Supporting Information

Design and Synthesis of Monobody Variants with Low Immunogenicity

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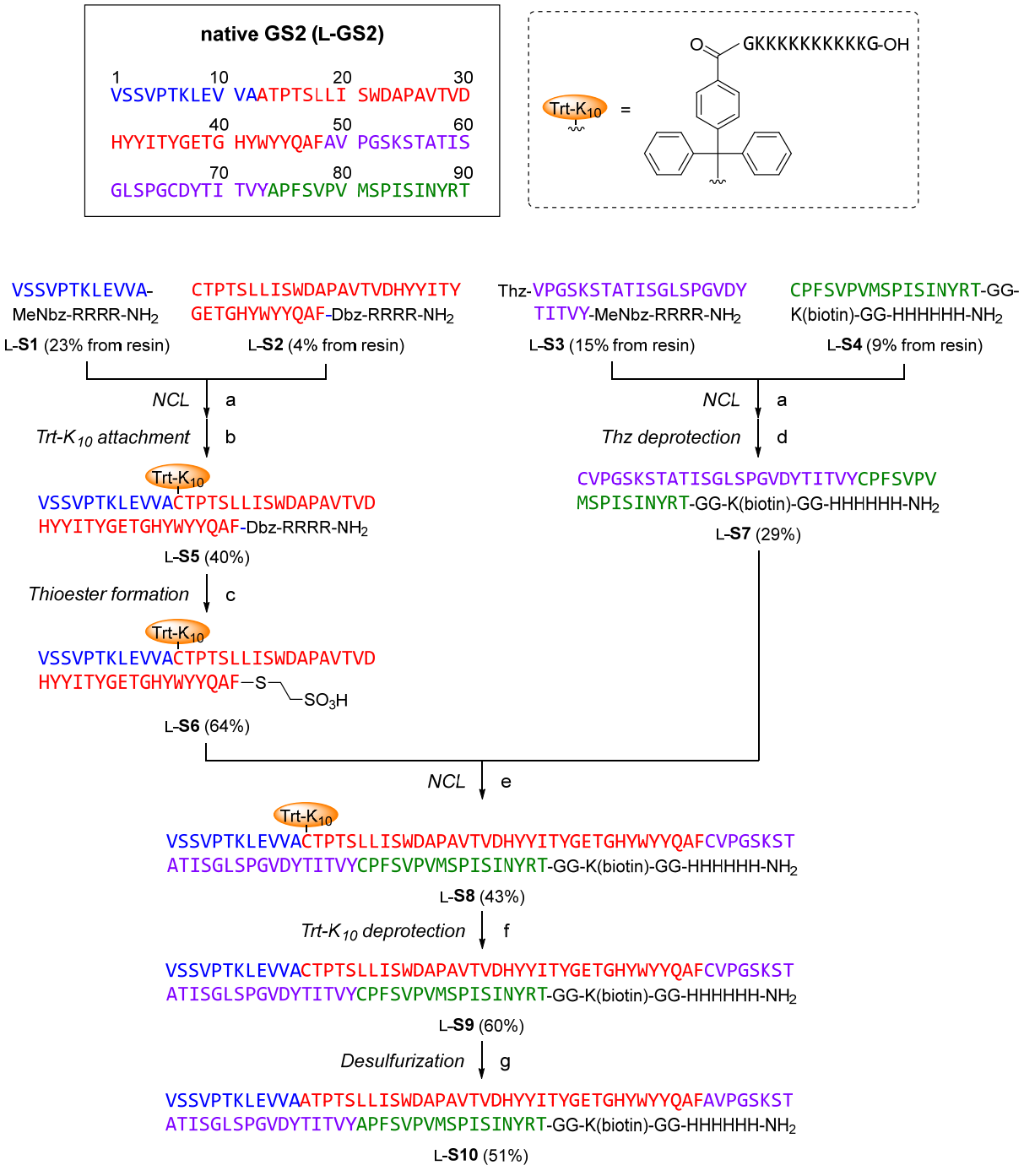
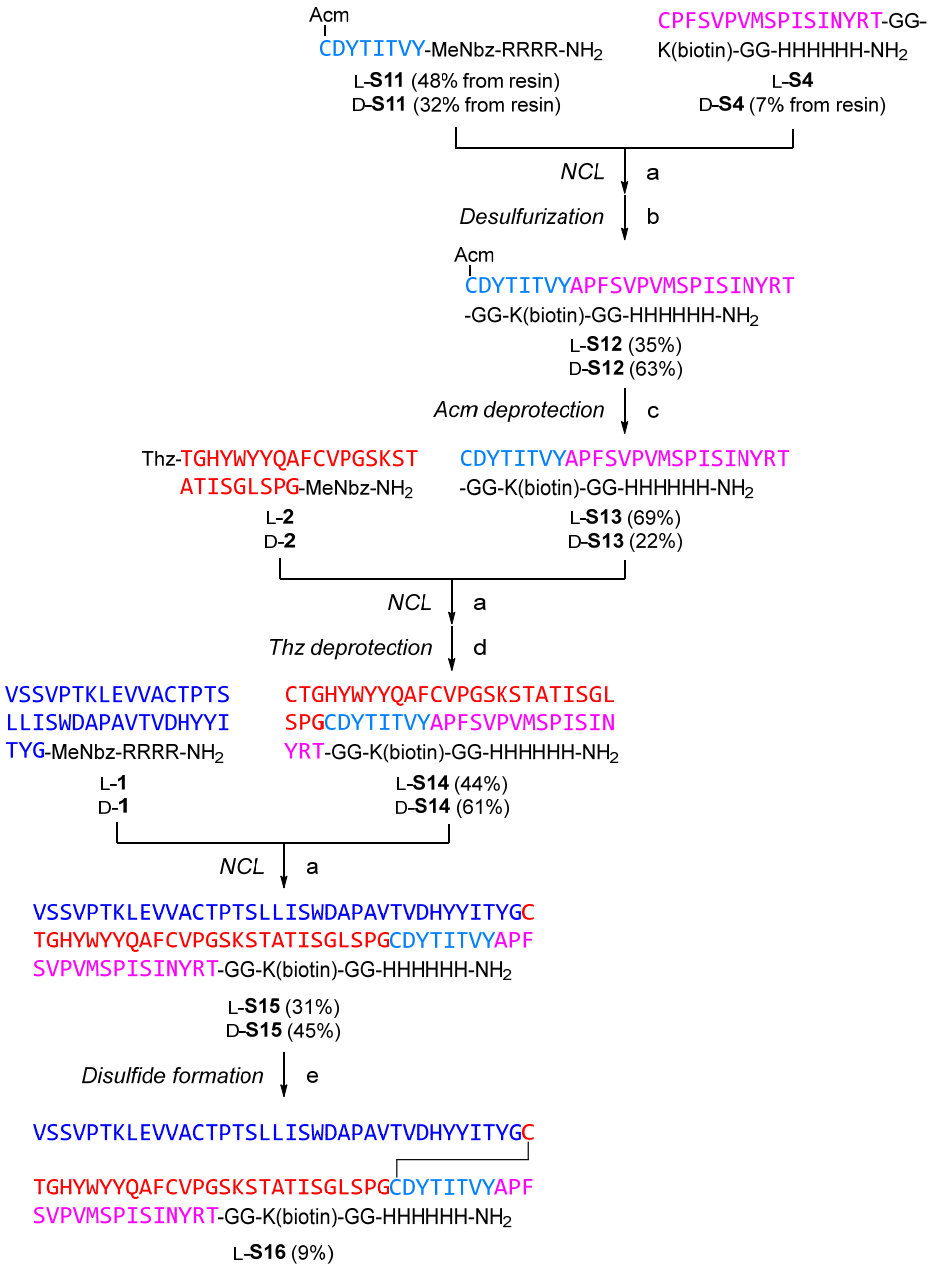


Scheme S1. Synthesis of biotinylated native GS2 [L-GS2^{biotin} (L-**S10**)]. *Reagents and conditions*: (a) 1,2,4-triazole, TCEP, 6 M guanidine (pH 7.0); (b) Trt(OH)-K_{10},[S1] TFA; (c) $NaNO_2$, 6 M guanidine, phosphate buffer (pH 3.0); and then MPAA, TCEP (pH 6.0); (d) methoxyamine; (e) MPAA, TCEP, 6 M guanidine, phosphate buffer (pH 6.6); (f) TFA/EDT (95:5); (g) VA-044, MESNa, TCEP, 6 M guanidine, phosphate buffer (pH 6.5).[S2]



Scheme S2. Synthesis of biotinylated L/D-mGS2 and L-mGS2^{SS} [L/D-mGS2^{biotin} (L/D-**S15**), L-mGS2$^{SS/biotin}$ (L-**S16**)]. *Reagents and conditions*: (a) 1,2,4-triazole, TCEP, 6 M guanidine (pH 7.0); (b) VA-044, MESNa, TCEP, 6 M guanidine, phosphate buffer (pH 6.5); (c) AgOTf, anisole, TFA;[S3] and then 10 mM DTT, 6 M guanidine, phosphate buffer (pH 7.4); (d) methoxyamine; (e) 2-PDS, 6 M guanidine, Tris buffer (pH 8.0).

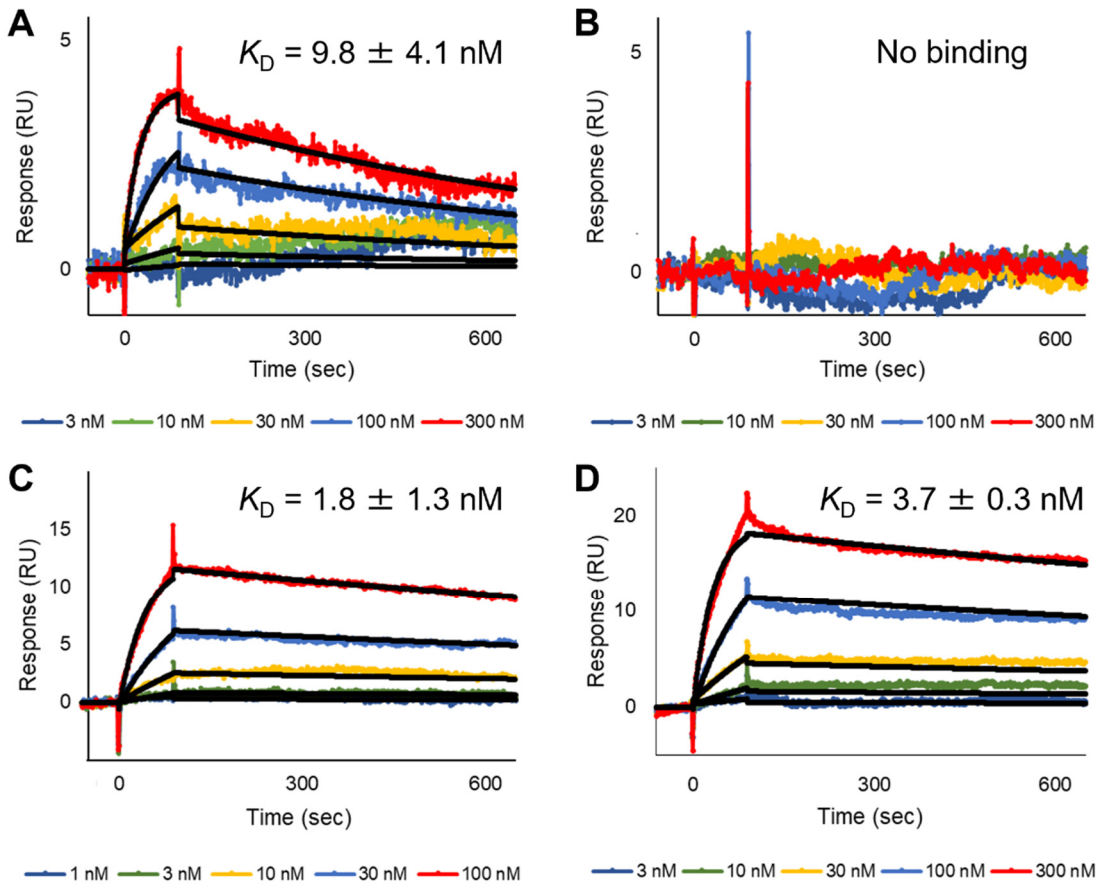


Figure S1. The binding affinity of synthetic monobody proteins toward GST-EGFP by SPR analysis. (A) L-mGS2^{biotin}, (B) D-mGS2^{bitoin}, (C) L-GS2^{biotin} and (D) L-mGS2$^{SS/biotin}$. Binding affinity was determined from triplicate assays.

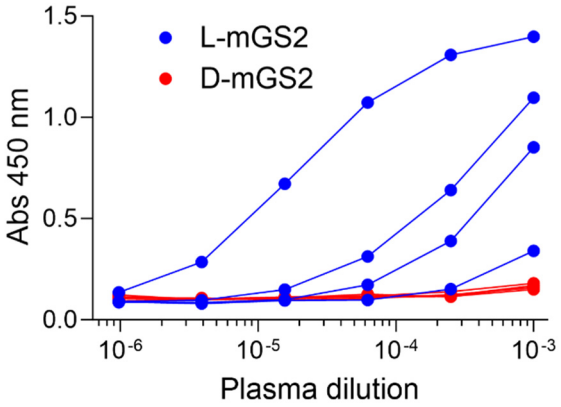
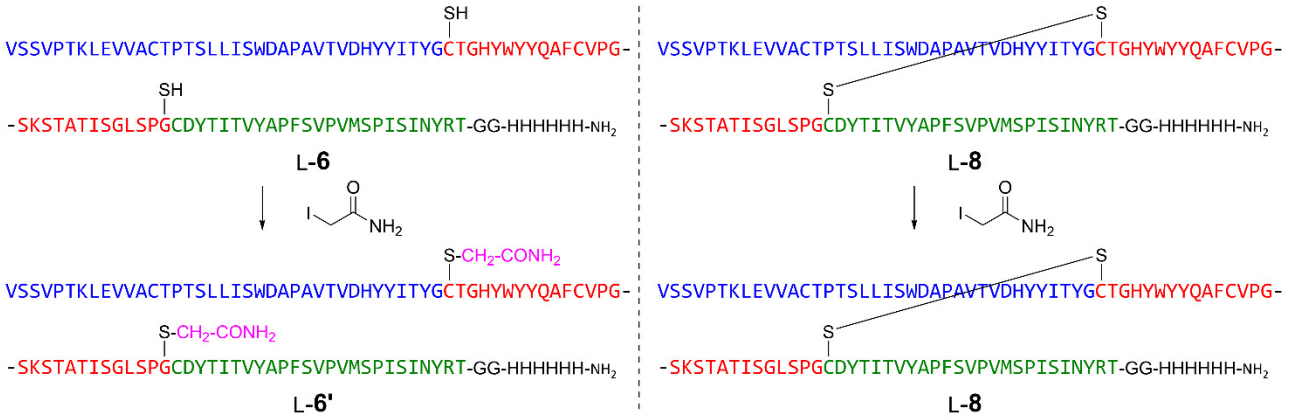


Figure S2. ADA production in mouse plasma at day 44 after injection of L-mGS2 and D-mGS2. ADA in serially diluted plasma was detected by ELISA. Each line connects the data of individual mice. Absorbance of 3,3',5,5'-tetramethylbenzidine (TMB) was measured at 450 nm.



Peptide	Formula	Calcd	Observed *m/z*
L-**6**	$C_{489}H_{718}N_{124}O_{139}S_3$	10647.21	$[M+7H]^{7+}$ = 1522.50, $[M+8H]^{8+}$ = 1332.29
L-**6'**	$C_{493}H_{724}N_{126}O_{141}S_3$	10761.25	$[M+7H]^{7+}$ = 1538.63, $[M+8H]^{8+}$ = 1346.55
L-**8**	$C_{489}H_{716}N_{124}O_{139}S_3$	10645.19	$[M+7H]^{7+}$ = 1522.31, $[M+8H]^{8+}$ = 1332.11
L-**8** after treatment	$C_{489}H_{716}N_{124}O_{139}S_3$	10645.19	$[M+7H]^{7+}$ = 1522.67, $[M+8H]^{8+}$ = 1331.63

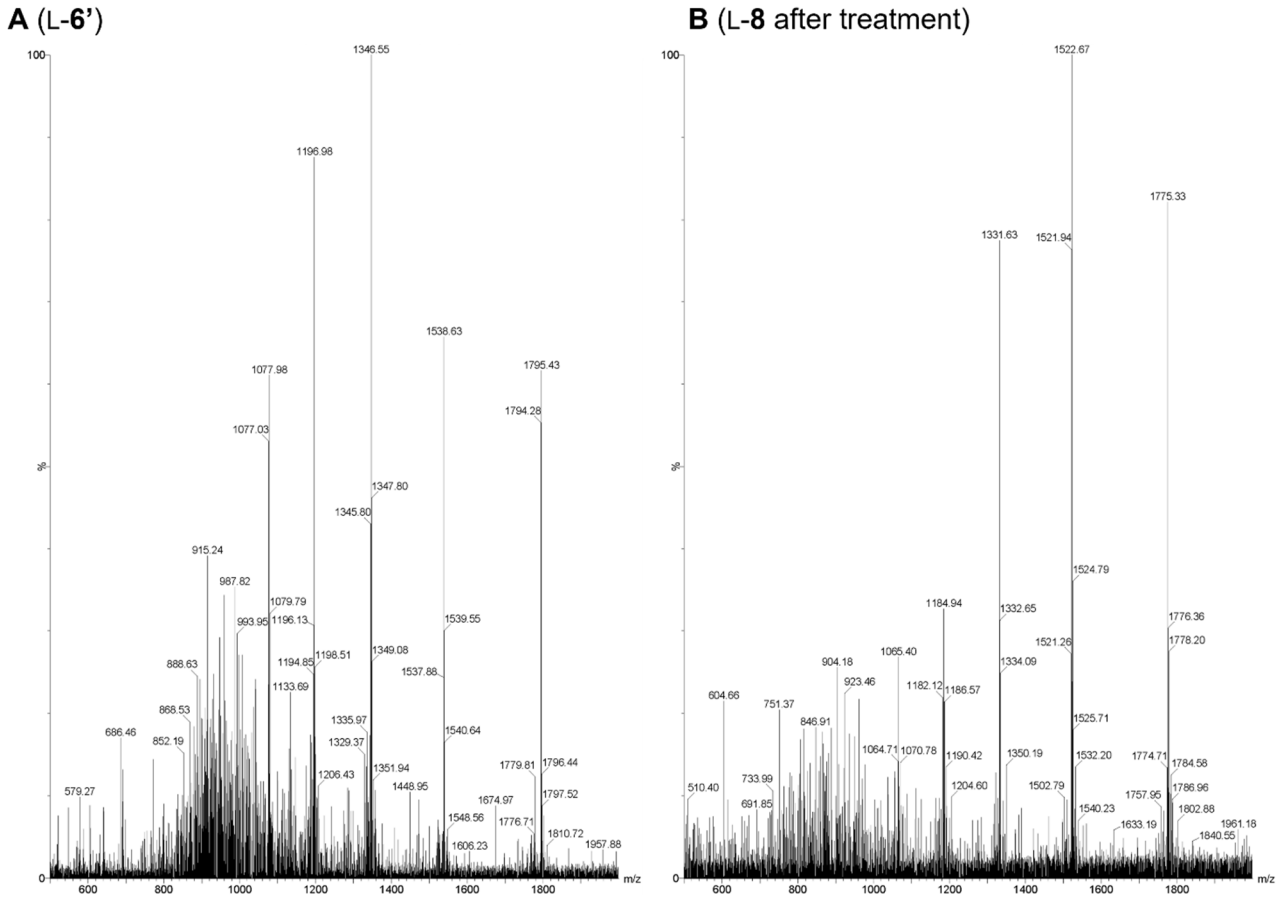


Figure S3. Confirmation of disulfide bond formation in L-mGS2 and L-mGS2^{SS}. After treating peptides L-mGS2 (L-**6**) or L-mGS2^{SS} (L-**8**) with iodoacetamide, the products were analyzed by LC-MS. (A) Mass spectrum of the product from L-**6** (L-**6'**). (B) Mass spectrum of the product from L-**8**.

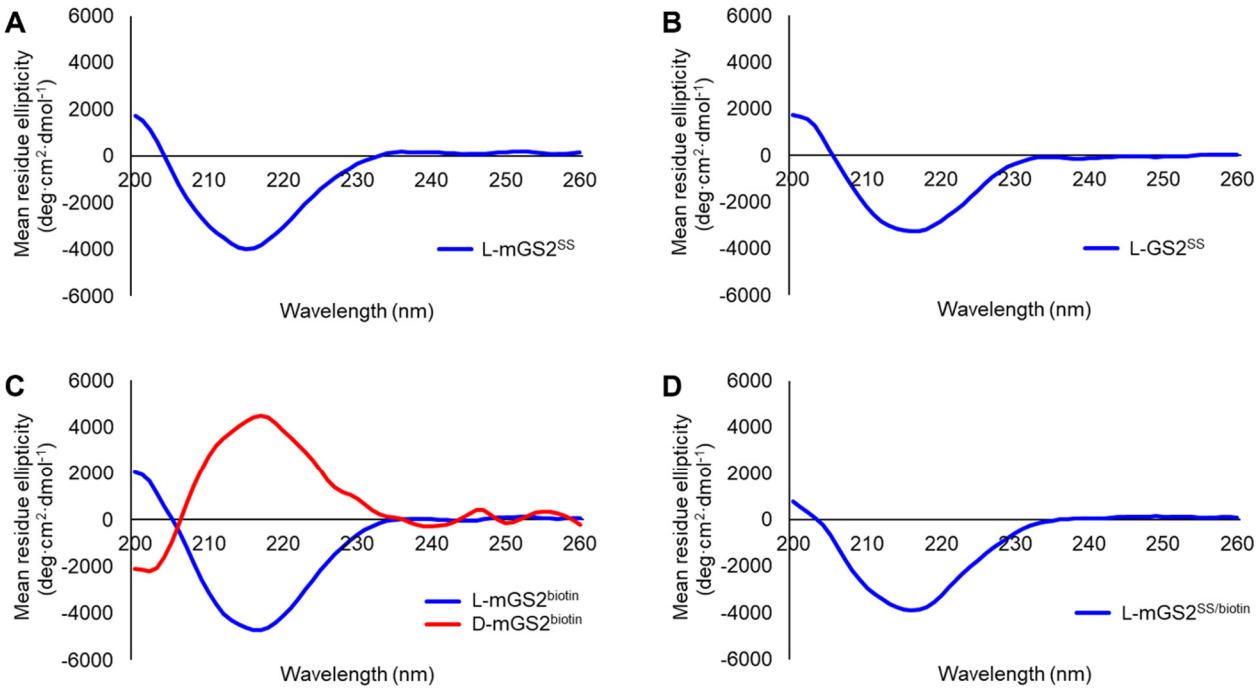


Figure S4. CD spectra of synthetic monobody proteins. (A) L-mGS2^{SS}, (B) L-GS2^{biotin}, (C) L/D-mGS2^{biotin} and (D) L-mGS2$^{SS/biotin}$.

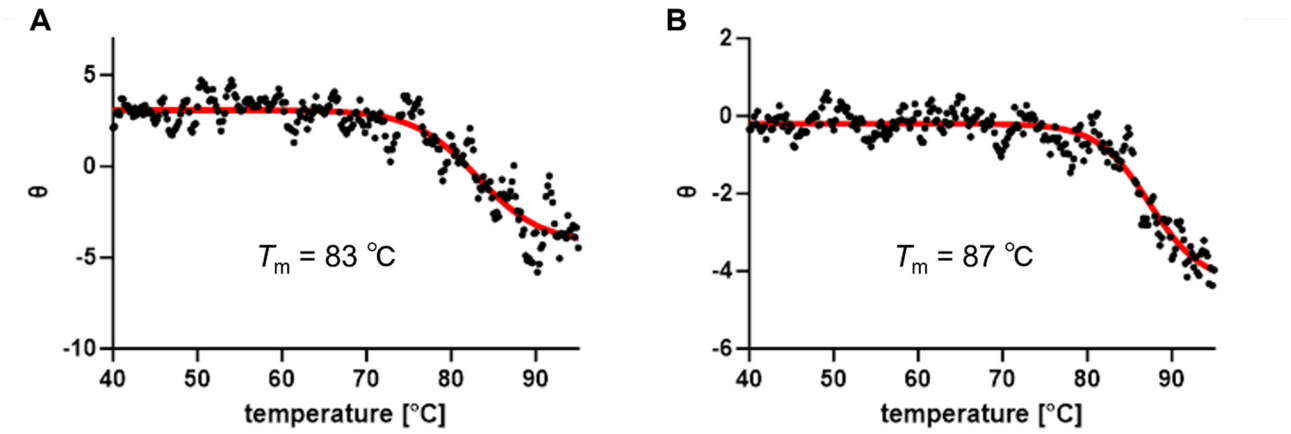


Figure S5. Thermal stability of (A) L-mGS2 and (B) L-mGS2^{SS}. The thermal unfolding transition was monitored by the CD signal at 203 nm. Data were smoothed with centered moving averages. The T_m of the unfolding curves was determined by data fitting to the Boltzmann sigmoidal curve using GraphPad Prism10.

