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Synthesis of a Glycosylated Peptide Thioester by the Boc Strategy and Its Application to Segment Condensation

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The synthesis of a chitobiosylated peptide thioester by the *t*-butoxycarbonyl (Boc) strategy is demonstrated. Boc-Asn carrying benzyl-protected chitobiose was introduced during application of the Boc mode solid-phase method. HF treatment of the resulting protected peptide resin gave the desired chitobiosylated peptide thioester. This thioester was used to prepare the peptide sequence derived from extracellular matrix metalloproteinase inducers (emmprin) (34-94), (34-118) and (22-118) by the thioester segment condensation method. The conformation of these glycopeptides is characterized by circular dichroism (CD) spectral measurement.

Key words: glycosylated peptide thioester; solid-phase synthesis; segment condensation

Peptide thioesters have been widely used as key intermediates for the preparation of polypeptides by segment condensation strategies such as the thioester method[1,2] and native chemical ligation method.[3,4] The former method involves the thioester group of the *N*-terminal partially-protected peptide being activated by silver ions and the amide bond formed with the *C*-terminal peptide. The latter uses the thioesterification reaction between the thioester group of the *N*-terminal peptide and the thiol group of the terminal cysteine residue of the other peptide, this being followed by the intramolecular *S* to *N* acyl migration reaction to form a native peptide bond. The preparation of peptide thioesters has been mainly carried out by the Boc strategy, in which the peptide chain is elongated by repetitive acid treatment to remove the Boc group and then deprotected by a stronger acid treatment such as with HF. Increasing interest in such post-translational modification as glycosylation has resulted in the development of thioester preparation by the Fmoc strategy which does not use a harsh acid treatment for the deprotection step.[5–16] These methods have been successfully used to prepare peptide thioesters carrying an *O*-linked single *N*-acetylgalactosamine,[7] seven *N*-acetylgalactosamines[17,18] and *N*-linked core pentasaccharide.[14,15] However, the Fmoc strategy does not always give satisfactory results. As an extreme case, Marcaurelle *et al.* have reported that they could not obtain a peptide thioester by the Fmoc strategy when using the safety catch linker because of failure of thiolysis at the final step, whereas the Boc strategy successfully gave the desired product.[19] Thus, depending on the peptide sequence, the Boc strategy is more reliable than Fmoc strategy. However, due to the sensitivity of glycosidic linkages to strong acids, the Boc strategy has seldom been applied to the preparation of glycosylated peptide thioesters.[20–23] Its applicability and limitations still remain unclear. We have briefly reported the preparation of a chitobiosylated thioester by the Boc strategy and its application to the synthesis of the extracellular matrix metalloproteinase inducer, emmprin[24,25] (34-94), by the thioester method.[23] We report here the full experimental details. In addition, the glycosylated peptide thioester was further applied to the synthesis of the larger glycopeptides, emmprin (34-118) **4** as well as (22-118) **5** by using repeated segment coupling by the thioester method (Fig. 1). The effect of chain length on the conformation was analyzed by CD spectral measurement of these glycopeptides.

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Abbreviations: Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; Boc-OSu, *N*-(*t*-butoxycarbonyloxy) succinimide; CD, circular dichroism; DCC, 1,3-dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HOOBt, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; Ig, immunoglobulin; MMP, matrix metalloproteinase; NMP, 1-methyl-2-pyrrolidinone; TFA, trifluoroacetic acid; THF, tetrahydrofuran

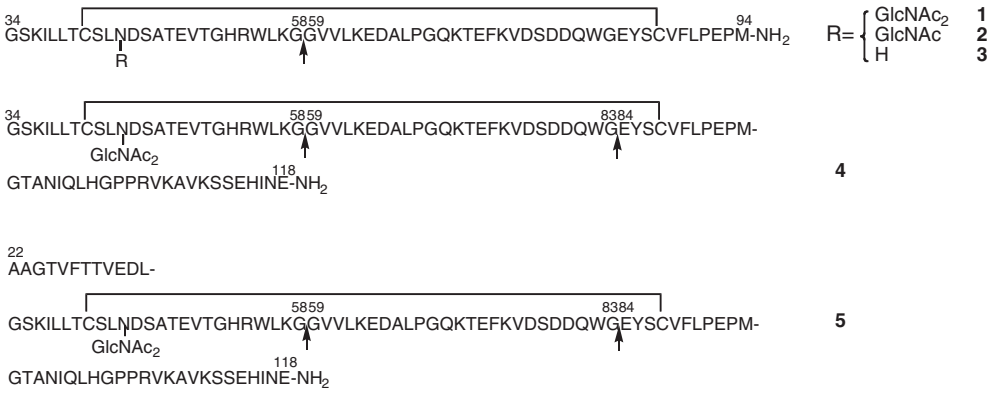

Fig. 1. Amino Acid Sequence of Emmprin (34-94), (34-118) and (22-118). The arrows indicate the site of segment coupling.

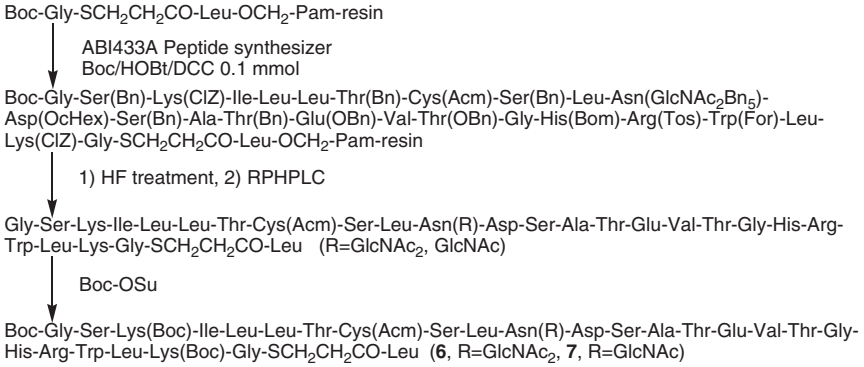

Fig. 2. Synthetic Route for Glycosylated Peptide Thioesters **6** and **7**.

Results and Discussion

Emmprin is a heavily glycosylated protein located on the surface of tumor cells. It stimulates nearby fibroblasts to produce MMPs, which are key enzymes for tumor metastasis.[24,25] In this respect, emmprin is viewed as a clinical target for the suppression of tumor metastasis. The functional site is presumed to be the first Ig domain (34-94) which has an *N*-glycosylation site at Asn^{44}. However, direct evidence that the first Ig domain itself possesses collagenase stimulation activity has not been presented so far. Moreover, the significance and the structure of the carbohydrate at Asn^{44} has not been clearly elucidated. To clarify these points and the mechanism for tumor metastasis, we synthesized the extracellular domains of emmprin carrying carbohydrates at Asn^{44}.

