Toward Fully Synthetic Homogeneous β-Human Follicle-Stimulating Hormone (β-hFSH) with a Biantennary N-Linked Dodecasaccharide. Synthesis of β-hFSH with Chitobiose Units at the Natural Linkage Sites

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Abstract: A highly convergent synthesis of the sialic acid-rich biantennary N-linked glycan found in human glycoprotein hormones and its use in the synthesis of a fragment derived from the β-domain of human Follicle-Stimulating Hormone (hFSH) are described. The synthesis highlights the use of the Sinaÿ radical glycosidation protocol for the simultaneous installation of both biantennary side-chains of the dodecasaccharide as well as the use of glycal chemistry to construct the tetrasaccharide core in an efficient manner. The synthetic glycan was used to prepare the glycosylated 20−27aa domain of the β-subunit of hFSH under a Lansbury aspartylation protocol. The proposed strategy for incorporating the prepared N-linked dodecasaccharide-containing 20−27aa domain into β-hFSH subunit was validated in the context of a model system, providing protected β-hFSH subunit functionalized with chitobiose at positions 7 and 24.

Introduction

Important recent developments in the fields of carbohydrate and polypeptide synthesis have now enabled organic chemists to consider the possibility, at various levels of realism, of gaining access to highly complex biomolecules through purely synthetic means.[1] In particular, advances in the area of protein synthesis, including the development of solid-phase peptide synthesis (SPPS)[2] as well as thiol-based ligation methods,[3] have served to enable the preparation of polypeptides and small proteins. In nature, many biologically important proteins undergo post translational modifications, often resulting in the formation of highly complex heterogeneous glycoforms. The chemical synthesis of such glycoproteins still remains a daunting task, due to the paucity of chemical methods available for the incorporation of glycans into proteins, not to speak of the difficulties inherent in the syntheses of the complex glycans.

Our laboratory has a long-standing interest in the development of enabling protocols addressed to accomplishing *de novo* syntheses of naturally occurring glycoproteins of demonstrated

therapeutic value. Each individual target will, of course, present its own unique set of synthetic challenges. Nonetheless, we are pursuing approaches for the general synthesis of glycoprotein target compounds. Our strategy for the assembly of N-linked glycoproteins is adumbrated in Figure 1.[4] Thus, the oligosaccharide unit, prepared through chemical synthesis, is functionalized in the form of an anomerically aminated glycan. Next, the aminoglycan is merged with an aspartic acid-containing peptide under Lansbury aspartylation conditions.[5] The resultant glycopeptide undergoes sequential ligations to provide the target glycoprotein. Indeed, we have been able to demonstrate the gross feasibility of this approach in the context of model systems. It is our ultimate goal to apply the convergent strategy outlined in Figure 1 to the total synthesis of highly complex glycoproteins (*vide infra*).

In investigating potential targets of N-linked glycoproteins with major biological activity, we first launched a program in the context of erythropoietin (EPO), used in the treatment of anemia.[6] More recently, we have been focused on Follicle-Stimulating Hormone (hFSH), which is used to treat anovulatory disorders associated with infertility.[7] Other targets in our workscope include Chorionic Gonadotropin (hCG), Luteinizing Hormone (hLH), and Thyroid Hormone (hTH).[8] Although these glycoproteins have varying structures and biological functions, all of them carry mono-, di-, tri-, and tetra-sialo N-linked carbohydrates as illustrated in Figure 2. These structures possess a common core (cf., Manα1−6[Manα1−3]Manβ1−4GlcNAcβ1−4[Fucα1−6]GlcNAc).[9] The defucosylated version of this core, **I**, previously synthesized by our group as well as by others,[10] has been employed in the preparation of highly branched glycans frequently expressed on the prostate-specific

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(1) For selected examples of some recent syntheses or semisyntheses, refer to: (a) Becker, C. F. W.; Liu, X.; Olschewski, D.; Castelli, R.; Seidel, R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2008**, *47*, 8215–8219. (b) Pentelute, B. L.; Gates, Z. P.; Dashnau, J. L.; Vanderkooi, J. M.; Kent, S. B. H. *J. Am. Chem. Soc.* **2008**, *130*, 9702–9707. (c) Yamamoto, N.; Tanabe, Y.; Okamoto, R.; Dawson, P. E.; Kajihara, Y. *J. Am. Chem. Soc.* **2008**, *130*, 501–510. (d) Torbeev, V. Y.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1667–1670. (e) Kochendoerfer, G. G.; et al. *Science* **2003**, *299*, 884–887. (f) Kochendoerfer, G. G.; Tack, J. M.; Cressman, S. *Bioconjugate Chem.* **2002**, *13*, 474–480.

(2) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
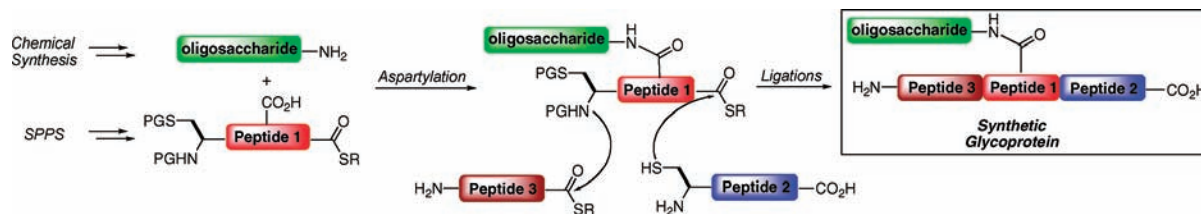
Figure 1. Convergent approach to the synthesis of glycoproteins.
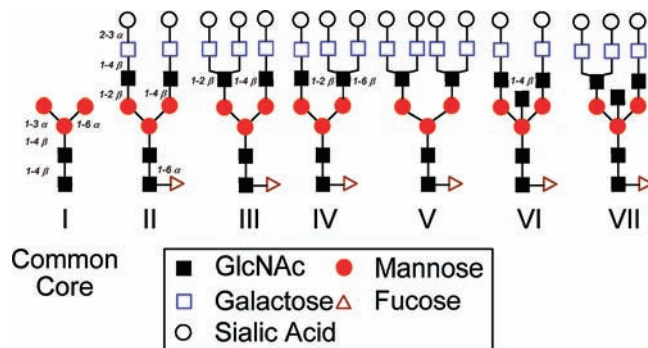
Figure 2. Sialylated N-linked carbohydrates found on some human glycoprotein hormones.

antigen (PSA)[11] and on gp120.[12] A chemical total synthesis of structures **II**–**VII** constitutes a formidable challenge, due to the presence of multiple *N*-acetyl neuraminic acid (NAN) linkages as well as the labile fucose branching. The need to accommodate these chemically sensitive substructures serves to limit the range of available options for synthesis. We hoped to meet the challenge by synthesizing the dibranched dodecasaccharide **II**,[8,9] a motif that has been found not only on all of the aforementioned hormones, but also on α-fetoprotein,[13] associated with human hepatocellular carcinoma, and on PSA.[14] Once prepared, carbohydrate **II** could then be used to prepare elaborate hormones by total synthesis according to the approach outlined in Figure 1.[15] We first describe a pleasingly concise route to the 12-mer. From there we show how the 12-mer is incorporated in an FSH-like peptidyl domain. Finally, we demonstrate the general viability of the proposed scheme to a total synthesis of β-hFSH, through the total synthesis of a congener in which chitobiose units are inserted at the mature 12-mer linkage sites of the wild type.

