Strand Selective Cleavage of DNA by Diastereomers of Hairpin Polyamide-*seco*-CBI Conjugates

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Abstract: Pyrrole-imidazole polyamides are synthetic ligands that bind predetermined DNA sequences with subnanomolar affinity. We report the synthesis and characterization of an eight-ring hairpin polyamide conjugated at the turn to both enantiomers of 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI), an alkylating moiety related to CC-1065. Alkylation yields and specificity were determined on a restriction fragment containing six base pair match and mismatch sites. Alkylation was observed at a single adenine flanking the polyamide binding site, and strand selective cleavage could be achieved based on the enantiomer of *seco*-CBI chosen. At 1 nM concentrations of polyamide-*seco*-CBI conjugate, near quantitative cleavage was observed after 12 h. These bifunctional molecules could be useful for targeting coding regions of genes and inhibition of transcription.

Introduction

Pyrrole-imidazole hairpin polyamides have affinity and specificity for DNA comparable to naturally occurring DNA binding proteins.[1−5] Sequence-specific DNA recognition in the minor groove depends on the sequence of side-by-side aromatic amino acid pairings oriented N to C with respect to the 5′ to 3′ direction of the DNA helix. Antiparallel pairing of imidazole opposite pyrrole (Im/Py) recognizes a G·C base pair, while a Py/Im combination recognizes C·G.[2,5−11] A Py/Py pair is partially degenerate and specifies for A·T or T·A base pairs.[12−15] A hydroxypyrrole-pyrrole pairing (Hp/Py) distinguishes T·A from A·T (Figure 1).[16,17] Eight-ring hairpin polyamides can bind a 6 base pair match sequence at subnanomolar concentrations with good sensitivity to mismatch sequences.[5] It has been demonstrated that hairpin polyamides permeate cellular and nuclear membranes of eukaryotes, and when targeted to promoter regions can inhibit specific gene expression.[18,19] In addition to competing with transcription factors in promoters, would it be possible to target a gene's coding region with small synthetic ligands? In unpublished work we have found that hairpin polyamides targeted to coding regions do not inhibit RNA polymerase II gene expression. We would be interested in asking whether bifunctional DNA binding hairpins that covalently interact with the minor groove of DNA could inhibit polymerase elongation. This could create a new class of gene specific "knockout" reagents which may be useful in biological disciplines.

Design. The design of sequence specific DNA alkylating agents requires the integration of two separate functional moieties for recognition and reaction. Hairpin Im-Py polyamides are suitable for DNA recognition due to their high affinity and specificity for the minor groove of DNA. The reactive moiety must specifically alkylate in the minor groove proximal to the hairpin polyamide target site, with covalent reaction yields that are near quantitative. To maximize stoichiometric reaction on the DNA, the "electrophilic functionality" must be reactive with DNA at 37 °C, be inert in aqueous media and buffer components, and not suffer unimolecular decomposition in competition with the desired reaction with DNA. The reactive moiety of the natural product (+)-CC-1065 and its analogues meets these criteria for our design of bifunctional molecules for sequence specific alkylation of DNA.

We report here our efforts in the design of bifunctional polyamides with covalently attached alkylating agents (Figure

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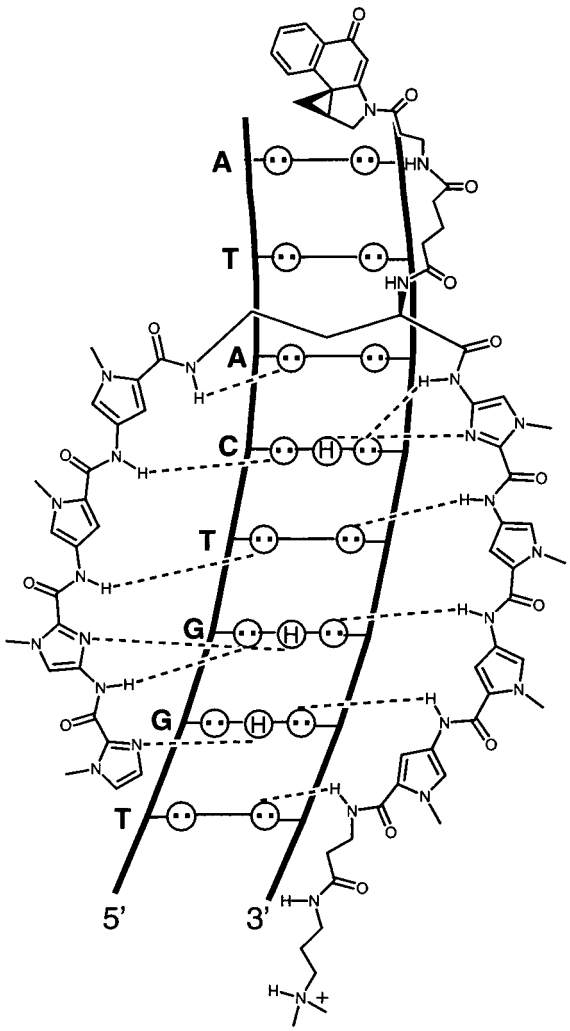

Figure 1. (Top) Hydrogen bond model of the polyamide−DNA complex formed by the polyamide ImImPyPy-$\gamma^{(S)-CBI}$-ImPyPyPy-β-Dp (**4S**) bound to the minor groove of 5′-TGGTCA-3′. Circles with dots represent lone pairs of N3 purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogen of G. Putative hydrogen bonds are illustrated by dotted lines. (Bottom) Binding model for polyamide ImImPyPy-$\gamma^{(S)-CBI}$-ImPyPyPy-β-Dp (**4S**) with a 5′-TGGTCA-3′ site. Shaded and nonshaded circles denote imidazole (Im) and pyrrole (Py) rings, respectively. Diamonds and hatched triangles represent β-alanine (β) and (*S*)-CBI, respectively. (*R*)-2,4-Diaminobutyric acid (γ) and dimethylaminopropylamine (Dp) are depicted as a curved line and a plus sign, respectively.

1). We have chosen 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI), a precursor to 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI),[20,21] an analogue of (+)-CC-1065. Boger and co-workers have shown that CBI affords increased reactivity to DNA as well as increased stability to solvolysis.[22] The *seco* agents readily close to the cyclopropane forms and have equivalent reactivity as compared to CBI, but have longer shelf lives.[23] In our design we attach the *seco*-CBI unit to the hairpin turn.[24] In addition, we chose to study the two different diastereomers which could afford *opposite strand reactivity* in the minor groove depending on mirror image (Figure 1).

A key feature in any attempt to construct gene knock-out reagents for the purpose of cell culture and whole animals experiments will be attention to the *yield of covalent modification*. Recent work by Sugiyama has attached Duocarmycin A, a different analogue of CC-1065, to the C-terminus of a hairpin polyamide in a report on this class of molecules. We believe that it will be interesting to compare the two.[25]

Results

Synthesis of Polyamides. An eight-ring hairpin polyamide of sequence composition ImImPyPy-(*R*)$^{H_2N}\gamma$-ImPyPyPy-β-Dp (**1**) was synthesized, which according to the pairing rules binds the sequence 5′-(A/T)GG(A/T)C(A/T)-3′. Polyamide **1** has been shown to bind to the sequence 5′-TGGTCA-3′ with a K_a of 1.3×10^{10} M^{-1}.[17] Polyamide **1** was prepared by manual solid-phase peptide synthesis.[26] After reverse phase HPLC purification, an NHS-activated glutaric acid linker was attached to the α-amino group on the γ-turn to afford modified polyamide **2**. After HPLC purification, the appropriate enantiomer of β-alanine linked *seco*-CBI (**3R** or **3S**) was coupled using DCC/NHS activation to give the corresponding polyamide-*seco*-CBI conjugates **4R** and **4S** (Figure 2).

