Carbohydrates

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# A Chemoenzymatic Total Synthesis of the Neurogenic Starfish Ganglioside LLG-3 Using an Engineered and Evolved Synthase

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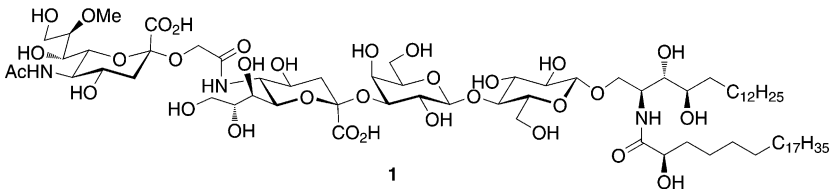
Sialic acid containing glycosphingolipids (GSLs), or gangliosides, are common to all vertebrate cells and play important roles in the pathology of a variety of human ailments, including Alzheimers disease, lysosomal storage disorders, and kidney disease, as well as in the function of the mammalian nervous system.[1] Accordingly, their synthesis has been the focus of considerable efforts.[2] Although the successful synthesis of gangliosides is still very much the domain of the specialist laboratory, the state of the art has advanced to the point where preparation of molecules appropriate for investigating the influence of subtle structural alterations on the function of GSLs is a reality.

Gangliosides are largely absent from the invertebrate lineage with the exception of the echinoderms, from which numerous molecules with rare or unique structural features have been identified in relative abundance.[3] Examples include GSLs containing modified sialic acids that are frequently attached to other sialic acid residues by either α(2–4) or α(2–11) linkages. Echinodermatous gangliosides are also characterized by highly heterogeneous sphingolipids, with structures uncommon in vertebrate systems predominating. Several gangliosides from starfish, including the tetrasaccharide LLG-3 from *Linckia laevigata* (**1**, Scheme 1),[4] show promising evidence of neuritogenic activity in cell culture and are thus of pharmacological interest.[5]




***Scheme 1.*** The predominant structural isomer of LLG-3 from *Linckia laevigata*.

LLG-3 from natural sources exists as a heterogeneous mixture of ceramides with variability in both the D-*ribo*-phytosphingosine and α-hydroxy acid components. Understanding how the structural features of LLG-3 contribute to neurotrophic activity demands access to structurally well-defined homogeneous material most readily provided by synthesis.

Echinodermatous gangliosides have attracted attention from synthetic chemists, in particular from Kiso and coworkers, who recently reported an impressive total synthesis of LLG-3.[6] We have been eager to develop an enzymatic synthesis of **1** wherein the attendant benefits of biocatalysis could be demonstrated, including high-yielding regio- and stereo-selective coupling reactions and a minimum of synthetic steps. However, the enzymes involved in the biosynthesis of LLG-3 are uncharacterized and, most importantly, no sialyltransferase (ST) has been found to catalyze the formation of the relatively uncommon α(2–11) sialosyl linkage. Herein we report a highly flexible chemoenzymatic synthesis of the ganglioside LLG-3, thus highlighting the suitability of an engineered and evolved endoglycoceramidase II glycosynthase for the synthesis of complex glycosphingolipids that feature natural heterogeneity in the sphingolipid, rare sphingolipids, and "unusual" structural modifications of the oligosaccharide.

