Supplementary Information for

Convergent Synthesis of Proteins Using Peptide-aminothiazoline

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Contents

Materials

All reagents were purchased from commercial suppliers and were used without further purification. Fmoc amino acids and Boc amino acids were purchased from Peptide Institute Inc., Merk Millipore, Bachem, or Sigma-Aldrich. 3-[(9-Fluorenylmethoxycarbonyl)amino]-4-(methylamino)benzoic acid (Fmoc-MeDbz)**,** 1-[bis-(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Peptide Institute Inc. Aminomethyl-ChemMatrix resin was purchased from Sigma-Aldrich. *N,N*-diisopropylethylamine (DIEA), *N,N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), guanidine hydrochloride (GuHCl), acetonitrile, and $CuSO_4$ were purchased from FUJIFILM Wako Pure Chemical. Tris(2-carboxyethyl)phosphine (TCEP), 2-mercaptoethanesulfonic acid sodium salt (MESNa), 2,2'-dipyridyl disulfide, triisopropylsilane (TIPS), *N*,*N*'-diisopropylcarbodiimide (DIC), and 2-aminothiazoline hydrochloride were purchased from Tokyo Chemical Industry. Boc-Leu-Pam resin was purchased from Bachem. Trifluoromethanesulfonic acid (TfOH) was purchased from Nakalai tesque. 1*H*-Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBop), 1-((dimethylamino)(dimethyliminio)methyl)-1*H*-[1,2,3]triazolo[4,5-*b*]pyridine 3-oxide hexafluorophosphate (HATU) and Fmoc-NH-SAL resin were purchased from Watanabe Chemical Industry. Amino-PEGA resin was purchased from Merk. Dichroromethane (DCM) was purchased from Kanto Chemical Co. Inc.

Analytical LCMS.

The purities of synthesized peptides, glycopeptides and glycoproteins were confirmed by analytical liquid chromatography-mass spectrometry (LCMS). Chromatographic separations were performed by using a linear gradient of solvent B in solvent A typically over 5-15 min (solvent A: 0.1% formic acid in water, solvent B: 0.09% formic acid in 90% acetonitrile and 10% water). C4 silica gel column (Proteonavi, Osaka soda) with column dimension either 2.0 x 50 mm and 2.0 x 150 mm, or C18 silica gel column (Cadenza CD-C18, Imtakt) with column dimension 2.0 x 50 mm, were used for the analytical columns. Eluent was monitoring by UV-absorbance at 218 nm with on-line Q-TOF ESI-MS (Bruker, Compact). Reported mass measurements are the sum of the ion currents across the major UV peak in each chromatogram.

Semipreparative HPLC.

Synthetic products were purified on C4 silica gel (Proteonavi, Osaka soda) or C18 silica gel (CAPCELLPAK C18, Osaka soda) with the column dimension 10 x 250 mm. Crude peptides were loaded onto the preparative column at a flow rate 2.5 mL/min. Then a shallow gradient of solvent B (0.1% TFA in 90% acetonitrile and 10% water) in solvent A (0.1% TFA) was used to elute the desired compound (e.g. typically increasing buffer B in 0.25~0.3 %/min). Fractions containing pure material were identified by analytical LCMS and combined then lyophilized. In the case of the purification of (glyco)peptide-At derivatives, all fractions containing target materials were kept in an ice bath until lyophilization. This manipulation aimed to avoid unexpected hydrolysis of At moiety under the acidic aqueous condition derived from the mobile phase of HPLC.

High-resolution mass spectrometry (HRMS)

HRMS was recorded on Q-TOF mass spectrometry (Bruker, Compact), or linear ion trap-Orbitrap (Thermo Fisher Scientific, LTQ Orbitrap XL) using electrospray-ionization (ESI) method.

Synthesis of pentapeptide-At 1

Pentapeptide-At **1** (LYRAA-At) was synthesized manually through Fmoc SPPS. Fmoc protected amino acids utilized were Fmoc-Tyr(tBu)-OH, Fmoc-Arg(pbf)-OH and Fmoc-Ala-OH. The N-terminal Cys residue was coupled by using Boc-Leu-OH. Peptides were synthesized on aminomethyl ChemMatrix resin (50 μmol) equipped with 3-Fmoc-4-diaminobenzoic acid (Fmoc-Dbz-OH) according to the report from the Dawson group.[1] Aminomethyl ChemMatrix resin was coupled with Fmoc-Dbz-OH (4-fold excess)[1] pre-activated with 0.2 M HBTU/HOBt (1:1 in DMF, 3.9-fold excess) and 0.2 M DIEA (in DMF, 6-fold excess) for 3 min. This coupling was performed for 40 min twice. The coupling of the first Fmoc-Ala-OH (6-fold excess) was performed by using HBTU (6-fold excess) and DIEA (9-fold excess) in DMF (1 mL) for 40 min twice. The following coupling of Fmoc-Ala-OH, Fmoc-Arg(pbf)-OH, Fmoc-Tyr(tBu)-OH and Boc-Leu-OH were performed using 4-fold excess. A solution of 0.2 M HBTU/HOBt (1:1 in DMF, 3.8-fold excess) and 0.2 M DIEA (in DMF, 6-fold excess) was added to the amino acid derivative, which was activated for 1 min prior to add to the resin. Each coupling was performed for 40 min. Removal of Fmoc group was performed by using 20% piperidine/DMF for 2 + 13 min. After the completion of the peptide assembly, the resin was washed with DCM, followed by treated with 50 mM 4-nitrophenyl chloroformate solution (in DCM, 5-fold excess) for 40 min. After washing with DCM, the resin was treated with 0.5 M DIEA/DMF (5 ml) for 15 min. After washing with DMF and DCM, the resin was dried in vacuo and was deprotected by treatment with an acid reagent cocktail: 95% TFA, 2.5% TIPS, 2.5% H_2O for 2 h. After removal or TFA solution by filtration, the resin was washed with DCM and DMF. To the resin was added sodium phosphate buffer (0.2 M, pH 7.1, 2.5 mL) containing 6 M GuHCl and 1 M 2-aminothiazoline hydrochloride for 4 hours. After filtration, the resin was washed with the same buffer (1 mL x 2 times). All filtrates were combined and subjected to the purification by semipreparative HPLC (Proteonavi C4 1.0 × 250 mm, a linear gradient 99:1 to 80:20 of 0.1% TFA : 90% CH_3CN, 0.09% TFA over 60 min at a flow rate of 2.5 ml/min), afforded **1** (8.5 mg, 25% isolated). HRMS for **1** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{30}H_{49}N_{10}O_6S$ 677.3552; Obsd 677.3556 (monoisotopic).

Synthesis of pentapeptide-PivGu 2

Pentapeptide-PivGu (LYRAA-PivGu, **2**) was synthesized manually by Boc SPPS according to our previous report.[2] Boc protected amino acids (Boc-Xaa-OH) utilized were Boc-Leu-OH, Boc-Tyr(Br-Z)-OH Boc-Arg(di-Z)-OH and Boc-Ala-OH. Peptides were synthesized on aminomethyl ChemMatrix resin (100 μmol). First, aminomethyl ChemMatrix resin was coupled with S-trityl-3-mercaptopropionic acid (4-fold excess)[3] pre-activated with 0.38 M HBTU/DMF (3.8-fold excess) and DIEA (8-fold excess) for 1 min prior to add to the resin. This coupling reaction was performed for 2 hours. Removal of Trt group was performed by using 2.5% TIPS/TFA for 2 min twice. Coupling of following Boc-Xaa-OH was performed using 4-fold excess. A solution of 0.38 M HBTU/DMF (3.8-fold excess) and DIEA (8-fold excess) was added to Boc-Xaa, which was preactivated for 1 min prior to add to the resin. Each coupling reaction was performed for 20 min. Removal of Boc group was performed by using TFA for 2 min twice.

