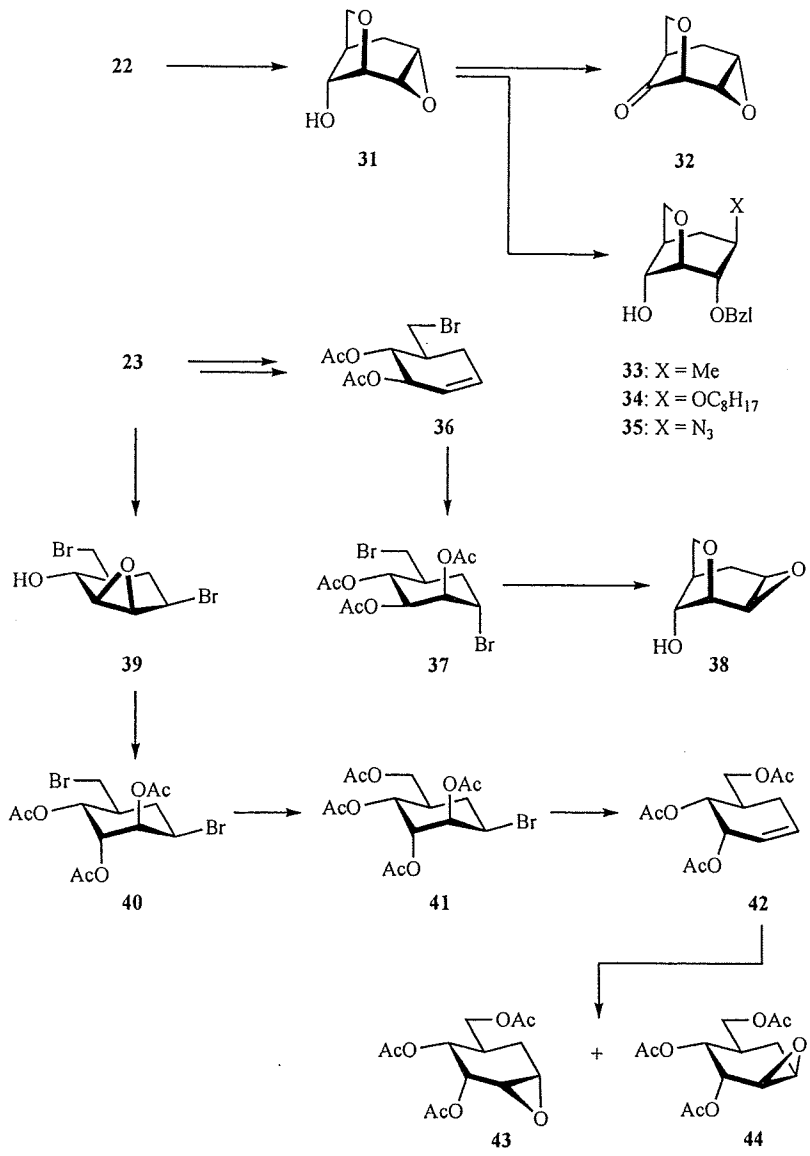


Fig. (5). Preparation of some anhydro and dianhydro-5a-carbahexopyranoses.

2-3. Systematic Synthesis from the *endo*-Adduct of Furan and Acrylic Acid

Peracid oxidation of the adduct **13** gave crystalline hydroxy lactone (**14**), which was reduced with LAH to give the triol, isolated as crystalline acetate (**16**) [16], Fig. (**3**). Similarly, through its bromo lactone (**15**), the bromodeoxy compound (**17**) was obtained. Acid cleavage of their 1,4-anhydro rings with H_2SO_4/AcOH afforded the corresponding 5a-carba-β-glucopyranose (**20** and **21**) and α-galacto-pyranose (**24** and **25**) in acceptable yields. Direct treatment of **14** and **15** with HBr/AcOH gave the bromodeoxy carba-uronic acids **18** and **19** [17]. On the other hand, similar bromination of **16** and **17** produced selectively the bromo-deoxy-5a-carbaglucopyranose derivatives **22** and **23** [18]. These bromo compounds underwent nucleophilic substi-tution with acetate, azide, and thiolate anions to produce precursors for aminodeoxy and deoxythio-5a-carbahexo-pyranoses. Stereochemical reaction courses were usually controlled on the basis of direct S_N1 in aprotic solvents and/or S_N2 reaction mechanisms through neighboring group participation. On other hand, elimination of **22** with DBU/ toluene afforded the conjugate 1,3-alkadiene, a versatile precursor for the 5,5a-unsaturated derivative, valienamine (**5**), and its analogues [19].

Anhydro and dianhydro derivatives, obtained by treatment of bromides with NaOMe/MeOH, provide access to new types of N- and O-linked carba-oligosaccharides. For example, coupling with protected carbasugars gave rise to a new type of 5a-carbadisaccharide, Fig. (**4**). Selective displacement of the 6-bromo group of **23** with an acetate ion gave the 1,2-dibromide **26**, which was debrominated with Zn/AcOH to afford the versatile carbaglucal-type derivative **27**. Peracid oxidation of **27** gave the α-gluco epoxide **29** as the major product, while the triol **28** gave the β-manno

epoxide **30** selectively through the *cis*-directing effect of the 3-hydroxyl group.

On treatment with NaOMe/MeOH, compounds **21** and **25** gave, through base-catalyzed epoxide-group migration, a hardly separable mixture of the epoxides.

Similar treatment of **22** with MeONa gave the versatile dianhydride **31**, which was transformed into the carbahexose intermediates **32–35**, Fig. (**5**). Starting from **23**, the isomeric dianhydride **38** was obtained through the alkene **36** and dibromide **37**. On the other hand, the isomeric dibromide **40** was provided through the epoxide **39**. The 1,2-anhydrides **43** and **44** were prepared through the alkene **42** derived from the bromide **41**.

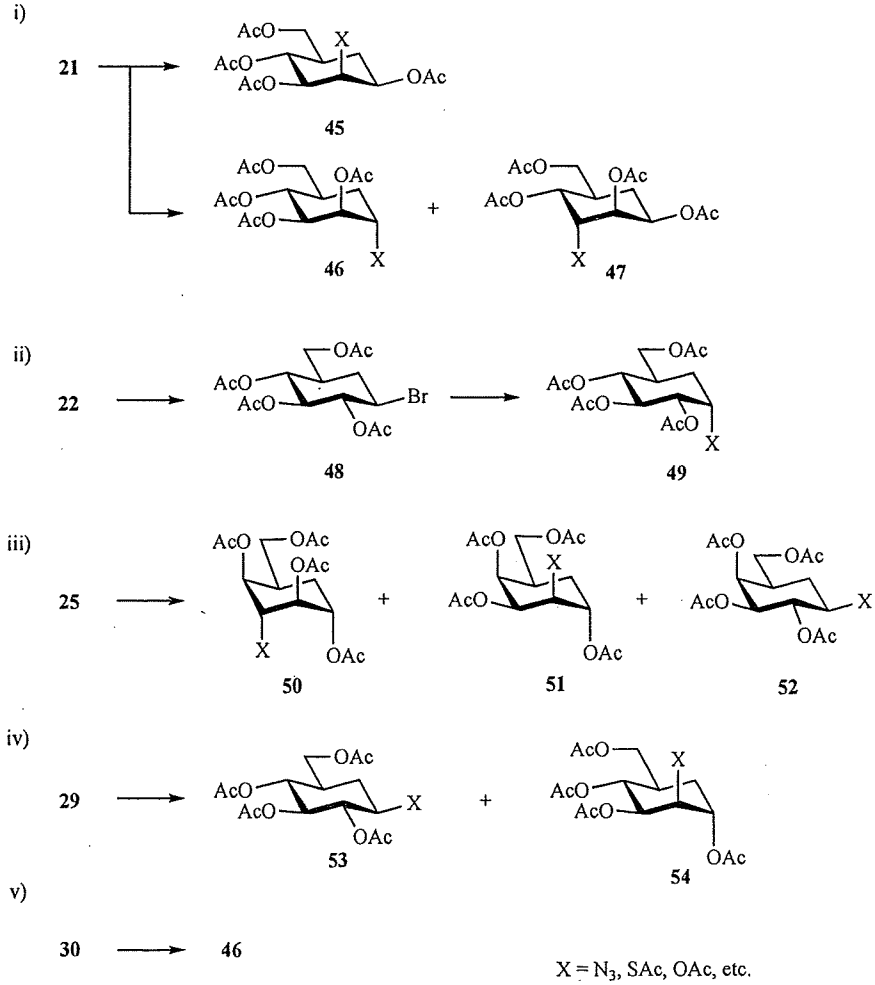
2-4. Design and Synthesis of Validamine and its Stereoisomers

These bromo and anhydro compounds can be used as reactive carbaglycosyl acceptors and/or donors in nucleophilic substitution reactions, as well as in coupling with NH_2 and OH unprotected carba and true sugar derivatives, affording N- and O-linked carba-oligosaccharides, Fig. (**6**).

Starting from the bromides **21**, **22**, and **25**, eight precursors **45-52** were generated for carbahexose derivatives, including α-manno **46**, α-gluco **49**, and β-galacto validamines **52**. The epoxy compounds **29** and **30** afforded β-*gluco* **53** and α-*manno* type compounds **46** and **54**.

Progressing from successful development of voglibose, Takeda Chemical Co. has been extensively engaged in chemical modification of the components supplied abundantly by degradation of validamycins.

The β-galacto [20], β-gluco [21], and α-manno validamines [22] (**4β**, **9β** and **10α**) were first synthesized by our group in racemic modification, starting from the *endo*-adduct **13**. Later, Kameda and coworkers prepared α-D-galacto and α-D-manno validamines (**D-9α** and **D-10α**) by chemical transformation of validamine, and assayed their inhibitory activity toward glycohydrolases in detail [23]. Compound **D-10α** was shown to be a moderate α-mannosidase inhibitor (IC_{50} = 56 μM, Jack beans; 36 μM, Almonds) and to possess high potency (K_i = 1.2 μM) against endoplasmic reticulum α-mannosidase. Furthermore, **D-9α** was found to be a weak α-galactosidase inhibitor.



* The reaction products were isolated and characterized as acetyl derivatives.

Fig. (6). Readily available 5a-carbahexopyranose derivatives* from synthetic precursors through nucleophilic substitution.

