Nucleic Acids

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An Isosteric and Fluorescent DNA Base Pair Consisting of 4-aminophthalimide and 2,4-diaminopyrimidine as C-Nucleosides

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Abstract: *A 13mer DNA duplex containing the artificial 4-aminophthalimide:2,4-diaminopyrimidine (4AP:DAP) base pair in the central position was characterized by optical and NMR spectroscopy. The fluorescence of 4AP in the duplex has a large Stokes shift of $\Delta\lambda = 124$ nm and a quantum yield of $\Phi_F = 24\,\%$. The NMR structure shows that two interstrand hydrogen bonds are formed and confirms the artificial base pairing. In contrast, the 4-N,N-dimethylaminophthalimide moiety prefers the syn conformation in DNA. The fluorescence intensity of this chromophore in DNA is very low and the NMR structure shows no significant interaction with DAP. Primer-extension experiments with DNA polymerases showed that not only is the 4AP C nucleotide incorporated at the desired position opposite DAP in the template, but also that the polymerase is able to progress past this position to give the full-length product. The observed selectivity supports the NMR results.*

Imaging of nucleic acids requires bright fluorescent base analogues that may be positioned precisely in any DNA or RNA sequence.[1] The isosteric replacement of DNA bases best meets the requirement that fluorescent probes should not alter the structure of double-stranded (ds) DNA and its biological functions. An extensively studied example is 2-aminopurine,[2] although this has the disadvantages that 1) the excitation lies just next to the DNA absorption and occurs with a low coefficient, and 2) the emission also lies in the UV-A region and is quenched in DNA. The absorption of the pteridine nucleosides developed by Hawkins et al. is better separated from that of DNA, and their fluorescence occurs in the visible region, but it is still quenched in DNA.[1a,3] Other important alternatives include ε-ethenoadenine,[4] pyrrolo-fused cytosine,[5] and cyclized 4-*N*-carbamoyl-2′-deoxycytidine derivatives.[6] Unfortunately these base surrogates also show low fluorescence quantum yields. The problem could be solved by increasing the size, as Wilhemsson et al. have demonstrated for 1,3-diaza-2-oxophenoxa/thiazines.[7] Hirao et al. have developed fluorescent base surrogates for the expansion of the genetic alphabet.[8] More recently, Tor et al. established fluorescent and isomorphic nucleoside surrogates based on thieno[3,4-*d*]-pyrimidines[9] that "conquer 2-aminopurine's deficiencies".[10] Luedtke et al. have also synthesized base surrogates[11] through fusion with *N*,*N*-dimethylaniline to combine canonical base pairing with environment-sensitive fluorescence.

4-Aminophthalimide (4AP)[12] can be excited at the border between the UV-A and visible region and shows fluorescence in the visible region that is well separated from the excitation by a large Stokes shift.[13] It exhibits strong solvatofluorochromism[14] and is thus a probe for solvent polarity and hydrogen bonding.[12,15] 4AP derivatives have been used as probes for different environments,[13,16] but only rarely as covalently attached labels for biopolymers,[17] for example, by Hocek et al. as a DNA base modification.[18] 4AP is approximately the size of a purine and should therefore be directly applicable as an isosteric DNA base surrogate.[15a] Theoretical calculations and NMR structures of dsDNA modified internally with C-nucleoside **1** opposite 2,4-aminopyrimidine (DAP) as C-nucleoside **4** revealed that out of three possible H bonds between them, only two are actually formed.[19] A more serious impediment to future use is the fact that a significant fraction of the duplexes forms only a single H bond between 4AP and DAP, and that the backbone structure adjacent to this artificial pair is distorted from native B-DNA. Obviously, **1** does not offer the correct angle between the glycosidic bond and the imide as a "base-pairing" interface. Hence the attachment of the 2′-deoxyribofuranoside was shifted from the 6-position (in **1**) to the 5-position (in **2**) of the 4AP chromophore. We show herein that the new geometry is properly adjusted, and that a unique fluorescent DNA base pair is formed with DAP that is well adapted to the overall B-DNA structure. This allows application in primer extensions with different DNA polymerases. Furthermore, the dimethylated derivative **3** shows increased steric hindrance at the amino substituent and thus serves as an important control for nucleoside *syn* conformation and its influence on fluorescence and base pairing.

