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Synthesis and antiviral activity of 1-*O*-octadecyl-2-*O*-alkyl-*sn*-glycero-3-foscarnet conjugates in human cytomegalovirus-infected cells

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Abstract

A series of new lipid prodrugs with the general structure, 1-*O*-octadecyl-2-*X*-*sn*-glycero-3-PFA were synthesized and evaluated for antiviral activity in HCMV-infected human lung fibroblasts (*X* is –H, –OH or an *O*-alkyl group of increasing chain length) in order to study structure–activity relationships of PFA lipid prodrugs. The EC_{50} values for the 2-*O*-octyl, 2-*O*-butyl, 2-H, 2-OH, 2-*O*-methyl and 2-*O*-ethyl substituted analogs were 1.96, 0.36, 1.0, 0.7, 0.53 and 0.18 μM respectively versus 40 μM for PFA, representing increases in antiviral activity of 20–220 fold. We also synthesized the enantiomer of ODG-PFA, 3-*O*-octadecyl-*sn*-glycero-1-PFA, and found that the antiviral activity of both enantiomers as well as the racemate were not significantly different, with EC_{50} values in the range of 0.67–0.71 μM. © 1997 Elsevier Science B.V.

Keywords: Antiviral agents; Cytomegalovirus; Lipids; Prodrugs; Structure–activity relationship

1. Introduction

Foscarnet (trisodium phosphonoformic acid, PFA) is currently in clinical use for the treatment of HCMV retinitis associated with AIDS (Jabs, 1994; Jacobson et al., 1994; Holland et al., 1995; Balfour et al., 1996). PFA is also effective against gastrointestinal HCMV disease (Blanshard et al., 1995) and has been reported to prolong life

Abbreviations: HIV, human immunodeficiency virus; HCMV, human cytomegalovirus; PFA, phosphonoformic acid; ODG-PFA, 1-*O*-octadecylglycero-3-PFA; NMR, nuclear magnetic resonance; AIDS, acquired immunodeficiency syndrome; TLC, thin layer chromatography.

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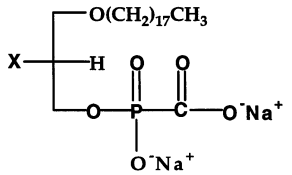


Fig. 1. General structure of 1,2-disubstituted alkylglycerol analogs of PFA, $X =$ –H; –OH; –O-CH_3; –OCH_2CH_3; –$O(CH_2)_3CH_3$; –$O(CH_2)_7CH_3$; –$O(CH_2)_{11}CH_3$ or O-$CH_2C_6H_5$.

(Kaiser et al., 1995) and lower serum HIV p24 and RNA levels in AIDS patients (Polis et al., 1993). However, its poor oral absorption and low bioavailability necessitate intravenous administration. In addition, the use of foscarnet has been associated with hypocalcemia and renal toxicity (Chrisp and Clissold, 1991).

We recently reported the synthesis of 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformic acid (ODG-PFA), a lipid prodrug of PFA. This compound exhibited a 93-fold increase in anti-HCMV activity relative to foscarnet (Hostetler et al., 1996). We also synthesized alkoxypropane phosphonoformates with varying alkyl chain lengths. Antiviral testing of these compounds in HCMV-infected cells indicated an optimum chain length of 16–18 carbons for the alkoxypropane moiety (Kini et al., 1997). To further evaluate the structure–activity relationships and to identify more potent prodrugs of PFA, we synthesized several analogs with various substituents at the *sn*-2-position of glycerol and having the optimal alkyl chain at the 1-position of glycerol (1-*O*-octadecyl glycerol) (Fig. 1). In addition we synthesized the enantiomer of 1-*O*-octadecyl-*sn*-glycero-3-PFA starting from optically pure 3-*O*-octadecyl-*sn*-glycerol. This series of compounds (1a–1f, 7b–7c) (Fig. 2) was tested in vitro for antiviral activity and selectivity in MRC-5 human lung fibroblasts infected with HCMV. In this paper we report the structure–activity data and evaluation of the optimal substituents at the *sn*-2 position of glycerol.

2. Materials and methods

2.1. Chemistry

Nuclear magnetic resonance spectra were obtained on a 300 MHz General Electric QE-300 spectrometer, with TMS as internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; bs, broad singlet). Flash chromatography was performed with silica gel EM Keiselgel 60 (230–400 mesh). Thin layer chromatography was carried out with Analtech Silica gel-GF (250 micron) plates. The products were visualized with UV light, phosphorus spray and charring.

