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A novel entry to 2′-*O*-aminopropyl modified nucleosides amenable for further modifications

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Abstract—2′-*O*-aminopropyl modified 4,6-difluoro-substituted benzimidazole nucleosides were synthesized in a two-step synthesis via Michael reaction and a Raney-nickel catalyzed reduction in high yields. These building blocks were used as a starting point for the conjugation of different carboxylic acids to enhance the lipophilicity or cationic character of oligonucleotides when used in biological assays or medical applications.
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Chemically modified RNAs are more and more in the focus of medical applications for gene silencing like, for example, antisense, antigene and RNA interference (RNAi).[1,2] Particularly, for siRNAs modifications appear to be necessary to increase efficacy of this evolutionary mechanism in humans.[3,4] Thus, chemical modifications of one or both strands are desired for therapeutic applications to enhance stability and to improve pharmacokinetic properties. There are many different possibilities to modify RNAs: well known are modifications to the phosphate-backbone, the nucleobase and at the 2′-O-position of the ribose.[5]

Especially the 2′-O-position is attractive for attaching functional groups. It is proven that modifications at this position in siRNAs are generally well tolerated since the 2′-OH are not involved in recognition of the catalytic ribonuclease activity of RNA-induced silencing complex (RISC).[3] Furthermore, 2′-O-alkylated RNAs bearing groups like 2′-*O*-methoxy, 2′-*O*-methoxyethyl or 2′-*O*-propargyl exhibit excellent nuclease resistance and affinity for the complementary oligonucleotides.[6–9]

Due to their negatively charged backbone, siRNAs usually show a poor passing through cell membranes. For therapeutic approaches, this property limits severely addressing the targeted tissue. Thus, considering a successful drug delivery, chemical modification could also help increase cellular-uptake of siRNAs.

In this context, modifications bearing cationic charges promise to have a pronounced effect.[10] The synthetic access to this sort of modifications is usually not straightforward; 2′-O-alkylation with strong bases like NaH to activate the 2′-OH-group often are the only possibility to obtain these derivatives.[11] These alkylations, though practically useful, often result in low yields and in a variety of side reactions.

Recently, we reported that the disubstituted fluorobenzimidazole (DFBi) **1** can be used as a universal nucleobase, which does not differentiate between the four natural nucleosides A, C, G and U in a duplex. The use of such universal nucleosides in terms of siRNA-modification could offer new possibilities to challenge point-mutations (‘escape-mutants’) in short RNAs, occurring for example in HIV-infections.[12,13] Moreover, the attachment of a 2′-*O*-aminoethyl residue **2** leads to an increased thermodynamic stability of RNA duplexes in comparison to 2′-O-unmodified difluorobenzimidazole nucleoside (Fig. 1).

Thus, once inserted into a siRNA, universal nucleosides could enhance the thermostability and pharmacokinetic properties as well as prevent possible point-mutations as mentioned above.

Keywords: Nucleosides; 2′-Modification; Michael addition; Oligonucleotides; siRNA.
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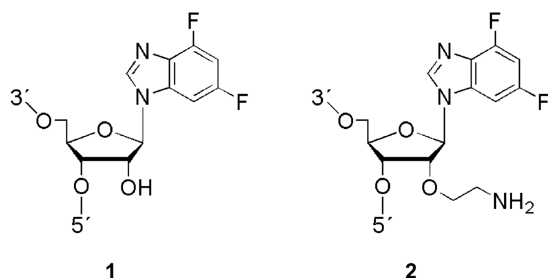


Figure 1. Universal nucleosides; 1′-deoxy-1′-(4,6-difluoro-1H-benzimidazol-1-yl)-β-D-ribofuranose **1**; 1′-deoxy-1′-(4,6-difluoro-1H-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose **2**.

We have already described the synthesis of 1′-deoxy-1′-(4,6-difluoro-1H-benzimidazol-1-yl)-2′-(β-aminoethyl)-β-D-ribofuranose nucleoside analogue elsewhere.[12,14] To obtain other cationic 2′-O-modified DFBi nucleosides, we have developed other strategies to introduce 2′-*O*-alkyl tether whereby new polycationic conjugates are accessible.

Here we present a novel synthetic pathway based on a Michael addition and reduction scheme to achieve 4,6-difluorobenzimidazole-nucleosides with a 2′-*O*-aminopropyl-linker in high yields which can be used as a precursor for the synthesis of a variety of conjugates to increase the cationic character of siRNAs. Amino acids, for example, as well as fatty acids can be subjected to coupling reactions according to peptide coupling conditions. To check the positional influence of the 2′-O-modifications, we derivatized the universal nucleoside 4,6-difluorobenzimidazole variously. With appropriately protected nucleobases, this method can also be adapted to natural nucleosides and used for the synthesis of modified siRNAs.