The control *seco*-CBI analogues (**7R** and **7S**) with the same charge as the polyamide analogues were prepared by coupling the enantiomerically pure *seco*-CBI (**5R** and **5S**)[27−29] to Boc-β-alanine. These β-alanine derivatives (**6R** and **6S**) were deprotected and coupled to *N*,*N*-dimethyl-γ-amino butyric acid, and purified by reverse phase HPLC to give compounds **7R** and **7S** (Figure 3).

Thermally Induced Cleavage Reactions. To examine the alkylation specificity and reaction yields of the polyamide-*seco*-CBI conjugates (**4R** and **4S**), thermally induced cleavage assays were performed on a 277 base pair restriction fragment containing two match sites, 5′-AGGACT-3′ and 5′-TGGTCA-3′, and one mismatch site, 5′-AGGAGT-3′ (mismatch underlined), with different A/T tracts flanking the 3′ side (Figure 4).[30] Polyamide **4R** at a concentration of 500 pM alkylates a single adenine on the 3′-labeled (bottom) strand two base pairs to the 3′ side of the match site 5′-AGGACT-3′ with a yield of 74.5% (Figure 5b). At 200 pM concentrations of polyamide **4R** a second cleavage site appears on the same strand at a single adenine proximal to the mismatch site 5′-AGGAGT-3′. At 1 nM concentrations of **4R** and 12 h of equilibration near quantitative cleavage (96%) of the intact DNA is observed (Figure 5b). Remarkably, the reaction appears to be strand specific. No cleavage appears on the 5′-labeled (top) strand (Figure 5a).

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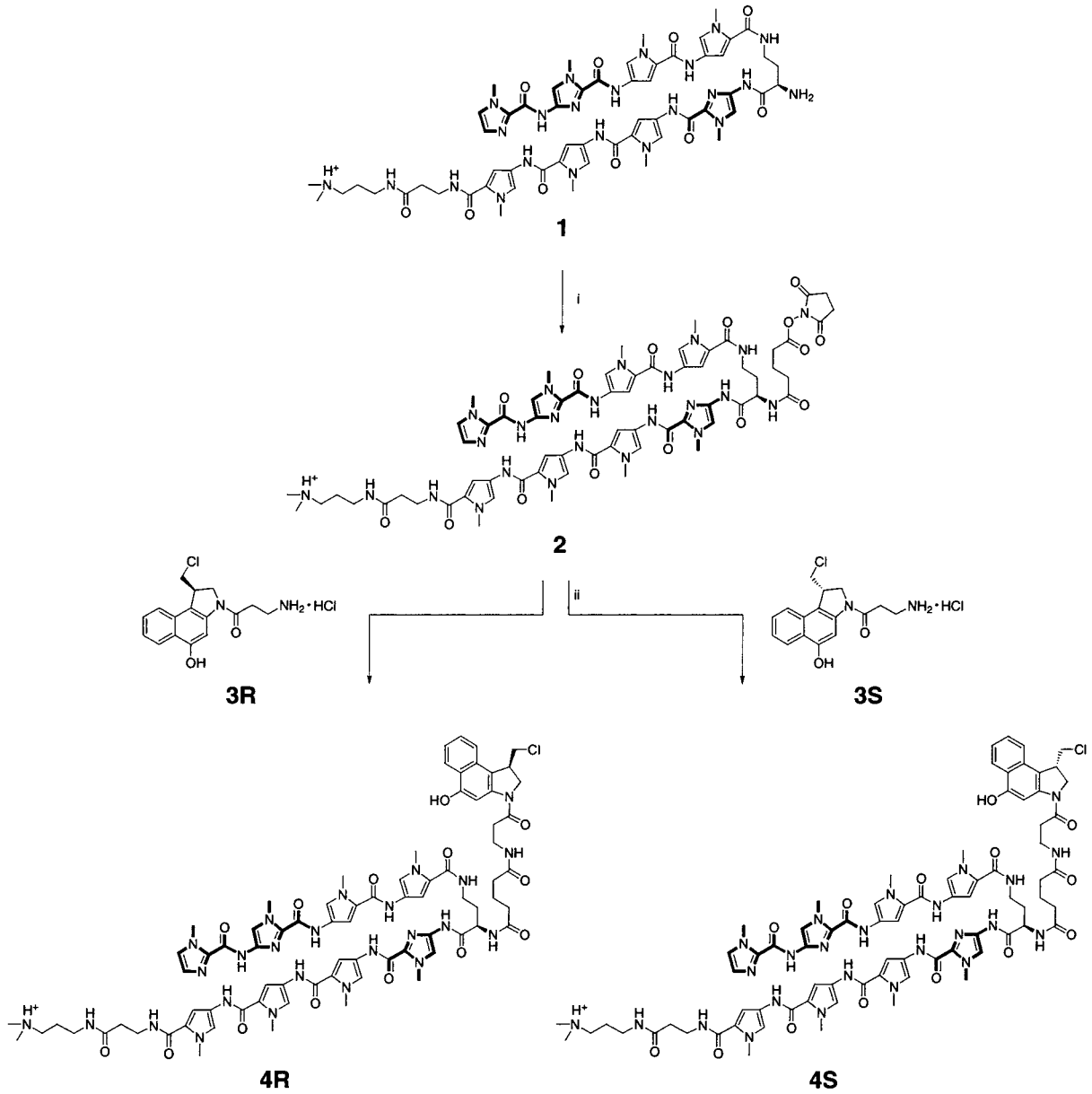

Figure 2. Synthetic scheme for the preparation of *seco*-CBI-polyamide conjugates **4R** and **4S**: (i) Disuccinimidyl glutarate, DMF, 2 h; (ii) **3R** or **3S**, DCC, NHS, DIEA, DMF, 3 h. Polyamide **1** was prepared by already disclosed methods.

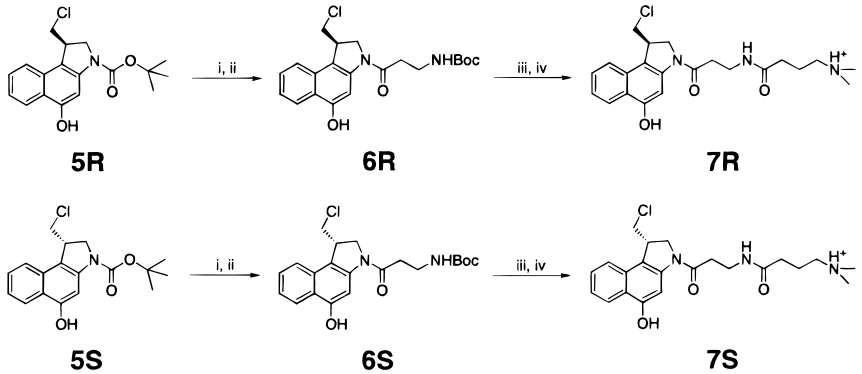

Figure 3. Synthetic scheme for charged control compounds **7R** and **7S**. (i) 3 M HCl/EtOAc, 30 min; (ii) Boc-β-alanine, HBTU, DIEA, DMF; (iii) 3 M HCl/EtOAc, 30 min; (iv) 4-dimethylamino butyric acid, HBTU, DIEA, DMF.

In constrast, polyamide **4S** at 50 pM concentrations affords one major cleavage site on the 5′-labeled (top) strand corresponding to the adenine two base pairs to the 3′ side of the polyamide match site 5′-TGGTCA-3′. At 1 nM concentrations of **4S** cleavage yields of 42% are observed (Figure 5a). On the 3′ labeled (bottom) strand, only minor alkylation and cleavage

Figure 4. Illustration of the 277 base pair *EcoRI/PvuII* restriction fragment with the position of the sequence indicated. The binding sites for polyamide **1**, 5′-AGGACT-3′, and 5′-TGGTCA-3′ are boxed. A single base pair mismatch site is also boxed, the mismatch shaded.

is observed at four sites in low yield (Figure 5b). The two sites which can be assigned are proximal to a match 5′-AGGACT-3′ and mismatch site 5′-AGGAGT-3′, respectively.