Synthesis of chitobiosylated Ig domain (34-94)-NH_2 by the thioester method

The first Ig domain (34-94) is composed of 61 amino acid residues, in which Asn^{44} is an *N*-glycosylation site (Fig. 1). To effectively obtain this long glycopeptide, the sequence was divided in two, and segment condensation by the thioester method was carried out. Gly^{58}-Gly^{59} was selected as a ligation site, since no precaution is required to prevent epimerization during segment coupling. Based on the reported stability of the chitobiose moiety to an HF treatment,[26] the *N*-terminal chitobiosylated peptide thioester was prepared by the Boc strategy as shown in Fig. 2. To introduce the chitobiose moiety, Boc-Asn($GlcNAc_2Bn_5$) **11** was prepared by coupling known protected chitobiose unit **9**[27] to the anhydride of the Boc-Asp derivative as shown in Fig. 3. Following the previous protocol for peptide thioester preparation,[23] the peptide chain was assembled on Boc-Gly-SCH_2CH_2CO-Leu-OCH_2-Pam-resin by an ABI 433A peptide synthesizer with the Boc/HOBt/DCC protocol. The protected peptide resin corresponding to the sequence of emmprin (45-58) was then reacted with the benzotriazolyl ester of Boc-Asn($GlcNAc_2Bn_5$) **11** (2 eq to the peptide on resin). The remaining sequence was again introduced by the synthesizer. After assembly of the sequence (34-58) had been completed, a part of the resin was stored for the synthesis of (22-58). The remaining resin was treated with HF at 0 °C for 1.5 h. Analysis of the crude peptide by RPHPLC showed that the desired chitobiosylated peptide thioester had been obtained in a good yield. In addition to the desired product, however, a peptide thioester carrying GlcNAc

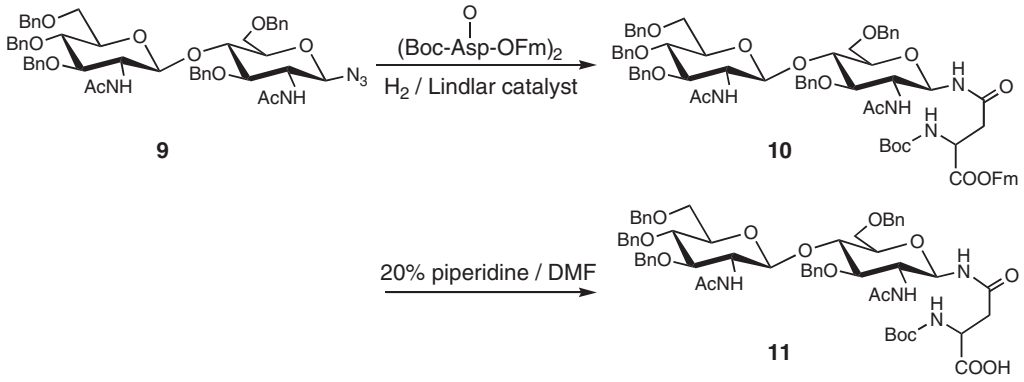

Fig. 3. Synthetic Procedure for Compound **11**.

Fmoc-Rink amide MBHA-resin (Fmoc-NH-resin)

↓ ABI433A peptide synthesizer system software 2.03
FastMoc 0.25 Ω MonPrevPk

Fmoc-Gly-Val-Val-Leu-Lys(Boc)-Glu(OBu^t)-Asp(OBu^t)-Ala-Leu-Pro-Gly-Gln(Trt)-Lys(Boc)-Thr(Bu^t)-Glu(OBu^t)-Phe-Lys(Boc)-Val-Asp(OBu^t)-Ser(Bu^t)-Asp(OBu^t)-Asp(OBu^t)-Gln(Trt)-Trp(Boc)-Gly-Glu(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Cys(Acm)-Val-Phe-Leu-Pro-Glu(OBu^t)-Pro-Met-NH-resin

↓ 1) Reagent K, 2) ion-exchange chromatography, 3) HPLC
↓ Boc-OSu, DIEA
↓ 5% piperidine / DMSO

Gly-Val-Val-Leu-Lys(Boc)-Glu-Asp-Ala-Leu-Pro-Gly-Gln-Lys(Boc)-Thr-Glu-Phe-Lys(Boc)-Val-Asp-Ser-Asp-Asp-Gln-Trp-Gly-Glu-Tyr-Ser-Cys-Val-Phe-Leu-Pro-Glu-Pro-Met-NH_2 **12**

Fig. 4. Synthesis of Peptide **12**.

was also obtained in about a two-thirds ratio, which might have been derived from cleavage of the GlcNAc-GlcNAc bond by the HF treatment. Thus, under the standard HF treatment conditions (the concentration of HF was more than 80%), the chitobiose did not retain complete stability. Treatment with a decreased concentration of an acid, such as a low-HF treatment,[28] might overcome this problem. However, it would be worth noting that the yield of the desired peptide was 6.7%, which is about 4 times higher than that of the same peptide thioester carrying an *N*-linked core pentasaccharide by the Fmoc strategy (1.8% yield).[14] This result demonstrates that, in the case of a peptide thioester carrying chitobiose, the Boc strategy would be the method of choice in respect of the yield of the product. The resin stored at (34-58) was used for further elongation by the Boc strategy to obtain the protected peptide resin of (22-58). HF treatent of this resin gave the desired peptide and the GlcNAc-truncated peptide in almost the same ratio as that of the (34-58) peptide thioester. This result indicates that the stability of chitobiose to HF seems to have been independent of its local environment within the peptide. The thioester method uses activation of the thioester group by silver ions to acylate the terminal amino group of the amino component. Thus, other amino and thiol groups were protected to suppress side reactions. Introduction of the Boc groups was easily achieved by Boc-OSu in the presence of DIEA. By following the same procedure, non-glycosylated peptide thioester **8** was obtained.

C-terminal peptide (59-94) **12** was prepared with the Fmoc strategy by the *FastMoc* protocol, which used HBTU as a coupling reagent, as shown in Fig. 4. The protected peptide resin was treated with TFA (Reagent K[29]), and the crude peptide was purified by RPHPLC. Synthesis of this peptide was accompanied by a significant amount of an aspartimide side product (about 30%) which could not be removed by RPHPLC. The peptide was further purified by anion-exchange to remove the by-product and was again desalted by RPHPLC. This two-step purification enabled a highly pure product to be obtained in a comparable yield to that of the *N*-terminal peptide thioester. Protection of the side-chain amino groups and subsequent removal of the terminal Fmoc group were then carried out for segment coupling with the *N*-terminal peptide thioester.

The segment coupling reaction by the thioester method was carried out as shown in Fig. 5. Peptides **6**

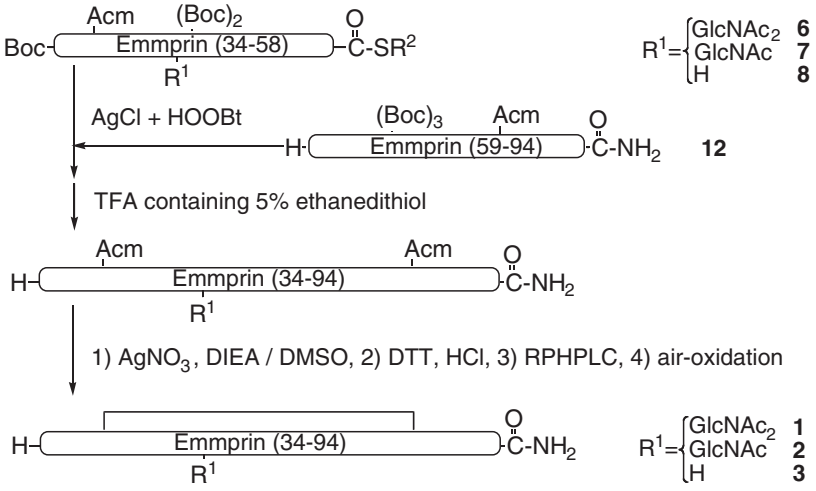

Fig. 5. Synthetic Procedure for Emmprin (34-94)-NH_2 **1**–**3**.
R^2 denotes-CH_2CH_2CO-Leu for **6** and **7**, and -CH_2CH_2CO-Nle-NH_2 for **8**.

and **12** were dissolved in DMSO containing HOOBt and DIEA. The thioester group was then activated by silver ions. The coupling reaction was almost completed within 6 h without significant side reactions. The Boc and Acm groups of the peptide were respectively removed by TFA and $AgNO_3$. The peptide was purified by RPHPLC, and the disulfide bond was formed by air-oxidation in 1% $AcONH_4$ (pH 7.8) overnight. Final product **1** was successfully obtained after RPHPLC purification. The chitobiose unit was stable throughout the segment coupling reaction. The glucosaminylated and non-glycosylated Ig domains (34-94)-NH_2 **2**, **3** were prepared by following the same procedure (Fig. 6).

