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New PDMP analogues inhibit process outgrowth in an insect cell line☆

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Abstract—D-*threo*-1-Phenyl-2-aminodecanoyl-3-morpholinopropanol (D-*threo*-PDMP) has previously been shown to inhibit the biosynthesis of glycosphingolipids (GSLs) in mammals and mammalian cell lines by the inhibition of glucosylceramide synthase. New D-*threo*-PDMP analogues were synthesized from D-serine, and found to suppress neurite extension in an embryonic insect cell line from the moth *Manduca sexta,* and in explanted neural tissue from insect pupae. Inhibition occurred at lower concentrations than D-*threo*-PDMP. The observed suppression of neurite formation was found to be reversible after the removal of the compounds. Due to their small size and short life cycle, *M. sexta* is shown to be an ideal model organism for studies of GSL effects in cellular development, and for drug development studies.

1. Introduction

The glycosyltransferase inhibitor D-*threo*-PDMP has displayed an ability to inhibit the enzyme glycosylceramide synthase,[1] which is responsible for the initial glycosylation of ceramide.[2] By inhibiting this glycosylation, D-*threo*-PDMP depletes the display of glycosphingolipids (GSLs) on cellular surfaces,[3] but also increases intracellular ceramide levels.[4] Many reports have suggested that ceramide plays an active role in cellular apoptosis (programmed cell death).[5,6]

Since D-*threo*-PDMP has revealed the ability to reduce GSL levels and amplify ceramide levels, D-*threo*-PDMP has been employed to halt cellular expansion and development. Treatment of embryonic rat hippocampal neurons with 50 μM D-*threo*-PDMP in culture significantly reduced the length of the total axon plexus and the number of axonal branch points after three days of administration.[7] Hynds et al.[8] have reported that D-*threo*-PDMP at 20 μM concentration inhibited neurite outgrowth from SH-SY5Y cells, a neuroblastoma cell line.

Given the biological utility and pharmacological potential of D-*threo*-PDMP, researchers have attempted to synthesize more potent inhibitors.[9,10] Shayman et al.[11] recently reported that electron-rich aromatic D-*threo*-PDMP analogues gave increased inhibition of glucosylceramide synthase. D-*threo*-4′-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and D-*threo*-(3′,4′-ethylenedioxy)-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol inhibited the glucosylceramide synthase from Madin–Darby canine kidney (MDCK) cell homogenates at an IC_{50} of 90 nM.

In this paper we report the synthesis and biological effects of D-*threo*-PDMP analogues (**7a–c**) that bear ether substituents on the aromatic ring; specifically, 4-methoxy (**7a**), 4-*tert*-butoxy (**7b**), and 3,4-methylenedioxy (**7c**). The analogues were tested against embryonic MRRL-CH1 cells from *M. sexta* to determine their effectiveness in neurite outgrowth suppression and cytotoxicity. Additionally, the effects on neurite extension from neuronal explants (antennal sensory neurons) were observed. All ether-bearing analogues suppressed cellular growth at lower concentrations than the lead compound, D-*threo*-PDMP, and the suppressive effects were reversible at lower concentrations (Scheme 1).

Because of its short life cycle, a relatively simple GSL series, and well characterized neuroanatomy and developmental profile, the insect *M. sexta* is an ideal model

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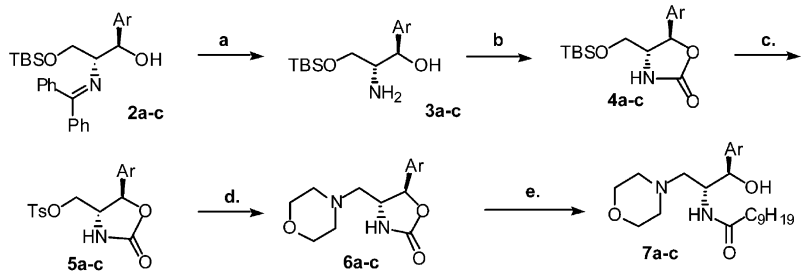


