Fluorescent Analogs of Biomolecular Building Blocks: Design, Properties, and Applications

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1. Introduction

Fluorescence spectroscopy, one of the most informative and sensitive analytical techniques, has played and continues to play key roles in modern research. Indeed, unraveling the inner workings of biomolecules, cells, and organisms relied on the development of fluorescence-based tools. As many of the players in these sophisticated interactions and exceedingly complex systems are not inherently emissive, researchers have relied on synthesizing fluorescent analogs of the building blocks found in biological macromolecules. These are the constituents of the cell surface and cell membrane, as well as proteins and nucleic acids. This review is dedicated to emissive analogs of these relatively small molecules.

For organizational purposes, we have arbitrarily selected to approach these diverse families of biomolecules by imagining "a journey into the center of the cell". Approaching the exterior of a cell, one first encounters oligosaccharides that decorate the cell surface and are involved in cell recognition and signaling. Next, we arrive at the cell membrane itself. This semipermeable envelope sets the cell boundaries and regulates its traffic. Several types of building blocks assemble this membrane, most notably among them are the phospholipids. Upon entering the cell, the cytosol reveals a plethora of small and large molecules, including proteins, as well as soluble RNA molecules and RNA-rich ribosomes. Within the cytosol of eukaryotes and prokaryotes lies the nucleus or nucleoid, respectively. This membrane-enclosed control center contains most of the cells' genetic material. DNA, the cellular blueprint, is permanently found in the nucleus, which also hosts diverse RNA molecules. Accordingly, we first discuss emissive carbohydrate deriva-

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Nicholas J. Greco, having earned his B.S. in Chemistry from Stonehill College (Easton, MA) in 2002, joined the laboratory of Professor Yitzhak Tor at the University of California, San Diego. In 2008, he earned his Ph.D. for his work on the design, synthesis, and incorporation of novel fluorescent nucleosides. After a postdoctoral stay at Boston College in the laboratory of Professor Larry W. McLaughlin, he moved to Western Connecticut State University where he is currently an Assistant Professor of Chemistry. His main research interests include novel nucleosides and their effect on the structure and function of biological systems.

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tives. We then present fluorescent membrane constituents, followed by emissive amino acids. Our journey ends by focusing on emissive analogs of nucleosides and nucleotides, the building blocks of nucleic acids.

The common biomolecular building blocks, excluding a few amino acids, lack appreciably useful fluorescence properties. This implies that structural modifications are required to impart such photophysical features. Ideally, a designer probe should closely resemble its natural counterpart in size and shape without the loss of the original function (a feature we refer to as "isomorphicity"). This presents a fundamental predicament, because any modification attempting to alter the electronic nature of a molecule, typically by including aromatic residues or extending conjugation, will also alter its steric bulk and therefore the interactions with its surroundings.

Clearly not all biomolecular building blocks can or need to accommodate strict isomorphic design criteria. The heterocycles found in nucleosides already provide a platform that facilitates the extension of π-conjugation, which is also true for some aromatic amino acids. In contrast, employing fluorescence spectroscopy to membrane research requires very creative probe designs. Saccharides can be viewed as the most restrictive in this context, because no chemical modification is conceivable without a major structural disruption and likely loss of function. Such aliphatic biomolecules accommodate labeling only, where an established fluorophore is covalently conjugated to provide an emissive derivative. We therefore reserve the term *probe* to molecular designs that are expected to furnish useful modified biomolecules capable of reliable reporting. Understandably, fluorescent probes must meet the most stringent isomorphic design principles to ensure a biologically meaningful readout. The isomorphic design principle is therefore a central theme of this review.

This review focuses on designing fluorescent probes for the four major families of macromolecular building blocks discussed above. Although not necessarily in chronological order, it spans roughly four decades of probe design with emphasis, when justified, on recent contributions. As the reader may imagine, this topic encapsulates a vast research field and cannot be comprehensively reviewed within the space limitation of *Chemical Reviews*. Nevertheless, we have attempted to summarize the most important and general contributions discussing fluorescent probes that were designed to shed light on biological processes and refer the reader to other resources.[1] Although a few examples have found their way into the text, we do not generally address here the development of small molecule fluorophores and sensors that are not part of biomolecular assemblies. We open this review with a brief overview of the key features of fluorescence spectroscopy, where essential theoretical, experimental, and practical elements are discussed.

2. Fluorescence Spectroscopy Techniques in a Nutshell

2.1. Essentials and Benefits of Fluorescence Spectroscopy

Any spectroscopy-based technique is associated with inherent sensitivity traits and time-scale features, which are dependent on the fundamental nature of the transitions involved. Optical excitation of a chromophore generates the Franck−Condon state extremely rapidly (within 10^{-15} s). The efficiency of this process is related to the chromophore's absorption cross-section (σ), which is proportional to its extinction coefficient (ε). Vibrational relaxation (within 10^{-12}−10^{-10} s) quickly populates the lowest vibronic state of the chromophore's excited state (Figure 2.1, Jablonski diagram). This relaxation process, generating the emissive state, accounts for the lower emission energy of a chromophore compared with its excitation energy (Stokes shift). Typical organic chromophores reside in their excited state for a period of (0.5−20) × 10^{-9} s. The excited state lifetime reflects the sum of the various radiative and nonradiative processes that the excited chromophore undergoes in decaying back to the ground state (τ_0). The fraction responsible for emitting a photon, or the fluorescence lifetime (τ), reflects the emission quantum yield of the chromophores ($Q = \Phi = \tau/\tau_0$). In some studies, the brightness ($\varepsilon\Phi$) of a fluorophore is reported, which is the product of the molar absorptivity (ε) and the fluorescence quantum yield (Φ). This becomes useful when comparing the utility of two fluorophores with similar fluorescence quantum yields but very different molar absorptivities.

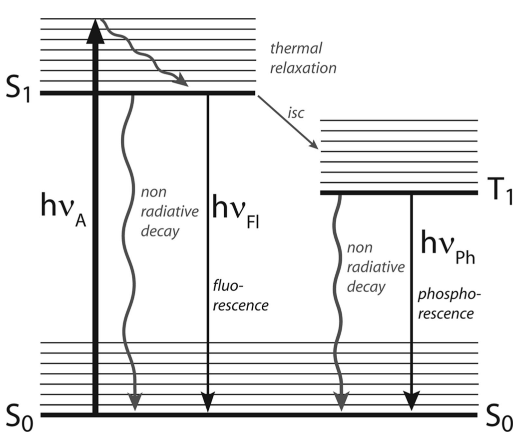


Figure 2.1. A simplified Jablonski diagram.

Fluorescence-based techniques are commonly appreciated for their versatility and sensitivity (up to a 1000-fold higher than absorption spectrophotometry). Creative probe design can provide chromophores with appropriate excitation and emission wavelengths, while minimizing interference by other emissive cellular constituents. Selective excitation coupled to the sensitivity of many chromophores to various environmental parameters (pH, polarity, viscosity, presence of quenchers, etc.) makes molecular fluorescence an extremely effective tool for *in vitro* biophysical and biochemical analyses, as well as *in vivo* cellular imaging, capable of providing spatial and temporal information.[2,3]

Before discussing the chromophoric biomolecular building blocks themselves, we first survey the most common techniques and tools used in fluorescence spectroscopy. For additional theoretical and technical details, the interested reader is referred to Valeur's *Molecular Fluorescence*,[4] Turro's *Modern Molecular Photochemistry of Organic Molecules*,[5] and Lakowicz's comprehensive monograph entitled *Principles of Fluorescence Spectroscopy*.[2]

2.2. Steady-State Fluorescence Spectroscopy

The simplest and most frequently used technique is steady-state fluorescence spectroscopy. Upon excitation of a chromophore (typically at its absorption maximum) with a light source providing a constant photon flow, an emission spectrum is recorded, revealing the energy maximum and intensity of emission. At low concentrations (absorbance < 10^{-2}), the emission intensity is typically proportional to the concentration of the chromophore (with approximately 1% deviation from linearity). While the emission maximum is an intrinsic characteristic of a chromophore, it is frequently sensitive to environmental perturbations.

Fluorophores with emission maxima that display sensitivity to polarity can be used to estimate the properties of the chromophore's microenvironment. If the dipole moment of the excited state is greater than that of the ground state, rearrangement of solvent molecules can lower the energy of the excited state prior to emission, resulting in a red shift of the emission maximum.[6,7] This phenomenon has been employed, for example, to investigate the local polarity in membranes,[8] proteins,[9,10] and DNA.[11] Dielectric constants (ε), reflecting a bulk property, were initially used to express polarity as orientational polarizability, Δf.[6,7] With the development of microscopic solvent polarity parameters (such as Reichardt's $E_T(30)$ scale), polarity could be quantified at the molecular level.[12] This is of significance for the study of confined cavities in biomolecules, where the local polarity is likely to differ dramatically from the aqueous bulk polarity. Indeed, microscopic polarity parameters, show a better linear correlation with Stokes shifts ($\nu_{abs} - \nu_{em}$), when compared with dielectric constants or orientational polarizability values (Figure 2.2).[13]

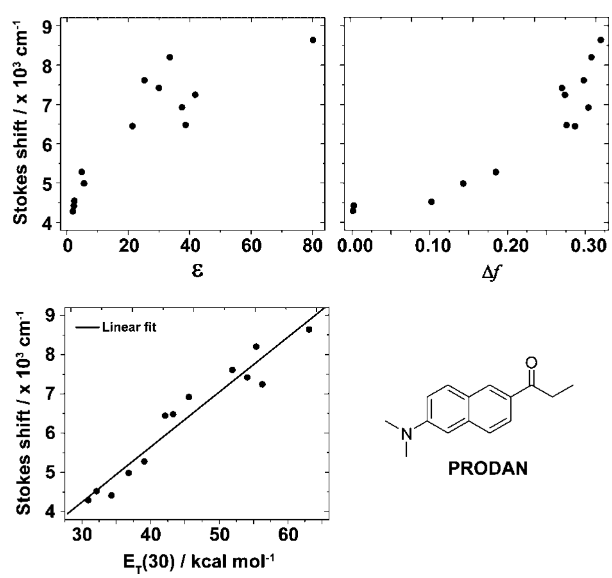


Figure 2.2. Correlation of solvent polarity and Stokes shift of PRODAN.

2.3. Fluorescence Quenching and Resonance Energy Transfer

Two common processes that cause loss of emission intensity are collisional and static quenching. The former process is described by the Stern−Volmer equation[14] and a modification thereof, the Lehrer equation.[15] Dynamic quenching is characterized by a linear correlation between the quenching effect and the quencher concentration. Deviation from linearity typically implies the involvement of static quenching, where a sphere of effective quenching exists or a nonfluorescent ground state complex is formed, as formulated by the Perrin model.[16] Quenching experiments have been used, for example, to study conformational and dynamic properties of proteins,[17,18] microdomains in membranes,[19] and RNA folding dynamics.[20] Despite their relative simplicity, the interpretation of quenching experiments can be complex.[18]

A more sophisticated, yet related, phenomenon involves resonance energy transfer (RET), a nonradiative transfer of excitation energy between distinct chromophores, typically referred to as donors and acceptors. Different mechanisms can facilitate energy transfer. The Dexter mechanism (or electron exchange) operates at short ranges and requires an intermolecular orbital overlap. The Förster mechanism, a Coulombic or dipolar interaction, operates at larger distances and is facilitated when the emission band of the donor overlaps with the absorption band of the acceptor.[2] Förster (commonly, albeit somewhat inaccurately, substituted for fluorescence) resonance energy transfer, FRET, therefore, results in a quenched donor emission and a concomitant increase in the lower energy emission of the acceptor. The strong dependence of the energy transfer rate on donor−acceptor distance ($k_{ET} \approx r^{-6}$) contributes to the utility of this phenomenon and facilitates the calculation of distances between interacting donors and acceptors.[21]

Resonance energy transfer experiments have been extensively used in biophysics and biology, where the participating partners are labeled with highly emissive and typically large donors and acceptors. Scattered and relatively recent examples include the study of protein folding, protein−protein interactions, and cellular signaling events in living cells.[17,22−24] FRET has also been used to elucidate folding and dynamics of RNA,[25] as well as the sequence-dependent structure, stability and dynamics of nucleosomes.[26] Membrane researchers have used FRET to study, for example, microdomain formation[19] and transmembrane peptides in surface-supported bilayers.[27]

2.4. Time-Resolved Fluorescence Spectroscopy

Steady-state measurements are instrumental in detecting changes in fluorescence intensity, as well as emission and excitation maxima. Steady-state spectra give, however, an average emission profile of all excited fluorophores present in the sample. This technique, therefore, cannot distinguish between individual fluorophores found in a heterogeneous population, such as those associated with different conformational states. Time-resolved measurements, yielding excited state lifetimes, provide insight into the excited state dynamics and the decay pathways of the excited chromophore. In this fashion, it is possible, for example, to extract information on different excited species in a single sample based on differences in their fluorescence lifetime. Time-resolved quenching experiments can distinguish between collisional (lifetime is affected) or static (lifetime is unaffected) quenching. Like steady-state, time-resolved fluorescence spectroscopy also gives an averaged profile of the excited chromophores in a sample. With deconvolution, however, it is possible to resolve more than one decay pathway, each of which represents an average across a population. Moreover, in contrast to steady-state analysis, time-resolved fluorescence spectroscopy is concentration independent.

2.5. Fluorescence Anisotropy

Within the short time window after excitation but before emission, the excited fluorophore undergoes Brownian motion. Its tumbling rate is affected by temperature, solvent viscosity, its size, and bound species. This can be investigated with polarized fluorescence spectroscopy, also called fluorescence anisotropy. Polarization (P) is defined as the difference between intensities of parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) polarized emission divided by the sum of the two and is interchangeable with anisotropy [$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$]. In a practical setup, optical polarizers for excitation and emission are used. Vertically polarized light is used for excitation, while the emission is detected once after vertical and once after horizontal polarization. A low molecular weight fluorophore by itself typically shows complete depolarization, since its rotational correlation time is normally much shorter than its excited state lifetime.[2] When attached to a larger (bio)molecule or when the viscosity of the medium is increased, its Brownian molecular rotation is slowed. As a result, the excited state remains partially aligned and its emission is polarized. This principle has been widely used to follow biomolecular binding events. Note that depending on the size and correlation times of the partners involved, fluorophores of different excited-state lifetimes are needed. For accurate polarization measurements with very large biomolecular complexes, probes with extended lifetimes (up to 10^{-6} s) are required.

Fluorescence anisotropy has been widely used in membrane studies with a particular emphasis on properties like fluidity and microviscosity[28] but also to determine aqueous bulk−membrane partition coefficients of fluorophores.[29] Protein dynamics,[30] protein−protein interactions,[31] and protein−nucleic acids interactions[32,33] have been studied with fluorescence anisotropy as well.

2.6. Fluorescence Microscopy and Single Molecule Spectroscopy

The sensitivity of fluorescence-based techniques, coupled to advances in instrumentation, has dramatically revolutionized cellular visualization techniques. Technical developments encompass total internal reflection, confocal, and two- or multiphoton fluorescence microscopy.[34−38] Single molecule spectroscopy has proven very useful, and combinations of these techniques have been extensively used in the study of membranes, proteins, and nucleic acids.[17,39−42] Although beyond the scope of this review, many of these studies have benefited greatly from the discovery and heterologous expression of the green fluorescent protein (GFP).[43] The development and use of fluorescent proteins is discussed in section 5.2 of this review.

2.7. *In Vivo* Fluorescence-Based Imaging

Nonfluorescence-based imaging techniques, including magnetic resonance imaging (MRI), X-ray, positron-emission tomotography (PET), and ultrasound are invaluable for modern medicine. They are, however, expensive, could suffer from poor resolution and contrast, and do not necessarily respond to specific physiological changes.[44] These limitations have triggered interest in optical-based techniques. Probes that absorb and emit in the ultraviolet and visible range of the electromagnetic spectrum, the main focus of this review, are ill-equipped for *in vivo* fluorescence-based imaging techniques, due to the absorption and light scattering of these frequencies by living tissues. Near-infrared (NIR) wavelengths (700−1000 nm), however, propagate efficiently through centimeters of living tissue due to minimized absorption by water and lipids, as well as oxy- and deoxyhemoglobin.[45−47]

Progress in fluorescence-based imaging techniques has benefited from both technological advances and new probe development. For example, differences in fluorescence lifetimes have been exploited to distinguish probe emission from the emission of tissue components.[48,49] From a probe design perspective, it is of great importance for the probe to have a low energy excitation wavelength in conjunction with a large Stokes shift. Examples of fluorescent probes suitable for *in vivo* (and *ex vivo*) fluorescence studies are diverse[50−52] and include modified amino acids[53,54] and nucleosides,[55−57] as well as high molecular weight entities such as nanoparticles, dendrimers, and quantum dots.[54,58,59] An infrared-fluorescent protein has recently been engineered by Tsien from bacterial phytochromes.[60] The low excitation energy employed to excite fluorescent NIR probes is typically harmless and therefore provides the prospect for whole-body fluorescence tomography.[61] This evolving field of NIR fluorescent probes, targeting strategies, and their application for *in vivo* imaging has been described in recent reviews.[44,47,61−64]

3. Fluorescent Analogs of Carbohydrates

3.1. Function of Carbohydrates in Biological Systems

Monosaccharides, $C_n(H_2O)_n$, are well appreciated for their roles in metabolism and energy storage. These essential building blocks make up the cell wall of plants, bacteria, and insects. Perhaps of more importance for this review, monosaccharides are the building blocks of complex oligosaccharides, also referred to as glycans. Abundant on cell surfaces and typically covalently linked to other biomolecules (e.g., proteins, lipids, etc.), glycans play essential roles in signaling, as well as in cell−cell and cell−pathogen recognition.[65−68] Oligosaccharides also serve numerous intracellular functions and impact protein folding and trafficking.[65,69] These highly significant biological roles are encoded in the fundamental chemistry of their building blocks.

A glimpse into the complex chemistry of carbohydrates is provided in Figure 3.1. A monosaccharide in solution can exist in a cyclic or acyclic form. Cyclization to a hemiacetal (or hemiketal) can generate either a five-membered ring (furanose) or a six-membered ring (pyranose). In addition, the newly formed chiral anomeric center can form the α- or *β*-anomers (Figure 3.1).[65] Furthermore, monosaccharides can be chemically strung by forming acetals (or ketals), named glycosidic bonds, where a hydroxyl group from one monosaccharide reacts with the anomeric center of another. Disaccharides, trisaccharides, and higher oligosaccharides are enzymatically fabricated and conjugated. Due to the large number of possible regioisomers, stereochemical combinations, and branching, as well as heterogeneity and additional chemical modification (e.g., sulfation), the chemical and structural diversity of oligosaccharides and glycans is vast.[65]

While fluorescent analogs of biopolymers, such as peptides and oligonucleotides, can be constructed and exploited, the situation is much more complex in the context of carbohydrates. It is apparent from the brief description of their chemistry that any modification of the carbohydrate skeleton is likely to impede its biological activity. Genuine emissive and biologically acceptable analogs of monosaccharide building blocks cannot be actually conceived. This section concisely discusses, therefore, methodologies for fluorescence-based saccharide sensing, oligosaccharide mapping, and cell-surface glycan labeling.

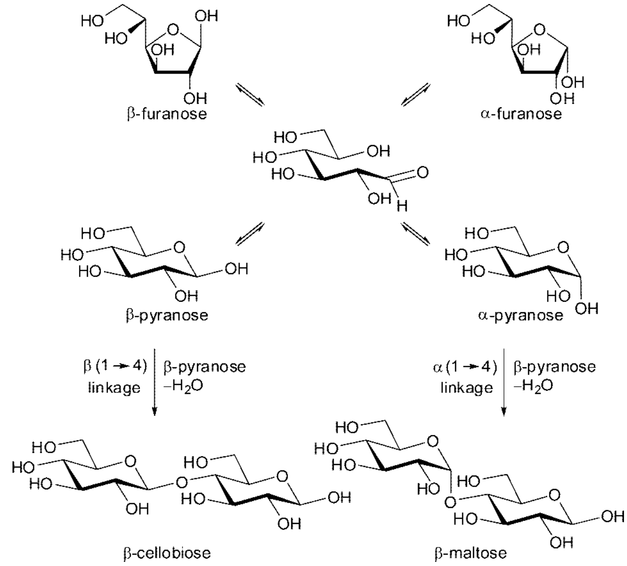


Figure 3.1. Cyclization of the acyclic form of D-glucose shown in the open, pyranose, and furanose forms. Hemiacetal formation produces both the α and *β* anomers (i.e., C-1 epimers). The diversity in oligosaccharide structures is illustrated here by the formation of two glucose-derived disaccharides differing only in the stereochemistry at the newly-formed anomeric center.

3.2. Sensors for Saccharides

Lectins are naturally occurring carbohydrate-binding proteins, with concanavalin A (Con A) being one of the archetypal examples.[70] Con A, extracted from jack beans, shows no appreciable fluorescence. Saturating its four binding sites with fluorescein-labeled high molecular weight dextran facilitates the evaluation of carbohydrate binding via competition experiments, where dextran displacement by competing saccharides results in increased emission.[71] This methodology was later improved by labeling Con A with rhodamine to facilitate FRET-based analysis. When the fluorescein-labeled dextran was competed off, energy transfer from fluorescein (the donor) to rhodamine (the acceptor) ceased.[72]

The biological significance of carbohydrates prompted the development of numerous synthetic saccharide sensors. Early work focused on the use of functionalized macrocycles, including decorated porphyrins.[73−76] Such noncovalent, supramolecular optical sensors for saccharides have been reviewed.[77]

The high affinity of boronic acids to diols has been exploited for the fabrication of numerous carbohydrate receptors and sensors. Boronic acids form five- or six-membered cyclic esters with 1,2 or 1,3 diols, respectively. Early receptors devised by Czarnik relied on photoinduced electron transfer (PET) processes to impact the fluorescence of a known fluorophore, such as anthracene (**3.1**), by attaching the boronic acids to the aromatic ring (Figure 3.2).[78]

Changes in fluorescence upon binding carbohydrates were modest and pH dependent. Incorporation of an anthracene-based chelating tertiary amine (**3.2**), as designed by Shinkai, demonstrated improved performance and resulted in fluorescence enhancement upon saccharide binding.[79−81] These design principles have been refined and advanced, resulting in a multitude of colorimetric and fluorometric sensors for carbohydrates. Examples include *N*-phenylnaphthalimide sensors (**3.3**),[82−84] and stilbenes (**3.4**),[81] as well as boronic acids derived from quinoline (**3.5**),[85] naphthalene (**3.6**),[86] nitrophenol (**3.7**),[87] and benzothiophene (**3.8**).[88] Their structures and properties are discussed in a number of overview articles.[86,89−94]

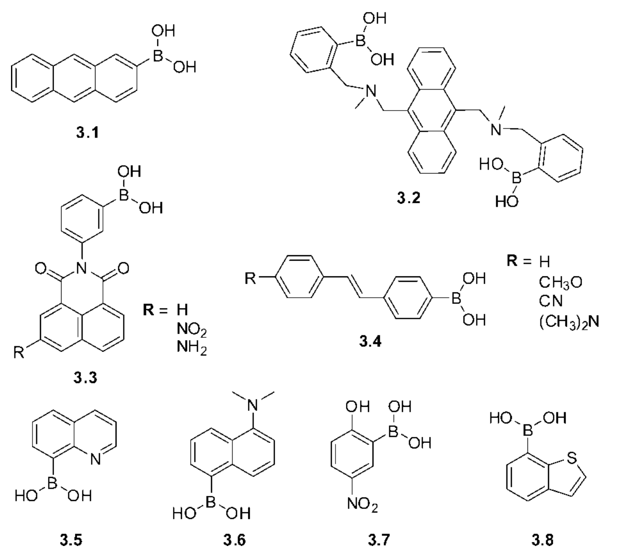


Figure 3.2. Structures of boronic acid-based saccharide sensors.