Experimental procedures

During all the experiments in this study, no unexpected or usually high safety hazards were encountered.

Peptide Synthesis. All reagents and solvents were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), Kokusan Chemical Industries, Ltd. (Kanagawa, Japan), Sigma-Aldrich JAPAN (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) or Nacalai Tesque, Inc. (Kyoto, Japan). All peptide segments were prepared by standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) using an automatic peptide synthesizer (PSSM-8, Shimadzu) unless otherwise stated. For side-chain protection, *t*-Bu ester was used for Asp and Glu; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) was used for Arg; *t*-Bu was used for Ser, Thr and Tyr; Boc was used for Lys; and Trt was used for Gln, Asn, His and Cys. In automatic peptide synthesis, Fmoc-amino acids were coupled using OxymaPure (5 equiv)/DIC (5 equiv) or HBTU (5 equiv)/HOBt·H_2O (5 equiv)/DIEA (10 equiv) in DMF for 60 min twice and Fmoc-deprotection was performed by 20% piperidine in DMF for 4 min twice. The resulting protected peptide resin was treated with TFA/H_2O/*m*-cresol/thioanisole/EDT (80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold Et_2O. The resulting powder was collected by centrifugation and washed with ice-cold dry Et_2O. Crude peptide segments were purified by preparative HPLC on a Cosmosil 5C18-AR300 preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 8 mL/min) or a Cosmosil 5C4-AR300 preparative column (Nacalai Tesque, 20 × 150 mm, flow rate 8 mL/min). For analytical HPLC of peptide segments, a Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm) or a Cosmosil 5C4-AR300 column (Nacalai Tesque, 4.6 × 150 mm) was employed with a linear gradient of CH_3CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min. All peptides were characterized by ESI-MS [micromass ZQ (Waters), LCMS-2020 (Shimadzu)].

Synthesis of Peptide Segments with a MeNbz Moiety at the C-terminus: Synthesis of L-mGS2$^{1\text{-}37}$ (L-1). Poly-arginine tag was manually loaded on SAL resin (0.57 mmol/g, 70 mg, 0.040 mmol) by Fmoc-Arg(Pbf)-OH (0.13 g, 0.20 mmol), OxymaPure (28 mg, 0.20 mmol) and DIC (31 μL, 0.20 mmol) in DMF for 2 h each step. MeDbz linker was then loaded on the resin by Fmoc-MeDbz-OH (78 mg, 0.20 mmol), HATU (76 mg, 0.20 mmol) and DIEA (70 μL, 0.40 mmol) in DMF for 3 h. The following Gly^{37} was coupled by Fmoc-Gly-OH (59 mg, 0.20 mmol), HATU (76 mg, 0.20 mmol) and DIEA (70 μL, 0.40 mmol) in DMF for 2 h. The remaining peptide sequence was constructed by a standard protocol of Fmoc-SPPS. Boc-Val-OH was employed for coupling of the N-terminal Val^{1}. After the solid-phase peptide synthesis, a solution of *p*-nitrophenyl chloroformate in CH_2Cl_2 (90

mg/mL, 1 mL) was added to the protected peptide resin, and the reaction was continued for 2 h at room temperature. Subsequently, the resin was treated with 1 M DIEA in DMF (1 mL) for 2 h at 37 °C twice. The resin was then treated with TFA/H_2O/*m*-cresol/thioanisole/EDT (80:5:5:5:5) for 2 h at room temperature. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et_2O. The precipitate was purified by preparative HPLC (a linear gradient of 26-56% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to afford L-**1** (23.6 mg, 11% from resin). MS(ESI) *m/z*, calcd for $C_{217}H_{338}N_{60}O_{61}$: 4760.52; observed: $[M+4H]^{4+}$ *m/z* = 1191.92, $[M+5H]^{5+}$ *m/z* = 953.76.

L-mGS2$^{38\text{-}65}$ (L-2a). By the standard protocol for peptide synthesis, peptide L-**2a** was synthesized (9.3 mg, 13% yield) from Rink amide resin (33 mg, 0.020 mmol). MS(ESI) *m/z*, calcd for $C_{166}H_{248}N_{52}O_{44}S$: 3705.85; observed: $[M+3H]^{3+}$ *m/z* = 1237.61, $[M+4H]^{4+}$ *m/z* = 928.38.

[Thz^{38}]-L-mGS2$^{38\text{-}65}$ (L-2b). By the same protocol for the synthesis of peptide L-**1**, peptide L-**2b** was synthesized (23.2 mg, 19% yield) from Fmoc-NH-SAL resin (70 mg, 0.040 mmol). MS(ESI) *m/z*, calcd for $C_{145}H_{200}N_{36}O_{41}S$: 3133.44; observed: $[M+2H]^{2+}$ *m/z* = 1568.60, $[M+3H]^{3+}$ *m/z* = 1046.15.

L-mGS2$^{66\text{-}90}$ (L-3). Peptide L-**3** was synthesized (11.3 mg, 10% yield) from TGR resin (0.10 g, 0.025 mmol) using microwave-assisted Fmoc-SPPS using Liberty Blue™ 2.0 (CEM Japan). MS(ESI) *m/z*, calcd for $C_{170}H_{246}N_{50}O_{45}S_2$: 3771.79; observed: $[M+2H]^{2+}$ *m/z* = 1258.67, $[M+3H]^{3+}$ *m/z* = 1887.71.

L-mGS2$^{1\text{-}65}$ (L-4). Peptides L-**1** (3.1 mg) and L-**2a** (2.2 mg) were dissolved in 1,2,4-triazole ligation buffer [2.5 M 1,2,4-triazole, 30 mM TCEP, 6 M guanidine (pH 7.0); 0.65 mL], and the reaction mixture was incubated for 1 h at 37 °C. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (a linear gradient of 27-57% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**4** (1.7 mg, 36% yield). MS(ESI) *m/z*, calcd for $C_{350}H_{529}N_{93}O_{99}S$: 7650.89; observed: $[M+4H]^{4+}$ *m/z* = 1915.16, $[M+5H]^{5+}$ *m/z* = 1532.32.

L-mGS2 (L-6, *N-to-C synthesis*). A $NaNO_2$ solution (200 mM, 4.3 μL) was added to a solution of L-**4** (0.38 mg) in activation buffer [6 M guanidine, 100 mM phosphate buffer (pH 3.0); 43 μL] at −20 °C, and the reaction was continued for 30 min. An MPAA solution [200 mM MPAA, 100 mM TCEP, 6 M guanidine, 200 mM phosphate buffer (pH 6.5); 15 μL] was then added to the reaction, and the reaction mixture was stirred for 5 min at −20 °C. After L-**3** (1.6 mg) in MPAA solution (28 μL, pH 7.9) was added, and the reaction mixture (pH 6.5) was incubated for 1 h at 37 °C. The mixture was purified by preparative HPLC [a linear gradient of 28-58% CH_3CN in H_2O containing 0.05% (v/v) TFA over 90 min, 5C4-AR300 preparative column (Nacalai Tesque, 20 × 150 mm, flow rate 8

mL/min)] to provide L-**6** (0.16 mg, ca. 30% yield). MS(ESI) *m/z*, calcd for $C_{489}H_{718}N_{124}O_{139}S_3$: 10647.21; observed: $[M+7H]^{7+}$ *m/z* = 1522.80, $[M+8H]^{8+}$ *m/z* = 1332.80, $[M+9H]^{9+}$ *m/z* = 1184.70, $[M+10H]^{10+}$ *m/z* = 1066.30.

L-mGS2$^{38\text{-}90}$ (L-7). Peptides L-**2b** (5.4 mg) and L-**3** (5.2 mg) were dissolved in 1,2,4-triazole ligation buffer (1.1 mL), and the reaction mixture was incubated for 1 h at 37 °C. Then, 2 M $MeONH_2$ (0.12 mL) was added, and the reaction mixture was incubated for 2 h at 37 °C. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (a linear gradient of 28-58% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**7** (4.9 mg, 56% yield). MS(ESI) *m/z*, calcd for $C_{305}H_{437}N_{83}O_{84}S_3$: 6702.16; observed: $[M+4H]^{4+}$ *m/z* = 1677.20, $[M+5H]^{5+}$ *m/z* = 1342.38.

L-mGS2 (L-6, *C-to-N synthesis*). Peptides L-**1** (10.8 mg) and L-**7** (10.6 mg) were dissolved in 1,2,4-triazole ligation buffer (2.5 mL), and the reaction mixture was incubated for 1 h at 37 °C. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (a linear gradient of 30-60% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**5** (10.0 mg, 62% yield). MS(ESI) *m/z*, calcd for $C_{489}H_{718}N_{124}O_{139}S_3$: 10647.21; observed: $[M+7H]^{7+}$ *m/z* = 1522.50, $[M+8H]^{8+}$ *m/z* = 1332.29, $[M+9H]^{9+}$ *m/z* = 1184.26, $[M+10H]^{10+}$ *m/z* = 1066.22.

L-mGS2^{SS} (L-8). 1 mM 2-PDS in MeOH (0.93 mL) was added dropwise to a solution of L-mGS2 (L-**6**, 0.93 mg) in denaturation buffer [6 M guanidine, 20 mM Tris buffer (pH 8.0); 9.3 mL]. The reaction mixture was incubated for 1 h on ice. The mixture was purified by preparative HPLC [a linear gradient of 30-60% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min, 5C4-AR300 preparative column (Nacalai Tesque, 20 × 150 mm, flow rate 8 mL/min)] to provide L-**8** (0.41 mg, 44% yield). MS(ESI) *m/z*, calcd for $C_{489}H_{716}N_{124}O_{139}S_3$: 10645.19; observed: $[M+6]^{6+}$ *m/z* = 1775.55, $[M+7H]^{7+}$ *m/z* = 1522.31, $[M+8H]^{8+}$ *m/z* = 1332.11, $[M+9H]^{9+}$ *m/z* = 1184.57.

D-mGS2$^{1\text{-}37}$ (D-1). By the identical procedure for the synthesis of peptide L-**1**, peptide D-**1** was synthesized (16.2 mg, 7% yield) from Fmoc-NH-SAL resin (70 mg, 0.040 mmol). MS(ESI) *m/z*, calcd for $C_{217}H_{338}N_{60}O_{61}$: 4760.52; observed: $[M+3H]^{3+}$ *m/z* = 1589.09, $[M+4H]^{4+}$ *m/z* = 1192.07, $[M+5H]^{5+}$ *m/z* = 953.92.

[Thz^{38}]-D-mGS2$^{38\text{-}65}$ (D-2b). By the identical procedure for the synthesis of peptide L-**2b**, peptide D-**2b** was synthesized (30.3 mg, 24% yield) from Fmoc-NH-SAL resin (70 mg, 0.040 mmol). MS(ESI)

m/z, calcd for $C_{145}H_{200}N_{36}O_{41}S$: 3133.44; observed: $[M+2H]^{2+}$ *m/z* = 1568.76, $[M+3H]^{3+}$ *m/z* = 1046.39.

D-mGS2$^{66-90}$ (D-3). By the identical procedure for the synthesis of peptide L-**3**, peptide D-**3** was synthesized (10.7 mg, 9% yield) from TGR resin (0.10 g, 0.025 mmol). MS(ESI) *m/z*, calcd for $C_{170}H_{246}N_{50}O_{45}S_2$: 3771.79; observed: $[M+2H]^{2+}$ *m/z* = 1258.58, $[M+3H]^{3+}$ *m/z* = 1887.97.

D-mGS2$^{38-90}$ (D-7). By the identical procedure for the synthesis of peptide L-**7**, peptide D-**7** was synthesized (5.6 mg, 59% yield) from D-**2b** (5.6 mg) and D-**3** (5.7 mg). MS(ESI) *m/z*, calcd for $C_{305}H_{437}N_{83}O_{84}S_3$: 6702.16; observed: $[M+4H]^{4+}$ *m/z* = 1677.05, $[M+5H]^{5+}$ *m/z* = 1341.67.

D-mGS2$^{1-90}$ (D-6). By the identical procedure for the synthesis of peptide L-**6**, peptide D-**6** was synthesized (4.3 mg, 63% yield) from D-**1** (5.0 mg) and D-**7** (5.3 mg). MS(ESI) *m/z*, calcd for $C_{489}H_{718}N_{124}O_{139}S_3$: 10647.21; observed: $[M+8H]^{8+}$ *m/z* = 1332.68, $[M+9H]^{9+}$ *m/z* = 1184.72, $[M+10H]^{10+}$ *m/z* = 1065.99, $[M+11H]^{11+}$ *m/z* = 968.93.

L-GS2$^{1-12}$ (L-S1). By the same protocol for the synthesis of peptide L-**1**, peptide L-**S1** was synthesized (50.1 mg, 23% yield) from Fmoc-NH SAL resin (0.14 g, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{88}H_{152}N_{32}O_{23}$: 2025.17; observed: $[M+2H]^{2+}$ *m/z* = 1013.60, $[M+3H]^{3+}$ *m/z* = 675.93.

L-GS2$^{13-48}$ (L-S2). By the standard protocol for peptide synthesis, peptide L-**S2** was synthesized (12.8 mg, 4% yield) from Fmoc-NH SAL resin (0.14 mg, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{232}H_{326}N_{62}O_{61}S$: 4988.40; observed: $[M+3H]^{3+}$ *m/z* = 1663.85, $[M+4H]^{4+}$ *m/z* = 1248.02, $[M+5H]^{5+}$ *m/z* = 998.90.

L-GS2$^{49-73}$ (L-S3). By the same protocol for the synthesis of peptide L-**1**, peptide L-**S3** was synthesized (47 mg, 15% yield) from Fmoc-NH SAL resin (0.14 mg, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{145}H_{233}N_{45}O_{43}S$: 3324.71; observed: $[M+3H]^{3+}$ *m/z* = 1109.68, $[M+4H]^{4+}$ *m/z* = 832.36.

L-GS2$^{74-90/biotin}$ (L-S4). By the standard protocol for peptide synthesis, peptide L-**S4** was synthesized (30.0 mg, 9% yield) from TGR resin (0.32 g, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{146}H_{216}N_{48}O_{36}S_3$: 3313.57; observed: $[M+2H]^{2+}$ *m/z* = 1657.55, $[M+3H]^{3+}$ *m/z* = 1105.72.

[Cys(Trt-K$_{10}$)^{13}]-L-GS2$^{1-48}$ (L-S5). Peptides L-**S1** (8.5 mg) and L-**S2** (9.6 mg) was dissolved in 1,2,4-triazole ligation buffer (3.0 mL), and the reaction mixture was incubated for 1 h at 37 °C. Then,

Trt(OH)-K_{10} (25 mg) and TFA (3.0 mL) were added, and the reaction mixture was incubated for 1 h at room temperature. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (a linear gradient of 30-60% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S5** (6.6 mg, 40% yield). MS(ESI) *m/z*, calcd for $C_{371}H_{561}N_{97}O_{92}S$: 7879.19; observed: $[M+5H]^{5+}$ *m/z* = 1576.86, $[M+6H]^{6+}$ *m/z* = 1314.67, $[M+7H]^{7+}$ *m/z* = 1126.94, $[M+8H]^{8+}$ *m/z* = 986.16.

[Cys(Trt-K_{10})^{13}]-L-GS2$^{1\text{-}48}$ MES ester (L-S6). A $NaNO_2$ solution (60 μL) was added to a solution of L-**S5** (6.1 mg) in activation buffer (0.60 mL) at −20 °C, and the reaction was continued for 30 min. An MESNa solution [200 mM MESNa, 50 mM TCEP, 6 M guanidine, 200 mM phosphate buffer (pH 6.5); 0.20 mL] was then added to the reaction, and the reaction mixture was stirred for 5 min at −20 °C. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (a linear gradient of 31-61% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S6** (3.4 mg, 64% yield). MS(ESI) *m/z*, calcd for $C_{342}H_{510}N_{78}O_{90}S_3$: 7245.69; observed: $[M+4H]^{4+}$ *m/z* = 1813.12, $[M+5H]^{5+}$ *m/z* = 1450.53, $[M+6H]^{6+}$ *m/z* = 1209.21.

[Cys^{49,74}]-L-GS2$^{49\text{-}90/biotin}$ (L-S7). By the same procedure for the synthesis of peptide L-**7**, peptide L-**S7** was synthesized (5.0 mg, 29% yield) from L-**S3** (13.4 mg) and L-**S4** (10.8 mg). MS(ESI) *m/z*, calcd for $C_{257}H_{392}N_{74}O_{73}S_4$: 5810.81; observed: $[M+4H]^{4+}$ *m/z* = 1454.07, $[M+5H]^{5+}$ *m/z* = 1163.24.

[Cys(Trt-K_{10})^{13}, Cys^{49,74}]-L-GS2^{biotin} (L-S8). Peptides L-**S6** (3.4 mg) and L-**S7** (2.5 mg) was dissolved in MPAA ligation buffer [100 mM MPAA, 50 mM TCEP, 6 M guanidine, 200 mM phosphate (pH 6.5); 0.72 mL], and the reaction mixture was incubated for 1 h at 37 °C. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (a linear gradient of 33-63% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S8** (2.4 mg, 43% yield). MS(ESI) *m/z*, calcd for $C_{597}H_{896}N_{152}O_{160}S_5$: 12914.53; observed: $[M+7H]^{7+}$ *m/z* = 1846.18, $[M+8H]^{8+}$ *m/z* = 1615.37, $[M+9H]^{9+}$ *m/z* = 1436.01, $[M+10H]^{10+}$ *m/z* = 1292.91, $[M+11H]^{11+}$ *m/z* = 1175.18, $[M+12H]^{12+}$ *m/z* = 1077.62.

[Cys^{13,49,74}]-L-GS2^{biotin} (L-S9). Peptide L-**S8** (2.1 mg) was dissolved in TFA/EDT (95/5) and the solution was incubated for 30 min at room temperature. The reaction mixture was poured into ice-cold dry Et_2O. The precipitate was dissolved in 6 M guanidine solution (0.20 mL), and purified by preparative HPLC (Cosmosil 5C4-AR300 column, a linear gradient of 31-61% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S9** (1.0 mg, 60%). MS(ESI) *m/z*, calcd for $C_{513}H_{756}N_{130}O_{146}S_5$: 11233.43; observed: $[M+7H]^{7+}$ *m/z* = 1606.33, $[M+8H]^{8+}$ *m/z* = 1406.13,

$[M+9H]^{9+}$ *m/z* = 1250.30, $[M+10H]^{10+}$ *m/z* = 1124.78.

L-GS2^{biotin} (L-S10). L-**S9** (1.0 mg) was dissolved in desulfurization buffer [20 mM VA-044, 100 mM MESNa, 250 mM TCEP, 6 M guanidine, 100 mM phosphate (pH 6.5); 0.42 mL], and incubated for 2 h at 37 °C. After H_2O/CH_3CN (1:1) containing 0.1% (v/v) TFA was added, the mixture was purified by preparative HPLC (Cosmosil 5C4-AR300 column, a linear gradient of 31-61% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S10** (0.53 mg, 51%). MS(ESI) *m/z*, calcd for $C_{513}H_{756}N_{130}O_{146}S_2$: 11137.52; observed: $[M+7H]^{7+}$ *m/z* = 1592.81, $[M+8H]^{8+}$ *m/z* = 1393.76, $[M+9H]^{9+}$ *m/z* = 1239.10, $[M+10H]^{10+}$ *m/z* = 1115.06.

L-mGS2$^{66\text{-}73}$ (L-S11). By the same protocol for the synthesis of peptide L-**1**, peptide L-**S11** was synthesized (92.0 mg, 48% yield) from Fmoc-NH SAL resin (0.14 g, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{80}H_{124}N_{28}O_{21}S$: 1844.92; observed: $[M+2H]^{2+}$ *m/z* = 923.64, $[M+3H]^{3+}$ *m/z* = 615.95.

[Cys(Acm)^{66}]-L-mGS2$^{66\text{-}90/biotin}$ (L-S12). Peptide**s** L-**S11** (17.1 mg) and L-**S4** (19.4 mg) were dissolved in 1,2,4-triazole ligation buffer (4.5 mL), and the reaction mixture was incubated for 1 h at 37 °C. Then, desulfurization buffer [450 mM TCEP, 80 mM MESNa, 6 M guanidine, 100 mM phosphate (pH 4.5); 4.5 mL] and 1 M VA-044 (0.45 mL) were added, and the pH was adjusted to 6.5. The reaction mixture was further incubated for 2 h at 37 °C. The product was purified by preparative HPLC (a linear gradient of 27-57% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S12** (8.4 mg, 35% yield). MS(ESI) *m/z*, calcd for $C_{193}H_{283}N_{57}O_{51}S_3$: 4311.05; observed: $[M+3H]^{3+}$ *m/z* = 1438.56, $[M+4H]^{4+}$ *m/z* = 1079.05, $[M+5H]^{5+}$ *m/z* = 863.53.

L-mGS2$^{66\text{-}90/biotin}$ (L-S13). Peptide L-**S12** (7.4 mg) was dissolved in deprotection cocktail [AgOTf (27 mg), anisole (10 μL), TFA (0.50 mL)], and the reaction mixture was incubated for 1.5 h on ice. The product was poured into ice-cold Et_2O, and the precipitate was collected by centrifugation. The precipitate was washed by reduction buffer [10 mM DTT, 6 M guanidine, 100 mM phosphate (pH 7.4)], and the supernatant was purified by preparative HPLC (a linear gradient of 27-57% CH_3CN in H_2O containing 0.1% (v/v) TFA over 90 min) to provide L-**S13** (5.0 mg, 69%). MS(ESI) *m/z*, calcd for $C_{190}H_{278}N_{56}O_{50}S_3$: 4240.01; observed: $[M+3H]^{3+}$ *m/z* = 1414.80, $[M+4H]^{4+}$ *m/z* = 1061.37.

L-mGS2$^{38\text{-}90/biotin}$ (L-S14). By the same procedure for the synthesis of peptide L-**7**, peptide L-**S14** was synthesized (3.2 mg, 44% yield) from L-**2b** (4.9 mg) and L-**S13** (4.6 mg). MS(ESI) *m/z*, calcd for $C_{325}H_{469}N_{89}O_{89}S_4$: 7170.38; observed: $[M+4H]^{4+}$ *m/z* = 1793.93, $[M+5H]^{5+}$ *m/z* = 1435.37, $[M+6H]^{6+}$ *m/z* = 1196.63.

L-mGS2^{biotin} (L-S15). By the same procedure for the synthesis of peptide L-**6**, peptide L-**S15** was synthesized (1.4 mg, 31% yield) from L-**1** (3.3 mg) and L-**S14** (3.0 mg). MS(ESI) *m/z*, calcd for $C_{509}H_{750}N_{130}O_{144}S_4$: 11115.42; observed: $[M+8H]^{8+}$ *m/z* = 1390.97, $[M+9H]^{9+}$ *m/z* = 1236.70, $[M+10H]^{10+}$ *m/z* = 1113.26, $[M+11H]^{11+}$ *m/z* = 1012.18.

L-mGS2$^{SS/biotin}$ (L-S16). By the same procedure for the synthesis of peptide L-**8**, peptide L-**S16** was synthesized (0.08 mg, 9% yield) from L-**S15** (0.93 mg). MS(ESI) *m/z*, calcd for $C_{509}H_{748}N_{130}O_{144}S_4$: 11113.41; observed: $[M+8H]^{8+}$ *m/z* = 1390.48, $[M+9H]^{9+}$ *m/z* = 1237.23, $[M+10H]^{10+}$ *m/z* = 1113.31, $[M+11H]^{11+}$ *m/z* = 1012.01.

D-mGS2$^{66-73}$ (D-S11). By the identical procedure for the synthesis of peptide L-**S11**, peptide D-**S11** was synthesized (63.1 mg, 32% yield) from Fmoc-NH SAL resin (0.14 g, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{80}H_{124}N_{28}O_{21}S$: 1844.92; observed: $[M+2H]^{2+}$ *m/z* = 923.66, $[M+3H]^{3+}$ *m/z* = 615.96.

D-mGS2$^{74-90/biotin}$ (D-S4). By the identical procedure for the synthesis of peptide L-**S4**, peptide D-**S4** was synthesized (24.4 mg, 7% yield) from TGR resin (0.32 g, 0.080 mmol). MS(ESI) *m/z*, calcd for $C_{146}H_{216}N_{48}O_{36}S_3$: 3313.57; observed: $[M+3H]^{3+}$ *m/z* = 1106.81, $[M+4H]^{4+}$ *m/z* = 830.17.

[Cys(Acm)^{66}]-D-mGS2$^{66-90/biotin}$ (D-S12). By the identical procedure for the synthesis of peptide L-**S12**, peptide D-**S12** was synthesized (18.0 mg, 63% yield) from D-**S11** (19.5 mg) and D-**S4** (23.2 mg). MS(ESI) *m/z*, calcd for $C_{193}H_{283}N_{57}O_{51}S_3$: 4311.05; observed: $[M+3H]^{3+}$ *m/z* = 1439.09, $[M+4H]^{4+}$ *m/z* = 1079.72, $[M+5H]^{5+}$ *m/z* = 863.88.

