Fluorescent DNA base replacements: reporters and sensors for biological systems

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We describe the design, synthesis, and properties of nucleoside monomers in which the DNA base is replaced by fluorescent hydrocarbons and heterocycles, and the assembly of these monomers into DNA-like molecules in which the all bases are fluorescent. As monomers, such molecules have useful applications as reporters in the DNA context. The use of fluorescent DNA bases, rather than more traditional fluorophores tethered to the DNA strand, gives a more predictable location and orientation, and yields a more direct response to changes that occur within the helix. In addition to uses as monomers, such compounds can be assembled into polychromophoric oligomers ("oligodeoxyfluorosides" or ODFs). ODFs are water soluble, discrete molecules and are easily arranged into specific sequences by use of a DNA synthesizer. They have displayed a number of properties not readily available in commercial fluorophores, including large Stokes shifts, tunable excitation and emission wavelengths, and sensing responses to physical changes or molecular species in solution. We describe an approach to assembling and screening large sets of oligofluorosides for rapid identification of molecules with desirable properties. Such compounds show promise for applications in biochemistry, biology, environmental and materials applications.

I. Introduction

Fluorescent DNA base analogues have been known for more than three decades.[1,2] Since the earliest reports there has been a steady increase in the study of this class of molecules. The rising interest in such compounds comes from several developments in organic chemistry, physics, engineering, biochemistry, and biology. First is the wide recognition of fluorescence as an extremely powerful tool in biochemistry and biology.[3] Much of the power of fluorescence comes from the large signal and low background signals that can be achieved. Also important in the development of fluorescence as a tool has been the development of a wide array of instruments that take advantage of fluorescence, including microscopy, flow cytometry, spectrometry, and of photophysical methods such as steady-state emission and/or excitation, time-resolved fluorescence, anisotropy, fluorescence resonance energy transfer (FRET), two-photon absorption, and quenching. Also enhancing interest in

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fluorescence has been the realization that this property can arise in a wide variety of molecules and structures, including many classes of organic frameworks (coumarins, fluoresceins, cyanines, bodipy dyes, to name just a few), in proteins such as green fluorescent protein, and even with inorganic particles such as quantum dots.[3,4] This variation in molecular and electronic structure leads to a wide range of photophysical properties such as molar absorptivity, quantum yield, Stokes shift, lifetime, and ability to respond to the environment and other electronic systems. Fluorescent DNA base analogues have begun to increasingly take advantage of this wide variety of properties and applications of fluorescence in general.

The topic of this Perspective is nucleoside compounds in which a natural DNA base is replaced by an entirely different structure having fluorescent emission. At first glance this may seem to be a rather narrow class of molecules; however, we hope to convince the reader that the opposite is true, and that an extremely broad array of molecules and properties can come from this type of structure. Indeed, almost the only thing in common for the entire class besides fluorescence is a sugar substituent; virtually everything about these molecules—their structure, their properties, and their applications, can vary widely. Moreover, we will demonstrate that they can be useful not only as individual structures, but also in oligomeric assemblies, where an even broader variation in molecules and properties can exist.

Until relatively recently, fluorescent DNA base analogues have been used most commonly in basic science applications. These compounds can be used in the context of DNA or RNA or mononucleotides, and since these species play a role in an impressively large number of biological processes, fluorescent DNA bases have been applied in the study of quite a number of these different biosystems. These fluorescent reporters have been used in structural studies of DNA and RNA, and in enzymatic processes involving DNA such as DNA repair and DNA replication. Some examples of these kinds of basic applications will be given below.

In addition to the basic science, very recently there have also appeared a number of molecular strategies for applying fluorescent DNA base analogues in biotechnological settings. Examples of such applications include sensing of single nucleotide polymorphisms (SNPs), and in sensing physical conditions and molecular species in solution. It seems likely that such uses will increase in the coming months and years. A few examples of this type of application will also be described here.

To be sure, there are numerous ways to label DNA, RNA and mononucleotides with fluorescence, and not all of those ways involve the use of fluorescent DNA base analogues. Indeed, it is quite common to tag synthetic oligonucleotides (ODNs) with fluorescence labels added at either end of the strand, and these tags can be added either during the synthesis of the oligonucleotide, or afterwards in a post-synthetic strategy. In addition, it is common to attach fluorophores to DNA bases by tethers attached typically at the 5-carbon of C or T(U) or at the 7-position of 7-deazapurines. This allows the positioning of fluorescent tags at many (if not all) sites along a DNA helix. One of the most important examples of the use of such a labeling strategy is in methods for sequencing DNA.[4a] Thus, given these other common strategies for labeling DNA, one may well ask why we need to replace DNA bases with fluorescent analogues. The answer to this lies in the fact that there are many different biological systems and applications in which fluorescence can be useful, and the different systems and structures place varied requirements on the reporter molecules being used. One chief difference between tethering known fluorophores to DNA, and replacing DNA bases directly with fluorescent analogues, is the tether itself. Typical tethers for attaching fluorophores to DNA involve several to as many as 1–2 dozen bonds, and often make use of simple linear alkyl chains. This flexible tethering results in the fluorophore having a good deal of mobility on a rapid timescale, and leads to uncertainty in the fluorophore's location and orientation. While this may not be a problem in some applications, it is directly important to a few where distance and structure play a central role. In addition, the tether and the attached fluorophore adds a considerably bulky group onto the DNA or especially onto a nucleobase, when it is attached there. This can easily negatively affect the biochemical or biological properties—again, not in all cases, but in some important ones.

In contrast to this, fluorescent DNA base replacements can be considerably smaller and less disruptive to local structure and to interactions with other biomolecules. As with natural DNA bases, the DNA backbone arranges fluorescent DNA base analogues in a position to potentially stack directly within the double helix. Not only does this place them at the "center of action" in a number of biological processes such as DNA repair and replication, but it also can give them a more certain, rigid and predictable location and orientation. This can be useful in applications such as in distance measurements with FRET, and in time-resolved studies. Finally, the lack of bulky sidechains and tethers also can open the possibility of retention of biological activities that might otherwise be blocked by the added bulk. Thus it seems certain that fluorescent DNA base replacements will continue to find useful applications in biological studies because of the unique properties that these compounds bring.

