Supporting Information

Fully Automated Synthesis of DNA-Binding Py-Im Polyamides Using a Triphosgene Coupling Strategy

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General Information

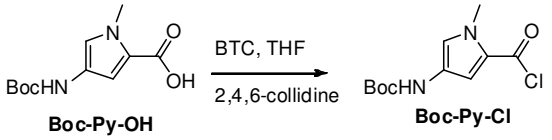
Abbreviations: BTC, bis-(trichloromethyl)-carbonate; CH_3CN, acetonitrile; DCC, N,N'-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, N,N'-diisopropylcarbodiimide; DIEA, N,N'-diisopropylethylamine; DMF, N,N'-dimethylformamide; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; HBTU, 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-aza-benzotriazole; HOBt, 1-hydroxybenzotriazole; PyBOP, benzotriazol-1-yl-oxytripyrrolidino- phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Dry DMF and Dry THF were purchased from Sigma-Aldrich. Boc-Py-OH, Fmoc-L-Dab(Boc)-OH, collidine (2,4,6-collidine) and the HPLC grade solvents (CH_3CN and MeOH) were purchased from J&K Scientific. Fmoc-GABA-OH, BTC, DIEA, TFA were purchased from Aladdin. Fmoc-hydrazinobenzoyl AM resin was purchased from Novabiochem. Fmoc-D-Dab(Boc)-OH was purchased from Alfa-Aesar. Boc-Im-OH, Fmoc-Py-OH and Fmoc-Im-OH were purchased from Atomax Chemicals. Fine powders of Boc-Im-OH, Fmoc-Py-OH and Fmoc-Im-OH were obtained after lyophilization to increase their solubility in THF. All other commercial reagents were used as received.

Analytical RP-HPLC was performed at room temperature on the Shimadzu LC 20 with UV detector SPD-20A using Inertsil ODS-SP column (4.6 x 250 mm, 5 μm, 100 Å). The RP-HPLC gradient was started at 10% of B (MeCN), then increased to 100% of B over 30 min (A: 0.1% TFA in water). Semi-preparative RP-HPLC was performed on the ULTIMAT 3000 Instrument (DIONEX). UV absorbance was measured using a photodiode array detector at 254 and 310 nm. The RP-HPLC gradient was started at 10% of B (MeCN), then increased to 100% of B over 30 min (A: 0.1% TFA in water). Mass spectra were measured on ABI Q-star Elite.

Experimental Procedure

1. Preparation of Boc-Py-Cl intermediate



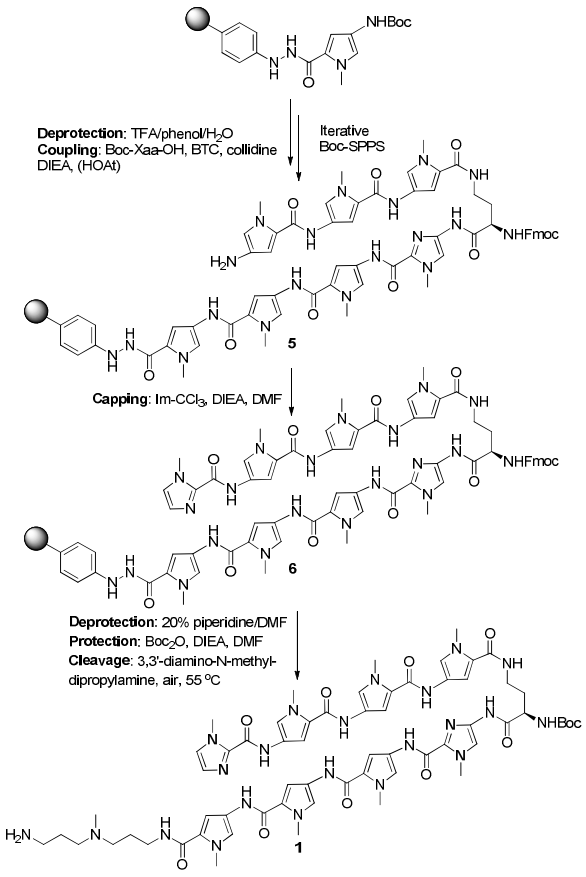
Boc-Py-OH **Boc-Py-Cl**

Boc-Py-OH (60 mg, 0.25 mmol, 1.0 eq) and BTC (24 mg, 0.33 eq.) were dissolved in dry THF (3 mL). To the resulting solution, collidine (14 eq.) was added slowly and the suspension was stirred for about 2 min. The resulting slurry was centrifuged and the solution was diluted with CH_3CN. A small portion was taken out for HRMS analysis. HRMS (ESI) *m/z*: calcd for $C_{11}H_{14}N_2O_3Cl$ $[M-H]^-$ (-TOF MS) 257.0698, found 257.0702; calcd for $C_{11}H_{15}N_2O_3$ $[M-Cl]^+$ (+TOF MS) 223.1077, found 223.1069.

2. Automated synthesis of Py-Im polyamides using Boc- chemistry

The automated synthesis was performed on a CS336X peptide synthesizer with a computer-controlled operation system. The synthesizer was programmed in the standard hardware configuration for DIC/HOBt (or HBTU/DIEA) protocols. Reagent position 1 was DMF, reagent position 2 was dry DMF, reagent position 3 was piperidine/DMF (20%), reagent position 4 was DCM, reagent position 5 was TFA/phenol/H_2O (92.5:5:2.5), reagent position 6 was DIEA/dry DMF (10%), reagent position 7 was 2,4,6-collidine/dry THF (15%), and reagent position 8 was dry THF. ***CAUTION: Phosgene and CO_2 gas were evolved, the peptide synthesizer was placed in a fume hood and the waste gas was treated with aqueous NaOH solution (20%) to destroy the excess phosgene.***

The synthesis was carried out on a 0.06 mmol scale (400 mg of the resin; 0.15 mmol/g). Each cycle of amino acid addition involved deprotection, amino acid activation and coupling. Two successive coupling cycles are employed when coupling pyrrole amino acids to imidazole amines; all other couplings are performed with single coupling cycles.



