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2′-*O*-Aminoethyl Oligoribonucleotides Containing Novel Base Analogues: Synthesis and Triple-Helix Formation At Pyrimidine/Purine Inversion Sites

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The synthesis of a common sugar intermediate for the 2′-aminoethyl-ribonucleoside synthesis in 9 steps and an overall yield of 33 % starting from D-ribose is described. This intermediate was successfully used for the synthesis of the 2′-aminoethyl-ribonucleosides containing the bases thymine (**t**), 5-methylcytosine (**c**), 5-methyl-2-pyrimidinone (**x**), 2-aminopurine (**ap**) and guanine (**g**). These were subsequently transformed into the corresponding cyanoethyl phosphoramidite building blocks for oligonucleotide synthesis. 2′-Aminoethyl oligonucleotide 15-mers were prepared with a DNA synthesizer, and an optimized post-synthetic deprotection protocol has been developed which prevents cyanoethylation of the 2′-amino side chains during conventional ammonia deprotection. A series of fully modified, triplex forming 2′-aminoethyl oligoribonucleotides (2′AE-TFOs) were prepared in which **x** was designed to bind to CG inversion sites and **ap** as well as **g** to TA inversion sites on a double-helical DNA target. The affinity of **x**-CG base-triple formation in different sequence contexts was assessed by UV- and CD melting analysis. It was found that TFO 15-mers containing up to 5 **x** residues still form stable triplexes even in the case where all **x** residues are consecutively arranged in the TFO. The nearest neighbor properties of **x** have been probed and it was found that triplex stability decreases in the local sequence order **-txt-** > **-txc-** >> **-cxc-**. TFOs containing **ap** and **g** were found to bind to their DNA targets with TA inversion sites with less affinity and less selectivity compared to TFOs containing the corresponding deoxyribonucleosides, irrespective whether they were incorporated in TFOs with a DNA or a 2′-AE-RNA backbone. The obtained data suggest that guanine-TA or aminopurine-TA base-triple formation is strongly sensitive to TFO conformation and more efficient in TFOs with a DNA than an RNA backbone.

Introduction

Triple-helix forming oligonucleotides (TFOs) received considerable attention in the past for their ability to target double-stranded DNA sequence-specifically and to modulate gene expression.[1–6] Two triple-helical binding motifs are known. In the parallel binding motif a pyrimidine-rich third strand runs parallel to the target purine strand and forms Hoogsteen C^+-GC and T-AT base triples. In the antiparallel motif a purine-rich third strand is oriented antiparallel to the duplex purine strand and forms reverse Hoogsteen A-AT or T-AT and G-GC base triples. Despite of the potential benefits, a number of drawbacks limite the use of TFOs. Among those are the low thermal stability of triplexes and the restriction of duplex recognition to homopurine/homopyrimidine sequence tracts.[7–9]

In the last years new concepts have successfully been developed and applied in order to overcome the stability problem. It was shown, for example, that conformationally constrained TFOs (e.g. locked nucleic acids, LNA) increase the thermal and thermodynamic stability of corresponding parallel triplexes at neutral pH, presumably by entropic stabilization.[10] Another successful approach consists in the dual recognition of a DNA duplex by specific base–base contacts and concomitant non-specific salt-bridge formation between a positively charged ammonium function on the TFO and a duplex phosphate group, as in the case of the 2′-*O*-(2-aminoethyl)-RNA (2′-AE-RNA) developed by Cuneoud and co-workers.[11] TFOs carrying this substitution showed enhanced kinetics of triplex formation and a strongly increased stability of the resulting complex at physiological pH and at low Mg^{2+} concentration. The dual recognition concept has meanwhile been applied to different sugar modifications by other groups and by ourselves.[12,13]

The sequence restriction problem is by far more difficult to solve because of the poor recognition of pyrimidine bases, which display only one H-bond donor- or acceptor site available for TFO binding in the major groove. The most stable base triples with natural bases in the TFO that recognize pyrimidines in the parallel motif are the T-CG

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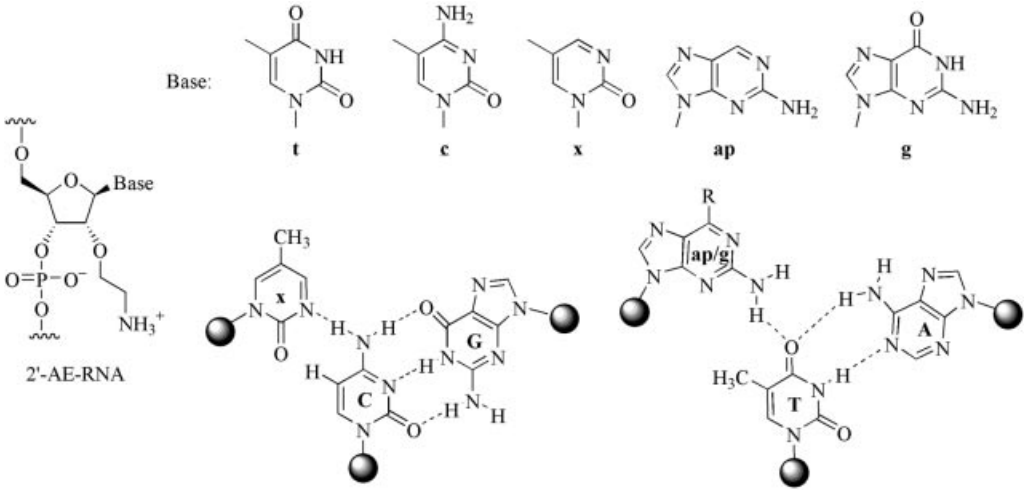


Figure 1. Chemical structures of the 2′-AE nucleotides prepared and used in this study together with the putative **x**-CG, and **g/ap**-TA base triple.

and the G-TA triple.[14–16] Their structures were firmly established by NMR analysis.[17,18] A search for novel nucleobase analogues identified a few interesting candidates as, for example, 5-methyl-2-pyrimidinone[19] or 2-pyridone[20] that recognize CG inversion sites with high selectivity. Triplex stability in these cases, however, is beyond what is needed for generating a biological response. Selective TA recognition is even more challenging because of the presence of the methyl group on thymine which sterically clashes into the backbone of the TFO. Modified bases are available also here,[21–23] but improvement, especially in terms of selectivity, is desperately needed.

The combination of such modified bases with the dual recognition concept was recently found to be a viable solution to the affinity problem in pyrimidine–purine inversion site recognition. In preliminary work, we have shown that the combination of the CG selective base 5-methyl-2-pyrimidinone with the 2′-AE-RNA backbone is formidably suited to overcome the low affinty of TFOs without compromising with selectivity, thus extending the amenable triplex recognition range from two to three base pairs.[24,25] More recently, Fox and co-workers found, that by careful combination of sugar and base-modified nucleosides, including the 2′-AE modification, all four natural bases can be recognized at nearly neutral pH.[26,27]

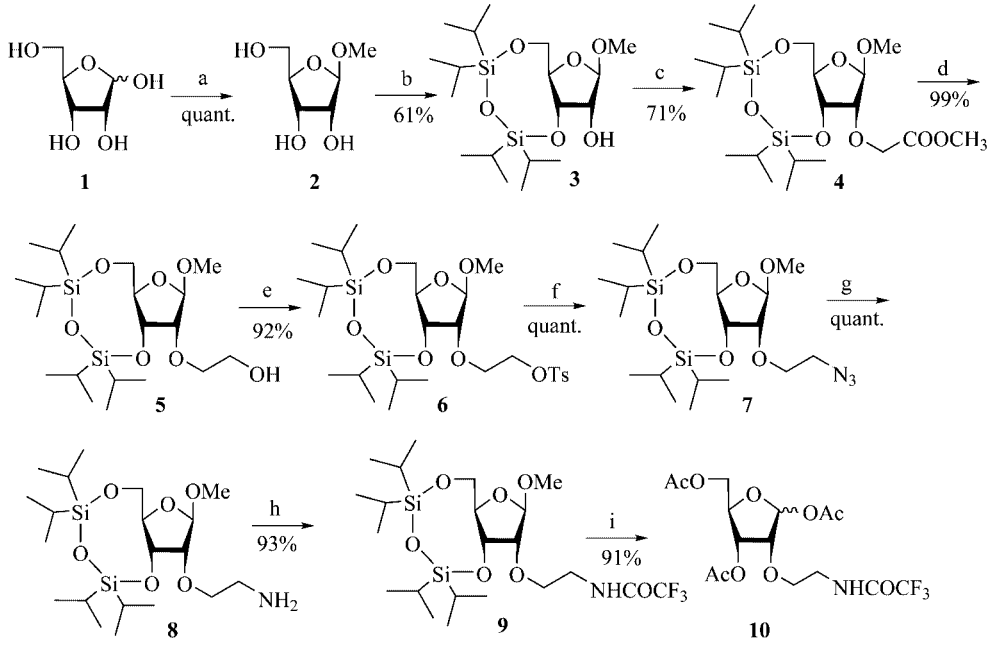
Here we present now a full account on the synthesis of the novel 2′-AE nucleosides **x**, **ap** and **g**, together with an alternative, more convergent synthesis of the already known 2′-AE monomers **t** and **c** (Figure 1). Furthermore, we report on the preparation of the corresponding 2′-AE oligoribonucleotides by standard phosphoramidite chemistry, an improved deprotection protocol for fully modified 2′-AE oligoribonucleotides as well as on the CG recognition properties of **x** in various sequence contexts and the TA-recognition properties of **ap** and **g**.

Results

Sugar Building Block

The synthesis of 2′-aminoethoxy (2′-AE) nucleosides was first reported in 1998 by Cuenoud and co-workers.[11] Their synthesis of the 2′-*O*-aminoethyl-modified thymidine and 5-methylcytidine building blocks started from protected ribothymidine. Initial attempts to prepare the monomer **x** by either modifying the base of a 2′-aminoethyl ribothymidine intermediate or by coupling 5-methyl-2-pyrimidinone to peracetylated ribose before introduction of the aminoethyl side-chain failed because of the instability of the base and/or the glycosidic bond during the following transformations. An alternative pathway was therefore developed based on the sugar intermediate **10**, which allows the introduction of various bases into the 2′-*O*-aminoethyl nucleoside scaffold at a late stage during synthesis. (Scheme 1).

The known methylriboside **2** was initially prepared from D-ribose (**1**) with 2% HCl or concd. H_2SO_4 in MeOH according to known protocols[28,29] but was only obtained in poor yields (20–50%). The use of a resin-bound acid (Dowex-50 H^+) in MeOH at 4 °C overnight greatly enhanced the practicality of this reaction and lead to riboside **2** (predominantly the β anomer) in almost quantitative yield. Compound **2** was then TIPS-protected in pyridine to afford the intermediate **3** (61%), which was subsequently alkylated using bromo methylacetate (→ **4**). Reduction of the methyl ester with $LiBH_4$ in THF/MeOH lead to compound **5** in excellent yield (99%). After activation of the alcohol by tosylation (→ **6**) and subsequent substitution with LiN_3 (→ **7**), the azide group was reduced to the amine **8** under Staudinger conditions. Subsequent TFA protection afforded compound **9** in very good yield (93%). Intermediate **9** was then treated with a mixture of $Ac_2O/AcOH/H_2SO_4$ and converted into the triacetate

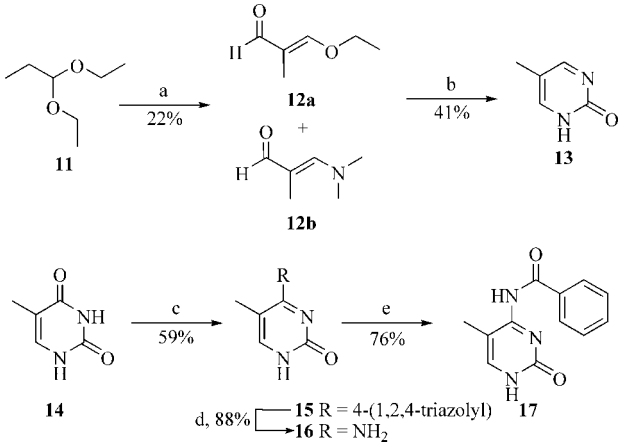


Scheme 1. Synthesis of the sugar building block **9**. a) Dowex-50 H^+, MeOH, overnight, 4 °C. b) TIPS-Cl, pyridine, 1 h, room temp. c) $BrCH_2CO_2Me$, NaH, DMF, 7 h, –10 °C → room temp. d) $LiBH_4$, THF/MeOH, 40 min, 0 °C. e) TsCl, NEt_3, DMAP, CH_2Cl_2, overnight, room temp. f) LiN_3, DMF, 1 h, 100 °C. g) i. Ph_3P, pyridine, 3 h, room temp. ii. NH_3 33%, 4 h, room temp. h) Et_3N, CF_3CO_2Et, 4 h, room temp. i) Ac_2O/AcOH/H_2SO_4, overnight, room temp.

10 (α/β 3:1), the central intermediate for the subsequent nucleosidations.[30,31]

Synthesis of 2′-AE Nucleosides and Phosphoramidite Building Blocks

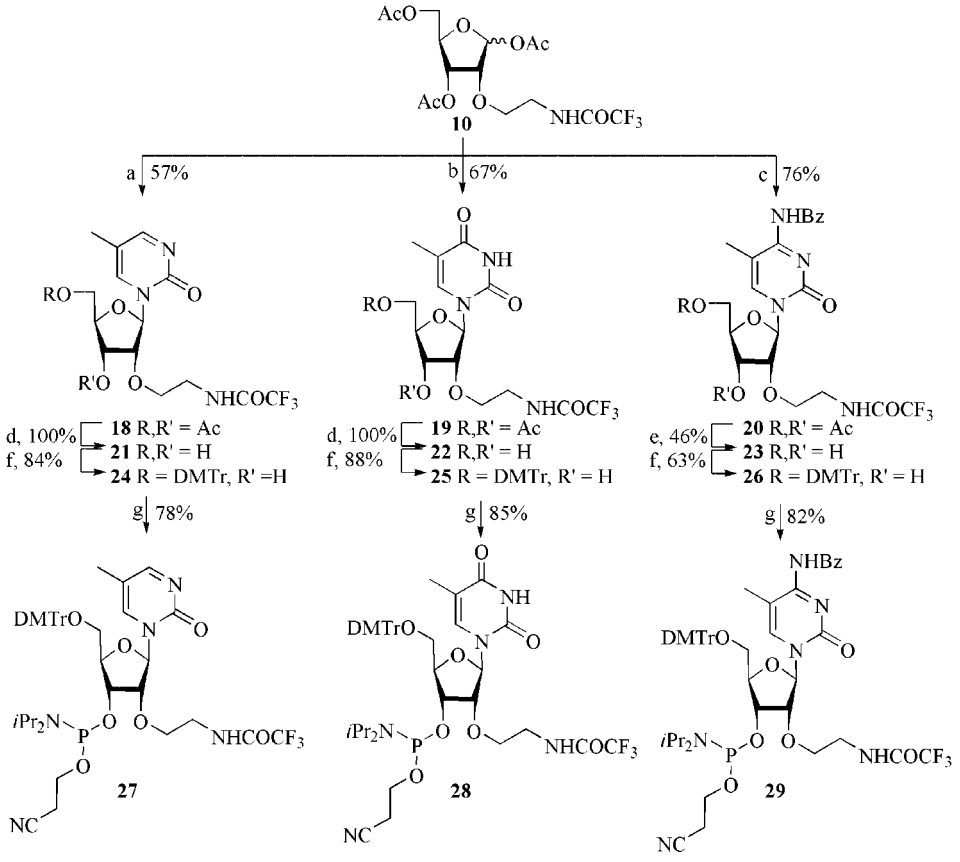
For the preparation of nucleoside **x** the corresponding base **13** had to be synthesized in two steps starting from commercially available propionaldehyde diethyl acetal (**11**) (Scheme 2).[32,33] A modified procedure involving the Vilsmeier–Haack–Arnold acylation[34] was used to prepare a mixture of **12a** and **12b** in a ratio of 1:2, respectively. The poor yield in this step could be compensated for by performing the reaction at a relatively high scale. This mixture was then treated with urea in the presence of Na in EtOH, followed by acidification to afford compound **13** in acceptable yield. The benzoyl-protected methylcytosine **17** was available in 3 steps from thymine (**14**) in an overall yield of 40% using a modified literature procedure.[35]



