Fluorescent amino acids: advances in protein-extrinsic fluorophores

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Fluorescent labeling is today of paramount importance to biological studies and numerous chemical dyes are used extensively to label biological specimens. This perspective highlights interesting aspects of fluorescent labeling by fluorescent peptides and small organic fluorophores that can be incorporated into proteins by genetic fusion to produce a fluorescent label. While many fluorescence applications rely on the use of intrinsic fluorophores, the development of new extrinsic fluorophores remains an essential element for the design of new fluorescent probes.

Introduction

Since biology entered the new post-genome era, the function of each gene present in a cell has attracted researchers' interests. Developments in organic chemistry, physics, engineering, biochemistry and biology have encouraged increased interest in specific classes of compounds as tools central to the study of localization, trafficking and expression levels of biomolecules in live cells. A representative list of biosensors and reporters useful in analyzing the molecular behavior and functions of living cells includes: fluorescent labels,[1a–j] isotopic markers,[2] radioactive tracers,[3] colorimetric biosensors,[4] photoswitchable biomaterials,[5a–d] photochromic compounds,[6a,b] and electrochemical sensors.[7a,b]

Fluorescent labels are very widely applied in biology, biotechnology and medicine[1f,g] and fluorophores are becoming increasingly useful in combinatorial chemistry and biology, both as encoders of individual library members and as reporters of chemical reactions.[1h–j] Current enhanced interest in fluorescent labeling has fed on the realization that this property is exhibited by a wide variety of molecules and structures, including many classes of organic frameworks[8a] (*e.g.*, coumarins,[8b] fluoresceins, cyanines, bodipy dyes), proteins such as green fluorescent protein,[9a] and even inorganic particles such as quantum dots.[9b,c] This diversity of molecular and electronic structures has led to the availability of fluorophores encompassing a wide range of photophysical properties including molar absorptivity, quantum yield, Stokes shift, lifetime and the ability to respond to the environment and other electronic systems.

New techniques for the labeling of the protein *in vitro* and *in vivo* are in active development.[10a–c] This perspective discusses advances in fluorescent probes for proteins focusing on small extrinsic organic fluorophores, and highlighting fluorescent amino acids.

Instrumentation for quantification of fluorescence

The development of a wide series of improved instruments has also assisted the applicability of fluorescence as a tool. Fluorescence

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microscopy was developed as a powerful technique to visualize the location of specific molecules in a cell.[11a] After the advent of Green Fluorescent Protein (GFP)-labeling, fluorescence microscopes including laser-confocal microscopes have been used in elucidating the molecular functions in cells.[11b–e] Fluorescent detection *via* single-molecule fluorescence spectroscopy (SMFS),[12] flow cytometry and spectrofluorometry together with flow cytometry (FACS) analysis, western blot assays and other immunoanalytical methods can localize the targets within a cell.

Fluorescence labeling techniques for proteins

New techniques for fluorescent labeling of molecules have been devised to directly monitor molecules in living cells. Fluorescent labeling techniques fall broadly into two categories: conventional or selective chemical fluorescent labeling and molecular tagging by introducing the genes of FPs (Fluorescent Protein) or specific binding motifs for fluorochromes by genetic engineering.[1b,13]

Green fluorescent protein

Many marine organisms are luminescent. Light-producing proteins comprise a primary light emitter and often a secondary photoprotein that red shifts the light for better penetration under water. Green Fluorescent Protein[9a] is one such secondary protein. It autocatalyzes the formation of its own fluorophore and thus can be expressed in a variety of organisms in its fluorescent form. GFP was originally isolated from the light-emitting organ of the jellyfish *Aequorea Victoria.*[14] Analogs of GFPs in a variety of colors have now been cloned from *Discosoma.*[15a] Such color variants of GFP can be fused genetically with high labeling specificity as fluorescent tags to a specific protein of interest.[15a–e] However, fluorescent proteins (FPs) have limitations since their relatively large size (238 amino acids for GFP) can potentially perturbate the structure and/or function of the host proteins leading to mislocalization and misexpression.

