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Original article

Synthesis and evaluation of A-*seco* type triterpenoids for anti-HIV-1 protease activity

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ABSTRACT

2,3-*Seco*-dioic acids derived from four different triterpene skeletons were prepared and evaluated for their anti-HIV-1 protease activity. Two A-*seco* derivatives showed potent inhibitory activity against HIV-1 protease (**3c** and **3e**, IC_{50} 5.7 and 3.9 μM, respectively), while four other derivatives showed moderate to weak inhibition (**3a**, **3b**, **3d** and **3f**, IC_{50} 15.7–88.1 μM). The combination of a 2,3-*seco*-2,3-dioic acid functional group in ring A and a free acid group at C-28 or C-30 significantly enhanced HIV-1 protease inhibitory activity (**3a**, **3c**–**3e**, IC_{50} 3.9–17.6 μM). On the other hand, all A-*seco* derivatives were found to be very weak inhibitors of HCV, renin and trypsin proteases ($IC_{50} > 80$ μM). These findings indicate that A-*seco* triterpenes with a carboxyl group at C-28 or C-30 are novel and highly selective HIV-1 protease inhibitors.

1. Introduction

Acquired immune deficiency syndrome (AIDS) is one of the most fatal infectious diseases in the world. The disease is caused by human immunodeficiency virus (HIV). A WHO study showed that there were nearly 35.7 million HIV-infected individuals, with more than 2.1 million individuals dying of AIDS worldwide in 2007 [1]. At present, highly active antiretroviral therapy (HAART) using a combination of HIV protease inhibitors (PI) and reverse transcriptase inhibitors (RTI) has markedly decreased the mortality of AIDS patients and improved the quality of life of HIV-infected individuals. However, the rapid emergence of drug resistance has increased the need for more diverse anti-HIV agents, with either new structures or mechanisms of action [2].

In our long-term screening of natural products and their derivatives as HIV-1 PR inhibitors [3–8], we have found that some triterpenes, such as ganoderic acid B and ganoderiol B [3], *N*-(3β-hydroxylolean-12-en-28-oyl)-6-aminohexanoic acid [4], hemiesters of ursolic acid [5], 3-oxotirucalla-7,24-dien-21-oic acid [6], and A-*seco* triterpenes, 16β-hydroxy-2,3-*seco*-lup-20(29)-en-2,3-dioic acid [7] and colossolactone V [8], showed potent inhibitory activity against HIV-1 PR. Our previous structure–activity relationship (SAR) investigation on triterpene derivatives against HIV-1 PR showed that oleanolic acid derivatives with a 6-aminohexanoic acid combination at the C-28 position (IC_{50} 1.7 μM) strongly enhanced activity as compared with the original oleanolic acid (IC_{50} 8.0 μM) [4]. Moreover, other research groups have reported that some naturally occurring triterpenes and their semisynthetic analogues possessed potent anti-HIV activity [9], such as 3,4-*seco*-ring A triterpene, lancilactone C [9], maslinic acid derivatives with several α- and ω-amino acids at C-28 [10], betulinic acid derivatives with benzyl amide at C-28 [11] and betulinic acid C-3 neoglycosides [12]. QSAR has been suggested for some of these compounds [13]. It is also reported that some valine-containing prodrugs derived from clinically used HIV PR inhibitors (saquinavir, nelfinavir and indinavir) were chemically more stable [14–16]. These results led us to synthesize a series of mono- or bi-functional 2,3-*seco*-2,3-dioic acid triterpene derivatives in order to develop compounds having more potent inhibitory activity against HIV-1 PR.

In the present work, we focused our attention on the pentacyclic triterpenes, including betulin, oleanolic acid (OA), ursolic acid (UA), glycyrrhetic acid (GA) and lupenone. These triterpenes occur abundantly in the plant kingdom and are reported to have interesting biological, pharmacological and medicinal properties, such as anti-HIV activity [17], anti-inflammatory activity [18] and cytotoxicity against tumor cell lines [19]. Nevertheless, no systematic bioactivity investigations of the A-ring *seco* derivatives of the pentacyclic triterpenes have been conducted to date.

Structurally, betulinic acid (BA), OA, GA and UA have a hydroxyl group at C-3 and also have a free acid group at C-28 or C-30. The former enables modification to 2,3-*seco*-2,3-dioic acid derivatives, and the latter allows conversion to an amide group at C-28 or C-30.

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A betulinic acid derivative, Berivimat (PA-457) has been undergoing second-phase clinical trials as an anti-AIDS drug candidate [20]. Interestingly, an α,α-dimethyl carboxyl moiety exists in the A-ring in both 16β-hydroxy-2,3-*seco*-lup-20(29)-en-2,3-dioic acid and PA-457. Here, we report the chemical modification of a hydroxyl group attached to the A-ring and a carboxyl group at C-28. The inhibitory activity was then screened for HIV-1 PR, and SARs were determined for 30 triterpene derivatives. In addition, all A-*seco* compounds were tested against other proteases, such as HCV, renin and trypsin PRs, to evaluate selectivity.

2. Results and discussion

2.1. Chemistry

For the purpose of preparing A-*seco* triterpene derivatives, betulin, OA, UA, GA and lupenone were subjected to stepwise oxidation to yield compounds **1a**–**3e**, according to the methods of Ma et al. [4] and Urban et al. [21] (Scheme 1). After screening with HIV-1 PR, the most active A-*seco* oleanoic acid was further converted to bi-functional A-*seco* derivative **3f**, A-nor compound (**7**) and triol (**8**), using the methods of Ma et al. [4] and Saxena et al. [22] (Schemes 1 and 2).

2.1.1. Synthesis of *A*-seco *type triterpenes*

A-*seco* derivatives **3a**–**3e** were prepared by stepwise oxidation (Scheme 1). First, oxidation of betulin, GA, OA and UA with pyridinium chlorochromate (PCC) in CH_2Cl_2 afforded the respective 3-oxo derivatives **1a** (81.7%), **1c** (21.2%), **1d** (32.4%) and **1e** (67.9%). Further oxidation was performed by introducing air into *tert*-butyl alcohol solutions of the respective 3-oxo derivatives and lupenone [7], in the presence of potassium *tert*-butoxide to yield 2-hydroxy-3-oxo derivatives **2a** (58.8%), **2b** (18.3%), **2c** (92.6%), **2d** (92.3%) and **2e** (44.3%). Cleavage of the A-ring of **2a**–**2e** was conducted by refluxing their MeOH solutions in the presence of hydrogen peroxide and KOH to afford the respective A-*seco* derivatives **3a** (34.5%), **3b** (17.4%), **3c** (17.0%), **3d** (47.8%) and **3e** (40.5%).

Scheme 1 shows the preparation of bi-functional A-*seco* derivatives. Initially, oleanolic acid was acetylated at C-3 with acetic anhydride in pyridine to afford 3-*O*-acetyloleanolic acid **4a** (100%). Thereafter, compound **4a** was further treated with oxalyl chloride in dry CH_2Cl_2 to yield 3β-acetoxyolean-12-en-28-oyl chloride **4b** in quantitative yield. Treatment of compound **4b** with L-valine methyl ester in the presence of triethylamine in CH_2Cl_2 afforded amide **5a** (70.7%). Hydrolysis of an acetyl group and a methyl ester of compound **5a** using aqueous sodium hydroxide yielded a mixture of *N*-[3β-hydroxyolean-12-en-28-oyl]-valine derivatives (**6a**/**6b**), which were further separated by HPLC to afford two isomers **6a** (21.6%) and **6b** (49.6%). As amino acid derivatives **6a** and **6b** were insoluble in dichloromethane, compounds **6a** and **6b** were treated with trimethylsilyldiazomethane in methanol to yield the corresponding methyl esters **6c** (50.8%) and **6d** (78.3%), which overcame the problem of solubility and let the total yield of next step rise up to 95%. Finally, the same stepwise oxidation reactions were used for preparation of isomers, which were separated by HPLC to yield the corresponding 3-oxo derivatives **1f** (34.4%), **1g** (60.6%), **1h** (18.8%) and **1i** (36.0%), and 2-hydroxy-3-oxo derivatives **2f** (7.2%) and **2g** (85.2%), as well as a 2,3-*seco*-2,3-dioic acid derivative **3f** (85.5%).

