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Pyrazolo[1,5-*a*]-1,3,5-triazine C-Nucleoside as Deoxyadenosine Analogue: Synthesis, Pairing, and Resistance to Hydrolysis

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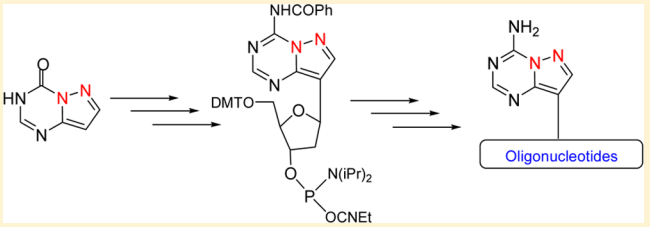
Supporting Information

ABSTRACT: The synthesis of a pyrazolo[1,5-*a*]-1,3,5-triazine C-nucleoside (dA^{PT}), designed to form two hydrogen bonds with a complementary dT residue, is reported. Oligonucleotides including this dA nucleoside analogue possess base-pairing properties similar to those of the parent oligonucleotide. This dA nucleoside analogue is more resistant to acid-catalyzed hydrolysis than dA.



Oligonucleotides (ONs) are widely used in different fields: molecular diagnostics,[1] molecular and mechanistic biological studies,[2] therapeutic development,[3] biotechnology and nanotechnology.[4] Most of these applications require the use of oligonucleotides with specific base-pairing ability and additional properties such as resistance to nucleases, cell-specific delivery, efficient uptake, and adequate intracellular distribution as well as possibility of detection. This has been made possible thanks to the development of numerous analogues involving chemically modified sugar, phosphate backbone, and nucleic bases as well as various conjugates. However, it appears that in some cases, such as, for example, to obtain efficient RNA interference activity, a fine-tuning of the binding affinity along the sequences is required.[3a,b,5] This can be achieved by site-specific incorporation of modified nucleosides with hybridization-stabilizing or -destabilizing effects. Although previous studies have shown that nucleoside analogues that retain Watson–Crick base-pairing ability are good candidates and identified steric restrictions, the understanding of effective nucleic base modifications remains limited.[2c,5] We do believe that other new modified nucleosides may be used in this field. We have focused our attention on C-nucleosides due to their increased stability toward hydrolysis.[6−8] Among the "purine-mimics" C-nucleoside previously synthesized,[6−8] the pyrazolo[1,5-*a*]-1,3,5-triazine derivatives are structurally close to the purine nucleus.[9,10] An adenosine[11] and several deoxyadenosine[9,10,12] analogues were previously described. However, in the deoxy series, a substituent was always present in position 2 of the base analogue, and to our knowledge, until now, none of these has been incorporated into oligodeoxyribonucleotides. We have designed a novel C-nucleoside involving a pyrazolo[1,5-*a*]-1,3,5-triazine ring as adenine mimic with base-pairing ability with thymine. This heterocycle is substituted in position 4 by an amine and is linked by position 8 to the 2′-deoxyribose through a C−C bond (Figure 1). In this paper, we report the synthesis of this deoxyadenosine C-nucleoside analogue (dA^{PT})

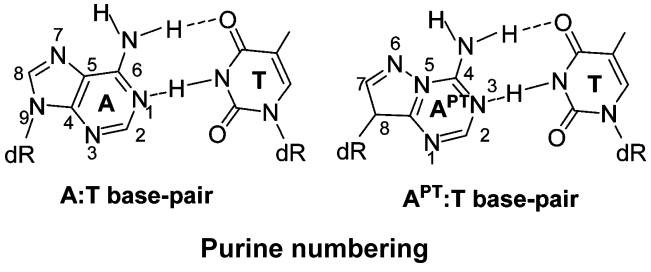


Figure 1. Structures of the natural A:T and modified A^{PT}:T base-pairs.

and its incorporation (1 and 2 incorporations) into oligodeoxyribonucleotides. The binding properties of these modified oligonucleotides have been studied by absorption spectroscopy and circular dichroism. The acid-catalyzed hydrolysis of the glycosidic bond has been also investigated.

Different strategies can be used to synthesize C-nucleosides.[7] They include the construction of an aglycon upon a carbohydrate residue, the construction of the carbohydrate upon the aglycon unit, the modification of the existing C-nucleosides or the direct coupling of preformed carbohydrate and aglycon units. According to previous reports on the synthesis of pyrazolotriazine nucleoside analogues, we chose to link a iodopyrazolotriazine aglycon with a glycal by the Heck palladium cross-coupling reaction, initially reported by Daves.[13,14] Due to the presence of the electron-rich double bond, the initial attack occurs from the less hindered face of the

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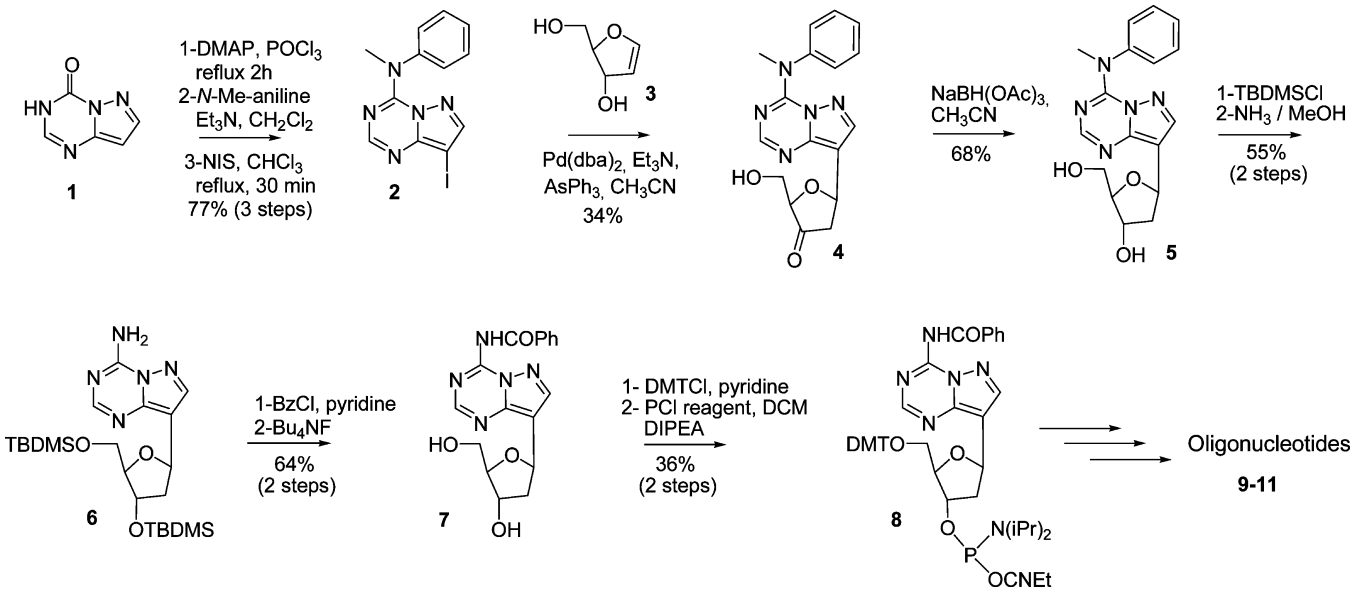
Scheme 1. Synthesis of the Pyrazolo[1, 5-*a*]-1,3,5-triazine C-Nucleoside Phosphoramidite