Labeling the proteins in living cells

Alternative chemoselective labeling techniques using low molecular weight compounds were developed to study protein biochemistry in living cells and overcome challenges in modern cell biology. Chemoselective labeling has been reviewed comprehensively covering the importance, and the advantages or disadvantages of the various methods.[1b,16a–g] The objective of this review is to describe concisely the main techniques of chemical-labeling, emphasizing fluorescent labeling by the incorporation of unnatural amino acids based on amber codon suppression mutagenesis.

In contrast to genetic encoding of fluorescent proteins (FPs) the selective chemical labeling technique fuses a small protein or peptide tag to a protein of interest (POI), reducing tag-POI potential interference.

In one method, a small protein is genetically fused with POI and specifically interacts with the small fluorophore containing the synthetically engineered ligand. The interaction between the receptor POI and the ligand can be covalent (*e.g.* in the case of hAGT and HaloTag protein)[17a] or noncovalent (*e.g.* DHFR, FKBP12).[17b] This method allows the incorporation of a variety of small-molecule fluorophores with infrared or near-infrared emission. Promising results for smaller protein labels have been achieved using fluorescein and rhodamine as organic fluorophores.[1c]

The next approach utilizes enzyme-mediated covalent attachment to a peptide tag. The protein expressed is fused to a peptide tag (*e.g.* an acceptor peptide). An enzyme (*e.g.* biotin ligase) labels the peptide tag directly with a small fluorescent molecule or with a functional handle to which the small molecule ligates through a bio-orthogonal reaction.[17c–i]

A further method based on metal-chelation coordination, together with enzymatic labeling, represents one of the most potent and successful ways for protein labeling.[1b,16a–g]

The first peptide tag/artificial probe pair utilizing multivalent coordination chemistry between peptide tag and multinuclear metal complexes was reported by Tsien's group in 1998.[18a] A biarsenical dye such as 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH) binds specifically with a short peptide sequence called the tetracysteine motif (CCPGCC) with high affinity. New generation of biarsenicals with unique functions[18b–d] as well as improvement of the tetracysteine tag[18e–f] has been developed by the Mayer and Miller groups. Vogel *et al.* applied the conventional His tag/Ni (II)-NTA pair for fluorescent labeling of a protein on a cell surface.[18g]

In 2006 Hamachi *et al.* developed an oligo-aspartate tag (D4 tag, (D4)n, n = 1–3) and multinuclear Zn (II) complexes (Zn(II)-DpaTyrs) for fluorescent imaging of a membrane-bound receptor protein.[18h,i]

Nagano *at. al.* based a novel protein labeling probe on quinine methide chemistry: a β-galactosidase labeling probe exhibits fluorescent wavelength changes after the labeling process, allowing rapid detection and precise quantification of the target protein.[18j]

Site-specific incorporation of unnatural amino acids based on suppressor tRNA technology is the approach for protein labeling discussed in the next topic.

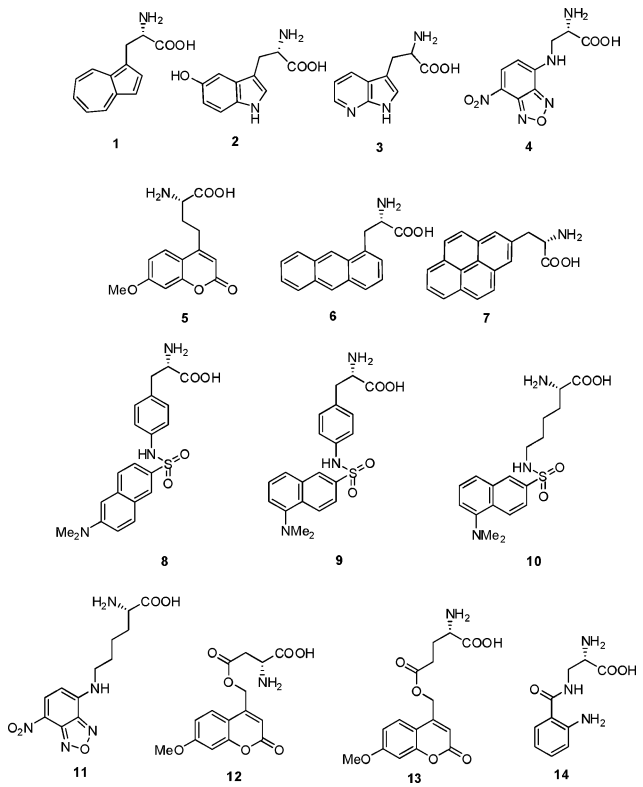
Small-molecule fluorescent labeling using nonnatural amino acids

