Supporting Information

Orthogonal γPNA dimerization domains empower DNA binders with cooperativity and versatility mimicking that of transcription factor pairs

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Table of Contents

MATERIALS AND METHODS

General

The reagents for polyamide syntheses such as Fmoc-Py-OH, Fmoc-Im-OH, Fmoc-Py-Im-OH, and Im-CCl_3, solid supports (Fmoc-Py-oxime resin and Fmoc-β Ala-Wang resin), *O*-(1*H*-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) and benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were from HiPep Laboratories (Kyoto, Japan). Trifluoroacetic acid (TFA), 3,3′-diamino-*N*-methyldipropylamine, *N*,*N*-diisopropylethylamine (DIEA), dichloromethane (DCM), methanol, acetic acid (AcOH), 1-methyl-2-prrrolidone (NMP), and *N*,*N*-dimethylformamide (DMF) were obtained from Nacalai Tesque (Kyoto, Japan). Fmoc-D-Dab (Boc)-OH and Fmoc-NH-$dPEG_3$-COOH were obtained from Iris Biotech GmbH (Marktredwitz, Deutschland). Polyamide-chain assembly was performed on an automated synthesizer, PSSM-8 (Shimadzu, Kyoto, Japan). HPLC grade acetonitrile (Nacalai tesque) was used for both analytical and preparative HPLC. Water was prepared by a Milli-Q apparatus (Millipore, Tokyo, Japan). All chemicals were used as received. Analyses by reversed-phase RP-HPLC were carried out online LCMS (Agilent 1100 ion-trap mass spectrometer, HCT ultra, Bruker Daltonics, Yokohama, Japan), with analytical RP-HPLC columns, UV spectra were measured on a NanoDrop 2000c (Thermo Fisher Scientific). ESI-MS and MALDI TOF-MS data for structural determination showed here are carried out in either Kyoto University or Carnegie Mellon University.

Polyamide Fmoc coupling procedure

Polyamides were prepared using PSSM-8 peptide synthesizer (Shimadzu, Kyoto) with a computer-assisted operation system at 43 mg of Fmoc-Pyrrol-oxime resin and β Ala-Wang resin (ca. 0.42 mmol/g, 100~200 mesh) by Fmoc solid-phase chemistry[1]. Reaction cycles were as follows: deblocking step for 4 min x 2, 20% piperidine in DMF; coupling step for 60 min, corresponding carboxylic acids, HCTU (88 mg), diisopropylethylamine (DIEA) (36 μL), 1-methyl-2-pyrrolidone (NMP); washing step for 1 min x 5, DMF. Each coupling reagents in steps were prepared in NMP solution of Fmoc-Py-COOH (77 mg), Fmoc-Im-COOH (77 mg), Fmoc-Py-Im-COOH (70 mg), Fmoc-β-COOH (66 mg), Fmoc-γ-COOH (69 mg) and Fmoc-mini PEG-COOH (69 mg). All other couplings were carried out with single-couple cycles with stirred by N_2 gas bubbling. Typically, resin (40 mg) was swollen in 1 mL of NMP in a 2.5-mL plastic reaction vessel for 30 min. 2-mL plastic centrifuge tubes with loading Fmoc-monomers with HCTU in NMP 1 mL were placed in programmed position. All lines were washed with NMP after solution transfers. After the completion of the synthesis by the peptide synthesizer, the resin was washed with DMF (1 mL × 2), methanol (1 mL × 2), and dried in a desiccator at room temperature in vacuo.

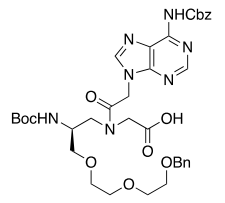
Resin cleavage and purification procedure

The resulting polyamide-oxime resin was cleaved from the solid support with *N*,*N*-dimethyl-1,3-propyldiamine for 3 h at 45 ℃. Polyamide-β Ala-Wang resin was cleaved from the solid support with 95% TFA, 2.5% triisopropylsilane, and 2.5% water for 30 min at room termperature. Resin was filtered off, and the resulting liquor was treated with diethyl ether. The

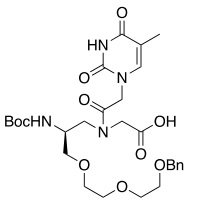
precipitated crude polyamide was washed three times with diethyl ether and analyzed by RP-HPLC. Crude polyamides were purified on a preparative column at 40 ℃. The purified peptides were assessed by the LC-MS system.

PNA monomer:

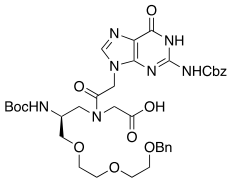
Detail synthetic route of each PNA monomer and PNA polymer can be found elsewhere of our previous work[2].



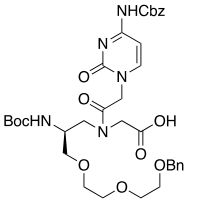
Chemical Formula: $C_{36}H_{45}N_7O_{10}$
Exact Mass: 735.3228
Molecular Weight: 735.7950



Chemical Formula: $C_{28}H_{40}N_4O_{10}$
Exact Mass: 592.2744
Molecular Weight: 592.6460



Chemical Formula: $C_{36}H_{45}N_7O_{11}$
Exact Mass: 751.3177
Molecular Weight: 751.7940



Chemical Formula: $C_{35}H_{45}N_5O_{11}$
Exact Mass: 711.3116
Molecular Weight: 711.7690

Monomer pA: ESI-HRMS: *m*/*z* calcd for $C_{36}H_{45}N_7NaO_{10}^+$ $[M+Na]^+$: 758.3126; found: 758.3114.

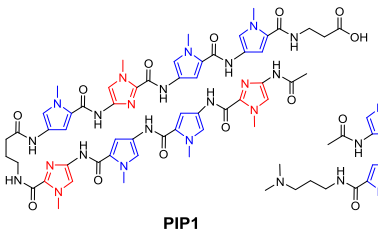
Monomer pT: ESI-HRMS: *m*/*z* calcd for $C_{28}H_{40}N_4NaO_{10}^+$ $[M+Na]^+$: 615.2642; found: 615.2628.

Monomer pG: ESI-HRMS: *m*/*z* calcd for $C_{36}H_{45}N_7NaO_{11}^+$ $[M+Na]^+$: 774.3075; found: 774.3060.

Monomer pC: ESI-HRMS: *m*/*z* calcd for $C_{35}H_{45}N_5NaO_{11}^+$ $[M+Na]^+$: 734.3013; found: 734.3006.

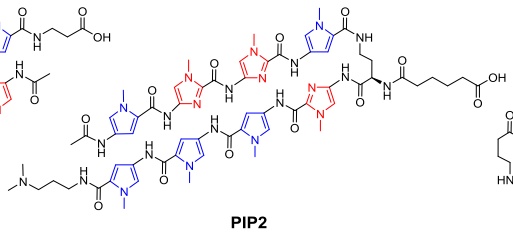
Synthesis of PIP1

Polyamide synthetic procedure has been described above. The resin cleavage and compound purification procedure have been described above. **PIP1** was obtained as a white powder. Overall yield is 4.5%. MALDI-TOF MS: *m*/*z* calcd for $C_{54}H_{61}N_{21}NaO_{12}^+$ $[M+Na]^+$: 1219.2068; found: 1218.608. HPLC: t_R=16.675 min (0.1 % TFA/MeCN, linear gradient 0–100 %, 0–40 min). (Mass data was attached in the bottom)



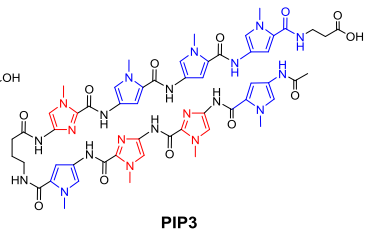
PIP1

Chemical Formula: $C_{54}H_{61}N_{21}O_{12}$
Exact Mass: 1195.4809
Molecular Weight: 1196.2170



PIP2

Chemical Formula: $C_{62}H_{77}N_{23}O_{13}$
Exact Mass: 1351.6071
Molecular Weight: 1352.4460



PIP3

Chemical Formula: $C_{54}H_{61}N_{21}O_{12}$
Exact Mass: 1195.4809
Molecular Weight: 1196.2170

