New Betulinic Acid Derivatives for Bevirimat-Resistant Human Immunodeficiency Virus Type-1

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Abstract

Bevirimat (**1**, BVM) is an anti-HIV agent that blocks HIV-1 replication by interfering with HIV-1 Gag-SP1 processing at a late stage of viral maturation. However, clinical trials of **1** have revealed a high baseline drug resistance that is attributed to naturally-occurring polymorphisms in HIV-1 Gag. To overcome the drug resistance, 28 new derivatives of **1** were synthesized and tested against compound **1**-resistant (BVM-R) HIV-1 variants. Among them, compound **6** exhibited much improved activity against several HIV-1 strains carrying BVM-R polymorphisms. Compound **6** was at least 20-fold more potent than **1** against the replication of NL4-3/V370A, which carries the most prevalent clinical BVM-R polymorphism in HIV-1 Gag-SP1. Thus, compound **6** merits further development as a potential anti-AIDS clinical trial candidate.

Keywords

Betulinic acid; Bevirimat; HIV-1; Maturation inhibitors; Bevirimat-resistance

INTRODUCTION

After three decades of battle against Acquired Immunodeficiency Syndrome (AIDS) and its causative agent human immunodeficiency virus type 1 (HIV-1), HIV-1 infection remains one of the leading causes of infectious diseases worldwide with more than 30 million people currently living with HIV/AIDS.[1] Although current anti-retroviral therapy (ART) has drastically improved the clinical outcome of AIDS, it fails to eradicate HIV-1 from patients.[2-4] Thus, long-term treatment is required to suppress HIV-1 replication in infected patients. The emergence of drug-resistant HIV strains and the side effects/toxicities associated with long-term drug treatment present additional obstacles for the clinical management of HIV-1 infection.[5,6] Therefore, identifying new drug candidates with novel mechanisms of action is vital to advancing AIDS therapy.

Bevirimat (**1**, BVM), also known as PA-457, DSB, and MPC-4326, is a first-in-class HIV-1 maturation inhibitor that interferes with the CA-SP1 cleavage step of Gag processing,

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resulting in the accumulation of p25 and immature viral cores.[7-10] Clinical trials indicated that **1** caused a significant and clinically relevant reduction of the viral load in both ART-experienced and naive patients.[11,12] However, the clinical trials also revealed a high baseline drug resistance to **1**. A significant fraction (40–50%) of treated patients had pre-existing resistant viruses that had significantly reduced sensitivity to **1**.[13-15] This BVM-resistance was linked to naturally-occurring HIV polymorphisms in the SP1 region of HIV-1 Gag. Polymorphisms within three amino acids, Gln-Val-Thr (Q-V-T), at Gag positions 369–371 of the SP1, such as 370A instead of V or deleted V or T, were shown to be associated with the BVM-resistance (Table 1). A polymorphism of 362I in the CA region of Gag was also reported to confer resistance to compound **1**.[16] Analysis of SP1 sequences indicated that 370A is the most prevalent polymorphism among HIV-1 isolates from subtype B and C viruses.[17] The high baseline drug resistance due to the existing polymorphisms poses serious limitations to the clinical potential and further development of compound **1**.

It has been shown that **1** does not bind to monomeric Gag, but rather binds to an immature Gag to interfere with the CA-SP1 cleavage. It was suggested that **1** associated with an alpha helical structure of SP1 of immature viral particles.[18,19] SP1 with BVM-R polymorphisms could disrupt the binding of **1**, leading to a loss of sensitivity to the compound. The goal of this study was to modify the structure of **1** to overcome the resistance. Our prior studies indicated that attachment of a side chain at the C28 position of **1** resulted in improved activity. For example compound **30** (*N*-[3β-*O*-(3′,3′-dimethylsuccinyl)lup-20(29)-en-28-oyl]-5-piperazinepentanoic acid), with a C28 piperazinepentanoic acid side chain on the compound **1** molecule, exhibited a greater than 10-fold increase in anti-HIV-1 maturation activity.[20] In spite of its increased activity against wild type virus, **30** was not effective against BVM-R viruses. Nevertheless, its improved anti-HIV-1 activity against the NL4-3 strain suggested that C28 modifications could impact drug-target interaction. Thus, the rationale of this study was that compound **1** analogs with optimized C28 side chains might improve drug-target interaction and overcome the resistance to compound **1**.

RESULTS

New analogs were synthesized with compound **1** as the scaffold and various spacer/R' groups at the C28 position (Figure 1). Compounds **2–18** contain an ω-aminoalkanoic acid spacer [H_2N-$(CH_2)n$-$COOH$], where n ranged from 6–9. Compounds **19–29** have a 1,ω-diaminoalkane [H_2N-$(CH_2)n$-NH_2] spacer, where n was 7–9.

As shown in Scheme 1, compounds **2–18** were synthesized by first coupling the C28 carboxylic acid of 3-*O*-acetate-BA with benzyl ω-aminoalkanoates (Table 2). The coupling reaction was then followed by alkaline hydrolysis to form intermediates **2a–4a** and **18a**, which are known compounds that have been previously reported for their activities against HIV-1 and HIV-2 viruses.[21-23] Further modifications at the C28 terminal carboxylic acid of the above intermediates with the corresponding amino acid ester in the presence of EDC, HOBt, and NMM resulted in **2b–4b** and **6b–18b**. Treatment of **2b–4b** and **6b–18b** with 2,2-dimethylsuccinic anhydride and DMAP in pyridine at 140 °C for 2 h using microwave radiation yielded the final compounds **2–4** and **6–18**. Compound **5** was obtained by alkaline hydrolysis of **4**.

Compounds **19–29** were synthesized by coupling the diaminoalkane with the C28 carboxylic acid of 3-*O*-acetate-BA to form amine intermediates **19a**, **21a** and **26a**.[21,23] Further coupling of the above amine intermediate with a corresponding N-protected amino acid, followed by alkaline hydrolysis of the esters at C3 resulted in compounds **19b–29b**. Esterification of **19b–29b** at the C3 hydroxyl formed the end products **19–29** (Scheme 2; Table 3).

Activity of Compound 1 Derivatives against BVM-R HIV-1 Variant V370A

To evaluate the effectiveness of the newly synthesized compound **1** analogs against BVM-R HIV-1 strains, a panel of NL4-3 mutants carrying BVM-R polymorphisms was constructed.[23] The NL4-3 sequences of the polymorphic site in SP1 and its flanking region are shown in Table 1 along with the sequences of BVM-R mutants. The synthesized compounds were tested initially against both the wild type HIV-1 NL4-3 and NL4-3/V370A, a variant that carries the most prevalent BVM-R polymorphism with an Ala at position 370 in the SP1 region.

As expected, **1** potently inhibited wild type NL4-3 with an IC_{50} of 0.076 μM, but was ineffective at up to 6.8 μM against the BVM-R variant NL4-3/V370A. Most of the newly synthesized analogs (**2–29**) were more potent than **1** against wild type NL4-3 (Tables 2 and 3). Twenty-four of the new compounds had IC_{50} values in the range of 0.01–0.05 μM, six in the range of 0.07–0.18 μM, and three were over 0.2 μM or inactive. In contrast to **1**, most of the new C28 modified analogs exhibited inhibitory activity against the BVM-R NL4-3/ V370A variant with IC_{50} values less than 3 μM. Ten of the tested compounds exhibited significant potency against this variant with IC_{50} values ranging from 0.16 to 0.47 μM. Among them, compounds **5, 6, 14, 15, 20, 21, 24**, and **29** also had improved potency (IC_{50} 0.05 μM) against wild type NL4-3 compared with **1**. Compound **6** with a C28 methyl nonanoyl-glutaminate side chain, exhibited the best anti-HIV potency against both wild type virus and the BVM-R NL4-3/V370A variant with IC_{50} values of 0.01 and 0.16 μM, respectively. The improved activity of these compounds over **1** was not accompanied with increased cytotoxicity, as their CC_{50} values were comparable to that of **1** (Tables 2 and 3).

Compound 6 Inhibited Multiple NL4-3 Variants Carrying BVM-R Polymorphisms

Due to its superior anti-HIV activity, compound **6** was tested against three more BVM-R variants that carry BVM-R polymorphisms (Table 1). While these viruses were not sensitive to **1** (Table 4), all four BVM-R variants with V370Δ, V370A, V362I, and T371Δ polymorphisms were sensitive to **6**. The IC_{50} values of **6** against these resistant variants ranged from 0.016 to 0.32 μM compared with 0.008 μM against wild type NL4-3 (Table 4). The rank order of sensitivity of the BVM-R variants to **6** was NL4-3/V362I > NL4-3/T371Δ > NL4-3/V370A > NL4-3/V370Δ.

