## 2'-O-[(4-CF<sub>3</sub>-triazol-1-yl)methyl] Uridine – A Sensitive <sup>19</sup>F-NMR Sensor for the Detection of RNA Secondary Structures

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## Abstract

A sensitive uridine-derived sensor {viz. 2'-O-[(4-CF<sub>3</sub>-triazol-1-yl)methyl]uridine, **1**} for <sup>19</sup>F NMR spectroscopic monitoring of RNA secondary structures is described. The applicability of **1** is demonstrated by monitoring the thermal denaturation of the double and triple helical RNA models [1) miR 215 hairpin, 2) poly U-A\*U-triple helix RNA (bearing two C-G\*C<sup>H+</sup>-interrupts) and 3) polyadenylated nuclear – nuclear retention element (PAN–ENE) complex]. In these RNA models, the <sup>19</sup>F NMR shift of the 2'-O-(CF<sub>3</sub>-triazolylmethyl) group shows high sensitivity to secondary structural arrangements. Moreover, **1** favours the desired *N*-conformation and its effect on both RNA duplex and triplex stabilities is marginal.

### INTRODUCTION

<sup>19</sup>F NMR spectroscopy has received considerable attention as a method for investigating the structure, dynamics and molecular interactions in oligonucleotides.<sup>1-21</sup> Compared to conventional spectrophotometric methods (UV-, CD- and fluorescence-based), sensitivity is low and the required <sup>19</sup>F-labelling itself is a limitation, yet the superiority of <sup>19</sup>F NMR may become distinct with more detailed information about the structure (especially in local environment) and molar ratios of secondary structural species. The characteristic shift of the <sup>19</sup>F nucleus, with wide chemical shift dispersion, is highly sensitive to local van der Waals interactions and electrostatic fields, which facilitates the detection of even minor secondary structural arrangements.<sup>22-26</sup> It may also be worth noting that remarkable breakthroughs have been made in NMR of hyperpolarized fluorine (HPF NMR, several thousand fold enhanced sensitivity) in studies of protein-ligand interactions.<sup>27</sup> There is a likelihood that HPF NMR may soon find applications in oligonucleotides as well.<sup>28</sup>

We and other research groups have focused on developing novel fluorine-labeled nucleoside derivatives, aimed at increasing the sensitivity and straightforwardness of <sup>19</sup>F NMR-detection of oligonucleotides.<sup>7,16,19,21</sup> The incorporation of a trifluoromethyl group via an appropriate proton coupling barrier into nucleosides is an obvious consequence in this design. The effect of the sensor on the native oligonucleotide structure should additionally be as marginal as possible, while the resulting <sup>19</sup>F signal shift should readily reflect secondary structural changes. <sup>19</sup>F NMR spectroscopic detection at micromolar oligonucleotide concentration may then be carried out rapidly and reliably by routine instrumentations without the need of <sup>1</sup>H decoupling. Micura et al. have recently successfully used 2'-trifluoromethylthio-modified ribonucleic acids as sensors for the <sup>19</sup>F NMR-spectroscopic detection of RNA-protein and RNA-small molecule interactions and of molar fractions of bistable RNAs.<sup>16,21</sup> While the shift of the 2'-trifluoromethylthio group was highly sensitive to secondary structural changes, this modification, unfortunately, markedly decreased the RNA duplex stability. This decrease was a result of strong *C*-2'-endo (*S*-conformation) preference

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of these ribonucleosides. We recently used 4-C-[(4-trifluoromethyl-1H-1,2,3-triazol-1-yl)methyl]thymidine (2) as a sensor for monitoring both DNA and RNA-secondary structures.<sup>20</sup> This deoxynucleoside, with predominant*S*-conformation, seemingly favored DNA environment, but it did not decrease RNA duplex stability either. The equilibrium between the*C*-2'- and*C*-3'- endo puckering of**2**was probably facile enough to adopt proper conformation for both types of helices (A and B). Although detailed <sup>19</sup>F NMR spectroscopic information by**2**may be gained, the*C*-4'-position for the label is, however, not the best possible for RNA. The*C*-4' site orients the label outward from the RNA helix, which may lead to a modest shift discrimination between the duplex and the single strand. Furthermore, this modification at*C*-4' may hardly be expanded to purine nucleosides.

In the present study we describe a new and promising <sup>19</sup>F NMR sensor for RNA: viz 2'-*O*-[(4-trifluoromethyl-1*H*-1,2,3-triazol-1-yl)methyl]uridine (**1**). The 2'-*O*-(CF<sub>3</sub>-triazolylmethyl) group offered a quasi-isolated <sup>19</sup>F spin system (as in **2**) and its <sup>19</sup>F resonance shift was highly sensitive to secondary structural changes. As expected, this modified nucleoside preferred *C*-3'-endo sugar puckering (*N*-conformation) and it neither affected the RNA duplex nor the RNA triplex stabilities (demonstrated by UV- and CD-melting profiles of the RNA models studied). Additionally, synthesis of the phosphoramidite derivate (**10**) was simple (Scheme 1) and its incorporation into RNA strands by an automated synthesizer was efficient. The <sup>19</sup>F NMR spectroscopic monitoring of thermal denaturation of RNA hairpins (<sup>19</sup>F-labelled models of miR 215<sup>29</sup> and PAN) and RNA-triple helices (<sup>19</sup>F-labelled models of poly U-A\*U-model, interrupted by two C-G\*C<sup>H+</sup> triplets and PAN-ENE-complex) was demonstrated. For the monitoring of thermal denaturation of an RNA hairpin (mir 215 model), the shift response of **1** was compared to those of two other potential sensors [**2** and **4'**-*C*-(4-trifluorophenyl)uridine **3**, the latter also synthesized in the present study].



Figure 1. Structures of the <sup>19</sup>F NMR sensors (1-3) studied

## **RESULTS AND DISCUSSION**

Synthesis of the phosphoramidite building blocks of 2'-O-[(4-trifluoromethyl-1H-triazol-1-4'-C-(4-trifluorophenvl)uridine 2'-0yl)methyl]uridine (10)and (20). (Azidomethyl)ribonucleosides may be prepared from the corresponding 2'-O-(methylthiomethyl)-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)ribonucleosides.<sup>30,31</sup> 2'-O-(Azidomethyl)-5'-O-(4,4'-dimethoxytrityl)uridine (8 in Scheme 1) was obtained following this reported protocol and treated with gaseous 3,3,3-trifluoroprop-1-yne in the presence of CuSO<sub>4</sub> and sodium ascorbate to yield 2'-O-[(4-trifluoromethyl-1H-1,2,3-triazol-1-yl)methyl]uridine **9** in 88% yield. It may be worth noting that 3,3,3-trifluoropropyne is able to react with alkylazides also in the copper-free conditions, but a mixture of 4- and 5-trifluoromethyl-1H-1,2,3-triazoles may be obtained.<sup>32</sup> Phosphitylation of the 3'-OH group gave the desired phosphoramidite 10. As seen, the synthesis of the phosphoramidite derivative of 1 was rather straightforward (six synthetic steps from commercially available 4, overall yield 47%).

 Scheme 1



(i) DMSO, Ac<sub>2</sub>O, AcOH; (ii); SO<sub>2</sub>Cl<sub>2</sub>, DCM; (iii) NaN<sub>3</sub>, DMF; (iv) TBAF, THF; (v) DMTrCl, Py;
(vi) 3,3,3-trifluoroprop-1-yne, CuSO<sub>4</sub>, sodium ascorbate, H<sub>2</sub>O, dioxane; (vii) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, Et<sub>3</sub>N, DCM.

