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Synthesis and biological activities of glycosphingolipid analogues from marine sponge *Aplysinella rhax*

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Abstract—A novel glycosphingolipid, β-D-GalNAc*p*(1 → 4)[α-D- Fuc*p*(1 → 3)]-β-D-GlcNAc*p*(1→)Cer (**1**), isolated from the marine sponge *Aplysinella rhax*, has a unique structure, with D-fucose and *N*-acetyl-D-galactosamine attached to a reducing-end *N*-acetyl-D-glucosamine through an α1 → 3 and β1 → 4 linkage, respectively. We synthesized glycolipid analogues carrying a 2-branched fatty alkyl residue or a 2-trimethylsilyl ethyl residue in place of ceramide (**2** and **3**), non-natural type trisaccharide analogue containing an L-fucose residue (**4**), and other analogues (**5** and **6**). Among these prepared compounds, **2** showed the most potent nitric oxide (NO) production inhibitory activity against LPS-activated J774.1 cells. In addition, their structure–activity relationships were established.

The structure and the biological functions of many gangliosides have been widely investigated and are reported in various reviews.[1,2] However, in our continuing systematic studies of the role and biological functions of glycolipids in various invertebrate animal species that do not have gangliosides, we have synthesized some novel glycolipid derivatives found in these invertebrates.[3–8] Recently, Zollo et al.[9] isolated and characterized a novel neutral glycosphingolipid (**1**, Fig. 1) from the marine sponge *Aplysinella rhax*, the carbohydrate structure of which features a D-fucose and an *N*-acetyl-D-galactosamine attached to a reducing-end *N*-acetyl-D-glucosamine through an α1 → 3 and a β1 → 4 linkage, respectively. This was the first report on glycolipids containing D-fucose. This compound, β-D-GalNAc*p*(1 → 4)[α-D-Fuc*p*(1 → 3)]-β-D-GlcNAc*p*(1→)Cer (**1**), has structural variations at the ceramide parts. Glycosphingolipids found in nature are classified into many types on the basis of their basic carbohydrate structure; these types include the globo-, lacto-, ganglio-, mollu-, arthro-, and gala series.[10] However, to the best of our knowledge, the carbohydrate structure, GalNAcβ1 → 4GlcNAcβ1→, represents a new type of glycolipid core structure. Furthermore, these glycolipids have been also found to exhibit significant inhibitory activity on LPS-induced NO release by J774.1 macrophages.[9] Therefore, in order to investigate the strength of these compounds, inhibitory against nitric oxide release, we synthesized the trisaccharide analogues **2** and **3**, containing a 2-branched fatty alkyl residue and a 2-(trimethylsilyl)ethyl (TMS-Et) residue, respectively, in place of ceramide; a non-natural-type trisaccharide analogue with an L-fucose residue (**4**); and other analogues **5** and **6** (Fig. 1).

The target glycolipid analogues were prepared by a conventional synthetic pathway (Scheme 1). Glycosyl acceptor **7** was prepared from the known compound 2-(trimethylsilyl)ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranoside[11] by deacetylation, benzylidenation, chloroacetylation, and reductive ring-opening of the benzylidene acetal to afford compound **7**. Glycosylation of **7** with **8**, which was prepared from 1,3,4,6,tetra-*O*-acetyl-2-deoxy-2-(2,2,2- trichloroethoxycarbonylamino)-β-D-galactopyranose,[11] was carried out in the presence of TMSOTf and 4Å molecular sieves (4A MS) in CH_2Cl_2. The reaction produced the desired disaccharide **9** in 80% yield, as evidenced by ^{1}H NMR spectroscopy (H-1′, 4.25 ppm, *J* 8.5 Hz).[12] The chloroacetyl group in **9** was removed by the action of thiourea to afford **10**, which was used in the subsequent coupling reaction. The D-fucopyranosyl donor **11**[13] was prepared in the same way as the L-fucopyranosyl donor phenyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside, as reported by Hasegawa

