Glycoconj J (2008) 25:545–553
DOI 10.1007/s10719-008-9111-2

A highly efficient construction of GM1 epitope tetrasaccharide and its conjugation with KLH

Takeru Yoshikawa · Yuki Kato · Nobuhiro Yuki · Tomio Yabe · Hideharu Ishida · Makoto Kiso

Received: 19 July 2007 / Revised: 7 December 2007 / Accepted: 15 January 2008 / Published online: 15 February 2008

Abstract GM1 epitope tetrasaccharide was synthesized by a condensation of sialyl-α(2–3)-gal acceptor and gal-β(1–3)-GalN donor in a highly efficient manner. After introduction of mercaptohexanol to the tetrasaccharide, it was coupled to maleimide-activated KLH carrier protein to give the desired GM1 epitope-KLH conjugate.

Keywords Lipo-oligosaccharide · GM1 · Guillain–Barré syndrome · Keyhole limpet hemocyanin

Introduction

Gangliosides are a large family of glycosphingolipids, predominantly distributed on the cell-surface membrane and anchored in the external leaflet of the lipid bilayer by a ceramide moiety. Sialylated oligosaccharides are exposed extracellularly. GM1 is a major ganglioside in the peripheral nervous system as well as in the central nervous system [1]. Guillain–Barré syndrome (GBS) is the most common cause of acute neuromuscular paralysis in developed countries [2]. One third of GBS patients develop after enteric infection by a Gram-negative bacterium *Campylobacter jejuni*, who have IgG autoantibodies against self ganglioside GM1. A series of studies has elucidated the molecular pathogenesis: (1) *C. jejuni* strains isolated from GBS patients carried lipo-oligosaccharides (LOS) bearing GM1-like structures, indicating the presence of molecular mimicry [3]; (2) rabbits sensitized with GM1-like LOS as well as GM1 developed anti-GM1 IgG antibodies and flaccid limb weakness [4, 5]; (3) anti-GM1 IgG antibodies produced complement-mediated disruption of sodium channel clusters in the peripheral nerves, causing muscle weakness [6].

The GBS model induced by sensitization of GM1 or GM1-like LOS with rabbits is helpful for developing new treatments [4, 5, 7], but higher induction rate and less inoculation time are required. Unconjugated *keyhole limpet hemocyanin* (KLH) with GM1 or GM1-like LOS was inoculated in the disease model, but GM1 conjugation with KLH as a carrier protein should be useful to obtain the disease model more efficiently.

In this report, we describe a highly efficient construction of GM1 epitope, and its conjugation with KLH as a carrier protein, to supply the material in an adequate amount for animal study.

Results and discussion

In the first part of this study, we describe an efficient construction of GM1 epitope tetrasaccharide, which can be

T. Yoshikawa · Y. Kato · H. Ishida (✉) · M. Kiso
Department of Applied Bio-organic Chemistry, Gifu University, Gifu 501-1193, Japan
e-mail: ishida@gifu-u.ac.jp

M. Kiso
e-mail: kiso@gifu-u.ac.jp

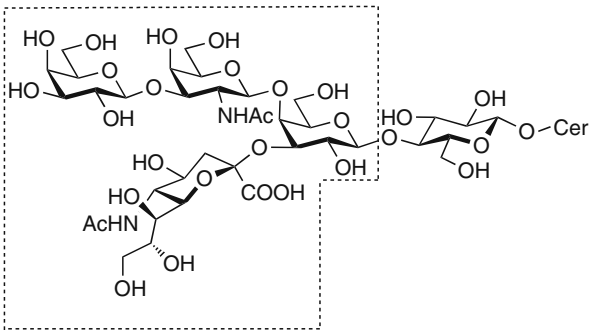
T. Yabe
Department of Food Science, Gifu University, Gifu 501-1193, Japan

H. Ishida · M. Kiso
CREST, Japan Science and Technology Agency (JST), Tokyo, Japan

N. Yuki
Department of Neurology and Research Institute for Neuroimmunological Diseases, Dokkyo Medical University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan

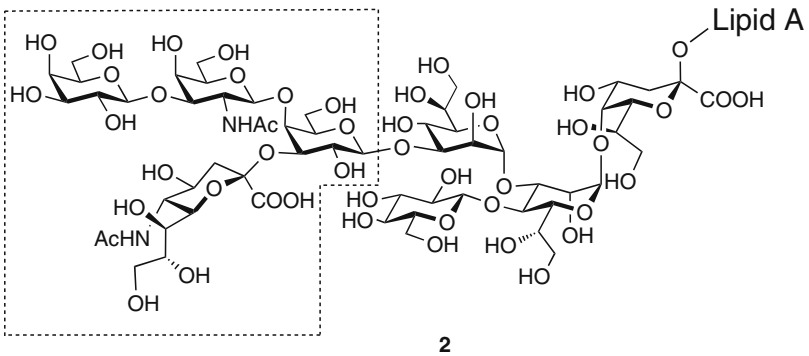
divided into *N*-acetylgalactosaminyl-β(1–4)-galactose derivative **5** and sialyl-α(2–3)-galactose derivative **6** by retrosynthetic analysis (Fig. 1). The disaccharide donor **5** was obtained by the deprotection of 4,6-*O*-di-*tert*-butylsilylene group followed by acetylation of known, phenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4,6-*O*-di-*tert*-butylsilylene-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-galactopyranoside (**4**) [8], which was synthesized by the glycosylation of readily accessible phenylthioglycoside of 2-*N*-Troc galactosamine with the commercially available per-*O*-acetylated galactose derivative. The disaccharide acceptor **6** was obtained as crystals [9] by the coupling of known *N*-Troc sialic acid thioglycoside donor and suitably protected *p*-methoxyphenyl galactoside (Scheme 1).

