Synthesis, Inhibitory Effects on Nitric Oxide and Structure-Activity Relationships of a Glycosphingolipid from the Marine Sponge Aplysinella rhax and Its Analogues

Yuzo Fujita 1, Naohiro Ohshima 1, Ai Hasegawa 1, Frank Schweizer 2, Tadahiro Takeda 1, Fumiyuki Kiuchi 1 and Noriyasu Hada 1,*

1 Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan
2 Department of Chemistry and Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, R3T 2N2, Canada

* Author to whom correspondence should be addressed; E-Mail: hada-nr@pha.keio.ac.jp; Tel.: +81-3-5400-2666; Fax: + 81-3-5400-2556.

Received: 9 December 2010; in revised form: 29 December 2010 / Accepted: 14 January 2011 / Published: 17 January 2011

Abstract: The novel glycosphingolipid, \( \beta\)-D-GalNAc\( p(1 \rightarrow 4)[\alpha\)-D-Fuc\( p(1 \rightarrow 3)]-\beta\)-D-GlcNAcp(1\( \rightarrow \))Cer (A), isolated from the marine sponge Aplysinella rhax has a unique structure, with D-fucose and N-acetyl-D-galactosamine moieties attached to a reducing-end N-acetyl-D-glucosamine through an \( \alpha 1 \rightarrow 3 \) and \( \beta 1 \rightarrow 4 \) linkage, respectively. We synthesized glycolipid 1 and some non-natural di- and trisaccharide analogues 2-6 containing a D-fucose residue. Among these compounds, the natural type showed the most potent nitric oxide (NO) production inhibitory activity against LPS-induced J774.1 cells. Our results indicate that both the presence of a D-Fuc\( \alpha 1 \)-3GlcNAc-linkage and the ceramide aglycon portion are crucial for optimal NO inhibition.

Keywords: glycosphingolipid; Aplysinella rhax; D-fucose; nitric oxide

1. Introduction

Carbohydrates in the form of glycoconjugates, for example glycoproteins, glycolipids and proteoglycans, play an important role in many intracellular and extracellular events including cell-cell
adhesion, cell differentiation, signal transduction, cancer metastasis and immune responses [1]. The majority of these studies have focused on higher animals and relatively little is known about the functions of glycoconjugates in lower animals [2]. In order to study the biological properties of glycans in glycoconjugates, over the past decade we have synthesized novel glycolipid and glycoprotein derivatives found in various invertebrates [3-12]. Organic synthesis is a powerful method to explore structure activity relationships by providing access to large amounts of homogeneous and structurally defined oligosaccharides including not only natural compounds, but also non-natural compounds [13]. Recently, Zollo et al. isolated and characterized a novel neutral glycosphingolipid (A, Figure 1) from the marine sponge *Aplysinella rhax* which features a D-fucose and an N-acetyl-D-galactosamine attached to a reducing-end N-acetyl-D-glucosamine through a α1→3 and a β1→4 linkages, respectively [14]. This was the first report on glycolipids containing D-fucose. Furthermore, these glycolipids have been found to exhibit significant inhibitory activity on LPS-induced nitric oxide (NO) release by J774.1 macrophages. In order to study the structure-activity relationships of these compounds inhibiting NO release, we previously reported the synthesis of β-D-GalNAc(1→4)[α-D-Fucp(1→3)]-β-D-GlcNAc(1→) aglycon trisaccharide analogues, containing a 2-branched fatty alkyl residue and a 2-(trimethylsilyl)ethyl (TMS-Et) residue, respectively [6]. Moreover, biological evaluation of these novel glycosphingolipid analogues using an LPS-induced NO release assay demonstrated that the presence of D-fucose is crucial for the NO inhibitory effect, while structural modifications at the aglycon moiety appeared to have little to no effect on LPS-induced NO release [6]. In this study, we describe for the first time the total synthesis of glycosphingolipid 1 and its structural analogues 3-6 to elucidate the structure activity relationships on LPS-induced NO production in more detail (Figure 1).

**Figure 1.** Target glycosphingolipid and the analogue compounds

2-(Trimethylsilyl)ethyl β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (3) was selected to explore how the presence of a terminal β-D-galacto-
Molecules 2011, 16

pyranosyl linkage instead of a 2-acetamido-2-deoxy-β-D-galactopyranosyl linkage affects the biological effect. Disaccharide-based regioisomers 4 and 5 were selected to explore differences in the connectivity of the α-D-fucopyranosyl moiety to the-β-D-GlcNAc portion while trisaccharide 6 was chosen to study the effect of two α-D-fucopyranosyl linkages linked to the core -D-GlcNAc moiety. The NO-inhibitory affect of commercially available ceramide 7 and known trisaccharide 2 was included in these experiments for comparison.

2. Results and Discussion

2.1. Chemical synthesis

Synthesis of glycosphingolipid 1: Glycosylation of phytoceramide acceptor 9 [15] with the glycosyl imidate 8 [6] was carried out in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) [16] and 4 Å molecular sieves (MS4 Å) to obtain the desired glycolipid derivative 10 in 33% yield with complete β-stereoselectivity. Deprotection of the Troc group was achieved with Zn in a mixture containing acetic anhydride and acetic acid, followed by catalytic hydrogenolysis over 10% Pd/C in MeOH/THF to provide 11 in 47% yield. Deacetylation of 11 using Zemplén conditions and purification by column chromatography on Sephadex LH-20 afforded target glycolipid 1 quantitatively (Scheme 1).

Scheme 1. Synthesis of glycosphingolipid 1.

Syntheses of oligosaccharides 3-6: Glycosylation of known disaccharide acceptor 12 [3] with the known D-fucopyranosyl donor 13 [6] in the presence of N-iodosuccinimide (NIS), trifluoromethanesulfonic acid (TfOH) [17] and MS4 Å in dichloromethane provided the desired α-glycoside 14 in 88% yield with complete α-stereoselectivity. The newly formed α-glycosidic linkage was confirmed by 1H-NMR spectroscopy. The anomeric proton of the fucose moiety in 14 appeared at 4.85 ppm as a doublet with a homonuclear proton-proton coupling constant of 3.7 Hz (H-1 of Fuc, δ = 4.85 ppm, J_{H1,H2} = 3.7 Hz). Deprotection of the Troc group in 14 was achieved with Zn in a mixture containing acetic anhydride and acetic acid, followed by catalytic hydrogenolysis over 10% Pd-C in MeOH and
acetylation to provide 15. Zemplén deacetylation and purification by column chromatography on Sephadex LH-20 produced trisaccharide 3 quantitatively (Scheme 2).

**Scheme 2. Synthesis of oligosaccharide 3.**

The synthesis of disaccharides 4 and 5 and trisaccharide 6 is outlined in Schemes 3-5. Glycosylation of known glycosyl acceptors 16, 19 [6] and 22 [18] with the D-fucopyranosyl donor 13 in the presence of NIS, TfOH and MS 4 Å in dichloromethane gave the desired α-glycosides 17 (68%), 20 (78%) and the trisaccharide 23 (42%) with complete α-steroselectivity, respectively. The newly formed α-glycosidic linkage was confirmed by $^1$H-NMR spectroscopy. The Troc-protecting group of 17 was converted into an acetamido group by reduction with Zn-AcOH followed by debenzylidation and debenzylation with catalytic hydrogenolysis over 10% Pd/C in MeOH-AcOH and acetylation to afford 18 in 44% yield. Finally, standard deacetylation and purification by column chromatography on Sephadex LH-20 furnished disaccharide 4 in 86% yield (Scheme 3).