The versatile biochemical technique of position-specific introduction of nonnatural amino acids into biosynthetic proteins enables protein engineering to insert a wide variety of artificial functions.[16b,19a,b] A variety of non-natural amino acids were incorporated using four base codon–anticodon pairs.[19c] For example, an amber stop codon (UAG) can be introduced by mutagenesis into the gene coding a protein of interest. During translation, a tRNA bearing the anticodon (AUC), which has been aminoacylated with an unnatural acid, suppresses the stop codon and enables production of the full-length protein containing the unnatural amino acid.

Fluorescent labeling of receptors, antibodies, and enzymes at specific positions was achieved by nonnatural mutagenesis.[20a–d] Use of appropriately labeled proteins enables detection of ligands, antigens, and inhibitors at extremely low concentrations. This requires fluorescent nonnatural amino acids that can be incorporated into proteins in high yields. Fluorescent tags must be highly sensitive to small changes of the microenvironment caused by the binding of small molecules. Furthermore, the nonnatural amino acids must be introduced at specific positions so that the binding ability of the protein is not suppressed.

To be efficiently incorporated into proteins, highly fluorescent amino acids should preferably absorb at wavelengths longer than

320 nm, where interference from the intrinsic tryptophan units is negligible. Compounds **1–13** (Scheme 1), satisfying these requirements, are among the fluorescent amino acids that have been incorporated with efficiencies of greater than 10% into proteins by *in vitro* protein synthesis.[19c,20b–d,21a–d] In particular, amino acids carrying 7-methoxycoumarinyl[21c] (**5**, **12**, **13**), β-anthraniloyl (**14**[20d]), 3-*N*-(7-nitro-2,1,3,benzooxadiazol-4-yl) (**4**,[21a] **11**[21d]) or dansyl (**8**–**10**[21c,20b]) fluorophores are highly sensitive to the microenvironment and thus well suited to the detection of ligand binding. By contrast, 2-anthrylalanine **6** emits fluorescence with similar quantum yields in both polar and less polar environments;[19c] therefore, although **6** is useful as a fluorescent tag to aid estimation of mutant protein concentration, it is insensitive to ligand binding, unless the ligand works as a quencher. This illustrates the importance of selecting an appropriate amino acid with the desired fluorescent properties in designing a probe protein.

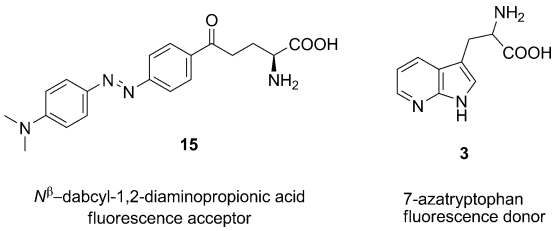


Scheme 1 Nonnatural fluorescent amino acids.

Very often, the amount of nonnatural mutants produced in the *in vitro* system is so small that the concentration of the fluorescent probe cannot be determined precisely. This is a serious drawback if fluorescence intensity is the only measure for quantitative determination of ligand concentrations. However, this disadvantage can be avoided by using fluorescence polarization or fluorescence decay-time as a measure of ligand–protein interactions, either of which can detect probe molecules down to nanomolar, or even picomolar concentrations.

