Synthesis of 2′-Aminoalkyl-Substituted Fluorinated Nucleobases and Their Influence on the Kinetic Properties of Hammerhead Ribozymes

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*Hammerhead ribozymes are ribonucleic acids that catalyse the hydrolytic cleavage of RNA. They interfere with gene expression in a highly specific manner and recognize the mRNA target through Watson–Crick base pairing. To overcome the problem of point mutations (Watson–Crick "mismatches") occurring in viral genomes, we developed 2′-aminoethyl-substituted fluorinated nucleosides, which are universal nucleobases. The highly efficient synthetic pathway, which features a direct phthaloylamination of a primary alcohol under Mitsunobu conditions, leads to modified phosphoroamidites. The 1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose nucleoside analogue does not differentiate between the four natural nucleosides and leads to a RNA duplex that is as stable as the unmodified parent duplex. Upon incorporation into a ribozyme, the analogue's catalytic activity is equal for all four possible substrates, and the cleavage rates for the modified ribozymes are significantly higher (up to a factor of 13) than for the natural Watson–Crick "mismatch" base pairs. In agreement with the thermodynamic data obtained by measurement of the T_m values of the RNA 12-mers, the cleavage rates for the 2′-substituted fluorinated benzimidazole derivative **4** are slightly higher than for the corresponding fluorinated benzene derivative **3**.*

Introduction

RNA is a central molecule in the chemistry of life and is involved in the cellular process of gene expression and protein biosynthesis.[1,2] RNA exhibits great structural diversity, and its secondary structures and three-dimensional conformations are mainly stabilized by hydrogen bonding and base stacking. Many efforts have been undertaken to elucidate structural changes through the incorporation of artificial nucleosides.[3]

As a result of our investigations on fluorinated nucleobase analogues,[4] the disubstituted fluorobenzene and -benzimidazole derivatives **1** and **2** appear to be universal nucleobases.[5] These nucleobase analogues do not differentiate thermodynamically between the four natural nucleosides A, C, G or U in duplexes. Furthermore, an enhanced base-stacking ability upon fluorination is observed for the 2,4-difluorobenzene nucleoside derivative. Nevertheless, a destabilization of modified RNA 12-mer duplexes results due to a lower solvation by H_2O molecules and the absence of hydrogen bonds between the modified and the natural nucleosides.

Based on these findings and owing to the special properties of "organic fluorine"[6,7] such as a high electronegativity, a small size and the often-discussed ability to form weak hydrogen bonds, we designed nucleosides which should stabilize RNA duplexes additionally through electrostatic interactions. With this approach we wanted to establish universal nucleobases which are able to overcome the problem of point mutations in biological applications. Here, oligonucleotides are simply deactivated by mutating bases. One example is the toleration of escape mutants in HIV.

Therefore, we synthesized the 2′-β-aminoethyl-substituted fluorinated nucleobase analogues **3** and **4** and investigated their ability to stabilize RNA duplexes. As the primary amino group is protonated under physiological conditions,[8] the intermolecular electrostatic interaction with the negatively charged RNA backbone of the second RNA strand may lead to increased stability of the RNA 12-mers (Scheme 1).

To test the artificial nucleosides in a biological system we incorporated these new modifications into hammerhead ribozymes that are directed against the integrase region of the human immunodeficiency virus.[9]

Hammerhead ribozymes are catalytically active ribonucleic acids that interfere with gene expression through the hydrolysis of the complementary mRNA.[10–14] Thus, they are potential therapeutics for gene therapy. Their recognition of viral mRNA and their catalytic activity is dependant on Watson–Crick base pairing and decreases dramatically in the presence of point mutations ("hot spots") in the target region. Therefore, we modified the hammerhead ribozymes with the above-mentioned universal nucleobases. To investigate their ability to tolerate point mutations in the target sequence without losing their catalytic activity, we analysed the kinetics of the cleavage reactions.

This article presents the synthesis of the 2′-substituted artificial nucleosides and their influence on the stability of RNA duplexes and the kinetic properties of hammerhead ribozymes.

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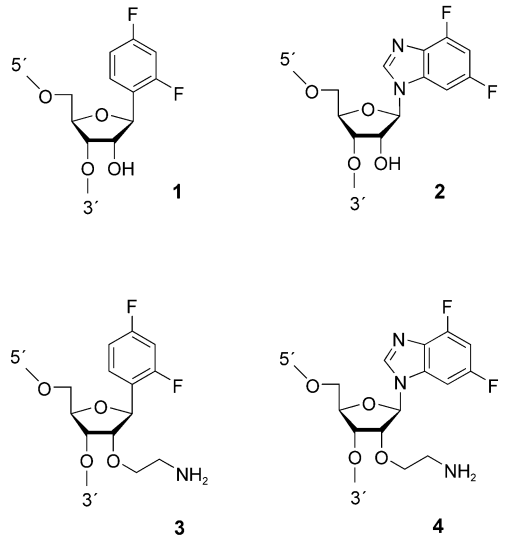
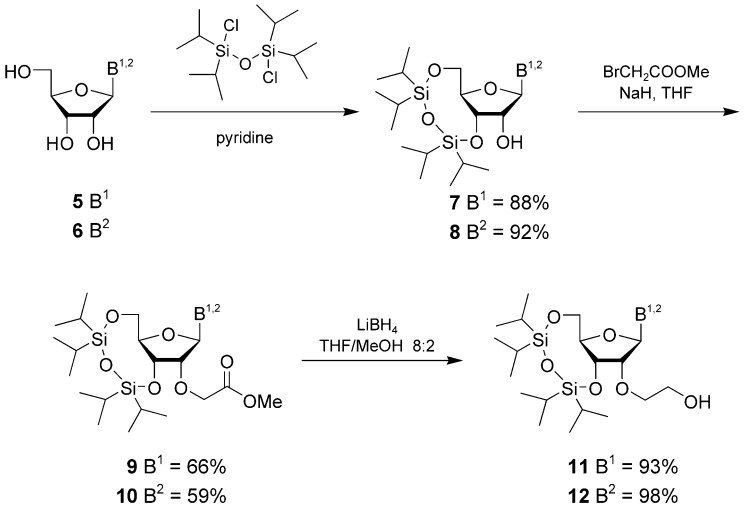


Scheme 1. *Modified fluorinated nucleoside analogues.*

Results and Discussion

Chemical synthesis

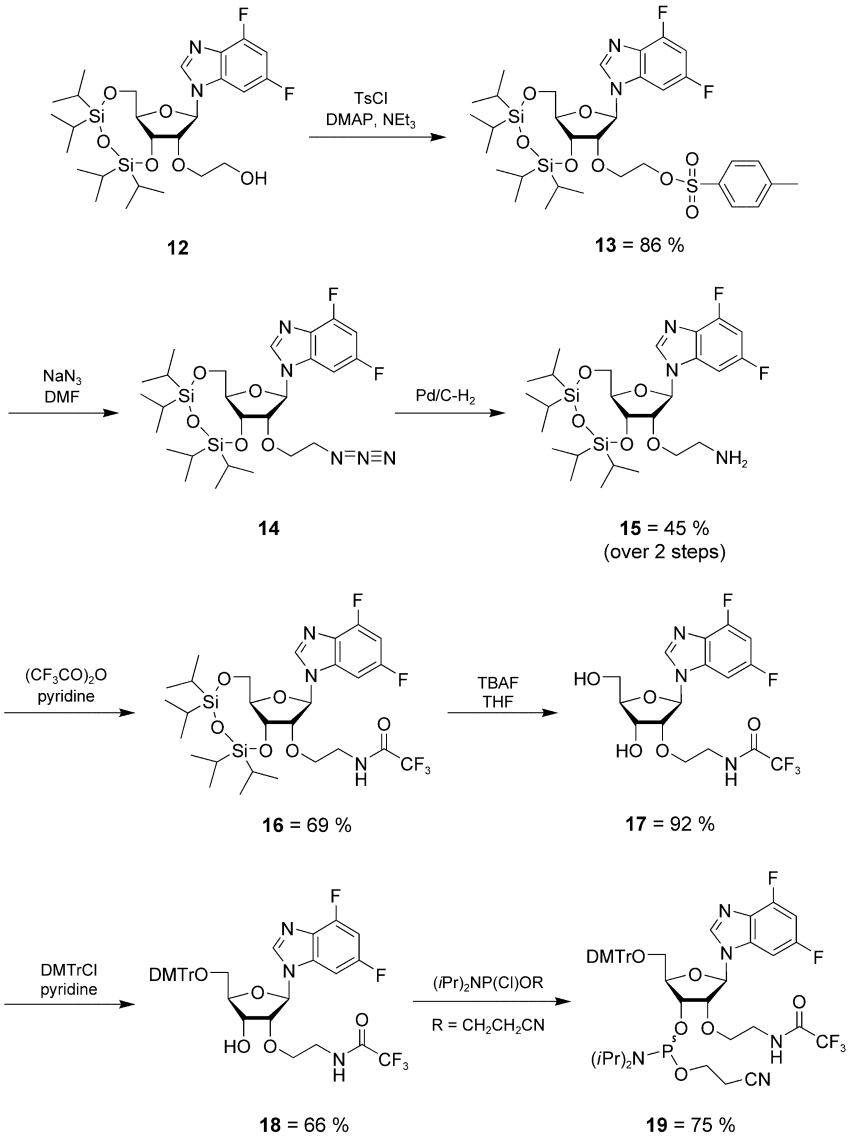
The fluorinated building blocks 1′-deoxy-1′-(2,4-difluorophenyl)-β-D-ribofuranose (**5**) and 1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-β-D-ribofuranose (**6**) were prepared according to the procedure established in our group, which is described in detail elsewhere by Parsch and Engels.[4] The simultaneous protection of the 5′- and 3′-hydroxyl group with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (Markiewicz reagent) was carried out in dry pyridine at room temperature and yielded the protected nucleosides **7** and **8** in 88% and 92%, respectively (Scheme 2). The Markiewicz reagent was synthesized by a Grignard reaction of 2-bromopropane, magnesium and trichlorosilane.[15] After the dimerisation of 1,1-diisopropylsilanol, the product 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane was obtained by subsequent chlorination in 81% overall yield. The alkylation of the 2′-position of the nucleosides **7** and **8** was performed with methyl bromoacetate and sodium hydride in anhydrous THF in 66% and 59% yield, respectively (Scheme 2). The reduction of the ester group to the primary alcohol was achieved by treatment with $LiBH_4$ in a mixture of dry THF/methanol (8:2) at 0°C. The desired nucleosides 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(β-hydroxyethyl)-β-D-ribofuranose (**11**) and 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-hydroxyethyl)-β-D-ribofuranose (**12**) were obtained in 93% and 98% yield, respectively.[16]