Time Course Experiments. To examine the time dependence of alkylation, reactions consisting of polyamides **4R** or **4S** at 1 nM concentrations were analyzed throughout a period of 10 h. For the 5′-labeled strand, alkylation by **4S** was first detected at 1 h of incubation at 37 °C. After 10 h, 24% of the DNA was cleaved proximal to the match site, 5′-TGGTCA-3′. **4R** shows no alkylation on this strand (Figure 6a). However, **4R** shows alkylation on the 3′-labeled strand after 15 min, and at 5 h it has 76% yield proximal to the match site 5′-AGGACT-3′ and 13% at the mismatch site 5′-AGGAGT-3′. After 10 h, **4S** reveals minor cleavage products, as described above for the titration experiments, each ranging from 1.5 to 17% yield (Figure 6b).

Comparison of Conjugates to Nonlinked Analogues. To study the ability of polyamides to direct the reactivity of CBI analogues, alkylations by the unlinked analogues were analyzed as controls (Figure 7). Analogue **7R** shows virtually no cleavage products at 1 μM concentrations, 3 orders of magnitude higher concentrations than that used in the conjugate studies. At 10 μM concentrations, **7R** shows several cleavage products, but with yields only between 1.1% and 5.1% on either strand. **7S** is shown to be a more efficient alkylating agent than **7R**. On the 5′-labeled strand, at 1 μM **7S**, the majority of the cleavage products are at 1% yield or lower. On the 3′-labeled strand, the efficiency ranges from less than 1% to 7.7% at the same concentrations. At 10 μM **7S**, the experiments show quantitative cleavage of the intact DNA for both strands, and the efficiency of alkylation on all sites increases accordingly. The yields range from 2 to 25% and 2 to 35% on the 3′- and 5′-labeled strands, respectively. The consensus sequence for both enantiomers of CBI is an adenine for alkylation, a >95% preference for an A·T base pair to the 5′ side of the adduct, and a 66% preference for a purine to the 3′ side.[21] The alkylation pattern observed for both **7R** and **7S** shows that the AT tracts are particularly reactive sites, but the selectivity is difficult to predict (Figure 8).

Discussion

Strand Selective Cleavage. One of the striking differences between polyamide-*seco*-CBI conjugates **4R** and **4S** is the strand selectivity of alkylation shown by the respective diastereomers (Figure 8). This is the result of combining the DNA binding properties of the polyamide and the opposite strand alkylation preference for each mirror image of CBI in the minor groove of DNA. The data support a model wherein the hairpin polyamide binds both match and mismatch sites and directs the chiral CBI moiety to A/T tracts proximal to the bound sites. The electrophilic cyclopropyl carbon of the *R*- and *S*-CBI reacts at different rates with the N3 of adenine on opposite strands on the floor of the minor groove. The specificity of reaction at specific adenines represents the ratios of two unimolecular rate constants: the dissociation rate of the polyamide from the minor groove of DNA (k_{OFF}) and the alkylation rate of CBI at N3 of adenine in the minor groove of DNA (k_{ALK}). When the polyamide binds to a match site, k_{OFF} is expected to be slow relative to mismatch sites. Similarly, k_{ALK} will be faster for sites which accommodate the orientation and sequence preferences of CBI as in pure A/T tracts, and slower for mixed sequences. These ratios of k_{OFF}/k_{ALK} at different DNA sites give the variation in alkylation yields seen in these experiments. On the 5′-labeled (top) strand, polyamide **4S** has one major cleavage site (Figures 5a and 6a) that is due to polyamide binding the match site 5′-TGGTCA-3′ (slow k_{OFF}) and an adenine in the appropriate position proximal to the match site for alkylation (fast k_{ALK}). Likewise, on the 3′-labeled (bottom) strand, polyamide **4R** has a major cleavage site (Figure 5b and 6b) due to polyamide binding the match site 5′-AGGACT-3′ (slow k_{OFF}) and an adenine on the opposite strand for alkylation (fast k_{ALK}). For the two DNA match sites bound by each diastereomeric polyamide-*seco*-CBI conjugate (**4R** and **4S**), k_{OFF} rates are expected to be similar. The variation in alkylation yields in these two instances most likely results from k_{ALK} being faster for the sequence proximal to the match site for **4R** than for that of **4S**.

Similar comparisons can be made for alkylation seen at mismatch sites. On the 3′-labeled (bottom) strand, polyamide **4R** has a minor cleavage site (Figures 5b and 6b) due to polyamide binding the single base pair mismatch site 5′-AGGAGT-3′ (fast k_{OFF}). But, because of favorable sequence contexts k_{ALK} is also fast. The alkylation at this site results from k_{ALK} being competitive with k_{OFF}, trapping that particular binding event. Also on the 3′-labeled (bottom) strand, polyamide **4S** has two minor cleavage sites that can be assigned (Figures 5b and 6b). For the alkylation seen adjacent to the match site 5′-AGGACT-3′, k_{OFF} is expected to be slow. In this case, k_{ALK} is also expected to be slow because the orientation preferences of *S*-*seco*-CBI are not optimal at this site. However, the binding event can be trapped because k_{ALK} may be competitive with k_{OFF} in this instance. **4S** also affords an alkylation product next to the single base pair mismatch site 5′-AGGAGT-3′. While k_{OFF} is expected to be faster in this case than for that of a match site, it can still be trapped for situations where k_{ALK} is faster than k_{OFF}.

It has been shown for CC-1065 and other analogues that the *S*, or (+), enantiomers are 10 times more reactive than the R, or (−), enantiomers.[22,31−33] The data shown for the control compounds **7R** and **7S** support this conclusion (Figure 7). It is

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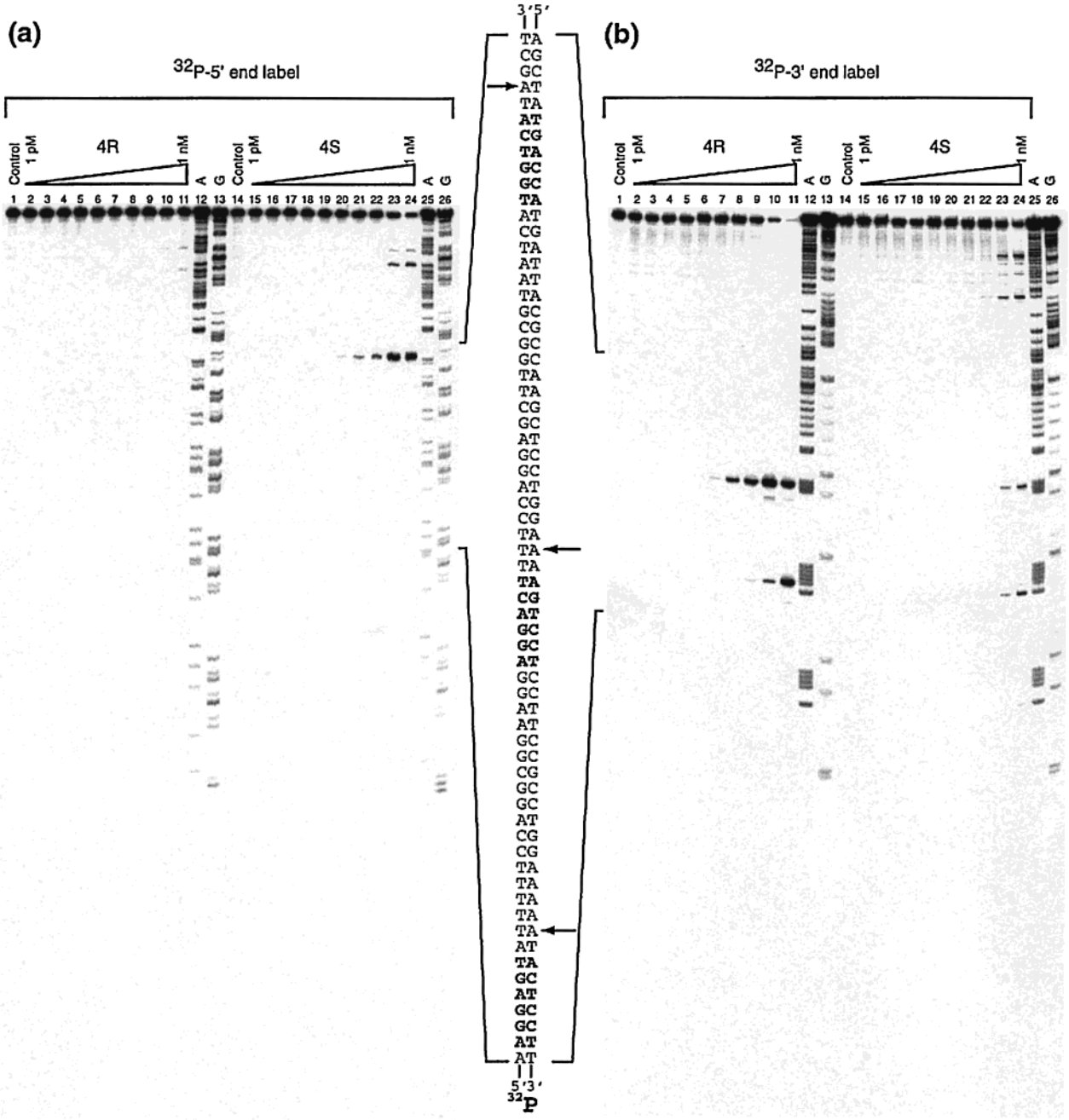

