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Synthesis and Properties of Aminopropyl Nucleic Acids

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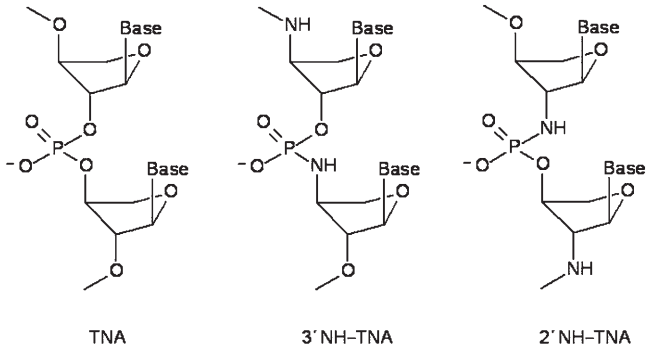
Oligonucleotides that contain up to three aminopropyl nucleoside analogues have been synthesized. Dimers of aminopropyl adenine and thymidine were prepared and used as building blocks by applying phosphoramidite chemistry. Both R *and* S *isomers of the aminopropyl nucleosides were used. This incorporation led to a reduction of thermal stability of double-stranded DNA. Furthermore, the (*R*)-adenine analogue, which yielded (*S*)-APNA, can be considered as a candidate for universal base pairing.*

Introduction

In an effort to explain the origin of nucleotides, several important observations have been made. It was shown that the four nucleobases of RNA could be generated under a variety of potentially prebiotic conditions.[1] Recent work has highlighted a chemical-assembly process for obtaining racemic phosphorylated ribose.[2] Reaction of two molecules, phosphorylated glycolaldehyde and formaldehyde [$2\times(C_2+C_1)$] gives rise to the formation of 2,4-*O*-diphosphorylated ribose, which is a potential precursor of natural nucleosides.[2] Later it was shown that glycolaldehyde could be very efficiently phosphorylated in aqueous solution and at extremely low concentrations by a novel phosphorylation process that uses amidotriphosphate (the ammonolysis product of metatriphosphate) as a sugar-selective phosphorylation agent.[3] Apart from these findings, a systematic investigation of potential nucleic-acid alternatives that contain various sugars and linkages has led to the discovery of some intriguing pairing systems. Most notable is threose nucleic acid (TNA), which is based on α-threofuranosyl units that are joined by 3′,2′-phosphodiester linkages. These have shown efficient and specific base paring as well as cross pairing with DNA and RNA.[4] TNAs are thought to be interesting nucleic acid alternatives not only because of their hybridization properties, but also because of their structural simplicity. Recently, work was extended to include oligonucleotides with nitrogen-linked C_4 backbone units (i.e., NH–TNA with phosphoramidate instead of phosphodiester links) that were derived from nitrogen-containing starting materials (Scheme 1).[5] These systems were found to behave in a similar manner to TNA.

Ribonucleosides have four chiral centres, while tetroses (TNA like) have three. TNA (C_2+C_2) is a simpler type of molecular system to generate than RNA. However, we wondered if it were possible to further reduce the number of carbon atoms and chiral centres in a nucleotide structure (C_2+C_1 style) without losing the ability for self- and cross-hybridization with RNA and DNA. In principle, such a nucleotide could be assembled from glycolaldehyde (C_2), formaldehyde (C_1) and a nucleobase. In the simplest prototypes, only one chiral centre is present; this raises the question of whether the chirality of carbon atoms still plays an important role in the strength and selectivity of hybridization (i.e., if nucleic acids composed of (*R*)- or (*S*)-nucleotides show similar hybridization properties). For this reason, we synthesized two new members of this family (Scheme 2) that possess a phosphoramidate linkage between the repeating units instead of the phosphodiester group, and which might have the ability to communicate with natural nucleic acids by intersystem cross pairing. Phosphoramidate was



Scheme 1. Base-pairing systems related to TNA: α-threofuranosyl oligonucleotides that contain phosphoramidate linkages.

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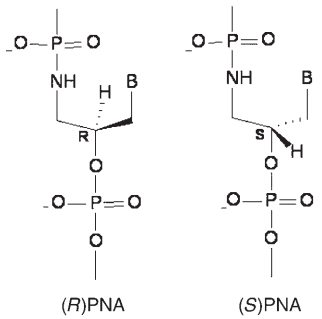
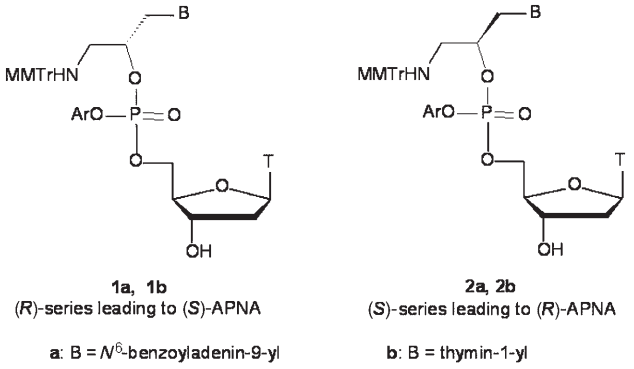
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: procedures for the synthesis of ***3a***, ***3b***, ***16a*** *and* ***16b***.



Scheme 2. Constitution and configuration of members of two APNA families; B = base.

selected due to its high nucleophilicity;[5] as demonstrated with NH–TNAs, nitrogen chemistry might be involved in prebiotic-assembly process that could lead to RNA ancestors.

