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Supporting Information

Epitope-Targeted Macrocyclic Peptide Ligand with Picomolar Cooperative Binding to Interleukin-17F

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Supporting Information

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SUPPLEMENTARY METHODS AND DATA

Screening for Macrocycles against IL-17F and IL-17A Epitopes

For IL-17F, screens were performed using a triazole-cyclized one-bead-one-compound (OBOC) library of the form H_2N-Pra-Cy(XXXXX)-Met-TG, where TG = TentaGel® S NH_2 resin (Rapp Polymere), X = one of 17 L-amino acids (lacking Cys, Met, and Ile), Pra = L-propargylglycine, and Cy( ) = triazole cyclization via flanking Pra and Az4 (= L-azidolysine) residues.

Pre-clear. Swelled library beads (500 mg) were blocked overnight with Blocking Buffer (25 mM Tris-HCl, 150 mM NaCl, 1% (w/v) BSA, and 0.05% (v/v) Tween-20, pH 7.6) at 4 ºC, then washed with Blocking Buffer three times. A 1:10,000 dilution of Streptavidin-Alkaline Phosphatase (V559C, Promega) in 5 mL Blocking Buffer was added to the beads and incubated with gentle shaking at room temperature for 1 h. The beads were subsequently washed with 3 x 3 mL TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.6) (1 min ea), 3 x 3 mL 0.1 M glycine pH 2.8 wash buffer, 3 x 3 mL TBS, then 3 x 3 mL Alkaline Phosphatase buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, pH 9) (5 min ea). Binding was visualized by incubating the beads in the presence of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (S3771, Promega) for 25 min. Purple beads indicated background binders and were removed by pipet and discarded. The remaining clear beads were collected and stripped with 7.5 M guanidine hydrochloride pH 2.0 for 30 min, washed ten times with water, and incubated in 1-methyl-2-pyrrolidinone (NMP) overnight to decolorize.

Product screen with IL-17F and IL-17A epitopes. Beads remaining from the pre-clear were washed with water ten times and TBS three times. Beads were then incubated with 3 mL of 100 μM IL-17F SynEp1 (Biotin-PEG_3-FFQKPES[Az4]PPVPGGS) in TBS for 1.5 h at room temperature to allow for an in situ click reaction to occur. The beads were washed with TBS ten

times and then incubated with 7.5 M guanidine hydrochloride pH 2.0 for 1 h to remove all IL-17F epitope not attached covalently to the beads. These beads were washed with TBS ten times and re-blocked with Blocking Buffer for 2 h. A 1:10,000 dilution of Streptavidin-Alkaline Phosphatase in 5 mL Blocking Buffer was added for 1 h to detect the presence of IL-17F epitope clicked to beads. The beads were subsequently washed with 3 x 3 mL TBS (1 min ea), 3 x 3 mL 0.1 M glycine pH 2.8 wash buffer, 3 x 3 mL TBS, then 3 x 3 mL Alkaline Phosphatase (pH 9) buffer (5 min ea). After this, the beads were developed with BCIP/NBT for 25 min as outlined in the pre-clear. Purple epitope-conjugated hit beads were selected by pipet and saved. These hits ***(25 total: 5 dark purple, 20 medium to light purple)*** were treated with 7.5 M guanidine hydrochloride pH 2.0 for 30 min to remove attached streptavidin, washed ten times with water, and incubated in NMP overnight to decolorize.

This screening protocol was repeated with IL-17F SynEp2 (Biotin-PEG_3-GI[Az4] NENQRVS). From this screen, 98 epitope-conjugated hits beads were selected ***(50 dark purple, 48 medium to light purple)***.

For IL-17A SynEp3 (Biotin-PEG_3-PNSEDKNFPRTVMVNL[Az4]), the same screening protocol was performed with a triazole-cyclized OBOC library of D-amino acids. From this screen, three epitope-conjugated hits beads were selected. Due to the small number of hit beads identified, no further IL-17A screens were performed.

Target screen with His-tagged IL-17F protein. The hits isolated from the IL-17F product screens were washed with water ten times and stored in TBS at 4 ºC. These beads were transferred to a Corning® 8162 Costar® Spin-X® centrifuge tube filter (cellulose acetate membrane) and incubated with Blocking Buffer for 3 h at room temperature. The beads were rinsed three times with Blocking Buffer and then incubated with 150 nM of full-length His-tagged

IL-17F protein (ab167911, Abcam) in Blocking Buffer (preparation: 0.5 µL His-tagged IL-17F protein in 200 µL Blocking Buffer) for 1 h at room temperature. The beads were washed three times with Blocking Buffer and then incubated with 500 µL of 1:10,000 Anti-6X His tag® antibody [HIS-1] (Alkaline Phosphatase-conjugated) (ab49746, Abcam) in Blocking Buffer for 1 h at room temperature. The beads were subsequently washed with 3 x 500 µL Blocking Buffer, 3 x 500 µL TBS, then 3 x 500 µL Alkaline Phosphatase (pH 9) buffer (centrifuging at 7000 rpm for 30 s after each wash). After this, the beads were developed with BCIP/NBT for 10 min. Purple hit beads bound to IL-17F protein were selected by pipet and saved. For SynEp1, ***20 beads were purple indicating binding to both the IL-17 epitope and protein, while 5 were clear indicating no binding to IL-17F protein.*** For SynEp2, ***53 beads were purple indicating binding to both the IL-17 epitope and protein, while 40 were clear indicating no binding to IL-17F protein.*** These target hits were treated with 7.5 M guanidine hydrochloride pH 2.0 for 30 min to remove bound proteins, washed ten times with water, and incubated in NMP overnight to decolorize.

Target screen with His-tagged IL-17F protein in diluted human serum. The hits isolated from the IL-17F target screens were washed with water ten times. These beads were incubated with Blocking Buffer for 7 h in a Corning® 8162 Costar® Spin-X® centrifuge tube filter (cellulose acetate membrane). The beads were rinsed three times with Blocking Buffer and then incubated with 150 nM of full-length His-tagged IL-17F protein (ab167911, Abcam) in Blocking Buffer containing 2% (v/v) human serum (HS-30, Omega Scientific) for 1 h at room temperature (preparation: 1.25 µL His-tagged IL-17F protein + 10 µL filtered serum + 490 µL Blocking Buffer). Note: Before the screen, particulate matter was removed from serum by centrifugation (7000 rpm, 30 s) using a Corning® 8162 Costar® Spin-X® tube filter. The beads were washed three times with Blocking Buffer and then incubated with 500 µL of 1:10,000 Anti-

6X His tag® antibody [HIS-1] (Alkaline Phosphatase-conjugated) (ab49746, Abcam) in Blocking Buffer for 1 h at room temperature. The beads were subsequently washed with 3 x 500 µL Blocking Buffer, 3 x 500 µL TBS, then 3 x 500 µL Alkaline Phosphatase (pH 9) buffer (centrifuging at 7000 rpm for 30 s after each wash). After this, the beads were developed with BCIP/NBT for 10 min. Purple hit beads were selected by pipet and saved. The ***2 hits (SynEp1) and 23 hits (SynEp2) whose binding to IL-17F protein was unperturbed by serum proteins*** were treated with 7.5 M guanidine hydrochloride pH 2.0 for 30 min to remove bound proteins, washed ten times with water, and incubated in NMP overnight to decolorize. The hits were finally washed with water ten times. The SynEp1 hits were subjected to sequencing analysis. For the SynEp2 hits, the target screen was repeated with 5% (v/v) human serum. Re-screening with an increased background of serum proteins resulted in ***6 hits (SynEp2)*** that were subsequently decolorized and subjected to sequencing analysis.

Hit sequencing was performed via Edman degradation. For the SynEp1 and SynEp2 hits, the Edman sequencer was unable to distinguish between 1) residues K (lysine) and L (leucine), and 2) residues Q (glutamine) and T (threonine). IL-17F sequencing results are shown in Tables S1 and S2 including the K/L and Q/T variants. IL-17A sequencing results are shown in Table S3.

Table S1. Sequences of macrocyclic peptide hits identified against IL-17F SynEp1.

	x2	x3	x4	x5	x6
hit1	F	Y	K	T	H
	F	Y	K	Q	H
	F	Y	L	T	H
	F	Y	L	Q	H

	x2	x3	x4	x5	x6
hit2	R	R	A	T	S
	R	R	A	Q	S

Table S2. Sequences of macrocyclic peptide hits identified against IL-17F SynEp2.

	x2	x3	x4	x5	x6
hit1	K	Y	G	E	V
	L	Y	G	E	V
	x2	x3	x4	x5	x6
hit2	V	H	K	S	G
	V	H	L	S	G
	x2	x3	x4	x5	x6
hit3	Q	K	H	G	P
	T	K	H	G	P
	Q	L	H	G	P
	T	L	H	G	P

	x2	x3	x4	x5	x6
hit4	Y	D	L	Q	R
	Y	D	L	T	R
	Y	D	K	Q	R
	Y	D	K	T	R
	x2	x3	x4	x5	x6
hit5	K	K	G	W	P
	K	L	G	W	P
	L	K	G	W	P
	L	L	G	W	P
	x2	x3	x4	x5	x6
hit6	R	S	Y	N	L
	R	S	Y	N	K

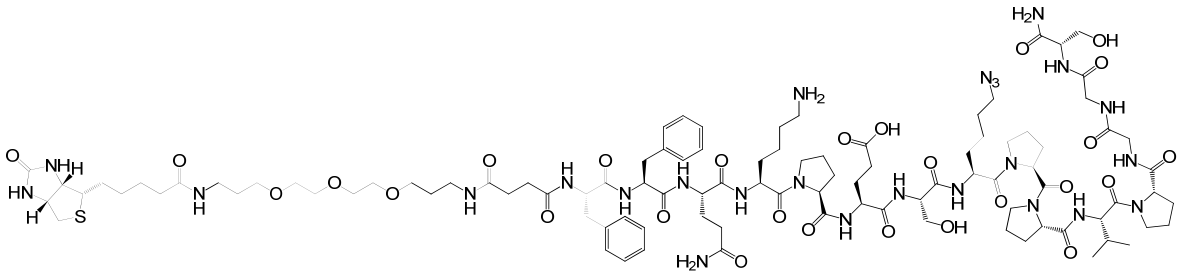
Table S3. Sequences of macrocyclic peptide hits identified against IL-17A SynEp3.

	x2	x3	x4	x5	x6
hit1	r	h	f	r	l
hit2	n	r	f	f	f
hit3	r	k	h	y	h

Characterization of Synthetic Epitopes (SynEps)

Figure S1. (A) Structure and (B) mass spectrum of IL-17F SynEp1.
Sequence: Biotin-PEG$_3$-FFQKPES[Az4]PPVPGGS. This epitope is substituted with Az4 at Cys-48 and appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{99}H_{151}N_{25}O_{27}S$ (M+H) 2155.09; found (M+H+O) = 2171.23.

A.



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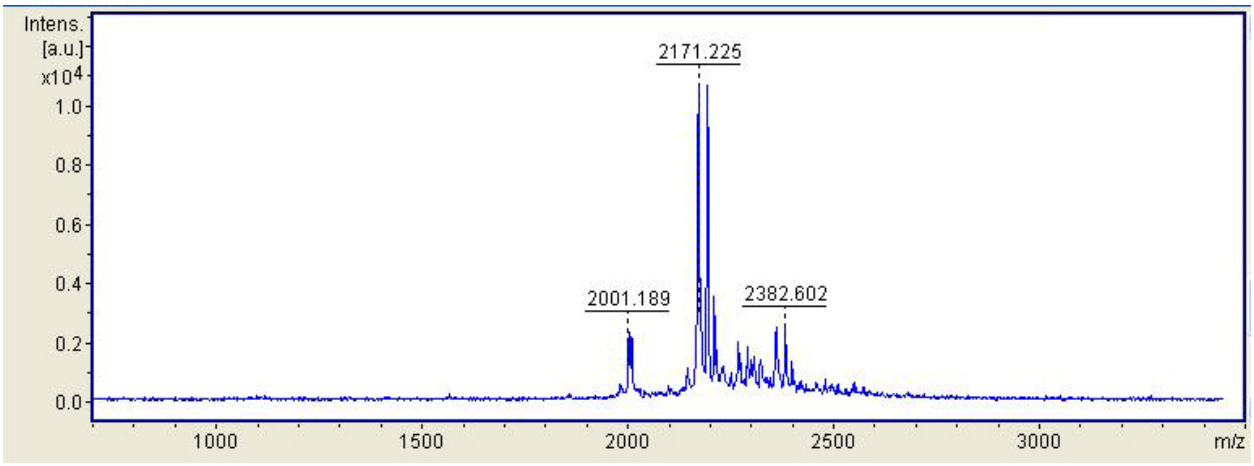
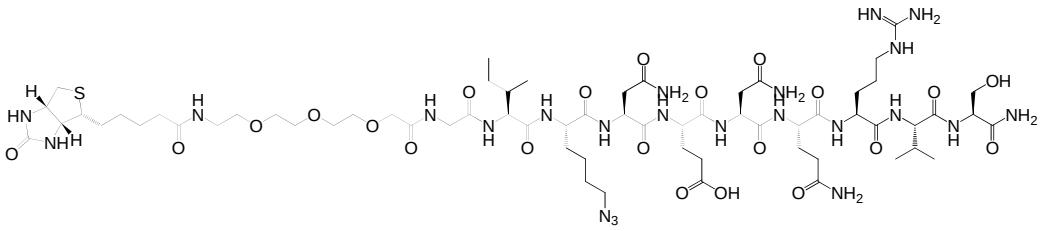


Figure S2. (A) Structure and (B) mass spectrum of IL-17F SynEp2.
Sequence: Biotin-PEG$_3$-GI[Az4]NENQRVS. This epitope is substituted with Az4 at Ile-62 and appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{64}H_{109}N_{23}O_{22}S$ (M+H) 1584.78; found (M+H+O) = 1600.69.

A.



