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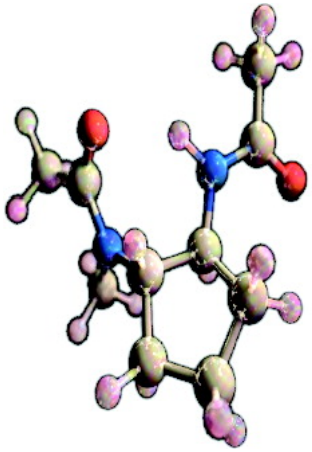

ΔT_m +5 °C
per PNA monomer

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(*S*,*S*)-*trans*-Cyclopentane-Constrained Peptide Nucleic Acids. A General Backbone Modification that Improves Binding Affinity and Sequence Specificity

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Abstract: Replacing the ethylenediamine portion of aminoethylglycine peptide nucleic acids (aegPNAs) with one or more (*S*,*S*)-*trans*-cyclopentane diamine units significantly increases binding affinity and sequence specificity to complementary DNA, making these modified PNAs ideal for use as nucleic acid probes in genomic analysis. The synthesis and study of this new class of PNAs (*t*cypPNAs) is described in which *trans*-cyclopentane diamine has been incorporated into several positions, and in varying number, within PNA backbones of mixed-base sequences.

Introduction

In this paper, we report that incorporating one or more (*S*,*S*)-*trans*-cyclopentane diamine units into aminoethylglycine peptide nucleic acids (aegPNAs)[1] significantly increases binding affinity and sequence specificity to complementary DNA. With the completion of the human genome sequence, and the sequences of several other organisms, genomic analysis can provide key diagnostic information about diseases and pathogens, as well as impact biochemical and biomedical research.[2] Central to the success of such studies is the availability of reliable diagnostic techniques to signal the presence or absence of specific DNA sequences. Many recent technological advances have shown that synthetic DNA oligomers can be coupled to highly sensitive assays so that the binding of a complementary DNA sequence can be detected.[3] These devices are typically rated according to their ability to detect low levels of DNA, discriminate between very similar sequences of DNA (i.e., identify single nucleotide polymorphisms (SNPs)), and provide useful information in a reasonable time frame. In particular, DNA microarrays are now routinely used to quantify gene expression and identify SNPs in a wide variety of biological studies;[4] however, limitations of the technology still exist.[5]

Replacing DNA probes with PNA has been shown to improve DNA-detection technology.[6] PNAs bind to complementary DNA sequences with significantly higher affinity than the corresponding DNA sequences,[7] which improves sensitivity. PNA is also significantly more stable on surfaces than DNA, yielding detection devices with longer shelf lives.[8] Most importantly, PNA-based probes can bind to complementary DNA at low salt concentrations in solution.[9] To form DNA−DNA duplexes, a high concentration of positively charged counterions is required to neutralize the charge−charge repulsion associated with bringing together two anionic backbones.[10] In addition, high salt concentration can promote formation of structures in single-stranded DNA via intramolecular hydrogen bonding, and promote formation of tertiary structures.[10d] Since PNAs consist of a neutral polyamide backbone, counterions are not required for the formation of PNA−DNA duplexes. By destabilizing DNA structures under low salt concentrations, one can create conditions that favor formation of DNA−PNA duplexes over DNA−DNA duplexes and tertiary structures. This arrangement should give PNA-based detection probes a distinct advantage over DNA probes because the chance for false negatives should be reduced. Despite the benefits that PNA can offer to DNA diagnostics, the use of PNA probes lags far behind that of DNA probes, most likely because the most commonly employed PNAs (derived from an aminoethylglycine backbone) do not have significantly improved properties over DNA to warrant the effort or funds necessary to purchase, license, or synthesize these oligomers.[11]

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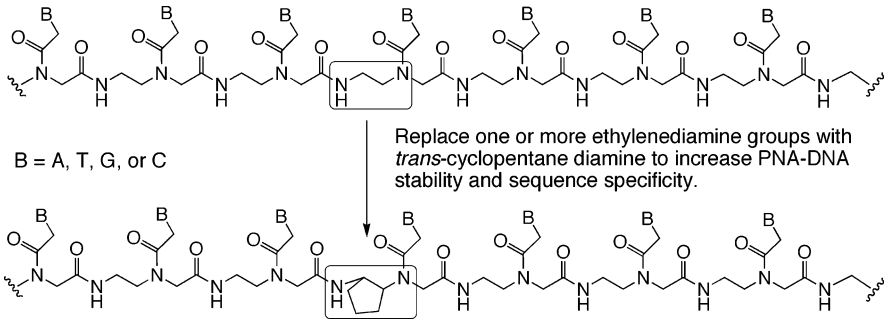

Figure 1. General strategy to insert *trans*-cyclopentane into the aegPNA backbone.

In this paper, we describe the synthesis and study of a new class of *trans*-cyclopentane-derived PNAs (*t*cypPNAs) in which *trans*-cyclopentane diamine has been incorporated into several positions, and in varying number, within PNA backbones of mixed-base sequences (Figure 1). Our initial publication on this research described a first-generation synthesis and initial binding studies of these modified PNAs.[12] We now report a more efficient, second-generation synthesis of *t*cypPNAs, and present binding data demonstrating the improvements that *trans*-cyclopentane incorporation conveys to PNAs. Compared to unmodified PNA, *t*cypPNA displays improved binding affinity (i.e., higher T_m's) and sequence specificity to complementary DNA, indicating that these modified PNAs possess favorable properties for use in DNA diagnostics. Furthermore, introduction of cyclopentanes into the PNA backbone affords PNAs with general and additive improvements in binding affinity to complementary DNA. While several other PNA backbone modifications have been explored, most lead to reduction in the stability of a PNA−DNA duplex.[13] Among the few modified PNAs that afford higher PNA−DNA stability, most are limited for one or more of the following reasons: the modified residues are synthetically challenging, the modification does not increase the stability of the duplex in a general manner, or cationic groups must be employed in order to promote binding affinity (which could ultimately lead to nonspecific binding to other DNA sequences or anionic biomolecules).[14,15] While our work was in progress, Kumar and Ganesh reported that *cis*-cyclopentane in oligothymine PNAs greatly stabilizes PNA_2DNA triplexes.[14e,i] The *trans*-cyclopentane modification described in our paper increases the stability and sequence specificity of PNA−DNA duplexes composed of mixed-base sequences, and provides a new class of probes that can be coupled to analytical devices for DNA detection.