The aim of this Perspective is to give a brief background on the historical development and applications of fluorescent DNA base replacements, and then to focus on recent literature and uses of this class of compounds. We will not cover the topic of natural DNA bases with known fluorophores tethered or attached to them as substituents; those compounds are part of the more classical strategy for labeling DNA, as mentioned above. In this Perspective there will be special emphasis on work in this area from the authors' laboratory, and so the review of the literature is not intended to be comprehensive. We hope that a brief glimpse into one laboratory's ongoing research will not only present one strategy for development of these molecules, but also pique the reader's interest in the field as a whole.

II. Fluorescent nucleobases: early examples

One of the earliest reports of fluorescent nucleobase substitutes was of adenosine analogs formycin (**1**), 2-aminopurine riboside (**2**) and 2,6 diaminopurine riboside (**3**) (Fig. 1).[5] In their pioneering study, Ward *et al.* explored the photophysical properties of the monomers and polynucleotides under different pH's, solvent polarities and temperatures (Table 1). Fluorescent ribosides **1–3** are characterized by absorbance maxima ranging from 280–303 nm—slightly red shifted compared with natural bases. Emission maxima ranged from 340–370 nm, which is also red shifted compared with natural bases with **1** and **3** being only weakly emissive, while **2** is highly emissive. Fluorescence studies of tRNA incorporating

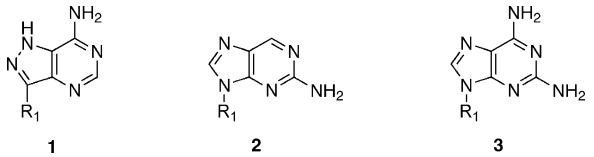

Fig. 1 Structures of fluorescent ribosides **1–3**; R_1 = ribose.

Table 1 Photophysical data[a] of fluorescent ribosides **1–3**[5]

	λ_{max}, abs/nm	λ_{max}, em/nm	Φ_{em}	τ/ns
1	295	340	0.06	<1
2	303	370	0.68	7
3	280	350	0.01	<1

[a] Data obtained in aqueous buffer, pH = 7.0.

1 at the nucleoside terminus showed significant dependence on temperature and pH demonstrating the utility of fluorescent nucleobases in determining interactions in biomacromolecules.

A fluorescent analogue of the coenzyme NAD+ was reported by Leonard.[1] Reaction of NAD+ with chloroacetylaldehyde generated nicotinamide 1,N6-etheno-modified adenine dinucleotide, (εNAD+, **4**) (Fig 2). The optical properties of εNAD+ and 5′ εAMP (**5**) were compared (Table 2). Both molecules have a maximum absorption between 265 and 275 nm, but a prominent shoulder of lower energy allows for longer wavelength excitation at 300 nm. The emission maximum of both molecules is 410 nm. This represents a Stokes shift of over 100 nm, pushing emission into the visible range when compared to earlier fluorescent nucleoside analogues. The quantum yield of **4** is only 0.07 while that of **5** is 0.56. The low quantum yield and shorter reported lifetime in the dinucleotide were indicative of quenching by the nicotinamide moiety. Hydrolysis by NADase resulted in increased fluorescence confirming the quenching by intermolecular interaction. The authors concluded that such systems would function as effective fluorogenic probes for enzymatic activity.

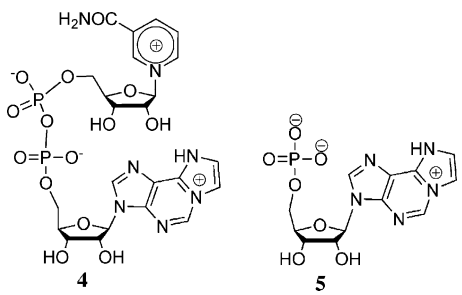

Fig. 2 Structures of fluorescent dinucleotide **4** and nucleotide **5**.

Table 2 Photophysical data[a] of fluorescent ribosides **4**, **5**[1]

	λ_{max}, abs/nm	λ_{max}, em/nm	Φ_{em}*	τ/ns
4	265	410	0.07	—
5	275	410	0.56	23

[a] Data obtained in aqueous buffer, pH = 7.0.

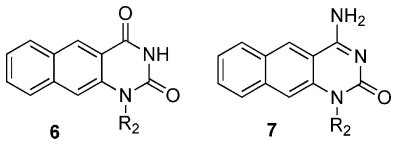

Fig. 3 Structures of **6** and **7**; R_2 = deoxyribose.

Table 3 Photophysical data of fluorescent ribosides **1–3**[2]

	λ_{max}, abs/nm	λ_{max}, em/nm	Φ_{em}
6	360	434	0.82
7	370	456	0.62

[a] Data obtained in aqueous buffer, pH = 7.0.

Godde *et al.* reported the synthesis of fluorescent thymidine and deoxycytidine derivatives and their emissive behavior in duplex and triplex formation (Fig. 3, Table 3).[2] A naphtho-substituted T-analogue, **6**, displays absorption maxima at longer wavelengths relative to dT with strong emission at 434 nm. Deoxycytidine analogue **7** was even further red shifted with respect to absorbance and emission spectra, with a slightly lower quantum yield. The emissions of both **6** and **7** were sensitive to neighboring bases and to oligonucleotide structure. When **6** was incorporated into strands containing only T and C, emission was lowered by only 15%, though, when placed between two Gs, emission intensity was lowered by 99%. Little change in the emission maxima or intensity of **6** was found upon duplex formation; however, changes observed upon triplex formation were more pronounced when the fluorescent base was on the Hoogsteen strand compared with the Watson–Crick strand. C analogue **7** was also shown to be sensitive to pH and thus served as a probe to determine protonation states in triplex formation.