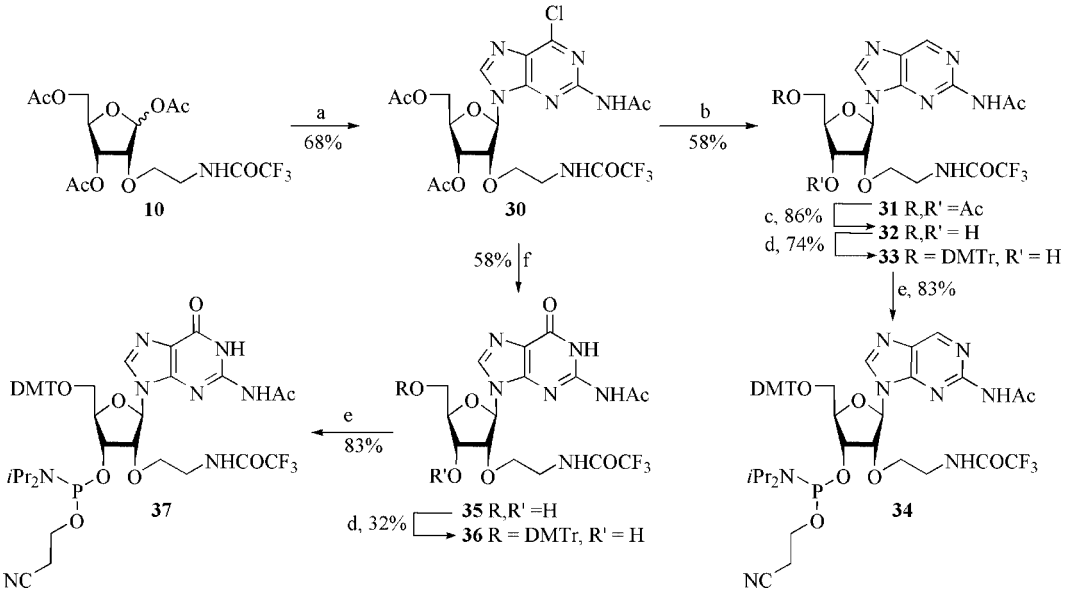
Scheme 2. Synthesis of bases. a) i. $POCl_3$, DMF, 2 h, 70 °C. ii. H_2O, K_2CO_3, overnight. b) i. Na, Urea, EtOH, 2d, reflux. ii. HCl 25%, 1 d, reflux. c) $POCl_3$, Et_3N, 1*H*-1,2,4-triazole, CH_3CN, 5–7 h, room temp. → reflux. d) NH_3 33%, 3 h, reflux. e) Bz_2O, DMAP, CH_3CN, 7 h, reflux.

Coupling of **13**, thymine (**14**) and 5-methyl-N^4-benzoylcytosine (**17**) with the key intermediate **10** under Vorbrüggen conditions afforded the corresponding nucleosides **18**–**20** in respectable yields (Scheme 3). After deacetylation, tritylation and phosphitylation, the phosphoramidite building blocks **27**–**29** were obtained. While these standard transformations worked out very well in the **x**- and **t** series we noted that the deacetylation of the **c** intermediate **20** proved more tedious than expected because of the instability of the benzoic amide function at N4. After considerable optimization, deacetylation in an ethanolic solution of NaOH in pyridine/EtOH for a few minutes worked best and led to the desired compound **23** in acceptable yield. All phosphoramidites were obtained as pure β anomers and the configuration at the anomeric center was confirmed by ^1H NMR-NOE experiments on the level of the acetylated nucleosides or of the phosphoramidites (see Experimental Section).

The 2′-AE-aminopurine **ap** nucleoside was prepared following a nucleosidation procedure developed earlier in our laboratory.[36] The N^2-acetyl-protected base 2-amino-6-chloro-9*H*-purine[37] was persilylated with BSA and subsequently coupled to the sugar **10** with TMSOTf as Lewis acid to afford the nucleoside **30** in good yield (Scheme 4). After dechlorination by catalytic hydrogenation, the pro-



Scheme 3. Synthesis of nucleosides and phosphoramidites **27**–**29**. a) **13**, BSA, $SnCl_4$, CH_3CN, 2 h, 0 °C. b) **14**, BSA, $SnCl_4$, CH_3CN, 4 h, 0 °C → room temp. c) **17**, HMDS, TMS-Cl, TMS-Tf, CH_3CN, 2 h, room temp. d) Na_2CO_3, MeOH, 15 min to 2 h, room temp. e) 50% ethanolic solution of NaOH (2 N NaOH/EtOH, 1:1), pyridine/EtOH, 1:2, 5–20 min, room temp. f) DMT-Cl, pyridine, 3 to 6 h, room temp. g) *i*Pr_2NEt, CEP-Cl, THF, 2 to 3 h, room temp.



Scheme 4. Synthesis of phosphoramidites **34** and **37**. a) i) 2-aminoacetyl-6-chloropurine, BSA, $ClCH_2CH_2Cl$, 80 °C, 20 min. ii) **1**, TMSOTf, toluene, 80 °C, 3 h. b) Et_3N, Pd/C, H_2, EtOAc, room temp., 5 h. c) Na_2CO_3, MeOH, 1 h, room temp. d) DMTr-Cl, mol. sieves, pyridine, 4 h, room temp. e) *i*Pr_2NEt, CEP-Cl, mol. sieves, THF, 1.5 to 3 h, room temp., 83%. f) $NC(CH_2)_2OH$, Cs_2CO_3, THF, 0 °C, 2 h.

tected nucleoside **31** was obtained. Assignment of the β-N^9-configuration was achieved by ^{13}C NMR, ^1H NMR-NOE experiments and by comparison with literature data.[36] After removal of the acetyl groups, the **ap** nucleoside **32** was converted into the phosphoramidite **34** under standard conditions.

The regioselectivity for the glycosylation of guanine under Vorbrüggen conditions is a known problem in nucleoside synthesis. Aware of the fact that the N^9 isomer is favored under thermodynamic control, we initially performed the nucleosidation reaction using similar conditions as for the synthesis of **31** with TMSOTf as Lewis acid in toluene at 80 °C for 2–6 hours and three different persilyated nucleobases: N^2-(acetyl)guanine, N^2-acetyl-O^6-(diphenylcarbamoyl)guanine and N^2-isobutyryl-O^6-[2-(*p*-nitrophenyl)-ethyl]guanine. The latter two nucleobase derivatives are known for their ability to direct the nucleosidation towards the N^9 position.[38,39] Unfortunately, in all cases only anomeric mixtures of N^7 nucleosides were observed. Increasing the reaction time from 6 to 15 hours in the case of N^2-(acetyl)guanine as base lead to the corresponding β-N^9 nucleoside, but only in 15% yield. Clearly an alternative had to be found.

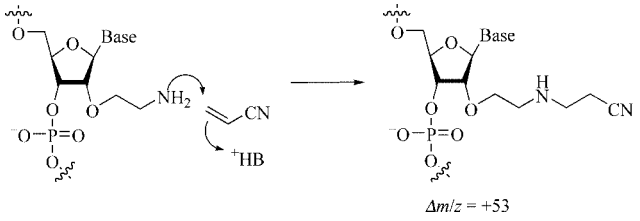
Rosenbohm et al. reported the preparation of guanine nucleosides starting from 2-amino-6-chloro-9*H*-purine derivatives by nucleophilic substitution of chlorine with cyanoethanol under basic conditions followed by in situ β elimination.[40] We applied these conditions to the intermediate **30** and isolated the *O*-deacetylated guanine nucleoside **35** in a remarkable 58% yield. Subsequent transformation into the phosphoramidite **37** proceeded smoothly on standard protocols.

Synthesis, Deprotection and Purification of 2′-AE Oligonucleotides

2′-AE-RNA oligonucleotides were synthesized on a 1.0 μmol scale on an automated DNA synthesizer using standard phosphoramidite chemistry. Higher concentrations (0.12 M) of the 2′-AE phosphoramidites were used for obtaining coupling yields >95%. Various difficulties were encountered during the deprotection of the 2′-AE-RNA oligonucleotides. One problem is the known lability of the 5-methyl-2-pyrimidinone base in **x** when treated with concd. ammonia.[41,42] The lability is most likely due to nucleophilic addition of ammonia at C6 followed by depyrimidination, leading to an abasic site prone to chain cleavage. This depyrimidination and strand cleavage could mostly be circumvented by using concd. NH_3 at room temperature for 2 hours or by using a 40% aqueous solution of CH_3NH_2 at room temperature for 3 hours.

Another complication was encountered during deprotection of fully modified 2′-AE oligonucleotides. The RP-HPLC traces of crude oligonucleotides indicated a complicated mixture of products, which could not be explained on the basis of the trityl assays of the corresponding syntheses (Figure 2, a).

MALDI-TOF MS analysis of the major peaks revealed a mixture of oligonucleotides with masses that were higher by *m*/*z* 53 or multiples of it compared to the expected mass. We hypothesized that the increase in molecular mass was due to one or multiple additions of acrylonitrile, arising from the deprotection of the cyanoethyl phosphate triester, to the already deprotected amine function of the aminoethyl side chain by Michael addition (Scheme 5).



Scheme 5. Proposed mechanism for the reaction of acrylonitrile with the amino side chain.

Two different strategies were tested in order to prevent this alkylation of the side chains by acrylonitrile during the ammonia treatment. The use of acrylonitrile scavengers (like for instance thiophenol and *t*BuNH$_2$) together with NH_3 was not satisfactory. Thus a two-step deprotection procedure was developed consisting of removal of the cyanoethyl phosphate protecting groups via β elimination with a non-nucleophilic, sterically hindered base, like TBD or DBU, prior to standard deprotection of the remaining pro-

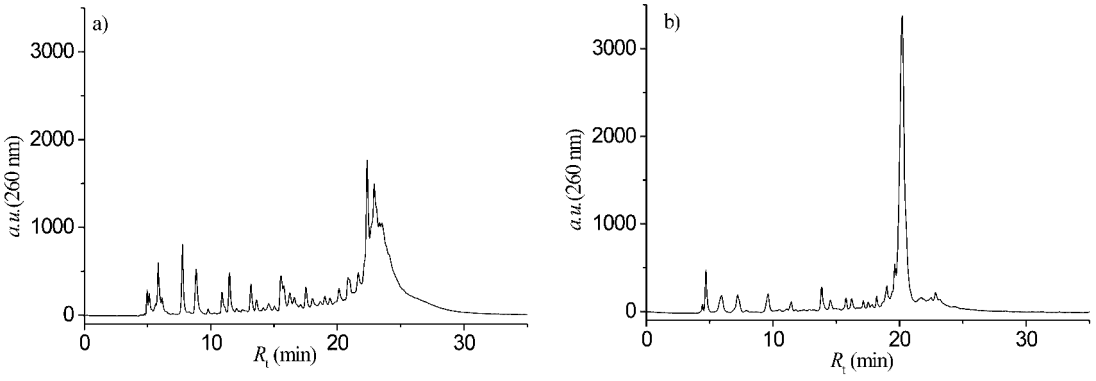


Figure 2. RP-HPLC traces of TFO **T2**: comparison between two different deprotection protocols. a) standard: NH_3 33%, room temp., 2 h. b) modified: DBU 1 M in CH_3CN, room temp., 2 h followed by NH_3 33%, room temp., 2 h.

tecting groups and cleavage from the solid support with NH_3. Indeed these new, fast and mild conditions (DBU 1 M or TBD 1 M in CH_3CN, room temp., 2 h followed by NH_3 33%, room temp., 2 h) allowed the exclusive formation of the expected deprotected oligonucleotides (Figure 2, b).

A further property of fully modified 2′-AE oligonucleotides, worth to mention, is their low solubility at neutral pH, due to their zwitterionic nature. The handling of fully modified 2′-AE-RNA oligonucleotides proved therefore to be quite difficult and often resulted in poor yields for that reason. We found it advantageous to heat stock solutions up to 90° for a few minutes before handling.

Recognition of CG Inversion Sites

T_m data and selectivity studies with the TFOs **T1**–**T3** were described before[25] and are reproduced in Table 1 for comparison only. In order to further explore scope and limitations of the generally very strong CG recognition by **x** in fully modified AE-TFOs it was necessary to address possible sequence effects. Therefore, a further series of 2′-AE-RNA TFOs were investigated with the aim of studying nearest neighbor effects of the bases 3′ and 5′ to **x** on triplex stability. Sequence information and T_m data of the investigated TFOs are summarized in Table 1.

With a UV melting-curve analysis we found that only TFO **T4** bound to its DNA target with relatively low affinity compared to all other TFOs. We reasoned that local repulsion of the accumulated positive charges at consecutive **c** residues in **T4** might be responsible for this. It is known that contiguous cytosine units in a TFO decrease the affinity of the third strand both for RNA and for DNA TFOs.[43] However, sequence effects on 2′-AE-RNA/DNA interactions even within the context of canonical base recognition (**t**-AT, $\mathbf{c}^+$-G-C) have not been addressed so far in the literature. In order to determine whether this T_m de-

Table 1. T_m data for third-strand melting from UV melting experiments (λ = 260 nm).[a]

TFO	Sequence	T_m [°C] Third strand[b]	T_m [°C] Duplex[c]
T1	5′-ttttt**x**t**x**t**x**t**x**t**x**T		
	5′-GCTAAAAAGA**C**AGAGAGATCG	77	63
	3′-CGATTTTTCT**G**TCTCTCTAGC		
T2	5′-ttttt**x**t**x**t**x**t**x**t**x**T		
	5′-GCTAAAAA**C**A**C**A**C**AGAGATCG	67	64
	3′-CGATTTTT**G**T**G**T**G**TCTCTAGC		
T3	5′-tt**x**tt**x**t**x**t**x**t**x**t**x**T		
	5′-GCTAA**C**AA**C**A**C**A**C**AGA**C**ATCG	67	67
	3′-CGATT**G**TT**G**T**G**T**G**TCT**G**TAGC		
T4	5′-ttt**x**c**x**c**x**c**x**c**x**ttT		
	5′-GCTAAAG**C**GG**C**GG**C**GAAATCG	20	73
	3′-CGATTTC**G**CC**G**CC**G**CTTTAGC		
T5	5′-cct**x**t**x**t**x**t**x**t**x**tT		
	5′-GCTGGAG**C**AG**C**AG**C**AGAATCG	60	72
	3′-CGACCTC**G**TC**G**TC**G**TCTTAGC		
T6	5′-TTT**x**T**x**C**x**T**x**C**x**T**x**TTT		
	5′-GCTAAAG**A**GG**A**GG**A**GAAATCG	34	58
	3′-CGATTTC**T**CC**T**CC**T**CTTTAGC		
T7	5′-ttt**x**t**x**c**x**t**x**c**x**t**x**ttT		
	5′-GCTAAAG**A**GG**A**GG**A**GAAATCG	69	58
	3′-CGATTTC**T**CC**T**CC**T**CTTTAGC		
T8	5′-**x**tt**x**t**xxx**t**x**c**x**t**x**T		
	5′-GCTGAAGA**CCC**GAGGAGATCG	62	71
	3′-CGACTTCT**GGG**CTCCTCTAGC		
T9	5′-**x**tt**x**t**xxxxx**c**x**t**x**T		
	5′-GCTGAAGA**CCCCC**GGAGATCG	76	73
	3′-CGACTTCT**GGGGG**CCTCTAGC		

[a] Capital letters = deoxynucleotides, lower case letters = 2′-aminoethyl nucleotides, **x** or **C** denotes 5-methylcytosine base; c (single strand) = 1.6 μM in 10 mM sodium cacodylate, 100 mM NaCl, 0.25 mM spermine. Data were obtained from the first derivative of the melting curve. [b] pH 6. [c] pH 7.

pression is base- or backbone-dependent we prepared the TFO **T7** and, as a DNA control, TFO **T6**. The melting profile of the natural sequence **T6** showed a relatively low T_m around 34 °C, which was still higher than the value determined for **T4** (ΔT_m = ca. –14 °C). Interestingly the fully modified AE-TFO **T7** formed a triplex (confirmed by gel electrophoresis and CD experiments, data not shown) having a much higher stability than the corresponding duplex (ΔT_m = +11 °C). On the basis of these observations we conclude that not the backbone but the base modification accounts for the sequence effect observed with TFO **T4**. Thus as a rule of thumb it emerges that **x** binds very strongly to CG interruptions when placed between two thymines, still strongly when placed between one thymine and one methylcytosine and relatively weakly when inserted between two 5-methylcytosines as nearest neighbor bases. Interstingly enough **x**-residues at the 3′- or 5′-side of **x**, as in the case of the consecutively modified TFOs **T8** and **T9** are also well tolerated.

