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Discrimination of Hairpin Polyamides with an α-Substituted-γ-aminobutyric Acid as a 5′-TG-3′ Reader in DNA Minor Groove

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Abstract: Pyrrole-imidazole (Py-Im) polyamides containing stereospecifically α-amino- or α-hydroxyl-substituted γ-aminobutyric acid as a 5′-TG-3′ recognition element were synthesized by machine-assisted Fmoc solid-phase synthesis. Their binding properties to predetermined DNA sequences containing a core binding site of 5′-TGC**N**CA-3′/3′-ACG**N′**GT-5′ (**N**·N′ = A·T, T·A, G·C, and C·G) were then systematically studied by surface plasmon resonance (SPR). SPR results revealed that the pairing of stereospecifically α-amino-/α-hydroxyl-substituted γ-aminobutyric acids, (*R* or *S*)-α,γ-diaminobutyric acid (γRN or γSN) and (*R* or *S*)-α-hydroxyl-γ-aminobutyric acid (γRO or γSO), side-by-side with β-alanine (β) in such polyamides significantly influenced the DNA binding affinity and recognition specificity of hairpin polyamides in the DNA minor groove compared with β/β, β/γ, and γ/β pairings. More importantly, the polyamide Ac-Im-γSO-ImPy-γ-ImPyβPy-β-Dp (β/γSO) favorably binds to a hairpin DNA containing a core binding site of 5′-TGC**N**CA-3′/3′-ACG**N′**GT-5′ (**N**·N′ = A·T) with dissociation equilibrium constant (K_D) of 1.9×10^{-7} M over **N**·N′ = T·A with $K_D = 3.7 \times 10^{-6}$ M, with a 19-fold specificity. By contrast, Ac-Im-γSN-ImPy-γ-ImPyβPy-β-Dp (β/γSN) binds to the above sequence with **N**·N′ = A·T with $K_D = 8.7 \times 10^{-7}$ M over **N**·N′ = T·A with $K_D = 8.4 \times 10^{-6}$ M, with a 9.6-fold specificity. The results also show that the stereochemistry of the α-substituent, as well as the α-substituent itself may greatly alter binding affinity and recognition selectivity of hairpin polyamides to different DNA sequences. Further, we carried out molecular modeling studies on the binding by an energy minimization method, suggesting that α-hydroxyl is very close to N3 of the 3′-terminal G to induce the formation of hydrogen bonding between hydroxyl and N3 in the recognition event of the polyamide Ac-Im-γSO-ImPy-γ-ImPyβPy-β-Dp (β/γSO) to 5′-TGC**N**CA-3′/3′-AC**GN′**GT-5′ (**N**·N′ = A·T). Therefore, SPR assays and molecular modeling studies collectively suggest that the (*S*)-α-hydroxyl-γ-aminobutyric acid (γSO) may act as a 5′-TG-3′ recognition unit.

Introduction

Py-Im polyamides composed of two or three different types of aromatic heterocyclic rings, analogous to the composition of the antibiotics, netropsin and distamycin, are currently among the most promising synthetic compounds for use in chemotherapy and biosensing.[1] They can recognize a predetermined DNA sequence in the minor groove of B-DNA,[2,3] alkylate DNA at a specific site when conjugated with an alkylating agent,[4,5] and be introduced into living cells with the aim of possible chemical regulation of gene expression.[6] In their hairpin structure, antiparallel side-by-side pairings of two aromatic amino acids bind to DNA sequences, with a polyamide ring packed specifically against each DNA base. *N*-Methylpyrrole (Py) favors T, A, and C bases, excluding G; *N*-methylimidazole (Im) is a G-reader; and 3-hydroxyl-*N*-methylpyrrol (Hp) is specific for thymine base. To date, all four Watson−Crick base pairs can be recognized using different pairings of three aromatic amino acids, with an Im/Py pairing reading G·C by symmetry, a Py/Im pairing reading C·G, an Hp/Py pairing being able to distinguish T·A from A·T, G·C, and C·G, and a Py/Py pairing nonspecifically discriminating both A·T and T·A from G·C and C·G.[7]

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There are disadvantages in using Hp as a T-specific recognition element. These disadvantages include:[8] (1) the Hp residue can degrade in solution over time in the presence of acid or free radicals; (2) it has a lower affinity for T bases relative to Py; and (3) Hp at the N-terminus of polyamides has almost no specificity for any base of the Watson−Crick base pairs.

For these reasons, researchers have been investigating other T-specific recognition elements as substitutes for Hp. Recently, Dervan's group has found that hydroxybenzimidazole (Hz) may replace Hp as a T-reader.[9] Although an Hz/Py pairing has an association equilibrium constant (K_A) of 5.7×10^8 M^{-1} for T·A, close to that of Hp/Py, and a specificity approximately 18-fold greater for T·A over A·T, and the Hz-containing polyamide is stable under acidic conditions, it only recognizes T base at the 3′ end of contiguous 5′-TT-3′ due to structural limitations of conjugates of Hz directly linked to Py.

In addition, it has been reported that the polyamide rise per aromatic amino acid residue basically matches the pitch of the B-DNA helix in the polyamide composed of not more than five contiguous aromatic rings, but the polyamides are overcurved compared to the curvature and twist of the DNA helix, suggesting that not more than five contiguous rings are better bound along the minor groove of B-DNA without loss of affinity and specificity.[10,11] Despite introducing β-alanine (β) as an aliphatic substitute for a Py ring in the construction of the polyamides, with the β residue taking advantage of its better flexibility to act like a "spring" to allow the crescent-shaped polyamide to match the curvature and twist of B-DNA, the β/β pairing still nonspecifically reads T·A and A·T pairs in the same way as Py/Py pairs.[12] This has led to attempts to identify an aliphatic monomer that can function as a base recognition element, as well as a "spring" to adjust the curvature and length of polyamides to fit to twist and pitch of B-DNA as a substitute for the aromatic ring. Floreancig and colleagues have shown that (*S*)-isoserine could serve as a T-recognition unit able to discriminate T specifically over A with 7.2-fold increased specificity.[13]

Subsequent attempts have been made to explore ideal aromatic B-DNA base binders binding in the minor groove, including T-specific binders, to adjust the curvature and length of the polyamide to fit the twist of the B-DNA helix by examining other types of single five-membered heterocyclic residues, including furan, thiazole, thiophene, 3-methylthiophene, 4-methylthiazole, 3-hydroxythiophene, 1H-pyrrol, and 1H-pyrazole; unfortunately, however, such hyperspecific rings have not yet been discovered.[8a]

To address the above problems, as shown in Figure 1, we designed four α-substituted γ-aminobutyric acid residues as T-specific recognition elements in the construction of polyamides: (*R*)-α,γ-diaminobutyric acid (γRN), (*S*)-α,γ-diaminobutyric acid (γSN), (*R*)-α-hydroxyl-γ-aminobutyric acid (γRO), and (*S*)-α-hydroxyl-γ-aminobutyric acid (γSO). These residues were based on the flexibility of the aliphatic γ-aminobutyric acid, the same covalence number as that between adjacent Py-Im aromatic ring carboxamides, and different stereochemical effects of substituents of functionalized β-alanine on the recognition of polyamides.[13] The binding properties of the designed polyamides to selected hairpin DNAs, including their binding affinity, specificity, stoichiometry, kinetics, and thermodynamics studied through equilibrium analysis [(K_A) or dissociation equilibrium constant (K_D)] and kinetic analysis [association rate constant (k_a) or dissociation rate constant (k_d)], were investigated using the Biacore SPR technique (Figures1 and 2).[14]

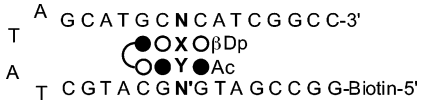

Figure 1. Molecular interaction model of polyamides with biotinylated hairpin DNAs in SPR assays. **X**/**Y** represents different aliphatic monomer pairings: β/β, β/γ, γ/β, γ/γ, β/γSO, β/γRO, γSO/β, γRO/β, β/γSN, β/γRN, γSN/β, γRN/β; **N**·**N**′ represents the four base pairs: T·A, A·T, C·G, G·C. The biotinylated hairpin DNA was incorporated by the 5′ end onto precoated streptavidin sensor chips. Im is indicated by the black circles, and Py the open circles; β represents β-alanine residue, Dp is 3-(dimethylamino)-propylamide, ⊂ represents the γ-aminobutanoic acid turn residue connecting the two subunits, and Ac is acetyl.

Results and Discussion

Monomer Synthesis. The protected optically active monomers **17a** (*S*) and **17b** (*R*) were synthesized according to the procedure in Scheme 1. (2*S*)-4-[(9-Fluorenylmethoxy)carbonylamino]-2-hydroxybutyric acid (**14a**) was prepared from the corresponding optically active starting material **13a** in a mixture of water and 1,2-dimethoxyethane in the presence of a small amount of sodium bicarbonate. The resultant Fmoc-protected 2-hydroxybutyric acid **14a** was suspended in DCM, and an excess of isobutene was introduced at a lower temperature. The reaction was carried out until a clear solution was formed, and after the mixture was worked up, the *tert*-butyl ester of the (2*S*)-4-[(9-fluorenylmethoxy)carbonylamino]-2-*tert*-butoxybutyric acid **15a** was obtained.[15] The cleavage of the *tert*-butyl ester from **15a** was accomplished in the presence of the high-activity silylating agent TMSOTf to give (2*S*)-2-*tert*-butyloxy-4-(9-fluorenylmethoxy) carbonylaminobutyric acid **(17a**) through a very labile intermediate **16a**.[16] **17b** was also synthesized using the above approach. The enantiomeric purity of these compounds based on ^{1}H NMR analysis of (*S*)-(−)-1-(1-naphthyl)-ethyl amide derivatives of **17a** and **17b** was shown to be more than 97%.