Inhibition of CA-SP1 Processing of BVM-R HIV-1 Variant by Compound 6

As a maturation inhibitor, compound **1** causes the accumulation of p25 by interfering with the HIV-1 Gag CA-SP1 cleavage.[7-10] To determine whether anti-HIV-1 maturation was indeed responsible for the observed anti-HIV activity, **6** was tested in a standard anti-maturation assay that measures p25 accumulation.[10,18] NL4-3 and the BVM-R variant NL4-3/V370A were produced by 293T cells in the presence of **1** or **6**. The Gag CA-SP1 (p25) protein and its processed product p24 were detected with Western blot analysis. As expected, **1** interfered with the cleavage of Gag CA-SP1 in wild type NL4-3 as shown by p25 accumulation, but did not inhibit the cleavage of Gag CA-SP1 in the drug resistant variant NL4-3/V370A under the same experimental conditions (*Figure 2a*). These results agree with the anti-viral assay results of **1**. On the other hand, treatment of the BVM-R NL4-3/V370A virus with **6** resulted in accumulation of CA-SP1 (p25), the signature event of inhibition of HIV-1 maturation. The accumulation of p25 was even more pronounced when the BVM-R NL4-3/V362I virus was produced in the presence of **6** (Figure 2b). This was consistent with the anti-viral results that NL4-3/V362I was most sensitive to **6** among the BVM-R mutants. These data indicated that **6** is a potent maturation inhibitor effective against both wild type and BVM-R mutants.

Compound 6 was Inactive against HIV-1 Entry

We and others have previously shown that betulinic acid (BA) derivatives with C28 modified side chains can block HIV-1 replication at the viral entry step.[25,26] To determine whether **6** also inhibits HIV-1 entry, **6** was tested in a fusion assay that was previously established for studying HIV-1 entry inhibitors.[23] A43D (**31**), a BA derivative with a C28 modified side chain but without the C3 side chain of **1**, was used as a positive control for inhibition of HIV-1 entry.[24] The results indicated that **1** and **6** do not inhibit HIV-1 entry, while **31** strongly inhibited the HIV-1 NL4-3 Env-mediated cell-cell fusion with an IC_{50} of 0.042 μM (Figure 3). These results suggested that **6** inhibited the maturation of BVM-R viruses without affecting viral entry.

DISCUSSION and CONCLUSIONS

This study demonstrates that structural optimization can result in analogs of **1** that are effective against BVM-R viruses. Compound **6** was at least 20-fold more potent than **1** against four BVM-R viruses. The improved potency against BVM-R viruses is due to the ability of the modified compounds to inhibit maturation of the viruses. Most of the derivatives of **1** that had increased potency against the resistant variants were also more potent against BVM-R viruses. However, improved potency against the sensitive virus alone was not sufficient to overcome resistance to compound **1**. For example, compound **30** was more potent than other tested compounds against the compound **1**-sensitive virus NL4-3, but it was ineffective against BVM-R variants. It appears that the improved activity against BVM-R viruses is associated with the BA scaffold modified at the C28 position with a long aliphatic side chain containing a spacer with 7–9 methylenes and terminated with a suitable amide group. This structural feature is shared by **5, 6, 14, 15, 20, 21, 24**, and **29**, which displayed significant improvements in potency against BVM-R viruses.

The BVM-R variants were not equally sensitive to **6**. The differential sensitivity is probably due to the variation in the amino acid residues at the drug resistant polymorphism site. In summary, it is possible to overcome resistance to **1**, since **6** showed markedly improved activity against the variant with the most prevalent BVM-R polymorphism, 370A in SP1 of HIV-1 Gag. However, optimal structural requirement(s) for inhibition of each BVM-R variant may vary, since the drug resistant polymorphisms involve multiple amino acid residues.

EXPERIMENTAL SECTION

General. Chemistry

All solvents and reagents were used as received from Sigma-Aldrich or other commercial sources. Positive or negative HR-FABMS were recorded on a Shimadzu LCMS-IT-TOF or a Joel SX-102 mass spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Varian Mercury 300 MHz spectrometer. Samples were dissolved in $CDCl_3$ with TMS as an internal standard unless specified. Silica gel chromatography was carried out on an ISCO CombiFlash Rf flash chromatograph system with a pre-packed Redi Sep Rf Si gel column (Teledyne ISCO), and MeOH/DCM (0–10% gradient) as mobile phase. Compounds were analyzed and purified (as needed) by using a Varian ProStar HPLC system with a PDA detector and Agilent Zorbax C18 columns (5 μM particle size, 4.6 × 250 mm and 9.4 × 250 mm for analytical and semi preparative scales, respectively). The mobile phase was composed of solution A (5% acetonitrile in water with 0.045% trifluoroacetic acid) and solution B (water/methanol/acetonitrile = 5:10:85 with 0.045% trifluoroacetic acid). A linear gradient of 80% to 100% of solution B with a flow rate at 1 mL/min or 4 mL/min was used in HPLC experiments. The compounds were analyzed with the UV absorption displayed at

220 nm and recorded at a range from 200 to 250 nm. All tested compounds have purity of 95% or above determined by HPLC analysis.

General Procedure for Preparation of Compounds 2–4, 6–18 (Scheme 1)

The intermediates **2b–4b** and **6b–18b** were synthesized using previously described methods.[21-23] Under enclosed conditions flushed with N_2, oxalyl chloride (2 mL, 20 mmol) was added to a stirring solution of betulinic acid-3-*O*-acetate (500 mg, 1 mmol) in DCM (anhydrous, 6 mL). After being stirred for 1 h at rt, the reaction mixture was concentrated and benzyl 9-amino nonanoate (315 mg, 1.2 mmol) in DCM (anhydrous, 6 mL) and Et_3N (0.85 mL, 6 mmol) were added. After being stirred overnight at rt, the reaction mixture was concentrated and chromatographed on Si-gel to yield 472 mg (63% yield) of intermediate. To a solution of this intermediate in THF (4 mL) and MeOH (4 mL), NaOH (2 mL, 4N) was added drop-wise. After being stirred overnight at rt, the reaction mixture was neutralized with HCl (1N). The precipitate was collected, washed with water, and dried in vacuum to give hydrolyzed intermediate **18a** (303 mg, 80% yield).

A solution of **18a** (45 mg, 0.07 mmol) in DCM (3 mL) was added to EDC-HCl (17 mg, 0.09 mmol), HOBt (11 mg, 0.09 mmol), NMM (17 mL, 0.15 mmol), and methyl glutaminate (33 mg, 0.21 mmol). After being stirred overnight at rt, the reaction mixture was diluted with DCM (40 mL), and then washed with water and brine. The organic layer was dried with Na_2SO_4 and concentrated in vacuum. The residue was chromatographed on Si-gel and reverse phase HPLC to yield compound **18b** (28 mg, 51% yield).

A mixture of intermediate **18b** (15 mg, 0.02 mmol), 2,2-dimethylsuccinic anhydride (35 mg, 0.27 mmol), and DMAP (3 mg, 0.025 mmol) in pyridine (anhydrous, 0.5 mL) was heated to 140 °C for 2 h in a microwave oven (Biotage initiator). The mixture was then concentrated under vacuum. The residue was chromatographed on Si-gel and reverse phase HPLC to yield compound **18** (6 mg, 34% yield).

The above method was used to synthesize compounds **2–4** and **6–17** using different reagents such as other alkanoates and/or amino acid derivatives instead of benzyl 9-amino nonanoate and methyl glutaminate, respectively.

Procedure for Preparation of Compound 5

NaOH (32 mg, 0.8 mmol) was added to a solution of **4** (4 mg, 0.005 mmol) in THF (1.4 mL), H_2O (0.7 mL) and MeOH (0.7 mL). After being stirred overnight at rt, the reaction mixture was neutralized with HCl (1N). The mixture was diluted with DCM (30 mL), and then washed with water and brine. The organic layer was dried with Na_2SO_4 and concentrated in vacuum. The residue was chromatographed on reverse phase HPLC to yield compound **5** (3 mg, yield 76%).

General Procedure for Preparation of Compounds 19–29 (Scheme 2)

Under enclosed conditions flushed with N_2, oxalyl chloride (12 mmol) was added to a stirring solution of betulinic acid-3-*O*-acetate (300 mg, 0.6 mmol) in DCM (anhydrous, 3 mL). After being stirred for 1 h at rt, the reaction was concentrated to dryness and was re-dissolved in DCM (anhydrous, 3 mL). The latter solution was added drop-wise to a stirring solution of 1,7-diaminoheptane (470 mg, 3.6 mmol) in DCM (10 mL). After being stirred overnight at rt, the reaction mixture was concentrated, re-dissolved in MeOH, and filtered. The filtrate was concentrated and chromatographed on Si-gel to yield **19a** (256 mg, yield 69%).

