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Oligonucleotides Containing 6-Aza-2′-deoxyuridine: Synthesis, Nucleobase Protection, pH-Dependent Duplex Stability, and Metal-DNA Formation

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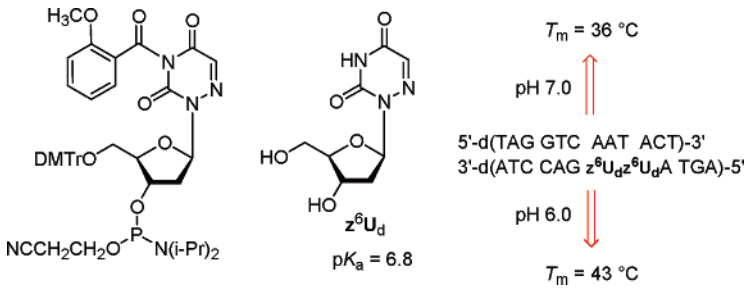

Oligodeoxyribonucleotides containing the nucleoside 6-aza-2′-deoxyuridine z^6U_d (**1a**) were prepared by using solid-phase synthesis. As the pK_a value of this nucleoside is 6.8, unwanted side reactions are observed. Consequently, nitrogen-3 was protected (*o*-anisoyl protection). The phosphoramidite of **1a** prepared on this route was as efficient as the building blocks of canonical nucleosides in allowing multiple incorporations into oligonucleotides. Oligonucleotide duplexes containing **1a** show a pH dependence of the T_m value. This is caused by nucleobase deprotonation occurring on compound **1a** already under neutral conditions. Metal (M)-DNA formation was studied in the presence of Zn^{+2} ions. It is demonstrated that 6-azauracil-modified duplexes form M-DNA already in neutral medium while alkaline conditions (above pH 8.5) are required for natural DNA. The conformational analysis of the sugar moiety of the nucleoside **1a** and its anisoyl derivative **5a** shows a preferred *N*-conformation in solution while an *S*-conformation for compound **1a** was obtained in the solid state (single-crystal X-ray analysis).

Introduction

6-Azauridine 5′-monophosphate (6-azaUMP) is a strong inhibitor of enzyme orotidine 5′-monophosphate decarboxylase[1] as the 6-position of the pyrimidine ring can serve as an enzyme binding site.[2] The polymer-linked 6-azauridine 5′-monophosphate was utilized as an affinity resin for the purification of this enzyme.[3] The 6-azauracil ribonucleoside is an antineoplastic and antimetabolite which interferes with the pyrimidine biosynthesis, thereby preventing the formation of cellular nucleic acids. In the triacetate form it is also effective as an antipsoriatic agent.[4] 6-Azauridine has been incorporated in oligoribonucleotides to study nucleic acid folding and activity of ribozymes.[5,6] The synthesis of 5-substituted-6-aza-2′-deoxyuridines was reported, resulting in compounds with antiviral activity.[7,8] Oligodeoxyribonucleotides containing 6-aza-2′-deoxyuridine are unknown, while corresponding oligoribonucleotides have been

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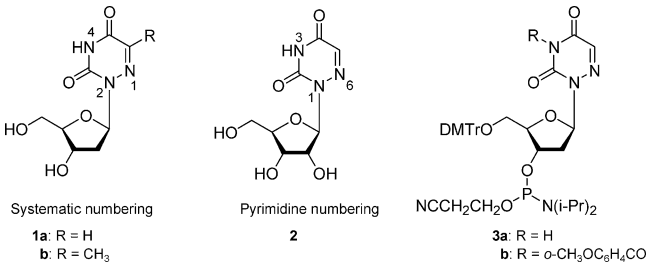
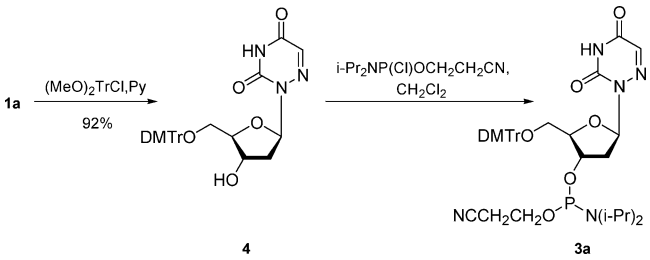
SCHEME 1. Structures of Nucleosides and Phosphoramidites 1−3



SCHEME 2. Transformation of Nucleoside 1a to the Phosphoramidite 3a



prepared.[5,6] The first chemical convergent synthesis of 6-azauridine **2** (ribonucleoside) as well as its large scale preparation using the *Streptococcus faecalis* was reported by Handschumacher.[9] The improved method was reported by Vorbrüggen using the Silyl−Hilbert−Johnson reaction in the presence of Friedel−Crafts catalysts such as $SnCl_4$ or $TiCl_4$.[10−12] The 2′-deoxy derivative **1a** was obtained via the anhydroribonucleoside[13,14] or enzymatically.[15] Later the stereochemical outcome of the chemical glycosylation reaction was controlled by the use of CuI as catalyst leading to the exclusive β-D-nucleoside formation.[16] 5-Substituted derivatives of 6-azauridine are prepared through Barton-deoxygenation of the ribonucleosides[17] or by convergent synthesis.[18]

Earlier work has shown that the incorporation of a nitrogen atom in the 6-position of the thymine moiety has an unfavorable effect on the duplex stability.[19] Our initial experiments on the synthesis of 6-aza-2′-deoxyuridine phosphoramidites performed with the unprotected nucleobase showed that the acidity of the nucleobase causes problems. This manuscript reports on the synthesis of the phosphoramidite building block **3b** with an anisoyl residue as N^3 protecting group (Scheme 1). The utilization of this building block allows multiple incorporations of **1a** in oligodeoxyribonucleotides with the same efficiency as the standard phosphoramidites. As the pK_a value of 6-aza-2′-deoxyuridine lies near pH 7 it is expected the base pair stability and the resulting oligonucleotide duplex stability is pH-dependent. This will be studied on oligonucleotides containing the nucleoside residue **1a** in place of dT. The ease of the base-deprotonation prompted us to investigate M-DNA (metal-DNA) formation. M-DNA represents a complex formed by B-DNA with certain divalent metal ions such as, Co^{2+}, Ni^{2+}, or Zn^{2+} at high pH values (>8). The replacement of the imino protons of guanine or uracil residues within the guanine-cytosine or uracil-adenine base pairs has been suggested by Lee;[20] the metal ions are expected to replace the imino protons of the guanine or the uracil moiety. However, other complex models have been suggested by Alexandre[21] and Lippert.[22] As the formation of M-DNA requires alkaline conditions the deprotonation of the nucleobases becomes likely. However, neither the planar nor the nonplanar model explains that Zn-DNA shows a metal-like conductivity which was the result of ab initio studies.[23] AFM measurements have been performed in the presence or absence of divalent ions such as Ni^{2+} or Co^{2+} ions showing that the DNA shrinks under those conditions.[24] Zn^{2+} concentrations higher than 0.5 mM lead to the precipitation of DNA.[25a] As the pK_a of the deprotonation of the lactam moiety of 6-aza-2′-deoxyuridine lies near pH 7, this modified nucleobase moiety might serve as an efficient Zn^{2+} acceptor within oligonucleotide duplexes already under neutral conditions. M-DNA is a good conductor, and the robust nature of electron transfer in M-DNA may have a remarkable potential for the development of nanoelectronic devices.[25b]

