Anti-AIDS Agents 49.[1] Synthesis, Anti-HIV, and Anti-Fusion Activities of IC9564 Analogues Based on Betulinic Acid

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Received February 11, 2002

The betulinic acid derivative IC9564 inhibits human immunodeficiency virus (HIV)-1 entry. Among a series of IC9564 derivatives, **5** and **20** were the most promising compounds against HIV infection with EC_{50} values of 0.33 and 0.46 μM, respectively. Both compounds inhibited syncytium formation with EC_{50} values of 0.40 and 0.33 μM, respectively. The comparable EC_{50} values in the two assays suggested that these compounds are fusion inhibitors. The structure−activity relationship data also indicated that a double bond in IC9564 can be eliminated and the statine moiety can be replaced with L-leucine while retaining anti-HIV activity.

Introduction

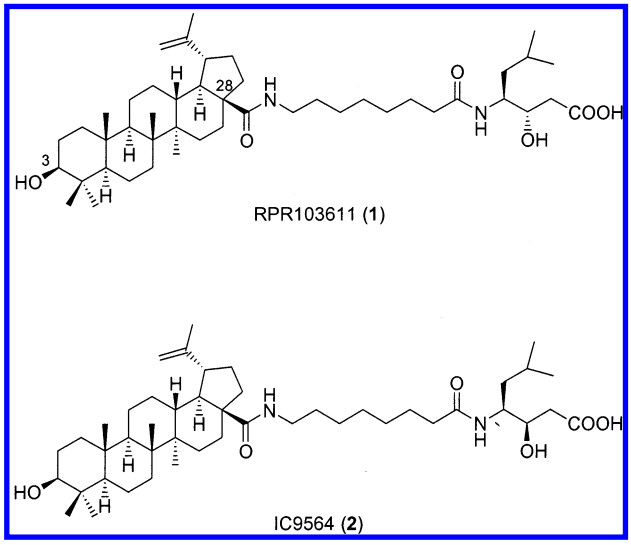
Triterpenes constitute a promising class of anti-HIV (human immunodeficiency virus) agents.[2−7] Their modes of action have been associated with several steps in the virus life cycle, including fusion,[8−11] reverse transcription,[12,13] and maturation.[14−16] Current HIV therapy lacks drugs targeted at the fusion step, although peptide mimetics (T20 and T1249), recombinant protein (PRO-542), and small molecular inhibitors (bicyclam AMD3100) have shown promise in clinical trials.[17]

Mayaux et al. reported that the triterpene fusion inhibitor RPR103611 (**1**, Chart 1) was active at a submicromolar level.[8] Subsequently, Labrosse et al. determined that the drug sensitivity was linked to a single amino acid change, I84S, on the loop region of HIV-1 transmembrane glycoprotein gp41[9], and to the stability of the gp120−gp41 complex.[10] IC9564 (**2**, Chart 1) is a stereoisomer of and equipotent with RPR103611. Results from a syncytium formation assay indicated that IC9564 inhibits HIV-1 at the membrane fusion step.[11] Further analysis showed that HIV-1 gp120 was the molecular target for IC9564. In an effort to discover more potent anti-fusion triterpenes based on betulinic acid, the present study has focused on modifications in the isopropylene and C28 side chain.

Chemistry

The common intermediates acid chlorides **3** and **6** were obtained by reacting dihydrobetulinic and betulinic acids with acetic anhydride followed by treating with oxalyl chloride. Acid chloride **3** (Scheme 1) was coupled with the readily prepared amine [4*S*-(8-aminooctanoylamino)-3*R*-hydroxy-6-methylheptanoic acid benzyl ester], synthesized as described in ref 11, in the presence of EDC/HOBT to give **4**. The protecting groups were cleaved with 4N sodium hydroxide in MeOH/tetrahydrofuran (THF) to yield **5**. Likewise, condensation of acid chloride **6** with L-leucine methyl ester gave **7**, which was converted to **8** by the same conditions as described for **5**.

Chart 1



Conjugation of acid chloride **6** (Scheme 2) with various amines (β-alanine, 8-aminocaprylic acid, and 11-aminoundecanoic acid methyl esters) in the presence of DMAP, potassium carbonate, triethylamine, and pyridine gave the corresponding amide derivatives **9**−**11**. Removal of the acetyl and methyl moieties using 4N sodium hydroxide produced the acids **12**−**14**. Compounds **15**−**17** were obtained similarly to **7** using EDC/HOBT in CH_2Cl_2 at room temperature in yields ranging from 83% to 93%. Finally, saponification of the methyl ester led to **18**−**20**.