After removal of Boc group of the N-terminal Leu residue, the peptidyl-resin was treated with TfOH/TFA/m-cresol/dimethylsulfide (1/5/1/3 (v/v), 3 mL) for 60 min on an ice bath. After removal of the acid solution by filtration, the peptidyl resin was washed with DCM and DMF. To the resin was added 0.2 M phosphate buffer (pH 8.0, 5.0 mL) containing 6 M GuHCl and 0.2 M *N*-pivaloylguanidine[2] and reacted

for 2 hours at ambient temperature. Purification of the resultant filtrate by preparative LC (Proteonavi C4 10 × 250 mm, a linear gradient from 90:10 to 70:30 of 0.1% TFA:90% CH_3CN, 0.09 % TFA over 60 min at a flow rate of 2.5 mL/min) afforded **3** (37.6 mg, 52% isolated). HRMS for **2** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{33}H_{56}N_{11}O_7$ 718.4377; Obsd 718.4359 (monoisotopic).

Synthesis of pentapeptide-thioester 3

Pentapeptide-thioester (LYRAA-thioester, **3**) was synthesized manually by Boc SPPS using the *in situ* neutralization protocol.[4] Boc-Xaa-OH utilized were Boc-Leu-OH, Boc-Tyr(Br-Z)-OH Boc-Arg(di-Z)-OH and Boc-Ala-OH. Peptides were synthesized on H_2N-Leu-Pam-resin, which was prepared from Boc-Leu-Pam-resin (100 μmol) by treatment with TFA (1 min x 2 times). First, H_2N-Leu-Pam-resin was coupled with S-trityl-3-mercaptopropionic acid (4-fold excess), which was preactivated with 0.38 M HBTU/DMF (3.8-fold excess) and DIEA (8-fold excess) for 1 min prior to add to the resin. This coupling reaction was performed for 2 hours. The removal of Trt group was performed by treatment with 2.5% TIPS/TFA for 2 min twice. Coupling of following Boc-Xaa-OH was performed using 4-fold excess. A solution of 0.38 M HBTU/DMF (3.8-fold excess) and DIEA (8-fold excess) was added to Boc-Xaa-OH and preactivated for 1 min prior to add to the resin. Each coupling was performed for 20 min. The removal of Boc group was performed by using TFA for 2 min twice.

After removal of Boc group of the N-terminal Leu residue, the peptidyl-resin was treated with TfOH/TFA/m-cresol/dimethylsulfide (1/5/1/3 (v/v), 3 mL) for 60 min on an ice bath. After removal of the acid solution by filtration, the peptidyl resin was subsequently treated with TfOH/TFA/EDT/thioanisole (2/20/1/2 (v/v), 5 mL) on an ice bath. The resulting solution was added over cold ether and precipitated by centrifugation. The supernatant was removed, and the residue was dissolved in 50% CH_3CN containing 0.1% TFA and lyophilized. Purification of the resultant crude material by semipreparative LC (Proteonavi C4 10 × 250 mm, a linear gradient 0.1% TFA : 90% CH_3CN, 0.09% TFA from 90:10 to 70:30 over 60 min at a flow rate of 2.5 mL/min) afforded **3** (28.9 mg, 36% isolated). HRMS for **2** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{36}H_{60}N_9O_9S$ 794.4229; Obsd 794.4210 (monoisotopic).

Synthesis of circular RGD protein

Synthesis of peptide-At 5

Peptide-At (C(Spy)GGRGDSA-At, **5**) was synthesized manually through Fmoc SPPS. Peptides were synthesized on Fmoc-NH-SAL resin (50 μmol) equipped with MeDbz as a linker according to the previous report from the Dawson group.[5] Protected amino acids used were Fmoc-Gly-OH, Fmoc-Arg(pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ala-OH, and Boc-Cys(Trt)-OH for the C-terminal Cys residue. After removal of Fmoc group of Fmoc-NH-SAL resin by 20% piperidin/DMF, the resin was coupled with Fmoc-Gly-OH (5-fold excess), which was pre-activated with 0.5 M HBTU/DMF (5-fold excess) and DIEA (5.5-fold excess) for 30 sec prior to add to the resin. This coupling was performed for 30 min. Next, coupling of Fmoc-MeDbz-OH (5-fold excess) was performed by the same manner. Subsequent coupling of Fmoc-Ala was also performed for 1 hour using the same reagents. Coupling of following Fmoc amino acids and Boc-Cys(Trt)-OH were performed by the same manner with coupling of the first Fmoc-Gly-OH. During the

synthesis, removal of Fmoc group was performed by treatment with 20% piperidine/DMF for 2 min + 13 min.

After completion of the peptide assembly, the resin was washed with DCM and was treated with a solution of 50 mM 4-nitrophenyl chloroformate/DCM (5 mL) for 60 min. After washing with DCM, the resin was treated with 0.5 M DIEA/DMF (5 mL) for 15 min followed by washed with DMF and DCM. The peptidyl-resin was treated with the acid reagent cocktail: 95% TFA, 2.5% TIPS, 2.5% H_2O (3 mL) and the resulting solution was added over cold ether and precipitated by centrifugation. The supernatant was removed, and the residue was dissolved in 50% CH_3CN containing 0.1% TFA and lyophilized. The resulting solid was then dissolved in sodium phosphate buffer (0.1 M, pH 7.0) containing 6 M GuHCl, 0.7 M 2-aminothiazoline hydrochloride, 20 mM 2,2'-dipyridyl disulfide and acetonitrile (10% v/v). After 3 hours, purification of the solution by semipreparative HPLC (CAPCELLPAK C18 10 × 250 mm, a linear gradient from 99:1 to 75:25 of 0.1% TFA : 90% CH_3CN, 0.09% TFA over 60 min at a flow rate of 2.5 mL/min) afforded **5** (12.3 mg, 26% isolated). HRMS for **5** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{33}H_{50}N_{14}O_{11}S_3$ 915.3018; Obsd 915.3055 (monoisotopic).

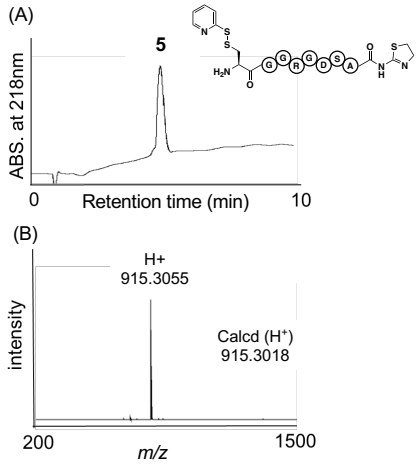


Figure S1. LCMS data for peptide-At **5**.

