Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Trivalent ligands for CXCR4 bearing polyproline linkers show specific recognition for cells with increased CXCR4 expression

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Supporting information

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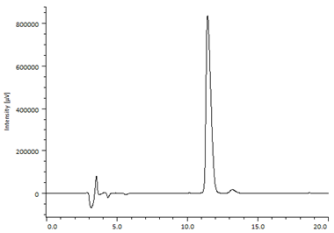
Synthesis of cFC131

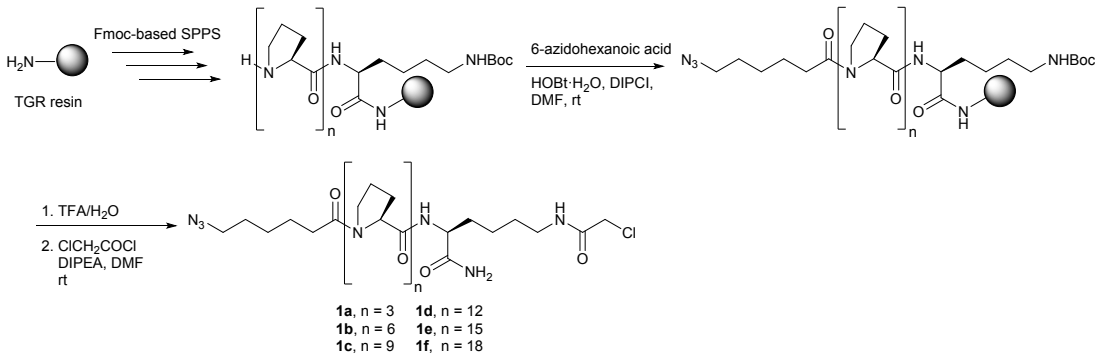
2-Chlorotrityl chloride resin (1.2 mmol/g, 1.0 g) was treated with Fmoc-D-Cys(Trt)-OH (0.50 mmol, 290 mg) and DIPEA (1.2 mmol, 350 μL) in dry DMF (5.0 mL) for 1 h. The resin was dried under vacuum after washing with dry DMF, CH_2Cl_2, and Et_2O. The loading was determined by measuring UV absorption at 301 nm of the piperidine treated Fmoc-D-Cys (Trt)-Trt(2-Cl)-resin (0.33 mmol/g). Unreacted chloride was capped by MeOH (2.0 mL) and DIPEA (510 μL, 0.94 mmol). The peptide chain of cFC131 was manually elongated on an Fmoc-D-Cys(Trt)-Trt(2-Cl)-resin (0.33 mmol/g, 0.84 mmol scale) by Fmoc-based SPPS. Each cycle involves (i) deprotection with 20% piperidine/DMF for 10 min and (ii) coupling with: Fmoc-amino acid (Fmoc-AA-OH) (3 eq.), 1-hydroxybenzotriazole (HOBt•H_2O) (5 eq.) and *N,N′*-diisopropylcarbodiimide (DIPCI) (5 equiv) in DMF for 50 min. Coupling efficiency was checked by the Kaiser ninhydrin test. In the case of a slightly positive Kaiser test, the coupling step was repeated (double coupling) using a mixture of Fmoc-protected amino acid (3 eq.), HOBt•H_2O (3 eq.), DIPEA (6 eq.), and *O*-benzotriazole-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) (2.9 eq.). After construction of the protected peptide on the resins, the resins were extensively washed with CH_2Cl_2, MeOH and Et_2O, and then dried in vacuum overnight. The synthetic peptide was cleaved from the resins by treatment with TFE-AcOH-CH_2Cl_2 (1:1:3, v/v) for 2 h. The reaction mixtures were filtered, and the resins were washed with CH_2Cl_2. The filtrates and washed solutions were evaporated under vacuum, and the protected peptides were precipitated as solid powder by an addition of cold Et_2O. After centrifugation, the supernatants were removed. The precipitations were washed with cold Et_2O. The obtained peptides were dried under vacuum overnight. To a mixture of protected liner peptide and $NaHCO_3$ (58 mg, 0.7 mmol) in DMF (40 mL) was added diphenylphosphoryl azide (86.7 μL, 0.40 mmol) at -40 °C. After incubation for 48 h with warming to room temperature, the reaction mixture was filtered. The filtrate was concentrated under reduced pressure, followed by chromatography over basic alumina column with $CHCl_3$-MeOH (9:1) to give the protected cyclic peptide. The obtained cyclic peptide was treated with TFA/thioanisole/*m*-cresol/ethanedithiol/ H_2O/triisopropylsilane (79/5/5/5/5/1, v/v) for 2 h. After concentration under reduced pressure, the residue was washed with cold Et_2O and dried under vacuum. Purification by preparative HPLC gave cFC131 (150 mg, 22%) as white powder: MS (ESI) m/z calculated for $C_{37}H_{50}N_{11}O_6S$ $[M+H]^+$ 776.4, found 776.3 (**Fig. S1**).



Retention time: 11.5 min with MeCN (27% isocratic)

Fig. S1. HPLC chart of cFC131.

Synthesis of polyproline linkers **1a-1f**.

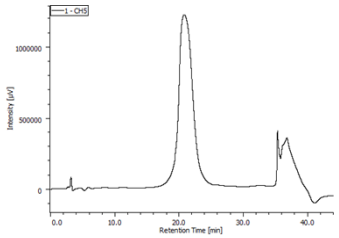


Polyproline linkers, **1a-1f**, were manually elongated on a NovaSyn® TGR resin (0.22 mmol/g, 0.91 g) by Fmoc-based SPPS. For the cleavage from the resin and deprotection, the protective peptide resin was treated with TFA (10 mL) and H_2O (0.5 mL) for 2 h. After filtering and concentration under reduced pressure, the residue was washed with cold Et_2O and dried under vacuum. To a solution of crude peptides in DMF (10 mL) were added chloroacetyl chloride (38 μL, 0.60 mmol) and DIPEA (160 μL, 1.2 mmol), and the mixture was stirred for 24 h. After concentration under reduced pressure, purification by preparative HPLC gave **1a-1f** (**Table S1**, **Fig. S2**). The UV-absorption was monitored at 220 nm.

Table S1. Yields of polyproline linkers **1a-1f**.