Scheme 1. Synthesis of polyamide **1**: (i) TFA/phenol/H_2O; (ii) Boc-Py-OH, BTC, Collidine, DIEA; (iii) TFA/phenol/H_2O; (iv) Boc-Py-OH, BTC, Collidine, DIEA; (v) TFA/phenol/H_2O; (vi) Boc-Im-OH, BTC, Collidine, DIEA, HOAt; (vii) TFA/phenol/H_2O; (viii) Fmoc-D-Dab(Boc)-OH, BTC, Collidine, DIEA, HOAt; (ix) TFA/phenol/H_2O; (x) Boc-Py-OH, BTC, Collidine, DIEA; (xi) TFA/phenol/H_2O; (xii) Boc-Py-OH, BTC, Collidine, DIEA; (xiii) TFA/phenol/H_2O; (xiv) Boc-Py-OH, BTC, Collidine, DIEA; (xv) TFA/phenol/H_2O; (xvi) Im-CCl_3, DIEA; (xvi) 20% piperidine/DMF; (xvii) Boc_2O, DIEA; (xvii) 3,3'-diamino-N-methyl-dipropylamine, air, 55 ℃, 16 h.

2.1 Deprocetion procedure

At the beginning of the synthesis, the needle was placed in an empty amino acid (AA) reservoir (the AA reservoirs loaded with the monomers and BTC (or HOAt) were arranged on the Wheel). Boc-Py-hydrazine resin (prepared manually from Boc-Py-OH and Fmoc-hydrazinobenzoyl AM resin) was loaded in the reaction vessel (RV) (10 mL). The resin was washed twice with DCM (5 mL, calibrated delivery from reagent bottle 4) and deprotected twice (for 2 min and 20 min, respectively) with

the mixture of TFA/phenol/H_2O (5 mL, calibrated delivery from reagent bottle 5). The RV was drained and the resin was treated with two DCM washes (5 mL from reagent bottle 4), a DMF wash (5 mL from reagent bottle 1), a DIEA/dry DMF wash (5 mL from reagent bottle 6, bypasing the AA reservoir), a dry THF wash (to wash the needle, 5 mL from reagent bottle 8, bypasing the AA reservoir), a DMF wash (5 mL from reagent bottle 1), and a dry DMF wash (5 mL from reagent bottle 2). The RV was drained and the needle was set up to be retracted and injected in the following AA reservoir loaded with the monomer and BTC solids (by "Advance_AA"). Then the resin was ready for the coupling reaction. The same deprocetion programs were applied at the initiation of each coupling cycle.

2.2 Amino acids activation

2.2.1 Activation of Boc-Py-OH

Boc-Py-OH (60 mg, 0.25 mmol) and BTC (37 mg, 0.125 mmol) were loaded as solids into an AA reservoir. After calibrated delivery of dry THF (1.25 mL from reagent bottle 8 bypassing amino acid metering vessel (MVAA)), the mixture in the AA reservoir was blended by nitrogen bubbles for 2 min (using the program "Bubble/shake_1Min"). The solution was activated by calibrated delivery of 2,4,6-collidine/dry THF (15%, 1.25 mL from reagent bottle 7 bypassing MVAA). The resulting slurry was blended by nitrogen bubbles for 2 min followed by calibrated delivery of DIEA/dry DMF (10%, 2.5 mL from reagent bottle 6 bypassing MVAA). After blended with nitrogen bubbles for 1 min, a clear pale yellow solution was formed.

Then the activator cycle was written to transfer activated monomer directly from the AA reservoir to RV, bypassing the metering vessel (MV) and transfer vessel (TV). All lines were emptied with nitrogen before and after solution transfers.

2.2.2 Activation of Boc-Im-OH

Boc-Im-OH (60 mg, 0.25 mmol) and BTC (37 mg, 0.125 mmol) were loaded as solids into one AA reservoir followed by another reservoir loaded with HOAt (34 mg, 0.25 mmol). The mixture of Boc-Im-OH and BTC was activated employing the same programs as that used for Boc-Py-OH to form a clear brown solution.

Then the activator cycle was written to transfer activated monomer from the AA reservoir to TV. The needle was set up to be retracted from the first AA reservoir and injected in the second one loaded with HOAt. After calibrated delivery of DIEA/dry DMF (10%, 1.25 mL from reagent bottle 6), the mixture in the AA reservoir was blended by nitrogen bubbles for 2 min. The solution was transferred from the AA reservoir to TV and mixed with the activated Im monomer. After 3 min (using the program "Shake_1Min"), the resulting solution was transferred to RV for the coupling reaction. All lines were emptied with nitrogen before and after solution transfers.

2.2.3 Activation of Fmoc-D-Dab(Boc)-OH

Fmoc-D-Dab(Boc)-OH (109 mg, 0.25 mmol) and BTC (37 mg, 0.125 mmol) were loaded as solids into one AA reservoir and HOAt (34 mg, 0.25 mmol) was loaded in another AA reservoir. The same programs as that used for Boc-Im-OH could be applied to the activation of Fmoc-D-Dab(Boc)-OH.

2.3 Amino acids coupling

After the amino acid was activated, it was transferred to RV loaded with the deprotected resin. The RV was shaked for 25 min (Shake_1Min) before it was drained. Then the resin was treated with two dry THF washes (to wash the needle and the AA reservoir, 5 mL from reagent bottle 8, bypasing the AA reservoir) and three DMF washes (5 mL from reagent bottle 1). The RV was drained and the resin was ready for the next coupling cycle.

2.4 Stepwise HPLC Analysis

During the coupling reaction, a resin sample (ca. 4 mg) was taken out and washed twice with DMF (1 mL). The resin was then treated with $Cu(OAc)_2$ and 3,3'-diamino-N-methyl-dipropylamine (20 μL) at room temperature for 30 min. The mixture was diluted with MeOH (100 μL) and filtered. A sample (20 μL) was analyzed by analytical HPLC at 310 nm.

