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Spontaneously Cleavable Glycosylated Linker Capable of Extended Release of its Conjugated Peptide

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Summary

Reversibly glycosylated conjugates were developed by adding complex-type *N*-linked oligosaccharides to peptides through self-cleavable linkers with the aim of increasing the solubility and stability of the peptides in plasma. The amino or carboxyl group of the peptide was connected to a glycosylated Ascendis or ester/thioester-type linker, respectively. Use of the linkers enabled extended release of the peptides depending on the pH and temperature of the buffer according to a first order reaction, and their cleavage rate was also affected by the structure of the peptide-linker coupling. This tunability will allow optimization towards the intended use of the peptides to be released. Furthermore, because glycosylation is a reliable method of greatly increasing the solubility of a peptide, the presented glycosylated linkers are expected to permit the preparation of antibodies in aqueous buffers even in the case of sparingly soluble antigen peptides.

Keywords self-cleavable linker; extended release; chemical glycosylation; bromoacetamide method; complex-type asparagine-linked oligosaccharide

Introduction

Peptides are involved in a wide variety of human physiological functions, including regulation of the immune system, metabolism and protein degradation, defense against microorganisms, and signal transmission. In general, biologically active peptides can induce a rapid and specific beneficial response at very low concentrations when they bind to specific cell surface receptors or ion channels and trigger intracellular effects. In addition, they are recognized as being relatively safe and well tolerated. Consequently, peptides that possess attractive pharmacological profiles and intrinsic properties can be potential therapeutic candidates.[1)] However, parenterally-administered bioactive peptides often have limited pharmacological efficacy due to their rapid degradation by enzymes, which leads to a short circulating plasma half-life. To solve these issues, several strategies that improve the plasma stability of peptides have been examined, such as polyethylene glycosylation (PEGylation), acylation/lipidation, and glycosylation as well as traditional rational design with the aid of peptide technology.[2)] One of the most promising methods is to link a glycan to the peptide of interest, since glycosylation is generally considered to provide peptides and proteins with prolonged circulating plasma half-life, improved solubility and/or lowered immunogenicity.[3-6)] Taking advantage of the beneficial characteristics of glycans, we have been focusing on covalently attaching human type *N*-linked glycans to bioactive peptides to improve not only their pharmacokinetic properties but also their solubility. However, glycopeptides developed via this approach sometimes suffer from a significant loss of their biological or pharmacological activity, especially when the attached glycan(s) have altered their structures and/or shield their active center, interfering with the interaction with the target receptors or proteins. To overcome this obstacle, in the present study, we attempted to develop a reversibly glycosylated peptide by coupling the parent peptide and glycan through a spontaneously cleavable linker. The resulting extended release of the parent peptide upon

cleavage of the linker in aqueous physiological conditions indicates that the present strategy should offer a useful method of enhancing the lifetime of peptides in vivo. Furthermore, because glycosylation is a reliable method of greatly increasing the solubility of peptides, this approach could be readily applied to antibody preparation even for antigen peptides that are sparingly soluble in water.

Results and Discussion

The linkers used to connect peptide to glycan needed to meet the following prerequisite conditions: they must be stable during the peptide synthesis procedures, but undergo cleavage at 37 ºC under neutral physiological conditions to gradually release the peptide in its non-modified, native active form.[7-9] Asparagine (Asn)-linked complex-type oligosaccharides, readily obtained from hen egg yolk,[10] were used as a glycan building block in the subsequent peptide synthesis. Using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry, incorporation of Fmoc-Asn(Glycan) onto the growing peptide chain was performed without any protection of its hydroxy groups, except for those of the sialic acid carboxyl groups.[11] In addition, a bromoacetamidyl glycan without protecting groups on its hydroxyl or carboxyl groups could be coupled with the sulfhydryl group on the linker molecule to preserve the native glycan structure. This derivative was prepared by coupling bromoacetic acid with the amino glycan produced by peptide *N*-glycosidase digestion, which cleaved the amide bond between the reducing end of the amino glycan and the carbonyl group of the side functional Asn.[12] This bromoacetamide method allows the structure of the conjugated glycan to be changed easily, facilitating a high-throughput synthesis system, because its attachment can be performed via a post-synthetic procedure.[13] A spontaneously cleavable conjugate was designed and prepared by combining a peptide and *N*-glycan through an ester, thioester or Ascendis-type linkage (Fig. 1). The utility of the *N*-glycan bearing cleavable linkers was

examined by attaching them to human epidermal growth factor receptor 2 (HER2, 8-16) (RWGLLLALL),[14] which is sparingly soluble in water, or chemerin-9 (YFPGQFAFS).[15]

Ester linkage

Ester linkages can be expected to be not only gradually cleaved under neutral conditions but also degraded by esterases within living organisms. 4-(Hydroxymethyl)benzoic acid (HMBA) was adopted as a linkage to connect the *C*-terminal of HER2(8-16) or chemerin-9 and the amino group of Cys-NH_2 through ester and amide bonds, respectively. It has been reported that such an HMBA ester is readily cleavable in alkaline solution. The HER2(8-16) conjugate was assembled by stepwise SPPS using an Fmoc strategy on HO-$CH_2C_6H_4$-CO-Cys(Trt)-Rink amide resin. After completion of the chain elongation, trifluoroacetic acid (TFA) treatment afforded the product in free peptide form. Disialoundecasaccharide (disialo) or asialononasaccharide (asialo) was introduced to the SH group of the *C*-terminal Cys by the bromoacetamide method to obtain the glycosylated conjugate (Chart 1). First, we examined the effect of chemical glycosylation on the solubility of the HER2(8-16) conjugates. The parent peptide is sparingly soluble in water. Upon introduction of the glycan to the molecule, however, the product was found to have dramatically increased solubility, as shown in Table 1.

Next, the self-cleavage behavior of the glycosylated conjugate was assessed by subjecting chemerin-9-HMBA-Cys(Disialo) conjugate **5** to various conditions. The chemerin-9 conjugate was synthesized using the same procedure as that for the HER2(8-16) conjugate. After dissolving compound **5** in a buffer, its recovery rate and the regeneration of the parent peptide were measured over time by RP-HPLC. A complementary relationship was found between the decrease in the starting material and the increase in the parent peptide. The half-life of compound **5** at 37 ºC was determined over 45 h in a phosphate buffered saline

(PBS, pH 7.4) and over 0.8 h in a boric-acid buffer (pH 9.0). No cleavage was observed in sodium acetate buffer (pH 4.0) (Fig. 2). Elevation of the pH and temperature of the buffer was found to increase the cleavage rate. Furthermore, the ester linkage of compound **5** also could be cleaved by human carboxylesterases (CES) such as CES1b and CES2, which are abundant in the liver but not in the blood.[16] Their cleavage rate was found to be slower than expected: the CES digestion only slightly shortened the half-life attributable to the self-cleavage of compound **5** in PBS (data not shown). This may be due to steric hindrance with the glycan chain. However, the parent peptide generated upon the ester cleavage was likely to be rapidly degraded by these enzymes.