Synthesis of peptide-thioester (6)

The peptide-thioester **6** (sequence: CAQRRFYEALHDPNLNEEQRNAKIKSIRDDA) was synthesized manually by Boc SPPS with an improved in situ neutralization protocol.[6] Boc amino acids (Boc-Xaa-OH) utilized were Boc-Gln-OH, Boc-Ala-OH, Boc-Ile-OH, Boc-Ser(Bzl)-OH, Boc-His(DNP)-OH, Boc-Asn(Xan)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Phe-OH, Boc-Asp(OBzl)-OH, Boc-Glu(OBzl)-OH, Boc-Tyr(Br-Z)-OH, Boc-Arg(di-Z)-OH and Boc-Thz-OH. Peptides were synthesized on aminomethyl ChemMatrix resin (100 μmol). Aminomethyl ChemMatrix resin was coupled with S-trityl-3-mercaptopropionic acid[3] (4-fold excess) pre-activated with 0.38 M HBTU/DMF (3.8-fold excess) and DIEA (8-fold excess) for 1 min prior to add to the resin. This coupling reaction was performed for 2 hours. Removal of Trt group was performed by using 5% TIPS/TFA for 2 min twice. Coupling of following Boc-Xaa-OH was performed using a 4-fold excess. A solution of 0.38 M HBTU/DMF (3.8-fold excess) and DIEA (8-fold excess) was added to Boc-Xaa, which was activated for 1 min prior to add to the resin. The resin was gently shaken (ca. 200 rpm) for 20 min. Removal of Boc group was performed by using TFA for 2 min twice. After removal of the N-terminal

Boc group, the peptidyl resin was treated with TfOH/TFA/m-cresol/dimethylsulfide (1/5/1/3 (v/v), 3 mL) for 60 min on an ice bath. After removal of the acid solution by filtration, the resin was subsequently treated with TfOH/TFA/EDT/thioanisole (2/20/1/2 (v/v), 5 mL) for 2 h on an ice bath. After removal of the acid solution by filtration, the peptidyl resin was washed with DCM and DMF. The peptidyl resin was treated with 0.1 M phosphate buffer (pH 7.0) containing 6 M GuHCl and 0.5 M MESNa for 1 hour under ambient temperature twice. All filtrates were combined and subjected to semipreparative HPLC (Proteonavi C4 10 × 250 mm, a linear gradient from 85:15 to 65:35 of 0.1% TFA : 90% CH_3CN, 0.09% TFA over 90 min at a flow rate of 2.5 mL/min), afforded **6** (30.5 mg, 7.9% isolated yield). HRMS for **6** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{159}H_{255}N_{52}O_{53}S_3$ 3836.8014; Obsd 33836.8179 (deconvoluted monoisotopic).

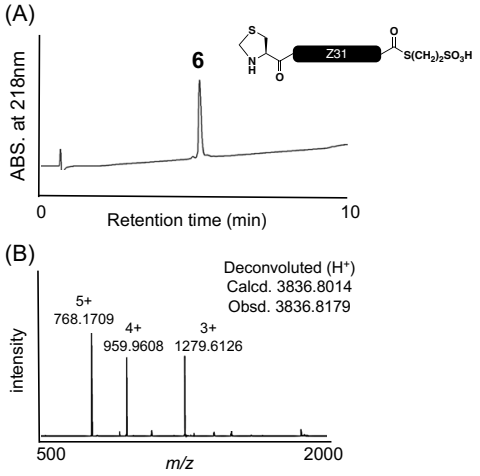


Figure S2. LCMS data for peptide-thioester **6**.

One-pot ligation for the synthesis of circular RGD protein 8

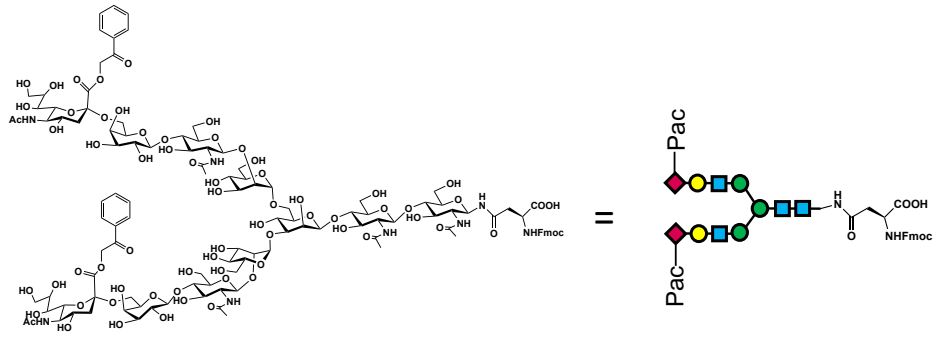
Peptide-thioester **6** (0.3 mg) and peptide-At **5** (0.15 mg) were dissolved in 0.1 M phosphate buffer (pH 7.0, 15 μL, the concentration of each peptide was 5 mM) containing 6 M GuHCl and 80 mM MPAA, 20 mM TCEP and reacted at ambient temperature (ca. 23℃). After 5 hours, to the reaction mixture was added a stock buffer solution (pH 7.0, 2 μL) containing 6 M GuHCl, 0.1 M phosphate, 620 mM $CuSO_4$ and 620 mM ascorbic acid sodium salt. After 1 hour, to the reaction mixture was added another stock aqueous solution (pH 7.0, 81 μL) containing 250 mM DTT and 300 MESNa, and reacted over night at ambient temperature. Purification of the reaction mixture by HPLC afforded **8** (0.19 mg, 56% isolated). HRMS for **8** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{181}H_{289}N_{63}O_{61}S_2$ 4386.0963; Obsd 4386.0726 (deconvoluted monoisotopic).

Synthesis of CCL1 glycoforms

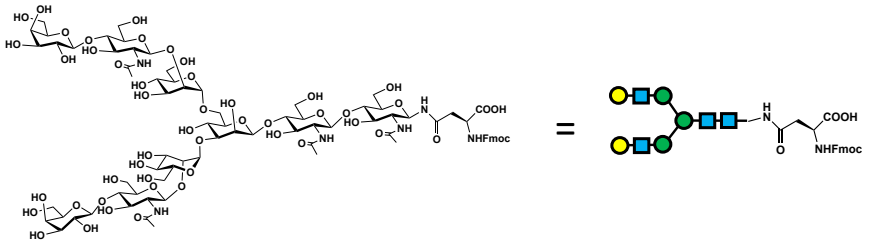
Glycan building blocks used in this study.

Glycan building blocks were prepared as a complex with an asparagine, of which amino group was protected with Fmoc group. These were prepared according to previous reports.[6-7] Structures are shown below.

Fmoc-Asn(diSia(diPac) glycan)-OH



Fmoc-Asn(diGal glycan)-OH



Fmoc-Asn(diGlcNAc glycan)-OH

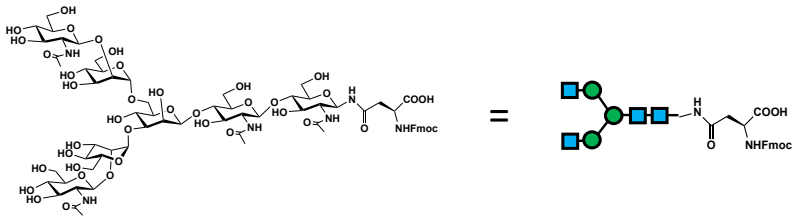


Figure S3. Structures of glycan building blocks used in this study.

