Intrinsically Fluorescent Base-Discriminating Nucleoside Analogs

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Abstract: Modified nucleosides possessing intrinsically fluorescent heterocycles as base surrogates that are capable of canonical base-pairing are becoming important biomolecular tools. These "base-discriminating fluorophores" (BDFs) find use in hybridization-based mismatch detection, elucidation of DNA damage and the study of localized structural phenomena as exemplified in the study of ribozymes and nucleic acid/ligand interactions. In this review, an historical perspective will be given along with some of the more recent highlights in this rapidly developing field.

Keywords: Base-discriminating fluorophore, SNP, DNA, luminescence, solvatochromatism.

INTRODUCTION

Classically, the central dogma of biology purports that the characteristics which make us unique are peculiar to the heritable material within almost every cell of our body: our DNA. It is therefore of no surprise that many disease states, both acquired and heritable, are derived from permutations within the genome. Mutations can make one more susceptible to heart disease, various carcinoma and are also the cause of diseases such as phenylketonurea, autism and cystic fibrosis, to name only a few. Diagnosis of mutations that make one, or one's progeny, susceptible to disease is a very desirable advancement of modern medicine and many research groups are currently working on making this process more rapid and economic. A few existing techniques for sequence analysis are outlined in the following paragraphs.

One of the most frequently used methods for the detection of permutations within a polynucleotide sequence employs molecular beacons based on fluorescent resonant energy transfer (FRET) [1]. The quintessential morphology of a molecular beacon consists of an oligonucleotide labeled at opposing termini with a fluorophore and a quenching moiety. Complementary sequences are placed at each terminus such that a hairpin structure is formed and the loop region is chosen to be complementary to the target oligonucleotide sequence to be analyzed. Upon hybridization of the interrogated sequence to the loop region, the stem opens and the fluorophore is no longer quenched as this quenching is distance dependent (Fig. **1**). This basic technique can be applied in a variety of different ways, the fluorophore and quencher may be appended to different sequences and FRET can be used to obtain emission at differing wavelengths [2].

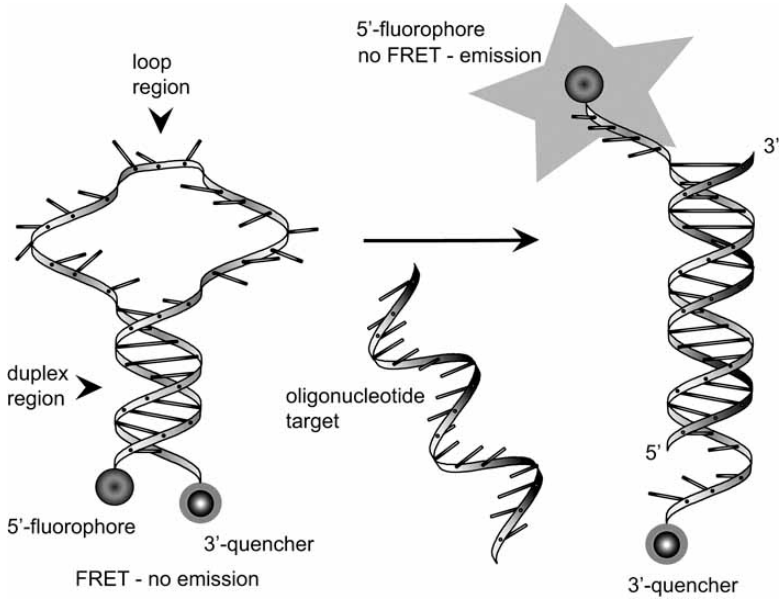

Fig. (1). Illustration of the underlying mechanism of molecular beacon operation based on FRET.

Detection of single nucleotide polymorphisms (SNPs) can oftentimes be difficult due to the sheer size of the genome. A point mutation may occur only in low abundance creating the problem of interference from the wild-type gene. In order to increase sensitivity, sequences can be examined and amplified through PCR-clamping using oligonucleotide analogs that will suppress the association of a primer to the template by steric hindrance. PCR clamping is most often peptide nucleic acid (PNA) mediated. Due to the unnatural *N*-(2-aminoethyl)glycine derived backbone, PNA is not recognized enzymatically and therefore does not act as a primer for DNA polymerase [3]. When a sequence of PNA is complementary to a PCR primer site it will virtually eliminate the formation of PCR product. PNA can be used in this manner to selectively silence or amplify genes differing by only one base. This technique, in conjunction with hybridization probes, has been used by several groups

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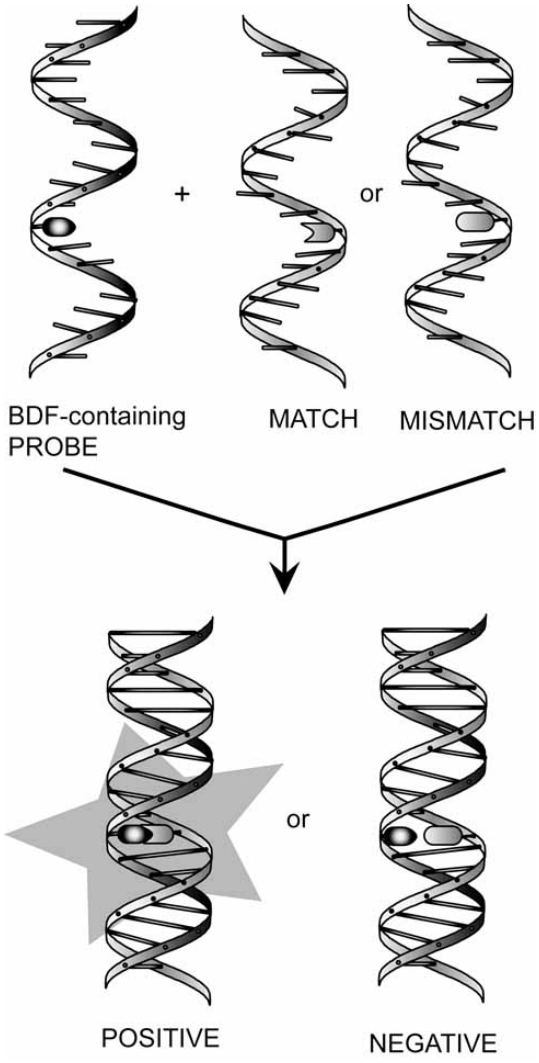


Fig. (2). Illustration of the principle of sequence readout by a base discriminating fluorophore (BDF) containing probe. The interaction of a BDF-containing probe with a complementary sequence leads to an observable property such as increased fluorescence (as indicated), but not in the case of a mismatched interaction.

to great effect. The detection of point mutations using a wild-type-specific PNA and a mutant-specific PCR primer has been carried out for oncogenes [4,5] and even on mitochondrial DNA [6].

Full sequencing of a PCR amplified gene is the archetypal method of SNP analysis; this technique is currently undesirable as it is costly and time consuming despite the significant advances that continue to be made in this area.

Electrochemical means may also be used for SNP detection. Many different approaches have been used, although most involve oligonucleotide modified electrodes that result in differing charge transfer rates upon complementary sequence binding. As an example of this, a recent paper has illustrated that a mismatch can be discriminated through labeling of an oligonucleotide immobilized on a gold surface with anthraquinone at the 2'- position, as the anthraquinone moiety intercalates the charge transfer rate decreases allowing for mismatch detection [7]. Electrochemical mismatch detection offers the advantages of simple readout and amenability to digitization. Microarray analysis of SNPs by electrochemical means remains limited as a mechanical connection is required to each electrode pad. This would make the manufacture of larger arrays, required for full sequence-screening, a difficult challenge [8].

The aforementioned techniques, molecular beacons in particular, are limited as they rely in differences in hybridization efficiency. These differences vary greatly with sequence context and strict hybridization conditions must be met [9]. In the context of oligonucleotide arrays, variations in packing density can affect hybridization efficiency therefore making the manufacture of high-throughput screening devices technically challenging [10].

Fluorescent base-pairing nucleoside analogs which may fluorometrically respond to hybridization when placed within an oligonucleotide sequence are of high interest as they overcome the reliance on hybridization efficiency and offer a conveniently measured parameter in the wavelength or intensity of light emission (Fig. **2**). These base-discriminating fluorophores (BDFs) may be used for a wide range of sensing applications including the detection of SNPs which may be used for the diagnosis of heritable diseases in a less costly manner than full sequencing.

The use of BDFs allows for the elucidation of localized events as opposed to other methodologies which monitor global structural phenomena (UV-Vis, CD spectroscopy). Other methods for detecting localized structural events do exist, such as NMR spectroscopy and gel electrophoresis of kinetically trapped radiolabeled products; however, these techniques are time consuming and limited with respect to reaction time scale.

As the natural nucleobases are essentially non-emissive [11] much work has been put towards the synthesis of base analogs which are luminescent. In the design of novel base-discriminating fluorophores several basic criteria should be met. The fluorophores should maintain a high degree of structural similarity with the natural nucleobases so as not to affect the hybridization efficiency, the compounds must obviously be fluorescent, their excitation wavelengths should not be within the absorption range of biological macromolecules, and the fluorescence should be sensitive to micro-environmental conditions. The fluorescence response may be a change in emission wavelength, an increase or decrease in quantum efficiency or length of the excited state lifetime; however, dramatic changes in emission wavelength or intensity are the most conveniently measured parameters.

