Double-Headed Nucleotides with Non-Native Nucleobases: Synthesis and Duplex Studies


Abstract: Double-headed nucleotides are DNA building blocks that store, in principle, twice as much information as native nucleotides due to the incorporation of an additional functional nucleobase. Herein, we present the development of two new double-headed nucleotides, U_2 and U_4, featuring a methylene-linked 5-aza-7-deazaxanthine (Z) and a propynyl-linked pseudouracil (W), respectively, attached to the 2'-position of arabinouridine. These analogues are evaluated in DNA duplexes for their ability to act as dinucleotides, and the base-pairing specificities of the 2'-nucleotides are compared to previous analogues. Although an improved discrimination was observed in the context of a cytosine mismatch using U_2, neither of the two new analogues gave rise to increased overall base-pairing fidelity possibly due to the formation of stable wobble pairs. Furthermore, we present an improved synthetic strategy for the preparation of the corresponding dianaminopurine analogue (U_2), in which the key fluoro-amine substitution is achieved post-synthetically to avoid the use of doubly protected dianaminopurines and complicated deprotections.

Introduction

The native nucleic acids form highly ordered structures in a predictable fashion, and yet, are composed of just a few simple monomeric building blocks, i.e. the nucleotides. Through chemical modification of these building blocks, the properties of nucleic acids have been fine-tuned for a wide selection of potential applications. This includes the use of nucleic acids as therapeutics in the form of antisense oligonucleotides, siRNAs and aptamers,[1-3] as well as within nanotechnology, where artificial genetic polymers termed xeno nucleic acids (XNAs) and systems with non-natural base pairs have been developed.[4-6] One type of modification involves the attachment of an additional nucleobase to the nucleotide scaffold, hence the concept of double-headed nucleotides.

In recent years, several double-headed nucleoside designs have been presented in the literature.[7-19] Our previous work has shown that depending on the site of attachment, the additional nucleobase can be positioned in the minor groove,[14-17] the major groove,[18-20] or the center of a DNA duplex.[21-23] In the latter case, given that the additional nucleobase is linked through a methylene to the 2'-position of 2'-deoxyuridine or arabinouridine (Fig. 1, U_x), the double-headed nucleotide has been shown to behave as a condensed dinucleotide. In practice, it can form two sets of Watson–Crick base pairs with two individual nucleotides of the opposite strand: one through its 1' base to give a 1'-1' base pair, and another through its 2' base to give a 2'-1' base pair.[24-26] This feature allows for the condensation of genetic information to smaller entities with fewer phosphates, which might be an effective approach for e.g. enhancing cellular uptake or target recognition in future nucleic acid-based therapeutics.

Currently, the full set of uridine-based analogues (U_c, U_o, U_r, U_A) have been presented and evaluated together with selected cytosine- and adenosine-based analogues in the form of C_c and A_r.[23-27]
These double-headed nucleotides are well-accommodated in DNA duplexes when incorporated in exchange for the corresponding dinucleotide (Fig. 1). For instance, when central 5'-UC or 5'-UG dinucleotides are replaced with the Uo or Us double-headed nucleotide monomers, respectively, the thermal duplex stability remains intact (Fig. 1, green bars).25 The 2'-bases of Uc and Ue also display a good mismatch discrimination comparable to that of mismatches involving natural nucleotides (Fig. 1, white bars). When Ur and Us monomers are incorporated, the duplex is slightly destabilized by 2.5 °C to 3.5 °C.25,27 While the 2'-1' mismatch discrimination capacity of Ur and Us against T, A or C also resemble those of the unmodified duplexes, the discrimination against G is insufficient (1.0–1.5 °C). Therefore, Ur and Us analogues with increased 2'-specificity are of interest.

One attempt has been the use of 2,6-diaminopurine (D) as an adenine analogue (Us, Fig. 1), due to its additional hydrogen bond donor that can exploit the unused hydrogen bond acceptor of thymine in the form of the 2-carbonyl.29 The Us monomer successfully functions as an improved Us analogue with increased discrimination of guanine (6.5 °C compared to 1.0 °C), a slightly higher thermal stability in the matched duplex (49.5 °C compared to 48.5 °C) and hereby high specificity for thymine. Its synthesis, on the other hand, currently requires an inconvenient 14-day treatment with methanolic ammonia to ensure complete removal of both benzoyl protecting groups of the 2,6-diaminopurine moiety after DNA synthesis.25,26 To improve this, we here implement a method developed by Manoharan and co-workers for the preparation of oligonucleotides containing 2-aminoadenosine monomers.29 With the use of 2'-fluoroadenine instead of 2,6-diaminopurine, the need for protecting groups prior to DNA synthesis is reduced. The 2'-fluoro group is then substituted with an amino group at the oligonucleotide level by treatment with methanolic ammonia to reveal the final diamino purine moiety.

