ORGANIC LETTERS

2009
Vol. 11, No. 11
2329−2332

Modulating the Hybridization Property of PNA with a Peptoid-Like Side Chain

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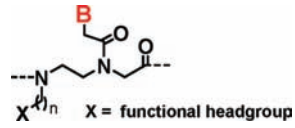
Received April 6, 2009

ABSTRACT



Modification on the γ-N of the PNA backbone yielded a PNA analogue with a peptoid-like side chain. We found that the length of the side chain was important in influencing the hybridization affinity of the modified PNA.

PNA is a DNA/RNA mimic with a poly-*N*-(2-aminoethyl)-glycine (aeg) backbone (Figure 1a) and binds to complementary oligonucleotides through Watson−Crick base pairing with high sequence specificity.[1,2] The structural simplicity, high hybridization affinity, and in vivo stability of PNA have made it an attractive agent for antisense and antigene applications in basic biology and medicine.[3−5] Since its advent in 1991, extensive modification work has been conducted on PNA in an effort to further improve its properties.[6−12] These modifications have been mainly focused on the carbons of PNA's aeg backbone,[6−11] and analogues with interesting pharmacological properties have been reported.[6−11] Modifications on these backbone carbons

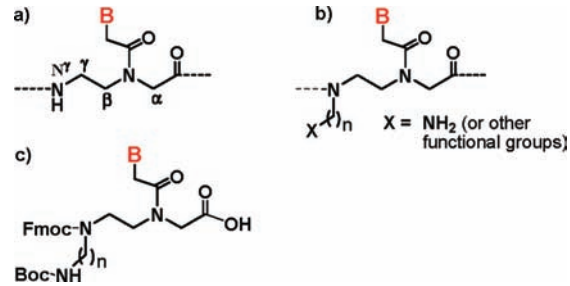


Figure 1. Structures of (a) an aegPNA residue, (b) a peptoid PNA residue (an aminopeptoid PNA residue, when X = NH_2), and (c) a protected aminopeptoid PNA monomer for oligomer synthesis. The red B denotes a nucleobase.

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10.1021/ol900587b CCC: $40.75
Published on Web 05/13/2009

Table 1. Thermal Stability Data (T_m, °C) of AP−PNA/DNA and AP−PNA/RNA Duplexes[a,i]

no.	name of PNA[b]	sequence[b]	DNA (anti-parallel)[c]	DNA (parallel)[d]	DNA mismatch[e]	RNA (anti-parallel)[f]	RNA mismatch[g]
1	aegPNA	Ac-GTAGATCACT-Gly-NH_2	48.6	34.9	36.4	51.9	42.7
2	AP-PNA 2-1	Ac-GTAGAT^{2a}CACT-Gly-NH_2	40.2	34.4	29[h]	48.9	41
3	AP-PNA 3-1	Ac-GTAGAT^{3a}CACT-Gly-NH_2	43.6	32.7	30[h]	50.1	40.3
4	AP-PNA 4-1	Ac-GTAGAT^{4a}CACT-Gly-NH_2	46.1	31.5	31.1[h]	51.5	42.2
5	AP-PNA 5-1	Ac-GTAGAT^{5a}CACT-Gly-NH_2	49.1	34.9	34.4[h]	52	43.3
6	AP-PNA 6-1	Ac-GTAGAT^{6a}CACT-Gly-NH_2	48.6	33.9	33.8[h]	51.8	39.3

[a] The concentrations of DNA/RNA, aegPNA, and AP−PNA oligomers were 2 μM each for duplex formation in 10 mM phosphate buffer (pH 7) containing 0.1 M NaCl and 0.1 mM EDTA. Thermal denaturation studies were conducted using UV monitoring at 260 nm scanning from 80 to 20 °C at the rate of 0.5 °C/min. All values are accurate to ± 1 °C. Experiments are repeated at least three times, and all values are the average of three valid measurements. [b] For nomenclature, AP−PNA is used for any PNA containing at least one aminopeptoid side chain. The first suffix number in the name specifies the length (the number of methylene carbons) of the side chain, and the second denotes the number of peptoid modifications in the PNA. Similarly in the sequence, the superscript number on a residue specifies the length of a peptoid side chain on that residue, and the superscript letter a denotes the amino headgroup of the peptoid side chain. [c] 5′d(AGTGATCTAC). [d] 5′d(CATCTAGTGA). [e] 5′d(AGTGGTCTAC). [f] 5′(AGUGAUCUAC. [g] 5′(AGUGGUCUAC). [h] The thermal transition of these strands was not obvious as compared with others, so this value was difficult to determine accurately. [i] The decamer AP−PNAs contain a single aminopeptoid side chain at the thymine residue at position 6.