Results and Discussion

1. Synthesis and Properties of the Monomers. The first convergent synthesis of anomeric 6-aza-2′-deoxyuridines was performed employing the mercury salt of 3-diphenylmethyl-6-azauracil and 2-deoxy- 3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranosyl chloride.[26] Anomeric mixtures are formed under these nonselective glycosylation conditions. The stereoselective glycosylation of silylated 6-azauracil with 2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranosyl chloride in the presence of CuI results in the exclusive formation of the protected β-D-nucleoside (pyrimidine numbering is used throughout the text; systematic numbering is used in the experimental section). Detoluoylation in 0.2 M NaOMe/MeOH afforded the free nucleoside **1a** (77% yield). Initial experiments using the unprotected nucleoside **1a** for phosphoramidite synthesis failed (Scheme 2). When the DMT-derivative **4** was phosphitylated

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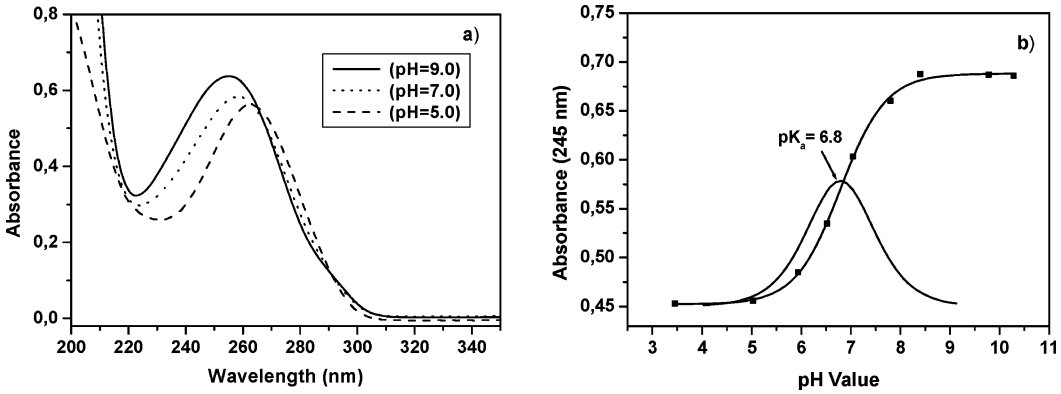
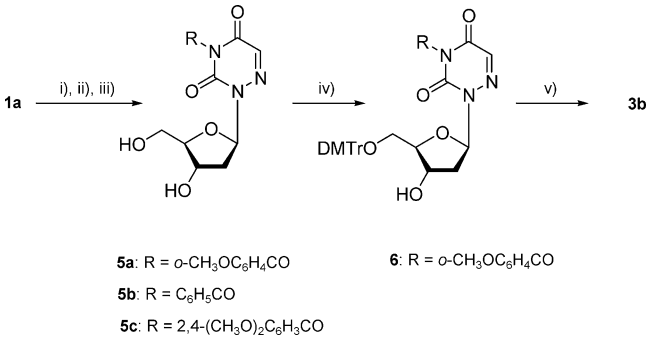


FIGURE 1. UV spectra of compound **1a** measured in phosphate buffer (7.8 g of $NaH_2PO_4 \cdot H_2O$ in 500 mL of H_2O) (a) at 260 nm from pH 5.0 to 9.0. (b) Absorbance of compound **1a** as a function of pH values measured at 245 nm from pH 1.5 to 12.5.

with ^{i}Pr$_2$NP(Cl)OCH$_2$CH$_2$CN/^{i}Pr$_2$EtN in CH_2Cl_2 at rt, the unprotected phosphoramidite **3a** was formed (TLC-monitoring). However, its chromatographical purification was impossible due to its degradation into unresolved products (Scheme 2). We also studied this phenomenon on the corresponding ribonucleoside (6-azauridine) phosphoramidite and encountered the same problem.[5] Recently, it was reported that the use of the more lipophilic 2′-O-ACE ortho ester protocol applied for HDV ribozymes synthesis[6] can overcome the difficulties appearing in oligoribonucleotide synthesis.

As we reasoned that this behavior results from deprotonation of the lactam moiety, the pK_a value of **1a** was determined by spectrophotometric titration[27] (Figure 1b) (pH 1.5−12.5) and was found to be 6.8, while its 5-methylated derivative **1b** shows a pK_a of 7.2. Both values are significantly lower than that of 2′-deoxyuridine (pK_a = 9.3). Consequently, the protection of the lactam moiety was considered employing a lipophilic protecting group. Earlier work performed on the lactam protection of 2′-deoxyuridines or 2′-deoxythymidines has shown that 4-*O*-aryl,[28] alkoxymethyl,[29] alkoxycarbonyl,[30] cyanoethyl,[31] benzoyl,[32,33] and anisoyl[34,35] groups are useful for this purpose. Thus, it was desirable to use such protecting groups on nitrogen-3 of the 6-azauracil moiety (Scheme 3). Here, the benzoyl, *o*-anisoyl, and the 2,4-dimethoxybenzoyl residues were introduced using the protocol of transient protection.[33] For that Et_3SiCl was employed in the presence of pyridine to protect the sugar hydroxyls. Then the acylating reagent was added in pyridine solution. The silyl groups were removed by treatment with 5% trifluoroacetic acid in dichloromethane/methanol (7:3). Among the various protecting groups, the 2,4-dimethoxy benzoyl was not considered for further manipulations because of the poor reaction yield. The N^3-anisoyl as well as the N^3-benzoyl protected nucleoside **5a**, **5b** were converted into the DMT derivatives. Only the anisoyl derivative **6** was isolated in sufficient yield, while the N^3-benzoyl residue was lost during the work up procedure. The anisoyl protected compound **6** was transformed into the phosphoramidite building block **3b** (Scheme 3), which was used for the solid-phase oligonucleotide synthesis. The structure of all synthesized compounds was characterized by ^{1}H-, ^{13}C- (Table 1), and ^{31}P-NMR spectroscopy (Supporting Information) as well as by elemental analysis. ^{1}H NMR data of compound **1a** were already unambiguously assigned.[36] The ^{13}C NMR chemical shifts of the N^3-derivatives show an up-field shift for C4 and C2 which is in agreement with earlier work reported by Hata.[37] The chemical shift assignment of C1′, C4′ is tentative for some compounds shown in Table 1. The position of acylation for the protected nucleoside **5a** was determined by X-ray analysis.