Endoglycoceramidase II (EGCase) from *Rhodococcus* sp. strain M-777 is a retaining glycoside hydrolase (GH) from CAZy family GH5 that catalyzes cleavage of glycosyl ceramides to liberate an intact reducing oligosaccharide and a ceramide.[7] This enzyme has been subjected to two rounds of protein engineering. Firstly, a glycosynthase (GS) mutant (E351S) was generated, giving glycosyl sphingosines through coupling of an oligosaccharide fluoride with a sphingosine.[8] Subsequently, the capacity of this GS to utilize diverse sphingolipid substrates, including D-*ribo*-phytosphingosine, was enhanced many thousand fold using directed evolution, resulting in the mutant E351S D314Y.[9] In addition to an engineered broad lipid specificity, EGCase GS has been demonstrated to utilize a range of structurally diverse glycosyl fluoride substrates for the synthesis of vertebrate GSLs, including $G_{M3}$, $G_{M1}$, and sialyl paragloboside.[10] The merits of an enzyme with flexibility toward both donor and acceptor substrates are obvious, and in this case access to such an enzyme permits ready synthesis of GSL libraries. Subsequent chemical or enzymatic N-acylation of *lyso*-glycosyl sphingosines, obtained by coupling of sphingosine(s) with glycosyl fluorides, generates additional structural diversity and permits the chemist to interrogate the importance of individual lipid structures observed within heterogeneous natural isolates.[1d] Our recent work has focused on establishing enzymatic methods for the synthesis of complex oligosaccharide fluorides from simpler glycosyl fluorides, and the use of these molecules with EGCase GS.

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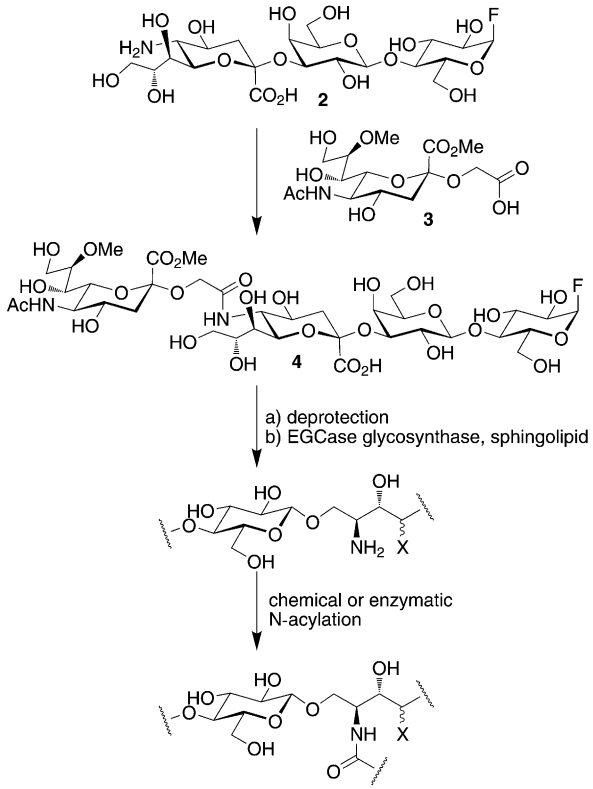
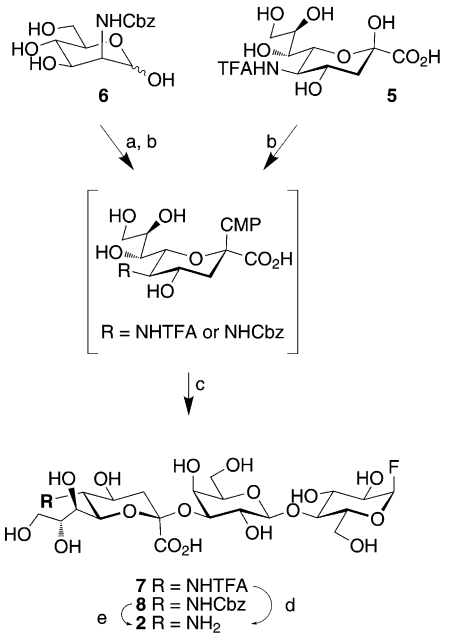
In the absence of a sialyltransferase capable of sialyltransfer to the hydroxy group of the glycolyl amide in Neu5Gc, enzymatic synthesis of this linkage remains impractical. Furthermore, methylation of the sialic acid is likely to occur after the glycosylation step in nature, and neither the requisite *S*-adenosyl-L-methionine:Neu5Ac 8-*O*-methyltransferase nor those membrane-bound enzymes observed in related organisms have been cloned.[11] Fortunately, methods for the chemical construction of the α(2–11) bond have been reported.[12,6] We envisioned an approach to LLG-3 in which chemoenzymatically generated Neuα(2,3)Galβ(1,4)Glcα-F **2** could first be coupled chemically with 8-OMe Neu5Acα-Gc **3**. After deprotection, **4** would serve as a donor substrate of EGCase GS for coupling to various lipids, potentially affording different glycosyl sphingosine variants of **1** in a remarkably short reaction sequence (Scheme 2).




