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Stabilisation of nucleic acid secondary structures by oligonucleotides with an additional nucleobase; synthesis and incorporation of 2′-deoxy-2′-*C*-(2-(thymine-1-yl)ethyl)uridine

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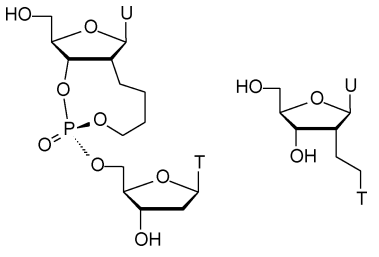
A nucleoside with two nucleobases is incorporated into oligonucleotides. The synthetic building block, 2′-deoxy-2′-*C*-(2-(thymine-1-yl)ethyl)uridine, **2**, is prepared from uridine *via* 5′,3′-TIPDS-protected 2′-deoxy-2′-*C*-allyluridine by an oxidative cleavage of the allyl group, a Mitsunobu reaction for the introduction of thymine and appropriate deprotection reactions. This compound is converted into a DMT-protected phosphoramidite and incorporated once into a 13-*mer* oligodeoxynucleotide sequence, once in an isosequential LNA-modified oligodeoxynucleotide and four times in the middle of a 12-*mer* oligodeoxynucleotide. These sequences are mixed with different complementary DNA and RNA sequences in order to study the effect of the additional nucleobase in duplexes, in bulged duplexes and in three-way junctions. The first additional thymine is found to be well-accommodated in a DNA–RNA duplex, whereas a DNA–DNA duplex was slightly destabilised. A three-way junction with the additional thymine in the branching point is found to be stabilised in both a DNA–DNA and a DNA–RNA context but destabilised where the modified LNA-sequence is used. In a Mg^{2+}-containing buffer, however, the relative stability of the three-way junctions is found to be opposite with especially the LNA-modified DNA–DNA complex being significantly stabilised by the additional nucleobase.

Introduction

Synthetic nucleic acid fragments have been designed for the study of secondary structures of DNA and RNA. Most efforts, however, have been concentrated on the stabilisation of A- and B-type duplexes by *e.g.* conformationally restricted nucleoside building blocks.[1–3] For the study of other secondary structures, cyclic dinucleotides have been constructed, *e.g.* as artificial models of bend motifs.[4–6] We have recently formulated a general strategy for the synthesis of cyclic dinucleotides based on ring-closing metathesis (RCM) methodology.[7–13] Hence, medium[7,8] or large ring connections[9–13] have been made in dinucleotides in order to form conformationally restricted nucleic acid fragments for the modelling or targeting of secondary nucleic acid structures.[7–13] Recently, the dinucleotide **1** containing a nine-membered ring forming a 2′-*C* to phosphate connection (Fig. 1) was incorporated into an oligonucleotide which was found to stabilise a three-way junction formed with a complementary RNA-sequence.[13] On the other hand, the cyclic moiety was found to destabilise duplex regions, bulged duplexes as well as a three-way junction with a complementary DNA-sequence.[13]



1 2

Fig. 1 A cyclic dinucleotide, **1**,[13] and a nucleoside with an additional nucleobase, **2**. T = thymin-1-yl, U = uracil-1-yl.

Herein, we describe a different and much simpler approach towards the induction as well as stabilisation of secondary nucleic acid structures. An additional nucleobase is introduced at a given nucleoside moiety in order to create oligonucleotides with additional base-pairing opportunities. This might stabilise secondary structures like bulges or three-way junctions by the compression of two base-pairing options at one nucleotide site. On the other hand, a nucleoside building block with two nucleobases (or "double-headed" nucleoside) can also be used for the creation of double-coding DNA—a DNA duplex with additional coding information on the outside of the duplex. The idea of positioning additional bases on a dsDNA has recently been approached by Herdewijn and co-workers who designed an achiral acyclic nucleoside building block with either two thymine or two adenine moieties.[14] We decided, on the other hand, to prepare a nucleoside with the additional nucleobase attached to a standard ribonucleoside structure in order to preserve the natural nucleic acid backbone. Other nucleosides with two nucleobases attached directly to the ribofuranose have been prepared as potential antiviral or anticancer compounds.[15–18] However, a 1′,5′- or a 1′,3′-positioning of the two nucleobases[15–17] does not fulfil our demand for a standard 5′–3′ incorporation into oligonucleotides. In a 1′,2′-positioning of the nucleobases,[16] the base-pairing options for the two bases were estimated to be very interdependent and too restricted for the purpose. Therefore, we designed a 2′-deoxyribonucleoside, **2**, with the additional nucleobase positioned at a 2′-alkyl group (Fig. 1). Although 2′-*O*-alkyl substituents are well-known to induce *N*-type conformations[19] of the nucleosides and to be well accommodated into duplexes,[20] we decided to use a 2′-*C*-alkyl group. Hereby, a target compound, **2**, with a shorter linker between the sugar moiety and the additional nucleobase is available. This nucleoside can be expected to accommodate an *S*-type conformation[19,20] and to show interesting features in nucleic acid secondary structures.