Figure 5. Thermally induced strand cleavage on the 5′-end labeled and 3′-end labeled 277 base pair restriction fragment by ImImPyPy-$\gamma^{(R)-seco-\mathrm{CBI}}$-ImPyPyPy-$\beta$-Dp (**4R**) and ImImPyPy-$\gamma^{(S)-seco-\mathrm{CBI}}$-ImPyPyPy-$\beta$-Dp (**4S**). Storage phosphor autoradiograms of 8% denaturing polyacrylamide gels used to separate the fragments generated by heat induced DNA cleavage at alkylation sites. All lanes contain 10 kcpm of either 5′ or 3′ radiolabeled DNA. Each reaction was equilibrated in TE, pH 7.5, at 37 °C for 12 h. The unbound polyamide was removed by precipitation, and then strand cleavage was induced by heating at 95 °C for 30 min. (a) 5′-^{32}P-end labeled restriction fragment. (b) 3′-^{32}P-end labeled restriction fragment. (a,b) Lanes 1 and 14, intact DNA; lanes 2−11, 15−24, 1 pM, 2 pM, 5 pM, 10 pM, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 nM respectively of the corresponding polyamide; lanes 12 and 25, A-specific reaction; lanes 13 and 26, G-specific reaction. (a,b) Match sites 5′-TGGTCA-3′ and 5′-AGGACT-3′ and single base pair mismatch site 5′-AGGAGT-3′ are indicated in bold on the sequence, with arrows indicating cleavage bands.

interesting then that polyamide conjugate **4R** shows higher alkylation efficiency and faster rates of alkylation than **4S**. Work by Lukhtanov and co-workers has shown that when an oligonucleotide-cyclopropapyrroloindole conjugate is hybridized to a variety of complementary oligonucleotide hairpins, rates of reaction can be quite rapid, and yields can be quantitative.[34] But the results are sequence dependent. For both enantiomers, pure A/T tracts allow for fast and efficient alkylation ($t_{1/2}$ as fast as 2 min), while mixed sequences seem to have slower and more unpredictable rates of reactivity. In our studies, the pure A/T tracts flanking the polyamide match sites differ and we may simply be observing sequence dependent differences in rates of reaction as a result of different adenine reactivities in various sequence contexts.

Comparison with Hairpin Polyamide Duocarmycin A Conjugates. Previous work by Sugiyama and co-workers has established that Duocarmycin A (Duo), an analogue of CC-1065, will alkylate a guanine preferentially when bound as a

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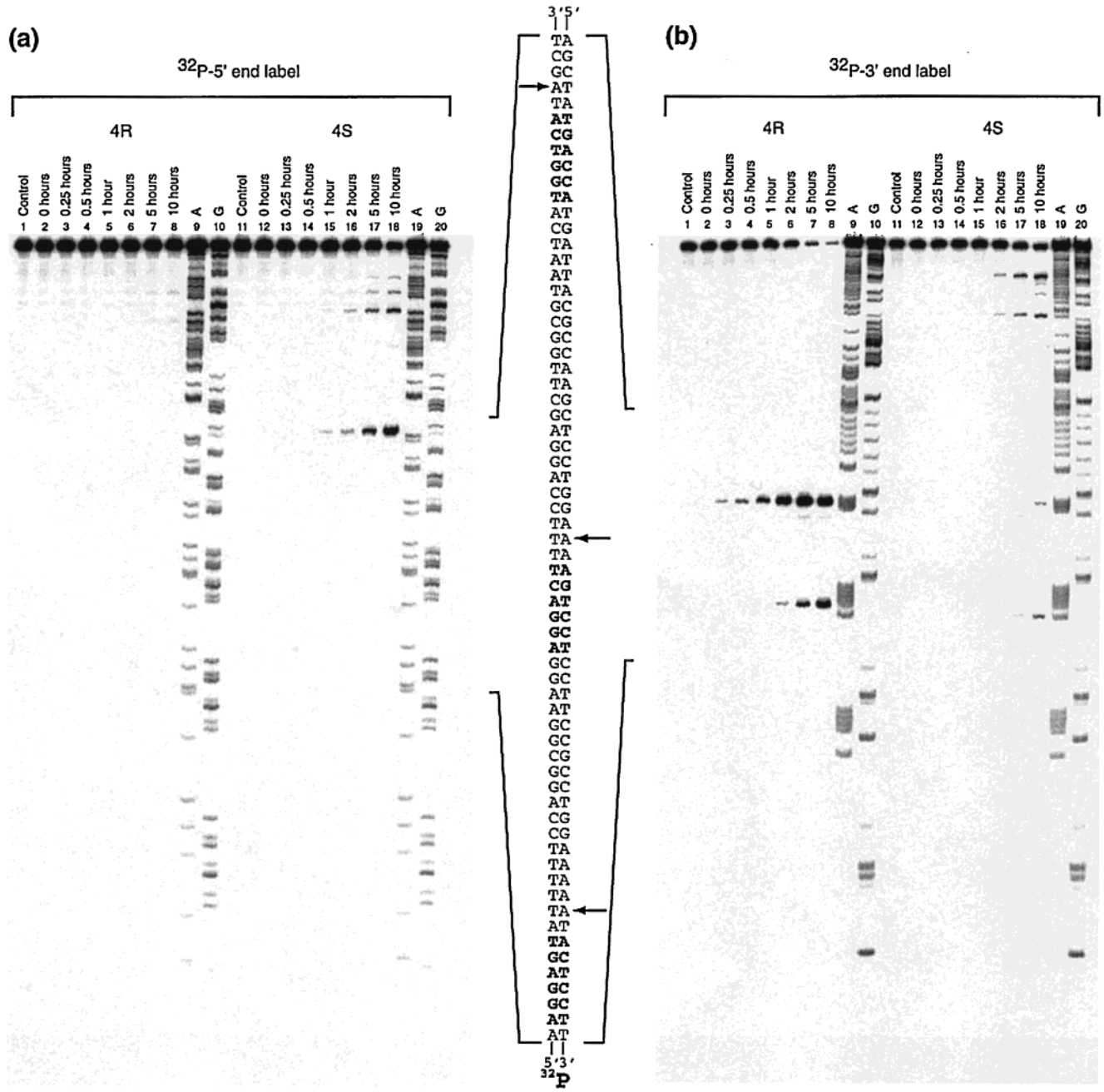

