Bioorganic & Medicinal Chemistry 17 (2009) 3003–3010

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of dammarane-type triterpene derivatives and their ability to inhibit HIV and HCV proteases

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ARTICLE INFO

Article history:
Received 8 February 2009
Revised 8 March 2009
Accepted 10 March 2009
Available online 14 March 2009

Keywords:
Dammarane
Triterpene
2,3-*seco*-Derivative
Succinyl derivative
HIV-1 protease
HCV protease

ABSTRACT

We synthesized dammarane-type triterpene derivatives and evaluated their ability to inhibit HIV-1 and HCV proteases to understand their structure–activity relationships. All of the mono- and di-succinyl derivatives (**5a**–**5f**) were powerful inhibitors of HIV-1 protease (IC_{50} < 10 μM). However, only di-succinyl (**5e**) and 2,3-*seco*-2,3-dioic acid (**3b**) derivatives similarly inhibited HCV protease (IC_{50} < 10 μM). A-nor dammarane-type triterpenes (**4a** and **4b**, IC_{50} 10.0 and 29.9 μM, respectively) inhibited HIV-1 protease moderately or strongly, but were inactive against HCV protease. All compounds that powerfully inhibited HIV-1 or HCV protease did not appreciably inhibit the general human proteases, renin and trypsin (IC_{50} > 1000 μM). These findings indicated that the mono-succinyl dammarane type derivatives (**5a**–**5d**) selectively inhibited HIV-1 protease and that the di-succinyl (**5e**, **5f**) as well as 2,3-*seco*-2,3-dioic acid (**3b**) derivatives preferably inhibited both viral proteases.

1. Introduction

The retroviral human immunodeficiency virus type-1 (HIV-1), is a causative agent of acquired immunodeficiency syndrome (AIDS). One important enzyme necessary for the maturation and infectivity of this virus is the aspartic protease, HIV-1 protease (HIV-1 PR), which functions in the dimeric form composed of monomers of 99 amino acids. Thus, HIV-1 PR is an important target of searches for anti-HIV drugs.[1]

On the other hand, the single-stranded RNA flavivirus hepatitis C virus (HCV), causes type C hepatitis, which often leads to liver cirrhosis, hepatic failure and hepatocellular carcinoma.[2] Its genome of about 9.6 kb encodes the structural proteins C, E1, E2, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. Since the NS3-NS4A protease plays a critical role in HCV viral replication, this dimeric serine protease has been viewed as a promising target for screening anti-HCV drugs.[3,4]

Currently approved anti-HIV drugs, including highly active antiretroviral therapy (HAART) with a combination of HIV PR inhibitors and reverse transcriptase inhibitors, have remarkably decreased the mortality of AIDS patients and improved the quality of life of those infected with HIV.[5,6] However, because HIV easily mutates and rapidly develops drug resistance, it remains a leading cause of death worldwide, resulting in more deaths than any other infectious disease due to inadequate access to HIV prevention and treatment.[7] As with hepatitis C, clinical options are currently limited, and the only available drugs are subcutaneous pegylated interferon (pegIFN-α) alone or in combination with oral ribavirin (Rbv), the therapeutic effectiveness of which is only 50% for HCV infected patients.[8–10] In light of the potential offered by chemoprevention, novel effective and safe agents are urgently required.[11] We reported that some natural products and their derivatives have anti-HIV-1 PR activity.[12] Of these, some triterpenes, such as ganoderic acid B and ganoderiol B,[13] *N*-(3β-hydroxylolean-12-en-28-oyl)-6-aminohexanoic acid,[14] hemiesters of ursolic acid,[15] 3-oxotirucalla-7,24-dien-21-oic acid,[16] 16β-hydroxy-2,3-*seco*-lup-20(29)-en-2,3-dioic acid[17] and colossolactone V,[18] are potent inhibitors of HIV-1 PR. We also reported that embelin, 5-*O*-methylembelin[19] as well as some indole derivatives[20] inhibited HCV PR. Several studies over the past decade have demonstrated that pentacyclic triterpenes especially oleanolic acid (OA), ursolic acid (UA), betulinic acid (BA) and glycyrrhetic acid (GA) as well as their derivatives inhibited HIV.[21,22] However, the abilities of tetracyclic triterpenes, especially dammarane-type triterpene derivatives to inhibit HIV and HCV PRs have not been reported. Ginseng roots contain abundant ginsenosides mainly consisting of dammarane-type triterpenes as the aglycones, such as protopanaxadiol and protopanaxatriol.[23] However, these aglycones convert to panaxadiol (PD) and panaxatriol (PT), respectively under strong acid conditions.[24] PD and PT have the same A ring moiety, but PT possesses one more hydroxyl group in B ring, that is, PT is 6α-hydroxy-PD. Panaxadiol has two

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doi:10.1016/j.bmc.2009.03.019

hydroxyl groups at C-3 and C-12 in the A and C rings, respectively, whereas PT has three hydroxyl groups at C-3, C-6 and C-12 in the A, B and C rings, respectively. These functional groups provide footholds to convert A—*seco* and acylated derivatives. Here, we describe the preparation of dammarane-type triterpene derivatives by modifying the hydroxyl groups in the A, B and C rings, and their abilities to inhibit HIV-1 and HCV PRs as well as the human aspartyl and serine proteases renin and trypsin, respectively.[25,26]

2. Results and discussion

2.1. Preparation of various dammarane type derivatives

Acid hydrolysis of a ginseng extract yielded (20*R*)-PD and (20*R*)-PT,[24] which were used as starting materials to prepare various 2,3-*seco*, A-nor and acylated dammarane-type triterpene derivatives by modification of the A, B and C rings.