The synthesis of 3′-aminopropyl nucleosides (APN) is very simple. However, the synthesis of aminopropyl nucleic acids (APNA) is a major problem due to competing side reactions during oligonucleotide synthesis, such as, intramolecular attack of nucleophilic nitrogen on the electrophilic phosphor atom. Therefore, we decided to prepare mixed DNA–APNA oligonucleotides with incorporation of the modified nucleotide as part of a dimer with a 2′-deoxynucleoside. Two different types of protected dimers were chosen to serve as intermediates in the chemical synthesis of the corresponding phosphoramidate oligomers and both contained either adenine or thymine as base moiety (Scheme 3).



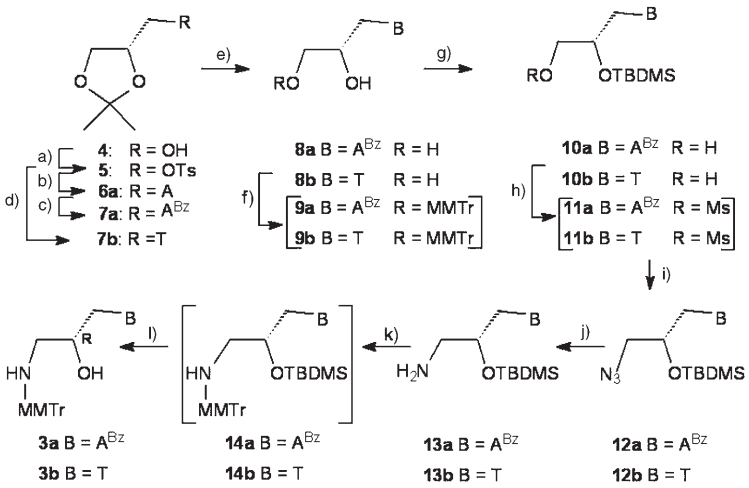
Scheme 3. Dimers used for oligonucleotide synthesis.

Results and Discussion

Optically active APNs with either an adenine or thymine base moiety were prepared from enantiomerically pure (*S*)-1,2-*O*-isopropylidene glycerol, which is less expensive than the *R* isomer.

Synthesis of protected (*R*)-acyclic nucleosides

The synthesis of **3a** and **3b** from the commercially available (*S*)-1,2-*O*-isopropylidene glycerol (**4**) is outlined in Scheme 4.



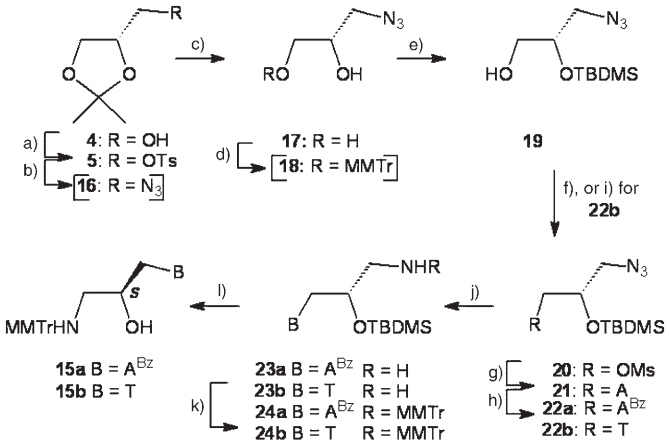
Scheme 4. Preparation of *R*-configured, protected nucleosides **3a** and **3b** from (*S*)-isopropylidene glycerol (**4**). a) TsCl, pyridine, 94%; b) A, NaH (60%), 80 °C, DMF, 1 h, 100 °C, 15 h, 68%; c) BzCl, pyridine, saturated NH_3 in MeOH, 0 °C, 79%; d) T^{Bz}, DIAD, Ph_3P, THF, saturated NH_3 in MeOH, 0 °C, not isolated; e) TFA (75%) in H_2O, **a**: 91%, **b**: 61%, over all steps; f) MMTrCl, pyridine, TBDMSCl, imidazole, DMF, not isolated, g) pTSOH (4%) in CH_2Cl_2 and MeOH, **a**: 54% over 2 steps, **b**: 47% over 2 steps; h) MsCl, pyridine, not isolated; i) NaN_3, DMF, 100 °C, **a**: 57% over 2 steps, **b**: 84% over both steps; j) Ph_3P, THF, H_2O (80%), **a**: 80%, **b**: 80%; k) MMTrCl, pyridine, not isolated; l) TBAF (1 M) in THF, **a**: 71% over 2 steps, **b**: 79% over 2 steps.

The adenine derivative **6a** was synthesized by nucleophilic substitution of (*R*)-1-*O*-tosyl-2,3-*O*-isopropylidene glycerol (**5**) with the sodium salt of adenine.[6] Note that the annotation of the chiral centres as *R* or *S* changes due to alteration of the substitution pattern, although the configuration of the asymmetric centre remains untouched. First, treatment of **4** with *p*-toluenesulfonyl chloride (TsCl) in pyridine yielded **5** quantitatively. This was then treated with the sodium salt of adenine, which was prepared in situ by treatment of adenine with sodium hydride, to give **6a** in 68% yield. Subsequent protection of the exocyclic amine with benzoyl chloride (BzCl) in pyridine was carried out to give **7a** (79%). Treatment of **4** with N^3-benzoylthymine (T^{Bz}) under Mitsunobu conditions[7] furnished the protected nucleoside, which was debenzoylated to obtain **7b**. Acid hydrolysis gave the free nucleosides **8a** and **8b**. In the following step, selective monotritylation of the primary hydroxyl group (3′-OH) was carried out with monomethoxytrityl chloride. This was followed by silylation of the secondary hydroxyl group (2′-OH) with *tert*-butyldimethylsilyl (TBDMS) chloride and imidazole in DMF. Subsequent cleavage of the 3′-*O*-MMTr group in the presence of *p*-toluenesulfonic acid (4%) in $CHCl_3$/MeOH (2:1, *v*/*v*) afforded the 2′-*O*-TBDMS derivatives **10a** (54%) and **10b** (47%). These reactions could be carried out conveniently in a single pot without the need for isolation of the intermediates **9a** and **9b**. Mesylation and displacement of the 3′-*O*-Ms group was achieved by heating **11a** and **11b** to 100 °C with 1.3 equiv of NaN_3 in DMF, overnight, to afford azido nucleosides **12a** (57%) and **12b** (84%), respectively. Reduction of the azide derivatives with PPh_3 in THF gave the free amino derivatives **13a** and **13b** in 80% yield. MMTr protection of the amino function yielded the fully protected intermediates **14a**/**b**, which were deprotected with tetrabutylammonium

