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New Thiazane and Thiazolidine PNA Monomers: Synthesis, Incorporation into PNAs and Hybridization Studies

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Abstract—New constrained PNA monomers containing a substituted thiazolidine or a thiazane ring were synthesized and incorporated in the center of a 9-mer homothymine PNA. The PNA/DNA hybrids stability was studied by UV-melting experiments which showed that the presence of the modified unit destabilizes the PNA/DNA triplexes.

Since their introduction in 1991 by Nielsen et al.,[1] peptide nucleic acids (PNAs) have reenergized the area of antisense and antigene strategies and have found applications as tools in molecular biology and genetic diagnostics. PNAs are DNA mimics in which the nucleobases are attached via a methylene carbonyl linker to a polyamide backbone composed of *N*-(2-aminoethyl)-glycine (aeg) units (Fig. 1). These polymers are resistant to cellular enzymes and bind to DNA and RNA with higher sequence specificity and affinity than non-modified oligonucleotides.[2]

Many chemical modifications have been proposed to improve the PNAs hybridization properties. Some of these imply the rigidification of the PNA backbone to favor the structure observed in the PNA/DNA or the PNA/RNA complexes.[3] We have recently reported the synthesis of the constrained units **U1** and **U2** containing a thiazolidine ring which restrains the fluctuation domain of the γ and δ torsion angles (Fig. 1, **U1**: γ and $\delta = 120° \pm 30$, **U2**: $\gamma = -120° \pm 30$, $\delta = 120° \pm 30$). The presence of these units in the center of a PNA was shown to destabilize the PNA/DNA triplex.[4] In order to further explore the influence of γ and δ values on the PNA hybridization properties we have designed the new units **U3** and **U4** (Fig. 1). The *anti* and *syn* **U3** units give γ and δ angles having opposite signs compared to the one obtained for **U1** and **U2**, respectively, due to the inversion of configuration of the C-4 center. The presence of the *gem*-dimethyl at position C-5 of the thiazolidine ring is expected to reduce the γ and δ fluctuation domains. The racemic *syn* thiazane **U4** unit in the chair conformation leads to γ and δ values of 180°. In contrast, for the racemic *anti* thiazane **U4** unit, four (γ, δ) sets are expected considering the two chair conformations of both enantiomers (180°, ±60°) or (±60°, 180°). We report here the synthesis of the new units **U3** and **U4**, their incorporation in a PNA and the hybridization studies with DNA. We have also

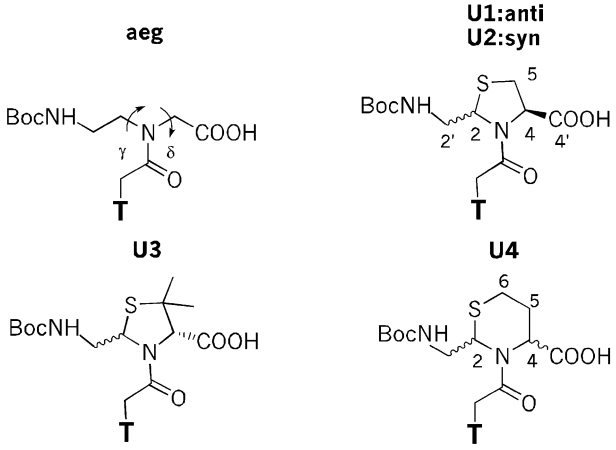

Figure 1. Structure of the different PNA units (T = thymine, Boc = *t*-butyloxycarbonyl). The atom numbering and the torsion angle nomenclature used here are indicated (γ: C-2′–C-2–*N*–C-4; δ: C-2–N–C-4–C-4′).