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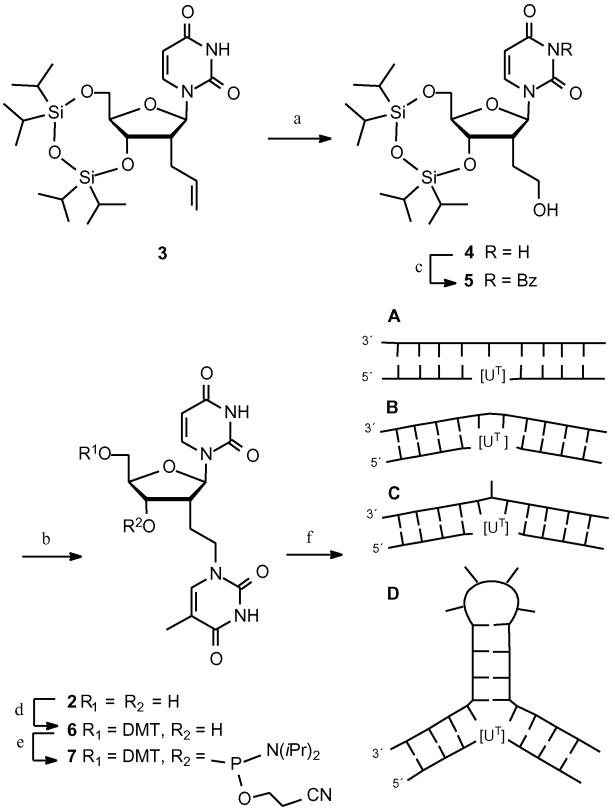
Results and discussion

Chemical synthesis

The target nucleoside **2** was constructed on the basis of a known and efficient methodology for preparing 2′-allyl-2′-deoxyuridine[21,22] that we also used in the preparation of the cyclic dinucleotide **1**.[13] Hence, the TIPDS-protected derivative **3** (Scheme 1) has been prepared in three steps from uridine.[21] The oxidative cleavage of the allylic chain to give the corresponding alcohol **4** was conducted in three steps slightly modified from the method described in the literature.[23,24] Dihydroxylation with a catalytic amount of OsO_4 in the presence of *N*-methylmorpholine oxide followed by the cleavage of the resulting diol with $NaIO_4$ and reduction of the aldehyde with $NaBH_4$ afforded **4** in a good overall yield. Substitution of the hydroxyl group with 3-*N*-benzoylthymine[25] was attempted by a standard Mitsunobu reaction.[26] Purification of the resulting nucleoside with two nucleobases proved difficult, and therefore, the protecting groups were removed before purification using TBAF for the TIPDS group and aqueous methanolic ammonia for the benzoyl group. The desired nucleoside **2** was isolated after a chromatographic separation in 37% yield over the three steps. MALDI-MS of the intermediate mixture indicated the competing formation of a seven-membered 2,2′-anhydro nucleoside species. Subsequently, the 3-*N* position of the uracil was protected before the Mitsunobu reaction. The alcohol **4** was treated with benzoyl chloride in a phase transfer method which has been previously useful in the selective 3-*N* protection of 3′,5′-TIPDS protected uridine leaving the 2′-hydroxyl group unprotected.[27] The reaction smoothly afforded the product **5** in a high 93% yield. The introduction of 3-*N*-benzoyl thymine by Mitsunobu conditions and deprotection with TBAF and aqueous methanolic ammonia resulted in a single product **2** in 67% yield over the three steps. The double headed nucleoside was confirmed by NMR and MALDI-MS demonstrating the loss of both the TIPDS group and the two benzoyl groups as well as the presence of the two pyrimidine moieties. The ^{1}H NMR spectrum of **2** also revealed a $J_{H1'H2'}$ coupling constant of 8.7 Hz as well as a very small $J_{H3'H4'}$ coupling constant. This confirms the expected strong preference of this nucleoside for adopting an *S*-type conformation. In order to incorporate **2** into oligonucleotides, the 5′-hydroxy group was protected as a 5′-*O*-dimethoxytrityl (DMT) ether, **6**, by application of DMT–Cl in pyridine. Due to the low solubility of **6** in acetonitrile, the phosphoramidite **7** was prepared in an unusual solvent mixture of DMF and acetonitrile. The cyanoethyl phosphordiamidite reagent was used with 4,5-dicyanoimidazole as the activator[28] to give **7** in 62% yield as an equimolar epimeric mixture as judged from ^{1}H and ^{31}P NMR.



Scheme 1 *Reagents and conditions*: a, i) OsO_4, NMO, aq. *t*-BuOH, ii) $NaIO_4$, aq. dioxane, iii) $NaBH_4$, MeOH, 64%; b, i) 3-*N*-benzoylthymine, PPh_3, DEAD, THF, ii) TBAF, THF, iii) NH_3, CH_3OH, 37% (from **4**), 67% (from **5**); c, BzCl, Bu_4NBr, Na_2CO_3 (aq), CH_2Cl_2, 93%; d, DMTCl, pyridine, 54%; e, $NC(CH_2)_2OP(N(iPr)_2)_2$, 4,5-dicyanoimidazole, CH_3CN, 62%; f, incorporation into (**A**) a duplex, (**B**) a bulged duplex with one additional nucleotide, (**C**) a bulged duplex with two additional nucleotides and (**D**) a three-way junction, respectively (see Table 2), [U^T] reflects the incorporation of **7** (see Tables 1 and 2). DMT = 4,4′-dimethoxytrityl.