Synthesis of PIP2

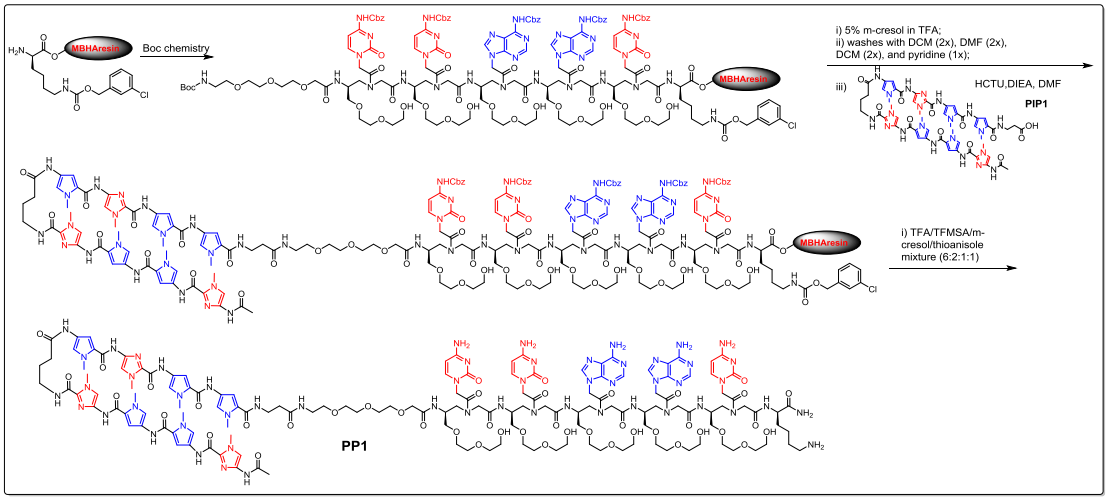
Polyamide synthetic procedure has been described above. The resin cleavage and compound purification procedure have been described above. **PIP2** was obtained as a white powder. Overall yield is 13.5%. MALDI-TOF MS: *m*/*z* calcd for $C_{62}H_{78}N_{23}O_{13}^+$ $[M+H]^+$: 1353.4540; found: 1351.968. HPLC: t_R=9.875 min (0.1 % TFA/MeCN, linear gradient 0–100 %, 0–20 min). (Mass data was attached in the bottom)

Synthesis of PIP3

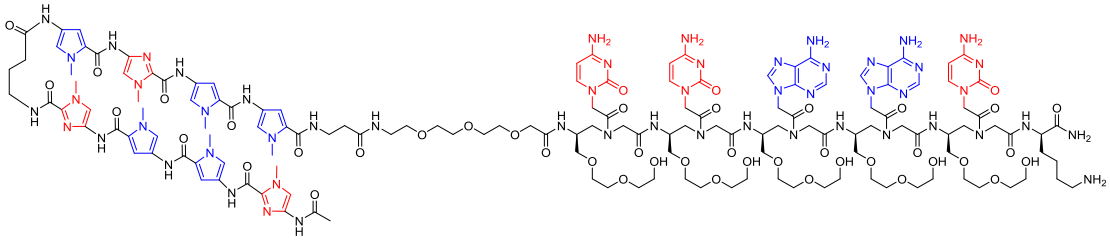
Polyamide synthetic procedure has been described above. The resin cleavage and compound purification procedure have been described above. **PIP3** was obtained as a white powder. Overall yield is 5.5%. MALDI-TOF MS: *m/z* calcd for $C_{54}H_{62}N_{21}O_{12}^{+}$ $[M+H]^{+}$: 1197.2250; found: 1196.898. HPLC: t_R=17.142 min (0.1 % TFA/MeCN, linear gradient 0–100 %, 0–40 min). (Mass data was attached in the bottom)

Pip-PNA synthesis[2]:

Synthetic route of PP1 (Applied to PP1-PP5):

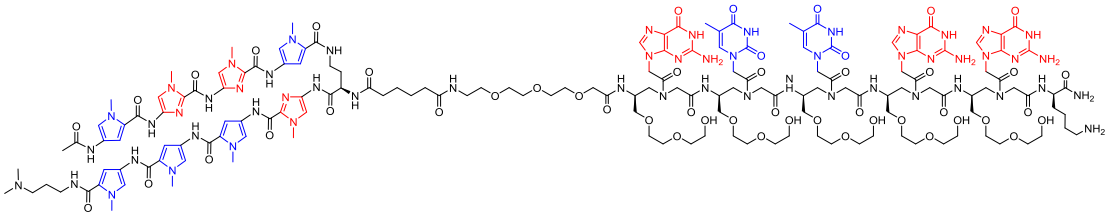


Synthesis of PP1



Synthetic route has been shown above. The resin cleavage and compound purification procedure have been described above. **PP1** was obtained as a white powder. Yield is 35.1%. MALDI-TOF MS: *m/z* calcd for $C_{145}H_{205}N_{54}O_{44}^{+}$ $[M+H]^{+}$: 3408.5690; found: 3405.703. HPLC: t_R=26.283 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min). (Mass data was attached in the bottom)

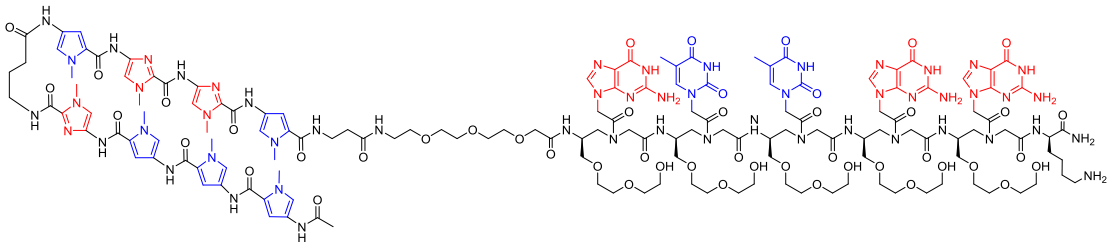
Synthesis of PP2



Chemical Formula: $C_{156}H_{222}N_{56}O_{49}$
Exact Mass: 3663.6601
Molecular Weight: 3665.8350

Synthetic route is similar with **PP1**. The resin cleavage and compound purification procedure have been described above. **PP2** was obtained as a white powder. Yield is 27.1%. MALDI-TOF MS: *m/z* calcd for $C_{156}H_{223}N_{56}O_{49}^{+}$ $[M+H]^{+}$: 3666.8430; found: 3664.700. HPLC: t_R=26.990 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min). (Mass data was attached in the bottom)

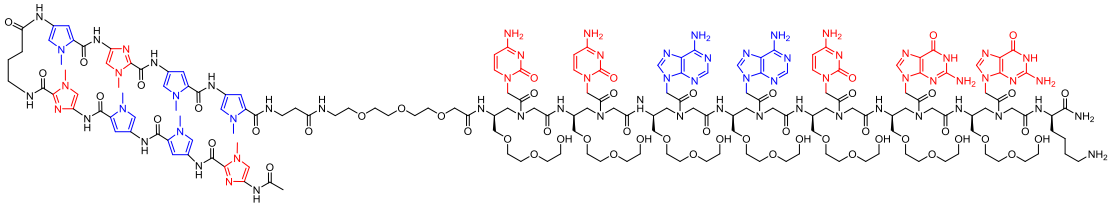
Synthesis of PP3



Chemical Formula: $C_{148}H_{206}N_{54}O_{48}$
Exact Mass: 3507.5339
Molecular Weight: 3509.6060

Synthetic route is similar with **PP1**. The resin cleavage and compound purification procedure have been described above. **PP3** was obtained as a white powder. Yield is 25.9%. MALDI-TOF MS: *m/z* calcd for $C_{148}H_{207}N_{54}O_{48}^{+}$ $[M+H]^{+}$: 3510.6140; found: 3509.891. HPLC: t_R=27.200 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min). (Mass data was attached in the bottom)

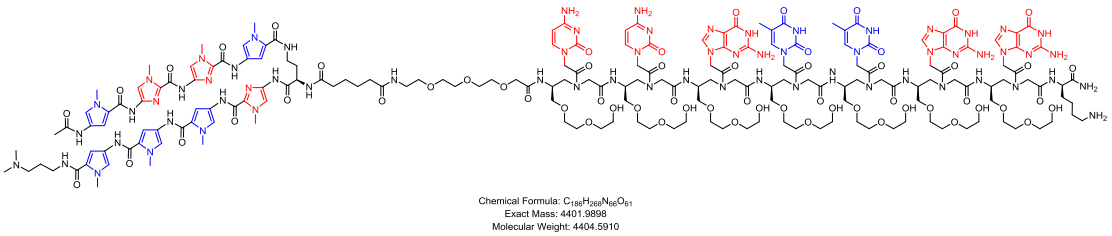
Synthesis of PP4



Chemical Formula: $C_{177}H_{260}N_{68}O_{56}$
Exact Mass: 4223.8805
Molecular Weight: 4226.3670

Synthetic route is similar with **PP1**. The resin cleavage and compound purification procedure have been described above. **PP4** was obtained as a white powder. Yield is 36.5%. MALDI-TOF MS: *m/z* calcd for $C_{177}H_{260}N_{68}O_{68}^{+}$ $[M+H]^{+}$: 4227.3750; found: 4226.890. HPLC: t_R=26.083 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min). (Mass data was attached in the bottom)

Synthesis of PP5



Synthetic route is similar with **PP1**. The resin cleavage and compound purification procedure have been described above. **PP5** was obtained as a white powder. Yield is 28.1%. MALDI-TOF MS: *m*/*z* calcd for $C_{186}H_{268}N_{66}O_{61}^{+}$ $[M+H]^{+}$: 4405.59900; found: 4405.149. HPLC: t_R=26.242 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min). (Mass data was attached in the bottom)

Compound solution preparation

Compounds were firstly dissolved in DMSO as the stock solution. PIPs and PIP-PNA conjugates concentrations were calculated with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) using an extinction coefficient of 9900 $M^{-1}cm^{-1}$ per one pyrrole or imidazole moiety at max near 310 nm[1a]. Concentrations of PNA oligomers were determined from the OD at 260 nm recorded at 90 ℃, using the following extinction coefficient: *T*=8600 M^{-1} cm^{-1}, *A*=13,700 M^{-1} cm^{-1}, *C*=6600 M^{-1} cm^{-1}, and *G*=11,700 $M^{-1}cm^{-1}$[2].