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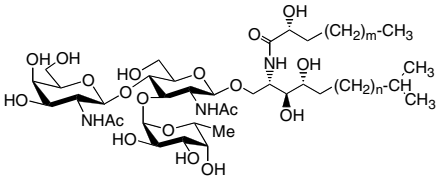


β-D-GalNAc*p*(1-4)-[α-D-Fuc*p*(1-3)]-β-D-GlcNAc*p*(1-)Cer **1**

natural products

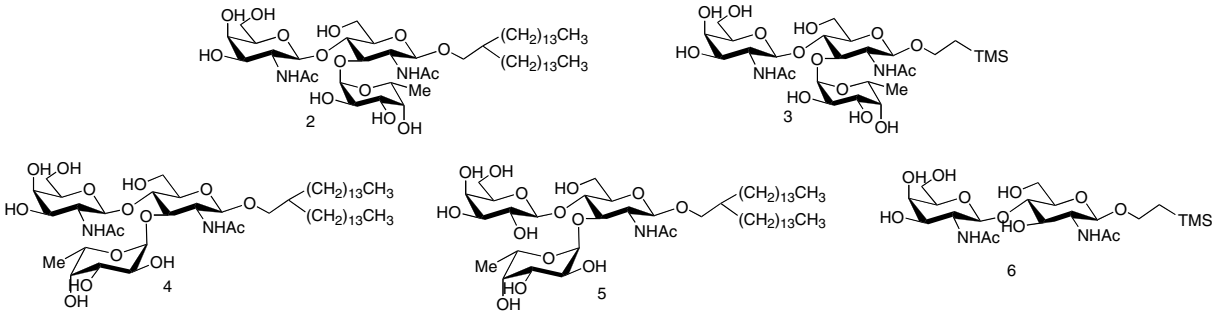
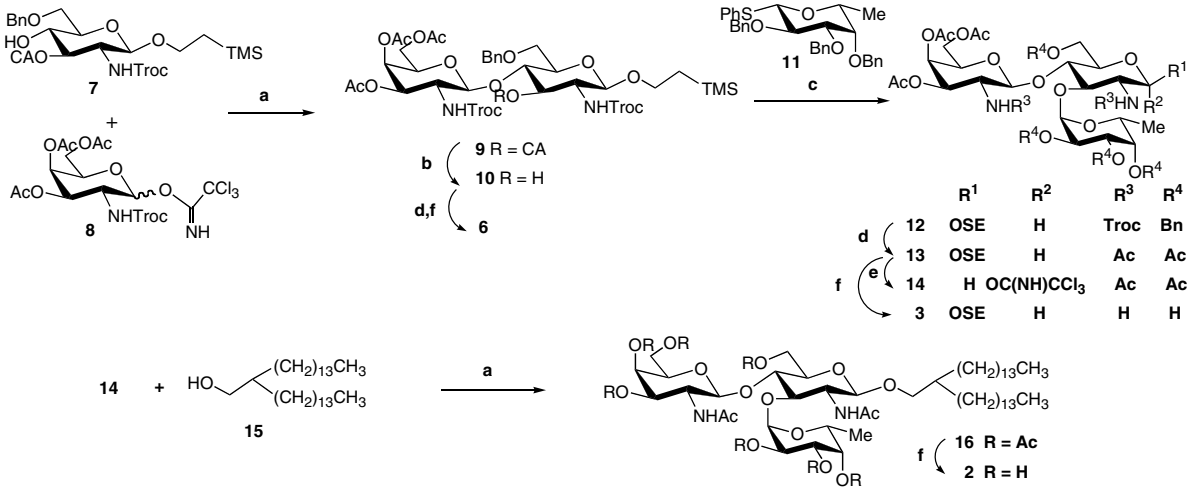
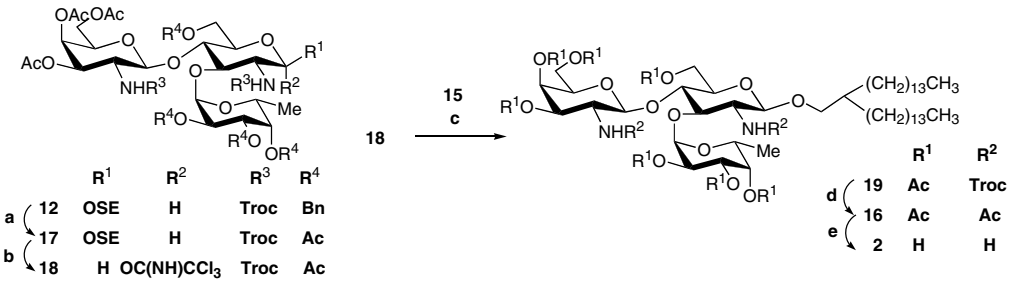


