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Chemoenzymatic Synthesis of Hydrophobic Glycoprotein: Synthesis of Saposin C Carrying Complex-Type Carbohydrate

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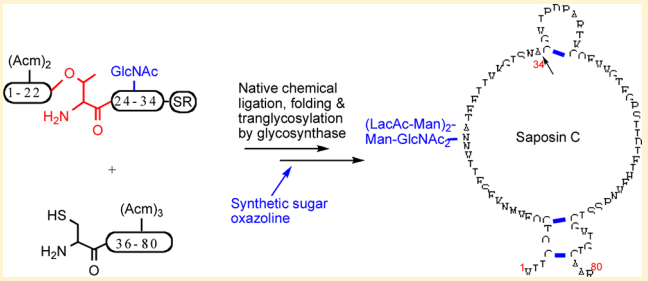
Supporting Information

ABSTRACT: The complex-type *N*-linked octasaccharide oxazoline having LacNAc as the nonreducing end sugar was efficiently synthesized using the benzyl-protected LacNAc, mannose, and *β*-mannosyl GlcNAc units as key building blocks. To achieve a highly *β*-selective glycosylation with the LacNAc unit, the *N*-trichloroacetyl group was used for the protection of the amino group in the LacNAc unit. After complete assembly of these units and deprotection, the obtained free sugar was successfully derivatized into the corresponding sugar oxazoline. On the other hand, the *N*-acetylglucosaminylated saposin C, a hydrophobic lipid-binding protein, was chemically synthesized by the native chemical ligation reaction. On the basis of the previous results related to the synthesis of the nonglycosylated saposin C, the *O*-acyl isopeptide structure was introduced to the N-terminal peptide thioester carrying GlcNAc to improve its solubility toward aqueous organic solvents. The ligation reaction efficiently proceeded with the simultaneous *O*- to *N*-acyl shift at the *O*-acyl isopeptide moiety. After the removal of the cysteine-protecting group and folding, saposin C carrying GlcNAc was successfully obtained. The synthetic sugar oxazoline was then transferred to this glycoprotein using the mutant of endo-*β*-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) (glycosynthase), and the saposin C carrying the complex-type nonasaccharide was successfully obtained.



INTRODUCTION

Glycosylation is a common post-translational modification of proteins. More than half of natural proteins are supposed to be glycosylated at the appropriate hydroxy groups of the Ser/Thr residues and/or amido groups of Asn located at the Asn-Xaa-Ser/Thr consensus motif.[1] However, the structure of these sugars is usually highly heterogeneous, making the functional and structural studies of glycoproteins a difficult task. On the basis of this fact, challenges toward the efficient preparation of homogeneous glycoproteins by chemical, chemoenzymatic, or semisynthetic methodologies have been performed by various researchers.[2] However, one of the problems of these methods is that the synthesis of a sufficient amount of the sugars is still far from routine, although the preparation of fairly large sugars, such as the biantennary complex-type *N*-glycan, have been reported.[3] *N*-Linked carbohydrates can also be isolated from natural sources; however, their structures are limited to the natural forms.[4] An efficient method for the glycan synthesis, which is applicable to various glycoforms, is still required.

We have been developing a facile method for the glycoprotein synthesis based on our benzyl-protection strategy for the carbohydrate portion combined with a ligation technique for the peptide chain assembly. Using this strategy, we have succeeded in the synthesis of various glycoproteins,[5] such as the extracellular first immunoglobulin domain of emmprin carrying the *N*-linked core pentasaccharide[5a] and 23 kDa mucin model carrying multiple *O*-GalNAcs.[5b]

To extend our strategy for the synthesis of *N*-glycoproteins carrying larger carbohydrates, we recently established an efficient route for the synthesis of 9-fluorenylmethoxycarbonyl (Fmoc)-Asn carrying a complex-type nonasaccharide suitable for the solid-phase peptide synthesis (SPPS) of a glycoprotein.[6] The key to this synthesis was the combination of the armed benzyl-protected sugar with the *N*-trichloroacetyl (TCA) group for the protection of the amino group of the LacNAc moiety. This protecting group showed a powerful stereocontrolling ability as well as a high reactivity, enabling the stereoselective formation of the *β*-LacNAc glycosidic linkage during the final stage of the synthesis.[7]

On the other hand, the chemoenzymatic synthesis of the *N*-glycosylated protein, which uses the transglycosylation ability of

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endo-β-*N*-acetylglucosaminidases (Endo A and M) to the GlcNAc residue on the polypeptide,[8] has attracted much attention, and the synthesis of glycopeptides as well as the conversion of natural glycoproteins carrying a heterogeneous sugar to the one with a homogeneous sugar has been demonstrated.[9] The major drawback of this method is that the yield of the product is low due to the hydrolysis of the product. This problem was recently solved by the development of the Endo A and M mutant,[10] although glycosyl oxazolines lacking the reducing end GlcNAc has to be prepared as glycosyl donors. This chemoenzymatic method has a great potential, since a single glycoprotein can be derivatized into various glycoforms, if we can prepare various glycosyl donors. Thus, we intended to apply our synthetic procedure to the preparation of the complex-type sugar oxazoline. Due to its highly convergent nature, our synthetic methodology could be easily used for the synthesis of the sugar oxazoline.

Saposins A–D are activators of glycosidases, which are engaged in the degradation of sphingolipids in lysosomes. Among them, saposin C helps in the degradation of glucosylceramide (GlcCer) by GlcCer-β-glucosidase (GCase) (Figure 1).[11] Saposin C deficiency in humans showed the

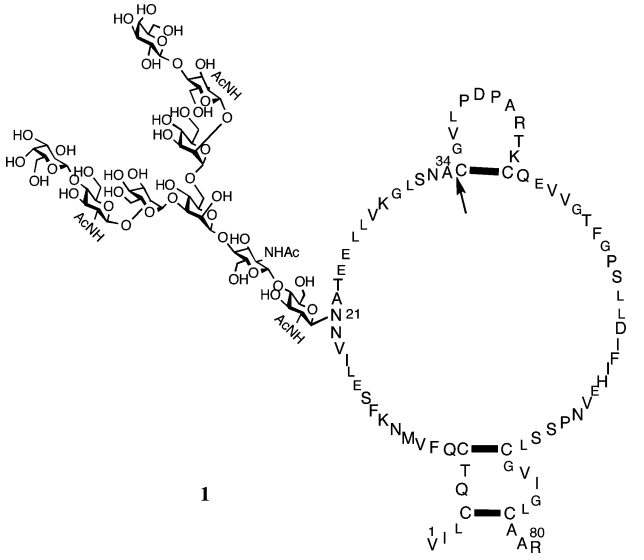


Figure 1. Structure of saposin C carrying complex-type non-asaccharide.

phenotype similar to Gaucher disease with the accumulation of GlcCer, which is due to a GCase deficiency. Saposin C-deficient mice cause neurodegenerative diseases with the patterned loss of cerebellar Purkinje cells and widespread axonal spheroids. Although saposin C is *N*-glycosylated at Asn,[21] its significance is still controversial. Recently, we completed the synthesis of the nonglycosylated saposin C.[12] In spite of the relatively small size of this protein, its synthesis by the native chemical ligation (NCL) method[13] was difficult due to the extremely low solubility of the N-terminal segment in organic and aqueous organic solvents. This fact might be due to the hydrophobic nature of saposin C, which binds to sphingolipid. Thus, we attempted to improve the solubility of the N-terminal segment by introducing the *O*-acyl isopeptide structure[14] and succeeded in its synthesis using the NCL method. Based on the synthetic route for the nonglycosylated saposin C, we now report the chemoenzymatic synthesis of glycosylated saposin C **1** (Figure 1) using the synthetic complex-type sugar oxazoline.

RESULTS AND DISCUSSION

Synthesis of Octasaccharide Oxazoline. Based on the previous synthesis of *N*-glycoasparagine **2** (Figure 2),[6] the

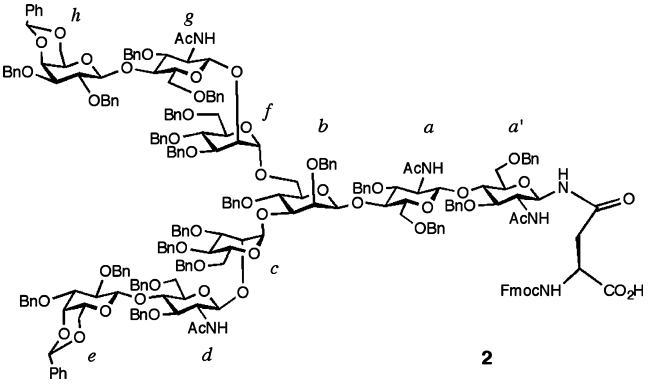
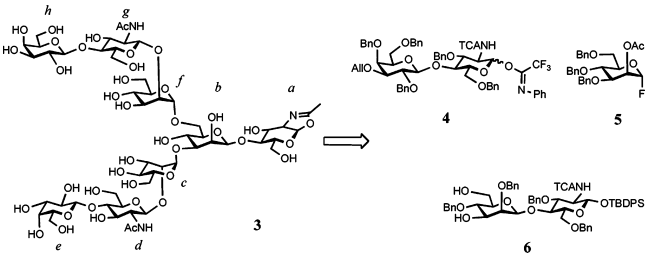