SCHEME 3. Synthesis of Protected Nucleosides 5a−c and the Phosphoramidite 3b[a]



[a] Reagents and conditions: (for **5a**) (i) Et_3SiCl, pyridine, *o*-anisoyl chloride, 5% CF_3COOH (CH_2Cl_2/MeOH 70:30) r.t., 82%; (**5b**) (ii) Et_3SiCl, pyridine, benzoyl chloride, 5% CF_3COOH (CH_2Cl_2/MeOH 70:30) rt, 69%; (**5c**) (iii) Et_3SiCl, pyridine, DMAP, 2,4-dimethoxy benzoyl chloride, 5% CF_3COOH (CH_2Cl_2/MeOH 70:30), 60 °C, 20%; (iv) $(MeO)_2TrCl$, pyridine, rt, 62%; (v) ^{i}Pr$_2$NP(Cl)OCH$_2$CH$_2$CN, ^{i}Pr$_2$EtN, CH_2Cl_2, rt, 61%.

2. Physical Properties of 6-Aza-2′-deoxyuridine and Its Derivatives. The calculation of the oligonucleotide concentration requires the values of extinction coefficients of the monomers in the medium of measurement. As deprotonation of **1a** occurs near pH 7 it was necessary to determine the UV maxima and extinction coefficients at various pH values. The

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TABLE 1. ^{13}C NMR Chemical Shifts of the 6-Aza-2′-deoxyuridine Derivatives 1−6[a]

compd	C(2)[b]	C(4)[b]	C(5)[b]	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)	MeO
1a	148.2	156.5	136.0	87.2	36.8	70.4	84.7	62.1	
5a	146.7	154.5	135.9	87.5	36.9	70.5	85.4	62.2	56.4
5b	146.8	154.9	136.1	87.5	37.0	70.4	85.5	62.0	
5c	146.7	154.5	135.8	87.3	36.8	70.4	85.3	62.1	56.5
4	148.1	156.5	135.7	85.2[c]	37.3	70.5	85.0[c]	64.4	55.0
6	146.6	154.4	135.7	85.3[c]	37.3	70.3	85.2[c]	63.9	56.4

[a] Measured in DMSO-d_6 at 25 °C. [b] Pyrimidine numbering. [c] Tentative.

TABLE 2. Half-Life Values of the N-3 Protected Nucleosides[a]

compd	τ [min][a]	λ [nm]
5a	9.2	260
5b	3.5	260
5c	9.7	280

[a] Measured UV-spectrophotometrically in 25% aq NH_3 at 25 °C.

UV maximum of **1a** is shifted bathochromically under acidic conditions (pH 5.0 = 262 nm, pH 7.0 = 258 nm, pH 9.0 = 255 nm) (Figure 1a). This is in agreement with data reported for 6-azauridine.[9] The extinction coefficients of 6-aza-2′-deoxyuridine measured in sodium phosphate buffer at 260 nm (near to the isosbestic point = 265 nm) are almost independent of the pH value (pH 5.0 = 6900, pH 7.0 = 6900, pH 9.0 = 7200) (Figure 1a). The UV spectrum of the N^3-protected nucleoside **5a** in MeOH shows two maxima at 258 and 325 nm.

As the benzoyl group can be used efficiently for the protection of dT[33] it was not clear why the benzoyl group was lost during dimethoxytritylation of **5b**. Therefore, the half-life values (τ) for the three protecting groups were determined. The reaction was followed UV-spectrophotometrically at 260 nm (25 °C) in 25% aqueous ammonia (Table 2). From the data it is obvious that the benzoyl group is too labile for the N-3 protection of 6-aza-2′-deoxyuridine while the anisoyl residue was suitable. This group was already used by Chattopadhyaya for uridine protection.[34] As it was reported that the acylation site (O-4 or N-3) was changed for 2′-deoxyuridine depending on the reaction conditions,[38] a single-crystal X-ray analysis of compound **5a**[39] was performed which immediately showed that nitrogen-3 is the only acylation site. Based on this X-ray analysis and the ^{13}C NMR chemical shifts (Table 1) it became apparent that all protecting groups are attached to nitrogen-3.

3. Nucleoside Conformation in Solution and in Solid State. The base moieties of the *ortho*-aza nucleoside **1a**, **5a** influence the *N/S* pseudorotational equilibrium of the sugar moiety. For this reason the conformational analysis was performed on the basis of ^3J(H,H) NMR coupling constants obtained from ^{1}H NMR spectra measured in D_2O, with the help of *PSEUROT* 6.3 program.[40] In this program, a minimization of the differences between the experimental and calculated couplings is accomplished by a nonlinear Newton−Raphson minimization; the quality of the fit is expressed by the root-mean-square (rms) differences. The input contained the following coupling constants: J(H1′,H2′), J(H1′,H2″), J(H2′,H3′), J(H2″,H3′), J(H3′,H4′). During the iterations either the puckering parameters (P, ψ_{max}) of the minor conformer (*N*) or the puckering amplitudes of both conformers were constrained. The coupling constants and the population of the nucleosides are shown in Table 3.

TABLE 3. $^3J_{H,H}$ Coupling Constants of the Sugar Moieties and *N/S*-Conformer Populations of the Nucleosides[a]

	$^3J_{H,H}$					conformation	
compd	1′,2′	1′,2″	2′,3′	2″,3′	3′,4′	%*N*	%*S*
dU[43]	6.70	6.60	6.50	4.30	4.10	30	70
1a[42]	4.82	7.28	6.10	5.80	5.93	58	42
5a	4.60	7.29	6.18	5.98	6.05	60	40
dA[44]	7.20	6.50	6.50	3.30	3.20	28	72
c^{7}z^{8}A$_d$[44,b]	6.55	6.70	6.45	4.00	3.70	37	63

[a] Measured in D_2O; rms < 0.4 Hz; $I\Delta J_{max}I$ < 0.5 Hz. [b] **c^{7}z^{8}A$_d$** = 8-Aza-7-deaza 2′-deoxyadenosine.

As the sugar conformation of nucleosides is controlled by stereo electronic and anomeric effects,[41] it was reasoned that the 6-aza-2′-deoxyuridine has a particular sugar conformation. Indeed, the nucleosides **1a, 5a** show a higher *N*-conformation population (60% *N*)[42] than the corresponding 2′-deoxyuridine (dU) (30% *N*).[43] The presence of an additional nitrogen atom at the position-6 of 6-aza-2′-deoxynucleoside is responsible for a conformational bias of the sugar moiety from *S*→*N*. The same observation has been made on other *ortho*-aza nucleosides such as of pyrazolo[3,4-*d*]pyrimidines;[44] 37% *N* for 8-aza-7-deaza-2′-deoxyadenosine (c^{7}z^{8}A$_d$) and 28% *N* for dA. The *N*⇆*S* equilibrium of protected compound **5a** does not show much influence on the conformation when compared to the unprotected **1a**.