fluoride (TBAF; 1 M) in THF to afford the desired *R* nucleosides **3a** (71%) and **3b** (79%). A shorter route, which involved the selective tosylation or triisopropylphenylsulfonylation of the primary hydroxyl group, proved less effective.

Synthesis of the *S* congeners

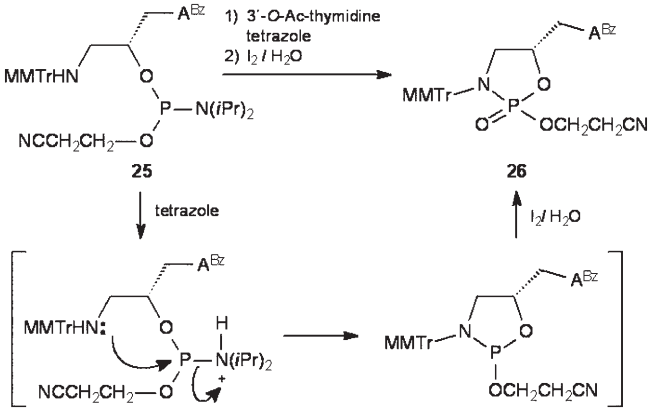
Acyclic (*S*)-nucleosides **15a** and **15b** were synthesized from intermediate **5** (Scheme 5). Nucleophilic substitution was carried out by heating NaN_3 to 100 °C in DMF. This was followed by acid hydrolysis to obtain **17**. Selective protection with MMTr, followed by treatment with TBDMS was carried out without isolation of the intermediate. Thereafter, the trityl group was selectively removed with *p*-toluenesulfonic acid (4%) in a $MeOH/CH_2Cl_2$ (1:3). The hydroxyl group of **19** was mesylated to allow the introduction of adenine. Thymine was introduced with the Mitsunobu reaction as described in Scheme 4. Ph_3P did not reduce the azido group of **19** during the Mitsunobu reaction, and the yield for compound **22b** was 77%. The following steps to give rise to the desired products **15a** and **15b** are described in Scheme 5.



Scheme 5. Preparation of *S*-configured, protected nucleosides **15a** and **15b** from (*S*)-isopropylidene glycerol (**4**). a) TsCl, pyridine, 94%; b) NaN_3, 100 °C, DMF, not isolated; c) TFA (80%) in H_2O, 33% over both steps; d) MMTrCl, pyridine, TBDMSCl, imidazole, DMF, not isolated; e) pTSOH (4%) in CH_2Cl_2 and MeOH, 37%; f) MsCl, pyridine, 81%; g) A, NaH (60%), 80 °C, DMF, 1 h, 100 °C, 15 h, 46%; h) BzCl, pyridine, saturated NH_3 in MeOH, 0 °C, 82%; i) T^{Bz}, DIAD, Ph_3P, THF, saturated NH_3 in MeOH, 0 °C, 77%; j) Ph_3P, THF, H_2O, **a**: 80%, **b**: 80%; k) MMTrCl, pyridine, not isolated; l) TBAF (1 M) in THF, yields over both steps **a**: 71%, **b**: 79%.

Synthesis of dimers with acyclic nucleosides

In order to verify the applicability of common DNA chemistry for the synthesis of oligonucleotides with the acyclic monomers, we tried to synthesize dimers from the monomer and natural 3′-*O*-acetylthymidine by using phosphoramidite chemistry. Treatment of **3a** with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite gave phosphoramidite **25**. Treatment of **25** with 3′-*O*-acetylthymidine in the presence of tetrazole, followed by oxidation with I_2/H_2O did not afford the desired dimer. Instead, **26** was obtained as a result of the intramolecular attack of the amino group on P^{III} (Scheme 6).



Scheme 6. Side reaction occurring during dimer synthesis when using the phosphoramidite approach.

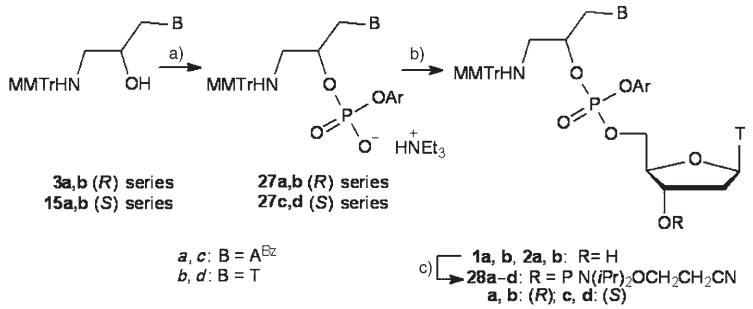
An attempt was made to emulate the phosphotriester method by converting **3a** into the stable phosphoramidate **27a** (73%).[8] However, the coupling of **27a** with **13a** was not observed in the presence of various activators, such as mesitylenesulfonyl chloride (MSCl) or 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in pyridine at room temperature. Instead the primary amino group was sulfonated in a side reaction. On the basis of these observations, attempts to generate oligonucleotide sequences that uniformly contained aminopropyl nucleotides were abandoned. However, the above results indicated that the phosphodiester building block **27a** was stable under phosphotriester conditions. Taking into account the modification of the phosphoramidate synthesis as described by Gryaznov,[9] we decided to couple these acyclic phosphodiesters with natural thymidine and use the dimers **28a**–**d** to synthesize oligomers by applying phosphoramidite methodology (Scheme 7).