Scheme 2. *Synthesis of the 2′-β-hydroxyethyl-substituted nucleoside building blocks (B^1 = 2,4-difluorobenzene; B^2 = 4,6-difluorobenzimidazole).*

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-hydroxyethyl)-β-D-ribofuranose (**12**) was then treated with *p*-toluenesulfonyl chloride, *N*,*N*′-dimethylaminopyridine and triethylamine in dry pyridine to afford compound **13** in 86% yield. After conversion into the azide **14** with NaN_3, the crude product was hydrogenated over Pd/C at room temperature for 4 h to yield the primary amine **15** (45% yield over two reaction steps). The primary amine group of 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose (**15**) was then protected with trifluoroacetic acid anhydride (69%), and the Markiewicz group was cleaved with tetrabutylammonium fluoride in THF to give 1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose (**17**) in 92% yield. The 5′-OH function of **17** was protected with 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl) in dry pyridine and Et_3N to afford the 5′-*O*-(4,4′-dimethoxytrityl) derivative **18** in 66% yield. The final phosphorylation of the 3′-position gave the phosphoroamidite **19** in 75% yield (Scheme 3). This building block can be directly incorporated into RNA oligoribonucleotides by solid-phase synthesis (Caruther's method).

The phosphoroamidite **23** of the 2′-substituted compound carrying the 2,4-difluorobenzene nucleobase analogue was synthesized by a shorter, optimised pathway. Thus, direct phthaloylamination of 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(β-hydroxyethyl)-β-D-ribofuranose (**11**) under Mitsunobu conditions[17] with diethylazodicarboxylate (DEAD), triphenylphosphine and phthalimide afforded the phthalimide-protected nucleoside **20** in excellent yield (79%). The advantage of this synthetic strategy is the orthogonality of the phthalimide and dimethoxytrityl protecting groups, which means that the phthalimide group can be used in solid-phase oligoribonucleotide synthesis.[18] This group is stable under the standard phosphoroamidite coupling conditions and can be cleaved simultaneously with the exocyclic amino protecting groups of the natural nucleobases in NH_3/MeOH (3:1). The Markiewicz group of compound **20** was cleaved with tetrabutylammonium fluoride in THF to give 1′-deoxy-1′-(2,4-difluorophenyl)-2′-(β-*N*-phthalimidoethyl)-β-D-ribofuranose (**21**) in 98% yield (Scheme 4). After protection of the 5′-hydroxyl group with 4,4′-dimethoxytrityl chloride (75%), the product



Scheme 3. *Synthesis of 1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-*D*-ribofuranose (**15**) and its conversion into the corresponding phosphoroamidite **19**.*

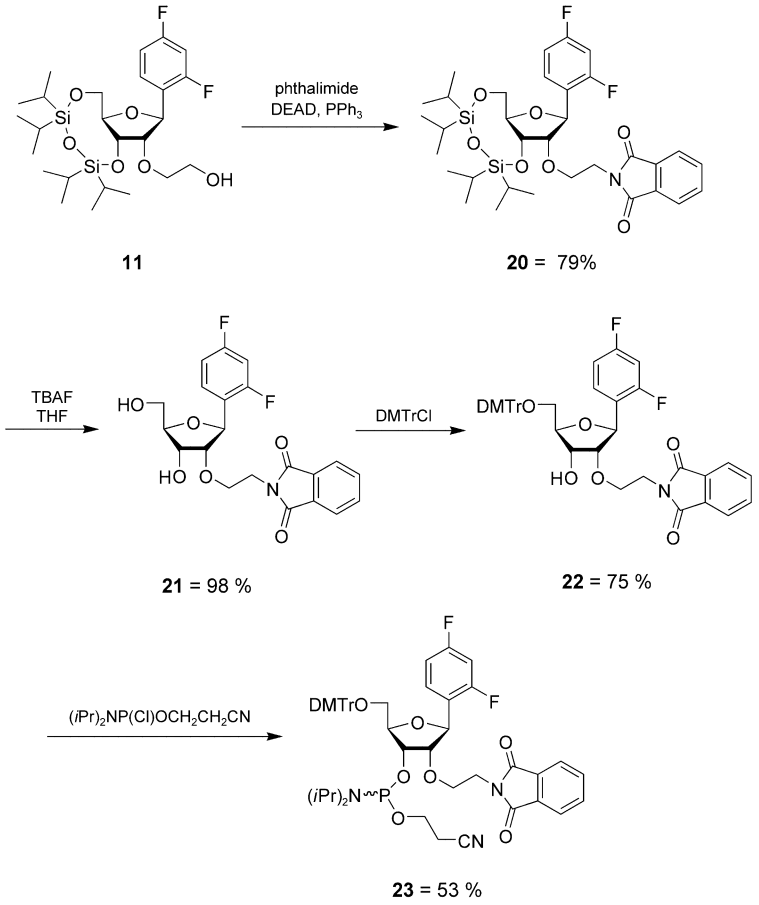
22 was phosphorylated to the corresponding phosphoroamidite **23** in 53% yield. The phthaloylamination also works well for the difluoro-substituted benzimidazole derivative (data not shown).

Thermal stabilities and thermodynamic data of modified RNA 12-mer duplexes

To investigate the influence of the modified nucleosides on the stability of RNA duplexes we measured the T_m values and calculated the thermodynamic properties of RNA 12-mers by UV/Vis melting studies. Therefore, we incorporated the modified phosphoroamidites into a defined A/U-rich RNA sequence (5′-CUU UUC **X** UU CUU-3′ paired with 3′-GAA AAG **N** AA GAA-5′). This sequence was also used in studies by Parsch and Engels;[4,19] thus, we are able to compare our results with those obtained for fluorinated nucleobase analogues without modification at the sugar moiety. The oligoribonucleotides were monomodified at the position marked with **X** and the two resulting strands were each hybridised with four different RNA strands carrying one of the four natural nucleobases opposite to the fluorinated nucleobase analogue (position marked with **N**, Table 1). With this

Table 1. *T_m values and the corresponding thermodynamic parameters of the mono-modified duplex RNAs (5′-CUU UUC **X** UU CUU-3′ paired with 3′-GAA AAG **N** AA-GAA-5′ in phosphate buffer containing 140 mM NaCl). ΔT_m is the difference in the T_m values compared to the corresponding 2′-unmodified fluorinated nucleobases **1** and **2**. The data for the unmodified duplexes (X = U) are published in ref. [4].*

Base pair X	N	T_m value [°C]	ΔT_m [°C]	$\Delta H°$ [kcal mol^{-1}]	$T\Delta S°$ [kcal mol^{-1}]	$\Delta G°$ [kcal mol^{-1}]
3	A	29.7 ± 0.2	+2.3	74.2 ± 3.5	65.0 ± 2.9	9.0 ± 0.3
	C	30.7 ± 0.2	+3.4	74.8 ± 1.2	65.2 ± 0.9	9.6 ± 0.1
	G	26.6 ± 0.3	−1.0	90.5 ± 2.1	81.7 ± 1.5	8.8 ± 0.1
	U	32.0 ± 0.2	+4.1	73.9 ± 4.7	64.0 ± 4.5	10.1 ± 0.2
4	A	35.9 ± 0.2	+7.5	72.2 ± 2.0	61.5 ± 2.0	10.8 ± 0.1
	C	36.7 ± 0.3	+8.0	74.3 ± 0.6	63.3 ± 0.6	10.9 ± 0.1
	G	37.3 ± 0.3	+7.9	72.6 ± 2.0	61.5 ± 1.8	11.1 ± 0.1
	U	38.3 ± 0.1	+9.0	83.3 ± 0.3	71.5 ± 0.3	11.8 ± 0.1
U	A	37.8 ± 0.1	–	87.8 ± 1.7	75.9 ± 1.6	11.9 ± 0.1
	C	30.4 ± 0.1	–	84.5 ± 1.3	74.8 ± 1.3	9.8 ± 0.1
	G	38.6 ± 0.2	–	83.0 ± 2.5	71.1 ± 2.4	11.9 ± 0.2
	U	30.1 ± 0.1	–	89.5 ± 1.6	79.8 ± 1.6	9.7 ± 0.1