The groups of Hecht[22] and Sisido[20a] first introduced a fluorophore–quencher pair at specific positions of a fusion protein by incorporating fluorescent donor and acceptor nonnatural amino acids.

Dihydrofolate reductase (DHFR) bearing a fusion peptide at its N terminus was labeled with unnatural amino acids incorporating a fluorescent acceptor and fluorescent donor which flanked an HIV-1 protease cleavage site. Energy transfer between the incorporated probes was illustrated, together with the feasibility of monitoring the special separation of the donor and acceptor in real time by the decrease in fluorescent energy transfer.[22] Dabcyl-diaminopropionic acid derivative **15** and 7-azatryptophan **3** were chosen for incorporation into the DHFR fusion peptide (Scheme 2).



Scheme 2

β-Anthraniloyl-L-α,β-diaminopropionic acid (antDap) **14** as a fluorescent amino acid and *p*-nitrophenylalanine (ntrPhe) as a quencher amino acid were incorporated in mutant streptavidins, synthesized through *Escherichia coli* in an *in vitro* protein synthesizing system.[20a] Intramolecular photoinduced electron transfer (ET) was monitored by decrease of intensity in steady state fluorescence spectroscopy and reported in terms of shortening of fluorescence decay times.

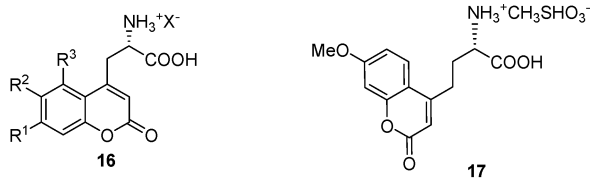
Coumarin labeled fluorescent amino acids

Coumarins (benzopyranones), the largest class of laser dyes for the "blue-green" region[23a–i] are highly sensitive. They have provided the most commercially acceptable categories of fluorescent derivatives with the advantages of an extended spectral range, high emission quantum yield, photostability, and good solubility in many solvents.

Previous enantioselective syntheses of coumarin-containing amino acids, have been carried out by diastereoselective alkylation of chiral glycine equivalents.[23f,24a,b] The synthesis of L-(6,7-dimethoxycoumarin-4-yl)alanine[23f,24a,b] and L-(7-methoxycoumarin-4-yl)alanine (L-Amp)[24c] were performed utilizing Oppolzer[24d] and Williams[24e] chiral auxiliaries respectively. These methods produced optically pure amino acids but required multiple steps which gave final products in moderate overall yields. The synthesis of coumarin amino acids was reported[25] utilizing protected aspartic and glutamic acids as chiral starting materials which led to coumaryl alanines **16** and β-(7-methoxycoumar-4-yl) ethylglycine **17** (Scheme 3) in a two- or a three-step process in yields of 16–71%.[25]

Coumarin-labeled lysines are of considerable general interest for the design and synthesis of fluorogenic triple-helical substrates for the analysis of matrix metalloproteinase family members.[23e,26a–c] Thus, N^{ε}-coumarin-labeled-N^{α}-Fmoc lysines allow successful labeling of peptide substrates by solid phase peptide synthesis for an extracellular matrix metalloprotease and present a powerful tool for proteolysis monitoring.[26]

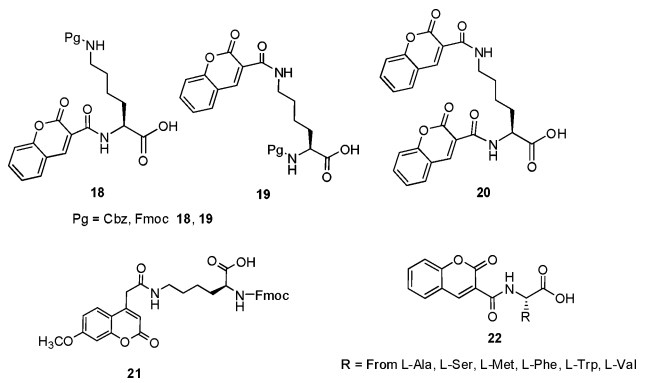
Recently our group has reported[27a,b] straightforward syntheses of coumarin-labeled amino acids and dipeptides which afford