inevitably generate a chiral center, and the two stereoisomers often exhibit distinct helical structures and binding behaviors in hybridizing with DNA and RNA sequences.[7−10,13] It is therefore of the utmost importance to preserve the chiral integrity of such PNA analogues during the synthetic process.[13b] Modifying the γ-nitrogen of the aeg backbone has also been considered.[12] However, in an early study by the Nielsen group, γ-N methylation was found to have a negative impact on the hybridizing affinity of the modified PNAs,[12a] which probably has deterred further attempts to modify at this position.

In our efforts to develop PNA-based agents, we decided to reinvestigate modifications at the γ-nitrogen by appending to it an alkyl side chain carrying a functional headgroup (Figure 1b). We name the side chain a peptoid-like side chain, as it is analoguous to the N^{α}-side chains in N-substituted oligoglycines or peptoids.[14] Modification on the γ-nitrogen preserves the achiral nature of PNA and therefore causes no stereochemistry complications synthetically. Introducing such a side chain may also bring about some of the beneficial effects observed of a similar side chain extended from the α- or γ-C. In addition, the functional headgroup could also serve as a suitable anchor point to attach various structural moieties of biophysical and biochemical interest. Furthermore, given the ease in choosing the length of the peptoid side chain and the nature of the functional headgroup, the electrosteric effects of such a side chain can be examined systematically. Interestingly, we find that the length of the peptoid-like side chain plays a critical role in determining the hybridization affinity of the modified PNA.

We devised a convenient synthetic method for the synthesis of AP−PNA monomers (Supporting Information), which was derived from the strategy used by the Nielsen group for the synthesis of N-methylated PNAs.[12a] We chose t-Boc to protect the peptoid side-chain amine and Fmoc for the backbone 2° amino group from which the PNA oligomer would elongate (Figure 1c). The PNA oligomers were synthesized on Rinkamide PEGA resin using standard solid-phase Fmoc peptide synthesis protocols. While coupling onto an aegPNA residue was easily effected using PyBOP as the coupling reagent, coupling onto an AP−PNA residue was more difficult owing to the steric hindrance of its backbone secondary amine and was achieved through the action of HATU for ~ 6 h. PNA oligomers containing aminopeptoid side chains were readily soluble in water, which facilitated their purification and hybridization studies.

A decamer PNA sequence with mixed pyrimidine and purine content was chosen in this study to examine duplex formation with complementary DNA and RNA. The amino termini of all PNA oligomers were acetylated. To determine whether the length of the aminopeptoid side chain has any influence on the thermal stability of the PNA/DNA or PNA/RNA duplex, a first set of five PNAs containing a single aminopeptoid thymine residue at the middle of the sequence were synthesized. The spacer length between the peptoid amino headgroup and the backbone in the five PNAs is of 2, 3, 4, 5, and 6 methylenes, respectively. Table 1 presents the hybridization data of these modified PNAs in comparison with the unmodified aegPNA. The data clearly show that the length of the aminopeptoid side chain plays a critical role in determining the thermal stability, i.e., the melting temperature (T_m), of the duplex formed between the modified PNA and the DNA. Introducing an aminopeptoid side chain of 5 or 6 methylene carbons, i.e., an N^{γ}-aminopentyl