Synthesis of diGal-glycopeptide-At (11)

The glycopeptide-At **11** (amino acid sequence: C(Spy)YRN(glycan)TSSI) was synthesized manually through Fmoc SPPS. Fmoc amino acids used were Fmoc-Gly-OH, Fmoc-Arg(pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH and Fmoc-Ala-OH. The N-terminal Cys residue was coupled by using Boc-Cys(Trt)-OH. Peptides were synthesized on amino-PEGA resin (40 μmol) equipped with Dbz as a linker according to the protocol reported in our previous study.[8] First, amino-PEGA resin was coupled with Fmoc-Dbz-OH (4-fold excess) pre-activated with 0.2 M HBTU·HOBt/DMF (800 μL, 4-fold excess) and 0.2 M DIEA/DMF (1.2 mL, 6-fold excess) for 3 min. This coupling reaction was performed for 40 min twice. Then, the Dbz linked resin was coupled with Fmoc-Ile (4-fold excess), which was pre-activated with HATU (4-fold excess) and DIEA (6-fold excess) in DMF (800 μL) for 2 min. This coupling reaction was performed for 1 hour twice.

The following coupling of Fmoc-Ser(tBu)-OH and Fmoc-Thr-(tBu)-OH were performed as single coupling by the same conditions of coupling of Fmoc-Dbz-OH. The peptidyl resin was then coupled with Fmoc-Asn(diGal glycan)-OH (1.5-fold excess)[7] using PyBop (4.5-fold excess), DIEA(4.5-fold excess) in DMSO/NMP (1:4 v/v, 2.0 mL) overnight (ca. 15 h). Coupling of following amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH and Boc-Cys(Trt)-OH) were performed using a diluted reaction condition as follows. Amino acid derivatives (5-fold excess) were preactivated with DIC (5-fold excess) and HOBt (5-fold excess) dissolved in DMF (5.0 mL, 40 mM for amino acid derivatives) for 10 min. The activated amino acid solution was added to the resin and reacted for 1 h. During the synthesis, removal of Fmoc group was performed by treatment with 20% piperidine/DMF (ca. 5 mL) for 2 min + 13 min.

After the completion of peptide assembly, the resin was washed with DCM and was treated with 50 mM 4-nitrophenyl chloroformate/DCM (4 mL) for 40 min twice. After washing with DCM, 0.5 M DIEA/DMF (5 ml) was added to the resin and reacted for 15 min. The resin was washed with DMF and DCM. The peptidyl resin was treated with an acid reagent cocktail: 95% TFA, 2.5% TIPS, 2.5% H_2O for 2 h. After removal of the acid solution by filtration, the peptidyl resin was washed with DCM and DMF. To the peptidyl resin was added sodium phosphate buffer (0.2 M, pH 7.5, 2.0 mL) containing 6 M GuHCl, 1 M 2-aminothiazoline hydrochloride, 20 mM 2,2'-dipyridyl disulfide and acetonitrile (10% v/v) for 3 h three times. All filtrates were combined and subjected to the purification by semipreparative HPLC (Proteonavi C4 1.0 × 250 mm, a linear gradient from 95:5 to 75:25 of 0.1% TFA : 90% CH_3CN, 0.09% TFA over 90 min at a flow rate of 2.5 mL/min), afforded **11** (18.4 mg, 16.6% isolated). HRMS for **11** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{108}H_{172}N_{19}O_{58}S_3$ 2759.0250; Obsd 2759.0177 (monoisotopic).

Synthesis of diGlcNAc-glycopeptide-At S1

DiGlcNAc-glycopeptide-At (**S1**) was synthesized through Fmoc SPPS using Fmoc-Asn(diGlcNAc-glycan)-OH[7] by essentially the same manner with the synthesis of **11.** Purification by semipreparative HPLC (Proteonavi C4 1.0 × 250 mm, a linear gradient from 95:5 to 75:25 of 0.1% TFA : 90% CH_3CN, 0.09% TFA over 90 min at a flow rate of 2.5 mL/min), afforded (4.0 mg, 16.4% isolated from 10 μmol scale synthesis). HRMS for **S1** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{96}H_{152}N_{19}O_{48}S_3$ 2434.9194; Obsd 2434.9094 (monoisotopic).

Synthesis of diSia-glycopeptide-At S2

The diSia-glycopeptide-At (**S2**) was synthesized using Fmoc-Asn(diSia(diPac)-glycan[6] by the same manner with the synthesis of **11** and **S1** except for the removal of Fmoc group after coupling of Fmoc-Asn(diSia(diPac)-glycan).[6] Removal of Fmoc groups after the Asn(diSia(diPac)-glycan) residue was performed by treatment with the cocktail: 2% DBU, 2% HOBt, 96% DMF (v/w/v) for 5 min + 25 min to avoid removal of the Pac ester groups of sialic acid residues during SPPS. Purification by semipreparative HPLC (Proteonavi C4 1.0 × 250 mm, a linear gradient from 90:10 to 70:30 of 0.1 % TFA : 90 % CH_3CN, 0.09 % TFA over 90 min at a flow rate of 2.5 mL/min) afforded **S2** (4.6 mg, 6.5% yield from 20 μmol scale synthesis). HRMS for **S2** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{146}H_{218}N_{21}O_{76}S_3$ 3577.2996; Obsd 3577.2811 (monoisotopic).

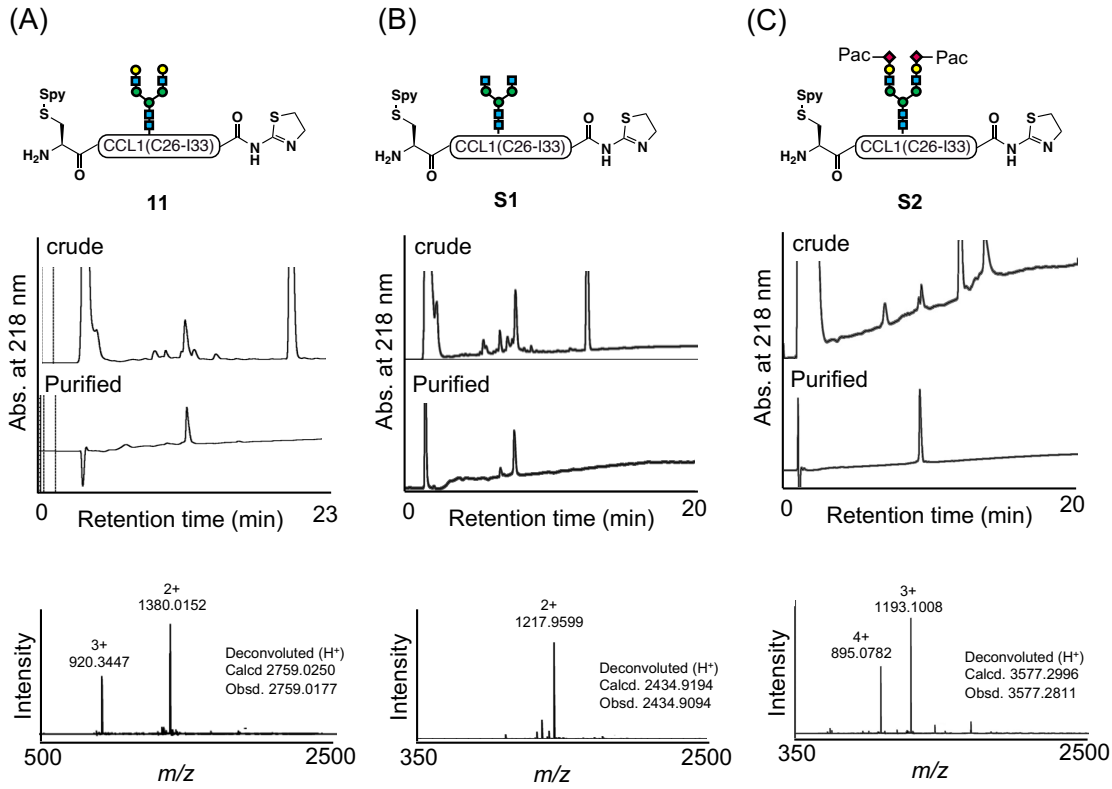


Figure S4. Analytical LCMS data for glycopeptide-At **11**, **S1**, and **S2**.