R^1 = OH, OMe, OEt; R^2 = H, OMe, Cl;
R^3 = H, OH, OMe; X- = Cl^-,$CH_3SO_3^-$, $CF_3CO_2^-$

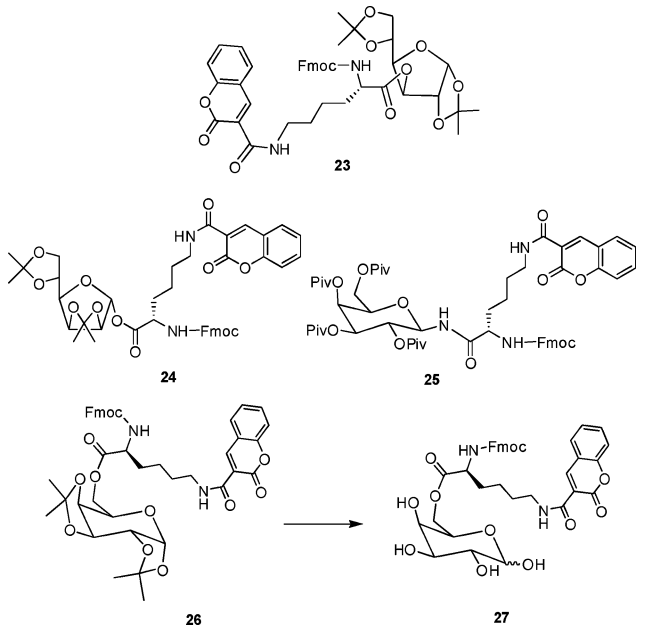
Scheme 3

enantiomerically pure fluorescent building blocks suitable for solid phase peptide synthesis (SPPS). Two-step synthetic route provides N^{α}- and N^{ε}-coumarin labeled Cbz- and Fmoc-L-lysines **19–21** and number of other amino acids **22** (Scheme 4) in 76–94% yields utilizing benzotriazole activated fluorogenic agents as coupling reagents.



Scheme 4

We also designed and synthesized a protected monosaccharide containing Fmoc-L-lysine scaffold based on fluorescent building blocks **23–26** (Scheme 5).[27c] After deprotection, **23–26** provide water soluble organic fluorophores **27** which could be useful for



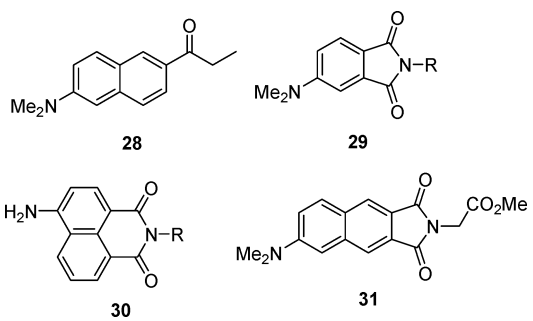
Scheme 5

peptide labeling at the *C*-terminus in inverse solid phase peptide synthesis (SPPS).[27d]

Environmental sensitive fluorophores and fluorescent amino acids

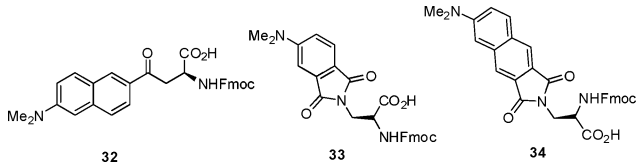
While many fluorescence applications rely on the use of intrinsic fluorophores, the development of new extrinsic fluorophores remains an essential element for the design of new fluorescent probes.