In the UV melting curves, the determination of third-strand dissociation was not completely unambiguous in many cases because of considerable overlap with target duplex melting. While we have already proven triplex formation by gel shift assays for the cases **T1**–**T3**,[17] we were in need for additional proof in the cases of **T5**, **T8**, and **T9**. CD spectroscopy is perfectly suited for monitoring triplex formation. Especially the range around 220 nm is very sensitive for triplex structures, which typically show strong negative cotton effects while duplexes do not. A CD melting curve of **T5** at 220 nm (Figure 3, top left) led to a sigmoidal curve with an approximated T_m value of 66 °C, which is in good agreement with the value determined by UV melting experiments. Temperature-dependent CD measurements were performed as well on triplex **T8**, containing three contiguous **x** units (Figure 3, bottom left). Also here a T_m of 66 °C, comparable to that measured by UV experiments (Table 1) was observed. The same experiment was repeated for triplex **T9**, containing five **x** units in a row. Monitoring of the temperature dependence of the CD intensity at 215 nm (Figure 3, top right) resulted again in a sigmoidal curve with a T_m comparable to that of the UV melting curve. In all cases the CD spectra of the triplexes differed markedly from the calculated sum of the spectra of the single strands and the duplexes alone at room temperature and they matched only at 85 °C, thus validating the triplex specificity for the observed transitions.

TA Recognition

We tested the TA recognition properties of the 2′-AE nucleosides **ap** and **g** and compared them to the corresponding deoxy-TFOs carrying a deoxynucleoside with the same base. Table 2 contains the sequence information and affinity data for the triplexes investigated. **T10** is a control TFO with the DNA backbone, containing a deoxy G residue instead of one of the modifications. As expected this TFO showed the highest affinity towards a target where G

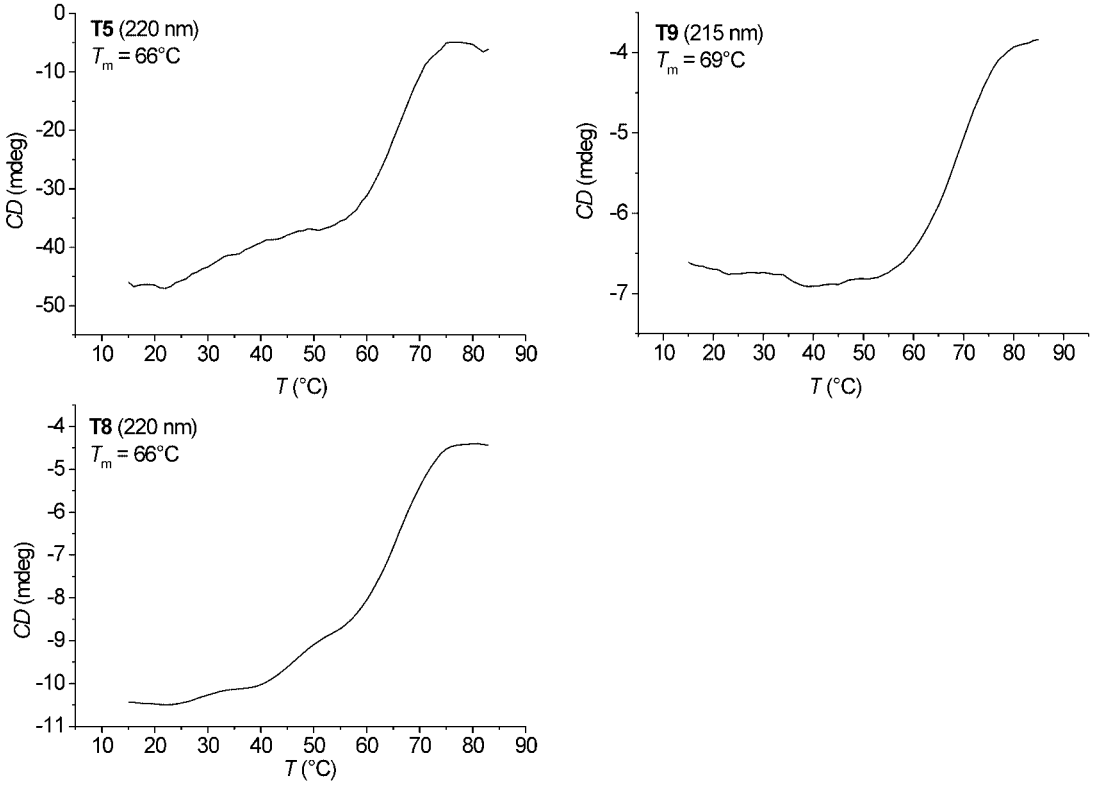


Figure 3. CD melting curves of triplex **T5** (top, left) **T8** (bottom left) and **T9** (top, right) at the indicated wavelength. Buffer: c (single strand) = 1.6 μM (**T5**), 3.8 μM (**T8**) and 1.6 μM (**T9**) in 10 mM sodium cacodylate, 100 mM NaCl, 0.25 mM spermine, pH 7.0.

is opposite the TA base pair with a third strand T_m of 41 °C. All other possible base pairs are discriminated by 5–11 °C in T_m. TFO **T11** is again a deoxyoligonucleotide containing a 2′-deoxy-2-aminopurine (**AP**) nucleoside. Relative to guanine it preserves the 2-amino function necessary for T recognition (Figure 1) but lacks the O6 carbonyl group. Also in this case the priority binding site of **AP** is a TA base pair with a T_m of 38 °C. All other base pairs are again discriminated by 4–6 °C. From these experiments we confirm the high selectivity of G for a corresponding TA base pair in a parallel TFO with the DNA backbone also in this particular sequence context. Furthermore we find that **AP** can act as a substitute for G showing essentially the same recognition pattern albeit with slightly lower affinity and lower selectivity.

In a next step, we investigated DNA-TFOs containing one insertion of either a **g** or an **ap** unit (**T12** and **T13**). We observed a drop in T_m by 9 °C and 4 °C, respectively, when positioning these residues opposite to a TA base pair. With this drop, the selectivity relative to any of the **Z/W** base pairs in the duplex target is abolished, indicating no specific **g**-TA or **ap**-TA interaction. In order to test the effect of multiple substitutions in a fully modified 2′-AE-TFO, **T14** and **T15** were hybridized to the corresponding DNA target showing TA base pairs opposite the **g** or **ap** residues. Only in the case of **T14** (**ap**) could third strand melting be observed while no such transition occurred in the case of **T15** (**g**).

Discussion and Conclusions

We demonstrated before that the base 5-methyl-2-pyrimidinone in the context of parallel binding deoxyribo-TFOs recognizes a CG base pair selectively albeit with low affinity.[14] We assume that this base recognizes cytosine by one conventional H-bond probably with the assistance of a non-conventional C–H···O hydrogen bond (Figure 1). We reasoned that the combination of this base with a 2′-AE ribose unit, as in **x**, might enhance triplex stability due to binding assistance from the aminoethyl side chain. Fully aminoethylated TFOs containing **x** confirmed the high selectivity for CG recognition and, remarkably enough, bound very strongly to their duplex target even in the presence of five **x** modifications corresponding to 33% pyrimidine content in the target site.[25] In this study, we further confirmed the high affinity of **x** for CG inversion sites in different sequence contexts with up to 5 **x** residues in a 15*mer* TFO. Interestingly, we found that the distribution of **x** within the sequence (scattered or consecutive) did not much alter the affinity pattern of a corresponding TFO. On the other hand, it appeared that nearest neighbor bases play an im-

Table 2. Sequence information and T_m data for third-strand melting from UV melting experiments (λ = 260 nm).[a]

Third strand	Triplex sequence	T_m [°C] third strand dissoc.[b] Z/W A/T	T/A	C/G	G/C
	5′-TTTTTCTGTCTCTCT				
T10	5′-GCTAAAAAGAZAGAGAGATCG	30	**41**	35	36
	3′-CGATTTTTCTWTCTCTCTAGC	(62)	(61)	(63)	(63)
	5′-TTTTTCTAPTCTCTCT				
T11	5′-GCTAAAAAGAZAGAGAGATCG	33	**38**	34	32
	3′-CGATTTTTCTWTCTCTCTAGC	(61)	(61)	(64)	(63)
	5′-TTTTTCTgTCTCTCT				
T12	5′-GCTAAAAAGAZAGAGAGATCG	28	**32**	31	32
	3′-CGATTTTTCTWTCTCTCTAGC	(61)	(61)	(63)	(62)
	5′-TTTTTCTapTCTCTCT				
T13	5′-GCTAAAAAGAZAGAGAGATCG	34	**34**	34	32
	3′-CGATTTTTCTWTCTCTCTAGC	(61)	(61)	(63)	(62)
	5′-ttttaptaptaptctcT				
T14	5′-GCTAAAAATATATAGAGATCG	n.d.	**ca. 11** (58)	n.d.	n.d.
	3′-CGATTTTTATATATCTCTAGC				
	5′-ttttgtgtgtctcT				
T15	5′-GCTAAAAATATATAGAGATCG	n.d.	**[c]** (57.8)	n.d.	n.d.
	3′-CGATTTTTATATATCTCTAGC				

[a] Capital letters = deoxynucleotides, lower-case letters = 2'-AE-nucleotides, c or C = 5-methylcytosine base; c (single strand) = 1.6 μM in 10 mM sodium cacodylate, 100 mM NaCl, 0.25 mM spermine, pH 6.5. Data were obtained from the first derivative of the melting curve. [b] T_m values for duplex melting are indicated in parentheses. [c] No T_m detectable; n.d. = not determined.

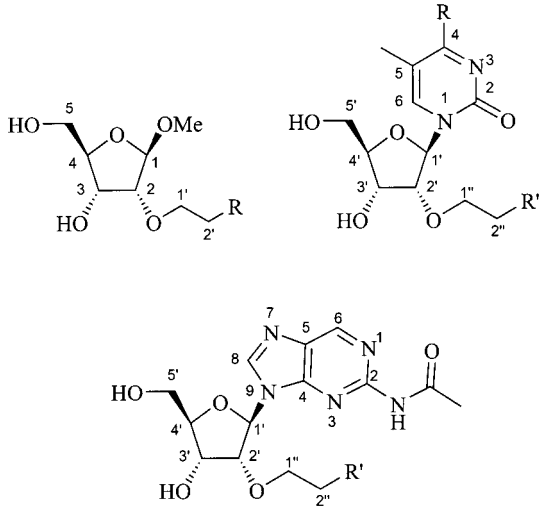
portant role, and triplex stability decreases in the following local sequence context: **-txt-** > **-txc-** >> **-cxc-**. From our investigations we can exclude this sequence dependence to be due to the 2′-AE-RNA backbone, as it is not observed in AE-TFOs with only regular bases **c** and **t**. Thus it is a direct consequence of the base modification. With the experiments reported here we have been able to define the basic rules for designing high-affinity TFOs for recognition of CG inversion sites by **x**. Further work is now concerned with its use in triplex-mediated regulation of cellular processes.

Contrary to the beneficial effect of the 2′AE modification for CG recognition were the findings with TFOs containing **ap** and **g** residues targeted to TA inversion sites. The pairing properties of **ap** and **g** to all four Watson–Crick base pairs on a DNA target duplex clearly showed that these bases in the context of the 2′AE-backbone have lost their selectivity and affinity towards the TA inversion site. From the described experiments it appears that local conformational effects in the TFO may play an important role in guanine-TA base-triple formation. For example, the change of sugar pucker from 2′-*endo* to 3′-*endo*, as in the case of the 2′AE ribonucleotides can lead to a local distortion of the TFO backbone, which is incompatible with the formation of the **g**-TA or **ap**-TA base triple and the salt bridge of the side chain with a duplex phosphate unit. Binding data on TFOs with an unmodified RNA backbone containing a rG-TA base triple are unfortunately not available as they would help identifying the effect of the aminoethyl side chain. It seems unlikely, however, that the side chain alone is responsible for this large drop in binding efficiency for steric reasons.

The O6 carbonyl group of guanine has some influence on the energetics of base-triple formation although it is not directly involved in specific H-bond formation. With TFOs in the DNA backbone, deletion of this functional group leads to reduced affinity and reduced selectivity. In the context of 2′-AE-TFOs the situation is opposite in the sense that the removal of O6 is beneficial for triplex formation. A clear explanation for this fact is elusive. However, it seems plausible that hydration effects in the major major groove, or direct or indirect contacts between third strand and pyrimidine-rich strand of the target duplex can account for this, given that the width and dimensions of this groove changes considerably by changing the third strand conformation from B-type to A-type. In order to interpret this fact in more detail, a high resolution structure of a G-TA triplex in which the Hoogsteen strand carries the RNA backbone is needed. Unfortunately such a structure has not yet been solved. In any case, to fully exploit the dual recognition features of 2′-AE-RNA with respect to recognition of TA inversion sites, the design of novel bases is clearly indicated.

Experimental Section

General: Reactions were carried out under Ar in anhydrous solvents. Solvents for extractions were technical grade and were distilled before use. Reagents were purchased from Fluka AG, Aldrich and Merck (highest quality available). NMR spectra were measured on a Bruker AC-300 or a Bruker DRX-400 spectrometer. ^{1}H NMR spectra were recorded at 300 MHz or 400 MHz: δ in ppm relative to residual non- or partially deuterated solvent ($CHCl_3$ = 7.27, [D_5]DMSO = 2.50, CD_2HOH = 3.35, HDO = 4.65), J in Hz. ^{13}C NMR spectra were recorded at 75 MHz or 100 MHz: δ in ppm relative to non- or partially deuterated solvent ($CHCl_3$ δ = 77.00, [D_5]DMSO δ = 39.70, CD_2HOH δ = 49.30). ^{19}F NMR spectra were recorded at 376 MHz: δ in ppm relative to 0.1% TFA/acetone as external standard. ^{31}P NMR spectra were recorded at 162 MHz: δ in ppm relative to 85% H_3PO_4 as external standard. Difference NOE experiments were recorded at 400 MHz. ESI-MS: VG platform Fisons instruments. HR-MS: Applied Biosystem, Sciex QSTAR Pulsar. TLC was performed using pre-coated silica gel plates SIL-G-25 UV_{254} from Macherey–Nagel. Visualization by UV (254 nm and/or 366 nm) and/or by $Ce(SO_4)_2$/phosphomolybdic acid staining.



