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Synthesis and anti-HIV activity of bi-functional betulinic acid derivatives

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Abstract—Betulinic acid (BA) derivatives with a side chain at C-3 can inhibit HIV-1 maturation. On the other hand, BA derivatives with a side chain at C-28 can block HIV-1 entry. In order to combine the anti-maturation and anti-entry activities in a single molecule, new bi-functional BA derivatives containing side chains at C-3 and C-28 have been synthesized. The most potent compound ([[*N*-[3β-*O*-(3′,3′-dimethylsuccinyl)-lup-20(29)-en-28-oyl]-7-aminoheptyl]-carbamoyl]methane) inhibited HIV-1 at an EC_{50} of 0.0026 μM and was at least 20 times more potent than either the anti-maturation lead compound DSB or the anti-entry lead compound IC9564. This bi-functional BA derivative was active against both HIV entry and maturation. These results suggest that bi-functional BA derivatives with dual mechanisms of action have the potential to become clinically useful for AIDS therapy.
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1. Introduction

Current clinical AIDS treatment relies on a multiple-drug combination regime called highly active antiretroviral therapy (HAART), which uses drugs targeting at least two viral proteins.[1,2] However, this existing drug therapy encounters problems such as the emergence of drug resistant viruses and toxicities caused by long-term drug usage.[3,4] Therefore, it is important to continually search for new drugs with novel mechanisms of action that can be added to the current anti-HIV therapy.

Betulinic acid (BA) derivatives have been studied for their potential as anti-HIV agents. Two classes of chemically modified BA derivatives are reported to inhibit HIV replication at nanomolar concentrations. Class I compounds, such as DSB (also designated as PA-457), possess side-chain modification at the C-3 position of BA.[5,6] Class II compounds, such as IC9564, possess side-chain modification at the C-28 position.[6–9] Although both classes of BA derivatives shared the same betulinic acid core, they exhibit very different modes of anti-HIV action. Previous studies suggested that the molecular mechanisms of action for both classes of BA derivatives were quite unique in comparison with currently known anti-HIV drugs that target HIV reverse transcriptase or protease.[5,9–11] For example, IC9564 was reported to inhibit HIV-1 envelope-mediated membrane fusion by targeting the HIV-1 envelope glycoproteins.[9,12–14] On the other hand, DSB was found to interfere with HIV-1 CA/SP1 processing. During HIV-1 maturation, the viral protease cleaves specific sites in p55 Gag polyprotein to form the structural proteins MA, CA (p24), NC, and p6 as well as the Gag spacer peptides SP1 (p2) and SP2.[15] DSB partially blocked the cleavage of CA/SP1, which resulted in the production of non-infectious HIV-1 particles.[10,11,16] Thus, DSB and IC9564 represent two distinct classes of BA derivatives with different anti-HIV profiles.

Previous studies on anti-HIV BA derivatives suggested that the C-3 side chain is the pharmacophore for anti-maturation activity, while the C-28 side chain is the pharmacophore for anti-entry activity.[5–8] Structurally, the C-3 and C-28 pharmacophores of these two compound classes are located at the opposite ends of the BA pentacyclic ring system. Therefore, a design that incorporates both pharmacophores into one BA molecule, such as **14**, is chemically feasible. As proof of principle, we previously synthesized two bi-functional BA

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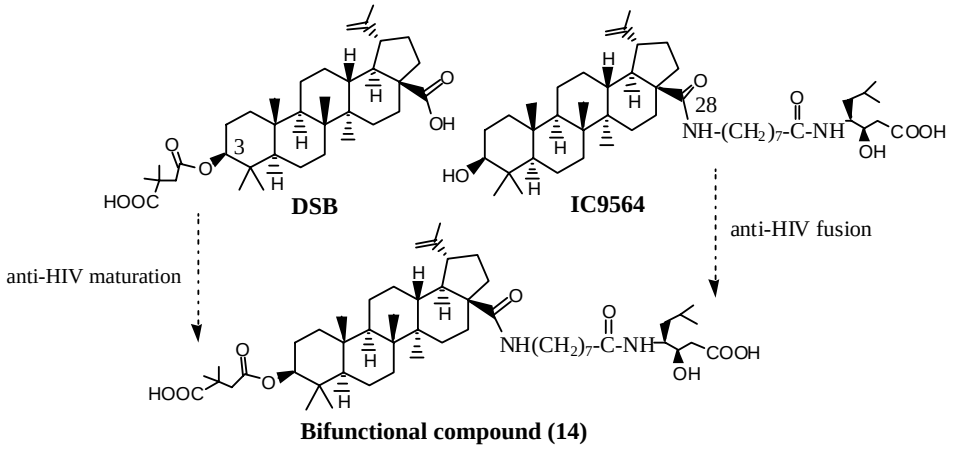


derivatives, LH15 (**4**) and LH55 (**8**), which exhibited improved anti-HIV-1 activity when compared to the mono-functional BA derivatives with a side chain either at the C-3 or the C-28 position.[17] The goal of this study was to develop new bi-functional BA derivatives that can potently inhibit HIV-1 replication by targeting both viral entry and maturation.

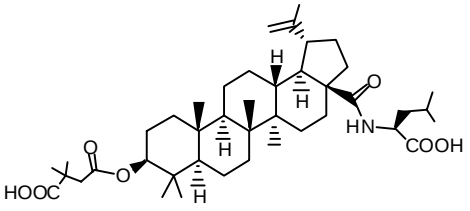
As previously shown by Hashimoto et al., 3′,3′-dimethylsuccinyl betulinic acid (DSB) is the most potent class I anti-HIV maturation BA derivative.[5] Other analogs with different C-3 side chains, including an isomer of DSB, 2′,2′-dimethylsuccinyl BA, showed a significantly lower anti-HIV activity. In contrast, IC9564, a class II anti-HIV entry BA derivative, has a much larger side chain (–CONH–$(CH_2)_7$-statine) attached to the C-28 position, which seems able to tolerate some changes without a drastic reduction in the anti-entry activity.[7,8] Therefore, we synthesized a series of new bi-functional BA derivatives possessing a 3′,3′-dimethylsuccinyl (or 2′,2′-dimethylsuccinyl) at C-3 and a variety of amide side chains at C-28. The range of length and bulkiness in the C-28 side chain was designed to generate molecules with optimal interactions with the putative viral binding targets.

Among the synthetic bi-functional compounds, **4**–**7** and **12** have relatively small C-28 side chains, such as leucine or glycinyl-leucine (Table 1). Conversely, compounds **8**–**10**, **13**–**15**, and **16**–**20** have a long C-28 side chain of –CONH–$(CH_2)n$–COOH, –CONH–$(CH_2)n$–COR (R = leucine, statine or 3-aminobenzoic acid, respectively), and –CONH–$(CH_2)n$–NH–$COCH_3$, respectively, with n ranging from 5 to 10. Compound **11** has a bulky bicyclic aromatic moiety. All synthetic bi-functional compounds contain two terminal carboxylic acids, one in each of the C-3 and C-28 side chains, except for compounds **11** and **16**–**20**, which have a terminal carboxylic acid in the C-3 but not the C-28 side chain.

2. Chemistry

Schemes 1–4 show the four general routes for synthesizing bi-functional BA derivatives **4**–**13** and **15**–**20**. Synthesis of these compounds began with modification at the C-28 position of either 3-OAc-BA or BA and ended with modification at the C-3 position. For the preparation of **4**–**10**, 3-OAc-BA was treated with oxalyl chloride, and the resulting acyl chloride was readily coupled with the appropriate amine. Saponification with 4 N NaOH in THF/MeOH yielded the corresponding C-28 derivatized BA intermediates **4a**, **6a**, **8a**, and **10a**. These intermediates were esterified at C-3 with 2,2-dimethylsuccinic anhydride, in the presence of dimethylaminopyridine (DMAP) and pyridine under reflux, to furnish the target bi-functional compounds **4**–**10** (Scheme 1).