**Scheme 3. Synthesis of oligosaccharide 4.**

Disaccharide 5 was synthesized from the disaccharide 20 in a six steps deblocking/blocking procedure (Scheme 4). At first, the chloroacetyl protecting group in 20 was deblocked with thiourea in an ethanol/pyridine solvent mixture before conversion of the Troc group into an acetamido group using
standard conditions. Debenzylation using catalytic hydrogenolysis followed by acetylation provided protected disaccharide 21 in 45% yield which was deprotected using standard conditions to provide disaccharide 5 (Scheme 4).

**Scheme 4. Synthesis of oligosaccharide 5**

The remaining trisaccharide 6 was prepared from protected trisaccharide 23 in a five steps procedure. Initially, the phthalimido-protecting group of 23 was removed using hydrazine monohydrate in ethanol followed by standard acetylation, debenzylation by catalytic hydrogenation over 10% Pd-C in MeOH-THF and acetylation to provide peracetylated trisaccharide 24 in 47% yield. Finally, standard deacetylation and purification by column chromatography on Sephadex LH-20 provided disaccharide 6 (Scheme 5). Oligosaccharide 2 was prepared according to a procedure previously reported by us [6].

**Scheme 5. Synthesis of oligosaccharide 6**
3.2. Inhibitory Effects of Synthetic Compounds on NO Production

The synthetic compounds were evaluated for their ability to inhibit nitric oxide (NO) production by LPS-induced macrophage-like J774.1 cells [19] (Figure 2). NO, a short living mediator is synthesized by a family of enzymes termed NO-synthase. Two types of NOS are recognized: constitutive isoforms and inducible isoforms (iNOS). iNOS is regulated by inflammatory mediators (LPS, cytokines) and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response [14]. The glycolipid 1 showed comparable NO inhibitory activity in high concentration (100 μM) to N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) used as the positive control. Related compounds having the common D-Fucα1-3GlcNAc structure (i.e. 2, 3 and 4) also showed significant inhibitory activity resulting in a 20% reduction of NO release at 50 μM and 100 μM concentrations. However, very little or no inhibition of NO release were seen at these concentrations for disaccharide 5 and trisaccharide 6 bearing an unnatural D-Fucα1-4GlcNAc linkage. Interestingly, glycosphingolipid 1 showed stronger activity than 2, suggesting that the ceramide-based aglycon contributes to the inhibition of NO release more efficiently than a 2-(trimethylsilyl) ethyl-based aglycon. Moreover, commercial ceramide 7 showed inhibitory activity at higher concentration (100 μM). However, the activity of ceramide is strongly enhanced by glycosylation to the trisaccharide β-D-GalNAcp(1→4)[α-D-Fucp(1→3)]-β-D-GlcNAc indicating that both the trisaccharide and ceramide-based aglycon portion of the glycosphingolipid contribute to the inhibition of cellular nitric oxide release.

**Figure 2.** Inhibitory effects on NO production in LPS-activated J774.1 cells of compounds 1-7. Each data represents the mean ± SD for quadruplet experimentals. P: Positive control (L-NMMA), 50 μM.
3. Experimental

3.1. General

Optical rotations were measured with a Jasco P-1020 digital polarimeter. $^1$H- and $^{13}$C-NMR spectra were recorded with JMN A500 and ECP 600 FT NMR spectrometers with Me$_4$Si as the internal standard for solutions in CDCl$_3$ and CD$_3$OD. MALDI-TOFMS was recorded on an Applied Biosystems Voyager DE RP mass spectrometer. High-resolution mass spectra were recorded on a JEOL JMS-700 under FAB conditions. TLC was performed on Silica Gel 60 F254 (E. Merck) with detection by quenching of UV fluorescence and by charring with 10% H$_2$SO$_4$. Column chromatography was carried out on Silica Gel 60 (E. Merck). The compounds 3,4,6-Tri-O-acetyl-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-β-D-galactopyranosyl-(1→4)[2,3,4-tri-O-acetyl-α-D-fucopyranosyl-(1→3)]-6-O-acetyl-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-D-glucopyranosyl trichloroacetimidate (8) [6], 2-(trimethylsilyl)ethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-6-O-benzyl-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-D-glucopyranoside (12) [3], phenyl 2,3,4-tri-O-benzyl-1-thio-β-D-fucopyranoside (13) [6], 2-(Trimethylsilyl)ethyl 4,6-O-benzylidene-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-β-D-glucopyranoside (16) [6], 2-(trimethylsilyl)ethyl 6-O-benzyl-3-O-chloroacetyl-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-β-D-glucopyranoside (19) [6] and 2-(trimethylsilyl)ethyl 6-O-benzyl-2-deoxy-2-phthalimide-β-D-glucopyranoside (22) [18] were prepared as reported. Benzyleceramide 9 was prepared by the conventional four-steps procedure [15] from phytosphingosine, which was purchased from Degussa (The Netherlands).

3,4,6-Tri-O-acetyl-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-β-D-galactopyranosyl-(1→4)-[2,3,4-tri-O-acetyl-α-D-fucopyranosyl-(1→3)]-6-O-acetyl-2-deoxy-2(2,2,2-trichloroethoxy carbonylamino)-β-D-glucopyranosyl-(1→1)-(2S,3S,4R)-3,4-di-O-benzoyl-2-hexadecanamido-octadecane-3,4-di-ol (10). Four Å molecular sieves (250 mg) were added to a solution of 8 (21 mg, 16.5 μmol) and (2S,3S,4R)-3-O-benzoyl-2-hexadecanamido-4-octadecene-1,3-diol 9 (24 mg, 30.0 μmol) in dry CH$_2$Cl$_2$ (0.5 mL) and the mixture was stirred for 16 h at room temperature, then cooled to 0 °C. TMSOTf (3 μL, 0.01 mmol) was added, and the mixture was stirred for 1 h at 0 °C, then neutralized with Et$_3$N. The solids were filtered off and washed with CHCl$_3$. The combined filtrate and washings were successively washed with water, dried (MgSO$_4$), and concentrated. The product was purified by silica gel column chromatography using 3:1 toluene-EtOAc as eluent to give 10 (10 mg, 33%). $[\alpha]_D^{23}$ = +18.2° (c 1.0, CHCl$_3$); $^1$H-NMR (500 MHz, CDCl$_3$): δ 4.94 (d, 1H, $J_{1,2}$=3.7Hz, H-1 of fuc), 4.50 (br. d, 1H, H-1 of GlcNAc), 4.34 (br. s, 1H, H-1, of GalNAc). MALDI-TOFMS: Caled for C$_{86}$H$_{129}$Cl$_6$N$_3$O$_{27}$Na [M+Na]$^+$: m/z 1868.7 Found: 1869.4.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-[2,3,4-tri-O-acetyl-α-D-fucopyranosyl-(1→3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→1)-(2S,3S,4R)-hexadecanamido-octadecane-3,4-di-ol (11). To a solution of 10 (31mg, 16.8 μmol) in acetic anhydride (2 mL) and AcOH (2 mL) was added zinc powder (100 mg). The reaction mixture was stirred for 16 h at room temperature. After completion of the reaction, the solids were filtered off and the filtrate was
concentrated with toluene. The solution of the product and Pd/C (10%, 100 mg) in 1:1 MeOH/THF (2.0 mL) was stirred for 16 h at room temperature under H₂, then filtered and concentrated. The product was purified by silica gel column chromatography using 2:1 toluene acetone as eluent to give \( \text{11} \) as an amorphous powder (11 mg, 47%). \([\alpha]_{D}^{23} = +5.3°\) (c 0.7, CHCl₃); \(^1\)H-NMR (500 MHz, CDCl₃): \( \delta \) 5.15 (d, 1H, \( J_{1,2} = 3.7\) Hz, H-1 of fuc), 4.33 (br. d, 1H, H-1 of GlcNAc), 4.30 (br. s, 1H, H-1, of GalNAc). MALDI-TOFMS: Calcd for C₇₀H₁₁₉N₃O₂₅Na [M+Na]⁺: m/z 1424.8 Found: 1424.5.