These results prompted us to investigate the conformation in solid state by single-crystal X-ray analysis of **1a** and **5a**. In the crystal structure of compound **1a**, the conformation of the glycosylic bond is between anti and high-anti with the O4′-C1′−N1−C2 torsion angle of χ = −94.0 (3)°, whereas compound **5a** has a high-anti conformation with χ = −86.4 (3)°.[39] Similar results were observed for the ribo analogue of 6-azauridine nucleoside which exhibits a high-anti orientation with a torsion angle of −93.3°.[45] In compound **1a** the sugar ring has the C(2′)-endo, C(3′)-exo ($^2T_3$) conformation (P = 188.1 (2)°, τ_m = 40.3 (2)°). Thus its pucker can be described as type-*S*, it is different from the behavior of the protected nucleoside

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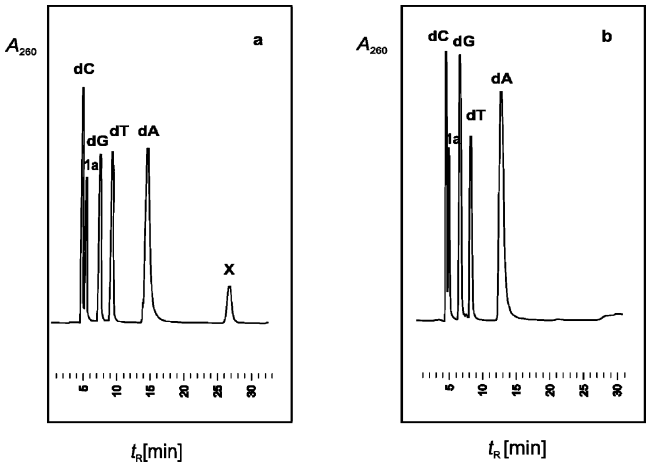


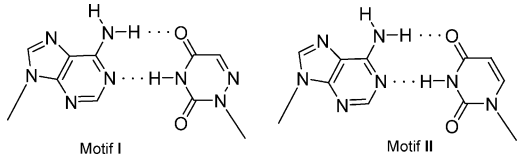
FIGURE 2. RP-18 HPLC profile of an enzymatic hydrolysis of the oligomer 5′-d(TAGG**1a**C AA**1a** ACT) after treatment with snake venom phosphodiesterase and alkaline phosphatase in 0.1 M Tris. HCl buffer (pH 7.0) at 37 °C, for (a) 4 h and for (b) 12 h.

5a which has a *N*-type sugar pucker with $P = 36.1\ (3)°$, $\tau_m = 33.5°$. From the above results it is apparent that **1a** shows *S*-type sugar pucker in the solid state while the preferred conformation is *N* in solution. The same solution conformation is found for **5a**.

4. Synthesis, Characterization, and Properties of Oligonucleotides. To investigate the influence of base-modified nucleosides on the duplex stability a series of oligonucleotides were synthesized. The oligonucleotides **7**−**14** were prepared by solid-phase synthesis using the protocol of phosphoramidite chemistry. The coupling yields were always higher than 95%. Deprotection of the oligomers was conducted in 25% aqueous ammonia at 60 °C for 16 h. The oligonucleotides were detritylated and purified by reversed-phase HPLC. The homogeneity of the oligonucleotides was proven by reversed-phase HPLC as well as by MALDI-TOF mass spectrometry. The detected masses were identical with the calculated values (Supporting Information, see Table 1). The composition of oligonucleotides containing **1a** was determined by the complete enzymatic hydrolysis using snake-venom phosphodiesterase followed by alkaline phosphatase and subsequent reversed-phase HPLC (RP-18). However, treatment under regular conditions did not lead to complete digestion. The incubation up to 4 h at 37 °C was not sufficient for the complete digestion, which is confirmed by the extra peak (X; nondigested oligonucleotide) found in the HPLC profile (Figure 2). After increasing the incubation time to 12 h at 37 °C, the additional peak disappeared, demonstrating the complete enzymatic hydrolysis. These results suggest that 6-azauridine containing oligonucleotides are partially protected from the nuclease digestion. Similar results were observed earlier for 6-aza-2′-deoxycytidine.[36]

To evaluate the influence of the base modification on the duplex stability, hybridization experiments were performed using the oligonucleotide duplex 5′-d(TAGGTCAATACT)·3′-d(ATCCAGTTATGA) (**7·8**) as a reference.[46] The modified residues were introduced at various positions in one or both strands of a DNA duplex. The replacement of the canonical thymine base in one strand of the duplex by a 6-azauracil residue results in a T_m decrease of 5 °C, when paired with dA (**7·11**, Scheme 4, motif **I**). According to Table 4, the T_m decrease is moderate when two modified residues are positioned in the flanking region (**7·10**) and stronger when they are located in the center of the duplex (**7·12**) or (**8·9**); similar observation was also found for natural 2′-deoxyuridine.[43] Nevertheless, the base pairing modes of **1a** and dU (Scheme 4, motif **I** and **II**) are expected to be the same.

SCHEME 4. Face to Face Base Pair Motifs of (I) dA-1a and (II) dA-dU



The strength of the base-pairing based on the pK_a difference (ΔpK_a) of the monomeric donor and acceptor involved in hydrogen bonding can be used to understand the relative base pairing strength of DNA−DNA and RNA−RNA duplexes.[47] The low pK_a value (pK_a = 6.8) of nucleoside **1a** has a significant influence on the base pair stability. At neutral pH the majority of the nucleoside molecules exist as negatively charged species. Alkali ions and water molecules can coordinate to the ring nitrogen. As a proton is required for base pair formation its absence destroys or weakens the base pair. At lower pH values (below 7.0) the proton necessary for base pairing is delivered from solution leading to the expected base pair formation thereby increasing the T_m value of duplex melting. To prove this, the duplex stability of the modified oligonucleotides containing **1a** was measured at pH of 6.0 (Table 4). At this pH, an increase in the T_m by 2.5 °C per modification was observed for two incorporations (**7·10**) when compared with the T_m value at pH 7.0; two successive modifications resulted in an increase of T_m by 7 °C. The T_m enhancement was 10 °C for four incorporations (from 28 °C to 38 °C). The reason for this is the partial deprotonation of the lactam moiety of **1a** at pH 7.0. In the absence of the N-3 proton WC base pairs cannot be formed at the modified sites which normally provide the necessary stability for duplex formation. The unmodified duplex **7·8** does not show a significant difference in the T_m values at different pH values.

These results clearly indicate that the destabilization of duplexes incorporating 6-aza-2′-deoxyuridine is caused mainly by the deprotonation of the nucleobase. In our comparison the influence of the 5-methyl group was neglected which causes an additional effect by 0.5−1.0 °C per modification.[36] If these two phenomena are taken into account there will be still a destabilizing effect left which does not result from nucleobase depotonation but which might result from the polar character of the nucleobase and/or the high anti conformation of the nucleoside as it was demonstrated by the single-crystal X-ray analysis of nucleoside **1a**. As we reasoned, that the ease of compound **1a** deprotonation will have a positive effect on the M-DNA formation this phenomenon was studied next.

