

Oligonucleotide Duplexes and Multistrand Assemblies with 8-Aza-2'-deoxyisoguanosine: A Fluorescent isoG_d Shape Mimic Expanding the Genetic Alphabet and Forming Ionophores

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Abstract: 8-Aza-2'-deoxyisoguanosine (**4**) is the first fluorescent shape mimic of 2'-deoxyisoguanosine (**1a**); its fluorescence is stronger in alkaline medium than under neutral conditions. Nucleoside **4**, which was synthesized from 8-aza-2'-deoxyguanosine via a 4,6-diamino intermediate after selective deamination, was incorporated in oligodeoxyribonucleotides using phosphoramidite **11**. Duplexes with **4**·m⁵iC_d (5-methyl-2'-deoxyisocytidine) base pairs are more stable than those incorporating dG·dC pairs, thereby expanding the genetic alphabet by a fluorescent orthogonal base pair. As demonstrated by *T_m* measurements, the base pair stability decreases in the order m⁵iC_d·**4** > dG·**4** > dT·**4** ≥ dC·**4** > dA·**4**. A better base pairing selectivity of **4** against the canonical nucleosides dT, dC, dA, and dG is observed than for the degenerated base pairing of **1a**. The base pair stability changes can be monitored by nucleobase anion fluorescence sensing. The fluorescence change correlates to the DNA base pair stability. Oligonucleotide 5'-d(T₄4T₄) (**22**), containing short runs of nucleoside **4**, forms stable multistranded assemblies (ionophores) with K⁺ in the central cavity. They are quite stable at elevated temperature but are destroyed at high pH value.

Introduction

Isoguanine is formed by oxidative stress of adenine either in DNA or on monomeric nucleotides.^{1–4} Isoguanine (purine numbering is used throughout the results and discussion section) occurs naturally in butterfly wing⁵ and the riboside (crotonoside) in croton beans⁶ and mollusks.⁷ Isoguanine forms an orthogonal base pair with isocytosine, thereby expanding the genetic alphabet.^{8,9} As a result of the tautomerism (2-hydroxyadenine vs 2-oxoadenine),^{10,11a} 2'-deoxyisoguanosine¹² (**1a**, Figure 1)

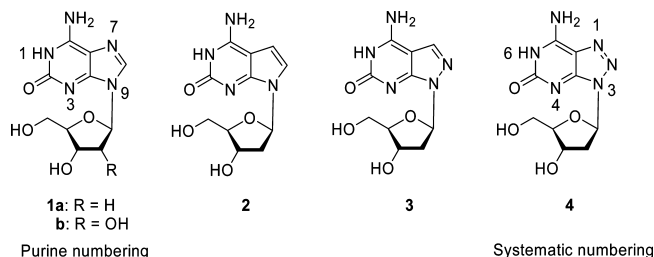


Figure 1

is a promiscuous nucleoside.^{13,14} Oligonucleotides containing 2'-deoxyisoguanosine (**1a**), which were synthesized by us^{15–17} and by others,^{18b,e–g} form duplexes and triplexes with parallel or antiparallel chain orientation.^{16,18} Multistranded assemblies

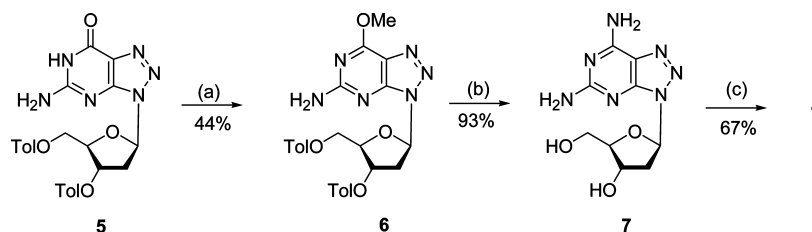
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Scheme 1^a

^a Reagents and conditions: (a) trifluoroacetic anhydride, pyridine, ice bath, 1 h; 0.1 M NaOMe/MeOH, overnight, rt; (b) NH₃/MeOH in autoclave, 24 h, 80 °C; (c) sodium nitrite, acetic acid, H₂O, 60 °C, 20 min; 25% aqueous NH₃ solution to pH 8.

are formed by oligonucleotides^{18a,19} and homopolynucleotides²⁰ containing short runs of isoguanine in the presence of alkali ions.^{18d,f} Whereas guanosine and 2'-deoxyguanosine form quartet structures,²¹ a pentameric structure was detected on a lipophilic isoguanosine derivative by single crystal X-ray analysis.²² Quadruplex and pentaplex assemblies were reported for oligonucleotides incorporating 2'-deoxyisoguanosine thereby forming ionophores.^{18d,e,23}

Shape mimics of 2'-deoxyisoguanosine, such as 7-deaza-2'-deoxyisoguanosine (2) and 8-aza-7-deaza-2'-deoxyisoguanosine (3), which were synthesized in our laboratory,^{12,24–29} show reduced base pair ambiguity when compared to 2'-deoxyisoguanosine.^{30,31} Those studies confirm that structural modifications in the five-membered ring of 2'-deoxyisoguanosine are tolerated as long as the Watson–Crick face of the nucleoside is not touched. This prompted us to study the unknown 8-aza-2'-deoxyisoguanosine (4). 8-Azapurine (3H-1,2,3-triazolo[4,5-d]pyrimidine) nucleosides³² are fluorescent as reported for 8-aza-2'-deoxyguanosine,³³ 8-azaguanosine,³⁴ and

8-aza-2'-deoxyinosine.³⁵ They are isosteric to purine nucleosides and act as purine nucleoside antimetabolites. Among the various isoguanine isosteres only 8-azaisoguanine shows intrinsic fluorescence.³⁶ The same was expected for 8-aza-2'-deoxyisoguanosine (4) as nucleoside or component of oligonucleotides.

This manuscript reports on the synthesis of nucleoside 4 and its conversion into a phosphoramidite building block for solid-phase oligonucleotide synthesis and describes the base pairing properties and the self-assembly of oligonucleotides containing compound 4. Thermal melting and nucleobase fluorescence sensing is used to measure the strength of base pairs; the potential of oligonucleotides with short runs of 8-aza-2'-deoxyisoguanosine (4) to form supramolecular assemblies was evaluated.

Results and Discussion

Monomers. Synthesis and Physical Properties of 8-Aza-2'-deoxyisoguanosine (4) and the Phosphoramidite 11.

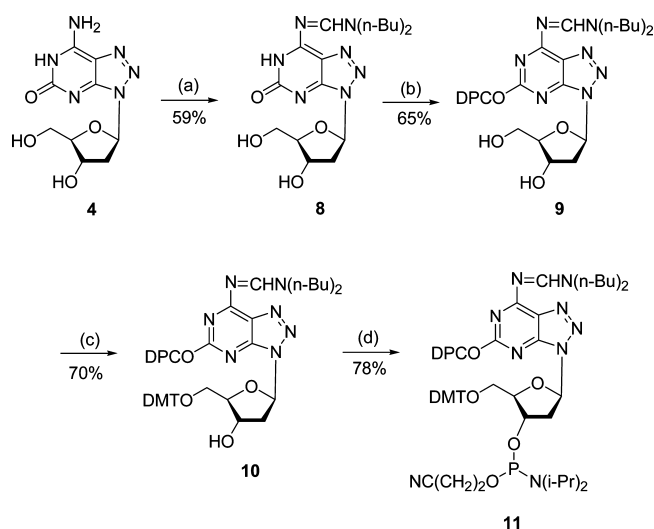
8-Aza-2'-deoxyisoguanosine (4) was prepared from the protected 8-aza-2'-deoxyguanosine (5)^{37,38} according to Scheme 1. After activation of 5 with trifluoroacetic anhydride, compound 5 was directly hydrolyzed in MeOH/NaOMe to give 6. The concentration of sodium methoxide was kept below 0.13 M to avoid degradation of the product.³⁹ Subsequent ammonolysis of 6 in methanolic ammonia afforded the diamino nucleoside 7. This was selectively deaminated^{28b} with sodium nitrite in diluted acetic acid to yield 8-aza-2'-deoxyisoguanosine (4).