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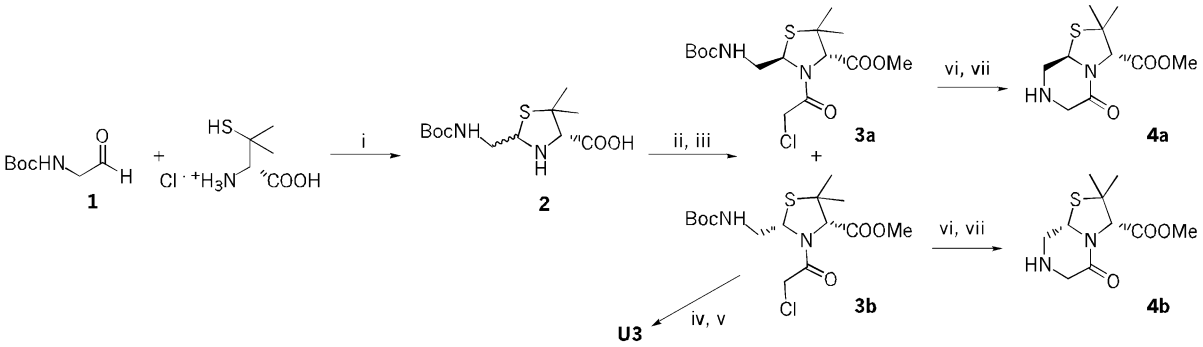
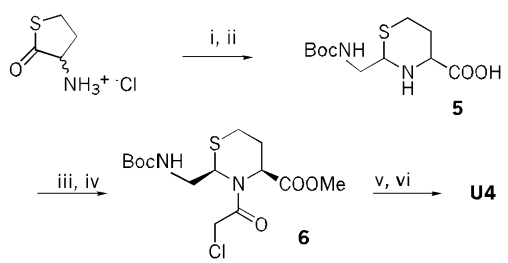


Scheme 1. (i) EtOH, H_2O; (ii) BTSA, CH_2Cl_2, $ClCH_2COCl$, pyridine; (iii) CH_2N_2, Et_2O; (iv) thymine, K_2CO_3, DMF; (v) LiOH, dioxane/H_2O; (vi) TFA/CH_2Cl_2; (vii) Et_3N, CH_2Cl_2.

examined the influence of the thiazolidine and thiazane sulfur oxidation on the PNA/DNA hybrid stability.

The PNA unit **U3** was prepared following the synthetic route outlined in Scheme 1. Condensation of *N*-Boc-glycine aldehyde **1**[5] with D-penicillamine was performed at room temperature in a mixture of ethanol/water (1:1) to give the thiazolidine-4-carboxylic acid **2**. After solvent removal and drying over P_2O_5 for 48 h, it was directly isolated without purification. Thiazolidine **2** was treated with *N*,*O*-bis(trimethylsilyl)acetamide (BTSA) to ensure its solubility in dichloromethane and reacted with chloroacetyl chloride in the presence of pyridine at −78 °C. Treatment with diazomethane in ether gave a mixture of two diastereomers **3a** and **3b** which were separated by flash chromatography and found to be present in a 60:40 ratio (51% yield starting from **1**).[6] The respective stereochemistry of **3a** and **3b** could not be determined by nuclear Overhauser effect (NOE) experiments. Indeed, no NOE was observed between protons H-2 and H-4. Furthermore, the complexity of the ^1H NMR spectra, due to peaks overlap and exchange magnetization transfer between the *cis*/*trans* amide bond isomers, prevented any clear interpretation of the other NOE observed. To circumvent the problem associated with the amide bond rotation, **3a** and **3b** were respectively converted into the bicyclic compounds **4a** (73%) and **4b** (80%) by Boc removal and triethylamine treatment of the resulting free amino compound.[7] In both cases, a single constrained product was formed. For both compounds **4a** and **4b**, no NOE was observed between protons H-2 and H-4. Thus, the determination of the stereochemistry of **4a** and **4b** was based on the analysis of the heteronuclear ^{13}C–^1H couplings and NOE involving the methyl groups. In the ^{13}C spectrum of compound **4b** recorded without proton decoupling, the two non-equivalent methyl groups exhibited different multiplicities: quadruplet of quintuplet (qqt) for the deshielded signal (CH_3a), quadruplet of quadruplet for the shielded one (CH_3b). This indicated that the coupling constant $^3J_{H\text{-}4,CH_3b}$ is close to 0 Hz showing the *anti* relationship between H-4 and CH_3b (torsion angle H-4–C-4–C-5–CH_3b close to 90° according to Karplus curves).[8] Furthermore, a NOE was observed between CH_3a and H-2 revealing their *syn* relationship. From these data, the *syn* relationship between H-2 and H-4 was assigned for compound **4b** and by deduction for compound **3b**. In contrast, the stereospecific assignment of the methyl groups of compound **4a** could not be deduced from the heteronuclear ^{13}C–^1H couplings since the two ^{13}C methyl groups signals exhibited the same multiplicity (qqt). However, NOE experiments were in agreement with an *anti* relationship between H-2 and H-4 for bicycle **4a** (the methyl group which gave a strong NOE with H-4 gave none with H-2). Thus the *anti* stereochemistry was assigned to **3a**. Compounds **3a** and **3b** were separately reacted with thymine in the presence of potassium carbonate in dimethylformamide at room temperature to give in each case a single coupling compound in 60% yield. The last step toward the synthesis of the PNA units involved the saponification of the methyl ester function by treatment with lithium hydroxide in a mixture of water/dioxane (2:3). The *syn* unit **U3** was formed quantitatively.[9] In contrast, the saponification of the *anti* compound led to a mixture of two diastereomers having the expected mass and another side product which prevented the isolation of the *anti* compound in a sufficient scale to allow incorporation into a PNA.