Experimental Section

General. Melting points (mp) were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations ($[\alpha]_D$) were measured on a Perkin-Elmer 241 polarimeter using sodium light (D line, 589.3 nm) and are reported in degrees; concentrations (*c*) are reported as g/100 mL. Infrared spectra (IR) were obtained on a Bio-Rad FTS60 FTIR. IR spectra were collected either as a thin film on a KBr disk or by preparing a pressed KBr pellet containing the title compound. Proton nuclear magnetic resonances (^{1}H NMR) were recorded in deuterated solvents on an iNOVA 500 (500 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane (δ 0.00). If tetramethylsilane was not present, the residual protio solvent is referenced ($CDCl_3$, δ 7.27; dimethyl sulfoxide-d_6 (DMSO), δ 2.50). ^{1}H NMR splitting patterns are designated as singlet (s), doublet (d), or quartet (q). Splitting patterns that could not be interpreted or easily visualized were designated as multiplet (m) or broad (br). Coupling constants are reported in hertz (Hz). Proton-decoupled (^{13}C NMR) spectra were obtained on an iNOVA 500 (125 MHz) spectrometer and are reported in ppm using the solvent as an internal standard ($CDCl_3$, δ 77.23; DMSO, δ 39.52). Low-resolution mass spectra (LRMS) were obtained using a Micromass Quattro II triple quadrupole HPLC/MS/MS mass spectrometer. High-resolution mass spectra (HRMS) were obtained on a Micromass Q-Tof Ultima at the University of Illinois at Urbana−Champaign Mass Spectrometry Center. Elemental analysis data were collected by Atlantic Microlab, Inc. Analytical thin-layer chromatography (TLC) was carried out on Sorbent Technologies TLC plates precoated with silica gel (250 μm layer thickness). Flash column chromatography was performed on EM Science silica gel 60 (230−400 mesh). Solvent mixtures used for TLC and flash column chromatography are reported in v/v ratios. Unless otherwise noted, all commercially available reagents and solvents were purchased from Aldrich and used without further purification. Tetrahydrofuran (THF) was distilled from sodium and benzophenone prior to use. Dimethylformamide (DMF) was purified by passage through a bed of activated alumina.[16] 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), HATU, and Boc-Lys-(2-Cl-Z)-OH were purchased from Advanced ChemTech. All aegPNA monomers were purchased from Applied Biosystems. Kaiser test solutions were purchased from Fluka.

Abbreviations: RBF, round-bottom flask; rt, room temperature; DMAP, dimethylaminopyridine; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; NMP, 1-methyl-2-pyrrolidinone; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; MDCHA, *N*-methyldicyclohexylamine; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; Ac_2O, acetic anhydride; TFMSA, trifluoromethanesulfonic acid; TFA, trifluoroacetic acid.

(1*S*,2*S*)-2-(*tert*-Butoxycarbonylamino)cyclopentyl Isocyanate (2). β-Amino acid **1**[17] (2.3 g, 10.1 mmol) was added to an oven-dried 250 mL RBF under N_2(g) atmosphere and dissolved in dry THF (50 mL) with stirring. The solution was cooled to 0 °C, and triethylamine (2.8 mL, 20.2 mmol, 2.0 equiv) and ethyl chloroformate (1.9 mL, 20.2

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mmol, 2.0 equiv) were added dropwise via syringe, forming a white precipitate. The heterogeneous mixture was stirred at 0 °C for 20 min. Sodium azide (2.0 g, 30.3 mmol, 3.0 equiv) was dissolved in H_2O (35 mL) and added to the reaction mixture. The biphasic mixture was stirred vigorously at 0 °C for 20 min. The ice bath was removed, and the mixture was allowed to warm to room temperature for 10 min. The mixture was diluted with H_2O (30 mL) and was extracted with ethyl acetate (3 × 30 mL). The combined organic phases were dried over Na_2SO_4 and concentrated (*not to dryness*!).[18] The residue was taken up in benzene (50 mL) and refluxed for 30 min. The solution was cooled, concentrated, and dried under vacuum to yield 2.15 g (94%) of **2** as a colorless, crystalline solid: mp = 73−75 °C; IR (KBr) 3700, 3379, 2981, 2279 (N═C═O stretch), 1687, 1517, 1458, 1366, 1345, 1314, 1290, 1250, 1164, 1047, 909, 780, 580 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.48 (br s, 1H, Boc−N*H*), 3.85 (br s, 1H, O═C═N−C*H*), 3.71 (br s, 1H, Boc−NH−C*H*), 2.19−2.11 (m, 1H, cyclopentane−*H*), 2.04−1.97 (m, 1H, cyclopentane−*H*), 1.81−1.65 (m, 3H, cyclopentane−*H*), 1.46 (s, 10H, *tert*-butyl−CH_3 + cyclopentane−*H*).

***tert*-Butyl (1*S*,2*S*)-2-(Benzyloxycarbonylamino)cyclopentylcarbamate (3).**[19] Copper(I) chloride (0.9 g, 9.5 mmol) was added to an oven-dried 100 mL RBF under N_2(g) atmosphere and suspended in dry DMF (35 mL) with stirring. Benzyl alcohol (0.9 mL, 9.5 mmol) was added dropwise via syringe and allowed to stir until a bright yellow-green suspension had formed (approximately 5 min). At this point, **2** (2.1 g, 9.5 mmol) was added and stirred at room temperature for 45 min. The dark green suspension was poured into H_2O (70 mL) and extracted with ethyl acetate (150 mL). The organic phase was washed with saturated aqueous NaCl (2 × 50 mL), dried over Na_2SO_4, concentrated, and dried under vacuum to yield 2.83 g (89%) of **3** as a colorless solid: R_f = 0.38 (2% MeOH/ CH_2Cl_2); mp = 150−151 °C; $[\alpha]^{23}_D$ = −8.0° (*c* = 1.0, EtOH, 100%); IR (film) 3341, 2971, 1680, 1528, 1304, 1270, 1233, 1169, 1041, 779, 741, 696, 639 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.34 (m, 5H, Ph−*H*), 5.30 (br s, 1H, Cbz−N*H*), 5.09 (s, 2H, Ph−CH_2), 4.81 (br s, 1H, Boc−N*H*), 3.68 (m, 2H, carbamate−NH−C*H*), 2.17−2.09 (m, 2H, cyclopentane−*H*), 1.70 (m, 2H, cyclopentane−*H*), 1.43 (s, 11H, *tert*-butyl−CH_3 + 2 cyclopentane−*H*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.9, 156.5, 136.7, 128.5, 128.0, 79.5, 66.6, 58.4, 57.2, 30.1, 29.8, 28.4, 19.7; LRMS (ESI-MS *m*/*z*): mass calcd for $C_{18}H_{27}N_2O_4$ $[M + H]^+$, 335.20, found 335.3. Anal. Calcd for $C_{18}H_{26}N_2O_4$: C, 64.65; H, 7.84; N, 8.38. Found: C, 64.45; H, 7.86; N, 8.29.