Figure 6. Time course experiment of thermally induced strand cleavage on the 5′-end labeled and 3′-end labeled 277 base pair restriction fragment by ImImPyPy-$\gamma^{(R)-seco-CBI}$-ImPyPyPy-β-Dp (**4R**) and ImImPyPy-$\gamma^{(S)-seco-CBI}$-ImPyPyPy-β-Dp (**4S**). Storage phosphor autoradiograms of 8% denaturing polyacrylamide gels used to separate the fragments generated by heat induced DNA cleavage at alkylation sites. All lanes contain 10 kcpm of either 5′ or 3′ radiolabeled DNA. Each reaction was equilibrated in TE, pH 7.5, at 37 °C from 0 to 10 h. The unbound polyamide was removed by precipitation, and then strand cleavage was induced by heating at 95 °C for 30 min. (a) 5′-^{32}P-end labeled restriction fragment. (b) 3′-^{32}P-end labeled restriction fragment. (a,b) Lanes 1 and 11, intact DNA; lanes 2−8, 1 nM of corresponding polyamide, equilibrations for 0, 15 min, 30 min, 1 h, 2 h, 5 h, and 10 h, respectively; lanes 9 and 19, A-specific reaction; lanes 10 and 20, G-specific reaction. (a,b) Match sites 5′-TGGTCA-3′ and 5′-AGGACT-3′ and single base pair mismatch site 5′-AGGAGT-3′ are indicated in bold on the sequence, with arrows indicating cleavage bands.

heterodimer with distamycin.[35] Sugiyama and co-workers have recently described pyrrole-imidazole hairpin polyamide conjugates which incorporate the natural enantiomer of Duo at the C-terminus.[25] These molecules demonstrate the same altered reactivity at guanine, and target purines proximal to a hairpin polyamide binding site. Our choice of using both enantiomers of *seco*-CBI was driven by the orientation preferences of CC-1065 necessary for alkylation. These orientation preferences suggested that it might be possible to target either strand of the DNA duplex, based on the binding orientation of *seco*-CBI at that site. We have shown that the same hairpin polyamide can be used to target opposite strands of the DNA depending on the enantiomer of *seco*-CBI we choose.

With the 6-ring hairpin polyamide-Duo conjugates, the highest yield Sugiyama and co-workers report is 7.4% cleavage at 800 nM polyamide for 7 days at room temperature.[25] Despite the rather slow reaction kinetics, this compound shows very good specificity for its match site. No other cleavage sites are seen for this compound on the fragment analyzed. Our 8-ring hairpin polyamide-*seco*-CBI conjugate shows higher yields of

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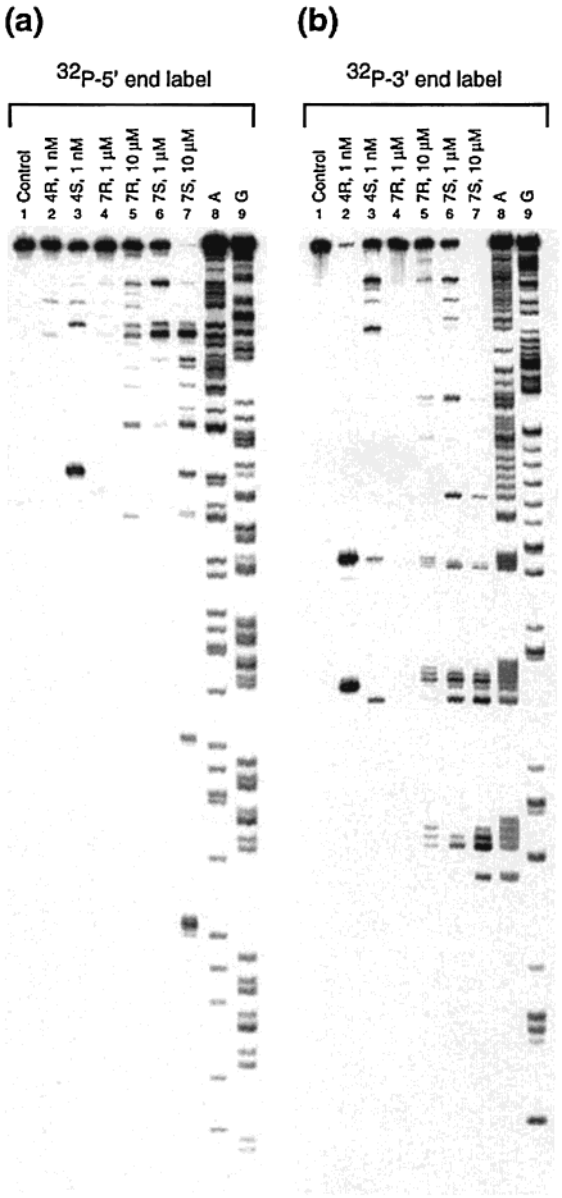

Figure 7. Thermally induced strand cleavage on the 5′-end labeled and 3′-end labeled 277 base pair restriction fragment by ImImPyPy-$\gamma^{(R)-seco-\mathrm{CBI}}$-ImPyPyPy-$\beta$-Dp (**4R**), ImImPyPy-$\gamma^{(S)-seco-\mathrm{CBI}}$-ImPyPyPy-$\beta$-Dp (**4S**), (*R*)-*seco*-CBI-β-dimethyl-γ (**7R**), and (*S*)-*seco*-CBI-β-dimethyl-γ (**7S**). Storage phosphor autoradiograms of 8% denaturing polyacrylamide gels used to separate the fragments generated by heat induced DNA cleavage at alkylation sites. All lanes contain 10 kcpm of either 5′ or 3′ radiolabeled DNA. Each reaction was equilibrated in TE, pH 7.5, at 37 °C for 12 h. The unbound polyamide or agent was removed by precipitation, and then strand cleavage was induced by heating at 95 °C for 30 min. (a) 5′-^{32}P-end labeled restriction fragment. (b) 3′-^{32}P-end labeled restriction fragment. (a,b) Lane 1, intact DNA; lane 2, **4R**, 1 nM; lane 3 **4S**, 1 nM; lanes 4−5, **7R**, 1 and 10 μM, respectively; lanes 6−7, **7S**, 1 and 10 μM, respectively; lane 8, A-specific reaction; lane 9, G-specific reaction.

alkylation at faster rates and lower concentrations of polyamide: 42% (at a single site) for **4S** at 1 nM after 12 h and 74.5% (at a single site) for **4R** at 500 pM after 12 h, both at 37 °C. But, we do see contributions from mismatch alkylation sites: 7.4% for **4R** at 500 pM after 12 h at 37 °C. Because the two systems are very distinct, it is difficult to interpret the differences in the results. It is unclear how differences in hairpin polyamide (6 ring versus 8 ring), place of attachment (C-terminus versus turn), linker length (amide versus longer methylene), and alkylation conditions (time, temperature, ligand concentration, DNA concentration) will affect the overall reaction. It is worth noting that both sets of results show alkylation directed by hairpin polyamides in a sequence specific fashion. Differences in yields and rates of reaction can be a consequence of many factors, which will require more studies for detailed understanding.

Conclusion. Hairpin polyamide-CBI conjugates have been shown to efficiently and selectively alkylate a single adenine adjacent to a polyamide match site. Because of the high efficiency of alkylation, these molecules should be useful in the design of reagents that target a single gene. It has already been shown that triplex forming oligonucleotide-psoralen and nitrogen mustard conjugates form covalent adducts in the major groove on the coding strand to inhibit elongation of transcription.[36] It remains to be seen whether this class of polyamide-CBI conjugates which react in the minor groove will be useful for functional genomics.

Experimental Section

Materials. ^{1}H NMR spectra were recorded on a General Electric-QE NMR spectrometer at 300 MHz and a Varian Inova NMR spectrometer at 500 MHz with chemical shifts reported in parts per million relative to residual solvent. UV spectra were measured in water on a Hewlett-Packard Model 8452A diode array spectrophotometer. Optical rotations were recorded on a JASCO Dip 1000 digital polarimeter. Matrix-assisted, laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was performed at the Protein and Peptide Microanalytical Facility at the California Institute of Technology. High-resolution mass spectometry was performed at the mass spectrometry facility at the University of California at Los Angeles. Preparatory reversed phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 × 100 mm, 300 Å C18 column equipped with a guard, 0.1% (wt/v) TFA, 0.25% acetonitrile/min.