III. Fluorescent hydrocarbons

Although the earliest examples of fluorescent DNA base replacements were heterocycles (like DNA bases themselves), we showed that polycyclic hydrocarbons could also function as base replacements. Nucleosides of polycyclic aromatics such as naphthalene, phenanthrene and pyrene (**8**) can be synthesized by coupling organocadmium or organozinc derivatives of the aromatic "bases" with Hoffer's chlorosugar.[6] The α-epimers can be obtained as major products, which can either be used in that form or subjected to epimerization to provide β-deoxyribosides. Early studies of pyrene and phenanthryl nucleosides revealed emission behavior similar to the parent fluorophores (*e.g.*, λ_{max} = 375 nm, Φ = 0.12 for **8**). However, when incorporated into oligonucleotides, **8** was significantly quenched due to interactions with neighboring bases.

More recently the collection of fluorescent hydrocarbon nucleosides has been expanded to include deoxyribosides of perylene (**9**), its O-linked variant oxoperylene (**10**), and benzopyrene (**11**) (Fig. 4, Table 4).[7] These compounds display blue emission with high quantum yields even in protic solvents (Φ = 0.88–0.98). Other groups have also utilized **9**: a set of DNAs incorporating both perylene nucleosides and "caps" was reported.[8] The perylene nucleosides were of two types, with the perylene either directly attached to the 1′ position (**9**) or attached *via* an alkyl tether. The

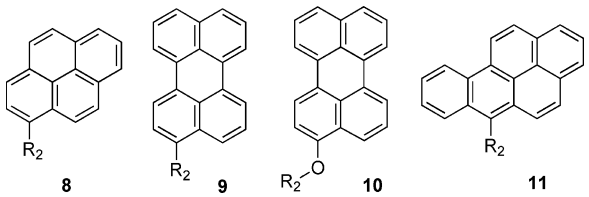

Fig. 4 Structures of fluorescent hydrocarbon nucleosides **8–11**; R_2 = deoxyribose.

Table 4 Photophysical data[a] of fluorescent ribosides **8–11**[6,7]

	λ_{max}, abs/nm	ε/cm^{-1}	λ_{max}, em/nm	Φ_{em}
8	241, 345	39 000	375, 395	0.12
9	440	39 200	443, 472	0.88
10	444	25 100	461, 487	0.81
11	394	28 200	408	0.98

[a] Data obtained in methanol.

inclusion of the large aromatic base at multiple positions was found in most cases to stabilize both duplexes and triplexes, with a larger stabilization found in the latter structure. Only small changes in fluorescence intensity were observed for the ODNs containing one perylene unit. Greater increases in fluorescence intensity were found when ODNs containing two perylenes were hybridized with their complements.

It has been reported that large aromatic non-hydrogen bonding bases can "pair" stably opposite deoxyribose residues lacking bases (*i.e.*, abasic sites) in dsDNA.[9] A pyrene–abasic pair was as stabilizing to the double helix as an A–T pair in some contexts. An NMR structure of dsDNA with a pyrene–abasic pair showed that the pyrene resides inside the helix opposite its abasic partner. This behavior emphasizes the central role that stacking of the bases plays in stabilization of duplex DNA.

An intercalating pyrene "pseudo-nucleotide" was reported to discriminate DNA and RNA based on fluorescence intensity and differences in T_ms.[10] A pyrene fluorophore linked to a glycerol backbone was incorporated into ODNs to create "intercalating nucleic acids" (INAs). When the "base" was included as a dangling end of a self-complementary 6-mer and 8-mer, it was found to increase T_ms substantially; however, when incorporated in place of a complementary base, it was found to decrease the T_m. When introduced as an intercalating bulge, a single pyrene increased T_m in duplex DNA. A double-bulge-containing DNA–INA complex could be discriminated from RNA–INA complexes based on substantial differences in T_ms as well as increased quenching in the DNA–INA complex.

IV. Other examples of fluorescent base replacements

Coleman reported the synthesis of C-linked coumarin deoxyriboside, **12**, as a fluorescent probe of DNA dynamics (Fig. 5, Table 5).[11a] Coumarin dyes are known for their high quantum yields, and coumarin 102 has an absorption maximum at 400 nm which allows for its selective excitation in the presence of natural DNA bases. The coumarin fluorophore was attached to the sugar *via* a palladium-catalyzed coupling of the triflate derivative of coumarin 102 to an alkenyl glycal. The resulting coumarin nucleoside was incorporated into ODNs using automated synthesis and

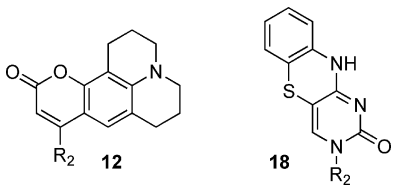

Fig. 5 Structures of fluorescent nucleosides **12** and **18**. R_2 = deoxyribose.