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Table 2. Effects of Multiple Aminopeptoid Modifications on the Thermal Stability (T_m, °C) of AP−PNA/DNA and AP−PNA/RNA Duplexes

no.	name of PNA	sequence	DNA (ap)[a]	DNA (p)[b]	DNA (m)[c]	RNA (ap)[d]	RNA (m)[e]
1	aegPNA	Ac-GTAGATCACT-Gly-NH_2	48.6	34.9	36.4	51.9	42.7
7	AP-PNA 2-2	Ac-GTAGA^{2a} TC^{2a} ACT-Gly-NH_2	39	31.9	31.6[f]	44.5	37.6
8	AP-PNA 2-3	Ac-G^{2a} TAGA^{2a} TC^{2a} ACT-Gly-NH_2	36.2	32.9	31.4[f]	42.3	35.6
9	AP-PNA 2-4	Ac-GT^{2a}AG^{2a}AT^{2a}CA^{2a}C T-Gly-NH_2	31.1	31.4	29[f]	36.9	33.9
10	AP-PNA 3-2	Ac-GTAGA^{3a} TC^{3a} ACT-Gly-NH_2	40.6	33.8	25.9[f]	50.4	40.9
11	AP-PNA 3-3	Ac-G^{3a} TAGA^{3a} TC^{3a} ACT-Gly-NH_2	37.3	34	23.1[f]	50.2	39.2
12	AP-PNA 3-4	Ac-GT^{3a}AG^{3a}AT^{3a}CA^{3a}C T-Gly-NH_2	34.3	29.5	32.4[f]	44.6	34.3
13	AP-PNA 4-2	Ac-GTAGA^{4a} TC^{4a} ACT-Gly-NH_2	46.6	34.3	31.9[f]	49.7	39.2
14	AP-PNA 4-3	Ac-G^{4a} TAGA^{4a} TC^{4a} ACT-Gly-NH_2	46	32.8	33.3[f]	50.1	41.1
15	AP-PNA 4-4	Ac-GT^{4a}AG^{4a}AT^{4a}CA^{4a}C T-Gly-NH_2	45.5	33.5	34.6[f]	47	34.6
16	AP-PNA 6-2	Ac-GTAGA^{6a} TC^{6a} ACT-Gly-NH_2	47.2	35.5	31.7[f]	52.9	43.9
17	AP-PNA 6-3	Ac-G^{6a} TAGA^{6a} TC^{6a} ACT-Gly-NH_2	46.6	35	31	52.4	43.4
18	AP-PNA 6-4	Ac-GT^{6a}AG^{6a}AT^{6a}CA^{6a}C T-Gly-NH_2	45.8	33.5	34.3	53.6	44
19	AP-PNA 6-5	Ac-GT^{6a}AG^{6a}AT^{6a}CA^{6a}C T^{6a} –Gly-NH_2	46.4	36	33.5	54.3	44

[a] 5′d(AGTGATCTAC). [b] 5′d(CATCTAGTGA). [c] 5′d(AGTGGTCTAC). [d] 5′(AGUGAUCUAC. [e] 5′(AGUGGUCUAC). [f] The thermal transition of these strands was not obvious as compared with others, therefore this value is difficult to determine accurately.

or N$^{\gamma}$-aminohexyl group, maintains the high thermal stability of the PNA/DNA duplexes. However, inclusion of a shorter aminopeptoid side chain (n = 4, 3, or 2) results in a decrease in thermal stability of the PNA/DNA duplex, and this negative effect becomes most detrimental with the shortest two-carbon spacer which causes an 8.4 °C drop in T_m of the AP−PNA/DNA(ap) duplex. On the binding of the modified PNA to RNA, there is also a spacer length-dependent effect on the thermal stabilities of AP−PNA/RNA duplexes, but the effect is less pronounced, as the negative impact of a short aminopeptoid side chain is less severe than in the case of AP−PNA/DNA duplexes (Table 1). Also, like aegPNA and other backbone-modified PNAs, these aminopeptoid-modified PNAs exhibit a higher binding affinity to complementary RNA than to the DNA. Most importantly, these AP−PNAs bind to antiparallel DNA or RNA containing a single-base mismatch with remarkably lower thermal stability than to the fully matched antiparallel DNA or RNA. This clearly shows that introducing a peptoid side chain onto aegPNA does not compromise the binding specificity of the modified PNA and that these AP−PNAs maintain the ability to discriminate between closely related sequences. This aminopeptoid modification also preserves the preference of PNA for binding to the DNA in the antiparallel orientation, as the AP−PNAs bind to the parallel DNA sequence with significantly reduced affinity.