D-mGS2$^{66-90/biotin}$ (D-S13). By the identical procedure for the synthesis of peptide L-**S13**, peptide D-**S13** was synthesized (2.2 mg, 22% yield) from D-**S12** (10.4 mg). MS(ESI) *m/z*, calcd for $C_{190}H_{278}N_{56}O_{50}S_3$: 4240.01; observed: $[M+3H]^{3+}$ *m/z* = 1415.72, $[M+4H]^{4+}$ *m/z* = 1061.76.

D-mGS2$^{38-90/biotin}$ (D-S14). By the identical procedure for the synthesis of peptide L-**S14**, peptide D-**S14** was synthesized (2.1 mg, 61% yield) from D-**2b** (2.2 mg) and D-**S13** (2.2 mg). MS(ESI) *m/z*, calcd for $C_{325}H_{469}N_{89}O_{89}S_4$: 7170.38; observed: $[M+5H]^{5+}$ *m/z* = 1436.29, $[M+6H]^{6+}$ *m/z* = 1196.82, $[M+7H]^{7+}$ *m/z* = 1026.18.

D-mGS2^{biotin} (D-S15). By the identical procedure for the synthesis of peptide L-**S15**, peptide D-**S15** was synthesized (1.3 mg, 45% yield) from D-**1** (2.0 mg) and D-**S14** (2.0 mg). MS(ESI) *m/z*, calcd for

$C_{509}H_{750}N_{130}O_{144}S_4$: 11115.42; observed: $[M+8H]^{8+}$ *m/z* = 1391.50, $[M+10H]^{10+}$ *m/z* = 1113.37, $[M+11H]^{11+}$ *m/z* = 1012.46.

Folding of L/D-mGS2. The lyophilized peptide was dissolved in guanidine solution [5 mM TCEP, 6 M guanidine, 20 mM Tris (pH 8); 0.3 mg/mL], and incubated for 1 h at room temperature. The denatured protein was subjected to dialysis using Slide-A-Lyzer G2 dialysis cassette (cutoff 3.5 kDa, Thermo) against a 200-fold volume of dialysis buffer [10 mM TCEP, 50 mM acetate buffer (pH 4.5)] for 1.5 h and 12 h at 4 °C. The folded proteins were used for the following experiments without purification.

Folding of L-mGS2^{SS}. The lyophilized peptide was dissolved in guanidine solution [6 M guanidine, 20 mM Tris (pH 8.0); 0.3 mg/mL], and incubated for 1 h at room temperature. The denatured protein was subjected to dialysis using Slide-A-Lyzer G2 dialysis cassette (cutoff 3.5 kDa, Thermo) against a 200-fold volume of dialysis buffer [50 mM acetate buffer (pH 4.5)] for 1.5 h and 12 h at 4 °C. The folded protein was used for the following experiments without purification.

Folding of L-GS2^{biotin}. The lyophilized peptide was dissolved in guanidine solution [6 M guanidine, 20 mM Tris (pH 8.0); 0.3 mg/mL], and incubated for 1 h at room temperature. The denatured protein was subjected to dialysis using Slide-A-Lyzer G2 dialysis cassette (cutoff 3.5 kDa, Thermo) against a 200-fold volume of dialysis buffer [50 mM acetate buffer (pH 4.5)] for 1.5 h and 12 h at 4 °C. The folded protein was used for the following experiments without purification.

CD Spectra. Refolded protein in acetate buffer was used for measurement. CD spectrum was recorded on a J-720 circular dichroism spectrometer (JASCO) at 20 °C. The thermal denaturation was measured by observing the transition of CD signal at 203 nm during heating at a rate of 1 °C/min (protein concentration: 20 μM).

Surface plasmon resonance (SPR) analysis. SPR analysis of monobody proteins binding to GST-EGFP was carried out using a Biacore X-100 (Cytiva). After the immobilization of biotinylated monobody proteins on a sensor chip SA, the analyte protein solution of GST-EGFP was flashed into the flow system in the running buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20] at 25 °C. All analytes were evaluated for 90 s as contact time, followed by 600 s dissociation at a flow rate of 50 μL/min. The triplicate data were analyzed.

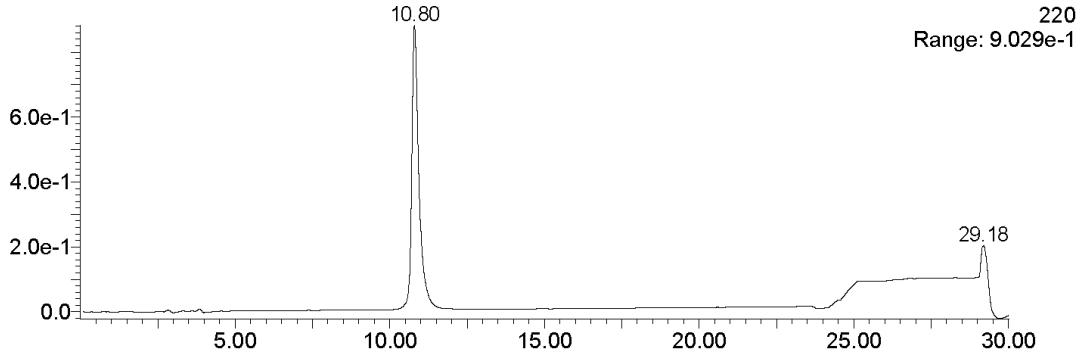
Immunogenicity Assays. All animal experiments were carried out in accordance with our

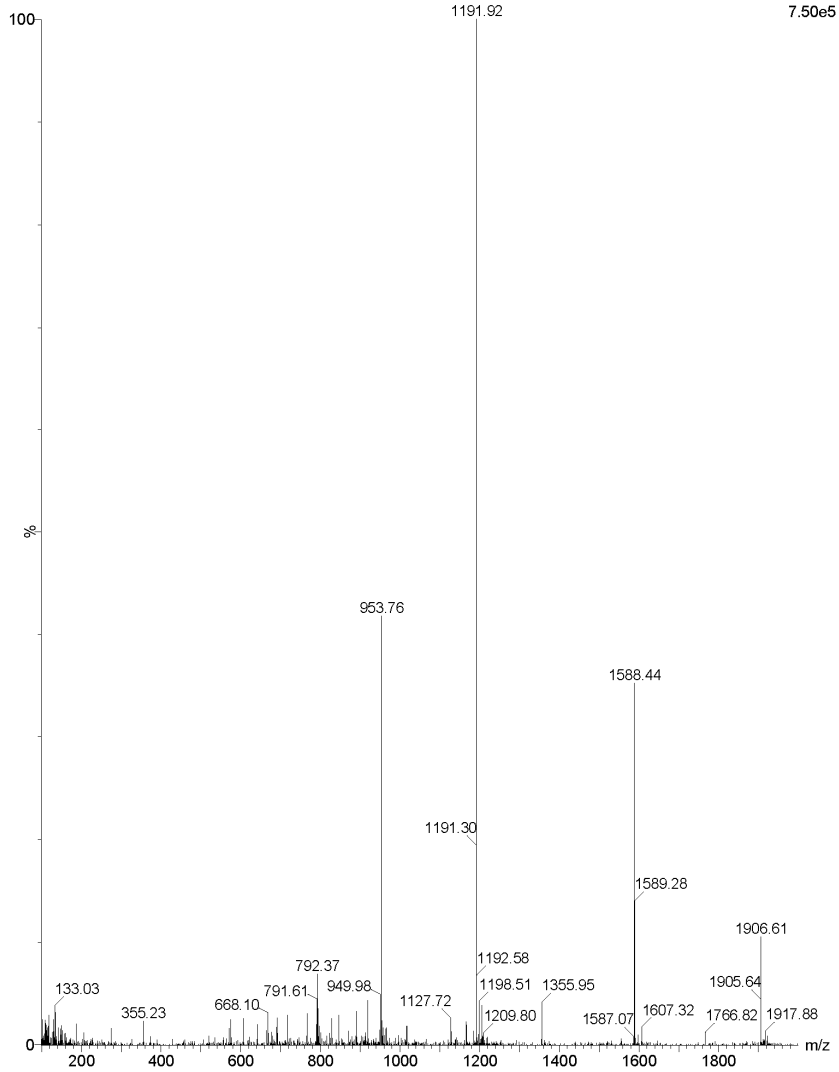
institutional guidelines and approved by the Kyoto University Animal Care Committee. BALB/c mice (female, 5 weeks old) were given intraperitoneal injections on day 0 with 50 μg/injection of synthetic L-mGS2 or D-mGS2 antigen emulsified in Freund's Complete Adjuvant (Wako) and on days 14 and 28 with 50 μg/injection of synthetic L-mGS2 or D-mGS2 antigen emulsified in Freund's Incomplete Adjuvant (Wako). Immune plasma samples were collected on days 0, 14, 28, 35 and 44.

ELISAs were performed in PBS (pH 7.4) containing 0.05% Tween 20 for all wash and dilution processes. 96-well Maxisorp microtiter plates (Thermo) were coated overnight at 4 °C with either L-mGS2 or D-mGS2 in 50 mM acetate buffer (pH 4.4) (50 μL/well; 1,000 ng/mL). After coating, wells were washed three times and blocked with PBS containing 3% BSA (150 μL/well) for 2 h. After three times washes, 1:1,000 dilution of immunized plasma from each mouse (50 μL/well; day 0, 14, 28, 35 and 44) or serial dilution of immunized plasma from each mouse (50 μL/well; day 44) was added and incubated for 1 h. After three times washes, 1:10,000 dilution of anti-mouse IgG (H+L), HRP conjugate (Jackson ImmunoResearch Laboratories) (50 μL/well) was added and incubated for 30 min. After five times washes, TMB solution was added and incubated for 2 min, then the reaction was stopped using 1 M H_2SO_4 (50 μL/well). Absorbance was measured at 450 nm using an infinite M200 Plate Reader (TECAN).

Analytical HPLC chromatograms and mass spectrometry data of synthetic peptides

L-mGS2$^{1\text{-}37}$ (L-1)

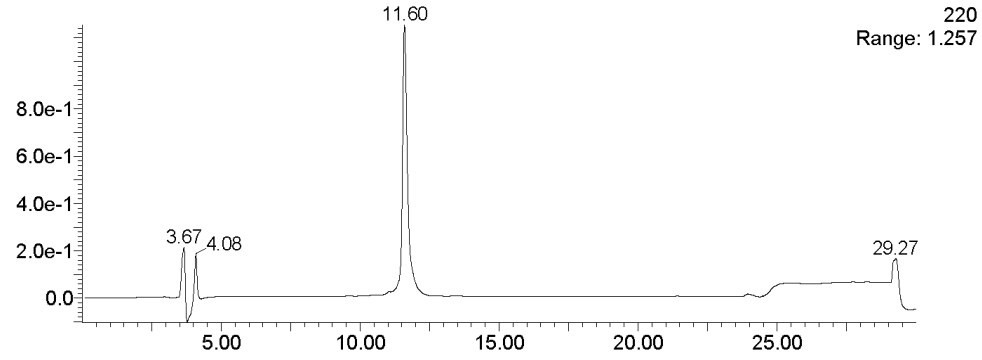


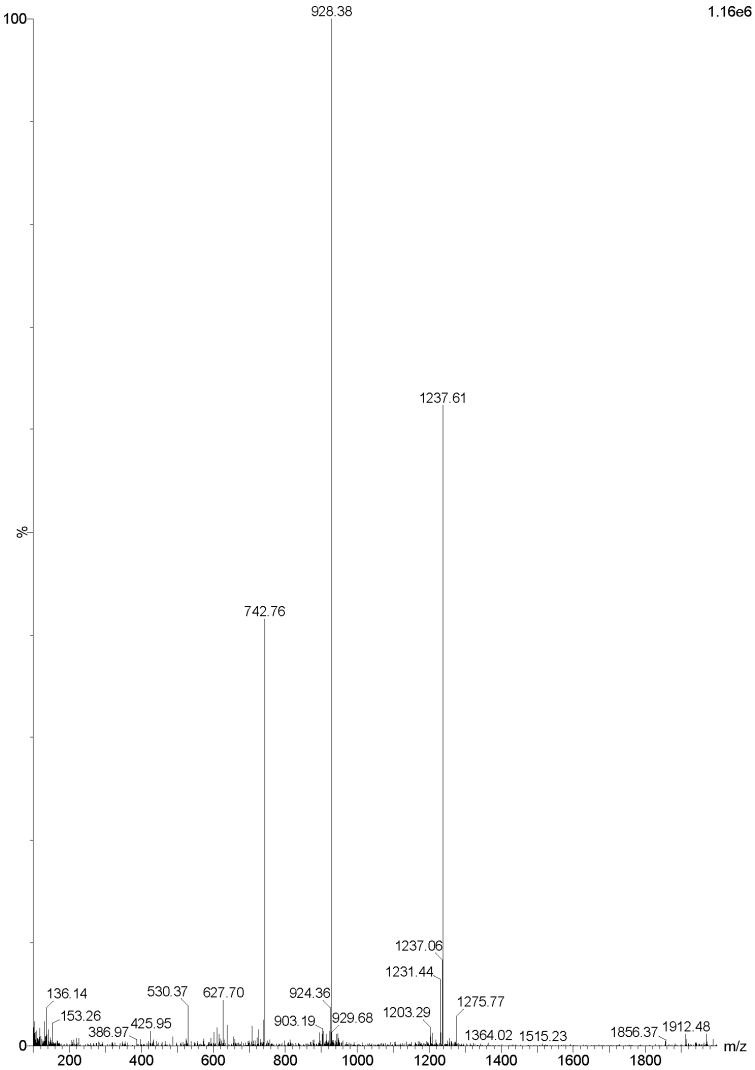


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4763.53 (MH^+): Major observed ions: 953.76 (+5), 1191.92 (+4), 1588.44 (+3).

L-mGS2$^{38-65}$ (L-2a)

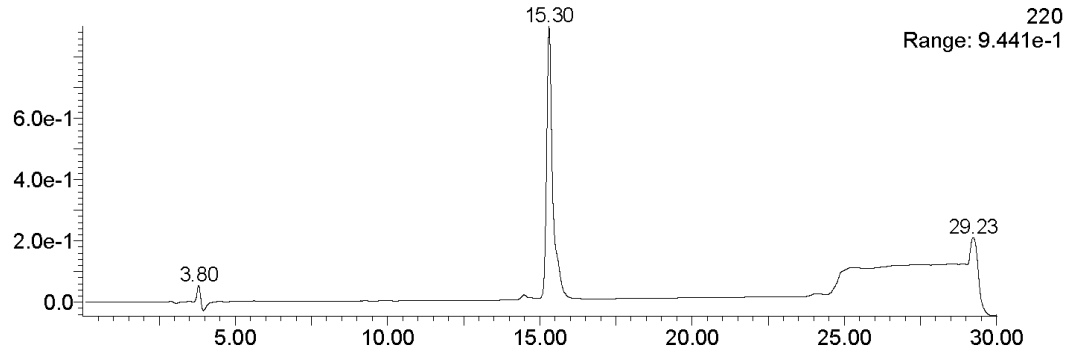


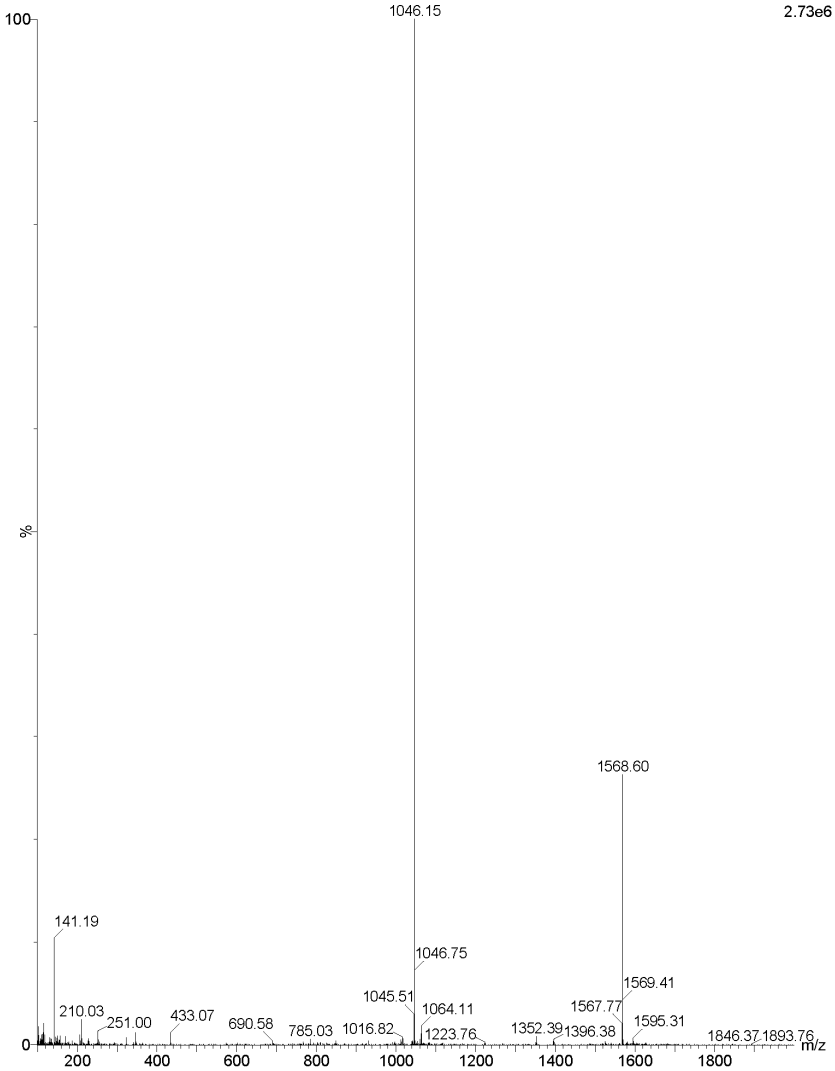


Analytical HPLC trace of the purified product: Cosmosil 5C4-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3707.86 (MH^+): Major observed ions: 742.76 (+5), 928.38 (+4), 1237.61 (+3).

[Thz^{38}]-L-mGS2$^{38-65}$ (L-2b)

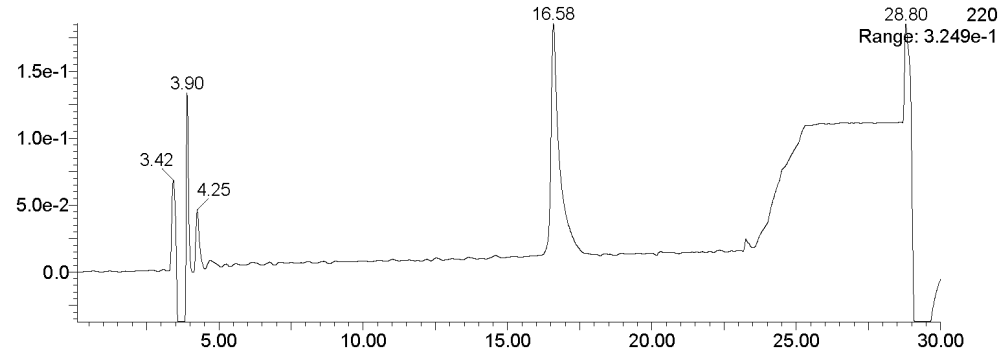


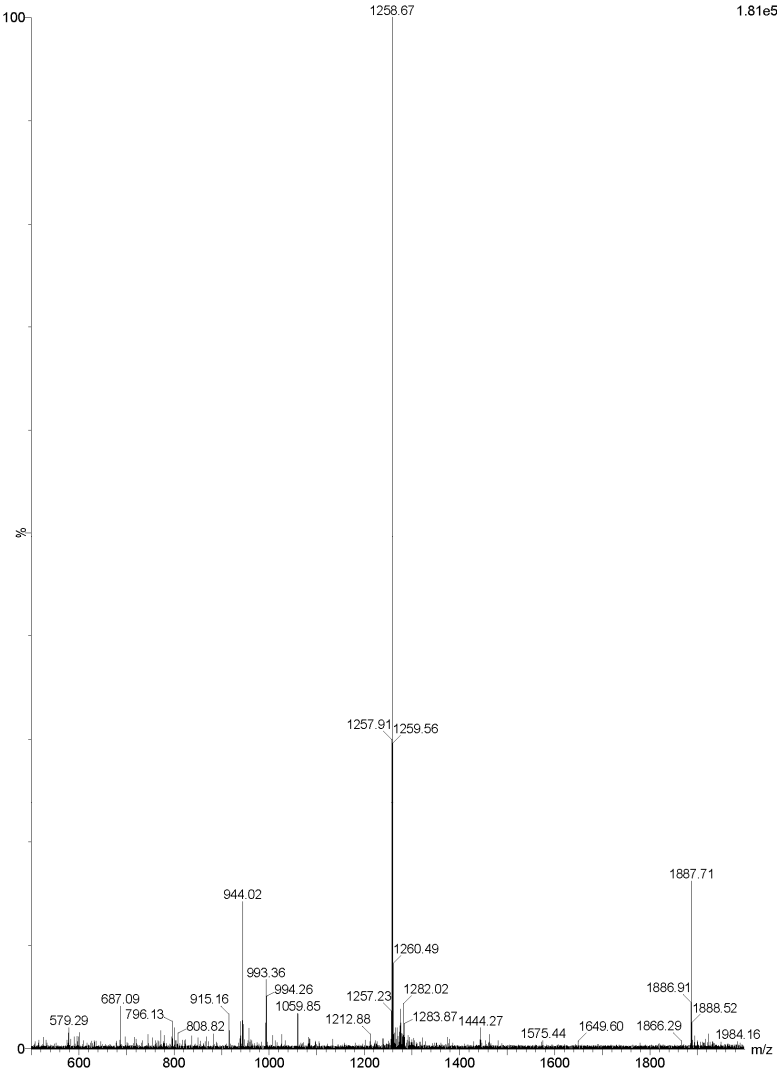


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3135.45 (MH^+): Major observed ions: 1046.15 (+3), 1568.60 (+2).

L-mGS2$^{66-90}$ (L-3)

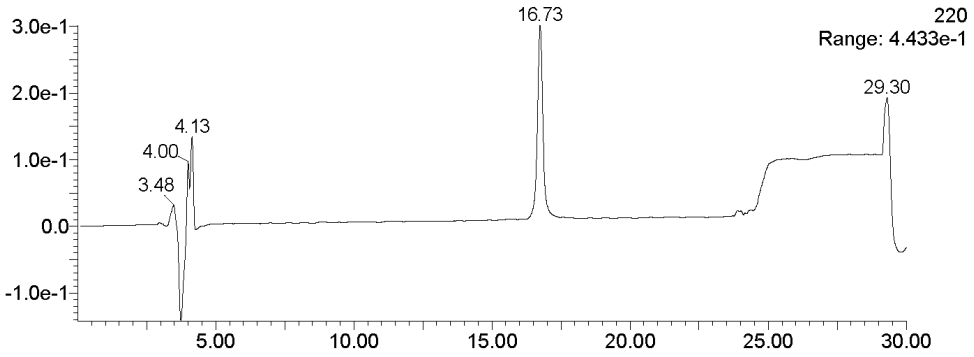


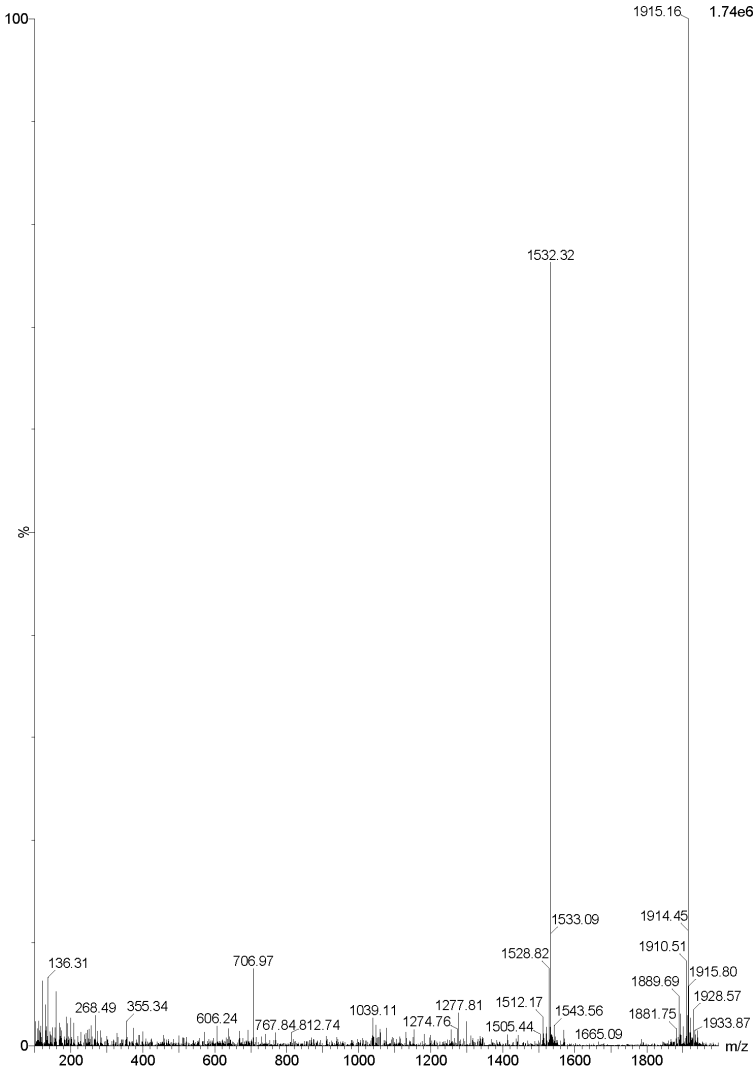


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3773.80 (MH^+): Major observed ions: 1258.67 (+3), 1887.71 (+2).