Hypoxanthine (H) has been previously introduced to the 2'-position of double-headed nucleotides to give Uh (Fig. 1), and whereas hypoxanthine in the natural 1'-position represents a guanine analogue, it binds preferentially to adenine (48.0 °C) in the 2'-1' setting.28 This observation demonstrates that double-headed nucleotides can accommodate 2'-1' purine-purine base pairs, which is also evident from the relatively high stability of the 2'-1' A-G mismatch (Fig. 1). Uh and Ur showed similar recognition of a cognate A opposite the 2'-base, and the discrimination of G was improved by 3.5 °C with Uh. Nevertheless, C was found to be only marginally discriminated (1.0 °C) when Uh is used (Fig. 1).29

In this report, we present the synthesis and evaluation of a new double-headed nucleotide monomer (Us) containing 5-aza-7-deazaxanthine (Z) in the 2'-position. This purine nucleobase has been introduced into natural 2'-deoxynucleotides by Dea and co-workers,38 and later explored by the Benner group aiming for alternative DNA base pairs.31-33 With its additional hydrogen bond acceptor in the form of a carbonyl in the 2'-position compared to hypoxanthine, we expect it to be able to discriminate C more effectively while retaining the high affinity towards A (Fig. 2), however, with a risk for stable wobble pairs.

An analogue with pseudouracil connected to the 2'-position through a propynyl linker (Uw) is also presented (Fig. 1). Artificial genetic systems have previously been introduced where modified nucleobases were attached to the 1'-position of a 2'-deoxyribose through an ethynyl linker.33,34 Altogether, Uh and Uw represent two double-headed nucleoside monomers each exposing a thymine Watson–Crick face differently within the duplex core compared to that of the original Ur monomer. Based on the apparently larger space in the 2'-1' setting allowing for instance purine-purine pairs, we hereby hope to find an alternative to Ur that allows stronger binding and/or more specific base pairing.

**Figure 2.** Suggested base pairs with the non-native nucleobases H and Z.

### Results and Discussion

#### Synthesis

The modified oligonucleotides were prepared using standard solid-phase DNA synthesis with commercial and modified phosphoramidite building blocks, and the syntheses of the latter are outlined in Scheme 1. For all three double-headed nucleotides, the synthesis started from the known 2'-ketone 1, which is prepared from uridine through an efficient two-step chromatography-free procedure.33

The synthesis of Us followed the same overall methodology as for previous Ur monomers, where ketone 1 was converted to the 2'-spiroepoxide 2 in a stereospecific manner via a Corey–Chaykovsky epoxidation.23,36 This epoxide was opened by deprotonated 2-fluoro adenine to give 3. M9-alkylation was confirmed by a 3JCH-coupling between C4' of the 2'-fluoro adenine moiety and the H6' protons in the HMBC spectrum. The silyl protecting group was removed using triethylamine trihydrofluoride to give the naked nucleoside 4. It was envisioned that the 2-fluoro group would be sufficiently electronegative to deactivate the 6-amino group of adenine, so that protection of the latter before DNA synthesis would be unnecessary.33,39 and therefore 4 was tritylated with DMTiCl to give 5. However, phosphorylation of 5 with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite proved infeasible. Instead, 4 was first treated with dimethylformamide dimethyloxalyl to install a 6-N-amidine protecting group followed by tritylation with DMTiCl to give 6. Phosphitylation of 6 then proceeded successfully to give the fully protected phosphoramidite 7. After incorporation into oligonucleotides, treatment with methanolic ammonia ensured replacement of the 2-fluoro group by an amino group (vide infra).

In the synthesis of Us, epoxide 2 was instead opened regioselectively by deprotonated 2-aminoimidazole to give nucleoside 8, for which N1-alkylation was confirmed by 1H NMR as well as by a 3JCH-coupling between C5' of the 2-aminoimidazole moiety and the H6' protons in the HMBC spectrum. The xanthine-derived nucleobase in 9 was then built through a cyclization of 8 with a yield of 55% using freshly distilled phenyl isocyanatoformate, which was prepared from phenyl carbamate according to the procedure from Lavigne and co-workers.37 The silyl protecting group of 9 was removed by treatment with triethylamine trihydrofluoride to give the corresponding naked nucleoside 10. Finally,
tritylation and phosphitylation of 10 was performed following standard procedures to give phosphoramidite 12.

