Synthesis and Evaluation of Acylated Derivatives of Hederagenin as Inhibitors of HIV-1 and HCV NS3/4A Proteases

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Abstract
Viral infection imposes a major threat to human health. To develop new potent antiviral agents, Hederagenin (HE), a known inhibitor of HIV-1 and HCV NS3/4A proteases, was used as a starting material to synthesize 4 types of HE derivatives, HE-3,23-diacyl, HES-3,23-diacyl, HES-3-acyl, and HES-3-oxo-23-acyl. We evaluated the in vitro inhibitory activities of the derivatives against HIV-1 and HCV NS3/4A proteases. (3β,23)-Di-O-diglutaroyl-hederagenin (1b) and (3β,23)-di-O-(3′,3′-dimethylsuccinyl-hederagenin ethyl ester (2b) exhibited potent inhibitory activities against the HIV-1 and/or HCV NS3/4A proteases with IC50 values < 10 μM, but did not appreciably inhibit general human proteases renin and trypsin. The SARs showed that dicarboxylic acid hemiesters of HE significantly enhance the antiviral activities when C3 or C23 are linked with 6 carbon acyl chains.

Keywords
acyl hederagenin, HIV-1 protease, HCV NS3/4A protease, enzyme inhibition

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Introduction
HIV and hepatitis C infections continue to be a major threat to human health worldwide. According to the World Health Organization, there were an estimated 37.7 million people living with HIV at the end of 2020, and hepatitis C killed about 290 000 people in 2019.1,2 Although antiviral therapy has significantly reduced the death rate for both HIV and hepatitis C, drug resistance has emerged to be a drawback for the therapy and new classes of antiviral drugs are needed to combat the drug-resistant strains.3 A key strategy in developing new drugs is to identify and modify bioactive compounds from natural resources.

The flower buds of *Lonicera fulvotomentosa* Hsu et S. C. Cheng, a widely used traditional Chinese medicine, have been shown to possess various biological effects, such as antiviral, antimicrobial, and liver-protection activities.4-7 The major active ingredients have been identified to be pentacyclic triterpenes and phenolic compounds, such as aglycones, fulvotomentoside A, chlorogenic acid, and caffeoylquinic acid derivatives. Previous studies have shown that some triterpenes, such as hemiesters of ursolic acid, 3-oxo-tirucalla-7,24-dien-21-oic acid, and 16β-hydroxy-2,3-seco-lup-20(29)-en-2,3-dioic acid, exhibit potent inhibitory activities towards HIV-1 protease (HIV PR).8-12

As a naturally occurring derivative of oleanolic acid, hederagenin (HE) is hydroxylated at C-23 (Figure 1) and possesses moderate inhibitory activities against HIV PR and HCV NS3/4A protease (HCV NS3/4A PR).12,13 Previous studies have shown that oleanolic acid and its derivatives inhibit both HIV-1 and HCV proteases.8,14-17 Furthermore, either 3-substitution or 3,28-disubstitutions can significantly increase the antiviral activity of oleanolic acid against HIV-1 PR.8-20 However, 23-substitution of HE has been rarely studied. Therefore, in the current study, we synthesized a series of C-3 and/or C-23 acylated derivatives of HE and HE-28-ethyl ester (HES) and
evaluated theirs in vitro inhibitory activities against HIV-1 PR and HCV NS3/4A PR.

Material and Methods

Instruments and Chemicals

An Autopol-1 polarimeter (Rudolph Research Analytical), Bruker Vector-22 FTIR spectrometer (Bruker BioSpin Corporation), Bruker DRX 600 spectrometer (Bruker BioSpin Corporation), and Micro TOF-QII mass spectrometer (Agilent Technologies), and Micro TOF-QII mass spectrometer (Bruker BioSpin Corporation), Bruker Vector-22 FTIR spectrometer (Bruker BioSpin Corporation), and Micro TOF-QII mass spectrometer (Agilent Technologies) were equipped with an ESI interface (Bruker Daltoniks GmbH) were used to analyze related parameters. Silica gel, octadecylsilane, and silica gel GF254 were used to purify the compounds. All reagents were obtained from Aladdin Chemistry Co. Ltd (Shanghai, P.R. China).