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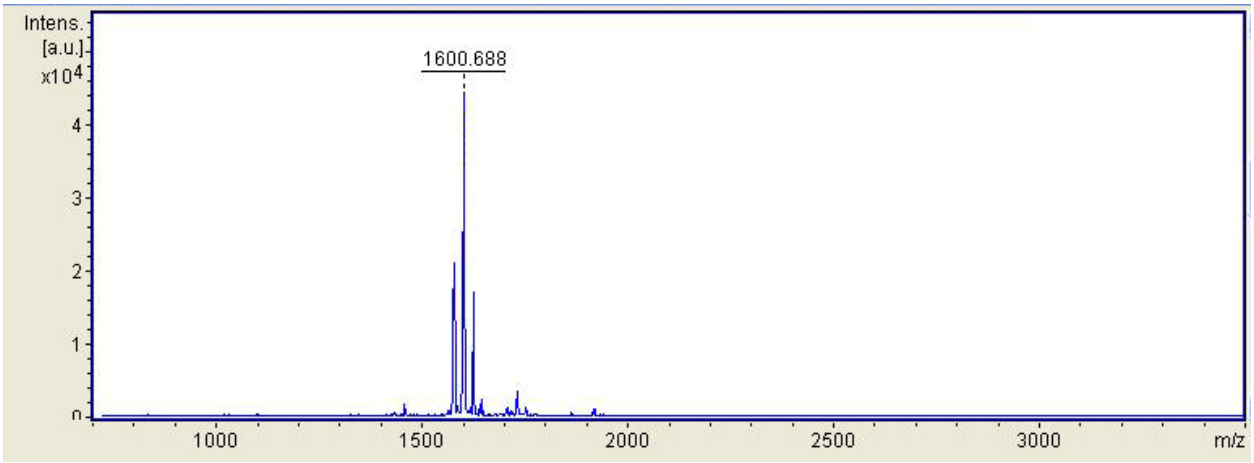
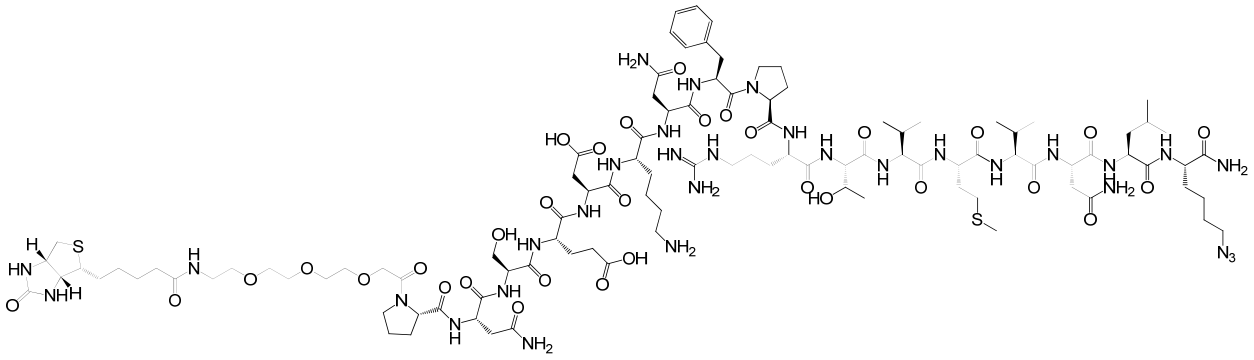


Figure S3. (A) Structure and (B) mass spectrum of IL-17A SynEp3.
Sequence: Biotin-PEG$_3$-PNSEDKNFPRTVMVNL[Az4]. This epitope is appended with Az4 at the C-terminus and has an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{104}H_{169}N_{31}O_{32}S_2$ (M+H) 2429.20; found 2429.73.

A.



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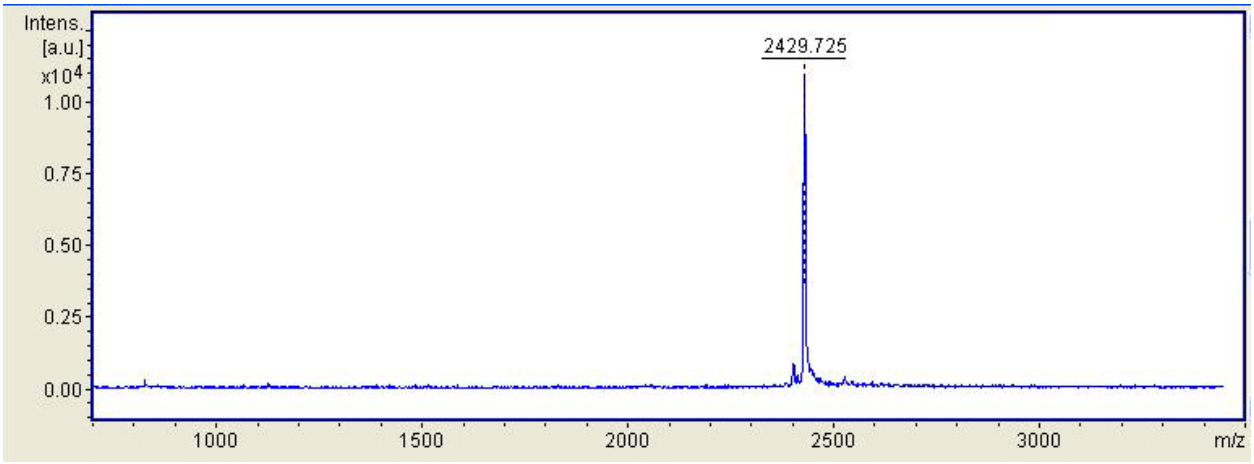
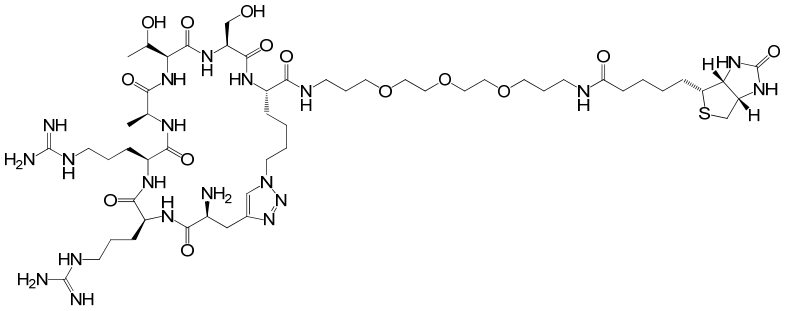


Characterization of Epitope-Targeted Peptide Ligands

Figure S4. (A) Structure and (B) mass spectrum of biotinylated L_F1.
Sequence: Cy(RRATS)-PEG_3-biotin. This ligand is appended with a C-terminal PEG_3-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{53}H_{94}N_{20}O_{14}S$ (M+H) 1267.50; found 1268.60.

A.



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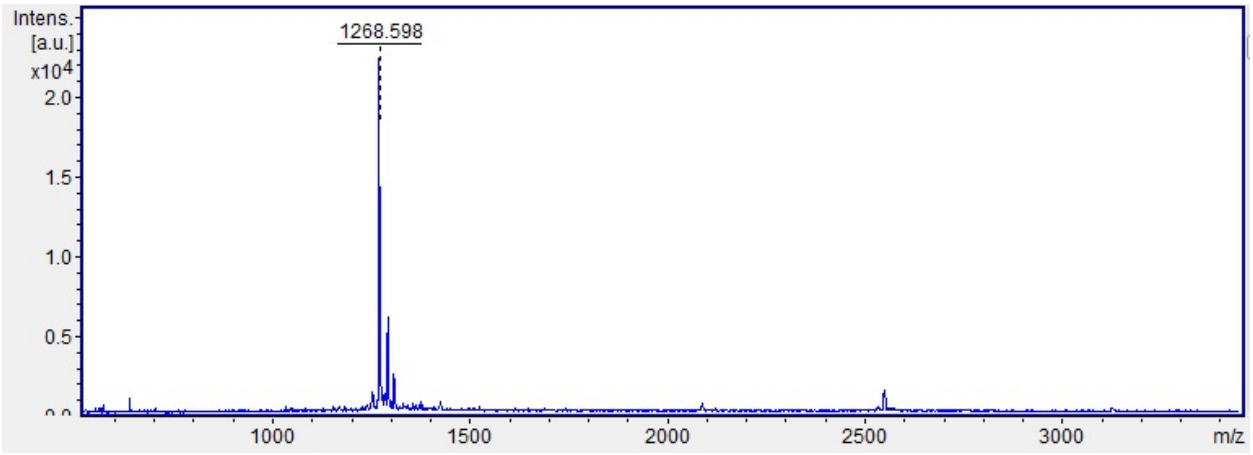
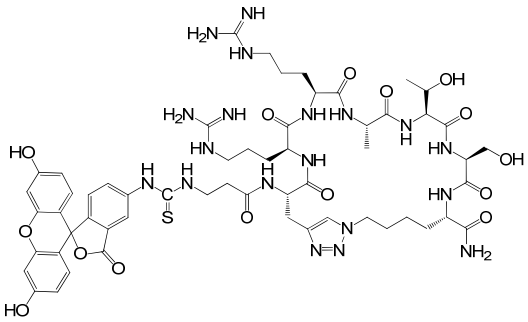


Figure S5. (A) Structure and (B) mass spectrum of 5-FITC-tagged L_F1.
Sequence: 5-FITC-β-Ala-Cy(RRATS). This ligand is appended with an N-terminal 5-FITC label. MALDI-TOF MS (m/z): calcd. for $C_{57}H_{75}N_{19}O_{15}S$ (M+H) 1298.54; found 1299.47.

A.



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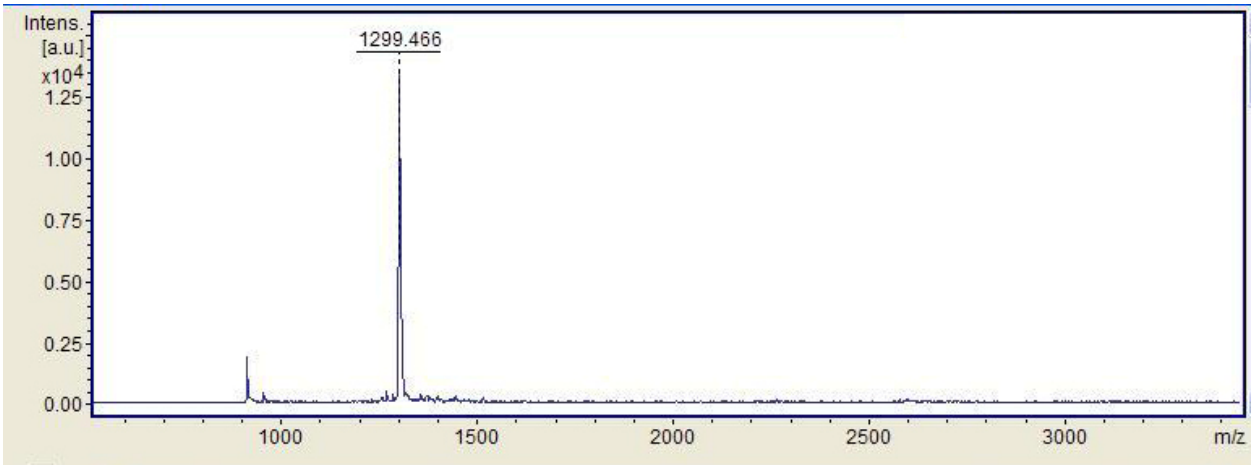
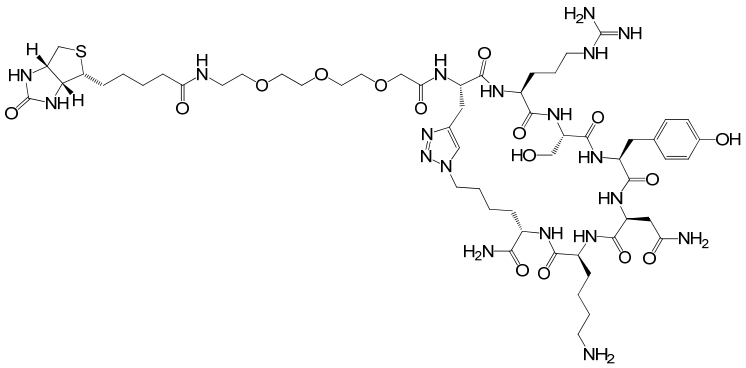


Figure S6. (A) Structure and (B) mass spectrum of biotinylated L_F2.
Sequence: Biotin-PEG_3-Cy(RSYNK). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{57}H_{91}N_{19}O_{16}S$ (M+H) 1330.52; found 1331.06.

A.



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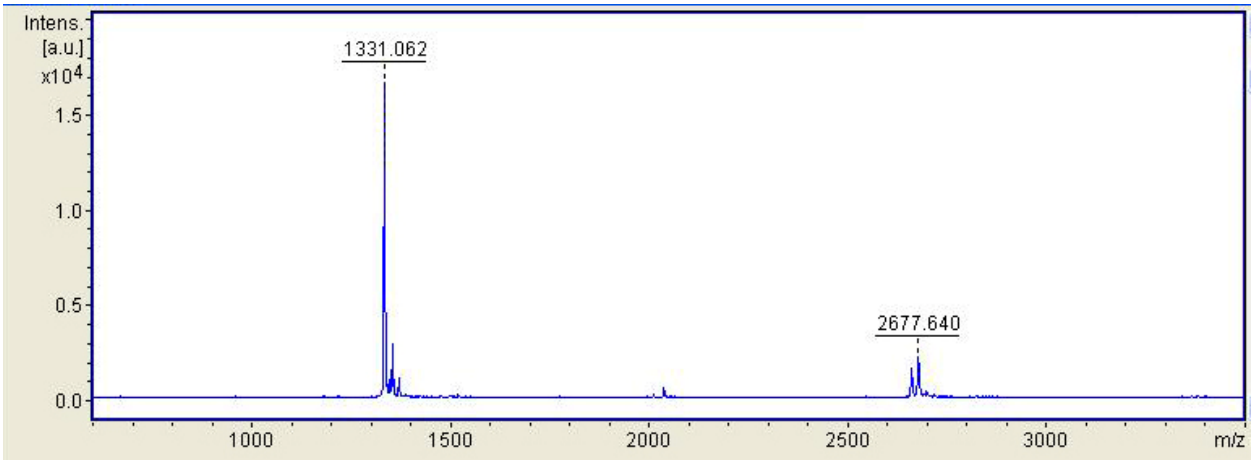
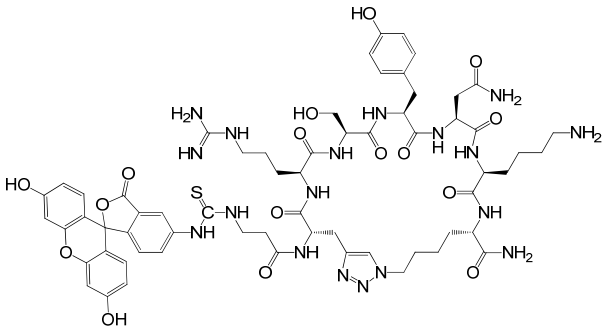


Figure S7. (A) Structure and (B) mass spectrum of 5-FITC-tagged L_F2.
Sequence: 5-FITC-β-Ala-Cy(RSYNK). This ligand is appended with an N-terminal 5-FITC label. MALDI-TOF MS (m/z): calcd. for $C_{63}H_{78}N_{18}O_{16}S$ (M+H) 1375.56; found 1376.40.

A.