Environment-sensitive fluorophores are a special class of chromophores that have spectroscopic behavior that is dependent on the physico-chemical properties of the surrounding environment.[28a] Particularly useful are solvatochromic fluorophores sensitive to the polarity of the local environment, such as 2-propionyl-6-dimethylaminonaphthalene (PRODAN) **28**,[28b] 4-dimethylaminophthalimide (4-DMAP) **29**,[28c] and 4-amino-1,8-naphthalimide derivatives **30**[28d] (Scheme 6). The synthesis and preliminary photophysical characterization of a new environment-sensitive fluorophore 6-*N*,*N*-dimethylamino-2,3-naphthalimide (6DMN) was described.[28e] These molecules generally exhibit a low quantum yield in aqueous solution, but become highly fluorescent in nonpolar solvents or when bound to hydrophobic sites in proteins or membranes. The 4-(*N*,*N*-dimethylamino)phthalimide system **29** (4-DMAP) has been recognized as a fluorophore being extraordinarily environment-sensitive highly responsive to the changes in polarity and viscosity often found in biological media. The application of 4-DMAP sensors to the study of complex biological processes has been limited by the lack of simple chemical methods for the specific introduction of the probe into relevant biological systems. PRODAN and derivatives still constitute the most widely used environment-sensitive fluorophores, regardless of certain limitations mainly resulting from the relatively intense fluorescence even in aqueous environment.[28b] The 6DMN fluorophore combines some of the advantageous fluorescence properties of PRODAN with the extreme sensitivity to the local polarity exhibited by the 4-aminophthalimide family of environment-sensitive fluorophores.[28e]



Scheme 6 Environment-sensitive fluorophores: PRODAN **28**, 4-DMAP **20**, 4-amino-1,8-naphthalimidine **30** and 6DMN-GlyOMe **31**.

Syntheses of amino acid analogs of **28**,[28b,f] **29**[29] and **31**[28e] were developed to overcome the limitations in simple chemical methods for the specific introduction of the environment-sensitive fluorescent probes into the protein. Their Fmoc protected analogs **32–34** (Scheme 7) are suitable for the incorporation of these fluorophores into peptides using standard Fmoc solid phase synthesis.

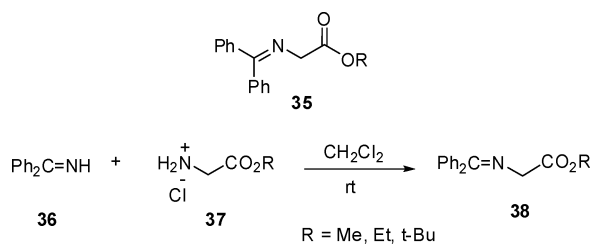


Scheme 7 Fmoc-protected derivatives of environment-sensitive fluorescent amino acids: ALADAN/DANA **32**, DAPA **33** and Dap(6DMN)-OH **34**.

The fluorescent amino acid 6-(2-dimethylaminonaphthoyl)-alanine (DANA),[28b] also called ALADAN[28f] is highly sensitive to the polarity of its surroundings and can be incorporated site-selectively at buried and exposed sites, of both soluble and membrane proteins[28f] in the form of Fmoc-DANA **32**. Showing similar fluorescent properties to PRODAN **28**, **32** has been useful for monitoring the phosphorylation-dependent binding of peptides to proteins,[28b] and for investigating protein–protein interactions.[28f] Recently, a series of novel fluorogenic probes that incorporate the environment-sensitive amino-acid analogs DANA and 4-DAPA was developed for monitoring peptide binding to class II MHC (Major Histocompatibility Complex) proteins in living cells, for immune response study.[28g] Syntheses of many other fluorescent amino acids have been published[21,28b,e,f,29] but until now none has the same degree of sensitivity to the environment as PRODAN.