Scheme 4. *Optimised synthetic pathway for the 2,4-difluorobenzene derivative **11** by the direct phthaloylamination of the alcohol to obtain the phosphoroamidite **23**.*

approach we mimic the point mutations in viral genomes and are able to investigate the effect of artificial nucleosides on the stability of RNA duplexes. The hybridization of the difluorobenzene- and difluorobenzimidazole-modified nucleosides **1** and **2** with all four natural nucleobases results in nearly identical T_m values (27.3–27.9 °C and 28.4–29.4 °C, light grey and white bars in Figure 1) for each set of the corresponding RNA duplexes. Hence, both nucleosides can be considered as universal bases which do not discriminate between the four natural ones in RNA duplexes. However, due to the absence of hydrogen bonds and the lower solvation of the modified bases by water molecules, the duplexes are less stable than the unmodified reference duplex (up to 2.4 kcal mol^{-1}). Regarding the natural base pairs, the T_m values for the Watson–Crick base pair (T_m = 37.8 °C) and the U·G Wobble base pair (T_m = 38.6 °C) are as expected significantly higher than for the mismatch base pairs U–C and U–U (T_m = 30.4 °C and T_m = 30.1 °C, respectively). The effect of the substitution of the aromatic hydrogen with fluorine atoms can be outlined by the measurement of the corresponding RNA modified with a benzene residue which shows significantly lower T_m values (dashed bars in Figure 1). Therefore, we improved the fluorinated universal bases by adding a 2′-(β-aminoethyl)group to the sugar moiety (**3** and **4** respectively, dark grey and black bars in Figure 1).

In the case of the 2′-(β-aminoethyl)-4,6-difluorobenzimidazole-modified nucleoside **4**, the protonated amino group leads to an enhanced stability which is equal to the unmodified parent duplex (black bars in Figure 1). This can be explained by electrostatic interactions with the negatively charged RNA backbone in the duplex. Furthermore, this artificial nucleoside can be considered as a universal nucleobase, too. It pairs with all four naturally occurring nucleosides without energy discrimination (Table 1). The maximum variation in the T_m values for the four modified duplexes is only 2.4 °C, which is a very small amount compared to literature data of universal nucleobases.[5] Compared to the Watson–Crick mismatch base pairs U–C and U–U, there is an increase of T_m values of up to 8.2 °C. A stabilization relative to the parent duplex is even observed in the case of the **4**–U modified base pair. The T_m values of the 12-mer RNA double strands modified with this nucleoside analogue are about 8 °C higher than the RNA duplex containing the fluorinated benzimidazole compound without 2′-modification. This enhanced stability of 2.2 kcal mol^{-1} can be assigned to the interaction of the protonated amino group.

RNA strands modified with 2′-(β-aminoethyl)-2,4-difluorobenzene-substituted nucleoside **3** behave completely differently. The analysed strands have much lower T_m values (26.6–32.0 °C, dark grey bars in Figure 1) but they still stabilize the RNA duplexes. Compared to the 2′-unmodified fluorinated benzenes and the Watson–Crick mismatch cases there is still a substantial stabilization of up to 4 °C except for the base pair **3**–G which destabilizes the RNA duplex by 1.0 °C. The conformation of the sugar moiety ("sugar puckering") or the geometry of the newly formed base pair and the accompanying possible restriction of the rotation of the nucleobase or the length of the 2′-alkyl chain may explain this loss of duplex stability, but this is still under investigation by means

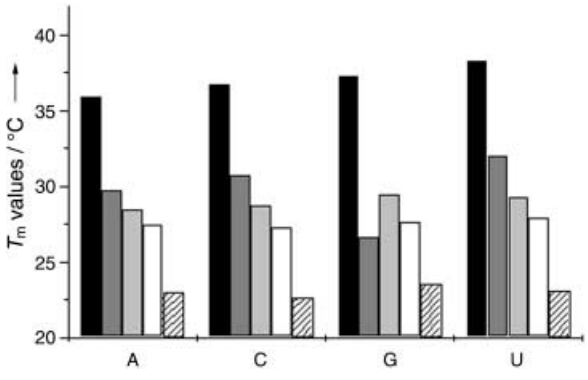


Figure 1. *T_m values of the mono-modified duplex RNAs (black bars = 1′-deoxy-1′-(4,6-difluoro-1H-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose (**4**), dark grey bars = 1′-deoxy-1′-(2,4-difluorophenyl)-2′-(β-aminoethyl)-β-D-ribofuranose (**3**), light grey bars = 1′-deoxy-1′-(4,6-difluoro-1H-benzimidazol-1-yl)-β-D-ribofuranose (**2**), white bars = 1′-deoxy-1′-(2,4-difluorophenyl)-β-D-ribofuranose (**1**), dashed bars = 1′-deoxy-1′-phenyl-β-D-ribofuranose.*

of NMR spectroscopy. Surprisingly, this modification differentiates between the nucleobases A, C, G and U. Paired with a pyrimidine nucleobase, the modification **3** results in distinctly higher T_m values (up to 2 °C) than those paired with purine nucleobases.

In summary, we have developed the new, artificial universal base **4** that stabilizes RNA duplexes as well as the natural Watson–Crick base pair U–A. By analogy with the results for the 2′-unmodified nucleobase analogues **1** and **2**, the RNA duplexes modified with 2′-substituted benzimidazole derivatives show higher stabilities than their modified counterparts based on the benzene modification.

Kinetic analysis of chemically modified hammerhead ribozymes

In order to compare the results obtained for the stability of modified RNA duplexes with the influence of the nucleoside analogues **3** and **4** on RNA cleavage reactions, we analysed the kinetic properties of chemically modified catalytic RNAs. In a previous study[20] we investigated the effect of the fluorinated nucleobase analogues **1** and **2** on the catalytic activity of hammerhead ribozymes. In correlation with the UV/Vis melting studies,[4] the mismatch variants of the hammerhead ribozyme–substrate complexes were still a little more active than the modified ribozymes. For an application of the fluorinated nucleosides in biological systems we needed to optimise them in order to enhance the catalytic activity of the ribozymes in such a way that they are superior to the Watson–Crick mismatch variants. For these further investigations we substituted the 2′-position as discussed in the previous chapters and we optimised the hammerhead ribozyme domains for the measurement under multiple turnover conditions (Figure 2). To reveal the influence of the artificial nucleobases, only one defined position in the hammerhead sequence was substituted. The hammerhead ribozyme was modified at the position 15.4[21] (marked **M** in Figure 2) and paired against any natural nucleobase **N** in a dye-labelled 17-mer substrate. Twelve different ribozyme–substrate complexes were analysed, which means that each of the two modifications **3** and **4** as well as the unmodified ribozyme were tested against the four naturally occurring substrates. The substrates were marked with the fluorescence dye Cy5. To avoid a possible interference of the dye with the ribozyme–substrate complex the dye was attached to the 5′-end of the substrate through a tetrathymidine-containing linker by using phosphoramidite chemistry. The cleavage products were detected with laser diodes in an ALF Express™ system.[22]

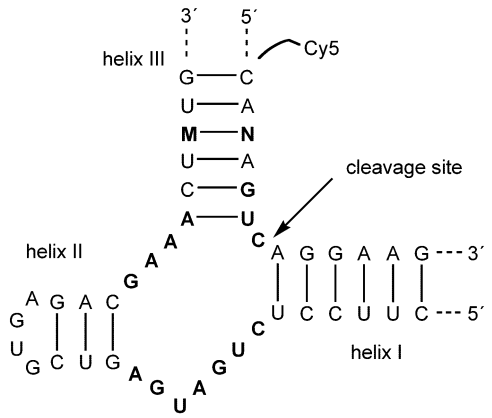


Figure 2. *Secondary structure of the chemically modified hammerhead ribozyme–substrate complex with highlighted sites of incorporation (**M** = A, **3**, **4**; **N** = A, C, G, U; Cy5 = fluorescence dye; bold black letters: highly conserved core structure).*

As expected, a great difference in the cleavage rates is observed when comparing the unmodified ribozyme (**M**–**N** = A–U) cleaving the Watson–Crick match substrate ($k_{cat} = 2.5\ min^{-1}$) and the three mismatch substrates (Table 2*)*. The kinetic analyses of the modified ribozymes reveal that the benzimidazole-modified ribozyme (**M** = **4**) cleaves all the four possible short synthetic RNA substrates quite effectively. The reaction rates are lower than for the unmodified ribozyme; however, focussing on the four different base pairs they do not vary very much ($k_{cat} = 0.19$–$0.39\ min^{-1}$, Table 2). These reaction rates are up to a factor 13 higher than those for the Watson–Crick mismatch ribozyme–substrate complexes. Thus, we have developed a hammerhead ribozyme which tolerates point mutations and does not differentiate between the four possible substrates. Interestingly, the ribozyme–substrate complex with a C opposite to the modification **4** (**M**–**N** = **4**–C) has the highest cleavage rates. One possible explanation for this result is that the 4,6-difluorobenzimidazole nucleobase acts as a steric mimic of guanosine, since the determined parameter fits well into that thought. As a result the hammerhead ribozyme modified with a 1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose nucleoside analogue **4** is a candidate for cell assays targeting HIV point mutations.

