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Efficient incorporation of 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-imidazole-4-carboxamide into DNA via a suitable convertible phosphoramidite derivative

Claudio Cadena-Amaro and Sylvie Pochet*

Unité de Chimie Organique, URA CNRS 2128, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris cedex 15, France

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Abstract—The synthetic scheme of 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-imidazole-4-carboxamide (**3**) is based on the ring contraction of pyrimidine (5-BrdU) into imidazolin-2-one. The rearrangement leads to the unexpected mixture of the deoxynucleoside **4β** and its α anomer. The mechanism of the anomerisation under basic conditions is proposed. Further conversion of 4-carboxylic acid into amide affords the title compound **3**. The conversion of 4-carboxylic acid into ethyl ester is preferred for the preparation of the phosphoramidite derivative **11** suitable for chemical incorporation of the modified nucleobase into DNA. Thermal denaturation studies show that 2-oxoY within the sequence used pairs more favorably with the purines than the pyrimidines.

1. Introduction

There has been a tremendous interest in the design and synthesis of DNA nucleoside mimics for a variety of explorations and applications. For example, the search for universal or ambiguous base has been intensive in recent years.[1] Among the candidates a family of azole carboxamide nucleosides **1** (Fig. 1) has been studied.[2–5] Because of the rotation about the glycosidic bond (*syn*/*anti*) and the exocyclic carboxamide function, members of the family **1** may adopt four different conformations, each could pair with one of the four canonical bases. However, experimental data have shown that these molecules do not use this potential but manifest a preference for some particular nucleobases.[6–8] Other related five membered rings have been synthesized, such as the imidazolin-2-one (**2**), which has been proposed as structural isomer of thymidine,[9] and 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-imidazole-4-carboxamide (**3**) as a 'lure' at ambiguous positions.[10]

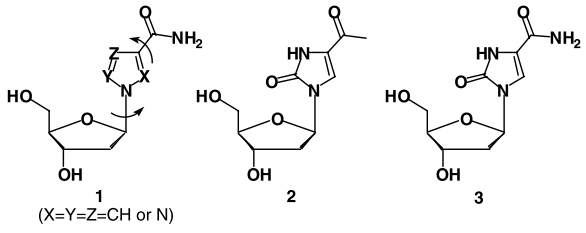

Figure 1. Structure of DNA nucleoside analogues.

We previously developed several artificial bases as mutagenic agents based on substituted five-membered ring.[6,11,12] Interestingly, nucleoside **3** (designed 2-oxodY) belonging to the imidazole carboxamide family **1**, features an added H-bond acceptor group at C-2 which might contribute to base pairing. Accordingly, 2-oxodY might have an enhanced potential for mutagenesis experiments. Although the synthesis of **3** and its DNA incorporation according to H-phosphonate chemistry have been previously reported,[10] a rapid access to oligonucleotides containing **3** via the phosphoramidite chemistry is still needed to study the ambiguous pairing potential of this artificial nucleobase.

Herein, we describe a new route for simple and efficient incorporation of the nucleobase 2-oxoY into DNA fragments via a convenient phosphoramidite derivative. During the synthesis of the nucleoside **3**, we observed the anomerisation of the intermediate 1-(2-deoxy-D-β-ribofuranosyl)-2-oxo-imidazole-4-carboxylic acid (**4**) and we propose a mechanism for this unexpected anomerisation.

Keywords: Nucleobase; Nucleoside; Base pairing; Anomerisaton; Conformation.
* Corresponding author. Tel.: +33 1 40 61 33 28; fax: +33 1 45 68 84 04; e-mail: spochet@pasteur.fr

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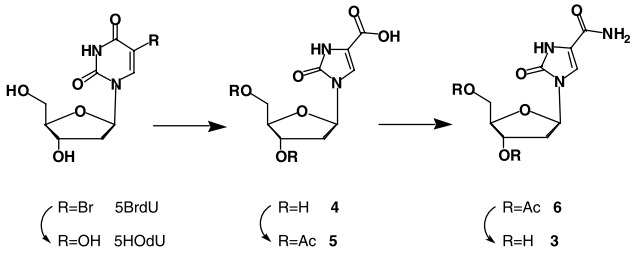
2. Results and discussion

2.1. Synthesis of 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-imidazole-4-carboxylic acid (4)

The synthetic scheme of **3** is based on the conversion of 5-halogeno-uridine into imidazoline nucleoside according to a known rearrangement.[13] Thus, mild alkaline treatment of 5-bromo-2′-deoxyuridine (5BrdU) results in the ring contraction of pyrimidine into imidazoline nucleoside **4** via the 5-hydroxy derivative (5OHdU) (Scheme 1).



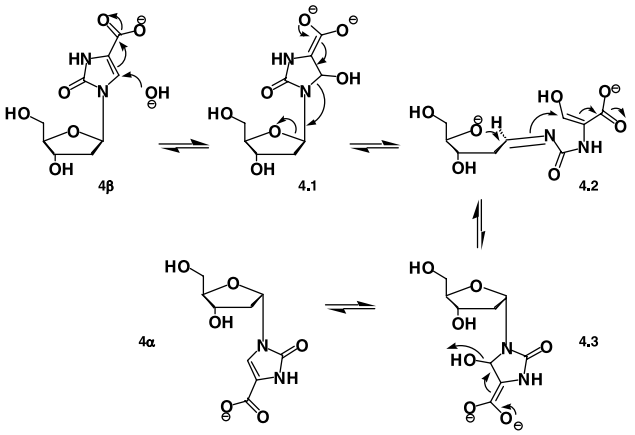
Scheme 1. Synthesis of 4-carboxamide-1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole (**3**).