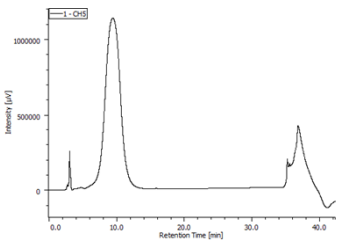
compd.	yield (%)
1a	38
1b	53
1c	20
1d	46
1e	55
1f	47

1a



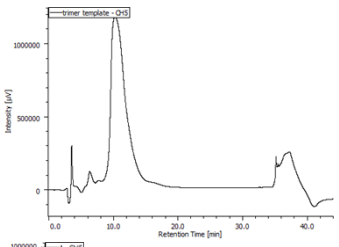
Retention time: 20.3 min with MeCN (20-25%)

1b



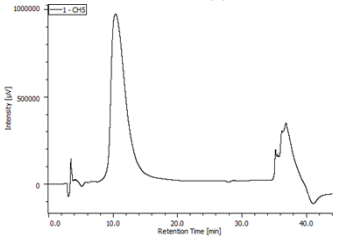
Retention time: 9.9 min with MeCN (25-30%)

1c



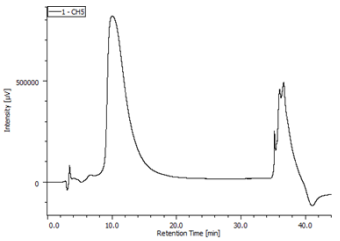
Retention time: 10.1 min with MeCN (25-30%)

1d



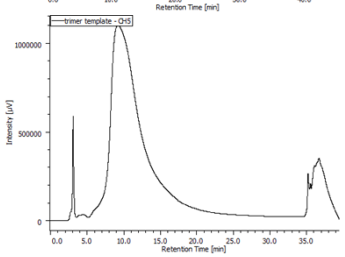
Retention time: 10.2 min with MeCN (25-30%)

1e



Retention time: 10.3 min with MeCN (25-30%)

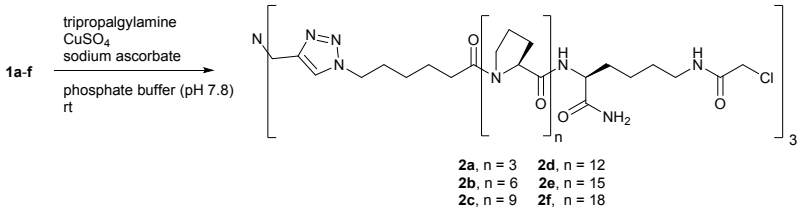
1f



Retention time: 10.3 min with MeCN (25-30%).

Fig. S2 HPLC charts of polyproline linkers **1a-1f**.

Synthesis of templates **2a-2f**.

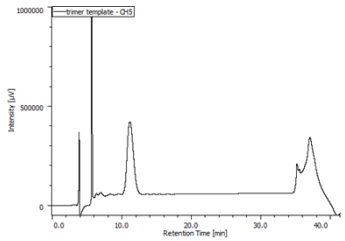


To a solution of polyproline chains **1a-1f** in 0.2 M phosphate buffer were added tripropargylamine (0.33 eq.), 100 mM aqueous solution of $CuSO_4$ (0.01 eq.), and 200 mM aqueous solution of sodium ascorbate (0.01 eq.), and the mixture was incubated for 24 h under room temperature. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **2a-2f** (**Table S2**, **Fig. S3**). The UV-absorption was monitored at 220 nm.

Table S2. Yields of trivalent polyproline templates **2a-2f**.

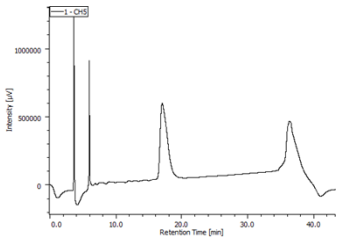
compd.	yield (%)
2a	81
2b	45
2c	25
2d	42
2e	77
2f	99

2a



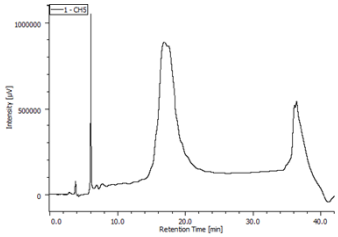
Retention time: 11.3 min with MeCN (25-40%)

2b



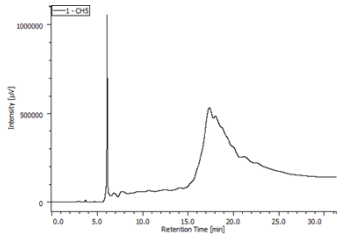
Retention time: 17.6 min with MeCN (15-45%)

2c



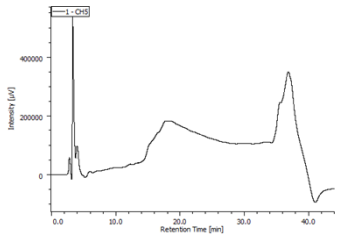
Retention time: 19.0 min with MeCN (15-45%)

2d



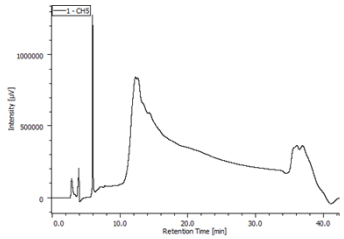
Retention time: 18.2 min with MeCN (15-45%)

2e



Retention time: 18.9 min with MeCN (15-45%)

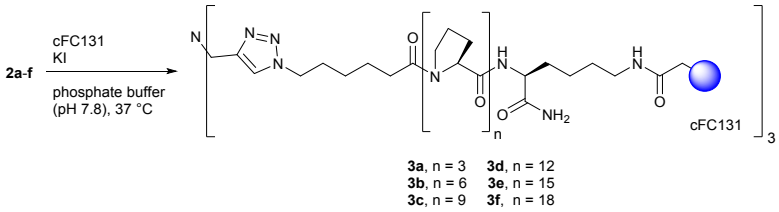
2f



Retention time: 13.5 min with MeCN (15-45%)

Fig. S3 HPLC chart of trivalent polyproline linkers **2a-2f**.

Synthesis of trivalent ligands **3a-3f**.



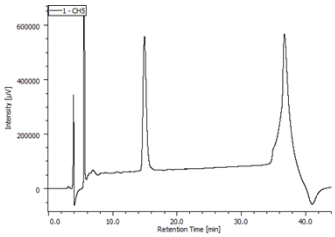
3a, n = 3 **3d**, n = 12
3b, n = 6 **3e**, n = 15
3c, n = 9 **3f**, n = 18

To a solution of trivalent polyproline linkers **2a-2f** in 0.1 M phosphate buffer were added potassium iodide (60 eq.) and cFC131 (3.3 eq.), and the mixture was degassed and stirred 24-72 h under 37 ºC. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **3a-3f** (**Table S3**, **Fig. S4**). The UV-absorption was monitored at 220 nm.