During the process of deacetylation, a portion of the L-form amino acid derivatives was isomerized to the D-form. The absolute amino acid configurations were determined by 2D-NMR. Before deacetylation of *N*-[3β-acetoxyolean-12-en-28-oyl]-L-valine methyl ester (**5a**), HMBC correlations between the carbon signal at δ_C 177.5 (C-28) and proton signals at δ 6.41 (NH), δ 4.45 (NH–*CH*, H-α) and δ 2.65 (H-18) as well as between the proton signal at δ 4.45 (NH–*CH*, H-α) and carbon signals at δ_C 172.2 ($COOCH_3$), δ_C 31.8 (C-β), δ_C 18.2 (C-γ_1) and δ_C 18.4 (C-γ_2), indicated that the chemical shift of C-α and C-β in **5a** were δ_C 57.2 and 31.8, respectively. After deacetylation at the C-3 position, differences in chemical shifts of C-α and C-β between the D- and L-type derivatives were observed, i.e., δ_C 57.4 and δ_C 30.5 for D-type **6a**, and δ_C 57.1 and δ_C 31.2 for L-type **6b**, respectively.

The optical rotation values of D-type compounds were usually larger than those of L-type compounds. On the other hand, D-type derivatives were usually eluted earlier, and peak areas of D-type were usually smaller than those of L-type derivatives after HPLC with an ODS column, suggesting that L-amino acid derivatives were the major products, except for target compound **3f**, owing to the strong base reaction conditions. A C-α signal of **3f** appeared at δ_C 57.5, and its optical rotation value was +66.7°. Based on this evidence, **3f** was determined to be a D-type derivative. In addition, different isomers were obtained in the potassium *tert*-butoxide oxidation step; *N*-[3-oxo-olean-12-en-28-oyl]-valines **1h** and **1i** were obtained when the molar ratio of the corresponding methyl esters (**1f**/**1g**) to potassium *tert*-butoxide was more than 36.4, thereafter, *N*-[2-hydroxy-3-oxo-oleana-1,12-dien-28-oyl]-valines (**2f** and **2g**) were obtained from **1h**/**1i** by treatment under the same reaction conditions as for preparing **1h** and **1i**. This indicates that hydrolysis of a 28-ester bond takes precedence over oxidation of a 3-hydroxyl.

2.1.2. Synthesis of 2-oxo-A-norolean-12-en-28-oic acid (7) and 2,3-seco-olean-12-en-2,3,28-triol (8)

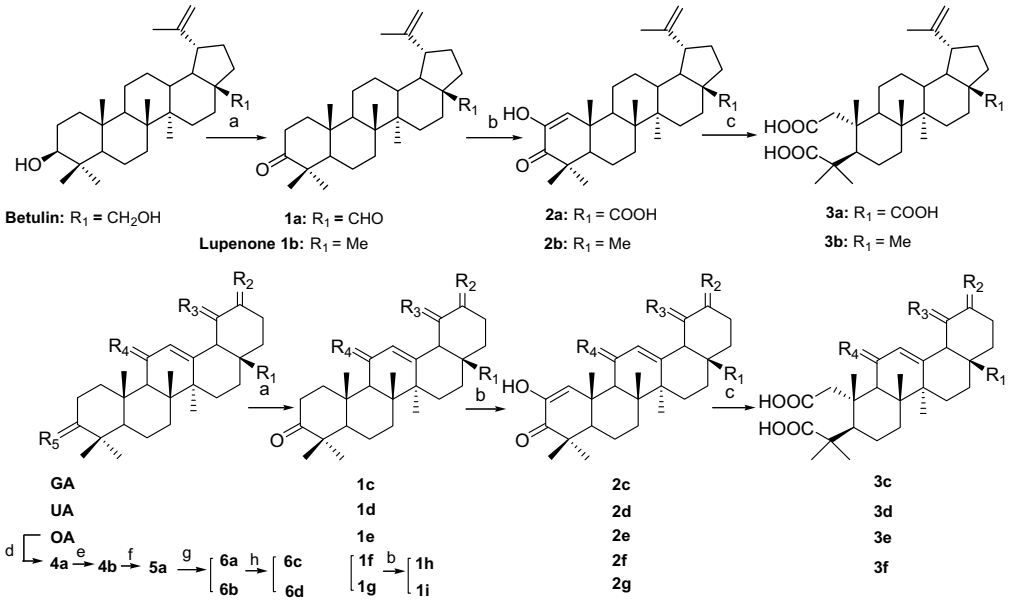
The most active A-*seco* tricarboxylic compound **3e** was further reduced with lithium aluminium hydride ($LiAlH_4$) under reflux in tetrahydrofuran (THF) to afford triol derivative **8** (33.0%). Moreover, **3e** was refluxed with acetic anhydride in pyridine to yield derivative **7** with a five-member A-ring (8.0%) (Scheme 2).

2.2. Inhibitory activity against HIV-1 PR

All compounds were evaluated for their inhibitory activities against HIV-1 PR using the methods previously reported by our group [7]. A known HIV-1 PR inhibitor, pepstatin A was used as a positive control with an IC_{50} value of 1.0 μM in the same assay [23] (Table 1). Based on the IC_{50} values, we regarded those compounds with IC_{50} <10 μM as strongly active, those with IC_{50} ranging from 10 to 50 μM as moderately active and those with IC_{50} ranging from 50 to 100 μM as weakly active. Other compounds with IC_{50} >100 μM were considered to be inactive.

2.2.1. Inhibitory activity of 2,3-seco *triterpenes*

Of A-ring modified triterpenes, two A-*seco* derivatives (**3c** and **3e**, IC_{50} 5.7 and 3.9 μM, respectively) displayed strong inhibitory activity against HIV-1 PR. The other four (**3a**, **3b**, **3d** and **3f**, IC_{50} 15.7–88.1 μM) showed moderate to weak inhibition. Compound **3e** (IC_{50} 3.9 μM) showed significant inhibitory activity against HIV-1 PR and this was much more potent than the lead compound 16β-hydroxy-2,3-*seco*-lup-20(29)-en-2,3-dioic acid (IC_{50} 10.9 μM) in the same assay. It is interesting to note that combination of a 2,3-*seco*-2,3-dioic acid group in ring A and a free acid group linked at C-28 or C-30 significantly enhanced activity in comparison with compounds containing the respective functional groups alone [**3a** (IC_{50} 15.7 μM) vs. **2a** (IC_{50}18.8 μM) and **3b** (IC_{50} 25.4 μM); **3e** (IC_{50} 3.9 μM) vs. oleanoic acid (IC_{50} 8.0 μM)] [4]. On the other hand, two triterpenes derived from the A-*seco* derivative **3e**, 2-oxo-A-nor-olean-12-en-28-oic acid (**7**) and 2,3-*seco*-olean-12-en-2,3,28-triol (**8**), which bear neutral functional groups in ring A showed moderate activity against HIV-1 PR with IC_{50} values of 28.6 and 30.4 μM, respectively, and were less active than **3e**, suggesting that A-*seco* triterpene derivatives with multi-acidic functional groups displayed potent inhibition on HIV-1 PR.



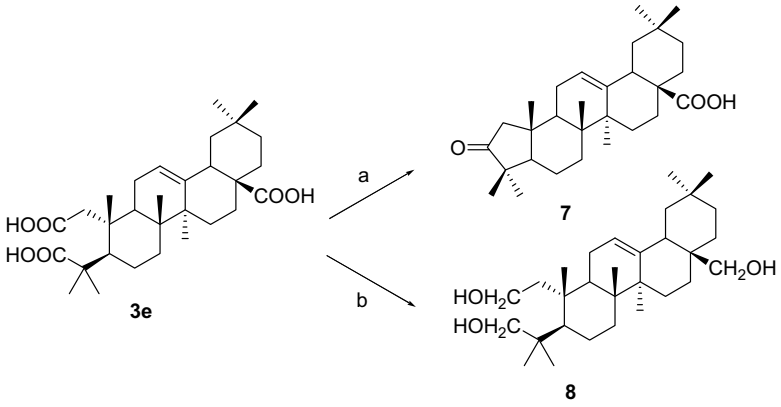
	R_1	R_2	R_3	R_4	R_5
GA:	Me	Me, β COOH	H, H	O	H, β OH
1c:	Me	Me, β COOH	H, H	O	
2c:	Me	Me, β COOH	H, H	O	
3c:	Me	Me, β COOH	H, H	O	
UA:	COOH	H, α Me	H, β Me	H, H	H, β OH
1d:	COOH	H, α Me	H, β Me	H, H	
2d:	COOH	H, α Me	H, β Me	H, H	
3d:	COOH	H, α Me	H, β Me	H, H	
OA:	COOH	Me, Me	Me, Me	H, H	H, β OH
1e:	COOH	Me, Me	Me, Me	H, H	
1f:	D-Val M	Me, Me	H, H	H, H	
1g:	L-Val M	Me, Me	H, H	H, H	
1h:	D-Val	Me, Me	H, H	H, H	
1i:	L-Val	Me, Me	H, H	H, H	
2e:	COOH	Me, Me	H, H	H, H	
2f:	D-Val	Me, Me	H, H	H, H	
2g:	L-Val	Me, Me	H, H	H, H	
3e:	COOH	Me, Me	H, H	H, H	
3f:	D-Val	Me, Me	H, H	H, H	
4a:	COOH	Me, Me	H, H	H, H	H, β OAc
4b:	COCl	Me, Me	H, H	H, H	H, β OAc
5a:	L-Val M	Me, Me	H, H	H, H	H, β OAc
6a:	D-Val	Me, Me	H, H	H, H	H, β OH
6b:	L-Val	Me, Me	H, H	H, H	H, β OH
6c:	D-Val M	Me, Me	H, H	H, H	H, β OH
6d:	L-Val M	Me, Me	H, H	H, H	H, β OH