Synthesis of the phosphoramidite **20** is outlined in Scheme 2. Ketone **11** could be prepared in a gram scale according to the literature.<sup>33</sup> Stereoselective Grignard reaction with 4bromobenzotrifluoride to **11** gave the desired addition product (**12**) in 73% yield (2*R*:2*S*, 7:1, *n/n*). The TBDMS group (**12**) was then replaced by the benzoyl group (**13**), since the premature exposing of the 5-OH group may lead to an undesired pyranose formation (cf. the forthcoming synthetic steps between **13**, **14** and **15**). In our preliminary attempt that took place with TBDMS protected 5-OH group upon the acid catalyzed isopropylidene removal (iv in Scheme 2). Oxidative release of the anomeric center (**14**), acid catalyzed removal of the isopropylidene group, perbenzoylation (**15**) and *N*-glycosidation gave 4'-*C*-[4-(trifluoromethyl)phenyl]uridine (**16**) in 38% overall isolated yield from **11**. The neighboring group participation *via* 2'-*O*-benzoyl group gave predominantly  $\beta$ -*N*-glycosidated product (1'*R*). A clear 2D NOESY correlation between H<sup>1'</sup> and aromatic protons of CF<sub>3</sub>Ph may be observed, which further verified the desired 4'*R*-configuration (see the Supporting information). 4,4'-Dimethoxytritylation of **17** was the bottleneck of the synthesis (**18** in 40% yield). Due to the steric hindrance of the CF<sub>3</sub>Ph group, the reactivity of the 5'-OH group was close to that of the 2'-OH group and a remarkable amount of 2',5'-bistritylated uridine was obtained. Several trials were conducted to overcome this problem. As an example, selective benzoyl removal from the primary hydroxyl group of **16** using [*t*BuSnOHCl]<sub>2</sub> in methanol,<sup>34</sup> followed by 4,4'-dimethoxytritylation and subsequent NaOMe-catalyzed removal of 2'-*O*- and 3'-*O*-acetyl groups gave **18** in 51% yield. In spite of the slight improvement of yield, this method was somewhat complex and repeating of the reaction from **17** to **18** (**17** readily recovered from the bistritylated uridine) turned out to be practically the best route for **18**. The 2'-OH could be selectively silylated by TOM-group (**19**) and phosphitylation of the 3'-OH (**27**) group gave finally **20**. The overall yield from **11** to **20** remained as low as 6%.

Scheme 2



(i) 4-bromobenzotrifluoride, Mg, Et<sub>2</sub>O; (ii) TBAF, THF; (iii) BzCl, DMAP, Py; (iv) 1: OsO<sub>4</sub>, 4methylmorpholine *N*-oxide, acetone; 2: H<sub>5</sub>IO<sub>6</sub>, THF; (v) HCl, H<sub>2</sub>O, dioxane; (vi) TMSOTf, 2,4bis(trimethylsilyloxy)uridine, MeCN; (vii) NaOMe, MeOH; (viii) DMTrCl, Py; (ix) [*t*BuSnOHCl]<sub>2</sub>, MeOH; (x) DIEA, Bu<sub>2</sub>SnCl<sub>2</sub>, triisopropylsilyloxymethylchloride, 1.2-dichloroethane; (xi) 2cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, Et<sub>3</sub>N, DCM.

Stability of 2'-O-[(4-trifluoromethyl-1*H*-1,2,3-triazol-1-yl)methyl]uridine and 4'-*C*-(4-trifluorophenyl)uridine in acidic and basic conditions. 2'-O-[(4-Trifluoromethyl-1*H*-1,2,3-triazol-1-yl)methyl]uridine and 4'-*C*-(4-trifluorophenyl)uridine may, in principle, undergo acid catalyzed hydrolysis of the 2'-O-methyltriazolyl group (1) and epimerization at the *C*-4'-position / ring opening of the ribose sugar (3), respectively. The stability of these nucleosides has been evaluated in 80% aqueous acetic acid at 25°C and additionally in concentrated aqueous ammonia at

55°C. Both nucleosides seemed virtually intact in these treatments after three days. Potential side reactions within the nucleoside units (**1** and **3**) are therefore hardly expected, for example in the treatments required for the automated RNA synthesis (*i.e.* removal of DMTr and nucleobase protections) or in the prolonged incubation at elevated temperature required for the <sup>19</sup>F NMR-melting temperature studies of the synthesized RNAs.

**Sugar conformation of the sensors.** Optimized Karplus relation<sup>35</sup> for <sup>1</sup>H NMR H1'-H2' coupling constants ( $J^{\text{H1'-H2'}}$ ) was used to evaluate the sugar puckering of the sensors (as nucleosides). Assuming a pure *N/S* (C3'/C2'-endo) equilibrium, 2'-*O*-(4-CF<sub>3</sub>-triazolylmethyl)uridine (cf. **1**) existed predominantly as a C3'-endo conformation (*N* 67%,  $J^{\text{H1'-H2'}} = 3.3$  H). The corresponding values for 4'-*C*-(4-CF<sub>3</sub>-triazolylmethyl)thymidine (cf. **2**), 4'-*C*-(4-trifluoromethylphenyl)uridine (cf. **3**) and uridine were S 72% ( $J_{\text{H1'-H2'}} = 7.3$  Hz), *N* 48% ( $J_{\text{H1'-H2'}} = 5.3$  Hz) and *N* 53% ( $J_{\text{H1'-H2'}} = 4.8$  Hz), respectively. While the duplex formation usually increases the favoured C3'-endo population of a single RNA residue (in a A-form RNA double helix),<sup>21</sup> relatively high C2'-endo populations for single–stranded residues may, however, be observed.<sup>16,21</sup> Short RNA-sequences (5'-AU1A-3' and 5'-AUUA-3') were additionally prepared and the *N/S*-ratio for each residue was determined. The C3'-endo population of **1** in AU1A sequence was now *N* 32% ( $J_{\text{H1'-H2'}} = 5.3$  Hz, A), 50% ( $J_{\text{H1'-H2'}} = 5.1$  Hz, A) and 46% ( $J_{\text{H1'-H2'}} = 5.5$  Hz, U). In AUUA model, the corresponding values (*N* %) were 50% ( $J_{\text{H1'-H2'}} = 5.1$  Hz, A), 46% ( $J_{\text{H1'-H2'}} = 5.5$  Hz, A), 44% ( $J_{\text{H1'-H2'}} = 5.7$  Hz, U) and 46% ( $J_{\text{H1'-H2'}} = 5.5$  Hz, U).

**Oligonucleotide synthesis.** Fluorine labelled oligoribonucleotides (**ORN 1 – 5**, see structures in Table 1 and Figures 3, 4 and 5) were synthesized on a 1.0  $\mu$ mol scale using an automatic DNA / RNA synthesizer. Benzylhiotetrazole as an activator and a coupling time of 300 s for the standard

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RNA building blocks and of 600 s for the fluorine labeled nucleosides (**10** and phosphoramidite derivative of **2**) were used. The coupling efficiency of **10** and the phosphoramidite derivative of **2** in the automatic synthesizer did not differ from those of the standard 2'-*O*-TBDMS-protected RNA-building blocks. A manual coupling (see the Experimental Procedures) with a higher (0.11 mol L<sup>-1</sup>) phosphoramidite concentration was however required to give an acceptable coupling yield (90% according to DMTr assay) for **20**. The oligoribonucleotides were released from the support with a mixture of concentrated ammonia and ethanol (3:1, *v/v*, 3h at 55°C + overnight at r.t.). The silyl protections were removed by a treatment with triethylamine trihydrofluoride followed by RP cartridge filtration. The filtrates were purified by RP HPLC and the authenticity of **ORN 1 – 5** was verified by MS (ESI-TOF) spectroscopy (Table 1). A representative example (**ORN 4**) of RP HPLC profiles of crude ORNs and of MS-spectra are shown in Figure 2. Isolated yields **ORN 1 – 5** ranged from 5 to 10 % (Table 1).

Table 1. The observed and calculated molecular masses of ORN 1-5.

ORN 1-3: 5'-CAC AGG AAA AUG ACU UCG GCC AAU AUU CUG UG-3'
ORN 4: 5'-AAA GAA AAG A $\sim$ UCU U $\mu$ U CUU U $\sim$ UUU CUU UUC-3'
ORN 5: 5'-GGC UGG GUU UUU CCU UCG AAA GAA GGU UUU UAU CCC AGU C-3'
ORN 1, ORN 4 and ORN 5: $U = 1$ ORN 2: $U = 2$ ORN 3: $U = 3$ bold letters are 2'-O-methyl ribonucleotides $\sim = \sum_{0}^{p} \sum_{0}^{c} \left\{ - \sum_{0}^{0} \right\}_{0}^{c}$

entry	<sup>19</sup> F-labelled RNA	Isolated yield	observed molecular	calculated average
			mass	molecular mass
1	ORN 1	7%	10438.9 <sup>a</sup>	10437.4
2	ORN 2	10%	10435.2 <sup>a</sup>	10435.4
3	ORN 3	5%	10431.9 <sup>a</sup>	10432.4
4	ORN 4	7%	10219.4 <sup>a</sup>	10219.2
5	ORN 5	7%	12989.4 <sup>b</sup>	12989.9

<sup>a</sup>calculated from the most intensive isotope combination at [(M-10H)/10]<sup>-10</sup>, <sup>b</sup>calculated from the

most intensive isotope combination at [(M-13H)/13]<sup>-13</sup>

entry	oligoribonucleotides	duplex T <sub>m</sub> /°C	triplex $T_{\rm m}$ /°C
1	ORN 1	52.9 (-0.7)	-
2	ORN 2	53.1 (-0.5)	-
3	ORN 3	53.4 (-0.2)	-
4	ORN 4	59.3 (-0.6)	21.8 (-1.6)
5	<b>ORN 4</b> <sup>a</sup>	60.4 (-0.6) <sup>a</sup>	26.5 (+0.1) <sup>a</sup>
6	ORN 5	68.8 (-0.2)	-
7	<b>ORN 5 +</b> A <sub>9</sub>	68.3 (-0.8)	n.d.

