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Expanding the Repertoire of Natural Product-Inspired Ring Pairs for Molecular Recognition of DNA

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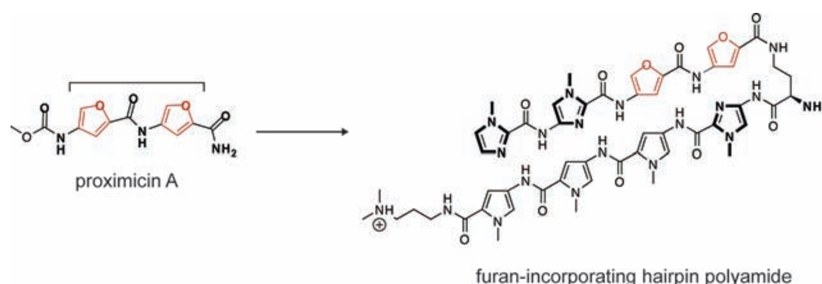
ABSTRACT



A furan amino acid, inspired by the recently discovered proximicin natural products, was incorporated into the scaffold of a DNA-binding hairpin polyamide. While unpaired oligomers of 2,4-disubstituted furan amino acids show poor DNA-binding activity, furan (Fn) carboxamides paired with *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) rings demonstrate excellent stabilization of duplex DNA as well as discrimination of noncognate sequences, consistent with function as a Py mimic according to the Py/Im polyamide pairing rules.

Small molecules capable of perturbing gene expression by the selective modulation of protein–DNA interfaces may have important applications in biology and human medicine. Hairpin pyrrole-imidazole polyamides are a class of synthetic minor groove-binding ligands that can be programmed to bind a broad repertoire of DNA sequences with affinities and specificities comparable to those of DNA-binding proteins (Figure 1A).[1] Cell-permeable polyamides have been shown to localize to the nuclei of living cells[2,3] and regulate endogenous gene expression by interfering with transcription factor/DNA interfaces.[4–8]

The structures of these programmable oligomers were inspired by the crescent-shaped natural products netropsin and distamycin (Figure 1B), which are comprised of two and three aromatic *N*-methylpyrrole heterocyclic rings, respectively.[9] Distamycin is known to bind the minor groove of A,T tracks of DNA with both 1:1 and 2:1 ligand/DNA stoichiometries.[10,11] Guided by structural and biophysical studies, synthetic analogues of distamycin were developed which applied single-atom changes to the *N*-methylpyrrole ring in order to rationally alter the A,T

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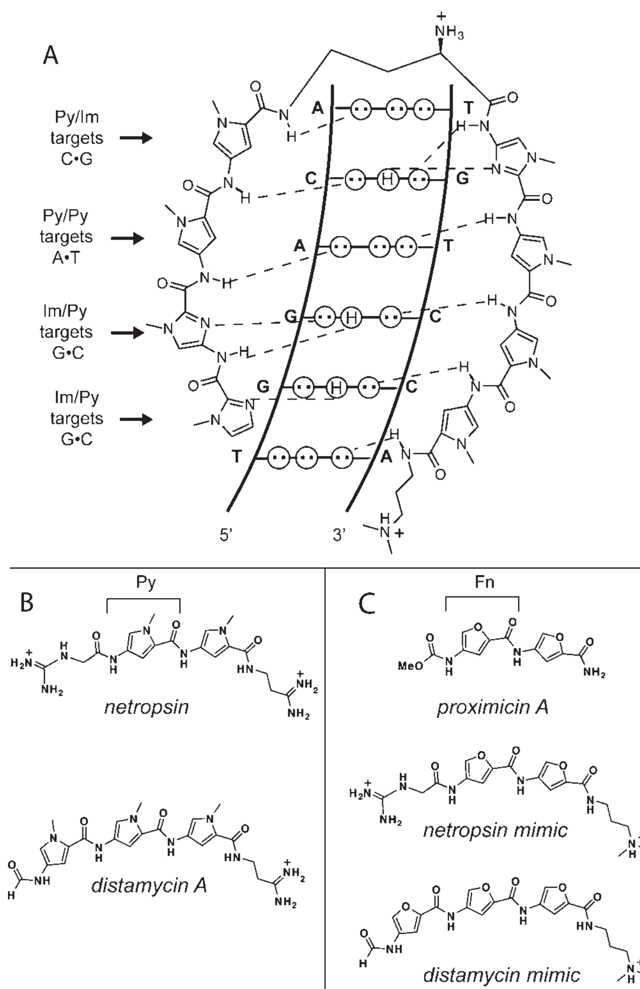

Figure 1. Natural and engineered small molecules for binding the minor groove of DNA.

