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Novel 3α-methoxyserrat-14-en-21β-ol (PJ-1) and 3β-methoxyserrat-14-en-21β-ol (PJ-2)-curcumin, kojic acid, quercetin, and baicalein conjugates as HIV agents

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ABSTRACT

Sixteen novel compounds; 3α-methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) and their curcumin, kojic acid, quercetin, and baicalein conjugates (**3**)–(**18**) were designed, synthesized, and evaluated for in vitro anti-HIV-1 reverse transcriptase (RT) activity in infected C8166-CCR5 cells, a human CD4$^+$ T-lymphocyte cell line. Among them, kojic acid derivatives, **9–12** showed significant biological activity. In particular, the compound **13**, the conjugate of two molecules of 3α-methoxyserrat-14-en-21β-ol (**1**) and one molecule of kojic acid, exerted significant anti-HIV activity with an EC50 value of 0.12 μg/mL.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) remains a formidable crisis because of both its emergent and long-term development. Although first generation drugs, such as zidovudine (AZT), zalcitabine (ddC), didanosine (ddI) and sanilvudine (d4T) have been used clinically,[1,2] the rapid development of the viral resistance to these nucleoside HIV-1 reverse transcriptase (RT) inhibitors is being recognized as a common problem to be resolved.[3] As anti-HIV triterpenoids, Chen et al., reported salaspermic acid inhibited HIV replication in H9 lymphocyte cells,[4] Li et al. disclosed suberosol showed anti-HIV replication activity in H9 lymphocyte cells (EC_{50} 3 μg/mL),[5] and Akihisa et al. indicated cycloartenyl ferulate (IC_{50} 2.2 μg/mL), 24-methylenecycloartenol ferulate (IC_{50} 1.9 μg/mL), lupenone (IC_{50} 2.1 μg/mL), betulin diacetate (IC_{50} 1.4 μg/mL), and karounidiol 29-benzoate (IC_{50} 2.2 μg/mL) inhibited HIV-1 RT activity.[6] Fujioka et al., found betulinic acid (EC_{50} 1.4 μM, IC_{50} 13 μg/mL) and platanic acid (EC_{50} 6.5 μM, IC_{50} 90 μg/mL) inhibited HIV replication in H9 lymphocyte cells,[7] and Kashiwada et al., showed oleanolic acid (EC_{50} 1.7 μg/mL) and pomolic acid (EC_{50} 1.4 μg/mL) inhibited HIV-replication in acutely infected H9 cells.[8] Xu et al. reported 2α,19α-dihydroxy-3-oxo-12-ursen-28-oic acid, ursolic acid, and maslinic acid showed potent inhibitory activity against HIV-1 protease (HIV-PR),[9] and Ito et al. demonstrated moronic acid showed anti-HIV activity (EC_{50} < 0.1 μg/mL, TI > 186),[10] while Takeoka et al. reported betulinic acid, oleanolic acid and ursolic acid showed anti-HIV activity.[11] Sun et al. found nigranoic acid showed activity in several anti-HIV RT and polymerase assays,[12] and Li et al. reported micrandilactone C possessed minimal cytotoxicity (CC_{50} > 200 μg/mL) to the tested human T cell leukemia cell line C8166 and the inhibitory activity on HIV-1IIIB-induced syncytium formation (EC_{50} 7.71 mg/mL).[13] Xiao et al. isolated lancifodilactone H (EC_{50} 16.6 μg/mL, CC_{50} > 104.9 μg/mL), lancifoic acid A (EC_{50} 16.2 μg/mL, CC_{50} > 104.9 μg/mL), and nigranoic acid (EC_{50} 10.3 μg/mL, CC_{50} > 88.0 μg/mL) showed anti-HIV activity in C8166 cells,[14] and lancfodlactone G showed anti-HIV activity (EC_{50} 95.47 ± 14.19 μg/mL, CC_{50} > 200 μg/mL).[15] Baltina

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et al. reported the conjugate of glycyrrizic acid 30-methylester with Lys(Z)-OH possessed anti-HIV activity in MT-4 cell culture (ID50 200 μg/mL; CD50 2000 μg/mL),[16] and the conjugates of glycyrrizic acid with α-D-glucosamine showed strong anti-HIV activity (CD_{50} 150 μg/mL; ID_{50} 1.5 μg/mL; IS = 100) and was more active than glycyrrhetic acid (IS = 9.6).[17] and the conjugate of glycyrrhizic acid 30-methyl ester with Lys(Z)-OH possessed anti-HIV activity.[18] Chen et al. disclosed cucurbitacin B showed potent anti-HIV-1 activity in C8166 cells (EC_{50} 0.09 μg/mL; SI 16.7).[19] Ma et al. noted triterpene–AZT conjugates showed potent anti-HIV activity and potent HIV-1 PR inhibitory activity, although AZT itself showed no PR inhibitory activity, and triterpene–anti-HIV alkaloid FK 3000 conjugates showed neither PR inhibitory activity nor anti-HIV activity,[20] and *N*-[3β-hydroxyolean-12-ene-28-oyl]-6-aminohexanoic acid (IC_{50} 1.7 μM) and *N*-[3β-*O*-adipoylolean- 12-en-28-oyl]-6-aminohexanoic acid (IC_{50} 1.7 μM) showed more than fourfold increase in activity against HIV-1 PR compared to oleanolic acid (IC_{50} 8 μM).[21] Ma et al. also reported ursolic acid and its hydrogen malonate inhibited HIV-1 PR activity (IC_{50} 8 μM and 6 μM)], and glutaryl hemiesters of ursolic acid, oleanolic acid,