**Table 2.** UV-melting experiments  $(T_m/^{\circ}C)$  of the <sup>19</sup>F-labelled oligoribonucleotides (**ORN 1-5**).

n.d: absorbance change referring to denaturation of **ORN 5** + A<sub>9</sub>-complex severely overlapped with background absorbance of **ORN 5**. Inflection point cannot be determined (profiles in the Supporting Information).  $\Delta T_{\rm m}$  (in parentheses) in comparison to those obtained with unmodified oligoribonucleotides (U = uridine). Conditions: 10 mmol L<sup>-1</sup> sodium cacodylate (pH = 7.0), 0.1 mol L<sup>-1</sup> NaCl, 2.0 µmol L<sup>-1</sup> of each oligonucleotide, UV-detection at 260 nm. <sup>a</sup>1.0 equiv neomycin was added to a mixture.



**Figure 2.** An example (**ORN 4**) of RP HPLC profiles of the crude product mixtures and of MS (ESI-TOF) spectra of the homogenized products.

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Melting temperature studies of fluorine labelled RNAs. UV-melting temperatures of the <sup>19</sup>Flabelled oligoribonucleotides ORN 1-4 and ORN 5 + A9-complex and the  $\Delta T_m$ -values in comparison to those obtained with unmodified oligoribonucleotides (U = 1 or uridine, cf. structures in Figures 3, 4 and 5) are shown in Table 2 (2 µmol L<sup>-1</sup> of each ORN in a mixture of 0.1 mol L<sup>-1</sup> NaCl and 10 mmol L<sup>-1</sup> sodium cacodylate at pH 7.0). As seen, none of the studied sensors (1, 2 and 3) affected the hairpin stability. The effect of 1 on the stability of triple helical structure of ORN 4 was also marginal (UV-melting profiles of ORN 1-3 are shown in Figure 3 and those of ORN 4 and ORN 5 + A<sub>9</sub>-complex in the Supporting Information)

<sup>19</sup>F NMR Measurements. From the <sup>19</sup>F NMR spectroscopic point of view, the minimal requirement for the sensor is the <sup>19</sup>F NMR shift, which responds well to hybridization. As a matter of fact, this shift discrimination (between the double helix and single strand) alone may be expanded for many more interesting <sup>19</sup>F NMR applications (cf. characterization of an RNA invasion<sup>9,12,20</sup> and determination of molar fractions of bistable RNAs<sup>3,16</sup>). As a first <sup>19</sup>F NMR experiment, 1, 2 and 3 were incorporated at the same site of a miR 215 hairpin model (ORN 1, **ORN 2** and **ORN 3**, U = 1, 2 and 3, respectively) and their <sup>19</sup>F NMR-applicability to detect thermal denaturation of this hairpin RNA was evaluated. The <sup>19</sup>F NMR measurements were carried out using RNA concentration of 50  $\mu$ mol L<sup>-1</sup> (**ORN 1**, **ORN 2** and **ORN 3** in a mixture of 0.1 mol L<sup>-1</sup> NaCl and 10 mmol L<sup>-1</sup> sodium cacodylate at pH 6.0). The <sup>19</sup>F NMR shift – versus – temperature profiles between 30 and 70°C are shown in Figure 3a, and the profiles of the shift differences – versus – temperature after subtraction of a passive temperature – dependent shift (0.014 ppm  $K^{-1}$ ) are shown in Figure 3b. Each sensor gave coalescence signal, which discriminated between the miR 215 hairpin (A) and the denaturated form (B). The signals referring to the hairpin (A) shifted to lower magnetic field and negative S-curves were obtained in each case. The inflection points showed nearly the same  $T_m^{A/B}$  value (ca 55°C), but 1 gave the largest shift dispersion (Figure 3b:

 $\Delta \delta$  1: 0.82 ppm, 2: 0.23 ppm and 3: 0.12 ppm between 30 and 70°C). The 4'-*C*-fluorine labels (2 and 3) were probably relatively naked at this site (*U*) in the stem region (*i.e.* the modest change in the local environment around *C*-4' of 2 and 3 upon hybridization led to a small shift response.). The large downfield shift of 1 was most likely related to the shielding of *H*-5',5''-protons of the preceding nucleotide in the helix. Due to the smallest shift dispersion obtained by 3 (together with the complex synthesis of 20 and the reduced coupling efficiency in the automated RNA synthesis) further effort to prove the potential of this seemingly promising sensor (3 bears free 2'-OH unlike 1 and 2) was excluded. Sensor 2 was, in turn, primarily designed for the DNA environment and its applicability has previously been described.<sup>20</sup> The superiority of 1 (the large shift dispersion, the simple synthesis of 10 and its efficient coupling) among these three sensors (1, 2 and 3) seemed obvious and its functionality was then further evaluated.

The <sup>19</sup>F NMR spectra obtained upon denaturation of **ORN 1** are shown in Figure 3c. In addition to main coalescence signal, there may be seen a minor signal (marked with asterisk \*). The shift of the minor signal followed mainly the passive temperature–dependent shift (slope: 0.014 ppm °C<sup>-1</sup>), but a modest downfield turn was observed on approaching the denaturation temperature ( $T_m^{A/B} = 55^{\circ}$ C). The downfield turn and the continuously reduced molar fraction in decreasing temperature refer to an incomplete coalescence signal (*i.e.* \* refers to **B**), but the minor signal may also partly be traced to hydrolytic cleavage products. Partial hydrolytic cleavage of **ORN 1** (and increase of the minor signal \*) was, in fact, observed after prolonged incubations at elevated temperatures.



**Figure 3.** <sup>19</sup>F NMR-shift *vs.* temperature – profiles obtained by sensors **1**, **2** and **3** upon thermal denaturation of a miR 215 hairpin model (**A/B**) (a and b); <sup>19</sup>F NMR spectra of **ORN 1** in different temperatures (50  $\mu$ mol L<sup>-1</sup> of **ORN 1** in 0.1 mol L<sup>-1</sup> NaCl and 10 mmol L<sup>-1</sup> sodium cacodylate at pH 6.0) (c); The corresponding UV-melting profiles of **ORN 1-3** (*T*<sub>m</sub>-values listed in Table 2) (d).

The scope of **1** was then expanded for <sup>19</sup>F NMR spectroscopic monitoring of RNA-triple helix/duplex/single strand conversion. **1** was incorporated to a previously studied poly U-A\*U model (interrupted by two C-G\*C<sup>H+</sup>-triplets, note: two pyrimidine strands and one purine strand are connected to each other by hexaethylene glycol spacers, **ORN 4**, Figure 4).<sup>36</sup> The triplex/duplex/single strand – conversion was monitored by <sup>19</sup>F NMR spectroscopy using 50 µmol L<sup>-1</sup> of **ORN 4** in a mixture of 0.1 mol L<sup>-1</sup> NaCl and 10 mmol L<sup>-1</sup> sodium cacodylate at pH 7.0. Like above with miR 215 model, a well-behaving coalescence signal was obtained for the melting of intramolecular double helix (Figure 4a: **D** *vs.* **E**). The <sup>19</sup>F NMR shift–versus–temperature profile as a negative *S*-curve is shown in Figure 4b, in which the inflection point shows  $T_m^{D/E} = 59^{\circ}$ C –value between the double helix (**D**) and the single strand (**E**). At a lower temperature (40°C) a separate

new <sup>19</sup>F NMR resonance signal appeared. When the temperature was decreased further, a relative peak area of this new signal increased and it was equal sized with the duplex signal at 32.5°C (Figure 4a). This new signal refers to a triplex, which denaturates at 32.5°C. Separate signals for the duplex and triplex, in turn, indicate equilibrium of an inter-molecular process (*i.e.* C). The <sup>19</sup>F NMR measurements were then additionally carried out at a lower **ORN 4** concentration. As seen in Figure 4a, the triplex formation was concentration–dependent: In the mixture of 50 µmol L<sup>-1</sup> of **ORN 4** the duplex and triplex signals were equal sized at 32.5°C ( $= T_m^{C/D}$ ), whereas in the mixture of 5 µmol L<sup>-1</sup> of **ORN 4**, only a trace of the triplex signal may be seen. Thus, the observed signal refers to a triple helical dimer of **ORN 4** (i.e. C) and not to an expected intra-molecular triplex (F).<sup>36</sup> Molar fractions of C, D and E, shown in Figure 4c, may be finally extracted from the relative peak areas of the signals (C vs. D, Figure 4a) and from the shift–versus–temperature profile (D vs. E, Figure 4b) after subtraction of the passive temperature–dependent shift.





