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Rapid Solid-Phase Synthesis of DNA-Binding Pyrrole-Imidazole Polyamides

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Abstract—Pyrrole-imidazole polyamides can be synthesized to target predetermined sequences of DNA with nanomolar affinity and high specificity, and have been shown to modulate gene transcription both in vitro and in vivo. To make polyamides more readily available to biological laboratories, we have developed a rapid solid-phase synthesis based on azabenzotriazole (OAt) activation that decreases synthesis time 60% compared to standard benzotriazole (OBt) techniques, without loss of yield or purity.

DNA minor groove-binding agents based on the naturally occurring distamycin and netropsin have recently received much attention due to their ability to 'read' sequences of DNA.[1] Among the analogues that have been developed, hairpin polyamides that employ an aliphatic linker between chains of aromatic pyrrole (Py) and imidazole (Im) groups have shown the greatest sequence specificity, selectivity, and affinity.[2] Based on the orientation and position of monomers, hairpin polyamides target specific sequences of DNA, and have been used in vitro to block the binding of various transcription factors[3] and inhibit restriction enzyme activity,[4] as well as in vivo to block HIV-1 replication,[5] 5S RNA gene transcription,[6] and modulate *Drosophila melanogaster* development.[7]

Solution-phase syntheses of polyamides are available, but their utility is compromised by long reaction times (due to low molar equivalents) and difficult stepwise purifications.[8] Solid-phase methods have become more widely used, benefiting from shorter reaction times (high molar equivalents), simple step-wise purification, and the diversity that is possible from combinatorial synthesis. Based on peptide synthesis techniques, both fluorenylmethoxycarbonyl (Fmoc) and *tert*-butoxycarbonyl (Boc) protection schemes have been employed for polyamide synthesis. Fmoc protection is amenable to a wide array of solid-phase resins available for post-synthetic modification and requires only mild deprotection conditions (20% piperidine/DMF), but it can result in relatively long coupling times (~3 h) with benzotriazole (OBt) activation methods (i.e., HBTU, DCC/HOBt).[9] Boc-based methods give faster coupling times (~45 min) with OBt activation but require lengthy trifluoroacetic acid (TFA) deprotections (~20 min) with the malodorous cation scavenger, thiophenol (PhSH).[10] In addition, both protection schemes typically cleave the product from the resin by an aminolysis reaction in neat dimethylaminopropylamine (DMPA), resulting in a sometimes-difficult separation of the crude polyamide from the polar amine.[11]

Herein, we describe a manual rapid Boc-based synthesis of polyamides with three critical modifications to previous procedures. First, azabenzotriazole (OAt) activation, by HATU or DCC/HOAt, is used, reducing reaction times from 45 to 15–20 min. Second, Boc deprotections of Py monomers are carried out for 5 min and use phenol and water as scavengers, instead of PhSH. Finally, the crude aminolysis product is first precipitated prior to HPLC purification, both extending column life time and simplifying purification. These modifications allow typical 6–8 ring polyamides (Fig. 1) to be synthesized more conveniently in as few as 3 h and in 20–40% yield, compared to 9–11 h with similar yields using previously published methods.[10]

The first parameter that was examined was the coupling reaction itself (Scheme 1b). Boc protection was chosen

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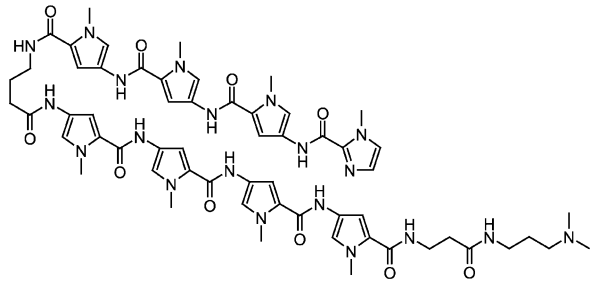
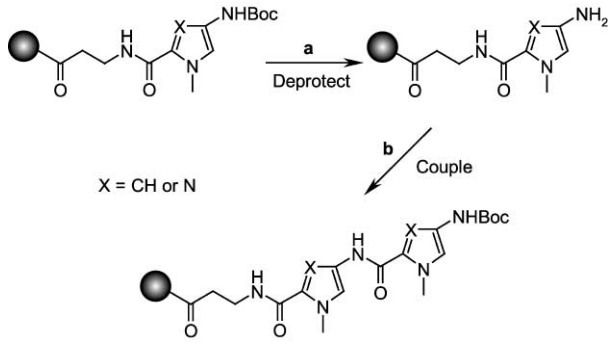


Figure 1. Hairpin polyamide: Im-Py-Py-Py-γ-Py-Py-Py-Py-β-Dp.