In our hands, we observed that the yield of the reaction and the anomeric purity of the target nucleoside were dependent on the reaction time and basic conditions used. Treatment of 5BrdU in a buffered sodium bicarbonate solution for 25 h (pH maintained to ~8 by addition of CO_2), followed by further heating of the resulting solution for another 16 h (pH raised to ~10) resulted in the formation of the expected imidazoline nucleoside **4** in good yield (75–86%). However, examination of ^{1}H and ^{13}C NMR spectra of **4** revealed the presence of another deoxynucleoside in a proportion varying from 6 to 11% depending on the experiments. The H-5, H2′ and H2″ signals, as well as C5 and sugar carbon signals indicated a mixture of the desired **4β** and the corresponding α anomer. In particular, the chemical shifts of H2″ proton (ddd for the β anomer and apparent dt for the α anomer) and H2′ proton (apparent septuplet for the β anomer and apparent quintuplet for the α anomer) are separated from 0.52 ppm for the α anomer compared to 0.14 ppm for the β anomer. Chromatographic separation of anomers at this stage was tedious resulting in a reduced yield (40–50%) of pure 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxylic acid (**4β**); moreover it was not possible to isolate pure anomer **4α**.

In another attempts to synthesize compound **4**, 5BrdU was treated with a 0.1 N NaOH solution (pH 13) at 100 °C.[13] The reaction was stopped after 6 h while some starting material and the intermediate 5OHdU were still detected on TLC. NMR analysis of an aliquot of the reaction mixture before chromatography revealed the absence of detectable α anomer. After chromatographic purification, pure **4β** was isolated in 55% yield.

2.2. Base catalysed anomerisation of 4

Formation of such a by-product in the ribo or deoxy series was not discussed in the previous reports.[10,13] We explain this unusual anomerisation (Scheme 2) by a mechanism



Scheme 2. Proposed mechanism for anomerisation of **4**.

similar to that observed during treatment of 5-formyl-uridine with alkaline conditions at room temperature.[14] Attack of hydroxide ion on C-5 of **4β** and subsequent migration of the double bond give the intermediate **4.1**, which undergoes by opening both the deoxyribose and imidazolin-2-one rings into the acyclic ureide **4.2**. Ring closure leads to the formation of both **4.1** and **4.3**. From the hydroxy intermediate **4.3**, nucleoside **4α** can be formed. As this base-catalysed anomerisation does not occur on treatment of 5-bromo-(2-deoxy)ribouridine, this reactivity is explained by the presence of the electron withdrawing group, 5-formyl in the case of pyrimidine or 4-carboxylic acid in the case of imidazoline ring.

2.3. Synthesis and properties of 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-imidazole-4-carboxamide (3)

The following steps for the preparation of **3** are the conversion of carboxylic acid into amide via activated ester. Acetylation of **4β** by acetic anhydride in pyridine afforded **5** in 84% yield (Scheme 1). Reaction of **5** in acetonitrile with pentafluorophenol in the presence of DCC, followed by treatment of the crude ester in 33% aqueous ammonia solution at 4 °C for 20 min gave **6** in 78% yield. The anomeric purity of **6** was confirmed by NMR analysis. When a mixture of α/β anomers was used in place of pure **4β**, the separation of **6α** and **6β** anomers is easier than of **4α** and **4β**, and pure acetylated **6β** was isolated in a good yield. Finally, ammonolysis of the acetyl group at room temperature (96% yield) gave the title nucleoside **3**, which was fully characterized by NMR, UV, HMRS and elemental analysis. These data are in agreement with those previously reported.[10] The route described here affords nucleoside **3** in a good overall yield (63% from **4**).

In order to assess the mutagenic potential of this nucleobase, we examined the possible occurrence of several tautomeric forms (acido–basic character) and of different base conformations about the deoxyribose.

The p*K*a value of nucleoside **3** was determined by pH-dependent NMR titration studies. ^{1}H NMR and HMBC NMR experiments were recorded at 400 MHz. The chemical shifts of aromatic proton (H-5) and carbons (C-2, C-4, C-5) were measured in the range of pH 6.5–12.6.

Table 1. Conformational parameters of **3** from PSEUROT

pH	P_N	P_S	Φ_N	Φ_S	p*N*	p*S*	r.m.s.
7.2	13.8	150.6	31.2	35.3	36%	64%	0.001
12.6	12.9	141.0	35.0	34.7	30%	70%	0.028

The marked shifts, noticed at C4 and C2, fitted to the equation of a single ionization equilibrium. The p*K*a value (N3) so obtained is 10.1 ± 0.2, while the p*K*a value (N3) for dT is 9.8.[15] This result indicates that the keto form predominates at physiological pH.

The conformational analysis of the furanose puckering of **3** was determined by ^{1}H NMR using vicinal spin-coupling constants of the sugar protons and PSEUROT program (version 6.1).[16] The values of the phase angle of pseudorotation (P), the amplitude of pucker (Φ) and the population of conformers are given Table 1. Calculations indicate that **3** preferentially adopts in solution a C2′-endo conformation (64% at pH 7.2 and 70% at pH 12.6).