One-pot Ligation: Synthesis of CCL1(Lys1-Asn29(diGal-glycan)-Lys73) 14

Peptide-thioester **10** (3.3 mg, 1.1 μmol, amino acid sequence: KSMQVPFSRCCFSFAEQEIPLRAIL),[9] and diGal-glycopeptide-At **11** (3.0 mg, 1.1 μmol) were dissolved in a phosphate buffer (0.2 M, pH 7.0, 54 μl) containing 6 M GuHCl, 20 mM MPAA and 100 mM TCEP. After 4.5 hours, to the solution was added the C-terminal peptide **13** (6.1 mg, 1.3 μmol, amino acid sequence: CSNEGLIFKLKRGKEACALDTVGWVQRHRKMLRHCPSKRK)[8] dissolved in a phosphate buffer (0.2 M, pH 7.0, 54 μl) containing 6 M GuHCl, 400 mM MPAA and 200 mM TCEP to achieve a final concentration 210 mM MPAA and 150 mM TCEP. After additional 72 hours, purification of the reaction mixture by HPLC afforded CCL1(Lys1-Asn29(diGal-glycan)-Lys73) **14** (2.5 mg, 23%). ESI-MS (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{432}H_{707}N_{118}O_{144}S_8$ 10114.0; Obsd 10113.8 (deconvoluted most intense isotope peak). HRMS (ESI-Orbitrap) *m/z:* $[M+9H]^{9+}$ calcd for $C_{432}H_{715}N_{118}O_{144}S_8$ 1123.9997; Obsd 1123.9990 (monoisotopic).

One-pot Ligation: Synthesis of CCL1(Lys1-Asn29(diSia-glycan)-Lys73) S3

One-pot ligation was performed by the same manner with the the synthesis of **14** using the N-terminal peptide-thioester **10** (2.5 mg, 0.8 μmol), diSia-glycopeptide-At **S2** (3.0 mg, 0.8 μmol) and C-terminal peptide **13** (5.9 mg, 1.3 μmol). Each ligation reaction was completed within 22 hours for the first ligation and 94 hours for the second ligation. Purification of the reaction mixture by HPLC afforded CCL1(Lys1-Asn29(diSia-glycan)-Lys73) **S3** (3.3 mg, 37%). ESIMS (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for

$C_{454}H_{747}N_{120}O_{160}S_8$ 10696.1; Obsd 10696.3 (deconvoluted most intense isotope peak). HRMS (ESI-Orbitrap) *m/z:* $[M+9H]^+$ calcd for $C_{454}H_{755}N_{120}O_{160}S_8$ 1188.6875; Obsd 1188.6886.

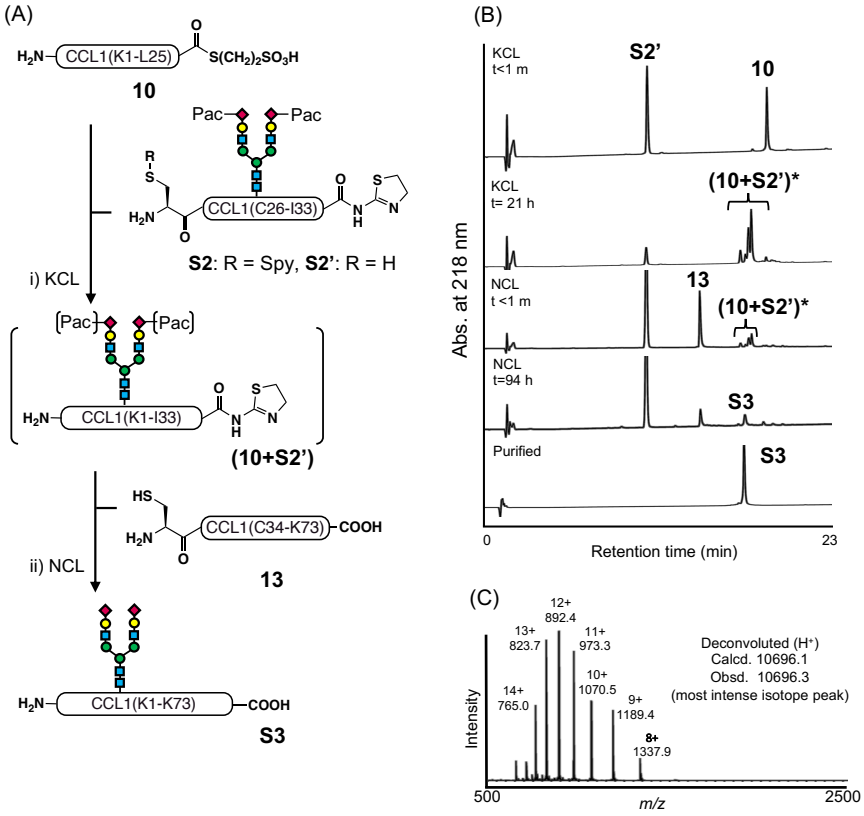


Figure S5. One-pot ligation for CCL1 glycoform having a diSia glycan. (A) Synthetic scheme. Spy group of **S2** was removed by a reducing agent TCEP after dissolving in the ligation buffer, affording **S2'**. (B) Analytical LC data and (C) MS from on-line Q-TOF ESI-MS of purified **S3.** *The Pac esters on sialic acid residues were deprotected during one-pot ligation reaction; After KCL reaction, the ligated product (**10**+**S2'**) afforded multiple LC peaks including mono-Pac, di-Pac and no Pac derivatives. These Pac groups were fully removed during the subsequent NCL reaction.

One-pot Ligation: Synthesis of CCL1(Lys1-Asn29(diGlcNAc-glycan)-Lys73) S4

One-pot ligation was performed by the same manner with the the synthesis of **14** using peptide-thioester **10** (1.8 mg, 0.59 μmol), diGlcNAc-glycopeptide-At **S1** (1.4 mg, 0.59 μmol) and peptide **13** (3.3 mg, 0.70 μmol). Each ligation reaction was completed within 9 hours for the first ligation (KCL) and 70 hours for the second ligation (NCL). Purification of the reaction mixture by HPLC afforded the desired glycosylated polypeptide CCL1(Lys1-Asn29(diGlcNAc-glycan)-Lys73) **S4** (1.6 mg, 29%). ESI-MS (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{420}H_{687}N_{118}O_{134}S_8$ 9789.8; Obsd 9789.7. HRMS (ESI-Orbitrap) *m/z:* $[M+9H]^{9+}$calcd for $C_{420}H_{695}N_{118}O_{134}S_8$ 1087.9879; Obsd 1087.9878.