The synthesis of the 2′-*O*-aminopropyl-linker succeeded in three steps in high yields starting from the unprotected nucleoside **3**. At first, the 3′- and 5′-OH functions are simultaneously protected using the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl group.[15]

Next, a 2′-*O*-cyanoethyl group is introduced via a Michael reaction of acrylonitrile with cesium carbonate as a weak base to activate the 2′-OH group selectively.[16,17] With *tert*-butyl alcohol as a solvent this reaction runs in excellent yield giving the desired 2′-O-cyanoethylated product **5**.[18] However, other conceivable molecules, like acrylamide, do not react with the 2′-OH function in that way. Presumably, due to the less electron-withdrawing effect of the amide group compared to the nitrile group, the double bound of acrylamide is not electrophilic enough to be attacked from the 2′-OH group.

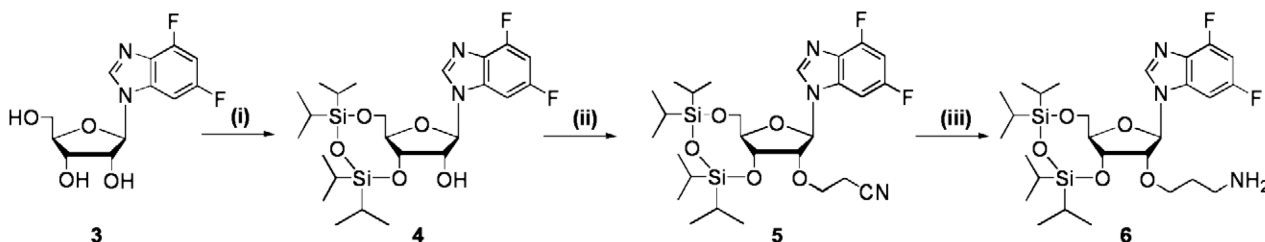
To convert the nitrile group into a primary amino-function, a hydrogenation applying 30 bar hydrogen pressure and Raney-nickel as catalyst was performed. Under usual nitrile-reduction conditions with $LiAlH_4$, an ether cleavage of the whole 2′-linker was observed. The aminopropyl-modified nucleoside **6** was obtained in quantitative yield.[19]

It is a primary benefit of this method that the crude amino compound **6** can be directly used for the next reaction step. Thus, after removal of the catalyst by filtration over Celite a further purification is not necessary.

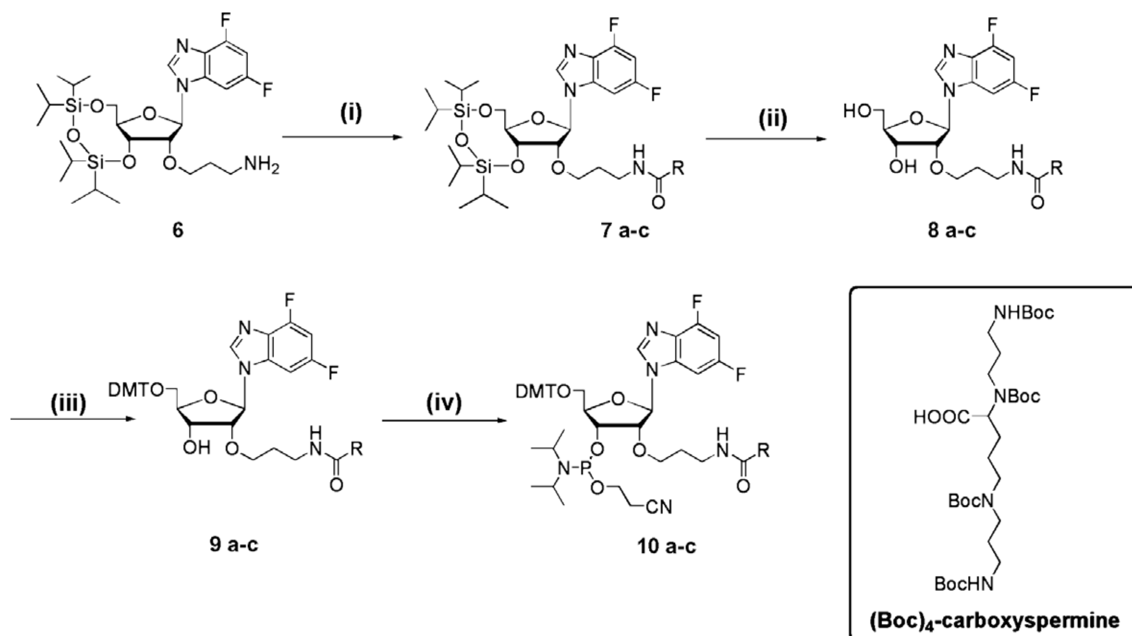
In general, it is possible to derivatize this 2′-*O*-aminopropyl-linker according to the common peptide-coupling chemistry with any carboxylic function using reactive ester reagents like carbodiimides and 1-hydroxybenzotriazoles. As a general procedure for this coupling reaction, 2′-*O*-(3-aminopropyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxy)-1′-deoxy-1′-(4,6-difluoro-1H-benzimidazol-1-yl)-β-D-ribofuranose **6** was dissolved in dry dichloromethane, HOBt and DIC and one equivalent of the coupling component was added. Stirring at room temperature for at least 48 h affords the desired product **7** in high crude yields. Since the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl protection group has to be cleaved afterwards, a further purification after coupling was not necessary (Scheme 1).

