

Published in final edited form as:

Bioorg Med Chem Lett. 2011 October 1; 21(19): 5944–5947. doi:10.1016/j.bmcl.2011.07.072.

New Betulinic Acid Derivatives as Potent Proteasome Inhibitors

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Abstract

In this study, 22 new betulinic acid (BA) derivatives were synthesized and tested for their inhibition of the chymotrypsin-like activity of 20S proteasome. From the SAR study, we concluded that the C-3 and C-30 positions are the pharmacophores for increasing the proteasome inhibition effects, and larger lipophilic or aromatic side chains are favored at these positions. Among the BA derivatives tested, compounds **13**, **20**, and **21** showed the best proteasome inhibition activity with IC₅₀ values of 1.42, 1.56, and 1.80 μM, respectively, which are three- to four-fold more potent than the proteasome inhibition controls LLM-F and lactacystin.

Betulinic acid (BA, 3β-hydroxy-lup-20(29)-en-28-oic acid, **1**) is a pentacyclic triterpene present in many plant species, e.g., *Triphyophyllum peltatum*, *Ancistrocladus heyneanus*, *Ziziphi fructus*, *Diospyros leucomelas*, *Tetracera boliviana* and *Syzygium formosanum*,^{1–3} and can be obtained in quantity from the bark of the London plane tree, *Platanus acerifolia*.^{4–6} BA (**1**) exhibits diverse biological activities including anti-retroviral, anti-bacterial, anti-malarial, anti-inflammatory, anti-cancer, anti-oxidant, and anthelmintic properties. Our previous research on **1** resulted in the discovery of bevirimat [DSB, 3-*O*-(3', 3'-dimethylsuccinyl)-betulinic acid, **2**] (Figure 1),⁷ which exhibits remarkable anti-HIV-1 activity with a mean EC₅₀ value of <10 nM against primary and drug-resistant HIV-1 isolates,^{7, 8} representing a unique first in a class of anti-HIV compounds termed maturation inhibitors (MIs).^{8, 9} Bevirimat has recently succeeded in Phase IIb clinical trials.^{6, 10–12} In the further evaluation of **2**, we discovered that HIV-1 virions produced in the presence of **2** contained higher amounts of the cellular protein APOBEC3G, which is usually degraded by cellular proteasomes. We then postulated that the elevated APOBEC3G level in these viral infected cells may be due to the inhibition of proteasomes by **2**.

Proteasomes are responsible for more than 80% of intracellular protein degradation.¹³ The most common form of the proteasome is known as 26S proteasome, which contains one 20S core particle structure and two 19S regulatory caps. All 20S particles consist of four stacked heptameric ring structures that are themselves composed of two different types of subunits,

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α and β . Alpha subunits are structural in nature, whereas β subunits are predominantly catalytic. The β subunits of proteasomes have three major proteolytic activities: chymotrypsin-like (β_5), trypsin-like (β_2), and caspase-like (β_1). These proteolytic activities enable the proteasome to cleave proteins into small peptides. Proteasomes are involved in many essential cellular functions, such as cell cycle regulation, cell differentiation, signal transduction pathways, antigen processing for appropriate immune responses, stress signaling, inflammatory responses, and apoptosis. Targeting the proteasome-ubiquitin pathway has thus been considered a novel strategy for the treatment of various disorders such as neurodegenerative diseases, cancers, and inflammatory diseases.^{14, 15}

In our initial screening, we found that **2** inhibited the chymotrypsin-like activity of 20S proteasome with an IC_{50} value of 9.2 μ M, but it did not inhibit the caspase-like activity, and had a very weak inhibitory effect (IC_{50} : 26.29 μ M) on the trypsin-like activity.¹⁶ These results are favorable because normal cells may suffer fewer consequences from treatment with **2**, as two of the main proteolytic activities of proteasomes are retained. Indeed, the anticancer drug bortezomib (PS341), the first successful proteasome inhibitor for the treatment of multiple myeloma, also showed differential inhibition of the three catalytic activities of proteasomes, which is believed to be critical for its clinical benefit in disease treatment.^{14, 17, 18} In addition, compound **2** did not inhibit the cellular proteases cathepsin B (cysteine protease) and DPPIV (DPP4, CD26, serine protease) in our screening.¹⁶ Consequently, it may have reduced side effects compared with other proteasome inhibitors such as peptide aldehydes and peptide boronates, which also inhibit these cellular proteases. Therefore, to better establish a structure-activity relationship (SAR) of the proteasome regulatory activity of BA derivatives, diverse substituted BA analogs were designed. This paper reports herein the synthesis and proteasome inhibitory activity of these BA derivatives.

