Pergamon

Tetrahedron 57 (2001) 9385–9391

TETRAHEDRON

Synthesis and anti-HIV activity of a bile acid analog of cosalane

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Received 8 August 2001; accepted 26 September 2001

Abstract—Cosalane is a novel anti-HIV agent that inhibits the attachment of gp120 to CD4. The therapeutic potential of cosalane is limited by poor oral absorption. In an attempt to target the ileal bile acid transporter and thus facilitate oral bioavailability, a cosalane analog was synthesized in which the disalicylmethane pharmacophore is attached to a bile acid through a linker chain appended to C-17 of the steroid nucleus. The resulting bile acid analog of cosalane retained antiviral activity vs. HIV-1_{IIIB} and HIV-2_{ROD} in MT-4 cells, but was less potent than cosalane.

1. Introduction

Cosalane (**1**) is an inhibitor of HIV replication with activity against both HIV-1 and HIV-2.[1,2] Cosalane acts primarily by inhibiting the binding of gp120 to CD4, as well as by hindering a post-binding fusion event.[2] Pharmacokinetic studies in male Sprague-Dawley rats have shown that cosalane follows a biphasic elimination with a very long half-life following intravenous administration.[3] The drug is metabolically very stable with low renal and biliary excretion. The poor oral bioavailability (<1%) of cosalane is mainly attributed to its poor permeation across the intestinal epithelium due to its very high lipophilicity and membrane interacting nature. There is therefore a need for cosalane analogs that would retain the anti-HIV activity of cosalane but would have enhanced oral bioavailability.

The utilization of carrier-mediated transport mechanisms offers a potentially useful strategy to enhance intestinal absorption. In order for this approach to be successful, the compound must be recognized and transported by a carrier system having high capacity and relatively low substrate specificity. Both of these specifications are satisfied by the bile acid transporters, which facilitate the daily absorption of 10–20 g of bile salts with >95% efficiency. Because of these facts, Ho first proposed using the bile acid carrier mechanisms to enhance hepatic and small intestinal absorption of drugs, and demonstrated that 3-tosylcholic acid is transported in the rat ileum almost as rapidly as cholic acid itself, and that for 3-benzoylcholic acid, the rates were identical.[4] In addition to high capacity, the bile acid transporters have been shown to accept and transport a variety of analogs that are derivatized at the 3- and 24-positions.[4,5] For example, a number of amino acids and peptides, attached to C-24 of cholic acid (**2**), have been shown to interact with the human bile acid transporter in a Caco-2 cell line used to model intestinal transport.[6,7] Peptides attached to the 3-position of cholic acid through an aminoethylene linker are transported in vitro in rabbit ileal vesicles as well as in vivo during ileum perfusion in rats, whereas the unconjugated peptides are not transported.[8] Similarly, the anticancer drug chlorambucil and the prolyl-4-hydroxylase inhibitor 4-nitrobenzo-2-oxo-1,3-diazol-β-Ala-Phe-5-oxaproline-Gly have been linked to the 3-position of the cholic acid nucleus and have been to shown interact with the ileal bile salt transport system in membrane vesicles prepared from rabbit ileum, and they also target the liver after intravenous administration.[9] In a related study, bile acid-derived HMG-CoA reductase inhibitors were demonstrated to interact with the ileal uptake system for bile acids.[10] Taken together, these studies have demonstrated conclusively that the bile acid transport system can be utilized to facilitate the intestinal absorption of poorly absorbable compounds.

The successful targeting of the intestinal bile acid transporter must take advantage of the known structural requirements for recognition, which include a negative charge located near the atom 24-to-29 region in the C-17 side chain of the bile acid or bile acid conjugate,[7,11,12] and at least one hydroxyl group at C-3, C-7, or C-12 of the steroid moiety. The rate of transport in the ileum depends on the number of hydroxyl groups, with trihydroxy>dihydroxy>monohydroxy[5,12–14]