2-5. Design and Synthesis of N-Linked Carba-oligosaccharides and Carbaglycosylceramides

In order to determine biochemical features of carbaglycosylamines related to validamine (**4α**), we first carried out chemical modification of the bioactive core structures of validamycins and acarbose, Fig. (7). Concerning trehalase inhibition, synthesized α,α-trehalose type symmetric bis(validamine) (**55**) [24] was shown to possess similar activity to validoxylamine A, indicating that validamine residues play a role in mimicking carbaglycosyl cations generated from α,α-trehalose. However, maltose type 5a'-carbadisaccharide (**56**) [25a] was found not to be a potent α-glucosidase inhibitor, revealing the transition-state type unsaturated carbaglycosylamine valienamine (**5**) to be indispensable [25b]. In line with our preparative interest in carbasugars, synthesis of 2-acetamido-2-deoxyvalidamine and derivatives, related to *N*-acetyl-5a-carbaglucosamine, was attempted. Chitobiose type 5a'-carbadisaccahrides (**57** and **58**) might have been expected to possess some inhibitory activity, *e.g.* chitinase, but this was not the case [26]. Only 5a-carbamannopyranosylamine derivatives **59–61** were found to be weak to moderate α-mannosidase inhibitors [27]. The N-linked carbalactoside **62**, and *N*-acetyl-carbalactosaminide **63** and isolactosaminide **64** were prepared and assayed for inhibitory activity against β-galactosidase. Rather interesting biological features were revealed as substrate analogues for fucosyltransferase [28].

In other attempts to replace hexopyranose residues with carbahexopyranose in bioactive compounds, Fig. (**8**), the 5a'-carbatrehazolin (**65**), derived from the trehalase inhibitor trehazolin, the α-glucosylamine residue of which was replaced with 5a-carbaglycosylamine, was shown to preserve strong activity [29]. In the case of bioactive glycosylamides, *N*-octadecyl-5a-carbaglucosylamide (**66**) was demonstrated to have similar activity to the parent, indicating that 5a-carbaglucose could be useful as a mimic of sugar moieties [30]. These successful results with carbaglycosylamides stimulated us to apply our preparative know-how to research on glycolipid chemistry.

First attempts to substitute glycopyranose residues of glycosylceramides with carbaglycopyranose provided us with 5a-carbaglucosyl (**67a**) and galactosylceramides (**67b**) [31]. Biological assays indicated these to possess weak to moderate inhibitory activity against gluco and galactocerebrosidases. The results further led to finding of the respective unsaturated analogues **67c,d**, possessing strong and specific potential toward the corresponding glycocere-

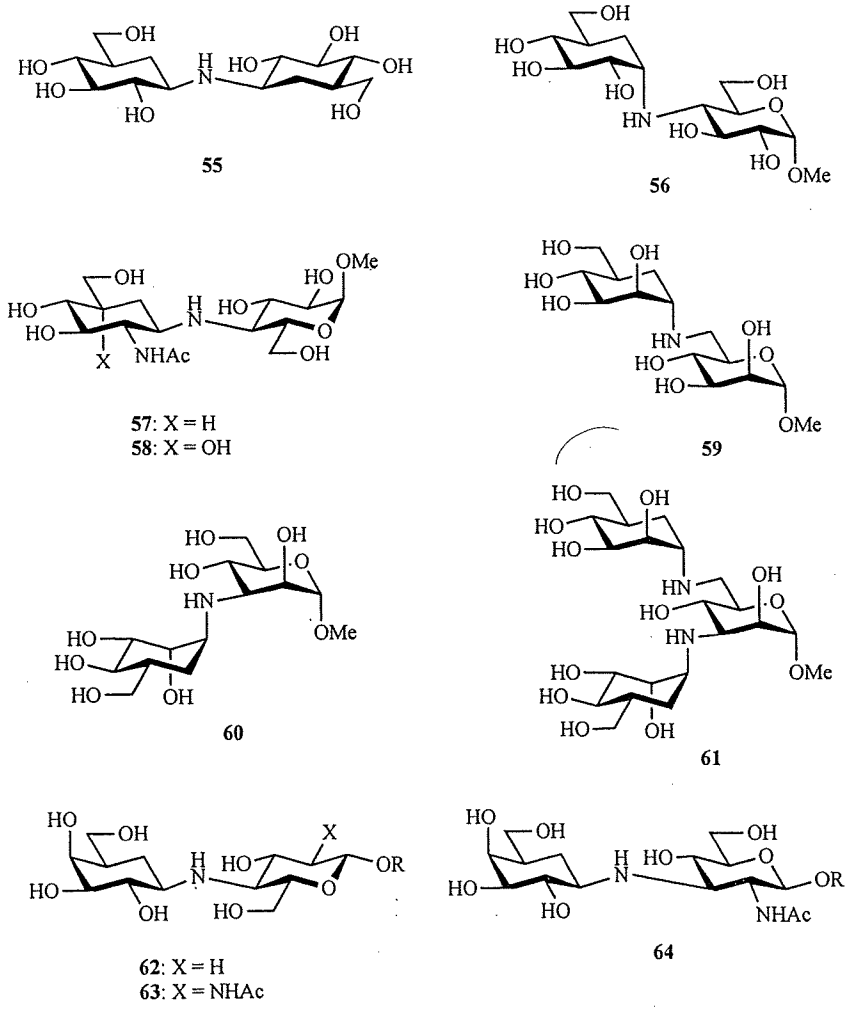


Fig. (7). Some N-linked 5a,5a'- and 5a'-carba-oligosaccharides classified as carbaglycosylamines.

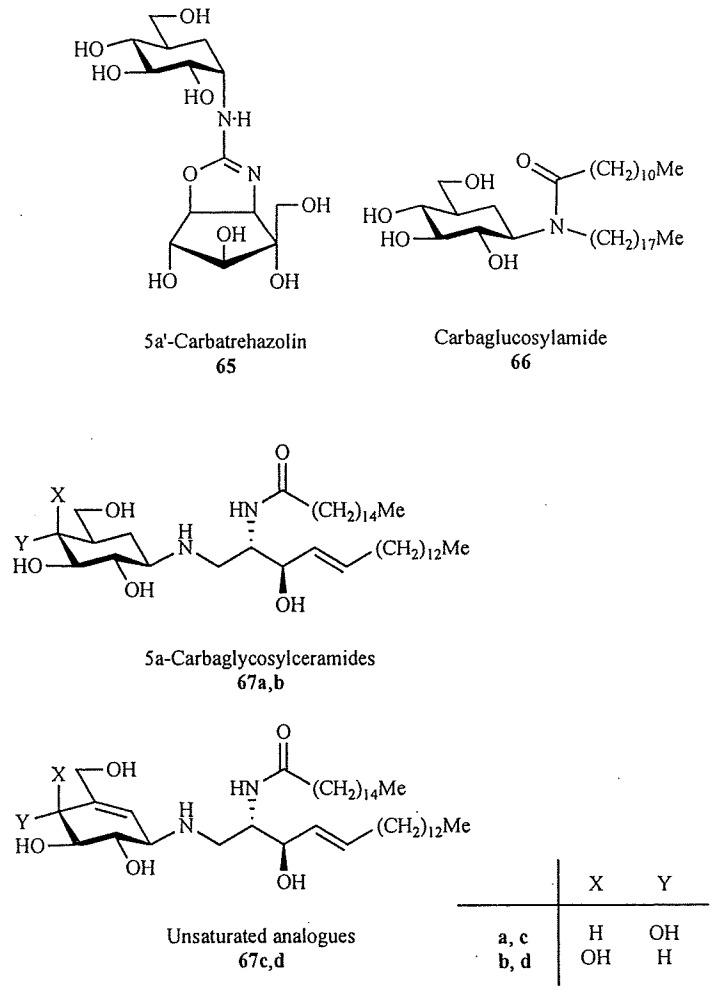


Fig. (8). 5a-Carbatrehazolin, carbaglucosylamide, and 5a-carbaglycosylceramides.

brosidases (mouse liver), IC_{50} 0.3 and 2.7 μM, respectively [32].

3. CARBAGLYCOSYLAMINE α-FUCOSIDASE INHIBITORS

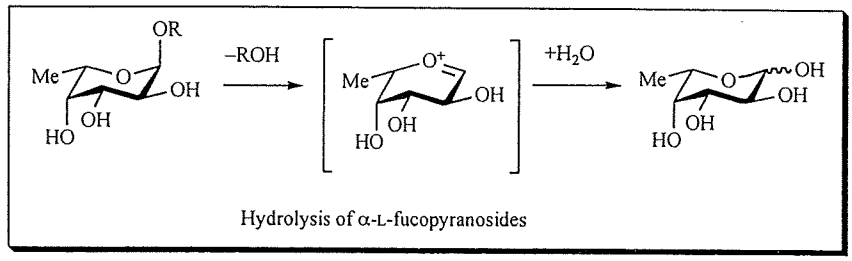
The inhibitory potency of validamine (**4**) stimulated our interest in studying biochemical features of other 5a-carbaglycosylamines with β-*gluco*-, α,β-*galacto*-, and αβ-*manno*- and α,β-*fuco* configurations, Fig. (**2**), expected to be potent inhibitors of some glycosidases. However, we were rather disappointed with the preliminary results that their free bases **4β**, **9α,β**, and **10α,β** did not possess any acceptable activity, except for the moderate α-mannosidase inhibitor 5a-carba-α-mannopyranosylamine **10α**. Especially, we were dissatisfied in that β-validamine **4β** and limited derivatives did not show any inhibitory activity toward β-glucosidase, contrary to expectations from analogy with the structural features of α-glucosidase inhibitors validamine **4α** and derivatives.

In the final stage of our work on 5a-carbaglycosylamine glycosidase inhibitors, α-fuco validamines (**11α**) remained for exploration owing to their synthetic inaccessibility. On the basis of the structure-inhibitory activity relationship as suggested by the postulated reaction mechanism for hydrolysis of α-fucopyranosides, two types of inhibitor were designed: the ground-state **11α,β** and the transition state mimics **68α,β**, Fig. (**9**). Furthermore, two analogues **69α,β** were added. These were 5a-carba-α- and β-fucopyranose derivatives featuring aminomethyl functions at C-1.

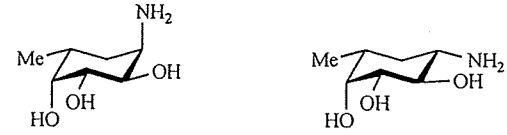
3-1. Synthesis of a α-Fuco Validamine, 5a-Carba-α-fucopyranosylamine

Synthesis of 5a-carba-α,β-fucopyranosylamines (**11α,β**) was planned in line with our systematic synthesis. Therefore, two or three preparative approaches [33,34] were carried out simultaneously for appropriate characterization of the intermediates appearing in the sequences.