Isolation of HE

HE was purified from flower buds of L. fulvotomentosa using our previously published protocol.21

Enzyme Assay Kits

Inhibitory activities of the acylated derivatives of HE and HES were evaluated against HIV-1 PR (Lot# AS-72028-5) using an HIV PR Assay Kit (Lot#71147-1028) and against HCV NS3/4A PR (Lot# AS-61017-10) using an HCV PR Assay Kit (Lot#AK 71145-1035). Both assay kits were purchased from AnaSpec Inc. (Fremont, CA, USA). The detailed experimental protocols have been previously reported.13,21,22

Synthesis

Synthesis of ethyl (3β,23)-dihydroxyolean-12-en-28-oate (HES)

HES (1.0 g, 2.12 mmol), iodoethane (0.68 mL, 8.40 mmol), and potassium carbonate (1451.1 mg, 10.50 mmol) were dissolved in 15 mL DMF at room temperature. The mixture was stirred overnight and extracted with EtOAc (2×100 mL).

After concentration under vacuum, the residue was separated using a silica gel column to obtain HES (800 mg, yield 76%) as a white amorphous powder; HRMS(EI) m/z 523.3758 [M + Na]+ (calcd. for C32H52O4Na, 523.3763); 1H and 13C NMR spectral data of HES were in agreement with those published in the literature.21

Synthesis of Other Compounds

The other compounds were synthesized according to the corresponding methods outlined below.

(β,23)-di-O-disuccinyl-hederagenin (1a). HE (100.0 mg, 0.21 mmol), succinic anhydride (372.0 mg, 3.72 mmol), and DMAP (146.4 mg, 1.20 mmol) were dissolved in pyridine (3 mL) overnight under reflux. The solution was diluted with EtOAc (50 mL) and washed with 1 M HCl and H2O. After concentration of the EtOAc layer, the residue was diluted with EtOAc (50 mL) and washed with 1 M HCl and H2O. After concentration of the EtOAc layer, the residue was separated by chromatography using an ODS column (1.5 cm×40 cm) with MeOH/H2O (5:5-100:0) and HPLC [MeOH-0.1% TFA/H2O, 1 mL/min, monitored at 208 nm] to yield 1a (40 mg, yield 30%). ESI-MS: m/z 723.7 [M-H]− (calcd. For C38H56O10Na, 723.7835); 1H NMR and 13C-NMR spectral data of 1a were in agreement with those published in the literature.23

(β,23)-di-O-diglutaryl-hederagenin (1b). Following the procedure described for 1a, 1b was obtained from HE as a white amorphous powder (25.7 mg, yield 17.5%). Compound 1b: ESI-MS: m/z 700.8 [M-H]− (calcd for C32H52O4Na, 700.8166); 1H and 13C NMR spectral data of 1a were in agreement with those published in the literature.23

(β,23)-di-O-disuccinyl-hederagenin ethyl ester (2a). HES (50.0 mg, 0.10 mmol), succinic anhydride (305.3 mg, 3.05 mmol), and DMAP (146.4 mg, 1.20 mmol) were dissolved in pyridine (2.0 mL) overnight under reflux. The purification procedure used was described for 1a to afford 2a (40 mg, yield 70%). Compound 2a: ESI-MS: m/z 723.7 [M+Na]+ (calcd for C38H56O10Na, 723.4084); 1H NMR and 13C-NMR spectral data of 2a were in agreement with those published in the literature.23

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ESI-MS: 