Glycosylation of **6** with **5** in dichloromethane for 1.5 h at −40°C in the presence of *N*-iodosuccinimide, trifluoromethanesulfonic acid and powdered 4 Å molecular sieves gave an 87% yield of the desired tetrasaccharide **7**. The stereochemistry of the newly formed glycosidic linkage was not decided at this stage, which was clearly demonstrated to be β after conversion into **18**, $J_{1,2}$=7.8 Hz. It is of worth to note that the tetrasaccharide was obtained in a large scale up to 10 g. The selective deprotection of Troc group of **7** with Zn–Cu complex acetic acid/dichloroethane [10] gave a free amine **8**, which, on successive treatment with acetic



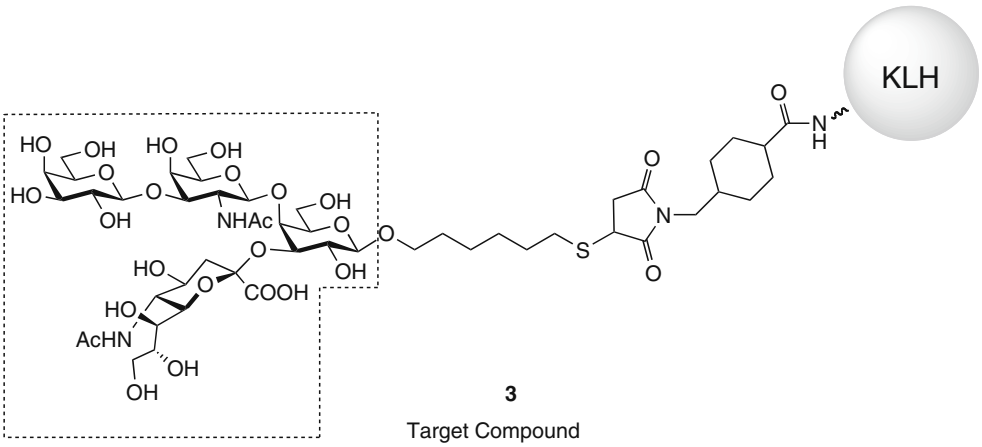
1

Ganglioside GM1



2

C. jejuni LOS

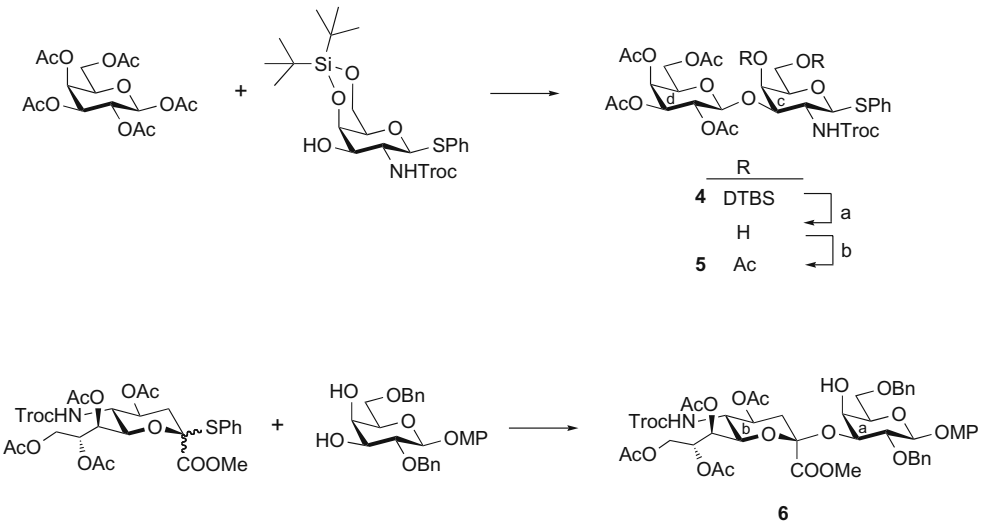


3

Target Compound

Fig. 1 Structures of GM1 epitope and GM1 epitope-KLH conjugate

Scheme 1 *a* TBAHF, r. t.; *b* Ac_2O, DMAP, pyr., r. t., 94%



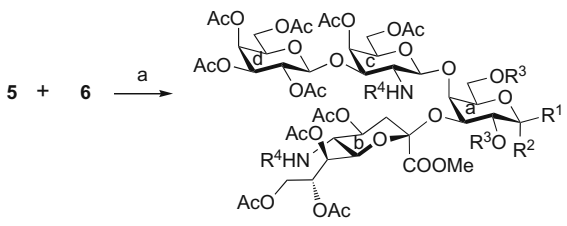
anhydride in pyridine afforded the corresponding acetamide **9**. Hydrogenolytic removal of the benzyl group in **9** and the following benzoylation gave **11**. Compound **11** was then transformed into the corresponding trichloroacetimidate **13** by selective deblocking of the *p*-methoxyphenyl group with ceric ammonium nitrate and subsequent imidate formation (Scheme 2). As a result, the tetrasaccharide **7** was converted into the corresponding glycosyl donor in 54% yield by the protecting group manipulation in seven steps.

In the second part of this work, we describe a conjugation of GM1 with and KLH. We used MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinamide ester), a heterobifunctional reagent, which cross-links thiol groups with amino groups [11]. Since maleimide-activated KLH is commercially available, we tried to introduce thiol function into the glycan part. 6-(*Tert*-butyldithio)hexanol (**16**) was prepared from 6-mercaptohexanol by a treatment with methoxycarbonylsulfenyl chloride [12] to give an asymmetrical disulfide **15** in 95%, which was transformed into the desired spacer part **16** by a treatment with 2-methyl-2-propanthiol in 99% yield (Scheme 3). The coupling of the glycosyl imidate **13** and the alcohol **16** was accomplished by a treatment with trimethylsilyl trifluoromethanesulfonate to give **17** in 87% yield. The stereochemistry of the new glycoside was again decided after conversion into intermediate **18** to be β, $J_{1,2}$=7.6 Hz. *O*-Deacylation of **17** with sodium methoxide in methanol, and subsequent saponification of the methyl ester group, yielded the key intermediate **18**.

Coupling of GM1 epitope with KLH was performed by a two-step procedure. First, the disulfide **18** was treated with tris(2-carboxyethyl)phosphine [13] to give the thioderivative **19**, which was then coupled to the maleimide-activated carrier protein MA-KLH [14] to give the GM1 epitope-KLH conjugate (Scheme 4). It is worth to note that the symmetric disulfide was not formed in sequence of the reactions. The hapten-carrier ratio (see "Experimental" section) was quantified in the range of 18:1 ratio. Inoculation of a rabbit with 500 μg of the conjugate two times effectively induced the production of anti-GM1 IgG antibodies, although it did not induce the development of paralysis (Phongsisay and Yuki, unpublished data).

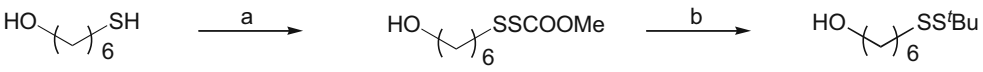
In conclusion we have succeeded in a highly efficient procedure to construct GM1 epitope tetrasaccharide, which makes the synthesis a variety of GM1-containing glycoconjugates really practical, including natural GM1 ganglioside as well as the derivatives conjugated with useful aglycons.