Tor has divided fluorescent base analogs into five basic categories: isomorphic base analogs **1**, pteridines as purine analogs **2**, extended nucleobases **3**, conjugated base analogs **4**, and aromatic hydrocarbon and chromophoric base analogs **5** (Fig. **3**) [12].

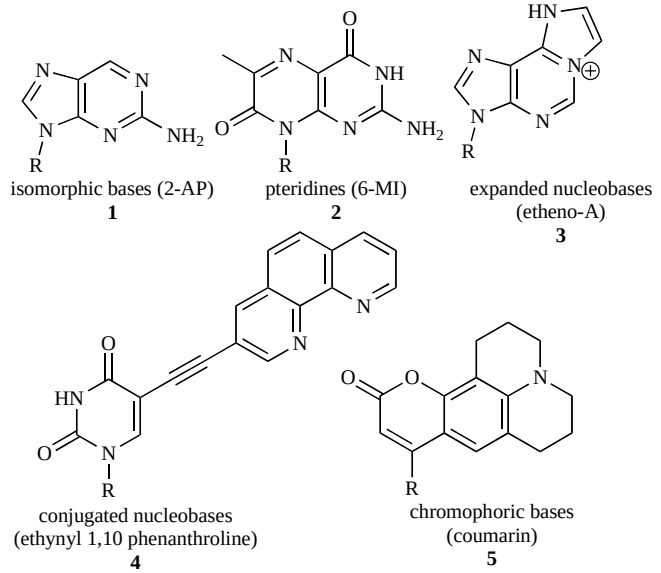
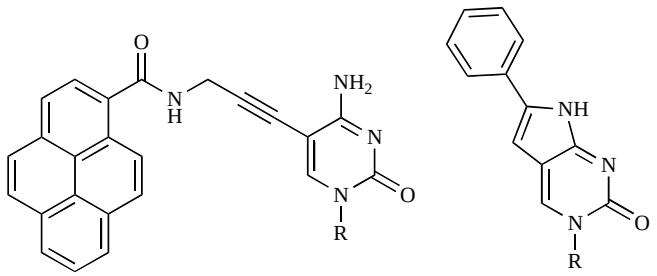


Fig. (3). Representative molecules of the different classes of BDFs defined by Tor [12].



6 **7**

Fig. (4). Representative molecules of the two basic fluorescent nucleobase analog designs: those containing a pendant fluorophore **6,** and the intrinsically fluorescent 6-phenylpyrrolocytosine **7** [13,14].

Fluorescent base analogs may be more selectively divided into two categories which are capable of canonical base pairing: those possessing pendant fluorophores and intrinsically fluorescent nucleoside analogs. Above are pictured representative molecules of these classes (Fig. **4**) [13,14]. Both of these molecules are able to hydrogen bond with natural bases and respond fluorometrically to hybridization. The former class often has an advantage in that higher overall brightness (brightness is defined by the quantum yield multiplied by the extinction coefficient at the excitation maximum) and greater quantum yields are often achieved through the attachment of a traditional chromophore. However, fluorescence spectroscopy is a fantastically sensitive technique and oftentimes high luminescence is not required for a reporter group to be practical and useful.

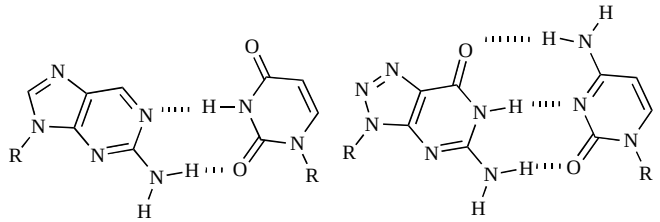
The use of conventional fluorescent moieties, attached to the base *via* a flexible linker, can allow independent movement of the fluorophore, this can make interpretation of results complicated as the fluorescence response is not necessarily originating from the area of interest. The intrinsically fluorescent BDF category has become attractive as it is the nucleobase itself whose environment is being monitored, they are synthetically accessible and also have potential for biological incorporation. Such intrinsically fluorescent, complementary base-discriminating/pairing nucleobase analogs are the subject of the present review. Whenever possible the photophysical parameters characterizing the BDFs ablility to act as a reporter group are given; however, for some analogs this information is not yet available.

INTRINSICALLY FLUORESCENT PURINE NUCLEOBASE ANALOGS: COMPLEMENTARITY AND RESPONSE TO PYRIMIDINES

Isomorphic Bases: 2-aminopurine and 8-azaguanine

The base analogs 2-aminopurine **1** (2-AP) and 8-azaguanine **8** (8-azaG) are historically two of the most often used isomorphic, fluorescent nucleobases. Their overall size matches that of the natural bases and they are able to form isostructural Watson-Crick base pairs (Fig. **5**). 2-AP, a constitutional isomer of adenine, has been used for almost 40 years [15]. Owing to its high quantum yield as the free nucleoside in aqueous solution ($\Phi = 0.68$) and extreme sensitivity to microenvironmental changes with drastically lower emission observed in single-stranded (ss) and double stranded (ds) oligonucleotides (as much as 100 fold decrease in emission in dsDNA) [16], 2-AP has been employed in many studies of nucleic acid structure and dynamics and in a wide array of biochemical assays. Examples include: real-time monitoring of hammerhead ribozyme folding, oligonucleotide cleavage and inhibition, nucleic acid/protein and nucleic acid/drug interactions [17]. 2-AP is also attractive because it can form Watson-Crick type base pairs with thymine, uracil or cytosine. It also has a red shifted absorption spectrum which allows differential excitation in the presence of biological macromolecules.

8-azaG **8** is another isomorphic purine analog which can be enzymatically incorporated into oligonucleotides. It is relatively emissive, exhibits a high degree of solvatofluorochromism and is quenched by adjacent nucleobases (Fig. **5**). 8-azaguan(os)ine has a high quantum yield when *N*1 is deprotonated at high pH ($\Phi = 0.55$) but much lower fluorescence when the natural Watson-Crick base-pairing face is presented. One of the more interesting and recent advances made with this BDF has been the development of a highly efficient enzymatic synthesis of the triphosphate from 8-azaguanine and the establishment of pH dependent fluorescence within a structured oligoribonucleotide (with implications towards ribozyme mechanism elucidation) [18].



2-AP **1** 8-azaG **8**

Fig. (5). The isosteric base analogs 2-aminopurine **1** and 8-azaguanine **8** in hybridization with their natural base complements uracil and cytosine, respectively.

Purines Modified at the 8-position

The 8-position of purines lends itself well to derivatization; 8-bromoadenosine is remarkably easy to prepare in high yield and can be used in a variety of transition metal-catalyzed cross-couplings. Deoxyadenosine and guanine derivatives with phenol directly attached at the 8-position can be accessed through the coupling of hydroxyphenylboronic acid with the appropriate 8-bromonucleoside in the palladium catalyzed Suzuki-Miyaura reaction [19]. These compounds are of interest for several reasons: the compounds are biomarkers for phenol exposure and are therefore of use for the study of mechanisms of carcinogenicity, the compounds exhibit pH dependent fluorescence [20] and the phenolate, anionic form of the modified base can act as a quencher through photoinduced electron transfer (PET) [21]. Although these nucleosides have not yet been incorporated into oligonucleotides, their high quantum yields at neutral pH ($\Phi = 0.25 - 0.56$) could make them quite useful as BDFs.

Various other moieties have been attached to purines in the same manner including bipyridine and phenanthroline for use as metal chelators (demonstrated for Ru^{II}) and various aryl substituents to tune fluorescence properties (Fig. **6**) [22].

Purines Modified at the 6-position: 2-Amino-6-(2-thienyl)purine and 2-amino-6-(2-thiazolyl)purine

The compounds shown in Fig. (**7**) have been known for some time and applications for the highly emissive base analogs continue to be found [23-25]. The unnatural base pairs of 2-amino-6-(2-thienyl)purine **14** with 2-oxopyridine and 2-amino-6-(2-thiazolyl) purine **15** with imidazolin-2-one remarkably function in transcription with good fidelity and hence may be placed specifically within an RNA transcript with ease. Both the thienyl and thiazolylpurines have quantum yields from 0.41-0.46 as the free 5'-monophosphates. These quantum yields are roughly halved upon incorporation into a ss oligonucleotide and halved again when in ds DNA/RNA. Used alone or in FRET experiments, with fluorescein derivatives (FAM) as an acceptor moiety, accurate descriptions of hybridization events

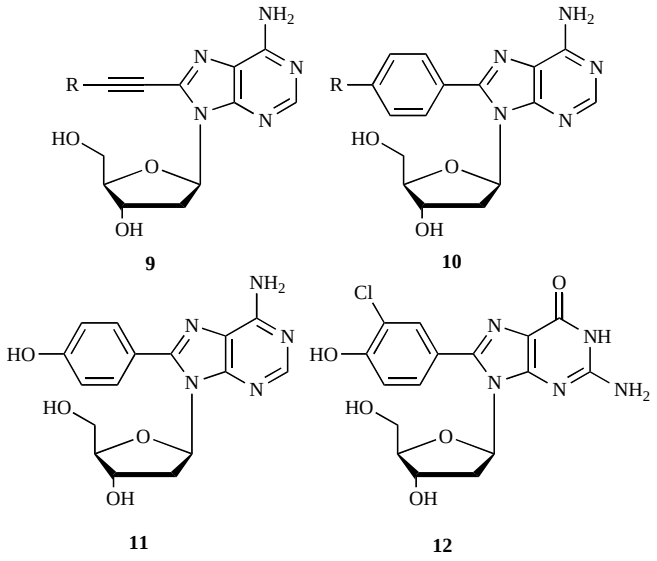


Fig. (6). Purine nucleosides derivatized at C-8 (R = H, bpy) [20,22].