\[ m/z = 751.8 \text{ [M} + \text{Na}]^+ \] (calcd for \( C_{42}H_{70}O_{7}SiNa \), 751.43972); \( [\alpha]_D^{21.3} = -1.6^\circ \) (C = 0.1, CH$_2$OH); IR \( \nu_{KBr} \text{ max cm}^{-1} = 2947, 1732, 1716, 1313, 11.1, 12.4, 12.9, 13.0 \) (3H each, s, 14X CH$_3$), 254, 268, 288 (1H each, d, \( J = 18.0 \text{ Hz} \)), 310 (d, \( J = 6.0 \text{ Hz} \)), 324 (d, \( J = 12.0 \text{ Hz} \)), 4.10 (m, 2H), 491 (d, \( J = 12.0 \text{ Hz} \)), 5.31 (1H, H-12); 13C-NMR (CDCl$_3$, 125 MHz) \( [\alpha]_D^{21.3} = -5.7, -5.9, 13.1, 14.2, 15.7, 17.0, 17.7, 18.0, 22.8, 23.0, 23.4, 23.6, 24.8, 25.6, 25.8, 25.8, 27.6, 30.8, 32.3, 32.9, 32.3, 36.4, 38.0, 39.3, 40.4, 41.4, 41.5, 41.8, 44.6, 45.9, 46.2, 46.7, 47.6, 60.1, 63.7, 75.6, 122.3, 143.7, 170.3, 178.1, 182.9.

ESI-MS: 

\[ m/z = 741.8 \text{ [M} + \text{H}]^- \] (calcd for \( C_{44}H_{82}O_{7}Si \), 742.52083); \( [\alpha]_D^{21.3} = -1.8^\circ \) (C = 0.1, CH$_2$OH); IR \( \nu_{KBr} \text{ max cm}^{-1} = 1732, 1472, 1389, 1364; 1^1\text{H-NMR (CDCl}_3, 600 MHz) \( \delta = 0.01, 0.01, 0.06, 0.76, 0.90, 0.99, 0.91, 0.92, 0.94, 0.95, 1.14, 1.24, 1.29, 1.30 \) (3H each, s, 14X CH$_3$), 2.54, 2.68, 2.88 (1H each, d, \( J = 18.0 \text{ Hz} \)), 3.10 (d, \( J = 6.0 \text{ Hz} \)), 3.24 (d, \( J = 12.0 \text{ Hz} \)), 4.10 (m, 2H), 4.91 (d, \( J = 12.0 \text{ Hz} \)), 5.31 (1H, H-12); 13C-NMR (CDCl$_3$, 125 MHz) \( [\alpha]_D^{21.3} = -5.7, -5.9, 13.1, 14.2, 15.7, 17.0, 17.7, 18.0, 22.8, 23.0, 23.4, 23.6, 24.8, 25.6, 25.8, 25.8, 27.6, 30.8, 32.3, 32.9, 32.3, 36.4, 38.0, 39.3, 40.4, 41.4, 41.5, 41.8, 44.6, 45.9, 46.2, 46.7, 47.6, 60.1, 63.7, 75.6, 122.3, 143.7, 170.3, 178.1, 182.9.

- **ESI-MS:** 
  \[ m/z = 713.7 \text{ [M} + \text{Na}]^+ \] (calcd for \( C_{42}H_{70}O_{7}SiNa \), 714.43972); \( [\alpha]_D^{21.3} = -10.8^\circ \) (C = 0.1, CH$_2$OH); IR \( \nu_{KBr} \text{ max cm}^{-1} = 2950, 2369, 1731; 1^1\text{H-NMR (CDCl}_3, 600 MHz) \( \delta = -0.04, -0.01, 0.69, 0.75, 0.88, 0.88, 0.89, 0.93, 0.94, 1.12, 1.23 \) (3H each, s, 12X CH$_3$), 2.57, 2.86 (1H each, d, \( J = 18.0 \text{ Hz} \)), 3.07 (d, \( J = 6.0 \text{ Hz} \)), 3.23, 4.88 (1H each, d, \( J = 12.0 \text{ Hz} \)), 4.10 (m, 2H), 5.28 (1H, H-12); 13C-NMR (CDCl$_3$, 125 MHz) \( [\alpha]_D^{21.3} = -5.7, -5.9, 13.1, 14.3, 15.7, 17.0, 17.6, 18.1, 22.8, 23.0, 23.4, 23.6, 25.6, 25.8, 25.8, 27.6, 29.6, 29.8, 30.7, 32.2, 32.4, 33.1, 33.9, 36.4, 37.9, 39.3, 41.4, 41.6, 41.8, 45.9, 46.3, 46.6, 47.6, 60.1, 63.9, 75.1, 122.3, 143.7, 172.0, 172.0, 177.8.