Thioester linkage

Thioester linkages are considered to be more labile than ester linkages under neutral conditions. HER2(8-16) was linked through its C-terminal carboxylic acid to the SH group on the linker bearing the glycan. Ethane dithiol and 4-mercaptophenylacetic acid were incorporated as linker moieties and were coupled with the acetamidyl asialoglycan and Asn(Asialo)-Gly through thioether and amide bonds, respectively (Chart 2). The water solubility of the resulting thioalkylester and thioarylester conjugates (> 75 mg/mL) was found to be dramatically improved compared with that of the parent peptide (0.71 mg/mL), as in the case of the ester-type conjugate. However, the cleavage rate of the thioester-type conjugates was faster than that of the ester-type conjugates (Fig. 3).

Ascendis-type linker

Ascendis-type linkers are cleavable linkers that can be bound to the amino group of peptides.[17] A chemerin-9 conjugate bearing the asialoglycan (**14**) was designed based on an Ascendis linker framework (Chart 3). Phthalic anhydride was subjected to aminolysis with

the *N*-terminal amino group of the side-chain protected chemerin-9 which had been assembled on a solid support and the resulting carboxyl group was coupled with the amino group of *N*-(2-aminoethyl)piperazine. Subsequently, Fmoc-Asn(Asialo) was introduced to the *N*-terminal imino group. The protected peptide resin **12** was treated by TFA to cleave the resin and all of the protecting groups, except for the N^{α}-Fmoc group. The Fmoc group on **13** was removed using 20% piperidine in DMF to produce the glycosylated conjugate **14**. It was supposed that the carbonyl group of the phthalic acid amide bound to the peptide could be attacked by the nitrogen atom of its other amide activated via an intramolecular catalytic event, resulting in autolysis to release the peptide. The time course of the development of the parent peptide from glycosylated conjugate **14** measured by RP-HPLC and its half-life determined under various conditions are summarized in Fig. 4. This linker was found to have a shorter half-life than the ester and thioester linkers, but it functioned in a similar fashion from the viewpoint that the cleavage rate of the linker increased with the pH and/or temperature of the buffer. Usually, immunization is performed using an antigen peptide emulsified in an adjuvant. For conjugate **14** to be applicable in the development of peptide vaccines, therefore, it must be self-cleavable not only in aqueous solution but also in emulsion. We examined the release rate of the parent peptide from conjugate **14** in an emulsion prepared by mixing equal volumes of the sodium acetate solution (pH 4.0) of conjugate **14** and an adjuvant based on a light mineral oil, Montanide™ ISA 206VG. Conjugate **14** was found to release the parent peptide even in this emulsion in accordance with the same first-order reaction observed in the sodium acetate buffer (pH 4.0). This clearly indicated that the present glycan-bearing Ascendis-type linkers could be used in antibody preparation. Next, to clarify the influence of the *N*-terminal structure in conjugate **14** on its self-cleavage efficiency, we prepared analogs by replacing the Asn(Asialo) residue as shown in Table 2. These analogs were synthesized using the same procedure as that for conjugate **14**.

Substitution of Cys(glycan) for Asn(glycan) resulted in alteration of the linkage structure which connected the glycan and the amino acid residue. This change in linkage structure as well as changes of the glycan structure showed no effect on the cleavage efficiency of the Ascendis-type linkers. When the *N*-terminal amino group was masked by an Fmoc or acetyl group, however, the half-life of the resulting conjugates in the sodium acetate buffer (pH 4.0) was significantly prolonged. In contrast, electron-withdrawing chlorine substitution on the benzene ring of the phthalic acid amide accelerated the cleavage of the linker. Decreasing the electron density of the carbonyl carbon bonded to the peptide could make it susceptible to nucleophilic attack by nitrogen. These results imply that modification of the self-cleavable linker molecule may control its peptide release behavior.

Conclusion

We have demonstrated the utility of ester/thioester and Ascendis-type linkers bearing biodegradable glycans which can be bound to peptides through their carboxyl and amino functions, respectively. In addition, the resulting chemical glycosylation efficiently improved the solubility of the conjugates even when the conjugated peptide itself was sparingly soluble under physiological conditions. The linkers enabled extended release of the conjugated peptides but were stable throughout the Fmoc chemistry-based synthetic process. The self-cleavage rate of the glycosylated linkers was dependent on their coupling structure. Furthermore, their cleavability could be controlled by modifying the electronic environment on their linker molecules. These features of the linkers are expected to allow optimization of the conjugates towards the intended use of the peptides to be released. Especially, such glycosylated linkers could function as a useful method of preparing the antibodies in aqueous buffers even for sparingly soluble antigen peptides.

It has been reported that glycan conjugates with certain sugar chain structures can

target particular organs or tissues.[18] Thus, we are currently aiming to develop conjugates containing a bioactive peptide and glycosylated self-cleavable linker that can not only be delivered to a selected target through the guidance of the glycan molecule but also release the parent peptide at that location.

Experimental

Materials

Bromoacetamidyl glycan derivatives having disialoundecasaccharide, asialononasaccharide, and GlcNAc moieties were obtained from GlyTech, Inc. (Kyoto, Japan). All other reagents and solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), Merck KGaA (Darmstadt, Germany), Peptide Institute, Inc. (Osaka, Japan) and Sigma-Aldrich Co. LLC.(St. Louis, MO).

HPLC and MS

Preparative HPLC was carried out on a Hitachi LaChrom Elite HPLC system with a SHISEIDO CAPCELL PAK C18 UG120 (20 × 250 mm) with a binary mixture of A (0.1% TFA in H_2O) and B (MeCN/H_2O/TFA (90/10/0.09)) mobile phases. Separation was performed using the described linear gradient, a flow rate of 7.0 mL/min and detection at 220 nm. Analytical HPLC was performed on a Hitachi LaChrom Elite HPLC system or a Shimadzu UFLC system with a SHISEIDO CAPCELL PAK C18 UG120 (4.6 × 250 mm) using a binary mixture of A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN/H_2O (90/10)) mobile phases. The analysis was performed using a linear gradient at a flow rate of 0.7 mL/min and detection at 220 nm. ESI-MS experiments were conducted on a Synapt HDMS mass spectrometer (Waters).