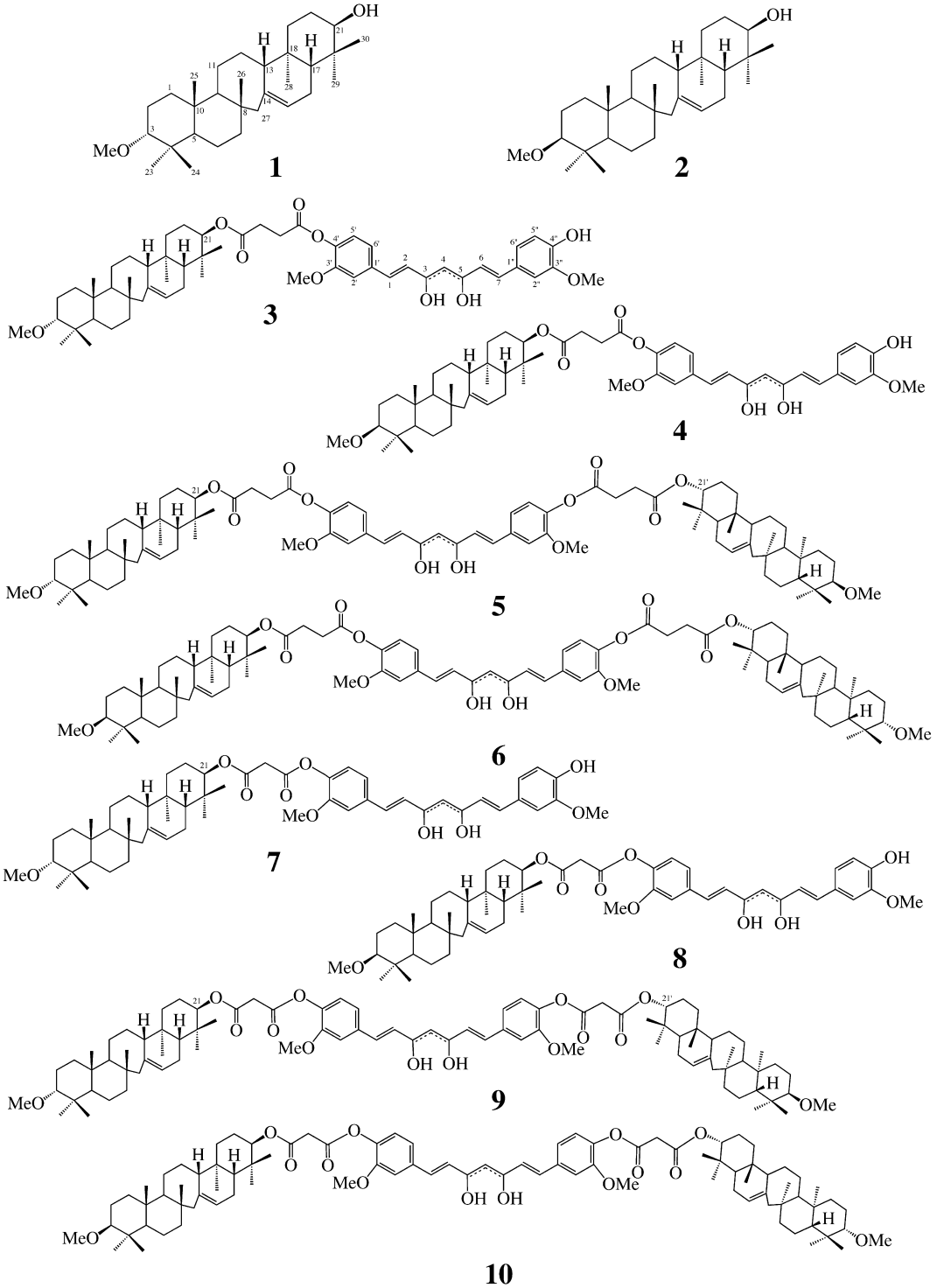

Figure 1. 3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) and their curcumin conjugates (**3**–**10**).

and betulinic acid showed increased activity (each IC_{50} 4 μM).[22] Kashiwada et al. reported 3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid (PA-457) inhibits HIV-1 mutation by interfering with HIV-1 P24/P25 processing,[23,24] but, IC9564 acts at an early stage of viral infection,[25] and moronic acid derivatives showed potent anti-HIV activity with EC_{50} values of 0.0085 μM against NL4-3, 0.021 μM against PI-R, and 0.13 μM against FHR-2.[26] Compounds possessing potent anti-HIV activity as well as novel structures and modes of action are urgently needed to add to the list of existing anti-HIV agents.

The genus *Picea* consists of approximately 40 species: this number is next to those of genera *Pinus* and *Abies* under the order *Pinales*. *Picea* species do not have any use besides construction materials. In our quest for biologically active constituents from natural sources, we found compounds showing significant anti-tumor promoting activities in an in vivo two-stage mouse-skin carcinogenesis assay using 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA): 13α, 14α-epoxy-3β-methoxyserratan-21β-ol and 21α-hydroxy-3β-methoxyserrat-14-en-29-al,[27] serrat-14-en-3β,21β-diol,[28] 3β-methoxyserrat-14-en-21β-ol (**1**),[29] and 14β,15β-epoxy-3β-methoxyserratan-21β-ol[30] from the bark of *Picea jezoensis* (Sieb. et Zucc) Carr. var. *jezoensis* (Pinaceae, Japanese name: Ezomatsu), and *P. jezoensis* (Sieb. et Zucc) Carr. var. *hondoensis* (Mayr) Rehder