- **ESI-MS:** 
  \[ m/z = 651.8 \text{ [M} + \text{Na}]^+ \] (calcd for \( C_{38}H_{66}O_{4}SiNa \), 651.42367); \( [\alpha]_D^{21.3} = -4.4^\circ \) (C = 0.1, CH$_2$OH); IR \( \nu_{KBr} \text{ max cm}^{-1} = 3432, 2946, 1726; 1^1\text{H-NMR (CDCl}_3, 600 MHz) \( \delta = 0.76, 0.78, 0.91, 0.95, 0.96, 1.15, 1.25, 1.26 \) (3H each, s, 12X CH$_3$), 2.66 (s, 1H), 2.88, 3.48 (1H each, d, \( J = 6.0 \text{ Hz} \)), 3.91 (d, \( J = 12.0 \text{ Hz} \)), 4.10 (m, 2H), 5.30 (1H, H-12); 13C-NMR (CDCl$_3$, 125 MHz) \( [\alpha]_D^{21.3} = -5.7, -5.9, 13.2, 14.3, 15.7, 17.0, 17.7, 18.1, 20.0, 22.9, 23.0, 23.4, 23.6, 25.6, 25.8, 25.8, 25.8, 27.6, 30.7, 32.2, 32.4, 33.0, 33.1, 33.6, 33.9, 37.9, 39.3, 41.4, 41.6, 41.8, 45.9, 46.3, 46.6, 47.6, 60.1, 64.0, 74.7, 122.3, 143.7, 172.1, 177.9, 178.5.

- **ESI-MS:** 
  \[ m/z = 567.3 \text{ [M} + \text{Na}]^+ \] (calcd for \( C_{35}H_{62}O_{7}Na \), 568.50473); \( [\alpha]_D^{21.3} = -1.2^\circ \) (C = 0.1, CH$_2$OH);
23-α-Butyldimethylsiloxy-3-oxo-hederagenin ethyl ester (5a). PCC (103.0 mg, 0.48 mmol) and 3a (50 mg, 0.08 mmol) were dissolved in CH₂Cl₂ (6.0 mL) at room temperature for 6 h. The mixture was diluted with H₂O and extracted with chloroform. The extract was concentrated to offer a residue which was chromatographed on a silica gel column (70 cm × 2.5 cm) eluting with hexane–acetone (95:5) to yield 5a (20.0 mg, yield 37.5%). Compound 5a: ESI-MS: m/z 635.8 [M + Na]+ (calcd for C₃₀H₅₄O₅Na, 635.44716); 1H-NMR (CDCl₃, 600 MHz): δ = 0.01, 0.01, 0.05, 0.06, 0.08, 0.08, 0.09, 0.09, 0.09, 0.10, 0.11, 0.12 (3H each, s, 7 × CH₃), 2.91 (d, J = 6.0, 12.0 Hz, 1H), 3.29, 3.66 (1H each, d, J = 12.0), 4.10 (m, 2H), 5.38 (1H, H-12); 13C-NMR (CDCl₃, 150 MHz): δ = 7.5, 5.5, 14.3, 14.9, 17.0, 17.5, 18.1, 19.6, 23.1, 23.6, 25.5, 25.8, 25.8, 25.8, 26.7, 29.7, 30.7, 32.1, 32.4, 33.1, 34.0, 35.9, 36.1, 37.1, 39.3, 41.6, 42.0, 45.9, 46.1, 46.6, 52.0, 60.1, 68.5, 124.3, 143.7, 177.7, 217.5.