Figure 1. Target analogue compounds.



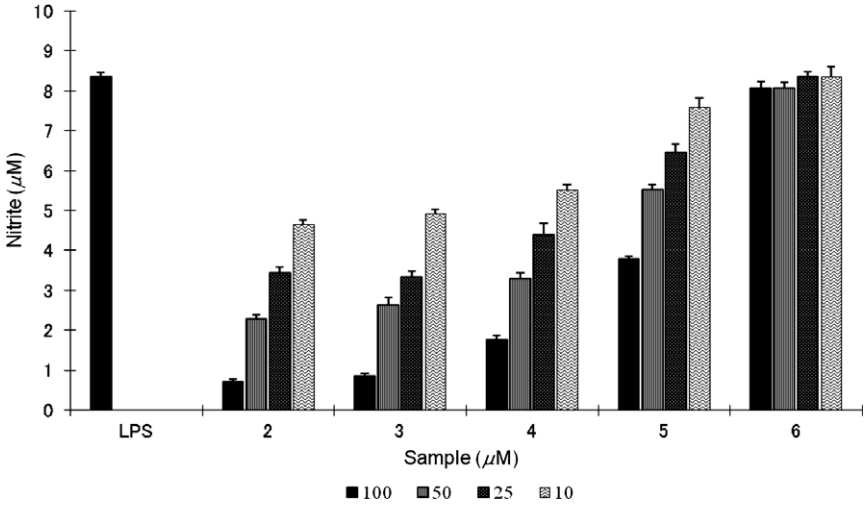
Scheme 1. Reagents: (a) TMSOTf, 4A MS, CH_2Cl_2, **9**: 80%, **16**: 12%; (b) thiourea, Pyr.–EtOH, 77%; (c) NIS, TfOH, MS4A CH_2Cl_2, 89%; (d) i—Zn–AcOH; ii—Ac_2O–Pyr.; iii—H_2, Pd–C, MeOH–AcOH; iv—Ac_2O–Pyr., 71% over four steps; (e) i—TFA, CH_2Cl_2; ii—CCl_3CN, DBU, CH_2Cl_2, 64%; (f) NaOMe, 1,4-dioxane–MeOH,**2**:86%, **3**: 94%.

