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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 article 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 semi-permeable 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 derivatives. 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, as 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,

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employing fluorescence spectroscopy to membrane research requires very creative probe designs. Saccharides can be viewed as the most restrictive in this context, as 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 read-out. The isomorphic design principle is therefore a central theme of this review.

This article 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 article 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} sec). 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} sec) 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 to its excitation energy (Stokes shift). Typical organic chromophores reside in their excited state for a period of 0.5–20×10^{-9} seconds. The excited state lifetime reflects the sum of the various radiative and non-radiative processes 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 ($\varepsilon \times \Phi$) 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.

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.), make 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]

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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 to Lakowicz's comprehensive monograph entitled the '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 to dielectric constants or orientational polarizability values (Figure 2.2).[13]

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 non-fluorescent 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 could be complex.[18]

A more sophisticated, yet related, phenomenon involves resonance energy transfer (RET), a non-radiative 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 ($k_{ET} \sim r^{-6}$) contributes to

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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 trans-membrane 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. As 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 representing 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 down. As a result, the excited state remains partially aligned and its emission 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 requiring probes with extended lifetimes (up to 10^{-6} sec).

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] and protein–protein

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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, two- or multi-photon fluorescence microscopy.[34–38] Single molecule spectroscopy has proven very useful, and combinations of these techniques have been extensively used in the study on membranes, proteins and nucleic acids.[17,39–42] Although beyond the scope of this article, 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 paragraph 5.2 of this review.

2.7. In Vivo Fluorescence-Based Imaging

Non-fluorescence-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 ultra-violet 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, 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.

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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 hemi-ketal) can generate either a 5-membered ring (furanose) or a 6-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 enzymaticaly 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, 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.

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 non-covalent, 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 5- or 6-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] 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]

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-

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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 as 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 irreversable (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]

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]

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 architetcture, 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) head-group, 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 is subject to change with age.[119] Both phospholipids have a similar polar head-group, 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 head-group. 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 to sphingomyelin. This structural distinction gives rise to a difference in the net dipole and ability to form hydrogen bonds, which ultimately impacts the the constitution and dynamics of lipid bilayers.[120–123]

The lamellar phase or lipid bilayer with a head to head distance of 35 and 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, 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 superlattices129 or lipid rafts,[131] has been described. The superlattice model proposes a regular, rather than random, distribution of membrane components, formed by favorable lipid packing, where steric and columbic 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.

Besides affecting the cell's membrane constitution, the type and ratio of its building blocks also determines its interaction with extra cellular 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] Biological include membranes, however, are much more complex by nature since their make up includes a divers constituents.

This section discuses 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 non-covalent 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.

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4.2. Non-Covalent Fluorescent Membrane Probes

The term non-covalent is somewhat ambiguous in this context since membranes themselves are non-covalent 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 1 lists their key photophysical parameters.

An example of an extensively studied non-covalent probe is diphenylhexatriene (DPH) (**4.1**), 120,144,145 which resides in the non-polar 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 phosphatidylcholines 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 ($\varepsilon = 10.4–12.3$ 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 non-covalent 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 non-viscous environments is ascribed to rotational relaxation, a dominating non-radiative 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] 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 head-group and the tail end of a phospholipid (Sections 4.3 and 4.4).[164,167]

The main advantage of employing non-covalent 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

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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 head-groups, at the end of the chain or within the hydrophobic chain.

4.3. Polar Head-Group 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 membrane-spanning bolaamphiphile fixates the fluorophore at the water–lipid interface. Two fundamental designs have been explored: (a) Labeling the head group with a fluorophore or replacing the head group with a charged fluorophore, and (b) utilizing a long hydrocarbon to connect two fluorescent residues. The two distinct approaches are exemplified with octadecyl naphtylamine 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 2 summarizes the spectroscopic properties of the corresponding fluorophores.

Although fatty acids functionalized with fluorescent probes have been reported, their phospholipids-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 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 non-emissive 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 head-group 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 to '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, ensuring a sufficient emission quantum yield. Not surprisingly, modifications of the 'non-covalent' probes discussed above with an apolar chain and polar head group generates many of these probes. Figure 4.5 depicts key examples, and Table 3 lists their primary spectroscopic characteristics.

Modifying DPH with a trimethylammonium head group 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 $M^{-1}cm^{-1}$) at 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 parenthesis 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 4.6 presents selected examples, and Table 4 provides primary spectroscopic characteristics for the chromophores most commonly used in this catagory.

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 (exdhPhenanthrene, **4.30**).[198] Extending the conjugation of the central polyaromatics tends to impart favorable photphysical 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 head-groups 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

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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 location.

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 5 summarizes the photophysical characteristics of the key chromophores discussed in this section. Note, the spectroscopy of polyenes, being "classical" chromophores, dates back to the 1930's,[207–211] and is discussed in later reviews as well.[28,212]

Designer polyenes originate form 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 natural 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 70's.[218]

Extending the conjugation by a single double bond gives all *trans*-penteanoic acid (*trans*-PA, **4.37**).[219] This polyene was 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 to membrane perturbing NBD and BODIPY tags.[220] To minimize mobility, implementation of the bolaamphiphile design principle gave all *trans*-penteanoic diacid (*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]

In spite of 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*-penteanoic acid (*trans*-PA, **4.37**) exhibits additional lower energy transitions and a fluorescence maximum around 470 nm.[219] This illustrates the tuneability 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 a clearly impossible. The brief discussion below and Table 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) and/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 familiar 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 are comprised of 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 proteins 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 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, is not only 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 labels141,[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

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clear if 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.

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 twenty 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 posttranslational modifications (e.g., 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 review articles have discussed non-canonical amino acids[292] and their fluorescent counterparts in particular.[293,294] Compared to 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 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).

About thirty 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 to 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 7).[43,299] Examples of engineered fluorescent proteins include

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topaz (**5.3**), a yellow fluorescent protein (YFP), whose emissive properties are attributed to a deprotonated tyrosine involved in π-π 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 non-invasive and provides the prospect for whole-body scale studies.306 The development of (near) infrared-fluorescent proteins (IFP's) 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 ($\varepsilon > 90\ M^{-1}\ cm^{-1}$) 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 X3Cys2X2Cys2X3 motif, facilitates *in vivo* labeling by exposing the cells to the permeable and non-emissive 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 cys 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 to GFP fusion, and has become popular in the last 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 8). 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 8). While it lacks significant sensitivity to its environmental polarity, its photophysics is pH dependent due to its acidic side chain (pK_a ~10). Deprotonation of the phenolic hydroxyl group results in a bathochromic shift of the emission maximum from 310 to 340 nm.

Tryptophan is by far a more favorable probe than phenylalanine or tyrosine, as it benefits from higher brightness ($\varepsilon \times \Phi_F$).[319] Due to the large dipole moment of its excited state, tryptophan's

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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, heme groups, and coenzymes, can all 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 review articles.[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 facing 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 to tryptophan and other tryptophan mimics (Table 9).[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, benzofuranyl alanine (BfAla, **5.13**)[333] and benzothiophenyl (BtAla, **5.14**),[334] only differ from tryptophan in their ring heteroatom.[335–337] Tirrell and coworkers 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 review articles.[293,294,327,339]

5.4.2. Side Chain Modification With Heterocyclic Chromophores—Attaching an established fluorophore to a side chain of a non-emissive amino acid such as Ala is a rational approach for the generation of fluorescent amino acids (Figure 5.4). Examples include 7-methoxy-coumarine 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] 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 fotodurability.[346,347] Interestingly, most of these probes have found limited use in exploring

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natural systems, but have been used in studying synthetic photoactive polypeptides (Tables 9 and 10).[348,349]

In contrast to the aforementioned fluorophores, some probes undergo chemical modification in the probing process. Sox (**5.23**), a quinoline derivative functionalized amino acid developed by the Imperiali group, shows a considerable fluorescence enhancement upon chelation of divalent zinc, a process termed "chelation-enhanced fluorescence" (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] as well as 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 9.

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] Their basic spectroscopic properties can be found in Table 9. Two phenylalanine-based designs, 62dansylPhe (**5.36**) and 52dansylPhe (**5.37**), have been proposed,[362] but have not, to our knowledge, been explored.

5.4.5. Diaminopropionic Acid Derivatives—Push-pull aromatic hydrocarbons 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 coworkers 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**) represents another example of this design (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 maxima (Table 9). 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

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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 shown 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 9).[374] Interestingly, introduction of a dimethylamino group gives non-fluorescent dabcyl-diaminopropionic acid (**5.43**),[375] which has been used as a fluorescent acceptor with 7azaAla (**5.12**, Figure 5.3) in FRET studies.

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 10 below 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 the 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 are analogous to the susceptibility of solvatochromic probes to changes in polarity. Such chromophores can be either used to estimate local polarity or to study binding events.

D. Incorporation—This application lists the *in vitro* or *in vivo* corporation 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 review articles.[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 scaffold 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.

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 messenger RNA molecules are all well regulated. Additionally, recent developments suggest significant roles for non-coding RNA sequences in cellular regulatory processes, adding to the multifaceted and intricate roles of these biomolecules in the cell.409–412

Nucleic acids are phosphodietser-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 non-emissive in neutral aqueous conditions. Accurate measurements reveal exceedingly low fluorescence quantum yields for the natural nucleobases ($\Phi_F = 0.5\times10^{-4} - 3\times10^{-4}$) associated with sub-picosecond excited state lifetimes (Table 11).[418] Quite expectedly, mother 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.

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 wyeosine 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

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of emissive nucleobase analogs, with ethenoadenosine (εA) 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 and systematically organized view of this rapidly evolving field. The diverse applications, summarized in table 20, 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 lacks the Watson-Crick (W-C) 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 12).[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 (figure 6.2),[440] having photophysical properties similar to its parent chromophore (Table 12),[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] A phenanthrenyl nucleoside, recently reported by Leumann, was 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 13). 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-MI457 and 6-MI) and A analogs (DMAP and 6-MAP), retain their overall absorption and emission characteristics 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-dependant 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 6.7).

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).

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Most retain their W-C 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 to their natural counterparts, emission bands near or in the visible range, and rather high emission quantum efficiencies, ranging from 0.2–0.82 (Table 14 and 15).

Leonard and co-workers first investigated etheno-A (εA, **6.25**)[462,463] and benzo-A (**6.27**)464 in the early 1970's following an initial report showing that adenine and cytosine could be cyclized to produce nucleobases with red shifted absorptions 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 notably 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 (9,917 cm^{-1}) and a relatively long lifetime for a small organic chromophore ($\tau = 20$ ns).[463,]467 εATP, identified as 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 the significantly improved photophysical properties compared to 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 6.5).476[,477] Both analogs display a strong emission band in the visible range ($\Phi = 0.82$, 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 6.7).

A cytidine analog, tC (**6.30**), originally synthesized for antisense applications by Matteucci, 478 forms W-C like base pairs with guanosine,479 and somewhat surprisingly, does not suffer a dramatic reduction in quantum efficiency upon in corporation into PNA480 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 ($\Phi = 0.17$) for this class of nucleobases. 480,482–484 Most recently, Millar and Tahmassebi have utilized tC along with a non-fluorescent 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), 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 6.7). [488–]490

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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 6.4) and (b) ring-expanded nucleobases. Benzopyridopyrimidine (BPP, **6.31**) is a cytidine analog that forms stable pairs with both A (wobble bp) and G (W-C bp).[492] BPP displays an isolated absorption band (347 nm), but its low quantum efficiency ($\Phi = 0.04$) 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 ($\Phi = 0.26$) (Table 15).[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 6.7).[494] Both ^{MD}A and ^{MD}I have rather large Stokes shifts in comparison to BPP/NPP, resulting from a blue shifted absorption and red shifted emission, although their quantum efficiency remains rather modest ($\Phi = 0.12$).[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 (~ 7,000 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, either via flexible or rigid linkers (Figure 6.6 and Figure 6.7, respectively). Connecting known chromophores via electronically non-conjugating 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.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 nm and 270 nm vs. 260 nm, respectively), they are not emissive, unlike the alkene- and alkyne-conjugated analogs (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 to adenosine, all due to the non-conjugated phenylalkyl substituents.[507] While the free nucleosides display very low emission quantum efficiencies and rather long lifetimes (Table 16), 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 non-conjugated systems.

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Netzel and co-workers have linked pyrene to the 5-position of 2′-deoxyuridine directly and via an amide or ketone linkages (Figure 6.6).[508,509] While primarily developed as tools to evaluate electron transfer processes in DNA, detailed analysis of their photophysical characteristics, including steady state and time-resolved studies provided 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.[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 has been utilized by numerous groups for a variety of applications (Section 6.7).[510,512,513] The corresponding 8-substituted purines, which can be viewed as rather perturbing analogs, have also found utility (Section 6.7).[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 16). Despite the distance between the pyrene and the pyrimidine core and the non-conjugating linker, these BDFs have been very successful probes for SNP analysis (Section 6.7).[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 16).[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 16). 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 multi-color 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 coworkers 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 6.7).[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., $(bpy)_2Ru(3\text{-Br-1,10-phen})^{2+}$] with 5-ethynyldU.[532–534] The electrochemical and photophysical properties of the resulting metal-containing nucleosides were investigated.[532–534] The presence of coordinately

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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 diasteromerically-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 non-metallated 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 17 and Figure 6.7: **6.114–6.125**) 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 6.7).[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 to 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 18). 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 1,600 contributions. The seminal paper by Reich and Stryer suggests 2-AP to be an ideal emissive nucleoside analog. Its ability to form W-C 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 (Figure 6.8), display substantially lower quantum efficiencies (0.01 and 0.06, respectively, Table 18).550 Other variations of 2-AP, including 7-

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deaza and 8-aza-7-deaza, have been studied by Seela and co-workers.[552] While these analogs display larger Stokes 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 A- or B-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 6.7), is sequence dependent. Adding to its complex photophysics, energy transfer processes have been documented for 2-AP containing duplexes.[557] A 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 to 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 to 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 5–10), displaying desirable properties as a probe (see Section 6.7). 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 nucleobases analogs.569[–571] The two purine analogs, 2-amino-6-(2-thienyl)purine and 2-amino-6-(2-thiazolyl)purine, 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 displays 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 6.7). In this context, it is useful to comment on additional novel base pairs, including the well-studied isoG–isoC system, as 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 (s or m^5K, **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 1980's.582,[583] Early photophysical studies described its isolated absorption (280 nm), and time resolved data were used to probe its stacking ability in a single stranded oligonucleotide (Table 18).[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 W-C base pair with adenine.

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 reveals, in addition to the typical pyrimidine transitions, a lower energy absorption band (~310 nm) that

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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 (8,400–9,700 cm^{-1}) for such small organic chromophores, while their quantum efficiency is relatively low (Φ = 0.01–0.035).[589–591] The responsive furan and thiophene analogs (**6.138, 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 6.7). 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 6.7).[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 to 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 (Φ = 0.69 and 0.57 for **6.145** and **6.148**, 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 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 (Φ = 0.02 – 0.058).[598] 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, Φ = 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 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 coworkers (Figure 6.8, Table 18).[600] Among the analogs **6.167–6.169**, the 5-methoxyquinazoline-2,4(1*H*,3*H*)-dione derivative (**6.168**, Φ = 0.16) was found to be an ideal FRET donor for chromophores derived from 7-diethylaminecoumarine-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 coumarine 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

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nucleobases. Its visible emission (460 nm) is reasonably intense (estimated as $\Phi = 0.2$), 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 6.7). 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, 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 18). 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 18, **6.139** vs. **6.151**). These isomorphic analogs are currently being used to develop assays for monitoring glycosyltransferases activity.[612]

Lastly, we discuss a series of nucleobase analogs that, although 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 to their acceptor-free analogs, including red shifted absorption maximum (20–50nm), enhanced quantum efficiency (approaching unity) and solvatochromatic effects (Table 19). 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 post synthetic 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'-DMT protecting group of the solid-supported nucleoside, (2): mild acidic activation of the phosphoramidite (e.g., using 1H-tetrazole), (3) coupling of the available 5'-hydroxyl to the activated phosphoramidite, (4) oxidation of the trivalent phosphorous 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).

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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 as 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 steps 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 review 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 20 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 (**6.123**), reported four decades ago by Reich and Stryer, are still finding vast utility in contemporary biophysics, while others, such as benzo-A (**6.123**), 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 Fluoroscent (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/

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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). As 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 nature of the distinct families of biomolecular building blocks inherently controls 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 require rather creative 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. Viewing the data summarized here and the challenge of designer fluorophores from a fundamental physical organic chemistry 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 nucleoside, does not always function optimally within oligonucleotides. This highlights certain fundamental challenges in this field, as the photophysical properties of any emissive biomolecular building block are further impacted when embedded within macromolecules by rigidification, de-solvation, 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

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ingenious probes, reflecting seemingly endless creativity, will always be embraced by chemists, biologists and biophysicists.

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Biographies

Renatus W. Sinkeldam earned his Ph.D. in 2006 under the supervision of Professor E. W. Meijer and Dr J. A. J. M. Vekemans at Eindhoven University of Technology in The Netherlands, for his work on the design, synthesis, and spectroscopic analysis of small organic dyes and helical foldamers. He is currently a postdoctoral fellow in the group of Professor Yitzhak Tor at the University of California, San Diego. His work focuses on isomorphic fluorescent nucleosides and their incorporation into DNA oligonucleotides. His main research interests include the design, synthesis, and spectroscopic evaluation of molecular probes that

are responsive to environmental parameters, and their application in supramolecular and biological molecules.