2-Acetamido-2-deoxy-\( \beta \)-D-galactopyranosyl-(1→4)-[\( \alpha \)-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-\( \beta \)-D-glucopyranosyl-(1→)(2S,3S,4R)-hexadecanamido-octadecane-3,4-di-ol (1). To a solution of \( \text{11} \) (11 mg, 7.8 \( \mu \)mol) in MeOH (2 mL) was added dioxane (2 mL) and NaOMe (25 mg) at 40 °C. The mixture was stirred for 2 h and then neutralized with Amberlite IR 120 [H⁺]. The mixture was filtered and concentrated. The product was purified by Sephadex LH-20 column chromatography in 1:1 CHCl₃-MeOH to give \( \text{1 as white solid (10 mg, quant.)}. \([\alpha]_{D}^{25} +17.0 \) (c 0.06, 1:1 CHCl₃-MeOH). \(^1\)H-NMR (500 MHz, 1:1 CDCl₃-CD₃OD): \( \delta \) 5.13 (d, 1H, \( J = 3.7\) Hz, H-1 of Fuc), 4.62 (d, 1H, \( J = 8.3\) Hz, H-1 of GlcNAc), 4.32 (d, 1H, d, 1H, \( J = 8.0\) Hz, H-1 of GalNAc). MALDI-TOFMS: Calcd for C₅₆H₁₀₅N₃O₁₈Na: m/z 1130.7 Found: 1130.4 \([\text{M+Na}]^{+}\). HR-FABMS: Calcd for C₅₆H₁₀₅N₃O₁₈Na: m/z 1130.7291. Found m/z 1130.7257 \([\text{M+Na}]^{+}\).

2-(Trimethylsilyl)ethyl 2,3,4,6-tetra-O-acetyl-\( \beta \)-D-galactopyranosyl-(1→4)-[2,3,4-tri-O-benzyl-\( \alpha \)-D-fucopyranosyl-(1→3)]-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxy-carbonylamino)-\( \beta \)-D-glucopyranoside (14). To a solution of \( \text{12} \) (99 mg, 0.11 mmol) and \( \text{13} \) (89 mg, 0.17 mmol) in dry CH₂Cl₂ (1.5 mL) was added powdered MS 4Å (200 mg), and the mixture was stirred for 2 h at room temperature, then cooled to -60 °C. NIS (57 mg, 0.03 mmol) and TfOH (1.5 \( \mu \)L, 0.01 mmol) were added to the mixture, which was stirred for 3 h at -60 °C, then neutralized with Et₃N. The solids were filtered off and washed with CHCl₃. The combined filtrate and washings were successively washed with aq Na₂S₂O₃ and water, dried (MgSO₄), and concentrated. The product was purified by silica gel column chromatography using 3:1 hexane-EtOAc as eluent to give \( \text{14} \) (128 mg, 88%). \([\alpha]_{D}^{24} +20.4 \) (c 0.7, CHCl₃); \(^1\)H-NMR (500 MHz, CDCl₃): \( \delta \) 7.28–7.14 (m, 20H, 4Ph), 5.74 (d, 1H, NH), 5.26 (d, 1H, H-4 of Gal), 5.11 (t, 1H, H-2 of Gal), 4.92–4.84 (m, 2H, H-3 of Gal, benzylmethylene), 4.85 (d, 1H, \( J_{1,2} = 7.3\) Hz, H-1 of GlcNAc), 4.38 (d, 1H, \( J_{1,2} = 7.9\) Hz, H-1 of Gal), 4.27 (dd, 2H, benzylmethylene \( \times 2 \)), 4.06–4.02 (m, 7H, benzylmethylene \( \times 5 \), CH₂CCl₃), 4.40 (d, 1H, \( J_{1,2} = 7.3\) Hz, H-1 of Fuc), 4.75–4.54 (m, 7H, benzylmethylene \( \times 5 \), CH₂CCl₃), 4.30 (d, 1H, \( J_{1,2} = 7.9\) Hz, H-1 of Gal), 3.97–3.75 (m, 9H, H-2, H-3, H-6b of GlcNAc, H-5, H-6 of Gal, H-2, H-3 of Fuc, C₆H₅CH₂Si(CH₃)₃), 3.66–3.62 (m, 3H, H-4, H-5 of GlcNAc, H-4 of Fuc), 3.88–3.32 (m, 1H, C₆H₅CH₂Si(CH₃)₃), 2.07–1.87 (m, 12H, CH₂CO \( \times 4 \)), 1.09 (d, 3H, H-6 of Fuc), 0.88–0.73 (m, 2H, C₆H₅CH₂Si(CH₃)₃), −0.09 (s, 9H, Si(CH₃)₃); \(^13\)C-NMR (125 MHz, CDCl₃): \( \delta \) 170.2, 170.0, 154.2, 139.0, 138.9, 138.7, 138.3, 128.4, 128.23, 128.20, 127.8, 127.74, 127.65, 127.59, 127.5, 127.3, 100.2 (C-1 of GlcNAc), 100.1 (C-1 of Gal), 99.3 (C-1 of Fuc), 95.8, 79.5, 77.8, 76.4, 76.3, 75.6, 74.8, 74.6, 74.1, 73.5, 73.2, 72.9, 71.0, 70.4, 69.0, 67.4, 66.8, 66.7, 61.1, 53.6, 29.7, 20.8, 20.62, 20.57, 18.2, 16.5, −1.4 (Si(CH₃)₃); MALDI-TOFMS: Calcd for C₆₂H₇₈Cl₃NO₂₆SiNa: m/z 1312.4 Found: 1312.9 [M+Na]⁺.
2-(Trimethylsilyl)ethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-[2,3,4-tri-O-acetyl-α-D-fucopyranosyl-(1→3)]-6-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside (15). To a solution of 14 (107 mg, 0.08 mmol) in acetic anhydride (6 mL) and AcOH (6 mL) was added zinc powder (150 mg). The reaction mixture was stirred for 12 h at 40 °C. After completion of the reaction, the solids were filtered off and the filtrate was concentrated with toluene. The solution of the product and Pd/C (10%, 100 mg) in MeOH (2.0 mL) was stirred for 16 h at room temperature under H2, then filtered and concentrated. The residue was acetylated with acetic anhydride (2 mL) in pyridine (3 mL) for 16 h at room temperature. The reaction mixture was poured into ice-water and extracted with CHCl3. The extract was washed sequentially with 5% HCl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The product was purified by silica gel column chromatography using 5:1 toluene-acetone as eluent to give 15 (37 mg, 46%) as an amorphous powder. \([\alpha]_D^{24} +11.8 \ (c \ 0.7, \text{CHCl}_3); 1H-\text{NMR} (500 \text{ MHz}, \text{CDCl}_3): \delta \ 6.47 \ (d, 1H, NH), 5.36 \ (d, 1H, H-4 of Gal), 5.23–5.21 \ (m, 2H, H-2 of Gal, H-4 of Fuc), 5.10–5.00 \ (m, 3H, H-3 of Gal, H-2, H-3 of Fuc), 5.08 \ (d, 1H, J_{1,2}=3.7 Hz, H-1 of Fuc), 4.57 \ (dd, 1H, H-6a of Gal), 4.52 \ (d, 1H, J_{1,2}=7.9 Hz, H-1 of Gal), 4.42 \ (dd, 1H, H-1 of GlcNAc), 4.37 \ (d, 1H, J_{1,2}=7.9 Hz, H-1 of Fuc), 4.32 \ (d, 1H, J_{1,2}=7.9 Hz, H-1 of GlcNAc), 4.33 \ (dd, 1H, H-6 of GlcNAc), 4.24 \ (dd, 1H, H-6 of GlcNAc), 4.08 \ (d, 1H, H-6 of GlcNAc), 4.07 \ (dd, 1H, H-6 of GlcNAc), 4.06 \ (d, 1H, H-6 of GlcNAc), 4.05 \ (d, 1H, H-6 of GlcNAc), 4.04 \ (d, 1H, H-6 of GlcNAc), 4.03 \ (d, 1H, H-6 of GlcNAc), 4.02 \ (s, 3H, Si(CH)_3); 13C-\text{NMR} (125 \text{ MHz}, \text{CDCl}_3): \delta \ 170.9, \ 170.7, \ 170.5, \ 170.3, \ 170.2, \ 170.0, \ 169.9, \ 169.5, \ 99.5 \ (C-1 of Gal), \ 99.4 \ (C-1 of GlcNAc), \ 97.3 \ (C-1 of Fuc), \ 74.8, \ 73.6, \ 72.7, \ 71.5, \ 70.90, \ 70.86, \ 68.5, \ 67.9, \ 62.9, \ 61.1, \ 56.7, \ 30.7, \ 23.5, \ 18.8, \ 16.8, \ -1.3 \ (\text{Si(CH}_3)_3); \text{MALDI-TOFMS}: \text{Calcd for C}_{41}\text{H}_{63}\text{NO}_{23}\text{SiNa: m/z 988.3 Found: 988.4 [M+Na]}.\]

