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OLIGONUCLEOTIDES CONTAINING 4'-*C*-AMINOMETHYL-2'-MODIFIED THYMIDINES SHOW INCREASED BINDING AFFINITY TOWARDS DNA AND RNA

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Abstract: Oligonucleotides containing 4'-*C*-aminomethyl-2'-*O*-methyl or 4'-*C*-aminomethyl-2'-deoxy-2'-fluoro modified thymidines have been synthesized. Compared with the corresponding oligodeoxynucleotide reference these novel oligonucleotide analogues display increased binding affinity towards complementary single stranded DNA as well as RNA. The possible effect of the positively charged 4'-*C*-aminomethyl group has been investigated.

In recent years a large number of chemically modified antisense oligonucleotides (ONs) have been evaluated in order to improve binding affinity towards complementary RNA and stability towards nucleases.[1a-c] In addition to 2'-deoxy-2'-fluoro modified ONs,[2a,b] several 2'-*O*-alkylated ONs including 2'-*O*-methyl have shown increased binding affinity towards RNA.[3a-d] This has been attributed to the preference of these nucleotides to adopt a C3'-endo (N-type) conformation leading to thermally stable A-type duplexes. ONs containing 4'-*C*-substituted nucleotides have likewise been reported showing in general small decreases in binding affinity towards RNA and small increases in binding affinity towards DNA[1c,4a-e] presumably caused by the tendency of these nucleotides to adopt a C2'-endo (S-type) conformation[5] generally found in B-type DNA:DNA duplexes. In order to investigate the possibility of increasing the binding affinity and nuclease resistance of ONs containing 4'-*C*-substituted nucleotides towards RNA we decided to synthesize and evaluate ONs containing 4'-*C*-aminomethyl-2'-*O*-methyl and 4'-*C*-aminomethyl-2'-deoxy-2'-fluoro modified thymidines. In this communication, synthesis of the required monomers and evaluation of the thermal stabilities of mixed sequence 9-mer ONs containing one, two or three of these modified thymidines are reported.

For synthesis of the 4'-*C*-aminomethyl-2'-*O*-methyl- and 4'-*C*-aminomethyl-2'-deoxy-2'-fluoro derivatized thymidine nucleosides[6] we chose a strategy starting from the well known 3,5-di-*O*-benzyl-4-*C*-hydroxymethyl-1,2-di-*O*-isopropylidene-α-D-ribofuranose **2** (Scheme) which can be synthesized from **1** via regioselective benzylation.[7a,b] We decided to use an azide as a "protected" amino group which could be unmasked later at an appropriate point in the synthesis. Thus, the 4-*C*-(trifluoromethanesulfonyl)oxymethyl derivative was

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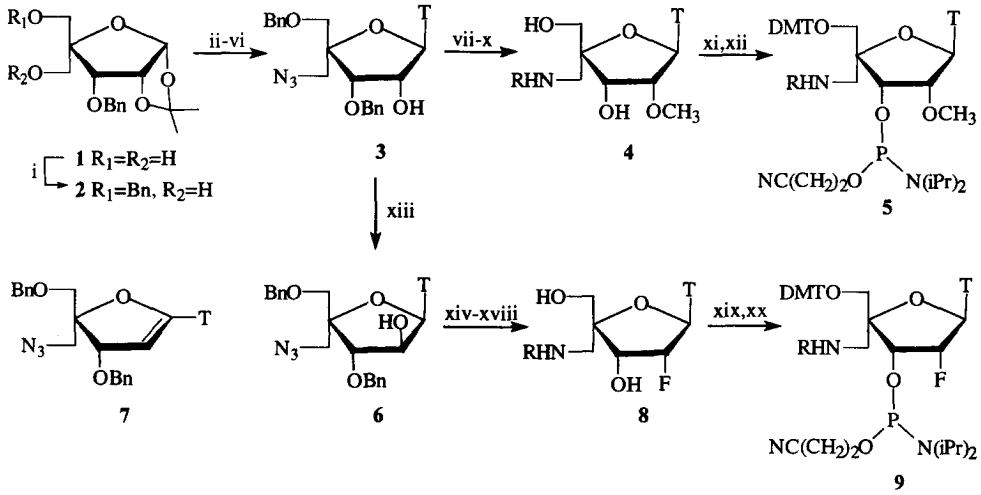
synthesized using standard chemistry and reacted with sodium azide in hot DMF to give the 4-*C*-azidomethyl derivative. Acidic hydrolysis, basic acetylation followed by coupling with silylated thymine[8a,b] afforded after deacetylation the nucleoside **3** in 49% yield from **2**. To obtain the 2'-*O*-methyl derivative **4**, nucleoside **3** was methylated in 90% yield under conditions described earlier for methylation of a 4'-*C*-hydroxymethyl modified thymidine derivative.[4b] The structure of the 2'-*O*-methyl nucleoside product was verified by ^{1}H NMR showing the presence of NH (δ 9.35 ppm), and by a signal at 59 ppm in the ^{13}C NMR spectrum indicating an *O*-methyl group. Chemoselective reduction of the azide with Lindlar's catalyst followed by trifluoroacetylation of the resulting amino group and debenzylation afforded nucleoside diol **4**[9] in 60% combined yield from **3**. To prepare for oligonucleotide synthesis, nucleoside **4** was regioselectively converted to the 5'-*O*-4,4'-dimethoxytrityl (5'-*O*-DMT) protected derivative and subsequently transformed into the phosphoramidite **5** in 39% yield (two steps). To obtain the corresponding 4'-*C*-aminomethyl-2'-deoxy-2'-fluoro nucleoside phosphoramidite **9** (Scheme), the configuration at the 2'-carbon atom of nucleoside **3** was inverted using the anhydro approach.[10]