Table 1 Prepared oligodeoxynucleotide sequences and their MS-data

	ODN sequences[a]	MW (found/calc.)[b]
8	5′-GCTCACTCTCCCA	—
9	5′-GCTCACTTCTCCCA	—
10	5′-GCTCAC[U^T]CTCCCA	3965/3969 $[MH]^+$
11	5′-GC^LTC^LAC^LTC^LTC^LCC^LA	—
12	5′-GC^LTC^LAC^LTTC^LTC^LCC^LA	—
13	5′-GC^LTC^LAC^L[U^T]C^LTC^LCC^LA	4218/4216 $[MH]^+$
14	5′-AGCTTTTTTCGC	—
15	5′-AGCT[$U^T U^T U^T U^T$]TCGC	4184/4186 $[MH]^+$

[a] [U^T] refers to the incorporation of **7**. C^L refers to the LNA 5-methylcytidine monomer. [b] MALDI-MS positive mode.

Preparation and evaluation of oligonucleotides

The phosphoramidite **7** was incorporated into three different oligonucleotides by standard automated solid phase DNA-synthesis using 17 minutes coupling time for **7** followed by prolonged capping time. The coupling efficiencies for **7** were in the range of 96–99%. The oligonucleotides prepared for this study are shown in Table 1, and the reference sequences as well as the complements are to a large extent the same as used in the study of the cyclic dinucleotide **1**.[13] Thus, the central thymidine of the standard 13-*mer* sequence **8** or the two central thymidines of the standard 14-*mer* sequence **9** were replaced by the double-headed nucleoside **2** in sequence **10**. Also an LNA–DNA mixmer sequence was studied securing an overall preference for A-type duplex formation for this sequence.‡[,30,31] Replacing the central T or TT in sequences **11** and **12**, respectively, with **2** corresponds to sequence **13**. Finally, a 12-*mer* sequence **14** with a central thymidine tract was studied with the four central thymidines replaced with four times **2** in sequence **15**. The constitution and purity of the oligonucleotides was verified by MALDI-MS (Table 1) and HPLC-profiles, respectively.

Hybridisation properties of oligonucleotides **8–15** with complementary DNA and RNA sequences were evaluated by thermal stability examinations (Table 2). First, the standard duplexes

‡ LNA (*locked nucleic acid*) has been introduced as oligonucleotides containing the LNA monomers with a bicyclic carbohydrate moiety locked in an *N*-type conformation[2,29,30] and with the ability of tuning the overall duplex conformation towards the A-type.[30,31] LNA sequences recognise complementary DNA and RNA with significantly increased affinity compared to unmodified oligonucleotides.[29,30] LNA monomers:

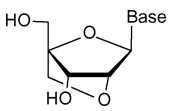


Table 2 Hybridisation data for the prepared oligonucleotides with different DNA- and RNA-complements[a]

A, fully matched complements

	DNA[b]	RNA[b]		DNA[c]	RNA[c]
8	50.5	57.8	**14**	49.3	44.3
10	46.3	57.0	**15**	14.5	22.8
	(−4.2)	(−0.8)		(−34.8)	(−21.5)
11	74.0	>90			
13	72.8	>90			
	(−1.2)				

Complements with bulges

	B, additional A		**C**, additional GA	
	DNA[d]	RNA[d]	DNA[e]	RNA[e]
8	41.0	48.0	40.8	47.5
9	52.0	58.5	40.5	47.8
10	42.0	46.0	39.0	46.3
	(+1.0)[g]	(−2.0)[g]	(−1.8)[g]	(−1.2)[g]
	(−10.0)[h]	(−12.5)[h]	(−1.5)[h]	(−1.5)[h]
11	67.5	83.8	65.5	84.3
12	76.3	>90	65.3	81.0
13	66.0	82.0	61.8	>85[k]
	(−1.5)[i]	(−1.8)[i]	(−3.7)[i]	(>+0.7)[i]
	(−10.3)[j]	(<−8)[j]	(−3.5)[j]	(>+4.0)[j]

D, complements with an intrastrand stem-loop

	DNA[f]			RNA[f]		
	Increasing [Mg^{2+}]			Increasing [Mg^{2+}]		
	0 mM	5 mM	10 mM	0 mM	5 mM	10 mM
8	25.3	31.8	33.3	33.5	40.8	42.8
9	24.8	32.5	34.0	34.8	44.0	44.5
10	29.5	34.0	34.5	36.0	43.3	44.5
	(+4.2)[g]	(+2.2)[g]	(+1.2)[g]	(+2.5)[g]	(+2.5)[g]	(+1.7)[g]
	(+4.7)[h]	(+1.5)[h]	(+0.5)[h]	(+1.2)[h]	(−0.7)[h]	(0.0)[h]
11	51.5	60.0	61.5	72.3	76.8	78.8
12	53.8	58.8	62.5	73.0	80.8	80.5
13	50.8	62.3	74.8	69.0	75.3	77.3
	(−0.7)[i]	(+2.3)[i]	(+13.3)[i]	(−3.3)[i]	(−1.5)[i]	(−1.5)[i]
	(−3.0)[j]	(+3.5)[j]	(+12.3)[j]	(−4.0)[j]	(−5.5)[j]	(−3.2)[j]

[a] Melting temperatures (T_m values/° C) obtained from the maxima of the first derivatives of the melting curves (A_{260} *vs.* temperature) recorded in a medium salt buffer (Na_2HPO_4 (15 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.0 μM concentrations of each strand. All T_m values are given as averages of double determinations. ΔT_m values are given in brackets. [b] DNA 3′-CGAGTGAGAGGGT, RNA 3′-CGAGUGAGAGGGU. [c] DNA 3′-TCGAAAAAAGCG, RNA 3′-UCGAAAAAAGCG. [d] DNA 3′-CGAGTGAAGAGGGT, RNA 3′-CGAGUGAAGAGGGU. [e] DNA 3′-CGAGTGA**G**AGAGGGT, RNA 3′-CGAGUGA**G**AGAGGGU. [f] DNA 3′-CGAGTGA**CGCGTTTTCGCG**AGAGGGT, RNA 3′-CGAGUGA**CGCGUUUUCGCG**AG-AGGGU, bold sequences are stem-loops. [g] Compared with **8**. [h] Compared with **9**. [i] Compared with **11**. [j] Compared with **12**. [k] Unclear transition.

