Fluorescent Labeling of Biomolecules with Organic Probes

M. Sameiro T. Gonçalves*

Centro de Química, Universidade do Minho, Gualtar, 4710-057 Braga, Portugal

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Contents

M. Sameiro T. Gonçalves was born in Melgaço, Portugal, in 1971. She obtained her Ph.D. degree in 1997, from the University of Minho, Braga, Portugal, and followed this with several months of postdoctoral research at the same university. Also in 1997 she was appointed as an Invited Auxiliary Professor at University of Oporto, Portugal. In 1998 she took the position of Assistant Teacher at University of Minho and Auxiliary Professor from 1999 to present. Her research interests include the synthesis, photophysical characterization, and investigation of the biological activity of oxygen and nitrogen fluorescent heterocyclic compounds with potential application in biomedicine as biomarkers, phototriggers, and photocleavable protecting groups either in solution or solid-phase organic synthesis.

1. Introduction

Many areas of modern technology are dependent on sensitive analytical techniques, and this is particularly true for environmental science, medicine, pharmacy, and cellular biology, where there have been many new developments in new techniques and reagents.

Labeling is one of the most common methodologies used for bioanalytical purposes, which can be carried out with radioactive materials or compounds with absorption and/or fluorescence from the ultraviolet to the near-infrared region of the electromagnetic spectrum.

Use of organic molecules in nonfluorescent labeling, in the ultraviolet and visible regions, is important in several applications. However, in recent years, detection based on fluorescence techniques has received special attention and notable progress has been made in both fluorescence instrumentation and synthesis of new fluorophores.

Fluorescence labeling can be extended over a wide range of wavelengths using semiconductor nanocrystals, fluorescent proteins, or organic molecules.

The organic fluorophores may form covalent or noncovalent linkages with the sample to be analyzed, producing the respective conjugates or complexes that can show fluorescence from short to very long wavelengths, depending on the marker used.

* To whom correspondence should be addressed. Fax: +351-253604382. E-mail: msameiro@quimica.uminho.pt.

In addition to the intrinsic interest of new fluorophores, development of fluorophores with absorption and emission at long wavelengths is of extreme importance for biological purposes.

The main aim of this review is to provide insight into the state of the art in the field of the synthesis and application of organic fluorescent markers for labeling of amino acids, peptides, proteins, DNA, and other biomolecules. Nanocrystal semiconductors[1−5] and fluorescent proteins[6,7] are not discussed since recent reviews on these subjects have already been published.

The organic fluorophores are considered in the order of their increasing absorption and emission wavelength values when conjugated to or complexed with an analyte. Thus, recent developments in compounds providing fluorescence from the near-ultraviolet to approximately 500 nm are first reviewed, and this includes oxobenzopyrans, naphthofurans, oligothiophenes, 4,7-phenanthroline-5,6-diones, benzooxadiazoles, dansyl chloride, naphthalene 2,3-dicarboxaldehyde, and 6-propionyl-2-(dimethylamino)naphthalene. In the case of organic fluorescent labels emitting between 500 nm and the near-infrared (ca. 900 nm), the chemical classes discussed comprise fluoresceins (including biarsenical dyes), rhodamines, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacenes (BODIPY dyes), squaraines, and cyanines.

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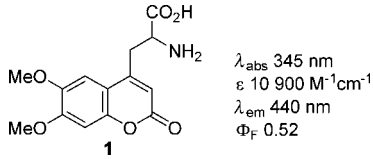


Figure 1. Structure of Dmca (**1**).

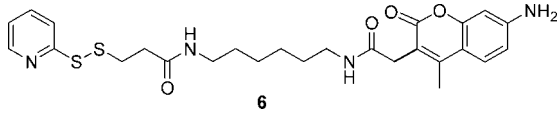


Figure 2. Structure of AMCA-HPDP (**6**).

Many oxazine derivatives, namely, phenoxazines and benzophenoxazines, which can fluoresce up to about 700 nm, have better photochemical stability than cyanine dyes, but most of them possess limited solubility in water. Examples of these compounds include Nile Red, Nile Blue, and their derivatives, and these have found numerous applications as molecular probes of biological interest. In recent years, efforts have been made to synthesize new analogues with improved water solubility and functional groups that allow for covalent linkage to the analyte. Despite their importance, these fluorophores are not discussed in this manuscript since use of benzophenoxazine-based fluorescent dyes for labeling biomolecules has recently been reviewed.[8]

In these overviews, the photophysical properties (for absorbance and emission) of the relevant labels are shown wherever possible, both before and after linkage to an analyte.

2. Organic Labels with Emission up to 500 nm

2.1. Fluorophores Based on Oxygen Heterocycles

3-Oxo-3*H*-benzopyrans, commonly designated as coumarins, present one of the most sensitive and commercially accepted categories of reagents for fluorescent derivatization. Many fluorophores of this type are mentioned in the literature, and an exceptionally high number of articles refer to determination of carboxylic acid compounds using such labels.[9,10]

Modified fluorogenic amino acids with oxobenzopyrans appear to be interesting molecules due to their extended spectral range, high emission quantum yields, photostability, and good solubility in several solvents.[11]

2-Amino-3-(6,7-dimethoxy-3-oxo-3*H*-benzopyran) propanoic acid (Dmca, **1**) (Figure 1) shows maximum absorption and emission wavelengths at 345 and 440 nm, respectively.[12−14] These properties enable selective determination of Dmca (**1**)-labeled peptides even when amino acid tryptophan (Trp, W) residues are present. Furthermore, compound **1** possesses a high fluorescent quantum yield (Φ_F) and large molar absorptivity (ϵ) (Φ_F 0.52 and ϵ 10 900 M^{-1} cm^{-1}), which facilitate detection of the labeled peptides on a picomolar scale, with a sensitivity similar to that of radiolabeling.

Déleris and collaborators[15] described the synthesis of two novel oxobenzopyran-labeled lysines. Carboxylic acids **2** and **3**, prepared according to modified well-known procedures,[16−22] were activated through the *N*-hydroxysuccinimide ester and coupled with Fmoc-Lys-OH (Scheme 1).

The wavelengths of the absorption and emission maxima (λ_{abs} /λ_{em}) for compounds **5a** and **5b** were 350/404 and 431/480 nm, respectively (Table 1). The linkage of heterocycles **2** and **3** to the lysine residue resulted in red shifts in the absorption values of 15 and 24 nm, respectively.

Scheme 1. Synthesis of the Reactive Fluorophores 4a and 4b and Their Use in the Labeling of Lysine

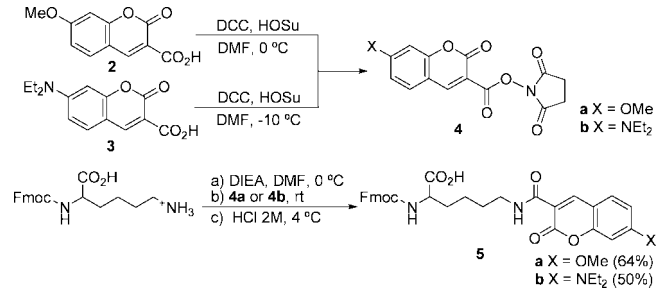


Table 1. Optical Properties of Compounds 2, 3, 5a, and 5b in 0.1 M TRIS-HCl Buffer, pH 9.0

compound	λ_{abs}, nm (ϵ, M^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	$\Delta\lambda$ (nm)
2	335(19 000)	335	400	65
3	407(32 000)	407	470	63
5a	350(17 500)	335	404	69
5b	431(42 000)	407	480	73

The authors suggested use of the labeled lysine **5a**,**b** as fluorescent building blocks for solid-phase peptide synthesis (SPPS).[23−25]

Protein S-nitrosylation (modification of a cysteine thiol group in a protein by a nitrosyl [NO]^2 group) operates as an important switch in regulating protein functions. A number of proteins have been documented to be S-nitrosylated in vitro or in vivo, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[26,27] protein disulfide isomerase (PDI),[28] parkin,[29] and β-actin.[30] In 2008, Chen et al.[31] reported a fluorescence-based method in which the S-nitrosylated cysteines are converted into 3-(7-amino-4-methyl-2-oxo-2*H*-benzopyran) ethanoic acid (AMCA) fluorophore-labeled cysteines: termed the AMCA switch method. AMCA-HPDP (**6**) (Figure 2) was used in the labeling step. The labeled proteins were then analyzed by nonreducing SDS−PAGE, and the S-nitrosylated proteins could be readily detected as brilliant blue bands under ultraviolet light. When combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) the method provided an accurate identification of nitrosocysteines with the AMCA adduct as the label.

Ivana and Pavol reported the synthesis of new fluorescent probes suitable for site-specific incorporation into oligonucleotides.[32] Oxobenzopyran derivatives (**7**−**10**) were used as sensitive fluorescent labels, and two linkers (**11** and **12**) were attached to the basic oxobenzopyran skeleton to produce the functionalized derivatives (**13**−**15**, **18**, and **19**) (Scheme 2). The hydroxy oxobenzopyran derivatives **10a** and **10b** are commercially available, and carboxylic acid heterocycles **7**−**9** were synthesized via the Pechman reaction.[33]

Optical properties of compounds **7**−**10**, **13**−**15**, **18**, and **19** were measured in methanol (Table 2). The results showed that conjugates **13**−**15** and **18**−**19** had emission maxima between 381 and 426 nm with fluorescence quantum yields in the range 0.02−0.30. Compound **14**, which presented the best fluorescent properties, was chosen for preparation of a benzopyranylphosphoramidite linker **20** suitable for direct incorporation into oligonucleotides.

In addition to their importance in the derivatization of amino acids,[15] peptides,[34] and nucleic acids,[35,36] these

Scheme 2. Synthesis of Oxobenzopyran-Based Fluorescent Probes 13−15, 18, and 19

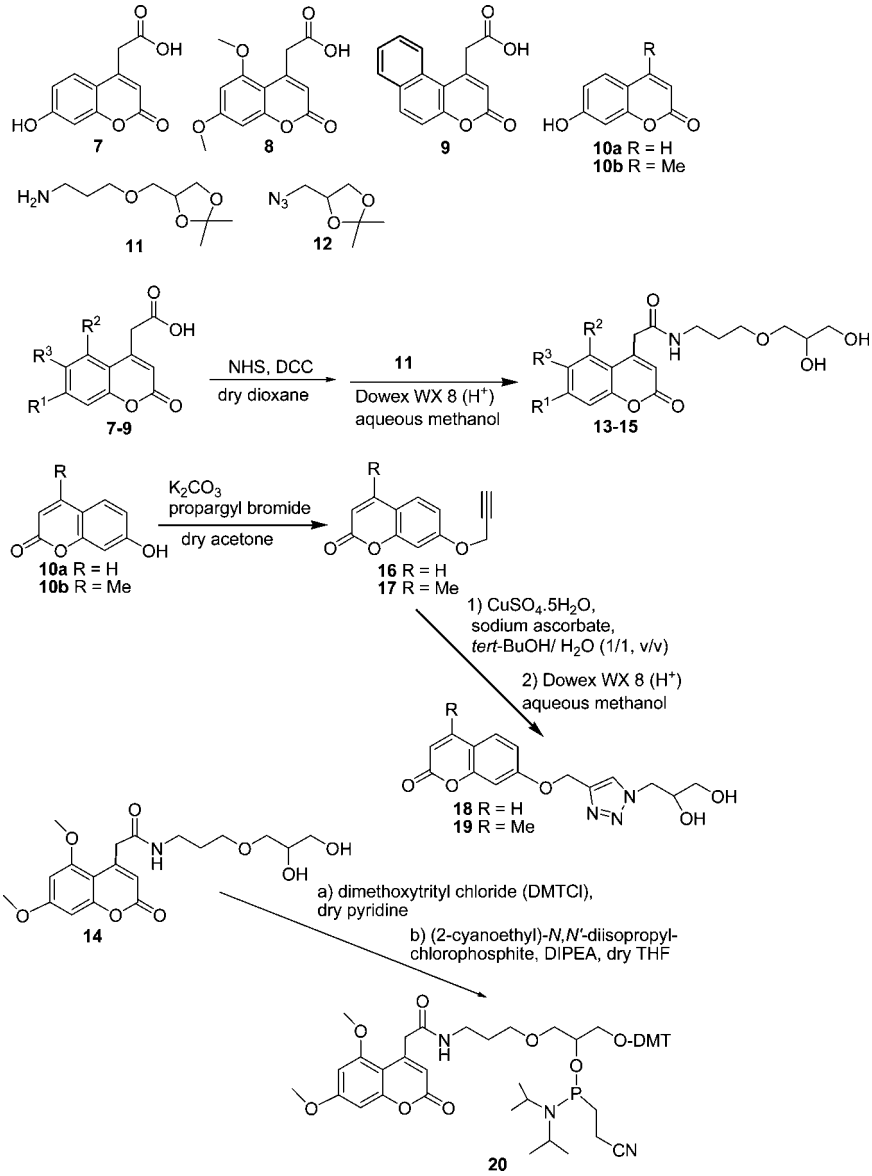
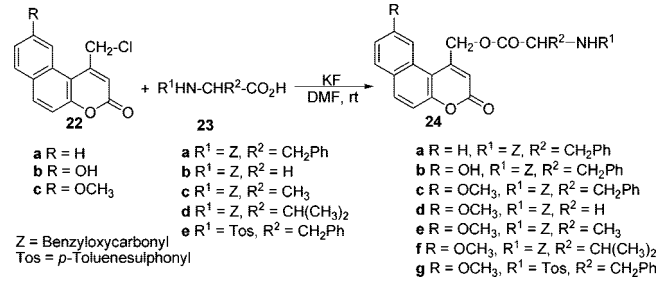


Table 2. Optical Properties of Compounds 13−15, 18, and 19

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

Scheme 3. Synthesis of Fluorescent Conjugates 24a−g



oxobenzopyran heterocycles have also been used as fluorophores in the study of enzymes.[37,38]

Shutes and Der[39] reported the labeling of phosphate-binding protein (PBP) with fluorophore **21** (MDCC) (Figure 3)[40,41] and its use in measuring GTPase activity. The MDCC-PBP probe provides an easy method of examining GTP hydrolysis through GTPases in vitro, in real time.

Recently, functionalized oxobenzo[*f*]benzopyrans **22a−c** were used for efficient preparation of several fluorescent α-amino acid derivatives (**24a−g**) (Scheme 3).[42] The resultant fluorescent conjugates (**24a−g**) showed high Stokes' shifts (66−131 nm) with λ_{abs} values in the range 345−360 nm and λ_{em} values in the range 411−478 nm (Table 3). Heterocycles **22a−c** displayed low Φ_F values (0.02−0.08), which increased dramatically upon reaction with the amino

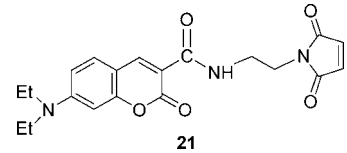
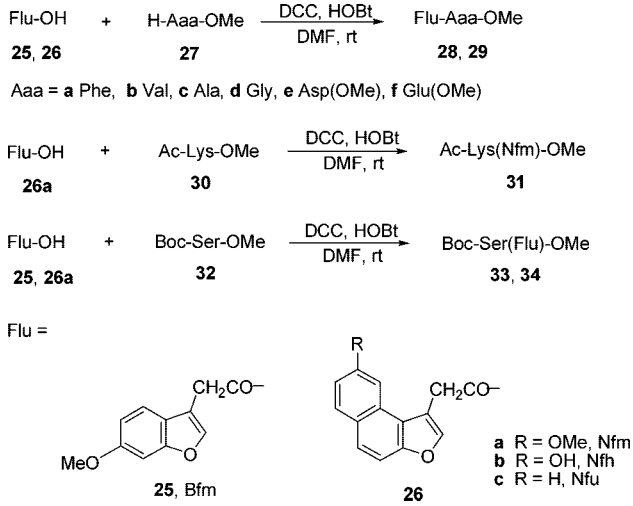


Figure 3. Structure of MDCC (**21**).

Table 3. Optical Properties 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)
22a	352(11 449)	418	0.08	66
22b	361(12 190)	462	0.02	101
22c	354(12 826)	472	0.03	118
24a	345(14 125)	411	0.42	66
24b	360(10 174)	456	0.13	96
24c	347(12 075)	478	0.59	131
24d	347(11 436)	471	0.70	124
24e	348(11 640)	477	0.66	129
24f	348(11 830)	478	0.58	130
24g	347(12 883)	475	0.53	128

Scheme 4. Synthesis of labeled Amino Acid Derivatives 28, 29, 31, 33, and 34



acids (Φ_F 0.13−0.70, **24a**−**g**). On the basis of their photophysical properties, it was suggested that heterocycles **22a**−**c** were potential candidates for the fluorescent labeling of peptides and other biomolecules.

Benzofurans and naphthofurans are other polycyclic oxygen heterocycles which have been reported as fluorescent markers for biomolecules.[43,44] The authors used the carboxylic benzofuran (**25**) and naphthofuran derivatives (**26a**−**c**) in the derivatization of α-amino acids (**27**, **30**, and **32**) located in the amine function of their main (**27**) or lateral (lysine, **30**) chains as well as in the hydroxyl group of the lateral chain (serine, **32**) (Scheme 4).

Considering the photophysical data of the fluorescent amino acid residues (**28a**,**b**, **29a**−**h**, **31**, **33**, and **34**), 8-methoxy-naphto[2,1-*b*]furan-1-yl ethanoic acid (**26a**) was found to be the most suitable derivative for labeling purposes. The amino acid−naphthofuran conjugates (**29a**−**h**, **31**, and **34**) had absorption and emission maxima at about 298 and 325−350 nm, respectively; the fluorescence quantum yields varied between 0.10 and 0.44 (Table 4).

2.2. Fluorophores Based on Sulfur Heterocycles

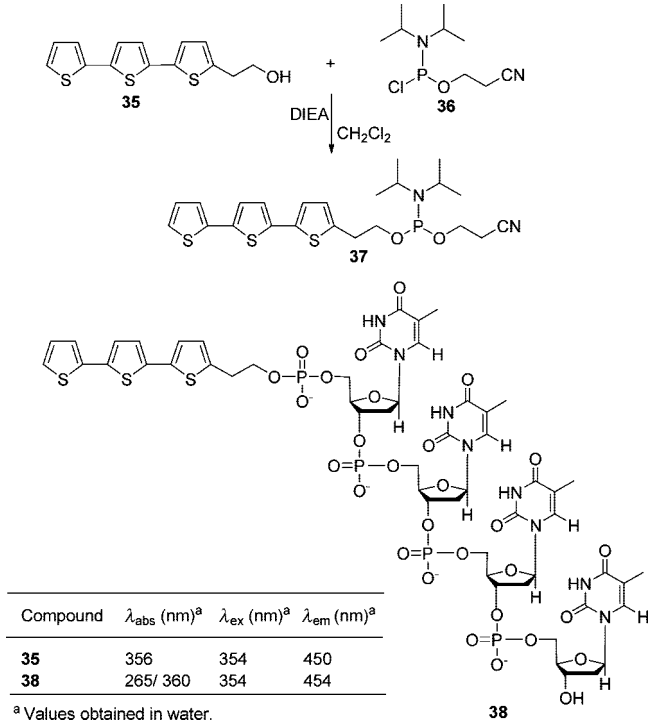
Oligothiophenes are compounds with intrinsic fluorescence whose properties can be easily modified synthetically, and they have attracted great interest in recent years.[45−47] Despite these characteristics, very little attention has been given to the development of fluorescent markers for biomolecules based on this heterocycle family.[48]

Capobianco and collaborators[49] demonstrated, for the first time, the suitability of an oligothiophene, namely, terthiophene **35**, for use as a fluorescent tag for oligonucleotides

Table 4. Optical Properties 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)
25	Bfm-OH	285	315	0.020	30
26a	Nfm-OH	298	349	0.20	51
26b	Nfh-OH	301	349	0.062	48
26c	Nfu-OH	293	340	0.076	47
28a	Bfm-Phe-OMe	288	315	0.064	27
28b	Bfm-Val-OMe	288	315	0.070	27
29a	Nfm-Phe-OMe	298	349	0.32	52
29b	Nfm-Val-OMe	298	346	0.37	49
29c	Nfh-Val-OMe	300	350	0.10	50
29d	Nfu-Val-OMe	292	325	0.13	33
29e	Nfm-Ala-OMe	298	349	0.24	49
29f	Nfm-Gly-OMe	297	343	0.24	46
29g	Nfm-Asp(OMe)-OMe	298	346	0.14	48
29h	Nfm-Glu(OMe)-OMe	298	347	0.14	49
31	Ac-Lys(Nfm)-OMe	297	347	0.44	50
33	Boc-Ser(Bfm)-OMe	287	314	0.064	27
34	Boc-Ser(Nfm)-OMe	298	349	0.13	51

Scheme 5. Preparation of Phosphoramidite of 35 and the Terthiophene-T_4 Conjugate (38) Prepared with 37



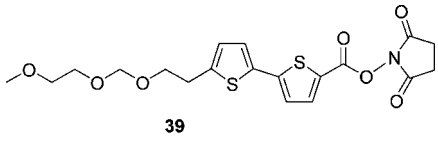
Compound	λ_{abs} (nm)[a]	λ_{ex} (nm)[a]	λ_{em} (nm)[a]
35	356	354	450
38	265/ 360	354	454

[a] Values obtained in water.

via phosphoramidite coupling. The authors reported the preparation of derivative **37**, starting with the oligothiophene **35**, and its incorporation into oligonucleotides for biochemical studies (Scheme 5).

