Table 1 Summary of clinical information and mutations identified in Japanese patients with Krabbe disease

Patient no.	Genotype	Phenotype	Age at onset	GALC activity		CSF protein (mg dL^{-1})	MCV (m s^{-1})	Refs.
				Lymphocytes (nmol mg^{-1} h^{-1})	Skin fibroblasts (nmol mg^{-1} h^{-1})			
A1[b]	**W115X**/P302A[a]	Infantile	6 months	Not described	Not described	ND	ND	Tatsumi et al. (1995)
A2	**R204X**/12Del3Ins[a]	Infantile	< 6 months	Not described	Not described	ND	ND	Tatsumi et al. (1995)
A3	**R204X**/I234T	Infantile	< 6 months	0.08	ND	ND	ND	
A4	**L364R**/T652P	Infantile	5 months	0	0.1	81	Undetectable	
A5	12Del3Ins/–	Infantile	6 months	ND	0.27	ND	ND	
A6	T652P/T652P	Infantile	4 months	0.07	0.3	244	Undetectable	
A7	12Del3Ins/T652P	Infantile	6 months	0.1	ND	169	ND	
A8	**393delT**/–	Infantile	4 months	0	ND	185	12	
A9	**S257F**/–	Infantile	6 months	0.3	0.4	236	18	
A10	P302A/L618S	Late-infantile	8 months	0	0.3	119	25	
A11	G270D/R515H	Late-infantile	2 years	0.03	0.08	71	50	
A12	I66M + I289V/ 12Del3Ins	Juvenile	5 years	0.09	0.14	95	24	
A13	I66M + I289V / W647X[a]	Juvenile	3 years	0.6	ND	124	Low	Fu et al. (1999)
A14	I66M + I289V/ **1719-1720insT**	Juvenile	3.5 years	0.09	0.23	75	Low	
A15	12Del3Ins/–	Juvenile	3.5 years	0.12	0.5	34	ND	
A16	G270D/G270D	Adult	69 years	0.2	0.8	ND	ND	
A17	I66M + I289V/ I66M + I289V	Adult	59 years	0.29	0.23	51	17	

Patient no.	Genotype	Phenotype	Age at onset	CSF protein (mg dL^{-1})	MCV (m s^{-1})	Refs.
B1	12Del3Ins/12Del3Ins	Infantile	4 months	ND	ND	Tatsumi et al. (1995)
B2	12Del3Ins/12Del3Ins	Infantile	3 months	106	Low	Tatsumi et al. (1995)
B3	S52F/W410G	Infantile	< 6 months	ND	ND	Fu et al. (1999)
B4	12Del3ins/T652P	Infantile	4 months	ND	13–18	Fu et al. (1999)
B5	R515H/R515H	Infantile	5 months	80	Low	Fu et al. (1999)
B6	T262I/12Del3Ins	Infantile	1 years	42	36	Fu et al. (1999)
B7	G270D/G270D	Adult	10–20 years	Not described	Not described	Furuya et al. (1997)
B8	I66M + I289V/ I66M + I289V	Adult	10–20 years	Not described	Not described	Furuya et al. (1997), Kukita et al. (1997-98)
B9	I66M + I289V/Y354X	Adult	10–20 years	Not described	Not described	Furuya et al. (1997), Kukita et al. (1997-98)
B10	L618S/IVS6 + 5G > A	Adult	10–20 years	Not described	Not described	Furuya et al. (1997), Kukita et al. (1997-98)
B11	L618S/L618S	Adult	51 years	Not described	Not described	Satoh et al. (1997)

Bold print in Genotype column denotes a novel mutation identified in this study
Normal ranges of GALC activity in lymphocytes and skin fibroblasts are 2.1 ± 0.29 and 4.5 ± 1.2 nmol mg^{-1} h^{-1}, respectively
"A" in patient no. column represents the subjects investigated in this study; "B" in patient no. column represents subjects reported previously
"–" in the Genotype column indicates no mutation was found in the second allele in our experiment
CSF cerebrospinal fluid, MCV motor nerve conduction velocity, ND not done
[a]Previously detected mutant allele
[b]The patient was previously reported as late-infantile type. This has, however, been corrected to infantile type on the basis of detailed clinical information

Table 2 Primer sequences used for PCR amplification of GALC gene, and DHPLC oven temperatures

Exon	Forward primer (5′ > 3′)	Reverse primer (5′ > 3′)	Size (bp)	Predicted temperature (°C)	DHPLC oven temperature (°C)
1	GGAGTCATGTGACCCACACA	CGCGTATCCCCGCAGCTT	242	56	55/56/57
2	GGTGTGCGTGAACACTGTAGA	CTATGGTGAAATTCACCATCC	215	54.8	54/55/56
3	GGATGGTGAATTTCACCATAG	TCACAGTCCATATGCTGAGGT	333	54.9	54/55/56
4	GGTCCTAGGAAGTACCATTCATG	CACCAACACGATTCAGAATTTAA	190	56.4	55/58/61
5	GTTTTATTTTCAATAGCGCCAGC	CCTCATGGCATAAAATGGTTAGTC	312	57	55/57/59
6	AATGGTATCGTAACGATAATCTG	TTTCTGTGTTAGGAACCATAAGG	190	54.5	54/55/56
7	CTATTATCCAGAACGCTGATTTG	GTAATCAAATGGGGAGAAGGC	335	54.4	53/55/56
8	GAAAACCTTGGAGAAGACTCGTA	GGCTGGAAGAATAAGGAATTCC	354	53.2	54/56/58
9	CTCCAGGTTTTTAGACATTTAC	CTGCTTTGTCTCTTAGAGAAGA	251	55.7	52/55/58
10	CAGACTCAAATTGATATACAGCT	GGCATCTGTCTGTATGCTTAT	337	53.1	54/56/58
11	TTCTGTTAATCTTGGGCATTAAC	CAGGGCCTCTGTCAATTCATA	317	54.4	52/54/56
12	CATTGGTACATTCTTGCTGGTACT	GTCACCATCCACCAAGACAAA	413	54.5	53.5/54.5/56.5
13	ATTCCAAGGGCCTTGATATTG	TTTGACAGCCACTCCATCATG	413	55.7	54/56/60
14	AATATCAGCAAGGAGAGCTTCTG	GGAGGACCATTGAAAACTCTTC	339	56.7	54/56/58
15	GCATGTGCTGTGAAATGACATA	CCCACAAATAACAAGTAGGTGCT	325	55.1	55.1/56.1/57.1
16	CAGATGCCACTCAAGAACCCC	CCCCTCCTATTTTATAACAG	251	55.1	52/54/55
17	CCATACATGATCAAGAAACAGAC	GAAACAAGAATTGGCTCTGAA	301	57.9	56.9/57.9/58.9

healthy individuals was performed with the primer pair for exon 8 and exon 10 (Table 2) and the product was digested with *Eco* 57I and *Aci* I, respectively. The normal allele of 257S should be digested by *Eco* 57I and the mutant allele of 364R should be digested by *Aci* I.

Results

All 27 mutations detected in this study are listed in Table 1. The analytical procedures for GALC mutation detection are described in detail below.

Screening for 12Del3Ins and I66M + I289V with restriction enzyme digestion

DNA isolated from peripheral blood leukocytes or cultured skin fibroblasts was tested for mutation detection. Screening for 12Del3Ins mutation by restriction enzyme digestion revealed that five patients (A2, A5, A7, A12, A15), including one patient (A2) reported previously, were heterozygous. As a result of screening for I66M + I289V, I66M was found to be homozygous in one patient (A17), heterozygous in three patients (A12, A13, and A14), and I289V was found to be homozygous

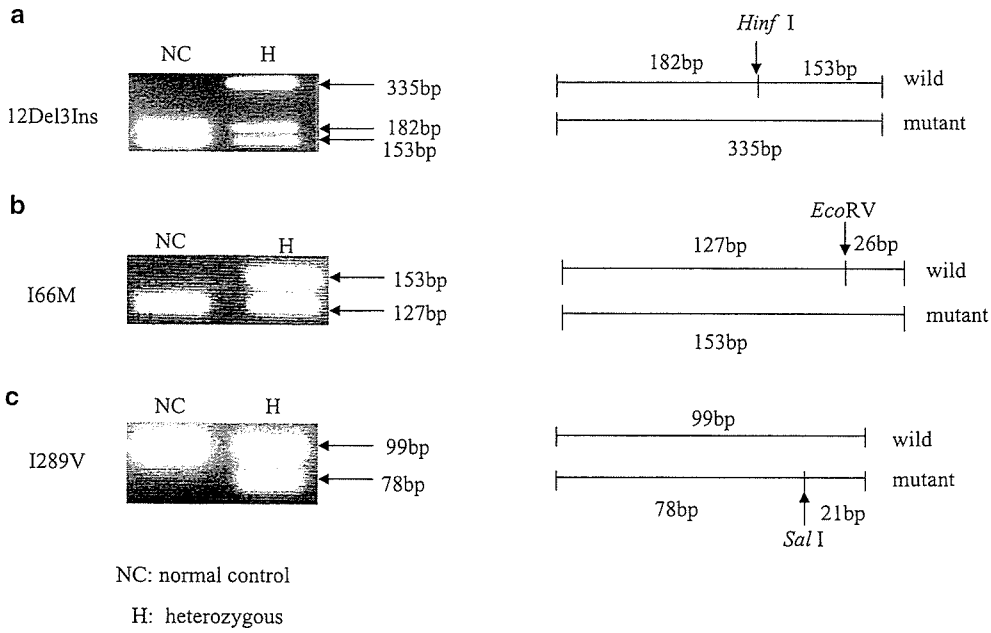

Fig. 1 Mutation detection for 12Del3Ins and I66M + I289V with restriction enzyme digestion. **a** Genomic DNA samples were amplified with the primer pair of exon 7; the product was digested with *Hin*fI and subjected to 3% Nusieve gel. Fragments with 12Del3Ins were not digested with *Hin*fI. **b** For detection of I66M, amplified fragments using a sense primer of exon 2 and a mismatch-antisense primer (5′-TCATTACCTTAAAGAGATAATCCGA-3′) were digested with *Eco*RV. **c** To detect I289V, amplified fragments with a sense primer of exon 9 and a mismatch-antisense primer (5′-ACTAGCCACTAAATTCCAGTCGA-3′) were digested with *Sal*I

in A14 and A17, and heterozygous in A12 and A13. In patients 14 and 17, I66M and I289V were easily confirmed to reside on the same allele. To confirm that I66M and I289V resided on the same allele in patients A12 and A13, their parents' DNA was also analyzed for I66M and I289V. In A12 and A13, both I66M and I289V were proved to reside on the same allele inherited from the father of A12 and the mother of A13, respectively. I289V substitution without I66M in patient A14 was regarded as a polymorphism. These mutations were also confirmed by direct sequencing analysis.

Screening for the 30 kb large deletion mutation

Shortened PCR products from the deleted allele were not detected in any of our patients. The most frequent mutation in Caucasians was not found in our Japanese patients.

Mutation screening with DHPLC and mutation detection with direct sequencing

DHPLC analysis was performed for 11 patients (A1–11). Seventeen abnormal DHPLC elution profiles (A1, exons 4, 9; A2, exon 7; A3, exon 7; A4, exons 10, 12, 17; A5, exon 7; A7, exons 5, 7, 17; A8, exon 4; A9, exons 14, 15; A10, exons 9, 16; A11, exon 14) were found. The corresponding amplicons were characterized by direct sequencing. As a result, four polymorphisms (A7, IVS5+71G>A; A9, IVS14+60C>T; A4, 1254C>T (S418S); and A9, 1637T>C (I546T)) and 15 mutations (A1, W115X, P302A; A2, R204X, 12Del3Ins; A3, R204X, I234T; A4, L364R, T652P; A5, 12Del3Ins; A7, 12Del3Ins, T652P; A8, 393delT; A10, P302A, L618S; A11, R515H) were identified in the above 17 amplicons by direct sequencing. Within four polymorphisms, two (IVS5+71G>A and IVS14+60C>T) were novel intronic polymorphisms and the other two (1254C>T (S418S) and 1637T>C (I546T)) were exonic polymorphisms which have previously been reported (Sakai et al. 1994; Kukita et al. 1997-98). Within 15 mutations, 11 mutations were first identified by DHPLC; the exceptions were two mutations (A1, P302A; A2, 12Del3Ins) identified in the previous study (Tatsumi et al. 1995) and two mutations (A5, A7, 12Del3Ins) already detected in the screening step for 12Del3Ins. Four different novel mutations (W115X, R204X, L364R, and 393delT) were found in this step.