2-(Trimethylsilyl)ethyl β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (3). To a solution of 15 (36 mg, 0.04 mmol) in MeOH (5 mL) NaOMe (25 mg) was added at 40 °C. The mixture was stirred for 2 h and then neutralized with Amberlite IR 120 (H\(^+\)). The mixture was filtered and concentrated. The product was purified by Sephadex LH-20 column chromatography in 1 : 1 CHCl3-MeOH to give 5 as white solid (24 mg, quant.). \([\alpha]_D^{24} +14.4 \ (c \ 0.3, \text{CH}_3\text{OH}); 1H-\text{NMR} (500 \text{ MHz}, \text{CD}_2\text{OD}): \delta \ 5.12 \ (d, 1H, J_{1,2}=4.3 Hz, H-1 of Fuc), 4.42 \ (d, 1H, J_{1,2}=7.9 Hz, H-1 of GlcNAc), 4.32 \ (d, 1H, J_{1,2}=7.9 Hz, H-1 of Gal); 13C-\text{NMR} (125 \text{ MHz}, \text{CD}_2\text{OD}): \delta \ 173.4, \ 104.1 \ (C-1 of GlcNAc), 102.2 \ (C-1 of Gal), 102.1 \ (C-1 of Fuc), 74.8, 73.6, 72.7, 71.5, 70.90, 70.86, 68.5, 67.9, 62.9, 61.1, 56.7, 30.7, 23.5, 18.8, 16.8, -1.3 \ (\text{Si(CH}_3)_3); \text{MALDI-TOFMS}: \text{Calcd for C}_{25}\text{H}_{47}\text{NO}_{15}\text{SiNa: m/z 652.3 Found: 652.6 [M+Na]+. HR-FABMS: Calcd for C}_{25}\text{H}_{47}\text{NO}_{15}\text{SiNa: m/z 652.2613. Found m/z 652.2642 [M+Na]+.}\]