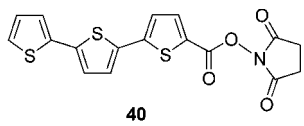
The resulting conjugate **38** showed absorption maxima at 265 and 360 nm and an emission maximum at 454 nm in water. The intensity of fluorescence of this conjugate allows for its easy detection at submicromolar concentrations such as those usually required in hybridization or microscopy studies. Furthermore, the emission signal is stable under prolonged ultraviolet irradiation, contrary to other bioconjugates labeled with many of the more widely used fluorophores, for example, fluorescein whose emission decays in few minutes.

Later, Sotgiu et al.[50] verified that oligothiophenes functionalized with the *N*-succinimidyl ester (**39** and **40**, Figure 4) reacted under mild conditions with both monoclonal antibodies and $-NH_2$-terminated oligonucleotides to produce bioconjugates that were photochemically and chemically very



39

Conjugate of **39** with monoclonal antibody anti-CD3:
λ_{abs} 350 nm, λ_{ex} 340 nm, λ_{em} 425 nm
(in sodium cacodylate buffer)

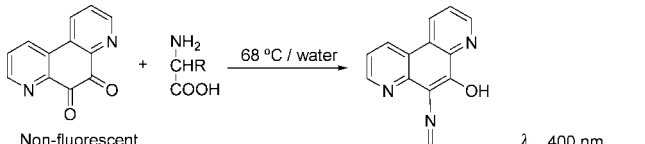


40

Conjugate of **40** with oligonucleotide
5' GCGGTAGTGTGGTTCGAAGGGTGGTACCGC-$(CH_2)_6$-3'-NH_2:
λ_{em} 480 nm (in sodium cacodylate buffer)

Figure 4. Oligothiophenes functionalized with *N*-succinimidyl ester.

Scheme 6. Derivatization Reaction of Amino Acids with 4,7-Phenanthroline-5,6-dione (Phanquinone) Reagent (41)



stable and could be used for cellular imaging and fluorescence resonance energy transfer experiments, respectively. The maximum emission wavelengths of the fluorescent conjugates of **39** with monoclonal antibodies anti-CD3 and **40** with the oligonucleotide functionalized with an amine terminal group at the 3′ position occurred at 425 (**39**) and 480 nm (**40**), respectively.

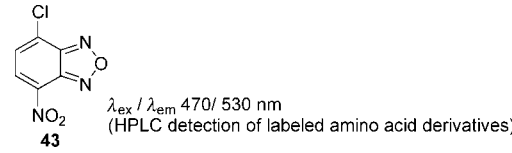
2.3. Fluorophores Based on Nitrogen Heterocycles

High-performance liquid chromatography (HPLC) associated with pre- or postcolumn chemical derivatization represents an efficient tool in the analysis of amino acids. Two classes of heterocyclic fluorescent derivatization reagents for amino acids and peptides reported in recent years are the 4,7-phenanthroline-5,6-diones (phanquinones) and benzooxadiazoles.[51−55]

Gatti and collaborators[51] used 4,7-phenanthroline-5,6-dione (**41**) as a fluorogenic labeling reagent in precolumn derivatization for the LC separation of amino acids. This reacted with the primary amino function of L-amino acids (for example, leucine, valine, and phenylalanine) to produce fluorescent derivatives (**42**) (Scheme 6), which could be separated by reverse-phase HPLC; these had $\lambda_{ex} = 400$ nm and $\lambda_{em} = 460$ nm.

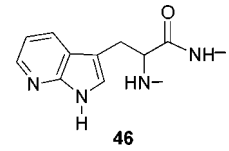
Gatti et al.[52] recently continued these studies and verified the applicability of phanquinone (**41**) to the analysis of amino acids, such as D,L-*p*-serine.

The benzooxadiazoles substituted in positions 4 and 7,[53] namely, the 4-chloro-7-nitrobenzooxadiazole (NBD-Cl), **43**, (Figure 5), have been used as derivatization reagents for amino acids in HPLC analysis[54,55] and also for proteins (for example, synthetic derivatives of the neurohormones oxytocin, vasopressin, and insulin) in electrophoresis.[56] Detection characteristics were ca. $\lambda_{ex} = 470$ nm and $\lambda_{em} = 530$ nm.



43

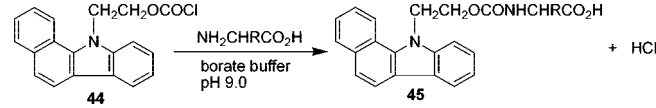
Figure 5. Structure of NBD-Cl (**43**).



46

Figure 6. Structure of 7-azatryptophan (AW, **46**).

Scheme 7. Derivatization of Amino Acids with BCEOC (44)



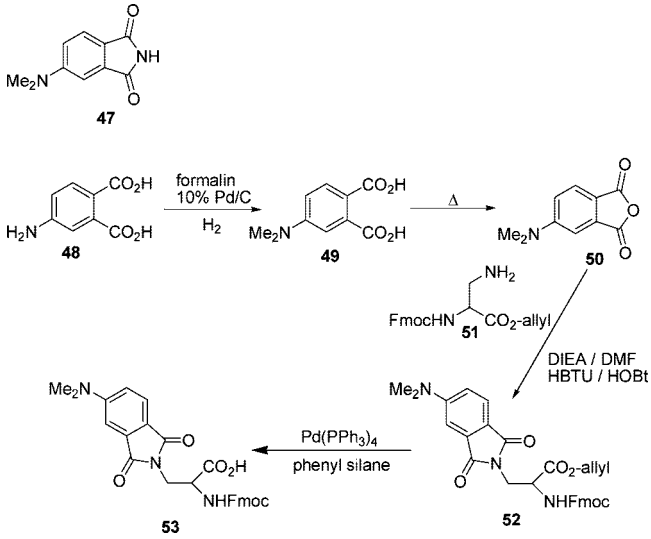
The 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC, **44**)[57,58] (Scheme 7) was recently used by You-Rui et al.[59] as a precolumn derivatization agent for determination of amino acids in serum from rats in three activity states (quiet, exhausted after exercise, and 12 h after exhaustion). The relationship between the exercise state and the serum content of excitatory amino acids (branched chain) and inhibitory amino acids (aromatic amino acids) was investigated.

The amino acid tryptophan has been widely used as an intrinsic fluorescent probe to study protein dynamics and ligand binding in solution.[60] However, its complex photophysics makes interpretation of fluorescence data difficult and can often inhibit universal interpretation of fluorescence data.[61] Furthermore, proteins usually possess more than one Trp residue, which complicates the assignment of a change in the fluorescence signal to a specific site. It is also difficult to interpret the spectral changes that result from intermolecular protein−protein association.[62] Development of covalent extrinsic fluorophores and their linkage to protein functional groups[63,64] has been used to solve this problem.

Among the noncoded W analogs studied (i.e., 5-hydroxy- and 5-methoxy-Trp, benzo[*b*]thiophenylalanine, and the spectrally silent fluorotryptophans), 7-azatryptophan (AW) (**46**) (Figure 6) exhibits interesting optical properties[65] and its incorporation into proteins has been used to study protein structure,[66,67] stability,[68,69] macromolecular recognition,[70] and protein conformational change.[71] Contrary to W, free AW (**46**) displays single-exponential fluorescence decay,[72] and the presence of a nitrogen atom at the 7-position in the indoyl nucleus resulted in a red shift of 10 nm in the absorption of AW (**46**) and 46 nm in its emission compared with Trp.[62] The fluorescence emission wavelength and quantum yield of 7-azaindole (7 AI) are strongly influenced by the polarity of the environment. In particular, when going from cyclohexane to water, the λ_{em} of 7AI shifts from 325 to 400 nm and the Φ_F increases from 0.01 in aqueous solution (pH 7) to 0.25 in acetonitrile.[62]

Fillipis et al. demonstrated that solid-phase synthesis is an appropriate method to induce AW (**46**) even in long polypeptide chains.[73] These authors reported the chemical synthesis of an analogue of the N-terminal proteolytic fragment 1−47 of hirudin (a disulfide-cross-linked polypeptide, known as the most highly potent and specific thrombin inhibitor) in which Tyr 3 was replaced by AW (**46**) and

Scheme 8. Synthesis of Compound 53



carried out its conformational and functional characterization. The interaction of Y3AW with thrombin was studied by exciting the sample at 320 nm and recording the change in fluorescence of Y3AW on binding to the enzyme. The results indicated that the fluorescence of AW (**46**) of hirudin 1−47 in the Y3AW−thrombin complex was strongly quenched, possibly due to the presence of two structural water molecules at the hirudin−thrombin interface that could promote the nonradiative decay of AW (**46**) in the excited state. On the basis of these results, Fillipis et al. suggested that incorporation of AW might have a broad applicability in the study of protein folding and protein−protein interaction.

The 4-(*N*,*N*-dimethylamino)-phthalimide system (4-DMAP) (**47**, Scheme 8) has been accepted as an extraordinarily environment-sensitive fluorophore whose optical properties are readily modified in response to changes in polarity and viscosity often found in organized media.[74−76] On changing the solvent from water to 1,4-dioxane,[77] this fluorescent sensor showed a 70-fold increase in its fluorescence quantum yield; the fluorescence maximum was shifted by as much as 100 nm. Although 4-DMAP (**47**) possesses the required photophysical properties for its use in biological applications, sensors based on this have only found limited use in complex biological systems.[78]

Imperiali et al. [79] reported the synthesis of a new 4-(*N*,*N*-dimethylamino) phthalimide (DAPA, **53**)-sensitive fluorescent building block for solid-phase peptide synthesis and its incorporation into peptides (Scheme 8).

Evaluation of compound **53** for monitoring the binding of peptides to 14−3−3 proteins (proteins involved in phosphoserine-dependent signaling and essential intermediates in cell cycle regulation)[80] was undertaken by incubation of the corresponding fluorescent peptide with 14−3−3 ζ protein. The fluorescence emission intensity at 510 nm was 6-fold greater when the peptide was complexed with 14−3−3 than when protein was absent, and the emission band shifted from 570 to 531 nm. This new fluorescent peptidic sensor for 14−3−3 proteins demonstrated great potential in monitoring protein/protein interactions.

2.4. Naphthalene Fluorophores

In addition to fluorophores based on heterocyclic rings, naphthalene-based fluorophores have been extensively investigated, for example, as labels for amino acids, peptides, and proteins.

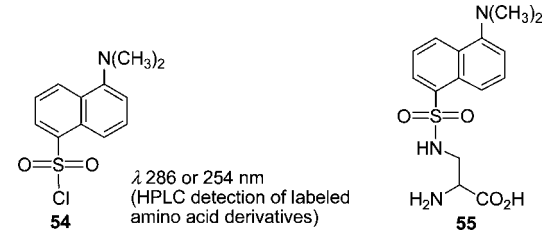


Figure 7. Structure of dansyl chloride (**54**) and a related fluorescent amino acid (**55**).

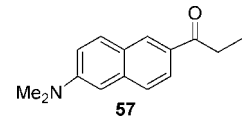
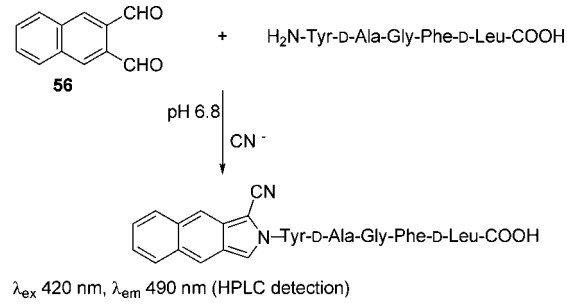


Figure 8. Structure of PRODAN (**57**).

Scheme 9. Derivatization Reaction of an Amino Terminal of a Peptide with NDA (56)



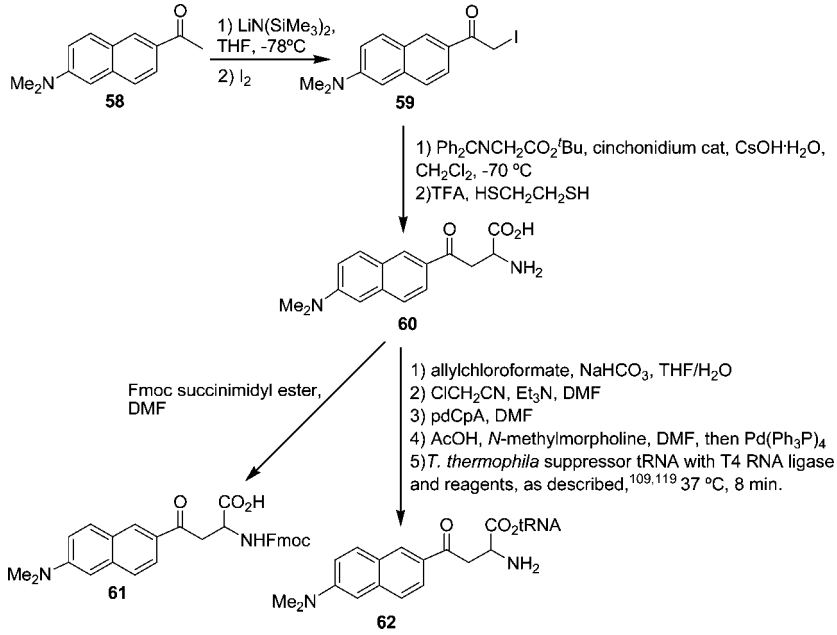
Chersie and collaborators used dansyl chloride (**54**, Figure 7) for direct "in synthesis" labeling of peptides.[81,82] More recently, several research studies were published relating to analysis of amino acids in biological samples through the HPLC technique ($\lambda = 286$ or 254 nm) using this compound as a precolumn derivatization reagent.[83,84]

Fluorescently labeled proteins are useful in a large number of bioanalytical applications including in vivo imaging, high-throughput screening, diagnostics, proteomics, and single-biomolecule spectroscopy.[85−89] Summerer et al. report a strategy for the selective and efficient biosynthetic incorporation of a low molecular weight fluorophore into proteins at defined sites.[90] Dansyl chloride (**54**, Figure 7) was used in the synthesis of the fluorescent amino acid 2-amino-3-[5-(dimethylamino)naphthalene-1-sulfonamide] propanoic acid (dansylalanine) (**55**), which was then incorporated into proteins in yeast at genetically specified sites in good yields and with high fidelity in response to the nonsense codon TAG.

The amino acid **55** is potentially useful for in vitro studies of protein structure, protein function, and bimolecular interactions, due to its relatively small size and its sensitivity to the local environment. Furthermore, it may be possible to extend this strategy to amino acids with increased fluorescent quantum yields or longer emission wavelengths, and this methodology could also be of value in direct imaging in mammalian cells.

Naphthalene-2,3-dicarboxaldehyde (NDA, **56**) has also been used as a fluorescent derivatization reagent for amino acids (including amino acid neurotransmitters) and peptides in HPLC and electrophoresis techniques that use fluorescence detection (Scheme 9).[91−100]

Scheme 10. Synthesis of ALADAN Derivatives 61 and 62



Recently, Clarke et al.[101] employed the HPLC technique using fluorescent precolumn derivatization with NDA (**56**) for quantification of amino acid neurotransmitters γ-aminobutyric (GABA) and glutamate ($\lambda_{ex}/\lambda_{em}$ 420/480 nm) in biological samples. Capillary electrophoresis has also recently been described for analysis of amino acids labeled with NDA.[102] The procedure was successfully applied in the determination of glutamic and aspartic acids in biological fluids, including human serum.

Fluorescent sensors incorporating environmentally sensitive fluorophores, which alter their spectral properties in response to changes in their environment, are of great importance in the study of biological processes. 6-Propionyl-2-(dimethylamino)naphthalene (PRODAN) (**57**) (Figure 8), first introduced by Weber and Farris,[103] is an example of an environmentally sensitive fluorophore.[104] Upon transfer to more hydrophobic environments there is a marked blue shift in the emission maximum wavelength and the fluorescence quantum yield increases.[105]

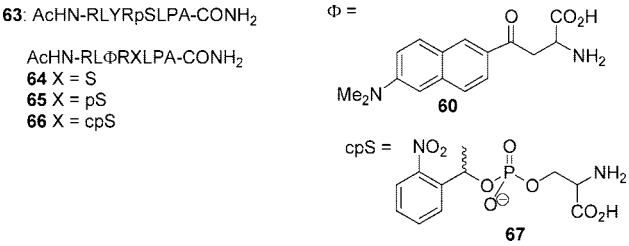
6-Dimethylamino-2-acylnaphthalene (DAN) (**58**) undergoes a large charge redistribution upon excitation and has the required properties for use as an environmental sensor.[103,106,107] This naphthalene compound has been used in the synthesis of the fluorescent PRODAN-based amino acid, 6-(2-dimethylaminonaphthoyl)alanine, DANA (**60**), which can probe the electrostatic character of a protein at multiple sites (Scheme 10).[108]

The alanine derivative (**60**) was converted into its Fmoc derivative (**61**) for solid-phase peptide synthesis or functionalized (**62**) for coupling to a *T. thermophila* suppressor tRNA[109] for nonsense suppression.

The study proved that DANA (**60**) presented some advantages over other probes in order to characterize protein dynamics, which included its keen environmental sensitivity and ability to site-specifically label proteins at buried, transmembrane, or interface positions, even in complex membrane proteins.

The non-natural alanine residue (**60**) possessing a fluorescent solvatochromic side chain has also been used for monitoring the phosphorylation-dependent binding of peptides to proteins.[104,110] Peptides **64**, **65**, and **66** incorporated

Scheme 11. Sequence of 14−3−3-Binding Peptides[a]



[a] **64**−**66**: peptides examined in this study (Φ = DANA (**60**) and cpS = cadged phosphoserine **67**).

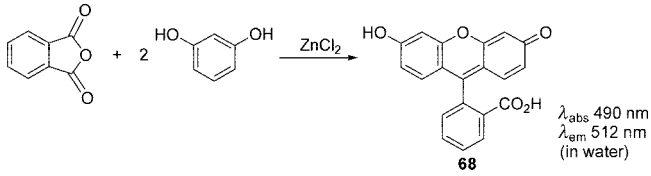
DANA (**60**) as well as the key serine residue in three forms: unmodified serine, phosphoserine, and caged phosphoserine were synthesized (Scheme 11).[111,112] Phosphoserine was caged as the 1-(2-nitrophenyl)ethyl derivative,[113−117] and this was photochemically releasable at above 300 nm.[118]

Incubation of 14−3−3 ζ isoform[119] with the unphosphorylated peptide **64** did not induce any change in the fluorescence spectrum. In contrast, phosphopeptide **65** showed a dramatic increase in the emission intensity upon addition of 14−3−3 ζ; moreover, a blue shift of the emission band was observed from 522 nm in the free form to 501 nm when bound to 14−3−3 ζ.