Results and Discussion

The synthetic logic toward dodecasaccharide **II** common to our targets is outlined in Scheme 1. The protecting group

strategy was designed to minimize the number of required steps, while hopefully maximizing the yield and diastereoselectivity for the glycosidation steps. It was anticipated that global deprotection would be executed toward the end of the synthesis, presumably by reductive and base-mediated steps.

To take advantage of the symmetry of **II**, and to make the synthesis optimally convergent, a late stage bis-glycosidation of the hexasaccharide **6** with the sialic acid-containing trisaccharide **7** was planned. In turn, the symmetry of **6** would allow for a bis-glycosidation reaction of the precursor tetrasaccharide, **5**. The latter would be assembled from the known building blocks **1**–**4**, through the addition of an α-fucosylation step to the synthesis of the core **I** motif (i.e., Manα1–6[Manα1–3]-Manβ1–4GlcNAcβ1–4GlcNAc), previously reported by our group.[10c,d]

The progression to tetrasaccharide **5** commenced with the coupling of glycal **1**[16] to the known glucosamine derivative **2**,[17] using MeOTf as a promoter (Scheme 2). The desired disaccharide was isolated in 55–72% yield as a 7:1 ratio of β:α anomers. This mixture was then treated with TBAF/AcOH in THF to remove the primary tri-isopropylsilyl (TIPS) protecting group. The resultant anomeric mixture was separable by column chromatography (98% yield). Next, the fucosylation of **8** was examined under various conditions. It was found that MeOTf-promoted[18] reaction of **8** with tri-*O*-benzyl-1-thioethylfucoside donor resulted in formation of a complex mixture of inseparable products (entry 1). When a mixture of anomeric fucosyl fluorides[19] was employed in the coupling reaction, it was discovered that only the β-anomeric fluoride (**3**) was reactive, while the corresponding α-anomeric fluoride survived intact. The use of (Cp)$_2$Zr(OTf)$_2$·THF as a promoter[20] (entry 3) gave poor levels of product diastereoselectivity at the newly fashioned glycosidic bond. Fortunately, however, Sn(OTf)$_2$ was found to be a more favorable promoter. Following optimization, the reaction of the β-anomeric fluoride **3** and acceptor **8** proceeded in good yields (65–75%) and acceptable diastereoselectivity (8:1 α:β, entry 4).[21] It was found that stoichiometric amounts of Lewis acid, administered in small portions, were required to drive the reaction to completion and to suppress yield erosion by isomerization of the β-anomeric fluoride to the more stable unreactive α-anomer. Superior to the fluoro method, the

(3) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779. (b) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Justus Liebigs Ann. Chem.* **1953**, *583*, 129–149. (c) Kemp, D. S.; Galakatos, N. G.; Bowen, B.; Tan, K. *J. Org. Chem.* **1986**, *51*, 1829–1838. (d) Tam, J. P.; Lu, Y.; Liu, C.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485–12489. (e) Liu, C.; Rao, C.; Tam, J. P. *Tetrahedron Lett.* **1996**, *37*, 933–966. (f) Tam, J. P.; Yu, Q. *Biopolymer* **1998**, *46*, 319–327. (g) Low, D. W.; Hill, M. G.; Carrasco, M. R.; Kent, S. B. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6554–6559. (h) Wu, B.; Chen, J.; Warren, J. D.; Chen, G.; Hua, Z.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 4116–4125. (i) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526–533. (j) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064–10065. (k) Brik, A.; Yang, Y.-Y.; Ficht, S.; Wong, C.-H. *J. Am. Chem. Soc.* **2006**, *128*, 5626–5627. (l) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248–9252. (m) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807–6810. (n) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J. L.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521–8524. (o) Wan, Q.; Chen, J.; Yuan, Y.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2008**, *130*, 15814–15816.

(4) Miller, J. S.; Dudkin, V. Y.; Lyon, G. J.; Muir, T. W.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2003**, *42*, 431–434.

(5) Cohen-Anisfeld, S. T.; Lansbury, P. T., Jr. *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537.

(6) (a) Szymkowski, D. E. *Curr. Opin. Drug Discovery Dev.* **2005**, *8*, 590–600. (b) Pavlou, A. K.; Rechert, J. M. *Nat. Biotechnol.* **2004**, *22*, 1513–1519. (c) Jelkmann, W.; Wagner, K. *Ann. Hematol.* **2004**, *83*, 673–686. (d) Ridley, D. M.; Dawkins, F.; Perlin, E. *J. Natl. Med. Assoc.* **1994**, *86*, 129–135.

(7) (a) Pang, S. C. *Women's Health* **2005**, *1*, 87–95. (b) Herbert, D. C. *Am. J. Anat.* **1975**, *144*, 379–385. (c) Dada, M. O.; Campbell, G. T.; Blake, C. A. *Endocrinology* **1983**, *113*, 970–984. (d) Rathnam, P.; Saxena, B. B. *J. Biol. Chem.* **1975**, *250*, 6735–6746. (e) Saxena, B. B.; Rathnam, P. *J. Biol. Chem.* **1975**, *251*, 993–1005.