5. Formation of M-DNA. Experiments with the aim to shift M-DNA formation to neutral pH values have been already performed using modified polynucleotides.[48] As we want to perform our experiments under defined conditions regarding DNA length and composition we are now using chemically

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TABLE 4. T_m Values and Thermodynamic Data of Oligonucleotide Duplexes Containing Regular and the Base-Modified Nucleoside at Different pH Values[a]

	pH 7.0		pH 6.0	
duplex	T_m [°C]	$\Delta G°_{310}$ [kcal/mol]	T_m [°C]	$\Delta G°_{310}$ [kcal/mol]
5′-d(TAG GTC AAT ACT)-3′ (**7**) · 3′-d(ATC CAG TTA TGA)-5′ (**8**)	50	−11.8	51	−12.1
5′-d(TAG G**1a**C AA**1a** ACT)-3′ (**9**) · 3′-d(ATC C A G TTA TGA)-5′ (**8**)	38	−7.5	–	–
5′-d(TAG GTC AAT ACT)-3′ (**7**) · 3′-d(A**1a**C CAG TTA **1a**GA)-5′ (**10**)	42	−8.6	47	−10.1
5′-d(TAG GTC AAT ACT)-3′ (**7**) · 3′-d(ATC CAG **1a**TA TGA)-5′ (**11**)	45	−10.0	–	–
5′-d(TAG GTC AAT ACT)-3′ (**7**) · 3′-d(ATC CAG **1a1a**A TGA)-5′ (**12**)	36	−7.0	43	−8.9
5′-d(TAG G**1a**C AA**1a** ACT)-3′ (**9**) · 3′-d(A**1a**C CAG TTA **1a**GA)-5′ (**10**)	28	−5.9	38	−8.1

[a] Measured at 260 nm in 1 M NaCl, 100 mM $MgCl_2$, and 60 mM Na-cacodylate (pH 7.0 and 6.0) with 5 μM single-strand concentration.

TABLE 5. Precipitation Assay Performed with 0.5 A_{260} Units (1.9 nmol) of the ss- (13) or ds-Oligonucleotides (7·8) in 1.0 mL of 10 mM TRIS-HCl, 0.2 mM Sodium Phosphate at pH = 8.5 at Various Zn^{+2} Concentrations at 25 °C

sample	Zn^{2+} concn (mM)	ss-oligonucleotide (**13**) (%)[a]	ds-oligonucleotide (**7·8**) (%)[b]
1	0.1	100	95
2	0.5	90	90
3	1	70	80
4	2	20	15
5	5	0	0

[a] [**13**] = 5′-d(TAGGTCAATACTTAGGTCAATACT). [b] [**7·8**] = 5′-d(TAGGTCAATACT)·3′-d(ATCCAGTTATGA).

TABLE 6. Recovery of ss- (13) or ds-Oligonucleotides (7·8) from Zn^{+2} Complex by Addition of 1.0 mL of 10 mM TRIS-HCl, 1 mM EDTA at pH = 8.5 at 25 °C

sample	Zn^{2+} concn (mM)	ss-oligonucleotide (%)	ds-oligonucleotide (%)
1	0.1	≤5	≤5
2	0.5	10	–
3	1	35	20
4	2	75	70
5	5	80	85

synthesized oligonucleotides. This allowed us to undertake studies on short DNA-fragments which are usually not available by enzymatic procedures. To our best knowledge these are the first experiments using chemically defined duplexes of 12-mers for this purpose. In a first series of experiments the solubility of the Zn^{+2} complexes was studied. This was necessary as it was reported for high-molecular weight DNA that Zn ions precipitate duplex DNA. This technique has the potential to separate DNA or even oligonucleotides from other biomolecules selectively. The precipitation experiments were preformed with the single-stranded oligonucleotide **13** as well as with the unmodified duplex **7·8**. At first the single-stranded (ss) oligonucleotide **13** as well as the duplex **7·8** were incubated for 20 min at various $ZnCl_2$ concentrations (0−5 mM) at pH 8.5 (for details, see Experimental Section). The samples were centrifuged, the supernatant was taken up, and the absorbance was read at 260 nm. As the volume was always the same (1 mL), the amount of DNA present in solution before and after Zn treatment could be compared (Experimental Section, Table 5). Next, the precipitate of each experiment was dissolved in 1 mL of buffer containing EDTA to recover the free DNA. EDTA complexes Zn^{+2} ions and leads to the release of B-DNA. The results of these experiments are shown in the Experimental Section (Table 6). From the data of the two tables (Experimental Section), it can be concluded that short oligonucleotides can be precipitated almost quantitatively with Zn^{+2} ions with a concentration higher than 5 mM; the DNA is almost completely recovered by the addition of EDTA. We noticed that ss-DNA start to precipitate at about the same Zn^{+2} concentrations as duplex DNA. This implies that DNA precipitation results from complex formation of Zn ions with the phosphodiester backbone leading to charge neutralization and shrinkage of the DNA.[24] Nevertheless, the precipitation process is faster in the case of ds-DNA.

M-DNA does not bind ethidium bromide, thus a 'fluorescence assay' in the presence of ethidium bromide (EB) can be used to monitor the M-DNA formation.[20] The qualitative and quantitative elements of this assay have been reviewed by Boger.[49] However, it is also possible to monitor EB binding by UV measurements. There are two possible binding sites for the Zn^{+2}−DNA interactions, the negatively charged phosphates of the backbone and/or the nucleobase binding sites.[50] To avoid precipitation of the complex, the Zn^{+2} concentration was always kept below 0.5 mM, preferentially in a concentration range of 0.2 to 0.25 mM Zn^{+2}. The UV absorption maximum of free EB is located at 480 nm and is not altered when Zn^{+2} ions and/or EDTA are added. However, it is shifted bathochromically to 525 nm when EB is bound to duplex DNA. When 0.25 mM $ZnCl_2$ was added to this complex, EB is released from DNA and the spectrum of the free EB is formed back with the UV maximum at 480 nm.[51] For comparison we performed this experiment on single-stranded DNA at pH 8.5 and 7.0; after addition of EB there is no shift in the absorbance due to non-intercalation of EB. Next, we used two different unmodified duplexes, the duplex of the 12-mers **7·8** and of the 24-mers **13·14** as well as the duplex of 12-mers **9·12** containing four 6-aza-2′-deoxyuridine residues. According to the Figures 3a,b, 4a,b, and 5a,b, the bathochromic shift of the EB maximum

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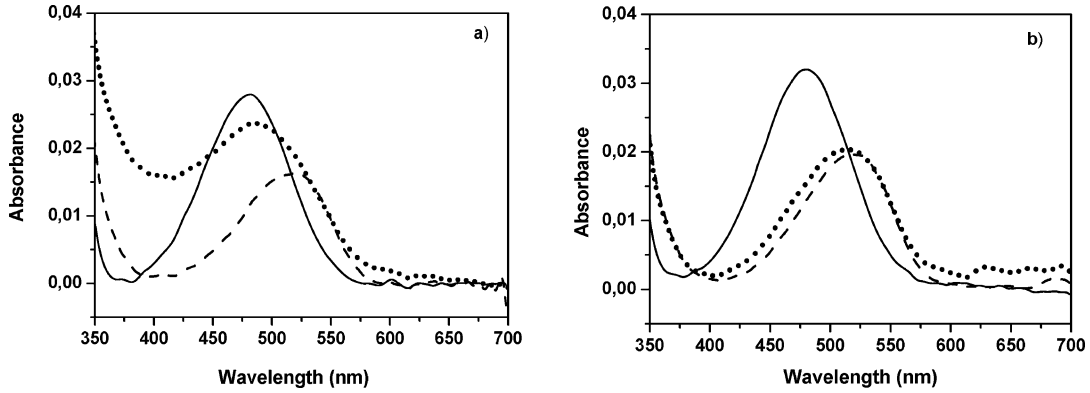

FIGURE 3. Absorption spectra of 5 μM ethidium bromide at (a) pH 8.5, (b) pH 7.0. Solid line, EB; dashed line, EB with 4 μM DNA (**7·8**) 5′-d(TAGGTCAATACT)·3′-d(ATCCAGTTATGA); dotted line, EB with DNA (**7·8**) and 0.25 mM $ZnCl_2$.