Scheme 1. Synthesis of ether-substituted D-*threo*-PDMP analogues.[14] (a) 2 equiv PPTS/THF/H_2O; (b) 1 equiv 1,1′-diimidazole carbonyl/THF; (c) (i) 4 equiv HF/H_2O/CH_3CN; (ii) 1 equiv TsCl/pyridine; (d) 5 equiv morpholine/THF/Δ (e) (i) 2M KOH (aq)/Δ; (ii) 1 equiv NO_2-C_6H_4-O_2C-C_9H_{19}/0.1 equiv HOBt/pyridine.

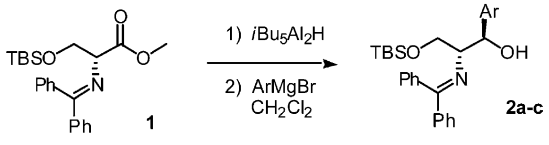
system for the study of GSL roles in neural development. Moreover, since this insect (tobacco hornworm, or Sphinx moth) has been extensively studied, drug effects can be observed at the cellular, tissue, morphological, and even behavioral studies on the adult moth become feasible. Pupation (metamorphosis) may represent an acceptable model for metastasis in mammalian tumors. The role of glycosphingolipids in insects (arthrosides) may be studied more conveniently in this organism than in mammals, where the glycosphingolipids are more numerous and structurally complex.

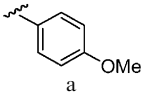
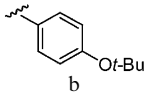
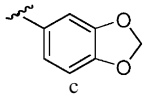
2. Synthesis of D-*threo*-PDMP analogues

Starting with the imino ester of D-serine[12] (**1**), the aromatic ring (yields given in Table 1) was introduced in a stereoselective manner via a tandem reductive-alkylation reaction to provide the desired *threo*-isomer.[13] The *threo* adducts were isolated by column chromatography and elaborated as described previously.[14]

The O'Donnell Schiff base of imines **2a–c** was removed with pyridinium *p*-toluenesulfonate in 70% yield in all cases. Treatment of β-amino alcohol **3a–c** with 1,1′-diimidazole carbonyl gave the carbamate **4a–c** in 55–64% yield. The silyl group was removed by aqueous HF to afford crude hydroxyl carbamate. The crude hydroxyl carbamate was treated with tosyl chloride in pyridine to furnish the tosylate **5a–c** in 48–83% yield over two steps. The tosyl group was displaced with morpholine in refluxing THF to afford the morpholino carbamate **6a–c** in >90% yield in all cases. Saponification and *N*-acylation completed the synthesis of the D-*threo*-PDMP analogues in 61–68% yield over two steps.[15]

Table 1. Yields of D-*threo* isomer from the tandem reductive-alkylation



Aromatic Ring	 a	 b	 c
Yield	48%	44%	39%

3. Biological assays on D-*threo*-PDMP analogues

In our analogue tests, MRRL-CH1 cells[16] were exposed to D-*threo*-PDMP analogues at 0.5, 1, and 5 μM for 48 h. The results of the 24 h exposure to the D-*threo*-PDMP analogues are illustrated in Figure 1. After 48 h of exposure to the analogues, the drug media was removed and fresh media was provided. Cells were allowed to grow for an additional 6 days to see if the inhibition was reversible. The results are illustrated in Figure 2. The reversibility of each analogue is summarized in Table 2.

Cells were originally exposed to the D-*threo*-PDMP analogues at concentrations varying from 5 to 20 μM for 48 h. In all cases, the analogues at these concentrations were deemed cytotoxic. This observation was intriguing because early testing had shown that D-*threo*-PDMP exhibited only weak inhibition at 20 μM (unpublished work). The dosage concentrations were then reduced to 1.0 μM and 0.5 μM.