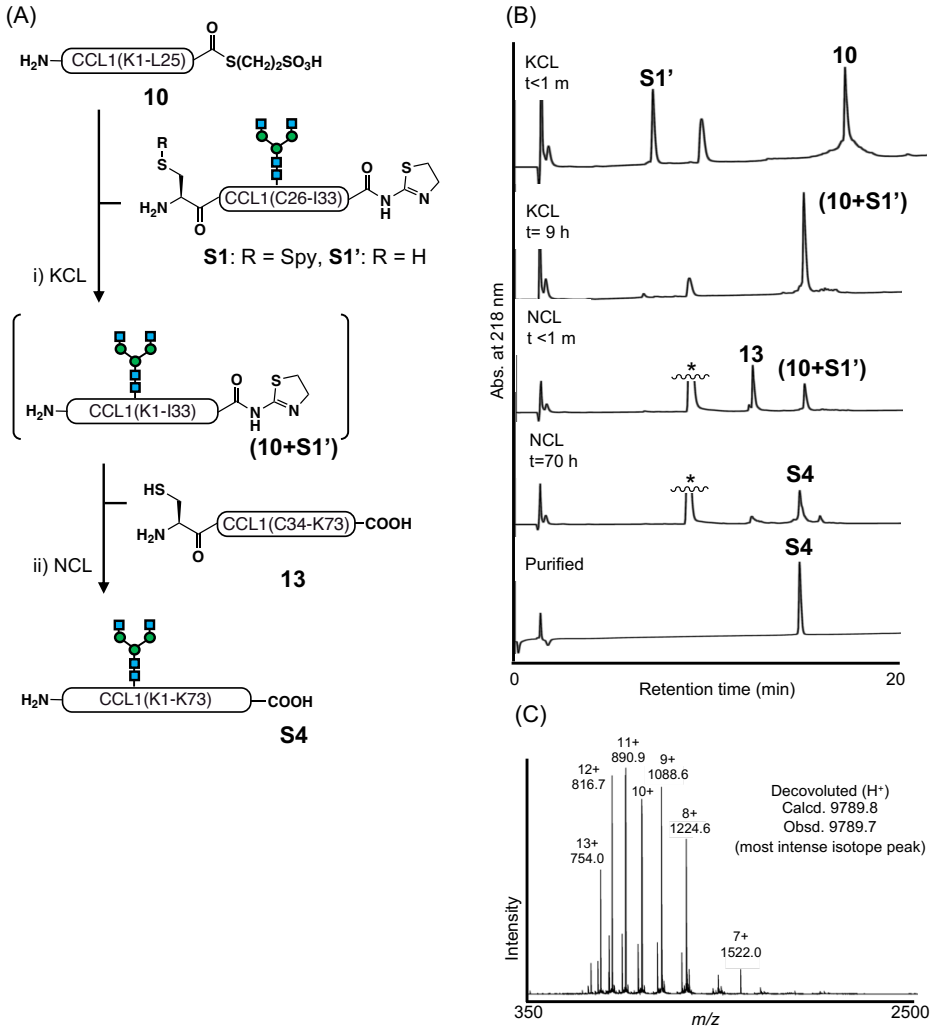


Figure S6. One-pot ligation for CCL1 glycoform having a diGlcNAc glycan. (A) Synthetic scheme. Spy group of **S1** was removed by a reducing agent TCEP after dissolving in the ligation buffer, affording **S1'**. (B) Analytical LC data and (C) MS from on-line Q-TOF ESI-MS of purified **S4.**

A typical procedure for the folding

The full length of glycopolypeptide of CCL1 **14** (2.5 mg) was dissolved in 6 M Gu-HCl solution (0.84 mL) followed by the addition of H_2O (0.84 mL) and the folding buffer:150 mM Tris-HCl containing 12 mM cysteine and 1.5 mM cystine (pH 8.0, 3.36 mL) to give a final folding reaction conditions of a glycopeptide solution (0.5 mg/mL) dissolved in 1 M GuHCl, 100 mM Tris, 8 mM cysteine, 1 mM cystine. After 5 h, purification of the reaction mixture by preparative LC afforded the diGal-CCL1 **15** (1.3 mg, 40%). By essentially the same manner, CCL1(Lys1-Asn29(diSia-glycan)-Lys73) **S3** (3.3 mg) afforded **16** (1.3 mg. 40%), and CCL1(Lys1-Asn29(diGlcNAc-glycan)-Lys73) **S4** (1.6 mg) afforded **17** (0.7 mg, 40%) respectively.

ESIMS for **15** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{432}H_{701}N_{118}O_{144}S_8$ 10107.9; Obsd 10107.6 (deconvoluted most intense isotope). HRMS for **15** (ESI-Orbitrap) *m/z:* $[M+9H]^{9+}$ calcd for $C_{432}H_{710}N_{118}O_{144}S_8$ 1123.3278; Obsd 1123.3269.

ESIMS for **16** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{454}H_{741}N_{120}O_{160}S_8$ 10690.1; Obsd 10690.1 (deconvoluted most intense isotope). HRMS for **16** (ESI-Orbitrap) *m/z:* $[M+9H]^+$ calcd for $C_{454}H_{750}N_{120}O_{160}S_8$ 1188.0157; Obsd 1188.0179.

ESIMS for **17** (ESI-QTOF) *m/z:* $[M+H]^+$ calcd for $C_{420}H_{681}N_{118}O_{134}S_8$ 9783.8; Obsd 9783.9 (deconvoluted most intense isotope). HRMS for **17** (ESI-Orbitrap) *m/z:* $[M+9H]^{9+}$calcd for $C_{420}H_{689}N_{118}O_{134}S_8$ 1087.3160; Obsd 1087.3162

