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Total chemical synthesis of monoglycosylated GM2 ganglioside activator using a novel cysteine surrogate†

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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We describe a novel peptide ligation/desulfurization strategy using β-mercapto-*N*-glycosylated asparagine derivative. The newly developed procedure is successfully applied to total chemical synthesis of GM2 ganglioside activator protein bearing a monosaccharide on the native glycosylation site.

GM2 ganglioside activator protein (GM2AP), consisting of 162 amino acid residues, is a lysosomal glycoprotein involved in degradation of GM2 ganglioside (GM2) by β-hexosaminidase A (HexA).[1] Functional deficiency of GM2AP causes abnormal accumulation of undegraded GM2, resulting in a fatal neurological disease known as the AB variant of GM2 gangliosidosis. Protein replacement therapy, based on supplying a functional protein as an alternative to the defective protein, has served as one potential therapeutic approach to such disorders.[2] Although GM2AP-based therapeutics have remained to be developed, GM2AP itself would be expected to have therapeutic effects against the AB variant. In addition, recent studies suggest that the use of GM2AP as a biomarker for lung cancer[3] and a stimulator of insulin secretion.[4] Thus, the development of GM2AP-based protein therapeutics and precisely uncovering their biological functions are crucial for understanding GM2AP-related disorders.

Achieving these objectives requires preparation of GM2AP with high homogeneity. Chemical synthesis is an attractive way to obtain such a homogenous glycoprotein.[5] We have recently established a fully chemical process to make a GM2AP analog in which Asn^{32} is replaced by Cys.[6] The synthetic strategy relies both on native chemical ligation (NCL), which is a powerful technique to assemble a peptide thioester with an N-terminal Cys peptide,[7] and on *N*-sulfanylethylanilide (SEAlide) chemistry which enables one-pot multi-peptide condensation as a crypto-thioester (Fig. 1B).[8] Despite the successful application of these techniques, total synthesis of native GM2AP remains a challenge because of the difficulty of preparing the natural type glycosylated Asn^{32}-containing thioester fragment with at least 67 residues for NCL. One potential approach to confer non-cysteinyl ligation sites is a ligation/desulfurization strategy using Cys surrogates (Fig. 1A).[9] This strategy is based on an NCL-like surrogate-mediated ligation followed by desulfurization of the surrogate. In the chemical synthesis of native GM2AP, a protocol similar to the established ligation/desulfurization protocol seems to be used and Asn^{32} may serve as a potential ligation site for the NCL-like reaction. Wong and co-workers have reported a sugar-assisted ligation (SAL) in which an *N*-glycosylated Asn derivative, with a thiol handle at the C-2 position, is used as a Cys surrogate.[10] In the SAL reaction, a reasonable reaction rate is obtained using an N-terminally one-residue extended glycopeptide instead of a non-extended glyco-Asn peptide, which is non-functional as a thioester acceptor.

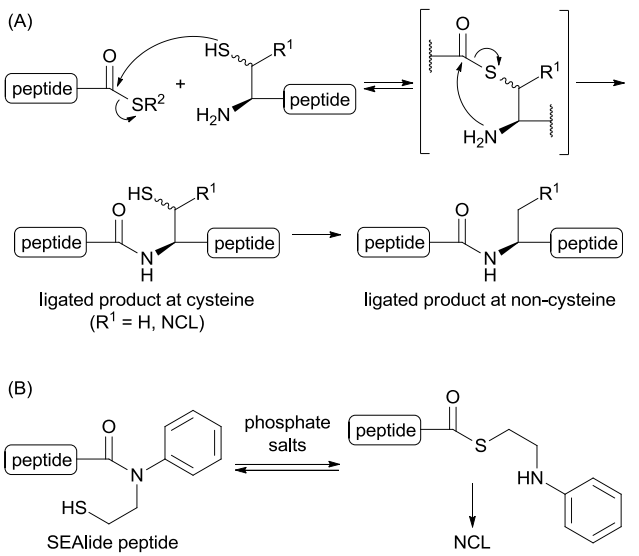


Fig. 1 (A) Ligation/desulfurization strategy using cysteine surrogates. (B) SEAlide peptide as a crypto-thioester.

