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2-Amino-α-2′-deoxyadenosine Increased Duplex Stability of Methoxyethylphosphoramidate α-Oligodeoxynucleotides with RNA Target

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Abstract—A new efficient synthesis of 2-amino-α-2′-deoxyadenosine and its incorporation into methoxyethylphosphoramidate α-oligodeoxynucleotides (ODNs) via *H*-phosphonate chemistry were reported. Thermal denaturation experiments demonstrated a significant stabilization of the complexes formed between these analogues and their RNA target ($+2\,^{\circ}C/^{NH2}A$) relative to adenosine-containing phosphoramidate α-oligonucleotides. Concerning the binding specificity of these modified ODNs, unlike natural ODNs, discrimination against G pairing is higher and against C pairing is lower.

Some years ago, we demonstrated that oligodeoxynucleotides (ODNs) combining two structural modifications, that is the inversion of the anomeric configuration in the sugar moieties (from β to α) and the substitution of phosphate diester by non-ionic *N*-(2-methoxyethyl)phosphoramidate (PNHME) linkages, formed stable duplexes with complementary single stranded RNA and DNA targets.[1] These backbone modified α-ODNs hybridized to their targets more tightly than the corresponding natural phosphodiester (PO) β-ODN (ΔT_m +9.6 °C for DNA and ΔT_m +1.4 °C for RNA). In this work, in order to increase still more the affinity of PNHME α-ODNs for RNA, we have replaced adenine by 2-aminoadenine. Indeed, this nucleobase can potentially form three hydrogen bonds with a complementary thymine or uracil base, unlike adenine, which forms only two hydrogen bonds and so ODNs containing 2-aminoadenine in place of adenine should bind more efficiently to their complementary DNA and RNA targets. The numerous reported data demonstrated that this additional bond contributed to the stabilization of the duplexes but other factors such as Π-stacking, water exclusion, backbone and sequence effects could have also beneficial effects[2–4] (for a review, see ref 5).

The non-natural nucleoside 2-amino-α-2′-deoxyadenosine (2-amino-α-dA) has never been incorporated into backbone-modified α-ODNs. Here we report a new efficient synthesis of a fully protected 2-amino α-dA 3′-H-phosphonate synthon and its incorporation into PNHME α-ODNs for studies of their binding properties to complementary DNA and RNA.

Synthesis of 2-Amino-α-2′-deoxyadenosine

In the past, the α-anomer was already obtained with very low yield during the synthesis of 2-amino β-dA from 2-amino-6-chloropurine[6] or 2-benzamido-*N*-benzoyladenine[7] by the chloromercuri procedure, or from 2,6-dichloropurine by fusion reaction.[7,8] In an effort to get a high yield of α-anomer of 2-amino-2′-deoxyadenosine, we used a phase transfer glycosylation method which afforded efficient regio (N-9) and stereospecificity (α-anomer predominant) in the condensation of natural nucleobases with 2-deoxysugars.[9] According to this approach, 1-*O*-acetyl-3,5-di-*p*-toluyl-2-deoxyribose was reacted with silylated 2,6-diaminopurine under phase transfer conditions using dibenzo-18-crown-6 and potassium iodide in an acetonitrile–toluene mixture (1/1, v/v) at reflux within 18 h. The glycosylation yield was 66% but unfortunately the α-anomer could not be separated from the β-one. Under the same conditions, silylated 2,6-dichloropurine which is more lipophilic than 2,6-diaminopurine was coupled with 1-*O*-acetyl-3,5-di-*p*-toluyl-2-deoxyribose (Scheme 1). After silica gel chromatography, N-9 α-nucleoside **1** was isolated among a mixture of anomers and regioisomers