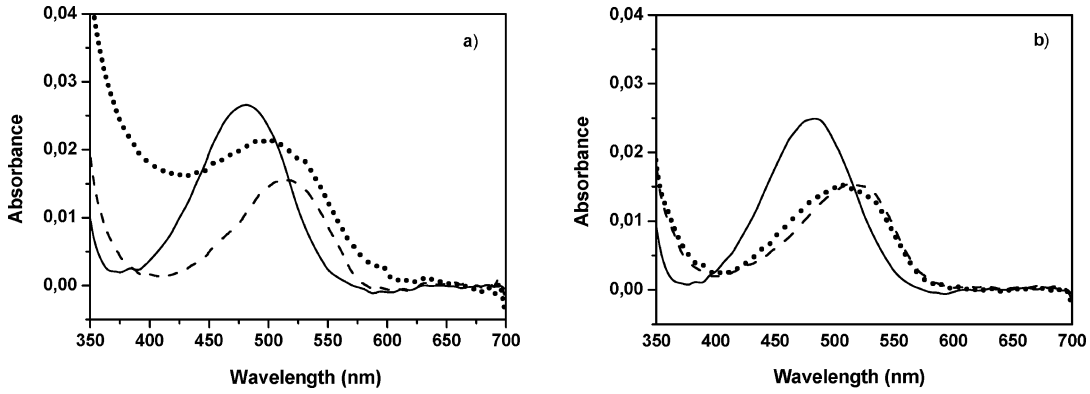

FIGURE 4. Absorption spectra of 5 μM ethidium bromide at (a) pH 8.5, (b) pH 7.0. Solid line, EB; dashed line, EB with 4 μM DNA (**13·14**) 5′-d(TAGGTCAATACTTAGGTCAATACT)·(ATCCAGTTATGA ATCCAGTTATGA); dotted line, EB with DNA (**13·14**) and 0.25 mM $ZnCl_2$.

induced by the addition of duplex DNA is apparent in all cases. From that it is concluded that the base modified duplex binds EB in a similar manner as the duplexes formed by the 12-mers or the 24-mers at pH 8.5 and 7.0. However, significant changes occur when Zn ions are added. At pH 8.5 the maxima of EB of the unmodified duplexes are shifted fully or partially to the maximum of free EB, indicating the release of the dye by the addition of Zn^{+2} ions. Nevertheless, the EB spectra are broadened due to a bathochromic shift of the second shorter EB band into the range of the longer wave length absorption. This shift of the EB spectrum is only observed in the presence of DNA at pH 8.5 but not at pH 7.0 (Figure 3b, 4b). This was explained earlier with the formation of a Zn-DNA complex at pH 8.5 in which Zn occupies positions in the core of the DNA helix thereby replacing the imino protons of the dG-dC and dA-dT base pairs.[20] The exact structure of this complex is unknown and other binding positions have been discussed.[23] However, all models involve the nucleobases and explain the release of EB by the interaction with Zn^{+2} ions which does not allow EB intercalation.

Next the spectral changes for the unmodified duplex **7·8** were compared with that containing 6-aza-2′-deoxyuridine. From Figure 3b it is obvious that M-DNA formation does not take place at pH 7.0 in the case of the unmodified duplex. This does not mean that Zn ion cannot be bound to duplex DNA as interactions with the negatively charged sugar phosphate backbone are still possible. However, according to Figure 3b EB is not released from duplex DNA. This is supported by the fact that free EB is not released by the addition of Zn^{+2} ions when the pH is 7.0 and the canonical nucleobases are not deprotonated, a phenomenon which seems to be required for the binding of Zn^{+2} ions (Figures 3b, 4b). This situation is different in the case of the base modified duplex **9·12**. At both pH 8.5 and 7.0 the maxima of the EB are restored after Zn^{+2} addition (Figure 5a,b). This implies that deprotonation of the nucleobases is required when Zn^{+2} will bind to the nucleobases.

According to the melting temperatures of the duplexes at pH 7.0 and 6.0 (see Table 4), the 6-azauracil base is partially deprotonated at neutral pH. However, duplex formation is still observed, as the unmodified base pairs are formed even under the low salt condition used for the EB experiments. The conversion of M-DNA to B-DNA is achieved by adding EDTA which chelates the metal ion and restoring the UV maxima to 525 nm (data not shown). Thus, the M-DNA formation at neural pH can be related to the lower pK_a values of the imino proton in the respective nucleobase; it is hypothesized that the lower pK_a of the base allows the imino proton to be replaced by the metal ion at lower pH. On the basis of these observations, compound **1a** shows favorable properties regarding M-DNA formation which is illustrated by the lower pK_a value (pK_a = 6.8) of **1a** compared to dU (pK_a =9.5).

Conclusions and Outlook

The N-3 protection of 6-aza-2′-deoxyuridine (**1a**) leads to phosphoramidite building block **3b** which gives the same high coupling yield as the phosphoramidites of the canonical bases. The additional ring nitrogen atom of **1a** causes a special glycosylic bond conformation (high-anti) which was studied in solution and in solid state.[39] DNA duplexes containing **1a**−dA

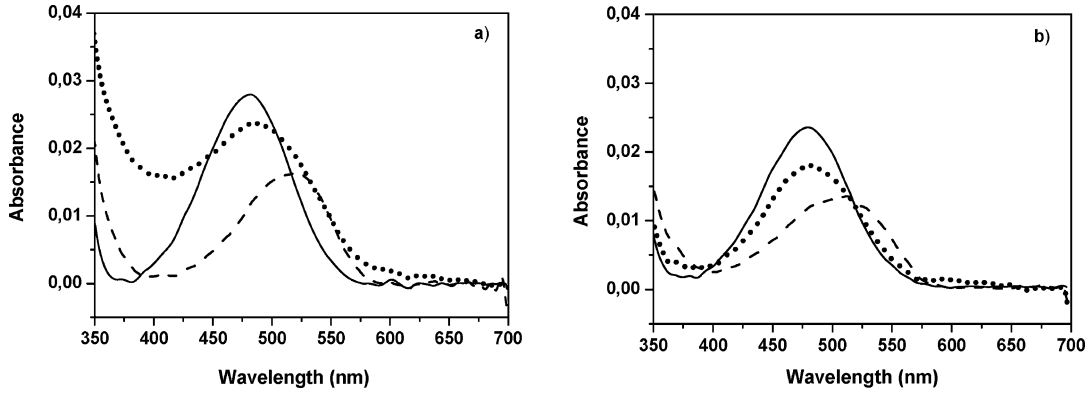

FIGURE 5. Absorption spectra of 5 μM ethidium bromide at (a) pH 8.5, (b) pH 7.0. Solid line, EB; dashed line, EB with 4 μM DNA (**9·12**) 5′-d(TAGG**1a**CAA**1a**ACT)·3′-d(ATCCAG**1a1a**ATGA); dotted line, EB with DNA (**9·12**) and 0.25 mM $ZnCl_2$.