Scheme 2 *a* NIS, TfOH, MS 4 Å, CH_2Cl_2, −40°C, 87%; *b* Zn (Cu), AcOH, $(CH_2Cl)_2$, r. t.; *c* Ac_2O, DMAP, pyr., r. t., 85% (two steps); *d* H_2, $Pd(OH)_2$, 1,4-dioxane, 40°C, 94%; *e* Bz_2O, DMAP, pyr., r. t., 96%; *f* CAN, MeCN, toluene, H_2O, 0°C, 88%; *g* CCl_3CN, DBU, CH_2Cl_2, 0°C, 93%



	R^1	R^2	R^3	R^4	
7	OMP	H	Bn	Troc	b
8	OMP	H	Bn	H	c
9	OMP	H	Bn	Ac	d
10	OMP	H	H	Ac	e
11	OMP	H	Bz	Ac	f
12	[OH , H]		Bz	Ac	g
13	H	$OC(NH)CCl_3$	Bz	Ac	

Scheme 3 *a* $CH_3OC(O)SCl$, CH_2Cl_2, r. t., 96%; *b* $(CH_3)_3CSH$, MeOH, r. t., 89%



14 **15** **16**

Experimental section

General methods

^1H and ^{13}C NMR spectra were recorded with Varian INOVA 400 and 500 or JEOL JNM-ECA-500 and 600. Chemical shifts are expressed in ppm (δ) relative to the signal of Me_4Si adjusted to 0.00 ppm. MALDI-TOF MS spectra were recorded in positive ion and negative ion mode on a Bruker Autoflex with the use of α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. Specific rotations were determined with a HORIBA SEPA-300 polarimeter at 25°C. Molecular sieves were purchased from Wako Chemical Inc. and dried at 300°C for 2 h in muffle furnace prior to use. Solvents as reaction media were dried over molecular sieves and used without purification. TLC analysis was performed on Merck TLC (silica gel $60F_{254}$ on glass plate). Silica gel (80 mesh and 300 mesh) manufactured by Fuji Silysia Co. was used for flash column chromatography. Solvent systems in chromatography were specified in *v/v*. Evaporation and concentration were carried out *in vacuo*.

Phenyl

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4,6-di-*O*-acetyl-2-deoxy-1-thio-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-galactopyranoside (**5**).

A solution of **4** (4.25 g, 4.63 mmol) in 1.0 M tributylamine hydrofluoride (TBA·HF; 45.0 ml) was stirred for 1 h 45 min at room temperature under argon atmosphere. The mixture was washed with water, 2 M hydrochloric acid, and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (60:1 chloroform–methanol) gave a residue with a trace amount of impurity. The residue was treated with pyridine (41.8 ml) and acetic anhydride (1.3 ml) and 4-dimethylaminopyridine (DMAP; 56.0 mg) for 18 h under argon atmosphere, then mixture was concentrated, and the residue was diluted with chloroform, successively washed with cold 2 M hydrochloric acid, water, saturated Na_2CO_3 and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (130:1 chloroform–methanol) of the residue gave **5** (3.93 g, 94%).

$[\alpha]_D$=+11.0° (*c* 1.1, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$): δ 1.96–2.14 (m, 18H, AcO), 3.66 (dd, 1H, $J_{1,2}$= 10.3 Hz, $J_{2,3}$=9.6 Hz, H-2c), 3.87–3.89 (m, 2H, H-5c,5d), 4.04–4.18 (m, 4H, H-6c,6′c,6d,6d′), 4.28 (d, 1H, $J_{2,3}$= 9.6 Hz H-3c), 4.64 (d, 1H, $J_{1,2}$=7.6 Hz, H-1d), 4.72–4.80 (dd, 2H, CH_2CCl_3), 4.93–4.96 (dd, 1H, $J_{2,3}$=8.6 Hz, $J_{3,4}$= 3.4 Hz, H-3d), 5.07–5.13 (m, 2H, $J_{1,2}$=10.3 Hz, H-1c, H-2d), 5.33 (d, 1H, $J_{3,4}$=3.4 Hz, H-4d), 5.43 (d, 1H, $J_{3,4}$= 3.5 Hz, H-4c), 5.63 (d, 1H, $J_{2,NH}$=8.2 Hz, NH), 7.29–7.51 (m, 5H, Ph).

^{13}C NMR (150 MHz, $CDCl_3$): δ 20.6, 20.7, 20.7, 20.8, 52.9, 61.1, 62.9, 66.9, 68.8, 68.9, 70.8, 74.4, 75.2, 76.1, 86.1, 95.5, 101.1, 128.0, 129.0, 132.3, 132.8, 153.8, 169.4, 169.9, 170.1, 170.4, 170.5, 170.6.

MALDI-TOF MS

Calculated for $C_{33}H_{40}Cl_3NO_{17}S$

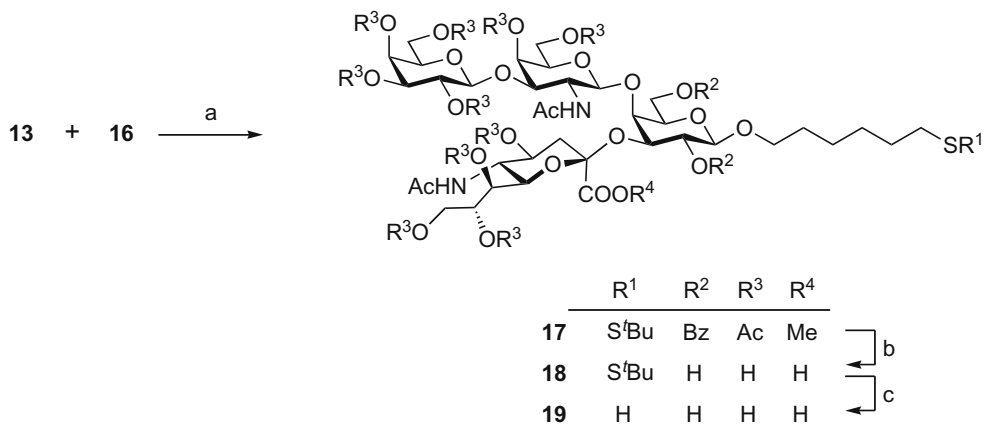
$[M+Na]^+$=882.10 $[M+K]^+$=898.07

Found=882.12, 898.10

p-Methoxyphenyl

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4,6-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-

Scheme 4 *a* TMSOTf, MS 4 Å, CH_2Cl_2, −20°C, 87%; *b* MeONa, MeOH, reflux, 85%; *c* tris(2-carboxyethyl)phosphine HCl, H_2O, r. t., 80%



	R^1	R^2	R^3	R^4	
17	S^{t}Bu	Bz	Ac	Me	b
18	S^{t}Bu	H	H	H	c
19	H	H	H	H	

β-D-galactopyranosyl-(1→4)-{methyl 4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-(2,2,2-trichloroethoxycarbonylamino)-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate-(2→3)}-2,6-di-*O*-benzyl-β-D-galactopyranoside (**7**).