23-Hydroxy-3-oxo-hederagenin ethyl ester (5b). Hydrochloric acid (10%, 2 mL) was added dropwise to a solution of 5a (50 mg, 0.08 mmol) in acetone (4.2 mL) at room temperature for 22 h. Aceton was then evaporated, and the mixture was adjusted to pH = 7 using 1 M NaOH. The mixture was poured into H₂O and extracted with ethyl acetate. Evaporation of the ethyl acetate layer yielded a residue, which was subsequently separated on silica gel to obtain 5b (40.0 mg, yield 90%). Compound 5b: ESI-MS: m/z 521.3 [M + Na]+ (calcd for C₂₃H₄₆O₂Na, 521.30686); 1H-NMR (CDCl₃, 600 MHz): δ = 0.82, 0.90, 0.93, 1.02, 1.14, 1.16, 1.24 (3H each, s, 7 × CH₃), 2.88, 3.42, 3.64 (1H each, d, J = 12.0 Hz), 4.10 (m, 2H), 5.30 (1H, H-12); 13C-NMR (CDCl₃, 150 MHz): δ = 14.2, 15.2, 16.8, 17.1, 19.1, 22.9, 23.6, 25.8, 27.6, 30.5, 32.1, 32.3, 33.1, 33.9, 35.2, 36.6, 38.8, 39.3, 41.3, 41.8, 45.8, 46.5, 46.8, 49.2, 52.4, 53.4, 60.0, 67.1, 121.7, 144.2, 177.7, 218.9.

3-Oxo-23-O-succinyl-hederagenin ethyl ester (5c). Following the procedure described for 1a, 5c was obtained from 5b as a white amorphous powder (20 mg, 33.4% yield). Compound 5c: ESI-MS: m/z 597.3 [M - H]⁻ (calcd for C₃₆H₅₂O₇, 598.38695); 1H-NMR (CDCl₃, 600 MHz): δ = 0.82, 0.91, 0.94, 1.02, 1.06, 1.19, 1.25 (3H each, s, 7 × CH₃), 2.87 (m, 3H), 4.11 (m, 4H), 5.52 (1H, H-12); 13C-NMR (CDCl₃, 125 MHz): δ = 14.3, 15.0, 17.0, 17.6, 19.5, 23.0, 23.6, 25.7, 27.6, 27.9, 28.8, 29.0, 30.7, 32.0, 32.4, 33.1, 33.9, 34.9, 36.4, 37.6, 39.3, 41.4, 41.9, 45.9, 46.5, 46.6, 48.2, 50.3, 60.2, 67.7, 122.0, 144.0, 171.7, 174.2, 177.8, 214.8.

3-Oxo-23-O-(3',4'-dimethylsucinyl)-hederagenin ethyl ester (5d). Following the procedure described for 1a, 5d was obtained from 5b as a white amorphous powder (10 mg, 16% yield). Compound 5d: ESI-MS: m/z 649.7 [M + Na]+ (calcd for C₃₈H₅₆O₇Na, 649.48028); 1H-NMR (CDCl₃, 600 MHz): δ = 0.82, 0.91, 0.95, 1.01, 1.09, 1.16, 1.25, 1.29, 1.31 (3H each, s, 9 × CH₃), 2.59, 2.66, 2.90 (1H each, d, J = 6.0, 18.0 Hz), 4.02 (d, J = 12.0 Hz, 1H), 4.11 (m, 4H), 5.33 (1H, H-12); 13C-NMR (CDCl₃, 150 MHz): δ = 14.3, 14.9, 17.1, 17.6, 19.3, 23.0, 23.5, 23.6, 25.2, 25.6, 27.6, 30.7, 32.0, 32.4, 33.1, 33.9, 34.8, 36.4, 37.5, 39.3, 40.5, 41.4, 41.9, 44.2, 45.9, 46.5, 46.6, 48.0, 50.3, 60.1, 67.4, 122.0, 143.9, 170.8, 177.7, 177.7, 214.8.

Molecular Docking

To gain insight into how HE derivatives inhibit HIV-1 and HCV NS3/4A PRs, (3β,23)-di-O-diglyceral-hederagenin (1b) and (3β,23)-di-O-(3',3'-dimethylsuccinyl)-hederagenin ethyl ester (2b) were docked to the active site of HIV-1 PR [PDB ID: 1QBS] and HCV NS3/4A PR [PDB ID: 6PJ2] with a grid box of 20 × 20 × 20 Å. Two FDA approved inhibitors, pepstatin A and boceprevir, were used as a positive docking control in the respective HIV-1 PR and HCV NS3/4A PR molecular dockings. All conformers of the 2 compounds and positive controls were included in the rigid receptor docking with OPLS2005 [GLIDE HTVS] force field by Schrödinger [Cambridge, MA, USA].