Table 2. *Kinetic parameters of the modified hammerhead ribozymes directed against the four possible substrates (k_{cat} = cleavage rate, K_m = Michaelis–Menten constant; **N** = A, C, G, U).*

M (ribozyme)	N (substrate)	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [$10^{-4} min^{-1} nM^{-1}$]
A	A	0.21 ± 0.12	2.09 ± 1.4	1.1 ± 0.2
	C	0.03 ± 0.01	0.11 ± 0.05	2.3 ± 0.2
	G	0.14 ± 0.06	0.93 ± 0.4	2.1 ± 0.7
	U	2.5 ± 0.8	2.2 ± 0.6	26 ± 16
3	A	0.21 ± 0.07	0.6 ± 0.37	5.3 ± 2.5
	C	0.15 ± 0.05	0.66 ± 0.4	2.7 ± 1.1
	G	0.14 ± 0.02	0.5 ± 0.1	2.9 ± 0.7
	U	0.11 ± 0.02	1.0 ± 0.5	1.3 ± 0.6
4	A	0.19 ± 0.1	2.4 ± 1.3	1.1 ± 0.5
	C	0.39 ± 0.12	2.03 ± 1.4	2.6 ± 1.3
	G	0.22 ± 0.12	3.08 ± 2.3	1 ± 0.7
	U	0.24 ± 0.1	0.93 ± 0.8	8.4 ± 4.1

For the difluorobenzene-modified ribozyme (**M** = **3**), the observed cleavage rates k_{cat} are only slightly higher than for the Watson–Crick mismatch cases. But as a result of smaller K_m values the catalytic efficiency remains in the range of those obtained for the difluorobenzimidazole modified ribozyme (**M** =

4). In correlation to the UV melting studies on the stability of RNA 12-mers, the improvement in the catalytic activity is not as distinct as for the benzimidazole derivative. In contrast to the low T_m value obtained for the base pair **3**–G, the kinetic analysis does not indicate a significant loss of activity.

Similar to the observation for the complex modified with the Watson–Crick "analogon" base pair (**4**–C), the highest reaction rate for the 2,4-difluorobenzene modification **3**, which is considered to be a steric mimic of uridine, is determined with the ribozyme–substrate complex containing the base pair **3**–A ($k_{cat} = 0.21\ min^{-1}$, Table 2). As the end point of all performed cleavage reactions is similar, the modifications seem to slightly decrease the reactions rates and it is not likely that they trap the ribozyme–substrate complex in an inactive conformation.

By comparing these results with the above-mentioned previous studies, it is evident that the incorporation of the 2′-aminoalkyl chain led to an enhancement of the cleavage rate in such a way that this new generation of chemically modified ribozymes tolerate point mutations and cleave short synthetic substrates substantially better than the Watson–Crick mismatch variants do.

Conclusion

A synthetic pathway leading to 2′-aminoethyl-substituted nucleoside analogues has been established and optimised through the application of the phthalimido group under Mitsunobu conditions. The artificial nucleobases were incorporated into RNA 12-mer duplexes in order to investigate their influence on the stability of RNA helices. As the difluorobenzimidazole derivative turned out to be a universal nucleobase, which stabilizes all four possible duplexes to the same extent as the Watson–Crick base pair does, the modified phosphoroamidites were introduced into a hammerhead ribozyme sequence, and their kinetic parameters were determined. Ribozymes modified with both **3** and **4** have better or at least equal catalytic activities relative to the Watson–Crick mismatches. This improvement can be assigned to the 2′-aminoethyl group owing to the fact that the 2′-unsubstituted fluorinated nucleosides have significantly lower catalytic rates than the Watson–Crick mismatch complexes. Thus, the most important ability for tolerating point mutations is implemented. The correlation of the results obtained in the UV/Vis melting studies with the kinetic analyses is challenging. In analogy to the thermodynamic properties, the 2′-substituted fluorinated benzimidazole modification **4** is significantly better than the Watson–Crick mismatch complexes and ribozymes modified with **3** have reaction rates comparable to the Watson–Crick mismatch ones. Nevertheless, the enhancement of the stability gained for the RNA duplexes modified with **4** in the UV/Vis melting studies is not so distinct in the kinetic studies. As the ribozyme activity is dependent on the formation of a precisely three-dimensionally folded structure between the ribozyme and its substrate, the introduced chemical modifications may have an influence on the geometry of the complex. Therefore, not only are the parameters investigated in 12-mer helices (such as base stacking, hydrogen bonding and solvation) of interest for the catalytic activity, but the steric demands of the modifications and the change of the overall conformation of the ribozyme–substrate complex also influence the kinetic parameters dramatically. Molecular dynamic simulations reveal that the C-F···H-C distance in base pairs containing fluorinated benzimidazoles and benzenes is about 0.5 Å longer than hydrogen bonds between natural nucleosides. Furthermore, the angle between the nucleobase and its adjacent nucleotide increases to an extent of 14° (50° instead of 36°, data not shown). This change leads to a closer contact which allows a better base stacking between the benzimidazole ring and its 5′-neighbouring nucleotide. The greater hydrophobicity of the 4,6-difluorobenzimidazole nucleobase may also be an explanation for its improved properties. It has been proposed[23] that nature inserts adenine so readily in non-instructional situations because of the greater hydrophobicity of adenine relative to the other normal bases. These studies may provide an explanation for the better ability of fluorinated benzimidazole nucleosides to stabilize RNA duplexes and to improve the cleavage rates of ribozymes compared to the fluorinated benzenes.

The presented chemical modifications with improved properties for RNA recognition can be easily introduced into different ribozymes making them applicable to a variety of different mRNA targets.

Experimental Section

Oligonucleotide synthesis: The RNA oligoribonucleotides (1.0 μmol scale) were synthesized on an Expedite 8900-DNA/RNA Synthesizer (Perseptive Biosystems) by phosphoroamidite chemistry with a coupling time of 12 min for the modified monomeric building blocks. The fully protected RNA strands were cleaved from the controlled-pore-glass (CPG) support with methanol in 25% aqueous NH_3 solution (1:3) at room temperature for 24 h. The 2′-silyl groups were deprotected with $Et_3N{\cdot}3HF$ within 24 h at room temperature. The crude RNA oligomers were precipitated with butanol at −20 °C and the fully deprotected RNAs were purified by sterile anion-exchange HPLC (JASCO-System, NucleoPac-PA-100 column from Dionex). The purified oligoribonucleotides were subsequently desalted over Sephadex-G25 columns (Amersham Biosciences). The purified hammerhead ribozymes were first desalted with ultracentrifugation membranes and then twice desalted with Sephadex columns (Amersham Biosciences). All oligoribonucleotides were characterized by MALDI-TOF mass spectrometry and the masses obtained were in good agreement with the calculated ones.

UV melting curves: UV melting profiles of the RNA duplexes were recorded with a CARY-1 UV-VIS Spectrophotometer (Varian) in 10 mm cuvettes. A phosphate buffer containing 140 mM NaCl (pH 7.0) was used at oligonucleotide concentrations of 2 μM for each strand and a wavelength of 260 nm. Each melting curve was determined three times. The temperature range was 10–70 °C with a heating rate of 0.5 °C min^{-1}. The thermodynamic data were calculated from the melting curves by means of a two-state model for the transition from duplex to single strand.[24]

Kinetic analysis: The cleavage reactions were carried out under multiple turnover conditions in 50 mM TRIS buffer (pH 8.0) with 10 mM $MgCl_2$. The substrate concentrations varied between 100 nM and 1250 nM; the ribozyme concentration was 50 nM or 25 nM, re-

spectively (ratio 1:2 to 1:50). The reaction mixtures were incubated at 37 °C and the reactions were stopped with formamide after definite times. The probes were then loaded on a 16% polyacrylamide gel (15% crosslinking, 1xTBE buffer, 7 M urea) and the Cy5-labeled cleavage products and substrates were detected with an ALF Express System (Amersham Biosciences) at 667 nm. Each kinetic analysis was performed at least three times and the Michaelis–Menten kinetics were calculated after the linearisation plots of Eadie–Hofstee and Lineweaver–Burk.