For the preparation of Uw, ketone 1 was treated with propargyl bromide under Barbier conditions to give 13 in a stereoselective fashion and with a yield of 88% by adjusting the protocol from Gonnade and co-workers,[38] who reported the equivalent reaction with a 3-ulos derivative as substrate. The 2’-stereocenter of 13 was confirmed by the presence of dipolar couplings between the H6’ protons and both H1’ and H4’ in the NOESY spectrum. Through a Sonogashira reaction, 13 was converted to the double-headed nucleoside 14 using 3-benzoyl-5-iodouracil, which was prepared from 5-iodouracil using the procedure from Montagu and co-workers.[39] The presence of a benzoyl protecting group was found to increase the yield of the cross-coupling notably from 17% to 56% for this specific reaction. Finally, the silyl protecting group was removed by treatment with triethylamine to give phosphoramidite 15 followed by standard tritylation to give 16 in an overall yield of 62%. Finally, standard phosphitylation afforded phosphoramidite 17.

All three modified phosphoramidites were incorporated into oligonucleotides using standard solid-phase DNA synthesis with 4,5-dicyanoimidazole as activator and with extended coupling times (15 min). Cleavage from the solid support and removal of the protecting groups were facilitated by treatment with aqueous ammonia at rt for 24 h (Uz, Uw) or methanolic ammonia at 65 °C for 44 h (U0). The crude oligonucleotides were purified using reversed-phase HPLC and ion-exchange chromatography, and the identity of the pure oligonucleotides were confirmed by MALDI-TOF mass spectrometry. In addition, HPLC profiles were used to characterize the U0 containing oligonucleotide, due to the very small difference of ~3 m/z units in the monoisotopic masses of the oligonucleotides that contain either a fluoro group or the desired amino group. In practice, the HPLC profile of the newly synthesized oligonucleotide and that of a reference oligonucleotide with the same sequence that was synthesized using the original protocol[25] were obtained and found to be identical by co-injection (see supporting information, Figs. S1–S3). Two separate peaks were observed when comparing the crude HPLC profiles obtained after treatment with aqueous ammonia at rt (24 h) and methanolic ammonia at 65 °C (44 h), respectively, demonstrating that the HPLC program is in fact capable of separating the fluoro and the amino analogues (see supporting information, Figs. S4 and S5). Finally, this 13-mer sequence containing U0 was hybridized to its 14-mer complementary strand and the melting temperature was determined. A clear correlation was observed between the melting temperature (50.0 °C) and that of the reference duplex (49.5 °C).[26] Altogether, we are confident that the fluoro-to-amino substitution has occurred.

Scheme 1. Reagents and conditions: (a) NaH, (CH3)2SO, THF, DMSO, 0 °C, 84%; (b) NaHMDS, 2-fluoroadenine, DMF, rt, 51%; (c) Et3N-3HF, THF, rt, 463%; 10 82%; (d) DMTrCl, 2,6-lutidine, DMSO, rt, 5 55%, 11 27%, 16 62% (two steps); (e) i. dimethylformamide dimethylacetal, DMF, rt; ii. DMTrCl, 2,6-lutidine, DMSO, rt, 51% (two steps from 4); f) 2-cyanoethyl N,N-disopropylchlorophosphoramidite, (i-Pr)2NEt, CH2Cl2, rt, 7 46%, 12 64%, 17 65%; (g) NaHMDS, 2-aminoimidazole, DMF, THF, 50 °C, 47%; (h) PhOC(O)O)NCO, DMF, 50 °C, 55%; (i) propargyl bromide, Zn, THF, rt, 88%; (j) 3-benzoyl-5-iodouracil, Pd(PPh3)4, Cul, DMF, Et3N, rt, 56%; (k) incorporation into DNA oligonucleotides. Deprotection conditions: 7 N methanolic ammonia, 44 h, 65 °C (U0) or 28% aq. NH3, 24 h, rt (Uz, Uw). TIPDS = tetraisopropylsiloxan-1,3-diyldimethylglycol.
Hybridization studies

The two new double-headed nucleotides, Uz and Uw, were each incorporated centrally into the previously studied[24-25] mixed 14-mer DNA strand (Fig. 1) in exchange for the corresponding 5′-UT dinucleotide (Fig. 3). The oligonucleotides were then hybridized to their unmodified complements in a medium salt buffer and the melting temperatures (Tm) were determined from the UV melting curve (A260 nm vs temperature) and compared to unmodified references. In order to evaluate the dinucleotide behaviour of Uz and Uw, as well as the base-pairing properties of the two modified 2′-bases, the melting temperature of both the fully matched duplexes and the 2′-base mispaired duplexes were recorded and compared to duplexes containing the UT and UW monomers in the same position (Fig. 3).

