cl$, Na_2CO_3, KI, CH_3CN, 25h; b) 48% aq. HBr, glacial AcOH, 135-140 ^oC, 22h; c) tetraflurophthalic anhydride, toluene, reflux, 5h; d) $POCl_3$, $ClCH_2CH_2Cl$, 63-65 ^oC, 3.5 h; e)$POCl_3$, $ClCH_2CH_2Cl$, reflux, 2.5 h; f) Et_3N, CH_3CN, rt; g) 10% aq. KOH, THF, O ^oC; h) 2M HCl in 1,4-dioxane, rt; i) 96-98% H_2SO_4, rt, 40h

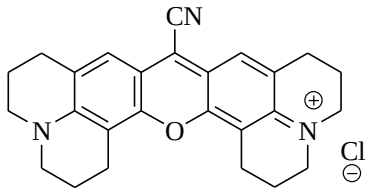
Scheme 18: Synthesis of lipophilic (55,57) and hydrophilic (56) rhodamines.

Table 4: Spectroscopic possessions of the synthetically designed fluorescent dyes

Compound	λ_{max} (abs) [nm]	λ_{max} (fl.) [nm]	Solvent	ε [$10^{-5}M^{-1}cm^{-1}$]	$\Phi_{fl.}$ [%] in H_2O	$\tau_{fl.}$ [ns]	$K_{ISC/T}$ [10^{-6} s^{-1}]
4	616	641	MeOH	-	63	-	-
7	632	655.5	MeOH	-	-	-	-
5-*t*Bu	638	661	MeOH	0.92	61	-	-
5-Me	638	661.5	MeOH	0.66	62	-	-
5-H	638	661.5	MeOH	0.73	53	3.6	-

6	637	660	H_2O	0.94	80	6.4	2.5/0.3
6D	637	660	H_2O	0.92	78	6.4	2.5/0.3

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



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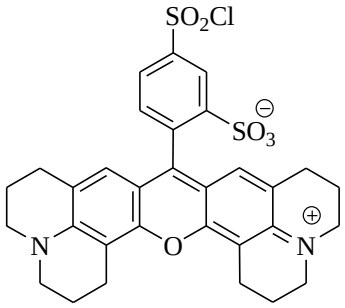
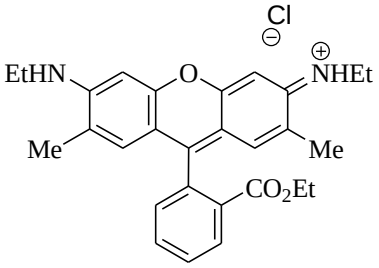


(59)

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) was also utilised in experiments related to enzymatic activity [98]. Lavis et al. reported the synthesis of the flexible ‘latent’ fluorophore 67 (Scheme 21), a Rhodamine 110 (60) derivative in which one of the nitrogens was changed as a urea and the other as a ‘trimethyl lock’ [99]. The first modification reduced rhodamine's half-fluorescence while

enabling conjugation with a target molecule. The second alteration allowed fluorescence to be completely unmasked by a single user-specified chemical reaction.



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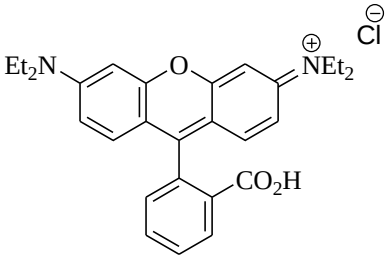

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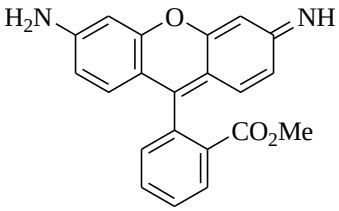
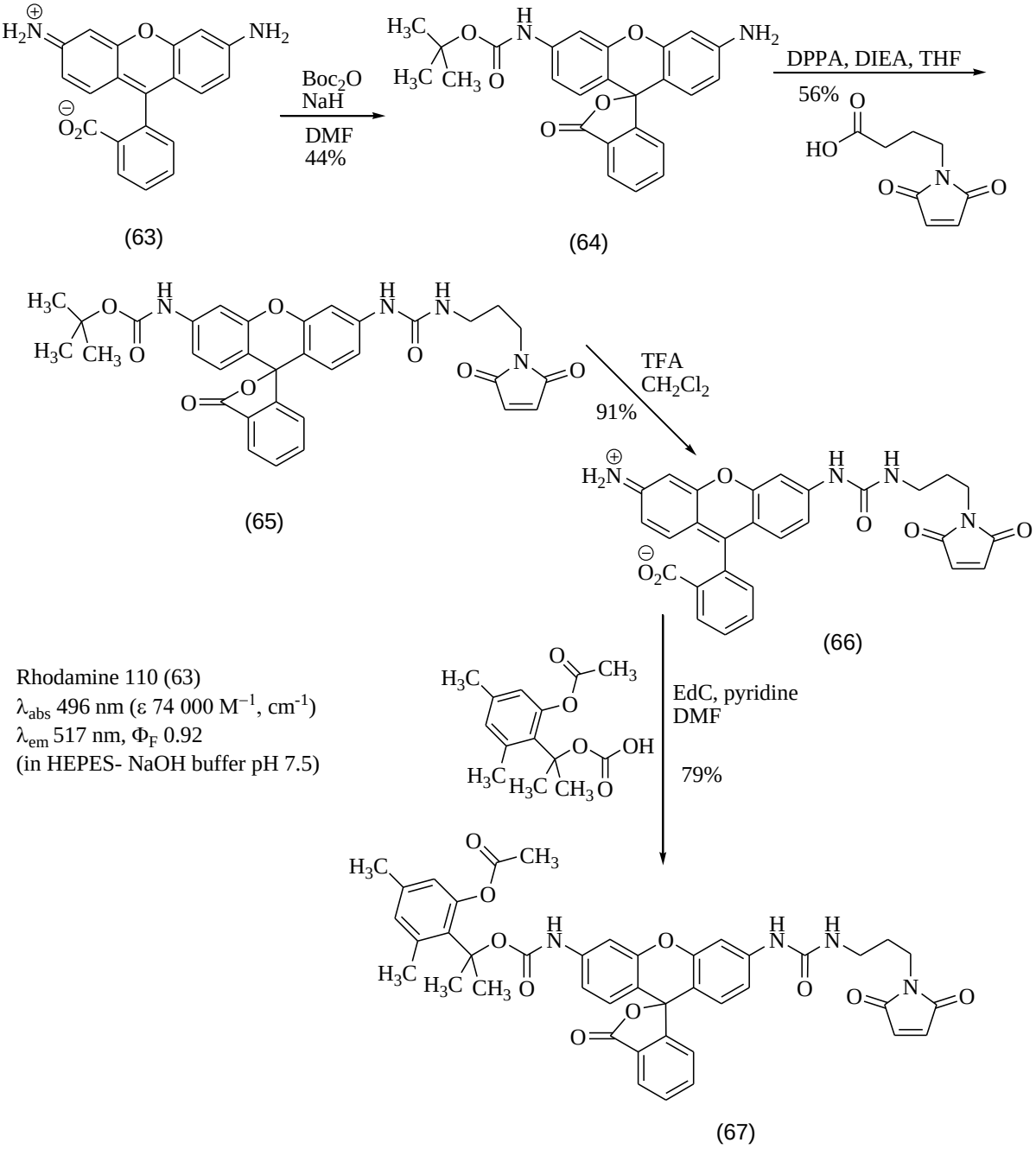