The orientation of the aromatic base with respect to the sugar moiety is determined by the glycosidic torsion angle χ defined as the O-4′-C-1′-N-1-C-2 torsion in pyrimidines and the O-4′-C-1′-N-9-C-4 torsion in purines. Although quantitative evaluation of interproton distances by mean of NOE measurement is widely used, most often (and in absence of proton at C2 position) the values of χ are determined from the measurement of three-bond carbon–proton scalar couplings $^3J_{CH}$ across the glycosidic bond and DFT analysis using Karplus equations.[17] Thus, the maximum magnitude of $^3J_{C2/4\text{-}H1'}$ for purines and pyrimidines in the *anti* glycosidic torsion ($\chi \approx -120$ °C) is observed between 2.0 and 2.7 Hz, while in the *syn* orientation ($\chi \approx 60$ °C) the maximum is between 5.5 and 5.7 Hz for dA, dG, dC and 6.9 Hz for T. A significant difference between purine and pyrimidine bases is found for $^3J_{C6/8\text{-}H1'}$; in the *anti* region, the maximal varied from 4.3 to 4.5 Hz for purines and from 3.9 to 4.0 Hz for pyrimidines, while in the *syn* region the maximum is 3.9 Hz for dA and dG, versus 5.7 Hz for dC and T.

The $^3J_{C2\text{-}H1'}$ and $^3J_{C5\text{-}H1'}$ values of nucleoside **3** were determined at 30 mM in D_2O (pH 7.2) by gradient selected ^3J-HMBC NMR experiments recorded at 600 MHz. The experimental values of $^3J_{CH}$ were 2.05 Hz for $^3J_{C2\text{-}H1'}$ and 4.20 Hz for $^3J_{C5\text{-}H1'}$. Comparison of these values with the $^3J_{CH}$ values reported for purines and pyrimidines[17] and the fact that $^3J_{C5\text{-}H1'} > {}^3J_{C2\text{-}H1'}$[18] led us to conclude that the preferred conformation of nucleoside **3** in solution is the *anti* conformation (Fig. 2).

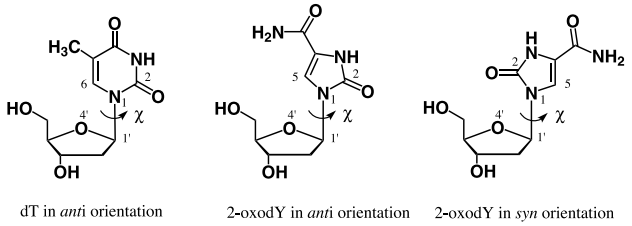
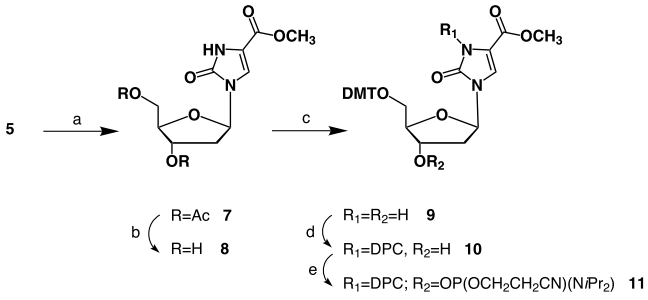


Figure 2. *Anti* and *syn*-χ conformers of 2-oxodY compared to dT (*anti*-χ).

2.4. Synthesis of the new phosphoramidite building block (11) and oligonucleotides containing the nucleoside analogue 2-oxodY

The nucleoside was next converted into a phosphoramidite building block for its chemical incorporation into DNA fragments. In order to circumvent the reactivity of the carboxamide group under phosphitylation conditions,[5] we prepared a convertible phosphoramidite suitable for automatic DNA synthesis (Scheme 3). The methyl ester was synthesized from the 3′,5′-*O*-diacetylated acid derivative **5** by reaction with methyl chloroformate in anhydrous dichloromethane in the presence of triethylamine and DMAP.[19] The reaction occurred via the formation of mixed anhydride and compound **7** was isolated in 75% yield. Treatment of **7** with a 0.5 N sodium methylate in methanol afforded methyl 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-imidazole-4-carboxylate (**8**) in nearly quantitative yield.



Scheme 3. Synthesis of the phosphoramidite derivative **11**. Reagents and conditions: (a) CH_3OCOCl, CH_2Cl_2, Et_3N, 1 h, then DMAP, 8 h; (b) $NaOCH_3$, 4 °C, 30 min, 75% from **5**; (c) DMTCl chloride, pyridine, 1.5 h, 78%; (d) diphenylcarbamoyl chloride, DIPEA, pyridine, 2 h, 65%; (e) 2-cyanoethyl-*N*,*N*-di*iso*propylchlorophosphoramidite, DIPEA, CH_2Cl_2, 30 min, 75%.

The expected conversion of the methyl ester into amide under oligonucleotide deprotection conditions (ammonia at 55 °C for 8 h) was checked at the nucleoside stage. Thus, complete amidation of **8** into **3** was confirmed by HPLC and NMR analyses. Moreover, no anomerisation was detected under these conditions.

The phosphoramidite **11** was obtained in three steps according to classical methodology.[20] The 5′-*O*-dimethoxytritylation of **8** (78% yield) followed by *N*-3 protection of resulting **9** by a diphenylcarbamoyl group gave compound **10** in 65% yield. When *N*-3 protection took place before 5′-*O*-dimethoxytritylation, **10** was isolated in a reduced global yield (29% in place of 51%). Finally, phosphitylation of **10** with 2-cyanoethyl-*N*,*N*-di*iso*propylchlorophosphoramidite in the presence of di*iso*propylethylamine in CH_2Cl_2 afforded the phosphoramidite **11** in 75% yield.