Figure 2. Structure of Asn-linked nonasaccharide **2**.

convergent route for the synthesis of oxazoline **3** was designed (see Scheme 1). The target was divided into the LacNAc unit **4**,

Scheme 1. Retrosynthetic Pathway to Sugar Oxazoline 3



mannosyl donor **5**, and β-mannosyl GlcNAc **6**. In the previous synthesis of the complex type nonasaccharide unit **2**, we first attempted the glycosylation of the LacNAc-mannosyl donor (*h*–*g*–*f* and *e*–*d*–*c* in Figure 2) to the β-mannosyl chitobiose unit (*b*–*a*–*a*′). However, we could not achieve a highly stereoselective di-α-glycosylation. Thus, the mannose and the LacNAc units were sequentially glycosylated with the β-mannosyl chitobiose unit to obtain unit **2**. This sequential glycosylation was also used in this synthesis. The phthaloyl group previously used for the amino protection of the chitobiose moiety was changed to the trichloroacetyl (TCA) group, which was also used for the amino protection at the LacNAc moieties. This change realizes the formation of all acetamido groups in one step by the zinc in acetic acid treatment. The 3-OH group of the Gal in the LacNAc unit was protected by an allyl group so that the same LacNAc unit can be used to construct the poly LacNAc structure or to achieve sialylation of the 3-OH group in the future studies. The obtained octasaccharide unit was completely deprotected and then derivatized into the sugar oxazoline **3**.

The synthesis was started with the construction of β-mannosyl GlcNAc unit **6** following Crich's method[15] as shown in Scheme 2. The mannosyl thioglycoside **7** was activated with 1-benzenesulfinyl piperidine (BSP), 2,4,6-tri-*tert*-butylpyrimidine (TTBP), and triflic anhydride (Tf_2O) at −60 °C and reacted overnight with GlcNAc unit **8** at −78 °C. The product

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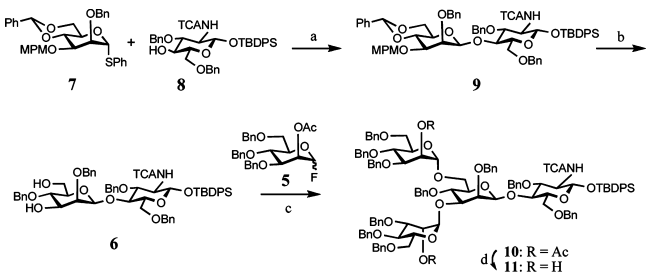
Scheme 2. Synthesis of Tetrasaccharide 11[a]

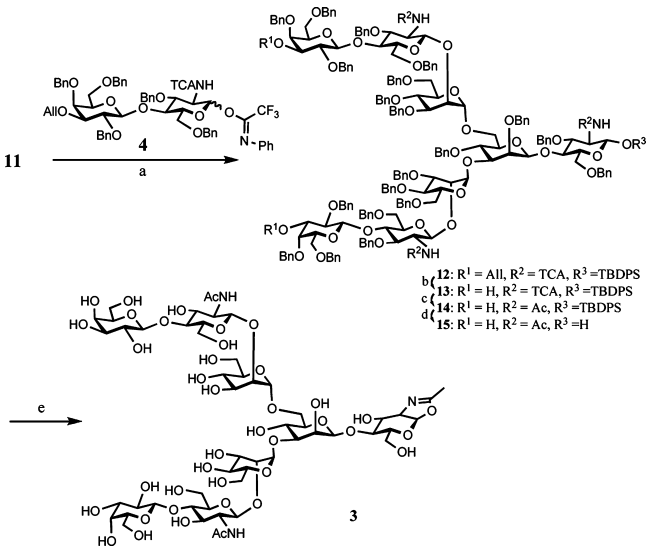


[a]Reagents and conditions: (a) BSP, TTBP, Tf_2O, MS4A, CH_2Cl_2, −78 °C, 1 d, 74%; (b) (1) Et_3SiH, $PhBCl_2$, MS4A, CH_2Cl_2, −78 °C, 0.5 h; (2) 90% TFA aq, CH_2Cl_2, −10 °C, 0.5 h, 87% (two steps); (c) Cp_2ZrCl_2, $AgClO_4$, MS4A, CH_2Cl_2, −40 to −15 °C, 1.5 h, 85%; (d) 1 M NaOMe, MeOH−THF (9:1), rt, 1 d, 88%.

was obtained as a mixture of stereoisomers (β/α = 5/1) in 87% yield. The β-isomer **9** (74% yield) was isolated and treated with Et_3SiH and $PhBCl_2$[16] to regioselectively reduce the benzylidene acetal. The MPM group was then removed by aqueous TFA to afford **6** in 87% yield (two steps). As in the previous synthesis, the mannosylation of **6** with mannosyl fluoride **5**[17] efficiently produced the α,α-dimannosylated tetrasaccharide **10** in 85% yield, which was readily deacetylated to the diol **11** in a yield of 88%.

The glycosylation of **11** with **4**[7b] (3.5 equiv) was promoted using a catalytic amount of trimethylsilyl triflate (TMSOTf) at −78 °C for 1 h and then at −40 °C for 1 h to give the desired octasaccharide **12** as a single isomer in 86% yield (Scheme 3). The protected octasaccharide was then deallylated by the Ir-complex treatment followed by the oxymercuration reaction to give compound **13** in 78% yield (two steps). The TCA groups were converted to Ac groups by Zn treatment in acetic acid and ethyl acetate (EtOAc). This reaction was efficiently promoted

Scheme 3. Synthesis of Sugar Oxazoline 3[a]

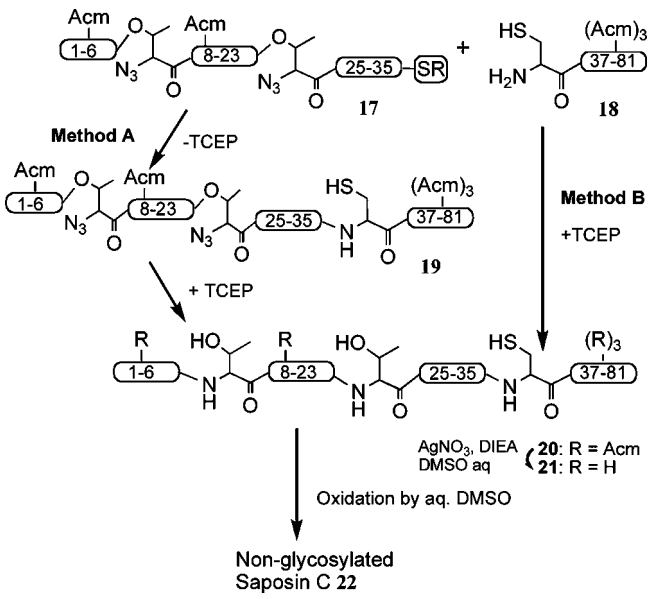


[a]Reagents and conditions: (a) TMSOTf, MS AW-300, CH_2Cl_2, −78 to −40 °C, 2 h, 86%; (b) (1) $Ir(COD)(PMe_2Ph)_2PF_6$, THF, t, 1 h; (2) $HgCl_2$, HgO, 90% acetone aq, rt, 1 h, 78% (two steps); (c) Zn, AcOH, EtOAc, microwave (150 w), reflux, 1 h, 79%; (d) TBAF, AcOH, THF, 0 °C to rt, 1 d, 86%; (e) (1) H_2, $Pd(OH)_2$, 70% THF aq, rt, 2.5 d, **16**: 96%, (2) DMC, Et_3N, H_2O, 0 °C, 6 h, 64%.

by microwave irradiation and the desired product **14** was obtained in 79% yield.[7] The *tert*-butyldiphenylsilyl (TBDPS) group was then removed by tetrabutylammonium fluoride (TBAF) in the presence of AcOH in 86% yield to give compound **15**. Finally, the Bn groups were removed by catalytic hydrogenation to give a completely free octasaccharide **16**. The conversion of this sugar to its oxazoline derivative **3** was successfully achieved by reacting with 2-chloro-1,3-dimethylimidozolium chloride (DMC) according to Shoda's method in 64% yield.[18] Overall, this synthetic route efficiently gave the desired product **3** with a highstereoselectivity. This route can be easily extended to the synthesis of other *N*-linked carbohydrates.