Circular dichroism (CD) experiment

All PIPs and Pip-PNA conjugated was quantified as previous established methods of PIPs[3]. The Pip-PNA samples (5 μM, 500 μL) for CD titration were prepared in 10 mM sodium phosphate, 0.1 mM EDTA, 100 mM NaCl, pH 7.2. Aliquots of master solution of compounds (1 mM in DMSO) were added continuously and incubated at least 3 min to reach the equilibrium. CD spectra were recorded at 22 ℃ over the range of 230-350 nm using JASCO J-805LST spectrometer in a 1-cm path length quartz cuvette.

Electrophoretic mobility shift assay (EMSA)

Preparation loading mixture[4]. The sequences of the DNAs used were purchased from Sigma-Aldrich. The analysis buffer is as follows: the aqueous solution of 10 mM sodium phosphate, 100 mM NaCl, pH 7.2 containing 0.25% v/v DMSO. The final concentrations of polyamides and dsDNA were clarified in the manuscript. Mixtures were placed at room temperature for 2 h before gel loading. Gel Loading Dye was Purple 6X, no SDS (B7025S, New England Bio lab).

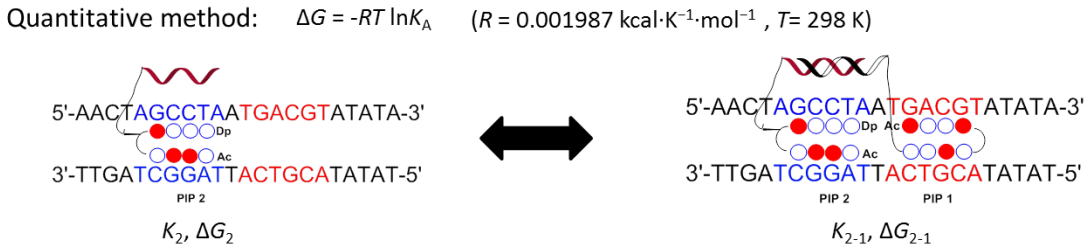
Preparation of gels. In a clean glass beaker the following reagents were mixture in the given order (10 ml system, reagent volume doubled for 20 ml system). 5.25 mL MiliQ, 1 mL 10× TBE, and 3.75 mL of 40% Acrylamide/Bis Solution (29 : 1), followed by gas-removing to ensure the removal of all air bubbles. Then 90 μL APS (10% w/w in MiliQ) and 100 μL TEMED (10% v/v in MiliQ) were then added to the mixture and mixed properly before pouring it gently along parallel glass plates. Sufficient time was given for polymerization (20 min).

Electrophoresis. A pre-run of the gels was performed prior to loading. Care was taken to see that the gel were properly immersed in 1× Tris-Borate-EDTA buffer (TBE buffer) and the loading

wells were free from any air bubbles. The wells were washed after the pre-run. Instrument settings: 120 V for 30 min at 4 ℃. 4 μL of the loading mixture was then loaded onto the wells. Pre-run again at 120 V for 30 minutes at 4 ℃. Then gel running as the instrument settings: 180 V for 160 min at 4 ℃.

Analysis of gels. The bands were stained with SYBR gold (10000× concentration in DMSO, from Thermofisher) and quantified with a FujiFilm FLA-3000G fluorescent imaging analyzer. FAM labeled forward strand (5'-FAM-AACTAGCCTAATGACGTATAT-3') was used for quantitative assay directly with a FujiFilm FLA-3000G fluorescent imaging analyzer without SYBR gold staining.

Quantitative determination of minimum cooperative binding energy



Quantitative EMSAs (FAM-labeled ODN) were performed to analyze the magnitude of cooperativity.[5] The experimental design involved measuring the equilibrium constants for binding of **PP1** to Mode C in the presence and absence of **PP2**. Fitting a Langmuir binding isotherm yielded the binding isotherms and equilibrium association constants of K_1 for **PP1** binding alone and $K_{1,2}$ for **PP1** in the presence of **PP2**. Based on the free-energy-of-binding equation, we can calculate that the ΔG_2 *and* $\Delta G_{2\text{-}1}$ for **PP1** in the presence and absence of **PP2**, respectively. From this, we can estimate that the minimum free energy of interaction ($\Delta G_{1,2} - \Delta G_1$). GraphPad Prism 5 were used for curve fitting lead to the calculation of equilibrium association constant. Gas constant (R) is 0.001987 kcal K^{-1} mol^{-1} and T= 298 K.

Statistical analysis

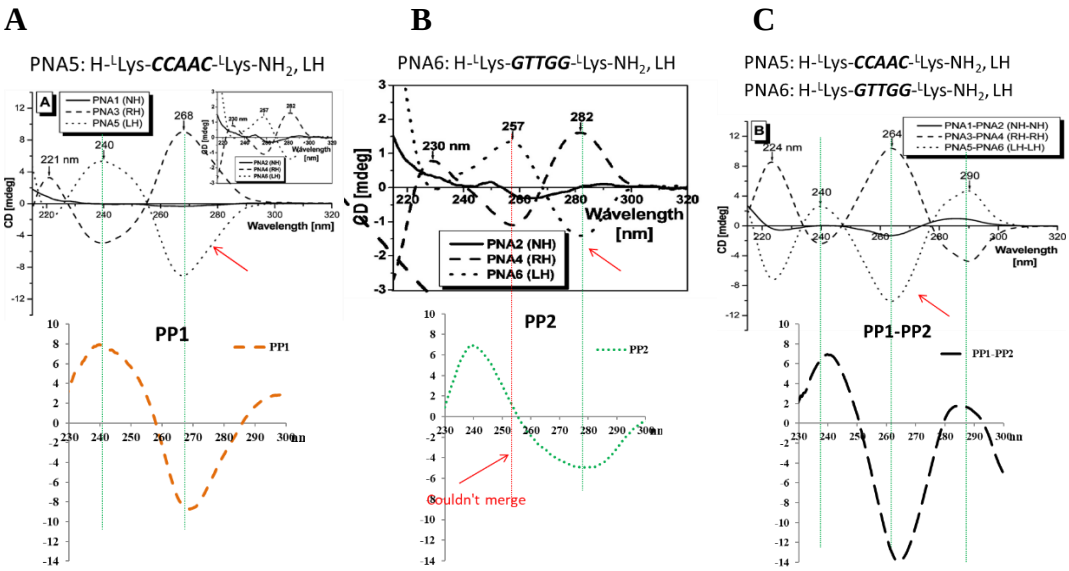
Results for continuous variables were presented as the mean ± standard error. Two-group differences in continuous variables were assessed by the unpaired T-test. Statistical analysis was performed by comparing treated samples with untreated controls. The statistical analyses were performed using GraphPad Prism 5.

SUPPORTING TABLES AND FIGURES

Table S1. Detailed information of the relationship among gap distance, moiety distance, and propeller angle.

DNA mode	Mode A									Mode B								
ODNs	1'P	0P	1P	2P	3P	4P	5P	6P	8P	1'N	0N	1N	2N	3N	4N	5N	6N	8N
Gap distance	-1	0	1	2	3	4	5	6	8	11	12	13	14	15	16	17	18	20
Spacing	1	2	3	4	5	6	7	8	10	1	2	3	4	5	6	7	8	10
Propeller angle	-	36	72	108	144	180	216	252	288	36	72	108	144	180	216	252	288	360

Figure S1. The comparison of CD spectra between LH γPNA modified with γ-*R-Me* and PIP-PNA modified with γ-*L-MP*



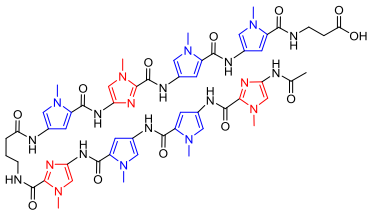
Upper: γPNA (dashed line with red arrow), they have same PNA sequence with PIP-PNA conjugates without PIPs.[6]

Down: PIP-PNA conjugates.