Both C-nucleosides (**2** and **3**) were synthesized and show significant solvatochromism and solvatofluorochromism in the UV/Vis absorption and fluorescence spectra (see Figures S3 and S4 in Supporting Information). The fluorescence quantum yields of **2** are in the range 0.60–0.70 in nonprotic solvents, but are very low in water (0.036 for **2**, 0.003 for **3**), which is typical for 4-APs.[17a] C-nucleosides **2** and **3** were incorporated into 13mer DNA**2** and DNA**3** and annealed with five different complementary counterstrands that vary in position Y (opposite position X; Scheme 1). The residues used at position Y were **4**; natural A, G, T, or C; or the abasic-

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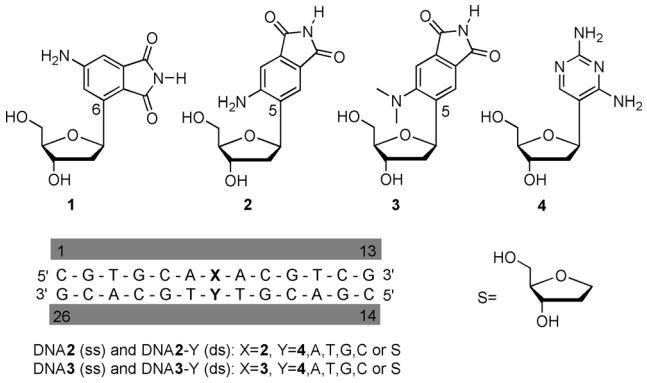


Scheme 1. C-nucleosides of 4-aminophthalimide (4AP; nucleosides **1**–**3**) and of 2,4-diaminopyrimidine (DAP; nucleoside **4**) in the modified DNA single strands DNA**2**, DNA**3**, and double strands DNA**2**-Y, DNA**3**-Y, with Y = **4**, A, T, G, C, or S. Labels for NMR structure determination are indicated in gray bars.

site analogue S, to give the 4AP nucleosides the best flexibility to intercalate within the DNA base stack. According to the polarity-dependent fluorescence of **2**, the fluorescence maximum of DNA**2**-Y (see Figure S5) shifts from 530 nm in the single-stranded (ss) DNA**2** (more access to water) to 506 nm in the dsDNA**2**-A. The quantum yield of ssDNA**2** is 0.065 and significantly increases up to 0.24 for DNA**2**-**4**. Melting temperature (T_m, Table 1) analysis revealed that DNA**2**-**4** shows the highest T_m of 54.4 °C, compared to 54.2–51.4 °C for DNA**2**-A/T/G/C. The T_m value of DNA**2**-S is the lowest (49.3 °C), which supports partial or complete base pairing of **2** in the other DNA duplexes, especially DNA**2**-**4**. Taken together, both the highest T_m and quantum yield of DNA**2**-**4** indicate that **4** acts as the "correct" counterbase to the 4AP moiety of **2** in this duplex. It is important to note here that this result stands in contrast to our previously published DNA samples modified with **1**.

Table 1: Melting temperatures (T_m) and quantum yields (Φ_F) of DNA**2**-Y and DNA**2**-Y; the T_m value of an unmodified duplex is 60.3 °C.[20]