Results

Preparation of HE Derivatives

Since 3,28-disubstitutions of oleanolic acid can significantly increase the antiviral activity against HIV-1 protease, we decided to investigate the inhibitory activities of HE derivatives by...
introducing more carboxyl groups into the A ring. **HE** was treated with iodoethane to yield hederagenin ethyl ester (**HES**). The 23-hydroxyl of **HES** was protected by t-butyldimethylchlorosilane (TBSCl) to offer 23-t-butyldimethylsilyloxy-3-β-hydroxyl-hederagenin ethyl ester (**3b**). The hydroxyl group of **3a** was oxidized by PCC to form 23-t-butyldimethylsilyloxy-3-oxo-hederagenin ethyl ester (**5a**). For deprotection of the 23-t-butyldimethylsilyloxy group, **5a** was treated with 10% hydrochloric acid to yield the 23-hydroxyl derivative **5b**. Four types of **HE** derivatives, **HE-3,23-diacyl**, **HES-3,23-diacyl**, **HES-3-acyl**, and **HES-3-Oxo-23-acyl**, were prepared by treatment of **HE**, **HES**, **3a**, and **5b** with the corresponding acid anhydride, as illustrated in Figure 2.

**Inhibitory Activities of HE Derivatives Against HIV-1 and HCV NS3/4A PRs**

Inhibitory activities of **HE** derivatives against HIV-1 and HCV NS3/4A PRs were measured and summarized in Table 1, with pepstatin A (PC A) and Emberlin (PC B) as the respective positive control in the HIV-1 PR and HCV NS3/4A PR assays. We
classified the inhibitory effects into 4 categories: potent inhibition (IC$_{50} < 10$ μM), moderate inhibition (IC$_{50} \sim 10-50$ μM), weak inhibition (IC$_{50} \sim 50-100$ μM) and no inhibition (IC$_{50} > 100$ μM). Clearly, HE showed a weak inhibitory effect against HIV-1 and HCV NS3/4A PRs with IC$_{50}$ values of 76.0 and 42.3 μM, respectively. HES had no inhibition against either protease. For the HE-3,23-diacyl derivatives, 1a showed moderate inhibition against both HIV-1 PR (IC$_{50} = 19.3$ μM) and HCV NS3/4A PR (IC$_{50} = 25.3$ μM); 1b exhibited potent inhibition of HIV-1 PR (IC$_{50} = 9.3$ μM) and moderate inhibition of HCV NS3/4A PR (IC$_{50} = 21.4$ μM). The inhibitory activity of 1b towards HIV-1 PR was increased nearly 2-fold in comparison with that of 1a. This indicated that the terminal COOH group in SA and GA confers the inhibitory effect for 1a and 1b and the longer linker chain is likely to be more favorable for the inhibition of HIV-1 PR. Further studies are warranted to optimize linker size (distance between COOH and C3) for the best inhibition of HIV-1 PR. Both 1a and 1b have similar IC$_{50}$s towards HCV NS3/4A PR, suggesting that HCV NS3/4A PR is likely to have a volume rather than a specific interaction (such as ionic interaction) requirement at C3 of HE. We observed similar patterns for the HES-3,23-diaryl derivatives.

Table 1. Inhibitory Activities of Acylated Derivatives of Hederagenin Against HIV-1 and HCV NS3/4A Proteases.