binding specificity of the natural product.[12,13] These efforts resulted in a set of pairing rules, in which amino acid oligomers composed of heterocyclic analogues of *N*-methylpyrrole oriented in a hairpin configuration can be combined as unsymmetrical ring pairs in order to read the minor groove of DNA.[14] Specifically, side-by-side stacked *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) carboxamides (Im/Py pairs) distinguish G·C from C·G base pairs, whereas *N*-methyl-3-hydroxypyrrole (Hp)/Py shows specificity for T·A over A·T. Finally, Py/Py pairs specify for both T·A and A·T (Figure 1A).[15] However, while Py/Py and Py/Im represent the most robust sets of sequence-specific pairs, continued efforts to optimize polyamide affinity and specificity have led to the evaluation of several

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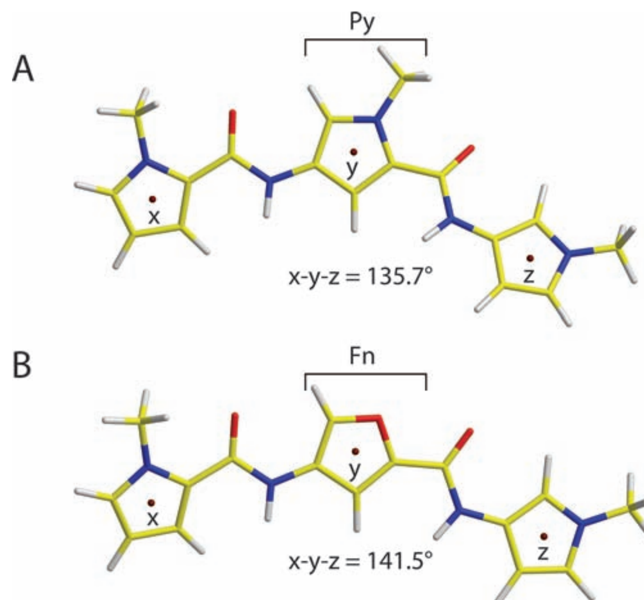

Figure 2. DFT-optimization (B3LYP/6-31G(d)) of the PyPyPy and PyFnPy trimers in the conformation relevant for DNA recogntion. The angle X-Y-Z defines the trimer curvature. Comparison with Im and Hp is provided in the Supporitng Information.

additional amino acid pairings.[16,17] For example, incorporation of the fused heterocycles hydroxybenzimidazole (Hz) and imidazopyridine (Ip) within the hairpin polyamide scaffold produces Py/Hz and Py/Ip ring pairs capable of functionally replacing Hp/Py and Py/Im in T·A and C·G recognition.[18] The continued exploration of new ring pairs is essential in order to further refine the ability of these molecules to recognize the sequence-dependent microstructures of DNA, and importantly to optimize their pharmacokinetic properties for biological applications.

Recently Sussmuth and co-workers revealed the discovery of the natural products proximicins A–C, which contain a central core of 2,4-disubstituted furan amino acid dipeptides (Figure 1C).[19] While these molecules show structural similarity to netropsin and distamycin, 4-aminofuran-2-carboxylates represent a new heterocyclic amino acid which had not been previously reported in the literature.[20–22] Notably, the proximicins show considerably greater antitumor activity and cytotoxicity than

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netropsin and distamycin, and the two families of molecules appear to exert their influence on different cellular targets.[23] However, in contrast to netropsin and distamycin, the proximicins do not stabilize the thermal melting of DNA duplexes. The recent development of a synthetic route to the proximicin core allowed synthesis of furan-based netropsin and distamycin mimics (Figure 1C). These cationic proximicin analogues show greatly reduced thermal stabilization of DNA relative to netropsin and distamycin.[24] Molecular modeling indicates replacement of the central Py of a PyPyPy trimer by furan (Fn) is accompanied by a significant reduction in overall molecular curvature, likely due to reduced repulsion of the amide carbonyl group by the Fn oxygen compared to the N-Me group of Py (Figure 2). This raises the question of how the Fn amino acid might behave in the context of ring pairs, as enforced in a hairpin polyamide–DNA complex. Specifically, does Fn/Py mimic the sequence preferences of Py/Py, and does Fn/Im mimic the sequence preferences Py/Im?

To this end, we synthesized Py/Im hairpin polyamide **1**, which targets the sequence 5′-WGGWCW′3′ (where W = A or T), as well as three analogues (**2**–**4**) with furan rings substituted at varying positions within the polyamide hairpin structure (Figure 3). This particular core sequence allows comparison of all three Py/Im/Fn ring pairs within a single series of analogues: by altering which position of the parent Py-Im polyamide **1** is substituted with a furan we can assess base pair specificity of both a Fn/Im (**2**) pair as well as a Fn/Py (**3**) pair. Furthermore, by incrementally increasing the number of furan rings from zero (**1**) to two (**4**) we can begin to assess the effects overall Fn content will have on the energetics of Py/Im/Fn polyamide DNA-binding.