2.5 The terminal Im capping

Method A: Im-CCl_3 was prepared according to Masiukiewicz' procedure (Organic Preparations and Procedures International: The New Journal for Organic Synthesis, 37:4, 403-405, DOI: 10.1080/00304940509354973). Im-CCl_3 (57 mg, 0.25 mmol)

was placed in an AA reservoir and dissolved by calibrated delivery of DIEA/dry DMF (10%, 2 x 2.5 mL from reagent bottle 6), the mixture in the AA reservoir was blended by nitrogen bubbles for 2 min before it was transferred to RV, bypassing MV and TV. The RV was shaked for 40 min ("shake_1Min) to enable the coupling. The resin was treated with two dry THF washes (to wash the needle and the AA reservoir, 2 x 2.5 mL from reagent bottle 8, bypasing the AA reservoir) and three DMF washes (5 mL from reagent bottle 1).

Method B: Im-OH (32 mg, 0.25 mmol) and PyBOP (130 mg, 0.25 mmol) were placed in an AA reservoir and dissolved by calibrated delivery of DIEA/ dry DMF (10%, 2 x 2.5 mL from reagent bottle 6), the mixture in the AA reservoir was blended by nitrogen bubbles for 5 min before it was transferred to RV, which was shaked for 120 min to accomplish the coupling. After treated following the same procedure as used in method A, the resin was taken out for cleavage.

2.6 Protecting group exchange

Deprection: The Fmoc group of the γ -turn unit was removed with 20% piperidine/DMF using the **"**Deprocetion procedure**"** of Fmoc- chemistry described below.

Boc- protection: Boc_2O (115 μL, 0.50 mmol) was placed in an AA reservoir. Following a similar procedure as used in the "terminal Im capping", the Boc_2O/DIEA/dry DMF mixture was transferred to RV. After shaking for 15 min ("shake_1Min), the resin was treated with two dry THF washes and three DMF washes. Then the resin was taken out for cleavage.

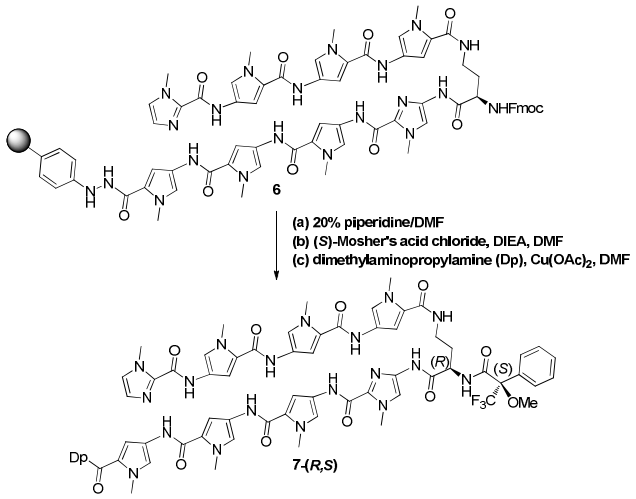
2.7 Resin cleavage

The resin (polyamide **6**) was divided in four parts. To one part, 3,3'-diamino-N-methyl-dipropylamine (100 μL) in DMF (200 μL) was added and the mixture was shaked at 55 °C for 16 h. After cooling to room temperature, the resin was removed by filtration through a disposable propylene filter and washed with MeOH (2 mL). The organic solution was concentrated under reduced pressure and the residue was precipitated by adding 10 volumes of cold diethyl ether. The crude peptide was collected by centrifugation and the light-yellow powders were dissolved

in 10% MeCN/H_2O/0.1%TFA and purified by semi-preparative RP-HPLC. After lyophilization, polyamide **1** was obtained as a pale yellow powder (3 mg, 15%). Analytical HPLC t_r = 17.7 min. HRMS (ESI) *m/z*: calcd for $C_{62}H_{81}N_{22}O_{11}$ $[M+H]^+$ 1309.6450, found 1309.6444.

Following a similar procedure, polyamide **2** was cleaved from the resin using dimethylaminopropylamine (1.6 mg, 9%). Analytical HPLC t_r = 15.9 min. HRMS (ESI) *m/z*: calcd for $C_{53}H_{66}N_{23}O_9$ $[M+H]^+$ 1168.5408, found 1168.5415.

2.8 Preparation of the Mosher amides



Scheme 2. Synthesis of Mosher amide **7-(*R,S*)**

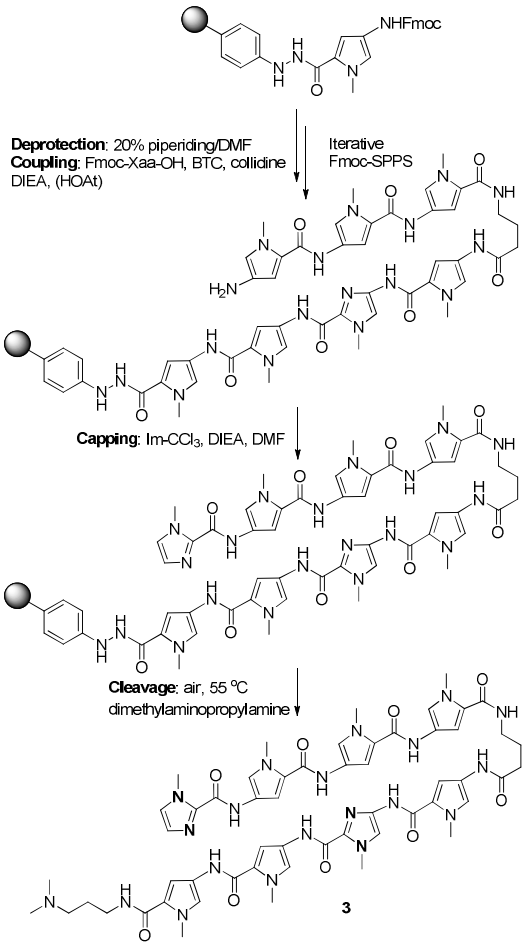
The Fmoc group of polyamide **6** (ca. 20 mg resin, 0.15 mmol/g) was removed using a mixture of 20% piperidine/DMF. After washing with DMF (4 x 2 mL) and dry DMF (2 mL), the resin was treated with a mixture of (*S*)-Mosher's acid chloride (15 mg, 0.06 mmol), DIEA (20 μL) and DMF (1 mL) for 15 min. The reaction mixture was drained and the resin was rinsed with DMF (4 x 2 mL). Then the resin was treated with $Cu(OAc)_2$ and dimethylaminopropylamine (20 μL) at room temperature for 3 h. The crude peptide was collected by centrifugation and the blue solution was diluted with 10% MeCN/H_2O/0.1% TFA and purified by semi-preparative RP-HPLC. A smaple of the collected solution of Mosher amide **7-(*R,S*)** in MeCN/0.1% TFA was taken for HPLC analysis. Chiral HPLC t_r = 10.9 min. HRMS (ESI) *m/z*: calcd for $C_{65}H_{75}F_3N_{21}O_{11}$ $[M+H]^+$ 1382.5902, found 1382.5898.