Numbering scheme for compounds **2**–**10** and **18**–**37**

Methyl D-Ribofuranoside (2):[28,29] To a solution of D-ribose (**1**) (20 g, 133 mmol) in MeOH (400 mL) was added Dowex-50 (H^+ form, 10–20% w/w) at 0 °C. The mixture was stirred at 4 °C overnight. The solution was then filtered through celite, concentrated to dryness and crude **2** (predominantly β anomer) was obtained as a colorless oil in quantitative yield. R_f (β) = 0.3 (EtOAc/EtOH, 9:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.25 (s, 3 H, OCH_3), 3.46 (dd, $J_{H,H}$ = 6.4, 12.5 Hz, 1 H, 5-H), 3.65 (dd, $J_{H,H}$ = 3.1, 12.5 Hz, 1 H, 5-H), 3.83–3.89 (m, 2 H, 3-H, 4-H), 4.01 (dd, $J_{H,H}$ = 4.8, 7.0 Hz, 1 H, 2-H), 4.75 (s, 1 H, 1-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.5 (s, OCH_3), 63.1 (C-5), 71.1 (C-3), 74.6 (C-2), 83.2 (C-4), 108.3 (C-1) ppm. HR-MS (ESI^+): 187.0586 [M^+ + Na] (calcd. for $C_6H_{12}NaO_5$: 187.0582).

Methyl 3,5-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranoside (3): Riboside **2** (3.65 g, 22.2 mmol) was dissolved in dry pyridine (60 mL). The solution was cooled to 0 °C and TIPS-Cl (8.42 g, 26.7 mmol) was added dropwise. After stirring for 1 h at room temp., the solution was diluted with EtOAc (300 mL) and washed with H_2O (2×180 mL) and a satd. $NaHCO_3$ (2×180 mL). The combined organic layers were dried with $MgSO_4$, filtered and concentrated to dryness and purified by column chromatography (EtOAc/hexane, 1:9) to give **3** (5.54 g, 61%) as colorless oil. R_f = 0.5 (EtOAc/hexane, 1:9). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.03–

1.11 (m, 28 H, TIPS), 3.33 (s, 3 H, OCH_3), 3.71–3.79 (m, 1 H, 5-H), 3.99–4.12 (m, 3 H, 5-H, 2-H, 4-H), 4.49–4.52 (m, 1 H, 3-H), 4.83 (s, 1 H, 1-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.2–18.2 (TIPS), 55.6 (OCH_3), 66.9 (C-5), 75.7 (C-3), 76.4 (C-2), 83.4 (C-4), 107.9 (C-1) ppm. HR-MS (ESI$^+$): 429.1781 [M$^+$ + Na] (calcd. for $C_{18}H_{38}NaO_6Si_2$: 429.2104).

Methyl 2-*O*-(Methoxycarbonylmethyl)-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranoside (4): To a solution of **3** (22.97 g, 56.5 mmol) in dry DMF (134 mL) was added at –10 °C $BrCH_2CO_2CH_3$ (26.2 mL, 282 mmol), followed by portionwise addition of NaH (4.97 g, 124 mmol) over 1.5 h. After stirring for 7 h, the reaction mixture was carefully quenched with satd. NH_4Cl (380 mL), extracted with EtOAc (350 mL) and the organic phase was washed five times with H_2O. After drying with $MgSO_4$, the organic layer was evaporated and the residue purified by chromatography (EtOAc/hexane, 1:6) to give compound **4** (19.18 g, 71%) as a white solid. R_f = 0.4 (EtOAc/hexane, 1:6). m.p. 61–64 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.04–1.08 (m, 28 H, TIPS), 3.34 (s, 3 H, OCH_3), 3.75 (s, 3 H, OCH_3 chain), 3.79–4.07 (m, 4 H, 2-H, 4-H, 5-H_2), 4.41 (dd, $J_{H,H}$ = 16.9, 37.1 Hz, 2 H, 1′-H), 4.50 (dd, $J_{H,H}$ = 4.2, 7.5 Hz, 1 H, 3-H), 4.88 (s, 1 H, 1-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.4–18.1 (TIPS), 52.5 (O*C*H$_3$ chain), 55.4 (O*C*H$_3$), 64.4 (C-5), 70.0 (C-1′), 74.8 (C-3), 81.5 (C-2), 83.9 (C-4), 106.6 (C-1), 171.6 (*C*O) ppm. HR-MS (ESI$^+$): 501.2168 [M$^+$ + Na] (calcd. for $C_{21}H_{42}NaO_8Si_2$: 501.2315).

Methyl 2-*O*-(2-Hydroxyethyl)-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranoside (5): Compound **4** (15.08 g, 31.5 mmol) was dissolved in dry MeOH/THF (120:30 mL). The solution was cooled to 0 °C and $LiBH_4$ (2.74 g, 125.8 mmol) was added portionwise over 40 min. After stirring for another hour, the reaction was carefully quenched with satd. NH_4Cl (400 mL) and extracted with EtOAc (240 mL). The organic layer was washed with a satd. NaCl (100 mL), dried with $MgSO_4$ and filtered. Evaporation of the solvent gave compound **5** (14.0 g, 99%) as a white solid, which could be used in the next step without further purification. R_f = 0.1 (EtOAc/hexane, 1:9). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.04–1.10 (m, 28 H, TIPS), 3.33 (s, 3 H, OCH_3), 3.71–3.76 (m, 3 H, 2-H, 2′-H), 3.80–3.91 (m, 3 H, 5-H, 1′-H), 3.97–4.05 (m, 2 H, 4-H, 5-H), 4.51 (dd, $J_{H,H}$ = 4.2, 7.8 Hz, 1 H, 3-H), 4.78 (s, 1 H, 1-H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 13.4–18.2 (TIPS), 55.3 (OCH_3), 62.4 (C-2′), 64.3 (C-5), 73.8 (C-1′), 74.2 (C-3), 81.7 (C-4), 84.3 (C-2), 106.6 (C-1) ppm. HR-MS (ESI$^+$): 451.2536 [M$^+$ + H] (calcd. for $C_{20}H_{42}O_7Si_2$: 451.2547).

Methyl 3,5-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-[2-*O*-(2-*p*-tolylsulfonyloxyethyl]-β-D-ribofuranoside (6): Compound **5** (24.03 g, 53.3 mmol) was dissolved in dry CH_2Cl_2 (420 mL). TsCl (22.3 g, 116.9 mmol), Et_3N (17.8 mL, 127.7 mmol) and DMAP (0.65 g, 5.3 mmol) were added to the yellow solution. After stirring overnight, the solution was diluted with H_2O (200 mL), extracted with CH_2Cl_2 (3 × 300 mL) and the organic layer was dried with $MgSO_4$. Evaporation of the solvent and purification by column chromatography (EtOAc/hexane, 1:8) afforded 6 (29.76 g, 92%) as a white solid. R_f = 0.4 (EtOAc/hexane, 1:6). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.98–1.07 (m, 28 H, TIPS), 2.45 (s, 3 H, CH_3-Ts), 3.33 (s, 3 H, OCH_3), 3.69 (d, $J_{H,H}$ = 4.0 Hz, 1 H, 2-H), 3.73–3.90 (2m, 3 H, 4-H, 5-H, 2′-H), 3.93–3.97 (m, 1 H, 5-H), 4.08–4.18 (m, 3 H, 1′-H, 2′-H), 4.42 (dd, $J_{H,H}$ = 4.2, 7.5 Hz, 1 H, 3-H), 4.61 (s, 1 H, 1-H), 7.33 (d, $J_{H,H}$ = 7.7 Hz, 2 H, H-Ts), 7.80 (d, $J_{H,H}$ = 8.4 H, 2 Hz, H-Ts) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.3–18.1 (TIPS), 22.3 (*C*H$_3$ Ts), 55.3 (O*C*H$_3$), 64.4 (C-5), 69.6 (C-2′), 70.8 (C-1′), 74.8 (C-3), 81.5 (C-4), 84.3 (C-2), 106.6 (C-1), 128.7–130.5 (Ts), 133.7 (*C*-SO$_2$), 145.4 (*C*-CH$_3$) ppm. HR-MS (ESI$^+$): 605.2610 [M$^+$ + H] (calcd. for $C_{27}H_{48}O_9SSi_2$: 605.2635).

Methyl 2-*O*-(2-Azidoethyl)-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranoside (7): Compound **6** (29.76 g, 49.2 mmol) was dissolved in dry DMF (350 mL). LiN_3 (4.91 g, 98.3 mmol) was added and the solution was heated to 90–100 °C for 1 h. The reaction mixture was cooled to room temp., diluted with MeOH (440 mL) and then evaporated to dryness. The residue was taken up with *tert*-butyl methyl ether (600 mL) and washed with H_2O. The organic layer was dried with $MgSO_4$ and evaporated to give compound **7** (23.8 g, quantitative yield) as a white solid. R_f = 0.5 (EtOAc/hexane, 1:8). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.04–1.10 (m, 28 H, TIPS), 3.28–3.46 (m, 2 H, 2′-H), 3.34 (s, 3 H, OCH_3), 3.71–3.75 (m, 1 H, 1′-H), 3.77 (d, $J_{H,H}$ = 4.0 Hz, 1 H, 2-H), 3.88 (dd, $J_{H,H}$ = 5.7, 11.9 H, 1 Hz, 5-H), 4.01 (dd, $J_{H,H}$ = 4.4, 15.4 Hz, 1 H, 5-H), 4.06 (dd, $J_{H,H}$ = 2.9, 5.9 Hz, 1 H, 4-H), 4.12–4.19 (m, 1 H, 1′-H), 4.49 (dd, $J_{H,H}$ = 4.4, 8.1 Hz, 1 H, 3-H), 4.79 (s, 1 H, 1-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.4–18.1 (TIPS), 51.7 (C-2′), 55.4 (O*C*H$_3$), 64.3 (C-5), 71.3 (C-1′), 74.6 (C-3), 81.5 (C-4), 84.4 (C-2), 106.6 (C-1) ppm. HR-MS (ESI$^+$): 498.2419 [M$^+$ + Na] (calcd. for $C_{20}H_{41}N_3O_6Si_2$: 498.2431).

Methyl 2-*O*-(2-Aminoethyl)-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranoside (8): To a solution of compound **7** (1.1 g, 2.3 mmol) in dry pyridine (10 mL) was added PPh_3 (1.5 g, 5.7 mmol). The evolution of a gas was observed indicating the forthcoming of the reaction. The resulting yellow solution was stirred at room temp. for 3 h, when concentrated NH_3 33% (3.5 mL) was added and stirring continued for another 4 h. The formed precipitate (Ph_3P=O) was filtered off, the solvent evaporated, the residue taken up with EtOAc (30 mL) and washed with H_2O (10 mL). The organic layer was dried with $MgSO_4$ and evaporated to give compound **8** as a white solid (quant.) that was used in the next transformation without further purification. R_f = 0.6 (CH_2Cl_2/MeOH, 5:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.03–1.09 (m, 28 H, TIPS), 2.79–2.92 (m, 2 H, 2′-H), 3.34 (s, 3 H, OCH_3), 3.60–3.66 (m, 1 H, 1′-H), 3.73 (d, $J_{H,H}$ = 4.4 Hz, 1 H, 2-H), 3.82–3.90 (m, 2 H, 5-H), 3.98–4.04 (m, 2 H, 4-H, 1′-H), 4.48 (dd, $J_{H,H}$ = 4.4, 7.7 Hz, 1 H, 3-H), 4.77 (s, 1 H, 1-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.4–18.2 (TIPS), 42.8 (C-2′), 55.3 (O*C*H$_3$), 64.6 (C-5), 74.4 (C-1′), 74.5 (C-3), 81.8 (C-4), 84.0 (C-2), 106.7 (C-1) ppm. MS (ES$^+$): *m*/*z* (%) 450 (5) [M$^+$ + H], 406 (94), 260 (46), 205 (53), 135 (57), 44 (100).

Methyl 3,5-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl-2-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranoside (9): Compound **8** (19.0 g, 42.2 mmol) was dissolved in CF_3CO_2Et (350 mL). Freshly distilled Et_3N (7 mL, 50.2 mmol) was added and the solution was stirred at room temp. for 4 h. After evaporation to dryness the residue was taken up with CH_2Cl_2 (300 mL), washed with H_2O (2 × 200 mL) and dried with $MgSO_4$. The crude product was purified by column chromatography (EtOAc/hexane, 1:5) to give compound **9** (21.45 g, 93%) as a white solid. R_f = 0.6 (EtOAc/hexane, 1:4). m.p. 50–52 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.01–1.09 (m, 28 H, TIPS), 3.33 (s, 3 H, OCH_3), 3.58 (dd, $J_{H,H}$ = 5.0, 10.1 Hz, 2 H, 2′-H), 3.73 (d, $J_{H,H}$ = 4.4 Hz, 1 H, 2-H), 3.75–3.81 (m, 1 H, 1′-H), 3.86–4.03 (m, 4 H, 4-H, 5-H, 1′-H), 4.51 (dd, $J_{H,H}$ = 4.4, 7.7 Hz, 1 H, 3-H), 4.75 (s, 1 H, 1-H), 6.92 (s, 1 H, N*H*) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.3–18.1 (TIPS), 40.6 (C-2′), 55.4 (O*C*H$_3$), 64.2 (C-5), 69.4 (C-1′), 74.3 (C-3), 81.8 (C-4), 83.8 (C-2), 106.2 (C-1), 118.4 (*C*F$_3$), 157.6 (*C*O) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.40 ppm. HR-MS (ESI$^+$): 568.2330 [M$^+$ + Na] (calcd. for $C_{22}H_{42}F_3NO_7Si_2$: 568.2349).

1,3,5-Tri-*O*-acetyl-2-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranoside (10): A solution of **9** (3.87 g, 7.1 mmol) in Ac_2O (80 mL) and AcOH (80 mL) was stirred for 1 h, before concd. H_2SO_4 (0.8 mL)

was added. The clear yellowish solution was then stirred at room temp. overnight. The reaction mixture was then added dropwise to 400 mL of satd. $NaHCO_3$ and extracted with $CHCl_3$ (3·1000 mL). The combined organic layers were dried with $MgSO_4$, the solvent was evaporated and the crude product was purified by column chromatography (EtOAc/CH_2Cl_2, 1:1) to yield compound **10** (2.68 g, 91%) in an anomeric ratio of α/β, 3:1. R_f = 0.6 (α anomer), 0.4 (β anomer) (EtOAc/ CH_2Cl_2 1:4). **α Anomer:** ^{1}H NMR (400 MHz, $CDCl_3$), δ = 2.09–2.14 (m, 9 H, CH_3 Ac), 3.53–3.82 (m, 4 H, 1′-H, 2′-H), 4.10–4.16(m, 2 H, 2-H, 5-H), 4.32–4.52 (m, 2 H, 4-H, 5-H), 5.07 (dd, $J_{H,H}$ = 5.2, 6.3 Hz, 1 H, 3-H), 6.11 (s, 1 H, 1-H), 6.78 (s, 1 H, N*H*) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4, 21.6, 21.8 (*C*H_3 Ac), 40.2 (C-2′), 64.4 (C-5), 69.5 (C-1′), 72.9 (C-3), 80.4 (C-2), 81.6(C-4), 99.8 (C-1), 118.4 (*C*F_3), 169.2, 170.3, 170.9 (*C*O of Ac) ppm. **β Anomer:** ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.10, 2.12, 2.14 (3s, 9 H, CH_3 Ac), 3.49 (dd, $J_{H,H}$ = 5.2, 9.6 Hz, 2 H, 2′-H), 3.73 (t, $J_{H,H}$ = 5.0 Hz, 2 H, 1′-H), 3.98 (dd, $J_{H,H}$ = 4.4, 6.6 Hz, 1 H, 2-H), 4.12–4.18 (m, 1 H, 5-H), 4.31 (dd, $J_{H,H}$ = 3.3, 12.1 Hz, 1 H, 5-H), 4.50 (dd, $J_{H,H}$ = 3.7, 7.0 Hz, 1 H, 4-H), 5.20 (dd, $J_{H,H}$ = 2.9, 6.6 Hz, 1 H, 3-H), 6.35 (d, $J_{H,H}$ = 6.4 Hz, 1 H, 1-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.3, 21.4, 21.7 (*C*H_3 Ac), 40.5 (C-2′), 64.1 (C-5), 70.6 (C-3), 71.1 (C-1′), 79.5 (C-2), 81.8(C-4), 95.9 (C-1), 171.0, 171.2, 171.7 (*C*O of Ac) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.20 ppm. MS (EI$^+$): *m*/*z* (%) = 416 (10) [M$^+$ + H], 356 (100), 279 (38), 236 (87).