Results and Discussion

Compounds **4**, **5**, **7**, and **9**−**20** were evaluated for anti-HIV-1 activity in a MAGI assay against the virus NL4-3. Compound **8** was also examined for inhibitory activity in H9 lymphocytes.[18] AZT was included during each experiment as a positive drug control. The data are presented in Table 1.

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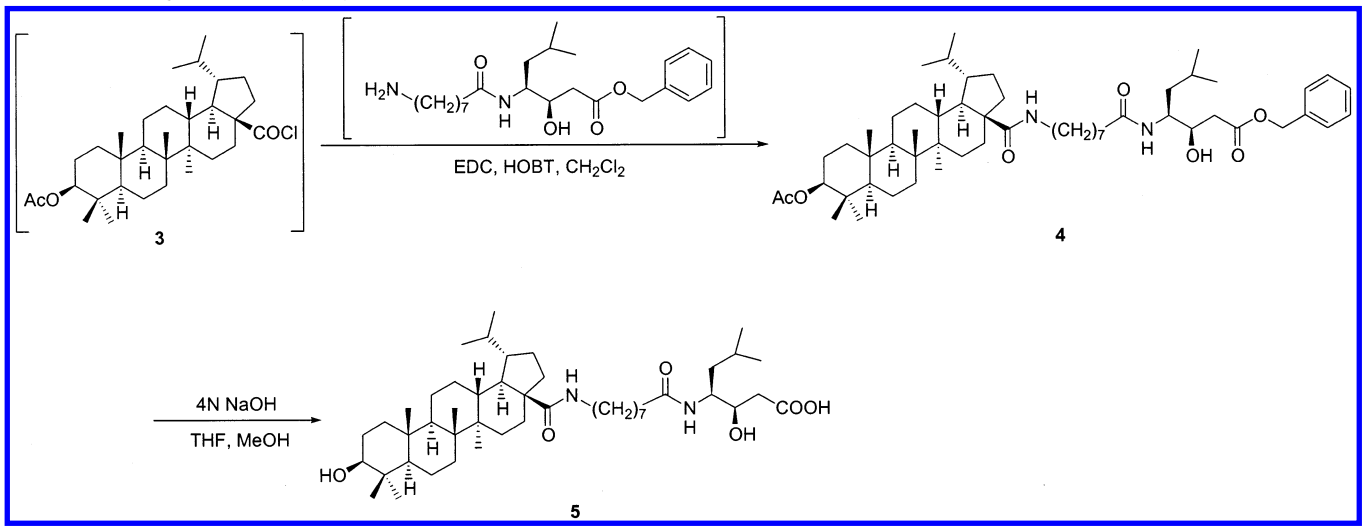
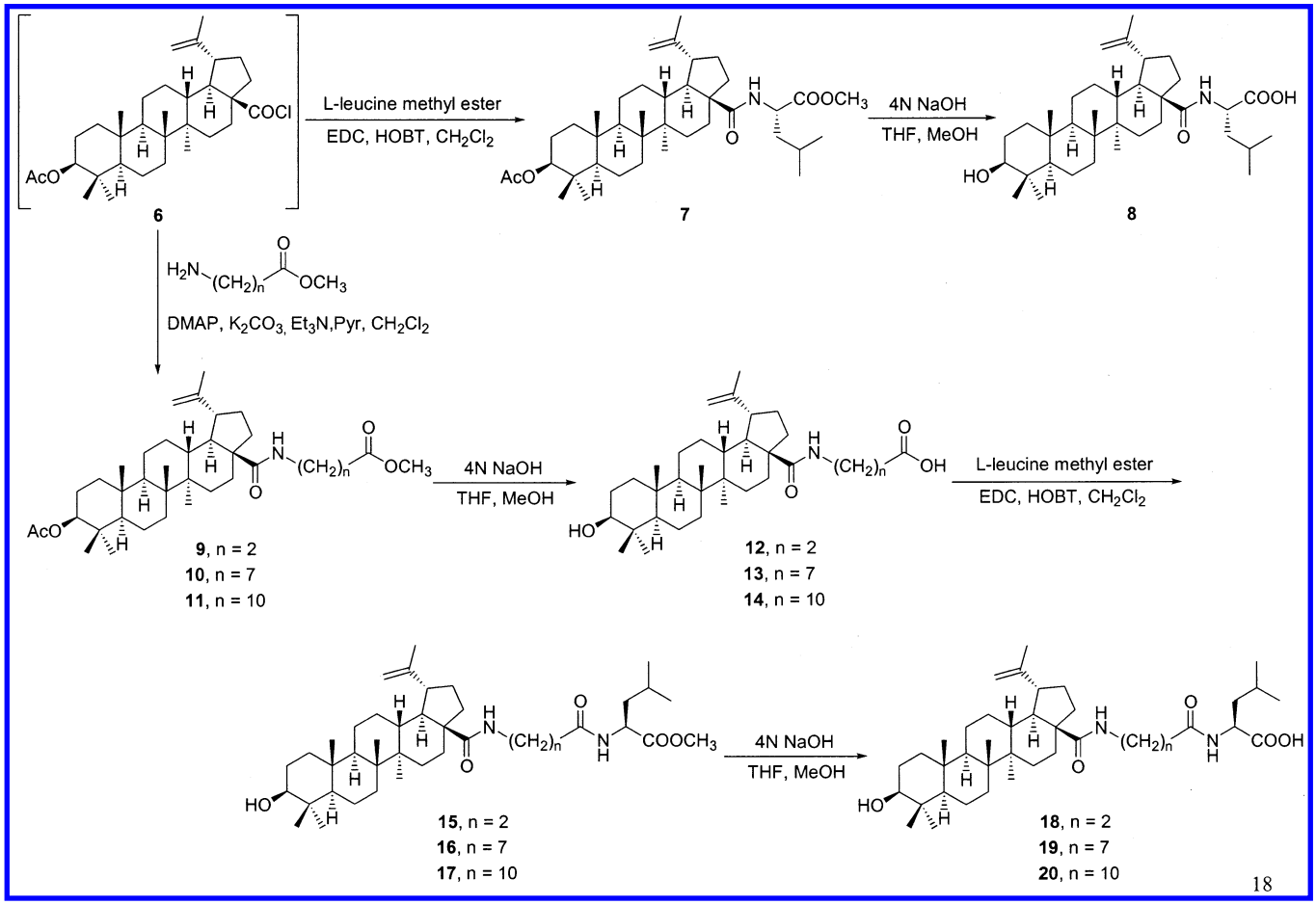
10.1021/jm020069c CCC: $22.00
Published on Web 09/05/2002