Following the same procedure, the sample of Mosher amide **7-(*S,S*)** was prepared

from Fmoc-L-Dab(Boc)-OH. Chiral HPLC t_r = 13.25 min. HRMS (ESI) *m/z*: calcd for $C_{65}H_{75}F_3N_{21}O_{11}$ $[M+H]^+$ 1382.5902, found 1382.5895.

The sample of **7-(*R,S*), 7-(*S,S*)** and a mixture of them were subjected to HPLC analysis using CHIRALPAK® ID column (0.46 cm I.D. × 25 cm L.× 5 μm) to separate the two diastereomers. (Shimadzu LC 20 with UV detector SPD-20A, at 310 nm.) The gradient was 45% CH_3CN in phosphate buffer solution (PH = 2.5) over 30 min at 0.4 mL/min.

3. Automated synthesis of polyamide 3 using Fmoc- chemistry



Scheme 3. Synthesis of polyamide **3**: (i) 20% piperidine/DMF; (ii) Fmoc-Py-OH, BTC, Collidine, DIEA; (iii) 20% piperidine/DMF; (iv) Fmoc-Im-OH, BTC, Collidine, DIEA, HOAt; (v) 20% piperidine/DMF; (vi) Fmoc-Py-OH, BTC, Collidine, DIEA; (vii) 20% piperidine/DMF; (viii) Fmoc-GABA-OH, BTC, Collidine, DIEA, HOAt; (ix) 20% piperidine/DMF; (x) Fmoc-Py-OH, BTC, Collidine, DIEA; (xi) 20% piperidine/DMF; (xii) Fmoc-Py-OH, BTC, Collidine, DIEA; (xiii) 20% piperidine/DMF; (xiv) Fmoc-Py-OH, BTC, Collidine, DIEA; (xv) 20% piperidine/DMF; (xvi) Im-CCl_3, DIEA; (xvii) dimethylaminopropylamine, air, 55 ℃, 16 h.

3.1 Deprocetion procedure

At the beginning of the synthesis, the needle was placed in an empty AA reservoir (the AA reservoirs loaded with the monomers and BTC (or HOAt) were arranged on the Wheel). Fmoc-Py-hydrazine resin (prepared manually from Fmoc-Py-OH and Fmoc-hydrazinobenzoyl AM resin) was loaded in RV (10 mL). The resin was deprotected twice (10 min each) with the mixture of 20% piperidine/DMF (5 mL from reagent bottle 3). The RV was drained and the resin was treated with two dry DMF washes (5 mL from reagent bottle 2), two dry THF washes (to wash the needle, 2 x 2.5 mL from reagent bottle 8, bypasing the AA reservoir), a DCM wash (5 mL from reagent bottle 4), a DMF wash (5 mL from reagent bottle 1), and a dry DMF wash (5 mL from reagent bottle 2). The RV was drained and the needle was set up to retracted and injected in the following AA reservoir loaded with the monomer and BTC solids (by "Advance_AA"). Then the resin was ready for the coupling reaction. The same deprocetion programs were applied at the initiation of each coupling cycle.

3.2 Amino acids activation

Fmoc-Py-OH, Fmoc-Im-OH and Fmoc-GABA-OH were activated according to the programs used to activate Boc-Py-OH, Boc-Im-OH and Boc-D-Dab(Boc)-OH, respectively.

3.3 Amino acids coupling

After the amino acid was activated, it was transferred to RV loaded with the deprotected resin. Two coupling cycles were needed to drive the reaction of Fmoc-Py-OH/Resin-Im-NH_2 to completion (60 min each). All other couplings were carried out with a single-coupling cycle with extended reaction time (40 min). Then the resin was treated with two dry THF washes (to wash the needle and the AA reservoir, 2 x 2.5 mL from reagent bottle 8, bypasing the AA reservoir) and three DMF washes (5 mL from reagent bottle 1). The RV was drained and the resin was ready for the next coupling cycle.

3.4 Stepwise HPLC Analysis

During the coupling reaction, a resin sample (ca. 4 mg) was taken out and washed with DMF (2 x 1 mL). The Fmoc group was removed using a mixture of 20%

piperidine/DMF. After washing with DMF (3 x 1 mL) and dry DMF (1 mL), the resin was treated with a mixture of Boc_2O/DIEA/DMF for 10 min. The reaction mixture was drained and the resin was rinsed with DMF (3 x 1 mL). Then the resin was treated with $Cu(OAc)_2$ and 3,3'-diamino-N-methyl-dipropylamine (20 μL) at room temperature for 30 min. The mixture was diluted with MeOH (100 μL) and filtered. A sample (20 μL) was analyzed by analytical HPLC at 310 nm.

3.5 The terminal Im capping

The terminal Im capping was carried out following the same procedure as used in Boc- chemistry.

3.6 Resin cleavage

Following a similar procedure as mentioned above, polyamide **3** was obtained after cleavage from the resin using dimethylaminopropylamine (2 mg, 12%). Analytical HPLC t_r = 17.3 min. HRMS (ESI) *m/z*: calcd for $C_{55}H_{67}N_{20}O_9$ $[M+H]^+$ 1151.5394, found 1151.5392.