(62)

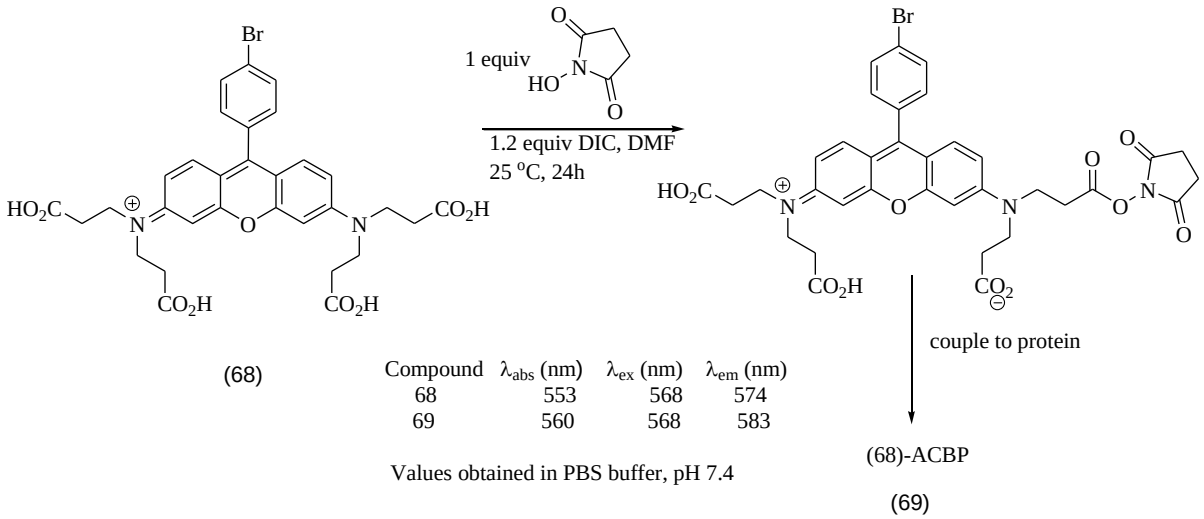
Scheme 20: Representative **s**tructures of Rhodamine 6G (60), Rhodamine 123 (61) and Rhodamine B (62).



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)