2.2. Synthesis of 1-*O*-octadecyl-3-*O*-trityl-*sn*-glycerol

To a solution of 1-*O*-octadecyl-*sn*-glycerol (8.5 g) in anhydrous dichloromethane (125 ml) was added triethylamine (7 ml), *N*,*N*-dimethylaminopyridine (DMAP, 250 mg), and chlorotriphenylmethane (8.5 g) and the resulting reaction mixture was stirred 18 h. Thin layer chromatography (in hexane:ethyl acetate::3:1) showed complete conversion to the product, thus the reaction mixture was adsorbed onto silica gel. Flash chromatography over silica gel with an increasing gradient of acetone in hexane (0–2%) eluted the proper fractions. The appropriate fractions were then combined and evaporated under reduced pressure to give 14.08 g (96%) of 1-*O*-octadecyl-3-*O*-trityl-*sn*-glycerol: ^{1}H NMR ($CDCl_3$): δ0.9 (t, 3H), 1.3 (bs, 30H), 1.55 (m, 4H) 3.2 (m, 2H), 3.4–3.6 (m, 3H), 3.95 (m, 1H) 7.2–7.5 (m, 15H).

2.3. General procedure for the synthesis of 1-*O*-octadecyl-2-*O*-alkyl-3-*O*-trityl-sn-glycerols (2c–g)

2.3.1. Example: 1-*O*-octadecyl-2-*O*-dodecyl-3-*O*-trityl-sn-glycerol (2g)

To a solution of 1-*O*-octadecyl-3-*O*-trityl-*sn*-glycerol (3.0 g) in *N*,*N*-dimethylformamide (25 ml), maintained under an atmosphere of nitrogen, was added sodium hydride (0.29 g), and the resulting mixture was stirred for 10 min. Dodecyl

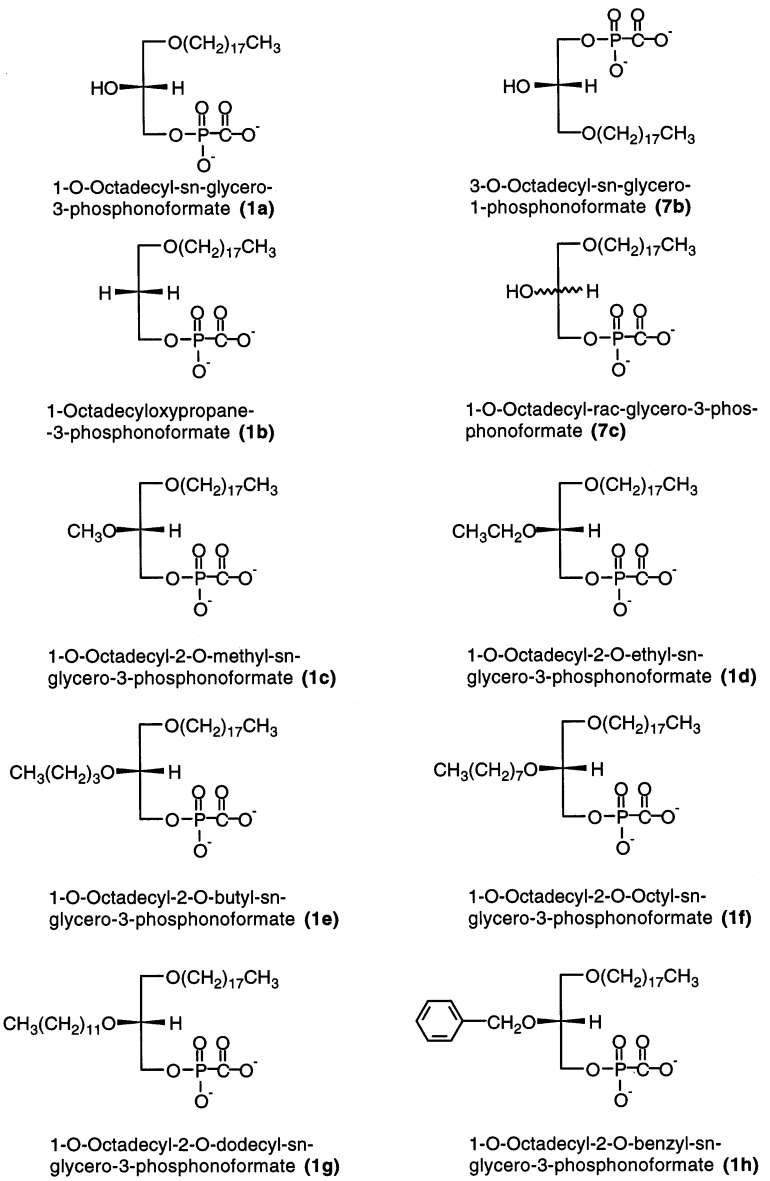


Fig. 2. Structures of compounds 1a–h, and 7b–c.

