Solid Phase Synthesis of Polyamides Containing Imidazole and Pyrrole Amino Acids

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Abstract: The solid phase synthesis of sequence specific DNA binding polyamides containing N-methylimidazole (Im) and N-methylpyrrole (Py) amino acids is described. Two monomer building blocks, Boc-Py-OBt ester and Boc-Im acid, are prepared on a 50 g scale without column chromatography. Using commercially available Boc-β-alanine-Pam resin, cycling protocols were optimized to afford high stepwise coupling yields (> 99%). Deprotection by aminolysis affords up to 100 mg quantities of polyamide. Solid phase methodology increases both the number and complexity of minor groove binding polyamides which can be synthesized and analyzed with regard to DNA binding affinity and sequence specificity. The solid phase synthesis of a representative eight-residue polyamide is reported.

Introduction

Efforts to discover a universal set of simple chemical rules for the digital readout of double-helical DNA by artificial molecules have met with encouraging success.1,2 Polyamides containing N-methylimidazole (Im) and N-methylpyrrole (Py) amino acids can be combined in antiparallel side-by-side dimeric complexes with the minor groove of DNA.2-4 The DNA sequence specificity of these small molecules can be controlled by the linear sequence of pyrrole and imidazole amino acids. An imidazole ring on one ligand complemented by a pyrrole ring on the second ligand recognizes a G-C base pair, while a pyrrole/ imidazole combination targets a C-G base pair.2,4 A pyrrole/pyrrole pair is degenerate for A-T or T-A base pairs.2-4 The utility of the 2:1 model is demonstrated by the four-ring polyamide ImPyImPy-Dp (Dp = (N,N-dimethylamino)propylamide), which binds the four base pair core sequence 5'-GGGC-3’, a complete reversal of the natural specificity of netropsin and distamycin.5

Covalently linking polyamide heterodimers and homodimers within the 2:1 motif has led to designed ligands with both increased affinity and specificity.5,7 A simple polyamide “hairpin” motif with γ-aminobutyric acid (γ) serving as a “turn monomer” provides a synthetically accessible method of linking polyamide units within the 2:1 motif.7 The polyamide ImPyPy-γ-PyPyPy-Dp (1) was found to bind the designated target site 5’-TGTATA-3’ with high specificity and an approximate 300-fold binding enhancement over the individual unlinked polyamide pair, ImPyPy and PyPyPy.7

While the limits of the 2:1 model for the design of polyamides for the recognition of any sequence of any site size are being explored, the synthetic effort emerged as a limiting step. The process of expanding the 2:1 motif to include larger sequences recognized by increasingly complex polyamides is demanding. For example, using previously described multistep solution phase chemistry, the total synthesis of hairpin polyamides such as ImPyPy-γ-PyPyPy-Dp (1) and ImPyPy-γ-PyPyPy-β-Dp (2) (β = β-alanine) would require more than a month’s effort for each polyamide (Figure 1). We report here general protocols for manual and machine-assisted Boc-chemistry solid phase synthesis of the pyrrole–imidazole polyamides which reduce the synthetic investment from months to days.

Synthesis of Pyrrole–Imidazole Polyamides. Distamycin and its analogs have previously been considered targets of traditional multistep synthetic chemistry.8 The repeating amide of distamycin is formed from an aromatic carboxylic acid and an aromatic amine. The aromatic acid is often unstable to decarboxylation, and the aromatic amines have been found to be air and light sensitive.9 The variable coupling yields, long reaction times (often >24 h), numerous side products, and reactive intermediates (acid chlorides and trichloro ketones) characteristic of the traditional solution phase coupling reactions make the synthesis of the aromatic carboxamides problematic.10

Solid Phase Synthesis. In order to implement an efficient solid phase methodology for the synthesis of the pyrrole-imidazole polyamides, the following components were developed: (1) a synthesis which provides large quantities of appropriately protected monomer or dimer building blocks in high purity, (2) optimized protocols for forming an amide in high yield from a support-bound aromatic amine and an aromatic carboxylic acid, (3) methods for monitoring reactions on the solid support, and (4) a stable resin linkage agent that can be cleaved in high yield upon completion of the synthesis.