glycal ring resulting in the selective new C-glycosidic bond formation at the anomeric position. When a bulky protective group is present at the 3′-position in combination with the bulky $AsPh_3$ ligand, the stereocontrol is enhanced and the exclusive formation of the β-anomer is observed. One example of Heck palladium coupling of 4-aminopyrazolo[1,5-*a*]-1,3,5-triazine, protected at the amino function with an isobutyloxycarbonyl group, with a 3′-*tert*-butyldiphenylsilylated glycal leading to a 3′-keto C-nucleoside has been reported.[15] A few examples of unexpected exclusive formation of the β-anomer with the use of the fully unprotected glycal were also described.[10,16] Following these results, we applied the method reported by Raboisson et al. which involves the use of the *N*-methyl-*N*-phenylamino group in position 4 of the iodo aglycon (Scheme 1). Starting from the previously reported 3*H*-pyrazolo[1,5-*a*]-1,3,5-triazin-4-one **1**,[17] the 8-iodo-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine **2** was obtained by a three-step reaction. Compound **1** was reacted with phosphorus oxychloride in the presence of DMAP, followed by reaction with *N*-methylaniline.[10] Subsequent treatment with *N*-iodosuccinimide led to the iodopyrazolotriazine **2**. The reaction between the aglycon **2** and the unprotected glycal **3**, easily obtained from thymidine,[18] was performed in the presence of bis(dibenzylideneacetone)-palladium(0), triphenylarsine, and triethylamine in acetonitrile to give the keto compound **4** with a yield of 34%. Changing the solvent (CH_3CN, DMF, dioxane), the palladium catalyst ($Pd(dba)_2$, $Pd_2(dba)_3$, Pd(OAc)), or the base (Bu_3N, Et_3N) did not improve the yield.

The incorporation of the modified pyrazolo[1,5-*a*]-1,3,5-triazine C-nucleoside into oligonucleotides requires the transient protection of its primary amino function by a group easily removable at the end of the oligonucleotide synthesis. One example of ammonium hydroxide displacement of the *N*-methyl-*N*-phenylamino group in position 4 of a C-nucleoside analogue has been reported but with a moderate yield (53%).[10] For this reason, the conversion into the amino function during the deprotection step by ammonia treatment after the oligonucleotide synthesis is not compatible with the incorporation of more than one dA-like nucleoside. Thus, we chose to protect the amino function of the nucleic base analogue with a benzoyl group commonly used in oligonucleotide synthesis.[19] The shortest route for the synthesis would have been to replace the *N*-methyl-*N*-phenylamino group by the benzoyl before the preparation of nucleoside. However, various attempts for coupling the deprotected base analogue or its mono- or bis-benzoylated derivatives with the glycal were unsuccessful. These results led us to proceed to the amino function protection of the nucleoside. The synthesis was performed according to Scheme 1. The selective reduction of the ketone in the 3′-position of **4** was achieved by treatment with tri(acetoxy)borohydride in acetonitrile to give nucleoside **5** in 68% yield. Then the 5′- and 3′-hydroxyl functions of **5** were protected by treatment with *tert*-butyldimethylsilyl chloride, and the amino function in the 4-position was obtained by methanolic ammonia treatment to give **6** in 55% yield. The amino function was protected by treatment with benzoyl chloride in pyridine, and then the desilylation of the 5′- and 3′-hydroxyl functions was performed by treatment with tetrabutylammonium fluoride to give the nucleoside **7** in 64% yield. The NOESY experiment showed two specific correlations: between H-1′ and H-4′ which confirms the β configuration and between H-3′ and H-5′ which confirms the configuration after the reduction of the 3′-ketone (Figure S27, Supporting Information). After selective protection of the 5′-hydroxyl function by treatment with dimethoxytrityl chloride and phosphitylation of the 3′-hydroxyl function with 2-cyanoethyl *N*,*N*-diisopropyl chlorophosphite, the phosphoramidite derivative **8** was obtained in 36% yield. Oligonucleotides 18 mer (ONs) including, either one dA^{PT} (ON **9**) or two dA^{PT} (ON **10**) incorporations were synthesized (see Table 1 for structures) in order to study the base-pairing ability of the new dA^{PT} nucleoside analogue with thymidine. Oligonucleotide **11** involving only one dA^{PT}, and no other purine, has been synthesized in order to study its stability under acid-catalyzed hydrolysis (Table 1). (See the Experimental Section for ON synthesis and Figures S39−S44 (Supporting Information) for characterizations).

The influence of dA replacement (one or two) by the pyrazolo[1,5-*a*]-1,3,5-triazine derivative (dA^{PT}) on duplex

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Table 1. Sequences of the Modified (9−11) and Unmodified (12−14) Oligonucleotides

ON	sequence
9	5′d-(TAC CGC GTG CAA^{PT} CCC TCT) 3′
10	5′d-(TA^{PT}C CGC GTG CAA^{PT} CCC TCT) 3′
11	5′d-(TTC CTC TTT CTA^{PT} CCC TCT) 3′
12	5′d-(ACA AGA GGG **X**TG CAC GCG GTA GGA)-3′ (**X** = A, C, G or T)
13	5′d-(TAC CGC GTG CAA CCC TCT) 3′
14	5′d-(TTC CTC TTT CTA CCC TCT) 3′

stability was investigated by thermal denaturation studies, followed by absorption spectroscopy of DNA duplexes obtained by mixing modified oligonucleotides **9** and **10** with a 24-mer single-stranded target DNA sequences **12** (X = T) (Table 1, Figures 2 and 3). The target sequence was chosen so