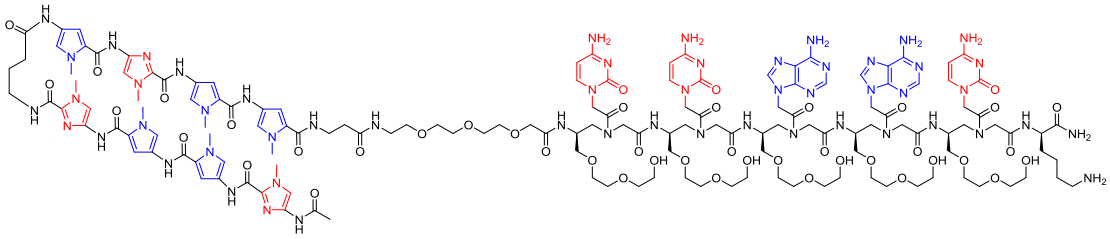
A. PNA5 and PP1; B. PNA6 and PP2; C. PNA5-PNA6 and PP1-PP2.

PP2 (PIP2-$^{R\text{-}Me}$γPNA2, contains two thymines) is less stable than $^{R\text{-}Me}$γPNA1, because thymine has the lowest base-stacking energy among the four nucleobases (*J. Am. Chem. Soc.* **2015**, *137*, 8603-8610). In another report (*J. Org. Chem.* **2011**, *76*, 5614-5627.), diethylene glycol (MP) substituent at γ-site enhance PNA pre-organization. Based on these results, we preclude that the higher stabilized LH conformation of PP2 (PIP2-$^{S\text{-}MP}$γPNA) is attributable to S-MP substituent at γ-site, by comparison with $^{R\text{-}Me}$γPNA2 (R-Me substituent). Moreover, we agree that PIP conjugation will promotes the changes of CD pattern. But such changes might be destabilize the LH confirmation, rather than stabilize.

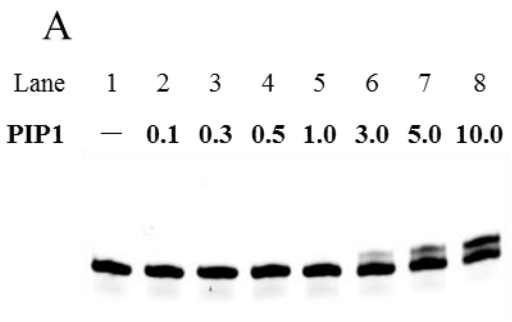
Figure S2. Gel shift assay of PIP1 and PP1, together with their respective chemical structures.



PIP1



PP1



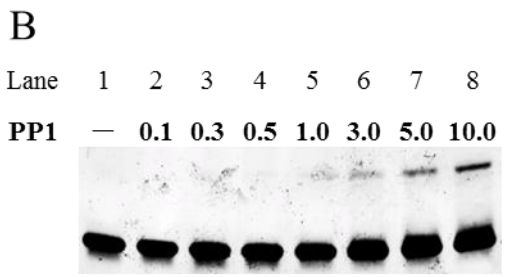
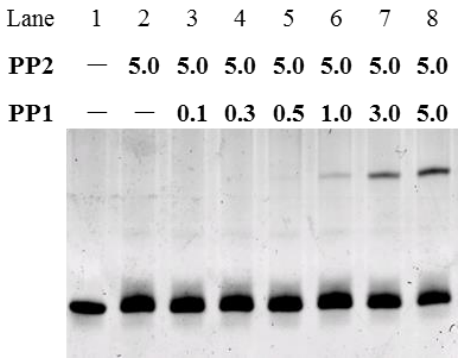


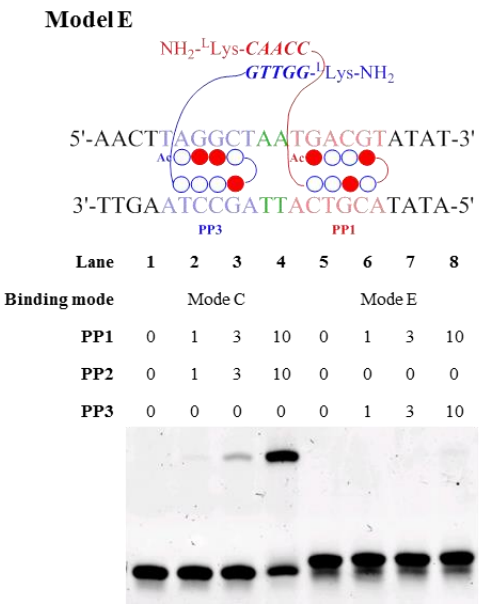
Figure S3. Gel shift assay of mismatch sequence with PP1-PP2



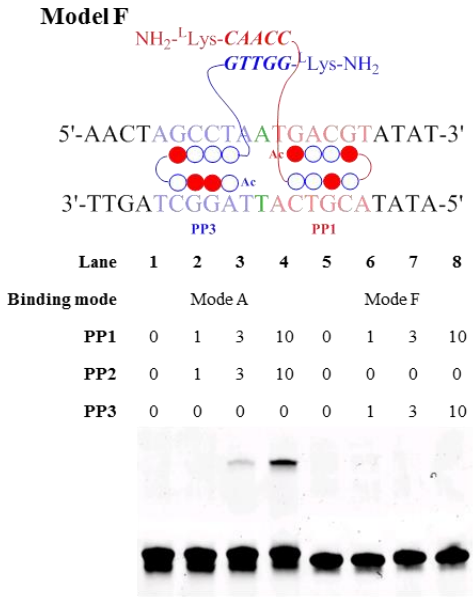
ODN-C: 5'-AACTAGGCTAATGACGTATAT-3' (reverse strand omitted)

ODN-CM: 5'-AACTAGTCTAATGACGTATAT-3' (reverse strand omitted)

Figure S4. Binding affinity comparison between PP3-PP2 and PP1-PP1 with variable binding modes.

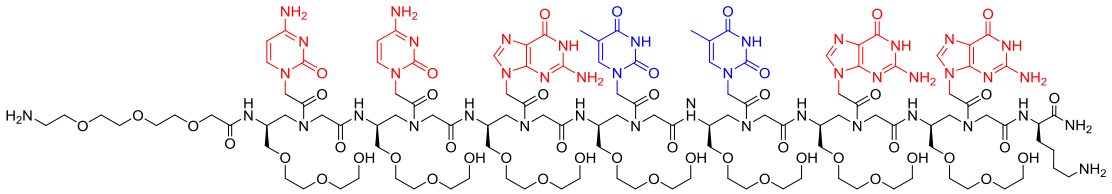


Lane	1	2	3	4	5	6	7	8
Binding mode		Mode C				Mode E		
PP1	0	1	3	10	0	1	3	10
PP2	0	1	3	10	0	0	0	0
PP3	0	0	0	0	0	1	3	10



Lane	1	2	3	4	5	6	7	8
Binding mode		Mode A				Mode F		
PP1	0	1	3	10	0	1	3	10
PP2	0	1	3	10	0	0	0	0
PP3	0	0	0	0	0	1	3	10

Figure S5. Chemical structures of $^{L\text{-MP}}\gamma$PNA5.