	With **1**[20]		With **2**			With **3**	
Y	DNA	T_m [°C]	DNA	T_m [°C]	Φ_F		T_m [°C]
4	DNA**1**-**4**	53.6	DNA**2**-**4**	54.4	0.24	DNA**3**-**4**	52.0
A	DNA**1**-A	56.0	DNA**2**-A	54.2	0.23	DNA**3**-A	53.8
T	DNA**1**-T	53.0	DNA**2**-T	53.0	0.15	DNA**3**-T	53.9
G	DNA**1**-G	53.5	DNA**2**-G	52.3	0.05	DNA**3**-G	53.1
C	DNA**1**-C	51.2	DNA**2**-C	51.4	0.23	DNA**3**-C	49.1
S	DNA**1**-S	50.0	DNA**2**-S	49.3	0.18	DNA**3**-S	48.6

In contrast to DNA**2**-Y, DNA**3**-Y does not show a significant increase in fluorescence quantum yield (see Figure S5). This is due to the *syn* conformation of **3** (as discussed below), which interferes with base pairing and inhibits proper stacking within the duplex. Nucleoside **3** bears two methyl groups that increase the electron density. Experimental results and theoretical calculations showed that there is a transition from the locally excited state to a twisted intramolecular charge-transfer state if this is sufficiently stabilized in polar solvents.[17a] If this state is populated, the deactivation into the S_0 state occurs in a radiationless manner. Except for DNA**3**-T,G. the T_m values of DNA**3**-Y are all lower compared to the corresponding duplexes of DNA**2**-Y, which raises the suspicion that the dimethylamino group of **3** perturbs both the double-helical conformation and the base pairing, especially in DNA**3**-**4**.

It is important to confirm the 4AP:DAP base pair with an NMR solution structure. The assignment of the NOESY spectra of DNA**2**–**4** was performed according to described methods.[21] Labels 01–26 for the nucleotides are given in Scheme 1 and in the Supporting Information, together with further details. In our previous work on the pairing properties of **1** and **4** in DNA**1**-**4**,[19] an additional imino proton signal was found that could only be assigned to a central A:T base pair, thus indicating that two conformations coexist in that case. For the current DNA**2**-**4**, we find no evidence that a second structure or conformation is hidden in the data. This is an important result since it shows that by changing the glycosidic connection of the 4AP from C6 (in **1**) to C5 (in **2**), the second conformation is eliminated, as we had predicted.[15] For structure determination of DNA**2**-**4**, simulated annealing was used to calculate an ensemble of 100 structures. An averaged structure was then built from the 10 violation-free structures with minimum energy (Figure 1). Remarkably, it shows a planar and untwisted 4AP:DAP base pair in a nearly perfect coplanar orientation within the base stack. The B-conformation of the dsDNA is only slightly perturbed (see below).

A closer look into the 4AP:DAP base pair, however, reveals that the H bonding interface is not completely closed; only two of the three potential H bonds are formed (Figure 2). The chemical structure of the 4AP chromophore in **2** is isosteric to purines, but has a reversed order of rings, meaning that the six-membered ring is connected to the 2′-deoxyribofuranoside and the five-membered ring faces towards the counter site. Presumably because of this geometrical difference, we observe only two H bonds. Based on the fact that 4AP:DAP is isosteric to a natural base pair and thereby provides shape complementarity,[22] it is important to mention here that these two H bonds are still enough to provide sufficient selectivity for the DNA polymerases in the primer extension (see below).

The 4AP:DAP base pair in the previously investigated DNA**1**-**4** is also able to form two H bonds, but only in one out of two coexisting conformations of the base pair and backbones.[15] The 4AP:DAP base pair in DNA**2**-**4** forms two H bonds homogeneously in a single conformation. As a consequence of switching the backbone from C6 to C5, the C1′–C1′ distance of the central base pair is now 11.71 Å (compared to only 9.23 Å in DNA**1**-**4**). The average is 10.4 Å in all cases. DNA**2**-**4** forms a well-defined B-DNA structure, although with a slightly wider center around the central 4AP:DAP pair. A view of the three central base pairs (Figure S1) reveals a large structural overlap of the 4AP moiety and the adjacent A residue in the 3′-direction and implies strong stacking interactions. This result is supported by the NOESY spectra, which show an upfield-shifted H8 signal of the corresponding A residue at position 8. This structural detail is consistent with