3-Ethoxy-2-methylprop-2-enal (12a) and 3-Dimethylamino-2-methylprop-2-enal (12b):[34] DMF (62 mL) was added dropwise at 0 °C to $POCl_3$ (64 mL) under vigorous stirring such that the temperature never exceeded 30 °C. The ice bath was then replaced by a water bath of 40 °C and propionaldehyde diethylacetal (53 mL) was added slowly accompanied with an exothermic reaction (30 °C → 85 °C). The reaction temperature was maintained at 60–70 °C by dropwise addition of propionaldehyde diethylacetal and under ice-cooling. The now dark brown solution was further heated to 70 °C for 2 h. After cooling to room temp. the mixture was poured onto ice (500 g), and left overnight. Anhydrous K_2CO_3 was added until pH ca. 9, followed by H_2O (350 mL) to dissolve the precipitated salts. The aqueous solution was then extracted with CH_2Cl_2 (5 × 100 mL). The combined organic layers were dried with K_2CO_3 and the solvents evaporated. A mixture of **12a** and **12b** in a ratio of 1:2 ester/amine (16.5 g, 22%) was obtained as a dark red liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.34 (t, $J_{H,H}$ = 7.2 Hz, 3 H, 5-H), 1.62 (s, 3 H, 6-H), 1.88 (s, 3 H, 12-H), 3.10 (s, 6 H, 10-H, 11-H), 4.12 (q, $J_{H,H}$ = 7.2 Hz, 2 H, 14.2 Hz, 4-H), 6.48 (s, 1 H, 9-H), 6.94 (s, 1 H, 3-H), 8.80 (s, 1 H, 7-H), 9.17 (s, 1 H, 1-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 6.2 (C-5), 8.5 (C-6), 15.1 (C-12), 42.9 (C-10, C-11), 70.8 (C-4), 108.7 (C-9), 119.8 (C-3), 159.9 (C-8), 167.7 (C-2), 190.9 (C-7), 191.6 (C-1) ppm.

5-Methyl-2-pyrimidinone (13): Dry EtOH (7 mL) was added to Na (0.3 g, 13 mmol) and the mixture was stirred at room temp. until the Na was dissolved. After dropwise addition of **12a** and **12b** (0.5 g) in EtOH (7 mL), followed by urea (0.3 g, 5 mmol), the mixture was refluxed for two days. HCl 25% was added to adjust the pH to ca. 2 and refluxing was continued for another day. The mixture was cooled to room temp., the insoluble residues were filtered off through celite and washed with a small amount of Et_2O. The filtrate was evaporated and purified by column chromatography (CH_2Cl_2/MeOH, 4:1). Recrystallization from MeOH afforded compound **13** (0.2 g, 41%) as brown solid. R_f = 0.5 (CH_2Cl_2/MeOH, 4:1). M.p. > 220 °C (n.d.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.28 (s, 3 H, CH_3), 8.72 (s, 2 H, 6-H, 4-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.9 (*C*H_3), 115.6 (C-5), 148.3 (C-2), 160.6 (C-4, C-6) ppm. HR-MS (ESI$^+$): 110.048 (M$^+$ + H) (calcd. for $C_5H_6N_2O$: 110.048).

4-Triazolylthymine (15):[35] 1*H*-1,2,4-Triazole (6.9 g, 99.9 mmol) was suspended in dry CH_3CN (80 mL). The suspension was cooled to 0 °C and $POCl_3$ (3 mL, 32.8 mmol) was added followed by dropwise addition of dry Et_3N (14 mL, 100 mmol). The suspension was stirred at 0 °C for about 40 min and then at room temp. for another 30 min. Thymine (**14**) (1.8 g, 14.3 mmol) was then added and the yellowish suspension was stirred for another 4 h at room temp. and 5 h under reflux. The mixture was cooled to room temp., H_2O (2.5 mL) was added and stirring was continued for 10 min. The mixture was then filtered and the yellow residue was resuspended in H_2O (40 mL) and stirred for 40 min. The filtrate was evaporated and the residue resuspended in H_2O (40 mL) and stirred for 20 min. The combined aqueous suspension were filtered and rinsed with H_2O. Further product could be isolated from the filtrate after standing overnight. Compound **15** was obtained in 59% yield (1.5 g) as a yellow solid. R_f = 0.4 (EtOAc/MeOH, 5:1). ^{1}H NMR (300 MHz, DMSO): δ = 2.25 (s, 3 H, CH_3), 8.10 (s, 1 H, 6-H), 8.34 (s, 1 H, triazole), 9.27 (s, 1 H, triazole) ppm. ^{13}C NMR (75 MHz, DMSO): δ = 15.8 (*C*H_3), 145.3 (C-6), 151.1 (C-4), 153.4 (triazole), 158.8 (C-2) ppm. MS (EI$^+$): *m*/*z* (%) = 177 (30) [M$^+$ + H], 86 (100), 69 (59).

5-Methylcytosine (16):[35] Compound **15** (1.6 g, 9.0 mmol) was suspended in concentrated NH_3 (33%, 51 mL) and refluxed for 3 h. The reaction mixture was cooled to room temp., evaporated and the residue dissolved in hot EtOH (the insoluble solid was filtered off). Cold acetone (40 mL) was added at room temp. and the formed precipitate collected by filtration to obtain **16** (1.0 g, 88%) as white solid. R_f = 0.1 (EtOAc/MeOH, 1:1). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 1.82 (s, 3 H, CH_3), 3.55 (s, 2 H, NH_2), 7.35 (s, 1 H, C6-H) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 12.7 (CH_3), 100.6 (C5), 141.5 (C6), 154.8 (C2), 164.8 (C4) ppm. MS (EI$^+$): *m*/*z* (%) = 125 (100) [M$^+$ + H], 81 (23), 70 (38), 54 (33).

***N*-Benzoyl-5-methylcytosine (17):**[35] To a suspension of **16** (1.0 g, 8.0 mmol) in dry CH_3CN (32 mL) was added Bz_2O (2.2 g, 9.7 mmol), followed by DMAP (0.2 g, 1.6 mmol). The reaction mixture was refluxed for 7 h, when EtOH (21 mL) was added to the hot solution. The mixture was cooled to room temp., the solid was filtered off and washed with EtOH and Et_2O. Compound **17** (1.4 g, 76%) was obtained as white solid. R_f = 0.1 (EtOAc/hexane, 1:1). ^{1}H NMR (300 MHz, DMSO): δ = 1.98 (d, $J_{H,H}$ = 0.7 Hz, 3 H, CH_3), 7.45–7.50 (m, 2 H, Bz-H *meta*), 7.56 (d, $J_{H,H}$ = 7.3 Hz, 1 H, Bz-H *para*), 7.65 (d, $J_{H,H}$ = 0.8 Hz, 1 H, 6-H), 8.18 (d, $J_{H,H}$ = 7.2 Hz, 2 H, Bz-H *ortho*), 11.53 (s, 1 H, N*H*), 12.98 (s, 1 H, N*H*) ppm. ^{13}C NMR (75 MHz, DMSO): δ = 12.8 (*C*H_3), 128.5 (Bz), 129.5 (Bz), 132.5 (C-6) ppm. MS (EI$^+$): *m*/*z* (%) = 229 (43) [M$^+$ + H], 152 (18), 105 (100), 77 (60).

1-[3′,5′-Di-*O*-acetyl-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]-5-methyl-2-pyrimidinone (18): 5-Methyl-2-pyrimidinone (**13**) (0.18 g, 1.6 mmol) was suspended in dry CH_3CN (5 mL), BSA (1 mL, 4.1 mmol) was added and the clear solution was stirred at room temp. for 1 h. The solution was then cooled to 0 °C and **10** (0.63 g, 1.5 mmol) in dry CH_3CN (12 mL) was added, followed by portionwise addition of $SnCl_4$ (0.21 mL, 1.8 mmol). After stirring for 2 h at 0 °C, CH_2Cl_2 (80 mL) was added and the solution washed with H_2O (40 mL) and satd. $NaHCO_3$ (40 mL). The organic layer was dried with $MgSO_4$, evaporated and the crude product purified by column chromatography (EtOAc). Compound **18** (0.40 g, 57%) was obtained as white foam. R_f = 0.1 (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.14–2.17 (m, 9 H, CH_3 Ac, CH_3 base), 3.53–3.62 (m, 2 H, 2′′-H), 3.89–3.96 (m, 2 H, 1′′-H), 4.23 (d, $J_{H,H}$

= 5.2 Hz, 1 H, 2′-H), 4.44 (m, 2 H, 5′-H), 4.51–4.54 (m, 1 H, 4′-H), 4.80 (dd, $J_{H,H}$ = 5.1, 8.8 Hz, 1 H, 3′-H), 5.87 (s, 1 H, 1′-H), 7.69 (s, 1 H, N*H*), 7.92 (d, $J_{H,H}$ = 2.6 Hz, 1 H, 6-H), 8.54 (d, $J_{H,H}$ = 2.9 Hz, 1 H, 4-H) ppm. ^{1}H NMR-difference NOE (400 MHz): δ = 4.23 (2′-H) → 3.89–3.96 (1′′-H, 5%), 4.80 (3′-H, 12%), 5.87 (1′-H, 5%), 7.92 (6-H, 1%), 4.44 (5′-H) → 4.80 (3′-H, 3%), 7.92 (6-H, 2%), 4.51–4.54 (4′-H) → 5.87 (1′-H, 3%), 7.92 (6-H, 1%), 4.80 (3′-H) → 4.23 (2′-H, 10%), 4.44 (5′-H, 1%), 7.92 (6-H, 4%), 5.87 (1′-H) → 3.89–3.96 (1′′-H, 2%), 4.23 (2′-H, 3%), 4.51–4.54 (4′-H, 2%), 7.69 (NH chain, 2%), 7.92 (H6, 2%), 7.92 (H6) → 2.17 (5-CH_3, 5%), 4.80 (3′-H, 4%), 5.87 (1′-H, 3%), 8.54 (4-H) → 2.17 (5-CH_3, 4%). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 15.2 (*C*H_3 base), 21.1, 21.5 (*C*H_3 Ac), 40.2 (C-2′′), 62.4 (C-5′), 69.8 (C-1′′), 70.1 (C-3′), 79.6 (C-4′), 81.2 (C-2′), 91.8 (C-1′), 114.2 (C-5), 118.5 (*C*F_3), 155.7 (C-2), 158.0 (*C*O chain), 164.4 (C-6), 169.0 (C-4), 170.7, 171.0 (*C*O of Ac) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.15 ppm. HR-MS (ESI$^+$): 488.1257 [M$^+$ + Na] (calcd. for $C_{18}H_{22}F_3N_3O_7$: 488.1256).

5-Methyl-1-[2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]-2-pyrimidinone (21): To a solution of **18** (1.84 g, 3.9 mmol) in dry MeOH (47 mL) was added anhydrous Na_2CO_3 (180 mg, 10% w/w) and the mixture was stirred at room temp. for 15 min. The reaction was then neutralized with DOWEX 50 (H$^+$ form, pH ca. 7), filtered through celite and the solvents evaporated. Compound **21** (1.5 g, quantitative yield) was obtained as a white solid and was used for the next step without further purification. R_f = 0.2 (EtOAc/MeOH, 10:1). ^{1}H NMR (400 MHz, MeOD): δ = 2.17 (s, 3 H, C*H*$_3$ base), 3.49–3.72 (2m, 2 H, 2′′-H), 3.83–3.92 (m, 2 H, 5′-H, 1′′-H), 3.97 (d, $J_{H,H}$ = 5.1 Hz, 1 H, 2′-H), 4.05–4.12 (m, 3 H, 5′-H, 4′-H, 1′′-H), 4.25 (dd, $J_{H,H}$ = 5.0, 9.5 Hz, 1 H, 3′-H), 5.92 (s, 1 H, 1′-H), 8.52 (d, $J_{H,H}$ = 3.3 Hz, 1 H, 6-H), 8.71 (d, $J_{H,H}$ = 2.2 Hz, 1 H, 4-H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 14.50 (*C*H_3 base), 41.0 (C-2′′), 60.5 (C-5′), 68.7 (C-3′), 70.2 (C-1′′), 84.4 (C-2′), 85.7 (C-4′), 91.4 (C-1′), 115.9 (C-5), 119.7 (*C*F_3), 144.5 (C-4), 157.2 (C-2), 162.2 (C-6) ppm. HR-MS (ESI$^+$): 382.0978 [M$^+$ + Na] (calcd. for $C_{14}H_{18}F_3N_3O_6$: 382.1225).

1-[-5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]-5-methyl-2-pyrimidinone (24): Compound **21** (1.5 g, 3.9 mmol), co-evaporated from pyridine and dried overnight at high vacuum, was dissolved in dry pyridine (21 mL) and DMTr-Cl (2.4 g, 7.1 mmol) was added portionwise over 1.5 h. Stirring was continued for 5 h in the presence of molecular sieves. CH_2Cl_2 was added and the organic layer was washed with satd. $NaHCO_3$, dried with $MgSO_4$ and the solvents evaporated. Column chromatography (EtOAc + 1% of Et_3N) of the crude product afforded compound **24** (2.25 g, 84%) as a white foam. R_f = 0.2 (EtOAc + 1% Et_3N). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.56 (s, 3 H, C*H*$_3$ base), 1.77 (s, 1 H, O*H*), 3.53–3.79 (m, 3 H, 5′-H, 2′′-H), 3.80 (s, 6 H, OC*H*$_3$ DMT), 4.03 (d, $J_{H,H}$ = 5.1 Hz, 1 H, 2′-H), 4.11–4.22 (m, 4 H, 4′-H, 5′-H, 1′′-H), 4.49 (dd, $J_{H,H}$ = 5.5, 8.8 Hz, 1 H, 3′-H), 5.85 (s, 1 H, 1′-H), 6.83–6.86 (m, 4 H, DMT), 7.24–7.43 (m, 9 H, DMT), 7.99 (s, 1 H, N*H*), 8.27 (d, $J_{H,H}$ = 2.9 Hz, 1 H, 6-H), 8.42 (d, $J_{H,H}$ = 3.3 Hz, 1 H, 4-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.4 (*C*H_3 base), 40.5 (C-2′′), 56.0 (O*C*H_3 DMT), 61.6 (C-5′), 68.8 (C-3′), 70.3 (C-1′′), 83.5 (C-2′), 83.9 (C-4′), 87.5 (DMT), 90.8 (C-1′), 114.0 (DMT), 114.5 (C-5), 127.8 (DMT), 128.8 (DMT), 130.8 (DMT), 136.0 (DMT), 141.6 (C-6), 145.1 (DMT), 155.9 (C-2), 159.4 (DMT), 168.7 (C-4) ppm. MS (ESI$^+$): *m*/*z* (%) = 684.22 (100) [M$^+$ + H], 303.03 (22).