Results and Discussion

Monomer Syntheses. The synthesis of Boc-Py-OBt ester has been previously described. Available procedures provide only milligram to gram quantities of monomer while requiring difficult column chromatography (Figure 2 and 3). Two dimeric building blocks have also been prepared, Boc-Py-Im acid and Boc-γ-Im acid monomers to be cleaved in a single-step which introduces a positive charge into the polyamide. The addition of an aliphatic amino acid at the C-terminus of the pyrrole-imidazole polyamides allows the use of Boc-β-alanine-Pam-resin which is commercially available in appropriate substitution levels (0.2 mmol/g) (Figure 1).Figure 1. ImPyPy-γ-PyPyPy-Dp prepared by multistep solution phase synthesis (top) and the solid phase analog ImPyPy-γ-PyPyPy-β-Dp (bottom) containing a C-terminal β-alanine residue to facilitate synthesis.23,24

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(1) The solid phase approach has been successfully developed for the synthesis of a variety of proteins, oligonucleotides, peptides, oligosacharides, and small non-polymers.16


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Figure 4. (i) 500 psi of H₂, 10% Pd/C, DMF; (ii) Boc—pyrrole acid (activated in situ with DCC/HOBt), DIEA, DMF, 60 °C; (iii) NaOH, MeOH, water, 60 °C; (iv) Boc—γ-aminobutyric acid (activated in situ with DCC/HOBt), DIEA, DMF, 60 °C; (v) NaOH, MeOH, water, 60 °C.

Figure 5. Protocol for the solid phase synthesis of a pyrrole—imidazole polyamide.

Table 1. Standard Protocol for Manual Solid Phase Synthesis of Pyrrole—Imidazole Polyamides

<table>
<thead>
<tr>
<th>synthesis cycle</th>
<th>time/mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. deprotect</td>
<td>80% TFA/DMF, 0.5 M PhSH</td>
</tr>
<tr>
<td>2. wash</td>
<td>DCM</td>
</tr>
<tr>
<td>3. couple</td>
<td>OBt ester, DIEA</td>
</tr>
<tr>
<td>4. wash</td>
<td>DMF</td>
</tr>
</tbody>
</table>

Solid Phase Polyamide Synthesis Protocols. Solid phase polyamide synthesis protocols were modified from the in situ neutralization Boc-chemistry protocols recently reported by Kent and co-workers. Coupling cycles are rapid, 72 min per residue for manual synthesis or 180 min per residue for machine-assisted synthesis, and require no special precautions beyond those used for ordinary solid phase peptide synthesis. Manual solid phase synthesis of a pyrrole—imidazole polyamide consists of a dichloromethane (DCM) wash, removal of the Boc group with trichloroacetic acid (TFA)/DMF/thiophenol (PhSH), a DCM wash, a DMF wash, taking a resin sample for analysis, addition of activated monomer, addition of DIEA if necessary, coupling for 45 min, taking a resin sample for analysis, and a final DMF wash (Figure 5, Table 1). In addition, the manual solid phase synthesis protocols for synthesis of pyrrole—imidazole polyamides have been adapted for use on an ABI 430A peptide synthesizer.

Monitoring the Synthesis. The aromatic amine of the pyrrole and imidazole do not react in the quantitative ninhydrin test. Stepwise cleavage of a sample of resin and analysis by HPLC indicates that high stepwise yields (>99%) are routinely achieved. We note that acylation of imidazole amine with Boc-Py-OBt ester was not satisfactory. However, acylation with Boc-Py symmetrical anhydride/DMAP ester (DCC, DMAP, DCM) proceeds to completion within 3 h. Alternatively, the preparation of a Boc-PyIm dimer unit avoids the difficult coupling of pyrrole to imidazole.

Synthesis of Eight-Residue Polyamide 2. ImPyPyγ-PyPyPyPyβ-Dp was prepared in 14 steps using the protocols described in the Experimental Section (Figure 6). The yield of each individual coupling step was established as >98% by HPLC analysis. The resin was cleaved in high yield (>90%) by aminolysis with (N,N-dimethylamino)propylamine. A single HPLC separation of the eight-residue polyamide was sufficient to obtain a final purity greater than 98% as determined by a combination of analytical HPLC, 1H NMR, and mass spectrosopy.

Conclusion. Pyrrole—imidazole polyamide—DNA complexes provide a potentially general model for the design of non-natural ligands for the sequence specific recognition of the minor groove of DNA. The large number of polyamides made available by solid phase synthetic methodology should accelerate the elucidation of the scope and limitations of this approach.

Experimental Section

Materials. Boc-β-alanine-(4-carbamoylaminoethyl)-benzyl-ester-copoly(styrene-divinylbenzene) resin (Boc-β-Pam-resin), dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Boc-glycine, and Boc-β-alanine were purchased from Peptides International, N,N-Diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), and DMDSO/NMP were purchased from Applied Biosystems. Boc—γ-aminobutyric acid was from NOVA Biochem, dichloromethane (DCM) and triethylamine (TEA) were in situ (activated in situ with DCC/HOBt), DIEA, DMF, 60 °C; (v) NaOH, MeOH, water, 60 °C. 