***tert*-Butyl (1*R*,2*R*)-2-(Benzyloxycarbonylamino)cyclopentylcarbamate.** Prepared via identical procedures used to prepare **3**, except with (*R*)-α-methylbenzylamine. All characterization data matched **3** except $[\alpha]^{23}_D$ = +8.0° (*c* = 1.0, EtOH, 100%).

***tert*-Butyl (1*S*,2*S*)-2-((Methoxycarbonyl)methylamino)cyclopentylcarbamate (4).** Compound **3** (1.5 g, 7.6 mmol) was dissolved in methanol (150 mL) and added to an oven-dried Parr flask containing 10% Pd/C (0.5 g). The flask was placed on a Parr apparatus under 55 psi of H_2(g) pressure, and was shaken for 12 h. TLC analysis revealed no starting material [R_f = 0.38 (2% MeOH/CH_2Cl_2)]. The suspension was filtered through Celite and concentrated. The residue was dissolved in dry DMF with stirring. Triethylamine (1.1 mL, 7.6 mmol) and methyl bromoacetate (0.6 mL, 6.8 mmol, 0.9 equiv) were added dropwise via syringe. The reaction was allowed to stir at room temperature for 3 h. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ (50 mL) and extracted with ethyl acetate (2 × 35 mL). The combined organic phases were washed with saturated aqueous NaCl (2 × 25 mL), dried over Na_2SO_4, concentrated, and dried under vacuum. The residue was purified by flash column chromatography [R_f = 0.30 (EtOAc)] to yield 1.26 g (67%) of **4** as a colorless oil, which formed a colorless, crystalline solid upon sitting at room temperature: R_f = 0.30 (EtOAc); mp = 49−52 °C; $[\alpha]^{23}_D$ = +5.0° (*c* = 1.0, EtOH, 100%); IR (film) 3343, 2960, 2871, 1741, 1703, 1522, 1438, 1392, 1367, 1244, 1207, 1173, 1044, 1021, 996, 778 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.56 (br s, 1H, Boc−N*H*), 3.73 (s, 3H, CO_2CH_3), 3.67 (br m, 1H, Boc−NH−C*H*), 3.48 (q, *J* = 13.5 Hz, 2H, NH−CH_2−CO_2Me), 2.85 (m, 1H, C*H*−NH), 2.13 (m, 1H, cyclopentane−*H*), 1.93 (m, 2H, cyclopentane−*H*), 1.77−1.68 (m, 1H, cyclopentane−*H*), 1.67−1.59 (m, 1H, cyclopentane−*H*), 1.45 (s, 9H, *tert*-butyl−CH_3), 1.42−1.34 (m, 1H, cyclopentane−*H*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.8, 155.5, 78.6, 64.7, 57.2, 51.4, 48.8, 31.1, 30.8, 28.1, 21.2; LRMS (ESI-MS *m*/*z*) mass calcd for $C_{13}H_{25}N_2O_4$ $[M + H]^+$, 273.18, found 273.2. Anal. Calcd for $C_{13}H_{24}N_2O_4$: C, 57.33; H, 8.88; N, 10.29. Found: C, 57.20; H, 8.96; N, 10.22.

General Procedure A for Coupling Bases to *t*cypPNA Backbone. Compound **4** (0.9 g, 3.5 mmol) was added to an oven-dried 50 mL RBF and dissolved in dry DMF (15 mL) with stirring. The solution was cooled to 0 °C. Nucleobase acetic acid (5.25 mmol, 1.5 equiv) and DMAP (100 mg, 0.9 mmol) were added. EDC (1.3 g, 7.0 mmol, 2.0 equiv) was added, and the reaction mixture was allowed to stir at 0 °C for 10 min. The ice bath was removed, and the reaction was stirred for 36 h at room temperature. The solution was diluted with H_2O (90 mL) and extracted with ethyl acetate (3 × 60 mL). The combined organic phases were washed with saturated aqueous NaCl (4 × 75 mL), dried over Na_2SO_4, concentrated, and dried under vacuum to yield base-coupled monomer esters as colorless solids.

Methyl *N*-[(2*S*)-*tert*-Butoxycarbonylaminocyclopent-(1*S*)-yl]-*N*-[thymin-1-ylacetyl]glycinate (5). Following general procedure A, *t*cypPNA backbone **4** (0.95 g, 3.5 mmol) was coupled to thymine acetic acid (0.96 g, 5.25 mmol) to yield 1.33 g (87%) of ester **5**. If necessary, the solid was purified by flash column chromatography: R_f = 0.38 (5% MeOH/CH_2Cl_2); ^1H NMR (500 MHz, DMSO) δ major rotamer 11.29 (s, 1H, imide−N*H*), 7.17 (s, 1H, thymine−*H*), 6.96 (d, *J* = 8.0 Hz, 1H, Boc−N*H*), 4.72 (q, *J* = 16.5 Hz, 2H, thymine−CH_2), 4.0−3.75 (m, 4H, NH−CH_2−CO_2Me + C*H*−NH), 3.59 (s, 3H, CO_2CH_3), 1.9−1.4 (m, 6H, cyclopentane−*H*), 1.75 (s, 3H, thymine−CH_3), 1.36 (s, 9H, *tert*-butyl−CH_3), minor rotamer 7.20 (s, 1H, thymine−*H*), 6.75 (d, *J* = 8.0 Hz, 1H, Boc−N*H*), 4.45 (q, *J* = 16.5 Hz, 2H, thymine−CH_2), 3.70 (s, 3H, CO_2CH_3); ^{13}C NMR (125 MHz, DMSO) δ 169.6, 167.3, 164.4, 155.4, 151.0, 141.7, 108.3, 77.9, 61.1, 52.7, 51.6, 47.9, 43.6, 28.2, 25.9, 18.8, 12.0; LRMS (ESI-MS *m*/*z*) mass calcd for $C_{20}H_{31}N_4O_7$ $[M + H]^+$, 439.22, found 439.2.