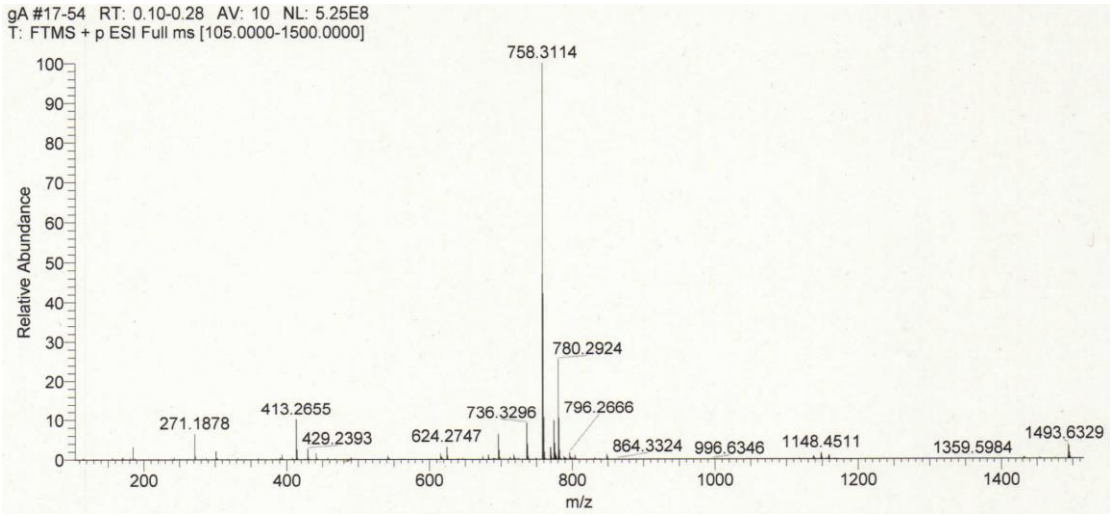


PNA5

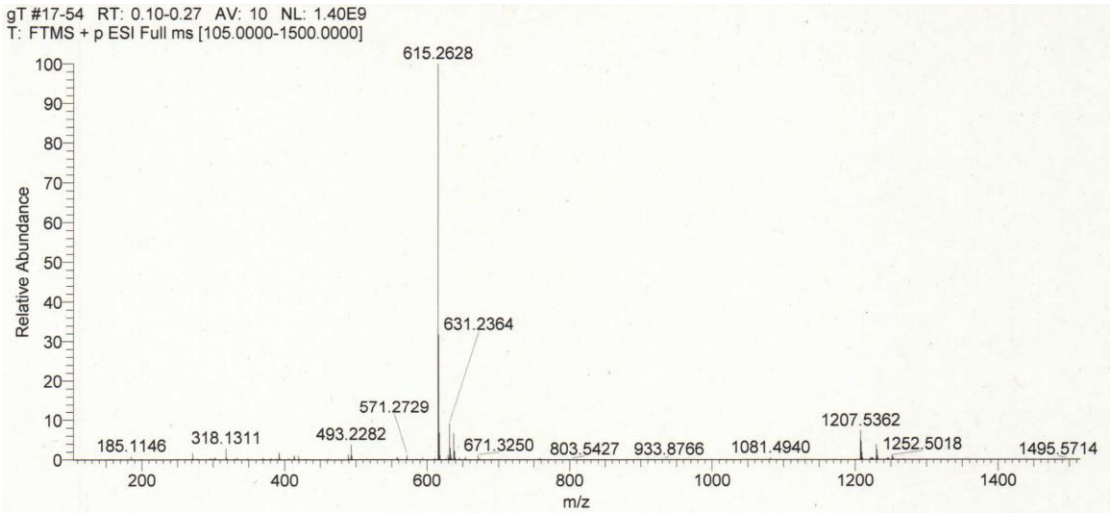
Chemical Formula: $C_{124}H_{193}N_{43}O_{49}$
Exact Mass: 3068.3932
Molecular Weight: 3070.1600