Scheme 1. Optimization approach and results. (a) TFA deprotection: TPW (92.5:5:2.5 TFA/phenol/water) for 1, 2, and 2 min for Boc-Py (X=CH) Boc-β-Ala, or Boc-γ-Abu; additional 10 min for Boc-Im (X=N); (b) monomer coupling: activation in 1:1 DMF/NMP, Boc-Py–OH, Boc-β-Ala–OH, or Boc-γ-Abu–OH, HATU, DIEA, 3–5 min; Boc-Im–OH, Im-OH, DCC/HOAt, 2 h, DIEA; coupling for 15–20 min.

over Fmoc protection primarily due to the increased rates of coupling previously observed, but also because of the relative ease of Boc monomer synthesis. Although several coupling reagents have been shown to enhance reaction rates (Bop, PyBOP, PyBroP, acyl chlorides and fluorides, etc.), we chose HATU because it has been used successfully in difficult couplings of hindered and aromatic amines.[12] When HATU was tested, it was found to give remarkably efficient amide formation in this system. Coupling of Boc-Py-OAt (4 equiv, ~0.15 M), formed in situ with HATU (3.6 equiv) and DIEA (12 equiv) for 3–5 min in 1:1 DMF/NMP, to resin bound Py–NH_2 was >99% complete in 10 min.[13–15] However, this rapid reaction time for the initial coupling was found to decrease as the growing polyamide chain is formed, possibly because subsequent coupling reactions are retarded by aromatic stacking and/or resin aggregation. The aliphatic monomers, Boc-γ-aminobutyric acid (γ) and Boc-β-alanine (β) are also efficiently activated by HATU and react rapidly with resin-bound Py–NH_2. Therefore, for shorter polyamides (i.e., six aromatic residues) a coupling time of 15 min was employed, while for longer (eight residue polyamides) 20 min reaction times were found to be most effective (Table 1).

Analogous to previous reports with OBt activation,[10] the OAt ester of Boc-Py–OH can be formed in solution and purified prior to synthesis. Thus, Boc-Py–OAt was formed from the corresponding free acid with EDCI and HOAt in DMF (68% yield).[16] Coupling with 4 equiv of the pre-formed OAt ester proceeded more rapidly than when it was formed in situ (15 instead of 20 min, Table 1). However, this method requires an extra step of solution-based synthesis that is somewhat wasteful of reagents. The in situ HATU activation, on the other hand, requires only commercially available reagents, and is readily performed prior to addition of the monomer to the resin.

Although HATU provides excellent activation for coupling Py, β-Ala, and γ-Abu monomers (the most common couplings), Im monomers are problematic. The electron-withdrawing effect of the aromatic nitrogen of the Im ring may reduce the nucleophilicity of both the aromatic amine and carboxyl groups, resulting in (1) less efficient activation with HATU (often leaving unreacted HATU in the reaction mixture) and (2) slow coupling reactions of Py and Im monomers to the Im amine that are prone to guanidinium formation.[13]

To overcome the problems of incomplete activation, collidine (4 equiv) was employed as base instead of DIEA, resulting in the full activation Boc-Im-OH in 1 h.[17] However, collidine could not be used alone because it did not promote full coupling, making the subsequent addition of DIEA (8 equiv) necessary prior to resin loading. Because under these conditions the activation was still somewhat prone to incomplete activation, DCC and HOAt were added to Boc-Im–OH for 2 h to produce the OAt ester. The advantage of DCC/HOAt is that activation is clearly visible by the formation of the insoluble DCU byproduct.

Using DCC/HOAt, Im monomers were efficiently coupled to resin-bound imidazole amine in 15–20 min. β-Ala and γ-Abu monomers (HATU activation) also couple to the imidazole amine, but require the use of 8 equiv relative to resin loading for complete reaction. Even with its enhanced reactivity, Boc-Py–OAt does not react with imidazole amines at an appreciable rate (<10% in 1 h). This sluggishness necessitates the formation of Boc-Py-Im–OH dimers in solution.[10] However, most polyamides do not employ the Py-Im step as it can generally be replaced by a β-Im step without loss of specificity or affinity.[2,18] If possible, it is recommended to synthesize the Py-Im, β-Im, and γ-Im dimers, as they promote simplified, rapid, and cleaner overall syntheses. The results of coupling optimization are summarized in Table 2.