Unprotected BA was used as the starting material for the synthesis of **11** and **12** (Scheme 2). In this route, mild conditions were used for the initial amide coupling, so that protection of the C-3 hydroxyl group as the acetate was not needed. After the initial coupling to assemble the C-28 side-chain, **11** was obtained by subsequent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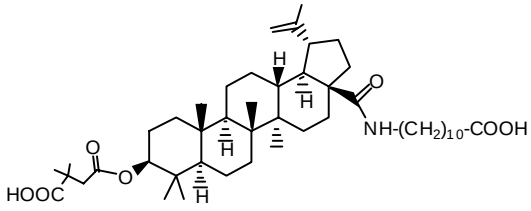
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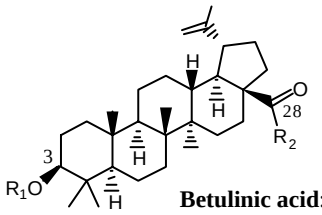
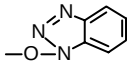
Table 1. The effect of BA derivatives against HIV-NL4-3 in MT4 cells[a]

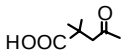
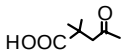
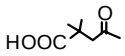


Betulinic acid: R_1 = H and R_2 = OH

Compound	R_1	R_2	EC_{50} (μM)[b]	IC_{50} (μM)[b]
1 (IC9564)	H	—NH$(CH_2)_7$-C(=O)-NH-CH(CH(CH$_3$)$_2$)-CH(OH)-COOH	0.053	>10
2 (DSB)	HOOC-C(=O)-C(CH$_3$)$_2$-	OH	0.075	7.5
3	HOOC-C(=O)-C(CH$_3$)$_2$-	OH	0.45	8
4	HOOC-C(=O)-C(CH$_3$)$_2$-	L-Leucine	0.015	10
5	HOOC-C(=O)-C(CH$_3$)$_2$-	L-Leucine	0.008	12
6	HOOC-C(=O)-C(CH$_3$)$_2$-	D-Leucine	0.02	8
7	HOOC-C(=O)-C(CH$_3$)$_2$-	D-Leucine	0.01	9
8	HOOC-C(=O)-C(CH$_3$)$_2$-	–NH–$(CH_2)_{10}$–COOH	0.012	6
9	HOOC-C(=O)-C(CH$_3$)$_2$-	–NH–$(CH_2)_{10}$–COOH	0.035	8
10	HOOC-C(=O)-C(CH$_3$)$_2$-	–NH–$(CH_2)_7$–COOH	0.077	>10
11	HOOC-C(=O)-C(CH$_3$)$_2$-		0.035	2.5
12	HOOC-C(=O)-C(CH$_3$)$_2$-	—NHCH$_2$-C(=O)-NH-CH(CH(CH$_3$)$_2$)-COOH	0.016	>10
13	HOOC-C(=O)-C(CH$_3$)$_2$-	—NH$(CH_2)_7$-C(=O)-NH-CH(CH(CH$_3$)$_2$)-COOH	0.087	>10
14	HOOC-C(=O)-C(CH$_3$)$_2$-	—NH$(CH_2)_7$-C(=O)-NH-CH(CH(CH$_3$)$_2$)-CH(OH)-COOH	0.096	>10
15	HOOC-C(=O)-C(CH$_3$)$_2$-	—NH$(CH_2)_7$-C(=O)-NH-C$_6$H$_4$-COOH	0.007	9.5
16	HOOC-C(=O)-C(CH$_3$)$_2$-	–NH–$(CH_2)_5$–NH–COCH$_3$	0.012	9
17	HOOC-C(=O)-C(CH$_3$)$_2$-	–NH–$(CH_2)_6$–NH–COCH$_3$	0.007	7.5

(*continued on next page*)

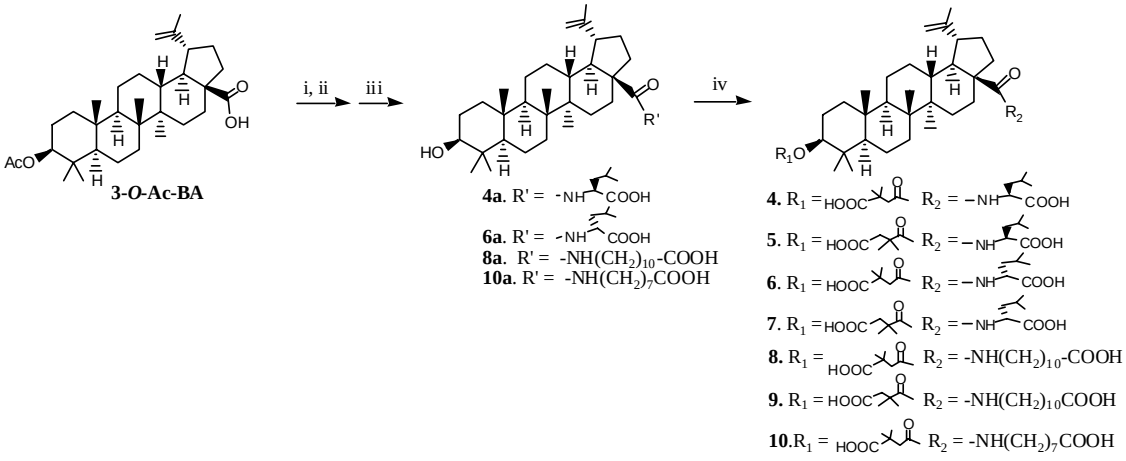
Table 1 (*continued*)

Compound	R_1	R_2	EC_{50} (μM)[b]	IC_{50} (μM)[b]
18 (A12-2)		–NH–$(CH_2)_7$–NH–$COCH_3$	0.0026	8
19		–NH–$(CH_2)_8$–NH–$COCH_3$	0.0036	7
20		–NH–$(CH_2)_9$–NH–$COCH_3$	0.012	7.4
18a (A43-D)	H	–NH–$(CH_2)_7$–NH–$COCH_3$	0.047	>10

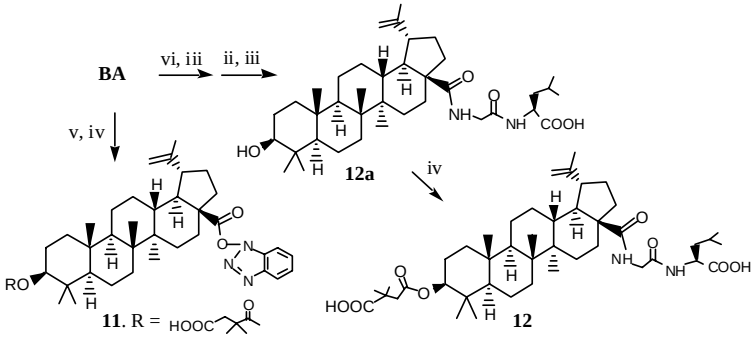
Anti-HIV activity of Bi-functional BA derivatives.

[a] See anti-HIV assay in Section 5 for experimental procedures.

[b] EC_{50} is the concentration that inhibits HIV-1 replication by 50%; IC_{50} is the drug concentration that resulted in a 50% reduction of viable cells in a 4-day assay. Data represent an average of at least two experiments.



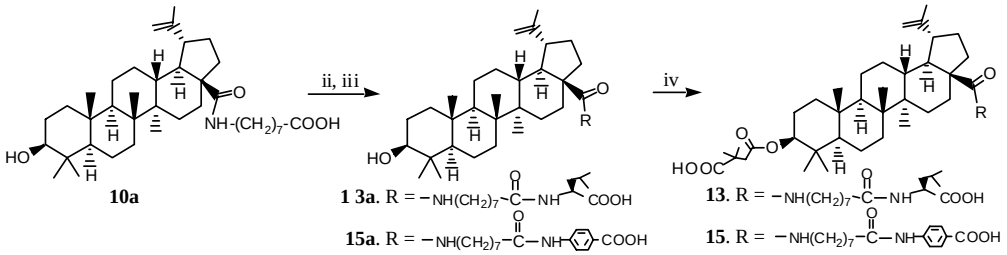
Scheme 1. Reagents and conditions: (i) $(CO)_2Cl_2/CH_2Cl_2$, rt, 10 min; (ii) H_2N–R′–COOMe/HOBT/TEA (or DIEA)/CH_2Cl_2, or H_2N–R′–COOMe/HOBT/CH_2Cl_2, rt, overnight; (iii) NaOH 4 N/THF/MeOH, rt, overnight; (iv) 2,2-dimethylsuccinic anhydride/Py/DMAP, reflux, overnight.