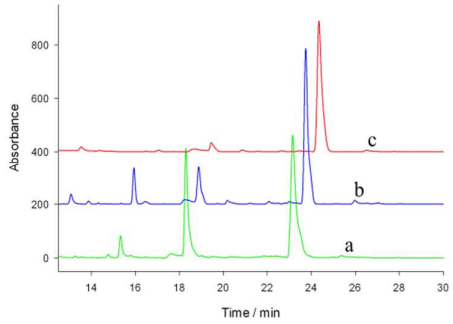
4. Coupling conditions used in the automated Py-Im Polyamide synthesis:

resin	monomer	BTC equiv	collidine equiv[a]	DIEA equiv[a]	HOAt equiv	PyBOP equiv	monomer in DMF/THF	coupling time (min)
Resin-Py-NH_2	Boc-Py-OH (4 equiv)	2	24	24	-	-	0.05 M	25
	Boc-Im-OH (4 equiv)	2	24	24	4	-	0.05 M	25
	Fmoc-D-Dab(Boc)-OH (4 equiv)	2	24	24	4	-	0.05 M	25
	Fmoc-Py-OH (6 equiv)	3	24	24		-	0.075	40
	Fmoc-Im-OH (6 equiv)	3	24	24	4	-	0.075	40
	Fmoc-GABA-OH (6 equiv)	3	24	24	4	-	0.075	40
	Im-CCl_3 (4 equiv)	-		24	-	-	0.05	40
	Im-OH (4 equiv)	-		24	-	4	0.05	120
Resin-Im-NH_2	Boc-Py-OH (4 equiv)	2	24	24	-	-	0.05	25 (twice)
	Boc-Im-OH (4 equiv)	2	24	24	4	-	0.05	25
	Fmoc-D-Dab(Boc)-OH (4 equiv)	2	24	24	4	-	0.05	25
	Fmoc-Py-OH (6 equiv)	3	24	24	-	-	0.075	60 (twice)
	Im-CCl_3 (4 equiv)	-		24	-	-	0.05	40
	Im-OH (4 equiv)	-		24	-	4	0.05	120

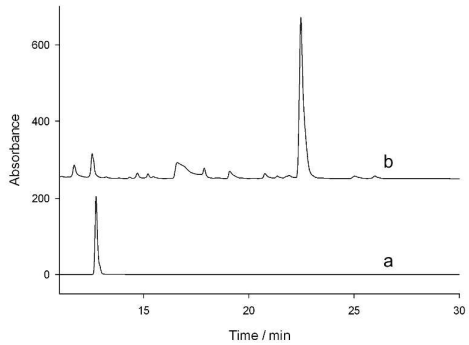
[a]Excess collidine and DIEA were used for two reasons: (1) to neutralize the acid resulted by the decomposition of excess BTC; (2) when more monomer and BTC were used in the synthesis, the volume of collidine/dry THF and DIEA/dry DMF can be kept unchanged

HPLC Analysis

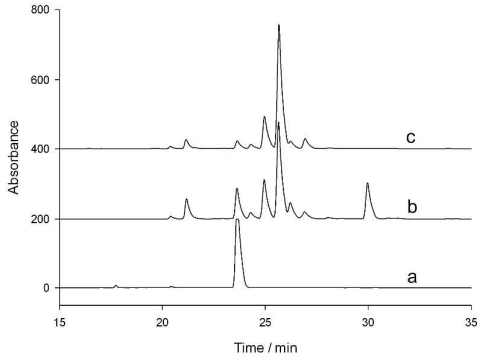
1. Amino acid activation



Activation of Boc-Py-OH with BTC (285 nm): (a) 0.33 equiv of BTC; (b) 0.41 equiv of BTC; (c) 0.50 equiv of BTC. t_r = 18.29 (Boc-Py-OH), t_r = 23.16 (the resulting amide) (1:15, estimated by integration).

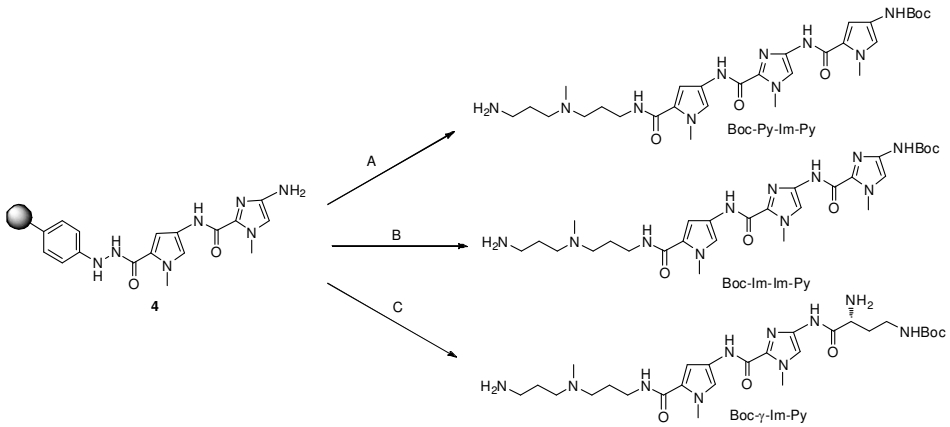


Activation of Boc-Im-OH (trace a) with 0.50 equiv BTC (trace b) (283 nm): t_r = 12.83 (Boc-Im-OH), t_r = 22.47 (the resulting amide), (1:9, estimated by integration)

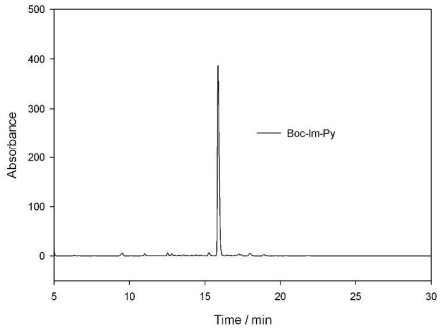


Activation of Fmoc-D-Dab(Boc)-OH (trace a) with 0.33 equiv BTC (trace b) and 0.5 equiv BTC(trace c) (254 nm): t_r = 23.68 (Fmoc-D-Dab(Boc)-OH), t_r = 25.66 (the resulting methyl ester), (1:17, estimated by integration)

