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Development of a New Inhibitor of Glucosylceramide Synthase[1]

Masayuki Jimbo,[*] Kiwamu Yamagishi,[*] Toshiaki Yamaki,[†] Katsuyuki Nunomura,[†] Kazuya Kabayama,[‡] Yasuyuki Igarashi,[‡] and Jin-ichi Inokuchi[‡,2]

[*]Tokyo Research Institute, Seikagaku Corp., Tateno, Higashiyamato, Tokyo 207-0021; [†]Department of Neurosurgery, Sapporo Medical University School of Medicine, S-1, W-16, Sapporo, Hokkaido 060-0812; and [‡]Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, N-12, W-6, Sapporo, Hokkaido 060-0812

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Analogs of the potent inhibitor of glucosylceramide (GlcCer) synthase, D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), based on substitutions in the palmitoyl group were made by means of a stereo-selective synthetic method in order to elucidate the role of the hydrophobic portion in both the inhibitory action toward the enzyme and the biological effects. While P4 strongly inhibited GlcCer synthase with an IC_{50} of 0.5 μM *in vitro*, it also inhibited cell growth by 50% at the concentration of 7 μM. The shorter *N*-acyl chain analogs including decanoyl, octanoyl, and hexanoyl groups showed similar IC_{50} values for GlcCer synthase (around 2 μM), but the hexanoyl analog exhibited only a slight inhibitory effect on cell growth, showing the dissociation between GlcCer depletion and cell growth. Several compounds which exhibit similar hydrophobicity to the hexanoyl analog of P4 were subsequently designed. We found that D-*threo*-1-phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol (PBPP) was a most potent inhibitor, showing an IC50 of 0.3 μM. In cultured cells, PBPP was able to deplete glycosphingolipids without affecting cell growth or the ceramide level.

Key words: ceramide, glucosylceramide synthase, glycosphingolipids, inhibitor, PBPP, PDMP.

Efforts to deplete cellular glycosphingolipids (GSLs) by means of the specific inhibitors of GSL biosynthesis are useful for determining the functions of endogenous GSLs. Nearly all GSLs are derived from glucosylceramide (GlcCer), which is synthesized from ceramide and UDP-glucose. The sequential addition of additional monosaccharide and sialic acid to GlcCer results in a complex family of structures, such as the ganglio, globo, isoglobo, neolacto, and lacto series. It has been demonstrated by Inokuchi and Radin (*1*) that an analog of ceramide, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), could inhibit UDP-glucose:*N*-acylsphingosine glucosyltransferase [EC 2.4.1.80] which forms GlcCer. Thus, D-PDMP has been used extensively to study the biological function of GSLs in living cells, including in cell growth, cell adhesion, and cell-signalling pathways (*2*). Because of the involvement of GlcCer and higher GSLs derived from GlcCer in multiple pathological processes (*e.g.*, tumorigenesis, drug resistance, and host-pathogen interactions) (*3–5*), GlcCer synthase has been suggested to be a potential target for therapeutic agents (*6*, *7*). Indeed, PDMP and related compounds have been shown to have several potentially useful effects including the inhibition of tumor metastasis (*8*), reduction of the tumorigenicity of Shope carcinoma cells (*9*), inhibition of the shedding of immunosuppressive gangliosides in neuroblastoma cells (*10*, *11*), and preferential killing of multidrug-resistant tumor cells (*12*).

In previously reported work, a series of PDMP analogs was synthesized (*13–16*). Replacement of the decanoyl moiety with a palmitoyl moiety enhanced the effectiveness of PDMP (*13*). In addition, replacement of the morpholino ring with a pyrrolidino ring, yielding D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), not only enhanced the potency as to the inhibition of GlcCer synthase but, at 2–8 μM, also exhibited a strong suppressive effect on cell growth (*15*). Recently, modifications of the phenyl ring of P4 to make it more polar yielded very potent inhibitors (D-*threo*-ethylenedioxy-P4 or D-*threo*-4′-hydorxy-P4) that act at nM concentrations without inhibiting cell growth (*17*). We report here the establishment of a stereo-selective synthetic method for these ceramide analogs, and have synthesized P4 analogs, replacing the *N*-palmitoyl chain with various less hydrophobic moieties. D-*threo*-1-Phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol (PBPP) was found to be a novel inhibitor of GlcCer synthase lacking cellular toxicity.

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[2] To whom correspondence should be addressed. Fax:+81-11-706-4986, E-mail: inokuchi@kinou02.pharm.hokudai.ac.jp

Abbreviations: GSLs, glycosphingolipids; GlcCer, glucosylceramide; DMEM, Dulbecco's modified Eagle medium; PAPP, D-*threo*-1-phenyl-2-amino-3-pyrrolidino-1-propanol; PBPP, D-*threo*-1-phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PDPP, D-*threo*-1-phenyl-2-decanoylamino-3-pyrrolidino-1-propanol; PHPP, D-*threo*-1-phenyl-2-hexanoylaminino-3-pyrrolidino-1-propanol; POPP, D-*threo*-1-phenyl-2-octanoylamino-3-pyrrolidino-1-propanol; PPPP-(P4), D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