2.1.1. Synthesis of 2,3-*seco* and A-nor dammarane type triterpene derivatives

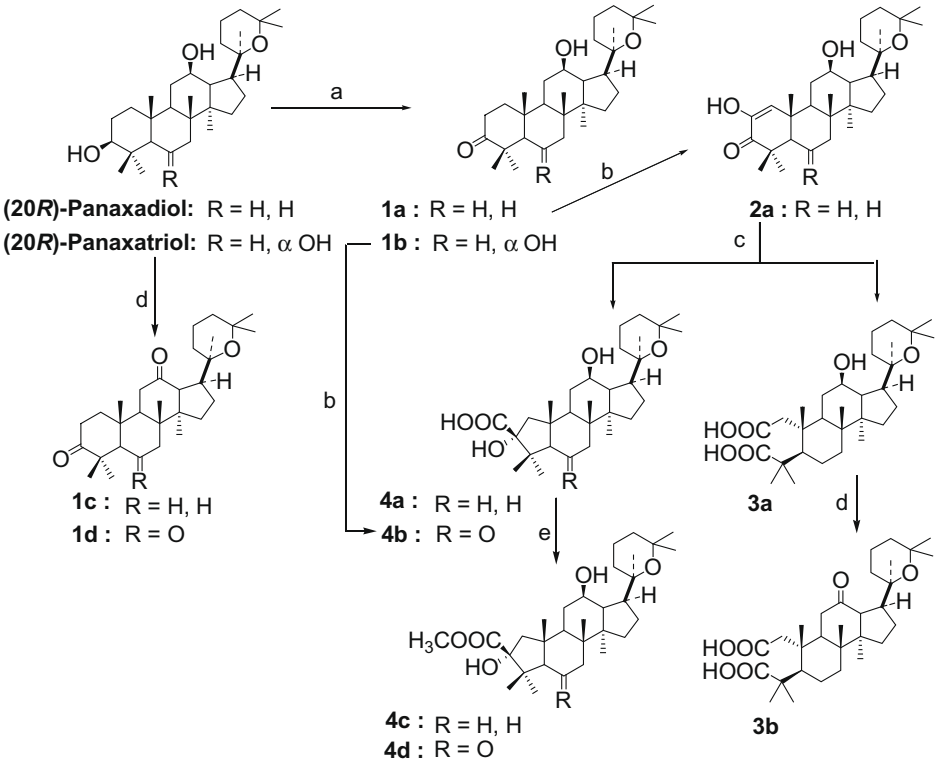
Scheme 1 shows the synthetic routes of various 2,3-*seco* and other oxidized derivatives of (20*R*)-PD and (20*R*)-PT that were essentially developed by Urban et al.[27] and Nagai et al.[28] We initially oxidized (20*R*)-PD or (20*R*)-PT to 3-oxo derivatives **1a** (92.0%) or **1b** (75.5%) using pyridinium chlorochromate (PCC). Thereafter, compound **1a** or **1b** was further oxidized at the A ring by introducing air into a *t*-BuOH solution of **1a** or **1b** in the presence of *t*-BuOK to afford the 2-hydroxy-3-oxo-1-en derivative **2a** (95.0%) or A-nor dammarane-type triterpene **4b** (6.2%). Compound **2a** was then oxidized by the combined action of hydrogen peroxide and KOH in refluxing MeOH to obtain the major products, which were the *seco*-derivatives **3a** (38.8%), and the minor product, the A-nor derivative **4a** (2.0%). Finally, the 2,3-*seco*-2,3-dioic acid derivative **3a** was treated with the Jones reagent to yield the 12-oxo-2,3-*seco*-2.3-dioic acid derivative **3b** (67.8%). To determine the orientation of the 2-carboxyl or 2-hydroxyl group of A-nor derivatives, compounds **4a** and **4b** were treated with trimethylsilyldiazomethane in methanol to afford the methyl esters **4c** (72.5%) and **4d** (48.2%), respectively. The orientation of a 2-methoxycarbonyl group in compounds **4c** and **4d** was confirmed by significant NOE correlations in a NOESY experiment, which identified the following correlations. Proton signals of the 2-methoxylcarboxyl group in compound **4c** correlated with methyl signals at δ 0.97 (H_3-29, β-orientation) and δ 0.89 (H_3-19, β-orientation), suggesting that the 2-methoxylcarboxyl is oriented to the β-face and that the 2-hydroxyl is consequently oriented to the α-face. A methoxyl signal of δ 3.78 (2-OCH_3) in compound **4d** correlated with proton signals at δ 2.65 (H-1b, β-orientation), δ 2.18 (H-9, β-orientation), δ 0.94 (H_3-19, β-orientation) and δ 0.89 (H_3-29, β-orientation), suggesting that a 2-$COOCH_3$ group is in the β-orientation and thus a 2-hydroxyl is in the α-orientation. Under the same reaction conditions described for preparation of the A-nor derivative **4a** from (20*R*)-PD, the corresponding compound **4b** was obtained from (20*R*)-PT simply by two-step oxidation.

2.1.2. Synthesis of (20*R*)-20,25-epoxy-dammaran-3,12-dione (1c) and (20*R*)-20,25-epoxy-dammaran-3,6,12-trione (1d)

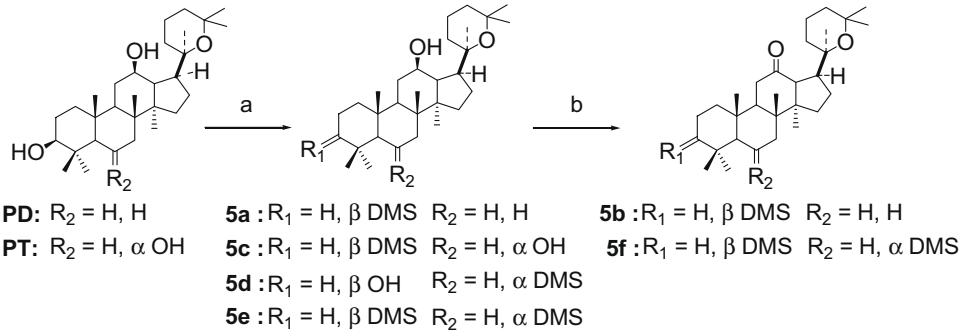
To investigate the effects of hydroxyl groups in (20*R*)-PD and (20*R*)-PT on inhibitory activity against HIV and HCV PRs, (20*R*)-PD and (20*R*)-PT were treated with the Jones reagent for 3 h at room temperature to afford the corresponding oxidized compounds **1c** (25.0%) and **1d** (25.3%) (Scheme 1).

2.1.3. Synthesis of 2,2-dimethylsuccinyl derivatives

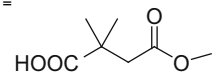
Scheme 2 shows the synthetic routes of acylated (20*R*)-PD and (20*R*)-PT with 2,2-dimethylsuccinic acid, followed by oxidation of a



Scheme 1. Synthesis of 2,3-*seco* and other oxidized derivatives of PD and PT. 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) Jones reagent, rt, 3 h; (e) MeOH, trimethylsilyldiazomethane, rt, 30 min.



Scheme 2. Synthesis of succinyl derivatives of PD and PT. Reagents and conditions: (a) 2,2-dimethylsuccinic anhydride, DMAP, Pyridine, reflux, 16 h; (b) Jones reagent, rt, 3 h. DMS =



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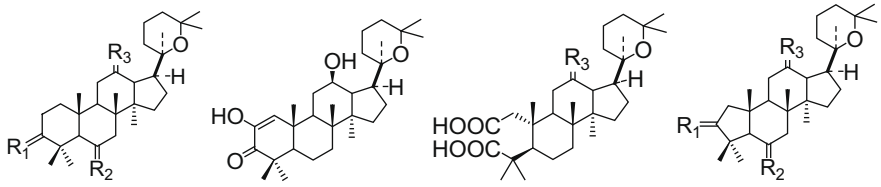
12-hydroxyl group. As described by Hashimoto et al.,[29] (20*R*)-PD and (20*R*)-PT were treated with 2,2-dimethylsuccinic anhydride in the presence of 4-(dimethylamino) pyridine (DMAP) in dry pyridine to afford the acylated derivatives, 3-*O*-acyl [**5a** (35.6%) and **5c** (31.4%)], 6-*O*-acyl [**5d** (12.4%)] and 3,6-di-*O*-acyl [**5e** (24.8%)] derivatives. Compounds **5a** and **5e** were further oxidized using the Jones reagent to afford 12-oxo derivatives **5b** (12%) and **5f** (9.8%), respectively. The structures of these compounds were determined by spectroscopic methodologies including MS, ^{1}H NMR, ^{13}C NMR and IR, and their spectral data are described in Section 4.