Table 2. Synthesized oligonucleotides using **11**

Sequences	Calculated mass	Measured mass
5′-GCAT**Y**^{oxo}GTCATAGCTGTTTCCTG-3′	6704.1133	6704.1315
5′-TGAC**Y**^{oxo}GTCATAGCTGTTTCCTG-3′	6704.1133	6704.1395
5′-ACTTGGCC**Y**^{oxo}CCATTTTG-3′	5124.8586	5124.8692
5′-CAAAATGG**Y**^{oxo}GGCCAAGT-3′	5240.9171	5240.9126

Four oligonucleotides (17- and 22-mers, Table 2) containing the modified base 2-oxoY were synthesized on an automated DNA synthesizer according to standard β-cyanoethyl phosphoramidite chemistry. The coupling efficiency of **11** was not different from those of the normal phosphoramidites. Conversion of the ester into amide occurred during the final deprotection step. The crude oligomers were purified by reverse phase HPLC at two stages (DMT on, then after detritylation). Integrity of the modified base was assessed by high-resolution mass spectrometry (MALDI-TOF) and by reversed phase HPLC analysis of nucleosides after enzymatic digestion.

2.5. Thermal denaturation studies

In order to assess the thermal stability of heteroduplexes containing 2-oxodY, UV thermal-denaturation experiments were conducted using a system composed of two complementary heptadecamers. Placing the artificial base in the ninth position of each strand allowed evaluation of the pairing preferences of 2-oxodY and the effects of local nearest-neighbor bases (2-oxodY within a C- or G-rich region). The melting temperatures (T_m) derived from respective melting curves are listed in Table 3.

All duplexes containing **3** have lower T_m values (entries 3–11) than the corresponding natural bases (A:T, entry 1 and C:G, entry 2). However, these values are significantly different according to the central base pairs involved. The most favorable T_m values are found when **3** is located opposite A ($\Delta T_m = -3$ °C) and in a lesser extent opposite G ($\Delta T_m = -6$ °C). Duplexes involving C opposite **3** are significantly less stable, though in the range of mismatches (entries 12–14). No significant context effect can be inferred from the different T_ms involving 2-oxodY. On the basis of melting temperatures, the nucleoside **3** shows a preference for pairing to the natural bases in the order **3**:A > **3**:G > **3**:T > **3**:C,**3**:**3**.

3. Conclusion

In summary, we have described an efficient access to oligonucleotides containing 2-oxodY using a suitable convertible phosphoramidite derivative. During the first step of the synthesis of 2-oxodY, we have observed an unexpected anomerisation under the basic conditions of the ring contraction reaction and we proposed a mechanism for the formation of the α anomer. Using synthesized modified oligodeoxynucleotides, we have measured the thermal stability of different base pairs involving 2-oxoY and each of the four canonical bases. In the studied context, the nucleobase 2-oxoY shows a preference for pairing to purines. Because replication depends not only on the characteristic of the base pairs, but also on those of the DNA polymerase, further work is under way to determine the enzymatic recognition of this artificial base and its mutagenic potential through alternative pairing schemes.

4. Experimental

4.1. General

NMR spectra were recorded on a Bruker Avance 400 instrument at 400.13, 100.62 and 161.98 MHz, for ^{1}H, ^{13}C and ^{31}P, respectively. ^{1}H and ^{13}C chemical shifts are given in ppm (δ) relative to residual solvent peak in the case of DMSO-d_6 or relative to TMS in the case of D_2O and $CDCl_3$. Chemical shifts are reported in ppm (δ) relative to 85% phosphoric acid as external standard for ^{31}P NMR spectra. Mass spectra were recorded by the mass spectroscopy laboratory (CNRS-ICSN, Gif-sur-Yvette). TLC was carried out on analytical silica plates (Kieselgel 60 F_{254}/0.2 mm thickness) and spots were visualized by UV light, then revealed by sulfuric acid-anisaldehyde spray followed by heating. Silica gel column chromatography was performed using Merck silica gel 60 (230–400 mesh). HPLC was performed on a Perkin Elmer system equipped with a diode array detector. Preparative reverse phase HPLC were carried out with a C18 column using a flow rate of

Table 3. Melting temperatures (T_m)[a] of heteroduplexes containing canonical bases and 2-oxodY
5′-ACTTGGCC**X**CCATTTTG-3′
3′-TGAACCGG**Y**GGTAAAAC-5′

Entry	**X**:**Y**	T_m (°C)	Entry	**X**:**Y**	T_m (°C)
1	C:G	59	**2**	A:T	58
3	G:Y^{oxo}	53	**4**	A:Y^{oxo}	55
5	Y^{oxo}:G	52	**6**	Y^{oxo}:A	55
7	C:Y^{oxo}	46	**8**	T:Y^{oxo}	51
9	Y^{oxo}:C	46	**10**	Y^{oxo}:T	50
11	Y^{oxo}:Y^{oxo}	51	**12**	G:T[b]	55
13	C:T[b]	48	**14**	C:A[b]	49

[a] Conditions: 10 mM sodium cacodylate, 0.1 M NaCl at pH 7.2, 1 μM of each strand.
[b] Data from literature.[5]

5.5 mL/min and a linear gradient of CH_3CN (A) in 10 mM triethylammonium acetate buffer (B) at pH 7.5 over 20 min. Purity of all tested compounds was checked by analytical HPLC on a Perkin Elmer system with a reverse phase column (C18) using a flow rate of 1 mL/min and a linear gradient of A in B at pH 7.5 over 20 min and using a diode array detector. Solvents were spectroscopic or HPLC grade and reagents used without purification.

