# pH-Independent Triple Helix Formation by an Oligonucleotide Containing a Pyrazine Donor−Donor−Acceptor Base

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Duplex DNA with purine-consecutive sequences can be targeted by oligonucleotides that bind as a third strand in the major groove to form a triple helix structure.[1] In the parallel binding motif, a thymine in the third strand is used to form a Hoogsteen pair with adenine at neutral pH. There is, however, no natural base that can bind in a Hoogsteen fashion to guanine in its predominant form at pH 7. Generally, protonated cytosine is used in this capacity (Figure 1). However, as the p$K_a$ of protonated cytosine is ~4.2 and the intracellular pH is ~7.4, protonated C has limited applicability as a component in a triple strand targeted against G−C base pairs inside a cell.

Some time ago, we noted that an expanded set of nucleoside bases could be designed that exploits the full structural possibilities of the Watson-Crick base pair (Figure 2).[2,3] One of these, the pyDDA base (where py indicates that the aglycon is a pyrimidine analog, and D and A refer to hydrogen bond donor and acceptor groups on the ring, respectively), presents the same hydrogen bonding pattern as protonated cytosine.[4] This suggested that the expanded genetic "alphabet" might not only allow the formation of the full set of Watson−Crick base pairs but should also complete the set of possible Hoogsteen base pairs. We report here that the pyDDA base does so, supporting triple helix formation independent of pH from pH 6.3 to 8.0 when incorporated into an appropriate oligonucleotide strand.

Benzoylation of pyrazine riboside **1**[5] yields the tribenzoate, which was converted to the monobenzoate via mild hydrolysis, and then to the *O*-allyl protected derivative **2** via a Mitsunobu reaction (Figure 3). The silyl, isopropylidene, and benzoyl protecting groups were then removed in two steps, and the exocyclic amino group of the pyrazine was protected as the dimethylformamidine to yield **3**.[6] The compound was then converted to its 3′,5′-tetraisopropyldisilyl derivative, the 2′-oxygen methylated, and the silyl protecting group removed. The product **4** was converted to a 5′-dimethoxytritylated phosphoramidite suitable for incorporation into an oligonucleotide by automated solid phase synthesis.[7]

To measure triplex formation, synthetic oligonucleotides with the sequence 5′-TTTTTZTTTTTTTTT-3′, where Z is either the pyDDA nonstandard base (as its 2′-OMe riboside) or 2′-deoxycytidine, were mixed in equimolar amounts with 5′-GCAAAAAGAAAAAAAAACG-3′ and 3′-CGTTTTTCTTTTTTTTTGC-5′. The ultraviolet absorbance at 260 nm was then followed as a function of temperature through multiple heating−cooling cycles in aqueous media at different pH values. Melting temperatures were extracted by computer analysis of the melting curves and are reported in Table 1. The melting curves were also obtained at 370 nm, where the pyrazine base (but not the standard bases) absorbs. The correspondence between the two experiments unambiguously assigned the melting transition observed at ~29 °C to the disassociation of the third strand (containing the pyDDA base) from the duplex.

The results in Table 1 show that the pyDDA base contributes to triplex stability in a pH-independent fashion. Interestingly, the heating and cooling curves were superimposable to within 10%, even after two heating−cooling cycles to 80 °C. This implies that the pyDDA 2′-OMe riboside does not epimerize more than ~10%, much less than the epimerization observed with the pyADD base.[3] This improved behavior may reflect

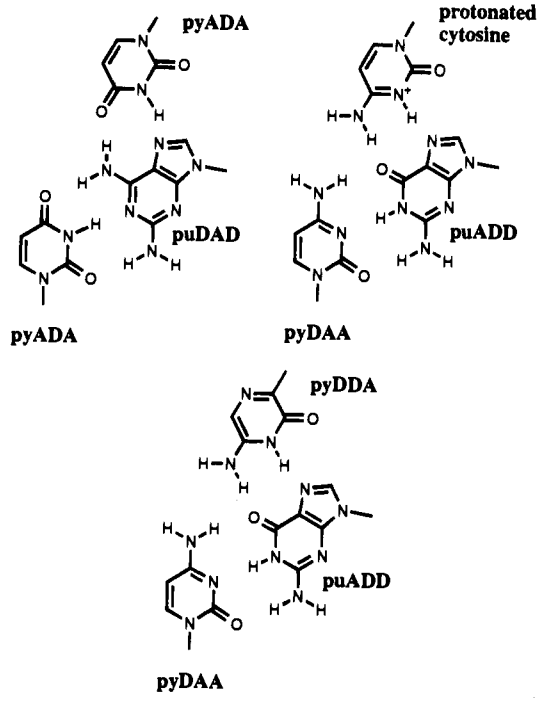


**Figure 1.** Triple helices formed between T and the A-T base pair, protonated C and the G-C base pair, and the nonstandard pyDAA and the G-C base pair.