Methyl *N*-[(2*S*)-*tert*-Butoxycarbonylaminocyclopent-(1*S*)-yl]-*N*-[4-*N*-(benzyloxycarbonyl)cytosin-1-ylacetyl]glycinate (6). Following general proedure A, *t*cypPNA backbone **4** (0.84 g, 2.94 mmol) was coupled to Cbz-cytosine acetic acid[20] (1.42 g, 4.70 mmol) to yield 0.88 g (52%) of ester **6**. If necessary, the solid was purified by flash column chromatography: R_f = 0.21 (1% MeOH/EtOAc); ^1H NMR (500 MHz, DMSO) δ major rotamer 10.77 (s, 1H, Cbz−N*H*), 7.78 (d, *J* = 7.0 Hz, 1H, cytosine−*H*), 7.41 (m, 5H, Ph−*H*), 7.01 (d, *J* = 7.0 Hz, 1H, cytosine−*H*), 6.95 (d, *J* = 8.5 Hz, 1H, Boc−N*H*), 5.19 (s, 2H, Ph−CH_2), 4.90 (q, *J* = 16.0 Hz, 2H, cytosine−CH_2), 4.35−3.75 (m, 4H, NH−CH_2−CO_2Me + C*H*−NH), 3.58 (s, 3H, CO_2CH_3), 1.90−1.40 (m, 6H, cyclopentane−*H*), 1.36 (s, 9H, *tert*-butyl−CH_3), minor rotamer 7.84 (d, *J* = 7.0 Hz, 1H, cytosine−*H*), 6.73 (d, *J* = 8.5 Hz, 1H, Boc−N*H*), 4.61 (q, *J* = 16.0 Hz, 2H, cytosine−CH_2), 3.70 (s, 3H, CO_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.5, 167.3, 163.0, 155.4, 155.0, 153.2, 150.5, 136.0, 128.5, 128.2, 127.9, 93.9, 77.9, 66.5, 61.1, 52.7, 51.6, 49.5, 43.7, 28.2, 26.0, 18.8; LRMS (ESI-MS *m*/*z*) mass calcd for $C_{27}H_{36}N_5O_8$ $[M + H]^+$, 558.26, found 558.1.

Methyl *N*-[(2*S*)-*tert*-Butoxycarbonylaminocyclopent-(1*S*)-yl]-*N*-[6-*N*-(benzyloxycarbonyl)adenin-9-ylacetyl]glycinate (7). Following general procedure A, *t*cypPNA backbone **4** (0.20 g, 0.70 mmol) was

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coupled to Cbz-adenine acetic acid[20] (0.34 g, 1.05 mmol) to yield 0.26 g (63%) of ester **7**. If necessary, the solid was purified by flash column chromatography: $R_f = 0.26$ (5% MeOH/CH_2Cl_2); ^{1}H NMR (500 MHz, DMSO) δ major rotamer 10.68 (s, 1H, Cbz−N*H*), 8.59 (s, 1H, adenine−*H*), 8.25 (s, 1H, adenine−*H*), 7.46 (d, $J = 7.5$ Hz, 2H, *o*-Ph−*H*), 7.40 (t, $J = 7.5$ Hz, 2H, *m*-Ph−*H*), 7.34 (t, $J = 7.5$ Hz, 1H, *p*-Ph−*H*), 7.08 (br d, $J = 8.5$ Hz, 1H, Boc−N*H*), 5.41 (q, $J = 17.0$ Hz, 2H, adenine−CH_2), 5.22 (s, 2H, Ph−CH_2), 4.43−3.81 (m, 4H, NH−CH_2−CO_2Me + C*H*−NH), 3.56 (s, 3H, CO_2CH_3), 1.97−1.46 (m, 6H, cyclopentane−*H*), 1.39 (s, 9H, *tert*-butyl−CH_3), minor rotamer 8.29 (s, 1H, adenine−*H*), 6.75 (d, $J = 8.5$ Hz, 1H, Boc−N*H*), 3.76 (s, 3H, CO_2CH_3), 1.36 (s, 9H, *tert*-butyl−CH_3); ^{13}C NMR (125 MHz, DMSO) δ 169.5, 166.6, 155.4, 152.4, 151.3, 149.1, 145.2, 136.3, 128.4, 128.0, 127.9, 122.0, 78.0, 66.5, 61.3, 52.8, 51.6, 44.3, 43.7, 28.2, 25.9, 18.8; LRMS (ESI-MS *m*/*z*) mass calcd for $C_{28}H_{36}N_7O_7$ $[M + H]^+$, 582.27, found 582.1.

General Procedure B for Saponification of Methyl Esters. A *t*cypPNA ester (0.9 g, 2.1 mmol) was dissolved in THF (33 mL) and cooled to 0 °C. Lithium hydroxide monohydrate (1.2 g, 28.4 mmol) was dissolved in H_2O (28 mL), and the solution was added to the reaction mixture over 5 min. The solution was allowed to warm to room temperature and stirred for 5 h. The mixture was diluted with H_2O (45 mL) and extracted with ethyl ether (3 × 50 mL). The aqueous layer was acidified with aqueous 3 N HCl to pH 1. The solution was extracted with ethyl acetate (5 × 70 mL). The combined organic phases were dried over Na_2SO_4, concentrated, and dried under vacuum to yield PNA monomers as colorless solids.

***N*-[(2*S*)-*tert*-Butoxycarbonylaminocyclopent-(1*S*)-yl]-*N*-[thymin-1-ylacetyl]glycine (8).** Following general procedure B, **5** (0.93 g, 2.12 mmol) was converted to 0.87 g (96%) of PNA monomer **8**: mp = 203 °C (dec); $[\alpha]^{23}_D = -36.5°$ ($c = 1.0$, MeOH, 100%); IR (KBr) 3351, 3178, 2976, 2611, 2531, 1682, 1530, 1455, 1365, 1244, 1167, 1045, 853, 782, 467 cm^{-1}; ^{1}H NMR (500 MHz, DMSO) δ major rotamer 12.41 (br s, 1H, CO_2H), 11.28 (s, 1H, imide−N*H*), 7.15 (s, 1H, thymine−*H*), 6.94 (d, $J = 8.0$ Hz, 1H, Boc−N*H*), 4.71 (q, $J = 17.0$ Hz, 2H, thymine−CH_2), 3.9−3.7 (m, 4H, NH−CH_2−CO_2Me + C*H*−NH), 1.9−1.4 (m, 6H, cyclopentane−*H*), 1.75 (s, 3H, thymine−CH_3), 1.36 (s, 9H, *tert*-butyl−CH_3), minor rotamer 6.75 (d, $J = 8.0$ Hz, 1H, Boc−N*H*), 4.44 (q, $J = 17.0$ Hz, 2H, thymine−CH_2); ^{13}C NMR (125 MHz, DMSO) δ 177.5, 167.1, 164.4, 155.4, 151.0, 141.8, 108.2, 77.9, 61.2, 52.7, 47.9, 43.8, 28.2, 26.0, 18.9, 12.0; HRMS (ESI-MS *m*/*z*) mass calcd for $C_{29}H_{29}N_4O_7$ $[M + H]^+$, 425.2036, found 425.2043.