***Scheme 2.*** Strategy for the construction of LLG-3 and structural variants.

Methods for the chemoenzymatic synthesis of the trisaccharide **2** were explored with the expectation that sialylation of lactosyl fluoride using ST Cst-I (*Campylobacter jejuni*) and a modified CMP-sialic acid donor would give an amine-masked precursor of **2** (Scheme 3). We identified the azide, trifluoroacetamide (TFA), and *N*-carboxybenzyl (Cbz) functionalities as potentially suitable amine protecting groups. Although 2-azido-2-deoxy-D-mannose could be transformed into 5-azido-sialylactose through the actions of Neu5Ac aldolase (*Escherichia coli*), CMP-sialic acid synthetase NSY-05 (*Neisseria meningitides* 406Y),[13] and α(2,3)-sialyltransferase Cst-I,[14] yields were much inferior to those obtained for



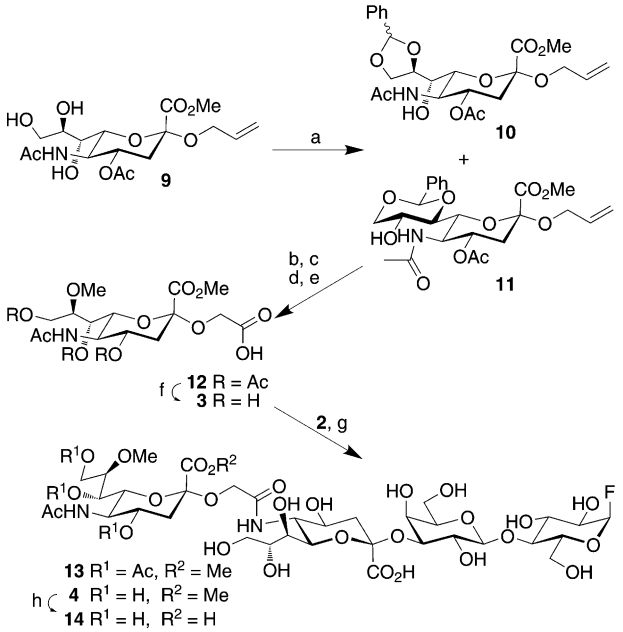

***Scheme 3.*** Preparation of trisaccharide **2**. a) Neu5Ac aldolase, pyruvic acid; b) CMP-Neu5Ac synthetase, cytidine-5'-triphosphate, inorganic pyrophosphatase; c) α-2,3-sialyltransferase, α-lactosyl-fluoride, alkaline phosphatase, 70% for **7** (2 steps), 57–94% for **8** (3 steps); d) NaOMe, MeOH, 44%; e) Pd/C, $H_2$, MeOH, 94%.

conversion of NeuTFA **5** to trisaccharide **7**. Unfortunately, yields for deprotection of **7** were low (44%). However, ManCbz **6** could be smoothly converted to the trisaccharide **8**, which was then isolated from a mixture of five enzymes and associated substrates and by-products in yields of up to 94% using solid-phase extraction (SPE). Hydrogenolysis of **8** over Pd/C afforded the amine **2**.