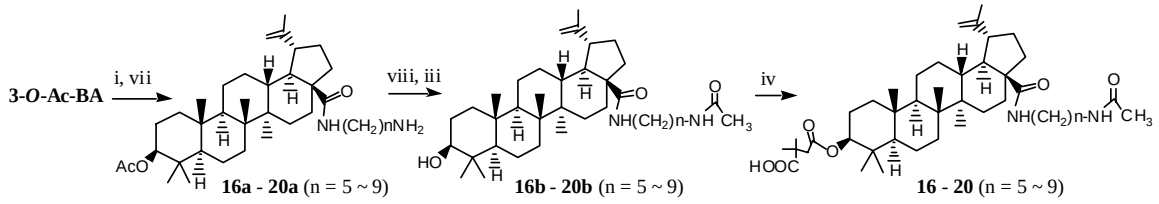
Scheme 2. Reagents and conditions: (ii) H_2N–R′–COOMe/HOBT/TEA (or DIEA)/CH_2Cl_2, or H_2N–R′–COOMe/HOBT/CH_2Cl_2, rt, overnight; (iii) NaOH 4 N/THF/MeOH, rt, overnight; (iv) 2,2-dimethylsuccinic anhydride/Py/DMAP, reflux, overnight; (v) PyBop/HOBT/DIEA/NH_4Cl/DMF, rt, 1 h; (vi) H_2N–CH_2–COOEt/DMAP/CH_2Cl_2, rt, overnight.

C-3 esterification with 2,2-dimethylsuccinic anhydride. The complete assembly of the C-28 side chain of **12** required two steps of coupling, each followed by a step of saponification to furnish the intermediate **12a**. The final C-3 esterification as described above completed the synthesis of **12**.

To synthesize **13** and **15** (Scheme 3), the intermediate **10a** was coupled with leucine or *p*-aminobenzoic acid, catalyzed by PyBop/HOBT/DIEA, to form the corresponding amide. Subsequent saponification furnished the intermediates **13a** and **15a**, which were finally modified at C-3 to give **13** and **15**, respectively.



Scheme 3. Reagents and conditions: (ii) H_2N–R′–COOMe/HOBT/TEA (or DIEA)/CH_2Cl_2, or H_2N–R′–COOMe/HOBT/CH_2Cl_2, rt, overnight; (iii) NaOH 4 N/THF/MeOH, rt, overnight; (iv) 2,2-dimethylsuccinic anhydride/Py/DMAP, reflux, overnight.

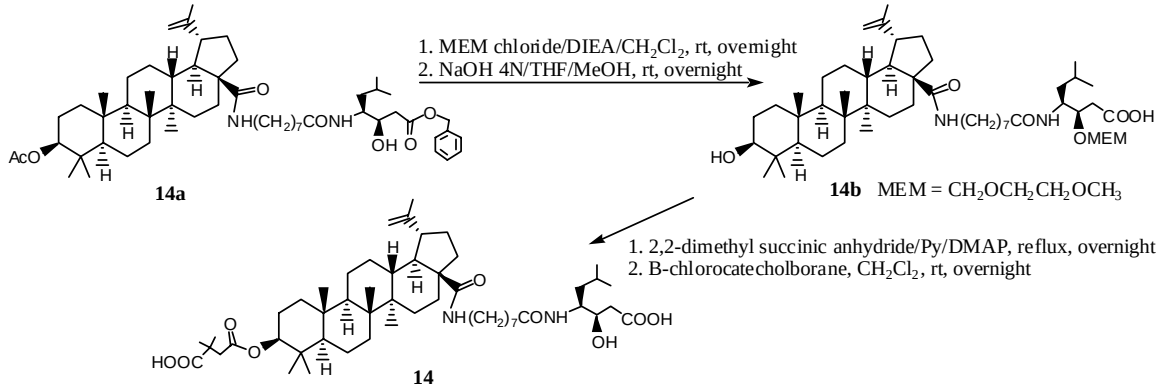


Scheme 4. Reagents and conditions: (i) $(CO)_2Cl_2/CH_2Cl_2$, rt, 10 min; (iii) NaOH 4 N/THF/MeOH, rt, overnight; (iv) 2,2-dimethylsuccinic anhydride/Py/DMAP, reflux, overnight; (vii) H_2N–$(CH_2)n$–NH_2/CH_2Cl_2, n=5–9; (viii) Ac_2O/DMAP/Py, rt, overnight.

Scheme 4 shows the synthetic steps for the preparation of **16**–**20**. The protected 3-OAc-BA was subjected to two successive coupling steps for the C-28 side chain assembly. The acyl chloride of 3-OAc-BA was first treated with excess alkyldiamine to form **16a**–**20a**, which were further coupled with acetic anhydride in the presence of DMAP and pyridine. Subsequent saponification resulted in **16b**–**20b**, which were finally esterified at C-3 to form the corresponding bi-functional compounds **16**–**20**, respectively.

To synthesize compound **14**, extra protection/de-protection steps were needed to preserve the statine hydroxyl group (Scheme 5). The MEM ether was chosen as it was stable during the following steps of saponification and C-3 acylation. However, the cleavage of the MEM ether did not occur as readily as anticipated. No reaction occurred after $ZnBr_2$ treatment in DCM for over 2 days; however, cleavage of the MEM ether was finally successful by using B-chlorocatecholborane.[18]

The final esterification of the C-3 hydroxyl involves reflux of a pyridine mixture of the BA intermediate and 2,2-dimethylsuccinic anhydride in the presence of DMAP. This reaction produced two isomers: 2′,2′-dimethyl and 3′,3′-dimethylsuccinyl BA derivatives. Under our synthetic conditions, the 3′,3′-dimethylsuccinyl isomer is the dominant product, especially for compounds with long C-28 side chains. Accordingly, with **12**–**20**, only the 3′,3′-dimethylsuccinyl BA derivatives was obtained. However, for **4a** and **6a**, which have relatively short C-28 side chains, approximately equal amount of both isomers (**4/5** and **6/7**, respectively) were obtained. Compound **8a** with C-28 undecanoic acid side chain also afforded two isomers (**8** and **9**) upon esterification, with **8** as the major and **9** as the minor products. Compound **11**, which has a C-28 aromatic bicyclic ring system, was the only compound that formed predominantly the 2′,2′-dimethylsuccinyl BA derivative. These results suggest that the C-3 acylation of BA derivatives was sterically influenced by its C-28 moiety. The two isomers formed after esterification were separated by reverse-phase HPLC. Extensive NMR experiments including HMQC and HMBC were performed on compound **7** to establish its structure as the 2′,2′-dimethyl isomer. The distinct proton signal patterns of the methylene



Scheme 5.

group, derived from 2′,2′-dimethyl (in **7**) or 3′,3′-dimethylsuccinyl (in **6**), were used as the basis to determine the structures of all remaining isomers.[5]

3. Results and discussion

3.1. Anti-HIV activity of bi-functional BA derivatives

As shown in Table 1, all new bi-functional compounds exhibited anti-HIV activity. Compounds **4**–**7**, which contain a C-28 leucine, showed EC_{50} values of around 0.02 μM. The leucine stereochemistry, either D or L, did not affect the anti-HIV potency. Bi-functional BA derivatives **5** and **7**, which have 3-*O*-2′,2′-dimethylsuccinyl substitution, were approximately twice as potent as their 3-*O*-3′,3′-dimethylsuccinyl isomers, **4** and **6**. However, this rank order of activity was not uniform among all derivatives. For example, with the two C-28 aminoundecanoic acid isomers, compound **8** with a 3-*O*-3′,3′-dimethylsuccinyl side chain was 3-fold more potent than its 3-*O*-2′,2′-dimethylsuccinyl isomer, **9**. This latter result corresponds to the prior and current results with the mono-functional BA derivatives. For example, DSB (**2**) contains a 3-*O*-3′,3′-dimethylsuccinyl side chain, is clearly much more potent than its 3-*O*-2′,2′-dimethylsuccinyl isomer (**3**). These data suggested that the C-28 side chain might affect the interaction between the C-3 pharmacophore and its target.