MATERIALS AND METHODS

Synthesis of PBPP and Its Analogs—Synthesis of (2*R*)-2-benzyloxycarbonylamino-3-hydroxy-1-phenyl-1-propanone (compound 2): To a solution of *N*-benzyloxycarbonyl-D-serine (compound 1,479 mg, 2.0 mmol) in tetrahydrofuran (2 ml), a solution of phenylmagnesium bromide in tetrahydrofuran (1 M, 16 ml) was added dropwise in an ice bath for 2 min under an argon atmosphere. After stirring overnight at room temperature, the reaction mixture was thrown into icy 1 N HCl and then extracted with ethyl acetate (50 ml × 3). The combined organic layer was washed with water, a sodium bicarbonate solution, water, and brine, 50 ml each, successively, dried over sodium sulfate, and then evaporated to obtain the crude product. The crude product was recrystallized from a mixture of ethyl acetate and *n*-hexane (1:1) to obtain compound 2 as a white solid (511.5 mg, 85.4% yield).

$[\alpha]_D^{20}$ + 5.7° (*c* = 1, methanol). ^{1}H-NMR ($CDCl_3$) δ: 7.80 (2H, d, *J* = 7.33 Hz, aromatic), 7.63 (1H, t, aromatic), 7.51 (2H, t, aromatic), 7.38–7.26 (5H, m, aromatic), 6.12 (1H, d, *J* = 5.86 Hz, NH), 5.40 (1H, m, H-2), 5.16 (2H, s, CH_2-O-CO), 4.08–4.04 (1H, m, H-3), 3.92–3.87 (1H, m, H-3)

Synthesis of (1*R*,2*R*)-2-benzyloxycarbonylamino-3-hydroxy-1-phenyl-1-propanol (compound 3): To a solution of compound 2 (598.9 mg, 2.0 mmol) in tetrahydrofuran (12 ml), a solution of diisobutylaluminum hydride in *n*-hexane (1 M, 8 ml) was added dropwise for 10 min under an argon atmosphere at –78°C, followed by stirring for 2 h, and then a further 1 h at room temperature.

1 N HCl (30 ml) was added to the reaction mixture, followed by extraction with ethyl acetate (70 ml × 3). The combined organic layer was washed with water and brine, dried over sodium sulfate, and then evaporated to obtain the crude product. The crude product was recrystallized from a mixture of ethyl acetate and *n*-hexane (1:1) to obtain compound 3 as a white solid (481.6 mg, 80.0% yield). ^{1}H-NMR ($CDCl_3$) δ: 7.36–7.25 (10H, m, aromatic), 5.00 (3H, m, H-1, CH_2-O-CO), 3.89–3.75 (3H, m, H-2, H-3)

Synthesis of (1*R*,2*R*)-2-benzyloxycarbonylamino-1-phenyl-1,3-propanediol-3-methanesulfonyl ester (compound 4): To a solution of (1*R*, 2*R*)-2-benzyloxycarbonylamino-1-phenyl-1,3-propanediol (compound 3, 21.2 g, 70.3 mmol) in pyridine (350 ml), methanesulfonyl chloride (5.6 ml, 72.3 mmol) was added dropwise in an ice bath for 5 min. The resulting mixture was stirred for 30 min in the ice bath and then overnight at room temperature. After checking the completion of the reaction by TLC (chloroform:methanol = 20:1, *n*-hexane:ethyl acetate = 1:1), the solvent was removed by evaporation and ethyl acetate (500 ml) was added to the residue, and then the organic layer was washed with 1 N HCl (250 ml × 3) and brine (250 ml), dried over sodium sulfate, and finally evaporated. The crude product was washed with a mixture of ethyl acetate and *n*-hexane (1:1) to obtain compound 4 as a white solid (25.3 g, 95.0% yield).

^{1}H-NMR ($CDCl_3$) δ: 7.35–7.26(10H, m, aromatic), 5.30 (1H, d, *J* = 7.81 Hz, NH), 5.02 (2H, s, CH_2-O-CO), 4.99 (1H, d, *J* = 3.91 Hz, C<u>H</u>-OH), 4.43–4.39 and 4.22–4.12 (3H, m, N-CH-CH_2), 2.98 (3H, s, SO_3CH_3)

Synthesis of (1*R*,2*R*)-1-phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol (compound 5; PBPP): To a solution of compound 4 (1.52 g, 4.01 mmol) in *N,N*-dimethylformamide (8 ml), pyrrolidine (1.14 g, 16.0 mmol) was added at room temperature, and then the mixture was stirred at 45°C for 18 h. After checking the completion of the reaction by TLC (chloroform:methanol = 20:1), a sodium bicarbonate solution (70 ml) and ethyl acetate (100 ml) were added to the mixture, and then the organic layer was washed with water (70 ml) and brine (70 ml), and dried over sodium sulfate. After removing the solvent by evaporation, the crude product was purified by silica gel column chromatography (chloroform:methanol = 20:1) to obtain compound 5 (PBPP) as a colorless oil (1.21 g, 85.5% yield).