Synthesis of dimers for oligonucleotide synthesis

Treatment of **3a**, **3b**, **15a** and **15b** with a slight excess of 2-chlorophenyl dichlorophosphate in the presence of 1,2,4-triazole gave the required phosphodiester building blocks **27a**–**d**, respectively. The dimers **1a**, **1b**, **2a** and **2b** were obtained by treatment of **27a**–**d**, respectively, with 3′-*O*-Fmoc-thymidine, mesitylenesulfonyl chloride and 1-methylimidazole (NMI) in pyridine (**1a**, **2a**: 62%; **1b**, **2b**: 49%; Scheme 7). The Fmoc protecting group was slowly removed during the condensation reaction. The desired dimers **1a**, **1b**, **2a** and **2b** were isolated and their structures were analyzed by mass and NMR spectroscopy.

Synthesis of oligonucleotides with ayclic 3′NH-phosphoramidate linkage

Phosphitylation was carried out with the monomethoxytritylated derivatives (**1a**, **1b**, **2a** and **2b**) in dichloromethane by using freshly distilled diisopropylethylamine and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite under argon to afford **28a**–**d**, respectively, in high yield. Several oligonucleotides



Scheme 7. Synthesis of dimer amidites **28** for oligonucleotide synthesis a) 2-chlorophenyl phosphorodichloridate, 1*H*-1,2,4-triazole, pyridine; **a** and **c**: 73%; **b** and **d**: 68%; b) MSCl, NMI, pyridine; **1a**, **2a** (*S*): 62%; **1b**, **2b** (*R*): 49%; c) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, diisopropylethylamine, DCM; **a**: 72%, **b**: 78%, **c**: 77%, **d**: 85%.

with one or three modified acyclic building blocks were synthesized with these phosphoramidites. In addition, the protocols developed for NH–TNA[10] were used, and a modified version of the phosphoramidite method described by Gryaznov for their analogues was taken into account.[11] The oligomers were isolated after ion-exchange chromatography and gel filtration. The purified oligonucleotides (sodium salts) were desalted by cation exchange beads and analyzed by mass spectrometry, and the correct incorporation of the acyclic building blocks was demonstrated.

Pairing properties of oligonucleotides that contain a 3′NH-phosphoramidate linkage

The pairing properties of the analogues were examined by hybridizing oligomers with their complementary DNA strands and determining the melting points of the hybrids by temperature-dependent UV spectroscopy. Table 1 shows the influence of aminopropyl thymine (T*) or aminopropyl adenine (A*) incorporation into the DNA oligomers on the T_m, which was determined at 260 nm in NaCl (0.1 M) buffer with KH_2PO_4 (20 mM, pH 7.5) and EDTA (0.1 mM) together with 4 μM of each oligomer complement. Likewise Table 1 shows the T_m for duplexes that contain T* or A* in each strand.

Incorporation of one T* or A* resulted in a strong decrease in duplex stability (ΔT_m/mod = −9.8 to −15.7), the absolute value being independent of the incorporation site. The decrease for T* (**30**) is larger than for A* substitutions (**29**, **31**). A larger ΔT_m/mod was observed when both strands contained a modified building block, albeit not in complementary positions (duplexes **4** and **5**). However, the destabilization per modification proved smaller. A large difference was also noticed between the adenine and thymine congeners, with 12 and 7 °C destabilization per modification, respectively.

Evaluation of potential for hybridization of complementary APNAs (duplexes **6** and **7**) within a dsDNA sequence indicated strong destabilization. Remarkably, however, there is a large difference in the stability of the two enantiomers which favours (*S*)-APNA or (*R*)-nucleoside incorporation (27 °C vs. 22 °C for A*–T* interaction; duplexes **6** and **7**). The mismatches that were created (duplexes **10** and **11**) might reflect the lack of sufficient width for the APNA modification in B-DNA for normal Watson–Crick base pairing: the bulky A*–A* pairing (duplex **10**) is more stable than the control A–A mismatch (duplex **9**); this is especially so for the incorporation of *R* dimers (33.4 °C vs. 27.5 °C). However, the smaller T*–T* mismatch further destabilizes the helix in comparison with a single T* modification (duplex **11** vs. **2**). Thus, we observed that the hybridization properties of *R* dimers were better than those of *S* dimers; this indicates that the chirality of the carbon atoms in APNA nucleotides plays an important role in the hybridization strength in a double-stranded DNA sequence.

Table 2 shows the T_m of DNA oligomers that contain three T* or A* (**37**, **38**) and that have been hybridized to their complement. The T_m value for these duplexes decreased too much and could no longer be detected. This result indicates that long stretches of such nucleoside analogues are difficult to accommodate in the DNA duplex.

To further evaluate the possible universal characteristics of T* and A*, a single modification was incorporated into a mixed sequence and hybridized to oligomers that contained either one of the four natural bases in the complementary position

Table 1. Sequences and stability studies of the oligonucleotide 5′-CCTTT**XYZ**TTTCC-3′ and its complement 3′-GGAAA**MLK**AAAGG-5′.