*Synthesis of chitobiosylated emmprin (34-118)-NH_2 **4** and (22-118)-NH_2 **5** by the thioester method*

The thioester method was further applied to the larger glycopeptides, emmprin (34-118) **4** and (22-118) **5**, by repetition of the segment coupling as shown in Fig. 7. To achieve repetition of the coupling, the terminal amino group of middle segment **14** was orthogonally protected by the Fmoc group to the side chain amino group. This peptide thioester was prepared by the Boc strategy. The *N*-terminal glycine was introduced by using Fmoc-Gly. *C*-terminal peptide **15** was synthesized by the Fmoc strategy according to the same procedure as that used for peptide **12**.

To synthesize of glycopeptide **4**, peptides **14** and **15** were condensed by the same procedure as that used for (34-94)-NH_2, using activation of the thioester group by silver ions. The reaction was completed without significant side reactions within 12 h. The terminal Fmoc group was then removed by piperidine. The crude peptide obtained was used for the second coupling reaction with glycopeptide thioester **6** without further purification. This reaction also proceeded smoothly (Fig. 8a). After the Boc and Acm groups had been removed (Fig. 8b), the crude peptide was purified by

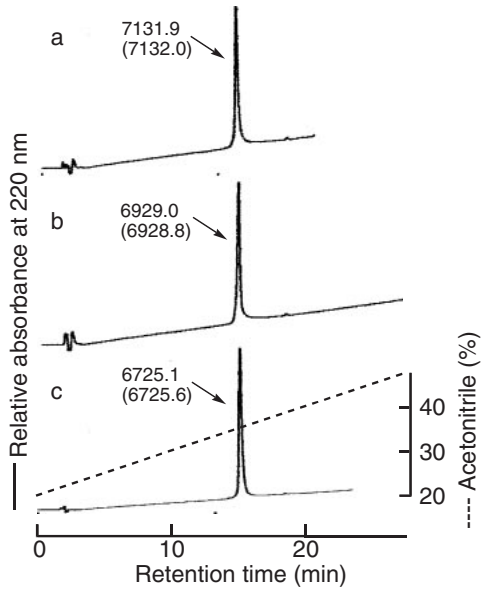

Fig. 6. RPHPLC Profiles of Emmprin (34-94)-NH_2.
a) glycopeptide **1**, b) glycopeptide **2**, c) peptide **3**. Elution conditions: column, Mightysil RP-18GP (4.6 × 150 mm) at a flow rate of 1 ml min^{-1}; eluent A, distilled water containing 0.1% TFA; eluent B, acetonitrile containing 0.1% TFA.

RPHPLC to obtain glycopeptide **17**. This reduced form of the product was air-oxidized to form a disulfide bond (Fig. 8c). After HPLC purification, final product **4** was successfully obtained with high purity as shown in Fig. 8d.

The thioester method was further applied to the synthesis of (22-118) which was composed of 97 amino acid residues. Ala^{22} of this peptide is presumed to be the *N*-terminal of the native emmprin. Peptides **14** and **15** were condensed as already described. Obtained crude peptide **16** was further coupled with glycopeptide thioester **13** by the thioester method. The reaction was

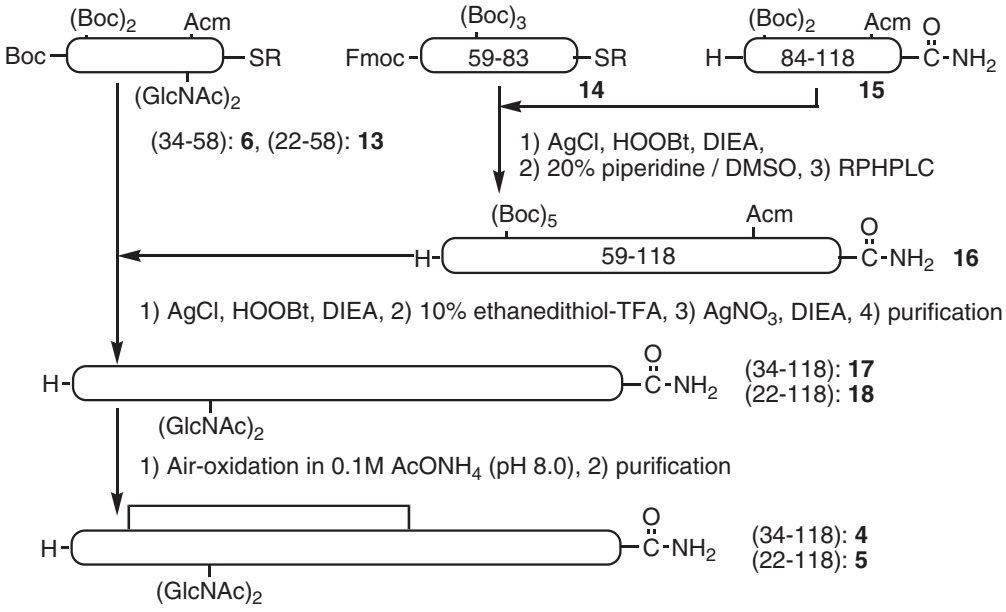

Fig. 7. Synthetic Procedure for Chitobiosylated Emmprin (34-118)-NH_2 **4** and (22-118)-NH_2 **5**.

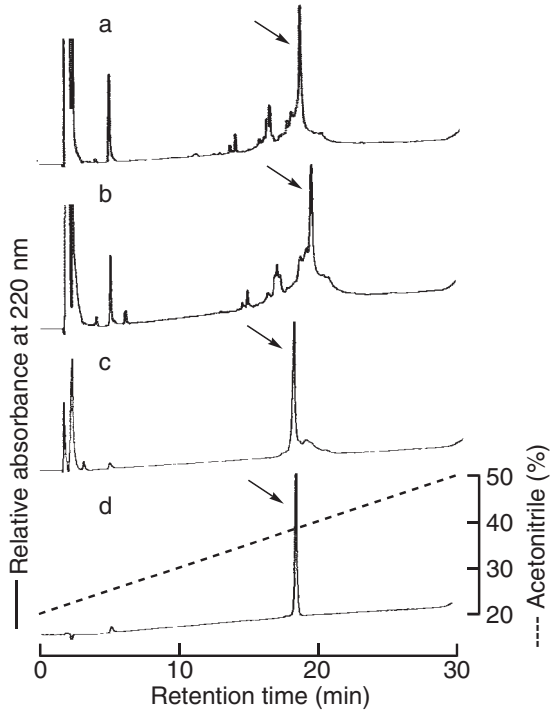

Fig. 8. RPHPLC Profiles of the Glycopeptides.
a) TFA-treated reaction mixture of the coupling between peptides **6** and **16**, b) crude peptide **17**, c) crude peptide **4**, d) purified peptide **4**. The elution conditions were the same as those described in Fig. 6.

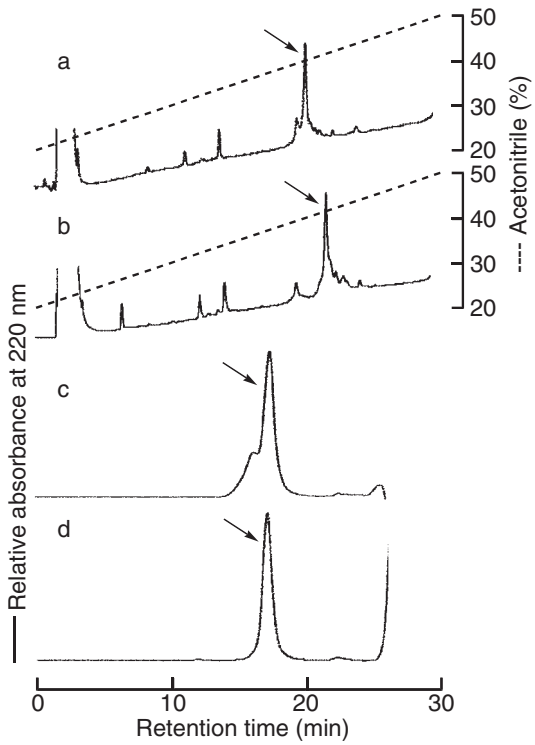

Fig. 9. HPLC Profiles of the Glycopeptides.
a) TFA-treated reaction mixture of the coupling between peptides **13** and **16**, b) crude peptide **18**, c) crude peptide **5**, d) purified peptide **5**. The elution conditions were the same as those described in Fig. 6.