^{1}H-NMR ($CDCl_3$) δ: 7.39–7.24 (10H, m, aromatic), 5.06–5.02 (2H, m, CH_2-O-CO), 4.99 (1H, d, *J* = 3.91 Hz, H-1), 4.07 (1H, m, H-2), 2.9–2.6 (6H, m, N $(CH_2)_3$), 1.83–1.74 (4H, m, H-3', H-4')

^{13}C-NMR ($CDCl_3$) δ:156.0, 140.8, 136.5, 128.4, 128.2, 128.0, 127.8, 127.4, 126.1, 75.7, 66.6, 58.1, 55.2, 53.4, 23.6

Synthesis of (1*R*,2*R*)-2-amino-1-phenyl-3-pyrrolidino-1-propanol (compound 6): A solution of compound 5 (PBPP, 394.2 mg, 1.11 mmol) in methanol (10 ml) was hydrogenated with 10% palladium carbon (124.8 mg, 10.6 mol%) under a hydrogen atmosphere. After checking the completion of the reaction by TLC (chloroform:methanol = 7:3 and ethyl acetate:methanol = 2:1), palladium carbon was removed by filtration, and then the filtrate was concentrated to obtain compound 6 as a colorless oil (250.2 mg). TLC R_f 0.1 (chloroform:methanol = 7:3), 0.1 (ethyl acetate: methanol = 2:1)

Synthesis of (1*R*,2*R*)-2-hexanoylamino-1-phenyl -3-pyrrolidino-1-propanol (compound 7; PHPP): To a solution of compound 6 (160.0 mg, 0.727 mmol) in methanol (4 ml), triethyl amine (242.8 μl, 1.745 mmol) and hexanoyl chloride (201.6 μl, 1.442 mmol) were added dropwise in an ice bath, followed by stirring at room temperature. After checking the completion of the reaction by TLC (chloroform:methanol = 7:3, ethyl acetate:methanol = 2:1), the solvent was removed by evaporation. To the residue, a sodium bicarbonate solution (25 ml) and ethyl acetate (40 ml) were added, and then the organic layer was washed with water (25 ml) and brine (25 ml), dried over sodium sulfate, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (ethyl acetate:methanol = 2:1) to obtain compound 7 (PHPP) as a colorless oil (21.7 mg, 9.4% yield).

^{1}H-NMR ($CDCl_3$) δ: 7.36–7.23 (5H, m, aromatic), 5.86 (1H, d, *J* = 7.33 Hz, NH), 5.04 (1H, d, *J* = 2.93 Hz, H-1), 4.27–4.22 (1H, m, H-2), 2.85–2.83 (2H, m), 2.71–2.63 (4H, m), 2.09–2.04 (2H, m, $COCH_2$), 1.79 (4H, m), 1.52–1.44 (2H, m, $COCH_2C\underline{H}_2$), 1.29–1.20 (2H, m), 1.17–1.11 (2H, m), 0.84 (3H, t, CH_3)

^{13}C-NMR ($CDCl_3$) δ: 173.4, 141.1, 128.2, 127.4, 125.9, 75.8, 58.1, 55.3, 52.4, 36.7, 31.2, 25.3, 23.7, 22.3, 13.8

Synthesis of (1*R*,2*R*)-2-octanoylamino-1-phenyl-3-pyrrolidino-1-propanol (compound 8; POPP): To a solution of compound 6 (99.3 mg, 0.451 mmol) in methanol (3 ml), triethyl amine (132.0 μl, 0.949 mmol) and octanoyl chloride (148.0 μl, 0.867 mmol) were added dropwise in an ice bath, followed by stirring at room temperature. After checking the completion of the reaction by TLC (chloroform:methanol = 7:3, ethyl acetate:methanol = 2:1), the solvent was removed by evaporation. To the residue, a sodium bicarbonate solution (20 ml) and chloroform (50 ml) were added, and then

the organic layer was washed with water (20 ml) and brine (20 ml), dried over sodium sulfate, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (ethyl acetate:methanol = 2:1) to obtain compound 8 (POPP) as a colorless oil (41.5 mg, 26.6% yield).

^{1}H-NMR ($CDCl_3$) δ: 7.36–7.23 (5H, m, aromatic), 5.88 (1H, d, J = 7.33 Hz, NH), 5.04 (1H, d, J = 3.41 Hz, H-1), 4.25 (1H, m, H-2), 2.84 (2H, d, J = 4.88 Hz, H-3), 2.68 (4H, m, N $(CH_2)_2$), 2.07 (2H, m, $COCH_2$), 1.80 (4H, m), 1.47 (2H, m, $COCH_2C\underline{H}_2$), 1.21 (8H, m, $(C\underline{H}_2)_4CH_3$), 0.87(3H, t, CH_3)

13C-NMR (CDCl3) δ: 173.4, 141.0, 128.2, 127.3, 125.9, 75.7, 58.0, 55.3, 52.3, 36.7, 31.6, 29.0, 28.9, 25.6, 23.6, 22.5, 14.0

Synthesis of (1*R*,2*R*)-2-decanoylamino-1-phenyl-3-pyrrolidino-1-propanol (compound 9; PDPP): To a solution of compound 6 (123.6 mg, 0.562 mmol) in methanol (4 ml), triethyl amine (187.6 μl, 1.348 mmol) and decanoyl chloride (225.2 μl, 1.098 mmol) were added dropwise in an ice bath, followed by stirring at room temperature. After checking the completion of the reaction by TLC (chloroform:methanol = 7:3), the solvent was removed by evaporation. To the residue, a sodium bicarbonate solution (15 ml) and chloroform (30 ml) were added, and then the organic layer was washed with water (15 ml) and brine (15 ml), dried over sodium sulfate, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (chloroform:methanol = 20:1) to obtain compound 9 (PDPP) as a colorless oil (39.6 mg, 18.8% yield).