**Figure 4.** <sup>19</sup>F NMR spectra of **ORN 4** in different temperatures (5 and 50  $\mu$ mol L<sup>-1</sup> of **ORN 4** in 0.1 mol L<sup>-1</sup> NaCl and 10 mmol L<sup>-1</sup> sodium cacodylate at pH 7.0) (a); <sup>19</sup>F NMR shift–versus–temperature profile of **ORN 4** (**D** *vs.* **E**) (b); Molar fractions of dimeric triple helix **C**, duplex **D** and single strand **E** of **ORN 4** (c).

Polyadenylated nuclear – nuclear retention element (PAN – ENE) complex has recently received considerable medical and biological attention.<sup>37,38</sup> PAN RNA is a long noncoding RNA produced by the oncogenic gammaherpesvirus KSHV, it accumulates extraordinary high levels during lytic infection and is required for the production of late viral proteins. Triple helix formation with ENE is essential for this high accumulation. Although spectrophotometric data of this complex have been

documented, the inflection point of the denaturation of PAN-ENE complex is usually severely overlapped by the strong background absorbance of PAN. More accurate determination of molar fractions of the secondary structural species throughout the denaturation is, however, required e.g. for the determination of thermodynamic parameters and therefore an alternative insight for the monitoring of this complex would be advisable. In the present study, applicability of <sup>19</sup>F NMR spectroscopy using 1 as a sensor for the more detailed denaturation analysis of a model of PAN-ENE complex has been demonstrated. ORN 5 (Figure 5) was mixed with A<sub>9</sub> and the triplex/duplex/single strand – conversion was monitored by  ${}^{19}$ F NMR spectroscopy (50 µmol L<sup>-1</sup> of **ORN 5** and A<sub>9</sub> in a mixture of 0.1 mol  $L^{-1}$  NaCl and 10 mmol  $L^{-1}$  sodium cacodylate at pH 7.0). The bipartite triple helical structure  $\mathbf{G}$  in Figure 5 has previously been described for the same PAN - ENE model system<sup>38</sup> (in our case **1** and four 2'-O-methyl ribonucleotides have been incorporated into the structure). As seen in <sup>19</sup>F NMR spectra (Figure 5a), the system (**ORN 5** +  $A_9$  in different temperatures) behaved in a similar manner as described above for ORN 4. A well-behaving coalescence signal was obtained for the hairpin melting (H vs. I), whereas the intermolecular complex formation (**ORN 5** +  $A_9$ ) gave a separate signal (G). Despite the labelling site that was clearly outside the expected binding region, 1 could distinguish surprisingly well between the bipartite triple helical structure of **ORN 5** +  $A_9$  (G) and **ORN 5** hairpin (H). Molar fractions of the secondary structures upon hairpin denaturation (**H** vs. **I**) may be extracted from the shift of the coalescence signal after subtraction of the passive temperature-dependent shift, whereas the molar fractions of **G** and **H** from the relative peak areas of the <sup>19</sup>F NMR resonance signals (Figure 5b). Melting temperatures of  $T_{\rm m}^{\rm G/H} = 20^{\circ}$ C and  $T_{\rm m}^{\rm H/I} = 68^{\circ}$ C were obtained. The measurements were additionally carried out in the presence of neomycin (5 equiv, a known groove binder for RNA triple helices<sup>39</sup>). A remarkable stabilization of the triple helical complex ( $T_m^{G'/H'} = 48^{\circ}C, \Delta T_m =$ 28°C) was expectedly observed, but the stability of hairpin was also affected by neomycin: the melting temperature ( $T_{\rm m}^{\rm H'/\Gamma}$  = 83°C) of hairpin **H** increased by  $\Delta T_{\rm m}$  = 16°C.



**Figure 5.** <sup>19</sup>F NMR spectra of **ORN 5** + A<sub>9</sub> in different temperatures in the presence and absence of neomycin (5 equiv) (50  $\mu$ mol L<sup>-1</sup> **ORN 5** and A<sub>9</sub> in 0.1 mol L<sup>-1</sup> NaCl and 10 mmol L<sup>-1</sup> sodium cacodylate at pH 7.0) (a); Molar fractions of different secondary structures in different temperatures (symbols **G**, **H**, **I** and solid lines refer to a mixture without neomycin, symbols **G'**, **H'**, **I'** and dotted lines to a mixture in the presence of neomycin) (b).

#### CONCLUSION

2'-O-[(4-Trifluoromethyl-1*H*-1,2,3-triazol-1-yl)methyl]uridine (1) proved to be an excellent <sup>19</sup>F NMR sensor for the characterization of RNA secondary structures. The advantages of 1 may be summarized as follows: 1) synthesis of the corresponding phosphoramidite (10) was straightforward, 2) the coupling efficiency of 10 in automated RNA-synthesis, 3) the <sup>19</sup>F-NMR

signal of **1** was sensitive to secondary structural changes with relatively wide shift dispersion and 4) **1** did not affect either the RNA duplex or RNA triplex stabilities (duplex  $\Delta T_{\rm m} < 1^{\circ}$ C and triplex  $\Delta T_{\rm m} < 2^{\circ}$ C). The applicability of **1** was demonstrated for the <sup>19</sup>F-NMR-spectroscopic monitoring of thermal denaturation of RNA hairpins (**ORN 1, ORN 4** and **ORN 5**) and triplex/duplex/single strand conversion upon denaturation of a poly U-A-U-model (interrupted by two C-G\*C<sup>H+</sup>-triplets, **ORN 4**) and a model of PAN–ENE complex (**ORN 5** + A<sub>9</sub>). The applicability of <sup>19</sup>F NMR spectroscopy for the monitoring of an RNA triple helix was described for the first time.

#### EXPERIMENTAL PROCEDURES

**General Remarks.** Dichloromethane and MeCN were dried over 3 Å molecular sieves and triethylamine over CaH<sub>2</sub>. The NMR spectra were recorded at 500 MHz. The chemical shifts in <sup>1</sup>H and <sup>13</sup>C NMR spectra are given in ppm from the residual signal of the deuterated solvents CD<sub>3</sub>OD and CD<sub>3</sub>CN for. <sup>31</sup>P-shifts are referenced to external H<sub>3</sub>PO<sub>4</sub> and <sup>19</sup>F-shifts to external CCl<sub>3</sub>F. The mass spectra were recorded using ESI ionization.