The next critical aspect of the synthesis that required optimization was Boc deprotection (Scheme 1a). Typically, deprotections are carried out with 80% TFA/DCM with 0.4 M PhSH for 30 s, 1 min, and finally 20 min.[10] This long deprotection scheme did not seem appropriate with the enhanced speed of coupling achieved with HATU, and PhSH is both toxic and extremely malodorous. To first monitor the rate of deprotection, resin bound Boc-Py-Py was treated with neat TFA (~0.5 mL/100 mg resin) for various time periods, emptying the vessel between each addition. It

Table 1. Yields obtained with various deprotection and activation methods by rapid OAt-based polyamide syntheses

Entry	Polyamide	TFA deprotection method[a]	Activation/base[b]	Coupling time (min)	Yield (%)[c]
1	I-P-P-γ-P-P-P-β-Dp	TPW	DIEA	15	41
2	I-P-P-γ-P-P-P-β-Dp	80%TFA/0.4 M PhSH	DIEA	15	36
3	I-P-P-P-γ-I-P-P-P-β-Dp	TPW	DIEA	20	23
4	I-P-P-P-γ-I-P-P-P-β-Dp	TPW	Collidine (4 equiv) DIEA (8 equiv)	20	17
5	I-P-P-P-γ-I-P-P-P-β-Dp	Neat TFA	DIEA	20	13
6	I-P-P-P-γ-P-P-P-P-β-Dp	TPW	DIEA	20	29
7	I-P-P-P-γ-P-P-P-P-β-Dp	Fast 80% TFA	DIEA	20	34
8	I-P-P-P-γ-P-P-P-P-β-Dp	80% TFA	Preformed OAt ester/DIEA	15	30

[a]TPW, 92.5:5:2.5 TFA/phenol/water. All deprotections were for 1, 2, 2 min, except for Boc-Im steps (extra 10 min) and fast 80% TFA, where cleavage mixture was added 3×15 s, 1 min, and 2 min.
[b]DIEA was added at 12 equiv Preformed OAt ester was synthesized by EDCI/HOAt and purified prior to use.
[c]Yield is based upon HPLC purified material and was determined by absorbance spectroscopy, using E∼8333 L/M/cm per aromatic ring.

Table 2. Activation and deprotection methods for Py, β, γ, and Im monomers

Monomer[a]	Activation[a]	TPW deprotection
Boc-Py–OH (4), Boc-β-Ala–OH (8), Boc-γ-Abu–OH (8)	HATU (3.6 or 7.2), DIEA (12), 3–5 min	1, 2, 2 min
Boc-Im–OH (4), Im–OH (4)[b]	DCC/HOAt (4), 2 h then DIEA (12)	1, 2, 2, 10 min

[a]Numbers in parentheses refer to equivalents of reagent relative to resin loading.
[b]TPW deprotection does not apply to Im–OH monomer.

was found that deprotection was complete within 4 min (1 min followed by 3 min addition). The aliphatic monomers, γ-Abu and β-Ala, are also completely deprotected in this time period. Therefore, deprotection was carried out for 1, 2, and 2 min to ensure completeness. However, Boc-Im resin was deprotected much more slowly, requiring an additional 10 min after the initial 5 min treatment to obtain >99% cleavage.

In all cases, the presence of a cation scavenger (i.e., PhSH) is absolutely necessary, as analysis of final crude reaction products by HPLC and MS indicated large amounts of *t*-butyl-containing by-product when no scavengers were employed. However, the stench of PhSH makes it undesirable to use outside of fume hoods or in automated synthesizers (or even within hoods if it can be avoided). Various scavenger mixtures have been used in place of PhSH, one of the most common being reagent B (87.5:5:5:2.5 TFA/phenol/triisoproylsilane/water).[19] Preliminary reactions showed that triisopropylsilane, or triethylsilane, did not reduce *t*-butylation to any great extent. Therefore, a modified reagent B, hereafter called TPW (92.5:5:2.5 TFA/phenol/water) was used. When compared directly to PhSH-containing TFA mixtures, TPW performs slightly better in final yield (Table 1). Not only is TPW less malodorous, it is also safer to use than the highly toxic PhSH. It is of interest to note that 80% TFA, without scavengers, was effective when cleavage was performed rapidly, 3×15 s, followed by 1 min, and 2 min (Table 1). This observation indicates that the formation of *t*-butyl adducts is rather slow, and can be reduced by performing multiple rapid TFA 'washes'. Yet, this is wasteful of reagents, and is not as amenable to automatic synthesizers, which currently deliver reagents more slowly.