Polyamides **1**–**4** were synthesized by solid-phase methods using Boc-protected monomeric units on Kaiser oxime resin. Incorporation of Boc-protected Py and Im rings followed established methods (Scheme S1).[25] However, while the reported route to 4-*tert*-butoxycarbonylamino-furan-2-carboxylic acid (Fn monomer) proved robust,[24] incorporation of the Fn monomer unit into eight-ring Py-Im polyamides was complicated by the sensitivity of the furan monomer to the repeated application of the harsh acidic conditions used for Boc removal. Increasing decomposition of the growing oligomer chain was observed following each trifluoroacetic acid (TFA) deprotection. Attempts to use Lewis acid deprotection strategies (boron trifluoride diethyl etherate or trimethylsilyl chloride) were also unsuccessful, resulting in negligible recovery of polyamides from the resin. Hairpins **2**–**4** represent a minimal panel of furan-containing polyamides that require (at most) only two cycles of exposure to TFA, and thus were accessible by this route, although in overall yields (0.5–7%) approximately 50% lower than those previously reported for polyamide oxime synthesis. Therefore we note that for future syntheses of hairpin polyamides incorporating Fn rings it may be prudent to utilize Fmoc-based peptide synthesis methodologies due to the milder conditions required in removal of the Fmoc protecting group. Critically, polyamides containing Fn rings, once isolated, appear to have photochemical and thermal stabilities identical to those for standard Py-Im polyamides and remain pure by HPLC analysis even after being in solution at room temperature for several days.

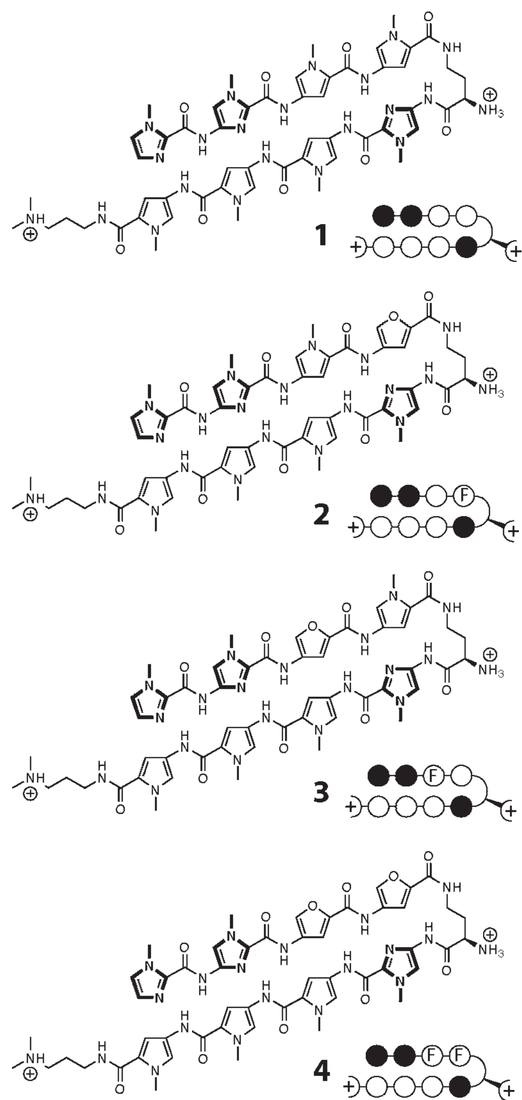

Figure 3. Chemical and ball and stick structures of polyamides containing furan amino acids. Ball and stick symbols are defined as follows: *N*-methylpyrrole is denoted by an open circle, *N*-methylimidazole is denoted by a filled circle, and furan is denoted by open circles with "F" inside.

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Following synthesis, polyamides **1**–**4** were analyzed for DNA binding affinity and specificity by melting temperature analysis[26] of 12-mer duplex DNA oligonucleotides containing the sequence 5′-TGGXCA-3′ where X was cycled through all four base pair possibilities (Table 1). Spectroscopic analyses show that all four hairpins considerably increase the thermal stability of each DNA duplex, indicative of polyamide-DNA binding. The relative shifts in DNA melting temperature induced by **1**–**3** for each of the two match DNA duplexes tested are similar ($\Delta T_m \approx 15$–18 °C), indicating that the binding affinity of hairpin polyamides containing Fn/Py or Fn/Im ring pairs remains intact relative to the traditional Py/Im scaffold (Table 1). This thermal stabilization is reduced when X is mutated to a C·G or G·C ($\Delta\Delta T_m \approx 5$–$7$ °C), demonstrating polyamide specificity is also unaffected by Fn substitution. Furthermore, multiple Fn pairs are tolerated when housed in the hairpin polyamide scaffold, as indicated by the marked stabilization of duplex DNA by polyamide **4**. Thus, despite the differences in Fn and Py curvature predicted from modeling studies, our experimental results indicate that 2,4-substituted furan amino acids can function as Py-mimics when paired in the hairpin polyamide–DNA complex.