***N*-[(2*R*)-*tert*-Butoxycarbonylaminocyclopent-(1*R*)-yl]-*N*-[thymin-1-ylacetyl]glycine.** Prepared through identical procedures used to prepare **8** utilizing (*R*,*R*)-diamine. All characterization data matched **8** except $[\alpha]^{23}_D = +36.5°$ ($c = 1.0$, MeOH, 100%).

***N*-[(2*S*)-*tert*-Butoxycarbonylaminocyclopent-(1*S*)-yl]-*N*-[4-*N*-(benzyloxycarbonyl)cytosin-1-ylacetyl]glycine (9).** Following general procedure B, **6** (0.88 g, 1.54 mmol) was converted to 0.67 g (80%) of PNA monomer **9**: mp = 155 °C (dec); $[\alpha]^{23}_D = -35.0°$ ($c = 1.0$, MeOH, 100%); IR (KBr) 3359, 3326, 2972, 1750, 1668, 1497, 1455, 1367, 1215, 1064, 1045, 1002, 783, 745, 695 cm^{-1}; ^{1}H NMR (500 MHz, DMSO) δ major rotamer 12.35 (br s, 1H, CO_2H), 10.78 (br s, 1H, Cbz−N*H*), 7.76 (d, $J = 7.0$ Hz, 1H, cytosine−*H*), 7.41 (m, 5H, Ph−*H*), 7.01 (d, $J = 7.0$ Hz, 1H, cytosine−*H*), 6.94 (d, $J = 7.5$ Hz, 1H, Boc−N*H*), 5.19 (s, 2H, Ph−CH_2), 4.89 (q, $J = 16.0$ Hz, 2H, cytosine−CH_2), 4.3−3.7 (m, 4H, NH−CH_2−CO_2H + C*H*−NH), 1.9−1.4 (m, 6H, cyclopentane−*H*), 1.36 (s, 9H, *tert*-butyl−CH_3), minor rotamer 7.80 (d, $J = 7.0$ Hz, 1H, cytosine−*H*), 6.74 (d, $J = 7.5$ Hz, 1H, Boc−N*H*), 4.60 (q, $J = 16.0$ Hz, 2H, cytosine−CH_2); ^{13}C NMR (125 MHz, DMSO) δ 171.2, 170.5, 167.0, 162.9, 155.4, 154.7, 153.2, 150.7, 136.0, 128.5, 128.2, 128.0, 93.9, 77.9, 66.6, 61.1, 52.8, 49.6, 43.8, 28.2, 26.1, 18.9; HRMS (ESI-MS *m*/*z*) mass calcd for $C_{26}H_{34}N_5O_8$ $[M + H]^+$, 544.2407, found 544.2405.

***N*-[(2*S*)-*tert*-Butoxycarbonylaminocyclopent-(1*S*)-yl]-*N*-[6-*N*-(benzyloxycarbonyl)adenin-9-ylacetyl]glycine (10).** Following general procedure B, **7** (0.20 g, 0.34 mmol) was converted to 0.19 g (95%) of PNA monomer **10**: mp = 135 °C (dec); $[\alpha]^{23}_D = -30.5°$ ($c = 1.0$, MeOH, 100%); IR (KBr) 3250, 3196, 3124, 2977, 1750, 1707, 1669, 1615, 1521, 1456, 1214, 1159, 996, 697, 640 cm^{-1}; ^{1}H NMR (500 MHz, DMSO) δ major rotamer 10.69 (s, 1H, Cbz−N*H*), 8.59 (s, 1H, adenine−*H*), 8.23 (s, 1H, adenine−*H*), 7.46 (d, $J = 7.5$ Hz, 2H, *o*-Ph−*H*), 7.40 (t, $J = 7.5$ Hz, 2H, *m*-Ph−*H*), 7.34 (t, $J = 7.5$ Hz, 1H, *p*-Ph−*H*), 7.07 (br d, $J = 8.5$ Hz, 1H, Boc−N*H*), 5.40 (q, $J = 17.0$ Hz, 2H, adenine−CH_2), 5.22 (s, 2H, Ph−CH_2), 4.40−3.78 (m, 4H, NH−CH_2−CO_2Me + C*H*−NH), 2.00−1.40 (m, 6H, cyclopentane−*H*), 1.39 (s, 9H, *tert*-butyl−CH_3), minor rotamer 6.76 (br d, $J = 8.5$ Hz, 1H, Boc−N*H*), 1.36 (s, 9H, *tert*-butyl−CH_3); ^{13}C NMR (125 MHz, DMSO) δ 170.5, 166.5, 155.5, 152.5, 152.3, 151.4, 145.1, 136.4, 128.5, 128.0, 127.9, 123.0, 78.0, 66.3, 61.4, 52.8, 44.1, 43.8, 28.2, 25.9, 18.8; HRMS (ESI-MS *m*/*z*) mass calcd for $C_{27}H_{34}N_7O_7$ $[M + H]^+$, 568.2520, found 568.2501.

Table 1. Mass Characterization Data for All PNAs

entry[a]	sequence	calculated	observed[b]
	Nielsen Sequence		
1	GTAGATCACT-Lys	2855.2	2855.6
2	GTAGA**T***CACT-Lys	2895.2	2895.9
3	GTAGATC**A***CT-Lys	2895.2	2895.9
4	GTAGAT**C***ACT-Lys	2895.2	2895.8
5	GTAGA**T*C***ACT-Lys	2935.3	2934.7
6	G**T***AGA**T***CACT-Lys	2935.3	2934.5
7	G**T***AGA**T***C**A***CT-Lys	2975.3	2976.6
8	GTAG**A*T*C***ACT-Lys	2975.3	2971.1
9	G**T***AG**A*T*C**A***CT-Lys	3015.3	3016.5
10	GTAGA**T*^{R}**CACT-Lys[c]	2895.2	2895.6
	p53		
11	GGCAGTGCCT-Lys	2872.2	2873.5
12	GGCAG**T***GCCT-Lys	2912.2	2913.3

[a] Cyclopentane stereochemistry is (*S*,*S*), unless indicated otherwise. **B*** = *t*cyp residue. [b] PNAs were characterized using a PerSeptive Biosystems Voyager DE MALDI-TOF system with 2′,4′,6′-trihydroxyacetophenone monohydrate matrix. Mass spectra were acquired using a N_2 laser (337 nm wavelength, 5 ns pulse), with at least 100 shots per sample. All PNA oligomers gave molecular ions consistent with the final product. [c] **B*^{R}** = *t*cyp with (*R*,*R*) stereochemistry.