It is worth noting that it was difficult to optimize the synthesis conditions to give rise to the optically active compound **17a** or **17b**, without side reactions and racemization, as a base-labile amino protective group, Fmoc, and an acid-labile hydroxy protective group, *tert*-butyl, are simultaneously present in the **17a** or **17b** monomer. Two key steps are necessary to enable

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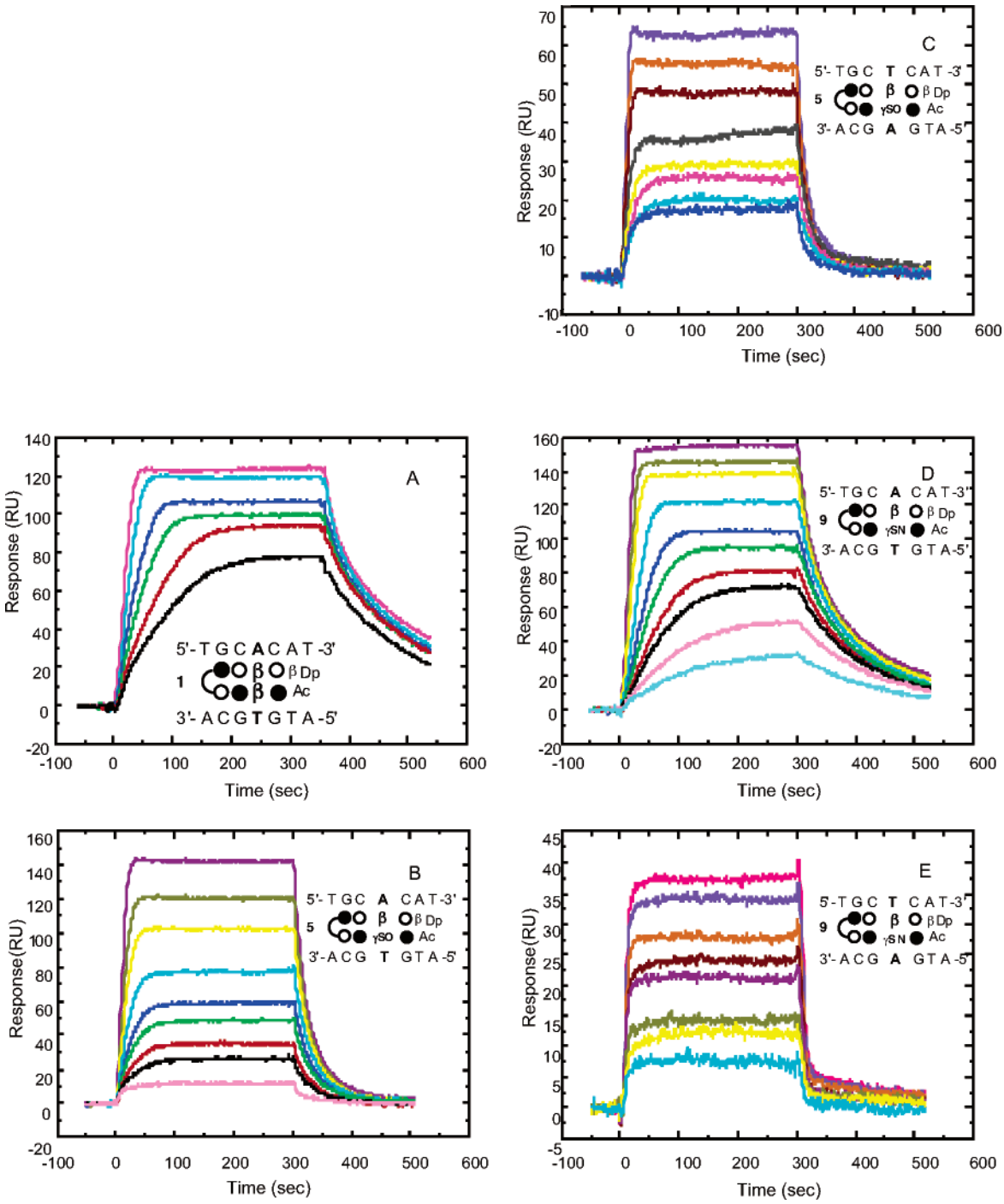
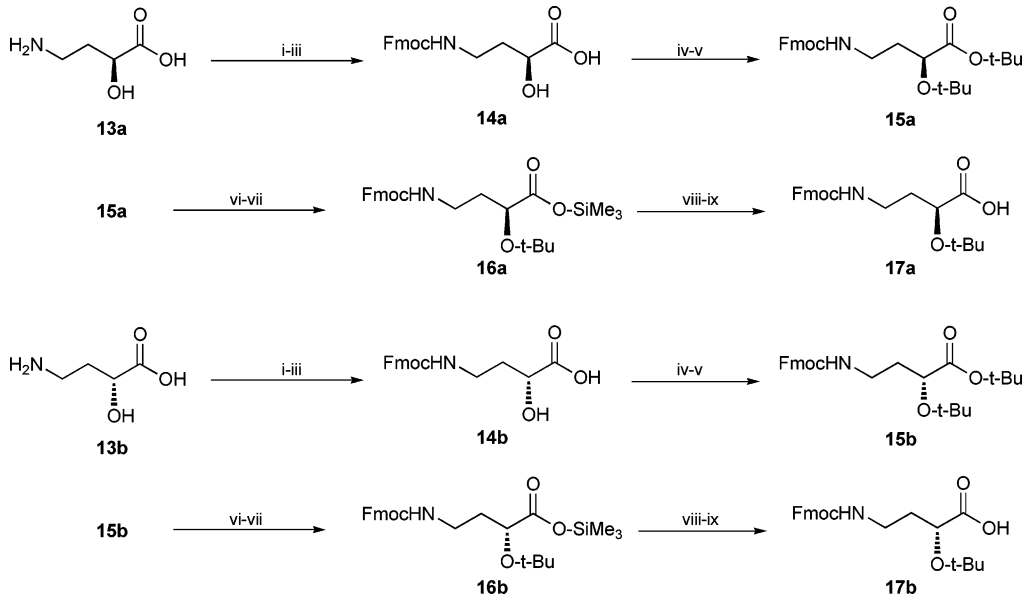


Figure 2. Typical SPR sensorgrams for the interaction of polyamides with hairpin DNAs immobilized on the surface of sensor chips. The binding response is changed over time. Experiments at each concentration were repeated three times, and the data were globally fitted to a 1:1 interaction model with mass transport. The association phase was allowed to come to equilibrium at the different concentrations of the polyamides. All experiments were done using the same DNA immobilization level sensor chips in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) with 0.1% DMSO at 25 °C. (A) Polyamide **1** with β/β pairing for the recognition of A·T base pairs at a concentration range from 3.75×10^{-8} M (lowest curve) to 2.00×10^{-7} M (highest curve). (B) Polyamide **5** with γ/γSO pairing for the recognition of A·T base pairs at a concentration range from 2.00×10^{-8} M (lowest curve) to 5.00×10^{-7} M (highest curve). (C) Polyamide **5** with γ/γSO pairing for the recognition of T·A base pairs at a concentration range from 1.00×10^{-7} M (lowest curve) to 1.00×10^{-6} M (highest curve). (D) Polyamide **9** with γ/γSN pairing for the recognition of A·T base pair at a concentration range from 1.00×10^{-8} M (lowest curve) to 5.00×10^{-7} M (highest curve). (E) Polyamide **9** with γ/γSN pairing for the recognition of T·A base pair at a concentration range from 1.50×10^{-7} M (lowest curve) to 1.25×10^{-6} M (highest curve).

our method to synthesize such optically active targets and to avoid side reactions and the concomitant danger of racemization. The first is the simultaneous formation of *tert*-butyl-ester and -ether and the second is the conversion of *tert*-butyl ester to the corresponding trimethylsilyl ester. The selective cleavage of *tert*-butyl ester in the presence of *tert*-butyl ether has rarely been reported.[16] Less than 3% racemization (see Experimental Section) was observed, and the final products **17a** and **17b** were afforded at overall yields of 69.5% and 67.2%, respectively, by the four steps in our experimental conditions, as shown in the Experimental Section. More importantly, our experiments showed that selective protection of the hydroxyl group by *tert*-

Scheme 1 . Synthesis of Two New Monomers[a]



[a] Reagents (i) (*S*)-(−)-4-amino-2-hydroxybutyric acid (**13a**) or (*R*)-(+)-amino-2-hydroxybutyric acid (**13b**), H_2O, $NaHCO_3$, (ii) FmocONSu, 1,2-dimethoxyethane. (iii) Concentrated hydrochloric acid. (iv) 98% H_2SO_4, DCM, then $CH_2{=}CMe_2$. (v) 2 M aqueous NaOH. (vi) TMSOTf, DIEA, dioxane. (vii) EtOAc/H_2O. (viii) 10% aqueous hydrochloric acid. (ix) 5% aqueous $KHSO_4$, and 10% aqueous K_2SO_4.

butyl and selective deprotection for *tert*-butyl ester with optically active hydroxyl amino acids are possible. Our method has potential for the synthesis of *tert*-butyl ethers of amino-protected hydroxyl amino acids, which are valuable building blocks for peptide synthesis.