With the introduction of the Uz monomer in the DNA duplex, the melting temperature was unexpectedly decreased by 2.0 °C to 46.0 °C compared to UT. On the other hand, cytosine was hypothesized well-discriminated giving a melting temperature of 41.5 °C for the duplex with ZC. However, ZG and ZT mismatches were no longer discriminated (Tm = 0.0-1.0 °C) compared to UT (Tm = 5.0 °C). These results show that the introduction of the additional 2-carbonyl on the purine base interrupts effective Watson–Crick base-pairing with an opposite cytosine as proposed (Fig. 2), however, we also realize that other base-pairing motifs are introduced. Specifically, it seems that the flexible nature of the double-headed nucleotide’s 2′-base allows the formation of stable 2′-1′ wobble base pairs. In a direct comparison of Uz and UT, it is apparent that H cannot form any stable wobble pairs with G and only a type 1 wobble pair with T, whereas Z can form a type 2 wobble pair with G and both wobble pair types with T (Fig. 3). In our previous work, we have established that base pairs exploiting only one hydrogen bond are thermodynamically unlikely to exist.[26] The proposed wobble base pairs explain the observed increase in thermal stability for G and T mismatches when comparing Uz to UT. Apparently, 2′-1′ purine-purine base pairs permit the formation of several base-pairing modes, which makes the development of a purine-scaffolded thymine analogue more challenging than first anticipated.

Next, we introduced the Uw monomer in the same position of the duplex and observed a Tm of 44.0 °C, which corresponds to a ΔTm of -6.5 °C compared to the unmodified reference duplex and a -4.0 °C higher destabilization than observed for the UT monomer.[28] However, this is not as pronounced as for previous 2′-ethyl (ΔTm = -10.0 °C)[40] or 2′-methyltriazolyl (ΔTm = -10.5 °C)[16] linked 2′-deoxy Ur analogues in the same duplex indicating a geometry advantage combined with some stacking stabilization from the extended π-system of the 5-ethynyluracil. Nevertheless, the three mismatched duplexes of Uw varied within ±0.5 °C from the matched duplex, demonstrating that the base-pairing specificity of the 2′-base has been significantly compromised. The melting temperatures are generally higher than those of well-discriminated mismatch duplexes, as a likely result of various weak and non-specific hydrogen bonds. It follows that the spatial organization of the nucleobase within the DNA core when using the propynyl linker is inferior compared to when the methylene linker is used.

Altogether, the introduction of Uz successfully solves the issue of low discrimination of the 2′-base towards an opposite 1′-cytosine, however, at the same time it presents new specificity challenges in the recognition of guanine and thymine. Uw, on the other hand, displays a complete loss in specificity declaring the propynyl linker unfit for the purpose. In future designs, non-extended thymine analogues such as e.g. 5-chlorouracil could be explored in the search for a thymine analogue that displays sufficient mismatch discrimination, as it has been shown to stabilize a 12-mer DNA duplex slightly (+0.4 °C), while also discriminating an opposite guanine more effectively (~1.1 °C) compared to a thymine in the 1′-position.[41] Overall, the double-headed nucleotides have proven to be a robust concept capable of forming stable DNA duplexes through predictable 2′-1′ and 2′-2′ base pairs. With the use of the now optimized preparation of Uz as a replacement of UT, the remaining building block where there is a need for further optimization of its base pairing specificity.

Conclusion

Two new double-headed nucleotides, Uz and Uw, containing a methylene-linked 5-aza-7-deazaxanthine and a propynyl-linked pseudouracil in the 2′-position were successfully constructed through cyclization of a 2-aminoimidazole moiety with phenyl isocyanatofomate or via a Sonogashira cross-coupling, respectively. They were then incorporated into oligonucleotides
for evaluation, and, as anticipated, U₂ was found to display increased mismatch discrimination of cytosine compared to the former U₉ analogue bearing hypoxanthine in the 2′-position. However, the stability of the matched 5′-U₂-3′-AA duplex was lower than expected, and the U₂ monomer also formed stable (probably wobble) pairs with guanine and thymine, which compromised the general base-pairing specificity of this monomer. In general, it seems that the flexibility of the nucleobases in the 2′-position permits the formation of stable Watson–Crick base pairs, yet it also enables several other competing modes of binding. For the U₉ monomer, a complete lack of specificity was observed. Also, the synthetic procedure for the known modified double-headed nucleotide, U₉, was improved. This was done using a 2′-fluoroadenine strategy. Altogether, the results presented here further expands the field of double-headed nucleotides by showing that modified analogues are readily accessible, yet a thymine analogue with full specificity still remains a challenge.