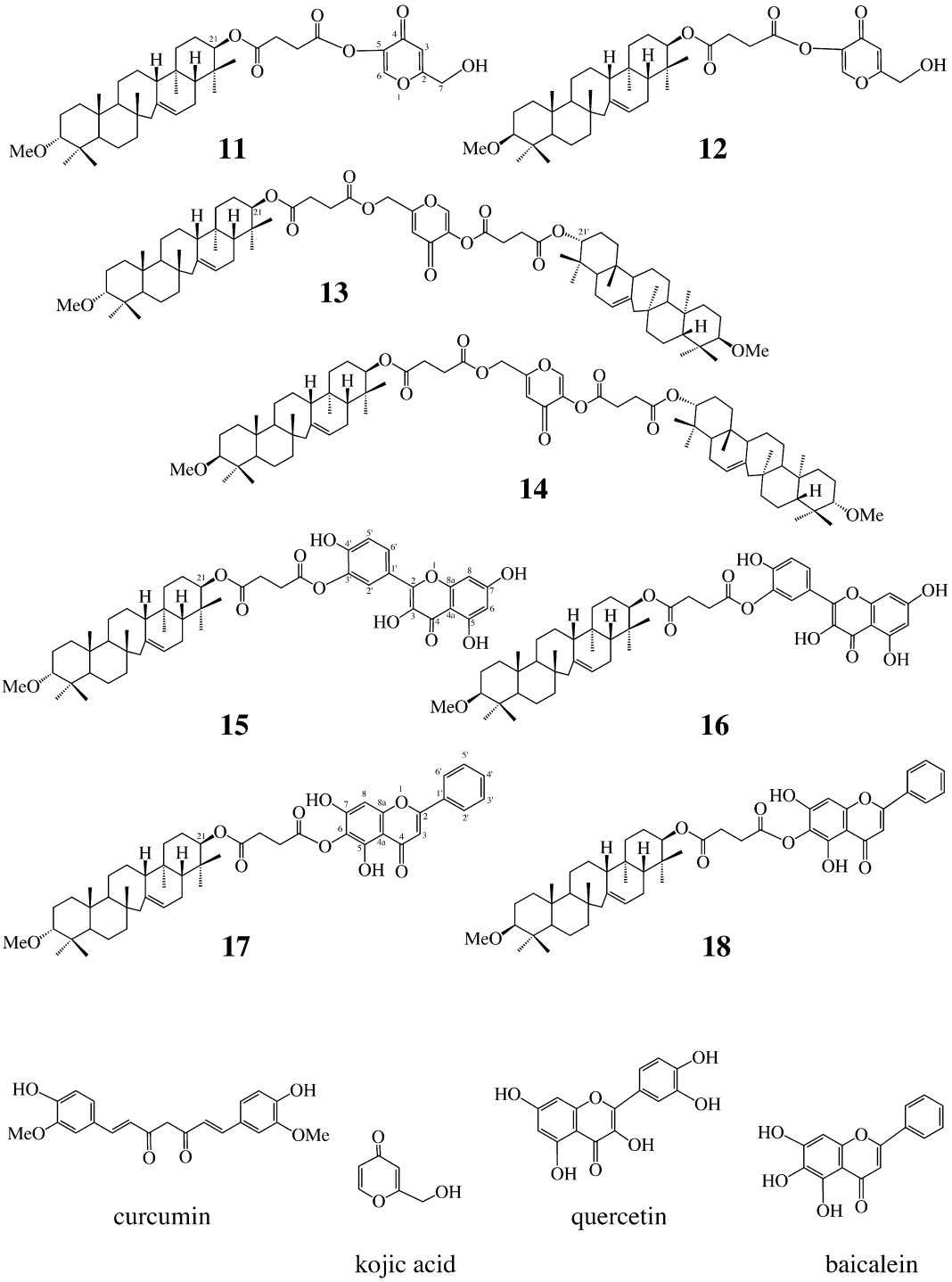

Figure 2. 3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) and their kojic acid, quercetin, and baicalein conjugates (**11**–**18**).

(Pinaceae, Japanese name Touhi). 3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) are the most abundant triterpenoid constituents from two the above two *Picea* plants and *Picea glehni* (Fr. Schm.) Masters (Japanese name: Akaezomatsu),[31] and the total yields of **1** and **2** accounted for more than 1/3 of the chloroform extract of the above three plants. It was recently reported that 3α-methoxyserrat-14-en-21β-ol (**1**) significantly decreased the size of adenomas and total tumors in a revised rat multi-organ carcinogenesis (DMBDD) model.[32]

Table 1
Anti-HIV and cytostatic activity of compounds **1**–**18**

Compounds	Anti HIV activity EC_{50}[a] (μg/ml)	Cytotoxicity CC_{50}[b] (μg/ml)	Selectivity index SI (CC_{50}/EC_{50})
1	>100	>100	–
2	>100	>100	–
3–10	>100	>100	–
11	4.13	22.2	5.4
12	6.75	>100	86.8
13	0.12	4.2	35
14	5.94	>100	>5000
15	26.57	>100	4.8
16	23.18	>100	>5000
17	21.40	>100	4.9
18	55.37	>100	2.3
AZT[c]	0.02	>100	>5000

[a] Effective concentration of 50% required to inhibit HIV-induced cytopathy.
[b] Cytostatic concentration of 50%.
[c] Positive control.