Val =  ; Val M = 

Scheme 1. Synthesis of A-*seco* derivatives. Reagents and conditions: (a) PCC, rt, 6 h; (b) O_2, *t*-BuOK, *t*-BuOH, 40 °C, 40 min; (c) H_2O_2, KOH, MeOH, reflux, 100 min; (d) Ac_2O, pyridine, reflux, 10 h; (e) $(COCl)_2$, $CHCl_3$, rt, 20 h; (f) L-Valine methyl ester, CH_2Cl_2, Et_3N, rt, overnight; (g) 4 N NaOH, CH_3OH–THF, rt, 18 h; (h) MeOH, trimethylsilyldiazomethane, rt, 30 min.

2.2.2. Inhibitory activity of 3-oxo and 2-hydroxyl-3-oxo derivatives

Most 3-oxo and 2-hydroxyl-3-oxo derivatives (**1a**–**1i** and **2a**–**2g**), having one free carboxyl group or amide residue at C-28 or C-30 showed moderate activity against HIV-1 PR and decreased inhibition when compared with the corresponding A-*seco* compound, suggesting that the number and position of carboxyl groups significantly affect inhibitory activity against HIV-1 PR. Meanwhile, another aspect to be considered is that 2-hydroxyl-3-oxo derivatives (**2a**–**2e**, IC_{50} 18.8–25.4 μM) showed more potent activity than their 3-oxo derivatives (**1a**–**1e**, IC_{50} 23.6–235.7 μM),



Scheme 2. Synthesis of **3e** derivatives. Reagents and conditions: (a) Ac_2O, pyridine, reflux, 10 h; (b) $LiAlH_4$, THF, reflux, 5 h.

suggesting that the multi-hydrogen bonding capacity of the A-ring is important for anti-HIV-1 PR activity. Of 3-oxo and 2-hydroxyl-3-oxo amides, L-configuration amides showed more activity than the corresponding D-type compounds against HIV-1 PR [**1g** (IC_{50} 37.0 μM) vs. **1f** (IC_{50} 41.4 μM), **1i** (IC_{50} 14.6 μM) vs. **1h** (IC_{50} 45.2 μM), and **2g** (IC_{50} 40.5 μM) vs. **2f** (IC_{50} 49.7 μM)].

2.2.3. Inhibitory activity of 3-acetyl derivatives

3-Acetyl-L-amide **5a** showed moderate inhibition of HIV-1 PR (IC_{50} 21.3 μM).

2.2.4. Inhibitory activity of 3-hydroxyl amide derivatives

Most 3-hydroxyl amides (**6a**–**6d**) showed moderate inhibition of HIV-1 PR (IC_{50} from 10.6 to 47.3 μM).

2.2.5. Inhibitory activity against other proteases

All *seco*-compounds were tested for their inhibitory activities against other proteases, such as HCV PR, renin and trypsin. HCV PR is a dimeric protease, like HIV-1 PR [24,25]. Renin is an aspartyl PR, like HIV-1 PR, and trypsin belongs to serine PR, like HCV PR [26,27]. All tested derivatives showed no inhibitory activity against HCV, renin or trypsin PRs ($IC_{50} > 80.3$ μM) (Table 2), suggesting that A-*seco* triterpenes are potent, highly selective HIV-1 PR inhibitors.

In summary, starting from four different pentacyclic triterpene skeletons, a series of A-*seco* triterpene derivatives were successfully synthesized using stepwise oxidation reactions. Two of the A-*seco* derivatives showed potent inhibitory activity against HIV-1 protease (**3c** and **3e**, IC_{50} 5.7 and 3.9 μM, respectively), while four other derivatives showed moderate to weak inhibition (**3a**, **3b**, **3d** and **3f**, IC_{50} 15.7–88.1 μM). In comparison with bi-functional A-*seco* triterpene derivative (**3f**), the mono-2,3-*seco*-2,3-dioic acid compounds (**3a**–**3e**) are more potent against HIV-1 PR. Some interesting SARs were determined as follows.

1. 2,3-*Seco*-2,3-dioic acid derivatives with a carboxyl group directly attached at C-28 or C-30 of four different skeletons, have significantly higher inhibitory activity against HIV-1 PR.
2. Of 2,3-*seco*-2,3-dioic acid derivatives, a 28-carboxyl group enhances the inhibitory activity when compared to a 28-methyl group (**3a** vs. **3b**).
3. Reduction of a 2,3-*seco*-2,3,28-trioic acid derivative (**3e**) to a 2,3-*seco*-2,3,28-triol derivative (**8**) results in decreased inhibitory activity against HIV-1 PR.
4. An A-nor type triterpene with an oxo group at C-2 has less inhibitory activity against HIV-1 PR than the original A-*seco* compound.
5. An A-*seco* derivative conjugated with D-valine at C-28 shows decreased inhibitory activity against HIV-1 PR.

In our previous paper, we demonstrated that triterpenes inhibited HIV-1 PR by dissociation of the dimeric enzyme (active form) to monomeric enzyme (inactive form) [4]. The present triterpene derivatives are thought to have the same mechanism of action, i.e., dimerization inhibitors of HIV PR. Further syntheses and biological evaluations of new triterpene derivatives are currently in progress in order to identify more potent anti-HIV-1 PR agents with preventive and therapeutic effects on AIDS.

3. Experimental

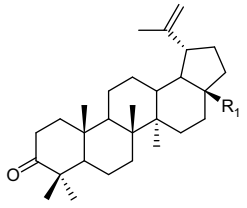
3.1. Instruments and chemicals

Optical rotations were measured on a Jasco DIP-360 digital automatic polarimeter at ambient temperature. IR spectra were measured with a Jasco FT/IR-230 infrared spectrometer. Melting points were determined on a Yanagimoto micro hot-stage melting point apparatus without correction. The ^1H and ^{13}C spectra were recorded on a Varian UNITY Plus 500 (^1H, 500 MHz; ^{13}C, 125 MHz) spectrometer. Conventional pulse sequences were used for COSY, HMBC and HMQC. All chemical shifts (δ) are given in parts per million (ppm) units with reference to tetramethylsilane (TMS) as an internal standard, and the coupling constants (J) are given in hertz (Hz). Electrospray ionization mass (ESI-MS) was obtained on an Esquire 3000^{plus} spectrometer (Bruker Daltonik GmbH, Bremen, Germany). HR-EI-MS and EI-MS were taken on a JMS-AX 505 HAD gc/ms system and a JMS DX-300 system at ionization voltage at 70 eV. HR-FAB-MS were measured on a Jeol JMS-700 apparatus with a resolution of 5000 and with *m*-nitrobenzyl alcohol as matrix. TLC was carried out on precoated silica gel 60 F_{254} (Merck). The following chromatographic carriers were used for isolation: silica gel BW-820MH (Fuji, Silysia); and ODS DM 1020T (Fuji Silysia). Prep HPLC was performed on a Tosoh CCPM-CCPM-II system (Tosoh Co.) equipped with a UV 8020 detector and a TSK gel ODS-80Ts column (21.5 × 300 mm, Tosoh Co.). Potassium *tert*-butoxide, *tert*-butyl alcohol, tetrahydrofuran, lithium aluminium hydride ($LAlH_4$) and pyridinium chlorochromate (PCC), acetic anhydride, pyridine, oxalyl dichloride, L-valine methyl ester, methylene dichloride, triethylamine, sodium hydroxide, trimethylsilyldiazomethane were purchased from Sigma–Aldrich Company. GA, OA and lupenone were isolated in our laboratory in a previous work [4,7].