Compound **12** with C-3 3′,3′-dimethylsuccinyl and C-28 glycinyl-leucine side chains showed similar potency to the leucine compounds **4**–**7**. Compound **11**, which contains a C-3 2′,2′-dimethylsuccinyl and a fused ring system at C-28, was less active and more toxic to the cells when compared to the bi-functional BA derivatives with short side chains at C-28 such as **4**–**7**.

Bi-functional BA derivatives **13**–**15** all have a 3′,3′-dimethylsuccinyl moiety at C-3 and a large C-28 amido side chain containing an aminooctanoyl moiety terminated with an amino acid [–CONH–$(CH_2)_7$–CONH–R, R = leucine, statine, or 3-aminobenzoic acid, respectively]. Although DSB and IC9564 are among the most potent mono-functional class I and II BA derivatives, respectively, bi-functional compound **14**, which has an identical side chain to that of IC9564 at C-28 and to that of DSB at C-3, exhibited a weaker anti-HIV activity than either mono-functional compound. The anti-HIV potency of **13** was about the same as **14**. On the other hand, **15**, the benzoic acid bi-functional derivative, was significantly more potent than the statine analog **14**, the leucine analog **13**, and both DSB and IC9564. These results suggested that the structure-activity relationships of the bi-functional BA derivatives are different from those reported for mono-functional BA derivatives.

Compounds **16**–**20** were also obtained as 3-*O*-3′,3′-dimethylsuccinyl BA derivatives. The C-28 side chain of **16**–**20** is *N*-acetyl diamino alkane [–CONH–$(CH_2)n$–$NHCOCH_3$]. Thus, their structures vary from those of **13**–**15** in the order and identity of the terminal amide group (–CONHR′–COOH for **13**–**15**; –$NHCOCH_3$ for **16**–**20**). By using a variety of diaminoalkanes with *n* ranging from 5 to 9, BA derivatives with various chain lengths attached to C-28 were obtained. Compounds **18** and **19** with *n* equal to 7 and 8, respectively, are the most active compounds obtained thus far in our study (EC_{50} = 0.0026 and 0.0036 μM, respectively).

3.2. Anti-fusion activity of bi-functional BA derivatives

To determine if the bi-functional BA derivatives indeed inhibit both HIV-1 entry and maturation activities, the most potent bi-functional BA derivative (**18**) was chosen for mechanistic studies. A luciferase-based fusion assay was used to determine the anti-HIV-1 entry activity of **18**. TZM cells that express luciferase upon fusion with envelope-expressing COS cells were used as fusion partners. A Promega luciferase assay kit was used to quantify luciferase activity in the fused cells using a Biotek luminometer. In the absence of inhibitors, HIV-1 envelope-mediated fusion of a sample in 96-well plates yielded an average of 70,000 relative luminescence units (control RLU). The drug concentration that reduced the control RLU by 50% is defined as 50% effective concentration (EC_{50}). The mono-functional class I BA derivative, DSB, did not exhibit significant anti-fusion activity (Fig. 1). On the other hand, the mono-functional class II BA derivative **18a** (A43-D in Fig. 1) is a potent anti-fusion inhibitor (EC_{50} 0.080 μM). The bi-functional analog **18** (A12-2 in Fig. 1) was also active (EC_{50} 0.22 μM) in the anti-fusion assay, although not as potent as **18a** (Fig. 1). Since **18a** and **18** share the same anti-fusion C-28 functional side chain, the results suggest that the side chain at C-3 has a negative impact on the anti-fusion activity of **18**.

3.3. The anti-maturation activity of bi-functional BA derivatives

Partial inhibition of p25 processing is believed to be responsible for the anti-maturation activity of DSB.[10,11] Thus, the anti-maturation activity of BA derivatives can be estimated by detecting p25 using Western blot analysis. Unlike the viral replication assay, detection of p25 accumulation allowed us to specifically

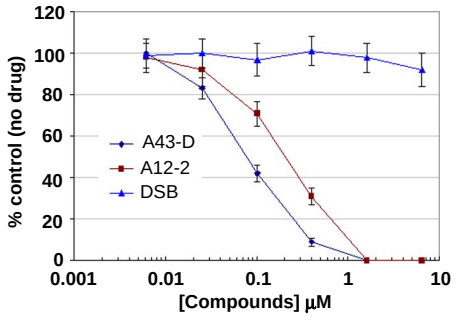


Figure 1. BA derivatives inhibited HIV-1 envelope-mediated membrane fusion. The fusion assay performed in the absence of drugs is defined as 100% control fusion. A43-D is compound **18a** and A12-2 is compound **18** described in the text. Each data point represents the mean ± SD of two duplicated experiments.

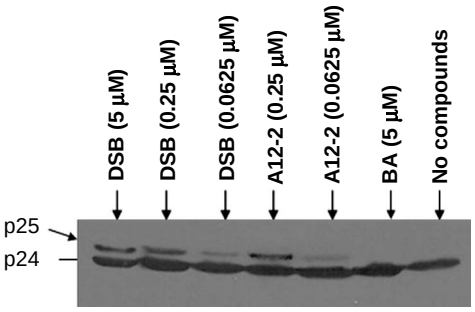


Figure 2. BA derivatives interfere with HIV-1 p25 processing. The partially processed p25 and matured p24 were detected with a rabbit anti-p24 antiserum on a Western blot. A12-2 is compound **18** described in the text.

evaluate the anti-maturation activity of BA derivatives. DSB at 5 μM was used as a marker for 100% inhibition, since DSB completely inhibited HIV NL4-3 replication at this concentration. BA was used as a negative control, since it did not inhibit NL4-3 replication. The results indicated that the bi-functional BA derivative **18** (A12-2 in Fig. 2) is comparable to DSB in inhibiting the processing of p25 (Fig. 2).

4. Conclusions

In this paper, we described a straightforward approach to obtain bi-functional BA derivatives by combining two pharmacophores from two classes of anti-HIV BA agents, each with its own unique mode of action, into one molecule. Two derivatives, **18** and **19**, were found to be at least 10- to 20-fold more potent than the leading anti-maturation mono-functional BA derivative DSB or the anti-entry mono-functional BA derivative IC9564. Further mechanistic study also proved that **18** was active in both anti-HIV entry and anti-HIV maturation assays. Both **18** and **19** contain the C-3 pharmacophore of DSB and a new C-28 pharmacophore –CONH–$(CH_2)n$NH–$COCH_3$. Based on the anti-HIV potency of **16**–**20**, *n* is optimally equal to 7. Prior reports suggested that a terminal carboxylic acid in the C-28 amido side chain was important for anti-HIV entry activity.[5–8] However, while both bi-functional **18** and its C-28 mono-functional analogs **18a** do not have a terminal carboxylic acid, they are quite potent against HIV-1 induced fusion (Fig. 1). Therefore, we propose that the terminal carboxylic acid at C-28 is not a requirement for anti-HIV entry activity. Instead, a carbonyl moiety near the terminal of C-28 side chain, such as the one in –CONH-$(CH_2)_7$NH–$COCH_3$, may be critical for potent anti-entry activity. In addition, the bi-functional analog **14**, which possesses the C-3 pharmacophore of DSB and the C-28 pharmacophore of IC9564, exhibited unexpectedly low anti-HIV potency. This result suggests that although the two pharmacophores at the C-3 and C-28 positions of BA are located far apart, they may interfere with each other in target binding. Indeed, the fact that the anti-fusion activity of bi-functional compound **18** was weaker than that of its mono-functional analog **18a**, also suggested that the presence of C-3 side chain can negatively affect the anti-fusion activity exerted by the C-28 pharmacophore. However, even though these two pharmacophores might interfere with each other, the overall potency of the bi-functional BA derivatives **18** was at least a log higher than the mono-functional **18a** or DSB in the HIV-1 replication assay (Table 1). Given the evidence that **18** was active in both HIV fusion and maturation assays, this improved antiviral potency is likely due to synergism between these two modes of action.