Table 5 Photophysical data[a] of fluorosides **19**, **27**[11,14b]

	λ_{max}, abs/nm	λ_{max}, em/nm	Φ_{em}
12	400	515	—
18	375	500	0.20

[a] Data obtained in aqueous buffer (**12**, pH = 7.2; **18**; pH = 7.5).

paired opposite a tetrahydrofuran abasic site in duplex DNA. This led to a moderate decrease in T_m. The coumarin "base" was subsequently employed as a fluorescent probe of the dynamics in the interior of the DNA duplex by time-resolved Stokes shift spectroscopy.[11b]

Saito has produced a number of fluorescent nucleosides which can roughly be divided into two types: novel expanded-ring-system nucleobases[12] and nucleobases with pendant fluorophores (Fig. 6, Table 6).[13] Attractive fluorescent properties in **13–15** are gained by fusing benzo- and naphtho-ring systems to hydrogen bonding aromatic rings. These bases are able to hydrogen bond opposite natural nucleobases and produce substantial emission responses

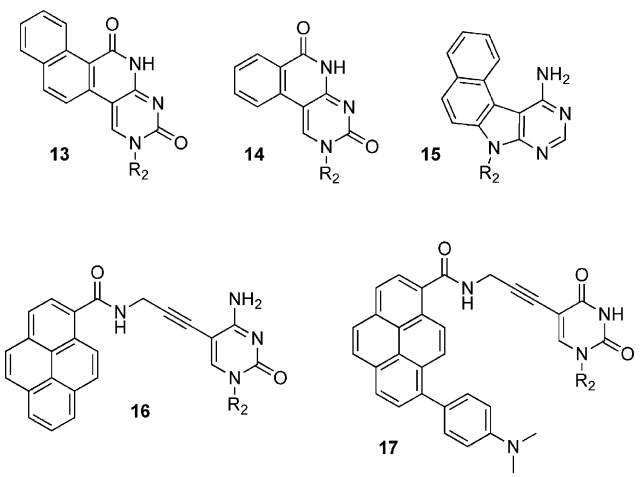

Fig. 6 Examples of fluorescent nucleobases reported by Saito. R_2 = deoxyribose.

Table 6 Photophysical data[a] of fluorosides **13–17**[12a,c,13a,d]

	λ_{max}, abs/nm	λ_{max}, em/nm	Φ_{em}
13	365	395	0.26
14	347	390	0.04
15	355	383	0.027
16	329	393	0.147
17	303	440 (LE), 540 (ICT)[b]	0.01

[a] Data obtained in aqueous buffer (pH = 7.0). [b] LE = locally excited state; ICT = intermolecular charge transfer state.

leading to their moniker as 'base-discriminating-fluorosides' (BDFs).

Saito has also reported several pendant fluorophore-labeled nucleobases which combine a fluorophore *via* a short tether to a natural nucleoside (**16**,**17**). This class of fluorescent nucleobase may also function as BDFs capable of single nucleotide discrimination: in the presence of a perfect match, the pyrene is located outside the helix, eliminating quenching interactions with neighboring bases. DNA dynamics can also be monitored by this type of construction using a donor–acceptor substituted pyrene tethered to a dU nucleoside (**17**).[13a] This fluorescent nucleoside probe was able to differentiate between ss and ds DNAs by modulating ICT *vs.* LE fluorescence, which correspond to emission at 540 and 440 respectively.

While many fluorescent nucleosides, such as 2-aminopurine, suffer from emission quenching when incorporated into ODNs or duplexes, **18** is reported to be largely insensitive to luminescence-diminishing interactions with neighboring bases (Fig 5, Table 5).[14] This expanded ring structure based on cytidine has a long wavelength absorption maximum at 375 nm and emits in the green region of the spectrum with a maximum at 505 nm. The lack of quenching of **18** in ODNs has been explained by its lower oxidation potential relative to G, which may eliminate PET as a quenching mechanism. Through NMR and anisotropic characterizations, **18** has been demonstrated to occupy a well-defined geometry within duplex DNA, and experiments have shown that it may increase the stability of duplexes in which it is substituted.[14a]

V. Fluorescent metal ligands

Organic ligands for metals are quite often constructed from flat aromatic heterocycles, and thus this structural feature is shared in common with DNA bases. A substantial number of such metal ligands can be fluorescent either alone or in complexes with metals. For example, the synthesis of a porphyrin deoxyriboside produced a DNA monomer that can function both as a fluorophore and as a metal ligand (Fig. 5, Table 5).[15] Porphyrins are characterized by high molar absorptivities making them interesting chromophores and sensors. Porphyrin nucleoside **19** has an absorbance maximum (Soret band) at 400 nm with additional less pronounced transitions at longer wavelengths (Fig. 7, Table 7). Upon photoexcitation, it fluoresces a deep red. Although the quantum yield is moderate, the emission intensity remains relatively constant regardless of the neighboring bases, which can quench the emission of other fluorescent bases such as 2-aminopurine by almost 100-fold. While the nucleoside was found to be destabilizing when situated in the middle of a duplex opposite a base or an abasic site, CD spectroscopy indicated that the porphyrin 'base' was located within the duplex, which is not unexpected given its large hydrophobic structure. Interestingly, the porphyrin nucleoside was found to increase T_ms moderately at the end of a duplex.

A number of authors have reported examples of metal "ligandosides" (a term coined by Tor in describing a bipyridyl nucleoside).[16–19] While it is not reported whether many of the bases are fluorescent, there often were changes in the absorption spectra upon complexation. For example, bipyridyl "base-pairs" are formed when complementary sequences containing them are complexed in the presence of Cu^{2+} giving rise to a red shoulder (*ca.* 310 nm) in the absorbance spectra and a concomitant increase in T_m.[16] A self-assembled array of metal ions was described by Tanaka *et al.*, utilizing hydroxypyridone nucleobases paired around a Cu^{2+} ion.[19] Duplex formation of sequences containing varying numbers (1–5) of the artificial "base" was confirmed by several methods including CD and UV–vis spectroscopy. Other authors have reported metal coordinating base pairs but changes in absorption or emission were not specifically noted.[17,18]

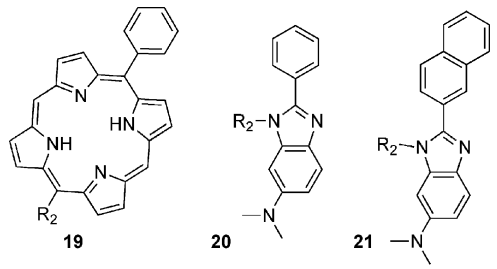

Fig. 7 Structures of fluorescent 'ligandosides' **19–21**. R_2 = deoxyribose.