The racemic **U4** unit was synthesized starting from homocysteine thiolactone chlorohydrate (Scheme 2). After ring opening in the presence of sodium hydroxide and condensation with *N*-Boc-glycine aldehyde **1**, the thiazane **5** was obtained as a single diastereomer (45% yield, two steps). This compound was treated successively with chloroacetyl chloride in the presence of BTSA and diazomethane to give the chloro derivative **6** as a single diastereomer (62%, two steps).[6,10] The analysis of the coupling constants in its ^1H NMR spectra showed that the thiazane ring adopts a twist conformation and the interpretation of the NOE experiments indicated the *syn* stereochemical relationship between



Scheme 2. (i) NaOH, H_2O; (ii) *N*-Boc-glycine aldehyde, EtOH, H_2O; (iii) BTSA, CH_2Cl_2, $ClCH_2COCl$, pyridine; (iv) CH_2N_2, Et_2O; (v) thymine, K_2CO_3, DMF; (vi) LiOH, dioxane/H_2O.

the C-2 and C-4 substituents. Thymine was alkylated by thiazane **6** and the resulting coupling compound was subjected to a saponification to give the *syn* unit **U4** in good yield.[11] The corresponding *anti* unit could not be obtained by this synthetic route.

The constrained units **U1** to **U4** were introduced in the center of a 9-mer homothymine aegPNA to give PNA **1** to **4** (Table 1). The reference PNA containing only aegT units (Fig. 1) was also synthesized. Lysine was introduced at the C-terminus of the PNAs to reduce their self-aggregation.[1] PNAs were assembled as described previously[4] on a 5 μmol scale applying with minor modifications the in situ neutralization protocol for Boc solid phase peptide chemistry.[12] Cleavage of the PNAs from the resin was performed by standard HF procedure and the PNAs were purified by reverse phase HPLC and characterized by MALDI-TOF mass spectroscopy.[13] Sulfur mono-oxidation of the constrained units was performed directly on the purified polymers (PNA **1** to **4**) by treatment with sodium periodate (10 equiv) in water at room temperature (48 h) to give PNA **1ox** to **4ox** (Table 1).[13] Although a single peak was always observed during preparative or analytical HPLC, the presence of the two sulfoxide epimers is not excluded.