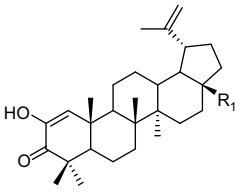
3.2. Enzyme assay kits

The HIV protease (Lot# 146125) was purchased from Bachem A. G., Switzerland. HIV protease substrate (Lot# 2000403) and Pepstatin A (Cat# 71147) were provided by Wako Pure Chemical Industries, Ltd. SensoLyte™ 520 HCV Protease Assay Kit *Fluorimetric* (Lot# AK

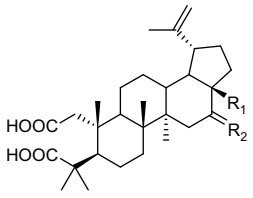
Table 1
Inhibitory activity of 31 triterpenes against HIV-1 PR.



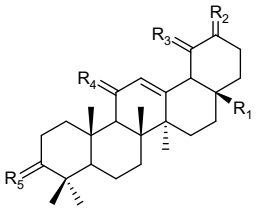
1a, 1b



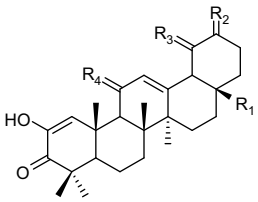
2a, 2b



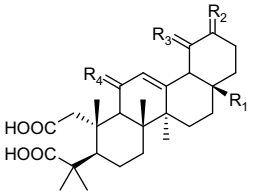
LC, **3a, 3b**



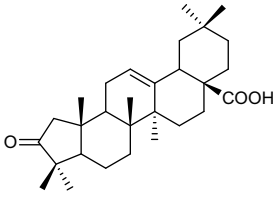
1c-1i, **4a**, **5a**, **6a-6d**



2c-2g



3c-3f



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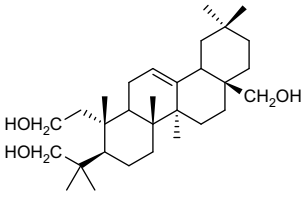
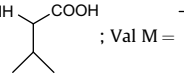
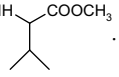


8

Comp.	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM) ± RSD% ($n = 3$)
						HIV PR
1a	CHO					31.9 ± 3.8
1b	Me					>235.6 ± 4.5
1c	Me	Me, β COOH	H, H	O	O	23.6 ± 8.5
1d	COOH	H, α Me	H, β Me	H, H	O	37.5 ± 1.3
1e	COOH	Me, Me	H, H	H, H	O	27.4 ± 4.9
1f	D-Val M	Me, Me	H, H	H, H	O	41.4 ± 4.2
1g	L-Val M	Me, Me	H, H	H, H	O	37.0 ± 3.4
1h	D-Val	Me, Me	H, H	H, H	O	45.2 ± 2.3
1i	L-Val	Me, Me	H, H	H, H	O	14.6 ± 4.3
2a	COOH					18.8 ± 4.3
2b	Me					24.6 ± 6.1
2c	Me	Me, β COOH	H, H	O		15.8 ± 6.0
2d	COOH	H, α Me	H, β Me	H, H		25.4 ± 4.5
2e	COOH	Me, Me	H, H	H, H		19.2 ± 2.8
2f	D-Val	Me, Me	H, H	H, H		49.7 ± 2.9
2g	L-Val	Me, Me	H, H	H, H		40.5 ± 6.5
LC	Me	H, β OH				10.9 ± 5.9
3a	COOH	H, H				15.7 ± 3.4
3b	Me	H, H				25.4 ± 5.1
3c	Me	Me, β COOH	H, H	O		5.7 ± 1.2
3d	COOH	H, α Me	H, β Me	H, H		17.6 ± 2.6
3e	COOH	Me, Me	H, H	H, H		3.9 ± 2.9
3f	D-Val	Me, Me	H, H	H, H		88.1 ± 8.6
4a	COOH	Me, Me	H, H	H, H	H, β OAc	>200.6 ± 3.4
5a	L-Val M	Me, Me	H, H	H, H	H, β OAc	21.3 ± 3.2
6a	D-Val	Me, Me	H, H	H, H	H, β OH	47.3 ± 5.2
6b	L-Val	Me, Me	H, H	H, H	H, β OH	10.6 ± 4.3
6c	D-Val M	Me, Me	H, H	H, H	H, β OH	32.3 ± 1.3
6d	L-Val M	Me, Me	H, H	H, H	H, β OH	24.6 ± 3.8
7						28.6 ± 2.4
8						30.4 ± 5.0
[a]**PC A**						1.0 ± 2.9

Data represent mean values ± RSD% for three independent experiments.
LC: lead compound, 16β-hydroxy-2,3-*seco*-lup-20(29)-en-2,3-dioic acid.

Val =  ; Val M =  .

[a] Pepstatin A: positive control for HIV PR.

Table 2
Inhibitory activity of A-*seco* triterpenes against HCV, renin and trypsin PRs.

Comp.	HCV PR IC_{50} (μM)	Renin PR IC_{50} (μM)	Trypsin PR IC_{50} (μM)
A-seco lupene type triterpenes			
LC	>204.6 ± 8.9	>2046.0 ± 5.7	>2046.0 ± 3.3
3a	>199.1 ± 6.6	>1991.0 ± 3.4	856.0 ± 3.2
3b	80.3 ± 6.8	>2117.0 ± 6.3	>2117.0 ± 6.3
A-seco oleanolic acid type triterpenes			
3c	>194.4 ± 1.2	>1935.0 ± 6.3	>1935.0 ± 3.5
3d	>199.1 ± 3.3	>1991.0 ± 0.7	>1991.0 ± 8.1
3e	>199.0 ± 3.7	>1990.0 ± 4.4	>1990.0 ± 2.7
3f	>166.3 ± 3.1	>1663.0 ± 5.1	>1663.0 ± 1.3
[a]**PC B**	0.5 ± 1.5	nt	nt
[b]**PC C**	nt	0.043 ± 4.5	nt
[c]**PC D**	nt	nt	0.040 ± 1.4

Data represent mean values (±RSD%) for three independent experiments.
LC: lead compound, 16β-hydroxy-2,3-*seco*-lup-20(29)-en-2,3-dioic acid.
nt: not tested.
[a] Hepatitis Virus C NS 3 Protease Inhibitor 2: positive control for HCV PR.
[b] [Ac-HPFV-(Sta)-LF-NH_2]: positive control for renin.
[c] Trypsin–chymotrypsin inhibitor from soybean: positive control for trypsin.

71145-1016), Hepatitis Virus C NS3 protease inhibitor 2 (Cat# 25346), HCV NS3/4A protease (Lot# 046-079), SensoLyte™ 520 Renin Assay Kit *Fluorimetric* (Lot# 72040) and SensoLyte™ Green Protease Assay Kit *Fluorimetric* (Lot# AK 71124-1009) were purchased from *AnaSpec. Inc.*, San Jose, CA, USA. The trypsin–chymotrypsin inhibitor from soybean was obtained from Sigma–Aldrich Co. The BD Falcon™ Microtest™ 384-well 120 μL black assay plates were purchased from Nonsterile, No Lid. Lot# 05 39 11 55, Germany.

3.3. General experimental procedure

3.3.1. Synthesis of 28-formyl-lup-20(29)-en-3-one (**1a**)

PCC (1317 mg, 6.1 mmol) was slowly added to a solution of betulin (900 mg, 2.0 mmol) in CH_2Cl_2 (5.2 mL) and stirred for 6 h at room temperature. The mixture was poured into H_2O and extracted with chloroform, and the extract was then washed with H_2O, saturated aqueous $NaHCO_3$, and H_2O again, followed by drying over $MgSO_4$, filtration, and evaporation of solvent under reduced pressure. The residue was subjected to silica gel column chromatography (70 cm × 2.5 cm) and eluted with hexane–acetone (95:5) to yield **1a** as colorless crystals (728 mg, 81.7% yield); mp 126–130 °C; $[\alpha]_D^{25}$ +35.2° (*c* 0.3, $CHCl_3$), lit. [28], $[\alpha]_D^{28}$ +35.7° (*c* 0.3, MeOH); ESI-MS *m*/*z* 439.4 $[M+H]^+$; ^1H and ^{13}C NMR spectral data of **1a** were in agreement with those published in the literature [28].

3.3.2. Synthesis of 2-hydroxy-3-oxo-lupa-1,20(29)-dien-28-oic acid (**2a**)

Air was introduced to a solution of **1a** (500 mg, 1.14 mmol) and potassium *tert*-butoxide (4.66 g, 41.5 mmol) in *tert*-butyl alcohol (45.3 mL), and the mixture was stirred at 40 °C for 40 min, and prepared in the same way as for **1a**. The crude product was separated by chromatography over an ODS column (20 cm × 4.5 cm) and eluted with MeOH–H_2O (50–100%) to yield compound **2a** as yellow meringue (519 mg, 58.8% yield); mp. 204–205 °C, lit. [16], 200–203 °C; $[\alpha]_D^{21}$ +12° (*c* 0.56, C_5H_5N), lit. [21], $[\alpha]_D^{28}$ +10.3° (*c* 1.01, MeOH); ESI-MS *m*/*z* 467.9 $[M-H]^-$; ^1H NMR spectral data of **2a** were in agreement with those published in the literature [21].