Compound 4 was converted into the phosphoramidite 11 (Scheme 2). The amino group was protected with *N,N*-dibutylformamide dimethyl acetal in methanol, furnishing compound 8. The 2-oxo function of 8 had to be protected with the diphenylcarbamoyl residue (\rightarrow 9) as the unprotected compound gives rise to side reactions in oligonucleotide synthesis. Conversion into the DMT compound 10 followed by phosphitylation furnished the phosphoramidite 11. All compounds were characterized by ¹H and ¹³C NMR spectra, and elemental analyses were performed.⁴¹

Among the various isoguanine 2'-deoxyribonucleoside derivatives, there is no fluorescent compound existing that can be considered as a true shape mimic of 2'-deoxyisoguanosine. Only

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Scheme 2^a

^a Reagents and conditions: (a) *N,N*-dibutylformamide dimethyl acetal, methanol, 1 h, rt; (b) *N,N*-diphenyl carbamoyl chloride, *N,N*-diisopropylethylamine, pyridine, 10 min, rt; (c) 4,4'-dimethoxytrityl chloride, pyridine, overnight, rt; (d) *N,N*-diisopropylethylamine, 2-cyanoethyl diisopropylphosphoramidite, CH₂Cl₂, 15 min, rt.

the hitherto unknown 8-aza-2'-deoxyisoguanosine (**4**) develops fluorescence at neutral pH, and the fluorescence intensity increases under alkaline conditions (from pH 7.2 to 9.5; 370 nm) about 10-fold (Figure 2a). This property is similar to other 8-azapurine nucleosides, e.g., 8-azaadenosine, 8-azainosine, or 8-azaguanosine, which are all fluorescent. The excitation and emission maxima are not significantly changed at different pH values (Figure 2a). The Stokes shift⁴⁰ of **4** amounts to about 86 nm. Compound **4** displays different UV spectra in dioxane and water (Figure 2b). Such a phenomenon was already observed in the case of compound **1a**, **2**, and **3** and was correlated to a population change of keto versus enol tautomer.^{11a,29–31} The pH-dependent fluorescence of **4** was used to determine the *pK_a* value of deprotonation, which was found to be 8.3 (Figure 3b) and is almost identical to the *pK_a* value (8.4) obtained using UV spectrophotometry (Figure 3a). Thus, nucleoside **4** is more acidic than 2'-deoxyisoguanosine (**1a**; *pK_a* = 9.9).¹²

Oligonucleotides. Duplex Stability and Mismatch Discrimination Determined by UV Melting and Fluorescence Sensing. In aqueous solution, the canonical nucleic acid constituents guanosine and 2'-deoxyguanosine exist predominantly in the keto (lactam) form (*K_{TAUT}* ≈ 10⁴–10⁵)^{11b} which leads to an almost perfect formation of the Watson–Crick base pair. As only one of 10⁴–10⁵ of the molecules is enolized, mispairing is rare. However, in 2'-deoxyisoguanosine (**1a**, iG_d) the enol tautomer content is about 10%.^{11a,30,31} This causes rather stable pairs (mismatches) with dT, dC, and dG in the center of oligonucleotide duplexes, first reported by our laboratory¹⁵ and also with dA at the dangling end of a duplex reported by Sugimoto.⁴² Due to this mispairing, DNA polymerases catalyze misincorporation of dTTP, dATP, and dGTP opposite to iG_d⁴³ thereby generating mutagenic events.⁴⁴ This limits the application of 2'-deoxyisoguanosine in polymerase-catalyzed

reactions.^{45–49} Copying errors occur during replication when 2'-deoxyisoguanosine (**1a**) is formed in damaged DNA. Oxidation, irradiation, and normal metabolic transformations of the adenine base have been reported.¹ Nevertheless, 2'-deoxyisoguanosine (**1a**) forms very stable base pairs with dC in parallel DNA and with 2'-deoxy-5-methylisocytidine (m⁵iC_d) in DNA with anti-parallel chain orientation.^{15,18b,19,50} Thus, this was used to expand the genetic alphabet.^{8,51,52} Since the keto–enol equilibrium of iG_d depends on the electronic character of the nucleobase and on the polarity of its microenvironment, one expects that the structural modification of the isoguanine base can influence the keto–enol content of 2'-deoxyisoguanosine (**1a**). Indeed, for 7-deaza-2'-deoxyisoguanosine (**2**)³¹ and its 7-halogenated derivatives, the enol content was found to be about 1/1000.^{29,30} Mismatch discrimination was increased for these analogues compared to 2'-deoxyisoguanosine (**1a**), thereby making the coding more stringent.

To investigate base pairing and mismatch discrimination of 8-aza-2'-deoxyisoguanosine (**4**) in duplex DNA, two protocols were used: thermal melting (Tables 1 and 2) and nucleobase anion fluorescence sensing³³ (Figure 4). For that, oligonucleotides⁵³ employing the conventional phosphoramidites and phosphoramidite **11** were synthesized, and characterization of the oligonucleotides was performed as previously reported.⁴¹ In both protocols, our reference duplex 5'-d(TAGGTCAATACT) (**12**)•3'-d(ATCCAGTTATGA) (**13**) was modified in a central position. Duplexes containing 2'-deoxyisoguanosine (**1a**) were measured for comparison (Table 1). Thermal melting experiments were performed in 1.0 M NaCl, 0.1 M MgCl₂, 60 mM Na-cacodylate at pH 7, and the thermodynamic data of duplex formation were calculated from each individual melting profile. As listed in Table 1, the matched duplex containing a **4**•m⁵iC_d base pair (**14**•**15**, *T_m* = 53.5 °C) is more stable than that with a dG•dC pair (**12**•**13**, *T_m* = 50.0 °C) but equally stable as that with a m⁵iC_d•iG_d pair (**14**•**16**, *T_m* = 54 °C). From that point of view, 8-aza-2'-deoxyisoguanosine (**4**) is a perfect surrogate of 2'-deoxyisoguanosine (**1a**) in a tridentate base pair.

In order to evaluate mismatch discrimination in detail, the four canonical nucleosides (X = dA, dG, dT, dC) were placed opposite to the modification site [duplexes 5'-d(TAGGTX-AATACT)•3'-d(ATCCA4TTATGA)], and the *T_m* values were measured (Table 1). In all cases the base discrimination of **4** against the four canonical DNA bases is significantly better (higher ΔT_m) than that of 2'-deoxyisoguanosine or even 7-deaza-2'-deoxyisoguanosine.^{24,30,31} We anticipate that this results from the reduced formation of the enol tautomer.^{30,31} However, we have not determined the keto–enol content of **4** as corresponding fixed methyl isomers that are used for this

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(53) In all oligonucleotides the modified components are always 2'-deoxyribonucleosides.