Because **2** is a C-3 modified BA derivative, we first synthesized eleven 3-*O*-acyl-BA analogs (**3–13**) to investigate the impacts of different C-3 modifications on the proteasome inhibitory activity of BA derivatives. As shown in Scheme 1, 3,4,5-trimethoxybenzoyl chloride was reacted with **1** in the presence of Et_3N in CH_2Cl_2 at room temperature to yield compound **3**. Diverse carboxylic acids were first treated with EDCI and DMAP in CH_2Cl_2 for 1 h, before compound **1** and Et_3N were added into the system. The mixtures were stirred at room temperature to yield compounds **4–10** and **13**. 1,2,3,6-Tetrahydrophthalic and diglycolic anhydrides were refluxed with **1** in the presence of DMAP in pyridine to provide compounds **11** and **12**, respectively. To investigate the impact of 28- and 30-substitutions on BA's proteasome inhibitory effects, nine 28,30-disubstituted BA analogs (**14–22**) were synthesized by the method reported previously (Scheme 2)¹⁹ and were also tested against 20S proteasome. Briefly, BA was first protected with acetic anhydride to yield **25**, which was treated with oxalyl chloride and then reacted with leucine methyl ester to furnish **26**. Allylic bromination of **26** was carried out using NBS in dilute acetonitrile to provide **27**. Saponification of **27** yielded **14**. Meanwhile, nucleophilic displacement of the bromide of **27** was conducted by treating varied nucleophilic compounds with NaH, followed by their reaction with **27** in a microwave apparatus at 120 °C. Saponification of the resulting mixtures in MeOH- H_2O converted the intermediate esters to carboxylic acids to yield the target compounds 15–22. Two compounds with only a C-28 side chain, LH15bh (**23**) and IC9564 (**24**) (Figure 1),^{20, 21} were also tested for their proteasome inhibition activity to investigate the influence of C-28 mono-substitution.

Table 1 lists the results for inhibition of the chymotrypsin-like activity of 20S proteasome shown by BA derivatives **3–24**²² together with compound **2**. Known proteasome inhibitors Ac-Leu-Leu-Met-CHO (LLM-F) and lactacystin were also included in the in vitro 20S proteasome assay²³ as controls. From the results with compounds **2–13**, we can conclude

that addition of a C-3 ester group to **1** can confer proteasome inhibition activity and the identity of the ester group can influence the potency. Comparing the activity of **3–10**, we found that compound **6** with an unsubstituted benzoate ester side chain and compound **5** with 2',4',5'-trimethoxy substituted benzoate ester side chain at the C-3 position showed moderate inhibition activity (IC₅₀: 11.06 and 10.91 μM, respectively) on the chymotrypsin-like activity of 20S proteasome. Compounds **3**, **4**, and **7** without *ortho*-methoxy substitution on benzoate ester side chain abolished or greatly decreased the proteasome inhibition activity. In a comparison of three compounds with different brominated benzoate side chains, bromide on the 2'- or 3'-position (**8** and **10**) was advantageous (IC₅₀: 7.99 and 7.05 μM, respectively), while bromide on the 4'-position (**9**) was detrimental. Interestingly, while compound **12**, with a polar oxygen atom in the middle of a diglycolic ester side chain was inactive, compounds with both a cyclohexene ring (**11**) and a phenyl ring (**13**), located centrally within a dicarboxylic side chain were significantly active. Compound **11**, with a 1,2,3,6-tetrahydrophthalate ester side chain, exhibited increased proteasome inhibition activity with an IC₅₀ value of 2.96 μM. Elongation of the C-3 side chain to 3'-acetylphenylacetate (**13**) further increased the proteasome inhibition activity to an IC₅₀ value of 1.42 μM, which is six-fold better than **2** and four-fold better than LLM-F and lactacystin. Among the synthesized 3-*O*-acyl BA analogs, compound **13** showed the best activity, suggesting that an aromatic lipophilic ester side chain with a carboxylic acid terminus at the C-3 position can significantly increase the proteasome inhibition activity of BA derivatives.