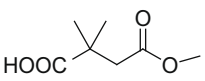
Table 1
Abilities of dammarane-type triterpene derivatives to inhibit HIV-1 and HCV PRs



PD, **PT**, **1a-1d**, **5a-5f** **2a** **3a-3b** **4a-4d**

Compound	R_1	R_2	R_3	IC_{50} (μM) ± RSD (%)	
				HIV PR	HCV PR
PD	H, β OH	H, H	H, β OH	>217.1 ± 1.3	>217.1 ± 1.3
PT	H, β OH	H, α OH	H, β OH	>209.9 ± 3.1	>209.9 ± 3.1
1a	O	H,H	H, β OH	39.4 ± 2.4	>218.0 ± 4.3
1b	O	O	H, β OH	42.3 ± 2.5	>213.0 ± 6.2
1c	O	H, H	O	>218.0 ± 4.3	72.3 ± 4.5
1d	O	O	O	24.2 ± 4.5	>213.0 ± 4.6
2a				28.5 ± 6.4	>212.0 ± 3.0
3a			H, β OH	11.9 ± 6.8	>198.0 ± 3.6
3b			O	22.8 ± 2.2	9.1 ± 5.4
4a	β COOH, α OH	H, H	H, β OH	10.0 ± 1.0	>204.0 ± 3.8
4b	β COOH, α OH	O	H, β OH	29.9 ± 5.2	>213.0 ± 7.2
4c	β $COOCH_3$, α OH	H, H	H, β OH	>198.0 ± 2.8	>198.0 ± 3.2
4d	β $COOCH_3$, α OH	O	H, β OH	>193.0±3.4	>193.0 ± 4.7
5a	H, β DMS	H, H	H, β OH	2.7 ± 4.3	30.4 ± 3.0
5b	H, β DMS	H, H	O	6.5 ± 0.1	62.2 ± 3.5
5c	H, β DMS	H, α OH	H, β OH	3.9 ± 0.1	>166.0 ± 0.4
5d	H, β OH	H, α DMS	H, β OH	2.7 ± 0.4	>166.0 ± 1.8
5e	H, β DMS	H, α DMS	H, β OH	5.4 ± 3.8	1.8 ± 2.6
5f	H, β DMS	H, α DMS	O	10.9 ± 1.5	18.9 ± ± 1.6
[a]PC A				1.3 ± 4.0	nt
[b]PCB				nt	0.5 ± 1.5

Data represent mean values ± RSD(%) for three independent experiments.
[a] PC A, Pepstatin A, positive control for HIV PR.
[b] PC B: Hepatitis Virus C NS 3 Protease Inhibitor 2, positive control for HCV PR; nt: not tested. DMS =



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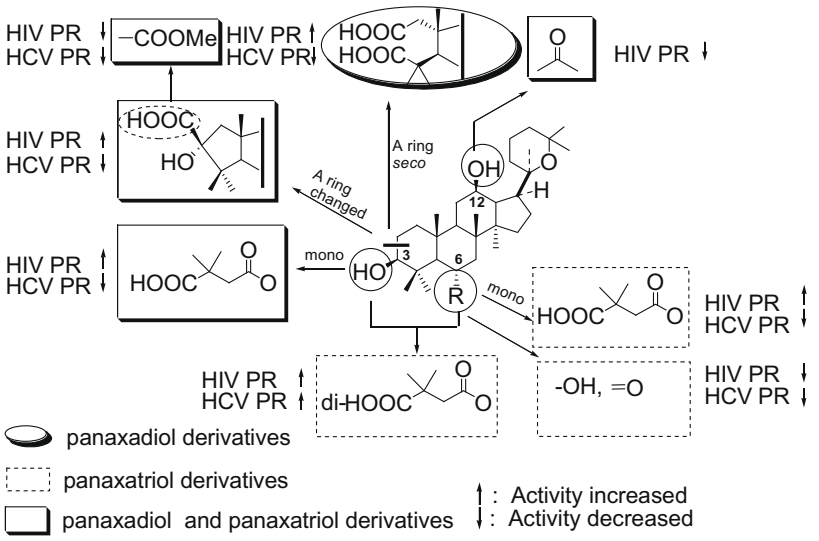

Figure 1. Structure–activity relationship of dammarane triterpene derivatives against HIV and HCV PRs.

2.2. Inhibitory activities of dammarane type derivatives against HIV-1, HCV and other proteases

Table 1 shows the 50% inhibitory concentrations (IC_{50}) in assays of various dammarane-type triterpene derivatives against HIV-1 and HCV PRs using the assay methods described previously.[17,30] The two known protease inhibitors, pepstatin A and hepatitis virus C NS 3 protease inhibitor 2, served as positive controls for HIV-1 and HCV PRs assays, their IC_{50} values being 1.3 and 0.5 μM, respectively.[31,32] We regarded compounds with IC_{50} < 10 μM, 10–50 μM and 50–100 μM as strongly, moderately and weakly inhibitory, respectively. Compounds with IC_{50} > 100 μM were considered inactive.

The starting materials, PD and PT, did not show inhibitory activity on either HIV protease[24] or HCV protease. Some of their derivatives showed potent activities and the structure–activity relationships are discussed as following and summarized in Figure 1.

2.2.1. 3-Oxo- and 2,3-*seco* derivatives

The inhibitory abilities of the dammarane-type triterpenes **1a**, **2a** and **1b** (IC_{50} 39.4, 28.5 and 42.3 μM, respectively) among the 3-oxo derivatives were moderate against HIV PR, but did not inhibit HCV PR. On the other hand, although both 2,3-*seco* derivatives (**3a** and **3b**, IC_{50}, 11.9 and 22.8 μM, respectively) moderately inhibited HIV PR, only compound **3b** was strongly inhibitory against HCV PR (IC_{50} 9.1 μM), suggesting that the 12-oxo group significantly increased the ability to inhibit HCV PR compared with the corresponding free hydroxyl group (**3a**, IC_{50} > 198 μM) in the 2,3-*seco* derivatives.

2.2.2. A-nor dammarane type derivatives

The A-nor dammarane type compounds **4a** and **4b**, with a carboxylic acid group attached to the A ring, moderately inhibited HIV PR with IC_{50} values of 10.0 and 29.9 μM, respectively, but they were inactive against HCV PR. The oxo group at C-6 in **4b** decreased the inhibitory activity against HIV PR. In addition, the methyl esters **4c** and **4d** were inactive against both PRs, suggesting that the free carboxylic acid group at C-2 of **4a** and **4b** is important for interaction with HIV PR.