After the cells were exposed to 0.5 μM of the D-*threo*-PDMP ether analogues, the effects were rapid. After 24 h, although normal cellular morphology and membrane integrity were maintained, cells appeared to be unable to form neurite extensions, and showed reduced adhesion to their substrate. Prior reports have stated that D-*threo*-PDMP effects on neurite extensions were not observed until 3 days after exposure.[7] Cellular adhesion decreased, resulting in an increase in cellular aggregation compared to the untreated cells.[8] The loss of adhesion may be a consequence of the decrease in *endogeneous* ganglioside levels.[17]

Table 2. Inhibitory effects of the analogues on MRRL-CH1 cell line outgrowth

Analogue	5.0 μM	1.0 μM	0.5 μM
D-*threo*-PDMP	No Effect	No Effect	No Effect
D-*threo*-ADMP	Irreversible	Irreversible	Reversible
D-*threo*-PipDMP	Irreversible	Irreversible	Reversible
D-*threo*-BDMP	Irreversible	Irreversible	Reversible

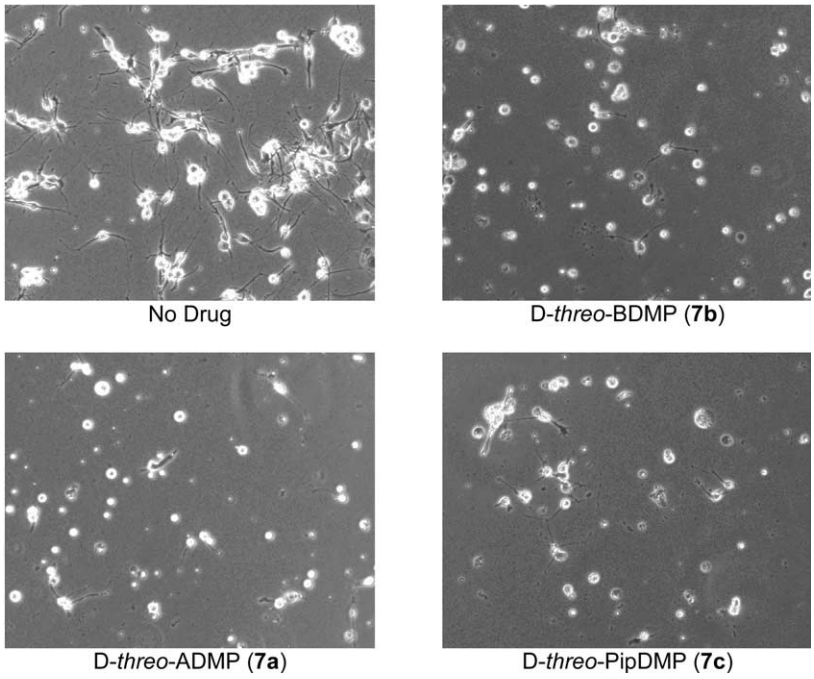


Figure 1. Effects of 0.5 μM D-*threo*-PDMP analogues on neurite outgrowth of MRRL-cell line.

