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Anti-AIDS Agents—XXVII. Synthesis and Anti-HIV Activity of Betulinic Acid and Dihydrobetulinic Acid Derivatives‡‡

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Abstract—Two series of lupane-type triterpenoic acid derivatives were synthesized and evaluated for their inhibitory activity against HIV- 1 replication in acutely infected H9 cells, based on the fact that betulinic acid (**1**) and dihydrobetulinic acid (**9**) were identified as anti-HIV agents. Among the derivatives, 3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid (**3**) and 3-*O*-(3′,3′-dimethylsuccinyl)-dihydrobetulinic acid (**11**) both demonstrated extremely potent inhibitory activity with EC_{50} values of $<3.5 \times 10^{-4}$ μM, and remarkable in vitro therapeutic index (TI) values of 20,000 and 14,000, respectively. 3-*O*-(3′,3′-dimethylglutaryl)-betulinic acid (**4**) and-dihydrobetulinic acid (**12**), 3-*O*-diglycolyl-betulinic acid (**5**) and -dihydrobetulinic acid (**13**) and 3-*O*-glutaryl-betulinic acid (**6**) were also potent inhibitors of HIV replication with EC_{50} values ranging from 0.04 to 2.3×10^{-3} μM and TI values from 292 to 2344. In addition, compounds **11** and **12** were also active against HIV replication in a monocyte cell line and in peripheral blood mononuclear cells. Our in vitro assay indicated that these compounds are not inhibitors of HIV-1 reverse transcriptase, whereas they inhibited syncytia formation completely in a concentration range of 20–40 μg/mL. However, 3-*O*-(2′,2′-dimethylsuccinyl)-betulinic acid (**2**) was also found to be an inhibitor of HIV-induced membrane fusion with an IC_{100} value of 20 μg/mL, though it displayed significantly lower anti-HIV activity than foregoing compounds with an EC_{50} value of 2.7 μM and TI of 6.7. Further study is underway to determine the mechanisms of action of these compounds. © 1997 Elsevier Science Ltd.

Introduction

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV) infection, is now rapidly spreading among many populations, and has become a serious global threat to human health and life. First generation drugs, such as AZT, ddC, ddI, and D4T[2,3] are used clinically, but rapid development of HIV resistance to these nucleoside HIV-1 reverse transcriptase (RT) inhibitors is common.[4–7] Moreover, these agents have limited or transient benefits due to their adverse side effects.[8] Therefore, many research approaches are currently underway to discover diverse anti-HIV agents with novel structures or mechanism(s) of action. Agents under development include inhibitors of HIV-1 RT, protease,[9–11] membrane fusion,[12,13] and integrase.[14,15]

Our continuing approach is to discover novel plant-derived natural products as potential new lead compounds for anti-HIV agents, and modify these new lead compounds to find still more potent anti-HIV agents. In our bioactivity-directed search for plant-derived naturally occurring compounds, we previously isolated betulinic acid (**1**) and plantanic acid (**30**) as anti-HIV principles from *Syzigium claviflorum* (Myrtaceae).[16] They exhibited inhibitory activities against HIV-1 replication in acutely infected H9 lymphocyte cells with EC_{50} values of 1.4 μM and 6.5 μM, respectively, and TI values of 9.3 and 14, respectively. Subsequent derivatization of betulinic acid (**1**) yielded dihydrobetulinic acid (**9**), which showed slightly more potent anti-HIV activity with an EC_{50} value of 0.9 μM and TI of 14. Based on these results, modifications of betulinic and dihydrobetulinic acid have been made. This paper deals with the synthesis of these new derivatives and evaluation of their anti-HIV activities.

Chemistry

The modifications described in this paper were focused on the introduction of an acyl group at the C-3 hydroxy

‡For part XXVI, see ref 1.
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Key words: betulinic acid derivatives, dihydrobetulinic acid derivatives, anti-HIV activity.

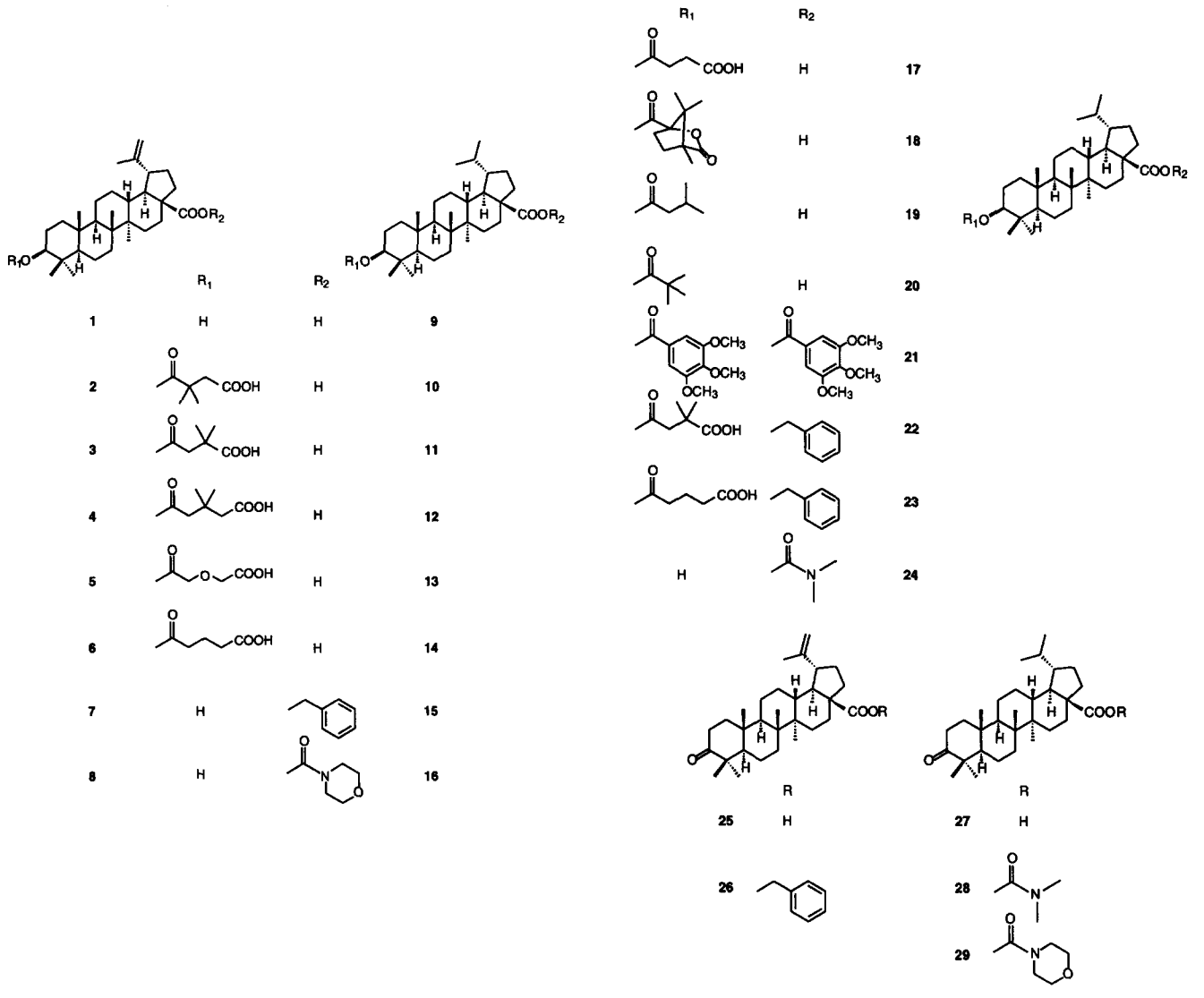


Figure 1. Structures of betulinic acid and dihydrobetulinic acid derivatives.

group or oxidation of the C-3 hydroxy group of betulinic acid and dihydrobetulinic acid. 3-*O*-acyl derivatives were obtained by treatment of triterpenes with an acid anhydride and 4-dimethylaminopyridine in pyridine or with an acid chloride in pyridine. It should be noted that treatment of triterpenes with each acid anhydride requires reagents in at least 2.5–10 times molar excess for completion of the reaction.