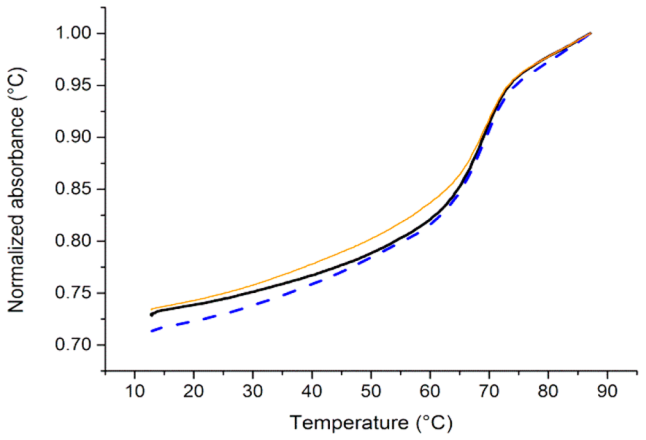

Figure 2. Thermal denaturation curves for modified duplexes including one dA^{PT} (dashed blue line, T_m = 67.9 °C), two dA^{PT} (plain orange line, T_m = 67.9 °C), and the unmodified duplex (bold black line, T_m = 68 °C). Conditions: 1 μM concentration (each strand) in 10 mM sodium phosphate, pH 7, buffer containing 150 mM NaCl and 1 mM EDTA. Estimated error in T_m = ± 1 °C.

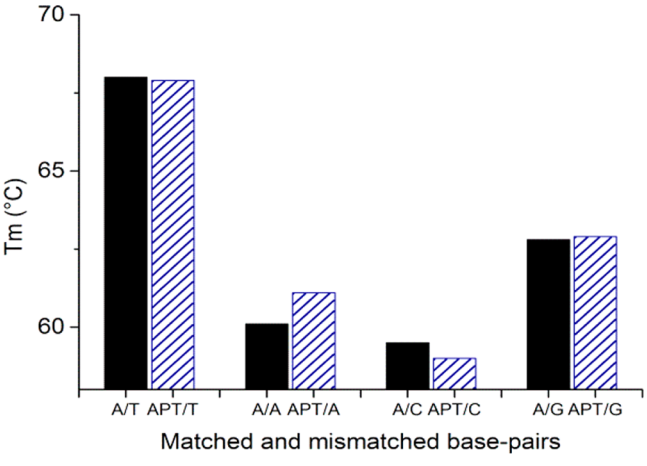

Figure 3. Comparison of T_m values for fully matched and mismatched duplexes containing dA (black bars) and dA^{PT} (hatched bars). Same conditions as for Figure 2

that, upon hybridization, three nucleotides would overhang on each side of the duplexes in order to mimic the interaction of ONs with full length targets.[20] 2′-Deoxyadenosine (dA) was also incorporated in the same positions for comparison (duplex **13** + **12**). Melting studies indicated that dA^{PT} forms base-pair with thymine that contribute to duplex stability about as much as the A−T base pair (Figure 2 and Table 2). T_m values are largely dependent on sequence and on the purine/pyrimidine ratio. The observed value for the reference is in accordance with literature data.[21,22]

Table 2. T_m Data and Thermodynamic Parameters of Duplex Formation

12	5′d-(ACA AGA GGG TTG CAC GCG GTA GGA)-3′				
ONs	T_m (°C)[a]	$\Delta T_{m/mod}$ (°C)	$-\Delta H^0$ (kcal/mol)	$-\Delta S^0$ (eu)	$-\Delta G^0$37 °C[b] (kcal/mol)
9	67.9	−0.1	−143.7	−392.5	−22.0
10	67.9	−0.05	−145.7	−398.4	−22.2
13	68		−147.8	−404.3	−22.4

[a]1 μM duplex concentrations in same buffer as for Figure 2. Estimated error in T_m = ± 1 °C. [b]Data obtained from $1/T_m$ vs log [C_m] plots using 8 × 10^{-6} M, 4 × 10^{-6} M, 2 × 10^{-6} M and 1 × 10^{-6} M duplex concentrations in same buffer as for Figure 2. Estimated error in $\Delta G^{37\ °C}$ = ± 15%.

Oligonucleotide **9** involving one dA^{PT} has been also hybridized with the target sequences **12** involving cytosine (X = C), guanine (X = G), or thymine (X = T) in position opposite to the modified nucleoside (Figure 3). From these experiments, it appears that the sequence studied involving dA^{PT} was able to recognize its target with selectivity equivalent to that obtained with dA.

The thermodynamic parameters of duplex formation for the modified ONs (**9**−**10**) and the reference (ON **13**) with their target (ON **12**) were determined from melting curves by the concentration variation method (Table 2).[23,24] Similar enthalpy and entropy values were observed for the three duplexes. The data observed for the reference are in the range of the literature values.[22,25]

In order to investigate the influence of the incorporation of one or two dA^{PT} nucleosides on the duplex structures, we have recorded CD spectra of modified (**9** and **10**) and unmodified (**13**) ONs in the presence of the target sequence (**12**). The results (Figure 4) indicated that the modified ONs involving one or two dA^{PT} were able to form duplex structures similar to that of the B-type DNA/DNA reference with only small

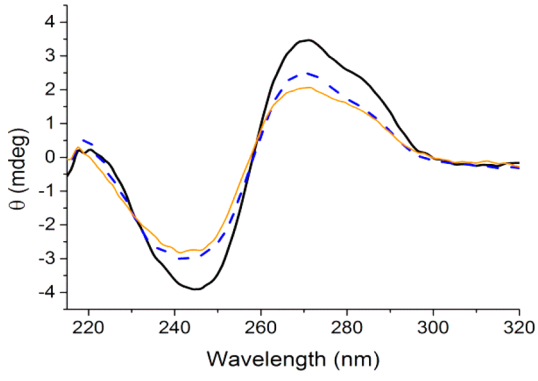

Figure 4. CD spectra for modified duplexes including one dA^{PT}(dashed blue line), two dA^{PT} (plain orange line), and the unmodified duplex (bold black line) recorded at 20 °C. Conditions: 1 μM concentration (each strand) and same buffer as for Figure 2.

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changes concerning intensity reduction of the positive and negative bands.