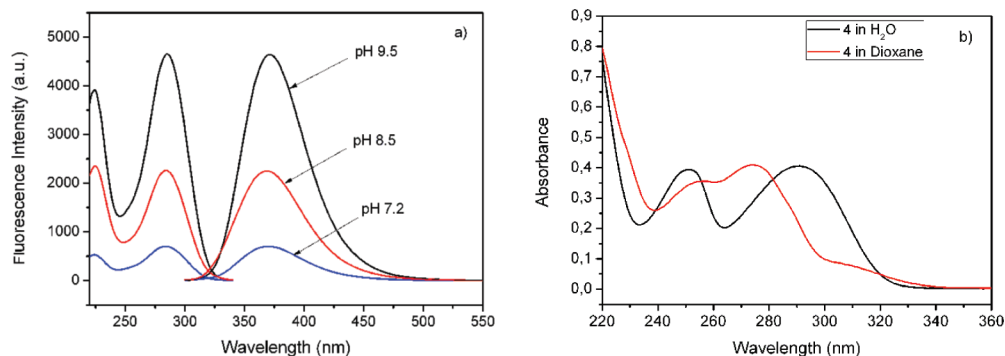


Figure 2. (a) Fluorescence excitation and emission spectra of 2.5 μM 8-aza-2'-deoxyisoguanosine (**4**) at the pH values shown in the figure. The spectra were measured in 1.0 M NaCl, 60 mM Na-cacodylate buffer. (b) UV spectra of compound **4** with 50 μM concentration measured in nanopure water, pH 6.5 (black curve) and in dioxane containing 0.5% water (red curve).

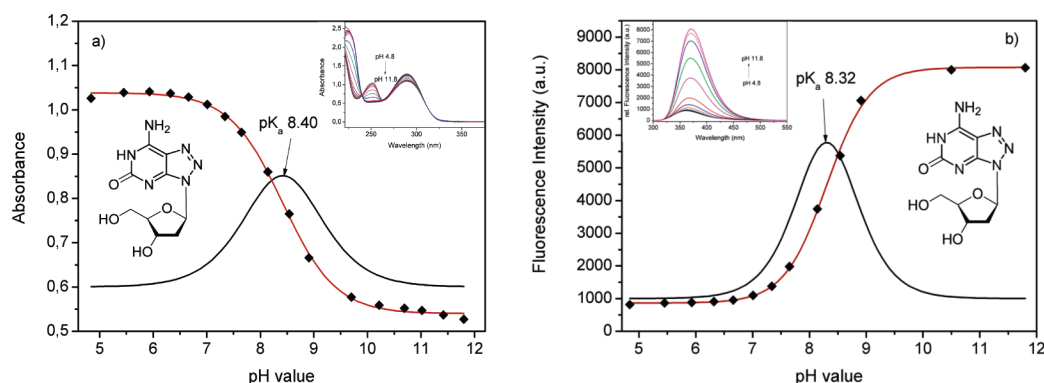


Figure 3. (a) UV absorbance at 260 nm versus pH (sigmoidal curve) and its first derivative using data from the UV spectroscopic change of 8-aza-2'-deoxyisoguanosine (**4**) with 100 μM concentration at various pH values measured in 0.1 M sodium phosphate buffer (inset).⁴¹ (b) Fluorescence emission at 370 nm against pH value (sigmoidal curve) and its first derivative using data from pH-dependent fluorescence spectra of 5 μM 8-aza-2'-deoxyisoguanosine (**4**) measured in 0.1 M sodium phosphate buffer (inset). The excitation wavelength was fixed at 286 nm.⁴¹

purpose were not available. On the other hand, the duplexes incorporating dT, dC, and dG opposite to **4** are almost equally stable. Only the base pair of **4** with dA is significantly less stable than the others (**19**•**15**, $\Delta T_m = -26.0$ °C), a fact that is also observed for 2'-deoxyisoguanosine (**1a**).¹⁵

8-Aza-2'-deoxyisoguanosine (**4**) has a lower pK_a value of deprotonation ($pK_a = 8.4$) compared to 2'-deoxyisoguanosine ($pK_a = 9.9$). Thus, melting experiments were also performed at higher pH values (pH 8.4 and 9.4, Table 2) in the absence of Mg^{2+} ions as the hydroxide precipitates at pH 9.4. The T_m value of the modified duplexes containing **4** decreases at higher pH values (9.4) compared to the lower pH (8.4) (Table 2). Altogether, duplex stability decreases in the order $m^5\text{iC}_d\cdot\mathbf{4} \gg \text{dG}\cdot\mathbf{4} > \text{dT}\cdot\mathbf{4} \geq \text{dC}\cdot\mathbf{4} \gg \text{dA}\cdot\mathbf{4}$ within each series of T_m measurements (pH 7.2, 8.4, 9.4). These results agree with data reported earlier for 2'-deoxyisoguanosine (**1a**) by our laboratory and by the Sugimoto group.^{15,42} According to Sugimoto, base pairing of dA with iG_d is sensitive to the position of incorporation. In the center, the iG_d -dA base pair is labile while it is rather stable at a dangling end position. Moreover, our data and those of the Sugimoto group support the observation that enzymatic incorporation of dT, dC, and dA opposite to iG_d depends at least in part on the base pair stability of 2'-deoxyisoguanosine with the canonical nucleotides.

Next, base pair discrimination was studied by fluorescence sensing. Fluorescent molecules are widely used for SNP (single

nucleoside polymorphism) detection.⁵⁴ Fluorescence measurements are common to detect small quantities of DNA. Fluorescent intercalating dyes are used for this purpose.⁵⁵ A more direct approach uses dyes attached to a nucleoside residue being part of an oligonucleotide.⁵⁶ However, space-demanding tags interfere with the size of the active center offered by polymerases. Thus, modified nucleosides with “built-in” fluorescence are valuable tools for such protocols. As 8-aza-2'-deoxyisoguanosine (**4**) shows intrinsic fluorescence, it offers the opportunity to monitor base pair stability (mismatch discrimination) in DNA by fluorescence measurements. For nucleobase anion fluorescence sensing,^{33,36} fluorescence spectra of oligonucleotide duplexes (ds) were measured in which the five nucleosides were placed opposite to the modification site (5'-d(TAGGTX-AATACT)•3'-d(ATCCA4TTATGA), X = dA, dG, dT, dC, $m^5\text{iC}_d$). The single-strand (ss) **15** was measured for comparison. Measurements were performed at three pH values (7.2, 8.4, and 9.4), as fluorescence of 8-aza-2'-deoxyisoguanosine (**4**) is significantly stronger in alkaline medium. The fluorescence emission curves for the experiments performed at pH 8.4, which is the pH used for PCR reactions, are shown in Figure 4a and in the corresponding bar diagrams in Figure 4b. For measurements performed at pH 7.2 and 9.4 see Figure S6 in Supporting