Scheme 1. Synthesis of (*R,S*)Statine Derivatives



Scheme 2. Synthesis of l-Leucine Derivatives



The saturated analogue **5** was equipotent (EC_{50} = 0.33 μM) with IC9564 (EC_{50} = 0.4 μM). In contrast, the EC_{50} value of its ester precursor **4** dropped to >28 μM. These data suggested that a free hydroxy group at C3 and free carboxylic acid in the C28 side chain are necessary for anti-HIV activity. This finding is consistent with the results reported previously for IC9564 and IC9563.[11] Our results also suggest that the double bond of the isopropylidene group might not play a key role in the HIV inhibitory activity of this compound type.

Replacing the statine moiety of IC9564 with L-leucine yielded the 4-fold less potent compound **19** (EC_{50} = 1.38 μM). However, the methyl ester (**16**) of **19** showed increased potency (EC_{50} = 0.52 μM). When the methylene chain was extended to 10 carbons, the free acid **20** showed equivalent activity (EC_{50} = 0.46 μM) to **16**. In contrast, the β-alanine derivative **18** totally lost anti-HIV activity (EC_{50} > 39 μM). Furthermore, the methyl esters (**15** and **17**) of **18** and **20** also lacked discernible anti-HIV activity. Complete omission of the aminoalkanoic chain (**8**) dropped the inhibitory activity 8-fold. Among the non-L-leucine intermediates, the esters **9**−**11** were active only at ≥10 μM. However, the deesterified compound **14** was quite active (EC_{50} = 0.7 μM), while

Table 1. Results of MAGI and Fusion Assays

compd	EC_{50} MAGI assay (μM)	EC_{50} fusion assay (μM)
4	>28	>22
5	0.33	0.40
7	>40	>32
8	3.22[a]	5.48
9	26.7	21.4
10	29.1	>31
11	10.2	>29
12	>47	>38
13		
14	0.70	0.31
15	>38	7.63
16	0.52	0.86
17	>32	>26
18	>39	>31
19	1.38	1.10
20	0.46	0.33
IC9564	0.40	0.66
DP178	0.014	0.019
AZT	0.075	>75

[a] Compound was tested in acutely infected H9 cells; see Experimental Section for details.

both compounds (**12** and **13**) with shorter aminoalkanoic acids were inactive. These results indicate that the statine group in the C28 side chain of IC9564 can be replaced by L-leucine and that the numbers of methylenes in the aminoalkanoic chain are crucial to optimal anti-HIV potency.