To a stirring solution of **19a** (43 mg, 0.07 mmol) in DCM (2 mL), EDC-HCl (53 mg, 0.27 mmol), HOBt (53 mg, 0.4 mmol), TEA (0.2 mmol), and Boc-glutamine (50 mg, 0.2 mmol) were added. After being stirred overnight at rt, the reaction mixture was diluted with DCM (40 mL), and then washed with water and brine. The organic layer was dried with Na_2SO_4 and concentrated in vacuum. The residue was chromatographed on Si-gel and reverse phase HPLC to yield intermediate (46 mg, 78% yield). To the mixture of this intermediate in THF (1 mL) and MeOH (1 mL), NaOH (0.5 mL, 4N) was added drop-wise. After being stirred for 5 h at rt, the reaction mixture was neutralized with HCl (1N), and then extracted with DCM. After removal of the organic solvent under vacuum, the residue was chromatographed on Si-gel to give **19b** (26 mg, 65% yield).

Using the same procedure described above to obtain **18** from **18b**, **19b** was further modified at the C3 position to furnish compound **19** (9 mg, 32%).

The above method (for obtaining **19** from betulinic acid-3-*O*-acetate) was used to synthesize compounds **20–29** using different reagents such as other 1,ω-diaminoalkane and/or amino acid derivatives instead of 1,7-diaminoheptane and Boc-glutamine, respectively.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-7-aminoheptanoyl]-L-glutaminate (2)—Yield 7 mg (22%). ^{1}H NMR (CDCl$_3$/Pyridine-d$_5$) δ 7.03 (bs, 1H, -CON*H*-), 6.81 and 5.68 (2bs, 1H each, -CON*H$_2$*), 5.83 (bs, 1H, -CON*H*-), 4.68 (s, 1H, =C*H*), 4.51–4.56 (m, 2H, -COC*H*-, =C*H*), 4.43 (dd, $J = 4.7$ Hz, $J = 11.1$ Hz, -C*H*- in 3), 3.67 (s, 3H, -OC*H$_3$*), 3.04–3.24 (m, 3H, -CONH-C*H$_2$*-, -C*H*- in 19), 2.66 and 2.55 (2d, $J = 15.3$ Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 1.62 (s, 3H, -C*H$_3$* in 30), 1.29 and 1.28 (2s, 3H each, -CO-C(C*H$_3$*)$_2$), 0.89 (s, 3H, -C*H$_3$*), 0.86 (s, 3H, -C*H$_3$*), 0.78 (s, 3H, -C*H$_3$*), 0.73 (s, 6H, 2 × -C*H$_3$*). Calcd for $C_{49}H_{79}N_3O_9Na$ (M+Na)[+]: 876.5714. Found: 876.5714.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-8-aminooctanoyl]-L-glutaminate (3)—Yield 10 mg (33%). ^{1}H NMR δ 6.95 and 6.88 (2bs, 1H each, -CON*H$_2$*), 6.64 (d, $J = 7.7$ Hz, 1H, -CON*H*-), 5.75 (t, $J = 5.4$ Hz, 1H, -CON*H*-), 4.72 (s, 1H, =C*H*), 4.55–4.62 (m, 2H, -COC*H*-, =C*H*), 4.47 (dd, $J = 6.5$ Hz, $J = 9.1$ Hz, 1H, -C*H*- in 3), 3.77 (s, 3H, -OC*H$_3$*), 3.04–3.30 (m, 3H, -CONH-C*H$_2$*-, -C*H*- in 19), 2.69 and 2.54 (2d, $J = 15.5$ Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 1.68 (s, 3H, -C*H$_3$* in 30), 1.29 and 1.27 (2s, 3H each, -CO-C(C*H$_3$*)$_2$), 0.96 (s, 3H, -C*H$_3$*), 0.91 (s, 3H, -C*H$_3$*), 0.82 (s, 6H, 2 × -C*H$_3$*), 0.78 (s, 3H, -C*H$_3$*). Calcd for $C_{50}H_{82}N_3O_9$ (M+H)[+]: 868.6046. Found: 868.6043.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-glycinate (4)—Yield 4 mg (34%). ^{1}H NMR δ 6.07 (bs, 1H, -CON*H*-), 5.60 (t, $J = 6.0$ Hz, 1H, -CON*H*-), 4.73 and 4.58 (2s, 1H each, =C*H$_2$*), 4.48 (m, 1H, -C*H*- in 3), 4.05 (d, $J = 5.2$ Hz, 2H, -CO-C*H$_2$*-NH-), 3.76 (s, 3H, -OC*H$_3$*), 3.10–3.30 (m, 3H, -CONH-C*H$_2$*-, -C*H*- in 19), 2.42–2.69 (m, 5H, -C(CH$_3$)$_2$-C*H$_2$*-, -CO-C*H$_2$*-, -C*H*- in 13), 1.67 (s, 3H, -C*H$_3$* in 30), 1.29 (m, 6H, -COC(C*H$_3$*)$_2$), 0.95 (s, 3H, -C*H$_3$*), 0.92 (s, 3H, -C*H$_3$*), 0.82 (s, 6H, 2 × -C*H$_3$*), 0.80 (s, 3H, -C*H$_3$*). Calcd for $C_{48}H_{79}N_2O_8$ (M+H)[+]: 811.5725. Found: 811.5729.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-glycine (5)—Yield 3 mg (76%). ^{1}H NMR (CDCl$_3$/Pyridine-d$_5$) δ 7.10 and 6.94 (2bs, 1H each, 2 × -CON*H*-), 4.80 and 4.63 (2s, 1H each, =C*H$_2$*), 4.54 (m, 1H, -C*H*- in 3), 4.15 (t, $J = 4.3$ Hz, 2H, -CO-C*H$_2$*NH-), 3.14–3.36 (m, 3H, -CONH-C*H$_2$*-, -C*H*- in 19), 2.64–2.79 (m, 5H, -C(CH$_3$)$_2$-C*H$_2$*-, -COC*H$_2$*-, -C*H*- in 13), 1.70 (s, 3H, -C*H$_3$* in 30), 1.30 (m, 6H, -CO-C(C*H$_3$*)$_2$), 0.97 (s, 6H, 2 × -C*H$_3$*), 0.87 (s, 3H, -C*H$_3$*), 0.82 (s, 3H, -C*H$_3$*), 0.79 (s, 3H, -C*H$_3$*). Calcd for $C_{47}H_{76}N_2O_8Na$ (M+Na)[+]: 819.5499. Found: 819.5470.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-glutaminate (6)—Yield 15 mg (40%). ^{1}H NMR (CDCl$_3$/Pyridine-d$_5$) δ 7.03 (d, J = 7.5 Hz, 1H, -CONH-), 6.86 (bs, 1H, 1H in -CONH_2), 5.85–5.89 (m, 2H, -CONH- and 1H in -CONH_2), 4.66 (s, 1H, =CH), 4.48–4.55 (m, 2H, -COCH-, =CH), 4.42 (dd, J = 4.5 Hz, J = 11.1 Hz, 1H, -CH- in 3), 3.64 (s, 3H, -OCH_3), 3.05–3.24 (m, 3H, -CONH-CH_2-, -CH- in 19), 2.65 and 2.53 (2d, J = 15.1 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.61 (s, 3H, -CH_3 in 30), 1.26 and 1.21 (2s, 3H each, -COC(CH_3)$_2$), 0.88 (s, 3H, -CH_3), 0.85 (s, 3H, -CH_3), 0.76 (s, 3H, -CH_3), 0.71 (s, 6H, 2 × -CH_3). ^{13}C NMR (CDCl$_3$/Pyridine-d$_5$, 75 MHz) δ 179.9, 176.3, 175.1,173.8, 172.7, 171.6, 151.2, 109.5, 81.3, 55.7, 55.6, 52.5, 52.1, 50.7, 50.3, 47.0, 45.3, 42.6, 40.9, 40.7, 39.3, 38.6 × 2, 37.9, 37.2, 36.5, 34.5, 33.9, 32.0, 31.1, 30.0, 29.9, 29.6, 29.3, 29.2 × 2, 28.0, 27.1, 26.1, 25.7 × 2, 25.6, 23.8, 21.1, 19.6, 18.3, 16.7, 16.3 × 2, 14.8. Calcd for C$_{51}$H$_{84}$N$_3$O$_9$ (M+H)$^+$: 882.6202. Found: 882.6203.

t-Butyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-glutaminate (7)—Yield 2 mg (17%). ^{1}H NMR δ 7.52, 6.91, and 5.64 (3bs, 1H each, -CONH-, -CONH_2), 6.23 (d, J = 7.8 Hz, 1H, -CONH-), 4.73 and 4.60 (2s, 1H each, =CH_2), 4.40–4.50 (m, 2H, -CO-CH-NH-, -CH- in 3), 2.94–3.16, 3.42–3.70 (m, 3H, -CONH-CH_2-, -CH- in 19), 2.79 and 2.47 (2d, J = 15.0 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.47 (s, 9H, -C(CH_3)$_3$), 1.28 (m, 6H, -CO-C(CH_3)$_2$), 0.96 (s, 3H, -CH_3), 0.92 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.78 (s, 3H, -CH_3), 0.77 (s, 3H, -CH_3). Calcd for C$_{54}$H$_{89}$N$_3$O$_9$ Na (M+Na)$^+$: 946.6496. Found: 946.6498.

Benzyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-glutaminate (8)—Yield 5 mg (28%). ^{1}H NMR δ 7.36 (bs, 5H, ArH), 7.22 (bs, 2H, -CONH_2-), 6.83 (bs, 1H, -CONH-), 6.59 (d, J = 6.9 Hz, 1H, -CONH-), 5.19 (s, 2H, -OCH_2-), 4.73 (s, 1H, =CH), 4.60 (m, 2H, -CO-CH-NH-, =CH), 4.71 (m, 1H, -CH- in 3), 3.30–3.42, 3.00–3.17 (m, 3H, -CONH-CH_2-, -CH- in 19), 2.71 and 2.54 (2d, J = 15.9 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.28 (m, 6H, -CO-C(CH_3)$_2$), 0.96 (s, 3H, -CH_3), 0.90 (s, 3H, -CH_3), 0.82 (s, 3H, -CH_3), 0.78 (s, 6H, 2 × -CH_3). Calcd for C$_{57}$H$_{87}$N$_3$O$_9$Na (M+Na)$^+$: 980.6340. Found: 980.6355.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-isoleucinate (9)—Yield 14 mg (48%). ^{1}H NMR δ 6.18 (d, J = 8.7 Hz, 1H, -CONH-), 5.71 (bs, 1H, -CONH-), 4.72 (s, 1H, =CH), 4.59–4.67 (m, 2H, -CONH-CH-, =CH), 4.48 (t, J = 6.0 Hz, -CH-in 3), 3.74 (s, 3H, -OCH_3), 3.05–3.26 (m, 3H, -CONH-CH_2-, -CH- in 19), 2.67 and 2.56 (2d, J = 15.9 Hz, 2H, -C(CH$_3$)$_2$-CH_2-), 2.39 (t, J = 10.2 Hz, 1H, -CH- in 13), 2.63 (t, J = 7.2 Hz, 2H, -CO-CH_2-), 1.67 (s, 3H, -CH_3 in 30), 1.29 (m, 6H, -CO-C(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.91 (s, 6H, 2 × -CH_3), 0.89 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{52}$H$_{86}$N$_2$O$_8$Na (M+Na)$^+$: 889.6282. Found: 889.6294.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-prolinate (10)—Yield 17 mg (59%). ^{1}H NMR δ 5.75 (bs, 1H, -CONH-), 4.72 and 4.59 (2s, 1H each, =CH_2), 4.45–4.54 (m, 2H, -CON-CH-, CH- in 3), 3.51-3.77 (m, 5H, -OCH_3, -CONCH_2-), 3.04–3.25 (m, 3H, -CONH-CH_2-, -CH- in 19), 2.67 and 2.56 (2d, J = 16.2 Hz, 2H, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.29 (m, 6H, -CO-C(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{51}$H$_{82}$N$_2$O$_8$Na (M+Na)$^+$: 873.5969. Found: 873.5989.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-phenylalaninate (11)—Yield 18 mg (67%). ^{1}H NMR δ 7.26–7.28 (m, 3H, ArH), 7.08 (d, 2H, J = 6.6 Hz, ArH), 5.92 (d, J = 7.2 Hz, 1H, -CONH-), 5.59 (bs,

1H, -CON*H*-), 4.90 (dd, *J* = 6.0 Hz, *J* = 12.9 Hz, 1H, -CO-C*H*-NH-), 4.72 and 4.58 (2s, 1H each, =C*H₂*), 4.47 (t, *J* = 6.3 Hz, 1H, -C*H*- in 3), 3.72 (s, 3H, -OC*H₃*), 3.67 (d, 2H, *J* = 6.9 Hz, -C*H₂*-C₆H₆), 3.05–3.30 (m, 3H, -CONHC*H₂*-, -C*H*- in 19), 2.52–2.69 (m, 2H, -C(CH₃)₂-C*H₂*-), 2.44 (t, *J* = 9.9 Hz, 1H, -C*H*- in 13), 1.67 (s, 3H, -C*H₃* in 30), 1.28 (m, 6H, -CO-C(C*H₃*)₂), 0.95 (s, 3H, -C*H₃*), 0.92 (s, 3H, -C*H₃*), 0.82 (s, 6H, 2 × -C*H₃*), 0.79 (s, 3H, -C*H₃*). Calcd for $C_{55}H_{84}N_2O_8Na$ (M+Na)⁺: 923.6125. Found: 923.6142.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-valinate (12)—Yield 8 mg (45%). ¹H NMR δ 6.09 (d, *J* = 8.4 Hz, -CON*H*-), 5.67 (m, 1H, -CON*H*-), 4.72 (s, 1H, =C*H*), 4.56–4.60 (m, 2H, =C*H*, -CO-C*H*-NH-), 4.48 (t, *J* = 6.9 Hz, 1H, -C*H*- in 3), 3.75 (s, 3H, -OC*H₃*), 3.07–3.28 (m, 3H, -CONH-C*H₂*-, -C*H*- in 19), 2.67 and 2.56 (2d, *J* = 15.9 Hz, 1H each, -C(CH₃)₂-C*H₂*-), 2.41 (t, *J* = 11.7 Hz, -C*H*- in 13), 2.27 (t, *J* = 7.5 Hz, 2H, -COC*H₂*-), 1.68 (s, 3H, -C*H₃* in 30), 1.28 (m, 6H, -CO-C(C*H₃*)₂), 0.95 (s, 3H, -C*H₃*), 0.92 (m, 6H, 2 × -C*H₃*), 0.89 (s, 3H, 2 × -C*H₃*), 0.82 (s, 6H, 2 × -C*H₃*), 0.79 (s, 3H, -C*H₃*). Calcd for $C_{51}H_{84}N_2O_8Na$ (M+Na)⁺: 875.6125. Found: 875.6118.

Dimethyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-glutamate (13)—Yield 12 mg (64%). ¹H NMR δ 6.57 (d, *J* = 7.8 Hz, -CON*H*-), 5.78 (bs, 1H, -CON*H*-), 4.72 and 4.59 (2s, 1H each, =C*H₂*), 4.61–4.66 (m, 1H, -CO-C*H*-NH-), 4.61 (t, *J* = 6.6 Hz, 1H, -C*H*- in 3), 3.76 and 3.69 (2s, 3H each, 2 × -OC*H₃*), 3.12–3.32 (m, 2H, -CONH-C*H₂*-), 3.03–3.09 (m, 1H, -C*H*- in 19), 2.67 and 2.57 (2d, *J* = 15.9 Hz, 1H each, -C(CH₃)₂-C*H₂*-), 2.15–2.48 (m, 7H, 2 × -COC*H₂*-, -NHCH-C*H₂*-, -C*H*- in 13), 1.68 (s, 3H, -C*H₃* in 30), 1.28 (m, 6H, -CO-C(C*H₃*)₂), 0.95 (s, 3H, -C*H₃*), 0.91 (s, 3H, -C*H₃*), 0.82 (s, 6H, 2 × -C*H₃*), 0.79 (s, 3H, -C*H₃*). Calcd for $C_{52}H_{84}N_2O_{10}Na$ (M+Na)⁺: 919.6024. Found: 919.6052.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-L-alaninate (14)—Yield 7 mg (42%). ¹H NMR δ 6.33 (d, *J* = 6.3 Hz, -CON*H*-CH-), 5.76 (bs, 1H, -CON*H*-), 4.73 (s, 1H, =C*H*), 4.53–4.63 (m, 2H, =C*H*, -CO-C*H*-NH-), 4.48 (t, *J* = 8.4 Hz, 1H, -C*H*- in 3), 3.77 (s, 3H, -OC*H₃*), 3.15–3.30 (m, 2H, -CONH-C*H₂*-), 3.07 (t, 1H, *J* = 8.4 Hz, 1H, -C*H*- in 19), 2.68 and 2.57 (2d, *J* = 15.6 Hz, 1H each, -C(CH₃)₂-C*H₂*-), 2.38 (t, *J* = 10.8 Hz, 1H, -C*H*- in 13), 2.27 (t, *J* = 7.2 Hz, 2H, -COC*H₂*-) 1.68 (s, 3H, -C*H₃* in 30), 1.43 and 1.41 (d, *J* = 7.5 Hz, 3H, -CH-C*H₃*), 1.30 (m, 6H, -CO-C(C*H₃*)₂), 0.96 (s, 3H, -C*H₃*), 0.91 (s, 3H, -C*H₃*), 0.82 (s, 6H, 2 × -C*H₃*), 0.80 (s, 3H, -C*H₃*). Calcd for $C_{49}H_{80}N_2O_8Na$ (M+Na)⁺: 847.5812. Found: 847.5813.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-aminoacetonitrile (15)—Yield 12 mg (46%). ¹H NMR δ 6.57 and 5.66 (2bs, 1H each, 2 × -CON*H*-), 4.72 and 4.59 (2s, 1H each, =C*H₂*), 4.48 (t, 1H, *J* = 6.0 Hz, -C*H*- in 3), 4.18 (d, *J* = 5.7 Hz, 2H, -C*H₂*-CN), 3.08–3.34 (m, 3H, -CONH-C*H₂*-, -C*H*- in 19), 2.67 and 2.55 (2d, *J* = 15.9 Hz, 2H, -C(CH₃)₂-C*H₂*-), 2.42 (t, *J* = 11.4 Hz, 1H, -C*H*- in 13), 2.25 (t, *J* = 7.5 Hz, 2H, -CO-C*H₂*-), 1.68 (s, 3H, -C*H₃* in 30), 1.29 (m, 6H, -CO-C(C*H₃*)₂), 0.96 (s, 3H, -C*H₃*), 0.92 (s, 3H, -C*H₃*), 0.82 (s, 6H, 2 × -C*H₃*), 0.80 (s, 3H, -C*H₃*). Calcd for $C_{47}H_{75}N_3O_6Na$ (M+Na)⁺: 800.5554. Found: 800.5535.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-aminopropionitrile (16)—Yield 11 mg (46%). ¹H NMR δ 6.40 (bs, 1H, -CON*H*-), 5.73 (t, *J* = 5.1 Hz, 1H, -CON*H*-), 4.71 and 4.59 (2s, 1H each, =C*H₂*), 4.48 (t, 1H, *J* = 6.9 Hz, -C*H*- in 3), 3.52 (dd, *J* = 6.0 Hz, *J* = 15.6 Hz, 2H, -CONH-C*H₂*-), 3.14–3.30 (m, 2H, -CONH-C*H₂*-), 3.07 (dt, *J* = 3.6 Hz, *J* = 14.7 Hz, 1H, -C*H*- in 19), 2.53–2.70 (m, 4H, -C(CH₃)₂-C*H₂*-, -C*H₂*-CN), 2.39 (t, *J* = 11.7 Hz, 1H, -C*H*- in 13), 2.24 (t, *J* = 8.1 Hz, 2H, -CO-C*H₂*-),