Synthesis of Saposin C carrying a Complex-Type Sugar. In the previous synthesis of the nonglycosylated saposin C **22** (Scheme 4), the SPPS of the N-terminal thioester as well

Scheme 4. Synthetic Route for Nonglycosylated Saposin C 22[12]



as the NCL reaction was hampered by the extreme low solubility of the thioester.[12] Sohma et al. reported that a peptide with the isopeptide structure retains a significantly higher solubility compared to the corresponding native peptide, which is due to the inhibition of the aggregation caused by the intermolecular β-sheet formation.[14] Thus, we applied this method to the synthesis of the N-terminal segment of saposin C using the azido group as an amino protecting group at the isopeptide site.[12] However, the desired peptide thioester **17** was not obtained by the Fmoc method, as the azido-protected isopeptide moiety was unexpectedly decomposed during the piperidine treatment. The use of the benzyloxylcarbonyl (Z) group instead of the azido group also failed to obtain the desired peptide having the Z-protected-*O*-acyl isopeptide structure. Thus, the synthesis of **17** was accomplished by the *t*-butoxylcarbonyl (Boc) strategy, which does not use a nucleophilic base during the synthesis. The NCL reaction with the C-terminal peptide **18** efficiently proceeded in the absence of tris(2-carboxyethyl)phosphine (TCEP) and the product carrying the azido-protected *O*-acyl isopeptide moieties **19** were successfully obtained (Scheme 4, method A). However, the ligation also efficiently proceeded even in the

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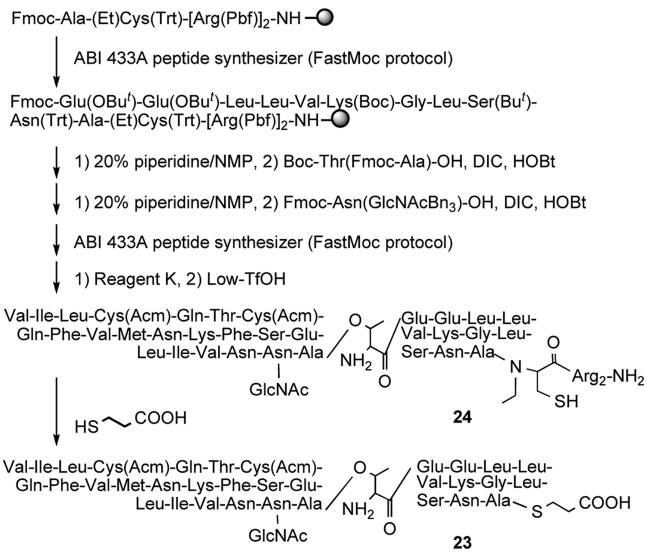
presence of TCEP, which quickly reduces the azido group and allows the *O*- to *N*-acyl shift reaction during the ligation (method B). This result was somewhat unexpected, as the acyl shift reaction would cause aggregation of the *N*-terminal segment, which would lower the efficiency of the ligation. This result might indicate that once the peptide is completely dissolved in a buffer, it remains soluble even after the *O*-acyl isopeptide structure is lost. Based on this speculation, we expected that the azido protection at the isopeptide moiety is not necessary. After removal of the Acm groups, the disulfide bond was formed by DMSO oxidation in Tris buffer to obtain the nonglycosylated saposin C **22**.

The glycosylated saposin C **1** was chemoenzymatically prepared based on the synthesis of the nonglycosylated one. First, the entire polypeptide chain of saposin C carrying GlcNAc was assembled by the NCL reaction, and then the enzymatic transfer of the sugar oxazoline was carried out to the reducing end GlcNAc on the polypeptide chain. It is noted that the N-terminal Asn in the nonglycosylated saposin C **22** was trimmed in this synthesis following a recent report.[11e] To achieve this strategy, the N-terminal peptide thioester carrying GlcNAc was synthesized as shown in Scheme 5. As the azido

Scheme 5. Synthesis of Peptide Thioester Carrying GlcNAc 23



protection at the acyl isopeptide moiety was omitted on the basis of the previous result, we returned to the use of the Fmoc method, which was routinely used for the synthesis of peptides having the *O*-acyl isopeptide structure.[14] To synthesize it as a thioester, our *N*-alkylcysteine (NAC) thioesterification method[19] was applied. This method realizes the efficient post-SPPS thioesterification reaction using the NAC introduced at the C-terminus of the peptide as an *N*- to *S*-acyl migration device. The NAC method is fully compatible with the conventional Fmoc method and produces the peptide thioester in excellent yield without any significant epimerization.[20]

For the synthesis of the N-terminal segment, the NAC moiety was introduced as a dipeptide with the C-terminal amino acid using Fmoc-Ala-(Et)Cys(Trt)-OH to the [Arg-$(Pbf)_2$]-Rink amide MBHA-resin using diisopropylcarbodiimide (DIC)-1-hydroxybenzotriazole (HOBt) method in CH_2Cl_2. The peptide chain was then elongated by the Fmoc strategy. During the synthesis, Boc-Thr(Fmoc-Ala)-OH and Fmoc-Asn($GlcNAcBn_3$)-OH were introduced. After the completion of the chain assembly, the peptide was cleaved from the resin by the TFA cocktail, and the benzyl group of the GlcNAc moiety was removed by the low-TfOH treatment.[21] The peptide was then treated with 3-mercaptopropionic acid (MPA) to perform the thioesterification by the NAC method. The desired peptide thioester **23** was successfully obtained in 3.8% yield with a purity comparable to the one obtained by the Boc strategy as shown in Figure 3. It seems that under the

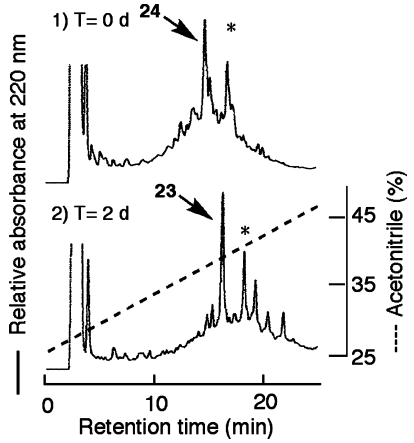


Figure 3. RPHPLC profile of the thioesterification reaction: (a) $T = 0$, (b) $T = 2$ d. Elution conditions: a column for protein purification (4.6 × 150 mm) at the flow rate of 1 mL/min and 50 °C; eluent, A, 0.1% aqueous TFA, B, 0.1% TFA in acetonitrile. The asterisked peaks were derived from the incomplete introduction of Asn[12] during SPPS by the synthesizer.

conditions of the Fmoc method, the *O*-acyl isopeptide structure is stabilized by the Boc-protection, but not by the azido- or Z-protection, the reason of which is not yet clear. The synthesis of the C-terminal segment **25** was achieved by the same procedure as described for the synthesis of the nonglycosylated saposin C.[12]

Prior to the NCL reaction, the N- and C-terminal segments were mixed in an equimolar amount in aqueous acetonitrile containing 0.1% TFA and a part of the mixture was analyzed by HPLC as shown in Figure 4 (a). After lyophilization, the ligation was carried out in a sodium phosphate buffer containing 6 M guanidium hydrochloride, 2% TCEP, and 2% 4-mercaptophenylacetic acid (MPAA)[22] at pH 8.5 as shown in Scheme 6. Even immediately after the peptides were dissolved in the buffer, the *O*-acyl isopeptide thioester **23** was converted to *N*-acyl peptide thioester **26** as shown by the comparison of the HPLC of the acidic mixture (a) and the $T = 0$ of the ligation reaction (b) in Figure 4. However, as we expected, the ligation efficiently proceeded and was almost complete within 3 h. The isolated yield of the product **27** was 30%. The Acm groups were removed by Ag^+ treatment in aq DMSO in the presence of *N*, *N*-diisopropylethylamine (DIEA). Finally, the disulfide bond was formed by DMSO oxidation in Tris buffer (pH 8.5). As in the case of the nonglycosylated saposin C,[12] the oxidized final product **29** was more hydrophobic than the reduced form **28**, which might be derived from the lipid-binding character of this glycoprotein (Figure 4).

Transglycosylation Reaction to the *N*-Acetylglucosaminylated Saposin C. According to the method of Huang et al.,[10b] the transglycosylation reaction was carried out (Scheme

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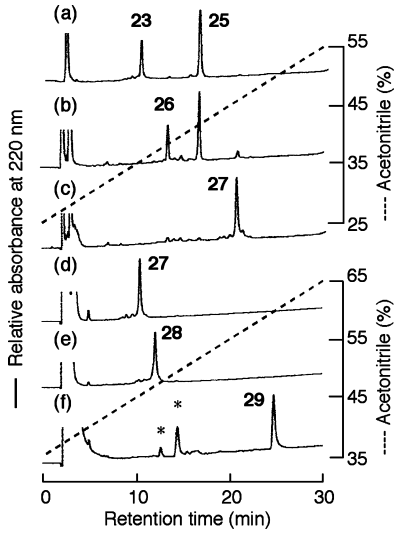
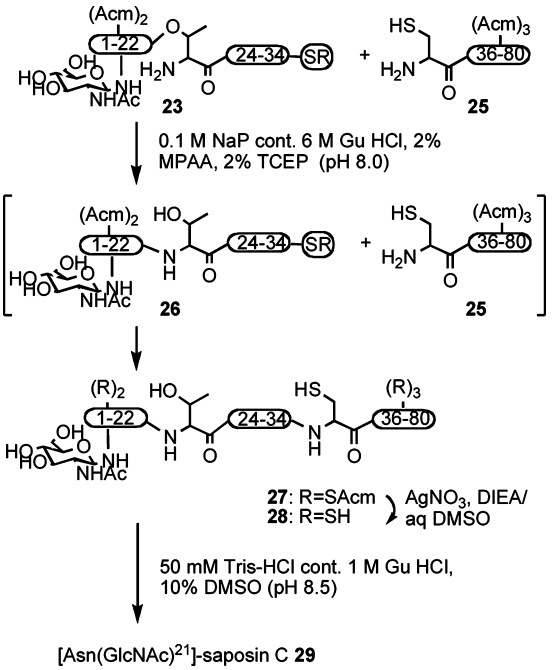