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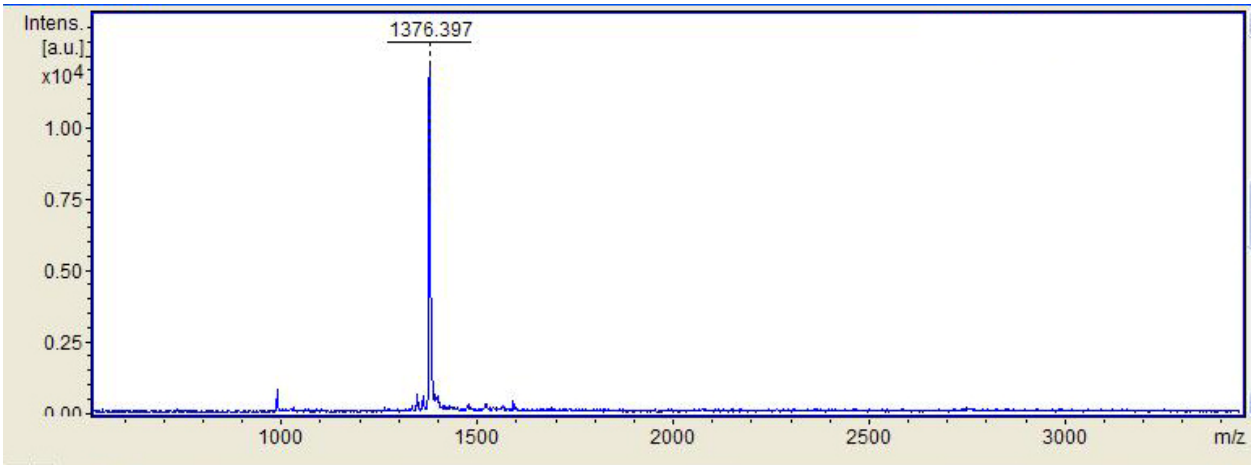
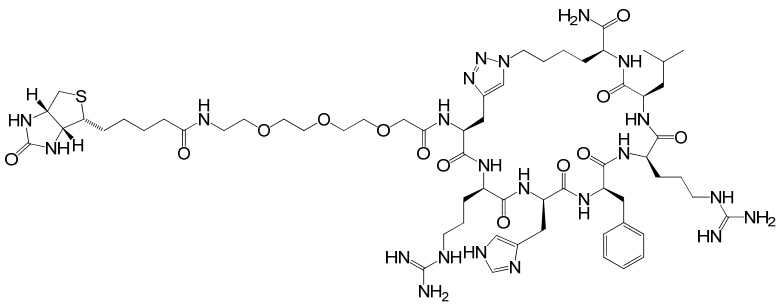


Figure S8. (A) Structure and (B) mass spectrum of biotinylated L_A3.
Sequence: Biotin-PEG_3-Cy(rhfrl). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{62}H_{98}N_{22}O_{13}S$ (M+H) 1391.74; found 1392.36.

A.



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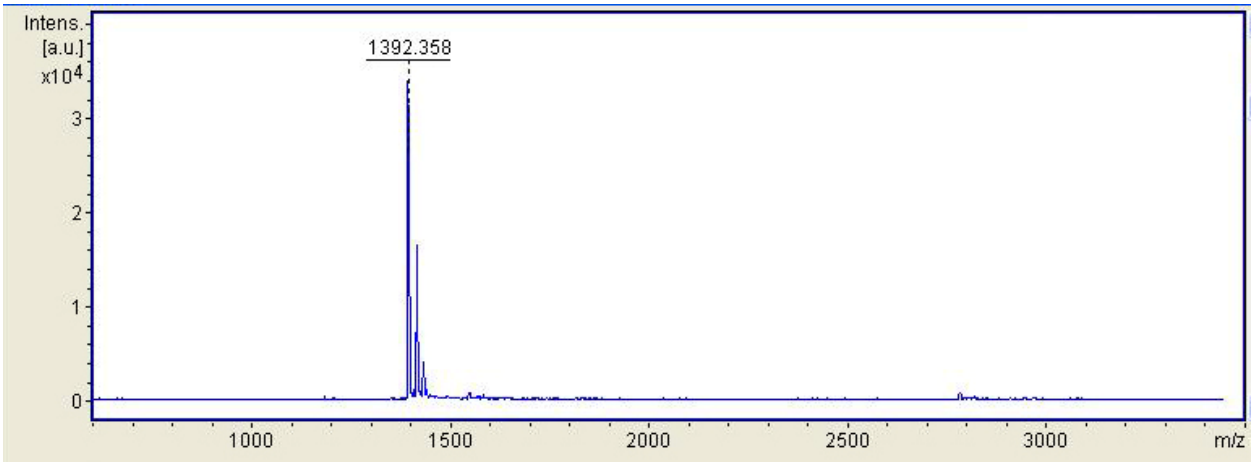
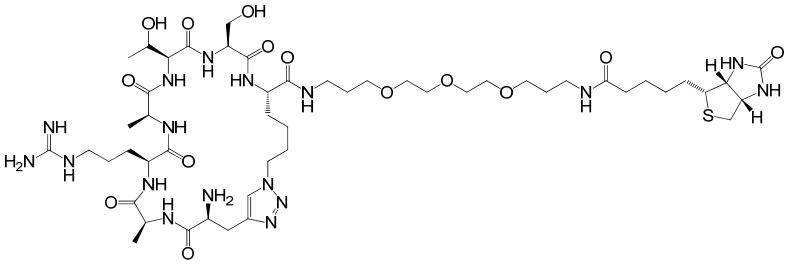


Figure S9. (A) Structure and (B) mass spectrum of L_F1 single-alanine mutant R1A (ARATS). Sequence: Cy(ARATS)-PEG_3-biotin. This ligand is appended with a C-terminal PEG_3-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{50}H_{87}N_{17}O_{14}S$ (M+H) 1182.40; found 1183.26.

A.



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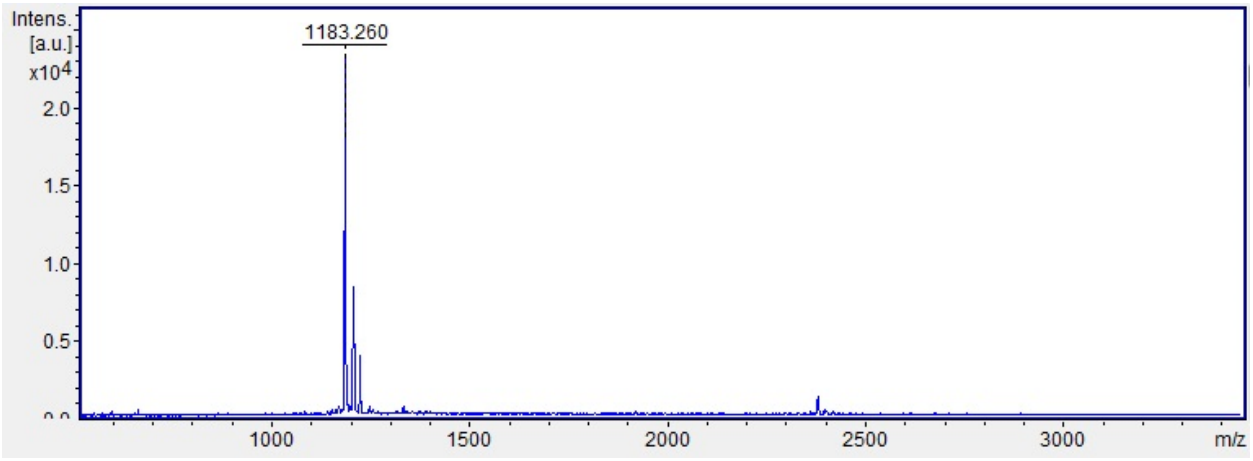
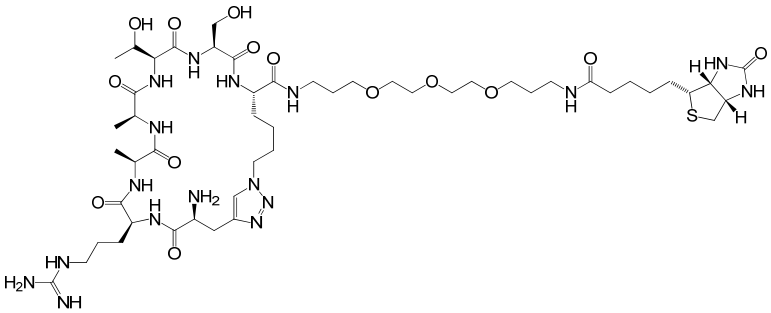


Figure S10. (A) Structure and (B) mass spectrum of L_F1 single-alanine mutant R2A (RAATS).

Sequence: Cy(RAATS)-PEG_3-biotin. This ligand is appended with a C-terminal PEG_3-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{50}H_{87}N_{17}O_{14}S$ (M+H) 1182.40; found 1184.20.

A.



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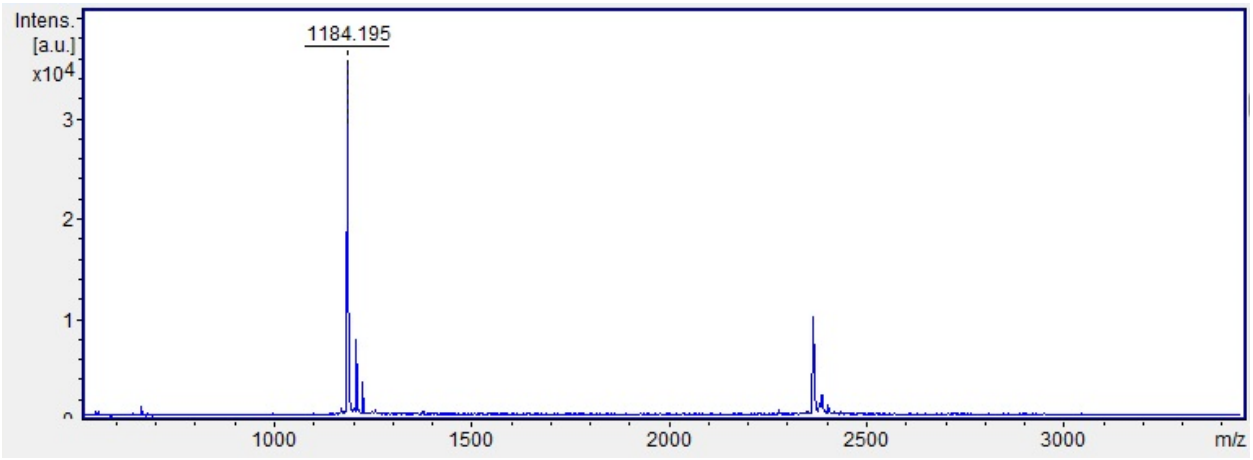
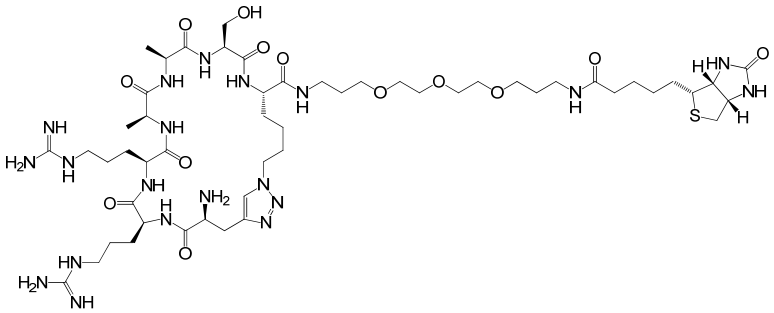


Figure S11. (A) Structure and (B) mass spectrum of L_F1 single-alanine mutant T4A (RRAAS).

Sequence: Cy(RRAAS)-PEG_3-biotin. This ligand is appended with a C-terminal PEG_3-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{52}H_{92}N_{20}O_{13}S$ (M+H) 1237.48; found 1239.37.

A.



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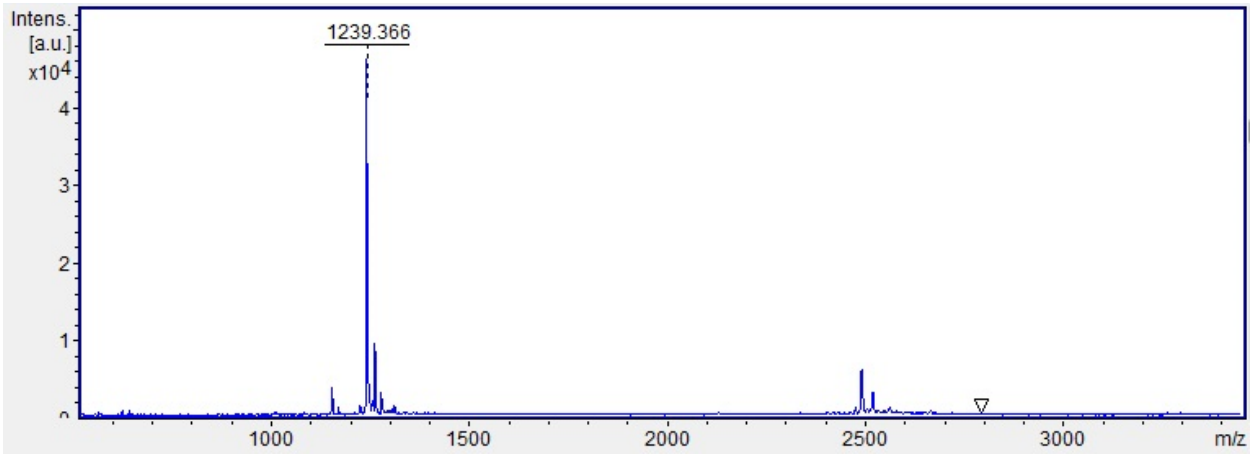
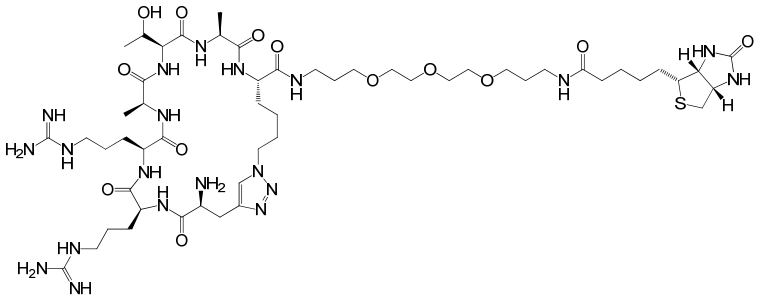


Figure S12. (A) Structure and (B) mass spectrum of L_F1 single-alanine mutant S5A (RRATA).

Sequence: Cy(RRATA)-PEG$_3$-biotin. This ligand is appended with a C-terminal PEG$_3$-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{53}H_{94}N_{20}O_{13}S$ (M+H) 1251.50; found 1253.17.

A.