Table S3. Yields of trivalent ligands **3a-3f**.

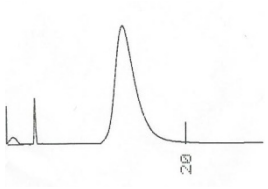
compd.	yield (%)
3a	7
3b	5
3c	4
3d	4
3e	6
3f	3

3a



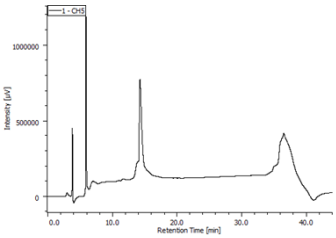
Retention time: 14.8 min with MeCN (25-40%)

3b



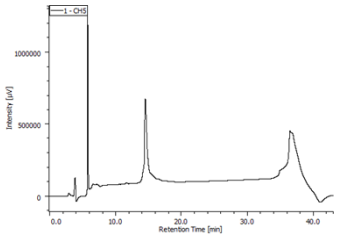
Retention time: 17.6 min with MeCN (15-45%)

3c



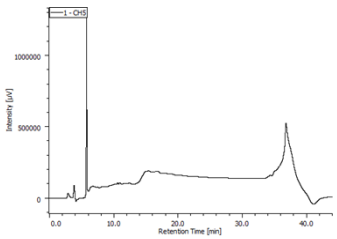
Retention time: 14.1 min with MeCN (25-40%)

3d



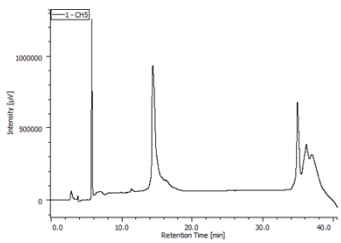
Retention time: 14.4 min with MeCN (25-40%)

3e



Retention time: 14.9 min with MeCN (25-40%)

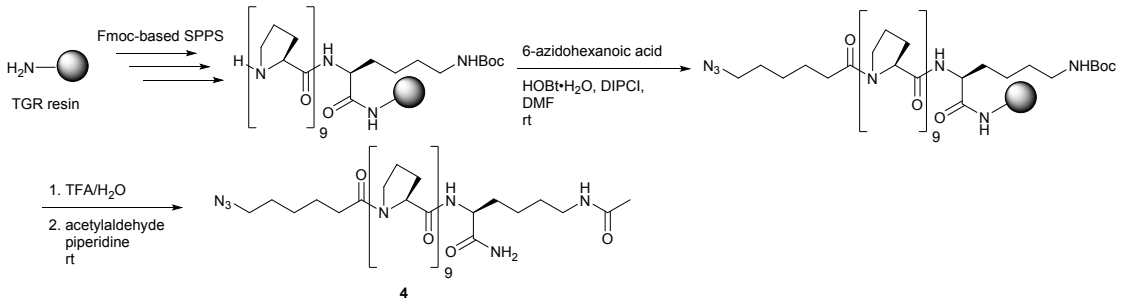
3f



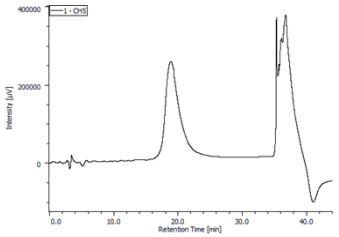
Retention time: 13.5 min with MeCN (25-40%)

Fig. S4 HPLC charts of trivalent ligands **3a-3f**.

Synthesis of polyproline linker (**4**).



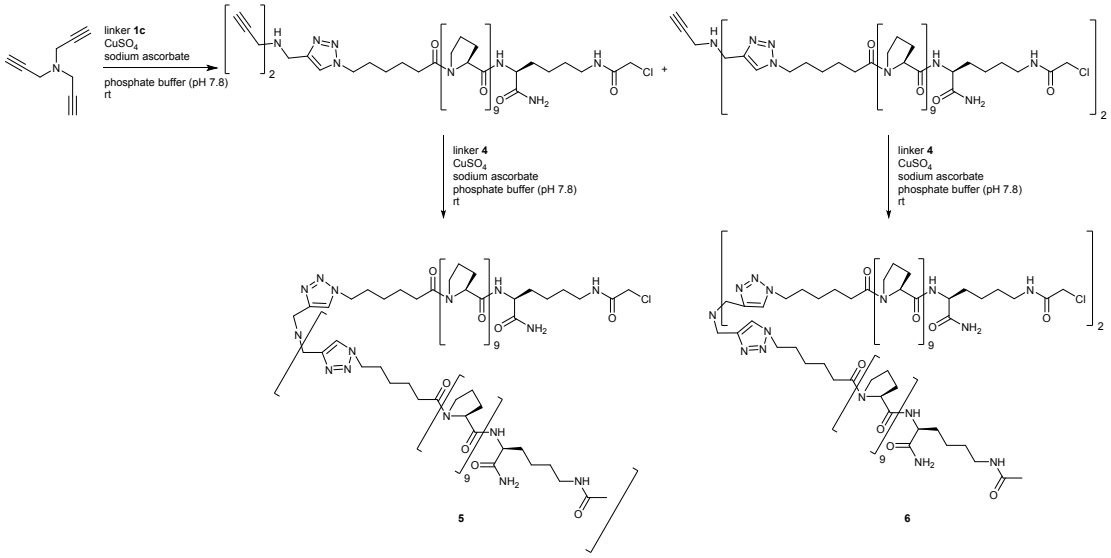
Polyproline linker **4** was manually elongated on a TGR resin (0.22 mmol/g, 0.91 g) by Fmoc-based SPPS. For the cleavage from the resin and deprotection, the protective peptide resin was treated with TFA (10 mL) and H_2O (0.5 mL) for 2 h. After filtering and concentration under reduced pressure, the residue was washed with cold Et_2O and dried under vacuum. To a solution of crude peptides in pyridine (10 mL) was added acetylanhydride (10 mL), and the mixture was stirred for 15 min. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **4** (55.7 mg, 46%) as white powder (**Fig. S5**). The UV-absorption was monitored at 220 nm.



Retention time: 18.3 min with MeCN (20-25%)

Fig. S5 HPLC chart of polyproline linker **4**.

Synthesis of templates for mono- and bi-valent ligands (**5** and **6**).