The resistance to depurination of dA^{PT} was tested by acid-catalyzed hydrolysis of two oligonucleotides involving either only one dA^{PT} (ON **11**) or one dA (ON **14**) and no other purine. The follow-up of the reaction was assessed by reversed-phase chromatography and MS MALDI-TOF analysis (see Experimental Section for conditions used and Figures S45−S53 (Supporting Information) for selected data). The results revealed that only 13% of the unmodified ON **14** was left unchanged after 2 h of incubation, and the degradation products corresponded mainly to the depurination process (m/z = 5187.8). A peak with retention time corresponding to that of adenine was also observed. As expected, additional alkali treatment generated cleavage fragments possibly corresponding to 3′-phosphorylated $^{5'}$TTC CTC TTT CT$^{-3'}$ and 5′-phosphorylated $^{5'}$CCC TCT$^{-3'}$ and different adducts. In contrast, at least 37% of ON **11** was still present after 18 h of acid-catalyzed hydrolysis and additional compounds with higher m/z values (m/z = 5345.7) that could correspond to acetylated ON and K adducts were observed. Additional alkali treatment generated also cleavage fragments that possibly correspond to 3′-phosphorylated $^{5'}$TTC CTC TTT CT$^{-3'}$ and 5′-phosphorylated $^{5'}$CCC TCT$^{-3'}$. The acid-catalyzed hydrolysis of ON **11** for 42 h followed by an alkaline treatment showed an increased amount of cleavage products with respect to ON **11**, the main compound detected still being ON **11**.

In summary, we have reported the synthesis of a new pyrazolo[1,5-*a*]-1,3,5-triazine C-nucleoside as deoxyadenosine analogue. Single and double incorporations of this new nucleoside have been performed in 18-mer oligonucleotides. For the sequence studied, the dA analogue is base-pairing with thymidine similarly to dA in terms of stability, specificity and thermodynamic. The modified ONs form B-DNA duplexes with only small changes in the structures, as suggested by the CD studies. Importantly, the modified nucleoside exhibits a strong stability increase toward acid-catalyzed hydrolysis. These different features confirm the potential of this new pyrazolo-[1,5-*a*]-1,3,5-triazine C-nucleoside as deoxyadenosine surrogate and make it worthy for further exploration for applications in oligonucleotide based therapeutic strategies.

EXPERIMENTAL SECTION

General Methods and Materials. All nonaqueous reactions were run in oven-dried glassware (120 °C) under argon pressure. Anhydrous tetrahydrofuran was obtained by passing through activated columns of alumina, while all other anhydrous solvents were bought on molecular sieves. Reagents were purchased and used without further purification. Yields refer to purified compounds unless otherwise stated. Reactions were monitored by thin-layer chromatography plates (TLC silica gel 60 F_{254}) that were visualized using a UV lamp (254 nm) and developed with aqueous solution of cerium ammonium molybdate or aqueous solution of $KMnO_4$. Column chromatography was performed on silica gel (63−200 μm). NMR spectra were recorded at 250 or 400 MHz using trimethylsilane (TMS) or residual undeuterated solvent as internal reference and reported in parts per million (ppm). The following abbreviations were used to explain multiplicities: s = singulet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets. Phase-sensitive 2D NOESY experiments were performed at 400 MHz (Figure S27, Supporting Information). Unmodified ONs were purchased from Eurogentec. MALDI-TOF MS analyses of oligonucleotides were performed using HPA-matrix. The molar extinction coefficient of ON **9**, determined by titration of its solution (in the same buffer as reported Figure 2) with the complementary sequence **12**, was found to be similar to that obtained for the corresponding unmodified ON **13** ($\varepsilon_{260\ nm}$ = 157700 L mol^{-1} cm^{-1}) (data not shown). From this result, the molar extinction coefficient of the modified nucleoside was estimated to be equivalent to that of 2′-deoxyadenosine. According to this, the same molar extinction coefficient was used for ONs **9** and **10** and the molar extinction coefficient for ON **11** ($\varepsilon_{260\ nm}$ = 147 700 L mol^{-1} cm^{-1}) was calculated according to ref 26. Melting experiments were performed with heating rate of 0.5 °C/min. T_m values were obtained by the baseline method.[27] CD measurements were carried out in optical cell with a path length of 1 cm at 20 ± 0.5 °C. with solutions of duplexes (1 μM each strand) prepared in a 10 mM NaH_2PO_4 buffer (pH 7.0) containing 150 mM NaCl and 1 mM EDTA. The reported spectra correspond to the average of three scans.

3*H*-Pyrazolo[1,5-*a*]-1,3,5-triazin-4-one (1). Compound **1** was obtained according to previously reported procedures.[17] To a cooled solution of sodium ethanolate [sodium (19.15g, 0.83 mol, 1.06 equiv) in EtOH (1 L)] was added drop by drop a solution of isoxazole (50 mL, 0.78 mol, 1 equiv) in EtOH (200 mL) over 2 h. After 30 min at rt, acetic acid (350 mL, 6.11 mol, 7.8 equiv) was added followed by semicarbazide hydrochloride (90 g, 0.81 mol, 1.04 equiv), and the mixture was heated at 95 °C for 2 h. After being cooled in an ice bath, the solution was neutralized with sodium hydroxide 2 N until pH 10. The aqueous layer was extracted with AcOEt three times. The organic layers were gathered, washed with brine, dried over magnesium sulfate, and evaporated to afford a yellow-brown solid (59 g, 52%) corresponding to the 5-aminopyrazole-1-carboxamide. ^{1}H NMR (DMSO-d_6, 250 MHz): δ 5.28 (d, 1H, J = 1.7 Hz), 6.37 (sl, 2H), 7.26 (d, 1H, J = 1.7 Hz), 7.48 (sl, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 87.3, 141.4, 150.3, 154.0. IR (KBr): ν 773, 908, 1422, 1602, 1745, 2300, 3250, 3310, 3417 cm^{-1}. MS(ESI +) m/z: calcd for $C_4H_7N_4O$ [M + H$^+$] 127.0615, found 127.061. To a solution of 5-aminopyrazole-1-carboxamide (20 g, 0.16 mol, 1 equiv) in CH_3CN (600 mL) was added triethyl orthoformate (35 mL, 0.21 mol, 1.3 equiv). The solution was heated at reflux for 24 h. Then the reaction was stirred at rt for 60 h. The solid which precipitated was filtrated, washed with CH_3CN, and then dried in a desiccator to afford a yellow-brown solid (18.24 g, 84%). Mp >250 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.52 (d, 1H, J = 1.9 Hz), 8.01 (s, 1H), 8.05 (d, 1H, J = 1.9 Hz). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 62.0, 106.9, 107.5, 108.5, 112.0. IR (KBr): ν 787, 1169, 1311, 1429, 1612, 1751, 3079 cm^{-1}. HRMS (ESI$^+$) m/z: calcd for $C_5H_5N_4O$: 137.0457 [M + H$^+$], found 137.0460.