This work by Imperiali et al.[110] has shown that the uncaging of 1-(2-nitrophenyl)ethyl phosphoserine peptides can be used to release bioactive species. The authors also demonstrated that DANA-containing peptides can be used to monitor binding events in vitro. Designed peptides containing these two unnatural amino acid probes represent valuable tools for the study of phosphorylation-dependent interaction within complex signaling networks since binding of such species can be modulated with spatial and temporal control through photolysis. At the same time, they can be localized using the reporting fluorescent functionality.

In conclusion, the mentioned fluorophores provide labels which are potentially applicable to many types of study with amino acids, peptides, proteins, or oligonucleotides involving general fluorescence spectroscopy techniques, HPLC, and electrophoresis.

Scheme 12. Synthesis of Fluorescein (68)



68

3. Organic Labels with Emission Beyond 500 nm

3.1. Fluoresceins

Fluorescein (**68**), a polycyclic fluorophore with absorption and fluorescence maxima in the visible region of the electromagnetic spectra (λ_{abs} 490 nm and λ_{em} 512 nm, in water), is one of the most common labels used in biological applications.[120] It may be synthesized from phthalic anhydride and 1,3-dihydroxy-benzene (resorcinol) in the presence of zinc chloride via the Friedel−Crafts reaction (Scheme 12).[121,122] Alternatively methanesulfonic acid may be used as the catalyst.[123]

Although there are many commercially available fluorescent probes for labeling proteins, through either covalent or noncovalent interactions,[120] the most widely used fluorophore for this purpose is an amine-reactive probe derived from fluorescein.[124] In addition to its relatively high molar absorptivity, excellent fluorescence quantum yield, and good solubility in water, fluorescein possesses the advantage of an excitation maximum at 494 nm, which is close to the 488 nm spectral line of the argon laser, thus making it an important fluorophore for applications involving confocal laser-scanning microscopy and flow cytometry. Furthermore, protein conjugates based on fluorescein are not very susceptible to precipitation and can thus be obtained at high purity levels.

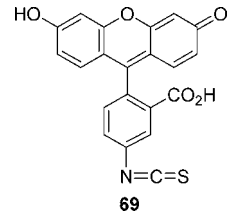
However, fluorophores derived from fluorescein and their macromolecular conjugates do have some disadvantages, namely: (i) a relatively high rate of photobleaching;[125−128] (ii) pH-sensitive fluorescence;[129−131] (iii) a relatively broad fluorescence emission spectrum, limiting their efficiency in multicolor applications;[132] (iv) a tendency to self-quench on conjugation to biopolymers, particularly at high degrees of substitution.[133,134]

The photobleaching and pH sensitivity of fluorescein make quantitative analysis with this fluorophore difficult. In particular, photobleaching limits sensitivity, which is undesirable for applications requiring ultrasensitive detection such as DNA sequencing, fluorescence in situ hybridization, and localization of low-abundance receptors. These limitations have encouraged the development of fluorescein derivatives with improved stability properties.

Some recent examples of the application of fluorescein and its derivatives in studies with amino acids, peptides, and proteins will be mentioned.

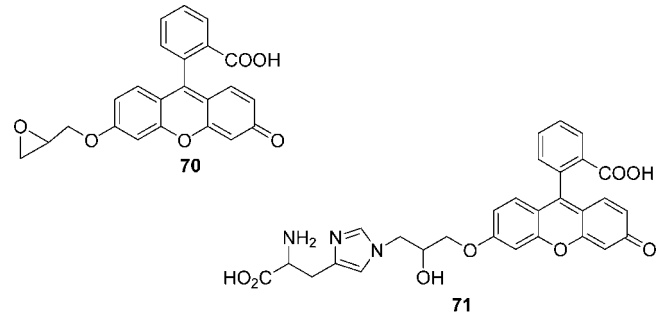
Labeling of peptides and proteins with fluorescein is usually carried out with commercially available reagents through amino or thiol groups of the lateral chain of lysine or cysteine residues.[120] Succinimidyl and maleidimyl fluorescein derivatives are used in the specific labeling of NH_2 and SH, respectively.[135,136] However, the most popular fluorophore used in the conjugation with proteins is fluorescein isothiocyanate (FITC) (**69**, Figure 9).[137,138]

The work developed by Kilár and Konecsni[139] exemplifies the use of the amine-reactive probe, fluorescein isothiocyanate, in the labeling of iron-free human serum transferrin,



69

Figure 9. Structrure of fluorescein isothiocyanate (FITC, **69**).



70 71

70, **71**
λ_{ex} 485 m, λ_{em} 513 nm
(aqueous solution, pH 12.0, NaOH)

Figure 10. 3-Epoxypropoxy fluorescein (EPF, **70**) and structure of the probable histidine fluorescent derivative (**71**).

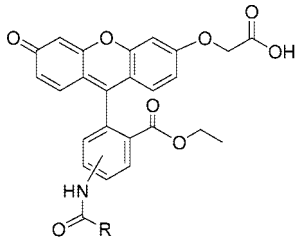
which was monitored using different dye−protein ratios. The degree of labeling was followed by capillary electophoresis. Transferrin is a monomeric serum glycoprotein which binds two ferric ions for delivery to vertebrate cells through receptor-mediated endocytosis. The purpose of this study was that of monitoring the labeling process of transferrin with FITC since the labeled protein can be used as a receptor-mediated endocytosis marker.[140]

Synthesis of a novel fluorescent probe, 3-epoxypropoxy fluorescein (EPF, **70**), was evaluated together with its properties for the selective labeling of histidine (Figure 10).[141] The probe included a fluorescein fluorophore with a long-wavelength response and an active epoxy labeling group. The EPF (**70**) had an excitation maximum at 485 nm and emission maximum at 513 nm, values which are very close to those of its parent fluorescein, but it also had a much lower quantum yield (0.27 compared to 0.95 for fluorescein). This may result from the fact that even in basic aqueous solution EPF (**70**) exists only in the form of the monoanion via ionization of its carboxylic acid functional group since the phenolic hydroxy group is blocked by epoxylation.

The authors verified that in alkaline media EPF (**70**) reacted selectively with histidine rather than with other amino acids (presumably generating compound **71**), producing a large increase in its intensity of fluorescence, which can be attributed to dianion formation of the labeling product via further ionization of carboxylic acid group of histidine.

The stability of the probe solutions and the reaction product with histidine (pH 12, NaOH) were both tested, and no significant alterations (<5%) in fluorescence intensity were observed within 1 month. As this proved to be a highly selective and sensitive method for histidine detection, it was developed and applied satisfactorily to histidine detection in human serum.

Giralt and Fernández−Carneado[142] studied the solid-phase fluorescent labeling of proline peptides (hexaproline P_6, dodecaproline P_{12}, and 18-proline P_{18})[143] in the N terminals with 5(6)-carboxyfluorescein (**72**) (Scheme 13). The authors suggested that the protocol described could find applications



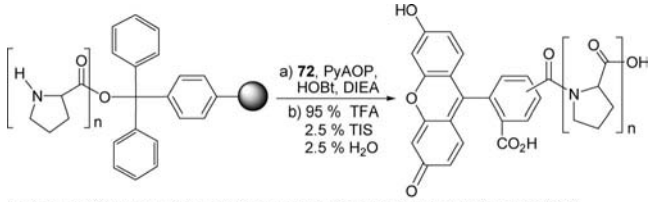
73

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-NH-Fmoc

λ_{abs} 456 and 481 nm, λ_{em} 520 nm
(sodium tetraborate, pH 9.8)

Figure 11. Fmoc-protected fluorescent amino acids.

Scheme 13. Conditions for the 5(6)-Carboxyfluoresceination Solid-Phase Reaction



7-Azabenzotriazolyoxytris(pyrrolidino) phosphonium hexafluorophosphate (PyAOP)

in other areas, such as solid-phase combinatorial synthesis of natural product-like molecules, polyamides, or PNAs, where fluorescent labeling for cell biology is also desirable.

Mannose-binding proteins on the surface of antigen-presenting cells (APCs) are able to recognize and internalize foreign agents in the early stages of immune response. These receptors offer a potential target for synthetic vaccines, especially those designed to stimulate T cells.[144,145]

Very recently, the 5(6)-carboxyfluorescein (**72**) has been used to prepare two sets of fluorescein-labeled peptides containing *O*-mannosylated serine units incorporated into peptide scaffolds with different numbers of alanines attached to a N^{α}-amino group or a side chain N^{ϵ}-amino group of lysine.[146] These *O*-mannosylated peptides will be tested in vitro for their ability to bind to mannose receptors or human APC subsets.

As noted previously, the low photostability and pH-dependent fluorescence of fluoresceins are the two most important factors that limit applications of these in bioanalysis.[147] Development of other heterocyclic derivatives with improved photochemical properties has therefore been the aim of several studies reported in the literature.[148]

The cost-efficient synthesis, photophysical characterization, and bioanalytical applications of new fluorescent amino acids have recently been described.[149] These compounds (**73a**−**c**, Figure 11), obtained from commercial fluorophores, possess the advantages over analogues (**68**) based on fluorescein of significantly increased photostability and pH-independent quantum yields.

Compounds **73a**−**c** exhibited two absorbance maxima at 456 and 481 nm. Their fluorescence emission spectra were broadened and red shifted (λ_{em} 520 nm) compared with those of fluorescein (λ_{em} 512 nm). The fluorescence quantum yields for **73** (Φ_F ca. 0.19) were notably lower than that for the fluorescein dianion (Φ_F ca. 0.92−0.93)[150−152] and consistent with the values reported for the 3-*O*-alkyl ethers (Φ_F ca. 0.13−0.31).[153,150] Broadening of emission spectra and decreasing quantum efficiency are probably due to modification of the D_{2h} molecular symmetry of the xanthene moiety in these compounds[154] and also to vibrational modes and the increased rate of nonradiative internal convertion from excited to ground states. The loss of D_{2h} symmetry in the fluorescein monoanion results in a similar broadening of its emission spectrum and a comparable drop in its fluorescence quantum yield (Φ_F ca. 0.25−0.37).[155,151] The fluorescence yields of Fmoc-protected amino acids (**73b**,**c**) (Φ_F ca. 0.04−0.12) are noticeably lower than that of the dye **73a**. This may result from fluorescence quenching induced by the Fmoc chromophore. Indeed, Fmoc-deprotection produced a significant increase in the fluorescence of the compounds **73b**,**c**.

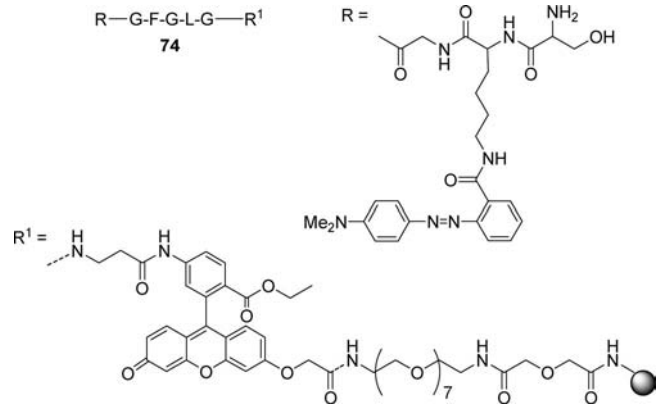


Figure 12. Structure of fluorogenic papain substrate (- - - indicates points of fluorophore attachment).

Consistent with the previous studies on 3-*O*-alkyl fluorescein 2′-esters[153,150,156] the fluorescence maxima and quantum yields for compounds **73** were pH independent. No significant changes were observed after incubation of fluorophores **73a** for several hours in aqueous pH 9.8 buffer. The pH independence exhibited by these fluorophores is of great benefit in applications requiring quantification and comparison of fluorescence intensity in different environments. Examples would include fluorescence measurements in acidic intracellular compartments[157,158] and studies of the pH dependence of enzymatic activity with fluorogenic substrates.[159,160] In such fluorescence methodologies as immunofluorescence when low concentrations of fluorescent molecules and/or small sample volumes are involved, irreversible destruction (photobleaching) of the fluorophore becomes a major limiting factor for fluorescence detectability.

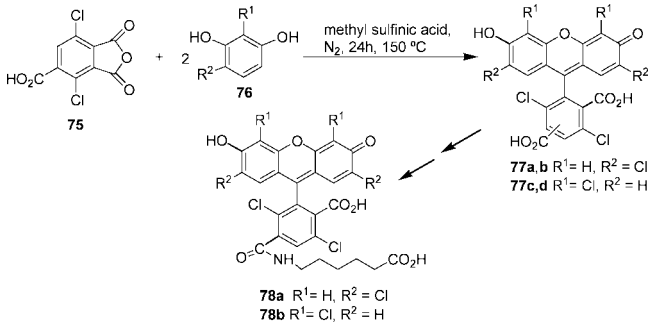
Under illumination conditions where fluorescein was rapidly bleached with a characteristic 50% decay time $t_{1/2} \approx 14$s the compound **73a** demonstrated remarkable photostability with only a ∼10% decrease in fluorescence for both fluorophores after 6 min irradiation.

Using one of these fluorophores and Methyl Red as a fluorescence quencher the authors also prepared the fluorogenic papain substrate (**74**, Figure 12) and demonstrated its applicability to the direct measurement of papain activity on TentaGel beads (the resin used in the synthesis process).

In fact, the fluorescent amino acids **73** synthesized by Balakirev et al.[149] are suitable for use in solid-phase organic synthesis and can be employed directly in the synthesis of fluorescent peptide ligands and fluorogenic protease substrates.[161,162] These dyes appear to have considerable potential for various bioanalytical applications.

Fluorescent probes with narrower fluorescence bands are a valuable tool in multicolor imaging, as, for example, in the high content analysis of live cells. Fluorescein derivatives

Scheme 14. Synthesis of Two New Chlorinated Fluoresceins 78a and 78b



tend to have relatively broad fluorescence bands, but selective substitution of fluoresceins by chlorine has been shown to be an effective way of producing probes with narrower emission bands.[163] In 2008, Tian and collaborators, reported the synthesis of two novel chlorinated fluoresceins 4,7,2′,7′-tetrachloro-6-(5-carboxypentyl)fluorescein (**78a**) and 4,7,4′,5′-tetra-chloro-6-(5-carboxypentyl)fluorescein (**78b**) as fluorescent probes for labeling proteins (Scheme 14).[164]

These two fluoresceins contain 6-aminohexanoic acid as a spacer linker. Compared to the fluorescein molecules that do not contain this linker the fluorescence of protein conjugates prepared from compounds **78a** and **78b** is not appreciably quenched, even at relatively high degrees of labeling. Furthermore, these fluorophores are more photostable than the nonchlorinated fluoresceins[163] and essentially pH insensitive in the physiological pH range.[165,166] These properties make the novel chlorinated fluoresceins interesting fluorophores for a variety of biological applications.

Succinimidyl esters, derivated from compounds **78a** and **78b**, were used for labeling of U2OS cells and Hela cells and found to exhibit strong fluorescence and good biocompatibility.

Although fluorescein derivatives have bulky and complex molecule structures, Kang et al. showed for the first time[167] the efficient incorporation of non-natural amino acids possessing these derivatives into a growing polypeptide, *Escherichia coli*.[168] However, the results showed that some dyes were not accepted in the eukaryotic in vitro expression system, indicating that the incorporation efficiency of nonnatural amino acids carrying fluorescent dyes varies according to the nature of the translational machinery and that nonnatural amino acids with fluorescein derivatives are not compatible with the eukaryotic biosynthetic machinery.

Study of the localization of proteins within live cells via fluorescence microscopy typically involves fusion of the required protein with a large fluorescent protein such as green fluorescent protein (GFP). Alternatively, fluorescent labeling technologies, such as the fluorescent biarsenical dye molecules (e.g., FlAsH and ReAsH), are desirable in the use of large fusion proteins.

First introduced by Griffin et al.,[169] biarsenical dyes were developed for the specific labeling of target peptides or proteins with small fluorescent dyes in vivo. In this method a genetically encodable motif of four cysteines in the sequence Cys-Cys-Xaa-Xaa-Cys-Cys (where Xaa is any amino acid except cysteine) attaches itself with high affinity to a fluorescent dye incorporating two arsenic moieties. The cell permeable fluorescent dye FlAsH [4′,5′-bis(1,3,2,-dithioarsolan-2-yl)fluorescein] (**79**) possesses two As(III) substituents that pair with the four cysteine thiol groups

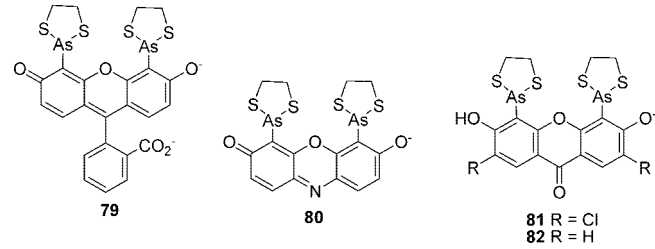


Figure 13. Structures of biarsenical dyes **79**−**82**.

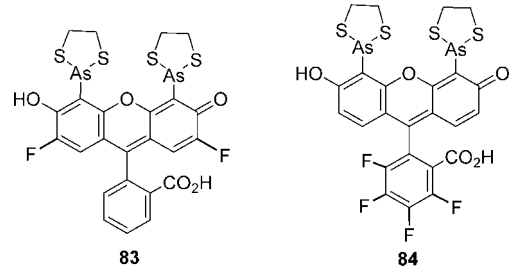


Figure 14. Structures of fluorinated fluorescein biarsenical dyes **83** and **84**.

located in the motif (Figure 13). The dye fluorescence intensity increases upon binding with the motif. An optimized tetracysteine (TC) sequence (-Cys-Cys-Pro-Gly-Cys-Cys-) provides high-affinity binding with a dissociation constant of 10 pM.[170] Quantitative labeling of the target occurs because of the specificity of the genetically encoded tetracysteine binding site as well as the high affinity of the binding itself.[169,170] The excitation and emission maxima of the FlAsH-TC complex are 508 and 528 nm, respectively.

The original FlAsH dyes were fluorescein derivatives. However, there are other cell-permeable biarsenical dyes available with different optical characteristics, namely, ReAsH (**80**) ($\lambda_{ex}/\lambda_{em}$ 593/608 nm), a phenoxazine derivative, and CHoXAsH (**81**) ($\lambda_{ex}/\lambda_{em}$ 380/430 nm), a xanthene derivative similar to the FlAsH compounds (Figure 13).[170]

The specific binding and membrane permeability properties of these biarsenical dyes offer important advantages for in-cells labeling compared to other conventional methods.

The combination of biarsenical dyes with visible fluorescence proteins (VFPs), as the Förster resonance energy transfer[171] donor−acceptor (DA) pairs, has been investigated by Jares-Erijman.[172] Because of the limited photostability and pH sensitivity of fluorescein derivatives in the physiological range, the authors introduced fluoro-substituted versions, F2FlAsH (**83**) and F4FlAsH (**84**) (Figure 14), and these proved to be significantly better in these respects compared to the original fluorescein derivative FlAsH (**79**).[173]

F2FlAsH (**83**) has a higher absorbance, larger Stokes' shift, higher fluorescence quantum yield, higher photostability, and reduced pH dependence compared to FlAsH (**79**). Emission of F4FlAsH (**84**) occurs in a wavelength region between that of FlAsH (**79**) and ReAsH (**80**)[170] and so provides a new color with excellent luminosity.

FlAsH-EDT_2 (**79**), F2FlAsH-EDT_2 (**83**), and F4FlAsH-EDT_2 (**84**) are almost nonfluorescent, but on formation of a complex between F2FlAsH (**83**) and a 12-mer peptidic sequence (P12) as a model target[173] a remarkable increase in fluorescence is observed, the emission peak occurring at 522 nm (Table 5). The absorption maximum of F2FlAsH-P12 (**86**) shifts 11 nm to the blue compared to FlAsH-P12 (**85**), whereas the maximum of F4FlAsH-P12 (**87**) is shifted 17 nm to the red. The Stokes' shift for F2FlAsH-P12 (**86**) is

Table 5. Optical Properties for the Biarsenical-P12 Complexes (85−87)

complex	λ_{abs}, nm (ϵ, M^{-1} cm^{-1})	λ_{em} (nm)	τ (ns)
FlAsH-P12 (**85**)	511(52 000)	527	4.88
F2FlAsH-P12 (**86**)	500 (65 500)	522	4.78
F4FlAsH-P12 (**87**)	528 (35 100)	544	5.18

22 nm; 6 nm greater than that of the FlAsH complex (**85**). The fluorescence intensity of the peptide adduct ($\lambda_{ex}/\lambda_{em}$ 490/522 nm) is 4 times brighter than that of the complex with the parent FlAsH probe (**85**). This enhancement is attributed to a larger extinction coefficient at 490 nm (2×) and a greater emission quantum yield (2×). The radiative lifetime of F2FlAsH-P12 (**86**) (4.78 ns) is similar to that of the corresponding FlAsH complex (**85**) (4.88 ns). The emission peak of F4FlAsH-P12 (**87**) at 544 nm expands the spectral range of the biarsenical dyes. In addition, the fluorescence lifetime increases to a value of 5.2 ns.