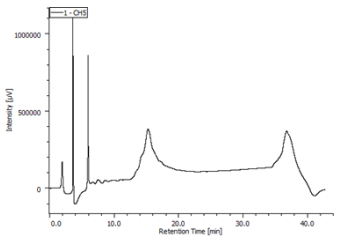


To a solution of tripropargylamine in 0.2 M phosphate buffer was added polyproline linker **1c** (1.5 eq.), 100 mM aqueous solution of $CuSO_4$ (0.01 eq.), and 200 mM aqueous solution of sodium ascorbate (0.01 eq.), and the mixture was incubated 24 h under room temperature. The mixture was added polyproline linker **4** (1.5 eq.), and the mixture was incubated 24 h under room temperature. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **5** and **6** (**Table S4**, **Fig. S6**). HPLC was monitored at 220 (nm).

Table S4. Yields of mono- and bi-valent polyproline linkers **5** and **6**.

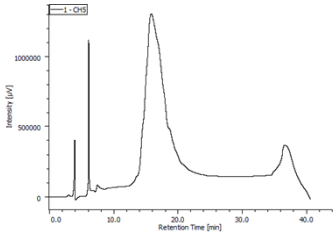
compd.	yield (%)
5	11
6	8

5



Retention time: 15.8 min with MeCN (15-45%)

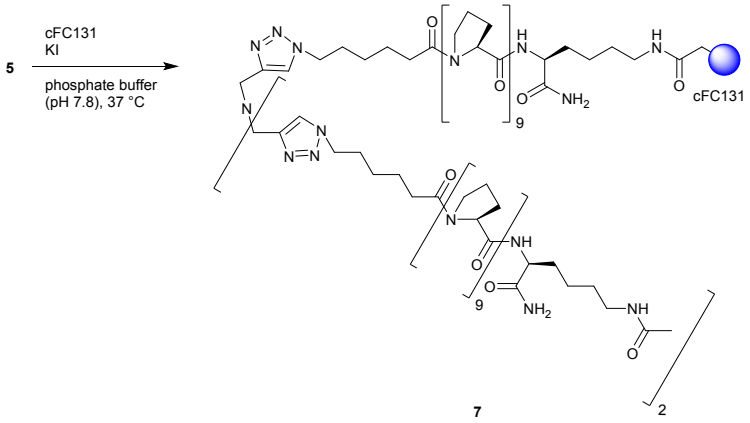
6



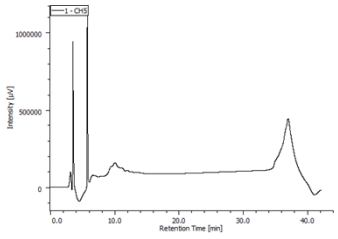
Retention time: 16.2 min with MeCN (15-45%)

Fig. S6 HPLC charts of mono- and bi-valent polyproline linkers **5** and **6**.

Synthesis of control monomer with the trivalent template (**7**).



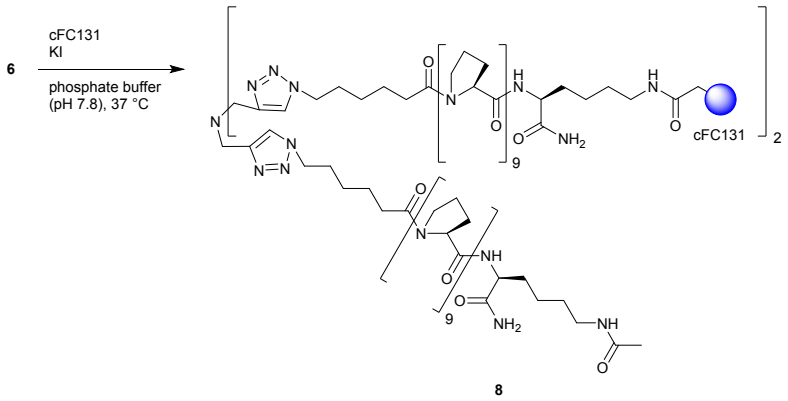
To a solution of trivalent polyproline linker **5** (2.8 mg, 0.74 μmol) in 0.1 M phosphate buffer was added potassium iodide (2.3 mg, 20 eq.) and cFC131 (0.63 mg, 1.1 eq.), and the mixture was degassed and stirred 24 h under 37 ºC. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **7** (1.05 mg, 31%) as white powder (**Fig. S7**). The UV-absorption was monitored at 220 nm.



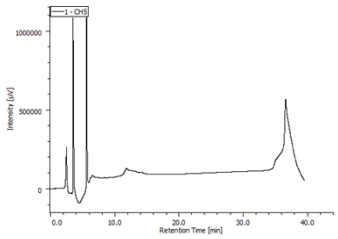
Retention time: 10.1 min with MeCN (25-40%)

Fig. S7 HPLC chart of monovalent ligand **7**.

Synthesis of bivalent control ligand with the trivalent template (**8**).



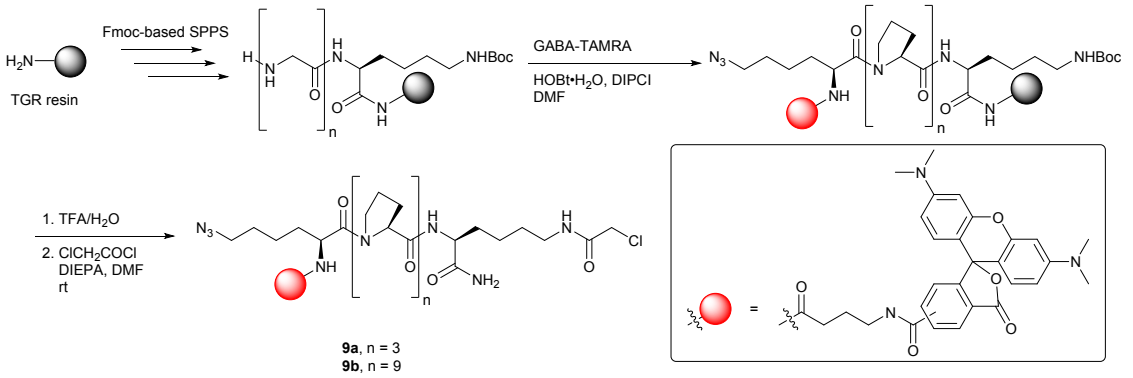
To a solution of trivalent polyproline linker **6** (2.3 mg, 0.61 μmol) in 0.1 M phosphate buffer was added potassium iodide (4.5 mg, 40 eq.) and cFC131 (1.0 mg, 2.2 eq.), and the mixture was degassed and stirred 24 h under 37 ºC. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **8** (0.64 mg, 20%) as white powder (**Fig. S8**). The UV-absorption was monitored at 220 nm.