2.2.3. 2,2-Dimethylsuccinyl derivatives

Of the mono-2,2-dimethylsuccinyl and di-2,2-dimeylsuccinyl derivatives of (20*R*)-PD and (20*R*)-PT, in which the hydroxyl groups at C-3 and C-6 are acylated with succinic acid, the mono- (**5a**–**5d**) and di- (**5e** and **5f**) acyl compounds strongly inhibited HIV-1 PR (IC_{50} 2.7–10.9 μM). The mono-acyl compounds were more inhibitory than the di-acyl compounds. On the other hand, the di-acyl compounds inhibited HCV PR, especially compound **5e**, with an IC_{50} value of 1.8 μM. A comparison of the mono- and di-acyl compounds (**5d** vs **5e** and **5b** vs **5f**) showed that the former preferably inhibited HIV PR, whereas the latter inhibited both proteases, indicating higher selectivity of the mono- than the di-acyl compounds. Moreover, the 12-hydroxyl derivatives (**5a** and **5e**) were more inhibitory than the 12-oxo derivatives (**5b** and **5f**) against both proteases.

We determined the selectivity of the eight compounds (**3a**, **3b** and **5a**–**5f**) with relatively high activity against viral proteases, by their ability to inhibit the human proteases, renin and trypsin. Renin is an aspartyl protease like HIV-1 PR and trypsin is a serine protease like HCV PR. None of the tested derivatives exerted any appreciable inhibitory activity against renin and trypsin PRs at concentrations up to 1 mg/mL (1365–2046 μM). These findings suggested that the dammarane-type triterpenes are highly selective and potent HIV-1 or HCV PR inhibitors that might not interfere with functional physiological reactions.

3. Conclusion

Starting from the artificial aglycones, (20*R*)-PD and (20*R*)-PT derived from an acid hydrolysate of a ginseng extract, we prepared a series of dammarane type derivatives bearing a 2,3-*seco*-2,3-dioic acid (**3a** and **3b**) moiety or 2,2-dimethylsuccinyl functional group(s) at C-3 (**5a**–**5c**) or C-6 (**5d**) or at both positions (**5e** and **5f**). Of seventeen tested compounds, eight were powerful inhibitors of HIV PR with IC_{50} values of 2.7–11.9 μM. Among these, the mono-2,2-dimethylsuccinyl compounds **5a** and **5d** were the most active with an IC_{50} of 2.7 μM. On the other hand, only compounds **3b** and **5e** were powerful inhibitors of HCV PR, (IC_{50} 9.1 and 1.8 μM, respectively), and compound **5a** was moderately inhibitory. The structure–activity relationships (SARs) of these compounds remarkably differed between the two viral proteases; most of the 2,2-dimethylsuccinyl derivatives (**5a**–**5e**) potently inhibited HIV PR, whereas only the di-2,2-dimethylsuccinyl (**5e**) and 12-oxo-2,3-*seco*-2,3-dioic acid (**3b**) derivatives potently inhibited HCV PR. Methylation of the carboxyl group in A-nor dammarane type compounds significantly decreased the inhibitory activities against both proteases. Thus, our study demonstrated that structural modification of dammarane-type triterpenes, especially

(20*R*)-PD and (20*R*)-PT, leads to derivatives with powerful inhibitory activity against either HIV or HCV PR. Compounds with high inhibitory activity on HIV or HCV PR were inactive against renin and trypsin, suggesting low physiological toxicity. Based on these findings, we believe that dammarane-type triterpene derivatives bearing a 2,3-*seco*-2,3-dioic acid moiety or 2,2-dimethylsuccinyl group(s) will provide valuable information for the development of new anti HIV and HCV agents.

4. Experimental

4.1. Instruments and chemicals

Melting points were measured using a Yanagimoto micro hot-stage melting point apparatus without correction. Optical rotation was measured using a Jasco DIP-360 automatic polarimeter. Infrared spectra (IR) were measured using a Jasco FT/IR-230 spectrometer. ^{1}H and ^{13}C NMR were measured using UNITY 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) and UNITY 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometers, the chemical shifts being represented as ppm with tetramethylsilane as the internal standard. Electrospray ionization mass (ESI-MS) spectra were obtained using an Esquire 3000^{plus} spectrometer (Bruker Daltonik GmbH, Bremen, Germany).

High-resolution-EI-MS and EI-MS spectra were obtained on a JMS-AX 505 HAD GC/MS system and a JMS DX-300 system at ionization voltage at 70 eV. High-resolution-FAB-MS were measured on a Jeol JMS-700 with a resolution of 5000 using *m*-nitrobenzyl alcohol as the matrix. Compounds were separated by TLC on plates recoated with Silica Gel 60 F_{254} (Merck). The following were the chromatographic carriers used for isolations, silica gel BW-820MH (Fuji Silysia Chemical Ltd) and ODS DM 1020T (Fuji Silysia). Preparative 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.).

t-BuOK, *t*-BuOH, 2,2-dimethylsuccinic anhydride, 4-dimethylaminorpyridine (DMAP), pyridinium chlorochromate (PCC) and chromium(VI) oxide were purchased from Sigma–Aldrich Company.

4.2. Enzyme assay kits

We purchased HIV protease (Lot# 146125) from Bachem AG., Switzerland and Wako Pure Chemical Industries Ltd provided the HIV protease substrate (Lot# 2000666) and Pepstatin A (Cat# 71147). SensoLyte™ 520 HCV Protease Assay Kit Fluorimetric (Lot# AK 71145-1013), HCV NS3/4A protease (Lot# 046-079), Hepatitis Virus C NS3 protease inhibitor 2 (Cat# 25346), 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. Soybean trypsin-chymotrypsin inhibitor was obtained from Sigma–Aldrich Co. Becton Dickinson Falcon™ Microtest™ 384-well 120 μL black assay plates were purchased from Nonsterile, No Lid. Lot# 06 04 11 55, Germany.

4.3. Isolation of (20*R*)-panaxadiol and (20*R*)-panaxatriol

Panax ginseng roots were extracted with hot methanol–H_2O (7:3) to obtain a crude saponin fraction. This fraction was hydrolyzed with 7% HCl and then separated by various types of column chromatography to yield the major aglycones, (20*R*)-panaxadiol (PD), (20*R*)-panaxatriol (PT) and oleanolic acid.[24]

4.3.1. (20*R*)-20,25-Epoxy-12β-hydroxydammaran-3-one (1a)

A CH_2Cl_2 (11.1 mL) solution of (20*R*)-panaxadiol (1.0 g, 2.17 mmol) and pyridinium chlorochromate (2812.1 mg, 13.0 mmol) was stirred for 6 h at room temperature. Work up was carried out in the following procedure. The mixture was poured into H_2O and extracted with chloroform. The organic layer was successively washed with H_2O, saturated aqueous $NaHCO_3$, once again with H_2O, and then dried over $MgSO_4$ and filtered. The filtrate was evaporated under reduced pressure, loaded onto a column (70 × 2.5 cm) containing silica gel and eluted with hexane–acetone (99:1–95:5) to afford compound **1a** as a white amorphous powder (920 mg, 92% yield); $[\alpha]_D^{26}$ +24.7 (*c* 1.24, $CHCl_3$), lit.[28], $[\alpha]_D^{24}$ + 35.2 (*c* 0.11, MeOH). ESI-MS *m/z* 459.4 $[M+H]^+$; IR $\nu_{max}^{CS_2}$ cm^{-1}: 3396, 1715. lit.[28], IR ν_{max}^{KBr} cm^{-1}: 3340, 2960, 1710, 1460, 1380.