5. Experimental

5.1. General experiment procedures

All melting points were determined with a Fisher–Johns melting point apparatus without correction. Positive and negative HR-FABMS were recorded on a Joel SX-102 spectrometer. ^{1}H and other NMR spectra were measured on a Varian Mercury 300 or Varian Inova 500 or Bruker DRX-400 spectrometer. Other than as noted, all samples were dissolved in $CDCl_3$ with TMS as internal standard. Si gel chromatography was carried out on a Biotage Horizon Flash chromatograph system with prepacked Si gel column. HPLC was performed on a Varian ProStar solvent delivery and PDA detector with Agilent Zorbax ODS or C-8 columns (4.6 mm × 25 cm and 9.4 mm × 25 cm for analytical and semipreparative scales, respectively).

5.2. Procedure for preparation of compounds 4–10

5.2.1. General procedure for coupling at C-28 position. A mixture of 3-*O*-Ac-BA (250–350 mg, 0.5–0.8 mmol) and oxalyl chloride (6 mmol, 2 M in CH_2Cl_2) was stirred for 10 min. After concentration under vacuum, the residual mixture was treated with L- or D-leucine methyl ester, 11-aminodecanoic acid methyl ester, or 7-aminoheptanoic acid methyl ester (1–2 mmol), HOBT (1 equiv) and triethylamine (0.3 mL) in dichloromethane. The mixture was then stirred at room temperature overnight, then diluted with CH_2Cl_2, washed with water and brine, dried over Na_2SO_4, and finally concentrated under vacuum. The residue was chromatographed on Si gel to yield the corresponding C-28 amide intermediates.

5.2.2. General procedure for saponification. To the solution of above intermediates in THF/MeOH (50%, 4 mL) was added aqueous NaOH (1 mL, 4 M). After stirring for 4–10 h at rt, the mixture was neutralized with 1 N HCl. The resulting precipitate was collected, washed with water, and dried under vacuum to yield the corresponding C-3 unprotected and C-28 amide intermediates **4a**, **6a**, **8a**, and **10a** with yields of 60%, 52%, 57%, and 80%, respectively, for the above two steps.

5.2.3. General procedure for coupling at C-3 position. The mixture of above intermediate (**4a**, **6a**, **8a**, or **10a**), 2,2-dimethylsuccinic anhydride (5–10 equiv), and DMAP (1 equiv) in pyridine (anhydrous, 4 mL) was refluxed overnight. The mixture was then concentrated

under vacuum, re-dissolved in MeOH, and purified with reverse-phase HPLC. Intermediates **4a**, **6a**, and **8a** each gave two isomers: **4** and **5**, **6** and **7**, and **8** and **9**, respectively. Compound **10** was the only isomer isolated after acylation of **10a**.

5.2.3.1. 3β-*O*-(3′,3′-Dimethylsuccinyl)-28-*N*-L-leucinyl-betulinic acid (4). Yield 32%. Mp 230–233 °C. Positive FABMS *m*/*z* 698 $(M+H)^+$; HR-FABMS calcd for $C_{42}H_{68}NO_7$ 698.4996, found 698.4998. ^1H NMR (500 MHz) δ 6.03 (1H, d, J = 7.8 Hz, –NH–), 4.74, 4.55 (each 1H, each s, $=CH_2$), 4.65 (1H, dd, J = 7.6 Hz, J = 13.0 Hz, –NH–C*H*–), 4.54 (1H, dd, J = 6.2 Hz, J = 10.0 Hz, H-3), 3.04 (1H, dt, J = 4.0 Hz, J = 11.0 Hz, H-19), 2.89, 2.37 (each d, each 1H, J = 14.5 Hz, –CH_2–COO–CH), 2.52–2.77 (m, 1H, H-13), 1.68 (3H, s, CH_3-30), 1.31, 1.21 (each 3H, each s, –C(CH_3)$_2$–COOH), 0.94–0.97 (6H, m, 2 × CH_3), 0.79 (6H, s, 2 × CH_3), 0.67 (3H, s, CH_3). A complete chemical shift assignment of carbon and proton of **4** derived from ^{13}C and HMQC NMR was provided as supplementary data (Table 2).

5.2.3.2. 3β-*O*-(2′,2′-Dimethylsuccinyl)-28-*N*-L-leucinyl-betulinic acid (5). Yield 26%. Mp 250–252 °C. Positive FABMS *m*/*z* 698 $(M+H)^+$; HR-FABMS calcd for $C_{42}H_{68}NO_7$ 698.4996, found 698.4996. ^1H NMR (400 MHz) δ 5.82 (1H, d, J = 7.6 Hz, –NH–), 4.70, 4.58 (each 1H, each s, $=CH_2$), 4.44–4.55 (2H, m, –NH–C*H*– and H-3), 3.05–3.10 (1H, dt, J = 4.0 Hz, J = 11 Hz, H-19), 2.66, 2.59 (each 1H, each d, J = 16.0 Hz, –CH_2–COOH), 2.44–2.50 (1H, dt, J = 3 Hz, J = 11 Hz, H-13), 1.67 (3H, s, CH_3-30), 1.30, 1.28 (each 3H, each s, –C(CH_3)$_2$–CH_2), 0.91–0.98 (9H, m, 3× CH_3), 0.83 (3H, d, J = 13 Hz, CH_3), 0.81 (3H, d, J = 11.0 Hz, CH_3).

5.2.3.3. 3β-*O*-(3′,3′-Dimethylsuccinyl)-28-*N*-D-leucinyl-betulinic acid (6). Yield 45%. Mp 205–207 °C. Positive FABMS *m*/*z* 698 $(M+H)^+$; HR-FABMS calcd for $C_{42}H_{68}NO_7$ 698.4996, found 698.4996. ^1H NMR (300 MHz) δ 6.04 (1H, d, J = 7.8 Hz, –NH–), 4.74, 4.59 (each 1H, each s, $=CH_2$), 4.65 (1H, dd, J = 7.6 Hz, J = 13.0 Hz, –NH–C*H*–), 4.53 (1H, dd, J = 6.1 Hz, J = 9.7 Hz, H-3), 3.03 (1H, dt, J = 4. 3 Hz, J = 10.0 Hz, H-19), 2.90, 2.35 (each 1H, each d, J = 14.5 Hz, –CH_2–COO–CH), 2.67 (1H, t, J = 13.8 Hz, H-13), 1.68 (3H, s, CH_3-30), 1.31, 1.21 (each 3H, each s, –C(CH_3)$_2$–COOH), 0.94–0.98 (6H, m, 2× CH_3), 0.79 (s, 6H, 2× CH_3), 0.67 (3H, s, CH_3).

5.2.3.4. 3β-*O*-(2′,2′-Dimethylsuccinyl)-28-D-leucinyl-betulinic acid (7). Yield 37%. Mp 235–237 °C. Positive FABMS *m*/*z* 698 $(M+H)^+$; HR-FABMS calcd for $C_{42}H_{68}NO_7$ 698.4996, found 698.4996. ^1H NMR (300 MHz) δ 5.81 (1H, d, J = 7.5 Hz, –NH–), 4.70, 4.58 (each 1H, each s, $=CH_2$), 4.48–4.56 (2H, m, –NH–C*H*– and H-3), 3.05 (1H, dt, J = 4.0 Hz, J = 11.0 Hz, H-19), 2.65, 2.58 (each 1H, each d, J = 16.0 Hz, –CH_2–COOH), 2.46 (1H, t, J = 12.0 Hz, H-13), 1.67 (3H, s, CH_3-30), 1.30, 1.28 (each 3H, each s, –C(CH_3)$_2$–CH_2), 0.91–0.98 (9H, m, 3× CH_3), 0.82–0.86 (6H, m, 2× CH_3).