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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Yitzhak Tor carried out his doctorate work at the Weizmann Institute of Science earning his Ph.D. in 1990. After a postdoctoral stay at the California Institute of Technology, he took his first faculty position at the University of Chicago. In 1994, he moved to the University of California, San Diego, where he is currently a Professor of Chemistry and Biochemistry and a Traylor Scholar in Organic Chemistry. His research interests include chemistry and biology of nucleic acids, the development of novel antiviral and antibacterial agents, the mechanisms and applications of low MW cellular delivery agents and the discovery and implementation of fluorescent nucleoside analogs.

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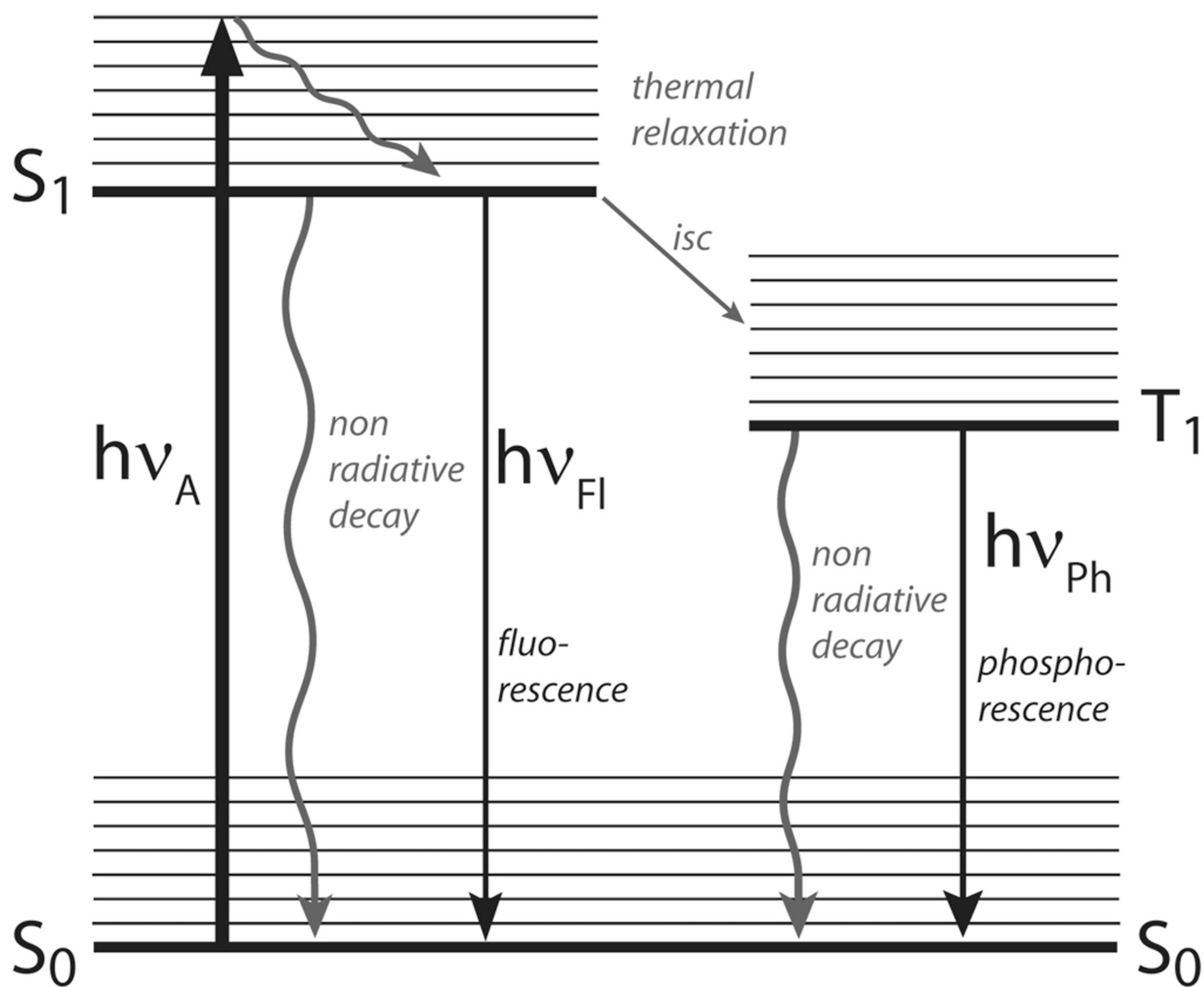

Figure 2.1.
A simplified Jablonski diagram.

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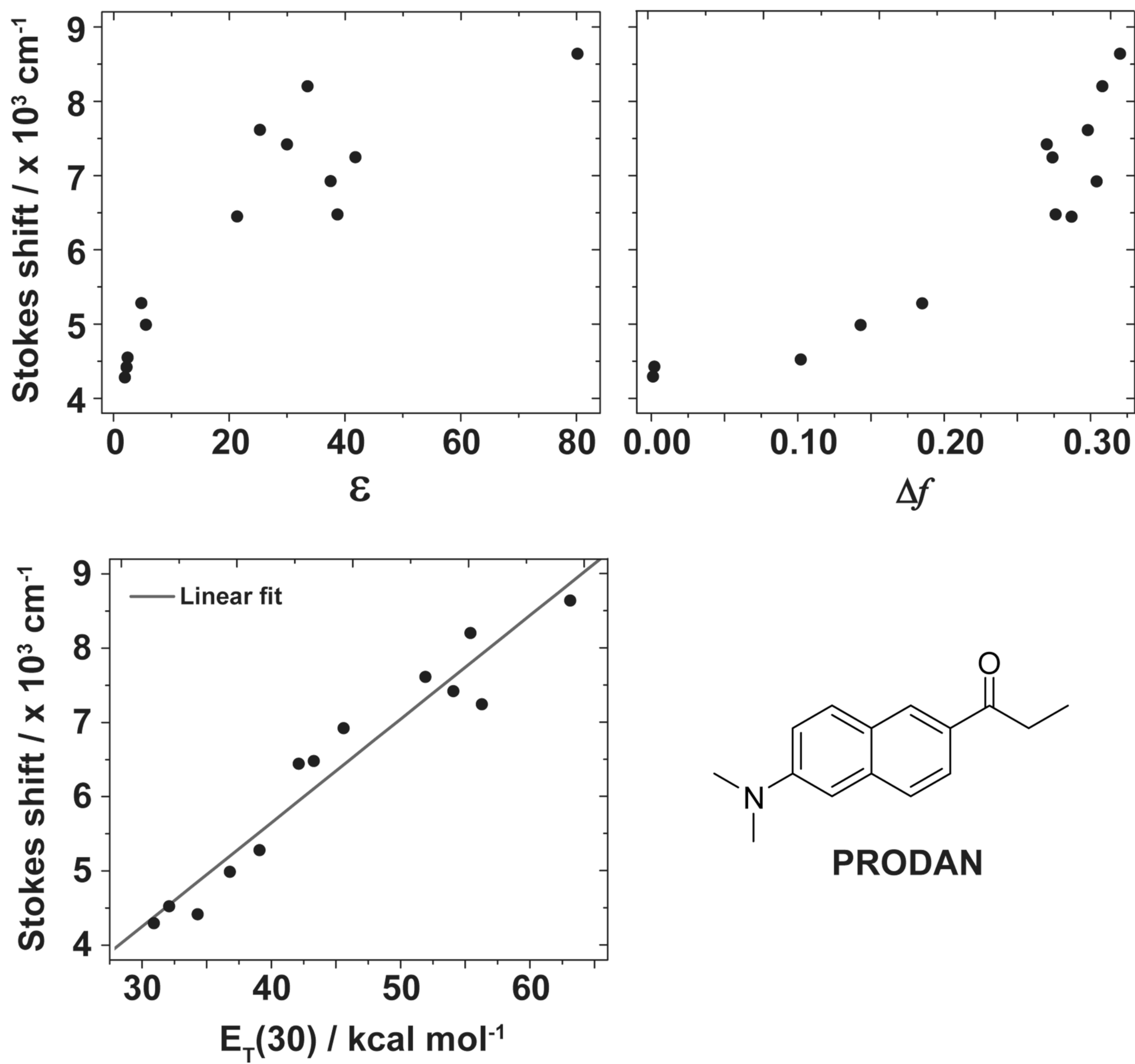

Figure 2.2.
Correlation of solvent polarity and Stokes shift of PRODAN.

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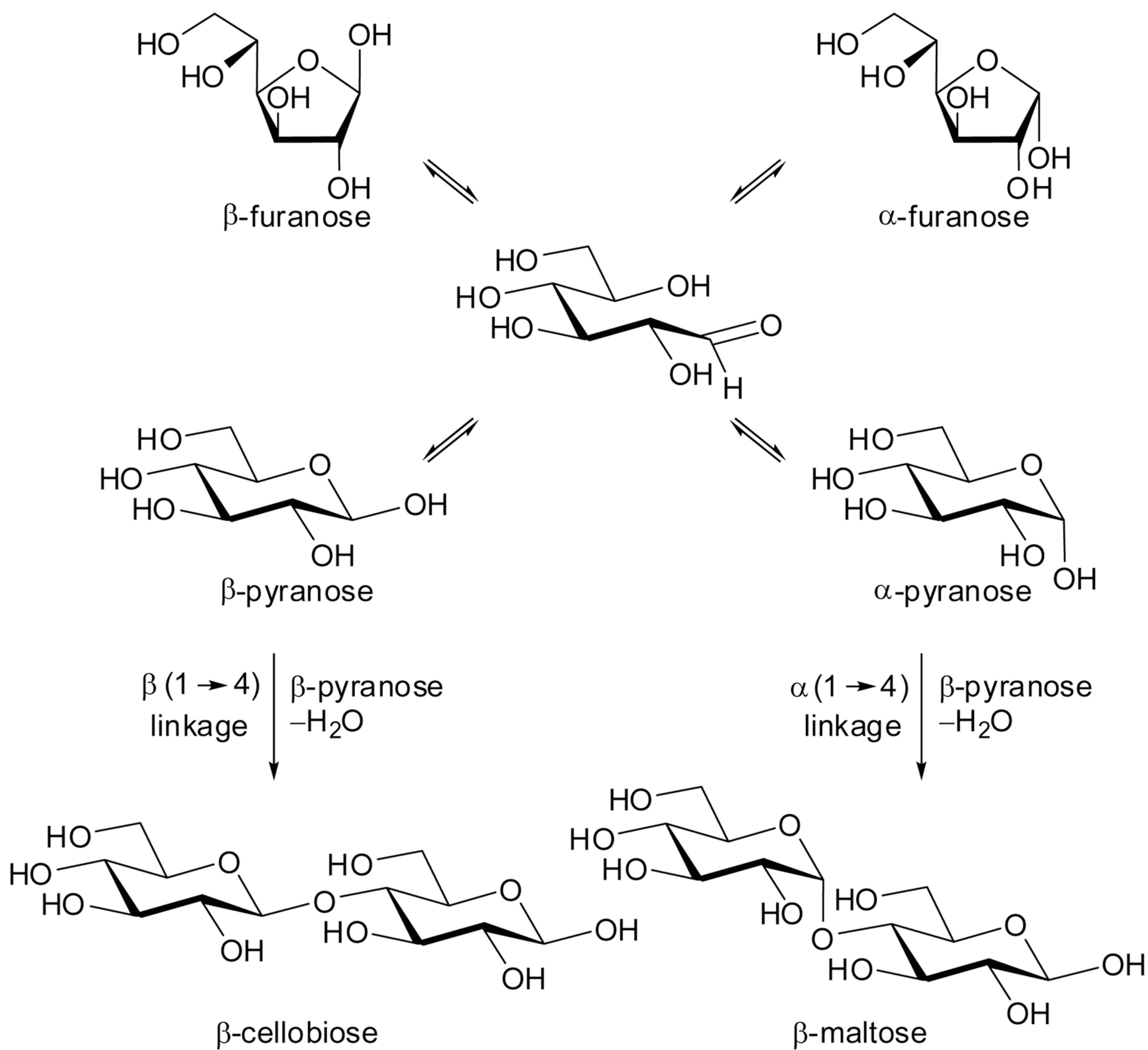

Figure 3.1.
Cyclization of the acyclic form of D-glucose shown in the open, pyranose, and furanose forms. Hemiacetal formation produces both the α and β anomers (i.e., C-1 epimers).

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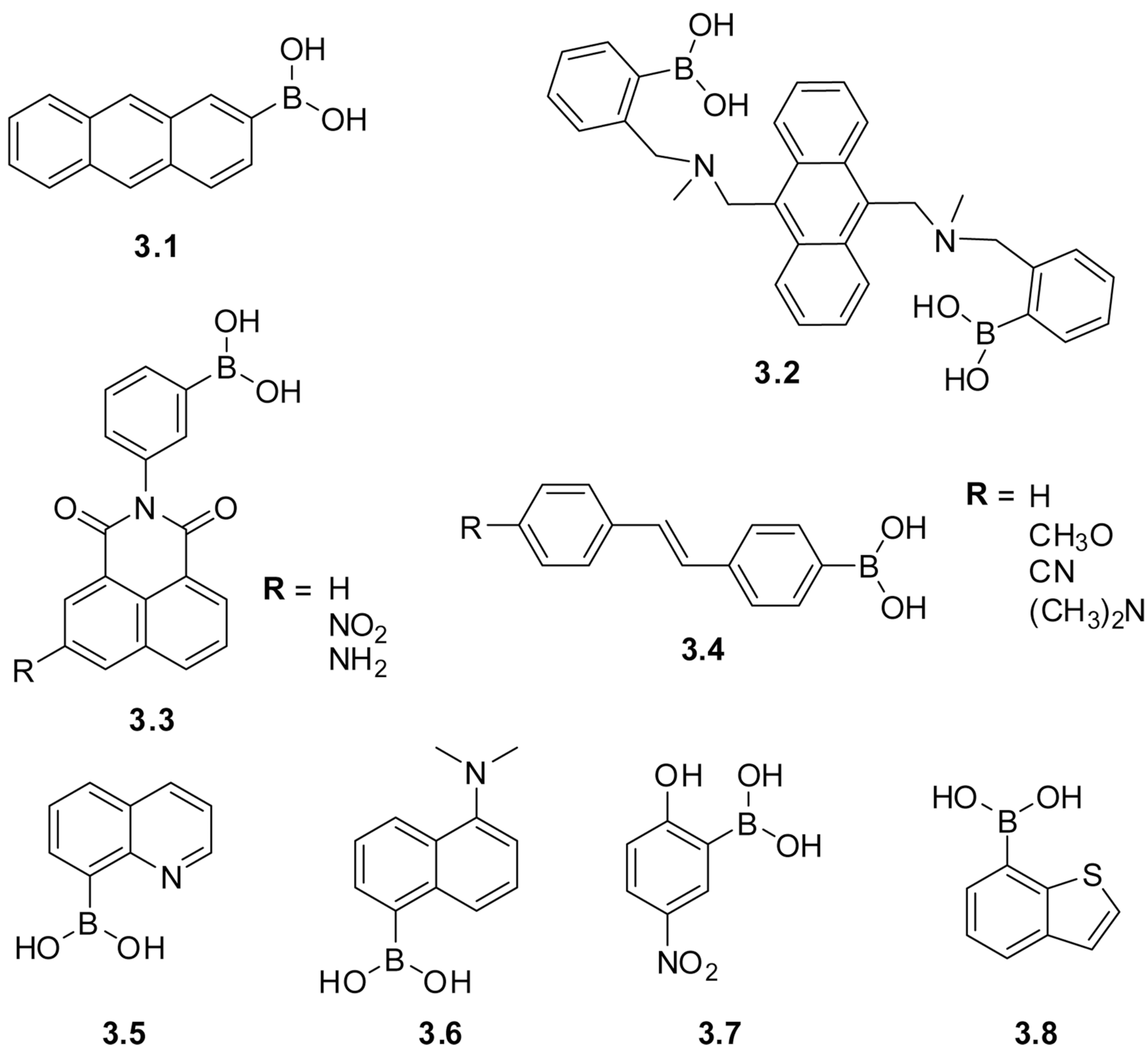

Figure 3.2.
Structures of boronic acid–based saccharide sensors.