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Table 1. T_m Values and Thermodynamic Data of Oligonucleotide Duplexes Containing 8-Aza-2'-deoxyisoguanosine **4** in a Match and Mismatch Situation at pH 7.0^{a,b}; Data of iG_d (**1a**) Given for Comparison¹⁵

| duplexes | | T_m^c [°C] | ΔT_m^d [°C] | ΔH^e [kcal/mol] | ΔS^e [cal/(mol K)] | ΔG_{310} [kcal/mol] |
|----------------------|-----------|-----------------|---------------------|-------------------------|----------------------------|-----------------------------|
| 5'-d(TAGGTCAACTACT) | 12 | 50.0 | −3.5 | −89 | −249 | −11.6 |
| 3'-d(ATCCAGTTATGA) | 13 | (47.0) | (−3.5) | (−93) | (−265) | (−10.9) |
| 5'-d(TAGGTiCAACTACT) | 14 | 53.5 | 0 | −90 | −251 | −12.5 |
| 3'-d(ATCCA4TTATGA) | 15 | (50.5) | (0) | (−84) | (−235) | (−11.5) |
| 5'-d(TAGGTiCAACTACT) | 14 | 54 ^e | +0.5 | (−95) | (−263) | (−13.4) |
| 3'-d(ATCCAiGTTATGA) | 16 | | | | | |
| 5'-d(TAGGTCAACTACT) | 12 | 39.5 | −14.0 | −70 | −197 | −8.5 |
| 3'-d(ATCCA4TTATGA) | 15 | (37.0) | (−13.5) | (−72) | (−208) | (−7.9) |
| 5'-d(TAGGTCAACTACT) | 12 | 44 ^e | −9.5 | −67 | 183 | −9.8 |
| 3'-d(ATCCAiGTTATGA) | 16 | | | | | |
| 5'-d(TAGGTGAATACT) | 17 | 40.5 | −13.0 | −66 | −183 | −8.7 |
| 3'-d(ATCCA4TTATGA) | 15 | (37.0) | (−13.5) | (−74) | (−211) | (−8.0) |
| 5'-d(TAGGTGAATACT) | 17 | 44 ^e | −9.5 | −68 | −189 | −9.8 |
| 3'-d(ATCCAiGTTATGA) | 16 | | | | | |
| 5'-d(TAGGTAAATACT) | 18 | 38.5 | −15.0 | −71 | −201 | −8.3 |
| 3'-d(ATCCA4TTATGA) | 15 | (35.0) | (−15.5) | (−79) | (−229) | (−7.5) |
| 5'-d(TAGGTAAATACT) | 18 | 42 ^e | −11.5 | −62 | −171 | −9.1 |
| 3'-d(ATCCAiGTTATGA) | 16 | | | | | |
| 5'-d(TAGGTAAATACT) | 19 | 27.5 | −26.0 | −81 | −253 | −5.3 |
| 3'-d(ATCCA4TTATGA) | 15 | <i>f</i> | <i>f</i> | <i>f</i> | <i>f</i> | <i>f</i> |
| 5'-d(TAGGTAAATACT) | 19 | 32 ^e | −21.5 | −42 | −113 | −7.2 |
| 3'-d(ATCCAiGTTATGA) | 16 | | | | | |

^a Measured at 260 nm in 1.0 M NaCl, 0.1 M MgCl₂, 60 mM Na-cacodylate buffer, pH = 7.0 with 5 μ M + 5 μ M single-strand concentration. Data in parentheses refer to measurements in 0.1 M NaCl, 10 mM MgCl₂, 10 mM Na-cacodylate, pH 7.0. ^b Thermodynamic values were calculated with the program MeltWin 3.0 and are within 10% error. ^c The T_m values were determined with ± 0.5 °C accuracy. ^d ΔT_m was calculated as $T_m^{\text{base mismatch}} - T_m^{\text{base match}}$ and within ± 0.5 °C error. ^e See Seela et al.¹⁵ d(iG): 2'-deoxyisoguanosine. d(iC): 2'-deoxy-5-methylisocytidine. ^f T_m value was too low for exact calculations.

Table 2. T_m Values of Oligonucleotide Duplexes Containing 8-Aza-2'-deoxyisoguanosine **4** at pH 7.2, 8.4, and 9.4^{a,b}

| duplexes | | pH 7.2 | | pH 8.4 | | pH 9.4 | |
|----------------------|-----------|--------------|---------------------|--------------|---------------------|--------------|---------------------|
| | | T_m^c [°C] | ΔT_m^d [°C] | T_m^c [°C] | ΔT_m^d [°C] | T_m^c [°C] | ΔT_m^d [°C] |
| 5'-d(TAGGTiCAACTACT) | 14 | 55.0 | | 51.0 | | 49.0 | |
| 3'-d(ATCCA4TTATGA) | 15 | | | | | | |
| 5'-d(TAGGTCAACTACT) | 12 | 40.0 | −15.0 | 35.0 | −16.0 | 35.0 | −14.0 |
| 3'-d(ATCCA4TTATGA) | 15 | | | | | | |
| 5'-d(TAGGTGAATACT) | 17 | 39.5 | −15.5 | 39.0 | −12.0 | 39.0 | −10.0 |
| 3'-d(ATCCA4TTATGA) | 15 | | | | | | |
| 5'-d(TAGGTAAATACT) | 18 | 37.0 | −18.0 | 36.0 | −15.0 | 35.0 | −14.0 |
| 3'-d(ATCCA4TTATGA) | 15 | | | | | | |
| 5'-d(TAGGTAAATACT) | 19 | 28.0 | −27.0 | 26.0 | −25.0 | 24.0 | −25.0 |
| 3'-d(ATCCA4TTATGA) | 15 | | | | | | |

^a Measured at 260 nm in 1.0 M NaCl, 60 mM Na-cacodylate buffer, with 5 μ M + 5 μ M single-strand concentration. ^b For thermodynamic data see Table S3 in Supporting Information. ^c The T_m values were determined with ± 0.5 °C accuracy. ^d ΔT_m was calculated as $T_m^{\text{base mismatch}} - T_m^{\text{base match}}$ and within ± 0.5 °C error. d(iC): 2'-deoxy-5-methylisocytidine.

Information. The fluorescence quenching is about 10-fold higher for the oligonucleotide duplex (ds **14**•**15**) containing the m⁵iC_d•**4** pair compared to the ss-oligonucleotide **15**; in the other four cases quenching is about 2- to 3-fold higher except for the duplex **19**•**15** containing dA•**4** (Figure 4). The fluorescence increases in the following order of base pairs: m⁵iC_d•**4** < dG•**4** < dT•**4** < dC•**4** \ll dA•**4**. This order corresponds to the decreased duplex stabilities of Table 2.

Self-Assembly of Oligonucleotides. Isoguanine nucleoside and oligonucleotide assemblies show entirely different H-bonding patterns^{18d,f,21} than guanine aggregates^{57,58} (G-quartet, motif I). The isoguanine assembly forms a cyclic structure that is stabilized by a hydrogen bond network with a N1–H and C=O

interaction and a HN6–H and N-3 hydrogen bond. Each isoguanine base acts as a double donor and a double acceptor for H-bonding within neighboring molecules. Nitrogen-7, which is involved in the H-bonding pattern of the G-quartet, is not participating in the H-bonding of the isoguanine assembly.²⁴ Only pyrimidine nitrogens and pyrimidine substituents are participating in base pairing. As a sterical consequence of the 67° angle of the donor–acceptor units of isoguanine^{18f,21}—the guanine quartet forms an angle of 90°—a diversity of aggregates including tetrads (motif II) and pentads (motif III) are formed (Figure 5).