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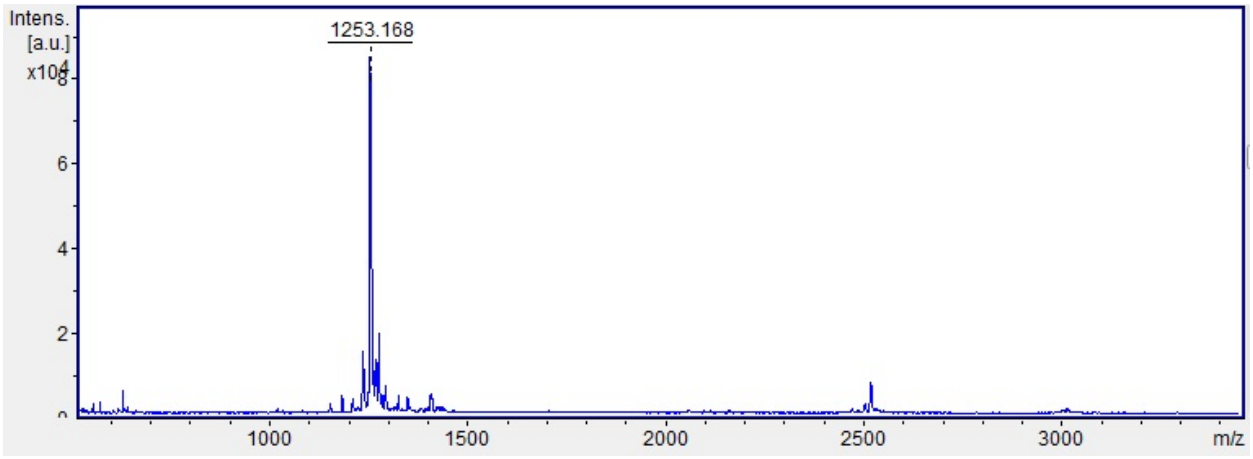
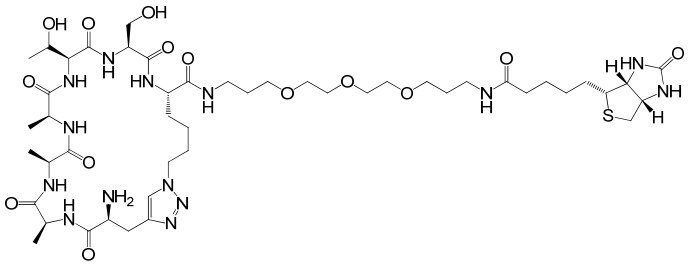


Figure S13. (A) Structure and (B) mass spectrum of L_F1 double-alanine mutant R1A/R2A (AAATS).

Sequence: Cy(AAATS)-PEG_3-biotin. This ligand is appended with a C-terminal PEG_3-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{47}H_{80}N_{14}O_{14}S$ (M+H) 1097.29; found 1099.18.

A.



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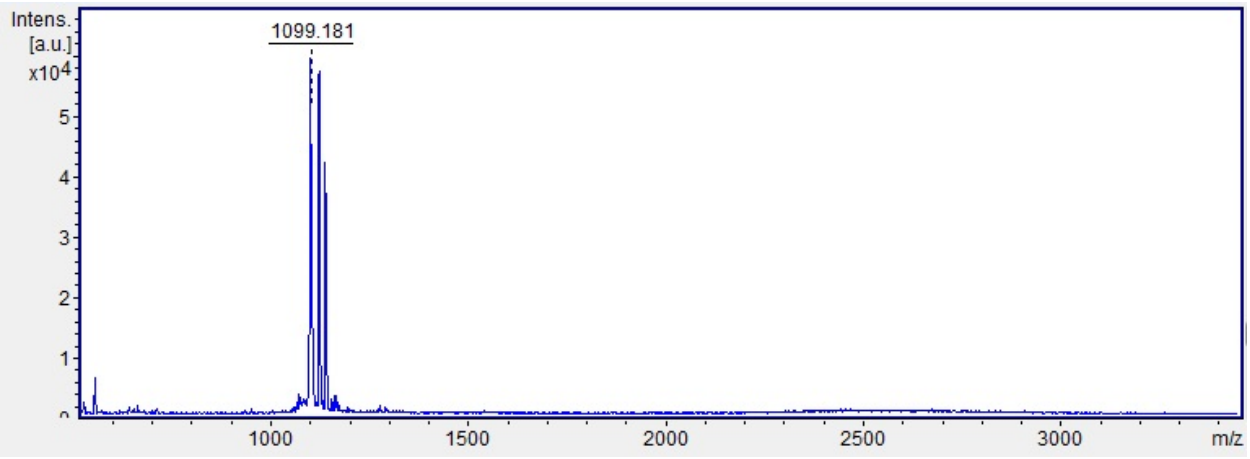
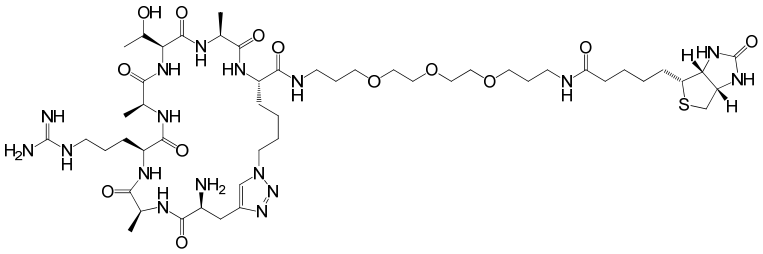


Figure S14. (A) Structure and (B) mass spectrum of L_F1 double-alanine mutant R1A/S5A (ARATA).

Sequence: Cy(ARATA)-PEG$_3$-biotin. This ligand is appended with a C-terminal PEG$_3$-biotin label. MALDI-TOF MS (m/z): calcd. for $C_{50}H_{87}N_{17}O_{13}S$ (M+H) 1166.40; found 1167.98.

A.



B.

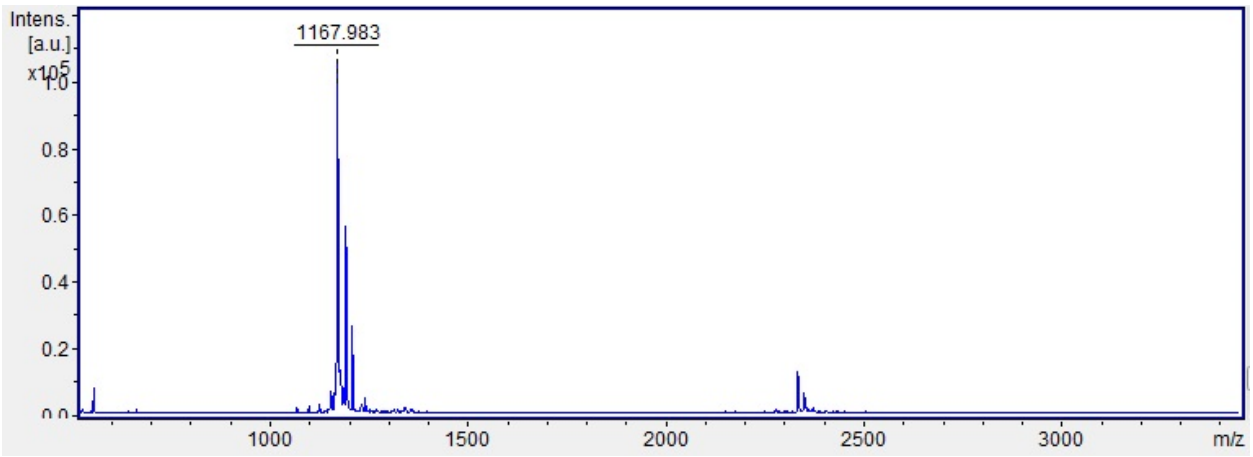
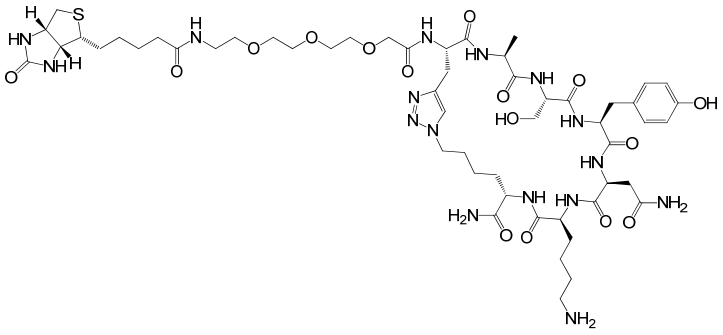


Figure S15. (A) Structure and (B) mass spectrum of L_F2 single-alanine mutant R1A (ASYNK).

Sequence: Biotin-PEG_3-Cy(ASYNK). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{54}H_{84}N_{16}O_{16}S$ (M+H) 1245.41; found 1246.40.

A.



B.

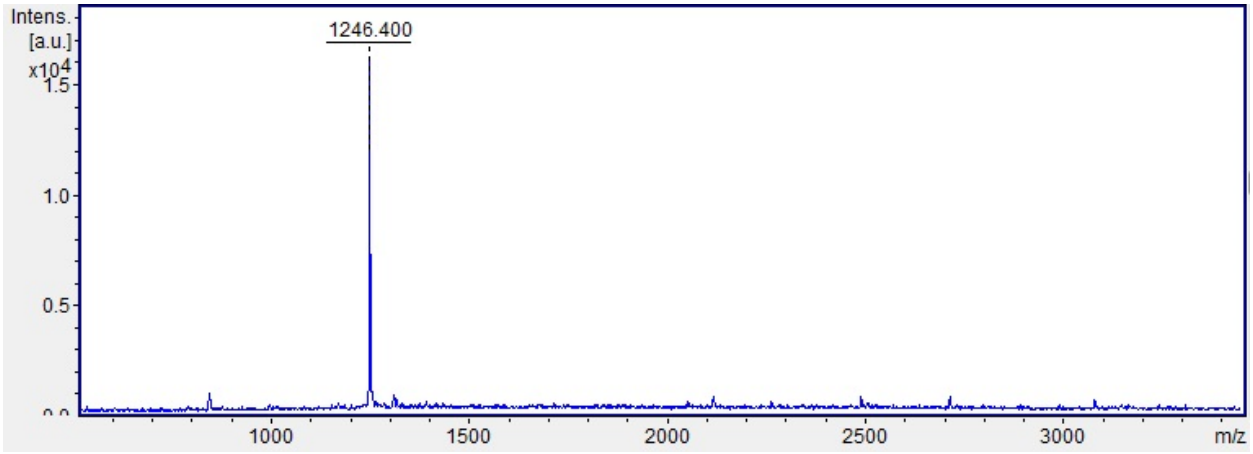
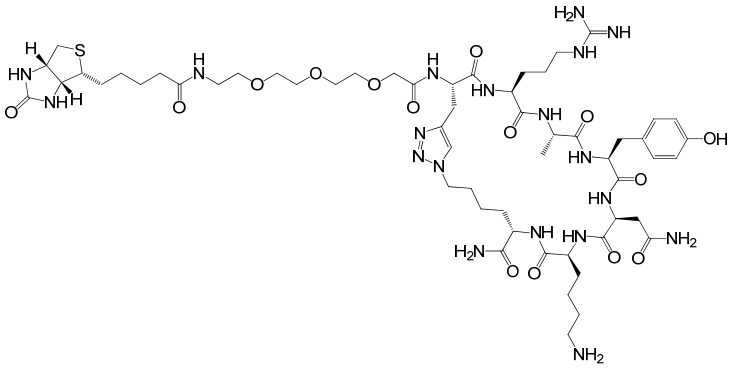


Figure S16. (A) Structure and (B) mass spectrum of L_F2 single-alanine mutant S2A (RAYNK).

Sequence: Biotin-PEG$_3$-Cy(RAYNK). This ligand is appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{57}H_{91}N_{19}O_{15}S$ (M+H) 1314.52; found 1315.72.

A.



B.

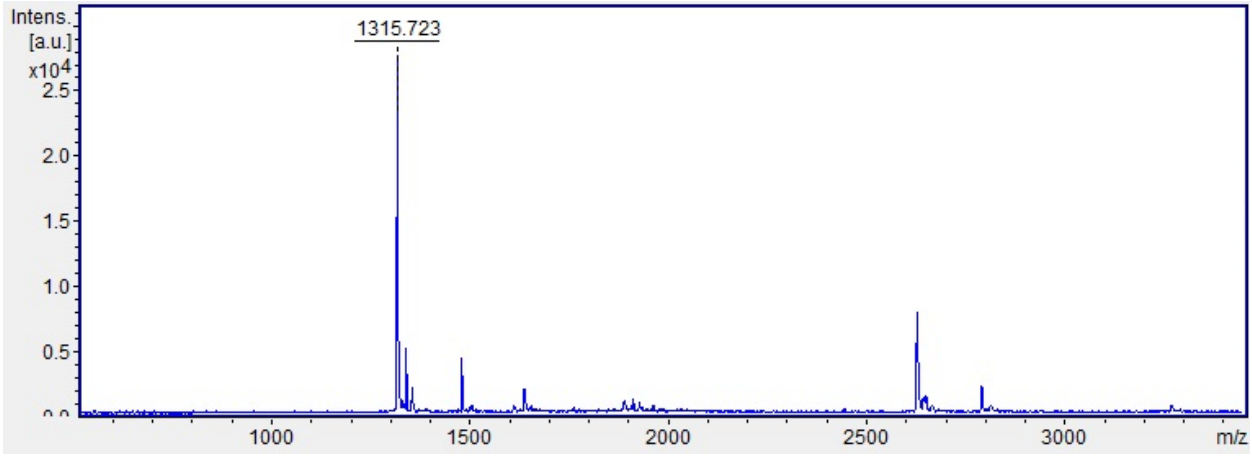
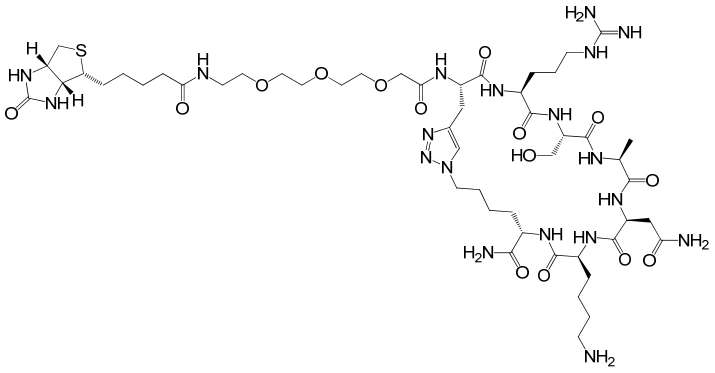


Figure S17. (A) Structure and (B) mass spectrum of L_F2 single-alanine mutant Y3A (RSANK).

Sequence: Biotin-PEG$_3$-Cy(RSANK). This ligand is appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{51}H_{87}N_{19}O_{15}S$ (M+H) 1238.42; found 1243.25.

A.



B.