8-O-Methyl modification of sialic acids is relatively rare, but nevertheless may be of considerable biological importance. For instance, LLG-3 contains 75 carbon atoms and differs from ganglioside LMG-4 (*Luidia maculata*) only by 8-O-methylation of Neu5Ac, yet LLG-3 shows significantly more potent neuritogenic activity toward the rat pheochromocytoma cell line PC-12.[5] This modification also clearly prevents establishment of the α(2–8) linkage that is characteristic of most polysialic acids. Both chemical and chemoenzymatic syntheses of 8-OMe sialic acid have been reported.[6,12e,15] Our synthesis of an 8-OMe sialic acid functionalized for coupling to **2** is partially illustrated in Scheme 4. Allyl glycoside **9** underwent acid-catalyzed transacetalization to afford **10** and **11**. Despite an almost quantitative yield, the 2:3 equilibrium ratio of regioisomers was unfavourable. Selective precipitation of **11** and subsequent re-equilibration of the dioxolane **10** gave **11** in 65% yield. A sequence of O-methylation, protecting group exchange, oxidation, and partial deprotection afforded **3**, the 8-OMe sialoside of glycolic acid, in good yield. Protection of C-1 was maintained to allow discrimination between the two carboxylic acids present.

Coupling of the amine **2** with acids **12** or **3** (Scheme 4) could be achieved under common peptide coupling condi-

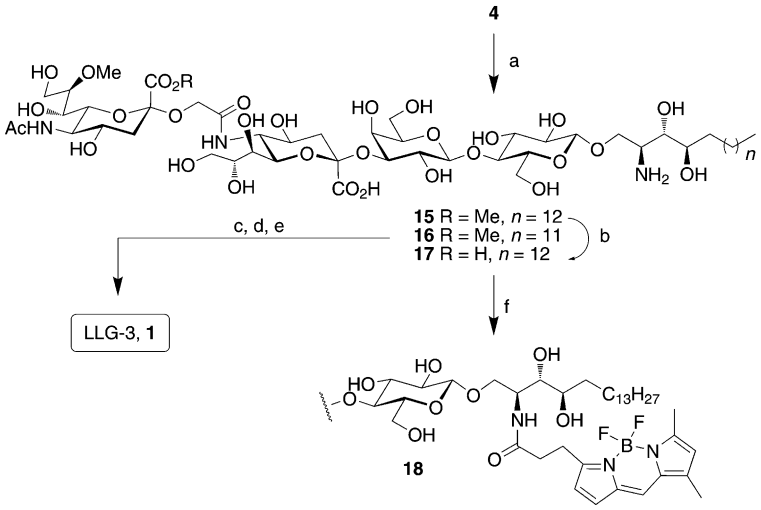



***Scheme 4.*** Synthesis of 8-OMe-Neu5Acα(2-11)Neu5Gcα(2–3)Galβ(1-4)GlcαF. a) $PhCH(OMe)_2$, MeCN, camphorsulfonic acid, 64%; b) MeI, NaH, DMF, −10°C, 85%; c) 80% AcOH, 55°C, 84%; d) $Ac_2O$, $CH_2Cl_2$, pyridine, 92%; e) $NaIO_4$, $RuCl_3$, $CCl_4$, MeCN, $H_2O$, 86%; f) NaOMe, MeOH, 96%; g) PyBOP, DMF, 0°C, then **2**, 78% for **13**, 81% for **4**; h) 30 mM **4**, 100 mM LiOH, $D_2O$, 75%. DMF = *N*,*N*-dimethylformamide, PyBOP = 1-benzotriazolyloxy-tris(pyrollidino)phosphonium hexafluorophosphate.