To obtain a 2′-O-modification with a weak cationic character, we coupled N_α-Boc-N_ε-trifluoroacetyl protected lysine to the primary amine. Furthermore, the sterically comparable fatty acid, lauric acid, which is more lipophilic, was also bound to the aminopropyl-linker in high yield. Moreover, to increase the cationic character, we chose to tether a carboxy-modified spermine (see Scheme 2), a natural polyamine derivative, too. Under physiological pH, three of their amino groups are protonated. All compounds were coupled corresponding to the procedures described above.

Compound **7** was treated with tetrabutylammonium fluoride in THF to cleave the 3′- and 5′-protecting



Scheme 1. Synthesis of the 2′-*O*-aminopropyl-DFBi **6** in three steps. Reagents and conditions: (i) $TIPDSCl_2$, pyridine, NEt_3; (ii) acrylonitrile, Cs_2CO_3, *tert*-butyl alcohol; (iii) Raney-nickel, H_2 (30 bar), methanol/NH_3.



Scheme 2. Peptide-like coupling of different carboxylic acid derivatives. a = lysine, b = lauric acid, c = $(Boc)_4$carboxyspermine. Reagents and conditions: (i) HOBt, DIC, CH_2Cl_2; (ii) TBAF, THF; (iii) DMTr-Cl, pyridine, NEt_3; (iv) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, *N*-methylimidazole, sym-collidine, CH_3CN.

groups. Afterwards the 5′-OH function of compound **8** was protected with 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl) in dry pyridine and Et_3N to afford the 5′-*O*-(4,4′-dimethoxytrityl)derivative **9**. Both steps provide good yields between 75% and 85%, respectively. Finally, all 2′-O-modified DFBi-nucleosides **9** were converted to the corresponding 3′-*O*-phosphoramidites **10** according to literature procedures.[20]

These building blocks can be directly incorporated into RNA oligoribonucleotides by solid-phase synthesis (Caruther's method). All synthesized phosphoramidites show solid-phase coupling reactivities comparable to the commercially available natural phosphoramidites A, C, G and U.

During the synthetic pathway, the acid-labile Boc-group was resistant under all conditions of RNA synthesis. When DMT-groups are cleaved during solid-phase RNA synthesis with trichloroacetic acid, the Boc-group will be quantitatively removed, too. In case of the lysine modification, the primary amine of the side chain was blocked as trifluoroacetamide. This protecting group can be cleaved under mild basic conditions, present for instance in the RNA workup after solid-phase synthesis.

Hence, all synthesized phosphoramidites **10a**–**c** were incorporated into RNA 12mer-oligonucleotides according to standard oligonucleotide procedures. As a testing sequence, we chose the following A-rich purine RNA sequence: 3′-AAG AAX GAA AAG-5′. The modified