3.3. Fluorescent Labeling of Reducing Saccharides

Carbohydrates, in contrast to other important biomolecular building blocks including certain amino acids, nucleosides, and even naturally occurring fatty acids, possess no conjugated π-system at all. This obviously eliminates any useful absorption and emission features. As pointed out above, significant structural modification is therefore required to confer useful photophysical properties upon saccharides. Fortunately, reducing carbohydrates, being hemiacetals or hemiketals, are chemically unique because they contain a masked carbonyl moiety (see Figures 3.1 and 3.3). As such, they are susceptible to condensation reactions with primary amines to form Schiff bases, a reversible reaction in an aqueous environment. Under reducing conditions (e.g., in the presence of $NaCNBH_3$), known as "reductive amination", the condensation becomes irreversible (Figure 3.3).[95,96] This unique feature has been exploited for labeling purposes by reacting reducing sugars with fluorescent amines, hydrazines, and aminooxi derivatives.[97−99] If no reducing ends are present, periodate-mediated oxidation of vicinal diols, naturally present in oligosaccharides, can be used to introduce reactive aldehydes. This approach has been applied to whole cells.[100,101]

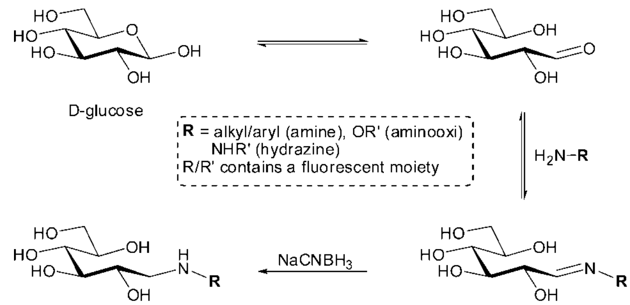


Figure 3.3. Labeling of reducing carbohydrates with amine-containing fluorophores.

3.4. Metabolic Saccharide Engineering: Exploiting the Sialic Acid Pathway

The tolerance of the sialic acid biosynthesis pathway to unnatural *N*-acyl substitutions, discovered in 1992,[102] facilitates cell-surface expression of modified oligosaccharides containing bioorthogonal groups (e.g., reactive ketones, azides), which can be further functionalized.[103] This pathway was used to decorate cell surfaces with membrane-anchored glycoproteins comprised of a ketone functionality by exposing cells to media enriched with *N*-levulinoyl-D-mannosamine (ManLev).[104,105] The newly introduced ketone can participate in a chemoselective cell-surface coupling to hydrazides, forming an acyl hydrazone, which can carry additional tags or labels (Figure 3.4).[104−106] It is worth noting that that hydrazone or oxime formation is a reversible condensation reaction in aqueous media, with its kinetics being dependent on concentration and pH.[107] A methodology for favoring imine formation at low concentrations, using aniline catalysis, has been developed[108−110] and applied to cells as well.[111]

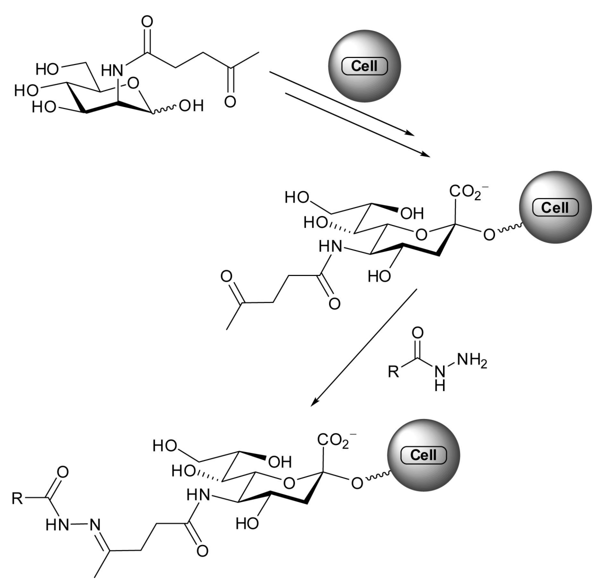


Figure 3.4. ManLev, its expression on the cell surface, and subsequent acylhydrazone formation.

The use of this biosynthetic pathway has been expanded in recent years to incorporate additional functional groups, particularly azides. This bioorthogonal entity, upon Staudinger reduction to the corresponding amine, can be engaged in condensation reactions, named Staudinger ligations.[112] Additionally, copper-mediated and copper-free "click chemistry" has been used to decorate cells of live zebrafish.[113] To further advance the scope of click chemistry, the sialic acid pathway has been utilized to express ethynyl functionalized glycans on cell surfaces in live mice.[114] Click chemistry could then be used to label and stain cells with a desired marker for fluorescence microscopy analysis.[113,114]

4. Fluorescent Analogs of Phospholipids and Fatty Acids

4.1. Biological Membranes

The lipid bilayer, discovered in 1925 by Grendel and Gorter,[115] is a key component of all biological membranes and, thereby, vital for sustaining cellular integrity and function. Formation of this fluid double layer structure,[116] a complex supramolecular architecture, is enabled by the special properties of amphipathic lipids. These structural building blocks constitute 50% of the mass of most animal cell membranes.[117]

Phospholipids span a range of sizes (MW 300−1200 Da) and are characterized by a polar (hydrophilic) headgroup connected to a phosphate-functionalized glycerol unit, which in turn links two apolar (hydrophobic) tails (Figure 4.1).[118] More than 50% of all phospholipids are sphingomyelin and lecithin, and their ratios vary widely per cell type and per species for the same cell type and are subject to change with age.[119] Both phospholipids have a similar polar headgroup, a choline for lecithin and a choline or aminoethanol for sphingomyelin, but differ significantly in their apolar moiety. Lecithin is comprised of two esters that connect the lipophilic fatty acid part to the polar headgroup. The ceramide unit in sphingomyelin has an acylated sphingosine moiety (Figure 4.1). In addition, the average length of the hydrocarbon chains in lecithin is shorter with a higher degree of unsaturation compared with sphingomyelin. This structural distinction gives rise to a difference in the net dipole and ability to form hydrogen bonds, which ultimately impacts the constitution and dynamics of lipid bilayers.[120−123]

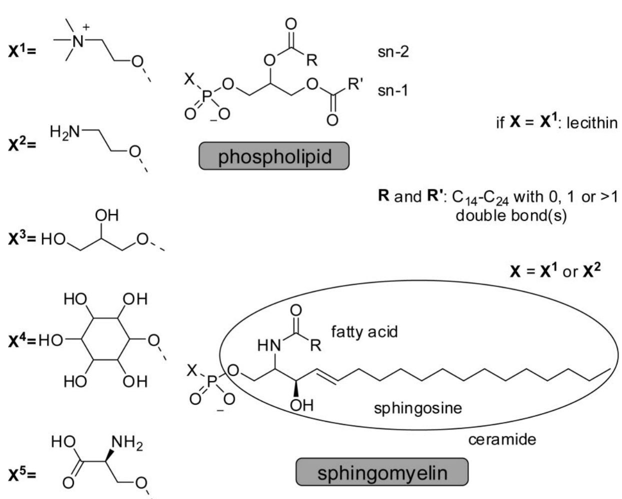


Figure 4.1. General structures of glycerophospholipids, sphingomyelin, and examples of natural head groups.

The lamellar phase or lipid bilayer with a head to head distance of 35 to 43 Å for dipalmitoylphosphatidylcholine (DPPC) vesicles[124−126] has been firmly established as the fundamental structural motif of all cellular membranes,[117] although pure lipids have been shown to organize into other assemblies in aqueous environments including planar bilayers or hexagonal or cubic phases (see Figure 4.2 for examples).[127] Membranes are not homogeneous. The formation and function of lipid assemblies within the homogeneous fluid bulk of the lipid bilayer,[128−130] referred to as superlattices[129] or lipid rafts,[131] have been described. The superlattice model proposes a regular, rather than random, distribution of membrane components, formed by favorable lipid packing, where steric and Coulombic interactions between phosphatidylcholine (PC), sphingomyelin (SM), and phosphatidylethanolamine (PE) building blocks are optimized.[129] Rafts are characterized by asymmetry with respect to the composition of their exoplasmic and cytoplasmic leaflets. The former is enriched with sphingomyelin and glycosphingolipids, and the latter mainly consist of glycerolipids.[131] Regardless of the two theories, the concept of phase-separated microdomains adds a new level of complexity to the already sophisticated role membranes play in biology.

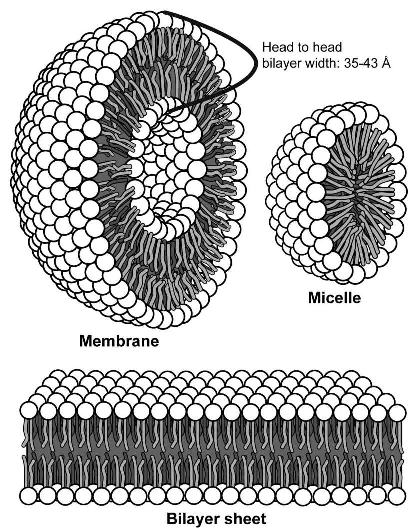


Figure 4.2. Phospholipid architectures in aqueous media.

Besides affecting the cell's membrane constitution, the type and ratio of its building blocks also determine its interaction with extracellular entities. The plasma membrane exterior of most mammalian cells, for instance, is characterized by the presence of zwitterionic phospholipids such as phosphatidylcholine and sphingomyelin,[132] while bacterial cells contain a high fraction of anionic phospholipids and related anionic amphiphiles on the outer surface.[133] This surface charge difference enhances the selectivity of positively charged antimicrobial agents to bacterial over mammalian cells.[134] Importantly, lipids are not merely structural elements of membranes but are involved in many important metabolic pathways and diseases. Sphingomyelin and glycerolipids can act as signaling molecules involved in differentiation, proliferation, and apoptosis (programmed cell death).[135−137] For its latter role in cancer cells, ceramide has been called the "tumor suppressor lipid".[138]

While learning about living cells is the ultimate goal, their heterogeneity and complex constitution make them less suitable for fundamental biophysical and biochemical studies. Instead, model membrane systems based on phospholipid bilayers and detergent-based micelles are commonly employed. Recent reviews discuss artificial membranes and giant unilamellar vesicles and their applications.[37,139] The application of membrane model systems comes with the predicament that they are comprised of an ideal two-phase system, each physically and chemically uniform, while in equilibrium with its monomeric building blocks.[140,141] Bio-

Table 4.1. Spectroscopic Properties of Selected "Noncovalent" Probes[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
4.1	DPH[b]	EtOH			0.24	2.2
		hexane	352, 370	430	0.64	15.7
4.2	M-9-A	MeOH	361 (7.1)	461	0.071	
		hexane		447		12.1
4.3	perylene[c]	EtOH	252, 408 (63.1), 434			
		dodecane			0.89	4.9
4.4	Pyrene[c,d]	EtOH	241 (79.4), 272, 334	376	0.65	410
4.5	ANS[e]	water	340	555	0.003	0.42
		dioxane		472	0.57	11.8
4.7	DCVJ	MeOH	455 (62)		0.0022	
		glycerol	469	508		
4.8	FCVJ	ethylene glycol	483	503		

[a] λ, ε, and τ are given in nm, 10^3 $M^{-1}cm^{-1}$, and ns respectively [b] Data from Bachilo et al.[168] and Cundall et al.[169] [c] ε is given only for the most intense λ_{abs}.[170] [d] λ_{em}, Φ_{fl}, and τ are from Hermetter.[142] [e] λ_{abs} is extracted from a graph; λ_{em} is highly solvent polarity sensitive; several values for λ_{em} and τ have been reported, some of which are contradicting.[154]

logical membranes, however, are much more complex by nature since their make up includes diverse constituents.

This section discusses the plethora of fluorescent probes, labels, and methodologies used in membrane research. While fluorescent analogs of phospholipids and sphingolipids are commercially available, the abundant literature in this field highlights the active development of custom-made probes to meet specific requirements.[142,143] Diverse approaches have been employed, including the use of noncovalent probes, as well as modification of distinct domains of the common building blocks. Since the position of the probe dictates, by and large, its function, this section is organized according to this criterion.

4.2. Noncovalent Fluorescent Membrane Probes

The term noncovalent is somewhat ambiguous in this context since membranes themselves are noncovalent architectures. For organization purposes, however, we distinguish between probes that are covalently linked to a membrane building block and probes that are lipophilic dyes that show no immediate structural likeliness to phospholipids. Figure 4.3 depicts prototypical examples of the latter, and Table 4.1 lists their key photophysical parameters.

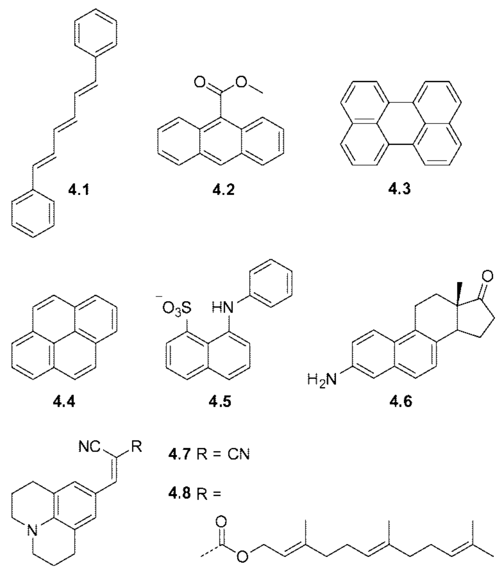


Figure 4.3. Noncovalent membrane probes that reside in the cell membrane interior.

An example of an extensively studied noncovalent probe is diphenylhexatriene (DPH) (**4.1**),[120,144,145] which resides in the nonpolar regions of the cell membrane.[146] DPH has been used in numerous studies concerned with molecular order and motion (also termed fluidity) within liposome bilayers.[28,147] Whereas the extended structure of DPH shows, albeit minimal, elements of similarity to lipid building blocks, it is clear that methyl-9-anthroate (M-9-A) (**4.2**) is, from a design perspective, nothing more than a lipophilic fluorophore. It is not as abundantly used as its counterparts attached to various positions of the alkyl chain of a lipid (*vide infra*). Together with anthranoyl labeled lipids, M-9-A has been used to study phase transitions of dipalmitoyl phosphatidylcholine[148] and to explore microviscosity barriers around the double bond in unsaturated phosphatidylcholine comprised bilayers.[149]

Other popular examples of lipophilic fluorophores used in countless membrane studies are perylene (**4.3**) and the smaller pyrene (**4.4**). Both are characterized by high emission quantum yields and long fluorescence lifetimes. At low concentrations, pyrene emits in the violet. At higher concentrations, easily reached in membranes, pyrene eximers, emitting in the green, are formed.[150] Polarity studies with 1-ethylpyrene within liposomes has indicated a much higher polarity in the hydrocarbon core of liposomes than expected (ε = 10.4−12.3 vs 1.9 and 80.2 for hexane and water, respectively).[8] The surface-residing probe 1-anilino-8-napthalene sulfonate (ANS) (**4.5**) has been used to probe dynamic behavior in model membranes,[151] as well as sulfate-dependent uptake processes in ascites tumor cells[152] and membrane fluidizing effects of paclitaxel (Taxol) with fluorescence anisotropy measurements.[153] Since ANS has been found to perturb membranes, its popularity has declined.[154,155] Aminodesoxyequilenin (EQ, **4.6**), a noncovalent probe resembling a steroidal skeleton, was used to study dynamics in model membranes.[151,156]

A membrane probe very different in design from the probes mentioned above is the intensely studied 4-(dicyanovinyl)julolidine (DCVJ, **4.7**).[157,158] This probe belongs to a family of chromophores coined molecular rotors, which are characterized by a twisted intramolecular charge transfer excited singlet state. The typical low quantum yield of these probes in nonviscous environments is ascribed to rotational relaxation, a dominating nonradiative decay pathway. Increasing the viscosity, however, impedes rotation around the single bond joining the two π-systems. The resulting structural rigidification causes a stark increase in the fluorescence quantum yield.[159−163] This property was utilized in membrane fluidity and microviscosity studies with DCVJ (**4.7**).[163−165] DCVJ was also found to bind to proteins[166]

Table 4.2. Spectroscopic Properties of Selected "Headgroup" Probes[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ
4.9	ONS	MeOH	349 (0.95)	427[b]	
4.10	Rh-101	EtOH	577 (90)	601[c]	0.9
4.11	DPE	MeOH	346 (3.6)[d]	514[e]	
4.12	NBD-PE[f]	MeOH	463 (21)	536	
4.13	coumarin				
4.14	head-CVJ	MeOH	320, 396, 470	490	
4.15	Rh-B	MeOH	560 (75)	581	

[a] λ and ε and are given in nm and 10^3 M^{-1} cm^{-1}, respectively. [b] Measured in water; λ_{em} in BuOH = 407 nm. [c] In MeOH, the fluorophore is also known as Texas Red.[150] [d] In EtOH, data from London et al.;[179] the values for *N*-dansyl ethylamine are 334 (4.6) in EtOH.[175] [e] λ_{em} in hexane is 443 nm and thus very polarity sensitive.[175] [f] Data from the Invitrogen Web site.[150]

facilitating its cellular uptake resulting in fluorescence from the cytoplasm, organelle membranes, and nucleolus.[164] To enhance localization in the membrane, a hydrophopic farnesyl chain has been connected to the julolidine core (FCVJ, **4.8**). Even better control over the positioning of the probe was obtained by connecting the core chromphore to the headgroup and the tail end of a phospholipid (sections 4.3 and 4.4).[164,167]

The main advantage of employing noncovalent fluorophores as probes is the minimal design and synthesis required. The location of a lipophilic probe at the membrane−water interface or deeper in the lipophilic inner domain in aqueous micellar suspensions is, however, ambiguous and might lead to multiple interpretations.[171−173] In addition, micelles and bilayers are able to compartmentalize lipophilic molecules, thereby jeopardizing proper readout.[174] These challenges could explain the limited use of some of the probes described above. Better certainty of the probe's localization is obtained by attaching it to a membrane building block. The following sections discuss such covalently modified phospholipids and their analogs, where the probe can be placed near the polar headgroups, at the end of the chain, or within the hydrophobic chain.

4.3. Polar Headgroup Labeling

To explore the outer cell surface, the polar head groups can be part of a charged fluorophore or be labeled with a known fluorophore (Figure 4.4). Such a membrane-spanning bolaamphiphile fixates the fluorophore at the water−lipid interface. Two fundamental designs have been explored: (a) labeling the headgroup with a fluorophore or replacing the headgroup with a charged fluorophore and (b) utilizing a long hydrocarbon to connect two fluorescent residues. The two distinct approaches are exemplified with octadecyl naphthylamine sulfonate, ONS (**4.9**),[175] and the bis rhodamine 101 labeled diacid Rh-101 (**4.10**),[176,177] respectively. The latter design requires the probe to span the head to head distance of a typical bilayer, ranging between 35 and 43 Å for dipalmitoylphosphatidylcholine (DPPC) vesicles.[124,125,178] Figure 4.4 provides typical examples, and Table 4.2 summarizes the spectroscopic properties of the corresponding fluorophores.

Although fatty acids functionalized with fluorescent probes have been reported, their phospholipid-based counterparts, many of which are commercially available, enjoy greater popularity. An early example is a dansyl-labeled phosphatidyl ethanolamine (DPE) (**4.11**).[175] This and related probes have been used to study the structure, dynamics, and local polarity

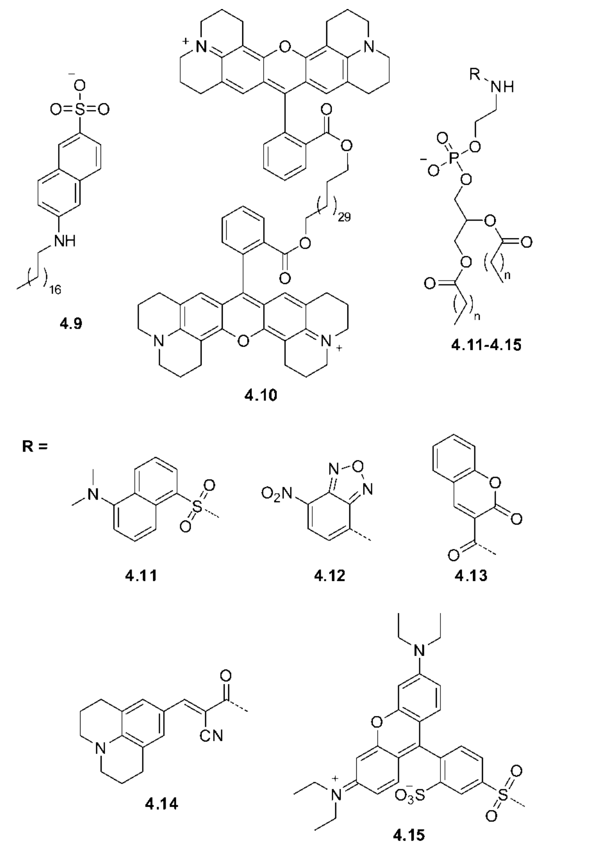


Figure 4.4. Headgroup-labeled membrane probes.

of biological membranes.[175,179] A commonly used probe is the commercially available nitrobenzoxadiazole-labeled phospholipid NBD-PE (**4.12**).[150] NBD is characterized by high quantum yields in apolar media but is nonemissive in aqueous media. Its emission maximum is polarity and pH sensitive. Moreover, NBD undergoes self-quenching at higher concentrations and has therefore been used in phase separation studies.[180] An example of a specific outer cell surface application is the headgroup-labeled phospholipid **4.13**, containing coumarin as a fluorophore, which has been used as an on/off fluorescence sensor for the detection of OH radicals.[181] The 4-(cyanovinyl)julolidine-functionalized phospholipid head-CVJ (**4.14**) is comprised of a molecular rotor moiety (section 4.2). Molecular rotors show a strong viscosity-dependent quantum yield. Since the probe is located on the membrane perimeter in this case, no response to membrane viscosity changes has been observed.[167] A study describing lipid bilayer organization and its perturbation employed the commercial rhodamine B furnished phospholipid, Rh-B (**4.15**).[176]

4.4. Chain-End and On-Chain Labeling

Introducing a probe at the very end of a lipophilic chain places it in the interior of the membrane with reasonable certainty. Two major design principles, "chain-end" and "on-chain", have been employed. A different impact on membrane stability is exerted, with the "chain-end" approach appearing to be less perturbing compared with "on-chain" placement. The former might suffer, however, from looping back of the chromophore, which could lead to ambiguity regarding its positioning within the bilayer.[182]

Polyaromatic hydrocarbons have been the chromophores of choice due to their apolar nature and rigid structure,

Table 4.3. Spectroscopic Properties of Selected "Chain-End" and "On-Chain" Probes[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
4.16	TMA-DPH	MeOH	354 (53)[b]	440[b]		0.27
4.17	dansyl-FA[c]	MeOH	335 (4.0)	518	0.23	
		toluene	336 (3.8)	475	0.40	
4.18	BODIPY-FA	MeOH	506 (>90)	512	0.94	
4.19	DPH-PC[d]	MeOH	354 (81)	428		
4.20	Anthr-PC	EtOH	378 (4.8)	~385, 410, 430		
4.21	pyrene-PC	MeOH	342 (37)[d]	376[d]	0.65[e]	410[e]
4.22	NBD-PC	EtOH	340, 460 (21)	525	0.39[f]	
4.23	fluorene-PC[g]	DMPC	290, 308, 316	319		3.0
4.24	Cor-PC[h]	DMPC	308, 344	448		98.2
4.25	tail-CVJ	MeOH	320, 396, 470	490		
4.26	12-AS	MeOH	362 (7.8)	458	0.071	1.6
		hexane		446		10.5

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. [b] Data from Thomas et al.[183] [c] Reported data is for 5-(dimethylamino)-*N*-methylnaphthalene-1-sulfonamide.[13] [d] Data from www.invitrogen.com.[150] Monomer and eximer emission in bilayers are 400 and 470 nm, respectively.[184] [e] In EtOH, data from Hermetter et al.[142] [f] Data from Chattopadhyay.[180] [g] Only a spectroscopic study in DMPC vesicles is reported, λ_{abs} and λ_{em} given are extracted from graphs showing more complexity; only the most contributing τ is given. [h] Only a spectroscopic study in DMPC vesicles is reported, the fluorescence spectrum is complex, and only the wavelength of the most intense fluorescence peak is given.

ensuring a sufficient emission quantum yield. Not surprisingly, modifications of the "noncovalent" probes discussed above with an apolar chain and polar headgroup generates many of these probes. Figure 4.5 depicts key examples, and Table 4.3 lists their primary spectroscopic characteristics.