5.2.3.5. *N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-11-aminoundecanoic acid (8). Yield 16%. Mp 115–120 °C. Positive FABMS *m*/*z* 768 $(M+H)^+$; HR-FABMS calcd for $C_{47}H_{78}NO_7$ 768.5778, found 768.5752. ^1H NMR (400 MHz) δ 5.76 (1H, t, J = 5.5 Hz, NH), 4.74, 4.53 (each 1H, each s, $=CH_2$), 4.51 (1H, dd, J = 5.0 Hz, J = 11.0 Hz, H-3), 3.30–3.41, 3.08–3.15 (2H, m, –CH_2NH), 3.05–3.08 (1H, m, H-19), 2.78, 2.47 (each 1H, each d, J = 15.0 Hz, –CH_2–COO–CH–), 2.23–2.37 (2H, m, –CH_2–COOH), 2.17 (1H, dt, J = 13.0 Hz, J = 3.4 Hz, H-13), 1.69 (3H, s, CH_3-30), 1.30, 1.24 (each 3H, each s, –C(CH_3)$_2$–COOH), 0.75, 0.82, 0.83, 0.94, 0.97 (each 3H, each s, 5× CH_3).

5.2.3.6. *N*-[3β-*O*-(2′,2′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-11-aminoundecanoic acid (9). Yield 3.2%. Mp 97–100 °C. Positive FABMS *m*/*z* 768 $(M+H)^+$; HR-FABMS calcd for $C_{47}H_{78}NO_7$ 768.5778, found 768.5752. ^1H NMR (400 MHz) δ 5.63 (1H, br s, –NH–), 4.73, 4.59 (each 1H, each s, $=CH_2$), 4.65–4.68 (1H, m, H-3), 3.27–3.33, 3.16–3.22 (2H, m, CH_2NH), 3.11 (1H, dt, J = 4 Hz, J = 11 Hz, H-19), 2.73 (2H, br s, –C(CH_3)$_2$–CH_2–COOH), 2.23–2.44 (1H, m, H-13), 2.35 (2H, t, J = 7.5 Hz, –CH_2–COOH), 1.68 (3H, s, CH_3-30), 1.28 (6H, s, –C(CH_3)$_2$–CH_2), 0.96 (6H, s, 2× CH_3), 0.93 (3H, s, CH_3), 0.87 (3H, d, J = 5.5 Hz, CH_3), 0.78 (3H, d, J = 24.0 Hz, CH_3).

5.2.3.7. *N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-8-aminooctanoic acid (10). Yield 70%. Mp 116–120 °C. Positive FABMS *m*/*z* 726 $(M+H)^+$; HR-FABMS calcd for $C_{44}H_{72}NO_7$ 726.5309, found 726.5309. ^1H NMR (300 MHz) δ 5.65 (1H, t, J = 5.8 Hz, –NH–), 4.73, 4.59 (each 1H, each s, $=CH_2$), 4.48 (1H, t, J = 7.8 Hz, H-3), 3.28–3.68, 3.12–3.18 (2H, m, –CH_2NH), 3.08–3.10 (1H, m, H-19), 2.67, 2.56 (each 1H, each d, J = 15.8 Hz, –CH_2–COO–CH–), 2.38–2.47 (1H, m, H-13), 2.34 (2H, t, J = 7.5 Hz, –CH_2–COOH), 1.68 (3H, s, CH_3-30), 1.30, 1.28 (each 3H, each s, –C(CH_3)$_2$–COOH), 0.79, 0.92, 0.95 (each 3H, each s, 3× CH_3), 0.82 (6H, s, 2× CH_3).

5.2.4. Preparation of 11. A mixture of betulinic acid (200 mg, 0.44 mmol), PyBop (343 mg, 0.66 mmol), HOBT (88 mg, 0.66 mmol), DIEA (0.26 mL, 1.76 mmol), and NH_4Cl (5 mg, 0.88 mmol) in DMF (4 mL) was stirred for 1 h at room temperature. The mixture was then diluted with EtOAc (50 mL), washed with diluted HCl (1 N) and brine, and then dried over Na_2SO_4. The organic layer was concentrated under vacuum and chromatographed on Si gel to yield the HOBT derivative of betulinic acid. The resulting intermediate was further modified following the general procedure of C-3 coupling described above to yield **11**.

5.2.4.1. *O*-[3β-*O*-(2′,2′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-1-hydroxybenzotriazole (11). Yield 64%. Mp 151–154 °C. Negative FABMS *m*/*z* 700 $(M-H)^-$; HR-FABMS calcd for $C_{42}H_{58}N_3O_6$ 700.4326, found 700.4326. ^1H NMR (300 MHz, Pyridine-d_5) δ 8.20 (1H, d, J = 8.5 Hz, Ar-H), 7.67 (s, 1H, Ar-H), 7.64 (1H, dd, J = 8.5 Hz, J = 15.0 Hz, Ar-H), 7.42 (1H, dt, J = 1.2 Hz, J = 6.6 Hz, Ar-H), 4.88, 4.75 (each 1H, each

s, =CH_2), 4.71 (1H, d, J = 4.7 Hz, H-3), 3.13 (1H, dd, J = 4.7 Hz, J = 11.0 Hz, H-19), 2.91 (2H, d, J = 6.0 Hz, –CH_2–COOH), 2.65 (1H, d, J = 2.8 Hz, CH), 2.38–2.47 (1H, m, CH), 2.31 (1H, t, J = 12.0 Hz, H-13), 2.06–2.20 (1H, m, CH), 1.72 (3H, s, CH_3-30), 1.53 (6H, s, –C(CH_3)$_2$–CH_2–COOH), 0.71, 0.92, 0.95, 0.96, 1.02, (each 3H, s, 5× CH_3).

5.2.5. Preparation of 12. A mixture of betulinic acid (300 mg, 0.66 mmol), DCC (1 mL, 1 M in dichloromethane), glycine ethylester (180 mg, 1.3 mmol), and DMAP (96 mg, 0.8 mmol) in dichloromethane (20 mL) was stirred overnight at room temperature. The mixture was then diluted with dichloromethane (50 mL), washed with water and brine, dried over Na_2SO_4, and then concentrated under vacuum. The residue was chromatographed on Si gel to yield the glycine ester derivative of betulinic acid, which was then subjected to hydrolysis (see Section 5.2.2) to yield the glycine BA. The resulting glycine BA (150 mg, 0.28 mmol) was treated with leucine methyl ester (100 mg, 0.7 mmol), PyBop (580 mg, 1.1 mmol), and HOBT (45 mg, 0.33 mmol) in CH_2Cl_2 (20 mL) at room temperature and stirred overnight. The reaction mixture was then diluted with methylene chloride (20 mL), washed with water and brine, dried over Na_2SO_4, and then concentrated under vacuum. Subsequent saponification (see Section 5.2.2) on the above glycinyl-leucine intermediate yielded **12a**, which was further modified at C-3 as described above to afford **12**.

5.2.5.1. *N*′-[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-L-glycinyl]-L-leucine (12). Yield 31% overall from betulinic acid. Mp 204–207 °C. Positive FABMS *m*/*z* 755 $(M+H)^+$; HR-FABMS calcd for $C_{44}H_{71}N_2O_8$ 755.5210, found 755.5210. ^{1}H NMR (300 MHz) δ 6.87 (1H, d, J = 8.0 Hz, NH), 6.63 (1H, br s, NH), 4.70, 4.55 (each 1H, each s, =CH_2), 4.60–4.66 (1H, m, –NH–CH–), 4.44 (1H, dd, J = 5.4 Hz, J = 11.0 Hz, H-3), 3.94 (2H, d, J = 4.8 Hz, –CH_2–NH–), 3.08 (1H, dt, J = 4.0 Hz, J = 11.0 Hz, H-19), 2.67, 2.56 (each d, each 1H, J = 15.3 Hz, –CH_2–COO–CH), 2.43 (t, 1H, J = 9.5 Hz, H-13), 1.64 (3H, s, CH_3-30), 1.30, 1.29 (each 3H, each s, –C(CH_3)$_2$–COOH), 0.91–0.95 (9H, m, 3× CH_3), 0.72, 0.73, 0.78, 0.86 (each 3H, each s, 4× CH_3).