With respect to the effects of 28- and 30-substitutions on the proteasome activity, we found that all nine 28,30-disubstituted BA analogs (**14–22**) showed inhibitory activity towards the chymotrypsin-like activity of 20S proteasome. However, the 28-substituted BA derivatives **23** and **24** did not inhibit the 20S proteasome to any degree. We therefore concluded that the proteasome inhibition activity of the 28,30-disubstituted BA compounds comes mainly from the contribution of the C-30 substituents. Within the C-30 side chains, larger ether substituents (**16–22**) were favored compared with bromide (**14**) or a smaller ethoxy group (**15**). Among **16–22**, the rank order of potency based on the C-30 ether substituent was 4'-bromophenethoxy (**20**) = 4'-chlorophenethoxy (**21**) > 4'-fluorophenethoxy (**19**) = phenethoxy (**17**) > propoxy (**16**) = 4'-methoxyphenethoxy (**18**) = 2'-morpholinoethoxy (**22**). Overall, compound **15** with an ethoxy substituent showed the lowest activity among the 28,30-disubstituted BAs with an IC₅₀ value of 13.21 μM, while compounds **20** and **21** with 4'-bromophenethoxy and 4'-chlorophenethoxy substituents, respectively, showed the highest activity with IC₅₀ values of 1.56 and 1.80 μM, respectively, three-fold better than LLM-F and lactacystin. Thus, longer C-30 side chains with aromatic substitutions were favored for the proteasome inhibition activity.

In summary, this study showed that substituted BA analogs can be proteasome inhibitors, and the C-3 and C-30 positions of BA are the pharmacophores for improving the proteasome inhibition activity. Within the C-3 and C-30 substitutions, larger side chains with lipophilic or aromatic side chains are favored for increased inhibition of the chymotrypsin-like activity of 20S proteasome. C-3 and C-30 modified BA compounds showed low toxicity in our previous anti-AIDS studies, and bevirimat (**2**), a C-3 substituted BA, has already succeeded in phase IIb clinical trials. Therefore, development of C-3 and/or C-30 modified BA derivatives as proteasome inhibitors might be able to provide new therapeutic agents for treating cancers and inflammatory diseases.

Acknowledgments

This investigation was supported by Grant GM-084337 from the National Institutes of General Medical Sciences (NIGMS) awarded to C.H.C and AI-077417 from the National Institute of Allergy and Infectious Diseases (NIAID)

awarded to K.H.L. This study was also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004). Efficient purification of all the synthetic BA analogs was performed with a Grace Reveleris® flash chromatography system equipped with RevealX(TM) detection allowing for multesignal (UV/ELSD) collection to low mg quantities. Reveleris® Navigator method optimizer and Grace Reveleris® flash silica cartridges were employed for high quality separations.

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22. *Materials and methods:* ¹H NMR spectra were measured on a 300 or 400 MHz Varian Gemini 2000 spectrometer using TMS as internal standard. The solvent used was CDCl₃. Mass spectra were measured on a Shimadzu LC-MS2010 instrument. Thin-layer chromatography (TLC) was performed on precoated silica gel GF plates purchased from Merck, Inc. All other chemicals were obtained from Aldrich, Inc. Fast purification of crude reaction mixtures was performed on the Grace Reveleris® flash system by prescreening the crude mixtures on TLC plates to select appropriate solvent pairs for purification. Method parameters were automatically loaded when the Reveleris® cartridge was inserted or when the Reveleris® Navigator method optimizer was employed to predict high purity or fastest separations, but were adjusted manually as desired.