2-(Trimethylsilyl)ethyl β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (17). To a solution of 16 (329 mg, 0.61 mmol) and 13 (479 mg, 0.91 mmol) in dry CH2Cl2 (1.5 mL) was added powdered 4Å MS (800 mg), and the mixture was stirred for 2 h at room temperature, then cooled to -60 °C. NIS (307 mg, 1.37 mmol) and TfOH (16 μL, 0.18 mmol) were added to the mixture, which was stirred for 3 h at -60 °C, then neutralized with Et3N. The solids were filtered off and washed with CHCl3. The combined filtrate and washings were successively washed withaq Na2S2O3 and water, dried (MgSO4), and...
concentrated. The product was purified by silica gel column chromatography using 7:1 hexane-EtOAc as eluent to give 17 (394 mg, 68%). $[\alpha]_D^{24} +18.9$ (c 2.4, CHCl$_3$); $^1$H-NMR (500 MHz, CDC$_3$): $\delta$ 7.41–6.89 (m, 20H, 4Ph), 5.49 (br s, 1H, H-1 of Fuc), 5.31 (s, 1H, OCHPh), 5.14 (br s, 1H, NH), 4.86–4.75 (m, 3H, benzylmethylene x 2, CH$_2$CCl$_3$), 4.64 (d, 1H, $J_{1,2}$=8.6 Hz, H-1 of GlcNAc), 4.60 (d, 1H, CH$_2$CCl$_3$), 4.48 (t, 2H, benzylmethylene x 2), 4.36–4.29 (m, 2H, H-3, H-6a of GlcNAc), 3.96–3.85 (m, 3H, CH$_2$CH$_2$Si(CH$_3$)$_3$, H-2, H-5 of Fuc), 3.81–3.67 (m, 3H, H-4, H-6b of GlcNAc, H-3 of Fuc), 3.52–3.40 (m, 4H, CH$_2$CH$_2$Si(CH$_3$)$_3$, H-2, H-5 of GlcNAc, H-4 of Fuc), 1.01 (d, 3H, H-6 of Fuc), 0.94–0.79 (m, 2H, CH$_2$CH$_2$Si(CH$_3$)$_3$), –0.07 (s, 9H, Si(CH$_3$)$_3$); $^{13}$C-NMR (125 MHz, CDCl$_3$): $\delta$ 153.7, 138.8, 138.4, 138.2, 136.9, 129.3, 128.5, 128.34, 128.27, 128.1, 128.0, 127.5, 127.3, 127.1, 126.2, 101.6, 100.8 (C-1 of GlcNAc), 97.0 (C-1 of Fuc), 95.4, 82.5, 78.6, 75.1, 74.8, 74.4, 73.6, 73.3, 71.4, 68.7, 67.6, 67.0, 65.8, 57.0, 29.6, 18.2, 16.7, –1.5 (Si(CH$_3$)$_3$); MALDI-TOFMS: Calcd for C$_{48}$H$_{58}$Cl$_3$NO$_{11}$SiNa $[M+Na]^+$: m/z 980.3. Found: 980.1.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-acetyl-\(\alpha\)-D-fucopyranosyl-(1→3)-2-acetamido-4,6-di-O-acetyl-2-deoxy-\(\beta\)-D-glucopyranoside (18). To a solution of 17 (113 mg, 0.12 mmol) in acetic anhydride (7 mL) and AcOH (7 mL) was added zinc powder (150 mg). The reaction mixture was stirred for 12 h at 40 °C. After completion of the reaction, the solids were filtered off and the filtrate was concentrated with toluene. The solution of the product and Pd/C (10%, 150 mg) in 3:1 MeOH-AcOH (2.0 mL) was stirred for 12 h at room temperature under H$_2$, then filtered and concentrated. The residue was acetylated with acetic anhydride (6 mL) in pyridine (10 mL) for 12 h at room temperature. The reaction mixture was poured into ice-water and extracted with CHCl$_3$. The extract was washed sequentially with 5% HCl, aq NaHCO$_3$ and water, dried (MgSO$_4$), and concentrated. The product was purified by silica gel column chromatography using 5:1 toluene-acetone as eluent to give 18 (35 mg, 44%) as an amorphous powder. $[\alpha]_D^{24} +74.9$ (c 0.3, CHCl$_3$); $^1$H-NMR (500 MHz, CDCl$_3$): $\delta$ 5.85 (d, 1H, NH), 5.25 (d, 1H, $J_{1,2}$=3.7 Hz, H-1 of Fuc), 5.26–5.23 (m, 2H, H-3, H-4 of Fuc), 5.08 (dd, 1H, H-2 of Fuc), 4.97 (dd, 1H, H-4 of GlcNAc), 4.67 (t, 1H, H-3 of GlcNAc), 4.67 (d, 1H, $J_{1,2}$=7.9 Hz H-1 of Fuc), 4.97 (dd, 1H, H-4 of GlcNAc), 4.67 (t, 1H, H-3 of GlcNAc), 4.25 (dd, 1H, H-5 of Fuc), 4.14 (dd, 1H, H-6a of Fuc), 3.99 (dd, 1H, H-6b of GlcNAc), 3.92–3.87 (m, 1H, CH$_2$CH$_2$Si(CH$_3$)$_3$), 3.61–3.51 (m, 2H, H-5 of GlcNAc, CH$_2$CH$_2$Si(CH$_3$)$_3$), 3.09–3.04 (m, 1H, H-2 of GlcNAc), 1.08 (d, 3H, H-6 of Fuc), 0.96–0.83 (m, 2H, CH$_2$CH$_2$Si(CH$_3$)$_3$), –0.02 (s, 9H, Si(CH$_3$)$_3$); $^{13}$C-NMR (125 MHz, CDCl$_3$): $\delta$ 170.7, 170.5, 98.1 (C-1 of GlcNAc), 95.8 (C-1 of Fuc), 73.7, 72.3, 71.5, 71.0, 67.6, 67.4, 67.2, 64.9, 62.5, 58.1, 23.8, 20.9, 20.8, 20.6, 18.1, 16.1 –1.4 (Si(CH$_3$)$_3$); MALDI-TOFMS: Caled for C$_{29}$H$_{47}$NO$_{15}$SiNa $[M+Na]^+$: m/z 700.3 Found: 700.5.

2-(Trimethylsilyl)ethyl $\alpha$-D-fucopyranosyl-(1→3)-2-acetamido-2-deoxy-$\beta$-D-glucopyranoside (4). Compound 4 was prepared from 18 (24 mg, 0.035 mmol) by the same method described for preparation of 3. The product was purified by Sephadex LH-20 column chromatography in 1:1 CHCl$_3$-MeOH to give 4 as white solid (14 mg, 86%). $[\alpha]_D^{24} +52.9$ (c 0.1, CH$_3$OH); $^1$H-NMR (500 MHz, CD$_3$OD): $\delta$ 4.93 (d, 1H, $J_{1,2}$=3.1 Hz, H-1 of Fuc), 4.30 (d, 1H, $J_{1,2}$=8.5 Hz H-1 of GlcNAc); $^{13}$C-NMR (125 MHz, CD$_3$OD): $\delta$ 173.3, 103.6 (C-1 of GlcNAc), 102.2 (C-1 of Fuc), 86.3, 79.5, 77.4, 73.7, 72.5, 71.6, 70.8, 68.5, 67.9, 62.6, 60.2, 55.9, 30.7, 23.4, 18.8, 16.9, –1.26 (Si(CH$_3$)$_3$); MALDI-TOFMS: Caled for C$_{19}$H$_{37}$NO$_{10}$SiNa: m/z 490.2 Found: 490.6 [M+Na]$^+$. HR-FABMS: Caled for C$_{19}$H$_{37}$NO$_{10}$SiNa: m/z 490.2084. Found m/z 490.2072 [M+Na]$^+$. 