3.3.3. Synthesis of 2,3-seco-lupa-20(29)-en-2,3,28-trioic acid (**3a**)

A solution of **2a** (500 mg, 1.1 mmol) and KOH (1.54 g) in MeOH (83.0 mL) was heated under reflux, and hydrogen peroxide (8.3 mL, 30%) was added over a 100-min period. The reaction mixture was then poured into cold H_2O, and the product was extracted with ethyl acetate (2 × 100 mL), followed by prep HPLC column chromatography (21.5 × 300 mm) with MeOH–0.1% TFA/H_2O, 5 mL/min, monitoring at 208 nm to yield **3a** (185 mg, 33.5% yield); mp. 276–277 °C, lit. [21], 270–274 °C; $[\alpha]_D^{28}$ +12.4° (*c* 2.06, MeOH); ESI-MS *m*/*z* 501.5 $[M-H]^-$; ^1H NMR spectral data of **3a** were in agreement with those published in the literature [21].

3.3.4. Synthesis of 2-hydroxy-lupa-1,20(29)-dien-3-one (**2b**)

This compound was prepared from lupenone (900 mg, 2.12 mmol) in the same manner as described for **2a**. Purification by chromatography on an ODS column (20 cm × 4.5 cm) with MeOH–H_2O (50–100%) yielded compound **2b** as an amorphous powder (169 mg, 18.3% yield); $[\alpha]_D^{28}$ +68.7° (*c* 1.01, MeOH), $[\alpha]_D$ +70.9° [29]; ESI-MS *m*/*z* 439.4 $[M+H]^+$.

3.3.5. Synthesis of 2,3-seco-lup-20(29)-en-2,3-dioic acid (**3b**)

This compound was prepared from **2b** (150 mg, 0.342 mmol) in the same manner as described for **3a**. Purification with an HPLC column (21.5 mm × 300 mm), with MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitoring at 208 nm, yielded compound **3b** as an amorphous powder (28 mg, 17.4% yield). $[\alpha]_D^{28}$ +40.5° (*c* 2.8, MeOH), $[\alpha]_D$ +49.6° [29]; ESI-MS *m*/*z* 471.5 $[M-H]^-$.

3.3.6. Synthesis of 3,11-dioxo-olean-12-en-30-oic acid (**1c**)

This compound was prepared from GA (520 mg, 1.11 mmol) in the same manner as described for **1a**. Purification by silica gel column chromatography (30 cm × 3.5 cm) with hexane–acetone (95:5) yielded compound **1c** as an amorphous powder (109.7 mg, 21.2% yield); $[\alpha]_D^{28}$ +243.4° (*c* 0.83, MeOH); ESI-MS *m*/*z* 467.2 $[M-H]^-$; ^1H and ^{13}C NMR spectral data of **1c** were in agreement with those published in the literature [30].

3.3.7. Synthesis of 2-hydroxy-3,11-dioxo-oleana-1,12-dien-30-oic acid (**2c**)

This compound was prepared from **1c** (109 mg, 0.233 mmol) in the same manner as described for **2a**. Purification by ODS column chromatography (20 cm × 4.5 cm) with MeOH–H_2O (50–100%) yielded compound **2c** as white needles (104.2 mg, 92.6% in yield); mp. 320–323 °C, lit. [31], 295–297 °C, $[\alpha]_D^{28}$ +124.2° (*c* 0.95, MeOH); ESI-MS *m*/*z* 481.3 $[M-H]^-$; ^1H NMR spectral data of **2c** were in agreement with those published in the literature [31].

3.3.8. Synthesis of 2,3-seco-11-oxo-olean-12-en-2,3,30-trioic acid (**3c**)

This compound was prepared from **2c** (115 mg, 0.239 mmol) in the same manner as described for **3a**. Purification by HPLC column chromatography (21.5 mm × 300 mm) [MeOH–0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] yielded compound **3c** as a white crystalline powder (21 mg, 17% yield); mp. 205–208 °C, lit. [32], 225–228 °C; $[\alpha]_D^{28}$ +108.8° (*c* 0.99, MeOH); ESI-MS *m*/*z* 515.4 $[M+H]^+$.

3.3.9. Synthesis of 3-oxo-urs-12-en-28-oic acid (**1d**)

This compound was prepared from UA (400 mg, 0.876 mmol) in the same manner as described for **1a**, and purified by silica gel column chromatography (70 cm × 2.5 cm) eluted with hexane–acetone (95:5) to yield **1d** as an amorphous powder (129 mg, 32.4% yield); mp. 271–275 °C, lit. [33], 272–275 °C; $[\alpha]_D^{28}$ +77.3° (*c* 1.22, MeOH); ESI-MS *m*/*z* 453.8 $[M-H]^-$. The ^1H NMR spectral data of **1d** were in agreement with those published in the literature [33].

3.3.10. Synthesis of 2-hydroxy-3-oxo-ursa-1,12-dien-28-oic acid (**2d**)

This compound was prepared from **1d** (100 mg, 0.22 mmol) in the same manner as described for **2a**, and purified by column

chromatography on ODS (20 cm × 4.5 cm) with MeOH–H_2O (50–100%) to yield **2d** as an amorphous powder (95.0 mg, 92.3% yield); $[\alpha]_D^{25}$ +99° (*c* 0.46, $CHCl_3$) lit. [34], $[\alpha]_D^{28}$ +77.2° (*c* 1.08, MeOH); ESI-MS *m*/*z* 467.2 $[M-H]^-$; ^1H and ^{13}C NMR spectral data of **2d** were in agreement with those published in the literature [34].

3.3.11. Synthesis of 2,3-seco-urs-12-en-2,3,28-trioic acid (3d)

This compound was prepared from **2d** (80 mg, 0.171 mmol) in the same manner as described for **3a**, and purified by HPLC (21.5 mm × 300 mm) [MeOH–0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] to yield **3d** as a white crystalline powder (41 mg, 47.8% yield); mp. 263–265.8 °C; $[\alpha]_D^{28}$ +37.2° (*c* 0.96, MeOH); HR-FAB-MS *m*/*z* 501.32236 $[M-H]^-$ (calcd for. $C_{30}H_{45}O_6$ 501.32162).

IR ν_{max}^{KBr} cm^{-1}: 3460, 2980, 2660, 1700, 1680, 1650, 1454, 1390; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.86 (3H, s, CH_3), 0.87 (3H, s, CH_3), 0.96 (3H, s, CH_3), 1.06 (3H, s, CH_3), 1.14 (3H, s, CH_3), 1.26 (6H, s, 2 × CH_3), 2.21 (1H, d, *J* = 11.0 Hz, H-18), 2.40 (1H, d, *J* = 18.0 Hz, H-1a), 2.55 (1H, d, *J* = 18.0 Hz, H-1b), 5.25 (1H, td, *J* = 3.5, 6.5 Hz, H-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 17.6, 18.0, 19.7, 21.5, 22.4, 23.8, 24.7, 24.9, 25.4, 27.4, 29.2, 31.8, 33.5, 38.1, 40.4, 40.5, 40.7, 42.8, 43.3, 44.0, 47.5, 48.5, 49.5, 54.4, 127.1, 139.3, 175.5, 181.7, 183.5.

3.3.12. Synthesis of 3-oxo-olean-12-en-28-oic acid (1e)

This compound was prepared from OA (1 g, 2.19 mmol) in the same manner as described for **1a**. Purification by silica gel column chromatography (70 cm × 2.5 cm) eluted with hexane–acetone (95:5) yielded **1e** as a white crystalline powder (675 mg, 67.9% yield); mp. 170–173 °C lit. [4], 168–171 °C; $[\alpha]_D^{28}$ +44.9° (*c* 1.03, MeOH); ESI-MS *m*/*z* 453.0 $[M-H]^-$. The ^1H NMR spectral data of **1e** were in agreement with those published in the literature [4].

3.3.13. Synthesis of 2-hydroxy-3-oxo-oleana-1,12-dien-28-oic acid (2e)

This compound was prepared from **1e** (647 mg, 1.42 mmol) in the same manner as described for **2a**. Purification by column chromatography (20 cm × 4.5 cm) on ODS with MeOH–H_2O (50–100%) yielded **2e** as an amorphous solid (294.6 mg, 44.3% yield): $[\alpha]_D^{25}$ +99° (*c* 0.46, $CHCl_3$) lit. [35], $[\alpha]_D^{28}$ +83.5° (*c* 0.95, MeOH); ESI-MS *m*/*z* 467.8 $[M-H]^-$; ^1H and ^{13}C NMR spectral data of **2e** were in agreement with those published in the literature [35].