**Experimental Section**

All commercial reagents were used as supplied except CH₃OH, which was distilled prior to use. Anhydrous solvents were dried over 3 Å (CH₃CN) or 4 Å (CH₃CO₂H, DCE, DMSO, DMF, Et₂N, 2,6-lutidine, petroleum ether, pyridine, THF) activated molecular sieves. Reactions were carried out under argon or nitrogen whenever anhydrous solvents were used. All reactions were monitored using TLC analysis with Merck silica gel plates (60 F₂₅₄). For visualisation, the TLC plates were exposed to UV light (254 nm) and/or immersed in a solution of 5% H₂SO₄ in methanol (v/v) followed by charring. Column chromatography was performed with Silica Gel 60 (particle size 0.040–0.063 μm, Merck). ¹H NMR, ¹³C NMR, ³¹P NMR and ¹⁹F NMR spectra were recorded at ambient temperature on a Bruker Avance III 400 instrument. Chemical shift values (δ) are reported in ppm relative to either tetramethylsilane (δMe: 0.0) or the deuterated solvents (CDCl₃: δC: 7.26, δC: 77.16; DMSO-d₆: δC: 2.50, δC: 39.52) and relative to 85% H₃PO₄ as external standard for ³¹P NMR. Assignment of NMR signals is based on 2D spectra and follow standard nucleoside convention. Signals assigned with a double prime belong to the nucleobase in the 2′-position. High resolution mass spectra were recorded on a Bruker microTOF-Q II (ESI) quadrupole time of flight instrument in positive ion mode.

**1-(2′-C-(2-Fluoroadenin-9-yl)methyl)-5′-O-(tetraisopropyl-disoxalane-1,3-diyil)-β-D-arabinofuranosyl)uracil (3):** NaHDMDS (1.0 M in THF, 1.82 mL, 1.82 mmol) was added to a solution of 2-fluoroadenine (0.28 g, 1.82 mmol) in anhydrous DMF (5 mL). The mixture was stirred under nitrogen for 30 min at rt, after which epoxide 2 (0.70 g, 1.40 mmol) was added. After stirring for 24 h at rt, the solvent was removed under reduced pressure and the residue was recrystallized from MeOH to give 3 (0.47 g, 0.72 mmol, 51%) as white crystals. ¹H NMR (400 MHz, DMSO-d₆) δ 11.26 (s, 1H, NH), 8.06 (s, 1H, H₈), 7.71 (br, 2H, NH₂), 7.50 (d, J = 8.2 Hz, 1H, H₅), 6.14 (s, 1H, 2′OH), 5.98 (s, 1H, H₁′), 5.53 (d, J = 8.2 Hz, 1H, H₅), 4.52 (d, J = 15.0 Hz, 1H, H₆′), 4.22 (d, J = 8.8 Hz, 1H, H₃), 4.12–4.06 (m, 1H, H₅S), 3.97–3.90 (m, 2H, H₄′, H₆′), 1.15–0.77 (m, 28H, TIPDS); ¹³C NMR (101 MHz, DMSO-d₆) δ 162.9 (C₄), 158.4 (d, δC = 203.4 Hz, C₂), 157.4 (d, δC = 21.2 Hz, C₆), 151.6 (d, δC = 19.9 Hz, C₄), 150.1 (C₂), 141.9 (d, δC = 2.0 Hz, C₇), 140.5 (C₆), 116.6 (d, δC = 3.7 Hz, C₅), 100.8 (C₅), 84.6 (C₁), 79.5 (C₄), 78.9 (C₂), 75.5 (C₃), 60.4 (C₅), 45.7 (C₆), 17.2, 17.1, 17.0, 16.7, 16.6, 16.6 (TIPDS CH₃), 12.7, 12.3, 12.3, 11.8 (TIPDS CH₃); ¹⁹F NMR (376 MHz, DMSO-d₆) δ −52.8; HRMS-ESI m/z 652.2674 [M + H]+; calcd (C₇₃H₇₅F₉N₉O₉) 652.2741.