et al.[14] Fucosyl donor **11** was then allowed to react with disaccharide acceptor **10** in the presence of *N*-iodosuccinimide (NIS), trifluoromethanesulfonic acid (TfOH),[15] and 4A MS in dichloromethane at −60 °C to afford the desired trisaccharide **12** (89%), which contains a newly introduced α-glycosidic linkage; this was confirmed by ^{1}H NMR spectroscopy (H-1″, 4.97 ppm, *J* 3.7 Hz).[16] Removal of the NTroc-protecting group in **12** was carried out via treatment with zinc powder in acetic acid, and the resulting crude amine was *N*-acetylated with acetic anhydride. Hydrogenation of the *N*-acetylated compound over a catalyst of 10% palladium on charcoal in methanol-acetic acid and subsequent acetylation gave the per-*O*-acetylated trisaccharide **13**. Complete deprotection of disaccharide derivative **10** and trisaccharide **13** were carried out to give analogue compounds **6** (without fucose) and **3** (with TMS-Et spacer), respectively. Next, compound **13** was converted into the trichloroacetimidate derivative **14**, which was then glycosylated with 2-tetradecylhexadecan-1-ol (**15**)[17] in the presence of trimethylsilyltrifrate (TMSOTf)[18] to give the desired β-glycoside **16** (12%). Finally, removal of all acetylated groups under basic conditions gave the target molecule **2**. Unfortunately, due to the formation of several side products, the imidate donor **14** gave a poor yield on coupling with **15**. Therefore, 2-Troc-protected imidate donor **18** was used instead of **14** (Scheme 2). Hydrogenolytic removal of the benzyl group of **12** over Pd–C in MeOH–AcOH, followed by removal of the TMS-Et group, yielded imidate donor **18**. Coupling of **18** with **15** in the presence of TMSOTf afforded the desired β-glycoside **19** in 40% yield. Treatment of **19** with zinc



Scheme 2. Reagents: (a) i—H_2, Pd–C, MeOH–AcOH; ii—Ac_2O–Pyr., 60%,; (b) i—TFA, CH_2Cl_2; ii—CCl_3CN, DBU, CH_2Cl_2, 92%; (c) TMSOTf, MS4A CH_2Cl_2, 40%; (d) i—Zn–AcOH; (ii) Ac_2O-Pyr., 58%; (e) NaOMe, 1,4-dioxane–MeOH, 94%.

Table 1. Inhibitory effects of **2**, **3**, **4**, **5**, and **6** on NO production in LPS-activated J774.1 cells



Each data represents the mean ± SD for quadruplet experiments.

powder in acetic acid cleaved the Troc group and subsequent *N*-acetylation gave **20**. Finally, removal of the *O*-acetyl groups of **20** under basic conditions followed by column chromatography using a Sephadex LH-20 gave the target glycolipid analogue **2** (Scheme 2). The structure of **2**, **3**, and **6** was demonstrated by ^{1}H NMR and MALDI-TOF-MS spectrometry.[19] Compound **4** was prepared by an analogous method using an L-fucose derivative instead of D-fucose.[20] Compound **5**, with a Le^X glycan structure, was also synthesized in a same manner, replacing *N*-acetyl-D-galactosamine with D-galactose.[21]

Nitric oxide (NO), which is naturally synthesized by a family of enzymes known as NO-synthase (NOS), is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes. Two forms of NOS are recognized: constitutive isoforms (endothelial NOS and neuronal NOS) and an inducible isoform for which mRNA translation and protein synthesis are required. When certain cells are activated by specific proinflammatory agents such as endotoxin, tumor necrosis factor (TNF), interferon-gamma (IFN-γ), or interleukin-1 (IL-1), NO is produced by inducible NOS (iNOS). The NO thus produced acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activity.[22] However, excessive production has detrimental effects on many organ systems of the body, which can lead to tissue damage and even to fatal development (septic shock).[23]

The inhibitory effect of glycosphingolipid (**1**) has been evaluated and reported in previous papers.[9] However, in order to reveal the effect of differences in carbohydrate structure on biological function, we examined inhibition of LPS-induced NO_2^- release by the synthesized compounds **2**–**6** (Table 1). The inhibition assays for **2**, **3**, and **6** suggested that the presence of fucose enhances the inhibitory effect, and that the aglycon moiety is not important in this process. In addition, comparison of the results obtained for **2**, **4**, and **5** revealed that the presence of D-fucose and GalNAc enhances inhibitory activity.