**Table 1.** Melting Temperatures for Triple and Double Helices[a]

| pH | Z | $T_m1$ | $T_m2$ |
|---|---|---|---|
| 6.3 | C | 27.1 | 62.3 |
| 7.0 | C | 25.0 | 61.1 |
| 7.3 | C | 22.6 | 61.4 |
| 7.7 | C | 22.5 | 60.9 |
| 8.0 | C | 18.5 | 60.5 |
| 6.3 | pyDDA | 28.8 | 62.2 |
| 7.0 | pyDDA | 30.0 | 62.6 |
| 7.3 | pyDDA | 27.9 | 62.2 |
| 7.7 | pyDDA | 28.5 | 61.8 |
| 8.0 | pyDDA | 28.2 | 62.3 |

[a] Melting temperatures are ±1.5 °C, and are extracted from primary data by computer fitting. The sequences used are 5′-TTTTTZTT TTTTTTT-3′, where Z is either the pyDDA nonstandard 2′-OMe riboside or 2′-deoxycytidine, 5′-GCAAAAAGAAAAAAAAACG-3′, and 3′-CGTTTTTCTTTTTTTTTGC-5′. $T_m1$ is assigned to the disassociation of the third strand; $T_m2$ is assigned to duplex disassociation. Data were collected in 10 mM $Na_2HPO_4$ buffer adjusted with $H_3PO_4$ to pH 6.3, 7.0, 7.3, 7.7, or 8.0 with 1 M NaCl.

(1) Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645−650.
(2) Benner, S. A.; Allemann, R. K.; Ellington, A. D.; Ge, L.; Glasfeld, A.; Leanz, G. F.; Krauch, T.; MacPherson, L. J.; Moroney, S. E.; Piccirilli, J. A.; Weinhold, E. G. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 53−63.
(3) Voegel, J. J.; Benner, S. A. *J. Am. Chem. Soc.* **1994**, *116*, 6929−6930.
(4) The p$K_a$ for deprotonation of the pyrazine was measured by titration to be 8.2.
(5) von Krosigk, U. Entwurf und Synthese eines neuen Basenpaars für das genetischen Alphabet. ETH Dissertation No. 10164, 1993. All synthetic intermediates were fully characterized.
(6) This protecting group strategy reflects the instability of the formamidine protecting group under the strong acidic conditions needed to remove the isopropylidine group and the incompatibility of the benzoyl protection with the methylation conditions.
(7) The tritylated product was released from the support via ammonia and purified by HPLC. The recovered product was detritylated (80% HOAc, 30 min), the product purified again by HPLC, and the allyl group removed in the final step. The product was purified again by HPLC. The oligonucleotide was degraded and the degradation products analyzed by HPLC; the pyrazine base was identified by its characteristic absorbance at 370 nm.

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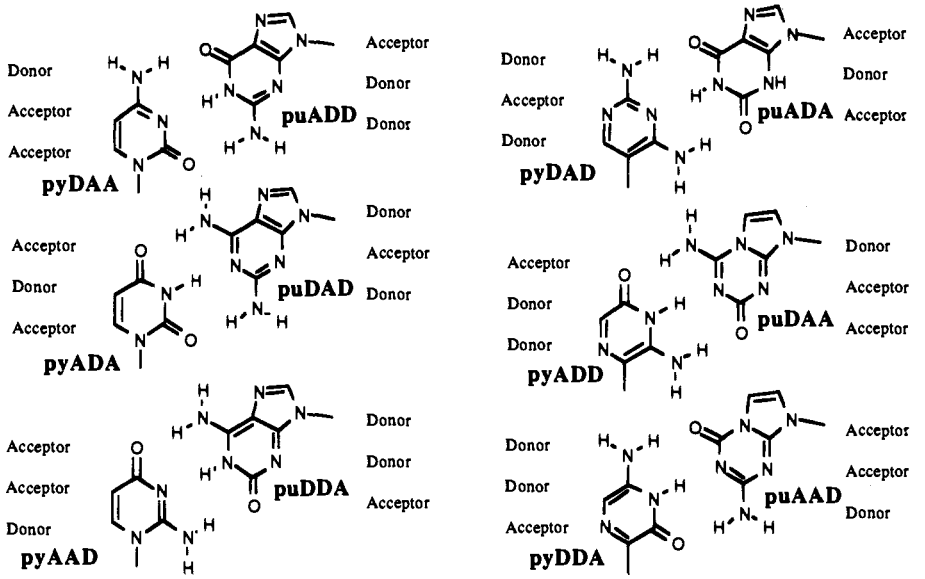