base pairs are less stable than those of dT−dA. We found that this destabilization is largely caused by the deprotonation of the 6-aza-2′-deoxyuridine residue which takes place already under neutral condition. A pH decrease of the buffer solution increases the duplex stability. Oligonucleotides containing **1a** show an enhanced stability against the 3′-exonucleases (snake-venom phosphodiesterase). The ease of deprotonation which causes an unfavorable effect on the duplex stability results in a very favorable behavior regarding M-DNA formation. This metal DNA is now formed under physiological as well as alkaline conditions unlike canonical DNA which is formed only at alkaline pH. Because of its conductive properties M-DNA has the capacity to act as a nanowire,[25b] and the use of the 6-aza-2′-deoxyuridine in place of dT can lead its application in the preparation of nano electronic devices being formed already under physiological conditions.

Experimental Section

M-DNA Sample Preparation. Ethidium bromide (5 μM) was dissolved in 1 mL of buffer solution (10 mM NaCl and 10 mM Tris-HCl, pH 8.5 or 7.0), and the UV absorbance was measured. To this 4 μM of DNA duplex (bases) (**7·8** or **13·14** or **9·12**) was added, and the absorbance was read. To the same solution was added 0.25 mM $ZnCl_2$, and the mixture was incubated for 20 min at rt for the formation of M-DNA followed by UV measurement. To convert back to B-DNA, 2 mM EDTA (20 μL of 100 mM stock solution in H_2O) was added to each sample and the absorbance was measured.

Precipitation of DNA Using Zn^{+2}. The oligonucleotides **13** or **7·8** (1.9 nmol) were dissolved in 1.0 mL of buffer (10 mM Tris-HCl, 0.2 mM sodium phosphate, pH 8.5). To this solution were added various concentrations of $ZnCl_2$ (0−5 mM, from 50 mM stock solution), the mixture was incubated for 20 min at room temperature, then the samples were centrifuged at 5000 rpm for 5 min, the supernatant was carefully taken out, and the absorbance was measured at 260 nm. Based on that, the amount of precipitated DNA was calculated (Table 5). To recover the DNA, the above precipitated samples were suspended in buffer (1.0 mL of 10 mM TRIS-HCl, 1 mM EDTA, pH 8.5), and UV absorbance was measured at 260 nm (Table 6).

2-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-N^4-(2-methoxybenzoyl)-1,2,4-triazin-3,5(2*H*,4*H*)-dione (5a). Compound **1a**[16] (0.5 g, 2.18 mmol) was treated with triethylsilyl chloride (0.94 mL, 5.61 mmol) in pyridine (3.5 mL) and stirred at room temperature for 1 h, and then *o*-anisoyl chloride (0.49 mL, 3.29 mmol) was added. After being stirred for 3 h, the mixture was evaporated and coevaporated with toluene to remove pyridine completely. The oily residue was further treated with 5% CF_3COOH in $CH_2Cl_2-CH_3OH$ (7:3, 20 mL), stirred at room temperature for 1 h, and evaporated. To this residue was added 5% aq $NaHCO_3$. The mixture was extracted with CH_2Cl_2, the extract was dried over Na_2SO_4 and evaporated, and the residue was applied to FC (silica gel, column 15 × 3 cm, CH_2Cl_2/MeOH, 98:2). Evaporation of the main zone afforded a colorless foam, which was recrystallized from MeOH to give **5a** as colorless crystals (0.65 g, 82%). mp = 141 °C, UV (MeOH); 258 (15200), 260 (15000), 325 (4300). TLC (silica gel, CH_2Cl_2/MeOH, 95:5): R_f 0.26. ^{1}H NMR (DMSO-d_6, 250 MHz): δ 2.15 [m, 1H, H_α−C(2′)], 2.50 [m, 1H, H_β−C(2′)], 3.48 [m, 2H, H−C(5′)], 3.74 [m, 1H, H−C(4′)], 3.78 [s, 3H, MeO], 4.30 [m, J = 4.78, 1H, H−C(3′)], 4.70 [t, J = 5.37, 1H, OH−C(5′)], 5.25 (d, J = 4.50, 1H, OH−C(3′)], 6.33 [t, J = 5.87, 1H, H−C(1′)], 7.18 [m, 2H, aromatic], 7.60 [m, 1H, aromatic], 7.84 [s, 1H, H−C(5)], 8.01 [m, 1H, aromatic]. Anal. calcd for $C_{16}H_{17}N_3O_7$ (363.32): C, 52.89; H, 4.72; N, 11.57. Found: C, 52.81; H, 4.75; N, 11.77.

2-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-N^4-benzoyl-1,2,4-triazin-3,5(2*H*,4*H*)-dione (5b). Compound (**1a**) (0.2 g, 0.87 mmol) was treated with triethylsilyl chloride (0.4 mL, 2.36 mmol) in pyridine (2 mL) and stirred at room temperature for 1 h and then benzoyl chloride (0.29 mL, 2.5 mmol) was added. After being stirred for 3 h, the mixture was evaporated and coevaporated with toluene to remove pyridine completely. The residue was further treated with 5% CF_3COOH in $CH_2Cl_2-CH_3OH$ (7:3, 8 mL) stirred at room temperature for 1 h and evaporated. To this residue was added 5% aq $NaHCO_3$, and the mixture was extracted with CH_2Cl_2, the extract was dried over Na_2SO_4 and evaporated, and the residue was applied to FC (silica gel, column 12 × 3 cm, CH_2Cl_2/MeOH, 95:5). Evaporation of the main fractions afforded **5b** as colorless foam (0.2 g, 69%). UV (MeOH); 254 (16000), 260 (14100). TLC (silica gel, CH_2Cl_2/MeOH 95:5): R_f 0.27. ^{1}H NMR (DMSO-d_6, 250 MHz): δ 2.08 [m, H_α−C(2′)], 2.49 [m, 1H, H_β−C(2′)], 3.73 [m, 2H, H−C(5′)], 4.12 [m, 1H, H−C(4′)], 4.29 [t, J = 4.9, 1H, H−C(3′)], 4.67 [t, J = 5.75, 1H, OH−C(5′)], 5.23 [t, J = 4.60, 1H, OH−C(3′)], 6.30 [t, J = 5.70, 1H, H−C(1′)], 7.59 [m, 2H, aromatic], 7.83 [s, 1H, H−C(5)], 8.12 [m, 2H, aromatic]. Anal. Calcd for $C_{15}H_{15}N_3O_6$ (333.30): C, 54.05; H, 4.54; N, 12.61 Found: C, 53.99; H, 4.50; N, 12.52.