# 5'-O-(4,4'-Dimethoxytrityl)-2'-O-[(4-trifluoromethyl-1*H*-1,2,3-triazol-1-yl)methyl]uridine (9). To a mixture of 5'-O-(4,4'-dimethoxytrityl)-2'-O-azidomethyluridine<sup>30,31</sup> (8, 0.33 g, 0.55 mmol) in DMSO (3.5 mL), aqueous solutions of CuSO<sub>4</sub> (0.1 mol L<sup>-1</sup>, 55 $\mu$ L, 5.5 $\mu$ mol) and sodium ascorbate (0.1 mol L<sup>-1</sup>, 0.55 mL, 55 $\mu$ mol) were added. The reaction was carried out in a sealed tube, placed in an ice bath and bubbled with gaseous 3,3,3-trifluoropropyne for 5 min. The reaction mixture was then stirred at room temperature for two days, during which time the gas was added seven times in an ice bath along with additions of the aqueous solutions of CuSO<sub>4</sub> and sodium ascorbate (the same amounts as above). The reaction mixture was then partitioned between dichloromethane and saturated aqueous NaHCO<sub>3</sub>. The organic phase was separated, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by silica gel chromatography (0.1 % Et<sub>3</sub>N, 5% MeOH in DCM)

to yield 0.34 g (88%) of the product **9** as a yellowish foam. <sup>1</sup>H NMR(500 MHz, CDCl<sub>3</sub>):  $\delta$  8.31 (s, 1H), 8.04 (d, 1H, J = 8.2 Hz), 7.39-7.38 (m, 2H), 7.32-7.23 (m, 7H), 6.87-6.85 (m, 4H), 6.17 (d, 1H, J = 11.1 Hz), 6.04 (d, 1H, J = 11.1 Hz), 5.93 (d, 1H,  $J^{1'2'}$  cant not be determined), 5.34 (d, 1H, J = 8.2 Hz), 4.49 (dd, 1H, J = 8.2 Hz and 5.2 Hz), 4.27 (dd, 1H , J = 5.2 Hz and  $J^{1',2'}$ ), 4.11 (m, 1H), 3.81 and 3.80 (2 × s, 2 x 3H), 3.61 (dd, 1H, J = 11.4 Hz and 2.0 Hz), 3.54 (dd, 1H, J = 11.3 Hz and 2.2 Hz); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  163.3, 158.8, 158.7, 150.9, 144.2, 139.5, 139.3 (q, J = 39.6 Hz), 135.2, 135.0, 130.15, 130.06, 128.10, 128.07, 127.2, 124.5, 120.2 (q, J = 268 Hz), 113.4, 102.5, 88.1, 87.2, 83.0, 81.6, 77.7, 68.05, 60.7, 55.3; <sup>19</sup>F NMR (470 MHz, CD<sub>3</sub>CN):  $\delta$  -63.51; HRMS (ESI-TOF) m/z: [M-H]<sup>-</sup> calculated for C<sub>34</sub>H<sub>31</sub>F<sub>3</sub>N<sub>5</sub>O<sub>8</sub> 694,2125, found 694.2119.

**3'-O-[(2-Cyanoethoxy)-(N,N-diisopropylamino)phosphinyl]-5'-O-(4,4'-dimethoxytrityl)-2'-O-[(4-trifluoromethyl-1H-1,2,3-triazol-1-yl)methyl]uridine (10).** Compound **9** (0.24 g, 0.35 mmol) was dried over P<sub>2</sub>O<sub>5</sub> in a vacuum desiccator and dissolved in dry dichloromethane (2.0 mL). Triethylamine (244  $\mu$ L, 1.75 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (0.18 mL, 0.80 mmol) were added and the mixture was stirred under nitrogen for 2 h. The reaction mixture was eluted through a short dried silica gel column (50-100 % EtOAc and 5% Et<sub>3</sub>N in hexane) to yield 0.33 g of **10** as white foam (87% yield, calculated by subtracting the mass of 2-cyanoethyl *N*,*N*-diisopropylphosphonamidate, the obtained side product according to the <sup>1</sup>H NMR spectrum). A 6:4 (*n/n*)-mixture of *Rp/Sp*-diastereomers was obtained [distinguished in the spectra as *I* (major diastereomer) and *II* (minor diastereomer)]. <sup>1</sup>H NMR(500 MHz, CD<sub>3</sub>CN):  $\delta$  8.52 (d, 0.6H, *J* = 0.7 Hz, *I*), 8.49 (d, 0.4H, *J* = 0.6 Hz, *II*), 7.86 (d, 0.4H, *J* = 8.2 Hz, *I*), 7.80 (d, 0.6H, *J* = 8.2 Hz, *II*), 6.12 (d, 0.6H, *J* = 11.4 Hz, *I*), 6.06 (d, 0.6H, *J* = 11.4Hz, *I*), 5.99 (d, 0.4H, *J* = 11.4, *II*), 5.91 (d, 0.4H, *J* = 2.2 Hz, *II*), 5.87 (d, 0.6H, *J* = 2.0 Hz, *I*), 5.17 (d, 0.4H, *J* = 8.2Hz, *II*), 5.15 (d, 0.6H, *J* = 8.2, *I*, *I*, 4.62-4.53 (m, 1.4H), 4.46 (dd, 0.6H, *J* = 5.0 and 2.1 Hz, *I*), 4.23 (ddd, 0.4H, *J* = 7.4, 2.5 and 2.3 Hz, II), 4.17 (m, 0.6H), 3.86-3.82 and 3.74-3.59 (both m, 2H), 3.804 and 3.802 (both s,  $0.4 \times 6H$ , II), 3.797 (s, 0.6 × 6H, I), 3.57-3.48 (m, 3H), 3.45 (dd, 0.4H, J = 11.3 and 2.8 Hz, II), 3.41 (dd, 0.6H, J = 11.2 and 3.2 Hz, I), 2.70 (m, 0.6 × 2H), 2.55 (m, 0.4 × 2H), 1.15 (d, 0.4 × 6H, J = 6.7 Hz, I), 1.14 (d, J = 6.7 Hz,  $0.6 \times 6H$ , II), 1.08 (d,  $0.4 \times 6H$ , II), 1.05 (d,  $0.6 \times 6H$ , I); <sup>13</sup>C NMR (125 MHz,  $CDCl_3$ :  $\delta$  162.9 (both I and II), 158.83 and 158.82 (both I and II), 150.5 (II) 150.4 (I), 144.7 (II), 144.6 (*I*), 139.6 (*I*), 139.5 (*II*), 138.1 (q, J = 39.0 Hz, both *I* and II), 135.5 and 135.4 (*II*), 135.3 and 135.2 (I), 130.21, 130.18, 128.1, 128.0 and 127.1 (both I and II), 125.5 (m, II), 125.4 (m, I), 120.9 (q, J = 266.7 Hz, both I and II), 118.5 (II), 118.0 (I), 101.6 (II), 101.5 (I), 88.5 (I), 88.1 (II), 86.71(I), 86.69 (II), 82.4 (d, J = 2.7 Hz, II), 81.8 (d, J = 6.4 Hz, I), 80.9 (I), 79.7 (d, J = 3.0 Hz, II), 78.1 (I), 77.7 (II), 69.6 (d, J = 14.0 Hz, I), 69.2 (d, J = 9.0 Hz, II), 60.99 (I), 60.93 (II), 58.2 (d, J = 7.3Hz, II), 58.1 (d, J = 8.1 Hz, I), 54.97 and 54.94 (both I and II), 43.0 (d, J = 4.3 Hz, II), 42.9 (d, J = 4.3 Hz, II), 43.9 (d, J = 4.3 Hz, II), 4.5 Hz, I), 24.05, 23.98, 23.82, 23.76, 23.74, 23.68, 20.07 20.02 and 19.97 (both I and II); <sup>31</sup>P NMR (200 MHz, CD<sub>3</sub>CN): δ 150.5 & 150.8, <sup>19</sup>F NMR (470 MHz, CD<sub>3</sub>CN): δ -61.52 & 61.60, HRMS  $(\text{ESI-TOF}) \text{ m/z: } [\text{M} + \text{H}]^+ \text{ calcd for } C_{43}\text{H}_{50}\text{F}_3\text{N}_7\text{O}_9\text{P} 896.3360, \text{ found } 896.3361.$ 1-O-tert-butyldimethylsilyl-3,4-O-isopropylidene-(2R,3R,4R)-6-methyl-2-[4-(trifluoromethyl)phenyl]hept-5-ene-1,2,3,4-tetraol (12) 4-bromobenzotrifluoride (3.4 g, 15 mmol) in dry diethyl ether (5 mL) was added slowly to a mixture of flakes of magnesium (0.37 g, 15 mmol) in dry diethyl ether (20 mL). The mixture was gently warmed up until it was spontaneously refluxed. The mixture was stirred for one hour and then ketone  $19^{33}$  (2.0 g, 6.1 

 spontaneously refluxed. The fluxture was suffed for one nour and then ketone 15° (2.0 g, 0.1 mmol) in dry diethyl ether (10 mL) was slowly added. The reaction was stirred for one hour at ambient temperature, acidified by aqueous HCl (1 mol L<sup>-1</sup>) to pH 3.0, diluted with diethyl ether and then washed with brine. The organic layer was separated, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The crude product was purified by silica gel chromatography (10% EtOAc in hexane) to give 2.1 g (73% yield) of **12** as a white foam (the undesired product with *S*-configured spiro carbon C-2 was obtained in 15% yield). <sup>1</sup>H NMR(500 MHz, CDCl<sub>3</sub>):  $\delta$  7.70 (m, 2H), 7.56

(m, 2H), 4.96 (dd, 1H, J = 10 Hz and 7.0 Hz), 4.60 (d, 1H, J = 7.0 Hz), 4.42 (m, 1H), 3.99 (d, 1H, J = 10.0 Hz), 3.55 (d, 1H, J = 10.0 Hz), 3.22 (s, 1H), 1.63 (b, 3H), 1.50 (s, 3H), 1.41 (s, 3H), 1.40 (b, 3H), 0.89 (s, 9H), 0.06 (s, 3H), 0.00 (s, 3H); <sup>13</sup>C NMR (125 MHz, CDCl3):  $\delta$  146.3, 135.2, 129.0 (q, J = 33 Hz), 126.8, 124.4 (q, J = 271 Hz), 124.0 (q, J = 2.5 Hz), 122.8, 108.0, 79.2, 75.0, 74.9, 68.9, 27.7, 25.9, 25.7, 25.2, 18.3, 17.8, -5.56, -5.57; <sup>19</sup>F NMR (470 MHz, CDCl<sub>3</sub>): $\delta$  -64.34; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C<sub>24</sub>H<sub>37</sub>F<sub>3</sub>NaO<sub>4</sub>Si 497.2311, found 497.2334.