On the other hand, a well-known phenolic natural products, curcumin[33] and kojic acid,[34] quercetin,[35] and baicalein[36] possess excellent activities such as anti-oxidant, anti-cancer, and anti-aging activities. The conjugation of two bioactive compounds is now accepted as an effective lead for the design of ligands, inhibitors, and other drugs.[37] Natural product hybrids play an important role in the development of drugs, especially for the treatment of infections and cancer, as well as immunosuppressive compounds.[38] Therefore, based on the diverse bioactivities of the triterpenoids as well as phenolic compounds having anti-oxidant activity, like curcumin, kojic acid, quercetin and baicalein, we started a study of triterpenoid-phenolic compounds conjugation on the basis of the hybrid drug strategy. Therefore, compounds **1** and **2**, and their curcumin, kojic acid, quercetin, and baicalein conjugates using succinic acid and malonic acid as linker (**3–18**) were synthesized (Figs. 1 and 2). Herein, we report the anti-HIV-1 RT activity of compounds **1**–**18** in C8166-CCR5 cells (Table 1).

2. Results and discussion

2.1. Chemistry

3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) are the predominant triterpenoid constituents in the chloroform extracts of *P. jezoensis* Carr. var. *jezoensis* and *P. jezoensis* Carr. var. *hondoensis*. Compounds **1** and **2** are C-3 methoxy epimers, constituting 25% and 10% of the extract of *P. jezoensis* Carr. var. *jezoensis* and 18% and 30% of the extract of *P. jezoensis* Carr. var. *hondoensis*, respectively. The distribution of serratane triterpenoids

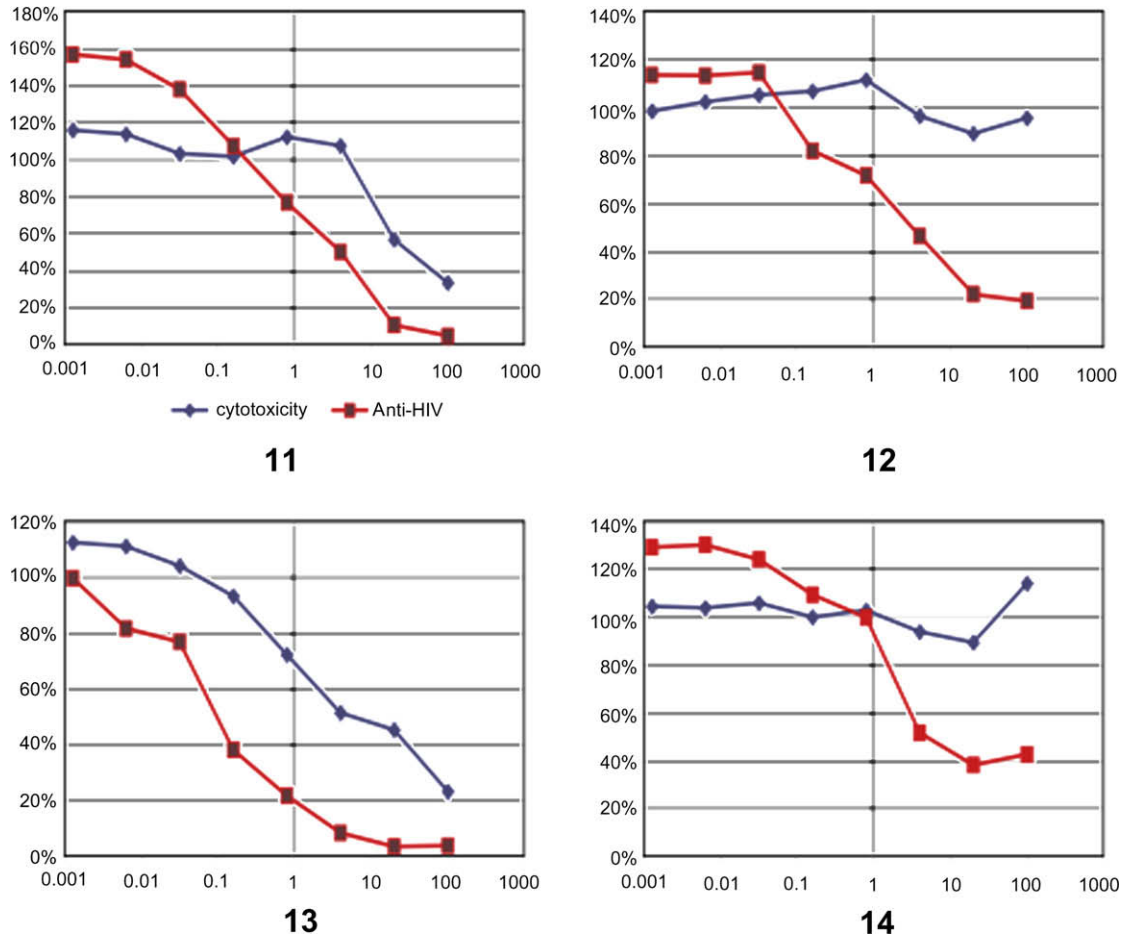

Figure 3. Anti-HIV-1 RT activity and cytotoxicity of compounds **11**–**14**.

is limited to *Picea*, *Pinus*, and some *Licopodium* plants. Serratane-type triterpenoids possess the rare 6–6–7–6–6-ring system, and the C-ring is flexible. Compounds **1** and **2** have the same serratane skeleton, the same C-21β-hydroxyl group, and the same trisubstituted double bond at C14–15; the only difference is that **1** has the C-3α-methoxy group while **2** has the C-3β-methoxy group. The isolation and characterization of **1** and **2** had been reported.[39]