It was observed that unlike the reference PNA the modified PNAs do not exhibit any tendency to self-aggregation during storage or manipulation. As previously reported for peptide sequences,[14] the presence of a thiazolidine motif in the PNA probably improves its solubility, as the thiazane also does. The PNAs were hybridized to the complementary DNA sequence dA_{10} or the mismatched sequence dA_4TA_4 and the thermal stabilities of the PNA/DNA hybrids were estimated by UV-melting experiments (Table 1).[15] A 2:1 ratio of PNA/DNA was used as homopyrimidine PNAs form PNA_2/DNA triplexes[2] and we observed previously that the incorporation of a single thiazolidine unit in the PNA does not prevent the triplex formation.[4] Well-defined heating (Fig. 2) and cooling curves were obtained for all the modified PNA/DNA hybrids indicating that the presence of the modified unit does not prevent the cooperative dissociation and association of the complexes. However, the thermal stabilities of the modified complexes were significantly reduced compared to that of the reference triplex PNA **ref**/dA_{10} (Table 1). The *anti* unit **U1** appeared to be better accommodated in the triplex than the different *syn* units which led to similar T_m values (17 °C reduction of the T_m with PNA **1**, 24–25 °C reduction with PNAs **2** to **4**). The thiazane ring of the synthetic intermediate **6** was shown by NMR studies to adopt a twist conformation. This unexpected result prompted us to perform molecular mechanic calculations on the *syn* racemic **U4** unit. The twist conformation was found to be the most stable leading to the (γ, δ) torsion angle sets ($-90° \pm 10$, $130° \pm 10$) and ($90° \pm 10$, $-130° \pm 10$). These angle values are close to those obtained with the *syn* thiazolidine units which could explain the similar T_m values observed for PNA **2** to **4**. The sulfur oxidation of the modified units did not improve the thermal stabilities of the hybrids. However, for PNA **1** and to a lesser extent PNA **2**, the hysteresis between the heating and cooling curves was reduced ($T_m - T_{as} = 2$ °C for PNA **1ox** instead of 9 °C for PNA **1** or 7.5 °C for PNA **ref**) suggesting that the triplex formation is accelerated. Finally, hybridization of the modified PNAs to the mismatched DNA sequence resulted in T_m reductions ranging from 9 to 15 °C (12 °C for PNA **ref**). As a conclusion, the presence of the modified units was shown to destabilize the PNA/DNA hybrids but maintain a base-pairing selectivity. Furthermore, it was observed that incorporation of the thiazolidine or thiazane units reduces PNA aggregation.

Table 1. Melting (T_m) and half re-association (T_{as}) temperatures of the PNA/DNA complexes[a]

PNA[b]	T_m PNA/dA_{10}	T_{as} PNA/dA_{10}	T_m PNA/dA_4TA_4
ref	66	58.5	54
1	49	40	39
1ox	48	46	39
2	42	33	27
2ox	41	36.5	27
3	41	25	29
3ox	39	22	25
4	42	29	30
4ox	41	31	31

[a]Temperatures are given in °C with estimated deviations of ±1 °C.
[b]PNAs sequences: PNA **ref** = H-$(aegT)_9$-Lys-NH2, PNA **n** = H-$(aegT)_4$-U**n**-$(aegT)_4$-Lys-NH_2, U**n** being one of the modified units presented in Figure 1. The corresponding oxidized PNAs are named PNA **nox**.

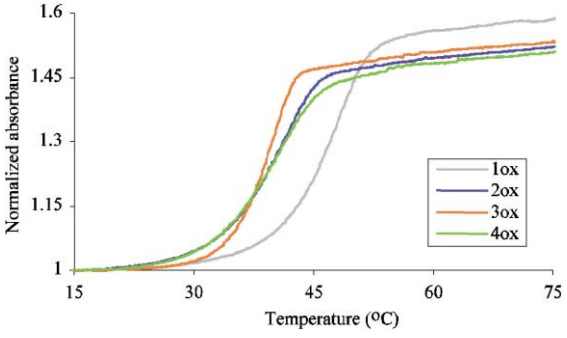


Figure 2. UV-melting curves of the PNA **ox**/DNA complexes.