Chemistry: The anhydrous solvents (e.g. THF, CH_2Cl_2 or pyridine) were obtained from Fluka and were used without further purification. Unless otherwise stated the reactions were performed under anhydrous conditions under an inert atmosphere. All reactions were monitored by thin-layer chromatography by using silica gel 60 F_{254} plates from Merck. Flash column chromatography (FC) was performed with Merck silica gel 60 (40–63 μm). NMR spectra were recorded on Bruker spectrometers: AM250 and AMX400 (^{1}H, ^{13}C, ^{31}P); primes refer to the sugar moiety. Mass Spectra were recorded on a Perseptive Biosystems MALDI-TOF spectrometer Voyager DE or with electrospray ionisation (ESI).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-β-D-ribofuranose (8): 1′-Deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-β-D-ribofuranose[3] (**6**) (4.1 g, 14.3 mmol) was dissolved in anhydrous pyridine (42.6 mL). After the dropwise addition of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (5.0 g, 15.7 mmol, 1.1 equiv), the reaction mixture was stirred for 5 h at room temperature. The reaction was stopped by the addition of water and the mixture was extracted with CH_2Cl_2. The combined organic phases were washed with water, dried ($MgSO_4$), filtered, concentrated in vacuo and the residue was coevaporated twice with toluene. Purification by FC (CH_2Cl_2/MeOH 95:5) yielded **8** (6.9 g, 13.2 mmol, 92%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 8.35$ (s, 1H; H2), 7.41 (dd, 1H, $J_{1,2} = 6.7$ Hz, $J_{1,3} = 8.8$ Hz; H7), 7.12 (dt, 1H, $J_{1,2} = 2.0$ Hz, $J_{1,3} = 10.7$ Hz; H5), 5.91 (d, 1H, $J_{1,2} = 2.0$ Hz; H1′), 5.74 (d, 1H, $J_{1,2} = 4.7$ Hz; 2′-OH), 4.34 (q, 1H, $J_{1,2} = 2.7$ Hz, $J_{1,3} = 8.0$ Hz; H2′), 2.26 (t, 1H; H3′), 4.14 (m, 1H; H5′), 4.05 (m, 1H; H4′), 3.93 (m, 1H; H5″), 1.30–0.91 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ([D$_6$]DMSO): $\delta =$ 158.00 (dd, C_F), 157.00 (dd, C_F), 149.54 (C2), 134.53 (C_{arom}), 129.01 (C_{arom}), 97.91 (t, $J_{1,2} = 22$ Hz; C5), 94.78 (dd, $J_{1,2} = 4$ Hz, $J_{1,3} = 28$ Hz; C7), 90.24 (C1′), 81.46 (C2′), 73.37 (C4′), 69.18 (C3′), 60.53 (C5′), 17.03 (SiCHCH_3), 12.32 ppm (SiCHCH$_3$); ESI(+) MS: *m/z* (%): 529.3 ([*M*+H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-methylacetate-β-D-ribofuranose (10): Sodium hydride (0.59 g, 25.4 mmol, 1.3 equiv) was added to a solution of 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-β-D-ribofuranose (**8**) (10 g, 18.9 mmol) in anhydrous THF (100 mL). After the dropwise addition of methyl bromoacetate (5.14 mL, 55.9 mmol, 3 equiv), the mixture was stirred overnight and then poured into a saturated solution of NaCl. The product was extracted with CH_2Cl_2 and the combined organic extracts were dried over $MgSO_4$. Purification by FC (CH_2Cl_2/MeOH 98:2) yielded the product **10** (6.7 g, 11.1 mmol, 59%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 8.38$ (s, 1H; H2), 7.45 (d, 1H, $J_{1,2} = 1.9$ Hz; H7), 7.17 (t, 1H, $J_{1,2} = 8.4$ Hz; H5), 6.12 (s, 1H; H1′), 4.59–4.46 (m, 3H; H2′, H3′, -CH_2-), 4.39 (m, 1H; -CH_2-), 4.14 (m, 1H; H5′), 4.05 (m, 1H; H4′), 3.95 (m, 1H; H5′), 3.60 ppm (s, 3H; CH_3), 1.12–0.92 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ESI(+) MS: *m/z* (%): 601.5 ([*M*+H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-hydroxyethyl)-β-D-ribofuranose (12): Compound **10** (6.68 g, 11.1 mmol) was dissolved in a mixture of THF and methanol (8:2) (200 mL) and cooled down to 0 °C. Lithium borohydride (1.94 g, 89.4 mmol, 8 equiv) was added and the mixture was stirred for 40 min. After quenching the reaction with a saturated solution of NH_4Cl, the mixture was extracted with CH_2Cl_2 and the combined organic extracts were dried over $MgSO_4$. Purification by FC (CH_2Cl_2/MeOH 98:2) yielded the white solid product **12** (6.22 g, 10.9 mmol, 98%). ^{1}H NMR ($CDCl_3$): $\delta = 8.26$ (s, 1H; H2), 6.95 (dd, 1H, $J_{1,2} = 6.3$ Hz, $J_{1,3} = 8.0$ Hz; H7), 6.83 (dt, 1H, $J_{1,2} =$ 2.1 Hz, $J_{1,3} = 10.1$ Hz; H5), 5.91 (s, 1H; H1′), 4.55 (m, 1H; -αCH_2-), 4.30 (d, 1H, $J_{1,2} = 13.4$ Hz; -βCH_2-), 4.20 (d, 1H, $J_{1,2} = 9.3$ Hz; -αCH_2-), 4.06 (d, 1H, $J_{1,2} = 10.9$ Hz; -βCH_2-), 3.95 (m, 3H; H2′, H3′, H4′), 3.81 (m, 2H; H5′, H5″), 2.60 (br s, 1H; OH), 1.18–0.98 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ([D$_6$]DMSO): $\delta = 158.17$ (dd, $J_{1,2} = 250$ Hz; C_F), 153.40 (dd, $J_{1,2} = 253$ Hz; C_F), 143.09 (C2), 135.39 (C_{arom}), 129.83 (C_{arom}), 98.76 (t, $J_{1,2} = 28$ Hz; C5), 95.35 (dd, $J_{1,2} = 5$ Hz, $J_{1,3} = 28$ Hz; C7), 89.60 (C1′), 82.16 (Cα), 73.81 (Cβ), 70.62 (C2′), 61.34 (C4′), 61.04 (C5′), 17.94 (SiCHCH_3), 13.02 ppm (SiCHCH$_3$); ESI(−) MS: *m/z* (%): 571.6 ([*M*−H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-[(β-(*para*-toluenesulfonyl)ethyl)]-β-D-ribofuranose (13): 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-hydroxyethyl)-β-D-ribofuranose (**12**) (6.22 g, 10.9 mmol) was dissolved in anhydrous CH_2Cl_2 (240 mL). *N*,*N*′-Dimethylaminopyridine (1.35 g, 1.05 mmol), triethylamine (5 mL) and 4-toluenesulfonyl chloride (6.53 g, 34.3 mmol) were added. While stirring at room temperature for 2 h the yellow solution turns orange. The reaction was stopped by adding a saturated solution of $NaHCO_3$ and the mixture was extracted with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$ and the crude product was purified by FC (CH_2Cl_2/MeOH 99:1) to afford compound **13** (6.82 g, 9.4 mmol, 86%) as a light yellow foam. ^{1}H NMR ($CDCl_3$): $\delta = 8.18$ (s, 1H; H2), 7.90 (d, 2H, $J_{1,2} = 8.3$ Hz; H_{tos}), 7.30 (d, 2H, $J_{1,2} = 8.0$ Hz; H_{tos}), 6.87 (dd, 1H, $J_{1,2} = 1.4$ Hz, $J_{1,3} =$ 2.2 Hz; H7), 6.80 (dt, 1H, $J_{1,2} = 2.2$ Hz, $J_{1,3} = 10.1$ Hz; H5), 5.74 (s, 1H; H1′), 4.45 (m, 1H; -αCH_2-), 4.28 (m, 4H; -αCH_2-, -βCH_2-, H2′), 4.05 (m, 2H; H3′, H4′), 3.84 (m, 2H; H5′, H5″), 2.41 (s, 3H; CH_3), 1.09–0.94 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): $\delta = 159.55$ (dd, $J_{1,2} = 243$ Hz, $J_{1,3} = 17$ Hz; C_F), 153.51 (dd, $J_{1,2} = 243$ Hz, $J_{1,3} = 23$ Hz; C_F), 144.95 (C_{tos}), 140.79 (C2), 134.06 (C_{arom}), 130.18 (C_{arom}), 129.82 (C_{tos}), 128.56 (C_{tos}), 98.67 (t, $J_{1,2} = 22$ Hz; C5), 93.12 (dd, $J_{1,2} = 23$ Hz, $J_{1,3} = 28$ Hz; C7), 89.72 (C1′), 82.09 (Cα), 81.21 (Cβ), 70.15, 70.08, 69.47 (C2′, C3′, C4′), 59.38 (C5′), 21.56 (C_{tos}), 17.21 (SiCHCH_3), 12.90 ppm (SiCHCH$_3$); ESI(+) MS: *m/z* (%): 741.4 ([*M*+2Li]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-azidoethyl)-β-D-ribofuranose (14): Sodium azide (1.44 g, 22.2 mmol) was added to a solution of **13** (6.8 g, 9.38 mmol) in DMF (20 mL). The mixture was stirred at 55 °C for 4 h and then stirred overnight at room temperature. The reaction was quenched by adding H_2O and CH_2Cl_2. After extraction the combined organic extracts were dried over $MgSO_4$ and the crude product was used directly for the synthesis of compound **15**. ^{1}H NMR ($CDCl_3$): $\delta = 8.44$ (s, 1H; H2), 7.05 (dd, 1H, $J_{1,2} = 6.0$ Hz, $J_{1,3} = 7.9$ Hz; H7), 6.89 (dt, 1H, $J_{1,2} = 2.1$ Hz, $J_{1,3} = 9.9$ Hz; H5), 5.92 (s, 1H; H1′), 4.53 (m, 1H; -αCH_2-), 4.29 (m, 2H; H2′, -CH_2-), 4.21 (m, 1H; -CH_2-), 4.05 (m, 1H; -CH_2-), 3.96 (m, 1H; H3′), 3.80 (m, 1H; H4′), 3.53 (m, 1H; H5′), 3.49 (m, 1H; H5″), 1.11–0.94 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): $\delta = 159.99$ (dd, $J_{1,2} = 243$ Hz, $J_{1,3} =$ 11 Hz; C_F), 154.00 (dd, C_F), 141.36 (C2), 129.05 (C_{arom}), 126.04 (C_{arom}), 98.68 (t, $J_{1,2} = 22$ Hz, C5), 93.32 (d, C7), 89.90 (C1′), 83.05 (Cα), 81.25 (C2′), 71.10, 70.08, 59.32 (C3′, C4′, C5′), 50.81 (Cβ), 16.77 (SiCHCH_3), 12.73 ppm (SiCHCH$_3$); ESI(+) MS: *m/z* (%): 598.3 ([*M*+H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose (15): 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-azidoethyl)-β-D-ribofuranose (**14**) was dissolved in a mixture of THF/MeOH (1:1, 50 mL). After addition of the hydrogenation catalyst (Pd/C) the flask was flushed with hydrogen and the mixture was stirred for 4 h at room temperature. The catalyst was removed by filtration and the solvents were evaporated. The crude product was purified by FC (CH_2Cl_2/MeOH 9:1) to afford compound **15** (2.39 g, 4.2 mmol, 45% starting from **13**) as a colourless solid. ^{1}H NMR ($CDCl_3$): $\delta = 8.25$ (s, 1H; H2), 6.93 (dd, 1H, $J_{1,2} = 6.5$ Hz, $J_{1,3} = 8.1$ Hz; H7), 6.68 (dt, 1H, $J_{1,2} = 2.0$ Hz, $J_{1,3} = 10.2$ Hz; H5), 5.90 (s, 1H; H1′), 4.46 (m, 1H; -αCH_2-), 4.19 (m, 1H; -αCH_2-), 4.07 (m, 1H; H2′), 3.98 (m, 2H; H3′, H4′), 3.87 (m, 1H; H5′), 3.72 (m, 1H; H5″), 2.97 (m, 2H; -βCH_2-), 2.90 (br s, 2H; NH_2), 1.05–0.92 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ESI(+) MS: *m/z* (%): 572.5 ([*M*+H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose (16): 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose (**15**) (2 g, 3.5 mmol) was dissolved in anhydrous pyridine (40 mL) and cooled down to 0°C. Trifluoroacetic anhydride (0.6 mL, 4.2 mmol) was added dropwise and the temperature was allowed to warm to 25°C. The reaction mixture was then stirred at room temperature overnight. After evaporation and coevaporation with toluene the residue was diluted with CH_2Cl_2 and H_2O. After extraction the combined organic extracts were dried over $MgSO_4$ and the crude product was purified by FC (CH_2Cl_2/MeOH 98:2→CH_2Cl_2/MeOH 95:5) to give **16** (1.60 g, 2.4 mmol, 69%) as a white foam. ^{1}H NMR ($CDCl_3$): $\delta = 8.41$ (s, 1H; H2), 7.09 (br s, 1H; N*H*), 6.93 (dd, 1H, $J_{1,2} = 6.1$ Hz, $J_{1,3} = 7.9$ Hz; H7), 6.84 (dt, 1H, $J_{1,2} = 2.1$ Hz, $J_{1,3} = 10.0$ Hz; H5), 5.88 (s, 1H; H1′), 4.55 (m, 1H; -αCH_2-), 4.29 (m, 1H; -αCH_2-), 4.13–4.03 (m, 3H; H2′, H3′, H4′), 3.88 (m, 2H; H5′, H5″), 3.65 (m, 2H; -βCH_2-), 1.13–0.94 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): $\delta = 158.36$ (dd, C_F), 157.47 (CO), 157.10 (CF_3), 154.85 (dd; C_F), 140.94 (C2), 131.00 (C_{arom}), 115.84 (C_{arom}), 98.96 (t, $J_{1,2} = 29$ Hz; C5), 93.1 (d, $J_{1,2} = 27$ Hz; C7), 89.73 (C1′), 82.65, 81.57 (C2′, C3′), 69.79 (Cα), 69.38 (C5′), 59.36 (C4′), 40.02 (Cβ), 17.10 (SiCHCH_3), 12.96 ppm (Si$CH$$CH_3$); ESI(+) MS: *m/z* (%): 668.5 ([*M*+H]$^+$, 100).