bromide (2.16 ml) was added and stirred for 2 h at which time TLC analysis (in hexane:ethyl acetate::9:1) showed the reaction to be incomplete. A second portion of sodium hydride was added (0.11 g) and stirred for 10 min, followed by the addition of dodecyl bromide (0.86 ml). The reaction mixture was left stirring overnight (still maintained under nitrogen) and TLC analysis the following morning showed the presence of starting material. A third portion of sodium hydride (0.06 g) was added and stirred for 10 min, followed by the addition of dodecyl bromide (0.434 ml). Two hours later TLC analysis showed the reaction to be complete, thus methanol (7.5 ml) was added and the reaction mixture was stirred for 10 min. The reaction mixture was then adsorbed onto silica gel and

purified by flash chromatography over silica gel. An increasing gradient of ethyl acetate in hexane (0–1%) eluted the proper fractions. The fractions were combined and evaporated under reduced pressure to yield 2.93 g (98%) of 1-*O*-octadecyl-2-*O*-dodecyl-3-*O*-trityl-*sn*-glycerol (2g); ^{1}H NMR: ($CDCl_3$) δ0.86 (t, 6H), 1.27 (bs, 48H), 1.43–1.65 (m, 5H) 3.18 (m, 2H), 3.41 (t, 2H), 3.5 (m, 4H), 7.18–7.5 (m, 15H).

2.3.2. 1-O-octadecyl-2-O-ethyl-3-O-trityl-sn-glycerol (2d)

Yield (88%); ^{1}H NMR: ($CDCl_3$) δ0.91 (t, 3H), 1.14–1.41 (m, 33H), 1.5 (t, 2H), 1.63 (s, 1H), 2.14 (m, 2H), 3.41 (t, 2H), 3.59 (m, 4H), 7.14–7.5 (m, 15H).

2.3.3. 1-O-octadecyl-2-O-butyl-3-O-trityl-sn-glycerol (2e)

Yield (81%); ^{1}H NMR: ($CDCl_3$) δ0.95 (m, 6H), 1.34 (bs, 32H), 1.4–1.64 (m, 5H), 3.16 (m, 2H), 3.41 (t, 2H), 3.55 (m, 4H), 7.18–7.55 (m, 15H).

2.4. General procedure for the synthesis of 1-O-octadecyl-2-O-alkyl-sn-glycerols (3c–g)

2.4.1. Example: 1-O-octadecyl-2-O-dodecyl-sn-glycerol (3g)

To a solution of 1-*O*-octadecyl-2-*O*-dodecyl-3-*O*-trityl-*sn*-glycerol (2.93 g) in anhydrous dichloromethane (25 ml) was added trifluoroacetic acid (32.0 ml) and the resulting bright yellow mixture was stirred for 1 h at room temperature. TLC analysis (in hexane:ethyl acetate::9:1) showed the reaction to be near completion. An additional hour of stirring showed no significant change in the TLC analysis, so the reaction mixture was poured onto cold saturated sodium bicarbonate (approximately 10 ml) and stirred for 15 min. The organic phase was separated and the aqueous phase extracted with dichloromethane (2 × 25 ml). The organic extracts were combined and washed with saturated sodium bicarbonate (2 × 25 ml). Magnesium sulfate was used to dry the combined organic extracts and the resulting mixture was adsorbed onto silica gel. Flash chromatography over silica gel with an increasing gradient of ethyl acetate in hexane (0–4%) eluted the proper fractions, which were collected and evaporated under reduced pressure giving 1.36 g (71%) of 1-*O*-octadecyl-2-*O*-dodecyl-*sn*-glycerol (3g); ^{1}H NMR ($CDCl_3$) δ0.9 (t, 6H), 1.29 (bs, 48H), 1.56 (m, 5H), 2.15 (t, 1H), 3.35–3.81 (m, 8H).

2.4.2. 1-O-octadecyl-2-O-methyl-sn-glycerol (3c)

(This compound was synthesized without isolating the tritylated intermediate. Yield from 1-*O*-octadecyl-3-*O*-trityl-*sn*-glycerol: 62.7%); ^{1}H NMR ($CDCl_3$) δ0.88 (t, 3H), 1.27 (bs, 32H), 1.53 (m, 2H), 3.29–3.8 (m, 8H).

2.4.3. 1-O-octadecyl-2-O-ethyl-sn-glycerol (3d)

Yield (36%); ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3H), 1.05–1.40 (M, 32H), 1.6 (m, 5H), 3.33–3.83 (m, 8H).

2.4.4. 1-O-octadecyl-2-O-butyl-sn-glycerol (3e)

Yield (43%); ^{1}H NMR ($CDCl_3$) δ0.92 (m, 6H), 1.33 (bs, 32H), 1.60 (m 5H), 3.33–4.33 (m, 8H).