8-Iodo-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (2). A solution of compound **1** (20.64 g, 0.15 mol, 1 equiv), phosphorus oxychloride (153 mL, 1.67 mol, 11 equiv), and *N*,*N*-dimethylaminopyridine (53.5 g, 0.44 mol, 3 equiv) was heated at reflux for 2 h. The volatile compounds were removed by evaporation. Then the mixture was dried under reduced pressure for 1 h. The black residue was dissolved in CH_2Cl_2 (800 mL) and cooled in an ice bath before the dropwise addition of *N*-methylaniline (64 mL, 0.59 mol, 4 equiv) followed by triethylamine (126 mL, 0.90 mol, 6 equiv). The solution was stirred at rt for 1 h and then hydrolyzed. The aqueous layer was extracted two more times with CH_2Cl_2. The organic layers were gathered and washed with brine, dried over magnesium sulfate, and evaporated over reduced pressure. The brown oily residue was stirred for 20 min with MeOH (80 mL) and then left on the bench 30 min more. The solid obtained was filtrated, washed with methanol (5 × 20 mL), and dried in a desiccator to afford a beige solid (33.6 g, 82%) corresponding to 4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine. Mp = 143 °C (mp = 141−142 °C).[28] ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.81 (s, 3H), 6.38 (d, 1H, J = 2.0 Hz), 7.19−7.43 (m, 5H), 7.77 (d, 1H, J = 2.0 Hz), 8.22 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 42.4, 95.6, 126.2, 126.2, 127.4, 129.2, 144.8, 150.2, 151.2, 152.7. IR (KBr): ν 703, 814, 905, 1095, 1566, 1604, 3104 cm^{-1}. MS(ESI$^+$) m/z: calcd for $C_{12}H_{11}N_5$ 225.25 [M + H$^+$], found 226. A solution of pyrazolotriazine (15 g, 66.59 mmol, 1 equiv) and *N*-iodosuccinimide (19.47 g, 86.57 mmol, 1.3 equiv) in chloroform (400 mL) was heated at reflux for 30 min. After being cooled at rt, the solution was hydrolyzed with $Na_2S_2O_5$ 1 M. The aqueous layer was

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extracted one more time with CH_2Cl_2. The organic layers were gathered, washed with brine, dried over magnesium sulfate, and evaporated over reduced pressure. AcOEt was added to the brown solid, which was filtrated, washed with a few quantities of AcOEt, and dried in a desiccator to afford **2** as a white solid (22.07g, 94%). Mp = 190−191 °C. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 3.81 (s, 3H), 7.17−7.46 (m, 5H), 7.76 (s, 1H), 8.31 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): 42.7 (CH_3), 48.8, 126.3, 127.7, 129.3, 144.4, 148.6, 150.1, 150.7, 154.0. IR (KBr): ν 750, 952, 1550, 1589 cm^{-1}. HRMS (ESI$^+$) m/z: calcd for $C_{12}H_{11}IN_5$ 352.0053 [M + H$^+$], found 352.0056.

1,4-Anhydro-2-deoxy-D-*erythro*-pent-1-enitol (3). A solution of thymidine (4.48 g, 18.5 mmol, 1 equiv), hexamethyldisilazane (25 mL), and ammonium sulfate (489 mg, 307 mmol, 0.2 equiv) was heated at reflux for 2 h. The volatile compounds were evaporated, and a mixture of ice and water was added to the residue until precipitation of a gummy solid. Cold CH_2Cl_2 (40 mL) was added, and the solid observed (thymine) was filtrated and washed with cold CH_2Cl_2. The organic layer was recovered after a rapid and cold extraction. The extraction was realized two more times with cold CH_2Cl_2. The organic layers were gathered, washed with cold brine (40 mL), dried over magnesium sulfate, and concentrated under reduced pressure. The brown oily residue was used without further purification. The silylated compound (1.74 g, 5.57 mmol, 1 equiv) was dissolved in THF (10 mL) before the addition of TBAF 1 M in THF (12.25 mL, 12.25 mmol, 2.2 equiv). The solution was stirred at rt for 3 h. The solvent was evaporated and the crude was purified by chromatography (eluent Et_2O/acetone 2/1) to afford colorless oil (363 mg, 56%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.67 (sl, 2H), 3.65 (dd, 1H, J = 11.9 Hz, J = 6.7 Hz), 3.70 (dd, 1H, J = 11.9 Hz, J = 4.4 Hz), 4.38−4.44 (m, 1H), 4.72−4.73 (m, 1H), 5.20 (t, 1H, J = 2.7 Hz, J = 2.7 Hz), 6.56 (dd, 1H, J = 2.7 Hz, J = 1.0 Hz). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 63.2, 75.5, 89.5, 103.9, 149.9. MS characterization has been unsuccessful.

8-(2′-Deoxy-β-D-*glycero*-pentofuran-3′-ulos-1′-yl)-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (4). A solution of bis(dibenzylideneacetone)palladium(0) (1.12 g, 1.94 mmol, 0.06 equiv) and triphenylarsine (1.19 g, 3.88 mmol, 0.12 equiv) in CH_3CN (200 mL) was stirred at rt for 1 h. This palladium complex was then added to a solution of iodopyrazolotriazine **2** (10.87 g, 30.96 mmol, 1 equiv), glycal **3** (7.19g, 61.92 mmol, 2 equiv), and triethylamine (5.18 mL, 35.15 mmol, 1.2 equiv) in CH_3CN (300 mL). The solution was stirred at 100 °C for 16 h. After cooling at rt, the palladium was filtrated over Celite and washed with CH_3CN until the filtrate became colorless. The filtrate was evaporated and purified by chromatography (eluent PE/AcOEt 95/5 to 2/8 then AcOEt) to afford **4** as a brown solid (1.70 g, 34%). The product was washed with AcOEt to obtain a beige solid. Mp = 157−158 °C. $[\alpha]^{20}_D$ = +1.46 (c = 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 2.78 (dd, 1H, J = 6.5 Hz, J = 17.8 Hz), 2.95 (dd, 1H J = 10.7 Hz, J = 17.8 Hz), 3.82 (s, 3H), 3.89 (dd, 1H, J = 1.8 Hz, J = 12.1 Hz), 3.96 (dd, 1H, J = 12.1 Hz, J = 2.3 Hz), 4.07−4.08 (m, 1H), 4.74 (sl, 1H), 5.42 (dd, 1H, J = 6.5 Hz, J = 10.7 Hz), 7.18−7.22 (m, 2H), 7.39−7.43 (m, 3H), 7.76 (s, 1H), 8.21 (s, 1H). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 42.6, 44.6, 62.7, 70.7, 82.3, 108.5, 126.3, 127.7, 129.2, 143.5, 144.3, 148.1, 150.0, 152.7, 214.3. IR (KBr): ν 1020, 1405, 1494, 1602 cm^{-1}. HRMS(ESI$^+$) m/z: calcd for $C_{17}H_{17}N_5O_3Na$ [M + Na$^+$] 362.1229, found 362.1227.