A known fusion inhibitor, DP178/T20, was used as a positive control in the fusion and MAGI assays.[19] Compounds **5**, **8**, **14**, **16**, **19**, and **20** all had EC_{50} values of $\leq$3.2 μM in the MAGI assay and were active against membrane fusion with EC_{50} values of <5.5 μM. In addition, the concentrations required to inhibit syncytium formation were comparable to those required to inhibit HIV-1 replication. Thus, HIV-1 envelope-induced membrane fusion appears to be the primary target of these compounds. Further mode of action studies will be determined.

Experimental Section

The melting points were measured with a Fisher–Johns melting point apparatus and are uncorrected. The proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained using a Bruker AC-300 NMR spectrometer. All chemical shifts are reported in parts per million from the internal standard Me_4Si (tetramethylsilane, TMS) with $CDCl_3$ or C_5D_5N as solvent. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Optical rotations were measured with a Jasco DIP-1000 polarimeter. Thin-layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F-254 plates. EM Kieselgel 60 (230–400 mesh ASTM) was used for column chromatography. All new target compounds were characterized by optical rotation, ^{1}H NMR, and elemental analysis.

Preparation of Acid Chlorides 3 and 6. Oxalyl chloride solution (2 M in CH_2Cl_2, 10 mL) was added to the 3-*O*-acetates of dihydrobetulinic acid and betulinic acid (0.5 mmol) and stirred for 2 h. Each mixture was concentrated to dryness under reduced pressure. The residues were diluted with dry CH_2Cl_2 (3 × 1 mL), concentrated to dryness under reduced pressure, and used without further purification.

General Procedure for Synthesizing Compounds 4, 7, and 15–17. A solution of amine ester (0.5 mmol) in dry CH_2Cl_2 (3 mL) was treated with EDC (1.5 equiv mol) and HOBT (1.5 equiv mol). To the mixture was added acid chloride (0.8 equiv mol) or free acid (0.8 equiv mol), and stirring was continued overnight. The solution was diluted with CH_2Cl_2 (20 mL) and then washed three times with water (50 mL). The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was chromatographed on silica gel and crystallized from *n*-hexane and EtOAc.

General Procedure for Synthesizing Compounds 9–11. A solution of amine ester (0.5 mmol) in dry CH_2Cl_2 (2 mL) was treated with 4-(dimethylamino)pyridine (1.5 equiv mol), K_2CO_3 (1.5 equiv mol), dry triethylamine (0.2 mL), and anhydrous pyridine (3 mL). To the mixture was added acid chloride **6** (0.8 equiv mol), and stirring was continued overnight. The solution was diluted with EtOAc (20 mL) and then washed three times with 10% HCl solution (50 mL). The EtOAc layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was chromatographed using a silica gel column and crystallized from *n*-hexane and EtOAc.

General Procedure for Synthesizing Compounds 5, 8, 12–14, and 18–20. A solution of starting material (0.25 mmol), MeOH (1 mL), and THF (1 mL) was treated with 4 N NaOH (0.5 mL). After the reaction was complete, the mixture was acidified with hydrochloric acid to pH 4 and extracted three times with $CHCl_3$ (20 mL). Followed by the routine workup, the residue was crystallized from *n*-hexane and EtOAc.

(3*R*,4*S*)-Benzyl *N*′-[*N*-[3*β*-Acetoxy-28-oyl]-8-aminooctanoyl]-4-amino-3-hydroxy-6-methylheptanoate (4). Yield 64%; an off-white amorphous powder; $[\alpha]_D^{25}$ −16° (*c* = 0.5, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.74, 0.85 (3H each, both d, *J* = 7 Hz; 20-(CH_3)$_2$), 0.83, 0.84, 0.85, 0.92, 0.94 (3H each, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 0.89, 0.92 (3H each, both d, *J* = 7 Hz; statine moiety −CH(CH_3)$_2$), 2.04 (3H, s; 3-OCOCH_3), 2.18 (2H, t, *J* = 7 Hz; −CH_2CONH−), 2.52 (2H, d, *J* = 6 Hz; −CH_2COO−), 3.16–3.25 (2H, m; −CONHCH_2−), 3.64 (1H, d, *J* = 4 Hz; −CHO*H*), 3.98–4.12 (2H, m; −NHC*H*-, −C*H*OH−), 4.47 (1H, dd, *J* = 5, 11 Hz; H-3), 5.15 (2H, s; −OC$H_2C_6H_5$), 5.55–5.59 (2H, m; −N*H*CH_2-, −N*H*CH−), 7.32–7.39 (5H, m; −C_6H_5). Anal. ($C_{55}H_{88}N_2O_7$) C, H, N.