Cosalane (**1**) consists of a disalicylmethane pharmacophore

Keywords: cosalane; HIV; AIDS; gp120; CD4; bile acid; antiviral therapy.
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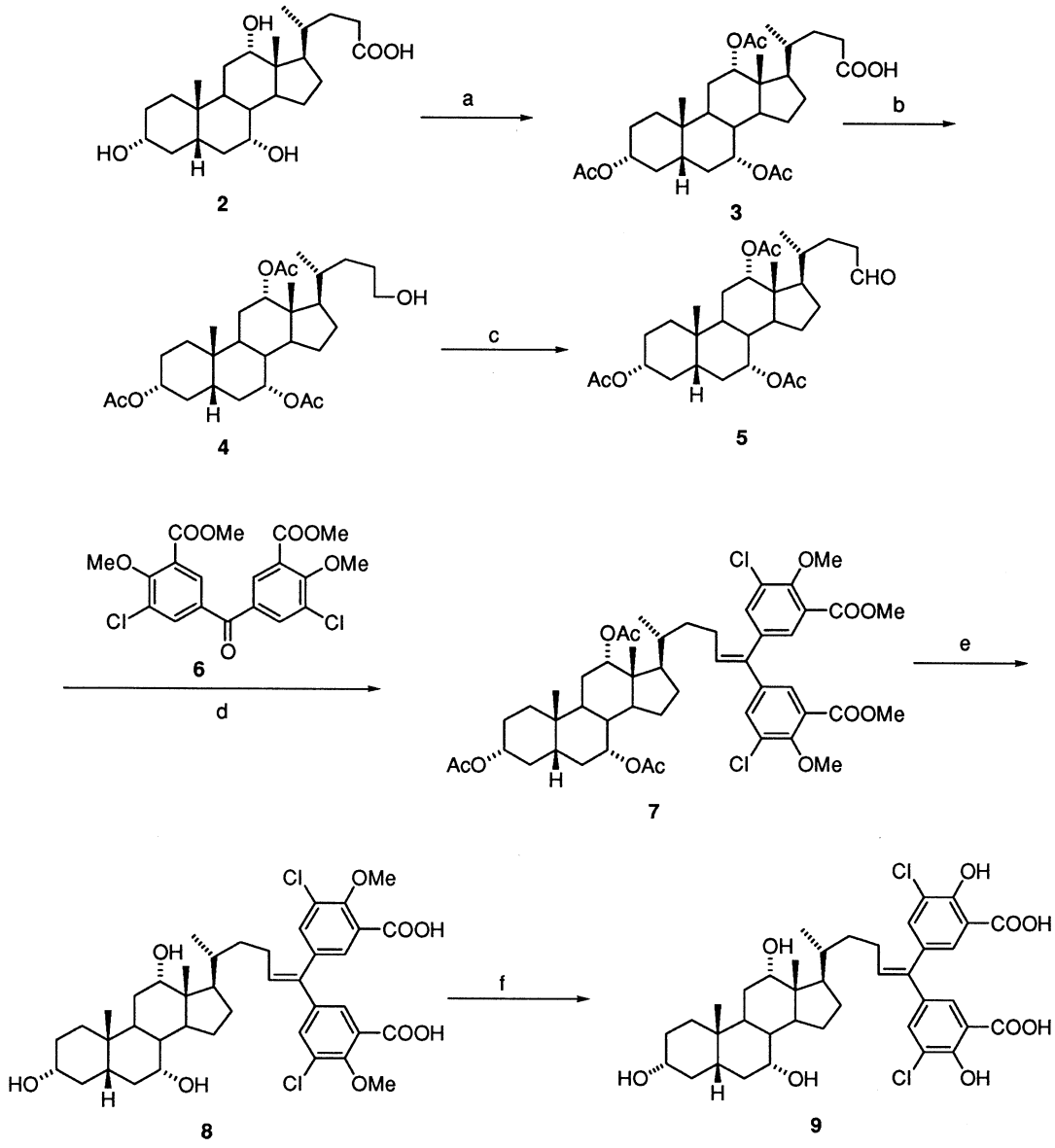
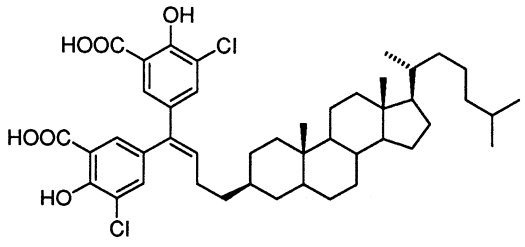
PII: S0040-4020(01)00955-3



Scheme 1. *Reagents and conditions*: (a) Ac_2O, Py, DMAP, CH_2Cl_2; (b) (i) Et_3N, ClCOOEt, (ii) $NaBH_4$, H_2O; (c) Dess-Martin reagent, CH_2Cl_2; (d) $TiCl_4{\cdot}2THF$, Zn, benzophenone **1**, THF, Δ; (e) KOH, MeOH, Δ, 24 h; (f) $BBr_3{\cdot}(CH_3)_2S$, $ClCH_2CH_2Cl$, 70°C, 40 h.

attached through a linker chain to a lipophilic steroid moiety that targets the compound to the cell membrane and viral envelope. Prior work has demonstrated that the point of attachment of the linker chain to the steroid can be moved from C-3 to C-6 or C-17 and antiviral activity maintained, and that a *trans* A/B ring fusion in the steroid is not critical for anti-HIV activity.[15,16] This suggests that a bile acid analog of cosalane with the pharmacophore attached at C-17 might retain antiviral activity and would also be a good choice for enhanced intestinal transport. The attachment could be accomplished while maintaining all three hydroxyls at C-3, C-7, and C-12, and as discussed earlier, bile acids having all three hydroxyl groups intact are in general transported faster than those having only two hydroxyl groups. The purpose of the present investigation was to determine the feasibility of attaching the cosalane pharmacophore to C-24 of a bile acid derivative while retaining the anti-HIV activity.