5.2.6. General procedure for preparation of 13 and 15. A mixture of **10a** (260 ~370 mg, 0.4–0.6 mmol) in dichloromethane (5 mL) was treated with leucine methyl ester or methyl-4-aminobenzoate (0.6 mmol), PyBop (312 mg, 0.6 mmol), HOBT (81 mg, 0.6 mmol), and DIEA (0.5 mL, 5.2 mmol) in dichloromethane (50 mL) and stirred overnight at room temperature. The mixture was diluted with 20 mL dichloromethane and washed with diluted HCl, water, and brine, and then dried over Na_2SO_4. After removal of organic solvent, the residue was saponified following the procedure described above. The collected precipitate was purified on Si gel to yield the corresponding C-28 mono-substituted betulinic acid derivatives **13a** and **15a** in 33% and 16% yields, respectively. Further modification using the general procedure for coupling at C-3, as described for compounds **4**–**10**, furnished the corresponding bi-functional compounds **13** and **15**.

5.2.6.1. *N*′-[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-8-aminooctanoyl]-L-leucine (13). Yield 37%. Mp 131–134 °C. Positive FABMS *m*/*z* 839 $(M+H)^+$; HR-FABMS calcd for $C_{50}H_{83}N_2O_8$ 839.6149, found 839.6194. ^{1}H NMR (300 MHz, Pyridine-d_5) δ 5.91 (1H, d, J = 7.0 Hz, NH), 5.21 (1H, br s, NH), 4.91, 4.74 (each 1H, each s, =CH_2), 4.73–4.77 (1H, m, H-3), 3.62 (1H, t, J = 11.0 Hz, CH–NH–), 3.45–3.55, 3.30–3.40 (each 1H, m, NH–CH_2), 3.07 (1H, t, J = 11.4 Hz, H-19), 2.95, 2.87 (2H, each d, J = 15.6 Hz, CH_2–COO–CH), 2.40–2.50 (2H, m, –CH_2–COO–NH), 1.77 (3H, s, CH_3-30), 1.52 (6H, s, –C(CH_3)$_2$–COOH), 0.75, 0.95, 1.03, 1.09 (each 3H, s, 4× CH_3), 0.99 (3H, d, J = 5.0 Hz, CH_3), 0.92 (6H, s, 2× CH_3).

5.2.6.2. *N*′-[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-8-aminooctanoyl]-3-aminobenzoic acid (15). Yield 63%. Mp 168–170 °C. Positive FABMS *m*/*z* 845 $(M+H)^+$; HR-FABMS calcd for $C_{51}H_{77}N_2O_8$ 845.5680, found 845.5680. ^{1}H NMR (300 MHz) δ 8.02 (2H, d, J = 8.5 Hz, Ar-H), 7.12 (1H, br s, CO–NH–Ar), 7.64 (2H, d, J = 7.5 Hz, Ar-H), 5.65 (1H, t, J = 5.8 Hz, –NH–), 4.71, 4.58 (each 1H, each s, =CH_2), 4.47 (1H, dd, J = 5.0 Hz, J = 11.0 Hz, H-3), 3.46–3.52, 2.93–2.99 (2H, m, –CH_2NH), 3.08–3.18 (1H, m, H-19), 2.78, 2.47 (each 1H, each d, J = 14.5 Hz, –CH_2–COO–CH–), 2.36–2.47 (1H, m, –CH), 2.16 (2H, s, CH_2CO–NH–), 1.67 (3H, s, CH_3-30), 1.32, 1.26 (each 3H, each s, –C(CH_3)$_2$–COOH), 0.41, 0.57, 0.76, 0.77, 0.92 (each 3H, each s, 5× CH_3).

5.2.7. Preparation of 14. A mixture of **14a**[12,19] (130 mg, 0.146 mmol), DIEA (0.2 mL, 1.1 mmol), and MEMCl (0.4 mL, 3.2 mmol) in anhydrous dichloromethane (5 mL) was stirred overnight at room temperature. The mixture was then diluted with EtOAc (50 mL), washed with saturated $NaHCO_3$ (2 × 25 mL) and brine, dried over Na_2SO_4, and concentrated under vacuum to give the MEM ether (160 mg, gum) without further purification.

To a solution of MEM ether (160 mg) in THF/MeOH (50%, 4 mL) was added NaOH (4 N, 1 mL). The mixture was stirred overnight, then neutralized with HCl (1 N), and extracted with EtOAc (2 × 25 mL). The organic layer was washed with water and brine, dried over Na_2SO_4, and then concentrated under vacuum to give **14b** (130 mg) without purification.

A mixture of **14b** (130 mg), DMAP (20 mg, 0.16 mmol), and 2,2-dimethylsuccinic anhydride (200 mg, 1.6 mmol) in anhydrous pyridine (5 mL) was refluxed overnight. After being concentrated in vacuo, the residue was diluted with dichloromethane, washed with water and brine, dried over Na_2SO_4, and then concentrated under vacuum. The residue was purified with Si gel chromatography to yield the 3,28-bi-functional BA derivative (40 mg, 28% yield in three steps).

A mixture of above bi-functional derivative MEM ether (40 mg, 0.04 mmol) and B-chlorocatecholborane (100 mg, 0.65 mmol) in dry dichloromethane (2 mL) was stirred overnight, after which water (1 mL) was added and the reaction mixture was stirred for 20 min. Then the mixture was diluted with dichloromethane (25 mL), washed with water and brine, dried over Na_2SO_4, and concentrated under vacuum. The residue was then purified with reverse-phase HPLC to yield **14** (10 mg).

5.2.7.1. (3*R*,4*S*)-*N*′-[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-8-aminooctanoyl]-4-amino-3-hydroxyl-6-methylheptanoic acid (14). Yield 28%. Mp 128–130 °C. Positive FABMS *m*/*z* 883 $(M+H)^+$; HR-FABMS calcd for $C_{52}H_{87}N_2O_9$ 883.6411, found 883.6412. ^{1}H NMR (300 MHz) δ 5.91 (1H, d, J = 7.0 Hz, NH), 5.82 (1H, br s, NH), 4.72, 4.60 (each 1H, each s, $=CH_2$), 4.89 (1H, dd, J = 5.2 Hz, J = 11.0 Hz, H-3), 4.39–4.10 (2H, m, C*H*–NH and C*H*–OH), 3.47–3.53, 2.91–2.98 (each 1H, m, NH–C*H*$_2$), 2.77, 2.47 (each 1H, d, J = 15.0 Hz, –C*H*$_2$–COO–CH), 1.68 (3H, s, CH_3-30), 1.29, 1.25 (each 3H, s, –C(C*H*$_3$)$_2$–COOH), 0.89–0.96 (12H, m, 4× CH_3), 0.73, 0.97, 0.81 (each 3H, s, 3× CH_3).

5.2.8. General procedure for preparation of 16–20. The synthesis of C-28 mono-modified BA was accomplished by following the procedures described previously by Dereu.[7,8] In brief, a mixture of 3-*O*-acyl betulinic acid (300 mg, 0.6 mmol) and oxalyl chloride (6 mmol, 2 M in CH_2Cl_2) was stirred for 10 min and then dried under vacuum. The residue was redissolved in dichloromethane and added slowly to a solution of the appropriate diaminoalkane (400 mg, 2.5–3.9 mmol) in dichloromethane (4 mL). After stirring overnight, the reaction mixture was concentrated, washed with water, redissolved in EtOH, and filtered to remove insoluble solid. The EtOH solution was concentrated under vacuum, and the residue was purified with Si gel chromatography to yield the corresponding amine intermediates **16a**–**20a**.

To a solution of the above amine intermediate (**16a**–**20a**) in anhydrous pyridine (4 mL) were added acetic anhydride (2 equiv) and DMAP (1 equiv). After stirring overnight at room temperature, the mixture was concentrated under vacuum. The residue was chromatographed on Si gel to yield the corresponding C-28 mono-substituted betulinic acid derivative, which was subjected to saponification as described above to yield the corresponding C-28 mono-modified intermediates **16b**–**20b**. Further modification of **16b**–**20b** using the general procedure for coupling at C-3 yielded the corresponding bi-functional compounds **16**–**20**.