Scheme 1. Synthetic Strategy toward **II**
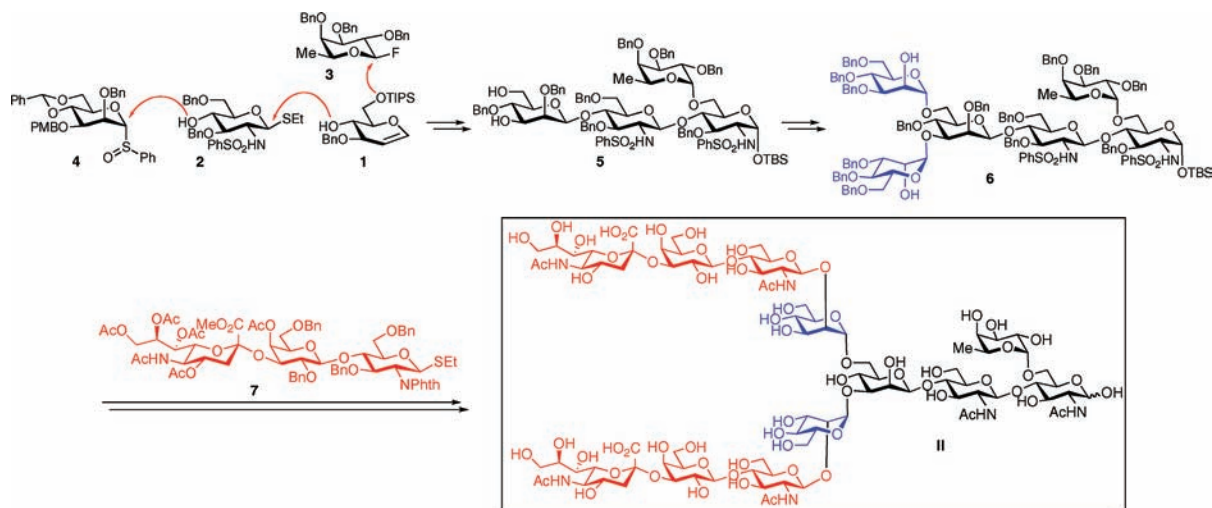
Scheme 2. Assembly of the Trisaccharide Core[a]
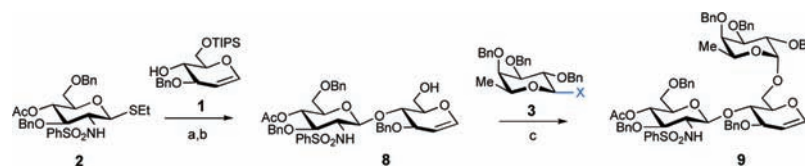
Entry	X	Promoter/T (°C)	Yield (dr)
1	EtS−	MeOTf/0	ND[a]
2	CCl₃C(=NH)−	Sn(OTf)₂/ −78	95(6:1)
3	F−	(Cp)₂Zr(OTf)₂·THF/+7	ND(1:1)
4	F−	Sn(OTf)₂/ −35	65-75(8:1)

[a] Complex mixture of products was isolated

[a] Reagents and conditions: (a) **1**, MeOTf, DTBP, DCM, MS 4Å, 0 °C, 55−72% (7:1 β:α); (b) TBAF, AcOH, THF, 98%; (c) **3**, Sn(OTf)₂ (4 × 0.25 equiv), DTBP, THF, MS 4Å, −35 °C, 65−75% (8:1 α:β).

Scheme 3. Synthesis of **6**[a]
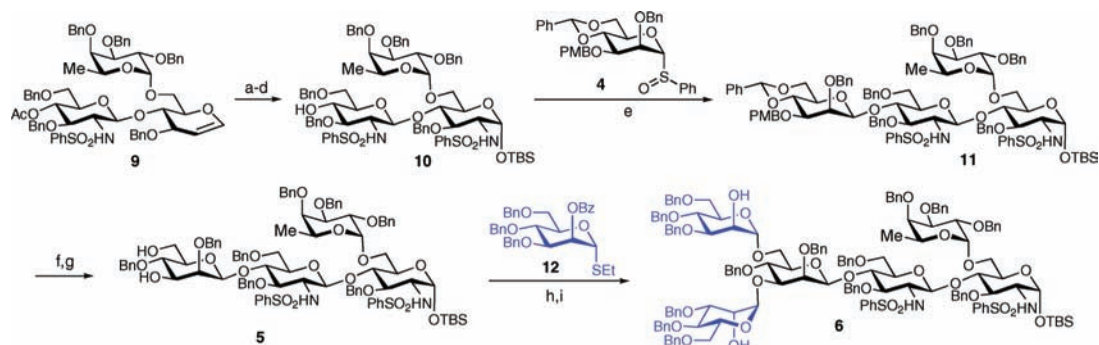
[a] Reagents and conditions: (a) IDCP, PhSO₂NH₂, THF, MS 4Å, 0 °C; (b) Et₃N, H₂O, THF; (c) TBSOTf, 2,6-lutidine, DCM; (d) NaOMe, MeOH, 45−55% (four steps); (e) Tf₂O, TBP, DCM, MS 4Å, 80−90% (6:1 α:β); (f) CAN, MeCN/H₂O, 65−70%; (g) Bu₂BOTf, BH₃·THF, DCM, 74−95%; (h) **10**, (BrC₆H₄)₃NSbCl₆, MeCN, MS 4Å, 10 °C to room temperature, 80−90%; (i) NaOMe−MeOH, 77−85%.

trichloroacetimidate derivative of tri-O-benzyl-L-fucose (entry 2) could be employed under Schmidt glycosidation conditions to provide the desired trisaccharide **9** in excellent yield (95%) and useful selectivity (6:1 α:β).[22]

Having established a reliable route to **9**, we next directed our attention to the synthesis of the tetrasaccharide unit **5** (Scheme 3). First, functionalization of the terminal glycal to the corresponding glucosamine derivative was achieved by the two-step procedure previously developed in our laboratory.[23] Toward this end, treatment of **9** with iodonium di-sym-collidin

perchlorate (IDCP) and benzenesulfonamide resulted in the formation of the 2-iodo-1-sulfonamide, which subsequently underwent a triethylamine-induced rearrangement in aqueous medium to the 2-sulfonamide-1-hydroxy derivative. The anomeric hydroxyl group of this product was protected, providing an anomeric silyl ether, exclusively as the α-anomer, and the acetate group was removed under Zemplèn conditions, furnishing the desired compound **10** (45−55%, four steps) as a single diastereomer. Subsequent triflate-mediated β-mannosylation of **10** proceeded smoothly under Kahne−Crich protocols[24] to

Scheme 4. Synthesis of the Sialic Acid-Containing Trisaccharide **7**[a]