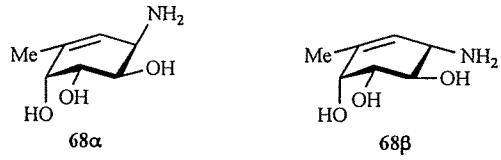
Compound **48** was prepared in a three-step process (**14→16→22→48**, overall yield 35%) from the *endo*-adduct **13** [35], Fig. (**10**). A mixture of **48** and NaN_3 in DMF was reacted at 90 °C to give an inseparable, ca. 4 :1 mixture of the azide **70** and the side product 5a,1-alkene. The mixture was de-*O*-acetylated (**→71**) under Zemplén conditions and subsequently treated with α,α-dimethoxytoluene and *p*-TsOH in DMF. After conventional acetylation, two products were found to be separable by silica gel chromatography,



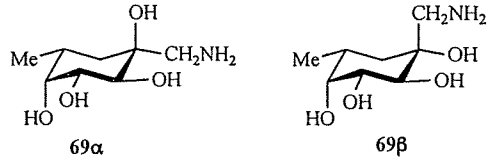
Hydrolysis of α-L-fucopyranosides



Ground-state mimics



Transition-state mimics



1-*C*-Aminomethyl-5α-carba-α,β-L-fucopyranoses

Fig. (9). Putative reaction mechanisms for hydrolysis of α-L-fucopyranosides, and some designed α-fucosidase inhibitors: fucose-type carbaglycosylamines.

giving **72** (60%). The direct S_N2 reaction occurred preferentially at C-1. Compound **72** was de-*O*-acetylated and then treated with NaH-benzyl bromide in DMF to give the dibenzyl ether **73** (89%). Treatment of **73** with 80% aq. AcOH gave the diol **74** (97%), which was mesylated to give the 4,6-dimesylate **75** (97%). Compound **75** was then treated with NaI in 2-butanone at 90 °C to afford selectively the 6-iodide **76** (85%), treatment of which with tributyltin hydride and AIBN in toluene at 120 °C gave the 6-deoxy derivative **77** (74%).

Compound **77** was treated with KOAc and 18-crown-6 ether in DMF to give the acetate **78** (99%) with an α-*galacto* configuration, the structure of which was confirmed on the basis of the ^{1}H NMR spectrum of its 4-OH derivative **79**. Hydrogenolysis of **79** in ethanol, containing 1 M HCl, in the presence of 10% Pd/C afforded, after purification by chromatography on a column of Dowex 50 W × 2 (H^+) resin with methanolic ammonia, syrupy **DL-11α** (8%).

As an alternative, the dianhydride **31** was benzylated to give the benzyl ether **80** (65%), Fig (**11**) [36]. Reaction of **80** with NaN_3 (→**81**), followed by tosylation, led to the azide tosylate **82** (75%), hydrogenation of which with Raney nickel in ethanol containing acetic anhydride gave the *N*-acetyl derivative **83** (80%). Cleavage of the anhydro ring, followed by simultaneous *O*-debenzylation, was conducted by treatment with HBr/AcOH to give the bromide **84** (80%), dehydrobromination of which with AgF in pyridine afforded the alkene **85** (48%), together with **84** (46%) unchanged. Hydrogenation of **85** with 5% Pd/C proceeded selectively to give the 6-deoxy derivative **86** (90%). Treatment of **86** with excess NaOMe/MeOH gave the 2,3-epoxide, which was hydrolyzed with acid, followed by acetylation, to afford the *N*,*O*-acetyl derivative **87** (80%). Removal of the protecting groups gave **11α** quantitatively.

3-2. Biological Assay and Chemical Modification of α-Fuco Validamine

Interestingly both 5a-carba-α-fucopyranosylamines **DL-** and **L-11α** were demonstrated to be very potent α-fucosidase inhibitors (K_i = 0.23 and 0.012 μM, bovine kidney), the effects being fully comparable to those of deoxyfuconojirimycin (DFJ) [37]. As expected, N-substitution with alkyl and phenylalkyl functions resulted in dramatic increase of their inhibitory potential. The 2-, 3-, and 4-deoxy

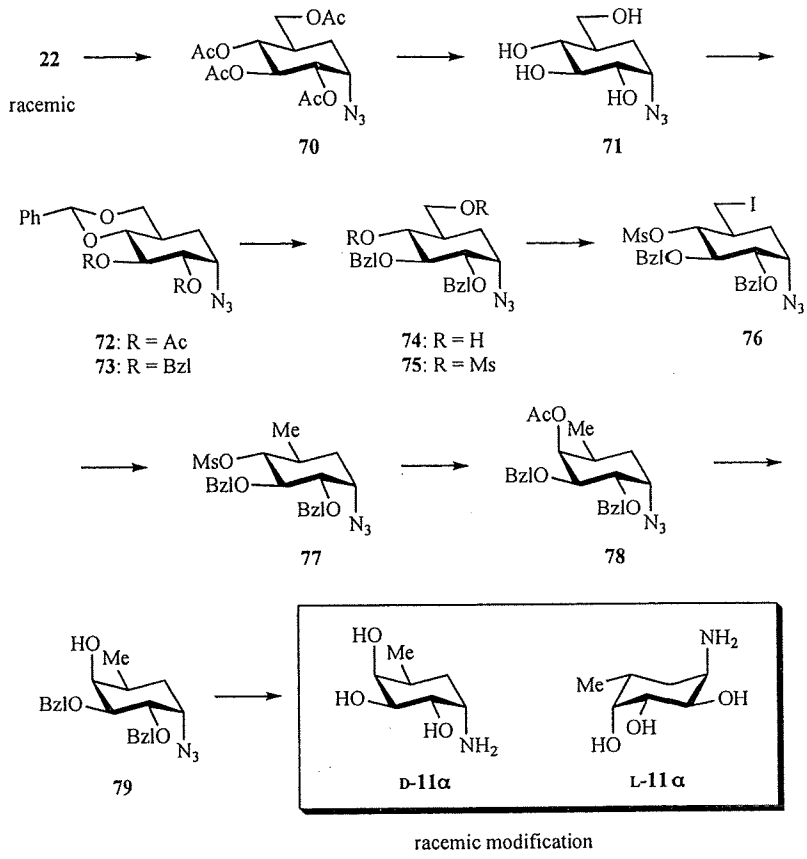


Fig. (10). Synthesis of 5a-carba-α-fucopyranosylamine.

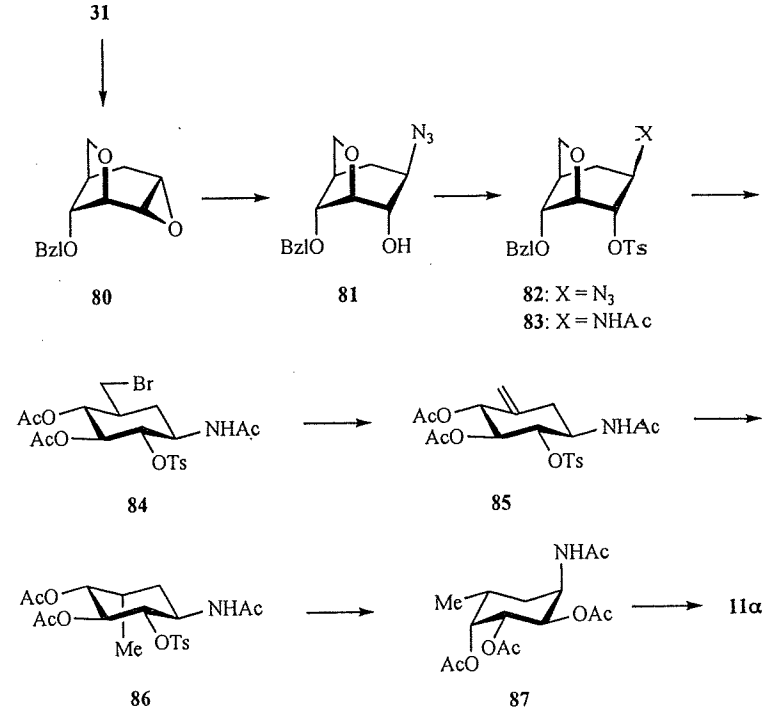


Fig. (11). Convenient synthesis of 5a-carba-α-fucopyranosylamine.

derivatives of 5a-carba-α-fucopyranosylamine were synthesized [38], and shown not to possess any detectable activity. The presence of all substituents with an α-*fuco* configuration, with one amino and three consecutive hydroxyl groups, constitutes the minimum core for α-fucosidase inhibitors.

Thus, the *N*-alkyl derivatives **90a-g** were initially prepared by LAH reduction of the corresponding amides **89a-g** from the protected amine **88** derived from the azide, Fig. (**12**). Alternatively, reductive alkylation of **88** with the corresponding aldehydes proceeded smoothly on treatment with sodium cyanoborohydride in THF under acidic conditions, leading to the *N*-alkyl and *N*-phenylalkyl derivatives in 45–70% yields.

Furthermore, an attempt was made to improve inhibitory potential by incorporation of a cyclic isourea function [36] with various N-substituents, thereby inducing change of the electron distribution and somewhat flattening the chair conformation without affecting the configurational arrangement of the two hydroxyl and methyl groups. Thus, treatment of **11α** with the corresponding alkyl and aryl isothiocyanates in aqueous 60% EtOH yielded the thioureas **91a-c** (ca. 100%), which, under influence of yellow mercuric oxide, were converted into the corresponding cyclic isoureas **92a-c**. (~100%).

Results of inhibition assay of several derivatives of **11α** toward α-L-fucosidase (bovine kidney) and four other glycosidases are listed in Table (**1**) [36]. None of the compounds, showed any significant activity against α-glucosidase (Baker's yeast and rat intestine), α-mannosidase (Jack beans), or α-galactosidase (green coffee beans and rat liver). As shown within the limited scope of racemic modifications of the compounds tested, the inhibitory activity against α-L-fucosidase was dramatically increased by incorporation of alkyl and phenylalkyl groups into the amino function of **11α**. Change of the *N*-ethyl on **11α** to a *N*-nonyl group improved the inhibitory potential, reaching a maximum with an aliphatic eight-carbon chain: *N*-octyl-5a-carba-α-DL-fucopyranosylamine (**90d**), shown to possess very strong and specific inhibitory activity against α-L-fucosidase, with *p*-nitrophenyl-α-L-fucopyranoside as the substrate. It is reasonable that the L-enantiomer should be several times more potent than DFJ, the strongest fucosidase inhibitor reported so far. The results suggest that the catalytic site of the enzyme can tolerate addition of various sizes of aliphatic chain to the basic portion involved in binding.