MASS data and HPLC data

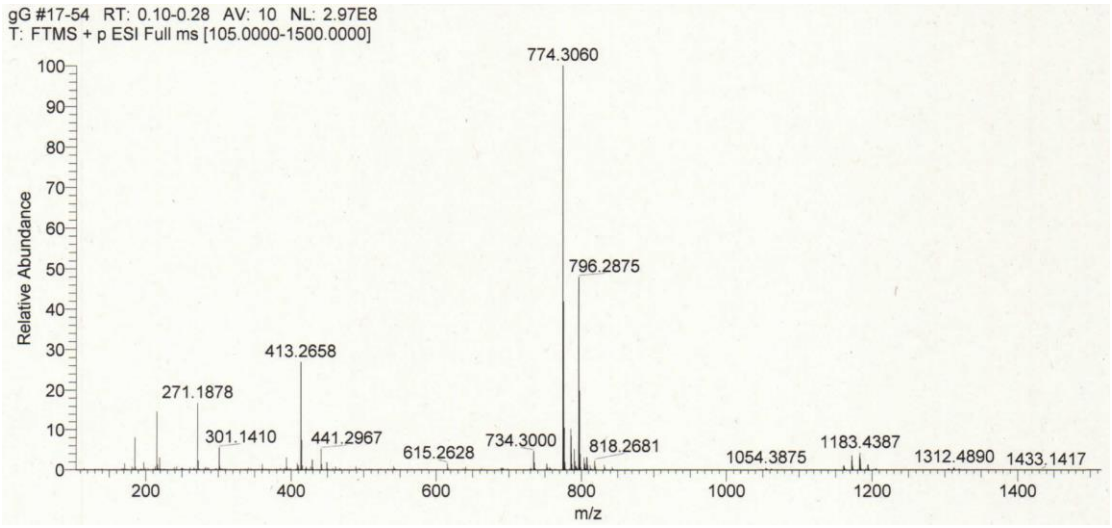
Monomer A



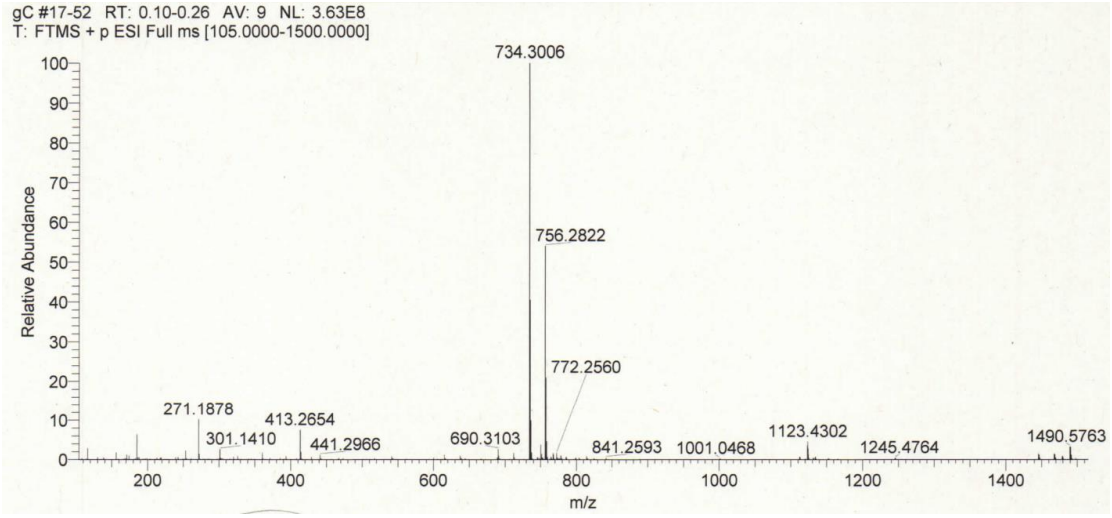
Monomer T



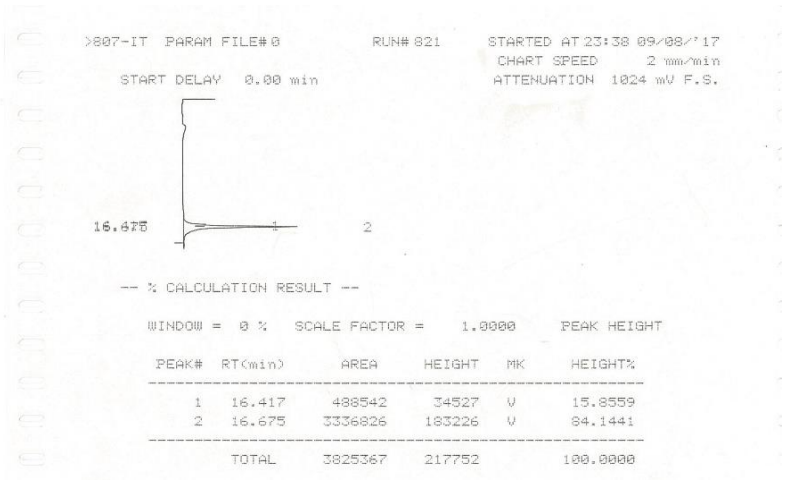
Monomer G

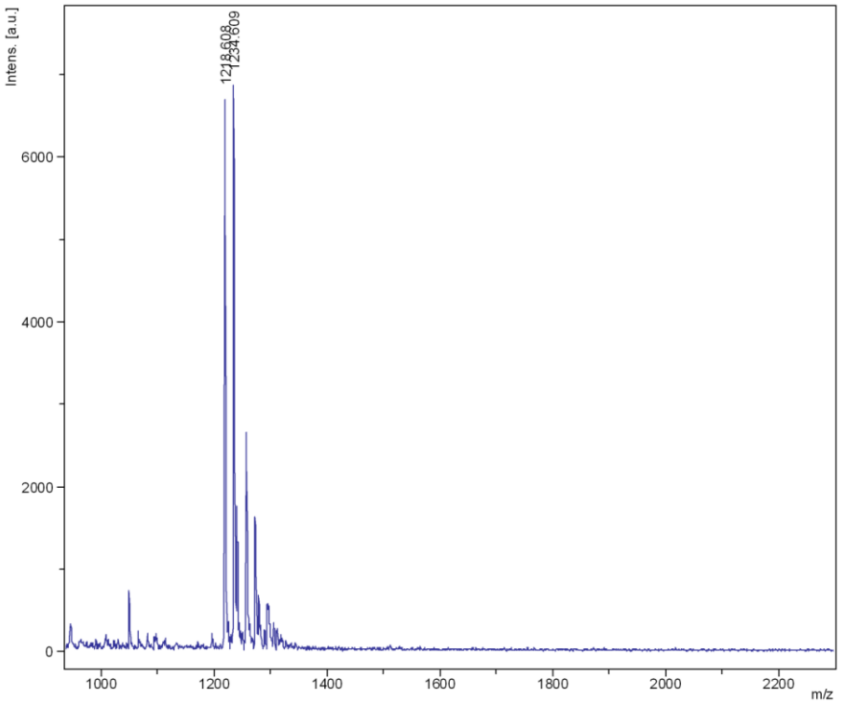


Monomer C

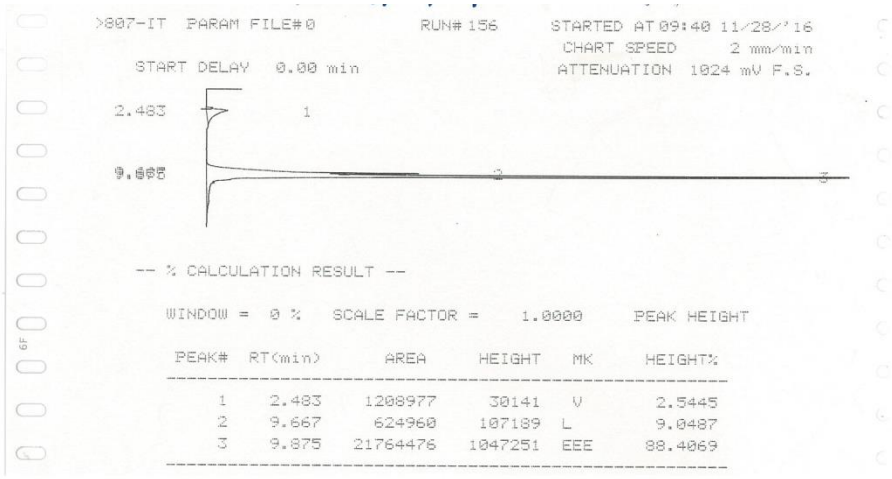


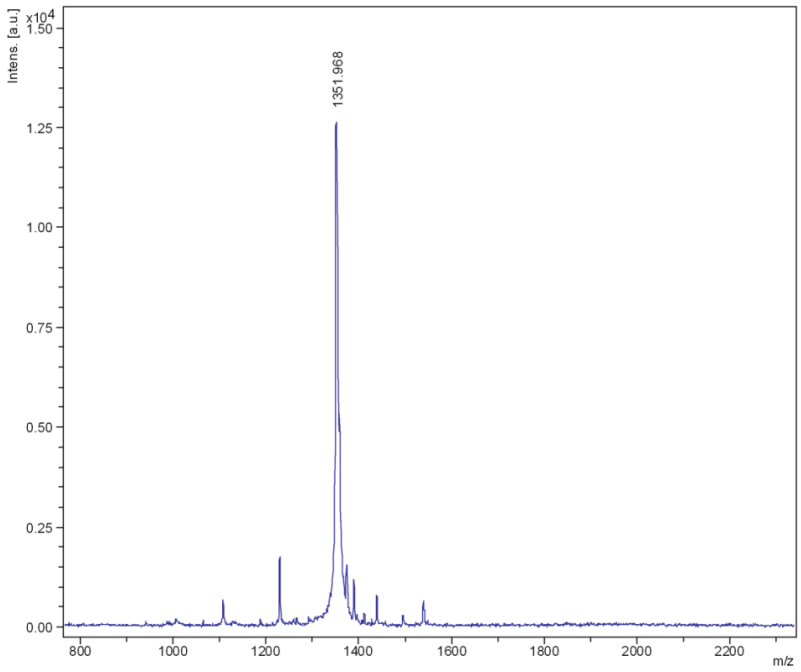
PIP1 was obtained as a white powder. Overall yield is 4.5%. MALDI-TOF MS: *m/z* calcd for $C_{54}H_{61}N_{21}NaO_{12}^{+}$ $[M+Na]^{+}$: 1219.2068; found: 1218.608. HPLC: t_R=16.675 min (0.1 % TFA/MeCN, linear gradient 0–100 %, 0–40 min).



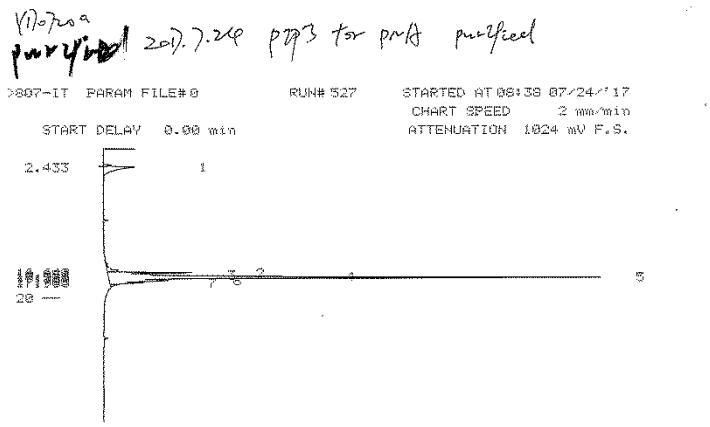


PIP2 was obtained as a white powder. Overall yield is 13.5%. MALDI-TOF MS: *m*/*z* calcd for $C_{62}H_{78}N_{23}O_{13}^{+}$ $[M+H]^{+}$: 1353.4540; found: 1351.968. HPLC: t_R=9.875 min (0.1 % TFA/MeCN, linear gradient 0–100 %, 0–20 min).





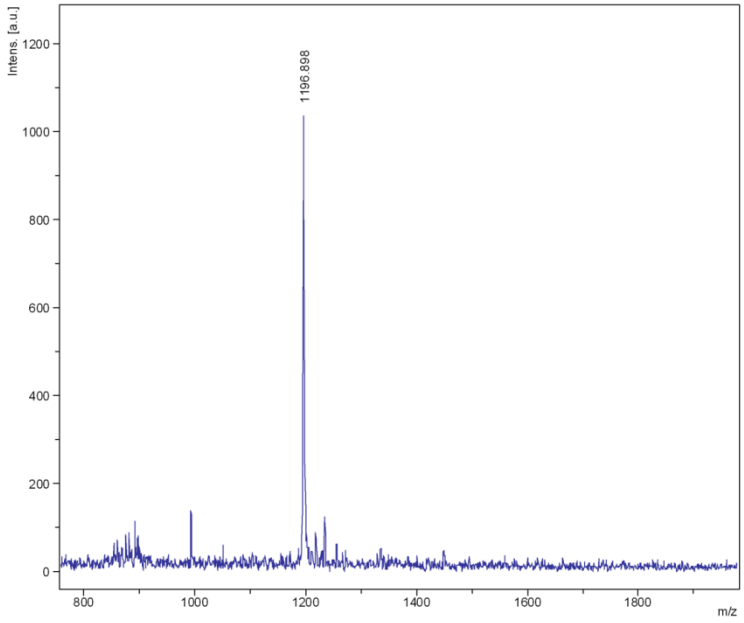
PIP3 was obtained as a white powder. Overall yield is 5.5%. MALDI-TOF MS: *m/z* calcd for $C_{54}H_{62}N_{21}O_{12}^{+}$ $[M+H]^{+}$: 1197.2250; found: 1196.898. HPLC: t_R=17.142 min (0.1 % TFA/MeCN, linear gradient 0–100 %, 0–40 min).



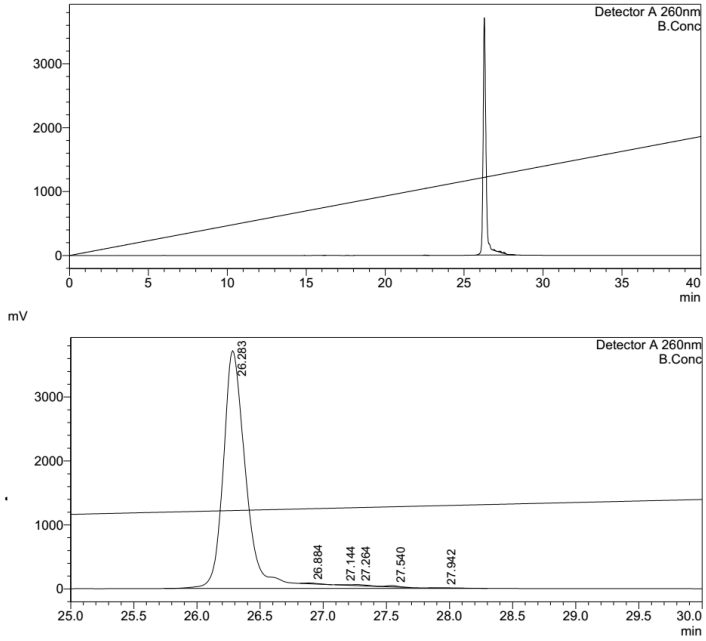
-- % CALCULATION RESULT --

WINDOW = 0 % SCALE FACTOR = 1.0000 PEAK HEIGHT

PEAK#	RT(min)	AREA	HEIGHT	MK	HEIGHT%
1	2.433	1439350	50824	V	3.3293
2	16.642	1380696	137596	V	9.0135
3	16.958	1015026	89325	V	5.8514
4	17.142	2463005	285749	V	18.7186