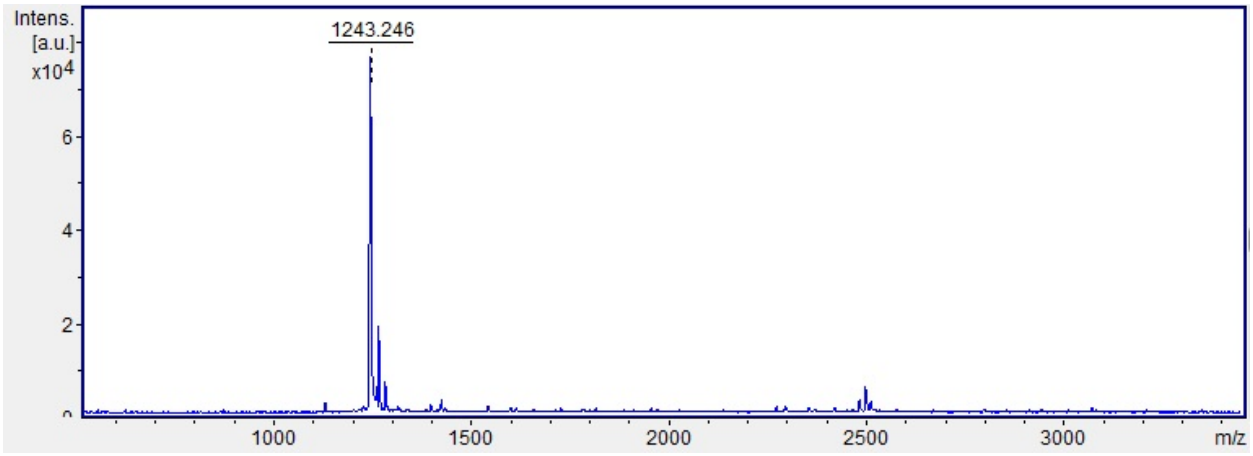
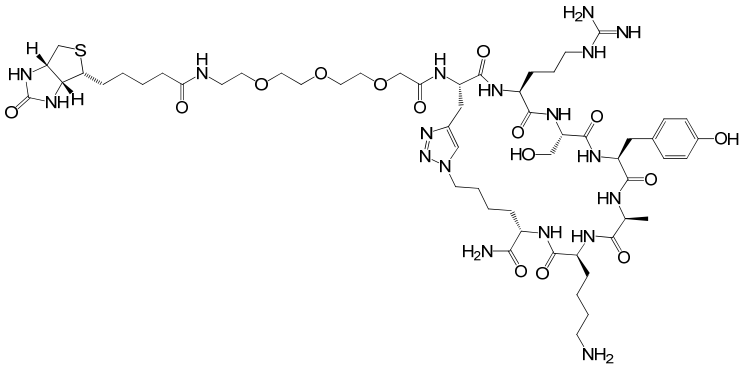


Figure S18. (A) Structure and (B) mass spectrum of L_F2 single-alanine mutant N4A (RSYAK).

Sequence: Biotin-PEG_3-Cy(RSYAK). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{56}H_{90}N_{18}O_{15}S$ (M+H) 1287.49; found 1288.25.

A.



B.

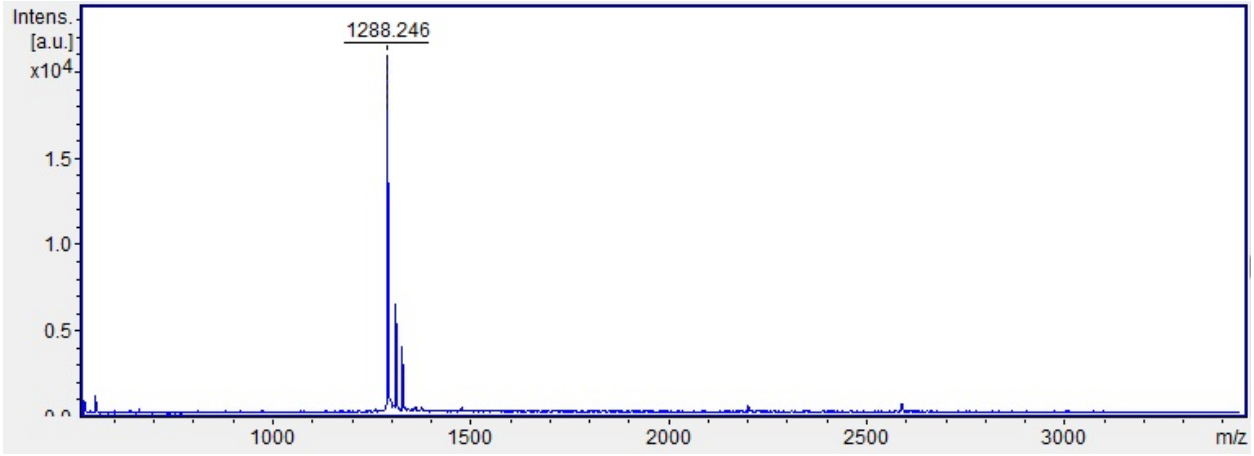
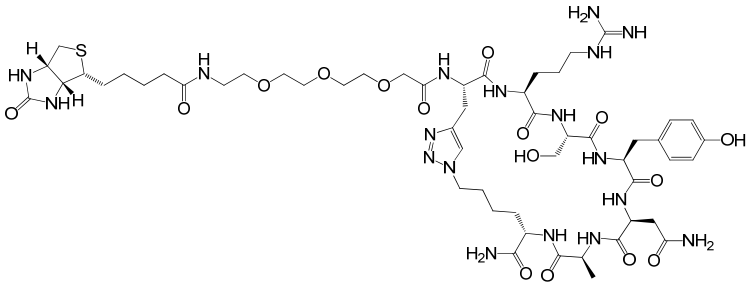


Figure S19. (A) Structure and (B) mass spectrum of L_F2 single-alanine mutant K5A (RSYNA).

Sequence: Biotin-PEG$_3$-Cy(RSYNA). This ligand is appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{54}H_{84}N_{18}O_{16}S$ (M+H) 1273.42; found 1274.50.

A.



B.

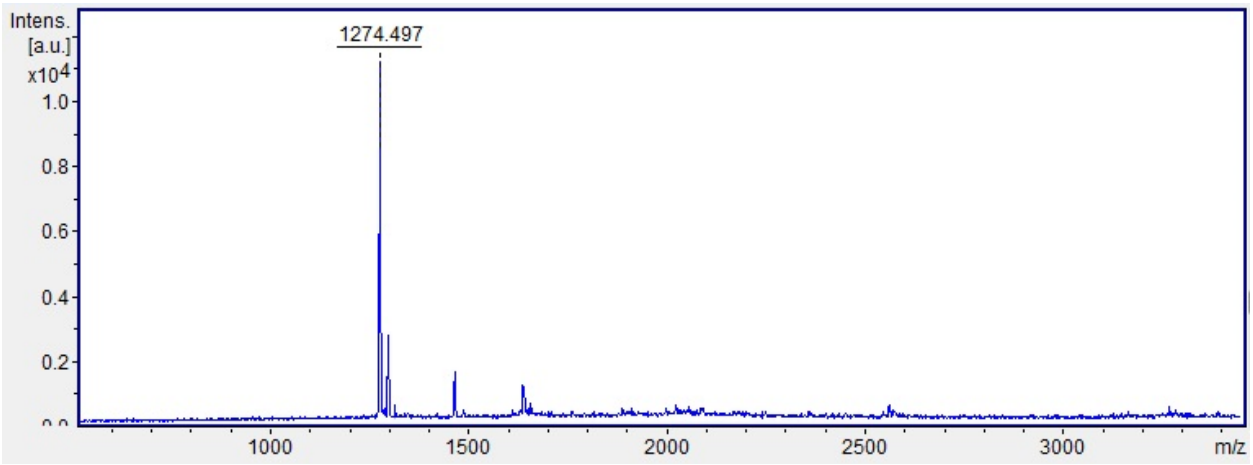
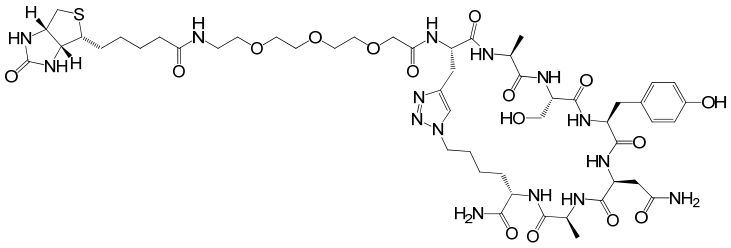


Figure S20. (A) Structure and (B) mass spectrum of L_F2 double-alanine mutant R1A/K5A (ASYNA).

Sequence: Biotin-PEG$_3$-Cy(ASYNA). This ligand is appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{51}H_{77}N_{15}O_{16}S$ (M+H) 1188.31; found 1190.23.

A.



B.

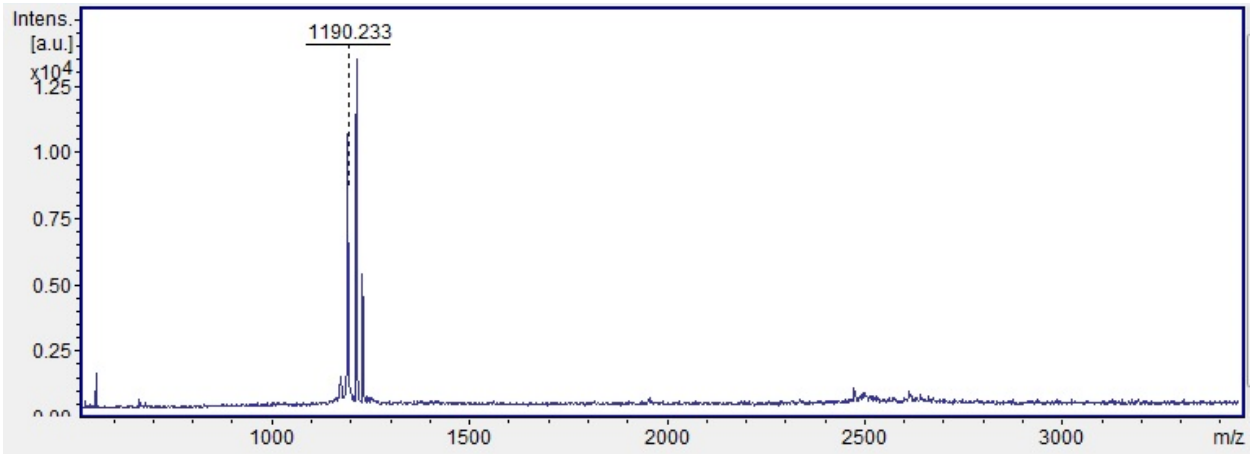
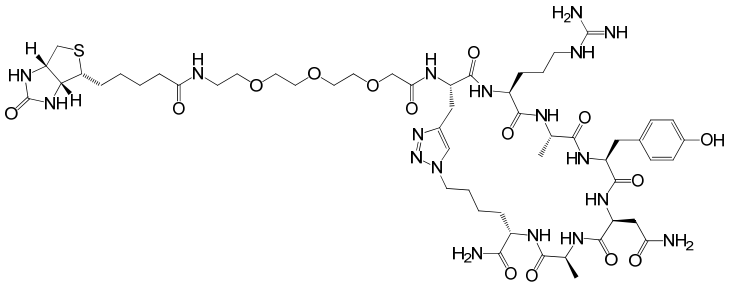


Figure S21. (A) Structure and (B) mass spectrum of L_F2 double-alanine mutant S2A/K5A (RAYNA).

Sequence: Biotin-PEG$_3$-Cy(RAYNA). This ligand is appended with an N-terminal Biotin-PEG$_3$ label. MALDI-TOF MS (m/z): calcd. for $C_{54}H_{84}N_{18}O_{15}S$ (M+H) 1257.42; found 1259.03.

A.



B.

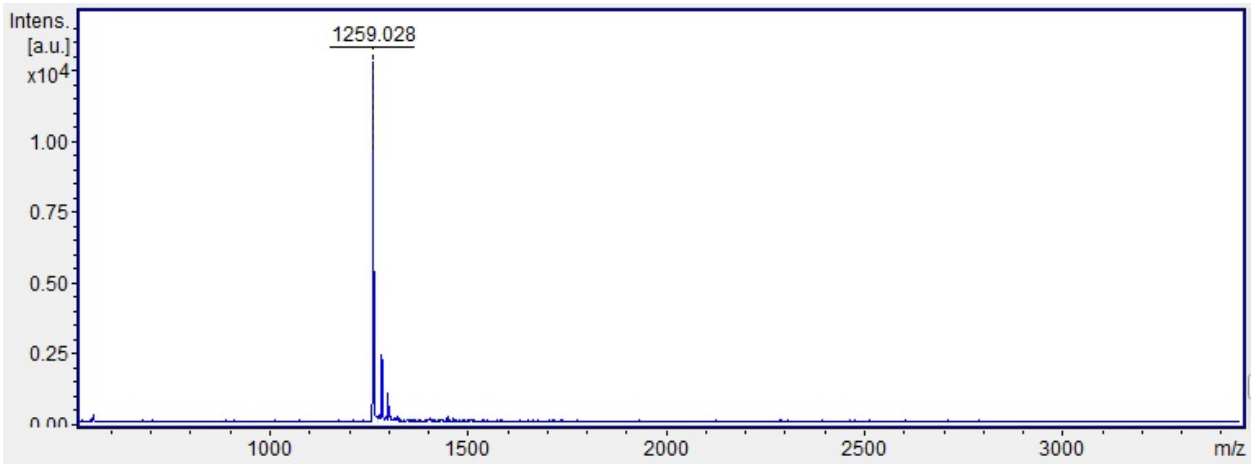
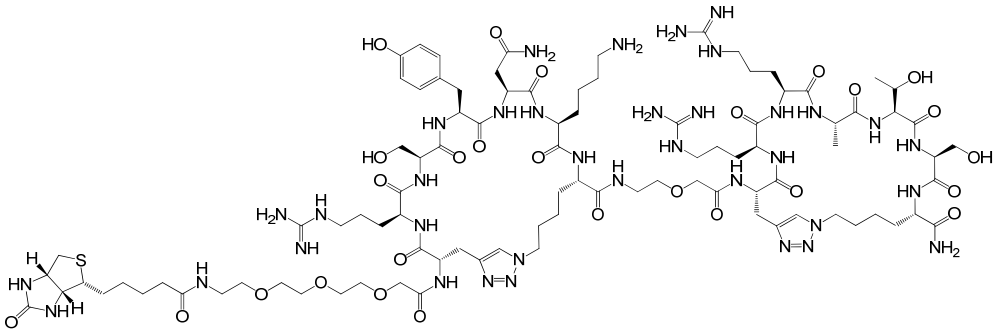


Figure S22. (A) Structure and (B) mass spectrum of biligand with PEG_1 linker.
Sequence: Biotin-PEG_3-Cy(RSYNK)-**PEG_1**-Cy(RRATS). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{94}H_{154}N_{36}O_{27}S$ (M+H) 2252.15; found 2252.53.

A.



B.