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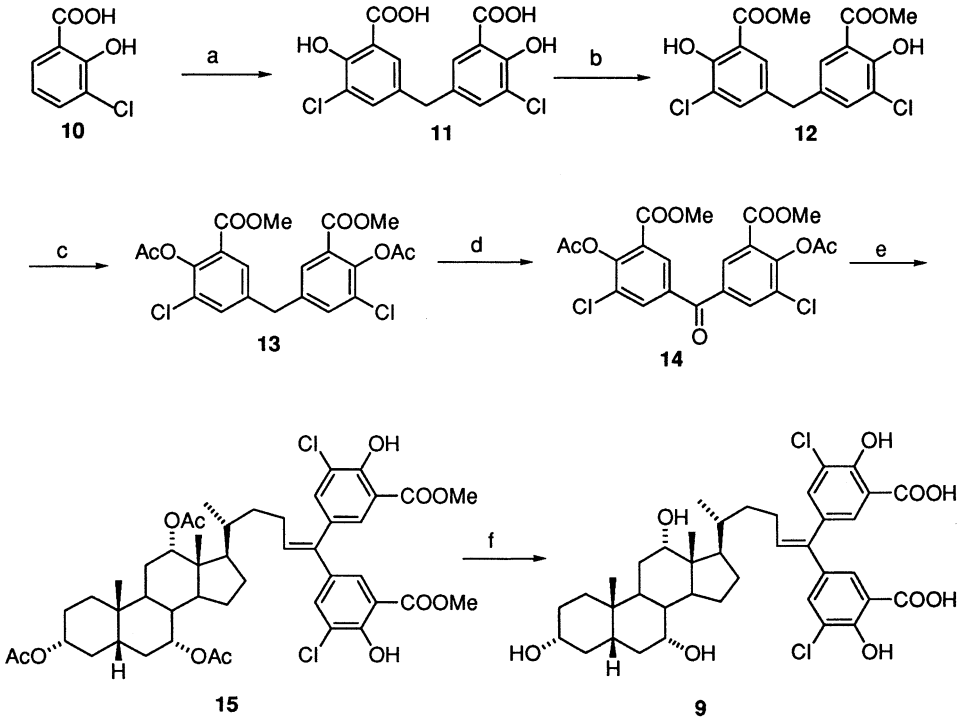
2. Results and discussion

In order to construct the desired cholic acid analog **9** of cosalane (**1**), the commercially available and inexpensive starting material cholic acid (**2**)[2] was employed as the starting material in a convergent synthesis in which the



Scheme 2. *Reagents and conditions*: (a) aq. CH_2O, H_2SO_4, MeOH, H_2O, Δ; (b) $TMSCHN_2$, MeOH, benzene; (c) Ac_2O, Py, DMAP, CH_2Cl_2; (d) CrO_3, AC_2O, (e) $TiCl_4$·2THF, Zn, aldehyde **5**, THF, Δ; (f) KOH, MeOH, Δ, 24 h.

key step was the McMurry coupling of the substituted benzophenone **6** with the intermediate aldehyde **5** (Scheme 1). As the first step, the three hydroxyl groups of cholic acid (**2**) were protected as acetates by treatment with acetic anhydride in pyridine in the presence of 4-dimethylamino-pyridine. The carboxyl group was converted to a mixed anhydride with ethyl chloroformate, which was then reduced to the alcohol **4** with sodium borohydride.[17] Oxidation of the free alcohol of **4** with Dess-Martin periodinane afforded the corresponding aldehyde **5**.[18] McMurry coupling of the aldehyde **5** with the ketone **6**[2] yielded the expected alkene **7**.[19,20] The two methyl esters and three acetates present in **7** were then hydrolyzed with potassium hydroxide in methanol to afford the diacid **8**, and the remaining two methyl ethers were cleaved using boron tribromide–dimethyl sulfide complex in 1,2-dichloroethane at 70°C.[21]

The two low-yielding steps in the synthesis outlined in Scheme 1 were the McMurry reaction (25%) and the ether cleavage (35%). The yield of the McMurry reaction is not expected to be ideal because of the formation of dimers in addition to the desired cross-coupling product. In our case, the isolation of the desired product required careful chromatography. The ether cleavage reaction required prolonged heating of intermediate **8** in the presence of a Lewis acid. In an attempt to improve the overall yield, the synthesis was modified by replacement of the methyl ether protection of the phenols with acetyl protection. The resulting route is shown in Scheme 2.