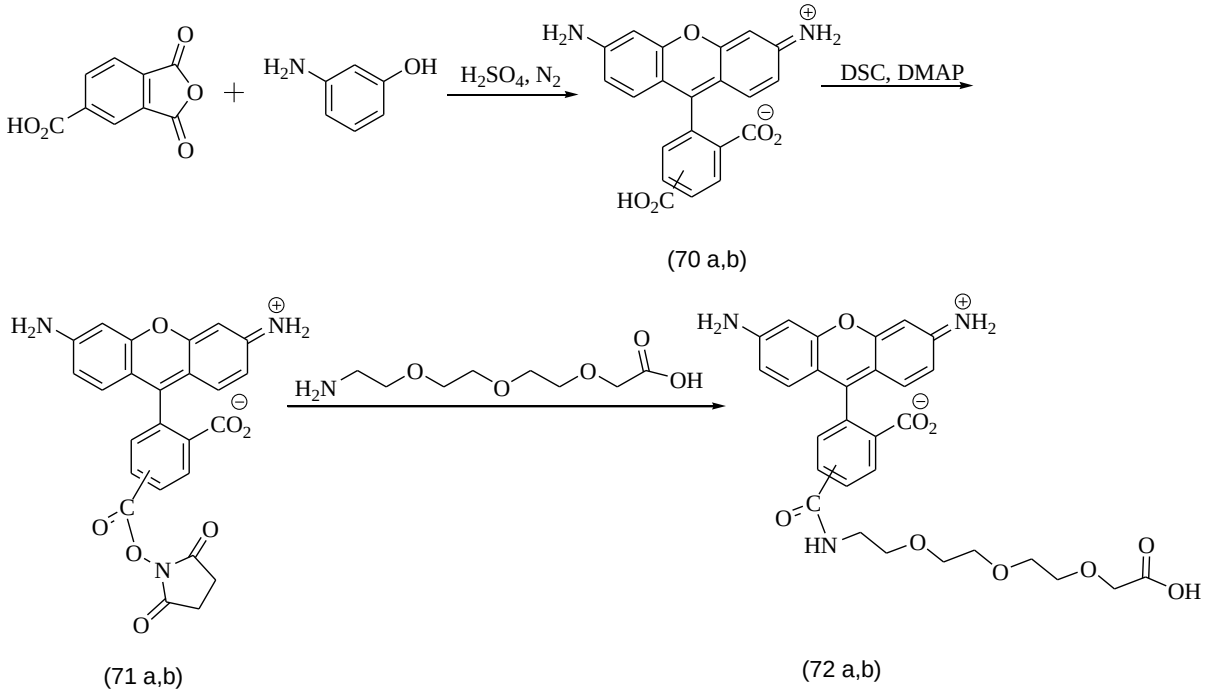
model protein. The features of this compound were investigated to ensure that the label did not considerably modify the protein's binding function to its natural ligand in vitro and that its secondary structure was not dramatically altered. Other experiments demonstrated the new Chariot-peptide carrier 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 fluorescently 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 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 alphatubulin 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 done 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 ultraviolet to the near-infrared, 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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IX. References

1. Albrecht, C. (2008). Joseph R. Lakowicz: Principles of fluorescence spectroscopy.
2. Mitra, A. K. (2019). I am the light beneath your eyes. *Resonance*, *24*(6), 623-632. [https://doi.org/10.1007/s12045-019-0821-5]
3. Valeur, B., & Berberan-Santos, M. N. (2011). A brief history of fluorescence and phosphorescence before the emergence of quantum theory. *Journal of Chemical Education*, *88*(6), 731-738.

[https://doi.org/10.1021/ed100182h]

4. Gonçalves, M. S. T. (2009). Fluorescent labeling of biomolecules with organic probes. *Chemical reviews*, *109*(1), 190-212. [https://doi.org/10.1021/cr0783840]
5. Fu, Y., & Finney, N. S. (2018). Small-molecule fluorescent probes and their design. *RSC advances*, *8*(51), 29051-29061. [https://doi.org/10.1039/C8RA02297F]
6. Terai, T., & Nagano, T. (2013). Small-molecule fluorophores and fluorescent probes for bioimaging. *Pflügers Archiv-European Journal of Physiology*, *465*, 347-359. [10.1007/s00424-013-1234-z.]
7. Sarkar, I., & Mishra, A. K. (2018). Fluorophore tagged bio-molecules and their applications: A brief review. *Applied Spectroscopy Reviews*, *53*(7), 552-601. [https://dx.doi.org/10.1080/05704928.2017.1376680]
8. Costa-Fernández, J. M., Pereiro, R., & Sanz-Medel, A. (2006). The use of luminescent quantum dots for optical sensing. *TrAC Trends in Analytical Chemistry*, *25*(3), 207-218. [http://dx.doi.org/10.1016/j.trac.2005.07.008]
9. Schneckenburger, H., Gschwend, M. H., Paul, R. J., Stepp, H. G., Rick, K., Betz, V., & Strauss, W. S. (1995, January). Time-gated spectroscopy of intrinsic fluorophores in cells and tissues. In *Optical Biopsy and Fluorescence Spectroscopy and Imaging* (Vol. 2324, pp. 187-195). SPIE.
10. Ammor, M. S. (2007). Recent advances in the use of intrinsic fluorescence for bacterial identification and characterization. *Journal of fluorescence*, *17*, 455-459. [https://doi.org/10.1007/s10895-007-0180-6].
11. Melavanki, R., Basanagouda, M. M., & Mogurampelly, S. (2021). Computational and spectroscopic studies of biologically active coumarin-based fluorophores. *Luminescence: the Journal of Biological and Chemical Luminescence*, *36*(3), 769-787. [https://doi.org/10.1002/bio.4002]
12. Sun, Y. F., Wang, H. P., Chen, Z. Y., & Duan, W. Z. (2013). Solid-state fluorescence emission and second-order nonlinear optical properties of coumarin-based fluorophores. *Journal of fluorescence*, *23*, 123-130. [https://doi.org/10.1007/s10895-012-1125-2]