completed within 12 h without serious side reactions as shown in Fig. 9a. Removal of the Boc and Acm groups was then carried out as described for glycopeptide **4**. This reaction proceeded smoothly (Fig. 9b). In contrast to glycopeptide **17**, the isolation of **18** was difficult, as it was highly adsorbed to the HPLC column. The reason for this low recovery might be attributable to the *N*-terminal sequence being abundant in hydrophobic amino acids. The purification was therefore carried out by gel filtration chromatography using 50% aqueous acetonitrile containing 0.1% TFA. The disulfide bond formation was first attempted in an ammonium acetate buffer

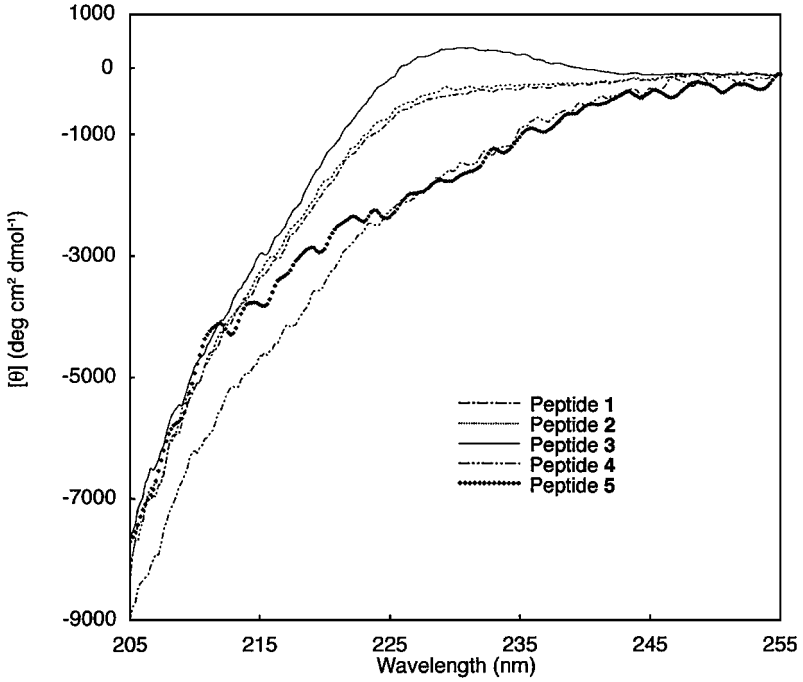

Fig. 10. CD Spectra of the Synthetic Glycopeptides.

(pH 8.0). However, most of the peptide was precipitated. This precipitate was dissolved in 6 M guanidine hydrochloride containing DTT. Glycopeptide **18** was recovered by gel filtration chromatography and oxidized for 2 days in ammonium acetate (pH 8.0) containing 6 M guanidine hydrochloride (Fig. 9c). The solution was loaded into a gel chromatography column and the desired product **5** was obtained (Fig. 9d). The MALDI-TOF mass analysis, and an amino acid composition analysis confirmed that the desired product had been successfully synthesized.

CD spectrum of the synthetic emmprin glycopeptides

The synthetic glycopeptides were dissolved in a sodium phosphate buffer (pH 7.0), and the secondary structure was characterized by CD spectral measurement. As shown in Fig. 10, there was a distinct difference between non-glycosylated peptide **3** and glycosylated peptides **1**, **2**, **4** and **5**. The biological activity of peptides **1**, **2** and **3** was preliminarily measured by Nabeshima *et al.* (unpublished data). The results show that the MMP stimulation activity increased as the sugar chain became longer from GlcNAc **2** to chitobiose **1**, whereas the Ig domain itself, **3**, had no activity. This result corresponds well with the CD spectrum. The [θ] values for peptide **4** and **5** were considerably larger in the negative direction than those of glycopeptides **1** and **2**, indicating that a more stable conformation was formed by elongation of the peptide chain. However, the spectra of these glycopeptides indicate that the peptide did not assume a structure rich in β-strand, which has been observed for immunoglobulins and Ig-like domains.[30] The reason for this result is not yet known. However, the result might indicate that other factors beside the peptide chain length also contributed to the folding of this peptide. Emmprin retains large carbohydrates with a molecular weight of about 30 kDa, which is comparable to the molecular weight of its polypeptide chain, 27 kDa. This fact might show that, at the small chitobiose level, glycosylation at Asn^{44} only altered the conformation around Asn^{44} to exert emmprin activity, but did not stabilize the entire Ig domains to take a particular conformation. It is therefore possible that a larger oligosaccharide chain would be required to assume the correct three-dimensional structure of emmprin. It might also be possible that further elongation of the peptide chain could induce a more stable comformation. Synthesis of the Ig domain carrying a more complex carbohydrate as well as synthesis of a longer extracellular chain are required to clarify this point.

In conclusion, a chitobiosylated peptide thioester was prepared by the Boc strategy and used for the preparation of chitobiosylated emmprin (34-94)-NH_2. The larger glycopeptides, (34-118)-NH_2 and (22-118)-NH_2, were successfully prepared by repetitive segment coupling, which demonstrates the usefulness of the thioester method for the synthesis of glycoproteins. Elongation of peptide chain contributed to a stabilized conformation. The biological activity of these glycosylated peptides is under study.

Experimental

Optical rotation values were determined with a DIP-

370 polarimeter (Jasco, Tokyo, Japan) for solutions in $CHCl_3$. ^{1}H-NMR spectra were recorded with an AL400 spectrometer (Jeol, Tokyo, Japan). Chemical shifts are expressed in ppm downfield from the signal for the internal Me_4Si standard in pyridine-d_5. TLC was performed on 60 F_{254} silica gel (Merck). MALDI-TOF mass spectra were recorded with a Voyager-DE PRO spectrometer (Applied Biosystems, CA, USA). The amino acid composition was determined with a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 6 M HCl at 150 °C for 2 h in an evacuated sealed tube. Calculation of the peptide content was based on the amino acid analysis data. Compound **9** was prepared as reported previously.[27)]

*N^2-(tert-Butoxycarbonyl)-N^4-[2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl]-L-asparagine fluorenylmethyl ester (**10**).* A mixture of Boc-Asp-OFm (180 mg, 0.44 mmol) and DCC (50 mg, 0.24 mmol) was dissolved in CH_2Cl_2 (0.44 ml) at 0 °C, and the solution was stirred for 30 min. The precipitate was filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in THF (5.2 ml) and added to a mixture of compound **9** (100 mg, 0.11 mmol) and a Lindlar catalyst (50 mg) under Ar. After the mixture had been stirred for 43 h under an H_2 atmosphere, the catalyst was filtered through Celite. The filtrate was purified on silica gel with $CHCl_3$–methanol (96:4) and then by gel chromatography on LH-60 with $CHCl_3$–methanol (1:1) to give compound **10** (140 mg, 0.11 mmol, 96%). R_f 0.41 (95:5 $CHCl_3$–methanol). $[\alpha]_D$ −3.2° (c 1.0, $CHCl_3$). ^{1}H-NMR (400 MHz, pyridine-d_5, TMS), δ: 10.25 (d, 1H, J = 9.3 Hz, Asn β-N<u>H</u>), 9.08 (d, 1H, J = 8.3 Hz, Ac-N<u>H</u>-b), 9.03 (d, 1H, J = 9.5 Hz, Ac-N<u>H</u>-a), 8.24 (d, 1H, J = 8.8 Hz, Asn α-N<u>H</u>), 5.81 (brt, 1H, J = 9.5 Hz, H-1a), 5.45 (m, 1H, Asn αH), 4.20 (m, 1H, H-2b), 4.07 (brt, 1H, J = 8.6 Hz, H-3a), 4.01 (dd, 1H, J = 4.0, 11.7 Hz, H-6a), 3.92 (brt, 1H, J = 9.2 Hz, H-4b), 3.65 (m, 1H, H-5b), 3.34 (dd, 1H, J = 6.4, 15.6 Hz, Asn βH), 3.25 (dd, 1H, J = 5.6, 15.6 Hz, Asn βH), 2.20 (s, 3H, C<u>H</u>$_3$CO), 2.12 (s, 3H, C<u>H</u>$_3$CO), 1.50 (s, 9H, *t*-Bu). HRFABMS m/z $(M+H)^+$: calcd. for $C_{74}H_{83}N_4O_{15}$, 1267.5855; found, 1267.5905.