tions, giving the tetrasaccharide-fluorides **13** and **4**, respectively. When PyBOP was added in greater excess, one or more tetrasaccharide lactone byproducts were observed. The "preactivated" spirolactone approach reported by Hindsgaul was not pursued, owing to anticipated formation of regioisomeric amides.[12c] Under standard conditions for transesterification or saponification, both **13** and **4** underwent rapid intramolecular transamidation, presumably via an intermediate cyclic imide.[12d] Careful control of the pH value and duration of the reaction allowed isolation of **14** with minimal (<10%) formation of the undesired amide. Unfortunately, conditions that were designed to prevent this side reaction altogether (LiCl, pyridine, elevated temperature) resulted in poor yields for the conversion of the glycosyl fluoride **4** to **14**. As characterization of a related GSL was reportedly facilitated through esterification, we opted to delay deprotection of the acid until after coupling of **4** with the sphingosine.[16]

Test reactions indicated that EGCase mutant E351S/D314Y catalyzed the coupling of **4** or **14** with D-*erythro*-sphingosine and the two most abundant forms identified in isolated LLG-3, the $C_{17}$ and $C_{18}$ D-*ribo*-phytosphingosines. In addition, the enzyme tolerated acetylated donor **13**, thus suggesting its utility in the preparation of the widely distributed O-acetylated gangliosides, for which synthetic methods are generally lacking. EGCase GS catalyzed the reaction of **4** with $C_{18}$ D-*ribo*-phytosphingosine to afford isolated methyl ester **15** in over 70% yield on a 20–40 mg scale (Scheme 5). Cleavage of the ester could be achieved with LiCl in pyridine to give the *lyso*-ganglioside **17**.[12d] A fluorescent analogue of $C_{18}$ LLG-3, **18**, was prepared by acylation of **15** with a BODIPY-FL-containing carboxylic acid. To obtain the main target LLG-3 (**1**), the EGCase GS product, $C_{17}$-*lyso*-LLG-3 **16**, was acylated with an appropriate α-hydroxy acid and deprotected by sequential cleavage of the methyl ester and saponification of the remaining acetyl group.

The structural complexity observed in GSLs from echinoderms is remarkable, as is their potential for studying nerve growth. We have developed a chemoenzymatic synthesis of LLG-3 that capitalizes on the structurally permissive nature of bacterial enzymes, namely Neu5Ac aldolase (*E. coli*), CMP-sialic acid synthetase NSY-05 (*N. meningitides* 406Y), and the α(2,3) sialyltransferase Cst-I (*C. jejuni*), for assembly of highly unusual tetrasaccharide fluorides. Furthermore, our synthesis exploits an endoglycoceramidase (*Rhodococcus sp.*) for coupling of the oligosaccharide with various sphingosines. This latter enzyme has been engineered to catalyze synthesis instead of degradation, and then further modified to build on its already liberal substrate specificity. Together, these catalysts have permitted an efficient synthesis of the LLG-3 ganglioside. Most importantly, this approach allows ready variation of each of the three principal constituents of gangliosides: the oligosaccharide, sphingosine, and N-acyl components. As a consequence, it is possible to produce




***Scheme 5.*** Reaction conditions: a) EGCase glycosynthase, sphingosine, 71% for **15**; b) LiCl, pyridine, 100°C, 72%; c) (*R*)-2-*O*-acetyl-tricosanoic acid, PyBOP, DMF, $Et_3N$; d) LiCl, pyridine, 100°C; e) NaOH, 30°C, 66% from **4**; f) Bodipy-FL-OSu, DMF, $Et_3N$, 40%.

structurally defined homogeneous samples of individual isoforms found among significantly heterogeneous natural isolates, and furthermore, to create new ganglioside analogues. Such molecules promise to be of use in delineating the structural basis for the neuritogenic nature of some GSLs, and could also assist in the identification of as-yet-unstudied biochemical pathways in echinoderms.