#### 1-O-benzoyl-3,4-O-isopropylidene-(2R,3R,4R)-6-methyl-2-[4-(trifluoromethyl)phenyl]hept-5-

ene-1,2,3,4-tetraol (13). Compound 12 (2.0 g, 4.2 mmol) was dissolved in THF (30 mL), 1 mol L<sup>-1</sup> of TBAF in THF (5.0 mL, 5.0 mmol) was added and the mixture was stirred overnight at ambient temperature. After completion of the reaction,  $CaCO_3$  (1 g), strong cation exchange resin (Dowex 50WX-200, 3 g) and methanol (3 mL) were added, the mixture was stirred for 1 hour and filtered through a celite column. The filtrate was evaporated to dryness, evaporated with dry pyridine and dissolved in dry pyridine (10 mL). A catalytic amount of DMAP and benzoyl chloride (0.54, 4.6 mmol) were added, the mixture was stirred over night at ambient temperature and extracted between saturated NaHCO<sub>3</sub> and dichloromethane. The organic layer was separated, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The crude product was purified by silica gel chromatography (30% EtOAc in hexane) to give 1.9 g (98% yield) of 13 a white foam. <sup>1</sup>H NMR(500 MHz, CDCl<sub>3</sub>):  $\delta$  7.90 (m, 2H), 7.77 (m, 2H), 7.63 (m, 2H), 7.60 (m, 1H), 7.43 (m, 2H), 5.03 (dd, 1H, J = 9.7 Hz & 6.7 Hz), 4.92 (m, 1H), 4.71 - 4.66 (m, 3H), 3.20 (s, 1H), 1.63 (s, 3H),1.61 (s, 3H), 1.55 (s, 3H), 1.42 (s, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 166.6, 145.6, 137.4, 133.4, 129.6, 129.5 (m), 128.5, 126.9, 124.6 (q, J = 3.6 Hz), 124.2 (q, J = 270 Hz), 121.6, 108.3, 80.2, 75.9, 74.4, 69.3, 27.3, 25.9, 24.8, 18.0; <sup>19</sup>F NMR (470 MHz, CDCl<sub>3</sub>):  $\delta$  -62.50; HRMS (ESI-TOF) m/z:  $[M + Na]^+$  calcd for C<sub>25</sub>H<sub>27</sub>F<sub>3</sub>NaO<sub>5</sub> 487.1708, found 487.1748.

**5-O-Benzoyl-4-C-(4-trifluorophenyl)-2,3-O-isopropylidene-D-ribose (14)**. A solution of osmium tetroxide (2.5 wt. % in 2-methyl-2-propanol, 2.5 mL, 0.25 mmol) was added portion wise to a mixture of **13** (2.0 g, 4.2 mmol) and 4-methylmorpholine *N*-oxide (1.4 g, 12 mmol) in acetone (50 mL). The mixture was stirred over night at ambient temperature and extracted between saturated Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and ethyl acetate. The organic layer was separated, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The residue (the vicinal diol intermediate) was dissolved in THF (25 mL) and H<sub>5</sub>IO<sub>6</sub> (0.96 g, 4.2 mmol) was added. The mixture was stirred for one hour at ambient temperature, concentrated and purified by silica gel chromatography (30% EtOAc in hexane) to give 1.7 g (92% yield) of **14** as a colorless oil. <sup>1</sup>H NMR(500 MHz, CDCl<sub>3</sub>): *δ* 7.87 (m, 2H), 7.64-7.59 (m, 5H), 7.54 (m, 1H), 7.39 (m, 2H), 5.69 (d, 1H, *J* = 3.0 Hz), 5.02 (d, 1H, *J* = 5.8 Hz), 4.89 (d, 1H, *J* = 11.7 Hz), 4.87 (d, 1H, *J* = 5.8 Hz), 4.59 (d, H, *J* = 11.7 Hz), 3.77 (d, 1H, *J* = 3.0 Hz), 1.27 (s, 3H), 1.02 (s, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): *δ* 166.6, 142.8, 133.4, 129.7, 129.5 (m), 128.5, 127.1, 124.6 (q, *J* = 3.6 Hz), 124.2 (q, *J* = 270 Hz), 113.2, 102.3, 90.6, 86.7, 83.0, 69.7, 25.8, 25.7; <sup>19</sup>F NMR (470 MHz, CDCl<sub>3</sub>): *δ* -64.38; HRMS (ESI-TOF) m/z: [M + Na]<sup>+</sup> calcd for C<sub>22</sub>H<sub>21</sub>F<sub>3</sub>NaO<sub>6</sub> 461.1188, found 461.1176.

**1,2,3,5-Tetra-***O***-benzoyl-4-***C***-(4-trifluorophenyl)-D-ribose (15) 14** (1.6 g, 3.6 mmol) was dissolved in dioxane (80 mL) and 1.0 mol L<sup>-1</sup> aq HCl (15 mL) was added. The mixture was stirred overnight at 55°C, neutralized by addition pyridine and evaporated to dryness. The residue was evaporated with dry pyridine and dissolved in the same solvent. Catalytic amount of DMAP and benzoyl chloride (2.0 mL, 17 mmol) were added at 0°C, the mixture was allowed to warm up, stirred for three hours at ambient temperature, quenched by methanol and extracted between saturated NaHCO<sub>3</sub> and ethyl acetate. The organic layers were combined, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The crude product was purified by silica gel chromatography (20% EtOAc in hexane) to give 2.1 g (81% yield) of **15** as a white foam ( $\alpha$ : $\beta$  = 1:4, *n/n*). <sup>1</sup>H NMR(500

MHz, CDCl<sub>3</sub>):  $\delta$  8.22-7.20 (m, 24H), 7.16 (d, 0.2H, *J* = 4.6 Hz, α), 6.94 (s, 0.8H, β-anomer), 6.50 (d, 0.2H, *J* = 5.7 Hz, α-anomer), 6.47 (d, 0.8H, *J* = 5.0 Hz, β-anomer), 6.22 (dd, 0.2H, *J* = 5.7 and 4.8 Hz, α-anomer), 6.13 (d, 0.8H, *J* = 5.0 Hz, β-anomer), 4.87 and 4.78 (2 × d, 2 × 0.8H, *J* = 12.3 Hz both, β-anomer), 4.80 and 4.71 (2 × d, 2 × 0.2H, *J* = 12.1 Hz both, α-anomer); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) for β-anomer only:  $\delta$  165.8, 165.1, 164.9, 164.8, 133.8, 133.73, 133.69, 133.3, 141.8, 130.5 (m), 130.1, 129.73, 129.65, 129.64, 129.5, 129.3, 128.87, 128.82, 128.58, 128.56, 128.4, 128.3, 127.2, 126.1 (q, *J* = 261 Hz), 125.0, 98.4, 87.6, 75.3, 72.2, 67.4; <sup>19</sup>F NMR (470 MHz, CDCl<sub>3</sub>):  $\delta$  -64.25 (β), -64.47 (α); HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C<sub>40</sub>H<sub>29</sub>F<sub>3</sub>NaO<sub>9</sub> 733.1661, found 733.1691.