Retention time: 11.8 min with MeCN (25-40%)

Fig. S8 HPLC chart of bivalent ligand **8**.

Synthesis of TAMRA-labeled polyproline linkers 9a and 9b.

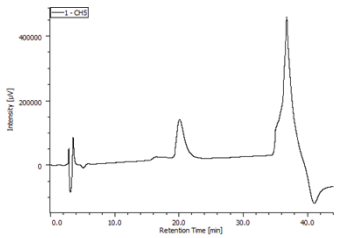


Polyproline linkers with TAMRA, **9a** and **9b**, were manually elongated on a NovaSyn® TGR resin (0.22 mmol/g, 0.91 g) by Fmoc-based SPPS. For the cleavage from the resin and deprotection, the protective peptide resin was treated with TFA (10 mL) and H_2O (0.5 mL) for 2 h. After filtering and concentration under reduced pressure, the residue was washed with cold Et_2O and dried under vacuum. To a solution of crude peptides in DMF (10 mL) were added chloroacetyl chloride (38 µL, 0.60 mmol) and DIPEA (160 µL, 1.2 mmol), and the mixture was stirred for 24 h. After concentration under reduced pressure, purification by preparative HPLC gave **9a** and **9b** (**Table S5**, **Fig. S9**). The UV-absorption was monitored at 220 nm.

Table S5. Yields of polyproline linkers **9a** and **9b**.

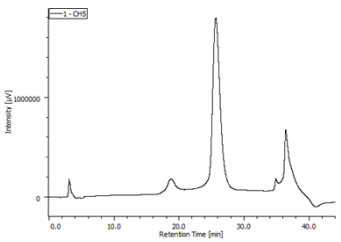
compd.	yield (%)
9a	9
9b	23

9a



Retention time: 20.1 min with MeCN (25-30%)

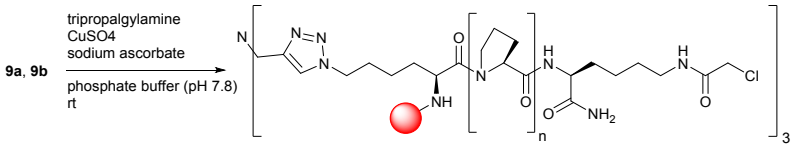
9b



Retention time: 25.8 min with MeCN (25-30%)

Fig. S9 HPLC charts of polyproline chains **9a** and **9b**.

Synthesis of TAMRA-labeled templates with polyproline linkers (**10a** and **10b**).



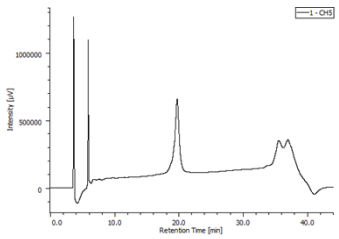
10a, n = 3
10b, n = 9

To a solution of polyproline linkers, **10a** and **10b**, in 0.2 M phosphate buffer were added tripropargylamine (0.33 eq.), 100 mM aqueous solution of $CuSO_4$ (0.01 eq.), and 200 mM aqueous solution of sodium ascorbate (0.01 eq.), and the mixture was incubated for 24 h under room temperature. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **10a** and **10b** (**Table S6**, **Fig. S10**). The UV-absorption was monitored at 220 nm.

Table S6. Yields of trivalent polyproline linkers **10a** and **10b**.

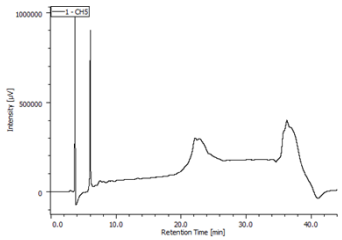
compd.	yield (%)
10a	69
10b	64

10a



Retention time: 19.7 min with MeCN (20-50%)

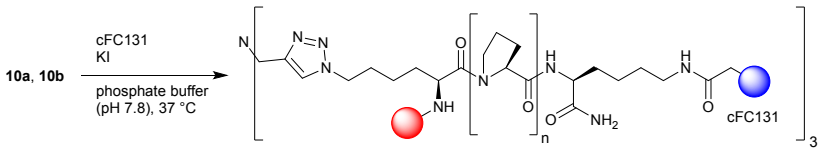
10b



Retention time: 22.9 min with MeCN (20-50%)

Fig. S10 HPLC chart of trivalent polyproline linkers **10a** and **10b**.

Synthesis of TAMRA-labeled trivalent ligands **11a** and **11b**.



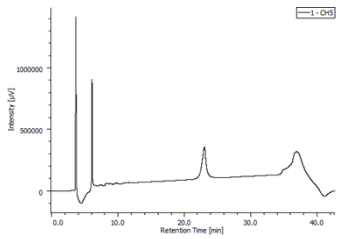
11a, n = 3
11b, n = 9

To a solution of trivalent polyproline linkers **11a** and **11b** in 0.1 M phosphate buffer were added potassium iodide (60 eq.) and cFC131 (3.3 eq.), and the mixture was degassed and stirred 24-72 h under 37 ºC. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **11a** and **11b** (**Table S7**, **Fig. S11**). The UV-absorption was monitored at 220 nm.

Table S7. Yields of trivalent ligands **11a** and **11b**.

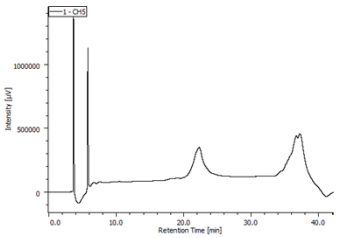
compd.	yield (%)
11a	3
11b	13

11a



Retention time: 23.8 min with MeCN (20-50%)

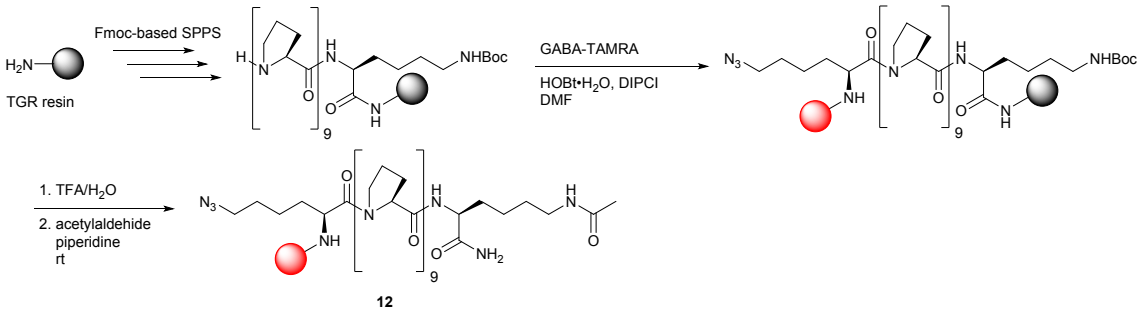
11b



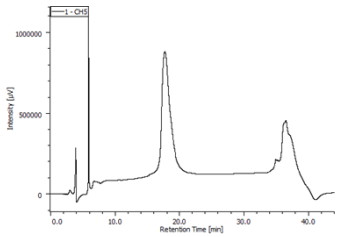
Retention time: 22.6 min with MeCN (25-40%)

Fig. S11 HPLC charts of trivalent ligands **11a** and **11b**.