13. Liu, C., & Scott, C. N. (2021). Design strategies to rhodamine analogue fluorophores for near-infrared II biological imaging applications. *Dyes and Pigments*, *196*, 109792. [https://doi.org/10.1016/j.dyepig.2021.109792]
14. Sam, B., George, L., N, S. Y., & Varghese, A. (2021). Fluorescein based fluorescence sensors for the selective sensing of various analytes. *Journal of Fluorescence*, *31*, 1251-1276. [https://doi.org/10.1007/s10895-021-02770-9]
15. Zhou, H. J., & Ren, T. B. (2022). Recent Progress of Cyanine Fluorophores for NIR-II Sensing and Imaging. *Chemistry–An Asian Journal*, *17*(8), e202200147. [https://doi.org/10.1002/asia.202200147]
16. McCann, T. E., Kosaka, N., Koide, Y., Mitsunaga, M., Choyke, P. L., Nagano, T., ... & Kobayashi, H. (2011). Activatable optical imaging with a silica-rhodamine based near infrared (SiR700) fluorophore: a comparison with cyanine based dyes. *Bioconjugate chemistry*, *22*(12), 2531-2538. [https://doi.org/10.1021/bc2003617]
17. Chandrasekaran, A. R. (2021). DNA-based ribonuclease detection assays. *Journal of Materials Chemistry B*, *9*(35), 7023-7029. [https://doi.org/10.1039/D1TB00300C]
18. Mitra, A. K. (2022). Synthesis, biological activity and photophysical studies of ellipticine and its derivatives: state of the art. *Chemistry of Heterocyclic Compounds*, *58*(4-5), 178-192. [https://doi.org/10.1007/s10593-022-03070-1]
19. Petriella, M., Deniz, E., Swaminathan, S., Roberti, M. J., Raymo, F. M., & Bossi, M. L. (2013). Superresolution imaging with switchable fluorophores based on oxazine auxochromes. *Photochemistry and photobiology*, *89*(6), 1391-1398. [https://doi.org/10.1111/php.12100]
20. Shang, J., Zhang, X., He, Z., Shen, S., Liu, D., Shi, W., & Ma, H. (2022). An Oxazine-Based Fluorogenic Probe with Changeable π-Conjugation to Eliminate False-Positive Interference of Albumin and Its Application to Sensing Aminopeptidase N. *Angewandte Chemie*, *134*(33), e202205043. [https://doi.org/10.1002/ange.202205043]
21. Moghal, Z. K. B., Manivannan, R., & Son, Y. A. (2022). Acridine-based fluorophores with improved lightfastness properties. *Dyes and Pigments*, *197*, 109924. [https://doi.org/10.1016/j.dyepig.2021.109924]

22. Adhikari, S., & Mitra, A. K. (2023). Perspective on acridine: a versatile heterocyclic biologically imperative framework. *Journal of the Iranian Chemical Society*, 1-57. [https://doi.org/10.1007/s13738-023-02840-8]
23. Wang, X. Q., Ling, Q. H., Wang, W., & Xu, L. (2020). Pyrene-based metallocycles and metallocages: more than fluorophores. *Materials Chemistry Frontiers*, *4*(11), 3190-3200. [https://doi.org/10.1039/D0QM00484G]
24. Ayyavoo, K., & Velusamy, P. (2021). Pyrene based materials as fluorescent probes in chemical and biological fields. *New Journal of Chemistry*, *45*(25), 10997-11017. [https://doi.org/10.1039/D1NJ00158B]
25. Dong, S., Li, Z., & Qin, J. (2009). New carbazole-based fluorophores: synthesis, characterization, and aggregation-induced emission enhancement. *The Journal of Physical Chemistry B*, *113*(2), 434-441. [https://doi.org/10.1021/jp807510a]
26. Mitra, A. K. (2022). The journey of 1-Keto-1, 2, 3, 4-tetrahydrocarbazole based fluorophores: from inception to implementation. *Journal of Fluorescence*, *32*(6), 2023-2052. [https://doi.org/10.1007/s10895-022-03004-2]
27. Mitra, A. K. (2021). Sesquicentennial birth anniversary of carbazole, a multifaceted wonder molecule: a revisit to its synthesis, photophysical and biological studies. *Journal of the Iranian Chemical Society*, 1-39. [https://doi.org/10.1007/s13738-021-02444-0]
28. Sarangi, M. K., Mitra, A. K., Sengupta, C., Ghosh, S., Chakraborty, S., Saha, C., & Basu, S. (2013). Hydrogen bond sensitive probe 5-methoxy-1-keto-1, 2, 3, 4-tetrahydro carbazole in the microheterogeneity of binary mixtures and reverse micelles. *The Journal of Physical Chemistry C*, *117*(5), 2166-2174. https://doi.org/10.1021/jp310923q
29. Mitra, A. K., Ghosh, S., Chakraborty, S., Basu, S., & Saha, C. (2013). Synthesis and spectroscopic exploration of carboxylic acid derivatives of 6-hydroxy-1-keto-1, 2, 3, 4-tetrahydrocarbazole: hydrogen bond sensitive fluorescent probes. *Journal of luminescence*, *143*, 693-703. https://doi.org/10.1016/j.jlumin.2013.06.021
30. Mitra, A. K., Ghosh, S., Chakraborty, S., Sarangi, M. K., Saha, C., & Basu, S. (2012). Photophysical properties of an environment sensitive fluorophore 1-keto-6, 7-

dimethoxy-1, 2, 3, 4-tetrahydrocarbazole and its excited state interaction with N, N-dimethylaniline: A spectroscopic investigation. *Journal of Photochemistry and Photobiology A: Chemistry*, *240*, 66-74. https://doi.org/10.1016/j.jphotochem.2012.05.006