Solid phase peptide synthesis

Automated peptide synthesis by Fmoc SPPS was carried out on a Prelude automated peptide synthesizer (Protein Technologies, Inc.). The peptide chain was elongated using the standard

Fmoc protocol of coupling with Fmoc-amino acid/1-[*bis*(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU)/*N*-methylmorpholine (5.3/5.0/20 equiv.) in *N*,*N*-dimethylformamide (DMF, 15 min, double coupling). Deprotection of N^{α}-Fmoc groups was performed using 20% piperidine/DMF (5 min × 2). During peptide synthesis, all washings after couplings and deprotections were performed with DMF. The following side chain-protecting groups were employed: *t*-butyl (tBu) for Tyr, *t*-butyloxycarbonyl (Boc) for Trp, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, trityl (Trt) for Cys and Gln.

Synthesis of glycosylated conjugates

*Glycosylated conjugate **2***

Rink amide PEGA resin (100 μmol) was placed in a solid-phase synthesis tube and washed with dichloromethane (DCM) and DMF. After the washing, a DMF (2.5 mL) solution containing Fmoc-Cys(Trt)-OH (234 mg, 0.399 mmol), HCTU (157 mg, 0.380 mmol), and 2,4,6-trimethylpyridine (79.6 μL, 0.600 mmol) was added thereto, and the mixture was shaken at room temperature.[19] After 10 min, the resin was washed with DMF, and the coupling reaction was repeated once again. The Fmoc group was removed by treatment with 20% piperidine in DMF to obtain Cys(Trt)-Rink amide PEGA resin, which was then washed with DMF. Next, a DMF (2.5 mL) solution of 4-hydroxymethyl benzoic acid (HMBA, 61.1 mg, 0.402 mmol), HCTU (157.8 mg, 0.381 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 104.5 μL, 0.600 mmol) was added, and the mixture was shaken at room temperature. After 1 h, the resin was washed with DMF and DCM to obtain HMBA-Cys(Trt) on the resin. To the resin (100 μmol) were added a DCM (5.0 mL) solution containing Fmoc-Leu-OH (176.7 mg, 0.500 mmol), 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT, 148.2 mg, 0.500

mmol), and N-methylimidazole (27.9 μL, 0.350 mmol), and the mixture was shaken at room temperature for 1 h. After the shaking, the resin was washed with DCM and DMF. The peptide chain was elongated on the Leu-HMBA-Cys(Trt)-Rink amide resin with a Prelude automated peptide synthesizer using the standard protocols. After the automated synthesis, the peptide resin was treated with TFA/triisopropylsilane (TIS)/H_2O/1,2-ethanedithiol (EDT) (90/5/2.5/2.5) at room temperature for 3 h to give a crude peptide (**1a**). The aliquot of the obtained peptide **1a** (15.5 mg) was then dissolved in a dimethyl sulfoxide (DMSO)/0.1 M phosphate buffer (pH 7.4) mixed solution (9/1, v/v, 240 μL) containing 50 mM 1,4-dithiothreitol (DTT). To this solution was added a DMSO/0.1 M phosphate buffer (pH 7.4) mixed solution (9/1, v/v, 946 μL) containing 30 mM of asialo-BrAc, and the resulting mixture was shaken at room temperature for 2 h. The reaction mixture was purified by preparative HPLC using a linear gradient of 40–60% B in 20 min to obtain a fraction containing glycosylated conjugate **2**. This fraction was further purified by HPLC [column: SHISEIDO CAPCELL PAK C18 UG120 (5 μm), 20 × 250 mm, flow rate: 7.0 mL/min, eluent A: 0.1% AcOH in H_2O, eluent B: 0.09% AcOH/10% H_2O/90% acetonitrile, A:B = 75:25→60:40 (30 min) linear gradient] to obtain glycosylated conjugate **2** (17.2 mg, 5.79 μmol). ESI-MS: *m/z* calcd for $C_{127}H_{204}N_{20}O_{58}S$ $[M+2H]^{2+}$ 1485.7, $[M+3H]^{3+}$ 990.8, $[M+4H]^{4+}$ 743.3; found 1485.7, 990.8, 743.3.

*Glycosylated conjugate **3***

This conjugate was prepared as described above for conjugate **2** to yield 21.8 mg (51%). ESI-MS: *m/z* calcd for $C_{149}H_{238}N_{22}O_{74}S$ $[M+2H]^{2+}$ 1776.8, $[M+3H]^{3+}$ 1184.8, $[M+4H]^{4+}$ 888.9; found 1776.8, 1184.8, 888.9.

*Glycosylated conjugate **5***

The peptide chain was elongated on the Ser(tBu)-HMBA-Cys(Trt)-Rink amide resin using the same procedure as that for conjugate **2**. After the chain assembly was completed, the peptide resin was treated with TFA/TIS/H_2O/EDT (90/5/2.5/2.5) at room temperature for 3 h to give the crude peptide (**1b**). The aliquot of the obtained peptide **1b** (14.2 mg), disialo-BrAc (41.6 mg, 17.7 μmol), and tris(2-carboxyethyl)phosphine (TCEP, 16.0 mg, 55.8 μmol) were dissolved in a 0.2 M phosphate buffer solution (pH 6.8, 1.15 mL) containing 7 M guanidine hydrochloride and reacted at room temperature. After 3 h, the reaction mixture was purified by HPLC [column: SHISEIDO CAPCELL PAK C18 UG120 (5 μm), 20 × 250 mm, flow rate: 7.0 mL/min, eluent A: 0.1% AcOH in H_2O, eluent B: 0.09% AcOH/10% H_2O/90% MeCN, A:B = 70:30→55:45 (10 min) linear gradient] to give glycosylated conjugate **5** (19.0 mg, 5.33 μmol, 49%). ESI-MS: *m/z* calcd for $C_{151}H_{217}N_{19}O_{77}S$ $[M+3H]^{3+}$ 1187.8, $[M+4H]^{4+}$ 891.1, $[M+5H]^{5+}$ 713.1; found 1187.8, 891.1, 713.1.

*Protected HER2(8-16) (**6**)*

The peptide chain was assembled onto Leu-HMPB-PEGA resin (200 μmol) using a Prelude automated peptide synthesizer. After completion of the chain elongation, the peptide resin was treated with AcOH/2,2,2-trifluoroethanol (TFE) (v/v, 1/1) for 30 min. This procedure was repeated and the combined filtrate was concentrated to dryness under reduced pressure to obtain Boc-Arg(Pbf)-Trp(Boc)-Gly-Leu-Leu-Leu-Ala-Leu-Leu (0.59 g).