4.2. Synthesis of 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxamide (3)

4.2.1. 1-(2-Deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxylic acid (4β). *Method A*. A solution of 5-bromo-2′-deoxyuridine (3.34 g, 10.89 mmol) in 0.1 N NaOH (385 mL) was refluxed for 6 h while the pH was maintained at 13 by addition of 1 N NaOH (2×0.5 mL, after 2 and 4 h, respectively). The cooled reaction was neutralized by addition of 0.1 N HCl, concentrated and the resulting solution was passed through a column of cationic resin (Dowex H^+). The product was eluated with water and the eluate was concentrated under vacuo. The crude product was purified by silica gel column chromatography (0–20% MeOH in CH_2Cl_2, then 0–10% CH_3COOH in 80:20 CH_2Cl_2/MeOH); first fractions were collected to give **4β** as a pale yellow powder (1.46 g, 55%). R_f (*i*PrOH/NH_4OH/H_2O: 70:10:20): 0.34. ^1H NMR (DMSO-d_6) δ: 1.88 (ddd, 1H, H2′, J=2.5, 6.0, 13.0 Hz), 2.12 (m, 1H, H2″), 3.31–3.40 (m, 2H, H5′ and H5″), 3.60 (m, 1H, H4′), 4.12 (m, 1H, H3′), 5.72 (dd, 1H, H1′, J=6.0, 8.3 Hz), 6.97 (s, 1H, H5), 10.20 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 39.74 (C2′), 62.81 (C5′), 71.81 (C3′), 82.54 (C1′), 87.82 (C4′), 114.15 (C5), 119.28 (C4), 153.15 (C2), 162.63 (COOH). HRMS (MALDI-TOF) *m/z* calcd for $C_9H_{12}N_2O_6$+Na 267.0593, found 267.0719.

Method B. A solution of 5-bromo-2′-deoxyuridine (5.0 g, 16.28 mmol) in 0.16 N $NaHCO_3$ (4.53 g in 0.33 mL) was refluxed for 25 h while the pH was maintained at 8.0 by addition of CO_2. The CO_2 arrival was suppressed and the pH raised rapidly to 10. After refluxing for 16 h, the cooled reaction was neutralized by addition of cationic resin (Dowex H^+), concentrated and the resulting solution was passed through a column of resin Dowex H^+. The product was eluated with water and the eluate was concentrated under vacuo. Purification by silica gel column chromatography afford compound **4** as a pale yellow powder (3.42 g, 86%) as a mixture of anomers β/α (95:5). ^1H NMR (D_2O) δ: 2.17 (dt, 0.05H, H2″α, J=3.7, 14.7 Hz), 2.27 (ddd, 0.95H, H2″β, J=3.6, 6.4, 14.0 Hz), 2.41 (ddd, 0.95H, H2′β, J=6.4, 7.4, 14.0 Hz), 2.69 (ddd, 0.05H, H2′α, J=7.4, 14.7 Hz), 3.59–3.70 (m, 2H, H5′ and H5″αβ), 3.92 (m, 0.95H, H4′β), 4.16 (m, 0.05H, H4′α), 4,36 (m, 0.05H, H3′α), 4.42 (m, 1H, 0.95H, H3′β), 5.94 (m, 1H, H1′αβ), 7.04 (s, 0.95H, H5β), 7.25 (s, 0.05H, H5α). ^{13}C NMR (D_2O) δ: 38.49 (C2′β), 39.02 (C2′α), 61.71 (C5′α), 62.07 (C5′β), 71.12 (C3′α), 71.47 (C3′β), 82.96 (C1′β), 83.57 (C1′α), 86.76 (C4′β), 87.40 (C4′α), 113.90 (C5β), 115.07 (C5α), 120.50 (C4), 153.51 (C2), 166.47 (COOH).

4.2.2. 1-(3,5-*O*-diacetyl-2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxylic acid (5). To compound **4** (1.46 g, 6.0 mmol) in dry pyridine (60 mL) was added acetic anhydride (2.26 mL, 24 mmol). After stirring for 3 h under argon, methanol (2 mL) was added. The reaction mixture was evaporated, the resulting residue adsorbed on silica gel and loaded onto a column (0–20% MeOH in CH_2Cl_2) to give **5** as a powder (1.65 g, 84%). Rf (*i*PrOH/NH_4OH/H_2O: 70:10:20): 0.56. ^1H NMR (DMSO-d_6) δ: 2.05 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.25 (m, 1H, H2″), 2.60 (m, 1H, H2′), 4.09 (m, 1H, H4′), 4.12 (m, 1H, H5′), 4.21 (m, 1H, H5″), 5.19 (m, 1H, H3′), 5.85 (dd, 1H, H1′, J=6.0, 8.7 Hz), 7.23 (s, 1H, H5), 10.51 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.40 (CH_3), 21.64 (CH_3), 35.86 (C2′), 64.63 (C5′), 75.20 (C3′), 81.76 (C4′), 82.81 (C1′), 115.64 (C4), 117.45 (C5), 153.14 (C2), 161.18 (4-COOH), 170.85 ($COOCH_3$), 170.98 ($COOCH_3$). MS (ESI-TOF) *m/z* 329.1 $(M+H)^+$, 351.1 $(M+Na)^+$, 367.0 $(M+K)^+$.

4.2.3. 1-(3,5-*O*-diacetyl-2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxamide (6). To compound **5** (1.57 g, 4.78 mmol) in anhydrous acetonitrile (50 mL) were added at 4 °C pentafluorophenol (1.32 g, 7.18 mmol) and DCC (1.48 g, 7.17 mmol). After stirring for 45 min at room temperature, the insolubles were filtered off and rinsed with acetonitrile. The filtrates were evaporated, taken up in acetonitrile (50 mL) and treated at 4 °C with 33% aqueous ammonia (8 mL). After 20 min, the solution was evaporated at low bath temperature and the resulting residue purified by silica gel column chromatography (0–15% MeOH in CH_2Cl_2) to give **6** as a white foam (1.22 g, 78%). R_f (CH_2Cl_2/MeOH 90:10): 0.28. ^1H NMR (DMSO-d_6) δ: 2.12 (s, 3H, CH_3), 2.13 (s, 3H, CH_3), 2.38 (oct, 1H, H2″, J=2.7, 6.1, 14.1 Hz), 2.51 (m, 1H, H2′), 4.13–4.20 (m, 2H, H4′ and H5′), 4.26 (m, 1H, H5″), 5.28 (m, 1H, H3′), 5.90 (dd, 1H, H1′, J=6.2, 8.1 Hz), 7.19 (bs, 1H, NH_2), 7.39 (d, 1H, H5, J=1.9 Hz), 7.43 (bs, 1H, NH_2), 10.58 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.43 (CH_3), 21.62 (CH_3), 36.23 (C2′), 64.63 (C5′), 79.92 (C3′), 81.54 (C4′), 82.63 (C1′), 113.04 (C5), 118.59 (C4), 152.93 (C2), 160.93 ($CONH_2$), 170.87 ($COOCH_3$), 171.05 ($COOCH_3$). MS (ESI-TOF) *m/z* 350.1 $(M+Na)^+$, 366.1 $(M+K)^+$.