*N^2-(tert-Butoxycarbonyl)-N^4-[2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl]-L-asparagine (**11**).* To a solution of compound **10** (50 mg, 39 μmol) in DMF (1.6 ml) was added piperidine (0.4 ml), and the solution was stirred for 30 min. The solvent was removed *in vacuo*, and the residue was dissolved in $CHCl_3$–methanol containing acetic acid (1:1:0.01). The solution was loaded onto LH-60 with $CHCl_3$–methanol (1:1). The major fraction was collected to give compound **11** (49 mg, quantitative). R_f 0.38 (9:1:0.1 $CHCl_3$–methanol–AcOH). $[\alpha]_D$ +11.0° (c 0.5, $CHCl_3$). ^{1}H-NMR (400 MHz, pyridine-d_5, TMS), δ: 10.14 (d, 1H, J = 9.0 Hz, Asn β-N<u>H</u>), 9.09 (d, 1H, J = 9.0 Hz, Ac-N<u>H</u>-b), 9.07 (d, 1H, J = 10.7 Hz, Ac-N<u>H</u>-a), 5.82 (brt, 1H, J = 9.3 Hz, H-1a), 4.94 (d, 1H, J = 11.2 Hz, Ph-C<u>H</u>$_2$-), 4.46 (m, 1H, H-3a), 4.23 (m, 1H, H-2a), 3.39 (dd, 1H, J = 6.4, 15.6 Hz, Asn βH), 3.27 (dd, 1H, J = 4.4, 15.6 Hz, Asn βH), 2.21 (s, 3H, C<u>H</u>$_3$CO), 2.13 (s, 3H, C<u>H</u>$_3$CO), 1.46 (s, 9H, *t*-Bu). HRFABMS m/z $(M+H)^+$: calcd. for $C_{60}H_{73}N_4O_{15}$, 1089.5072; found, 1089.5114.

*Boc-[Asn(GlcNAc$_n$)^{44}, Cys(Acm)^{41}, Lys(Boc)^{36,57}]-emmprin (34-58)-SCH$_2$CH$_2$CO-Leu (**6**, n = 2; **7**, n = 1).* Starting from Boc-Gly-SCH_2CH_2CO-Leu-OCH_2-Pam-resin (0.74 mmol/g, 0.14 g), the peptide chain was elongated by a 433A peptide synthesizer (Applied Biosystems, CA, USA), using 0.1 mmol Boc/HOBt/DCC. The brief procedure for this protocol is as follows: 1) TFA deprotection (25% TFA/CH_2Cl_2 for 3 min, 50% TFA/CH_2Cl_2 for 11 min), 2) CH_2Cl_2 wash (×4), 3) DIEA neutralization (5% DIEA/NMP for 0.5 min ×2), 4) NMP wash (×5), 5) Boc-amino acid HOBt ester (1 mmol) in a 15% DMSO-NMP solution for 8 min and then for 4 min in the presence of 3.8 eq of DIEA, 6) capping (10% Ac_2O, 5% DIEA in NMP for 5 min), 7) CH_2Cl_2 wash (×4). This protocol enabled Boc-Gly-Ser(Bn)-Lys(ClZ)-Ile-Leu-Leu-Thr(Bn)-Cys(Acm)-Ser(Bn)-Leu-Asn(GlcNAc$_2$Bn$_5$)-Asp(O*c*Hex)-Ser(Bn)-Ala-Thr(Bn)-Glu(OBn)-Val-Thr(Bn)-Gly-His(Bom)-Arg(Tos)-Trp(For)-Leu-Lys(ClZ)-Gly-SCH_2CH_2CO-Leu-OCH_2-Pam-resin (0.58 g) to be obtained. To introduce Asn^{44}, Boc-Asn(GlcNAc$_2$Bn$_5$) **11** (220 mg, 0.2 mmol), 1 M HOBt/NMP (0.2 ml) and 1 M DCC/NMP (0.2 ml) were mixed for 30 min, and the benzotriazolyl-active ester generated was added to the resin. A part of the resin (200 mg) was treated with HF (5.4 ml) containing anisole (0.4 ml) and 1,4-butanedithiol (0.8 ml) at 0 °C for 1.5 h. After HF had been evaporated *in vacuo*, the crude peptide was washed with ether (×3), extracted with 50% aqueous acetonitrile containing 0.1% TFA and lyophilized. The crude peptide obtained (120 mg) was purified by RPHPLC, and [Asn(GlcNAc$_2$)^{44}, Cys(Acm)^{41}]-emmprin (34-58)-SCH_2CH_2CO-Leu (2.3 μmol, 6.7% based on the Gly content of the initial resin) and [Asn(GlcNAc)^{44}, Cys(Acm)^{41}]-emmprin (34-58)-SCH_2CH_2CO-Leu (1.4 μmol, 4.1% based on the Gly content of the initial resin) were separately collected. [Asn(GlcNAc$_2$)^{44}, Cys(Acm)^{41}]-emmprin (34-58)-SCH_2CH_2CO-Leu (6.5 μmol) was dissolved in DMSO (0.2 ml), and Boc-OSu (7.6 mg, 35 μmol) and DIEA (6.2 μl, 36 μmol) were added. After the reaction mixture had stood for 2 h at room temperature, the product was precipitated by ether, washed twice with ethyl acetate and lyophilized from the dioxane suspension to give peptide **6** (4.8 μmol, 74%). MALDI-TOF MS: found, m/z 3665.1 $(M+H)^+$; calcd. for $(M+H)^+$, 3664.8. Amino acid analysis: $Asp_{1.99}Thr_{2.78}Ser_{2.59}Glu_{1.04}Gly_3$-$Ala_{1.02}Val_{1.40}Ile_{0.90}Leu_{5.01}Lys_{1.97}His_{1.01}Arg_{1.01}$. Peptide **7** was obtained by the same procedure in a 92% yield. MALDI-TOF MS: found, m/z 3461.8 $(M+H)^+$; calcd.

for $(M+H)^+$, 3461.8. Amino acid analysis: $Asp_{1.97}$-$Thr_{2.73}Ser_{2.59}Glu_{1.01}Gly_3Ala_{1.00}Val_{1.30}Ile_{0.88}Leu_{5.08}$-$Lys_{1.97}His_{1.01}Arg_{0.97}$.

