BIOLOGICAL ACTIVITIES OF CYCLOPHELLITOL

SONOKO ATSUMI, HIRONOBU IINUMA, CHISATO NOSAKA and KAZUO UMEZAWA†

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
†Department of Applied Chemistry, Faculty of Science and Technology, Keio University,
3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223, Japan

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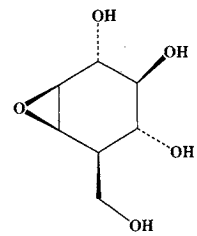
Cyclophellitol ((1*S*,2*R*,3*S*,4*R*,5*R*,6*R*)-5-hydroxymethyl-7-oxabicyclo[4,1,0]heptane-2,3,4-triol) was tested against 9 glycosidases and found to be a specific inhibitor of almond β-glucosidase. Cyclophellitol inhibited almond β-glucosidase activity by 50% at 0.8 μg/ml and was a competitive inhibitor of almond β-glucosidase as revealed by Lineweaver-Burk plot. Cyclophellitol was inactive against yeast α-glucosidase, β-galactosidase, β-glucuronidase, α-L-fucosidase, end-β-*N*-acetyl glucosaminidase, α-mannosidase, and cellulase. It was weakly active toward fungal β-xylosidase. Cyclophellitol-treated almond β-glucosidase was equally suppressed after dialysis; thus cyclophellitol is likely to bind to almond β-glucosidase irreversibly. The inhibitor was found by fluorimetric assay to be active against β-glucosidase but inactive toward α-glucosidase in Molt-4 microsomal fraction. It also inhibited Molt-4 β-glucocerebrosidase completely at 2 μg/ml when the enzyme was assayed with a synthetic labeled substrate, and the inhibitory activity was more than one hundred times higher than that of nojirimycin, castanospermine, or of deoxynojirimycin. Mice administered 1 mg of cyclophellitol daily for 5 days began to exhibit severe abnormalities of nervous system similar to those found in GAUCHER's mouse.

Recent studies have revealed various biological effects of glycosidase inhibitors, and these effects were thought to arise from their trimming action on glycoproteins.[1,2] HADWIGER-FANGMEIER *et al.* reported rat kidney (NRK) cells transformed by the v-*sis* oncogene lost serum- and anchorage-independence after treatment with castanospermine, an α-glucosidases I, II, and β-glucosidase inhibitor.[3] NICHOLS *et al.* described that v-*fms*-transformed Fisher rat embryo (FRE) cells grown in the presence of castanospermine reverted to normal cell morphology, anchorage dependence, contact inhibition of growth, and lowered hexose uptake.[4] Glucosidase inhibitors such as swainsonine,[5] castanospermine,[6] and glucarolactam[7] have been reported to inhibit the experimental metastasis of B16-melanoma cells. Deoxynojirimycin,[8] an α-glucosidases I, II, and β-glucosidase inhibitor, and castanospermine[9] inhibit cell fusion activity of human immunodeficiency virus (HIV).

β-Glucocerebrosidase is a key enzyme in GAUCHER's disease,[10] which is one of the sphingolipidoses that arises as an autosomal recessive disorder. Medical treatment required for essential healing is not obvious for this disease. KANFER *et al.*[11] and DAS *et al.*[12] produced an animal model and cells, respectively of GAUCHER's disease by using conduritol B epoxide, which is a cyclitol inhibiting glucocerebrosidase.[13] But because of the low efficiency of conduritol B epoxide, long-term daily injection of the inhibitor into experimental animals is required.[11]

Fig. 1. Structure of cyclophellitol.



Recently we isolated cyclophellitol (Fig. 1) from *Phellinus* sp.[14] as a novel inhibitor of almond β-glucosidase. In this report, we describe its activity

on various sugar-hydrolyzing enzymes including human β-glucocerebrosidase and the inhibitory mechanism against almond β-glucosidase.