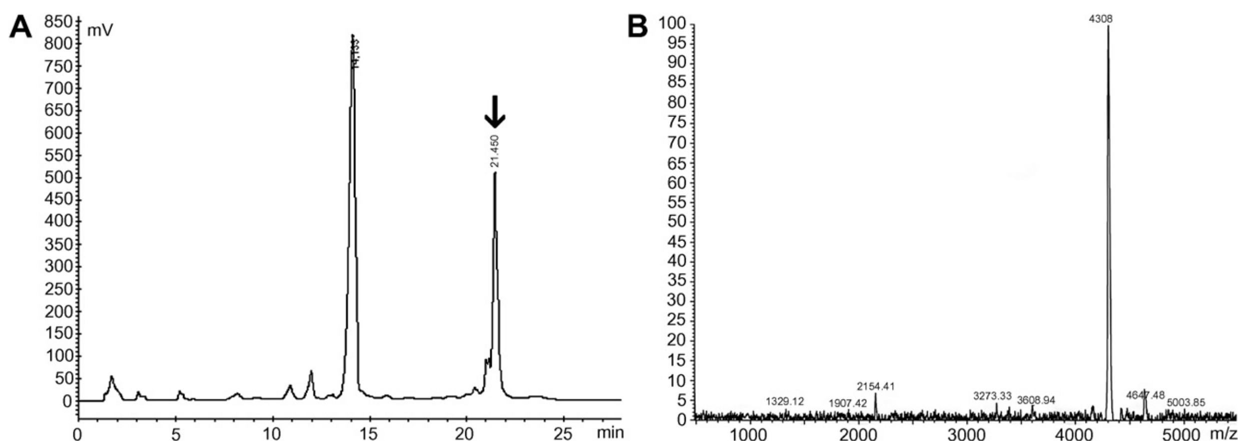


Figure 2. Anion-exchange HPLC profile of 3′-AAG AAX GAA AAG-5′ 12mer. X = DFBi-2′-O-propylamino-carboxyspermine (Panel A). Desired 12mer has a retention-time of 21 min (arrow). Correct oligomer was confirmed via MALDI-TOF MS; calcd mass ($C_{136}H_{164}N_{62}O_{79}F_2P_{11}$): 4309 g/mol (Panel B).

difluorobenzimidazole nucleosides **10a–c** were incorporated in the middle of this particular strand at the position designated with 'X'. Even an incorporation of the bulky spermine-derivative **10c** succeeded in acceptable yields of about 40% (Fig. 2).

In conclusion, we have developed a short and efficient way for 2′-*O*-propylamino-modified difluorobenzimidazole nucleosides, which can be used for further peptide-like coupling reactions. The overall yield for these seven synthetic steps starting from the free DFBi-nucleoside **3** to the corresponding phosphoramidite **10** varies between 25% and 35% depending on the coupling component.

Thus, a pool of novel 2′-O-modifications are easily accessible and their properties for RNAi can be investigated. This method can directly be transferred to natural nucleosides when appropriately protected.

Acknowledgements

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18. *Preparation of the 2′-O-cyanoethyl-linker* (**5**): Compound **4** (897 mg, 1.69 mmol) was dissolved in *t*-butanol (8 mL). After addition of acrylonitrile (2.2 mL, 33.9 mmol) and cesium carbonate (554 mg, 1.69 mmol) the mixture was vigorously stirred at room temperature for 2 h. The mixture was filtered over silica gel and dried under vacuo. The residue was chromatographed on a silica gel column with CH_2Cl_2/MeOH 98:2 to give compound **5** as a yellowish solid (810 mg, 82%).
   ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.25 (s, 1H), 6.99 (ddd, 1H, $J = 0.7$ Hz, $J = 2.2$ Hz, $J = 10.2$ Hz), 6.83 (dt, 1H, $J = 2.1$ Hz, $J = 10.0$ Hz), 5.90 (br s, 1H), 4.57–4.52 (m, 1H), 4.34–4.26 (m, 2H), 4.20–4.16 (m, 1H), 4.05 (dd, 1H, $J = 2.6$ Hz), 3.93 (m, 1H), 3.87–3.78 (m, 1H), 2.74–2.68 (m, 2H), 1.12–0.98 (m, 28H). HRMS (Micromass LCT) Calcd for $C_{27}H_{42}N_3O_5Si_2F_2$: 582.2631. Found: 582.2637.
19. *Preparation of the 2′-O-aminopropyl-linker* (**6**): Compound **5** (500 mg, 0.86 mmol) was dissolved in dry methanol (3 mL). Raney-nickel (approx. 300 mg), which was thoroughly washed with methanol several times, and a saturated solution of ammonia in methanol (3 mL) were added. The mixture was hydrogenated at 30 bar hydrogen-pressure for 5 h at room temp. Afterwards, the catalyst was filtrated over a sintered-glass filter and washed with methanol. After evaporation in vacuo product **6** was obtained as an orange oil (503 mg, quant.).
   ^{1}H NMR ($CDCl_3$): δ 8.16 (s, 1 H), 6.86 (dd, 1H, $J = 1.9$ Hz, $J = 8.1$ Hz), 6.75 (dt, 1H, $J = 2.1$ Hz, $J = 10.1$ Hz), 5.77 (br s, 1H), 4.46–4.22 (m, 1H), 4.20 (d, 1H, $J = 13.3$ Hz), 4.10–4.06 (m, 1H), 4.00–3.94 (m, 2H), 3.76 (d, 1H, $J = 4.5$), 3.72–3.67 (m, 1H), 3.50–3.47 (m, 1H), 2.83–2.78 (m, 2H), 1.76–1.70 (m, 2H), 1.12–0.98 (m, 28H). HRMS (Micromass LCT) Calcd for $C_{27}H_{46}N_3O_5$-Si_2F_2: 586.2944. Found: 586.2942.
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