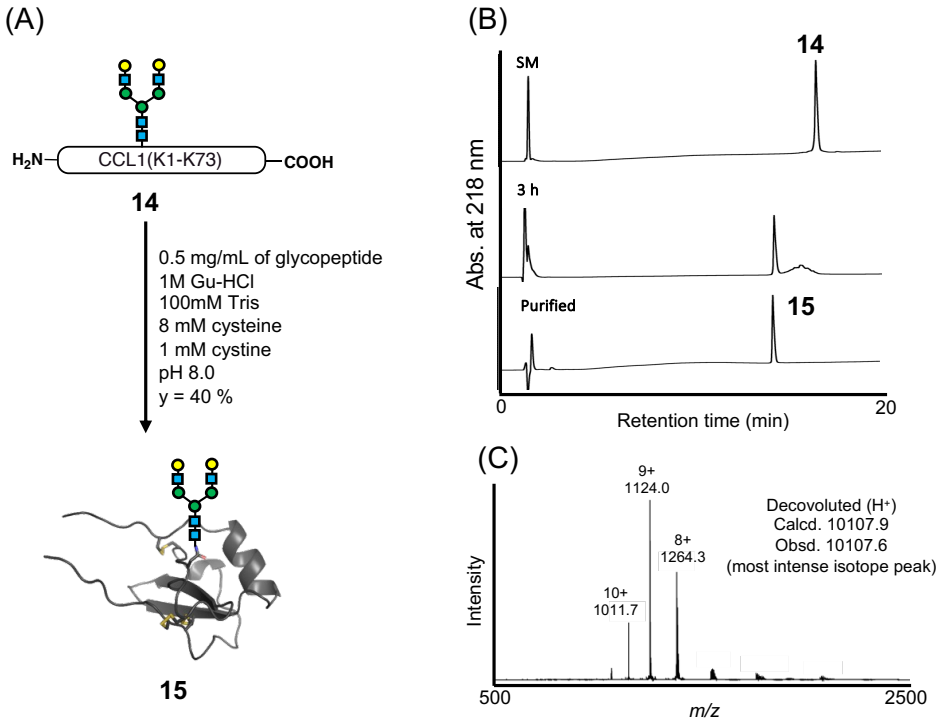


Figure S7. Folding of CCL1 glycoform having diGal-glycan. (A) Scheme for folding reaction, (B) analytical LC data, and (c) MS from on-line Q-TOF ESI-MS of purified **15**.

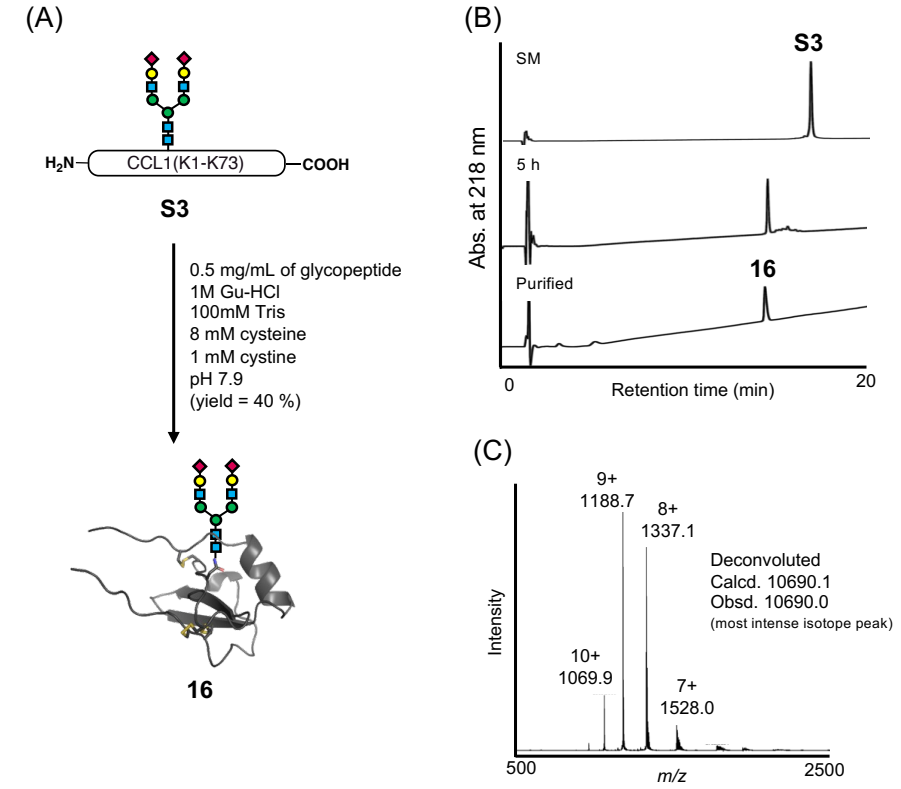


Figure S8. Folding of CCL1 glycoform having diSia-glycan. (A) Scheme for folding reaction, (B) analytical LC data, and (c) MS from on-line Q-TOF ESI-MS of purified **16**.

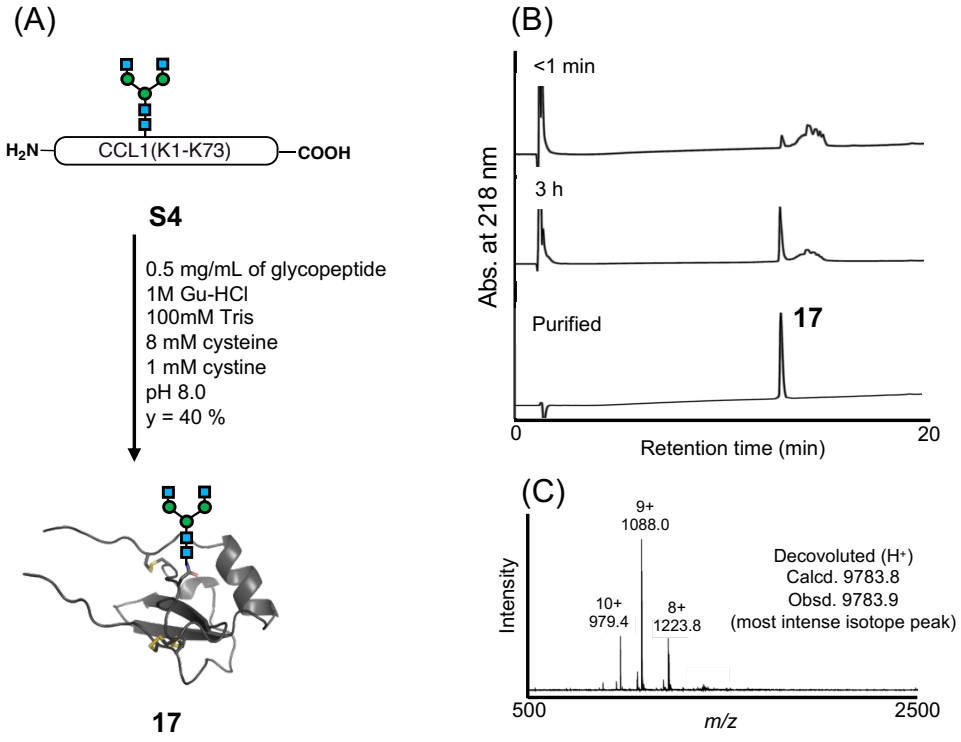


Figure S9. Folding of CCL1 glycoform having diGlcNAc-glycan. (A) Scheme for folding reaction, (B) analytical LC data, and (c) MS from on-line Q-TOF ESI-MS of purified **17**.

One-pot ligation and folding.

The N-terminal peptide-thioester **10** (4.5 mg, 1.5 μmol) and asialoglycosylated peptide-At **11** (4.1 mg, 1.5 μmol) was dissolved in a phosphate buffer (0.2 M, pH 7.3, 75 μL) containing 6 M GuHCl, 20 mM MPAA and 100 mM TCEP. After 14 h, to the solution was added the C-terminal Cys-peptide **13** (10.3 mg, 2.3 μmol) dissolved in a phosphate buffer (0.2 M, pH 7.1) containing 6 M GuHCl, 400 mM MPAA and 200 mM TCEP (300 μL). After 6 days, the reaction mixture was diluted with Tris-HCl buffer (100 mM, pH 7.5) containing 6 M GuHCl and poured into the dialysis tubing (MWCO at 3,500 Spectra/Por®). The micture was subsequently dialyzed against the first external buffer (3 M GuHCl, 100 mM Tris-HCl, pH 8.5) containing 4 mM cysteine and 0.5 mM cystine for 21 h at 4°C. Then, the external buffer solution was replaced by the second folding buffer solution (1 M GuHCl, 100 mM Tris-HCl, pH 8.0) and dialysis was performed for 13 h. Finally, the external buffer was replaced by the third buffer solution (10 mM Tris-HCl, pH 7.0) and dialysis was performed for 16 h. Purification of the resulting mixture by semipreparative LC afforded diGal-CCL1 **15** (2.3 mg, 17% overall yield).