**2',3',5'-tri-0-benzoyl-4'-C-(4-trifluoromethylphenyl)uridine** (16). Trimethylsilyltriflate (0.5 mL, 2.8 mmol) was added in drops to a mixture of 2,4-bis(trimethylsilyloxy)pyrimidine (0.55 g, 2.1 mmol) and **15** (0.26 g, 0.36 mmol) in dry acetonitrile (4 mL) under nitrogen (0°C). The mixture was allowed to warm up, stirred overnight at room temperature, poured to cold saturated NaHCO<sub>3</sub> and extracted with chloroform. The combined organic layers were dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The residue was purified by silica gel chromatography (30% EtOAc in hexane) to give 0.18 g (72%) of **16** as a white foam. <sup>1</sup>H NMR(500 MHz, CDCl<sub>3</sub>):  $\delta$  9.21 (b, 1H), 8.13 (d, 1H, *J* = 7.3 Hz), 7.69 (m, 4H), 7.63-7.46 (m, 10H), 7.27-7.23 (m, 4H), 6.61 (d, 1H, *J* = 6.0 Hz), 6.45 (d, 1H, *J* = 5.5 Hz), 6.08 (dd, 1H, *J* = 6.0 Hz and 5.5 Hz), 5.57 (dd, 1H, *J* = 8.0 Hz and 1.5 Hz), 4.85 (d, 1H, *J* = 12.5 Hz), 4.76 (d, 1H, *J* = 12.5 Hz); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  165.8, 164.9, 164.8, 162.6, 150.3, 140.8, 139.0, 134.0, 133.9, 133.8, 130.8 (q, *J* = 33 Hz), 129.8, 129.7, 129.5, 129.0, 128.9, 128.5, 128.4, 128.2, 128.1, 126.4, 125.6 (q, *J* = 3.6 Hz), 123.8 (q, *J* = 270 Hz), 103.7, 87.5, 86.3, 73.4, 72.3, 68.3; <sup>19</sup>F NMR (470 MHz, CD<sub>3</sub>CN):  $\delta$ -64.75; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C<sub>37</sub>H<sub>27</sub>F<sub>3</sub>KN<sub>2</sub>O<sub>9</sub> 739.1306, found 739.1311.

5'-O-(4,4'-dimetoxytrityl)-4'-C-(4-trifluoromethylphenyl)uridine (18). 16 (0.49 g, 0.70 mmol) was dissolved in a solution of 0.05 mol  $L^{-1}$  NaOMe in MeOH (10 mL). The mixture was stirred for three hours at ambient temperature, neutralized by strong cation exchange resin and evaporated to dryness. The residue (17) was washed with hexane, evaporated with dry pyridine and dissolved in a mixture of dichloromethane and pyridine (1:1, v/v, 5 mL). 4.4'-Dimethoxytritylchloride (0.26 g, 0.77 mmol) was slowly added and the mixture was stirred overnight at ambient temperature. The reaction was quenched by methanol and the mixture was extracted between dichloromethane and saturated NaHCO<sub>3</sub>. The organic layer was separated, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The residue was purified by silica gel chromatography to give 0.51 g (40%) of 18 as a white foam. <sup>1</sup>H NMR(500 MHz, MeOD):  $\delta$  8.00 (d, 1H, J = 8.0 Hz), 7.54 (d, 2H, J = 8.5 Hz), 7.49 (d, 2H, J = 8.5 Hz), 7.35-7.22 (m, 9H), 6.84 (m, 4H), 6.17 (d, 1H, J = 4.5 Hz), 5.30 (d, 1H, J = 8.0 Hz)Hz), 4.76 (d, 1H, J = 5.0 Hz), 4.50 (dd, 1H, J = 5.0 Hz and 4.5 Hz), 3.78 and 3.77 (2 x s, 2 x 3H), 3.60 (d, 1H, J = 11.0 Hz) and 3.37 (d, 1H, J = 11.0 Hz); <sup>13</sup>C NMR (125 MHz, CDCl3):  $\delta$  163.6, 158.8, 151.4, 144.0, 142.7, 140.4, 134.9, 134.7, 130.2, 130.1, 129.6 (q, J = 33 Hz), 128.1, 128.1, 127.3, 126.8, 124.8 (b), 124.1 (q, J = 270 Hz), 113.4, 113.3, 102.5, 90.2, 89.5, 87.6, 75.8, 72.4, 68.5 and 55.2; <sup>19</sup>F NMR (470 MHz, CDCl<sub>3</sub>): $\delta$  -64.11; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C<sub>37</sub>H<sub>33</sub>F<sub>3</sub>N<sub>2</sub>NaO<sub>8</sub> 713.2087, found 713.2089.

#### 5'-O-(4,4'-dimetoxytrityl)-4'-C-(4-trifluoromethylphenyl)-2'-O-(triisopropylsilyloxymethyl)-

**uridine** (19). A mixture of 18 (0.11 g, 0.16 mmol), DIEA (68  $\mu$ L, 0.40 mmol) and Bu<sub>2</sub>SnCl<sub>2</sub> (56 mg, 0.17 mmol) in 1,2-dichloroethane (2 mL) was stirred for one hour at ambient temperature. The mixture was then warmed up to 80°C and triisopropylsilyloxymethylchloride (TOMCl, 52  $\mu$ L, 0.21 mmol) was added over 90 min. The completed reaction mixture was diluted with dichloromethane, washed with saturated NaHCO<sub>3</sub>, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated to dryness. The residue was purified by silica gel chromatography (30% EtOAc in hexane) to give 67 mg (47%) of 19 as a

white foam. <sup>1</sup>H NMR(500 MHz, CD<sub>3</sub>CN):  $\delta$  7.67-7.65 (m, 3H), 7.54 (d, 2H, J = 8.3 Hz), 7.38-7.24 (m, 9H), 6.89-6.86 (m, 4H), 6.19 (d, 1H, J = 6.1 Hz), 5.49 (d, 1H, J = 8.2 Hz), 4.96 (s, 2H), 4.66 (d, 1H, J = 4.9 Hz), 4.58 (dd, 1H, J = 6.1 Hz and 4.9 Hz), 3.783 and 3.779 (2 x s, 2 x 3H), 3.56 (d, 1H, J = 10.6 Hz), 3.34 (d, 1H, J = 10.6 Hz), 1.10-1.00 (m, 21H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>CN):  $\delta$  162.8, 158.8, 144.4, 144.0, 140.3, 135.2, 135.1, 130.08, 130.06, 128.6 (q, J = 32 Hz), 128.02, 127.98, 127.2, 127.1, 124.5 (q, J = 270 Hz), 124.5 (b), 113.2, 102.4, 89.8, 89.5, 87.2, 86.7, 80.6, 71.6, 69.1, 60.0, 54.9, 17.2 and 11.7; <sup>19</sup>F NMR (470 MHz, CD<sub>3</sub>CN): $\delta$  -64.59; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C<sub>47</sub>H<sub>55</sub>F<sub>3</sub>N<sub>2</sub>NaO<sub>9</sub>Si 899.3527, found 899.3480.

3'-O-[(2-cyanoethoxy)-(N,N-diisopropylamino)phosphinyl]-5'-O-(4,4'-dimetoxytrityl)-4'-C-(4trifluoromethylphenyl)-2'-O-(triisopropylsilyloxymethyl)uridine (20). 19 (50 mg, 57 µmol) was dried over  $P_2O_5$  in a vacuum desiccator and dissolved in dry dichloromethane (0.3 mL). Triethylamine (55  $\mu$ L, 0.4 mmol) and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite (32  $\mu$ L, 0.14 mmol) were added and the mixture was stirred overnight at ambient temperature under nitrogen. The completed reaction mixture was eluted through a short silica gel column to yield 51 mg (83%) of the product (20) as a white foam [a mixture of diastereomers I(major)/II(minor): 7:3, n/n]. <sup>1</sup>H NMR(500 MHz, CD<sub>3</sub>CN):  $\delta$  7.71 (d, 0.7H, J = 8.2 Hz, I), 7.68-7.56 (m, 4.3H), 7.41-7.24 (m, 9H), 6.9-6.85 (m, 4H), 6.23 (d, 0.7H, J = 4.1 Hz), 6.17 (d, 0.3 H, J = 7.6 Hz), 5.55 (d, 0.3H, J = 7.6 Hz),8.1 Hz), 5.43 (d, 0.7H, J = 8.1 Hz), 4.95 and 4.93 (2 × d, 2 × 0.3H, J = 8.3 Hz both, II), 4.92 and 4.91 (2 × d, 2 × 0.7H, J = 8.9 Hz both, I), 4.82 (dd, 0.7H, J = 10.3 and 5.3 Hz, I), 4.71 (dd, 0.3H, J = 7.5 and 4.6 Hz, II), 4.58 (dd, 0.3H, J = 12.4 and 4.5 Hz, II), 4.54 (dd, 0.7H, J = 4.8 and 4.6 Hz, I), 3.87-3.67 (m, 2H), 3.793, 3.786 and 3.781 (each s, 6H), 3.66 and 3.47 (2 × d, 2 × 0.3H, J = 10.8Hz, both, II), 3.59 and 3.44 (2 × d, 2 × 0.7H, J = 10.8 Hz both, I), 3.59-3.53 and 3.02-2.97 (m, 2H), 2.74-2.59 (m, 2H), 1.12-0.85 (m, 33H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>CN): δ 162.9 (I), 162.7 (II), 158.82 (II), 158.79 (I), 150.6 (II), 150.4 (I), 144.5 (I), 144.4 (II), 144.1 (I), 144.0 (II), 141.0 (I),