Materials and Methods

Materials

Cyclophellitol was isolated from culture filtrates of *Phellinus* sp. as previously described.[14] All enzymes listed in Table 1, *p*-nitrophenyl glycosides, 4-methylumbelliferyl glycosides, 1-β-D-glucosylsphingosine, and β-glucocerebroside were purchased from Sigma Chemical Company. Deoxynojirimycin and castanospermine were purchased from Genzyme Corporation. Nojirimycin bisulfite was kindly provided by Meiji Seika Kaisha, Ltd. Labeled stearic acid was purchased from New England Nuclear Corporation, and Unisil activated silicic acid (100~200 mesh), from Clarkson Chemical Company, Inc. CDF_1 mice were supplied from Charles River Japan. Molt-4 cells were kindly provided by Dr. HIROO HOSHINO (Gunma University, School of Medicine).

Assay of Commercially Available Enzymes

Commercially available glycosidases except cellulase were assayed with the appropriate *p*-nitrophenyl glycosides following the method by SOUL *et al.*[15] with a slight modification. The reaction mixture contained 25 mM sodium acetate buffer (pH 5.2), 5 mM *p*-nitrophenyl glycoside, and enzyme in a final volume of 0.5 ml. We used enough enzyme to give an optical density change of 1.0 to 2.0 during the incubation period. In experiments with almond β-glucosidase, we used 1.7 μg/ml enzyme in the reaction mixture unless otherwise described. After incubation at 37°C for 10 minutes, 2.5 ml of 0.4 M glycine buffer (pH 10.4) was added, and liberated *p*-nitrophenol was measured at 410 nm. We did not preincubate the inhibitor-enzyme mixture unless stated otherwise in the text.

Cellulase was assayed in a 3-ml reaction mixture containing 25 mM sodium acetate buffer (pH 5.2), 6 mg of filter paper, and 10 mg/ml enzyme. After incubation at 37°C for 90 minutes, the amount of reducing sugars in the reaction mixture was measured according to the method described by AVIGAD.[16]

Preparation of Molt-4 Lysate and the Microsomal Fraction

Molt-4 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. For preparation of Molt-4 lysate, the cells (5×10^6) were washed and collected by centrifugation, and resuspended in 3 ml of physiological saline (0.9% (w/v) NaCl). The cell suspension was stored at −80°C and homogenized with an Ultraturrax immediately before use.

For preparation of the microsomal fraction, the cells (8×10^8) were harvested and washed with 1.15% (w/v) KCl. They were suspended in 15 ml of 1.15% KCl and homogenized with a Potter homogenizer. After centrifugation at $10{,}000 \times g$ for 15 minutes at 4°C, the precipitate was resuspended, homogenized, and centrifugated again. The supernatants were combined and centrifuged at $100{,}000 \times g$ for 60 minutes at 4°C. The precipitate was finally suspended in 3 ml of phosphate-buffered saline (PBS) and used as α- *or* β-glucosidase.

Assay for Molt-4 Microsomal Glucosidases

The reaction was started by the addition of 10 μl Molt-4 microsomal fraction to the reaction mixture (0.49 ml) containing 25 mM sodium acetate buffer (pH 5.5) and 5 mM 4-methylumbelliferyl glucoside. After incubation for 30~60 minutes at 37°C, the reaction was stopped by addition of 0.4 M glycine buffer (pH 10.7, 2.5 ml). The release of 4-methylumbelliferone was measured by fluorescence spectrophotometry with excitation and emission of 363 and 444 nm, respectively.

Preparation of Labeled Glucocerebroside

The substrate of β-glucocerebrosidase was prepared according to the method of ERICKSON and RADIN[17] with slight modification. The labeled stearic acid (0.5 mg, 100 μCi) was mixed with thionylchloride and stood for 60 minutes at 75°C. The reaction mixture was then evaporated to dryness under vacuum and 1 mg (24% excess) of 1-β-D-glucosylsphingosine in 0.3 ml of tetrahydrofuran and 0.15 ml of sodium acetate

50% (w/v) were added to the vessel. The mixture was stirred for 30 minutes, diluted with 0.5 ml of water, and washed twice by partitioning with 1 ml of $CHCl_3$-MeOH (9:1). The lower layer was concentrated under reduced pressure, and the resulting residue was purified by preparative TLC (Merck Kieselgel 60 F_{254}, $CHCl_3$-MeOH, 4:1) to give 0.7 mg of *N*-[^{14}C]stearoyl-glucosylsphingosine. The labeled glucocerebroside was diluted with cold β-glucocerebroside to 700 cpm/nmol and used as a substrate for the β-glucocerebrosidase.