1H NMR spectra were recorded on a GE 300 instrument operating at 300 MHz. Chemical shifts are reported in parts per million relative to the solvent residual signal. UV spectra were measured on a Hewlett-Packard Model 8452A diode array spectrophotometer. IR spectra were recorded on a Perkin-Elmer FTIR spectrometer. High-resolution FAB mass spectra were recorded at the Mass Spectroscopy Laboratory at the University of California, Riverside. Matrix-assisted, laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was carried out at the Protein and Peptide Microanalytical Facility at the California Institute of Technology. HPLC analysis was performed either on a HP 1090M analytical HPLC or on a Beckman Gold system using a RAINEN C18, Microsorb MV, 5 µm filtered. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 precoated plates. Reagent-grade chemicals were used unless otherwise stated.

Monomer Syntheses. 4-Nitro-2-(trichloroacetyl)-1-methylpyrrole (3). To a well-stirred solution of trichloroacetyl chloride (1 kg, 5.5 mol) in 1.5 L of ethyl ether was added dropwise over a period of 3 h a solution of N-methylpyrrole (0.45 kg, 5.5 mol) in 1.5 L of anhydrous ethyl ether. The reaction mixture was stirred for an additional 3 h, and the reaction was quenched by the dropwise addition of a solution of 400 g of potassium carbonate in 1.5 L of water. The layers were separated, and the ether layer was concentrated.

in vacuo to provide 2-(trichloroacetyl)pyrrole (1.2 kg, 5.1 mol) as a yellow crystalline solid sufficiently pure to be used without further purification. To a cooled (−40 °C) solution of 2-(trichloroacetyl)pyrrole (1.2 kg, 5.1 mol) in acetic anhydride (6 L) in a 12 L flask equipped with a mechanical stirrer was added acetic acid (440 mL) over a period of 4 h. The mixture was cooled to −20 °C for 30 min, during which time a white precipitate formed. The solution was allowed to stand for 15 min and the resulting precipitate collected by vacuum filtration to provide 3 (0.8 kg, 54% yield): TLC (7:2 benzene/ethyl acetate) Rf 0.7; 1H NMR (DMSO-d6) δ 8.55 (d, 1 H, J = 1.7 Hz), 7.77 (d, 1 H, J = 1.7 Hz), 3.98 (s, 3 H), 3.75 (s, 3 H); 13C NMR (DMSO-d6) δ 173.3, 134.7, 133.2, 121.1, 116.9, 95.0, 51.5; IR (KBr) 3095, 2693, 1709, 1548, 1448, 1317, 1226, 1195, 1116, 751; FABMS m/e 3148, 1718, 1541, 1425, 1317, 1226, 1195, 1116, 753; FABMS m/e 184.048 (M + H 184.048 calcd for C₇H₇NO₂Cl).  

Methyl 4-Nitropyrole-2-carboxylate Hydrochloride (5). Methyl 4-nitropyrole-2-carboxylate (4) (450 g, 2.8 mol) was dissolved in ethyl acetate (8 L). A slurry of 40 g of 10% Pd/C in 800 mL of ethyl acetate was then added and the mixture stirred under a slight positive pressure of hydrogen (ca. 1.1 atm) for 48 h. Pd/C was removed by filtration through Celite and washed with 1 × 50 mL ethyl acetate, and the volume of the mixture was reduced to ca. 500 mL; 7 L of cold ethyl ether was added and HCl gas gently bubbled through the mixture. The precipitated amine hydrochloride was then collected by vacuum filtration to yield 380 g (81.6%) of 5 as a white powder: TLC (ethyl acetate) Rf (amine) 0.6, Rf salt (0.0); 1H NMR (DMSO-d6) δ 10.23 (br s, 3 H), 7.24 (d, 1 H, J = 1.9 Hz), 6.79 (d, 1 H, J = 2.0 Hz), 3.83 (s, 3 H), 3.72 (s, 3 H); 13C NMR (DMSO-d6) δ 160.8, 124.3, 121.2, 113.4, 112.0, 51.8, 37.1; IR (KBr) 3095, 2693, 1709, 1548, 1448, 1266, 1102, 802, 751; FABMS m/e 154.075 (154.074 calcd for C₇H₇NO₂).  