Polyamide Synthesis. To examine the ability of α-substituted γ-butyric acid to recognize T bases, 12 polyamides were synthesized by the Fmoc-chemistry solid-phase synthesis method, starting with Fmoc-β-Ala-clear-acid resin and using the monomers shown in Scheme 2. Wurtz et al. first reported general protocols for machine-assisted Fmoc-chemistry solid-phase synthesis of Py-Im polyamides starting from Fmoc-β-alanine-Wang resin.[17] Here, *N*-methylpyrrolidone (NMP) was replaced with DMF as solvent, and HBTU, with HATU as activator. To minimize side products in the synthesis of our polyamides, capping was used for each cycle after coupling.[18] After the first Fmoc-Py acid **19** was activated by HATU in the presence of DIEA, it was readily coupled to swollen resin **18** in the synthesizer column for 60 min, in accordance with the established protocols shown in the Experimental Section. Coupling of the remaining aromatic monomers **19** and **20** was also completed using this procedure. Coupling of modified and unmodified aliphatic monomers **17a**, **17b**, **21**, **22**, **23**, and **24** was accomplished with HATU using conditions the same as those in the coupling of aromatic monomers. No effects were found in the polyamide solid-phase synthesis by the respective *tert*-butyloxy and *tert*-butyloxycarbonylamino groups in the aliphatic monomers **17a**, **17b** and **23**, **24**. Interestingly, to obtain higher recoveries of polyamides successfully, a double-couple cycle must be employed upon coupling of any monomer to imidazole and the coupling of imidazole itself, but their mechanism is not well understood.

After the completion of machine-assisted solid-phase synthesis, the polyamides were cleaved from resins **1a**−**12a** by aminolysis with 3-(dimethylamino)propylamine (Dp), to produce the desired polyamides **1**, **2**, **3**, **4**, and *tert*-butyl-protected **5**, **6**, **7**, **8**, and Boc-protected **9**, **10**, **11**, **12**. The deprotection of protected **5**, **6**, **7**, **8**, **9**, **10**, **11**, and **12** was readily achieved using an optimal deprotecting agent, TFA/TMS/TIS/H_2O (91/3/3/3, v/v), to give the final products **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**. No racemization occurred as the removal of *tert*-butyloxy was realized by breaking the oxygen-*tert*-butyl bond.[15a] A final purity for all synthesized polyamides greater than 98% was determined by a combination of analytical HPLC, ^{1}H NMR, and mass spectroscopy.

SPR Assay. By designing and using a self-complementary biotinylated oligonucleotide, 5′-biotin-GGCCGATG**N**′GCATGCTATAGCATGC**N**CATCGGCC-3′ (34 bases, **N**·**N**′: T·A, A·T, C·G, G·C) that was noncovalently bound to streptavidin-functionalized Biacore sensor chips and that formed double-stranded hairpin DNA including the target binding sites of the polyamides with a T-specific recognition unit (Figure 1), the kinetic and thermodynamic properties of polyamide binding to DNA, evaluated from binding affinity, specificity, and stoichiometry, can be obtained using SPR assays (Figure 2).

All SPR experiments for polyamides **1**−**12** were performed using a Biacore *X* instrument with a sensor chip SA in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) with 0.1% DMSO at 25 °C. The dissociation equilibrium constants (K_D, M) were obtained by fitting the resulting sensorgrams (Figure 2) to a theoretical model, providing the affinity and specificity of binding for the functionalized γ-monomer/β against all four base pairs: T·A, A·T, G·C, C·G. In addition, standard free energy change ($\Delta G°$, kcal/mol^{-1}) for binding was calculated from K_D.

To obtain rational and accurate binding constants, varied concentration ranges were used for different analytes in the SPR experiments. The stoichiometry for polyamide binding to hairpin DNA was determined by comparison of the maximum response for binding one molecule of polyamide per binding site, RU_{max},

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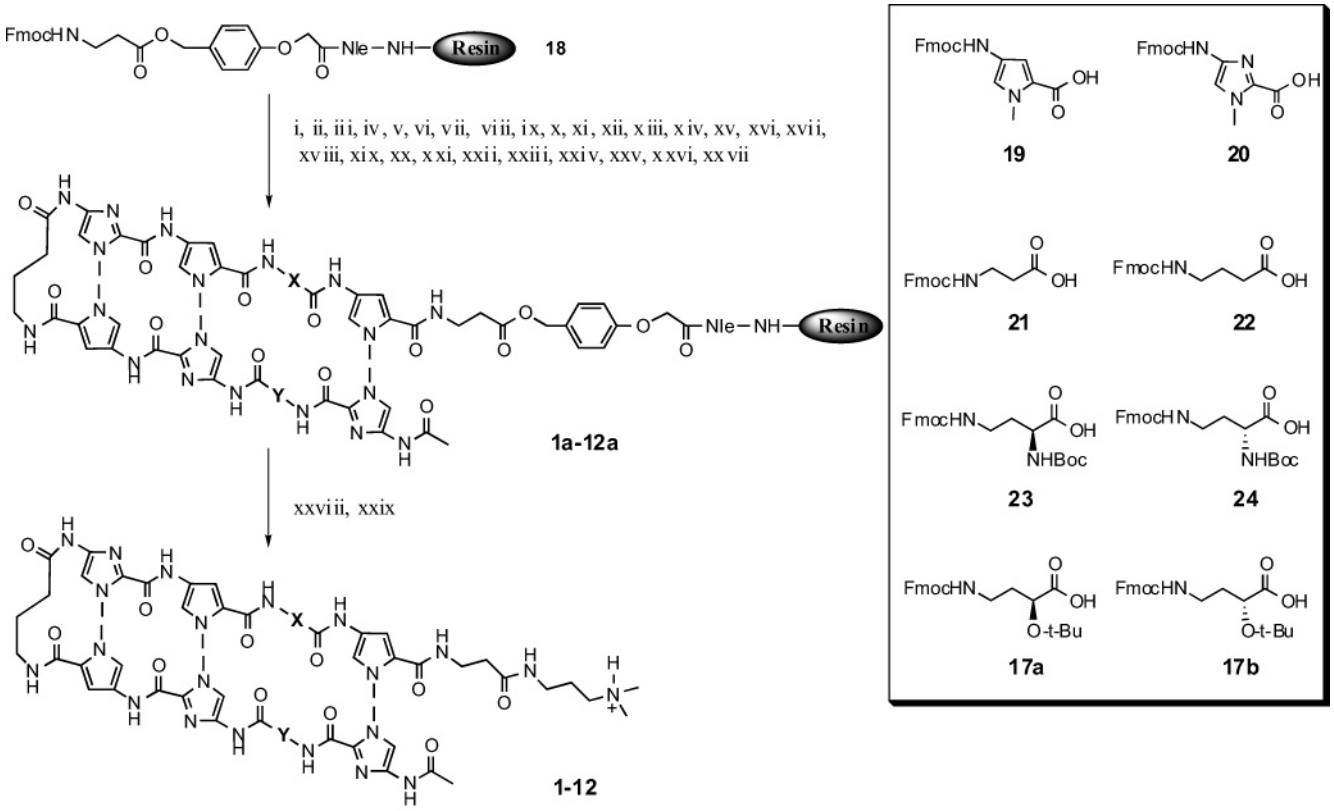
Scheme 2 · Fmoc Solid-Phase Synthesis of Polyamides Starting from Commercially Available Fmoc-β-Ala-Clear-Acid Resin **18**[a]



1: X = β, Y = β; **2**: X = β, Y = γ; **3**: X = γ, Y = β; **4**: X = γ, Y = γ;
5: X = β, Y = γSO; **6**: X = β, Y = γRO; **7**: X = γSO, Y = β; **8**: X = γRO, Y = β;
9: X = β, Y = γSN; **10**: X = β, Y = γRN; **11**: X = γSN, Y = β; **12**: X = γRN, Y = β