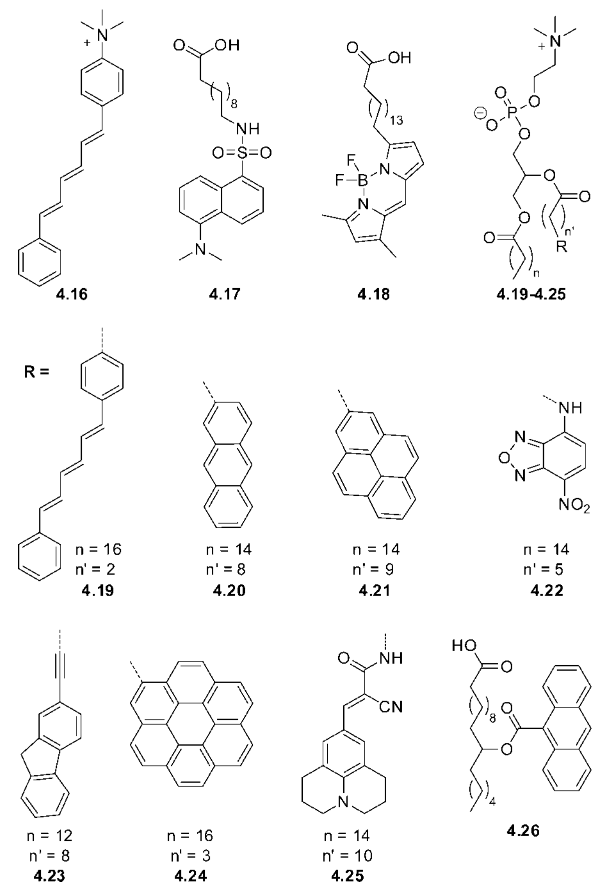


Figure 4.5. Examples of "chain-end" and "on-chain" labeling.

Modifying DPH with a trimethylammonium headgroup to give TMA-DPH (**4.16**) facilitates a more accurate positioning within the bilayer.[185] Examples of functionalized fatty acids include dansyl-FA (**4.17**),[179] and BODIPY-FA (**4.18**). In contrast to the minimal use of dansyl-FA (**4.17**), studies using BODIPY-FA are very abundant.[186,187] The BODIPY fluorophore has high molar absorptivity (>90 000 M^{-1} cm^{-1}) and a long emission wavelength (>500 nm) and shows a concentration-dependent eximer emission.[150]

A more common practice is the modification of phosphatidyl choline with fluorophores to mimic the naturally occurring membrane building blocks. Examples include DPH-PC (**4.19**),[188] Anthr-PC (**4.20**),[189-191] pyrene-PC (**4.21**),[8,184,192] and NBD-PC (**4.22**).[193] Due to its polarity-sensitive emission maximum and high quantum yields, NBD-labeled probes are often used to assess location within membranes.[180] Unfortunately, a "chain-end" NBD-labeled phospholipid can loop back, making its location within the membrane uncertrain.[180] More recent examples include the use of fluorene-PC (**4.23**)[194] and a coronene adduct of phosphatidyl choline (Cor-PC) (**4.24**).[195] Other fluorescent chain-end modified PCs are commercially available.

Similarly to the chain-end labeling, "on-chain" fluorophores must be accommodated by the highly apolar environment of the inner membrane. Tail-CVJ (**4.25**) represents an example of a phospholipid functionalized with a molecular rotor. The viscosity-dependent quantum yield of the chromophore was used to probe changes in membrane viscosity.[167] A common fluorophore for "on-chain" labeling of fatty acids is anthracene. Examples include 12-(9-anthoyloxy) stearic acid (12-AS) (**4.26**),[175] and 9-(9-anthroyloxy) stearic acid,[141,148] where the number preceding the parentheses indicates the position of the fluorophore on the chain. Despite the covalent attachment, the linker typically permits ample rotational freedom in the highly accommodating fluid lipid phase,[196] thereby complicating spectroscopic analysis.[191] The orientation and motion of various probes, including chain-end and on-chain anthracene-labeled fatty acids, has been studied with fluorescence polarization and has demonstrated sensitivity to structural changes induced by cholesterol addition, lipid type, or temperature.[151]

4.5. In-Chain Labeling

Making the dangling "on-chain" and "chain-end" fluorophore part of the fatty acid chain, as in the "in-chain" labeling strategy, minimizes probe-induced membrane perturbation. Fluorophores related to the ones discussed above can be employed as long as they accommodate functionalization on either side. Symmetrical modification tends to minimize membrane disruption and chain length selection ultimately impacting the depth of the probe within the bilayer. Figure

Table 4.4. Spectroscopic Properties of Selected "In-Chain" Probes[a]

compd no.	name	solvent	λ_{max} (ε)[b]	λ_{em}	Φ	τ
4.27	BA-Anthr-FE[c]	$CHCl_3$	334, 350, 364 (5.1), 384	396, 418, 442	0.19	3.9
4.28	BA-exAnthr-FE[c]	$CHCl_3$	342, 358, 378 (13.0), 399	408, 430, 457	0.28	3.1
4.29	BA-exFluorene-PC[d]	DMPC	308, 329	334		1.3
4.31	C8A-Fl-C4	MeOH	270 (38.0), 297, 309	319	0.65	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns respectively [b] Only ε values for the most intense peak are given. [c] Data extracted from graphs.[197] [d] Only a spectroscopic study in dimyristoylphosphatidylcholine (DMPC) vesicles is reported, λ_{abs} and λ_{em} are extracted from graphs, and only the most contributing τ is given.

Table 4.5. Spectroscopic Properties of Selected Polyenes[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
4.34[b]	retinol	MeOH			0.007	1.5
		cyclohexane	325	520	0.020	5.0
4.35[c]	*cis*-PnA	MeOH	318.6, 303.8 (79)[b]	"432"[d]	0.017	1.3
		decane	320,8, 305.8 (74)	432	0.054	5.2
4.36[e]	*trans*-PnA	MeOH	313.0, 298.6 (92)	"422"[d]	0.031	<1
		decane	315.3, 300.7 (88)	422	0.009	3.1
4.37[d]	*trans*-PA	EtOH	344.7 (103)	468	0.075	10.7
		dioxane	348.9 (81)	468	0.14	13.7
4.38[e]	*trans*-PdA	$CHCl_3$	353 (92), 335 (95), 320 (60)	474	0.14[f]	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively [b] Data from Radda and Smith.[205] [c] Data from Diamond et al.[213] [d] The emission maximum is virtually solvent independent.[213] [e] Data from Acuna et al., average lifetimes given.[214] [f] Quantum yield in DMPC vesicles.[215]

4.6 presents selected examples, and Table 4.4 provides primary spectroscopic characteristics for the chromophores most commonly used in this catagory.

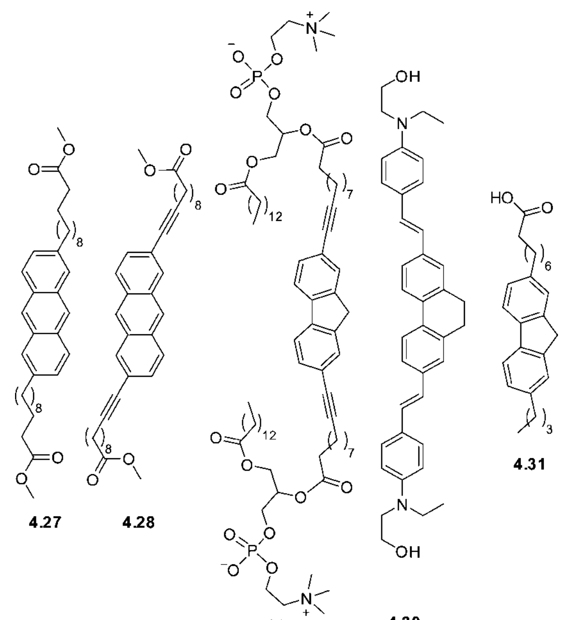


Figure 4.6. Examples of "in-chain" labeled chromophores.

Known fluorophores that have been incorporated into symmetrical bolaamphiphiles are anthracene (BA-Anthr-FE, **4.27**),[197] ethynyl-extended anthracene (BA-exAnthr-FE, **4.28**),[197] ethynyl-extended fluorene (BA-exFluorene-PC, **4.29**),[194] and vinyl-extended dihydrophenanthrene (exdh-Phenanthrene, **4.30**).[198] Extending the conjugation of the central polyaromatics tends to impart favorable photophysical features upon the chromophore (e.g., higher emission quantum yield), in addition to the structural rigidification imposed. The membrane spanning bolaamphiphile design is of specific interest, since the polar headgroups serve as anchors, thereby limiting longitudinal and transverse maneuverability of the probes, resulting in a higher accuracy of the probes' positioning.[199−201] A somewhat unique example is the asymmetrically substituted fluorene fatty acid (C8A-FL-C4, **4.31**).[202]

4.6. Polyene Fatty Acids

Polyenes are linear hydrocarbon chains characterized by conjugated multiple double or triple bonds. These minimally perturbing chromophores are a valuable substitution for saturated alkyl chains, which possess no useful emissive qualities. In addition, the high degree of unsaturation introduces rigidity, virtually preventing looping or folding of the probe. If the chain length matches the membrane width (referred to as "biomimetic membrane-spanning")[124] and is equipped with polar groups on either side, a bolaamphiphile is obtained, which is accurately positioned in a transverse fashion.

Early membrane studies with polyenes made use of rather large and structurally complex natural products. Examples include the macrolide polyene antibiotics filipin (**4.32**) and amphotericin (**4.33**),[203] which are known to cause cell lyses (Figure 4.7).[204] Examples of linear naturally occurring polyenes include retinol (**4.34**),[205] retinal, and other carotenoids.[206] Table 4.5 summarizes the photophysical characteristics of the key chromophores discussed in this section. Note that the spectroscopy of polyenes, being "classical" chromophores, dates back to the 1930s[207−211] and is discussed in later reviews as well.[28,212]

Designer polyenes originate from another naturally occurring polyene, α-parinaric acid (**4.35**), isolated for the first time from *Parinari laurinum* in 1933, to be identified two decades later as the (*Z*,*E*,*E*,*Z*)-isomer (Figure 4.8).[216] This compound clearly resembles membrane lipids. Treatment of the naturally occurring α-parinaric acid (*cis*-PnA, **4.35**) with iodine gave β-parinaric acid (*trans*-PnA, **4.36**) with all double bonds in the (*E*)-configuration.[217] *trans*-PnA (**4.36**), spectroscopically characterized in 1977,[213] got considerable attention for its use as a fluorescent probe in the research on synthetic phospholipid membranes in the late 1970s.[218]

Extending the conjugation by one double bond gives all *trans*-pentaenoic acid (*trans*-PA, **4.37**).[219] This polyene was

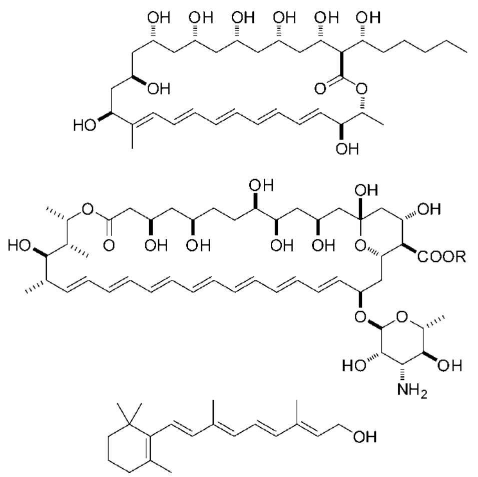


Figure 4.7. Naturally occurring polyenes.

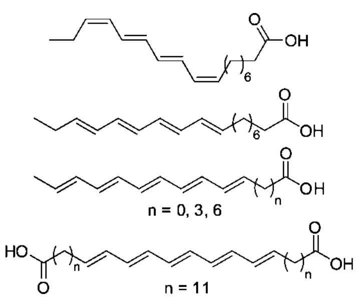


Figure 4.8. Structures of naturally occurring α-parinaric acid (**4.35**) and synthetic all *trans*-PnA (**4.36**), *trans*-PA (**4.37**), and all *trans*-PdA (**4.38**).

used for studying protein−lipid interactions by functioning as a FRET acceptor for tryptophan emission.[214] More recently, polyene lipids have been used as probes in live cells, highlighting their superior properties compared with membrane perturbing NBD and BODIPY tags.[220] To minimize mobility, implementation of the bolaamphiphile design principle gave all *trans*-pentaenoic (di)acid (*trans*-PdA, **4.38**). This probe has been used in polarized two-photon fluorescence microscopy to allow direct observation of the emission transition moment orientation of the probes in lipid bilayers.[125]

Modern synthetic methods, in particular, Pd-mediated sp^2−sp^2 cross-coupling reactions facilitate the synthesis of such polyenes.[221,222] This is frequently replaced by an alternative approach comprised of sp−sp^2 transition-metal-mediated cross-coupling reactions to give an ene−yne, followed by partial reduction of the alkyne.[223] A more classical approach involves consecutive Wittig and Wadsworth−Horner−Emmons reactions.[219,220] Regardless of the synthetic approach, the final polyene must be isomerized to the all-(*E*) isomer, typically by the use of iodine.[217,224]

Despite their relatively simple and short π-system, polyenes are characterized by high molar extinction coefficients and multiple emission maxima. Both are typically polarity independent.[213] Both *cis*-PnA (**4.35**) and *trans*-PnA (**4.36**) display solvent-independent fluorescence maxima around 425 nm with a solvent-dependent fluorescence lifetime ranging from 4 to 11 ns. Emission quantum yields vary from 0.020 (chloroform) to 0.054 (decane) for **4.35** and 0.010 (chloroform) to 0.031 (decane) for **4.36**.[212,213] As expected, the extended all *trans*-pentaenoic acid (*trans*-PA, **4.37**) exhibits additional lower energy transitions and a fluorescence maximum around 470 nm.[219] This illustrates the tunability of the spectroscopic properties of these isomorphic fluorescent membrane analogs.[222] It is worth noting that the addition of small amounts of polyunsaturated fatty acids can stabilize artificial phospholipid membranes, whereas larger amounts can cause destabilization.[225]

4.7. Applications

Fluorescent probes have greatly contributed to our understanding of the properties and function of biological membranes. While categorizing the plethora of membrane studies has been previously attempted,[226−228] capturing over 40 years of membrane research using fluorescent spectroscopy is clearly impossible. The brief discussion below and Table 4.6 summarize key studies involving the probes presented in this section.

A. Membrane polarity. Suitable environmentally sensitive probes located in biomolecular cavities can be used to approximate local polarity by changes in their fluorescence quantum yield (hyperchromic or hypochromic effects) or emission maxima (hypsochromic or bathochromic shifts). The correlation between the spectroscopic characteristics of polarity-sensitive probes and empirical polarity parameters and scales has recently been discussed.[13]

B. Fluidity. Fluidity gradient, or membrane lipid dynamics, is a fundamental physical characteristic of biomembranes encompassing the concepts of packing, average orientation, motion, and lateral movement of phospholipid chains.[148,191] These features can influence the bilayer permeability and optimal activity of membrane-bound proteins.[194] It is worth noting that the term "fluidity" and what it encompasses remains under debate.[28,229,230] A number of studies fall into this category:

B1. Microviscosity. Measuring the rotational freedom of a probe with fluorescence polarization could facilitate the determination of its local viscosity.

B2. Lateral diffusion. The fluid mosaic nature of membranes[116] suggests high rates of lateral diffusion of lipids and proteins. This parameter is considered to be the most important in the description of membrane mobility.[230]

B3. Influence of temperature. The "main" or chain-melting transition temperature describes the transition from a highly ordered quasi-two-dimensional crystalline solid to a quasi-two-dimensional liquid and is a reflection of membrane lipid composition.[128]

B4. Effect of cholesterol. Due to their flat and rigid molecular structure, sterols induce conformational ordering in neighboring aliphatic lipid chains. Cholesterol, being the most common sterol in animals, controls many aspects of membrane structure. It influences acyl chain dynamics[231] and function[128] and is involved in inhibition of membrane ion release.[232] Moreover, cholesterol can facilitate phase segregation, generating microdomains.[128]

B5. Microdomains. Membrane microdomains form as a result of the packing of the long saturated alkyl chains of sphingolipids. Their formation is dependent (lipid raft) or independent (superlattice) of local cholesterol concentration. These microdomains are thought to be involved in specific protein attachment, membrane transport, and intercellular signaling.[129,131]

C. Depth. Depth analysis is concerned with membrane penetration and localization of, for example, membrane-bound proteins, peptides,[233] or cholesterol[231,234,235] as well as the topology of phospholipids.[182,186] The depth of the probe within the membrane is related to the polarity of its

Table 4.6. Fluorescent Membrane Probes and Selected Applications

compd no.	name	application	description/remarks
		Noncovalent	
4.1	DPH	B[147,246,247]	study of membrane fluidity
		B1[144]	comparison of normal lymphocytes vs malignant lymphoma cells
		B4[144]	comparison of normal lymphocytes vs malignant lymphoma cells
		B4[145,231]	in artificial phospholipid membranes
		C[146]	in model lipid bilayer membrane systems
		B, E[248]	lipid mobility influence on prothrombinase complex activity
		E[230]	effect of propoxycaine·HCl on the properties of neuronal membranes
		F[249]	effect of H_2O_2 on *Saccharomyces cerevisiae* membrane permeability
4.2	M-9-A	B[148]	properties and membrane locations of fluidity probes
		B1[149]	influence of unsaturated acyl chains on membrane microviscosity
4.3	perylene	B1[250,251]	cholesterol influence on membrane microviscosity and order
		B4[251]	cholesterol influence on membrane microviscosity and order
4.4	pyrene	A[8]	heteroexcimers in single bilayer liposomes with 1-ethylpyrene
		A, B2, B4[252]	organization and dynamics of hippocampal membranes
4.5	ANS	B[153]	membrane fluidizing effect of taxol
		D[151]	dynamic fluorescent probes' behavior in a model lipid bilayer
		F, E[253]	*Escherichia coli* membrane disruption by granulysin derived G15
4.7	DCVJ	B[165]	study of dynamical properties of lipid membranes
		B1[254]	temperature-dependent viscosity changes and phase transition study
		B1[163]	microviscosity measurements of phospholipid-bilayers
		B1[255]	shear-stress induced viscosity changes in membranes
		Headgroup	
4.9	ONS	A[175]	polarity studies in phosphatidyl choline bilayers
		D[151]	dynamic fluorescent probes' behavior in a model lipid bilayer
4.10	Rh-101	D[178]	membrane spanning probe behavior in model membrane
4.11	DPE	A[175]	polarity studies in phosphatidyl choline bilayers
		A, B4[256]	local polarity estimation at the polar head region in lipid vesicles
		E[257]	influence of mono- and divalent cations on hemolysis
		E[258]	influence of bee venom and cytolysin A-III on hemolysis
4.12	NBD-PE	B2, B4[259]	organization and dynamics of bovine hippocampal membranes
		D[260]	probe location in model membranes
		E[261]	lipid interactions with human antiphospholipid antibody
		F[262]	hemolytic effect of merulinic acid on biomembranes
4.13	coumarin	G[181]	hydroxyl radical sensing on the membrane outer surface
4.15	Rh−B	D[176]	probe influence on bilayer organization
		Chain-End and On-Chain	
4.16	TMA-DPH	B[263]	order−disorder transitions in complexes of glycerophosphocholines
		B4[264]	influence of cholesterol and ergosterol on membrane dynamics
		C[186]	localization of DPH and its derivatives within membranes
		E[265]	interactions of TAT-PTD peptide with model lipid membranes
		F[266]	effect of lactose permease on the anisotropy of liposomes
		F[262]	hemolytic effect of merulinic acid on biomembranes
4.17	dansyl-FA	C[179]	membrane location of dansyl and related probes
4.18	BODIPY-FA	C[186]	localization of probe within membranes
		E[187]	study of probe binding to fatty acid-binding proteins
		G[267]	characterization of DNA/lipid complexes by FRET
4.19	DPH-PC	B[268]	hydration and order in lipid bilayers
		B4[269,270]	lateral distribution of cholesterol and superlattice domain formation
		B5[271]	superlattice domains in phosphatidylcholine bilayers
		G[267]	characterization of DNA/lipid complexes by FRET
4.20	anthra-PC	B[191]	study of phospholipid molecular motion in the gel phase
		B2[190]	the study of lateral diffusion of lipids in membranes
		D[189]	probe behavior in egg phosphatidylcholine liposomes
		F[272]	membrane penetration and localization of adriamycin
4.21	pyrene-PC	B[184]	lateral organization of phospholipids in synthetic membranes
		B4[273]	cholesterol's influence on the interdigitation in phosphatidylethanols
		B5[274−276]	studies on regularity in lipid distribution
		E[277]	protein-catalyzed import of phosphatidylcholine
		F[278]	free fatty acid influence on membrane permeability
4.22	NBD-PC	B, B2, C[279]	location and dynamics of NBD-labeled phosphatidylcholine
		B5[280]	role of ceramides in the maintenance of membrane microdomains
		E[281]	membrane fluidizing effect of annexin V
		F[282]	defining lipid transport pathways in animal cells
		F[278]	free fatty acid influence on membrane permeability
4.23	fluorene-PC	B3[283]	temperature influence on probe behavior in model membranes
		D[283]	probe behavior in model membranes

Table 4.6 Continued

compd no.	name	application	description/remarks
4.24	Cor-PC	B[195]	investigation of submicrosecomd lipid fluctuations
4.26	12-AS	A, C[175]	polarity/localiztion studies in phosphatidyl choline bilayers
		B, C[141,148]	properties and membrane locations of fluidity probes
		E[284]	viral membrane protein association with lipid bilayer
		In-Chain	
4.27	BA-Anthr-FE	D[197]	probe orientation in vesicles
4.28	BA-exAnthr-FE	D[197]	probe orientation in vesicles
4.29	BA-fluorene-PC	B3[283]	probe behavior in model membranes
		D[283]	probe behavior in model membranes
4.30	exdhPhenanthrene	G[198]	membrane imaging
4.31	C8A-Fl-C4	C[182,202]	depth analysis in membranes
		Polyenes	
4.32	filipin	B[203]	filipin III interaction with vesicles and membranes
		C[285]	probe localization with spin-label
4.33	amphotericin	B[203]	amphotericin B interaction with vesicles and membranes
4.34	retinol	B[205]	absorption and fluorescence of retinol in membranes
4.36	*trans*-PnA	B[286]	study on lipid clustering in bilayers
		B[287]	phase transition studies
		D[213,218]	probe characterization and behavior in membranes
		E[288]	probing of binding domains in neutrophil elastase
		E[289]	study of the probe binding to bovine serum albumin
4.37	(*trans*-PA)	B, D, E[214]	fluidity, probe behavior and FRET studies with gramicidin
		G[220]	fluorescence microscopy on cells with pentaene comprised sphingomyelin
4.38	(*trans*-PdA)	B, D[215]	dynamics of bolaamphiphilic fluorescent polyenes in lipid bilayers

microenvironment. Depth analysis typically relies on the comparison of emission maxima and fluorescence lifetimes of the probe in pure solvents of different polarity to that observed when incorporated into membranes.[175] The emission maximum, however, not only is related to the depth but also reflects probe-specific interactions with its surrounding and probe-induced polarity perturbation.[2,236] Moreover, an isotropic solvent does not resemble an organized, yet dynamic, architecture like a bilayer.[237] Others used dipole−dipole (Förster) energy transfer for depth analysis studies,[146,238−241] which have proven to be rather complex. Additional approaches used spin labels[141,242] or brominated probes[243−245] in fluorescence quenching experiments.