8-(2′-Deoxy-β-D-ribofuranosyl)-4-(*N*-methyl-*N*-phenylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (5). To a solution of the keto compound **4** (1.69 g, 4.98 mmol, 1 equiv) in CH_3CN (60 mL) was added sodium tri(acetoxy)borohydride (4.22 g, 19.92 mmol, 4 equiv) portionwise. After 5 h of stirring at rt, the solvent was evaporated. The product was purified by chromatography (eluent CH_2Cl_2/MeOH 95/5 to 9/1) to afford **5** as a white solid (1.16 g, 68%). Mp = 196−198 °C (degradation). $[\alpha]^{20}_D$ = +4.2 (c = 1, DMF). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.61 (sl, 1H), 2.11 (dd, 1H, J = 13.1 Hz, J = 5.4 Hz), 2.59 (ddd, 1H, J = 13.1 Hz, J = 11.2 Hz, J = 4.9 Hz), 3.73 (dd, 1H, J = 12.6 Hz, J = 1.9 Hz), 3.80 (s, 3H), 3.94 (dd, 1H, J = 12.6 Hz, J = 2.04), 4.11 (sl, 1H), 4.66 (d, 1H, J = 4.9 Hz), 5.37 (dd, 1H, J = 11.2 Hz, J = 5.4 Hz), 5.77 (sl, 1H), 7.17 (m, 2H), 7.40 (m, 3H), 7.71 (s, 1H), 8.18 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 41.0, 43.7, 64.2, 73.9, 75.8, 88.8, 109.1, 126.3, 127.7, 129.3, 144.1, 144.6, 147.9, 150.2, 152.2. IR (ATR-Ge): ν 1353, 1590, 2360, 3393 cm^{-1}. HRMS (ESI$^+$) m/z: calcd for $C_{17}H_{20}N_5O_3$ 342.1560 [M + H$^+$], found 342.1565.

8-(2′-Deoxy-3′,5′-bis(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl)-4-aminopyrazolo[1,5-*a*]-1,3,5-triazine (6). To a solution of nucleoside **5** (1.21g, 3.54 mmol, 1 equiv) and imidazole (2.89 g, 42.53 mmol, 12 equiv) in *N*,*N*-dimethylformamide (25 mL) was added *tert*-butyldimethylsilyl chloride (3.20 g, 21.27 mmol, 6 equiv). The solution was stirred at rt for 60 h. Then the solvent was evaporated. The crude material was purified by chromatography (eluent PE/AcOEt 98/2 then 95/5). The product was obtained as yellow oil which crystallized after addition/evaporation of MeOH to afford a white solid (1.43g, 71%). Mp = 94−95 °C. $[\alpha]^{20}_D$ = −18.0 (c = 1, $CHCl_3$) ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.07 (s, 3H), 0.08 (s, 3H), 0.14 (s, 6H), 0.91 (s, 9H), 0.94 (s, 9H), 2.09 (ddd, 1H, J = 1.4 Hz, J = 5.6 Hz, J = 12.7 Hz), 2.30 (ddd, 1H, J = 5.3 Hz, J = 10.6 Hz, J = 12.7 Hz), 3.63 (dd, 1H, J = 6.5 Hz, J = 10.6 Hz), 3.71 (dd, 1H, J = 4.4 Hz, J = 10.6 Hz), 3.79 (s, 3H), 3.86 (ddd, 1H, J = 1.7 Hz, J = 4.4 Hz, J = 6.5 Hz), 4.51−4.55 (m, 1H, J = 5.2 Hz), 5.38 (dd, 1H, J = 5.6 Hz, J = 10.5 Hz), 7.29−7.40 (m, 5H), 7.81 (s, 1H), 8.12 (s, 1H). ^{13}C NMR (acetone-d_6, 100 MHz): δ −5.2, −5.2, −4.4, −4.4, 26.2, 26.3, 42.1, 42.5, 64.8, 71.8, 75.5, 88.4, 110.1, 127.3, 127.7, 129.6, 144.2, 146.0, 148.9, 150.8, 152.8. IR (ATR-Ge): ν 695, 772, 832, 1088, 1252, 1536, 1613, 2856, 2952 cm^{-1}. HRMS (ESI$^+$) m/z: calcd for $C_{29}H_{48}N_5O_3Si_2$ 570.3290, found 570.3290. To a solution of this product (1.43 g, 2.5 mmol, 1 equiv) in MeOH (50 mL) in a sealed tube was added a solution of ammonia (7 N) in MeOH (4 mL, 28 mmol, 11.2 equiv). The sealed tube was closed, and the mixture was heated at 100 °C for 19 h. After the mixture was cooled at rt, the ammonia and solvent were removed by evaporation. The crude product was purified by chromatography (eluent PE/AcOEt 95/5 to 70/30) to afford **6** as a white solid (0.92 g, 77%). Mp = 126−128 °C. $[\alpha]^{20}_D$ = −1.5 (c = 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 250 MHz): δ −0.04 (s, 3H), −0.04 (s, 3H), 0.00 (s, 6H), 0.80 (s, 9H), 0.81 (s, 9H), 2.07 (dd, 1H, J = 4.5 Hz, J = 11.9 Hz), 2.20−2.27 (m, 1H), 3.52 (dd, 1H, J = 10.6 Hz, J = 6.3 Hz), 3.62 (dd, 1H, J = 10.6 Hz, J = 3.9 Hz), 3.85−3.86 (m, 1H), 4.39−4.40 (m, 1H), 5.37 (dd, 1H, J = 5.2 Hz, J = 10.4 Hz), 6.95 (sl, 2H), 7.96 (s, 1H), 8.05 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ −5.3, −5.2, −4.5, −4.5, 18.2, 18.5, 26.0, 26.1, 41.5, 64.0, 71.3, 74.5, 87.9, 111.1, 145.0, 146.1, 150.9, 152.9. IR (ATR-Ge): ν 773, 832, 1092, 1251, 1548, 2929 cm^{-1}. HRMS (ESI$^+$) m/z: calcd for $C_{22}H_{42}N_5O_3Si_2$ 480.2820 [M + H$^+$], found 480.2823.