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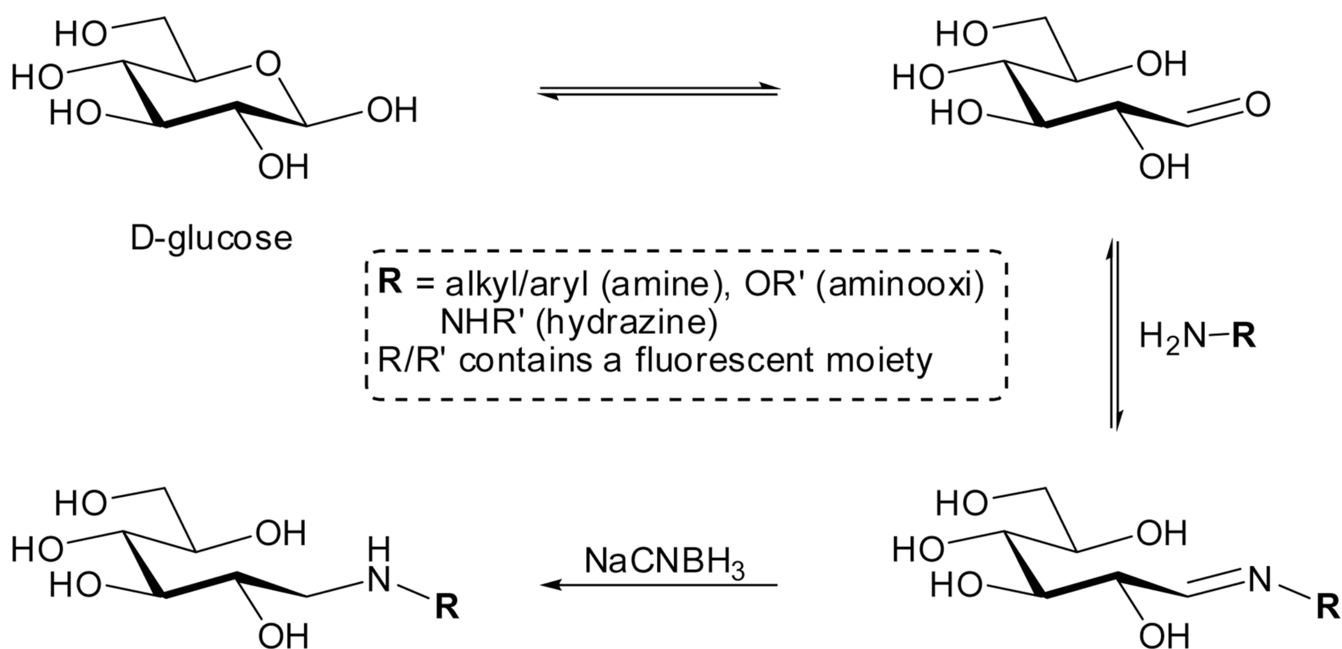

Figure 3.3.
Labeling of reducing carbohydrates with amine–containing fluorophores.

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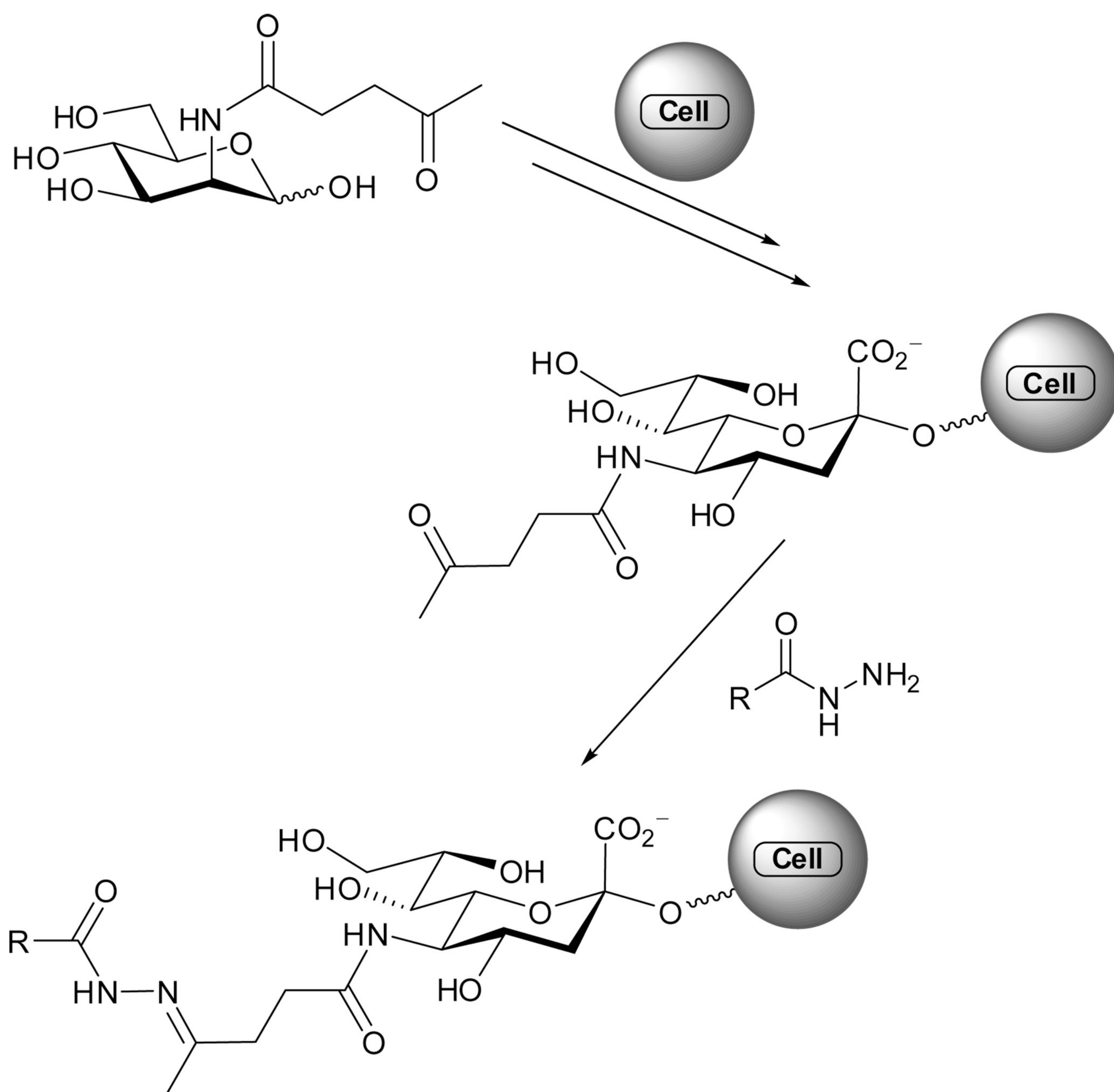

Figure 3.4.
ManLev, its expression on the cell-surface and subsequent acylhydrazone formation.

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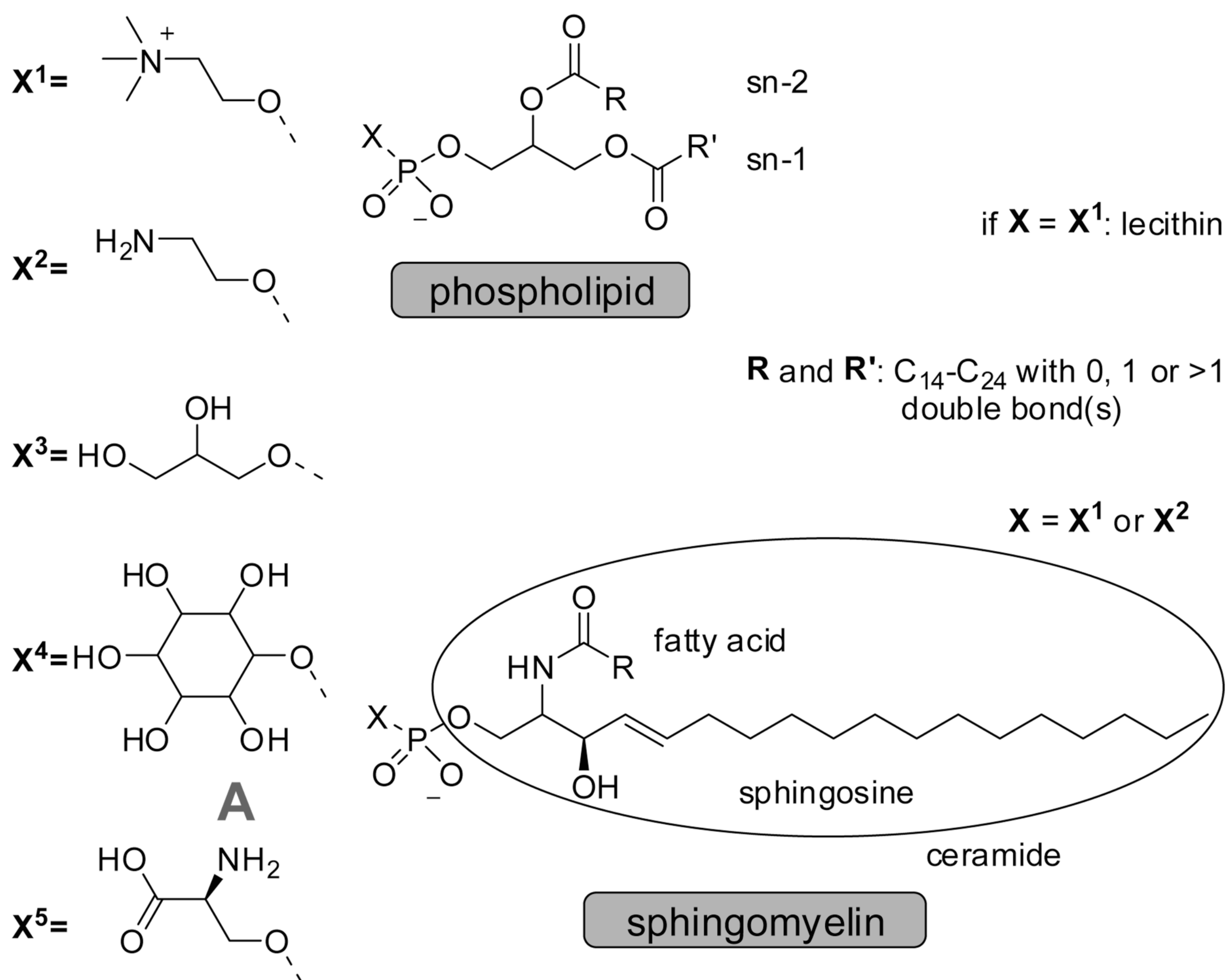

Figure 4.1.

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

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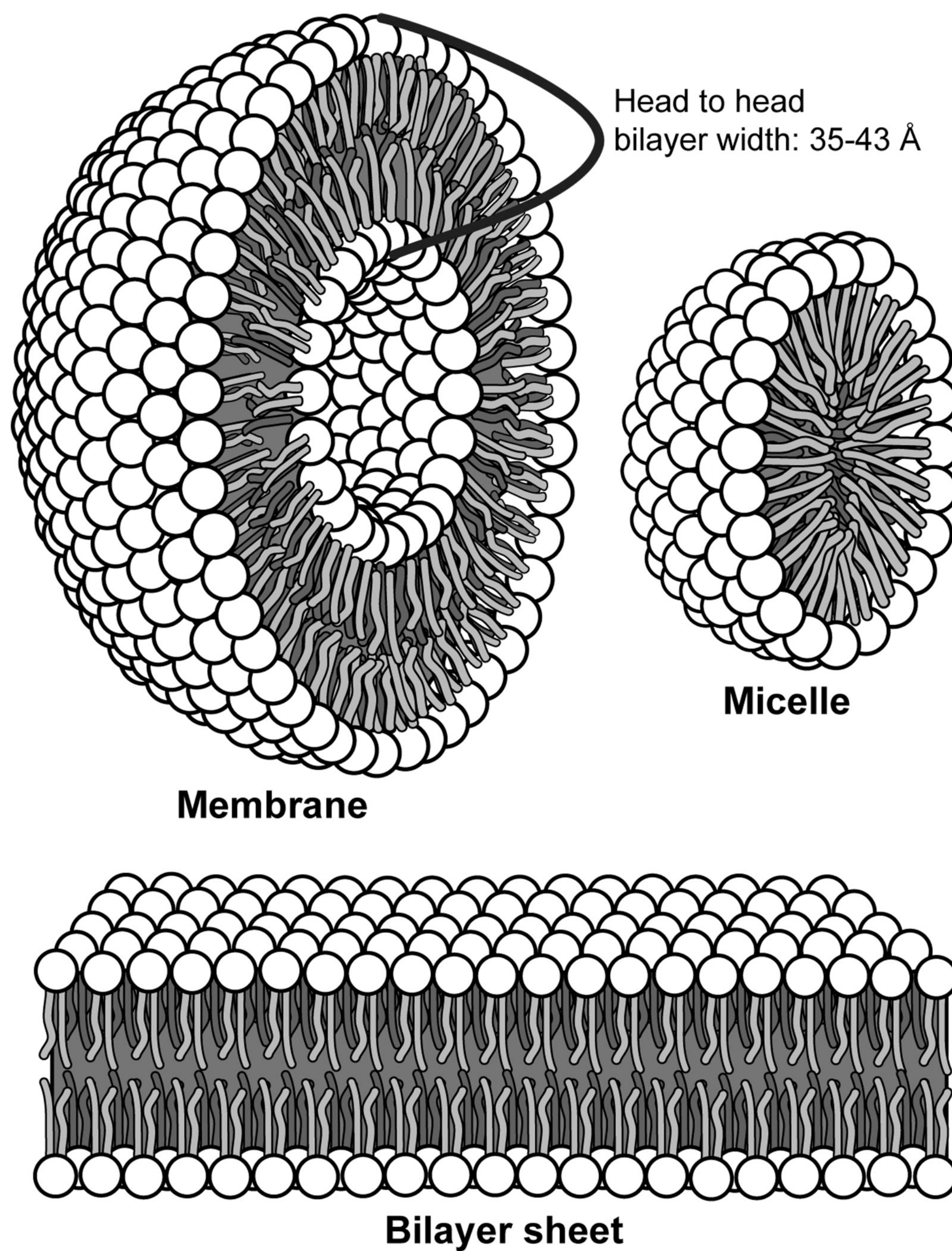

Figure 4.2.
Phospholipid architectures in aqueous media.

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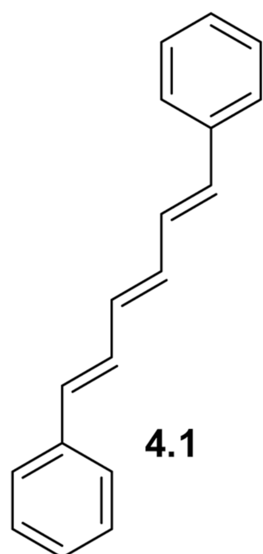

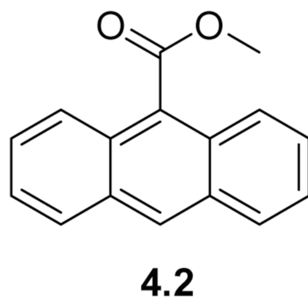

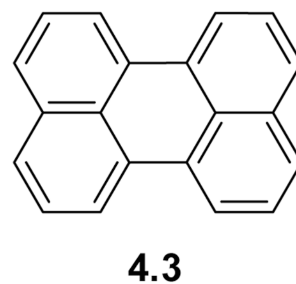

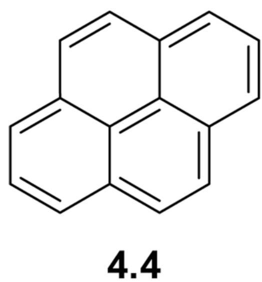

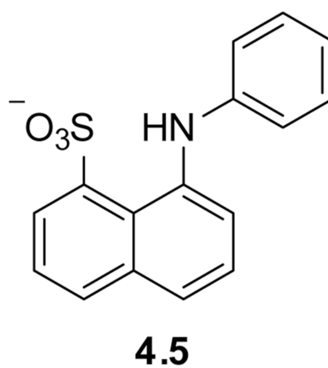

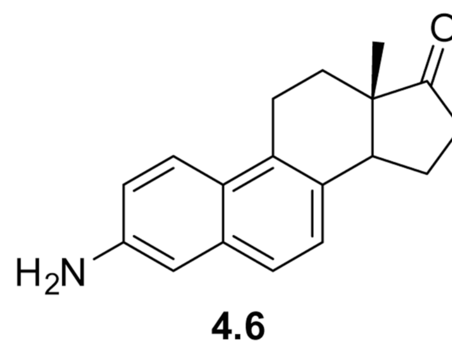

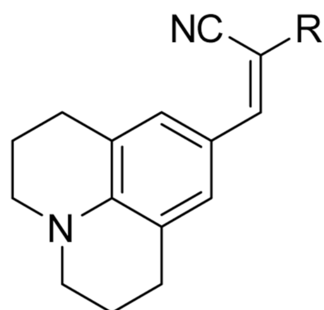

4.7 R = CN

4.8 R =

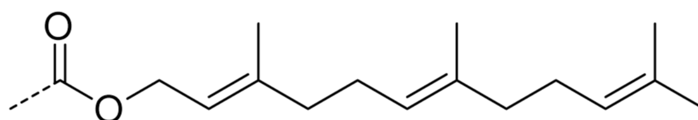

Figure 4.3.
Non-covalent membrane probes that reside in the cell membrane interior.