The *N*-octyl group seems to act as a structurally efficient hydrophobic spacer, leading to appropriate electron-release to the nitrogen atom for docking at the active site of the enzyme. Interestingly, the results are in line with those observed for inhibition of glucocerebrosidase by a series of *N*-alkyl-β-valienamines [39]. Thus, incorporation of *N*-alkyl functions could be clearly demonstrated to influence the activity of the inhibitors of this kind, suggesting that there is

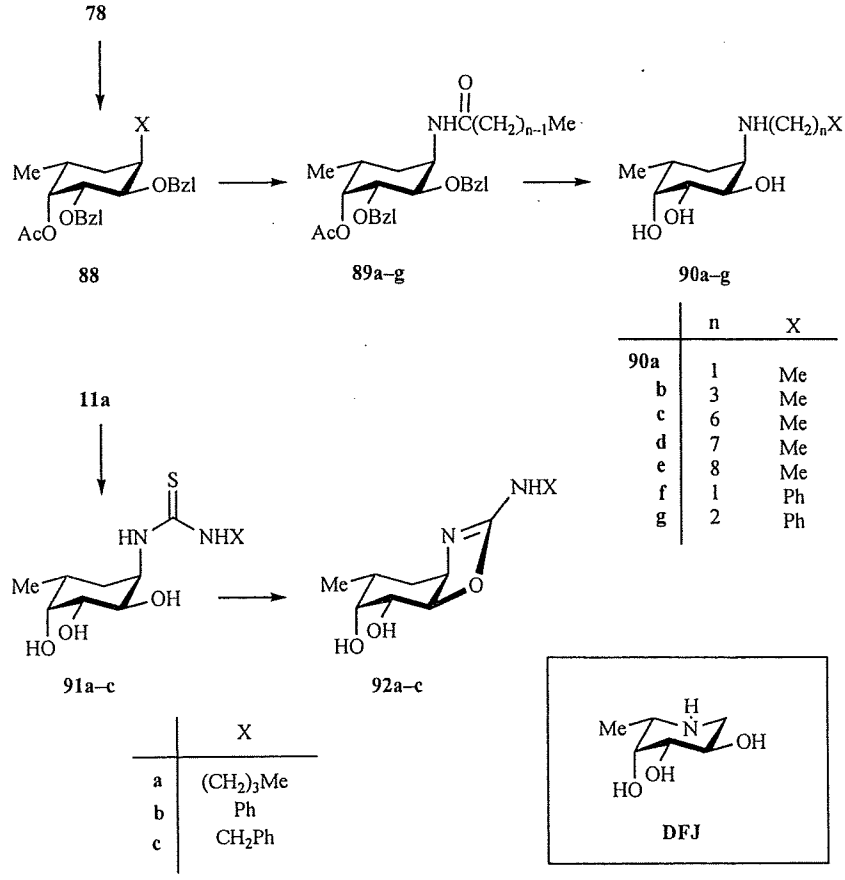
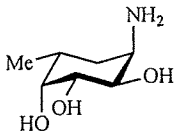
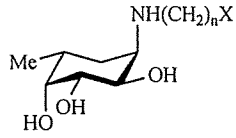


Fig. (12). Preparation of some N-substituted 5a-carba-α-DL-fucopyranosylamines and cyclic-isourea derivatives.

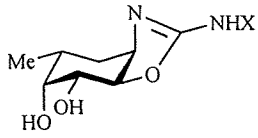
Table 1. Inhibitory Activity [IC_{50} (K_i) μM] of some 5a-Carba-α-DL-fucopyranose Derivatives against α-Fucosidase (Bovine kidney)



11α



90a–g



92a–c

Compd.	Inhibitory Activity
	IC_{50} (K_i) μM
11α	9.3 (1.0)
90a	21 (0.18)
b	0.92 (0.074)
c	0.27 (0.048)
d	0.11 (0.016)
e	0.24 (0.11)
f	1.0 (0.069)
g	0.24 (0.032)
92a	140
b	7.6
c	72
DFJ	0.41 (0.031)

All compounds except for **11α** and **DFJ** are racemic.

All compounds, except for **90d** (IC_{50} = 37 μM), were found to possess almost no activity toward β-galactosidase (bovine liver).

much room for development of potent new forms by further chemical modification.

On the other hand, the 1-*C*-aminomethyl analogues L-**69α,β** of **11α,β** were shown to possess strong inhibitory potential (K_i = 2.8 and 0.3 μM) toward α-fucosidase, implying their use as novel leads in chemical modification [34].

Similar change of the potential has been observed for three cyclic isourea derivatives **92a-c**. Although the *N*-phenyl derivative **92b** showed medium inhibitory activity, due to a possible stacking effect of the spacer phenyl group, both basic feature of nitrogen atoms and the free hydroxyl group at C-2 seem to play an important role in attaining high potential.

3-3. Synthesis and Inhibitory Activity of β-Fuco Validamine and Derivatives

The unexpected findings for biochemical features of the 1-*C*-aminomethyl-5a-carbafucopyranoses **69α,β** prompted us to prepare β-fuco validamine **11β** and evaluate its possible α-fucosidase inhibitory activity in detail.

Treatment of **23** with excess of Zn dust in DMF at 80 °C gave the alkene (**93**, 59%), together with the 6-debromo compound **94** (32%), Fig. (**13**) [44]. When the reaction time was prolonged, **94** was mainly obtained but in poor yield, owing to its simultaneous decomposition. Compound **93** isolated was debrominated with tributyltinhydride to give **94** (86%). *O*-Deacetylation of **93**, followed by selective benzoylation of the allylic hydroxyl group (→**94**) and subsequent mesylation, afforded the mesylate **95** (64%). Direct nucleophilic substitution of **95** with a benzoate anion in DMF and successive deprotection gave **96** (93%). Blocking the two hydroxyls with a cyclohexylidene group (→**97**, 85%), followed by epoxidation with *m*CPBA in a phosphate buffer solution (pH ca. 6), gave the single β-epoxide **98** (90%). Rear attack of the peracid seems to be restricted by the presence of a bulky cyclohexylidene group.

Reaction of the racemic **98** with alkylamines or phenylalkylamies in 2-propanol at 120 °C proceeded slowly but almost regio-selectively to afford the respective N-substituted 5a-carba-β-fucopyranosylamines **99a-f** (70–90%), which were deprotected with 80% aqueous AcOH, and the resulting amine acetates were purified over a column of Dowex 50 W × 2 (H^+) resin with 1% methanolic ammonia to give the free bases **100a-f** (ca. 100%). The optically active **100b-d,f** were similarly prepared from D- and L-**98**.

Compounds DL- and L-**11β**, and four *N*-alkyl and two *N*-phenylalkyl derivatives **100a-f** were assayed for activity against seven glycosidases: α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase, and α-fucosidase [44]. All compounds were shown to exhibit appropriate inhibitory potential toward β-galactosidase, β-glucosidase, and α-fucosidase, as listed in Table (**2**). Compared to the corresponding derivatives of the α-anomers, they possessed about one tenth of the inhibitory activity against α-fucosidase and the potential was thought to be attributable to β-L-fucopyranose mimicking enantiomers, as verified by assaying newly prepared pure L-enantiomers L-**100b-d**.

It is worthy of note that all N-substituted derivatives possess very strong activity toward both β-galactosidase and β-glucosidase, reaching a maximum with an aliphatic twelve-carbon dodecyl group. Their high cross-inhibitory potential could be demonstrated to be largely due to the respective D-enantiomers, viz. *N*-alkyl-6-deoxy-5a-carba-β-D-galactopyranosylamines. In fact, the L-enantiomers, *N*-alkyl-5a-carba-β-L-fucopyranosylamines had decreased activity toward these two enzymes as shown for L-**100b**, L-**100c**, and L-**100f**. For example, DL-**100e** has been demonstrated to be a strong inhibitor possessing characteristic pH dependent activity against β-glucosidase (almond): K_i = 0.39 μM, at pH 5.5, e.g. calystegine [41] (K_i = 0.75 μM, pH independent). Thus, N-substituted 6-deoxy-5a-carba-β-D-

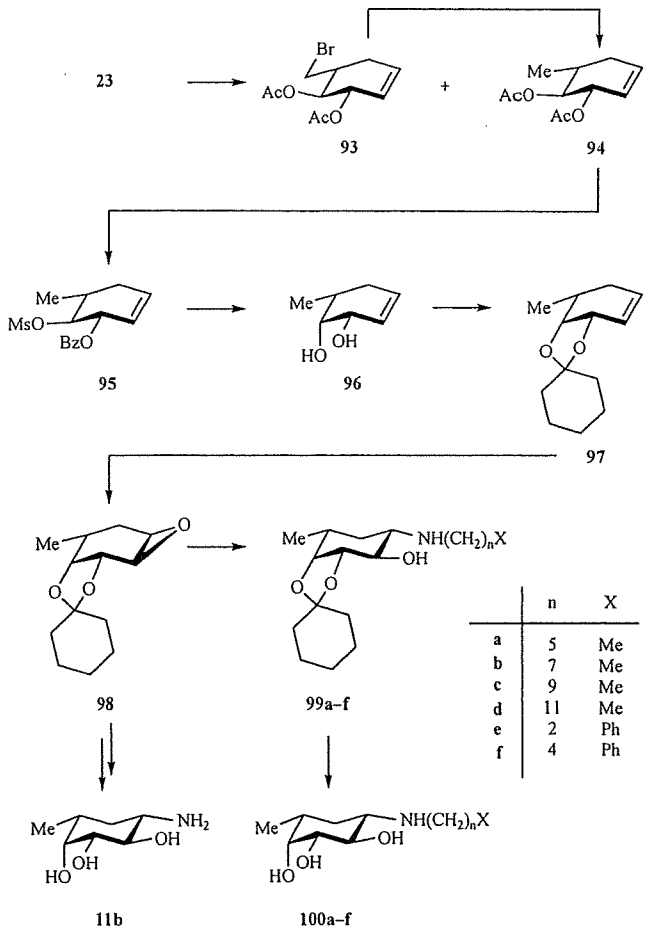


Fig. (13). Preparation of N-substituted 5a-carba-β-fucopyranosylamines.

galactopyranosylamines may be promising lead compounds for new β-galactosidase and β-glucosidase inhibitors.