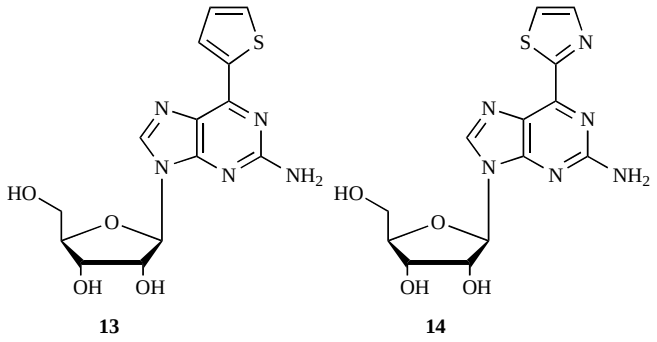


Fig. (7). 2-amino-6-(2-thienyl)purine **13** and 2-amino-6-(2-thiazolyl)purine **14** used as guanosine mimics despite not presenting an isostructural Watson-Crick face [23-25].

have been obtained. When placed adjacent to one another, the modified bases exhibit self-quenching behaviour but may still allow for the excitation of a nearby FAM moiety. The authors suggest that this may be desirable as it would enable the use of excess probe to test sequence in biological assays [25].

Pteridine Nucleoside Analogs

Pteridines are naturally occurring compounds that have been known for over a century. Pteridine derivatives have had many pharmaceutical applications, particularly as anti-cancer drugs. Pteridines have more recently been utilized as nucleoside analogs; they are highly fluorescent and are synthetically accessible through the condensation of the appropriate triaminopyrimidine with the desired pyruvate derivative (Fig. **8**) [26].

Once the base analog has been synthesized, *N*-glycosylation can be carried out to yield the desired nucleoside, this in turn can be converted to the corresponding phosphoramidite by well established methods.

The pteridines described by the Hawkins group are structurally similar to the natural purine bases, containing the appropriate hydrogen bond donor and acceptor components, with one exception (*vide infra*). The compounds 3-methylisoxanthopterin **15** (3MI) and 6-methylisoxanthopterin **2** (6MI) have been employed as guanosine analogs while 4-amino-6-methyl-8-(2'-deoxyribofuranosyl)-7(8*H*)-pteridone **16** (6MAP) and 4-amino-2,6-dimethyl-(2'-deoxyribofuranosyl)-7(8*H*)-pteridone **17** (DMAP) have been used as adenosine mimics (Fig. **8**) [27]. While other derivatives have been synthesized, it is the aforementioned compounds that have found the greatest success in the elucidation of DNA structural events.

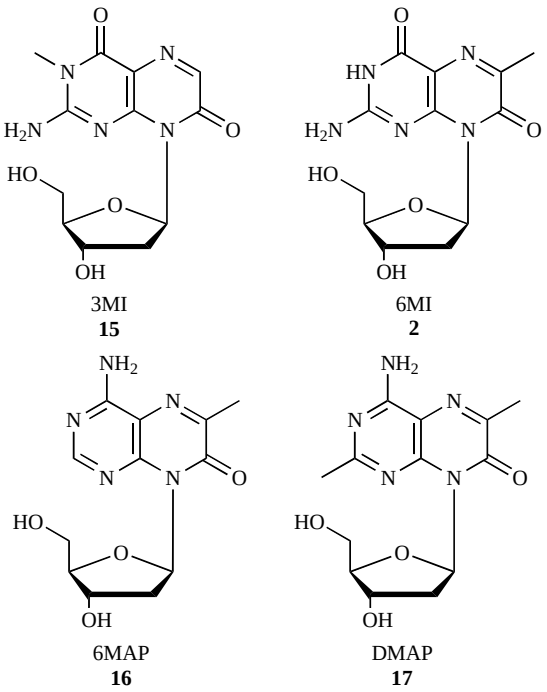


Fig. (8). Pteridine guanosine analogs 3-methylisoxanthopterin **15** (3MI), 6-methylisoxanthopterin **2** (6MI) and adenosine analogs 4-amino-6-methyl-8-(2'-deoxyribofuranosyl)-7(8*H*)-pteridone **16** (6MAP), 4-amino-2,6-dimethyl(2'-deoxyribofuranosyl)-7(8*H*)-pteridone **17** (DMAP) [27].

The applications of the pteridines as BDFs are diverse as the compounds are highly fluorescent (Φ = 0.77-0.88 for the adenosine analogs and 0.39-0.48 for the guanosine) and highly sensitive to microenvironmental conditions. All compounds are well tolerated within the duplex excepting 3MI due to the methyl group at the 3-position interfering with hydrogen bonding. This methylation destabilizes the duplex to a similar extent as would a single mismatch.

Pteridines have been used in the following applications: HIV-1 integrase activity assays, alkyl transferase coupled assays, use as hydridization probes, HU protein binding detection, "A-tract" structure detection, RNA polymerase activity and intracellular oligonucleotide transport analysis [27].

Intrinscially Fluorescent Pyrimidine Nucleobase Analogs: Complementarity and Response to Purines

5-Ethynyluridine

Variously substituted 5-ethynyluridine derivatives have been exploited in our laboratory for mismatch detection. These structurally simple and compact fluorophores are able to report hybridization events by "turning on" in the presence of a match sequence when placed internally within the modified strand. The greatest fluorescence response, out of a small selection of compounds, was observed for a *p*-methoxyphenylethynyluracil **18** containing probe (Fig. **9**) giving a six-fold increase in fluorescence upon encountering a match sequence [28].

The mismatch duplexes offer surprising results: when the mismatch is guanine the fluorophore is quenched considerably, when it is thymine, the chromophore suffers an intermediate degree of quenching and cytosine mismatch duplexes are not quenched significantly. These data imply that the fluorophores are exquisitely

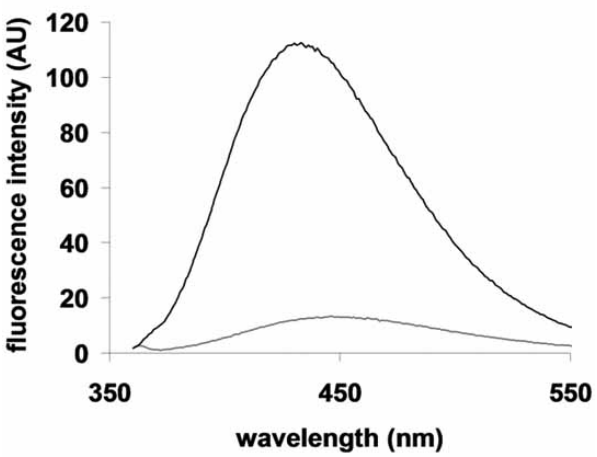


Fig. (9). 5-(*p*-methoxyphenylethynyl)uracil **18** containing oligonucleotide in the presence of a match sequence (black) as compared to ss (grey) [28].

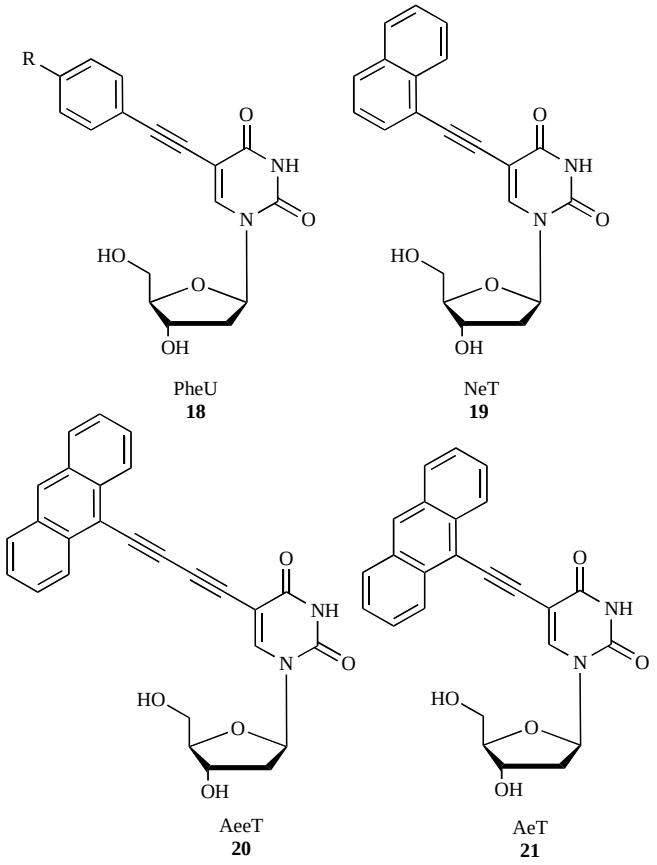


Fig. (10). Phenyl, naphthalenyl and anthracenyl-alkyne derivatized deoxyuridines used for mismatch detection (R = H, OMe) [28,30].

responsive to their local environment and may therefore find further use in determining DNA structural characteristics and ligand/DNA interactions.