1.68 (s, 3H, -CH_3 in 30), 1.29 (m, 6H, -CO-C(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{48}$H$_{77}$N$_3$O$_6$Na (M+Na)[+]: 814.5710. Found: 814.5719.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononanoyl]-N-methylaminoacetonitrile (17)—Yield 7 mg (39%). ^{1}H NMR δ 5.75 (bs, 1H, -CONH-), 4.72 and 4.59 (2s, 1H each, =CH_2), 4.46 (t, 1H, J = 6.6 Hz, -CH- in 3), 4.37 (s, 2H, -CH_2-CN), 3.04–3.28 (m, 6H, -CONH-CH_2-, -N-CH_3, -CH- in 19), 2.67 and 2.55 (2d, J = 15.9 Hz, 2H each, -C(CH$_3$)$_2$-CH_2-), 2.34–2.39 (m, 3H, -NCO-CH_2-, -CH- in 13), 1.67 (s, 3H, -CH_3 in 30), 1.28 (m, 6H, -COC(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{48}$H$_{78}$N$_3$O$_6$ (M+H)[+]: 792.5891. Found: 792.5923.

Methyl N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-10-aminodecanoyl]-L-glutaminate (18)—Yield 6 mg (34%). ^{1}H NMR δ 7.16 and 6.91 (2bs, 1H each, -CONH_2-) 6.56 (d, J = 6.9 Hz, 1H, -CONH-), 5.71 (bs, 1H, -CONH-), 4.72 (1s, 1H each, =CH), 4.59 (m, 2H, -CONH-CH-, =CH), 4.48 (m, 1H, -CH- in 3), 3.77 (s, 3H, -OCH_3-), 3.33–3.42 and 3.02–3.34 (m, 3H, -CO-CH_2-, -CH- in 19), 2.71 and 2.55 (2d, J = 15.6 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.29 (m, 6H, -CO-C(CH_3)$_2$), 0.96 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.82 (s, 3H, -CH_3), 0.80 (s, 3H, -CH_3), 0.79 (s, 3H, -CH). Calcd for C$_{52}$H$_{85}$N$_3$O$_9$Na (M+Na)[+]: 918.6183. Found: 918.6173.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-7-aminoheptyl]-N^a-boc-L-glutaminamide (19)—Yield 9 mg (32%). ^{1}H NMR δ 6.96 (d, J = 15.3 Hz, 1H, -CONH-), 6.73 (bs, 1H, -CONH-), 6.06 (bs, 2H, -CONH_2), 5.71–5.81 (m, 1H, -CONH-), 4.72 and 4.59 (2s, 1H each, =CH_2), 4.45 (t, 1H, J = 8.4 Hz, -CH- in 3), 4.10–4.23 (m, 1H, -COCH-), 3.03–3.35 (m, 5H, 2 × -CONH-CH_2-, -CH- in 19), 2.69 and 2.54 (2d, J = 15.9 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 2.32–2.39 (m, 3H, -CH_2-CONH$_2$, -CH- in 13), 1.67 (s, 3H, -CH_3 in 30), 1.43 (s, 9H, -C(CH_3)$_3$), 1.29 (m, 6H, -CO-C(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.81 (s, 6H, 2 × -CH_3), 0.78 (s, 3H, -CH_3). Calcd for C$_{53}$H$_{89}$N$_4$O$_9$ (M+H)[+]: 925.6630. Found: 925.6591.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-7-aminoheptyl]-N^a-boc-L-alaninamide (20)—Yield 6 mg (23%). ^{1}H NMR δ 6.53 (t, J = 4.8 Hz, 1H, -CONH-), 5.77 (t, J = 5.4 Hz, 1H, -CONH-), 5.30 (bs, 1H, -CONH-), 4.72 and 4.59 (2s, 1H each, =CH_2), 4.68 (t, 1H, J = 6.9 Hz, -CH- in 3), 4.16–4.23 (m, 1H, -COCH-), 3.03–3.28 (m, 5H, 2 × -CONH-CH_2-, -CH- in 19), 2.67 and 2.56 (2d, J = 16.2 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 2.38 (t, J = 12.0 Hz, 1H, -CH- in 13), 1.67 (s, 3H, -CH_3 in 30), 1.44 (s, 9H, -C(CH_3)$_3$), 1.35 (d, J = 6.9 Hz, 3H, -CH-CH_3), 1.28 (m, 6H, -CO-C(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.90 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{51}$H$_{86}$N$_3$O$_8$ (M+H)[+]: 868.6415. Found: 868.6462.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-8-aminooctyl]-N^a-boc-L-glutaminamide (21)—Yield 8 mg (35%). ^{1}H NMR δ 7.17, 6.86, 6.60, 5.73 and (4bs, 1H each, 3 × -CONH-, 1 H in -CONH_2-), 4.72 and 4.59 (2s, 1H each, =CH_2), 4.47 (t, 1H, J = 6.6 Hz, -CH- in 3), 4.13 (m, 1H, -COCH-), 3.34–3.42 (m, 1H, 1H in -CONH-CH_2-), 3.23 (dd, J = 6.3 Hz, J = 13.8 Hz, 2H, -CONH-CH_2-), 3.02–3.12 (m, 2H, 1H in -CONH-CH_2-, -CH- in 19), 2.71 and 2.54 (2d, J = 15.6 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.43 (s, 9H, -C(CH_3)$_3$), 1.28 (m, 6H, -CO-C(CH_3)$_2$), 0.96 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{54}$H$_{90}$N$_4$O$_9$Na (M+Na)[+]: 961.6605. Found: 961.6623.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-8-aminooctyl]-N^a-boc-L-valinamide (22)—Yield 11 mg (41%). ^{1}H NMR δ 6.45 (bs, 1H, -CON*H*-), 5.74 (t, *J* = 5.7 Hz, 1H, -CON*H*-), 5.40 (bs, 1H, -CON*H*-), 4.72 and 4.58 (2s, 1H each, =C*H$_2$*), 4.47 (t, 1H, *J* = 7.2 Hz, -C*H*- in 3), 3.84 (t, *J* = 7.8 Hz, 1H, -COC*H*-), 3.06–3.29 (m, 6H, 2 × -CONH-C*H$_2$*-, -C*H*-(CH$_3$)$_2$, -C*H*- in 19), 2.67 and 2.56 (2d, *J* = 15.9 Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 2.38 (t, *J* = 9.9 Hz, 1H, -C*H*- in 13), 1.67 (s, 3H, -C*H$_3$* in 30), 1.43 (s, 9H, -C(C*H$_3$)$_3$*), 1.35 (d, *J* = 6.9 Hz, 3H, -CH-C*H$_3$*), 1.28 (m, 12H, -CH-(CH$_3$)$_2$, -CO-C(C*H$_3$)$_2$*), 0.95 (s, 3H, -C*H$_3$*), 0.91 (s, 3H, -C*H$_3$*), 0.81 (s, 6H, 2 × -C*H$_3$*), 0.79 (s, 3H, -C*H$_3$*). Calcd for C$_{54}$H$_{91}$N$_3$O$_8$Na (M+Na)$^+$: 932.6704. Found: 932.6708.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-8-aminooctyl]-N^a-boc-L-alaninamide (23)—Yield 3 mg (17%). ^{1}H NMR δ 6.36 (bs, 1H, -CON*H*-), 5.71 (t, *J* = 5.4 Hz, 1H, -CON*H*-), 5.18 (bs, 1H, -CON*H*-), 4.72 and 4.59 (2s, 1H each, =C*H$_2$*), 4.48 (t, 1H, *J* = 6.3 Hz, -C*H*- in 3), 4.16–4.25 (m, 1H, -COC*H*-), 3.06–3.30 (m, 5H, 2 × -CONH-C*H$_2$*-, -C*H*- in 19), 2.67 and 2.56 (2d, *J* = 15.9 Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 1.68 (s, 3H, -C*H$_3$* in 30), 1.44 (s, 9H, -C(C*H$_3$)$_3$*), 1.35 (d, *J* = 7.2 Hz, 3H, -CH-C*H$_3$*), 1.29 (m, 6H, -CO-C(C*H$_3$)$_2$*), 0.95 (s, 3H, -C*H$_3$*), 0.89 (s, 3H, -C*H$_3$*), 0.82 (s, 6H, 2 × -C*H$_3$*), 0.80 (s, 3H, -C*H$_3$*). Calcd for C$_{52}$H$_{86}$N$_3$O$_8$ (M-H)$^+$: 880.6415. Found: 880.6389.