*Boc-[Asn(GlcNAc$_2$)^{44}, Cys(Acm)^{41}, Lys(Boc)^{36,57}]-emmprin (22-58)-SCH$_2$CH$_2$CO-Leu (**13**).* Using the resin stored at Gly^{34} during the synthesis of **6** and **7** (13 μmol), the peptide chain was further elongated by the 0.1 mmol Boc/HOBt/DCC protocol to obtain Boc-Ala-Ala-Gly-Thr(Bn)-Val-Phe-Thr(Bn)-Thr(Bn)-Val-Glu(OBn)-Asp(O*c*Hex)-Leu-Gly-Ser(Bn)-Lys(ClZ)-Ile-Leu-Leu-Thr(Bn)-Cys(Acm)-Ser(Bn)-Leu-Asn(GlcNAc$_2$Bn$_5$)-Asp(O*c*Hex)-Ser(Bn)-Ala-Thr(Bn)-Glu(OBn)-Val-Thr(Bn)-Gly-His(Bom)-Arg(Tos)-Trp(For)-Leu-Lys(ClZ)-Gly-SCH$_2$CH$_2$CO-Leu-OCH$_2$-Pam-resin (86 mg). This resin (86 mg) was treated with HF (2.5 ml) containing anisole (0.1 ml) and 1,4-butanedithiol (0.4 ml) at 0 °C for 1.5 h. After HF had been evaporated *in vacuo*, the crude peptide was washed with ether (×3), extracted with 50% aqueous acetonitrile containing 0.1% TFA and lyophilized. The analysis of the crude peptide showed that the desired product and GlcNAc-truncated peptide thioester had been obtained in 3:2 ratio. After purification by RPHPLC, [Asn(GlcNAc$_2$)^{44}, Cys(Acm)^{41}]-emmprin (22-58)-SCH$_2$CH$_2$CO-Leu (50 nmol, 0.4% based on the Gly content of the initial resin) was obtained. [Asn(GlcNAc$_2$)^{44}, Cys(Acm)^{41}]-emmprin (22-58)-SCH$_2$CH$_2$CO-Leu (50 nmol) was dissolved in DMSO (20 μl), and Boc-OSu (0.3 mg, 1.4 μmol) and DIEA (0.2 μl, 1.2 μmol) were added. After the reaction mixture had stood for 2 h at room temperature, the product was precipitated by ether, washed twice with ethyl acetate and lyophilized from the dioxane suspension to give peptide **13** (quantitative). MALDI-TOF MS: found, m/z 4868.8 $(M+H)^+$; calcd. for $(M+H)^+$, 4869.4. Amino acid analysis: $Asp_{2.99}$-$Thr_{5.43}Ser_{2.83}Glu_{1.18}Gly_4Ala_{2.85}Val_{2.51}Ile_{0.81}Leu_{5.68}$-$Phe_{0.80}Lys_{2.13}His_{0.97}Arg_{0.90}$.

*Boc-[Cys(Acm)^{41}, Lys(Boc)^{36,57}]-emmprin (34-58)-SCH$_2$CH$_2$CO-Nle-NH$_2$ (**8**).* Starting from Boc-Gly-SCH$_2$CH$_2$CO-Nle-NH-resin (0.42 mmol/g, 1.2 g), the peptide chain was elongated by an ABI 433A peptide synthesizer, using 0.5 mmol Boc/HOBt/DCC, and Boc-Gly-Ser(Bn)-Lys(ClZ)-Ile-Leu-Leu-Thr(Bn)-Cys(Acm)-Ser(Bn)-Leu-Asn-Asp(O*c*Hex)-Ser(Bn)-Ala-Thr(Bn)-Glu(OBn)-Val-Thr(Bn)-Gly-His(Bom)-Arg(Tos)-Trp(For)-Leu-Lys(ClZ)-Gly-SCH$_2$CH$_2$CO-Nle-NH-resin (3.4 g) was obtained. A part of this resin (600 mg) was treated with HF (10 ml) containing anisole (0.5 ml) and 1,4-butanedithiol (1.5 ml) at 0 °C for 1.5 h. After HF had been evaporated *in vacuo*, the crude peptide was washed with ether (×3), extracted with 50% aqueous acetonitrile containing 0.1% TFA and lyophilized. The crude peptide obtained (260 mg) was purified by RPHPLC, and [Cys(Acm)^{41}]-emmprin (34-58)-SCH$_2$CH$_2$CO-Nle-NH$_2$ (4.2 μmol, 4.8% based on the Gly content of the initial resin) was obtained. This peptide (3.7 μmol) was dissolved in DMSO (0.15 ml), and Boc-OSu (8.3 mg, 39 μmol) and DIEA (6.7 μl, 39 μmol) were added. The solution was kept at room temperature for 3 h, and the product was precipitated by ether, washed with ethyl acetate and lyophilized from the dioxane suspension to give peptide **8** (3.6 μmol, 97%). MALDI-TOF MS: found, m/z 3258.0 $(M+H)^+$; calcd. for $(M+H)^+$, 3257.7. Amino acid analysis: $Asp_{2.02}Thr_{2.80}Ser_{2.68}$-$Glu_{1.04}Gly_3Ala_{1.03}Val_{1.02}Ile_{0.88}Leu_{3.93}Lys_{2.11}His_{0.97}$-$Arg_{1.01}$.

*[Cys(Acm)^{87}, Lys(Boc)^{63,71,75}]-emmprin (59-94)-NH$_2$ (**12**).* Starting from Fmoc-Rink amide-MBHA-resin (0.54 mmol/g, 0.46 g), the peptide chain was elongated by an ABI 433A peptide synthesizer, using the *FastMoc* protocol. The brief procedure for this protocol (0.25 mmol scale) is as follows: 1) piperidine deprotection (18% piperidine/NMP for 3 min and 20% piperidine/NMP for 3 min. If the Fmoc removal is incomplete, the deprotection is repeated), 2) NMP wash (×5), 3) coupling with Fmoc-amino acid (1 mmol) activated by HBTU/HOBt (0.9 mmol each) and DIEA (2 mmol) for 20 min. The protected peptide resin, Fmoc-Gly-Val-Val-Leu-Lys(Boc)-Glu(OBu^t)-Asp(OBu^t)-Ala-Leu-Pro-Gly-Gln(Trt)-Lys(Boc)-Thr(Bu^t)-Glu(OBu^t)-Phe-Lys(Boc)-Val-Asp(OBu^t)-Ser(Bu^t)-Asp(OBu^t)-Asp(OBu^t)-Gln(Trt)-Trp(Boc)-Gly-Glu(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Cys(Acm)-Val-Phe-Leu-Pro-Glu(OBu^t)-Pro-Met-NH-resin (1.9 g), was obtained by this protocol. A part of the resin (400 mg) was treated with Reagent K[29] (5.0 ml) for 2 h at room temperature. TFA was removed in a nitrogen stream, and the peptide was precipitated by ether. The peptide was dissolved in a 0.1 M sodium phosphate buffer (pH 6.0) and loaded into an anion-exchange column (ICE DEAE-825, 8 mm × 75 mm; Showa Denko, Tokyo, Japan) which had been equilibrated with 0.1 M sodium phosphate (pH 6.0), before eluting with an increasing NaCl concentration in the buffer. The peptide was desalted by RPHPLC to give Fmoc-[Cys(Acm)^{87}]-emmprin (59-94)-NH$_2$ (5.6 μmol, 10% based on the amino groups in the initial resin). This peptide (5.6 μmol) was dissolved in DMSO (0.2 ml), and Boc-OSu (12 mg, 56 μmol) and DIEA (9.4 μl, 54 μmol) were added. After the solution had been kept at room temperature for 5 h, the product was precipitated by ether and washed with ethyl acetate. The obtained peptide was dissolved in DMSO (0.2 ml) containing 5% piperidine. The solution was kept at room temperature for 1 h, and the product was precipitated by ether and dried. The peptide was washed with 0.1% TFA and lyophilized to give peptide **12** (4.8 μmol, 8.9% based on the amino groups in the initial resin). MALDI-TOF MS: found, m/z 4453.6 $(M+Na)^+$ (average); calcd. for $(M+Na)^+$, 4452.0 (average). Amino acid analysis: $Asp_{4.05}Thr_{0.94}Ser_{1.75}Glu_{6.21}Pro_{3.03}Gly_3Ala_{1.02}Val_{2.92}$-$Met_{0.82}Leu_{3.01}Tyr_{0.88}Phe_{1.92}Lys_{2.97}$.