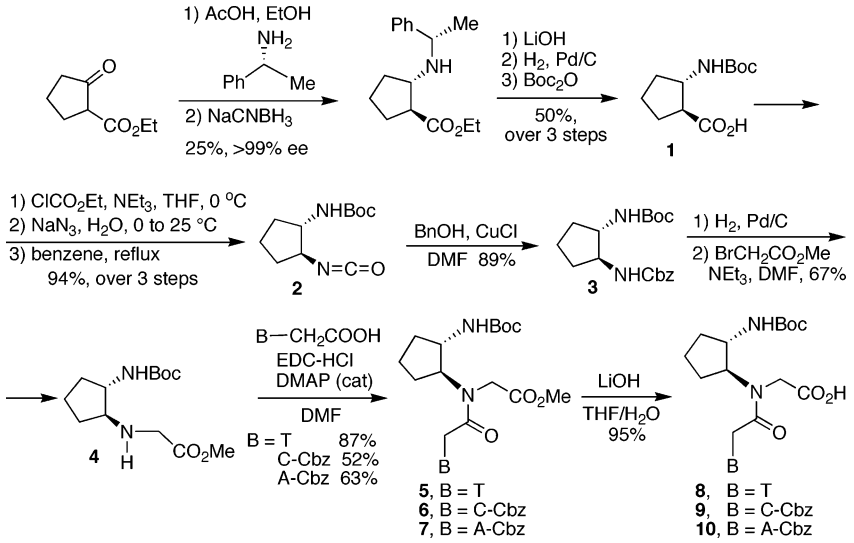
Synthesis of Peptide Nucleic Acids (PNAs). See Supporting Information for detailed procedures. All PNAs were purified by reversed-phase HPLC, and were characterized by MALDI-TOF mass spectrometry as shown in Table 1.

Results

The synthesis of *t*cypPNAs requires access to significant quantities of *trans*-cyclopentane diamine. Our initial, published synthesis was employed so that cycloalkane rings ranging from cyclopropane to cyclopentane could be incorporated into the PNA backbone.[12] Since then, cyclopentane has emerged as the optimal ring size. Therefore, we have focused our synthetic efforts to exclusively make cyclopentane PNA monomers. A central limitation of the previous route was the low-yielding reactions that were required to differentiate between the two identical nitrogens of *trans*-1,2-cyclopentane diamine. A more efficient synthesis yields the same diamine, but with the two nitrogens introduced in a stepwise fashion onto the five-membered ring so that orthogonal protecting groups can be placed on the nitrogens.

Such a route was developed on the basis of recent progress in cyclopentane β-amino acid synthesis developed by Gellman and co-workers (Scheme 1).[17] Starting from ethyl 2-oxocyclopentanecarboxylate, conversion to the Boc-protected β-amino acid **1** was accomplished via reductive amination, recrystallization to a single diastereomer, and deprotection/reprotection

Scheme 1



following the published procedures. From **1**, a Curtius rearrangement, followed by trapping the intermediate isocyanate **2** with benzyl alcohol, in the presence of stoichiometric CuCl as a Lewis acid activator,[19] afforded cyclopentane diamine **3**, in which the two nitrogens are conveniently protected with orthogonal protecting groups. Deprotection of the Cbz-protected nitrogen, followed by alkylation with a bromoacetate ester, afforded **4**, the essential backbone to make *t*cypPNA monomers. From this intermediate, all four DNA bases were attached using established procedures for the synthesis of PNA monomers. In this paper, we report the synthesis and complete characterization of cyclopentane PNA monomers with thymine, adenine, and cytosine. Therefore, esters **5**, **6**, and **7** were synthesized, and ester hydrolysis afforded *t*cypPNA monomers **8**, **9**, and **10** that were used directly in solid-phase peptide synthesis. The synthesis of the corresponding *t*cypPNA monomer with guanine was particularly challenging, and involved new chemistry that will be the subject of a future publication.

Using modified manual solid-phase peptide synthesis procedures,[21] 12 different PNAs were synthesized to probe the effects of cyclopentane incorporation into PNA. In general, cyclopentane PNA monomers could be readily inserted into a PNA sequence one or multiple times without difficulty (see Supporting Information for details). After cleavage from the resin, each PNA was readily purified by reversed-phase HPLC and characterized by MALDI-TOF mass spectrometry. Even though addition of cyclopentanes into a PNA should increase the hydrophobicity of the PNA and could reduce aqueous solubility, all the synthesized *t*cypPNAs were completely soluble under the aqueous conditions used in this work (up to 300 μM).

The effects of *trans*-cyclopentane incorporation on PNA−DNA stability were studied within a 10-residue, mixed-base PNA sequence that has been extensively studied in the literature (by Nielsen and co-workers in particular).[7,22] Data from variable-temperature UV absorbance were used to determine the influence of *trans*-cyclopentane on binding affinity relative to unmodified PNA. Incorporation of one (*S*,*S*)-*trans*-cyclopentane into several different positions within this PNA sequence results in an increase in the melting temperature (T_m) when bound to complementary DNA (Table 2, entries 1−4). Importantly, this increase is consistent regardless of the sequence position or nucleobase composition of the cyclopentane-modified residue. Furthermore, the benefit to binding is additive when multiple cyclopentanes are incorporated (Table 2, entries 5−9, Figure 2). Based on the increasing T_m's, each cyclopentane in the PNA increases the T_m by an average value of +5 °C.

The stereochemistry of the cyclopentane ring is critical to attain improved stability in the PNA−DNA duplex. Increases in T_m's are clearly observed when the stereochemistry of the cyclopentane ring is (*S*,*S*). In contrast, there is no well-defined melting transition in the UV analysis if the stereochemistry is changed to (*R*,*R*) (entry 10).