Crude samples were dissolved in the appropriate solvent before being injected automatically via solid loading. Spectroscopic data for target compounds are shown as follows. **Compound 3:** ^1H NMR (400 MHz, CDCl_3): δ 7.29, 7.27 (1H each, s, Ar-H), 4.76, 4.64 (1H each, s, H-29), 4.60 (1H, m, H-3), 3.94 (3H, s, Ar-OCH₃), 3.91 (6H, s, 2 \times Ar-OCH₃), 3.06 (1H, m, H-19), 1.71 (3H, s, H-30), 0.97, 0.90, 0.83, 0.76, 0.70 (3H each, s, 5 \times CH₃), MS m/z 649.50 ($\text{M}^- - 1$); **Compound 4:** ^1H NMR (400 MHz, CDCl_3): δ 7.64 (1H, d, $J = 8.0$ Hz, Ar-H-6'), 7.45 (1H, s, Ar-H-2'), 6.84 (1H, d, $J = 8.0$ Hz, Ar-H-5'), 6.03 (2H, s, -OCH₂O-), 4.73, 4.60 (1H each, s, H-29), 4.65 (1H, m, H-3), 3.10 (1H, m, H-19), 1.70 (3H, s, H-30), 0.99, 0.97, 0.96, 0.90, 0.89 (3H each, s, 5 \times CH₃), MS m/z 603.40 ($\text{M}^- - 1$); **Compound 5:** ^1H NMR (400 MHz, CDCl_3): δ 7.44 (1H, s, Ar-H-6'), 6.52 (1H, s, Ar-H-3'), 4.75, 4.62 (1H each, s, H-29), 4.69 (1H, dd, $J = 8.0, 4.0$ Hz, H-3), 3.93, 3.88, 3.86 (3H each, s, 3 \times Ar-OCH₃), 3.01 (1H, m, H-19), 1.70 (3H, s, H-30), 1.00, 0.97, 0.96, 0.95, 0.89 (3H each, s, 5 \times CH₃), MS m/z 649.70 ($\text{M}^- - 1$); **Compound 6:** ^1H NMR (400 MHz, CDCl_3): δ 8.04 (2H, d, $J = 8.0$ Hz, Ar-H-2', 6'), 7.55 (1H, m, Ar-H-4'), 7.45 (1H, m, Ar-H-3', 5'), 4.75, 4.62 (1H each, s, H-29), 4.71 (1H, m, H-3), 3.02 (1H, m, H-19), 1.71 (3H, s, H-30), 1.00, 1.00, 0.96, 0.93, 0.91 (3H each, s, 5 \times CH₃), MS m/z 559.45 ($\text{M}^- - 1$); **Compound 7:** ^1H NMR (400 MHz, CDCl_3): δ 7.68 (1H, d, $J = 4.0$ Hz, Ar-H-6'), 7.56 (1H, s, Ar-H-2'), 6.88 (1H, d, $J = 4.0$ Hz, Ar-H-5'), 4.75, 4.62 (1H each, s, H-29), 4.69 (1H, m, H-3), 3.96, 3.93 (3H each, s, 2 \times Ar-OCH₃), 3.02 (1H, m, H-19), 1.71 (3H, s, H-30), 1.00, 0.99, 0.96, 0.92, 0.90 (3H each, s, 5 \times CH₃), MS m/z 619.45 ($\text{M}^- - 1$); **Compound 8:** ^1H NMR (400 MHz, CDCl_3): δ 7.75 (1H, d, $J = 8.0$ Hz, Ar-H-6'), 7.64 (1H, d, $J = 8.0$ Hz, Ar-H-3'), 7.37-7.28 (2H, m, Ar-H-4', 5'), 4.78 (1H, m, H-3), 4.75, 4.62 (1H each, s, H-29), 3.01 (1H, m, H-19), 1.71 (3H, s, H-30), 1.00, 0.97, 0.95, 0.94, 0.89 (3H each, s, 5 \times CH₃), MS m/z 639.40 ($\text{M}^+ + 1$); **Compound 9:** ^1H NMR (400 MHz, CDCl_3): δ 7.89 (2H, d, $J = 8.0$ Hz, Ar-H-2', 6'), 7.59 (2H, d, $J = 8.0$ Hz, Ar-H-3', 5'), 4.73, 4.60 (1H each, s, H-29), 4.70 (1H, m, H-3), 3.02 (1H, m, H-19), 1.70 (3H, s, H-30), 1.00, 0.99, 0.97, 0.91, 0.90 (3H each, s, 5 \times CH₃), MS m/z 637.65 ($\text{M}^- - 1$); **Compound 10:** ^1H NMR (400 MHz, CDCl_3): δ 8.15 (1H, s, Ar-H-2'), 7.95 (1H, d, $J = 8.0$ Hz, Ar-H-6'), 7.67 (1H, d, $J = 8.0$ Hz, Ar-H-4'), 7.31 (1H, m, Ar-H-5'), 4.75, 4.62 (1H each, s, H-29), 4.71 (1H, m, H-3), 3.01 (1H, m, H-19), 1.71 (3H, s, H-30), 1.00, 0.99, 0.96, 0.92, 0.91 (3H each, s, 5 \times CH₃), MS m/z 637.50 ($\text{M}^- - 1$); **Compound 11:** ^1H NMR (300 MHz, CDCl_3): δ 