2.4.5. 1-O-octadecyl-2-O-octyl-sn-glycerol (3f)

(This compound was synthesized without isolating the tritylated intermediate. Yield from 1-*O*-octadecyl-3-*O*-trityl-*sn*-glycerol: 52%); ^{1}H NMR ($CDCl_3$) δ0.89 (t, 6H), 1.27 (bs, 40H), 1.61 (m, 5H), 2.20 (m, 1H), 3.39–3.82 (m, 8H).

2.5. General procedure for the synthesis of 1-O-octadecyl-2-O-alkyl-sn-glycero-3-phosphonoformate methyl ester (4c–g)

2.5.1. Example: 1-O-octadecyl-2-O-butyl-sn-glycero-3-phosphonoformate (methyl ester) (1e)

To a solution of 1-*O*-octadecyl-2-*O*-butyl-*sn*-glycerol (0.65 g) in pyridine was added methyl phosphonoformate (0.36 g) and the reaction mixture was stirred at 0°C in an ice bath. *N*,*N*,-dicyclohexylcarbodiimide (0.94 g) in dichloromethane was added dropwise with stirring. The reaction was run overnight and TLC analysis (in 80:20:1:1::Chloroform:Methanol:Ammonia:Water) showed the reaction to be complete. The precipitate was isolated by vacuum filtration and the resulting filtrate was then adsorbed onto silica gel. Flash chromatography over silica gel with an increasing gradient of methanol in dichloro-

methane (0–10%) eluted the proper fractions, which were then combined and evaporated under reduced pressure giving 0.79 g (93%) of 1-*O*-octadecyl-2-*O*-butyl-*sn*-glycero-3-phosphonoformate methyl ester (1e); ^{1}H NMR ($CDCl_3$) δ0.92 (m, 6H), 1.25 (bs, 30H), 1.54 (m, 4H), 3.33–3.57 (m, 9H), 3.75 (s, 3H), 4.04 (m, 2H).

2.5.2. 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphonoformate (methyl ester) (1c)

Yield (75%); ^{1}H NMR ($CDCl_3$) δ0.93 (t, 3H), 1.3 (bs, 30H), 1.6 (m, 2H), 3.23–3.65 (m, 9H), 3.76 (s, 3H), 4.10 (m, 2H).

2.5.3. 1-O-octadecyl-2-O-ethyl-sn-glycero-3-phosphonoformate (methyl ester) (1d)

Yield (76%); ^{1}H NMR ($CDCl_3$) δ0.88 (t, 3H), 1.23 (bs, 32H), 1.54 (m, 2H), 3.46 (m, 4H), 3.65 (m, 4H), 3.77 (s, 3H), 4.06 (m, 2H).

2.5.4. 1-O-octadecyl-2-O-octyl-sn-glycero-3-phosphonoformate (methyl ester) (1f)

Yield (41%); ^{1}H NMR ($CDCl_3$) δ0.92 (t, 6H), 1.29 (bs, 40H), 1.56 (m, 4H), 3.35–3.68 (m, 7H), 3.75 (s, 3H), 4.06 (q, 2H).

2.5.5. 1-O-octadecyl-2-O-dodecyl-sn-glycero-3-phosphonoformate (methyl ester) (1g)

Yield (83%); ^{1}H NMR ($CDC1_3$) δ0.91 (m, 6H), 1.27 (bs, 44H), 1.55 (m, 5H), 3.27–3.68 (m, 8H), 3.75 (s, 3H), 4.07 (m, 2H).

2.5.6. 1-O-octadecyl-sn-glycero-3-phosphonoformate (ethyl ester) (6a)

This compound was synthesized by the general procedure described in Section 2.5 using 1-*O*-octadecyl-*sn*-glycerol (5a) (purchased from Bachem) and ethyl phosphonoformate. Yield (70%); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3H), 1.25 (bs, 30H), 1.51 (m, 2H), 3.40 (m, 3H), 3.98 (m, 1H), 4.21 (q, 2H).

2.5.7. 3-O-octadecyl-sn-glycero-1-phosphonoformate (ethyl ester) (6b)

This compound was synthesized by the general procedure described in Section 2.5 using 3-*O*-octadecyl-*sn*-glycerol (5b, purchased from Bachem) and ethyl phosphonoformate. Yield (74%); ^{1}H NMR ($CDCl_3$) δ 0.91 (t, 3H), 1.25 (bs, 30H), 1.52 (m, 2H), 3.35 (m, 4H), 3.93 (bs, 1H), 4.19 (q, 2H).

2.5.8. Racemic 1-O-octadecyl-sn-glycero-3-phosphonoformate (ethyl ester) (6c)

This compound was synthesized by the general procedure described in Section 2.5, using racemic octadecyl-*sn*-glycerol (5c, purchased from Bachem) and ethyl phosphonoformate. Yield (75%); ^{1}H NMR (DMSO-d_6) δ0.85 (t, 3H), 1.20 (m, 35H), 1.47 (m, 2H), 3.3 (m, 3H), 3.67 (m, 1H), 3.77 (m, 2H), 4.03 (q, 2H).