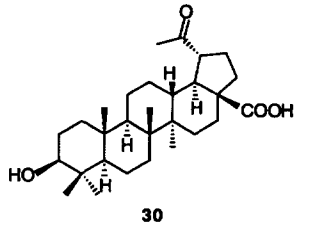
Betulinic acid (**1**) and dihydrobetulinic acid (**9**) were treated with 3,3-dimethylglutaric, diglycolic, glutaric or succinic anhydrides in pyridine in the presence of 4-dimethylaminopyridine to furnish the corresponding 3-*O*-acyl derivatives (**4–6, 12–14** and cf. 3-*O*-succinyl betulinic acid, see ref 16). In contrast, similar treatment of **1** and **9** with 2,2-dimethylsuccinic anhydride afforded a mixture of 3-*O*-(2′,2′-dimethylsuccinyl)- and 3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid (**2** and **3**) and -dihydrobetulinic acid (**10** and **11**), respectively, although **3** and **11** were the major products in each case. The proportions for the isomers were shown by HPLC to be ca. 5:95 and ca. 1:9 for the mixture of **2** and **3**, and **10** and **11**, respectively. These different yields were considered to be caused by the electron-donating effect of dimethyl groups in 2,2-dimethylsuccinic anhydride, and thus, the 3′,3′-dimethylsuccinyl derivative was expected to be the major product. The mixture was successfully separated by preparative scale HPLC yielding the corresponding pure derivatives.

The orientation of the dimethylsuccinyl group attached to the C-3 hydroxy group of **11** was established by long-range ^{1}H–^{13}C COSY examination. Thus, the ^{13}C NMR spectrum of **11** exhibited three carbonyl carbon signals at δ 171.6, 179.0, and 179.3. The latter two were ascribable to carboxylic acid resonances, while the signal at δ 171.6 was due to the ester carbonyl carbon resonance, based on their chemical shifts. The observation of ^{1}H–^{13}C long-range correlations between the signals at δ 2.89 and 2.97 (each 1H, d, J = 15.5 Hz),

ascribable to the methylene group of the dimethylsuccinyl group, and the resonances at δ 171.6 and 179.3 established the assignments of these carbonyl carbon resonances to be C-1′ and C-4′ of the dimethylsuccinate moiety, respectively. Furthermore, the resonance at δ 179.3 also displayed ^{1}H–^{13}C long-range correlation with the dimethylsuccinate methyl signal at δ 1.55 (6H, s) through a three-bond coupling. This observation indicated that the dimethyl group in **11** was at C-3′ as we expected, and the structure of **11** was determined to be 3-*O*-(3′,3′-dimethylsuccinyl)-dihydrobetulinic acid.

The ^{13}C NMR spectrum of **10,** the isomer of **11,** was quite similar to that of **11,** except for the chemical shifts for the carbonyl carbon resonances (δ 174.1, 176.7, and 177.8). The assignments for these signals were established by ^{1}H–^{13}C long-range COSY examination to be C-1′, C-4′, and C-28, respectively, and the structure of **10** was concluded to be 3-*O*-(2′,2′-dimethylsuccinyl)-dihydrobetulinic acid based on the observation of the similar long-range correlations. Although the ^{13}C NMR spectra of **10** and **11** exhibited different signal patterns in the carbonyl carbon region, comparison of the methylene proton signals due to the dimethylsuccinyl moiety in the ^{1}H NMR spectra made it easy to distinguish these two isomers. Thus, the ^{1}H NMR spectrum of **11** exhibited the methylene signals as AB-type doublets [δ 2.89 and 2.97 (each 1H, d, J = 15.5 Hz)], while that of **10** appeared as a singlet [δ 2.95 (2H, s)].

The ^{1}H NMR spectra of **2** displayed a two-proton singlet signal at δ 2.94 ascribable to dimethylsuccinyl methylene protons, whereas **3** showed methylene signals at δ 2.89 and 2.97 (each 1H, d, J = 15.5 Hz) as AB-type doublets. Since these signal patterns resembled those of **10** and **11,** respectively, and since **3** was the major product, the structures of **2** and **3** were concluded to be 3-*O*-(2′,2′-dimethylsuccinyl)-betulinic acid (**2**) and 3-*O*-(3′,3′-dimethsuccinyl)-betulinic acid (**3**), respectively.



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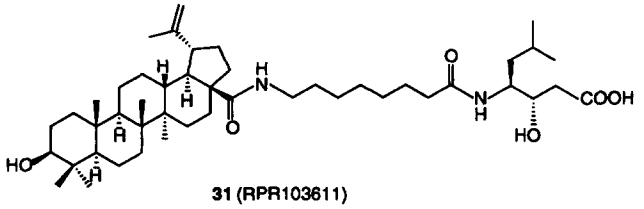

31 (RPR103611)

Figure 2. Structures of plantanic acid (**30**) and RPR103611 (**31**).

Treatment of betulinic and dihydrobetulinic acids with benzyl bromide and K_2CO_3 in dry acetone gave 28-*O*-benzyl esters (**7** and **15**). Further treatment of **7** with 2,2-dimethylsuccinic anhydride in pyridine in the presence of 4-dimethylaminopyridine was expected to afford two isomers as for **10** and **11,** but furnished only one product (**22**). The ^{1}H NMR examination of **22** revealed that **22** contained a 3′,3′-dimethylsuccinyl ester group. Compound **7** was also treated with glutaric anhydride in pyridine in the presence of 4-dimethylaminopyridine to yield **23.**

Similar treatment of **9** with 1-*S*-(−)-camphanic chloride in dry pyridine yielded 3-*O*-(1′-*S*)-(−)-camphanoyl-dihydrobetulinic acid (**18**). Moreover, dihydrobetulinic acid was treated with isovaleryl and *tert*-butyl chlorides in pyridine to furnish 3-*O*-acyl derivatives (**19** and **20,** respectively). Dihydrobetulinic acid was also treated with 3,4,5-trimethoxybenzoyl chloride in pyridine to afford a product (**21**). The observation of two two-proton aromatic singlets at δ 7.54 and 7.64 in the ^{1}H NMR spectrum as well as the presence of three carbonyl carbon resonances (δ 163.2, 166.0, and 172.3) in the ^{13}C NMR spectrum suggested that **21** contained two 3,4,5-trimethoxybenzoyl moieties in the molecule. An upfield shift of the C-28 signal as compared with that (δ 178.9) of dihydrobetulinic acid suggested that a 3,4,5-trimethoxybenzoate was introduced, in addition to the C-3 hydroxyl group, at the 28-carboxylic acid group to form the anhydride.