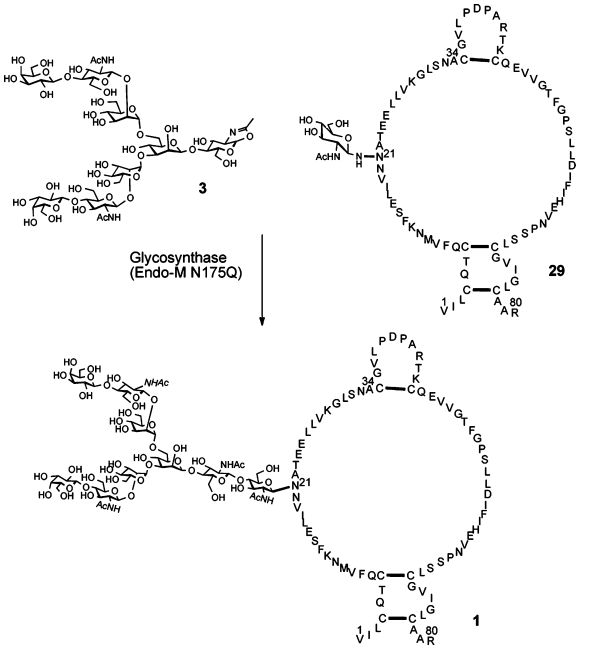
Figure 4. RPHPLC profile of the synthesis of saposin C carrying GlcNAc **29**: (a) acidic mixture of peptides **23** and **25**, (b) T = 0 h of the ligation mixture, (c) T = 3 h of the ligation mixture, (d) purified glycoprotein **27**, (e) reaction mixture after the removal of Acm groups, (f) reaction mixture after DMSO oxidation. The asterisked peaks in (f) are nonpeptidic components. Elution conditions are the same as those of Figure 3.

Scheme 6. Synthesis of Saposin C Carrying GlcNAc 29



7). Saposin C carrying GlcNAc **29** and the sugar oxazoline **3** (10 equiv to **29**) were dissolved in phosphate buffer containing 20% DMSO (pH 7.0), and then glycosynthase was added. After the solution was kept at room temperature for 1 h, an additional 10 equiv of oxazoline **3** (10 equiv) was added, and the reaction was continued for another 3 h. As shown in Figure S1 (Supporting Information), HPLC analysis of the reaction mixture showed the appearance of a slightly hydrophilic peak. The matrix assisted-laser desorption ionization-time-of-flight (MALDI-TOF) mass analysis of this peak showed a value that well corresponds to the theoretical one of the desired product

Scheme 7. Synthesis of Saposin C Carrying Nonasaccharide 1



1, indicating a successful reaction. The conversion ratio of GlcNAc to the nonasaccaride was 64% based on a comparison of the HPLC peak areas of both glycopeptides. By further optimization, the amount of the sugar oxazoline used in this reaction would be decreased. However, it should be pointed out that the total amount of the sugar oxazoline was only a small portion of the total amount of the synthetic sugar.

CD Spectrum Measurement of Saposin C. The nona- and monoglycosylated saposin C **1**, **29**, and nonglycosylated saposin C **30** (see the Experimental Section for its synthesis) were dissolved in 5 mM sodium phosphate containing 50 mM NaCl (pH 4.5), and the CD spectrum was measured. As shown in Figure 5, all of the saposin C's showed a very similar spectrum rich in an α-helical structure, which is in good agreement with those previously reported by other researchers.[23] On the basis of these spectra, the carbohydrates on Asn,[21]

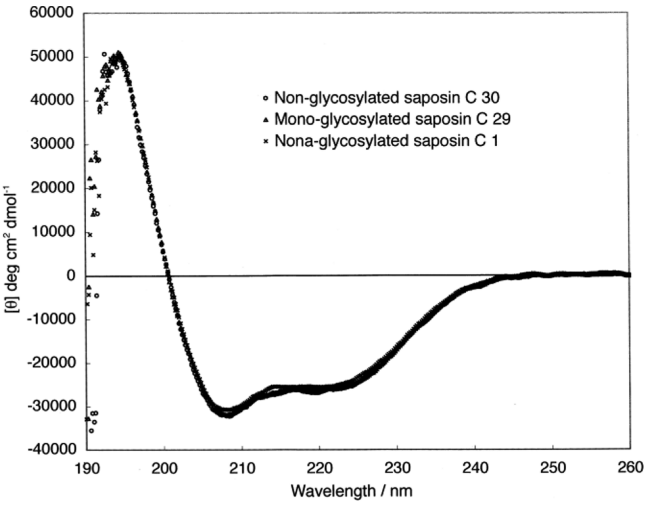


Figure 5. CD spectrum of the synthetic saposin C's in 5 mM sodium phosphate buffer containing 50 mM NaCl at pH 4.5.

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regardless of their size, did not have specific effects on the tertiary structure of saposin C. However, we noticed that the nonglycosylated saposin C tends to form aggregates when purified lyophilized powder was dissolved in an aqueous buffer. Thus, one of the significant features of the glycosylation in saposin C would be the increase in its solubility.

Analysis of GCase Activity. Activation of GCase by the synthetic saposin C was analyzed using 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) as the substrate in a detergent-free system with the negatively charged lipid, L-α-phosphatidylserine (PS), as previously described with minor modifications.[24] The activities of GCase increased 30- to 40-fold in the presence of both PS and the saposin C (nonglycosylated saposin C: 37-fold, monoglycosylated saposin C: 29-fold, nonaglycosylated saposin C: 32-fold) compared to those in the presence of only PS (Table 1). These activations of GCase were somewhat greater than the reported value which showed a 12-fold activation of the GCase activity by the 8 mM concentration of the nonglycosylated recombinant saposin C.[24b] In this assay system with 4MU-Glc as the substrate, the presence or absence of *N*-glycans on saposin C did not affect the degree of GCase activation. Thus, in this in vitro assay system, we could not find a significance of the glycan moiety of saposin C, which was reported by the comparison of the activity between the native and recombinant saposin Cs.[24b] We are now doing further studies to clarify the biological role of saposin C as well as its carbohydrate using our synthetic saposin C's.

Table 1. Effect of Chemically Synthesized Saposin C on GCase Activity

	GCase activity (nmol/h/ng GCase)[a]
PS only	0.0704 ± 0.007
PS+nonglycosylated saposin C **30**	2.59 ± 0.03[b]
PS+monoglycosylated saposin C **29**	2.06 ± 0.04[b]
PS+nona-glycosylated saposin C **1**	2.23 ± 0.02[b]

[a]Values (nmol/h/ng GCase) are presented as mean ± SE (n = 3). [b]P < 0.05.

CONCLUSION

Our convergent approach was successfully applied to the synthesis of the *N*-linked complex-type sugar oxazoline. On the other hand, the *N*-acetylglucosaminylated saposin C was prepared by the NCL reaction incorporating the *O*-acyl isopeptide structure as a solubility enhancing element. The sugar oxazoline was then transferred to the saposin C carrying GlcNAc by the tranglycosylation reaction using the Endo M mutant (glycosythase) and the saposin C carrying the *N*-linked complex-type nonasaccharide was successfully obtained. This chemoenzymatic approach could be useful for the synthesis of other hydrophobic glycoproteins. The synthetic saposins all showed very similar α-helical structures in their CD spectra, which indicates that the glycosylation does not have a specific effect on the conformation of saposin C. We also did not observe a significant difference in the GCase activation activity among the synthetic saposin Cs. Further studies on the significance of the glycosylation in saposin C is currently underway.

EXPERIMENTAL SECTION

General Experimental Methods. The optical rotation values were determined using a polarimeter for the solutions in $CHCl_3$, unless noted otherwise. The ^1H and ^{13}C NMR spectra were recorded by a spectrometer (^1H at 400 MHz and ^{13}C at 100 MHz). Chemical shifts are expressed in ppm downfield from the signal for the internal Me_4Si for solutions in $CDCl_3$. For a description of the NMR data, each sugar residue in the oligosaccharide is indicated by an alphabetical mark as shown in Scheme 1. Peak assignments were performed by H−H and C−H COSY measurements. The MALDI TOF mass spectra were obtained using a spectrometer using 2,5-dihydroxybenzoic acid as a matrix. HPLC was performed in ODS columns (4.6 × 150 mm for analysis, 10 × 250 mm and 20 × 250 mm for preparation). The glycopeptide yield was determined by an amino acid analysis after the samples were hydrolyzed in a sealed tube with 20% HCl and 0.5% phenol at 150 °C for 2 h. The amino acids were analyzed by an amino acid analyzer. Glycosynthase was purchased from a commercial supplier.