31. Ghosh, S., Mitra, A. K., Saha, C., & Basu, S. (2013). Tuning the solution phase photophysics of two de novo designed hydrogen bond sensitive 9-methyl-2, 3, 4, 9-tetrahydro-1H-carbazol-1-one derivatives. *Journal of fluorescence*, *23*(6), 1179-1195. https://doi.org/10.1007/s10895-013-1249-z
32. Ghosh, S., Mitra, A. K., Basu, S., Chakraborty, S., & Saha, C. (2014). 5, 6, 7, 9-Tetrahydro-[1, 3] dioxolo [4, 5-h] carbazol-8-one: A solvatochromic PET-acceptor fluorescent probe. *Journal of luminescence*, *153*, 296-303. https://doi.org/10.1016/j.jlumin.2014.03.046
33. Mitra, A. K., Ghosh, S., Sarangi, M. K., Sau, A., Saha, C., & Basu, S. (2015). Influence of microheterogeneity on the solution phase photophysics of a newly synthesised, environment sensitive fluorophore 2-((7, 8-dimethyl-1-oxo-2, 3, 4, 9-tetrahydro-1H-carbazol-6-yl) oxy) acetic acid and its tagged derivative. *Journal of Photochemistry and Photobiology A: Chemistry*, *296*, 66-79. https://doi.org/10.1016/j.jphotochem.2014.09.009
34. Ghosh, S., Mitra, A. K., Pal, U., Basu, S., & Saha, C. (2017). Evidence of two structurally related solvatochromic probes complexed with β-cyclodextrin by using spectroscopic methods. *Journal of Molecular Structure*, *1130*, 810-817. https://doi.org/10.1016/j.molstruc.2016.10.097
35. Mitra, A. K., Ghosh, S., Sarangi, M. K., Chakraborty, S., Saha, C., & Basu, S. (2014). Photophysics of a solvent sensitive keto-tetrahydrocarbazole based fluorophore and its interaction with triethylamine: a spectroscopic inquest under surfactant and β-CD confinement. *Journal of Molecular Structure*, *1074*, 617-628. https://doi.org/10.1016/j.molstruc.2014.06.038
36. Mitra, A. K., Ghosh, S., Sau, A., Saha, C., & Basu, S. (2015). Solution phase photophysics of 5, 7-dimethoxy-2, 3, 4, 9-tetrahydro-1H-carbazol-1-one: Analysing the lineaments of a new fluorosensor to probe different micro-environments. *Journal of Luminescence*, *167*, 233-248. https://doi.org/10.1016/j.jlumin.2015.06.048
37. Mitra, A. K., Sau, A., Pal, U., Saha, C., & Basu, S. (2017). Constrained Photophysics of 5, 7-dimethoxy-2, 3, 4, 9-tetrahydro-1H-carbazol-1-one in the Bioenvironment of Serum Albumins: A Spectroscopic Endeavour Supported by Molecular Docking

Analysis. *Journal of Fluorescence*, *27*(4), 1547-1558. https://doi.org/10.1007/s10895-017-2094-2

38. Mitra, A. K., Sau, A., Bera, S. C., Chakraborty, S., Saha, C., & Basu, S. (2015). Monitoring the Competence of a New Keto-tetrahydrocarbazole Based Fluorosensor Under Homogeneous, Micro-Heterogeneous and Serum Albumin Environments. *Journal of fluorescence*, *25*(6), 1931-1949. https://doi.org/10.1007/s10895-015-1685-z
39. Niu, C. G., Guan, A. L., Zeng, G. M., Liu, Y. G., Huang, G. H., Gao, P. F., & Gui, X. Q. (2005). A ratiometric fluorescence halide sensor based on covalently immobilization of quinine and benzothioxanthene. *Analytica chimica acta*, *547*(2), 221-228. [https://doi.org/10.1016/j.aca.2005.05.031]
40. Mule, S. J., & Hushin, P. L. (1971). Semiautomated fluorometric assay for submicrogram quantities of morphine and quinine in human biological material. *Analytical Chemistry*, *43*(6), 708-711. [10.1021/ac60301a002]
41. Bai, L., Sun, P., Liu, Y., Zhang, H., Hu, W., Zhang, W., ... & Huang, W. (2019). Novel aza-BODIPY based small molecular NIR-II fluorophores for in vivo imaging. *Chemical Communications*, *55*(73), 10920-10923. [https://doi.org/10.1039/C9CC03378E]
42. Bennett, F. A., Barlow, D. J., Dodoo, A. N., Hider, R. C., Lansley, A. B., Lawrence, M. J., ... & Bansal, S. S. (1999). Synthesis and properties of (6, 7-dimethoxy-4-coumaryl) alanine: a fluorescent peptide label. *Analytical biochemistry*, *270*(1), 15-23. [https://doi.org/10.1006/abio.1999.4081]
43. Wang, W., & Li, H. (2004). An efficient synthesis of the intrinsic fluorescent peptide labels,(S)-and (R)-(6, 7-dimethoxy-4-coumaryl) alanines via asymmetric hydrogenations. *Tetrahedron letters*, *45*(46), 8479-8481. [https://doi.org/10.1016/j.tetlet.2004.09.093]
44. Sui, G., Kele, P., Orbulescu, J., Huo, Q., & Leblanc, R. M. (2001). Synthesis of a coumarin based fluorescent amino acid. *Letters in Peptide Science*, *8*, 47-51. [https://doi.org/10.1023/A:1014280017576]
45. Berthelot, T., Laïn, G., Latxague, L., & Déleris, G. (2004). Synthesis of novel fluorogenic L-Fmoc lysine derivatives as potential tools for imaging cells. *Journal of Fluorescence*, *14*, 671-675. [https://doi.org/10.1023/B:JOFL.0000047216.37306.79]
46. Clinton, R. O., & Laskowski, S. C. (1949). Coumarins. I. Derivatives of coumarin-3- and 4-carboxylic acids. *Journal of the American Chemical Society*, *71*(11), 3602-3606. [https://doi.org/10.1021/ja01179a007]