Treatment of betulinic and dihydrobetulinic acids with 4-morpholinocarbonyl chlorides yielded **8** and **16,** respectively, which were expected to be 3-*O*-carbamoyl derivatives. The ^{1}H NMR spectra of **8** and **16** exhibited H-3 signals at δ 3.48 and 3.45, respectively, whose chemical shifts suggested the absence of the carbamoyl group at C-3. Furthermore, the observation of the carbon resonance due to C-28 at δ 172.5 in each case indicated that the carbamoyl group was introduced at the C-28 carboxyl group. Similar treatment of **9** with dimethylcarbamyl chloride also furnished a 28-carbamoyl derivative (**24**). Introduction of a carbamoyl group at C-3 was not successful even under drastic conditions.

On the other hand, betulinic acid (**1**) and its benzyl derivative (**7**) were oxidized by pyridium chlorochromate (PCC) in CH_2Cl_2, giving the corresponding

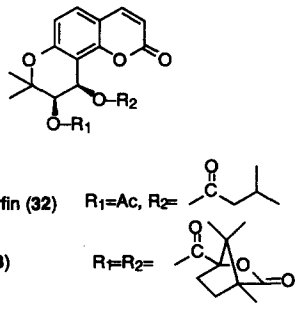

Figure 3. Suksdorfin (**32**) and DCK (**33**).

3-deoxy-3-oxo-derivatives (**25** and **26**, respectively). Hydrogenation of **26** with H_2/Pd–C in EtOAc afforded 3-deoxy-3-oxo-dihydrobetulinic acid (**27**). Compound **27** was further treated with dimethylcarbamyl or 4-morpholinocarbonyl chlorides to yield the 3-deoxy-3-oxo-28-carbamoyl derivatives (**28** and **29**, respectively).

Results and Discussion

Our preliminary investigation showed that betulinic acid and dihydrobetulinic acid were potential lead compounds for new anti-HIV agents. This finding prompted our further modification of betulinic acid and dihydrobetulinic acid.[17] In parallel anti-HIV studies, we recently reported 3′,4′-di-*O*-(−)-camphanoyl-(+)-*cis*-khellactone (DCK) (**33**) as an extremely potent anti-HIV agent ($EC_{50} = 4 \times 10^{-4}$ μM, TI = 136,719).[18] This compound was prepared by modifying the ester groups of the model compound, suksdorfin (**32**), which was identified as an anti-HIV principle from the fruits of *Lomatium suksudorfii*. In a similar fashion, our first approach in this study was to introduce an ester group at the C-3 hydroxy group of betulinic acid or dihydrobetulinic acid. These acyl groups included 1-*S*-(−)-camphanoyl, isovaleryl, trimethylacetyl, 3,3- and 2,2-dimethylsuccinyl, 3,3-dimethylglutaryl, and dimethylcarbamoyl groups.

The anti-HIV activities of betulinic and dihydrobetulinic acid derivatives are shown in Table 1. Among them, 3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid (**3**) and -dihydrobetulinic acid (**11**) both demonstrated extremely potent anti-HIV activity in acutely infected H9 lymphocytes with EC_{50} values of $<3.5 \times 10^{-4}$ μM. They also exhibited remarkable TI values of 20,000 and 14,000, respectively. In contrast, compounds **2** and **10**, the 2′,2′-dimethyl isomers of the corresponding compounds **3** and **11**, showed significantly lower anti-HIV activities with EC_{50} values of 2.7 and 0.56 μM, respectively, and TI values of 6.7 and 13.8, respectively. Compounds **4–6**, **12**, and **13** also exhibited potent anti-HIV activities with EC_{50} values ranging from 0.04 to 2.3 $\times 10^{-3}$ μM, and TI values from 292 to 2344. Compounds **19**, **22**, and **23** inhibited HIV replication with EC_{50} values of 1.5, 0.23, and 0.5 μM, respectively, and TI values of 56, 56, and 19, respectively, but were not as potent as the foregoing compounds.

The two compound pairs (**3**:**11** and **4**:**12**) exhibiting the most potent anti-HIV activity both contain an isovaleryl domain incorporated in 3,3-dimethyl-succinyl or -glutaryl moieties. However, compounds **2** and **10**, which contain an isobutyryl domain instead of an isovaleryl domain, were much less active. On the other hand, while the less potent compound **19** and the inactive compounds **18** and **20** do have an isovaleryl domain in the molecule, but they do not include a terminal carboxylic acid group. Thus, two fragment structures might be required for selective anti-HIV activity. In addition to the 3,3-dimethyl-succinyl and -glutaryl esters, quite active compounds were found with two other similar aryl groups containing terminal acid functionalities: glutaryl (**6**) and diglycoyl (**5**, **13**). Compound **14**, a dihydrobetulinic acid, was less active than its betulinic acid counterpart, compound **6**; however, reasonable activity correlation was found between the two series with the pairs **3**:**11**, **4**:**12**, and **5**:**13**.

3-Deoxy-3-oxo-derivatives of betulinic and dihydrobetulinic acid (**25–29**) displayed relatively strong cytotoxicity, resulting in small TI values. Compounds **23** and **22**, which are the 28-*O*-benzyl ester derivatives of **6** and **11**, respectively, exhibited decreased potency against HIV. Introduction of a 4-morpholinocarbonyl or dimethylcarbamoyl into the C-28 carboxylic acid group of **1**, **9**, or **27** as seen in compounds **8**, **16**, **24**, **28**, and **29** yielded toxic or inactive compounds.

Table 1. Anti-HIV activities of betulinic and dihydrobetulinic acid derivatives in acutely infected H9 lymphocytes

Compound	TC_{50} (μM)[a]	EC_{50} (μM)[b]	TI[c]
1	13.0	1.4	9.3[e]
2	15.9	2.7	6.7[e]
3	7	$<3.5 \times 10^{-4}$	20,000
4	4.5	2.3×10^{-3}	1974
5	11.7	0.01	1172
6	12.8	4.4×10^{-2}	292
7	—[d]	—[d]	—[d]
8	11.4	3.2	3.6[e]
9	12.6	0.9	14[h]
10	7.7	0.56	13.8[e]
11	4.9	$<3.5 \times 10^{-4}$	14,000
12	5.8	5.7×10^{-3}	1017
13	13.1	5.6×10^{-3}	2344
14	7.9	0.9	9[e]
15	>180[f]	100	>1.8[e]
16	35	26	1.3[e]
17	13.4	1.8	7.5[e]
18	1	0.5	2[e]
19	83	1.5	56[h]
20	83	—[g]	—
21	53	—[g]	—
22	13.0	0.23	56[h]
23	9.3	0.5	19
24	6.6	6.6	1[e]
25	1.8	0.22	8[e]
26	—[d]	—[d]	—[d]
27	3.2×10^{-2}	2×10^{-3}	16
28	13.3	3.8	3.5[e]
29	—[d]	—[d]	—[d]
31	2.7	0.33	35
AZT	500	0.02	25,000

[a]Concentration of agent that is cytotoxic to 50% of the H9 cells. In previous manuscripts, it was referred to as IC_{50}.
[b]Concentration of agent that inhibits viral replication in H9 cells by 50%.
[c]In vitro TI, ratio of TC_{50}:EC_{50}. Represents data from at least two separate assays.
[d]The agent did not dissolve in DMSO; therefore it was not tested.
[e]TI is <10; therefore, it is considered not to be suppressive.
[f]Could not test the agent at a higher concentration due to the inhibitory effects of DMSO at concentrations above 1%.
[g]The agent did not inhibit HIV replication.