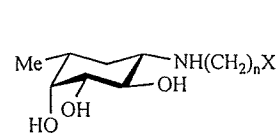
3-4. Synthesis and Inhibitory Activity of β-Galacto Validamine and Derivatives

The strong cross-inhibitory activity exhibited by β-fuco validamine (**11β**) stimulated our interest in biochemical features of its parent β-D-galacto validamine (**9β**). This compound had been assayed once for β-galactosidase, but its unexpected low potency did not point to advantages for further modification.

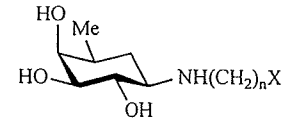
Epoxidation of the alkene **D-101**, derived from **21** through multi-step sequences, gave the α-galacto 1,2-epoxide **102** [30], Fig. (**14**). Treatment of **102** with some alkylamines, followed by deprotection, afforded four *N*-alkyl derivatives **D-103a-d**. Interestingly, as shown by e.g. the *N*-octyl derivative **D-103b**, they were found to be moderate inhibitors of α-galactosidase, as well as, β-galactosidase and β-glucosidase, Table **3**. In contrast, the L-enantiomers, as indicated by **L-103b**, were found to lack inhibitory activity against all enzymes [42].

It is noteworthy that the free bases **D-9β** and **L-11β** were found to be medium but specific inhibitors against α-galactosidase and α-fucosidase, matching the respective stereochemistry of the substrates. However, the *N*-alkyl derivatives **D-103b-d** are all moderate inhibitors of α- and β-galactosidases, and β-glucosidase, so that the N-substituents as well as the hydrophobic area conferred in by the 5-methyl branching on the carbocyclic ring are likely to control and enhance binding potential at the active sites of enzymes.

Table 2. Inhibitory Activity [IC_{50} (K_i), μM] of some *N*-Substituted 5a-Carba-β-fucopyranosylamines 100a-f toward Three Glycosidases*



L-100a–f



D-100a–f

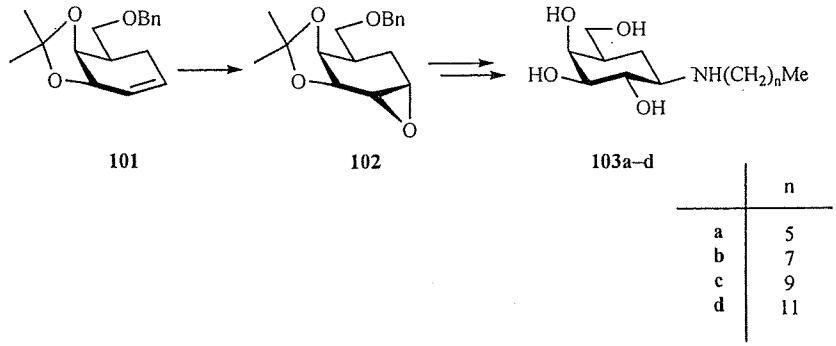
Compd.	Inhibitory activity, IC_{50} (K_i) μM		
	α-Fuc	β-Gal	β-Glc
L-11β	4.3 (0.2)	NI	NI
DL-100a	8.2	3.7	0.73
DL-100b	5.5	0.7 (0.11)	1.5 (3.2)
L-100b	1.8	3.7	15
DL-100c	2.7	0.2 (0.009)	2.0 (0.4)
L-100c	0.7	1.0	17
DL-100d	3.8	0.02 (0.0045)	0.50 (0.53)
L-100d	0.91	0.46	6.1
DL-100e	7.5	5.7	0.38 (0.057)
DL-100f	5.1	0.9 (0.046)	1.4 (1.2)
L-100f	1.2	2.4	10

* α-Fucosidae (bovine kidney); β-Galactosidase (bovine liver); β-Glucosidase (rat intestine). NI: no inhibition < 10^{-3} M

Table 3. Inhibitory Activity (IC_{50}, μM) of some *N*-Substituted 5a-Carba-β-D-galactosylamines Against Three Glycosidases*

Compd.	n	Inhibitory activity, IC_{50} μM		
		α-Gal	β-Gal	β-Gul
D-9β	-	2.8	NI	NI
D-103a	3	39	NI	13
b	7	5.2	8	14
c	9	25	16	25
d	11	35	5.8	8.7

* α-Galactosidase (green coffee beans); β-Galactosidase (bovine liver); β-Glucosidase (rat intestine).
NI: no inhibition < 10^{-3} M



	n
a	5
b	7
c	9
d	11

Fig. (14). Preparation of some N-substituted 5a-carba-β-D-galactopyranosylamines.

3-5. Synthesis of N-Linked Carbadisaccharides Composed of β-Fuco and β-Galacto Validamine Moieties

Carbadisaccharides containing the β-fuco and β-galacto validamine moieties were designed in order to elucidate the influence of the sugar and/or sugar like aglycones on the activity originating in the carbaglycosylamine moieties, Fig (**15**).

Coupling of **DL-11α** and the dianhydro azide **104** [33] in 2-propanol at 120°C afforded, after separation, new type carbadisaccharides **D-** and **L-105α** (48 and 44%), which were demonstrated to possess specific inhibitory activity (K_i = 3.1 and 0.13 μM) toward α-fucosidase [33]. The analogous β-carbadisaccharide **L-105β** (93%), derived from **L-11β** and **104**, was also shown to be a specific α-fucosidase inhibitor (IC_{50} = 3.9 μM), with a similar tendency as observed for **L-11β** [44]. It is promising that these disaccharide mimics may constitute a new group of specific α-fucosidase inhibitors suitable for further modification. Compounds **105α,β** were initially designed to provide precursors for carbadisaccharides of biological interest, e.g. N-linked *N*-acetyl-5a'-carbalactosaminide derivatives,

Reductive amination of the ketone **32** with the amine **9β** proceeded in stereoselective fashion, affording N-linked β(1→4)-5a,5a'-dicarbadisaccharide **106**, featuring a versatile 1,2:3,6-dianhydro-5a-carba-α-glucopyranose residue [43]. The free base **9β** was first converted into the hydrochloride and then subjected to reductive coupling with the ketone **32**. Thus, reaction of **32** (2 molar equiv) and **9β** HCl was conducted in aqueous methanol in the presence of sodium cyanoborohydride (2 molar equiv) and anhydrous $MgSO_4$ at reflux temperature. The product was readily isolated as the tetra-*O*-acetyl derivative (47%) of **106**. Therefore, the analogous ketones **107a–c** were prepared by oxidation of **33–35** and subjected to similar coupling with **9β**, giving the respective 5a,5a'-dicarbadisaccharide derivatives **108a-c** (60%, 41%, and 50%). *O*-Debenzylation of **108a** gave N-linked methyl 3,6-anhydro-5a,5a'-dicarba-β-D-lactoside **109** (91%), which could be shown to possess specific inhibitory activity against α-galactosidase (IC_{50} = 1.2 μM, green coffee beans). Acetolysis of **109** would be expected to give rise to the N-linked 5a,5a'-dicarba-β-lactoside derivative.

Further preparative utility of the dianhydride **31** is summarized in Fig. (**16**), demonstrating access to 5a-carba-galacto and glucopyranosylamine derivatives [43]. Compound **31** was converted into the azide mesylate (**110**) through mesylation followed by azidolysis. Acid catalyzed cleavage of the 3,6-anhydride produced the azide **111** with β-gluco configuration. Acetolysis of **111** proceeded through a direct S_N2 mechanism to give rise to the azide **112** with β-*galacto* configuration. On the other hand, nucleophilic opening of the 1,2-anhydride was easily conducted by treatment with octylamine in 2-propanol to give the amine **113** (ca. 90%), treatment of which with HBr/AcOH, followed by *O*-deacetylation, afforded the *N*-octyl-6-bromo-6-deoxy-carbaglucopyranosylamine **114a**. Conventionally **114a** could be transformed into the 6-hydroxyl **114b** and 6-deoxy derivatives **114c**.

We seem to have taken a roundabout course to finally elucidate the biochemical interactions of *N*-alkyl derivatives of β-validamine **4β** with glycosidases. Compounds **114b,c** have been assayed for activity toward β-glucosidase and β-galactosidase [44]. Contrary to our expectation, these compounds were demonstrated to be medium β-galactosidase inhibitors (IC_{50} = 10 and 18 μM) and did not exhibit any activity against β-glucosidase. These results suggest that only the β-*galacto* configuration may be required for inhibitory interaction toward β-glucosidase. It seems difficult to explain rationally the fact that β-validamine lacked inhibitory activity toward the enzyme in spite of close resemblance of the substrate structures.

3-6. Transition-state Mimic 5,5a-Unsaturated Derivatives of α,β-Fuco Validamines

Compounds **68α** and **68β** were synthesized in a racemic modification for rough assays of activity toward α-L-fucosidase [40], Fig. (**17**). Compound **115** was prepared in 50–60% yield by treatment of **40** with NaOAc in HMPA at 120 °C. The protecting groups were initially replaced with methoxymethyl groups, in order to eliminate neighboring participation reactions, thus converting **115** into the trimethoxymethyl ether **116** (ca. 100%). Treatment of **116** with bromine in CCl_4 gave the 1,4-addition products (**117α** and **117β**) in 21 and 48% yields, respectively. Selective debromination of the major **117β** was conducted by treatment with NBH in HMPA to give a 1:2 mixture of the bromides (**118α,β**, 57%), together with **117α,β** (ca. 45% recovered). Direct substitution with a bromide ion generated in situ is likely to occur at allylic carbon atom, resulting in epimerization. Therefore, a mixture of **118α,β** should be furnished directly from a crude mixture of the dibromides. The mixture was treated with NaN_3 in DMF to give a 1:2