2-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-N^4-(2,4-dimethoxybenzoyl)-1,2,4-triazin-3,5(2*H*,4*H*)-dione (5c). Compound (**1a**) (0.15 g, 0.65 mmol) was treated with triethylsilyl chloride (0.70 mL, 4.19 mmol) in pyridine (3.0 mL) and stirred at room temperature for 1 h, and then 2,4-dimethoxybenzoyl chloride (36 mg, 0.18 mmol) and DMAP were added. The reaction mixture was stirred at 60 °C for overnight. The mixture was evaporated and coevaporated with toluene to remove pyridine completely. The residue was further treated with 5% CF_3COOH in $CH_2Cl_2-CH_3OH$ (7:3, 6 mL), stirred at room temperature for 1 h, and evaporated. To this residue was added 5% aq $NaHCO_3$, and the mixture was

extracted with CH_2Cl_2. The extract was dried over Na_2SO_4, and the evaporated residue was applied to FC (silica gel, column 12 × 3 cm, CH_2Cl_2/MeOH, 98:2). Evaporation of the main fraction afforded **5c**; colorless solid (51 mg, 20%). UV (MeOH); 317 (8400), 277 (15800). ^{1}H NMR (DMSO-d_6, 250 MHz): δ 2.16 [m, 1H, H_α−C(2′)], 2.45 [m, 1H, H_β−C(2′)], 3.46 [m, 2H, H−C(5′)], 3.72 [m, 1H, H−C(4′)], 3.89 [s, 3H, MeO], 4.28 [m, 1H, H−C(3′)], 4.74 [t, 1H, OH−C(5′)], 5.27 [d, J = 4.5, 1H, OH−C(3′)], 6.32 [t, J = 5.50, 1H, H−C(1′)], 6.66 [s, 1H, aromatic], 6.74 [d, 1H, aromatic], 7.81 [s, 1H, H−C(5)], 7.97 [d, 2H, aromatic].

2-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-1,2,4-triazin-3,5(2*H*,4*H*)-dione (4). Compound **1a** (0.08 g, 0.35 mmol) was suspended in anhyd pyridine (1.5 mL), 4,4′-dimethoxytrityl chloride (0.14 g, 0.42 mmol) was added in portions, and the mixture was stirred at room temperature for 3 h. The reaction was quenched by the addition of MeOH, and the mixture was evaporated to dryness. The residue was dissolved in CH_2Cl_2, washed with H_2O, and dried over Na_2SO_4. The organic layer was coevaporated with toluene and applied to FC (silica gel, column 12 × 3 cm, CH_2Cl_2/MeOH, 90:10), furnishing a colorless foam (0.17 g, 92%). UV (MeOH): 233 (20300), 260 (6400), 267 (6800). TLC (silica gel, CH_2Cl_2/MeOH, 9:1): R_f 0.8. ^{1}H NMR (DMSO-d_6, 250 MHz): δ 2.11 [m, 1H, H_α−C(2′)], 2.35 [m, 1H, H_β−C(2′)], 3.01 [m, 2H, H−C(5′)], 3.72 [s, 3H, 2MeO], 3.86 [m, 1H, H−C(4′)], 4.24 [m, 1H, H−C(3′)], 5.27 [d, J = 4.75, 1H, OH−C(3′)], 6.34 [t, 1H, H−C(1′)], 6.83 [m, 4H, aromatic], 7.21−7.40 [m, 10H, aromatic, H−C(5)], 12.28 [s, NH]. Anal. Calcd for $C_{29}H_{29}N_3O_7$ (531.56): C, 65.53; H, 5.50; N, 7.91. Found: C, 65.41; H, 5.21; N, 8.00.

2-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-*N*^{4}-(2-methoxybenzoyl)-1,2,4-triazin-3,5(2*H*,4*H*)-dione (6). As described for **4**, compound **5a** (0.3 g, 0.83 mmol) in anhyd pyridine (4 mL) was treated with 4,4′-dimethoxytrityl chloride (0.33 g, 0.99 mmol). The organic layer was evaporated and applied to FC (silica gel, column 10 × 3 cm, CH_2Cl_2/acetone 98:2) to afford **6** as colorless foam (0.34 g, 62%). UV (MeOH): 235 (25300), 260 (14800), 324 (3800). TLC (silica gel, CH_2Cl_2/acetone, 95:5): R_f 0.64. ^{1}H NMR (DMSO-d_6, 250 MHz): δ 2.18 [m, 1H, H_α−C(2′)], 2.45 [m, 1H, H_β−C(2′)], 3.10 [m, 2H, H−C(5′)], 3.72 [s, 3MeO], 3.90 [m, 1H, H−C(4′)], 4.31 [m, 1H, H−C(3′)], 5.29 [d, J = 5.25, 1H, OH−C(3′)], 6.37 [t, J = 3.62, 1H, H−C(1′)], 6.86 [m, 4H, aromatic], 7.24−8.00 [m, 13H, aromatic, H−C(5)]. Anal. Calcd for $C_{37}H_{35}N_3O_9$ (665.69): C, 66.76; H, 5.30; N, 6.31. Found: C, 66.54; H, 5.15; N, 6.35.

2-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentofuranosyl]-*N*^{4}-(2-methoxybenzoyl)-1,2,4-triazin-3,5(2*H*,4*H*)-dione 3′-(2-Cyanoethyl Diisopropylphosphoramidite) (3b). A stirred solution of **6** (0.20 g, 0.30 mmol) in anhyd CH_2Cl_2 (5 mL) was pre-flushed with Ar and treated with (*i*-Pr)$_2$EtN (0.08 mL, 0.51 mmol) followed by 2-cyanoethyl diisopropyl phosphoramidochloridite (0.08 mL, 0.35 mmol) at room temperature. After 30 min, the mixture was diluted with CH_2Cl_2 (20 mL), washed with aq 5% $NaHCO_3$ (10 mL), dried over Na_2SO_4, and evaporated to an oil. This residue was applied to FC (silica gel, column 10 × 3 cm, CH_2Cl_2/acetone/Et_3N, 95:5:0.2) to give compound **3b** as a colorless foam (0.16 g, 61%). TLC (silica gel, CH_2Cl_2/acetone, 95:5): R_f 0.87. ^{31}P NMR ($CDCl_3$): 149.85, 149.69.

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Supporting Information Available: Synthesis, purification, and characterization of oligonucleotides **7**−**14**; molecular masses ([M + H]$^+$) of oligonucleotides **7**−**14** measured by MALDI-TOF mass spectrometry (Table 1); ^{1}H NMR and ^{13}C NMR of new compounds **4**−**6**; and ^{31}P NMR data of phosphoramidite **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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