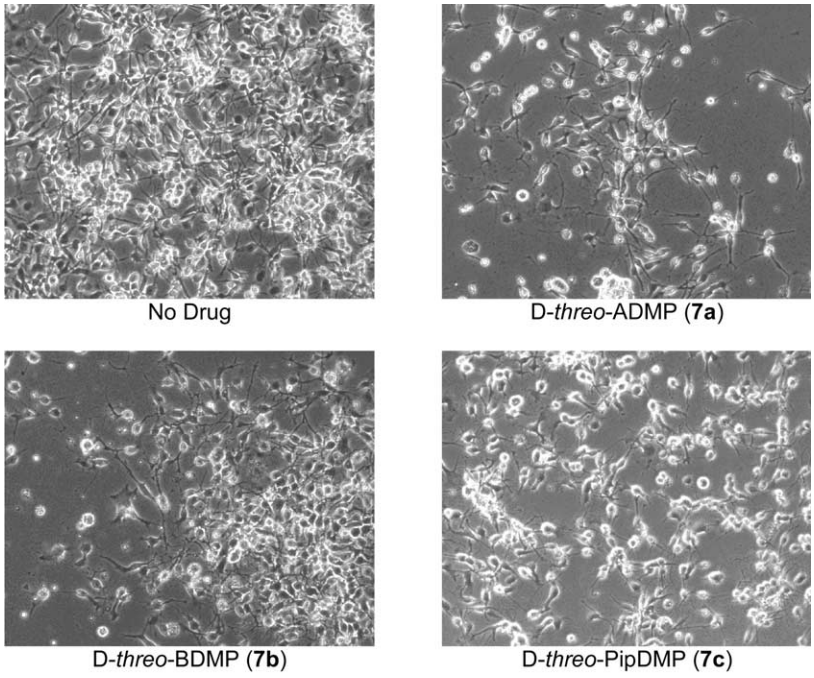


Figure 2. Growth is normal six days after the removal of the D-*threo*-PDMP analogues.

After removal of the D-*threo*-PDMP ether analogues, a majority of the cells were able to develop healthy extensions, similar to the neurite extensions observed in the untreated cells. At 1 μM, suppression of outgrowth was also observed, and recovery was reduced. Some recovery was observed where cells were exposed to D-*threo*-PipDMP and D-*threo*-ADMP, but not D-*threo*-BDMP. D-*threo*-BDMP appeared to be cytotoxic even at 1 μM.

D-*threo*-PDMP has been shown to deplete cells of *endogeneous* glycolipids, which can suppress cellular outgrowth and reduce cellular adhesion. We have synthesized three novel D-*threo*-PDMP ether analogues from D-serine that reduce cellular extensions at lower concentrations than D-*threo*-PDMP. The suppression of outgrowth is reversible after the removal of the analogue. Shayman et al.[11] found that their novel D-*threo*-PDMP ether analogues, which have a pyrrolidine head group in

place of the morpholine, depleted *endogeneous* glycolipids *without* affecting cellular growth. This observation convolutes the understanding of the true effect(s) of D-*threo*-PDMP and its analogues and the role of cell-surface carbohydrates. Isolation of GSLs from the *Manduca sexta* is also in progress.[18] In the future, we hope to gain an understanding of the perturbations of GSL expression caused by the D-*threo*-PDMP ether analogues.