Table 7 Photophysical data of fluorescent deoxyribosides **19–21**[15,20]

	λ_{max}, abs/nm	ε/cm^{-1} M^{-1}	λ_{max}, em/nm	Φ_{em}	τ/ns
19	400	32 400	629	0.11	—
20	360	13 700	494	0.05	0.45
21	370	13 600	539	0.009	0.36

[a] Data obtained in methanol.

We have reported pyridobenzimidazole nucleosides (**20**, **21**) which function both as metal ligands and as fluorescent nucleobases with the goal of detecting a range of metal ions through various emissive responses (Fig. 7, Table 7).[20] Nucleoside **20** has an absorption maximum at 360 nm and the benzo-expanded derivative **21** has a slightly longer absorption maximum at 370. Both compounds emitted in the visible range with maxima at 494 and 539 nm, respectively. The nucleosides were screened individually for emission responses to various transition metal ions. The fluorescent nucleobases also demonstrated cooperative behavior when combined in dsDNA sequences as "base-pairs", leading to emission responses that were in some cases entirely different than the responses of either ligandoside alone.

VI. Expanded-size DNA bases

Expanding the π system of DNA bases can result in attractive optical properties such as longer wavelength excitation as well as enhanced emission. When the hydrogen bonding groups of natural bases are retained, this can lead to useful features. One of the first reported examples of this type of fluorescent base, was Leonard's *lin*-benzoadenosine (**22**), in which the purine ring system was increased in size by insertion of a benzene ring (Fig. 8, Table 8).[21b,c] The benzo-expanded π system displayed red shifted absorption maxima relative to dA (348 nm *vs.* 280 nm) as well as bathochromically shifted emission maxima (372 *vs.* 315 nm).[22] Importantly, **22** has a relatively high quantum yield compared to dA, which has a negligible quantum yield in solution at room temperature.

More recently we described the synthesis of a complete set of analogous benzo-expanded nucleosides (**23–26**).[23a,c] The base-pairing and helix forming properties of these expanded DNA

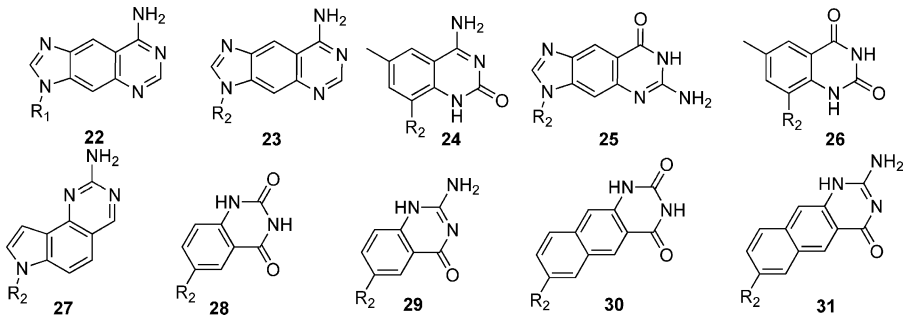

Fig. 8 Structures of expanded fluorescent nucleosides which maintain the hydrogen bonding patterns of natural DNA. Top row, 'xDNA', bottom row 'yDNA' and 'yyDNA'. R_1 = ribose; R_2 = deoxyribose.

Table 8 Summary of optical data[a] for expanded nucleobases **22–31**[21,23–25]

	λ_{max}, abs/nm	ε/cm^{-1} M^{-1}	λ_{max}, em/nm	Φ_{em}
22	340, 356	—	358, 379, 395	0.44
23	231, 260, 333	11 000	393 530 (excimer)	0.44
24	330	4100	388	0.52
25	320	3400	413	—
26	320	3400	377	0.30
27	262, 355	—	433	—
28	231, 312	33 900, 2780	375	0.40
29	221, 315	38 100, 3040	390	0.54
30	262, 362	30 100, 1510	446	0.67
31	262, 371	51 500, 1690	433	0.35

[a] Data obtained in methanol except **22** measured in aqueous buffer (pH = 7.0).

("xDNA") bases have been studied in detail.[23b,c,d] Their optical properties make them quite interesting as fluorophores with potential applications in DNA sensing. The longer-wavelength absorption (Table 8) allows them to be selectively excited in the presence of natural DNA bases. They also display relatively high quantum yields, another desirable feature of fluorescent nucleobases. The longest wavelength emission for an xDNA base is that of dxG (**25**) which emits at 413 nm.

Another set of stretched nucleobases, called yDNA (short for "wide DNA") has also been reported.[24] These too display longer-wavelength absorption and emission. In particular, yA (**27**) has an emission maximum of 433 nm, which is 40 nm further red shifted than its xDNA counterpart. The emission maxima of yC (**29**) and yT (**28**) are only slightly shifted relative to xC and xT. Other interesting emission properties have also been noted; for example, sequences containing multiple xAs exhibit an additional low-energy emission peak centered at ~510 nm. This peak increases in intensity with an increasing number of xA residues included in the ODN strand and could be ascribed to excited state interactions such as excimer formation between neighboring xA chromophores.

Very recently, we have reported examples of naphtho-expanded derivatives of C and T bases, yyC (**31**) and yyT (**30**).[25] These doubly expanded analogues exhibit absorption maxima even further red shifted from the singly-expanded nucleobases and exhibit intense blue emission (λ_{max} = 433 and 446 nm, respectively).