4.3.2. (20*R*)-20,25-Epoxy-dammaran-3,12-dione (1c)

The Jones reagent (0.29 mL) was reacted with a solution of (20*R*)-panaxadiol (59.0 mg, 0.1281 mmol) in acetone (5.88 mL) for 4 h at room temperature. The mixture was worked up in the same way as for **1a** and eluted through a column (45 × 2.0 cm) of silica gel with hexane–acetone (99:1–97:3) to afford compound **1c** as a white amorphous powder (5.0 mg, 8.6% yield); $[\alpha]_D^{25}$ +71.8 ($CHCl_3$), lit.[33], $[\alpha]_D^{24}$ +72 (*c* 0.03, MeOH). ESI-MS *m/z* 457.5 $[M+H]^+$; IR $\nu_{max}^{CS_2}$ cm^{-1}: 1717. lit.[33], IR ν_{max}^{KBr} cm^{-1}: 2980, 2870, 1710, 1650, 1460.

4.3.3. (20*R*)-20,25-Epoxy-2,12-dihydroxydammaran-1-en-3-one (2a)

Air was introduced to a solution of **1a** (860 mg, 1.87 mmol) and *t*-BuOK (7.65 g) in *t*-BuOH (74.4 mL) and the mixture was stirred for 40 min at 40 °C and worked up as usual. The crude product was eluted through an ODS column (20 × 4.5 cm) with MeOH–H_2O (50–100%) to afford compound **2a** as white crystals (884 mg, 100% yield); mp. 193–196 °C; $[\alpha]_D^{24}$ +45.9 (*c* 0.08, MeOH). HR-EI-MS *m/z* 472.35780 $[M+H]^+$ (calcd for $C_{30}H_{48}O_4$, 472.35526); IR ν_{max}^{KBr} cm^{-1}: 3360, 3100, 2960, 2880, 2800, 2500, 2200, 1900, 1660; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.81 (3H, s), 0.97 (3H, s), 1.05 (3H, s), 1.11 (3H, s), 1.12 (3H, s), 1.13 (3H, s), 1.16 (3H, s), 120 (3H, s), 3.50 (1H, m, H-12), 5.98 (1H, br, OH-2), 6.36 (1H, s, H-1), 6.44 (1H, s, OH-12); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ:* 16.0, 16.2, 16.9, 17.1, 18.7, 19.3, 20.2, 21.5, 25.1, 27.1, 30.4, 30.9, 33.0, 34.3, 35.6, 36.4, 38.5, 40.5, 44.0, 45.0, 49.2, 51.2, 54.4, 54.5, 69.5, 73.1, 76.6, 128.7, 143.8, 200.9.

4.3.4. (20*R*)-20,25-Epoxy-2*a*,12β-dihydroxy-A-nordammaran-2-carboxylic acid (4a)

A solution of **1c** (841.0 mg, 1.78 mmol) and KOH (2.57 g) in MeOH (138.4 mL) was heated under reflux, and hydrogen peroxide (13.8 mL, 30%) was added over a period of 100 min. The solution was then poured into cold H_2O and the products were extracted with ethyl acetate (2 × 100 mL), followed by preparative HPLC [MeOH–0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] to afford **3a** (350 mg, 38.8% yield) as a major product and **4a** (12 mg, 1.4% yield) as a minor product. Compound **4a**: mp. 120–123.8 °C; $[\alpha]_D^{24}$ +17.5 (*c* 0.07, MeOH). HR-FAB-MS *m/z* 491.37219 $[M+H]^+$ (calcd for $C_{30}H_{51}O_5$, 491.37365); IR ν_{max}^{KBr} cm^{-1}: 3260, 2940, 2880, 1710, 1690; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.93 (3H, s), 0.97 (6H, s), 1.01 (3H, s), 1.10 (3H, s), 1.18 (3H, s), 1.21 (3H, s), 1.28 (3H, s), 1.81 (1H, d, *J* = 13.0 Hz, H-1a), 1.96 (1H, ddd, *J* = 7.5, 11.0 Hz), 2.13 (1H, d, *J* = 13.0 Hz, H-1b), 3.64 (1H, ddd, *J* = 5.5, 10.5, 15.5 Hz, H-12); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ:* 15.7, 16.2, 16.7, 17.4, 18.8, 19.3, 20.9, 25.1, 27.0, 27.2, 31.3, 32.4, 32.8, 34.8, 35.7, 36.4, 40.6, 43.6, 48.2, 48.8, 49.8, 51.4, 54.4, 54.5, 62.5, 70.1, 73.6, 76.7, 86.7, 179.2.

4.3.5. (20*R*)-20,25-Epoxy-2a,12β-dihydroxy-A-nordammaran-2-carboxylic acid methyl ester (4c)

Trimethylsilyldiazomethane (0.5 mL) was added to a solution of **4a** (20 mg, 0.041 mmol) in MeOH (3.0 mL) until the mixture became yellow. After stirring for 30 min at room temperature monitoring by TLC, the mixture was worked up as usual and then eluted

through an ODS column (16 × 2.4 cm) with methanol–H_2O (4:1–100:0) to afford **4c** as a white amorphous powder (15 mg, 72.5% yield); $[\alpha]_D^{24}$ + 21.9 (*c* 0.10, MeOH). ESI-MS *m/z* 505.5 $[M+H]^+$ (calcd for $C_{31}H_{52}O_5$, 504.38147); IR ν_{max}^{KBr} cm^{-1}: 3460, 2960, 2870; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.88 (3H, s), 0.92 (6H, s), 0.96 (3H, s), 0.97 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.81 (1H, d, *J* = 13.0 Hz, H-1a), 1.94 (1H, ddd, *J* = 3.5, 11.0, 14.0 Hz, H-17), 2.08 (1H, d, *J* = 13.0 Hz, H-1b), 3.60 (1H, ddd, *J* = 5.0, 11.0, 16.0 Hz, H-12), 3.78 (1H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ:* 15.8, 16.3, 16.4, 17.3, 18.8, 19.3, 20.8, 25.1, 27.0, 27.1, 31.2, 32.7, 32.9, 34.8, 35.7, 36.4, 40.6, 43.6, 48.7, 49.1, 49.7, 51.2, 52.2, 53.3, 54.7, 62.5, 69.9, 73.2, 76.7, 87.1, 177.3 (C-2, $COOCH_3$).