Related alkynyluracils have been exploited for mismatch detection by Brown and co-workers. Again using Sonogashira cross-couplings, anthracenyl-ethynyl (Ae) and naphthalenyl-ethynyl (Ne) moieties have been appended to deoxyuridine at the 5-position **21** (Fig. **10**) [29,30]. The corresponding anthracenyl -diyne **20** (Aee) was also synthesized and these compounds were found to be only marginally destabilizing towards duplex formation. As is typically the case with fluorophores appended to pyrimidines at the 5-position, an increase in fluorescence was observed on duplex formation due to a change in the polarity of the microenvironment when the chromophore is projected into the aquated major groove of dsDNA *versus* its position in ss form [29,30].

Alkynylnaphthalene analog of deoxythymidine **20** (AeT) containing oligomers showed the greatest increase in emission upon binding to a match sequence; however, when presented with a C mismatch the fluorescence was also much increased (quantum yields not reported). The results were very similar with the anthracene containing oligomer only with overall lower fluorescence. The diyne compound gave surprising results, the fluorescence increased greatest in the case of a C or T mismatch, the T mismatch increase in emission intensity was also accompanied by a bathochromic shift of 12 nm. It can be said that all three of the compounds tested could potentially be used to effect in A/G SNP typing [30].

A similar modification, but for cytidine, was reported earlier by Sessler and coworkers [31]. They prepared a pair of base-modified ribonucleosides to examine photoinduced electron transfer in a hydrogen bonded ensemble. They chose to prepare a *C*8-modified guanosine **24** and a *C*5-modified cytidine **23** (Fig. **11**).

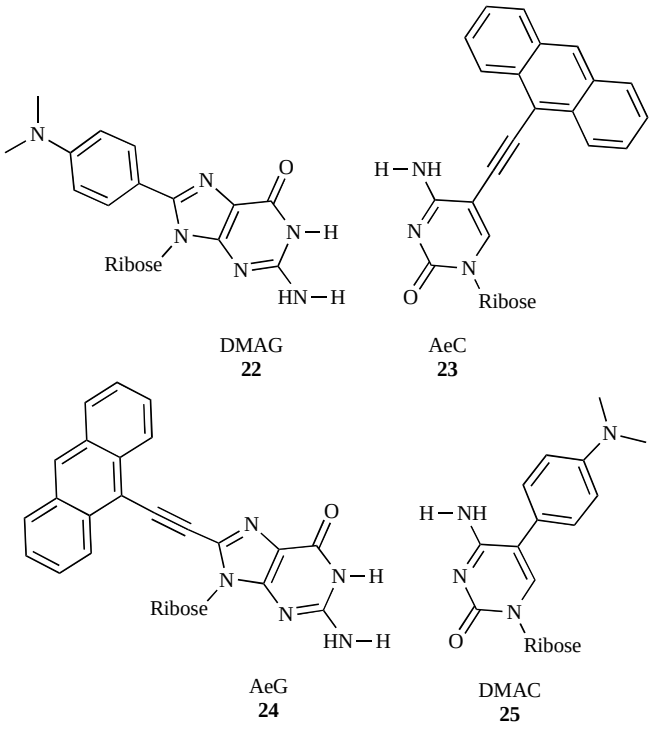


Fig. (11). Anthracenyl-ethynyl (Ae) derivatized riboguanosine **24** (G) and ribocytidine **23 (**C) and dimethylaniline-(DMA) modified bases **22** and **25** [31].

Although the purpose of the study was not base-discrimination, the fluorescence spectra of AeC **23** and AeG **24** were reported. The fluorescence was mostly characteristic of anthracene, yet each modified base had slightly different photophysical properties. Given the conjugated nature of the linkage of the luminophore to the nucleobase, it is reasonable to suspect that they may be responsive to base pairing in the context of an oligonucleotide. Likwise, the dimethylaniline-modified bases, especially DMAG, is structurally related to modified purines already presented (Fig. **6**). A characterization of the luminescence of DMAG **22** or DMAC **25** was not disclosed in this work, yet based on structurally similar modifications it would be unsurprising if these bases were fluorescent and possess fluorescence that was pH dependent.

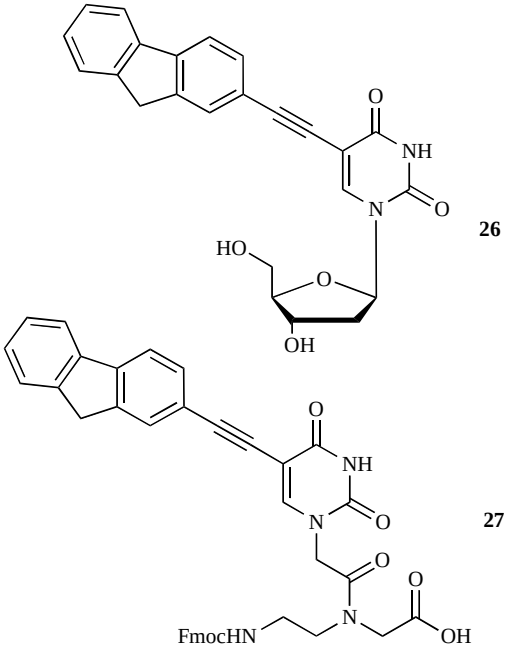


Fig. (12). Fluorenylalkynyluridine **26** [32] and fluorenylalkynyluracil PNA monomer **27** [33].

A fluorene derivatized deoxyuridine [32] nucleoside **26** and the analogous PNA monomer **27** [33] (Fig. **12**) have both been synthesized and were found to be moderately fluorescent ($\Phi = 0.14$ for the free nucleoside). Oligonucleotides containing the nucleoside analog were amenable to SNP typing, selectively fluorescing in the presence of a match sequence in a quencher-free molecular beacon construct [32].

Although the fluorescence of the PNA monomer **29** was found to be *ca*. 50 times greater than the structurally similar 5-phenylethynyluracil derivative **28** [34], (Fig. **13**) the fluorescence response on duplex formation was very modest. This highlights the principle of making only minor structural changes to the nucleobases to maintain sensitivity to the microenvironment. It also highlights the differences in the structures on ssPNA versus ssDNA and the challenges associated with portability of modifications/technologies to oligonucleotide analogs.

Pyrrolocytosine

The heteroannulation of 5-alkynyluridine, resulting in furanouridine **31** [34] was described more than a quarter of a century ago; however, **31** is of limited use as its Watson-Crick base-pairing face is not complementary to any natural nucleobase. Treatment of **31** with concentrated aqueous ammonia resulted in its conversion to pyrrolocytosine **32** which may be used as a cytosine analog. Pyrrolocytosine is one of the rarer isosteric pyrimidine analogs and has been used as an emissive C analog in mismatch detection [13], and also in the study of RNA secondary structure [35]. Pyrrolocytosine is only modestly emissive as the monomer and is quenched successively on incorporation into single-stranded and double-stranded oligonucleotides [36]. Able to base-pair effectively with guanine, it can be stabilizing or destabilizing to the duplex depending on the identity of the substituent at the 6-position of the pyrrole and the sequence context. 6-Methylpyrroloctosine, first synthesized in 1987 [37], remained unexploited as a BDF until its rediscovery in 2001 [36]. Since that time, it has been increasingly investigated due to its quenching upon hybridization and more recent commercial availability despite its low fluorescence efficiency ($\Phi = 0.05$). Although initially synthesized by an alternate route, it is

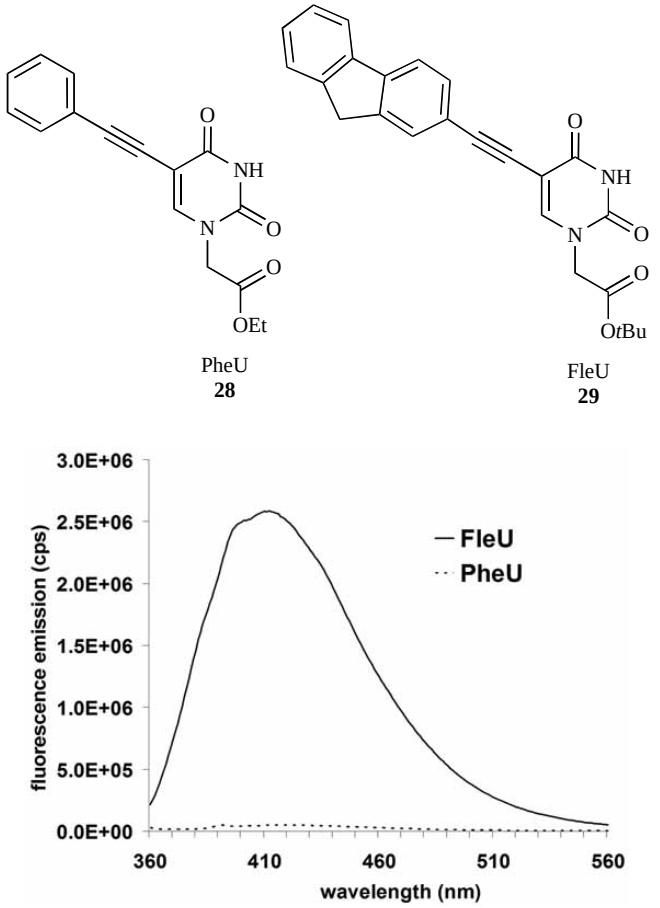


Fig. (13). Comparison of fluorescence intensity of 2.5 μM solution (CH_2Cl_2) of 5-fluorenylalkynyluracil and 5-phenylethynyluracil PNA monomer [33].