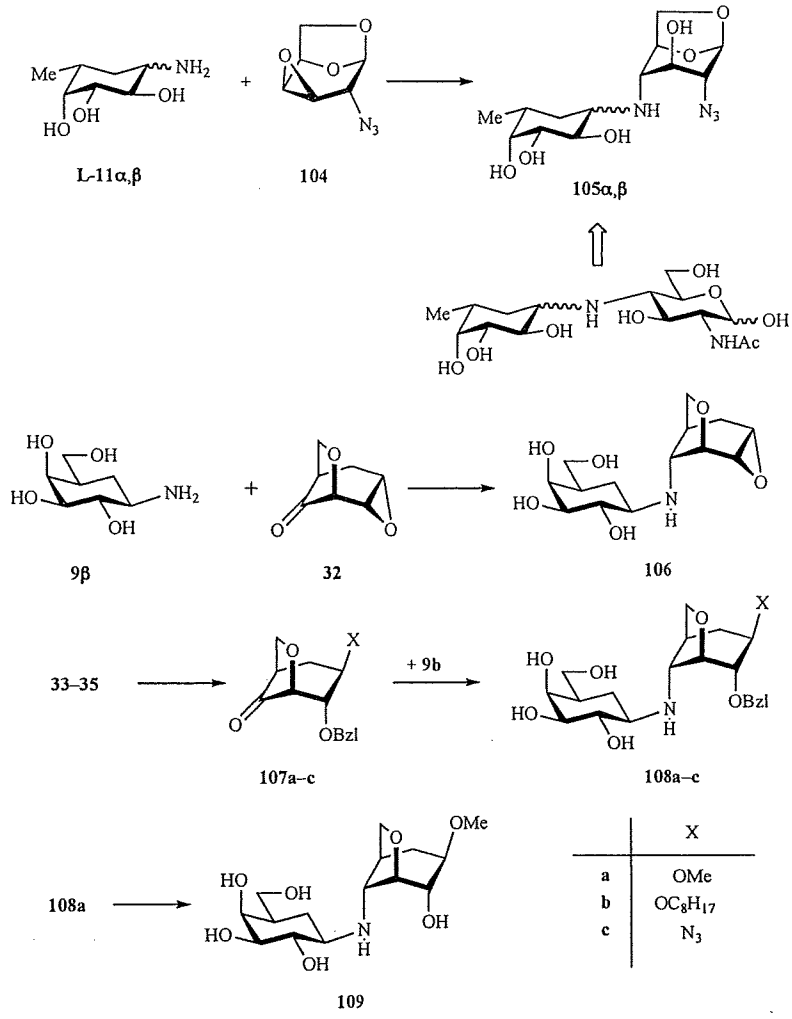


Fig. 15. Preparation of some precursors of N-linked 5a-carba-β-L-fucopyranosyl GlcNAc and 5a,5a'-dicarba-β-D-lactose derivatives.

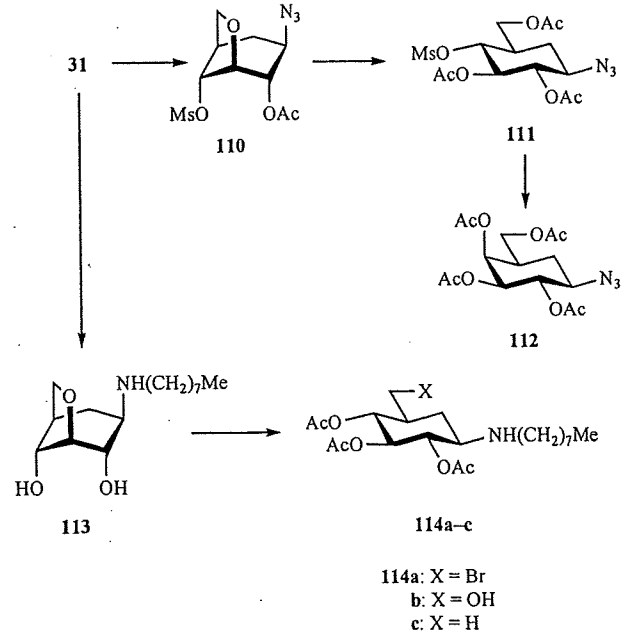


Fig. (16). Convenient preparation of 5a-carba-β-galacto and glucopyranosylamine derivatives from the dianhydride.

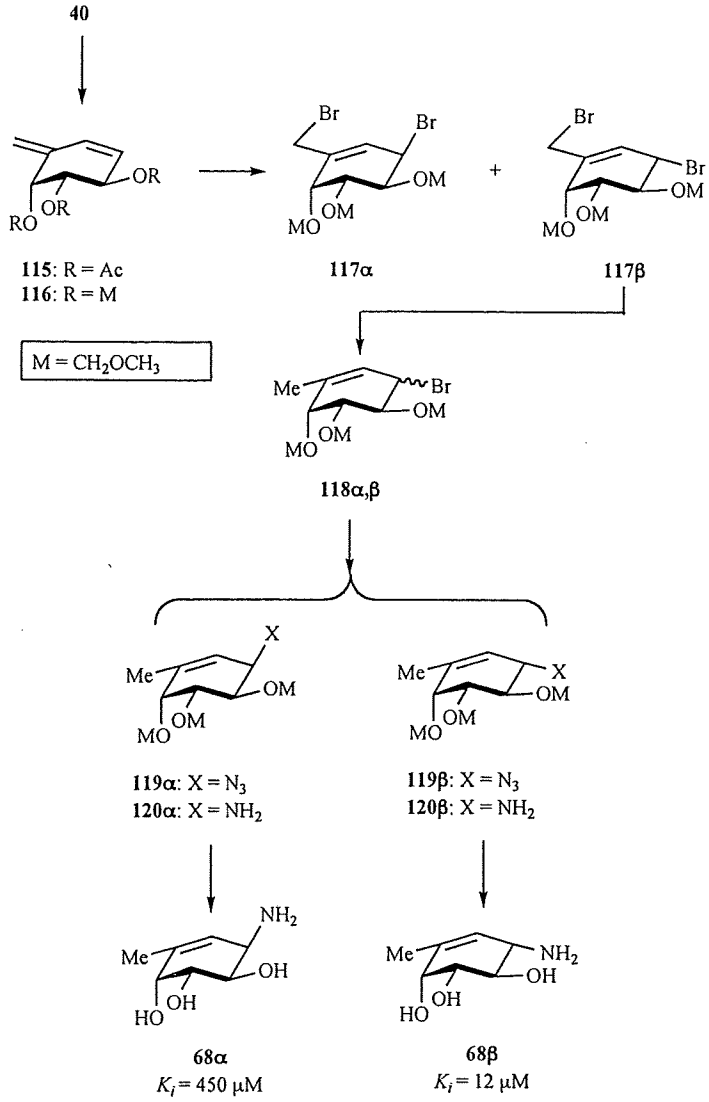


Fig. (17). Synthesis of transition state analogues of 5a-carba-α,β-fucopyranosylamines and constants for their enzyme inhibitory activity against α-fucosidase (bovine liver).

mixture of the azides **119α,β** (88%). Reduction with Ph_3P in aqueous THF gave a mixture of the amines **120α,β**, and subsequent treatment with 4 M HCl afforded the respective free bases **68α** and **68β** (ca. 100%). Contrary to expectation, racemic **68α** and **68β** were found to be rather weak fucosidase inhibitors, with K_i values of 45 and 1.2×10^{-5} M, respectively. Throughout our studies on carbaglycosylamine glycosidase inhibitors, these results are the first instance of transition-state mimics of postulated fucopyranosyl cations being less potent than ground-state mimics, owing to possible differences in binding to the active site of the enzyme. Hydrolysis of α-fucopyranosides seems to mechanistically provide a somewhat different stereoelectronic course, compared with other hexopyranosides having the hydroxyl function at C-6. The present results indirectly suggest that the hydrolytic reaction of the α-L-fucosidase (bovine kidney) features an S_N2-type mechanism with nucleophilic displacement rather than an S_N1-type one through an oxocarbenium ion intermediate.

3-7. Related Aminocyclopentanetriol Glycosidase Inhibitors

(Hydroxymethyl)aminocyclopentitol glycosidase inhibitors **122a-d**, **123**, and **124** , and several derivatives thereof have already been synthesized and their biological features investigated extensively [45, 46]. Actually these compounds are considered to be ring-contract models derived from validamine (**4**), valiolamine (**6**), and their stereoisomers **9** and **121**, Fig (**18**), and their structure and inhibitory activity relationship has been discussed in detail [47]. In general their inhibitory potential appears to be comparable with that of structurally related parent 5a-carbaglycopyranosylamines, probably depending on close resemblance between their structures and those of the ground states of the corresponding substrates. Interestingly, cross-inhibitory activity toward β-galactosidase and β-glucosidase, exhibited by the β-galacto **D-4β** and β-fuco validamines **D-9β**, has also been

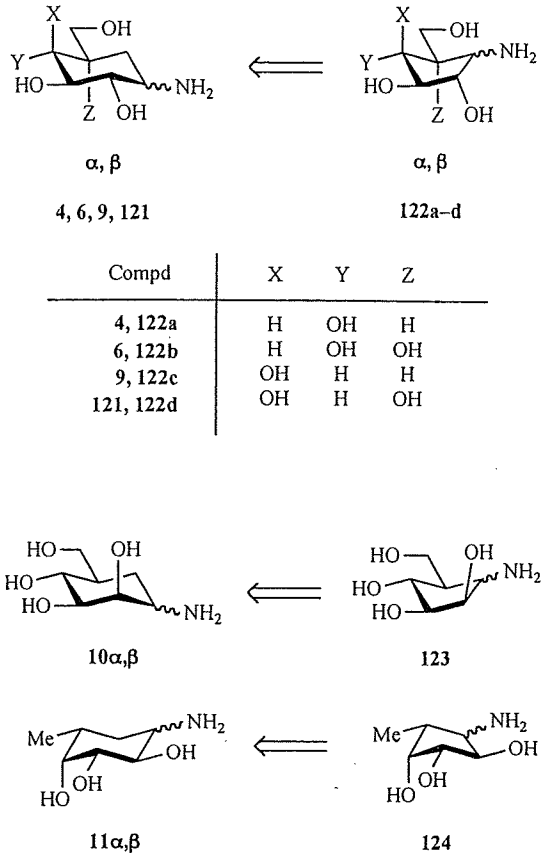


Fig. (18). Potent glycosidase inhibitors, amino(hydroxymethyl)-cyclopentanetriols, the ring-contract analogues derived from validamine and valiolamine, and their stereoisomers.

observed for the corresponding ring-contract analogues **122aβ** and **122cβ** [48], indicating that configuration of the 4-hydroxyl group is ignored by the enzymes. However, in the case of structurally more rigid carbahexopyranosylamine inhibitors, the configuration of the 4-hydroxyl seems to play a role of enhancing binding interaction toward the enzymes, although it is difficult to explain rationally solely on the close resemblance of the substrate structures. Therefore, enzymatic action of a certain glycosidase may depend on the structure and activity relationship of these characteristic inhibitors.