formed between the modified sequences and their fully matched complements were examined (Table 2, entry **A**). Comparing the duplexes formed by sequences **8** and **10**, respectively, the nucleotide with an additional nucleobase induced a drop in T_m of 4.2 °C in a DNA–DNA duplex and only a very small drop in T_m of 0.8 °C in a DNA–RNA duplex. This indicates that the additional nucleobase is relatively well-accommodated in a standard duplex. On the other hand, this was not the case with four additional nucleobases introduced in a thymidine tract. Thus, comparing **14** and **15**, a drop in T_m for the duplexes of 5–9 °C for each modified moiety was observed. In an LNA-modified (and therefore A-type)[30,31] duplex, a smaller drop in T_m of 1.2 °C in a DNA–DNA duplex compared to a standard sequence was observed (**11** and **13** compared), whereas the LNA-modified DNA–RNA duplex was too stable (T_m above the detection limit) due to the LNA-monomers,‡[2,29,30] to allow direct comparison.

Next, a duplex extended with one adenosine bulge in the complementary sequence was studied (Table 2, entry **B**). In other words, the ability of **2** to behave as a UT-dinucleotide opposite an AA-complement was investigated. Comparing **10** (or **13**) with **9** (or **12**) containing an unmodified TT-sequence, however, demonstrated large drops in T_m of the complexes of 8–13 °C in all cases with both DNA and RNA complements. Hence, the additional thymine in **2** does not seem to base-pair with the additional adenine in the complements indicating that this forms a real bulge. When comparing **10** (or **13**) with **8** (or **11**) containing only one thymidine instead of **2**, only small changes in T_m between −2 and +1 °C were detected. Thus the additional thymine is well-accommodated into the bulged duplexes. On the other hand, no stabilisation (except diminutively in the DNA–DNA context) due to base-pairing or stacking from the additional thymine was seen.

In another bulged duplex in which an AGA-sequence is opposing the dinucleotide **2**, similar results were observed (Table 2, entry **C**). Thus, the comparison of **10** with **8** or **9** revealed in all cases very small differences in T_m between −1.2 and −1.8 °C. The exact small changes in T_m were more or less the same as determined before with the smaller bulge, with **2** or T against the AA-sequence. In other words, the additional thymine is not influencing the structure significantly. With the LNA–DNA mixmer, the situation is slightly different. With **13** compared to **11** or **12**, slightly larger decreases in T_m of approx. −3.6 °C were seen with the bulged DNA-complement. With the RNA-complement, the stability is slightly increased compared to one thymidine and more increased compared to two thymidines (∼ +4 °C). Though these changes are small and the detected melting temperature somewhat unclear, the additional nucleobase seems to influence the structure to some extent.

Finally, we examined the stability of a three-way junction (TWJ) composed of a standard stable stem-loop sequence with two single stranded regions being complementary to the oligonucleotides **8–13** (Table 2, entry **D**). For **10** and **13**, the nucleotide with an additional nucleobase **2** is positioned in the branching point (see Scheme 1). This is compared with a standard situation with two thymidines (**9** and **12**) in the branching point. As shown in the study of **1**,[13] the standard TWJ is in general not very stable with a T_m of 24.8 °C in a complete DNA-context and 34.8 °C with a DNA–RNA hybrid, though significantly more stable with six LNA-monomers (53.8 and 73.0 °C, respectively).§ Most probably, one or both of the two adenosines are not base-paired with the two thymidines. This is reflected by the fact that nearly equal melting temperatures were detected when one of the thymidines was removed (compare **9** and **12** with **8** and **11**). When introducing the moiety with an additional nucleobase, the situation changes significantly. Hence, a large increase in T_m of 4.7 °C was observed (comparing **10** with **9**) in the DNA–DNA context and a smaller increase of 1.2 °C in the DNA–RNA context. For the LNA-sequence, the TWJ was found to be destabilised by the nucleotide with an additional nucleobase in both contexts (−3.0 to −4.0 °C, comparing **13** and **12**).

The TWJs were thereafter stabilised significantly by the addition of Mg^{2+}. Hence, a 5 mM concentration of Mg^{2+} was in general found to increase the thermal stabilities of the standard TWJs (**9** or **12** with DNA or RNA) with 5–9 °C. Increasing this to a 10 mM Mg^{2+} concentration led to significant further stabilisation only in the DNA–DNA context.§ The addition of Mg^{2+} changed, on the other hand, the situation for the TWJs

§ Small deviations in T_m compared to the data reported in ref. 13 are seen. Thus, these have been determined again for the present study on new equipment.

modified with **2**. In the DNA–DNA as well as the DNA–RNA context, the stabilisation induced with the additional nucleobase seems to vanish. In the LNA–RNA context, the destabilisation found before seems to be more or less unaffected. In the LNA–DNA context, on the other hand, a significant stabilisation of the TWJ was detected changing from −3.0 °C without Mg^{2+} to +3.5 °C with a 5 mM and +12.3 °C with a 10 mM Mg^{2+}-concentration. This indicates a Mg^{2+}-induced change in the secondary structure of this TWJ towards a more stable complex in which the additional nucleobase is taking an active part.