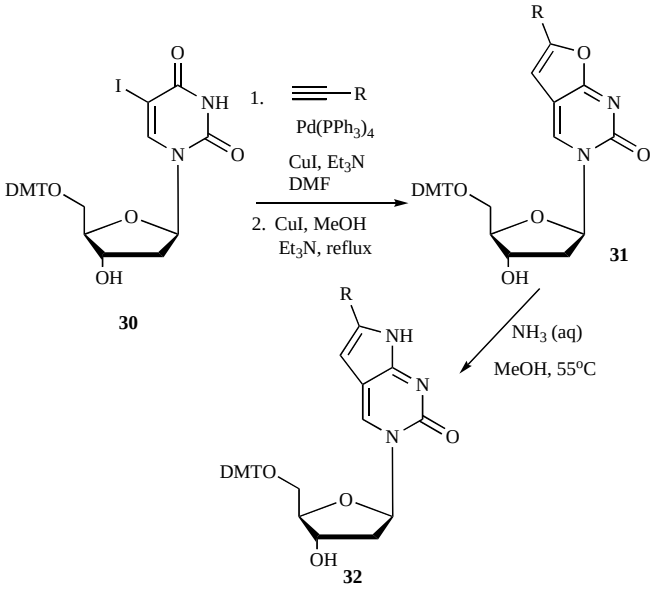


Fig. (14). Synthetic route to pyrrolocytosine by Sonogashira chemistry followed by a 5-endo-dig cyclization and *O→N* atom exchange in aqueous ammonia [38].

now commonly synthesized from uridine *via* Sonogashira coupling chemistry after iodination at *C*5 as shown in Fig. (**14**) [38].

Although the 6-methyl-substituted pyrrolocytosine has found success as a base-discriminating fluorophore, by variation of R

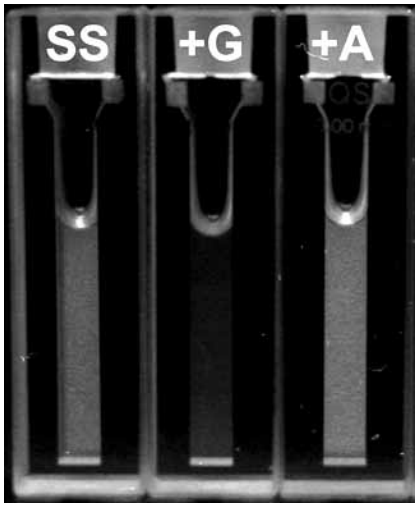


Fig. (15). SNP analysis using a 6-phenylpyrrolocytosine containing oligonucleotide. From left to right, single-stranded, G match sequence, A mismatch. Note: quenching occurs only on hybridization to the perfectly matched sequence. Even though the A-mismatch exists as a duplex at the analysis temperature, no quenching is observed [13].

group identity, greater emission and increased fluorescence response can be achieved. Substituting for a phenyl group yields a compound that is much more emissive than methylpyrrolocytosine and has a higher degree of quenching upon hybridization (85% *versus* 50% although this varies with sequence context). The overall greater luminescence of 6-phenylpyrrolocytosine can be demonstrated by the visual discrimination of mismatches which was not possible at comparable concentrations of the conventional luminophore (Fig. **15**) [13]. The greater emission intensity and increased responsiveness may translate to lower detection limits in SNP analysis.

6-Dimethylamino-2-acylnaphthalene Appended Nucleosides

6-Dimethylamino-2-acylnaphthalene (DAN) has been used for almost 30 years as a fluorescence reporter in biological systems [39]. Its utility as a reporter group is due to the fluorophore undergoing charge redistribution dependent on the polarity of the environment. DAN has been appended to guanosine and cytidine *via* the exocyclic amino group in order to monitor the polarity of the minor and major grooves of DNA (in both B and Z-form) respectively [40,41]. This same fluorophore has recently been appended to 2'-deoxyuridine by Saito's group in different ways as shown in Fig. (**16**) [42].

The fluorescence of these nucleoside analogs was measured with respect to solvent polarity. The fully conjugated system (Fig. **16**, compound **33**) was accessed from a Suzuki-Miyaura coupling of the DAN borate ester with 5'-*O*-DMT-5-iodouridine and showed the greatest degree of solvatofluorochromicity. Surprisingly, the pendant chromophore **34** (electronically separated from the base) and cross-conjugated compounds **35** (Fig. **16**) showed very different fluorescence properties with the **35** being the least fluorescent of the three and the propynoyl linked compound **34** being the most, the highest quantum yield being 0.26. The high degree of solvatofluorochromicity displayed by these compounds implies that there is strong charge transfer character to the relevant excited state [42].

C5-Furan Substituted Uridine

Purines, typically modified at *C*8, and pyrimidines modified at *C*5 are prominent in the literature due to a minimal thermodynamic penalty upon hybridization and ease of synthesis. A particularly rewarding modification at *C*5 of uridine was the appending of a

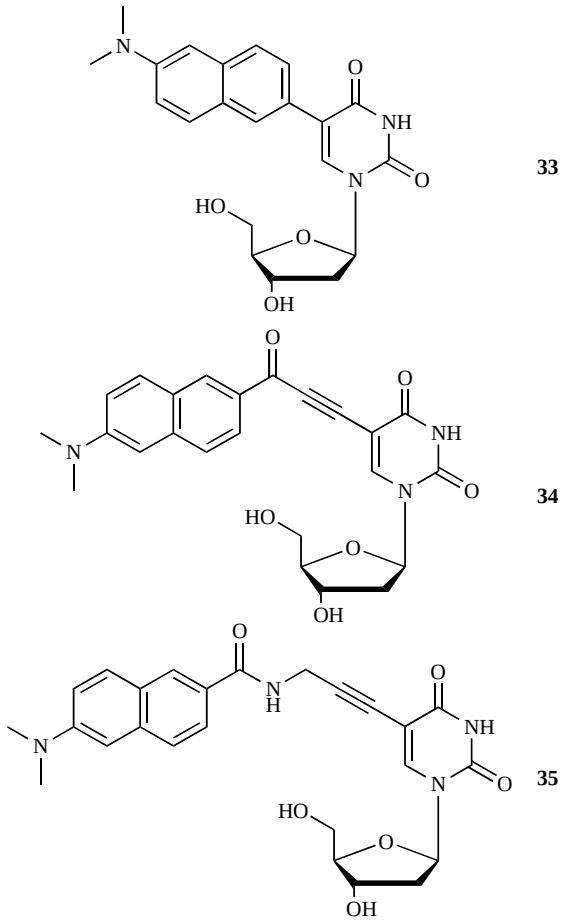
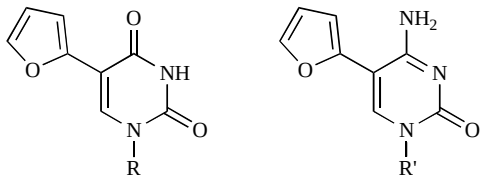


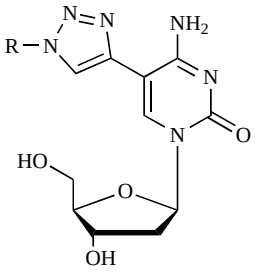
Fig. (16). DAN appended nucleosides differing by linkage strategy: directly attached **33**, propanoyl **34,** and carboxamide linkages **35** [39].

furan ring *via* the 2-position of furan. This was done by the Stille coupling of 2-(tributylstannyl)furan with 5-iodouracil and was first reported in 1991 [12,43]. The furanyluracil **36** was found to have a relatively low quantum yield (Φ=0.03 in H_2O) but demonstrated interesting bathochromic and hyperchromic shifts upon increase in solvent polarity (ether→water). Despite the low luminescence, the sensitivity to microenvironment implies that the compound may be useful in mismatch detection; indeed, the compound has been used for the successful detection of abasic sites in DNA [29]. In the same paper, nucleosides modified with oxazole, thiazole and thiophene showed less favourable photophysical properties. In addition to the favourable photophysical properties displayed by 5-furanyluridine it was also later found that the 5'-triphosphate was able to be incorporated by T7 RNA polymerase in the place of thymidine with good fidelity [44]. The fluorescence of the monomer has been shown to be dependent on microscopic polarity; a well definined linear plot of fluorescence intensity versus experimentally determined $E_T(30)$ values [45] has been obtained. The use of microscopic polarity scales, such as $E_T(30)$ values, has often been a more accurate parameter for such research than the use of dielectric constants [46]. The furan modified nucleobase has been incorporated into the potential pharmaceutical target: the bacterial decoding site known as the A-site and was used to screen aminoglycoside antibiotics. Although the fluorescent response on binding of the aminoglycoside was less than that exhibited by 2-aminopurine, when used in conjunction with 2-aminopurine an effective assay was developed. The need for new RNA structural probes still exists as potent inhibitors of this site do not induce a proportional change in fluorescence when using the currently available fluorophores. This fluorophore has also been used to examine the polarity of the major



furanyl-U **36** furanyl-C **37**

Fig. (17). Furan-decorated nucleosides. R = deoxyribose or ribose, R' = deoxyribose.