Reverse-transcription PCR and direct sequencing

Two mutations were identified in nine patients (A1, 2, 3, 4, 7, 10, 12, 13, 17) by use of the screening methods described above. Only one mutation (12Del3Ins, 393delT, R515H, I66M+I289V, and 12Del3Ins) was found in five patients (A5, 12Del3Ins; A8, 393delT; A11, R515H; A14, I66M+I289V; A15, 12Del3Ins). In the remaining three patients (A6, 9, 16), no mutations were found. To identify other mutations, mutation analysis by RT-PCR and direct sequencing were performed for eight patients (A5, 6, 8, 9, 11, 14, 15, 16), and four different mutations, the exceptions being the mutations detected by use of the above methods, were detected in five patients (T652P homozygous in A6, S257F heterozygous in A9, G270D heterozygous in A11, 1719-1720insT heterozygous in A14, and G270D homozygous in A16). In patient A14, I289V was also confirmed on the same allele as 1719-1720insT; I289V is regarded as a polymorphism, however, because substitution of I289V without I66M has been proven to be polymorphism (Furuya et al. 1997) and the 1719-1720insT resulted in frame shift leading to a premature stop codon. Two mutations (S257F and 1719-1720insT) were novel. These mutations were also confirmed by direct sequencing of genomic DNA.

Screening for S257F and L364R in healthy individuals

Of the six novel mutations (W115X, R204X, S257F, L364R, 393delT, and 1719-1720insT), two missense mutations (S257F, L364R) were screened by restriction enzyme digestion. These mutations were not detected in 100 healthy controls.

Genotype–phenotype correlation

The detected mutations are summarized in Table 1 with reference to reported mutations in Japanese patients. Table 1 includes other clinical information about the patients. Fourteen different mutations identified in this study were found in 27 detected alleles of 17 patients, including nine missense mutations (I66M+I289V, I234T, S257F, G270D, P302A, L364R, L618S, T652P), two nonsense mutations (W115X and R204X), one small deletion (393delT), one small insertion (1719-1720T), and one deletion/insertion (12Del3Ins). Of these mutations, six were novel (W115X, R204X, S257F, L364R, 393delT, and 1719-1720insT). All GALC mutations in Japanese patients, with their frequencies, including those previously reported, are summarized in Table 3.

The distribution of the clinical phenotype for each mutation is summarized in Table 4. For rare mutations, those detected less than twice, the genotype–phenotype correlation remains indeterminate; for rather common mutations, however, the tendency between genotype and phenotype was observed. The phenotype for 12Del3Ins, T652P, and R515H was observed mostly in infantile-type Krabbe disease whereas I66M+I289V, G270H, and L618S were mostly detected in the adult type and never in the infantile form of the disease. Concerning the regional distribution of patients, there was no specific region for each mutation.

Table 3 Summary of mutations identified in 28 Japanese patients including 11 patients previously characterized

No.	Exon/intron	Nucleotide change	Amino acid change	Allele frequency	Allele number
1	2	198A > G +865A > G	I66M + I289V	0.15	8
2	2	155C > T	S52F	0.02	1
3	4	**344G > A**	**W115X**	0.02	1
4	4	**393delT**	**L130fs; 154X**	0.02	1
5	IVS6	IVS6 + 5G > A	179–191 skipping	0.02	1
6	7	**610C > T**	**R204X**	0.04	2
7	7	635-646 del/ins CTC (12Del3Ins)	212-216 del (NLWES)/ins (TP)	0.22	11
8	7	701T > C	I234T	0.02	1
9	8	**770C > T**	**S257F**	0.02	1
10	8	785C > T	T262I	0.02	1
11	8	809G > A	G270D	0.1	5
12	9	904C > G	P302A	0.04	2
13	10	1062C > G	Y354X	0.02	1
14	10	**1091T > G**	**L364R**	0.02	1
15	12	1228T > G	W410G	0.02	1
16	14	1544G > A	R515H	0.06	3
17	15	**1719-1720insT**	**R574X**	0.02	1
18	16	1853T > C	L618S	0.08	4
19	17	1941G > A	W647X	0.02	1
20	17	1954A > C	T652P	0.1	5
Total				1	52

Bold print denotes novel mutations identified in this study. "fs" indicates a frameshift starting after the respective codon

Discussion

In this work we detected 27 mutant alleles in 17 patients. We found six novel mutations of the GALC gene in Japanese patients with Krabbe disease. Two, W115X and R204X, were nonsense mutations whereas 393delT and 1719-1720insT resulted in frame shifts. It was obvious all four were pathogenic mutations. The final two, S257F and L364R, were missense mutations that were not detected in 100 healthy controls. These two loci are well conserved in different species, including monkey, dog, and mouse (Luzi et al. 1997). This evidence, when taken together, suggests that these two missense mutations may be regarded as causative of Krabbe disease.

Table 4 Correlation between genotype and phenotype in 28 Japanese subjects

Mutation	Infantile	Late-infantile	Juvenile	Adult	Total allele
12Del3Ins	9	0	2	0	11
T652P	5	0	0	0	5
R515H	2	1	0	0	3
R204X	2	0	0	0	2
P302A	1	1	0	0	2
S52F	1	0	0	0	1
W115X	1	0	0	0	1
393delT	1	0	0	0	1
I234T	1	0	0	0	1
S257F	1	0	0	0	1
T262I	1	0	0	0	1
L364R	1	0	0	0	1
W410G	1	0	0	0	1
1719-1720insT	0	0	1	0	1
W647X	0	0	1	0	1
IVS6 + 5G > A	0	0	0	1	1
Y354X	0	0	0	1	1
I66M + I289V	0	0	3	5	8
G270H	0	1	0	4	5
L618S	0	1	0	3	4
Total allele	27	4	7	14	52

In four patients, only one mutant allele was detected. Because our screening method covered the coding region and the exon–intron boundary, undetected mutations may lie outside the analyzed region, for example in promoter and enhancer regions. We also did not analyze large-scale genomic rearrangement, other than the screened 30 kb deletion.

As reported in the literature, mutations of the GALC gene in Krabbe disease are very heterogeneous. In Caucasians, the 30 kb large deletion reported by Rafi et al. (1995) and Luzi et al. (1995) was widespread within the patient population, having a frequency of 40–50%. Other mutations followed heterogeneous patterning. As in Caucasians, to date, examination of Japanese patients revealed 14 mutations with a very heterogeneous distribution. It was considered hard to screen the GALC mutation, because there is no common mutation in Japanese patients; as our results show, however, there are several common mutations. The most common mutation in Japanese patients is 12Del3Ins with a 0.22 allele frequency. The second most frequent mutation, I66M + I289V, has 0.15 prevalence. To date, these two mutations have been identified in Japanese patients only. These two mutations are also unique and difficult to conceive as recurrently occurring mutations. They might be derived from a founder; parents' samples necessary for haplotype analysis were unavailable from most of the families, however.

Referring to Tables 1 and 4, the most common mutation (12Del3Ins) and the two other mutations

(T652P and R515H) in the homozygous state (B1, B2, A6, and B5) resulted in the classic infantile phenotype. The second most common mutation, I66M + I289V, contributed to late-onset-type Krabbe disease, because the homozygous state of this mutation was found only in the adult type (patients A17 and B8), the mildest form of the disease, whereas the heterozygous state was detected in the juvenile or adult form (patients A12, A13, A14, and B9). This concordance strongly suggests that the existence of this mutation leads to the mild clinical phenotype. Because screening for this mutation might directly reveal a mild phenotype of Krabbe disease in Japanese patients, we propose a viable screening method using restriction enzyme digestion with PCR fragments for I66M (Fig. 1b) and I289V (Fig. 1c). For the missense mutations G270D and L618S, similar concordance is shown in Tables 1 and 4, demonstrating that it will be effective to screen for this mutation to estimate the mild phenotype.

For most enzyme-deficiency diseases it is generally believed there is a correlation between residual enzyme activity and clinical severity; this does not appear to be true for Krabbe disease, however. The expression experiment did not always reveal better residual activity in late-onset patients. Harzer et al. (2002) analyzed substrate specificity for several mutations and, although the G270D mutation led to loss of enzymatic activity for galactocerebroside as its natural substrate, nearly normal activity for psychosine, its second substrate, was preserved. This paper supports the idea that measurement enzyme activity with one substrate does not necessarily lead to identification of an essential defect. It might be important to analyze the substrate specificity of L618S and I66M + I289V to elucidate genotype–phenotype correlation.

In a previous study, Furuya et al. (1997) investigated I66M + I289V allele expression, and found reduced enzymatic activity only when these two amino acid changes occurred on the same allele. We likewise confirmed that I66M and I289V occurred on the same strand and same peptide in all four patients. That I66M and I289V reside on the same strand suggests that each amino acid contributes to a different function, for example reaction center or substrate binding. Analysis of the structure of the crystallized protein might be important for future understanding of the mechanism of this mutation.

Acknowledgements This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and the Health and Labor Science Research.

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Histidine-Tagged Shiga Toxin B Subunit Binding Assay: Simple and Specific Determination of Gb3 Content in Mammalian Cells

In-Sun Shin,[a,b] Kiyotaka Nishikawa,[c,d] Hiroki Maruyama,[e] and Satoshi Ishii*,[a]

[a] *Department of Agricultural and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine; Obihiro 080–8555, Japan:* [b] *Course of Science of Bioresources, The United Graduate School of Agricultural Sciences, Iwate University; Morioka 020–8550, Japan:* [c] *Department of Clinical Pharmacology, Research Institute, International Medical Center of Japan; Tokyo 162–8655, Japan:* [d] *PRESTO, Japan Science and Technology Agency; Saitama 332–0012, Japan: and* [e] *Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences; Niigata 951–8510, Japan.*

Received November 22, 2005; accepted January 11, 2006; published online January 18, 2006

A two-step binding assay for globotriaosylceramide (Gb3) content was developed by histidine-tagging strategy, which is a well-established method for the purification of recombinant proteins. The complete binding of the recombinant His-tagged Shiga toxin 1B subunit (1B-His) (1 μg/ml) to the standard Gb3 adsorbed on a multi-well H type plate was observed within 30 min at 37 °C; and its binding could be visualized by the following applications of HisProbe-HRP (8 μg/ml) and tetramethylbenzidine (TMB) peroxidase substrate. The 1B-His binding assay was linear over the range of 1 to 100 ng of Gb3 per well. The binding of 1B-His was specific to Gb3 separated from HeLa cells, and no major cross-reactivity of other glycolipids in Folch's lower fractions extracted from HeLa cells was detected. The glycolipids in Folch's lower fractions from HeLa cells, human fibroblasts and mouse heart were suitable for this assay, but the further purification was needed for glycolipids from human plasma, thus sample preparation is critical factor for the reliable determination of Gb3 content. The 1B-His binding to Gb3 was inhibited by the addition of galactose, but not mannose. This 1B-His binding assay will be useful not only for the determination of Gb3 content, but also for screening for the compounds which inhibit the toxin-binding to Gb3. The strategy of our present method may be applicable for other binding assay, such as Cholera toxin B-subunit for ganglioside GM1.