8-(2′-Deoxy-β-D-ribofuranosyl)-4-(*N*-benzoylamino)pyrazolo[1,5-*a*]-1,3,5-triazine (7). Compound **6** (0.99g, 2.06 mmol, 1 equiv) was solubilized in pyridine (11 mL) and cooled at 0 °C, and benzoyl chloride (1.2 mL, 10.32 mmol, 5 equiv) was added dropwise. The solution was stirred at rt for 2 h 30 and the solution cooled again at 0 °C. After dropwise addition of water (10 mL) and then NH_4OH (15 mL), the solution was stirred at rt for 50 min. After evaporation of the solvent and then coevaporation with toluene, water (50 mL) was added, and the product was extracted with CH_2Cl_2 (2 × 50 mL). The organic layers were gathered, washed with brine (40 mL), dried over magnesium sulfate, and evaporated under reduced pressure. After purification by chromatography (eluent PE/AcOEt 90/10 to 70/30) a white solid was obtained (0.92 g, 80%). Mp = 70−72 °C. $[\alpha]^{20}_D$ = −10.8 (c = 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.06 (s, 3H, $SiCH_3$), 0.07 (s, 3H, $SiCH_3$), 0.11 (s, 6H, $Si(CH_3)_2$), 0.91 (s, 9H, Si−$C(CH_3)_3$), 0.93 (s, 9H, $SiC(CH_3)_3$), 2.22 (ddd, 1H, H-2′, $J_{2'a\text{-}3'}$ = 1.5 Hz, $J_{2'a\text{-}1'}$ = 5.4 Hz, $J_{2'a\text{-}2'b}$ = 12.6 Hz), 2.34 (ddd, 1H, H-2′b, $J_{2'b\text{-}3'}$ = 5.2 Hz, $J_{2'b\text{-}1'}$ = 10.6 Hz, $J_{2'b\text{-}2'a}$ = 12.6 Hz), 3.64 (dd, 1H, H-5′a, $J_{5'a\text{-}4'}$ = 5.9 Hz, $J_{5'a\text{-}5'b}$ = 10.7 Hz), 3.73 (dd, 1H, H-5′b, $J_{H5'b\text{-}4'}$ = 3.8 Hz, $J_{5'b\text{-}5'a}$ = 10.7 Hz), 3.98 (ddd, 1H, H-4′, $J_{4'\text{-}3'}$ = 1.8 Hz, $J_{4'\text{-}5'b}$ = 3.8 Hz, $J_{4'\text{-}5'a}$ = 5.8 Hz), 4.50−5.51 (m, 1H, H-3′), 5.50 (dd, 1H, H-1′, $J_{1'\text{-}2'a}$ = 5.4 Hz, $J_{1'\text{-}2'b}$ = 10.6 Hz), 7.57 (t, 2H, Hm Bz, ^3J = 1.9 Hz), 7.67 (t, 1H, Hp Bz, ^3J = 1.9 Hz), 8.09 (d, 2H, Ho Bz, ^3J = 1.9 Hz), 8.18 (s, 1H, H-7), 8.47 (sl, 1H, H-2), 9.95 (sl, 1H, NH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ −5.3 ($SiCH_3$), −5.2 ($SiCH_3$), −4.6 ($SiCH_3$), −4.5 ($SiCH_3$), 18.2 (Cq *t*Bu), 18.5 (Cq *t*Bu), 25.9 (3 CH_3 *t*Bu), 26.0 (3 CH_3 *t*Bu), 41.7 (C-2′), 63.9 (C-5′), 71.2 (C-1′), 74.4 (C-3′), 88.1 (C-4′), 112.9 (Cq), 128.3 (2 CH Bz), 129.3 (2 CH Bz), 132.6 (Cq), 133.8 (CH Bz), 145.2 (CH), 145.6 (Cq), 145.7 (Cq), 152.6 (CH), 163.4 (C═O). IR(ATR-

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Ge): ν 718, 1415, 1540, 1596, 2340, 2360 cm^{-1}. HRMS (ESI^+) m/z: calcd for $C_{29}H_{46}N_5O_4Si_2$ 584.3082 [M + H^+], found 584.3080.

To a solution of the previous compound (261 mg, 1.45 mmol, 1 equiv) in THF (3 mL) was added tetrabutylammonium fluoride 1 M in THF (1.3 mL, 1.3 mmol, 3 equiv). The solution was stirred at rt for 6 h, and then the solvent was evaporated and the residue purified by chromatography (eluent CH_2Cl_2/MeOH 95/5 to 85/15). The solid obtained was washed with water to give **7** as a white solid (127 mg, 80%). Mp = 144−145 °C. ^1H NMR (MeOD, 250 MHz): δ 2.24 (ddd, 1H, H-2′a, $J_{1'\text{-}2'a}$ = 5.4 Hz, $J_{2'a\text{-}2'b}$ = 12.9 Hz, $J_{2'a\text{-}3'}$ = 1.5 Hz), 2.38 (ddd, 1H, H-2′b, $J_{1'\text{-}2'b}$ = 10.5 Hz, $J_{2'a\text{-}2'b}$ = 12.9 Hz, $J_{2'b\text{-}3'}$ = 5.6 Hz), 3.67 (dd, 1H, H-5′a, $J_{H5'a\text{-}H5'b}$ = 11.8 Hz, $J_{H5'a\text{-}H4'}$ = 4.3 Hz), 3.73 (dd, 1H, H-5′b, $J_{H5'a\text{-}H5'b}$ = 11.8 Hz, $J_{H5'b\text{-}H4'}$ = 3.9 Hz), 3.95−3.99 (m, 1H, H-4′), 4.42−4.44 (m, 1H, H-3′), 5.47 (dd, 1H, H-1′, $J_{1'\text{-}2'a}$ = 5.4 Hz, $J_{1'\text{-}2'b}$ = 10.5 Hz), 7.51−7.68 (m, 3H, H arom), 8.19−8.22 (m, 2H, H arom), 8.26 (s, 1H, H-7), 8.31 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 43.7(C-2′), 59.4 (C-5′), 64.1 (C-1′), 72.7 (C-3′), 89.2 (C-4′), 114.3 (Cq), 129.3 (2 C arom), 130.0 (2 C arom), 131.8 (C-7), 133.6 (C arom), 136.5 (Cq), 145.7 (C-2), 146.1 (Cq), 147.7 (Cq), 164.7 (C═O). IR(ATR-Ge): ν 1071, 1287, 1469, 1620, 1648, 2927, 3366 cm^{-1}. HRMS (ESI^+) m/z: calcd for $C_{17}H_{18}N_5O_4$ 356.1353 [M + H^+], found 356.1354.