R = Ph, Bn, EtPh, thiophenyl, bithiophenyl, fluorenyl.
38

Fig. (18). 1,4-disubstituted triazolylcytidines synthesized for use as potential BDFs.

grooves of A and B-form nucleic acids due to its strong solvatochromic behaviour [47]. The *C*5-furan modified deoxycytidine has also been prepared, but it is approximately 3-fold less emissive than furanyl-U (Fig. **17**). Despite this lower emission the base has been employed in the successful discrimination between 8-Oxoguanosine, G and T. 8-oxoguanosine is a biomarker for oxidative stress and has been found to cause transversion mutations during DNA replication; therefore, its detection/quantification within the genome is desirable [48].

Due to the success found with furan-modified uridine, Tor and co-workers prepared the *C*8-furan modified adenosine and guanosine. Even though the nucleosides were highly emissive with quantum yields of 0.57 and 0.69, respectively, they possessed maximal emission in the near UV (375 nm) which displayed little solvent polarity dependence. Likely because of these suboptimal properties, investigation of their use as BDFs in oligonucleotides has not yet appeared [12].

Triazole Appended Nucleosides

Although there are many examples utilizing the Huisgen 3+2 azide/alkyne cycloaddition [49] for the modification of pre- and post-oligomerized nucleic acids, little attention has been paid towards the fluorescent properties of the resultant molecules. The most popular substrate for this copper(I) catalyzed "click" reaction has been 5-alkynyluracil as it can be accessed readily from the Sonogashira cross-coupling of 5-iodouridine with trimethylsilylacetylene, followed by desilylation. Various azides have been used and it has recently been found that stacking between adjacent triazole modified bases can be stabilizing towards the duplex [50]. A full set of fluorescent nucleobase analogs have also been developed through the "clicking" of azidocoumarin with 7-deaza-7-alkynyldeoxyguanosine, 7-deaza-7-alkynyldeoxyadenosine, 5-alkynyldeoxyuridine and 5-alkynyldeoxycytidine to form 1,4-disubstituted triazoles [51]. These compounds are not "inherently fluorescent" as categorized by the present review as they possess an independently fluorescent moiety attached to the nucleoside. Carell and co-workers have also used this reaction as a means of modifying DNA post-synthetically; the applications of this strategy abound [52].

Benzyltriazolyluridines have also been synthesized for use as small molecule hydrogelators [53,54] and our group has synthesized the corresponding cytidine analogs **38** that were found to be moderately fluorescent (Fig. **18**) (unpublished results). A variety of azides have been utilized including benzyl, phenyl, ethylphenyl, fluorenyl, trimethylsilyl, thiophenyl (connected *via* the 3-position of thiophene) and bithiophenyl azide. These monomers are currently being studied as potential base-discriminating fluorophores.

Thiophene Appended Nucleosides

5-(Thiophen-2-yl)uridine was first synthesized in 1994 with the aim of increasing duplex stability through stacking interactions [55]. However, only a modest increase in melting temperature resulted. The fluorescent properties of this and related molecules have been since exploited for mismatch detection. Bi- and terthiophene moieties appended to deoxyuridine with or without an ethynyl spacer (Fig. **19**) all bestow fluorescent properties on the nucleosides with varying suitability towards practical application [56].

It was found that there was only a small thermodynamic penalty associated with duplex formation; however, high ss emission and complex fluorometric data make the nucleoside analogs **39**-**42** of limited use for mismatch detection.

FUSED RING SYSTEMS: FLUORESCENT ANALOGS OF PURINES AND PYRIMIDINES

Benzo- and Naphtho-pyridopyrimidines

Some base discriminating nucleobases of particular interest have been described by Saito and co-workers (Fig. **20**). Benzopyridopyrimidine **43** (BPP) is quenched effectively by G but fluoresces in the presence of A; however, low overall quantum yield and high ss emission prompted the researchers to further elaborate on this scaffold thus giving rise to naphthopyridopyrimidine **44** (NPP) (Fig. **20**) [9]. The latter modified base was found to have a markedly higher quantum yield and less intense ss emission and has therefore found use in A/G SNP typing. Two other fluorescent bases of importance devised by the same group are methoxybenzodeazaadenosine **45** (^{MD}A) and methoxybenzodeazainosine **46** (^{MD}I) (Fig. **20**) [9].

Probes containing these bases are quenched in the presence of T and C complementary oligonucleotides respectively.

Size Expanded Nucleobases

A discussion of fluorescent, modified nucleobases would not be complete without mention of the pioneering work by Leonard and subsequent elaboration by Kool on size expanded nucleobases [57,58]. Leonard's *lin*-benzoadenosine **47** was one of the first examples of size expanded nucleobases which retain the natural hydrogen-bonding characteristics of the parent base (Fig. **21**). Through the insertion of benzene into the purine ring, desirable photopysical properties may be achieved. Kool then applied this ideology to the remaining three natural bases and acquired a full set of benzo-expanded nucleosides and incorporated them into an oligomer which was coined "xDNA" (Fig. **21**) [59].

This work has been developed further by the creation of "yDNA", which differs by the point of insertion of the benzene into the natural base, and naphtho-expanded nucleobases, both of which are highly fluorescent [60]. Although these fluorophores are not well tolerated with natural nucleotides in the same strand, full sequences of the modified bases show interesting fluorescent properties. New emission maxima appear within certain sequence contexts due to excited state interactions and these may be exploited in the future for SNP analysis or other sensing applications.

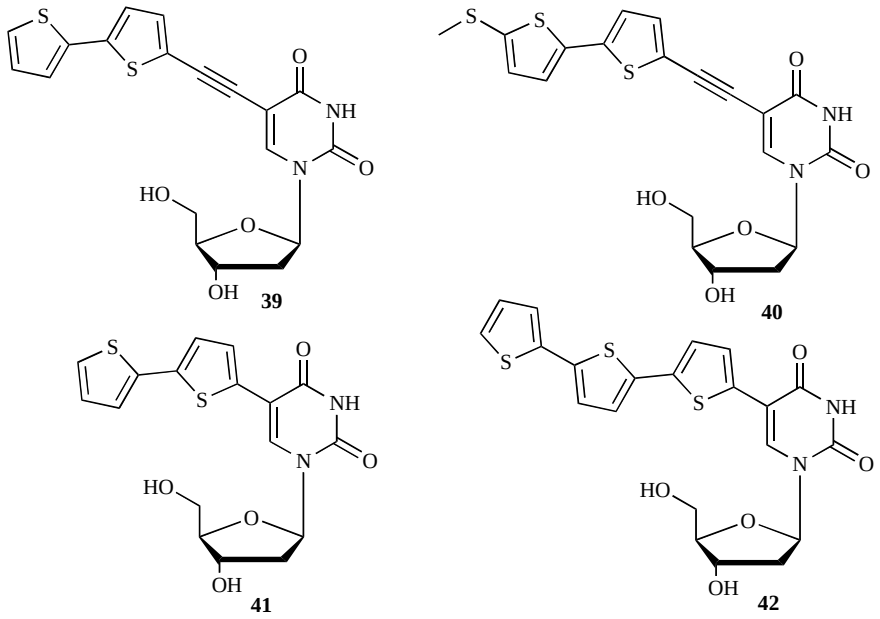


Fig. (19). Bi- and terthiophene derivatized uridines synthesized for use as BDFs [56].

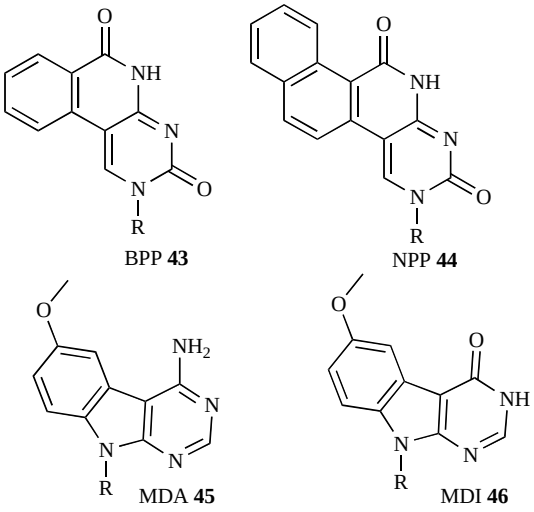


Fig. (20). Expanded-ring-system fluorescent nucleobases developed by Saito and coworkers. benzopyridopyrimidine **43** (BPP), naphthopyridopyrimidine **44** (NPP), methoxybenzodeazainosine **45** (^{MD}I) and methoxybenzodeazaadenosine **46** (^{MD}A), where R = 2'-deoxyribose [9].

Thieno[3,2d]pyrimidine

A particularly elegant, divergent synthesis of isomorphic fluorescent base analogs was recently described by Tor which relies on modification of the thieno[3,2*d*]pyrimidine core **51**. Glycosylation at *N*-1 provides a pyrimidine analog **52**, while *C* glycosylation at the β position of thiophene yields the purine analog **53** (Fig. **22**) [61]. The functional groups can then be manipulated to yield isosteric nucleobase analogs that maintain hydrogen bonding with the natural bases.