*MPAA-Asn(Asialo)-Gly-NH_2 (**7**)*

Trityl chloride (2.0 g, 7.1 mmol) was reacted with 4-mercaptophenylacetic acid (MPAA, 1.0 g, 6.1 mmol) in DCM (35 mL) at room temperature for 2.5 h. The reaction mixture was washed with H_2O, and dried over anhydrous sodium sulfate. After removal of the organic solvent *in vacuo*, the residue was triturated with ether to give a solid material (2.8 g), which

was used for the subsequent reaction without further purification. Next, a DMF (2.0 mL) solution containing the Trt-MPAA (320 μmol), 1-hydroxybenzotriazole (HOBt, 50.7 mg, 375 μmol), and *N,N'*-diisopropylcarbodiimide (DIC, 54 μL, 522 μmol) was added to Asn(asialo)-Gly-Rink-Amide-PEGA resin, and the mixture was shaken at room temperature for 1 h. The obtained peptide resin was washed with DMF and DCM, dried, and then treated with TFA/TIS/H_2O (92.5/5/2.5) for 3 h at room temperature. Then, the filtrate was concentrated under reduced pressure to obtain compound **7** (19.6 mg, 10 μmol, 16%). ESI-MS: *m/z* calcd for $C_{76}H_{120}N_8O_{49}S$ $[M+2H]^{2+}$ 981.34; found 981.37.

*Glycosylated conjugate **8***

The glycosylated linker compound **7** (8.6 mg, 4.4 μmol), protected peptide **6** (35.0 mg, 23.0 μmol), and 1*H*-benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP, 22.8 mg, 43.8 μmol) were dissolved in DMF (0.4 mL) and cooled to −15 °C under nitrogen atmosphere. To this solution was added DIPEA (7.5 μL, 76.4 μmol), and the mixture was stirred at −5 °C to −10 °C. After 2.5 h, TFA (50 μL) was added, and the mixture was concentrated to dryness under reduced pressure. A TFA/H_2O (95/5) solution (1 mL) was added to the obtained residue, and the mixture was stirred for 3 h. Ether was added to the resulting solution to obtain the crude peptide as a precipitate, which was purified by preparative HPLC using a linear gradient of 35–55% B in 30 min to obtain glycosylated conjugate **8** (2.3 mg, 18%). ESI-MS: *m/z* calcd for $C_{128}H_{205}N_{21}O_{58}S_1$ $[M+2H]^{2+}$ 1500.09, $[M+3H]^{3+}$ 1000.39; found 1499.67, 1000.08.

*Glycosylated conjugate **10***

Asialo-BrAc (131.7 mg, 75 μmol) in a phosphate buffer solution (0.1 M, pH 6.72, 4.0 mL) was added dropwise to EDT (63 μL, 750 μmol, 10 eq.) in a phosphate buffer solution (0.1 M,

pH 6.72, 4.0 mL), and reacted at room temperature for 40 min. After completion of the reaction was confirmed by HPLC, the product was purified by preparative HPLC (gradient A:B = 99:1 (0–1 min)→80:20 (30 min)) to obtain product **9** (118.3 mg, yield: 89%). ESI-MS: *m/z* calcd for $C_{66}H_{111}N_5O_{46}S_2$ $[M+2H]^{2+}$ 888.36; found 888.34.

Next, the protected peptide **6** (63.5 mg, 41.8 μmol), the glycosylated linker **9** (41.5 mg, 23.4 μmol), and PyBOP (121.8 mg, 234 μmol) were dissolved in DMF (1 mL) and cooled to −15 °C under nitrogen atmosphere. DIPEA (40.0 μL, 40.7 μmol) was then added to this solution and the mixture was stirred at −15 °C. After 3 h, TFA (100 μL) was added, and the mixture was concentrated to dryness under reduced pressure. A TFA/H_2O (95/5) solution (1 mL) was added to the obtained residue, and the mixture was stirred for 3 h. Et_2O was then added to obtain the crude peptide conjugate as a precipitate. The crude peptide conjugate was purified by preparative HPLC using a linear gradient of 35–55% B in 30 min to obtain the thioalkyl-type glycosylated linker-HER2(8-16) (6.9 mg, yield: 10.5%). ESI-MS: *m/z* calcd for $C_{118}H_{196}N_{18}O_{55}S_2$ $[M+2H]^{2+}$ 1406.52, $[M+3H]^{3+}$ 938.01; found 1406.10, 937.73.

*Glycosylated conjugate **13***

Amino-PEGA resin (200 μmol) was placed in a solid-phase synthesis tube and washed with DMF. A DMF (5.0 mL) solution containing HMPB (121.2 mg, 0.504 mmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU, 161.7 mg, 0.504 mmol), and *N*-ethylmorpholine (57.3 μL, 0.495 mmol) was added, and the mixture was shaken at room temperature for 3 h. After washing with DMF and DCM, a DCM (10 mL) solution containing Fmoc-Ser(tBu)-OH (383.1 mg, 1.00 mmol), MSNT (296.4 mg, 1.00 mmol) and N-methylimidazole (55.8 μL, 0.700 mmol) was added, and the mixture was shaken at room temperature for 3 h. After washing with DCM and DMF, DMF (4 mL), pyridine (1.2 mL) and acetic anhydride (189 μL, 2.00 mmol) were added sequentially, and the

mixture was shaken at room temperature. After 1 h, the resin was washed with DMF and DCM. Fmoc groups in an aliquot (100 μmol) of the resin thus obtained were then removed by treatment with 20% piperidine in DMF for 15 min. The peptide chain was elongated using the same procedure as that for conjugate **2**. The resin (70 μmol) was treated with 20% piperidine in DMF to remove the Fmoc group. After washing with DMF and DCM, phthalic anhydride (104 mg, 0.702 mmol), DCM (2.1 mL), and pyridine (126 μL) were added sequentially, and the mixture was shaken at room temperature for 3 h. After washing with DCM and DMF, a DMF (1.75 mL) solution containing HOBt (47.3 mg, 0.35 mmol) and DIC (51.2 μL, 0.33 mmol) was added, and the mixture was shaken at room temperature. After 15 min, *N*-(2-aminoethyl)piperazine (46.1 μL, 0.35 mmol) was added, and the mixture was shaken at room temperature for 1 h. The coupling reaction was repeated once, and the resin was washed with DMF to give the peptide resin **11**. To an aliquot (20 μmol) of the obtained resin, Fmoc-Asn(asialo) (75 mg, 38 μmol) in a DMSO-DMF (1/1, v/v, 833 μL) solution, TBTU (16.1 mg, 50 μmol) and DIPEA (13.1 μL, 75.2 μmol) were added sequentially, and the mixture was shaken at room temperature for 4.5 h. The resin was then washed with DMF and DCM. Next, TFA/TIS/H_2O/EDT (90/5/2.5/2.5) was added to the resin, and the mixture was shaken at room temperature for 3 h. The resulting filtrate was collected and cooled Et_2O was added to obtain the target compound as a precipitate. The compound was purified by preparative HPLC using a linear gradient of 35–60% B in 30 min to obtain the glycosylated conjugate **13** (8.6 mg). ESI-MS: *m/z* calcd for $C_{149}H_{201}N_{19}O_{64}$ $[M+3H]^{3+}$ 1094.4, $[M+4H]^{4+}$ 821.1; found 1094.4, 821.1.