(3*R*,4*S*)-*N*′-[*N*-[3*β*-Hydroxy-28-oyl]-8-aminooctanoyl]-4-amino-3-hydroxy-6-methylheptanoic Acid (5). Yield 88%; a white powder; mp 114 °C (dec); $[\alpha]_D^{25}$ −25° (*c* = 2.34, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.74, 0.85 (3H each, both d, *J* = 7 Hz; 20-(CH_3)$_2$), 0.76, 0.83, 0.94, 0.96, 0.97 (3H each, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 0.91, 0.94 (3H each, both d, *J* = 7 Hz; statine moiety −CH(CH_3)$_2$), 2.22 (2H, dt, *J* = 3, 7 Hz; −CH_2CONH−), 2.50 (2H, d, *J* = 6 Hz; −CH_2COOH), 3.13–3.28 (3H, m; −NHCH_2-, −C*H*OH−), 3.64 (1H, d, *J* = 4 Hz; −CHO*H*), 4.0–4.12 (2H, m; H-3, −NHC*H*−), 5.69 (1H, t, *J* = 6 Hz; −N*H*CH_2−), 5.94 (1H, d, *J* = 8 Hz; −N*H*CH−). Anal. ($C_{46}H_{80}N_2O_6$) C, H, N.

Methyl *N*-[3*β*-Acetoxy-20(29)-en-28-oyl]-L-leucinate (7). Yield 75%; an off-white amorphous powder; mp 115–117 °C; $[\alpha]_D^{25}$ −2.4° (*c* = 0.25, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.83, 0.84 (6H), 0.92, 0.95, 0.96 (6H) (3H each, except 0.84, 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.04 (3H, s; 3-OCOCH_3), 2.45 (1H, dt, *J* = 3, 12 Hz; H-13), 3.11 (1H, dt, *J* = 4, 11 Hz; H-19), 3.73 (3H, s; −COOCH_3), 4.47 (1H, t, *J* = 8 Hz; H-3), 4.58 (1H, s; H-29), 4.64 (1H, dt, *J* = 5, 8 Hz; −NHC*H*−), 4.72 (1H, d, *J* = 2 Hz; H-29), 5.87 (1H, d, *J* = 8 Hz; −CON*H*−). Anal. ($C_{39}H_{63}NO_5$) C, H, N.

***N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-L-leucine (8).** Yield 40%; an off-white amorphous powder; mp 243–244 °C; $[\alpha]_D^{25}$ −17.2° (*c* = 1.40, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.75, 0.82, 0.91, 0.96, 0.97 (9H) (3H each, except 0.97, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.42 (1H, dt, *J* = 3, 12 Hz; H-13), 3.10 (1H, dt, *J* = 4, 11 Hz; H-19), 3.19 (1H, dd, *J* = 5, 11 Hz; H-3), 4.59 (1H, s; H-29), 4.56–4.63 (1H, m; −NHC*H*−), 4.73 (1H, d, *J* = 2 Hz; H-29), 5.88 (1H, d, *J* = 8 Hz; −CON*H*−). Anal. ($C_{36}H_{59}NO_4$) C, H, N.

Methyl *N*-[3*β*-Acetoxy-20(29)-en-28-oyl]-3-aminopropanoate (9). Yield 76%; a white powder; mp 112–114 °C; $[\alpha]_D^{25}$ +20.3° (*c* = 1.43, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.83, 0.84

(6H), 0.91, 0.95 (3H each, except 0.84, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.68 (3H, s; H-30), 2.04 (3H, s; 3-OCOCH_3), 2.41 (1H, dt, J = 3, 12 Hz; H-13), 2.54−2.58 (2H, m; −CH_2-COOCH$_3$), 3.10 (1H, dt, J = 4, 11 Hz; H-19), 3.42−3.61 (2H, m; −CONHCH_2−), 3.70 (3H, s; −COOCH_3), 4.47 (1H, dd, J = 5, 11 Hz; H-3), 4.59 (1H, s; H-29), 4.74 (1H, d, J = 2 Hz; H-29), 6.20 (1H, t, J = 6 Hz; −CONH−). Anal. ($C_{36}H_{57}NO_5$) C, H, N.