2.6. General procedure for synthesis of 1-O-octadecyl-2-O-alkyl-sn-glycero-3-phosphono-formates (disodium salts) (1c–h)

2.6.1. Example: 1-O-octadecyl-2-O-ethyl-sn-glycero-3-phosphonoformate (disodium salt) (1d)

To a solution of 1-*O*-octadecyl-2-*O*-ethyl-*sn*-glycero-3-phosphonoformate (methyl ester) (0.33 g) in ethyl alcohol (20 ml) and ethyl ether (approximately 2 ml) was added 1 N sodium hydroxide (1.3 ml). The reaction mixture was stirred for 1 h at which time TLC analysis (in 80:20:1:1: :$CHCl_3$MeOH:NH_3:H_2O) showed the reaction to be complete. The solid was isolated by vacuum filtration, washed thoroughly with ethyl alcohol (3 × 5 ml), and then dried in vacuo to afford 0.31 g (88%) of 1-*O*-octadecyl-2-*O*-ethyl-*sn*-glycero-3-phosphonoformate (disodium salt) (1d); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.93 (bs, 3H), 1.29 (bs, 34H), 1.57 (m, 2H), 3.45 (m, 2H), 3.67 (m, 2H), 3.93 (bs, 2H).

2.6.2. 1-O-octadecyl-sn-glycero-3-phosphonoformate (disodium salt) (1a)

Yield (85%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.91 (t, 3H), 1.29 (bs, 30H), 1.57 (m, 2H), 3.45 (m, 2H), 3.82 (m, 1H).

2.6.3. 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphonoformate (disodium salt) (1c)

Yield (60%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.83 (S, 3H), 1.30 (bs, 32H), 1.36 (bs, 2H), 3.5 (m, 5H), 3.65 (m, 2H), 4.0 (q, 2H).

2.6.4. 1-O-octadecyl-2-O-butyl-sn-glycero-3-phosphonoformate (disodium salt) (1e)

Yield (82%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.92 (t, 6H), 1.43 (bs, 34H), 1.59 (m, 4H), 3.5 (m, 3H), 3.66 (m, 4H), 3.95 (m, 2H).

2.6.5. 1-O-octadecyl-2-O-octyl-sn-glycero-3-phosphonoformate (disodium salt) (1f)

Yield (35%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.95 (t, 6H), 1.34 (bs, 40H), 1.64 (m, 4H), 3.5 (m, 4H), 3.7 (m, 4H), 4.0 (m, 2H).

2.6.6. 1-O-octadecyl-2-O-dodecyl-sn-glycero-3-phosphonoformate (disodium salt) (1g)

Yield (77%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.93 (t, 6H), 1.3 (bs, 50H), 1.57 (m, 4H), 3.50 (m, 2H), 3.70 (m, 2H), 3.93 (m, 2H).

2.6.7. 1-O-octadecyl-2-O-benzyl-sn-glycero-3-phosphonoformate (disodium salt) (1h)

This compound was synthesized by the method described in Section 2.6, using the previously reported (Hostetler et al., 1996) 1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycero-3-phosphonoformate ethyl ester. Yield (80%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.89 (t, 3H), 1.30 (bs, 32H), 1.56 (m, 2H), 3.46 (t, 2H), 3.89 (m, 2H), 4.09 (m, 2H), 7.17–7.48 (m, 5H).

2.6.8. 3-O-octadecyl-sn-glycero-1-phosphonoformate (disodium salt) (7b)

Yield (41%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.90 (t, 3H), 1.26 (bs, 30H), 1.57 (m, 2H), 3.50 (m, 2H).

2.6.9. 1-O-octadecyl-rac-glycero-3-phosphonoformate (disodium salt) (7c)

Yield (60%); ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O::2:3:1) δ 0.88 (t, 3H), 1.26 (bs, 30H), 1.57 (m, 2H), 3.5 (m, 4H), 3.95 (m, 1H).