Although oleanolic acid, ulsoric acid, and betulinic acid were often modified in various forms, serratane-type triterpenoids, such as **1** and **2**, have not been studied so far. Therefore, we conjugated **1** and **2** with curcumin, kojic acid, quercetin, and baicalein (Figs. 1 and 2). Compounds **3** and **4** are conjugates of one molecule of compounds **1** or **2** and one molecule of curcumin using succinic acid as linker, and compounds **5** and **6** are conjugates of two molecules of **1** or **2** and one molecule of curcumin using succinic acid as linker. Compounds **7** and **8** are conjugates of each one molecule of **1** or **2** and one molecule of curcumin using malonic acid as linker, and compounds **9** and **10** are conjugates two molecules of **1** or **2** and one molecule of curcumin using malonic acid as linker. Compounds **11** and **12** are conjugates of one molecule of **1** or **2** and one molecule of kojic acid using succinic acid as linker, and compounds **13** and **14** are conjugates of two molecules of **1** or **2** and one molecule of kojic acid using succinic acid as linker. Compounds **15** and **16** are conjugates of one molecule of **1** or **2** and one molecule of quercetin using succinic acid as linker. Compounds **17** and **18** are conjugates of one molecule of **1** or **2** and one molecule of baicalein using succinic acid as linker. The synthesized novel compounds (**3** –**18**) along with compounds **1** and **2** were evaluated for their in vitro anti-HIV-1 RT activity. The chemical structures and the results of in vitro RT activation test are shown in Figures 1 and 2 and Table 1, respectively. Synthetic compounds **3–18** had purities >99%.

2.2. Biological evaluation

The RT activity and cytotoxicity of compounds **1–18** (Table 1) were evaluated in C8166-CCR5 cells, a human $CD4^+$ T-lymphocyte cell line. 3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) showed CC_{50} value over 100 μg/mL, but no anti-HIV activity even at high dose (100 μM/mL). Therefore, compounds **1**, **2** and conjugates **3–10** had neither RT activity nor cytotoxicity. On the other hand, compound **13** exerted significant anti-HIV RT activity with EC50 of 0.12 μg/mL, CC50 of 4.2 μg/mL, and selectivity index (SI: CC50/EC50) of 35 (Table 1, Fig. 3). Compound **13** possesses two molecules of compound **1** and one molecule of kojic acid linked by two succinic acids. In addition, compound **14** showed decent anti-HIV activity with EC50 of 5.94 μg/mL, but no cytotoxicity with CC50 > 100 μg/mL, thus SI > 5000 (Table 1, Fig. 3). Compound **14** possesses two molecules of compound **2** and one molecule of kojic acid linked by two succinic acids. Compounds **11** and **12** showed anti-HIV RT inhibitory activity with EC50 value of 4.13 and 6.75 μg/mL, while CC50 was 22.2 and 585.6 μg/mL, and SI was 5.4 and 86.8, respectively (Table 1, Fig. 3). Compounds **11** and **13** are conjugates of compound **1** and kojic acid, whereas compounds **12** and **14** are conjugates of compound **2** and kojic acid. The compound **1**—kojic acid conjugates **11** and **13** showed increased anti-HIV RT activity but decreased cytotoxicity compared with the compound **2**—kojic acid conjugates, **12** and **14**. Compounds **12**, **15**, **17** and **18** showed CC_{50} value over 100 μg/mL (Table 1), but reduced concentration from each decline curve showed CC_{50} value as 585.6, 126.6, 105.8, and 128.7 μg/mL, respectively. This study is the first to show the anti-HIV activity of serratane-type triterpenoids, **1** and **2**, and their conjugates **3–18**. Although compounds **1**, and **2** themselves, and the conjugates of compounds **1** or **2** and curcumin, **3–10**, were inactive, the conjugates of serratane type triterpenoids, **1** or **2** and kojic acid, such as **11–14**, showed potential as anti-HIV agents. Judging from these results, there is a possibility that kojic acid could work as a key element of anti-HIV activity in hybrid drugs. In response to the consideration, further studies on structure–activity relationships with conversion of kojic acid, various device of linker are now in progress.

3. Experimental

3.1. Chemistry

3.1.1. General

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1720X FTIR spectrophotometer. ^{1}H- and ^{13}C NMR spectra were measured with Varian INOVA 500, Varian Unity INOVA-400, JEOL JNM-AL300, or Varian GEMINI 2000/200 spectrometer with TMS as the internal standard. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively. EIMS were recorded on a HITACHI 4000H double-focusing mass spectrometer (70 eV). Column chromatography was carried out over silica gel (70–230 mesh, Merck) and medium-pressure liquid chromatography (MPLC) was carried out over silica gel (230–400 mesh, Merck). HPLC was run on a JASCO PU-1586 instrument equipped with a differential refractometer (RI 1531). Fractions obtained from column chromatography were monitored by TLC (silica gel 60 F_{254}, Merck). Preparative TLC was carried out on Merck silica gel F_{254} plates (20 × 20 cm, 0.5 mm thick).

3.1.2. Chemicals

Curcumin, kojic acid, quercetin, baicalein (GR) were purchased from Nacalai Tesque, Inc., Kyoto, Japan. Succinic anhydride, malonic acid mono *t*-butyl ester, carbonyldiimidazole, diisopropylethylamine, and 4-dimethylaminopyridine (DMAP), and dicyclohexylcarbodiimide (DCC) were purchased from Wako pure Chemical Industries, Ltd, Osaka, Japan. Succinic anhydride and *t*-butylmalonate were obtained Sigma–Aldrich Com., USA.