While the isoguanine pentamer is nearly planar and is held together by 10 hydrogen bonds and coordination forces to the central cation,^{18f,21} the tetrameric assembly leads to a bowl-shaped structure with the cation at the bottom.^{59a} Quantum mechanical calculations and energy minimization have compared

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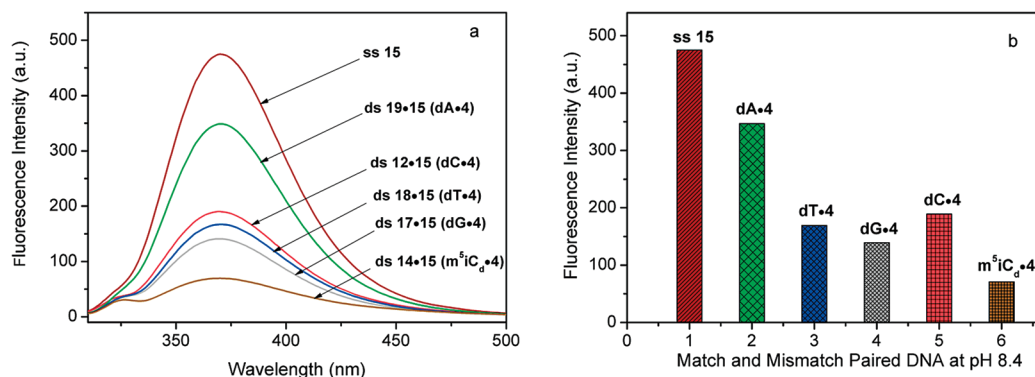


Figure 4. (a) Fluorescence emission spectra (excitation at 286 nm) of 8-aza-2'-deoxyisoguanosine (**4**) as constituent of the single-stranded oligonucleotide **15** and in paired duplexes (**12•15**, **14•15**, **17•15**, **18•15**, **19•15**) at pH 8.4 and (b) bar diagrams showing the fluorescence intensities of **4** (data taken from a). The curves were measured in 1.0 M NaCl, 60 mM Na-cacodylate buffer, pH 8.4 with 5 μ M + 5 μ M single-strand concentration. The results for the fluorescence sensing experiments performed at pH 7.2 and 9.4 are shown in Supporting Information.

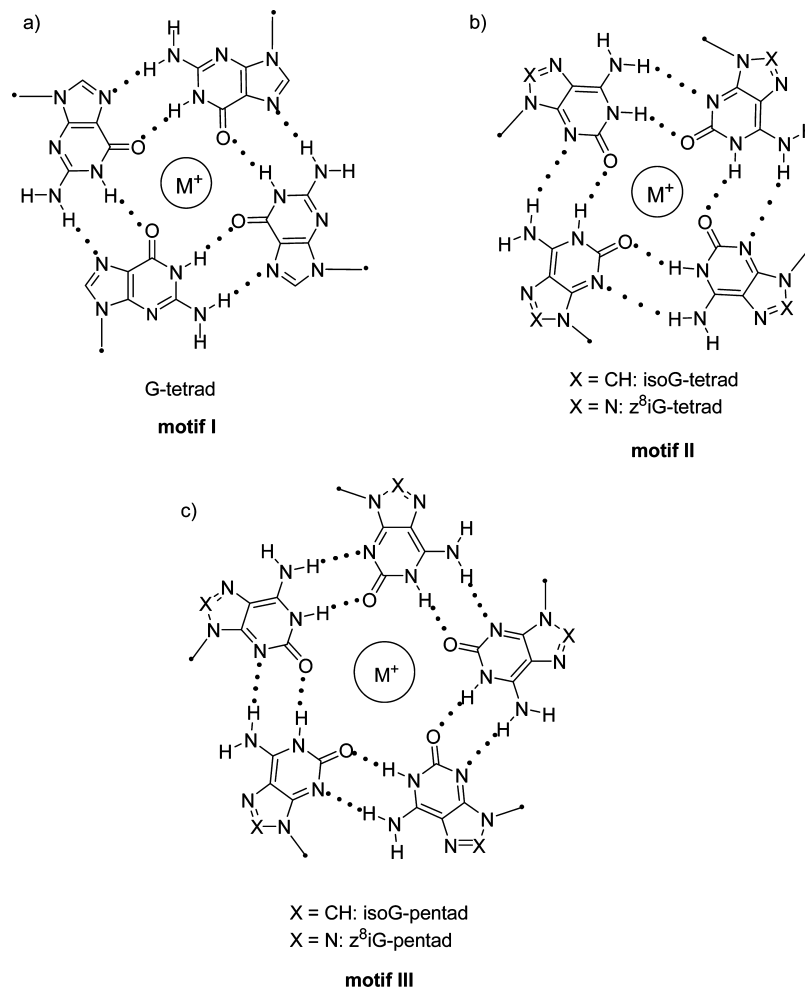


Figure 5. Tetrad versus pentad motifs.

the various stabilities of pentads versus tetrads.⁵⁹ From these studies and experimental data it was concluded that the central cation controls the complex stoichiometry in both cases. Although experimental evidence exists pointing toward the formation of isoguanine quadruplexes and pentaplexes, only pentaplexes were confirmed by single crystal X-ray analysis

performed on nucleosides.²² Recently, the oligonucleotide 5'-d(TiGiGiGiGTTT) was analyzed by ESI mass spectrometry.⁶⁰ The oligonucleotide was annealed in the presence of various cations, desalted, and treated with ammonium acetate prior to mass spectrometry. In almost all cases, pentaplexes with ammonium cations as central ions were detected, making it not possible to draw a conclusion on the aggregates formed in

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(b) Meyer, M.; Steinke, T.; Sühnel, J. *J. Mol. Model.* **2007**, *13*, 335–345.

(60) Pierce, S. E.; Wang, J.; Jayawickramarajah, J.; Hamilton, A. D.; Brodbelt, J. S. *Chem.—Eur. J.* **2009**, *15*, 11244–11255.

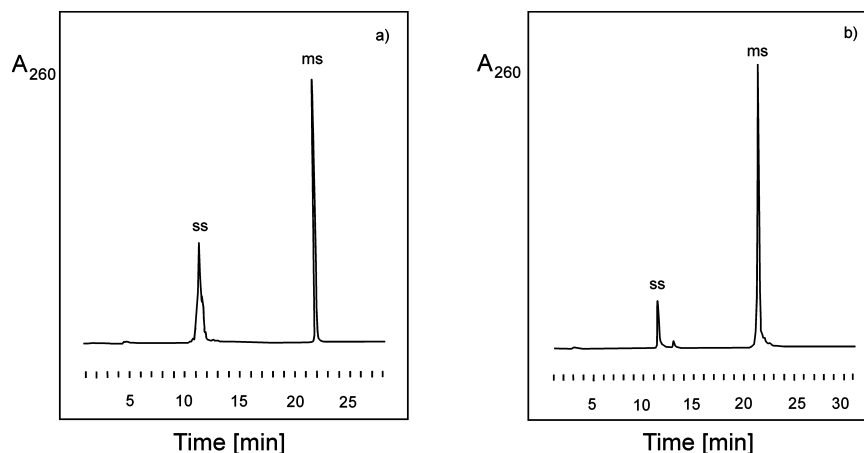


Figure 6. Ion-exchange HPLC elution profiles (260 nm) of 5'-d(T₄4T₄) (**22**) (a) and 5'-d(T₄G₄T₄) (**21**) (b) on a 4 mm × 250 mm DNAPac PA-100 column using the following buffer system: (C) 25 mM Tris-HCl, 10% MeCN, pH 7.0; (D) 25 mM Tris-HCl, 1.0 M KCl, and 10% MeCN, pH 7.0. Eluting gradient: 0–30 min 20–80% D in C with a flow rate of 0.75 cm³ min⁻¹. The oligonucleotide was annealed in an aqueous 1.0 M KCl solution. For details see Supporting Information; ss refers to the single-stranded species, and ms refers to the multistranded assembly.

solution with the original cations. Nevertheless, quartet and quintet structures were detected by gel electrophoresis^{18f} and ion-exchange HPLC.^{18d,25,23} The advantage of anion exchange chromatography is based on the fact that mobility depends on the number of negative charges (phosphodiester anions) of the oligonucleotides including duplexes or multistrand assemblies and can be performed at high concentration of the particular cation used for oligonucleotide assembly. The obtained data will not be obscured by other counterions.