To a mixture of the donor **5** (2.10 g, 2.48 mmol) and the acceptor **6** (1.77 g, 1.65 mmol) in dichloromethane (25 ml) was added molecular sieves 4 Å (3.90 g), and the mixture was stirred for 30 min at room temperature under argon atmosphere, then cooled to −40°C. *N*-Iodosuccinimide (NIS; 835 mg, 3.71 mmol) and trifluoromethanesulfonic acid (TfOH; 22 μl, 250 μmol) were added, and this was stirred for 1.5 h at −40°C. The insoluble materials were filtered off and washed with chloroform. The filtrate and washings were combined, successively washed with saturated Na_2CO_3, saturated $Na_2S_2O_3$ and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (100:1 chloroform–methanol) of the residue gave **7** (2.58 g, 87%).

$[\alpha]_D$=−2.8° (*c* 1.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.88–2.17 (m, 21H, AcO, H-3b$_{ax}$), 2.44 (dd, 1H, $J_{3ax,3eq}$=13.8 Hz, $J_{3eq,4}$=5.0 Hz, H-3b$_{eq}$), 3.40 (m, 1H, H-5b), 3.65–3.86 (m, 6H, H-5a,6a,6′a,6b,2c,6c), 3.77 (s, 6H, COOMe, MeO), 3.87–4.17 (m, 9H, H-2a,4a, 9b,9′b,3c,5c,5d,6d,6′d), 3.93 (s, 2H, *CH*$_2$Ph), 4.23 (dd, 1H, $J_{5,6}$=5.6 Hz, J6,6 $J_{6,6'}$=10.9 Hz, H-6c), 4.45 (d, 1H, CO*CH*$_2$Cl$_3$), 4.54 (d, 2H, *CH*$_2$Ph), 4.62 (d, 1H, CO*CH*$_2$Cl$_3$), 4.68–4.69 (m, 2H, H-1a,1c), 4.87–4.93 (m, 4H, H-3a,7b,4c, NHb), 4.94–4.99 (m, 2H, CO*CH*$_2$Cl$_3$), 5.12 (dd, 1H, $J_{1,2}$=8.1 Hz, $J_{2,3}$=10.5 Hz, H-2d), 5.27–5.37 (m, 5H, H-4b,8b,1d,3d,4d), 5.90 (d, 1H, NHc), 6.87–7.01 (m, 4H, Ph), 7.22–7.34 (m, 10H, Ph).

^{13}C NMR (125 MHz, $CDCl_3$): δ 20.8, 21.0, 21.3, 51.8, 53.6, 54.4, 55.8, 60.7, 61.8, 62.6, 66.8, 67.0, 68.1, 68.3, 68.9, 70.1, 70.7, 70.8, 71.4, 73.9, 74.1, 74.6, 75.6, 94.6, 95.6, 99.4, 101.3, 101.6, 103.1, 114.7, 118.8, 127.8, 127.9, 128.5, 128.6, 138.5, 151.6, 184.2, 154.5, 155.5, 168.9, 169.6, 169.8, 170.0, 170.3, 170.6, 170.7.

MALDI-TOF MS

Calculated for $C_{75}H_{90}Cl_6N_2O_{37}$

$[M+Na]^+$=1,843.33 $[M+K]^+$=1,859.43

Found=1,843.30, 1,859.30

p-Methoxyphenyl

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)}-2,6-di-*O*-benzyl-β-D-galactopyranoside (**9**).

To a solution of **7** (3.04 g, 1.69 mmol) in dichloroethane (16.8 ml) were added acetic acid (50.4 ml) and Zn–Cu complex (30.4 g), and the mixture was stirred for 3.5 h at room temperature. The insoluble materials were filtered off and washed with chloroform. The filtrate and washings were combined, successively washed with water, saturated Na_2CO_3 and brine, dried (Na_2SO_4) and concentrated to give **8** with a trace amount of impurity. The residue was treated with pyridine (16.9 ml) and acetic anhydride (478 μl) for 15 h, then cooled to 0°C. Methanol was added and the mixture was concentrated, and the residue was diluted with chloroform, successively washed with cold 2 M HCl, water, saturated Na_2CO_3 and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (60:1 chloroform–methanol) of the residue gave **9** (2.23 g, 85%).

$[\alpha]_D$=−12.5° (*c* 1.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.84–2.19 (m, 37H, AcO, AcN, H-3b$_{ax}$) 2.55 (dd, 1H, $J_{3ax,3eq}$=13.7 Hz, $J_{3eq,4}$=4.9 Hz, H-3b$_{eq}$) 3.69–4.22 (m, 22H, COOMe, MeO, *CH*$_2$Ph, H-5a,6a,6′a,5b,6b,9b,2c,3c,5c,6c,6′c,5d,6d,6′d), 4.48 (dd, 1H, $J_{8,9}$= 3.3 Hz, $J_{9,9'}$=10.9 Hz, H-9b), 4.54 (d, 2H, *CH*$_2$Ph), 4.64 (d, 1H, $J_{1,2}$=7.8 Hz, H-1a), 4.94–4.99 (m, 5H, H-3a,4a,1c,2d,4d), 5.03 (m, 1H, H-4b), 5.09 (dd, 1H, $J_{1,2}$=7.8 Hz, $J_{2,3}$=10.7 Hz, H-2a), 5.15 (d, 1H, $J_{1,2}$=9.8 Hz, H-1d), 5.27 (dd, 1H, $J_{2,3}$= 8.8 Hz, $J_{3,4}$=10.7 Hz, H-3d), 5.34–5.42 (m, 4H, H-7b,8b,4c, NHb), 6.07 (d, 1H, NHc), 6.77–6.80 (m, 2H, Ph), 7.01–7.05 (m, 2H, Ph), 7.22–7.36 (m, 10H, Ph).

^{13}C NMR (125 MHz, $CDCl_3$): δ 20.7, 20.7, 20.8, 20.9, 21.3, 23.3, 23.6, 29.8, 36.1, 49.6, 53.1, 53.6, 55.7, 60.8, 62.1, 62.5, 66.8, 67.0, 67.2, 68.4, 68.9, 70.5, 70.6, 70.9, 72.2, 73.6, 73.8, 75.0, 75.5, 76.0, 77.9, 98.9, 100.7, 101.2, 102.9, 114.5, 118.4, 118.6, 127.5, 127.6, 127.8, 128.3, 128.4, 128.4, 138.4, 138.8, 151.5, 155.3, 168.7, 169.5, 169.9, 170.3, 170.4, 170.5, 170.9.