5.70 (2H, m, H-5', 6'), 4.72, 4.60 (1H each, s, H-29), 4.48 (1H, m, H-3), 3.02 (1H, m, H-19), 2.83, 2.72 (1H each, m, H-2', 3'), 2.32-2.27 (4H, m, 2 \times CH₂, H-4', 7'), 1.68 (3H, s, H-30), 1.01, 0.97 (3H each, s, 2 \times CH₃), 0.89, 0.83, 0.81 (3H each, s, 3 \times CH₃), MS m/z 607.40 ($\text{M}^- - 1$); **Compound 12:** ^1H NMR (300 MHz, CDCl_3): δ 4.72, 4.60 (1H each, s, H-29), 4.46 (1H, m, H-3), 4.23, 4.21 (2H each, s, H-2', 4'), 3.01 (1H, m, H-19), 1.71 (3H, s, H-30), 1.00, 0.98, 0.79, 0.88, 0.87 (3H each, s, 5 \times CH₃), MS m/z 571.60 ($\text{M}^- - 1$); **Compound 13:** ^1H NMR (400 MHz, CDCl_3): δ 7.82 (1H, s, Ar-H-2'), 7.57-7.50 (2H, m, Ar-H-4', 6'), 7.30 (1H, m, Ar-H-5'), 4.72, 4.60 (1H each, s, H-29), 4.40 (1H, m, H-3), 3.63, 3.60 (2H each, s, 2 \times Ar-CH₂-CO-), 2.98 (1H, m, H-19), 1.68 (3H, s, H-30), 0.95, 0.90, 0.82, 0.72, 0.68 (3H each, s, 5 \times CH₃), MS m/z 631.78 ($\text{M}^- - 1$); **Compound 14:** ^1H NMR (300 MHz, CDCl_3): δ 5.86 (1H, d, $J = 8.0$ Hz, -CONH-), 5.11, 5.02 (1H each, s, H-29), 4.65 (1H, m, -NHCH-), 3.90 (2H, s, H₂-30), 3.17 (1H, dd, $J = 9.7, 5.4$ Hz, H-3), 3.10-3.03 (1H, m, H-19), 1.00 (6H, s, leucine moiety -(CH₃)₂), 0.96 (6H, s, 2 \times CH₃), 0.83, 0.80, 0.79 (3H each, s, 3 \times CH₃), MS m/z 646.41, 648.39 ($\text{M}^- - 1$); **Compound 15:** ^1H NMR (300 MHz, CDCl_3): δ 5.88 (1H, d, $J = 8.0$ Hz, -CONH-), 4.93, 4.92 (2H, br s, H-29), 4.63-4.58 (1H, m, -NHCH-), 3.90 (2H, s, H₂-30), 3.47 (2H, m, 30-OCH₂CH₃), 3.18 (1H, dd, $J = 11.1, 5.4$ Hz, H-3), 2.99 (1H, m, H-19), 2.50-2.32 (1H, m, H-13), 1.00 (9H, br s, 30-OCH₂CH₃, leucine moiety -(CH₃)₂), 0.96 (6H, s, 2 \times CH₃), 0.89, 0.86, 0.85 (3H each, s, 3 \times CH₃), MS m/z 612.40 ($\text{M}^- - 1$); **Compound 16:** ^1H NMR (300 MHz, CDCl_3): δ 6.13 (1H, br s, -CONH-), 4.91, 4.90 (2H, br s, H-29), 4.52 (1H, m, -NHCH-), 3.90 (2H, s, H₂-30), 3.36 (2H, t, $J = 6.9$ Hz, 30-OCH₂CH₂CH₃), 3.18 (1H, dd, $J = 11.1, 5.4$ Hz, H-3), 2.99 (1H, m, H-19), 0.96, 0.94, 0.92, 0.89 (15H, m, 30-O(CH₂)₂CH₃, leucine moiety -(CH₃)₂, CH₃-23, 24), 0.82, 0.81, 0.79 (3H each, s, 3 \times CH₃), MS m/z 626.50 ($\text{M}^- - 1$); **Compound 17:** ^1H NMR (300 MHz, CDCl_3): δ 7.68-7.62 (2H, m, Ar-H-3'), 7.28-7.20 (3H, m, Ar-H-2', 4'), 5.97 (1H, br s, -CONH-), 4.91, 4.90 (2H, br s, H-29), 4.44 (1H, m, -NHCH-), 3.93 (2H, s, H₂-30), 3.64 (2H, t, $J = 7.2$ Hz, 30-OCH₂CH₂Ph), 3.17 (1H, dd, $J = 11.1, 5.4$ Hz, H-3), 2.91 (1H, m, H-19), 2.57 (2H, m, 30-OCH₂CH₂Ph), 0.95 (12H, s, leucine moiety -(CH₃)₂, CH₃-23, 24), 0.89, 0.78, 0.74 (3H each, s, 3 \times CH₃), MS m/z 688.41 ($\text{M}^- - 1$); **Compound 18:** ^1H NMR (300 MHz, CDCl_3): δ 7.27, 7.16-7.13, 6.85-6.82 (4H, m, Ar-H-2', 3'), 5.97 (1H, br s, -CONH-), 4.91, 4.89 (H each, br s, H-29), 4.48 (1H, m, -NHCH-), 3.93 (2H, s, H₂-30), 3.79 (3H, s, Ar-OCH₃), 3.60 (2H, t, $J = 7.2$ Hz, 30-OCH₂CH₂Ph(*p*-OCH₃)), 3.17 (1H, dd,