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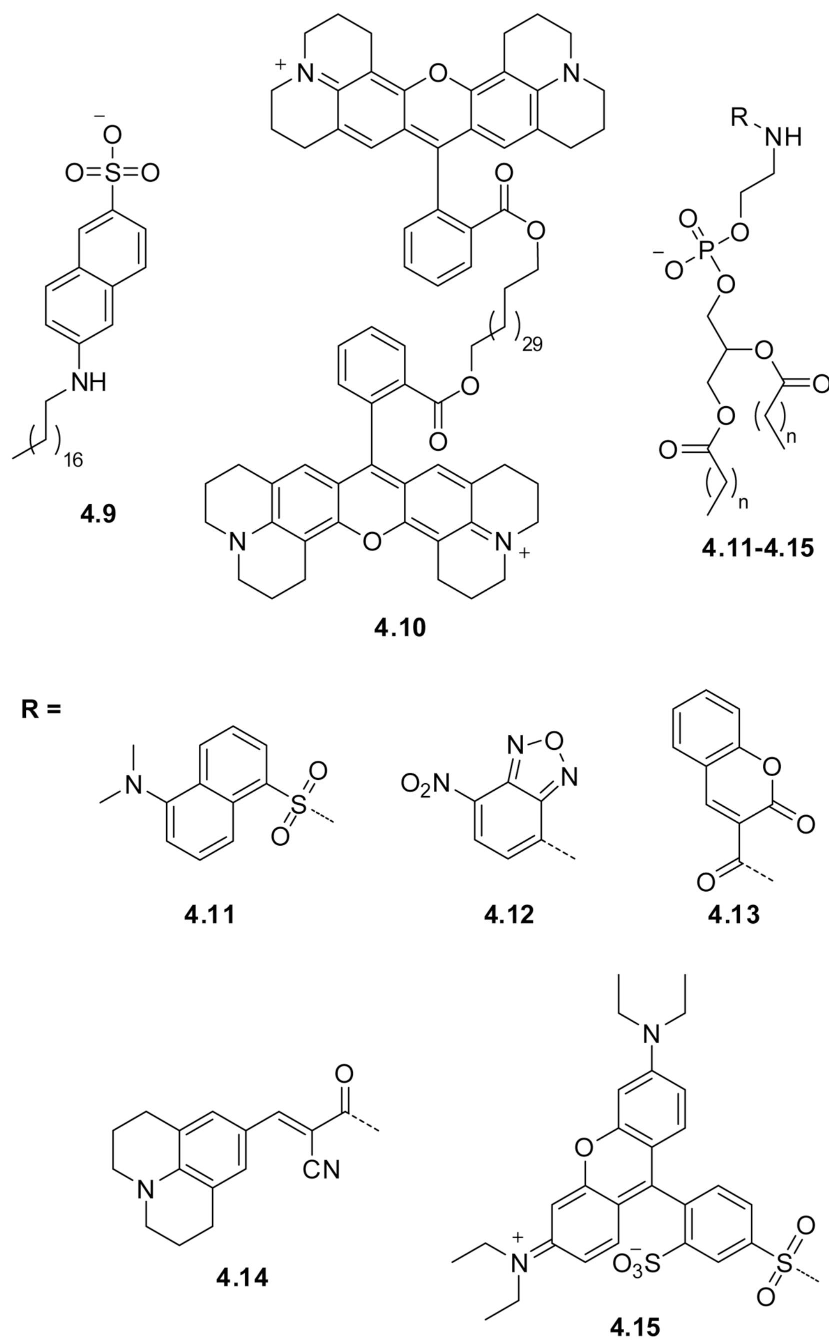

Figure 4.4.
Head–group labeled mamebrane probes.

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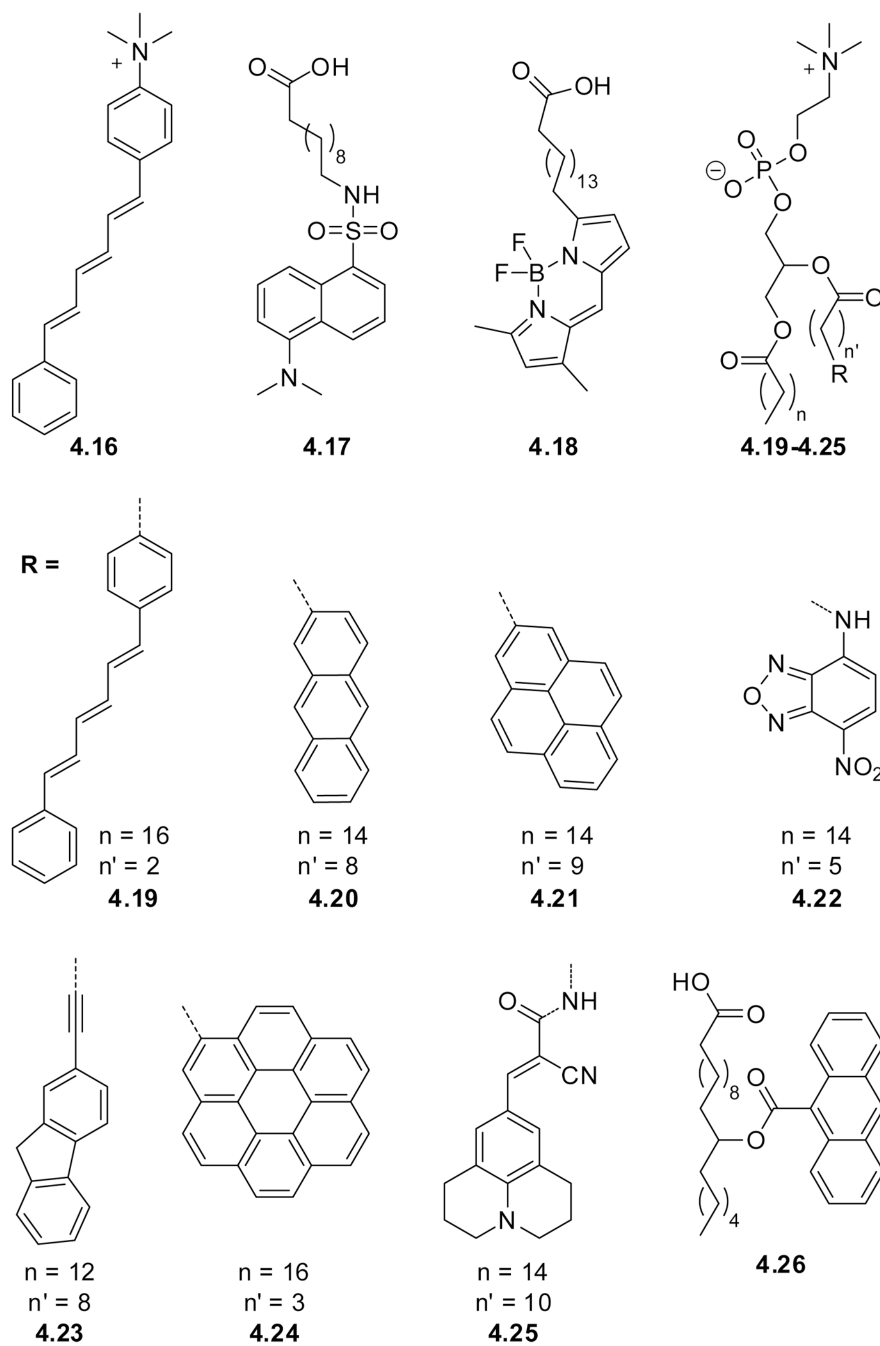

Figure 4.5.

Examples of 'chain-end' (**4.13–4.21**) and 'on-chain' (**4.22**) labeling.

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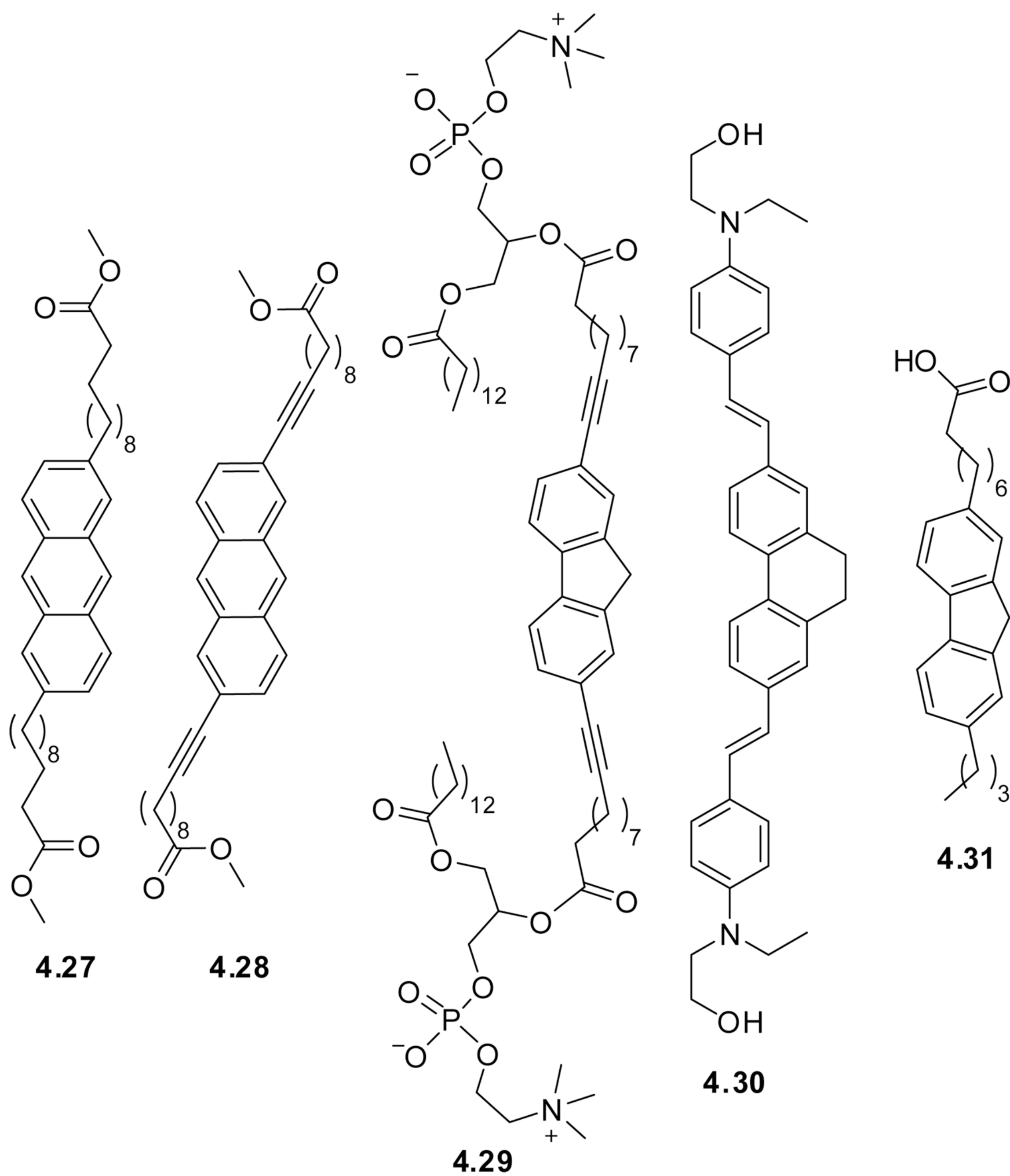

Figure 4.6.
Examples of 'in-chain' labeled chromophores.

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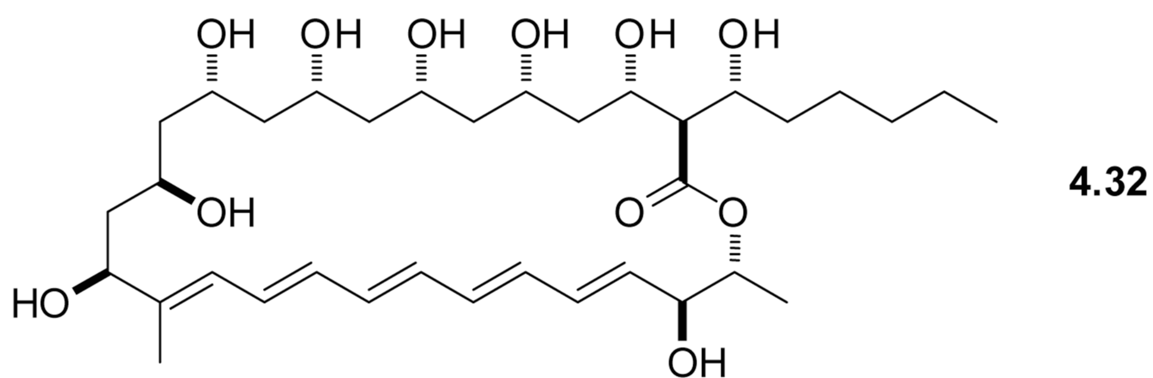

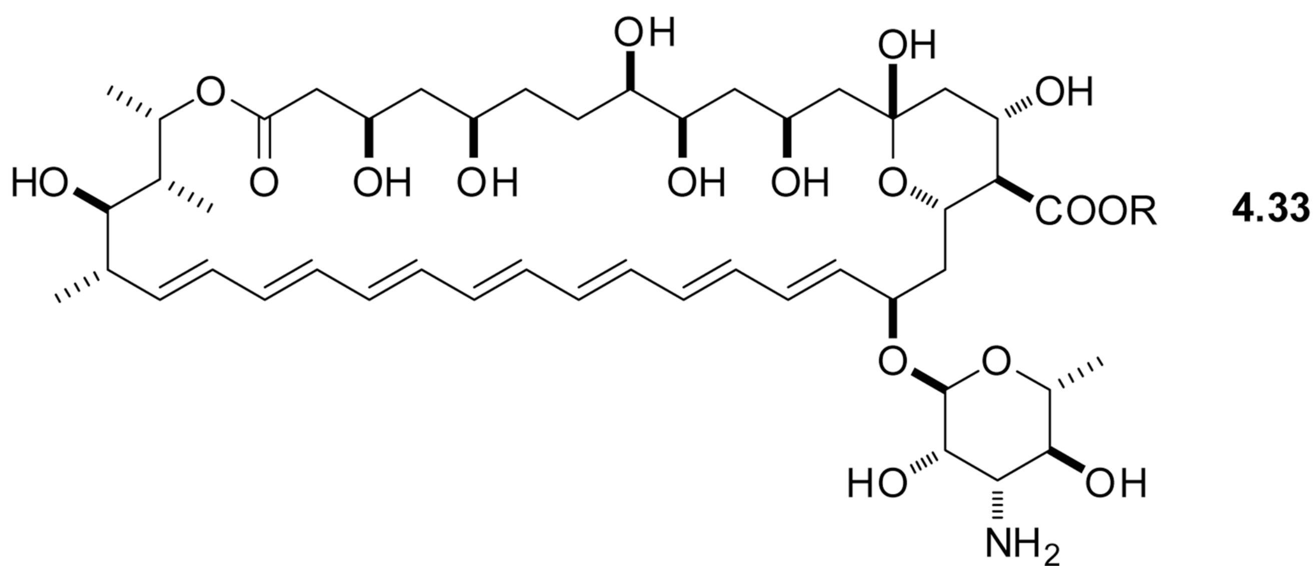

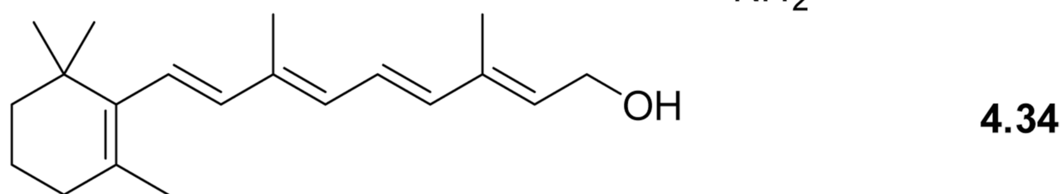

Figure 4.7.
Naturally occurring polyenes.