Next, PNAs containing multiple aminopeptoid side chains were prepared to study the cumulative effect of N$^{\gamma}$-aminoalkylation. Summarized in Table 2 are the data obtained on AP−PNAs of the 2-, 3-, 4-, and 6-C side-chain spacer series. From these data, one can see that although the negative effect of a short 2- or 3-C aminopeptoid side chain on the DNA-hybridizing affinity increases with increasing number of modifications the effect is not additive. This finding is similar to what was observed by the Nielson group on N$^{\gamma}$-methylated PNAs.[12a] One should point out that when the cumulative effect of multiple N$^{\gamma}$-aminoethylation or aminopropylation lowers the hybridizing affinity of the modified PNAs to a certain level it also almost abolishes their binding specificity, as seen in the cases of PNA **9** and PNA **12**. For the 6- and 4-C series, only a very small or no decrease in thermal stability of the AP−PNA/DNA(ap) duplex is seen when the number of peptoid modifications is increased from 1 to 4 or even 5. For the binding to the full-match antiparallel RNA, it is worth noticing that all members of the 6-C series display a slightly higher affinity (in T_m) than aegPNA and that their binding affinity is not affected by the increasing number of N$^{\gamma}$-aminohexyl modifications. With only a few exceptions (PNA **9** and PNA **12** basically), most of these AP−PNAs bind to complementary antiparallel DNA and RNA in a highly sequence-specific manner, as seen from their lower binding affinities toward the single base-mismatched DNA or RNA (Table 2).

It is worth pointing out that, despite the negative impact of a short peptoid side chain on hybridizing affinity, almost all the AP−PNAs listed in Table 2 still bind to complementary antiparallel DNA or RNA with a higher affinity than the corresponding DNA oligonucleotide (T_m of a comparable DNA/DNA or DNA/RNA duplex is about 33−34 °C[15]). The moderate hybridizing affinity of some of the peptoid PNAs, such as the 4-C series, may have some practical value for antisense applications and be exploited for designing antisense agents with optimal binding affinty and kinetics at physiological temperature.[16] Our data suggest that by choosing an appropriate side-chain length and/or degree of peptoid modification it is possible to design PNA-based agents with tailored hybridization strength. One can even envisage using several mixed peptoid side chains of different spacer lengths to fine-tune the hybridization behaviors of PNA. Our data illustrate again the excellent versatility of PNA's aeg backbone, which allows it to be easily modified to suit a variety of applications.

To further examine the effect of side-chain length on thermal stability, the amino headgroups of four mono-N^{γ}-aminoalkylated PNAs (PNA **2**, **3**, **4**, and **6** from Table 1) were modified by an aminopropionyl or β-alanyl group, which extended the side chain by three atoms, to give PNA **20**, **21**, **22**, and **23**, respectively. As seen from Table 3, aminopropionylation significantly increased the DNA-binding affinity of the

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Table 3. Effects of Modifying the Peptoid Side-Chain Amine on Hybridization Property

no.	name of PNA	sequence	DNA (ap)[a]	DNA (m)[b]	RNA (ap)[c]	RNA (m)[d]
1	aegPNA	Ac-GTAGATCACT-Gly-NH_2	48.6	36.4	51.9	42.7
20	AP-PNA 2-1(βAla)	Ac-GTAGA$T^{2a(\beta Ala)}$CACT-Gly-NH_2	46	32.5[e]	52.6	42.6
21	AP-PNA 3-1(βAla)	Ac-GTAGA$T^{3a(\beta Ala)}$CACT-Gly-NH_2	47.7	33.8[e]	52.4	43.2
22	AP-PNA 4-1(βAla)	Ac-GTAGA$T^{4a(\beta Ala)}$CACT-Gly-NH_2	48.7	32[e]	53.4	41.8
23	AP-PNA 6-1(βAla)	Ac-GTAGA$T^{6a(\beta Ala)}$CACT-Gly-NH_2	48.6	31.8[e]	53.9	44
24	AP-PNA 6-1(Ac)	Ac-GTAGA$T^{6a(Ac)}$CACT-Gly-NH_2	48.9	40.8[e]	53.9	44.5
25	AP-PNA 6-1(biotin)	Ac- GTAGA$T^{6a(Biotin)}$CACT-Gly-NH_2	48.6	40[e]	53.9	44.4

[a] 5′d(AGTGATCTAC). [b] 5′d(AGTGGTCTAC). [c] 5′(AGUGAUCUAC. [d] 5′(AGUGGUCUAC). [e] The thermal transition of these strands was not obvious as compared with others, therefore this value was difficult to determine accurately.