MALDI-TOF MS

Calculated for $C_{73}H_{92}N_2O_{32}$

$[M+Na]^+$=1,580.49 $[M+K]^+$=1,596.60

Found=1,580.39, 1,596.39

p-Methoxyphenyl

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)}-2,6-di-*O*-benzoyl-β-D-galactopyranoside (**11**)

A solution of **9** (1.80 g, 1.16 mmol) in 1,4-dioxane (46.4 ml) was vigorously stirred over palladium hydroxide [$Pd(OH)_2$; 1.80 g] for 6.5 h at room temperature under hydrogen atmosphere. The combined filtrate and washings was concentrated and the residue was chromatographed on a column of silica gel (50:1 chloroform–methanol) to give **10** (1.50 g) as a crude syrup.

To a solution of **10** (1.48 g, 1.07 mmol) in pyridine (5.37 ml) were added DMAP (6.6 mg) and benzoic anhydride (0.970 g, 4.29 mmol), and the mixture was stirred for 18 h at room temperature under argon atmo-

sphere, then cooled to 0°C. Methanol was added and the mixture was concentrated to a residue, which was extracted with chloroform, successively washed with 2 M HCl, water, saturated Na_2CO_3 and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (40:1 chloroform–methanol) of the residue afforded **11** (1.63 g, 89%).

$[\alpha]_D$=+9.2° (*c* 1.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.73–2.17 (m, 37H, AcO, AcN, H-3b$_{ax}$), 2.82 (dd, 1H, $J_{3ax,3eq}$=13.0 Hz, $J_{3eq,4}$=4.4 Hz, H-3b$_{eq}$), 3.32 (m, 1H, H-2c), 3.66–4.00 (m, 15H, COOMe, MeO, H-3a,5a,5b,6b,5c,6c,6′c,5d,6d), 4.03 (m, 1H, H-6′d), 4.13 (d, 1H, H-4a), 4.22 (dd, 1H, H-9b), 4.46 (dd, 1H, H-6a), 4.56 (dd, 1H, H-9′b), 4.61 (d, 1H, H-1d), 4.65 (dd, 1H, H-6′a), 4.78 (m, 1H, H-4b), 4.95–4.98 (m, 2H, H-3d, NHb), 5.06 (dd, 1H, H-3c), 5.12 (m, 1H, H-2d), 5.17–5.24 (m, 3H, H-1a,7b,1c), 5.34–5.37 (m, 2H, H-4c,4d), 5.59–5.64 (m, 2H, H-2a,8b), 6.04 (d, 1H, NHc), 6.62–6.65 (m, 2H, Ph), 6.88–6.92 (m, 2H, Ph), 7.44–8.13 (m, 10H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ 20.4, 20.7, 20.8, 20.9, 21.0, 21.2, 21.6, 23.3, 24.2, 37.2, 49.4, 53.0, 55.6, 55.7, 61.1, 62.6, 62.8, 63.8, 66.7, 67.0, 67.5, 69.0, 69.2, 70.7, 71.0, 71.1, 72.1, 72.3, 73.1, 73.4, 73.8, 77.6, 97.8, 98.9, 101.2, 101.3, 114.5, 119.2, 128.6, 129.8, 130.2, 130.3, 130.4, 133.4, 151.6, 155.7, 165.6, 166.1, 168.6, 169.3, 170.1, 170.2, 170.3, 170.6, 170.7, 170.8, 171.8, 172.5.

MALDI-TOF MS

Calculated for $C_{73}H_{88}N_2O_{37}$

$[M+Na]^+$=1,607.50 $[M+K]^+$=1,623.47

Found=1,607.53, 1,623.2

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)}-2,6-di-*O*-benzoyl-α,β-D-galactopyranose (**12**).

To a solution of **11** (144 mg, 90.8 μmol) in a mixture of toluene (650 μl), acetonitrile (780 μl) and water (390 μl) was added ceric ammonium nitrate (CAN; 498 mg, 908 μmol), and the mixture was stirred for 1.5 h at 0°C and extracted with ethyl acetate. The extract was successively washed with water, saturated $NaHCO_3$ and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (50:1 chloroform–methanol) of the residue afforded **12** (118 mg, 88%) as an anomeric mixture.

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate-(2→3)}-2,6-di-*O*-benzoyl-α,β-D-galactopyranosyl trichloroacetimidate (**13**).

To a solution of **12** (118 mg, 79.8 μmol) in dichloromethane (800 μl) were added trichloroacetonitrile (80 μl, 798 μmol) and 1,8-diazabicyclo[5,4,0]undec-7-ene (12 μl, 79.8 μmol) at 0°C under argon atmosphere, and the mixture was stirred for 80 min at 0°C and concentrated. Silica gel column chromatography (40:1 chloroform–methanol) of the residue gave **13** (120 mg, 93%) as an anomeric mixture.

*6-(Methoxycarbonyldithio)hexanol (**15**)*

To a solution of **14** (5.10 ml, 37.2 mmol) in dichloromethane (100 ml) was added methoxycarbonylsulfenyl chloride (4.03 ml, 44.6 mmol), and the mixture was stirred for 30 min at room temperature under argon atmosphere and concentrated. Silica gel column chromatography (1:4 ethyl acetate–hexane) of the residue gave **15** (8.34 g, 95%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.38–1.46 (m, 4H, $CH_{22}CH_2SS$), 1.57 (m, 2H, $HOCH_2CH_2$), 1.69 (m, 2H, $HOCH_2CH_2CH_2$), 2.79 (t, 2H, CH_2SS), 3.62 (t, 2H, $HOCH_2$), 3.89 (s, 3H, $C(O)OCH_3$).

^{13}C NMR (100 MHz; $CDCl_3$): δ 25.3, 28.1, 28.5, 32.5, 39.0, 55.4, 62.6, 170.4.

*6-(tert-Butyldithio)hexanol (**16**)*

To a solution of **15** (7.50 g, 33.5 mmol) in methanol (100 ml) was added 2-methyl-2-propanethiol (15.1 ml, 134 mmol), and the mixture was stirred for 6 h at room temperature under argon atmosphere and concentrated. Silica gel column chromatography (1:5 ethyl acetate–hexane) of the residue gave **10** (7.32 g, 99%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.33 (s, 9H, S^t*Bu*), 1.34–1.42 (m, 4H, $CH_{22}CH_2SS$), 1.58 (m, 2H, $HOCH_2CH_2$), 1.67 (m, 2H, $HOCH_2CH_2CH_2$), 2.70 (t, 2H, CH_2SS), 3.64 (t, 2H, $HOCH_2$).