On the basis of the results of this study the authors suggested that the two compounds (**83** and **84**) would form an excellent FRET pair with a large critical distance. Furthermore, they predicted that the observed properties should facilitate improved structural and dynamic studies of proteins in living cells.

In view of the fact that the biarsenical-TC complex is stable under the denaturing conditions typically used for gel electrophoresis of proteins and has a molecular weight of less than 2 kDa,when bound to the biarsenical dye,[170] Allbritton et al.[174] recently investigated the biarsenical dyes as fluorescent probes for in vitro and cellular peptide and protein studies using capillary electrophoresis.

Recombinant protein or synthesized peptides containing the optimized tetracysteine motif (-Cys-Cys-Pro-Gly-Cys-Cys-) were labeled with biarsenical dyes and then analyzed through micellar electrokinetic capillary chromatography (MEKC). The biarsenical−tetracysteine complex was stable and remained fluorescent under standard MEKC conditions for peptide and protein separations. The detection limit following electrophoresis in a capillary was less than 3×10^{-20} mol with a simple laser-induced fluorescence system. A mixture of multiple biarsenical-labeled peptides and a protein was easily resolved. Demonstrating that the label did not interfere with bioactivity, a peptide-based enzyme substrate conjugated to the tetracysteine motif and labeled with a biarsenical dye retained its ability to be phosphorylated by the parent kinase. The capability of using this label for chemical cytometry experiments was shown by intracellular labeling and subsequent analysis of a recombinant protein possessing the tetracysteine motif expressed in living cells.

Although introduction of biarsenical dyes for specific labeling of target peptides or proteins has occurred only recently,[169,175,176] several studies have already appeared showing their applicability[177] and advantages, and it appears that these fluorescent dyes offer a promising alternative to "conventional" procedures in this field.

In conclusion, fluorescein derivatives, including the biarsenical fluorophores, continue to provide one of the most commonly used and widely reported fluorophore classes for protein studies.

3.2. Rhodamines

Rhodamine dyes are among the oldest synthetic dyes used for the dyeing of fabrics, and they belong to the xanthene class of dyes. They generally have high molar absorptivities in the visible region, and many derivatives are strongly fluorescent. The absorption and emission properties are strongly influenced by substituents in the xanthene nucleus,[178] and the rhodamine dyes find use as not only colorants but also fluorescent markers in structural microscopic studies, photosensitizers, and laser dyes.[179]

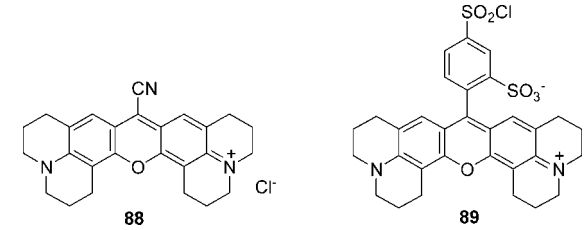


Figure 15. Structure of Rhodamine 800 (**88**) and Texas Red (**89**).

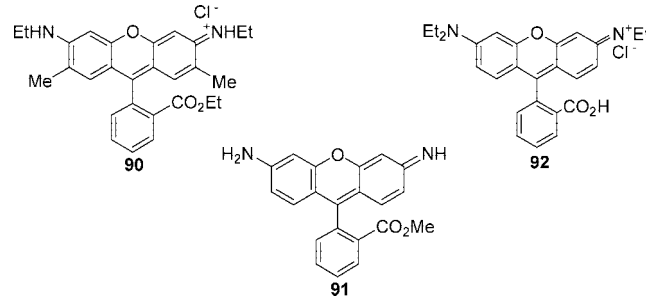


Figure 16. Structures of Rhodamine 6G (**90**), Rhodamine 123 (**91**), and Rhodamine B (**92**).

Two modern rhodamine derivatives used specifically in bioanalysis, either as noncovalent or as covalent labels, are Rhodamine 800 (**88**)[180,181] and Texas Red (**89**)[182,183] (Figure 15). Texas Red was used by Horneffer and co-workers as a fluorescent marker for localizing proteins in matrix-assisted laser desorption/ionization (MALDI) preparations by confocal laser scanning microscopy.[183] The limited pH dependence of this fluorophore surpasses the potential quenching of fluorescence by the acidic environments of typical MALDI matrices, such as 2,5- and 2,6-dihydroxybenzoic acids.

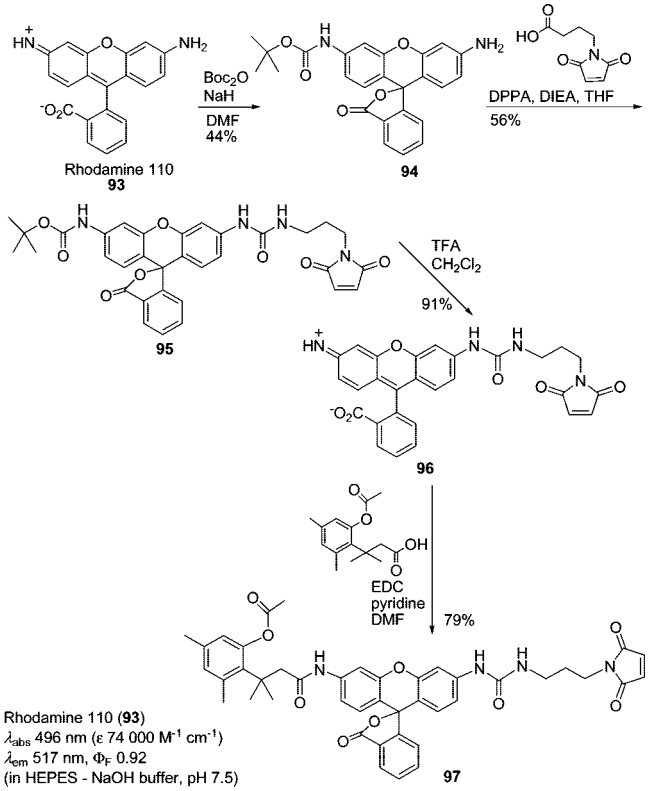
Other xanthene fluorophores, such as Rhodamine 6G (**90**),[184] Rhodamine 123 (**91**),[185] and Rhodamine B (**92**)[186] (Figure 16), have also been reported as fluorescent probes in biological studies

Rhodamine 110 (**93**) has been used in studies of enzymatic activity.[187] Recently, Raines and co-workers[188] reported the synthesis of the versatile "latent" fluorophore **97** (Scheme 15), a derivative of Rhodamine 110 (**93**), in which one of the nitrogens was modified as a urea and the other as a "trimethyl lock". The first alteration produced suppression of half-fluorescence of rhodamine while facilitating conjugation with a target molecule. The second modification enabled fluorescence to be unmasked fully by a single user-designated chemical reaction.

Fluorophore **97** was covalently attached to a cationic protein (a thiol-containing variant of bovine pancreatic ribonuclease, RNase A),[189] and the conjugate obtained did not fluoresce in the absence of esterases.

Since traditional fluorophores are always fluorescent, bulk fluorescence can obscure valuable information. "Latent" fluorophore **97** overcomes this limitation. Furthermore, the modular design of this "fluorogenic label" allows for easy synthesis of a range of small-molecule probes suitable for labeling of numerous biochemical and biological cell processes.

Scheme 15. Synthetic Route to Fluorogenic Label 97



It was also in 2006 that Burgess et al.[190] synthesized the new fluorescent rhodamine derivative **98** (Scheme 16). This probe is different from other rhodamines in that it possesses several (four) carboxylic acid functionalities to promote water solubility and facilitate conjugation to proteins. It also possesses an aryl bromide functionality that could be used for linking other moieties to the fluorescent dye via palladium-catalyzed reactions (e.g., Suzuki[191] and Sonogashira[192] couplings).

Dye **98** was bonded to a model protein called ACBP (acyl-CoA binding protein). The properties of this conjugate were tested to establish that the label did not significantly perturb the binding function of the protein to its natural ligand in vitro and confirm that its secondary structure was not significantly altered. Other studies showed that **98**-ACBP (**99**) could be imported into living cells using the novel Chariot-peptide carrier system wherein it accumulates, at least partially, in the nucleus. This result allows for the fluorescent use of conjugate **99** in vivo studies.

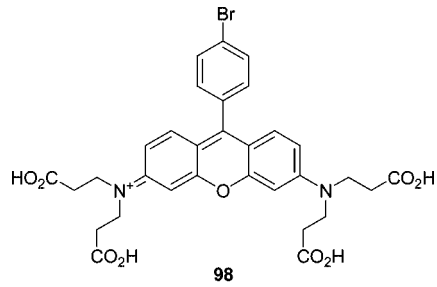
Concerning the wavelengths of absorption and emission maxima (in aqueous solution, PBS pH 7.4) of dye **98** and conjugate **99** the authors verified that a red shift occurs after linkage of the dye to the protein (λ_{abs} 553 nm (**98**)/560 nm (**99**); λ_{em} 574 nm (**98**)/583 nm (**99**) with λ_{ex} 568 nm).

In the use of rhodamine-based fluorescent probe dyes as target molecules in biochemical separation procedures or in biological processes it is extremely important to establish the extent to which the dye changes the behavior of target molecules. If such a change does in fact occur, the uniformity of the change in the target molecules is of great significance. 6-Aminohexanoic acid linker was used to reduce the potential interaction between rhodamine and the pharmacophore of melanocortin receptors.[193]

In order to minimize such potential steric problems, in 2007 Shi and co-workers investigated the incorporation of a linker molecule (11-amino-3,6,9-trioxaundecanoic acid) to 5- or 6-carboxy-rhodamine **110** (Scheme 17).[194]

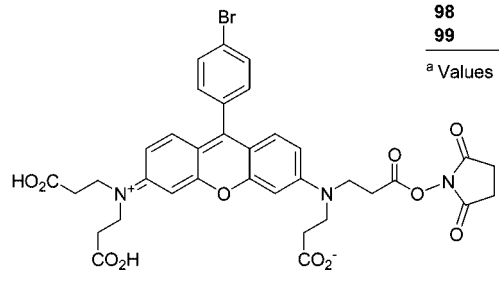
Evaluation of the effectiveness of this probe was carried out using it to label goat-antimouse IgG and detect alpha-tubulin in the endothelial cells of a bovine pulmonary artery. The results showed that compounds **102a** and **102b** were excellent green fluorescent probes, showing strong fluorescence and good biocompatibility, and suitable as labels for a variety of proteins and other biopolymers.

Scheme 16. Fluorescent Labeling of ACBP Protein with Dye 98

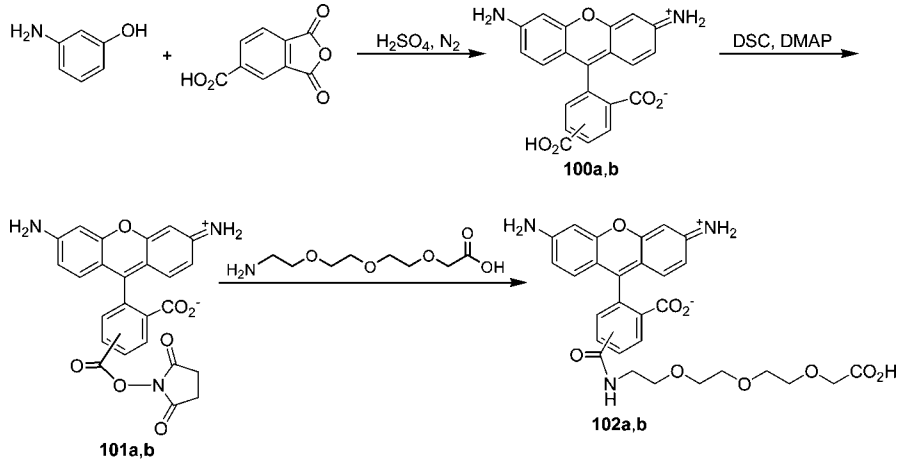


compound	λ_{abs} (nm)[a]	λ_{ex} (nm)[a]	λ_{em} (nm)[a]
98	553	568	574
99	560	568	583

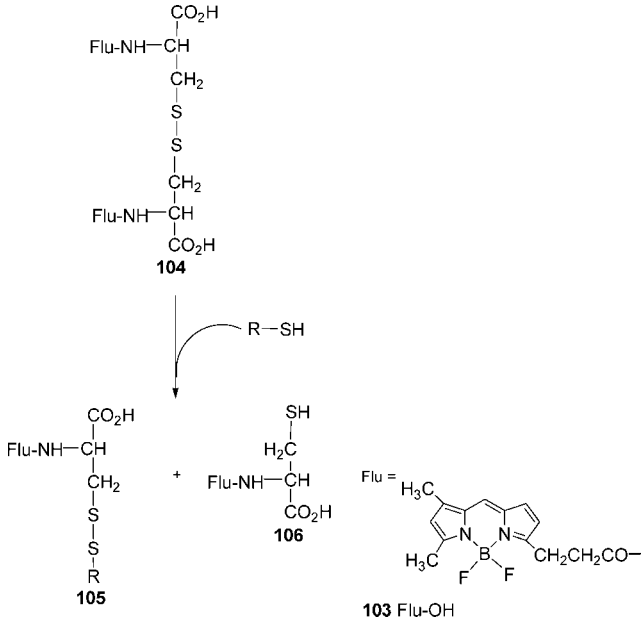
[a] Values obtained in PBS buffer, pH 7.4.



Scheme 17. Synthesis of Rhodamine Derivatives 102a and 102b



Scheme 18. Reaction of Intramolecularly Quenched BODIPY L-Cystine (104) with a Thiol, Yielding Fluorescent Compounds 105 and 106



It is evident from the forgoing that novel fluophores based on the rhodamine system provide an interesting and valuable class of compounds for the biolabeling area.

3.3. BODIPY Fluorophores

Fluorophores based on the 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene molecule, which can be modified at carbon positions 1, 3, 5, 7, and 8 are commonly designated as BODIPY fluorophores (BODIPY is a registered trademark of Molecular Probes, Inc.) (Figure 17).[120,195−202]

They exhibit optical properties that are often superior to fluorescein (**68**), tetramethylrhodamine, Texas Red (**89**), and other longer-wavelength dyes. BODIPY dyes are proving to be extremely versatile and useful in many biological applications. Commercially available BODIPY dyes can be linked to peptides, proteins, oligonucleotides, dextran, lipids, and polystyrene microspheres.[120,198−208]

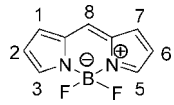


Figure 17. Basic structure of BODIPY fluorophores, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene.

The first BODIPY chromophore was synthesized in 1968 by Treibs and Kreuzer.[196] BODIPY compounds designed specifically for labeling biomolecules have been prepared by Kang and Haugland.[120,197] The synthesis and spectroscopic properties of BODIPY dyes and their derivatives have been recently reviewed by Burgess and Loudet.[209]

Interest in BODIPY-based probes can be attributed to their useful photophysical characteristics. Thus, their visible excitation and emission can be tuned by modification of the pyrrole core;[209−211] they have high molar absorption coefficients (typically log $\epsilon_{max} > 8.8$), high fluorescence quantum yields ($\Phi_F \approx 1.0$, even in water), good photostability, and narrow emission band widths (important in multicolor applications). Additional advantages are their lack of a net ionic charge and the general insensitivity of their fluorescence to solvent or pH.[195−197]

BODIPY dyes present an affinity in their ligand conjugates for receptors, which is sometimes enhanced by their electrically neutral and relatively nonpolar structures. This occurs only when the overall conjugate is not too lipophilic. BODIPY conjugates with low molecular weight molecules also tend to be more permanent in living cells than the conjugates of charged fluorophores. Oligonucleotide conjugates of BODIPY fluorophores have also been used in DNA sequencing[212−214] in part because the dye exhibits a minimal effect on the mobility of the fragment during electrophoresis.[215]

BODIPY dyes are also among the most readily detectable amine-derivatization reagents available for HPLC and capillary electrophoresis due to their high peak intensity.[215,216]

BODIPY fluorophores, either as reactive dyes or as conjugates, are covered by several patents issued to Molecular Probes.[197,217,218] Commercially available, amine-reactive BODIPY fluorophores include succinimidyl esters of BODIPY acids and carboxylic acids that can be converted into fluorescent esters,[219] acid halides, or amides by standard chemical methods. BODIPY dye−aliphatic amine derivatives are also suitable as carboxylic acid reactive reagents.[120] Thiol-reactive BODIPY reagents contain maleimido, iodoacetamido, and bromomethyl functionality.[220] These dyes are useful in the labeling of cysteine residues in proteins and thiolated oligonucleotides as well as in detection of thiolato conjugates separated by HPLC and capillary electrophoresis using ultrasensitive laser-scanning techniques.[221]

Scheme 19. Synthesis of Compounds 108−116

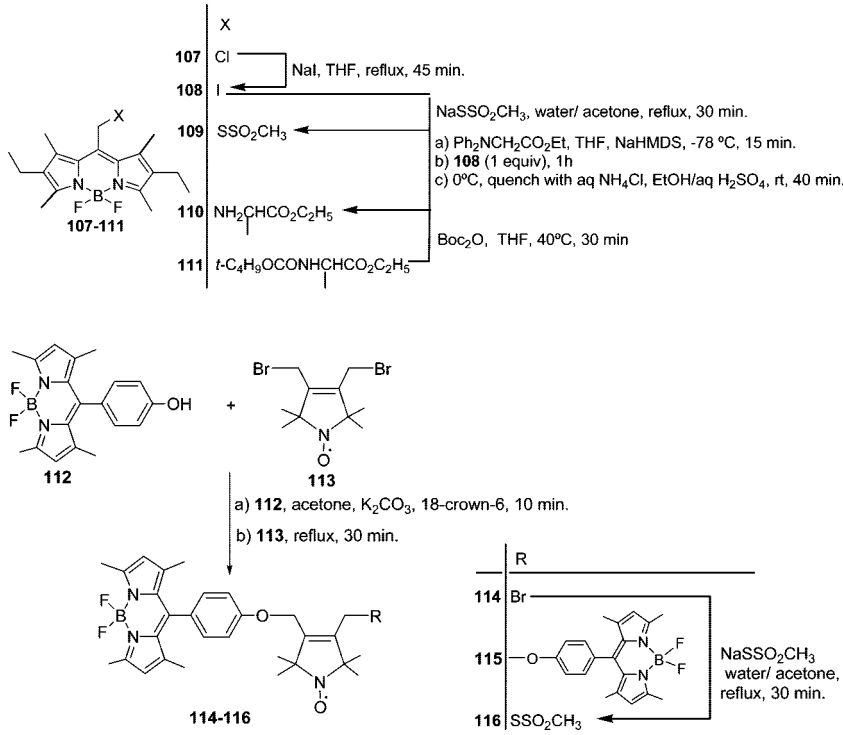


Table 6. Optical Properties of the BODIPY Derivatives 109, 111, and 116 in Acetonitrile

compound	λ_{abs}, nm (ϵ, M^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ_F
109	543 (3.37×10^4)	543	555	0.80
111	529 (3.54×10^4)	530	543	0.86
116	498 (4.35×10^4)	497	508	0.22

The BODIPY fluorophore derivative **103** has been linked to the amino groups of disulfide-linked amino acid cystine to produce a reagent for reversible, thiol-specific labeling of proteins, thiolated oligonucleotides, and cells (Scheme 18).[222] BODIPY L-cystine (**104**) is virtually nonfluorescent due to interaction between the two fluorophores; however, thiol-specific exchange to form a mixed disulfide (compounds **105** and **106**) results in a significant enhancement of the green fluorescence.[120]

Hideg and Kálai[223] reported the synthesis of a new BOBIPY derivative as a thiol-reactive fluorescent label as well as a fluorescent amino acid and a double (spin and fluorescence) label (Scheme 19).