***tert*-Butyldiphenylsilyl 2-*O*-Benzyl-4,6-*O*-benzylidene-3-*O*-(4-methoxyphenyl)methyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (9).** A stirred mixture of compound **7** (154 mg, 0.27 mmol), BSP (85 mg, 0.41 mmol), TTBP (134 mg, 0.54 mmol), and dried molecular sieves (MS) 4A (1.1 g) in anhydrous CH_2Cl_2 (2.5 mL) was cooled at −60 °C for 30 min. The mixture was stirred for a further 20 min after the addition of Tf_2O (51 μL, 0.30 mmol). The solution was cooled at −78 °C, and a solution of acceptor **8** (100 mg, 0.13 mmol) in anhydrous CH_2Cl_2 (2.5 mL) was slowly added to the mixture through a cannula. The mixture was then stirred overnight at the same temperature before the reaction was quenched by addition of saturated $NaHCO_3$ aq. The mixture was diluted with EtOAc and filtered through Celite. The organic layer of the combined filtrate was successively washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel with toluene−EtOAc (49:1) to give the disaccharide **9** (141 mg, 0.12 mmol, 87%, α/β = 17:83). $[\alpha]_D$ = −23.0 (c 1.0). R_f = 0.63 (7:1 hexane−EtOAc). ^1H NMR: δ 7.62−7.56 (m, 4H, Ar), 7.40−7.02 (m, 28H, Ar), 6.83 (d, 1H, J = 8.3 Hz, −N*H*), 6.74−6.71 (m, 2H, Ar), 5.41 (s, 1H, PhC*H*<), 4.97 (d, 1H, J = 10.7 Hz, −CH_2Ph), 4.88 (d, 1H, J = 7.8 Hz, H-1a), 4.68 (d, 1H, J = 11.7 Hz, −CH_2Ph), 4.63 (d, 1H, J=12.2 Hz, −CH_2Ph), 4.55 (d, 1H, J = 11.7 Hz, −CH_2Ph), 4.44 (brs, 1H, H-1b), 4.41 (d, 1H, J = 11.2 Hz, −CH_2Ph), 4.40 (d, 1H, J = 10.7 Hz, −CH_2Ph), 4.32 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.11 (d, 1H, J = 12.2 Hz, −CH_2Ph), 3.98−3.88 (m, 3H, H-4a, H-4b, H-6b), 3.77 (brt, 1H, J = 9.0 Hz, H-3a), 3.68−3.60 (m, 5H, H-2a, H-2b, −COCH_3 × 3), 3.39 (t, 1H, J = 10.2 Hz, H-6b), 3.34 (dd, 1H, J = 3.2, 9.5 Hz, H-3b), 3.25 (dd, 1H, J = 2.9, 11.2 Hz, H-6a), 3.18 (dd, 1H, J = 2.4, 11.2 Hz, H-6a), 3.02 (ddd, 1H, J= 4.9, 9.8, 14.2 Hz, H-5b), 2.92 (m, 1H, H-5a), 0.98 (s, 9H, Bu^t). ^{13}C NMR: δ 101.5 ($^1J_{CH}$ = 159.3 Hz, C-1b), 101.2 (PhC*H*<), 94.8 ($^1J_{CH}$ = 163.0 Hz, C-1a), 92.5 (−CCl_3). Anal. Calcd for $C_{66}H_{70}Cl_3NO_{12}Si$: C, 65.86; H, 5.86; N, 1.16. Found: C, 65.88; H, 5.96; N, 1.24.

***tert*-Butyldiphenylsilyl 2,4-Di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside 6.** To a stirred mixture of **9** (2.3 g, 1.9 mmol) and dried MS 4A (7.2 g) in anhydrous CH_2Cl_2 (170 mL) were successively added Et_3SiH (0.92 mL, 5.7 mmol) and $PhBCl_2$ (0.87 mL, 6.7 mmol) at −78 °C. The mixture was stirred for 30 min at that temperature. The reaction was quenched by addition of Et_3N and MeOH. The resulting mixture was diluted with EtOAc and then filtered through Celite. The combined filtrate and washings were successively washed with saturated $NaHCO_3$ aq, water, and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (65 mL) and stirred with 90% aq TFA (65 mL) at −10 °C for 45 min. The acidic mixture was then carefully neutralized with saturated $NaHCO_3$ aq and extracted with EtOAc. The organic layer was successively washed with saturated $NaHCO_3$ aq, water, and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel with toluene-EtOAc (7:1) followed by gel-permeation chromatography (Bio-Beads S-X1, toluene) to afford **6** (1.8 g, 1.7 mmol, 87% in two steps). $[\alpha]_D$ = −22.3 (c 1.0). R_f = 0.39 (7:1 hexane−EtOAc). ^1H NMR: δ 7.67 (m, 2H, Ar), 7.60 (m, 2H, Ar), 6.93 (d, 1H, J = 8.3 Hz, -N*H*), 5.01 (d, 1H, J = 10.7 Hz,

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−CH_2Ph), 4.92 (d, 1H, J = 7.8 Hz, H-1a), 4.87 (d, 1H, J = 11.7 Hz, −CH_2Ph), 4.76 (d, 1H, J = 11.2 Hz, −CH_2Ph), 4.52−4.46 (m, 4H, −CH_2Ph × 3, H-1b), 4.43 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.22 (d, 1H, J = 11.7 Hz, −CH_2Ph), 3.95 (brt, 1H, J = 9.0 Hz, H-4a), 3.87 (brt, 1H, J = 9.3 Hz, H-3a), 3.69 (m, 1H, H-2a), 2.01 (brs, −O*H* × 2), 1.01 (s, 9H, Bu^t). ^{13}C NMR: δ 100.9 (C-1b), 94.8 (C-1a), 92.5 (−CCl_3). Anal. Calcd for $C_{58}H_{64}Cl_3NO_{11}Si$: C, 64.17; H, 5.94; N, 1.29. Found: C, 64.08; H, 5.95; N, 1.25.

***tert*-Butyldiphenylsilyl 2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (10).** A mixture of Cp_2ZrCl_2 (153 mg, 0.52 mmol), $AgClO_4$ (218 mg, 1.1 mmol), and dried MS 4A (1.1 g) in anhydrous CH_2Cl_2 (3.5 mL) was stirred under Ar at room temperature for 30 min and then cooled at −40 °C. To the stirred mixture was added a mixture of **5** (261 mg, 0.53 mmol) and **6** (191 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (7.0 mL) through a cannula. The resulting mixture was stirred at −15 °C for 1.5 h before the reaction was quenched by the addition of saturated $NaHCO_3$ aq. The mixture was diluted with EtOAc and filtered through Celite. The organic layer was successively washed with saturated $NaHCO_3$ aq, water, and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product was chromatographed on Bio-Beads S-X1 with toluene and then on silica gel with toluene−EtOAc (14:1) to afford **10** (303 mg, 0.15 mmol, 85%). $[\alpha]_D$ = +13.0 (c 1.0). R_f = 0.32 (7:1 toluene−EtOAc). ^{1}H NMR: δ 7.61 (d, 2H, J = 6.8 Hz, Ar), 7.54 (d, 2H, J = 6.8 Hz, Ar), 6.72 (d, 1H, J = 7.8 Hz, -N*H*), 5.37 (brs, 1H, H-2c), 5.26 (brs, 1H, H-2d), 5.03 (brs, 1H, H-1c), 4.94 (d, 1H, J = 10.7 Hz, −CH_2Ph), 4.83 (d, 1H, J = 7.8 Hz, H-1a), 4.80 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.76 (d, 1H, J = 10.7 Hz, −CH_2Ph), 4.71 (d, 1H, J = 11.2 Hz, −CH_2Ph), 4.68 (brs, 1H, H-1d), 4.63 (d, 1H, J = 11.2 Hz, −CH_2Ph), 4.59 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.51 (brs, 1H, H-1b), 4.51 (d, 1H, J = 10.7 Hz, −CH_2Ph), 4.46 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.44 (d, 1H, J = 11.7 Hz, −CH_2Ph), 4.24 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.14 (d, 1H, J = 10.2 Hz, −CH_2Ph), 4.11 (d, 1H, J=11.7 Hz, −CH_2Ph), 3.91 (t, 1H, J = 8.8 Hz, H-4a), 3.85 (dd, 1H, J = 3.2, 9.0 Hz, H-3c), 3.10 (brd, 1H, J = 10.2 Hz, H-6a), 2.82 (brd, 1H, J = 9.3 Hz, H-5a), 1.99 (s, 3H, −CH_3 × 3), 1.93 (s, 3H, −CH_3 × 3), 0.96 (s, 9H, Bu^t). ^{13}C NMR: δ 101.0 ($^1J_{CH}$ = 157.2 Hz, C-1b), 99.7 ($^1J_{CH}$ = 172.1 Hz, C-1c), 97.5 ($^1J_{CH}$ = 171.3 Hz, C-1d), 94.9 ($^1J_{CH}$ = 166.3 Hz, C-1a), 92.4 (−CCl_3 × 3). Anal. Calcd for $C_{116}H_{124}Cl_3NO_{23}Si$: C, 68.48; H, 6.14; N, 0.69. Found: C, 68.22; H, 6.23; N, 0.66.