47. Bonsignore, L., Cottiglia, F., Maccioni, A. M., Secci, D., & Lavagna, S. M. (1995). Synthesis of coumarin-3-O-acylisoureas by dicyclohexylcarbodiimide. *Journal of heterocyclic chemistry*, *32*(2), 573-577. [https://doi.org/10.1002/jhet.5570320234]
48. Corrie, J. E., Munasinghe, V. R. N., & Rettig, W. (2000). Synthesis and fluorescence properties of substituted 7-aminocoumarin-3-carboxylate derivatives. *Journal of Heterocyclic Chemistry*, *37*(6), 1447-1455. [https://doi.org/10.1002/jhet.5570370608]
49. Amblard, M., Fehrentz, J. A., Martinez, J., & Subra, G. (2005). Fundamentals of modern peptide synthesis. *Peptide synthesis and applications*, 3-24. [https://doi.org/10.1385/1-59259-877-3:003]
50. Mitchell, A. R. (2017). Biopolymers (Pept Sci), 2008, 90, 175. AJ Mijalis, DA Thomas, MD Simon, A. Adamo, R. Beaumont, KF Jensen and BL Pentelute. *Nat. Chem. Biol*, *13*, 464.
51. Hara, M. R., Agrawal, N., Kim, S. F., Cascio, M. B., Fujimuro, M., Ozeki, Y., ... & Sawa, A. (2005). S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nature cell biology*, *7*(7), 665-674. [https://doi.org/10.1038/ncb1268]
52. Hara, M. R., & Snyder, S. H. (2006). Nitric oxide–GAPDH–Siah: a novel cell death cascade. *Cellular and molecular neurobiology*, *26*, 525-536. [https://doi.org/10.1007/s10571-006-9011-6]
53. Uehara, T., Nakamura, T., Yao, D., Shi, Z. Q., Gu, Z., Ma, Y., ... & Lipton, S. A. (2006). S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature*, *441*(7092), 513-517. [https://doi.org/10.1038/nature04782]
54. Yao, D., Gu, Z., Nakamura, T., Shi, Z. Q., Ma, Y., Gaston, B., ... & Lipton, S. A. (2004). Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proceedings of the National Academy of Sciences*, *101*(29), 10810-10814. [https://doi.org/10.1073/pnas.040416110]
55. Dalle-donne, I., Milzani/snm>, A., Giustarini, D., Simplicio, P. D., Colombo, R., & Rossi, R. (2000). S-NO-actin: S-nitrosylation kinetics and the effect on isolated vascular smooth muscle. *Journal of Muscle Research & Cell Motility*, *21*, 171-181. [https://doi.org/10.1023/A:1005671319604]

56. Han, P., Zhou, X., Huang, B., Zhang, X., & Chen, C. (2008). On-gel fluorescent visualization and the site identification of S-nitrosylated proteins. *Analytical biochemistry*, *377*(2), 150-155. [https://doi.org/10.1016/j.ab.2008.03.023]
57. Ivana, K.; Pavol, K. 10th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-10), 2006, a027; http://www.usc.es/congresos/ecsoc/10/ECSOC10.htm.
58. Tao, Z. F., Qian, X., & Fan, M. (1997). Regioselective synthesis and photooxygenations of furonaphthopyrones starting from 2, 7-naphthalenediol. *Tetrahedron*, *53*(39), 13329-13338. [https://doi.org/10.1016/S0040-4020(97)00845-4]
59. Kosiova, I., Janicova, A., & Kois, P. (2006). Synthesis of coumarin or ferrocene labeled nucleosides via Staudinger ligation. *Beilstein journal of organic chemistry*, *2*(1), 23. [https://doi.org/10.1186/1860-5397-2-23]
60. SATO, E., MATSUHISA, A., SAKASHITA, M., & KANAOKA, Y. (1988). New water-soluble fluorogenic amine.: 7-Aminocoumarin-4-methanesulfonic acid (ACMS) and related substrates for proteinases. *Chemical and pharmaceutical bulletin*, *36*(9), 3496-3502. [https://doi.org/10.1248/cpb.36.3496]
61. Shutes, A., & Der, C. J. (2005). Real-time in vitro measurement of GTP hydrolysis. *Methods*, *37*(2), 183-189. [https://doi.org/10.1016/j.ymeth.2005.05.019]
62. Piloto, A. M., Rovira, D., Costa, S. P., & Gonçalves, M. S. T. (2006). Oxobenzo [f] benzopyrans as new fluorescent photolabile protecting groups for the carboxylic function. *Tetrahedron*, *62*(51), 11955-11962. [https://doi.org/10.1016/j.tet.2006.09.085]
63. Piloto, A. M., Fonseca, A. S., Costa, S. P., & Gonçalves, M. S. T. (2006). Carboxylic fused furans for amino acid fluorescent labelling. *Tetrahedron*, *62*(39), 9258-9267. [https://doi.org/10.1016/j.tet.2006.07.003]
64. Piloto, A. M., Costa, S. P., & Gonçalves, M. S. T. (2005). A naphtho [2, 1-b] furan as a new fluorescent label: synthesis and spectral characterisation. *Tetrahedron letters*, *46*(28), 4757-4760. [https://doi.org/10.1016/j.tetlet.2005.05.035]