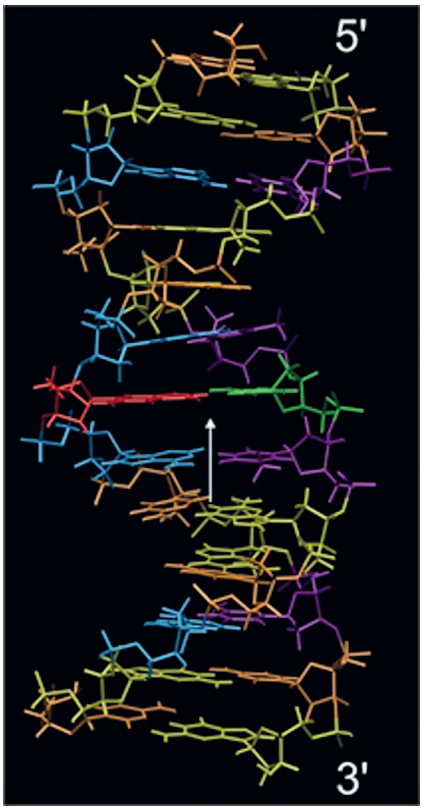


Figure 1. NMR structure of DNA**2**-**4**. Residue **2** is shown red; **4** in green; and A, G, C, and T in blue, yellow, orange, and violet, respectively. White arrows indicate the direction of view of the central base pair in Figure 2 and Figure S2. The RMSD for all H atoms (except methyl) is 0.54 Å.

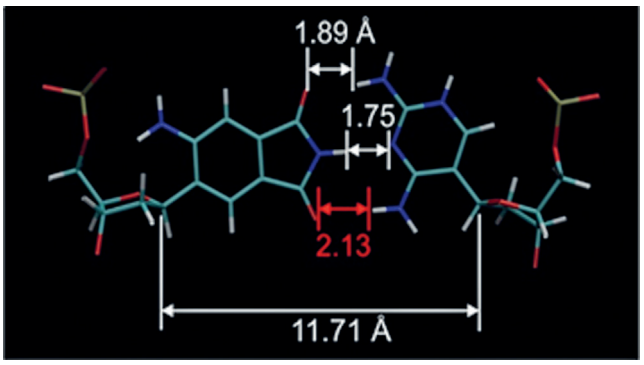
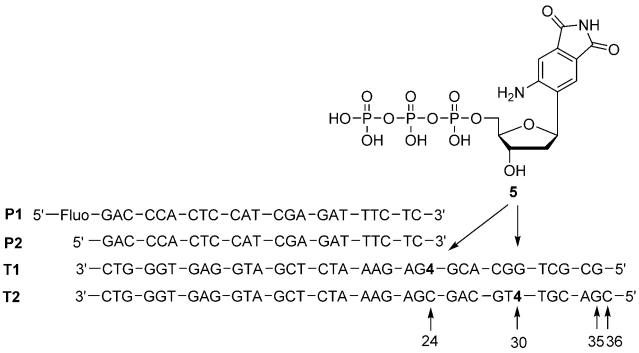


Figure 2. View of the central base pair of DNA**2**-**4** as indicated in Figure 1. The 4AP nucleoside **2** is on the left and the DAP nucleoside **4** on the right.

the observation that the T_m of DNA**2**-**4** is the highest in the duplex set DNA**2**-Y. The sugar conformations of 4AP and DAP are C2′-*endo*-C3′-*exo*, which are typical for B-DNA. This is in contrast to our previously published DNA**1**-**4**, in which the C3′-*endo* (RNA-like) conformation dominates in the conformation with two hydrogen bonds.[15] Hence, we conclude that the artificial 4AP:DAP pair of the current DNA**2**-**4** comes as close to a native base-pair structure as is possible with the 4AP chromophore through slight widening of the duplex and adaptation of the sugar pucker.