3.3.14. Synthesis of 2,3-seco-olean-12-en-2,3,28-trioic acid (3e)

This compound was prepared from **2e** (278 mg, 0.594 mmol) in the same manner as described for **3a**. Purification by HPLC [MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] yielded **3e** as a white crystalline powder (121 mg, 40.5% yield), mp. 294–296 °C lit. [36], 285–288 °C; $[\alpha]_D^{28}$ +70.7° (*c* 1.24, MeOH). ESI-MS *m*/*z* 501.5 $[M-H]^-$.

3.3.15. Synthesis of 2-oxo-A-norolean-12-en-28-oic acid (7)

Acetic anhydride (0.40 mL, 3.88 mmol) was added to a solution of a *seco* derivative **3e** (50 mg, 0.099 mmol) in pyridine (2 mL). After 10 h reflux, the mixture was poured into cold H_2O and worked up. The crude product was chromatographed over an ODS column (20 cm × 4.5 cm) eluted with MeOH–H_2O (50–100%) to afford A-nor triterpene **7** as an amorphous powder (3 mg, 8.0% yield); $[\alpha]_D^{28}$ +65.2° (*c* 0.7, MeOH) lit. [36]. ESI-MS *m*/*z* 439.1 $[M-H]^-$.

3.3.16. Synthesis of 2,3-seco-olean-12-en-2,3,28-triol (8)

$LiAlH_4$ (99 mg, 2.61 mmol) was added to a solution of triacid **3e** (50 mg, 0.099 mmol) in tetrahydrofuran (3.0 mL), and the mixture was refluxed for 5 h. Ethyl acetate (5.0 mL) was slowly added to decompose residual $LiAlH_4$, and the mixture was poured into cold H_2O followed by working up as usual. The crude product was chromatographed over an ODS column (20 cm × 4.5 cm) eluted with MeOH–H_2O (50–100%) to afford triol **8** (15 mg) as white power, 33.0% yield; mp. 252–255 °C, $[\alpha]_D^{28}$ +102.8° (*c* 0.91, MeOH); HR-EI-MS *m*/*z* 460.39094 $[M+H]^+$ (calcd for $C_{30}H_{52}O_3$, 460.39165); IR ν_{max}^{KBr} cm^{-1}: 3400, 2960, 2880, 1454; ^1H NMR (pyridine-d_5, 500 MHz) δ: 0.93 (3H, s, H_3-29), 0.96 (3H, s, H_3-30), 1.01 (3H, s, H_3-26), 1.03 (3H, s, H_3-24), 1.10 (3H, s, H_3-25), 1.18 (3H, s, H_3-27), 1.44 (3H, s, H_3-23), 1.80 (1H, m, *J* = 14.0 Hz, H-1a), 2.28 (1H, m, *J* = 14.0 Hz, H-1b), 3.51 (1H, dd, *J* = 5.0, 10.5 Hz, H-3a), 3.59 (1H, overlapping, *J* = 4.5 Hz, H-28a), 3.83 (1H, overlapping, *J* = 4.5 Hz, H-28b), 3.87 (1H, overlapping, *J* = 5.5, 10.5 Hz, H-3b), 4.11 (1H, m, *J* = 5.5, 10.0 Hz, H-2a), 4.43 (1H, m, *J* = 4.5, 10.0 Hz, H-2b), 5.26 (1H, t-like, *J* = 3.5 Hz, H-12), 5.81 (1H, t-like, *J* = 5.0 Hz, 2-OH), 5.98 (1H, t-like, *J* = 5.5, 10.5 Hz, 28-OH), 6.32 (1H, t-like, *J* = 5.0, 10.5 Hz, 3-OH). ^{13}C NMR (pyridine-d_5, 125 MHz) δ: 17.2, 20.4, 21.8, 22.9, 23.8, 24.2, 24.5, 25.7, 25.9, 26.2, 31.2, 31.7, 32.5, 33.4, 34.6, 37.6, 40.1, 40.4, 41.0, 42.3, 42.3,42.6, 42.7, 45.4, 47.0, 58.4, 68.6, 72.3, 123.3, 144.6.

3.3.17. Synthesis of N-(3β-acetoxyolean-12-en-28-oyl)-L-valine methyl ester (5a)

Oxalyl chloride (1.28 mL, 8.58 mmol) was added to a solution of 3-*O*-acetyloleanolic acid (1 g, 2.01 mmol) in $CHCl_3$. The reaction mixture was stirred at room temperature for 20 h and the solvent was removed. Cyclohexane (30 mL) was added to the residue, and the mixture was evaporated to dryness. This was repeated twice to yield crude 3β-acetoxylolean-12-en-28-oyl chloride (1.13, 2.19 mmol). A CH_2Cl_2 solution (50 mL) of the crude chloride (450 mg, 0.872 mmol) and L-valine methyl ester (167.6 mg, 1.0 mmol) was stirred in the presence of triethylamine (0.3 mL, 2.15 mmol) at room temperature over night. The reaction mixture was concentrated under vacuum, worked up as usual and chromatographed over ODS with H_2O–MeOH. The MeOH eluate was further purified by SiO_2 column chromatography (3.5 cm × 55 cm) with hexane–acetone (10:1–7:1) to yield **5a** as white powder (387 mg, 71.2% yield), $[\alpha]_D^{28}$ +33.8° (*c* 0.06, MeOH) lit. [37], ESI-MS *m*/*z* 610.7 $[M-H]^-$.

3.3.18. Synthesis of N-[3β-hydroxyolean-12-en-28-oyl]-D-valine (6a) and N-[3β-hydroxyolean-12-en-28-oyl]-L-valine (6b)

A solution of *N*-(3β-acetoxyolean-12-en-28-oyl)-L-valine methyl ester (377 mg, 0.62 mmol) and aqueous NaOH (4 N, 3.5 mL) in CH_3OH–THF (1:1.5, 14.5 mL) was stirred for 18 h at room temperature and then concentrated in vacuo. The residue was worked up and purified by HPLC [MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] to yield **6a** (74 mg, 21.6% yield) and **6b** (170 mg, 49.6% yield) as a white crystalline powder. Compound **6a**: mp. 270–271.8 °C. $[\alpha]_D^{28}$ +49.9° (*c* 0.05, MeOH); HR-FAB-MS *m*/*z* 554.42157 $[M-H]^+$ (calcd for $C_{35}H_{56}NO_4$, 554.42093).

IR ν_{max}^{KBr} cm^{-1}: 3480, 2960, 1740, 1710, 1690, 1650, 1520, 1390; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.76 (3H, s), 0.77 (3H, s), 0.90 (3H, s), 0.91 (3H, s), 0.94 (3H, s), 0.94 (3H, s), 0.95 (3H, s), 0.98 (3H, s), 1.16 (3H, s), 2.71 (1H, d, *J* = 12.5 Hz, H-18), 3.20 (1H, dd, *J* = 5.0, 9.5 Hz, H-3), 4.32 (1H, m, *J* = 5.0, 7.5, 9.0 Hz, –NH–*CH*–), 5.36 (1H, t-like, *J* = 3.0, 6.5 Hz, H-12), 6.37 (1H, d, *J* = 8.0 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.2, 15.4, 16.9, 17.1, 18.1, 18.1, 18.7, 23.2, 23.3, 25.6, 26.7, 27.1, 27.9, 30.5, 30.7, 32.7, 32.9, 33.1, 33.9, 36.8, 38.4, 38.6, 39.2, 41.9, 42.1, 46.2, 46.6, 47.4, 55.0, 57.5, 78.7, 122.5, 143.8, 173.8, 178.3.

Compound **6b**: mp. 277–278.9 °C. $[\alpha]_D^{28}$ +45.2° (*c* 0.11, MeOH) lit. [37]. ESI-MS *m*/*z* 554.5 $[M-H]^-$.

3.3.19. Synthesis of N-(3β-hydroxyolean-12-en-28-oyl)-D-valine methyl ester (6c) and N-[3β-hydroxyolean-12-en-28-oyl]-L-valine methyl ester (6d)

These compounds were prepared from valine conjugates (**6a**/**6b**). Trimethylsilyldiazomethane was added to a solution of mixture of **6a** and **6b** (210 mg, 0.38 mmol) with stirring for 30 min

at room temperature. The reaction mixture was purified by HPLC [MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] to yield **6c** (69.0 mg, 32.0% yield) as a white powder and **6d** (168.5 mg, 78.3% yield) as a white crystalline powder.