2.7. Antiviral testing

2.7.1. HCMV DNA reduction assay

Liposomes containing dioleoylphosphatidylcholine, dioleoylphosphatidylglycerol, cholesterol and drug at a molar ratio of 50/10/30/10 respectively, were prepared as previously described (Hostetler et al., 1996) to obtain a final drug concentration of 5 mM (stock). Blank liposomes without drug were similarly prepared and added to provide 1 mM liposomal lipid (equivalent to 100 μM drug) as a control. Subconfluent MRC-5 (human lung fibroblast) cells were pretreated for 24 h with the indicated concentrations of drug in MEM medium containing 2% fetal bovine serum and antibiotics. The medium was removed, HCMV (AD-169 strain) added at a dilution that resulted in a 3–4+ cytopathic effect in the no drug wells in 5 days, or an increase in HCMV DNA 31 times the control blank value and incubated at 37°C for 1 h. This was removed and replaced with drug dilutions and incubated for 5 days (2–3 cycles). The HCMV DNA present was quantified by nucleic acid hybridization using a CMV antiviral susceptibility test kit from Diagnostic Hybrids (Athens, OH). The medium was removed and the cells were lysed and adsorbed onto Hybriwix™ filters. The filters were hybridized overnight at 60°C, then washed for 30 min at 73°C and counted in a gamma counter. The data are expressed as a percentage of the no drug control HCMV DNA levels (mean $\pm$ S.D.).

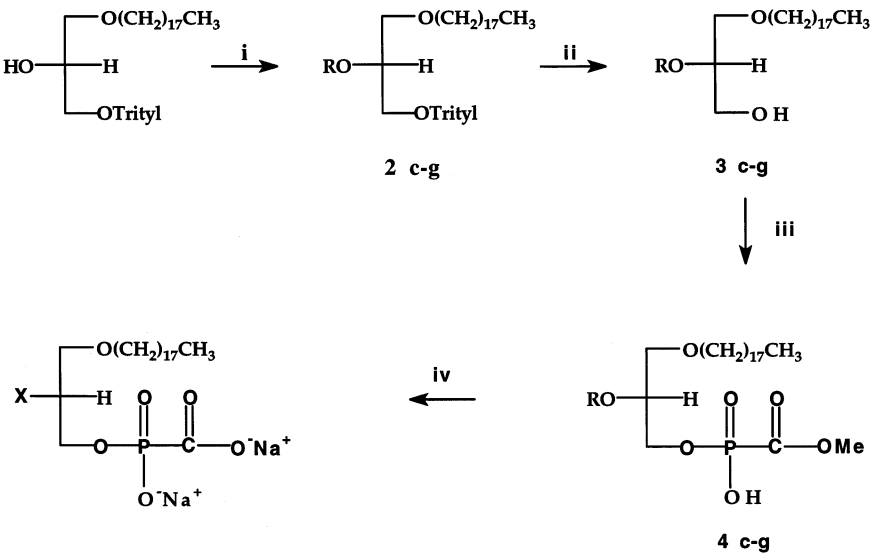
2.8. Cytotoxicity testing

Subconfluent MRC-5 cells were exposed to drugs for 5 days and evaluated by a visual grading system as previously described (Hostetler et al., 1996). Rapidly dividing human T-lymphoblast cells (CEM) were exposed to drug and the number of viable cells was determined after 72 h by flow cytometry after staining with propidium iodide as previously described (Kini et al., 1997). The cytotoxic concentration of drug which reduced viable cell number by 50% (TC_{50}) was determined.

3. Results

3.1. Chemistry

The synthesis of compounds 1 (c–g) is outlined in Fig. 3. 1-*O*-octadecyl-3-*O*-trityl-*sn*-glycerol was synthesized by treatment of 1-*O*-octadecyl-3-*sn*-glycerol with trityl chloride in pyridine. The product was isolated by flash chromatography with an increasing gradient of ethyl acetate in hexanes (0–5%). It was then alkylated by reaction with the appropriate alkyl bromide in the presence of sodium hydride in DMF. Compounds 2 (c–g) were isolated by flash chromatography in



1a : 1-O-Octadecyl-sn-glycero-3-phosphonoformate (X=OH)
1b : 1-O-Octadecylpropanediol-3-phosphonoformate (X=H)
1c : 1-O-Octadecyl-2-O-methyl-sn-glycero-3-phosphonoformate (X=OMe)
1d : 1-O-Octadecyl-2-O-ethyl-sn-glycero-3-phosphonoformate (X=OEt)
1e : 1-O-Octadecyl-2-O-butyl-sn-glycero-3-phosphonoformate (X=OBut)
1f : 1-O-Octadecyl-2-O-octyl-sn-glycero-3-phosphonoformate (X=OOct)
1g : 1-O-Octadecyl-2-O-dodecyl-sn-glycero-3-phosphonoformate (X=ODod)
1h : 1-O-Octadecyl-2-O-benzyl-sn-glycero-3-phosphonoformate (X=OBn)

* The synthesis of 1 b has been reported elsewhere.

i. NaH, R-Br, DMF; ii. 10% TFA in CH_2Cl_2; iii. DCC, methyl phosphonoformate, pyridine, CH_2Cl_2; iv. 1N NaOH, ethanol.