3.2. Test compounds

Natural serratane-type triterpenoids, 3α-methoxyserrat-14-en-21β-ol (**1**) and, 3β-methoxyserrat-14-en-21β-ol (**2**), were isolated from the stem bark of *P. jezoensis* Carr. var. *hondoensis and P. jezoensis* Carr. var. *jezoensis* (Pinaceae) which were collected at Aizuwakamatsu City (Fukushima Prefecture) and Sapporo City (Hokkaido), Japan. Voucher specimens were deposited at the laboratory of Medicinal chemistry, Osaka University of Pharmaceutical Sciences.

3.2.1. 3α-Methoxyserrat-14-en-21β-ol (1)

Compound **1** was isolated from two picea plants: *P. jezoensis* Carr. var. *jezoensis* and *P. jezoensis* Carr. var. *hondoensis*.[39] Mp 277–279 °C, $[\alpha]_D$ −57.

3.2.2. 3β-Methoxyserrat-14-en-21β-ol (2)

Compound **2** was isolated from two picea plants: *P. jezoensis* Carr. var. *jezoensis* and *P. jezoensis* Carr. var. *hondoensis*.[39] Mp 305–307 °C, $[\alpha]_D$ −2.

3.2.3. Compound 1 succinic acid

A mixture of 3α-methoxyserrat-14-en-21β-ol (**1**) (1.0 g, 2.2 mmol), succinic anhydride (2.2 g, 21.9 mmol) and 4-dimethylaminopyridine (DMAP) (270 mg, 2.2 mmol) in pyridine (10 mL)

was reacted at 100 °C for 15 h under nitrogen atmosphere. The reaction mixture was concentrated to remove pyridine. To the residue was added ethyl acetate and the mixture was filtered through Hyfro Super-CelR. The residue was washed with EtOAc. The filtrate and washings were combined and the mixture was washed with 1 N-HCl, brine, dried with magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography. (hexane/EtOAc = 4:1–2:1) affording compound **1** succinic acid (1.1 g, 88%) as a colorless powder.

3.2.4. Compound 2 succinic acid

3β-Methoxyserrat-14-en-21β-ol (**2**) succinic acid (1.26 g, 75%) was obtained from compound **2** (1.37 g). The reaction method employed was similar to that of compound **1** succinic acid.

3.2.5. Compounds 3 and 5

To a solution of compound **1**succinic acid (100.0 mg, 0.18 mmol) in $CHCl_3$ (2 mL) were successively added curcumin (72.0 mg, 0.2 mmol), dicyclohexylcarbodiimide (DCC) (37.1 mg, 0.22 mmol), and DMAP (catalyst), and the mixture was stirred at room temperature for 17 h under nitrogen atmosphere. The precipitate was filtered and the filtrate was concentrated. To the residue were added water and ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 5:1–2:1) to give compound **3** (77 mg, 29%) and compound **5** (70 mg, 43%). Compound **3**, mp 191–194 °C; $[\alpha]_D$ −141.0 (*c* 0.074); ^{1}H NMR δ 2.80 and 2.95 (each 2H, td, *J* = 6.8, 1.5 Hz, O=C–C*H*$_2$–C*H*$_2$–C=O), 3.87 (3H, s, C-3′ OMe), 3.95 (3H, s, C-3″ OMe), 4.73 (1H, t, *J* = 2.7 Hz, H-21), 5.83 (1H, s, H-4), 6.49 (1H, d, *J* = 15.8 Hz, H-6), 6.55 (1H, d, *J* = 15.8 Hz, H-2), 6.94 (1H, d, *J* = 8.2 Hz, H-5″), 7.06 (1H, d, *J* = 1.8 Hz, H-2″), 7.07 (1H, d, *J* = 8.2 Hz, H-5′), 7.11 (1H, d, *J* = 1.9 Hz, H-2′), 7.13 (1H, dd, *J* = 8.2, 1.8 Hz, H-6″), 7.15 (1H, dd, *J* = 8.2, 1.9 Hz, H-6′), 7.60 (1H, d, *J* = 15.8 Hz, H-1), 7.61 (1H, d, *J* = 15.8 Hz, H-7); ^{13}C NMR δ 29.5 and 29.8 (O=C–*C*H$_2$–*C*H$_2$–C=O), 79.0 (C-21), 101.5 (C-4), 109.7 (C-2″), 111.4 (C-2′), 114.9 (C-5″), 120.9 (C-6′), 121.7 (C-6), 123.0 (C-6″), 123.2 (C-5′), 124.2 (C-2), 127.5 (C-1″), 134.1 (C-1′), 139.4 (C-1), 141.1 (C-7), 146.8 (C-3″), 148.0 (C-4″), 151.3 (C-3′), 156.8 (C-4′), 170.3 and 171.5 (O=*C*–CH$_2$–CH$_2$–C=O), 181.7 (C-3), 184.5 (C-5). HRMS (FAB) calcd for $C_{56}H_{74}O_{10}Na$ [M+Na]$^+$, 929.5280; found, 929.5173. Compound **5**: mp 172–174 °C; $[\alpha]_D$ −119.8 (*c* 0.071); ^{1}H NMR δ 2.80 and 2.95 (each 4H, t, *J* = 7.4 Hz, O=C–C*H*$_2$–C*H*$_2$–C=O), 3.87 (6H, s, C-3′ and C-3″ OMe), 4.73 (2H, t, *J* = 2.1 Hz, H-21 and H-21′), 5.86 (1H, s, H-4), 6.56 (2H, d, *J* = 15.9 Hz, H-2 and H-6), 7.08 (2H, d, *J* = 8.2 Hz, H-5′ and H-5″), 7.12 (2H, d, *J* = 1.9 Hz, H-2′ and H-2″), 7.16 (2H, dd, *J* = 8.2, 1.9 Hz, H-6′ and H-6″), 7.62 (2H, d, *J* = 15.9 Hz, H-1 and H-7). ^{13}C NMR δ 29.1 and 29.5 (O=C–*C*H$_2$–*C*H$_2$–C=O), 79.0 (C-21, C-21′), 101.7 (C-4), 111.5 (C-2′, C-2″), 121.0 (C-6′, C-6″), 123.3 (C-5′, C-5″), 124.2 (C-2, C-6), 133.9 (C-1′, C-1″), 139.9 (C-1, C-7), 141.2 (C-4′, C-4″), 151.3 (C-3′, C-3″), 170.2 and 171.5 (O=*C*–CH$_2$–CH$_2$–*C*=O), 183.1 (C-3, C-5). HRMS (FAB) calcd for $C_{91}H_{128}O_{14}Na$ [M+Na]$^+$, 1467.9202; found, 1467.9210.