Molecules 2011, 16 646
2-(Trimethylsilyl)ethyl 2,3,4-tri-O-benzyl-α-D-fucopyranosyl-(1→4)-6-O-benzyl-3-O-chloroacetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamo)-β-D-glucopyranoside (20). Compound 20 was prepared from 19 (226 mg, 0.36 mmol) and 13 (383 mg, 0.73 mmol) by the same method described for preparation of 17. The product was purified by silica gel column chromatography using 10:1 hexane-EtOAc as eluent to give 20 as syrup (296 mg, 78%). [α]D24 +11.4 (c 4.0, CHCl3); 1H-NMR (500 MHz, CDCl3): δ 7.37–7.10 (m, 20H, 4 Ph), 5.49 (d, 1H, NH), 5.22 (t, 1H, H-3 of GlcNAc), 4.89 (d, 1H, benzylmethylene), 4.87 (d, 1H, J1,2=3.7 Hz, H-1 of Fuc), 4.76–4.53 (m, 9H, H-4 of Fuc, benzylmethylene x 6, CH2Cl2), 4.50 (d, 1H, J1,2=7.3 Hz, H-1 of GlcNAc), 4.45 (d, 1H, benzylmethylene), 3.92–3.79 (m, 5H, H-4, 6a of GlcNAc, H-2, 5 of Fuc), 3.75–3.62 (m, 5H, H-2, H-6b of GlcNAc, H-3 of Fuc, ClCH2CO), 3.51–3.46 (m, 2H, CH2CH2Si(CH3)3), 3.51–3.46 (m, 2H, H-2, H-6b of GlcNAc, H-3 of Fuc, ClCH2Si(CH3)3), 3.46–3.40 (m, 2H, CH2CH2Si(CH3)3, H-5 of GlcNAc), 0.97 (d, 3H, H-6 of Fuc), 0.96–0.83 (m, 2H, CH2CH2Si(CH3)3), –0.09 (s, 9H, Si(CH3)3); 13C-NMR (125 MHz, CDCl3): δ 167.1, 154.1, 138.5, 138.4, 138.0, 128.6, 128.5, 128.4, 128.3, 128.2, 127.6, 127.5, 127.4, 127.3, 100.1 (C-1 of Fuc), 98.8 (C-1 of GlcNAc), 95.5, 78.9, 77.7, 75.6, 74.9, 74.8, 74.6, 74.2, 74.0, 73.3, 73.2, 68.7, 67.6, 67.1, 55.5, 40.8, 18.1, 16.6, –1.32 (Si(CH3)3); MALDI-TOFMS: Calcd for C50H61Cl4NO12SiNa [M+Na]+: m/z 1058.3 Found: 1059.2.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-acetyl-α-D-fucopyranosyl-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (21). To a solution of 20 (296 mg, 0.29 mmol) in EtOH (2.5 mL) was added pyridine (1.5 mL) and thiourea (173 mg, 2.32 mmol). The reaction mixture was stirred for 6 h at 80 °C. The mixture was diluted with CHCl3, washed with aq 5%HCl, aq NaHCO3 and brine, dried (MgSO4) and concentrated. The solution of the residue in AcOH (2 mL) was added zinc powder (350 mg). The reaction mixture was stirred for 12 h at 60 °C. After completion of the reaction, the solids were filtered off and the filtrate was concentrated with toluene. The residue was acetylated with acetic anhydride (4 mL) in pyridine (7 mL). The reaction mixture was poured into ice-water and extracted with CHCl3. The extract was washed sequentially with 5% HCl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The solution of the product in MeOH (1.5 mL) and THF (0.5 mL) was hydrogenolysed under hydrogen in the presence of 10% Pd/C (150 mg) for 16 h at room temperature, then filtered and concentrated. The residue was acetylated with acetic anhydride (3 mL) in pyridine (5 mL). The reaction mixture was poured into ice-water and extracted with CHCl3. The extract was washed sequentially with 5% HCl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The product was purified by silica gel column chromatography using 5:1 toluene-acetone as eluent to give 21 as an amorphous powder (86 mg, 45%). [α]D24 +49.6 (c 0.5, CHCl3); 1H-NMR (500 MHz, CDCl3): δ 5.81 (d, 1H, NH), 5.34 (d, 1H, J1,2=3.7 Hz, H-1 of Fuc), 5.22–5.05 (m, 4H, H-3 of GlcNAc, H-2, H-3, H-4 of Fuc), 4.58 (d, 1H, J1,2=7.9 Hz H-1 of GlcNAc), 4.46 (dd, 1H, H-6a of GlcNAc), 4.08–4.01 (m, 2H, H-6b of GlcNAc, H-5 of Fuc), 3.95 (t, 1H, H-4 of GlcNAc), 3.88–3.83 (m, 1H, CH2CH2Si(CH3)3), 3.78 (dd, 1H, H-2 of GlcNAc), 3.61–3.57 (m, 1H, H-5 of GlcNAc), 3.53–3.48 (m, 1H, CH2CH2Si(CH3)3), 2.11–1.82 (m, 18H, CH3CO x 6), 1.04 (d, 3H, H-6 of Fuc), 0.91–0.78 (m, 2H, CH2CH2Si(CH3)3), –0.07 (s, 9H, Si(CH3)3); 13C-NMR (125 MHz, CDCl3): δ 171.0, 170.7, 170.5, 170.3, 170.1, 99.8 (C-1 of GlcNAc), 96.0 (C-1 of Fuc), 75.5, 72.1, 71.8, 70.9, 67.3, 67.2, 66.9, 65.5, 62.7, 54.6, 29.6, 23.1, 20.9, 20.8, 20.7, 20.6, 20.5, 17.8, 15.8, –1.5 (Si(CH3)3); MALDI-TOFMS: Calcd for C50H61Cl4NO12SiNa [M+Na]+: m/z 1058.3 Found: 1059.2.
2-(Trimethylsilyl)ethyl $\alpha$-D-fucopyranosyl-(1→4)-2-acetamido-2-deoxy-\(\beta\)-D-glucopyranoside (5). Compound 5 was prepared from 21 (86 mg, 0.13 mmol) by the same method described for preparation of 3. The product was purified by Sephadex LH-20 column chromatography in 1:1 CHCl₃-MeOH to give 5 as white solid (61 mg, quant.). [α]D²⁴ +33.9 (c 0.3, CH₃OH); ¹H-NMR (500 MHz, CD₃OD): δ 4.98 (d, 1H, J₁,₂=3.7 Hz, H-1 of Fuc), 4.32 (d, 1H, J₁,₂=7.9 Hz H-1 of GlcNAc); ¹³C-NMR (125 MHz, CD₃OD): δ 173.5, 103.5 (C-1 of Fuc), 102.0 (C-1 of GlcNAc), 82.2, 76.9, 75.9, 73.5, 71.7, 70.6, 68.6, 67.9, 62.5, 56.8, 30.7, 23.0, 18.8, 16.7, –1.3 (Si(CH₃)₃); MALDI-TOFMS: Calcd for C₁₉H₃₇NO₁₀SiNa: m/z 490.2 Found: 491.0 [M+Na].