[a] Reaction conditions: (i) 20% piperidine/DMF; (ii) FmocPyCOOH, HATU, DIEA; (iii) 5% acetic anhydride + 5% pyridine/DMF; (iv) 20% piperidine/DMF; (v) Fmoc-γ-Ala-OH for **1a**, **2a**, **5a**, **6a**, **9a**, **10a**, HATU, DIEA; Fmoc-γ-Abu-COOH for **3a**, **4a**, HATU, DIEA; **17a** for **7a**, HATU, DIEA; **17b** for **8a**, HATU, DIEA; **23** for **11a**, HATU, DIEA; **24** for **12a**, HATU, DIEA; (vi) 5% acetic anhydride + 5% pyridine/DMF; (vii) 20% piperidine/DMF; (viii) FmocPyCOOH, HATU, DIEA; (ix) 5% acetic anhydride + 5% pyridine/DMF; (x) 20% piperidine/DMF; (xi) FmocImCOOH, HATU, DIEA; (xii) 5% acetic anhydride + 5% pyridine/DMF; (xiii) 20% piperidine/DMF; (xiv) Fmoc-γ-Abu-COOH, HATU, DIEA; (xv) 5% acetic anhydride + 5% pyridine/DMF; (xvi) 20% piperidine/DMF; (xvii) FmocPyCOOH, HATU, DIEA; (xviii) 5% acetic anhydride + 5% pyridine/DMF; (xix) 20% piperidine/DMF; (xx) FmocImCOOH, HATU, DIEA; (xxi) 5% acetic anhydride + 5% pyridine/DMF; (xxii) 20% piperidine/DMF; (xxiii) Fmoc-γ-Ala-OH for **1a**, **3a**, **7a**, **8a**, **11a**, **12a**, HATU, DIEA; Fmoc-γ-Abu-COOH for **2a**, **4a**, HATU, DIEA; **17a** for **5a**, HATU, DIEA; **17b** for **6a**, HATU, DIEA; **23** for **9a**, HATU, DIEA; **24** for **10a**, HATU, DIEA; (xxiv) 5% acetic anhydride + 5% pyridine/DMF; (xxv) 20% piperidine/DMF; (xxvi) FmocImCOOH, HATU, DIEA; (xxvii) 5% acetic anhydride + 5% pyridine/DMF; (xxviii) *N*,*N*-dimethylaminopropylamine for **1**−**4** and Boc and *tert*-butyl-protected **5**−**12**, 55 °C, overnight; (xxix) TFA/TMS/TIS/H2O (91/3/3/23, v/v) for **5**−**12**

with the RU value at saturation. For a single-site model, RU_{max} can be calculated using the following equation:

$$RU_{max} = (RU_{DNA}/MW_{DNA}) \times MW_{polyamide} \times RII$$

where RU_{DNA} is the amount of hairpin DNA immobilized on the sensor chip in response units (RU), MW is the molecular weight of the polyamide and the hairpin DNA, and RII is the refractive index increment ratio of the compound to the refractive index of the DNA. Here, RII values for these compounds should range from 1.25 to 1.40.[19−21]

Effects of Stereochemistry of the Hydroxy Substituent on Recognition and Binding Stoichiometry. To evaluate the recognition ability of polyamides **1**−**12** with an aliphatic monomer pairing to all four possible base pairs T·A, A·T, C·G, and G·C in the DNA minor groove, their binding properties were examined by SPR (Figure 2, Table 1 and Supporting Information Figures 1 and 2). It is noteworthy that almost no binding of all examined polyamides **1**−**12** to 5′-TGC**N**CA-3′ (**N** = C, G) was observed at low concentrations of polyamides, but there was large nonspecific binding at higher concentrations, resulting in the inability to obtain precise dissociation equilibrium constants. For polyamides **1**, **2**, **3**, and **4** with an unsubstituted aliphatic amino acid pairing, almost no specificity for 5′-TGC**N**CA-3′/3′-ACG**N**′GT-5′ (**N**·**N**′ = T·A and A·T) was found under our experimental conditions. However, a phenomenon that should be noted is that the introduction of γ into **2** and **3** to pair with β reduces the binding affinity to A·T by 35-fold and 4.7-fold, respectively, and to T·A by 30-fold and 4.5-fold, respectively, when compared to **1** with a β/β pairing. In addition, the substitution of two γ residues for the two β monomers significantly decreased the binding affinity to the sequence 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ by approximately 846-fold and to 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ by 724-fold. These observations indicate that introduction of a single γ to pair with β to allow

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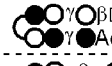
Table 1. Dissociation Equilibrium Constants for Polyamides **1**−**12**

Polyamide	Pair	5′-CAC-3′ 3′-GTG-5′ K_D (A•T)[a]	5′-CTC-3′ 3′-GAG-5′ K_D (T•A)	Specificity[b]
1 	β / β	2.6(±0.2) x10^{-8}	2.9(±0.4) x10^{-8}	1.1
2 	β / γ	9.4(±0.5) x10^{-7}	9.0(±0.3) x10^{-7}	0.96
3 	γ / β	1.5(±0.2) x10^{-7}	1.6(±0.3) x10^{-7}	1.1
4 	γ / γ	2.2(±0.3) x10^{-5}	2.1(±0.2) x10^{-5}	0.95
5 	β / γSO	1.9(±0.3) x10^{-7}	3.7(±0.1) x10^{-6}	>19
6 	β / γRO	8.5(±0.2) x10^{-7}	4.1(±0.2) x10^{-6}	4.8
7 	γSO / β	8.3(±0.6) x10^{-8}	1.1(±0.2) x10^{-7}	1.3
8 	γRO / β	2.6(±0.1) x10^{-7}	2.8(±0.2) x10^{-7}	1.1
9 	β / γSN	8.7(±0.2) x10^{-7}	8.4(±0.3) x10^{-6}	9.6
10 	β / γRN	9.0(±0.3) x10^{-7}	2.1(±0.1) x10^{-6}	2.3
11 	γSN / β	5.2(±0.3) x10^{-8}	7.5(±0.4) x10^{-8}	1.4
12 	γRN / β	5.6(±0.2) x10^{-8}	6.7(±0.3) x10^{-8}	1.2

[a] K_D: dissociation equilibrium constant with a unit of M. The standard deviation is given in parentheses.
[b] Specificity is defined as K_D(T·A)/K_D(A·T).

recognition of A•T and T•A did reduce the binding affinity, but not greatly.

In this study, polyamide **5**, with a *β*/*γ*SO, showed a strong binding preference for 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ (**N**•**N**′ = A•T) over 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ (**N**•N′ = T•A) with a 19-fold higher specificity (Table 1). Comparison of the binding affinities of polyamides with opposing paired aliphatic monomers, such as *β*/*γ*SO and *γ*SO/*β* in polyamides **5** and **7**, respectively, to the above sequence with A•T by polyamides **5**−**12**, showed that the α-hydroxy-/α-amino-substituted *γ* monomer is not able to simply recognize T or A by hydrogen bonding between the OH/NH_2 and O2 of T, but quite probably simultaneously recognizes two bases, that is, 5′-TG-3′, through strong hydrogen bonding between the OH/NH_2 and N3 of the 3′-G base adjacent to the T (Table 1). Furthermore, the α-position of the hydroxy substituent in the *γ*-monomer leads us to assume that an additional hydrogen bond occurs between the N3 of the 3′-terminal guanine adjacent to T and the hydroxy group, which may be responsible for the binding specificity of polyamide **5** to 5′-TGC**N**CA-3′/3′-ACG**N**′GT-5′ (**N**•**N**′ = T•A and A•T) (Figure 3). In addition, van der Waals contacts exist between the protons at the α-position of the *γ* residue and H1′ and/or H4′ of the deoxyribose of guanine.[22,23] These combined factors imply that binding affinity and specificity might be directly related to adjacent bases.

In this case, when polyamide **5** with a *β*/*γ*SO pairing binds to 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′, great steric hindrance by the A base makes the additional hydrogen bond between N3 of 3′-terminal guanine and α-hydroxyl group weak and leads in turn to a weaker binding affinity. Therefore, monomer *γ*SO actually acts as a 5′-TG-3′ reader in the recognition events.

To characterize the binding differences quantitatively among these polyamides, we obtained the thermodynamic parameter, standard free energy change (Δ*G*°) upon the formation of the complexes, as derived from the SPR results (Supporting Information Table 1). The binding of **5** to the sequence with A•T base pair is ∼2 kcal/mol more favorable than binding to the sequence with a T•A base pair. The improved binding stability to sequence 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ is correlated with much slower dissociation kinetics relative to the sequence 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ (B and C of Figure 2; data for dissociation rate constants not shown). The slow dissociation kinetics for the complex of polyamide **5**−5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ could be governed mainly by the breakage of the relatively strong additional H-bond between the OH and N3 of the G and maybe a possible factor for the release/reuptake of water molecules in the DNA minor groove. In contrast, only a 4.8-fold increase in specificity was observed in the recognition of the polyamide **6** with an *γ*RO monomer to 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ and to 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ (Table 1 and Supporting Information Figure 1, A and B) and replacing *γ*SO of **5** by *γ*RO at the same position to give **6** increases destabilization of binding to 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ and 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′, particularly with *γ*RO opposite T, with a 4.3-fold reduction in affinity. One