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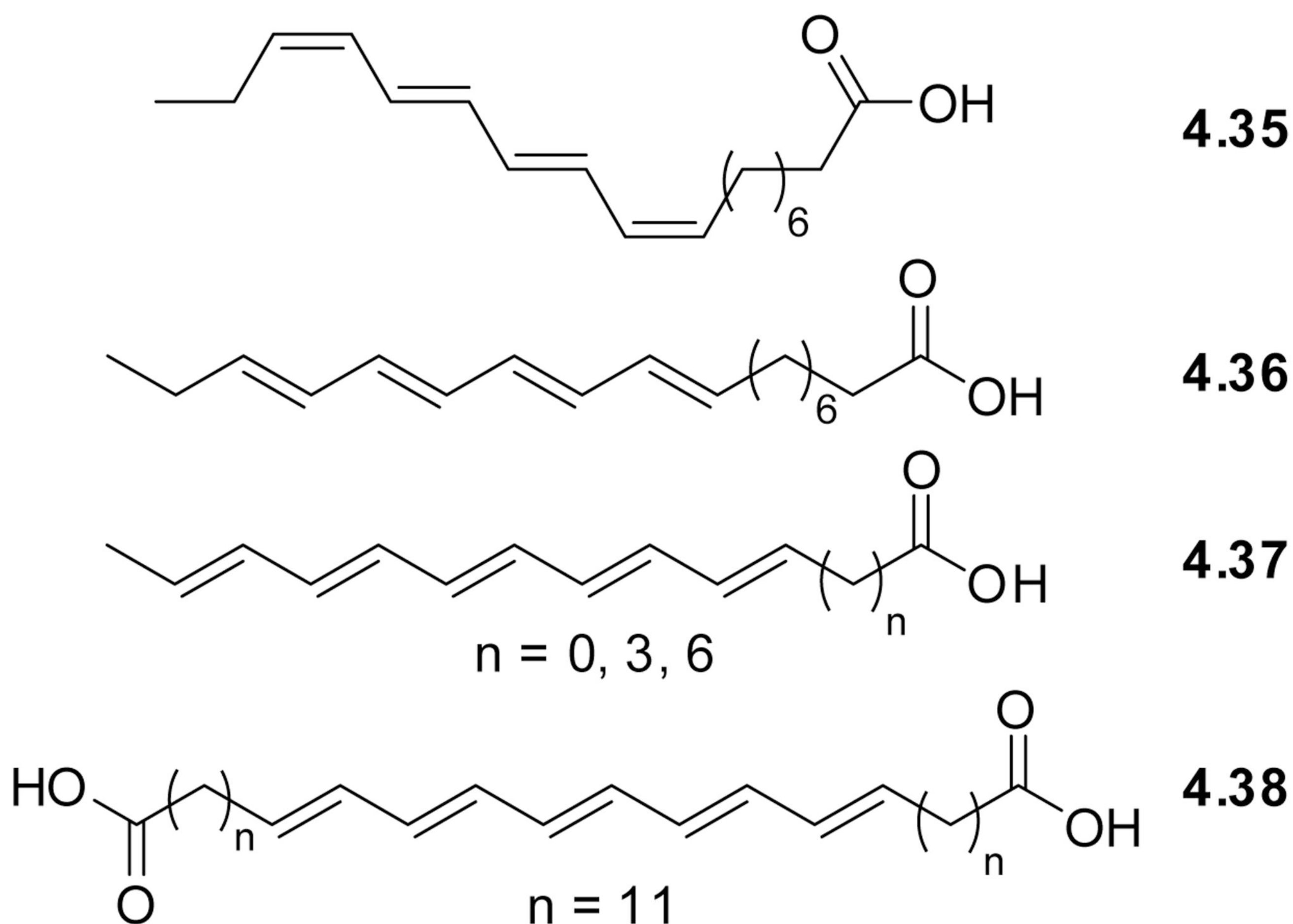

Figure 4.8.
Structures of naturally occurring α-parinaric acid (**4.31**) and synthetic all *trans*-PnA (**4.32**), *trans*-PA (**4.33**), and all *trans*-PdA (**4.34**).

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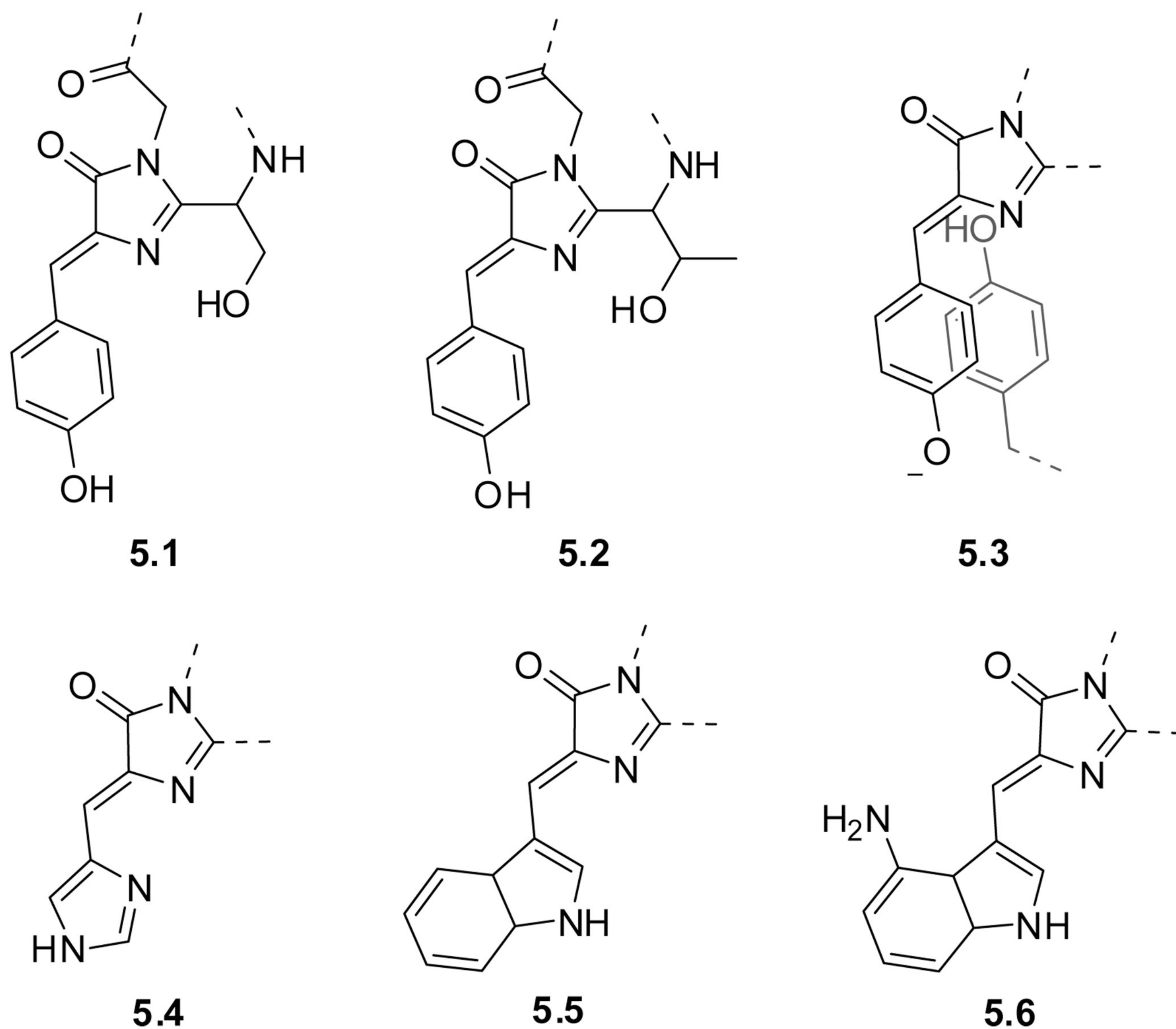
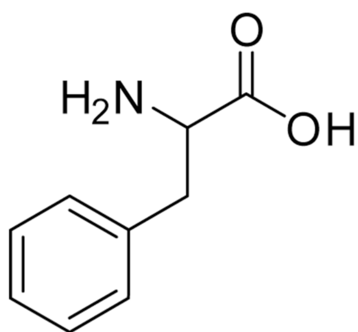
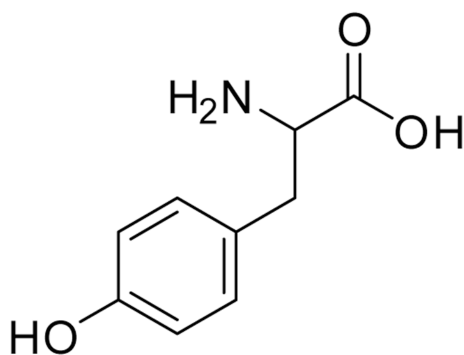


Figure 5.1.

Fluorophores found in fluorescent proteins. Wildtype 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**).





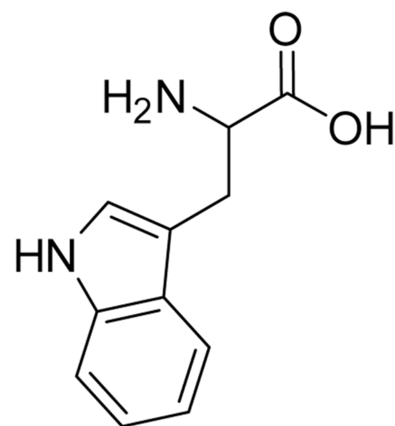


Figure 5.2.
Naturally occurring fluorescent amino acids.

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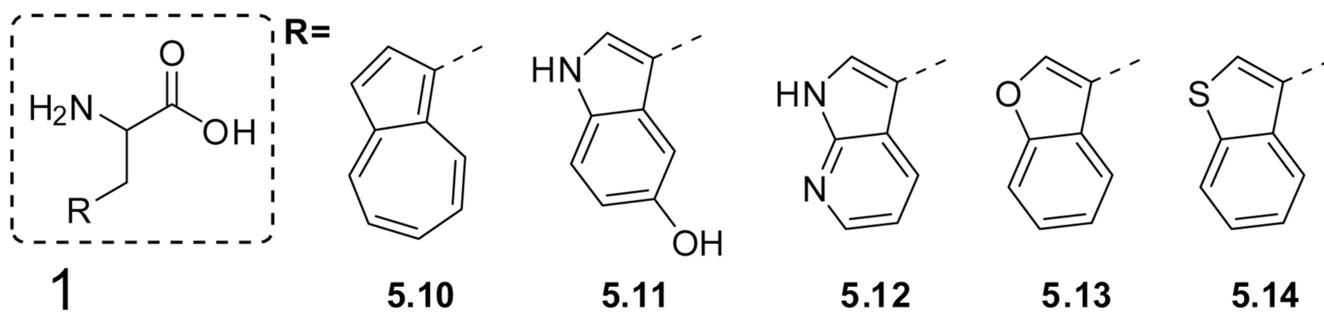

Figure 5.3.
Tryptophan mimics.

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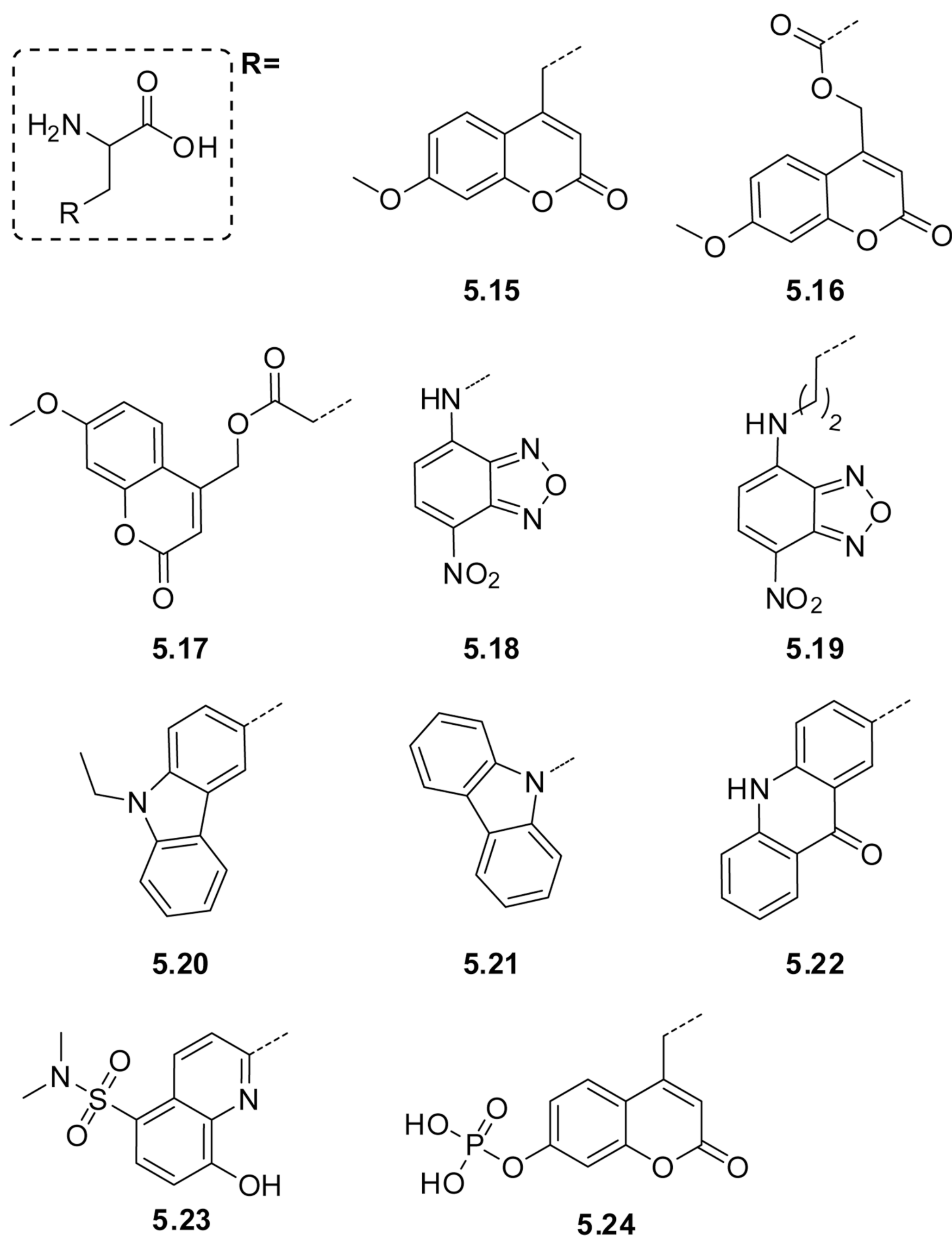

Figure 5.4.
Examples of amino acids containing heterocyclic chromophores.

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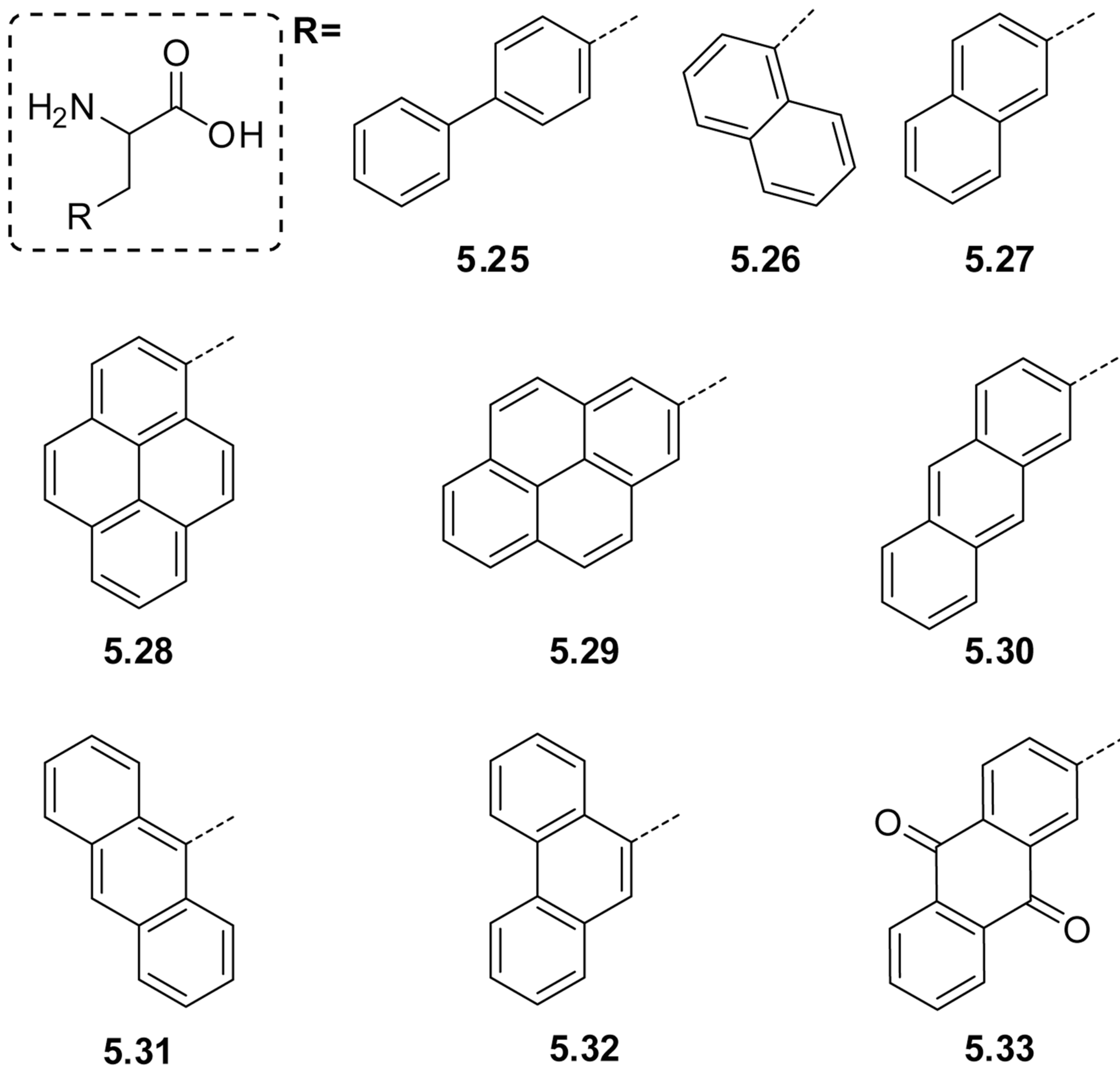

Figure 5.5.
Amino acids labeled with emissive heterocarbons.