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-benzyl-$\alpha$-D-fucopyranosyl-(1→3)-[2,3,4-tri-O-benzyl-$\alpha$-D-fucopyranosyl-(1→4)]-6-O-benzyl-2-deoxy-2-phthalimido-\(\beta\)-D-glucopyranoside (23). Compound 23 was prepared from 22 (89 mg, 0.18 mmol) and 13 (757 mg, 1.44 mmol) by the same method described for preparation of 14. The product was purified by silica gel column chromatography using 10:1 hexane-EtOAc as eluent to give 23 as syrup (99 mg, 42%). [α]D²⁴ +48.3 (c 1.2, CHCl₃); ¹H-NMR (600 MHz, CDCl₃): δ 7.90–7.26 (m, 39H, NPhth, 8 Ph), 6.20 (d, 1H, J₁,₂=3.6 Hz, H-1 of Fuc b), 5.19 (d, 2H, J₁,₂=8.5 Hz H-1 of GlcNAc, J₁,₂=4.4 Hz H-1 of Fuc a), 5.07 (d, 1H, benzylmethylene), 5.01 (dd, 1H, H-3 of GlcNAc, J₁,₂=8.5 Hz H-1 of Fuc b), 4.10 (dd, 1H, H-3 of Fuc a), 4.07–3.99 (m, 2H, CH₂CH₂Si(CH₃)₃, H-5 of Fuc a), 3.97–3.84 (m, 6H, H-5, H-6 of GlcNAc, H-2, H-5 of Fuc a, H-3 of Fuc b), 3.63–3.57 (m, 2H, H-4 of Fuc b, CH₂CH₂Si(CH₃)₃), 3.47(br.d, 1H, H-4 of Fuc a), 1.19 (d, 3H, H-6 of Fuc b) 0.95 (d, 3H, H-6 of Fuc a.), 0.93–0.81 (m, 2H, CH₂CH₂Si(CH₃)₃), –0.01 (s, 9H, Si(CH₃)₃); ¹³C-NMR (150 MHz, CDCl₃): δ 139.1, 138.7, 138.52, 138.50, 138.3, 133.8, 128.3, 128.23, 128.12, 128.05, 128.03, 127.93, 127.87, 127.7, 127.6, 127.5, 127.42, 127.37, 127.35, 127.28, 127.23, 127.1, 123.1, 169.3, 9, 97.6 (C-1 of GlcNAc), 97.5 (C-1 of Fuc a), 95.0 (C-1 of Fuc b), 78.8, 78.7, 78.4, 78.1, 78.0, 76.4, 76.3, 74.9, 74.6, 73.5, 73.3, 73.1, 73.0, 72.9, 72.8, 69.7, 67.7, 67.0, 66.7, 56.3, 29.7, 17.8, 16.6, 15.8, –1.5 (Si(CH₃)₃); MALDI-TOFMS: Calcd for C₈₀H₈₉NO₁₅SiNa [M+Na]: m/z 1354.6 Found: 1354.8.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-acetyl-$\alpha$-D-fucopyranosyl-(1→3)-[2,3,4-tri-O-acetyl-$\alpha$-D-fucopyranosyl-(1→4)]-2-acetamido-6-O-acetyl-2-deoxy-$\beta$-D-glucopyranoside (24). To a solution of 23 (60 mg, 0.05 mmol in EtOH (10 mL)) was added hydrazine monohydrate (3.3 mL, 0.07 mmol). The reaction mixture was refluxed for 3 h, then concentrated. The residue was acetylated with Ac₂O (3 mL) in pyridine (5 mL). The mixture was poured into ice-water and extracted with CHCl₃. The extract was washed sequentially with 5% HCl, NaHCO₃ and water, dried (MgSO₄), and concentrated. The solution of the product in MeOH (1 mL) and THF (1 mL) was hydrogenolysed under hydrogen in the presence of 10% Pd/C (100 mg) for 15 h at room temperature, then filtered and concentrated. The residue was acetylated with acetic anhydride (5 mL) in pyridine (7 mL). The reaction mixture was poured into ice-water and extracted with CHCl₃. The extract was washed sequentially with 5% HCl, NaHCO₃ and water, dried (MgSO₄), and concentrated. The product was purified by silica gel column chromatography using 9:1 toluene-acetone as eluent to give 24 as syrup (19 mg, 47%). [α]D²⁴ +75.2 (c 0.5 CHCl₃); ¹H-NMR (500 MHz, CDCl₃): δ 6.43 (d, 1H, NH), 5.28 (dd, 1H, H-3 of Fuc b), 5.23–5.18
(m, 3H, H-3, H-4 of Fuc a, H-4 of Fuc b), 5.13 (d, 1H, J_{1,2}=3.7 Hz, H-1 of Fuc b), 5.08–5.03 (m, 2H, H-3 of GlcNAc, H-2 of Fuc b), 5.04 (d, 1H, J_{1,2}=9.8 Hz H-1 of GlcNAc), 4.66 (d, 1H, J_{1,2}=3.7 Hz H-1 of Fuc a), 4.52 (dd, 1H, H-6a of GlcNAc), 4.43–4.36 (m, 2H, H-6b of GlcNAc, H-5 of Fuc b), 4.13–4.08 (m, 2H, H-2 of GlcNAc, H-5 of Fuc a), 3.98–3.85 (m, 1H, H-2 of Fuc a), 3.91–3.85 (m, 1H, CH₂CH₂Si(CH₃)₃), 3.60–3.55 (m, 2H, H-4 of GlcNAc, H-2 of Fuc a), 3.46–3.40 (m, 1H, CH₂CH₂Si(CH₃)₃), 2.13–1.87 (m, 21H, CH₃CO) 1.10 (d, 3H, H-6 of Fuc b) 1.07 (d, 3H, H-6 of Fuc a), 0.95–0.80 (m, 2H, CH₂CH₂Si(CH₃)₃), –0.03 (s, 9H, Si(CH₃)₃); 13C-NMR (125 MHz, CDCl₃): δ 170.5, 170.43, 170.36, 170.31, 169.9, 169.5, 169.3, 98.5 (C-1 of Fuc a), 98.2 (C-1 of Fuc b), 97.6 (C-1 of GlcNAc), 76.9, 76.8, 76.7, 74.1, 74.0, 73.7, 73.5, 71.2, 70.9, 68.3, 68.00, 67.97, 67.3, 66.5, 65.8, 65.7, 64.5, 49.3, 29.7, 23.4, 21.4, 20.9, 20.7, 20.6, 18.0, 16.0, 15.8, 15.7, 14.1, –1.5 (Si(CH₃)₃); MALDI-TOFMS: Calcd for C₃₉H₆₁NO₂₁SiNa [M+Na]+: m/z 930.3 Found: 930.5.

2-(Trimethylsilyl)ethyl α-D-fucopyranosyl-(1→3)-[α-D-fucopyranosyl-(1→4)]-2-acetamido-2-deoxy-β-D-glucopyranoside (6). Compound 6 was prepared from 24 (16 mg, 0.03 mmol) by the same method described for preparation of 3. The product was purified by Sephadex LH-20 column chromatography in 1:1 CHCl₃-MeOH to give 6 as white solid (10 mg, 93%). [α]D²⁴ +72.2 (c 0.2 CH₃OH); ¹H-NMR (600 MHz, CD₃OD): δ 4.97 (d, 1H, J_{1,2}=3.9 Hz H-1 of Fuc b), 4.66 (d, 1H, J_{1,2}=2.8 Hz H-1 of Fuc a), 4.38 (d, 1H, J_{1,2}=6.1 Hz H-1 of GlcNAc), –0.09 (s, 9H, Si(CH₃)₃); ¹³C-NMR (150 MHz, CD₃OD): δ 172.8, 101.8 (C-1 of GlcNAc), 101.6 (C-1 of Fuc a), 100.1 (C-1 of Fuc b), 79.3, 78.3, 74.8, 73.7, 73.5, 71.4, 70.1, 69.8, 68.5, 68.3, 67.6, 63.1, 54.7, 27.0, 23.1, 18.9, 16.7, –1.3 (Si(CH₃)₃); MALDI-TOFMS: Caled for C₂₅H₄₇NO₁₄SiNa [M+Na]+: m/z 636.3 Found: 636.7 [M+Na]+. HR-FABMS: Calcd for C₂₅H₄₇NO₁₄SiNa: m/z 636.266. Found m/z 636.2681 [M+Na]+.