Path A:
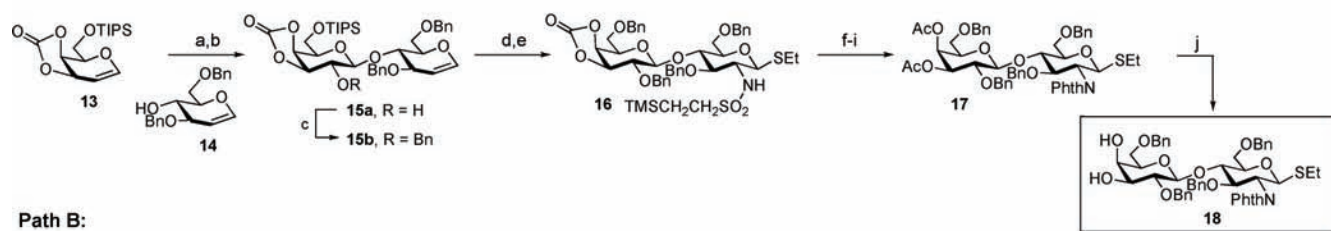
Path B:
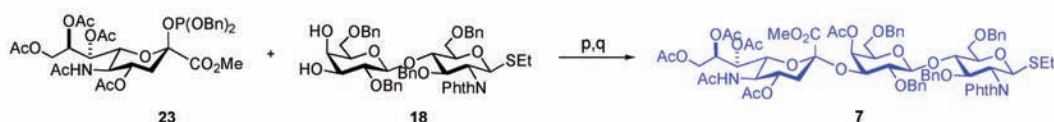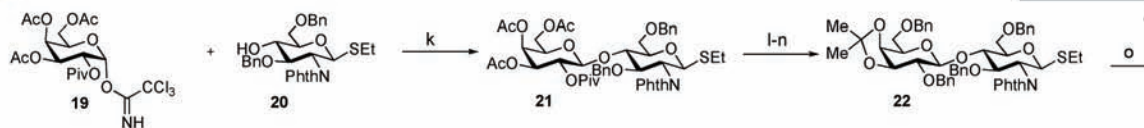
[a] Reagents and conditions: (a) DMDO, DCM, 0 °C; (b) **14**, ZnCl₂, THF, MS 4Å, 81% (two steps); (c) BnBr, NaH, DMF, 70−80%; (d) IDCP, TMSCH₂CH₂SO₂NH₂, MS 4Å, THF; (e) LHMDS, EtSH, DMF, 92% (two steps); (f) NaOMe, MeOH, 90%; (g) Ac₂O, Py, 92%; (h) CsF, DMF, 64−77%; (i) phthalic anhydride, Py, Piv₂O, 74−85%; (j) NaOMe, MeOH, 86−90%; (k) TMSOTf, DCM, MS 4Å, −78 °C, 79% (>95:5 β:α); (l) NaOMe, MeOH, 97%; (m) (MeO)₂CMe₂, CSA, 68−80%; (n) NaH, BnBr, TBAI, THF, 80%; (o) HOAc (aq), 60 °C, 92%; (p) TMSOTf, MeCN, MS 4Å, −40 °C (3.5:1 α:β); (q) Ac₂O, Py, 35−45% (two steps).

provide tetrasaccharide **11** in 80−90% yield as a 6:1 β:α mixture of anomers. The undesired α-isomer could be removed during the deprotection of the C₄ PMB group with cerium ammonium nitrate (CAN), to yield the desired product as a single isomer. Regioselective cleavage of the benzylidene acetal with BH₃·THF in the presence of dibutyl boron triflate afforded the 3,6-diol, **5**. The 2-fold glycosidation of the latter with the mannose derivative, **12**, was efficiently accomplished through the Sinaÿ radical activation protocol[25] (80−90% yield), in analogy with the corresponding transformation employed in the PSA synthesis.[11] The synthesis of hexasaccharide **6** was then completed through removal of the benzoate esters with NaOMe/MeOH (77−85% yield).

With hexasaccharide **6** in hand, we next turned to the synthesis of the sialic acid containing trisaccharide thiol donor, **7** (Scheme 4). The key disaccharide intermediate **18** could be prepared through two alternative pathways, as shown. According to path A, the synthesis of **18** began with the known glycal intermediates, **13** and **14**.[26] Activation of **13** with DMDO,[27] followed by reaction of the resultant oxirane with **14**, provided disaccharide **15a** in 81% yield (two steps). This product was benzylated to provide a fully protected glycal **15b**, which

underwent iodosulfonamidation and the subsequent "roll-over" reaction to yield the glucosamine derivative, **16** (92%, two steps).[23] Compound **16** was next elaborated to the target disaccharide, **18**, through a series of protecting group manipulations, which included (1) replacement of the cyclic carbonate with acetate groups, (2) changing the sulfonamide group to the corresponding phthalimide moiety, and (3) removal of the acetate moieties through the action of sodium methoxide in methanol.

Alternatively, the target disaccharide, **18**, was reached through path B, which was found to be more amenable to scale-up. The synthesis commenced with Schmidt glycosidation of galactose donor **19**[28] with the known glucosamine acceptor **20**,[29] to provide the disaccharide **21**[30] in good yield and diastereoselectivity (79%, >95:5 β:α). This disaccharide was converted to **22** through NaOMe-mediated cleavage of the ester protecting groups, followed by selective reprotection of the 3,4-diol of the galactose as an acetonide, culminating in dibenzylation of the remaining hydroxyl groups (56%, three steps).[31] The synthesis of the desired diol **18** was completed through removal of the acetonide function with aqueous acetic acid (92% yield). Finally, the coupling of **18** and known donor **23** was accomplished using TMSOTf as a promoter, following the protocol previously

(8) (a) Gharib, S. D.; Wierman, M. E.; Shupnik, M. A.; Chin, W. W. *Endocr. Rev.* **1990**, *11*, 177–199. (b) Pierce, J. G.; Parsons, T. F. *Annu. Rev. Biochem.* **1981**, *50*, 465–495.

(9) (a) Sairam, M. R. *FASEB J.* **1989**, *3*, 1915–1926. (b) Mutsaers, J. H. G. M.; Kamerling, J. P.; Devos, R.; Guisez, Y.; Fiers, W.; Vliegenthart, J. F. G. *Eur. J. Biochem.* **1986**, *156*, 651–654. (c) Damm, J. B. L.; Kamerling, J. P.; Van Dedem, G. W. K.; Vliegenthart, J. F. G. *Glycoconjugates* **1987**, *4*, 129–144. (d) Hokke, C. H.; Berwer, A. A.; van Dedem, G. W. K.; van Ostrum, J. V.; Kamerling, J. P.; Vliegenthart, J. F. G. *FEBS Lett.* **1990**, *275*, 9–14. (e) Green, E. D.; Baenziger, J. U. *J. Biol. Chem.* **1988**, *263*, 25–35.

(10) (a) Paulsen, H.; Lebuhn, R. *Carbohydr. Res.* **1984**, *130*, 85–101. (b) Matsuo, J.; Nakahara, Y.; Ito, Y.; Nukada, T.; Nakahara, Y.; Ogawa, T. *Bioorg. Med. Chem.* **1995**, *3*, 1455–1463. (c) Danishefsky, S. J.; Hu, S.; Cirillo, P. F.; Eckhardt, M.; Seeberger, P. H. *Chem.-Eur. J.* **1997**, *3*, 1617–1628. (d) Dudkin, V. Y.; Miller, J. S.; Danishefsky, S. J. *Tetrahedron Lett.* **2003**, *44*, 1791–1793. (e) Totani, K.; Matsuo, I.; Ito, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2285–2289.

(11) (a) Dudkin, V. Y.; Miller, J. S.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 736–738. (b) Dudkin, V. Y.; Miller, J. S.; Dudkina, A. S.; Antczak, C.; Scheinberg, D. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2008**, *130*, 13598–13607.