*[Asn(GlcNAc$_2$)^{44}]-emmprin (34-94)-NH$_2$ (**1**).* Pep-

tides **6** (2.3 μmol) and **12** (1.9 μmol) were dissolved in DMSO (0.1 ml) containing HOOBt (15 mg, 92 μmol) and DIEA (10 μl, 58 μmol), and AgCl (1.3 mg, 9.1 μmol) were added. The reaction mixture was stood overnight in the dark. The product was precipitated with ether, washed twice with ethyl acetate and dried. The obtained powder was treated for 10 min with TFA containing 5% ethanedithiol (0.2 ml) to remove the Boc groups. TFA was removed in a nitrogen stream, and the product was precipitated with ether. The crude peptide was extracted with 50% aqueous acetonitrile and lyophilized. The powder was dissolved in DMSO (0.2 ml), and $AgNO_3$ (3.6 mg, 21 μmol) and DIEA (7.3 μl, 42 μmol) were added. After the reaction mixture had been kept in the dark for 2 h, DTT (44 mg, 0.29 mmol) was added. The solution was acidified by 0.5 M HCl, and the product was purified by RPHPLC to give linear [Asn(GlcNAc$_2$)^{44}]-emmprin (34-94)-NH_2 (1.2 μmol, 63% based on peptide **12**). This obtained peptide (1.2 μmol) was dissolved in DMSO (3 ml) and diluted with 0.1 M $AcONH_4$ (pH 7.8, 27 ml). The solution was stirred for 2 days. After purification by RPHPLC, peptide **1** (0.82 μmol, 68%) was obtained. MALDI-TOF MS: found, *m/z* 7131.9 $(M + H)^+$ (average); calcd. for $(M + H)^+$, 7132.0 (average). Amino acid analysis: $Asp_{5.99}Thr_{3.74}Ser_{4.47}Glu_{7.33}Pro_{3.13}Gly_6Ala_{2.10}Val_{4.51}Cys_{0.26}Met_{0.84}Ile_{0.90}Leu_{7.52}Tyr_{1.07}Phe_{1.92}Lys_{4.84}His_{1.02}Arg_{0.98}$.

*[Asn(GlcNAc)^{44}]-emmprin (34-94)-NH_2 (**2**).* Peptides **7** (2.9 μmol) and **12** (3.2 μmol) were dissolved in DMSO (0.15 ml) containing HOOBt (16 mg, 98 μmol) and DIEA (11 μl, 63 μmol). AgCl (1.4 mg, 9.8 μmol) was added, and the reaction mixture was stood overnight in the dark. The product was precipitated with ether, washed with ethyl acetate and treated with TFA containing 5% ethanedithiol (0.25 ml) for 10 min to remove the Boc groups. TFA was removed in a nitrogen stream, and the product was precipitated with ether. To the product dissolved in DMSO (0.2 ml), $AgNO_3$ (3.8 mg, 22 μmol) and DIEA (7.8 μl, 45 μmol) were added. After the reaction mixture had been kept in the dark for 1 h, DTT (44 mg, 0.29 mmol) and 0.5 M HCl (0.6 ml) were added. The product was purified by RPHPLC to give linear [Asn(GlcNAc)^{44}]-emmprin (34-94)-NH_2 (0.77 μmol, 27% based on peptide **7**). The obtained peptide (0.77 μmol) was dissolved in DMSO (1.5 ml) and diluted with 1% $AcONH_4$ (pH 7.8, 14 ml). After the solution had been stirred at room temperature for 2 days, the product was purified by RPHPLC to give peptide **2** (0.50 μmol, 65%). MALDI-TOF MS: found, *m/z* 6929.0 $(M + H)^+$ (average); calcd. for $(M + H)^+$, 6928.8 (average). Amino acid analysis: $Asp_{6.01}Thr_{3.79}Ser_{4.49}Glu_{7.39}Pro_{3.25}Gly_6Ala_{2.07}Val_{4.37}Met_{0.84}Ile_{0.92}Leu_{7.58}Tyr_{1.09}Phe_{1.94}Lys_{4.88}His_{1.03}Arg_{1.04}$.

*Emmprin (34-94)-NH_2 (**3**).* Peptides **8** (1.8 μmol) and **12** (1.5 μmol) were dissolved in DMSO (0.1 ml) containing HOOBt (15 mg, 92 μmol) and DIEA (10 μl, 58 μmol). AgCl (1.3 mg, 9.1 μmol) was added, and the reaction mixture was stood overnight in the dark. The product was precipitated with ether, washed with ethyl acetate and treated with TFA containing 5% ethanedithiol (0.25 ml) for 10 min to remove the Boc groups. TFA was removed in a nitrogen stream, and the product was precipitated with ether. The precipitate was purified by RPHPLC. The obtained powder was dissolved in DMSO (0.2 ml), and $AgNO_3$ (1.7 mg, 10 μmol) and DIEA (3.5 μl, 20 μmol) were added. After the reaction mixture had been kept in the dark for 1.5 h, DTT (22 mg, 0.14 mmol) and 0.5 M HCl (0.3 ml) were added. The product was purified by RPHPLC. The obtained linear emmprin (34-94)-NH_2 was dissolved DMSO (0.9 ml) and diluted with 1% $AcONH_4$ (pH 7.8, 6 ml). After the solution had been stirred at room temperature for 4 days, the product was purified by RPHPLC to give peptide **3** (0.35 μmol, 23% based on peptide **12**). MALDI-TOF MS: found, *m/z* 6725.1 $(M + H)^+$ (average); calcd. for $(M + H)^+$, 6725.6 (average). Amino acid analysis: $Asp_{5.98}Thr_{3.45}Ser_{3.77}Glu_{6.94}Pro_{2.88}Gly_6Ala_{2.04}Val_{4.54}Met_{1.22}Ile_{0.97}Leu_{7.19}Tyr_{0.80}Phe_{2.13}Lys_{4.79}His_{1.00}Arg_{0.95}$.

*Fmoc-[Lys(Boc)^{63,71,75}]-Emmprin (59-83)-SCH_2CH_2-CO-Leu (**14**).* Starting from Boc-Gly-SCH_2CH_2CO-Leu-OCH_2-Pam-resin (0.45 mmol/g, 1.1 g), the peptide chain was elongated with an ABI 433A peptide synthesizer by using Boc/HOBt/DCC, and protected peptide resin, Fmoc-Gly-Val-Val-Leu-Lys(ClZ)-Glu(OBn)-Asp(O*c*Hex)-Ala-Leu-Pro-Gly-Gln-Lys(ClZ)-Thr(Bn)-Glu(OBn)-Phe-Lys(ClZ)-Val-Asp(O*c*Hex)-Ser(Bn)-Asp(O*c*Hex)-Asp(O*c*Hex)-Gln-Trp(For)-Gly-SCH_2CH_2CO-Leu-OCH_2-Pam-resin (1.9 g), was obtained. The final amino acid was introduced by using Fmoc-Gly. Part of the resin (650 mg) was treated for 1.5 h with HF (10 ml) containing anisole (0.5 ml) and 1,4-butanedithiol (1.5 ml) at 0 °C. HF was removed *in vacuo*, and the residue was washed with ether. The peptide was extracted with aqueous acetonitrile containing 0.1% TFA, lyophilized and purified by RPHPLC to obtain Fmoc-emmprin (59-83)-SCH_2CH_2CO-Leu (2.4 μmol, 1.4% based on the Gly content of the initial resin). This peptide (2.4 μmol) was dissolved in DMSO (0.3 ml), and Boc-OSu (18 mg, 84 μmol) and DIEA (15 μl, 86 μmol) were added. After the resulting solution had beeen stood for 2 h, the product was precipitated by ether, washed twice with ethyl acetate and lyophilized from the dioxane suspension to give peptide **14** (1.7 μmol, 71%). MALDI-TOF MS: found, *m/z* 3509.0 (average); calcd. for $(M + Na)^+$, 3508.9 (average). Amino acid analysis: $Asp_{4.20}Thr_{0.89}Ser_{0.75}Glu_{4.08}Pro_{1.07}Gly_3Ala_{1.00}Val_{2.67}Leu_{3.03}Phe_{1.00}Lys_{3.13}$.