Although the SAL seems to be applicable to the synthesis of native GM2AP, ligation between prolyl thioester and Gly^{31}-Asn^{32}(Glyco-SH) is required, where NCL reaction at the prolyl thioester site is expected to proceed in low efficiency and with formation of two-residue deleted side product.[11] Therefore, we attempted to develop a novel protocol that allows ligations

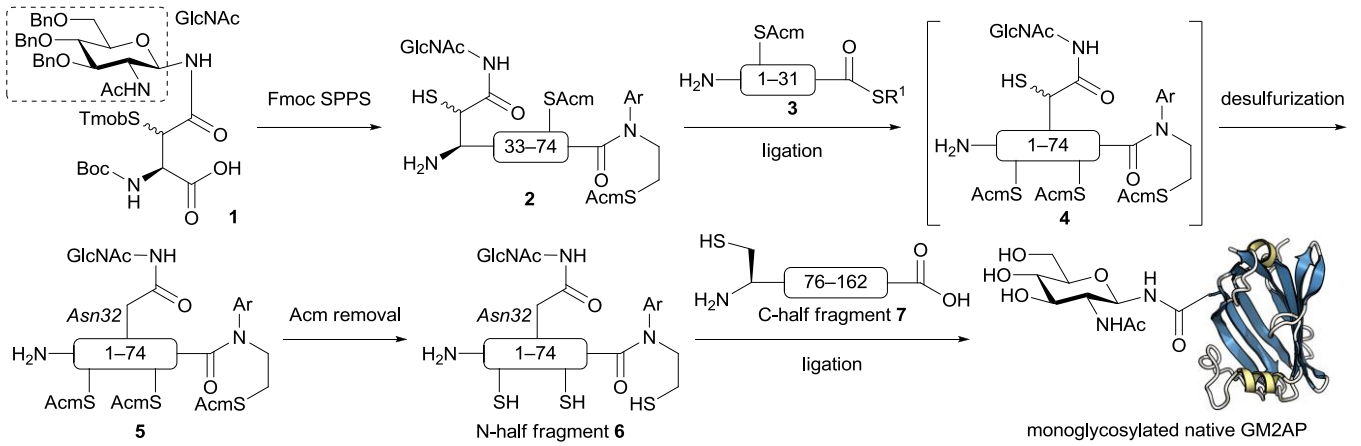


Fig. 2 Synthetic plan for monoglycosylated native GM2AP. Ar: *p*-C_6H_4-CO-Leu-NH_2; Acm: acetamidomethyl.

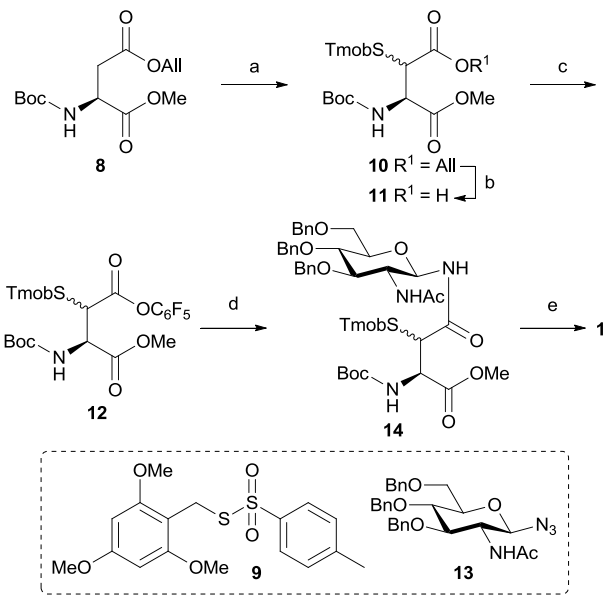
between an *N*-glycosylated Asn residue and its neighbouring N-terminal residue. Additionally, the ligation at such junction enabled us to use our synthetic platform for GM2AP analog.[6]

The envisioned strategy is shown in Fig. 2. A newly designed Asn derivative **1** bears a protected thiol group at the β-position. The thiol unit would mimic the Cys side chain to enable chemoselective condensation with a peptide thioester. The ligation of a peptide **2** possessing the β-mercapto-*N*-glycosylated Asn residue with a peptide thioester **3** would afford a ligated product **4** with an additional thiol function. Subsequent desulfurization of the additional thiol group would yield a product **5** ligated between native *N*-glycosylated Asn and its neighbouring *N*-terminal amino acid.