Methyl *N*-[3*β*-Acetoxy-20(29)-en-28-oyl]-8-aminooctanoate (10). Yield 69%; a yellow gum; mp 83−85 °C; $[\alpha]_D^{25}$ +17.4° (c = 0.3, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.83, 0.84 (6H), 0.93, 0.96 (3H each, except 0.84, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.68 (3H, s; H-30), 2.04 (3H, s; 3-OCOCH_3), 2.31 (2H, t, J = 7 Hz; −CH_2COOCH$_3$), 2.46 (1H, dt, J = 3, 12 Hz; H-13), 3.10−3.32 (3H, m; H-19, −CONHCH_2−), 3.67 (3H, s; −COOCH_3), 4.47 (1H, t, J = 8 Hz; H-3), 4.59 (1H, s; H-29), 4.73 (1H, d, J = 2 Hz; H-29), 5.57 (1H, t, J = 6 Hz; −CONH−). Anal. ($C_{41}H_{67}NO_5$) C, H, N.

Methyl *N*-[3*β*-Acetoxy-20(29)-en-28-oyl]-11-aminoundecanoate (11). Yield 65%; an off-white amorphous powder; mp 75−77 °C; $[\alpha]_D^{25}$ +15.1° (c = 0.49, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.83, 0.84 (6H), 0.93, 0.96 (3H each, except 0.84, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.68 (3H, s; H-30), 2.04 (3H, s; 3-OCOCH_3), 2.30 (2H, t, J = 8 Hz; −CH_2COOCH$_3$), 2.45 (1H, dt, J = 3, 12 Hz; H-13), 3.10−3.36 (3H, m; H-19, −CONHCH_2−), 3.67 (3H, s; −COOCH_3), 4.46 (1H, t, J = 8 Hz; H-3), 4.59 (1H, s; H-29), 4.73 (1H, d, J = 2 Hz; H-29), 5.57 (1H, t, J = 6 Hz; −CONH−). Anal. ($C_{44}H_{73}NO_5$) C, H, N.

***N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-3-aminopropanoic Acid (12).** Yield 87%; a white powder; $[\alpha]_D^{25}$ +25.0° (c = 0.58, C$_5$H$_5$N). ^{1}H NMR (C$_5$D$_5$N): δ 0.83, 0.97, 1.02, 1.08, 1.17 (3H each, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.75 (3H, s; H-30), 2.85−2.93 (3H, m; H-13, −CH_2COOH), 3.39 (1H, t, J = 8 Hz; H-3), 3.51 (1H, dt, J = 4, 11 Hz; H-19), 3.68−3.84 (2H, m; −NHCH_2−), 4.71 (1H, s; H-29), 4.89 (1H, d, J = 2 Hz; H-29), 8.05 (1H, br s; −CONH−). Anal. ($C_{33}H_{53}NO_4$) C, H, N.

***N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-8-aminooctanoic Acid (13).** Yield 82%; an off-white amorphous powder; mp 114−116 °C; $[\alpha]_D^{25}$ +5.1° (c = 0.65, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.81, 0.93, 0.96 (6H) (3H each, except 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.68 (3H, s; H-30), 2.35 (2H, t, J = 8 Hz; −CH_2COOH), 2.45 (1H, dt, J = 3, 12 Hz; H-13), 3.09−3.25 (4H, m; H-3, H-19, −CONHCH_2−), 4.59 (1H, s; H-29), 4.73 (1H, d, J = 2 Hz; H-29), 5.58 (1H, t, J = 6 Hz; −CONH−). Anal. ($C_{38}H_{63}NO_4$) C, H, N.

***N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-11-aminoundecanoic Acid (14).** Yield 81%; an off-white amorphous powder; mp 102−104 °C; $[\alpha]_D^{25}$ +8.5° (c = 0.33, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.82, 0.93, 0.96 (6H) (3H each, except 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.68 (3H, s; H-30), 2.35 (2H, t, J = 7 Hz; −CH_2COOH), 2.45 (1H, dt, J = 3, 12 Hz; H-13), 3.10−3.36 (4H, m; H-3, H-19, −CONHCH_2−), 4.59 (1H, s; H-29), 4.74 (1H, d, J = 2 Hz; H-29), 5.59 (1H, t, J = 6 Hz; −CONH−). Anal. ($C_{41}H_{69}NO_4$) C, H, N.