4.3.6. (20*R*)-20,25-Epoxy-2,3-*seco*-dammaran-2,3-dioc acid (3a)

This compound was prepared from **2a** in the same manner as described for **4a**. Compound **3a**: mp 208–210.5 °C; $[\alpha]_D^{25}$ +10.7 (*c* 0.14, MeOH). HR-FAB-MS *m/z* 507.36360 $[M+H]^+$ (calcd for $C_{30}H_{51}O_6$, 507.36857); IR ν_{max}^{KBr} cm^{-1}: 3500, 3260, 2980, 2800, 1710, 1690, 1650; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.90 (3H, s), 0.98 (6H, s), 1.18 (3H, s), 1.20 (3H, s), 1.21 (3H, s), 1.24 (3H, s), 1.25 (6H, s), 3.56 (1H, m, H-12), 6.59 (1H, s, 12-OH); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ:* 15.5, 16.3, 17.1, 19.4, 20.2, 22.2, 25.3, 27.1, 28.7, 31.0, 31.2, 33.0, 34.0, 35.7, 36.5, 39.6, 41.0, 41.6, 45.9, 48.6, 49.1, 51.6, 54.5, 69.7, 73.2, 176.7, 185.6.

4.3.7. (20*R*)-20,25-Epoxy-12-oxo-2,3-*seco*-dammaran-2,3-dioc acid (3b)

The Jones reagent (0.23 mL) was reacted with a solution of **3a** (100 mg, 0.198 mmol) in acetone (9.0 mL) for 4 h at room temperature. The mixture was worked up as usual and then purified by HPLC [column chromatography (21.5 × 300 mm) with MeOH–0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] to afford **3b** as a colorless crystal (10 mg, 10% yield); mp 127–130.9 °C. $[\alpha]_D^{25}$ +149.8 (*c* 0.04, MeOH). HR-FAB-MS *m/z* 503.33579 $[M-H]^-$ (calcd for $C_{30}H_{47}O_6$, 503.33727); IR ν_{max}^{KBr} cm^{-1}: 3440, 3180, 2960, 2620, 2040, 1710; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.73 (3H, s), 1.03 (3H, s), 1.09 (3H, s), 1.16 (9H, s), 1.18 (3H, s), 1.24 (3H, s), 2.31 (1H, d, *J* = 18.5 Hz, H-1a), 2.60 (1H, d, *J* = 18.5 Hz, H-1b), 3.31 (1H, m); ^{13}C NMR ($CDCl_3$, 300 MHz) *δ:* 15.6, 16.4, 16.9, 19.8, 20.7, 21.8, 24.3, 25.9, 27.4, 29.1, 32.2, 33.3, 33.6, 37.0, 40.0, 40.2, 41.1, 41.9, 45.7, 45.9, 46.0, 48.6, 55.7, 56.1, 70.7, 74.8, 76.6, 174.7, 186.1, 214.8.

4.3.8. (20*R*)-20,25-Epoxy-12β-hydroxydammaran-3,6-dione (1b)

This compound was prepared from PT (1.0 g, 2.10 mmol) in the same manner as described for **1a**. Purification by elution through silica gel (50 × 3.0 cm) with hexane–acetone (99:1 **[v/v]** to pure acetone) afforded **1b** as a white amorphous powder (749 mg, 75.5% yield). $[\alpha]_D^{25}$ −25.1 (*c* 0.11, MeOH). ESI-MS *m/z* 473.5 $[M+H]^+$. IR ν_{max}^{CCl4} cm^{-1}: 3327, 1718, lit.[34] IR ν_{max}^{KBr} cm^{-1}: 3380, 2940, 2880, 1710.

4.3.9. (20*R*)-20,25-Epoxy-dammaran-3,6,12-trione (1d)

This compound was prepared from PT (206 mg, 0.433 mmol) in the same manner as described for **3b**. Chromatographic separation by hexane–acetone (99:1–95:5) elution through a column (50 × 3.0 cm) containing silica gel afforded **1d** as a colorless amorphous powder (50 mg, 25.3% yield); $[\alpha]_D^{25}$ +25.9 (*c* 0.06, MeOH). ESI-MS *m/z* 471.5 $[M+H]^+$; IR ν_{max}^{KBr} cm^{-1}: 2960, 2800, 2300, 1710. The ^1H and ^{13}C NMR spectral data of **1d** were in agreement with those published in the literature.[35]

4.3.10. (20*R*)-20,25-Epoxy-2*a*,12β-dihydroxy-6-oxo-A-nordammaran-2-carboxylic acid (4b)

This compound was prepared from **1b** (679 mg, 1.44 mmol) in the same manner as described for **2a**. Purification by HPLC (column, 21.5 × 300 mm; MeOH-0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] afforded **4b** as a white amorphous powder (44.8 mg, 6.2% yield); $[\alpha]_D^{25}$ −26.1 (*c* 0.15, MeOH). HR-FAB-MS *m/z* 503.33396 $[M-H]^-$ (calcd for $C_{30}H_{47}O_6$, 503.33727); IR ν_{max}^{KBr} cm^{-1}: 3400, 3280, 2980, 2860, 1760, 1680; ^1H NMR (MeOD, 500 MHz) δ 0.95 (3H, s, Me-28), 0.97 (3H, s, Me-19), 0.98 (3H, s, Me-30), 1.12 (3H, s, Me-29), 1.22 (6H, s, Me-21, Me-26), 1.25 (3H, s, Me-18), 1.30 (3H, s, Me-27), 1.95 (1H, d, *J* = 14.0 Hz, H-1a), 2.08 (1H, d, *J* = 14.5 Hz, H-7a), 2.28 (1H, dd, *J* = 3.0, 13.5 Hz, H-9), 2.47 (1H, d, *J* = 14.5 Hz, H-5), 2.73 (1H, d, *J* = 14.5 Hz, H-1b), 3.64 (1H, dd, *J* = 5.0, 10.0 Hz, H-12); ^{13}C NMR (MeOD, 125 MHz) *δ:* 16.9, 17.1, 17.5, 19.9, 22.1, 24.4, 25.8, 27.6, 28.6, 32.5, 33.5, 35.2, 36.7, 37.4, 44.3, 45.0, 48.5, 50.2, 51.7, 52.7, 53.1, 54.7, 56.0, 68.6, 71.0, 74.7, 78.1, 86.5, 176.5, 217.1.

4.3.11. (20*R*)-20,25-Epoxy-2*a*,12β-dihydroxy-6-oxo-A-nordammaran-2-carboxylic acid methyl ester (4d)

This compound was prepared from **4b** (22.4 mg, 0.044 mmol) in the same manner as described for **4c**. Purification by HPLC [column, 21.5 × 300 mm; eluted with MeOH-0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] afforded **4d** as a colorless powder (11 mg, 48.2% yield); mp. 124–126.2 °C. $[\alpha]_D^{25}$ + 53.3 (*c* 0.04, MeOH). ESI-MS *m/z* 519.7 $[M+H]^+$ (calcd for $C_{31}H_{50}O_6$, 518.36074); IR ν_{max}^{KBr} cm^{-1}: 3400, 2980, 2860, 1780, 1710, 1690; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.89 (3H, s), 0.94 (6H, s), 0.97 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.23 (3H, s), 1.24 (3H, s), 1.28 (3H, s), 1.98 (1H, d, *J* = 14.5 Hz, H-1a), 2.13 (1H, d, *J* = 14.5 Hz, H-7a), 2.18 (1H, dd, *J* = 3.5, 13.5 Hz, H-9), 2.37 (1H, d, *J* = 14.5 Hz, H-7b), 2.58 (1H, s, H-5), 2.65 (1H, d, *J* = 14.0 Hz, H-1b), 3.62 (1H, dd, *J* = 4.0, 10.0 Hz, H-12), 3.78 (1H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ:* 16.2, 16.4, 17.2, 19.4, 21.3, 23.7, 24.8, 27.0, 27.8, 32.5, 31.6, 32.9, 34.3, 35.7, 36.4, 42.9, 44.1, 47.8, 49.0, 50.5, 51.7, 52.4, 52.7, 52.8, 54.6, 66.6, 69.3, 73.4, 76.5, 86.2, 173.8, 214.4.