3.2.6. Compounds 4 and 6

Compounds **4** (65%) and **6** (30%) were synthesized from compound **2** succinic acid similarly to compounds **3** and **5**. Compound **4**: mp 212–215 °C; $[\alpha]_D$ −0.8 (*c* 0.098); ^{1}H NMR δ 2.80 and 2.95 (each 2H, td, *J* = 7.4, 1.8 Hz, O=C–C*H*$_2$–C*H*$_2$–C=O), 3.88 (3H, s, C-3′ OMe), 3.95 (3H, s, C-3″ OMe), 4.73 (1H, t, *J* = 2.3 Hz, H-21), 5.83 (1H, s, H-4), 6.49 (1H, d, *J* = 15.8 Hz, H-6), 6.55 (1H, d, *J* = 15.8 Hz, H-2), 6.94 (1H, d, *J* = 8.1 Hz, H-5″), 7.06 (1H, d, *J* = 2.1 Hz, H-2″), 7.08 (1H, d, *J* = 8.1 Hz, H-5′), 7.11 (1H, d, *J* = 2.1 Hz, H-2′), 7.13 (1H, dd, *J* = 8.1, 2.1 Hz, H-6″), 7.15 (1H, dd, *J* = 8.1, 2.1 Hz, H-6′), 7.60 (1H, d, *J* = 15.8 Hz, H-1), 7.61 (1H, d, *J* = 15.8 Hz, H-7). ^{13}C NMR δ 29.1 and 29.5 (O=C–*C*H$_2$–*C*H$_2$–C=O), 79.0 (C-21), 101.5 (C-4), 109.7 (C-2″), 111.4 (C-2′), 114.9 (C-5″), 120.9 (C-6′), 121.7 (C-6), 123.0 (C-6″), 123.2 (C-5′), 124.2 (C-2), 127.5 (C-1″), 134.1 (C-1′), 139.3 (C-1), 141.1 (C-7), 146.8 (C-3″), 148.1 (C-4″), 151.3 (C-3′), 156.8 (C-4′), 170.2 and 171.5 (O=*C*–CH$_2$–CH$_2$–*C*=O), 181.7 (C-3), 184.5 (C-5). HRMS (FAB) calcd for $C_{56}H_{74}O_{10}Na$ [M+Na]$^+$, 929.5180; found, 929.5186. Compound **6**: mp 183–186 °C; $[\alpha]_D$ −21.3 (*c* 0.116); ^{1}H NMR δ 2.80 and 2.95 (each 4H, td, *J* = 7.5, 1.4 Hz, O=C–C*H*$_2$–C*H*$_2$–C=O), 3.87 (6H, s, C-3′ and C-3″ OMe), 4.73 (2H, t, *J* = 2.2 Hz, H-21 and H-21′), 5.86 (1H, s, H-4), 6.57 (2H, d, *J* = 15.8 Hz, H-2 and H-6), 7.08 (2H, d, *J* = 8.2 Hz, H-5′ and H-5″), 7.12 (2H, d, *J* = 1.8 Hz, H-2′ and H-2″), 7.16 (2H, dd, *J* = 8.2, 1.8 Hz, H-6′ and H-6″), 7.62 (2H, d, *J* = 15.8 Hz, H-1 and H-7). ^{13}C NMR δ 29.1 and 29.5 (O=C–*C*H$_2$–*C*H$_2$–C=O), 79.0 (C-21, C-21′), 101.8 (C-4), 111.4 (C-2′, C-2″), 121.1 (C-6′, C-6″), 123.3 (C-5′, C-5″), 124.2 (C-2, C-6), 133.9 (C-1′, C-1″), 139.9 (C-1, C-7), 141.2 (C-4′, C-4″), 151.3 (C-3′, C-3″), 170.2 and 171.5 (O=*C*–CH$_2$–CH$_2$–*C*=O), 183.0 (C-3, C-5). HRMS (FAB) calcd for $C_{91}H_{128}O_{14}Na$ [M+Na]$^+$, 1467.9202; found, 1467.9210.

3.2.7. Compound 1 *t*-butylmalonate

To a solution of compound **1** (228 mg, 0.5 mmol) in DMF (2 mL) were added malonic acid mono *t*-butyl ester (166 mg, 1 mmol), carbonyldiimidazole (178 mg, 1.1 mmol), diisopropylethylamine (0.10 mL, 0.6 mmol) and DMAP (catalyst), and the mixture was stirred at 100 °C for 2 h under nitrogen atmosphere. The reaction mixture was concentrated to remove solvent, to which was added water and extracted with EtOAc. The extract was washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by recrystallization with $CHCl_3$ and MeOH, giving compound **1** malonic acid mono *t*-butyl ester (146.6 mg, 83%).