The solution structure of DNA**3**-**4** was also determined and is briefly described (see WATERGATE spectra in Figure S2). The results show that **3** primarily takes the *syn* conformation, as can be concluded directly from the NOESY spectra. Full assignment of protons around the central base pair is only possible in case of a 4AP with *syn* conformation. For example, cross-peaks between the 4AP methyl protons and the H2 protons of the A residues at positions 6 and 8 can be found in the NOESY spectrum, and these groups would be too far away in the *anti* conformation. A unique side effect is that the amino groups of DAP in DNA**3**-**4** are visible in the NOESY spectrum. Usually, only the "slow-exchanging" amino groups of C residues can be detected. Structure calculations based on simulated annealing confirmed our direct conclusion.

The base-pairing properties of **2** were further elucidated in primer-extension experiments. The DAP nucleoside **4** was placed into templates **T1** and **T2** as a counterbase to **2** (Scheme 2). These templates were nearly equally long (35 and 36 nucleotides), and were designed for standing- and running-start experiments, respectively. The primers **P1** and **P2** contain 23 nucleotides; only **P1** carries a fluorescein label at the 5′-end to facilitate detection by PAGE. Nucleoside **2** was directly converted into the corresponding triphosphates **5** in 5% yield.[23]



Scheme 2. Nucleotide triphosphate **5**, primers **P1** (labeled with fluorescein at the 5′end) and **P2** (unlabeled), and templates **T1** (standing start) and **T2** (running start) for primer-extension experiments.

An initial screening of DNA polymerases (see Figure S6) in standing-start experiments with **P1** and **T1** revealed that a broad variety of enzymes, especially the Vent(*-exo*), Deep Vent(*-exo*) and Therminator polymerases, tolerate **5** and extend **P1**. In the absence of **5**, **P1** is also extended with any of the four natural dNTPs but not as efficiently as with **5** (see Figure S7 in comparison to Figure S6). **P2** (which lacks the fluorescein label) allows the detection of 4AP through its intrinsic fluorescence in the extension product. In fact, the products with **P2** showed 4AP fluorescence (510–560 nm) when excited in the UV-A region (see Figure S8). This is an important observation since it proves that the 4AP moiety is not only successfully incorporated by the DNA polymerase but also remains chemically intact. Representative standing-start experiments performed with the positively evaluated

Vent(*-exo*), Deep Vent(*-exo*), and Therminator polymerases, in the presence of **5** and the four natural dNTPs, revealed complete extension to the full-length product (35mer; Figure 3).

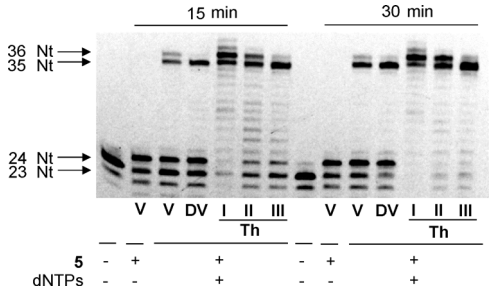


Figure 3. PAGE analysis of polymerase screening for in the presence (+) and in absence (−) of **5** and dNTPs. **P1** (750 nM); **T1** (900 nM); **5** and dNTPs (200 μM); polymerases (1.0 μL): V = Vent(*-exo*), DV = Deep Vent(*-exo*), Th I-III = Therminator I-III; reaction at 70 °C; $\lambda_{exc} = 470 \pm 20$ nm, $\lambda_{em} = 535 \pm 20$ nm. The band smaller than 23-nt is an N−1 contamination.