Synthesis of polyproline linker **12**.



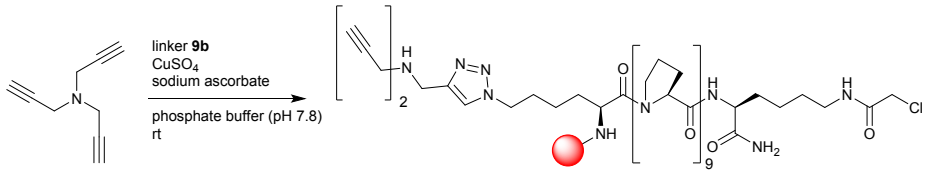
Polyproline linker **12** was manually elongated on a TGR resin (0.22 mmol/g, 0.91 g) by Fmoc-based SPPS. For the cleavage from the resin and deprotection, the protective peptide resin was treated with TFA (10 mL) and H_2O (0.5 mL) for 2 h. After filtering and concentration under reduced pressure, the residue was washed with cold Et_2O and dried under vacuum. To a solution of crude peptides in pyridine (10 mL) was added acetylanhydride (10 mL), and the mixture was stirred for 15 min. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **12** (10.0 mg, 3%) as red powder (**Fig. S12**). The UV-absorption was monitored at 220 nm.



Retention time: 18.0 min with MeCN (25-30%)

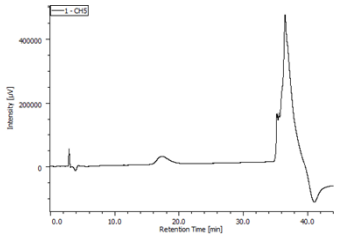
Fig. S12 HPLC chart of polyproline linker **12**.

Synthesis of TAMRA-labeled linker **13**.



13

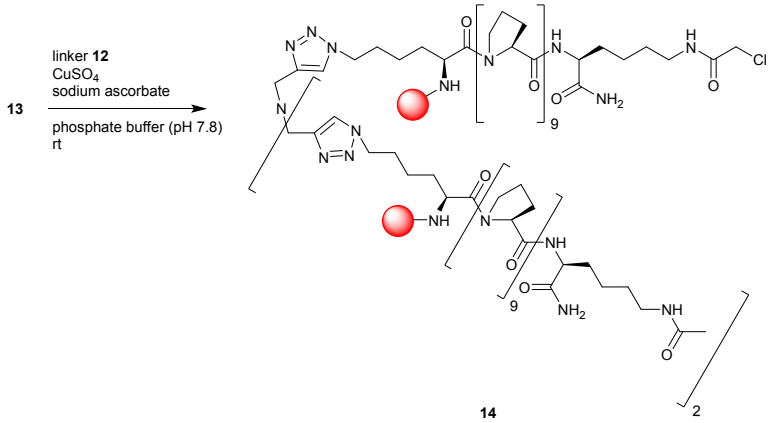
To a solution of tripropargylamine in 0.2 M phosphate buffer was added polyproline linker **9b** (7.0 eq.), 100 mM aqueous solution of $CuSO_4$ (0.01 eq.), and 200 mM aqueous solution of sodium ascorbate (0.01 eq.), and the mixture was incubated 24 h under room temperature. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **13** (3.1 mg, 22%) as red powder (**Fig. S13**). The UV-absorption was monitored at 220 nm.



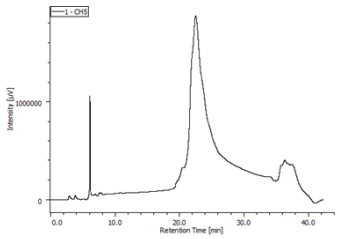
Retention time: 17.3 min with MeCN (25-30%)

Fig. S13 HPLC chart of polyproline linker **13**.

Synthesis of TAMRA-labeled template **14**.



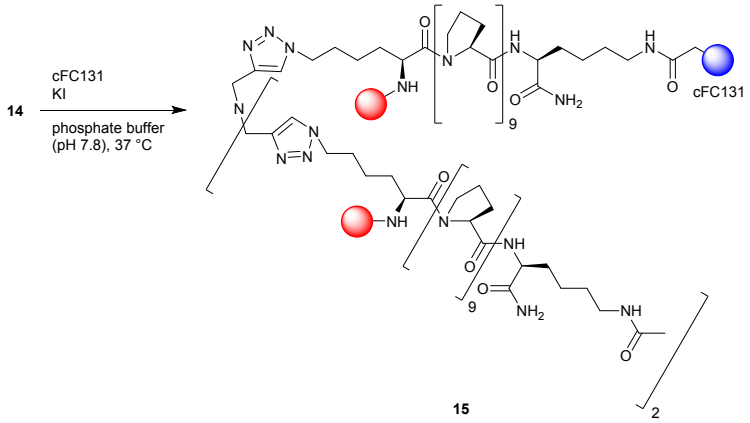
To a solution of polyproline linker **13** in 0.2 M phosphate buffer was added polyproline linker **12** (2.0 eq.), 100 mM aqueous solution of $CuSO_4$ (0.01 eq.), and 200 mM aqueous solution of sodium ascorbate (0.01 eq.), and the mixture was incubated 24 h under room temperature. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **14** (3.5 mg, 41%) as red powder (**Fig. S14**). The UV-absorption was monitored at 220 nm.



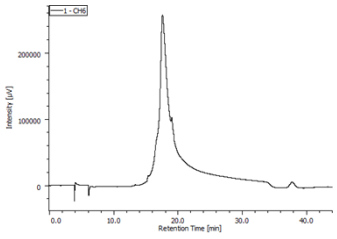
Retention time: 23.1 min with MeCN (15-45%)

Fig. S14 HPLC chart of polyproline linker **14**.

Synthesis of TAMRA-labeled monomer ligand with trivalent template **15**.