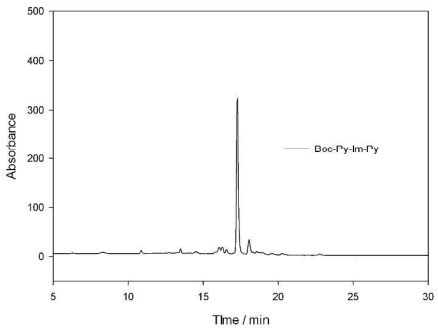
2. Coupling of monomers to Im amine 4



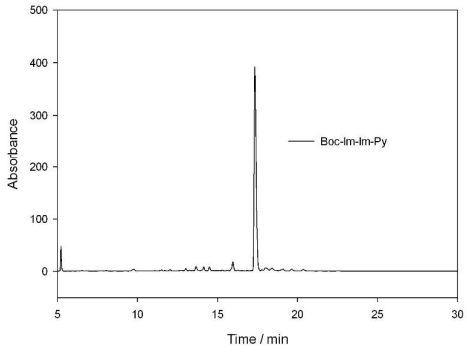
Scheme 4. Coupling of Boc-Py-OH, Boc-Im-OH and Fmoc-D-Dab(Boc)-OH to the resin bound Im amine **4**: A. (1) Boc-Py-OH, BTC, Collidine, DIEA; (2) 3,3'-diamino-N-methyl-dipropylamine, $Cu(OAc)_2$. B. (1) Boc-Im-OH, BTC, Collidine, DIEA, HOAt; (2) 3,3'-diamino-N-methyl-dipropylamine, $Cu(OAc)_2$. C. (1) Fmoc-D-Dab(Boc)-OH, BTC, Collidine, DIEA, HOAt; (2) 3,3'-diamino-N-methyl- dipropylamine, $Cu(OAc)_2$.



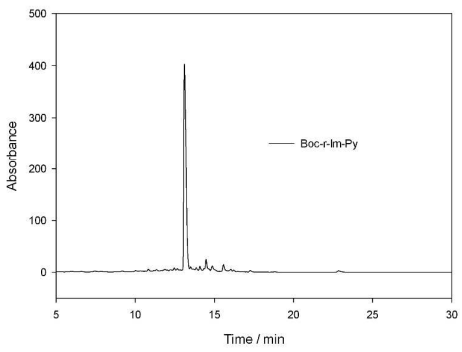
crude Boc-Im-Py (t_r = 15.86 min)



crude Boc-Py-Im-Py (t_r = 17.29 min)

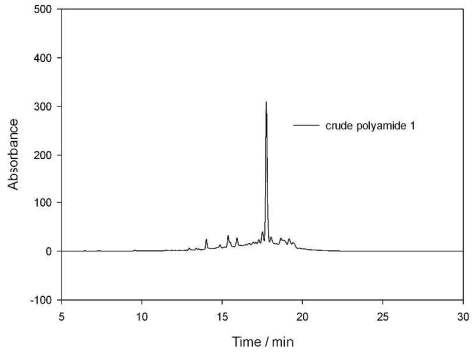


crude Boc-Im-Im-Py (t_r = 17.33 min)

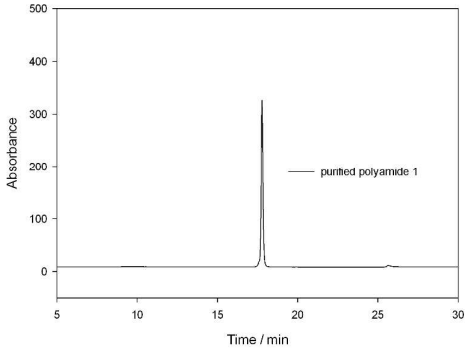


crude Boc- γ - Im-Py (t_r = 13.10 min)

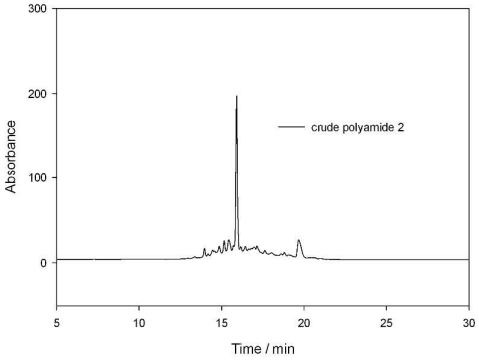
3. Polyamide 1, 2 and 3



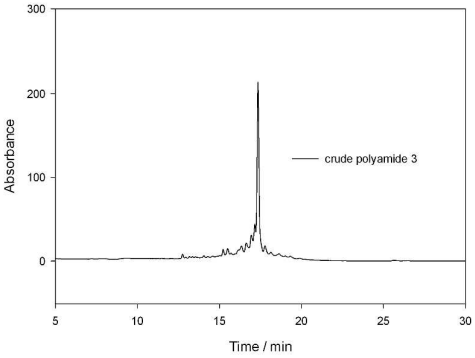
crude polyamide **1** (t_r = 17.74 min)



purified polyamide **1** (t_r = 17.78 min)

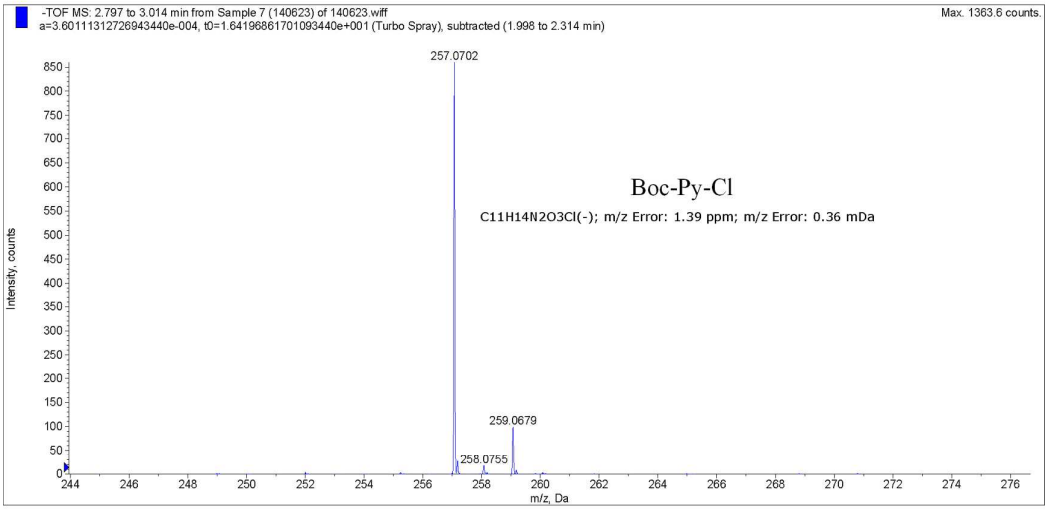


crude polyamide **2** (t_r = 15.90 min)