*Glycosylated conjugate **14***

Compound **13** (4.0 mg, 1.2 μmol) was treated with 20% piperidine in DMF (60 μL) for 5 min. After addition of AcOH (57.5 μL) and 0.1% TFA in H_2O (1.3 mL), the compound was

purified by preparative HPLC using a linear gradient of 20–50% B in 30 min to yield the glycosylated conjugate **14** (3.5 mg, 1.1 μmol, 92%). ESI-MS: *m/z* calcd for $C_{134}H_{191}N_{19}O_{62}$ $[M+3H]^{3+}$ 1020.4, $[M+4H]^{4+}$ 765.6; found 1020.4, 765.6.

*Glycosylated conjugate **15***

The peptide resin **12** (15 μmol) was treated with 20% piperidine in DMF. After washing with DMF, a solution of AcOH (4.3 μL), HOBt (10.1 mg, 75 μmol), and DIC (11.0 μL, 71 μmol) in DMF (375 μL) was added, and the mixture was shaken at room temperature for 1 h. After washing with DMF, the coupling reaction was repeated once again. After washing with DMF and DCM, the peptide resin was treated with TFA/H_2O/TIS/EDT (90/2.5/5.0/2.5) at room temperature for 3 h. Cooled Et_2O was then added to the filtrate to obtain the product as a precipitate. Preparative HPLC of the crude product using a linear gradient of 25–33% B in 8 min to yield 5.0 mg of glycosylated conjugate **15**. ESI-MS: *m/z* calcd for $C_{136}H_{193}N_{19}O_{63}$ $[M+2H]^{2+}$ 1551.1, $[M+3H]^{3+}$ 1034.4; found 1551.1, 1034.4.

*Glycosylated conjugate **20***

This conjugate was prepared as described above for conjugate **14** to yield 1.1 mg. ESI-MS: *m/z* calcd for $C_{134}H_{187}C_{14}N_{19}O_{62}C_4$ $[M+2H]^{2+}$ 1598.0, $[M+3H]^{3+}$ 1065.7; found 1598.0, 1065.7.

*Glycosylated conjugate **16***

To the peptide resin **11** (50 μmol) was added a DMF (1.25 mL) solution containing Fmoc-Cys(Trt)-OH (146.8 mg, 0.251 mmol), HOBt (33.8 mg, 0.250 mmol), and DIC (36.6 μL, 0.238 mmol), and the mixture was shaken at room temperature. After washing with DMF, this procedure was repeated once. After washing with DMF and DCM, the peptide resin was

treated with TFA/H_2O/TIS/EDT (90/2.5/5.0/2.5) at room temperature for 3 h. Cooled Et_2O was then added to the filtrate to obtain the product as a precipitate. The crude product was purified by preparative HPLC using a linear gradient of 50–57.3% B in 22 min to yield 6.6 mg of the Fmoc-Cys-chemerin-9 conjugate connected via the Ascendis-type linker. ESI-MS: *m/z* calcd for $C_{86}H_{98}N_{14}O_{18}S$ $[M+2H]^{2+}$ 824.4; found 824.4.

The obtained peptide (6.6 mg, 4.0 μmol) was treated with 20% piperidine in DMF (1.0 mL). After addition of AcOH (1.0 mL) and 0.1% TFA in H_2O (2.0 mL), the resulting compound was purified by preparative HPLC using a linear gradient of 30–50% B in 30 min to yield Cys-chemerin-9 conjugate connected via the Ascendis-type linker (3.4 mg, 60%). ESI-MS: *m/z* calcd for $C_{71}H_{88}N_{14}O_{16}S$ $[M+2H]^{2+}$ 713.3; found 713.3.

The obtained peptide (2.2 mg) and GlcNAc-BrAc (2.7 mg) were dissolved in 50 mM phosphate buffer solution (pH 7.4, 316 μL) containing 4.9 mM DTT and reacted at room temperature for 50 min. The reaction solution was purified by preparative HPLC using a linear gradient of 25–55% B in 20 min to obtain the glycosylated conjugate **16** (1.8 mg, 73%). ESI-MS: *m/z* calcd for $C_{81}H_{104}N_{16}O_{22}S$ $[M+2H]^{2+}$ 843.4; found 843.4.

*Glycosylated conjugate **17***

This conjugate was prepared as described above for conjugate **16**. ESI-MS: *m/z* calcd for $C_{135}H_{193}N_{19}O_{62}S$ $[M+2H]^{2+}$ 1553.1, $[M+3H]^{3+}$ 1035.7; found 1553.2, 1035.8.

*Glycosylated conjugate **18***

This conjugate was prepared as described above for conjugate **16** to yield 0.9 mg. ESI-MS: *m/z* calcd for $C_{172}H_{237}N_{21}O_{80}S$ $[M+3H]^{3+}$ 1303.8, $[M+4H]^{4+}$ 978.1; found 1308.8, 978.1.

*Glycosylated conjugate **19***

This conjugate was prepared as described above for conjugate **16**. ESI-MS: *m/z* calcd for $C_{157}H_{227}N_{21}O_{78}S$ $[M+3H]^{3+}$ 1229.8, $[M+4H]^{4+}$ 922.6; found 1229.8, 922.6.