8-[(3′-((2-Cyanoethyl)-(*N*,*N*-diisopropyl))phosphoramidite-2′-deoxy-5′-dimethoxytrityl-*β*-D-ribofuranosyl]-4-(*N*-benzoyl-amino)pyrazolo[1,5-*a*]-1,3,5-triazine (8). In a dry 50 mL flask under argon was dissolved compound **7** (591 mg, 1.66 mmol, 1 equiv) in dry pyridine (10 mL). The solution was cooled at 0 °C before the dropwise addition of a DMT-Cl solution (620 mg, 1.83 mmol, 1.1 equiv) in dry pyridine (10 mL) over 1 h. After 18 h of stirring at rt, pyridine was removed by evaporation. Water (10 mL) was added to the residue, and the product was extracted with CH_2Cl_2 (2 × 15 mL). The organic phases were gathered and washed with brine, dried over magnesium sulfate, and evaporated. The crude product was purified by chromatography (eluent CH_2Cl_2/MeOH 98/2 to 95/5) to give a white solid (655 mg, 60%). ^1H NMR($CDCl_3$, 400 MHz): δ 2.25−2.30 (ddd, 1H, J = 1.6 Hz, J = 5.6 Hz, J = 7.2 Hz), 2.40−2.46 (m, 1H), 3.25 (d, 1H, J = 4.8 Hz), 3.77 (s, 6H), 4.08−4.11 (m, 1H), 4.29 (sl, 1H), 4.48 (m, 1H), 5.49 (dd, 1H, J = 5.6 Hz, J = 10.4 Hz), 6.85 (dd, 4H), 7.18−7.65 (m, 12H), 8.23−8.31 (m, 4H). ^{13}C NMR (acetone-d_6, 100 MHz): δ 42.7, 55.5, 65.8, 71.8, 74.5, 86.7, 87.5, 113.8, 127.4, 128.5, 129.0, 129.4, 130.1, 130.9, 131.0, 133.8, 137.0, 137.1, 146.3, 159.5. HRMS(ESI^+) m/z: calcd for $C_{38}H_{36}N_5O_6$ 658.2660 [M + H^+], found 658.2655. To a mixture of the 5′-*O*-dimethoxytritylated nucleoside (144 mg, 0.26 mmol, 1 equiv) and 4,5-dicyanoimidazole 0.25 M in CH_3CN (0.68 μL, 0.17 mmol, 0.67 equiv) into freshly distilled CH_2Cl_2 (2 mL) was added dropwise 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-phosphorodiamidite (9 μL, 0.31 mmol) under argon. The reaction was stirred for 1 h at rt, and then the mixture was hydrolyzed with crushed ice and rapidly extracted with CH_2Cl_2 (2 × 15 mL). Organic phases were gathered and washed with cold brine, dried over magnesium sulfate, and evaporated. The crude product was purified by chromatography (eluent pentane/AcOEt/Et_3N 60/39/1) to give **8** as a white solid (133 mg, 60%). ^1H NMR ($CDCl_3$, 250 MHz): δ 1.11−1.21 (m, 12H), 2.44−2.52 (m, 3H), 2.62 (t, 1H, J = 6.5 Hz), 3.22−3.37 (m, 2H), 3.56−3.89 (m, 4H), 3.77 (s, 6H), 4.24−4.28 (m, 1H), 4.60−4.67 (m, 1H), 5.52 (dd, 1H, J = 5.7 Hz, J = 9.5 Hz), 6.78−6.82 (m, 4H), 7.19−7.48 (m, 9H), 7.52−7.68 (m, 3H), 8.09 (d, 2H, J = 7.2 Hz), 8.21 (s, 1H), 8.42 (s, 1H). ^{31}P NMR ($CDCl_3$, 162 MHz): δ 148.02. IR (ATR-Ge): ν 703, 726, 1030, 1176, 1248, 1508, 1597, 1719, 2247, 2966 cm^{-1}. MS(ESI^-) m/z: calcd for $C_{47}H_{52}N_7O_7P$ 857.95 [M + H^+], found 856.4.

Oligonucleotide Syntheses. Modified ONs **9**−**11** were prepared from modified nucleoside phosphoramidite **8** and commercially available dC, dA, dG, and dT phosphoramidites (Biosolve) and deoxynucleoside-CPG (1 μmol, Glen Research) by standard solid-phase phosphoramidite chemistry except for the modified phosphoramidite **8**. The coupling time for **8** was increased to 10 min. Tetrazole (0.45 M in CH_3CN, Biosolve) was used as coupling reagent and 3% dichloroacetic acid in 1,2-dichloroethane for the detritylation step. Coupling efficiency was estimated from the trityl assay and was >95%. After chain elongation and final detritylation, the oligomers were cleaved from the resin and deprotected by treatment with 1 mL of concd aq ammonia solution (25%) at 55 °C during 16 h and filtered through syringe filters with 0.45 μm GHP membrane (Pall). The ammonia solution was then removed by evaporation. Oligonucleotide purification and analyses were performed by reversed-phase HPLC on a Lichrospher 100 RP 18 (5 μm) column (125 mm × 4.6 mm) from Merck using a linear gradient of CH_3CN (0−38.5% over 45 min) in 0.1 M aqueous triethylammonium acetate buffer, pH = 7, with a flow rate of 1 mL/min. Detection λ = 260 nm. The yields were 17% (ON **9**), 12% (ON **10**), and 14% (ON **11**). The integrity of all oligonucleotides was confirmed by MALDI-TOF mass spectrometry (Figures S39−S44, Supporting Information).

Acid-Catalyzed Hydrolysis. The modified oligonucleotide **11** and the parent unmodified **14**, used as a reference (2 OD each), were separately incubated in 80% acetic acid (0.4 mL) for different times at 65 °C. Acid was then removed under reduced pressure. The residues were analyzed by reversed-phase chromatography (using the same column and buffer as above and a linear gradient of CH_3CN (5 to 27.5% over 30 min) and MALDI-TOF mass spectrometry (see Figures S45−S53, Supporting Information, for selected data). Then the residues were incubated with 30% aqueous ammonium hydroxide solutions (1 mL) for 8 h at 65 °C. After removal of the ammonia solution and lyophilization the residues were analyzed by mass spectrometry.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1HNMR, ^{13}CNMR, and ^{31}PNMR spectra, MS spectra for compounds **1**−**8**. HPLC reversed-phase analyses and MS characterizations for oligonucleotides **9**−**11** and selected data for the acid-catalyzed studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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