Scheme. i) ref. 7a,b; ii) Tf_2O, pyridine, CH_2Cl_2; iii) NaN_3, DMF, 59% (two steps); iv) a. 50% AcOH, b. Ac_2O, pyridine, 90%; v) Thymine, BSA, TMS-triflate, CH_3CN, 93%; vi) NH_3, MeOH, 97%; vii) CH_3I, NaH, THF, 90%; viii) H_2, Lindlar's catalyst, EtOH, 75%; ix) CF_3COOEt, NEt_3, CH_2Cl_2, 97%; x) H_2, Pd/C, EtOH, 92%; xi) DMTCl, pyridine, 91%; xii) $NC(CH_2)_2OP(Cl)N(iPr)_2$, $NEt(iPr)_2$, CH_2Cl_2, 43%; xiii) a. MsCl, pyridine, b. NaOH, EtOH, H_2O, 94% (two steps); xiv) Tf_2O, DMAP, pyridine, CH_2Cl_2, 77%; xv) TBAF, THF, 37% yield of the 2'-deoxy-2'-fluoro compound and 30% yield of **7**; xvi) H_2, Lindlar's catalyst, dioxane, EtOH, 64%; xvii) CF_3COOEt, NEt_3, CH_2Cl_2, 89%; xviii) H_2, Pd/C, EtOH, 93%; xix) DMTCl, Pyr., 87%; xx) $NC(CH_2)_2OP(Cl)N(iPr)_2$, $NEt(iPr)_2$, CH_2Cl_2, 76%. T = thymin-1-yl. R = trifluoroacetyl.

Thus, mesylation of *ribo*-configured **3** followed by reaction with aqueous sodium hydroxide afforded the *arabino*-configurated nucleoside **6** in 94% yield (two steps). Under strictly anhydrous conditions the desired 2'-deoxy-2'-fluoro derivative could be synthesized in 28% yield from nucleoside **6** using carefully dried tetrabutylammonium fluoride (TBAF).[11a,b] In addition, the *N*,*O*-ketene acetal **7** was isolated as a by-product in 23% yield *via trans*-elimination of the 2'-trifluoromethanesulfonyloxy group. The structure of **7** was verified by

MS and NMR and by comparison with other structurally similar 1',2'-unsaturated nucleoside derivatives.[12a,b] The assumed C2'-endo conformation of *arabino*-configured nucleoside **6** arranges the 1'-hydrogen atom and the 2'-triflyloxy group with *trans* axial geometry advantageous for E2 elimination offering an explanation of the almost 1:1 ratio of substitution *versus* elimination. Following the same *modus operandi* as for the 2'-*O*-methyl nucleoside, the 4'-*C*-azidomethyl-2'-deoxy-2'-fluoro derivative was converted into nucleoside diol **8**[9] in a combined yield of 53% from **6**. Eventually, nucleoside **8** was transformed into the 5'-*O*-4,4'-dimethoxytrityl 3'-*O*-phosphoramidite derivative **9** in 66% yield (two steps).