General procedure for tracing of hydrolysis behavior in aqueous solution

The hydrolysis behaviors of the glycan-conjugated peptides were traced. The hydrolysis reaction was started by the addition of a buffer solution (acetate buffer solution (pH 4.0) or PBS (pH 7.4)) prewarmed to the reaction temperature (25 °C or 37 °C) to each of the freeze-dried glycan-conjugated peptides. The reaction mixtures were kept at a constant temperature (25 °C or 37 °C) using a block incubator. A given amount of each solution was injected into the HPLC at appropriate time intervals to trace the hydrolysis reaction. The relative starting material concentration was determined from the area of the HPLC peak corresponding to the starting material. The natural logarithm of relative concentration of the starting material was plotted against incubation time. A linear plot was obtained, indicating that the hydrolysis reaction was the first order reaction. Also, the half-life ($t_{1/2}$) of the starting material was calculated according to the expression $t_{1/2} = \ln(2)/k$ (wherein k represents the slope of the linear plot).

General procedure for solubility measurement

Peptide solubilities in aqueous solution were determined as the maximum supernatant concentration in supersaturated peptide solutions. Samples of supersaturated peptide solution (~30 μL) were prepared by adding small aliquots of aqueous solution to approximately 4.5 mg of lyophilized peptide in a microtube. The microtube was shaken at 25 °C for 15 min and then centrifuged at 16100 ×g at 25 °C for 10 min to separate the insoluble and soluble fractions. The concentration of peptide in the supernatant was determined from the extinction coefficient of the solution at 280 nm (ε_{280}).

The molar extinction coefficients of the glycan-peptide conjugates at 280 nm were determined by dividing the absorbance of the conjugate peptide at 280 nm by the concentration determined by amino acid analysis. The molar absorption coefficient ε_{280} of the unmodified peptide at 280 nm was calculated according to the following expression:

ε_{280} ($L \cdot mol^{-1} \cdot cm^{-1}$) = $n_{Trp} \times 5500 + n_{Tyr} \times 1490 + n_{ss} \times 125$

wherein n_{Trp} represents the number of tryptophan residues, n_{Tyr} represents the number of tyrosine residues, and n_{ss} represents the number of disulfide bonds.[20)]

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Conflict of Interest

The authors declare no conflict of interest.

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Legends for Charts and Figures

Chart 1. Synthesis of the glycan-HER2(8-16) conjugates **2**, **3**, and **5** connected via the ester linker.

Chart 2. Synthesis of the glycan-HER2(8-16) conjugates **8** and **10** connected via the thioarylester linker and the thioalkylester linker, respectively. Reagents and conditions: (a) PyBOP, DIPEA, DMF; (b) TFA/H_2O (v/v, 95/5).

Chart 3. Synthesis of the glycan-chemerin-9 conjugate **14** connected via the Ascendis-type linker.

Fig. 1. (A) Structures of the glycan-peptide conjugates and their spontaneously cleavable glycosylated linkers; (B) Structure of the complex-type N-linked oligosaccharide and its symbolic representation.

Fig. 2. Time course of the recovery rate of glycan-HMBA-chemerin-9 conjugate **5** in acetate buffer (pH 4.0), PBS (pH 7.4), and borate buffer (pH 9.0). The conversion reaction was carried out at 25 or 37 °C.

Fig. 3. Time course of the recovery rate of glycan-HER2(8-16) conjugates **8** and **10** containing thioarylester and thioarylester linkers, respectively, in acetate buffer (pH 4.0) and PBS (pH 7.4). The conversion reaction was carried out at 37 °C.

Fig. 4. Time course of the recovery rate of glycan-chemerin-9 **14** containing Ascendis-type linker in acetate buffer (pH 4.0) and PBS (pH 7.4) at 25 or 37 °C.

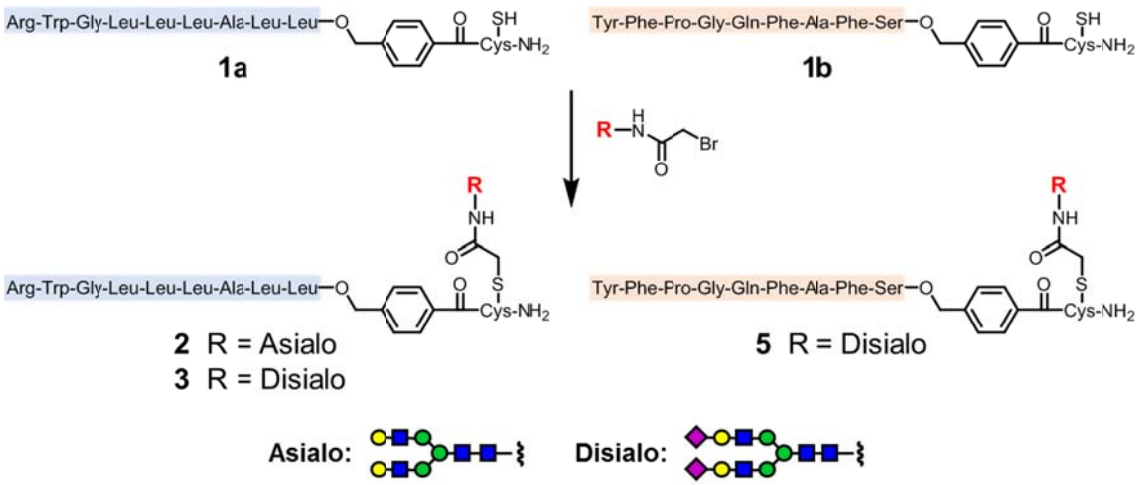


Chart 1.

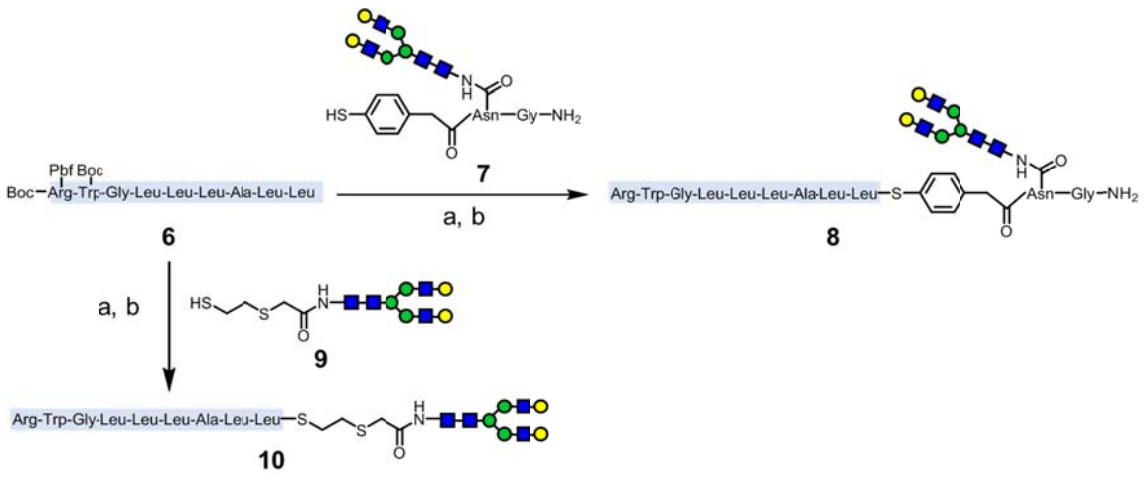


Chart 2.