Methyl 4-Amino-1-methylpyrrole-2-carboxylic Acid (6). The hydrochloride salt of the pyrrole amine 5 (340 g, 1.8 mol) was dissolved in 1 L of 10% aqueous sodium carbonate in 3 L flask equipped with a mechanical stirrer; di-tert-butyl dicarbonate (400 g, 2.0 mmol) slurried in 500 mL of dioxane was added over a period of 30 min while a temperature of 20 °C was maintained. The reaction was allowed to proceed for 3 h and was determined complete by TLC: the mixture was cooled to 5 °C for 2 h and the resulting white precipitate collected by vacuum filtration. The Boc-pyrrole ester contaminated with Boc-anhydride was dissolved in 700 mL of MeOH; 700 mL of 2 M NaOH was added and the solution heated at 60 °C for 6 h. The
reaction was cooled to room temperature and washed with ethyl ether (4 x 1000 mL), the aqueous layer of ca. 3 with 10% (v/v) H2SO4, and the mixture was extracted with ethyl acetate (4 x 1000 mL). The combined ethyl acetate extracts were dried (sodium sulfate) and concentrated in vacuo to provide a tan foam. The foam was dissolved in 500 mL of DCM and 2 L petroleum ether added, and the resulting slurry was concentrated in vacuo. The reaction mixture was redissolved and concentrated three additional times to provide 320 g (78% yield) of 6 as a fine white powder: TLC (7:2 benzene/ethyl acetate v/v) Rf (ester) 0.8, Rf (acid) 0.6; 1H NMR (DMSO-d6) δ 12.10 (s, 1 H), 9.05 (s, 1 H), 7.02 (s, 1 H), 6.55 (s, 1 H), 3.75 (s, 3H), 1.41 (s, 9 H); 13C NMR (DMSO-d6) δ 162.4, 153.2, 123.3, 121.0, 119.2, 107.9, 78.9, 36.6, 28.7; IR(KBr) 3350, 2978, 1700, 1670, 1586, 1458, 1368, 1247, 1112, 878, 779; FABMS m/z 241.119 (M + H 241.119 calcd for C8H10O3).

1,2,3-Benzotriazol-1-yl-4-[[tert-Butoxy carbonylamino]-1-methylpyrrole-2-carboxylic acid (7). Boc-Pyr-acid 6 (31 g, 129 mmol) was dissolved in 500 mL of DCM and HOBt (17 g, 129 mmol) was added followed by DCC (34 g, 129 mmol). The reaction mixture was stirred for 24 h and then filtered dropwise into a well-stirred solution of 5 L of ice water. The precipitate was allowed to sit for 15 min at 0°C and then collected by filtration. The wet cake was dissolved in 500 mL of DCM, and the organic layer was added slowly to a stirred solution of reduced pressure (2 Torr, 102°C) hydrogen chloride was removed by filtration and the solution concentrated in vacuo to provide 75 g (78% yield): TLC (7:2 benzene/ethyl acetate v/v) Rf (amine) 0.3, Rf (salt) 0.0; 1H NMR (DMSO-d6) δ 10.11 (br s, 3 H), 7.43 (s, 1 H), 4.28 (q, 2 H, J = 7.1 Hz), 3.92 (s, 1 H), 1.28 (t, 3 H, J = 7.1 Hz); 13C NMR (DMSO-d6) δ 157.6, 132.6, 117.4, 117.3, 63.8, 36.6, 14.5; IR(KBr) 3139, 2883, 1707, 1655, 1492, 1420, 1314, 1255, 1152, 1057, 837, 776; FABMS m/z 169.085 (169.084 calcd for C6H7N2O).