β-Glucocerebrosidase Assay

The assay was carried out as described by MILLER *et al.* with modification.[18] The reaction was started by the addition of 30 μl of Molt-4 lysate to the substrate mixture (70 μl) containing *N*-[^{14}C]stearoyl-glucocerebroside (12,000 cpm), 0.2 M sodium acetate (pH 5.5), and 1.2% (w/v) sodium taurocholate. After incubation with vigorous shaking for 1 hour at 37°C, the reaction was stopped by the addition of 1% (w/v) KCl (0.5 ml) and $CHCl_3$-MeOH (2:1, 1.5 ml). The lower phase containing the substrate and the product was evaporated to dryness. The residue was dissolved in 2% methanol in chloroform and applied to a 0.5-ml Unicil silicic acid column pre-equilibrated with the same solvent. Under these conditions, the substrate but not the product, binds to the column. Then, the column was washed with the same solvent to elute the product, *N*-[^{14}C]stearoyl-glucosylsphingosine. The solvent was evaporated, and the residue was counted by liquid scintillation.

Animal Toxicity

We administered 1, 0.33 or 0.11 mg of cyclophellitol to each of four CDF_1 mice (5 weeks old, female) daily for 5 days by intraperitoneal injection. The control mice were given by 250 μl of 0.9% saline. Each mouse was weighed and observed for behavior everyday.

Results

Inhibitory Activity against Glucosidases

As shown in Table 1, cyclophellitol inhibited almond β-glucosidase completely at 100 μg/ml (568 μM), but it showed no marked inhibitory activity against the other 8 enzymes tested. Nojirimycin also inhibited only almond β-glucosidase. Thus, the inhibition spectrum is closely related to that of nojirimycin.

Inhibition of Almond β-Glucosidase by Cyclophellitol

The IC_{50} of cyclophellitol on almond β-glucosidase was 0.85 μg/ml, while the IC_{50}'s of nojirimycin,[19] 1-deoxynojirimycin,[19] and castanospermine[15] were 2.5 μg/ml, 43 μg/ml, and 12 μg/ml, respectively.

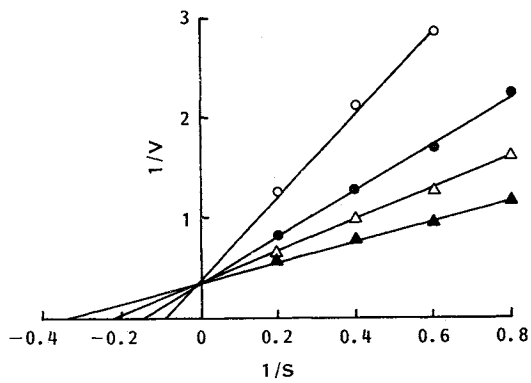
Table 1. Effect of cyclophellitol on sugar-hydrolyzing enzymes.

Enzyme tested	Inhibition at 100 μg/ml (%)	
	Cyclophellitol	Nojirimycin
β-Glucosidase (Almond)	99	99
β-Xylosidase (*Aspergillus niger*)	43	26
α-Glucosidase (Yeast)	15	27
β-Galactosidase (*Escherichia coli*)	9	29
β-Glucuronidase (*E. coli*)	0	20
α-L-Fucosidase (Bovine kidney)	0	10
End-β-*N*-acetyl glucosaminidase (Bovine kidney)	0	0
α-Mannosidase (Jack bean)	0	0
Cellulase (*A. niger*)	0	ND

ND: Not determined.

Fig. 2. Lineweaver-Burk plot of almond β-glucosidase reaction with cyclophellitol.

○ 10 μM inhibitor, ● 5 μM, △ 2.5 μM, ▲ control.



Inhibition of almond β-glucosidase by cyclophellitol was shown to be competitive in the double-reciprocal plot of the enzyme activity (Fig. 2). The *Ki* value was deduced to be 2.8 μM from the Dixon plot.