2-(Boc-amino)-4-cyano-N-[N'-[3β-O-(3',3'-dimethylsuccinyl)-betulinic acid-28-oyl]-8-aminooctyl]butanamide (24)—Yield 2 mg (22%). ^{1}H NMR δ 6.32 (bs, 1H, -CON*H*-), 5.68 (bs, 1H, -CON*H*-), 5.30 (bs, 1H, -CON*H*-), 4.72 and 4.59 (2s, 1H each, =C*H$_2$*), 4.46 (m, 1H, -C*H*- in 3), 4.16–4.27 (m, 5H, -C*H$_2$*-C*H$_2$*-CN, -COC*H*-), 3.10–3.30 (m, 5H, 2 × -CONH-C*H$_2$*-, -C*H*- in 19), 2.68 and 2.56 (2d, *J* = 15.0 Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 1.68 (s, 3H, -C*H$_3$* in 30), 1.45 (s, 9H, -C(C*H$_3$)$_3$*), 1.30 (m, 6H, -CO-C(C*H$_3$)$_2$*), 0.92 (s, 6H, 2 × -C*H$_3$*), 0.89 (s, 3H, -C*H$_3$*), 0.83 (s, 6H, 2 × -C*H$_3$*), 0.80 (s, 3H, -C*H$_3$*). Calcd for C$_{54}$H$_{87}$N$_4$O$_8$ (M-H)$^-$: 919.6524. Found: 919.6481.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-8-aminooctyl]-N^a-boc-L-prolinamide (25)—Yield 9 mg (34%). ^{1}H NMR δ 6.90 and 6.48 (2bs, 1H each, 2 × -CON*H*-), 4.72 and 4.59 (2s, 1H each, =C*H$_2$*), 4.75 (t, 1H, *J* = 9.6 Hz, -C*H*- in 3), 4.22 (t, *J* = 4.5 Hz, 1H, -COC*H*-), 3.06–3.50 (m, 7H, 2 × -CONH-C*H$_2$*-, -CON-C*H$_2$*-, -C*H*- in 19), 2.66 and 2.56 (2d, *J* = 16.2 Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 2.39 (t, *J* = 10.8 Hz, 1H, -C*H*- in 13), 1.68 (s, 3H, -C*H$_3$* in 30), 1.45 (s, 9H, -C(C*H$_3$)$_3$*), 1.25 (s, 6H, -CO-C(C*H$_3$)$_2$*), 0.95 (s, 3H, -C*H$_3$*), 0.91 (s, 3H, -C*H$_3$*), 0.82 (s, 6H, 2 × -C*H$_3$*), 0.80 (s, 3H, -C*H$_3$*). Calcd for C$_{54}$H$_{88}$N$_3$O$_8$ (M-H)$^-$: 906.6571. Found: 906.6523.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononactyl]-N^a-boc-L-glutaminamide (26)—Yield 6 mg (21%). ^{1}H NMR δ 7.10, 6.93 and 6.63 (3s, 1H each, 2 × -CON*H*-, 1H in -CON*H$_2$*), 5.75 (t, *J* = 5.4 Hz, 1H, -CON*H*-), 5.71 (bs, 1H, 1H in -CON*H$_2$*), 4.72 and 4.59 (2s, 1H each, =C*H$_2$*), 4.48 (dd, 1H, *J* = 6.0 Hz, *J* = 9.6 Hz, -C*H*- in 3), 4.21 (dd, *J* = 4.2 Hz, *J* = 6.0 Hz, 1H, -COC*H*-), 3.05–3.40 (m, 5H, 2 × -CONH-C*H$_2$*-, -C*H*- in 19), 2.75 and 2.53 (2d, *J* = 15.9 Hz, 1H each, -C(CH$_3$)$_2$-C*H$_2$*-), 1.68 (s, 3H, -C*H$_3$* in 30), 1.43 (s, 9H, -C(C*H$_3$)$_3$*), 1.29 and 1.27 (2s, 3H each, -CO-C(C*H$_3$)$_2$*), 1.11 (s, 3H, -C*H$_3$*), 0.96 (s, 3H, -C*H$_3$*), 0.91 (s, 3H, -C*H$_3$*), 0.82 (s, 6H, 2 × -C*H$_3$*), 0.79 (s, 3H, -C*H$_3$*). Calcd for C$_{55}$H$_{91}$N$_4$O$_9$ (M-H)$^-$: 951.6786. Found: 951.6762.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononactyl]-N^a-boc-L-alaninamide (27)—Yield 12 mg (45%). ^{1}H NMR δ 6.42 and 5.50 (2bs, 1H each, 2 × -CON*H*-), 5.70 (t, *J* = 5.4 Hz, 1H, -CON*H*-), 4.72 and 4.59 (2s, 1H each, =C*H$_2$*), 4.47 (dd, 1H, *J* = 6.0 Hz, *J* = 9.6 Hz, -C*H*- in 3), 4.21 (dd, *J* = 3.3 Hz, *J* = 6.3 Hz, 1H, -COC*H*-), 3.02–3.30 (m, 5H, 2 × -CONH-C*H$_2$*-, -C*H*- in 19), 2.67 and 2.55 (2d, *J* = 15.6 Hz,

1H each, -C(CH$_3$)$_2$-CH_2-), 2.38 (t, J = 6.0 Hz, 1H, -CH- in 13), 1.67 (s, 3H, -CH_3 in 30), 1.43 (s, 9H, -C(CH_3)$_3$), 1.35 (d, J = 7.2 Hz, 3H, -CHCH_3), 1.28 and 1.27 (2s, 3H each, -CO-C(CH_3)$_2$), 0.95 (s, 3H, -CH_3), 0.91 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.79 (s, 3H, -CH_3). Calcd for C$_{53}$H$_{90}$N$_3$O$_8$ (M+H)$^+$: 896.6728. Found: 896.6773.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononactyl]-N^a-boc-L-glycinamide (28)—Yield 6 mg (34%). ^{1}H NMR δ 6.41, 5.72 and 5.34 (3bs, 1H each, 3 × -CONH-), 4.73 and 4.59 (2s, 1H each, =CH_2), 4.46–4.51 (m, 1H, -CH- in 3), 3.80 (d, J = 3.6 Hz, 2H, -COCH_2-NH-), 3.02–3.36 (m, 5H, 2 × -CONH-CH_2-, -CH- in 19), 2.70 and 2.56 (2d, J = 15.9 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.45 (s, 9H, -C(CH_3)$_3$), 1.25 (m, 6H, -COC(CH_3)$_2$), 0.96 (s, 3H, -CH_3), 0.92 (s, 3H, -CH_3), 0.82 (s, 6H, 2 × -CH_3), 0.80 (s, 3H, -CH_3). Calcd for C$_{52}$H$_{88}$N$_3$O$_8$ (M+H)$^+$: 882.6571. Found: 882.6553.