The present data reflect the sensitive behaviour of secondary nucleic acid structures and demonstrate how easily these can be influenced by simple chemical modification. It is remarkable that a TWJ can be stabilised significantly by the introduction of one additional thymine ($\Delta T_m \sim +4.7$ °C), though other features in the modification including the conformational behaviour might contribute to this effect. The power of this stabilisation decreases by the introduction of Mg^{2+}. The corresponding TWJ with a few LNA-monomers incorporated demonstrates entirely opposite behaviour when the additional thymine is introduced ($\Delta T_m \sim -3.0$ °C). Nevertheless, Mg^{2+} seems to induce a conformational shift in the TWJ towards a different structure in which the additional thymine plays a role and stabilises the structure significantly ($\Delta T_m \sim +12$ °C).

The structural basis for the observed stabilisation of the TWJs by the additional nucleobase motif is not immediately deducible from the hybridisation data. However, the evident but small destabilisation of bulged duplexes observed suggests that the additional nucleobase is not able to perform Watson–Crick base-pairing with a bulged nucleobase in the complementary sequence. Hence, the stabilisation of a standard DNA TWJ is more likely due to a stacking effect of the thymine and not a base-pairing. On the other hand, the significantly stabilised structure of the LNA–DNA constituted TWJ might contain a base-pairing as well as a stacking effect from the additional thymine. In the future, therefore, the replacement of the thymine in **2** with other nucleobases, natural or unnatural, or with other aromatic moieties can be performed. This will allow direct comparison of the effects as a purine nucleobase or an aromatic intercalator should increase the stacking effect but remove the possibilities of base-pairing. Efficient stabilisation of a DNA TWJ has been previously demonstrated with an intercalating moiety incorporated in an oligodeoxynucleotide.[32]

In comparison to the effects in similar sequence contexts obtained by the introduction of the cyclic dinucleotide **1**,[13] the effects of **2** are significantly stronger and more encouraging. The best effect observed with **1** was a slight stabilisation of a DNA–RNA TWJ. Taking the synthetic effort into account, the preparation of **2** and of the corresponding oligonucleotides is a significantly simpler task compared to the RCM-based preparation of **1**. However, **1** and **2** are examples of very different approaches. In **1**, the effect on the secondary structures is first of all conformational with the introduction of an artificial bend or, at least, some restriction into the oligonucleotide. The introduction of **2**, on the other hand, disposes additional stacking and hydrogen-bonding opportunities in the structure with the conformational restriction towards an *S*-type conformation being a secondary effect. The Mg^{2+}-induced effects demonstrate the dynamic behaviour of the TWJ and the ability of **2** to participate and influence this behaviour.

The application of **2** for the introduction of additional nucleobases on the outside of a duplex is an intriguing possibility. However, the relatively large decreases in duplex stability observed with several successive incorporations of **2** suggest that **2** might not be the best candidate for this purpose. A similar approach by Herdewijn and co-workers using a more flexible acyclic double-headed nucleoside did, in fact, in one case demonstrate a more stable duplex with more than one additional base than we obtained with **2**.[14] With single incorporations, on the other hand, a more significant destabilisation than with **2** was observed.[14] In the future, the ideal building block for presenting additional nucleobases on the outside of nucleic acid duplexes might be found in other nucleosides with two nucleobases in which the additional base is conjugated to other positions of a standard nucleoside.

The present results demonstrate the potential for mimicking and stabilising secondary structures like TWJs by the introduction of additional nucleobases on the 2′-position of the nucleotide. More thorough structural investigations might throw light on the observed stabilised TWJs and the role of the double-headed nucleoside moiety. The double headed nucleotide motif induced by **2**, however, might not be the optimal for the secondary structures investigated in this study. Obviously, other positions for the additional nucleobase, other combinations of nucleobases as well as other linker-lengths are topics for further investigations. Molecular modelling investigations of biologically important sequences in combination with the present approach will be performed in the near future. Thus, the observed stabilisations of a TWJ are encouraging, and we expect the idea of introducing additional nucleobases to be a powerful tool for the future in nucleic acid chemical biology and Ångström-scale engineering.[33]

Conclusion

In conclusion, a nucleoside with two nucleobases, 2′-deoxy-2′-*C*-(2-(thymine-1-yl)ethyl)uridine, has been easily synthesised from uridine and used as a building block in the preparation of synthetic nucleic acid secondary structures. The "double-headed" nucleoside moiety was found to be reasonably well-accommodated in duplexes and to stabilise three-way junctions. Significant conformational changes in these secondary structures have also been induced. These results demonstrate the potential of nucleosides conjugated to additional nucleobases as simple tools towards the engineering of functional or therapeutic synthetic nucleic acid architectures such as dynamic secondary structures or double-coding DNA with potentially base-pairing nucleobases presented on the outside of the duplex.

Experimental

All commercial reagents were used as supplied. When necessary, reactions were performed under an atmosphere of nitrogen. Column chromatography was carried out on glass columns using silica gel 60 (0.040–0.063 mm). NMR spectra were recorded on a Varian Gemini 2000 spectrometer. ^{1}H NMR spectra were recorded at 300 MHz, ^{13}C NMR spectra were recorded at 62.5 MHz and ^{31}P NMR spectra were recorded at 121.5 MHz. The values for δ are in ppm relative to tetramethylsilane as internal standard or 85% H_3PO_4 as external standard. HRMALDI mass spectra were recorded on an Ionspec Ultima Fourier Transform mass spectrometer with a DHB-matrix. Assignments of NMR spectra are based on ^{1}H, ^{1}H-COSY and/or DEPT spectra and follow standard carbohydrate and nucleoside style; *i.e.* the carbon atom next to a nucleobase is assigned as C-1′.