D. Probe behavior. These fundamental studies are concerned with the orientation and mobility of a fluorescent probe and its locally provoked perturbation upon incorporation into lipid bilayers.[151]

E. Protein−lipid interactions. Membrane proteins can be located using FRET experiments between tryptophan and an appropriate acceptor (e.g., pentaenoic acid).[214] While membrane protein function can be influenced by membrane-permeable drugs, it is not always clear whether the observed effect is due to a specific drug−protein interaction or a drug-induced change in local lipid composition.[230]

F. Membrane permeability. These studies are concerned with transport across the cell membrane and cellular-uptake.

G. Miscellaneous. This category encompasses studies that do not fit in the categories listed above. A brief description is given in the last column of the table.

5. Fluorescent Analogs of Amino Acids

5.1. The Chemistry and Biology of Proteins and Peptides

In the grand scheme of biological macromolecules, recapitulated in the central dogma of biology, proteins appear last but are responsible for the majority of cellular functions. Diversity in structure and function is encoded in their sequence, a linear string of 20 different α-amino acids, their fundamental building blocks, which are linked through amide (also called peptide) bonds.[290,291] Protein recognition, function, cell localization, and fate, in addition to being primarily dependent on their three-dimensional fold, are also susceptible to environmental factors (e.g., polarity, ionic strength, etc.) and post-translational modifications (glycosylation, phosphorylation, acetylation, etc.). The central role proteins play in modern biology has stimulated extensive exploration of their biochemistry and biophysics. Not surprisingly, fluorescence spectroscopy has proven extremely instrumental in shedding light on their intricacies.

This section provides an overview of amino acid analogs that display favorable spectroscopic properties. A number of reviews have discussed noncanonical amino acids[292] and their fluorescent counterparts in particular.[293,294] Compared with fluorescent analogs of phospholipids and nucleosides, most α-amino acid-based probes show limited diversity in their design. The acute dependency of protein function on its correct fold is likely to constrain the structural modifications that can be tolerated, thus prohibiting radical structural redesign of the fundamental building blocks. In this section, the probes have been organized based on their structural features. Their basic spectroscopic properties and diverse applications have been tabulated to facilitate comparison. The modified amino acid overview section is preceded by a brief discussion of fluorescent proteins and inherently fluorescent native amino acids, illustrating that Nature has set the bar relatively high when it comes to the generation of useful fluorophores.

5.2. Fluorescent Proteins

One cannot discuss fluorescent amino acids without addressing fluorescent proteins, best exemplified by the green fluorescent protein (GFP). This "spontaneously generated" and highly emissive chromophore has become one of the most useful tools in modern biology and was instrumental in enabling live-cell imaging.[43,295] The isolation of GFP from the jellyfish *Aequorea* was first reported in 1962[296] and was soon followed by characterization of its remarkable spectral

Table 5.1. Spectroscopic Properties of Selected Fluorescent Proteins[a]

compd no.	name	λ_{max} (ε)	λ_{em}	Φ
5.1	GFP wild-type	396 (27.5)	504	0.79
5.2	EGFP	489 (55)	510	0.64
5.3	YFP, topaz	514 (94.5)	527	0.6
5.4	P4-3	382 (22.3)	446	0.3
5.5	ECFP	452	505	
5.6	GdFP	466 (23.4)	574	

[a] λ, ε, and τ are in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. Data from Tsien[43] and Budisa et al.[300] and are averaged if a range is given.

properties.[297] The actual GFP fluorophore component is *p*-hydroxybenzylideneimidazolinone (**5.1**) formed, in case of the wild type, by condensation of a three-residue sequence, Ser-Tyr-Gly (Figure 5.1).

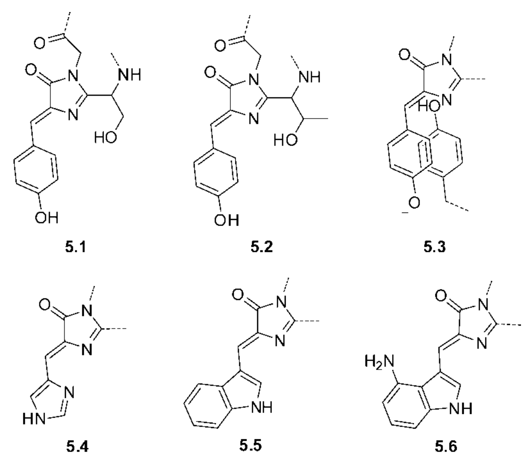


Figure 5.1. Fluorophores found in fluorescent proteins. Wild-type GFP (**5.1**) and the S65T point mutation EGFP (**5.2**), topaz (**5.3**), P4-3 (**5.4**), ECFP (**5.5**), and GdFP (**5.6**).

About 30 years after its discovery, Tsien et al. published the first major improvement with a single point mutation (S65T) resulting in an emissive protein with enhanced quantum yield and better photostability compared with GFP (EGFP, **5.2**).[298] Color mutants developed later clearly show how the chemical structure of the chromophore impacts its spectroscopic properties (Figure 5.1 and Table 5.1).[43,299] Examples of engineered fluorescent proteins include topaz (**5.3**), a yellow fluorescent protein (YFP), whose emissive properties are attributed to a deprotonated tyrosine involved in $\pi-\pi$ stacking.[43,299] Substitution of the phenol ring by an imidazole changes the emission to blue (P4-3, **5.4**), while substitution with an indole moiety gives an enhanced cyan fluorescent protein (ECFP, **5.5**). Moreover, modification of the indole ring at the 4-position with an amine group results in GdFP (**5.6**), possessing a "golden" emission.[300] Advances in visibly fluorescent proteins and their applications have been discussed in various reviews.[43,299,301−305]

Although a great tool in molecular and cell biology, the use of fluorescent proteins in intact animals is limited due to poor tissue penetration of visible light. This hurdle can be overcome by imaging with far-red and near-infrared probes (section 2.7).[44,62] The low excitation energy employed is noninvasive and provides the prospect for whole-body scale studies.[306] The development of (near) infrared-fluorescent proteins (IFPs) is, therefore, an active area of exploration. A recent example is the engineering of tetrapyrrolic biliverdin-containing *Deinococcus radiodurans* resulting in an IFP characterized by an excitation maximum of 684 nm (ε > 90 M^{-1} cm^{-1}) and concomitant emission maximum of 708 nm with a quantum yield of 0.07.[60]

While GFP and its variants have found unprecedented utility in modern cell biology as intracellular labels,[307,308] it is worth noting that their size (~28 kD or ~230 amino acids) could alter the location, stability, and functionality of their specific fusion partners.[295]

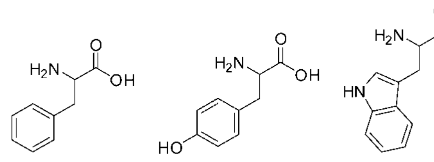
In this context, it is worthwhile to briefly discuss an elegant exogenous labeling procedure for recombinant proteins. The protocol, developed by Tsien, facilitates genetically targeted labeling with a low molecular weight fluorophore in living cells.[309] Fusing the protein of interest to a string of amino acids that contains four cysteine residues in an $X_3Cys_2X_2Cys_2X_3$ motif, facilitates *in vivo* labeling by exposing the cells to the permeable and nonemissive 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (named FLASH-EDT2 for fluorescein arsenical helix binder bis-EDT adduct).[309] Upon ligand displacement with the uniquely spaced cysteine residues, a highly emissive peptide−fluorophore complex is formed (Φ = 0.49). This *in situ* and versatile labeling technique increases the mass of the protein of interest only slightly, compared with GFP fusion, and has become popular in the past decade.[310−312]

The fluorophore in fluorescent proteins is formed by a complex intramolecular reaction involving the peptide backbone, which is consequently compromised.[303] Nevertheless, from a fluorophore design perspective, it shows that small fluorophores can be chemically modified to obtain and tune desirable spectroscopic qualities.[313−316] Indeed, synthetic analogs inspired by the GFP fluorophore have been reported.[317] In designing fluorescent amino acids analogs, however, the backbone is typically left intact to ensure proper incorporation and folding.

5.3. Naturally Occurring Fluorescent Amino Acids

Due to their aromatic side chains, the native amino acids phenylalanine (**5.7**), tyrosine (**5.8**), and tryptophan (**5.9**) possess favorable spectroscopic properties and have been frequently employed as "built in" fluorescent probes (Figure 5.2 and Table 5.2). The combination of its low quantum yield and low molar extinction coefficient makes phenylalanine detectable only in proteins that are deficient in tryptophan or tyrosine. Tyrosine, however, does possess a reasonable quantum yield (Table 5.2). While it lacks significant sensitivity to its environmental polarity, its photophysics is pH dependent due to its acidic side chain ($pK_a \approx 10$). Deprotonation of the phenolic hydroxyl group results in a bathochromic shift of the emission maximum from 310 to 340 nm.1

Tryptophan is by far a more favorable probe than phenylalanine or tyrosine, as it benefits from higher brightness ($\varepsilon\Phi_F$).[319] Due to the large dipole moment of its excited state, tryptophan's fluorescence quantum yield and emission maximum are highly sensitive to polarity.[322] As an apolar amino acid, tryptophan is most often located in the hydrophobic interior of a protein,[323] where it emits at 309 nm.[324] Changes in tertiary structures, induced, for instance, by unfolding, can expose tryptophan to more polar aqueous environments, with concomitant red shift of its emission maximum to 355 nm.[323] The wide range of quantum yields displayed by tryptophan is attributed to the diverse surroundings the chromophoric indole ring can experience. In addition, diverse quenchers, including disulfide bonds, protonated histidines, and peptide bonds, as well as metal ions,



5.7 5.8 5.9

Figure 5.2. Naturally occurring fluorescent amino acids.

Table 5.2. Spectroscopic Properties of Emissive Native Amino Acids[a]

compd no.	name	λ_{max} (ε)	λ_{em}	Φ	τ
5.7	Phe	258 (0.20)	282	0.024	
5.8	Tyr	275 (1.41)	310[b]	0.14	3.3−3.8
5.9	Trp	279 (5.58)	365	0.01−0.4[c]	

[a] λ, ε, and τ are in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively, and mostly from Dean's Handbook of Organic Chemistry[318] and Jameson and Ross.[319] [b] If deprotonated, λ_{em} = 340 nm.[320] [c] Data from Eftink.[321]

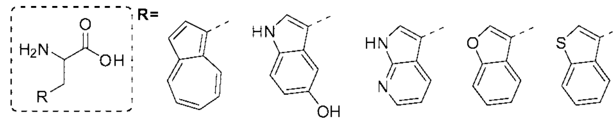
heme groups, and coenzymes, can affect the excited state of tryptophan. For this reason, unfolding of a tryptophan-containing protein typically results in a consecutive red shift of the absorption maximum and significant alteration of the fluorescence quantum yield. These sensitive spectroscopic properties of tryptophan have been widely used to explore protein dynamics, folding, and ligand binding, as discussed in a number of reviews.[321,323,325,326]

Despite tryptophan's inherent favorable photophysical properties and its relative low abundance in proteins, the presence of multiple residues in different environments within a single protein can complicate the resulting spectroscopy. This might necessitate site-directed mutagenesis of all but one tryptophan residue with tyrosine or phenylalanine to mitigate tryptophan emission while minimizing structural perturbations. Another approach involves the introduction of a non-natural amino acid with distinct spectroscopic characteristics. Such modification, however, can potentially perturb protein folding and hence function. Even the substitution of all three tryptophan residues in barstar by 4-aminotryptophan, a relatively small analog, can result in protein destabilization and compromised function.[327] This illustrates the challenges faced by protein chemists who attempt to design benign yet spectroscopically useful modified amino acids as discussed below.

5.4. Side-Chain-Modified Amino Acids

5.4.1. Tryptophan Mimics

Even though intrinsic probes (native amino acids) facilitate the biophysical study of proteins without the need for chemical modification, extrinsic probes (modified amino acids) have been employed due to their distinct spectroscopic parameters. To minimize potential perturbation upon incorporation of modified fluorescent amino acids, mimicking the size and polarity of tryptophan is a logical approach. Examples of such structures are shown in Figure 5.3 and include the blue-emitting azulene (azuAla, **5.10**).[328] The spectroscopic properties of two other tryptophan derivatives, 5-hydroxytryptophan (5OHTrp, **5.11**)[329] and 7-azatryptophan (7azaTrp, **5.12**)[329,330] have been conveniently compared with tryptophan and other tryptophan mimics (Table 5.3).[331] Both 5OHTrp (**5.11**) and 7azaTrp (**5.12**) display a 20 nm bathochromic shift of their absorption maximum relative to tryptophan, facilitating selective excitation.[332] Two other examples, benzofuranylalanine (BfAla, **5.13**)[333] and benzothiophenyl (BtAla, **5.14**),[334] only differ from tryptophan in their ring heteroatom.[335−337] Tirrell and co-workers have incorporated BtAla (**5.14**) and other tryptophan mimics to modify the spectral properties of fluorescent proteins.[338] These and other tryptophan mimics have been discussed in reviews.[293,294,327,339]

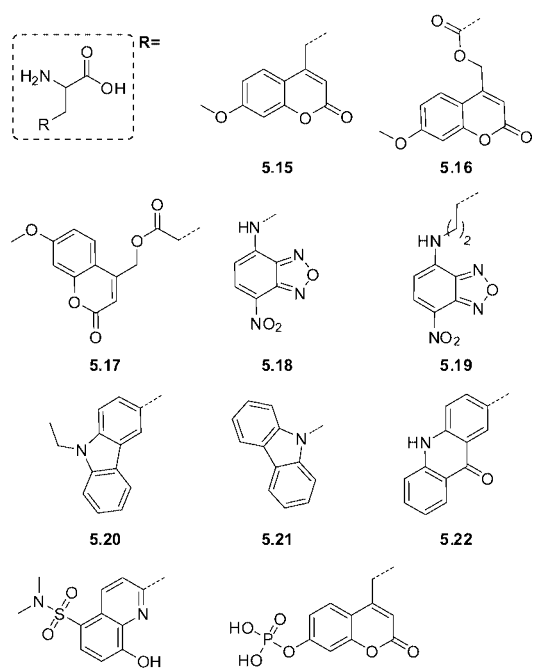


5.10 5.11 5.12 5.13 5.14

Figure 5.3. Tryptophan mimics.

5.4.2. Side Chain Modification with Heterocyclic Chromophores

Attaching an established fluorophore to a side chain of a nonemissive amino acid such as alanine is a rational approach for the generation of fluorescent amino acids (Figure 5.4). Examples include 7-methoxy-coumarin-labeled alanine (mchAla, **5.15**), aspartic acid (Asp(OMc), **5.16**),[340] and glutamic acid (Glu(OMc), **5.17**).[340] Other examples include NBD-labeled alanine (NBDAla, **5.18**),[341] NBD-labeled lysine (NBDLys, **5.19**),[342,343] and carbazole-labeled alanines, 3-(9-ethylcarbazolyl)alanine (EtcbzAla, **5.20**)[344] and 9-carbazolylalanine (cbzAla, **5.21**).[345] A recent addition is the 2-acrydonylalanine (acroAla, **5.22**), which is reported to possess high photodurability.[346,347] Interestingly, most of these probes have found limited use in exploring natural systems, but have been used in studying synthetic photoactive polypeptides (Tables 5.3 and 5.4).[348,349]



5.15 5.16 5.17 5.18 5.19 5.20 5.21 5.22 5.23 5.24

Figure 5.4. Examples of amino acids containing heterocyclic chromophores.

In contrast to the aforementioned fluorophores, some probes undergo chemical modification in the probing process. Sox (**5.23**), a hydroxyquinoline functionalized amino acid developed by the Imperiali group, shows a considerable fluorescence enhancement upon chelation of divalent zinc, a process termed "chelation-enhanced fluo-

Table 5.3. Basic Spectroscopic Properties of Selected Modified Amino Acids[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
			Trp Mimics			
5.10	azuAla	K_3PO_4 buffer	276, 339	381	0.031[b]	
5.11	5OHTrp[c]	water	279, 297	336	0.27	3.46
5.12	7azaTrp[c]	water	291	391	0.01	1.24
		MeOH	297	366	0.01	
5.14	BtAla[d]	EtOH	228, 297		0.019	0.28
			Heterocycles			
5.15	mchAla[e]	buffer	325 (1.4)		0.36	
5.16	Asp(OMc)[e]	buffer	325 (1.4)		0.36	
5.17	Glu(OMc)[e]	buffer	325 (1.4)		0.36	
5.18	NBDAla[f]	EtOH	264, 330, 462 (19.7)	532	0.38	
5.19	NBDLys[f]	EtOH	264, 330, 462 (19.7)	532	0.38	
5.20	EtcbzAla[g]	EtOH	340	437		7.8
5.21	cbzAla[g]	EtOH	340	437		7.8
5.22	acroAla	water	388 (5.6), 407	420	0.95	
		THF	378, 395 (6.3)	422	0.21	
5.23	Sox	in peptide −Zn		500	<0.005	
		in peptide +Zn	360 (6.2)	500	0.16	
5.24	pCAP	in peptide				
	CAP	in peptide	334	460	pCAP × 10^4	
			Hydrocarbons			
5.25	pbpAla[h]	$(CH_3O)_3PO$	256 (2.2)	302, 319	0.14	
5.26	1napAla[h]	$(CH_3O)_3PO$	272, 283 (0.8), 295	326		
5.27	2napAla	$(CH_3O)_3PO$		340		
5.28	1pyrAla[i]	EtOH	241 (79.4), 272, 334	376	0.65	410
5.29	2pyrAla[i]	EtOH	241 (79.4), 272, 334	376	0.65	410
5.30	9antAla[j]	EtOH	252 (199.5), 338, 357, 376	398	0.30	
5.31	2antAla	water	342 (5.2)	384	0.11	
5.32	9phantAla[k]	EtOH	250 (50.1), 293, 330, 346			
5.33	anthrAla[l]	EtOH	253 (50.1), 326, 405			
			Dansyl			
5.34	51dansylAla[m]	MeOH	335 (4.0)	518	0.23	
		dioxane	335 (4.1)	479	0.54	
5.35	52dansylLys[m]	MeOH	335 (4.0)	518	0.23	
		dioxane	335 (4.1)	479	0.54	
			Charge Transfer Chromophores			
5.38	4DAPA[n]	water	408	562		
		dioxane	378	457	0.62	14.9
5.39	6DMNA[o]	water	388	592	0.002	
		dioxane	372	498	0.22	
5.40	4DMNA[p]	MeOH	422	524	0.01	0.2
		dioxane	403	500	0.76	9.2
5.41	Aladan[q]	water	364 (14.5)	531		2.1
		cyclohexane	342	401		1.6
			Miscellaneous			
5.42	azoAla	water	350	450		

[a] Wavelength maxima are in nm, ε in 10^4 M^{-1} cm^{-1}, and τ in ns. ε and λ_{em} are only given for the most intense peaks. For a number of probes, no spectroscopic data could be found. In such cases, the relevant parameter of the actual fluorophore is given. [b] Azulene in EtOH.[376] [c] More solvents are given; τ is the mean lifetime.[331] [d] Values for benzothiophene.[377] [e] Values for 7-methoxycoumarin-4-acetic acid. [f] Values are for 7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole.[378] [g] Values for *N*-ethylcarbazole.[318] [h] Values for the modification in a central position of oligoglycine. [i] Values for pyrene.[142,170] [j] Values for anthracene.[379,380] [k] Values for phenanthrene.[381] [l] Values for anthraquinone.[382] [m] Data reported is for 5-(dimethylamino)-*N*-methylnaphthalene-1-sulfonamide.[13] [n] Values for 4-(*N*,*N*-dimethylamino)-phthalimide.[383] [o] Values for model compound 6DMN-GlyOMe. [p] Values for dimethylaminonaphthalimide.[384] [q] Values for PRODAN.[385]

rescence" (CHEF).[350] This property has been utilized in the study of protein kinase activity. Although Sox alone is capable of chelating Mg^{2+} with concomitant fluorescent enhancement, phosphorylation of a nearby serine, threonine, or tyrosine in a β-turn sequence, results in a ~10-fold enhancement of the binding affinity and thus a strong increase in the fluorescence signal.[351,352] Improved Sox-based probes appeared shortly thereafter.[353,354] Another example of probing based on chemical modification is the dephosphorylation of pCAP (**5.24**), a phosphorylated coumarin derivative functionalized amino acid.[355] Enzyme-mediated dephosphorylation leads to augmented fluorescence intensity, which has been used in protein tyrosine phosphatase studies.[356,357]

5.4.3. Labeling with Aromatic Hydrocarbons

Aromatic hydrocarbons are often highly emissive and can therefore be attached to an amino acid side chain to provide fluorescent building blocks (Figure 5.5). Examples include *p*-biphenyl-labeled alanine (pbpAla, **5.25**),[358] 1-naphthyl- (1napAla, **5.26**)[358] and 2-naphthyl-labeled alanine (2napAla, **5.27**),[359] and 1-pyrenyl- (1pyrAla, **5.28**)[360] and 2-pyrenyl-labeled alanine (2pyrAla, **5.29**).[345] The anthracene-functionalized alanine 2-anthryl (2antAla, **5.30**)[345,361] has very comparable spectroscopic properties in polar and apolar environments, which makes it useful as a fluorescent tag but hampers its use as a reporting probe.[362] Other examples include 9-anthryl-modified alanine (9antAla, **5.31**),[363,364] 9-phenanthryl-labeled alanine (9phantAla, **5.32**),[364] and anthraquinone-based alanine (anthrAla, **5.33**).[365] Basic spectroscopic properties of various fluorescent amino acid analogs can be found in Table 5.3.

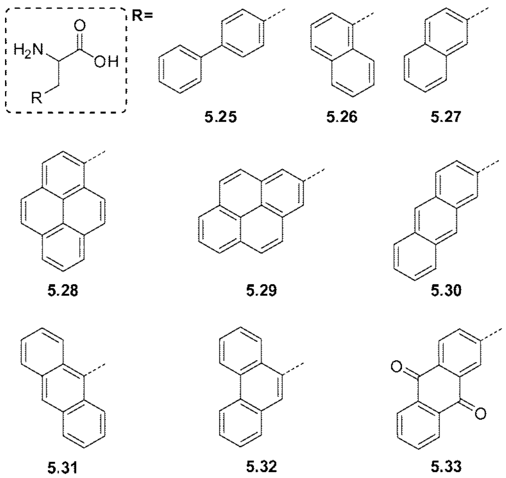


Figure 5.5. Amino acids labeled with emissive heterocarbons.

5.4.4. Dansyl-Modified Amino Acids

Due to its spectroscopic qualities, the dansyl fluorophore has been used as a probe with most biomolecules, including amino acids (Figure 5.6). The dansyl-modified alanine, 51dansylAla (**5.34**), is probably the most studied (Figure 5.6).[329,366] Dansyl-modified lysine (51dansylLys, **5.35**) has been used as well.[367] The basic spectroscopic properties of these push−pull chromophores can be found in Table 5.3. Two phenylalanine-based designs, 62dansylPhe (**5.36**) and 52dansylPhe (**5.37**), have been proposed[362] but have not, to our knowledge, been explored. Note that the latter chromophore lacks a push−pull feature and might therefore possess different spectroscopic properties.

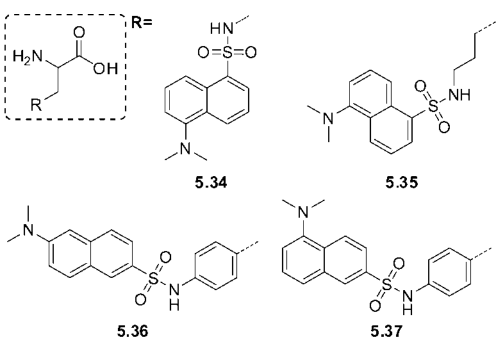


Figure 5.6. Examples of dansyl-decorated amino acids.