Key words globotriaosylceramide; Shiga toxin; binding assay method; His-tagged protein

Globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1Cer; Gb3) is the major neutral glycosphingolipid accumulating in patients with Fabry disease, which is an inherited disease caused by a deficiency of α-galactosidase A,[1,2] and is also known as the receptor for the Shiga toxin (Stx, also called verotoxin), which is a class of protein toxin produced by Stx-producing strains of *Escherichia coli* (STEC) associated with diarrhea, hemorrhagic colitis,[3] and hemolytic uremic syndrome (HUS).[4,5] Stx is an approximately 70-kDa complex comprised of one A-subunit (32 kDa) and five B-subunits (7.5 kDa each).[6] The holotoxin binds *via* B-subunit pentamer with high affinity to the terminal digalactose of Gb3.[7,8] Although the cytotoxicity of Stx was mainly caused by the A-subunit, which has an RNA-*N*-glycosidase activity, resulting in inhibition of protein synthesis,[7] Jacewicz *et al.*[9] suggested that the basal levels of Gb3 in human cells may relate to their sensitivity toward Stx. The assay of Gb3 content in mammalian cells is important for the study on the mechanism of Stx-cytotoxicity and on the pathogenesis of Fabry disease.

Various assay methods for Gb3 content have been previously reported, such as HPLC analysis of benzoylated glycolipids,[10] TLC-orcinol staining,[11] liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis,[12] and matrix-assisted laser desorption ionisation with time-of-flight (MALDI-TOF) mass spectrometry.[13] High cost equipment is sometimes required for these methods. In contrast, the simple assay method has been reported as an enzyme-linked immunosorbent assay (ELISA) method using verotoxin B subunit.[14] This method has many benefits including no special equipment is required, many samples can be assayed at once, and a specific Gb3 assay can be accomplished because of the high affinity of the verotoxin B subunit toward Gb3.

The purpose of this study was to simplify the ELISA method using the verotoxin B subunit and to determine the specificity of Gb3 assay in various sources. In this study, we used the recombinant histidine-tagged B subunit of Stx 1 (1B-His), because 1B-His has a higher affinity toward Gb3 in comparison with His-tagged Stx 2B subunit (2B-His).[15] The two-step binding assay was established by using 1B-His and HisProbe-HRP, which is a nickel (Ni^{2+}) activated derivative of horseradish peroxidase (HRP) and is specifically bound to the poly-histidine residue; and the sample preparation of glycosphingolipid suitable for this Gb3 assay was studied in different sources. Other applications of this binding assay were also suggested in this study.

Experimental

Preparation of Recombinant 1B-His The recombinant 1B-His, in which 6 histidine residues were added at the carboxy termini of the B subunits, was prepared as described previously.[15] The BL21DE cells expressing 1B-His were cultured in 300 ml of LB broth (Difco laboratories, Detroit, MI, U.S.A.) supplemented with 50 μg/ml kanamycin (Nacalai Tesque, Inc., Kyoto, Japan) at 37 °C for 2 h. The cells were subsequently treated with 1.0 mmol/l isopropyl β-D(−)thiogalactopyranoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37 °C for 4 h. Cells were pooled by centrifugation at 6000 rpm for 15 min at 4 °C. The 1B-His was extracted from cell pellets with the Bugbuster protein extraction reagent (Novagen, Madison, WI, U.S.A.) and purified by using the His-bind purification kit (Novagen) according to manufacturer's recommendations. Purified 1B-His fractions were applied to an NAP10 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS). The purified 1B-His was revealed as a single band on SDS-PAGE (data not shown) and its aliquots were stored at −20 °C.

Materials HisProbe-HRP was purchased from Pierce Biotechnology,

* To whom correspondence should be addressed. e-mail: ishiis@obihiro.ac.jp.

Inc. (Rockford, IL, U.S.A.). Purified glycosphingolipids, Gb3 from porcine erythrocytes and globotetraosylceramide (Gb4) from porcine erythrocytes were purchased from Nacalai Tesque, Inc. and Wako Pure Chemical Industries, Ltd., respectively.

Cell Lines and Specimen HeLa cell line (JCRB9004) was purchased from Human Science Research Resources Bank (Osaka, Japan) and human fibroblast cell line was established from a heterozygous patient with Fabry disease. Cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.) supplemented with 10% fetal calf serum (Biological Industries, Haemek, Israel) under a humidified 5% CO_2 atmosphere. Cultured cells were washed with PBS and then harvested by a plastic scraper. Cells were precipitated by centrifugation at 2000 rpm for 5 min. Human plasma was prepared from the heparinized blood of a heterozygous patient with Fabry disease by centrifugation at 3000×*g* for 5 min. All samples were stored at −20 °C.

Glycosphingolipid Extraction Cell pellets and minced heart from an α-galactosidase A-knock out (KO) mouse[16] were homogenized using a handy micro homogenizer (Physcotron, Niti-on, Inc., Chiba, Japan) in 0.2-ml H_2O. After the determination of protein content with a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.), cellular lipids were extracted from homogenates containing 5—10 mg protein with 20 volumes of chloroform–methanol (2 : 1, v/v). The lipid from human plasma was also extracted with 20-fold excess volume of chloroform–methanol (2 : 1, v/v). Following filtration by 90-mm 5A filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), crude extracts were dried and stored as a crude extract fraction. Crude extracts were further treated with 1 ml of methanolic NaOH (0.2 M NaOH in methanol) at 40 °C for 2 h. After neutralizing the solution with glacial acetic acid, glycosphingolipids were further subjected to the Folch's partition (chloroform–methanol–H_2O, 8 : 4 : 3 in v/v/v).[17] Glycosphingolipids recovered in the lower phase were pooled and designated as a Folch's lower fraction. The glycosphingolipids in a Folch's lower fraction were further purified by the Iatrobeads (Iatron Laboratories, Tokyo, Japan) column chromatography. Glycosphingolipids suspended in 1 ml of chloroform–methanol (2 : 1, v/v) were applied to an Iatrobeads column (0.5×2 cm) equilibrated with isopropanol–hexane (55 : 45, v/v). The bound glycolipids were eluted with isopropanol–hexane–water (55 : 30 : 15, v/v/v). Eluates were pooled and used as an Iatrobeads-binding fraction.

Separation of Glycosphingolipids by HPLC Glycosphingolipids in Folch's lower fraction extracted from HeLa cells were separated by HPLC (LC-10AD, Shimadzu Corporation, Kyoto, Japan) according to the method of Kannagi *et al.*[18] Folch's lower fraction from HeLa cells was dissolved in 2 ml of chloroform–methanol (2 : 1, v/v), and applied to a packed column of Iatrobeads (6RS-8010, Iatron Laboratories; 1×50 cm) equilibrated with isopropanol–hexane (55 : 45, v/v). Glycosphingolipids were eluted with a gradient of isopropanol–hexane–water from 55 : 45 : 0 to 55 : 30 : 15 (v/v/v). The flow rate was set at 2 ml/min by applying approximately 60—70 kgf/cm^2. Fractions were collected by a fraction collector (FC 203B, Gilson Inc., Middleton, WI, U.S.A.) at 2-min intervals and the total volume of eluate was 400 ml. Each fraction was dried and resuspended in chloroform–methanol (2 : 1, v/v). A small aliquot of each fraction was applied to TLC analysis, and 7 major lipid components were pooled in 7 fractions.

1B-His Binding Assay The 1B-His binding assay for the determination of Gb3 content was performed as follows. Samples containing glycosphingolipids in 10 μl of 100% methanol were plated onto a multi-well H type plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The plates were placed to evaporate methanol at room temperature for 1 h, and then 200 μl of 1% bovine serum albumin solution in PBS (BSA-PBS) was added to each well and incubated at 37 °C for 1 h. Plates were washed once with 400 μl of 0.2% BSA-PBS, and then incubated with purified 1B-His (1 μg/ml) in 0.2% BSA-PBS at 37 °C for 30 min. Following washing three times with 200 μl of PBS, the plates were incubated with the HisProbe-HRP (8 μg/ml) in 0.2% BSA-PBS at 37 °C for 30 min. The plates were washed with 200 μl of PBS and then visualized with tetramethylbenzidine (TMB) peroxidase substrate system (100 μl/well) (KPL, Gaithersburg, MD, U.S.A.) at room temperature for 10—15 min. Reactions were terminated by the addition of 100 μl of 1 M phosphoric acid and gentle shaking. The absorbance of each well at 415 nm was determined using a micro plate reader (MPR-A4, Tosoh Co., Tokyo, Japan). For the determination of Gb3 content, 10—100 ng of standard Gb3 was consistently applied on the same plate, and absorbances were determined at each assay.

TLC and TLC-Blotting TLC analysis was quantitatively performed with high-performance TLC (HPTLC)-Silica gel 60 plates (Merck & Co., Inc., Whitehouse Station, NJ, U.S.A.) using a solvent system of chloroform–methanol–water (60 : 35 : 8, v/v/v). Glycosphingolipids were visualized by spraying orcinol-sulfuric acid reagent; and their band intensities were determined by the image processing software Scion Image (Scion Corporation, Frederick, MD, U.S.A.). TLC-blotting with 1B-His was performed by the method of Taki *et al.*[19] Glycosphingolipids were separated on a TLC plate as described above. The plate was sunk in 0.4% polyisobutylmethacrylate (PIM) solution (2.5% PIM in chloroform was diluted to 0.4% with hexane) and followed by blocking with 1% BSA-PBS. After incubation with 1B-His at 1 μg/ml at room temperature for 30 min, the plate was washed with PBS. After further incubation with HisPorbe-HRP at 8 μg/ml at room temperature for 1 h and the following final washing, 1B-His binding was visualized with an enhanced chemiluminescent substrate (Pierce Biotechnology, Inc.).

Results

1B-His Binding Assay with Standard Glycosphingolipids To establish the 1B-His binding assay, we first used standard glycosphingolipids (Gb3 and Gb4) in the amount of 1—100 ng/well (Fig. 1). Absorbance (abs.) at 415 nm and amount of Gb3 were good corresponding in the range of 1 to 100 ng of Gb3. 1B-His binding was partially observed when a higher amount of Gb4 was used. One hundred nanograms of Gb4 showed 0.062 abs. units; and 7.5% of 0.823 abs. units was observed with 100 ng of Gb3. The assay was highly reproducible, had a very low background (<0.05 abs. units), and there was minimal variation between three independent determinations for each Gb3 concentration. The effect of incubation time on 1B-His binding to standard Gb3 was analyzed. The 1B-His solution (1 μg/ml) in 0.2% BSA-PBS was applied to multi-well H type plates, coated with standard Gb3 (100 ng), and incubated for 5 different lengths (10, 20, 30, 60, 120 min) at 37 °C. The 1B-His binding was low for the shortest incubation, and reached a plateau at 30 min; and the effect of incubation time on the 1B-His binding was similar for lower Gb3 content (data not shown). This data suggested that at least 30-min incubation at 37 °C was necessary for the completion of 1B-His binding to Gb3. In Fig. 2, the optimum concentration of 1B-His and HisProbe-HRP was determined. The Gb3-dependent binding of 1B-His increased, according to the concentration of 1B-His, and reached a plateau at 1 μg/ml (Fig. 2A). 1B-His binding activity was also dependent on the addition of HisProbe-HRP, and reached a plateau at 8 μg/ml (Fig. 2B). These data indicated that the optimum concentrations of 1B-His and HisProbe-HRP for the detection of Gb3 content were 1 μg/ml and 8 μg/ml, respectively.