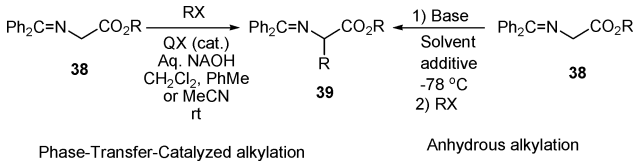
Synthetic strategies for non-natural fluorescent amino acids

The synthesis of chiral nonnatural fluorescent amino acids is an important part for the future development of fluorescent labels and remains a considerable challenge. We therefore discuss potential approaches to nonnatural fluorescent amino acids. Several strategies have been developed for the enantioselective synthesis of different types of α-amino acids.[30a,b] Benzophenone imines of glycine derivatives **35** have been used extensively as glycine anion equivalents for the preparation of optically active α-amino acids.[30a–m] The transimination under mild reaction conditions of glycine alkyl ester salt **37** with benzophenone imine **36** gives more reactive benzophenone equivalent **38** (Scheme 8). Substrates **38** are active methylene compounds that can be deprotonated using mildly basic conditions. Also phase-transfer-catalyzed (PTC) reactions of **38** with electrophiles provide an attractive route to the higher amino acid derivatives **39** (Scheme 9).



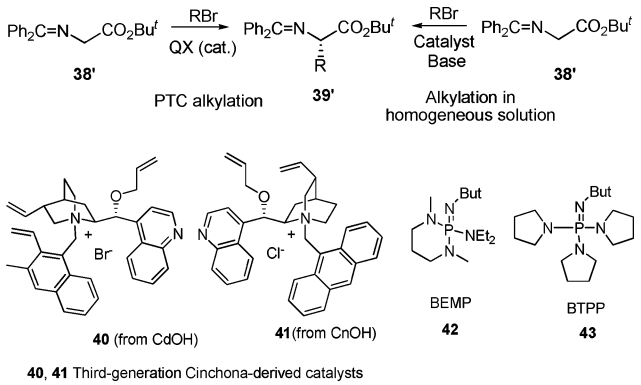
Scheme 8

Recent novel chiral catalysts for enantioselective, phase-transfer reactions have led to the increased use of this methodology.[30a–m] Catalytic enantioselective reactions were pioneered by the Merck group using phase-transfer catalysts, derived by N-alkylation of the *Cinchona* alkaloids, for the preparation of alkylated indanone



Scheme 9 PTC and anhydrous alkylation of benzophenone imine derivatives.

derivatives.[30c] Investigations performed in 1989–1997 by the groups of O'Donnell,[30d–f] Lygo[30g,h] and Corey[30i,j] for the catalytic enantioselective PTC alkylation of imine **38′** utilized the first and third generation of cinchonine, cinchonidine and cinchona-derived catalysts (Scheme 10). The highest enantioselectivities reported were 94% ee (with benzyl bromide) for the Lygo system and 99.5% ee (with either *n*-hexyl iodide or benzhydryl bromide) using catalyst **40** as reported by Corey (Scheme 10). This method can be extended to the implementation of aromatic α-halo ketones as the electrophilic species. Utilizing catalyst **40**, both high yield and excellent enantioselectivity were achieved in the synthesis of DANA.[28b]



Scheme 10

These catalytic enantioselective reactions were also reported[30k] in *homogeneous* solution by using the Schwesinger bases, 2-*tert*-butylimino-2-diethylamino-1,3-dimethyl-perhydro-1,3,2-diazaphosphorine (BEMP) **42** or *tert*-butylimino-tri(pyrrolidino)-phosphorane (BTPP) **43**, in conjunction with catalyst **40** or its pseudoenantiomer **41**.[30k] The highest enantioselectivity (97% ee) was obtained using isobutyl bromide as the alkyl halide. The enantioselective solid-phase synthesis of α-amino acid derivatives was also reported using the *Cinchona*-derived reagents, **40** and **41**, together with the Schwesinger bases, BEMP or BTP, where enantioselectivities of up to 89% ee were obtained.[30l,m]

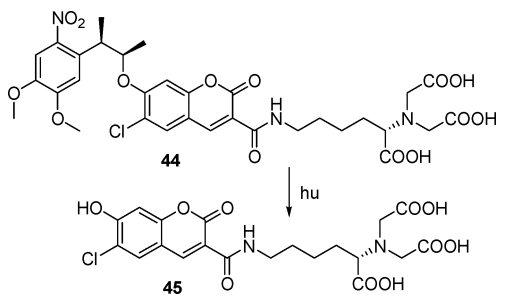
Other reactions for the enantioselective synthesis of different types of α-amino acids utilizing benzophenone imines of glycine derivatives include: Michael addition, aldol reactions, stereoselective reactions involving chiral auxiliaries and so on.[30a]