5.4.5. Diaminopropionic Acid Derivatives

Other charge transfer chromophores have been linked to the peptide backbone through an imide- or amide-derived 2,3-diaminopropanoic acid, maintaining the α-amino acid core (Figure 5.7). Imperiali and co-workers have developed three probes, 4DAPA (**5.38**),[368] 6DMNA (**5.39**),[369] and 4DMNA (**5.40**),[370] based on this design principle. PRODAN-based Aladan (**5.41**), albeit not a diaminopropionic acid derivative, represents another example of a push−pull chromophore (Figure 5.7).[9,371,372]

These charge transfer dyes impart upon the resulting amino acid their characteristic dependency on environmental polarity, which is manifested in their absorption and emission

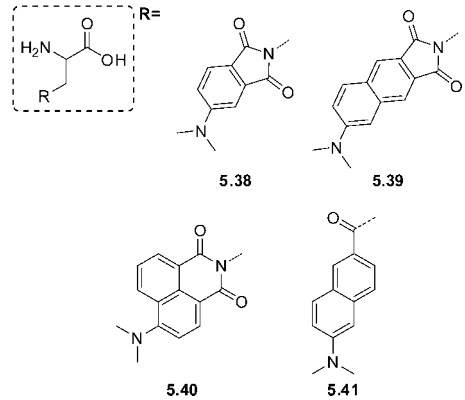


Figure 5.7. Amino acids containing a charge transfer chromophore.

maxima (Table 5.3). This useful spectroscopic property, in combination with knowledge of the probe's location after incorporation into a peptide or protein, makes these probes effective for investigating binding events.[368,369,371] The Imperiali group has recently reported a comparative study of the responsiveness of 4DAPA (**5.38**), 6DMNA (**5.39**), 4DMNA (**5.40**), NBD (**5.18**), 51dansylAla (**5.34**), and Aladan (**5.41**) when incorporated in the fourth position of a six-residue sequence.[370] While each probe had its own favorable properties, 4DMNA (**5.40**) was found to be of particular interest, due to its chemical stability and minimal structural perturbation upon incorporation.[370] Similarly, Aladan (**5.41**) has been employed to estimate the local polarity in proteins.[9] Despite its desirable spectral characteristics, it has been suggested to be destabilizing.[10]

5.4.6. Modification with a Photoswitch

A chromophore that undergoes a reversible light-induced structural change (cis−trans isomerization), for instance, *p*-phenylazophenylalanine (azoAla, **5.42**), can be used to control enzymatic activity[373] and polypeptide conformation (Figure 5.8, Table 5.3).[374] Interestingly, introduction of a dimethylamino group gives nonfluorescent dabcyl-diaminopropionic acid (**5.43**),[375] which has been used as a fluorescence acceptor with 7azaTrp (**5.12**, Figure 5.3) in FRET studies.

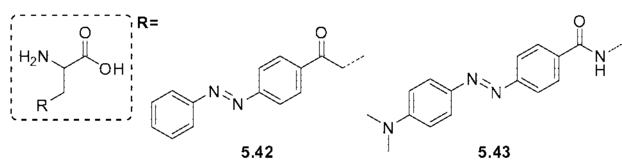


Figure 5.8. Selected photoswitchable probes.

5.5. Incorporation of Modified Amino Acids

Incorporating modified amino acids into peptides or small proteins can be done by solid phase peptide synthesis, using the appropriately protected modified building blocks. Longer peptides or proteins can be obtained by means of native chemical ligation of two peptides,[386] independently synthesized.

Strategies utilizing the translational machinery have also been developed for the incorporation of modified amino acids into proteins.[387,388] These include *in vitro* translation reactions in cell-free extracts or, alternatively, *in vivo* expression of modified proteins. Note that these techniques require unique codon−anticodon interactions (achieved using, for example, Amber codon suppression,[389,390] orthogonal nucleobases,[391,392] or extended 4- and 5-base codons[340,343,362,393]). Additionally, the corresponding tRNAs have to be synthetically charged (in the case of *in vitro* translation) or be a substrate for a specific synthetase (for *in vivo* applications). Despite their complexity, these techniques have found extensive applications in modern chemical biology.[292,394,395]

5.6. Applications

The fluorescent probes discussed in this section were, in most cases, designed for specific applications. Table 5.4 is not all encompassing but correlates chromophore design, properties, and applications. Listed below are the main areas where these probes have found applications.

A. Structure, conformation and function. Protein function relies on its structure and proper conformation. Probes in this category are used to study the conformational behavior or to investigate the influence of the modified amino acids on the protein's native function.

B. Folding/unfolding. Unfolding (i.e., denaturation) increases exposure to bulk water. Fluorophores with spectroscopic characteristics that are dependent on polarity (i.e., solvatochromic probes) are ideal for such purposes. Tryptophan is a classical example.

C. Electrostatics/polarity. Electrostatics plays a key role in virtually all aspects of protein structure and activity and is of particular relevance for proteins whose function involves charge stabilization.[9] The sensitivity of Coulombic interactions to polarity is analogous to the susceptibility of solvatochromic probes to changes in polarity. Such chromophores can be used to either estimate local polarity or to study binding events.

D. Incorporation. This application lists the *in vitro* or *in vivo* incorporation of emissive probes into peptides and proteins. The incorporation of a large number of non-natural amino acids, tabulated below, has been discussed in a couple of reviews.[362,394]

E. Photoswitching. A photoswitchable chromophore is employed to photocontrol a biological function.

F. Binding events. Since binding sites typically become less exposed to the aqueous bulk upon ligand binding, polarity-sensitive probes can be used to monitor such processes.

G. Synthetic polypeptides. Synthetic peptides have found numerous applications as protein models in biophysical studies and in material sciences. Well-defined secondary structures (e.g., α-helix) provide useful scaffolds for placing chromophores and exploring their interactions.[348]

H. Miscellaneous. Studies that do not fit in any of the categories listed above are included here. A brief description is provided in the last column of the table.

6. Fluorescent Nucleoside Analogs

6.1. Introduction

As we complete our "journey into the center of the cell", we approach nucleosides, nucleotides, and oligonucleotides, molecules of utmost importance in cell biology. While originally regarded as molecules of heredity, our contemporary view of the flow of biochemical information from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) and then to proteins suggests much more complex roles for nucleic acids.[408] Briefly, DNA sequences coding for specific genes are transcribed in the cell nucleus to yield heterogeneous nuclear RNAs. The resulting primary RNA transcripts are processed, yielding mature RNAs, which are exported to the cytoplasm where ribosome-based protein synthesis takes place. The transport, localization, stability, and translation efficiency of individual mRNA molecules are all well regulated. Additionally, recent developments suggest significant roles for noncoding RNA sequences in cellular regulatory processes, adding to the multifaceted and intricate roles of these biomolecules in the cell.[409−412]

Nucleic acids are phosphodiester-based biopolymers composed of several different building blocks in no particular or repeating linear sequence. The minimal building blocks, or monomers, are nucleotides, which can be viewed as phosphorylated nucleosides (Figure 6.1). Nucleosides, in turn, are composed of a five-carbon monosaccharide (D-ribose) linked to nitrogenous heterocyclic rings (pyrimidines and purines). While a handful of rare naturally occurring nucleosides are emissive[413−416] and despite very early studies suggesting that nucleobases and nucleic acids are fluorescent,[417] the purines and pyrimidines commonly found in nucleic acids are practically nonemissive in neutral aqueous conditions. Accurate measurements reveal exceedingly low fluorescence quantum yields for the natural nucleobases ($\Phi_F = (0.5-3) \times 10^{-4}$) associated with subpicosecond excited state lifetimes (Table 6.1).[418] Quite expectedly, Nature has selected the building blocks for its precious genetic material to rapidly decay back to their ground state upon photochemical excitation.[419] This property has presented, however, a major challenge to the biophysical community interested in exploring nucleic acids, which stimulated an extensive search for emissive nucleoside analogs, as discussed in this section.

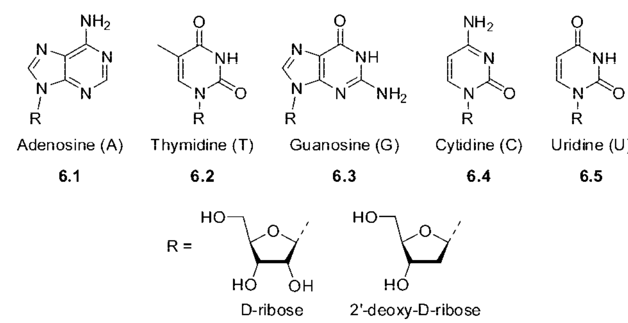


Figure 6.1. The naturally occurring ribo- and deoxyribonucleosides. Note that in RNA, the bases A, T, G, C, and U are connected to D-ribose at the 1′-position whereas the sugar moiety in DNA is 2′-deoxy-D-ribose.

Unlike the majority of biomolecular building blocks discussed in previous sections, the pyrimidines and purines present a fertile ground for synthetic organic chemists. These aromatic heterocycles are receptive to diverse modifications, where minimal structural and electronic perturbations can, in certain cases, dramatically alter their photophysical characteristics. Early work was inspired by naturally occurring emissive heterocycles, such as the wyosine bases, a family of tricyclic guanine derivatives. Leonard's pioneering work, where an etheno bridge was constructed across the H-bonding face of the purines and pyrimidines, furnished a series of emissive nucleobase analogs, with ethenoadenosine (εA, **6.25**) becoming one of the most useful early emissive nucleosides.[420] The advance of solid-phase oligonucleotide synthesis, facilitating the incorporation of modified nucleoside into oligomers, further propelled nucleoside chemists to explore new analogs. As presented in this section, the contemporary landscape of fluorescent nucleoside analogs is vast. We attempt to provide the reader with an up to date

Table 5.4. Applications of Selected Fluorescent Amino Acids

compd no.	name	applications	brief description
			Trp Mimics
5.10	azuAla	D[328]	incorporation by solid phase assisted peptide synthesis
5.11	5OHTrp	D1[329]	incorporation in β-galactosidase
		D1[332]	incorporation in mannitol transporter (EIImtl), a membrane protein
		F[339]	DNA−protein binding
5.12	7azaTrp	D1[329]	incorporation in β-galactosidase
		D1[332]	incorporation in mannitol transporter (EIImtl), a membrane protein
		F[339]	DNA−protein binding
5.13	BfAla	D[396,397]	site-specific *in vivo* incorporation
		H[336]	used as a competitive inhibitor for indoleamine 2,3-dioxygenase
5.14	BtAla	A[398]	substitutions in the 3-position of cyclic β-casomorphin analogs
		D[338]	incorporation in cyan fluorescent protein (CFP6)
		H[336]	used as a competitive inhibitor for indoleamine 2,3-dioxygenase
			Heterocycles
5.15	mchAla[e]	D[340]	incorporation in streptavidin
5.16	Asp(OMc)[e]	D[340]	incorporation in streptavidin
5.17	Glu(OMc)[e]	D[340]	incorporation in streptavidin
5.18	NBDAla	A[341]	probing structure and function of the tachykinin neurokinin-2 receptor
		D[370]	incorporation into a hexapeptide
		D[394]	review
5.19	NBDLys	D[345]	incorporation in streptavidin
5.20	NEtcarbazole	G[349]	study of photoinduced electron transfer in synthetic polypeptides
5.21	carbazole	D[345]	incorporation in streptavidin
5.23	Sox	H[350]	chemosensor scaffold for divalent zinc
		H[351−354]	protein kinase activity (Sox and Sox derivatives)
5.24	pCAP	H[355−357]	tyrosine phosphatase activity
			Hydrocarbons
5.25	pbpAla	D[345]	incorporation in streptavidin
5.26	1napAla	D[399]	*in vivo* incorporation using elongation factor Tu mutants
		G[348]	review
		G[358]	singlet energy transfer
		H[400]	pressor and antidiuretic peptides
		H[363]	photoenergy trapping in vesicles
5.27	2napAla	D[342]	streptavidin
		D[399]	*in vivo* incorporation using elongation factor Tu mutants
		G[359]	CD spectroscopy on synthetic polypeptides
		G[348]	review
		H[400]	pressor and antidiuretic peptides
		H[363]	photoenergy trapping in vesicles
5.28	1pyrAla	D[399]	*in vivo* incorporation using elongation factor Tu mutants
		F[401]	enkephalin−opiate receptor interaction
		F[402]	peptide−antibody interaction
		G[349]	PET in synthetic polypeptides
		G[360]	eximer formation
		G[403]	helix−helix interaction
5.30	9antAla	D[399]	*in vivo* incorporation using elongation factor Tu mutants
		G[348]	review
		H[363,364]	photoenergy trapping in vesicles
5.31	2antAla	D[340,345]	incorporation in streptavidin
5.32	9phantAla	H[363]	photoenergy trapping in vesicles
5.33	anthrAla	D[399]	*in vivo* incorporation using elongation factor Tu mutants
		D[361]	λ-Cro repressor protein
		G[348]	review
		G[365,404]	synthesis and conformation of poly(L-2-anthraquinonylalanine)
		H[361]	duplex DNA photocleavage
			Dansyl
5.34	51dansylAla	B[366]	monitoring of the unfolding of human superoxide dismutase
		C1[370]	incorporation into a hexapeptide
		D1[329]	β-galactosidase
		D[366]	superoxide dismutase
		F[405,406]	peptide−protein binding
		G[348]	review
			Charge Transfer Chromophores
5.38	4DAPA	C1[370]	incorporation into a hexapeptide
		F[368]	peptide−protein
5.39	6DMNA	C1[370]	incorporation into a hexapeptide
		F[369]	peptide−protein binding
5.40	4DMNA	C1[370]	incorporation into a hexapeptide
5.41	Aladan	C1[370]	incorporation into a hexapeptide
		C2[9]	petide−protein binding
		F[372]	investigation of phosphorylation-dependent protein associations
			Miscellaneous
5.42	azoAla	A[374,407]	studies on a series of azopolypeptides
		E[373]	photoswitching of an NAD^+-mediated enzyme reaction

Table 6.1. Spectroscopic Properties of Native Nucleosides and Nucleotides in Water[a]

compd no.	name	λ_{max} (ε)	λ_{em}	Φ	τ
6.1	A	260 (14.9)	310	5×10^{-5}	0.53
	AMP	259 (15.4)	312	5×10^{-5}	0.52
6.2	T	267 (9.7)	327	10×10^{-5}	0.70
	TMP	267 (10.2)	330	12×10^{-5}	0.98
6.3	G	253 (13.6)			0.69
	GMP	252 (13.7)	340	0.8×10^{-5}	0.86
6.4	C	271 (9.1)	324	7×10^{-5}	0.76
	CMP	271 (9.1)	330	12×10^{-5}	0.95

[a] λ, ε, and τ are in nm, 10^3 M^{-1} cm^{-1}, and ps, respectively; data from Johnson and Sprecher,[425] Callis[426] and Peon and Zewail.[418]

Table 6.2. Spectroscopic Properties of Selected Chromophoric Base Analogs[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
6.6	pyrene[b,f]	MeOH	241, 345 (39.0)	375, 395	0.12	
6.7	perylene[b,f]	MeOH	440 (39.2)	433, 472	0.88	
6.8	benzopyrene[b,f]	MeOH	394 (28.2)	408	0.98	
6.9	coumarin[b,c]	buffer[d]	400	515		7.4
6.10	terthiophene[e,f]	MeOH	358 (31.4)	432	0.059	
6.11	benzoterthiophene[e,f]	MeOH	437 (18.3)	536	0.67	
6.12	terphenyl[e,f]	MeOH	285 (40.1)	345	0.42	
6.13	stilbene[e,f]	MeOH	301 (21.1)	356	0.055	
6.16	1-anthracene[g]	N/A				
6.17	2-anthracene[g]	N/A				
6.18	Phen	MeOH	212 (32.0), 252 (73.2), 293 (10.5)			
6.19	PhenNH2[i]	MeOH	264 (70.9), 293 (14.9)			
6.20	Nile red nucleoside[h]	MeOH	557 (3.38)	632	0.09	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns respectively. [b] Data from Wilson and Kool[427] [c] τ is reported for oligo form from Coleman, Murphy and Berg et al.[428] [d] data obtained in aqueous buffer at pH 7.2. [e] Data from Kool et al.[429] [f] α-nucleosides [g] No photophysical data available.[430] See Table 5.3 for photophysical data of anthracene. [h] Data from Okamoto et al.[431] [i] Data for 2-amino-7-[2′-deoxy-3′,5′-di-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl-1]phenanthrene.

Table 6.3. Spectroscopic Properties of Pteridine Nucleoside Analogs in Buffer[a]

compd no.	name	λ_{max} (ε)	λ_{em}	Φ	τ
6.21	3-MI	254 (4.9), 350 (13.5)[b]	430	0.88	6.5
6.22	6-MI	340	431	0.70	6.4
6.23	DMAP	250 (12.0), 333 (8.9)[c]	430	0.48	4.8
6.24	6-MAP	248 (12.3), 329 (8.5)[c]	430	0.39	3.8

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively, from Hawkins.[456] All spectra were recorded in Tris buffer, pH = 7.5, at room temperature. [b] In MeOH, from Hawkins.[457] [c] In MeOH, from Hawkins.[458]

and systematically organized view of this rapidly evolving field. The diverse applications, summarized in Table 6.10 illustrate the breadth of this growing field and the great utility of judiciously implemented fluorescent nucleosides. Although beyond the scope of this review, fluorescent nucleoside mimics and surrogates have also been explored.[421−424]

6.2. Chromophoric Base Analogs

Replacing the natural nucleobases with established fluorophores, typically polycyclic aromatic hydrocarbons (PAH), yields an unusual family of chromophoric base analogs that lack the Watson−Crick (WC) hydrogen bonding face (Figure 6.2). Many of these fluorescent nucleobases have isolated absorption bands (≥345 nm) that facilitate selective excitation in the presence of the natural nucleobases and high emission quantum efficiencies approaching unity (Table 6.2).[427]

Kool and co-workers have utilized such PAH analogs for the investigation of enzyme−substrate recognition, demonstrating that size and shape are important factors in these template-directed events.[432] When linked via phosphodiester bonds to form oligomeric structures resembling DNA, these oligodeoxyfluorosides yield unique water-soluble fluorophores, where the photophysical properties are dictated by the composition and sequence of the individual chromophores. The complex electronic interactions between the stacked chromophores lead to fluorophores that typically display large Stokes shifts and a wide range of emission wavelengths and quantum yields.[429,433−439] A coumarin 102 containing nucleoside (**6.9**) (Figure 6.2),[440] having photophysical properties similar to its parent chromophore (Table 6.2),[441−443] was designed to pair with an abasic site in DNA. It was used to explore environmental and dynamics features of DNA oligonucleotides.[428,444−452] Phenanthrenyl nucleosides (**6.18** and **6.19**), recently reported by Leumann, were used to explore electron transfer in DNA.[453,454]

6.3. Pteridines

Pteridines are naturally occurring, highly emissive heterocycles whose structures are related to that of the purines (Figure 6.3, Table 6.3). Their intense (Φ = 0.39−0.88) and visible fluorescence (∼430 nm), characterized by a relatively long excited state lifetime (τ = 3.8−6.5 ns), results from an isolated absorption band above 300 nm. The development of the pteridines as fluorescent nucleoside analogs was initiated and advanced almost exclusively by Hawkins and co-workers.[455,456]

All four pteridine analogs (Figure 6.3), namely, the G analogs (3-MI[457] and 6-MI, **6.21** and **6.22**, respectively) and A analogs (DMAP and 6-MAP, **6.23** and **6.24**, respectively), retain their overall absorption and emission characteristics

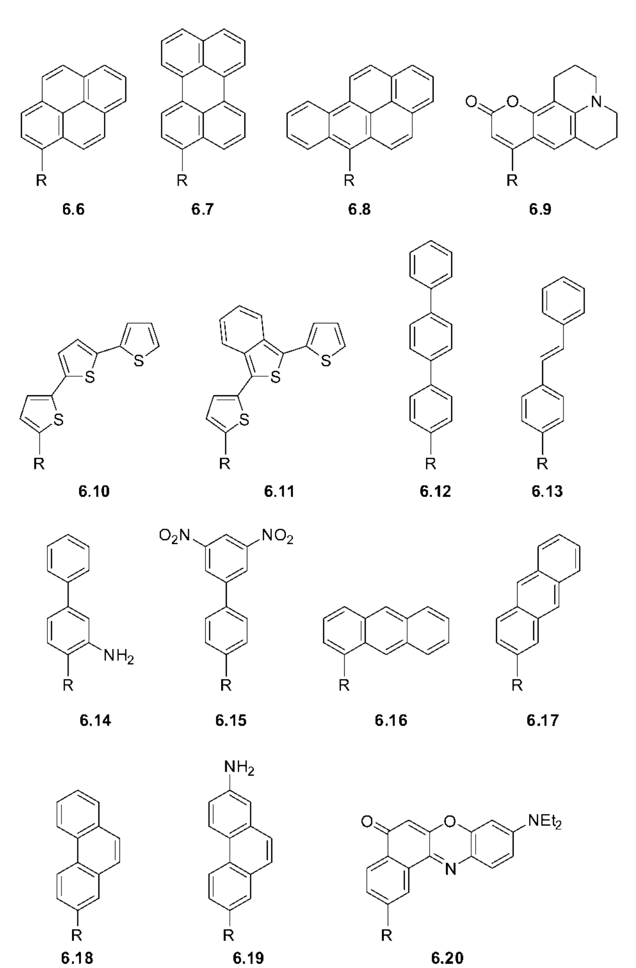


Figure 6.2. Selected examples of chromophoric base analogs, where R = 2′-deoxyribose.

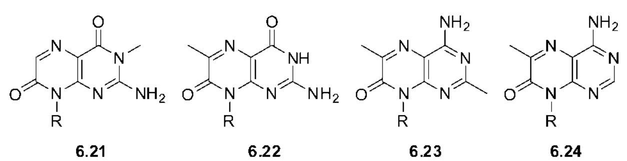


Figure 6.3. Selected examples of pteridines (R = 2′-deoxyribose or ribose).

upon incorporation into oligonucleotides. Significant sequence-dependent quenching has been observed, however, with purines being more effective quenchers than pyrimidines.[459] Incorporation of these modified nucleosides, except for 6-MI, typically results in sequence-dependent destabilizing effects similar to that of a single base pair mismatch.[460] Nevertheless, these fluorescent nucleosides have found numerous applications and remain very useful due to their high quantum efficiency, well-documented quenching effects, and commercial availability (section 6.8).

6.4. Nucleosides Containing Expanded Nucleobases

Extending the conjugation of the natural nucleobases by fusing additional aromatic rings onto the pyrimidine and purine nuclei generates diverse expanded nucleobases (Figures 6.4 and 6.5). Most retain their WC hydrogen bonding face (εA being an exception), although their large surface area could structurally perturb the resulting oligonucleotides. Having an extended aromatic surface typically results in favorable photophysical properties, with red-shifted absorption bands compared with their natural counterparts, emission bands near or in the visible range, and rather high emission quantum efficiencies, ranging from 0.2 to 0.82 (Tables 6.4 and 6.5).

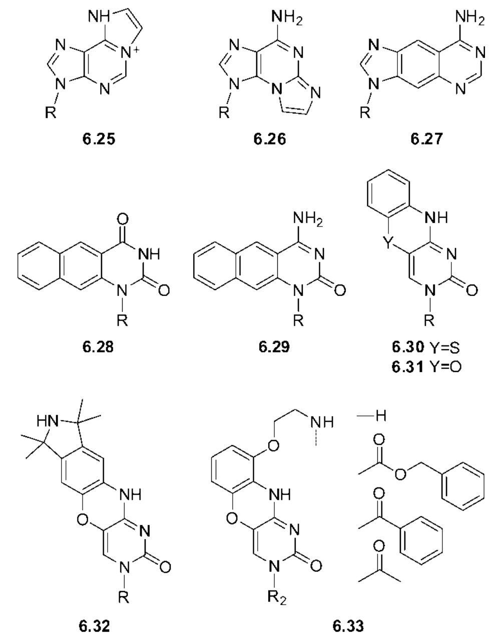


Figure 6.4. Examples of expanded nucleobase analogs (R = 2′-deoxyribose, 2′-OMe ribose, or ribose and R_2 = 3′,5′-O-TBDMS-2′-deoxyribose).