4. CONCLUSION

The door to the new possibility of carbasugars being developed as potent glycosidase inhibitor, is now open by analogy with the preceding discovery of very strong 5a-carba-α-fucopyranosylamine α-fucosidase inhibitor. Furthermore, the fact that α-fuco valienamines designed as related transition-state mimicking inhibitors of α-fucosidase unexpectedly possessed only moderate activity motivated us to compile this review article on validamines as ground-state type inhibitors. The interesting biochemical and biological features found for simple 5a-carbaglycopyranosylamines provide a promising basis for further development [51] of biologically active sugar mimics of this type.

Recently, *N*-octyl-β-valienamine (NOV) [39] and its 4-epimer (NOEV) [49] were found to induce remarkable expression of mutant lysosomal enzymes and to correct pathological effects of plasmic storage of substrates in some human disorders. This stimulated a systematic survey of such kind of glycosidase inhibitors has been carried out [50]. Very recently, NOEV was found to be a good candidate new molecular therapeutic (chemical chaperone therapy) for G_{M1}-gangliosidosis caused by β-galactosidase deficiency [52]. Since some derivatives of 5a-carbaglycosylamines with α-*fuco* and β-*galacto* configurations have been recognized as strong and specific inhibitors of fucosidase or/and nonspecific but very strong cross-inhibitors of β-galactosidase and β-glucosidase, our efforts should continue for development of medicines of this kind, adopting these substances as leads and target compounds.

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Original article

Intracerebral cell transplantation therapy for murine GM1 gangliosidosis

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Abstract

We performed a cell transplantation study to treat the brain involvement in lysosomal storage diseases. We used acid β-galactosidase knock-out mice (BKO) from C57BL/6 as recipients. To minimize immune responses, we used cells derived from transgenic mice of C57BL/6 overexpressing the normal human β-galactosidase. Fetal brain cells (FBC), bone marrow-derived mesenchymal stem cells (MSC), and mixed FBC and MSC cells were prepared and injected into the ventricle of newborn BKO mouse brain. The mice were examined at 1, 2, 4, and 8 weeks and 6 months after injection. In each experiment, the injected cells migrated into the whole brain effectively and survived for at least 8 weeks. Decrease in ganglioside GM1 level was also observed. FBC could survive for 6 months in recipient brain. However, the number of transplanted FBC decreased. In the brains of MSC- or mixed cell-treated mice, no grafted cells could be found at 6 months. To achieve sufficient long-term effects on the brain, a method of steering the immune response away from cytotoxic responses or of inducing tolerance to the products of therapeutic genes must be developed.

Keywords: GM1-gangliosidosis; Cell transplantation; Fetal brain cell; Mesenchymal stem cell

1. Introduction

Enzyme replacement therapy (ERT), hematopoeitic stem cell transplantation (HSCT), and gene transfer have been studied in animals and in humans with lysosomal storage disease (LSD). ERT is now available clinically for Gaucher disease, Fabry disease, Pompe disease, and MPS I, II, and VI in many countries, and has been successful in visceral organs. HSCT is also effective against the somatic involvements in Gaucher disease and MPS I, II, and VI. However, HSCT exhibits little efficacy in conditions such as Fabry disease and Pompe disease, when enzyme secretion from donor cells is poor or the uptake of enzyme proteins by the affected host cells is inadequate. In addition, efficacy in individual organs differs markedly, in both ERT and HSCT, depending on accessibility of blood flow and the density of mannose-6-phosphate receptors. Neither HSCT nor ERT exhibits efficacy against the brain involvement in Gaucher or MPSs because of the poor access due to the blood–brain barrier.

Many experimental studies have been carried out, involving methods such as gene therapy [1–5], cell

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therapy [6–9], or intrathecal administration of enzymes [10,11], for treatment of the brain in LSDs. Such treatments were able to overcome the blood–brain barrier to access brain tissue and exhibit considerable efficacy in brain. However, it is difficult to maintain such efficacy for long periods of time. Repetition of these treatments is not practical because intracranial administration is required for them. On the other hand, the usefulness of intravenous administration is limited because of the blood–brain barrier, except in newborn mice which have an immature barrier. It has been reported that intravenous administration of extremely high doses of enzymes [12–14] or of enzymes that remain in the circulation for long periods [15,16] yielded slight passage through the blood–brain barrier, though with increase in the risk of immune response.

Oral administration of small molecules would be a good and convenient method of treatment of the brain for prolonged periods, such as substrate reduction therapy with *N*-butyldeoxynojirimycin or *N*-butyldeoxygalactonojirimycin for glycosphingolipidoses [17–19] or genistein for mucopolysaccharidoses [20], and chemical chaperone therapy for Fabry disease [21] or GM1-gangliosidosis [22]. However, the efficacy of substrate reduction therapies has thus far been quite limited, and chemical chaperone therapies are not applicable for every type of gene mutation.

GM1 gangliosidosis is an LSD and a progressive neurological disease in humans caused by a genetic defect of lysosomal acid β-galactosidase, which hydrolyses the terminal β-galactosidic residue of ganglioside GM1 and other glycoconjugates. The defects in β-galactosidase activity result in accumulation of ganglioside GM1 in various organs, especially the brain, causing progressive neurodegeneration. In our previous study [2], we injected recombinant adenovirus encoding mouse β-galactosidase cDNA intravenously in β-galactosidase-deficient newborn mice, and showed that vector-mediated β-galactosidase-producing brain cells could reduce ganglioside GM1 accumulation. We showed that β-galactosidase enzyme protein could be secreted as well as taken up by the brain cells and function effectively. However, the efficacy obtained was transient. If sufficient amounts of the defective enzyme could be permanently secreted by cells in the brain, injury of the brain could be prevented. To examine the possibility of long-term cell treatment of the brain in LSDs, we carried out a transplantation experiment in the brain of a GM1-gangliosidosis mouse model (acid β-galactosidase knock-out mouse) using fetal brain cells (FBC) and mesenchymal stem cells (MSC) from bone marrow. These cells used for transplantation were derived from mice of the same genetic background as recipient mice except for possession of the human β-galactosidase gene.

2. Materials and methods

2.1. Knock-out and transgenic mice

A mouse model of GM1 gangliosidosis (BKO mouse) was generated by targeting of the β-galactosidase gene at exon 15 in ES cells as previously described [23]. Newborn mice were obtained by mating heterozygous female mice with homozygous male mice. Identification of newborn mutants was accomplished by quantitative analysis of β-galactosidase activity in tail tip homogenates on the day of birth. Mice with high β-galactosidase activity (TG mice) [24] were generated by introducing the human β-galactosidase gene as a transgene in ES cells obtained from the BKO mouse, which has several copies of the human β-galactosidase gene without the mouse β-galactosidase background. Age-matched wild-type mice of C57BL/6 strain were used as a control.

2.2. Cell preparations for transplantation

Cultured mesenchymal stem cells (MSC) were obtained from the bone marrow of the tibias and femurs of 5–8 month-old TG mice according to the method of Meirelles et al. [25] with some modifications. Dulbecco's modified Eagle's medium (DMEM: Sigma Chemical Co., St Louis, MO) containing 10% fetal bovine serum (Medical and Biological Laboratories, Nagoya, Japan) was used for culture.

Fetal brain cells (FBC) were obtained from the fetal cerebral cortex of TG mice at 13 days of gestation according to the method of Meberg and Miller [26]. The brain tissue was disrupted in a Pasteur glass pipette by gentle stroking several times (uncultured FBC), and then cultured for 4 h in Neurobasal medium (Invitrogen, Carlsbad, CA, No. 12348-07) containing 2 mM glutamine and 10% FBS, followed by two days in Neurobasal medium containing 2 mM glutamine and B27 supplement (Invitrogen, No. 14175-095) (cultured FBC).

2.3. Transplantation of cells into newborn mouse brain

Each BKO mouse received a single injection of 0.5–1.0 $\times 10^5$ of the cells prepared as described above in the right cerebral ventricle from 24 to 48 hours after birth. Study groups were as follows: uncultured FBC ($n = 18$), cultured FBC ($n = 10$), MSC ($n = 17$), and mixed MSC and FBC (1:1) ($n = 15$). Mice of each experimental group were divided into three subgroups for X-gal staining, β-galactosidase assay and ganglioside GM1 analysis. Mice were examined at one, two, four, and eight weeks and 6 months after injection as shown in Table 1.

For biochemical analysis, mice were anesthetized with diethylether and the blood was washed out with normal saline by perfusion through the heart, and the

brains were removed and kept at −80 °C until use. For histological studies, the brains were fixed by perfusion through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB) for 20 min., after washing out the blood with normal saline. To obtain frozen sections, the brains were placed in 0.1 M phosphate buffer pH 7.4 containing 30% sucrose, and frozen in liquid nitrogen.

All surgical and care procedures were carried out in accordance with the Guidelines for Use and Care of Experimental Animals approved by the Animal Committee of Osaka City University School of Medicine.

2.4. X-Gal staining

Frozen sections (16 μm thick) were reacted with X-gal using the β-gal staining Kit (Invitrogen Corp., Carlsbad, CA) to visualize β-galactosidase activity.

2.5. β-Galactosidase assay

β-Galactosidase activity was analyzed in the tissue homogenate with the artificial substrate 2 mM 4-methylumbelliferyl β-galactoside at pH 4.0 in 0.1 M sodium citrate-phosphate buffer according to the method described by Suzuki [27]. Protein was analyzed using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA) with the method of Bradford [28].

2.6. Analysis of ganglioside GM1

Amounts of ganglioside GM1 were measured by immunoblot assay using anti-GM1 ganglioside monoclonal antibody (Code: 370685, Seikagaku Corp., Tokyo, Japan) by the method of Michikawa et al. [29] with some modifications.