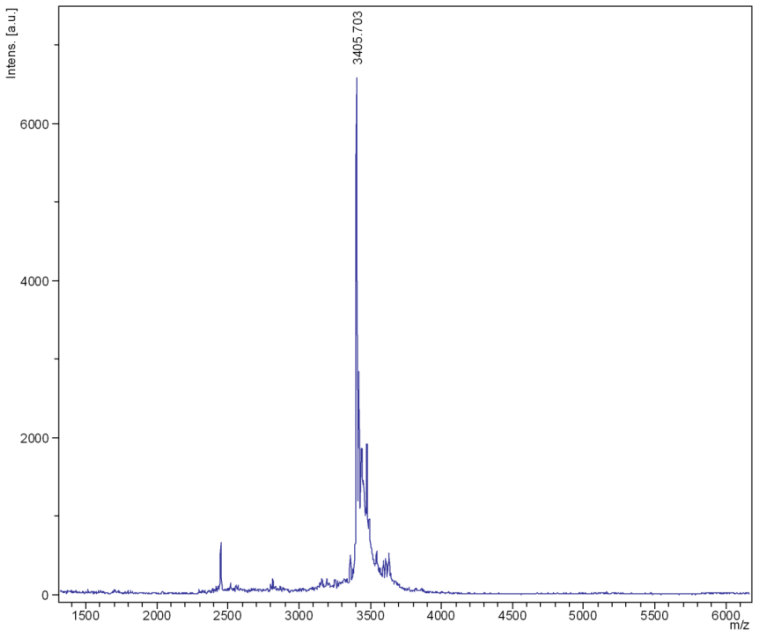


PP1 was obtained as a white powder. Yield is 35.1%. MALDI-TOF MS: *m*/*z* calcd for $C_{145}H_{205}N_{54}O_{44}^{+}$ $[M+H]^{+}$: 3408.5690; found: 3405.703. HPLC: t_R=26.283 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min).

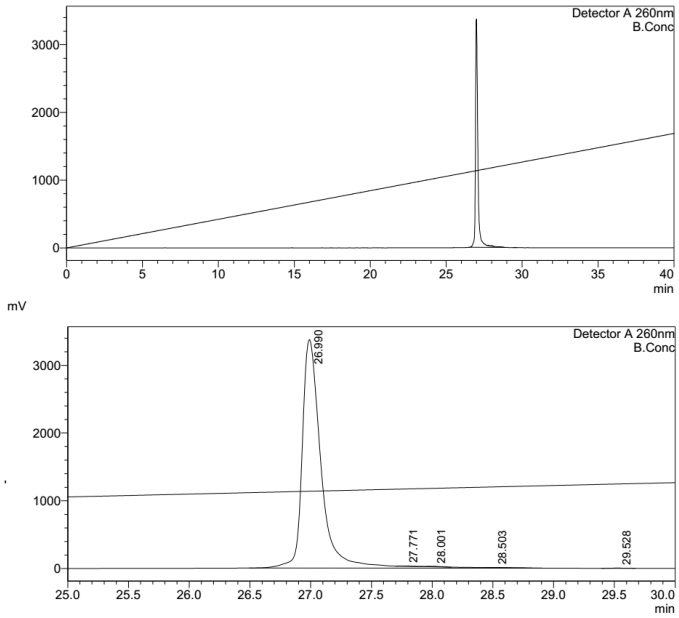


Detector A 260nm

Peak#	Ret. Time	Area	Area%	Conc.	Unit	Mark	Name
1	16.117	10962	0.023	0.023			
2	22.538	34364	0.071	0.071			
3	26.283	48194723	98.938	98.938		S	
4	26.884	86163	0.177	0.177		T	
5	27.144	29194	0.060	0.060		T	
6	27.264	145916	0.300	0.300		TV	
7	27.540	130775	0.268	0.268		TV	
8	27.942	22440	0.046	0.046		T	
9	64.160	57618	0.118	0.118			
Total		48712155	100.000				



PP2 was obtained as a white powder. Yield is 27.1%. MALDI-TOF MS: *m*/*z* calcd for $C_{156}H_{223}N_{56}O_{49}^{+}$ $[M+H]^{+}$: 3666.8430; found: 3664.700. HPLC: t_R=26.990 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min).

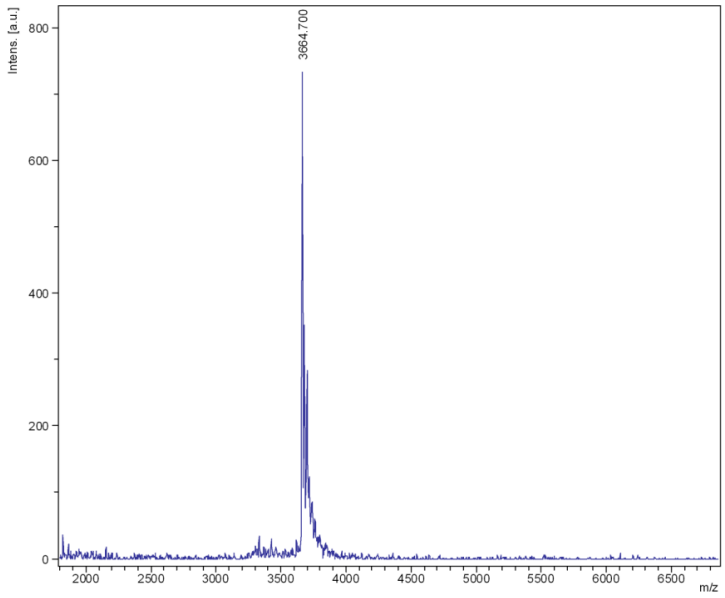


<<LC Time Program>>

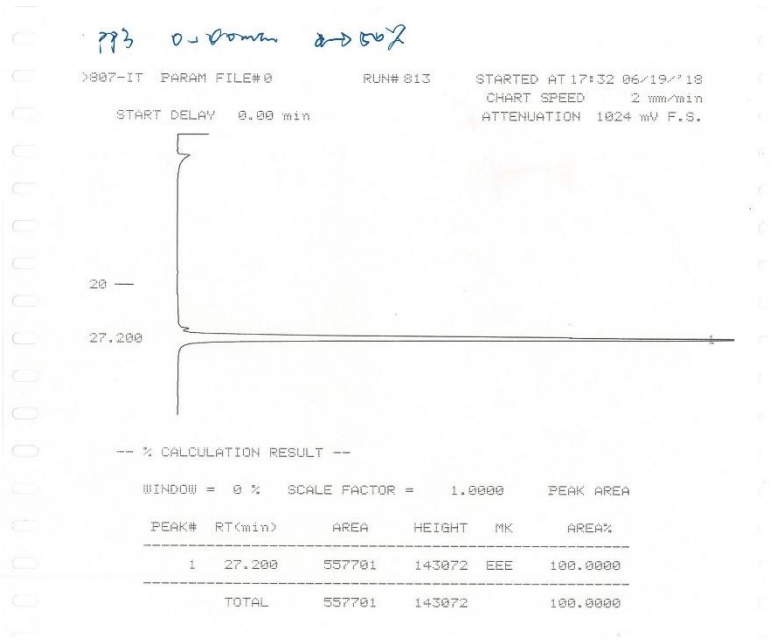
Time	Module	Command	Value
0.01	Pumps	Pump B Conc.	1
40.00	Pumps	Pump B Conc.	50
42.00	Pumps	Pump B Conc.	95
57.00	Pumps	Pump B Conc.	95
59.00	Pumps	Pump B Conc.	1
74.00	Pumps	Pump B Conc.	1
74.01	Controller	Stop	