Compound **6c**: $[\alpha]_D^{28}$ +35.9° (*c* 0.04, MeOH); HR-EI-MS *m*/*z* 569.44153 $[M+H]^+$ (calcd for $C_{36}H_{59}NO_4$, 569.44441); IR ν_{max}^{KBr} cm^{-1}: 3500, 2950, 1740, 1660, 1650, 1520, 1460, 1390, 1370, 1210; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.75 (3H, s), 0.78 (3H, s), 0.90 (3H, s), 0.91 (6H, s), 0.92 (3H, s), 0.94 (3H, s), 0.99 (3H, s), 1.16 (3H, s), 2.74 (1 H, dd, *J* = 2.5, 13.0 Hz, H-18), 3.22 (1H, dd, *J* = 4.0, 11.0 Hz, H-3), 3.73 (3 H, s, $COOCH_3$), 4.41 (1H, dd, *J* = 5.0, 7.5 Hz, –NH–*CH*–), 5.36 (1H, t-like, *J* = 3.5, 7.0 Hz, H-12), 6.20 (1H, d, *J* = 8.0 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.4, 15.6, 17.3, 18.2, 18.3, 18.9, 23.4, 23.5, 23.6, 25.7, 27.1, 27.3, 28.1, 30.7, 31.1, 32.8, 33.0, 33.3, 34.1, 37.0, 38.5, 38.7, 39.4, 42.1, 42.1, 46.3, 46.7, 47.5, 52.0, 55.1, 57.4, 79.0, 122.5, 144.1, 172.7, 177.7.

Compound **6d**: mp. 196–198.2 °C. $[\alpha]_D^{28}$ +26.1° (*c* 0.05, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3480, 3420, 2960, 1740, 1660, 1650, 1510, 1460, 1390, 1360, 1250; HR-EI-MS *m*/*z* 569.44519 $[M+H]^+$ (calcd for $C_{36}H_{59}NO_4$, 569.44441); ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.68 (3H, s), 0.77 (3H, s), 0.90 (3H, s), 0.90 (3H, s), 0.92 (6H, s), 0.94 (3H, s), 0.99 (3H, s), 1.16 (3H, s), 2.65 (1H, dd, *J* = 3.0, 12.5 Hz, H-18), 3.22 (1H, dd, *J* = 4.0, 11.0 Hz, H-3), 3.71 (3H, s, $COOCH_3$), 4.32 (1H, dd, *J* = 5.5, 7.5 Hz, –NH–*CH*–), 5.44 (1H, t-like, *J* = 3.5, 7.0 Hz, H-12), 6.44 (1H, d, *J* = 7.0 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.3, 15.5, 16.6, 18.2, 18.3, 18.7, 23.5, 23.5, 23.6, 25.7, 27.1, 27.4, 28.1, 30.7, 31.7, 32.6, 32.9, 33.2, 34.1, 36.9, 38.4, 38.7, 39.4, 42.0, 42.1, 46.5, 46.6, 47.5, 51.9, 55.1, 57.2, 78.9, 123.3, 143.6, 172.4, 177.8.

3.3.20. Synthesis of N-(3-oxo-olean-12-en-28-oyl)-D-valine methyl ester (**1f**) and N-(3-oxo-olean-12-en-28-oyl)-L-valine methyl ester (**1g**)

These compounds were prepared from a mixture of valine methyl esters (**6c**/**6d**) (190 mg, 0.33 mmol) in the same manner as described for **1a** and purified by HPLC [MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] to yield **1f** (70.9 mg, 39.4% yield) and **1g** (109 mg, 60.6% yield) as a white powder.

Compound **1f**: $[\alpha]_D^{28}$ +81.7° (*c* 0.05, MeOH); HR-EI-MS *m*/*z* 567.43011 $[M+H]^+$ (calcd for $C_{36}H_{57}NO_4$, 567.42876); IR ν_{max}^{KBr} cm^{-1}: 3460, 3200, 2940, 2660, 2400, 2080, 1740, 1710, 1690, 1660, 1650, 1510, 1460, 1390, 1370, 1260, 1200; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.80 (3H, s), 0.91–0.94 (12H, s), 1.03 (3H, s), 1.04 (3H, s), 1.09 (3H, s), 1.17 (3H, s), 2.37 (1H, m, *J* = 3.5, 7.0, 10.0 Hz, H-2a), 2.54 (1H, m, *J* = 4.0, 9.0, 15.5 Hz, H-2b), 2.77 (1H, d, *J* = 10.0 Hz, H-18), 3.74 (3H, s, $COOCH_3$), 4.43 (1H, dd, *J* = 4.5, 12.5 Hz, –NH–*CH*–), 5.38 (1H, t-like, H-12), 6.19 (1H, d, *J* = 7.5 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.4, 17.2, 17.2, 17.4, 17.5, 18.6, 19.2, 19.8, 21.7, 23.8, 23.8, 25.9, 26.8, 27.5, 31.0, 31.4, 32.6, 33.3, 33.6, 34.4, 37.0, 39.4, 39.6, 42.4, 46.5, 46.9, 47.1, 47.7, 52.3, 55.5, 57.6, 122.5, 144.4, 173.0, 177.9, 218.1.

Compound **1g**: $[\alpha]_D^{28}$ +40.3° (*c* 0.06, MeOH); HR-EI-MS *m*/*z* 567.43109 $[M+H]^+$ (calcd for $C_{36}H_{57}NO_4$, 567.42876); IR ν_{max}^{KBr} cm^{-1}: 3500, 2960, 2660, 2400, 2100, 1740, 1710, 1690, 1660, 1650, 1510, 1460, 1390, 1370, 1260, 1200; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.74 (3H, s), 0.92–0.94 (12H, s), 1.04 (6H, s), 1.09 (3H, s), 1.17 (3H, s), 2.37 (1H, m, *J* = 13.5 Hz, H-2a), 2.67 (1H, m, *J* = 6.5, 15.5 Hz, H-2b), 2.67 (1 H, d, *J* = 12.0 Hz, H-18), 3.72 (3H, s, $COOCH_3$), 4.46 (1H, dd, *J* = 5.5, 11.5 Hz, –NH–*CH*–), 5.46 (1H, t-like, H-12), 6.42 (1H, d, *J* = 7.0 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.0, 16.6, 16.8, 18.2, 18.7, 19.5, 21.4, 23.5, 23.6, 23.7, 25.5, 26.4, 27.3, 30.7, 31.7, 32.1, 32.9, 33.2, 34.1, 36.6, 39.1, 39.3, 42.1, 42.2, 46.4, 46.6, 46.8, 47.4, 51.9, 55.2, 57.2, 123.0, 143.7, 172.4, 177.7, 217.7.

3.3.21. Synthesis of N-(3-oxo-olean-12-en-28-oyl)-D-valine (**1h**) and N-(3-oxo-olean-12-en-28-oyl)-L-valine (**1i**)

These compounds were prepared from *N*-[3-oxo-olean-12-en-28-oyl]-valine methyl ester (**1f**/**1g**) (150 mg, 0.264 mmol) in the same manner as described for **2a** and purified by HPLC column (21.5 mm × 300 mm) [MeOH–0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] to yield **1h** (27.0 mg, 18.8% yield) and **1i** (52.5 mg, 36.0% yield) as a white powder.

Compound **1h**: $[\alpha]_D^{23.6}$ +64.1° (*c* 0.06, MeOH); HR-FAB-MS *m*/*z* 552.40371 $[M-H]^-$ (calcd for $C_{35}H_{54}NO_4$, 552.40528); IR ν_{max}^{KBr} cm^{-1}: 3500, 2940, 1740, 1710, 1690, 1650, 1520, 1460, 1390, 1200; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.81 (3H, s), 0.92 (3H, s), 0.93 (3H, s), 0.98–1.00 (6H, s), 1.03 (3H, s), 1.04 (3H, s), 1.09 (3H, s), 1.17 (3H, s), 2.38 (1H, m, *J* = 3.5, 7.0, 10.5 Hz, H-2a), 2.55 (1H, m, *J* = 3.5, 8.5, 19.0 Hz, H-2b), 2.70 (1 H, d, *J* = 9.5 Hz, H-18), 4.28 (1H, t-like, *J* = 6.0, 12.0 Hz, –NH–*CH*–), 5.39 (1H, t-like, *J* = 3.5 Hz, H-12), 6.29 (1H, d, *J* = 7.0 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.1, 17.0, 17.1, 17.4, 17.9, 18.4, 19.0, 19.5, 21.4, 23.5, 25.6, 26.5, 27.2, 30.2, 30.7, 32.2, 33.0, 33.0, 34.0, 34.1, 36.7, 39.2, 39.3, 42.2, 42.2, 46.3, 46.7, 47.4, 55.2, 58.6, 122.6, 144.1, 173.5, 178.9, 217.9.