Functional analysis of synthetic proteins

Circular dichroism (CD) analysis of circular RGD protein 8.

Circular dichroism analysis was performed using JASCO J-805 spectrometer. The synthetic circular RGD protein 8 was prepared at a concentration of 0.25 mg.mL in H_2O. CD spectrum was recorded at 25 ºC in a 0.1 cm path length quartz cuvette.

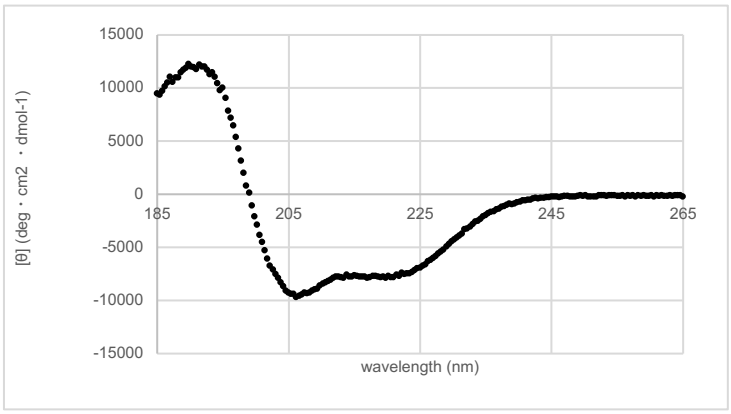


Figure S10. The CD spectrum of circular RGD protein **8**.

Evaluation of the inhibition activity of circular RGD protein 8

To the suspension of HeLa cells, labelled with calcein AM (Cellstain®, DOJINDO, Japan), in D-PBS(+) (270 µL) was added circular RGD protein **8** (30 µL for each) dissolved in water at a concentration of 1000, 100, 10 and 1 µM, achieving 100, 10, 1, 0.1 µM as a final concentration of compound **8**. A 100 µL/well (n=3) of the resultant suspension containing HeLa cells and synthetic compound was added to the fibronectin coated 96 wells (BioCoat™) and incubated for 30 min. Non-adherent cells were removed by suctioning, and each well was gently washed with 250 µL of D-PBS (+) 5 times. The absorbance (λex = 490 nm, λem = 515 nm) of Calcein in HeLa cells attached to fibronectin on wells was measured with a microplate reader (BioTek Cytation5, Agilent, Japan). The inhibition activity of synthetic compounds was evaluated as the percentage of attached cells, (absorbance of attachment cells in the well/absorbance of total cells added to the well) x 100.

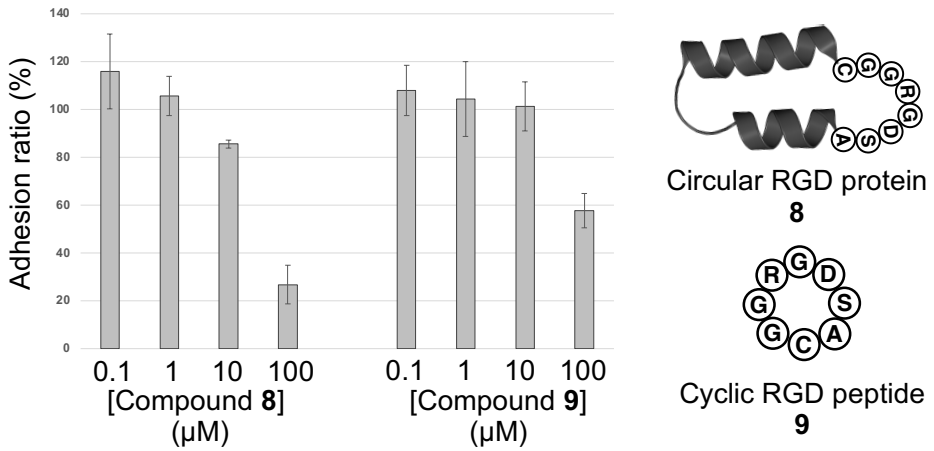


Figure S11. Result for the inhibition assay for cell-adhesion using synthetic **8** and its control cyclic peptide **9**. Inhibition activity for **8** or **9** increased according to their concentrations.

Calcium flux assay for CCL1 glycoforms

THP-1 cells (1.5 x 10^6 cells) in 150 µL of PBS with 1% FBS, were labeled for 30 min at 37˚C with 4 µM Fluo-8 acetoxylmethylester (AAT Bioquest). After washing with PBS twice, cells were diluted in 1 mL of PBS containing 10 mM HEPES, and were left for one hour at 37˚C in the dark. Before analysis, Aliquot 135 µL of cell solution into a 96-well plate, the Fluo-8 fluorescence intensity was measured for 1 min (490/525) by the imaging plate reader (BioTek Cytation 5, Agilent). 15 µL of each synthesized CCL1 or commercial CCL1 were added at final concentration of 1 x 10^{-12} M, Fluo-8 fluorescence intensity was measured for 660 second.

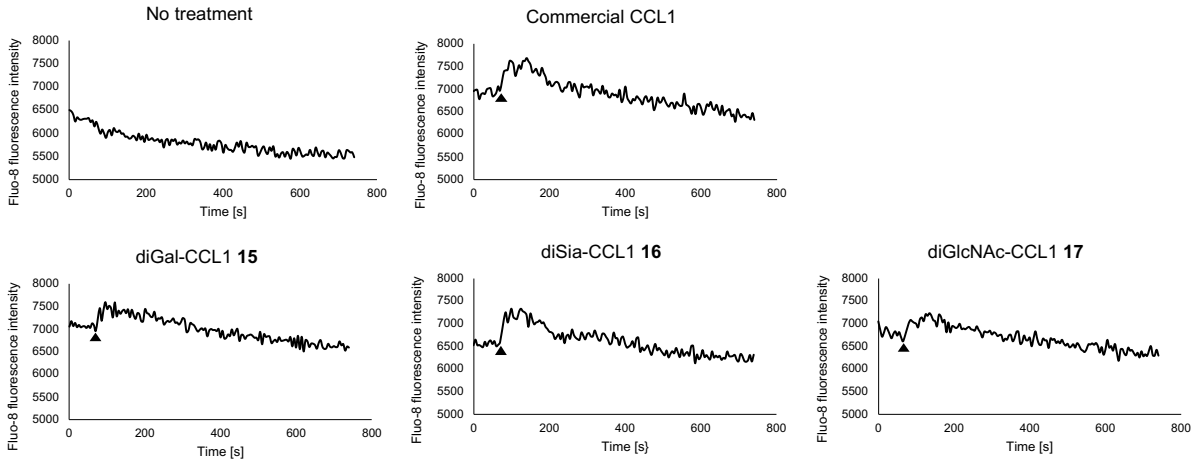


Figure S12. Calcium flux assay for CCL1 glycoforms. Each CCL1 derivative was added at the time indicated by a black triangle.

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