Fig. 3. Synthetic scheme for 1-*o*-octadecyl-2-*o*-alkyl-*sn*-glycero-3-PFA analogs.

good yields, and subsequently detritylated by treatment with trifluoroacetic acid to yield the intermediates 3 (c–g). After chromatographic purification, these were coupled to methyl phosphonoformate with *N*,*N*-dicyclohexylcarbodiimide in a mixture of dichloromethane and pyridine. The esterified products 4 (c–g) were isolated by flash chromatography over silica gel in yields ranging from 57–93%. The methyl esters 4 (c–g) were saponified with aqueous sodium hydroxide in ethanol to yield the target compounds 1 (c–g) as the disodium salts. These salts were purified by washing with ethanol to yield analytically pure amorphous solids in yields ranging from 35–89%.

Fig. 4 outlines the synthesis of stereoisomeric forms, 1a, 7b and 7c. The appropriate isomer of alkyl glycerol (5a–c) was coupled to ethyl phosphonoformate with *N*,*N*-dicyclohexyl carbodiimide in a mixture of dichloromethane and pyridine. The product (6a–c) was isolated by flash chromatography over silica gel in yields ranging from 70–85%. The ester (6a–c) was saponified with aqueous sodium hydroxide in ethanol to yield the target compound (1a, 7b or 7c) as the disodium salt. This salt was purified by washing with ethanol to yield analytically pure (1a, 7b or 7c) as an amorphous solid in yields ranging from 60–80%.

3.2. Antiviral testing

The purpose of the structure–activity studies was to evaluate the effect of various types of substitutions at the *sn*-2 hydroxyl of glycerol. ODG-PFA (1a) which has a free *sn*-2 hydroxyl, was significantly more active than PFA, EC_{50} of 0.70 ± 0.36 μM versus 40 ± 16 μM ($P < 0.0001$). Replacement of the *sn*-2 hydroxyl with hydrogen

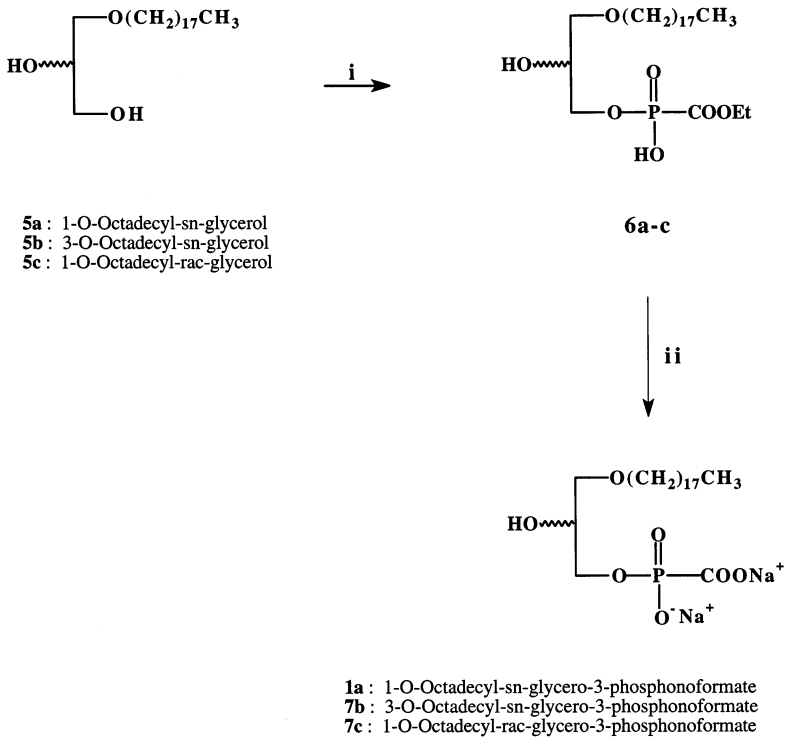


Fig. 4. Synthetic scheme for stereoisomers of ODG-PFA.

(1b) results in a propanediol compound with an EC_{50} of 1.0 ± 0.43 μM, not significantly different from ODG-PFA (1a). We prepared a series of 1-*O*-octadecylglycerol ethers having at the *sn*-2 hydroxyl alkyl moieties ranging from one to 12 carbons in length. Alkyl ethers of one, two, four and eight carbons at the *sn*-2 position had EC_{50} values of 0.55, 0.18, 0.36 and 1.96 μM, respectively, indicating that *sn*-2 ethers of two carbons provide optimal activity and chain length of eight or greater reduce activity dramatically. 1-*O*-octadecyl-2-*O*-ethyl-*sn*-glycero-3-PFA and the 2-*O*-butyl-analog were significantly more active than 1-*O*-octadecylglycero-3-PFA ($P < 0.05$). The 2-*O*-benzyl analog (EC_{50} 0.24 μM) was intermediate between 2-*O*-octyl and 2-*O*-butyl analogs and was also statistically more active than ODG-PFA. The 2-*O*-octyl analog was less active than ODG-PFA, EC_{50} 1.96 ± 0.75 ($P < 0.001$) and the 2-*O*-dodecyl analog was substantially less active than ODG-PFA, $EC_{50} > 31.6$ μM (Table 1).