**1-(2′-C-(2-Fluoro-6-N-(dimethylaminomethylene)adenin-9-yl)-methyl)-5′-O-(4,4′-dimethoxythiophenyl)-β-D-arabinofuranosyl)uracil (5):** Nucleoside 4 (180 mg, 0.44 mmol) was co-evaporated with anhydrous pyridine (2 × 5 mL) and dissolved in a mixture of anhydrous DMF (5 mL) and anhydrous 2,6-lutidine (0.15 mL). DMTrCl (0.30 g, 0.88 mmol) was added and the mixture was stirred under nitrogen for 24 h at rt. Water (10 mL) was added and the mixture was extracted with EtOAc (3 × 15 mL). The combined organic phase was dried (MgSO₄) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0–10% MeOH in CH₂Cl₂, v/v) to give 5 (206 mg, 0.24 mmol, 55%) as a foam containing 1.7 eq. DMSO. ¹H NMR (400 MHz, DMSO-d₆) δ 1.130 (d, J = 2.0 Hz, 1H, NH), 7.97 (s, 1H, H₈), 7.79 (br s, 2H, NH₂), 7.43 (d, J = 8.2 Hz, 1H, H₆), 7.43–7.36 (m, 2H, DMTr), 7.32 (t, J = 7.6 Hz, 2H, DMTr), 7.29–7.19 (m, 5H, DMTr), 6.90 (d, J = 9.0 Hz, 4H, DMTr), 6.18 (d, J = 5.1 Hz, 1H, 3'OH), 6.06 (s, 1H, H₁), 5.77 (s, 1H, 2′OH), 5.42 (d, J = 8.2, 2.0 Hz, 1H, H₅), 4.35 (d, J = 14.8 Hz, 1H, H₆'), 4.17 (d, J = 14.8 Hz, 1H, H₇'), 4.14–4.06 (m, 1H, H₄), 3.02 (dd, J = 4.8, 4.0 Hz, 1H, H₇), 3.74 (s, 3H, OCH₃), 3.74 (s, 3H, OCH₃), 3.34–3.27 (m, 1H, H₅S), 3.21 (dd, J = 10.3, 2.9 Hz, 1H, H₆'), 151.2 (d, δC = 19.3 Hz, C₄), 150.4 (C₂), 144.6 (DMTr), 142.0 (d, δC = 1.9 Hz, C₈), 141.7 (C₁), 135.4, 135.3, 129.7, 129.6, 127.8, 127.6, 126.7, 126.3 (DMTr), 116.7 (d, δC = 3.9 Hz, C₅), 113.1 (DMTr), 100.4 (C₅), 85.4 (C₁), 83.5 (C₄), 80.2 (C₂), 75.8 (C₃), 63.6 (C₅), 55.0 (OCH₃), 44.6 (C₆); ¹⁹F NMR (376 MHz, DMSO-d₆) δ −52.7; HRMS-ESI m/z 734.2273 [M + Na]+; calcd (C₇₃H₇₇F₉N₉O₉Na) 734.2345.

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1-2'-C-(5-Aza-7-deazaxanthin-9-yl)methyl-3',5'-O-(tetraisopropyl disiloxane-1,3-diyl)-beta-D-arabinofuranosyl]uracil (11): Nucleoside 10 (100 mg, 0.24 mmol) was co-evaporated with anhydrous pyridine (2 x 5 mL) and then re-dissolved in anhydrous DMSO (4 mL). 2.6-Lutidine (0.15 mL, 1.29 mmol) was added followed by DMTQ (137 mg, 0.40 mmol) and the mixture was stirred at rt for 18 h. The mixture was concentrated under reduced pressure, co-evaporated with anhydrous pyridine (2 x 5 mL) and the residue was then dissolved in anhydrous pyridine (5 mL). A second portion of DMTQ (130 mg, 0.39 mmol) was added together with DMAP (9 mg, 73 µmol). The mixture was stirred for 22 h at rt, after which few drops of 99.9% EtOH was added. The solvent was removed under vacuum, and the residue was redissolved in EtOAc (15 mL) and washed with water (20 mL). The aqueous phase was extracted with EtOAc (3 x 15 mL), and the combined organic phase was dried (Na2SO4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0–5% MeOH in CH2Cl2, v/v) to give the product 11 (56 mg, 64 µmol, 27%) containing 1.6 eq. EtN. 1H NMR (400 MHz, DMSO-d6) δ 11.28 (br s, 2H, 2 x NH), 7.44–7.38 (m, 3H, H6, DMTr), 7.36–7.30 (m, 3H, H7, DMTr), 7.28–7.21 (m, 2H, DMTr), 7.16 (d, J = 2.8, 1H, H8), 6.90 (d, J = 9.0 Hz, 4H, DMTr), 6.37 (br s, 1H, 1’OH), 6.01 (s, 1H, H7), 5.89 (s, 1H, 2’OH), 5.42 (d, J = 8.1, 1H, H5), 4.09–4.03 (m, 3H, H4, H6a, H6b), 3.86 (br s, 1H, H3), 3.74 (s, 3H, OCH3), 3.32 (d, J = 10.3, 7.0 Hz, 2H, H5), 3.23 (dd, J = 10.3, 2.7 Hz, 1H, H5); 13C NMR (101 MHz, DMSO-d6) δ 162.2 (C4), 158.1 (154.5) (DMTr), 154.5 (C2), 150.9 (C4), 150.5 (C2), 145.7 (C6), 144.7 (DMTr), 141.7 (C7), 135.5, 135.3, 129.7, 127.9, 127.7, 126.7 (DMTr), 120.7 (C8), 113.2 (DMTr), 106.9 (C7), 100.5 (C5), 85.6 (DMTr), 85.3 (C1), 82.7 (C4), 80.7 (C2), 75.9 (C3), 63.6 (C5), 55.0 (OCH3), 46.1 (C6); HRMS-ESI m/z 733.2211 [M + Na]+; calcd (C43H36N10O8Na) 733.2229.