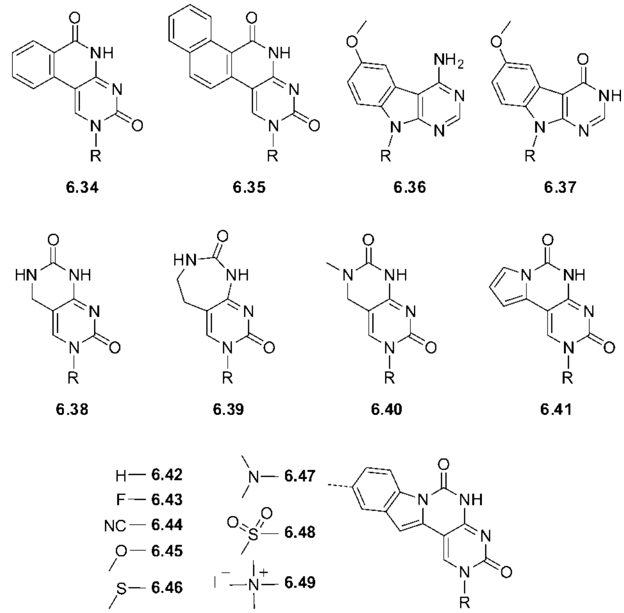


Figure 6.5. Expanded nucleobase analogs (R = 2′-deoxyribose).

Leonard and co-workers first investigated etheno-A (εA, **6.25**)[462,463] and benzo-A (**6.27**)[464] in the early 1970s following an initial report showing that adenine and cytosine could be cyclized to produce nucleobases with red-shifted absorption bands.[465] While the fused structure of εA, reminiscent of the naturally occurring fluorescent nucleoside wyosine,[466] masks the hydrogen bonding face, it also improves the photophysical properties. This is most notable with a red-shifted absorption (294 nm) and an intense emission band in the visible (415 nm, $\Phi = 0.56$), which is associated with a rather large Stokes shift (9917 cm^{-1}) and a relatively long lifetime for a small organic chromophore ($\tau = 20$ ns).[463,467] εATP,

Table 6.4. Spectroscopic Properties of Expanded Nucleoside Analogs[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
6.25	etheno-A	buffer[c]	258 (5.0), 265 (6.0), 275 (6.0), 294 (3.1)	415	0.6	20
6.27	benzo-A[b]	buffer[c]	340, 356	358, 379, 395	0.44	
6.28	BgQ[b]	buffer[c]	260 (49.8), 292 (16.0), 360 (14.4)	434	0.82	
6.29	C_f[b]	buffer[c]	370	456	0.62	
6.30	tC	buffer[d]	375 (4.0)	500	0.17	3.7
6.33	G-clamp and derivatives	$CHCl_3$	∼365[e]	∼450		

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. [b] Data from Wilson and Kool[427] and Moreau.[461] [c] Photophysical properties measured in buffer at pH = 7.0. [d] Photophysical properties measured in buffer at pH = 7.5. [e] Excitation wavelength.

Table 6.5. Spectroscopic Properties of Expanded Nucleosides[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
6.34	BPP[e]	buffer[b]	347	390	0.04	
6.35	NPP[e]	buffer[b]	365	395	0.26	
6.36	^{MD}A	buffer[b]	327	397, 427	0.118	
6.37	^{MD}I	buffer[b]	315	442	0.117	
6.38	dC^{hpp}	buffer[b]	300	360	0.12	
6.39	dC^{hpd}	buffer[b]	300	360		
6.40	dC^{mpp}	buffer[b]	300	360		
6.41	dC^{ppp}	buffer[c]	369 (4.76)	490	0.105	
6.42	dC^{ppi}	buffer[c]	374 (4.157)	513	0.006	
6.43	3-fluoro-dC^{PPI}	buffer[c]	371 (6.284)	505	0.019	
6.44	3-cyano-dC^{PPI}	buffer[c]	369 (6.998)	487	0.061	
6.45	3-methoxy-dC^{PPI}	buffer[c]	375 (5.321)	511	0.005	
6.46	3-methlythio-dC^{PPI}	buffer[c]	372 (4.11)	511	0.002	
6.47	3-*N*,*N*-dimethylamino-dC^{PPI}	buffer[d]	366 (9.05)	486	0.068	
6.48	3-methylsulfonyl-dC^{PPI}	buffer[c]	367 (7.38)	484	0.078	
6.49	3-*N*,*N*,*N*-trimethylammoniumiodido-dC^{PPI}	buffer[c]	368 (8.468)	483	0.096	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. All spectra taken in aqueous buffer. [b] pH = 7.0. [c] pH = 7.4. [d] pH = 3.0. [e] Data from Wilson and Kool.[427]

a fluorescent replacement for ATP, is recognized as a substrate by AMP/ATP binding enzymes.[463] Seela and co-workers have investigated a 7-deaza analog of εA, which displays a larger Stokes shift and similar quantum efficiency. The 7-deaza εA derivative, however, shows higher stability over a larger range of pHs and can be used to monitor oligonucleotide denaturation.[468]

Benzo-A (**6.27**) retains the hydrogen bonding face of adenine. The extended heterocycle is responsible, however, for significantly improved photophysical properties compared with adenosine. A structural and photophysical comparison of εA and benzo-A reveals the intricacies shown by modified nucleosides. Fusing a benzene ring into the purine core, as in benzo-A (**6.27**), results in a tremendous red shift of the absorption bands, leading to a rather small Stokes shift (∼40 nm) for emission and lower quantum efficiency in comparison to εA. These nucleoside analogs have found unique applications in recent years, beyond the realm of fluorescence-based applications, particularly in exploring size-expanded DNAs.[469−475]

Two naptho-expanded nucleosides, BgQ (**6.28**) and C_f (**6.29**), are relatively recent additions to this class of fluorescent nucleosides (Figure 6.4).[476,477] Both analogs display a strong emission band in the visible range (Φ = 0.82, 0.62, respectively), resulting from a red-shifted absorption band (360 and 370 nm, respectively).[476,477] Due to their desirable photophysical properties and large surface area, BgQ and C_f were employed for the study of double- and triple-stranded oligonucleotides (see section 6.8).

A cytidine analog, tC (**6.30**), originally synthesized for antisense applications by Matteucci,[478] forms WC-like base pairs with guanosine[479] and, somewhat surprisingly, does not suffer a dramatic reduction in quantum efficiency upon in corporation into PNA[480] or DNA,[481] unlike most other fluorescent nucleosides. Like many expanded analogs, tC emits in the visible (500 nm) with a somewhat low quantum efficiency (Φ = 0.17) for this class of nucleobases.[480,482−484] Most recently, Millar and Tahmassebi have utilized tC analogs with a nonfluorescent quencher (TEMPO) to demonstrate the utility of a fluorescent nucleoside/quencher combination.[485]

Sasaki and co-workers have utilized an emissive expanded base analog (G-clamp, **6.33**), first introduced by Matteucci.[486,487] The photophysical properties of G-clamp and derivatives are similar to that of tC, with an absorption maximum around 365 nm and a corresponding emission maximum of 450 nm.[488,489] The ability of the protected G-clamp nucleoside (8-oxoG-Clamp) and its derivatives to detect the presence of 8-oxodG have been explored (see section 6.8).[488−490]

In 2003, Saito and co-workers introduced base-discriminating fluorescent (BDF) nucleosides, designed primarily for single nucleotide polymorphism (SNP) analysis.[491] Such emissive nucleoside analogs can be divided into two main categories: (a) nucleobases with pendent fluorophores (extended nucleobases, see section 6.5) and (b) ring-expanded nucleobases. Benzopyridopyrimidine (BPP, **6.34**) is a cytidine analog that forms stable pairs with both A (wobble bp) and G (WC bp).[492] BPP displays an isolated absorption band (347 nm), but its low quantum efficiency (Φ = 0.04) prompted the synthesis of naphthopyridopyrimidine (NPP, **6.35**) (Figure 6.5). While showing similar absorption and emission wavelengths as BPP, NPP displays a substantially higher emission quantum efficiency (Φ = 0.26) (Table 6.5).[493] With an acceptable purine-discriminating fluorescent nucleoside in hand, Saito and co-workers designed adenosine (^{MD}A, **6.36**) and inosine (^{MD}I, **6.37**) analogs as pyrimidine-discriminating fluorescent nucleosides (section 6.8).[494] Both ^{MD}A and ^{MD}I have rather large Stokes shifts in comparison to BPP/NPP, resulting from a blue-shifted absorption and

Table 6.6. Spectroscopic Properties of Conjugated Nucleoside Analogs[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
6.50	8-aza-7-deaza-7-phenylethynyl	water[b]	294	360	0.08	0.55
6.51	7-deaza-7-phenylethynyl	water[b]	296	412	0.02	0.69
6.52	2-phenylpropyl dA	water	215, 262	385	0.011	6.22
6.53	2-phenylbutyl dA	water	215, 262	396	0.007	7.13
6.54	^{PY}U	buffer[c]	341	397	0.2	
6.55	^{PY}C	buffer[c]	329	393	0.15	
6.56	DMAP-^{PY}U	buffer[c]	303	440 LE, 540 ICT	0.01	
6.57	5-(1-pyrenyl)-dU	MeOH	342 (28.5)	474	0.027	
6.59	5-(1-pyrenoyl)-dU	MeOH	357 (6.68)	395	0.002	
6.60	5-(1-ethynylpyrenyl)-dU (U^{P})	MeOH	392	400, 424		
6.66	ethynyl-FL	buffer[d]	320, 492 (23.9, 57.0)	520	0.53	
6.67	phenylethynyl-FL	buffer[d]	322, 492 (39.2, 63.3)	516		
6.68	ethynylphenylethynyl-FL	buffer[d]	330, 492 (55.0, 63.7)	520		
6.71	^{MMe}U	water	350[e]	450[e]		
6.72	^{Ph}U	water	320[e]	400[e]		
6.73	^{MeOPh}U	water	330[e]	450[e]		
6.75	5(DAN)-dU[f]	CH_2Cl_2	341 (1.15)	507	0.032	
6.76	5(DANethylyl-one)-dU[f]	CH_2Cl_2	410 (2.11)	532	0.041	
6.77	^{DNC}U[f]	CH_2Cl_2	332 (1.42)	421	0.126	
6.78	^{PDN}U[g]	water	380	524	0.033	
6.82	^{dan}C[h]	water	321	459		
6.84	5-aminodansyl-dU[i]	water	330	523		
6.90	U^{FL}[j]	CH_3Cl	325	405	0.139	
6.91[k]	5(difluoroBODIPY)phenyl-dU	MeOH	496 (69.3)	507	0.42	
6.92[k]	5(fluoromethoxyBODIPY)phenyl-dU	MeOH	497 (69.1)	508	0.42	
6.93[k]	5(dimethoxyBODIPY)phenyl-dU	MeOH	498 (70.8)	509	0.47	
6.94[k]	5(difluorodiethylBODIPY)phenyl-dU	MeOH	522 (57.5)	534	0.61	
6.95[k]	5 (fluoromethoxydiethylBODIPY)phenyl-dU	MeOH	523 (55.2)	535	0.62	
6.96[k]	5(dimethoxydiethylBODIPY)phenyl-dU	MeOH	523 (53.6)	535	0.59	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. [b] Spectra were collected in doubly distilled water. [c] Spectra were collected in aqueous phosphate buffer (pH = 7.0).[427] [d] Spectra were collected in aqueous phosphate buffer (pH = 7.2). [e] Excitation and emission data are for single-stranded oligomers (13-mer), where the modified nucleoside is located in the center.[497] [f] Data from Saito and Okamoto et al.[498] [g] Data from Okamoto et al.[499] [h] Data from Majima et al.[500] [i] Data from Barawkar and Ganesh[501] [j] Data from Kim et al.[502] [k] Data from Ehrenschwender and Wagenknecht.[503]

red-shifted emission, although their quantum efficiency remains rather modest (Φ = 0.12).[494]

Sekine and co-workers have investigated cyclized dC analogs, which maintain the H bonding face of the parent nucleoside but extend the heterocycle surface by linking the 4 and 5 positions on the pyrimidine core. Early derivatives included dC^{hpp} (**6.38**), dC^{hpd} (**6.39**), and dC^{mpp} (**6.40**) whose absorption band around 300 nm resulted in an emission near the visible range (375 nm). Extending these bicyclic systems into a tricyclic system (dC^{ppp}, **6.41**) resulted in a large red shift in both absorption (369 nm) and emission (490 nm).[495] The photophysical properties of the further expanded dC^{ppi} system (**6.42**), a family of dC analogs that can be viewed as having a fused indole ring, can be tuned by altering the remote 3 position of the heterocycle.[496] These analogs all display very large Stokes shifts (~7000 cm^{-1}), which grow with increasing polarity from toluene to methanol and then decrease again with further increase in polarity. A detailed investigation of the solvatochromatic effects of these nucleoside analogs revealed complex trends in the sensitivity of absorption, emission, and quantum yields to solvent polarity, suggesting susceptibility to a multitude of factors.[496]

6.5. Nucleosides Containing Extended Nucleobases

Extended fluorescent nucleoside analogs are distinguished by fluorescent moieties that are linked or conjugated to the natural nucleobases, via either flexible or rigid linkers (Figures 6.6 and 6.7, respectively). Connecting known chromophores via electronically nonconjugating linkers yields nucleoside analogs with photophysical features that are normally very similar to that of the parent fluorophore. Extending the purines and pyrimidines by electronically conjugating them to additional aromatic moieties typically generates a new chromophore with unique, and somewhat unpredictable, photophysical characteristics.

Seela and co-workers have diligently investigated the impact of extending the conjugation of 7-deaza-adenosine and 8-aza-7-deaza-adenosine on the photophysical characteristics by attaching functionalized alkenes and alkynes to the 7-position (e.g., **6.50** and **6.51**).[504,505] Although the absorption spectra of the parent compounds, 7-deaza-adenosine and 8-aza-7-deaza-adenosine, are slightly red-shifted in comparison to adenosine (270 and 270 nm vs 260 nm, respectively), they are not emissive, unlike the alkene- and alkyne-conjugated analogs (**6.50** and **6.51**, Figure 6.6).[505]

Unusual 2-substituted adenosine analogs, **6.52** and **6.53**, have been recently explored by Baranger and co-workers (Figure 6.6).[506] These probes show dramatic photophysical changes when compared with adenosine, all due to the nonconjugated phenylalkyl substituents.[507] While the free nucleosides display very low emission quantum efficiencies and rather long lifetimes (Table 6.6), incorporation into RNA hairpins results in significant increase in quantum efficiency and slight shortening of the excited state lifetimes. It is worth noting that such enhancement of quantum efficiency upon incorporation into oligonucleotides is very rarely seen in emissive nucleoside analogs, let alone those with such nonconjugated systems.

Netzel and co-workers have linked pyrene to the 5-position of 2′-deoxyuridine directly (**6.57**) and via amide or ketone (**6.59**) linkages (Figure 6.6).[508,509] While primarily developed as tools to evaluate electron transfer processes in DNA,

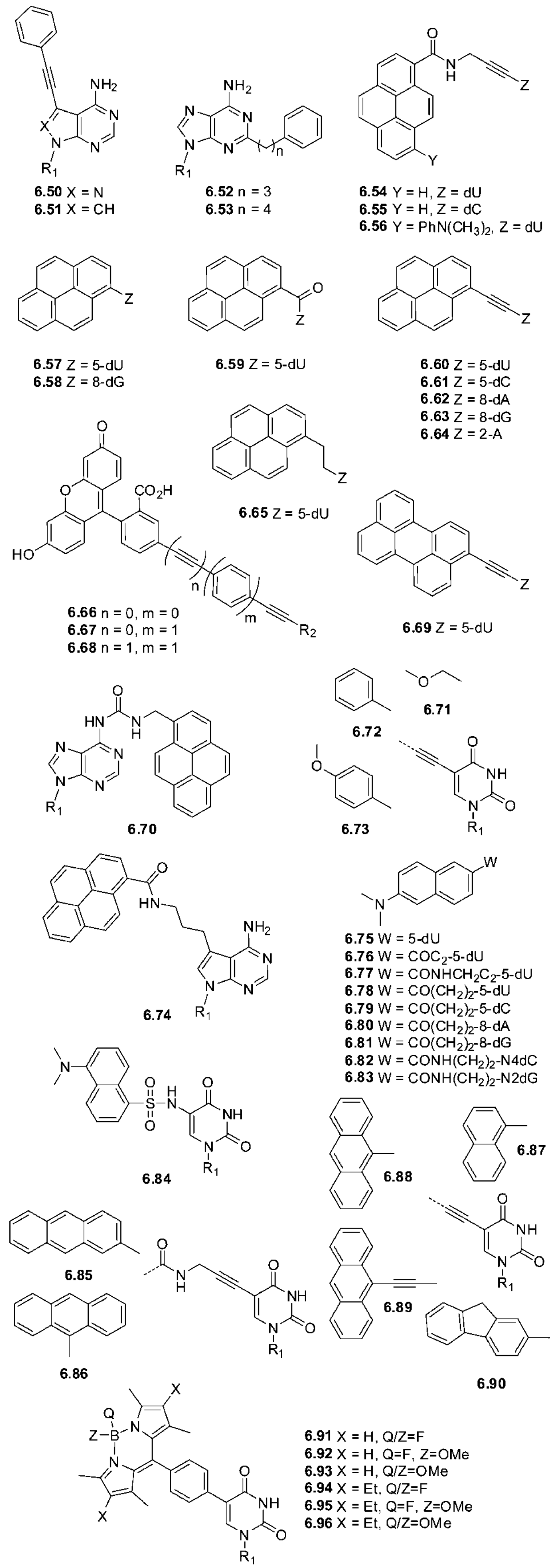


Figure 6.6. Selected examples of extended base analogs (R_1 = 2′-deoxyribose and R_2 = 2′-deoxyuridine or 2′,3′-dideoxyuridine).

detailed analysis of their photophysical characteristics, including steady state and time-resolved studies provided

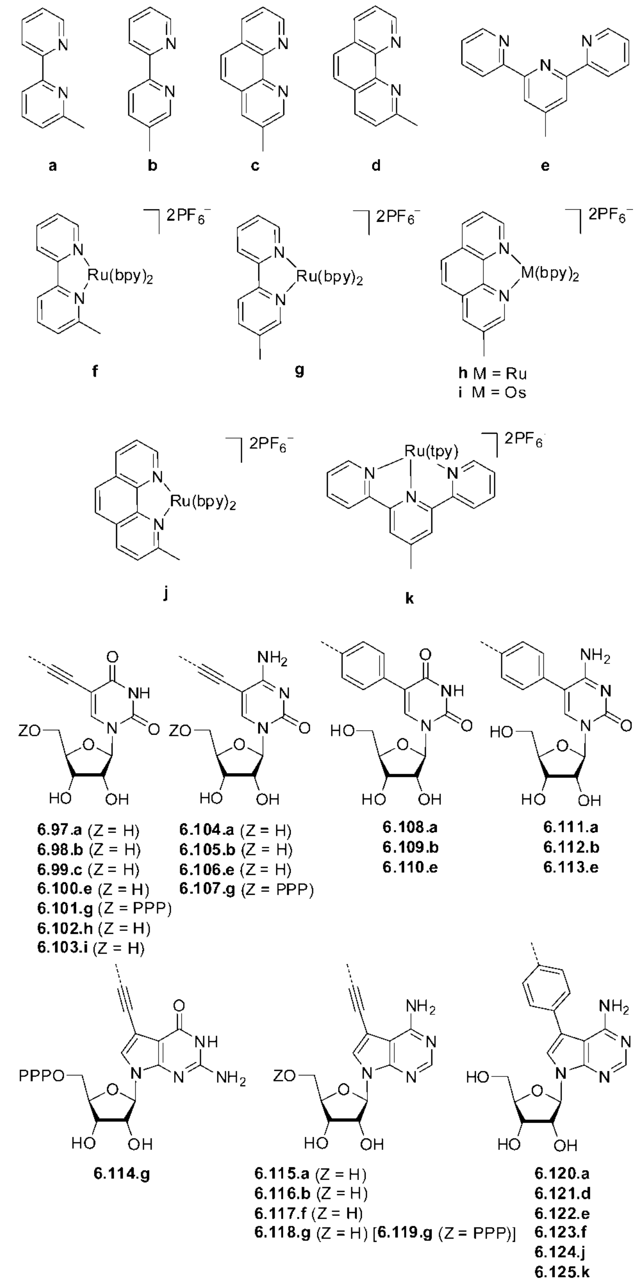


Figure 6.7. Examples of extended nucleobase analogs.

evidence that such nucleosides can be responsive to changes in their microenvironment.[508−510] Similarly, Berlin and co-workers linked pyrene via an ethynyl linkage to the 5-position of dU (**6.60**).[511] All pyrene nucleosides share similar photophysical properties with red-shifted absorption in comparison to the native nucleobases (342−392 nm) and a weak emission band (Φ = 0.002−0.027) near the visible range (395−474 nm). 5-(1-Ethynylpyrenyl)-dU (**6.60**) has been utilized by numerous groups for a variety of applications (section 6.8).[510,512,513] The corresponding 8-substituted purines (**6.62** and **6.63**), which can be viewed as rather perturbing analogs, have also found utility (section 6.8).[512,513]

In pursuit of fluorescent nucleosides capable of SNP detection, Saito and co-workers have bridged pyrene to 2′-deoxyuridine or cytosine via a propargylamide linker (Figure 6.6). Nucleosides **6.54** and **6.55** retain the hydrogen bonding face of U/C and the typical photophysical properties of pyrene, showing an isolated absorption band at ∼335 nm and a relatively intense emission band around ∼400 nm (Φ

= ~0.2, Table 6.6). Despite the distance between the pyrene and the pyrimidine core and the nonconjugating linker, these BDFs have been very successful probes for SNP analysis (section 6.8).[491] Modifying the pyrene moiety with a dimethylaminopyridine group (DMAP-^{Py}U, **6.56**) generates a probe with dual emission, resulting from either locally excited or charge transfer states populated via a rather energetic absorption band (Table 6.6).[514]

While diverse xanthene-type fluorophores (e.g., fluorescein) have been linked to dideoxynucleotides for sequencing applications, only recently has fluorescein been rigidly conjugated to dU and ddU by Burgess (Figure 6.6).[515] Upon excitation at 320 nm, nucleoside **6.66**, where the fluorophore is conjugated via an ethynyl linkage, emits at 520 nm (Table 6.6). Extending the rigid linker by a phenyl or a phenylethynyl moiety increases the extinction coefficient of the nucleoside without red shifting the excitation wavelength (Table 6.6). Preparation of the triphosphates for exploring polymerase-based incorporation reactions was accomplished by synthesizing 5-iodo-UTP, followed by cross-coupling reactions with ethynyl-linked fluorescein derivatives. Only the derivative with the longest linker (**6.68**), either in the deoxy or dideoxy form, showed acceptable levels of enzymatic incorporation, albeit lower than the commonly used rhodamine-based probe (6-TAMRA-ddTTP).[515] In addition to the classically multicolor fluorescent nucleoside triphosphates made by flexibly conjugating established fluorophores to nucleobases (e.g., FAM, TAMRA), emissive nucleosides and fluorophore/quencher pairs have been developed for molecular beacon and sequencing applications.[516−528]

Having shown that the emission of 3- and 3,8-arylethynyl-extended 1,10-phenanthroline derivatives respond to polarity changes,[529,530] Tor and co-workers have attached this moiety to the 5-position of dU, using Pd-mediated cross-coupling reactions.[531] Nucleoside **6.99.c** (dU^{phen}) displays an absorption band at 333 nm, which was insensitive to solvent polarity. Excitation of dU^{phen} results in emission ranging from 385 nm (dichloromethane) to 408 nm (water).[531] The sensitivity of this emissive nucleoside to its environment has been used to explore its utility as a SNP probe upon incorporation into oligonucleotides (section 6.8).[531]

Utilizing the phenanthroline-extended dU as a core structure, the corresponding polypyridine Ru^{II}- and Os^{II}-containing nucleosides (**6.102.h** and **6.103.i**) were prepared by cross-coupling the brominated metal-containing polypyridyl precursors [e.g., $(bpy)_2Ru(3\text{-Br-}1,10\text{-phen})^{2+}$] with 5-ethynyl-dU.[532−534] The electrochemical and photophysical properties of the resulting metal-containing nucleosides were investigated.[532−534] The presence of coordinately saturated polypyridine complexes in these nucleosides results in typical visible MLCT absorption bands (~460 nm). The Ru^{II}-based nucleoside shows a moderately strong luminescence (Φ = 0.137 at 629 nm) and a rather long excited state lifetime (2.8×10^3 ns), while the Os^{II}-containing nucleoside displays a very weak luminescence (Φ = 0.0003 at 749 nm), which is associated with a very short excited state lifetime (78 ns). Incorporation of these nucleosides into oligonucleotides results in minimal duplex destabilization. This facilitated a thorough investigation of donor−acceptor interactions in systematically Ru/Os-modified oligonucleotides.[533] It is worth noting that the diastereomerically pure nucleosides were also synthesized and incorporated into oligonucleotides.[534] While the photophysical characteristics of the diastereomerically pure **Δ-6.102.h** and **Λ-6.102.h** nucleosides are essentially identical, analysis of time-resolved data suggests the Δ-$(bpy)_2$Ru(phen) metal center is better accommodated within the major groove of a DNA duplex.[534]

Hocek and co-workers have recently investigated the emissive properties of both pyrimidine and purine analogs with conjugated bipyridine, terpyridine, and phenanthroline moieties.[535−537] The chelators were attached through ethynyl and phenyl linkages to the 5 position on pyrimidines and the 7 position on 7-deazapurine. The nonmetalated nucleoside analogs were prepared via cross-coupling reactions of the ethynyl/phenyl-modified polypyridyl arm with the unprotected halo-nucleosides. These conjugated chromophores show an isolated absorption in the 306−329 nm range, with corresponding emission bands between 389 and 451 nm (Table 6.7 and Figure 6.7, **6.97**−**6.125**, R = **a**, **b**, **d**, and **e**). Diverse metal-containing nucleoside and nucleotide triphosphates have also been prepared (Figure 6.7).[536−538] Enzymatic incorporation of these metal-containing triphosphates (**6.101.g**, **6.107.g**, **6.114.g**, and **6.119.g**), by vent(exo-) and Pwo polymerases, produced modified oligonucleotides that were employed for SNP detection using luminescence (in case of Ru^{II}-containing oligonucleotides) or electrochemical detection (for Os^{II}-containing oligonucleotides) (section 6.8).[536−538] Ru complexes have also been connected through a propargylamide linker to the 5-position of dU, yielding nucleosides with photophysical properties similar to the ones listed above.[539,540] Tuning the redox potential of Ru- and Os-containing nucleotides has been discussed.[541]

6.6. Isomorphic Nucleobases

Isomorphic nucleobase analogs are heterocycles that closely resemble the corresponding natural nucleobases with respect to their overall dimensions, hydrogen bonding patterns, and ability to form isostructural W−C base pairs (Figures 6.8 and 6.9). A clear advantage of these analogs is their strong similarity to the native nucleosides and minimally perturbing nature, when compared with the diverse analogs discussed above. Since favorable photophysical characteristics (e.g., red-shifted absorption and high emission quantum efficiencies) are typically associated with significant structural perturbation and extended conjugation, isomorphic fluorescent nucleosides are the most challenging to design.