4.2.4. 1-(2-Deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxamide (3). To compound **6** (1.22 g, 3.74 mmol) in MeOH (85 mL) was added 33% aqueous ammonia (85 mL). After stirring for 20 min at room temperature, solvents were removed and the residue purified by silica gel column chromatography (0–25% MeOH in CH_2Cl_2). Compound **3** was obtained as a white powder (0.90 g, 95%). R_f (CH_2Cl_2/MeOH 90:10): 0.38. Mp 160–162 °C. R_t (0–20% A in B): 9.21 min. ^1H NMR (DMSO-d_6) δ: 2.06 (ddd, 1H, H2″, J=3.1, 6.2, 13.2 Hz), 2.15 (ddd, 1H, H2′, J=5.8, 7.9, 13.2 Hz), 3.47 (m, 2H, H5′ and H5″), 3.72 (m, 1H, H4′), 4.24 (m, 1H, H3′), 4.85 (t, 1H, 5′OH, J=5.6 Hz), 5.21 (d, 1H, 3′OH, J=4.3 Hz), 5.83 (dd, 1H, H1′, J=6.2, 7.8 Hz), 7.20 (bs, 1H, NH_2), 7.31 (s, 1H, H5), 7.35 (bs, 1H, NH_2), 10.43 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 39.22 (C2′), 62.45 (C5′), 71.17 (C3′), 81.87 (C1′), 87.31 (C4′), 112.89 (C5), 117.66 (C4), 152.50 (C2), 160.52 ($CONH_2$). MS (ESI-TOF) *m/z* 244.2 $(M+H)^+$, 266.1 (M+Na), 282.1 $M+K)^+$. HRMS (ESI-TOF) *m/z* calcd for $C_9H_{13}N_3O_5$+Na 266.0753, found 266.0738. Anal. calcd for $C_9H_{13}N_3O_5$+3/4H_2O: C, 42.11; H, 5.31; N, 16.37, found C, 42.46; H, 5.34; N, 16.24. UV (H_2O/pH 6.2) λ_{max} 263 nm (ε

8920); (H_2O–NaOH/pH 12.6) λ_{max} 283 nm (ε 7340). UV (H_2O/pH 7.0) λ 260 nm (ε 8720).

4.3. Synthesis of the phosphoramidite unit (11)

4.3.1. Methyl 1-(3,5-*O*-diacetyl-2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-*1H*-imidazole-4-carboxylate (7). To a stirred solution of compound **5** (1.80 g, 5.48 mmol) in anhydrous CH_2Cl_2 (20 mL) and Et_3N (1.46 mL, 8.22 mmol) was added slowly at 0 °C methyl chloroformate (0.64 mL, 8.23 mmol). After 1 h at room temperature, the anhydride intermediate was totally formed (as judged by TLC). DMAP (0.80 g, 6.58 mmol) was added and the stirring was maintained for 8 h. The reaction was quenched with MeOH (5 mL), evaporated and purified by silica gel column chromatography (0–10% MeOH in CH_2Cl_2) to give **7** as a white powder (1.41 g, 75%). R_f (CH_2Cl_2/MeOH 90:10): 0.60. ^{1}H NMR (DMSO-d_6) δ: 2.05 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 2.29 (m, 1H, H2′), 2.65 (m, 1H, H2″), 3.75 (s, 3H, OCH_3), 4.10–4.18 (m, 2H, H4′ and H5′), 4.22 (m, 1H, H5″), 5.20 (m, 1H, H3′), 5.87 (dd, 1H, H1′, J=6.0, 8.6 Hz), 7.60 (d, 1H, H5, J=1.7 Hz), 11.00 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.38 (CH_3), 21.63 (CH_3), 35.85 (C2′), 52.41 (OCH_3), 64.61 (C5′), 75.15 (C3′), 81.81 (C4′), 82.87 (C1′), 114.57 (C4), 118.27 (C5), 153.06 (C2), 160.15 (4-$COOCH_3$), 170.84 ($COOCH_3$), 170.98 ($COOCH_3$). MS (ESI-TOF) m/z 343.0 $(M+H)^+$, 365.1 $(M+Na)^+$, 381.1 $(M+K)^+$.