1-2'-C-(5-Aza-7-deazaxanthin-9-yl)methyl-3',5'-O-(tetraisopropyl disiloxane-1,3-diyl)-beta-D-arabinofuranosyl]uracil (12): Nucleoside 11 (56 mg, 79 µmol) was co-evaporated with anhydrous DCE (2 mL), anhydrous pyridine (2 mL) and again anhydrous DCE (2 mL) and then re-dissolved in anhydrous DCE (1.5 mL). DPEA (0.06 mL, 0.34 mmol) was added followed by N,N-dimethylchlorophosphoramide (0.05 mm, 0.24 µmol), and the mixture was stirred for 2 h at rt, and then the reaction mixture was purified directly using silica gel column chromatography (0–4% MeOH in CH2Cl2, v/v) to give the phosphoramidite 12 (46 mg, 50 µmol, 64%). 13P NMR (162 MHz, CDCl3) δ 152.5, 150.8; HRMS-ESI m/z 1012.4715 [M + EtNH]+; calcd (C43H36N10O8) 1012.4692.

1-2'-C-(5-Aza-7-deazaxanthin-9-yl)methyl-3',5'-O-(tetraisopropyl disiloxane-1,3-diyl)2'-C-(prop-2-yn-1-yl)-beta-D-arabinofuranosyl]uracil (13): The ketone 1 (2.0 g, 4.2 mmol)
was dissolved in anhydrous THF (10 mL) and added to a degassed mixture of zinc (2.28 g, 35.6 mmol) and propargyl bromide (1.22 mL, 12.4 mmol) in anhydrous THF (15 mL). The mixture was stirred under nitrogen for 40 h at rt, and then a saturated aqueous solution of NH4Cl (12 mL) was added.

The mixture was filtered through celite and concentrated under reduced pressure. The residue was redissolved in CH2Cl2 (25 mL) and washed with water (2 × 50 mL) and brine (50 mL). The combined aqueous phase was extracted with CH2Cl2 (2 × 25 mL) and the combined organic phase was dried (MgSO4) and concentrated under reduced pressure.

The residue was purified by silica gel column chromatography (0–6% MeOH in CH2Cl2, v/v) to give 13 (1.93 g, 3.68 mmol, 88%) as a white crystalline foam. 1H NMR (400 MHz, CDCl3) δ 8.60 – 8.56 (m, 1H, H6), 5.70 – 5.67 (m, 1H, H5), 4.28 (d, J = 8.6 Hz, 1H, H3), 4.12 (d, J = 13.2, 2.8 Hz, 1H, H5'), 4.00 (d, J = 12.8, 2.8 Hz, 1H, H5''), 3.87 (dt, J = 8.6, 2.8 Hz, 1H, H4'), 3.14 (br s, 1H, 2CH2), 2.92 (dd, J = 17.0, 2.8 Hz, 1H, H5'''), 2.65 (dd, J = 17.0, 2.6 Hz, 1H, H5''', 2.30 (t, J = 2.6 Hz, 1H, CH3OH), 1.11 – 1.00 (m, 28H, TIPSCH3), 13.5, 13.1, 13.0, 12.5 (TIPSCH3); HRMS-ESI m/z 252.2434 (M + H+); calcd (C19H19NO4Si2) = 252.2447.