VII. Applications of fluorescent nucleobases in DNA sensing

Fluorescent nucleobases have recently played an important role in the development of new strategies for detecting DNA hybridization and discriminating between SNPs. Multiple modes of detection are possible including emission enhancement, quenching or changes in emission wavelength. For example, many large aromatic hydrocarbons are known to form excited state dimers (excimers) when two or more molecules are in close proximity and one is promoted to a photoexcited state. Pyrene excimer switching has recently been used in a molecular beacon type construct to detect target DNAs.[26]

Using the previously reported pyrene nucleoside **8**,[6] we designed an excimer-based scheme for detecting DNA sequences. In this strategy, two adjacent strands containing pyrene nucleobases each form part of a complementary strand to a DNA target.[27] When both strands hybridize on the target, the excimer fluorescence can be detected. A set of targets was designed to explore the distance and conformation dependence for excimer formation. It was found that having "dangling" bases formed excimers most efficiently, apparently allowing the bases to achieve the right orientation for excimer formation. This sensor route successfully detected a known *ras* codon 12 point mutation *in vitro* as a proof-of-principle demonstration.

Saito has introduced a set of base-discriminating fluorescent nucleobases (BDFs). BDFs constructed from pyrene-labeled U (**17**) and C (**16**) selectively bond to their Watson–Crick partner, A and G respectively.[13d] When confronted with a mismatch, the pyrene instead of the nucleobase, is believed to be inserted into the duplex and emission is subsequently quenched. When properly paired, the pyrene is located outside the duplex and is not subject to quenching by neighboring bases. This results in so-called "turn-on" fluorescent sensors. Another example of a pyrene-labeled nucleoside is PyA which is actually quenched in the presence of T.[13b] However, a turn-on sensor for T was achieved by using the slightly altered 8PyA.[13c] BPP nucleoside can also discriminate between A and G.[12a] In the presence of A, it is brightly fluorescent. A naphtho-expanded A analogue selectively detected C by emission enhancement as a FRET donor to fluorescein.[12b] Taken together, the BDF nucleobases represent an impressive collection of fluorescent base replacements that can be used to detect SNPs and base-mismatches.

VIII. Applications in enzymatic activity

Much of the interest in fluorophore nucleobase replacements is centered around their ability to mimic natural nucleobases while at the same time reporting the activity of enzymes acting upon them by optical means. Some of the earliest examples were reported by Leonard, who employed adenosine analogues as fluorescent reporters.[1] Here the long wavelength absorption and emission properties of these novel nucleobases were employed to eliminate interference from UV active amino acid residues and natural nucleobases. εATP was found to be a suitable substrate for ATP in several enzyme systems including adenylate kinase, phospho-fructokinase pyruvate kinase, and hexokinase. Binding and activity were on the same order of magnitude as the natural substrate for all of these enzymes and in one case could be followed by a simple TLC of the reaction monitoring the spots by their fluorescence. Expanded adenosine analogue **22** was found to be a suitable substrate for calf intestinal adenosine deaminase, producing the *lin*-benzohypoxanthine derivative of **22**. This compound, in turn, was found to be an active metabolite for xanthine oxidase.

RNA polymers containing the ATP analogues **1** or **2** were prepared using synthetic templates and *E. coli* RNA polymerase to produce alternating copolymers of ATP analogues and uracil derivatives.[5] The emission intensity of the polynucleotides was 2 to 3 orders of magnitude less than the corresponding monomers. Furthermore, it was found that the residual fluorescence of the polynucleotides resulted from 2-aminopurine residues near the terminus of the chains. Thermal denaturation of the double helices increased the emission intensity of the polynucleotides by a factor of 3 to 10. The authors speculated on the mechanisms of quenching suggesting effects due to the close packing of the bases and concluded that fluorescent analogues of ATP would allow investigations into the structural dynamics of nucleic acids as well as their interactions with enzymes.

A set of fluorescent analogues based on a pteridine ring system was reported by Pfleiderer including A (**32**) and G (**33**) analogues as well as benzo-, naphtho- and anthra-appended analogues (**34–36**) (Fig. 9, Table 9).[28] The fluorescent nucleosides were incorporated into oligonucleotides by automated synthesis. The larger ring systems in **34–36** were found to have a small stabilizing effect on duplex T_ms, possibly due to increased stacking ability, while **32** and **33** were slightly destabilizing. Quenching of the fluorophores occurred when incorporated into oligonucleotides; however, this allowed for so-called "turn-on" emission-based assays when the fluorescent nucleotides were released from the parent oligonucleotide. Guanosine analogue **33** was used in a real-time fluorescence assay for activity of HIV-1 integrase while adenosine analogue **32** was employed in a P1 nuclease digestion assay.

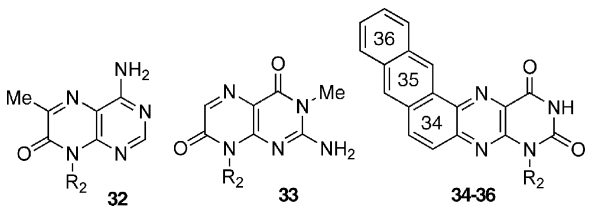

Fig. 9 Structures of fluorescent nucleosides **32–36**. R_2 = deoxyribose.

Table 9 Photophysical data[a] of fluorescent deoxyribosides **32**, **33**[28]

	λ_{max}, abs/nm	ε/cm^{-1} M^{-1}	λ_{max}, em/nm	Φ_{em}	τ/ns
32	310	—	430	0.39	3.8
33	350	41 000	425	0.88	—

[a] Data obtained in aqueous buffer (**32**, pH = 7.5; **33**, pH = 7.6).

Large polycyclic aromatic bases such as pyrene can function as effective base pairs for abasic sites and can even be incorporated into a growing strand of DNA during replication.[29] The Klenow fragment of *E. coli* DNA polymerase I was shown to pair the 5′-triphosphate derivative of **8**, dPTP, opposite an abasic site with high fidelity and efficiency, even when given the option of natural nucleotides. Polymerase activities around TT photodimers in DNA have also been explored utilizing dPTP.[30] In a direct competition assay, dPMP was found to be preferentially inserted over dAMP opposite the 3′ T of most TT dimers. Thus, derivatives of **8** appear to be attractive mechanistic probes of enzyme responses to DNA damage involving abasic sites or their analogues.