Duplex (oligo pair)	Sequence **XYZ/MLK**	T_m [°C] (*R*)-APNA	(*S*)-APNA	ΔT_m/mod [°C][a] (*R*)-APNA	(*S*)-APNA	T_m [°C] B-DNA
1 (**29**/**41**)	TA*T/ATA	30.8	30.8	−10	−10	40.8
2 (**30**/**42**)	TT*T/AAA	26.7	26.2	−15.5	−15.7	42.2
3 (**43**/**31**)	TAT/ATA*	29.8	31.0	−11	−9.8	40.8
4 (**29**/**31**)	TA*T/ATA*	25.6	25.7	−7.6	−7.6	40.8
5 (**32**/**33**)	AAT*/TT*A	16.2	16.1	−12.3	−12.3	40.8
6 (**34**/**33**)	AA*T/TT*A	21.7	27.1	–[b]	–[b]	–[b]
7 (**35**/**36**)	AT*T/TA*A	22.2	26.7	–[b]	–[b]	–[b]
8 (**30**/**41**)	TT*T/ATA	20.7	24.9	−9.1	−4.9	29.8
9 (**29**/**42**)	TA*T/AAA	23.8	24.1	−3.7	−2.6	27.5
10 (**34**/**36**)	AA*T/TA*A	28.2	33.4	–[b]	–[b]	–[b]
11 (**35**/**33**)	AT*T/TT*A	20.2	22.0	–[b]	–[b]	–[b]

[a] ΔT_m between unmodified and modified nucleotides. [b] Not determined.

Table 2. Thermal stability of three T* or A* residues incorporated in DNA oligomers.

Sequence	(*R*)-APNA	(*S*)-APNA	Unmodified control
5′-CCTTT*TT*TT*TTCC-3′ (**37**)	no[a]	no[a]	42.2
5′-CCTTA*TA*TA*TTCC-3′ (**38**)	<10	<10	39.0

[a] Temperatures too low for measurement.

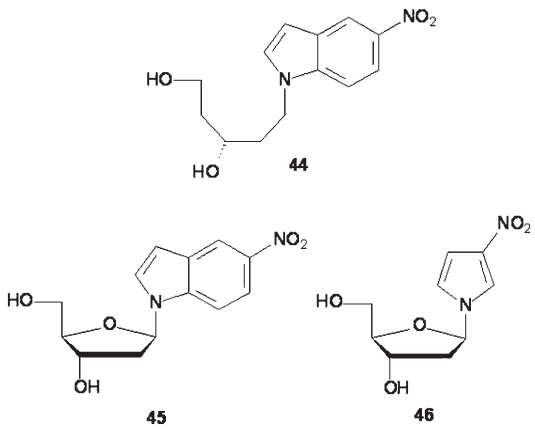
(Y; Table 3). As with A/T homopolymer stretches, both T* and A* destabilized the matched sequence to a large extent (approximately 12 °C and 8.5 °C, respectively) with both the *R* and *S* series. Mismatches, however, did not increase the destabilization of the duplexes to a large extent, and in some cases (A*) even proved advantageous compared to the control mismatch. The T_m range for the different mismatches proved smallest for the A* congeners, and again the best results were obtained with the (*R*)-A* nucleoside analogue ((*S*)-APNA series). This modified building block displayed almost universal or ambiguous characteristics in hybridizing to all four natural bases with about equal strength, with a range of only 2.7 °C. Unfortunately, there was also an average destabilization of 9.3 °C compared to the control matched sequence (A–T, 57.4 °C). However, this is comparable to other common "universal bases" (Scheme 8, **44**–**46**).

Table 3. Hybridization studies of a single T* or A* incorporation into a mixed sequence 5′-CACCG**X**TGCTACC-3′ and its complement 3′-GTGGC**Y**ACGATGG-5′, including mismatches.

	X					
Y	T* (*S*, **39**)		T* (*R*, **39**)		T	
	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
A	44.8	–[a]	45.8	–[a]	57.0	–[a]
T	41.1	−3.7	44.9	−0.9	46.8	−10.2
C	38.8	−6.0	41.2	−4.6	44.4	−12.6
G	44.5	−0.3	49.5	+4.6	50.9	−6.1
	A* (*S*, **40**)		A* (*R*, **40**)		A	
	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
T	48.7	–[a]	49.2	–[a]	57.4	–[a]
A	45.4	−3.3	47.8	−1.4	46.6	−10.8
C	44.5	−4.2	46.5	−2.7	44.7	−12.7
G	48.3	−0.4	49.0	−0.6	53.5	−3.9

[a] Did not match control sequence.

The acyclic 5-nitroindazole derivative **44** displayed a spread of 2.2 °C with an average destabilization of 7.8 °C in an analogous sequence.[12] The 5-nitroindole nucleoside analogue **45** displayed a spread of only 1.0 °C with 8.1 °C average destabilization.[13] The commercially available nitropyrrole congener **46** had a detrimental spread of 5.1 °C and a ΔT_m average of 12.0 °C for the same sequence.[14]



Scheme 8. Structure of three "universal" nucleoside analogues.

Conclusion

In conclusion, all four modifications studied considerably destabilized the double helix upon a single or multiple incorporations. This might be due to large constitutional and conformational irregularities that arise at the insertion site when mixed oligonucleotides are synthesized. Better results might be obtained with fully modified APNA as has recently been demonstrated with (*S*)-glycol nucleic acids (GNA). During the preparation of this manuscript, an article describing the successful hybridization of GNA with RNA was published.[15]

The (*S*)-APNA series is slightly better accommodated, and especially an A*–A* interaction seems favourable. The (*R*)-A* monomer modification behaves almost like the best ambiguous nucleoside analogues known to-date and therefore the system deserves further study. Aminopropyl nucleoside analogues are not substitutes for deoxynucleotides when incorporated into DNA. APNA itself as a simple nucleic acid alternative and its potential to hybridize with DNA or RNA, could not be evaluated so far due to the difficulty of obtaining APNA oligomers with the present phosphoramidite method.