Compound **107**, obtained from 3-ethyl-2,4-dimethylpyrrole and chloroacetylchloride,[224] was converted to the more reactive iodomethyl derivative **108**, which was then transformed into the methanethiosulfonate derivative **109** (MTS). The resulting compound is a thiol-specific, reversible fluorescent dye with 555 nm emission (Table 6), suitable for producing the S−S linkage with a cysteine SH group, and a good candidate for site-directed fluorescent labeling of peptides.[225] Compound **108** was used in the preparation of the fluorescent amino acid ester **110**, which was then reacted with di-*tert*-butyldicarbonate to produce the more stable *N*-Boc protected D,L-amino acid ester **111**. Compounds **109** and **111** are capable of fluorescent modification of the peptide by labeling at the cysteine side chain or incorporation of the fluorescent amino acid through solid-phase peptide synthesis. A double (spin and fluorescence) label, capable of simultaneous labeling at the same site of a protein, was also synthesized from BODIPY dye **112**[226] with a paramagnetic homobifunctional alkylating nitroxide **113**.[227]

Compound **116** was a SH-specific double (fluorescent and spin) label with a green emission (508 nm), reduced quantum yield (Φ_F 0.22), and 2.5 ns lifetime, which is about one-half of the regular BODIPY lifetime. [119]

The detailed elucidation of protein folding cooperativity and nucleation requires site-specific measurements of intramolecular distances and their changes upon induction of structural transitions. Johansson et al. demonstrate that tryptophan and BODIPY is a suitable donor−acceptor (D−A) pair for electronic energy-transfer experiments in folding studies.[228] The suitability of the Trp−BODIPY energy transfer is exemplified in the extensively characterized two-state protein, S6. For a comprehensive structural coverage individual BODIPY molecules were anchored by Cys insertions at four different positions on the S6 surface, where communication with a donor Trp can occur at position 33 or 62.

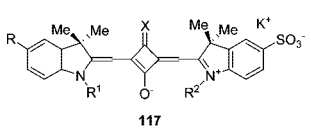
Interest in BODIPY-based fluorophores has grown over the past few years because of their advantageous photophysical properties. They provide valuable alternatives to other heterocycle skeleton fluorophores that fluoresce at wavelengths beyond 500 nm.

3.4. Squaraines

Squaraines, a subclass of polymethine dyes with a zwitterionic structure, are one of the most interesting classes of dyes which can be used as fluorescent probes in the near-infrared.[229−231]

These compounds possess unique physical−chemical properties, such as extremely intense and sharp absorption bands, that can be in the visible and near-infrared regions,[232] sharp and intense fluorescence bands.[233] They are also unusual in exhibiting photoconductivity.[234] Their photophysical characteristics make squarylium fluorophores appropriate for the

Scheme 20. Structure of Several Unsymmetrical and Symmetrical Squaraine Dyes (117)



117

a X = S, R = H, R^1 = Me, R^2 = $(CH_2)_5CO_2H$
b X = C(CN)$_2$, R = H, R^1 = Me, R^2 = $(CH_2)_5CO_2H$
c X = O, R = H, R^1 = Me, R^2 = $(CH_2)_5CO_2H$
d X = C(CN)$_2$, R = H, R^1 = $(CH_2)_3CO_2H$, R^2 = Et
e X = S, R = $SO_3^-K^+$, R^1 = R^2 = $(CH_2)_5CO_2H$
f X = C(CN)$_2$, R = $SO_3^-K^+$, R^1 = R^2 = $(CH_2)_5CO_2H$
g X = O, R = $SO_3^-K^+$, R^1 = R^2 = $(CH_2)_5CO_2H$

Table 7. Optical Properties of Squaraine Dyes 117a−g in the Absence and Presence of 6 mg/mL BSA and When Covalently Bound to BSA in Phosphate Buffer, pH 7.4

dye	environment	λ_{abs}, nm (ϵ, M^{-1} cm^{-1})	λ_{em} (nm)	Φ_F (%)[a]	$\Delta\nu$ (cm^{-1})	τ (ns)
117a	PB	628 (163 000), 373 (21 500)	642	3	350	0.15
	BSA/PB	649	661	35	280	
	conjugate	646	662	9	370	1.30
117b	PB	659 (207 000), 385 (44 500)	676	2	380	0.29
	BSA/PB	673	693	30	430	
	conjugate	671	694	3	490	2.61
117c	PB	624 (245 000)	635	2	280	0.16
	BSA/PB	639	648	40	220	
	conjugate	638	650	19	290	2.26
117d	PB	658 (182 000), 385 (34 000)	677	3	430	0.29
	BSA/PB	679	695	45	340	
	conjugate	676	695	13	400	3.32
117e	PB	636 (216 000), 370 (22 000)	648	6	290	0.41
	BSA/PB	655	666	28	250	
	conjugate	648	666	4	420	2.44
117f	PB	667 (188 000), 380 (26 500)	685	7	390	0.84
	BSA/PB	683	699	44	340	
	conjugate	681	702	24	440	3.26
117g	PB	632 (265 000)	642	6	250	0.44
	BSA/PB	645	654	38	210	
	conjugate	642	655	27	310	1.84

selective fluorescent detection of biomolecules, such as proteins in biological and biomedical applications.

Structural modifications in these compounds can be achieved by introduction of substituents into the aromatic ring or on the N atom of the terminal heterocyclic moiety. It is also possible to modify the squaraine ring, but this is more difficult. Such changes can be used to produce a red shift of the absorption and fluorescence bands.

The synthesis and characterization of water-soluble symmetrical and asymmetrical squaraines, of the general structure shown in Scheme 20, were recently described by Terpetschnig and co-workers.[235] Studies of the covalent and noncovalent binding of these dyes to bovine serum albumin (BSA) were also carried out.

The compounds obtained (**117a−g**) exhibited long-wavelength absorption in the range 624−667 nm with emission maxima in the range 642−685 nm. They also displayed high molar absorptivities (between 163 000 and 265 000 M^{-1} cm^{-1}) (Table 7). The absorption and emission maxima of these dyes shift toward longer wavelengths in the order: X = oxo < monothio < dicyanomethylene. When compared to dioxo-squaraines, the monothio dyes were red shifted by only 4−7 nm, whereas the shift for the dicyanomethylene-squaraines was on the order of 35−43 nm. In addition, the absorption and emission spectra of the symmetrical dyes **117e−g** were always red shifted relative to the unsymmetrical dyes **117a−d**.

The Stokes shifts of squaraine dyes **117a−g** increased in the same order, oxo < monothio < dicyanomethylene, but there was no evidence of the relationship between the Stokes shifts of the symmetrical dyes (**117e−g**) and the unsymmetrical squaraines (**117a−d**).

The absorption spectra of dicyanomethylene- and thio-substituted squaraines (**117a**, **117b**, **117e**, and **117f**) exhibited an additional absorption maximum near 400 nm with molar absorptivities of 22 000 M^{-1} cm^{-1} for the monothio- and 26 500−44 500 M^{-1} cm^{-1} for the dicyanomethylene squaraines.

The noncovalent attachment of squaraine dyes to protein causes a noticeable red shift (12−21 nm) of the absorption and emission maxima. However, when compared to noncovalently bound dyes, the absorption maxima of the dye−conjugates were found to shift slightly to shorter wavelengths by about 2−7 nm, while the emission maxima were unchanged or red-shifted slightly by about 3 nm. The Stokes shifts for the dye−BSA conjugates, with the exception of **117d**, were larger than those for the noncovalently bound or free dyes.

The fluorescence lifetimes (τ) of the monosulfonated dyes **117a−d** measured in PB occurred in the range between 0.15 and 0.29 ns, while the lifetimes of the disulfonated dyes **117e−g** were obtained from 0.41 to 0.84 ns (Table 7). The lifetimes of the squaraine dyes increases several times upon covalent attachment to BSA. The increase is more pronounced (8.7−14.1 times) for monosulfonated dyes **117a−d**, as in disulfonated dyes **117e−g** (3.9−6.0 times). No clear correlation between the fluorescence lifetimes and quantum yields of dye−BSA conjugates was observed. The dicyanomethylene dye conjugates of **117b**, **117d**, and **117f** exhibit the longest lifetimes.

The protein conjugates exhibited higher quantum yields, longer fluorescence lifetimes, and better photostability than conventional polymethines. In addition, in contrast to most other dyes, some of the described squaraines (**117a**, **117b**, **117d**, **117e**, and **117f**) could be excited by both a red laser and blue light from a laser or light-emitting diodes because of their additional intense absorption band at about 400 nm. The authors suggested that these fluorophores would be of particular value for fluorescence lifetime-based biomedical applications.

Following on from this work, Yarmoluk and co-workers[236] recently described the synthesis of the symmetrical and asymmetrical squaraines (**120**, **122**, and **124a−d**), and they evaluated their suitability for the fluorescence detection of a variety of proteins, such as BSA, human serum albumin (HSA), ovalbumin, avidin, trypsin, and lysozyme. The influence of the structure of the dye molecule on protein selectivity was investigated. The synthetic route used to prepare these dyes is summarized in Scheme 21. The free dyes in aqueous solution (0.005 M TRIS-HCl, pH 8.0) showed absorption and emission maxima in the ranges 605−685 and 634−702 nm, respectively (Table 8).

All the squaraines showed a considerable increase in fluorescence intensity (by a factor of up to 190) in the presence of BSA, whereas in contrast, the fluorescence enhancement in the presence of other albumins was significantly lower (increasing by a factor of about 24). Although, the Stokes shifts were not high, there was a slight increase in the case of dyes **120** and **124a−d**.

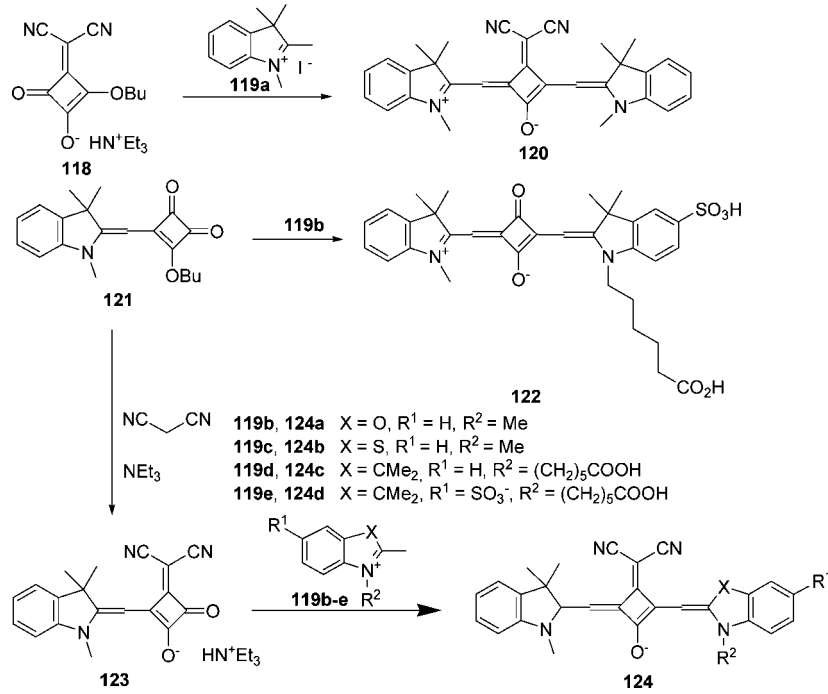
Novel bis-squaraine fluorophores, in which two squaraine nuclei are conjugatively linked by a phenyl or biphenyl unit, have been described by Yagi et al.[237] More recently, the same research group reported the synthesis of new bis-squaraine

Table 8. Optical Properties of Squaraines 120, 122, and 124a−d[a]

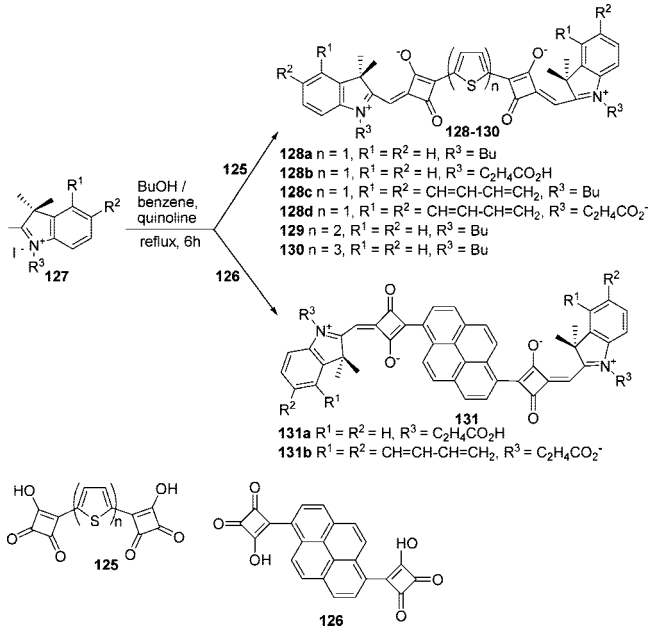
	MeOH	buffer					in the presence of BSA					
dye	λ_{abs}[b]	λ_{abs}[b]	λ_{ex}[b]	λ_{em}[b]	I_0[c]	$\triangle\lambda$[b]	λ_{abs}[b]	λ_{ex}[b]	λ_{em}[b]	I^{BSA}[c]	$\triangle\lambda$[b]	I^{BSA}/I_0
120	661	636 685[d]	690	700	11	10	637[d]	684	698	502	14	46
122	630	620	632	643	269	11	625	646	655	6573	9	24
124a	625	605	622	634	9	12	601[d]	654	671	1723	17	191
124b	665	676	688	702	1	14	676	686	702	121	16	121
124c	665	616[d]	654	669	84	15	630[d]	689	708	4246	19	51
124d	670	617[d]	661	675	199	14	672	683	696	2850	13	24

[a] In methanol, aqueous buffer, and the presence of BSA. I_0 (I^{BSA}) fluorescence intensity of dye in buffer (and in the presence of BSA). [b] Units nm. [c] Units a.u. [d] Absorption spectra shoulders.

Scheme 21. Synthetic Route for Preparation of Squaraine Dyes 120, 122, and 124



Scheme 22. Synthesis of Bis-Squaraine Dyes Linked by Thiophene (128−130) or Pyrene (131) Spacer



dyes linked by either a thiophene or a pyrene spacer unit (Scheme 22).[238]

Bis-squaraine dyes linked by a monothiophene (**128**) or pyrene (**131**) spacer and bonded to HSA and BSA as noncovalent labeling probes exhibited enhanced fluorescence in the near-infrared region when used to form 1:1 dye−protein complexes. Although the fluorescence quantum yields of these dyes were low, there was an increase in their values for all dyes studied with BSA and HSA. The most notable of these was dye **131b**, where the fluorescence efficiency was about 14 times higher for dye−BSA than that for the free dye. The absorption and emission maxima of dyes **128b**, **128d**, and **131a**,**b** linked to HSA and BSA were in the wavelength ranges 658−772 and 750−790 nm, respectively, in aqueous solution (TRIS-HCl buffer, pH 7.4) (Table 9).

The synthesis and photophysical characterization of squaraines **134a−d** containing amphiphilic substituents have been reported by Ramaiah and Arun (Scheme 23).[239] These dyes exhibited absorption bands in the range 640−649 nm in aqueous solution with high molar absorptivities (ϵ 100 000−300 000 M^{-1} cm^{-1}) (Table 10). The corresponding fluorescence bands had maxima between 663 and 675 nm depending on the nature of the substituents. The fluorescence quantum yields were in the range 0.15−0.21 in ethanol, but these values decreased by an order of magnitude in an aqueous medium (Φ_F 0.01−0.02). In micelles, formed, for example, with cetyltrimethylammonium bromide, sodium dodecyl sulfate, or Triton X-100, **134a−d** showed a slight enhancement (5−10 fold) in their fluorescence yields.

Table 9. Optical Properties of Bis-Squaraine Dyes 128b, 128d, 131a, and 131b in the Absence and Presence of HSA or BSA

	free form[a]		with HSA[b]					with BSA[b]				
dye	λ_{abs}[d]	Φ_F[c,e]	λ_{abs}[d]	λ_{ex}[d]	λ_{em}[d]	Φ_F[c,e]	$\triangle\lambda$[d]	λ_{abs}[d]	λ_{ex}[d]	λ_{em}[d]	Φ_F[c,e]	$\triangle\lambda$[d]
128b	695, 633	8.0	742	717	750	9.6	33	754	722	771	8.3	49
128d	727, 670	0.45	772	740	790	1.1	50	769	728	780	1.0	52
131a	636	3.0	658	700	757	13	57	668	700	750	7.8	50
131b	647	0.95	671	690	763	0.70	73	681	690	763	13	73

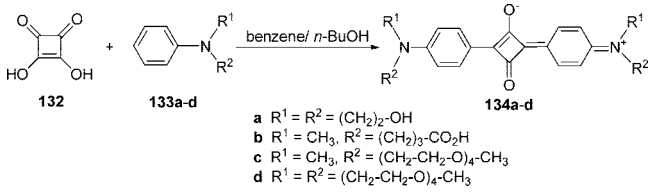
[a] 5.0×10^{-7} M solution of dye dissolved in trizma buffer (pH 7.4) at 298 K. [b] 5.0×10^{-7} M solution of dye dissolved in trizma buffer (pH 7.4) with 5.0×10^{-6} M HSA or BSA at 298 K. [c] The quantum yield was determined relative to that of Rhodamine B (0.97). Excitations at 717 (722), 740 (728), 700 (700), and 690 (690) nm for **128b**, **128d**, **131a**, and **131b** in the presence of HSA, respectively, and in parentheses of BSA. [d] Units: nm. [e] $\times 10^{-3}$.

Table 10. Optical Properties of Dyes 134a−d in Ethanol and 10% (vol/vol) Ethanol−Water Mixture and in the Presence of Micelles such as Cetyltrimethylammonium Bromide (CTAB), Sodium Dodecyl Sulphate (SDS), and Triton X-100 (TX-100)[a,b]

	134a			**134b**			**134c**			**134d**		
additive	λ_{abs}[c]	λ_{em}[c]	Φ_F	λ_{abs}[c]	λ_{em}[c]	Φ_F	λ_{abs}[c]	λ_{em}[c]	Φ_F	λ_{abs}[c]	λ_{em}[c]	Φ_F
EtOH	639	663	0.15	636	662	0.18	637	660	0.19	638	662	0.21
EtOH−H_2O[d]	644	671	0.021	647	673	0.015	645	674	0.018	646	675	0.026
CTAB[e]	642	667	0.11	644	667	0.14	642	663	0.12	644	667	0.096
SDS[f]	641	672	0.098	*g*	*g*	*g*	640	663	0.15	643	668	0.16
TX-100[h]	647	671	0.15	649	672	0.12	645	669	0.12	646	671	0.14

[a] Average of more than two experiments. [b] Error involved in Φ_F values ca. ±5%. [c] Units: nm. [d] 10% EtOH−H_2O. [e] [CTAB] = 129, 8, 21, and 110 mM for dyes **134a**, **134b**, **134c**, and **134d**, respectively. [f] [SDS] = 28, 21, and 22 mM for dyes **134a**, **134c**, and **134d**, respectively. [g] Negligible changes were observed for dye **134b** in the presence of SDS. [h] [TX-100] = 109, 116, 118, and 135 mM for dyes **134a**, **134b**, **134c**, and **134d**, respectively.

Scheme 23. Synthesis of Amphiphilic Squaraine Dyes 134a−d



These novel amphiphilic squaraines combine favorable photophysical properties with good solubility in aqueous media and in addition interact efficiently with micelles, and thus, the authors suggested that dyes **134a−d** had potential as near-infrared fluorescent bioanalytical sensors.

Because of their useful photophysical characteristics, both in their free form and after linkage to proteins, the squarylium dyes are a subject of particular current interest. This is evidenced by the high number of new derivatives and studies that have been reported in the recent literature. In particular, the squaraines represent an important class of probes to be considered for the near-infrared region.