Brain tissue cells were disrupted by sonication and solubilized in 20 mM Tris–HCl buffer pH 8.0 containing 137 mM NaCl, 10% glycerol, and a protease inhibitor cocktail (Complete, Mini, Cat No. 11836153001, Roche Diagnostics, Mannheim, Germany). Five micrograms of tissue protein was applied onto Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (0.45 μm pore size, Code: 162-0117, Bio-Rad Laboratories) through the slots of a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories). The membrane was reacted with anti-GM1 ganglioside monoclonal antibody diluted 1:500, after blocking with 5% skim milk in PBS solution for 1 h at room temperature, and then with horseradish peroxidase-linked anti-mouse IgG sheep antibody (Code: NA931, GE Healthcare UK Ltd., Buckinghamshire, UK) diluted 1:1,000. The washing solution used was 0.1 M Tris buffered saline pH 7.5 containing 0.1% Tween 20 (TTBS). Bound antibody was detected using ECL after reaction with ECL™ Western Blotting Detection Reagents (Code: RPN2209, GE Healthcare UK Ltd.) and visualized on X-ray film. Densitometric quantification of immunoreactive signal was performed using the Kodak Digital Science™ EDAS 120 system with 1D Image Analysis software (Eastman Kodak Company, NY). The values obtained were compared with those of quantification of histological immunoreactivity with Leica Control Software as previously described [30], and the same ratios were obtained among the samples (data not shown). The assay was performed three times and in duplicate for each sample independently, and mean values were calculated.

3. Results

3.1. X-Gal staining

Layered staining of the transplanted cells was observed over the entire ventricular surface on both sides of the cerebral hemispheres in treated mice at one week after injection (data not shown). Positive cells had spread into the brain tissue by two weeks (Fig. 1c and f) in the mice treated with cultured FBC ($n = 1$), uncultured FBC ($n = 1$), and MSC ($n = 2$) in the same amounts. The cells had spread further and had reached every part of the brain by 4 weeks in the mice of all experimental groups (Fig. 1d, g and i). Less positive cells were found in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1g and i) than in the mice treated with cultured ($n = 3$) or uncultured FBC ($n = 3$) (Fig. 1d). The number of the X-Gal positive cells increased gradually until 4 weeks after injection in every experimental mouse. At 8 weeks after injection, positive cells still existed in the cultured FBC- ($n = 3$) and uncultured FBC-treated ($n = 3$) mice (Fig. 1e) in the same numbers with a similar distribution as at 4 weeks. However, a significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1h and j). In the mice treated with mixed MSC and FBC, positive cells existed in higher numbers in deep areas than in the mice treated with MSC alone. In the mice treated with cultured ($n = 2$) and uncultured FBC ($n = 2$), small numbers of positive cells with strong staining still existed in many parts of the brain, especially around the striatum and lateral globs pallidus (Fig. 1k and l), at 6 months after injection. No grafted cells were found in the mice treated with MSC ($n = 1$) or mixed MSC and FBC ($n = 1$) at 6 months. No significant differences were noted among the mice within each experimental group at each stage.

3.2. β-Galactosidase activity

The β-galactosidase activity in FBC and MSC derived from TG mice were 214.5–227.5 nmol/mg/h ($n = 4$) and 143.0–121.4 nmol/mg/h ($n = 3$), respec-

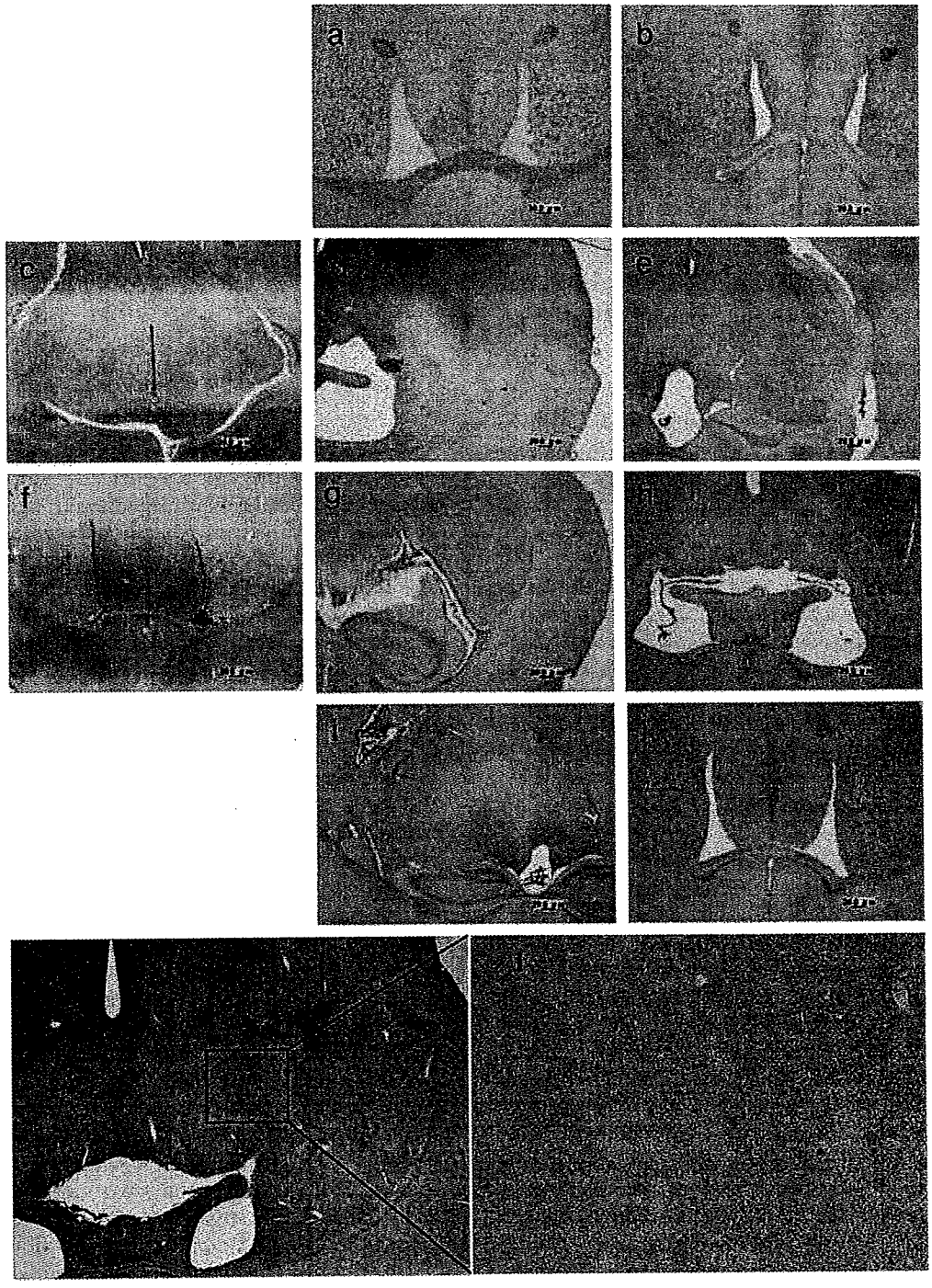

Fig. 1. X-Gal staining of brain coronal sections at +0.8 mm to −2.0 mm of bregma. (a and b) Non-treated BKO mouse at 4 and 8 weeks old, respectively; (c–e) Treated with FBC at 2, 4, and 8 weeks after injection; (f–h) Treated with MSC at 2, 4, and 8 weeks after injection; (i and j) Treated with mixed MSC and FBC at 4 and 8 weeks after injection; (k) FBC-treated brain at 6 months after injection; (l) Magnification of figure k. Positive cells had spread into the brain tissue by two weeks (c and f). The cells had spread further by 4 weeks (d, g and i). Less positive cells were found in the mice treated with MSC or mixed MSC and FBC (g and i) than in the mice treated with FBC (d). At 8 weeks, positive cells still existed in FBC-treated mouse (e) as at 4 weeks (d). A significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC (h) or mixed MSC and FBC (j). Strong positive staining cells still existed at 6 months in the brain of FBC-treated mouse (k and l).

tively, while the activity in FBC and in MSC derived from wild-type mice were 54.9–69.1 ($n = 2$) and 63.0 ($n = 1$), respectively.

The results of brain β-galactosidase activity in transplantation experiments are shown in Table 2. Increases in β-galactosidase activity were found in the brains of each experimental group at 4 weeks after injection. Activity in the FBC-treated mice was definitely increased at 4 weeks as well as at 8 weeks, while activity at 8 weeks in the MSC-treated mice and mixed MSC and FBC-treated mice was almost the same level as that in the untreated mice. These findings were consistent with those in the X-Gal staining study.

3.3. Immunoassay of ganglioside GM1

Immunoassay of accumulated ganglioside GM1 was performed for each mouse using anti-GM1 ganglioside monoclonal antibody. Values are ratios to the amounts in age-matched normal control mice. The results are shown in Fig. 2 and Tables 3. At 4 weeks after injection, remarkable decrease in ganglioside GM1 accumulation

Table 1
Mouse numbers used for each experiment.

Time after injection	1 week	2 weeks	4 weeks	8 weeks	6 months
	[X-Gal staining]				
Uncultured FBC	1	1	3	3	2
Cultured FBC	1	1	3	3	2
MSC		2	3	3	1
Mixed MSC and FBC			3	3	1
	[β-galactosidase activity]				
Uncultured FBC			2	2	
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	
	[Immunoblot assay of ganglioside GM1 amount]				
Uncultured FBC			1	1	1
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	

Table 2
β-Galactosidase activity.

	4 weeks	8 weeks
Age-matched normal control (mean ± SD)	197 ± 61 ($n = 7$)	159 ± 56 ($n = 7$)
Non-treated (mean ± SD)	4.38 ± 0.35 ($n = 5$)	4.10 ± 0.47 ($n = 5$)
Treated with uncultured FBC	Mouse 1	Mouse 7
	Rt: 6.65[a]	Rt: 4.94
	Lt: 5.31[a]	Lt: 6.03[a]
	Mouse 2	Mouse 8
	Rt: 7.36[a]	Rt: 5.58[a]
	Lt: 5.33[a]	Lt: 5.05[a]
Treated with MSC	Mouse 3	Mouse 9
	Rt: 6.30[a]	Rt: 4.13
	Lt: 5.95[a]	Lt: 3.67
	Mouse 4	Mouse 10
	Rt: 5.74[a]	Rt: 4.19
	Lt: 5.12[a]	Lt: 5.05[a]
Treated with mixed MSC and FBC	Mouse 5	Mouse 11
	Rt: 5.80[a]	4.13 (mix of both hemispheres)
	Lt: 5.40[a]	
	Mouse 6	Mouse 12
	Rt: 5.06	Rt: 4.85
	Lt: 4.52	Lt: 5.02

Values are in nmol/mg/h. Each sample was tested in duplicate and results are mean values. Rt, right hemisphere; Lt, left hemisphere.
[a] Increase of activity over mean + 2SD of non-treated mice.