Of the 4 isosteric base analogs synthesized, the thymine mimic **52** showed the greatest fluorometric response to changes in solvent polarity. Upon titration with the four natural 5'-monophosphates this analogue showed quenching in the presence of all bases excepting the complementary nucleotide AMP [61]. Such quenching may also occur in ss form making SNP analysis troublesome; indeed, it is yet to be shown if this fluorescence response can be replicated in the context of oligomers.

More recently, a constitutional isomer of the aforementioned compound, based on the thieno[3,4-*d*]pyrimidine core, has been synthesized. Due to the apparent hypsochromic effects observed in lower polarity solvents, the triphosphate was incorporated enzymatically into an RNA transcript. This oligomer was shown to report a C mismatch by an increase in emission [62]. Introduction of this monomer into a specific RNA hairpin the detection of the heterodimeric protein ricin was possible. Ricin catalyses the depurination of ribosomal RNA at specific sites and therefore causes a halt in translation and cell death. Upon cleavage of the unnatural nucleobase an increase in fluorescence is observed. This is a useful contribution to the field as a practical field test for a potential biological warfare agent can be envisaged in the near future [63].

Benzoquinazolines

Benzoquinazoline derivatives have been used as probes for duplex and triplex formation and can be synthesized either by the condensation of an α-aminocyanonaphthalene **56** or an α-aminonaphthanoic acid **58** [64] with urea (Fig. **23**) [65]. The reaction of α-aminocyanonaphthalene with urea results in a cytosine analog **57** which can be converted to the thymine mimic by treatment with conc. HCl in DMF.

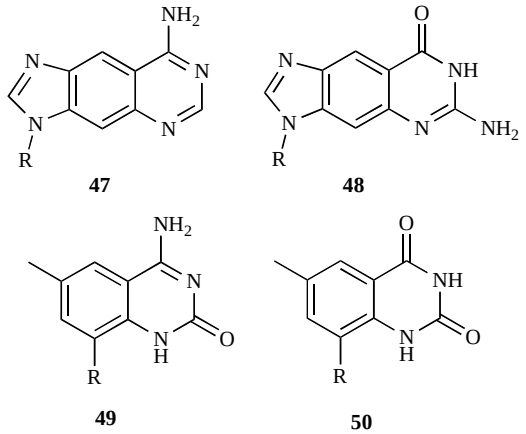


Fig. (21). The full set of "xDNA" benzo-expanded fluorescent bases which maintain canonical Watson-Crick hydrogen bonding [57-59].

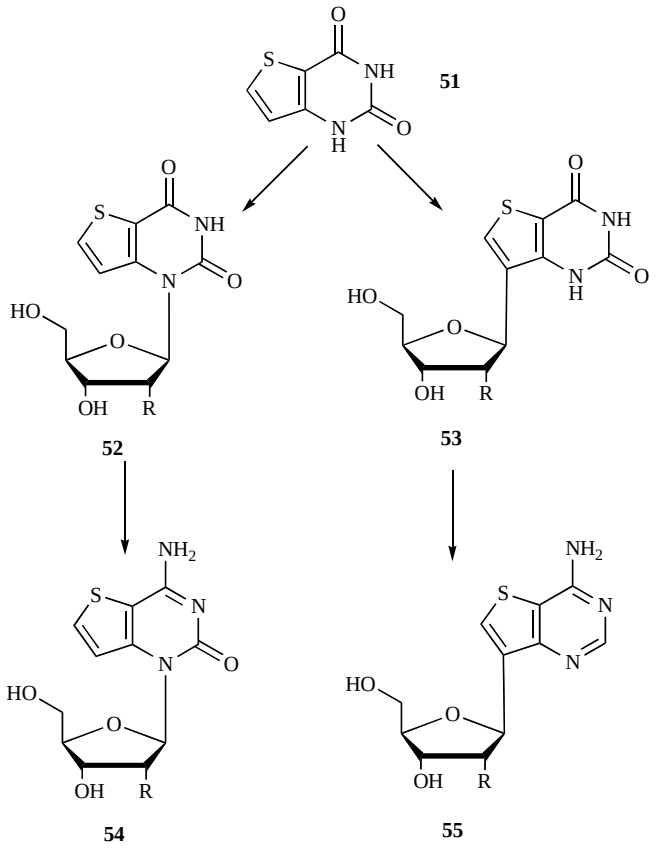


Fig. (22). Alternative glycosylation of the thieno[3,2*d*] pyrimidine core with a protected ribofuranoside yields both pyrimidine (left) and purine (right) analogs that are emissive and can be further manipulated to maintain hydrogen bonding with the natural bases (R = H, OH). See reference for reaction conditions [61].

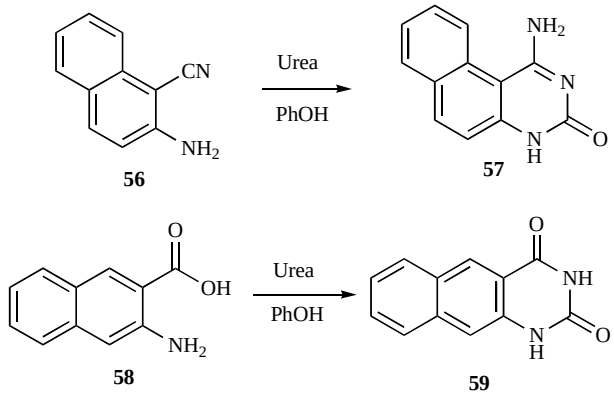


Fig. (23). Synthesis of benzoquinazolines for use as fluorescent pyrimidine mimics [64,65].

Amongst other uses, the benzoquinazolines have been successfully employed as triple helix probes. Homopurine tracts are requisite for triple-helix formation whereby a third strand binds in the major groove of the duplex; in parallel triplexes the third strand, composed of pyrimidines, binds to the purine strand of the duplex in the major groove by Hoogsteen base-pairing [66,67]. Benzoquinazoline thymidine mimics (Fig. **20**) were incorporated into an oligopyrimidine sequence, they were found to be stabilizing towards triple-helix formation (due to more favourable stacking interactions) and, more relevantly, they were also found to respond fluorometrically to hybridization, particularly when incorporated into the Hoogsteen strand. Fluorescence maxima were blue-shifted

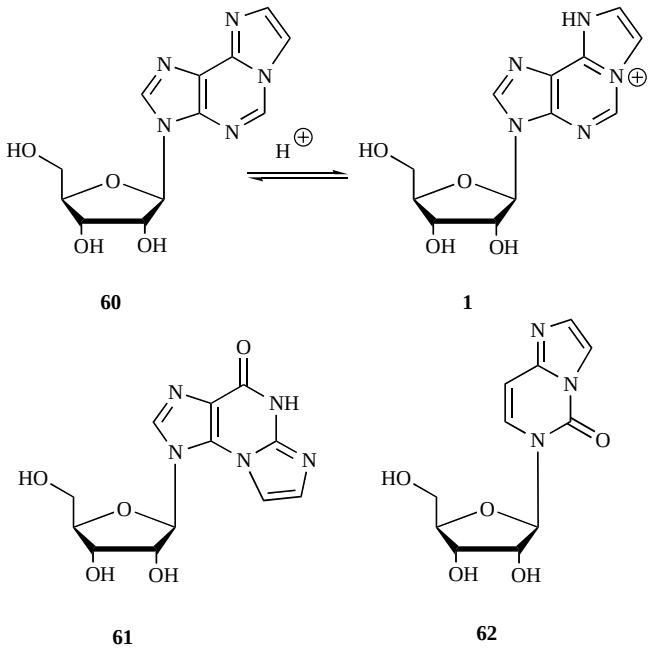


Fig. (24). Etheno(ε)-bridged bases: ε-adenosine **60** in equilibrium with the cationic form **3** responsible for its desirable photophysical properties, ε-guanosine **61** and ε-cytidine **62**.

and decreased in intensity while the excited state lifetime increased upon triple-helix formation. As the formation of parallel triplexes are pH sensitive (relying in part on CG*C^+ triplets) the fluorescence observed was also subject to pH [65].

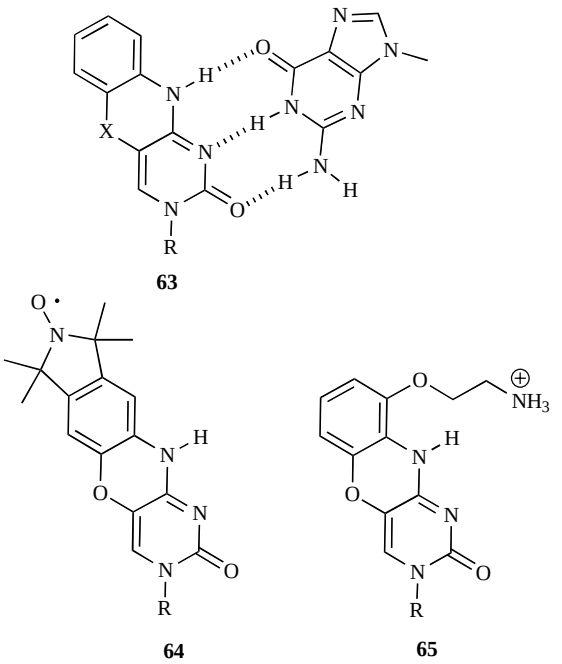
Etheno(ε)-Bridged Bases

Although structurally similar to the natural bases, etheno(ε)-bridged bases possess strikingly different photophysical properties (Fig. **24**). These base analogs can be formed *in vivo* through the action of various carcinogens such as vinyl chloride and ethyl carbamate on the natural exocyclic amine-containing bases [68].