^{13}C NMR (100 MHz; $CDCl_3$): δ 25.4, 28.3, 29.2, 29.9, 32.6, 40.8, 47.7, 62.9.

6-(tert-Butyldithio)hexyl

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranosyl-(1→4)-{(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,6-di-*O*-benzoyl-β-D-galactopyranoside (**17**).

To a solution of **13** (513 mg, 316 μmol) and **16** (107 mg, 480 μmol) in dichloromethane (8.00 ml) was added molecular sieves 4 Å (630 mg), and the mixture was stirred for 1 h at room temperature, then cooled to −20°C. Trimethylsilyl trifluoromethanesulfonate (5.8 μl, 32 μmol) was added to the mixture and this was stirred for 2 h at −20°C. Insoluble materials were filtered off and washed with chloroform. The combined filtrate and washings was successively washed with water, saturated Na_2CO_3 and brine, dried (Na_2SO_4) and concentrated. Silica gel column chromatography (50:1 chloroform–methanol) of the concentrate afforded **17** (464 mg, 87%).

$[\alpha]_D$=+18.0° (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.09–1.18 (m, 4H, $OCH_2CH_2CH_{22}CH_2CH_2S$), 1.26–1.38 (m, 11H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$, S^t*Bu*), 1.39–1.47 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 1.79–2.19 (m, 37H, AcO, AcN, H-3b$_{ax}$), 2.48 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 2.81 (dd, 1H, $J_{3ax,3eq}$= 12.9 Hz, $J_{3eq,4}$=4.4 Hz, H-3b$_{eq}$), 3.30 (m, 1H, H-2c), 3.47 (m, 1H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 3.71–4.00 (m, 11H, COOMe, H-4a,5a,5b, 6b,5c,6c,6′c,5d,6d), 4.03 (m, 1H, H-6′b), 4.13 (d, 1H, $J_{3,4}$=6.8 Hz, H-4a), 4.22 (dd, 1H, $J_{8,9}$= 2.4 Hz, $J_{9,9'}$=12.4 Hz, H-9b), 4.46 (dd, 1H, $J_{5,6}$=7.1 Hz, $J_{6,6'}$=11.5 Hz, H-6a), 4.56 (dd, 1H, $J_{8,9'}$=12.4 Hz, H-9′b), 4.61 (d, 1H, H-1d), 4.65 (dd, 1H, $J_{5,6'}$=7.1 Hz, H-6′a), 4.78 (m, 1H, H-4b), 4.95–4.98 (m, 2H, H-3d, NHb), 5.06 (dd, 1H, $J_{2,3}$=12.0 Hz, $J_{3,4}$=3.4 Hz, H-3c), 5.12 (m, 1H, H-2d), 5.17–5.24 (m, 3H, H-1a, H-1c, H-7b), 5.34–5.37 (m, 2H, H-4c,4d), 5.59–5.64 (m, 2H, H-2a,8b), 6.04 (d, 1H, NHc), 6.62–6.65 (m, 2H, Ph), 6.88–6.92 (m, 2H, Ph), 7.44–8.13 (m, 10H, Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ 20.3, 20.4, 20.5, 20.7, 20.8, 20.9, 20.9, 20.9, 21.0, 21.5, 23.2, 24.1, 25.7, 28.3, 29.2, 29.4, 29.8, 30.1, 32.0, 37.0, 40.8, 46.1, 47.7, 49.2, 52.8, 55.4, 91.1, 62.6, 62.8, 63.8, 66.7, 67.0, 67.6, 69.1, 69.2, 70.5, 70.6, 70.8, 71.0, 71.0, 71.9, 72.0, 73.4, 73.9, 77.1, 97.8, 98.9, 101.2, 101.6, 128.5, 128.6, 129.7, 130.2, 130.2, 133.3, 165.3, 165.4, 165.9, 166.0, 168.5, 169.3, 170.1, 170.1, 170.2, 170.4, 170.5, 170.5, 170.7, 170.9, 172.3.

MALDI-TOF MS

Calculated for $C_{72}H_{93}N_2O_{36}S_2$

$[M+Na]^+$=1,705.56 $[M+K]^+$=1,721.53

Found=1,705.56, 1,721.60

6-(tert-Butyldithio)hexyl

β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-{(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)}-β-D-galactopyranoside (**18**).

To a solution of **17** (281 mg, 167 μmol) in methanol (2.0 ml) was added 28% sodium methoxide in methanol (96 mg, 1.00 mmol), and the mixture was stirred for 10 h under reflux. Water (2.0 ml) was added and the mixture was stirred for 16 h at room temperature, and then neutralized with Dowex (HCR-W2-H). After filtration, the filtrate was concentrated. Sephadex LH-20 column chromatography (methanol) of the residue gave **18** (147 mg, 85%).

$[\alpha]_D$=+51.3° (*c* 1.9, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.33–1.57 (m, 13H, $OCH_2CH_2CH_{22}CH_2CH_2S$, S^t*Bu*), 1.65–1.72 (m, 4H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 1.95 (t, 1H, $J_{3ax,3eq}$=$J_{3ax,4}$=12.7 Hz, H-3b$_{ax}$), 2.30–2.36 (m, 6H, 2AcN), 2.73–2.80 (m,3H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$, H-3b$_{eq}$), 3.33–3.95 (m, 25H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$, H-2a,3a,4a,5a,6a,6′a, 4b,5b,6b,7b,8b,9b,9′b,2c,5c,6c,6′c,3d,5d,6d,6′d, NHb, NHc), 4.02–4.04 (m, 2H, H-3c,4d), 4.12–4.25 (m, 2H, H-4c,2d), 4.31 (d, 1H, $J_{1,2}$=7.8 Hz, H-1c), 4.49 (d, 1H, $J_{1,2}$=7.6 Hz, H-1a), 4.98 (d, 1H, $J_{1,2}$ =8.8 Hz, H-1d).

^{13}C NMR (125 MHz, $CDCl_3$): δ 13.0, 21.1, 22.3, 25.2, 27.9, 28.8, 28.9, 29.0, 29.2, 29.3, 37.1, 40.2, 51.3, 52.3, 60.0, 61.0, 64.0, 68.3, 68.4, 68.8, 69.0, 69.1, 69.6, 71.0, 72.0, 73.1, 73.5, 73.6, 74.5, 75.0, 75.1, 77.7, 81.7, 102.0, 102.6, 103.0, 105.2, 173.3, 173.8, 174.2.