Experimental Section

For all reactions analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware (100 °C) under a nitrogen atmosphere. ^{1}H NMR spectroscopy was carried out with a Varian Unity 500 MHz spectrometer with tetramethylsilane (TMS) as internal standard. A 200 MHz Varian Gemini apparatus was used for ^{13}C NMR spectrum determinations in the solvents [D_6]DMSO (39.6 ppm) or $CDCl_3$ (76.9 ppm) by using the solvent peak as reference. Exact mass measurements were performed on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard ESI interface; samples were infused in *i*PrOH/H_2O (1:1) at 3 μL min^{-1}. TLC was performed with aluminum sheets (Merck, Silica gel 60 F_{254}). The spots were examined with UV light, or sprayed with ethanol/sulfuric acid/anisaldehyde (70:5:5) or potassium permanganate (1%) solution. Column chromatography was performed on ICN silica gel 60 A 60–200. The names of the compounds accorded to IUPAC rules and were verified with a nomenclature program (ACD-Labs, Version 4.08, Sept. 1999, Adv. Chem. Dev. Inc., Toronto, Canada).

(*R*)-2-[6-(benzoylamino)-9*H*-purin-9-yl]-1-({[(4-methoxyphenyl)diphenylmethyl]amino}methyl)ethyl-2-cyanoethyl diisopropylamidophosphite (25): Compound **3a** (0.32 g, 0.55 mmol) was treated with freshly distilled diisopropylethylamine (0.28 mL, 1.6 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.20 mL, 0.88 mmol) in CH_2Cl_2 under argon atmosphere. The reaction mixture was stirred at room temperature for 45 min after which TLC analysis indicated complete reaction. Water (3 mL) was added, the solution was stirred for 10 min and partitioned between CH_2Cl_2 (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic phase was washed with brine (3×30 mL) and the aqueous phases were back extracted with CH_2Cl_2 (30 mL). Evaporation of the organics left an oil, which was purified by flash chromatography (25 g silica, *n*-hexane/acetone/TEA, (67:32:1)) to afford the product as foam. Dissolution in CH_2Cl_2 (2 mL) and precipitation in cold hexane (160 mL; −60 °C) was carried out twice to afford **25** as a white powder. The product was dried in vacuo and stored under nitrogen at −20 °C until use for oligonucleotide synthesis. Yield: 0.37 g (86%); ^{31}P NMR: $\delta = 149.20, 150.32$; ESMS calcd for $C_{44}H_{50}N_8O_4P$: 785.3692 $[M+H]^+$; found: 785.3679.

(*S*)-*N*-[9-({2-(2-cyanoethoxy)-3-[(4-methoxyphenyl)diphenylmethyl]-2-oxido-1,3,2-oxazaphospholidin-5-yl}methyl)-9*H*-purin-6-yl]-benzamide (26): Compound **25** (0.070 g, 0.09 mmol) and 3′-*O*-acetylthymidine (0.011 g, 0.04 mmol) were dissolved in CH_3CN (1 mL) and tetrazole in CH_3CN (0.5 M, 1 mL) was added. The reaction mixture was stirred for 10 min, I_2 (0.05 M) in THF/H_2O/pyridine (1 mL) was added and the mixture was stirred for a further 20 min. CH_2Cl_2 (25 mL) was added, and, after 10 min, the mixture was partitioned between CH_2Cl_2 (50 mL) and aqueous $NaHCO_3$ (5 mL). The organic phase was washed with brine (3×30 mL), and the aqueous phases were extracted with CH_2Cl_2 (2×25 mL). Evaporation of the organics left an oil, which was purified by flash chromatography (10 g silica, CH_2Cl_2/MEOH (97:3)). Yield: 0.03 g (44%); ^{31}P NMR: $\delta = 17.44, 18.26$; ESMS calcd for $C_{38}H_{35}N_7O_5P$: 700.2437 $[M+H]^+$; found: 700.2440.