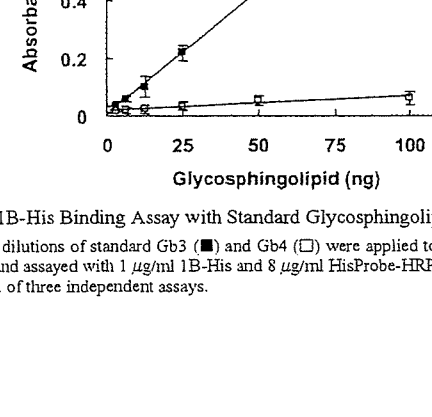

Fig. 1. 1B-His Binding Assay with Standard Glycosphingolipids

Doubled dilutions of standard Gb3 (■) and Gb4 (□) were applied to a multi-well H type plate and assayed with 1 μg/ml 1B-His and 8 μg/ml HisProbe-HRP. Results are the mean ± S.D. of three independent assays.

Specificity of the 1B-His Binding Assay In order to determine the specificity of the 1B-His binding assay, Folch's lower fraction from HeLa cells was used as the source of various lipids. As shown in Fig. 3A, more than 6 different glycolipids and a group of cholesterol were present in this fraction. All major lipid components were separated by an HPLC attached Iatrobeads column, and pooled into 7 fractions (Fig. 3A). Fraction 1 was the pass through fraction using Iatrobeads column chromatography, and Fractions 2 to 7 were once bound and then eluted with different solvent conditions. The highest abs. units (1.238) in 1B-His binding was observed in Fraction 5, which contained Gb3. A partial 1B-His binding (0.104 abs. units) was detected in Fraction 6, which contained Gb4, but other fractions were less than background level (<0.05 abs. units) (Fig. 3B). To clarify whether the presence of non-Gb3 lipids negatively or positively affect the 1B-His binding assay, all 6 fractions other than Fraction 5 were combined, and a different concentration of standard Gb3 (Fig. 4A) was added to the pooled lipids (Fig. 4B). The abs. unit in the 1B-His binding simply corresponded to the content of standard Gb3, and no significant difference was observed between samples with or without combined lipids (Fig. 4C). These data indicated that no marked effect of other lipids on the 1B-His binding assay and low Gb3 content in HeLa cells can be correctly determined. The specificity of the 1B-His binding was further determined by TLC-blotting (Fig. 5). Iatrobeads-binding fractions from human cells, human plasma and KO-mouse heart were applied to TLC plate. More than 5 kinds of neutral glycolipids were detected in all Iatrobeads-binding fractions by the orcinol-staining

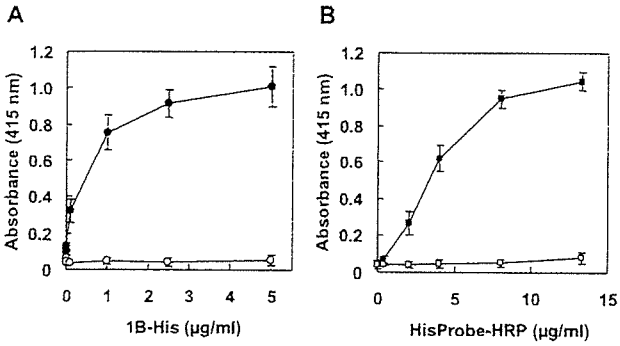

Fig. 2. Optimum Concentration of 1B-His (A) and HisProbe-HRP (B)

In A, wells with (●) or without (○) 100 ng of standard Gb3 were treated with indicated concentrations of 1B-His for 30 min. Bound 1B-His was detected by 8 μg/ml HisProbe-HRP. In B, wells coated with 100 ng of Gb3 were treated with (■) or without (□) 1 μg/ml of 1B-His. Bound 1B-His was determined by indicated concentrations of HisProbe-HRP. The error bars represent S.D. ($n=3$).

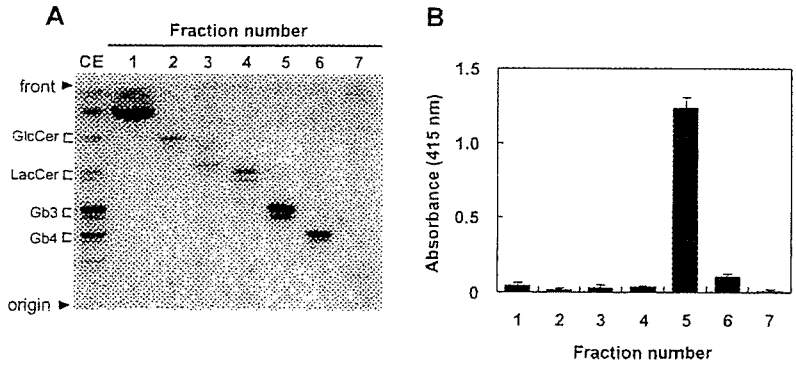

Fig. 3. 1B-His Binding to Glycosphingolipids Fractionated by HPLC from HeLa Cells

In A, glycosphingolipids in Folch's lower fraction extracted from HeLa cells were separated through low-pressure HPLC on an Iatrobeads 6RS-8010 column. TLC analysis was quantitatively performed with a solvent system of chloroform–methanol–water (60 : 35 : 8), and glycosphingolipids were visualized by orcinol reagent as described in Experimental. CE is crude extract in Folch's lower fraction extracted from HeLa cells. In B, the 1B-His binding assay of each glycosphingolipid fraction was performed. 1/10 aliquot of each fraction suspended in 100 μl of methanol was applied on a multi-well H type plate, and assayed as described in the legend to Fig. 1. Experiments were performed in triplicate, and the means ± S.D. of the values are indicated.

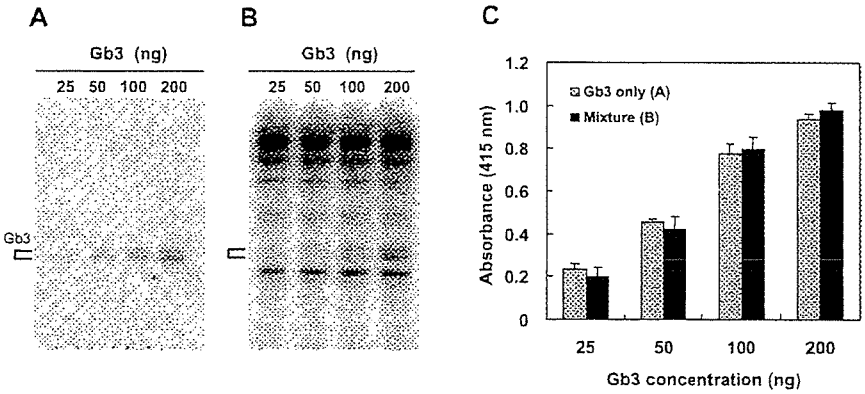

Fig. 4. Effect of All Components Other Than Gb3 in Folch's Lower Fraction from HeLa Cells on the 1B-His Binding Assay

Standard Gb3 at indicated concentrations with (B) or without (A) all components other than Gb3 in Folch's lower fraction from HeLa cells were applied onto TLC, and after its development with chloroform–methanol–water (60 : 35 : 8) TLC plates were stained with orcinol reagent. In C, the 1B-His binding assay of the same samples used in A and B was performed as described in the legend to Fig. 1. Experiments were performed in triplicate and the means ± S.D. of the values indicated.

(Fig. 5A). 1B-His strongly bound to Gb3 in all samples, and weak binding to Gb4 was also observed in HeLa and human fibroblasts; but no other binding was observed in the glycolipids from human and mouse sources (Fig. 5B).

Effect of Sample Preparation on the 1B-His Binding Assay For a practical application of the 1B-His binding assay, the effect of a sample preparation on the assay condition was studied (Fig. 6). The glycolipids were sequentially prepared from human cells and plasma, and mouse tissue, and pooled as a crude extract fraction, a Folch's lower fraction and an Iatrobeads-binding fraction. All Iatrobeads-binding fractions showed good correlation between sample concentration and the 1B-His binding, but no correlation was observed in all crude extract fractions. Although Folch's lower fraction from human plasma did not show linear correlation, Folch's lower fractions from human cells and mouse tissue corresponded well with Iatrobeads-binding fractions from respective sources. This data indicated that a Folch's lower fraction may be suitable preparation for the 1B-His binding assay, but not for human plasma.

Comparison with Another Assay Method Gb3 content was determined in 4 different sources by the 1B-His binding assay and the results were compared to the data from the TLC-orcinol method (Table 1). The mean of Gb3 content determined by the 1B-His binding assay in HeLa cells, human fibroblasts and human plasma from a heterozygous patient with Fabry disease, and a heart from a KO mouse (27.3 μg/mg protein, 37.4 μg/mg protein, 11.1 μg/ml and 7.5 μg/mg protein, respectively), corresponded well with the values measured by the TLC-orcinol method (25.9 μg/mg protein, 34.7 μg/mg protein, 13.9 μg/ml and 6.3 μg/mg protein, respectively). Although the TLC-orcinol method was reproducible, a high amount of Gb3 (100—500 ng) was necessary for the assay; while, 10—50 ng Gb3 was enough for the 1B-His binding assay.

Other Applications of the 1B-His Binding Assay Blocking of 1B-His binding to Gb3 occurred with the addition of a high concentration of galactose but not mannose (Fig. 7). The abs. unit was decreased to 40% of the control by the addition of 300 mM galactose. These inhibition data indicated that 1B-His binding to Gb3 was specific to galactose.

The possibility that the 1B-His binding assay will be applicable to the measurement of Gb3 synthase (α1,4-galactosyltransferase) activity was studied. HeLa-cell homogenate was incubated with 0.5 mM UDP-Gal and 0.2 mM lactosylceramide (LacCer) at 37 °C for the indicated period. Folch's lower fraction was then prepared from each reaction solution and Gb3 content determined by the 1B-His binding assay (Fig. 8). The increase in Gb3 content was 3.2, 6.8, and 9.4 (μg/mg protein), after incubation for 1, 2 and 4 h, respec-

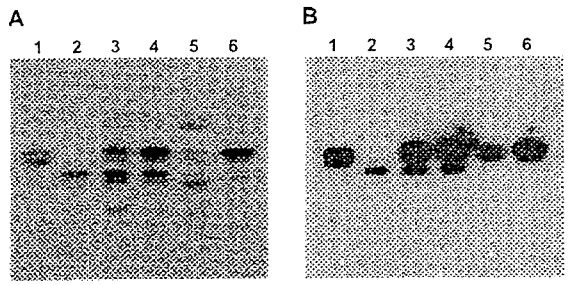

Fig. 5. 1B-His Binding on Glycosphingolipids Separated by TLC of Iatrobeads-Binding Fractions from Human Cells and Plasma, and Mouse Heart

Glycosphingolipid samples were applied to a HPTLC plate, and separated by a solvent system of chloroform : methanol : water (60 : 35 : 8). After the fixation of the plate with 0.4% PIM solution, TLC-blotting with 1B-His (B) was performed as described in Experimental. After the determination of 1B-His binding with chemiluminescent substrate, the same TLC plate was stained with orcinol reagent (A). Lanes 1 and 2, 500 ng of standard Gb3 and Gb4, respectively: lanes 3, 4, 5 and 6, Iatrobeads-binding fractions from HeLa cells, human fibroblasts, human plasma and the heart from KO mouse, respectively.

Table 1. Comparison with Gb3 Contents Determined by the Assay Methods of TLC-Orcinol and the 1B-His Binding

Sample	TLC-orcinol	1B-His binding
HeLa cells	25.89±2.94	27.28±0.88
Human fibroblasts	34.68±3.08	37.36±0.64
Human plasma	13.91±2.66	11.13±1.70
Mouse heart	6.28±1.58	7.52±0.57

Folch's lower fractions of human cells and mouse heart, and the Iatrobeads-binding fraction of human plasma were used as sample sources for both assays. The determination of Gb3 content by the TLC-orcinol and 1B-His binding assays was performed as described in Experimental. Values are expressed as μg/mg of protein±S.D. for human cells and mouse heart, and as μg/ml of plasma±S.D. for human plasma.