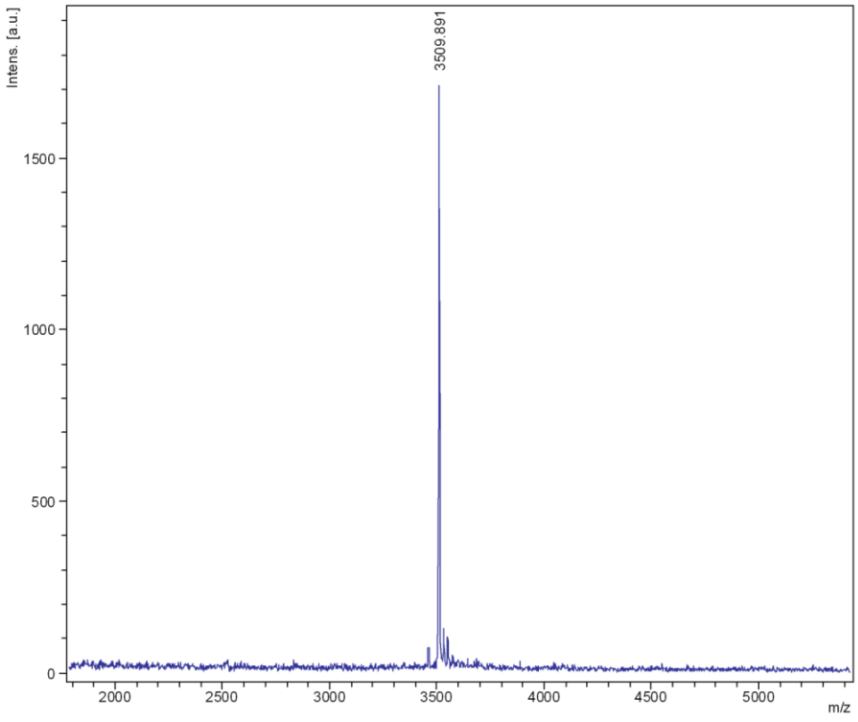
Detector A 260nm

Peak#	Ret. Time	Area	Area%	Conc.	Unit	Mark	Name
1	26.990	36862899	99.507	99.507		S	
2	27.771	29959	0.081	0.081		T	
3	28.001	57142	0.154	0.154		TV	
4	28.503	17348	0.047	0.047		T	
5	29.528	23849	0.064	0.064			
6	64.224	54161	0.146	0.146			
Total		37045357	100.000				

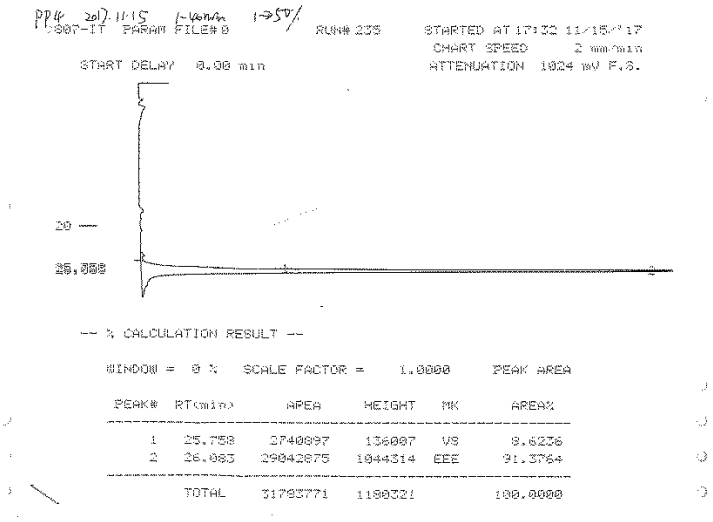


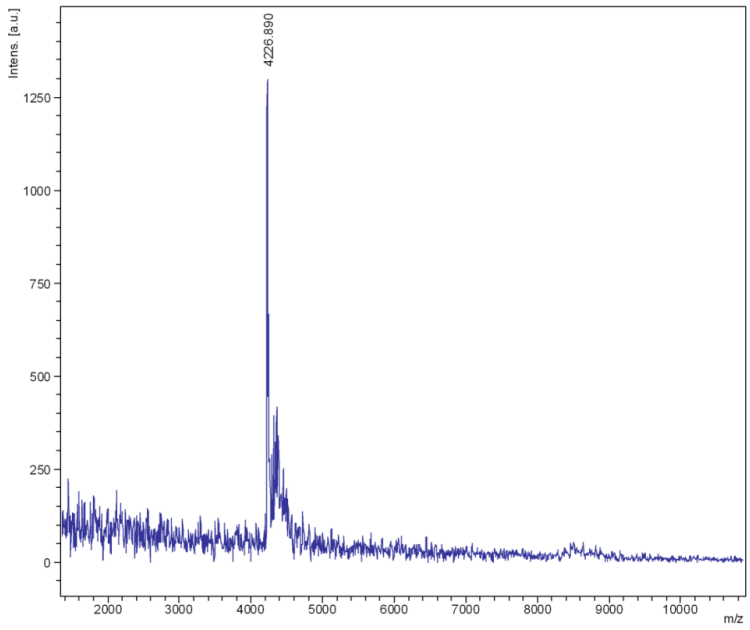
PP3 was obtained as a white powder. Yield is 25.9%. MALDI-TOF MS: *m/z* calcd for $C_{148}H_{207}N_{54}O_{48}^{+}$ $[M+H]^{+}$: 3510.6140; found: 3509.891. HPLC: t_R=27.200 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min).



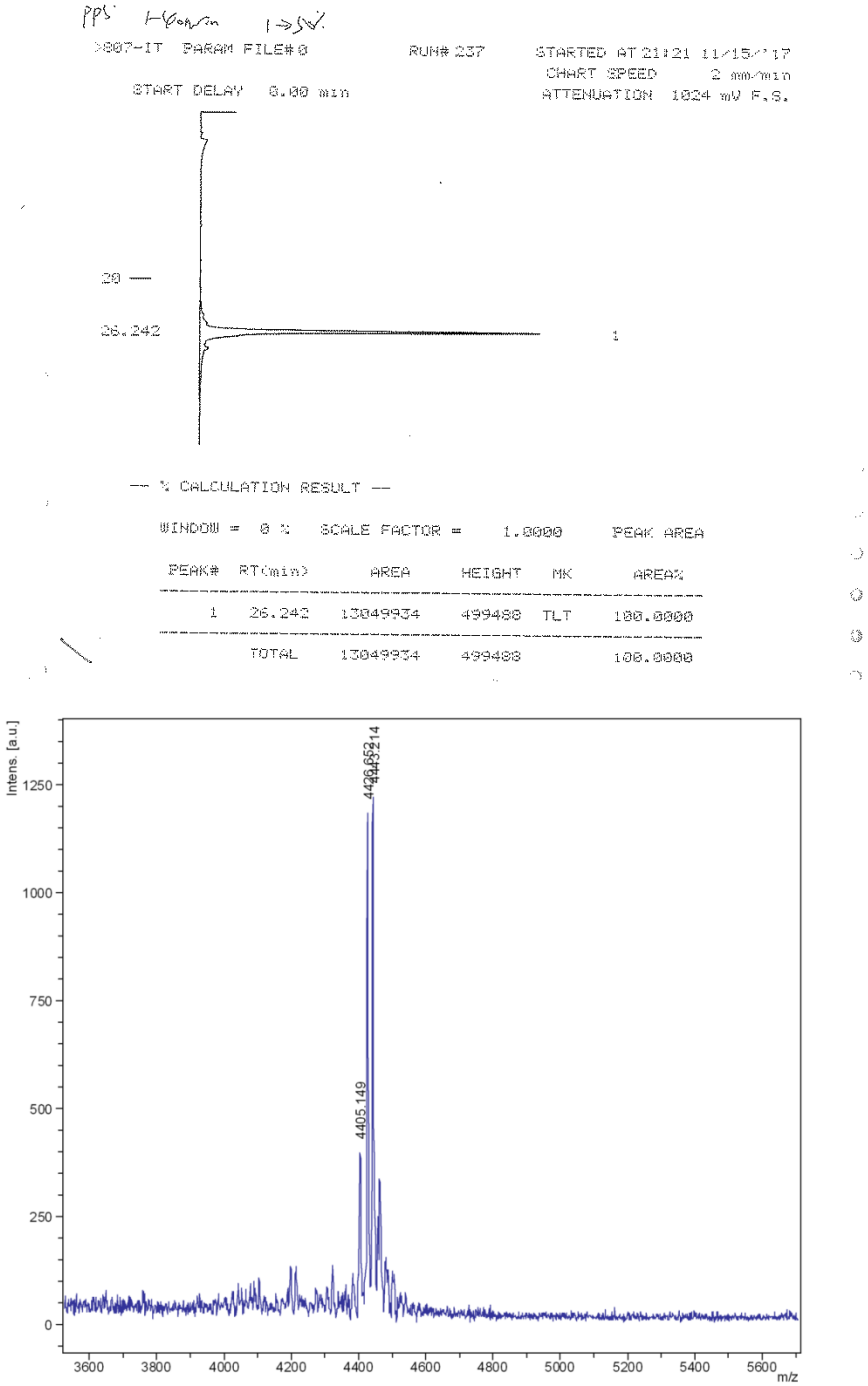


PP4 was obtained as a white powder. Yield is 36.5%. MALDI-TOF MS: *m*/*z* calcd for $C_{177}H_{260}N_{68}O_{68}^{+}$ $[M+H]^{+}$: 4227.3750; found: 4226.890. HPLC: t_R=26.083 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min).





PP5 was obtained as a white powder. Yield is 28.1%. MALDI-TOF MS: *m/z* calcd for $C_{186}H_{268}N_{66}O_{61}^{+}$ $[M+H]^{+}$: 4405.59900; found: 4405.149. HPLC: t_R=26.242 min (0.1 % TFA/MeCN, linear gradient 0–50 %, 0–40 min).



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