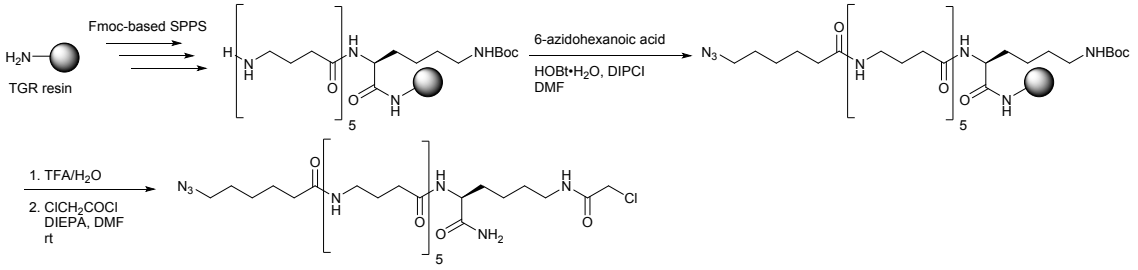
To a solution of trivalent polyproline linker **14** in 0.1 M phosphate buffer were added potassium iodide (20 eq.) and cFC131 (1.1 eq.), and the mixture was degassed and stirred 24 h under 37 ºC. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **15** (0.52 mg, 13%) as red powder (**Fig. S15**). The UV-absorption was monitored at 220 nm.



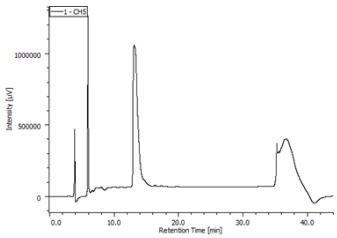
Retention time: 17.8 min with MeCN (25-40%)

Fig. S15 HPLC chart of monovalent ligand **15**.

Synthesis of penta-GABA linker **16**.



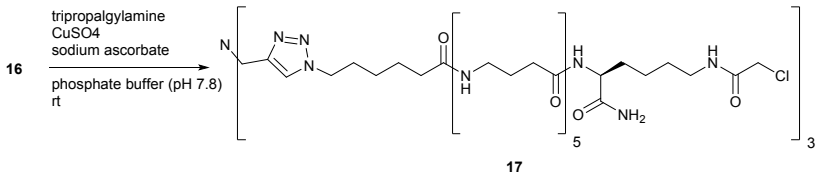
Polyproline linker **16** was manually elongated on a NovaSyn® TGR resin (0.22 mmol/g, 0.91 g) by Fmoc-based SPPS. For the cleavage from the resin and deprotection, the protective peptide resin was treated with TFA (10 mL) and H_2O (0.5 mL) for 2 h. After filtering and concentration under reduced pressure, the residue was washed with cold Et_2O and dried under vacuum. To a solution of crude peptides in DMF (10 mL) were added chloroacetyl chloride (38 µL, 0.60 mmol) and DIPEA (160 µL, 1.2 mmol), and the mixture was stirred for 24 h. After concentration under reduced pressure, purification by preparative HPLC gave **16** (58.0 mg, 37%) as red powde (**Fig. S16**). The UV-absorption was monitored at 220 nm.



Retention time: 13.6 min with MeCN (20-25%)

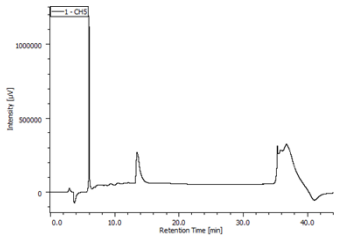
Fig. S16 HPLC chart of penta-GABA linker **16**.

Synthesis of trivalent template with penta-GABA linkers **17**.



17

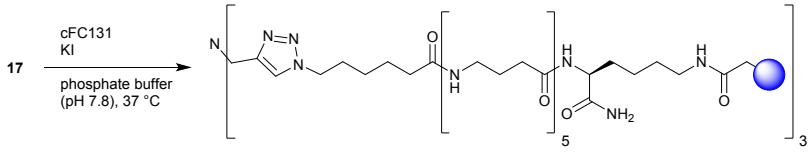
To a solution of polyproline linker **16** in 0.2 M phosphate buffer were added tripropargylamine (0.33 eq.), 100 mM aqueous solution of $CuSO_4$ (0.01 eq.), and 200 mM aqueous solution of sodium ascorbate (0.01 eq.), and the mixture was incubated for 24 h under room temperature. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **17** (0.52 mg, 13%) as red powder (**Fig. S17**). The UV-absorption was monitored at 220 nm.



Retention time: 13.5 min with MeCN (15-45%)

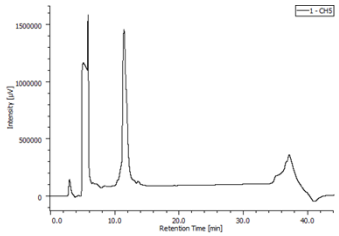
Fig. S17 HPLC chart of trivalent template with penta-GABA linkers **17**.

Synthesis of trivalent ligand with penta-GABA linker **18**.



18

To a solution of trivalent penta-GABA linker **17** in 0.1 M phosphate buffer were added potassium iodide (60 eq.) and cFC131 (3.3 eq.), and the mixture was degassed and stirred 24 h under 37 ºC. After being diluted with 0.1% aqueous TFA, purification by preparative HPLC gave **18** (0.57 mg, 22%) as red powder (**Fig. S18**). The UV-absorption was monitored at 220 nm.



Retention time: 17.8 min with MeCN (25-40%)

Fig. S18 HPLC chart of trivalent ligand **18**.

Table S8. HRMS of newly constructed ligands.

Compound	Formular	Calculated[a]	Found[a]
3a	$C_{207}H_{291}N_{61}O_{36}S_3$	1436.4070 $(M/3+H)^{3+}$	1436.4062
3b	$C_{252}H_{354}N_{70}O_{45}S_3$	1295.9318 $(M/4+H)^{4+}$	1295.9318
3c	$C_{297}H_{417}N_{79}O_{54}S_3$	1211.6420 $(M/5+H)^{5+}$	1211.6395
3d	$C_{342}H_{480}N_{88}O_{63}S_3$	1386.3370 $(M/5+H)^{5+}$	1386.3377
3e	$C_{387}H_{543}N_{97}O_{72}S_3$	1301.0279 $(M/6+H)^{6+}$	1301.0306
3f	$C_{432}H_{606}N_{106}O_{81}S_3$	1240.0929 $(M/7+H)^{7+}$	1240.0979
7	$C_{223}H_{324}N_{57}O_{42}S$	1502.4975 $(M/3+H)^{3+}$	1502.4935
8	$C_{260}H_{373}N_{68}O_{48}S_2$	1320.9646 $(M/4+H)^{4+}$	1320.9611
11a	$C_{294}H_{375}N_{73}O_{51}S_3$	1169.5756 $(M/5+H)^{5+}$	1169.5707
11b	$C_{384}H_{501}N_{91}O_{69}S_3$	1265.9726 $(M/6+H)^{6+}$	1265.9676
15	$C_{310}H_{410}N_{69}O_{57}S$	1209.2252 $(M/5+H)^{5+}$	1209.2231
18	$C_{222}H_{333}N_{67}O_{42}S_3$	1177.3864 $(M/4+H)^{4+}$	1177.3884

[a] HRMS was recorded on a micrOTOF-2focus (Bruker Daltonics) mass spectrometer.