MALDI-TOF MS

Calculated for $C_{41}H_{72}N_2O_{24}S_2$

$[M]^-$=1,039.39 $[M+Na]^-$=1,062.38 $[M+K]^-$=1,078.36

Found=1,039.66

6-Mercaptohexyl

β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-{(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)}-β-D-galactopyranoside (**19**).

To a solution of **18** (55 mg, 52.8 μmol) in water (2.0 ml) was added tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl; 23 mg, 79.3 μmol), and the mixture was stirred for 49 h at room temperature under argon atmosphere. The reaction mixture was chromatographed on a Sephadex LH-20 (H_2O) to give **19** (40 mg, 80%).

$[\alpha]_D$=+10.4° (*c* 1.2 MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.29–1.36 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 1.39–1.45 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 1.60–1.64 (m, 4H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 1.91–2.02 (m, 7H, 2AcN, H-3b$_{ax}$), 2.50 (t, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$), 2.73 (dd, 1H, $J_{3ax,3eq}$ =12.7 Hz, $J_{3eq,4}$ =4.5 Hz, H-3b$_{eq}$), 3.38–3.90 (m, 25H, $OCH_2CH_2CH_2CH_2CH_2CH_2S$, H-2a,3a,4a,5a,6a,6′a,4b,5b,6b,7b,8b,9b,9′b,2c,5c,6c, 6′c,3d,5d,6d,6′d, NHb, NHc), 3.99–4.11 (m, 2H, H-3c, H-4d), 4.16–4.28 (m, 2H, H-4c, H-2d), 4.27 (d, 1H, $J_{1,2}$= 7.8 Hz, H-1c), 4.43 (d, 1H, $J_{1,2}$=7.6 Hz, H-1a), 4.92 (d, 1H, $J_{1,2}$=8.8 Hz, H-1d).

^{13}C NMR (125 MHz, $CDCl_3$): δ=13.2, 17.2, 21.4, 22.5, 22.6, 23.7, 25.4, 25.6, 28.0, 29.5, 29.6, 31.7, 34.0, 37.4, 51.7, 52.6, 60.3, 61.2, 64.2, 68.5, 68.6, 69.0, 69.3, 39.4, 69.8, 71.3, 72.2, 73.4, 73.8, 73.9, 74.8, 75.3, 75.4, 77.8, 81.8, 102.1, 102.8, 103.3, 105.4, 173.6, 174.0, 174.4.

MALDI-TOF MS

Calculated for $C_{37}H_{64}N_2O_{24}S$

$[M]^-$=952.97 $[M+Na]^+$=975.95 $[M+K]^+$=992.06

Found=975.87

General procedure for the coupling of **21** to MA-KLH

To a solution of maleimide-KLH (2.00 mg: PIERCE) in water (200 μl) was added a solution of **21** (2.00 mg, 2.10 μmol) in 10 mM phosphate buffer saline (PBS:

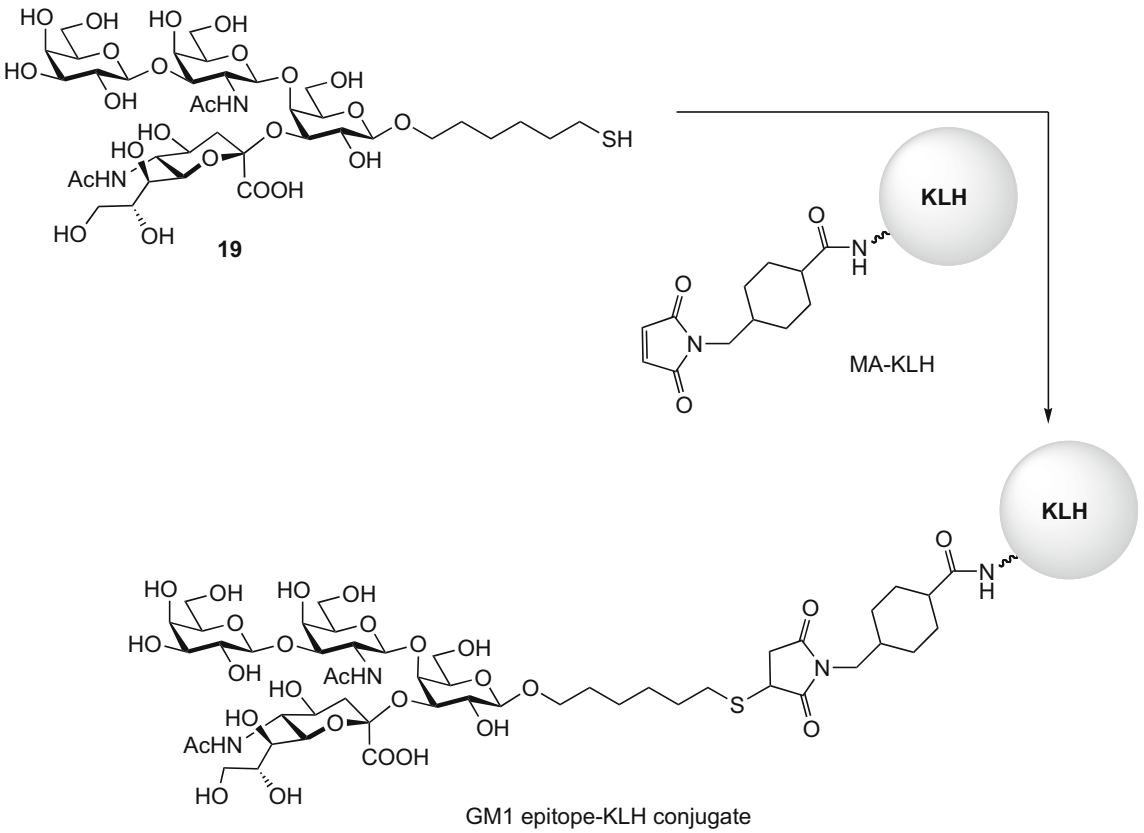
pH 7.2) and the mixture was gently stirred for 2 h at room temperature. Unreacted **21** was separated from the modified KLH by gel filtration on Sephadex G-25 (GE Healthcare) in PBS. The fractions containing GM1 epitope-KLH were pooled (6.5 ml). The number of hapten molecules per KLH was estimated by resorcinol reaction method and ELISA assay. 180 Hapten of GM1 epitope was found to conjugate with per mole of KLH (MW=3,000,000) (Scheme 5).