Access to the protected β-mercapto Asn derivative **1** from allyl (All)-protected aspartic acid Boc-Asp(OAll)-OMe **8** is shown in Scheme 1. A thiol unit protected with 2,4,6-trimethoxybenzyl (Tmob) was introduced at the β-position of **8** using sulfenylation reagent **9**.[12] The Asp derivative **10** was obtained in 81% yield as a 7:2 diastereomeric mixture, determined by ^{1}H-NMR. These diastereomers would be eventually converted to the same desulfurized Asn residue; therefore, the diastereomeric mixture was used in the next steps without separation. Palladium-catalyzed deprotection of the allyl ester in the side chain followed by condensation with pentafluorophenol afforded the active ester **12** in 73% yield over two steps as a 1:1 diastereomeric mixture. The activated ester was reacted with an amine generated in *in situ* reaction from *O*-benzyl-protected *N*-acetyl glucosamine azide **13** and *n*-tributyl phosphine to afford the glycosylated Asn derivative **14** in 48% yield as a 4:1 diastereomeric mixture after reprecipitation.[13] Finally, trimethyltin hydroxide-mediated hydrolysis of the methyl ester gave the desired β-mercapto-*N*-glycosylated Asn derivative **1** in 69% yield as a 4:1 diastereomeric mixture.[14]

The Asn derivative **1** was successfully introduced by solid-phase peptide synthesis (SPPS) into the N-terminus of the resin-bound peptide chain under benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate (PyBop)-diisopropylethylamine conditions. After the introduction, global deprotection and cleavage from the solid phase were carried out with the aid of 1 M trimethylsilyl bromide-thioanisole in trifluoroacetic acid (TFA),[15] which enables all protecting groups on the glycopeptide except for acetamidomethyl (Acm) groups to be removed, to afford the partially Acm-protected SEAlide peptide **2** as an inseparable diastereomeric mixture. Requisite peptide fragments (**3** and **7**) were prepared by SPPS and the SPPS was followed by the N-to-C-directive one-pot/sequential NCL, respectively.[6]

The reaction progress for the synthesis of the monoglycosylated native GM2AP is summarized in Fig. 3. A one-pot ligation/desulfurization protocol using trifluoroethane thiol (TFET) as a thiol additive was attempted to ligate **2** with **3** (Fig. 3A and B).[16] These peptides were chemoselectively assembled in 6 M guanidine (Gn)·HCl-0.1 M sodium phosphate buffer in the presence of 3% (v/v) TFET and successfully yielded the ligated peptide **4** within 8 hours. After treatment of the reaction mixture with a nitrogen stream to remove excess TFET, desulfurization of the thiol function on **4** was carried out (Fig. 3C). To the reaction mixture was added 6 M Gn·HCl-0.4



Scheme 1 Synthesis of Asn derivative **1**. (a) LiHMDS (2 eq.), **9**, THF, -78 °C, 81%. (b) $Pd(PPh_3)_4$, *N*-methylaniline, THF, 90%. (c) C_6F_5OH, EDC·HCl, CH_2Cl_2, 81%. (d) *n*-Bu_3P, **13**, HOOBt, 2% (v/v) H_2O/THF, 48%. (e) Me_3SnOH, 1,2-dichloroethane, 50 °C, 69%.