^{1}H-NMR ($CDCl_3$) δ: 7.36–7.23 (5H, m, aromatic), 5.91 (1H, d, J = 7.8 Hz, NH), 5.05 (1H, d, J = 3.4 Hz, H-1), 4.26 (1H, m, H-2), 2.86 (2H, d, J = 5.4 Hz, H-3), 2.70 (4H, m), 2.07 (2H, m, COCH2), 1.81 (4H, m), 1.47 (2H, m, $COCH_2C\underline{H}_2$), 1.3–1.1 (12H, m, $(C\underline{H}_2)_6CH_3$), 0.88 (3H, t, CH_3)

^{13}C-NMR ($CDCl_3$) δ: 173.5, 141.0, 128.2, 127.4, 125.9, 75.4, 57.9, 55.2, 52.3, 36.7, 31.8, 29.3, 29.2, 29.0, 25.6, 23.6, 22.6, 14.0

Synthesis of (1*R*,2*R*)-2-palmitoylamino-1-phenyl-3-pyrrolidino-1-propanol (compound 10; PPPP): To a solution of compound 6 (94.0 mg, 0.427 mmol) in methanol (4 ml), triethyl amine (213.9 μl, 1.538 mmol), and palmitoyl chloride (352.2 μl, 1.161 mmol) were added dropwise in an ice bath, followed by stirring at room temperature. After checking the completion of the reaction by TLC (chloroform:methanol = 7:3), the solvent was removed by evaporation. To the residue, a sodium bicarbonate solution (25 ml) and ethyl acetate (40 ml) were added, and then the organic layer was washed with water (25 ml) and brine (25 ml), dried over sodium sulfate, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (ethyl acetate:methanol = 4:1) to obtain compound 10 (PPPP) as a colorless oil (46.2 mg, 23.6% yield).

^{1}H-NMR ($CDCl_3$) δ: 7.36–7.23 (5H, m, aromatic), 5.91 (1H, d, J = 7.81 Hz, NH), 5.05 (1H, d, J = 3.42 Hz, H-1), 4.26 (1H, m, H-2), 2.88 (2H, d, J = 4.89 Hz, H-3), 2.72 (4H, m), 2.07 (2H, m, $COCH_2$), 1.82 (4H, m), 1.47 (2H, m, $COCH_2C\underline{H}_2$), 1.26 (24H, m, $(C\underline{H}_2)_{12}CH_3$), 0.88 (3H, t, CH_3)

^{13}C-NMR ($CDCl_3$) δ: 173.5, 141.0, 128.3, 127.4, 125.9, 75.7, 58.0, 55.3, 52.3, 36.7, 31.9, 29.7, 29.6, 29.4, 29.3, 29.1, 25.6, 23.6, 22.7, 14.1

GlcCer Synthase Assay—The ceramide substrate was prepared by evaporating to dryness a solution of 4 μmol (1.70 mg) *N*-octanoylsphingosine, 11.3 mg of dioleoyl phosphatidylcholine, and 2 mg of brain sulfatide in chloroform/methanol. The residue was suspended in cyclohexane and then lyophilized overnight. The resultant powder was dispersed in 1 ml of water by sonication with a probe immersed to ~2 cm, with the tube in an ice bath. The power cycle was 60 s on and 30 s off, for a total of 7.5 min. The almost clear suspension could be refrigerated and used for a week, provided it was resonicated the same way just before use.

The assaying of GlcCer synthase was performed as described previously (*1*). In brief, the 200 μl assay mixture contained 20 mM β-NAD (20 μl), 10 mM dithiothreitol (20 μl), 20 mM Na EDTA (20 μl), 200 mM $MgCl_2$ (10 μl), 1 M Tris-HCl (20 μl), mouse microsomal protein (10 μl), H_2O (10 μl), inhibitors (10 μl), liposomal ceramide (50 μl), and UDP-[^{3}H]glucose (30 μl, 111 KBq/20 nmol). The mixture was incubated at 37°C for 1 h. The reaction was stopped by the addition of 1 ml of isopropanol and 0.8 ml of aqueous Na_2SO_4 (40 mg anhydrous). After brief vortexing, 6 ml of *t*-butyl methyl ether was added, follwed by vortexing for 1 min. The mixture was centrifuged for 10 min at 1,500 ×*g*. Six of the 7 ml in the upper layer was transferred with a volumetric pipet to a scintillation vial and then evaporated to dryness under an air-flow in a 45°C dry block heater. The radioactivity of the residue was counted after dissolving it in 0.5 ml of water and 5 ml of ACSII (Amersham).