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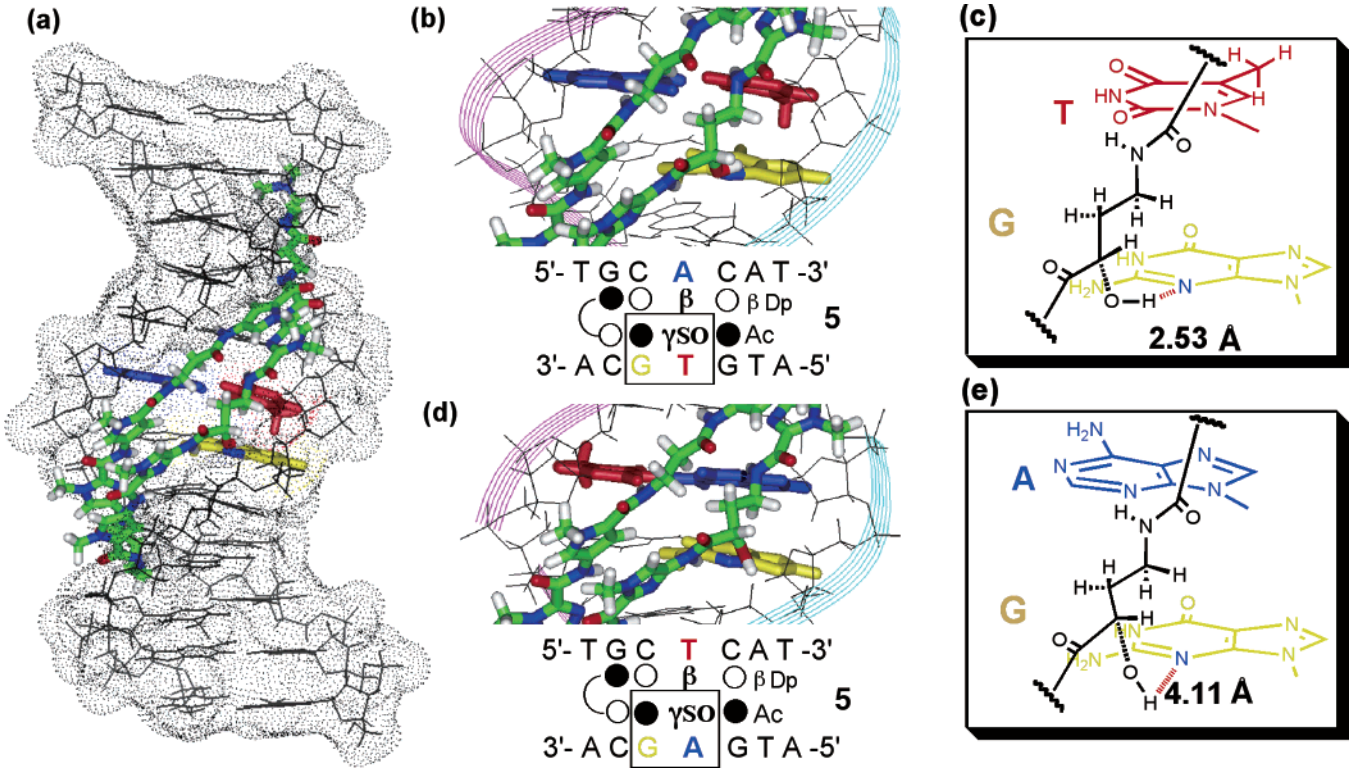

Figure 3. Molecular modeling study of the recognition by the polyamide with β/γSO of the sequence 5′-GCATGC**A**/**T**CATCG-3′/5′-CGATG**A**/**T**GCATGC-3′. (a) Molecular model of the 1:1 complex of polyamide **5** with the sequence 5′-GCATGC**A**CATCG-3′/5′-CGATG**T**GCATGC-3′ using a computer-assisted molecular simulation was obtained by energy minimization with the use of semiquantitative distance restraints derived from NOESY. The polyamide is shown as a stick model, with the A base in blue, T base in red, G base in yellow, but N3 in blue; in the polyamide, oxygen is represented in red, hydrogen is in gray, nitrogen is in blue, and covalent bonds are green. (b) Partial intermolecular contacts between the hairpin polyamide **5** with a β/γSO pairing and the sequence with an A·T base pair. (c) Schematic for (b) with hydrogen bond between OH and N3 of G shown as red dashed lines. (d) Partial intermolecular contacts between the hairpin polyamide **5** with a β/γSO pairing and the sequence with a T·A base pair. (e) Schematic for (d) with a hydrogen bond between OH and N3 of G shown as red dashed line.

explanation for this result is that the (*R*)-α-hydroxy substituent points toward the wall of the minor groove of B-DNA, causing a steric clash with the minor groove, leading to increased destabilization, whereas the (*S*)-α-hydroxy is in a favorable orientation, increasing binding affinity. Conversely, polyamides **7** and **8** with a γSO/β and a γRO/β pairing, respectively, almost nonspecifically bind to 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ and to 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′. However, their binding affinities all increase relative to the corresponding polyamides **5,** with a β/γSO pairing, and **6,** with a β/γRO pairing. This implies that van der Waals interactions between the hydroxyl-modified γ-monomer and flanking bases were also playing an important role in the discrimination events.

In addition, destabilization of polyamides **5**−**12** with a substituted γ residue increases for recognition of 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ and 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ relative to binding of the polyamide **1** with a β/β pairing to the above sequences, but compared with their parent polyamides destabilization of some polyamides enhances while that of others decreases destabilization (Table 1). This result illustrates that in some cases specificity in the recognition of sequence-specific DNAs by polyamides may indeed come at the cost of binding affinity.[7c,13]

As expected, all results were a better fit to a model with 1:1 polyamide to DNA stoichiometry, except for the recognition of G·C and C·G. No 2:1 binding motif of polyamides to hairpin DNAs was found despite high concentrations being used (Figure 2, Supporting Information Figures 1 and 2).

Effects of the Amino Substituent on Binding. Polyamide **9**, with a β/γSN pairing, prefers to bind the sequence 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ over 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ by a factor of 9.6, while **10**, with a β/γRN pairing, displays a 2.3-fold preference for the sequence 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ (Table 1). This difference in specificity may result from the reasons indicated above, that is, the different binding orientation of the *S*- and *R*-amino substituents could explain the differences in recognition. In addition, the SPR results shown in D and E of Figure 2 indicate that the faster dissociation kinetics for the complex of **9** and the sequence with T·A could be responsible for the difference in binding affinity between sequences with A·T and T·A (data for dissociation rate constants not shown). Likewise, ΔG values may also account for the binding preference of polyamide **9** to A·T over T·A (Supporting Information Table 1).

Similarly, almost equal binding affinities for **11**, with an γSN/β, and **12**, with an γRN/β, to A·T and T·A were found, but their binding stability increases relative to the corresponding **9**, with an γSN/β, and **10**, with an γRN/β. Furthermore, it was significant that polyamide **5**, with a β/γSO pairing, has a higher binding affinity to both 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ and to 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ than **9**, with a β/γSN, in particular, with the γSO monomer on the T side giving an approximately 4.6-fold increase. This difference may be caused by at least two factors. One is that (*S*)-α-hydroxy has a stronger ability to form hydrogen bonds with the N3 of guanine than the protonated (*S*)-α-amino; the other is that the bulkier

protonated amino has a steric clash with the minor groove, such as the NH of guanine, or induces serious distortion of the minor groove of the DNA. In short, these observations suggest that, although hydrogen-bonding ability is a key factor, other factors such as the surface complementarity of the DNA minor groove and the hairpin polyamide in the complex and van der Waals interactions may also play a role in DNA minor groove recognition by polyamides.

Molecular Modeling. To understand more clearly the mechanism for the strong and preferential binding of **5** to A·T over T·A, a plausible molecular model of the **5**−DNA complexes was estimated by energy minimization (Figure 3).[22a,24] As shown in Figure 3a, the hairpin polyamide **5** is significantly better docked in an antiparallel manner into the sequence 5′-GCAT-GC**A**CATCG-3′/5′-CGATG**T**GCATGC-3′, with A·T in the DNA minor groove, to make close contact with the bases recognized. As expected, b and d of Figure 3 both show that the α-hydroxy of the γ-monomer is indeed close to the N3 of the 3′-G and the proximity and the match between the γ residue and the G base in this conformation may induce the formation of a hydrogen bond between the OH and N3 of the G with bond lengths of 2.53 and 4.11 Å in the recognition by **5** of the sequences with A·T and T·A, respectively (Figure 3, c and e).

However, as can be seen in Figure 3d, the bulkier steric structure of the A base pushes the γ-monomer away from the floor of the minor groove to prevent the hydroxyl from forming a strong H-bonding interaction of the type seen in the **5**−A·T complex (Figure 3b) and/or influences more favorable aromatic overlap between polyamide monomers and the DNA bases in the **5**−T·A complex. These combined factors result in weaker binding affinity of **5** to the sequence with T·A. In summary, molecular modeling studies are in agreement with the SPR experimental results and provide a molecular rationale for the thermodynamic observations.