L-mGS2$^{1\text{-}65}$ (L-4)

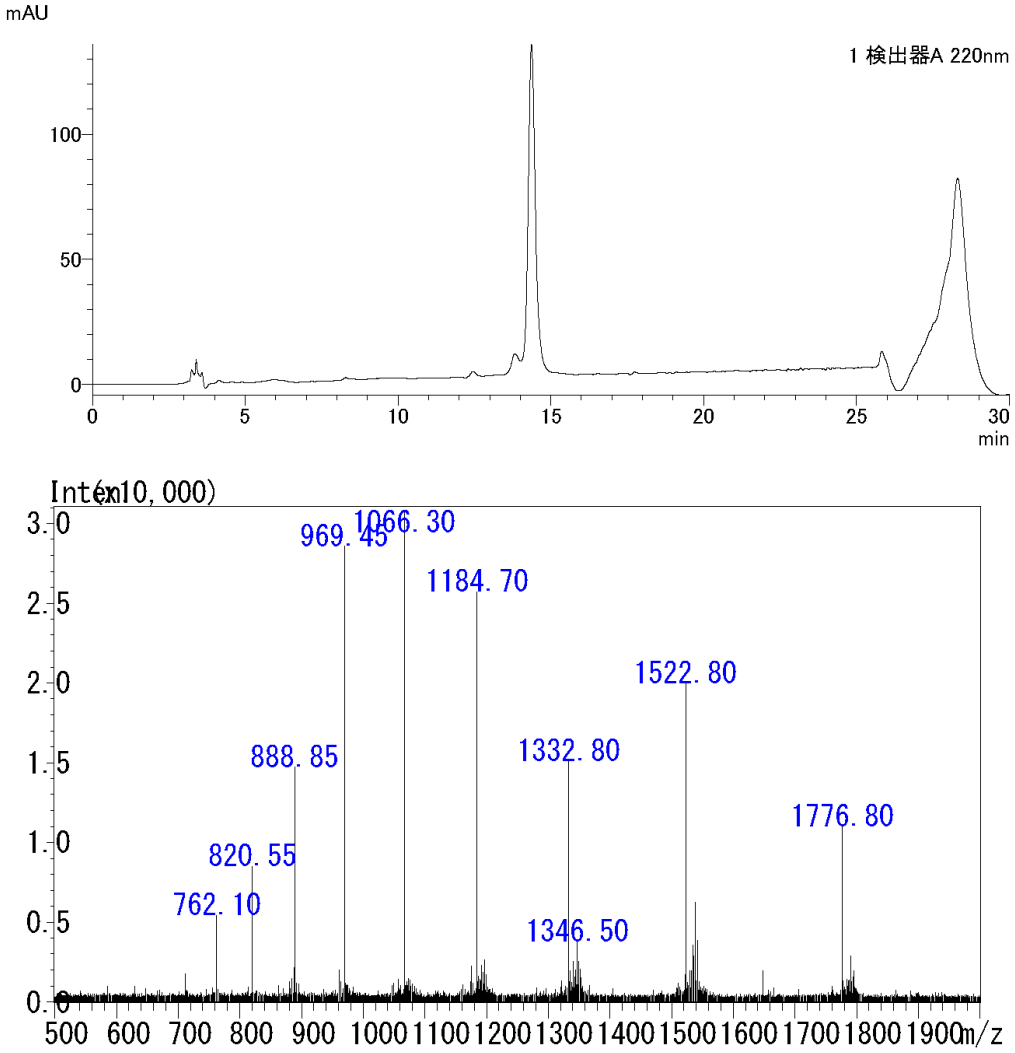




Analytical HPLC trace of the purified product: Cosmosil 5C4-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 7654.91 (MH^+): Major observed ions: 1532.32 (+5), 1915.16 (+4).

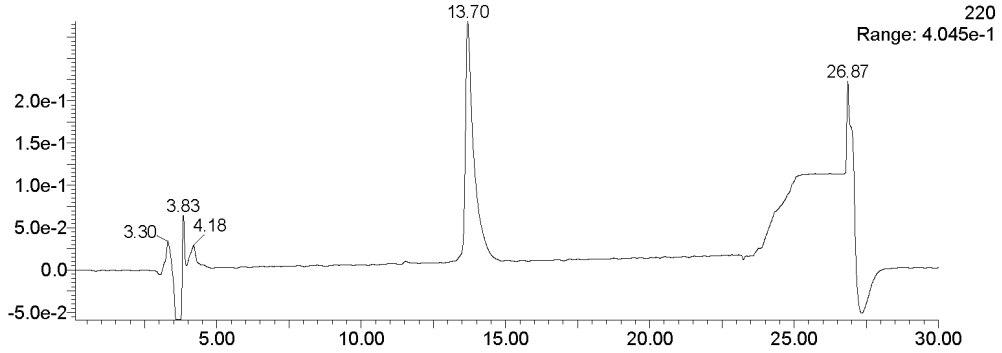
L-mGS2 (L-6, *N-to-C synthesis*)

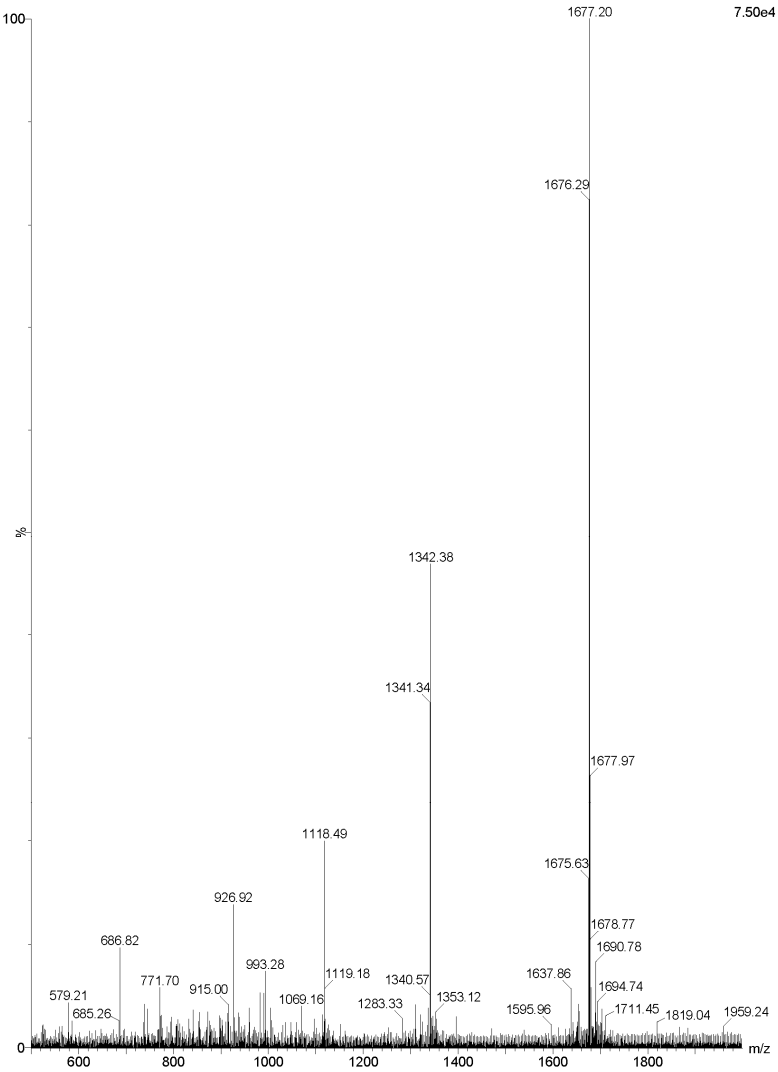


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.05% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 10653.23 (MH^+): Major observed ions: 1066.30 (+10), 1184.70 (+9), 1332.80 (+8), 1522.80 (+7).

L-mGS2$^{38\text{-}90}$ (L-7)

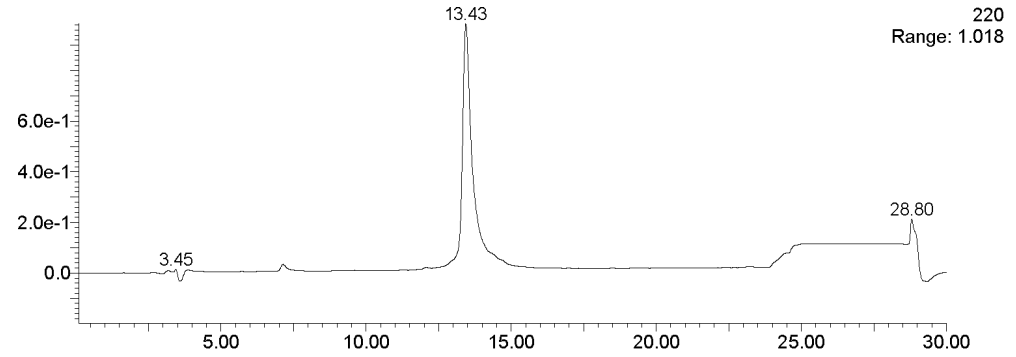


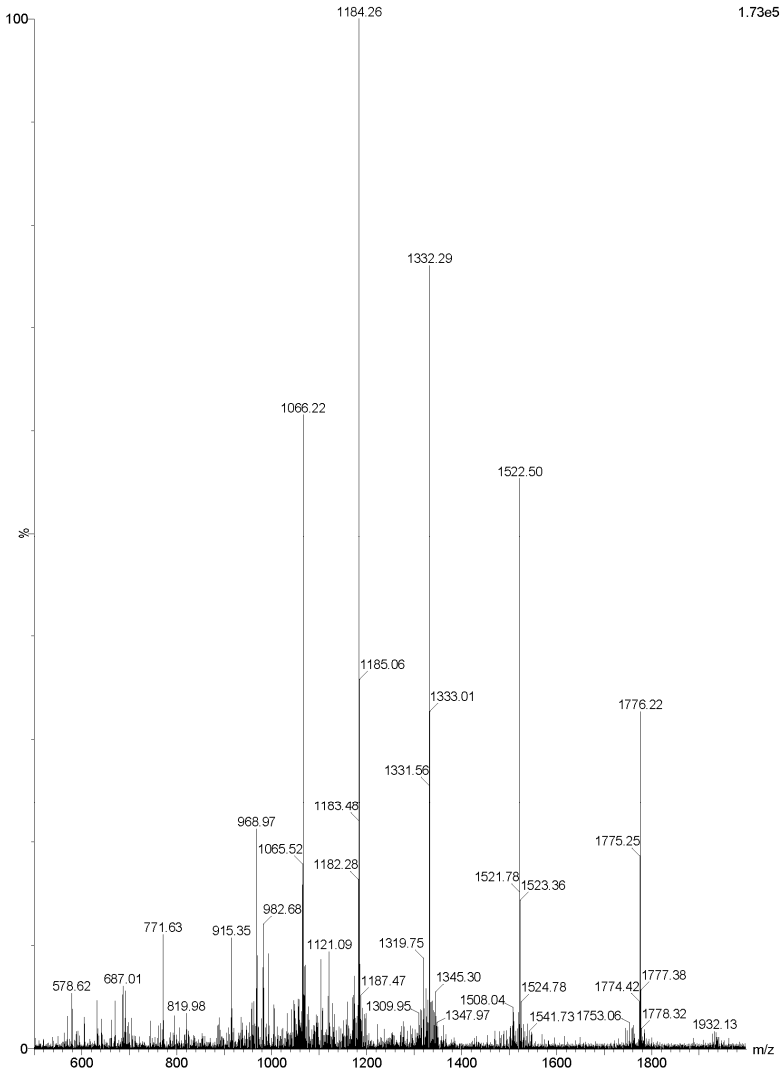


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 6705.18 (MH^+): Major observed ions: 1342.38 (+5), 1677.20 (+4).

L-mGS2 (L-6, *C-to-N synthesis*)

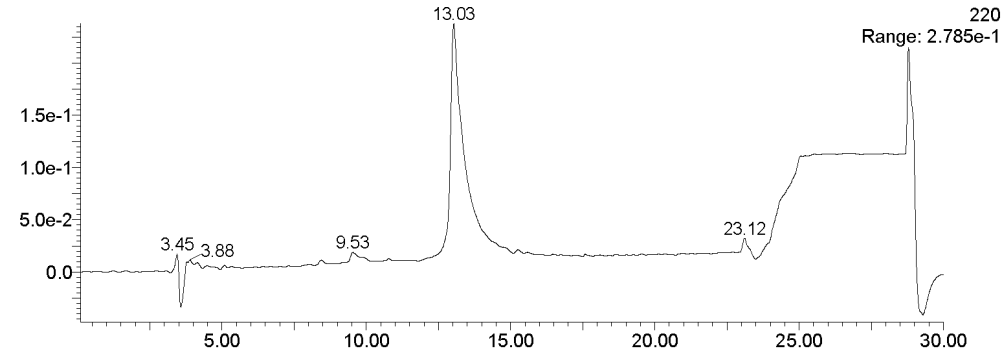


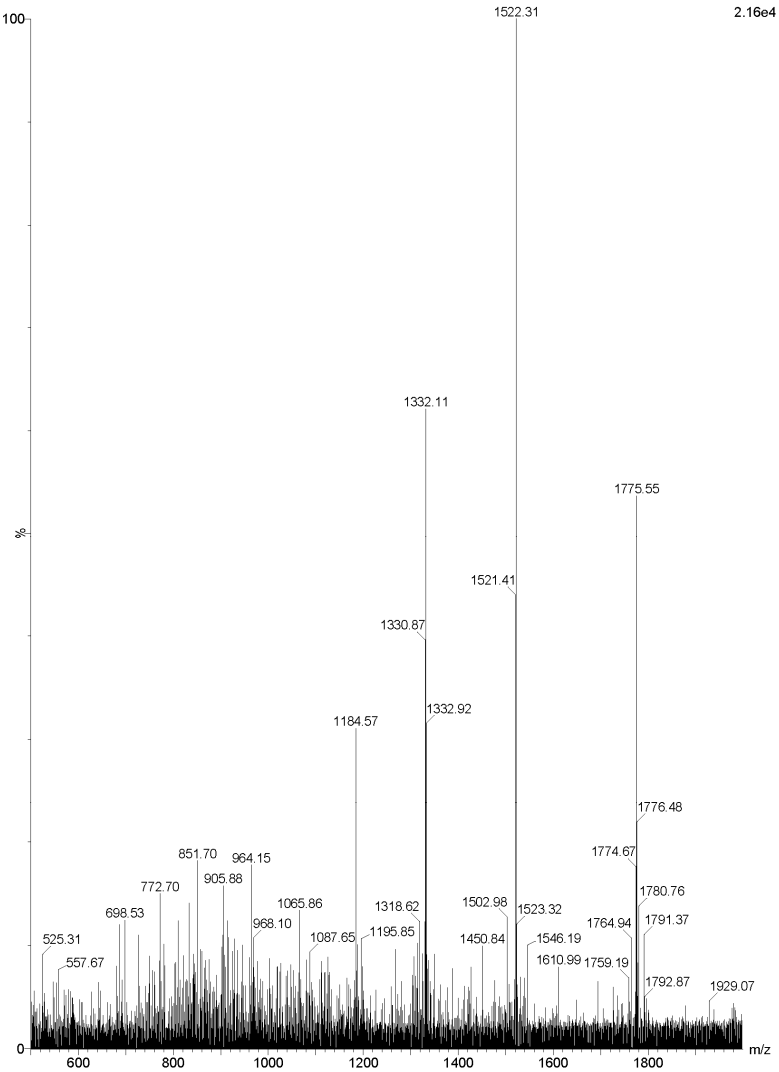


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 10653.23 (MH^+): Major observed ions: 1066.22 (+10), 1184.26 (+9), 1332.29 (+8), 1522.50 (+7).

L-mGS2^{SS} (L-8)

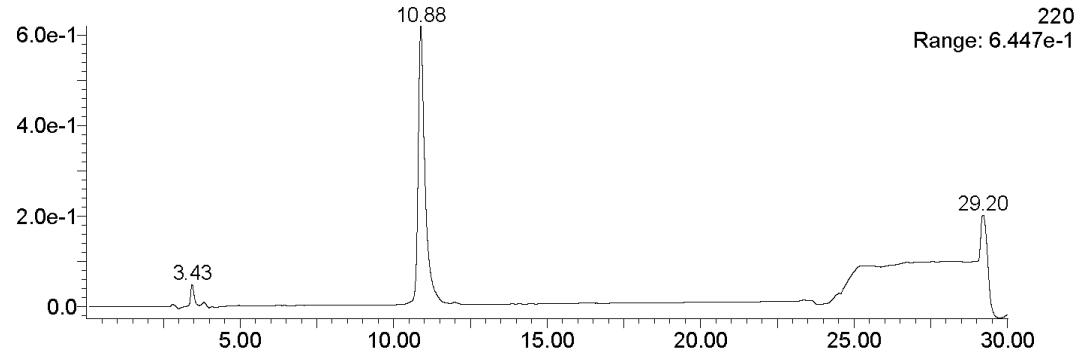


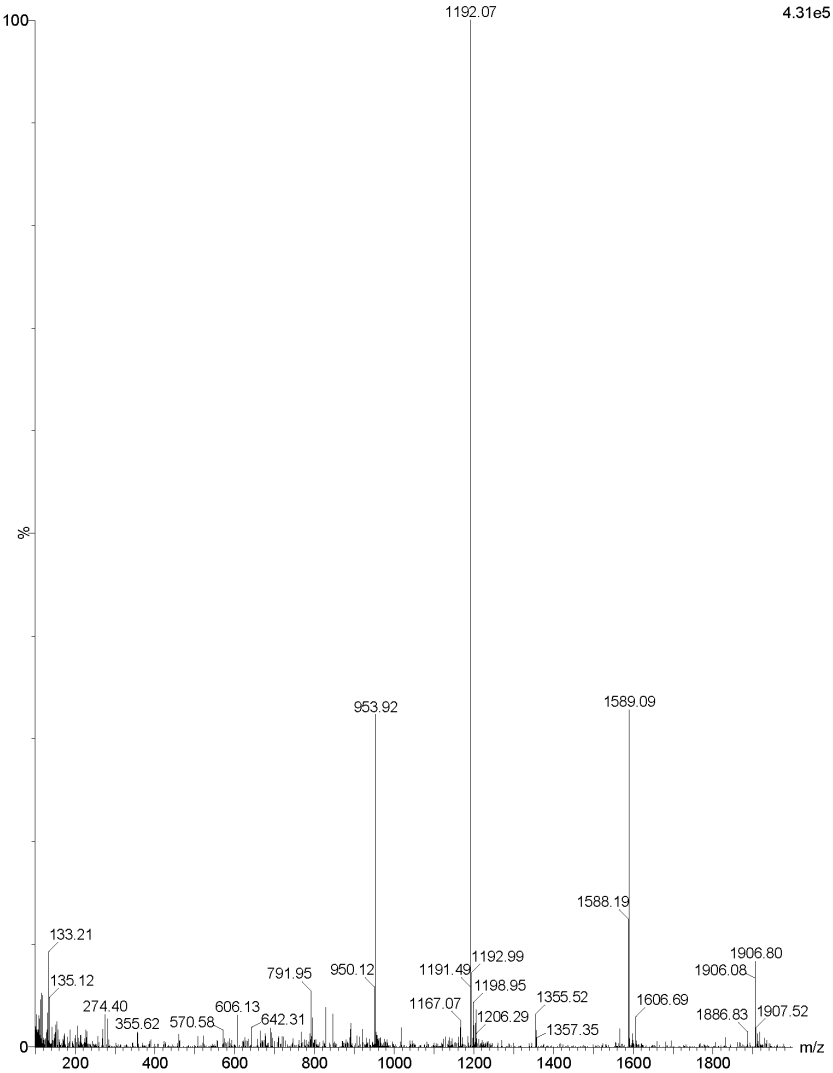


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 10651.22 (MH^+): Major observed ions: 1184.57 (+9), 1332.11 (+8), 1522.31 (+7), 1775.55 (+6).

D-mGS2$^{1-37}$ (D-1)

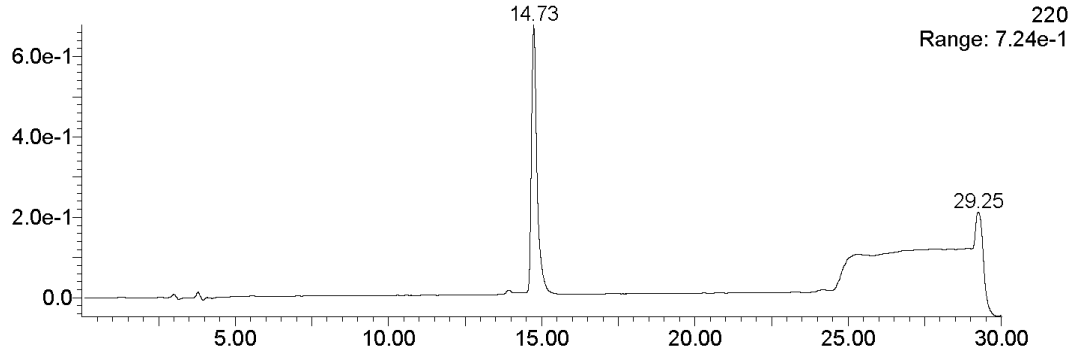


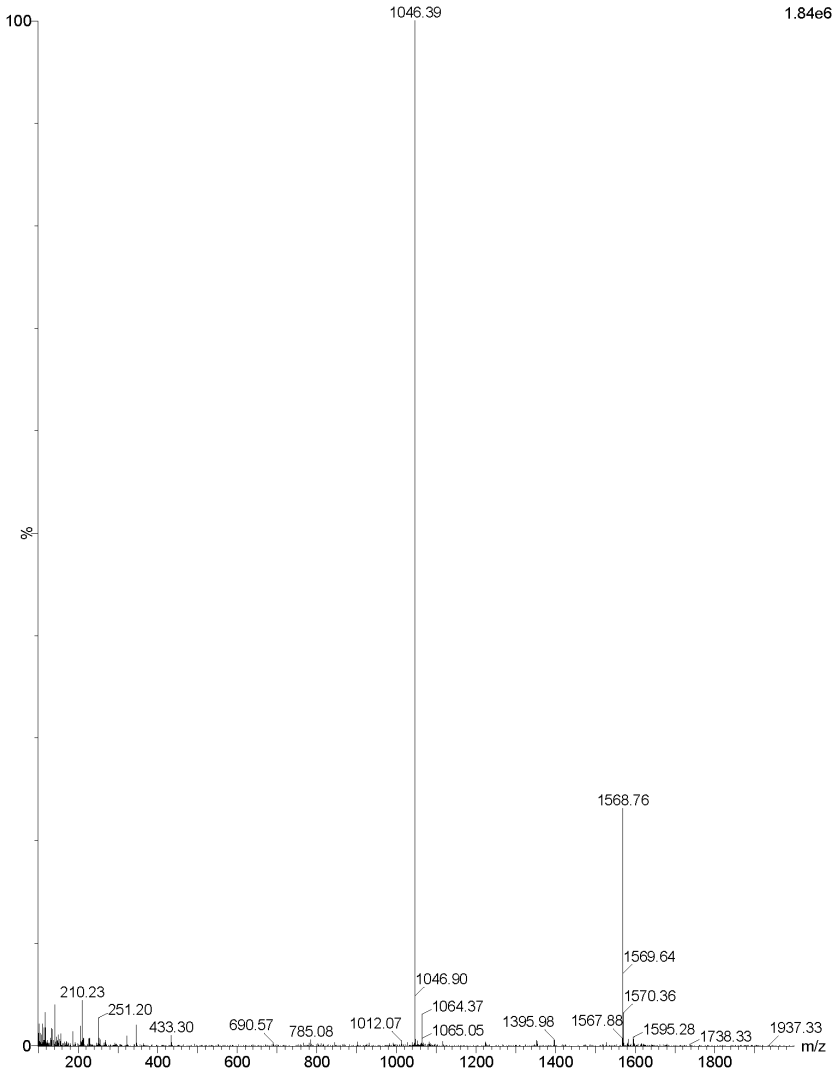


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4763.53 (MH^+): Major observed ions: 953.92 (+5), 1192.07 (+4), 1589.09 (+3).