Methyl *N*′-[*N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-3-aminopropanoyl]-L-leucinate (15). Yield 83%; a white powder; mp 134−136 °C; $[\alpha]_D^{25}$ +3.1° (c = 0.45, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.82, 0.93 (6H), 0.96 (9H) (3H each, except 0.93, 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.35−2.53 (3H, m; H-13, −CH_2CONH−), 3.11 (1H, dt, J = 4, 11 Hz; H-19), 3.17 (1H, dd, J = 5, 11 Hz; H-3), 3.49−3.57 (2H, m; −CONHCH_2−), 3.74 (3H, s; −COOCH_3), 4.59 (1H, s; H-29), 4.57−4.64 (1H, m; −NHCH(CH$_2$−)CO−), 4.73 (1H, d, J = 2 Hz; H-29), 5.91 (1H, d, J = 8 Hz; −CONHCH(CH$_2$−)CO−), 6.42 (1H, t, J = 6 Hz; −CONHC-H$_2$−). Anal. ($C_{40}H_{66}N_2O_5$) C, H, N.

Methyl *N*′-[*N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-8-aminooctanoyl]-L-leucinate (16). Yield 93%; an off-white amorphous powder; mp 107−108 °C; $[\alpha]_D^{25}$ +3.7° (c = 1.0, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.82, 0.93 (6H), 0.96 (9H) (3H each, except 0.93, 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.18−2.24 (2H, m; −CH_2-CONH−), 2.45 (1H, dt, J = 3, 12 Hz; H-13), 3.11−3.33 (4H, m; H-3, H-19, −CONHCH_2−), 3.73 (3H, s; −COOCH_3), 4.59 (1H, s; H-29), 4.65 (1H, dt, J = 5, 8 Hz; −NHCH(CH$_2$−)CO−), 4.74 (1H, d, J = 2 Hz; H-29), 5.60 (1H, t, J = 6 Hz; −CONHCH$_2$−), 5.81 (1H, d, J = 8 Hz; −CONHCH(CH$_2$−)-CO−). Anal. ($C_{45}H_{76}N_2O_5$) C, H, N.

Methyl *N*′-[*N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-11-aminoundecanoyl]-L-leucinate (17). Yield 87%; a yellow gum; mp 92−94 °C; $[\alpha]_D^{25}$ +3.4° (c = 0.93, CHCl$_3$). ^{1}H NMR (CDCl$_3$) δ 0.75, 0.82, 0.94 (6H), 0.96 (9H) (3H each, except 0.94, 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.18−2.24 (2H, m; −CH_2CONH−), 2.45 (1H, dt, J = 3, 12 Hz; H-13), 3.11−3.34 (4H, m; H-3, H-19, −CONHCH_2−), 3.73 (3H, s; −COOCH_3), 4.59 (1H, s; H-29), 4.66 (1H, dt, J = 5, 8 Hz; −NHCH(CH$_2$−)CO−), 4.73 (1H, d, J = 2 Hz; H-29), 5.58 (1H, t, J = 6 Hz; −CONHCH$_2$−), 5.81 (1H, d, J = 8 Hz; −CONHCH(CH$_2$−)CO−). Anal. ($C_{48}H_{82}N_2O_5$) C, H, N.

***N*′-[*N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-3-aminopropanoyl]-L-leucine (18).** Yield 77%; a white powder; mp 180−182 °C; $[\alpha]_D^{25}$ +3.6° (c = 0.28, C$_5$H$_5$N). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.81, 0.91, 0.96 (12H) (3H each, except 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.35−2.52 (3H, m; H-13, −CH_2CONH−), 3.08 (1H, dt, J = 4, 11 Hz; H-19), 3.18 (1H, dd, J = 5, 11 Hz; H-3), 3.45−3.57 (2H, m; −CONHCH_2−), 4.53−4.59 (2H, m; −NHCH(CH$_2$−)CO-, H-29), 4.73 (1H, br s; H-29), 6.32 (1H, d, J = 8 Hz; −CONHCH$_2$−), 6.40 (1H, t, J = 6 Hz; −CONHCH(CH$_2$−)CO−). Anal. ($C_{39}H_{64}N_2O_5$) C, H, N.