Reaction of 3-chlorosalicylic acid (**10**)[22] with formaldehyde under acidic conditions afforded the disalicylmethane derivative **11**[2]. Treatment of **11** with trimethylsilyldiazomethane[23] resulted in selective methylation of both carboxylic acids to yield intermediate **12**. The two phenolic hydroxyl groups of **12** were then acetylated with acetic anhydride in pyridine, resulting in the expected diacetate **13**, which was oxidized with chromium trioxide in acetic anhydride to afford the benzophenone **14**. The McMurry coupling of **14** with aldehyde **5**, followed by the usual acidic

Table 1. Anti-HIV activities of cosalane (**1**) and analogs

	EC_{50} (μM)[a]			CC_{50} (μM)[b]	
Compound	HIV-1_{RF}[c]	HIV-1_{IIIB}[d]	HIV-2_{ROD}[d]	CEM-SS cells	MT-4 cells
1	5.1±2.1	3.0±0.18	4.0±2.1	>200	>125
8	53.1	>50	>54.1	130	>54.1
9	>150	62.3±13.9	67.2±20.1	152	>125
16	28.0	27.2	>31.3	69.5	31.4

[a] Concentration required to reduce the cytopathic effect of the virus by 50%.
[b] Concentration required for a 50% reduction in cellular viability of uninfected cells.
[c] Determined in CEM-SS cells.
[d] Determined in MT-4 cells.

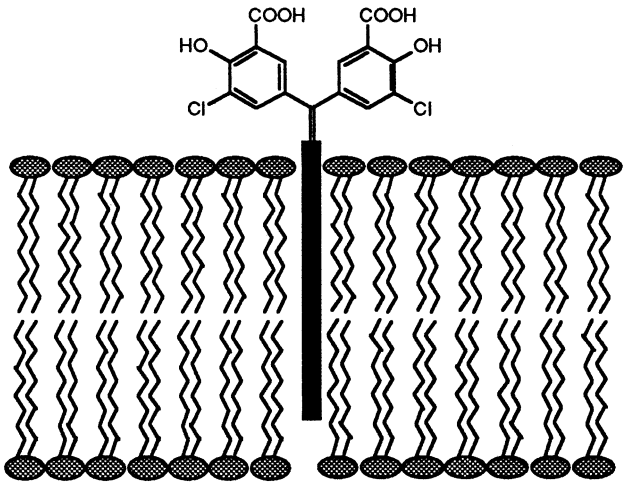
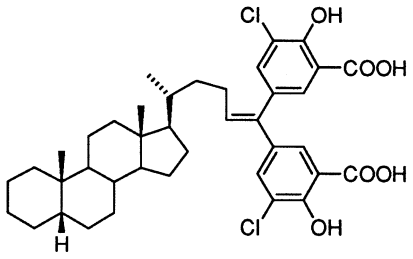


Figure 1. Schematic representation of a hypothetical model of the anchoring of the disalicylmethane moiety of cosalane and cosalane analogs to the cell membrane and viral envelope by the steroid fragment. Reprinted with permission from (see Ref. 24). Copyright 1996 American Chemical Society.

workup with hydrochloric acid, resulted in **15**, in which the two phenolic acetates had been selectively hydrolyzed. The hydrolysis of the phenolic acetates presumably occurred under the acidic conditions employed in the workup of the reaction mixture, and not during the McMurry reaction itself. The remaining three acetates present in **15** were then hydrolyzed with potassium hydroxide in methanol to provide the desired compound **9**. The overall yield of the route shown in Scheme 2 was higher than that in Scheme 1, mainly because it avoided the low-yielding methyl ether cleavage reaction.

The bile acid cosalane analog **9** was evaluated for inhibition of the cytopathic effect of HIV-1_{RF} in CEM-SS cells, and of HIV-1_{IIIB} and HIV-2_{ROD} in MT-4 cells (Table 1). The compound proved to be inactive against HIV-1_{RF} in CEM-SS cells (EC_{50}>150 μM), and was also cytotoxic in uninfected CEM-SS cells (CC_{50}: 152 μM). However, compound **9** did display some activity against HIV-1_{IIIB} in MT-4 cells (EC_{50}: 62.3±13.9 μM), and was also active vs. HIV-2_{ROD} in MT-4 cells (EC_{50}: 67.2±20.0 μM). It was not cytotoxic in uninfected MT-4 cells at a concentration of 125 μM (CC_{50}>125 μM). In contrast to these results, the di(methyl ether) **8** proved to be active against HIV-1_{RF} in CEM-SS cells (EC_{50}: 53.1 μM), and it was also cytotoxic in uninfected CEM-SS cells (CC_{50}: 130.0 μM). Compound **8** was not active against HIV-1_{IIIB} (EC_{50}>50 μM) or HIV-2_{ROD} in MT-4 cells (EC_{50}>54.1 μM), and it was not cytotoxic in uninfected MT-4 cells at a concentration of 54.1 μM (CC_{50}>54.1 μM).