[Thz^{38}]-D-mGS2$^{38-65}$ (D-2b)

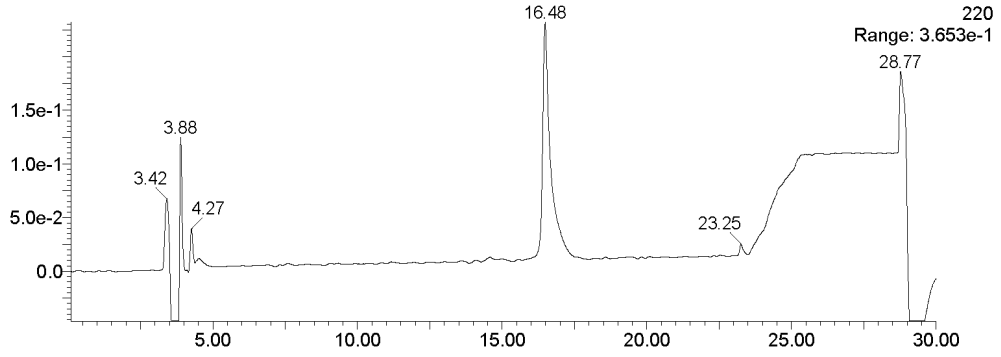


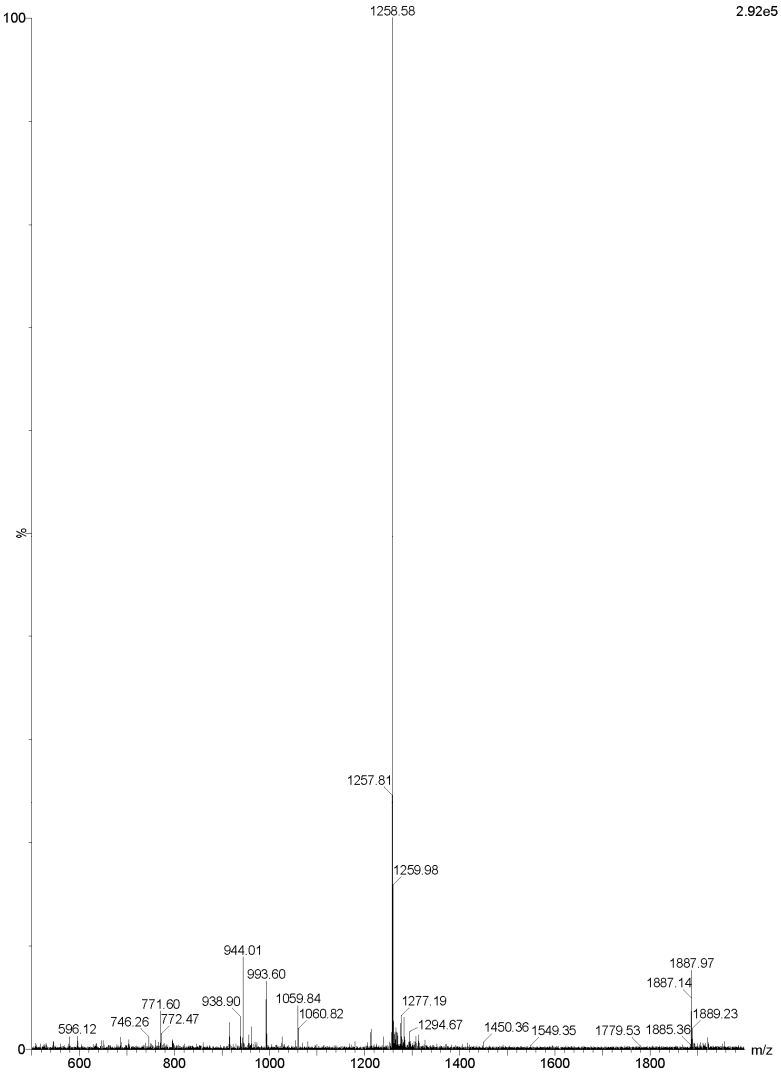


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3135.45 (MH^+): Major observed ions: 1046.39 (+3), 1568.76 (+2).

D-mGS2$^{66-90}$ (D-3)

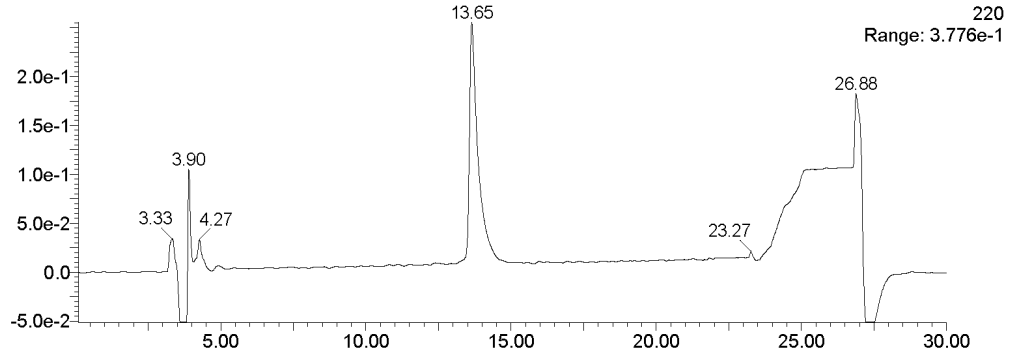


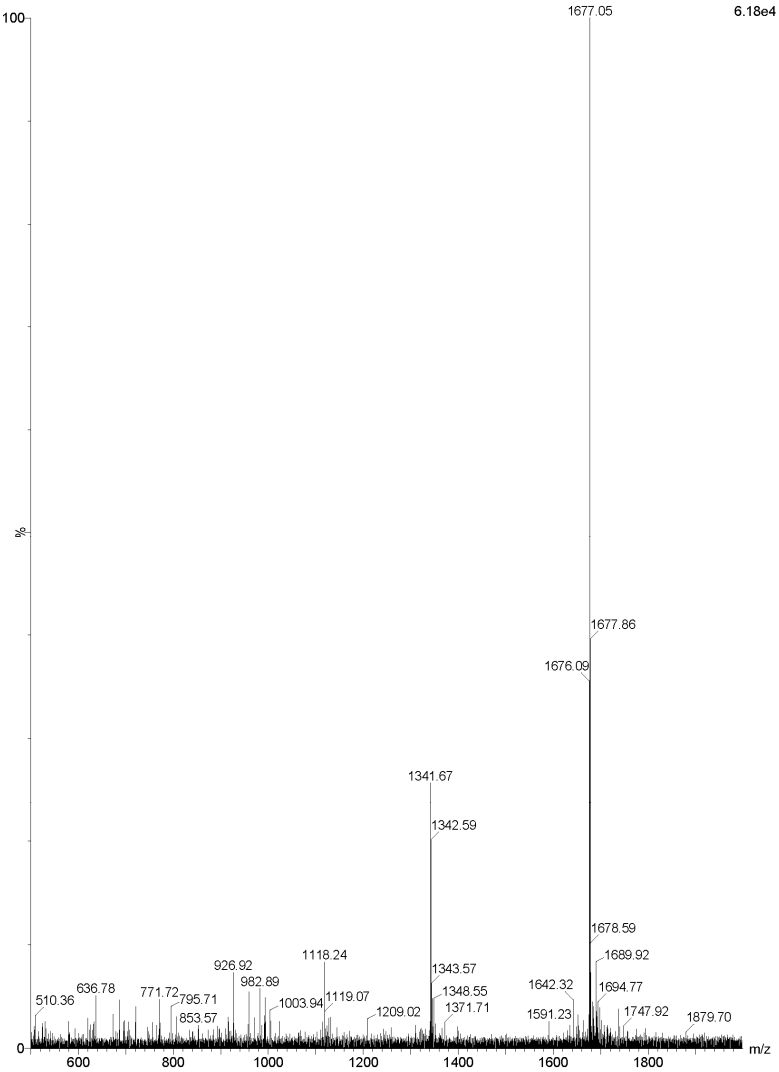


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3773.80 (MH^+): Major observed ions: 1258.58 (+3), 1887.97 (+2).

D-mGS2$^{38-90}$ (D-7)

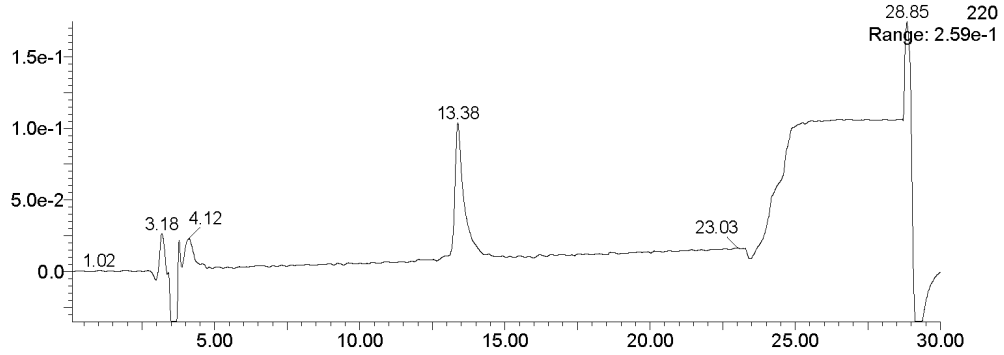


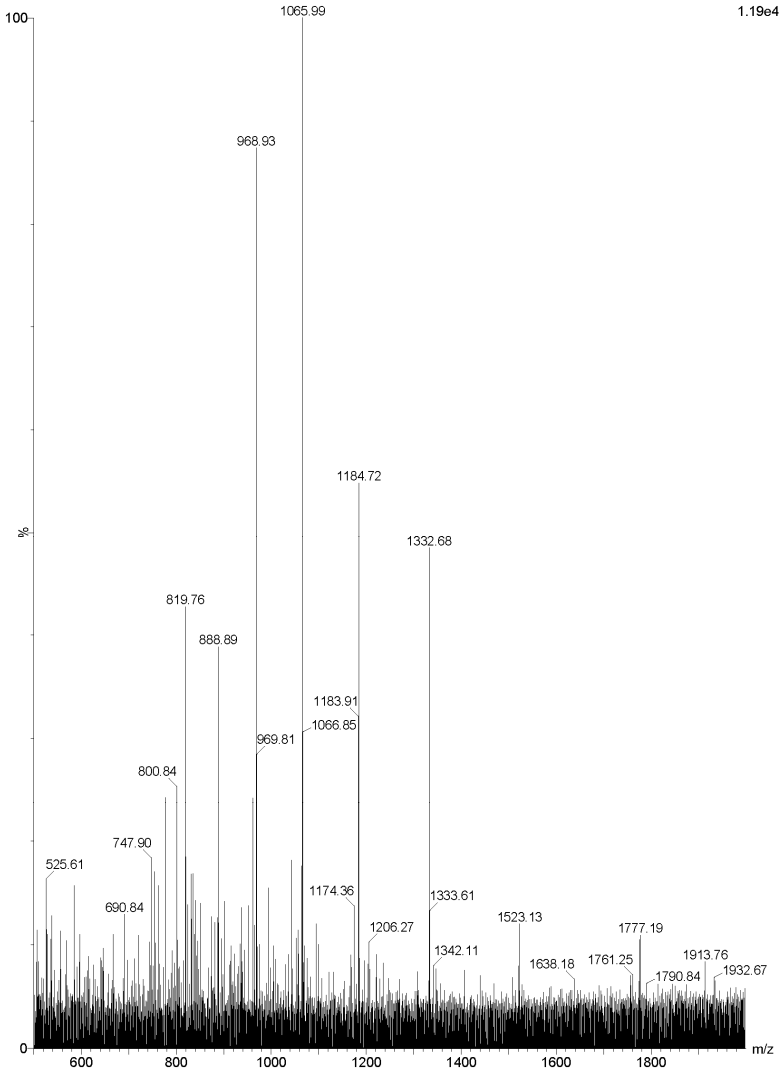


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 6705.18 (MH^+): Major observed ions: 1341.67 (+5), 1677.05 (+4).

D-mGS2 (D-6)

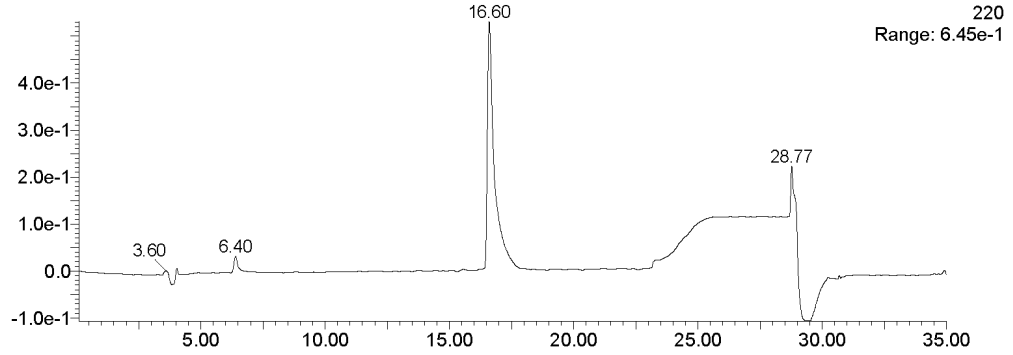


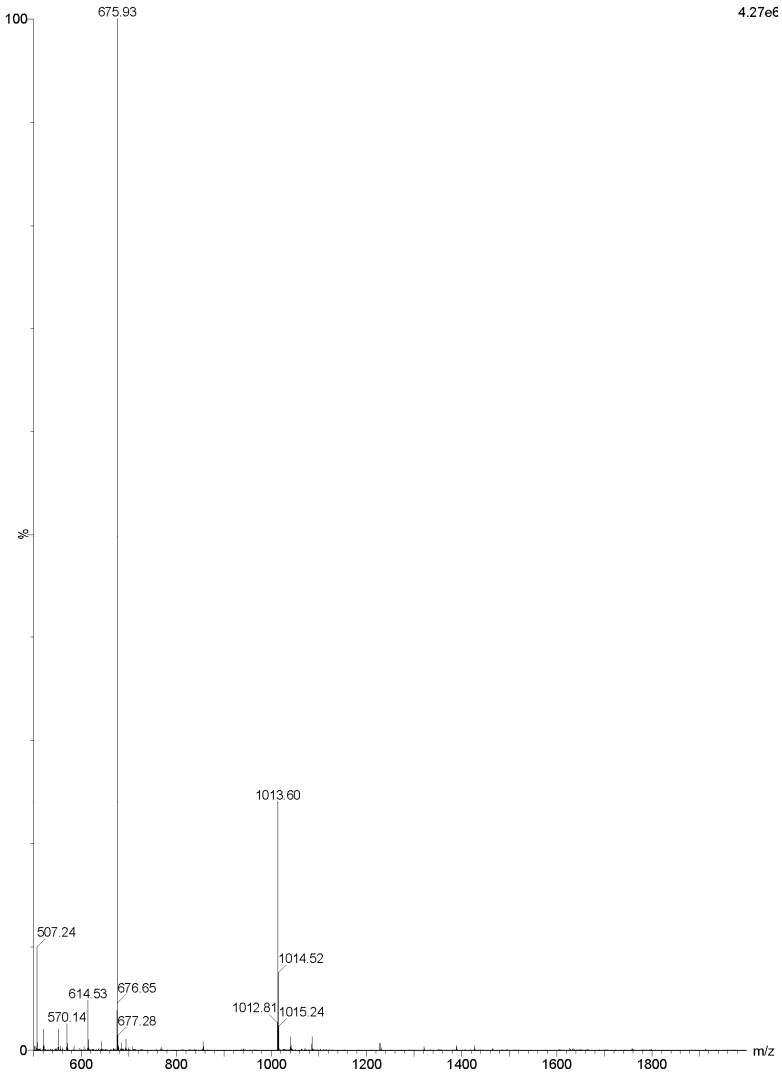


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 10653.23 (MH^+): Major observed ions: 968.93 (+11), 1065.99 (+10), 1184.72 (+9), 1332.68 (+8).

L-GS2$^{1\text{-}12}$ (L-S1)

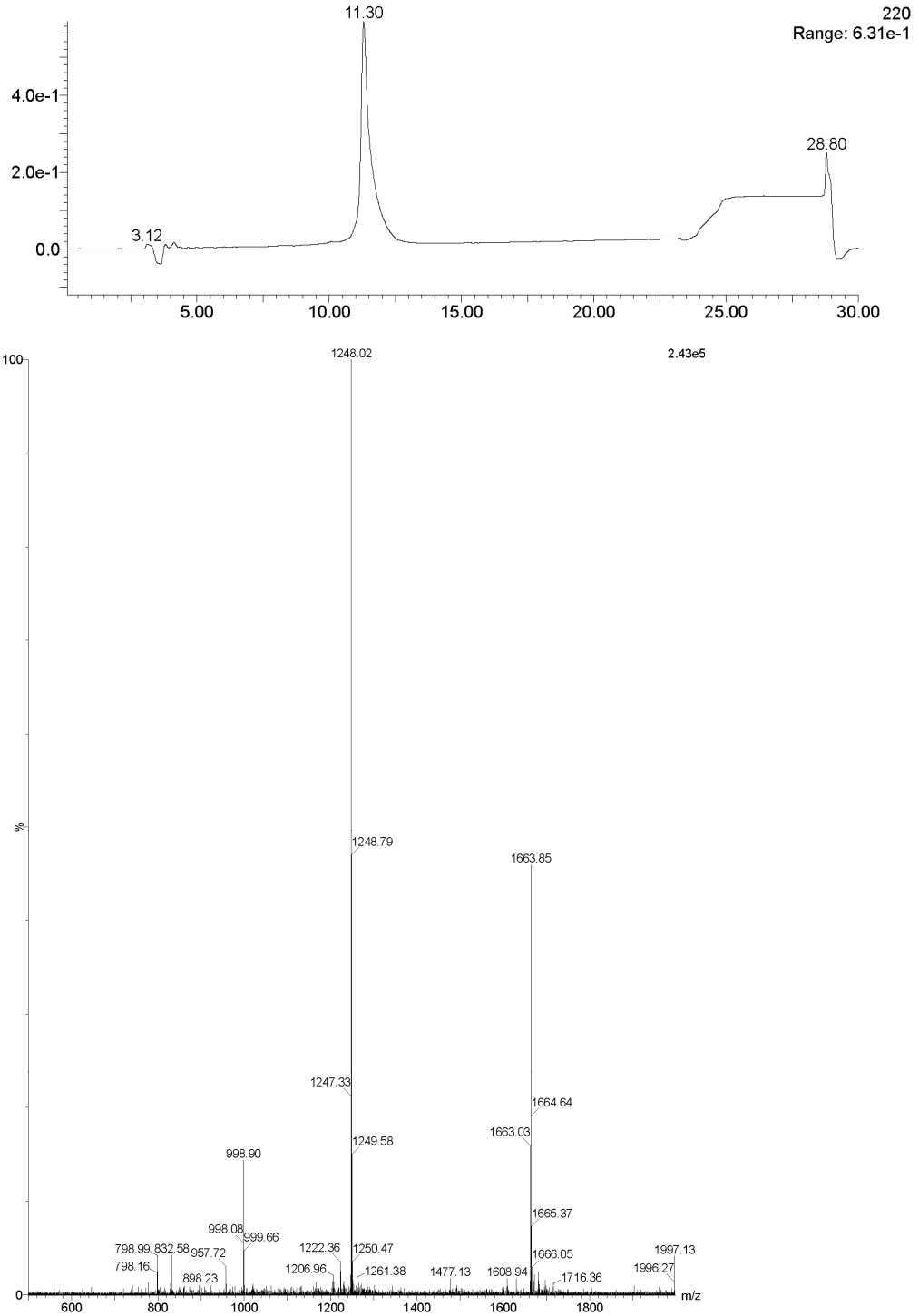




Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 10-30% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 2026.18 (MH^+): Major observed ions: 675.93 (+3), 1013.60 (+2).

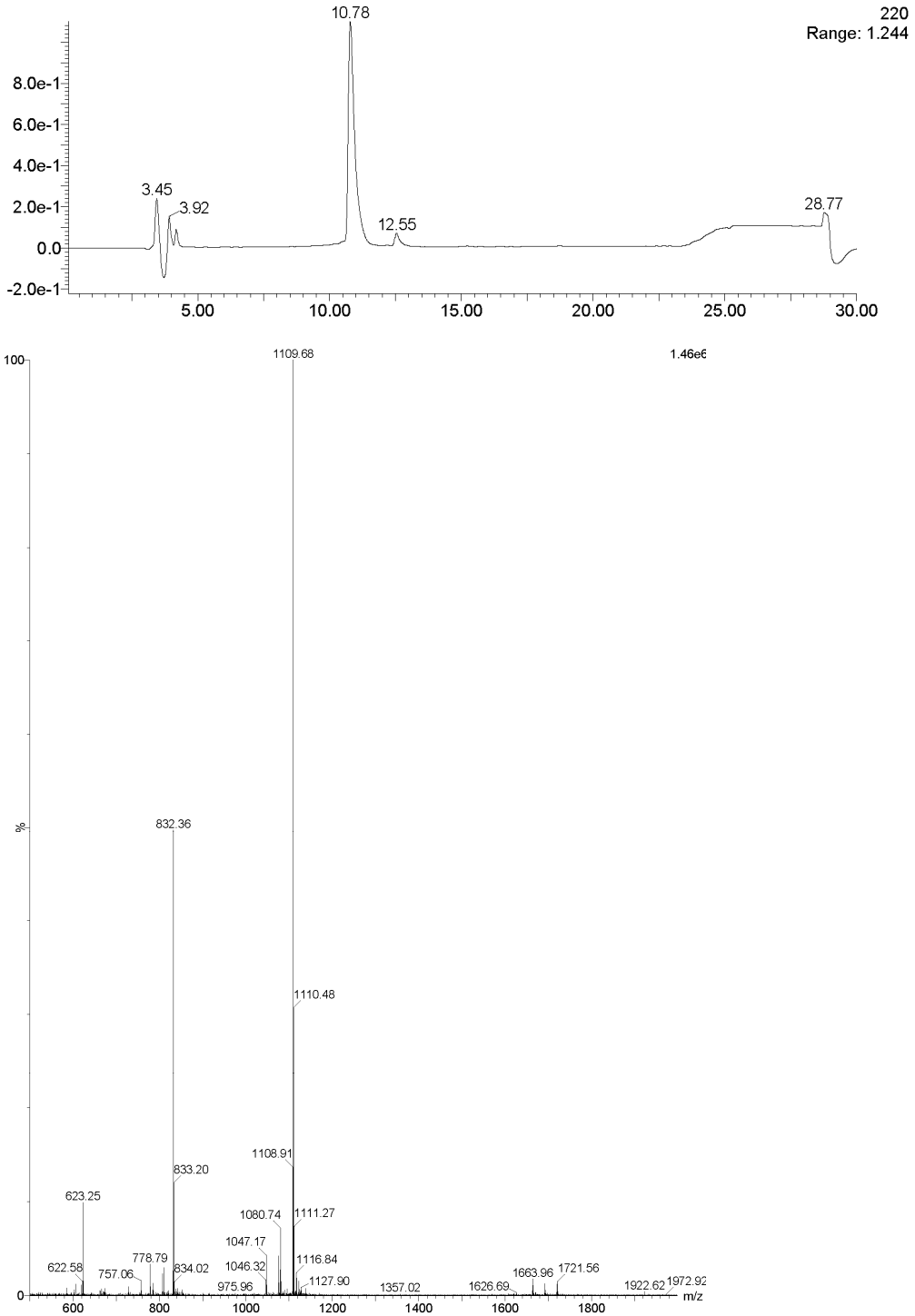
L-GS2$^{13-48}$ (L-S2)



Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4991.42 (MH^+): Major observed ions: 998.90 (+5), 1248.02 (+4), 1663.85 (+3).