Inhibitory activity of cyclophellitol was increased remarkably by a long period of preincubation with the enzyme. Cyclophellitol at 0.1 μg/ml inhibited only 20% of the enzyme activity without preincubation, while it inhibited 70% and 94% with preincubation at room temperature for 1 hour and 3 hours, respectively (data not shown). Therefore, in order to investigate the irreversible binding between the enzyme and cyclophellitol, we examined the influence of dialysis on the enzyme inhibition. As shown in Fig. 3, cyclophellitol-treated enzyme was equally suppressed after dialysis; while nojirimycin, although reported to be a tight-binding inhibitor,[20] lost its inhibitory activity after dialysis.

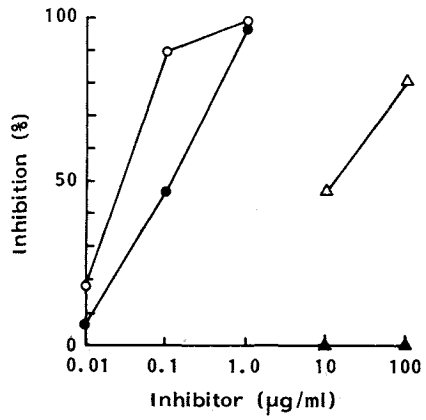
Inhibitory Activity on Molt-4 Cell-associated Glucosidase

The IC_{50} value of cyclophellitol against β-glucosidase activity in Molt-4 microsomal fraction was 0.015 μg/ml, which was smaller than those of the amino sugar inhibitors (Table 2). The activity of castanospermine, which was reported to inhibit β-glucosidase in human fibroblast,[15] was also over two hundred times lower than that of cyclophellitol.

On the other hand, cyclophellitol had no activity against α-glucosidase in the Molt-4 microsomal fraction at 100 μg/ml. Thus, it was distinguishable from the amino sugars or castanospermine, as their IC_{50}'s were under 0.01 μg/ml (Table 2).

Fig. 3. Effect of dialysis on β-glucosidase inhibition by cyclophellitol or nojirimycin.

Cyclophellitol (●, ○) or nojirimycin (▲, △) were added to the enzyme solution containing 10 μg/ml almond β-glucosidase and 25 mM sodium acetate (pH 5.2).



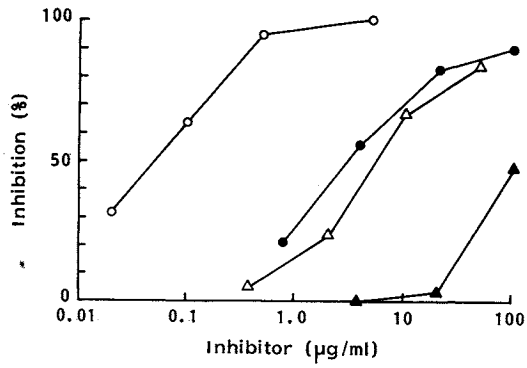
After incubation at room temperature for 60 minutes, one half volume was dialyzed against the same sodium acetate buffer for 12 hours at 4°C (●, ▲). The rest was stood for 12 hours at 4°C without dialysis (○, △). The dialyzed and control samples were tested equally for the enzyme activities as described in the text. Values are means of duplicate samples.

Table 2. Inhibitory activity against Molt-4 microsomal glucosidases.

Inhibitor	IC_{50} (μg/ml)	
	α-Glucosidase	β-Glucosidase
Cyclophellitol	>100	0.015
Nojirimycin	<0.01	1.0
Deoxynojirimycin	<0.01	20
Castanospermine	<0.01	3.6

Fig. 4. Inhibition of Molt-4 glucocerebrosidase by glucosidase inhibitors.