A biologically relevant sequence was also investigated to determine whether the cyclopentane modification is sequence independent. A PNA sequence was constructed that is complementary to a portion of DNA encoding a mutant p53 protein

Table 2. Melting Temperature Data for PNA−DNA Complexes

entry[a]	sequence	T_m (°C) for DNA[b]	ΔT_m (°C)[c]
	Nielsen Sequence		
1	GTAGATCACT-Lys	48.9	
2	GTAGA**T***CACT-Lys	54.9	6.0
3	GTAGATC**A***CT-Lys	54.5	5.6
4	GTAGAT**C***ACT-Lys	54.2	5.3
5	GTAGA**T*C***ACT-Lys	60.2	11.3
6	G**T***AGA**T***CACT-Lys	59.6	10.7
7	G**T***AGA**T***C**A***CT-Lys	63.2	14.3
8	GTAG**A*T*C***ACT-Lys	64.4	15.5
9	G**T***AG**A*T***C**A***CT-Lys	70.3	21.4
10	GTAGA**T*^{R}**CACT-Lys[d]	na[e]	na
	p53		
11	GGCAGTGCCT-Lys	57.7	
12	GGCAG**T***GCCT-Lys	65.7	8.0

[a] Cyclopentane stereochemistry is (*S*,*S*), unless indicated otherwise. **B*** = *t*cyp residue. [b] Conditions for T_m measurement: 150 mM NaCl, 10 mM phosphate buffer, pH 7.0, 0.1 mM EDTA, UV measured at 260 nm from 90 to 25 °C, in 1 °C increments. All values are averages from two or more experiments. Approximate error for T_m's is ±0.6 °C. [c] ΔT_m is the difference in melting temperature between unmodified PNA (entry 1) and cyclopentane modified PNA, unless indicated otherwise. [d] **B*^{R}** = *t*cyp with (*R*,*R*) stereochemistry [e] No detectable melting transition observed.

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(22) (a) Tomac, S.; Sarkar, M.; Ratilainen, T.; Wittung, P.; Nielsen, P. E.; Norden, B.; Graslund, A. *J. Am. Chem. Soc.* **1996**, *118*, 5544. (b) Ratilainen, T.; Holmen, A.; Tuite, E.; Haaima, G.; Christensen, L.; Nielsen, P. E.; Norden, B. *Biochemistry* **1998**, *37*, 12331.

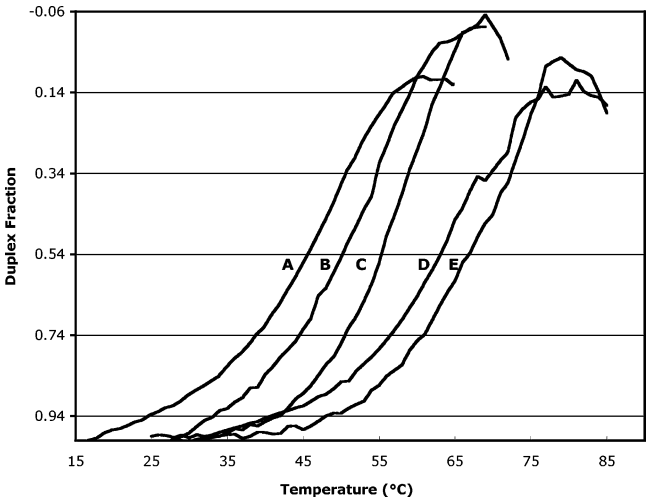

Figure 2. Calculated fraction of PNA duplexes as a function of temperature, with zero (A) to four (E) (*S*,*S*)-cyclopentanes incorporated into the PNA sequence. The curves represent the indicated sequences listed in Table 1: A = entry 1; B = entry 2; C = entry 5; D = entry 8; E = entry 9.

Table 3. Thermodynamic Parameters for *t*cypPNA (entry 8, Table 1) Compared to aegPNA

entry[a]	sequence	ΔH (kcal/mol)[b]	ΔS (cal/mol·K)[b]	ΔG (kcal/mol)[b]
1	GTAGATCACT-Lys	-74 ± 9	-203 ± 25	-13 ± 9
8	GTAGA*T**C*ACT-Lys	-66 ± 4	-170 ± 9	-15 ± 4

[a] Entry numbers correlate to the entries in Table 1 with identical PNA sequences. B* = *t*cyp residue with (*S*,*S*) stereochemistry. [b] Values are calculated from a line fit to a plot of $1/T_m$ vs $\ln(C_t)$. Errors are calculated on the basis of the line fit. ΔS and ΔG are calculated at $T = 298$ K.

that is prevalent in many different cancer cell lines. Specifically, the PNA sequence is complementary to the G-to-A mutation at codon 175 of the p53 protein.[23] Recognition of such a DNA sequence could become an important component for diagnosis of specific types of cancer. As shown for the previous PNA sequences, a consistent increase in the T_m was observed (entry 12). Taken together, these data show the general benefits of cyclopentane incorporation to the stability of PNA−DNA duplexes. Such benefits should offer more sensitive probes that can be used in diagnostic assays for DNA.

Several additional characteristics of the cyclopentane modification have also been investigated relative to unmodified PNA. A Job plot indicates that a *t*cypPNA with three cyclopentanes (entry 8, Table 2) has a 1:1 stoichiometry when bound to complementary DNA. In addition, both *t*cypPNA and the unmodified PNA have similar single-stranded melting profiles, and the degrees of hysteresis for heating and cooling runs in single strand and in PNA−DNA duplex melting experiments are similar (see Supporting Information for the data).

To understand how cyclopentane improves binding affinity, the thermodynamic values of binding to DNA were determined for PNA vs a *t*cypPNA. Variable-concentration T_m data were obtained for each PNA−DNA duplex and subjected to a Van't Hoff analysis. The calculated thermodynamic values for binding are presented in Table 3 (see Supporting Information for the data). These data indicate that each cyclopentane contributes approximately 0.7 kcal/mol to the overall stability of the PNA−DNA duplex, and that this stability is largely derived from more favorable entropy of binding.

Table 4. Discrimination of Single Base Mismatches and Improvements in *t*cypPNA

		T_m (ΔT_m)[b]		
entry[a]	sequence	TT mismatch	TC mismatch	TG mismatch
1	GTAGA***T***CACT-Lys	36.0 (−12.9)	32.0 (−16.9)	34.0 (−14.9)
2	GTAGA***T****CACT-Lys	34.7 (−20.5)	29.4 (−25.8)	36.8 (−18.3)
6	GT*AGA***T****CACT-Lys	42.5 (−17.1)	29.6 (−29.5)	40.1 (−19.7)
7	GT*AGA***T****CA*CT-Lys	36.5 (−26.7)	30.1 (−33.0)	38.6 (−24.5)
8	GTAGA****T****C*ACT-Lys	37.8 (−26.6)	29.5 (−34.9)	40.7 (−23.7)
9	GT*AGA****T****CA*CT-Lys	38.8 (−31.5)	32.3 (−38.0)	42.8 (−27.5)

[a] Entry numbers correlate to the entries in Table 1 with identical PNA sequences. B* = *t*cyp residue with (*S*,*S*) stereochemistry. [b] All values reported in units of °C. ΔT_m represents the difference in melting temperature between the complementary DNA and the DNA with the indicated mismatch. All mismatches were opposite to residue symbolized with ***B*** or ***B****.