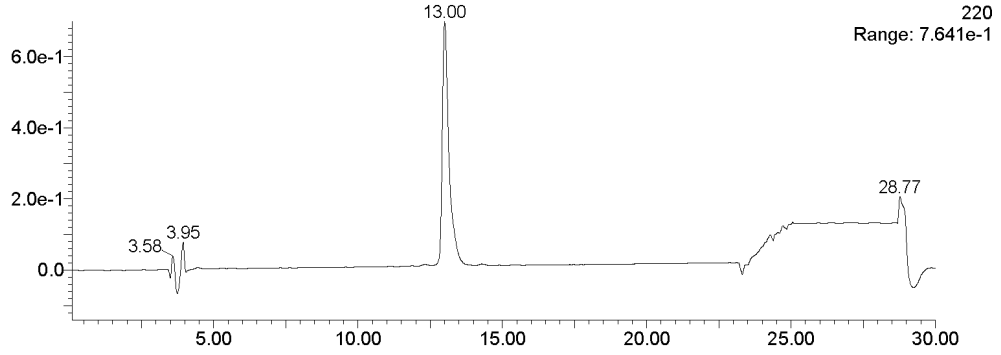
L-GS2$^{49\text{-}73}$ (L-S3)

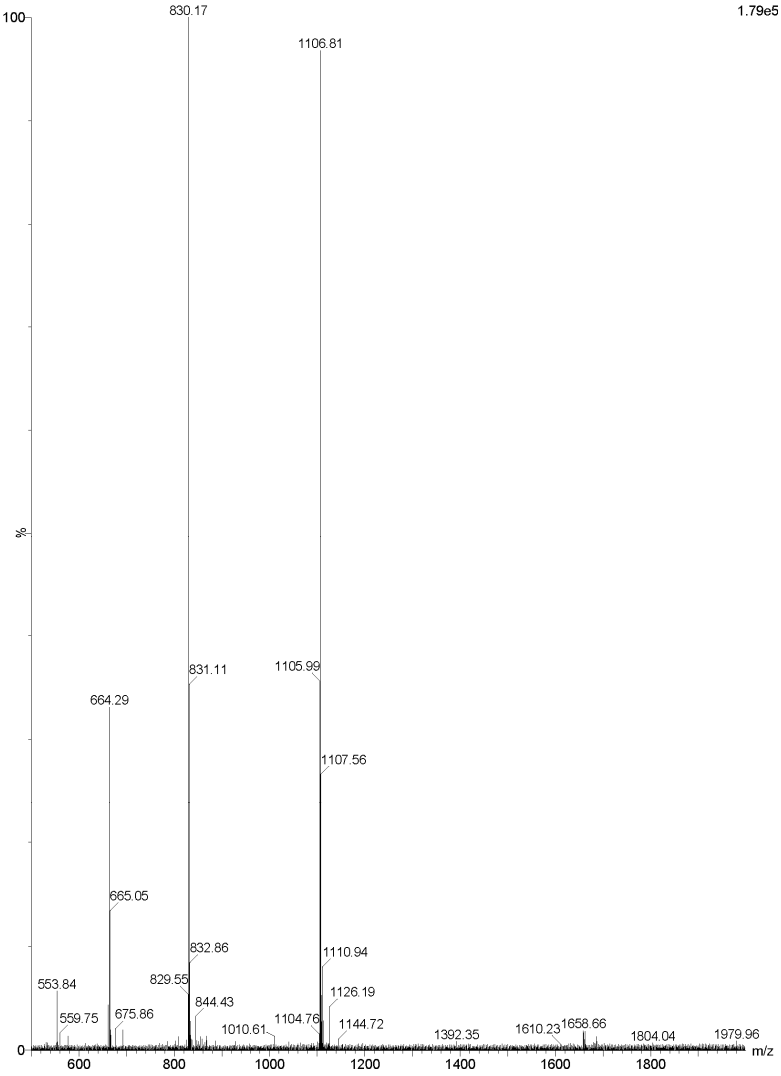


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3326.73 (MH^+): Major observed ions: 832.36 (+4), 1109.68 (+3).

L-GS2$^{74\text{-}90/biotin}$ (L-S4)

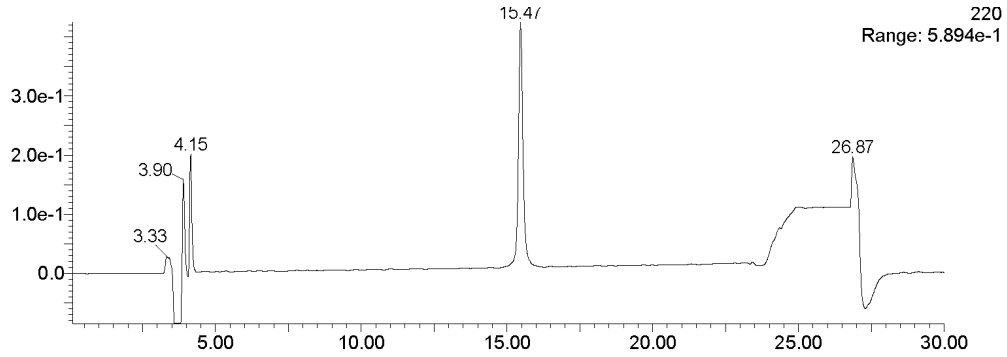


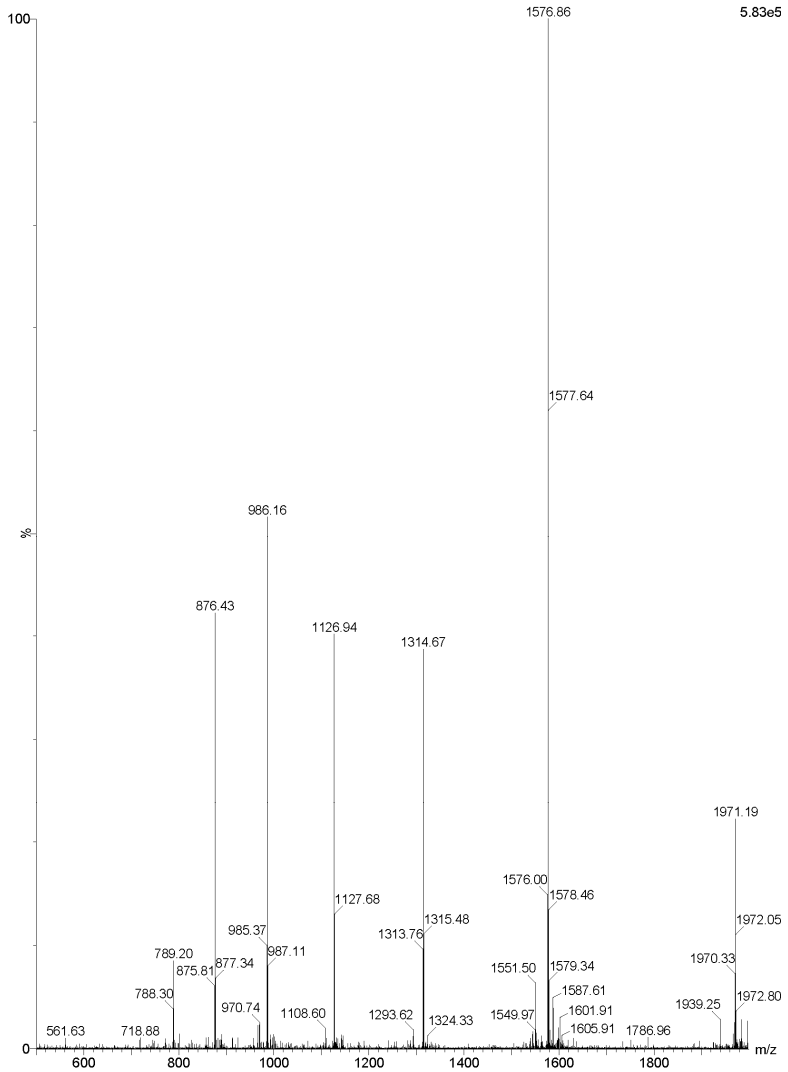


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3315.58 (MH^+): Major observed ions: 829.51 (+4), 1105.72 (+3), 1657.55 (+2).

[Cys(Trt-K_{10})^{13}]-L-GS2$^{1-48}$ (L-S5)

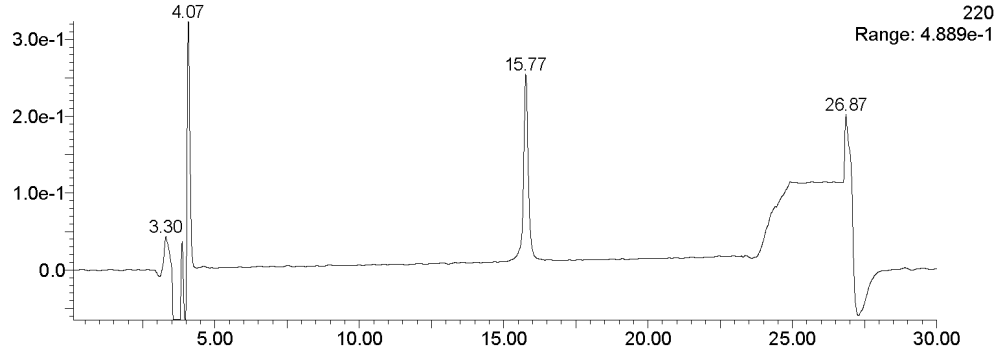


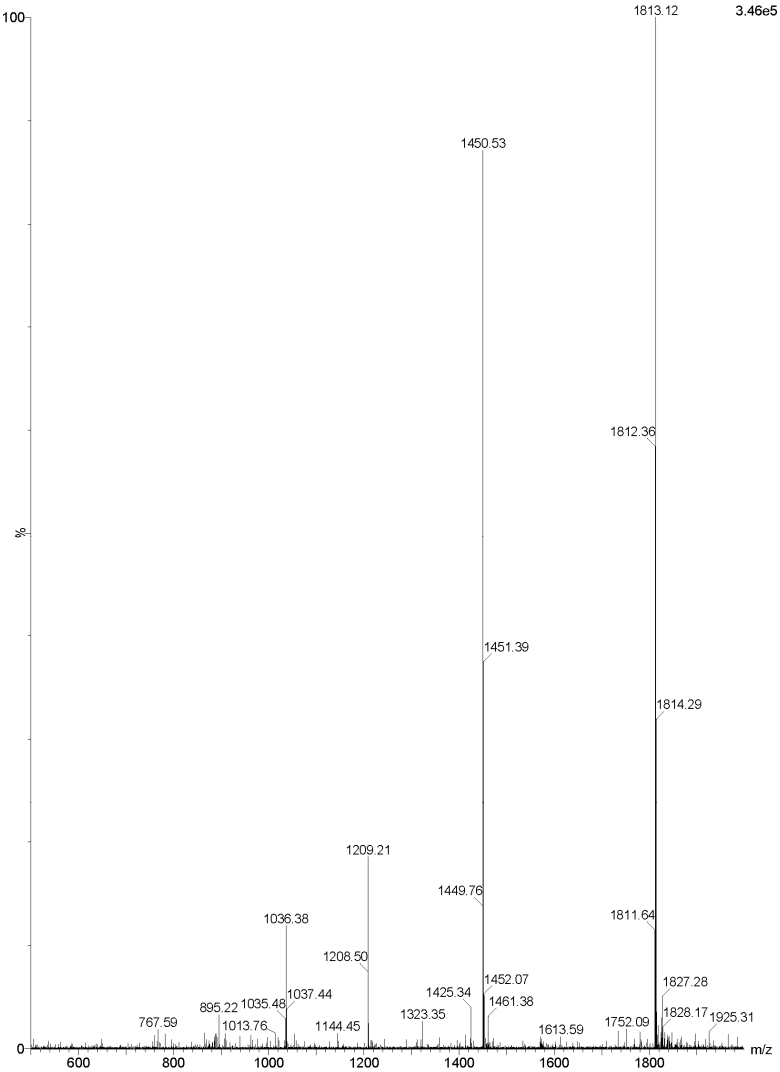


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 7884.21 (MH^+): Major observed ions: 986.16 (+8), 1126.94 (+7), 1314.67 (+6), 1576.86 (+5).

[Cys(Trt-K_{10})^{13}]-L-GS2$^{1-48}$ MES ester (L-S6)

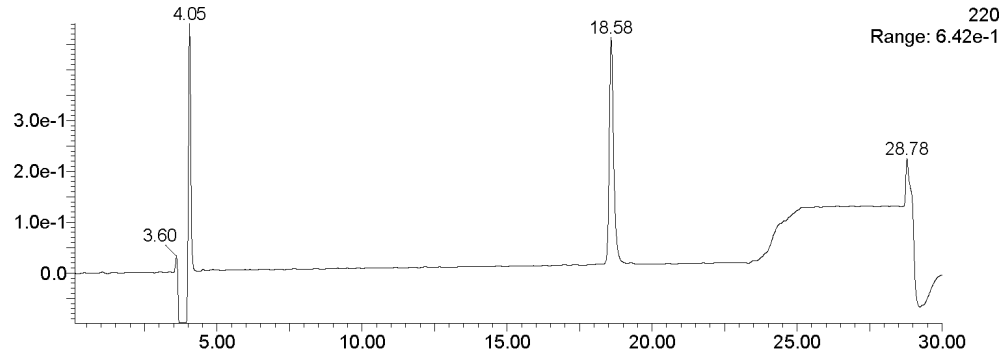


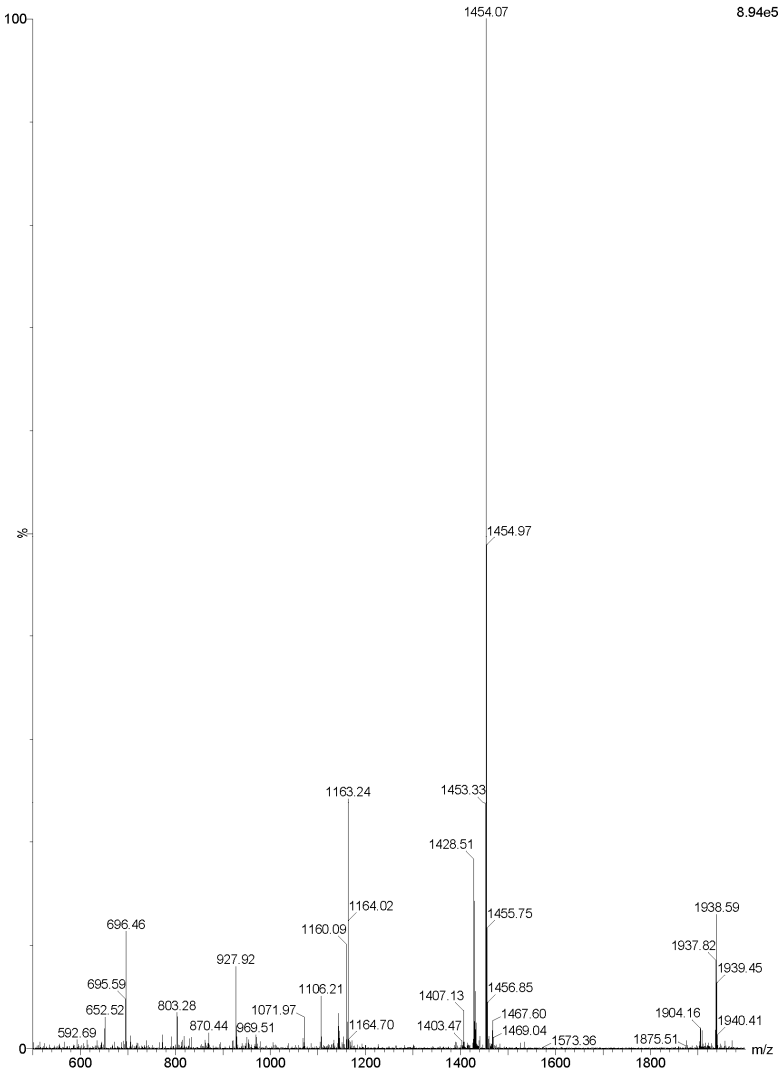


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 7249.71 (MH^+): Major observed ions: 1209.21 (+6), 1450.53 (+5), 1813.12 (+4).

[Cys$^{49, 74}$]-L-GS2$^{49\text{-}90/\text{biotin}}$ (L-S7)

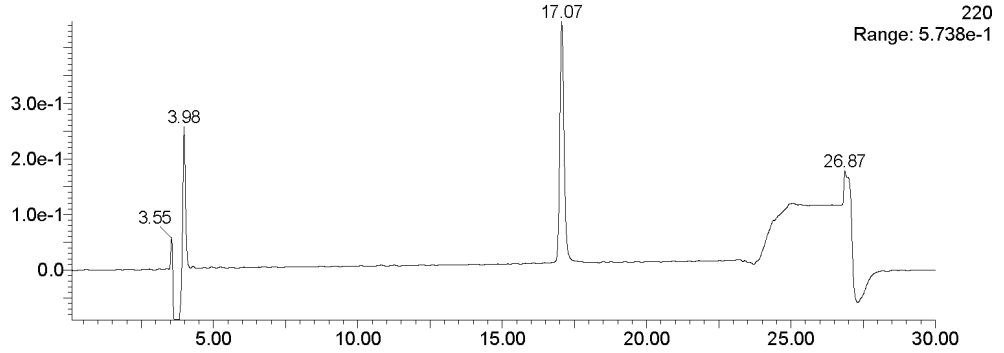


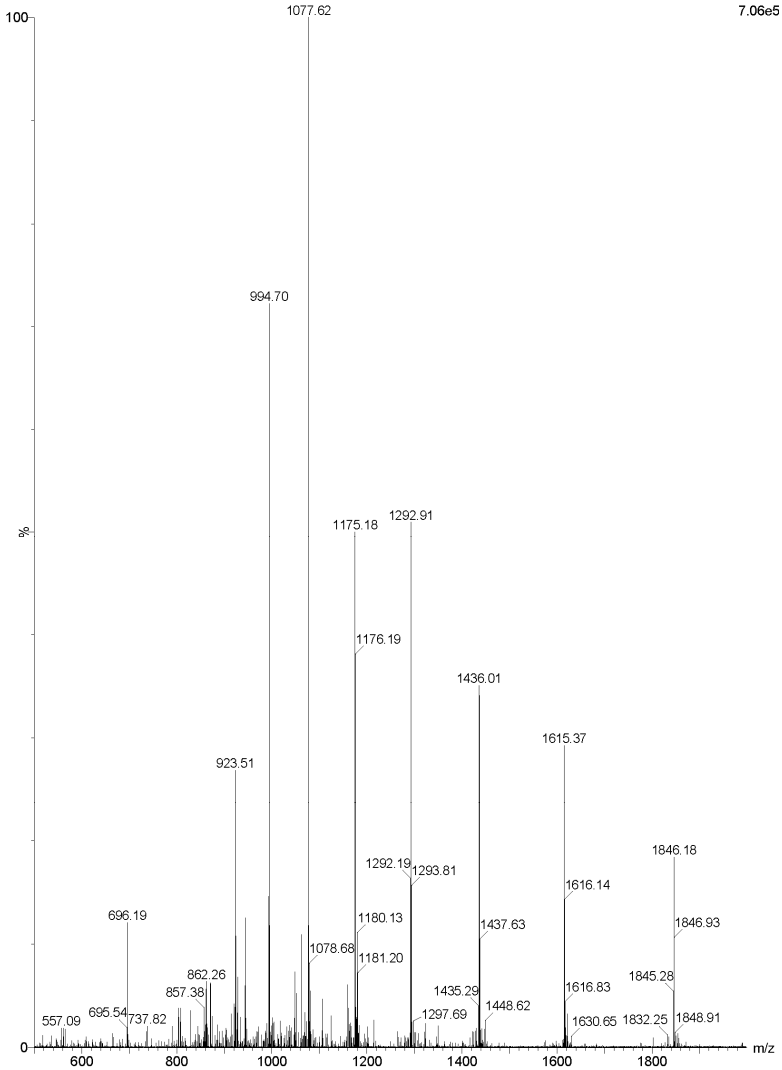


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 5813.83 (MH^+): Major observed ions: 1163.24 (+5), 1454.07 (+4), 1938.59 (+3).

[Cys(Trt-K_{10})^{13}, Cys$^{49, 74}$]-L-GS2^{biotin} (L-S8)

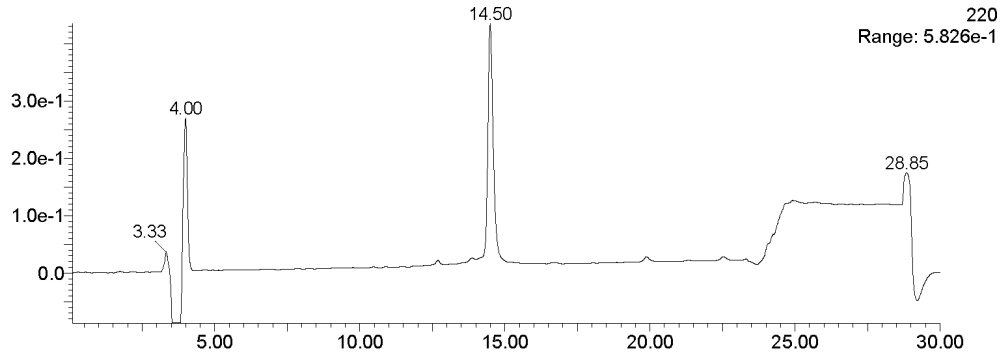


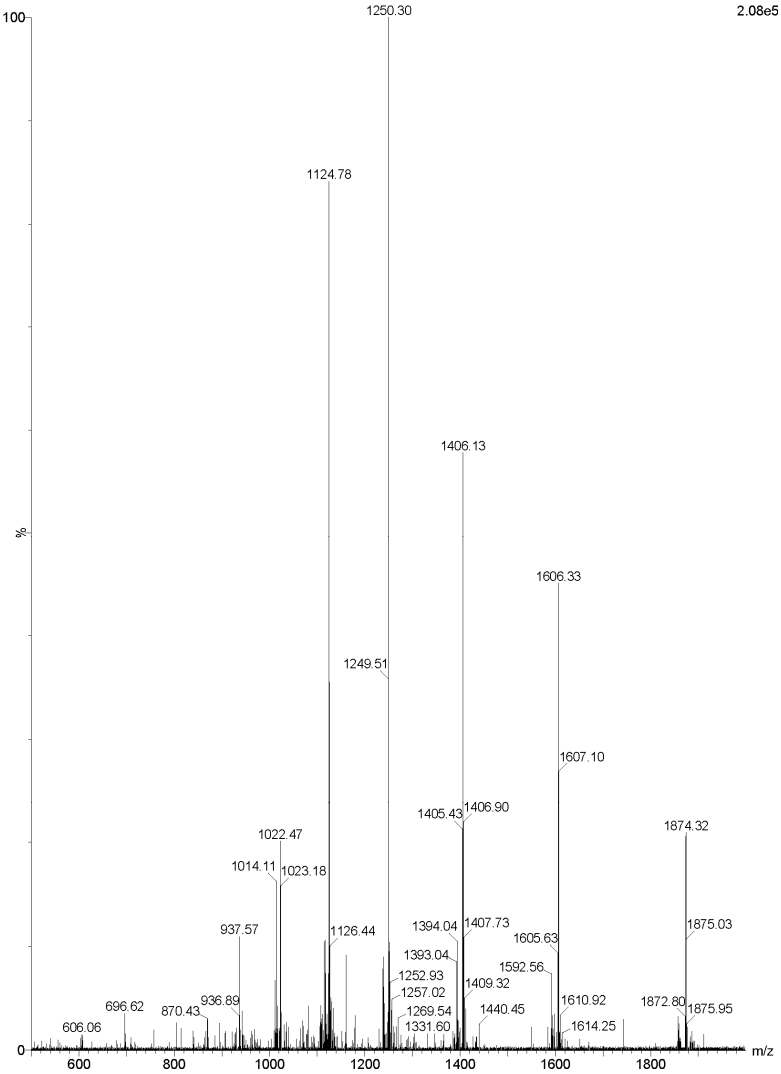


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 12920.55 (MH^+): Major observed ions: 994.70 (+13), 1077.62 (+12), 1175.18 (+11), 1292.91 (+10).

[Cys$^{13, 49, 74}$]-L-GS2^{biotin} (L-S9)

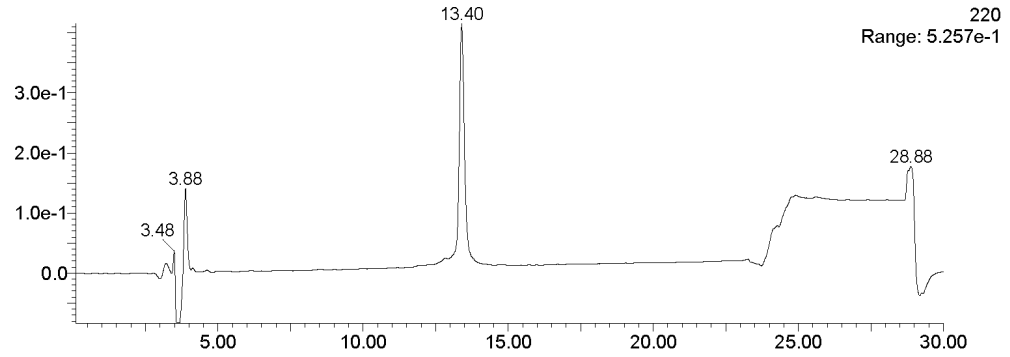


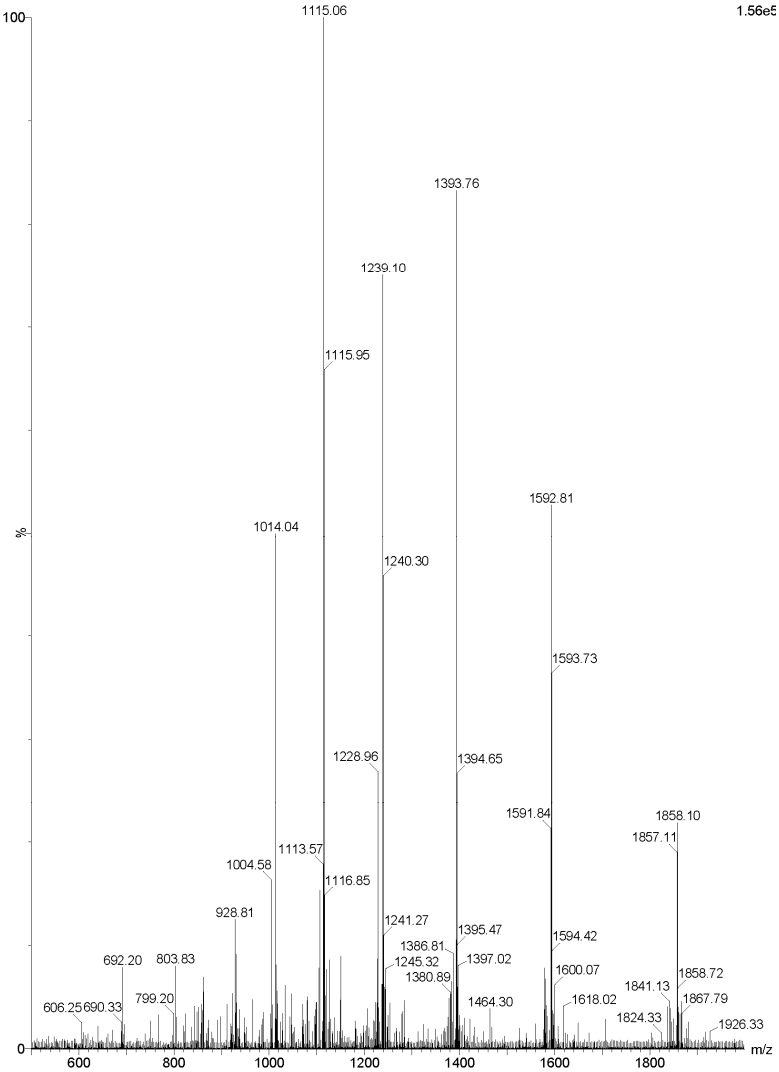


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 11239.46 (MH^+): Major observed ions: 1124.78 (+11), 1250.30 (+10), 1406.13 (+9), 1606.33 (+8).