$J = 11.1, 5.4$ Hz, $H-3$), 2.85 (1H, t, $J = 7.5$ Hz, $H-19$), 2.39 (3H, m, 30-OCH₂CH₂Ph(p -OCH₃), $H-13$), 0.95, 0.93, 0.90 (15H, s, leucine moiety -(CH₃)₂, 3 × CH₃), 0.79, 0.75 (3H each, s, 2 × CH₃), MS m/z 718.50 ($M^- - 1$); **Compound 19**: ¹H NMR (300 MHz, CDCl₃): δ 7.16-6.80 (4H, m, Ar- $H-2'$, 3'), 5.96 (1H, br s, -CONH-), 4.90, 4.89 (2H, br s, $H-29$), 4.48 (1H, m, -NHCH-), 3.92 (2H, s, H_2-30), 3.61 (2H, t, $J = 7.2$ Hz, 30-OCH₂CH₂Ph(p -F)), 3.17 (1H, m, $H-3$), 2.87 (1H, t, $J = 7.5$ Hz, $H-19$), 2.36-2.10 (3H, m, 30-OCH₂CH₂Ph(p -F), $H-13$), 0.95, 0.88 (15H, s, leucine moiety -(CH₃)₂, 3 × CH₃), 0.75, 0.73 (3H each, s, 2 × CH₃), MS m/z 706.40 ($M^- - 1$); **Compound 20**: ¹H NMR (300 MHz, CDCl₃): δ 7.56-7.28 (4H, m, Ar- $H-2'$, 3'), 5.96 (1H, br s, -CONH-), 4.91, 4.90 (2H, br s, $H-29$), 4.48 (1H, m, -NHCH-), 3.91 (2H, s, H_2-30), 3.60 (2H, t, $J = 7.0$ Hz, 30-OCH₂CH₂Ph(p -Br)), 3.17 (1H, dd, $J = 11.0, 5.6$ Hz, $H-3$), 2.89 (1H, t, $J = 7.5$ Hz, $H-19$), 2.39 (1H, m, 30-OCH₂CH₂Ph(p -Br)), 0.96 (12H, s, leucine moiety -(CH₃)₂, 2 × CH₃), 0.82, 0.79, 0.75 (3H each, s, 3 × CH₃), MS m/z 766.38 ($M^- - 1$); **Compound 21**: ¹H NMR (300 MHz, CDCl₃): δ 7.18-6.87 (4H, m, Ar- $H-2'$, 3'), 5.96 (1H, br s, -CONH-), 4.91, 4.90 (2H, br s, $H-29$), 4.48 (1H, m, -NHCH-), 3.91 (2H, s, H_2-30), 3.62 (2H, t, $J = 6.8$ Hz, 30-OCH₂CH₂Ph(p -Cl)), 3.17 (1H, dd, $J = 11.0, 5.6$ Hz, $H-3$), 2.87 (1H, t, $J = 7.5$ Hz, $H-19$), 2.36-2.06 (3H, m, 30-OCH₂CH₂Ph(p -Cl), $H-13$), 0.96 (15H, s, leucine moiety -(CH₃)₂, 3 × CH₃), 0.81, 0.76 (3H each, s, 2 × CH₃), MS m/z 722.40 ($M^- - 1$); **Compound 22**: ¹H NMR (300 MHz, CDCl₃): δ 5.61 (1H, d, $J = 8$ Hz, -CONH-), 4.92, 4.90 (H each, s, $H-29$), 4.59 (1H, m, -NHCH-), 3.94 (2H, s, H_2-30), 3.72 (4H, m, -N(CH₂CH₂)₂O), 3.58 (2H, t, $J = 5.7$ Hz, 30-OCH₂CH₂-morpholine), 3.18 (1H, dd, $J = 11.4, 4.6$ Hz, $H-3$), 3.01 (1H, m, $H-19$), 2.60 (2H, t, $J = 5.4$ Hz, 30-OCH₂CH₂-morpholine), 2.53 (4H, m, -N(CH₂CH₂)₂O), 1.00 (6H, s, leucine moiety -(CH₃)₂), 0.96 (6H, s, 2 × CH₃), 0.89, 0.85, 0.80 (3H each, s, 3 × CH₃), MS m/z 697.40 ($M^- - 1$).