Both analogs **8** and **9** were less potent than cosalane **1** (EC_{50}: 5.1±2.1 μM against HIV-1_{RF} in CEM-SS cells, EC_{50}: 3.0±0.18 μM against HIV-1_{IIIB} in MT-4 cells, and EC_{50}: 4.0±2.1 μM vs. HIV-2_{ROD} in MT-4 cells). It is thought that the antiviral activity of cosalane might result, in part, from the insertion of the steroid fragment in viral and cellular membranes, with the disalicylmethane moiety pointing outward in an obstructive mode (Fig. 1). This could result in inhibition of the fusion of the viral envelope with the cell membrane. Previous studies have demonstrated a correlation of anti-HIV potency with lipophilicity in a series of cosalane analogs having normal alkenyl and phosphodiester chains as cholestane replacements.[24] Moreover, recent evidence indicates that cosalane may in fact accumulate in the lipid bilayer of the hepatocyte membranes.[25] The decrease in lipophilicity of the steroid fragment of the bile acid analog **9** versus cosalane **1** may therefore be at least partially responsible for the observed decrease in antiviral potency. This hypothesis is also in agreement with the fact that the previously reported cosalane analog **16**, which lacks the three hydroxyl groups present in **9** but is otherwise identical, is more active than **9** as an anti-HIV agent (EC_{50} of **16** against HIV-1_{RF} in CEM-SS cells, 28.0 μM; against HIV-1_{IIIB} in MT-4 cells, 27.2 μM; and against HIV-1_{ROD} in MT-4 cells, >31.3 μM).[16]



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3. Experimental

3.1. General procedures

Melting points were determined in capillary tubes on a Mel-Temp apparatus and were uncorrected. Spectra were obtained as follows: CI mass spectra on a Finnegan 4000 spectrometer; FAB mass spectra and EI mass spectra on a Kratos MS50 spectrometer; and ^{1}H NMR spectra on a Varian VXR-500S or Bruker ARX-300 spectrometers. Microanalyses were performed at the Purdue Microanalysis Laboratory, and all values were within ±0.4% of the calculated compositions. Silica gel used for column chromatography was 230–400 mesh.

3.1.1. 3α,7α,12α-Triacetoxy-5β-cholane-24-al (5). A solution of 3α,7α,12α-triacetoxy-5β-cholane-24-ol (**4**)[17] (520 mg, 1 mmol) in methylene chloride (5 mL) was added to a solution of Dess-Martin reagent (466 mg, 1.1 mmol) in methylene chloride (10 mL) with stirring. After stirring for 2 h, the reaction mixture was diluted with ether (50 mL) and the resulting suspension was added to a solution of sodium bicarbonate (2 g) and sodium thiosulfate (7 g) in water (50 mL). The resulting mixture was stirred for 20 min and the layers were separated. The organic layer was washed with aq. sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was dried with Na_2SO_4, and evaporated, and the residue was purified by flash chromatography on silica gel (20 g), eluting with hexanes–EtOAc (3:1), to give a white solid **5**[26–28] (414 mg, 80%): mp 55°C. IR (KBr) 2949, 2874, 1734, 1444, 1377, 1248, 1025 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.74 (s, 1H), 5.07 (s, 1H), 4.89 (bs, 1H), 4.58–4.52 (m, 1H),

2.43–2.30 (m, 2H), 2.12 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01–1.04 (m, 22H), 0.90 (s, 3H), 0.80 (d, J=6.5 Hz, 3H), 0.67 (s, 3H). ESIMS m/z 541 (MNa^+). Anal. calcd for $C_{30}H_{46}O_7$: C, 69.45; H, 8.94. Found: C, 69.53; H, 8.96.