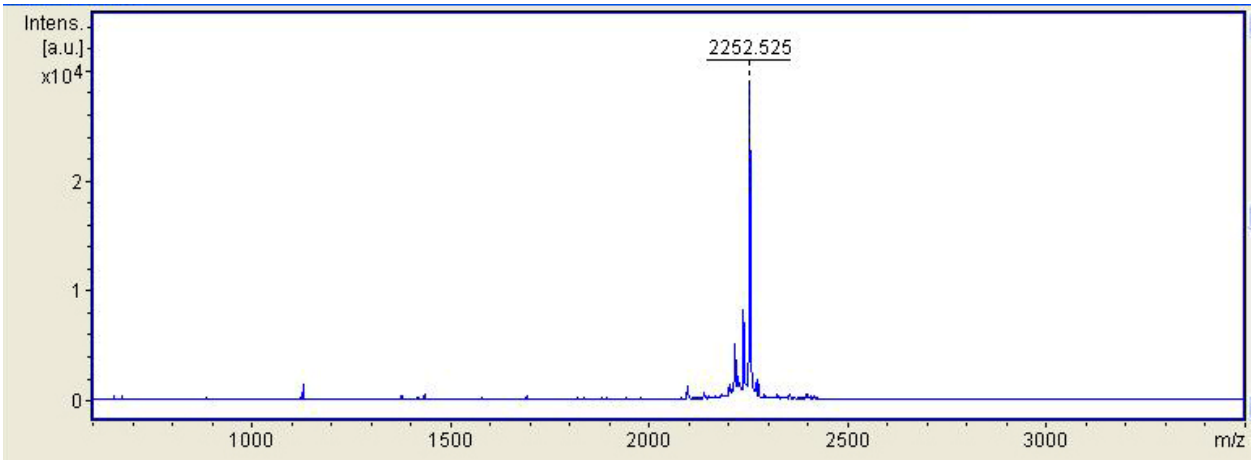
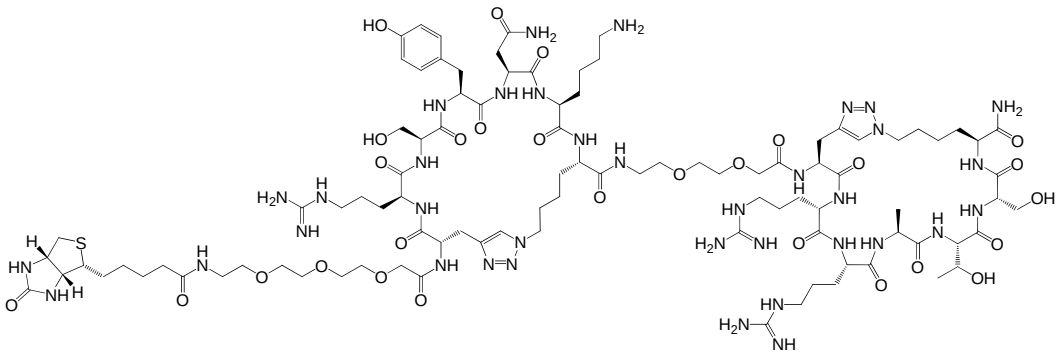


Figure S23. (A) Structure and (B) mass spectrum of biligand with PEG_2 linker.
Sequence: Biotin-PEG_3-Cy(RSYNK)-**PEG_2**-Cy(RRATS). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{96}H_{158}N_{36}O_{28}S$ (M+H) 2296.18; found 2296.61.

A.



B.

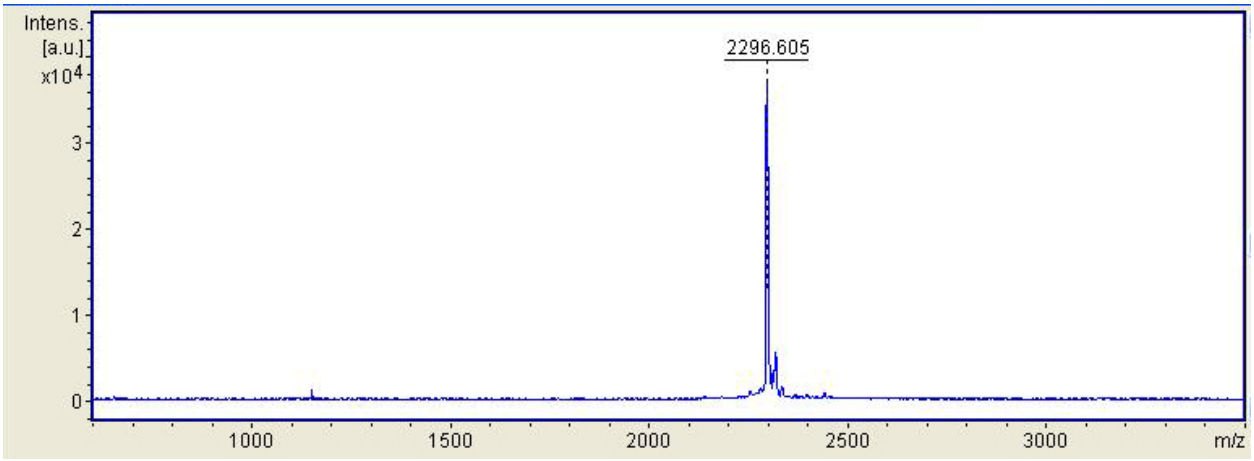
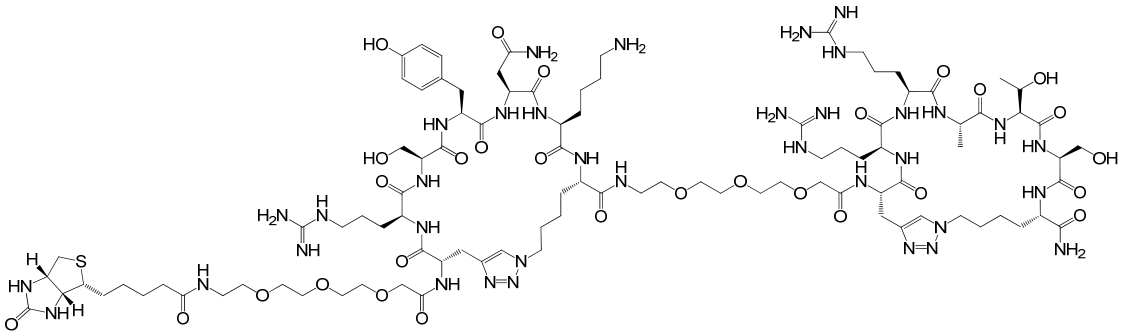


Figure S24. Characterization of biotinylated biligand with PEG_3 linker, Bi-L_F.
Sequence: Biotin-PEG_3-Cy(RSYNK)-**PEG_3**-Cy(RRATS). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{98}H_{162}N_{36}O_{29}S$ (M+H) 2340.20; found 2340.26. (A) Structure. (B) Mass spectrum.

A.



B.

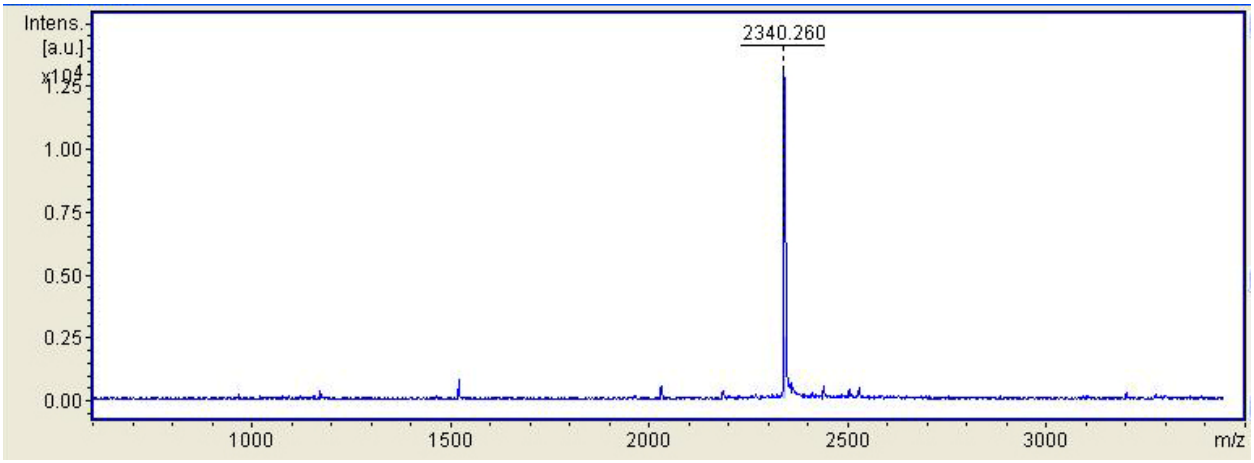
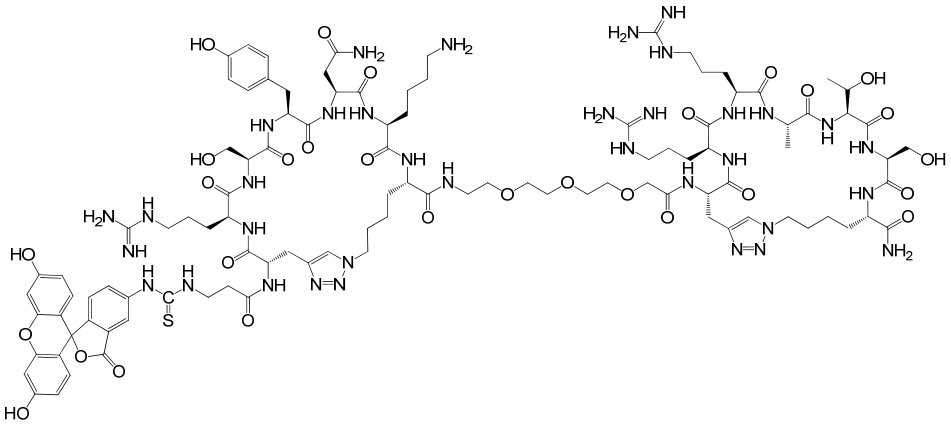


Figure S25. Characterization of 5-FITC-tagged biligand with PEG_3 linker, Bi-L_F.
Sequence: 5-FITC-β-Ala-Cy(RSYNK)-**PEG_3**-Cy(RRATS). This ligand is appended with an N-terminal 5-FITC label. MALDI-TOF MS (m/z): calcd. for $C_{104}H_{149}N_{35}O_{29}S$ (M+H) 2385.10; found 2387.66. (A) Structure. (B) Mass spectrum.

A.



B.

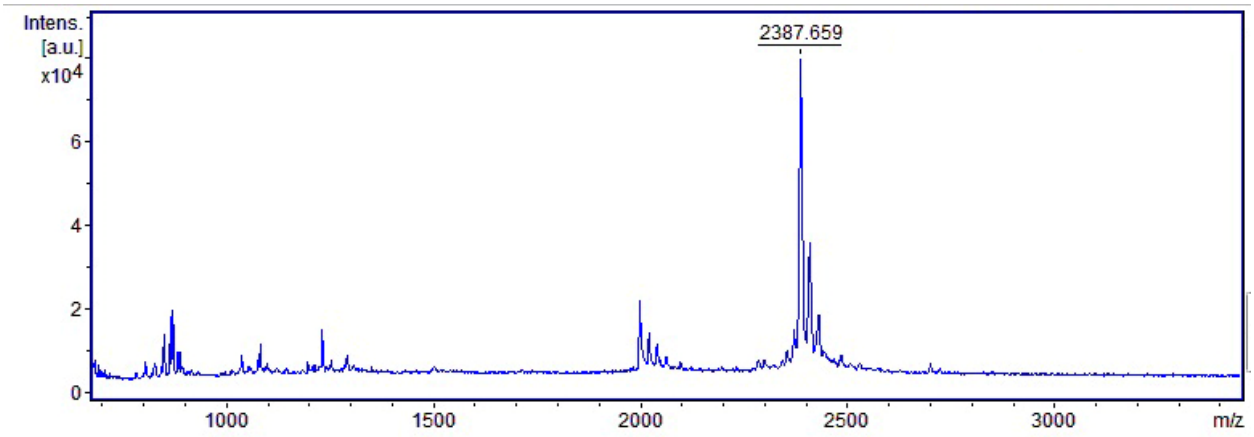
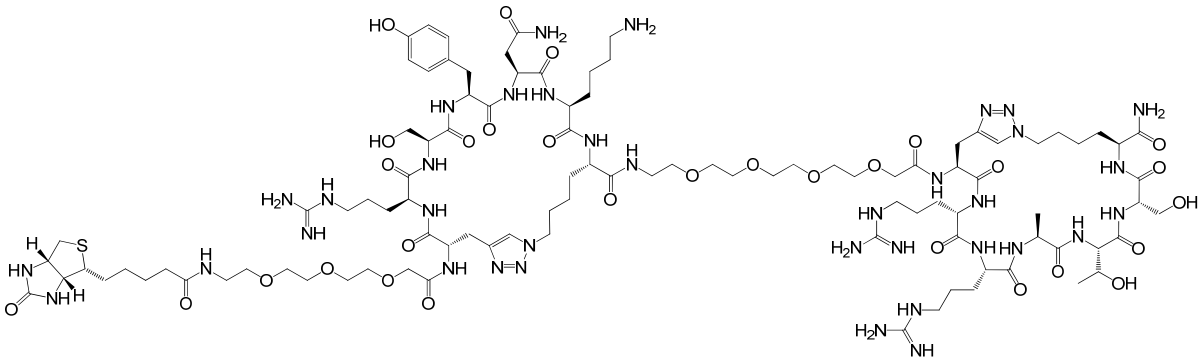


Figure S26. (A) Structure and (B) mass spectrum of biligand with PEG_4 linker.
Sequence: Biotin-PEG_3-Cy(RSYNK)-**PEG_4**-Cy(RRATS). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{100}H_{166}N_{36}O_{30}S$ (M+H) 2384.23; found 2384.24.

A.



B.

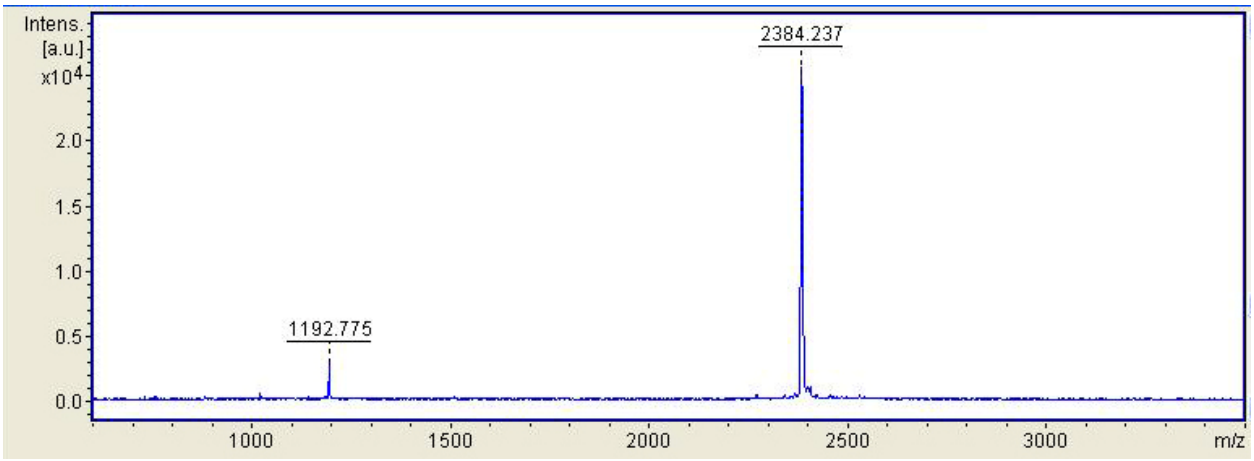
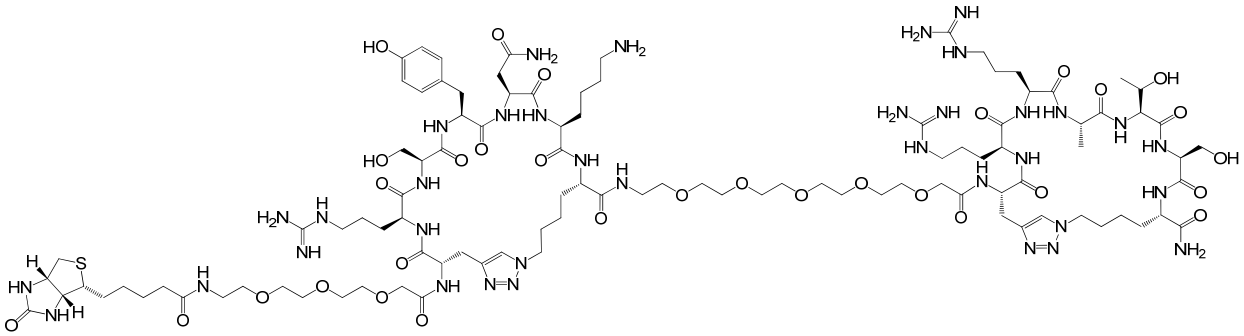


Figure S27. (A) Structure and (B) mass spectrum of biligand with PEG_5 linker.
Sequence: Biotin-PEG_3-Cy(RSYNK)-**PEG_5**-Cy(RRATS). This ligand is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{102}H_{170}N_{36}O_{31}S$ (M+H) 2428.26; found 2428.98.