In conclusion, 12 new polyamides with different aliphatic amino acid pairings, *β*/*β*, *β*/γ, γ/*β*, γ/γ, *β*/γSO, *β*/γRO, γSO/*β*, γRO/*β*, *β*/γSN, *β*/γRN, γSN/*β*, γRN/*β*, have been designed and synthesized and their binding kinetic properties to hairpin DNA examined by SPR. Importantly, we found that the steric configuration of the α-substituent of the γ-aminobutyric acid residue in the polyamides could lead to alteration of their binding properties to predetermined DNA sequences relative to unmodified *β*-alanine or γ-aminobutyric acid. More importantly, the polyamide with the *β*/γSO pairing preferably bound to 5′-TGC**A**CA-3′/3′-ACG**T**GT-5′ over 5′-TGC**T**CA-3′/3′-ACG**A**GT-5′ with a 19-fold higher specificity, while the polyamide with the *β*/γSN has a moderate selectivity of 9.6-fold in the discrimination of these sequences. We found that the recognition by hairpin polyamides with an α-substituted-γ-aminobutyric acid in the DNA minor groove was significantly affected by the environment, here mainly the flanking bases, as well as the stereochemistry, steric bulk, electronic and hydrogen-binding ability of the substituent at the α-position of γ-aminobutyric acid. The combined results from the SPR and molecular modeling indicated that (*S*)-α-hydroxyl-γ-aminobutyric acid (γSO) may not only serve as a flexible subunit in the construction of polyamides but also as a 5′-TG-3′ recognition unit in the hairpin polyamides examined. The synthesis and examination of the recognition properties of longer polyamides containing the γSO monomer(s) as a "spring" to target longer specific DNA sequences are in progress.

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Experimental Section

General. The following abbreviations are used: Fmoc-ONSu: 9-fluorenyl succinimidyl carbonate, HOBt: 1-hydroxybenzotriazole, DCC: dicyclohexylcarbodiimide, DCM: dichloromethane, DMF: *N*,*N*-dimethylformamide, DIEA: *N*,*N*-diisopropylethylamine, TFA: trifluoroacetic acid, HATU: *N*-{(dimethylamino)-1H-1,2,3-triazolo[4,5-*b*]-pyridino-1-ylmethylene}-*N*-methylmethanaminium hexafluorophosphate, HOAc: acetic acid, Boc: *tert*-butoxycarbonyl, Fmoc: fluorenylmethoxycarbonyl, DMSO: dimethyl sulfoxide, *β* or *β*-head: *β*-alanine, γ or γ-turn: γ-aminobutyric acid, Hp: *N*-methyl-3-hydroxypyrrole, Py: *N*-methylpyrrole, Im: *N*-methylimidazole, Dp: 3-(dimethylamino)-propylamine, bp: base pairs, Hz: hydroxybenzimidazole, Ac: acetyl, γSN: (*S*)-α, γ-diaminobutyric acid, γRN: (*R*)-α, γ-diaminobutyric acid, γSO: (*S*)-α-hydroxyl-γ-aminobutyric acid, γRO: (*R*)-α-hydroxyl-γ-aminobutyric acid, TMSOTf: trimethylsilyl trifluoromethanesulfonate, TIS: triisopropylsilane, and DMS: dimethyl sulfide.

^{1}H NMR spectra were recorded on a JEOL JNM-FX400 Superconducting NMR spectrometer 400 MHz, with chemical shifts reported in parts per million relative to residual solvent and coupling constants in Hertz. The following abbreviations were applied to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), and br (broad). UV spectra were measured in HBS-EP buffer on a Beckman Coulter DU 650 diode array spectrophotometer. Mass spectra were recorded on the following mass spectrometers using a positive ionization mode (unless otherwise stated): matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with a BioTOF II from Bruker Daltonics and electrospray ionization mass spectrometry (ESI-Mass) with an API 165 from PE SCIEX (APPLIED BIOSYSTEMS). Biacore assays were performed on a Biacore *X* system (Sweden), and processing of data was carried out using BIAevaluation version 4.1. Glass plates precoated with silica gel 60F254 (Merck) were used for thin-layer chromatography (TLC) and silica gel 60 (63−200 μm) for column chromatography. Visualization was realized by UV exposure and/or with ninhydrin spray. High-performance liquid chromatography (HPLC) analysis was performed on a Tosoh CCP & 8020 series system using a 150 × 10 mm ChemcoPak Chemcoband 5-ODS-H reversed phase column in 0.1% AcOH in water with acetonitrile as eluent at a flow rate of 3.0 mL/min, and a linear gradient elution of 0 to 100% acetonitrile over 30 min with detection at 254 nm (unless otherwise stated). Preparatory reversed phase HPLC was performed on Tosoh CCP & 8020 series system using a 150 mm × 20 mm YMC-Pack R & D ODS D-ODS-5-ST S-5 μm reversed phase column in 0.1% AcOH in water with acetonitrile as the eluent at a flow rate of 6.0 mL/min, appropriate gradient elution conditions, and detection at 254 or 350 nm. Collected fractions were analyzed by ESI-Mass (PE SCIEX) or TOF-Mass (Bruker). All HPLC retention times quoted refer to the analytical column. A Millipore MilliQ water purification system was used to produce 18 MΩ water, and all buffers for Biacore assays were filtered at 0.22 μm and degassed. Hairpin DNAs were obtained from Proligo and used without further purification. HBS-EP buffer (0.01 M HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20) and sensor chip SA were purchased from Biacore AB (Sweden). Clear resin (0.52 mequiv/g), (*S*)-α-*tert*-butyloxycarbonylamino-γ-(9-fluorenylmethoxy)carbonylaminobutyric acid and (*R*)-α-*tert*-butyloxycarbonylamino-γ-(9-fluorenylmethoxy)carbonylaminobutyric acid were purchased from Peptides International. Fmoc-ImCOOH, Fmoc-PyCOOH, DMF, DMSO, piperidine, and pyridine were purchased from Wako, and Fmoc-*β*-Ala-OH, Fmoc-γ-Abu-COOH, and HOBt were from Novabiochem. DIEA, acetic anhydride, and *N*,*N*-dimethyl-1,3-propanediamine were from Nacalai Tesque, Inc. TFA was from Kanto Chemical Co., Inc. HATU was purchased from Peptide Institute, Inc. (*S*)-α-Hydroxyl-γ-aminobutyric acid (γSO) was from

Aldrich and (*R*)-α-hydroxyl-γ-aminobutyric acid (γRO) from Xiamen Forever Green Source Bio-Chem Technology Co., Ltd. DCC was fromTIC of Japan. TMSOTf was from Acros Organics. All other reagents and materials were from standard suppliers (highest quality available).

Monomer Synthesis. (2*S*)-4-[(9-Fluorenylmethoxy)carbonylamino]-2-hydroxybutyric Acid (14a) and (2*R*)-4-[(9-Fluorenylmethoxy)-carbonylamino]-2-hydroxybutyric Acid (14b). A solution of Fmoc-ONSu (6.72 g, 24 mmol) in 90 mL of 1,2-dimethyoxyethane was added dropwise to a solution of (*S*)-(−)-4-amino-2-hydroxybutyric acid (**13a**) (2.38 g, 20 mmol) and sodium hydrogen carbonate (2.0 g) in 30 mL of water with stirring at room temperature over 45 min, and the resultant solution was stirred for a further 15 h. The reaction was monitored by TLC with a mixture of DCM/methanol/acetic acid (4/1/0.2, v/v) as the developing solvent. The solution was directly acidified with concentrated hydrochloric acid and concentrated on a rotary evaporator. After the addition of 20 mL of ice water to the residue, the suspension was filtered, and then washed with a small amount of cold water to obtain a white solid. The filtrate was continuously concentrated, cold water was added, and the precipitate again was collected by filtration. The combined products were dried to remove traces of water to afford a white solid at a yield of 98.2%. The product was analyzed and shown to be 98% pure by HPLC and confirmed by ESI-Mass to give **14a**. Data for **14a**: R_f 0.70 (DCM/methanol/acetic acid 4/1/0.2, v/v). Analytical HPLC: t_R = 19.7 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.87 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.67 (d, $^3J_{H,H}$ = 7.2 Hz, 2H, Fluoren. H), 7.40 (m, 2H, Fluoren. H), 7.32 (m, 2H, Fluoren. H), 4.25 (m, 2H, OCH_2), 4.22 (m, 1H, Fluoren. H), 3.97 (dd, $^3J_{H,H}$ = 8.8 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, CH_2CH), 3.10 (m, 2H, $NHCH_2$), 1.78 and 1.59 (m, 2H, CH_2CH). ESI-Mass: calculated *m*/*z* for $C_{19}H_{19}NO_5$: 341.13; found: 342.12 $[M + H]^+$.

14b was synthesized according to the above procedure with a yield of 95.8% and 98% minimum purity by HPLC. Data for **14b**: R_f 0.71 (DCM/methanol/acetic acid 4/1/0.2, v/v). Analytical HPLC: t_R 19.75 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.87 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.67 (d, $^3J_{H,H}$ = 7.2 Hz, 2H, Fluoren. H), 7.40 (m, 2H, Fluoren. H), 7.32 (m, 2H, Fluoren. H), 4.26 (m, 2H, OCH_2), 4.20 (m, 1H, Fluoren. H), 3.96 (dd, $^3J_{H,H}$ = 8.7 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, CH_2CH), 3.08 (m, 2H, $NHCH_2$), 1.79 and 1.59 (m, 2H, CH_2CH). ESI-Mass: calculated *m*/*z* for $C_{19}H_{19}NO_5$: 341.13; found: 342.12 $[M + H]^+$, 364.12 $[M + Na]^+$, 380.10 $[M + K]^+$.