Cell Lines—Nitrosourea-induced rat glioma cell lines C6 and RG2, and human glioma cell line A172 were provided by the Japanese Cancer Research Resources Bank. MRC5 cells (human diploid fibroblast cell line) were obtained from the Riken Cell Bank. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate, without the addition of antibiotics, at 37°C under a 5% CO_2 atmosphere.

Assessment of In Vitro Cell Proliferation and Survival—The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) method was used for this purpose, as reported previously (*1*). Briefly, 200 μl aliquots of MRC5 cells at 2.5×10^4/ml (5,000 cells) were distributed into flat-bottomed 96-well plates (Falcon 3072, NJ), and then incubated for 24 h, various doses of P4 and its analogs being administered to the cultures. The cells were treated for 24 h and then their growth was assessed by adding 10 μl of a solution of MTT dissolved in PBS at the concentration of 5 mg/ml. After incubating the plates for 30 min at 37°C, the medium in each well was removed and replaced with 100 μl of a 0.04 N HCl-isopropanol solution. After mixing, the plates were read with a micro plate reader (Novapath Microplate Reader, Bio-Rad Laboratories) using a test wavelength of 550 nm and a reference wavelength of 630 nm. The plates were normally read within 1 h after the addition of isopropanol.

Glycolipid Analysis—The inhibitory effects of PBPP and related compounds on the glycolipid synthesis were examined. C6, RG2, and A172 glioma cells were plated at 5×10^6 cells per culture dish of 15 cm in diameter (145 cm^2, Nunc, Sweden). On the next day, the cells were further cultured in the presence or absence of an inhibitor for 4 days. After the culture, the cells were scraped from the dishes and glycolipid analysis was performed. Briefly, the total lipid was extracted with chloroform/methanol/water (4:8:3, v/v/v) for

2 consecutive 24 h periods, followed by 4 h extraction at 40°C. Glycerolipids were decomposed by saponification and the nonsaponifible lipids were separated into neutral and acidic fractions by ion-exchange column chromatography (DEAE Sephadex A-25; Pharmacia). Thin-layer chromatography (TLC) was carried out on Silica Gel 60 HPTLC plates (Merck) with the following solvent systems: chloroform/methanol/water (65: 25:4) for neutral GSLs, chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10) for acidic GSLs (gangliosides), chloroform/methanol/acetic acid/formic acid/water (42:18:7.2:2.4:1.2) for sphingomyelin, and chloroform/methanol/acetic acid (91:2:7) for ceramide. After TLC separation, the bands were visualized with 0.2% orcinol in 2 M sulfric acid for neutral GSLs, resorcinol-HCl reagent for gangliosides or 3% cupric acetate–8% H_3PO_4 for sphingomyelin and ceramide, and then densitometry was carried out with a dual wavelength flying spot scanner (CS 9000; Shimadzu, Kyoto). To assess the rates of synthesis of GSLs by metabolic radiolabeling, C6 glioma cells were treated with or without PBPP for 2 days, and for the last 6 h, D-[1,6-^{3}H]glucosamine (1,491.1 GBq/mmol; NEN, Boston) and D-[1-^{14}C-galactose (1.93 GBq/mmol; NEN, Boston) were added to the culture. The extraction and analysis of GSLs by HPTLC were performed in the same manner as described above, and the radioactive bands were identified with an imaging analyzer (BAS 2000; Fuji Film).

Protein Assay—Protein was determined with bicinchoninic acid protein assay kits (Pierce) using bovine serum albumin as the standard.