3.1.2. 24-[Bis[5′-chloro-4′-methoxy-3′-(methoxycarbonyl)-phenyl]methylene]3α,7α,12α-triacetoxycholane (7). THF (20 mL) was added to a mixture of titanium(IV) chloride tetrahydrofuran complex (1:2) (1.32 g, 4 mmol) and zinc dust (0. 45 g, 6 mmol) kept under argon atmosphere and the mixture was heated at reflux for 3 h. A solution of benzophenone **6** (427 mg, 1 mmol) and the aldehyde **5** (518 mg, 1 mmol) in THF (15 mL) was added to the reaction mixture, which was heated at reflux for 12 h. The reaction mixture was cooled to room temperature, 1N HCl (10 mL) was added, and the reaction mixture was extracted with EtOAc (3×40 mL) and washed with brine (50 mL). The organic layer was dried with Na_2SO_4, and evaporated, and the residue was purified by flash chromatography on silica gel (20 g), eluting with hexanes–EtOAc (3:1), to give a white solid **7** (228 mg, 25%): mp (starts to soften at 55°C) 90°C. IR (KBr) 2951, 2872, 1735, 1477, 1438, 1373, 1248, 1024 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.26 (d, J=2.3 Hz, 1H), 7.22 (d, J=2.3 Hz, 1H), 7.07 (d, J=2.1 Hz, 1H), 7.02 (d, J=2.3 Hz, 1H), 5.78 (t, J=7.6 Hz, 1H), 4.79 (s, 1H), 4.64 (bs, 1H), 4.31–4.27 (m, 1H), 3.72 (s, 3H), 3.65 (s, 3H), 3.64 (s, 3H), 3.63 (s, 3H), 1.84 (s, 3H), 1.82 (s, 3H), 1.77 (s, 3H), 1.74–0.77 (m, 24H), 0.77 (s, 3H), 0.48 (d, J=6.4 Hz, 3H), 0.45 (s, 3H). ESIMS m/z 935/937 (MNa^+).

3.1.3. 24-[Bis[5′-chloro-4′-methoxy-3′-(carbonyl)phenyl]-methylene]3α,7α,12α-trihydroxycholane (8). 24-[Bis[5′-chloro-4′-methoxy-3′-(methoxycarbonyl)-phenyl]methylene]-3α,7α,12α-triacetoxy cholane (**7**, 93.5 mg, 0.1 mmol) was taken up with stirring in methanol (30 mL). KOH (200 mg) was added, and the resulting solution was heated under reflux for 24 h. The reaction mixture was poured into water (75 mL) and acidified to pH 1 by dropwise addition of 1N HCl. The resulting solution was extracted with EtOAc (3×50 mL). The organic extracts were combined and dried over Na_2SO_4 and concentrated to give the triol **8** (68 mg, 90%): mp (starts to soften at 35°C) 175°C. IR (KBr) 3507–3389, 2936, 2861, 1711, 1475, 1425, 1374, 1254, 1079, 1002 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, J=2.1 Hz, 2H), 7.51 (d, J=2.1 Hz, 1H), 7.49 (d, J=2.3 Hz, 1H), 6.30 (t, J=7.7 Hz, 1H), 3.96 (s, 3H), 3.90 (s, 4H), 3.78 (s, 1H), 3.37–3.26 (m, 1H), 2.35–1.06 (m, 29H), 0.96 (s, J=6.5 Hz, 3H), 0.88 (s, 3H), 0.68 (s, 3H). ESIMS m/z 759/761(MH^+), 743/741, 725/723, 707/705. Anal. calcd for $C_{41}H_{52}Cl_2O_9 \cdot 0.5H_2O$: C, 64.09; H,6.95. Found: C, 64.07; H, 7.18.

3.1.4. 24-[Bis[5′-chloro-4′-hydroxy-3′-(carbonyl)-phenyl]-methylene]3α,7α,12α-trihydroxycholane (9). Boron-tribromide–dimethyl sulfide complex (1 M solution in methylene chloride, 0.61 mL) was added dropwise to a stirred solution of diacid **8** (0.15 g, 0.12 mmol) in dichloroethane (25 mL), and the mixture was stirred at 70°C for 40 h and at room temperature overnight. It was cooled in ice, the reaction quenched with water (10 mL), and the mixture was stirred for 1 h. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (3×20 mL). The combined organic extracts were washed with water, dried over Na_2SO_4 and evaporated to dryness. The solid was recrystallized from a mixture of methylene chloride and acetone to yield diacid **9** (50 mg, 35%): mp 152°C dec. IR (KBr) 3500–2600, 2930, 2865, 1682, 1459, 1360, 1233, 1187 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ 7.79 (d, J=1.9 Hz, 1H), 7.76 (d, J=2.1 Hz, 1H), 7.61 (d, J=2.1 Hz, 1H), 7.55 (d, J=1.9 Hz, 1H), 6.24 (t, J=7.6 Hz, 1H), 4.00 (s, 1H), 3.87 (s, 1H), 3.44–3.41 (m, 1H), 2.35–1.06 (m, 29H), 1.02 (d, J=6.5 Hz, 3H), 0.95 (s, 3H), 0.75 (s, 3H). ESIMS m/z 733/731(MH^+), 715/713, 697/699, 679/677. Anal. calcd for $C_{39}H_{48}Cl_2O_9 \cdot 2.5H_2O$: C, 60.37; H, 6.89. Found: C, 60.51; H, 7.03.