AP−PNAs with a short side chain, AP−PNA 2−1 and AP−PNA 3−1 notably, but not that of AP−PNA 6−1 which already has a long side-chain spacer. For example, PNA **20**, whose side-chain spacer is extended from three atoms in AP−PNA 2−1 to six atoms, gains ∼6 °C in T_m as compared to AP−PNA 2−1. The fact that PNA **20** is still not as good as AP−PNA 6−1 despite having a spacer with the same number of atoms suggests that, in addition to the spacer length, factors such as intrachain hydrogen-bonding ability and/or conformational rigidity of the peptoid side chain may also, albeit in a minor way, affect the binding affinity of AP−PNA toward complementary oligonucleotides.

The above data illustrate again the beneficial effects of a long side chain in AP−PNA, although the effects seem capped at the 5- or 6-C spacer length. This observed spacer length-dependent effect on the hybridizing affinity of AP−PNA is intriguing. From the data obtained in this study and those of Nielsen et al. showing the negative impact of N^{γ}-methylation, one might be enticed to postulate a favorable role of the positive charge of the peptoid amine for charge−charge interactions with the phosphodiester backbone which would compensate for the negative effect of an N^{γ}-alkyl group, and a longer 5- or 6-C spacer would provide the optimal distance for such interactions. However, acetylation of the positive charge-bearing peptoid amine of AP−PNA 6−1 causes no reduction in the hybridization affinity of the resultant PNA **24** (Table 3). Also, when the side-chain amine of AP−PNA 6−1 was acylated with biotin, a useful biochemical probe, the resultant PNA **25** exhibits the same hybridizing affinity as does AP−PNA 6−1. Therefore, the positive charge on the amino headgroup of the peptoid side chain does not seem to play a role in contributing to the hybridizing affinity of AP−PNA. This observation is also similar to what was found in a previous study on PNAs with a γ-C side chain.[11] The present data do not allow us to pinpoint the cause of this length-dependent effect, and it seems that factors other than charge−charge interactions are at play. One possible factor is that a short aminopeptoid side chain might induce a higher content of E configuration about the tertiary amide due to hydrogen bonding potential of the peptoid ammonium with the amide carbonyl, whereas PNA's backbone amide is known to adopt a Z configuration for oligonucleotide binding.[17] It is also possible that a longer side chain may have a stabilizing effect on the helical structure of the AP−PNA/DNA duplex, as previous studies on peptoids have shown that increasing the size of the peptoid side chain can impose restrictions on the conformational flexibility of the oligoglycine backbone and be used to induce its helicity.[18] However, future structural investigations by NMR or X-ray crystallography are needed to find out whether these or any other factors are involved. The data on PNA **24** and particularly PNA **25** also confirm that one can indeed use the peptoid side-chain amine in an AP−PNA as the attachment site to introduce a relatively large molecular moiety for preparing PNA conjugates with potential diagnostic and therapeutic interest.

In conclusion, we have shown that the aeg backbone of PNA can be modified at the γ-nitrogen with a long side chain while maintaining the high hybridizing affinity and specificity of PNA. A systematic structure−activity study reveals an interesting relationship between the hybridizing affinity and the length of the side chain of the peptoid PNA, which has not been previously described for other backbone-modified PNA analogues. The amino headgroup in AP−PNAs can be modified in various ways without affecting the binding affinity, suggesting that the peptoid side chain can bear a variety of functional moieties. These findings indicate that modification on the γ-N of the PNA backbone is capable of generating PNA-based agents whose hybridization and pharmacological properties can be modulated, through changing the length and headgroup of the peptoid side chain, to meet the specific requirements in different applications.

Acknowledgment. The authors thank the Ministry of Education (MoE) of Singapore for financial support as well as Nanyang Technological University.

Supporting Information Available: Experimental procedures, ^{1}H and ^{13}C NMR, HPLC profiles, MS data, and thermal denaturation curves. This material is available free of charge via the Internet at http://pubs.acs.org.

OL900587B

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