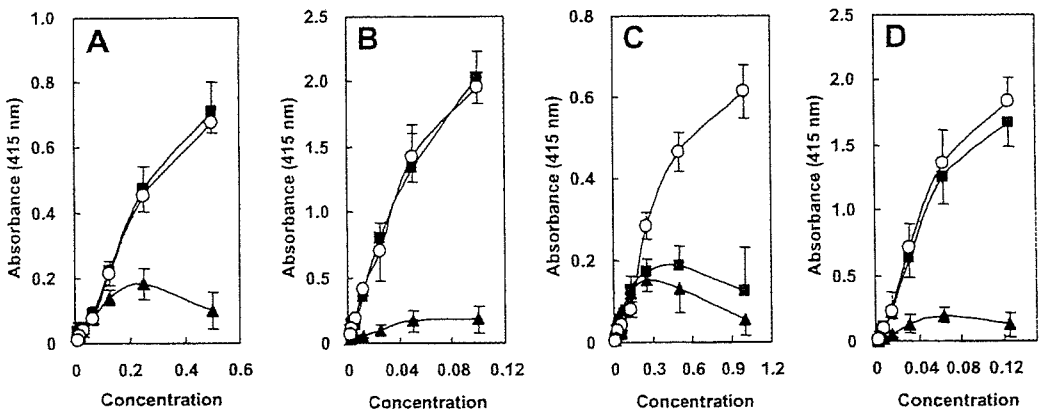

Fig. 6. Effect of Preparation of Lipid Samples on 1B-His Binding Assay

Lipids were extracted from HeLa cells (A), human fibroblasts (B), human plasma (C) and heart from KO mouse (D). Preparation of crude extract fraction (▲), Folch's lower fraction (■) and Iatrobeads-binding fraction (○) was performed as described in Experimental. Doubled dilutions of lipid samples were dried on the plate and then assayed by 1B-His binding. Concentration 1 was corresponding to 5 ng protein content of HeLa cells, fibroblasts, mouse heart and 5 μl of human plasma. Results are mean±S.D.

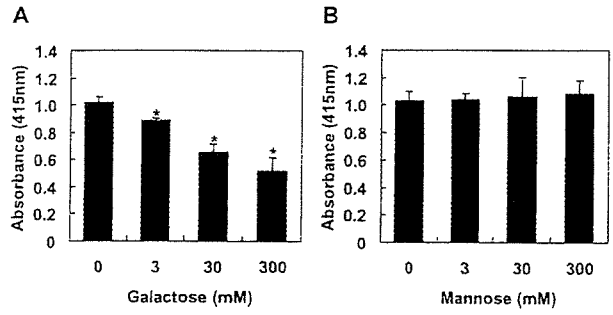

Fig. 7. Effect of Galactose and Mannose Addition in 1B-His Binding

1B-His in 0.2% BSA-PBS solutions (1 μg/ml) containing galactose (A) or mannose (B) at indicated concentrations was applied to a plate coated with 100 ng of standard Gb3. After incubation at 37°C for 30 min, bound 1B-His was assayed as described in the legend to Fig. 1. Data represent mean±S.D. of three different experiments per point. $*p<0.05$ (compared with concentration=0).

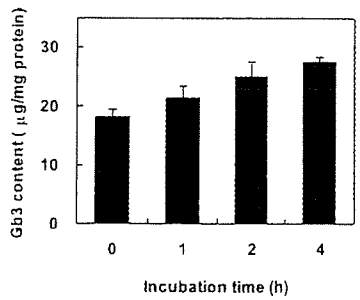

Fig. 8. The Application for Gb3 Synthase Assay

100 μl of homogenate from HeLa cells (5×10^6) was incubated at 37°C in assay mixture containing 25 mM citrate/phosphate (pH 6.0), 10 mM $MgCl_2$, 0.2 mM LacCer, 0.3% Triton X-100, 3 mM phosphatidylglycerol, 0.5 mM UDP-Gal. Folch's lower fractions extracted from incubated samples were applied to a multi-well H type plate, and the Gb3 content was standardized by the protein amount. The error bars represent the S.D. of independent assays ($n=5$).

tively. The linearity of the enzyme reaction was observed until 2 h of incubation. Gb3 synthase activity in HeLa cells was calculated at 2 h incubation as 3.4 μg of Gb3 production/h/mg protein. This result indicates that the 1B-His binding assay may be applicable for the determination of Gb3 synthase activity.

Discussion

Recent studies on Stx 1B structure revealed that three sites may be involved in the recognition of Gb3[20]; and one of the 3 sites around Phe30 might play an important role for the high affinity toward Gb3 binding.[21] Although AB_5 class toxin is a complex of an A-subunit and 5 B-subunits, the stability and structure of B-subunits were not altered by the binding of the A-subunit,[22] and the intracellular trafficking of B-subunits was independent of the A-subunit.[23] From the above information, 6 histidine residues at the carboxy termini of His-tagged Stx B may not disturb its binding. We used 1B-His for the detection of Gb3 because a higher affinity of 1B-His toward Gb3 was observed in comparison with 2B-His.[15] The His-tagging strategy is useful for the purification of recombinant proteins[24] and we tried to apply for their detection in our present study. HisProbe-HRP is a unique product for the detection of His-tagged protein. Both 1B-His and HisProbe-HRP provide us simple and specific methods for the determination of Gb3 content. The strategy of this assay method may be applicable for other binding assays, such as Cholera toxin B-subunit for ganglioside GM1.[25]

Although the ELISA method using verotoxin B subunit for the determination of Gb3 content was already established,[14] the assay procedure in our method (only two-step binding procedure) was simpler than the ELISA method (three steps). We believe minimum steps in a procedure have benefits not only for the quick assay but also for the specific assay. In a paper by Zeidner *et al.*,[14] cross reactivity was determined only with some glycosphingolipids (glucosylceramide, LacCer and Gb4); however we determined the effect of all components in Folch's lower fraction and proved the specificity of our assay method for Gb3 in HeLa cells (Fig. 4), and proved its specificity. For the practical application, we also determined the effect of sample preparation on the 1B-His binding assay. Surprisingly, the sample preparation was critical for the correct determination of Gb3 content by the 1B-His binding assay (Fig. 6). Folch's lower fraction was a suitable preparation for determination in human cells and mouse heart, but further purification was needed in human plasma. Correct binding may be disturbed by lipids other than glycosphingolipids in human plasma. We also confirmed that more than 95% of Gb3 in HeLa cells was recovered in Folch's lower fraction by the TLC-orcinol method (data not shown). From this information, we concluded that a simple and specific determination of Gb3 content in HeLa cells can be performed by 1B-His binding assay with Folch's lower fraction.

Gb3 is one of the potential glycosphingolipid receptors for Stx.[26] The cytotoxicity of Stx is highly selective toward cells that express Gb3 on the cell surface; and it is widely believed that endothelial cells expressing Gb3 are the main targets for Stx in STEC infection.[27,28] To prevent Stx cytotoxicity, Stx absorbents or binding neutralizers have been developed and were studied for clinical application.[29—31] In our present study, 1B-His binding with Gb3 was blocked by galactose but not mannose (Fig. 7). This data indicated that this 1B-His binding method can be applicable for the screening of Stx-binding neutralizer.

Another application of 1B-His binding was described in Fig. 8. The assay of Gb3 synthase activity was usually performed with radioactive materials.[32,33] Our present study indicated that a non-radioactive assay may be possible with the 1B-His binding assay, because the increase in Gb3 content was linear up to 2 h.

In this study, we established a new Gb3 assay method using a recombinant His-tagged Stx 1B-subunit. The data were reproducible and it was proved that a specific assay can be performed with Folch's lower fraction from HeLa cells. This simple and specific Gb3 assay method is very useful for the determination of effectiveness of various approaches for the treatment of Fabry disease. Recently, we described a new therapeutic strategy, active-site specific chaperone therapy for this disease.[34,35] The assay method established by this study will provide a crucial benefit for the preclinical and clinical trial of this treatment strategy. Knowledge of the physiological and pathological role of Gb3 will increase using this assay method.

Acknowledgments This work was supported in part by a research grant from the Ministry of Health, Labour and Welfare of Japan (S.I.).

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Bioorganic & Medicinal Chemistry Letters

Convenient synthesis of 3- and 6-deoxy-D-*myo*-inositol phosphate analogues from (+)-*epi*- and (−)-*vibo*-quercitols

Seiichiro Ogawa* and Yoji Tezuka

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

Received 26 December 2005; revised 7 April 2006; accepted 26 June 2006
Available online 10 August 2006

Abstract—Starting from (+)-*epi*- and (−)-*vibo*-quercitols readily produced by bioconversion of *myo*-inositol, some biologically interesting phosphate and polyphosphate analogues, including the Ins(1,4,5)P_3 derivatives of 3-deoxy- and 6-deoxy-D-*myo*-inositol, could be readily prepared in a conventional manner. In addition, chemical modification at C-2 of the 3-deoxy Ins(1,4,5)P_3 provided 2-epimer, and 2-deoxy and 2-deoxy-2-fluoro forms. Eight polyphosphate analogues obtained were assayed for biological activity against PDH-Pase and PDH-K, and G6Pase, but none proved positive.

In recent years, D-*myo*-inositol-1,4,5-trisphosphate Ins(1,4,5)P_3, as well as its bis and tetrakisphosphates, have been demonstrated[1] to play important roles as second messengers which control many cellular processes by generating internal calcium signals, which then diffuse through the cytosol and bind to receptors on the endoplasmic reticulum causing the release of calcium ions (Ca^{2+}) into the cytosol. Therefore, it is feasible that inhibitors of enzymes of the phosphoinositide cascade, involved in biosynthesis and degradation of Ins(1,4,5)P_3, could be of medicinal interest and also invaluable tools to elucidate the individual roles of metabolites in the regulation of cell function. In order to study biochemical and medicinal properties of these polyphosphates, a large number of analogues and derivatives have so far been synthesized[2] and their biological activity tested. Recent findings[3] of insulin-like and anti-inflammatory properties have also stimulated us to develop means for routine synthesis of these compounds (Fig. 1).

Bioconversion[4] of *myo*-inositol readily provides some inaccessible optically active deoxyinositols,[5] such as (+)-*epi*- and (−)-*vibo*-quercitols (**1** and **2**), in quantity, which might allow their application as starting materials for development of novel biologically active cyclitol derivatives. In preceding papers, we reported the synthesis of several anhydro[5] and some *C*-(aminomethyl)deoxyinositols,[6] and *O*-methyl-deoxyinosamines[7] as

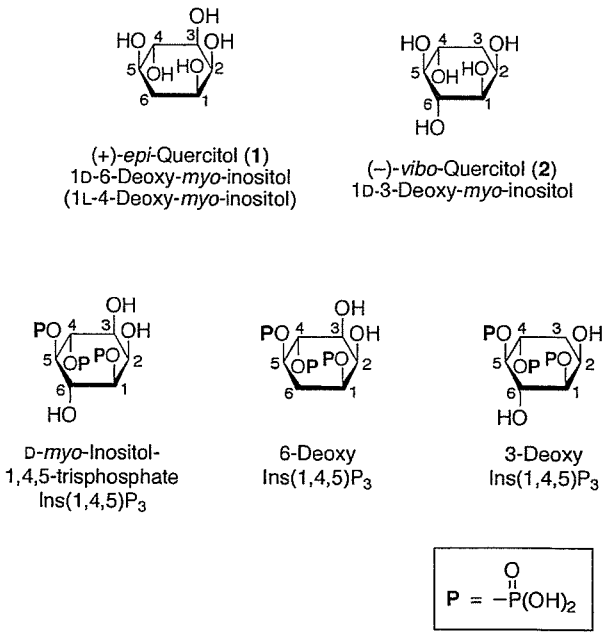

Figure 1. Deoxyinositols **1** and **2**, and Ins(1,4,5)P_3 and its deoxy derivatives.