3.2. Nitric Oxide Inhibitory Assay

J774.1 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO) and cultured at 37°C in humidified 5% CO₂/95% air. The cells were suspended in medium, plated on 96-well culture plates (Falcon) at a density of 5.0 × 10⁵ cells/mL/well, volume of 200 μL/well and allowed to adhere for 24 h. Then, the medium was replaced with fresh medium, containing LPS (1 μg/mL) from *E. coli* (Sigma) and test compounds dissolved in DMSO at various concentrations (13, 25, 50, 100 μM) were incubated for 24 h. NO production was determined by measuring the accumulation of nitrite (a stable metabolite of NO) in the culture supernatant using Griess reagent [20]. Briefly, 50 μL of the supernatant from incubates were mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylendiamine dihydrochloride in 5% H₃PO₄) and were allowed to stand for 10 minutes at room temperature. Absorbance at 550 nm was measured using a MTP-810 Microplate Reader (Corona Co.). The blank correction was carried out by subtracting the absorbance due to medium from the absorbance reading of each well. The reaction percentage was calculated as follows: % of control = [As/Ac] × 100, where As and Ac are absorbance of a run treated with LPS and a sample, and that treated with LPS alone, respectively. In this assay, N⁵-monomethyl-L-arginine (L-NMMA, IC₅₀ 32.0 μM), a non-selective nitric oxide synthase (NOS) inhibitor, was used as a positive control [21].
4. Conclusions

We have succeeded for the first time in carrying out the total syntheses of D-fucose-containing glycosphingolipids found in invertebrate species. Both the presence of a D-Fucα1-3GlcNAc-linkage and the ceramide aglycon portion resulted in a significant enhancement of their ability to inhibit NO production by LPS-induced macrophage-like J774.1 cells. The prepared glycolipids are easily-accessible target compounds in the field of carbohydrate chemistry and may serve as chemical probes to explore glycosphingolipid-mediated anti-inflammatory processes in biology and medicine.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (No. 19590011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and the (MEXT) Open Research Center project. The authors are grateful to J. Hada for providing HR-FABMS data.

References and Notes

1. Seeberger, P. H.; Werz, D. B. Synthesis and medical applications of oligosaccharides. Nature 2007, 446, 1046-1051.
2. Galonic, D. P.; Gin, D. Y. Chemical glycosylation in the synthesis of glycoconjugate antitumour vaccines. Nature 2007, 446, 1000-1007.
3. Itonori, S.; Sugita, M. Glycophylogenetic aspects of lower animals. In Comprehensive Glycoscience; Kamerling, J.P., Ed.; Elsevier Ltd.: Amsterdam, The Netherlands, 2007; Volume 3, pp. 253-284.
4. Kanaya, T.; Yagi, S.; Schweizer, F.; Takeda, T.; Kiuchi, F.; Hada, N. Synthetic studies on glycosphingolipids from Protostomia phyla: synthesis of glycosphingolipids from the parasite Schistosoma mansoni. Chem. Pharm. Bull. 2010, 58, 811-817.
5. Koizumi, A.; Hada, N.; Kaburaki, A.; Yamano, K.; Schweizer, F.; Takeda, T. Synthetic studies on the carbohydrate moiety of the antigen from the parasite Echinococcus multilocularis. Carbohydr. Res. 2009, 344, 856-868.
6. Hada, N.; Shida, Y.; Shimamura, H.; Sonooda, Y.; Kasahara, T.; Sugita, M.; Takeda, T. Synthetic studies on glycosphingolipids from Protostomia phyla: syntheses and biological activities of amphoteric glycolipids containing a phosphocholine residue from the earthworm Phretima hilgendorfi. Carbohydr. Res. 2008, 343, 2221-2228.
7. Hada, N.; Nakashima, T; Shrestha, S. P; Masui, R; Narukawa, Y; Tani, K; Takeda, T. Synthesis and biological activities of glycosphingolipid analogues from marine sponge Aplysinella rhax. Bioorg. Med. Chem. Lett. 2007, 17, 5912-5915.
8. Hada, N.; Sonoda, Y.; Takeda, T. Synthesis of a novel glycosphingolipid from the millipede, Parafontaria laminata armigera and the clusterization of the carbohydrate residue. Carbohydr. Res. 2006, 341, 1341-1352.
9. Yamamura, T.; Hada, N.; Kaburaki, A.; Yamano, K.; Takeda, T. Synthetic studies on glycosphingolipids from Protostomia phyla: total syntheses of glycosphingolipids from the parasite, Echinococcus multilocularis. Carbohydr. Res. 2004, 339, 2749-2759
10. Ohtsuka, I.; Hada, N.; Sugita, M.; Takeda, T. Synthetic studies on glycosphingolipids from Protostomia phyla: synthesis of arthro-series glycosphingolipids. *Carbohydr. Res.* **2003**, *337*, 2037-2047.

11. Ohtsuka, I.; Hada, N.; Ohtaka, H.; Sugita, M.; Takeda, T. Synthetic studies on glycosphingolipids from Protostomia phyla: synthesis of amphoteric glycolipid analogues from the porcine nematode, *Ascaris suum*. *Chem. Pharm. Bull.* **2002**, *50*, 600-604.

12. Hada, N.; Ohtsuka, I.; Sugita, M.; Takeda, T. Synthetic studies on novel fucosylated glycosphingolipids from the millipede, *Parafontaria laminata armigera*. *Tetrahedoron Lett.* **2000**, *41*, 9065-9068.

13. Koester, D. C.; Holkenbrink, A.; Werz, D. B. Recent advances in the synthesis of carbohydrate mimetics. *Synthesis* **2010**, 3217-3242.

14. Borbone, N.; Marino, S. D.; Iorizzi, M.; Zollo, F.; Debitus, C.; Ianaro, A.; Pisano, B. New glycosphingolipids from the marine sponge *Aplysinella rhax* and their potential as nitric oxide release inhibitors. *Eur. J. Org. Chem.* **2001**, 4651.

15. Xia, C.; Yao, Q.; Schümann, J.; Rossy, E.; Chen, W.; Zhu, L.; Zhang, W.; De Libero, G.; Wang, P.G. Synthesis and biological evaluation of α-galactosylceramide (KRN7000) and isoglobo trihexosylceramide (iGb3). *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2195-2199.

16. Schmidt, R. R. New methods for the synthesis of glycosides and oligosaccharides—are there alternatives to the Koenigs-Knorr method? [new synthetic methods (56)]. *Angew. Chem. Int. Ed.* **1986**, *25*, 212-235.

17. Konradsson, P.; Udodong, U. E.; Fraser-Reid B. Iodonium promoted reactions of disarmed thioglycosides. *Tetrahedron Lett.* **1990**, *31*, 4313-4316.

18. Ellervik, U.; Magnusson, G. A high yielding chemical synthesis of sialyl lewis x tetrasaccharide and lewis x trisaccharide; examples of regio- and stereodifferentiated glycosylations. *J. Org. Chem.* **1998**, *63*, 9314-9322.

19. Salvemini, D.; Korbut, R.; Vane, J. NG-Monomethyl-L-arginine inhibits release of a nitric oxide-like substance induced by E. coli lipopolysaccharide in the mouse macrophage cell line, J774. *International Congress Series* **1990**, *897*, 267-273.

20. Dirsch, V. M.; Stuppner, H.; Vollmar, A. M. The griess assay: suitable for a bio-guided fractionation of anti-inflammatory plant extracts? *Planta Med.* **1998**, *64*, 423-426.

21. Park, K. H.; Park, M. P.; Choi, S. E.; Jeong, M. S.; Kwon, J. H.; Oh, H. M.; Choi, K. H.; Seo, J. S.; Lee, W. M. The Anti-oxidative and anti-inflammatory effects of caffeoyl derivatives from the roots of *Aconitum koreanum* R. RAYMOND. *Biol. Pharm. Bull.* **2009**, *32*, 2029-2033.

© 2011 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).