General procedure for the ELISA assay

Each well of a 96-well Maxisorp surface plate (Nunc, Roskilde, Denmark) was coated with 50 μl of the GM1 epitope-KLH conjugate (308 μg/ml) in phosphate buffer saline (PBS; 10 mM, pH 7.0) at different dilutions (1/2 to 1/1,024) and incubated at 4°C overnight [15]. The plate was washed three times with PBS. A solution of BSA (5% in PBS, 200 μl) was added to the wells and the plate was incubated for 2 h at room temperature. The plate was washed three times with anti-GM1 plasma from a GBS rabbit [5] (1/500 diluted by PBS containing 5% BSA, 100 μl) was added and the plate was incubated for 2 h at room temperature. Washing with PBS was repeated four times and a solution of peroxidase-conjugated anti-rabbit IgG (GE Healthcare UK Ltd., Buckinghamshire, England; 1/1,000 diluted by PBS containing 5% BSA, 50 μl) was added to each well. The plate was incubated for 1 h at room temperature and washed four times with PBS. To each well was added 100 μl of a solution of *o*-phenylenediamine (0.4 mg/ml) in 0.1 M sodium citrate (pH 5.5) containing 30% H_2O_2. The color was allowed to develop for 20 min at room temperature in the dark and 50 μl of 2 M H_2SO_4 were added to stop the reaction. The absorbance at 490 nm was measured using a microtiter plate reader (immuno-mini NJ-2300: Inter Med, Japan). A solution of KLH (Wako, Japan) without GM1 epitope was used as a control.

General procedure for resorcinol reaction

GM1 epitope-KLH conjugate solution (308 μg/ml) at different dilutions (1/2 to 1/6) was added resorcinol reaction reagent (0.02% of $FeCl_3$ and resorcinol in 8 M HCl). The mixture was heated on a boiling-water bath for 15 min, cooled 0°C for 15 min and amyl alcohol (2-methoxyethanol) was added. The absorbance at 580 nm was measured using a spectrophotometer (UV mini-1240: SHIMADZU, Japan).



Scheme 5 Coupling of GM1 epitope to MA-KLH

Acknowledgements This work was partly supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (Grant-in-Aid for Scientific Research to M. Kiso, No. 17101007) and CREST of JST (Japan Science and Technology Corporation). We thank Ms. Kiyoko Ito for technical assistance.

References

1. Ogawa-Goto, K., Abe, T.: Gangliosides and glycosphingolipids of peripheral nervous system myelins—a minireview. Neurochem. Res. **23**, 305–310 (1998)
2. Yuki, N.: Infectious origins of, and molecular mimicry in, Guillain–Barré and Fisher syndromes. Lancet, Infect. Dis. **1**, 29–37 (2001)
3. Yuki, N., Taki, T., Inagaki, F., Kasama, T., Takahashi, M., Saito, K., Handa, S., Miyatake, T.: A bacterium lipopolysaccharide that elicits Guillain–Barré syndrome has a GM1 ganglioside-like structure. J. Exp. Med. **178**, 1771–1775 (1993)
4. Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Odaka, M., Hirata, K., Taguchi, K., Miyatake, T., Furukawa, K., Kobata, T., Yamada, M.: Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain–Barré syndrome. Proc. Natl. Acad. Sci. U. S. A. **101**, 11404–11409 (2004)
5. Yuki, N., Yamada, M., Koga, M., Odaka, M., Suzuki, K., Tagawa, Y., Ueda, S., Kasama, T., Ohnishi, A., Hayashi, S., Takahashi, H., Kamijo, M., Hirata, K.: Animal model of axonal Guillain–Barré syndrome induced by sensitization with GM1 ganglioside. Ann. Neurol. **49**, 712–720 (2001)
6. Susuki, K., Rasband, M.N., Tohyama, K., Koibuchi, K., Okamoto, S., Funakoshi, K., Hirata, K., Baba, H., Yuki, N.: Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. J. Neurosci. **27**, 3956–3967 (2007)
7. Nishimoto, Y., Koga, M., Kamijo, M., Hirata, K., Yuki, N.: Immunoglobulin improves a model of acute motor axonal neuropathy by preventing axonal degeneration. Neurology **62**, 1939–1944 (2004)
8. Imamura, A., Ando, H., Ishida, H., Kiso, M.: Di-*tert*-butylsilylene (DTBS)-directed α-selective synthesis of 4-methylumbelliferyl T-antigen. Org. Lett. **7**, 4415–4418 (2005)
9. Fuse, T., Ando, H., Imamura, A., Sawada, N., Ishida, H., Kiso, M., Ando, T., Li, S.-C., Li, Y.-T.: Synthesis and enzymatic susceptibility of a series of novel GM2 analogs. Glycoconj. J. **23**, 329–343 (2006)
10. Cook, A.F.: The use of β,β,β-tribromoethyl chloroformate for the protection of nucleoside hydroxyl groups. J. Org. Chem. **33**, 3589–3593 (1968)
11. Kuduk, S.D., Schwarz, J.B., Chen, X.-T., Glunz, P.W., Sames, D., Ragupathi, G., Livingston, P.O., Danishefsky, S.J.: Synthetic and immunological studies on clustered modes of mucin-related Tn and TF O-linked antigens: the preparation of a glycopeptide-based vaccine for clinical trials against prostate. J. Am. Chem. Soc. **120**, 12474–12485 (1998)
12. Field, L., Hanley, W.S., McVeigh, I.: Organic disulfides and related substances. 32. Preparation and decomposition of β-substituted ethyl acetyl disulfides. J. Org. Chem. **36**, 2735–2739 (1971)
13. Gets, E.B., Xiao, M., Chakrabarty, T., Cooke, R., Selvin, P.R.: A comparison between the sulfhydryl reductants tris(2-carboxyethyl) phosphine and dithiothreitol for use in protein biochemistry. Anal. Biochem. **273**, 73–80 (1999)
14. Scherrmann, M., Boutboul, A., Estramareix, B., Hoffmann, A., Lubineau, A.: Binding propreties and esterase activity of monoclonal antibodies elicited against sucrose 6-heptylphosphonate. Carbohydr. Res. **334**, 295–307 (2001)
15. O'Sullivan, M.J., Gnemmi, E., Morris, D., Chieregatti, G., Simmonds, A.D., Simmons, M., Bridges, J.W., Marks, V.: Comparison of two methods of preparing enzyme-antibody conjugates: application of these conjugate for enzyme immunoassay. Ann. Biochem. **100**, 100–108 (1979)