| Comp.          | R$_1$ | R$_2$ | R$_3$ | IC$_{50}$ (μM) ± RSD% (n = 3) |
|----------------|------|------|------|-----------------------------|
|                |      |      |      | HIV PR | HCV PR          |
| HE             | H, β OH | OH | H    | 76.0 ± 3.20* | 42.3 ± 3.57 |
| HES            | H, β OH | OH | Et   | >100 ± 3.89 | > 100 ± 6.78 |
| HE-3,23-diacyl derivatives | | | | |
| 1a             | H, β SA | SA | H    | 19.3 ± 3.03 | 25.3 ± 4.39 |
| 1b             | H, β GA | GA | H    | 9.3 ± 4.16  | 21.4 ± 3.25 |
| HES-3,23-diacyl derivatives | | | | |
| 2a             | H, β SA | SA | Et   | 25.7 ± 6.42 | 45.7 ± 1.87 |
| 2b             | H, β DMS | DMS | Et   | 17.2 ± 5.30 | 8.6 ± 3.92  |
| 2c             | H, β GA | GA | Et   | 27.4 ± 4.53 | 34.3 ± 8.63 |
| HES-TBS derivatives | | | | |
| 3a             | H, β OH | TBS | Et   | > 100 ± 0.72 | > 100 ± 1.10 |
| HES-3-acyl derivatives | | | | |
| 3b             | H, β SA | TBS | Et   | 46.1 ± 1.55 | 39.2 ± 2.09 |
| 3c             | H, β DMS | TBS | Et   | 35.0 ± 5.57 | 17.8 ± 1.55 |
| 3d             | H, β GA | TBS | Et   | 38.2 ± 4.33 | 16.4 ± 6.60 |
| 4a             | H, β DMS | OH | Et   | 24.5 ± 3.69 | 20.7 ± 1.04 |
| 4b             | H, β GA | OH | Et   | 26.0 ± 5.19 | 37.4 ± 8.10 |
| HES-3-oxo-23-acyl derivatives | | | | |
| 5a             | O     | TBS | Et   | > 100 ± 2.19 | > 100 ± 2.19 |
| 5b             | O     | OH  | Et   | > 100 ± 4.67 | > 100 ± 2.78 |
| 5c             | O     | SA  | Et   | 66.8 ± 5.47 | 30.9 ± 5.19 |
| 5d             | O     | DMS | Et   | 35.1 ± 4.11 | 23.9 ± 5.94 |
| 5e             | O     | GA  | Et   | 27.7 ± 1.89 | 32.6 ± 2.90 |
| PC A           |       |     |      | 0.55 ± 2.02 | nt            |
| PC B           |       |     |      | nt     | 4.8 ± 3.48    |

*HPLC method test.
2a and 2c are moderate inhibitors of both HIV-1 and HCV NS3/4A PRs. Obviously, their inhibitory effects were weaker than those of HE derivatives, indicating that the 28-ethyl group decreased the inhibition of HIV-1 and HCV NS3/4A PRs. Our results are consistent with previous studies showing that small groups at C28 diminish and large groups enhance the inhibitory effects towards HIV protease.\textsuperscript{14,19} Furthermore, compared to 2a and 2c, 2b is a stronger inhibitor with moderate inhibition of HIV-1 PR (IC\textsubscript{50} = 17.2 μM) and potent inhibition of HCV NS3/4A PR (IC\textsubscript{50} = 8.6 μM), implying that branching of the linker between the COOH group and HES might provide better binding to both proteases.

We further decided to investigate the effect of 23-substitution on the antiviral activity of HE and HES. We synthesized 4a and 4b, leaving 23-OH unsubstituted. For HIV-1 PR, 4a and 4b showed similar inhibition (IC\textsubscript{50} of 24.5 and 26.0 μM, respectively). Towards HCV NS3/4A PR, 4a (IC\textsubscript{50} = 20.7 μM) is more potent than 4b (IC\textsubscript{50} = 37.4 μM). These observations show that the substitution of dicarboxylic acid hemiesters (such as DMS) at C23 will strengthen the anti-viral activities of HE and HES derivatives.

Next, we evaluated how the COOH moiety in the 23-substituted functional groups affects the antiviral activity. A series of 23-TBS derivatives of HES was synthesized. As shown in Table 1, 3a did not show any inhibitory activity towards HIV-1 and HCV NS3/4A PRs, but 3b, 3c, and 3d exhibited moderate inhibition of both HIV-1 PR and HCV NS3/4A PR. This study suggests that a dicarboxylic acid hemiester-containing group at C23 is likely to enhance the inhibitory activities of HE and HES derivatives against HIV-1 PR, but may exhibit different effects against HCV NS3/4A PR. Thus, further studies are needed to identify the structural requirement of 23-substitution on HE or HES for potent anti-HCV PR activity.