***tert*-Butyldiphenylsilyl 3,4,6-Tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (11).** Compound **10** (226 mg, 0.11 mmol) was dissolved in MeOH−THF (9:1, 1.1 mL), and 1 M NaOMe/MeOH (11 μL) was slowly added with stirring. After the stirring was continued overnight, the solution was neutralized by adding Amberlyst, filtered, and concentrated in vacuo. The residue was purified by silica gel with toluene−EtOAc (4:1) to give **11** (190 mg, 98 μmol, 88%). $[\alpha]_D$ = +11.8 (c 1.0). R_f = 0.32 (3:1 toluene−EtOAc). ^{1}H NMR: δ 7.69 (d, 2H, J = 6.8 Hz, Ar), 7.63 (d, 2H, J = 6.8 Hz, Ar), 6.81 (d, 1H, J = 7.3 Hz, -N*H*), 5.13 (brs, 1H, H-1c or -1d), 5.09 (d, 1H, J = 11.2 Hz, −CH_2Ph), 4.89 (d, 1H, J = 11.7 Hz, −CH_2Ph), 4.88 (d, 1H, J = 7.8 Hz, H-1a), 4.81 (brs, 1H, H-1b), 4.80 (d, 1H, J = 10.7 Hz, −CH_2Ph), 4.77 (d, 1H, J = 11.7 Hz, −CH_2Ph), 4.67 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.62 (d, 1H, J = 11.2 Hz, −CH_2Ph), 4.34 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.21 (d, 1H, J = 12.2 Hz, −CH_2Ph), 4.03 (t, 1H, J = 8.8 Hz, H-4a), 3.93 (brs, 1H, H-2c or 2d), 3.32 (dd, 1H, J=2.4 11.7 Hz, H-6a), 2.88 (brd, 1H, J = 9.3 Hz, H-5a), 1.91 (brs, 2H, -O*H* × 2), 1.05 (s, 9H, Bu^t). ^{13}C NMR: δ 101.4 (C-1c or 1d), 100.9 (C-1c or 1d), 99.6 (C-1b), 94.9 (C-1a), 92.4 (−CCl_3). Anal. Calcd for $C_{112}H_{120}Cl_3NO_{21}Si$: C, 68.96; H, 6.20; N, 0.72. Found: C, 68.82; H, 6.25; N, 0.72.

***tert*-Butyldiphenylsilyl 3-*O*-Allyl-2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[3-*O*-allyl-2,4,6-tri-*O*-benzyl-β-D- galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*- benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2- trichloroacetamido-β-D-glucopyranoside (12).** A stirred mixture of **4** (285 mg, 0.25 mmol), **11** (139 mg, 71 μmol), and dried MS AW-300 (0.71 g) in anhydrous CH_2Cl_2 (3.6 mL) was cooled at −78 °C. TMSOTf (2.3 μL, 13 μmol) was then added to the mixture. The stirring was continued for 1 h at −78 °C and for another 1 h at −40 °C. After the reaction was quenched by addition of saturated $NaHCO_3$ aq, the mixture was diluted with EtOAc and filtered through Celite. The organic layer was successively washed with saturated $NaHCO_3$ aq, water, and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product was chromatographed on Bio-Beads S-X1 with toluene and then on silica gel with toluene-EtOAc (19:1) to afford unit **12** (236 mg, 61 μmol, 86%). $[\alpha]_D$ = +8.3 (c 1.0). R_f = 0.42 (2:1 hexane−EtOAc). ^{1}H NMR: δ 7.65 (d, 2H, J = 7.8 Hz, Ar), 7.61 (d, 2H, J = 7.8 Hz, Ar), 6.73 (d, 1H, J = 7.3 Hz, −N*H*), 6.68 (d, 1H, J = 7.3 Hz, −N*H*), 5.96−5.87 (m, 2H, −C*H*=CH$_2$ × 2), 5.35−5.29 (m, 2H, −CH=C*H*$_2$), 5.18 (d, 1H, J = 10.7 Hz, −CH=C*H*$_2$), 5.17 (d, 1H, J = 10.2 Hz, −CH=C*H*$_2$), 5.02 (s, 1H, H-1c), 4.84 (d, 1H, J = 7.8 Hz, H-1a), 4.70 (s, 1H, H-1d), 2.83 (d, 1H, J = 9.3 Hz, H-5a), 1.02 (s, 9H, Bu^t). ^{13}C NMR: δ 102.8 and 102.7 ($^1J_{CH}$ = 161.8 Hz, C-1e and 1 h), 100.8 ($^1J_{CH}$ = 158.1 Hz, C-1b), 100.1 ($^1J_{CH}$ = 169.6 Hz, C-1c or 1f), 98.8 ($^1J_{CH}$ = 171.3 Hz, C-1c or 1f), 98.1 ($^1J_{CH}$ = 163.8 Hz, C-1d or 1 g), 97.3 (C-1d or 1 g), 94.8 ($^1J_{CH}$ = 163.8 Hz, C-1a), 92.6, 92.5, and 92.4 (−CCl_3 × 3). Anal. Calcd for $C_{216}H_{228}Cl_9N_3O_{41}Si$: C, 67.05; H, 5.94; N, 1.09. Found: C, 67.00; H, 5.98; N, 1.06.

***tert*-Butyldiphenylsilyl 2,4,6-Tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (13).** A mixture of Ir(COD)(PMe$_2$Ph)$_2$PF$_6$ (58 mg, 70 μmol) in freshly distilled THF (0.70 mL) was stirred at room temperature for 15 min under H_2, and the atmosphere was replaced by Ar. To the mixture of the activated Ir complex in THF was added a solution of **12** (338 mg, 87 μmol) in THF (0.70 mL) under Ar. After the mixture was stirred for 1 h, it was evaporated under vacuo. The residue was dissolved in 90% aq acetone (1.0 mL), and $HgCl_2$ (113 mg, 0.42 mmol) and HgO (11 mg, 51 μmol) were then added. The mixture was stirred for 1.5 h, diluted with EtOAc, and filtered through Celite. The combined filtrate and washings were successively washed with aq potassium iodide, water, and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel with toluene−EtOAc (6:1) to afford **13** (256 mg, 68 μmol, 78%). $[\alpha]_D$ = +11.8 (c 0.5). R_f = 0.31 (4:1 toluene−EtOAc). ^{1}H NMR: δ 7.57 (d, 2H, J = 8.3 Hz, Ar), 7.53 (d, 2H, J = 7.8 Hz, Ar), 6.72 (d, 1H, J = 7.3 Hz, −N*H*), 6.61 (d, 1H, J = 7.8 Hz, −N*H*), 2.77 (d, 1H, J = 9.8 Hz, H-5a), 0.95 (s, 9H, Bu^t). ^{13}C NMR: δ 102.7 and 102.6 (C-1e and 1 h), 100.9 (C-1b), 100.1 (C-1c or 1f), 98.9 (C-1c or 1f), 97.8 (C-1d or 1 g), 97.2 (C-1d or 1 g), 94.9 (C-1a), 92.6, 92.5, and 92.4 (−CCl_3 × 3).

***tert*-Butyldiphenylsilyl 2,4,6-Tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D- mannopyranosyl-(1→3)-[2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (14).** A mixture of **13** (109 mg, 28 μmol), powdered Zn (1.4 g), and AcOH (1.4 mL) in EtOAc (14 mL) was stirred under microwave irradiation at 150 W for 1 h. The microwave unit was controlled to allow gentle refluxing during this reaction period. After cooling, the insoluble materials were removed by filtration through Celite, and the filtrate was concentrated in vacuo. The residual crude product was purified by chromatography on silica gel with toluene−EtOAc (2:1−5:3) to afford **14** (78 mg, 22 μmol, 79%). $[\alpha]_D$ = +12.1 (c 0.5). R_f = 0.39 (3:2 toluene-EtOAc). ^{1}H NMR: δ 7.57 (d, 2H, J = 7.3 Hz, Ar), 7.51 (d, 2H, J = 7.3 Hz, Ar), 5.58 (d,

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1H, J = 6.3 Hz, -N*H*), 5.18 (d, 1H, J = 7.8 Hz, -N*H*), 5.08 (d, 1H, J = 7.3 Hz, -N*H*), 1.62 (s, 3H, $-COCH_3 \times 3$), 1.58 (s, 3H, $-COCH_3 \times 3$), 1.41 (s, 3H, $-COCH_3 \times 3$), 0.96 (s, 9H, Bu^t). ^{13}C NMR: δ 102.8 and 102.6 (C-1e and 1 h), 100.7 (C-1b), 99.6 (C-1c or 1f), 98.1, 98.0, and 97.9 (C-1c or 1f, d and g), 94.9 (C-1a). HRMS: calcd for $C_{210}H_{229}N_3O_{41}Si$ $[M + 2Na]^{2+}$, m/z 1762.2772 (100), found 1762.2761.

2,4,6-Tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2- acetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)-[2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido- β-D-glucopyranose (15). To a stirred mixture of **14** (77 mg, 22 μmol) and AcOH (13 μL, 0.23 mmol) in freshly distilled THF (0.40 mL) was added 1 M tetra-*n*-butylammonium fluoride (TBAF)/THF (88 μL, 88 μmol) at 0 °C. The mixture was stirred at room temperature for 20 h. The THF was evaporated in vacuo, and the product was extracted with EtOAc. The extract was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on HPLC (JAYGEL 2H, 20 × 600 mm, CH_2Cl_2) to give compound **15** (62 mg, 19 μmol, 86%). R_f = 0.36, 0.27 (29:1 $CHCl_3$–MeOH). ^1H NMR: δ 5.70 (δ, 1H, J = 6.3 Hz, −N*H*), 5.32 (d, 1H, J = 7.3 Hz, −N*H*. HRMS: calcd for $C_{194}H_{211}N_3O_{41}$ $[M + 2Na]^{2+}$, m/z 1643.2184 (100), found 1643.2173.