A more subtle finding of the melting temperature study is that polyamides incorporating the Fn/Im pair (**2**,**4**) exhibit slightly decreased thermal stabilization of DNA duplexes when compared to polyamides containing a Py/Im pair at this position (**1**, **3**). While the magnitude of this decrease is close to the limit of error of the melting temperature assay, a consistent trend was observed for all four duplexes (Table 1). It was unclear whether this reduction in DNA-binding affinity is an inherent property of the Fn/Im pair, a positional effect of Fn-incorporation adjacent to the turn residue, or an indication of repulsive effects between the Fn oxygen, which projects outward from the minor groove, and the negatively charged DNA backbone.[24] We explored this by synthesizing polyamides in which the Fn rings of **2**–**4** were replaced with desmethylpyrrole (Ds) amino acids (Figure S1, **5**–**7**), which provide a more polar, less bulky alternative to Py but offer a hydrogen instead of a lone pair for interaction with the DNA backbone.[27] Thermal denaturation analysis indicates Ds-containing polyamides stabilize duplex DNA with similar magnitudes and specificity as Py/Im and Py/Im/Fn-polyamides **1**–**4** (Table S1). As with the Fn series, Ds/Im containing polyamide **5** exhibits slightly decreased thermal stabilization relative to Py/Im polyamide **1**, despite its drastically different electronic and hydrogen bonding properties. Therefore, the small loss in affinity for Fn/Im at this position is likely a consequence of loss of the hydrophobic *N*-methyl of Py and not any repulsive interactions of the Fn monomer.

Recent in vitro ADMET analysis of a hairpin Py/Im polyamide revealed high levels of plasma protein binding,

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Table 1. Melting Temperatures of DNA/Polyamide Complexes for Cognate and Mismatch Duplexes[a]

5′ – C G A **T G G X C A** A G C – 3′
3′ – G C T **A C C Y G T** T C G – 5′

	match DNA		mismatch DNA	
X-Y	**A-T**	**T-A**	**G-C**	**C-G**
DNA	58.0 ± 0.2	58.2 ± 0.5	59.6 ± 0.1	59.8 ± 0.3
1	76.2 ± 1.9 [18.2]	76.2 ± 2.3 [17.9]	71.2 ± 0.2 [11.7]	72.3 ± 0.3 [12.4]
2	73.8 ± 0.3 [15.8]	75.2 ± 1.5 [17.0]	69.9 ± 0.1 [10.3]	70.0 ± 0.4 [10.1]
3	76.6 ± 1.1 [18.6]	77.1 ± 0.8 [18.9]	70.8 ± 0.6 [11.9]	72.9 ± 0.3 [13.1]
4	73.2 ± 1.3 [15.2]	74.6 ± 1.0 [16.3]	68.5 ± 0.5 [8.6]	69.1 ± 0.3 [9.3]

[a] All values are derived from at least three independent experiments. Shift in melting temperature temperature (ΔT_m) is reported in brackets below the absolute value.

an oftentimes negative pharamacokinetic determinant characteristic of a highly lipophilic compound.[28] This led us to briefly explore the ability of alternative ring pairs to modulate polyamide lipophilicity. Our anticipation was that incorporation of the more polar Fn ring (and removal of the corresponding *N*-methyl group) would decrease lipophilicity. However, substitution of Py by one or two Fn rings (compounds **3**, **4**) does not significantly alter the lipophilicity of parent polyamide **1** as measured by its LogD in a miniaturized shake-flask octanol/water partition assay (Table S2). In contrast, compounds **5**–**7** demonstrated a considerable decrease in LogD value compared to parent compound **1**, representative of increased water solubility. While the greater polarity of the Ds-containing polyamides is consistent with previous observations,[27] the lack of effect of Fn incorporation on LogD is surprising. The difficulty in predicting such properties a priori is an indication that DNA-binding polyamides may benefit from a systematic effort to define their pharmacokinetic properties, as has been done for cellular uptake, and is thus an area of active research.

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Supporting Information Available. Supplementary data associated with this article, including synthetic procedures and characterization data, thermal DNA stabilization data, computational analyses, and polyamide LogD values can be found in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.