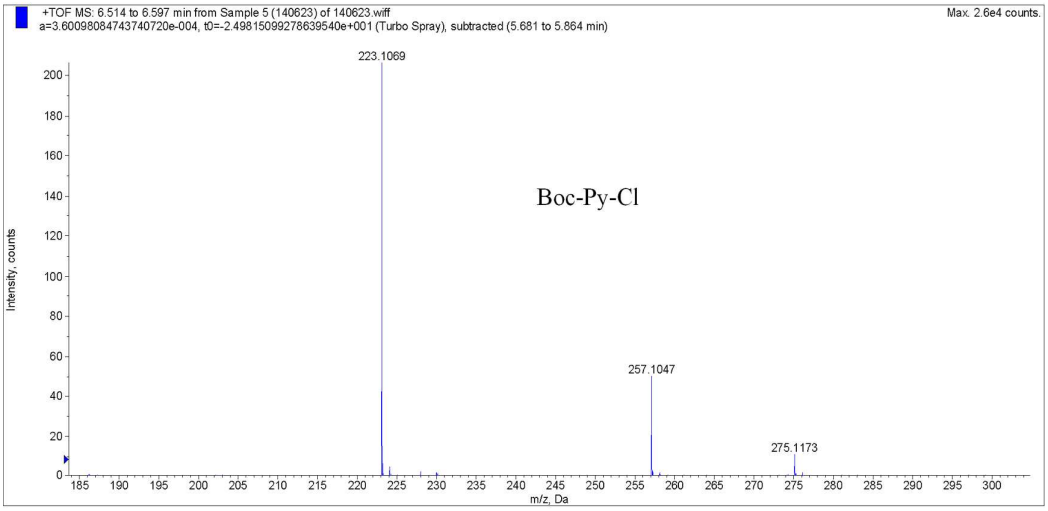


crude polyamide **3** (t_r = 17.32 min)