Acknowledgements

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15. **(1*R*,2*R*)-(+)-1-*para*-Methoxyphenyl-2-decanoylamino-3-morpholinopropan-ol [D-threo-ADMP] (7a).** $[\alpha]_D^{25} = +7.0°$ (*c* 1.0, $CHCl_3$). HRMS (FAB + H) calcd: 421.3066, found: 421.3076. ^1H NMR (300 MHz, $CDCl_3$) δ 7.3 (d, $J = 8.8$ Hz, 2H), 6.9 (d, $J = 8.8$ Hz, 2H), 5.8 (d, $J = 7.3$ Hz, 1H), 4.9 (d, $J = 2.9$ Hz, 1H), 4.2 (m, 1H), 3.8 (s, 3H), 3.7 (t, $J = 4.6$ Hz, 4H), 2.7 (m, 6H), 2.05 (t, $J = 7.8$ Hz, 2H), 1.50 (m, 2H), 1.25 (bs, 12H), 0.90 (t, $J = 5.8$ Hz, 3H). ^{13}C NMR (75.0 MHz, $CDCl_3$) δ 174, 155, 136, 126, 124, 79, 75, 66, 60, 54, 50, 36, 32, 30, 29, 24, 22, 21, 15. **(1*R*,2*R*)-(+)-1-*para*-*t*-Butoxyphenyl-2-decanoylamino-3-(N-morpholino)-1-propanol [D-*threo*-BDMP] (7b).** $[\alpha]_D^{25} = +4.5°$ (*c* 1.0, $CHCl_3$). HRMS (FAB + H) calcd: 463.3536, found: 463.3549. ^1H NMR (300 MHz, $CDCl_3$) δ 7.3 (d, $J = 8.8$ Hz, 2H), 6.9 (d, $J = 8.8$ Hz, 2H), 5.9 (d, $J = 7.3$ Hz, 1H), 4.9 (d, $J = 2.9$ Hz, 1H), 4.2 (m, 1H), 3.7 (t, $J = 4.6$ Hz, 4H), 2.7 (m, 6H), 2.1 (t, $J = 7.8$ Hz, 2H), 1.5 (m, 2H), 1.3 (s, 3H), 1.25 (s, 12H), 0.9 (t, $J = 5.8$ Hz, 3H). ^{13}C NMR (75.0 MHz, $CDCl_3$): δ 174, 155, 136, 127, 124, 79, 76, 67, 60, 54, 51, 37, 32, 29, 26, 23, 14. **(1*R*,2*R*)-(+)-1-(3,4-Methylenedioxy)-phenyl-2-decanoylamino-3-(N-morpholino)-1-propanol [D-*threo*-PipDMP] (7c).** $[\alpha]_D^{25} = +3.8°$ (*c* 1.0, $CHCl_3$). HRMS (FAB + H) calcd: 435.2859, found: 435.2844. ^1H NMR (300 MHz, $CDCl_3$): δ 7.4–7.1 (m, 4H), 6.05 (d, $J = 7.3$ Hz, 1H), 6.0 (s, 2H), 5.22 (d, $J = 2.9$ Hz, 1H), 4.18 (m, 1H), 3.71 (t, $J = 4.6$ Hz, 4H), 2.70 (dd, $J = 13.1$, 6.2 Hz, 1H), 2.65 (m, 5H), 2.33 (s, 3H), 2.05 (t, $J = 7.8$ Hz, 2H), 1.50 (m, 2H), 1.25 (s, 12H), 0.90 (t, $J = 5.8$ Hz, 3H). ^{13}C NMR (75.0 MHz, $CDCl_3$) 173, 139, 134, 130, 128, 125.9, 125.7, 72, 66, 62, 60, 55, 50, 37, 32, 29.4, 29.3, 29.2, 29.1, 26, 23, 19, 14.
16. **RRL-CH1 cell line procedure**: The cell line, MRRL-CH1, which was derived originally from embryonic Manduca sexta by Dwight Lynn (USDA), is maintained in a 10-mL volume in 50-mL Falcon flasks in a 27° incubator in room air. The cells grow attached to the bottom of the flask, and are passaged approximately every 3–4 weeks by using the outflow of a medium-filled pipette to dislodge cells that have grown to confluence, then seeding a new flask containing 10 mL fresh DL medium with approximately 300 μL of the cell suspension.
For the current experiments, 1.2 mL DL medium and 120 μL of cell suspension from the 50-mL flask were added to each well of a sterile 12-well plate. Analogues were added 2–4 days after initiation of the well cultures. 48 h after the drugs were added, 0.9 mL of medium were removed from each well and replaced with 0.9 mL fresh medium, and the step was repeated to decrease the concentration of the remaining analogue by 16-fold. Digital photographs were taken just before adding the analogue, at 24 and 48 h after analogue administration, and at 24, 48, and 144 h after analogue removal.
Because the PDMP analogues were lipophilic, each analogue was dissolved in DMSO in an amount appropriate to yield a 100 mM stock solution, and then diluted in 1 mL DL medium to yield a 100 μM solution. Warming, vortexing, and sonication were necessary to force the analogues into solution, and the analogue-containing solutions were used immediately after preparation. Further dilutions from the 100 μM solution were made by removing from each well the volume of medium-containing analogue to be added. All doses were tested in duplicate. Vehicle control wells were given an amount of DMSO equal to the amount of DMSO that the cells exposed to the highest analogue dose tested received.
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