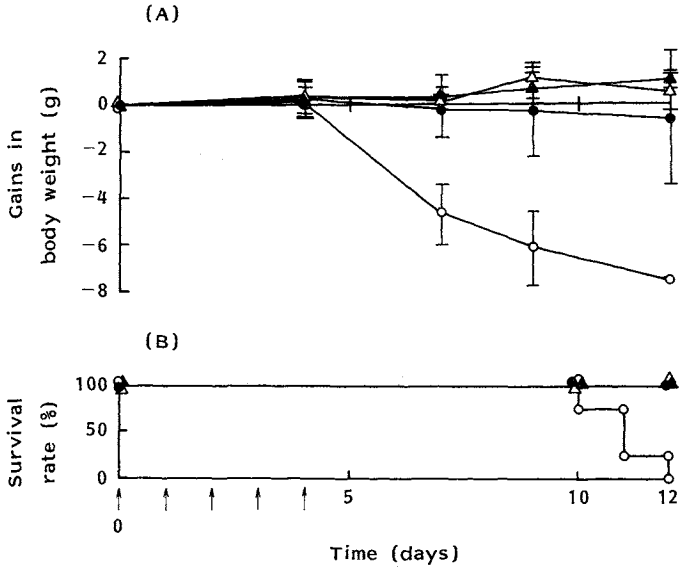
○ Cyclophellitol, ● nojirimycin, △ castanospermine, or ▲ deoxynojirimycin was added to the reaction mixture for Molt-4 glucocerebrosidase.



Values are means of duplicated samples.

Fig. 5. Effect of cyclophellitol on body weight and survival rate of mice.

(A) Body weight, (B) survival rate. Mice were intraperitoneally injected with cyclophellitol at 1 mg/mouse (○), 0.33 mg/mouse (●), 0.11 mg/mouse (△), or 0.9% saline (▲).



Values are means and standard deviation of 4 mice except that of 1 mg/mouse administration of cyclophellitol on day 12 in (A).

Arrows indicate times of the injections.

Inhibitory Activity on Molt-4 Lysate β-Glucocerebrosidase

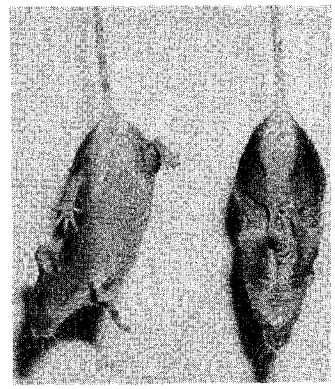
As shown in Fig. 4, cyclophellitol inhibited Molt-4 β-glucocerebrosidase completely at 2 μg/ml. The activity was more than one hundred times higher than that of castanospermine which was earlier reported to inhibit β-glucocerebrosidase.[15] Nojirimycin inhibited the enzyme only weakly, and deoxynojirimycin showed the lowest inhibitory activity in our assay system.

In Vivo Toxicity of Cyclophellitol

Cyclophellitol did not show cytotoxicity in cultured NIH3T3, P388 and Molt-4 cells even at 100 μg/ml. On the other hand, in the course of our screening of antitumor compounds we found that cyclophellitol showed severe toxicity in mice, accompanied by abnormal behaviors. Then, we observed in detail mice administered with cyclophellitol.

Fig. 6. Contractile convulsion induced by cyclophellitol on day 6.

Right mouse administrated 1 mg of cyclophellitol, left mouse administrated 0.9% saline.



Mice daily administered 1 mg of cyclophellitol for 5 days lost their body weight (Fig. 5A), and began to exhibit severe abnormalities on day 6 (Fig. 6). They all died on day 10～12 (Fig. 5B). Mice administered 0.33 mg of cyclophellitol lost body weight only slightly, and began to exhibit these abnormalities on day

8~10. Cyclophellitol at 0.11 mg per mouse did not show either toxicity or abnormal movements until day 14.

Discussion

We have shown that cyclophellitol is a specific inhibitor of almond and human Molt-4 lysate β-glucosidase. Furthermore, it has no activity against α-glucosidase in Molt-4 cells. GRUTERS *et al.*[8] and WALKER *et al.*[9] observed that deoxynojirimycin and castanospermine inhibit the cytostatic effect of HIV by perturbing its gp120 protein glycosylation. Deoxynojirimycin[21,22] and castanospermine[23,24] were shown to inhibit α-glucosidase I and α-glucosidase II. Cyclophellitol had no anti-HIV activity on infected CEM cell line (National Cancer Institute Development Therapeutics Program *in vitro* testing results) and on MT-4 cells (personal communication from Prof. H. HOSHINO, Gunma University). So, the anti-HIV activity of deoxynojirimycin and castanospermine may be due to their ability to inhibit α-glucosidase but not β-glucosidase.