*[Cys(Acm)^{87}, Lys(Boc)^{108,111}]-Emmprin (84-118)-NH_2 (**15**).* Starting from Fmoc-Rink amide-MBHA-resin (0.73 mmol/g, 0.34 g), the peptide chain was elongated with an ABI 433A peptide synthesizer by using the *FastMoc* protocol, and the protected peptide resin,

Fmoc-Glu(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Cys(Acm)-Val-Phe-Leu-Pro-Glu(OBu^t)-Pro-Met-Gly-Thr(Bu^t)-Ala-Asn(Trt)-Ile-Gln(Trt)-Leu-His(Trt)-Gly-Pro-Pro-Arg(Pbf)-Val-Lys(Boc)-Ala-Val-Lys(Boc)-Ser(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-His(Trt)-Ile-Asn(Trt)-Glu(OBu^t)-NH-resin (1.87 g), was obtained. Part of the resin (930 mg) was treated with Reagent K (9 ml) for 2 h. TFA was removed in a nitrogen stream, and the peptide was precipitated with ether. The residue was dissolved in aqueous acetonitrile and purified by RPHPLC to obtain Fmoc-[Cys(Acm)^{87}]-emmprin (84-118)-NH_2 (13 μmol, 11% based on the amino group of the initial resin). To a solution of this peptide (13 μmol) in DMSO (0.4 ml), Boc-OSu (26 mg, 120 μmol) and DIEA (21 μl, 120 μmol) were added, and the mixture was stood for 1 h. The peptide was precipitated by ether, washed with ethyl acetate and lyophilized from the dioxane suspension. The peptide was dissolved in DMSO (0.4 ml) containing 5% piperidine, and the solution was kept for 30 min. The product, precipitated by ether, was purified by RPHPLC, and peptide **15** was obtained (11 μmol, 85%). MALDI-TOF MS: found, m/z 4148.3 $(M + H)^+$; calcd. for $(M + H)^+$, 4148.1. Amino acid analysis: $Asp_{1.98}Thr_{0.84}Ser_{1.81}Glu_{4.75}Pro_{4.41}Gly_2Ala_{1.98}Val_{2.87}Met_{0.95}Ile_{1.87}Leu_{1.99}Tyr_{1.02}Phe_{0.97}Lys_{1.94}His_{1.96}Arg_{1.01}$.

*Emmprin (34-118)-NH_2 (**4**).* Peptides **14** (1.1 μmol) and **15** (1.6 μmol) were dissolved in DMSO (85 μl) containing HOOBt (7.7 mg, 47 μmol) and DIEA (5.5 μl, 32 μmol). AgCl (0.7 mg, 4.9 μmol) was added, and the reaction mixture was stirred overnight in the dark. DTT (15 mg, 97 μmol) was added, and the peptide was precipitated by distilled water containing 0.1% TFA. The precipitate was dried *in vacuo* and dissolved in DMSO (0.1 ml) containing 5% piperidine and DTT (500 μg, 3.2 μmol). After the solution had been stood for 1 h at room temperature, the product was precipitated by ether and successively washed with acetonitrile and 0.1% TFA. Lyophilized crude [Cys(Acm)^{87}, Lys(Boc)^{63,71,75,108,111}]-emmprin (59-118)-NH_2 **16** and peptide **6** (1.1 μmol) were dissolved in DMSO (0.1 ml) containing HOOBt (5.4 mg, 33 μmol) and DIEA (3.8 μl, 22 μmol). To the solution, AgCl (0.47 mg, 3.3 μmol) was added, and resulting mixture was stirred overnight in the dark. Ether was added to form a precipitate, which was washed twice with the same solvent and dried *in vacuo*. The powder was treated with 10% ethanedithiol TFA (0.2 ml) for 10 min. TFA was removed in an N_2 stream, and the product was precipitated with ether and dried *in vacuo*. The product was extracted with 50% aqueous acetonitrile containing 0.1% TFA and lyophilized. The obtained powder was dissolved in DMSO (0.1 ml) containing DIEA (1.9 μl, 11 μmol), and $AgNO_3$ (0.93 mg, 5.5 μmol) was added. After the solution had been kept in the dark for 1 h, DTT (10 mg, 65 μmol) and 0.5 M HCl (0.1 ml) were added to the solution, and the product was purified by RPHPLC to give peptide **17**. The obtained reduced form of the peptide was air-oxidized overnight in 10 mM $AcONH_4$ containing 6 M guanidine HCl (pH 8.0, 5 ml). The product was purified by RPHPLC, and peptide **4** (83 nmol, 7.5% based on peptide **14**) was obtained. MALDI-TOF MS: found, m/z 9696.5 (average); calcd. for $(M + H)^+$, m/z 9696.9 (average). Amino acid analysis: $Asp_{8.05}Thr_{4.55}Ser_{6.36}Glu_{10.03}Pro_{5.08}Gly_{8.29}Ala_{4.33}Val_{6.28}Met_{0.90}Ile_{2.70}Leu_8Tyr_{1.10}Phe_{2.03}Lys_{6.70}His_{2.85}Arg_{2.00}$.

*Emmprin (22-118)-NH_2 (**5**).* Peptides **14** (160 nmol) and **15** (240 nmol) were condensed in the same manner as that described for peptide **4**. Obtained crude peptides **16** and **13** (48 nmol) were dissolved in DMSO (20 μl) containing HOOBt (1.0 mg, 6.1 μmol) and DIEA (0.7 μl, 4.0 μmol). To the solution, AgCl (0.1 mg, 0.7 μmol) was added, and the resulting mixture was stirred overnight in the dark. Ether was added to form a precipitate, which was washed twice with the same solvent and dried *in vacuo*. The powder was treated with 10% ethanedithiol TFA (0.1 ml) for 10 min. TFA was removed in an N_2 stream, and the product was precipitated with ether and dried *in vacuo*. The product was extracted with 50% aqueous acetonitrile containing 0.1% TFA and lyophilized. The obtained powder was dissolved in DMSO (40 μl) containing DIEA (0.4 μl, 2.3 μmol), and $AgNO_3$ (0.17 mg, 1.0 μmol) was added. After the solution had been kept in the dark for 2 h, DTT (1.5 mg, 9.7 μmol) and 0.5 M HCl (20 μl) were added, and the product was purified in a G3000PW_{XL} column (7.8 mm × 300 mm, Tosoh, Japan), using 50% aqueous acetonitrile containing 0.1% TFA at a flow rate of 0.5 ml/min, to give peptide **18** (20 nmol). The peptide was dissolved in 5 μl of a 6 M guanidine HCl solution and dropped into 0.01 M sodium phosphate (pH 7.0, 400 μl) for 2 days. The precipitated peptide was again dissolved in 6 M guanidine hydrochloride (500 μl) containing DTT (1.0 mg, 6.5 μmol) and kept at 37 °C for 30 min. The solution was applied to the G3000PW_{XL} column, and peptide **18** was recovered. The peptide was dissolved in 0.1 M $AcONH_4$, pH 8.0, 200 μl), and the solution was gently vortexed for 2 days. This solution was applied to the G3000PW_{XL} column and the fraction containing peptide **5** was collected (3.4 nmol). MALDI-TOF MS: found, m/z 10899.8 (average); calcd. for $(M + H)^+$, m/z 10900.2 (average). Amino acid analysis: $Asp_{9.34}Thr_{7.36}Ser_{6.32}Glu_{11.35}Pro_{4.77}Gly_{9.28}Ala_6Val_{8.45}Met_{0.63}Ile_{2.91}Leu_{9.34}Tyr_{1.37}Phe_{3.02}Lys_{7.47}His_{2.87}Arg_{1.73}$.

CD spectral measurement. Each peptide was dissolved in a 10 mM sodium phosphate buffer (pH 7.0) at a concentration of 0.5 to 1 mg/ml. The CD spectrum was recorded between 205 nm and 260 nm by a J-720 instrument (Jasco, Tokyo, Japan) at room temperature, using a 1-mm-path-length cell.

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