**Figure 2.** Expanded set of nucleotide bases.

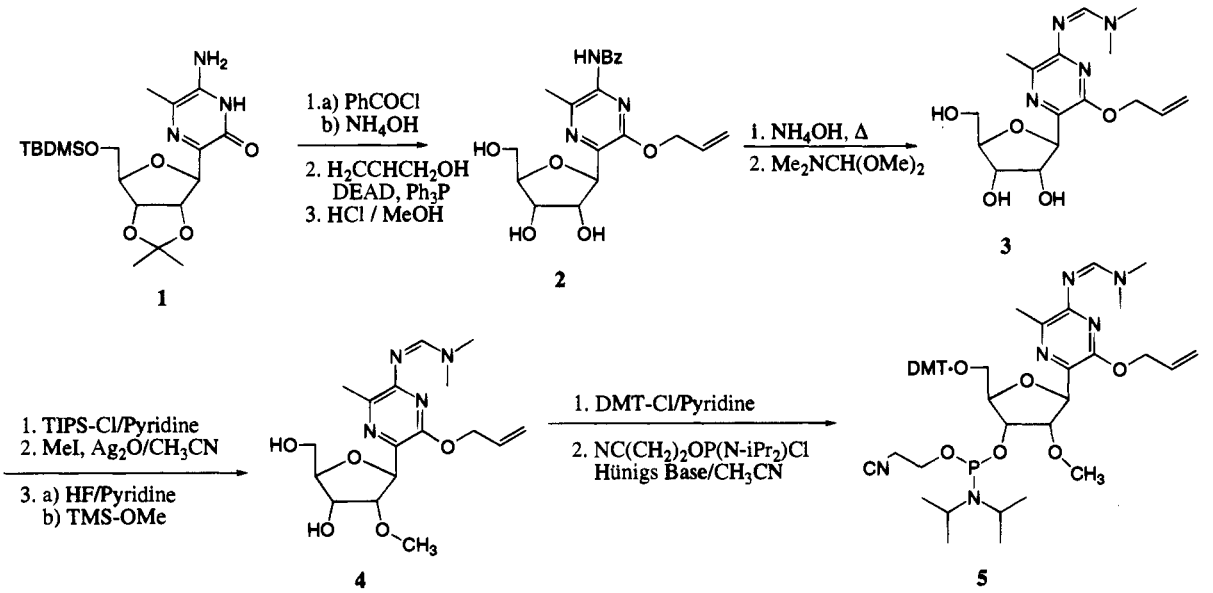


**Figure 3.** Synthesis of a nucleoside derivative, suitable for solid phase oligonucleotide synthesis, bearing the pyrazine pyDDA base, which presents a hydrogen bond donor–donor–acceptor hydrogen bonding pattern to a complementary oligonucleotide.

an increased $\beta/\alpha$ equilibrium constant due to steric hindrance arising from the 2′-OMe group.

Some time ago, Ono *et al.*[8] suggested that pseudoisocytosine might also serve as an analog of protonated cytosine in a triplex forming oligonucleotide. After this work was accepted for publication, Xiang *et al.* introduced the 5-methyl-2,6-(1*H*,3*H*)-pyrimidione as a potentially useful analog of protonated cytosine.[9] Pseudoisocytosine was not included into the base set in Zurich because it is tautomerically ambiguous; by shifting a proton, it can present either a pyDDA or a pyDAA hydrogen bonding pattern to a complementary strand. This tautomeric ambiguity should not create serious problems in the binding of pseudoisocytosine to duplex DNA in the major groove. However, it should make it difficult to incorporate pseudoisocytosine at a specific sequence into an oligonucleotide synthesized by template-directed polymerization.[10] Thus, the pyDDA base reported here may have advantages as a recognition element to expand the ability of oligonucleotides or their membrane permeable analogs[11] to form major groove binding elements. This should increase the facility with which triplex strategies are pursued in intracellular gene targeting.

**Supplementary Material Available:** Experimental details and characterization data (31 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(8) Ono, A.; Ts'o, P. O. P.; Kan, L.-S. *J. Am. Chem. Soc.* **1991**, *113*, 4032–4033; *J. Org. Chem.* **1992**, *57*, 3225–3230.

(9) Xiang, G., Soussou, W., McLaughlin, L. W. *J. Am. Chem. Soc.* **1994**, *116*, 11155–11156.

(10) (a) Switzer, C. Y.; Moroney, S. E.; Benner, S. A. *J. Am. Chem. Soc.* **1989**, *111*, 8322–8323. (b) Piccirilli, J. A.; Krauch, T. , Moroney, S. E.; Benner, S. A. *Nature* **1990**, *343*, 33–37. (c) Switzer, C. Y.; Moroney, S. E.; Benner, S. A. *Biochemistry* **1993**, *32*, 10489–10496.

(11) Huang, Z.; Schneider, K. C.; Benner, S. A. *J. Org. Chem.* **1991**, *56*, 3869–3882.