Keywords: Inositol phosphates; *myo*-Inositol-1,4,5-trisphosphate; Deoxyinositol trisphosphate analogues; *myo*-Inositol bioconversion.
* Corresponding author. Tel.: +81 45 566 1788; fax: +81 45 566 1789; e-mail addresses: sogawa379@ybb.ne.jp; ogawa@bio.keio.ac.jp

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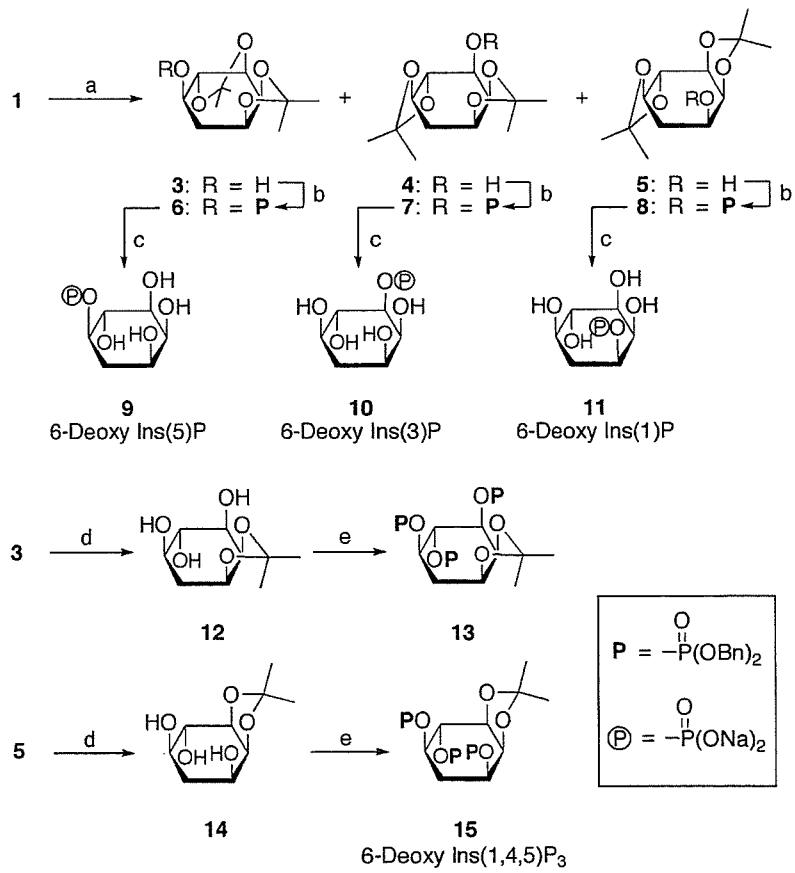
doi:10.1016/j.bmcl.2006.06.096

potential candidate new glycosidase inhibitors. In this letter, we describe convenient synthesis of a number of mono-, tris-, and tetrakisphosphate derivatives of 3- and 6-deoxy-D-*myo*-inositols. In addition, chemical modification at C-2 of the 3-deoxy Ins(1,4,5)P_3 was carried out to prepare 2-epimer, and 2-deoxy and 2-deoxy-2-fluoro derivatives. Furthermore, in order to provide certain trisphosphate mimics[8] designed by analogy with adenophostines,[9] the most potent agonists of Ins(1,4,5)P_3 receptor, the 1-phosphate function of the 3-deoxy Ins(1,4,5)P_3 was replaced with (phosphinyl)alkyloxyl groups (Schemes 1 and 2).

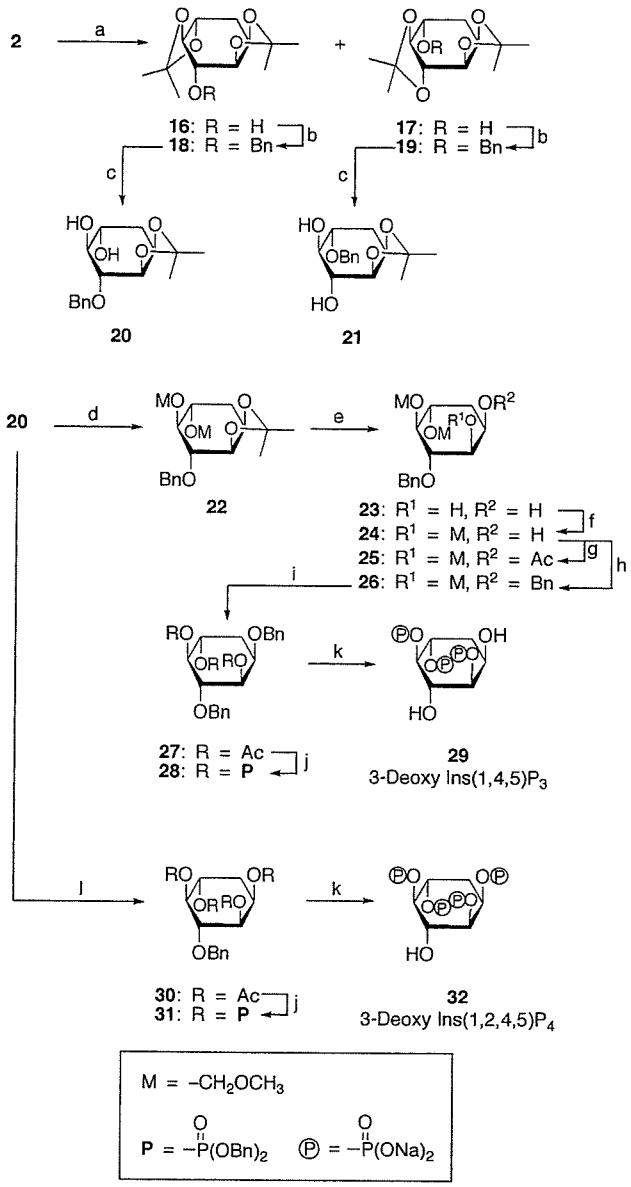
Recently, synthesis of several polyphosphate derivatives of 6-deoxy-D-*myo*-inositol[10] (**1**) has been elaborated[11] from precursors derived from D-galactose, and their biological activity assayed. 6-Deoxy Ins(1,4,5)P_3 is recognized by the highly selective 3-kinase,[12] the kinetics of its metabolism indicate that it is a substrate for this enzyme, with resultant competitive inhibition of phosphorylation of Ins(1,4,5)P_3.

We here describe a convenient preparative route from (+)-*epi*-quercitol[4] (**1**). Isopropylidenation[6] of **1** with an excess of 2,2-dimethoxypropane and TsOH in DMF was carried out at room temperature to give three readily separable di-*O*-isopropylidene derivatives **3** (24%), **4** (26%), and **5** (31%). These were treated with dibenzylphosphoryl chloride in pyridine at room temperature to give the respective dibenzylphosphates **6** (77%), **7** (86%), and **8** (87%), hydrogenolysis of which with PtO_2 in ethanol produced, after neutralization with cyclohexylamine, the respective phosphates as crystalline amine salts. Treatment of the salts with Dowex 50 W × 2 (H^+) resin gave the free phosphates **9** (88%), **10** (70%), and **11** (80%), isolated as bis-sodium salts.

Furthermore, compounds **3** and **5** could be partially de-O-isopropylidenated with TsOH in EtOH at 0 °C to give the triols **12** (70%) and **14** (78%), respectively. Possible contamination of **12** and **14** due to acid-catalyzed migration of the *cis*-isopropylidene groups was not observed. Compound **14** was similarly phosphorylated to give the protected precursor **15** (60%) of 6-deoxy Ins(1,4,5)P_3.[11] The structure of **15** was indirectly confirmed by the fact that ^1H NMR spectrum of isomeric trisphosphate **13** obtained for reference from **12** showed coupled signals (δ 4.31, ddd, J = 5.6, 8.9, and 13.9 Hz) and (δ 4.62, J = 5.1, and 8.9 Hz) due to H-1 and H-2 bonded to carbon atoms of the O-isopropylidene group. Thus, five biologically interesting phosphate analogues could be demonstrated to be readily available from (+)-*epi*-quercitol (**1**).



Scheme 1. Synthesis of some mono- and trisphosphates of 6-deoxy-D-*myo*-inositol. Reagents and conditions: (a) $(MeO)_2CMe_2$–DMF (1:2, v/v), TsOH, 6 h, rt; (b) $(PhO)_2POCl$ (1.5 M equiv), pyridine, 1 h, rt; aqueous 80% AcOH; (c) H_2, PtO_2, EtOH, rt; $C_6H_{11}NH_2$; Dowex 50 W × 2 (H^+) resin, 1 M NaOMe, MeOH; (d) TsOH, EtOH, pH ~ 4, 0 °C; (e) $(PhO)_2POCl$, DMAP, pyridine, rt; aqueous 80% AcOH.



Scheme 2. Synthesis of some tris- and tetrakisphosphates of 3-deoxy-*myo*-inositol. Reagents and conditions: (a) CH_2=C(OMe)CH_3, TsOH (0.1 M equiv), DMF, 3 h, rt; (b) NaH, BnBr, DMF; (c) CSA, MeOH, pH ~ 4, rt; (d) $MeOCH_2Cl$ (4 M equiv), diisopropylethylamine, DMF; (e) aqueous 80% AcOH; (f) Bu_2SnO (2 M equiv), tetrabutylammonium bromide; (g) Ac_2O, pyridine; (h) NaH, BnBr, DMF, rt; (i) 4 M HCl, 50 °C; Ac_2O, pyridine; (j) NaOMe, MeOH; i-$Pr_2NP(OBn)_2$ (6 M equiv), DMF, rt; *m*CPBA (10 M equiv), rt; (k) H_2, 10% Pd/C, aqueous EtOH; $C_6H_{11}NH_2$; Dowex 50 W × 2 (H^+) resin, NaOMe, MeOH; (l) aqueous 80% AcOH, 50 °C.

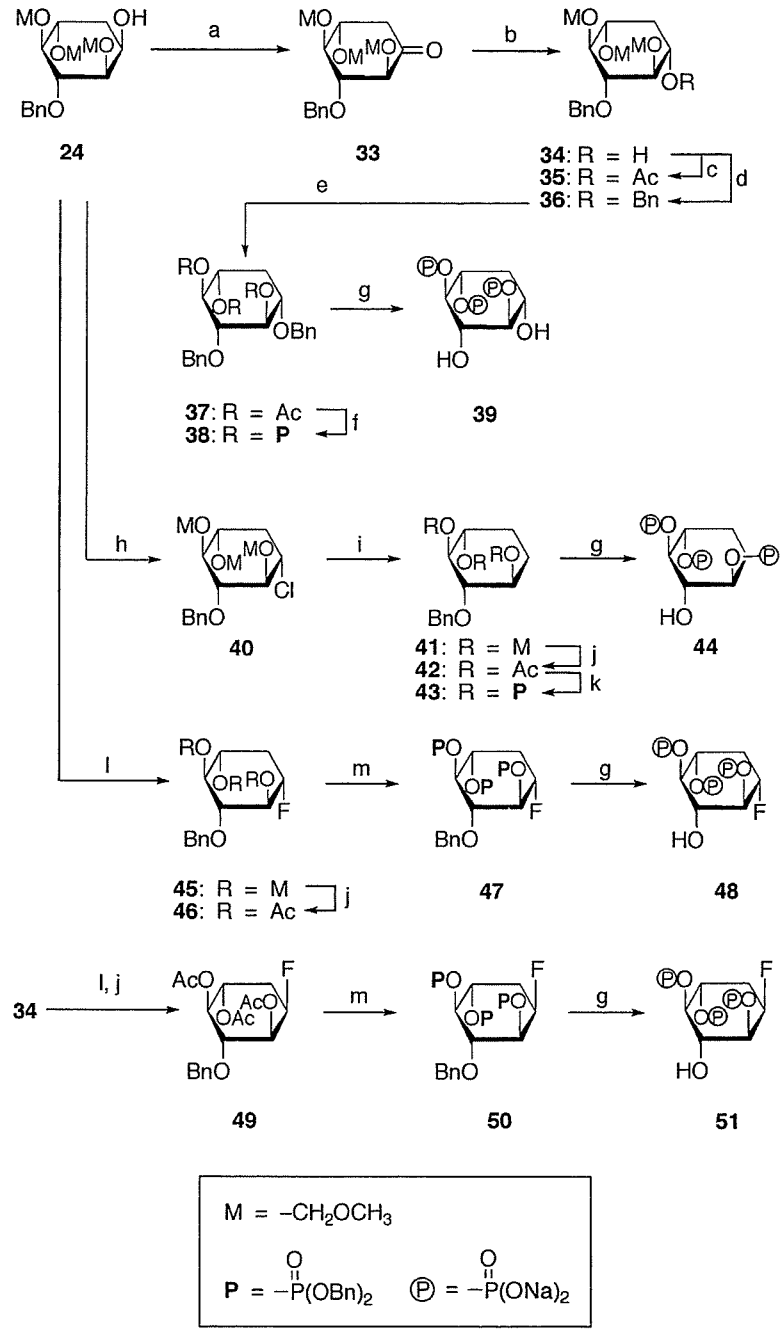
3-Deoxy-D-*myo*-inositol-(1,4,5)P_3 was first synthesized[13,14] from L-quebrachitol through a multi-step sequence and shown to be a good substrate of Ins(1,4,5)P_3-5-phosphatase (Schemes 2 and 3).