***N*′-[*N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-8-aminooctanoyl]-L-leucine (19).** Yield 64%; a white powder; mp 147−149 °C; $[\alpha]_D^{25}$ −2.9° (c = 1.37, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.82, 0.92, 0.96 (12H) (3H each, except 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.18−2.29 (2H, m; −CH_2CONH−), 2.41 (1H, dt, J = 3, 12 Hz; H-13), 3.07−3.31 (4H, m; H-3, H-19, −CONHCH_2−), 4.59−4.66 (2H, m; −NHCH(CH$_2$−)CO-, H-29), 4.73 (1H, br s; H-29), 5.77 (1H, t, J = 6 Hz; −CONHCH$_2$−), 6.25 (1H, d, J = 8 Hz; −CONHCH(CH$_2$−)CO−). Anal. ($C_{44}H_{74}N_2O_5$) C, H, N.

***N*′-[*N*-[3*β*-Hydroxy-20(29)-en-28-oyl]-11-aminoundecanoyl]-L-leucine (20).** Yield 91%; an off-white amorphous powder; $[\alpha]_D^{25}$ −0.9° (c = 2.87, CHCl$_3$). ^{1}H NMR (CDCl$_3$): δ 0.75, 0.82, 0.92, 0.95, 0.96 (6H), 0.97 (3H each, except 0.96, all s; 4-(CH_3)$_2$, 8-CH_3, 10-CH_3, 14-CH_3, leucine moiety -(CH_3)$_2$), 1.68 (3H, s; H-30), 2.16−2.32 (2H, m; −CH_2CONH−), 2.40 (1H, dt, J = 3, 12 Hz; H-13), 3.10 (1H, dt, J = 4, 11 Hz; H-19), 3.16−3.30 (3H, m; H-19, −CONHCH_2−), 4.59−4.66 (2H, m; −NHCH(CH$_2$−)CO-, H-29), 4.72 (1H, br s; H-29), 5.71 (1H, t, J = 6 Hz; −CONHCH$_2$−), 6.02 (1H, d, J = 8 Hz; −CONHCH-(CH$_2$−)CO−). Anal. ($C_{47}H_{80}N_2O_5$) C, H, N.

MAGI Assay.[20] Hela-CD4/CCR5/*β*-gal cells were plated on a 48 well plates at 10 000 cells/well and cultured in DMEM medium containing 500 μg/mL of G418 and 250 μg/mL of hygromycin for 1 day. The cells were infected with virus dilutions in the presence of various concentrations of anti-HIV agents and incubated for 2 days at 37 °C. The infected cells were stained blue by adding X-gal at 0.4 mg/mL to the culture. The cells were fixed with a solution containing 1% formaldehyde and 0.2% glutaraldehyde before staining. The numbers of infected cells were counted by using an AlphaImager (Alpha Innotech). An anti-HIV compound that inhibits 50% of virus infection is defined by its ability to reduce the number of infected cells by 50%, for example, from 400 blue cells to 200 blue cells. The MAGI assay detects the presence of the HIV-1 transactivating protein, Tat, during the viral replication. It is a fast and accurate assay for HIV-1 entry inhibitors such as IC9564 and its derivatives.

Cell Fusion Assay.[11] The fusion assays were performed by transfecting the monkey kidney cells (COS) with the expression vector pSRHS that contains the HIV-1 NL4-3 envelope gene. The original HXB envelope in pSRHS was replaced with the NL4-3 envelope. Electroporation was performed to express the HIV-1 envelope on COS cells. Briefly, COS cells (10^6) in culture medium were incubated with 2 μg of the envelope expression vector on ice for 10 min. The electroporation was performed using a gene pulser (BioRad, Hercules, CA) with capacitance set at 950 μF and a voltage at

150 mA. The transfected COS cells were cultured for 1 day before mixing with the fusion partner MOLT-4 cells. MOLT-4 cells (7×10^4) were incubated with the envelope-transfected cells (10^4) in 96 well half-area flat-bottomed plates (Costar) in 100 μL of culture medium. Compounds to be tested at various concentrations in 10 μL of culture medium were incubated with the cell mixtures at 37 °C for 24 h. Multinucleated syncytia were enumerated by microscopic examination of the entire contents of each well.

Acknowledgment. This investigation was supported by Grants AI-33066 and AI-49096 from the National Institute of Allergy and Infectious Diseases awarded to K.-H.L. and C.-H.C., respectively.

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JM020069C