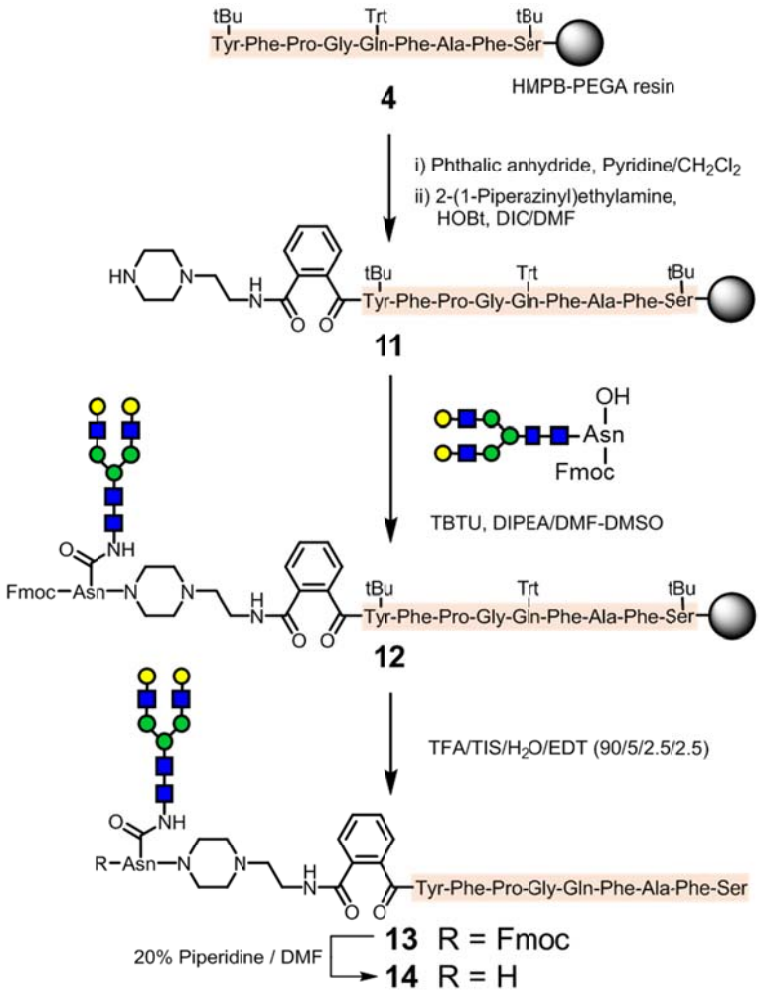

tBu
Trt
tBu
Tyr-Phe-Pro-Gly-Gln-Phe-Ala-Phe-Ser
HMPB-PEGA resin
4
i) Phthalic anhydride, Pyridine/CH2Cl2
ii) 2-(1-Piperazinyl)ethylamine,
HOBt, DIC/DMF
11
OH
Asn
Fmoc
TBTU, DIPEA/DMF-DMSO
12
TFA/TIS/H2O/EDT (90/5/2.5/2.5)
Tyr-Phe-Pro-Gly-Gln-Phe-Ala-Phe-Ser
20% Piperidine / DMF
13 R = Fmoc
14 R = H

Chart 3.

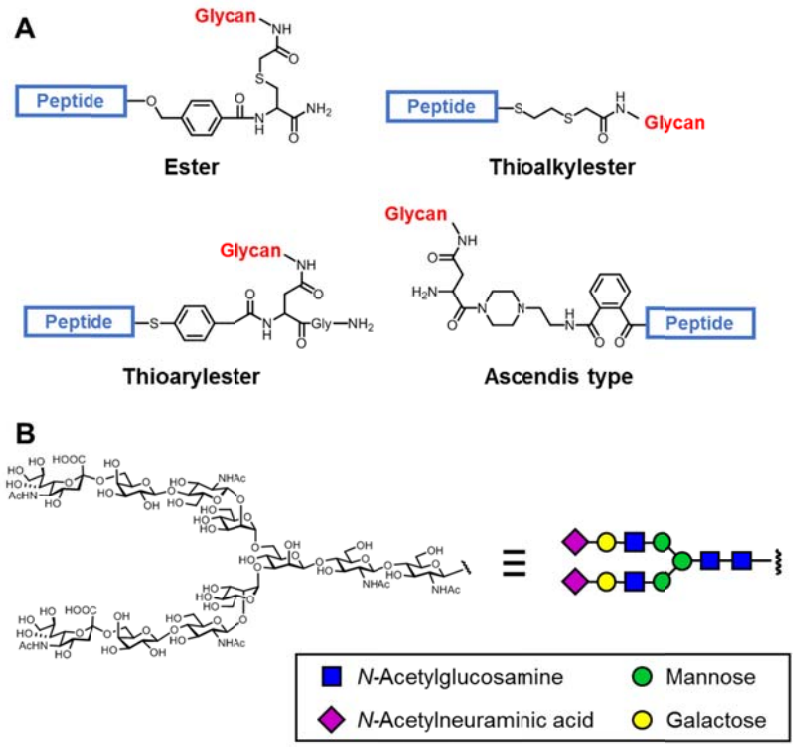

A
Glycan
Peptide
Ester
Peptide
Glycan
Thioalkylester
Glycan
Peptide
Thioarylester
Glycan
Peptide
Ascendis type
B
N-Acetylglucosamine
Mannose
N-Acetylneuraminic acid
Galactose

Fig. 1.