(12) (a) Mandal, M.; Dudkin, V. Y.; Geng, X.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2004**, *43*, 2557–2561. (b) Geng, X.; Dudkin, V. Y.; Mandal, M.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2004**, *43*, 2562–2565.

(13) Taketa, K. *Electrophoresis* **1998**, *19*, 2595–2602.

(14) Tajiri, M.; Ohyama, C.; Wada, Y. *Glycobiology* **2008**, *18*, 2–8.

(15) For the completed syntheses of **II**, refer to: (a) Wu, B.; Hua, Z.; Warren, J. D.; Ranganathan, K.; Wan, Q.; Chen, G.; Tan, Z.; Chen, J.; Endo, A.; Danishefsky, S. J. *Tetrahedron Lett.* **2006**, *47*, 5577–5579. (b) Wu, B.; Tan, Z.; Chen, G.; Chen, J.; Hua, Z.; Wan, Q.; Ranganathan, K.; Danishefsky, S. J. *Tetrahedron Lett.* **2006**, *47*, 8009–8011. (c) Sun, B.; Srinivasan, B.; Huang, X. *Chem.-Eur. J.* **2008**, *14*, 7072–7081.

Scheme 5. Synthesis of the Protected Dodecasaccharide[a]
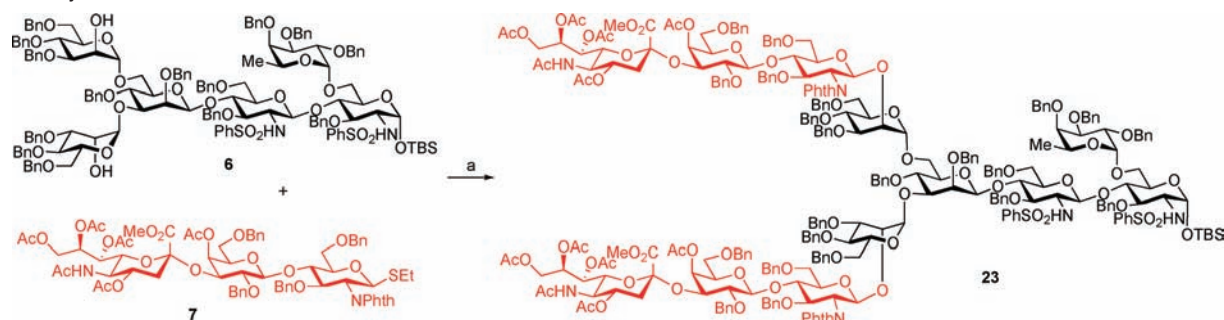
[a] Reagents and conditions: (a) (BrC$_6$H$_4$)$_3$NSbCl$_6$, MeCN, MS 4Å, 0 °C to room temperature, 74%.

Scheme 6. Deprotection of the Dodecasaccharide[a]
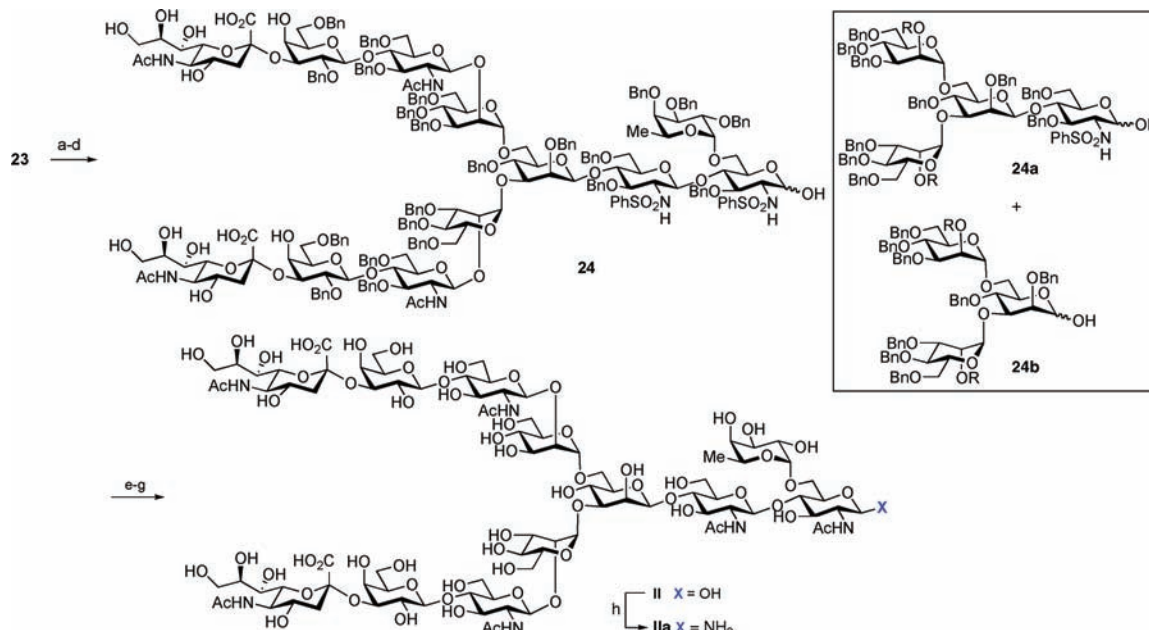
[a] Reagents and conditions: (a) NaOMe, MeOH; (b) NH$_2$CH$_2$CH$_2$NH$_2$, toluene, *n*-BuOH, 90 °C; (c) Ac$_2$O, Py; (d) TBAF, AcOH, THF; (e) NaOMe, H$_2$O, MeOH; (f) Na, NH$_3$, THF, −78 °C; (g) Ac$_2$O, NaHCO$_3$, H$_2$O; (h) NH$_4$HCO$_3$, H$_2$O, 40 °C (54−63% overall yield).

reported by our group.[32] The resultant trisaccharide product was obtained as a 3.5:1 mixture of anomers, which was acetylated and purified to furnish **7** as a pure α-anomer (35−45%, two steps).

Having established routes to the key intermediates in the synthesis of **II**, the final bis-glycosidation of the hexasaccharide **6** with trisaccharide **7** was evaluated (Scheme 5). Of the various conditions examined, the Sinaÿ radical activation protocol[25] was the most effective, producing the fully protected dodecasac-charide **23** in 74% yield, together with ca. 10−15% of the monoglycosidated nonasaccharide.