The inhibitory activities of **11** and **12** were also evaluated against PHA-stimulated peripheral blood mononuclear cells (PBMCs)-infected with HIV-1_{IIIB}. These freshly isolated cells were chosen because of their clinical relevance. The same virus stock (IIIB) and multiplicity of infection (moi) were used for these experiments, so that a comparison could be made to HIV-1 infected H9 cells. Results with the HIV-1 infected PHA-stimulated PBMCs indicate that **11** and **12** displayed potent inhibitory activity. In fact, **11**s therapeutic index value (2286) was tenfold greater than that mediated by **12** and comparable to that obtained when AZT was tested in the same system (2000). As shown in Table 2, **11**'s EC_{50} value was approximately 12-fold lower than that mediated by AZT, but AZT's TC_{50} value was approximately 11-fold greater than **11**, thus explaining the similarity in TI values for both agents. Outside independent confirmatory experiments have also been completed using low passage clinical isolates along with fresh cells in the presence of **11**. Significant suppressive activity was mediated by **11** with two different clinical isolates (data not shown), further supporting the anti-viral activity of this family of agents.

In this paper, we have shown the inhibitory effect of betulinic acid and its analogues on an acute HIV-1_{IIIB} infection. We also evaluated two of these agents (**11** and **12**) with cells that are chronically HIV-1_{LAV}-infected. The cell lines we used were ACH-2 (a chronically infected T cell) and Ul (a chronically infected monocytic cell). These two cell lines have the following advantages: (1) They are very sensitive to substances that can induce virus replication, so they are useful for evaluating the in vitro response that agents may have on virus expression. (2) They constitutively produce a low level of virus replication that is increased in the presence of the phorbol ester, PMA. Since agents such as interferon-alpha have been shown to inhibit PMA-induced virus expression,[18] these chronically infected cells are useful for evaluating whether an agent induces virus replication or is capable of inhibiting a stimulatory signal such as PMA. In general, data obtained by culturing chronically HIV-infected cell lines with a test drug can give a sense of how these agents may function in vivo when given to individuals that are chronically/latently HIV-infected. There was no increase in virus expression from either cell line when either drug was added to the cells alone. Even when both chronically HIV-1 infected cell lines were cultured in the presence of a known virus inducer such as the phorbol ester, PMA (phorbol 12-myristate 13-acetate), there was no alteration in the level of virus expression (Tables 4 and 5). Thus, **11** and **12** did not increase or decrease virus expression from the chronically HIV-infected cells either when cultured alone or in the presence of PMA, respectively.

As a mechanism(s) of action study, the inhibitory activity of compounds **1–5**, **9**, and **11–13** against HIV-1 RT were investigated (Table 3). These compounds did not inhibit HIV-RT activity at a concentration of 100 μg/mL in assays using polyA as a template. On the other hand, ddCTP, a known HIV-RT inhibitor, inhibited RT activity by 50% at 8 μg/mL in the same experiment.

Since other betulinic acid derivatives, such as RPR103611 (**31**), were recently reported as anti-HIV agents and demonstrated to inhibit HIV-induced membrane fusion,[19] compounds **1–5**, **9**, and **11–13** were also evaluated for inhibitory activity against HIV-induced membrane fusion. Compounds **2–5** and **11–13** inhibited syncytia formation in a concentration range of 20–40 μg/mL (Table 3), suggesting that an inhibitory effect against HIV-induced membrane fusion could be involved in their mechanism(s) of action. However, although compounds **2** and **3** inhibited syncytia formation at the same concentration, the anti-HIV activity of **3** is 7700 times greater than that of **2**. Moreover, RPR103611 (**31**), while less potent against HIV-1 infection, is at least 100-fold more potent than compound **3** in inhibiting HIV-1_{IIIB} induced syncytia formation (data not shown). This result supports the notion that the potent anti-HIV-1 activity of the triterpene derivatives described here is due to a novel mechanism distinct from the other class of triterpene derivatives which includes RPR103611.

Table 2. Anti-HIV activities of compounds **11** and **12** in acutely infected PHA-stimulated peripheral blood mononuclear cells (PBMCs)

Compound	TC_{50} (μM)[a]	EC_{50} (μM)[b]	TI[c]
11[d]	6.8	0.00298	2286
12	6.6	0.0291	228.6
AZT	74.8	0.037	2000

[a]Concentration which was toxic to 50% of the PHA-stimulated PBMCs. In previous manuscripts, it was referred to as IC50.
[b]Concentration which inhibited 50% of the virus replication from the acutely HIV-1 infected PHA-stimulated PBMCs.
[c]In vitro therapeutic index (TI), ratio of TC_{50}:EC_{50}.
[d]The suppressive anti-HIV activity has been confirmed by outside testing with fresh clinical isolates and freshly activated (PHA-stimulated) PBMCs.

Table 3. HIV-RT and fusion assay for betulinic and dihydrobetulinic acid derivatives

Compound	HIV-RT assay IC_{50} (μM)[a]	Fusion assay IC_{100} (μM)[b]
1	>219	>219
2	>171	34
3	>171	34
4	>167	50
5	>175	70
9	>218	>218
10	NT[c]	NT[c]
11	>171	34
12	>174	70
31	No inhibition[d]	3.97[d]

[a]Concentration required to inhibit 50% of HIV-1 RT activity.
[b]Concentration required to completely inhibit HIV-1 induced syncytia.
[c]NT = not tested.
[d]Data taken from ref. 20.

Experimental

General experiment procedures

Melting points were measured with a Fisher–Johns melting point apparatus and are uncorrected. Optical rotations were determined with a Rudolph Research Autopol III polarimeter. ^1H and ^{13}C NMR spectra were obtained using a Bruker AC-300 instrument with Me_4Si (TMS) as an internal standard, and chemical shifts are given in δ (ppm). Elemental analyses were performed by Atlantic MicroLab, Inc., Norcross, GA. HR-FABMS, positive and negative FABMS were recorded on a HX- 110 JEOL spectrometer. Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60 F_{254} plates (0.20 mm, Merck), and spots were detected by UV illumination and spraying with 10% aqueous H_2SO_4 solution. Silica gel (230–400 mesh) from Aldrich, Inc. was used for column chromatography. Betulinic acid (**1**) was purchased from Aldrich, Inc., and dihydrobetulinic acid (**9**) was prepared from betulinic acid as described in ref 16.

Preparation of 28-*O*-benzyl-betulinic acid (7) and -dihydrobetulinic acid (15). A solution of betulinic acid (**1**) or dihydrobetulinic acid (**9**) (each ca. 200 mg), benzyl bromide (1 mL), and potassium carbonate (400 mg) in anhydrous acetone (20 mL) was refluxed for 2 h. The inorganic salt was filtered off and the filtrate was evaporated in vacuo. The residue was chromatographed using a silica gel column to afford the product.

28-*O*-benzyl-betulinic acid (7). Starting with 207.5 mg of **1** crystallization from MeOH–H_2O gave colorless needles; yield 93.6%; mp 184–185 °C; $[\alpha]^{20}_D$ +13.2 ° (*c* 0.87; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.85, 0.92, 1.01, 1.04, 1.23, 1.75 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3, 20-CH_3), 3.46 (lH, br s, 3-H), 4.76, 4.91 (each 1H, br s, 30-H), 5.30, 5.36 (each 1H, d, J = 12.4 Hz, benzyl H_2-1′), 7.34–7.56 (5H, m, benzyl-H_5), anal. calcd for $C_{37}H_{50}O_3$: C, 81.27; H, 9.95. Found: C, 81.36; H, 10.00%.