N-[N'-[3β-O-(3',3'-Dimethylsuccinyl)-betulinic acid-28-oyl]-9-aminononactyl]-N^a-boc-L-asparaginamide (29)—Yield 8 mg (41%). ^{1}H NMR δ 6.72, 6.21, 6.13 and 5.69 (4bs, 1H each, 3 × -CONH-, 1H in -CO-NH_2), 4.73 and 4.59 (2s, 1H each, =CH_2), 4.42–4.50 (m, 2H, -COCH-, -CH- in 3), 3.64–3.24 and 2.84–3.20 (m, 7H, 2 × -CONH-CH_2-, -COCH_2-, -CH- in 19), 2.78 and 2.47 (2d, J = 15.0 Hz, 1H each, -C(CH$_3$)$_2$-CH_2-), 1.68 (s, 3H, -CH_3 in 30), 1.46 (s, 9H, -C(CH_3)$_3$), 1.25 (m, 6H, -CO-C(CH_3)$_2$), 0.96 (s, 3H, -CH_3), 0.92 (s, 3H, -CH_3), 0.82 (s, 3H, -CH_3), 0.81 (s, 3H, -CH_3), 0.75 (s, 3H, -CH). Calcd for C$_{54}$H$_{89}$N$_4$O$_9$ (M-H)$^-$: 937.6630. Found: 937.6595.

Construction of BVM-R Variants

Construction of these mutants was achieved by using a QuickChange site-directed mutagenesis kit purchased from Stratagene as previously described.[23] The plasmid, pNL4-3, was used as a template to create all the mutants listed in Table 1. Each mutation was introduced into pNL4-3 following the protocol provided by Stratagene.

Multi-Cycle Viral Replication in MT4 Cell Assay

HIV-1 NL4-3 or the resistant variants with a multiplicity of infection (MOI) of 0.001 TCID$_{50}$/cell was used to infect MT4 cells in the presence of compounds at various concentrations. On day 4 post-infection, supernatant samples were harvested and assayed for p24 using an ELISA kit from Perkin Elmer. The antiviral potency is defined as the drug concentration that reduces the amount of p24 by 50% (IC$_{50}$).

Cytotoxicity Assay

A CytoTox-Glo™ cytotoxicity assay (Promega) was used to determine the cytotoxicity of the synthesized BA derivatives. MT4 cells were cultured in the presence of various concentrations of the compounds for 4 days. Percent of viable cells was determined by following the protocol provided by the manufacturer. The 50% cytotoxic concentration (CC$_{50}$) was defined as the concentration that caused a 50% reduction of cell viability.

Determination of Anti-HIV-1 Maturation Activity

Analysis of anti-maturation activity was performed as described previously.[10,18] Briefly, 293T cells were transfected with plasmids with corresponding proviruses, such as pNL4-3. The culture supernatant was harvested for Western blot analysis two days after transfection. The signature event of anti-maturation activity of compound **1** is a partial inhibition of CA-SP1 (p25) cleavage that occurs without interfering with other Gag cleavage sites. Inhibition of CA-SP1 cleavage resulted in an accumulation of p25. Accumulation of CA-SP1 (p25)

was analyzed with a Western blot analysis using a monoclonal antibody produced by the HIV-1 p24 hybridoma, 183-H12-5C (NIH AIDS Reagent Program).

Fusion Assay

The fusion assay used in this study was described previously.[24] The fusion assay was performed by transfecting monkey kidney cells (COS) with the expression vector pars that contained HIV-1 Env and tat genes. COS cells (1×10^6 cells/mL) were mixed with 5 μg of the Env-expressing vector and incubated on ice for 10 minutes. Electroporation was performed using a gene pulsar (Bio-Rad, Hercules, CA) with capacitance set at 950 F and voltage at 150 V. The transfected COS cells were cultured for one day and then mixed with TZM-bl cells. TZM-bl cells were incubated with the Env-expressing COS cells in the presence of inhibitors in 96-well flat-bottom plates (Costar) overnight. Fusion was measured by quantifying luciferase activity in the fused cells using a Bright-Glow luciferase assay kit (Promega, Luis Obispo, CA). Inhibition of the Env-mediated membrane fusion was expressed as a percentage of the control (Env-mediated membrane fusion in the absence of inhibitors).

Acknowledgments

ABBREVIATIONS USED

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
BA	betulinic acid
BVM-R	bevirimat-resistant
DCM	dichloromethane
DMAP	4-Dimethylaminopyridine
DSB	3-*O*-(3',3'-dimethylsuccinyl)-betulinic acid
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
HIV-1	human immunodeficiency virus type 1
HOBt	hydroxybenzotriazole
NMM	*N*-methylmorpholine
THF	tetrahydrofuran

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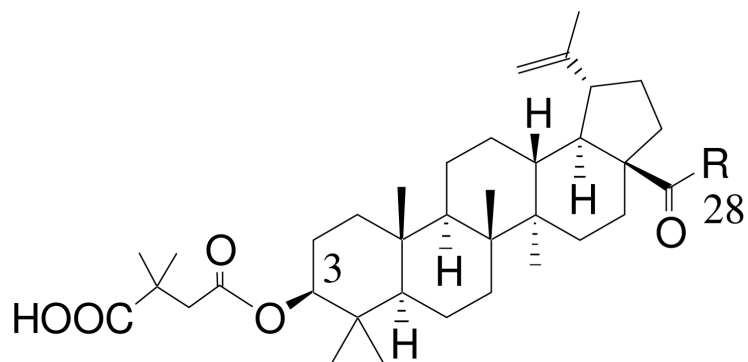
1 (Bevirimat): R = OH

New analogs:

2-18 R = -H₂N-(CH₂)n-COR', n = 6 - 9

19-29 R = -H₂N-(CH₂)n-NHR' n = 7 - 9

Figure 1.
Structure of compound **1** analogs.
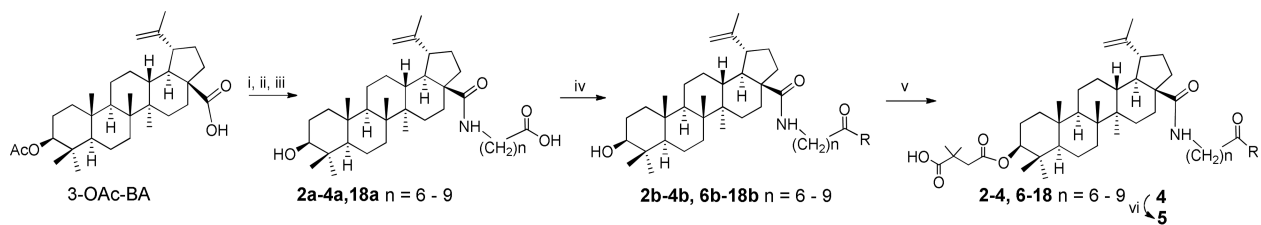
^{a}Reagents and conditions: (i) oxalyl chloride, DCM, 1h; (ii) $NH_2(CH_2)nCOOBn$, Et_3N, DCM, overnight; (iii) NaOH, THF, MeOH, 5h; (iv) R-H, HOBt, EDC, NMM, DCM, overnight; (v) 2,2-dimethylsuccinic anhydride, DMAP, pyridine, microwave, 140 °C, 2h; (vi) NaOH, THF, MeOH, overnight.

Scheme 1.
Synthesis of Compounds 2-18^a
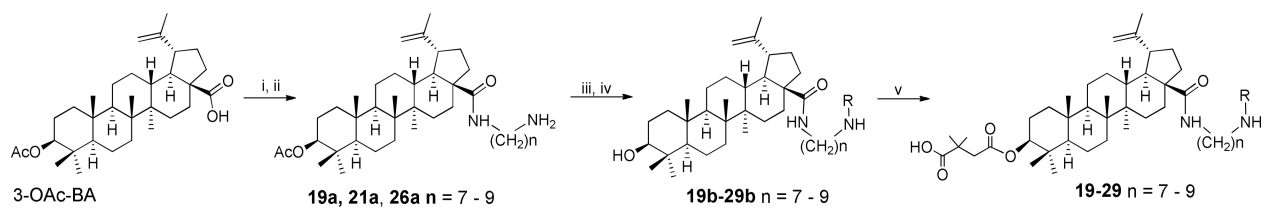
^{a}Reagents and conditions: (i) oxalyl chloride, DCM, 1h; (ii) NH$_2$(CH$_2$)nNH$_2$, DCM, overnight; (iii) R-H, HOBt, EDC, Et$_3$N, DCM, overnight; (iv) NaOH, THF, MeOH, 3h; (v) 2,2-dimethylsuccinic anhydride, DMAP, pyridine, microwave, 140 °C, 2h.