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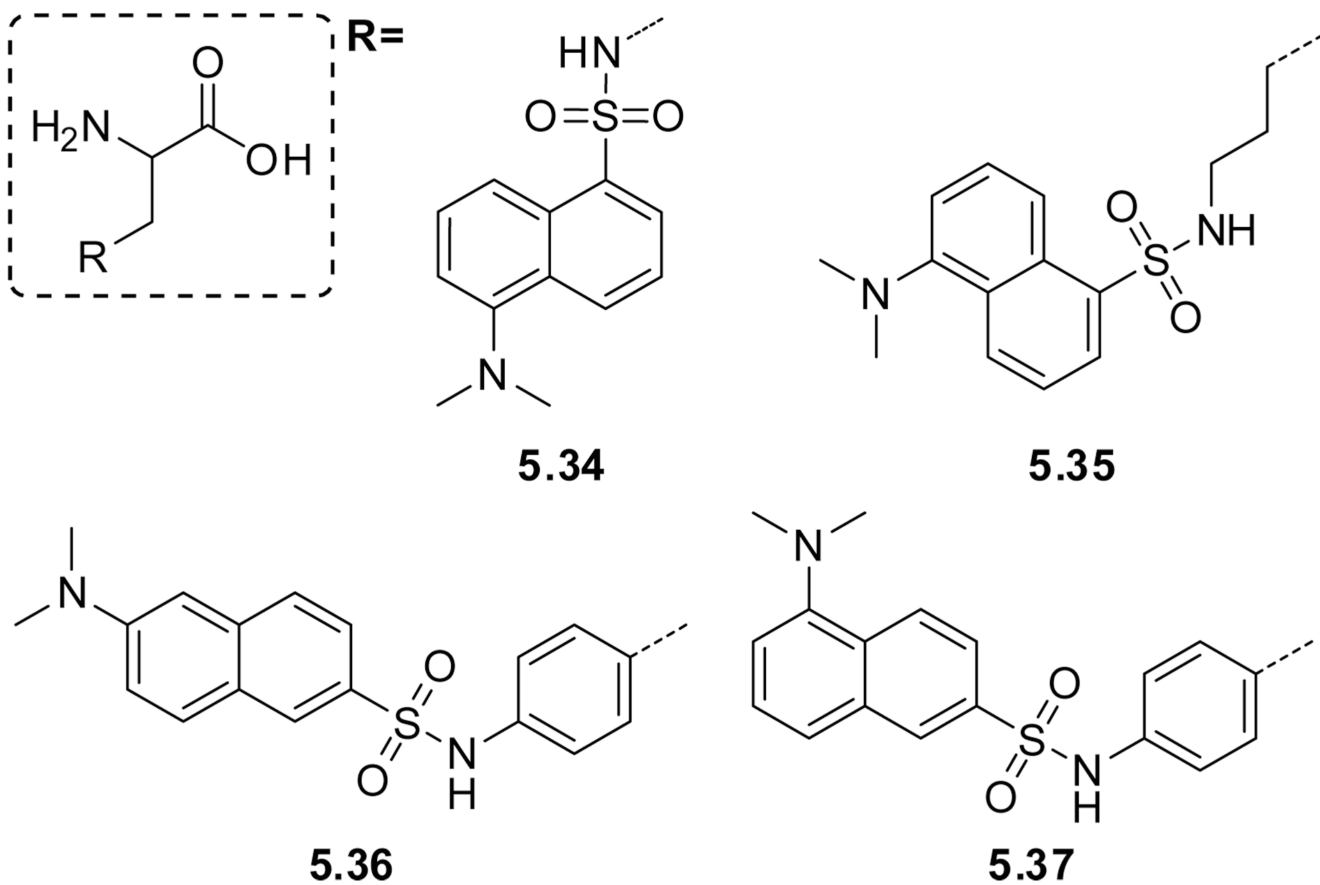

Figure 5.6.
Examples of dansyl decorated amino acids.

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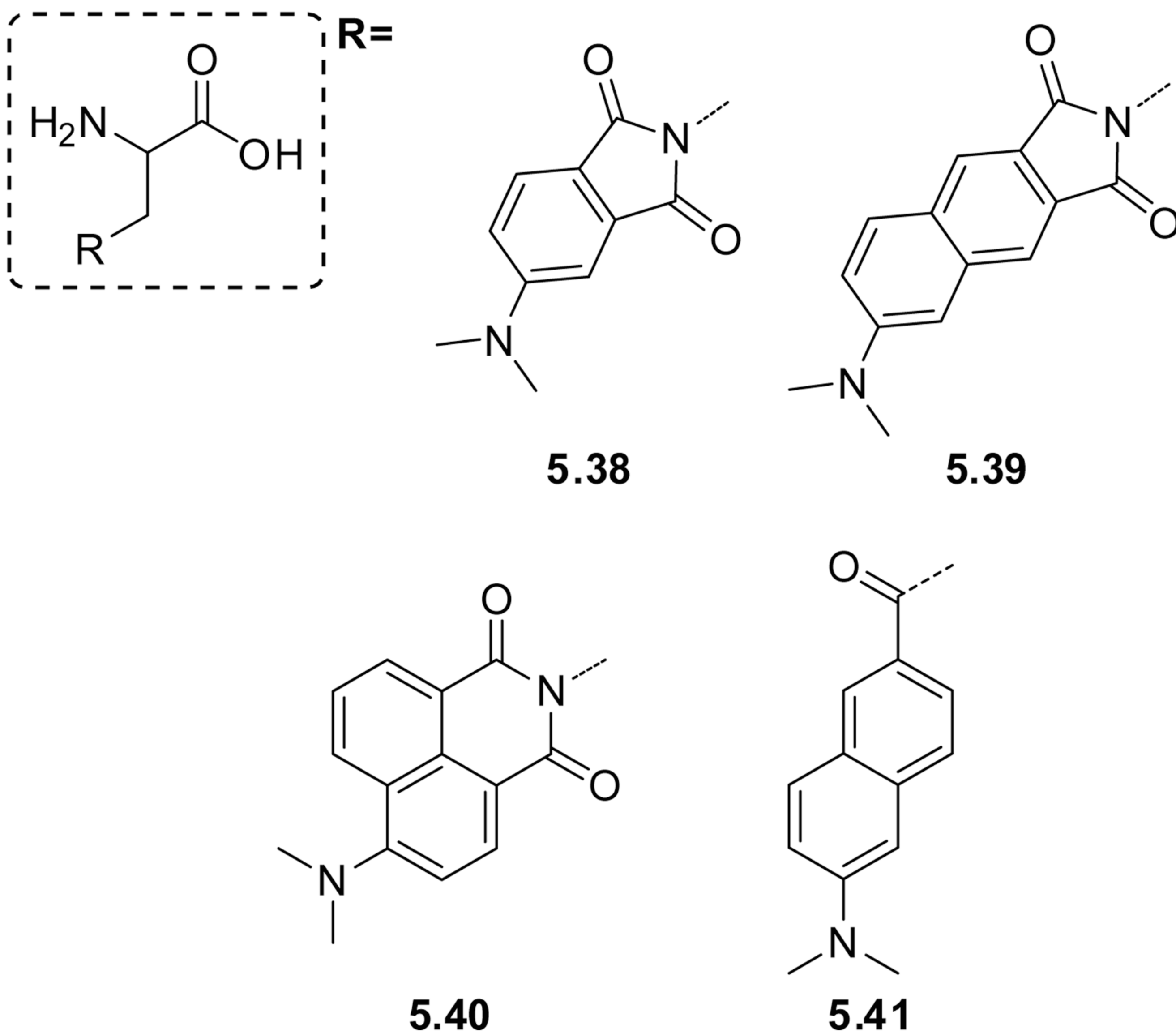

Figure 5.7.
Polarity sensitive phthalimide analogs.

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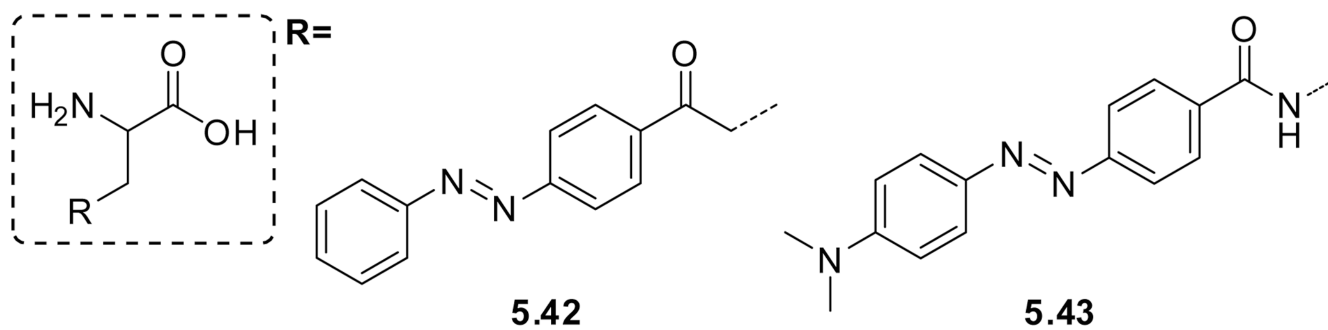

Figure 5.8.
Photo–switchable probes **5.40** and **5.41**.

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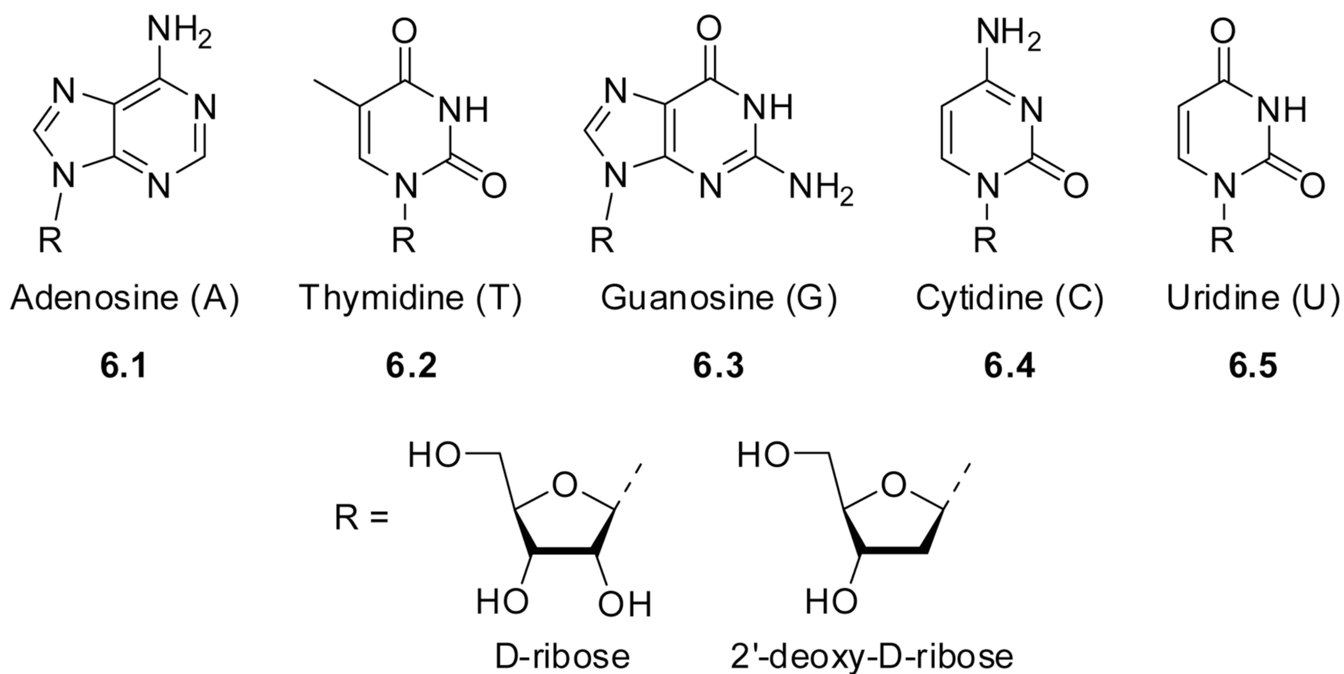

Figure 6.1.

The naturally occurring ribo- and deoxyribo-nucleosides. Note: in RNA, the bases A, T, G, C and U are connected to D-ribose at the 1'-position, where the sugar moiety in DNA is 2'-deoxy-D-ribose.

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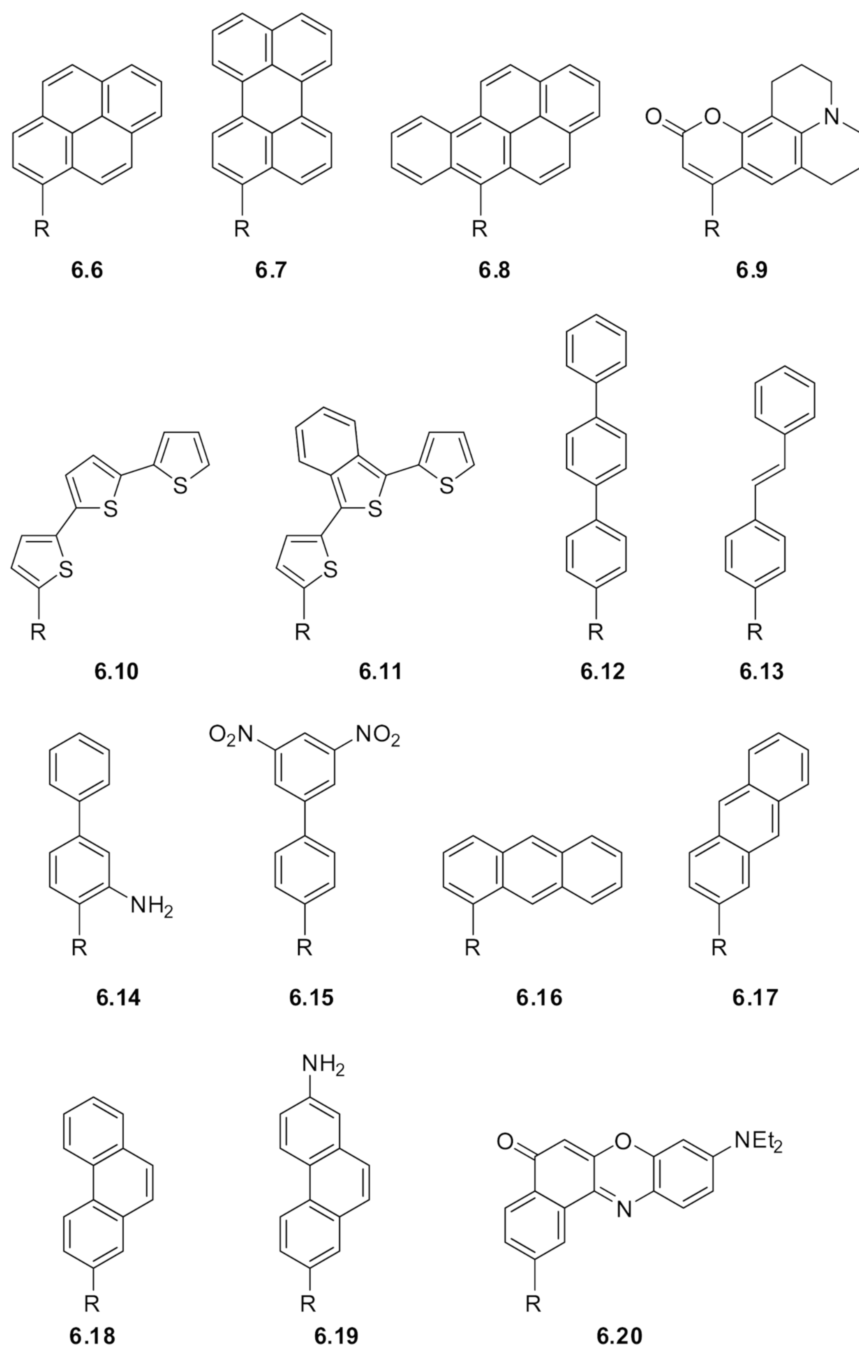

Figure 6.2.
Selected examples of chromophoric base analogs, where R = 2′-deoxyribose.

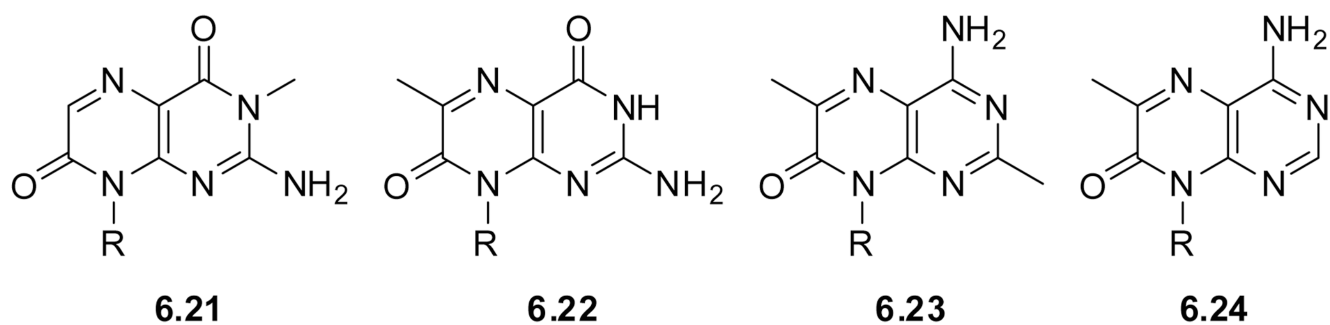

Figure 6.3.