Because of the high degree of functionalization of dodeca-saccharide **23**, the sequence for protecting group removal had to be evaluated with great care. Because the sialic acid methyl ester group would not be compatible with the usual conditions adopted for the removal of the phthalimide protecting group, the concomitant NaOMe-mediated hydrolysis of the methyl esters and acetyl protecting groups was undertaken first (Scheme 6). The resultant diacid was treated with ethylenediamine to remove the phthalimide protecting groups. The unmasked amines were reprotected through simultaneous reacetylation of the sialic acid portion.[11] The subsequent removal of the anomeric *tert*-butyldimethylsilyl (TBS) protecting group (TBAF, AcOH)

was followed by cleavage of the acetates with sodium meth-oxide. Opening of the lactones with sodium hydroxide resulted in the formation of compound **24**, together with the "peeling reaction" products **24a** and **24b**.[33] Fortunately, the formation of **24a** and **24b** could be drastically reduced when the hydrolysis step was performed with NaOH *in lieu* of NaOMe. Finally, the benzyl and sulfonamide protecting groups were cleaved under

(16) (a) Nicolaou, K. C.; Trujillo, J. I.; Chibale, K. *Tetrahedron* **1997**, *53*, 8751–8778. (b) Lohman, G. J. S.; Seeberger, P. H. *J. Org. Chem.* **2003**, *68*, 7541–7543. (c) Schell, P.; Orgueira, H. A.; Roehrig, S.; Seeberger, P. H. *Tetrahedron Lett.* **2001**, *42*, 3811–3814.
(17) Wang, Z.-G.; Warren, J. D.; Dudkin, V. Y.; Zhang, X.; Iserloh, U.; Visser, M.; Eckhardt, M.; Seeberger, P. H.; Danishefsky, S. J. *Tetrahedron* **2006**, *62*, 4954–4978.
(18) Liao, L.; Auzanneau, F.-I. *Carbohydr. Res.* **2006**, *341*, 2426–2433.
(19) Nicolaou, K. C.; Caulfield, T. J.; Kataoka, H.; Stylianides, N. A. *J. Am. Chem. Soc.* **1990**, *112*, 3693–3695.
(20) Allen, J. R.; Allen, J. G.; Zhang, X.-F.; Williams, L. J.; Zatorski, A.; Ragupathi, G.; Livingston, P. O.; Danishefsky, S. J. *Chem.-Eur. J.* **2000**, *6*, 1366–1375.
(21) Spassova, M. K.; Bornmann, W. G.; Ragupathi, G.; Sukenick, G.; Livingston, P. O.; Danishefsky, S. J. *J. Org. Chem.* **2005**, *70*, 3383–3395.
(22) Wegmann, B.; Schmidt, R. R. *Carbohydr. Res.* **1988**, *184*, 254–261.
(23) Griffith, D. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 5811–5819.

**Scheme 7.** Coupling of the Dodecasaccharide with the 20−27aa Domain of hFSH β-Subunit[a]
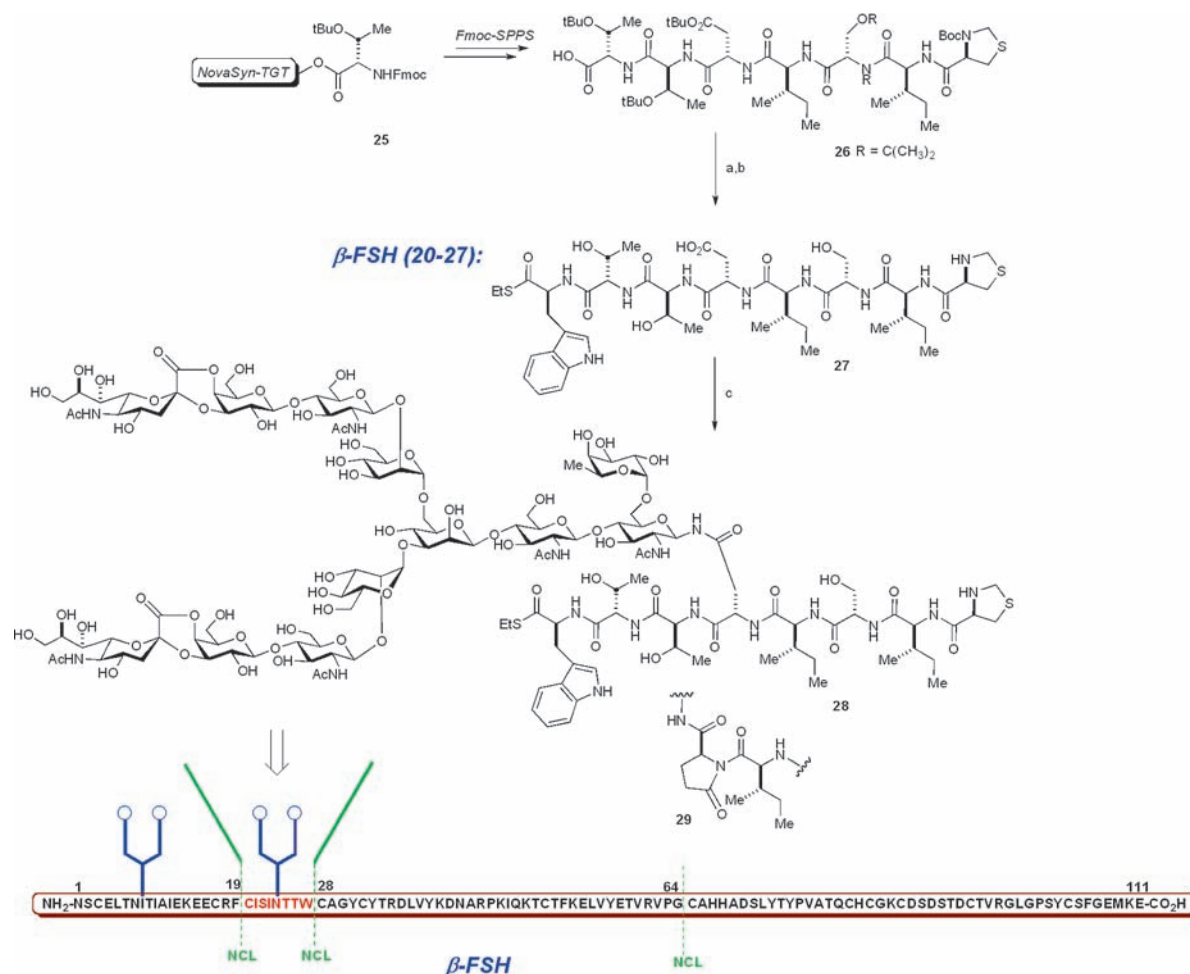
[a] Reagents and conditions: (a) H-Trp(SEt), EDC, HOOBt, TFE/CHCl₃ (1:3); (b) TFA, PhOH, TIPS, H₂O; 21% from **25**; (c) **IIa**, HATU, DIEA, DMSO, ca. 25−35%.