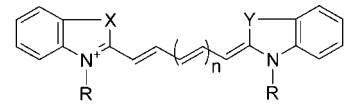
3.5. Cyanines

Cyanine dyes are fluorescent compounds which have found numerous technical applications since their discovery, including their use as photographic sensitizers,[240] nonlinear optical materials,[241] and, more recently, fluorescent probes for biomolecular labeling.[242−244] Applications of these in genetic analysis, DNA sequencing,[245] in vivo imaging,[246] and proteomics[247] are increasing dramatically.

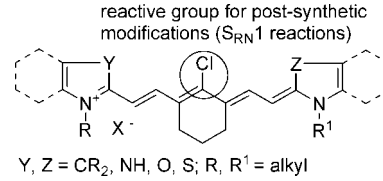
The basic structure of cyanine dyes includes two aromatic or heterocyclic rings linked by a polymethine chain with conjugated carbon−carbon double bonds (Scheme 24).

Nowadays, these compounds can be considered to be the main source of organic long-wavelength fluorophores and provide excitation bands in the range 600−900 nm. A further large bathochromic shift can be obtained by addition of a

Scheme 24. Basic Structure of Cyanine Dyes



Scheme 25. General Structure of Convertible Cyanine Dyes



vinyl group (−CH═CH−) to the polymethine chain. Much of their interest stems from their straightforward synthesis,[248,249] broad wavelength tunability, and, in particular, high near-infrared absorption and emission and large molar absorptivities.

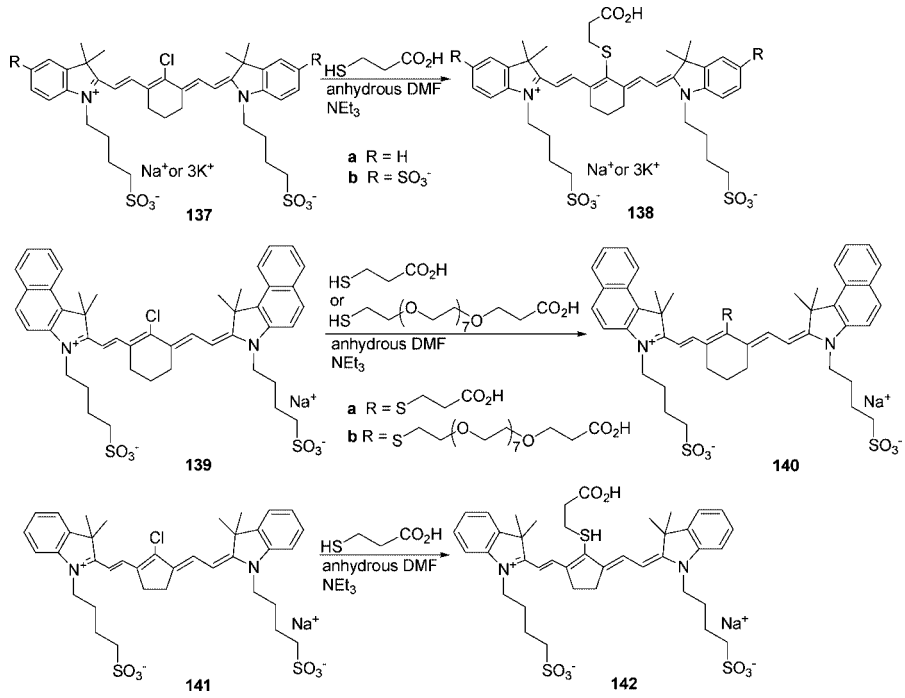
Some drawbacks in the analytical use of cyanine dyes are also known. For example, the number of these available as probes for labeling is very limited at present. In addition, most cyanines have short fluorescence lifetimes and low fluorescence quantum yields and undergo extensive aggregation in aqueous solution, leading to low fluorescence intensities.[250] Nevertheless, the photophysical properties of these dyes can be improved in solutions containing macromolecules or when organic solvents are present. Alteration of the molecular structure of the dye, for example, through introduction of

Table 11. Optical Properties of Dyes 138a,b, 140a,b, and 142 in PBS pH 7.4

dye	λ_{abs}, nm (ϵ, M^{-1} cm^{-1})	λ_{em} (nm)
138a	777(130 000)	812
138b	783(174 000)	814
140a[a]	822(128 000)	845
140b[a]	823(116 000)	847
142	807(120 000)	839

[a] In PBS, pH 7.4, with 20% DMSO.

Scheme 26. Synthetic Methodology for Synthesis of Dyes 138, 140, and 142



alkyl sulfonate groups, can help increase water solubility, fluorescence quantum yield, and photochemical stability. Addition of functional groups, such as isothiocyanato, makes the fluorophore useful for covalent labeling.

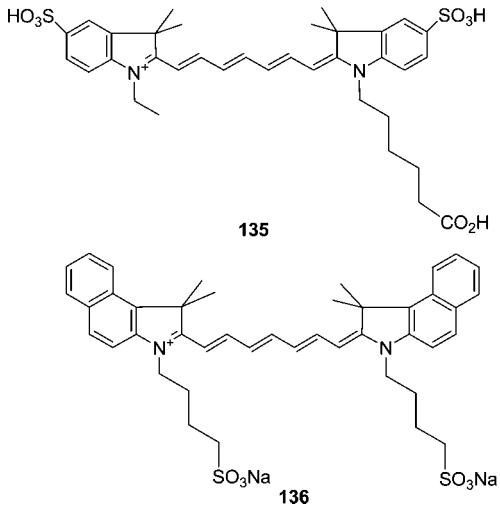


Figure 18. Structure of Cys (**135**) and ICG (**136**) dyes.

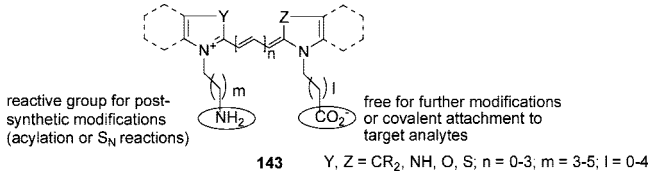


Figure 19. Structure of convertible cyanine dyes.

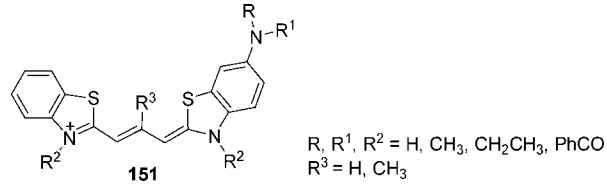
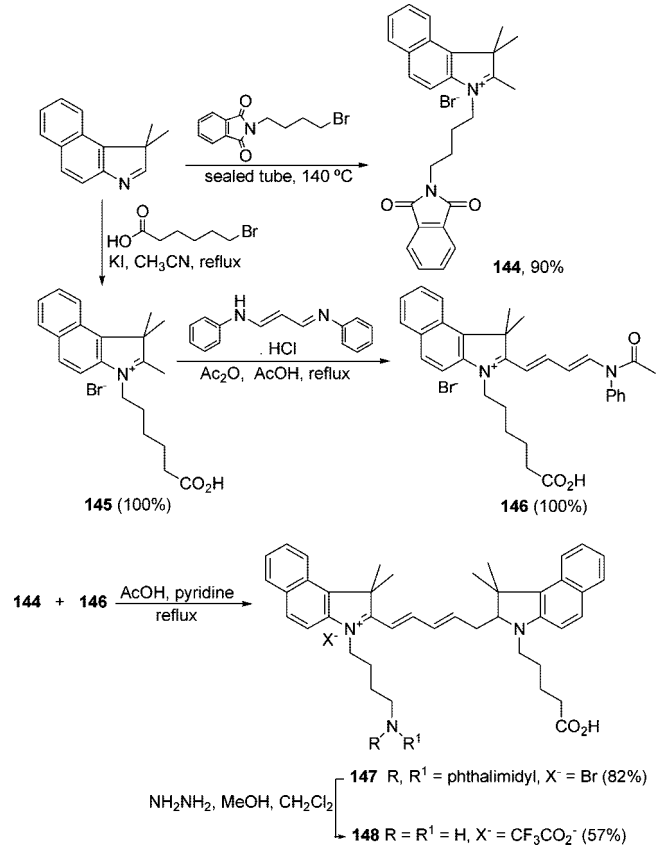


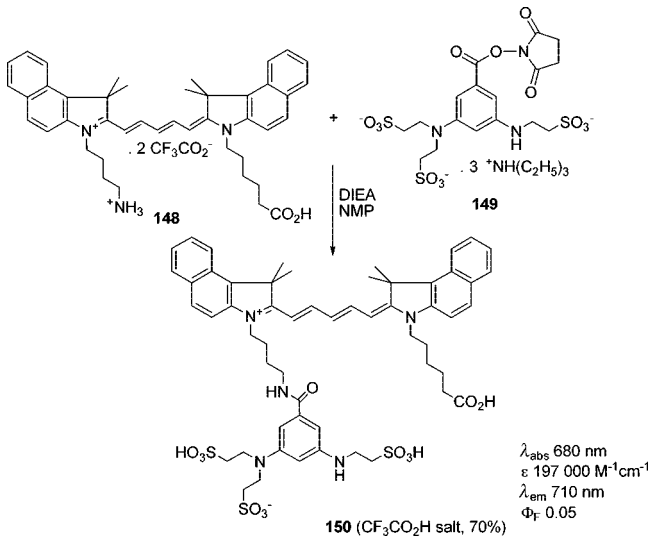
Figure 20. Structure of cyanine dyes **151**.

Scheme 27. Synthesis of the Cyanine-Based Amino Acid



Compounds Cy5 (**135**) and Indocyanine Green (ICG) (also referred as IR-125) (**136**) are two cyanine dyes commonly used for analytical purposes (Figure 18). Cy5 (**135**) has been used in capillary electrophoresis[251] and for sensor applications.[252,253] ICG (**136**) is a water-soluble dye that was initially used in the field of medical imaging as it is nontoxic in humans. Although this compound has a very low fluorescence

Scheme 28. Synthesis of the Novel Water-Soluble NIR Dye



efficiency in aqueous solution, this can increase dramatically when the dye is bound to macromolecules such as proteins.[250]

In the synthesis of cyanine dyes, preparation of a functionalized precursor of the fluorophore cyanine, termed as "convertible cyanine dye" (Scheme 25), is important. Through a chemical reaction it can be modified to give the fluorophore containing the required reactive groups.

Use of precursors with a labile chlorine atom at the central meso position, which can easily be replaced by various nucleophiles (such as metal alcoholates,[254] amines,[255] and thiols[256]) has allowed the synthesis of new markers which possess reactivity and optical properties appropriate for in vivo imaging[257] and DNA sequencing.[254]

Using this principle and addressing the need for cyanine labels with carboxylic acid functionality,[258−260] Tung and collaborators[257] developed an efficient synthesis of the cyanine monocarboxylates **138**, **140**, and **142** (Scheme 26).

Their dyes had absorption and emission maxima in the ranges 777−823 and 812−847 nm, respectively, in aqueous solution (PBS, pH 7.4) (Table 11). Interestingly, several of these fluorophores showed no tendency to aggregate in an aqueous solution.

Despite the importance of the synthetic methodology previously described for preparation of carboxylic acid fluorophores, this has been limited to heptamethine cyanine dyes containing a chloro-cyclohexenyl (or cyclopentenyl) moiety within the polyene chain. It would be of interest to explore a postsynthetic method applicable to a wider range

Table 12. Optical Properties of Cyanine Dyes 154a−f in Methanol

dye	λ_{abs}, nm ($\epsilon \times 10^5$ M^{-1} cm^{-1})	λ_{em} (nm)	Φ_F	$\triangle\lambda$ (nm)
154a	786 (2.5)	814	0.1097	29
154b	782 (2.3)	808	0.1188	26
154c	785 (2.2)	813	0.1315	28
154d	784 (2.2)	809	0.1484	25
154e	785 (2.2)	807	0.1009	22
154f	784 (2.3)	813	0.1168	29

of cyanine dyes. In addition, some of the applications of these fluorophores (namely, DNA sequencing by means of energy-transfer terminators or fluorescence-labeling biopolymers using solid-phase synthesis techniques)[244] are only possible with heterobifunctional derivatives of cyanine dyes. It was in 2006 that Romieu et al.[261] first reported the preparation of cyanine dyes with two orthogonal reactive groups, namely, amino and carboxylic acid functionality. The study presented an original synthetic route to functionalized cyanine dyes based on the use of an unnatural amino acid as a convertible cyanine dye (**143**) (Figure 19).

The structure of this precursor was designed to possess a scaffold with two orthogonal reactive amino and carboxylic acid groups, which could be modified readily and selectively. The resultant dye **148** (Scheme 27) possessed an amino group that could be used either to bond to a second fluorescent molecule to facilitate its use in FRET[262] or to introduce a further functional group that could modulate the hydrophobic/hydrophilic character or the spectroscopic properties of the cyanine nucleus. The carboxylic acid could be kept free for subsequent covalent linkage to biomolecules.

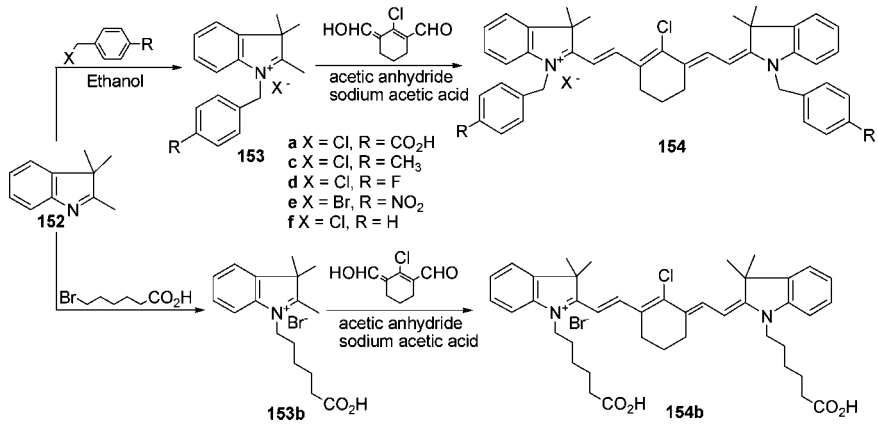
The selective derivatization of the amino group of dye **148** with an original trisulfonated linker (**149**) produced another water-soluble dye (**150**) suitable for the covalent labeling of biomolecules in the infrared region (Scheme 28).

Although cyanine dyes are more noted for possessing long methine chains, which results in excitation and emission at long wavelengths, the synthesis and bioapplications of cyanine derivatives with short chains, including monomethine cyanines, have also been reported recently.[263,264]

Yarmoluk and co-workers[264] studied the influence of several substituents in the benzothiazole heterocycle of trimethinic cyanines (**151**, Figure 20) on the photophysical properties of the dye in its free form and in the presence of DNA, RNA, and BSA.

One of the limitations of the cyanine dyes is their photostability, which decreases as the emission wavelength shifts to the red. Studies focusing on the relationships

Scheme 29. Synthetic Route for Preparation of Cyanine Dyes 154



between the molecular structure and the properties of this type of compound have been carried out.[265,266,259] It was recently reported that replacement of the central chlorine atom in the cyclohexenylene chain by an electron-donor group can increase the photostability of these dyes.[267]

Peng et al.[268] showed the effect of N substitution of 3*H*-indolenine on the photostability of the dyes: electron-donating groups at the N atom of 3*H*-indolenine rings were more effective in obtaining greater resistance to photobleaching than electron-withdrawing groups. The dyes **154a**−**f** were synthesized and characterized (Scheme 29) in this study.

These compounds exhibited absorption and emission maxima at 782−786 and 807−814 nm, respectively, with fluorescence quantum yields ranging from 0.10 to 0.15 in methanol (Table 12). The photostability of dyes **154a**−**f** with different substitutions at the N positions was found to decrease in the order **154f**, **154c** > **154d** > **154a** > **154b** > **154e**.

Studies of the relationship between molecular structure and photophysical properties are extremely important in the design of new cyanine dyes with good photostability. Thus, this fluorophore family continues to occupy a prominent position in the group of markers with long-wavelength emission for biological applications.

It will be evident from a consideration of the studies covered in this review that the synthesis and application of organic fluorophores with absorption and emission at long wavelengths is perceived among researchers to be of particular practical importance. In fact, as the emission of fluorophores shifts to the red, the potential bioanalytical applications of fluorescent labels become wider in range, and this can be attributed to the decrease in possible interferences from biological material at these wavelengths.[269−272]

4. Conclusions and Perspectives

As we have seen, fluorescence detection can be used in a wide range of studies related to life sciences. The number of established fluorescence probes is high; as discussed here there are also many newer systems available, and thus, it is now possible, in almost all cases, to choose a fluorophore that is optimal for the characteristics of the material to be labeled and the objectives of the study in question. However, in spite of the inherent advantages of the fluorophores covered in this review, there is still much work to be done to improve specific properties or circumvent some of their limitations.

Although known organic fluorophore labels include compounds with emission from the ultraviolet to the near-infrared in the electromagnetic spectrum, there are still limitations with the longer wavelength probes, and yet these are of particular importance for many biological applications. Thus, there is a strong need for new fluorophores or derivatives of known fluorophores with improved water solubility, where the excitation and emission maxima lie beyond about 600 nm and the fluorescence quantum yields are high.

The most demanding challenge is undoubtedly that of the design and synthesis of fluorophores possessing these characteristics. There is, of course, the additional requirement that these compounds must also include a functional group capable of efficient covalent bonding to biomolecules.

5. Abbreviations

Ala	alanine
Boc_2O	di-*tert*-butyl pyrocarbonate
BSA	bovine serum albumin
Cys	cysteine
DIC	diisopropylcarbodiimide
DIEA	diisopropylethylamine
DPPA	diphenylphosphoric azide
$\Delta\lambda$	Stokes' shift in nm
EDC	*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide
EDT	ethanedithiol
ε	molar absorptivity (M^{-1} cm^{-1})
Φ_F	fluorescent quantum yield
FRET	fluorescence resonance energy transfer
Glu	glutamic acid
Gly	glycine
HOBt	1-hydroxybenzotriazole
HOSu	*N*-hydroxyssuccinimide
HSA	human serum albumin
LC	liquid chromatography
Lys	lysine
λ_{abs}	wavelength of maximum absorption
λ_{em}	wavelength of maximum emission
λ_{ex}	wavelength of maximum excitation
MeOH	methanol
NHS	*N*-hydroxysuccinimide
NIR	near-infrared
$\Delta\nu$	Stokes' shift in cm^{-1}
PBS	phosphate-buffered saline
Phe	phenylalanine
PNAs	peptide nucleic acids
Pro	proline
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPPS	solid-phase peptide synthesis
*t*Bu	*tert*-butyl
TIS	triisopropylsilane
TRIS	tris(hydroxymethyl)aminomethane
Val	valine
τ	fluorescence lifetimes

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7. References

(1) Costa-Fernández, J. M.; Pereiro, R.; Sanz-Medel, A. *Trends Anal. Chem.* **2006**, *25*, 207.
(2) Yu, W. W.; Chang, E.; Drezek, R.; Colvin, V. L. *Biochem. Biophys. Res. Commun.* **2006**, *348*, 781.
(3) Pinaud, F.; Michalet, X.; Bentolila, L. A.; Tsay, J. M.; Doose, S.; Li, J. J.; Iyer, G.; Weiss, S. *Biomaterials* **2006**, *27*, 1679.
(4) Fu, A.; Gu, W.; Larabell, C.; Alivisatos, A. P. *Curr. Opin. Neurobiol.* **2005**, *15*, 568.
(5) Grecco, H. E.; Lidke, K. A.; Heintzmann, R.; Lidke, D. S.; Spagnuolo, C.; Martinez, O. E.; Jares-Erijman, E. A.; Jovin, T. M. *Microsc. Res. Technol.* **2004**, *65*, 169.
(6) Hechler, D.; Nitsch, R.; Hendrix, S. *Brain Res. Rev.* **2006**, *52*, 160.
(7) Stewart Jr, C. N. *Trends Biotechnol.* **2006**, *24*, 155.
(8) Jose, J.; Burgess, K. *Tetrahedron* **2006**, *62*, 11021.
(9) Toyo'oka, T. *Anal. Chim. Acta* **2002**, *465*, 111.
(10) Gikas, E.; Parissi-Poulou, M.; Kazanis, M.; Vavagianis, A. *Anal. Chim. Acta* **2003**, *489*, 153.
(11) Ammar, H.; Fery-Forgues, S.; El Gharbi, R. *Dyes Pigm.* **2003**, *57*, 259.