Compound **1i**: $[\alpha]_D^{28}$ +59.2° (*c* 0.05, MeOH); HR-FAB-MS *m*/*z* 552.40569 $[M-H]^-$ (calcd for $C_{35}H_{54}NO_4$, 552.40528); IR ν_{max}^{KBr} cm^{-1}: 3420, 2960, 1740, 1710, 1690, 1650, 1510, 1460, 1430, 1390; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.76 (3H, s), 0.92 (6H, s), 0.98 (3H, s), 0.99 (3H, s), 1.02 (3H, s), 1.03 (3H, s), 1.09 (3H, s), 1.17 (3H, s), 2.38 (1H, m, *J* = 2.5, 6.0, 15.5 Hz, H-2a), 2.55 (1H, m, *J* = 3.0, 9.0, 15.5 Hz, H-2b), 2.63 (1 H, d, *J* = 11.0 Hz, H-18), 4.47 (1H, dd, *J* = 5.5, 11.0 Hz, –NH–*CH*–), 5.47 (1H, t-like, H-12), 6.53 (1H, d, *J* = 6.5 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.1, 16.7, 17.9, 17.9, 18.9, 19.5, 21.4, 23.4, 23.6, 25.5, 26.4, 27.3, 30.7, 31.0, 32.1, 32.9, 33.0, 34.0, 34.1, 36.6, 39.2, 39.4, 42.2, 42.3, 46.4, 46.7, 46.8, 47.4, 55.2, 57.2, 123.3, 143.7, 175.2, 178.9, 218.0.

3.3.22. Synthesis of N-(2-hydroxy-3-oxo-oleana-1,12-dien-28-oyl)-D-valine (**2f**) and N-(2-hydroxy-3-oxo-oleana-1,12-dien-28-oyl)-L-valine (**2g**)

These compounds were prepared from a mixture of **1h** and **1i** (68 mg, 0.123 mmol) in the same manner as described for **2a** and purified by HPLC (column, 21.5 mm × 300 mm) [MeOH–0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] to yield **2f** (5 mg, 7.2% yield) and **2g** (29 mg, 85.2.0% yield) as a white powder.

Compound **2f**: $[\alpha]_D^{23.6}$ +115.8° (*c* 0.04, MeOH); HR-FAB-MS *m*/*z* 566.38077 $[M-H]^-$ (calcd for $C_{35}H_{52}NO_5$, 566.38454); IR ν_{max}^{KBr} cm^{-1}: 3420, 3200, 2950, 2620, 2080, 1740, 1730, 1710, 1690, 1650, 1520, 1460, 1390, 1240; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.83 (3H, s), 0.92 (3H, s), 0.94 (3H, s), 0.98–1.00 (6H, s), 1.12 (3H, s), 1.16 (3H, s), 1.21 (3H, s), 1.22 (3H, s), 2.70 (1H, d, *J* = 13.5 Hz, H-18), 4.25 (1H, t-like, *J* = 6.0, 12.0 Hz, –NH–*CH*–), 5.43 (1H, t-like, H-12), 5.91 (1H, bs, OH), 6.27 (1H, d, *J* = 7.5 Hz, –NH–), 6.33 (1H, s, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 17.1, 17.2, 17.7, 18.4, 19.1, 19.7, 21.8, 23.5, 23.5, 25.7, 27.2, 27.2, 30.7, 31.3, 32.4, 33.0, 33.0, 34.0, 38.3, 40.1, 42.3, 42.4, 43.0, 43.9, 46.2, 46.8, 53.8, 58.7, 122.2, 128.0, 143.7, 144.5, 174.0, 178.9, 201.1.

Compound **2g**: $[\alpha]_D^{28}$ +48.1° (*c* 0.04, MeOH); HR-FAB-MS *m*/*z* 566.38430 $[M-H]^-$ (calcd for $C_{35}H_{52}NO_5$, 566.38454); IR ν_{max}^{KBr} cm^{-1}: 3420, 2980, 1740, 1730, 1710, 1690, 1650, 1510, 1460, 1390, 1240; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.78 (3H, s), 0.93 (6H, s), 0.98–1.00 (6H, s), 1.11 (3H, s), 1.16 (3H, s), 1.20 (3H, s), 1.22 (3H, s), 2.64 (1 H, d, *J* = 10.0 Hz, H-18), 4.47 (1H, t-like, *J* = 6.0, 12.0 Hz, –NH–*CH*–), 5.50 (1H, t-like, H-12), 5.95 (1H, bs, OH), 6.34 (1H, s, H-1), 6.51 (1H, d, *J* = 3.5 Hz, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 17.1, 17.2, 17.9, 18.6, 19.0, 19.6, 21.8, 23.4, 23.5, 25.6, 27.2, 27.2, 30.7, 30.9, 32.4, 32.9, 33.0, 34.0, 38.3, 40.1, 42.3, 42.4, 42.9, 43.9, 46.2, 46.8, 53.7, 57.2, 122.9, 128.1, 143.7, 144.1, 175.2, 179.0, 201.1.

3.3.23. Synthesis of N-(2,3-seco-olean-12-en-28-oyl)-D-valine acid (**3f**)

This compound was prepared from **2g** (20 mg, 0. 035 mmol) in the same manner as described for **3a**. Purification by HPLC [MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] yielded **3f** as a white powder (18.0 mg, 85.5% yield). $[\alpha]_D^{28}$ +66.7° (*c* 0.06, MeOH);

HR-FAB-MS m/z 600.38786 $[M-H]^-$ (calcd for $C_{35}H_{55}NO_7$, 600.39002); IR ν_{max}^{KBr} cm^{-1}: 3410, 2960, 2660, 1730, 1710, 1690, 1650, 1510, 1460, 1390, 1260, 1200; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.73 (3H, s), 0.91 (6H, s), 0.94–0.97 (6H, s), 1.00 (3H, s), 1.18 (3H, s), 1.21 (3H, s), 1.25 (3H, s), 2.38–2.45 (2H, d, $J = 18.5$ Hz, H-1a, H-1b), 2.67 (1H, m, $J = 12.0$ Hz, H-18), 4.37 (1H, m, $J = 2.5$, 5.0 Hz, –NH–*CH*–), 5.43 (1H, t-like, H-12), 6.58 (1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 17.0, 18.2, 18.3, 18.9, 19.2, 20.8, 23.2, 23.6, 23.8, 23.9, 25.3, 27.5, 28.2, 30.8, 31.5, 32.1, 33.1, 33.4, 34.3, 39.3, 39.6, 41.8, 42.3, 42.9, 46.0, 46.4, 47.0, 48.9, 57.4, 123.7, 143.2, 173.8, 174.6, 178.8, 183.5.

3.4. HIV PR assay

Inhibitory activity was tested with HIV-1 PR using a PR-HPLC method, as described previously [7].

3.5. HCV PR assay

Inhibitory activity was tested with HCV PR using a fluorometric method, as described previously [38]. Hepatitis Virus C NS 3 Protease Inhibitor 2 was used as a positive control with an IC_{50} value of 0.5 μM in the same assay [39].

3.6. Renin PR assay

Compound solution (2.5 μL; DMSO as solvent; final concentration, 10%) was placed in a 384-well format microplate, and then 14.0 μL of rec-renine PR (0.5 μg/mL) was added to the wells containing sample, and the plate was agitated to ensure good mixing. Finally, 8.5 μL of freshly diluted rennin substrate [Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Il2-His-Thr-Lys(Dabcyl)-Arg] was added with sequential rotational shaking. The reaction mixture was incubated for 30 min at 37 °C. A renin inhibitor [Ac-HPFV-(Sta)-LF-NH_2] was used as positive control [40]. The methods of fluorometric analyses and inhibitory calculation were the same as described for HCV PR assay [38].

3.7. Green PR assay

Compound solution (2.5 μL; DMSO as solvent; final concentration, 10%) was placed in a 384-well format microplate, then 17.5 μL of an assay buffer and 2.5 μL of trypsin (0.1 U/μL) were added to the wells containing sample and the plate was agitated. Finally, 2.5 μL of freshly diluted protease substrate, HiLyte Fluor™ 488-labeled casein, was added with sequential rotational shaking. The reaction mixture was incubated for 30 min at 37 °C. A trypsin–chymotrypsin inhibitor from soybean was used as a positive control [41]. The methods of fluorometric analysis and inhibitory calculation were the same as described for HCV PR assay [38].

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