5.2.8.1. [[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-5-aminopentyl]-carbamoyl]methane (16). Yield 27%. Mp 137 °C (dec). Positive FABMS *m*/*z* 711 $(M+H)^+$; HR-FABMS calcd for $C_{43}H_{71}N_2O_6$ 711.5312, found 711.5313. ^{1}H NMR (500 MHz) δ 6.02 (1H, br s, NH), 5.67 (1H, t, J = 6.0 Hz, NH), 4.72, 4.59 (each 1H, each s, $=CH_2$), 4.48 (1H, dd, J = 6.0 Hz, J = 10.0 Hz, H-3), 3.16–3.32 (4H, m, 2× C*H*$_2$–NH), 3.12 (1H, dt, J = 4.0 Hz, J = 11.0 Hz, H-19), 2.56, 2.67 (each 1H, d, J = 16.0 Hz, –C*H*$_2$–COO–CH), 2.43 (1H, dt, J = 2.5 Hz, J = 12.0 Hz, H-13), 2.01 (3H, s, $COCH_3$), 1.68 (3H, s, CH_3-30), 1.28, 1.30 (each 3H, s, –C(C*H*$_3$)$_2$–COOH), 0.80, 0.91, 0.96 (each 3H, s, 3× CH_3) 0.83 (6H, s, 2× CH_3). A complete chemical shift assignment of carbon and proton of **16** derived from ^{13}C and HMQC NMR was provided as Supplementary data (Table 2).

5.2.8.2. [[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-6-aminohexyl]-carbamoyl]methane (17). Yield 17%. Mp 137 °C (dec). Positive FABMS *m*/*z* 725 $(M+H)^+$; HR-FABMS calcd for $C_{44}H_{73}N_2O_6$ 725.5474, found 725.5469. ^{1}H NMR (300 MHz) δ 5.89 (1H, br s, NH), 5.70 (1H, t, J = 6.0 Hz, NH), 4.70, 4.58 (each 1H, each s, $=CH_2$), 4.47 (1H, dd, J = 6.5 Hz, J = 10.5 Hz, H-3), 3.17–3.29 (4H, m, 2× C*H*$_2$–NH), 3.10 (1H, dt, J = 3.5 Hz, J = 14.0 Hz, H-19), 2.56, 2.66 (each 1H, d, J = 16.0 Hz, –C*H*$_2$–COO–CH), 2.43 (1H, t, J = 12.0 Hz, H-13), 1.99 (3H, s, $COCH_3$), 1.67 (3H, s, CH_3-30), 1.27, 1.29 (each 3H, s, –C(C*H*$_3$)$_2$–COOH), 0.79, 0.91, 0.94 (each 3H, s, 3× CH_3) 0.81 (6H, s, 2× CH_3).

5.2.8.3. [[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-7-aminoheptyl]-carbamoyl]methane (18). Yield 29%. Mp 117–120 °C. Positive FABMS *m*/*z* 739 $(M+H)^+$; HR-FABMS calcd for $C_{45}H_{75}N_2O_6$ 739.5625, found 739.5615. ^{1}H NMR (300 MHz) δ 5.62 (2H, t, J = 5.0 Hz, 2× NH), 4.72, 4.59 (each 1H, each s, $=CH_2$), 4.49 (1H, dd, J = 6.5 Hz, J = 10.0 Hz, H-3), 3.09–3.30 (5H, m, 2× C*H*$_2$–NH and H-19), 2.55, 2.67 (each 1H, d, J = 16.0 Hz, –C*H*$_2$–COO–CH), 2.46 (1H, t, J = 11.0 Hz, H-13), 1.99 (3H, s, $COCH_3$), 1.68 (3H, s, CH_3-30), 1.29, 1.30 (each 3H, s, –C(C*H*$_3$)$_2$–COOH), 0.80, 0.93, 0.96 (each 3H, s, 3× CH_3) 0.83 (6H, s, 2× CH_3).

5.2.8.4. [[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-8-aminooctyl]-carbamoyl]methane (19). Yield 59%. Mp 113–115 °C. Positive FABMS *m*/*z* 753 $(M+H)^+$; HR-FABMS calcd for $C_{46}H_{77}N_2O_6$ 753.5781, found 753.5779. ^{1}H NMR (300 MHz) δ 5.83 (1H, br s, NH), 5.67 (1H, t, J = 5.5 Hz, NH), 4.72, 4.58 (each 1H, each s, $=CH_2$), 4.47 (1H, t, J = 7.0 Hz, H-3), 3.06–3.29 (5H, m, 2× C*H*$_2$–NH and H-19), 2.55, 2.66 (each 1H, d, J = 16.0 Hz, –C*H*$_2$–COO–CH), 2.43 (1H, t, J = 10.0 Hz, H-13), 2.00 (3H, s, $COCH_3$), 1.67 (3H, s, CH_3-30), 1.27, 1.29 (each 3H, s, –C(C*H*$_3$)$_2$–COOH), 0.79, 0.91, 0.95 (each 3H, s, 3× CH_3) 0.82 (6H, s, 2× CH_3).

5.2.8.5. [[*N*-[3β-*O*-(3′,3′-Dimethylsuccinyl)-lup-20(29)-en-28-oyl]-9-aminonoyl]-carbamoyl]methane (20). Yield 40%. Mp 120–122 °C. Positive FABMS *m*/*z* 698 $(M+H)^+$; HR-FABMS calcd for $C_{47}H_{79}N_2O_6$ 767.5938, found 767.5944. ^{1}H NMR (300 MHz) δ 5.72 (1H, br s, NH), 5.64 (1H, t, J = 5.5 Hz, NH), 4.72, 4.59 (each 1H, each s, $=CH_2$), 4.48 (1H, t, J = 7.0 Hz, H-3), 3.08–3.31 (5H, m, 2× C*H*$_2$–NH and H-19), 2.55, 2.67 (each 1H, d, J = 16.0 Hz, –C*H*$_2$–COO–CH), 2.42 (1H, t, J = 12.0 Hz, H-13), 2.00 (3H, s, $COCH_3$), 1.68 (3H, s, CH_3-30), 1.28, 1.29 (each 3H, s,

–C(CH_3)$_2$–COOH), 0.80, 0.92, 0.95 (each 3H, s, 3× CH_3) 0.82 (6H, s, 2× CH_3).

5.3. Anti-HIV assay

An HIV-1 infectivity assay previously described was used in the experiments.[20] A 96-well microtiter plate was used to set up the HIV-1 NL4-3 replication assay. HIV-1 NL4-3 at a multiplicity of infection (MOI) of 0.01 was used to infect MT4 cells. Culture supernatants were collected on day 4 post infection for the p24 assay using an ELISA kit from ZeptoMetrix Corporation (Buffalo, New York).

5.4. Cell fusion assay

TZM cells that express luciferase upon fusion with envelope-expressing COS cells were used as fusion partners. The fusion assays were performed by transfecting monkey kidney cells (COS) with the expression vector pSRHS that contains the HIV-1 NL4-3 envelope genes. 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. Electroporation was performed using a gene pulser (BioRad, Hercules, CA) with capacitance set at 950 μF and voltage at 150 V. The transfected COS cells were cultured for one day before mixing with TZM cells. TZM cells (10×10^4) were incubated with COS cells (10^4) in 96-well flat-bottomed plates (Costar) in 100 μL culture medium. Compounds to be tested at various concentrations in 10 μL culture medium were incubated with the cell mixtures at 37 °C for 24 hours. A Promega luciferase assay kit was used to quantify luciferase activity in the fused cells using a Biotek luminometer. The drug concentration that reduced the control HIV-1 envelope-mediated fusion by 50% is defined as 50% effective concentration (EC_{50}).

5.5. HIV-1 maturation assay

MT4 cells (10^5 cells/mL) were infected with HIV-1 NL4-3 at a multiplicity of infection (MOI) of 0.01 infectious units/cells for three days. The culture medium was removed and the infected cells were resuspended at 5×10^5 cells/mL and treated with BA derivatives at various concentrations. The virions in the culture supernatant were collected one day after drug treatment. The viral particles were spun down and subjected to Western blot analysis of p24 and p25 as previously described.[10,16]

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Supplementary data

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