Isopropylidenation[5] of (−)-*vibo*-quercitol (**2**) was carried out by treatment with 2-methoxypropene in the presence of TsOH in DMF at room temperature. A mixture of the 1,2:4,5- **16** and 1,2:5,6-di-*O*-isopropylidene derivatives **17** was, without separation, treated with NaH in DMF and then with an excess of BnBr to give the benzyl ethers **18** and **19**, which were partially de-O-isopropylidenated under the influence of CSA in MeOH to afford, after separation over a silica gel column, the 4- and 6-*O*-benzyl derivatives **20** (55%) and **21** (42%). Compound **20** was treated with chloromethyl methyl ether and diisopropylethylamine to give the di-*O*-methoxymethyl derivative **22** (89%), de-O-isopropylidenation of which with 80% aqueous acetic acid gave the diol **23** (88%). Treatment of **23** with dibutyltin oxide and tetrabutyl ammonium bromide at 120 °C, and subsequent similar etherification, gave crude methoxymethyl ether **24** (87%). The structure of **24** could be fully characterized with the ^{1}H NMR spectrum of the *O*-acetyl derivative **25** (~100%) obtained. In addition, **24** was conventionally benzylated to give the 2-*O*-benzyl derivative **26** (91%). The methoxymethyl groups of **26** were removed by treatment with 4 M hydrochloric acid, and the product was subsequently acetylated to give the tri-*O*-acetyl derivative **27** (90% over-all yield). Compound **27** was treated with methanolic sodium methoxide under Zemplén conditions, and the resulting triol was phosphorylated with dibenzyl diisopropylphosphoro-amidite in DMF, and, then the reaction mixture was treated with *m*CPBA. The product was isolated by chromatography on silica gel to afford the 1,4,5-tris(dibenzylphosphate) **28** (93% over-all yield). Hydrogenolysis of **28** in the presence of 10% Pd/C in aqueous ethanol at room temperature gave the trisphosphate, which was purified by treatment with cyclohexylamine to produce a crystalline salt. This compound was deaminated by passage through a column of Dowex 50 × 2 (H^+) resin to afford the free phosphate isolated as a bis-sodium salt **29** (97%).

The tetra-*O*-acetyl derivative **30** (98%), obtained similarly from **20**, was deacetylated and a crude alcohol was dibenzylphosphorylated to give tetrakisbenzylphosphate **31** (70% over-all yield). It was deprotected and the product was obtained as a bis-sodium salt[15] **32** (83%).

Oxidation of compound **24** with pyridinium chlorochromate in the presence of molecular sieves 4 Å in CH_2Cl_2 gave the deoxyinososе derivative **33** (96%), which was reduced with sodium borohydride to give the 2-epimer **34** (56%) as a major product. The structure of **34** was confirmed by the ^{1}H NMR spectrum of the 2-*O*-acetyl derivative **35**. Compound **34** was converted into the 2-epimer, 3-deoxy-*scyllo* Ins(1,4,5)P_3 **39**, following the standard sequence of reactions [→**36** (95%) →**37** (90%) →**38** (56%) →**39** (97%)].

Treatment of **24** with sulfuryl chloride in pyridine in the presence of DAMP gave the chloride **40** (70%). This compound was treated with tributyltin hydride in the presence of AIBN to provide the 2-deoxy derivative **41** (94%). Starting from **41**, 2,3-dideoxy Ins(1,4,5)(P_3)[14] **44** was obtained [→**42** (96%) →**43** (86%) →**44** (~100%)] in a conventional manner. Fluorination of **24** with dimethyl amino sulfur trifluoride (DAST) in CH_2Cl_2 afforded the 2-deoxy-2-fluoro derivative **45** (70%), which was converted into the tri-*O*-acetyl derivative **46** (95%). This acetate **46** was deacylated and conventionally phosphorylated to give the trisphosphate **47**, which was deprotec-

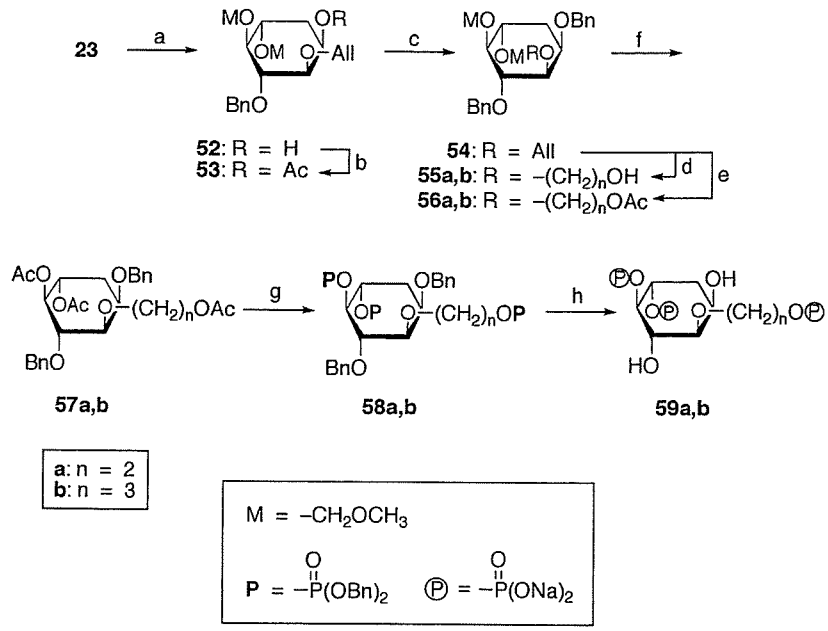


Scheme 3. Chemical modification at C-2 of 3-deoxy Ins(1,4,5)P_3. Synthesis of deoxy and deoxyfluoro derivatives. Reagents and conditions: (a) PCC, MS 4 Å, CH_2Cl_2, rt; (b) $NaBH_4$, EtOH; (c) Ac_2O, pyridine; (d) NaH, BnBr, DMF; (e) $MeOCH_2Cl$, diisopropylethylamime, DMF; Ac_2O, pyridine; (f) NaOMe, MeOH; dibenzyldiisopropylphosphoro-amidite, DMF; (g) H_2, 10% Pd/C, aqueous EtOH; $C_6H_{11}NH_2$, Dowex 50 W × 2 (H^+) resin, NaOMe, MeOH; (h) $SOCl_2$, DAMP, pyridine; (i) Bu_3SnH, AIBN, toluene, 120 °C; (j) Ac_2O, pyridine; (k) 4 M HCl, THF; phosphorylation; (l) DAST, CH_2Cl_2, rt; (m) aqueous 80% AcOH; $(PhO)_2POCl$.

ted to give the deoxyfluoro derivative[15] **48** (70% over-all yield). Similarly, the alcohol **34** was transformed into the deoxyfluoro derivative[15] **51** (80% over-all yield) via **49** and **50**.

Next, adenophosphine[9] analogues of 3-deoxy Ins(1,4,5)P_3 were prepared. Several permeant analogues of Ins(1,4,5)P_3 have been synthesized[8] and their ability to cross the membrane tested with vasopressin cells (Scheme 4).

Compound **23** was treated with dibutyltin oxide in toluene, and then after addition of allyl bromide, the mixture was heated at reflux temperature to give the allyl ether **52** (90%), which was characterized as the acetate **53** (96%) as a syrup. Compound **52** was converted into the benzyl ether (→**54**, 82%), which was subjected to ozonolysis in CH_2Cl_2/MeOH, followed by reduction with $NaBH_4$ (→**55a**) and conventional acetylation to give the 2-acetoxyethyl derivative **56a** (60% over-all yield). Hydrolysis of **56a** with 4 M



Scheme 4. Chemical modification of the C-2 function of 3-deoxy Ins(1,4,5)P_3. Reagents and conditions: (a) Bu_2SnO, TBAB, toluene, 120 °C; CH_2=CHBr; (b) Ac_2O, pyridine; (c) NaOMe, MeOH; NaH, BnBr, DMF; (d) (→**55a**) O_3, CH_2Cl_2/MeOH, −78 °C; $NaBH_4$, MeOH; (→**55b**) BH_3/THF, THF; H_2O_2; (e) Ac_2O, pyridine; (f) 4 M HCl, THF; Ac_2O, pyridine; (g) NaOMe, MeOH; i-$Pr_2NP(OBn)_2$, 1*H*-Tetrazole, DMF; mCPBA; (h) H_2, 10% Pd/C, aqueous 30% EtOH; $C_6H_{11}NH_2$, Dowex 50 W × 2 (H^+) resin, 1 M NaOMe, MeOH.

hydrochloric acid in THF gave, after conventional acetylation, the acetate **57a** (80%). Conventional de-O-acetylation of **57a** gave the diol, which was treated with 1*H*-tetrazole in DMF and then with dibenzyliso-propylphosphoro-amidite to give the protected tris-phosphate **58a** (72% over-all yield). Hydrogenolysis of **58a** in aqueous ethanol in the presence of 10% Pd/C gave the trisphosphate[15] **59a** (~100%) isolated as a syrupy sodium salt.

Hydroboration of **54** was accomplished with boran–THF complex in THF, and then the reaction was quenched by addition of water, followed by treatment with 35% aqueous hydroperoxide for 30 min (→**55b**). The product was isolated as the acetate **56b** (~100%) and similarly converted in turn into the trisphosphate[15] **59b**.

None of the eight compounds **29**, **32**, **39**, **44**, **48**, **51**, and **59a,b** activated pyruvate dehydrogenase phosphatase (PDH-Pase), or inhibited pyruvate dehydrogenase kinase (PDH-K) significantly. None of the compounds tested inhibited glucose 6-phosphatase (G6Pase) significantly.

Compounds **59a** and **59b** appeared to inhibit lipogenesis (Multi-Well Plate cell based assay). However, the inhibition decreased with increasing concentrations of the test compounds. When tested in vivo in streptozotocin diabetic mice (a model of type I diabetes) at a dose of 1 mg/kg, neither **59a** nor **59b** had any acute lowering effect on blood glucose. Furthermore, when administered chronically at a dose of 1 mg/kg/day for 10 days, **59a** did not significantly lower blood glucose.

Acknowledgments

The authors sincerely thank Ms. Lili Zhao for performing elemental analyses, Drs. Atsushi Takahashi and Kiyoshi Sato (Hokko Chemical Industry Co. Ltd, Atsugi, Japan) for providing (+)-*epi*- and (−)-*vibo*-quercitols, Drs. James W. Stygall and Khalid Gumaa (Rodaris Pharmaceuticals Ltd, UK) for carrying out a part of the biological assays, and Ms. Miki Kanto for her assistance in preparing this manuscript.