Various shape mimics of 2'-deoxyisoguanosine (**1a**) such as 7-deaza-2'-deoxyisoguanosine (**2**) and 8-aza-7-deaza-2'-deoxyisoguanosine (**3**) have been incorporated into oligonucleotides, yielding similar multistranded assemblies as observed for 2'-deoxyisoguanosine (**1a**).^{18d,23,25}

The multistranded assemblies are ion-dependent and form a central cavity.^{19b} The favorable properties of 8-aza-2'-deoxyisoguanosine (**4**) prompted us to study the behavior of this nucleoside in multistranded assemblies by ion-exchange HPLC analysis. Consequently, the dodecanucleotide 5'-d(T₄4T₄) (**22**) containing four consecutive nucleoside **4** residues was prepared. The oligonucleotide was annealed in 1.0 M KCl at -20 °C for 16 h as described earlier,⁴¹ and aggregation was analyzed by ion-exchange HPLC (elution with a gradient of KCl).^{18d,19b,41} The same experiment was performed with 5'-d(T₄G₄T₄) (**21**).

According to Figure 6, a similar profile was observed for 5'-d(T₄4T₄) (**22**) and 5'-d(T₄G₄T₄) (**21**). These findings are in line with earlier ion-exchange HPLC experiments performed with 5'-d(T₄iG₄T₄) (**20**) and 5'-d(T₄G₄T₄) (**21**) both showing well-separated peaks for the monomer and the aggregate.^{18d} Mobility shift analyses based on phosphate charge differences of oligonucleotides bound to the cationic ion-exchange resin were carried out to distinguish between pentad and tetrad formation. From this, it was concluded that **22** forms a tetrad (motif II) and not a pentad (motif III) in KCl solution: a cyclic structure with a central cavity similar to that of the tetrameric assembly formed by 5'-d(T₄iG₄T₄). A detailed description of the mobility shift analyses is presented in Supporting Information. Nevertheless, only a single-crystal X-ray structure analysis or rigorous NMR assignment can solve the details of the structure.

Next, the thermal stability of the assembly of 5'-d(T₄4T₄) (**22**) was investigated.⁴¹ The ion-exchange HPLC elution profiles derived from these experiments are shown in Figure S8 in

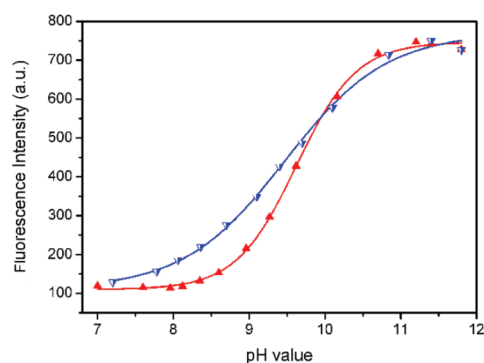


Figure 7. pH-dependent fluorescence of the oligonucleotide tetraplex 5'-d(T₄4T₄)₄ containing **4** as constituent in the presence of K⁺. Measurements were performed in 0.6 M KCl, 25 mM Tris·HCl, 10% MeCN with 1.2 μM ss concentration. The red curve represents the pH-increasing procedure, and the blue curve represents the pH-decreasing procedure. Excitation wavelength = 286 nm with emission at 370 nm.

Supporting Information. Only part of the assembly is destroyed during heating, indicating that the complex is quite stable. This observation is supported by hybridization experiments. We added the complementary strand 5'-d(A₄C₄A₄) (**23**) to a solution of the assembly of 5'-d(T₄4T₄) (**22**) and expected formation of the duplex **22**·**23**. However, the melting experiment did not yield sigmoidal melting profiles, indicating that the multistranded assembly is kinetically too stable to form the duplex **22**·**23**. Only when the assembly was first destroyed at high pH, the complementary strand 5'-d(A₄C₄A₄) (**23**) was added, and the solution was neutralized. Afterwards, a duplex melting profile was detected due to rehybridization forming the parallel-stranded duplex **22**·**23** (*T*_m = 37 °C). The UV melting profile is shown in Figure S9 in Supporting Information.⁴¹

Next, strand separation and p*K*_a values were investigated by pH-induced disassembly monitored by fluorescence change (Figure 7). We started this experiment at pH 7.0 with the assembled aggregate to give the single-stranded species at pH 12.⁴¹ From Figure 7, it is apparent that both curves are not superimposing. The red curve (filled triangles) represents the fluorescence increase due to the formation of the compound **4** anion and the dissociation of the assembly of 5'-d(T₄4T₄) into single strands of 5'-d(T₄4T₄); more and more single strands are formed by increasing the pH, with a complete separation of

the assembly to single strands (**22**) at pH 12. From that, an apparent pK_a value (9.6) was calculated. This value is about 1 unit higher than that determined for the monomeric **4** (8.3) or the single-stranded oligonucleotide **15** (8.4; data not shown) with one incorporation of nucleoside **4**. The blue curve (back-titration with acid) shows hysteresis and yields an apparent pK_a value of 9.4. Apparently, the equilibration process during the assembly is slower than during disassembly. From that, one can conclude that the assembly of 5'-d(T₄4T₄) has a higher pK_a value than oligonucleotide **15** with one incorporation of nucleoside **4**. The above-mentioned fluorescence hysteresis of the blue curve versus the red curve indicates that the fluorescence of compound **4** is quenched in the assembly when compared to single-stranded species.

Conclusion and Outlook

8-Aza-2'-deoxyisoguanosine (**4**) represents the first fluorescent shape mimic of 2'-deoxyisoguanosine (**1a**) expanding the genetic alphabet by a fluorophore. It is fluorescent under neutral condition and its fluorescence increases in alkaline medium. Therefore, fluorescence sensing offers a way to detect mismatches alternatively to T_m determination by thermal melting. For nucleoside **4**, the stability of base pairs decreases in the order $m^5iC_4 \cdot 4 \gg dG \cdot 4 > dT \cdot 4 \geq dC \cdot 4 \gg dA \cdot 4$ as determined from nucleobase anion fluorescence sensing and UV melting. Thus, the base pair stability is reflected by the fluorescence intensity. The base pairing of **4** is more stringent than that of 2'-deoxyisoguanosine.

Oligonucleotide 5'-d(T₄4T₄) (**22**) containing short runs of nucleoside **4** forms multistranded assemblies in the presence of K^+ that are stable and can be purified by ion-exchange HPLC. Mobility shift analysis suggests tetrad formation. The 8-azaisoguanine K^+ is quite stable at elevated temperature.