β-D-Galactopyranosyl-(1→4)-2-deoxy-2-acetamido-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-β-D- glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-β-D-mannopyranosyl-(1→4)-2-deoxy-2-acetamido-β-D-glucopyranose (16). Compound **15** (56 mg, 17 μmol) was dissolved in 70% aq THF (8.5 mL), and $Pd(OH)_2$ (56 mg) was added. After the resulting mixture was stirred for 2.5 d under a H_2 atmosphere, the product was extracted by THF–H_2O and filtered through Celite, and the filtrate was concentrated in vacuo. The crude product was purified by RPHPLC using an ODS column (10 × 250 mm) in distilled water to give compound **16** (24 mg, 17 μmol, 96%). MALDI-TOF mass, found m/z 1460.68, calcd for $(M + Na)^+$ 1460.50. The NMR spectrum of compound **16** was identical to a previously reported one (see the Supporting Information).[9b] MALDI-TOF MS: found m/z 1460.56 $(M + Na)^+$, calcd for $(M + Na)^+$ 1460.50.

β-D-Galactopyranosyl-(1→4)-2-deoxy-2-acetamido-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→4)-2-deoxy-2-acetamido-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-β-D-mannopyranosyl-(1→4)-1,2-dideoxy-β-D-glucopyrano-2,1-D-2-oxazoline (3). Compound **16** (20 mg, 14 μmol) was dissolved in distilled water (0.40 mL) containing Et_3N (84 μL, 0.61 mmol), and DMC (34 mg, 0.20 mmol) was added at 0 °C. After the reaction mixture was stored at 0 °C for 1 h, the solution was applied to an ODS column (10 × 250 mm at a flow rate of 2.5 mL/min) using a linear gradient of acetonitrile concentration in distilled water from 1% to 15% in 14 min, and the fraction containing the desired product was collected. After 1 M NaOH (0.2 equiv to the compound **16** used) was added, the solution was lyophilized to obtain the product **3** (12 mg, 8.7 μmol, 64%). The NMR spectrum of compound **3** was identical with a previously reported one (see the Supporting Information).[9b]

Synthesis of Saposin C Carrying GlcNAc. *[Asn(GlcNAc)^{21}, Cys(Acm)^{4,7}]-Saposin C (1–34)-SCH$_2$CH$_2$COOH (**23**).* Fmoc-Rink amide MBHA resin (0.74 g, 0.25 mmol) was subjected to automated synthesis by the FastMoc protocol to produce the H-Arg(Pbf)-Arg(Pbf)-NH-resin. To this resin, Fmoc-alanyl *N*-ethyl-*S*-tritylcysteine (0.30 g, 0.44 mmol), activated by DIC (0.10 mL, 0.66 mmol) and HOBt (89 mg, 0.66 mmol) in dichloromethane for 30 min at room temperature, was added. After the mixture was shaken overnight, the peptide chain was elongated by a synthesizer using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as an activator and H-Glu(OBu^t)-Glu(OBu^t)-Leu-Leu-Val-Lys(Boc)-Gly-Leu-Ser(Bu^t)-Asn(Trt)-Ala-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin was obtained. Boc-Thr(Fmoc-Ala)-OH (230 mg, 0.45 mmol), activated by DIC (0.077 mL, 0.50 mmol) and HOBt (62 mg, 0.45 mmol) in dichloromethane for 30 min at room temperature, was added, and the mixture was shaken for 3 h at room temperature. The Fmoc group was removed by 20% piperidine/NMP treatment for 5 min, followed by a 15-min treatment using the new reagent. Fmoc-Asn(GlcNAcBn$_3$)-OH (0.26 g, 0.31 mmol), activated by DCC (93 mg, 0.45 mmol) and HOBt (62 mg, 0.45 mmol) in NMP for 30 min at room temperature, was added, and the mixture was shaken overnight at room temperature. The obtained resin was again subjected to automated synthesis to produce Boc-Thr[H-Val-Ile-Leu-Cys(Acm)-Gln(Trt)-Thr(Bu^t)-Cys(Acm)-Gln(Trt)-Phe-Val-Met-Asn(Trt)-Lys-(Boc)-Phe-Ser(Bu^t)-Glu(OBu^t)-Leu-Ile-Val-Asn(Trt)-Asn-(GlcNAcBn$_3$)-Ala]-Glu(OBu^t)-Glu(OBu^t)-Leu-Leu-Val-Lys(Boc)-Gly-Leu-Ser(Bu^t)-Asn(Trt)-Ala-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (2.1 g). A part of the resin (0.50 g) was treated with TFA containing 2.5% TIS and 2.5% H_2O (5.0 mL) at room temperature for 1 h. After the reaction mixture was filtered, the TFA was removed by a nitrogen stream, and the crude peptide was precipitated by diethyl ether and dried in vacuo. The product was then treated with the low-TfOH (TfOH/TFA/dimethyl sulfide/m-cresol, 10:50:30:10, 2 mL) at −15 °C for 2 h. The product was precipitated by cold diethyl ether, washed two times with the same solvent, and dried in vacuo. The residue was dissolved in 50% aqueous acetonitrile containing 6 M urea and 5% MPA (10 mL), and an aliquot of the solution was analyzed by RPHPLC. Peptide **24** was identified by MALDI-TOF mass analysis (found: m/z 4558.4 (average) $(M + H)^+$, calcd for $(M + H)^+$ 4559.4 (average)). The solution was stored overnight at 40 °C and loaded on a RPHPLC column (Mightysil 5C18, 10 × 250 mm). The fraction containing the product was isolated and lyophilized to give the desired peptide thioester **23** (2.3 μmol, 3.8% based on the C-terminal Ala residue on the initial resin). MALDI-TOF mass: found m/z 4204.5 (average), calcd for $(M + H)^+$ 4204.9 (average). Amino acid analysis: $Asp_{3.73}Thr_{1.30}Ser_{1.69}Glu_{4.74}Gly_{1.01}Ala_2Val_{2.97}Met_{0.94}Ile_{1.11}Leu_{4.68}Phe_{1.86}$-$Lys_{2.02}$.

*[Cys(Acm)^{71,77}]-Saposin C (35–80)-NH$_2$ (**25**).* Fmoc-Rink amide MBHA resin (0.74 g, 0.25 mmol) was subjected to an automated synthesis to produce H-Cys(Trt)-Gly-Val-Leu-Pro-Asp(OBu^t)-Pro-Ala-Arg(Pbf)-Thr(Bu^t)-Lys(Boc)-Cys(Acm)-Gln(Trt)-Glu(OBu^t)-Val-Val-Gly-Thr(Bu^t)-Phe-Gly-Pro-Ser(Bu^t)-Leu-Leu-Asp(OBu^t)-Ile-Phe-Ile-His(Trt)-Glu(OBu^t)-Val-Asn(Trt)-Pro-Ser(Bu^t)-Ser(Bu^t)-Leu-Cys(Acm)-Gly-Val-Ile-Gly-Leu-Cys(Acm)-Ala-Ala-Arg(Pbf)-NH-resin (2.4 g). A part of the resin (0.94 g) was treated with Reagent K (15 mL) for 2 h at room temperature. The TFA was removed by a nitrogen stream, and the product was precipitated by ether, which was washed twice with the same solvent and dried in vacuo. The powder obtained was purified by RPHPLC to obtain **25** (14 μmol, 15%). MALDI-TOF MS: found m/z 4994.9 $(M + H)^+$, calcd for $(M + H)^+$ 4994.6. Amino acid analysis: $Asp_{2.91}Thr_{1.80}Ser_{2.47}Glu_{3.04}Pro_{3.96}Gly_5$-$Ala_{3.06}Val_{4.27}Ile_{2.54}Leu_{5.02}Phe_{2.06}Lys_{1.02}His_{0.91}Arg_{1.97}$.

*[Asn(GlcNAc)^{21}, Cys(Acm)^{4,7,46,71,77}]-Saposin C (**27**).* The peptide thioester **23** and peptide **25** (240 nmol each) were dissolved in 0.1 M sodium phosphate containing 2% TCEP, 2% MPAA, and 6 M guanidine HCl (120 μL), and the solution was stored at room temperature. At T = 0, an aliquot of the solution was analyzed by RPHPLC and peptide thioester **26** was identified (found m/z 4225.9 (average) $(M + H)^+$, calcd for $(M + H)^+$ 4226.9 (average)). After 3 h, the product was purified by gel filtration chromatography to obtain the glycoprotein **27** (70 nmol, 30%). MALDI-TOF MS: found m/z 9094.1 (average) $(M + H)^+$, calcd for $(M + H)^+$ 9095.7 (average). Amino acid analysis: $Asp_{6.89}Thr_{3.33}Ser_{4.41}Glu_{7.90}Pro_{3.96}Gly_{6.02}Ala_5Val_{6.86}Met_{0.89}$-$Ile_{3.21}Leu_{9.84}Phe_{3.78}Lys_{3.20}His_{0.79}Arg_{2.03}$.