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[1] a) X. Zhang, F. L. Kiechle, *Ann. Clin. Lab. Sci.* **2004**, *34*, 3; b) T. Kolter, *Chem. Phys. Lipids* **2011**, *164*, 590; c) A. R. Mather, L. J. Siskind, *Adv. Exp. Med. Biol.* **2011**, *721*, 121; d) C. A. Lingwood, *Cold Spring Harb. Perspect. Biol.* **2011**, DOI: 10.1101/cshperspect.a004788.
[2] Y. D. Vankar, R. R. Schmidt, *Chem. Soc. Rev.* **2000**, *29*, 201.
[3] R. Higuchi, M. Inagaki, K. Yamada, T. Miyamoto, *J. Nat. Med.* **2007**, *61*, 367.
[4] M. Inagaki, R. Isobe, R. Higuchi, *Eur. J. Org. Chem.* **1999**, 771.
[5] M. Kaneko, K. Yamada, T. Miyamoto, M. Inagaki, R. Higuchi, *Chem. Pharm. Bull.* **2007**, *55*, 462.
[6] H. Tamai, H. Ando, H.-N. Tanaka, T. Hosoda-Yabe, T. Yabe, H. Ishida, M. Kiso, *Angew. Chem.* **2011**, *123*, 2378; *Angew. Chem. Int. Ed.* **2011**, *50*, 2330.
[7] H. Izu, Y. Izumi, Y. Kurome, M. Sano, A. Kondo, I. Kato, M. Ito, *J. Biol. Chem.* **1997**, *272*, 19846.
[8] M. D. Vaughan, K. Johnson, S. Defrees, X. Tang, R. A. J. Warren, S. G. Withers, *J. Am. Chem. Soc.* **2006**, *128*, 6300.
[9] S. M. Hancock, J. R. Rich, M. E. Caines, N. C. J. Strynadka, S. G. Withers, *Nat. Chem. Biol.* **2009**, *5*, 508.
[10] J. R. Rich, A. Cunningham, M. Gilbert, S. G. Withers, *Chem. Commun.* **2011**, *47*, 10806.
[11] a) A. A. Bergwerff, S. H. D. Hulleman, J. P. Kamerling, J. F. G. Vliegenthart, L. Shaw, G. Reuter, R. Schauer, *Biochimie* **1992**, *74*, 25; b) A. Kelm, L. Shaw, R. Schauer, G. Reuter, *Eur. J. Biochem.* **1998**, *251*, 874.
[12] a) C.-T. Ren, C.-S. Chen, S.-H. Wu, *J. Org. Chem.* **2002**, *67*, 1376; b) G.-T. Fan, C.-C. Lee, C. C. Lin, J.-M. Fang, *J. Org. Chem.* **2002**, *67*, 7565; c) J. C. McAuliffe, D. Rabuka, O. Hindsgaul, *Org. Lett.* **2002**, *4*, 3067; d) C.-T. Ren, C.-S. Chen, Y.-P. Yu, Y.-F. Tsai, P.-Y. Lin, Y.-J. Chen, W. Zou, S.-H. Wu, *Chem. Eur. J.* **2003**, *9*, 1085; e) S. Hanashima, D. Ishikawa, S. Akai, K.-i. Sato, *Carbohydr. Res.* **2009**, *344*, 747.
[13] M.-F. Karwaski, W. W. Wakarchuk, M. Gilbert, *Protein Expression Purif.* **2002**, *25*, 237.
[14] C. P. Chiu, L. L. Lairson, M. Gilbert, W. W. Wakarchuk, S. G. Withers, N. C. J. Strynadka, *Biochemistry* **2007**, *46*, 7196.
[15] a) G. Dufner, R. Schworer, B. Muller, R. R. Schmidt, *Eur. J. Org. Chem.* **2000**, 1467; b) T. J. Morley, S. G. Withers, *J. Am. Chem. Soc.* **2010**, *132*, 9430; c) H. Yu, H. Cao, V. M. Tiwari, Y. Li, X. Chen, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5037; d) A. Y. Khorlin, I. M. Privalova, *Carbohydr. Res.* **1970**, *13*, 373.
[16] M. Inagaki, T. Miyamoto, R. Isobe, R. Higuchi, *Chem. Pharm. Bull.* **2005**, *53*, 1551.

  