The final aspect of polyamide synthesis that was examined is the aminolysis reaction that cleaves the product from the resin. Typical aminolysis is carried out with DMPA (∼0.7 mL/100 mg resin) for 15 h at 55 °C.[10] It was found that shorter polyamides (six ring) can be cleaved efficiently in 5 h at 70 °C (used for entries 1 and 2 in Table 1), while longer (eight ring) polyamides are more effectively cleaved overnight at 50–55 °C. The major difficulty however, lies in purification of the polyamide from the DMPA mixture that results. To this end, a slightly modified version of precipitation used previously was employed.[11] Thus, the crude polyamide was precipitated twice with DCM and ether prior to HPLC purification.[20] This greatly enhances both the lifetime of the HPLC column and the purity of the product that results. Without precipitation, products purified by HPLC are often still contaminated by amines, clearly visible due to their oily nature.

In conclusion, a rapid solid-phase synthesis of polyamides has been described, utilizing OAt activation and shortened Boc deprotection. This combination leads to a dramatic increase in speed, shortening overall synthesis by ∼60% (Table 3).

Though many factors were examined, improvements are still possible. Resin aggregation or other factors appear to have a large deleterious effect on coupling rates as the polyamide chain grows longer, and the investigation of

Table 3. Comparison of rapid –OAt and previously reported –OBt based polyamide syntheses

Step	–OAt-based	–OBt-based[a]
Deprotection	1, 2, 2 min	30 s, 1, 20 min
Wash	1 min DCM, 30 s DMF	
Couple	20 min	45 min
Wash	1 min DMF, 30 s DCM	
Total stepwise	28 min	70 min
Nine-step synthesis	4.2 h	10.5 h

[a]See ref 10.

resins other than polystyrene-based (such as PEG-based) may prove worthwhile in terms of yield and overall efficiency. Yet, the speed at which polyamides can now be synthesized makes their production attractive to biological laboratories that have access to peptide synthesis facilities.

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14. All syntheses described were performed manually in a 10 mL peptide synthesis vessel with three-way stopcock on 200–400 mg Boc-β-Ala-PAM resin (Peptides International, Louisville, KY, USA). No special precautions were taken to maintain an inert atmosphere. The syntheses worked equally well in an Argonaut Technologies Quest 210 Parallel Synthesizer; however, wash steps are inherently slower and increase coupling cycles to 35 min. Monomers were synthesized as described previously.[10] HATU was from Perseptive Biosystems, and other reagents/solvents were reagent grade. Monomers were dissolved in DMF, and HATU in NMP prior to synthesis and combined with DIEA during the activation step (3–5 min for HATU) to yield a 1:1 DMF/NMP mixture. In situ neutralization was used throughout.[13] All polyamides were RP-HPLC purified to greater than 95% purity and characterized by analytical HPLC, ^{1}HNMR, and ES–MS. All data are consistent with literature values, and I-P-P-P-γ-P-P-P-P-β-Dp was found identical to an authentic sample from P. B. Dervan.
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16. Boc-Py–OAt was synthesized as follows: Boc-Py–OH (2.4 g, 10 mmol), EDCI (1.92 g, 10 mmol) and HOAt (1.38 g, 10 mmol) were dissolved in 30 mL DMF and stirred overnight. The resulting suspension was filtered through Celite into 500 mL ice-cold water. The precipitate was filtered, suspended in acetone, and added dropwise to cold petroleum ether. The resulting precipitate was suspended in DCM, added to pet ether, and filtered. Boc-Py–OAt (3.59 g, 68%) was obtained as a tan solid ^{1}H NMR: 9.44 (s, 1H), 8.85 (d, 1H, $J=4$ Hz), 8.75 (d, 1H, $J=8$ Hz), 7.68 (q, 1H, $J=4$ Hz), 7.58 (s, 1H), 7.23 (s, 1H), 3.86 (s, 3H), 1.50 (s, 9H).
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20. The DMPA/resin/polyamide mixture was filtered to remove resin, the resin washed two times with two volumes (relative to DMPA) of DCM each, then precipitated by adding 8–10 volumes diethyl ether and cooling to −20 (or 0) °C. The suspension was then centrifuged in a table top centrifuge at maximum speed, the solid resuspended in DCM, and precipitated as before. The crude product obtained was dried under vacuum for at least 30 min to produce a powdery solid, largely free of the aminolysis reagent. The product was dissolved in 15% acetonitrile/ 0.1% TFA/H_2O and purified by reversed-phase HPLC.