(2*S*)-2-*tert*-Butyloxy-4-(9-fluorenylmethoxy)carbonylaminobutyrate *tert*-Butyl Ester (15a) and (2*R*)-2-*tert*-Butyloxy-4-(9-fluorenylmethoxy)carbonylaminobutyrate *tert*-Butyl Ester (15b). Isobutylene (20 mL) was introduced to a solution of **14a** (1 g, 2.93 mmol) in 20 mL of dry DCM in the presence of 40 μL of concentrated sulfuric acid (98%) in a pressure flask with stirring at low temperature. The mixture was then very slowly warmed to room temperature and continuously stirred for another 40 h. The reaction was monitored by TLC with a mixture of ethyl acetate/hexane (1/2, v/v). After completion of the reaction, the solution was exposed to air at room temperature to remove excess isobutylene. Then, the solution was transferred to a separatory funnel, washed with 3 mL of water, and adjusted to pH 7.0 with 2 M aqueous sodium hydroxide. The dichloromethane layer was separated and the organic solvent carefully removed by rotary evaporation. Ethyl acetate (5 mL) and 3 mL of water were added to the residue, and the organic layer was washed with 3 mL of brine, dried with anhydrous sodium sulfate for 5 h, filtered, and dried after the removal of solvents to obtain 1.41 g of crude product as a light-yellow oil. The product was subjected to flash column chromatography on 63−200 μm silica gel using gradient elution of a mixture of ethyl acetate/hexane from 1/3 to 1/2 as eluents to afford finally 1.01 g of light-yellow oil with a yield of 76.5% at a 98% minimum purity by HPLC. The final product was confirmed by ESI-Mass to be the desired compound **15a**. Data for **15a**: R_f 0.52 (ethyl acetate/hexane, 1/2, v/v). Analytical HPLC: t_R = 29.70 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (d, $^3J_{H,H}$ = 7.2 Hz, 2H, Fluoren. H), 7.67 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.40 (m, 2H, Fluoren. H), 7.31 (m, 2H, Fluoren. H), 7.24 (t, $^3J_{H,H}$ = 4.8 Hz, 1H, NH), 4.28 (m, 2H, OCH_2), 4.20 (m, 1H, Fluoren. H), 3.88 (dd, $^3J_{H,H}$ = 8.8 Hz, $^3J_{H,H}$ = 3.6 Hz, 1H, CH_2CH), 3.04 (m, 2H, $NHCH_2$), 1.67 and 1.58 (m, 2H, CH_2CH), 1.39 (s, 9H, $COOCMe_3$), 1.09 (s, 9H, $OCMe_3$). ESI-Mass: *m*/*z* calculated for $C_{27}H_{35}NO_5$: 453.25; found: 454.26 $[M + H]^+$.

15b was synthesized according to the above procedure with a yield of 79.6% at 98% minimum purity by HPLC. Data for **15b**: R_f 0.53 (ethyl acetate/hexane, 1/2, v/v). Analytical HPLC: t_R = 29.70 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.66 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.40 (t, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.31 (m, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.23 (t, $^3J_{H,H}$ = 4.8 Hz,1H, NH), 4.28 (m, 2H, OCH_2), 4.19 (t, $^3J_{H,H}$ = 6.8 Hz, 1H, Fluoren. H), 3.89 (dd, $^3J_{H,H}$ = 8.8 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, CH_2CH), 3.03 (m, 2H, $NHCH_2$), 1.66 & 1.57 (m, 2H, CH_2CH), 1.39 (s, 9H, $COOCMe_3$), 1.08 (s, 9H, $OCMe_3$). ESI-Mass: *m*/*z* calculated for $C_{27}H_{35}NO_5$: 453.25; found: 454.27 $[M + H]^+$.

(2*S*)-2-*tert*-Butyloxy-4-(9-fluorenylmethoxy)carbonylaminobutyric Acid (17a) and (2*R*)-2-*tert*-Butyloxy-4-(9-fluorennylmethoxy)-carbonylaminobutyric Acid (17b). Trimethylsilyl trifluoromethanesulfonate (TMSOTf, 215.6 mg, 0.97 mmol) was added dropwise with stirring to a solution of **15a** (295.5 mg, 0.65 mmol) in 6.5 mL of dioxane in the presence of 150 μL of DIEA over 25 min at room temperature, and the resultant solution was continuously stirred for a further 50 min. The reaction was monitored by TLC with a mixture of ethyl acetate/hexane/acetic acid (2/1/0.01, v/v). After completion of the reaction, the reaction mix was cooled to room temperature; 5 mL of water and 8 mL of ethyl acetate were added promptly to the reaction mixture in an ice−water bath, and the solution was transferred to a separatory funnel. The organic layer was separated and washed with 5 mL of 5% aqueous sodium hydrogen carbonate. Because intermediate **16a** is very unstable and hydrolyzes easily to the target compound **17a** during the process of workup, the resulting mixture without further separation and purification was directly stirred for another 2 h at pH 5, adjusted with 10% aqueous hydrochloric acid. The reaction solution was then washed with 5 mL of 5% aqueous potassium hydrogen sulfate and 10% aqueous potassium sulfate, and 5 mL of brine, was dried over sodium sulfate, filtered and dried after the removal of solvents to give 239.3 mg of a white solid with a yield of 92.6% and 98% minimum purity by HPLC. The final product was confirmed by ESI-Mass to be the desired **17a**. Data for **16a**: R_f 0.46 (acetate/hexane/acetic acid, 2/1/0.01, v/v). Analytical HPLC: t_R = 25.83 min. ESI-Mass: *m*/*z* calculated for $C_{26}H_{35}NO_5Si$: 469.23; found: 470.21 $[M + H]^+$, 492.23 $[M + Na]^+$. Data for **17a**: R_f 0.28 (acetate/hexane/acetic acid, 2/1/0.01, v/v). Analytical HPLC: t_R = 24.01 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.3 (br, 1H, COOH), 7.87 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.67 (d, $^3J_{H,H}$ = 7.2 Hz, 2H, Fluoren. H), 7.40 (t,$^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.30 (t, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.25 (t, $^3J_{H,H}$ = 4.8 Hz,-1H, NH), 4.27 (d, $^3J_{H,H}$ = 6.4 Hz, 2H, OCH_2), 4.19 (t, $^3J_{H,H}$ = 6.8 Hz, 1H, Fluoren. H), 3.94 (dd, $^3J_{H,H}$ = 8.4 Hz, $^3J_{H,H}$ = 3.6 Hz, 1H, CH_2CH), 3.04 (m, 2H, $NHCH_2$), 1.71 & 1.60 (m, 2H, CH_2CH), 1.13 (s, 9H, $OCMe_3$). ESI-Mass: *m*/*z* calculated for $C_{23}H_{27}NO_5$: 397.19; found: 398.26 $[M + H]^+$, 420.17 $[M + Na]^+$; negative ionization, 396.18 $[M - H]^-$.

17b was synthesized according to the above procedure at a yield of 88% and 98% minimum purity by HPLC. Data for **16b**: R_f 0.48 (acetate/hexane/acetic acid, 2/1/0.01, v/v). Analytical HPLC: t_R = 25.9 min. ESI-Mass: *m*/*z* calculated for $C_{26}H_{35}NO_5Si$: 469.23; found: 470.22 $[M + H]^+$, 492.25 $[M + Na]^+$. Data for **17b**: R_f 0.28 (acetate/hexane/acetic acid, 2/1/0.01, v/v). Analytical HPLC: t_R = 24.0 min. $_1$H NMR (400 MHz, DMSO-d_6): δ 12.3 (br, 1H, COOH), 7.88 (d, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.67 (d, $^3J_{H,H}$ = 7.2 Hz, 2H, Fluoren. H), 7.40 (t,$^3J_{H,H}$ = 7.4 Hz, 2H, Fluoren. H), 7.31 (t, $^3J_{H,H}$ = 7.6 Hz, 2H, Fluoren. H), 7.26 (t, $^3J_{H,H}$ = 4.6 Hz,1H, NH), 4.28 (m, 2H, OCH_2), 4.19 (t, $^3J_{H,H}$ = 6.0 Hz, 1H, Fluoren. H), 3.95 (dd, $^3J_{H,H}$ = 8.2 Hz,

$^3J_{H,H}$ = 3.6 Hz, 1H, CH_2CH), 3.05 (m, 2H, $NHCH_2$), 1.71 & 1.60 (m, 2H, CH_2CH), 1.11 (s, 9H, $OCMe_3$). ESI-Mass: *m*/*z* calculated for $C_{23}H_{27}NO_5$: 397.19; found: 398.23 $[M + H]^+$, 420.16 $[M + Na]^+$; negative ionization, 396.17 $[M - H]^-$.