On an automated DNA-synthesizer the phosphoramidites **5** and **9** were incorporated into mixed sequence 9-mer ONs in combinations with unmodified 2'-deoxynucleotides (sequences **II-VII**, Table). The stepwise

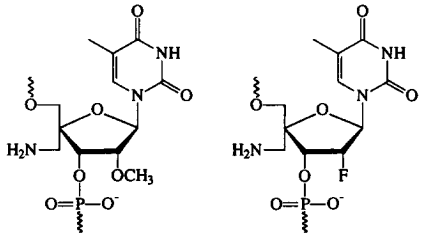


Table. Melting experiments of oligonucleotides containing monomer **Y** and **Z**

		Complementary ssDNA		Complementary ssRNA	
		Medium salt[a]	High salt[b]	Medium salt[a]	High salt[b]
	Oligonucleotide	$T_m/\Delta T_m$ per mod/°C[c]		$T_m/\Delta T_m$ per mod/°C[c]	
I	5'-GTGATATGC	31.0	36.5	29.5	34.0
II	5'-GTGAYATGC	+1.5	-0.5	+2.5	+2.5
III	5'-GTGAYAYGC	+1.5	-0.3	+1.3	+1.3
IV	5'-GYGAYAYGC	-0.3	-1.7	+0.8	+1.0
V	5'-GTGAZATGC	+1.5	-0.5	+0.5	±0.0
VI	5'-GTGAZAZGC	+1.8	±0.0	+1.5	+0.8
VII	5'-GZGAZAZGC	+0.3	-0.8	+1.0	+0.7

[a]Measured at 260 nm in medium salt buffer: 1 mM EDTA, 10 mM sodium phosphate, 140 mM sodium chloride, pH = 7.1. [b]Measured at 260 nm in high salt buffer: 1 mM EDTA, 10 mM sodium phosphate, 740 mM sodium chloride, pH = 7.1. [c]T_m is shown for oligodeoxynucleotide **I**. ΔT_m per mod is shown for oligonucleotides **II-VII** with **I** as reference. T = thymidine monomer; G = 2'-deoxy-guanosine monomer; A = 2'-deoxyadenosine monomer; C = 2'-deoxycytidine monomer; **Y** = 1-[4-*C*-aminomethyl-2-*O*-methyl-β-D-ribofuranosyl]thymine monomer; **Z** = 1-[4-*C*-aminomethyl-2-deoxy-2-fluoro-β-D-ribofuranosyl]thymine monomer. T_m = melting temperature determined as the maximum of the first derivative of the absorbance *vs* temperature curve. ΔT_m per mod = change in T_m per modification compared to the unmodified reference oligodeoxynucleotide I.

coupling yield for phosphoramidite **5** was approximately 40% (2 x 12 min couplings) and >99% for unmodified 2'-deoxynucleoside phosphoramidites using standard tetrazole coupling conditions. However, stepwise coupling

yields of >95% for amidite **5** and >80% for amidite **9** were obtained with pyridine hydrochloride[13a-c] as activator (10 min couplings). After cleavage from the solid support and deprotection using concentrated aqueous ammonia (see below), the 5'-*O*-DMT-ON oligonucleotides were purified by use of reversed-phase chromatography cartridges. The purity (>90%) of all oligonucleotides **I-VII** was verified by capillary gel electrophoresis and their composition confirmed by MALDI-MS. Earlier, Kawasaki *et al.*[2a] have observed significant conversion of 2'-deoxy-2'-fluoropyrimidine nucleotide monomers in ONs *via* intramolecular nucleophilic attack from the pyrimidine nucleobase followed by ring-opening of the resulting 2,2'-anhydro intermediate under the usual oligonucleotide deprotection conditions (55 °C, 12 h) leading to the suggestion to use instead methanolic ammonia (RT, 24 h). However, under these conditions only approximately 50% of the trifluoroacetyl protecting groups of monomers **Y** and **Z** in ONs **II-VII** were removed according to MALDI-MS analysis. Furthermore, the 2-*N*-isobutyryl protecting groups of the 2'-deoxyguanosine monomers remained intact following this suggested procedure. Instead, we performed cleavage and deprotection using the standard conditions (55 °C, 12 h). In fact, MALDI MS analysis did not indicate any kind of degradation of ONs **II-VII** as also verified by capillary gel electrophoresis. This result is consistent with observations reported by others.[14a,b]

The hybridization properties of the modified oligonucleotides (ONs **II-VII**) and of the reference oligodeoxynucleotide (ON **I**) towards complementary single stranded DNA and RNA were measured under conditions of medium and high ionic strength (Table). Oligonucleotides **II**, **III**, **V** and **VI** containing either **Y** or **Z** once or twice showed significantly increased binding affinity towards complementary DNA (ΔT_m per mod = +1.3 °C to +2.5 °C) compared with the unmodified reference ON **I** when measured in the medium salt buffer (140 mM NaCl). For ONs **IV** and **VII** containing three **Y** or **Z** modifications, a small decrease (ΔT_m per mod = -0.3 °C) or increase (ΔT_m per mod = +0.3 °C), respectively, in binding affinity towards DNA was observed. The 2'-methyloxy group and the 2'-fluoro group both direct the sugar pucker towards a C3'-endo conformation for monomers **Y** and **Z** as indicated[15] by the fact that the coupling constant $^3J_{H1'H2'}$ in general is less than 5 Hz for the corresponding nucleosides. This fact was expected to induce an increase in the thermal stability of duplexes towards complementary RNA (A-type duplexes). The observed increased thermal stability of the duplexes formed between ONs **II** and **III** and **V-VII** towards complementary DNA could be caused by a reduction in the electrostatic repulsion between interstrand phosphate moieties by the presence of the aminomethyl group expected to exist mainly in the protonated form under the applied experimental conditions. To confirm this hypothesis the duplex stabilities were additionally evaluated under conditions of high ionic strength (740 mM NaCl, see Table) leading, as expected, to an increased binding affinity of the unmodified reference ON **I** towards complementary DNA. However, compared with the T_m value of 36.5 °C observed for ON **I**, ONs **II-IV** containing monomer **Y** and ONs **V-VII** containing monomer **Z** displayed reduced binding affinity which confirms the positive effect of the protonated aminomethyl group on the binding affinity towards complementary DNA at medium ionic strength.