Polycyclic aromatic bases were found to stabilize base-flipping DNA–methyltransferase M*TaqI complexes when paired opposite the flipped base.[31] When incorporated into dsDNA, **8** was found to stabilize the enzyme–DNA complex by a factor of 400, perhaps owing to its large size and increased stacking interactions. Similarly, studies of uracil DNA glycosylase acting upon a U–Y base pair, showed that the pyrene nucleotide "wedge" flipped the U outside the helix as effectively as the enzyme's native Leu191 wedge.[32]

Singleton employed a fluorescent pyrimidine analogue as a probe for the dynamics of *E. coli.* RecA complexation with ssDNA.[33] While inherently emissive as the free nucleoside, when incorporated into ODNs, fluorescence was, for the most part, quenched. Emission quenching was only partially reversed upon complexation with RecA protein, but the increase in polarized emission was far more prominent.

IX. Fluorescent nucleobases in oligomeric form

Oligomers of chromophores have recently attracted interest as light harvesting antennae, energy transfer systems as well as models for electron and hole transport. A number of molecular scaffolds have been employed, including polymers, polypeptides as well as nucleic acids.[34–36] DNA is a particularly attractive platform for assembling arrays of chromophores and fluorophores due to the ease of synthesis, water solubility, and well-defined structure offered by the backbone. Recently multistep energy transfer *via* FRET between four dyes tethered to a DNA backbone was reported.[36b]

We hypothesized that multiple modes of energy transfer including FRET as well as charge transfer, excimer and exciplex formation would occur in oligomers of fluorescent nucleobases. Rather than tethering fluorophores to natural bases, we planned to encourage close contacts (3.4 Å) found in natural DNA that lead to π–π interactions. A number of photophysical outcomes could result from photoexcitation of such a system, including antenna effects leading to increased brightness, quenching, and hypsochromic and bathochromic shifts in emission wavelength.

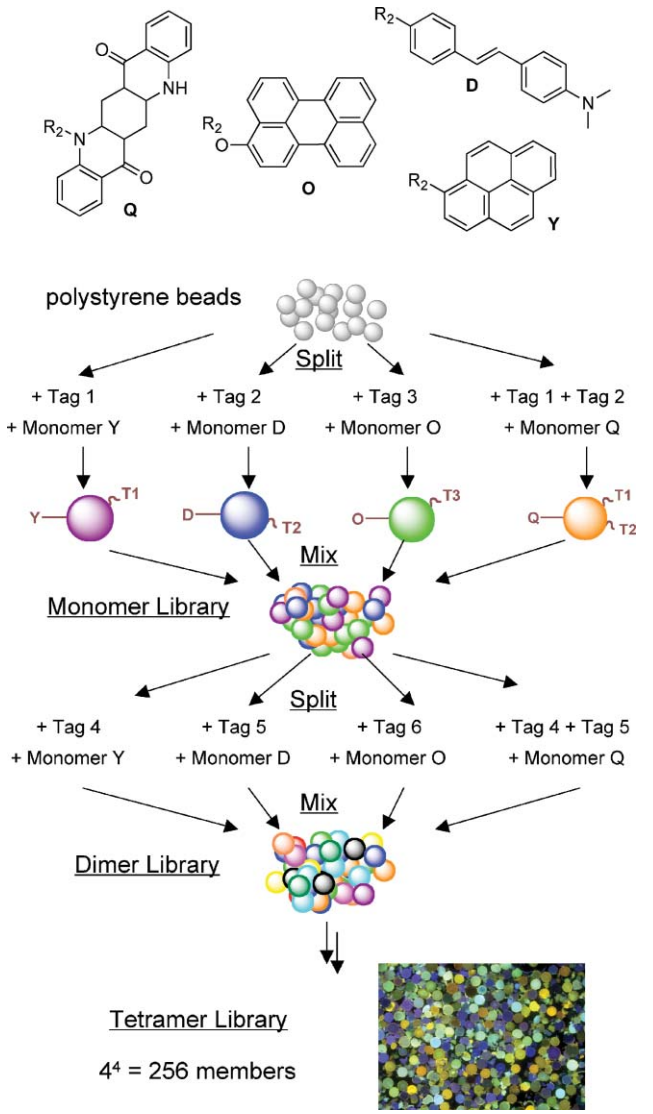

Fig. 10 Scheme for the construction of a library of polyfluors: using only a small family of fluorescent nucleosides (top, R_2 = deoxyribose), the combinatorial synthesis yields a 256-member library of tetramers with a broad array of colors (bottom).

Using a small set of fluorophore nucleosides (**Q**, **O**, **D**, **Y**, Fig. 10), a library of polyfluors was constructed using a 'split and pool' strategy on a DNA synthesizer employing phosphoramidite chemistry (Fig. 10).[7a] Each coupling reaction was encoded using a molecular tagging system so that composition and sequence could be determined after selecting individual beads. The resulting library demonstrated a number of attractive properties. Almost 50 colors and hues were found ranging from violet to orange despite only using four individual fluorophores. Most remarkably, this diversity was achieved with a narrow range of UV excitation (340–380 nm) owing to the large Stokes shifts that resulted from the oligomeric fluorophores (Fig. 11). A set of fluorophores with large Stokes shifts and single wavelength excitation would be desirable in bioassays where a single, relatively inexpensive LED lamp could function as an excitation source for multiple reporter dye colors.

A second, larger library of over 14 000 members was constructed more recently using 11 nucleosides in oligomers four units long. The library was screened for color changes upon exposure to light.[7b] While many fluorophores demonstrate photobleaching upon extended light exposure, certain oligodeoxyfluorosides in this library exhibited large hypsochromic shifts in their emission spectra after exposure to UV light. This demonstrated the use of oligodeoxyfluorosides in sensing.