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15. ^{1}H NMR (300 MHz) data for compound **32** (in D_2O); $[\alpha]_D^{21}$ −19 (*c* 3.7, H_2O); δ 4.50–4.47 (m, 1H, H-2), 4.23–4.12 (m, 1H, H-4), 3.90–3.81 (m, 2H, H-1, H-5), 3.70 (dd, 1H, $J_{5,6}$ = 9.2 Hz, $J_{1,6}$ = 9.6 Hz, H-6), 2.27 (ddd, 1H, $J_{2,3eq}$ = 4.4 Hz, $J_{4,3eq}$ = 4.7 Hz, J_{gem} = 14.4 Hz, H-3eq), 1.52 (m, 1H, H-3ax). [M−H] *m/z* 482, [M+Na−2H] *m/z* 505, $[M-2H]_2$ *m/z* 241; for compound **48** (in D_2O); $[\alpha]_D^{21}$ +7.3 (*c* 2.5, H_2O); δ 4.53–4.29 (m, 1H, H-5), 4.06–3.85 (m, 3H, H-1, H-2, H-4), 3.43 (dd, 1H, $J_{2,3}$ = 8.5 Hz, $J_{3,4}$ = 8.8 Hz, H-3), 2.41–2.35 (m, 1H, H-6eq), 1.75–1.65 (m, 1H, H-6ax). [M−H] *m/z* 405, [M+Na−2H] *m/z* 427, $[M-2H]_2$ *m/z* 202; for compound **51** (in D_2O); $[\alpha]_D^{21}$ +7.5 (*c* 1.4, H_2O); δ 4.90 (d, 1H, $J_{2,F}$ = 48.6 Hz, H-2), 4.16–4.02 (m, 1H, H-4), 3.98–3.82 (m, 2H, H-1, H-5), 3.67 (dd, 1H, $J_{1,6} = J_{5,6}$ = 9.5 Hz, H-6), 2.41–2.30 (m, 1H, H-3eq), 1.60 (dddd, 1H, $J_{2,3ax}$ = 2.2 Hz, $J_{3ax,4}$ = 12.9 Hz, J_{gem} = 13.7 Hz, $J_{3ax,F}$ = 46.9 Hz, H-3ax). [M−H] *m/z* 405, [M+Na−2H] *m/z* 427, $[M-2H]_2$ *m/z* 202; for compound **59a** (in $CDCl_3$); $[\alpha]_D^{23}$ +17 (*c* 2.4, $CHCl_3$); δ 7.34–7.01 (m, 40H, 8 × Ph), 5.08 and 4.44 (m, 18H, H-4, H-5, 8 × CH_2Ph), 4.03–3.98 (m, 2H, 2 × H-2′), 3.89 (dd, 1H, $J_{3,4}$ = 9.4 Hz, $J_{2,3}$ = 9.8 Hz, H-3), 3.78 (br, 1H, H-1), 3.60–3.55 (m, 2H, 2 × H-1′), 3.26 (dd, 1H, $J_{1,2}$ = 2.7 Hz, $J_{2,3}$ = 9.8 Hz, H-2), 2.61 (ddd, 1H, $J_{1,6eq} = J_{5,6eq}$ = 4.4 Hz, J_{gem} = 13.9 Hz, H-6eq), 1.39–1.28 (m, 1H, H-6ax); and for compound **59b** (in $CDCl_3$); $[\alpha]_D^{26}$ −129 (*c* 1.3, $CHCl_3$); δ 7.36–7.01 (m, 40H, 8 × Ph), 5.08 and 4.56 (m, 17H, H-5, 8 × CH_2Ph), 4.52 (dd, 1H, $J_{4,5}$ = 9.0 Hz, $J_{3,4}$ = 9.3 Hz, H-4), 4.05–3.95 (m, 2H, 2 × H-3′), 3.87 (dd, 1H, $J_{3,4}$ = 9.3 Hz, $J_{2,3}$ = 9.5 Hz, H-3), 3.76 (br, 1H, H-1), 3.75–3.38 (m, 2H, 2 × H-1′), 3.22 (dd, 1H, $J_{1,2}$ = 2.7 Hz, $J_{2,3}$ = 9,5 Hz, H-2), 2.65 (ddd, 1H, $J_{1,6eq} = J_{5,6eq}$ = 4.4 Hz, J_{gem} = 14.4 Hz, H-6eq), 1.83–1.79 (m, 2H, 2 × H-2′), 1.39–1.30 (m, 1H, H-6ax).

Journal of Carbohydrate Chemistry, 25:69–93, 2006

ISSN: 0732-8303 print 1532-2327 online
DOI: 10.1080/07328300600572907

Preparation of Building Blocks for Carba-Oligosaccharides: Some Protected 5a′-Carba-D-hexopyranosyl-1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitols, and 5a,5a′-Dicarba Congeners Thereof

Seiichiro Ogawa and Yoshiyuki Senba

Department of Biosciences and Informatics, Faculty of Science and Engineering, Keio University, Yokohama, Japan

Four configurational types of two protected *O*-linked (5a-carba-D-hexopyranosyl)-D-glucal and carba-D-glucal derivatives were prepared in order to provide versatile synthetic intermediates readily convertible into carba-oligosaccharides of biological interest. These compounds may also find application as donors for elongation of carba-oligosaccharide chains having *O*-linked carbahexopyranose residues at nonreducing ends.

Keywords Carbohydrate mimics, Carbasugars, Carba-oligosaccharides, 5a′-Carba- and 5a,5a′-dicarbadisaccharides, *O*-linked

INTRODUCTION

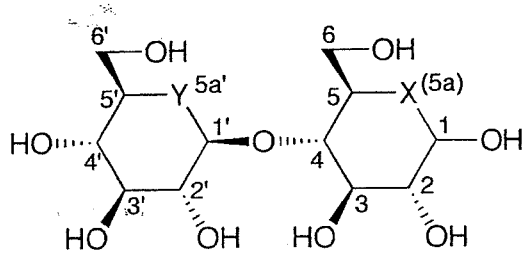
Recently, 5a-carbasugars[1–3] as well as *N*- and *O*-linked 5a′-carbadisaccharides have been shown to act as substrate analogs[4–6] and/or inhibitors[7] for some

Received September 27, 2005; accepted January 5, 2006.
Address correspondence to Seiichiro Ogawa, Department of Biosciences and Informatics, Faculty of Science and Engineering, Keio University, Hiyoshi, Kohoku-ku, Yokohama, 223-8522 Japan. E-mail: sogawa379@ybb.ne.jp

glycosyltransferases involved in oligosaccharide chain synthesis. Therefore, taking advantage of these distinct features of carba-oligosaccharides, much attention has been focused on the development of new glycosyltransferase inhibitors.

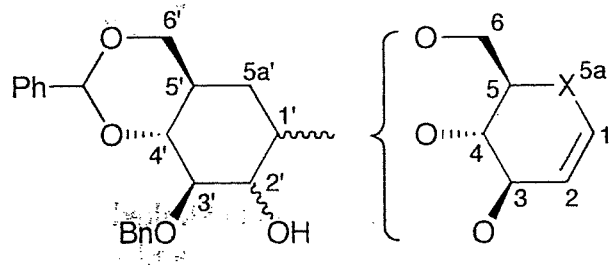
There are three types of carba-disaccharides; taking carbacellobiose as an example, type A is composed of two carbaglucopyranoses, and types B and C consist of one carba- and one true-glucopyranoses (Fig. 1). In the present study, versatile protected intermediates were prepared systematically, aiming at provision of conventional sequences for design of biologically interesting carba-oligosaccharides of types A and B, resistant to enzymatic hydrolysis. For strategic reasons the use of reactive D-glucal and carba-D-glucal as precursors for reducing ends is desirable to minimize the crucial glycosylation steps for elongation of oligosaccharide chains.

Attempts were made to prepare carba-disaccharides by allowing 1,2-anhydro-3-*O*-benzyl-4,6-*O*-benzylidene-5a-carba-β-D-mannopyranose[8] (**1**) to couple with some protected glucal and carbaglucal derivatives **2**–**6** (Fig. 2).



Three types of *O*-linked carbadisaccharides:

A: 5a,5a'-Dicarba: X = Y = CH_2
B: 5a'-Carba: X = O, Y = CH_2
C: 5a-Carba: X = CH_2, Y = O



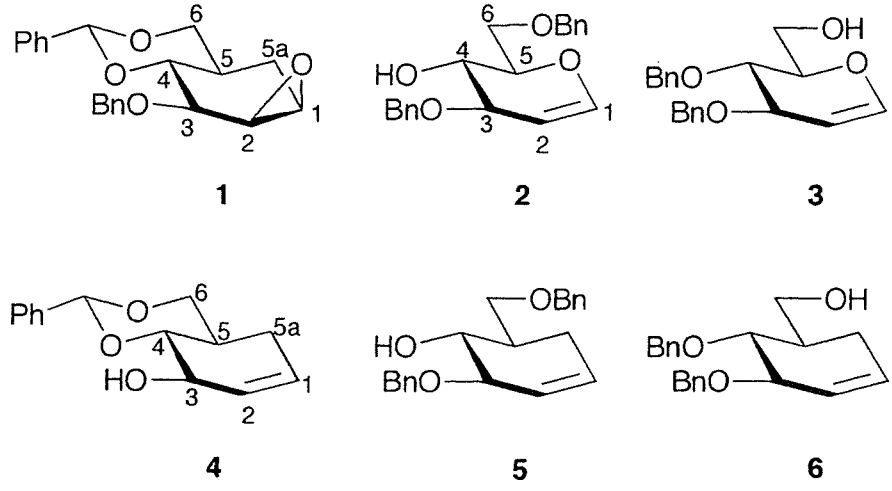
α,β-5aCGlc

α,β-5aCMan

X = O
X = CH_2

Protecting groups:
benzyls or benzylidene

Figure 1: Some protected 5a'-carba- and 5a,5a'-dicarba-disaccharide derivatives synthesized in the present study.



1 **2** **3**

4 **5** **6**

Figure 2: The 5a-carba-D-mannopyranosyl donor **1** and five acceptors **2**–**6**.

The configurations of 5a-carba-α-D-mannopyranose residues in the obtained condensates were then structurally modified through consecutive steps: first oxidation of the 2′-hydroxyl group, then base-catalyzed epimerization at C-1′, and finally selective reduction, to construct 5a-carba-α- and β-mannosyl and α-and β-glucosyl-glucal derivatives. These protected compounds may find use as acceptors or donors for chemical and/or biochemical preparation of complex carba-oligosaccharides of biological interest.

RESULTS AND DISCUSSION

Preparation of 5a′-Carbadisaccharide Derivatives

First, 4- and 6-*O*-unprotected derivatives of D-glucal were chosen as acceptors for coupling. Thus, 3,6-di-*O*-benzyl-D-glucal (**2**) was prepared from D-glucal under standard conditions.[9] Also 3,4-di-*O*-benzyl-D-glucal[10] (**3**) was generated from 6-*O*-(*tert*-butyldiphenylsilyl)-D-glucal.

Coupling of the epoxide **1** (1.5 molar equiv) and the oxide anion generated from compound **2** by treatment with an excess of NaH in dry DMF was carried out in the presence of 15-crown-5 ether at 60°C (Sch. 1). After treatment of the reaction mixture with acetic anhydride in pyridine, a mixture of products was easily separable by a silica gel column and the major product was isolated as the *O*-acetyl derivative **8a** (44%) in 87% yield based on **2** consumed. *O*-Deacylation of **8a** with a catalytic amount of methanolic sodium methoxide in methanol gave the alcohol **7a** (97%), which was shown to be identical with the major product observed in the coupling reaction mixture by TLC. The assigned structure of **8a** could be confirmed by ^{1}H NMR spectroscopy: resonated broad double doublets were present at δ 4.09 and 4.17 for H-1′ and H-2′ with