3.1.5. 3,3′-Dicarbomethoxy-5,5′-dichloro-4,4′-dihydroxy-diphenylmethane (12). 3,3′-Dicarboxy-5,5′-dichloro-4,4′-dihydroxydiphenylmethane (**11**,[2] 0.5 g, 1.40 mmol) was taken up in methanol (10 mL) and benzene (25 mL). $TMSCHN_2$ (2.5 mL, 5.0 mL, 2.0 M solution in hexanes) was added dropwise to the stirring solution. The solution was allowed to stir for 10 h after addition was completed. The reaction mixture was concentrated and the crude product was purified by column chromatography on silica gel (50 g) using ethyl acetate–hexanes (1:4) as the eluent to give a white solid **12** (0.25 g, 45%): mp 175°C. IR (KBr) 3093, 2950, 1735, 1676, 1445, 1344, 1294, 1198 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 11.23 (s, 2H), 7.55 (d, J=2.3 Hz, 2H), 7.35 (d, J=2.2 Hz, 2H), 3.96 (s, 6H), 3.81 (s, 2H). Anal. calcd for $C_{17}H_{14}Cl_2O_6$: C, 53.01; H, 3.66. Found: C, 52.81; H, 3.77.

3.1.6. 4,4′-Diacetoxy-3,3′-dicarbomethoxy-5,5′-dichloro-diphenylmethane (13). 3,3′-Dicarbomethoxy-5,5′-dichloro-4,4′-dihydroxydiphenylmethane (12, 0.5 g, 1.30 mmol) was taken up in acetic anhydride (8 mL), pyridine (4 mL) and DMAP (25 mg) with stirring. Stirring was continued for 24 h. The reaction mixture was diluted with ethyl acetate (40 mL) and the resulting mixture was washed successively with 5% aqueous HCl solution (2×25 mL), 5% aqueous $NaHCO_3$ solution (20 mL) and water (20 mL). The organic layer was dried with Na_2SO_4, and concentrated, and the residue was flash chromatographed on silica gel (20 g), eluting with hexanes–EtOAc (4:1), to give a white powder **13** (0.35 g, 58%): mp 155°C. IR (KBr) 2953, 1765, 1723, 1576, 1467, 1364, 1277 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.71 (d, J=2.2 Hz, 2H), 7.41 (d, J=2.2 Hz, 2H), 3.96 (s, 2H), 3.87 (s, 6H), 2.38 (s, 6H). Found: C, 52.81; H, 3.77. Anal. calcd for $C_{21}H_{18}Cl_2O_8$: C, 53.75; H, 3.87. Found: C, 54.00; H, 3.20.

3.1.7. Di[4-acetoxy-3-chloro-5-(methoxycarbonyl)phenyl] ketone (14). 3,3′-Dicarbomethoxy-5,5′-dichloro-4,4′-dihydroxydiphenylmethane (**13**, 0.40 g, 0.85 mmol) was taken up in acetic anhydride (20 mL) with stirring. CrO_3 (0.357 g, 3.57 mmol) was added in one portion to the stirring mixture, and the resulting mixture was stirred under argon at room temperature for 12 h. The reaction mixture was filtered through a pad of celite in a sintered glass funnel. The celite pad was washed with ethyl acetate (40 mL). The filtrate and washes were combined, and the solvent was removed on a rotary evaporator. The crude product was flash chromatographed on silica gel (20 g), eluting with hexanes–EtOAc (4:1), to give a white solid **14** (0.40 g, 97%): mp 160°C. IR (KBr) 3080, 2955, 1780,

1732, 1657, 1597, 1460, 1368, 1270 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.28 (d, J=2.0 Hz, 2H), 8.07 (d, J=2.0 Hz, 2H), 3.90 (s, 6H), 2.44 (s, 6H). Anal. calcd for $C_{21}H_{16}Cl_2O_9$: C, 52.19; H, 3.34. Found: C, 52.11; H, 3.20.