1-{3′-*O*-[2-Cyanoethyl(diisopropylamino)phosphanyl]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl}-5-methyl-2-pyrimidinone (27): To a solution of **24** (2.13 g, 3.1 mmol) in dry THF (60 mL), *i*Pr$_2$NEt (1.6 mL, 9.3 mmol) was added, followed by CEP-Cl (1 mL, 4.5 mmol). After stirring for 2 h at room temp., CH_2Cl_2 (200 mL) was added and the organic phase was washed with satd. $NaHCO_3$ (3 × 150 mL), dried with $MgSO_4$ and the solvents evaporated. Column chromatography (EtOAc + 1% Et_3N) afforded compound **27** (2.15 g, 78%) as a yellowish foam. R_f = 0.4, 0.3 (EtOAc + 1% TEA). M.p. 70–74 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.98 (d, $J_{H,H}$ = 6.6 Hz, 2 H, C*H*$_3$ *i*Pr), 1.10, 1.17 (2d, $J_{H,H}$ = 7.0 Hz, 10 H, C*H*$_3$ *i*Pr), 1.45, 1.50 (2s, 3 H, C*H*$_3$ base), 2.39, 2.58 (2t, $J_{H,H}$ = 6.1 and 5.9 Hz, 2 H, C*H*$_2$CN), 3.35–3.42 (m, 1 H, 5′-H), 3.45–3.68 (m, 7 H, 5′-H, C*H* *i*Pr, 2′′-H, OC*H*$_2$ CEP), 3.79, 3.80 (2s, 6 H, OC*H*$_3$ DMT), 4.06–4.16 (m, 3 H, 2′-H, 1′′-H), 4.25–4.34 (m, 1 H, 4′-H), 4.56–4.63 (m, 1 H, 3′-H), 5.88, 5.90 (2s, 1 H, 1′-H), 6.82–6.87 (m, 4 H, DMT), 7.28–7.36 (m, 7 H, DMT), 7.42–7.46 (m, 2 H, DMT), 7.91 (m,1 H, N*H* chain), 8.33 (m, 1 H, 6-H), 8.41–8.44 (m, 1 H, 4-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.3–14.4 (*C*H_3 base), 20.8–20.9 (*C*H_2CN), 25.1–25.4 (*C*H_3 *i*Pr), 40.5–40.6 (C-2′′), 43.8–43.9 (*C*H *i*Pr), 55.9–56.0 (O*C*H_3 DMT), 58.1–58.8 (O*C*H_2 CEP), 60.9 (C-5′), 69.5 (C-1′′), 69.6–69.8 (C-3′), 81.8 (C-2′), 82.6 (C-4′), 87.4 (DMT), 91.8–92.3 (C-1′), 113.7–113.9 (DMT), 114.5–114.6 (C-5), 118.1 (*C*F_3), 127.8–127.9 (DMT), 128.6–129.0 (DMT), 130.9–131.0 (DMT), 136.0 (DMT), 141.4–141.5 (C-6), 145.0 (DMT), 156.0 (*C*O chain), 159.4–159.5 (DMT), 168.6–168.7 (C-4) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.06, –76.02 ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 150.12, 152.54 ppm. HR-MS (ESI$^+$): 884.3609 (M$^+$ + H) (calcd. for $C_{44}H_{53}F_3N_5O_9P$: 884.3611).

[3′,5′-Di-*O*-diacetyl-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]thymine (19): Thymine (**14**) (1.3 g, 10.3 mmol) was suspended in dry CH_3CN (50 mL) and BSA (6.5 mL, 26.6 mmol) was added. The resulting clear solution was stirred for 1 h at room temp. Compound **10** (3.55 g, 8.5 mmol) in dry CH_3CN (100 mL) was then added at 0 °C, followed by portionwise addition of $SnCl_4$ (1.32 mL, 11.2 mmol). After stirring for 3 h at room temp., CH_2Cl_2 (150 mL) was added and the solution was washed with satd. $NaHCO_3$ (80 mL). The organic layer was dried with $MgSO_4$, evaporated to dryness and the crude product purified by column chromatography (EtOAc/hexane, 3:1) to give compound **19** (2.77 g, 67%) as white foam. R_f = 0.3 (EtOAc/hexane, 4:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.94 (d, $J_{H,H}$ = 1.1 Hz, 3 H, C*H*$_3$ base), 2.16 (s, 6 H, C*H*$_3$ Ac), 3.53–3.57 (m, 2 H, 2′′-H), 3.78–3.82 (m, 2 H, 1′′-H), 4.12–4.17 (m, 1 H, 2′-H), 4.39–4.45 (m, 3 H, 5′-H, 4′-H), 4.95 (t, $J_{H,H}$ = 6.0 Hz, 1 H, 3′-H), 5.89 (d, $J_{H,H}$ = 3.2 Hz, 1 H, 1′-H), 7.33 (s, 1 H, N*H* chain), 7.36 (d, $J_{H,H}$ = 1.3 Hz, 1 H, 6-H), 9.36 (s, 1 H, N*H* base).^{1}H NMR-difference NOE (400 MHz): δ = 3.78–3.82 (1′′-H) → 3.53–3.57 (2′′-H, 6%), 4.12–4.17 (2′-H, 4%), 5.89 (1′-H, 3%), 4.12–4.17 (2′-H) → 3.78–3.82 (1′′-H, 5%), 4.95 (3′-H, 7%), 5.89 (1′-H, 3%), 7.36 (6-H, 3%), 4.39–4.45 (5′-H) → δ = 4.95 (3′-H, 2%), 5.89 (1′-H, 1%), 7.36 (6-H, 1%), 4.39–4.45 (4′-H) → δ = 4.95 (3′-H, 3%), 5.89 (1′-H, 2%), 4.95 (3′-H) → 1.94 (Me base, 1%), 4.12–4.17 (2′-H, 7%), 4.39–4.45 (5′-H, 6%), 7.36 (6-H, 2%), 5.89 (1′-H) → 3.78–3.82 (1′′-H, 2%), 4.12–4.17 (2′-H, 2%), 4.39–4.45 (4′-H, 2%), 7.36 (6-H, 3%), 7.36 (6-H) → 1.94 (Me base, 3%), 4.12–4.17 (2′-H, 2%), 4.95 (3′-H, 1%), 5.89 (1′-H, 2%). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.4 (*C*H_3 base), 21.2, 21.5 (*C*H_3 Ac), 40.2 (C-2′′), 62.9 (C-5′), 69.9 (C-1′′), 70.7 (C-3′), 79.8 (C-4′), 81.7 (C-2′), 89.6 (C-1′), 112.4 (C-5), 134.9 (C-6), 151.4 (C-2), 157.9 (*C*O chain), 163.9 (C-4), 170.7, 171.2 (*C*O Ac). ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.18. HR-MS (ESI$^+$): 482.1373 (M$^+$ + H) (calcd. for $C_{18}H_{22}F_3N_3O_9$: 482.1386).

[2′-*O*-(2-Trifluoroacetamido)ethyl-β-D-ribofuranosyl]thymine (22): Compound **19** (2.77 g, 5.7 mmol) was dissolved in dry MeOH (60 mL), Na_2CO_3 anhydrous (277 mg, 10% w/w) was added and

the mixture was stirred at room temp. for 2 h. The reaction mixture was neutralized with DOWEX-50 (H^+-form, pH ca. 7), filtered through celite, washed with warm MeOH and the solvents evaporated. Compound **22** (2.52 g, quantitative yield) was obtained as a white solid, which could be used for the next step without further purification. R_f = 0.1 (EtOAc/hexane, 20:1). ^{1}H NMR (400 MHz, MeOD): δ = 1.90 (d, $J_{H,H}$ = 1.1 Hz, 3 H, CH_3 base), 3.44–3.63 (m, 2 H, 2′′-H), 3.74–3.81 (m, 2 H, 5′-H, 1′′-H), 3.84–3.96 (m, 2 H, 5′-H, 1′′-H), 3.99–4.04 (m, 2 H, 2′-H, 4′-H), 4.30 (t, $J_{H,H}$ = 5.5 H, 1 Hz, 3′-H), 5.95 (d, $J_{H,H}$ = 3.6 Hz, 1 H, 1′-H), 7.96 (d, $J_{H,H}$ = 1.1 Hz, 1 H, 6-H) ppm. ^{13}C NMR (75 MHz, MeOD): δ = 12.8(*C*H_3 base), 41.1 (C-2′′), 61.8 (C-5′), 69.8 (C-1′′), 70.1 (C-3′), 83.9 (C-4′), 86.3 (C-2′), 89.3 (C-1′), 111.6 (C-5), 138.3 (C-6), 152.7 (C-2), 166.7 (*C*O) ppm. ^{19}F NMR (376 MHz, MeOD): δ = –73.86 ppm. HR-MS (ESI$^+$): 398.1185 (M^+ + H) (calcd. for $C_{14}H_{18}F_3N_3O_7$: 398.1175).

[5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]thymine (25): Compound **22** (2.29 g, 5.8 mmol), co-evaporated from pyridine and dried overnight at the high vacuum, was dissolved in dry pyridine (30 mL) and DMT-Cl (2.9 g, 8.6 mmol) was added portionwise over 1.5 h. After stirring for another 2 h, CH_2Cl_2 (200 mL, filtered trough basic Alox) was added and the mixture was washed with satd. $NaHCO_3$ (2 × 100 mL) and dried with $MgSO_4$. Evaporation of the solvent and purification by column chromatography (EtOAc/hexane, 5:1 + 1% of Et_3N) gave compound **25** (3.56 g, 88%) as a white foam. R_f = 0.1 (EtOAc/hexane, 5:1 + 1% Et_3N). ^{1}H NMR (400 MHz, $CDCl_3$): δ — 1.37 (s, 3 H, CH_3 base), 2.94 (s, 1 H, O*H*), 3.45 (dd, $J_{H,H}$ = 2.6, 11.3 Hz, 1 H, 5′-H), 3.56-.372 (m, 3 H, 5′-H, 2′′-H), 3.80 (s, 6 H, OCH_3 DMT), 3.99–4.04 (m, 3 H, 2′-H, 1′′-H), 4.09 (d, $J_{H,H}$ = 7.0 Hz, 1 H, 4′-H), 4.44–4.46 (m, 1 H, 3′-H), 5.90 (d, $J_{H,H}$ = 1.9 Hz, 1 H, 1′-H), 6.85 (d, $J_{H,H}$ = 8.1 Hz, 4 H, DMT), 7.30–7.32 (m, 7 H, DMT), 7.40–7.42 (m, 2 H, DMT), 7.74 (d, $J_{H,H}$ = 1.0 Hz, 1 H, 6-H), 7.89 (t, $J_{H,H}$ = 5.5 Hz, 1 H, N*H* chain), 10.12 (s, 1 H, N*H* base) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.4 (*C*H_3 base), 40.3 (C-2′′), 55.9 (O*C*H_3 DMT), 62.2 (C-5′), 69.4 (C-3′), 69.8 (C-1′′), 83.9 (C-2′), 84.1 (C-4′), 87.6 (DMT), 88.9 (C-1′), 112.3(C-5), 113.9 (DMT), 118.5 (*C*F_3), 127.9 (DMT), 128.7–128–8 (DMT), 130.7–130.8 (DMT), 135.7 (C-6), 135.9–136.0 (DMT), 144.9 (DMT), 151.9 (*C*O chain), 158.4, 158.9, 159.4, 164.6 (DMT) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.15 ppm. HR-MS (ESI$^+$): 722.2276 [M^+ + Na] (calcd. for $C_{35}H_{36}F_3N_3NaO_9$: 722.2301).

{3′-*O*-[2-Cyanoethyl(diisopropylamino)phosphanyl]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl}thymine (28): To a solution of **25** (3.6 g, 5.1 mmol) in dry THF (100 mL) was added *i*Pr_2NEt (2.9 mL, 16.9 mmol), followed by CEP-Cl (1.9 mL, 8.5 mmol). After stirring for 3 h, CH_2Cl_2 (50 mL) was added and the mixture was washed with satd. $NaHCO_3$ (2 × 30 mL), dried with $MgSO_4$ and the solvents evaporated. Column chromatography (EtOAc/hexane, 2:1 + 1% Et_3N) afforded compound 28 (3.8 g, 82%) as a white foam. R_f = 0.4, 0.3 (EtOAc/hexane, 3:1 + 1% Et_3N). ^{1}H NMR (400 MHz, $CDCl_3$): δ — 1.00 (d, $J_{H,H}$ = 7.0 Hz, 2 H, CH_3 *i*Pr), 1.16 (dd, $J_{H,H}$ = 6.8, 9.0 Hz, 10 H, CH_3 *i*Pr), 1.33, 1.36 (2s, 3 H, CH_3 base), 2.42, 2.64 (2t, $J_{H,H}$ = 6.1 Hz and 5.9 Hz, 2 H, CH_2CN), 3.32–3.39 (m, 1 H, 5′-H), 3.52-.373 (m, 7 H, 5′-H, C*H* *i*Pr, 2′′-H, OCH_2 CEP), 3.80, 3.81 (2s, 6 H, OCH_3 DMT), 3.85–4.02 (m, 2 H, 1′′-H), 4.11–4.14 (m, 1 H, 2′-H), 4.21–4.28 (m, 1 H, 4′-H), 4.48–4.62 (m, 1 H, 3′-H), 5.92–5.94 (m, 1 H, 1′-H), 6.82–6.86 (m, 4 H, DMT), 7.27–7.34 (m, 7 H, DMT), 7.40–7.44 (m, 2 H, DMT), 7.61 (m, 1 H, N*H* chain), 7.76 (2d, $J_{H,H}$ = 1.1 Hz, 1 H, 6-H), 9.04 (s, 1 H, N*H* base) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.3–12.4 (*C*H_3 base), 20.9–21.2 (*C*H_2CN), 25.1–25.4 (*C*H_3 *i*Pr), 40.4–40.5 (C-2′′), 43.8–44.0 (*C*H *i*Pr), 55.9 (O*C*H_3 DMT), 58.3–58.7 (O*C*H_2 CEP), 61.8–62.0 (C-5′), 69.4 (C-1′′), 70.6–71.3 (C-3′), 82.6 (C-2′), 83.2 (C-4′), 87.5–87.6 (DMT), 89.4–89.7 (C-1′), 112.3(C-5), 113.9 (DMT), 118.2–118.4 (*C*F_3), 127.9 (DMT), 128.7–129.0 (DMT), 130.9–131.0 (DMT), 135.6 (C-6), 135.9–136.0 (DMT), 144.9 (DMT), 151.4 (*C*O chain), 159.4, 164.3 (DMT) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.06, –76.11 ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 149.18, 150.58 ppm. HR-MS (ESI$^+$): 900.3265 (M^+ + H) (calcd. for $C_{44}H_{53}F_3N_5O_{10}P$: 900.3560).

***N*-Benzoyl-5-methyl -[3′,5′-Di-*O*-acetyl-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]-cytosine (20):** To a solution of **10** (4.04 g, 9.7 mmol) in dry CH_3CN (80 mL) was added *N*-benzoyl-5-methylcytosine **17** (2.68 g, 11.7 mmol), followed by HMDS (2.4 mL, 11.5 mmol) and TMS-Cl (5.9 mL, 46.6 mmol). The mixture was cooled to –10 °C and $SnCl_4$ (1.2 mL, 10.2 mmol) was added. After stirring for 1 h at –10 °C and another 2 h at room temp., the mixture was evaporated, CH_2Cl_2 (200 mL) was added and the solution was washed with H_2O (90 mL) and satd. $NaHCO_3$ (2 × 90 mL). The organic layer was dried with $MgSO_4$, evaporated to dryness and the crude product purified by column chromatography (EtOAc/hexane, 1:1) to give compound **20** (4.33 g, 76%) as a white foam. R_f = 0.5 (EtOAc/hexane, 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.15 (d, $J_{H,H}$ = 0.9 Hz, 3 H, CH_3 base), 2.16, 2.18 (2s, 6 H, CH_3 Ac), 3.46–3.64 (m, 2 H, 2′′-H), 3.80 (t, $J_{H,H}$ = 5.0 Hz, 2 H, 1′′-H), 4.19 (dd, $J_{H,H}$ = 3.4, 5.5 Hz, 1 H, 2′-H), 4.41–4.46 (m, 3 H, 4′-H, 5′-H), 5.00 (t, $J_{H,H}$ = 5.9 Hz, 1 H, 3′-H), 5.93 (d, $J_{H,H}$ = 3.6 Hz, 1 H, 1′-H), 7.07 (s, 1 H, N*H* chain), 7.43–7.54 (m, 4 H, Bz, 6-H), 8.33 (d, $J_{H,H}$ = 7.2 Hz, 2 H, Bz), 13.38 (s, 1 H, N*H* base) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.5 (*C*H_3 base), 21.2, 21.5 (*C*H_3 Ac), 40.2 (C-2′′), 63.0 (C-5′), 70.0 (C-1′′), 70.9 (C-3′), 79.9 (C-4′), 81.5 (C-2′), 89.8 (C-1′), 113.2 (C-5), 118.4 (*C*F_3), 128.9, 130.7 (Bz), 133.4 (C-6), 148.7 (*C*O Bz), 157.8 (C-2), 159.8 (*C*O chain), 170.7, 171.1 (*C*O Ac) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.22 ppm. HR-MS (ESI$^+$): 585.1818 (M^+ + H) (calcd. for $C_{25}H_{27}F_3N_4O_9$: 585.1808).