M tris(2-carboxyethyl)phosphine (TCEP)·HCl aq., pH 7.0, with reduced glutathione as a hydrogen atom source and VA-044 as a radical initiator (final concentrations: 6 M, 0.2 M, 40 mM, and 20 mM, respectively).[16] The desulfurization went to completion within 18 hours, yielding the Acm-protected SEAlide peptide **5** in 53% isolated yield over two steps. The Acm groups on the resulting peptide **5** were removed by the action of AgOTf-anisole in TFA followed by incubation in the presence of dithiothreitol.[17] After HPLC purification, the native N-half 74-residue glycopeptide fragment **6** was obtained in 54% isolated yield.

Convergent assembly of **6** and the 88-residue C-half fragment **7**[6] was accomplished by SEAlide-mediated NCL in the presence of phosphate salts (Fig. 3D). The NCL went almost to completion within 30 hours to afford the monoglycosylated 162-residue peptide **15** in 40% isolated yield, also affording the epimerized product **15'** at ligation site Ser (13%).[18] After the main product **15** was folded (Fig. 3E) and purified by HPLC, a product with molecular weight identical to that of the folded monoglycosylated native GM2AP (calcd: 17,784.3; found: 17,783.8) was obtained in 52% isolated yield.

CD spectrum of the folded product was similar to that of previously synthesized GM2AP analog (see ESI†).[19] Assay of GM2-degradation using the synthesized native GM2AP in the presence of HexA clearly exhibited the conversion of GM2 to GM3 (Fig. 3F), indicating that the synthetic protein should be correctly folded and work similarly to intrinsic GM2AP.

In conclusion, we developed a novel ligation/desulfurization procedure enabling *N*-glycosylated Asn site ligations. This ligation procedure allowed the total synthesis of the monoglycosylated native GM2AP. Combining this newly developed ligation method with the chemoenzymatic strategy should open new avenues for the preparation of homogeneous glycoproteins.[6] Chemistry-based derivatizations of GM2AP for protein therapeutics and protein probes are ongoing in our laboratory.

This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) and research grants from the Takeda Science and the Uehara Memorial Foundations. K.S. is grateful for a scholarship from the Yoshida Scholarship Foundation.

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† Electronic Supplementary Information (ESI) available: Experimental details of syntheses including HPLC charts and CD spectrum. See DOI: 10.1039/b000000x/

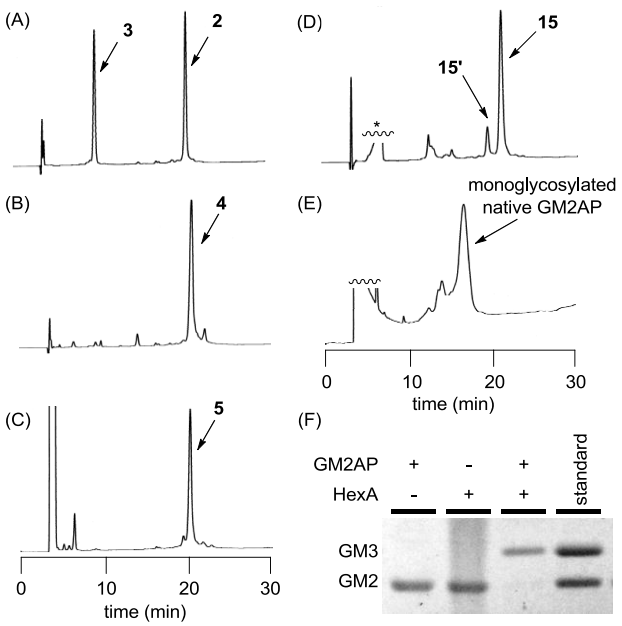

Fig. 3 HPLC analysis of reactions for **6**: (A) ligation of **2** and **3** (t < 5 min); (B) ligation of **2** and **3** (t = 8 h); (C) desulfurization of **4** (t = 18 h). HPLC analysis of reactions for monoglycosylated native GM2AP: (D) NCL of **6** and **7** (t = 30 h); (E) folding of **15** (t = 24 h). (F) TLC monitoring of degradation of GM2 with HexA and synthesized GM2AP. HPLC and TLC conditions: see ESI.† *MPAA.

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