4.3.2. Methyl 1-(2-deoxy-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-1*H*-imidazole-4-carboxylate (8). To compound **7** (1.40 g, 4.09 mmol) in MeOH (40 mL) was added at 0 °C a 0.5 N solution of MeONa in MeOH (20 mL). After 30 min, the solution was neutralized by addition of Dowex H^+. The resin was filtered off, rinsed with MeOH and the filtrates evaporated. Compound **8** was isolated by chromatography on a silica gel column as a white foam (1.06 g, 98%). R_f (CH_2Cl_2/MeOH: 90:10): 0.18. ^{1}H NMR (DMSO-d_6) δ: 2.03 (m, 1H, H2′), 2.27 (m, 1H, H2″), 3.43–3.55 (m, 2H, H5′ and H5″), 3.74–3.76 (m, 4H, OCH_3 and H4′), 4.24 (m, 1H, H3′), 4.91 (t, 1H, 5′OH, J=5.6 Hz), 5.19 (d, 1H, 3′OH, J=4.1 Hz), 5.85 (dd, 1H, H1′, J=6.0, 8.2 Hz), 7.57 (s, 1H, H5), 10.91 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 39.95 (C2′), 52.32 (OCH_3), 62.61 (C5′), 71.73 (C3′), 82.81 (C1′), 88.10 (C4′), 114.12 (C4), 118.52 (C5), 153.04 (C2), 160.21 (4-$COOCH_3$). MS (ESI-TOF) m/z 259.1 $(M+H)^+$, 281.2 $(M+Na)^+$, 297.2 $(M+K)^+$. HRMS (ESI-TOF) m/z calcd for $C_{10}H_{14}N_2O_6$+Na 281.0750, found 281.0748.

4.3.3. Methyl 1-(2-deoxy-5-*O*-dimethoxytrityl-β-D-ribofuranosyl)-2-oxo-2,3-dihydro-*1H*-imidazole-4-carboxylate (9). Compound **8** (0.42 g, 1.63 mmol) was coevaporated with pyridine (3×5 mL) and resuspended in anhydrous pyridine (10 mL/mmol). To this was added DMTCl (1.15 equiv) and the resulting solution was allowed to stir at room temperature until the complete disappearance of the starting material as judged by TLC (less than 4 h). The reaction mixture was extracted with CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$, water, dried over Na_2SO_4, and evaporated. The crude product was purified by chromatography on silica gel to give **9** (0.71 g, 78%) as a pale yellow foam. R_f (dichloromethane/MeOH 90:10): 0.50. ^{1}H NMR (DMSO-d_6) δ: 2.10 (m, 1H, H2′), 2.36 (m, 1H, H2″), 3.05 (dd, 1H, H5′, J=3.5, 10.1 Hz), 3.16 (dd, 1H, H5″, J=6.0, 10.1 Hz), 3.68 (s, 3H, $COOCH_3$), 3.74 (2 s, 6H, OCH_3), 3.86 (m, 1H, H4′), 4.25 (m, 1H, H3′), 5.27 (d, 1H, 3′OH, J=4.5 Hz), 5.87 (t, 1H, H1′, J=6.9 Hz), 6.87 (m, 4H, H arom. DMT), 7.20–7.32 (m, 7H, H arom. DMT), 7.39 (m, 2H, H arom. DMT), 7.40 (d, 1H, H5), 10.95 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 37.30 (C2′), 49.85 (OCH_3), 53.35 (OCH_3–DMT), 53.45 (OCH_3–DMT), 62.67 (C5′), 69.13 (C3′), 80.16 (C1′), 83.76 (C4′), 83.97 (Cq DMT), 111.61 (CH DMT), 111.86 (C4), 115.78 (C5), 125.07 (Cq DMT), 126.13, 126.24, 128.12, 128.16 (CH DMT), 133.98, 134.07 and 143.36 (Cq DMT), 150.59 (C2), 156.49 and 156.51 (Cq DMT), 157.74 ($COOCH_3$). MS (ESI-TOF) m/z 259.1 $(M+H)^+$, 281.2 $(M+Na)^+$, 297.2 $(M+K)^+$.

4.3.4. Methyl 1-(2-deoxy-5-*O*-dimethoxytrityl-β-D-ribofuranosyl)-N^3-diphenylcarbamoyl-2-oxo-2,3-dihydro-*1H*-imidazole-4-carboxylate (10). Compound **9** (0.70 g, 1.25 mmol) was coevaporated with pyridine (3×5 mL) and resuspended in anhydrous pyridine (10 mL/mmol). To this solution wad added diphenylcarbamoyl chloride (1.2 equiv) and the resulting solution was allowed to stir at room temperature until the complete disappearance of the starting material as judged by TLC (less than 4 h). The reaction mixture was extracted with CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$, water, dried over Na_2SO_4, and evaporated. The crude product was purified by chromatography on silica gel to afford the title compound **10** as a foam (0.61 g, 65% yield). ^{1}H NMR (DMSO-d_6) δ: 2.30 (m, 1H, H2′), 2.41 (m, 1H, H2″), 3.10 (m, 1H, H5′), 3.16 (m, 1H, H5″), 3.68 (s, 3H, $COOCH_3$), 3.72 (s, 6H, OCH_3), 3.97 (m, 1H, H4′), 4.33 (m, 1H, H3′), 5.41 (d, 1H, 3′-OH, J=4.6 Hz), 5.99 (t, 1H, H1′, J=6.3 Hz), 6.84 (m, 4H, H arom. DMT), 7.17–7.29 (m, 7H, H arom. DMT), 7.36 (m, 4H, H arom. DMT and DPC), 7.38–7.55 (m, 8H, H arom. DPC), 7.86 (s, 1H, H5). ^{13}C NMR (DMSO-d_6) δ: 40.69 (C2′), 51.49 ($COOCH_3$), 55.34 (OCH_3), 64.02 (C5′), 70.39 (C3′), 84.35 (C1′), 85.93 (Cq DMT), 86.37 (C4′), 113.50 (CH DMT), 122.20 (C5), 126.96 (Cq DMT), 128.00 and 128.15 (CH DMT), 128.26 (C4), 129.77 (CH DPC), 130.00 and 130.07 (CH DMT), 135.80 and 135.82 (Cq DMT), 143.57 (C2), 145.11 (Cq DMT), 150.39 (CO DPC), 158.37 and 158.40 (Cq DMT), 162.28 ($COOCH_3$). HRMS (MALDI-TOF) m/z calcd for $C_{44}H_{41}N_3O_9$+Na 778.2740, found 778.2761.