Standing-start (**T1**) and running-start (**T2**) experiments were performed with **P2** (Figure 4). The full-length extension products were detectable through their 4AP fluorescence in all experiments with **5** and the four dNTPs, which shows that Therminator polymerases I-III, in particular, are able not only to incorporate 4AP but also to progress past the 4AP modification site. Especially in the running-start experiments, the 30mer intermediate product is detectable. The DNA polymerase may pause after 4AP incorporation, and progression through the 4AP site is slower, which lowers the yield of full-length product. On the other hand, this observation indicates that **5** is only incorporated opposite DAP, as desired. In the case of significant amounts of misincorporation of **5** opposite A, G, T, or C (in **T1** or **T2**), oligonucleotide products of different lengths should be detectable from their 4AP fluorescence. In standing-start experiments (**T1**) in the presence of **5** but absence of the four natural dNTPs (lanes 1 and 4 in Figure 4), a small band is observed that shows similar electrophoretic mobility as the full-length product. We assume that this band results from non-templated 3′-transferase activity, which is typical for these polymerases.

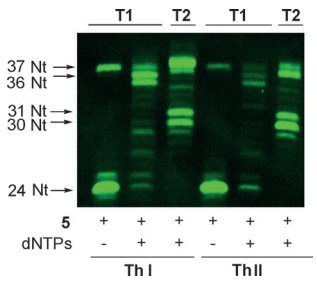


Figure 4. PAGE analysis of primer extensions in the presence (+) and absence (−) of **5** and dNTPs. **P2** (750 nM), **T1** and **T2** (900 nM); **5** and dNTPs (200 μM); polymerases (1.0 μL): Th I and II = Therminator type I and II; $\lambda_{exc} = 312$ nm, $\lambda_{em} = 535 \pm 20$ nm. Note that **P2** is not labeled and cannot be seen on the gel; the visible 24mer is the extended primer (+1).

In conclusion, the C-nucleosides of 4AP (**2**) and 4-*N*,*N*-dimethylaminophthalimide (**3**), were synthesized as isosteric DNA base surrogates. The key structural detail is that both C-nucleosides bear the 2′-deoxyribofuranoside in the 5-position of the 4AP chromophore to allow specific base pairing with the C-nucleoside of DAP (**4**) in the counterstrand. The fluorescence of DNA**2**-**4** shows a quantum yield of 24%, a brightness of $800\,M^{-1}cm^{-1}$, and a large Stokes shift of 124 nm. These are remarkable optical properties considering the very small size of this artificial base pair, and compared to other isosteric fluorescent DNA base surrogates.[10,11] The NMR structure reveals two interstrand H bonds that provide selectivity in the 4AP:DAP base pair. Primer extensions were performed with the triphosphate **5**. In standing- and running-start experiments with the Therminator DNA polymerases, both specific incorporation of **2** at the desired position opposite **4** and polymerase progression past this position was successfully demonstrated. This clearly shows that the two H bonds that are observable in the NMR solution structure between 4AP and DAP are enough to provide sufficient selectivity in primer extension. Remarkably, the fluorescence of the 4AP chromophore was detectable in the extension products. It follows that the 4AP fluorophore is chemically stable enough to be applied under physiological conditions. Our observations show that the 4AP:DAP base pair is not only isosteric and selective but also fluorescent. It has significant potential for applications in chemical biology, especially with respect to the molecular imaging of living cells.

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Keywords: DNA · DNA polymerase · fluoroescence · NMR spectroscopy · oligonucleotide

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Communications

Nucleic Acids

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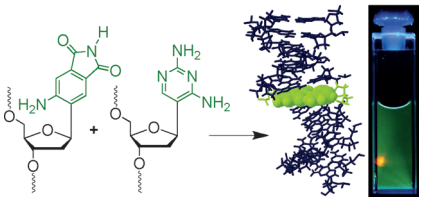
An Isosteric and Fluorescent DNA Base Pair Consisting of 4-aminophthalimide and 2,4-diaminopyrimidine as C-Nucleosides



Let's get together and feel all bright: The C-nucleosides of 4-aminophthalimide (4-AP) and 2,4-diaminopyrimidine (DAP) form a stable and brightly fluorescent base pair in double-stranded DNA, which shows great potential for molecular imaging in living cells. Primer-extension experiments showed that DNA polymerases can selectively incorporate the 4-AP nucleotide opposite DAP in the template and continue on to give the full-length product.