28-*O*-benzyl-dihydrobetulinic acid (15). Starting with 233.2 mg of **9** crystallization from MeOH gave colorless needles; yield 89.6%; mp 203 °C; $[\alpha]^{20}_D$ 28.1 ° (*c* 0.54; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.81, 0.85 (each 3H, d, J = 6.7 Hz, 20-$(CH_3)_2$), 0.87, 0.91, 0.98, 1.05, 1.24 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 3.47 (1H, br t, J = 8.0Hz, 3-H), 5.30, 5.35 (each 1H, d, J = 12.4 Hz, benzyl H_2-1′), 7.33–7.56 (5H, m, benzyl-H_5), anal. calcd for $C_{37}H_{56}O_3$: C, 80.97; H, 10.28. Found: C, 80.90; H, 10.32%.

Table 4. Effects of compounds **11** and **12** on chronically infected U1 cells

Sample identification*	p24 pg/mL +Media	+PMA
U1 cells + **11**		
20	0	44
4	0	2600
0.8	0	3512
0.16	0	4160
0.032	0	3776
0.0064	0	3744
0.00128	0	3712
0.000256	0	6586
U1 cells + **12**		
20	0	133
4	0	2362
0.8	0	1512
0.16	0	3480
0.032	0	2506
0.0064	0	3307
0.00128	12	5469
0.000256	0	2622
U1 cells + AZT		
100	2	1976
10	0	2328
1	4	4139
0.1	0	2606
U1 cells + media	0	4205

*Compound concentrations in μg/mL. The results presented in this table are from a single experiment that has been confirmed in a separate experiment.

Table 5. Effects of compounds **11** and **12** on chronically infected ACH-2 cells

Sample identification*	p24 pg/mL +Media	+PMA
ACH-2 cells + **11**		
20	498	21,606
4	189	26,752
0.8	167	26,202
0.16	148	26,637
0.032	146	27,558
0.0064	146	26,470
0.00128	161	28,634
0.000256	160	35,686
ACH-2 cells + **12**		
20	396	39,987
4	189	30,669
0.8	164	25,549
0.16	131	26,714
0.032	135	27,533
0.0064	125	29,158
0.00128	120	26,176
0.000256	126	21,773
ACH-2 cells + AZT		
100	272	19,302
10	158	24,294
1	94	26,586
0.1	217	23,245
ACH-2 cells + media	134	24,243

*Compound concentrations in μg/mL. The results presented in this table are from a single experiment that has been confirmed in a separate experiment.

Procedure for 3-*O*-acyl derivatives of betulinic acid and dihydrobetulinic acid (2–6, 10–14, 17, 22, 23) with acid anhydride. A solution of betulinic acid (**1**), dihydrobetulinic acid (**9**), 28-*O*-benzylbetulinic acid (**7**), or 28-*O*-benzyldihydrobetulinic acid (**15**) with dimethylaminopyridine (1 equiv mol) and an appropriate anhydride (2.5–10 equiv mol) in anhydrous pyridine (5–10 mL) was refluxed overnight. The reaction mixture was diluted with ice–water and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed using silica gel column or semi-preparative-scale HPLC to afford the product.

3-*O*-(2′,2′-dimethylsuccinyl)-betulinic acid (2). Starting with 542 mg of **1** crystallization from MeOH gave colorless needles; yield 3.1%; mp 279–280 °C; $[\alpha]^{19}_D$ +36.2° (*c* 0.35; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.75, 0.93, 1.03 (× 2), 1.06 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.49 (6H, s, 2′-CH_3 × 2), 1.80 (3H, s, 20-CH_3), 2.94 (2H, s, H_2-3′), 3.55 (1H, m, H-19), 4.77 (1H, dd, *J* = 5.0, 11.5 Hz, H-3), 4.79, 4.95 (each 1H, br s, H-30). Positive FABMS *m/z* 585 $(M+H)^+$; negative FABMS *m/z* 583 $(M-H)^-$; HR-FABMS calcd for $C_{36}H_{57}O_6$ 585.4155, found *m/z* 585.4156.

3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid (3). Starting with 542 mg of **1** crystallization from MeOH gave colorless needles; yield 70.0%; mp 274–276 °C; $[\alpha]^{19}_D$ +23.5° (*c* 0.71; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.73, 0.92, 0.97, 1.01, 1.05 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.55 (6H, s, 3′-CH_3 × 2), 1.80 (3H, s, 20-CH_3), 2.89, 2.97 (each 1H, d, *J* = 15.5 Hz, H-2′), 3.53 (1H, m, H-19), 4.76 (1H, dd, *J* = 5.0, 11.5 Hz, H-3), 4.78, 4.95 (each 1H, br s, H-30). Positive FABMS *m/z* 585 $(M+H)^+$; negative FABMS *m/z* 583 $(M-H)^-$; HR-FABMS calcd for $C_{36}H_{57}O_6$ 585.4155, found *m/z* 585.4161.

3-*O*-(3′,3′-dimethylglutaryl)-betulinic acid (4). Starting with 51.3 mg of **1** crystallization from MeOH–H_2O gave colorless needles; yield 59.5%; mp 214–215 °C; $[\alpha]^{20}_D$ +9.8° (*c* 1.2; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.77, 0.91, 0.95, 1.03, 1.07, 1.80 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3, 20-CH_3), 1.36, 1.37 (each 3H, s, 3′-CH_3 × 2), 4.73 (1H, dd, *J* = 4.5, 11.5 Hz, H-3), 4.78, 4.95 (each 1H, br s, H-30). Anal. calcd for $C_{37}H_{58}O_6 \cdot 4H_2O$: C, 66.24; H, 9.92. Found: C, 65.91; H, 9.76%.

3-*O*-diglycolyl-betulinic acid (5). Starting with 49.6 mg of **1** an amorphous powder yield 84.7%, $[\alpha]^{20}_D$ +2.1° (*c* 1.1, $CHCl_3$:MeOH [1:1]); ^1H NMR (methanol-d_4-$CDCl_3$ [1:1]) 0.85, 0.88 (× 2), 0.97, 1.00 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.69 (20-CH_3), 4.21, 4.23 (each 2H, s; H_2-2′ and 4′), 4.65–4.75 (1H, br s, H-3), 4.60, 4.73 (each 1H, br s, H-30). Anal. calcd for $C_{34}H_{52}O_7 \cdot H_2O$: C, 69.12; H, 9.21. Found: C, 69.60; H, 9.03%.

3-*O*-glutaryl-betulinic acid (6). Starting with 49.7 mg of **1** crystallization from MeOH-H_2O gave colorless needles; yield 67.5%; mp 275–277 °C (dec.); $[\alpha]^{20}_D$ +16.9° (*c* 0.49; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.76, 0.88, 0.92, 1.03, 1.08, 1.80 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3, 20-CH_3), 2.24 (2H, d, *J*= 7.0 Hz, H_2-3′), 2.62, 2.64 (each 2H, t, *J* = 7.0 Hz; H_2-2′ and 4′), 4.74 (1H, dd, *J* = 4.9, 11.3 Hz, 3-H), 4.78, 4.96 (each 1H, br s, 30-H). Anal. calcd for $C_{35}H_{54}O_6$: C, 73.65; H, 9.54. Found: C, 73.68; H, 9.61%.