The self-assembled 8-azaisoguanine has properties of an ionophore thereby forming an ion channel.^{61,62} The parent nucleoside 2'-deoxyisoguanosine was recently used in an 5'-d(TiG₄T) pentad to assemble a pentameric protein in CsCl solution.⁶³ The unique fluorescence properties of **4** that were employed in fluorescence sensing for match and mismatch recognition in duplex DNA have the potential to be applied to complex DNA architectures. It might be used as fluorescence sensor for duplex DNA (match and mismatch recognition). It has the potential to act as fluorescence sensor for particular cations moving through ion channels formed by 8-azaisoguanine. The modified base can be either integrated as such or in a polymer, in a membrane, in a DNA assembly acting as an ionophore, or as a scaffold to assemble biopolymers.⁶³

Experimental Section

For a complete description of the experimental procedures see Supporting Information.

5-Amino-3-[2-deoxy-3,5-di-O-(4-toluoyl)- β -D-erythro-pentofuranosyl]-7-methoxy-3H-1,2,3-triazolo[4,5-d]pyrimidine (6). Compound **5** (5.6 g, 11 mmol) was dried by repeated coevaporation with anhydrous pyridine (3 \times 10 mL) and then suspended in anhydrous pyridine (130 mL). Trifluoroacetic anhydride (6.3 mL, 44 mmol) was added dropwise while cooling the solution in an ice bath. After 1 h, NaOMe/MeOH (0.08 mol/L, 700

mL) was added. The reaction mixture was stirred at room temperature overnight. The solution was evaporated, and the residue was applied to FC (silica gel, column 4 cm \times 10 cm). Elution with CH₂Cl₂/MeOH, 99:1 to 97:3 afforded **6** as a colorless foam (2.5 g, 44%) and the recovered starting material **5** (2.2 g, 40%). ¹H NMR (DMSO-*d*₆, 300 MHz) δ 2.37, 2.40 (6H, 2 s, 2 CH₃), 2.83–2.88 (1H, m, 2'-H_a), 3.39–3.46 (1H, m, 2'-H_b), 4.06 (3H, s, OCH₃), 4.39–4.60 (3H, m, 4'-H, 5'-H₂), 5.84–5.89 (1H, m, 3'-H), 6.59–6.63 (1H, t, *J* = 6.3 Hz, 1'-H), 7.26–8.02 (10H, m, NH₂, H-arom). For elemental analysis, R_f and UV see ref 37.

5,7-Diamino-3-(2-deoxy- β -D-erythro-pentofuranosyl)-3H-1,2,3-triazolo[4,5-d]pyrimidine (7). Compound **6** (2.5 g, 4.8 mmol) in a steel bomb was treated with NH₃/MeOH (250 mL, saturated at 0 °C) at 80 °C for 24 h. After evaporation of the solvent, the residue was applied to FC (silica gel, column 4 cm \times 10 cm, eluted with CH₂Cl₂/MeOH, 99:1 to 4:1) giving **7** as a white powder (1.2 g, 93%). ¹H NMR (DMSO-*d*₆, 300 MHz) δ 2.26–2.36 (1H, m, 2'-H_a), 2.90–2.99 (1H, m, 2'-H_b), 3.39–3.60 (2H, m, 5'-H₂), 3.82–3.87 (1H, m, 4'-H), 4.40–4.49 (1H, m, 3'-H), 4.89–4.93 (1H, t, *J* = 6.0 Hz, 5'-OH), 5.31–5.33 (1H, d, *J* = 5.4 Hz, 3'-OH), 6.32–6.37 (1H, t, *J* = 6.5 Hz, 1'-H), 6.42 (2H, s, NH₂), 7.33–7.84 (2H, br s, NH₂). For elemental analysis, R_f and UV see ref 37.

7-Amino-3-(2-deoxy- β -D-erythro-pentofuranosyl)-3H-1,2,3-triazolo[4,5-d]pyrimidin-5(6H)-one (4). To a solution of **7** (1.8 g, 6.7 mmol) and sodium nitrite (1.9 g, 27 mmol) in water (70 mL) was added glacial acetic acid (2.7 mL) dropwise at 60 °C under stirring. The stirring was continued for 20 min, and the pH of the solution was adjusted to 8.0 with 25% aqueous NH₃ solution. The crude precipitated material was filtered, and the solid was dissolved in water (100 mL, 60 °C) and applied to Serdolit AD-4 (4 cm \times 20 cm, resin 0.1–0.2 mm, Serva, Germany). The column was washed with water (500 mL), and the product was eluted with H₂O/*i*-PrOH, 99:1. Compound **4** was obtained as a light yellowish powder (1.2 g, 67%). TLC (silica gel, 25% aq NH₃/*i*-PrOH/H₂O, 1:7:2) R_f 0.6; UV λ_{max} (MeOH)/nm (ϵ /dm³ mol⁻¹ cm⁻¹) 251 (7600), 285 (6060); ¹H NMR (DMSO-*d*₆, 300 MHz) δ 2.28–2.30 (1H, m, 2'-H_a), 2.83–2.87 (1H, m, 2'-H_b), 3.36 (2H, m, 5'-H₂), 3.53–3.55 (1H, m, 4'-H), 3.85–3.88 (1H, m, 3'-H), 4.45 (1H, s, 5'-OH), 5.33–5.34 (1H, d, *J* = 3.9 Hz, 3'-OH), 6.34–6.28 (1H, t, *J* = 6.0 Hz, 1'-H), 8.94 (2H, br s, NH₂). Anal. Calcd for C₉H₁₂N₆O₄ (268.23): C 40.30, H 4.51, N 31.33. Found: C 40.09, H 4.63, N 30.79.

3-(2-Deoxy- β -D-erythro-pentofuranosyl)-7-(*N,N*-dibutylaminomethylidene)-3H-1,2,3-triazolo[4,5-d]pyrimidin-5(6H)-one (8). The dried compound **4** (270 mg, 1 mmol) was suspended in MeOH (10 mL). *N,N*-Dibutylformamide dimethyl acetal (500 μ L, 4.2 mmol) was added, and the reaction mixture was stirred for 1 h at room temperature. After evaporation of the solvent, the residue was applied to FC (silica gel, column 2 cm \times 10 cm, elution with CH₂Cl₂/MeOH 99:1 to 97:3) furnishing a colorless foam of **8** (240 mg, 59%). TLC (silica gel, CH₂Cl₂/MeOH, 10:1) R_f 0.3; UV λ_{max} (MeOH)/nm (ϵ /dm³ mol⁻¹ cm⁻¹) 228 (14400), 256 (9070), 276 (10000), 346 (14600). ¹H NMR (DMSO-*d*₆, 300 MHz) δ 0.83–0.94 (6H, m, 2 CH₃), 1.25–1.41 (4H, m, 2 CH₂), 1.62–1.66 (4H, m, 2 CH₂), 2.22–2.31 (1H, m, 2'-H_a), 2.84–2.89 (1H, m, 2'-H_b), 3.38–3.44 (2H, m, 5'-H₂), 3.52–3.64 (5H, m, 4'-H, 2 CH₂), 3.86–3.89 (1H, m, 3'-H), 4.46 (1H, s, 5'-OH), 5.34 (1H, br s, 3'-OH), 6.33–6.38 (1H, t, *J* = 6.6 Hz, 1'-H), 9.22 (1H, s, CH), 11.42 (1H, br s, NH). Anal. Calcd for C₁₈H₂₉N₇O₄ (407.47): C 53.06, H 7.17. Found: C 53.65, H 7.45.