References and Notes

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6. General procedure for the acylation reaction: To the carboxylic acid derivative suspended in dry CH_2Cl_2, BTSA (1 equiv) was added dropwise under argon. The reaction mixture was stirred at rt until complete dissolution then cooled to −78 °C, pyridine (1.5 equiv) and chloroacetyl chloride (1 equiv) were added successively. After 30 min, CH_2Cl_2 was added. The organic layer was washed twice with a 10% citric acid aqueous solution and brine, dried over $MgSO_4$ and concentrated. The resulting residue was dissolved in ether before careful addition of freshly prepared diazomethane. When the starting material was consumed, argon was bubbled through the solution in order to remove diazomethane excess. The reaction mixture was concentrated under vacuum and the residue was purified by flash chromatography on silica gel (EtOAc/cyclohexane).
7. Bicyclic compounds **4a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (dd, 1H, ^3J = 10.2, 4.0 Hz, H-2), 4.74 (s, 1H, H-4), 3.75 (s, 3H, OCH_3), 3.66, 3.49 (2d, 2H, ^2J = 18.2 Hz, NHCH_2CO), 3.43 (dd, 1H, ^2J = 13.4 Hz, ^3J = 4.0 Hz, NHCH*H*), (dd, 1H, ^2J = 13.4 Hz, ^3J = 10.2 Hz, NHC*H*H), 1.60, 1.45 (2s, 6H, C(CH_3)$_2$). ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 169.0, 169.0 (*C*OCH$_3$, N*C*OCH$_2$), 71.0 (C-4), 62.3 (C-2), 52.4 (OCH_3), 51.5 (*C*(CH$_3$)$_2$), 48.5 (NH*C*H$_2$CO), 48.4 (NHCH$_2$), 30.1, 26.6 (C(*C*H$_3$)$_2$). **4b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.02 (dd, 1H, ^3J = 10.1, 3.0 Hz, H-2), 4.35 (s, 1H, H-4), 3.72 (s, 3H, OCH_3), 3.61, 3.52 (2d, 2H, ^2J = 17.8 Hz, NHCH_2CO), 3.43 (dd, 1H, ^2J = 12.7 Hz, ^3J = 3.0 Hz, NHCH*H*), (dd, 1H, ^2J = 12.7 Hz, ^3J = 10.1 Hz, NHC*H*H), 1.59, 1.37 (2s, 6H, C(CH_3)$_2$). ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 169.4, 167.4 (*C*OCH$_3$, N*C*OCH$_2$), 71.8 (C-4), 62.0 (C-2), 52.2 (OCH_3), 51.0 (*C*(CH$_3$)$_2$), 48.1 (NH*C*H$_2$CO), 47.3 (NHCH$_2$), 31.5, 23.8 (C(*C*H$_3$)$_2$).
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9. *syn* PNA unit **U3**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.37, 11.31 (2s, 1H, NH T), 7.63, 7.33 (2m, 1H, N*H*Boc), 7.47, 7.39 (2s, 1H, CH T), 5.44 (dd, 0.5H, ^3J = 6.2, <1 Hz, H-2), 5.37 (m, 0.5H, H-2), 4.80, 4.71 (2d, 2H, ^2J = 16.4 Hz, T-CH_2), 4.65 (s, 0.5H, H-4), 4.58, 4.23 (2d, 2H, ^2J = 16.4 Hz, T-CH_2), 4.45 (s, 0.5H, H-4), 3.85–3.30 (m, 2H, NHCH_2), 1.76, 1.74 (2s, 3H, CH_3 T), 1.50–1.40 (4s, 6H, C(CH_3)$_2$), 1.38, 1.35 (2s, 9H, CH_3 Boc). ^{13}C NMR (100.61 MHz, DMSO-d_6) 