1′-Deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose (17): Compound **16** (1.6 g, 2.4 mmol) was dissolved in anhydrous THF (100 mL). A solution of tetrabutylammonium fluoride (0.42 mL, 0.42 mmol, 2.1eq, 1 M in THF) was added dropwise. After stirring for 15 min at room temperature the solvents were evaporated and the residue was purified by FC (CH_2Cl_2/MeOH 98:2→CH_2Cl_2/MeOH 9:1) to give a **17** (1.31 g, 3.1 mmol, 88%) as a white foam. ^{1}H NMR ($CDCl_3$): $\delta = 8.60$ (s, 1H; H2), 8.08 (*t*, 1H; N*H*), 7.01 (dd, 1H, $J_{1,2} = 8.2$ Hz; H7), 6.75 (dt, 1H, $J_{1,2} = 2.0$ Hz, $J_{1,3} = 9.9$ Hz; H5), 5.87 (d, 1H, $J_{1,2} = 4.1$ Hz; H1′), 4.58 (t, 1H, $J_{1,2} = 5.0$ Hz; 5′-OH), 4.16 (m, 2H, 3′-OH; -αCH_2-), 3.90 (m, 2H; H2′, -αCH_2-), 3.50 (m, 2H; H3′, H4′), 3.18 (m, 2H; H5′, H5″), 2.91 ppm (m, 2H; -βCH_2-); ESI(−) MS: *m/z* (%): 424.1 ([*M*−H]$^+$, 100).

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose (18): 1′-Deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose (**17**) (1.3 g, 3.1 mmol) was dissolved in anhydrous pyridine (20 mL). After addition of triethylamine (0.7 mL) and 4,4′-dimethoxytriphenylmethyl chloride (1.21 g, 3.8 mmol, 1.2 equiv) the mixture was stirred at room temperature for 3 h. The reaction was quenched by adding methanol. After evaporating the solvent and coevaporation with toluene the residue was purified by FC (CH_2Cl_2/MeOH 98:2) to afford **18** (1.48 g, 2.03 mmol, 66%) as a yellow foam. ^{1}H NMR ($CDCl_3$): $\delta = 8.29$ (t, 1H; N*H*), 8.08 (s, 1H; H2), 7.44–7.19 (m, 10H; H_{arom}), 7.08 (dd, 1H, $J_{1,2} = 6.1$ Hz, $J_{1,3} = 8.2$ Hz; H7), 6.86–6.79 (m, 4H; H5, H_{arom}), 5.93 (d, 1H, $J_{1,2} = 5.6$ Hz; H1′), 4.53 (m, 1H; -αCH_2-), 4.33 (m, 2H; H2′, -αCH_2-), 3.79 (s, 3H; OMe), 3.78 (s, 3H; OMe), 3.72 (m, 1H; H3′), 3.59 (m, 1H; H3′), 3.48 ppm (m, 4H; H5′, H5″, -βCH_2-); ^{13}C NMR ($CDCl_3$): $\delta = 158.90$ (C_F), 158.57 (CO), 158.52 (CF_3), 157.00 (dd, C_F), 141.23 (C2), 129.92, 129.08, 127.95, 127.89, 127.74, 127.72, 126.99 126.95(C_{arom}), 123.94 (C_{arom}), 113.18, 113.06 (C_{arom}), 98.92 (C5), 94.50 (C7), 88.12 (C1′), 84.64, 82.30 (C2′, C3′), 69.71 (C4′), 68.75 (Cα), 63.04 (Cβ), 55.16 (OCH_3), 40.02 ppm (C5′); ESI(−) MS: *m/z* (%): 726.8 ([*M*−H]$^+$, 100).