The EC_{50} values for the enantiomer of ODG-PFA (1a), 3-*O*-octadecyl-*sn*-glycero-1-phosphonoformate (7b) and the racemate (7c) were comparable, in the range of 0.67 to 0.70 μM (Table 2).

4. Discussion

In an earlier study we synthesized alkoxypropane-PFA analogs having saturated alkyl chains ranging in length from eight to 22 carbon atoms; optimal antiviral activity was observed at 16–18 carbons (Kini et al., 1997). Recently we prepared a series of alkylthioglycerol-PFA compounds with chain length ranging from eight to 22 carbon atoms. This study also showed that the

Table 1
Antiviral activity of drugs against HCMV

Compound	*X*	HCMV EC_{50}	MRC-5 TC_{50}	CEM TC_{50}
PFA		40 ± 16 (6)*	1000	827
1a	–OH	0.70 ± 0.36 (24)	1000	350
1b	–H	1.0 ± 0.4 (3)	320–1000	169
1c	$-OCH_3$	0.55 ± 0.2 (4)	320–1000	135
1d	$-OCH_2CH_3$	0.18 ± 0.02 (3)*	1000	357
1e	$-O(CH_2)_3CH_3$	0.36 ± 0.26 (3)	1000	1130
1f	$-O(CH_2)_7CH_3$	1.96 ± 0.75 (3)**	>1000	368
1g	$-O(CH_2)_{11}CH_3$	>31.6 (3)	1000	507
1h	$-OCH_2C_6H_5$	0.24 ± 0.17 (5)[a]	1000	182

[a] EC_{50} of compounds 1a–1f and 1h was significantly less than PFA, $P<0.005$ or less.
* $P<0.05$ vs. compound 1a by student paired *t*-test
** $P<0.0001$ vs. compound 1a by student paired *t*-test
X, nature of the substituted moiety at carbon 2 of glycerol
Mean ± S.D.; EC_{50}, TC_{50} refers to the μM concentration of drug which reduces viral DNA or viable cell number by 50%. TC_{50} data are the average of two or three replicates.

optimal chain length for antiviral activity and selectivity in HCMV-, HSV-1 and HIV-1 infected cells was 16–18 carbon atoms (Beadle et al., 1997). In this paper we have prepared a series of 2-substituted analogs of 1-*O*-octadecyl-*sn*-glycerol-3-PFA to determine which type of substitutions provide optimal antiviral activity. Our results in HCMV-infected cells indicate that short *O*-alkyl or *O*-benzyl substitutions at the *sn*-2 hydroxyl of glycerol provide analogs with the highest degree of antiviral activity. The optimal 2-substitutions appear to be *O*-ethyl, *O*-butyl and *O*-benzyl, while longer chains lose antiviral activity substantially. In MRC-5 human lung fibroblasts, toxicity assessed by visual grading was low with TC_{50} values of 1000 μM and greater. However, the compounds are more toxic when evaluated in rapidly dividing T-lymphoblastic leukemia (CEM) cells. TC_{50} values range from 135 to 1130 μM in CEM versus 1000 μM in MRC-5 cells. There is a suggestion that compounds having 2-*O*-alkyl substitutions of 2–12 carbons (1d, 1e, 1f) are less cytotoxic than the 2-H, 2-*O*-CH_3 and 2-*O*-benzyl analogs. In conclusion, of the lipid analogs synthesized to date, the most active and selective for HCMV have 16 or 18 carbon alkyl ether at *sn*-1 and –ethyl, –butyl or –benzyl ethers at the *sn*-2 of glycerol. In addition, the stereochemistry at the *sn*-2 position of the glycerol may not be a factor in the antiviral activity of these analogs.

Table 2
Antiviral activity of stereoisomers of 1-*O*-octadecyl-glyero-3-PFA in HCMV-infected cells

Compound	HCMV EC_{50}
PFA	40 ± 16 (6)
1-*O*-octadecyl-*sn*-glycero-3-PFA (1a)	0.70 ± 0.36 (24)
3-*O*-octadecyl-*sn*-glycero-1-PFA (7b)	0.67 ± 0.05 (3)
1-*O*-octadecyl-rac-glycerol-3-PFA (7c)	0.71 ± 0.10 (3)

Mean ± S.D., all compounds significantly different from PFA, $P<0.0001$
EC_{50}, μM concentration which reduces HCMV DNA by 50%

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