We have shown that cyclophellitol is a competitive and irreversible inhibitor of β-glucosidase and also that it inhibits mammalian β-glucocerebrosidase. These biological properties are distinguishable from those of amino sugar derivative inhibitors or castanospermine, but are similar to these of conduritol B epoxide.[13,25~28] They share a common structure as epoxide cyclitols.

As mentioned earlier KANFER *et al.*[11] have prepared an animal model of GAUCHER's disease by using conduritol B epoxide. They administered 100 mg/kg body weight of conduritol B epoxide over a 3-week period to C57/Bl mice to obtain "GAUCHER's" mice. We have shown that cyclophellitol can induce abnormalities in the nervous system, as are found in GAUCHER's mouse (Fig. 6) with daily administration for 5 days of 16.5 mg/kg body weight. Though the mouse strain was different, the doses of cyclophellitol to induce GAUCHER's-like disease in CDF_1 mice were apparently lower than those of conduritol B epoxide required in C57/Bl mice.[11,25] The mechanism of symptom induction by cyclophellitol may be due to the inhibition of β-glucocerebrosidase, as for conduritol B epoxide.

Cyclophellitol appears to be useful as an inhibitor of β-glucosidases from a variety of sources. Furthermore, cyclophellitol may be useful in induction of GAUCHER's-like disease in mice, through inhibition of glucocerebrosidase activity.

Acknowledgments

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References

1) DATEMA, R.; S. OLOFSSON & P. A. ROMERO: Inhibitors of protein glycosylation and glycoprotein processing in viral systems. Pharmacol. Ther. 33: 221~286, 1987
2) KLENK, H.-D. & R. T. SCHWARZ: Viral glycoprotein metabolism as a target for antiviral substances. Antiviral Res. 2: 177~190, 1982
3) HADWIGER-FANGMEIER, A.; H. NIEMANN & T. TAMURA: Inhibition of glycosylation processing alters the growth parameters of cells transformed by the oncogene of Simian Sarcoma virus. Arch. Virol. 104: 339~345, 1989
4) NICHOLS, E. V.; R. MANGER, S. HAKOMORI & L. R. ROHRSCHNEIDER: Transformation by the oncogene v-*fms*: The effect of castanospermine on transformation-related parameters. Exp. Cell Res. 173: 486~495, 1987
5) HUMPHRIES, M. J.; K. MATSUMOTO, S. L. WHITE & K. OLDEN: Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proc. Natl. Acad. Sci. U.S.A. 83: 1752~1756, 1986
6) HUMPHRIES, M. J.; K. MATSUMOTO, S. L. WHITE & K. OLDEN: Inhibition of experimental metastasis by castanospermine in mice: Blockage of two distinct stages of tumor colonization by oligosaccharide processing inhibitors. Cancer Res. 46: 5215~5222, 1986
7) TSURUOKA, T.; H. FUKUYASU & K. KAWAHARAJO: Inhibition of pulmonary metastasis of murine melanoma by glucarolactam. Proceedings of the Japanese Cancer Association, 48th Annual Meeting, p. 216, Nagoya, Oct.