(12) Bennett, F. A.; Barlow, D. J.; Dodoo, A. N. O.; Hider, R. C.; Lansley, A. B.; Lawrence, M. J.; Marriott, C.; Bansal, S. S. *Anal. Biochem.* **1999**, *270*, 15.
(13) Wang, W.; Li, H. *Tetrahedron Lett.* **2004**, *45*, 8479.
(14) Sui, G.; Kele, P.; Orbulescu, J.; Huo, Q.; Leblanc, R. M. *Lett. Pept. Sci.* **2002**, *8*, 47.
(15) Berthelot, T.; Lain, G.; Latxague, L.; Déleris, G. *J. Fluoresc.* **2004**, *14*, 671.
(16) Clinton, R. O.; Laskowski, S. C. *J. Am. Chem. Soc.* **1949**, *71*, 3602.
(17) Bonsignore, L.; Cottiglia, F; Maccioni, A. M.; Secci, D.; Lavagna, S. M. *J. Heterocycl.Chem.* **1995**, *32*, 573.
(18) Corrie, J. E. T.; Munsasinghe, V. R. N. *J. Heterocycl. Chem.* **2000**, *37*, 1447.
(19) Takechi, H. K.; Machida, M. *Chem. Pharm. Bull.* **1996**, *44*, 793.
(20) Takadate, A.Y. I.; Irikura, M.; Fujino, H.; Goya, S. *Chem. Pharm. Bull.* **1989**, *37*, 373.
(21) Muller, C. E. P.; Viriot, M.-L.; Carré, M.-C. *Helv. Chim. Acta* **2001**, *84*, 3735.
(22) Van Gompel, J. S.; Schuster, G. B. *J. Org. Chem.* **1987**, *52*, 1465.
(23) Dörwald, F. Z. *Organic Synthesis on Solid Phase: Supports, Linkers, Reactions*; Wiley-VCH Verlag GmbH: Weinheim, 2002.
(24) Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G. In *Peptide Synthesis and Applications*; Howl J., Ed.; Humana Press: Totowa, 2005; p 3.
(25) Mitchell, A. R. *Pept. Sci.* **2008**, *90*, 175.
(26) Hara, M. R.; Agrawal, N.; Kim, S. F.; Cascio, M. B.; Fujimuro, M.; Ozeki, Y.; Takahashi, M.; Cheah, J. H.; Tankou, S. K.; Hester, L. D.; Ferris, C. D.; Hayward, S. D.; Snyder, S. H.; Sawa, A. *Nat. Cell Biol.* **2005**, *7*, 665.
(27) Hara, M. R.; Snyder, S. H. *Cell. Mol. Neurobiol.* **2006**, *26*, 527.
(28) Uehara, T.; Nakamura, T.; Yao, D.; Shi, Z. Q.; Gu, Z.; Ma, Y.; Masliah, E.; Nomura, Y.; Lipton, S. A. *Nature* **2006**, *441*, 513.
(29) Yao, D.; Gu, Z.; Nakamura, T.; Shi, Z. Q.; Ma, Y.; Gaston, B.; Palmer, L. A.; Rockenstein, E. M.; Zhang, Z.; Masliah, E.; Uehara, T.; Lipton, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10810.
(30) Dalle-Donne, I.; Milzani, A.; Giustarini, D.; Di, S. P.; Colombo, R.; Rossi, R. *J. Muscle Res. Cell Motil.* **2000**, *21*, 171.
(31) Han, P.; Zhou, X.; Huang, B.; Zhang, X.; Chen, C. *Anal. Biochem.* **2008**, *377*, 150.
(32) 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.
(33) Tao, Z. F.; Qian, X.; Fan, M. *Tetrahedron* **1997**, *53*, 13329.
(34) Ohuchi, Y.; Katayama, Y.; Maeda, M. *Anal. Sci.* **2001**, *17*, i1465.
(35) Kosiova, I.; Janicova, A.; Kois, P. *Beilstein J. Org. Chem* **2006**, *2*, 2.
(36) Webb, M. R.; Corrie, J. E. T. *Biophys. J.* **2001**, *81*, 1562.
(37) Bissell, E. R.; Mitchell, A. R.; Pearson, K. W.; Smith, R. E. U.S. Patent 4,388,233, 1983.
(38) Sato, E.; Matsuhisa, A.; Sakashita, M.; Kanaoka, Y. *Chem. Pharm. Bull.* **1988**, *36*, 3496.
(39) Shutes, A.; Der, C. J. *Methods* **2005**, *37*, 183.
(40) Brune, M.; Hunter, J.; Corrie, J.; Webb, M. *Biochemistry* **1994**, *33*, 8262.
(41) Hirshberg, M.; Henrick, K.; Haire, L. L.; Vasisht, N.; Brune, M.; Corrie, J. E. T.; Webb, M. R. *Biochemistry* **1998**, *37*, 10381.
(42) Piloto, A. M.; Rovira, D.; Costa, S. P. G.; Gonçalves, M. S. T. *Tetrahedron* **2006**, *62*, 11955.
(43) Piloto, A. M.; Fonseca, A. S. C.; Costa, S. P. G.; Gonçalves, M. S. T. *Tetrahedron* **2006**, *62*, 9258.
(44) Piloto, A. M.; Costa, S. P. G.; Gonçalves, M. S. T. *Tetrahedron Lett.* **2005**, *46*, 4757.
(45) Doré, K.; Dubus, S.; Ho, H. A.; Lévesque, I.; Brunette, M.; Corbeil, G.; Boissinot, M.; Boivin, G.; Bergeron, M. G.; Boudreau, D.; Leclerc, M. *J. Am. Chem. Soc.* **2004**, *126*, 4240.
(46) Jousselme, B.; Blanchard, P.; Levillain, E.; Delaunay, J.; Allain, M.; Richomme, P.; Rondeau, D.; Gallego-Planas, N.; Roncali, J. *J. Am. Chem. Soc.* **2003**, *125*, 1363.
(47) Melucci, M.; Gazzano, M.; Barbarella, G.; Cavallini, M.; Biscarini, F.; Maccagnani, P.; Ostoja, P. *J. Am. Chem. Soc.* **2003**, *125*, 10266.
(48) Barbarella, G.; Zambianchi, M.; Pudova, O.; Paladini, V.; Ventola, A.; Cipriani, F.; Gigli, G.; Cingolani, R.; Citro, G. *J. Am. Chem. Soc.* **2001**, *123*, 11600.
(49) Capobianco, M. L.; Naldi, M.; Zambianchi, M.; Barbarella, G. *Tetrahedron Lett.* **2005**, *46*, 8181.
(50) Barbarella, G.; Zambianchi, M.; Sotgiu, G.; Ventola, A.; Galeotti, M.; Gigli, G.; Cazzato, A.; Capobianco, M. L. *J. Non-Cryst. Solids* **2006**, *352*, 2465.
(51) Gatti, R.; Gioia, M. G.; Di Pietra, A. M. *Anal. Chim. Acta* **2002**, *474*, 11.
(52) Gatti, M.; Gioia, M. G.; Andreatta, P.; Pentassuglia, G. *J. Pharm. Biomed. Anal.* **2004**, *35*, 339.
(53) Uchiyama, S.; Takehira, K.; Kohtani, S.; Imai, K.; Nakagaki, R.; Tobita, S.; Santa, T. *Org. Biomol. Chem.* **2003**, *1*, 1067.
(54) Nemati, M.; Oveisi, M. R.; Abdollahi, H.; Sabzevari, O. *J. Pharm. Biomed. Anal.* **2004**, *34*, 485.
(55) Dufau, I.; Mazerguil, H. *Tetrahedron Lett.* **2000**, *41*, 6063.
(56) Solínová, V.; Kasicka, V.; Koval, D.; Barth, T.; Ciencialová, A.; Záková, L. *J. Chromatogr. B* **2004**, *808*, 75.
(57) Shi, Y. W.; Ming, Y. F.; Wang, C. P.; Zhao, X. E.; Zhang, R; You, J. M. *Chin. J. Anal. Chem* **2006**, *34*, 503.
(58) You, J. M.; Ming, Y. F.; Shi, Y. W.; Zhao, X. E.; Suo, Y. R. *Talanta* **2005**, *68*, 448.
(59) Xian-En, Z.; Jin-Mao, Y.; Hong-Zhen, L.; You-Rui, S. *Chin. J. Anal. Chem.* **2007**, *35*, 938.
(60) Eftink, M. R.; Shastry, M. C. R. *Methods Enzymol.* **1997**, *278*, 258.
(61) Lacowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic/Plenum: New York, 1999.
(62) Ross, A.; Szabo, A. G.; Hogue, C. W. V. *Methods Enzymol.* **1997**, *278*, 151.
(63) Wu, P.; Brand, L. *Anal. Biochem.* **1994**, *18*, 1.
(64) Buckler, D. R.; Haas, E.; Scheraga, H. A. *Biochemistry* **1995**, *34*, 15965.
(65) Twine, S. M.; Szabo, A. G. *Methods Enzymol.* **2003**, *360*, 104.
(66) Schlesinger, S. *J. Biol. Chem.* **1968**, *243*, 3877.
(67) Scott, D. J.; Leejeerajumnean, S.; Brannigan, J. A.; Lewis, R. J.; Wilkinson, A. J.; Hoggett, J. G. *J. Mol. Biol.* **1999**, *293*, 997.
(68) Soumillon, P.; Jespers, L.; Vervoort, J.; Fastrez, J. *Protein Eng.* **1995**, *8*, 451.
(69) Wong, C.-Y.; Eftink, M. R. *Protein Sci.* **1997**, *6*, 689.
(70) Mohammadi, F.; Prentice, G. A.; Merrill, A. R. *Biochemistry* **2001**, *40*, 10273.
(71) Blouse, G. E.; Perron, M. J.; Thompson, J. H.; Day, D. E.; Link, C. A.; Shore, J. D. *Biochemistry* **2002**, *41*, 11997.
(72) Chen, Y.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1994**, *98*, 2203.
(73) Filippis, V.; Boni, S.; Dea, E.; Dalzoppo, D.; Grandi, C.; Fontana, A. *Protein Sci.* **2004**, *13*, 1489.
(74) Saroja, G.; Ramachandram, B.; Saha, S.; Samanta, A. *J. Phys. Chem. B* **1999**, *103*, 2906.
(75) Soujanya, T.; Krishna, T. S. R.; Samanta, A. *J. Phys. Chem.* **1992**, *96*, 8544.
(76) Saroja, G.; Soujanya, T.; Ramachandram, B.; Samanta, A. *J. Fluoresc.* **1998**, *8*, 405.
(77) Soujanya, T.; Fessenden, R. W.; Samanta, A. *J. Phys. Chem.* **1996**, *100*, 3507.
(78) Mandal, D.; Sen, S.; Sukul, D.; Bhattacharyya, K.; Mandal, A. K.; Banerjee, R.; Roy, S. *J. Phys. Chem. B* **2002**, *106*, 10741.
(79) Vázquez, M. E.; Rothman, D. M.; Imperiali, B. *Org. Biomol. Chem.* **2004**, 1965.
(80) Muslin, A. J.; Tanner, J. W.; Allen, P. M.; Shaw, A. S. *Cell* **1996**, *84*, 889.
(81) Chersi, A.; Modugno, F.; Rosanò, L. *Biochim. Biophys. Acta* **1997**, *1336*, 83.
(82) Chersi, A.; Sezzi, M. L.; Romano, T. F.; Evangelista, M.; Nista, A. *Biochim. Biophys. Acta* **1990**, *1034*, 330.
(83) Naval, M. V.; Gómez-Serranillos, M. P.; Carretero, M. E.; De Arce, C. *J. Chromatogr. A* **2006**, *1121*, 242.
(84) Kang, X.; Xião, J.; Huang, X.; Gu, Z. *Clin. Chim. Acta* **2006**, *366*, 352.
(85) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906.
(86) Ting, A. Y.; Kain, K. H.; Klemke, R. L.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 15003.
(87) Gaietta, G.; Deerinck, T. J.; Adams, S. R.; Bouwer, J.; Tour, O.; Laird, D. W.; Sosinsky, G. E.; Tsien, R. Y.; Ellisman, M. H. *Science* **2002**, *296*, 503.
(88) Sako, Y.; Yanagida, T *Nat. Rev. Mol. Cell Biol.* **2003**, Suppl. SS1.
(89) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760.
(90) Summerer, D.; Chen, S.; Wu, N.; Deiters, A.; Chin, J. W.; Schultz, P. G. *PNAS* **2006**, *103*, 9785.
(91) Yang, Q.; Zhang, X.-L.; Ma, M.; Huang, K.-J.; Zhang, J.-X.; Ni, W.-Z.; Fang, C.-X.; Zheng, C.-Y. *J. Chromatogr. A* **2007**, *1146*, 23.
(92) Yang, J. Z.; Bastian, K. C.; Moore, R. D.; Stobaugh, J. F.; Borchardt, R. T. *J. Chromatogr. B* **2002**, *780*, 269.
(93) Manica, D. P.; Lapos, J. A.; Jones, A. D.; Ewing, A. G. *Anal. Biochem.* **2003**, *322*, 68.
(94) Dawson, L. A.; Organ, A. J.; Winter, P.; Lacroix, L. P.; Shilliam, C. S.; Heidbreder, C.; Shah, A. J. *J. Chromatogr. B* **2004**, *807*, 235.
(95) Parrot, S.; Sauvinet, V.; Riban, V.; Depaulis, A.; Renaud, B.; Denoroy, L. *J. Neurosci. Methods* **2004**, *140*, 29.
(96) Benturquia, N.; Parrot, S.; Sauvinet, V.; Renaud, B.; Denoroy, L. *J. Chromatogr. B* **2004**, *806*, 237.
(97) Zhang, L.-Y.; Sun, M.-X. *J. Chromatogr. A* **2004**, *1040*, 133.
(98) Lu, M.-J.; Chiu, T.-C.; Chang, P.-L.; Ho, H.-T.; Chang, H.-T. *Anal. Chim. Acta* **2005**, *538*, 143.