Determination of the Enantiomeric Purity of 17a and 17b. To **17a** or **17b** (7.95 mg, 0.02 mmol) in DMF (0.45 mL) were added HATU (22.8 mg, 0.06 mmol) and DIEA (4.5 μL). The resultant mixture was stirred at room temperature for approximately 15 min, and then (*S*)-(−)-1-(1-naphthyl)ethylamine (7.78 mg, 0.0454 mmol) was added. The mixture was continuously stirred at room temperature for 2 h. The reaction was monitored by TLC with a mixture of ethyl acetate/hexane (1/1, v/v). After the completion of the reaction, the solution was diluted with 1 mL of ethyl acetate, washed with 10% aqueous citric acid (2 × 1 mL) and brine (1 × 1 mL), respectively. The resulting organic layer was dried (anhydrous Na_2SO_4), filtered, concentrated, and chromatographed on a column packed with 63−200 μm silica gel using a mixture of ethyl acetate/hexane (2/1, v/v) to obtain 10.9 mg of the amide from **17a** (*S*) at a yield of 99% and 10.8 mg of the amide from **17b** (*R*) at a yield of 99% as white solids. The final products were analyzed by ^{1}H NMR (400 MHz, DMSO-d_6) and confirmed to be targets by ESI-Mass. The amide from **17a** (*S*) showed a triplet (J = 5.4) at 7.17 ppm and a doublet (J = 8.0) at 6.83 ppm, and the amide from **17b** (*R*) showed a triplet (J = 5.6) at 7.23 ppm and a doublet (J = 7.6) at 6.96 ppm. In both spectra, almost no evidence of diastereomeric contamination (<3%) was observed.

Polyamide Synthesis by Fmoc Chemistry. Machine-Assisted Solid-Phase Protocols. All machine-assisted polyamide syntheses were performed on a Pioneer Peptide Synthesizer (Applied Biosystems) with a computer-assisted operation system at a 0.10 mmol scale (200 mg of clear resin, 0.52 mequiv/g) by using Fmoc chemistry. Reagent positions were as follows: wash position, DMF; deblock position, 20% piperidine in DMF; activator position 1, 0.5 M HATU in DMF; activator position 2, 1.0 M DIEA in DMF; auxiliary reagent position 1, methanol; and auxiliary reagent position 3, 5% acetic anhydride and 5% pyridine in DMF. The following conditions were used in all polyamide solid-phase syntheses for each cycle: deblocking for 5 min with 20% piperidine/DMF, activating for 2 min with 0.5 M HATU/DMF and 1.0 M DIEA/DMF, coupling for 60 min, capping for 5 min with 5% acetic anhydride/5% pyridine/DMF, and final washing with methanol. A double-couple cycle was employed when coupling any monomer to imidazole and coupling of imidazole itself; all other couplings were carried out with single-couple cycles.

Typically, Fmoc-β-Ala-clear-acid resin (200 mg, 0.10 mmol) was swollen in 3 mL of DMF in a 15-mL plastic centrifuge tube for 30 min and loaded onto the reaction column. Glass scintillation tubes with loading monomers in DMF were placed in the racks, in order, from the front position to the back position. The synthesis was then started, controlled by the computer using the established program. All lines were emptied with argon before and after solution transfers.

After the completion of the synthesis on the peptide synthesizer, the resin was washed with a mixture of methanol/DCM (1/1, v/v) (3 × 1 mL) and dried in a desiccator at room temperature in vacuo. The resin was then placed in a 20-mL glass scintillation vial, 5 mL of (dimethylamino)propylamine was added, and the solution stirred at 55 °C overnight. Resin was removed by filtration through a pad of Celite and washed thoroughly with methanol/DCM (1/1, v/v). The resultant filtrate was acidified to pH 4−5 with 10% aqueous hydrochloric acid, and solvents were removed in vacuo. The residue was dissolved in approximately 0.5 mL of DMF, and insoluble substances were removed carefully using a 0.45 μm disposable syringe filter unit. The resulting polyamide solution was analyzed and purified by analytical HPLC at 254 nm and preparatory HPLC at 254 or 350 nm, respectively. After confirmation by ^{1}H NMR and ESI-Mass or TOF-Mass, appropriate fractions were lyophilized to give the final desired polyamides **1**, **2**, **3**, and **4** as white or light-yellow powders.

Manual Synthesis Protocol. To obtain polyamides **5**−12 with amino- or hydroxyl-modified monomers, the following additional step was involved. After polyamides were cleaved from resins **5a**−**12a** in the presence of (dimethylamino)propylamine, purified by HPLC and dried, Boc- or *tert*-butyl-protected polyamides were obtained and confirmed by ^{1}H NMR and ESI-Mass or TOF-Mass. Then, the products in the presence of a mixture of TFA/TIS/TMS/H_2O (91/3/3/3, v/v) (0.5 μL) were stirred at room temperature for 30 min under nitrogen, and solvents were removed by coevaporation with DCM in vacuo. Hydrochloric acid (0.5 mL, 5 M) was added to the residue, and solvent was removed in vacuo with a cold trap containing potassium hydroxide. The residue was subjected to analysis, then separation by HPLC, confirmation by ^{1}H NMR and ESI-Mass or TOF-Mass, and finally lyophilization to yield the final desired polyamides **5**−12 with amino- or hydroxyl-modified monomers as white or light-yellow powders.

Surface Plasmon Resonance (SPR) Assay. All SPR experiments were performed on a Biacore *X* instrument at 25 °C. The biotinylated hairpin DNAs: 5′-biotin-GGCCGATG**N**′GCATGCTATAGCATGC-**N**CATCGGCC-3′ (**N**·N′: T·A, A·T, G·C, and C·G) were obtained from Proligo. Streptavidin-functionalized SA sensor chips were purchased from Biacore. Immobilization of these hairpin DNAs onto the sensorchips, measurements of binding curves of polyamides to the hairpin DNAs, and data processing were performed according to following procedure.[25]

Immobilization of Biotinylated DNAs. After docking the streptavidin-coated sensor chip SA into the instrument, priming the system three times with HBS-EP buffer, normalizing the system with BIACORE normalizing solution (40% (w/w) glycerol in H_2O), and allowing the buffer to run for about 10 min at a rate of 10 μL/min until the baseline was stable, the sensor chip SA was preconditioned to remove any unbound streptavidin from the surface of the sensor chip with a solution of 1 M NaCl/50 mM NaOH at a rate of 10 μL/min with injection of 10 μL three to five times, and then allowing the buffer to run for 10 min to remove any trace of NaOH until the baseline was stable. Simultaneously, 20 μL of buffer was injected three times to wash the loop at the same flow rate. Before immobilizing DNA on the sensor chip, 100 μL of 5′-biotinylated DNA solution in running buffer without DMSO was renatured by heating to 85 °C for 2 min and slowly cooled to room temperature. DNA was then immobilized onto the chosen flow cell by injecting appropriate amounts of DNA solution at a flow rate of 2 μL/min to obtain the wanted immobilization level. After the DNA injection was complete, the buffer was kept running through the cell until the response units on the sensorgram became stable. An unmodified flow cell was used as a control for matrix effects.

General Procedures for SPR Binding Studies. Different concentrations of polyamide solutions in HBS-EP buffer with 0.1% DMSO were prepared by dilutions from 10 mM of stock solution in DMSO, and concentrations were checked by UV spectroscopy at 294 nm. Unless otherwise noted, all binding experiments were carried out using HBS-EP buffer with 0.1% DMSO as running buffer, and the running buffer or 10 mM glycine-HCl (pH = 1.5) was used as regeneration solution. Typically, the buffer was injected at a flow rate of 5 μL/min as a blank control after a stable baseline was obtained, and then samples were injected under identical conditions to those of buffer injection at concentrations ranging from 5 nM to 5 μM. To minimize carryover, samples were injected in the order of increasing concentration. Kinetic information was obtained by global fitting of the response units vs time using a model with mass transport effects with the BIA evaluation 4.1 program.

Molecular Modeling Studies. Minimizations were performed with the Discover (MSI, San Diego, CA) program using CVFF force-field

(25) (a) Supekova, L.; Pezacki, J. P.; Su, A. I.; Loweth, C. J.; Riedl, R.; Geierstanger, B.; Schultz, P. G.; Wemmer, D. E. *Chem. Biol.* **2002**, *9*, 821−827. (b) Wong, C. H.; Hendrix, M.; Manning, D. D.; Rosenbohm, C.; Greenberg, W. A. *J. Am. Chem. Soc.* **1998**, *120*, 8319−8327. (c) Rich, R. L.; Myszka, D. G. *Curr. Opin. Biotechnol.* **2000**, *11*, 54−61.

parameters. The starting structure was built based on the NMR structure of the ImPyPy-γ-PyPyPy-5′-d(CGCTAACAGGC)-3′/5′-d(GCCTGTTAGCG)-3′ complex.[22a] Aliphatic monomers were inserted into the polyamide by using standard bond lengths and angles. Twenty-two Na cations were placed at the bifurcating position of the O−P−O angle at a distance of 2.21 Å from the phosphorus atom. The resulting complex was soaked in a 10 Å layer of water. The whole system was minimized without constraints to the stage where the RMS was less than 0.001 kcal/mol Å.

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Supporting Information Available: Data for synthesis and characterization of polyamides **1**−**12**, sensorgrams of polyamides **6** and **10** for recognition of 5′-TGC**N**CA-3′ (**N** = T, A), and the free energy changes before and after binding of polyamides **1**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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