23～25, 1989

8) GRUTERS, R. A.; J. J. NEEFJES, M. TERSMETTE, R. E. Y. DE GOEDE, A. TULP, H. G. HUISMAN, F. MIEDEMA & H. L. PLOEGH: Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature 330: 74～77, 1987
9) WALKER B. D.; M. KOWALSKI, W. C. GOH, K. KOZARSKY, M. KRIEGER, G. ROSEN, L. ROHRSCHNEIDER, W. A. HASELTINE & J. SODROSKI: Inhibition of human immunodeficiency virus syncytium formation and virus replication by castanospermine. Proc. Natl. Acad. Sci. U.S.A. 84: 8120～8124, 1987
10) BRADY, R. O.; J. N. KANFER & D. SHAPIRO: Metabolism of glucocerebrosidase II. Evidence of an enzymatic deficiency in Gaucher's disease. Biochem. Biophys. Res. Commun. 18: 221～225, 1965
11) KANFER, J. N.; G. LEGLER, J. SULLIVAN, S. S. RAGHAVAN & R. A. MUMFORD: The Gaucher mouse. Biochem. Biophys. Res. Commun. 67: 85～90, 1975
12) DAS, P. K.; G. J. MURRAY, A. E. GAL & J. A. BARRANGER: Glucocerebrosidase deficiency and lysosomal strage of glucocerebroside induced in cultured macrophages. Exp. Cell Res. 168: 463～474, 1987
13) DANIELS, L. B.; R. H. GLEW, N. S. RADIN & R. R. VUNNAM: A revised fluorometric assay for Gaucher's disease using conduritol-β-epoxide with liver as the source of β-glucosidase. Clin. Chim. Acta. 106: 155～163, 1980
14) ATSUMI, S.; K. UMEZAWA, H. IINUMA, H. NAGANAWA, H. NAKAMURA, Y. IITAKA & T. TAKEUCHI: Production, isolation and structure determination of a novel β-glucosidase inhibitor, cyclophellitol, from *Phellinus* sp. J. Antibiotics 43: 49～53, 1990
15) SAUL, R.; J. P. CHAMBERS, R. J. MOLYNEUX & A. D. ELBEIN: Castanospermine, a tetrahydroxylated alkaloid that inhibits β-glucosidase and β-glucocerebrosidase. Arch. Biochem. Biophys. 221: 593～597, 1983
16) AVIGAD, G.: Colorimetric ultramicro assay for reducing sugars. *In* Method in Enzymology. Vol. 41. *Ed.*, W. A. WOOD, pp. 27～29, Academic Press, 1975
17) ERICKSON, J. S. & N. S. RADIN: *N*-Hexyl-*O*-glucosyl sphingosine, an inhibitor of glucosyl ceramide β-glucosidase. J. Lipid Res. 14: 133～137, 1973
18) MILLER, R. R.; S. P. PETERS, M. S. KUHLENSCHMIDT & R. H. GLEW: The use of ion-exchange resins in the application of protein samples to gel filtration colums. Anal. Biochem. 72: 45～48, 1976
19) NIWA, T.; S. INOUYE, T. TSURUOKA, Y. KOAZE & T. NIIDA: "Nojirimycin" as a potent inhibitor of glucosidase. Agric. Biol. Chem. 34: 966～968, 1970
20) GROVER, A. K. & R. J. CUSHLEY: Studies on almond emulsin β-D-glucosidase II. Kinetic evidence for independent glucosidase and galactosidase sites. Biochem. Biophys. Acta 482: 109～124, 1977
21) HETTKAMP, H.; E. BAUSE & G. LEGLER: Inhibition by nojirimycin and 1-deoxynojirimycin of microsomal glucosidases from calf liver acting on the glycoprotein oligosaccharides Glc_{1-3} $Man_9GlcNac_2$. Biosci. Rep. 2: 899～906, 1982
22) SAUNIER, B.; R. D. KILKER, Jr., J. S. TKACZ, A. QUARONI & A. HERSCOVICS: Inhibition of *N*-linked complex oligosaccharide formation by 1-deoxynojirimycin, an inhibitor of processing glucosidases. J. Biol. Chem. 257: 14155～14161, 1982
23) PAN, Y. T.; H. HORI, R. SAUL, B. A. SANFORD, R. J. MOLYNEUX & A. D. ELBEIN: Castanospermine inhibits the processing of the oligosaccharide portion of the influenza viral hemagglutinin. Biochemistry 22: 3975～3984, 1983
24) PALAMARCZYK, G. & A. D. ELBEIN: The effect of castanospermine on the oligosaccharide structures of glycoproteins from lymphoma cell lines. Biochem. J. 227: 795～804, 1985
25) KANFER, J. N.; M. C. STEPHENS, H. SINGH & G. LEGLER: The Gaucher mouse. Prog. Clin. Biol. Res. 95: 627～644, 1982
26) LEGLER, G.: Action Mechanism of glucoside-splitting enzymes. I. Preparation and properties of specific deactivators. Z. Phisiol. Chem. 345: 197～214, 1966
27) LEGLER, G.: Labeilling of the active centre of a β-glucosidase. Biochim. Biophys. Acta 151: 728～729, 1968
28) LEGLER, G. & E. BIEBERICH: Active site direct inhibition of a cytosolic β-glucosidase from calf liver by bromoconduritol B epoxide and bromoconduritol F. Arch. Biochem. Biophys. 260: 437～442, 1988