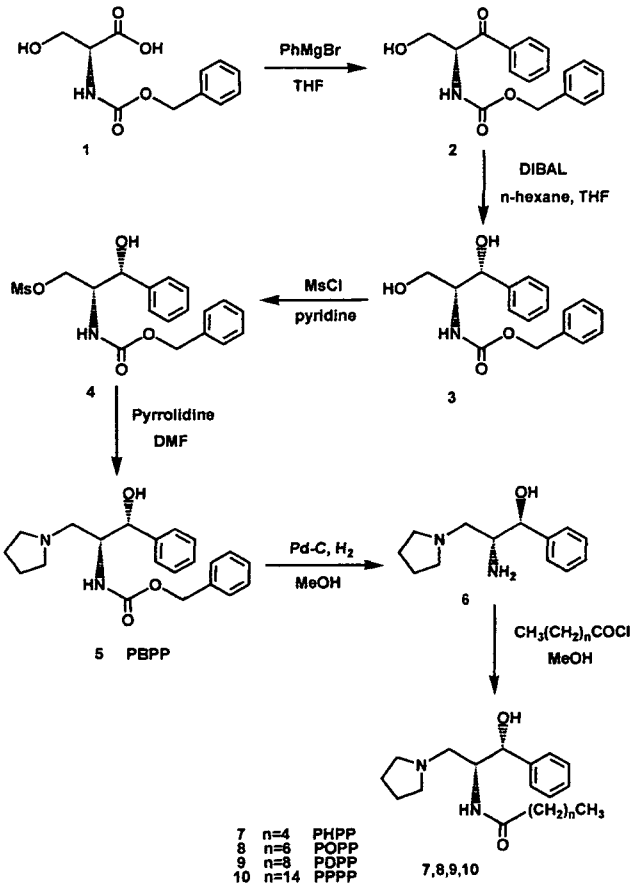


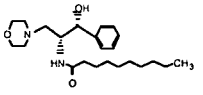
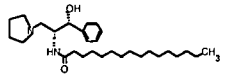
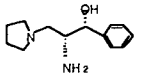
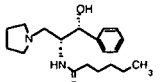
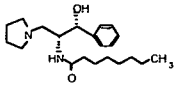
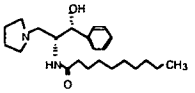
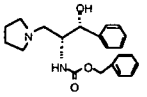
Fig. 1. **Stereo-selective synthesis of P4 analogs.**

RESULTS

Stereo-Selective Synthesis of P4 Analogs—We demonstrated previously that only one of the four stereo isomers of PDMP, D-*threo*-PDMP, was active in inhibiting GlcCer synthase (*1*). However, the previous synthetic method for PDMP was not stereo-selective and one has to separate the D-isomer from the mixture of four isomers. So we decided to develop a new synthetic route to D-PDMP and its analogs (Fig. 1). In this way, we could synthesize PDMP analogs in which morpholino and decanoyl groups are substituted with various amines and hydrophobic moieties, in a stereo-selective manner. In the new synthetic route (Fig.1), *N*-benzyloxycarbonyl-D-serine (compound 1) was treated with phenylmagnesium bromide to obtain compound 2, and then stereo-selective reduction of compound 2 was performed using diisobutylaluminum hydride (DIBAL-H) to obtain compound 3. Compound 3 was converted to a mesylester in pyridine, and then the mesyl group was substituted with a pyrrolidino group to obtain PBPP (compound 5). The *N*-protecting group of compound 5 was removed by hydrogenation to obtain compound 6, which was then acylated with an activated acyl group to obtain compounds 7, 8, 9, and 10.

Inhibition of GlcCer Synthase Activity by P4 Analogs—In previous work, we synthesized homologs of PDMP having different acyl chains (C6–C18), and compared their effectiveness as inhibitors of GlcCer synthase and of cell prolif-

TABLE I. **Structures of PDMP and P4 analogs and their inhibitory activity toward GlcCer synthase.**

Abbreviation	Structure	Inhibition of GlcCer synthase IC_{50}(μM) *
D-PDMP		23
PPPP (P4)		0.5
PAPP		No Inhibition
PHPP		2.3
POPP		2.2
PDPP		2.4
PBPP		0.3

*The IC_{50} values are the means of duplicate determinations, and the differences in the values in each group were less than 10%.

eration (*13*). We found that their inhibitory activity toward GlcCer synthase as well as cell growth decreased with decreasing fatty acyl chain length. In the present study, we first synthesized homologs of P4 having different acyl chains (C6–C16), and then examined their inhibitory potential as to GlcCer synthase *in vitro* (Table I). The C6–C10 homologs of P4 exhibited similar inhibitory potentials, although their IC_{50} values (2.2–2.4 μM) were about five times larger than that of P4 (Table I). Unlike the C6 homolog of PDMP (*13*), the C6 homolog of P4 still exhibited relatively potent inhibitory activity toward GlcCer synthase. This finding led us to further design P4 analogs whose hydrophobicity was similar to that of the *N*-hexanoyl moiety. Among those tested, D-*threo*-1-phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol (PBPP) was a most potent inhibitor, showing an IC_{50} of 0.3 μM.

Effects of P4 Analogs on Cell Proliferation—While P4 strongly inhibited GlcCer synthase with an IC_{50} of 0.5 μM (Table I), potent cell growth inhibition has been reported with similar concentrations (*17*). We have evaluated the effects of the P4 analogs synthesized here on cell proliferation and survival. As shown in Fig. 2, while P4 inhibited MRC5 cell growth by 50% at the concentration of 7 μM, PBPP did not inhibit the cell growth even at 160 μM, showing the dissociation of GlcCer depletion and cell proliferation. The cytotoxic effect of the hexanoyl analog of P4 was also significantly reduced (Fig. 2). Similar results were obtained with the other cell types, B16 melanoma cells and three glioma cell lines (C6, A172, and RG2 cells).

Effects of PBPP on the Biosynthesis and Cellular Contents of GSLs—Metabolic labeling of GSLs with D-[1,6-^{3}H]glucosamine and D-[1-^{14}C]galactose in C6 glioma cells treated with PBPP showed that there was significant dose-dependent inhibition of GlcCer, LacCer, GM3, and GM2 biosynthesis (Fig. 3). Next we analyzed the effect of PBPP on the cellular contents of GSLs of the three glioma cell lines, C6, A172, and RG2 cells, to confirm the general applicability of the synthase inhibitor. When C6, A172, and RG2 cells were treated with 10 μM PBPP for 48 h, their GSL contents including GlcCer, LacCer, GM3, and GM2 were significantly reduced, there being a 50 to 80% reduction of the total GSL contents (Table II).