3-*O*-(2′,2′-dimethylsuccinyl)-dihydrobetulinic acid (10). Starting with 155.9 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 4.8%; mp 297–298 °C; $[\alpha]^{17}_D$ −32.2° (*c* 0.21; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.85, 0.94 (each 3H, d, *J* = 6.7 Hz, 20-$(CH_3)_2$), 0.77, 0.94, 1.03 (× 2), 1.04 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.50 (6H, s; 2′-CH_3 × 2), 2.95 (2H, s, H_2-3′), 4.79 (^1H, dd, *J* = 4.5, 11.5 Hz, 3-H). Positive FABMS *m/z* 587 $(M+H)^+$; negative FABMS *m/z* 585 $(M-H^-$; HR-FABMS calcd for $C_{36}H_{59}O_6$ 587.4311, found *m/z* 587.4308.

3-*O*-(3′,3′-dimethylsuccinyl)-dihydrobetulinic acid (11). Starting with 155.9 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 24.5%; mp 291–292 °C; $[\alpha]^{20}_D$ −13.4° (*c* 1.1; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.85, 0.94 (each 3H, d, *J* = 7.0 Hz, 20-$(CH_3)_2$), 0.75, 0.93, 0.97, 1.01, 1.03 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.55 (6H, s, 3′-CH_3 × 2), 2.89, 2.97 (each 1H, d, *J* = 15.5 Hz, H-2′), 4.77 (1H, dd, *J* = 5.0, 11.0 Hz, 3-H), anal. calcd for $C_{36}H_{58}O_6 \cdot 2.5\ H_2O$: C, 68.43; H, 10.04. Found: C, 68.64; H, 9.78%.

3-*O*-(3′,3′-dimethylgiutaryl)-dihydrobetulinic acid (12). Starting with 100.5 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 93.3%; mp 287–289 °C; $[\alpha]^{20}_D$ −17.9° (*c* 0.5; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.86, 0.93 (each 3H, d, *J* = 6.5 Hz, 20-$(CH_3)_2$), 0.78, 0.92, 0.96, 1.02, 1.05 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, l0-CH_3, 14-CH_3), 1.38, 1.39 (each 3H, s, 3′-CH_3 × 2), 2.78 (4H, m; H_2-2′ and 4′), 4.76 (1H, dd, *J* = 4.5, 11.5 Hz, 3-H). Anal. calcd for $C_{37}H_{60}O_6$: C, 73.96; H, 10.06. Found: C, 73.83; H, 10.10%.

3-*O*-diglycolyl-dihydrobetulinic acid (13). Starting with 103.5 mg of **9** an off-white amorphous powder; yield 79.2%; $[\alpha]^{20}_D$ −9.8° (*c* 1.1; $CHCl_3$:MeOH [1:1]); ^1H NMR (methanol-d_4) 0.79, 0.87 (each 3H, d, *J* = 6.5 Hz, 20-$(CH_3)_2$), 0.87, 0.88, 0.91, 0.98, 1.01 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 4.21, 4.23 (each 2H, s; H_2-2′ and 4′), 4.57 (1H, dd, *J* = 6.5, 10.0 Hz, 3-H). Anal. calcd for $C_{34}H_{54}O_7 \cdot 2H_2O$: C, 66.85; H, 9.57. Found: C, 67.21; H, 9.33%.

3-*O*-glutaryl-dihydrobetulinic acid (14). Starting with 49.6 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 96.5%; mp 291–294 °C; $[\alpha]^{20}_D$

15.4° (*c* 0.8 1; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.86, 0.94 (each 3H, d, *J* = 6.8 Hz; 20-$(CH_3)_2$), 0.78, 0.89, 0.92, 1.03, 1.06 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 2.24 (2H, d, *J* = 7.2Hz, H_2-3′), 2.62, 2.66 (each 2H, t, *J* = 7.2 Hz; H_2-2′ and 4′), 4.75 (1H, dd, *J* = 4.9, 11.1 Hz, 3-H). Anal. calcd for $C_{35}H_{56}O_6 \cdot H_2O$: C, 73.13; H, 10.17. Found: C, 73.46; H, 9.83%.

3-*O*-succinyl-dihydrobetulinic acid (17). Starting with 100.0 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 57.5%; mp 296–299 °C (dec.); $[\alpha]^{20}_D$ −30.7° (*c* 0.32; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.85, 0.94 (each 3H, d, *J* = 6.8 Hz, (20-$(CH_3)_2$), 0.77, 0.91, 0.97, 1.01, 1.04 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 2.93 (4H, m; H_2-2′ and 3′), 4.79 (1H, dd, *J* = 4.9, 11.2 Hz, 3-H), 7.54, 7.64 (each 2H, s, 2′,6′,2″,6″-H). Anal. calcd for $C_{34}H_{54}O_6$: C, 73.08; H, 9.74. Found: C, 72.82; H, 9.66%.

28-*O*-benzyl-3-*O*-(3′,3′-dimethylsuccinyl)-dihydrobetulinic acid (22). Starting with 120.0 mg of **15** crystallization from MeOH gave colorless needles; yield 49.8%; mp 217 °C; $[\alpha]^{20}_D$ −10.2° (*c* 0.79, $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.79, 0.89 (each 3H, d, *J* = 6.8 Hz, 20-$(CH_3)_2$), 0.77, 0.85, 0.95, 0.96, 0.97 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.55 (6H, 5, 3′-CH_3 × 2), 2.89, 2.97 (each 1H, d, *J* = 15.6 Hz, H-2′), 4.77 (1H, dd, *J* = 4.8, 11.4Hz, 3-H), 5.29, 5.34 (each 1H, d, *J* = 12.4 Hz, benzyl H_2-1″), 7.33–7.56 (5H, m, benzyl-H_5). Anal. calcd for $C_{43}H_{64}O_6$: C, 76.29; H, 9.53. Found: C, 76.23; H, 9.46%.

28-*O*-benzyl-3-*O*-glutaryl-dihydrobetulinic acid (23). Starting with 102.0 mg of **15** crystallization from $CHCl_3$:MeOH–H_2O gave colorless needles; yield 54.6%; mp 160–161 °C; $[\alpha]^{20}_D$ −3.8° (*c* 0.88; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.81, 0.90 (each 3H, d, *J* = 6.7 Hz, 20-$(CH_3)_2$), 0.79, 0.87, 0.90, 0.92, 0.99 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 2.24 (2H, d, *J* = 7.3 Hz, H_2-3′), 2.63, 2.67 (each 2H, t, *J* = 7.3 Hz; H_2-2′ and 4′), 4.74 (1H, dd, *J* = 5.1, 11.1 Hz, 3-H), 5.30, 5.35 (each 1H, d, *J* = 12.4 Hz, benzyl H_2-1″), 7.36–7.57 (5H, m, benzyl-H_5). Anal. calcd for $C_{41}H_{62}O_6$: C, 75.65; H, 9.60. Found: C, 76.10; H, 9.68%.

Procedure for preparing dihydrobetulinic acid 3-*O*-esters with acid chloride (18–21). To a solution of dihydrobetulinic acid (**9**) (54.7–151.3 mg) in pyridine (5–10 mL) was added dropwise an appropriate acid chloride (2.5–5 equiv mol) at room temperature. The reaction mixture was diluted with ice–water and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed using a silica gel column to afford the product.