our previously described Birch reduction conditions. The reader will note that this massive reductive deprotection can be conducted without reduction of the "reducing end" of the 12-mer.[34] The product diamine was acetylated to yield the desired product, **II**. Our ultimate goal was to employ **II** for the construction of naturally occurring glycoprotein hormones. Accordingly, it was subjected to prolonged treatment with a saturated solution of NH₄HCO₃ at 40 °C.[35] Excess of ammonium bicarbonate and water was removed by repetitive lyophilization. This protocol provided glycosyl amine **IIa** in 54−63% yield over the entire deprotection sequence. Care was taken to minimize the exposure of **IIa** to aqueous medium, as anomeric amines are known to be unstable under these conditions.[36]

With the synthetic dodecasaccharide anomeric amine **IIa** in hand, we sought to evaluate its likely utility for the construction of the FSH β-subunit (Scheme 7).[37] The complexity of the glycans, and a relatively long sequence consisting of 111 amino acids, serves to identify this glycoprotein as an extremely ambitious target for total synthesis. Fortunately, a relatively high frequency of cysteine throughout the sequence allows one to consider bisecting this structure into simpler fragments, which could be assembled in the forward direction outlined in Figure 1. Accordingly, we defined as a target glycopeptide **28** (Scheme 7), which could be constructed by a direct coupling of dodecasaccharide anomeric amine **IIa** and peptide **27** under modified Lansbury conditions.[4,5] This glycopeptide contains a C-terminal thioester and an N-terminal Thz-protected cysteine that can be exploited to enable elaboration of **28** to the β-subunit of FSH. The synthesis of **28** commenced with Fmoc SPPS of the protected peptide **26**, starting from commercially available resin **25**. After cleavage from the resin, **26** was coupled with

(24) (a) Kahne, D.; Walker, S.; Cheng, Y.; Van Engen, D. *J. Am. Chem. Soc.* **1989**, *111*, 6881−6882. (b) Crich, D.; Sun, S. X. *J. Am. Chem. Soc.* **1998**, *120*, 435.

(25) (a) Zhang, Y. M.; Mallet, J. M.; Sinay, P. *Carbohydr. Res.* **1992**, *236*, 73−78. (b) Marra, A.; Mallet, J. M.; Amatore, C.; Sinay, P. *Synlett* **1990**, 572−574.

(26) Deshpande, P. P.; Kim, H. M.; Zatorski, A.; Park, T.-K.; Ragupathi, G.; Livingston, P. O.; Live, D.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 1600−1614.

(27) Halcomb, R.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1989**, *111*, 6661−6666.

(28) (a) Seki, M.; Kayo, A.; Mori, K. *Tetrahedron Lett.* **2001**, *42*, 2357−2360. (b) Schnabel, M.; Römpp, B.; Ruckdeschel, D.; Unverzagt, C. *Tetrahedron Lett.* **2004**, *45*, 295−297.

(29) Macindoe, W. M.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1995**, *271*, 207−216.

(30) van Seeventer, P. B.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1997**, *299*, 181−195.

(31) Spijker, N. M.; Keuning, C. A.; Hooglugt, M.; Veeneman, G. H.; van Boeckel, C. A. A. *Tetrahedron* **1996**, *52*, 5945−5960.

(32) Bhattacharya, S. K.; Danishefsky, S. J. *J. Org. Chem.* **2000**, *65*, 144−151.

Scheme 8. Assembly of the β-FSH Subunit with N-Linked Chitobiose[a]
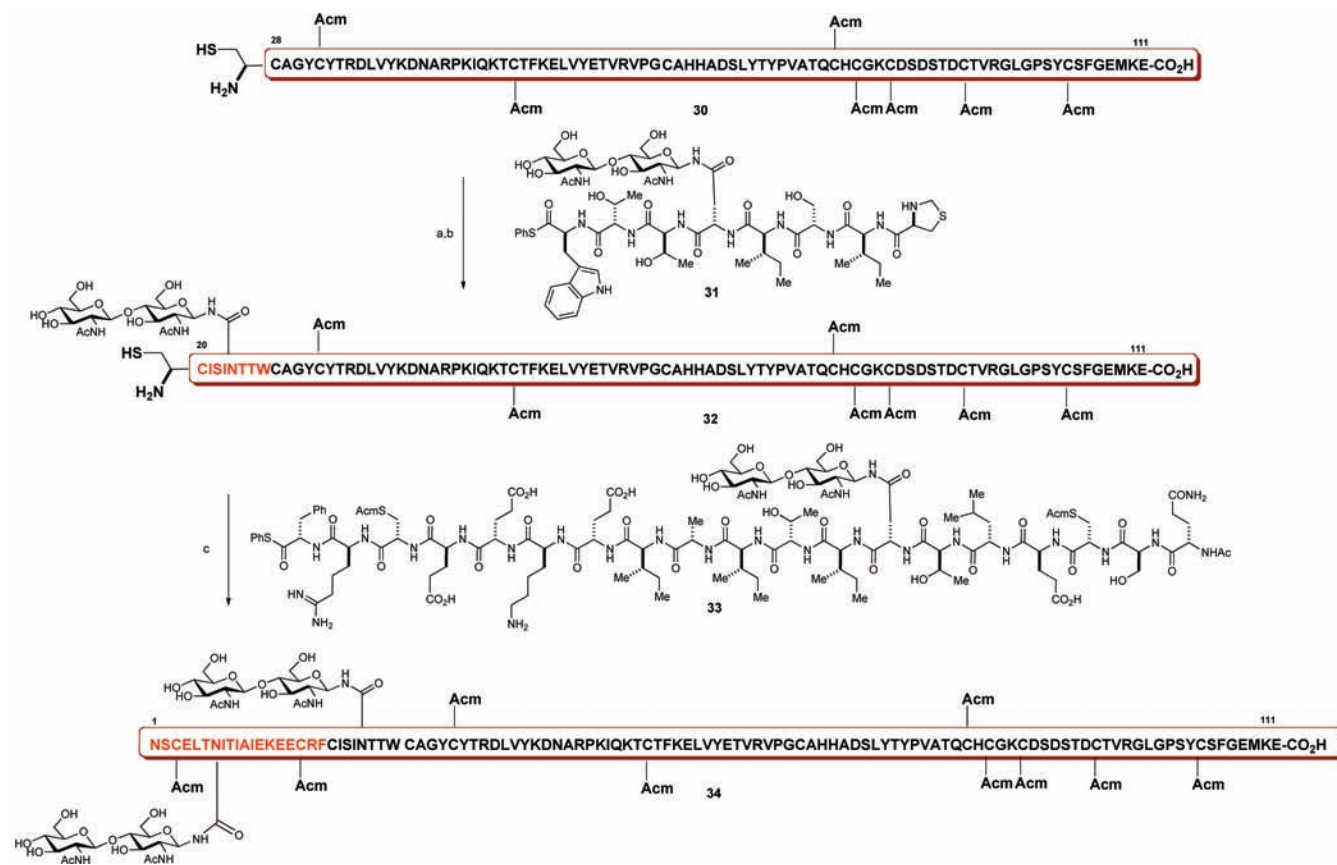
[a] Reagents and conditions: (a) PhSH, Gnd·HCl, Na$_2$HPO$_4$, TCEP·HCl (pH = 7.2); (b) NH$_2$OMe·HCl (pH = 4.6); 75% conversion, 27% yield; (c) PhSH, Gnd·HCl, Na$_2$HPO$_4$, TCEP·HCl (pH = 7.2), 64% yield.

tryptophane ethyl thioester under Sakakibara conditions with no epimerization being detected.[38] Next, the peptide was treated with cocktail B (trifluoroacetic acid, phenol, triisopropylsilane, H$_2$O) to provide the desired precursor **27** (21% from **25**).