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

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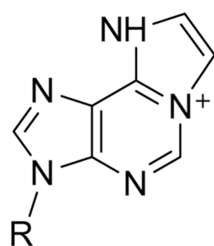
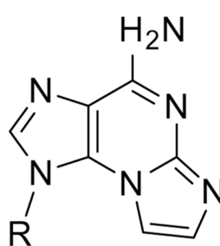


6.25



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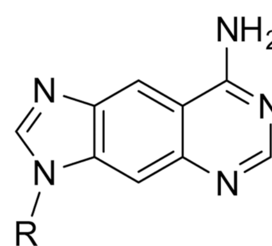

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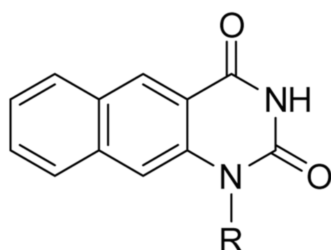

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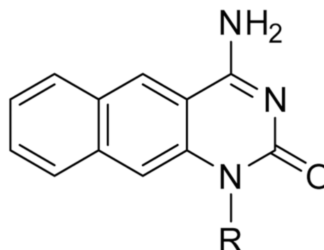
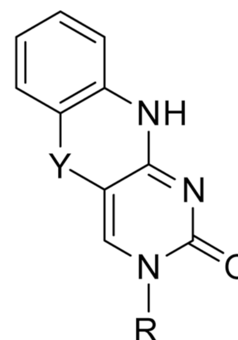
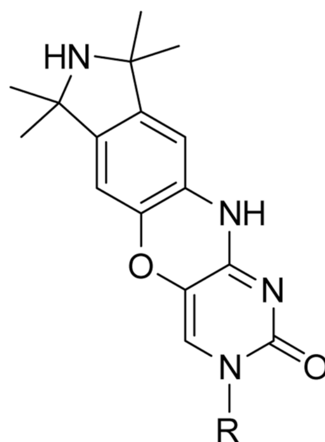


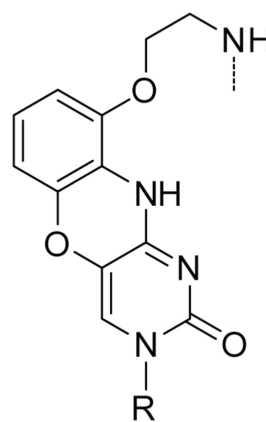
6.29



6.30 Y=S
6.31 Y=O



6.32



—H

6.33

Figure 6.4.

Examples of expanded nucleobase analogs (R= 2’-deoxyribose, 2’-OMe ribose or ribose and R2=3’,5’-O-TBDMS-2’-deoxyribose).

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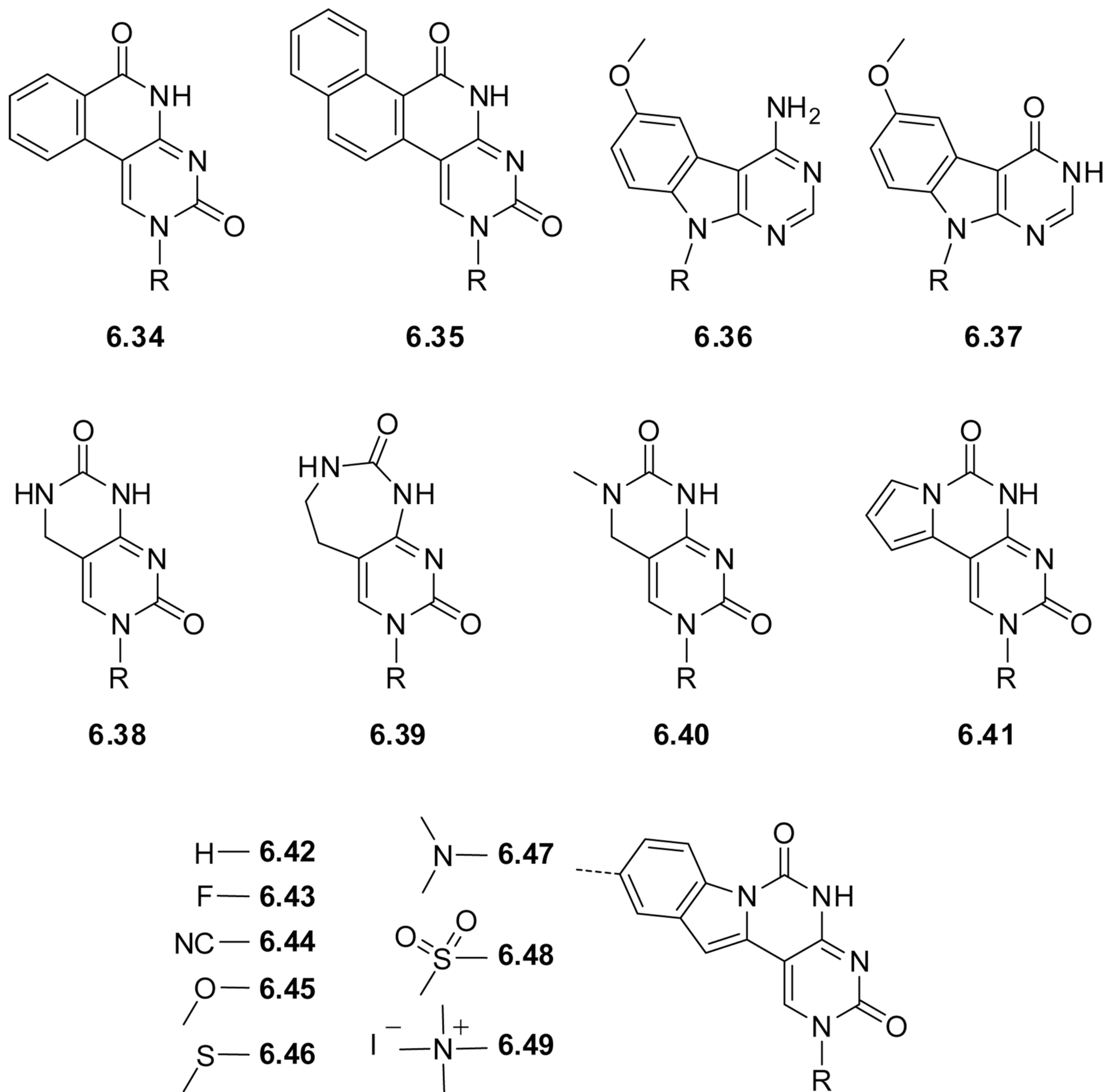

Figure 6.5.
Expanded nucleobase analogs (R = 2′-deoxyribose).

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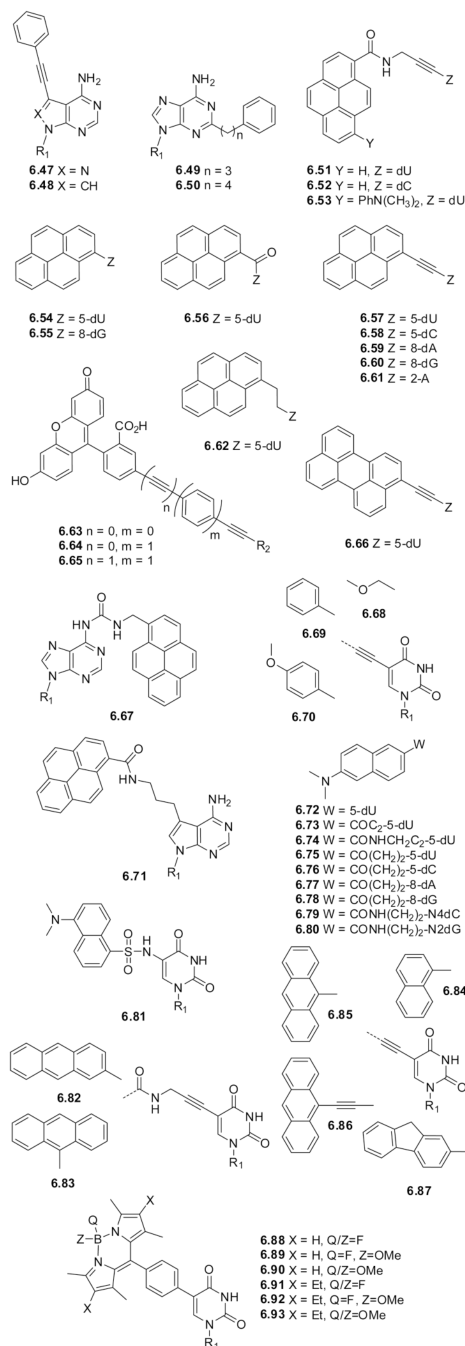

Figure 6.6.

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

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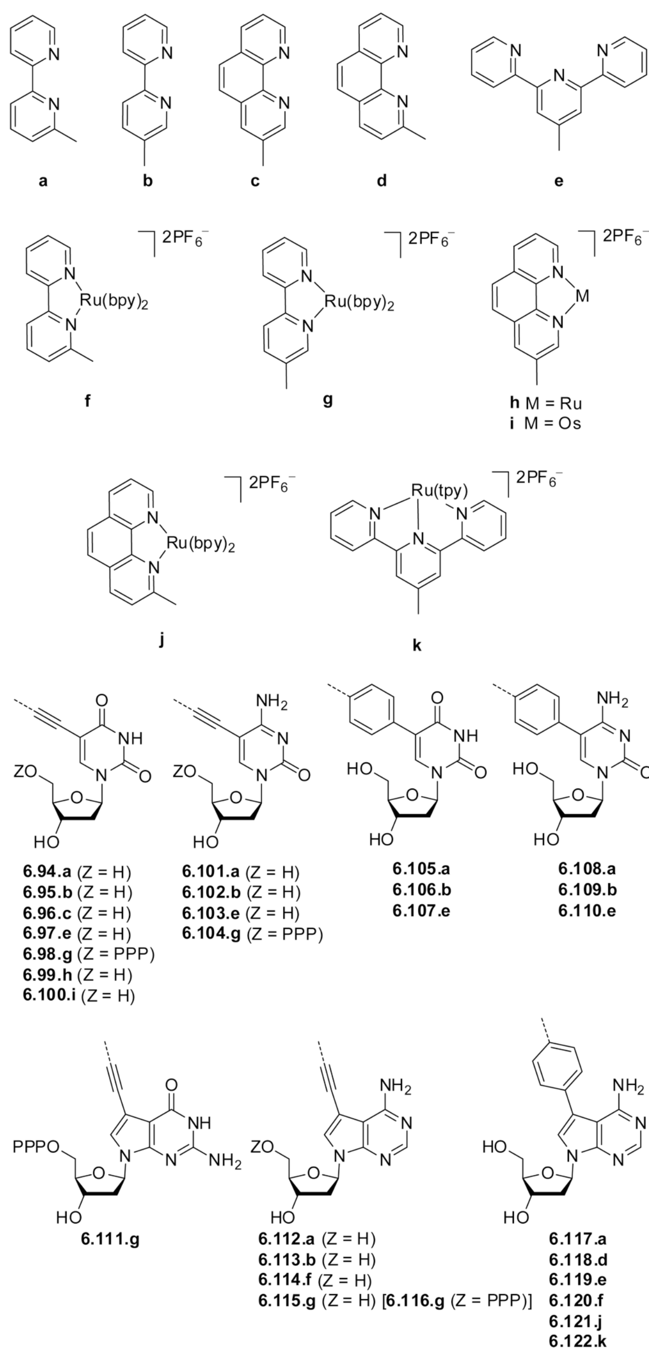

Figure 6.7.

Examples of extended nucleobase analogs.

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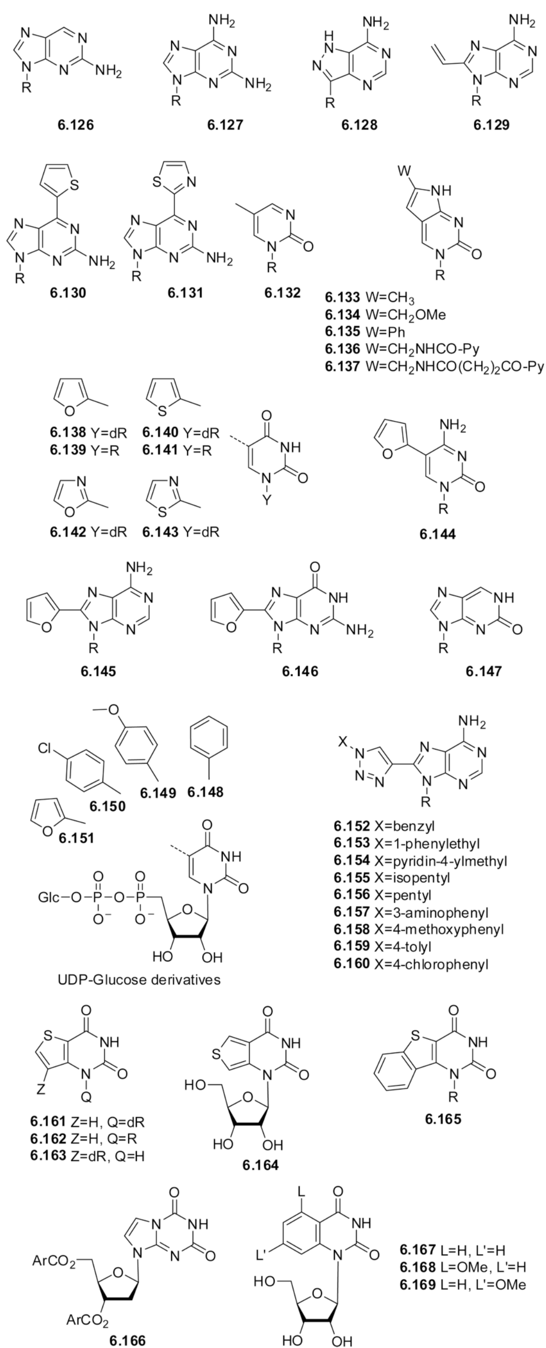

Figure 6.8.

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

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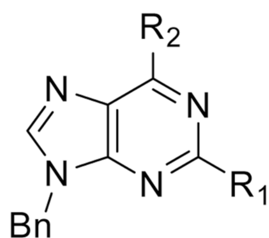
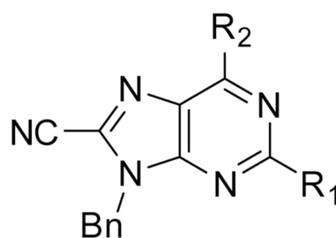
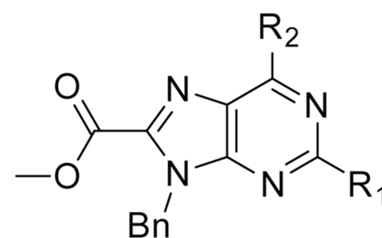


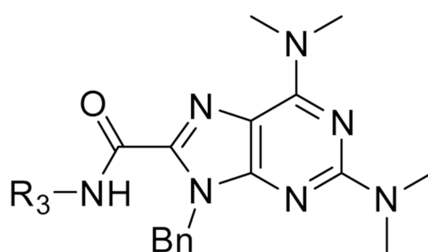




	R_1=	R_2=
6.170	NH_2	H
6.171	$NHCH_3$	H
6.172	$N(CH_3)_2$	H
6.173	NH_2	OBn
6.174	NH_2	$N(CH_3)_2$
6.175	$N(CH_3)_2$	NH_2
6.176	$N(CH_3)_2$	$N(CH_3)_2$

	R_1=	R_2=
6.177	NH_2	H
6.178	$NHCH_3$	H
6.179	$N(CH_3)_2$	H
6.180	NH_2	OBn
6.181	NH_2	$N(CH_3)_2$
6.182	$N(CH_3)_2$	NH_2
6.183	$N(CH_3)_2$	$N(CH_3)_2$

	R_1=	R_2=
6.184	NH_2	H
6.185	$NHCH_3$	H
6.186	$N(CH_3)_2$	H
6.187	NH_2	OBn
6.188	NH_2	$N(CH_3)_2$
6.189	$N(CH_3)_2$	NH_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.