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose-3′-(2-cyanoethyl diisopropylaminophosphoroamidite) (19): Collidine (2,4,6-trimethylpyridine, 1.4 mL, 10.7 mmol), 1-methyl-1*H*-imidazole (45 μL, 0.56 mmol) and were added to a solution of 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-1′-deoxy-1′-(4,6-difluoro-1*H*-benzimidazol-1-yl)-2′-(β-*N*-trifluoroacetamidoethyl)-β-D-ribofuranose (**18**) (0.8 g, 1.1 mmol) in anhydrous MeCN (45 mL). The mixture was cooled down to 0°C, and 2-cyanoethyl diisopropylphosphoramidochloridite (0.41 mL, 1.84 mmol, 1.7 equiv) was added slowly. The reaction mixture was stirred for 30 min at 0°C. The reaction was quenched by the addition of a saturated aqueous solution of $NaHCO_3$. After extraction with CH_2Cl_2, the combined organic extracts were dried over $MgSO_4$, and the crude product was rapidly purified by FC (CH_2Cl_2/MeOH 98:2) to give compound **19** (0.76 g, 0.82 mmol, 75%) as a white foam. ^{1}H NMR ($CDCl_3$): $\delta = 8.05$ (s, 1H; H2), 7.75 (br s, 1H; N*H*), 7.39–7.21 (m, 7H; H_{arom}), 7.06 (m, 1H; H7), 6.87–6.76 (m, 7H; H5, H_{arom}), 5.93 (d, 1H, $J_{1,2} = 6.6$ Hz; H1′), 4.38–4.12 (m, 4H; H2′, H4′, -αCH_2-), 3.88 (t, 1H, $J_{1,2} = 6.2$ Hz; H3′), 3.78 (s, 3H; OMe), 3.77 (s, 3H; OMe), 3.70 (m, 1H; -OCH_2CH_2CN-), 3.63–3.37 (m, 6H; H5′, H5″, -βCH_2-, –OCH_2CH_2CN), (t, 1H, $J_{1,2} = 6.3$ Hz; –OCH_2CH_2CN), 1.32–1.18 ppm (m, 14H; 2*i*Pr); ^{31}P NMR ($CDCl_3$): $\delta = 138.73$, 138.38 ppm (ratio 1:1.4); ESI(+) MS: *m/z* (%): 928.5 ([*M*+H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-β-D-ribofuranose (7): 1′-Deoxy-1′-(2,4-difluorophenyl)-β-D-ribofuranose[3] (**5**) (10.39 g, 42.2 mmol) was dissolved in anhydrous pyridine (126 mL). After the dropwise addition of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (14.8 g, 46.6 mmol, 1.1 equiv) the reaction mixture was stirred for 16 h at room temperature. The yellow reaction mixture was stopped by the addition of water and the mixture was extracted with CH_2Cl_2. The combined organic phases were washed with water, dried ($MgSO_4$), filtered, concentrated in vacuo and the residue was coevaporated twice with toluene. Purification by FC (CH_2Cl_2/MeOH 97:3) yielded **7** (18.1 g, 37.0 mmol, 88%). ^{1}H NMR ($CDCl_3$): $\delta = 7.55$ (q, 1H, $J_{1,2} = 6.6$ Hz, $J_{1,3} = 14.7$ Hz; H6), 6.83 (m, 2H; H3, H5), 5.10 (d, 1H, $J_{1,2} = 2.1$ Hz; H1′), 4.37 (dd, $J_{1,2} = 2.3$ Hz, $J_{1,3} = 7.7$ Hz; H2′), 4.13 (m, 2H; H3′, H4′), 4.03 (m, 2H; H5′, H5″), 2.57 (br s, 1H; OH), 0.97–1.18 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): $\delta = 163.10$ (dd, $J_{1,2} = 235$ Hz, $J_{1,3} = 12$ Hz, C_F), 160.42 (dd, $J_{1,2} = 236$ Hz, $J_{1,3} = 12$ Hz; C_F), 129.36 (C6), 123.95 (C1), 111.69 (d, $J_{1,2} = 21$ Hz; C5), 104.30 (t, $J_{1,2} = 25$ Hz; C3), 82.05 (C5′), 80.70 (C3′), 77.08 (C1′), 71.42 (C2′), 62.10 (C4′), 17.78 (SiCHCH_3), 13.92, 13.63, 13.34, 13.17 ppm (Si$CH$$CH_3$); ESI(+) MS: *m/z* (%): 506.4 ([*M*+NH_4]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-methylacetate-β-D-ribofuranose (9): Sodium hydride (0.43 g, 18.5 mmol, 1.3 equiv) was added to a solution of 3′,5′-*O*-

(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-β-D-ribofuranose (**7**) (8 g, 16.3 mmol) in anhydrous THF (80 mL). After the dropwise addition of methyl bromoacetate (3.94 mL, 42.9 mmol, 3 equiv) the mixture was stirred for 4 h and then poured into a saturated aqueous solution of NaCl. The product was extracted with CH_2Cl_2 and the combined organic extracts were dried over $MgSO_4$. Purification by FC (CH_2Cl_2) yielded the product **9** (6.02 g, 10.74 mmol, 66%). ^{1}H NMR ([D$_6$]DMSO): δ = 7.61 (q, 1H, $J_{1,2}$ = 6.9 Hz, $J_{1,3}$ = 15.5 Hz; H6), 7.23 (t, 1H, $J_{1,2}$ = 9.8 Hz; H3), 7.01 (t, 1H, $J_{1,2}$ = 7.3 Hz; H5), 5.10 (s, 1H; H1′), 4.40 (m, 2H; H2′, H3′), 4.21 (m, 1H; -CH_2-), 4.13 (m, 1H; -CH_2-), 3.98–3.87 (m, 3H; H4′, H5′, H5″), 3.61 (s, 3H; CH_3), 1.04–0.91 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): δ = 131.18 (C_{arom}), 128.09 (C_{arom}), 111.48 (C3), 103.86 (C5), 83.81, 81.97, 80.51 (Cα, Cβ, C1′), 72.93, 72.07, 71.11 (C2′, C3′, C5′), 62.44 (C4′), 16.77 (SiCHCH_3), 13.51 ppm (Si$CHCH_3$); ESI(+) MS: *m/z* (%): 561.3 ([*M*+H]$^+$, 100).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(*β*-hydroxyethyl)-*β*-D-ribofuranose (11): Compound **9** (6 g, 10.7 mmol) was dissolved in anhydrous THF (120 mL). After the addition of methanol (30 mL) the mixture was cooled down to 0 °C. Lithium borohydride (1.8 g, 83.1 mmol, 8 equiv) was added and the mixture was stirred for 3 h. The mixture was then stirred at room temperature for 1 h. After quenching the reaction with a saturated aqueous solution of NH_4Cl the mixture was extracted with CH_2Cl_2 and the combined organic extracts were dried over $MgSO_4$. Purification by FC (CH_2Cl_2/MeOH 98:2) yielded the white foam **12** (5.29 g, 9.94 mmol, 93%). ^{1}H NMR ($CDCl_3$): δ = 7.71 (q, 1H, $J_{1,2}$ = 6.7 Hz, $J_{1,3}$ = 8.4 Hz; H6), 6.82 (m, 2H; H3, H5), 5.20 (s, 1H; H1′), 4.27 (m, 2H; H2′, -αCH_2-), 4.05 (m, 1H; -αCH_2-), 3.96 (m, 2H; H3′, -βCH_2-), 3.83 (m, 1H; -βCH_2-), 3.75 (m, 3H; H4′, H5′, H5″), 2.57 (br s, 1H; OH), 1.15–0.91 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): δ = 162.62 (dd, $J_{1,2}$ = 235 Hz, $J_{1,3}$ = 12 Hz; C_F), 159.03 (dd, $J_{1,2}$ = 234 Hz, $J_{1,3}$ = 12 Hz; C_F), 128.41 (C6), 124.165 (C1), 111.07 (d, $J_{1,2}$ = 21 Hz; C5), 103.50 (t, $J_{1,2}$ = 21 Hz; C3), 85.15 (C5′), 80.36 (C3′), 79.18 (C1′), 72.79 (Cβ), 69.63 (C2′), 61.65 (C4′), 59.93 (Cα), 17.21 (SiCHCH_3), 13.41, 13.00, 12.83, 12.65 ppm (Si$CHCH_3$); ESI(+) MS: *m/z* (%): 550.5 ([*M*+NH_4]$^+$, 100)