***N*-Benzoyl-5-methyl-[2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]cytosine (23):** Compound **20** (0.66 g, 1.1 mmol) was suspended in absolute EtOH (3 mL) and pyridine (1.5 mL) was added. To the clear solution a 50% ethanolic NaOH solution (3 mL, NaOH 2 N/ EtOH abs. 1:1) was added and stirring was continued for 10 min at room temp. The solution was then neutralized with DOWEX-50 (H^+-form, pHca. 7), filtered through celite, washed with warm EtOH and the solvents evaporated. The crude product was purified by column chromatography (EtOAc) to give compound **23** (0.26 g, 46%) as a yellowish foam. R_f = 0.5 (EtOAc). ^{1}H NMR (300 MHz, MeOD): δ = 2.14 (s, 3 H, CH_3 base), 3.51–3.67 (m, 2 H, 2′′-H), 3.83–3.87 (m, 2 H, 1′′-H), 3.94–4.13 (m, 4 H, 2′-H, 4′-H, 5′-H), 4.33 (dd, $J_{H,H}$ = 5.5, 7.0 Hz, 1 H, 3′-H), 5.99 (d, $J_{H,H}$ = 2.6 Hz, 1 H, 1′-H), 7.50–7.60 (m, 4 H, Bz, 6-H), 8.29 (m, 2 H, Bz) ppm. HR-MS (ESI$^+$): 501.1581 (M^+ + H) (calcd. for $C_{21}H_{23}F_3N_4O_7$: 501.1597).

***N*-Benzoyl-5-methyl-[5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]-cytosine (26):** Compound **23** (0.8 g, 1.6 mmol), co-evaporated from dry pyridine and dried overnight at high vacuum, was dissolved in dry pyridine (16 mL). DMTr-Cl (1.08 g, 3.2 mmol) was added portionwise during 1.5 h and stirring was continued for another 2 h. CH_2Cl_2 (100 mL filtered through basic Alox) was added and the organic layer was washed with satd. $NaHCO_3$ (2 × 50 mL), dried with $MgSO_4$ and the solvents evaporated. The crude product was purified by column chromatography (EtOAc/hexane, 1:1 + 1% of Et_3N) to give compound **26** (0.81 g, 63%) as a yellowish foam. R_f = 0.3 (EtOAc/

hexane, 1:1 + 1% Et_3N). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.60 (s, 3 H, CH_3 base), 3.45–3.79 (m, 4 H, 2′′-H, 5′-H), 3.81 (s, 6 H, OCH_3 DMT), 3.95–4.26 (m, 4 H, 2′-H, 4′-H, 1′′-H), 4.47 (dd, $J_{H,H}$ = 5.5, 7.4 Hz, 1 H, 3′-H), 5.94 (d, $J_{H,H}$ = 2.2 Hz, 1 H, 1′-H), 6.87 (dd, $J_{H,H}$ = 1.5, 8.8 H, 4 Hz, DMT), 7.30–7.34 (m, 7 H, DMT), 7.42–7.47 (m, 4 H, Bz, DMT), 7.52–7.56 (m, 1 H, Bz), 7.88 (s, 1 H, 6-H), 8.30 (d, $J_{H,H}$ = 7.4 Hz, 2 H, Bz) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.7 (*C*H_3 base), 40.5 (C-2′′), 55.9 (O*C*H_3 DMT), 62.2 (C-5′), 69.7 (C-3′), 70.1 (C-1′′), 83.7 (C-2′), 84.1 (C-4′), 87.7 (DMT), 89.3 (C-1′), 113.0 (C-5), 114.0 (DMT), 127.9, 128.8–128.9, 130.6–130.8 (DMT, Bz), 133.3 (C-6), 135.9, 136.0, 144.9, 159.5, 159.5 (DMT, Bz) ppm. HR-MS (ESI$^+$): 804.2878 (M^+ + H) (calcd. for $C_{41}H_{42}F_3N_4O_9$: 803.2903).

***N*-Benzoyl-5-methyl-{3′-*O*-[2-cyanoethyl(diisopropylamino)phosphanyl]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)-ethyl-β-D-ribofuranosyl}cytosine (29):** To a solution of **26** (0.81 g, 1 mmol) in dry THF (25 mL) was added *i*Pr_2NEt (0.52 mL, 3.0 mmol) followed by CEP-Cl (0.16 mL, 1.5 mmol). After stirring for 3 h at room temp., CH_2Cl_2 (90 mL) was added and the organic phase was washed with satd. $NaHCO_3$ (2 × 30 mL), dried with $MgSO_4$ and the solvents evaporated. Column chromatography (EtOAc/hexane, 1:1 + 1% Et_3N) of the crude compound yielded 29 (0.86 g, 85%) as a yellowish foam. R_f = 0.5 (EtOAc/hexane, 1:1 + 1% Et_3N). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.00 (d, $J_{H,H}$ = 6.8 Hz, 2 H, CH_3 *i*Pr), 1.15 (dd, $J_{H,H}$ = 6.8, 9.6 Hz, 10 H, CH_3 *i*Pr), 1.50, 1.54 (2d, $J_{H,H}$ = 0.8 Hz, 3 H, CH_3 base), 2.41, 2.62 (2t, $J_{H,H}$ = 5.9 and 6.2 Hz, 2 H, CH_2CN), 3.33–3.41 (m, 1 H, 5′-H), 3.52-.374 (m, 7 H, 5′-H, C*H* *i*Pr, 2′′-H, OCH_2 CEP), 3.81–3.82 (m, 6 H, OCH_3 DMT), 3.92–3.95 (m, 2 H, 1′′-H), 4.13–4.17 (m, 1 H, 2′-H), 4.23–4.31 (m, 1 H, 4′-H), 4.52–4.65 (m, 1 H, 3′-H), 5.98 (d, $J_{H,H}$ = 2.3 Hz, 1 H, 1′-H), 6.83–6.89 (m, 4 H, DMT), 7.27–7.37 (m, 7 H, DMT), 7.41–7.47 (m, 2 H, DMT), 7.50–7.55 (m, 1 H, Bz), 7.59 (s, 1 H, N*H* chain), 7.93–7.95 (m, 1 H, 6-H), 8.30 (d, $J_{H,H}$ = 7.1 Hz, 2 H, Bz), 13.43 (s, 1 H, N*H* Bz) ppm. ^1H NMR-difference NOE (400 MHz): δ = 3.33–3.41 (5′-H) → 3.52-.374 (5′-H, 17%), 4.23–4.31 (4′-H, 6%), 4.52–4.65 (3′-H, 2%), 7.41–7.47 and 7.55 7.93 (DMT, Bz, 6%), 7.93–7.95 (6-H, 1%), 8.30 (Bz, 1%), 4.52–4.65 (3′-H) → 4.13–4.17 (2′-H, 7%), 7.41–7.47 and 7.55 7.93 (DMT, Bz, 3% and 4%), 7.93–7.95 (6-H, 3%), 8.30 (Bz, 1%), 5.98 (1′-H) → 4.13–4.17 (2′-H, 2%), 7.93–7.95 (6-H, 1%), 7.93–7.95 (6-H) → 1.50, 1.54 (Me base, 5%), 4.13–4.17 (2′-H, 2%), 4.52–4.65 (H3′, 3%), 5.97–5.98 (1′-H, 2%), 7.41–7.47 and 7.55 7.93 (DMT, Bz, 4% and 7%). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.53 (*C*H_3 base), 20.9–21.2 (*C*H_2CN), 25.2–25.3 (*C*H_3 *i*Pr), 40.5–40.6 (C-2′′), 43.8–44.0 (*C*H *i*Pr), 56.0 (O*C*H_3 DMT), 58.4–58.7 (O*C*H_2 CEP), 61.8–61.9 (C-5′), 69.4–69.5 (C-1′′), 70.6–70.7 (C-3′), 82.5 (C-2′), 83.2 (C-4′), 87.6 (DMT), 89.8 (C-1′), 113.0 (C-5), 113.9 (DMT), 118.1 (*C*F_3), 127.9 (DMT), 128.7–133.1 (DMT, Bz), 135.9 (C-6), 136.9 (DMT), 144.9 (DMT), 159.4 (DMT), 160.4 (*C*O) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.14, –76.10 ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 149.34, 150.88 ppm. HR-MS (ESI$^+$): 1003.3960 (M^+ + H) (calcd. for $C_{51}H_{58}F_3N_6O_{10}P$: 1003.3982).

***N*²-Acetyl-6-chloro-9-[3′,5′-di-*O*-acetyl-2′-*O*-(2-trifluoroacetamido)-ethyl-β-D-ribofuranosyl]purine (30):** BSA (0.74 mL, 3.0 mmol) was added to a suspension of the nucleobase 2-acetylamino-6-chloropurine (0.256 g, 1.2 mmol) in dry 1,2-dichloroethane (10 mL). The mixture was heated to 80 °C for 30 min and then evaporated to dryness. The residue was dissolved in dry toluene (5 mL) and **10** (0.457 g, 2.4 mmol) in dry toluene (5 mL) was added, followed by TMS-Tf (0.44 mL, 2.4 mmol). The solution was stirred at 80 °C for 2.5 h. The mixture was cooled to room temp., EtOAc (20 mL) was added and the organic phase was washed with satd. $NaHCO_3$ (2 × 10 mL) and finally dried with $MgSO_4$. The crude product was purified by column chromatography (EtOAc/hexane, 10:1) to give compound **30** (0.424 g, 68% yield) as a white foam. R_f = 0.4 (EtOAc/hexane, 10:1). ^1H NMR (400 MHz, $CDCl_3$): δ = 2.14, 2.17 (2s, 6 H, CH_3 Ac), 2.30 (s, 3 H, CH_3 base), 3.54–3.64 (m, 2 H, 2′′-H), 3.96–4.02 (m, 2 H, 1′′-H), 4.39–4.51 (m, 2 H, 5′-H), 4.53–4.58 (m, 1 H, 4′), 4.89 (dd, $J_{H,H}$ = 2.8, 5.0 Hz, 1 H, 2′-H), 5.07 (dd, $J_{H,H}$ = 5.3, 6.8 Hz, 1 H, 3′-H), 6.08 (d, $J_{H,H}$ = 2.6 Hz, 1 H, 1′-H), 7.81 (s, 1 H, N*H* chain), 8.28 (s, 1 H, 8-H), 8.35 (s, 1 H, N*H* Ac) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.2, 21.5 (*C*H_3 Ac), 25.7 (*C*H_3 base), 40.1 (C-2′′), 62.7 (C-5′), 69.5 (C-1′′), 70.9 (C-3′), 80.4 (C-4′), 81.0 (C-2′), 89.6 (C-1′), 130.0, 143.2 (C-8), 152.0, 152.1, 152.3, 169.1, 171.0, 171.1 (*C*O Ac, chain) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.32 ppm.

***N*²-Acetyl-9-[3′,5′-di-*O*-acetyl-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]purine (31):** Compound **30** (0.33 g, 0.6 mmol) was dissolved in dry MeOH (10 mL). Et_3N (0.1 mL, 0.7 mmol) and Pd/C 10% (0.16 g, 50% w/w) was added and the mixture stirred for 5 h under H_2. The catalyst was filtered off through celite, the solvent evaporated and the crude product purified by column chromatography (EtOAc) to give compound **31** (0.18 g, 58%) as a white solid. R_f = 0.2 (EtOAc). ^1H NMR (300 MHz, $CDCl_3$): δ = 2.14, 2.17 (2s, 6 H, CH_3 Ac), 2.34 (s, 3 H, CH_3 base), 3.54–3.62 (m, 2 H, 2′′-H), 3.90–4.03 (m, 2 H, 1′′-H), 4.38–4.49 (m, 2 H, 5′-H), 4.51–4.56 (m, 1 H, 4′-H), 4.93 (t, $J_{H,H}$ = 4.2 Hz, 1 H, 2′-H), 5.13 (t, $J_{H,H}$ = 5.7 Hz, 1 H, 3′-H), 6.11 (d, $J_{H,H}$ = 3.3 Hz, 1 H, 1′-H), 7.93 (s, 1 H, N*H* chain), 8.24 (s, 1 H, 8-H), 8.98 (s, 1 H, 6-H), 9.02 (s, 1 H, N*H* Ac) ppm. ^1H NMR-difference NOE (400 MHz): δ = 3.90–4.03 (1′′-H) → 3.54–3.62 (2′′-H, 4%), 4.91–4.94 (2′-H, 5%), 6.10–6.11 (1′-H, 3%), 7.93 (NH chain, 1%), 4.38–4.49 (5′-H) → 4.51–4.56 (4′-H, 4%), 5.13 (H3′, 3%), 4.51–4.56 (4′-H) → 5.13 (3′-H, 2%), 6.11 (1′-H, 2%), 4.91–4.94 (2′-H) → 3.90–4.03 (1′′-H, 5%), 5.13 (3′-H, 4%), 6.11 (1′-H, 4%), 8.24 (8-H, 1%), 5.13 (3′-H) → 4.38–4.49 (5′-H, 2%), 4.51–4.56 (4′-H, 2%), 4.91–4.94 (2′-H, 1%), 8.24 (8-H, 1%), 6.11 (1′-H) → 3.90–4.03 (1′′-H, 2%), 4.91–4.94 (2′-H, 2%), 8.24 (8-H, 2%), 8.24 (8-H) → 6.11 (1′-H, 3%), 5.13 (3′-H, 1%). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.2, 21.4 (*C*H_3 Ac), 25.7 (*C*H_3 base), 40.2 (C-2′′), 63.0 (C-5′), 69.5 (C-1′′), 71.1 (C-3′), 80.4 (C-4′), 80.9 (C-2′), 89.1 (C-1′), 118.5 (*C*F_3), 132.4, 143.2 (C-8), 150.5 (C-6), 151.9, 153.1, 158.0, 158.4, 171.0, 171.1 (*C*O Ac, chain) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.31 ppm.

***N*²-Acetyl-6-chloro-9-[2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]purine (32):** To a solution of **31** (0.18 g, 0.3 mmol) in dry MeOH (4 mL) was added anhydrous Na_2CO_3 (22 mg, 0.2 mmol, 12% w/w) and the suspension was stirred at room temp. for 1 h. The mixture was then neutralized by addition of DOWEX-50 (H^+-form), filtered through celite and washed with warm MeOH. Evaporation of the solvent afforded compound **32** (0.13 g, 86%) as a white solid that was used without further purification. R_f = 0.1 (EtOAc/MeOH, 10:1). ^1H NMR (300 MHz, $CDCl_3$): δ = 2.32 (s, 3 H, CH_3 base), 3.50–3.65 (m, 3 H, 1 × 1′′-H, 2′′-H), 3.81–3.99 (m, 5 H, 2′-H, 4′-H, 5′-H, 1 × 1′′-H), 4.15 (dd, $J_{H,H}$ = 4.2, 7.2 Hz, 1 H, 3′-H), 6.27 (d, J = 2.9 Hz, 1 H, 1′-H), 8.72 (s, 1 H, 8-H), 8.94 (s, 1 H, 6-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 24.7 (*C*H_3 base), 41.1 (C-2′′), 62.3 (C-5′), 70.0 (C-1′′), 70.5 (C-3′), 83.9 (C-4′), 87.1 (C-2′), 89.0 (C-1′), 119.7 (*C*F_3), 132.5, 146.2 (C-8), 150.2 (C-6), 153.1, 154.5, 159.1, 172.3 ppm.