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in 32% yield. Surprisingly, the N-7 regioisomer was obtained with 15% yield whereas it was not detected in the preparation of α-2′-deoxyguanosine.[9] Then chlorine atoms of **1** were replaced by azide groups by reaction with NaN_3 in refluxing ethanol for 2 h to give compound **2** in 82% yield.[7] The azide groups of **2** were reduced by hydrogenation in the presence of 10% Pd/C to afford **3** in 90% yield. Nucleoside 2-amino-α-dA **4** was finally obtained in 95% yield by heating **3** with methanolic ammonia at 60 °C overnight in a bomb and was crystallized from H_2O. 2-Amino-α-2′-deoxyadenosine **4** was fully characterized: ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) 7.97 (s, 1H, H-8), 6.72 (br s, 2H, NH_2), 6.13 (dd, 1H, H-1′), 5.77 (br s, 3H, NH_2, OH), 4.81 (t, 1H, H-3′), 4.26 (s, 1H, OH), 4.05 (q, 1H, H-4′), 3.42 (m, 2H, H-5′, H-5″), 2.69 (m, 1H, H-2′), 2.20 (dd, 1H, H-2″). ^{13}C NMR (DMSO-d_6, 400 MHz) δ (ppm) 160.96 (C-2), 157.0 (C-6), 152.03 (C-4), 137.12 (C-8), 114.04 (C-5), 89.04 (C-4′), 83.58 (C-1′), 71.68 (C-3′), 62.58 (C-5′), 40.4 (C-2′). FAB mass spectra (positive and negative mode, thioglycerol): 267 $[M+H]^+$, 265 $[M-H]^-$. UV λ_{max} 256, 279 nm. Mp 229 °C.

Synthesis of 2-Amino-α-dA 3′-H-Phosphonate and Preparation of PNHME α-ODNs

Fully protected 2-amino-α-dA 3′-H-phosphonate **5** was prepared from nucleoside **4** in three steps (Scheme 1). Due to the presence of two nucleophilic amino groups, incorporation of 2-amino-α-dA into ODNs via *H*-phosphonate chemistry requires the double protection of the base to avoid unwanted side reactions. We chose to protect **4** with two diisobutylformamidine groups which would be more easily removed than acyl groups by ammonia treatment after ODN elongation and have been shown to impart depurination resistance.[10,11] Furthermore, with these amidine protections, there is no requirement to transiently protect the 3′- and 5′-hydroxyls of **4** which directly reacted with *N*,*N*-diisobutylformamide dimethyl acetal[10] in anhydrous methanol for 43 h at room temperature, then for 12 h at 40 °C to carry the reaction to completion (**6**, 72% yield). Tritylation of the 5′-hydroxyl of **6** was performed in the presence of DMAP and triethylamine with 50% yield. Phosphitylation by standard procedures[12] afforded the 3′-H-phosphonate synthon **5**.

We incorporated 2-amino α-dA into PNHME α-ODNs via *H*-phosphonate chemistry using the appropriately protected α-nucleoside *H*-phosphonate synthons[1] following a standard procedure.[13] The *H*-phosphonate diester linkages were then oxidized by CCl_4 in the presence of 2-methoxyethylamine. The influence of base modifications (1 or 3) on the thermal stability of PNHME α-ODNs/DNA or RNA complexes was determined by UV thermal denaturation (T_m) experiments with ODNs **6**–**9** (Fig. 1 and Table 1).

DNA targets β 5′ AGAA**X**TGGGTGT 3′ with **X** = T, A, G, C
RNA targets β 5′ AGAA**X**UGGGUGU 3′ with **X** = U, A, G, C

ODN **6** β 5′ ACACCCAATTCT 3′
ODN **7** α 5′ T*p*C*p*T*p*T*p*A*p*A*p*C*p*C*p*C*p*A*p*C*p*A 3′
ODN **8** α 5′ T*p*C*p*T*p*T*p*^{NH2}A*p*A*p*C*p*C*p*C*p*A*p*C*p*C*p*A 3′
ODN **9** α 5′ T*p*C*p*T*p*T*p*^{NH2}A*p*^{NH2}A*p*C*p*C*p*C*p*^{NH2}A*p*C*p*C*p*A 3′