3-*O*-(1′-*S*)-(−)-camphanoyl-dihydrobetulinic acid (18). Starting with 101.5 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 61.2%; mp 276–277 °C; $[\alpha]^{20}_D$ −15.9° (*c* 0.93; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.87, 0.95 (each 3H, d, *J* = 6.7 Hz, 20-$(CH_3)_2$), 0.79, 0.91, 0.95, 1.04, 1.06 (× 2) (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3, 10′-CH_3), 1.08, 1.10 (each 3H, s; 8′-CH_3, 9′-CH_3). Anal. calcd for $C_{39}H_{60}O_6$: C, 74.96; H, 9.68. Found: C, 74.74; H, 9.73%.

3-*O*-isovaleryl-dihydrobetulinic acid (19). Starting with 57.5 mg of **9** crystallization from $CHCl_3$–MeOH–H_2O gave colorless needles; yield 48.8%; mp 261–262 °C (dec.); $[\alpha]^{20}_D$ −34.4° (*c* 0.29; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.86,0.95 (each 3H, d, *J* = 6.7 Hz, 20-$(CH_3)_2$), 0.80, 0.91, 0.98, 1.04, 1.06 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 0.95 (6H, br d, *J* = 3.5 Hz, Isovaleryl-$(CH_3)_2$), 4.74 (1H, dd, *J* = 4.9, 11.2 Hz, 3-H). Anal. calcd for $C_{35}H_{58}O_4$: C, 77.44; H, 10.77. Found: C, 77.32; H, 10.84%.

3-*O*-*tert*-butyl-dihydrobetulinic acid (20). Starting with 54.7 mg of **9** crystallization from $CHCl_3$–MeOH–H_2O gave colorless needles; yield 29.0%; mp 279–281 °C (dec.); $[\alpha]^{20}_D$ −45.8 ° (*c* 0.19; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.86,0.95 (each 3H, d, *J* = 6.7 Hz, 20-$(CH_3)_2$), 0.80, 0.92, 0.95, 1.03, 1.06 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, l0-CH_3, 14-CH_3), 1.10 (9H, s, *tert*-butyl-$(CH_3)_3$), 4.72 (1H, dd, *J* = 4.9, 11.3 Hz, 3-H). Anal. calcd for $C_{35}H_{58}O_4$: C, 77.44; H, 10.77. Found: C, 77.48; H, 10.84%.

3,28-Di-*O*-(3′,4′,5′-trimethoxy)-benzoyl-dihydrobetulinic anhydride (21). Starting with 151.3 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 71.9%; mp 132–133 °C; $[\alpha]^{20}_D$ +13.80° (*c* 0.73; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.85, 0.92 (each 3H, d, *J* = 6.7 Hz, 20-$(CH_3)_2$), 0.88, 1.04, 1.11 (× 2), 1.15 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 3.83, 3.85 (each 6H, s, benzoyl 3,5″-OCH_3 × 2), 3.97, 3.98 (each 3H, s, benzoyl 4′-OCH_3), 5.02 (1H, dd, *J* = 4.7, 11.5Hz, 3-H). Anal. calcd for $C_{50}H_{70}O_{11}$: C, 70.89; H, 8.33. Found: C, 70.73; H, 8.41%.

Procedure for preparation of 3-deoxy-3-oxo-betulinic acid derivatives (25 and 26). To a solution of betulinic acid (**1**) or 28-*O*-benzyl-betulinic acid (**7**) in CH_2Cl_2 (5 mL) was added dropwise pyridinium chlorochromate (PCC, 1.2–1.5 equiv mol) at room temperature. The reaction mixture was filtered and extracted with CH_2Cl_2. The organic layer was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed using silica gel column to afford the product.

3-Deoxy-3-oxo-betulinic acid (25). Starting with 50.0 mg of **1** an off-white amorphous powder; yield 86.5% $[\alpha]^{20}_D$ +30.3° (*c* 0.2; $CHCl_3$:MeOH [1:1]); ^1H NMR (pyridine-d_5) 0.82, 1.02, 1.04 , 1.06, 1.13, 1.80 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 4.79, 4.96 (each 1H, br d, *J* = 2.1 Hz, 30-H). Anal. calcd for

$C_{30}H_{46}O_3 \cdot 0.5\ H_2O$: C, 77.70; H, 10.21. Found: C, 77.67; H, 10.11%.

28-*O*-benzyl-3-deoxy-3-oxo-betulinic acid (26). Starting with 304.1 mg of **7** an off-white amorphous powder; yield 95.6%; $[\alpha]^{20}_D$ +47.7° (*c* 0.93; $CHCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.83, 0.90, 0.98, 1.03, 1.13, 1.75 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3, 20-CH_3), 4.76, 4.77 (each 1H, d, J = 1.4 Hz, 30-H), 5.29, 5.35 (each 1H, d, J = 7.0 Hz, benzyl H_2-1′), 7.34–7.56 (5H, m, benzyl-H_5). Anal. calcd for $C_{37}H_{52}O_3 \cdot 0.66H_2O$: C, 79.81; H, 9.65. Found: C, 79.65; H, 9.51%.

3-Deoxy-3-oxo-dihydrobetulinic acid (27). A mixture of 28-*O*-benzyl-3-deoxy-oxo-betulinic acid (**26**) (223.0 mg), 5% Pd-C (200 mg) with H_2 in EtOAc (100 mL) was stirred overnight at room temperature. After filtration of the reaction mixture, the filtrate was evaporated under reduced pressure. The residue was chromatographed using a silica gel column to afford **28**: yield 96.7%; crystallization from MeOH–H_2O gave colorless needles; mp 265–267 °C; $[\alpha]^{20}_D$ +9.3° (*c* 0.8; $CHCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.86, 0.95 (each 3H, d, J = 6.7 Hz, 20-$(CH_3)_2$), 0.84, 1.02, 1.04, 1.14, 1.37 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3). Anal. calcd for $C_{30}H_{48}O_3$: C, 78.89; H, 10.59. Found: C, 78.48; H, 10.78%.

Procedure for synthesizing betulinic, dihydrobetulinic and 3-deoxy-3-oxo-dihydrobetulinic acid 28-anhydrides (8, 16, 24, 28, 29). To a solution of betulinic acid (**1**), dihydrobetulinic acid (**9**) or 3-deoxy-3-oxo-dihydrobetulinic acid (**27**) in CH_2Cl_2 (3 mL) and pyridine (3 mL) was added dropwise an appropriate carbamyl or carbamoyl chloride (0.5 mL) at room temperature. The reaction mixture was diluted with ice–water and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed using a silica gel column to afford the product.

Betulinic acid 28-*O*-(4′-morpholine)-carbonic anhydride (8). Starting with 150.0 mg of **1** crystallization from hexane–EtOAc gave colorless needles; yield 86.8%; mp 213–215 °C; $[\alpha]^{20}_D$ −12.0° (*c* 0.92; $CHCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.84, 1.03 (× 2), 1.09, 1.24, 1.76 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3, 20-CH_3), 3.48 (lH, dd, J = 8.4, 16.1 Hz, 3-H), 3.53–3.73 (8H, m; 4′-morpholine H_2-2′, 3′ × 2), 4.77, 4.90 (each 1H, d, J = 2.0 Hz; 30-H). Anal. calcd for $C_{35}H_{55}O_5N$: C, 73.77; H, 9.73; N, 2.46. Found: C, 73.80; H, 9.72; N, 2.51%.