A.



B.

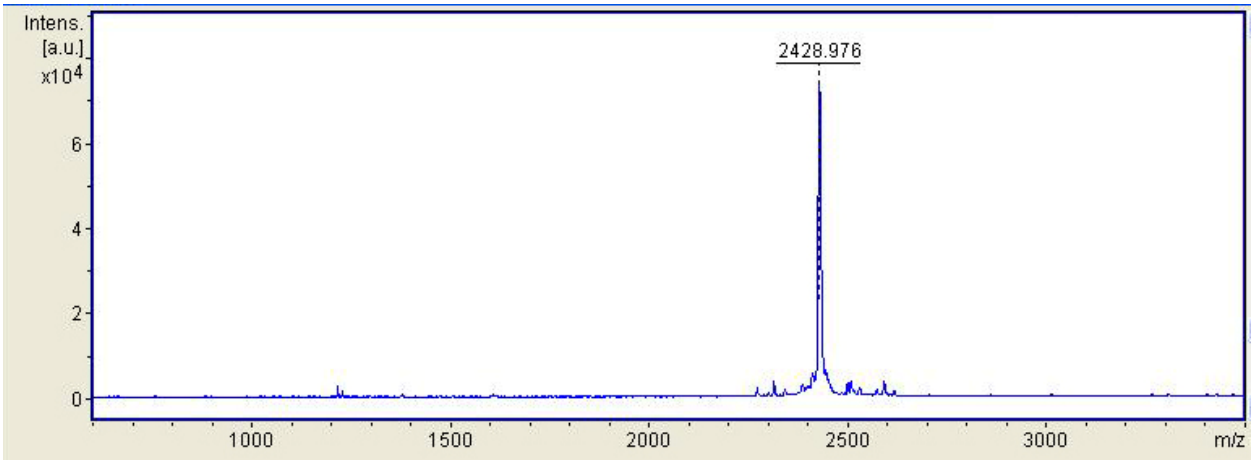
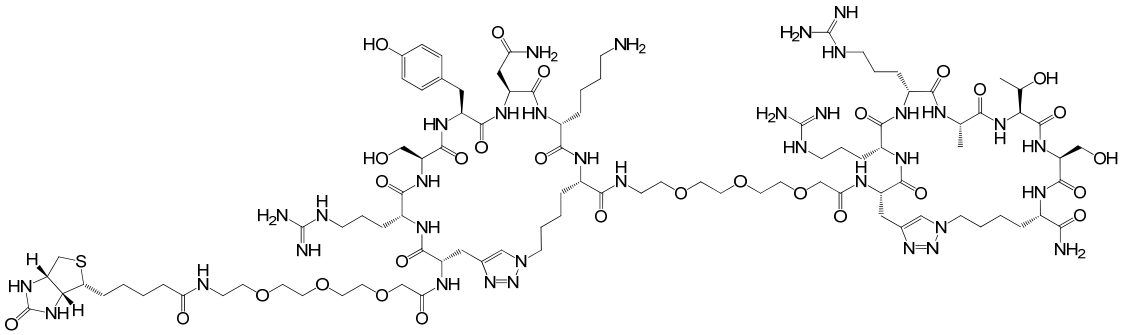


Figure S28. (A) Structure and (B) mass spectrum of Bi-L_S.
Sequence: Biotin-PEG_3-Cy(rSYNk)-**PEG_3**-Cy(rrATS). This ligand contains strategic substitution of the trypsin-sensitive amino acids with D-amino acids and is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{98}H_{162}N_{36}O_{29}S$ (M+H) 2340.20; found 2340.47.

A.



B.

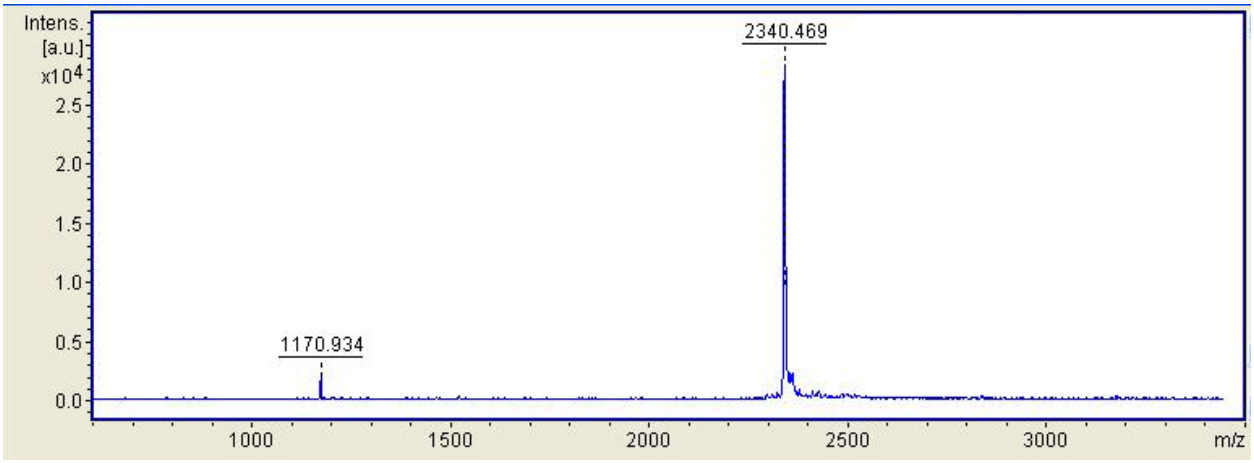
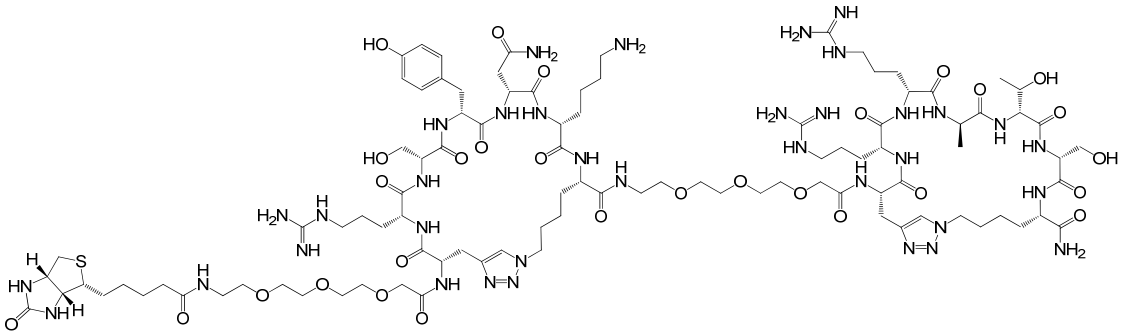


Figure S29. (A) Structure and (B) mass spectrum of Bi-L_G.
Sequence: Biotin-PEG_3-Cy(rsynk)-**PEG_3**-Cy(rrats). This ligand contains global substitution of D-amino acids and is appended with an N-terminal Biotin-PEG_3 label. MALDI-TOF MS (m/z): calcd. for $C_{98}H_{162}N_{36}O_{29}S$ (M+H) 2340.20; found 2341.05.

A.



B.

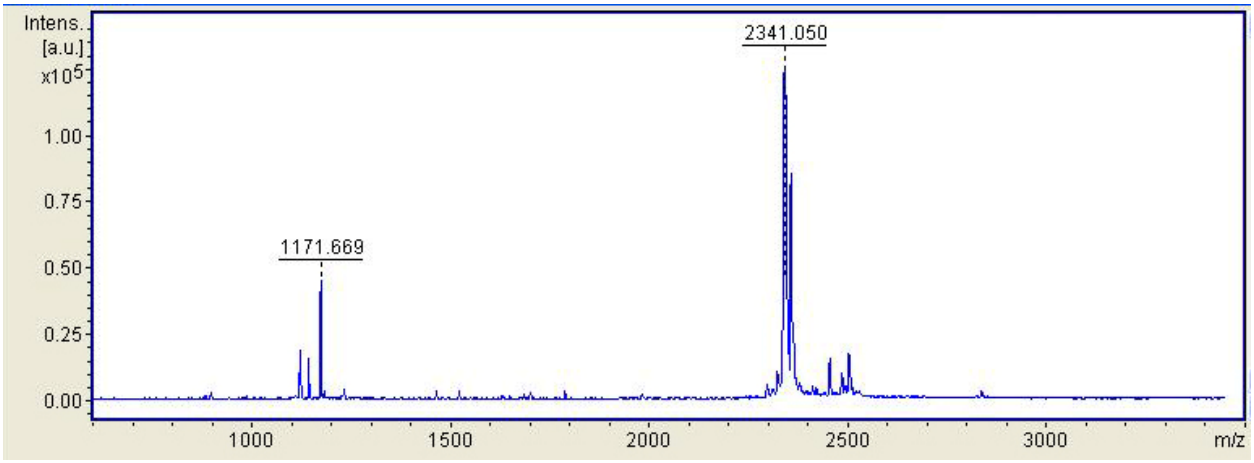
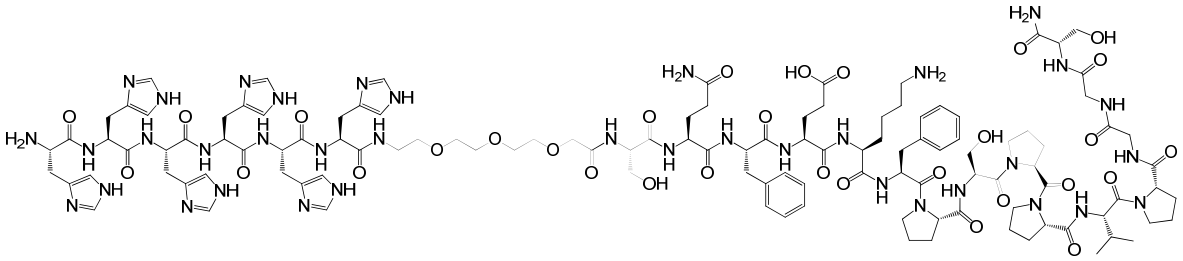
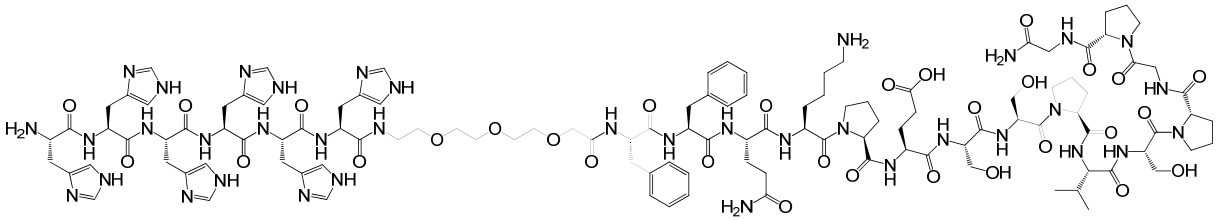


Figure S30. Sequences, structures, and mass spectrum of His-tagged IL-17F SynEp1 peptides. To determine the orientation of macrocycle binding to IL-17F SynEp1, this epitope was re-synthesized with a His_6 assay handle, C48S substitution instead of a click handle, and strategic scrambling of the sequences either N-terminal or C-terminal to C48S. The scrambled amino acids are shown in italics.

1) Sequence: His_6-PEG_3-*SQFEKFP*SPPVPGGS (scrambled N-terminal to click handle).



2) Sequence: His_6-PEG_3-FFQKPESS*PVSPGPG* (scrambled C-terminal to click handle).



MALDI-TOF MS (m/z): calcd. for $C_{116}H_{163}N_{37}O_{31}$ (M+H) 2571.23; found 2571.57.