Recently Mayer-Enthart and Wagenknecht introduced a helical array of pyrene-based fluorophores assembled on a DNA backbone.[37] Directly conjugating the pyrene moiety to the C-5 position of deoxyuridine (as opposed to linking *via* a flexible tether) results in electronic coupling and modification of the emission spectra of pyrene. Hybridization of the labeled ss-DNA with its complementary strand to form a duplex results in a dramatic enhancement of emission intensity as well as a hypsochromic shift.

As described above, derivatives of **8** have been shown to be suitable substrates for certain enzymes. We recently employed terminal deoxynucleotidyl transferase to synthesize polyfluors using dPTP, the 5′ triphosphate derivative of **8**.[38] Surprisingly, both α and β anomers functioned as suitable substrates for elongation of a DNA primer. Extension terminated with either 3 or 4 pyrene nucleotide additions for the α and β anomers, respectively. The resulting trifluorophore and tetrafluorophore deoxyriboside oligomers displayed the characteristic blue-green emission of the

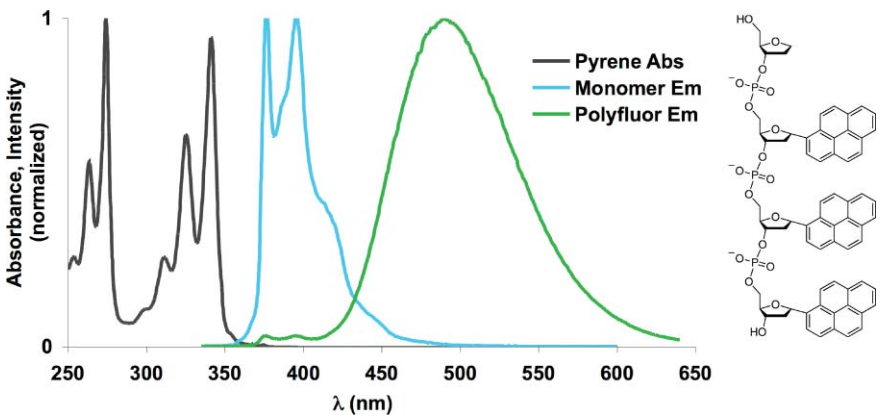

Fig. 11 Example of the large Stokes shifts found in certain sequences of oligodeoxyfluorosides. UV-excitation (345 nm) of the tri-pyrene sequence produces pyrene excimer emission centered at 490 nm. See ref. 7*a* for additional examples.

pyrene excimer (λ_{max} = 475 nm). Thus, using the TdT enzyme, one could generate homogeneous excimer labels with the possibility of applications in bioassays.

X. Conclusions and future prospects

Even a short survey of the literature makes it quite clear that there is a surprising variety of fluorescent DNA base replacements, and that they can be useful in many ways. There are now several broad classes of fluorescent DNA base replacements. Some of these closely resemble natural bases; this includes the classical case of 2-aminopurine and the recently studied cytosine analogues, and the substituted analogues of Saito. Some use heterocyclic frameworks that differ from the natural ones, such as those of Pfleiderer and Hawkins, but still retain the ability to undergo hydrogen bonding similar to their natural congeners. Some examples resemble natural DNA bases in their hydrogen bonding arrangements, but rely on added size to add fluorescent properties; this includes the benzoadenine of Leonard and the "xDNA" and "yDNA" nucleobases from our laboratory. Finally, many of the new cases do not resemble natural DNA bases at all, except that they include (as with DNA) a flat, aromatic structure. This brings in several new classes of fluorescent compounds, some that are simple hydrocarbons (such as pyrene) or heterocycles (such as oligothiophene), and a broad set from several labs that act as ligands for metals. It is virtually certain that this variety will increase further as more scientists participate in this field.

Fluorescent DNA base replacements have already been used in a variety of biochemical and biotechnological applications, and the number of these applications is likely to rise. We have outlined several examples of uses of these reporters in the basic study of the biochemistry of nucleotide use, in the study of DNA synthesis by DNA polymerase enzymes, and in examination of structures involved in DNA repair mechanisms. Moreover, the biotechnological applications of these molecules are increasing; to date the main use has been in the detection of specific DNA sequences in solution, but it seems likely that other applications will be reported soon.

In addition to the use of fluorescent DNA base replacements as individual molecules and single labels, these monomeric species can be assembled into oligomeric form, either by use of a DNA synthesizer, and likely by enzymatic methods as well. By combining different combinations of sequences, this can yield large sets of molecules of moderate length (*e.g.*, trimers or tetramers) that are water-soluble and which have an exceptionally large range of emissive properties. Although the studies of these molecules are in their early stages, it is clear that the assembly into oligomers ("oligofluorosides") can yield properties that do not exist either in the monomeric components or in commercially available fluorescent labels. Some of the novel properties include tunable excitation and emission wavelengths, exceptionally large Stokes shifts, and the ability to act as sensors. The early results suggest that additional exploration of this molecular strategy is warranted.

What is to be expected for the future of fluorescent DNA base replacements? First, new kinds of fluorophores are still needed. This could increase the utility and spectrum of applications of these compounds. Compounds with new wavelengths of absorption and emission, and emission intensities will add to the breadth of properties that are currently available, and will increase the utility of the compounds in their applications. In addition, a number of fluorophores in the broader literature are known to be highly sensitive to their environment; however, this kind of environmental sensitivity has not been explored much in fluorescent DNA base replacements. Thus we may expect to see future examples that show sensitivity to pH or polarity of environment, and this development will bring about some interesting applications in biology.

Finally, it is certain that new ranges of applications will be explored in the future with this class of molecules. For example, since the compounds are based on a biological structure, it seems certain that we will begin to see applications of such compounds not just in the biochemical *in vitro* setting, but also in living cellular systems. In that setting they may well provide useful tracking, probing, imaging and sensing applications. In addition, it is quite possible that more applications in the basic sciences will also be developed, including not only biophysical systems but also potentially even in non-biological systems where capture and transfer of energy, starting in the energy of photons, takes place.

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