Finally, we pursued the direct coupling of the dodecasaccharide with peptide **27**. After some experimentation, the coupling was found to provide the desired product **28** in ca. 25−35% yield with one of the major side products being an aspartimide **29**. Although **28** was isolated as a bis-lactone at the sialic acid termini, we believe that the required functionalities will be restored in the consequent ligation steps.[15b] A crucial observation made during the preparation of **28** was the finding that the efficiency of the aspartylation reaction is drastically dependent on the quality of the anomeric amine after the Kochetkov amination. Thus, the use of highly pure batches of ammonium bicarbonate, prolonged lyophilization times, and filtration of the insoluble residue were critical in providing batches of reactive amine **IIa**.

It is known that ammonium bicarbonate is degraded during the course of such treatment, producing variable amounts of ammonium carbonate and ureas. These basic byproducts promote the formation of aspartimide, which is a serious impediment in the complex step.[35,39] Not surprisingly, due to different conformational restrictions imposed by the aspartic acid residue, various peptide sequences exhibit different susceptibilities to such impurities. Only inorganic impurity-free batches of **IIa** exhibited consistent reactivity in the Lansbury aspartylation of various peptides.

With the key dodecasaccharide-containing domain being successfully synthesized, we turned our attention to validating

the proposed synthesis of β-FSH subunit (Scheme 7), using the corresponding fragments functionalized with a more available disaccharide chitobiose (Scheme 8). The glycopeptides **31** and **33** were prepared by methods similar to those described earlier for the preparation of compound **28** (Scheme 7).[40]

The coupling studies commenced with construction of the 20−111aa subunit **32** by ligation of peptides **30** and **31**. Thus, **31** and **30** underwent native chemical ligation in PBS (pH = 7.2) providing the desired product, which was treated in situ with O-methylhydroxylamine (pH = 4.6) to yield glycopeptide **32** (75% conversion, 27% yield after HPLC). The glycopeptide was isolated with its terminal cysteine residue deprotected and ready for coupling with glycopeptide **33**. The all-crucial coupling step was conducted under standard NCL conditions (3% v/v PhSH, 6 M Gnd·HCl, 0.1 M PBS, pH = 7.2).[3] The ligation between **33** and **32** proceeded smoothly. The desired product **34** was obtained in 64% yield after purification by HPLC, together with the side product that presumably arises from the Met109 oxidation (ca. 25%).[40] We found this demonstration to be quite gratifying. Thus, the work shown here provides a

(33) Alkaline degradation of polysaccharides is a well-known process albeit rarely observed on the fully protected glycans. For an example of such degradations, refer to: Aspinall, G. O.; Krishnamurthy, T. N. *Can. J. Chem.* **1975**, *53*, 2171–2177.

(34) Iserloh, U.; Dudkin, V.; Wang, Z.-G.; Danishefsky, S. J. *Tetrahedron Lett.* **2002**, *43*, 7027–7030.

(35) Likhosherstov, L. M.; Novikova, O. S.; Derevitskaja, V.; Kochetkov, N. K. *Carbohydr. Res.* **1986**, *146*, C1−C5.

(36) Isbell, H. S.; Frush, H. L. *J. Org. Chem.* **1958**, *23*, 1309–1319.

(37) For the application of dodecasaccharide **II** in the synthesis of homogeneous erythropoietin (EPO) fragments, see ref 15b.

plausible prospectus for reaching FSH itself. We now have significant confidence in the ability to generate the β-FSH with fixed carbohydrates at wild type sites. Although the present demonstration involved a lactosamine model system, we are hopeful that these protocols will be applicable to inclusion of the dodecamer. Of course, from the stage corresponding to compound **34**, in the 12-mer oligosaccharide series, we still have to master the intricacies of global deprotection and glycoprotein folding.

Conclusion

In summary, we have developed a highly convergent and stereospecific synthesis of the sialic acid-rich biantennary N-linked glycan found in human glycoprotein hormones such as human Erythropoietin (hEPO), Follicle-Stimulating Hormone (hFSH), and Chorionic Gonadotropin (hCG), as well as in many other medicine-relevant glycoprotein targets. Our synthesis highlights the use of the Sinaÿ radical glycosidation protocol for the simultaneous installment of both biantennary side-chains, a task that is very difficult to achieve with complex carbohydrate systems. Moreover, it highlights the value of glycal chemistry first pioneered in these laboratories to construct the tetrasaccharide core (**5**) in an efficient manner. A carefully selected deprotection strategy allowed us to overcome the difficulties associated with the presence of the multiple sialic acid and labile fucose linkages, thus enabling access to significant quantities of the deprotected dodecasaccharide **II**.

We have also demonstrated that the presence of the multiple sialic acids as well as fucose branching does not pre-empt usage

of the Kochetkov amination reaction or of subsequent Lansbury aspartylation of the resultant amine. The synthetic dodecasaccharide was used to prepare the glycosidated 20−27 domain of β-subunit of hFSH, which comprises an essential part of our program directed toward the synthesis of this glycoprotein hormone. Finally, the possibility of constructing the β-FSH subunit functionalized with N-linked dodecasaccharide was investigated using chitobiose-containing glycoproteins as a model. These studies demonstrate that the selected retrosynthetic bisections proposed for FSH could be successfully utilized for the preparation of the β-FSH subunit. Further synthetic efforts toward the construction of the homogeneous β-FSH with N-linked biantannary dodecasaccharide will be reported in due course.

Supporting Information Available: Experimental procedures and spectroscopic and analytical data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(38) (a) Kuroda, H.; Chen, Y. N.; Kimura, T.; Sakakibara, S. *Int. J. Pept. Protein Res.* **1992**, *40*, 294–299. (b) Sakakibara, S. *Biopolymers* **1995**, *37*, 17–28.
(39) Vetter, D.; Gallop, M. A. *Bioconjugate Chem.* **1995**, *6*, 316–318.
(40) Refer to the Supporting Information for details.