These analogs are highly fluorescent (quantum yields as high as 0.6) and also very pH sensitive, in the deprotonated state the compounds are only weakly fluorescent, this pH sensitivity has been exploited in related derivatives to gauge the acidity of nucleotide binding sites [69], and with excitation wavelengths of 290-300 nm, their usage in the presence of absorbing biological macromolecules is permitted.

Described by Leonard in the early 1970s, ε-adenosine has been used in numerous applications. Its first biologically significant uses were as the bridged ε-adenine dinucleotide (εNAD+) and ε-adenosine triphosphate (εATP) [70,71]. The synthetic NAD+ analog was found to be an active coenzyme in 4 different dehydrogenase-catalyzed reactions and was also an effective substrate for NADase and phosphodiesterase I. Although the emission intensity of εNAD+ is only 8% of the parent monophosphate, full emission is restored upon phosphodiester cleavage allowing the real-time monitoring of such hydrolyses. εATP was found to be a versatile analog of ATP, it is an allosteric effector of several enzymes and a co-substrate of kinases [71].

It has been found recently that ε-7-deaza-2'-deoxyadenosine has a higher quantum yield than the corresponding ε-adenosine and is more stable to extreme pH [72]. Despite the solution of a crystal structure containing a putative ε-A – G base pair [73] evidence for similar behaviour in solution is scant as both ε-A and its corresponding deaza-compound are destabilizing towards duplex formation when placed within an oligonucleotide sequence. This lack of



63 **64** **65**

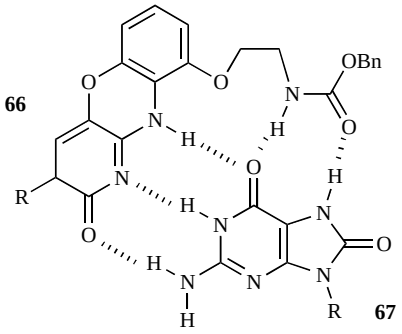
Fig. (25). 3,5-diaza-4-oxophenothiazine (tC) (X = S, **35**) and 3,5-diaza-4-phenoxazine (tC^{O}) (X = O, **35**) in hybridization with guanine. Elaboration of the $tC^{(O)}$ scaffold has yielded a nitroxide spin labeled compound **64** that may be used for EPR measurements and the "G-clamp" **65** which has increased binding affinity to guanine [74-84].

base-pairing competency obviously limits their potential use as BDFs.

Tricyclic Analogs of Cytosine: 3,5-Diaza-4-oxophenothiazine (tC) and 3,5-diaza-4-phenoxazine (tC^{O})

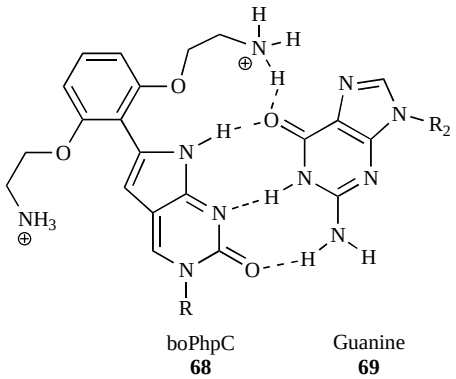
These base analogs have been known for over a decade and were the first example of tricyclic pyrimidine base analogs capable of complementary base-pairing and compounds of similar structures continue to be pursued (Fig. **25**). These tricyclic cytidine analogs base-pair with guanine effectively and also impart greater stability towards the duplex, purportedly due to increased stacking interactions; indeed, when two phenoxazine or phenothiazine tricycles are neighbouring one another the duplex stability is further increased [74]. The compounds are highly fluorescent, tC **63** (X=S) as the free nucleobase analog (which can be prepared according to Roth and Schloemer [75]) is only very slightly more emissive than when incorporated into an oligonucleotide. A small increase in anistropy is found upon hybridization to a complementary strand, brought about by the increase in effective molecular mass. The long emission wavelength of tC means it may be used in FRET experiments coupled with chromophores such as rhodamine as the acceptor moiety [76]. Unfortunately, there is little change in the emission spectrum upon hybridization to a complementary strand and only a small increase in the fluorescence lifetime [77]. The phenoxazine **63** (X=O) (tC^{O}) has very similar properties to the phenothiazine although it is slightly more emissive and has had the honour of "brightest DNA-incorporated base analog" bestowed on it by the authors of a recent puclication, as judged by its quantum yield in the duplex state [78]. This striking luminescence was put to use in probing the conformational dynamics of DNA polymerase. Using an Alexa-555 labeled cysteine residue as the FRET acceptor, movement of a subdomain was measured using stopped-flow experiments on nucleotide binding [79].

The phenoxazine derivative, first prepared by Matteucci and coworkers, has been used more recently by Sigurdsson as a structural



66 **67**

Fig. (26). Purported hydrogen-bonding motif between Cbz-protected "G-clamp" **66** and 8-oxoguanosine **67** (R=2'-deoxyribose) [88].



boPhpC **68** Guanine **69**

Fig. (27). The proposed interaction of [bis-*o*-(aminoethoxy) phenyl]pyrrolocytosine to guan(os)ine via simultaneous Watson-Crick and Hoogsteen binding [89].

scaffold for nitroxide spin labeling [80] which is useful for EPR studies (Fig. **25**). The nitroxide, quite expectedly, quenched the fluorophore; however, fluorescence could be re-introduced by reduction of the nitroxide to the sulphonate ester or the hydroxylamine (the latter of which is fully reversible) while incorporated within an oligonucleotide. This switchable behaviour has obvious benefits, fluorescence and EPR measurements may be made within the same vessel. The use of this compound for SNP typing has been investigated in some simple sequences, it is possible to distinguish between single stranded, match sequence and various mismatches based on emission intensity. Matches with G result in a *ca*. 60% decrease in intensity from the single stranded form with mismatches being flanked by the two extremes of emission. When placed opposite A several maxima appear in the emission spectrum for reasons, as of yet, unclear [81].

Another elaboration of the phenoxazine has been the introduction of an ethoxyamino group to the terminal aromatic ring. The primary ammonium is capable of forming an additional hydrogen bond to guanine on the Hoogsteen face, further increasing duplex stability while maintaining desirable photophysical properties [82] this additional hydrogen bonding inspired the moniker "G-clamp". The G-clamp has been introduced into both DNA and PNA and shows a high degree of sequence dependence with regards to binding affinity [83,84]. The increased binding affinity of the G-clamp has been exploited to sterically inhibit HIV-1 Tat-dependent *trans*-activation *in vivo* when incorporated into a 2'-*O*-Me oligoribonucleotide [84]. Replacing the primary amine of the "G-clamp" with a guanidine functionality further stabilizes the duplex through an additional hydrogen bond with the *N*7 of guanine. These modified bases have been incorporated into PNA, 2'-*O*-Me oligoribonucleotides, 2'-*O*-methoxyethyl phosphorothioate oligoribonucleotides and DNA primarily for stability purposes and the fluorescence response data have not been hitherto reported [85-87].

G-clamp derivatives have been used for the fluorometric detection of 8-oxoguanosine [88]. Protection of the pendant amino functionality of the "G-clamp" with a Cbz group allowed for selective quenching in the presence of 8-oxoguanosine. This is purportedly due a hydrogen bonding interaction of the carbonyl oxygen of the Cbz group with the *N*7 hydrogen of 8-oxoguanosine **67** (Fig. **26**).

Quenching was found to occur only on titration of 8-oxoguanosine, the natural nucleosides, A, C and T, did not affect fluorescence appreciably.

Mechanistically similar to the "G-clamp", [bis-*o*-(aminoethoxy) phenyl]pyrrolocytosine (boPhpC) was designed to interact with the Hoogsteen face of guanine (Fig. **27**).

This base has been incorporated into PNA and was shown to increase the thermal stability of duplexes with natural nucleic acid oligomers by *ca.* 10°C although this does vary with sequence context. The compound, like the "G-clamp", has a very high quantum yield (Φ = 0.32 for boPhpC in H_2O); however, unlike the "G-clamp" boPhpC is highly responsive to microenvironmental conditions. Upon hybridization to a match sequence the emission intensity is decreased by greater than 50%. The high quantum yield, responsiveness and stabilizing properties of this base replacement give it potential for antisense/antigene applications and as a reporter group.

CONCLUSIONS

Although much progress has been made, the repertoire of useful fluorescent nucleotides must continue to be expanded as no universal system exists. SNP analysis remains one of the primary goals in this field; however, new applications continue to be found. The ability to monitor gene regulation, transcription and protein/nucleic acid interactions in general is becoming increasingly accessible. Combinations of a BDF with an oligonucleotide analog which provides high affinity and discriminating binding, such as LNA, PNA or morpholinos has yet to be fully explored.

Certain examples deemed of interest by the authors were highlighted in order to give a broad overview of the field. Due to the scope of this area of research this report was not intended to be fully comprehensive, for a more complete overview of the literature the reader is directed to several excellent reviews [9,42,90-92].

ACKNOWLEDGEMENT

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