1-(2′-C-(3′-N-Benzoyluracil-5-yl)prop-2-yn-1-yl)-3′,5″-O-(tetraisopropylsiloxane-1,3-diyl)-β-D-arabinofuranosyluracil (14): 3-Benzoyl-5-iodouracil (0.64 g, 2.44 mmol), CuI (75 mg, 0.40 mmol) and Pd(PPh3)4 (0.26 g, 0.24 mmol) were dissolved in anhydrous DMF (4 mL) and anhydrous Et2O (1.2 mL), which had both been degassed with argon. A solution of nucleoside 13 (0.64 g, 2.13 mmol) in anhydrous DMF (8 mL) was added over 4 h. The mixture was stirred for 40 h at rt under argon, and then concentrated under reduced pressure and co-evaporated with xylene (2 × 5 mL). Water (20 mL) was added, and the suspension was extracted with EtOAc (2 × 50 mL). The combined organic phase was washed with brine (2 × 25 mL) and water (2 × 15 mL) and then dried (MgSO4). The residue was purified by silica gel column chromatography (0–10% MeOH in CH2Cl2, v/v) to give 14 (0.51 g, 0.69 mmol, 56%) as slightly yellow foam. 1H NMR (400 MHz, CDCl3) δ 7.11 – 7.09 (m, 1H, H6), 7.06 (t, J = 7.5 Hz, 1H, H5), 7.46 (t, J = 7.5 Hz, 2H, Bz), 6.29 (s, 1H, H1′), 5.75 (br s, 1H, H5'), 4.26 (d, J = 9.0 Hz, 1H, H3'), 4.13 – 4.06 (m, 1H, H5'''), 3.95 (d, J = 13.2, 2.4 Hz, 1H, H5''), 3.73 – 3.67 (m, 1H, H4'), 3.03 (d, J = 17.6 Hz, 1H, H5''), 2.82 (d, J = 17.6 Hz, 1H, H6'), 1.11 – 0.97 (m, 28H, TIPSCH3), 1.10 – 1.00 (m, 28H, TIPSCH3) 13.5, 13.1, 13.0, 12.5 (TIPSCH3); HRMS-ESI m/z 276.2124 [M + Na+]; calcd (C20H21NO5Si2Na) = 276.2145.

Oligonucleotide synthesis: Oligonucleotide synthesis was carried out on an automated DNA synthesizer following the phosphoramidite approach. The synthesis was performed on a 0.2 µmol scale by using the amidites 7, 12 and 17 as well as the corresponding commercial 2-cyanoethyl phosphoramidites of the natural deoxy nucleosides. The synthesis followed the regular protocol for the DNA synthesizer. However, for modified amides a prolonged coupling time of 15 min was used. 4,5-Dicyanomiazole was used as the activator. The 5′-O-DMT oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia at rt for 24 h (U, U2, U3, U4, U5) and methanolic ammonia at 65 °C for 44 h (Ua), which also removed the protecting groups. All oligonucleotides were deprotected by treatment with an 80% aqueous solution of acetic acid for 30 min. Aqueous solutions of sodium acetate (3 M, 15 µL) and sodium perchlorate (5 M, 15 µL) were added, followed by acetone (1 mL). The pure oligonucleotides precipitated overnight at −20 °C. After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was removed, and the pellet was washed with cold acetone (2 × 1 mL), dried for 30 min at 35 °C and dissolved in pure water (1000 µL). The concentration was determined by optical density at 260 nm, the purity was confirmed by ion-exchange chromatography and the identity was confirmed by MALDI-TOF mass spectrometry.

Thermal denaturation experiments: UV melting experiments were carried out on a PerkinElmer 35 UV/Vis spectrophotometer. The samples were comprised of a medium salt buffer containing 2.5 mM Na2HPO4, 5 mM NaH2PO4, 100 mM NaCl, and 0.1 mM EDTA at pH = 7.0 with 1.5 µM concentrations of the two complementary sequences. Before measuring, the oligonucleotides were annealed by heating the samples to 75 °C followed by controlled cooling to 10 °C. The absorbance at 260 nm was recorded as a function of the temperature (10 °C to 75 °C, 1 °C/min) by means of a PTP-6 Peltier Temperature Programmer. The melting temperatures (Tm) were determined as the local maximum of the first derivatives of the absorbance vs. temperature curves. All determinations are averages of at least duplicates within ±0.5 °C. For all duplexes, a clear melting temperature was found, and the process of denaturation was reversible upon cooling.
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Keywords: Cyclization • DNA • Nucleobases • Nucleosides • Oligonucleotides

References

New double-headed nucleotide analogues featuring non-native nucleobases Z and W attached to the 2’-position of arabinouridine were synthesized. Their base-pairing specificities were evaluated in DNA duplexes, however, stable wobble pairs challenged the specificity of Z. Also, the synthesis of a double-headed nucleotide containing dianinopurine in the 2’-position was improved using a substitution from 2-fluoroadenine at the oligonucleotide level.