65. Zheng, H., Zhan, X. Q., Bian, Q. N., & Zhang, X. J. (2013). Advances in modifying fluorescein and rhodamine fluorophores as fluorescent chemosensors. *Chemical communications*, *49*(5), 429-447. [https://doi.org/10.1039/C2CC35997A]
66. Fang, Y., Good, G. N., Zhou, X., & Stains, C. I. (2019). Phosphinate-containing rhodol and fluorescein scaffolds for the development of bioprobes. *Chemical communications (Cambridge, England)*, *55*(42), 5962. [10.1039/c9cc02492a]
67. Haugland, R. P. (2002). *Handbook of fluorescent probes and research products*. Molecular probes.
68. Sun, W. C., Gee, K. R., Klaubert, D. H., & Haugland, R. P. (1997). Synthesis of fluorinated fluoresceins. *The Journal of Organic Chemistry*, *62*(19), 6469-6475. [https://doi.org/10.1021/jo9706178]
69. Udenfriend, S., Stein, S., Boehlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972). Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science*, *178*(4063), 871-872. [https://doi.org/10.1126/science.178.4063.871]
70. Neckers, D. C., & Valdes-Aguilera, O. M. (1993). In Advances in Photochemistry; Volman, DH, Hammond, GS, Neckers, DC, Eds.
71. Talhavini, M., & Atvars, T. D. Z. (1998). Dye-polymer interactions controlling the kinetics of fluorescein photobleaching reactions in poly (vinyl alcohol). *Journal of Photochemistry and Photobiology A: Chemistry*, *114*(1), 65-73. [https://doi.org/10.1016/S1010-6030(97)00307-9]
72. Talhavini, M., & Atvars, T. D. Z. (1999). Photostability of xanthene molecules trapped in poly (vinyl alcohol)(PVA) matrices. *Journal of Photochemistry and Photobiology A: Chemistry*, *120*(2), 141-149. [https://doi.org/10.1016/S1010-6030(98)00416-X]
73. Weigele, M., DeBernardo, S., & Leimgruber, W. (1973). Fluorometric assay of secondary amino acids. *Biochemical and biophysical research communications*, *50*(2), 352-356. [https://doi.org/10.1016/0006-291X(73)90847-4]
74. Geisow, M. J. (1984). Fluorescein conjugates as indicators of subcellular pH: a critical evaluation. *Experimental cell research*, *150*(1), 29-35. [https://doi.org/10.1016/0014-4827(84)90698-0]

75. Vivès, E., & Lebleu, B. (2003). One-pot labeling and purification of peptides and proteins with fluorescein maleimide. *Tetrahedron letters*, *44*(29), 5389-5391. [https://doi.org/10.1016/S0040-4039(03)01304-2]
76. Lau, S. K., Zaccardo, F., Little, M., & Banks, P. (1998). Nanomolar derivatizations with 5-carboxyfluorescein succinimidyl ester for fluorescence detection in capillary electrophoresis. *Journal of Chromatography A*, *809*(1-2), 203-210. [https://doi.org/10.1016/S0021-9673(98)00165-4]
77. Fülöp, L., Penke, B., & Zarándi, M. (2001). Synthesis and fluorescent labeling of beta-amyloid peptides. *Journal of Peptide Science: An Official Publication of the European Peptide Society*, *7*(8), 397-401. [https://doi.org/10.1002/psc.346]
78. Hoffmann, C., Leroy-Dudal, J., Patel, S., Gallet, O., & Pauthe, E. (2008). Fluorescein isothiocyanate-labeled human plasma fibronectin in extracellular matrix remodeling. *Analytical biochemistry*, *372*(1), 62-71. [https://doi.org/10.1016/j.ab.2007.07.027]
79. Twining, S. S. (1984). Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Analytical biochemistry*, *143*(1), 30-34. [https://doi.org/10.1016/0003-2697(84)90553-0]
80. Konecsni, T., & Kilár, F. (2004). Monitoring of the conjugation reaction between human serum transferrin and fluorescein isothiocyanate by capillary electrophoresis. *Journal of Chromatography A*, *1051*(1-2), 135-139. [https://doi.org/10.1016/j.chroma.2004.06.056]
81. Li, X., Ma, H., Dong, S., Duan, X., & Liang, S. (2004). Selective labeling of histidine by a designed fluorescein-based probe. *Talanta*, *62*(2), 367-371. [https://doi.org/10.1016/j.talanta.2003.08.004]
82. Brimble, M. A., Kowalczyk, R., Harris, P. W., Dunbar, P. R., & Muir, V. J. (2008). Synthesis of fluorescein-labelled O-mannosylated peptides as components for synthetic vaccines: comparison of two synthetic strategies. *Organic & biomolecular chemistry*, *6*(1), 112-121. [https://doi.org/10.1039/B712926B]
83. Fernández-Carneado, J., & Giralt, E. (2004). An efficient method for the solid-phase synthesis of fluorescently labelled peptides. *Tetrahedron letters*, *45*(31), 6079-6081. [https://doi.org/10.1016/j.tetlet.2004.05.081]