In all melting experiments towards complementary RNA, enhanced binding affinities were observed for the modified ONs **II-VII** (Table) compared with the reference ON **I**. The effect of the two modifications **Y** and **Z** differs as ΔT_m per modification is reduced when 2'-*O*-methylated monomer **Y** is incorporated two or three times (ΔT_m per mod = +2.5 °C for one modification; = +0.8 °C for three modifications; 140 mM NaCl) whereas a more than additive effect on the binding affinity was observed for 2'-fluoro monomer **Z** (ΔT_m per mod = +0.5 °C for one modification; = +1.0 °C for three modifications; 140 mM NaCl). The relative thermal stability of the duplexes involving complementary RNA appears to be unaffected by the change from medium ionic strength to high ionic strength. This indicates that there is no significant electrostatic interaction between the 4'-*C*-aminomethyl group and the negatively charged phosphate backbones.

Due to the larger gauche effect of the 2'-fluoro substituent compared with the 2'-methyloxy substituent we expected the substitution of the 2'-*O*-methyl monomer **Y** with the 2'-deoxy-2'-fluoro monomer **Z** to cause an increase in binding affinity towards RNA. Accordingly, ONs **VI** and **VII** containing **Z** showed slightly increased binding affinities towards RNA compared with the corresponding oligonucleotides **III** and **IV** containing monomer **Y** (Table). However, towards the RNA complement monomer **Y** proved significantly more stabilizing than monomer **Z** for the singly modified ONs **II** and **V**. The effect of the two different modifications in melting studies towards complementary DNA proved very similar and the stabilizations observed in medium salt buffer appear to be of electrostatic origin. The observed increased binding affinities obtained for these partly modified sequences, also compared with sequences containing similar 4'-*C*-branched-2'-deoxy monomers without a 2'-substituent,[1c] illustrate the importance of conformational steering of the furanose ring in ON analogues. In fact, the 2'-*O*-methyl-4'-*C*-aminomethyl and the 4'-C-aminomethyl-2'-deoxy-2'-fluoro functionalized monomers **Y** and **Z** introduced herein are among the modifications synthesized so far of the natural monocyclic pentofuranose-phosphordiester backbone structural class that display the highest binding affinities towards complementary DNA as well as RNA.[1a-c] However, so far only few sequences have been investigated and it remains to be seen whether this trend holds in general for other sequences, including fully modified ones.

In conclusion, 4'-*C*-aminomethyl-2'-modified nucleosides have been synthesized by a convergent approach, converted to the corresponding phosphoramidites, and incorporated into mixed sequence 9-mer oligonucleotides. Compared with the corresponding 4'-*C*-branched-2'-deoxy derivatives, the introduction of the electronegative 2'-substituents induces a conformational shift towards a C3'-endo type furanose conformation and increased binding affinities towards complementary DNA and RNA under physiologically relevant ionic strength. These results suggest the novel 4'-aminomethyl-2'-modified oligonucleotide analogues to be useful as monomeric constituents, *e.g.* conjugation sites, in high-affinity antisense oligonucleotides.

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9. ^{13}C NMR for compound **4** (CD_3OD): 12.4, 42.1, 59.1, 64.3, 71.1, 84.9, 88.7, 89.0, 111.7, 117.5 (q, *J* 286 Hz), 138.4, 152.4, 159.4 (q, *J* 35.4 Hz), 166.3. ^{13}C NMR for compound **8** (CD_3OD): 12.3, 41.7 (d, *J* 4.1 Hz), 63.7, 71.1 (d, *J* 15.3 Hz), 88.8, 89.7 (d, *J* 34 Hz), 95.1 (d, *J* 190 Hz), 111.8, 117.6 (q, *J* 287 Hz), 138.8, 152.4, 159.8 (q, *J* 37 Hz), 166.6.
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