Synthesis of CBI-Polyamide Conjugates: ImImPyPy-$(R)^{H_2N}\gamma$-ImPyPyPy-β-Dp (1). ImImPyPy-$(R)^{H_2N}\gamma$-ImPyPyPy-β-Pam resin was synthesized in a stepwise fashion by Boc-chemistry manual solid-phase protocols. A sample of resin was treated with neat (dimethylamino)-propylamine (2 mL), heated (55 °C, 24 h), and purified by reversed phase HPLC. ImImPyPy-$(R)^{H_2N}\gamma$-ImPyPyPy-β-Dp was recovered as a white powder upon lyophilization of the appropriate fraction (14.3 mg, 11.6 mmol, 4.8% recovery). UV (H_2O) λ_{max} (ϵ) 312 nm (66 600). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.009 (s, 1H), 10.325 (s, 1H), 10.073 (s, 1H), 9.947 (s, 1H), 9.936 (s, 1H), 9.870 (s, 1H), 9.07 (s, 1H), 9.23 (br s, 1H, CF_3COOH), 8.334 (s, 3H), 8.173 (t, 1H, J = 6 Hz), 8.040 (t, 1H, J = 6 Hz), 8.012 (t, 1H, J = 6 Hz), 7.567 (s, 1H), 7.530 (s, 1H), 7.457 (s, 1H), 7.270 (s, 1H), 7.256 (s, 1H), 7.200 (s, 1H), 7.177 (s, 1H), 7.164 (s, 1H), 7.155 (s, 1H), 7.142 (s, 1H), 7.071 (m, 2H), 6.964 (s, 1H), 6.876 (s, 1H), 4.002 (m, 6H), 3.978 (s, 3H), 3.853 (s, 3H), 3.842 (s, 3H), 3.833 (s, 3H), 3.807 (s, 3H), 3.792 (s, 3H), 3.375 (q, 2H, J = 5.5 Hz), 3.294 (m, 2H, J = 5.5 Hz), 3.106 (q, 2H, J = 6.5 Hz), 2.997 (m, 3H), 2.735 (s, 3H), 2.725 (s, 3H), 2.343 (t, 2H, J = 7 Hz), 1.738 (m, 2H), 1.633 (m, 2H). MALDI-TOF-MS (monoisotopic) [M + H] 1238.83 (calcd 1238.58 for $C_{57}H_{72}N_{23}O_{10}$).

ImImPyPy-$(R)^{Glu-NHS}\gamma$-ImPyPyPy-β-Dp (2). To a solution of disuccinimidyl glutarate (41.9 mg, 120 μmol) in 2.5 mL of DMF was added 100 μL of a 14.3 mM solution of **1** (15.9 mg, 12.8 μmol) in DMF (800 μL) and DIEA (100 μL). One-hundred microliters of the solution was added every 15 min with stirring. Following the completion of the addition of **1**, the reaction was stirred for 2 h. The reaction was diluted with 0.1% TFA (15 mL) and purified by reversed phase HPLC. ImImPyPy-$(R)^{Glu-NHS}\gamma$-ImPyPyPy-β-Dp was recovered as a white powder upon lyophilization of the appropriate fraction (8.8 mg, 6.1 μmol, 47.3% recovery). UV (H_2O) λ_{max} (ϵ) 312 nm (66 600). ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ 10.320 (s, 1H), 10.242 (s, 1H), 10.097

(36) Ebbinghaus, S. W.; Fortinberry, H.; Gamper, H. B. *Biochemistry* **1999**, *38*, 619−628.

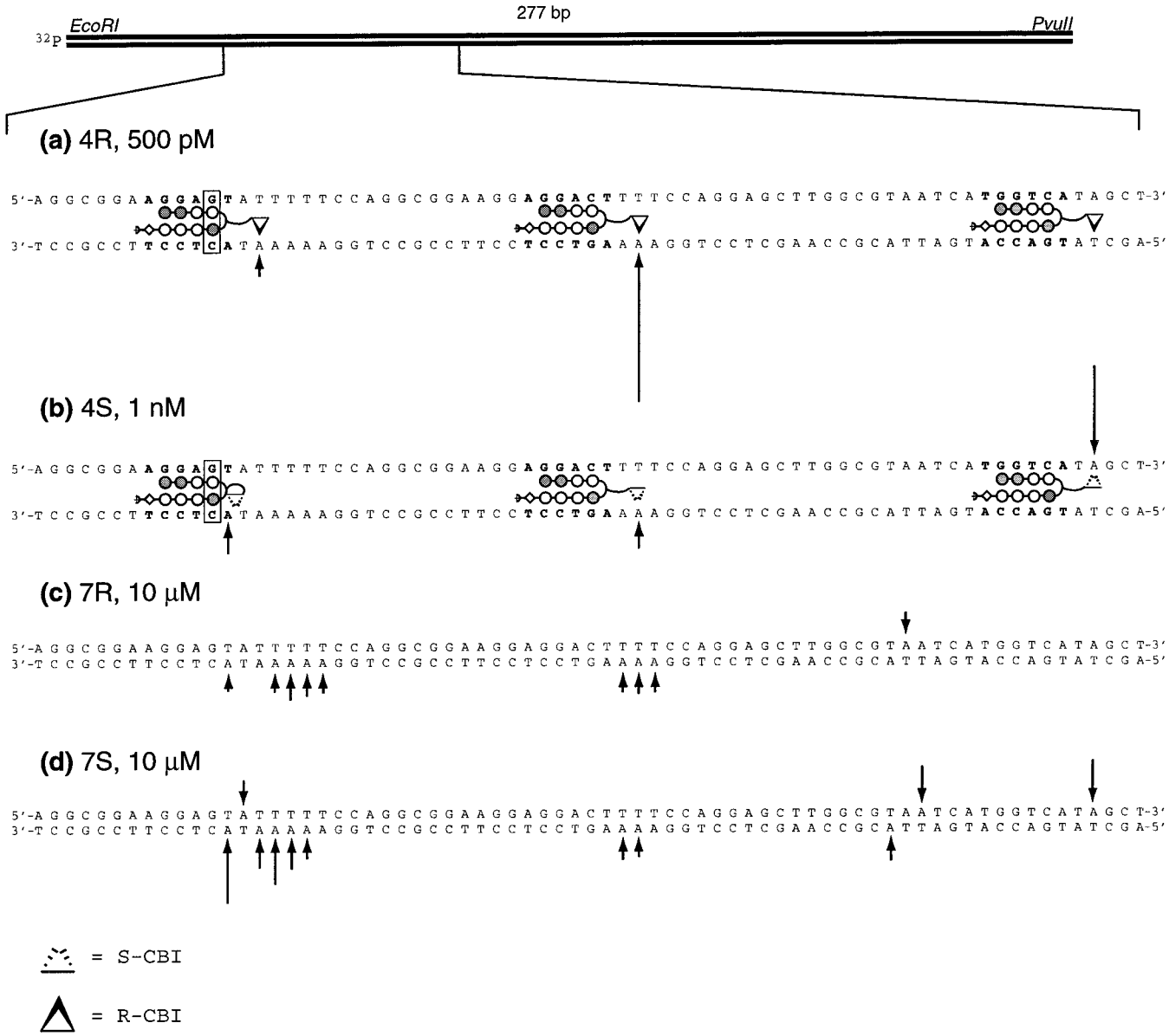

Figure 8. Illustration of the 277 bp restriction fragment with the position of the sequence indicated. Cleavage patterns are shown for (a) ImImPyPy-$\gamma^{(R)-seco-\mathrm{CBI}}$-ImPyPyPy-$\beta$-Dp (**4R**), 500 pM; (b) ImImPyPy-$\gamma^{(S)-seco-\mathrm{CBI}}$-ImPyPyPy-$\beta$-Dp (**4S**), 1 nM; (c) (*R*)-*seco*-CBI-β-dimethyl-γ (**7R**), 10 μM; and (d) (*S*)-*seco*-CBI-β-dimethyl-γ (**7S**), 10 μM. Match sites 5′-TGGTCA-3′ and 5′-AGGACT-3′ and single base pair mismatch site 5′-AGGAGT-3′ are indicated in bold on the sequence, with arrows indicating cleavage bands. The single base pair mismatch is indicated by a rectangle. The polyamide is shaded as in Figure 1, with the bold triangle representing (*R*)-CBI.