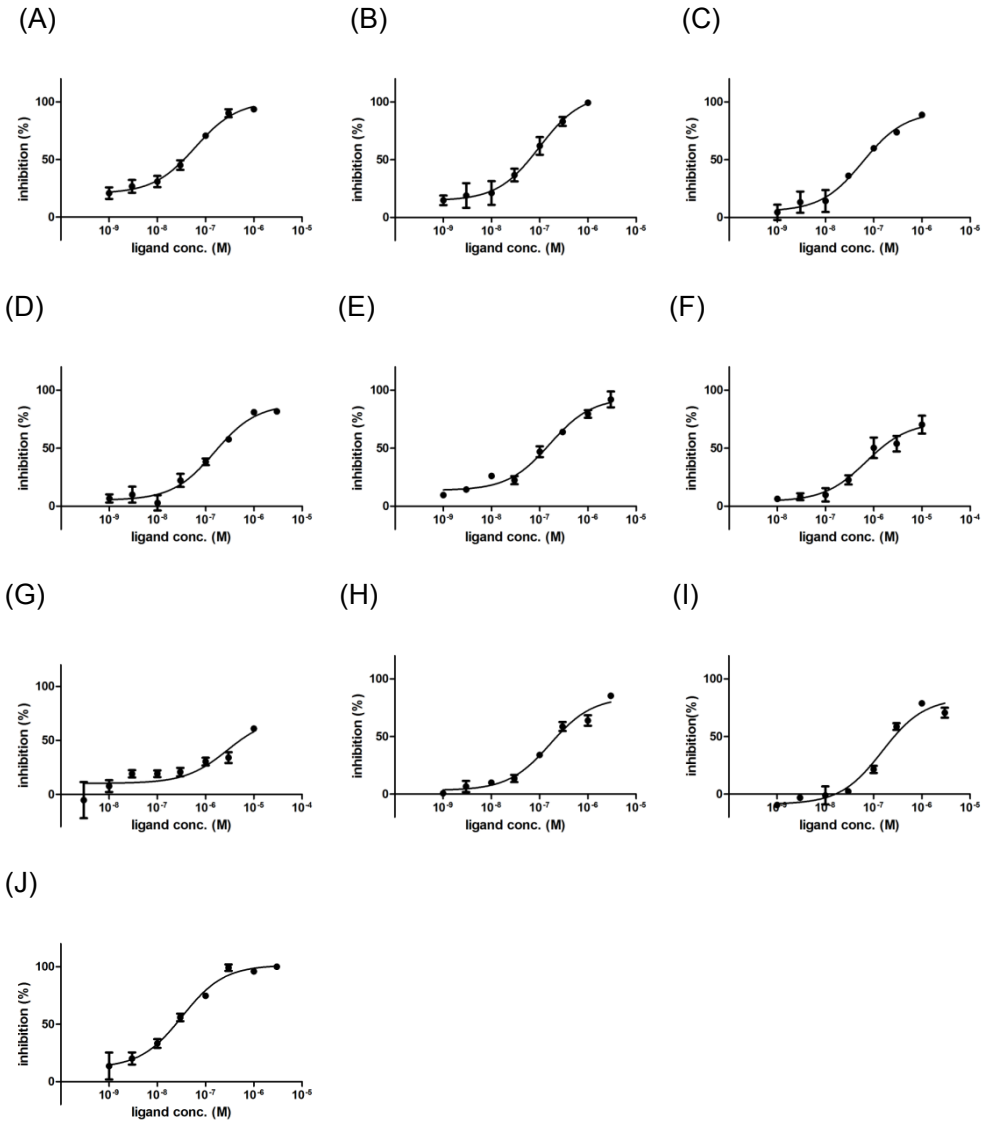


Fig. S19 Curve fittings of competitive binding analysis of CXCR4 ligand candidates. Panels (A-J) show the results of compounds **3a-f**, **7**, **8**, **18**, and FC131, respectively. The error bars represent S.E.M. of three independent experiments.

Table S9. Standard errors of IC_{50} for CXCR4 ligands

Compound	$logIC_{50}$ ± SEM
3a	-6.819 ± 0.0993
3b	-7.009 ± 0.153
3c	-7.223 ± 0.133
3d	-6.818 ± 0.105
3e	-6.793 ± 0.0977
3f	-6.158 ± 0.181
7	-5.550 ± 0.364
8	-6.776 ± 0.0913
18	-6.819 ± 0.0993
FC131	-7.486 ± 0.1016

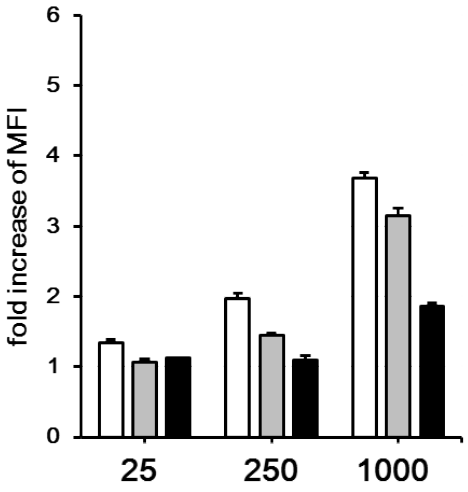


Fig. S20 The results of flow cytometry analysis for binding of TAMRA-labeled trivalent ligand with 3-proline linkers (**11a**). The bars represent the fold increase of fluorescence at the indicated ligand concentration (nM). Each bar shows the results of Jurkat (white), HeLa (gray), and K562 (black).

Reference

1. T. Tanaka, W. Nomura, T. Narumi, A. Masuda, H. Tamamura, *J. Am. Chem. Soc.* 2010, **132**, 15899.