L-GS2^{biotin} (L-S10)

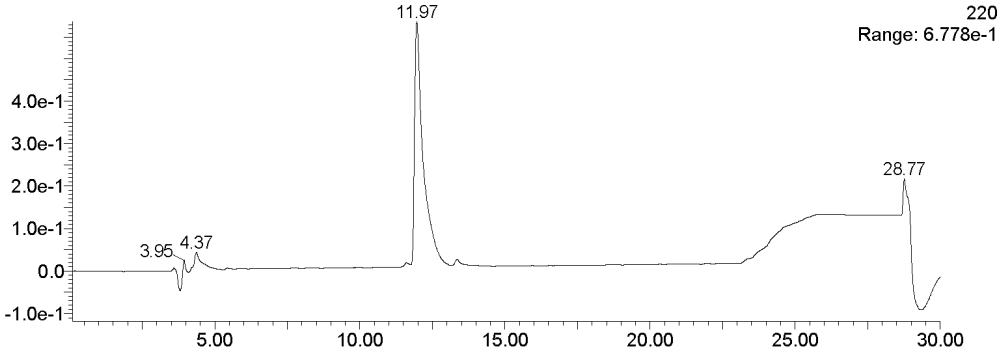


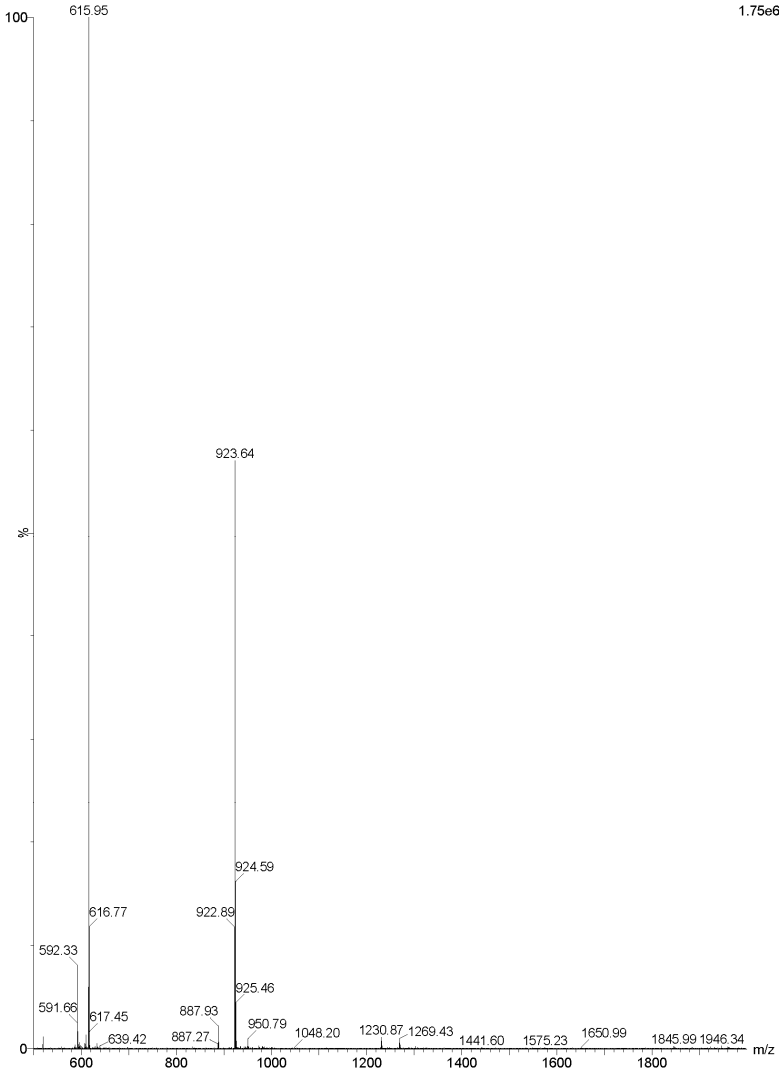


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 11143.54 (MH^+): Major observed ions: 1115.06 (+10), 1239.10 (+9), 1393.76 (+8), 1592.81 (+7).

L-mGS2$^{67\text{-}73}$ (L-S11)

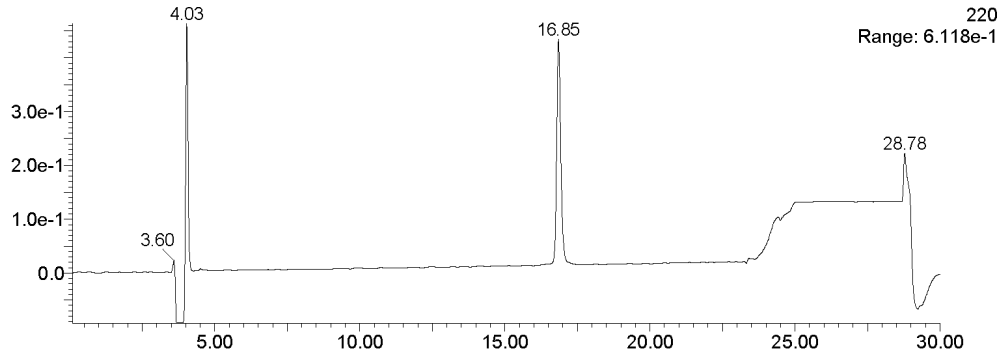


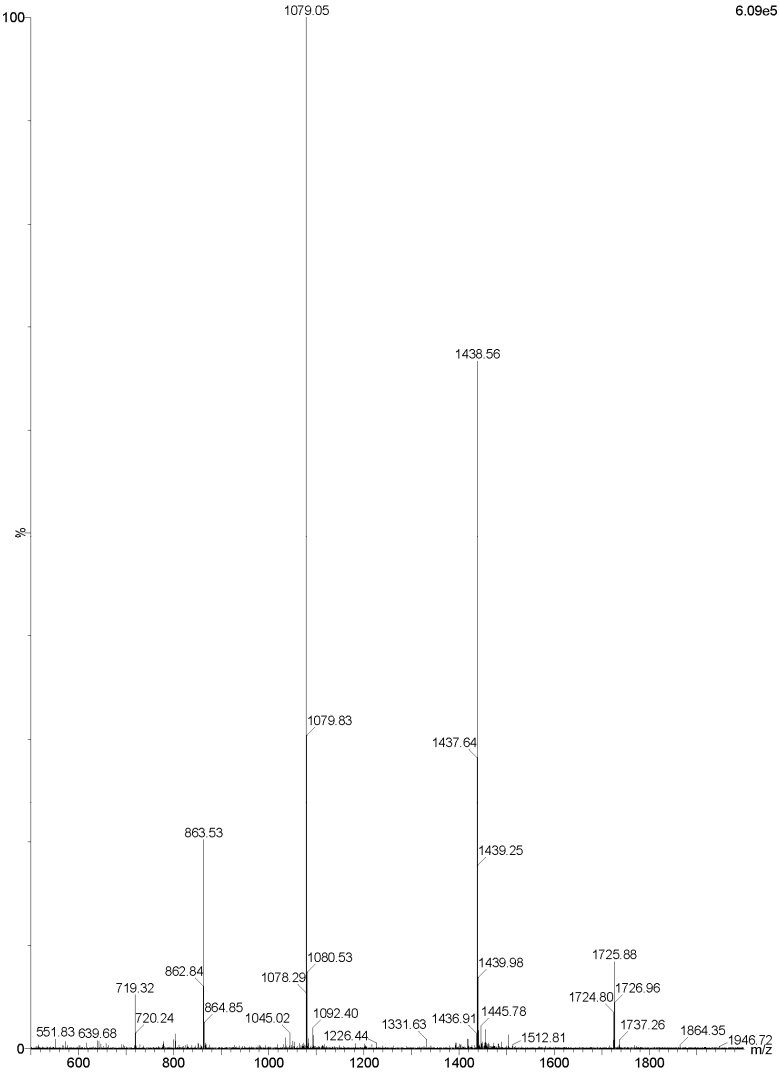


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 15-35% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 1845.93 (MH^+): Major observed ions: 615.95 (+3), 923.64 (+2).

[Cys(Acm)^{66}]-L-mGS2$^{66\text{-}90/\text{biotin}}$ (L-S12)

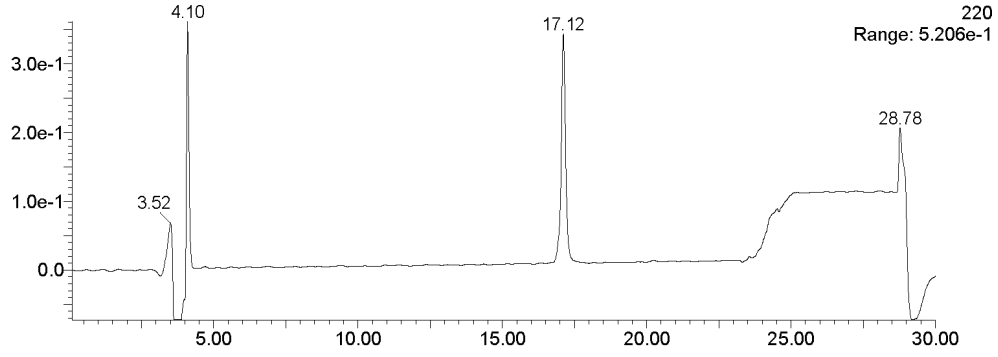


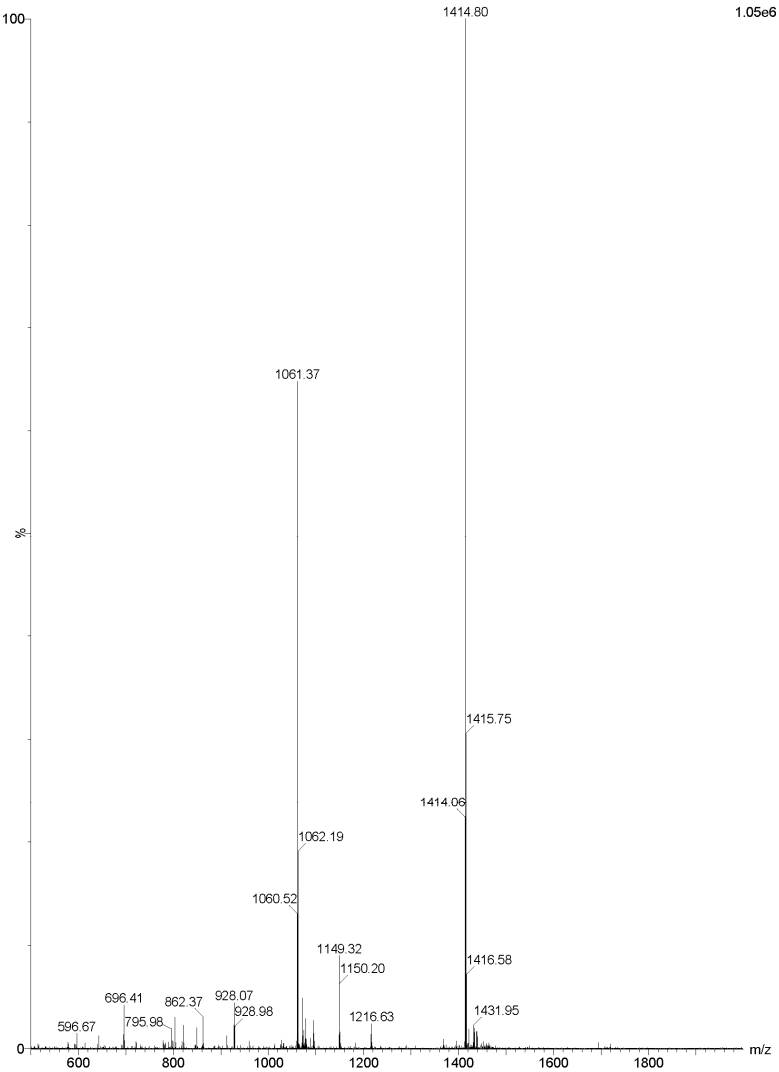


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4314.06 (MH^+): Major observed ions: 863.53 (+5), 1079.05 (+4), 1438.56 (+3).

L-mGS2$^{66\text{-}90/biotin}$ (L-S13)

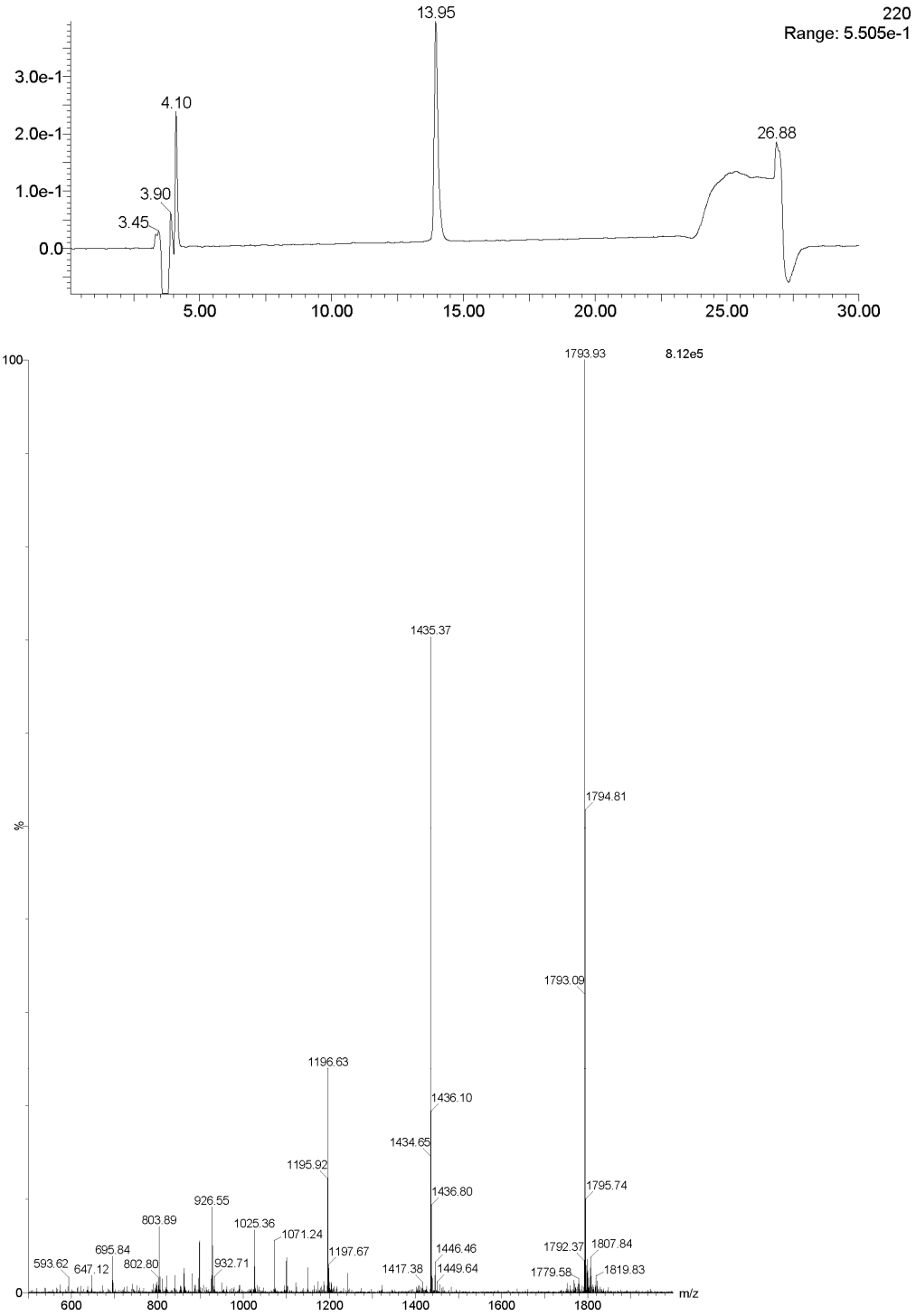




Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4243.02 (MH^+): Major observed ions: 1061.37 (+4), 1414.80 (+3).

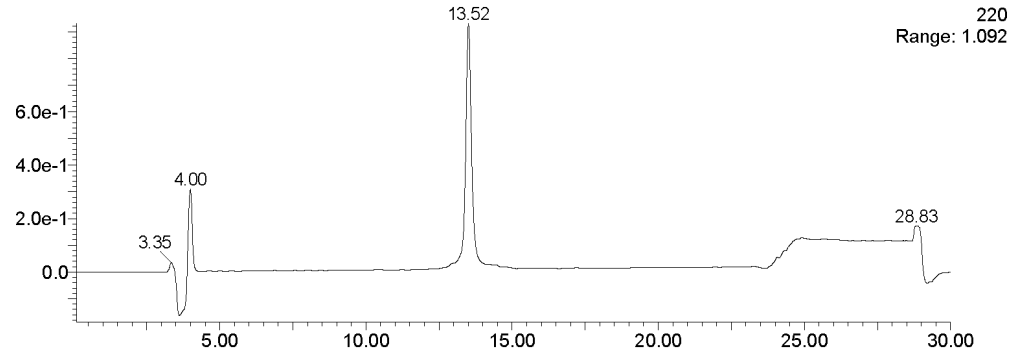
L-mGS2$^{38\text{-}90/biotin}$ (L-S14)

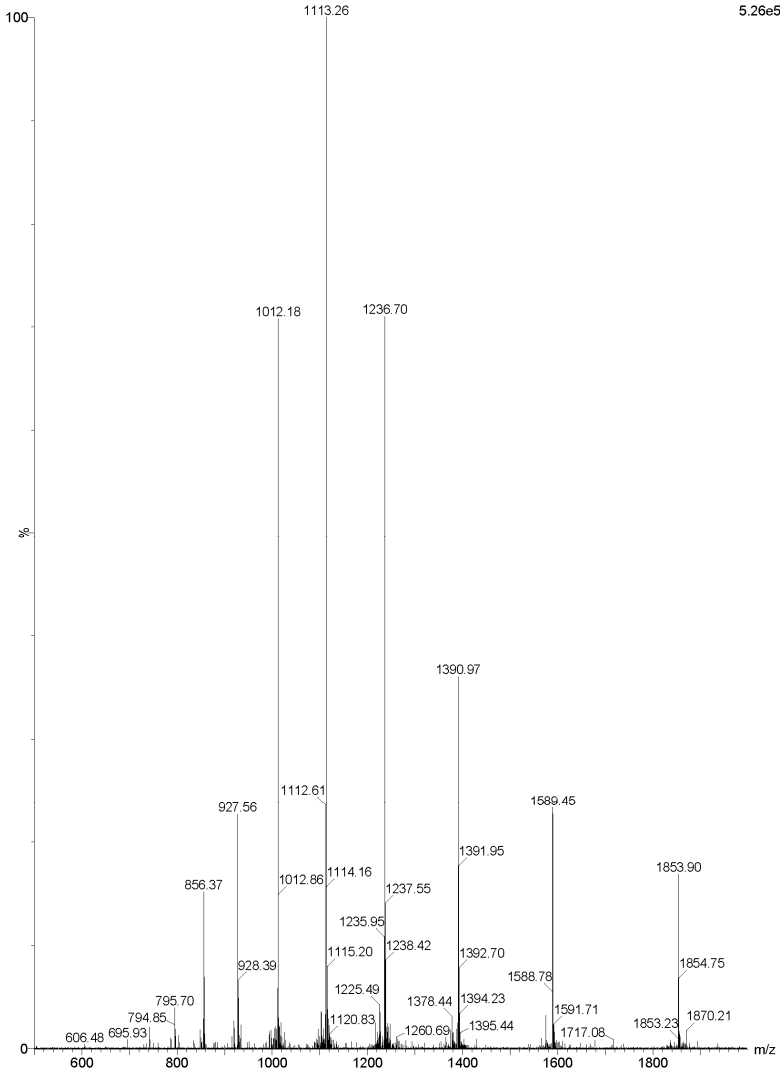


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 7174.40 (MH^+): Major observed ions: 1196.63 (+6), 1435.37 (+5), 1793.93 (+4).

L-mGS2^{biotin} (L-S15)

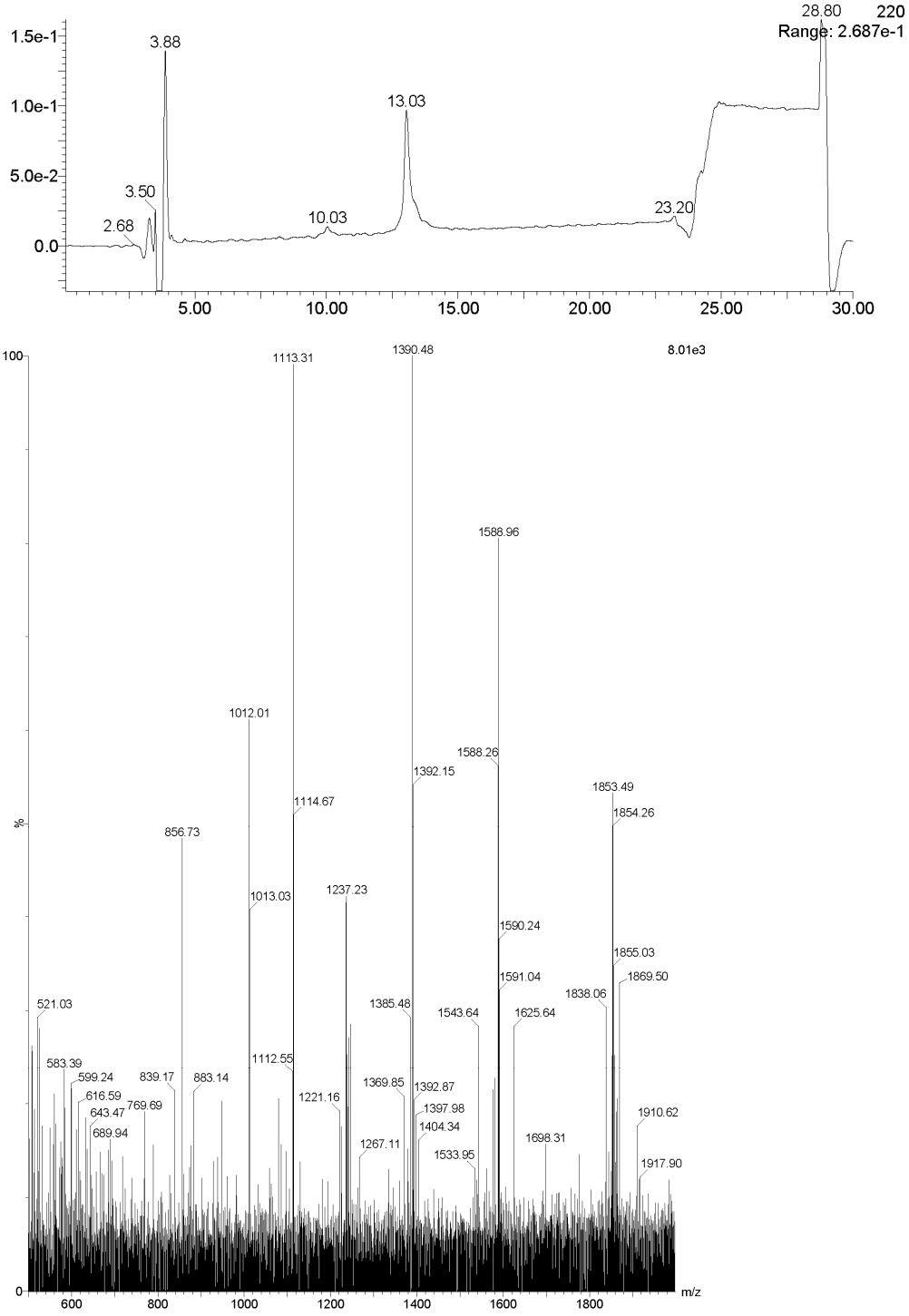




Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 11121.45 (MH^+): Major observed ions: 1012.18 (+11), 1113.26 (+10), 1236.70 (+9), 1390.97 (+8).