3.1.8. 24-[Bis[5′-chloro-4′-hydroxy-3′-(methoxycarbonyl)-phenyl]methylene]3α,7α,12α-triacetoxycholane (15). THF (20 mL) was added to a mixture of titanium(IV) chloride tetrahydrofuran complex (1:2) (1.32 g, 4 mmol) and zinc dust (0.45 g, 6 mmol) kept under argon atmosphere and the mixture was heated at reflux for 3 h. A solution of di[4-acetoxy-3-chloro-5-(methoxycarbonyl)phenyl] ketone (**14**, 350 mg, 0.73 mmol) and the aldehyde **5** (375 mg, 0.73 mmol) in THF (15 mL) was added to the reaction mixture, which was heated at reflux for 24 h. The reaction mixture was cooled to room temperature, 1N HCl (10 mL) was added, and the reaction mixture was extracted with EtOAc (3×40 mL) and washed with brine (50 mL). The organic layer was dried with Na_2SO_4 and evaporated to give the crude product. The crude product was flash chromatographed on silica gel (20 g), eluting with hexane–EtOAc (4:1), to give a sticky material **15** (0.16 g, 25%): mp 82°C dec. IR (KBr) 3500–3400, 2936, 2861, 1710, 1475, 1425, 1374, 1254, 1079, 1002 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 11.38 (s, 1H), 11.29 (s, 1H), 7.51 (d, J=2.0 Hz, 1H), 7.49 (d, J=2.1 Hz, 1H), 7.37 (d, J=2.1 Hz, 1H), 7.33 (d, J=2.0 Hz, 1H), 5.92 (t, J=7.5 Hz, 1H), 5.05 (s, 1H), 4.90 (s, 1H), 4.48–4.44 (m, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.95–0.90 (m, 29 H), 0.89 (s, 3H), 0.73 (d, J=6.5 Hz, 3H), 0.70 (s, 3H). ESIMS m/z 885/887(MH^+). Anal. calcd for $C_{47}H_{58}Cl_2O_{12}$: C, 63.78; H, 6.61. Found: C, 63.96; H, 6.50.

3.1.9. 24-[Bis[5′-chloro-4′-hydroxy-3′-(carbonyl)phenyl]-methylene]3α,7α,12α-trihydroxycholane (9). 24-[Bis[5′-chloro-4′-hydroxy-3′-(methoxycarbonyl)phenyl]methylene]-3α,7α,12α-triacetoxycholane (**15**, 88 mg, 0.1 mmol) was taken up with stirring in methanol (30 mL). KOH (200 mg) was added, and the resulting solution was heated under reflux for 24 h. The reaction mixture was poured into water (75 mL) and was acidified to pH 1 by dropwise addition of 1N HCl. The resulting solution was extracted with EtOAc (3×50 mL). The organic extracts were combined and dried over Na_2SO_4 and concentrated to give the diacid **9** (58 mg, 80%).

3.1.10. Anti-HIV assays. The HIV-inhibitory activity of compounds was evaluated as previously described for HIV-1_{Rf} in CEM-SS[36] cells and HIV-1_{IIIB} and HIV-2_{ROD} in MT-4 cells.[29] These are microtiter assays which quantitate the ability of a compound to inhibit HIV-1- and HIV-2-induced cell killing via syncytium formation. Antiviral and toxicity data are reported as the concentration of compound required to inhibit 50% virus-induced cell killing (50% effective concentration [EC_{50}]) and the concentration of compound required to reduce cell viability by 50% (cytotoxicity CC_{50}). All data are derived from three tests.

For the assays involving HIV-1_{IIIB} and HIV-2_{ROD}, stock solutions (10×final concentration) of test compounds were added to 25 μL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock- and HIV-infected cells. Serial five-fold dilutions of test compounds were made directly in flat bottom 96-well plastic microtiter trays using a Biomek 2000 robot (Beckman Instruments, Fullerton, CA). Untreated control HIV- and mock-infected cell samples were included for each sample.

HIV-1_{IIIB}[30] or HIV-2_{ROD}[31] stock (50 μL) at 100–300 $CCID_{50}$ (cell culture infectious dose) or culture medium was added to either the infected or mock-infected well of the microtiter tray. Uninfected (mock-infected) cells were used to evaluate the cytotoxic effect of the test compounds. Exponentially growing MT-4 cells[32] were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/mL, using slight magnetic stirring, and 50 μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells were examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a formazan that can be measured spectrophotometrically. The absorbances were read in Multiskan Ascent Reader (Labsystems, Helsinki, Finland), at a wavelengths 540 nm, with a reference wavelength of 690 nm. The EC_{50} and CC_{50} values were then calculated.

Acknowledgements

This research was made possible by NIH grant AI 36624, awarded by the National Institutes of Health, DHHS, and by grants from the (Belgian) Fonds voor Wetenschappelijk Onderzoek (FWO Grant G.0104.98) and the (Flemish) Geconcerteerde Onderzoeksacties (GOA 00/12). We thank ISEP/FORTIS for financial support. We also thank Kristien Erven for excellent technical assistance.

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