4.3.12. (20*R*)-20,25-Epoxy-dammaran-3β,12β-diol-3-(3′,3′-dimethyl) succinate (5a)

A mixture of (20*R*)-panaxadiol (50 mg, 0.109 mmol), 2,2-dimethylsuccinic anhydride (85.3 mg, 0.67 mmol) and 4-dimethylamino pyridine (DMAP), (26.5 mg, 0.217 mmol) was heated in pyridine (2.0 mL) overnight under reflux. Ethyl acetate (50 mL) was added and then the mixture was washed with 2 N HCl and H_2O. Evaporating the EtOAc yielded a mixture of the starting material and products that were separated by elution through an ODS column with MeOH/H_2O (9:1) and by HPLC [MeOH-0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] to afford **5a** as a white crystalline powder (22.0 mg, 34.3% yield); mp. 145–147.7 °C; $[\alpha]_D^{25}$ +14.5 (*c* 0.08, MeOH). HR-FAB-MS *m/z* 589.44494 $[M+H]^+$ (calcd for $C_{36}H_{61}O_6$. 589.44682); IR ν_{max}^{KBr} cm^{-1}: 3440, 2980, 2880, 1730; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.83 (3H, s), 0.84 (3H, s), 0.87 (3H, s), 0.89 (3H, s), 0.97 (3H, s), 1.18 (3H, s), 1.21 (3H, s), 1.26 (1H, s), 1.27 (1H, s), 1.29 (1H, s), 1.92 (1H, m, H-17), 2.56 (1H, d, *J* = 15.5 Hz, H-2′), 2.66 (1H, d, *J* = 15.5 Hz, H-2″), 3.55 (1H, m, *J* = 5.0, 10.5 Hz, H-12), 4.49 (1H, m, *J* = 6.0, 11.5 Hz, H-3), 6.43 (1H, s, 12-OH); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ:* 15.4, 15.9, 16.0, 16.3, 16.8, 18.0, 19.2, 23.4, 25.0, 25.1, 25.4, 26.9, 27.7, 30.0, 31.0, 32.7, 34.6, 35.5, 36.2, 36.8, 37.6, 38.4, 39.6, 39.6, 44.7, 48.8, 49.6, 51.1, 54.5, 55.8, 69.9, 73.1, 76.6, 81.3, 171.7, 181.7.

4.3.13. (20*R*)-20,25-Epoxy-3β-hydroxydammaran-12-one-3-(3′,3′-dimethyl) succinate (5b)

This compound was prepared from **5a** (102 mg, 0.17 mmol) in the same manner as described for compound **1c**. Purification by HPLC [column, 21.5 × 300 mm; MeOH-0.1% TFA/H_2O, 5.0 mL/min, monitored at 208 nm] afforded compound **5b** as a white amorphous powder: (12.0 mg, 12% yield). $[\alpha]_D^{25}$ +46.7 (*c* 0.03, MeOH), ESI-MS *m/z* 587.7 $[M+H]^+$ (calcd for $C_{36}H_{58}O_6$, 586.42334); IR

ν_{max}^{KBr} cm^{-1}: 3460, 2980, 2880, 2680, 1710, 1650; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.89 (3H, s), 0.95 (3H, s), 1.04 (3H, s), 1.06 (3H, s), 1.12 (3H, s), 1.16 (3H, s), 1.17 (3H, s), 1.22 (1H, s), 1.32 (1H, s), 1.38 (1H, s), 2.39–2.49 (2H, m, H-2′, H-2″), 4.64 (1H, dd, J = 4.5, 12.0 Hz, H-3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 16.4, 17.0, 17.7, 18.4, 18.7, 19.5, 22.8, 23.1, 24.8, 24.9, 27.2, 27.9, 31.3, 32.8, 34.7, 35.4, 36.4, 36.8, 37.8, 38.2, 40.0, 40.3, 42.0, 42.9, 48.5, 50.9, 54.0, 55.7, 56.2, 58.5, 71.2, 75.2, 80.7, 171.0, 183.4, 210.0.

4.3.14. (20*R*)-20,25-Epoxy-dammaran-3β,6α,12ββ-triol-3-(3′,3′-dimethyl) succinate (5c)

This compound was prepared from (20*R*)-panaxatriol (50 mg, 0.105 mmol) in the same manner as described for **5a**. Purification by HPLC [MeOH–0.1% TFA/H_2O, 5 mL/min, monitored at 208 nm] afforded **5c** (20.0 mg, 31.4% yield), **5d** (8.0 mg, 12.4% in yield) and **5e** (19.0 mg, 24.8% in yield) as white amorphous powders.

$[\alpha]_D^{25}$ +7.89 (c 0.08, MeOH), HR-FAB-MS m/z 605.44166 $[M+H]^+$ (calcd for $C_{36}H_{61}O_7$, 605.44173); IR ν_{max}^{KBr} cm^{-1}: 3440, 3340, 2960, 2860, 2600, 1730, 1710; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.90 (3H, s), 0.95 (3H, s), 1.04 (3H, s), 1.06 (3H, s), 1.16 (3H, s), 1.18 (3H, s), 1.21 (3H, s), 1.26 (3H, s), 1.28 (3H, s), 1.29 (3H, s) (10 × CH_3), 1.91 (1H, m, H-17), 2.56 (1H, d, J = 16.0 Hz, H-2′), 2.69 (1H, J = 16.0 Hz, H-2″), 3.54 (1H, m, J = 5.5, 10.5, 15.5 Hz, H-12), 4.10 (1H, m, J = 4.0, 9.5 Hz, H-6), 4.47 (1H, dd, J = 5.5, 11.0 Hz, H-3), 6.45 (1H, s, OH-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 16.2, 16.6, 17.0, 17.1, 17.2, 17.9, 19.3, 20.9, 23.3, 25.1, 25.8, 27.1, 28.4, 30.2, 30.6, 31.1, 33.0, 35.7, 36.4, 38.1, 38.3, 39.0, 40.9, 44.7, 46.8, 48.6, 49.2, 51.0, 54.6, 55.2, 61.2, 68.4, 69.8, 73.2, 76.6, 81.3, 170.5, 181.7.