Comparison between the Effects of PBPP and D-PDMP on the Cellular Sphingolipid Levels—Previous work with the parent GlcCer synthase inhibitor, D-PDMP, revealed in the depletion of all GlcCer-based GSLs and the accumulation of

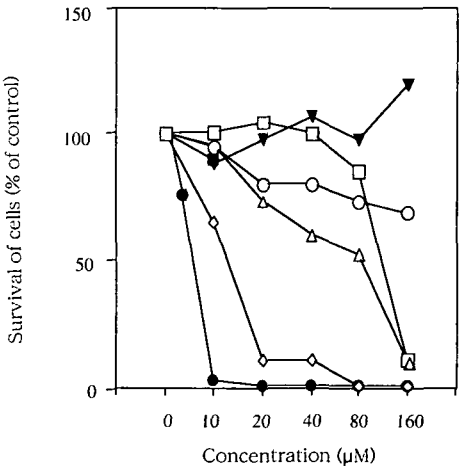


Fig. 2. **Effects of P4 analogs on the growth of normal human fibroblasts, MRC5 cells.** The MTT assay was performed as described under "MATERIALS AND METHODS." □, PDMP (D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol); ●, PPPP (D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol); ○, PHPP (D-*threo*-1-phenyl-2-hexanoylaminino-3-pyrrolidino-1-propanol); △, POPP (D-*threo*-1-phenyl-2-octanoylamino-3-pyrrolidino-1-propanol); ◇, PDPP (D-*threo*-1-phenyl-2-decanoylamino-3-pyrrolidino-1-propanol); ▼, PBPP (D-*threo*-1-phenyl-2-benzyloxycarbonylamino-3-pyrrolidino-1-propanol). The values are the means of duplicate determinations and the differences in the values in each group were less than 12%.

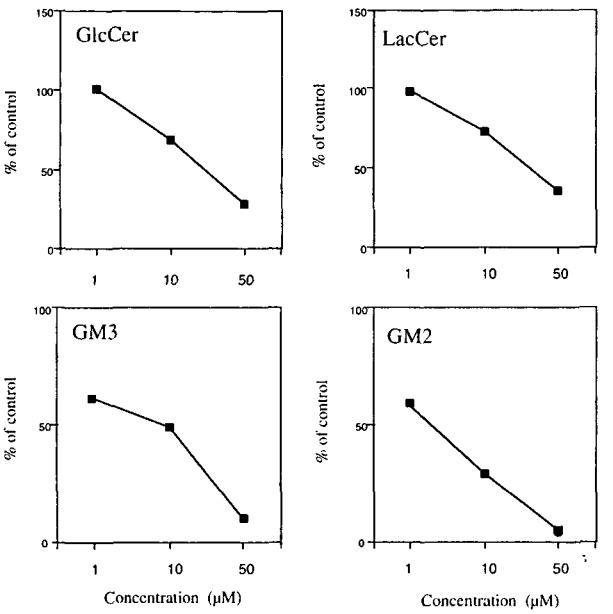


Fig. 3. **Effects of PBPP on the biosynthesis of GSLs in C6 glioma cells.** Cells were cultured with 1, 10, and 50 μM PBPP for 2 days, [^{3}H]glucosamine and [^{14}C]galactose being added to the culture for the last 6 h. The radioactivity incorporated into the neutral and acidic GSLs fractions was measured after TLC as described under "MATERIALS AND METHODS." The values are the means of duplicate determinations and the differences in the values in each group were less than 10%.

TABLE II. **Reduction of GSL contents caused by PBPP in glioma cell lines.**

Cells/treatment	GSL contents (μg /100 mg wet wt.)				
	GlcCer	LacCer	GM3	GM2	Total
C6					
Control	0.40	ND[a]	1.30	0.05	1.75
PBPP	0.13	ND	0.62	0.05	0.80 (46%)[b]
A172					
Conrol	0.84	ND	0.41	1.58	2.53
PBPPT	0.08	ND	0.09	0.76	0.93 (35%)
RG2					
Control	0.56	1.12	0.83	0.14	2.65
PBPP	0.21	0.30	0.31	0.01	0.83 (31%)

Each lot of cultured cells were treated with or without 10 μM PBPP for 2 days. The GSL extraction and analysis were performed as described under "MATERIALS AND METHODS." The data are the means of duplicate determinations, and the differences in the values in each group were less than 10%. [a]Not detectable. [b]% of respective control.

TABLE III. **Comparison between the effects of PBPP and D-PDMP on the sphingolipid levels in C6 glioma cells.**

Sphingolipid	Content (μg/mg protein)		
	control	20 μM D-PDMP	20 μM PBPP
Ceramide	0.58	0.71	0.52
Sphingomyelin	3.84	5.03	3.92
GlcCer	0.76	0.26	0.19
GM3	2.48	1.91	0.77

C6 glioma cells were treated with or without 20 μM PBPP or D-PDMP for 4 days. The lipid extraction and analyses were performed as described under "MATERIALS AND METHODS." The data are the means of duplicate determinations and the differences in the values in each group were less than 10%.

ceramide, probably as a result of substrate accumulation and/or inhibition of ceramide metabolizing enzymes (*15*, *18*; see also "DISCUSSION"). Examination by means of TLC of GSLs of C6 glioma cells after 48 h incubation disclosed that PBPP was more effective than D-PDMP in lowering the GlcCer and GM3 levels, as expected from its greater effectiveness *in vitro* as a GlcCer synthase inhibitor (Tables I and III). As expected, the accumulation of cellular ceramide and sphingomyelin occurred with D-PDMP, however, the PBPP treatment did not affect these sphingolipid levels (Table III), suggesting an additional site of action for P4 analogs including PBPP on sphingolipid metabolism other than the action of D-PDMP.