3-(2-Deoxy- β -D-erythro-pentofuranosyl)-7-(*N,N*-dibutylaminomethylidene)-5-[(diphenylcarbamoyl)oxy]-3H-1,2,3-triazolo[4,5-d]pyrimidine (9). Compound **8** (500 mg, 1.23 mmol) was dried by repeated coevaporation with anhydrous pyridine (3 \times 2 mL) and then suspended in anhydrous pyridine (10 mL). *N,N*-Diphenyl carbamoyl chloride (DPC-Cl) (510 mg, 2.2 mmol) was added in the presence of *N,N*-diisopropylethylamine (320 μ L). The reaction mixture was stirred for 10 min at room temperature. The excess of DPC-Cl was hydrolyzed with crushed ice. Then, the

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mixture was poured into 5% NaHCO₃ and extracted with CH₂Cl₂ (3 × 30 mL). The combined organic layer was dried with Na₂SO₄ and filtered. After evaporation of solution, the residue was applied to FC (silica gel, column 2 cm × 15 cm). Elution with CH₂Cl₂/MeOH (99:1 to 97:3) gave a colorless foam of **6** (480 mg, 65%). TLC (silica gel, CH₂Cl₂/MeOH, 9:1) *R_f* 0.5; UV λ_{max} (MeOH)/nm (ε/dm³ mol⁻¹ cm⁻¹) 235 (34800), 330 (25600); ¹H NMR (DMSO-*d*₆, 300 MHz) δ 0.88–0.94 (6H, m, 2 CH₃), 1.26–1.36 (4H, m, 2 CH₂), 1.62–1.64 (4H, m, 2 CH₂), 2.41–2.48 (1H, m, 2'-H_α), 2.95–2.99 (1H, m, 2'-H_β), 3.52–3.64 (5H, m, 5'-H₂, 2 CH₂), 3.87–3.90 (1H, m, 4'-H), 4.53–4.54 (1H, t, *J* = 4.8 Hz, 3'-H), 4.80–4.83 (1H, t, *J* = 5.7 Hz, 5'-OH), 5.43–5.45 (1H, d, *J* = 5.8 Hz, 3'-OH), 6.54–6.58 (1H, t, *J* = 6.3 Hz, 1'-H), 7.23–7.44 (10H, m, H-phenyl), 9.00 (1H, s, CH). Anal. Calcd for C₃₁H₃₈N₈O₅ (602.68): C 61.78, H 6.36, N 18.59. Found: C 61.76, H 6.37, N 18.40.

3-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-7-(*N,N*-dibutylaminomethylidene)-5-[(diphenylcarbamoyl)oxy]-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (10). Compound **9** (220 mg, 0.36 mmol) was dried by repeated coevaporation with anhydrous pyridine (3 × 2 mL) and then suspended in anhydrous pyridine (1.3 mL). The solution was treated with 4,4'-dimethoxytrityl chloride (160 mg, 0.47 mmol) at room temperature under stirring overnight. After evaporation of the solvent, the residue was applied to FC (silica gel, column 2 cm × 10 cm, eluted with CH₂Cl₂/acetone 400:1 to 10:1) affording **10** as a colorless foam (230 mg, 70%). TLC (silica gel, CH₂Cl₂/acetone, 9:1) *R_f* 0.5; UV λ_{max} (MeOH)/nm (ε/dm³ mol⁻¹ cm⁻¹) 238 (46700), 329 (58300); ¹H NMR (DMSO-*d*₆, 300 MHz) δ 0.90–0.96 (6H, m, 2 CH₃), 1.30–1.36 (4H, m, 2 CH₂), 1.63–1.65 (4H, m, 2 CH₂), 2.93–3.09 (2H, m, 2'-H_α, 2'-H_β), 3.54–3.59 (1H, m, 5'-H₂), 3.57–3.68 (10H, m, 2 CH₂, 2 OCH₃), 3.98–4.03 (1H, m, 4'-H), 4.62–4.67 (1H, t, *J* = 5.7 Hz, 3'-H), 5.43–5.45 (1H, d, *J* = 5.1 Hz, 3'-OH), 6.64–6.67 (1H, m, 1'-H), 6.70–7.46 (23H, m, H-arom), 9.00 (1H, s, CH). Anal. Calcd for C₅₂H₅₆N₈O₇ (905.05): C 69.01, H 6.24, N 12.38. Found: C 68.87, H 6.26, N 12.17.

3-[2-Deoxy-5-*O*-(4,4'-dimethoxytriphenylmethyl)-β-D-erythro-pentofuranosyl]-7-(*N,N*-dibutylaminomethylidene)-5-[(diphenylcarbamoyl)oxy]-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine 3'-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (11**).** To a solution of **10** (230 mg, 0.25 mmol) in dry CH₂Cl₂ (2 mL) were added *N,N*-diisopropylethylamine (120 μL) and 2-cyanoethyl-diisopropyl-

phosphoramido chloride (170 μL, 0.76 mmol), and the mixture was stirred for 15 min at room temperature. Then the reaction mixture was washed with 5% NaHCO₃ (10 mL) and extracted with CH₂Cl₂ (2 × 15 mL). The combined organic layer was dried with Na₂SO₄, filtered, and evaporated. The residue was applied to FC (silica gel, column 2 × 10 cm, eluted with CH₂Cl₂/acetone, 10:1), yielding **11** as a colorless foam (220 mg, 78%); TLC (silica gel, CH₂Cl₂/acetone, 95:5) *R_f* 0.6; ³¹P NMR (CDCl₃, 121.5 MHz) δ 148.78, 148.99.

Acknowledgment. We thank Mr. N. Q. Tran for the oligonucleotide synthesis and Dr. R. Thiele from Roche Diagnostics, Penzberg for the measurement of the MALDI spectra. We thank Dr. S. Budow for her continuous support throughout the preparation of the manuscript and appreciate critical reading of the manuscript by Dr. P. Leonard and Mr. S. Pujari. Financial support by the Roche Diagnostics GmbH, Penzberg and ChemBiotech, Münster, Germany, and a China Scholarship (CSC), Beijing are gratefully acknowledged.

Supporting Information Available: Materials and general procedures. ¹³C NMR chemical shifts of the monomers. Oligonucleotide synthesis and characterization of oligonucleotides. *T_m* values and thermodynamic data of oligonucleotide duplexes containing 8-aza-2'-deoxy-isoguanosine **4** determined at pH 7.2, 8.4, and 9.4. Original melting curves of oligonucleotide duplexes. *pK_a* determination by fluorescence and UV. Fluorescence measurements of forward and reverse pH titration of 5'-d(T₄T₄T₄) (**22**) aggregate complex in the presence of K⁺. Fluorescence measurements of ss- and ds-oligonucleotides. Ion-exchange experiments to investigate the behavior of **4** in multistranded assemblies. Mobility shift analyses. Ion-exchange experiments to investigate the stability of the assembly of oligonucleotide 5'-d(T₄T₄T₄) (**22**). Hybridization experiments of 5'-d(A₄C₄A₄) (**23**) and 5'-d(T₄T₄T₄) (**22**). Proposed base pairs formed by nucleoside **4**. ¹H and ¹³C NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA910020N