(s, 1H), 9.930 (s, 2H), 9.872 (s, 1H), 9.740 (s, 1H), 9.23 (br s, 1H, CF_3COOH), 8.183 (d, 1H, $J = 8$ Hz), 8.034 (t, 1H, $J = 5.5$ Hz), 8.010 (t, 1H, $J = 5.5$ Hz), 7.946 (t, 1H, $J = 5.3$ Hz), 7.563 (s, 1H), 7.454 (s, 1H), 7.272 (s, 1H), 7.263 (s, 1H), 7.215 (s, 1H), 7.181 (s, 1H), 7.144 (m, 4H), 7.080 (s, 1H), 7.061 (s, 1H), 6.887 (s, 1H), 6.870 (s, 1H), 4.001 (s, 6H), 3.957 (s, 3H), 3.848 (s, 3H), 3.842 (s, 3H), 3.833 (s, 3H), 3.793 (s, 3H), 3.790 (s, 3H), 3.7−3.9 (br, m, 3H), 3.378 (q, 2H, $J = 5.5$ Hz), 3.228 (m, 2H), 3.107 (q, 2H, $J = 6$ Hz), 2.998 (m, 2H), 2.741 (s, 3H), 2.731 (s, 3H), 2.345 (t, 2H, $J = 7$ Hz), 2.228 (m, 2H), 1.728 (m, 4H). MALDI-TOF-MS (monoisotopic) [M + H] 1449.63 (calcd 1449.62 for $C_{66}H_{81}N_{24}O_{15}$).

ImImPyPy-(*R*)$^{(R)-\mathrm{CBI}}\gamma$-ImPyPyPy-$\beta$-Dp (4R). To a solution of **2** (6.9 mg, 4.75 μmol) in dry DMF was added 47.5 μL of a 1 μM solution of DCC (10 equiv) and 9.5 μL of a 0.5 M solution of *N*-hydroxysuccinimide (1 equiv). The solution was stirred for 2 h. Separately, a solution of **6R** (2 mg, 1 equiv) was deprotected with 3 M HCl/ethyl acetate (5 mL) for 30 min under argon. The ethyl acetate was then removed by vacuum and coevaporated twice from dichloromethane to yield **3R**. The gray solid was then dissolved in 50 μL of dry DMF and added to the polyamide solution. DIEA (8 μL, 10 equiv) was then added and the reaction was stirred for 3 h under argon. Upon completion, the reaction was diluted with 0.1% TFA (2 mL) and the reaction was purified by reversed phase HPLC. ImImPyPy-(*R*)$^{(R)-\mathrm{CBI}}\gamma$-ImPyPyPy-$\beta$-Dp was recovered as a white powder upon lyophilization of the appropriate fraction (1.4 mg, 17.8% recovery). UV (H_2O) λ_{max} (ϵ) 314 nm (73 854). ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ 10.342 (s, 1H), 10.316 (s, 1H), 10.248 (s, 1H), 10.096 (s, 1H), 9.928 (s, 2H), 9.869 (s, 1H), 9.691 (s, 1H), 9.17 (br s, 1H, CF_3COOH), 8.172 (d, 1H, $J = 7.5$ Hz), 8.063 (d, 1H, $J = 8$ Hz), 8.029 (t, 1H, $J = 6$ Hz), 8.008 (t, 1H, $J = 5.5$ Hz), 7.962 (m, 2H), 7.894 (t, 1H, $J = 5.5$ Hz), 7.747 (d, 1H, $J = 8$ Hz), 7.556 (s, 1H), 7.464 (t, 1H, $J = 7.5$ Hz), 7.449 (s, 2H), 7.299 (t, 1H, $J = 7$), 7.267 (s, 1H), 7.254 (s, 1H), 7.206 (s, 1H), 7.172 (s, 1H), 7.145 (m, 3H), 7.059 (s, 2H), 6.892 (s, 1H), 6.870 (s, 1H), 4.526 (q, 1H, $J = 7$ Hz), 4.287 (t, 1H, $J = 10.5$ Hz), 4.111 (m, 2H), 3.998 (s, 6H), 3.948 (s, 3H), 3.844 (s, 3H), 3.837 (s, 3H), 3.831 (s, 3H), 3.791 (s, 3H), 3.787 (s, 3H), 3.106 (q, 2H, $J = 6.5$ Hz), 3.004 (m, 4H), 2.739 (s, 3H), 2.729 (s, 3H), 2.343 (t, 2H, $J = 7.3$ Hz), 2.181 (t, 2H, $J = 7$ Hz), 2.098 (t, 2H, $J = 7.8$ Hz), 1.981 (m, 2H), 1.741 (m, 4H), 1.631 (m, 2H), 1.540 (m, 1H). MALDI-TOF-MS (monoisotopic) [M + H] 1638.72 (calcd 1638.69 for $C_{78}H_{93}N_{25}O_{14}$).

ImImPyPy-(*R*)$^{(S)-CBI}$γ-ImPyPyPy-β-Dp (4S). ImImPyPy-(*R*)$^{(S)-CBI}$γ-ImPyPyPy-β-Dp was prepared from **2** as described for **4R** (1.4 mg, 30.4% recovery). UV (H_2O) λ_{max} (ε) 314 nm (73 854). ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ 10.343 (s, 1H), 10.316 (s, 1H), 10.248 (s, 1H), 10.095 (s, 1H), 9.929 (s, 2H), 9.870 (s, 1H), 9.689 (s, 1H), 9.19 (br s, 1H, CF_3COOH), 8.173 (d, 1H, J = 7.5 Hz), 8.064 (d, 1H, J = 8 Hz), 8.029 (m, 2H), 7.963 (m, 2H, C4−H), 7.895 (t, 1H, J = 5.5 Hz), 7.747 (d, 1H, J = 8 Hz), 7.556 (s, 1H), 7.464 (t, 1H, J = 8 Hz), 7.451 (s, 1H), 7.445 (s, 1H), 7.299 (t, 1H, J = 7.8 Hz), 7.266 (s, 1H), 7.255 (s, 1H), 7.206 (s, 1H), 7.175 (s, 1H), 7.145 (m, 3H), 7.060 (s, 2H), 6.891 (s, 1H), 6.870 (s, 1H), 4.525 (q, 1H, J = 7 Hz), 4.285 (t, 1H, J = 10.5 Hz), 4.111 (d, 2H, J = 8.5 Hz), 3.998 (s, 6H), 3.951 (s, 3H), 3.844 (s, 3H), 3.837 (s, 3H), 3.831 (s, 3H), 3.792 (s, 3H), 3.787 (s, 3H), 3.105 (q, 2H, J = 6 Hz), 3.000 (m, 4H), 2.737 (s, 3H), 2.729 (s, 3H), 2.343 (t, 2H, J = 6.8 Hz), 2.180 (t, 2H, J = 8 Hz), 2.099 (t, 2H, J = 8 Hz), 1.997 (m, 2H), 1.727 (m, 2H), 1.631 (m, 2H), 1.540 (m, 1H). MALDI-TOF-MS (monoisotopic) [M + H] 1638.71 (calcd 1638.69 for $C_{78}H_{93}N_{25}O_{14}$).