Preparation of 2′-deoxy-2′-*C*-(2-hydroxyethyl)-3′,5′-*O*-(1,1,3,3-tetraisopropylsiloxan-1,3-diyl)uridine (4)

To a solution of compound **3**[21] (1.81 g, 3.54 mmol) in a mixture of acetone (15 mL) and water (4 mL) was added *N*-methylmorpholine *N*-oxide (584 mg, 4.49 mmol) and a 2.5% w/v solution of OsO_4 in *tert*-butanol (1.58 mL, 0.156 mmol). The reaction mixture was stirred at room temperature for 1.5 h. The reaction was quenched by the addition of a 5% aqueous solution of sodium thiosulfate (15 mL) and the mixture was diluted with ethyl acetate (50 mL). The mixture was concentrated under reduced pressure to approx. one third of the volume and diluted again with ethyl acetate (50 mL). The mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 × 40 mL) and brine (40 mL), dried ($MgSO_4$) and concentrated

under reduced pressure to give the crude diol (1.85 g) as a white foam. This product was dissolved in a mixture of dioxane (30 mL) and water (10 mL) and $NaIO_4$ (1.51 g, 7.08 mmol) was added. The mixture was stirred for 40 minutes and then diluted with ethyl acetate (70 mL). The mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated under reduced pressure to give the crude aldehyde (2.00 g) as a colourless oil. This product was dissolved in cold methanol (8 mL) and added dropwise to a stirring suspension of sodium borohydride (774 mg, 20.46 mmol) in methanol at 0 °C. The solution was stirred for 40 minutes and then neutralized by the addition of solid citric acid. The mixture was concentrated under reduced pressure and then redissolved in dichloromethane (50 mL). The mixture was washed with water (25 mL), a saturated aqueous solution of $NaHCO_3$ (2 × 25 mL) and brine (2 × 25 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography (1–3% CH_3OH in CH_2Cl_2) to give the product as a white foam (1.17 g, 64%). R_f 0.23 (3 × 2.5% CH_3OH in CH_2Cl_2). δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.95 (1H, s, NH), 7.97 (1H, d, *J* 8.2 Hz, H-6), 5.88 (1H, s, H-1′), 5.72 (1H, d, *J* 8.2 Hz, H-5), 4.44 (1H, m, H-3′), 4.22 (1H, d, *J* 13.4 Hz, H-5′), 4.03–3.78 (4H, m, H-4′, H-5′, 2′-CH_2CH_2OH), 3.51 (1H, t, *J* 6.0 Hz, OH), 2.47 (1H, m, H-2′), 2.14 (1H, m, 2′-CH_2CH_2OH), 1.64 (1H, m, CH_2CH_2OH), 1.22–0.84 (28H, m, $CH(CH_3)_2$). δ_C (75 MHz; $CDCl_3$; Me_4Si) 163.7 (C-4), 151.0 (C-2), 139.6 (C-6), 101.8 (C-5), 89.0 (C-1′), 83.0 (C-4′), 67.2 (C-3′), 60.5, 59.7 (C-5′, 2′-CH_2CH_2OH), 46.6 (C-2′), 27.8 (2′-CH_2CH_2OH), 17.5, 17.4, 17.3, 17.2, 17.0, 17.0, 16.9, 16.8, 13.4, 13.1, 12.9, 12.5 ($CH(CH_3)_2$). HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 537.2423/537.2404.

Preparation of 2′-deoxy-2′-*C*-(2-(thymine-1-yl)ethyl)uridine (2)

Method I. Compound **4** (322 mg, 0.645 mmol), 3-*N*-benzoylthymine (331 mg, 1.44 mmol) and PPh_3 (371 mg, 1.41 mmol) were dissolved in anhydrous THF (10 mL) and the solution was stirred at 0 °C. Diethyl azodicarboxylate (0.31 mL, 1.42 mmol) was added slowly. The solution was stirred at room temperature for 18 h and concentrated under reduced pressure. The residue was purified by column chromatography (0–3% CH_3OH in CH_2Cl_2). The residue was redissolved in anhydrous THF (4 mL) and a 1 M solution of TBAF in THF (1.42 mL) was added over 5 minutes. The solution was stirred at room temperature for 40 minutes and a mixture of pyridine, methanol and water (3 : 1 : 1 v/v, 8 mL) was added. The reaction mixture was treated with amberlite IR-120® (5 g) in the same mixture of pyridine, methanol and water (3 : 1 : 1 v/v, 24 mL) and stirred for 30 minutes. The suspension was filtered and concentrated under reduced pressure. The residue was redissolved in methanol (5 mL) and a 25% aqueous solution of ammonia (2 mL) was added. The mixture was stirred for another 2 h and concentrated under reduced pressure. The residue was coevaporated with toluene and purified by column chromatography (petrol ether and then 0–9% CH_3OH in CH_2Cl_2) to give the product (90 mg, 37%) as a white foam. R_f 0.35 (20% CH_3OH in CH_2Cl_2). δ_H (300 MHz; CD_3OD; Me_4Si) 7.92 (1H, d, *J* 8.1 Hz, UH-6), 7.36 (1H, d, *J* 1.2 Hz, T-H-6), 5.99 (1H, d, *J* 8.7 Hz, H-1′), 5.67 (1H, d, *J* 8.1 Hz, U-H-5), 4.29 (1H, dd, *J* 1.6 Hz, *J* 5.6 Hz, H-3′), 3.96 (1H, m, H-4′), 3.84–3.64 (4H, m, H-5′, 2′-CH_2CH_2T), 2.23 (1H, m, H-2′), 2.03 (1H, m, 2′-CH_2CH_2T), 1.81 (3H, d, *J* 1.1 Hz, CH_3), 1.66 (1H, m, 2′-CH_2CH_2T). δ_C (75 MHz; DMSO-d6; Me_4Si) 164.2, 163.0 (U-C-4, T-C-4), 150.9, 150.8 (U-C-2, T-C-2), 141.0, 140.4 (U-C-6, T-C-6), 108.6 (T-C-5), 102.3 (U-C-5), 87.4, 87.2 (C-1′, C-4′), 71.2 (C-3′), 61.7 (C-5′), 45.2, 45.1 (C-2′, 2′-CH_2CH_2T), 23.2 (2′-CH_2CH_2T), 11.9 (CH_3). HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 403.1224/403.1223.