23. **20S proteasome assay**: 20S proteasome assay kits were purchased from Calbiochem, San Diego, CA. Measurement of the hydrolysis of the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC in the presence of the proteasome activator PA28 (16 μg/mL) is used to detect the chymotrypsin-like activity of the human 20S proteasome. Fluorescence generated from the proteolytic reaction in the presence of various concentrations of BA derivatives was measured using a BioTek fluorometer (Winooski, Vermont). The 50% inhibitory concentration (IC₅₀) is defined as the inhibitor concentration that reduces the reaction rate by 50%. The velocity of reaction (ΔRFU(360/460)/min) was plotted against the log-concentration of the inhibitor to determine the IC₅₀.

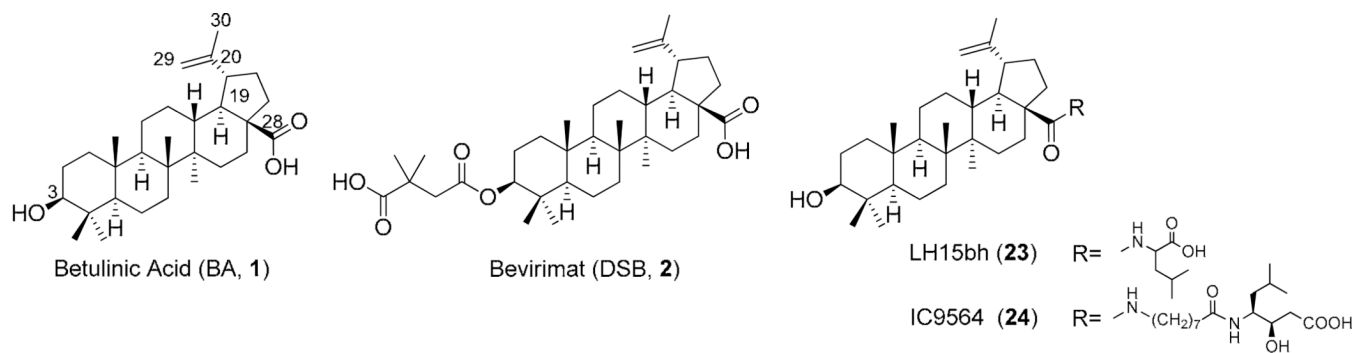
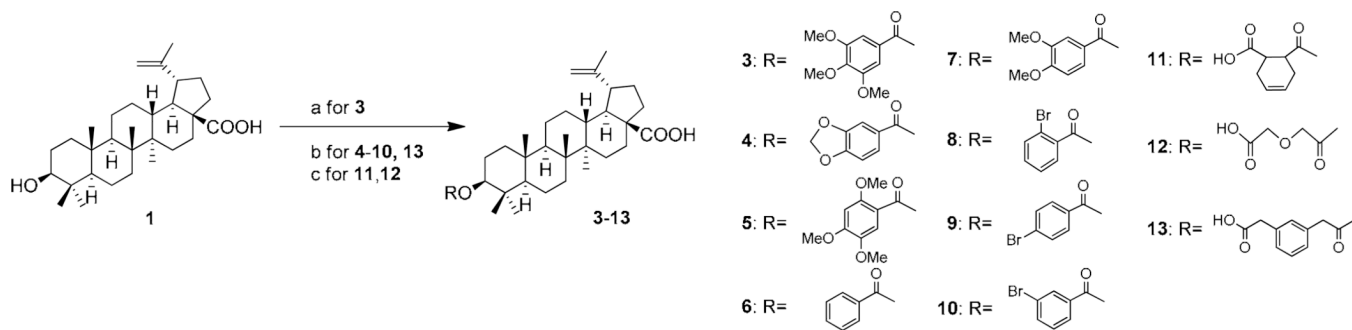
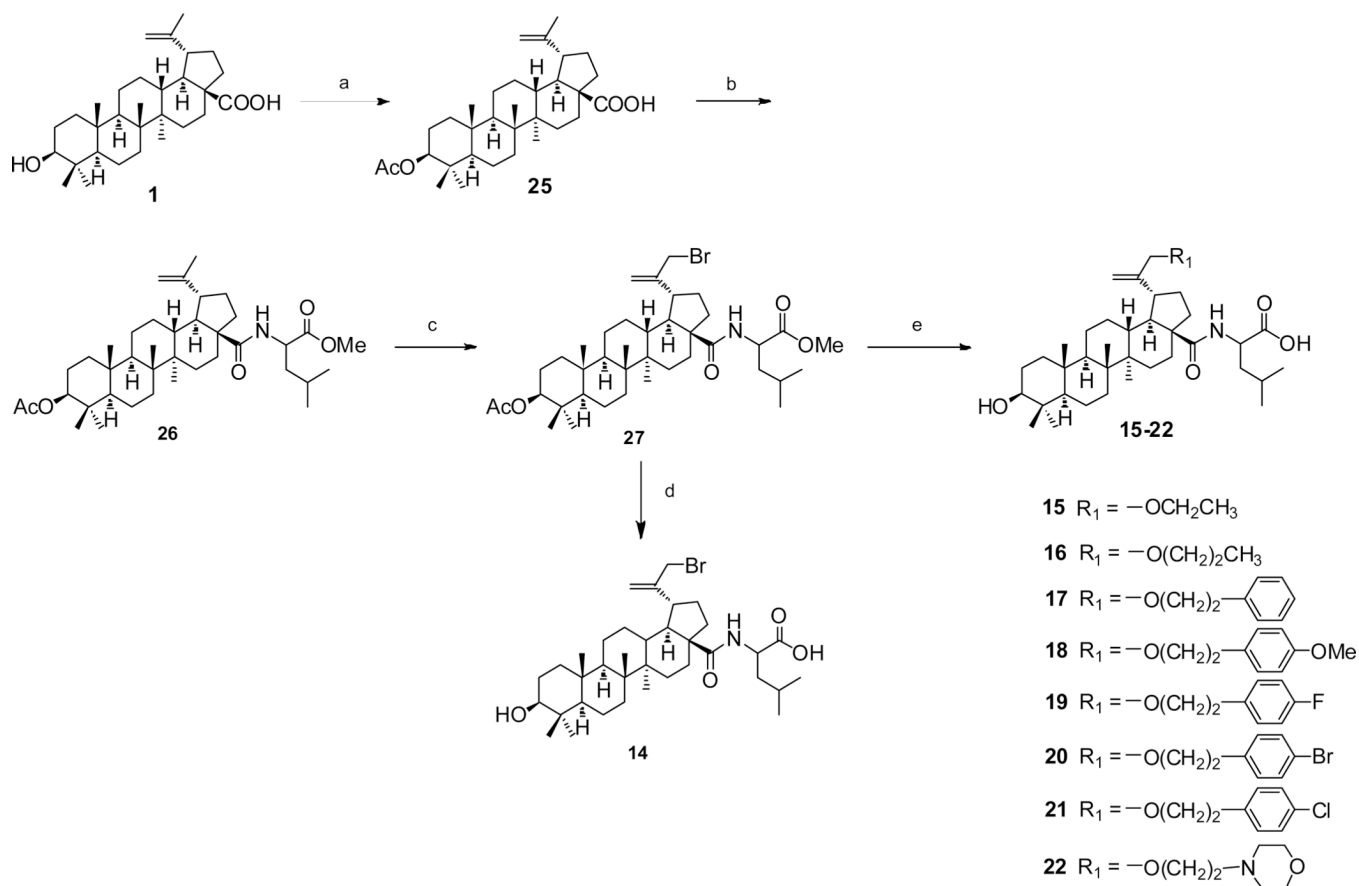