84. Tsien, R. Y. (1989). Fluorescent indicators of ion concentrations. *Methods in cell biology*, *30*, 127-156. [https://doi.org/10.1016/S0091-679X(08)60978-4]
85. Adamczyk, M., Grote, J., & Moore, J. A. (1999). Chemoenzymatic Synthesis of 3 '-O-(Carboxyalkyl) fluorescein Labels. *Bioconjugate chemistry*, *10*(3), 544-547. [https://doi.org/10.1021/bc990009u]
86. Martin, M. M., & Lindqvist, L. (1975). The pH dependence of fluorescein fluorescence. *Journal of Luminescence*, *10*(6), 381-390. [https://doi.org/10.1016/0022-2313(75)90003-4]
87. Fleming, G. R., Knight, A. W. E., Morris, J. M., Morrison, R. J. S., & Robinson, G. W. (1977). Picosecond fluorescence studies of xanthene dyes. *Journal of the American Chemical Society*, *99*(13), 4306-4311. [https://doi.org/10.1021/ja00455a017]
88. Burchak, O. N., Mugherli, L., Chatelain, F., & Balakirev, M. Y. (2006). Fluorescein-based amino acids for solid phase synthesis of fluorogenic protease substrates. *Bioorganic & Medicinal Chemistry*, *14*(8), 2559-2568. [https://doi.org/10.1016/j.bmc.2005.11.037]
89. Tian, M., Wu, X. L., Zhang, B., Li, J. L., & Shi, Z. (2008). Synthesis of chlorinated fluoresceins for labeling proteins. *Bioorganic & medicinal chemistry letters*, *18*(6), 1977-1979. [https://doi.org/10.1016/j.bmcl.2008.01.114]
90. Griffin, B. A., Adams, S. R., & Tsien, R. Y. (1998). Specific covalent labeling of recombinant protein molecules inside live cells. *Science*, *281*(5374), 269-272. [https://doi.org/10.1126/science.281.5374.269]
91. Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., ... & Tsien, R. Y. (2002). New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *Journal of the American Chemical Society*, *124*(21), 6063-6076. [https://doi.org/10.1021/ja017687n]
92. Spagnuolo, C. C., Vermeij, R. J., & Jares-Erijman, E. A. (2006). Improved Photostable FRET-competent biarsenical− tetracysteine probes based on fluorinated fluoresceins. *Journal of the American Chemical Society*, *128*(37), 12040-12041. [https://doi.org/10.1021/ja063212q]

93. Martin, B. R., Giepmans, B. N., Adams, S. R., & Tsien, R. Y. (2005). Mammalian cell–based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nature biotechnology*, *23*(10), 1308-1314. [https://doi.org/10.1038/nbt1136]
94. Wang, L., Du, W., Hu, Z., Uvdal, K., Li, L., & Huang, W. (2019). Hybrid rhodamine fluorophores in the visible/NIR region for biological imaging. *Angewandte Chemie International Edition*, *58*(40), 14026-14043. [https://doi.org/10.1002/anie.201901061]
95. Kolmakov, K., Belov, V. N., Bierwagen, J., Ringemann, C., Müller, V., Eggeling, C., & Hell, S. W. (2010). Red-emitting rhodamine dyes for fluorescence microscopy and nanoscopy. *Chemistry–A European Journal*, *16*(1), 158-166. [https://doi.org/10.1002/chem.200902309]
96. Wippersteg, V., Ribeiro, F., Liedtke, S., Kusel, J. R., & Grevelding, C. G. (2003). The uptake of Texas Red-BSA in the excretory system of schistosomes and its colocalisation with ER60 promoter-induced GFP in transiently transformed adult males. *International Journal for Parasitology*, *33*(11), 1139-1143. [https://doi.org/10.1016/S0020-7519(03)00168-1]
97. Horneffer, V., Forsmann, A., Strupat, K., Hillenkamp, F., & Kubitscheck, U. (2001). Localization of analyte molecules in MALDI preparations by confocal laser scanning microscopy. *Analytical chemistry*, *73*(5), 1016-1022. [https://doi.org/10.1021/ac000499f]
98. Guzikowski, A. P., Naleway, J. J., Shipp, C. T., & Schutte, R. C. (2000). Synthesis of a macrocyclic rhodamine 110 enzyme substrate as an intracellular probe for caspase 3 activity. *Tetrahedron Letters*, *41*(24), 4733-4735. [https://doi.org/10.1016/S0040-4039(00)00708-5]
99. Lavis, L. D., Chao, T. Y., & Raines, R. T. (2006). Fluorogenic label for biomolecular imaging. *ACS chemical biology*, *1*(4), 252-260. [https://doi.org/10.1021/cb600132m]
100. Bandichhor, R., Petrescu, A. D., Vespa, A., Kier, A. B., Schroeder, F., & Burgess, K. (2006). Water-soluble through-bond energy transfer cassettes for intracellular imaging. *Journal of the American Chemical Society*, *128*(33), 10688-10689. [https://doi.org/10.1021/ja063784a]

101. Gao, Y., Li, J. L., & Shi, Z. (2007). Synthesis and application of rhodamine-based fluorescent probe dyes with spacer linker arm. *Chinese Chemical Letters*, *18*(6), 667-669.
[https://doi.org/10.1016/j.cclet.2007.04.007]

Abbreviations used in this chapter:

PNAs: peptide nucleic acids
Glu: glutamic acid
Cys: cysteine
τ: fluorescence lifetimes
Gly: glycine
Val: valine

BSA: bovine serum albumin
PBS: phosphate-buffered saline
NHS: *N*-hydroxysuccinimide
Φ_F: fluorescent quantum yield
ε: molar absorptivity (M^{-1} cm^{-1})
SPPS: solid-phase peptide synthesis
DIEA: diisopropylethylamine
TIS: triisopropylsilane
HOBt: 1-hydroxybenzotriazole
Boc_2O: di-*tert*-butyl pyrocarbonate
DIC: diisopropylcarbodiimide
$\Delta\lambda$: Stokes' shift in nm
HOSu: *N*-hydroxyssuccinimide
HSA: human serum albumin
LC: liquid chromatography
DPPA: diphenylphosphoric azide
Phe: phenylalanine
NIR: near-infrared
MeOH: methanol
EDT: ethanedithiol
Pro: proline
Lys: lysine
Ala: alanine
*t*Bu: *tert*-butyl

FRET: fluorescence resonance energy transfer
λ_{abs}: wavelength of maximum absorption
λ_{ex}: wavelength of maximum excitation
TRIS: tris(hydroxymethyl)aminomethane
λ_{em}: wavelength of maximum emission
$\Delta\nu$: Stokes' shift in cm^{-1}