4-[[tert-Butoxy carbonylamino]-1-methylimidazole-2-carboxylic acid (11). The imidazole amine 10 (75 g, 395 mmol) was dissolved in 200 mL of DMF. DIEA (45 mL, 491 mmol) was added followed by di-tert-butyl dicarbonate (99 g, 491 mmol). The mixture was shaken at 60°C for 18 h, allowed to assume room temperature, and partitioned between 500 mL of brine and 500 mL of ethyl ether. The ether layer was extracted (2 x 200 mL each) with 10% (v/v) acetic acid, brine, saturated sodium bicarbonate, and brine over sodium sulfate, and concentrated in vacuo to yield the Boc-ester contaminated with 20% Boc-anhydride as indicated by 1H NMR. The Boc-ester, used without further purification, was dissolved in 200 mL of 1 M NaOH. The reaction mixture was allowed to stand for 3 h at 60°C with occasional agitation. The reaction mixture was cooled to 0°C and carefully neutralized with 1 M HCl to pH 2, at which time a white gel formed. The gel was collected by vacuum filtration and frozen before drying, and remaining water was lyophilized to yield 10 as a white powder (51 g, 54% yield): 1H NMR (DMSO-d6) δ 9.47 (s, 1 H), 7.13 (s, 1 H), 3.85 (s, 3 H), 1.41 (s, 9 H); 13C NMR (DMSO-d6) δ 160.9, 152.9, 137.5, 134.5, 112.4, 79.5, 35.7, 28.6; IR (KBr) 3448, 2982, 1734, 1654, 1638, 1578, 1537, 1321, 1149, 1263; FABMS m/z 241.105 (241.106 calcd for C6H7N2O).
Activation of Boc—Imidazole Acid. Boc—imidazole acid (257 mg, 1 mmol) and HOBt (135 mg, 1 mmol) were dissolved in 2 mL of DMF, DCC (202 mg, 1 mmol) was then added, and the solution was allowed to stand for at least 5 min.

Activation of Boc—γ—Imidazole Acid and Boc—Pyrrole—Imidazole Acid. The appropriate dimer (1 mmol) and HBTU (378 mg, 1 mmol) were combined in 2 mL of DMF. DIEA (1 mL) was then added, and the reaction mixture was allowed to stand for 5 min.

Activation of Boc—Pyrrole Acid (for Coupling to Imidazole Amine). Boc—pyrrole acid (514 mg, 2 mmol) was dissolved in 2 mL of dichloromethane, DCC (420 mg, 2 mmol) was added, the solution was allowed to stand for 10 min, DMAP (101 mg, 1 mmol) was added, and the solution was allowed to stand for 1 min.

Acetylation Mix. DMF (2 mL), DIEA (710 µL, 4.0 mmol), and acetic anhydride (380 µL, 4.0 mmol) were combined immediately before use.

Manual Synthesis Protocol. Boc-β-alanine-Pam-resin (1.25 g, 0.25 mmol) was placed in a 20 mL glass reaction vessel and shaken in DMF for 5 min, and the reaction vessel was drained. The resin was washed with DCM (2 × 30 s) and the Boc group removed with 80% TFA/DCM/0.5 M PthSH, 1 × 30 s, 1 × 20 min. The resin was washed with DCM (2 × 30 s) followed by DMF (1 × 30 s). A resin sample (5–10 mg) was taken for analysis. The vessel was drained completely and activated monomer added, followed by DIEA if necessary. The reaction vessel was shaken vigorously to make a slurry. The coupling was allowed to proceed for 45 min, and a resin sample was taken. The reaction vessel was then washed with DCM, followed by DMF.

Machine-Assisted Protocols. Machine-assisted synthesis was performed on an ABI 430A synthesizer on a 0.18 mmol scale (900 mg of resin; 0.2 mmol/g). Each cycle of amino acid addition involved deprotection with approximately 80% TFA/DCM/0.4 M PthSH for 3 min, draining the reaction vessel, and then deprotection for 17 min; two dichloromethane flow washes; an NMP flow wash; draining the reaction vessel; coupling for 1 h with in situ neutralization, addition of dimethyl sulfoxide (DMSO/NMP), coupling for 30 min, addition of DIEA, coupling for 30 min; draining the reaction vessel; washing with DCM, taking a resin sample for evaluation of the progress of the synthesis by HPLC analysis; capping with acetic anhydride/DIEA in DCM for 6 min; and washing with DCM. A double couple cycle is employed when coupling aliphatic amino acids to imidazole; all other couplings are performed with single couple cycles.

The ABI 430A synthesizer was left in the standard hardware configuration for NMP—HOBt protocols. Reagent positions 1 and 7 were DIEA, reagent position 2 was TFA/0.5 M thioenol, reagent position 3 was 70% ethanalamine/methanol, reagent position 4 was acetic anhydride, reagent position 5 was DMSO/NMP, reagent position 6 was methanol, and reagent position 8 was DMF. New activator functions were written, one for direct transfer of the cartridge contents to the concentrator (switch list 21, 25, 26, 35, 37, 44), and a second for transfer of reagent position 8 directly to the cartridge (switch list 37, 39, 45, 46).

Boc-Py-OBt ester (357 mg, 1 mmol) was dissolved in 2 mL of DMF and filtered into a synthesis cartridge. Boc-Im acid monomer was activated (DCC/HOBt), filtered, and placed in a synthesis cartridge.