Figure 1.
Structures of BA (**1**) and its derivatives (**2**, **23**, **24**).

**Scheme 1.**

Reactions and conditions: (a) acid chloride, Et₃N, CH₂Cl₂, rt; (b) acid, EDCI, DMAP, Et₃N, CH₂Cl₂, rt; (c) acid anhydride, DMAP, pyridine, reflux.

**Scheme 2.**

Reactions and conditions: (a) Ac_2O , pyridine, $78^\circ C$, 4h; (b) $(CO_2)Cl_2$, leucine methyl ester, Et_3N , CH_2Cl_2 , rt; (c) NBS, acetonitrile, rt; (d) 2N NaOH, MeOH/THF, rt; (e) step 1: nucleophilic compounds, NaH, THF; step 2: MeOH- H_2O .

Table 1

The inhibition on the chymotrypsin-like activity of 20S proteasome of **3–24** with **2**, LLM-F, and lactacystin as controls.

Compound	IC ₅₀ (μM) ^a	Compound	IC ₅₀ (μM) ^a
3	> 40	16	3.67
4	> 40	17	2.47
5	10.91	18	3.34
6	11.06	19	2.40
7	> 40	20	1.56
8	7.99	21	1.80
9	> 40	22	3.13
10	7.05	23	> 40
11	2.96	24	> 40
12	> 40	2	9.20
13	1.42	LLM-F	5.25
14	5.85	lactacystin	5.60
15	13.21		

^aThe IC₅₀ values in the table are the averages from three experiments.