4.3.5. Phosphoramidite building block (11). To a solution of compound **10** (0.29 g, 0.39 mmol) and DIEA (0.27 mL, 1.56 mmol) in CH_2Cl_2 (2 mL) was added 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.1 mL, 0.43 mmol). After stirring for 1 h at room temperature, the reaction mixture was diluted in CH_2Cl_2 (3 mL) and washed in turn with 10% sodium carbonate (2×2 mL). The organic layer was dried over Na_2SO_4, concentrated and purified by silica gel column chromatography (AcOEt/Et_3N/CH_2Cl_2 2:1:17). Compound **11** was isolated after precipitation by CH_2Cl_2/hexane (2:30 mL) at −78 °C as a powder (0.28 g, 75%). ^{1}H NMR ($CDCl_3$) δ: 1.03–1.13 (7s, 12H, CH–CH_3), 2.24–2.32 (m, 1H, H2′), 2.36 (m, 1H, CH_2CN), 2.40–2.56 (m, 2H, H2′ and CH_2CN), 3.16–3.26 (m, 2H, H5′ and H5″), 3.48–3.67 (m, 4H, *CH*–CH_3 and OCH_2), 3.71 (2s, 9H, $COOCH_3$ and OCH_3), 4.16 (m, 1H, H4′), 4.51 (m, 1H, H3′),

5.84 (m, 1H, H1′), 6.72–6.78 (m, 4H, H arom. DMT), 7.13–7.35 (m, 19H, H arom. DMT and DPC), 7.57 and 7.59 (each s, 1H, H5). ^{31}P NMR ($CDCl_3$) δ: 150.17 and 150.58. MS (ESI-TOF) *m/z* 957.4 $(M+H)^+$, 978.6 $(M+Na)^+$, 994.8 $(M+K)^+$. HRMS (MALDI-TOF) *m/z* calcd for $C_{53}H_{58}N_5O_{10}P+Na$ 978.3819, found 978.3800.

4.4. Oligonucleotide syntheses

Oligonucleotides were synthesized on an Expedite Millipore DNA synthesizer according to standard β-cyanoethyl phosphoramidite chemistry on 1 μmol scale trityl-on mode using a 0.15 M solution of **11** in CH_3CN with a lengthened coupling time (10 min). Conversion of ester into amide was achieved during the standard deprotection conditions used (33% aqueous ammonia solution at 55 °C for 8 h). The synthesized oligonucleotides were separated from failure sequences at DMT stage by preparative HPLC on a Perkin Elmer system with a reverse phase column (C18) using a flow rate of 5.5 mL/min and a linear gradient of CH_3CN (A) in 10 mM triethylammonium acetate buffer (B) at pH 7.5 over 20 min. The corresponding fractions (detection at 254 nm) were concentrated and treated with 80% acetic acid for 15 min at room temperature. The detritylated oligonucleotides were further purified by reverse phase HPLC. Retention times after purification were determined on a C18 column using a 5–25% linear gradient of CH_3CN (A) in 10 mM triethylammonium acetate buffer (B) at pH 7.5 over 20 min. Oligomers were quantified by UV absorption at 260 nm. The molar extinction coefficient of oligonucleotides containing 2-oxodY were approximated by calculating the molar extinction coefficients of oligomers according to the nearest neighbor method where the analog was replaced by thymidine, since their ε were very similar: $\varepsilon_{260}=8400$ (pH 7.0) for **3** and $\varepsilon_{260}=8700$ (pH 7.0) for d*T*. Intact sequences of the four synthesized oligomers were confirmed by HRMS (MALDI-TOF) analysis. 5′-ACTTGGCC**Y^{oxo}**C CATTTTG-3′ (44% yield) $R_t=8.64$ min, *m/z* calcd for $C_{164}H_{211}N_{55}O_{105}P_{16}+Na$ 5124.8586, found 5124.8692. 5′-CAAAATGG**Y^{oxo}**GGCCAAGT-3′ (40% yield) $R_t=7.85$ min, *m/z* calcd for $C_{166}H_{207}N_{71}O_{97}P_{16}+Na$ 5240.9171, found 5240.9126. 5′-GCAT**Y^{oxo}**GTCA TAGCTGTTTCCTG-3′ (39% yield) $R_t=8.29$ min, *m/z* calcd for $C_{214}H_{273}N_{74}O_{136}P_{21}+Na$ 6704.1133, found 6704.1315. 5′-TGAC**Y^{oxo}**GTCATAGCTGTTTCCTG-3′ (39% yield) $R_t=8.09$ min, *m/z* calcd for $C_{214}H_{273}N_{74}O_{136}P_{21}+Na$ 6704.1133, found 6704.1395.

4.5. p*K*a Determination

Solutions of nucleoside **3** completely exchanged by deuterium were prepared in D_2O at 10 mM concentration. The pD of the solution has been adjusted by addition of microliter volumes of NaOD solutions (0.5, 0.1 and 0.01 M) and the pH value measured with a pH meter calibrated with standard buffers in H_2O. ^{1}H and ^{13}C spectra at different pH were recorded at 298 K using 64 scans on a Bruker Avance 400 spectrometer. pH-titration study consists of 12–15 data points over the range $6.5<pH<12.6$. The p*K* value so obtained in D_2O was converted to value in H_2O by subtracting 0.2 unit to allow the isotopic effect.

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