Finally, we synthesized a series of 3-oxo-23-acyl derivatives of HES. As shown in Table 1, 5a and 5b did not inhibit either HIV-1 PR or HCV NS3/4A PR. 5c, 5d, and 5e moderately inhibited HIV-1 and HCV NS3/4A PRs, except 5c, which exhibited weak inhibition of HIV-1 PR. Thus, a dicarboxylic acid hemiester-containing group at C23 is favorable for the antiviral activity of HE, HES, and their derivatives.

**Molecular Docking and Interaction**

Molecular docking showed that (3\textbeta,23)-di-O-diglutaryl-hedera-genin (1b) formed one hydrogen bond with residue Gly48 (B) of HIV PR via the carboxyl moiety of its 23-O-diglutaryl group (Figure 3A) and formed one hydrogen bond with residue Ala1157 of the HCV NS3/4A PR via the carbonyl oxygen atom of the 3-O-diglutaryl group and 2 hydrogen bonds with Gly1137 and Ser1139 of HCV NS3/4A PR via the carboxyl group of the 23-O-diglutaryl group (Figure 4A). Compound 2b formed 5 hydrogen bonds and 1 salt bridge with residues in the binding pocket of HIV-1PR (Figure 3B) and 4 hydrogen bonds and 1 salt bridge with residues in the binding pocket of HCV NS3/4A PR (Figure 4B). For HIV-1 inhibition, 1 hydrogen bond and salt bridge were formed between the carboxyl moiety of the 3-O-diglutaryl group and residue Arg8 (B). 2 hydrogen bonds between the carboxyl moiety of the 23-O-diglutaryl group and residues Asp29 (A) and Asp30 (A), and 2 hydrogens between the carbonyl oxygen of the 28-ethyl ester group and residues Asp29 (B) and Asp8 (A). For HCV NS3/4A inhibition, the carboxyl moiety of the 3-O-diglutaryl group formed 2 hydrogen bonds with residues Ser1138 and Ser1139, and the carboxyl moiety of the 23-O-diglutaryl group formed 2 hydrogen bonds and 1 salt bridge with residues Lys1136 and Gly1137. The more and stronger hydrogen bonding formed by compound 2b is

![Image](image-url)

**Figure 3.** 2D Ligand interaction plot highlighting compounds 1b (3A), 2b (3B), and pepstatin A (3C) with the active site of HIV protease (PDB ID: 1QBS). In the figures above, hydrophobic interactions are depicted in green, polar interactions in cyan, positively charged as red/blue lines, and hydrogen bonds as purple arrows.
consistent with the protease activity assays that showed that 2b is a more potent inhibitor of HIV-1 and HCV NS3/4A PRs than 1b. Furthermore, the molecular docking revealed that the (3β,23)-di-O-(3′,3′-dimethylsuccinyl) fragments of compound 2b were well buried into the binding sites of HIV-1 and HCV NS3/4A PRs via extensive hydrophobic interactions. As for the 2 FDA approved positive controls, pepstatin A formed 2 hydrogen bonds and 1 salt bridge with HIV-1 PR (Figure 3C), and boceprevir formed 5 hydrogen bonds with HCV NS3/4A PR (Figure 4C). Specifically for HIV-1 PR, the binding contribution comes from residues 1 to 9 and 94 to 99 [N- and C-termini], 21 to 32 [active site core], 47 to 56 [flap region], and 78 to 88 [substrate-binding region]. Resistant mutations are usually evolved from these conserved regions, especially in the flap and substrate-binding region.24 Thus, compounds 1b and 2b might be alternatives to the current anti-HIV protease inhibitors to circumvent cross-resistance.

Conclusion

In this study, we synthesized 16 3-acyl and 23-acyl derivatives of either HE or HES. Inhibitory activity assays against HIV-1 and HCV NS3/4A PRs showed that dicarboxylic acid hemiester-containing groups with 6 carbons at C3 and/or C23 strengthen the antiviral activity. 3,23-di-GA-HE (1b) and 3,23-di-DMS-HES (2b) could be used as lead compounds in developing new potent HE-based antiviral agents.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent

Not applicable, because this article does not contain any studies with human or animal subjects.

Trial Registration

Not applicable, because this article does not contain any clinical trials.

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Supplemental Material

Supplemental material for this article is available online.

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