***N*²-Acetyl-9-[5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]purine (33):** Compound **32** (0.13 g, 0.3 mmol) was dissolved in dry pyridine (2 mL) and DMTr-Cl (0.22 g, 0.6 mmol) was added portionwise over 1.5 h. After stirring for 4 h at room temp., CH_2Cl_2 (20 mL, filtered through basic Alox) was added. The organic layer was washed with satd. $NaHCO_3$

(2×10 mL), dried with $MgSO_4$ and the solvents evaporated. The crude product was purified by column chromatography (EtOAc + 1% of Et_3N) to give compound **33** (0.16 g, 74%) as a white foam. R_f = 0.4 (EtOAc + 1% Et_3N). ^1H NMR (300 MHz, $CDCl_3$): δ = 2.27 (s, 3 H, C*H*$_3$ base), 2.85 (s, 1 H, O*H*), 3.27–3.72, 4.03–4.21, 4.39–4.49 (3m, 9 H, 2′-H, 3′-H, 4′-H, 5′-H, 1′′-H, 2′′-H), 3.79 (s, 6 H, OC*H*$_3$ DMT), 6.11 (d, $J_{H,H}$ = 2.2 Hz, 1 H, H1′), 6.75 (d, $J_{H,H}$ = 8.5 Hz, 4 H, DMT), 7.19–7.35 (m, 7 H, DMT), 7.36 (d, $J_{H,H}$ = 7.3 Hz, 2 H, DMT), 8.21 (s, 1 H, 8-H), 8.27 (s, 1 H, N*H* chain), 8.94 (s, 1 H, 6-H), 9.39 (s, 1 H, N*H* base) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.6 (*C*H$_3$ base), 40.3 (C-2′′), 55.9 (OCH$_3$ DMT), 63.2 (C-5′), 69.6 (C-1′′), 70.2 (C-3′), 83.7 (C-2′), 84.3 (C-4′), 87.4 (DMT), 88.5 (C-1′), 114.0 (DMT), 127.7, 128.7–128.8, 130.7 (DMT), 132.2 (base), 136.1, 136.2 (DMT), 143.6 (C-8), 145.1, 150.2 (C-6), 152.0, 153.1 (base), 159.3 (*C*O chain) ppm. MS (ESI^+): *m*/*z* (%) = 751.23 (100) [M^+ + H].

N^2-Acetyl-9-[3′-*O*-(2-cyanoethyl(diisopropylamino)phosphanyl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]purine (34): Compound **33** (0.2 g, 0.3 mmol) was dissolved in dry THF (8 mL) and *i*Pr_2NEt (0.14 mL, 0.8 mmol) was added, followed by CEP-Cl (0.09 mL, 0.4 mmol). After stirring for 3 h at room temp., CH_2Cl_2 (40 mL) was added and the organic phase was washed with satd. $NaHCO_3$ (2×10 mL), dried with $MgSO_4$ and the solvents evaporated. Column chromatography (EtOAc/hexane, 1:1 + 1% Et_3N) gave compound 34 (0.21 g, 83%) as a white foam. R_f = 0.5, 0.6 (EtOAc/hexane, 1:1 + 1% Et_3N). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.04 (d, $J_{H,H}$ = 6.8 Hz, 2 H, C*H*$_3$ *i*Pr), 1.15–1.18 (m, 10 H, C*H*$_3$ *i*Pr), 2.26 (s, 3 H, C*H*$_3$ base), 2.37, 2.61 (2t, $J_{H,H}$ = 7.0 and 6.0 Hz, 2 H, CH_2CN), 3.67–3.74 (m, 8 H, H5′, CH *i*Pr, H2′′, OCH_2 Phosph.), 3.79 (m, 6 H, OC*H*$_3$ DMT), 3.84–4.06 (m, 2 H, 1′′-H), 4.35–4.39, 4.46–4.51, 4.61–4.64 (3m, 3 H, 2′-H, 3′-H, 4′-H), 6.14–6.16 (m, 1 H, 1′-H), 6.80–6.84 (m, 4 H, DMT), 7.27–7.37 (m, 9 H, DMT), 7.73 (s, 1 H, N*H* chain), 8.23–8.30 (s, 1 H, 8-H), 8.59 (s, 1 H, N*H* base), 8.96–8.97 (s, 1 H, 6-H). ^{19}F NMR (376 MHz, $CDCl_3$): δ = –76.20, –76.26 ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 150.70, 151.10 ppm. HR-MS (ESI^+): 973.3609 (M^+ + H) (calcd. for $C_{46}H_{54}F_3N_8O_9P$: 973.3601).

N^2-Acetyl-9-[3′,5′-di-*O*-acetyl-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]guanine (35): To a solution of cyanoethanol (0.5 mL, 7.3 mmol) in dry THF (4 mL), Cs_2CO_3 (4×0.35 g, 4.3 mmol) was added at 0 °C. The mixture was stirred at room temp. for about 30 min, then it was cooled to 0 °C and **30** (0.41 g, 0.7 mmol) in dry THF (4 mL) was added dropwise. After stirring at 0 °C for 1.5 h, the solution was evaporated to dryness. The crude product was purified by column chromatography (CH_2Cl_2/MeOH, 10:1 → 5:1) to give compound **35** (0.195 g, 58%) as a white solid. R_f = 0.3 (CH_2Cl_2/MeOH, 5:1). ^1H NMR (300 MHz, $CDCl_3$): δ = 2.27 (s, 3 H, C*H*$_3$ base), 3.48–3.58 (m, 2 H, 2′′-H), 3.80–3.92 (m, 4 H, 1′′-H, 5′-H), 4.07–4.11 (m, 1 H, 4′-H), 4.38 (t, $J_{H,H}$ = 4.0 Hz, 1 H, 2′-H), 4.46 (t, $J_{H,H}$ = 5.1 Hz, 1 H, 3′-H), 6.05 (d, $J_{H,H}$ = 3.9 Hz, 1 H, 1′-H), 8.35 (s, 1 H, 8-H) ppm. HR-MS (ESI^+): 465.1348 (M^+ + H) (calcd. for $C_{16}H_{19}F_3N_6O_7$: 465.1345).

N^2-Acetyl-9-[5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl]guanine (36): Compound **35** (0.22 g, 0.5 mmol) was dissolved in dry pyridine (2.4 mL) and DMTr-Cl (0.19 g, 0.6 mmol) was added portionwise over 1.5 h. After stirring for 2 h at room temp., CH_2Cl_2 (20 mL, filtered through basic Alox) was added. The organic layer was washed with satd. $NaHCO_3$ (2×10 mL), dried with $MgSO_4$ and the solvents evaporated. The crude product was purified by column chromatography (CH_2Cl_2/MeOH, 20:1 + 1% of Et_3N) to give compound **36** (0.117 g, 32%) as a yellowish foam. R_f = 0.30 (CH_2Cl_2/MeOH, 20:1). ^1H NMR (300 MHz, $CDCl_3$): δ = 2.24 (s, 3 H, C*H*$_3$ base), 3.36 (dd, $J_{H,H}$ = 4.5, 10.9 Hz, 1 H, 1×5′-H), 3.48–3.54 (m, 1 H, 1×5′-H), 3.67–3.68 (m, 2 H, 2′′-H), 3.77 (s, 6 H, OC*H*$_3$ DMT), 3.99–4.11 (m, 2 H, 1′′-H), 4.15–4.19 (m, 1 H, 2′-H), 4.22 (dd, $J_{H,H}$ = 1.9, 5.3 Hz, 1 H, 4′-H), 4.51 (dd, $J_{H,H}$ = 5.5, 7.5 Hz, 1 H, 3′-H), 5.91 (d, $J_{H,H}$ = 1.9 Hz, 1 H, 1′-H), 6.80 (dd, $J_{H,H}$ = 1.7, 8.8 Hz, 4 H, DMT), 7.19–7.33 (m, 7 H, DMT), 7.43 (d, $J_{H,H}$ = 7.0 Hz, 2 H, DMT), 7.89 (s, 1 H, 8-H), 8.63 (s, 1 H, N*H* chain) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 24.7 (*C*H$_3$ base), 40.9 (C-2′′), 55.9 (OCH$_3$ DMT), 63.5 (C-5′), 69.5 (C-1′′), 70.2 (C-3′), 83.5 (C-2′), 83.9 (C-4′), 87.2 (DMT), 89.1 (C-1′), 113.9 (DMT), 122.2 (C-5), 127.7, 128.6–128.8, 130.7 (DMT), 136.4, 137.8 (DMT), 145.2 (C-8), 148.3, 148.5, 156.6, 159.3, 173.5 (quaternary C) ppm. HR-MS (ESI^+): 767.2672 (M^+ + H) (calcd. for $C_{37}H_{37}F_3N_6O_9$: 767.2652).

N^2-Acetyl-9-[3′-*O*-(2-Cyanoethyl(diisopropylamino)phosphanyl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-trifluoroacetamido)ethyl-β-D-ribofuranosyl}guanine (37): Compound **36** (0.117 g, 0.15 mmol) was dissolved in dry THF (4 mL) and *i*Pr_2NEt (0.08 mL, 0.5 mmol) was added, followed by CEP-Cl (0.05 mL, 0.2 mmol). After stirring for 1.5 h at room temp., CH_2Cl_2 (40 mL) was added and the organic phase was washed with satd. $NaHCO_3$ (2×10 mL), dried with $MgSO_4$ and the solvents evaporated. Column chromatography (CH_2Cl_2/MeOH, 30:1 + 1% of Et_3N) gave compound 37 (0.21 g, 83%) as a white foam. R_f = 0.3, 0.4 (CH_2Cl_2/MeOH, 20:1). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.00 (d, $J_{H,H}$ = 6.8 Hz, 2 H, C*H*$_3$ *i*Pr), 1.12–1.18 (m, 10 H, C*H*$_3$ *i*Pr), 2.05, 2.12 (2s, 3 H, C*H*$_3$ base), 2.36, 2.62 (2t, $J_{H,H}$ = 6.2 and 6.2 Hz, 2 H, CH_2CN), 3.52–3.74 (m, 8 H, 5′-H, C*H* *i*Pr, 2′′-H, OCH_2 CEP), 3.79 (m, 6 H, OC*H*$_3$ DMT), 3.86–3.92 (m, 2 H, 1′′-H), 4.08–4.25, 4.33–4.42, 4.50–4.54 (3m, 3 H, 2′-H, 3′-H, 4′-H), 5.86, 5.93 (2d, $J_{H,H}$ = 2.6 and 3.6 Hz, 1 H, 1′-H), 6.81–6.84 (m, 4 H, DMT), 7.29–7.50 (m, 9 H, DMT), 7.86, 7.88 (2s, 1 H, 8-H) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 148.88, 149.72 ppm. MS (ESI^+) *m*/*z* 967.37 (M^+ + H).

Oligonucleotides: All 2′-AE oligonucleotides were prepared from the modified phosphoramidites **27**–**29** and commercially available dT solid support (Glen Research) on an Applied Biosystems Expedite 8909 DNA synthesizer by standard solid-phase phosphoramidite chemistry on the 1 μmol scale in the trityl-off mode. The following modifications relative to the standard protocols were made: i) the concentrations for 2′-AE phosphoramidites were 0.1–0.12 mM; ii) 5-ethylthio-1*H*-tetrazole (0.25 M in CH_3CN) was used as the coupling agent; iii) the coupling time for phosphoramidites **27**–**29**, **34**, and **37** was extended to 6 min. Deprotection and detachment from solid support was effected in a two step procedure. First, the solid support was treated with 1 mL of a 1 M solution of DBU in CH_3CN at room temp. for 1.5 h to remove the cyanoethyl protecting groups, followed by washing of the solid support with CH_3CN and H_2O. Then deprotection of the bases and detachment from solid support was performed by treatment with 1 mL concd. aqueous NH_3 solution at room temp. for 2 h. The crude oligonucleotides were purified by RP-HPLC (Aquapore RP-300, 7 μm, 220×4.6 mm, Brownlee or NUCLEOSIL 100–5, C18, 220×5 mm, Macherey–Nagel) and desalted over Sep-Pack C18 cartridges (Waters). The integrity of all oligonucleotides was confirmed by ESI-MS. Table 3 shows MS data of the 2′-AE oligonucleotides prepared in this way. All oligodeoxynucleotides for the target duplexes were prepared and HPLC-purified by standard techniques and routinely analyzed by ESI-MS.

Thermal Denaturation Experiments: UV melting experiments were performed with a Varian Cary 100 UV/Vis spectrophotometer equipped with a temperature controller. Data were collected with the Cary WinUV software. All measurements were conducted in a

Table 3. Experimental and calculated masses of TFOs.

TFO	Sequence	*m/z* (found)	*m/z* (calcd.)
T1	5′-ttttt**c**t**x**t**c**t**c**t**c**T	5308.2	5307.9
T2	5′-ttttt**x**t**x**t**x**t**c**t**c**T	5278.3	5278.0
T3	5′-tt**x**tt**x**t**x**t**x**t**c**t**x**T	5247.2	5246.9
T4	5′-ttt**c**x**cc**x**cc**x**c**ttT	5277.2	5274.2
T5	5′-**cc**t**c**x**t**c**x**t**c**x**t**c**tT	5275.4	5274.2
T6	5′-TTT**C**T**CC**T**CC**T**C**TTT	4495.0	4495.0
T7	5′-ttt**c**t**cc**t**cc**t**c**ttT	5358.3 [M+K$^+$]	5361.0 [M+K$^+$]
T8	5′-**c**tt**c**t**xxx**c**t**cc**t**c**T	5275.3	5274.2
T9	5′-**c**ttc**c**t**xxxxx**cc**t**c**	5244.2	5243.2
T10	5′-TTTTT**C**T**ap**T**C**T**C**T**C**T	4564.6	4565.1
T11	5′-TTTTT**C**TGT**C**T**C**T**C**T	4521.3	4522.0
T12	5′-TTTTT**C**T**AP**T**C**T**C**T**C**T	4505.6	4505.9
T13	5'-TTTTT**C**T**g**T**C**T**C**T**C**T	4579.1	4578.8
T14	5′-ttttt**ap**t**ap**t**ap**t**c**t**c**T	5352.2	5353.0
T15	5'-ttttt**g**t**g**t**g**t**c**t**c**T	5401.6	5400.9

buffer containing 10 mM sodium cacodylate, 100 mM NaCl, 0.25 mM spermine at the requisite pH. Oligonucleotides were mixed to 1:1:1 stoichiometry with 1.6 μM single-strand oligonucleotide concentration; the pH was measured directly in the sample. Melting curves were recorded at 260 nm in a consecutive heating-cooling-heating cycle (0–90 °C) with a temperature gradient of 0.5 °C/min. For temperatures < 20 °C, the spectrophotometer was flushed with nitrogen. To avoid evaporation of the solutions, 6–8 drops of dimethylpolysiloxane were added on top of the samples. T_m values were determined from the first derivative of the melting curve with the software package Origin™ V5.0.

Circular Dichroism: A Jasco J-715 spectropolarimeter equipped with a Jasco PFO-350S temperature controller was used. The temperature was measured directly in the sample. Subsequently, the graphs were smoothed with a noise filter.

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