(99) Zhao, S.; Song, Y.; Liu, Y.-M. *Talanta* **2005**, *67*, 212.
(100) Gyimesi-Forrás, K.; Leitner, A.; Akasaka, K.; Lindner, W. *J. Chromatogr. A* **2005**, *1083*, 80.
(101) Clarke, G.; O'Mahony, S.; Malone, G.; Dian, T. G. *J. Neurosci. Methods* **2007**, *160*, 223.
(102) Wang, C.; Zhao, S.; Yuan, H.; Xião, D. *J. Chromatogr. B* **2006**, *833*, 129.
(103) Weber, G.; Farris, F. J. *J. Biochem.* **1979**, *18*, 3075.
(104) Valeur, B. *Molecular Fluorescence, Principles and Applications*; Wiley-VCH: Weinheim, 2002.
(105) Nitz, M.; Mezo, A. R.; Ali, M. H.; Imperiali, B. *Chem. Commun.* **2002**, 1912.
(106) Macgregor, R. B.; Weber, G. *Nature* **1986**, *319*, 70.
(107) Pierce, D. W.; Boxer, S. G. *J. Phys. Chem.* **1992**, *96*, 5560.
(108) Cohen, B. E.; McAnaney, T. B.; Park, E. S.; Jan, Y. N.; Boxer, S. G.; Jan, L. Y. *Science* **2002**, *296*, 1700.
(109) Nowak, M. W.; Gallivan, J. P.; Silverman, S. K.; Labarca, C. G.; Dougherty, D. A.; Lester, H. A. *Methods Enzymol.* **1998**, *293*, 504.
(110) Vázquez, M. E.; Nitz, M.; Stehn, J.; Yaffe, M. B.; Imperiali, B. *J. Am. Chem. Soc.* **2003**, *125*, 10150.
(111) Rothman, D. M.; Vázquez, M. E.; Vogel, E. M.; Imperiali, B. *Org. Lett.* **2002**, *4*, 2865.
(112) Sewald, N.; Jakubke, H. *Peptides: Chemistry and Biology*; Wiley-VCH: Weinheim, 2002.
(113) Ellis-Davies, G. C. R.; Kaplan, J. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6571.
(114) Park, C.; Givens, R. S. *J. Am. Chem. Soc.* **1997**, *119*, 2453.
(115) Wood, J. S.; Koszelak, M.; Liu, J.; Lawrence, D. S. *J. Am. Chem. Soc.* **1998**, *120*, 7145.
(116) Walker, J. W.; Gilbert, S. H.; Drummond, R. M.; Yamada, M.; Sreekumar, R.; Carraway, R. E.; Ikebe, M.; Fay, F. S *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1568.
(117) Curley, K.; Lawrence, D. S. *Curr. Opin. Chem. Biol.* **1999**, *3*, 84.
(118) Kaplan, J. H.; Forbush, G., III; Hoffman, J. F. *Biochemistry* **1978**, *17*, 1929.
(119) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910.
(120) Haugland, R. P. *Handbook of Fluorescent Probes and Research Products*; Molecular Probes: Eugene, OR, 2002.
(121) Sun, W. C.; Gee, K. R.; Klaubert, D. H.; Haugland, R. P. *J. Org. Chem.* **1997**, *62*, 6469.
(122) Ueno, Y.; Jiao, G.-S.; Burgess, K. *Pract. Synth. Proc.* **2004**, *31*, 2591.
(123) Confalone, P. N. *J. Heterocycl. Chem.* **1990**, *27*, 31.
(124) Wu, S.; Dovichi, N. J. *J. Chromatogr.* **1989**, *480*, 141.
(125) Udenfriend, S.; Stein, S.; Böhlen, P.; Dairman, W.; Leimgruber, W.; Weigele, M. *Science* **1972**, *178*, 871.
(126) Neckers, D. C.; Valdes-Aguilera, O. M. In *Advances in Photochemistry*; Volman, D., Hammond, G. S., Neckers, D. C., Eds.; Wiley Interscience: New York, 1993; Vol. 18, p 315.
(127) Talhavini, M.; Atvars, T. D. Z. *J. Photochem. Photobiol. A: Chem.* **1998**, *114*, 65.
(128) Talhavini, M.; Atvars, T. D. Z. *J. Photochem. Photobiol. A: Chem.* **1999**, *120*, 141.
(129) Weigele, M.; DeBernardo, S.; Leimgruber, W. *Biochem. Biophys. Res. Commun.* **1973**, *50*, 352.
(130) Geisow, M. J. *Exp. Cell. Res.* **1984**, *150*, 29.
(131) Carvell, M.; Robb, I. D.; Small, P. W. *Polymer* **1998**, *39*, 393.
(132) Martin, M. M. *Chem. Phys. Lett.* **1975**, *35*, 105.
(133) Stein, S.; Böhlen, P.; Udenfriend, S. *Arch. Biochem. Biophys.* **1974**, *163*, 400.
(134) Bridges, M. A.; McErlane, K. M.; Kwong, E.; Katz, S.; Applegarth, D. A. *Clin. Chim. Acta* **1986**, *157*, 73.
(135) Vivès, E.; Lebleu, B. *Tetrahedron Lett.* **2003**, *44*, 5389.
(136) Lau, S. K.; Zaccardo, F.; Little, M.; Banks, P. *J. Chromatogr. A* **1998**, *809*, 203.
(137) Fulop, L.; Penke, B.; Zarandi, M. *J. Pept. Sci.* **2001**, *7*, 397.
(138) Hoffmann, C.; Leroy-Dudal, J.; Patel, S.; Gallet, O.; Pauthe, E. *Anal. Biochem.* **2008**, *372*, 62.
(139) Konecsni, T.; Kilár, F. *J. Chromatogr. A* **2004**, *1051*, 135.
(140) Laird, V.; Spiess, M. *Exp. Cell Res.* **2000**, *260*, 340.
(141) Li, X.; Ma, H.; Dong, S.; Duan, X.; Liang, S. *Talanta* **2004**, *62*, 367.
(142) Fernández-Carneado, J.; Giralt, E. *Tetrahedron Lett.* **2004**, *45*, 6079.
(143) Fernández-Carneado, J.; Kogan, M. J.; Castelo, S.; Giralt, E. *Angew. Chem., Int. Ed* **2004**, *43*, 1811.
(144) Engering, A. J.; Cella, M.; Fluitsma, D. M.; Hoefsmit, E. C. M.; Lanzavecchia, A.; Pietres, J. *Adv. Exp. Med. Biol.* **1997**, *417*, 183.
(145) van der Bruggen, P.; van den Eynde, B. J. *Curr. Opin. Immunol.* **2006**, *18*, 98.
(146) Brimble, M. A.; Kowalczyk, R.; Harris, P. W. R.; Dunbar, P. R.; Muir, V. J. *Org. Biomol. Chem.* **2008**, *6*, 112.
(147) Tsien, R. Y. *Methods Cell. Biol.* **1989**, *30*, 127.
(148) Gao, J.; Wang, P.; Giese, R. W. *Anal. Chem.* **2002**, *74*, 6397.
(149) Burchak, O. N.; Mugherli, L.; Chatelain, F.; Balakirev, M. Y. *Bioorg. Med. Chem.* **2006**, *14*, 2559.
(150) Adamczyk, M.; Grote, J.; Moore, J. A. *Bioconjugate Chem.* **1999**, *10*, 544.
(151) Martin, M. M.; Lindqvist, L. *J. Luminesc.* **1975**, *10*, 381.
(152) Flemming, G. R.; Knight, A. W. E.; Morris, J. M.; Morrison, R. J. S.; Robinson, G. W. *J. Am. Chem. Soc.* **1977**, *99*, 4306.
(153) Lohse, J.; Nielsen, P. E.; Harrit, N.; Dahl, O. *Bioconjugate Chem.* **1997**, *8*, 503.
(154) Wang, L.; Roitberg, A.; Meuse, C.; Gaigalas, A. K. *Spectrochim. Acta, Part A* **2001**, *57*, 1781.
(155) Klonis, N.; Sawyer, W. H. *J. Fluoresc.* **1996**, *6*, 147.
(156) Gao, J.; Wang, P.; Giese, R. W. *Anal. Chem.* **2002**, *74*, 6397.
(157) Falck, J. R.; Krieger, M.; Goldstein, J. L.; Brown, M. S. *J. Am. Chem. Soc.* **1981**, *103*, 7396.
(158) Galloway, C. J.; Dean, G. E.; Marsh, M.; Rudnick, G.; Mellman, I. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3334.
(159) Andrews, P. C.; Hines, C. M.; Dixon, J. E. *Biochemistry* **1980**, *19*, 5494.
(160) Fiorucci, L.; Erba, F.; Bolognesi, M.; Coletta, M.; Ascoli, F. *FEBS Lett.* **1997**, *408*, 85.
(161) Wei, A.-P.; Williams, M. G. U.S. Patent 10,930,522, 2007.
(162) Gosalia, D. N.; Salisbury, C. M.; Maly, D. J.; Ellman, J. A.; Diamond, S. L. *Proteomics* **2005**, *5*, 1292.
(163) Menchen, S. M.; Lee, L. G.; Connel, C. R.; Hershey, N. D.; Chakerian, V.; Woo, S. L.; Fung, S. U.S. Patent 6,649,598, 2003.
(164) Tian, M.; Wu, X.-L.; Zhang, B.; Li, J.-L.; Shi, Z. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1977.
(165) Hourdez, S.; Lallier, F. H.; De Cian, M.-C.; Green, B. N.; Weber, R. E.; Toulmo, A. *Physiol. Biochem. Zool.* **2000**, *73*, 365.
(166) Khanna, P. L.; Ullman, E. F. U.S. Patent 4,318,846, 1982.
(167) Hohsaka, T.; Sisido, M. *Curr. Opin. Chem. Biol.* **2002**, *6*, 809.
(168) Kang, S.-H.; Jun, S.-Y; Kim, D.-M. *Anal. Biochem.* **2007**, *360*, 1.
(169) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269.
(170) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063.
(171) Hoffmann, C.; Gaietta, G.; Bunemann, M.; Adams, S. R.; Oberdorff-Maass, S.; Behr, B.; Vilardaga, J. P.; Tsien, R. Y.; Eisman, M. H.; Lohse, M. J. *Nat. Methods* **2005**, *2*, 171.
(172) Spagnuolo, C. C.; Vermeij, R. J.; Jares-Erijman, E. A. *J. Am. Chem. Soc.* **2006**, *128*, 12040.
(173) Martin, B. R.; Giepmans, B. N. G.; Adams, S. R.; Tsien, R. Y. *Nat. Biotechnol.* **2005**, *23*, 1308.
(174) Kottegoda, S.; Aoto, P. C.; Sims, C. E.; Allbritton N. L. *Anal. Chem.* **2008**, *80*, 5358.
(175) Liu, B.; Archer, C. T.; Burdine, L.; Gillette, T. G.; Kodadek, T. *J. Am. Chem. Soc.* **2007**, *129*, 12348.
(176) Zhang, X.-Y.; Bishop, A. C. *J. Am. Chem. Soc.* **2007**, *129*, 3812.
(177) Hearps, A. C.; Pryor, M. J.; Kuusisto, H. V.; Rawlinson, S. M.; Piller, S. C.; Jans, D. A. *J. Fluoresc.* **2007**, *17*, 593.
(178) Arbeloa, I. L.; Ojeda, P. R. *Chem. Phys. Lett.* **1981**, *79*, 347.
(179) Amat-Guerri, F.; Costela, A.; Figuera, J. M.; Florido, F.; Sastre, R. *Chem. Phys. Lett.* **1993**, *209*, 352.
(180) Walsh, R. J.; Reinot, T.; Hayes, J. M.; Kalli, K. R.; Hartmann, L. C.; Small, G. J. *J. Lumin.* **2002**, *98*, 115.
(181) Abugo, O. O.; Nair, R.; Lakowicz, J. R. *Anal. Biochem.* **2000**, *279*, 142.
(182) Wippersteg, V.; Ribeiro, F.; Liedtke, S.; Kusel, J. R.; Grevelding, C. G. *Int. J. Parasitol.* **2003**, *33*, 1139.
(183) Horneffer, V.; Forsmann, A.; Strupat, K.; Hillenkamp, F.; Kubitscheck, U. *Anal. Chem.* **2001**, *73*, 1016.
(184) Mandalá, M.; Serck-Hanssen, G.; Martino, G.; Helle, K. B. *Anal. Biochem.* **1999**, *274*, 1.
(185) Wang, Y.; Hao, D.; Stein, W. D.; Yang, L. *Biochim. Biophys. Acta* **2006**, *1758*, 1671.
(186) Meng, Q.; Yu, M.; Zhang, H.; Ren, J.; Huang, D. *Dyes Pigm.* **2007**, *73*, 254.
(187) Guzikowski, A. P.; Naleway, J. J.; Shipp, C. T.; Schutte, R. C. *Tetrahedron Lett.* **2000**, *41*, 4733.
(188) Lavis, L. D.; Chao, T.-Y.; Raines, R. T. *ACS Chem. Biol.* **2006**, *1*, 252.
(189) Raines, R. T. *Chem. Rev.* **1998**, *98*, 1045.
(190) Bandichhor, R.; Petrescu, A. D.; Vespa, A.; Kier, A. B.; Schroeder, F.; Burgess, K. *Bioconjugate Chem.* **2006**, *17*, 1219.
(191) Miyaura, N.; Suzuk, A. *Chem. Rev.* **1995**, *95*, 2457.
(192) Sonogashira, K.; Tohda, Y.; Hagihara, N. *Tetrahedron Lett.* **1975**, *16*, 4467.
(193) Cai, M.; Stankova, M.; Pond, S. J. K.; Mayorov, A. V.; Perry, J. W.; Yamamura, H. I.; Trivedi, D.; Hruby, V. J. *J. Am. Chem. Soc.* **2004**, *126*, 7160.
(194) Gao, Y.; Li, J. L.; Shi, Z. *Chin. Chem. Lett.* **2007**, *18*, 667.

(195) Sathyamoorthi, G.; Boyer, J. H.; Allik, T. H.; Chandra, S. *Heteroat. Chem.* **1994**, *5*, 403.
(196) Treibs, A.; Kreuzer, F.-H. *Liebigs Ann. Chem.* **1968**, *718*, 203.
(197) Haugland, R. P.; Kang, H. C. U.S. Patent 4,774,339, 1988.
(198) Johnson, I. D.; Kang, H. C.; Haugland, R. P. *Anal. Biochem.* **1991**, *198*, 228.
(199) Karolin, J.; Johansson, L. B. A.; Strandberg, L.; Ny, T. *J. Am. Chem. Soc.* **1994**, *116*, 7801.
(200) Keller, R. C. A.; Silvius, J. R.; De Kruijff, B. *Biochem. Biophys. Res. Commun.* **1995**, *207*, 508.
(201) Schade, S. Z.; Jolley, M. E.; Sarauer, B. J.; Simonson, L. G. *Anal. Biochem.* **1996**, *243*, 1.
(202) Jones, L. J.; Upson, R. H.; Haugland, R. P.; Panchuk-Voloshina, N.; Zhou, M.; Haugland, R. P. *Anal. Biochem.* **1997**, *251*, 144.
(203) Durand, J.; Kojic, L.; Wang, Y.; Lee, P.; Cynader, M. S.; Gu, Q. *Neuroscience* **2000**, *97*, 11.
(204) Marmé, N.; Knemeyer, J.-P.; Sauer, M.; Wolfrum, J. *Bioconjugate Chem.* **2003**, *14*, 1133.
(205) Traverso, G.; Diehl, F.; Hurst, R.; Shuber, A.; Whitney, D.; Johnson, C.; Levin, B.; Kinzler, K. W.; Vogelstein, B. *Nat. Biotechnol.* **2003**, *21*, 1093.
(206) Yang, P.; Whelan, R. J.; Jameson, E. E.; Kurzer, J. H.; Argetsinger, L. S.; Carter-Su, C.; Kabir, A.; Malik, A.; Kennedy, R. T. *Anal. Chem.* **2005**, *77*, 2482.
(207) Epand, R. F.; Sayer, B. G.; Epand, R. M. *FEBS J.* **2005**, *272*, 1792.
(208) Jang, H. G.; Park, M.; Wishnok, J. S.; Tannenbaum, S. R.; Wogan, G. N. *Anal. Biochem.* **2006**, *359*, 151.
(209) Loudet, A.; Burgess, K. *Chem. Rev.* **2007**, *107*, 4891.
(210) Thoresen, L. H.; Kim, H.; Welch, M. B.; Burghart, A.; Burgess, K. *Synlett* **1998**, 1276.
(211) Rurack, K.; Kollmannsberger, M.; Daub, J. *New J. Chem.* **2001**, *25*, 289.
(212) Metzker, M. L.; Ansari-Lari, M. A.; Liu, X.-M.; Holder, D. J.; Gibbs, R. A. *Biotechniques* **1998**, *25*, 446.
(213) Metzker, M. L.; Lu, J.; Gibbs, R. A. *Science* **1996**, *271*, 1420.
(214) Lee, L. G.; Connell, C. R.; Woo, S. L.; Cheng, R. D.; McArdle, B. F.; Fuller, C. W.; Halloran, N. D.; Wilson, R. K. *Nucleic Acids Res.* **1992**, *20*, 2471.
(215) Timpennan, A. T.; Khatib, K.; Sweedler, J. V. *Anal. Chem.* **1995**, *67*, 139.
(216) Li, J.-S.; Wang, H.; Cao, L.-W.; Zhang, H.-S. *Talanta* **2006**, *69*, 1190.
(217) Kang, H. C.; Haugland, R. P. U.S. Patent 5,433,869, 1995.
(218) Kang, H. C.; Haugland, R. P. U.S. Patent 5,451,663, 1995.
(219) Staros, J. V.; Wright, R. W.; Swingle, D. M. *Anal. Biochem.* **1986**, *156*, 220.
(220) Baker, G. A.; Pandey, S.; Kane, M. A.; Maloney, T. D.; Hartnett, A. M.; Bright, F. V. *Biopolymers* **2001**, *59*, 502.
(221) Karolin, J.; Johansson, L. B.-A.; Strandberg, L.; Ny, T. *J. Am. Chem. Soc.* **1994**, *116*, 7801.
(222) Daly, T. J.; Olson, J. S.; Matthews, K. S. *Biochemistry* **1986**, *25*, 5468.
(223) Kálai, T.; Hideg, K. *Tetrahedron* **2006**, *62*, 10352.
(224) Guerri-Amat, F.; Liras, M.; Carrascoso, M. L.; Sastre, R. *Photochem. Photobiol.* **2003**, *77*, 577.
(225) Hubbell, W. L.; Altenbach, C.; Hubbell, C. M.; Khorana, H. G. *Adv. Protein Chem.* **2003**, *63*, 243.
(226) Coskun, A.; Deniz, E.; Akkaya, E. U. *Org. Lett.* **2005**, *7*, 5187.
(227) Kálai, T.; Balog, M.; Jekõ, J.; Hideg, K. *Synthesis* **1999**, 973.
(228) Olofsson, M.; Kalinin, S.; Zdunek, J.; Oliveberg, M.; Johansson, L. B. *Phys. Chem. Chem. Phys.* **2006**, *8*, 3130.
(229) Oswald, B.; Simon, L.; Lehmann, G.; Terpetsching, E.; Wolfbeis, O. S. *Anal. Biochem.* **2000**, *280*, 272.
(230) Kukrer, B.; Akkaya, E. U. *Tetrahedron Lett.* **1999**, *40*, 9125.
(231) Dilek, G.; Akkaya, E. U. *Tetrahedron Lett.* **2000**, *413*, 721.
(232) Welder, F.; Paul, B.; Nakazumi, H.; Yagi, S.; Colyer, C. L. *J. Chromatogr. B* **2003**, *793*, 93.
(233) Kim, S. H.; Kim, J. H.; Cui, J. Z.; Gal, Y. S.; Jin, S. H.; Koh, K. *Dyes Pigm.* **2002**, *55*, 1.
(234) Hyodo, Y.; Nakazumi, H.; Yagi, S. *Dyes Pigm.* **2002**, *54*, 163.
(235) Tatarets, A. L.; Fedyunyayeva, I. A.; Dyubko, T. S.; Povrozin, Y. A.; Doroshenko, A. O.; Terpetschnig, E. A.; Patsenker, L. D. *Anal. Chim. Acta* **2006**, *570*, 214.
(236) Volkova, K. D.; Kovalska, V. B.; Tatarets, A. L.; Patsenker, L. D.; Kryvorotenko, D. V.; Yarmoluk, S. M. *Dyes Pigm.* **2007**, *72*, 285.
(237) Yagi, S.; Hyodo, Y.; Matsumoto, S.; Takahashi, N.; Kono, H.; Nakazumi, H. *J. Chem. Soc., Perkin Trans.* **2002**, *1*, 1417.
(238) Nakazumi, H.; Ohta, T.; Etoh, H.; Uno, T.; Colyer, C. L.; Hyodo, Y.; Yagi, S. *Synth. Met.* **2005**, *153*, 33.
(239) Arun, K. T.; Ramaiah, D. *J. Phys. Chem. A* **2005**, *109*, 5571.
(240) Almeida, P. *Química* **1999**, *73*, 9.
(241) Williams, D. J. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 690.
(242) Gómez-Hens, A.; Aguilar-Caballos, M. P. *Trends Anal. Chem.* **2004**, *23*, 127.
(243) Patonay, G.; Salon, J.; Sowell, J.; Strekowski, L. *Molecules* **2004**, *9*, 40.
(244) Tung, C.-H. *Biopolymers* **2004**, *76*, 391.
(245) Mitra, R. D.; Shendure, J.; Olejnik, J.; Krzymanska-Olejnik, E.; Church, G. M. *Anal. Biochem.* **2003**, *320*, 55.
(246) Weissleder, R.; Ntziachristos, V. *Nat. Med.* **2003**, *9*, 123.
(247) Tonge, R.; Shaw, J.; Middleton, B.; Rowlinson, R.; Rayner, S.; Young, J.; Pognan, F.; Hawkins, E.; Currie, I.; Davison, M. *Proteomics* **2001**, *1*, 337.
(248) Mason, S. J.; Balasubramanian, S. *Org. Lett.* **2002**, *4*, 4261.
(249) Mason, S. J.; Hake, J. L.; Nairne, J.; Cummins, W. J.; Balasubramanian, S. J. *J. Org. Chem.* **2005**, *70*, 2939.
(250) McCorquodale, E. M.; Colyer, C. L. *Electrophoresis* **2001**, *22*, 2403.
(251) Jiang, G.; Attiya, S.; Ocvirk, G.; Lee, W. E.; Harrison, D. J. *Biosens. Bioelectron.* **2000**, *14*, 861.
(252) Charles, P. T.; Gauger, P. R., Jr.; Kusterbeck, A. W. *Environ. Sci. Technol.* **2000**, *34*, 4641.
(253) Goldman, E. R.; Pazirandeh, M. P.; Charles, P. T.; Balighian, E. D.; Anderson, G. P. *Anal. Chim. Acta* **2002**, *457*, 13.
(254) Flanagan, J. H.; Menchen, S. H.; Khan, S.; Soper, S. A.; Hammer, R. P. *Bioconjugate Chem.* **1997**, *8*, 751.
(255) Peng, X.; Song, F.; Lu, E.; Wang, Y.; Zhou, W.; Fan, J.; Gao, Y. *J. Am. Chem. Soc.* **2005**, *127*, 4170.
(256) Song, F.; Peng, X.; Lu, E.; Wang, Y.; Zhou, W.; Fan, J. *Tetrahedron Lett.* **2005**, *46*, 4817.
(257) Hilderbrand, S. A.; Kelly, K. A.; Weissleder, R.; Tung, C.-H. *Bioconjugate Chem.* **2005**, *16*, 1275.
(258) Lin, Y.; Weissleder, R.; Tung, C.-H. *Bioconjugate Chem.* **2002**, *13*, 605.
(259) Mader, O.; Reiner, K.; Egelhaaf, H. J.; Fisher, R.; Brock, R. *Bioconjugate Chem.* **2004**, *15*, 70.
(260) Pham, Z.; Medarova, W.; Moore, A. *Bioconjugate Chem.* **2005**, *16*, 735.
(261) Chipon, B.; Clavé, G.; Bouteiller, C.; Massonneau, M.; Renard, P.-Y.; Romieu, A. *Tetrahedron Lett.* **2006**, *47*, 8279.
(262) Jiao, G-S.; Thoresen, L. H.; Burgess, K. *J. Am. Chem. Soc.* **2003**, *125*, 14668.
(263) Vassilev, A.; Deligeorgiev, T.; Gadjev, N.; Drexhage, K.-H. *Dyes Pigm.* **2005**, *66*, 135.
(264) Kovalska, V. B.; Volkova, K. D.; Losytskyy, M. Y.; Tolmachev, O. I.; Balanda, A. O.; Yarmoluk, S. M. *Spectrochim. Acta, Part A* **2006**, *65*, 271.
(265) Murphy, S.; Schuster, G. B. *J. Phys. Chem.* **1995**, *99*, 8516.
(266) Guether, R.; Reddington, M. V. *Tetrahedron Lett.* **1997**, *38*, 6167.
(267) Song, F.; Peng, X.; Lu, E.; Zhang, R.; Chen, X.; Song, B. *J. Photochem. Photobiol., A* **2004**, *168*, 53.
(268) Chen, X.; Peng, X.; Cui, A.; Wang, B.; Wang, L.; Zhang, R. *J. Photochem. Photobiol., A* **2006**, *181*, 79.
(269) Aubin, J. *J. Histochem. Cytochem.* **1979**, *27*, 36.
(270) Benson, R. C.; Meyer, R. A.; Zauba, M. E.; McKhann, G. M. *J. Histochem. Cytochem.* **1979**, *27*, 44.
(271) Dellinger, M.; Geze, M.; Santus, R.; Kohen, E.; Kohen, C.; Hirschberg, J. G.; Monti, M. *Biotechnol. Appl. Biochem.* **1998**, *28*, 25.
(272) Billinton, N.; Knight, W. A. *Anal. Biochem.* **2001**, *291*, 175.

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