To properly identify SNPs, any DNA probe must be very effective at discriminating between single base mismatches. Therefore, the sequence specificity of cyclopentane-modified PNA was examined by determining the change in T_m of a DNA−PNA duplex when a single base mismatch is present. Incorporation of (*S*,*S*)-*trans*-cyclopentane residues generally improves the mismatch discrimination when using the Nielsen PNA sequence (Table 4). The mismatches were positioned opposite to a cyclopentane residue at the sixth position in the sequence, and a measure of the sequence specificity was determined by taking the difference in the melting temperature between the fully matched DNA−PNA duplex and the duplex with a single base mismatch (this is expressed as ΔT_m in Table 4). One *trans*-cyclopentane residue in the Nielsen sequence was found to improve mismatch discrimination over unmodified PNA, and each subsequent addition of cyclopentane residues further improves discrimination (except in the case of entry 6 when studied in the context of the TT mismatch). The fact that this trend is observed for a TG mismatch is of particular importance, as this mismatch is generally considered to be the most difficult to discriminate against due to the facile formation of a wobble base pair.[24] The large differences in melting temperatures observed for *t*cypPNAs bound to complementary vs noncomplementary DNA allows one to control hybridization conditions to exclude binding of noncomplementary DNA by adjusting the temperature.

One of the benefits that PNA can offer to DNA detection is the ability to hybridize to DNA under low salt conditions. Under such conditions, DNA secondary structure can be denatured[10] so that all DNA binding sites are available for detection. Typically, PNAs bind with higher affinity at lower salt concentrations than at higher concentrations. For the *t*cypPNAs in Table 5, this trend is similar to that observed for the aegPNAs.[22a]

Discussion

In this paper, we have reported the benefits of incorporating *trans*-cyclopentane units into an aegPNA backbone. The *t*cypPNAs bind with higher affinity and better sequence specificity compared to unmodified PNA, and the incorporation of multiple cyclopentanes improves these properties further. From variable-

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Table 5. Ionic Effects on the Thermal Stability (T_m) of Modified PNAs

		[NaCl][b]		
entry[a]	sequence	0 mM	150 mM	300 mM
2	GTAGA**T***CACT-Lys	60.8	55.1	53.2
4	GTAGAT**C***ACT-Lys	60.1	54.2	52.2

[a] Entry numbers correlate to the entries in Table 1 with identical PNA sequences. **B*** = *t*cyp residue. All T_m values reported in units of °C. [b] [NaCl] refers to the amount of exogenous salt added to 10 mM sodium phosphate buffer.

concentration T_m data, the increase in DNA binding affinity of a cyclopentane-modified PNA is due largely to an entropic effect, indicating that replacing the ethylenediamine portion of a PNA with a carbocyclic ring significantly reduces the conformational flexibility of an unbound PNA, lowering the entropic cost associated with formation of a PNA−DNA complex.[25] The enthalpy of binding calculated from these data is consistent with a *t*cypPNA−DNA duplex that is similar in structure to an aegPNA−DNA duplex.[22a,26]

The stereochemistry and size of the ring incorporated at this position also impact the ability of PNA to bind DNA. For instance, previously published studies that examined the effects of replacing the ethylenediamine portions of PNAs with *trans*-cyclohexane did not report the same benefits as we report for *trans*-cyclopentane.[27] Furthermore, both studies have found that the (*S*,*S*) stereochemistry in five- and six-membered rings is more compatible than (*R*,*R*) in the PNA−DNA duplex. We speculate that, in order for a carbocyclic ring to promote PNA binding to oligonucleotides, both ring size and stereochemistry must restrict the PNA to access only the range of dihedral angles necessary for binding, while excluding conformations that are irrelevant to duplex formation. In a PNA−DNA duplex, the dihedral angles about the ethylenediamine units adopt a range of values depending on the position of each unit in the sequence. From an NMR-derived PNA−DNA structure, the approximate range for this dihedral angle is 130−165°.[28] In crystal structures, the same dihedral angle has an average value of 73° in the Watson−Crick portion of a PNA_2−DNA triplex,[29] and a range of 60−83° in a D-Lys-modified PNA−DNA duplex.[30] In our previous publication, we used molecular modeling to demonstrate that the conformations of a five-membered ring should allow PNAs to access the requisite conformations for DNA binding to a greater extent than a six-membered ring.[12] Therefore, the success of *trans*-cyclopentane compared to *trans*-cyclohexane is most likely due to conformational differences between the two rings. The recent success with *cis*-cyclopentane in stabilizing oligothymine PNA_2−DNA triplexes indicates that the cis stereochemistry about the cyclopentane ring is also compatible with the requisite conformations to promote PNA binding to DNA.[14e,i] However, further work will be required to determine whether the *cis*- or *trans*-cyclopentane stereochemistry is the most beneficial in terms of stability and sequence specificity for a large number of different PNA−DNA duplexes.

The properties of *t*cypPNAs could impact the design of new probes for genomic analysis. DNA detection with PNA-based systems could lead to new diagnostics that are more sensitive, faster, and more durable than the current state-of-the-art systems. Clearly, the standard aegPNAs have not replaced DNA oligomers as hybridization probes in most diagnostic applications, most likely due to limitations in the properties and the synthesis of aegPNAs. Furthermore, there are currently no standard backbone modifications available that can be used to adjust the binding properties of aegPNA. In contrast, there are a large number of modified analogues of the natural nucleosides (such as the locked nucleic acids) that are routinely used to fine-tune the binding properties of DNA and RNA oligomers for different applications.[31] The development of new PNA monomers that improve the binding properties of aegPNAs could spark the widespread use of such oligomers in a wide variety of applications. The *t*cypPNAs described in this paper allow one to tune the oligomers' DNA binding properties by calculating the number of cyclopentanes required. In particular, using these molecules as DNA binding probes could make current diagnostic techniques significantly more reliable. We are currently examining the benefits of using *t*cypPNAs in PCR-[32] and microarray-based applications.

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Supporting Information Available: Procedures for solid-phase synthesis of all PNAs, mass spectra to characterize all PNAs, ^1H and ^{13}C spectra for all compounds listed in the Experimental Section, data for single-strand melting experiments, hysteresis between heating and cooling melting experiments, plot of $1/T_m$ vs ln C_t from variable-concentration melting temperature data, and Job plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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