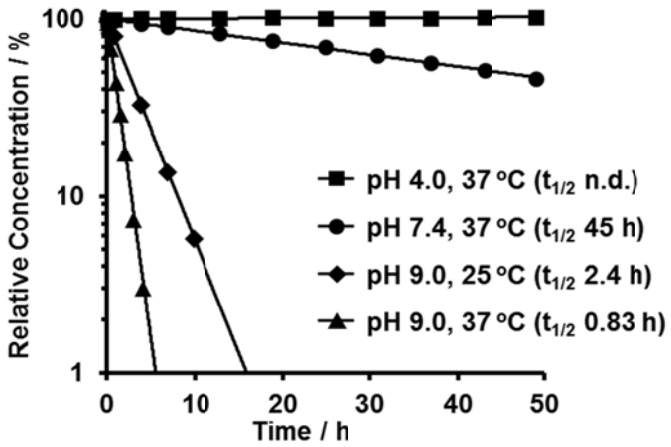

Relative Concentration / %
100
10
1
0
10
20
30
40
50
Time / h
pH 4.0, 37 °C (t1/2 n.d.)
pH 7.4, 37 °C (t1/2 45 h)
pH 9.0, 25 °C (t1/2 2.4 h)
pH 9.0, 37 °C (t1/2 0.83 h)

Fig. 2.

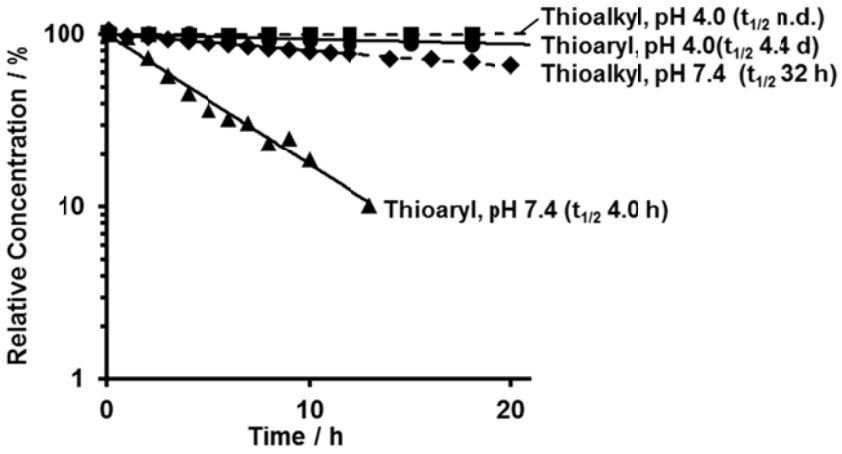

Thioalkyl, pH 4.0 ($t_{1/2}$ n.d.)
Thioaryl, pH 4.0($t_{1/2}$ 4.4 d)
Thioalkyl, pH 7.4 ($t_{1/2}$ 32 h)
Thioaryl, pH 7.4 ($t_{1/2}$ 4.0 h)
Relative Concentration / %
100
10
1
0
10
20
Time / h

Fig. 3.

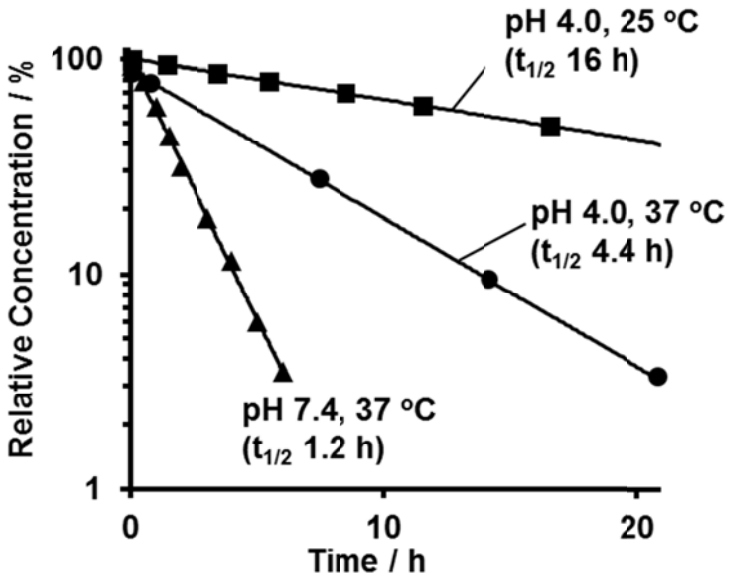
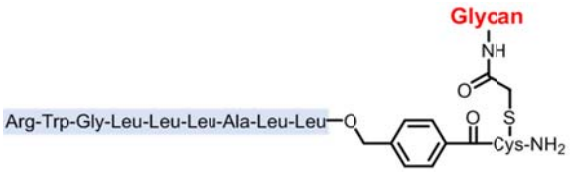

pH 4.0, 25 °C
($t_{1/2}$ 16 h)
pH 4.0, 37 °C
($t_{1/2}$ 4.4 h)
pH 7.4, 37 °C
($t_{1/2}$ 1.2 h)
Relative Concentration / %
100
10
1
0
10
20
Time / h

Fig. 4.

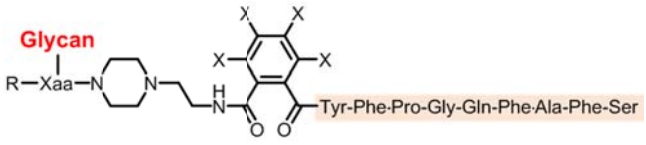
Table 1. Water solubility of HER2 (8-16) peptide and its glycan conjugates.



Conjugate	Glycan	Solubility [a)]
	(parent peptide without linker)	0.71 mg/mL (0.68 mM)
2	Asialo	> 121 mg/mL (> 41 mM)
3	Disialo	> 157 mg/mL (> 44 mM)

[a)] Determined by measuring the peptide content in the supernatant by UV analysis. Glycopeptide solutions were clearly homogeneous at the above concentrations.

Table 2. Cleavage half-life of glycan-chemerin-9 conjugates containing the Ascendis-type linker.



Conjugate	Xaa(Glycan)	R	X	Half-Life (h) [a]	
				pH 4.0	pH 7.4
14	Asn(Asialo)	H	H	4.4	1.2
16	Cys(GlcNAc)	H	H	4.3	1.4
17	Cys(Asialo)	H	H	4.5	1.3
19	Cys(Disialo)	H	H	4.8	1.4
13	Asn(Asialo)	Fmoc	H	12.2	2.6
18	Cys(Disialo)	Fmoc	H	10.5	2.5
15	Asn(Asialo)	Ac	H	12.7	1.4
20	Asn(Asialo)	H	Cl	1.3	0.25
14	Asn(Asialo)	H	H	3.8 [b]	n.t.

[a] Values were calculated from the respective degradation profiles. Tests were performed in sodium acetate buffer (pH 4.0) or PBS (pH 7.4) at 37 °C. [b] The emulsion was prepared by mixing equal volumes of the conjugate solution in sodium acetate buffer (pH 4.0) and the mineral oil adjuvant.