*[Asn(GlcNAc)^{21}]-Saposin C (**29**).* The peptide **27** (45 nmol) was dissolved in DMSO (90 μL) containing DIEA (0.15 μL, 0.86 μmol), and $AgNO_3$ (1.1 mg, 6.5 μmol) dissolved in distilled water (15 μL) was added. The reaction mixture was stored at 50 °C for 2 h in the dark. After the silver ions were quenched by DTT (10 mg), the product was isolated by gel filtration chromatography and lyophilized. The obtained peptide **28** (found m/z 8739.5 (average) $(M + H)^+$, calcd for $(M + H)^+$ 8738.2 (average)) was dissolved in 6 M guanidine hydrochloride (100 μL), which was dropped in 50 mM Tris-HCl

buffer (3.0 mL) containing 0.5 M guanidine hydrochloride and 10% DMSO (pH 8.5). After the solution was kept at 5 °C for 2 d, the product was isolated by RPHPLC to give [Asn(GlcNAc)^{21}]-saposin C (**29**) (13 nmol, 29%). MALDI-TOF MS: found m/z 8734.4 (average) $(M + H)^+$, calcd for $(M + H)^+$ 8734.2 (average). Amino acid analysis: $Asp_{7.01}Thr_{3.22}Ser_{3.27}Glu_{7.90}Pro_{3.93}Gly_6Ala_{4.93}Val_{8.30}Met_{0.66}Ile_{4.44}Leu_{9.95}Phe_{3.94}Lys_{3.00}His_{0.96}Arg_{2.00}$.

Enzymatic Transfer of Complex-Type Sugar to Saposin C. *{Asn[(Gal-GlcNAc-Man)$_2$Man-GlcNAc$_2$]^{21}}-Saposin C (**1**).* [Asn-(GlcNAc)^{21}]-Saposin C **29** (12 nmol) was dissolved in 50 mM sodium phosphate containing 20% DMSO (pH 7.0, 5.0 μL). Octasaccharide oxazoline **3** (0.17 mg, 0.12 μmol) dissolved in distilled water (3 μL), glycosynthase (5 munit) was added, and the resulting solution was stored for 3 h at room temperature. Octasaccharide oxazoline (0.17 mg, 0.12 μmol) and glycosynthase (3 munit) were further added, and the solution was stored for another 1 h. The product was purified by an RPHPLC column for protein purification (4.6 × 150 mm at a flow rate of 1.0 mL/min and at 50 °C) using a linear gradient of acetonitrile concentration in distilled water containing 0.1% TFA from 40% to 50% in 20 min and the fraction containing the desired product were collected. The glycoprotein **1** (4.9 nmol, 41%) was obtained as a powder after lyophilization. MALDI-TOF MS: found m/z 10156.0 (average) $(M + H)^+$, calcd for $(M + H)^+$ 10154.5 (average). Amino acid analysis: $Asp_{6.87}Thr_{3.28}Ser_{3.55}Glu_{7.80}Pro_{3.79}Gly_6Ala_{4.88}Val_{8.00}Met_{0.70}Ile_{4.24}Leu_{9.81}Phe_{3.91}Lys_{2.95}His_{0.97}Arg_{1.94}$.

Synthesis of Nonglycosylated Saposin C. *[Cys(Acm)^{4,7}]-Saposin C (1−34)-SCH$_2$CH$_2$COOH (**31**).* The Fmoc-Rink amide MBHA resin (0.29 g, 0.10 mmol) was subjected to automated synthesis by the FastMoc protocol to produce the H-Arg(Pbf)-Arg(Pbf)-NH-resin. To this resin, Fmoc-alanyl *N*-ethyl-*S*-tritylcysteine (0.10 g, 0.15 mmol), activated by DIC (50 μL, 0.30 mmol) and HOBt (27 mg, 0.20 mmol) in dichloroethane (0.50 mL) for 30 min at room temperature, was added. After the mixture was shaken overnight, the peptide chain was elongated by an synthesizer and H-Glu(OBu^t)-Glu(OBu^t)-Leu-Leu-Val-Lys(Boc)-Gly-Leu-Ser(Bu^t)-Asn(Trt)-Ala-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin was obtained. After Boc-Thr(Fmoc-Ala)-OH (77 mg, 0.15 mmol), activated by DIC (50 mL, 0.30 mmol) and HOBt (27 mg, 0.20 mmol) in dichloromethane for 30 min at room temperature, was added and the mixture was shaken for 1 h at 40 °C, the peptide chain was again elongated by the FastMoc protocol to produce Boc-Thr[H-Val-Ile-Leu-Cys(Acm)-Gln(Trt)-Thr-(Bu^t)-Cys(Acm)-Gln(Trt)-Phe-Val-Met-Asn(Trt)-Lys(Boc)-Phe-Ser-(Bu^t)-Glu(OBu^t)-Leu-Ile-Val-Asn(Trt)-Asn(Trt)-Ala]-Glu(OBu^t)-Glu(OBu^t)-Leu-Leu-Val-Lys(Boc)-Gly-Leu-Ser(Bu^t)-Asn(Trt)-Ala-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (720 mg). A part of the resin (150 mg) was treated with TFA containing 2.5% TIS and 2.5% H_2O (2.0 mL) at room temperature for 2 h. After the reaction mixture was filtered, TFA was removed by a nitrogen stream, and the crude peptide was precipitated by diethyl ether and dried in vacuo. The residue was dissolved in 50% aqueous acetonitrile containing 6 M urea and 5% MPA (5.0 mL), then stored at 40 °C for 2 d. The solution was loaded on a RPHPLC column (Mightysil 5C18, 10 × 250 mm) and the fraction containing the product was isolated and lyophilized to give the desired peptide thioester **31** (344 nmol, 1.7% based on the C-terminal Ala residue in the initial resin). MALDI-TOF mass: found m/z 4001.3 (average), calcd for $(M + H)^+$ 4001.7 (average). Amino acid analysis: $Asp_{3.24}Thr_{1.20}Ser_{1.57}Glu_{4.08}Gly_{1.06}Ala_2Val_{2.54}Met_{0.68}Ile_{0.85}Leu_{4.45}Phe_{1.51}Lys_{1.74}$.

*[Cys(Acm)^{4,7,46,71,77}]-Saposin C (**32**).* The peptide thioester **31** and peptide **25** (220 nmol each) were dissolved in 0.1 M sodium phosphate containing 2% TCEP, 2% MPAA and 6 M guanidine HCl (100 μL), then the solution was stored at room temperature. After 6 h, the product was purified by RPHPLC to obtain **32** (61 nmol, 27%). MALDI-TOF MS: found m/z 8890.9 (average) $(M + H)^+$, calcd for $(M + H)^+$ 8892.5 (average). Amino acid analysis: $Asp_{6.63}Thr_{1.81}Ser_{4.13}Glu_{7.70}Pro_{3.79}Gly_6Ala_{4.95}Val_{7.17}Met_{1.02}Ile_{3.52}Leu_{9.45}Phe_{3.70}Lys_{2.88}His_{0.86}Arg_{1.93}$.

*Saposin C (**30**).* The peptide **32** (53 nmol) was dissolved in DMSO (50 μL) containing DIEA (0.09 μL, 0.52 μmol), and $AgNO_3$ (1.4 mg, 8.2 μmol) dissolved in distilled water (10 μL) was added. The reaction mixture was stored at 50 °C for 2 h in the dark. After the silver ions were quenched by DTT (3.6 mg, 23 μmol), the product was isolated by gel filtration chromatography and lyophilized. The obtained powder was dissolved in 6 M guanidine hydrochloride (100 μL), which was dropped in 50 mM Tris-HCl buffer (6.0 mL) containing 0.5 M guanidine hydrochloride and 10% DMSO (pH 8.5). After the solution was stored at 5 °C for 2 d, the product was isolated by RPHPLC to give the protein **30** (8.8 nmol, 17%). MALDI-TOF MS: found m/z 8734.4 (average) $(M + H)^+$, calcd for $(M + H)^+$ 8734.2 (average). Amino acid analysis: $Asp_{6.77}Thr_{3.44}Ser_{4.56}Glu_{7.86}Pro_{4.16}Gly_6Ala_{5.11}Val_{6.65}Met_{0.66}Ile_{3.32}Leu_{9.37}$ $Phe_{3.76}Lys_{2.89}His_{0.72}Arg_{1.91}$.

CD Spectrum Measurements. The synthetic saposin C's were dissolved in 5 mM sodium phosphate containing 50 mM NaCl (pH 4.5) at a concentration of 50−100 μg/mL. The CD spectrum was measured between 190 nm and 260 nm by a spectropolarimeter at room temperature using a 1 mm path-length cell. The concentration of the samples was determined by the amino acid analysis of each sample.

GCase Activation Assay. Activation of GCase by the chemically synthesized saposin C was analyzed with 4MU-Glc as the substrate in a detergent-free system with PS. Imiglucerase was used as the GCase source. The enzyme assay mixture contained the final concentrations of 200 mM sodium citrate phosphate buffer (pH 5.5), 20 μg/mL PS, 1 nM imiglucerase, 2.5 mM 4MU-Glc, and 2 mM of each chemically synthesized saposin C (**1**), **29**, and **30** in a 100 μL volume. Prior to the addition of 4MU-Glc, the imiglucerase was preincubated at pH 5.5 with PS vesicles and each saposin C for 30 min at room temperature to facilitate the interaction of the enzyme, PS, and saposin C. 4MU-Glc was then added to the assay mixture and incubated for 30 min at 37 °C. The GCase activities were determined by the amount of fluorescence of the liberated 4MU with Ex/Em 460/515 nm. The data were analyzed by Mann−Whitney's U-test.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **6**, **9**, **11**−**16**, and **3** and HPLC profile of the transglycosylation reaction (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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