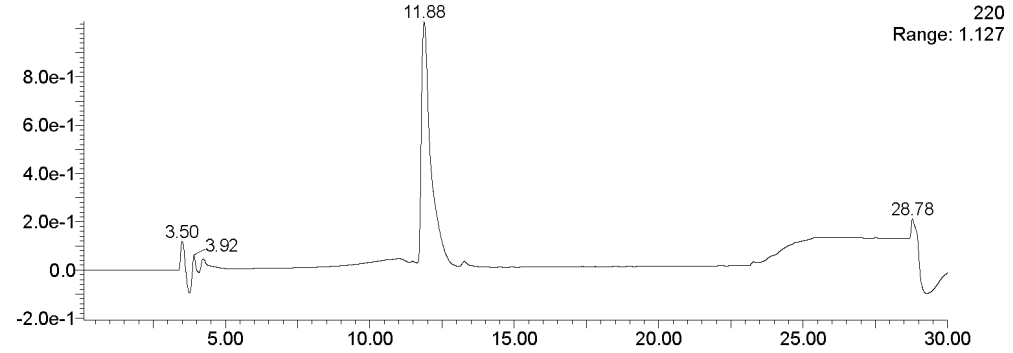
L-mGS2$^{SS/biotin}$ (L-S16)

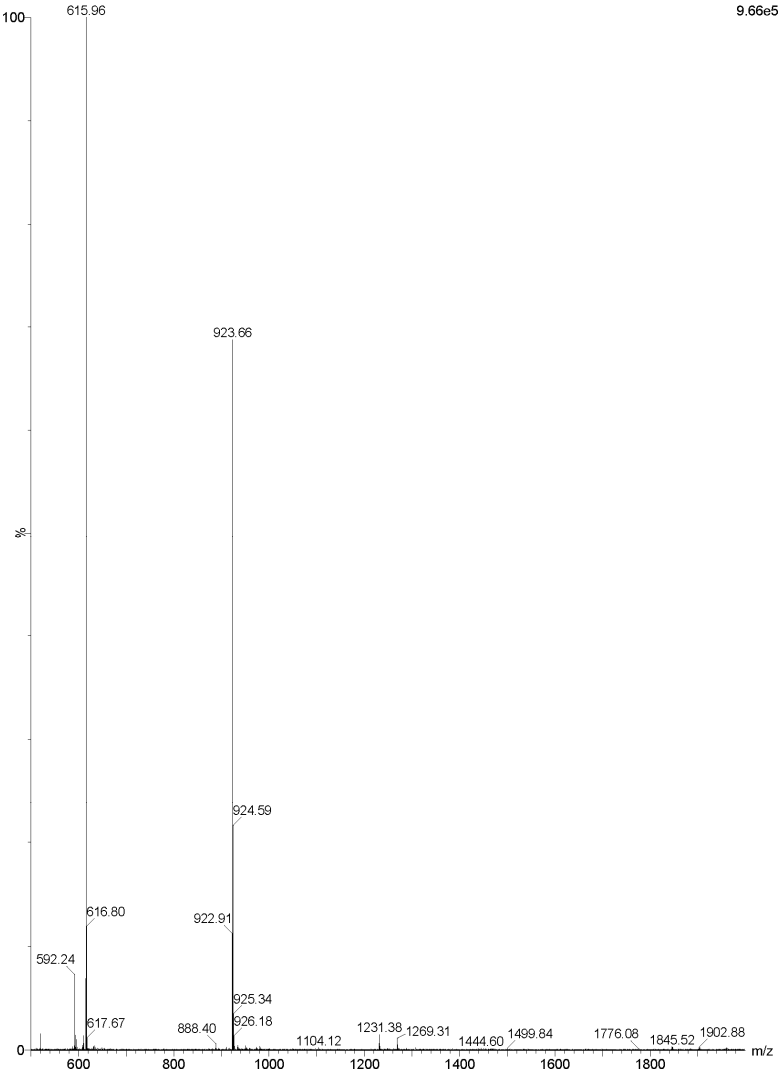


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 11119.43 (MH^+): Major observed ions: 1012.01 (+11), 1113.31 (+10), 1237.23 (+9), 1390.48 (+8), 1588.96 (+7).

D-mGS2$^{67-73}$ (D-S11)

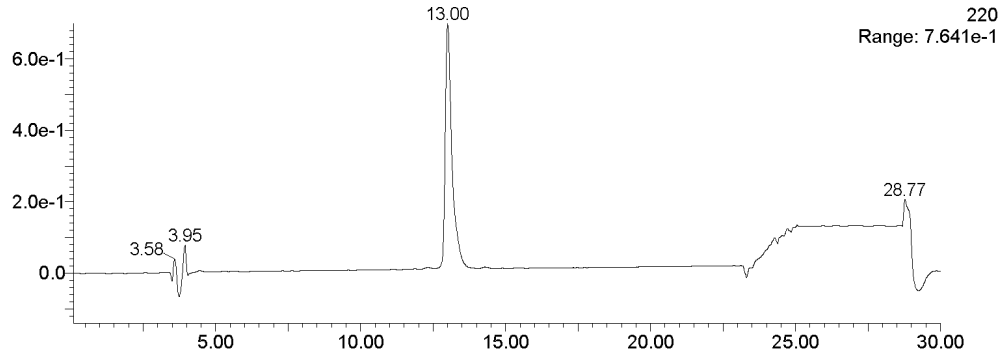


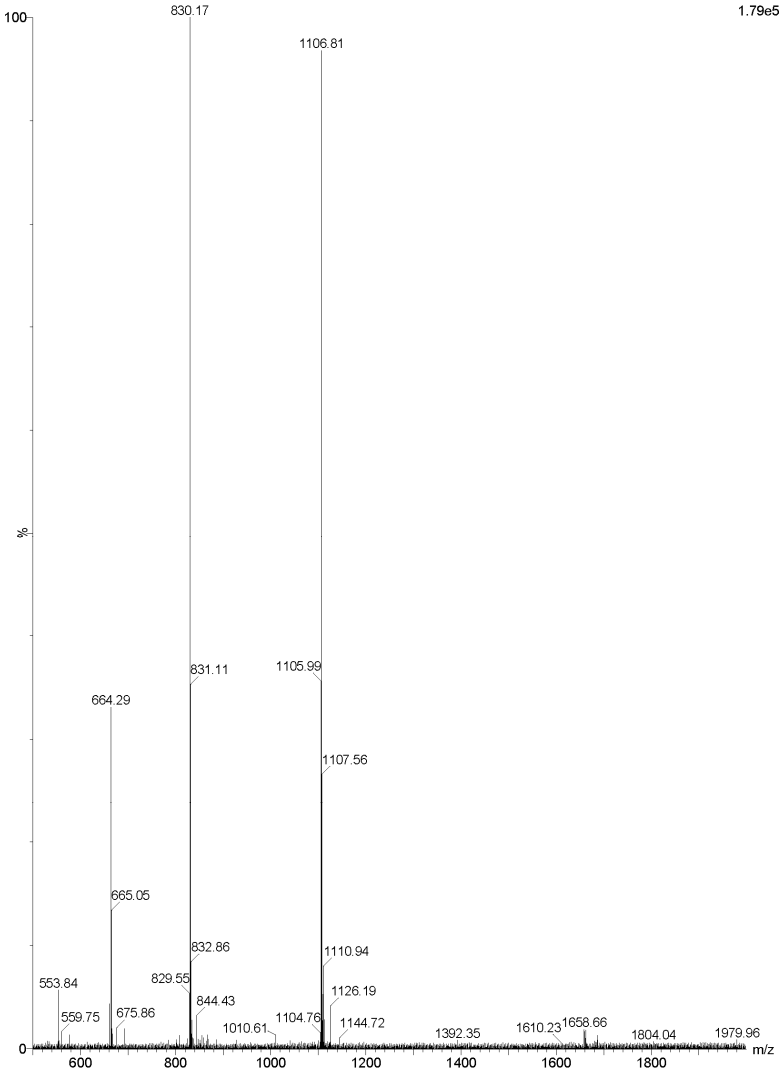


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 15-35% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 1845.93 (MH^+): Major observed ions: 615.96 (+3), 923.66 (+2).

D-mGS2$^{74\text{-}90/biotin}$ (D-S4)

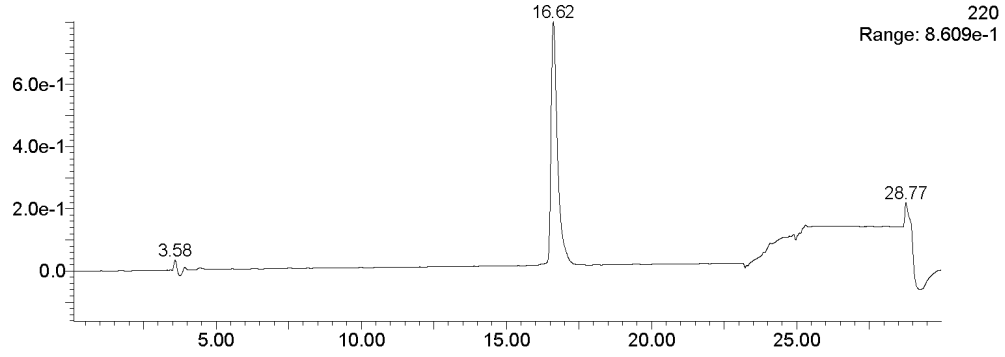


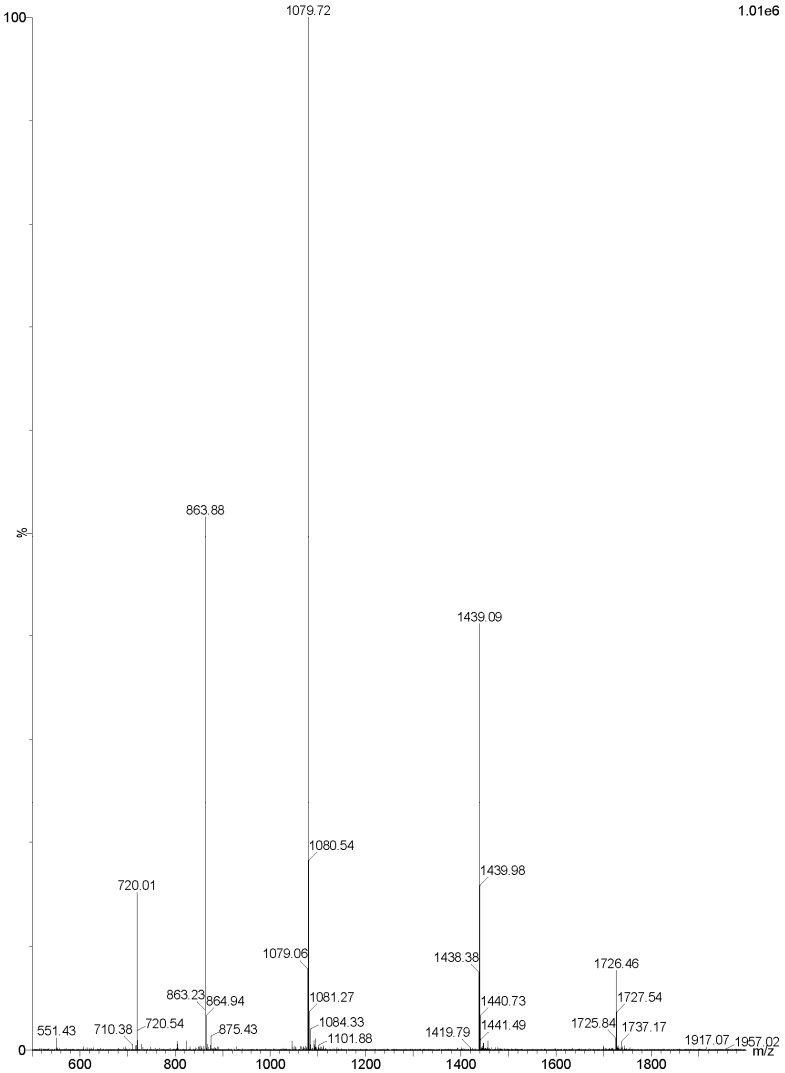


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 3315.58 (MH^+): Major observed ions: 830.17 (+4), 1106.81 (+3).

[Cys(Acm)^{66}]-D-mGS2$^{66\text{-}90/\text{biotin}}$ (D-S12)

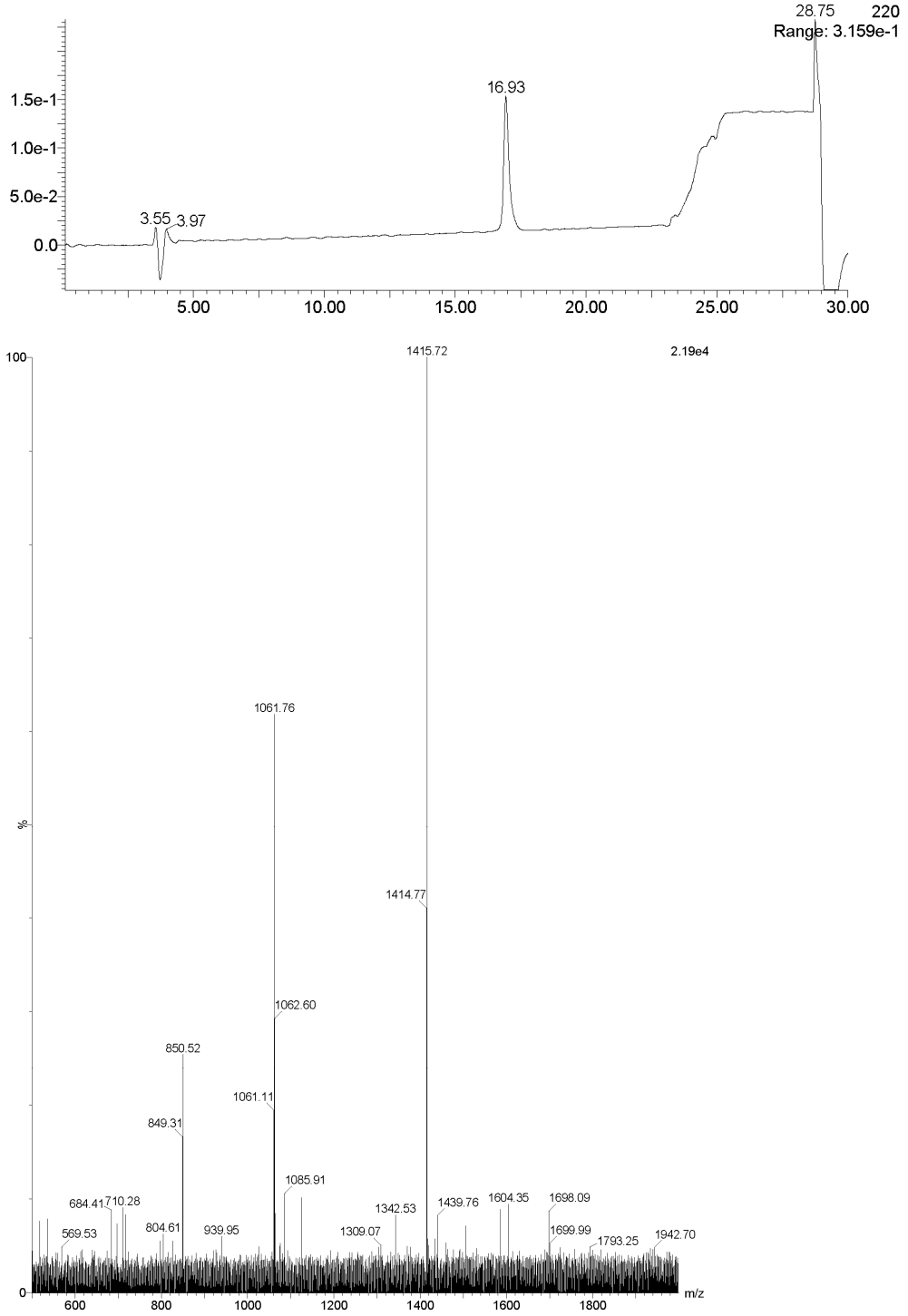




Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4314.06 (MH^+): Major observed ions: 863.88 (+5), 1079.72 (+4), 1439.09 (+3).

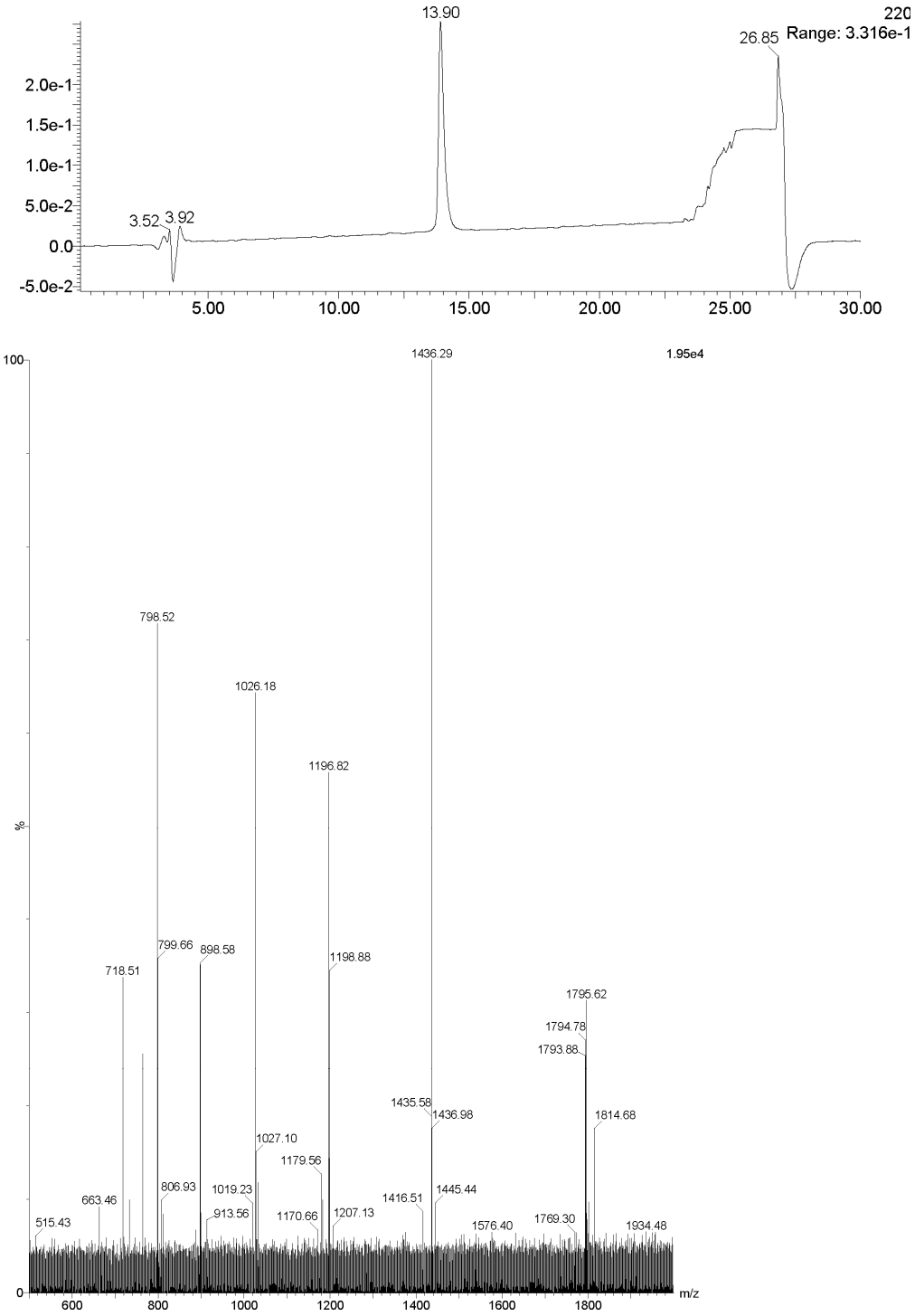
D-mGS2$^{66\text{-}90/biotin}$ (D-S13)



Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 20-40% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 4243.02 (MH^+): Major observed ions: 1061.76 (+4), 1415.72 (+3).

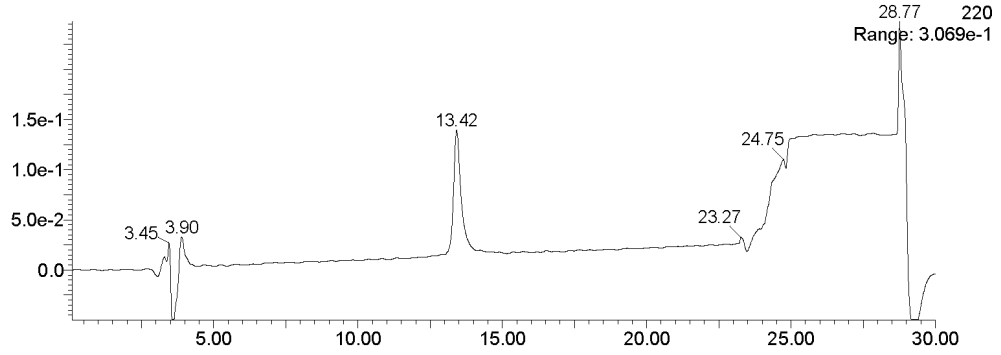
D-mGS2$^{38\text{-}90/biotin}$ (D-S14)

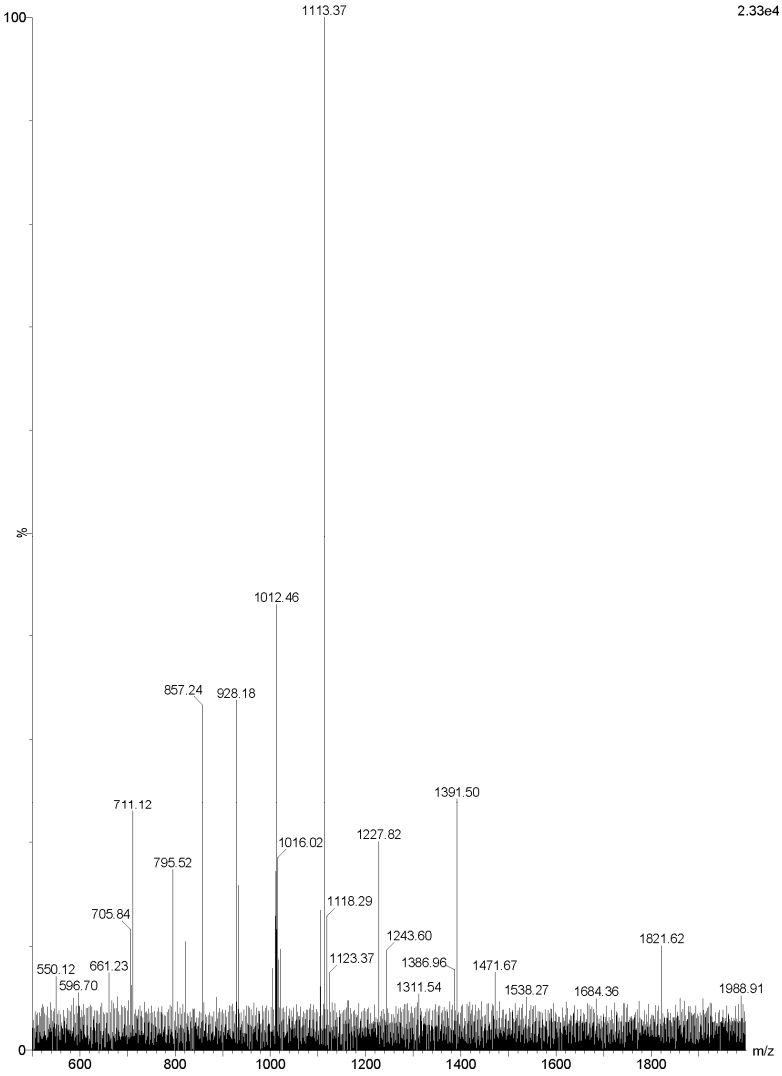


Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 25-45% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 7174.40 (MH^+): Major observed ions: 1026.18 (+7), 1196.82 (+6), 1436.29 (+5).

D-mGS2^{biotin} (D-S15)





Analytical HPLC trace of the purified product: Cosmosil 5C18-AR300 column (Nacalai Tesque, 4.6 × 250 mm). Linear gradient of 30-50% CH_3CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Mass spec analysis of the purified product: Expected mass based on the sequence: 11121.45 (MH^+): Major observed ions: 928.16 (+12), 1012.46 (+11), 1113.37 (+10), 1391.50 (+8).

References

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S2 Wan, Q.; Danishefsky, S. J. Free-radical-based, specific desulfurization of cysteines: a powerful advance in the synthesis of polypeptides and glycopolypeptides. *Angew. Chem. Int. Ed.* **2007**, *46*, 9248-9252.

S3 Hunter, M. J.; Komives, E. A. Deprotection of *S*-acetamidemethyl cysteine-containing peptides by silver. *Anal. Biochem.* **1995**, *228*, 173-177.