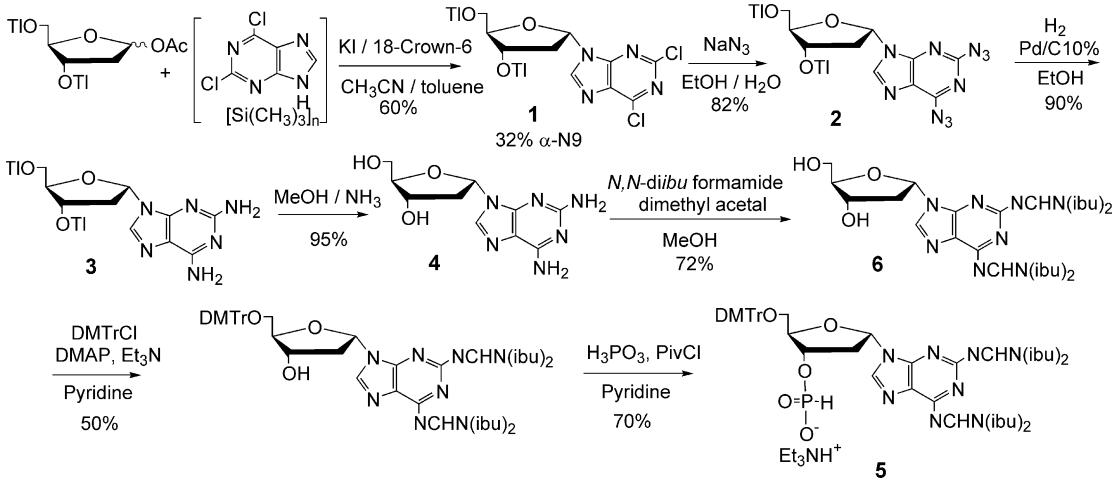
Figure 1. ODNs which are complementary to the splice acceptor site of the mRNA coding for HIV-1 tat protein. *p*, methoxyethylphosphoramidate; ^{NH2}A, 2-amino-α-2′-deoxyadenosine.

Hybridization Properties of PNHME α-ODNs Containing 2-Amino-α-dA

The binding properties of PNHME α-oligonucleotides **7**–**9** to DNA and RNA targets were characterized in

Table 1. Thermal stability (T_m and ΔT_m with respect to α-ODN **7**) of duplexes formed between PO oligonucleotide **6** or PNHME α-oligonucleotides **7**–**9** containing or not 2-amino-α-dA and their complementary DNA or RNA targets

ODNs	DNA target with X = T		RNA target with X = U	
	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
6	45.4		44.6	
7	55.0		46.0	
8	54.0	−1.0	48.3	+2.3
9	57.5	+2.5	52.0	+6.0



Scheme 1.

Table 2. Influence of a single mismatch on the T_m of duplexes with phosphodiester β-oligonucleotide **6** and PNHME α-oligonucleotides **7** and **8** ($\Delta T_m = T_m$ mismatch $- T_m$ match)

ODNs	T_m °C (ΔT_m) DNA target, X=			T_m °C (ΔT_m) RNA target, X=		
	C	A	G	C	A	G
6	30.5 (−14.9)	32.3 (−13.1)	37.7 (−7.7)	33.3 (−11.3)	33.8 (−10.8)	36.8 (−7.8)
7	38.0 (−17.0)	39.3 (−15.7)	41.5 (−13.5)	35.5 (−10.5)	35.0 (−11.0)	34.3 (−11.7)
8	44.0 (−10.0)	43.0 (−11.0)	43.0 (−11.0)	43.0 (−5.3)	36.8 (−12.0)	35.3 (−13.0)

term of affinity with the perfectly matched duplexes (X = T for DNA and U for RNA targets, respectively). As reported, these modified oligonucleotides form stable parallel-stranded duplexes. Introduction of one 2-aminoadenine modification (α-ODN **8**) had a destabilizing effect (ΔT_m −1 °C) on the DNA hybrid (X = T), whereas the replacement of three α-dA in α-ODN **7** by three 2-amino α-dA in α-ODN **9** slightly stabilized the corresponding duplex (ΔT_m/^{NH2}A +0.8 °C). With RNA duplexes (X = U), a strong positive effect on binding was found when one (ΔT_m + 2.3 °C) and three 2-amino-α-dA (ΔT_m +6 °C) were incorporated into PNHME α-oligonucleotides.