DISCUSSION

In previous searches for a potent inhibitor of GlcCer synthase (*19*), based on phenyl analogs of the ceramide structure, modifcation of the structure to include an amine group was found to enhance compound's effectiveness. The most intensively studied cyclic amine, morpholine, was positioned so as to interact with the glucose-binding site and thus the well known GlcCer synthase inhibitor, D-*threo*-PDMP (PDMP), was developed (*1*). A later study (*15*) showed that the nature and size of the cyclic amine have a strong influence on GlcCer synthase, a 5-membered ring being most active. Replacing the morpholino ring with a pyrrolidino ring, yielding D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), not only enhanced the potency as to the inhibition of GlcCer synthase but also exhibited a strong suppressive effect on cell growth (*15*, *17*). Recently, modifications of the phenyl ring of P4 (D-*threo*-ethylenedioxy-P4 or D-*threo*-4'-hydorxy-P4) yielded very potent inhibitors, which are effective at low nM concentrations without cell growth inhibition (*17*). In addition, an aliphatic analog of P4 based on sphingosine instead of phenylpropanol also proved to be a strong inhibitor, yet showed no effect on cell growth (*14*). We report here the establishment of a stereo-selective synthetic method for these ceramide analogs, and have synthesized P4 analogs, replacing the *N*-palmitoyl chain with various less hydrophobic moieties. PBPP was found to be a novel inhibitor of GlcCer synthase lacking cellular toxicity. Thus, these studies have shown that there is no direct relationship between GlcCer synthase inhibition and cell growth inhibition.

We have shown here that PBPP did not cause the accumulation of ceramide. Many studies have shown a connection between increased ceramide levels and growth inhibition or apoptosis (*6*). Thus, the lack of inhibition of cell growth by PBPP might be attributed to the lack of ceramide accumulation. It was previously reported that although D-PDMP and some P4 analogs (D-*threo*-ethylenedioxy-P4, D-*threo*-4'-hydroxy-P4 or an aliphatic analog of P4) are inhibitors of GlcCer synthase, PDMP causes a decrease in GlcCer levels and an increase in ceramide levels (*6*), whereas the P4 analogs also cause in a decrease in the GlcCer level without a detectable increase in the ceramide level (*15*, *17*). The ceramide accumulation by GlcCer synthase inhibitors could be considered as the result of substrate accumulation, however, the findings described above suggested a second site of action for these inhibitors that is independent of the inhibition of GlcCer synthase. It is possible that some cell types (a) do not make ceramide rapidly, (b) have a relatively high level of ceramidase, (c) direct accumulated ceramide to enhanced synthesis of sphingomyelin or 1-*O*-acylceramide (*18*), or (d) possess a strong feedback control system that slows ceramide formation when it is not needed for GlcCer synthesis. Perhaps these GlcCer synthase inhibitors interfere with or enhance one of these properties in different manners. Another explanation for the differences between inhibitors is that some of them exert a direct effect on ceramide that results in its accumulation. This could be (a) stimulation or induction of ceramide synthase (like in the case of the anti-multidrug resistance drug, PSC 833 [Ref. *20*]), (b) inhibition or down-regulation of ceramidase, or (c) stimulation or induction of sphingomyelin hydrolase. These possibilities need further evaluation.

The pharmacological blockade of GSL synthesis has proven to be a valuable approach for understanding the metabolism and functions of GSLs as well as a target for chemotherapeutic agents for a number of diseases, including sphingolipidoses, infections and cancer (*6*, *7*, *12*, *21*, *22*). Recently, treatment of sphingolipid storage disorders by means of inhibition of GlcCer synthase was reported, *i.e.* reversal of the Tay-Sachs phenotype in knockout mice treated with the inhibitor *N*-butyldeoxynojirimycin (*21*). It has been reported that the accumulation of GlcCer is a characteristic of some multidrug-resistant cancer cells (*22*), and that the transfection of the GlcCer synthase gene into MCF7 breast cancer cells induces resistance to adriamycin (*4*). Moreover, a reduction in the GlcCer level through the inhibition of GlcCer synthase inhibitors, PDMP and P4, caused much greater induction of apoptosis in multidrug-resistant KB cell lines than in a drug-sensitive cell line (*12*). While PDMP and P4 reduced the viability and growth rate of normal human fibroblast cells, PBPP does not affect the cell growth behavior, suggesting that further studies on this newly developed GlcCer synthase inhibitor or related analogs might open up possibilities for clinical application.

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