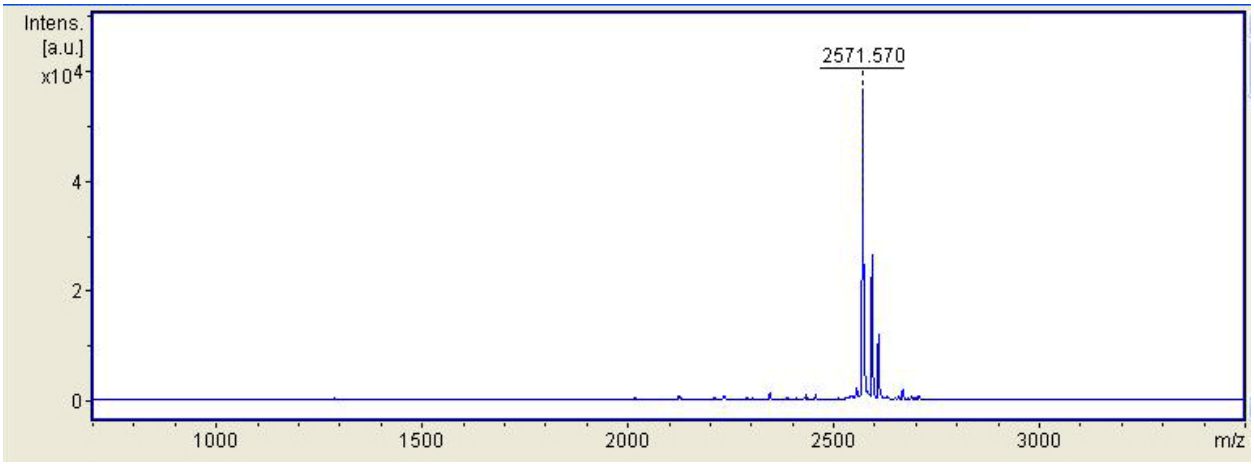
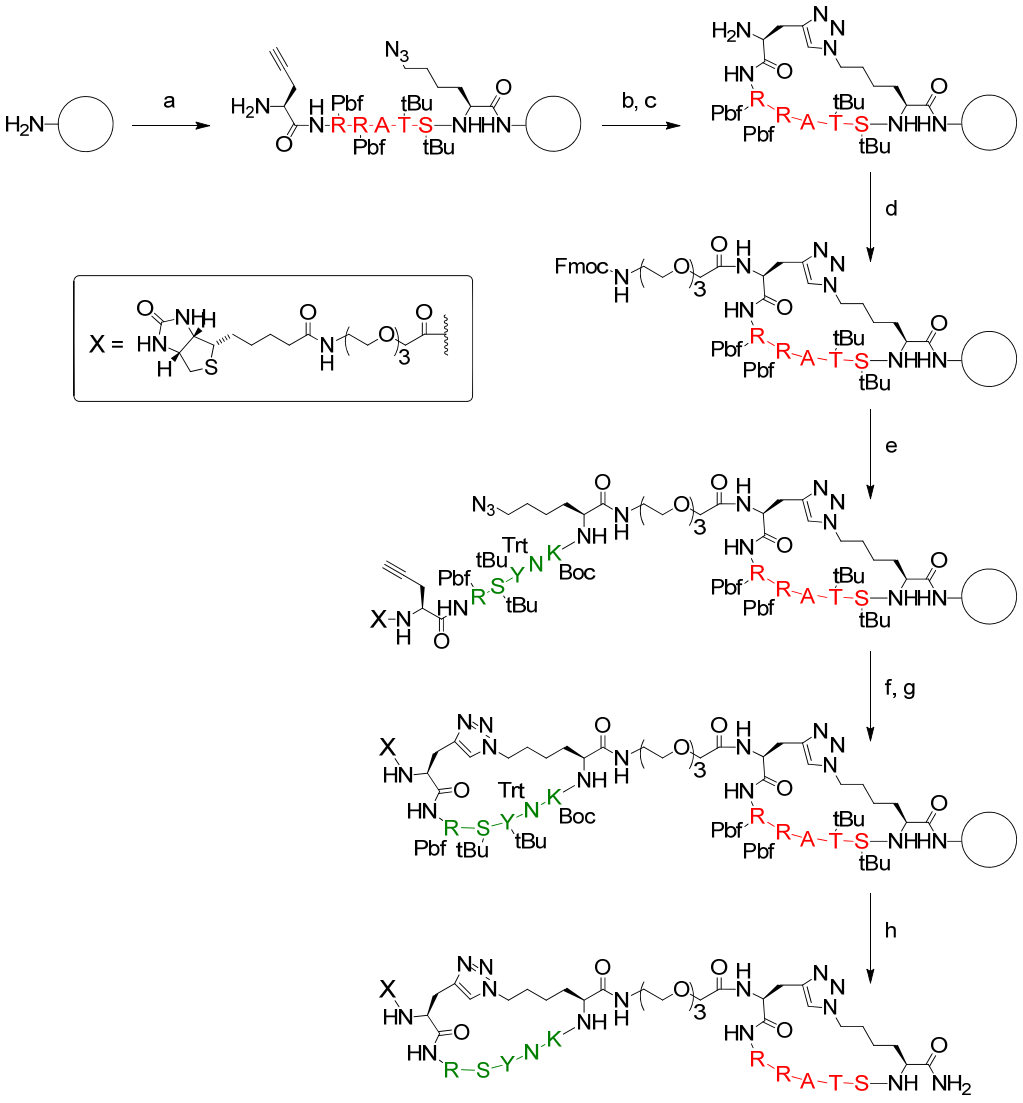


Figure S31. Solid-phase synthesis of biotinylated Bi-L_F. Amino acids are shown in one-letter code, with L-amino acids in uppercase and D-amino acids in lowercase. *Reagents and conditions:* (a) standard Fmoc/HBTU chemistry; (b) copper(I) iodide (1.5 eq.) and L-ascorbic acid (5 eq.) in 4:1 NMP:piperidine, overnight; (c) 5% (w/v) sodium diethyldithiocarbamate trihydrate and 5% (v/v) DIEA in NMP for 5 min, followed by thorough washes with NMP; (d) Fmoc-NH-PEG_3-CH_2COOH (2 eq.), HATU (1.9 eq.), DIEA (5 eq.), 2 h; (e) standard Fmoc/HBTU chemistry; (f) copper(I) iodide (1.5 eq.) and L-ascorbic acid (5 eq.) in 4:1 NMP:piperidine, overnight; (g) 5% (w/v) sodium diethyldithiocarbamate trihydrate and 5% (v/v) DIEA in NMP for 5 min, followed by thorough washes with NMP; (h) TFA/H_2O/TIS/DODT (92.5/2.5/2.5/2.5) for 2 h, followed by ether precipitation.



Peptide Synthesis

SynEps and epitope-targeted peptide ligands were synthesized on Rink amide resin using Fmoc-based solid-phase synthesis on either a Titan 357 automated peptide synthesizer (AAPPTEC) or Liberty 1 microwave peptide synthesizer (CEM Corporation). After acidic deprotection and resin cleavage, peptides were purified on a preparative scale Shimadzu HPLC with a reverse-phase C_{18} column (Phenomenex). All peptides were analyzed for the correct mass using matrix-assisted laser-desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) with a Bruker ultrafleXtreme instrument. Peptides were lyophilized to a powder for long-term storage. Concentrated peptide stocks were made by dissolving powder in DMSO and measuring the A280 absorbance via Nanodrop to determine the concentration.

Binding Assays

Enzyme-linked immunosorbent assay (ELISA). A black 96-well NeutrAvidin Coated High Binding Capacity plate (15510, Pierce) was coated with 2 μM biotinylated macrocyclic peptide ligand in TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 2 h at room temperature. Biotinylated monoclonal anti-IL17F (TA319597, Origene) was coated at 4 μg/mL in TBS as a control. The plate was aspirated and then washed with TBS (5 ×) and Wash Buffer (0.05% (v/v) Tween-20 in PBS, 1 ×). Full-length His-tagged IL-17F protein (ab167911, Abcam) was serially diluted in Wash Buffer (from 800 to 0 nM) and incubated in the designated microwells for 90 min at room temperature. Microwells were aspirated and then washed with Wash Buffer (10 ×). To detect the bound IL-17F protein, Alkaline Phosphatase (AP)-conjugated Anti-6X His tag® antibody [HIS-1] (ab49746, Abcam) was prepared at 1:10,000 dilution and added to the microwells for 1 h at room temperature. The plate was aspirated and washed with Wash Buffer (11 ×). AttoPhos® AP Fluorescent Substrate System (S1000, Promega) was employed to develop the

microwells. Using an excitation wavelength of 430 nm, fluorescent emission at 535 nm was recorded by Beckman Coulter DTX880 multimode microplate reader. Titration curves were fit using a four-parameter regression curve fitting program (Origin 8.5) to determine EC_{50} values. The same protocol was employed to characterize the binding affinities of anti-IL-17A macrocyclic peptide ligands using His-tagged IL-17A (ab166882, Abcam). Biotinylated monoclonal anti-IL17A (TA700015, Origene) was used as a control.

Point ELISA (IL-17F vs. IL-17A selectivity assay). A black 96-well NeutrAvidin Coated High Binding Capacity plate (15510, Pierce) was coated with 2 μM biotinylated macrocyclic peptide ligand in TBS (pH 7.6) for 2 h at room temperature. The plate was aspirated and then washed with TBS (5 ×) and Wash Buffer (0.05% (v/v) Tween-20 in PBS, 1 ×). Full-length His-tagged IL-17F (ab167911, Abcam) and IL-17A (ab166882, Abcam) proteins were prepared at 100 and 10 nM in Wash Buffer and incubated in the designated microwells for 90 min at room temperature. Microwells were aspirated and then washed with Wash Buffer (10 ×). To detect the bound IL-17F and IL-17A proteins, Alkaline Phosphatase (AP)-conjugated Anti-6X His tag® antibody [HIS-1] (ab49746, Abcam) was prepared at 1:10,000 dilution and added to the microwells for 1 h at room temperature. The plate was aspirated and washed with Wash Buffer (11 ×). AttoPhos® AP Fluorescent Substrate System (S1000, Promega) was employed to develop the microwells. Using an excitation wavelength of 430 nm, fluorescent emission at 535 nm was recorded by Beckman Coulter DTX880 multimode microplate reader.

Assay to determine orientation of macrocycle binding to IL-17F SynEp1. A black 96-well NeutrAvidin Coated High Binding Capacity plate (15510, Pierce) was coated with 2 μM biotinylated macrocyclic peptide ligand in TBS (pH 7.6) for 2 h at room temperature. Biotinylated monoclonal anti-IL17F (TA319597, Origene) was coated at 4 μg/mL in TBS as a control. The

plate was aspirated and then washed with TBS (5 ×) and Wash Buffer (0.05% (v/v) Tween-20 in PBS, 1 ×). Chemically synthesized His-tagged IL-17F epitopes were prepared at 2 μM in Wash Buffer and incubated in the designated microwells for 90 min at room temperature. Wash Buffer without epitope was added as a control. Microwells were aspirated and subsequently washed with Wash Buffer (10 ×). To detect the bound IL-17F epitopes, Alkaline Phosphatase (AP)-conjugated Anti-6X His tag® antibody [HIS-1] (ab49746, Abcam) was prepared at 1:10,000 dilution and added to the microwells for 1 h at room temperature. The plate was aspirated and washed with Wash Buffer (11 ×). AttoPhos® AP Fluorescent Substrate System (S1000, Promega) was employed to develop the microwells. Using an excitation wavelength of 430 nm, fluorescent emission at 535 nm was recorded by Beckman Coulter DTX880 multimode microplate reader. Data are shown after subtraction of the no-epitope background.

Fluorescence polarization to determine binding affinities in solution. To conduct the fluorescence polarization experiments, **L_F1**, **L_F2**, and **Bi-L_F** were synthesized with fluorescein 5-isothiocyanate (5-FITC; 204607, ChemPep) coupled to the N-terminus of each ligand. Experimental samples were generated by diluting human recombinant IL-17F with 50 nM of 5-FITC-tagged ligands in 0.1% bovine serum albumin (BSA) in PBS. These solutions were then pipetted into a black 96-well Flat Bottom Polystyrene NBS Microplate (3991, Corning). The plate was then incubated at 25 °C for 30 min. Using the excitation wavelength of 485 nm, polarized emission at 535 nm was recorded by Beckman Coulter DTX880 multimode plate reader. Titration curves were fit using a four-parameter regression curve fitting program (Origin 8.5) to determine the K_D values.

Point ELISA (serum selectivity assay). A black 96-well NeutrAvidin Coated High Binding Capacity plate was coated with biotinylated **L_F1**, **L_F2**, and **Bi-L_F**. Human serum (HS-30, Omega Scientific) was centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was utilized in an ELISA. Full-length His-tagged IL-17F (ab167911, Abcam) protein was prepared at 100 nM in Wash Buffer with 0.1%, 1%, 10%, 25%, 50%, and 100% human serum, and incubated in the designated microwells for 90 min at room temperature. To detect the bound IL-17F protein, a 1:10,000 dilution of Alkaline Phosphatase (AP)-conjugated Anti-6X His tag® antibody [HIS-1] (ab49746, Abcam) and AttoPhos® AP Fluorescent Substrate System (S1000, Promega) were employed. The resulting fluorescence was measured in a Beckman Coulter DTX880 multimode plate reader (excitation at 430 nm, emission at 535 nm).

Figure S32. ELISA curves comparing the biotinylated macrocycles and anti-IL-17F mAb.

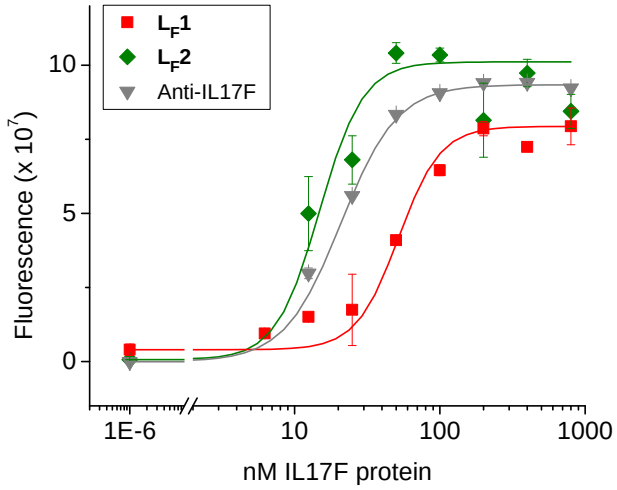


Figure S33. Point ELISAs for IL-17F protein against the **(A)** macrocycles **L_F1**, **L_F2**, and **(B)** biligand **Bi-L_F** in human serum solutions (% v/v).

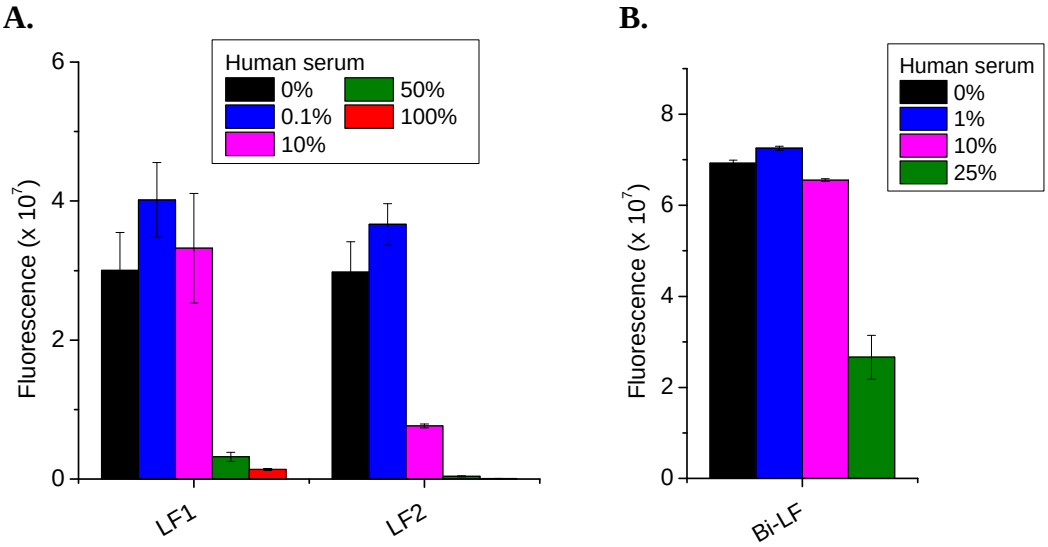


Figure S34. ELISA curves comparing the biotinylated biligands Bi-L_S and Bi-L_G.