140.2 (*II*), 135.2 (I), 135.1 (II), 130.2, 130.14, 130.08, 128.7 (q, J = 32.1 Hz, *I*), 128.5 (q, J = 31.9 Hz, *II*), 128.12, 128.08, 128.0, 127.6 (*II*), 127.4 (*I*), 127.1 (*II*), 127.0 (*I*), 124.6 (q, J = 271 Hz, *II*), 124.5 (q, J = 271 Hz, *I*), 124.4 (m), 118.8 (*II*), 118.6 (*I*), 113.28 and 113.26 (*II*), 113.16 and 113.14 (*I*), 102.7 (*II*), 102.2 (*I*), 89.8 (*I*), 89.8 (*I*), 89.2 (d, J = 10.1 Hz, *II*), 88.7 (*I*), 88.4 (d, J = 5.9 Hz, *I*), 87.4 (*II*), 86.8 (*I*), 85.6 (*II*), 79.0 (*I*), 77.8 (*II*), 73.6 (d, J = 7.0 Hz, *II*), 73.4 (d, J = 11.1 Hz, *I*), 69.07 (*II*), 67.7 (*I*), 58.2 (d, J = 20.8 Hz, *I*), 57.7 (d, J = 22.0 Hz, *II*), 54.96 (*II*), 54.92 (*I*), 43.0 (d, J = 12.7 Hz, I), 42.7 (d, J = 12.2 Hz, *II*), 23.95, 23.87, 23.84, 23.78, 23.65, 23.60, 20.08, 20.02, 19.95, 17.23, 17.21, 11.73, 11.70; <sup>31</sup>P NMR (200 MHz, CD<sub>3</sub>CN):  $\delta$  151.18 (*II*) and 149.96 (*I*); <sup>19</sup>F NMR (470 MHz, CD<sub>3</sub>CN): $\delta$  -64.57 (*II*) and -64.62 (*I*); HRMS (ESI-TOF) m/z: [M + H]<sup>+</sup> calcd for C<sub>56</sub>H<sub>73</sub>F<sub>3</sub>N<sub>4</sub>O<sub>10</sub>PSi 1077.4786, found 1077.4742.

**Oligonucleotide synthesis.** Oligoribonucleotides **ORN 1-5** were synthesized in 1.0  $\mu$ mol scale using an automatic DNA/RNA-synthesizer. Benzylthiotetrazol was used as an activator. 0.11 mol L<sup>-1</sup> solutions of **1** and **2** were used to loading of the synthesizer vessels. Coupling time of 300 s was used for the standard 2'-O-TBDMS- and 2'-O-Me RNA building blocks and 600 s for **1** and **2**. According to the DMTr assay, the coupling efficiency (> 95%) of **1** and **2** was equal with those of the standard building blocks. Manual coupling was required for the coupling of **3**: A 0.20 mol L<sup>-1</sup> solution of **3** (50 µL, 10 µmol) was mixed with a solution of benzylthiotetrazol (0.25 mol L<sup>-1</sup> in dry acetonitrile, 40 µL, 10 µmol) and suspended with the CPG-support (bearing the sequence before **3**, 1 µmol). The suspension was mixed for 10 min under nitrogen at ambient temperature, loaded to the synthesize column and filtered. The coupling was repeated and the synthesis column was set to the synthesizer. The automatic chain elongation was then continued. According to the DMTr assay, **3** could be coupled in 90% yield. After the chain elongation, the oligonucleotides were released from supports (in columns) by a mixture of concentrated ammonia and ethanol (3 : 1,  $\nu/\nu$ ) for 3 hours at 55°C. The supports were removed by filtration and the deprotection was then continued in the same

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mixture overnight at room temperature. The mixtures were evaporated to dryness and the residues were dissolved in a mixture of triethylamine trihydrofluoride (75 µL), triethylamine (60 µL) and DMSO (115 µL) (for 2.5 hours at 65°C). 0.1 mol L<sup>-1</sup> NaOAc (10 mL) was added to each mixture and loading to an RP cartridge was carried out. 0.1 mol L<sup>-1</sup> aqueous Et<sub>3</sub>N<sup>+</sup>AcO<sup>-</sup> (6.0 mL, pH = 7.0) was eluted though the cartridges, the crude RNAs were released by elution with 60% aqueous acetonitrile and the RNA – fractions were evaporated to dryness. The crude RNAs were dissolved in sterilized water and then RP HPLC was carried out. After RP HPLC purification (a semipreparative RP HPLC column (C-18, 250 × 10 mm, 5 µm) with a gradient elution 0-90% acetonitrile in 0.1 mol L<sup>-1</sup> triethylammonium acetate in 25 min, detection at 260 nm) the homogenized RNAs were lyophilized and their authenticity was verified by MS (ESI-TOF)spectroscopy (Table 1). Isolated yields (Table 1) for **ORN 1** (7%), **ORN 2** (10%), **ORN 3** (5%), **ORN 4** (7%) and **ORN 5** (7%) were determined from the UV-absorbance at  $\lambda = 260$  nm.

<sup>19</sup>F NMR spectroscopy studies. The samples for the <sup>19</sup>F NMR-measurements were prepared as we previously reported.<sup>20</sup> Similarly, the <sup>19</sup>F NMR parameters were the same: ORN 1, ORN 2, ORN 3, ORN 4 or ORN 5 (25 nmol, as triethylammonium salts) was dissolved in a mixture of 10 mmol L<sup>-1</sup> sodium cacodylate and 0.1 mol L<sup>-1</sup> NaCl in D<sub>2</sub>O-H<sub>2</sub>O (1:9  $\nu/\nu$ ), pH = 6.0 or 7.0. All samples were heated to 90 °C, allowed to cool down to room temperature and then the NMR measurements were carried out in target temperatures. Spectra were recorded at a frequency of 470.6 MHz. Typical experimental parameters were chosen as follows: <sup>19</sup>F excitation pulse 4.0 µs, acquisition time 1.17 s, prescan delay 6.0 µs, relaxation delay 0.8 s and usual number of scan was 2048 or 1024. The parameters were optimized to gain the signals with the longest relaxation rate. A macro command was used for automatic temperature ramps using a 20 min equilibration time for each temperature.

**UV- and CD-measurements.** The melting curves (absorbance *versus* temperature) were measured at 260 nm on a UV-Vis spectrometer equipped with a multiple cell holder and a Peltier temperature-controller. The temperature was changed at a rate of 0.5 °C / min (between 10 and 90 °C). The measurements were performed in 10 mmol L<sup>-1</sup> sodium cacodylate (pH 6.0 or 7.0). **ORN 1-5** were used at a concentration of 2  $\mu$ mol L<sup>-1</sup>. *T*<sub>m</sub>-values (Table 2) were determined as the maximum of the first derivate of the melting curve.

The CD-spectra were measured using the same mixtures as used to obtain UV-melting profiles. The sample temperature was changed at a rate of 0.5  $^{\circ}$ C/ min (see Supporting Information).

#### ASSOCIATED CONTENT

#### **Supporting Information.**

NMR spectra for 8-10, 12-20, RP HPLC profiles and MS (ESI-TOF) spectra for ORN 1-5, UVmelting profiles of ORN 4 and ORN 5 + A<sub>9</sub>, CD-profiles of ORN 1, ORN 4 and ORN 5, <sup>1</sup>H NMR data to determine the sugar puckering of 1, further <sup>19</sup>F NMR data for concentration-dependent triplex formation of ORN 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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