2-Aminopurine (2-AP, **6.126**), one of the first and most widely utilized fluorescent nucleosides, is a constitutional isomer of adenine with substantially enhanced photophysical features (Figure 6.8, Table 6.8). Since the initial publication in 1969 describing its fluorescence properties as a nucleoside or within oligonucleotides,[550] 2-AP has been reported in more than 1600 contributions. The seminal paper by Reich and Stryer suggests 2-AP to be an ideal emissive nucleoside analog. Its ability to form WC-like base pairs with dT/U, high quantum efficiency (Φ = 0.68 in water), isolated absorption band (303 nm), minimal sensitivity to pH changes, and importantly, sensitivity to environmental polarity, all contribute to its great utility.[550] Specifically, 2-AP's emission, and to a lesser extent its absorption, undergo a bathochromic shift with increasing solvent polarity.[551] Interestingly, 2,6-diaminopurine and formycin, two related emissive adenosine analogs (**6.127**, **6.128**, respectively, Figure 6.8), display substantially lower quantum efficiencies (0.01 and 0.06, respectively, Table 6.8).[550] Other variations of 2-AP, including 7-deaza and 8-aza-7-deaza, have been studied by Seela and co-workers.[552] While these analogs display larger Stokes

Table 6.7. Spectroscopic Properties of Conjugated Base Analogs[a]

compd no.	name	solvent	λ_{max} (ε)	λ_{em}	Φ	τ
6.97.a	5-[6(bpy)ethynyl]dU	MeOH	316 (31.0)	389	<0.01	
6.98.b	5-[5(bpy)ethynyl]dU	MeOH	327 (39.0)	421	0.16	
6.99.c	5-[3(phen)ethynyl]dU	H_2O	248 (19.0), 260 (18.0), 278 (20.0), 296 (18.0), 330 (20.0), 345 (18.0)	408	0.16	
6.100.e	5-[4(tpy)ethynyl]dU	MeOH	282 (44.0)	408	0.01	
6.101.g	5-[5-Ru(bpy)$_3$ethynyl]dU-TP	buffer[b]		645	0.0156	
6.102.h	5-[3(phen)Ru(bpy)$_2$-ethynyl]dU	CH_3CN	242 (40.0), 256 (39.0), 286 (69.0), 352 (26.0), 450 (13.0)	629	0.137	2.8×10^3
6.103.i	5-[3(phen)Os(bpy)$_2$-ethynyl]dU	CH_3CN	242 (38.0), 254 (36.0), 290 (64.0), 358 (26.0), 436 (11.0), 484 (11.0)	749	0.0003	78
6.104.a	5-[6(bpy)ethynyl]dC	MeOH	326 (21.0)	397	<0.01	
6.105.b	5-[5(bpy)ethynyl]dC	MeOH	329 (54.0)	435	0.11	
6.106.e	5-[4(tpy)ethynyl]dC	MeOH	283 (37.0)	445	0.02	
6.107.g	5-[5-Ru(bpy)$_3$ethynyl]dC-TP	buffer[a]		646	0.0132	
6.108.a	5-[6(bpy)phenyl]dU	MeOH	309 (36.0)	399	<0.01	
6.109.b	5-[5(bpy)phenyl]dU	MeOH	315 (53.0)	437	0.29	
6.110.e	5-[4(tpy)phenyl]dU	MeOH	285 (34.0)	427	0.08	
6.111.a	5-[6(bpy)phenyl]dC	MeOH	286 (39.0)	389	<0.01	
6.112.b	5-[5(bpy)phenyl]dC	MeOH	306 (37.0)	451	0.18	
6.113.e	5-[4(tpy)phenyl]dC	MeOH	286 (12.0)	444	0.04	
6.114.g	7-[7-deaza-5-Ru(bpy)$_3$-ethynyl]dG-TP	buffer[b]		635	0.0111	
6.115.a	7-[7-deaza-6(bpy)ethynyl]dA	$CHCl_3$	282 (28.0), 321 (24.0)	406		
6.116.b	7-[7-deaza-5(bpy)ethynyl]dA	$CHCl_3$	285 (35.0), 336 (25.0)	427		
6.117.f	7-[7-deaza-6-Ru(bpy)$_3$-ethynyl]dA	CH_3CN	288 (82.0), 488 (14.0)	639	0.00021	
6.118.g	7-[7-deaza-5-Ru(bpy)$_3$-ethynyl]dA	CH_3CN	254 (36.0), 287 (98.0), 384 (26.0)	665	0.0289	
[6.119.g]	[TP analog]	[buffer[b]]		[642]	[0.0187]	
6.120.a	7-[7-deaza-6(bpy)phenyl]dA	$CHCl_3$	291 (31.0), 325 (30.0)	395		
6.121.d	7-[7-deaza-2(phen)phenyl]dA	$CHCl_3$	283 (51.0), 322 (45.0)	424		
6.122.e	7-[7-deaza-4(tpy)phenyl]dA	$CHCl_3$	253 (47.0), 284 (54.0)	425		
6.123.f	7-[7-deaza-6-Ru(bpy)$_3$-phenyl]dA	CH_3CN	245 (33.0), 289 (73.0), 450 (12.0)	667	0.00019	
6.124.j	7-[7-deaza-2(phen)Ru(bpy)$_2$-phenyl]dA	CH_3CN	287 (71.0), 448 (10.0)	648	0.00043	
6.125.k	7-[7-deaza-4(tpy)Ru(tpy)phenyl]dA	CH_3CN	273 (51.0), 308 (64.0), 485 (23.0)	633	0.00024	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. [b] Buffer = 3.3 mM Tris-Cl, pH = 8.5.

shifts, their emission quantum efficiencies are lower in comparison to 2-AP (0.47 and 0.53 vs 0.68, respectively).[552]

While 2-AP pairing with T/U does not disturb the secondary structure of either B- or A-form DNA/RNA,[553] it can also pair with cytosine in various forms depending upon the pH.[554−556] When incorporated into oligonucleotides, 2-AP's emission is significantly quenched. This phenomenon, which has been exploited in numerous assays (see section 6.8), is sequence dependent. Adding to its complex photophysics, energy transfer processes have been documented for 2-AP-containing duplexes.[557] Adenine is the most efficient donor among the native nucleobases, while energy transfer from C/T or G is very inefficient, except in one particular case, when 2-AP is found at the end of a G pentamer.[558−561] Numerous theoretical and experimental approaches have probed the electronic structure of 2-AP and the origin of its unique photophysical characteristics.[562−566]

Substituting the hydrogen at the 8 position of adenine with a vinyl moiety results in remarkable photophysical changes compared with the parent nucleobase.[567,568] Upon excitation of 8-vinyl-2′-deoxyadenosine (8vdA, **6.129**) at its absorption maximum (290 nm), an intense emission is observed at 382 nm. 8vdA, thus, exhibits a significantly larger Stokes shift (8300 cm^{-1}) compared with 2-AP (5970 cm^{-1}) with a comparable quantum yield ($\Phi = 0.66$).[567] The emission of 8vdA was shown to be responsive to changes in temperature and solvent, while insensitive to pH changes (between 5 and 10), displaying desirable properties as a probe (see section 6.8). Incorporation into oligonucleotides showed sequence dependent, albeit minimal, disruption to duplex stability.[567] Much like 2-AP, the intense emission of 8vdA is quenched upon incorporation into oligonucleotides, albeit to a lesser extent.[567]

Hirao and co-workers have demonstrated a site-specific fluorescent labeling of RNA via an unnatural base pair, in which both components are fluorescent nucleobase analogs.[569−571] The two purine analogs, 2-amino-6-(2-thienyl)purine **(6.130)** and 2-amino-6-(2-thiazolyl)purine **(6.131)**, are isomorphic fluorescent nucleosides that can be viewed as 2-AP derivatives. The incorporation of a thiophene or thiazole ring to the 6-position results in a red-shifted absorption band in comparison to 2-AP (~355 nm), and a strong emission ($\Phi \approx 0.4$) in the visible range (~450 nm).[571] The pyrimidine pairing partner was an extended nucleobase analog where known chromophores (FAM, TAMRA, and Dansyl) were attached via a linker to the 5-position of a 2-pyrimidinone core.[570] This unnatural base pair facilitates enzymatic labeling of RNA for various applications (section 6.8). In this context, it is useful to comment on additional novel base pairs, including the well-studied isoG−isoC system, because they facilitate the incorporation of various emissive analogs (Figure 6.8).[572−579] Some of these derivatives, developed primarily to expand the genetic alphabet, including 5-aza-7-deazapurine-2′-deoxyriboside,[549] are emissive.

5-Methyl-2-pyrimidione (d5 or m^{5}K, **6.132**) has been explored as a T analog (Figure 6.8).[580,581] Its synthesis and incorporation into oligonucleotides were initially reported in the late 1980s.[582,583] Early photophysical studies described its isolated absorption maximum (280 nm), and time-resolved experiments were used to probe its stacking ability in a single-stranded oligonucleotide (Table 6.8).[544] The recent interest in d5 as a non-natural base for enzymatic incorporation[584−586] or as a base in triple helix motifs,[587,588] and not as a fluorescent probe, stems likely from its inability to form an adequate WC base pair with adenosine.

Table 6.8. Spectroscopic Properties of Isomorphic Nucleoside Analogs in Water[a]

compd no.	name	λ_{max} (ε)	λ_{em}	Φ	τ
6.126	2-aminopurine	303 (6.8)	370	0.68	7.0
6.127	2,6-diaminopurine	280 (10.0[b])	350	0.01	
6.128	formycin	295 (0.8[c])	340	0.06	<1
6.129	8-vinyl-6-aminopurine[d]	290 (12.6)	382	0.66	4.7
6.130	2-amino-6-(2-thienyl)purine[e]	297 (9.9), 348 (14.0)	434	0.41	
6.131	2-amino-6-(2-thiazolyl)purine[e]	297 (8.8), 359 (9.6)	461	0.46	
6.132	5-methyl-2-pyrimidinone (m^5K)	280	400		4.09[f]
6.133	pyrrolo-dC (pC)[e]	350 (5.9)[d]	460	0.2	
6.138	5-(fur-2-yl)dU	316 (11.0)	431	0.03	1.0
6.139	5-(fur-2-yl)U	254 (12.3), 316	440	0.035	
6.140	5-(thiophen-2-yl)dU	314 (9.0)	434	0.01	
6.141	5-(thiophen-2-yl)U	315 (8.7)	439	0.024	
6.142	5-(oxazol-2-yl)dU	296 (10.0)	400	<0.01	
6.143	5-(thiazol-2-yl)dU	316 (11.5)	404	<0.01	
6.144	5-(fur-2-yl)dC	310 (5.0)	443	0.02	
6.145	8-(fur-2-yl)A	304 (18.0)	374	0.69	
6.146	8-(fur-2-yl)G	294 (16.0)	378	0.57	
6.147	2′-deoxyisoinosine[g]	242 (2.9), 314 (4.6),	382		
6.148	phenyl-UDP-Glc	278	403		
6.149	4-methoxyphenyl-UDP-Glc	278	444		
6.150	4-chlorophenyl-UDP-Glc	281	398		
6.151	2-furyl-UDP-Glc	314	437		
6.152	8-(benzyltriazol-4-yl)A[h]	290 (16.0)	344	0.64	
6.153	8-(phenylethyltriazol-4-yl)A[h]	289 (21.0)	342	0.63	
6.154	8-(pyridin-4-ylmethyltriazol-4-yl)A[h]	289 (16.0)	346	0.49	
6.155	8-(isopentyltriazol-4-yl)A[h]	289 (18.0)	343	0.62	
6.156	8-(pentyltriazol-4-yl)A[h]	289 (17.0)	342	0.62	
6.157	8-(3-aminophenyltriazol-4-yl)A[h]	296 (24.0)	402	0.38	
6.158	8-(4-methoxyphenyltriazol-4-yl)A[h]	294 (23.0)	370	0.03	
6.159	8-(4-tolyltriazol-4-yl)A[h]	294 (21.0)	368	0.05	
6.160	8-(4-chlorophenyltriazol-4-yl)A[h]	294 (21.0)	396	0.05	
6.161	*N*-thieno[3,2-*d*]-dR[i]	293	350	0.02	
6.162	*N*-thieno[3,2-*d*]-R	292	351	0.058	
6.163	4-thieno[3,2-*d*]-dR	294	351	0.037	
6.164	thieno[3,4-*d*]-U	304 (3.65)	412	0.48	
6.165	^{BT}U[j]	333, 344	383	0.48	
6.166	5-aza-7-deazapurine-2′-deoxyriboside[k]	250	410, 500		
6.167	quinazolinedione	307[l] (2.34)	370	0.31	
		306[m] (2.07)	354		
6.168	5-MeO-quinazolinedione	320[l] (6.27)	395	0.16	
		314[m] (6.13)	362		
6.169	3-MeO-quinazolinedione	298[l] (6.33)	356	0.08	
		297[m] (5.00), 306[m] (4.82)	335		

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively. [b] For ε_{282}.[542] [c] For ε_{318}.[543] [d] Spectra were collected in HEPES buffer pH = 7.5. [e] Spectra were collected in HEPES buffer pH = 7.0. [f] For singly modified oligonucleotides; stated to be similar to the free nucleoside.[544] [g] Data from Seela and Chen.[545] [h] Data from Grotli et al.[546] [i] Data from Seaman.[547] [j] Data was collected in MeOH.[548] [k] Data was collected in MeCN.[549] [l] Data was collected in phosphate buffer (pH = 7.0). [m] Data was collected in dioxane.

Tor and co-workers have developed a series of isomorphic fluorescent T/C analogs by conjugating aromatic five-membered heterocycles at the pyrimidine's 5-position (**6.138**−**6.144**, Figure 6.8).[589−591] The absorption spectra of each modified nucleoside reveal, in addition to the typical pyrimidine transitions, a lower energy absorption band (∼310 nm) that remains practically unchanged upon changing solvent polarity. In contrast, the emission profile of the conjugated nucleosides is much more sensitive to the chromophore's microenvironment, resulting in both bathochromic and hyperchromic shifts upon increasing solvent polarity.[589−591] These nucleosides have very large Stokes shifts (8400−9700 cm^{-1}) for such small organic chromophores, while their quantum efficiency is relatively low ($\Phi = 0.01-0.035$).[589−591] The responsive furan and thiophene analogs (**6.138** and **6.140**, respectively), having the most desirable properties in terms of emission wavelength, quantum yield, and sensitivity to microenvironmental polarity, were selected as probes (section 6.8). These nucleosides can be incorporated into oligonucleotides using either solid-phase or enzymatic syntheses,[592−594] causing no destabilization of the resulting oligonucleotides.[589−591,595] Their simple synthesis and useful properties have facilitated diverse applications, including the detection of abasic sites and the monitoring of RNA−ligand interactions (section 6.8).[11,589,591,595,596]

Incorporating a furan ring at the 8-position of dG and dA resulted in highly emissive nucleosides with remarkably different properties when compared with their pyrimidine cousins.[590] The purine analogs (**6.145** and **6.146**) lack a separate absorption band but, instead, display one major red-shifted transition around ∼300 nm, which is largely unaffected by changes in solvent polarity.[590] In contrast to the corresponding modified pyrimidine analogs, the substituted purines display a very strong emission centered around 375 nm ($\Phi = 0.69$ and 0.57 for **6.145** and **6.146**, respectively),[590] which displays limited susceptibility to changes in solvent polarity.

Tor and co-workers have also investigated fused thiophene derivatives (**6.161**−**6.164**), where the fusion position of the thiophene ring shows to have a striking effect on the photophysical properties of the resulting analogs.[597,598] The [3,2] isomeric nucleosides, prepared as both a pyrimidine and purine analog (*N*-nucleoside **6.161/162** and *C*-nucleoside **6.163**, respectively), show an absorption at ∼290 nm with a corresponding weak emission centered at ∼350 nm ($\Phi = 0.02-0.058$).[598]

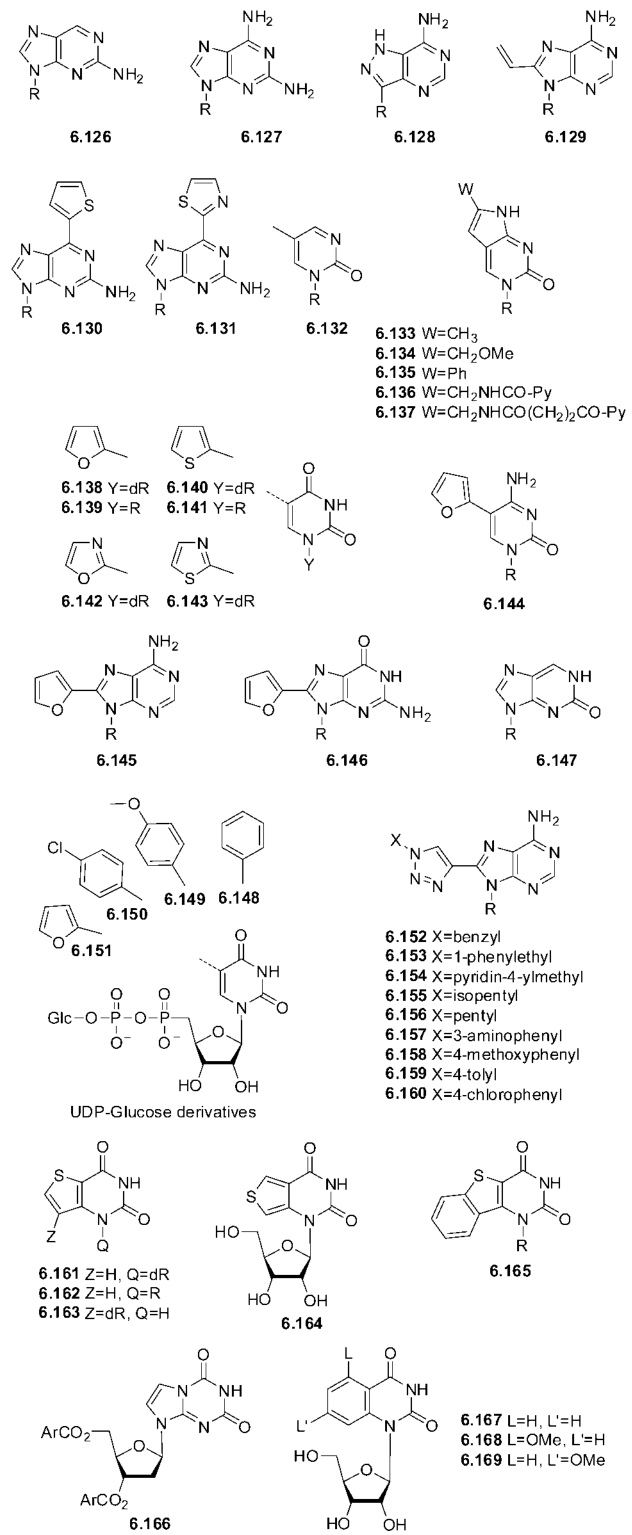
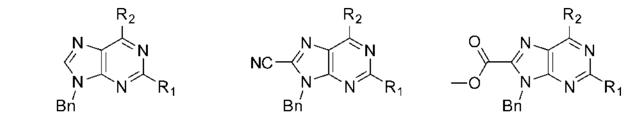
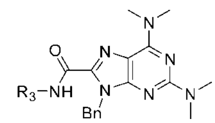


Figure 6.8. Examples of isomorphic nucleobase analogs, where R = 2′-deoxyribose or ribose.