(*R*)- and (*S*)-1-[6-(benzoylamino)-9*H*-purin-9-yl]-2-{[(4-methoxyphenyl)diphenylmethyl]amino}ethyl-2-chlorophenylphosphate triethylammonium salt (27a, 27c): 2-Chlorophenyl phosphorodichloridate (0.42 mL, 3.08 mmol) was slowly added to an ice-cooled solution of 1*H*-1,2,4-triazole (0.95 g, 12.3 mmol) in dry pyridine (10 mL). After stirring for 5 min, **3a** and **15a** (0.90 g, 1.54 mmol) which were dissolved in dry pyridine (1 mL) were slowly injected, respectively. After stirring for another 5 min the reaction mixture was quenched with TEAB (0.1 M, 20 mL). The mixture was extracted with chloroform (3×30 mL). The organic layer was dried with Na_2SO_4, evaporated and purified by column chromatography (silica, 100 g, CH_2Cl_2/MeOH/Et_3N 95:4.5:0.5). Yield: 0.99 g (73%); $R_f = 0.53$ (CH_2Cl_2/MeOH/Et_3N 95:4.5:0.5); ^{1}H NMR ($CDCl_3$): $\delta = 1.14$–1.21 (t, $J = 7.2$ Hz, 9H; 3CH_3-Et_3N), 2.02–2.37 (dd, $J_{(3'A,2')} = 6.4$, $J_{(gem)} = 12.2$ Hz, 1H; H-3A), 2.29–3.37 (dd, $J_{(3'B,2')} = 5.4$, $J_{(gem)} = 12.2$ Hz, 1H; H-3′B), 2.84–2.96 (q, $J = 7.2$, $J_{(germ)} = 14.6$ Hz, 6H; 3CH_2), 3.76 (s, 3H; CH_3-MMTr), 4.65–4.84 (m, 2H; H-1′), 4.97 (m, 1H; H-2′), 6.7–6.75 (t, 2H; $J_{(3,4)} = 8.8$ Hz, 2H-4-MMTr), 6.90–7.46 (m, 15H; H-MMTr), 7.49–7.65 (m, 4H; 3,4,5H-Bz, H-MMTr), 8.04–8.09 (d, $J = 6.6$ Hz, 2H; 2,6H-Bz), 8.27 (s, 1H; H-8), 8.65 (s, 1H; H-2); ^{13}C NMR ([D_6]DMSO): $\delta = 8.5$ (CH_3-Et_3N), 45.3 (C-3′, C-1′), 55.1 (CH_3-MMTr), 70.1 (NH-C-MMTr), 74.6 (C-2′, *J*(C-P coupling) = 5.5 Hz), 121.1 (C-4-P-Ar), 122.2 (C-6-P-Ar), 123.5 (C-2-P-Ar), 124.8 (C-5), 126.2 (C-MMTR), 127.5 (C-5-P-Ar), 127.7, 127.8 (C-MMTr), 128.4 (3,5C-Bz), 128.8 (2,6CH-Bz), 129.7 (C-MMTr), 132.6 (4C-Bz), 133.9 (1C-Bz), 137.9, 145.0 (C-MMTr), 146.0 (C-8), 149.1 (C-4), 152.2 (C-2, C-1-P-Ar), 153.2 (C-6), 157.6 (C-MMTr), 164.8 (C=O). ESMS calcd for $C_{41}H_{37}N_6O_6PCl$ $[M+H]^+$: 775.2200; found: 775.2216.

(*R*)- and (*S*)-2-chlorophenyl-2-{[(4-methoxyphenyl)diphenylmethyl]amino}-1-[(5-methyl-2,4-dioxo-3,4-dihydro-1(2*H*)-pyrimidinyl)methyl]ethylphosphate triethylammonium salt (27b, 27d): Following the method described for **27a** and **27c**, derivatives **27b** and **27d** were obtained in 68% and 70% yield, respectively. $R_f = 0.53$ (CH_2Cl_2/MeOH/Et_3N 95:4.5:0.5); ^{1}H NMR ($CDCl_3$): $\delta = 1.14$–1.18 (t, $J = 7.2$ Hz, 9H; 3CH_3-Et_3N), 1.79 (s, 3H; CH_3-T), 2.27–2.39 (m, 1H; H-3), 2.78–2.89 (q, $J = 7.2$, $J_{(germ)} = 14.6$ Hz, 6H; 3CH_2), 3.74 (s, 3H; CH_3-MMTr), 4.03–4.19 (m, 2H; H-1′), 4.79–4.89 (m, 1H; H-2′), 6.71–6.75 (d, 2H; 2H = MMTr), 6.86–6.89 (m, 1H; H-Ar-O), 6.90–7.42 (m, 15H; H-Ar-O, H-MMTr, H-6-T); ^{13}C NMR ([D_6]DMSO): $\delta = 8.5$ (CH_3-Et_3N), 11.9 (CH_3-T), 45.4 (CH_2-Et_3N), 50.2 (C-3′), 55.0 (CH_3-MMTr), 70.0 (NH-C-MMTr), 74.7–74.8 (C-2′, *J*(c,p) = 6.1 Hz), 109.3 (C-5), 113.2 (C-MMTr), 120.9, 123.3 (C-Ar-O-P), 125.9, 127.4, 127.6, 128.4, 129.7, 137.7, 146.0 (C-MMTr), 142.2 (C-6), 146.1 (C-Ar-O-P), 149.0–149.2 (d, $J = 6.1$ Hz, C-Ar-O-P), 151.3 (C-Ar-O-P), 157.4 (C-2), 164.5 (C-4); ESMS calcd for $C_{34}H_{32}N_3O_7PCl$: 660.1666 $[M-H]^-$; found: 660.1661.

Dimers (*R*)-1a and (*S*)-2a: Compound **27a** or **27c** (0.80 g, 0.91 mmol) was coevaporated with 3′-*O*-Fmoc-thymidine (0.42 g, 0.91 mmol) with pyridine (2×10 mL) and finally dissolved in dry pyridine (10 mL). MSCl (0.40 g, 1.82 mmol) and NMI (0.72 mL, 9.1 mmol) were added under nitrogen protection. After 2 h, the reaction was quenched with TEAB (0.1 M, 10 mL), and the mixture was extracted with CH_2Cl_2 (2×15 mL). The organic layer was dried over Na_2SO_4, evaporated under reduced pressure and purified by column chromatography (50 g silica, CH_2Cl_2/MeOH/pyridine 95:4.5:0.5). Yield: 0.55 g (62%); $R_f = 0.48$ (CH_2Cl_2/MeOH 95:5); ESMS calcd for $C_{51}H_{49}N_8O_{10}PCl$: 998.2920 $[M+H]^+$; found: 998.2995.

Dimers (*R*)-1b and (*S*)-2b: **27b** or **27d** (0.80 g, 1.05 mmol) was treated with 3′-O-Fmoc-thymidine (0.46 g, 0.99 mmol) in dry pyridine (10 mL) as described for **1a** and **2a**. Yield: 0.46 g (49%); $R_f = 0.31$ (CH_2Cl_2/MeOH 95:5); ESMS calcd for $C_{44}H_{45}N_5O_{11}PCl$: 886.2620 $[M+H]^+$; found: 886.2629.