Method II. The same procedure was used with compound **5** (865 mg, 1.40 mmol) as the starting material using 3-*N*-benzoylthymine (710 mg, 3.09 mmol), PPh_3 (807 mg, 3.08 mmol) and diethyl azocarboxylate (0.67 mL, 3.08 mmol) in anhydrous THF (50 mL) followed by a 1 M solution of TBAF in THF (3.1 mL, 3.08 mmol) and anhydrous THF (15 mL), and thereafter, amberlite IR-120® (5 g) in a mixture of pyridine, methanol and water (3 : 1 : 1 v/v, total 32 mL), and finally, a 25% aqueous solution of ammonia (4.0 mL) to give the product (358 mg, 67%) as a white foam.

Preparation of 3-*N*-benzoyl-2′-deoxy-2′-*C*-(2-hydroxyethyl)-3′,5′-*O*-(1,1,3,3-tetraisopropylsiloxan-1,3-diyl)uridine (5)

Compound **4** (1.48 mg, 2.88 mmol) was dissolved in a mixture of dichloromethane (40 mL) and a 0.2 M aqueous solution of Na_2CO_3 (80 mL). Bu_4NBr (2.4 mg, 7.44 μmol) was added and benzoyl chloride (0.43 mL, 3.74 mmol) was added dropwise over a period of 30 minutes. The mixture was stirred at room temperature for 16 h and then washed with water. The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (0–1% CH_3OH/CH_2Cl_2) to give the product (1.66 g, 93%) as a white foam. R_f 0.45 (5% CH_3OH in CH_2Cl_2). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.07 (1H, d, *J* 8.2 Hz, H-6), 7.92 (2H, m, Ph), 7.64 (1H, m, Ph), 7.49 (2H, m, Ph), 5.88 (1H, s, H-1′), 5.82 (1H, d, *J* 8.2 Hz, H-5), 4.48 (1H, m, H-3′), 4.26 (1H, d, *J* 13.7 Hz, H-5′), 4.06–3.92 (2H, m, H-4′, H-5′), 3.89–3.73 (2H, m, 2′-CH_2CH_2OH), 3.20 (1H, br s, OH), 2.47 (1H, m, H-2′), 2.10 (1H, m, 2′-CH_2CH_2OH), 1.64 (1H, m, 2′-CH_2CH_2OH), 1.21–0.92 (28H, m, $CH(CH_3)_2$). δ_C (75 MHz; $CDCl_3$; Me_4Si) 168.5 (Ph*C*O), 162.1 (C-4), 149.7 (C-2), 139.3 (C-6), 135.3, 131.2, 130.5, 129.2 (Ph), 101.6 (C-5), 89.3 (C-1′), 83.1 (C-4′), 67.2 (C-3′), 60.9, 59.7 (C-5′, 2′-CH_2CH_2OH), 47.1 (C-2′), 28.0 (2′-CH_2CH_2OH), 17.5, 17.4, 17.3, 17.2, 17.1, 17.0, 16.9, 16.8, 13.4, 13.1, 12.9, 12.5 ($CH(CH_3)_2$). MALDI-MS *m/z* (M + Na) 641.

Preparation of 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-*C*-(2-(thymin-1-yl)ethyl)uridine (6)

Compound **2** (187 mg, 492 μmol) was coevaporated twice with anhydrous pyridine and redissolved in the same solvent (1.5 mL). DMT–Cl (214 mg, 632 μmol) was added and the solution was stirred for 48 h at room temperature. The mixture was concentrated under reduced pressure, and the residue was purified by column chromatography (0.5–3% CH_3OH, 1% pyridine in CH_2Cl_2) to give the product (181 mg, 54%) as a white foam. R_f 0.55 (10% CH_3OH and traces of Et_3N in CH_2Cl_2). δ_H (300 MHz; DMSO-d6; Me_4Si) 11.36 (1H, s, NH), 11.20 (1H, s, NH), 7.56 (1H, d, *J* 8.2 Hz, U-H-6), 7.50 (1H, s, T-H-6), 7.39–7.19 (9H, m, Ph), 7.86–6.93 (4H, m, Ph), 5.88 (1H, d, *J* 8.3 Hz, H-1′), 5.42 (1H, d, *J* 5.2 Hz, OH), 5.33 (1H, d, *J* 8.2 Hz, U-H-5), 4.25 (1H, m, H-3′), 3.95 (1H, m, H-4′), 3.79–3.70 (8H, m, H-5′, OCH_3), 3.40–3.07 (2H, m, 2′-CH_2CH_2T), 2.20 (1H, m, H-2′), 1.89 (1H, m, 2′-CH_2CH_2T), 1.76–1.55 (4H, m, 2′-CH_2CH_2T, CH_3). HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 705.255/725.254.