Dihydrobetulinic acid 28-*O*-(4′-morpholine)-carbonic anhydride (16). Starting with 54.8 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 79.6%; mp 211–212 °C; $[\alpha]^{20}_D$ −37° (*c* 0.87; $CHCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.81, 0.90 (each 3H, d, J = 6.7 Hz, 20-$(CH_3)_2$), 0.87, 1.01, 1.05, 1.10, 1.25 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 3.45 (1H, t, J = 8.2 Hz, 3-H), 3.52–3.72 (8H, m, 4′-morpholine H_2-2′, 3′ × 2). Anal. calcd for $C_{35}H_{57}O_5N$: C, 73.51; H, 10.05; N, 2.45. Found: C, 73.38; H, 10.08; N, 2.38%.

Dihydrobetulinic acid 28-*O*-dimethylcarbamic anhydride (24). Starting with 55.2 mg of **9** crystallization from MeOH–H_2O gave colorless needles; yield 76.4%; mp 221–222 °C; $[\alpha]^{20}_D$ −35.6° (*c* 0.82; $CHCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.80, 0.90 (each 3H, d, J = 6.7 Hz, 20-$(CH_3)_2$), 0.86, 1.01, 1.04, 1.10, 1.24 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, l0-CH_3, 14-CH_3), 2.86, 2.89 (each 3H, s, carbamyl-CH_3 × 2), 3.47 (1H, t, J = 7.9 Hz, 3-H). Anal. calcd for $C_{33}H_{55}O_4N$: C, 74.81; H, 10.46; N, 2.64. Found: C, 74.77; H, 10.52; N, 2.84%.

3-Deoxy-3-oxo-dihydrobetulinic acid 28-dimethylcarbamic anhydride (28). Starting with 48.9 mg of **27** crystallization from MeOH–H_2O gave colorless needles; yield 97.5%; mp 126–127 °C; $[\alpha]^{20}_D$ −15.4° (*c* 0.43; $CHCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.82, 0.90 (each 3H, d, J = 6.8 Hz, 20-$(CH_3)_2$), 0.85, 0.99, 1.04, 1.07, 1.14 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 2.88, 2.92 (each 3H, s, carbamyl-CH_3 × 2). Anal. calcd for $C_{33}H_{53}O_4N \cdot 0.5H_2O$: C, 73.84; H, 10.14; N, 2.61. Found: C, 73.97; H, 10.44; N, 2.60%.

3-Deoxy-3-oxo-dihydrobetulinic acid 28-*O*-(4′-morpholine)-carbonic anhydride (29). Starting with 45.2 mg of **27** crystallization from MeOH–H_2O gave colorless needles; yield 97.7% mp 200–201 °C; $[\alpha]^{20}_D$ −11.9° (*c* 0.57; $CDCl_3$:MeOH [1:1]); ^{1}H NMR (pyridine-d_5) 0.82, 0.90 (each 3H, d, J = 6.8 Hz, 20-$(CH_3)_2$), 0.86, 0,99, 1.04, 1.07, 1.15 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 3.53–3.75 (8H, m, 4′-morpholine H_2-2′, 3′ × 2). Anal. calcd for $C_{35}H_{55}O_5N$: C, 73.77; H, 9.72; N, 2.45. Found: C, 73.71; H, 9.76; N, 2.37%.

Anti-HIV assay. The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10% fetal calf serum [FCS] supplemented with L-glutamine) at 5% CO_2 and 37 °C. Aliquots of this cell line were only used in experiments when in log-phase of growth. Uninfected peripheral blood mononuclear cells (PBMCs) from healthy HIV negative donors were stimulated with PHA (1 μg/mL) for three days.

Test samples are first dissolved in dimethyl sulfoxide (DMSO). The following are the final drug concentrations routinely used for screening: 100, 20, 4, and 0.8 μg/mL. For active agents, additional dilutions are prepared for subsequent testing so that an accurate EC_{50} value (see definition below) can be achieved.

As the test samples are being prepared, an aliquot of H9 cells or PHA-stimulated PBMCs is infected with HIV-1 (IIIB isolate) while another aliquot is mock-infected with complete medium. The mock-infected is used for toxicity determinations (IC_{50}, see definition

below). The stock virus used for these studies typically has a $TCID_{50}$ value of 10^4 infectious units (IU)/mL. The appropriate amount of virus for a multiplicity of infection (moi) between 0.1 and 0.01 IU/cell is added to the first aliquot of cells. The other aliquot of cells only receives culture medium and then is incubated under identical conditions as the HIV-infected cells. After a 4 h incubation at 37 °C and 5% CO_2, both cell populations are washed three times with fresh medium and then added to the appropriate wells of a 24 well-plate containing the various concentrations of the test drug or culture medium (positive infected control/ negative drug control). In addition, AZT is also assayed during each experiment as a positive drug control. The plates are incubated at 37 °C and 5% CO_2 for four days. Cell-free supernatants are collected on day four and tested by an in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. Toxicity is determined by performing cell counts by a Coulter Counter on the mock-infected cells which had either received culture medium (no toxicity) or test sample or AZT. If a test sample has suppressive capability and is not toxic, its effects are reported in the following terms: TC_{50}, the concentration of test sample which is toxic to 50% of the mock-infected cells; EC_{50}, the concentration of the test sample which is able to suppress HIV replication by 50%; and therapeutic index (TI), the ratio of TC_{50} to EC_{50}.

HIV-1 reverse transcriptase assay. HIV-1 reverse transcriptase microassay was adapted from ref 17. Briefly, 10 mL of virion-associated $HIV\text{-}1_{IIIB}$ reverse transcriptase in 1% Triton X-100 was mixed with 50 μL of a reaction cocktail containing 50 mM Tris–HCl (pH 7.8), 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, poly (A) (5 mg/mL; Pharmacia), oligo (dT) (0.25 unit/mL; Pharmacia), 0.05 % Nonidet P40, and ^{32}P-dTTP (10 mCi/mL) in the presence of various concentrations of test compounds. After incubating 1 h at 37 °C, 40 μL of the reaction mixture was applied to a Schleicher & Schuell NA 45 membrane saturated with 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) in a Schleicher & Minifold over one sheet of GBOO3 filter paper. Each well of the minifold was washed four times with 2 × SSC. Autoradiography was performed and radioactivity was quantified with a Packard Matrix (Meriden, CT) 9600 direct beta counter.

Cell fusion assay. Cell fusion assays were performed as previously described in ref 20. MOLT4 cells (7×10^4) were incubated with $HIV\text{-}1_{LAI}$ chronically infected CEM cells (10^4) in 96-well half-area flat-bottomed plates (Costar) in 100 μL culture medium. Test compounds at various concentration 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.

Chronically HIV-infected cell line. HIV-1 chronically infected T cell line, ACH-2,[21] and HIV-1 chronically infected promonocytic cell line, U1,[22] were continuously maintained in RPMI 1640 with 10% fetal calf serum. For the experiments, the cell lines were only used in log-phase of growth. Cells (1×10^6 cells/well) and either various concentrations of **11**, **12**, AZT, or media alone were added to 24-well plates in the presence or absence of PMA (10^{-8} M). After 72 h at 37 °C and 5% CO_2, an aliquot of the cell-free supernatants was collected and analyzed for p24 antigen by ELISA.

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