Photoactivated fluorophores

Light activated fluorescent molecules (also called "caged" fluorophores), including photoswitchable GFP's[31a] are important research tools for tracking the spatiotemporal dynamics of molecular events in biological systems[32b] where caged fluorescence has been used to study cytoskeletal proteins in locomoting cells. Such

markers prompted innovative cell biology work.[31c] The general strategy in masking fluorescence is to perturb the electronic structure by attaching of the photoremovable group to make the molecule either non-fluorescent or very weakly so. Photoactivation removes the protecting group and abruptly switches on the fluorescence of the parental dye. Desirable properties for the "caged" fluorophores include fast release of the fluorophore and large enhancement of the fluorescence in response to brief irradiation plus ability of the released dye to resist photobleaching. The resultant fluorescent mark is monitored with time to provide quantitative dynamic information. The initial "caged" fluorophores, derivatives of fluorescein[31b] and rhodamine,[31d] have been reviewed.[31b] Subsequently, 7-hydroxycoumarin derivatives, "caged" as *o*-nitrobenzyl ethers by Williamson coupling reactions[32a] offered 200-fold fluorescence enhancement after UV photolysis together with increased uncaging cross sections as compared to previously reported "caged" fluorophores.[32a] Novel ortho-nitrobenzyl fluorescein derivatives ("caged" Tokyo-Greens) also display a large fluorescence enhancement.[32b]

A recent class of "caged" 7-hydroxycoumarin fluorophores[33] possess 3-(3,4-dimethoxyphenyl)butan-2-ol (DMNPB) ether links as photoremovable protection and a Ni-nitrilotriacetic (Ni^{2+}-NTA) His–tag recognition motif. The UV analysis of the photolytic release of **45** from precursor **44** after irradiation showed the decrease in absorbance at 355 nm and increase at 406 nm, corresponding to the disappearance of **44** and the formation of **45** (Scheme 11). An almost quantitative formation of **45** after full conversion of **44** was demonstrated by HPLC. Fluorescence correlation spectroscopy (FCS) of cell membrane permeable Ni^{2+}-NTA dyes demonstrated that Ni^{2+}-NTA-coumarin (derived from **44**) binds a His-tagged protein, thus changing the physical properties of diffusion of the fluorescent probe.



Scheme 11 Conversion of 7-DMNPB-coumarin-NTA to coumarin-NTA after near UV (315–406 nm) activation.

Conclusions and future prospects

The short literature survey now presented illustrates the rapid contemporary development of completely synthetic fluorescent probes for a wide range of physiological parameters and demonstrates the fast expansion in both the number and sophistication of fluorescent protein biosensors, and the molecular techniques available to prepare fluorescent analogues of proteins.

What can we expect for future fluorescent probes? New kinds of fluorophores are still needed to increase their utility and range of applications. Ability to tune the wavelengths and intensities of absorption and emission of fluorophores will add to the range of properties that are currently available, and will increase their utility and range of applications.

Novel environment-sensitive fluorophores, designed to manifest modified emission properties in response to changes in the surrounding environment are of great interest. Thus we may expect future pH sensitivity examples reporting changes in living cells associated with some pathological processes or some other applications in biology. Recent advances in the area of new chiral catalysts for enantioselective, phase-transfer reactions will doubtless lead to the increased use of this methodology for the preparation of the new chiral fluorescent α-amino acids.

The number of nonnatural fluorescent amino acids that can efficiently be incorporated into proteins is still limited, thus new structures incorporating polycyclic hydrocarbon structures such as perylene, benzopyrene and so on, could have the potential to display fluorescent emission and high quantum yields even in protic solvents.

Acknowledgements

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