General procedure for phosphoramidite synthesis (28a–d): Phosphitylation was carried out with **1a** (0.54 mmol), **1b** (0.30 mmol), **2a** (0.60 mmol) and **2b** (0.56 mmol) in CH_2Cl_2 by using freshly distilled diisopropylethylamine and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite under argon atmosphere. The mixture was stirred at room temperature for 60 min, after which the reaction was found by TLC analysis to be complete. Water (3 mL) was added, and the solution was stirred for another 10 min and partitioned between CH_2Cl_2 (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic layer was washed with brine (3×30 mL), and the aqueous phases were back extracted with CH_2Cl_2 (30 mL). After evaporation of the solvent the resulting oil was purified by flash chromatography (45 g silica, *n*-hexane/acetone/pyr 55:45:1). Dissolution in CH_2Cl_2 (3 mL) and precipitation in cold hexane (160 mL, −60 °C) was carried out twice to afford the desired product as a white powder. The product was dried in vacuo and stored under nitrogen at −20 °C. **28a**: 0.47 g (72%); ^{31}P NMR: $\delta = -6.88, -7.02, 149.44, 149.54, 149.72, 149.98$; ESMS calcd for $C_{60}H_{65}N_{10}O_{11}P_2Cl$: 1199.4076 $[M+H]^+$; found: 1199.4117. **28c**: 0.47 g (78%); ^{31}P NMR: $\delta = -6.40, -7.27, -7.32, 14.48, 14.53, 149.43, 149.49, 149.85, 149.93$; ESMS calcd for $C_{60}H_{65}N_{10}O_{11}P_2Cl$: 1199.4076 $[M+H]^+$; found: 1199.41171. **28b**: 0.50 g (77%); ^{31}P NMR: $\delta = -6.49, -6.66, -6.98, -7.03, 14.48, 14.52, 149.16, 149.46, 149.90$; ESMS calcd for $C_{53}H_{62}N_7O_{12}P_2Cl$: 1086.3698 $[M+H]^+$; found: 1086.3705. **28d**: 0.53 g (85%); ^{31}P NMR: $\delta = -6.77, 14.47, 149.60, 150.01$; ESMS calcd for $C_{53}H_{62}N_7O_{12}P_2Cl$: 1086.3698; $[M+H]^+$ found: 1086.3688.

Synthesis of oligonucleotides: Oligonucleotide assembly was performed on an Expedite DNA synthesizer (Applied Biosystems) by

using the phosphoramidite approach. The standard DNA assembly protocol was used, except that 10 min coupling time was employed for the newly synthesized unnatural amidites (0.08 M) with ethylthiotetrazole (ETT) as the activator. The oligomers were deprotected and cleaved from the solid support by treatment with concentrated aqueous ammonia (55 °C, 16 h). After gel filtration on a NAP-10® column (Sephadex G25 DNA grade; Pharmacia) with water as eluent, the crude was analyzed on a Mono-Q® HR 5/5 anion exchange column. Purification was then achieved on a Mono-Q® HR 10/10 column (Pharmacia) with the following gradient system: A = NaOH (10 mM, pH 12.0), NaCl (0.1 M); B = NaOH (10 mM, pH 12.0), NaCl (0.9 M).

The low-pressure liquid chromatography system consisted of a Merck-Hitachi L6200A intelligent pump, a Mono Q® HR10/10 column (Pharmacia), a Uvicord SII 2138 UV detector (Pharmacia-LKB) and a recorder. The product-containing fraction was desalted on a NAP-10® column and lyophilized.

Oligonucleotides were characterized and their purity was checked by HPLC-MS on a capillary chromatograph (CapLC, Waters, Milford, MA). Columns of 150 mm × 0.3 mm length (LCPackings, San Francisco, CA) were used. Oligonucleotides were eluted with an acetonitrile gradient in triethylammonium (50 mM) adjusted to pH 8.0 with 1,1,1,3,3,3-hexafluoropropan-2-ol. The flow rate was 5 $\mu L\,min^{-1}$. Electrospray spectra were acquired on an orthogonal acceleration/time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) in negative-ion mode; scan time was 2 s. The combined spectra from a chromatographic peak were deconvoluted by using the MaxEnt algorithm of the software (Masslynx 3.4, Micromass, Manchester, UK). Theoretical oligonucleotide masses were calculated by using the monoisotopic element masses.

Melting temperatures: Oligomers were dissolved in NaCl (0.1 M), potassium phosphate (0.02 M, pH 7.5), EDTA (0.1 mM). The concentration was determined by measuring the absorbance in MilliQ water at 260 nm at 80 °C, and by assuming that the acyclic-nucleoside analogues had the same extinction coefficients per base moiety in the denatured state as the natural nucleosides (A* ε = 15000; T* ε = 8500). The concentration for each strand was 4 μM in all experiments unless otherwise stated. Melting curves were determined with a Varian Cary 300 BIO spectrophotometer. Cuvettes were maintained at constant temperature by water circulation through the cuvette holder. The temperature of the solution was measured with a thermistor that was directly immersed in the cuvette. Temperature control and data acquisition were carried out automatically with an IBM-compatible computer by using Cary WinUV thermal application software. A quick heating and cooling cycle was carried out to allow proper annealing of both strands. The samples were then heated from 10 °C to 80 °C at a rate of 0.2 $^{\circ}C\,min^{-1}$, and were cooled again at the same speed. Melting temperatures were determined by plotting the first derivative of the absorbance as a function of temperature; data plotted were the average of two runs. Up and down curves in general showed identical T_m values.

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