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(*β*-*N*-phthalimidoethyl)-*β*-D-ribofuranose (20): The primary alcohol **11** (1 g, 1.88 mmol) was dissolved in anhydrous THF (66 mL) and phthalimide (0.28 g, 1.88 mmol) and triphenylphosphine (0.74 g, 1.8 mmol) were added. Diethylazodicarboxylate (0.44 mL, 1.8 mmol) was added dropwise and the reaction mixture was stirred for 16 h. After quenching the reaction with a saturated aqueous solution of $NaHCO_3$ the mixture was extracted with CH_2Cl_2 and the combined organic extracts were dried over $MgSO_4$. Purification by FC (CH_2Cl_2→CH_2Cl_2/MeOH 98:2) yielded the white foam **20** (0.99 g, 1.49 mmol,79%). ^{1}H NMR ($CDCl_3$): δ = 7.85 (m, 2H; H_{phth}), 7.72 (m, 2H; H_{phth}), 7.63 (q, 1H, $J_{1,2}$ = 7.1 Hz, $J_{1,3}$ = 15.2 Hz; H6), 6.78 (dt, 1H, $J_{1,2}$ = 2.3 Hz, $J_{1,3}$ = 8.1 Hz; H5), 6.68 (dt, 1H, $J_{1,2}$ = 1.7 Hz, $J_{1,3}$ = 8.8 Hz; H3), 5.07 (s, 1H; H1′), 4.20 (m, 2H, H3′; -αCH_2-), 4.12 (m, 1H; -αCH_2-), 4.02–3.91 (m, 5H; H4′, H5′, H5″, -βCH_2-), 3.69 (d, 1H, $J_{1,2}$ = 4.1 Hz; H2′), 1.15–0.92 ppm (m, 28H; Si–CH_3, Si–*i*Pr); ^{13}C NMR ($CDCl_3$): δ = 168.21 (CO), 162.24 (dd, $J_{1,2}$ = 235 Hz, $J_{1,3}$ = 12 Hz; C_F), 159.12 (dd, $J_{1,2}$ = 235 Hz, $J_{1,3}$ = 12 Hz; C_F), 133.76 (CH_{phth}), 132.17 (C_{phth}), 128.56 (dd, $J_{1,2}$ = 4 Hz, $J_{1,3}$ = 9 Hz; C6), 124.25 (C1), 123.16 (CH_{phth}), 110.89 (d, $J_{1,2}$ = 19 Hz; C5), 103.38 (t, $J_{1,2}$ = 26 Hz; C3), 84.98 (C2′), 80.10 (C5′), 78.76 (C1′), 69.87 (C3′), 67.99 (Cβ), 59.96 (Cα), 38.21 (C4′), 17.30 (SiCHCH_3), 13.37, 13.00, 12.80, 12.50 ppm (Si$CHCH_3$); ESI(+) MS: *m/z* (%): 679.5 ([*M*+NH_4]$^+$, 100).

1′-Deoxy-1′-(2,4-difluorophenyl)-2′-(β-*N*-phthalimidoethyl)-*β*-D-ribofuranose (21): 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxy)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(β-*N*-phthalimidoethyl)-β-D-ribofuranose (**20**; 1.5 g, 2.27 mmol) was dissolved in anhydrous THF. Tetrabutylammonium fluoride (1 M solution in THF, 4.76 mL, 4.76 mmol, 2.1 equiv) was added dropwise. After stirring for 20 min at room temperature the solvent was evaporated and the residue was purified by FC (CH_2Cl_2/MeOH 98:2→CH_2Cl_2/MeOH 9:1) to give **21** (0.93 g, 2.22 mmol, 98%) as a white foam. ^{1}H NMR ($CDCl_3$): δ = 7.80 (m, 4H; H_{phth}), 7.52 (q, 1H, $J_{1,2}$ = 6.8 Hz, $J_{1,3}$ = 15.4 Hz; H6), 6.88 (dt, 1H, $J_{1,2}$ = 2.4 Hz, $J_{1,3}$ = 8.5 Hz; H5), 6.80 (dt, 1H, $J_{1,2}$ = 1.3 Hz, $J_{1,3}$ = 9.5 Hz; H3), 4.89 (d, 1H, $J_{1,2}$ = 5.65 Hz; H1′), 4.83 (m, 2H; 3′-OH, 5′-OH), 4.01 (q, 1H, $J_{1,2}$ = 5.2 Hz, $J_{1,3}$ = 4.9 Hz; H2′), 3.85 (m, 1H, -βCH_2-), 3.75 (m, 2H; H3′, -αCH_2-), 3.69–3.55 (m, 4H; -αCH_2-, -βCH_2-, H4′, H5′), 3.52 ppm (m, 1H; H5″); ^{13}C NMR ([D$_6$]DMSO): δ = 167.76 (CO), 161.0 (dd, C_F), 158.80 (dd, C_F), 134.29 (CH_{phth}), 131.55 (C_{phth}), 129.38 (dd, $J_{1,2}$ = 4 Hz, $J_{1,3}$ = 6 Hz; C6), 123.83 (C1), 123.72 (CH_{phth}), 111.26 (d, $J_{1,2}$ = 21 Hz; C5), 103.21 (t, $J_{1,2}$ = 26 Hz; C3), 85.14 (C4′), 85.11 (C3′), 74.93 (C1′), 69.62 (C2′), 66.46 (Cβ), 61.41 (C5′), 37.55 (Cα), 17.29 (SiCHCH_3), 13.64, 13.54, 12.97, 12.60 ppm (Si$CHCH_3$); ESI(+) MS: *m/z* (%): 420.0 ([*M*+H]$^+$, 100).

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(*β*-*N*-phthalimidoethyl)-*β*-D-ribofuranose (22): 1′-Deoxy-1′-(2,4-difluorophenyl)-2′-(β-*N*-phthalimidoethyl)-β-D-ribofuranose (**21**) (0.93 g, 2.2 mmol) was dissolved in anhydrous pyridine (14 mL). After addition of triethylamine (0.5 mL) and 4,4′-dimethoxytriphenylmethyl chloride (0.86 g, 2.7 mmol, 1.2 equiv) the mixture was stirred at room temperature for 3 h. The reaction was quenched by adding methanol. After evaporating the solvent and coevaporation with toluene the residue was purified by FC (CH_2Cl_2/MeOH 98:2→CH_2Cl_2/MeOH 95:5) to afford **22** (1.20 g, 1.66 mmol, 75%) as a yellow foam. ^{1}H NMR ($CDCl_3$): δ = 7.75 (m, 2H; H_{phth}), 7.60 (m, 2H; H_{phth}), 7.50 (q, 1H; H6), 7.35 (m, 3H; H_{arom}), 7.3–7.05 (m, 7H; H_{arom}), 6.95 (t, 1H; H3), 6.70 (m, 3H; H_{arom}), 6.58 (m, 1H; H5), 5.06 (s, 1H; H1′), 4.05–3.93 (m, 3H; H2′,-CH_2-), 3.90–3.79 (m, 3H; H3′,-CH_2-), 3.67 (s, 6H; OMe), 3.59 (m, 1H; H4′), 3.35 (m, 1H; H5′), 3.24 ppm (m, 1H; H5″); ^{13}C NMR ([D$_6$]DMSO): δ = 168.74 (CO), 159.03, 158.97 (C_F), 135.04 (CH_{phth}), 132.30 (C_{phth}), 128.55, 128.49, 128.40 ($C_{arom.}$), 123.62 (CH_{phth}), 113.85 (t, $J_{1,2}$ = 27 Hz; C3), 76.11 (C1′), 70.27 (C2′), 66.92 (Cβ), 63.96 (C5′), 55.36 (C4′), 51.62 (C3′), 37.67 (Cα), 17.07 ppm (OMe); ESI(+) MS: *m/z* (%): 744.2 ([*M*+Na]$^+$, 55).

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(*β*-*N*-phthalimidoethyl)-*β*-D-ribofuranose-3′-(2-cyanoethyl diisopropylphosphoroamidite) (23): Collidine (2,4,6-trimethylpyridine, 1.05 mL, 8.0 mmol) and 1-methyl-1*H*-imidazole (34 μL, 0.42 mmol) were added to a solution of 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-1′-deoxy-1′-(2,4-difluorophenyl)-2′-(β-*N*-phthalimidoethyl)-β-D-ribofuranose (**22**) (0.60 g, 0.83 mmol) in anhydrous MeCN (34 mL). The mixture was cooled down to 0 °C and 2-cyanoethyl diisopropylphosphoramidochloridite (0.31 mL, 1.39 mmol, 1.7 equiv) was added slowly. The reaction mixture was stirred for 40 min at 0 °C. The reaction was quenched by the addition of a saturated aqueous solution of $NaHCO_3$. After extraction with CH_2Cl_2 the combined organic extracts were dried over $MgSO_4$ and the crude product was rapidly purified by FC (CH_2Cl_2/MeOH 98:2) to give compound **23** (0.40 g, 0.44 mmol, 53%) as a white foam. ^{1}H NMR ($CDCl_3$): δ = 7.80 (m, 2H; H_{phth}), 7.71 (m, 2H; H_{phth}), 7.50 (q, 1H; H6), 7.52–7.17 (m, 6H; H_{arom}), 6.82 (m, 7H; H_{arom}), 6.62 (dt, 1H, $J_{1,2}$ = 2.0 Hz, $J_{1,3}$ = 8.4 Hz; H3), 6.36 (dt, 1H, $J_{1,2}$ = 0.7 Hz, $J_{1,3}$ = 10.4 Hz; H5), 5.09 (d, 1H, $J_{1,2}$ = 6.7 Hz; H1′), 4.22 (m, 2H; H2′, H3′), 4.0–3.87 (m, 3H; H4′, -CH_2-), 3.85 (m, 2H; -CH_2-), 3.79 (s, 3H; OMe), 3.78 (s, 3H; OMe), 3.75 (m, 2H; -OCH_2CH$_2$CN-), 3.40 (m, 1H; H5′), 3.24 (m, 1H; H5″), 2.31 (t, 2H, $J_{1,2}$ = 6.6 Hz; –OCH$_2$CH_2CN), 1.13 (m,

7H; *i*Pr), 0.98 ppm (m, 7H, *i*Pr); ^{31}P NMR ($CDCl_3$): $\delta = 150.81$, 150.51 ppm (ratio 1:2.4); ESI(+) MS: *m/z* (%): 922.8 ($[M+H]^+$, 100).

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