Acknowledgments

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12. Analytical data for the compound **9**: $[\alpha]_D^{23}$ −19.4° (*c* 2.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 7.37–7.49 (m, 5H, Ph), 5.23 (d, 1H, $J_{3,4}$ = 3.7 Hz, H-4′), 5.19 (dd, 1H, *J* = 9.2 Hz, 10.4 Hz, H-3), 5.13 (d, 1H, $J_{2,NH}$ = 10.4 Hz, NH), 4.83 (d, 1H, *J* = 12.2 Hz, benzyl methylene), 4.63–4.76 (m, 6H, H-3′, $CH_2CCl_3 \times 2$), 4.51 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1), 4.37 (d, 1H, *J* = 12.2 Hz, benzyl methylene), 4.25 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1′), 4.08 (dd, 2H, *J* = 14.7 Hz, *J* = 19.9 Hz, $ClCH_2CO$), 4.05–4.12 (m, 1H, H-6′a), 3.89–4.00 (m, 3H, H-6′b, H-4, $CH_2CH_2Si(CH_3)_3$), 3.74 (br. t, 1H, H-6a), 3.50 ∼ 3.66 (m, 5H, H-2, H-6b, H-2′, H-5′, $CH_2CH_2Si(CH_3)_3$), 3.47 (br. d, 1H, H-5), 2.11, 2.08, 1.95 (each s, 3H, Ac), 0.87–0.95 (m, 2H, $CH_2CH_2Si(CH_3)_3$), −0.012 (s, 9H, $Si(CH_3)_3$). ^{13}C NMR ($CDCl_3$): δ 170.4 (NHCO), 170.1 (NHCO), 167.1 ($COCH_2Cl$), 153.8, 137.4, 129.3, 129.1, 128.8, 100.4 (C-1), 100.2 (C-1′), 95.5 ($CCl_3 \times 2$), 74.5 ($CH_2CCl_3 \times 2$), 74.2 (C-5), 74.0 (C-3), 73.7 (benzyl methylene), 70.5 (C-5′), 69.8 (C-3′), 67.5 (C-4′), 67.0 (C-6), 66.2 (C-4′), 61.2 (C-6′), 56.2 (C-2), 52.4 (C-2′), 40.8 (CH_2Cl), 20.7, 20.7, 20.6, 18.1, −1.38. MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{38}H_{52}Cl_7N_2O_{17}Si$: 1104.1; found: *m/z* 1104.3.
13. Analytical data for the compound **11**: $[\alpha]_D^{23}$ +5.8° (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 7.53–7.18 (m, 20H, Ph × 4), 5.02–4.66 (m, 6H, benzyl methylene × 3), 4.60 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-1), 3.93 (t, 1H, $J_{2,3}$ = 9.7 Hz, H-2), 3.63 (d, 1H, $J_{3,4}$ = 2.4 Hz, H-4), 3.59 (m, dd, H-3), 3.52 (q, 1H, H-5), 1.26 (d, 3H, $J_{5,6}$ = 6.1 Hz, H-6); ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.7, 138.4, 138.3, 134.3, 131.5, 128.7, 128.4, 128.3, 128.1, 127.9, 127.6, 127.5, 127.4, 126.9, 87.5, 84.5, 77.1, 76.6, 75.5, 74.6, 72.8, 17.3; MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{33}H_{34}O_4SNa$: 549.2; found: 549.4.