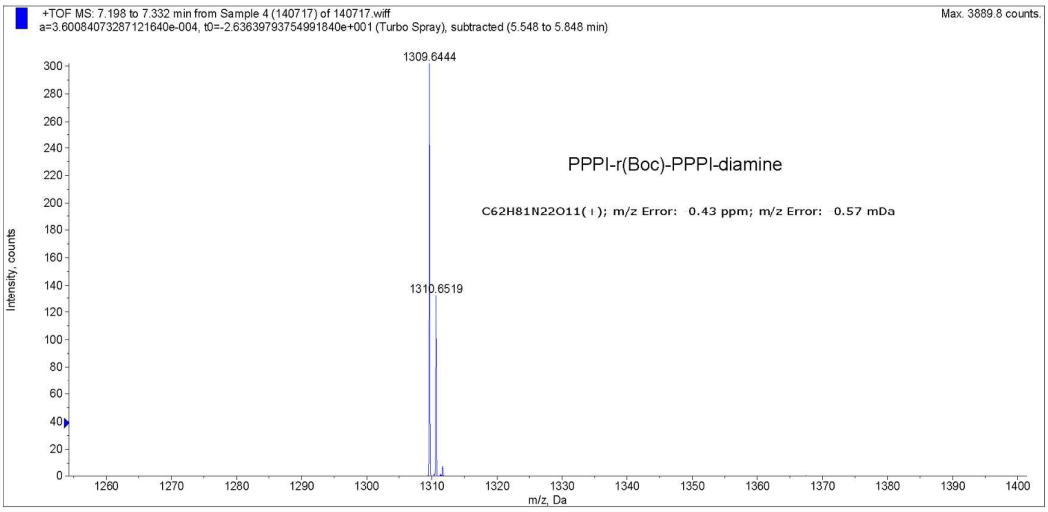
Copies of HRMS Spectrums



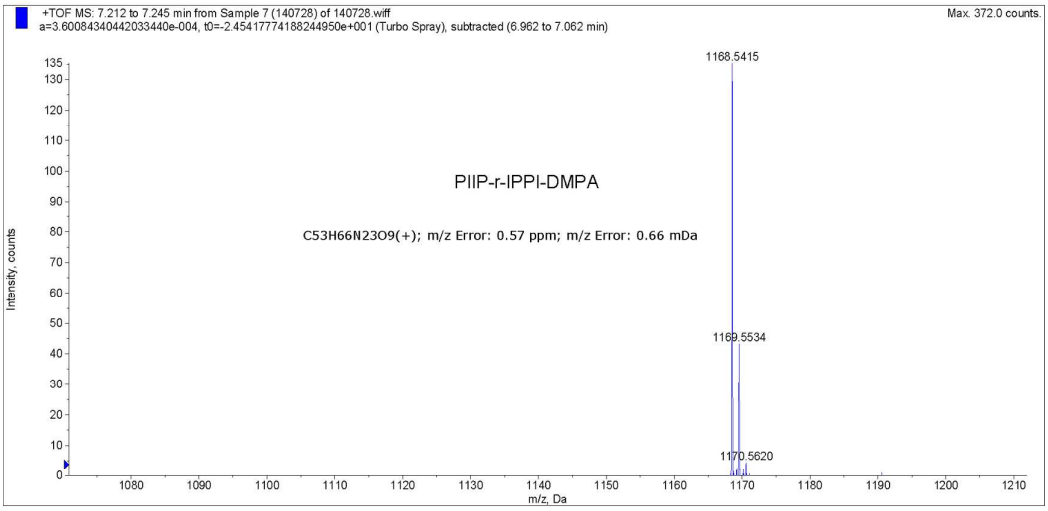
HRMS spectra (-TOF MS) of Boc-Py-Cl



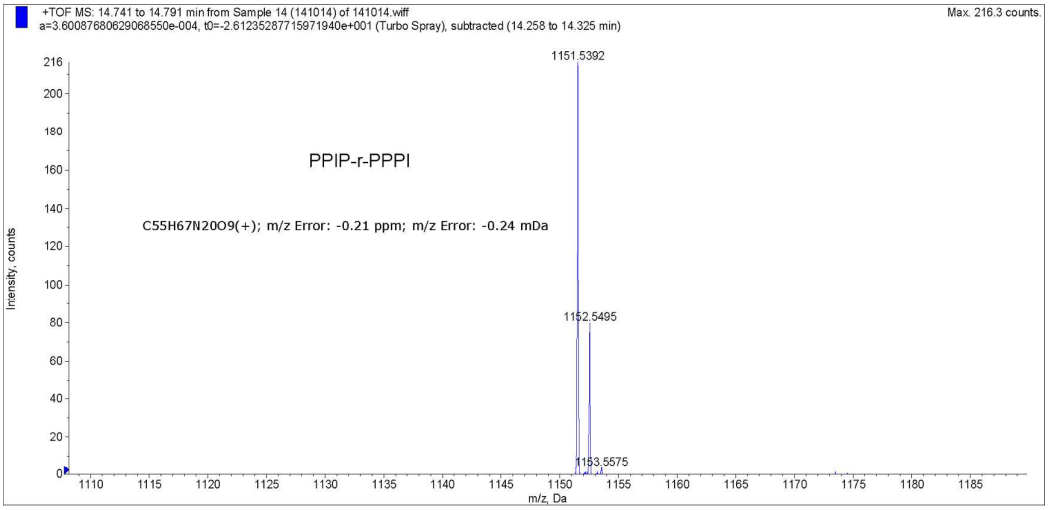
HRMS spectra (+TOF MS) of Boc-Py-Cl



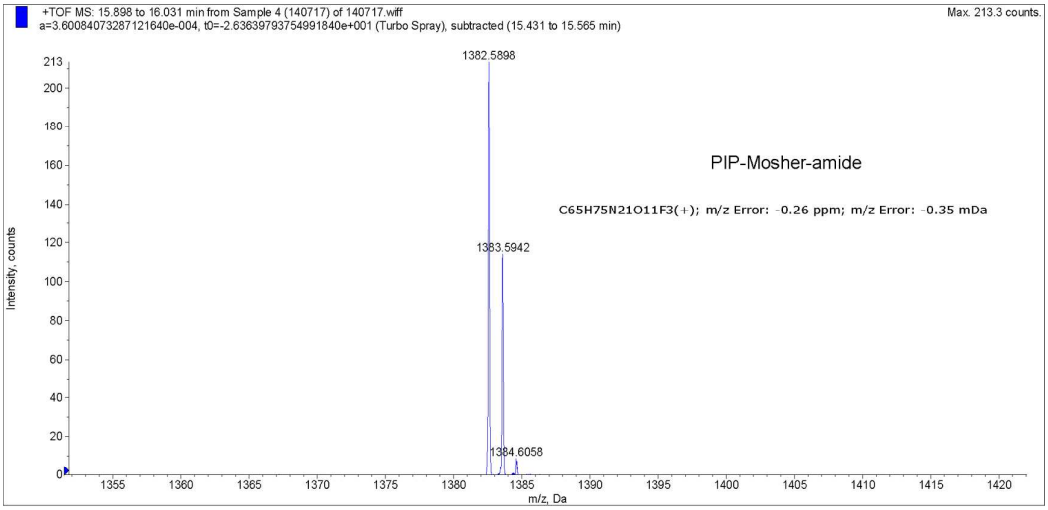
HRMS spectra of Py-Im polyamide **1**



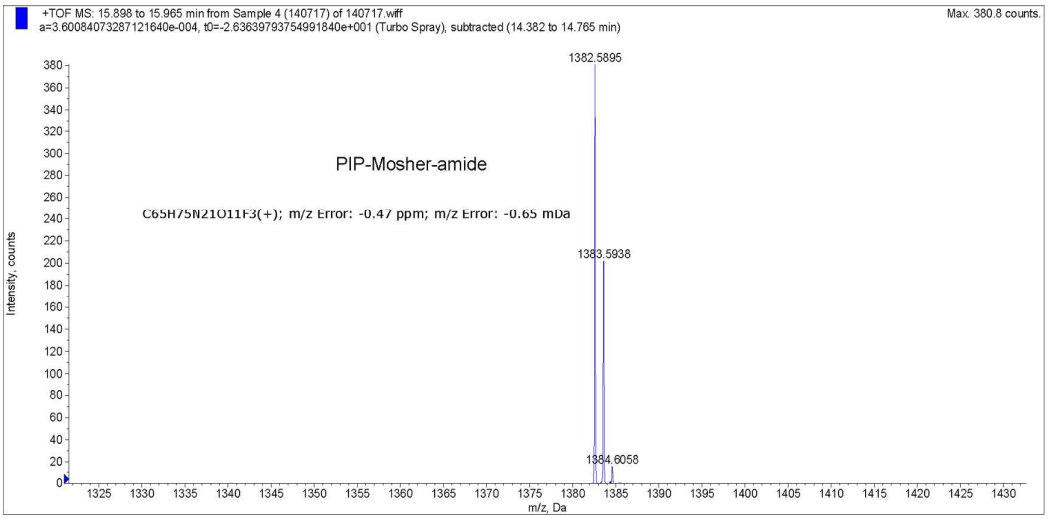
HRMS spectra of Py-Im polyamide **2**



HRMS spectra of Py-Im polyamide **3**



HRMS spectra of Mosher amide **7-(*R,S*)**



HRMS spectra of Mosher amide **7-(*S,S*)**