4.3.15. (20*R*)-20,25-Epoxy-dammaran-3β,6α,12β-triol-6-(3′,3′-dimethyl) succinate (5d)

$[\alpha]_D^{25}$ +108.8 (c 0.99, MeOH), HR-FAB-MS m/z 605.43693 $[M+H]^+$ (calcd for $C_{36}H_{61}O_7$, 605.44173); IR ν_{max}^{KBr} cm^{-1}: 3480, 2980, 2880, 2600, 1730, 1710; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.82 (3H, s), 0.89 (3H, s), 0.97 (3H, s), 1.10 (3H, s), 1.17 (3H, s), 1.18 (3H, s), 1.21 (3H, s), 1.26 (3H, s), 1.30 (3H, s), 1.33 (3H, s) (10 × CH_3), 2.54 (1H, d, J = 16.5 Hz, H-2′), 2.61 (1H, d, J = 16.5 Hz, H-2″), 3.19 (1H, dd, J = 4.5, 11.5 Hz, H-3), 3.53 (1H, m, J = 5.0, 10.0 Hz, H-12), 5.36 (1H, ddd, J = 4.5, 10.5, 15.0 Hz, H-6), 6.40 (1H, s, OH-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 15.5, 16.2, 16.8, 17.0, 17.0, 19.4, 25.1, 25.3, 25.5, 26.9, 27.1, 30.2, 30.5, 31.1, 33.0, 35.7, 36.4, 38.5, 38.7, 39.4, 40.5, 40.8, 42.4, 44.8, 48.6, 49.4, 51.0, 54.6, 58.7, 69.8, 71.1, 73.2, 76.6, 78.3, 170.6, 181.0.

4.3.16. (20*R*)-20,25-Epoxy-dammaran-3β,6α,12β-triol-3,6-di-(3′,3′-dimethyl) succinate (5e)

$[\alpha]_D^{25}$ +40.0 (c 0.03, MeOH), HR-FAB-MS m/z 733.48796 $[M+H]^+$ (calcd for $C_{42}H_{69}O_{10}$, 733.48903); IR ν_{max}^{KBr} cm^{-1}: 3460, 3380, 2960, 2880, 2600, 1730, 1710; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.89 (3H, s), 0.90 (3H, s), 1.01 (6H, s), 1.07 (3H, s), 1.21 (6H, s), 1.26 (12H, s), 1.29 (3H, s) (12 × CH_3), 2.49 (1H, d, J = 15.5 Hz, H-2a′), 2.56 (1H, d, J = 15.5 Hz, H-2a″), 2.62 (1H, d, J = 16.0 Hz, H-2b′), 2.72 (1H, d, J = 16.0 Hz, H-2b″), 3.53 (1H, m, J = 5.0, 10.0 Hz, H-12), 4.51 (1H, m, H-3), 5.39 (1H, m, J = 3.0, 10.5, 14.5 Hz, H-6), 6.46 (1H, s, OH-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 16.2, 16.5, 16.9, 17.2, 19.5, 23.2, 24.9, 25.1, 25.2, 25.6, 25.8, 27.1, 30.1, 30.1, 31.1, 33.0, 35.7, 36.4, 37.6, 38.2, 39.2, 40.6, 40.7, 40.7, 42.5, 45.1, 45.4, 48.6, 49.2, 51.0, 54.6, 58.7, 69.8, 70.4, 73.2, 76.7, 81.2, 170.0, 170.8, 183.1, 183.1.

4.3.17. (20*R*)-20,25-Epoxy-3,6-di-*O*-(3′,3′-dimethylsuccinyl)-dammaran-12-one (5f)

This compound was prepared from **5e** (150 mg, 0.205 mmol) in the same manner as described for **1c**. Purification by ODS column chromatography (20 × 4.5 cm) with MeOH-H_2O (50–100%) afforded **5f** as an amorphous solid (15 mg, 9.8% yield); $[\alpha]_D^{25}$ +126.1 (c 0.04, MeOH). ESI-MS m/z: 729.9 $[M-H]^-$ (calcd for $C_{42}H_{66}O_{10}$, 730.46450); IR ν_{max}^{KBr} cm^{-1}: 3460, 2980, 2880, 2700, 1730, 1710; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.74 (3H, s), 0.91 (3H, s), 1.02 (6H, s), 1.07 (6H, s), 1.16 (3H, s), 1.18 (3H, s), 1.26 (6H, s), 1.29 (3H, s), 1.30 (3H, s), 1.32 (3H, s), (12 × CH_3), 2.46 (1H, d, J = 15.5 Hz, H-2a′), 2.57 (1H, d, J = 15.5 Hz, H-2a″), 2.66 (1H, d, J = 8.0 Hz, H-2b′), 2.69 (1H, d, J = 8.0 Hz, H-2b″), 3.02 (1H, d, J = 8.5 Hz, H-11), 4.50 (1H, dd, J = 4.5, 11.5 Hz, H-3), 5.47 (1H, m, J = 5.5, 10.0 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 16.3, 16.5, 16.7, 17.0, 17.1, 22.9, 24.1, 24.8, 24.9, 25.7, 25.7, 25.8, 27.3, 30.0, 32.1, 33.5, 37.0, 37.5, 37.7, 39.3, 39.6, 40.6, 40.8, 41.1, 41.7, 45.1, 45.4, 45.8, 53.2, 55.2, 55.8, 58.3, 70.2, 70.7, 74.7, 80.7, 170.3, 170.5, 183.5, 183.5, 212.0.

4.4. HIV protease assay

Inhibitory activity against HIV PR was tested using HPLC method as described.[17]

4.5. HCV protease assay

Inhibitory activity against HCV PR was tested using a fluorometric method as described.[30]

4.6. Renin protease assay

A compound dissolved in DMSO (2.5 μl; final concentration, 10%) was placed in the wells of 384-well microplates, then 14.0 μL of rec-renine PR (0.5 μg/mL) was added and the plate was agitated. Thereafter, 8.5 μL of fresh dilutions of the renin substrate [Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Il2-His-Thr-Lys (Dabcyl)-Arg] was added and the mixture was rotated for 30 min at 37 °C. A renin inhibitor [Ac-HPFV-(Sta)-LF-NH_2] served as the positive control.[36] The methods of fluorometric analyses and inhibitory calculation were as described for the HCV protease assay.

4.7. Green protease assay

A compound dissolved in DMSO (2.5 μL; final concentration: 10%) was placed in the wells of 384-well microplates, then 17.5 μL of assay buffer and 2.5 μL of trypsin (0.1 U/μL) were added to the wells and the plate was agitated. Finally, fresh 2.5 μL dilutions of the protease substrate, HiLyte Fluor™ 488-labeled casein, were added under sequential rotary shaking incubated at 37 °C for 30 min. The positive control was soybean trypsin–chymotrypsin inhibitor.[37] The methods for the fluorometric analysis and inhibitory calculations were the same as described for the HCV protease assay.

Acknowledgment

This study received financial support from the Japan China Medical Association (NO. 103, Japan).

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