170.6, 170.3, 166.4, 166.1, 164.4, 164.4, 156.0, 155.8, 151.0, 151.0 (CO T, *C*OOH, TCH$_2$*C*O, CO Boc), 142.2, 142.2 (CH T), 108.2, 108.0 (*C*CH$_3$ T), 78.2, 77.4 (*C*(CH$_3$)$_3$), 72.8, 72.0 (C-4), 63.3, 61.8 (C-2), 52.7, 51.4 (*C*(CH$_3$)$_2$) 49.0, 48.3 (T-CH$_2$), 46.7, 42.9 (NHCH$_2$), 32.7, 32.7, 25.7, 25.7 (C(*C*H$_3$)$_2$), 28.3, 28.2 (C(*C*H$_3$)$_3$), 11.9, 11.9 (CH_3 T).
10. Thiazane **6**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.70 (m, 1H, N*H*Boc), 5.25 (t, 1H, ^3J = 6.2 Hz, H-4), 4.84 (dd, 1H, ^3J = 9.8, 5.5 Hz, H-2), 4.29, 4.11 (2d, 2H, ^2J = 13.3 Hz, ClCH_2), 3.72 (s, 3H, CH_3O), 3.53, 3.19 (2m, 2H, NHCH_2), 2.81 (m, 1H, ^3J = 9.9, 3.3 Hz, ^2J = 13.5 Hz, H-6), 2.51 (m, 1H, ^2J = 13.5 Hz, H-6), 2.42 (m, 1H, H-5), 2.20 (m, 1H, H-5), 1.40 (s, 9H, CH_3 Boc). ^{13}C NMR (100.61 MHz, $CDCl_3$) *δ* 172.2 (*C*OOCH$_3$), 167.1 (ClCH$_2$*C*O), 155.9 (CO Boc), 79.9 (*C*(CH$_3$)$_3$), 53.4 (C-2), 52.8 (CH_3O), 51.3 (C-4), 45.7 (NH*C*H$_2$), 41.3 (Cl*C*H$_2$), 28.2 (C(*C*H$_3$)$_3$), 26.8 (C-5), 20.3 (C-6).
11. *syn* PNA unit **U4** (amide bond isomers, 1:1): ^{1}H NMR (500 MHz, CD_3OD) δ 7.33, 7.30 (2s, 1H, CH T), 5.68 (m, 0.5H, H-2), 5.32 (m, 0.5H, H-4), 5.00–4.55 (m, 3H, H-2, H-4, T-CH_2), 3.80–3.15 (m, 2H, NHCH_2), 3.12, 3.04 (2t, 1H, ^2J = ^3J = 12.5 Hz, ^2J = ^3J = 11.6 Hz, H-6), 2.75–2.47 (m, 2H, H-6, H-5), 2.23 (m, 1H, H-5), 1.91 (s, 3H, CH_3 T), 1.48, 1.43 (2s, 9H, CH_3 Boc). ^{13}C NMR (125.73 MHz, CD_3OD) δ 175.2, 175.0 (*C*OOH), 169.8, 169.5, 167.5, 158.7, 153.5 (CO), 144.1, 143.9 (CH T), 111.6, 111.5 (*C*CH$_3$ T), 81.4, 80.9 (*C*(CH$_3$)$_3$), 55.6, 54.9 (C-4), 52.8, 52.0 (C-2), 50.5, 50.4 (T-*C*H$_2$), 46.3, 44.1 (NH*C*H$_2$), 29.2 (C(*C*H$_3$)$_3$), 28.9, 28.4 (C-5), 21.5, 20.8 (C-6), 12.8, 12.7 (CH_3 T).
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13. PNA characterization by MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid as matrix. [MH]$^+$: PNA **ref** 2541.2 (calcd 2541.0); PNA **1** 2584.9, PNA **2** 2585.3 (calcd 2585.0); PNA **1ox** 2601.0, PNA **2ox** 2600.9 (calcd 2601.0); PNA **3** 2612.9 (calcd 2613.0); PNA **3ox** 2629.1 (calcd 2629.0); PNA **4** 2598.8 (calcd 2599.0); PNA **4ox** 2615.0 (calcd 2615.0).
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15. UV-melting experiments were performed in 10 mM sodium phosphate buffer (pH = 7), 100 mM NaCl with [PNA] = 4 μM and [DNA] = 2 μM. The mixture was heated for 5 min at 85 °C, slowly cooled and stored overnight at R.T and incubated 30 min at 15 °C before T_m measurements (heating and cooling rates = 0.5 °C/min).