Scheme 2.
Synthesis of Compounds 19-29^a

Figure 2.
Inhibition of CA-SP1 processing. The anti-maturation activity of **1** and **6** was determined by transfecting 293T cells with the following plasmids: (a) pNL4-3 or pNL4-3/V370A; (b) pNL4-3/V362I. The viruses produced in the presence of the compounds were collected two days after transfection.
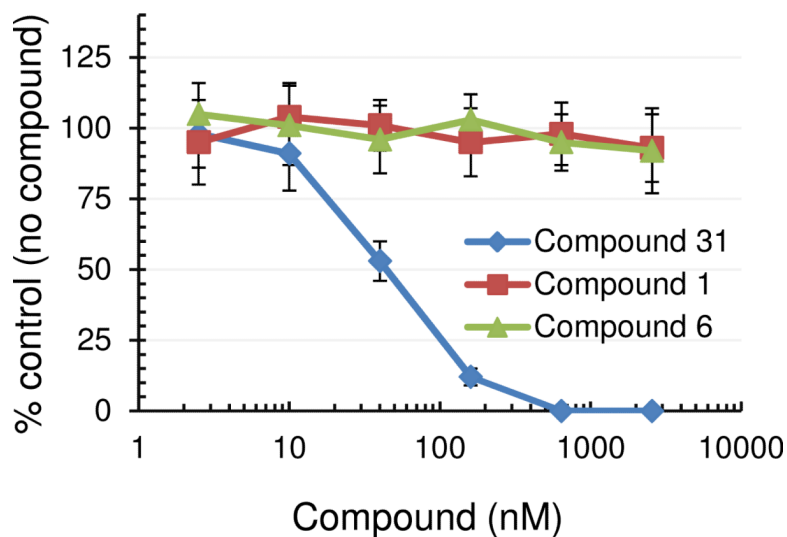
Figure 3.
Compound **6** did not inhibit HIV-1 Env–mediated cell-cell fusion. COS cells (1×10^6 cells/mL) were transfected by electroporation with HIV-1 NL4-3 Env-expressing vector (2 μg) for one day before mixing with TZM-bl cells for fusion. The luciferase expression measured as relative luciferase units (RLU) in the fusion cells was quantified 24 hours after cell mixing.[24] HIV-1 Env-mediated cell-cell fusion in the absence of compounds is defined as 100% control. Each data point in the figure represents mean +/- SD of three tests.

Table 1
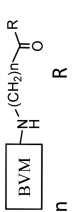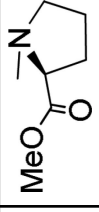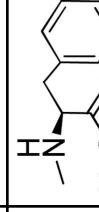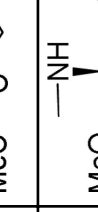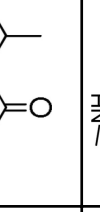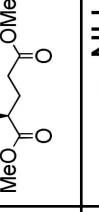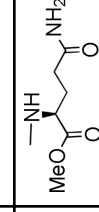
Genotypes of NL4-3 Variants Carrying BVM-R Polymorphisms

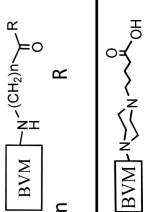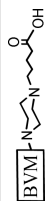| Virus (Gag)	CA	SP1	
	362	369	371
NL4-3	V L	A E A M S Q V T N S A	
NL4-3/V370Δ	V L	A E A M S Q Δ T N S A	
NL4-3/V370A	V L	A E A M S Q A T N S A	
NL4-3/T371Δ	V L	A E A M S Q V Δ N S A	
NL4-3/V362I	V I	A E A M S Q V T N S A	

Table 2

Activities of Compounds 2–18 against HIV-1 NL4-3 and NL4-3/V370A

Compound	n	R	IC$_{50}$[a] (μM)		CC$_{50}$[b] (μM)
			NL4-3	NL4-3/V370A	
1			0.076 +/- 0.018 (5)	>5	>10
2	6		0.05 +/- 0.014 (3)	2.46 ± 1.10 (3)	>10
3	7		0.05 +/- 0.017 (2)	>5	>10
4	8		0.01 ± 0.0032 (2)	1.51 ± 0.125 (2)	>10
5	8		0.04 ± 0.016 (2)	0.48 ± 0.32 (2)	>10
6	8		0.009 ± 0.002 (5)	0.16 ± 0.048 (5)	>10
7	8		0.01 ± 0.003 (3)	0.81 ± 0.28 (3)	>10
8	8		0.04 ± 0.013 (2)	2.54 ± 0.95 (2)	>10
9	8		0.04 ± 0.017 (2)	2.78 ± 0.93 (2)	>10

Compound	n	R	IC$_{50}$^{a} (µM) NL4-3	IC$_{50}$^{a} (µM) NL4-3/V370A	CC$_{50}$^{b} (µM)
10	8		0.10 ± 0.019 (2)	0.88 ± 0.31 (2)	>10
11	8		0.07 ± 0.042 (2)	1.29 ± 0.47 (2)	>10
12	8		0.01 ± 0.005 (2)	1.24 ± 0.052 (2)	>10
13	8		0.18 ± 0.073 (2)	2.16 ± 1.07 (2)	>10
14	8		0.01 ± 0.0036 (3)	0.42 ± 0.085 (3)	>10
15	8	—NHCH$_2$CN	0.03 ± 0.014 (2)	0.46 ± 0.22 (3)	6.49 ± 0.64 (2)
16	8	—NHCH$_2$CH$_2$CN	0.15 ± 0.062 (2)	0.33 ± 0.14 (2)	>10
17	8		0.04 ± 0.018 (2)	2.08 ± 0.88 (2)	>10
18	9		0.10 ± 0.045 (2)	0.70 ± 0.31 (2)	>10
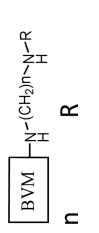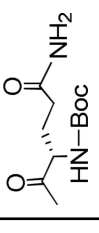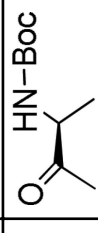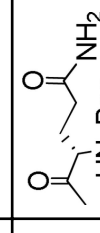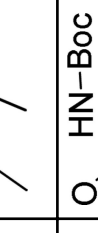
Compound	n		IC$_{50}$^{a} (μM)		CC$_{50}$^{b} (μM)
	R	R	NL4-3	NL4-3/V370A	
30			0.006 ± 0.0016 (3)	>3.3	>10

^{a}IC50: concentration that inhibited HIV-1 replication by 50%; presented as mean +/- standard deviation (SD).

^{b}CC50: concentration that reduced cell viability by 50%. IC50 and CC50 were determined by using CalcuSyn (Biosoft). The numbers in the parentheses represent the number of tests.

Table 3

Activities of Compounds 19–29 against HIV-1 NL4-3 and NL4-3/V370A

$BVM-N^{-(CH_2)n}-N^{-R}$ (H, H)

Compound	n	R	IC_{50}[a] (μM) NL4-3	IC_{50}[a] (μM) NL4-3/V370A	CC_{50}[b] (μM)
19	7		0.01 ± 0.004 (2)	2.6 ± 1.2 (2)	>10
20	7		0.01 ± 0.003 (2)	0.37 ± 0.15 (2)	7.9 ± 0.65 (2)
21	8		0.03 ± 0.007 (3)	0.36 ± 0.13 (3)	>10
22	8		0.15 ± 0.05 (2)	0.33 ± 0.04 (2)	>10
23	8		0.02 ± 0.0072 (2)	0.92 ± 0.21 (2)	>10
24	8		0.03 ± 0.014 (2)	0.33 ± 0.25 (2)	>10
25	8		0.04 ± 0.018 (2)	0.95 ± 0.34 (2)	>10

BVM—N(H)—(CH$_2$)$_n$—N(H)—N(H)—R

Compound	n	R	IC$_{50}$ ^{a} (μM)		CC$_{50}$ ^{b} (μM)
			NL4-3	NL4-3/V370A	
26	9		0.02 ± 0.008 (2)	1.20 ± 0.13 (2)	>10
27	9		0.05 ± 0.016 (2)	1.58 ± 0.33 (2)	>10
28	9		0.02 ± 0.007 (2)	1.04 ± 0.42 (2)	>10
29	9		0.04 ± 0.019 (2)	0.34 ± 0.22 (2)	8.3± 0.56 (2)

^{a}C50: concentration that inhibited HIV-1 replication by 50%; presented as mean +/- SD.

^{b}CC50: concentration that reduced cell viability by 50%. IC50 and CC50 were determined by using CalcuSyn (Biosoft). The numbers in the parentheses represent the number of tests.

Table 4

Activities of Compound 6 against BVM-R HIV-1 Variants.

Virus	$IC_{50}{}^{a}$ (μM)	
	Compound 1	**Compound 6**
NL4-3	0.076 ± 0.019	0.008 ± 0.0017
NL4-3/V370Δ	> 6.8	0.32 ± 0.077
NL4-3/V370Δ	> 6.8	0. 158 ± 0.048
NL4-3/T371Δ	> 6.8	0.067 ± 0.012
NL4-3/V362I	> 6.8	0.016±0.0042

[a]IC_{50}: the numbers in the table are mean +/- SD from three independent experiments.