In contrast, the isomeric [3,4] analog (**6.164**), while displaying a similar absorption band (304 nm), gives rise to a strong and red-shifted visible emission (412 nm, $\Phi = 0.48$).[597] The most important property of these fused analogs is the sensitivity of their photophysical parameters to polarity. When incorporated into oligonucleotides and hybridized to perfect complements, the highly responsive analog **6.164** displayed significant emission quenching. In contrast, substantial fluorescence enhancement was observed when the modified oligonucleotide was hybridized to complementary



	R_1=	R_2=		R_1=	R_2=		R_1=	R_2=
6.170	NH_2	H	**6.177**	NH_2	H	**6.184**	NH_2	H
6.171	$NHCH_3$	H	**6.178**	$NHCH_3$	H	**6.185**	$NHCH_3$	H
6.172	$N(CH_3)_2$	H	**6.179**	$N(CH_3)_2$	H	**6.186**	$N(CH_3)_2$	H
6.173	NH_2	OBn	**6.180**	NH_2	OBn	**6.187**	NH_2	OBn
6.174	NH_2	$N(CH_3)_2$	**6.181**	NH_2	$N(CH_3)_2$	**6.188**	NH_2	$N(CH_3)_2$
6.175	$N(CH_3)_2$	NH_2	**6.182**	$N(CH_3)_2$	NH_2	**6.189**	$N(CH_3)_2$	NH_2
6.176	$N(CH_3)_2$	$N(CH_3)_2$	**6.183**	$N(CH_3)_2$	$N(CH_3)_2$	**6.190**	$N(CH_3)_2$	$N(CH_3)_2$



R_3=
6.191 $(CH_2)_3CH_3$
6.192 $CH_2CO_2CH_3$

Figure 6.9. Examples of isomorphic nucleobase analogs.

oligonucleotides that contain an abasic site opposite the reporter. This key observation inspired the development of a new fluorescence-based approach for monitoring the depurination activity of toxic ribosome inactivating proteins (see section 6.8).[599]

A series of expanded isomorphic U analogs based on a quinazoline-2,4(1*H*,3*H*)-dione core (**6.167**) has recently been introduced by Tor and co-workers (Figure 6.8, Table 6.8).[600] Among the analogs **6.167**−**6.169**, the 5-methoxyquinazoline-2,4(1*H*,3*H*)-dione derivative (**6.168**, $\Phi = 0.16$) was found to be an ideal FRET donor for chromophores derived from 7-diethylaminecoumarin-3-carboxylic acid (with a critical Förster radius, R_0, of 27 Å). The pair was used to devise a robust analysis and discovery platform for antibiotics targeting the bacterial rRNA A-site, by placing the new emissive U surrogate into the RNA construct and labeling the aminoglycosides with the coumarin chromophore.[600]

PyrroloC (pC, **6.133**), an emissive C analog, was originally discovered following ammonolysis of furo[2,3-*d*]pyrimidinone, a side product in the Pd-mediated Sonogashira coupling reactions of terminal alkynes with 5-iodo-U.[601−603] Initial investigations were focused on the biological activity of the furo and pyrrolo nucleosides analogs.[604,605] Once the fluorescent properties of pC were recognized, its potential utility as a fluorescent probe became clear.[20,606] PyrroloC's low-energy absorption (350 nm) is considerably isolated from that of the native nucleobases. Its visible emission (460 nm) is reasonably intense (estimated as $\Phi \sim 0.2$),[607] although it appears to be significantly quenched upon incorporation into single-stranded oligonucleotides and further quenched upon duplex formation.[20,607] These favorable photophysical properties, along with its minimally perturbing structure, have resulted in a variety of applications (section 6.8). Modified pC analogs have also been developed and implemented in recent years.[608−611]

Wagner and co-workers have recently developed a series of emissive 5-substituted UDP glucose analogs (**6.148**−**6.151**), whose design was inspired by Tor and co-workers' original reports.[612] Their method relies on the conversion of the U nucleus to an emissive analog at a late stage of the synthesis, thereby facilitating the preparation of a number of analogs for diverse applications (Figure 6.8). A range of absorption (278−314 nm) and emission (403−444 nm) maxima can be obtained by varying the 5-aryl moiety (Table 6.8). The fluorescent properties of small isomorphic analogs, such as those containing the 2-furyl moiety are unaffected by substituents attached to the 5′ hydroxyl (Table 6.8, **6.139** vs

Table 6.9. Spectroscopic Properties of Isomorphic Nucleoside Analogs in Dichloromethane[a]

compd no.	name	λ_{max} (ε)	λ_{em}	Φ	τ
6.170	2-amino	304 (7.9)	357	0.20	
6.171	2-methylamino	317 (6.3)	386	0.25	
6.172	2-dimethylamino	330 (6.3)	393	0.76	
6.173	2-amino-6-*O*-benzyl	281 (10.0)	360	0.033	
6.174	2-amino-6-dimethylamino	286 (12.6)	351	0.013	
6.175	2-dimethylamino-6-amino	299 (7.9)	360	0.12	
6.176	2,6-tetramethylamino	297 (12.6)	392	0.033	
6.177	2-amino-8-carbonitrile	326 (1.6)	371	0.20	0.5
6.178	2-methylamino-8-carbonitrile	341 (6.3)	383	0.58	
6.179	2-dimethylamino-8-carbonitrile	361 (15.8)	429	0.90	4.25
6.180	2-amino-6-*O*-benzyl-8-carbonitrile	311 (20.0)	355	0.81	3.1
6.181	2-amino-6-dimethylamino-8-carbonitrile	324 (15.8)	375	0.30	1.6
6.182	2-dimethylamino-6-amino-8-carbonitrile	336 (12.6)	387	>0.95	3.2
6.183	2,6-tetramethylamino-8-carbonitrile	348 (25.1)	388	0.20	
6.184	2-amino-8-carboxylate	328 (12.6)	379	0.42	1.8
6.185	2-methylamino-8-carboxylate	345 (1.6)	403	0.65	
6.186	2-dimethylamino-8-carboxylate	362 (12.6)	433	0.81	3.37
6.187	2-amino-6-*O*-benzyl-8-carboxylate	315 (20.0)	371	>0.95	2.5
6.188	2-amino-6-dimethylamino-8-carboxylate	330 (15.8)	393	>0.95	3.1
6.189	2-dimethylamino-6-amino-8-carboxylate	338 (12.6)	409	>0.95	3.3
6.190	2,6-tetramethylamino-8-carboxylate	351 (20.0)	409	0.90	2.67
6.191	2,6-tetramethylamino-8-butylcarboxamide	338 (15.8)	402	0.87	2.27
6.192	2,6-tetramethylamino-8-butylcarboxamide	343 (20.0)	407	0.91	

[a] λ, ε, and τ are given in nm, 10^3 M^{-1} cm^{-1}, and ns, respectively.

6.151). These isomorphic analogs are currently being used to develop assays for monitoring glycosyltransferase activity.[612]

Lastly, we discuss a series of nucleobase analogs that, although they have not been incorporated into oligonucleotides, show intriguing properties. Castellano and co-workers have used the purine nucleus as a scaffold for the placement of diverse donor and acceptor groups, generating "push−pull" purines (Figure 6.9).[613,614] The basic design consists of attaching amino, methylamino, and dimethylamino donor moieties to the 2 and 6 positions, while placing cyano, methyl ester, and carboxyamide acceptor groups at the 8 position. These substituted purines show certain desirable photophysical properties when compared with their acceptor-free analogs, including red-shifted absorption maximum (20−50 nm), enhanced quantum efficiency (approaching unity), and solvatochromatic effects (Table 6.9). While such analogs might be too perturbing for incorporation into nucleic acids, they may find utility in material sciences and biosensing applications.[613,614]

6.7. Incorporation of Modified Nucleosides into Oligonucleotides

A number of approaches, including solid-phase assisted synthesis, enzymatic incorporation, and postsynthetic modification are available to arrive at fluorescent oligonucleotides.[615−617] The first two approaches are most relevant to the fluorescent nucleoside probes discussed herein.

Most popular and versatile is the incorporation of modified nucleosides into oligonucleotides using solid-phase-assisted synthesis, which facilitates modification of oligonucleotides at virtually any position in any sequence. The predominantly employed techniques rely on a 3′-phosphoramidite group for the introduction of the phosphate group in both DNA and RNA oligonucleotides.[618−620] For DNA, the standard 5′-dimethoxytrityl (DMTr) group and acyl or *N*,*N*-dimethylformamidine[621] protection of the exocyclic amines is typically utilized. RNA oligonucleotide synthesis requires additional protection of the 2′-hydroxyl group. Two "modern" approaches are available: (i) the 5′-*O*-DMTr-2′-*O*-[(trisisopropylsilyl)-oxy]methyl (2′-*O*-TOM) protecting scheme developed by Pitsch[622] and (ii) the 5′-*O*-silyl-2′-*O*-orthoester (2′-ACE) protecting group scheme, introduced by Scaringe and Caruthers.[623,624] The cycle employed in the standard solid-phase phosphoramidite chemistry is shown in Figure 6.10. Key steps include (1) acidic removal of the 5′-DMTr protecting group of the solid-supported nucleoside, (2) mild acidic activation of the phosphoramidite (e.g., using 1*H*-tetrazole), (3) coupling of the available 5′-hydroxyl to the activated phosphoramidite, (4) oxidation of the trivalent phosphorus to the pentavalent phosphorus, and (5) capping of unreacted hydroxyl groups. This cycle continues until termination and removal of the full-length oligonucleotide from the solid support, typically under basic/nucleophilic conditions (e.g., ammonium hydroxide).

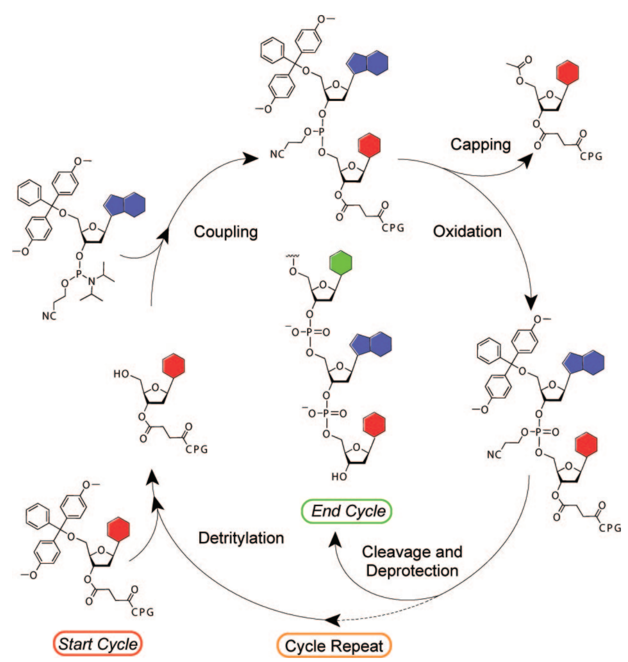


Figure 6.10. Common cycle for solid-phase-assisted phosphoramidite oligonucleotide synthesis.

Alternatively, enzymatic incorporation of fluorescent nucleotides can, in certain cases, be utilized. Unlike the almost universal solid-phase-assisted synthesis, enzymatic incorporation has to be evaluated on a case by case basis because DNA and RNA polymerases of different origin display diverse tolerance levels to unnatural nucleotides.[483,594] Conversion of the modified nucleosides into the corresponding triphosphate is, of course, necessary for implementation of this approach.[575,591,593,625−627] Fortunately, the synthesis of many triphosphates does not require any protection of the exocyclic groups and is performed in a one-pot/two-step procedure following established methods.[628−630] Additionally, enzymatic incorporation of fluorescent nucleotides has been achieved through non-natural base pairs,[569−575,631−635] the use of terminal transferases,[636,637] or a reactive amino group on the recently incorporated nucleoside able to undergo conjugation with an appropriately selected tag.[638,639]

6.8. Applications of Fluorescent Nucleosides

Fluorescent nucleosides have greatly contributed to our still growing understanding of nucleic acid folding, structure, recognition, and function. While numerous reviews and overview articles have previously appeared,[427,455,456,467,491,640−645] capturing over 40 years of fluorescent nucleoside research is clearly a daunting and almost impossible task. We have therefore classified below the main areas where fluorescent nucleoside analogs have found utility. Table 6.10 lists the various nucleoside analogs developed over the years and their main applications. It is worth noting that "classical" emissive nucleosides, such as 2-AP (**6.126**), reported four decades ago by Reich and Stryer, are still finding vast utility in contemporary biophysics, while others, such as benzo-A (**6.27**), originally reported by Leonard, are finding new applications distinctly different from their original use.[469,470]

A. **Single nucleotide polymorphism (SNP) detection.** The detection of single base substitutions located either within or outside a gene has attracted attention due to their relevance to human health and ultimately for the development of personalized medicine.[516,646−650] To identify SNPs, emissive oligonucleotides, complementary to the domain of interest, are hybridized to their target DNA. The fluorescent nucleoside probes are typically placed across from the base of interest, yielding, under ideal circumstances, markedly different signals depending upon their pairing partner. Saito and co-workers have named such fluorescent nucleosides, base discriminating fluorescent (BDF) nucleosides.[494,651]

B. **Nucleic acid structure and function.** Nucleic acids can be found in different forms, aggregated states, and polymorphs, which could be related to a specific cellular function.[652,653] Appropriately selected fluorescent nucleosides, strategically placed within the nucleic acid strands, can photophysically signal hybridization, folding, and conformational changes. Additionally, fluorescent nucleoside analogs have been used to fabricate assays that monitor enzymes operating on nucleic acids (e.g., polymerases) or nucleic acid-based enzymes (e.g., ribozymes).

C. **Nucleic acid microenvironment.** Nucleic acids experience a variety of reversible and irreversible perturbations, which may include nucleobase damage, depurination/depyrimidination events, or base flipping. Fluorescent nucleoside analogs that are sensitive to their local microenvironment have become powerful tools for investigating these perturbations. In addition, emissive fluorescent nucleosides have been employed to assess the polarity of nucleic acid grooves.

D. **Ligand binding.** Responsive fluorescent nucleoside analogs find utility as reporters in diverse DNA and RNA discovery or biophysical assays, particularly for monitoring ligand binding (both high and low molecular weight). Special attention is given to isomorphic/isosteric nucleosides, which are unlikely to perturb the native fold and recognition patterns characteristics of the target of interest.

E. **Miscellaneous.** Applications that do not fall into the above categories are grouped here. When possible, a footnote is added to the table to list the specific utilization of the probe.

7. Epilogue

Biochemists and biophysicists have long relied on fluorescence-based techniques to explore the fundamental structural, folding, recognition, and reactivity characteristics of biomolecules, as well as their cellular localization and dynamics. While this review encompasses fluorescent analogs of all major biomolecular building blocks (lipids, monosaccharides, amino acids, and nucleosides), it is apparent that distinct approaches have to be implemented for each category. This ultimately reflects the fundamental burden of their chemical structure and the tolerance level of the relevant biological context. In an ideal situation, an emissive analog of any naturally occurring biomolecular building block should closely resemble its natural counterpart and retain the original function (analogs we refer to as isosteric or isomorphic). Because most parent, naturally occurring molecules (excluding a few amino acids) do not display appreciably useful fluorescence properties, structural alteration is necessary to impart such features. This predicament ultimately leads to fluorescent analogs of diverse range in utility and applications, some having a rather limited scope.

As apparent from this review, not all applications require the strict imposition of isomorphic design criteria. Furthermore, the different chemical natures of the distinct families of biomolecular building blocks inherently control the possible structural and electronic changes. As evident from the size of the last section discussing fluorescent nucleoside analogs, the heterocyclic nucleobases provide a fertile platform for modifications that easily alter the photophysical characteristics. This also holds true for certain aromatic amino acids. In contrast, turning phospholipids or monosaccharides into emissive analogs requires rather creative and sometimes drastic modifications, with saccharides being viewed as the most limiting in this respect.

Inspiration for emissive analogs had come, in many cases, from Nature. In other cases, rational approaches have been implemented, the simplest being the conjugation of established fluorophores to the biomolecular core. When the data summarized here and the challenges associated with designer fluorophores and their implementation are viewed from a fundamental physical organic perspective, it is apparent that predicting the emissive properties of small organic molecules based on their structure is, at this stage, unrealistic. Probe design and implementation remain, for the most part, an empirical task. Very few chromophores have enjoyed a systematic and thorough exploration of their properties by experimentalists, as well as theoreticians. Even 2-AP, one of the most commonly used and investigated isomorphic fluorescent nucleosides, does not always function optimally within oligonucleotides. This highlights certain fundamental challenges in this field, because the photophysical properties of any emissive biomolecular building block are further

Table 6.10. Fluorescent Nucleosides and Some of Their Typical Applications

compd no.	probe name	application	remarks
		Chromophoric Base Analogs	
6.9	coumarin		time resolved Stokes shift:
		C[428]	helix interior
		C[446]	sequence dependence
		C[447,448]	counterions
		C[450]	structural changes/probe position
		C[444,445,451]	probe position
		B[449]	endonuclease APE1
6.14	M	B[654]	denaturation
6.15	O	B[654]	denaturation
6.20	Nile red nucleoside	E[431]	influence of β-cyclodextrin on emission maximum
		Pteridines	
6.21	3-MI	B[456,457]	HIV-1 integrase activity
		B/C[456,655]	DNA−HU interactions
		B/C[456]	interaction of DNA with RNA polymerase
		D[656]	aminoglycoside/RNA binding
		B[456,643]	detection of complementary strand
		C[456]	alkyl transferase
		E[456,643]	cellular uptake of oligonucleotides
6.22	6-MI	B[657]	RecA−DNA interactions
		B/C[456]	interaction of DNA with RNA polymerase
		D[656]	monitoring aminoglycoside/RNA binding
6.24	6-MAP	C[456,658]	investigating A-tracts
		Expanded Nucleobases	
6.25	etheno-A	B[657]	RecA−DNA interactions
6.27	benzo-A	C[470]	base pairing/stacking effects
6.28	BgQ	B[476,477]	triplex formation
		D[659]	Tat−TAR binding
6.29	C_f	B[476,477]	triplex formation
		D[660]	bleomycin analogs cleavage of DNA
6.30	tC	B[483,485]	Klenow fragment DNA polymerase
		B[661]	structural measurements on DNA using FRET
6.31	tC^{o}	B[483]	Klenow fragment DNA polymerase
		B[661]	structural measurements on DNA using FRET
6.32	Ç^{f}	A[662]	discrimination of all four bases
6.33	8-oxoG-Clamp	C[488−490]	detection of 8-oxoG (free nucleoside and incorporated into an oligonucleotide)
6.34	BPP	A[492]	A vs G detection
6.35	NPP	A[493,663]	A vs G detection
6.36	^{MD}A	A[494]	T vs C detection
6.37	^{MD}I	A[494]	T vs C detection
6.38	dC^{hpp}	A[664]	A vs G detection
6.42	dC^{ppi}	A[665]	A vs G detection
		Extended Nucleobases	
6.54	^{PY}U	A[666]	A vs C detection
		B[667]	RNA poly(A) tracts
		C[668]	detection of T•Hg•T base pairs
6.55	^{PY}C	A[666]	G vs T detection[a]
		B[669]	B to Z transition
6.56	DMAP-^{PY}U	B[514]	dual emission
6.57	5-(1-pyrenyl)-dU	B[670]	multiple consecutive modified nucleosides
6.58	8-(1-pyrenyl)-dG	A/B[422]	mismatch vs perfect complement; detection/ charge transfer studies
6.60	U^{P}	B[671]	B to Z transition
		B[672]	molecular beacons
		B[673]	probe base pairing interactions
		C[510,674,675]	charge transfer studies
		C[676]	HIV TAR RNA base conformations
		B[677]	hybridization detection
		A[678]	mismatch vs perfect complement detection
		B[679]	multiple consecutive modified nucleosides
		A[680]	A vs C/G/T detection
		C[681]	ultrafast structural dynamics
		B[682]	duplex stability
		A[683]	mismatch detection
		E[684]	white-light-emitting DNA
6.61	C^{P}	B[685]	molecular beacon/thermal denaturation
6.62	A^{P}	B[671]	B to Z transition
		B[672,686]	molecular beacons
		B[673]	probe base pairing interactions
		B[687,688]	G-quadruplex transition
		A[680]	A vs C/G/T detection
		A[689]	homoadenine signaling system for SNP typing

Table 6.10 Continued

compd no.	probe name	application	remarks
		B[690]	photophysics of modified poly-A oligonucleotides
6.63	G^{P}	B[685]	molecular beacon/thermal denaturation
		B[669]	B to Z transition
6.64	A^{PY}	B[691]	RNA hybridization
6.65	U^{PE}	C[675,692]	charge transfer studies
6.69	5-(perylen-3-ylethynyl)dU	B[682]	duplex stability
		A[683]	mismatch detection
6.70	dA^{pymcm}	A and B[693]	mismatch vs perfect complement detection
			hanging vs blunt ends detection
6.71	^{MMe}U	B[497]	hybridization detection
6.72	^{Ph}U	B[497]	hybridization detection
6.73	$^{MeOPh}U$	B[497]	hybridization detection
6.74	^{PY}A	A[694]	C vs T detection
6.77	^{DNC}U	B/C[695]	polarity inside DNA-binding protein (KF)
6.78	^{PDN}U	A[499]	mismatch vs perfect complement detection
6.79	^{PDN}C	A[499]	mismatch vs perfect complement detection
6.80	^{PDN}A	A[499]	mismatch vs perfect complement detection
6.81	^{PDN}G	A[499]	mismatch vs perfect complement detection
6.82	^{dan}C	B/C[500]	groove polarity (minor and major of A/B DNA)
		B/C[696]	groove polarity (minor and major of Z-DNA)
6.83	^{dan}G	B/C[500]	groove polarity (minor and major of A/B DNA)
		B/C[696]	groove polarity (minor and major of Z-DNA
6.84	5-aminodansyl-dU	B/C[501,697,698]	groove polarity (major/minor interactions and sequence dependence)
6.85	2-anthracenecarboxamide-dU	A[699]	mismatch vs perfect complement detection
6.86	9-anthracenecarboxamide-dU	A[699]	mismatch vs perfect complement detection
6.87	NeT	A/B[700]	A vs G detection/hybridization detection
6.88	AeT	A/B[700]	A vs G detection/hybridization detection
6.89	AeeT	A/B[700]	A vs G detection/hybridization detection
6.90	U^{FL}	A[502]	mismatch vs perfect complement detection
6.99.c	5-[3(phen)ethynyl]dU	A[531]	discrimination of all four bases
6.101.g	5-[5-Ru(bpy)$_3$ethynyl]dU-TP	A[538]	luminescent and electrochemical detection
6.107.g	5-[5-Ru(bpy)$_3$ethynyl]dC-TP	A[538]	luminescent and electrochemical detection
6.114.g	7-[7-deaza-5-Ru(bpy)$_3$-ethynyl]dG-TP	A[538]	luminescent and electrochemical detection
6.119.g	7-[7-deaza-5-Ru(bpy)$_3$-ethynyl]dA-TP	A[538]	luminescent and electrochemical detection
		Isomorphic Nucleobases	
6.126	2-AP	B[701]	molecular beacon
		B[702]	duplex denaturation
		B[703]	helicase activity
		B[704]	hammerhead ribozyme
		B[705]	DNA polymerase fidelity *in vitro*
		B[657]	RecA−DNA interactions
		B[706]	HIV-I DIS RNA structure
		B/D[707]	G-quadruplex structure and ligand binding
		C[708]	local conformation via CD
		C[702,709]	base flipping
		C[710−712]	abasic site structure/dynamics
		C[713]	strength of BP interactions
		D[656,714−717]	aminoglycoside/RNA binding
		B/C[718−720]	interaction of DNA with RNA polymerase
6.129	8-vinyl-6-aminopurine	A[721]	short/long excited state lifetimes
6.130	2-amino-6-(2-thienyl)purine	B[571]	detection of fluorescently labeled complementary strand
		B[569]	denaturation of RNA (hairpin and tRNA)
6.131	2-amino-6-(2-thiazolyl)purine	B[571]	detection of fluorescently labeled complementary strand
6.132	m^5K	B[657,722]	ssDNA−*Escherichia coli* RecA binding
6.133	pC	A[609]	mismatch vs perfect complement detection
		B[20]	duplex denaturation
		B[701]	molecular beacon
		B[608]	hybridization detection
		C[723]	local conformation via CD
		C/D[724]	metal ion biosensors
		B[725]	DNA−reverse transcriptase binding
		B[607,726]	T-7 RNA polymerase
		C[727]	base flipping
		B[728]	tRNA conformations
6.134	^{MMe}pC	B[608]	hybridization detection
6.135	^{Ph}pC	A[609]	mismatch vs perfect complement detection
6.136	^{PyI}pC	B[729]	molecular beacon
6.137	^{PyII}pC	B[729]	molecular beacon
6.138	5-(fur-2-yl)dU	C[589]	abasic site detection
		B/C[11]	major groove polarity
6.139	5-(fur-2-yl)U	D[595]	Tat/TAR binding
		D[591]	aminoglycoside/A-site binding
6.144	5-(fur-2-yl)dC	C[596]	detection of 8-oxoG and its transverse mutation product
6.164	thieno[3,4-*d*]-U	A[597]	C mismatch detection
		B/C[599]	activity of toxic ribosome-inactivating protiens

Table 6.10 Continued

compd no.	probe name	application	remarks
6.165	^{BT}U	B^{548}	hybridization detection
6.168	5-MeO-quinazolinedione	D^{600}	FRET-enabled detection of aminglycoside−RNA binding

[a] Amide moiety is vital to probe sensitivity.

impacted when embedded within macromolecules by rigidification, desolvation, and excited-state processes involving neighboring chromophores.

We do not end, however, on a pessimistic note. Rather, we view these challenges as stimulating and past accomplishments as a celebration of creativity. We note that this rather extensive review reflects multiple decennia of development. The numerous publications are testimony to the power of fluorescent spectroscopy in unraveling the intricacies of biological macromolecular structures themselves and their interaction with their complex environment. The great number of recent articles cited indicates that the field is blooming, and many more advancements are to be expected. The future of this colorful field is clearly bright! New ingenious probes, reflecting seemingly endless creativity, will always be embraced by chemists, biologists, and biophysicists.

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