Preparation of 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite)-2′-*C*-(2-(thymin-1-yl)ethyl)uridine (7)

Compound **6** (134 mg, 196 μmol) was dissolved in anhydrous DMF (1 mL) and CH_3CN (0.3 mL). A 0.5 M solution of 4,5-dicyanoimidazole in CH_3CN (0.55 mL, 275 μmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphoramidite (0.125 mL, 393 μmol) were added, and the reaction mixture was stirred at room temperature for 20 h. The solution was diluted with EtOAc (40 mL) and washed with a half saturated aqueous solution of $NaHCO_3$ (2 × 40 mL) and brine (40 mL). The combined washings were extracted with EtOAc (40 mL), and the combined organic fractions were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified

by column chromatography (0.5–2.5% CH_3OH and 1% pyridine in CH_2Cl_2) to give the product (108 mg, 62%) as a white foam. R_f 0.75 (10% CH_3OH and traces of Et_3N in CH_2Cl_2). δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.30–8.90 (2H, br s, NH), 7.86–7.74 (1H, m, U-H-6), 7.40–7.34 (1H, m, T-H-6), 7.33–7.20 (9H, m, Ph), 6.87–6.77 (4 H, m, Ph), 6.10–6.01 (1H, m, H-1′), 5.40–5.29 (1H, m, U-H-5), 4.71–4.58 (1H, m, H-3′), 4.30–4.15 (1H, m, H-4′), 4.13–3.36 (12H, m, OCH_3, CH_2OP, H-5′, 2′-CH_2CH_2T), 2.68–2.57 (1H, m, H-2′), 2.44–2.29 (2H, m, CH_2CN), 2.28–2.03 (1H, m, 2′-CH_2CH_2T), 1.96–1.68 (4H, m, 2′-CH_2CH_2T, CH_3), 1.23–1.05 (14H, m, $CH(CH_3)_2$). δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) 151.9, 149.9. HiRes MALDI FT-MS m/z (M + Na) found/calc. 905.3610/905.3588.

Preparation of oligodeoxynucleotides

Oligonucleotide synthesis was carried out by using an Expedite™ 8900 nucleic acid synthesis system from PerSeptive Biosystems Inc. following the phosphoramidite approach. Synthesis of oligonucleotides **8–15** was performed on a 0.2 μmol scale by using 2-cyanoethyl phosphoramidites of standard 2′-deoxynucleosides as well as the LNA–^{me}C monomer in combination with the modified phosphoramidite **7**. The synthesis followed the regular protocol employing standard CPG supports. However, for the LNA monomer, a prolonged coupling time of 4 minutes was used, and for **7**, a manual coupling in 17 minutes followed by 2 × 60 s capping. Coupling yields for **7** were in the range of 96–99%. The 5′-*O*-DMT-ON oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia at room temperature for 48 h, which also removed the protecting groups. Purification using reversed-phase HPLC was performed on a Waters 600 system using a X_{terra} prep MS C_{18}; 10 μm; 7.8 × 150 mm column; A buffer: 0.05 M triethylammonium acetate; B buffer: 75% CH_3CN and 25% water; 0–2 minutes 100% A, 2–40 minutes 30% A, 70% B, 40–50 minutes 100% B, 50–60 minutes 100% A. All fractions containing 5′-*O*-DMT protected oligonucleotide (retention time 20–30 minutes) were collected and concentrated. The oligonucleotides were precipitated by treatment with 100 μl 80% CH_3COOH for 30 minutes followed by the addition of 100 μL UHQ water, 50 μl 3 M aqueous CH_3COONa and 600 μL 99.9% ethanol. The mixture was left at −18 °C for 1 h followed by centrifugation for 20 minutes at 4 °C. The supernatant was removed and the oligonucleotide was washed with cold 99.9% ethanol, dried and dissolved in water. MALDI MS m/z (M + H) found/calc.: **10** 3965/3969; **13** 4218/4216; **15** 4184/4186.

Melting experiments

UV melting experiments were carried out on a Perkin-Elmer Lambda 35 UV–VIS spectrometer with a PTP-6 Peltier system and data were processed with Templab version 2.00 and UV WINLAB version 2.85.04. Samples were dissolved in a medium salt buffer containing NaH_2PO_4 (10 mM), Na_2HPO_4 (5 mM), NaCl (100 mM) and EDTA (0.1 mM), pH 7.0 with 1 μM concentrations of the two complementary sequences. The extinction coefficients were calculated assuming the extinction coefficients for nucleotide **2** to equal one thymidine and one uridine. The increase in absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 10 to 90 °C at a rate of 1.0 °C/min. The melting temperature was determined as the local maximum of the first derivatives of the absorbance *versus* temperature curve. For melting experiments with Mg^{2+}-concentrations of 5 or 10 mM, $MgCl_2$ (1.0 μL and 2.0 μL, respectively, of a 5.0 M solution) was added to the samples.

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