This different behavior of 2-aminoadenine towards DNA and RNA duplex formation was also reported for phosphodiester β-oligonucleotides[2] and for N3′→P5′ phosphoramidate ODNs.[4] Thus, PNHME α-ODN **9** containing 2-aminoadenine formed more stable duplexes with DNA target (ΔT_m +12.1 °C) and RNA target (ΔT_m +7.4 °C) than did the natural PO β-ODN **6**.

The sequence specificity of binding of PO β-ODN **6** and PNHME α-ODNs **7** (containing A) and **8** (containing one 2-amino A) was determined by comparison of singly mismatched duplexes (DNA and RNA, X = A, G, C) to the perfectly matched duplexes (DNA, X = T; RNA, X = U) (Table 2). The stabilities of base pairs formed with the PO β-ODN **6** decreased as T (or U) A > > G A > A A > > C A. According to the literature,[14] the most stable mismatches were those containing guanine and the least stable were those containing cytosine.

Concerning PNHME α-ODN **7**/DNA duplexes, the stability of base pairs followed same grading than observed for the PO β-ODN **6**. However, a higher discrimination between T A and X A base pairs was observed for the modified α-ODN **7** (average ΔT_m/mismatch −15.4 °C) compared to the natural one **6** (average ΔT_m/mismatch −11.9 °C). The most noticeable effect was the difference between discrimination of T and G by A in α-ODN **7** (ΔT_m −13.5 °C) compared to the same discrimination in β-ODN **6** (ΔT_m −7.7 °C).

Surprisingly, with the RNA target, there was only a very little influence on the thermal stability regardless which mismatch was opposite to α-dA in PNHME α-ODN **7**. Here again, a difference between α-ODNs **6** and **7** was noticeable with the G A mismatch, more stable for the regular PO ODN and less stable for the modified one.

The data obtained with α-ODN **8** targeted against DNA showed that the introduction of a 2-amino group in adenine reduced discrimination between mismatches (average ΔT_m/mismatch −10.8 °C) when compared to adenine in PNHME α-ODN **7** (average ΔT_m/mismatch −15.4 °C) as well as in phosphodiester β-ODN **6** (average ΔT_m/mismatch −11.9 °C). With α-ODN **8**/RNA hybrids, the most stable mismatch was C ^{NH2}A and the least stable was G ^{NH2}A. Thus the stability of base pairs decreased as U ^{NH2}A > > C ^{NH2}A > A ^{NH2}A > > G ^{NH2}A. The discrimination between U ^{NH2}A and A (or G) ^{NH2}A base pairs was enhanced compared to the discrimination of U A and A (or G) A in PNHME α-ODN **7** or PO β-ODN **6**/RNA duplexes. On the contrary, we observed a significant reduction of discrimination against C pairing with ^{NH2}A when compared with A in ODNs **6** and **7**/RNA duplexes. Formation of an additional hydrogen bond between cytosine and 2-aminoadenine could be responsible of this lower specificity of ^{NH2}A compared to A. Further investigations including NMR and molecular modeling studies will be needed to evaluate the structural features of the 2-aminoadenine containing PNHME α-ODNs duplexes which result in the observed stabilization effects particularly with RNA target.

In conclusion, we have described a new efficient synthesis of 2-amino α-dA using a phase transfer glycosylation method. The exocyclic amino functions of this nucleoside were suitably protected by diisobutylformamidine groups to be converted in its 3′-*H*-phosphonate synthon which was successfully incorporated into PNHME α-ODNs. The replacement of adenine by 2-aminoadenine in PNHME α-ODNs really improved their affinity to RNA while keeping a good specifity for nucleic acids. To explain these data, a full structural elucidation is currently under investigation. Such modified oligonucleotides are very promising for antisense and diagnostic purposes in terms of binding to nucleic acid targets. Therefore, we are presently evaluating their cellular uptake for further biological applications.

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