3-(*tert*-Butoxycarbonyl)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI-BOC, 5) was synthesized and enantiomers separated by already published protocols.[27−29]

***seco*-CBI-β-alanine-BOC (6R and 6S). 5** (85 mg, 0.255 mmol) was deprotected in 3 M HCl/ethyl acetate (10 mL) for 30 min under argon. After the ethyl acetate was removed by evaporation, the deprotected *seco*-CBI was coevaporated twice from dichloromethane. BOC-β-alanine (96.4 mg, 2 equiv) and EDC (293.3 mg, 6 equiv) were added with DMF (5 mL). The solution was stirred overnight under argon. After the reaction was complete, 15 mL of water was added, and the reaction was extracted 6 times with ethyl ether. The ether was washed with brine, dried with Na_2SO_4, and purified by flash chromatography (5% methanol/dichloromethane) to yield **6** as an off-white powder (90 mg, 86%).

(*R*)-*seco*-CBI-β-alanine-BOC (6R): ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 9.37 (s, 1H), 8.27 (m, 2H), 7.62 (d, 1H, J = 8.1 Hz), 7.52 (t, 1H, J = 6.6 Hz), 7.38 (t, 1H, J = 7.5 Hz), 5.55 (br t, 1H), 4.19 (d, 1H, J = 10.2 Hz), 4.06 (t, 1H, J = 9.9 Hz), 3.93 (m, 2H), 3.62 (m, 2H), 3.38 (t, 1H, J = 10.5 Hz), 2.75 (m, 2H), 1.44 (s, 9H). $[\alpha]^{29}_D$ +25.8° (*c* 0.1). HRMS calcd for $C_{21}H_{25}ClN_2O_4$ 404.1503, found 404.1496.

***(S)*-*seco*-CBI-β-alanine-BOC (6S):** ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 9.37 (s, 1H), 8.27 (m, 2H), 7.63 (d, 1H, J = 8.1 Hz), 7.53 (t, 1H, J = 6.6 Hz), 7.40 (t, 1H, J = 7.5 Hz), 5.55 (br t, 1H), 4.21 (d, 1H, J = 10.2 Hz), 4.06 (t, 1H, J = 9.9 Hz), 3.96 (m, 2H), 3.64 (m, 2H), 3.40 (t, 1H, J = 10.5 Hz), 2.75 (m, 2H), 1.44 (s, 9H). $[\alpha]^{29}_D$ −29.4° (*c* 0.1). HRMS calcd for $C_{21}H_{25}ClN_2O_4$ 404.1503, found 404.1490.

***seco*-CBI-β-alanine-(dimethyl)-γ-amino Butyric Acid (7R and 7S). 6** (5 mg, 12.3 μmol) was deprotected in 3 M HCl/ethyl acetate (5 mL) for 30 min under argon. The ethyl acetate was removed by evaporation, and the deprotected *seco*-CBI-β-alanine was coevaporated twice from dichloromethane. Separately, a solution of (dimethyl)-γ-aminobutyric acid (4.1 mg, 2 equiv) in DMF (200 μL) was stirred in a flame-dried flask with DCC (25.4 mg, 10 equiv) and *N*-hydroxysuccinimide (1.4 mg, 1 equiv) for 1 h under argon. This was added to the deprotected *seco*-CBI-β-alanine and DIEA (7 μL, 3 equiv) was added. The solution was stirred for 1 h under argon and purified by reversed phase HPLC. *seco*-CBI-β-alanine-(dimethyl)-γ-aminobutyric acid was recovered as a white powder upon lyophilization of the appropriate fraction.

(*R*)-*seco*-CBI-β-alanine-(dimethyl)-γ-aminobutyric acid (7R): 2.5 mg, 48% (recovery). ^{1}H NMR (300 MHz, CD_3CN, 25 °C): δ 10.9 (br s, 1H), 8.12 (d, 1H, J = 7.5 Hz), 8.02 (s, 1H), 7.69 (d, 1H, J = 8.4 Hz), 7.48 (t, 1H, J = 8.4 Hz), 7.33 (t, 1H, J = 6.9 Hz), 7.09 (br s, 1H), 4.15 (m, 2H), 3.93 (d, 3H), 3.63 (t, 2H), 3.51 (m, 2H), 3.04 (m, 4H), 2.71 (s, 6H). HRMS calcd for $C_{22}H_{29}ClN_3O_3$ (M + H) 418.1897, found 418.1889.

(*S*)-*seco*-CBI-β-alanine-(dimethyl)-γ-aminobutyric acid (7S): 3.4 mg, 66% (recovery). ^{1}H NMR (300 MHz, CD_3CN, 25 °C): δ 10.9 (br s, 1H), 8.13 (d, 1H, J = 8.1 Hz), 8.00 (s, 1H), 7.74 (d, 1H, J = 8.4 Hz), 7.50 (t, 1H, J = 7.5 Hz), 7.34 (t, 1H, J = 7.5 Hz), 7.00 (br s, 1H), 4.23 (m, 2H), 4.20 (m, 1H), 3.93 (d, 2H), 3.67 (t, 2H), 3.51 (m, 2H), 3.04 (t, 2H), 2.75 (s, 6H). HRMS calcd for $C_{22}H_{29}ClN_3O_3$ (M + H) 418.1897, found 418.1907.

DNA Reagents and Materials. Enzymes were purchased from Boehringer-Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P]triphosphates were obtained from Dupont/New England Nuclear, and deoxyadenosine 5′-[γ-^{32}P]triphosphates were purchased from I.C.N. Sonicated, deproteinized calf thymus DNA was acquired from Pharmacia. RNase free water was obtained from USB and used for all reactions. All other reagents and materials were used as received. All DNA manipulations were performed according to standard protocols.

Construction of Plasmid DNA. The plasmid pAC1 was constructed using previously described methods. Fluorescent sequencing was performed at the DNA Sequencing Facility at the California Institute of Technology and was used to verify the presence of the desired insert. Concentration of the prepared plasmid was determined at 260 nm from the relationship of 1 OD unit = 50 μg mL^{-1} duplex DNA.

PCR Labeling to Generate 5′-End-Labeled Restriction Fragments. Two 21 mer primers were synthesized for PCR amplification: primer A (labeled) 5′-AATTCGAGCTCGGTACCCGGG-3′ and primer B (unlabeled) 5′-CTGGCACGACAGGTTTCCCGA-3′. Primer A was treated with T4 polynucleotide kinase and deoxyadenosine 5′-[γ-^{32}P]-triphosphate as previously described. PCR reactions containing 60 pmol of each primer, 10 μL of PCR buffer (Boehringer-Mannheim), 3.7 μL of template (0.003 μg/mL), 2 μL of dNTP mix (each at 10 mM), 1 μL of 100X BSA (New England Biolabs), and 83 μL of water were heated at 70 C for 5 min. Four units of Taq Polymerase was added (Boehringer-Mannheim). Thirty amplification cycles were performed, each cycle consisting of the following segments: 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1.5 min. Following the last cycle, 10 min of extension at 72 °C completed the reaction. The PCR products were gel purified as previously reported for 3′-end labeling protocols.

Cleavage Reactions. All reactions were carried out in a volume of 50 μL. A polyamide or *seco*-CBI-dimethyl gaba stock solution or water (for reference lane) was added to an assay buffer of TE (pH 7.5) and 20 kcpm of 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 12 h or the appropriate time (for time course reactions) at 37 °C. The reactions were stopped with 60 μL of a solution containing NaOAc (600 mM), EDTA at pH 8.0 (12.5 mM), calf thymus DNA (150 μM base pair), glycogen (0.8 mg mL^{-1}), and NaCl (2 M). Ethanol was added to remove unbound polyamide and precipitate the products. The reactions were resuspended in 20 μL of TE (pH 7.5) and cleavage was initiated by heating at 95 °C for 30 min. The cleavage products were precipitated with 150 μL of ethanol, resuspended in 100 mM trisborate-EDTA/80% formamide loading buffer, and denatured and loaded onto polyacrylamide gels as previously reported. The gels were quantitated by the use of storage phosphor technology. The yield or efficiency of alkylation was determined as the ratio between the volume integration assigned to the products and the sum of the volumes of all the products in the lane.

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