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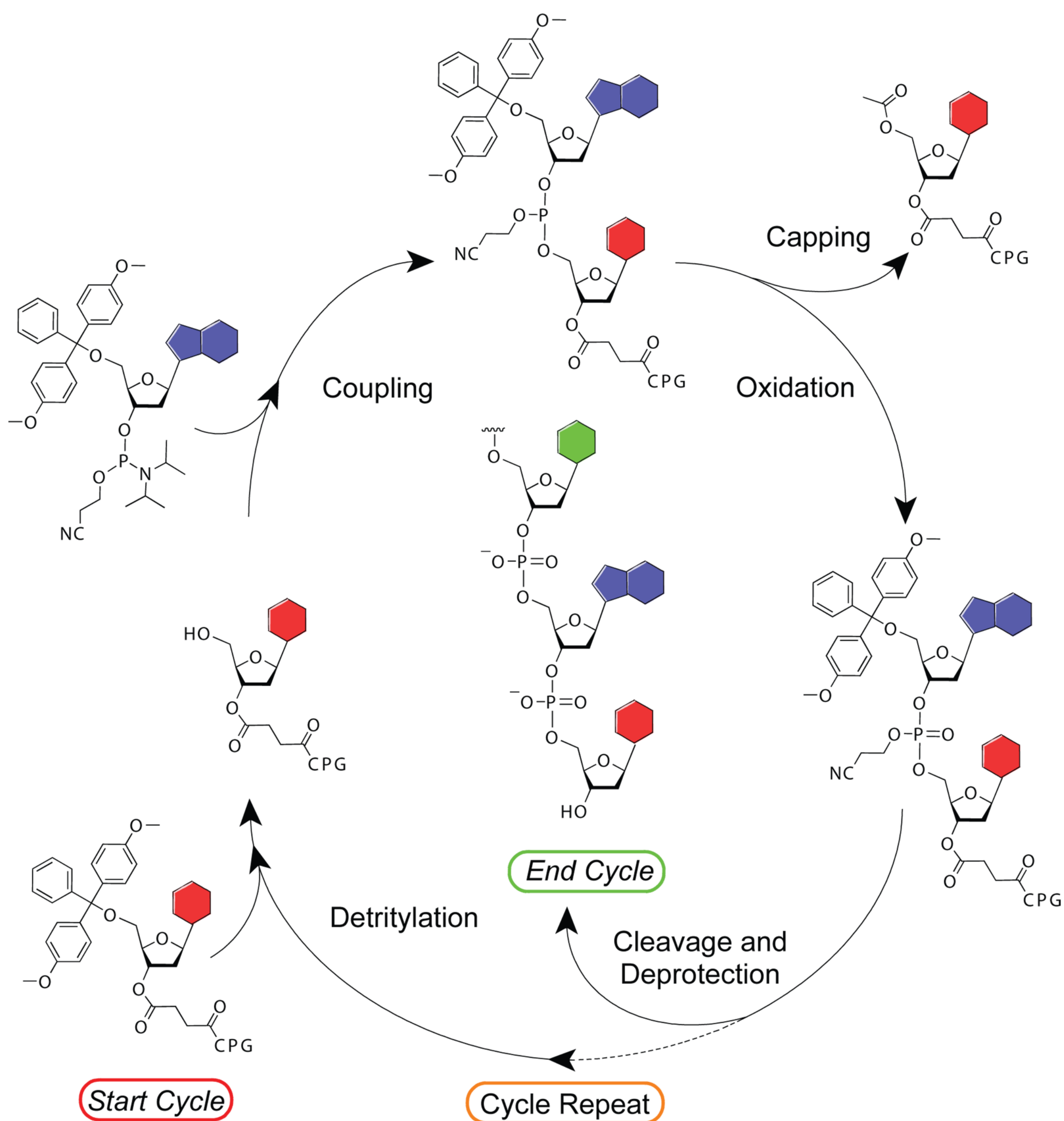

Figure 6.10.
Common cycle for solid-phase assisted phosphoramidite oligonucleotide synthesis.

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Table 1

Spectroscopic Properties of Selected 'Non-Covalent' Probes[a]

#	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]

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Table 2

Spectroscopic Properties of Selected 'Head-Group' Probes[a]

#	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, 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™ website.[150]

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Table 3

Spectroscopic Properties of Selected 'Chain-End' and 'On-Chain Probes[a]

#	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 eximers 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.

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Table 4

Spectroscopic Properties of Selected 'In-Chain' Probes[a]

#	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
4.30	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.

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Table 5

Spectroscopic Properties of Selected Polyenes[a]

#	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[g]	-

[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 as virtually solvent independent.[213]

[e] Data from Acuna *et al.*, average lifetimes given.[214]

[f] Data from Acuna *et al.*[215]

[g] Quantum yield in DMPC vesicles.[215]

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Table 6

Fluorescent Membrane Probes and Selected Applications

	#	Name	Application	Description/Remarks
Non-covalent	**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]	E.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
Head-group	**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

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	#	Name	Application	Description/Remarks
	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]	Haemolytic 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 & 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]	Haemolytic 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

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	#	Name	Application	Description/Remarks
			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 acids' 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 acids' 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
	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	
	4.27	BA-Anthr-FE	D[197]	Probe orientation in vesicles
	4.28	BA-exAnthr-FE	D[197]	Probe orientation in vesicles
In-chain	**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

#	Name	Application	Description/Remarks
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

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Table 7

Spectroscopic Properties of Selected Fluorescent Proteins[a]

#	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.

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Table 8

Spectroscopic Properties of Emissive Native Amino Acids[a]

#	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]

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Table 9

Basic Spectroscopic Properties of Selected Modified Amino Acids[a]

	#	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]	"	"	"	"	"
	5.17	Glu(OMc)[e]	"	"	"	"	"
	5.18	NBDAla[f]	EtOH	264, 330, 462 (19.7)	532	0.38	-
	5.19	NBDLys[f]	"	"	"	"	"
	5.20	EtcbzAla[g]	EtOH	340	437	-	7.8
	5.21	cbzAla[g]	"	"	"	-	"
	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]	"	"	"	"	"
	5.30	9antAla[j]	EtOH	252 (199.5), 338, 357, 376	398	0.30	
	5.31	2antAla	Water	342 (5.2)	384	0.11	-

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	#	Name	Solvent	λ_{max} (ε)	λ_{em}	Φ	τ
	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	-
Imides	**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
	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-methoxycoumarine-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 oligo-glycine.

[i]Values for pyrene.[142,170]

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[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]

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Table 10

Applications of Selected Fluorescent Amino Acids

	#	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 poly peptides
	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

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	#	Name	Applications	Brief description
			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 poly peptides
			G[360]	Eximers 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
Dansy	**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

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	#	Name	Applications	Brief description
Imides	**5.38**	4DAPA	Cl[370]	Incorporation ito 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
	5.42	azoAla	A[374,407]	Studies on a series of azopolypeptides
			E[373]	Photoswitching of an NAD+–mediated enzyme reaction

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Table 11

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

#	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, data from Johnson and Sprecher,[425] Callis[426] and Peon and Zewail[418], respectively.

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Table 12

Spectroscopic Properties of Selected Chromophoric Base Analogs[a]

#	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	-	-	-	-	-
6.19	PhenNH2	-	-	-	-	-
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 9 for photophysical data of anthracene.

[h] Data from Okamoto *et al.*[431]

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Table 13

Spectroscopic Properties of Pteridine Nucleoside Analogs in Buffer[a]

#	Name	λ_{max} (ε)	λ_{em}	Φ	τ
6.21	3-MI	348 254 (3.69), 350 (4.13)[b]	430 -	0.88 -	6.5 -
6.22	6-MI	340 (11.0)	431	0.70	6.4
6.23	DMAP	330	430	0.48	4.8
6.24	6-MAP	310	430	0.39	3.8
		329 (8.51)[c]	-	-	-

[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] In MeOH, from Hawkins.[458]

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Table 14

Spectroscopic Properties of Expanded Nucleoside Analogs[a]

#	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] Photophysical properties measured in buffer at:

[c] pH = 7.0 and

[d] 7.5.

[e] Excitation wavelength.

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Table 15

Spectroscopic Properties of Size Expanded Nucleosides[a]

#	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, and

[d] pH = 3.0.

[e] Data from Wilson and Kool.[427]

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Table 16

Spectroscopic Properties of Conjugated Nucleoside Analogs[a]

#	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	5(difluoroBODIPY)phenyl-dU	MeOH	496 (69.3)	507	0.42	-
6.92	5(fluoromethoxyBODIPY)phenyl-dU	MeOH	497 (69.1)	508	0.42	-
6.93	5(dimethoxyBODIPY)phenyl-dU	MeOH	498 (70.8)	509	0.47	-

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#	Name	Solvent	λ_{max} (ε)	λ_{em}	Φ	τ
6.94	5(difluorodiethylBODIPY)phenyl-dU	MeOH	522 (57.5)	534	0.61	-
6.95	5(fluoromethoxydiethylBODIPY)phenyl-dU	MeOH	523 (55.2)	535	0.62	-
6.96	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. All spectra were collected in

[b] doubly distilled water,

[c] aqueous phosphate buffer (pH=7.0)[427], or

[d] 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] Okamoto *et al.*[499]

[h] Majima *et al.*[500]

[i] Barawkar and Ganesh[501]

[j] Kim *et al.*[502]

[k] Data from Ehrenschwender and Wagenknecht.[503]

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Table 17

Spectroscopic Properties of Conjugated Base Analogs[a]

#	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 **[6.119.g]**	7-[7-deaza-5-Ru(bpy)$_3$-ethynyl]dA [TP analog]	CH_3CN [Buffer[b]]	254 (36.0), 287 (98.0), 384 (26.0) [−]	665 [642]	0.0289 [0.0187]	- [−]

#	Name	Solvent	λ_{max} (ε)	λ_{em}	Φ	τ
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.

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Table 18

Spectroscopic Properties of Isomorphic Nucleoside Analogs in Water[a]

#	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	-

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#	Name	λ_{max} (ε)	λ_{em}	Φ	τ
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) 306[m] (2.07)	370 354	0.31 -	- -
6.168	5-MeOQuinazolinedione	320[l] (6.27) 314[m] (6.13)	395 362	0.16 -	- -
6.169	3-MeOQuinazolinedione	298[l] (6.33) 297[m] (5.00), 306[m] (4.82)	356 335	0.08 -	- -

[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] Spectra were collected in buffer

[d] pH=7.5 (HEPES) and

[e] 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]

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[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 collected in dioxane.

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Table 19

Spectroscopic Properties of Isomorphic Nucleoside Analogs in Dichloromethane[a]

#	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.

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Table 20

Fluorescent Nucleosides and Some of Their Typical Applications

	#	Probe name	Application[ref]	Remarks
Chromophoric base analogs	**6.9**	Coumarin		Time resolved Stokes shift:
			C[428]	Helix interior
			C[446]	Sequence dependence
			C[447,448]	Counter ions
			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
	6.18	Nile red nucleoside	E[431]	Influence of β-cyclodextrin on emission maximum
Pteridines	**6.19**	3-MI	B[456,457]	HIV-1 Intergrase 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
	6.20	6-MI	B[657]	RecA-DNA interactions
			B/C[456]	Interaction of DNA with RNA polymerase
			D[656]	Monitoring Aminoglycoside/RNA binding
	6.22	6-MAP	C[456,658]	Investigating A-tracts
Expanded nucleobases	**6.23**	Etheno-A	B[657]	RecA-DNA interactions
	6.24	Benzo-A	C[470]	Base pairing/stacking effects
	6.25	BgQ	B[476,477]	Triplex formation
			D[659]	Tat–TAR binding
	6.26	C_f	B[476,477]	Triplex formation
			D[660]	Bleomycin analogs cleavage of DNA
	6.27	tC	B[483,485]	Klenow fragment DNA polymerase

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	#	Probe name	Application[ref]	Remarks
			B[661]	Structural measurements on DNA using FRET
	6.28	tC°	B[483]	Klenow fragment DNA polymerase
			B[661]	Structural measurements on DNA using FRET
	6.29	Ç[f]	A[662]	Discrimination of all 4 bases
	6.30	8-oxoG-Clamp	C[488–490]	Detection of 8-oxoG (free nucleoside and incorporated into an oligonucleotide)
	6.31	BPP	A[492]	A vs. G detection
	6.32	NPP	A[493,663]	A vs. G detection
	6.33	^{MD}A	A[494]	T vs. C detection
	6.34	^{MD}I	A[494]	T vs. C detection
	6.35	dC^{hpp}	A[664]	A vs. G detection
	6.39	dC^{ppi}	A[665]	A vs. G detection
Extended nucleobases	6.51	^{PY}U	A[666]	A vs. C detection
			B[667]	RNA poly(A) tracts
			C[668]	Detection of T•Hg•T base pairs
	6.52	^{PY}C	A[666]	G vs. T detection[a]
			B[669]	B to Z transition
	6.53	DMAP-^{PY}U	B[514]	Dual emission
	6.54	5-(1-pyrenyl)-dU	B[670]	Multiple consecutive modified nucleosides
	6.55	8-(1-pyrenyl)-dG	A/B[422]	Mismatch vs. perfect complement Detection/Charge transfer studies
	6.57	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

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#	Probe name	Application[ref]	Remarks
		C[681]	Ultrafast structural dynamics
		B[682]	Duplex stability
		A[683]	Mismatch detection
		E[684]	White-light-emitting DNA
6.58	C^P	B[685]	Molecular beacon/Thermal denaturation
6.59	A^P	B[671]	B to Z transition
		B[672,686]	Molecular beacons
		B[673]	Probe base pairing interactions
		B[678,688]	G-quadruplex transition
		A[680]	A vs. C/G/T detection
		A[689]	Homoadenine signalling system for SNP typing
		B[690]	Photophysics of modified poly-A oligonucleotides
6.60	G^P	B[685]	Molecular beacon/Thermal denaturation
		B[669]	B to Z transition
6.61	A^{PY}	B[691]	RNA hybridization
6.62	U^{PE}	C[675,692]	Charge transfer studies
6.66	5-(perylen-3-ylethynyl)dU	B[682]	Duplex stability
		A[683]	Mismatch detection
6.67	dA^{pymcm}	A and B[693]	Mismatch vs. perfect complement detection Hanging vs. blunt ends detection
6.68	^{MMe}U	B[497]	Hybridization detection
6.69	^{PH}U	B[497]	Hybridization detection
6.70	$^{MeOPh}U$	B[497]	Hybridization detection
6.71	^{PY}A	A[694]	C vs. T detection
6.74	^{DNC}U	B/C[695]	Polarity inside DNA-binding protein (KF)
6.75	^{PDN}U	A[499]	Mismatch vs. perfect complement detection
6.76	^{PDN}C	A[499]	Mismatch vs. perfect complement detection
6.77	^{PDN}A	A[499]	Mismatch vs. perfect complement detection
6.78	^{PDN}G	A[499]	Mismatch vs. perfect complement detection

	#	Probe name	Application[ref]	Remarks
	6.79	^{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)
	6.80	^{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)
	6.81	5-aminodansyl-dU	B/C[501,697,698]	Groove polarity (major/minor interactions and sequence dependence)
	6.82	2-Anthracenecarboxamide-dU	A[699]	Mismatch vs. perfect complement detection
	6.83	9-Anthracenecarboxamide-dU	A[699]	Mismatch vs. perfect complement detection
	6.84	NeT	A/B[700]	A vs. G detection/Hybridization detection
	6.85	AeT	A/B[700]	A vs. G detection/Hybridization detection
	6.86	AeeT	A/B[700]	A vs. G detection/Hybridization detection
	6.87	U^{FL}	A[502]	Mismatch vs. perfect complement detection
	6.96.c	5-[3(phen)ethynyl]dU	A[531]	Discrimination of all 4 bases
	6.98.g	5-[5-Ru(bpy)$_3$ethynyl] dU-TP	A[538]	Luminescent and electrochemical detection
	6.104.g	5-[5-Ru(bpy)$_3$ethynyl] dC-TP	A[538]	Luminescent and electrochemical detection
	6.111.g	7-[7-deaza-5-Ru(bpy)$_3$ ethynyl]dG-TP	A[538]	Luminescent and electrochemical detection
	6.116.g	7-[7-deaza-5-Ru(bpy)$_3$ ethynyl]dA-TP	A[538]	Luminescent and electrochemical detection
Isomorphic nucleobases	**6.123**	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

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#	Probe name	Application[ref]	Remarks
		C[713]	Strength of BP interactions
		D[656,714–717]	Aminoglycoside/RNA binding
		B/C[718–720]	Interaction of DNA with RNA polymerase
6.126	8-vinyl-6-aminopurine	A[721]	short/long excited state lifetimes
6.127	2-amino-6-(2-thienyl)purine	B[571]	Detection of fluorescently labeled complementary strand
		B[569]	Denaturation of RNA (hairpin and tRNA)
6.128	2-amino-6-(2-thiazolyl)purine	B[571]	Detection of fluorescently labeled complementary strand
6.129	m^{5}K	B[657,722]	ssDNA–*Escherichia coli* RecA binding
6.130	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
6.131	^{MMe}pC	B[608]	Hybridization detection
6.132	^{Ph}pC	A[609]	Mismatch vs. perfect complement detection
6.133	^{PyI}pC	B[729]	Molecular beacon
6.134	^{PyII}pC	B[729]	Molecular beacon
6.135	5-(fur-2-yl)dU	C[589]	Abasic site detection
		B/C[11]	Major groove polarity
6.136	5-(fur-2-yl)U	D[595]	Tat/TAR binding
		D[591]	aminoglycoside/A-site binding
6.141	5-(fur-2-yl)dC	C[596]	Detection of 8-oxoG and its transverse mutation product
6.161	Thieno[3,4-*d*]-U	A[597]	C mismatch detection
		B/C[599]	Activity of toxic ribosome-inactivating protiens

#	Probe name	Application[ref]	Remarks
6.162	^{BT}U	B[548]	Hybridization detection
6.168	5-MeOQuinazolinedione	D[600]	FRET enabled detection of aminglycoside-RNA binding

[a]Amide moiety is vital to probe sensitivity.

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