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16. Analytical data for the compound **12**: $[\alpha]_D^{23}$ +15.2° (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 4.97 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of fuc), 4.52 (br. d, 1H, H-1 of GlcNAc), 4.46 (br. s, 1H, H-1, of GalNAc). ^{13}C NMR (125 MHz, $CDCl_3$): δ: 170.5, 170.3, 170.2, 170.1, 154.2, 138.8, 138.7, 138.5, 138.4, 138.33, 138.28, 138.2, 129.0, 128.7, 128.6, 128.4, 128.3, 128.2, 128.16, 128.13, 128.0, 127.93, 127.85, 127.7, 127.6, 127.5, 127.3, 127.2, 127.1, 125.2, 100.2, 99.6, 98.8, 95.8, 95.3, 79.4, 77.6, 76.4, 76.2, 75.2, 74.8, 74.5, 74.4, 73.5, 73.4, 73.31, 73.25, 73.2, 72.8, 70.8, 70.6, 70.3, 69.6, 67.3, 66.7, 66.2, 61.3, 61.1, 54.2, 52.8, 52.5, 21.4, 20.62, 20.59, 20.5, 18.1, 17.9, 17.2, 16.6, 16.5, −0.1, −1.4. MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{63}H_{78}Cl_6N_2O_{20}SiNa$: 1443.3; found: 1443.1.
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19. Analytical data for the compound **2**: $[\alpha]_D^{23}$ −21.4° (*c* 0.1, 1:1 $CHCl_3$-MeOH); ^1H NMR (500 MHz, $CDCl_3$-CD_3OD): δ 5.15 (d, 1H, *J* = 3.6 Hz, H-1 of Fuc), 4.59 (d, 1H, *J* = 8.3 Hz, H-1 of GlcNAc), 4.32 (d, 1H, d, 1H, *J* = 8.0 Hz, H-1 of GalNAc). ^{13}C NMR (125 MHz, $CDCl_3$-CD_3OD): δ 173.5, 172.6, 102.7, 101.8, 101.3, 80.7, 76.4, 76.1, 75.3, 73.6, 73.0, 72.3, 71.1, 70.3, 69.6, 68.0, 62.4, 61.3, 55.7, 54.2, 49.6, 38.9, 32.6, 31.8, 31.5, 30.8, 30.7, 30.3, 30.0, 27.5, 27.3, 23.4, 23.3, 23.0, 16.5, 14.4. MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{52}H_{98}N_2O_{15}$-Na: 1014.7; found: 1014.4. Analytical data for the compound **3**: $[\alpha]_D^{23}$ +8.5° (*c* 0.3, MeOH); ^1H NMR (500 MHz, $CDCl_3$-CD_3OD): δ 5.25 (d, 1H, *J* = 3.7 Hz, H-1 of Fuc), 4.58 (d, 1H, *J* = 8.0 Hz, H-1 of GlcNAc), 4.38 (d, 1H, d, 1H, *J* = 8.0 Hz, H-1 of GalNAc). MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{27}H_{50}N_2O_{15}SiNa$: 693.3; found: 693.5. Analytical data for the compound **6**: ^1H NMR (500 MHz, CD_3OD): δ 4.45 (d, 1H, *J* = 8.5 Hz, H-1 of GlcNAc), 4.37 (d, 1H, d, 1H, *J* = 8.5 Hz, H-1 of GalNAc).). MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{21}H_{40}N_2O_{11}SiNa$: 547.2; found: 547.7.
20. Analytical data for the compound **4**: $[\alpha]_D^{23}$ −54.3° (*c* 0.1, 1:1 $CHCl_3$-MeOH); ^1H NMR (500 MHz, $CDCl_3$-CD_3OD): MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{52}H_{98}N_2O_{15}Na$: 1014.7; found: 1014.4.
21. Analytical data for the compound **5**: $[\alpha]_D^{23} = -33.9°$ (*c* 0.3, 1:1 $CHCl_3$-MeOH); ^1H NMR (500 MHz, $CDCl_3$-CD_3OD): δ 5.01 (d, 1H, *J* = 3.6 Hz, H-1 of Fuc), 4.46 (d, 1H, *J* = 8.3 Hz, H-1 of Gal), 4.44 (d, 1H, d, 1H, *J* = 8.0 Hz, H-1 of GlcNAc). MALDI-TOF-MS: *m/z* $[M+Na]^+$ calcd for $C_{50}H_{95}NO_{15}Na$: 972.7; found: 972.8.
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