3.2.8. Compound 2 *t*-butylmalonate

Compound **2** *t*-butylmalonate (252 mg, 65%) was obtained from compound **2** (300 mg). The reaction method employed was similar to that of compound **1** *t*-butylmalonate.

3.2.9. Compound 1 malonic acid

To a solution of compound **1** *t*-butylmalonate (237 mg, 0.39 mmol) in DMF (3 mL) was added methanesulfonic acid (0.7 mL, 7.8 mmol), and the mixture was stirred at 100 °C for 22.5 h under nitrogen atmosphere. The reaction mixture was concentrated to remove solvent, to which was added water and extracted with EtOAc. The extract was washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 3:2) to give compound **1** malonic acid (100 mg, 47%).

3.2.10. Compound 2 malonic acid

Compound **2** malonic acid (58 mg, 90%) was synthesized from compound **2**. The reaction method employed was similar to that of compound **1** malonic acid.

3.2.11. Compounds 7 and 9

To a solution of compound **1** malonic acid (30.0 mg, 0.055 mmol) in $CHCl_3$ (1 mL) were added curcumin (22.4 mg, 0.061 mmol), WSC·HCl (16.5 mg, 0.66 mmol) and DMAP (catalyst), and the mixture was stirred at 60 °C for 4 h under nitrogen atmosphere. To the reaction mixture was added water, the whole was extracted with EtOAc. The extract was washed with brine, dried over magnesium sulfate, and concentrated. The residue was purified by PTLC (hexane/EtOAc = 2:1) to give compounds **7** (24.2 mg, 49%) and **9** (12.5 mg, 32%). Compound **7**: mp 145–148 °C; $[\alpha]_D$ −46.0 (*c* 0.123); ^{1}H NMR δ 3.68 (2H, d, *J* = 1.8 Hz, O=C-C*H*$_2$-C=O), 3.87 (3H, s, C-3′ OMe), 3.95 (3H, s, C-3″ OMe), 4.80 (1H, t,

J = 2.1 Hz, H-21), 5.82 (1H, s, H-4), 6.49 (1H, d, J = 15.8 Hz, H-6), 6.54 (1H, d, J = 15.7 Hz, H-2), 6.94 (1H, d, J = 8.2 Hz, H-5″), 7.05 (1H, d, J = 1.8 Hz, H-2″), 7.11 (1H, d, J = 7.6 Hz, H-5′), 7.12 (1H, s, H-2′), 7.13 (1H, dd, J = 8.2, 1.8 Hz, H-6″), 7.16 (1H, d, J = 7.6 Hz, H-6′), 7.59 (1H, d, J = 15.7 Hz, H-1), 7.61 (1H, d, J = 15.8 Hz, H-7); ^{13}C NMR δ 41.6 (O=C–*C*H$_2$–C=O), 80.4 (C-21), 101.5 (C-4), 109.6 (C-2″), 111.6 (C-2′), 114.9 (C-5″), 120.8 (C-6′), 121.7 (C-6), 123.0 (C-5′ and C-6″), 124.4 (C-2), 127.5 (C-1″), 134.4 (C-1′), 139.2 (C-1), 140.7 (C-4′), 141.2 (C-7), 146.8 (C-3″), 148.0 (C-4″), 151.2 (C-3′), 164.5 and 165.5 (O=*C*–CH$_2$–*C*=O), 181.6 (C-3), 184.6 (C-5). HRMS (FAB) calcd for $C_{55}H_{72}O_{10}Na$ [M+Na]$^+$, 915.5023; found, 915.5030. Compound **9**: mp 173–176 °C; $[\alpha]_D$ + 0.3 (c 0.081); ^{1}H NMR δ 3.69 (4H, d, J = 1.6 Hz, O=C–*CH*$_2$–C=O), 3.88 (6H, s, C-3′ and C-3″ OMe), 4.80 (2H, t, J = 2.1 Hz, H-21 and H-21′), 5.85 (1H, s, H-4), 6.56 (2H, d, J = 15.8 Hz, H-2 and H-6), 7.11 (2H, d, J = 7.1 Hz, H-5′ and H-5″), 7.12 (2H, s, H-2′ and H-2″), 7.16 (2H, d, J = 7.1 Hz, H-6′ and H-6″), 7.62 (2H, d, J = 15.8 Hz, H-1 and H-7)); ^{13}C NMR δ 41.6 (O=C-*C*H$_2$-C=O), 80.4 (C-21, C-21′), 101.9 (C-4), 111.6 (C-2′, C-2″), 120.9 (C-6′, C-6″), 123.1 (C-5′, C-5″), 124.4 (C-2, C-6), 134.2 (C-1′, C-1″), 139.9 (C-1, C-7), 140.9 (C-4′, C-4″), 151.2 (C-3′, C-3″), 164.5 (O=*C*–CH$_2$–*C*=O), 165.5 (O=*C*–CH$_2$–*C*=O), 183.0 (C-3, C-5). HRMS (FAB) calcd for $C_{89}H_{124}O_{14}Na$ [M+Na]$^+$, 1439.8889; found, 1439.8882.

3.2.12. Compounds 8 and 10

Compounds **8** (137.0 mg, 49%) and **10** (52.1 mg, 20%) were obtained from compound **2** malonic acid (200 mg, 0.37 mmol). The reaction method employed was similar to that of compounds **7** and **8**. Compound **8**, mp 150–153 °C; $[\alpha]_D$ +6.8 (c 0.138); ^{1}H NMR δ 3.69 (2H, d, J = 1.6 Hz, O=C–*CH*$_2$–C=O), 3.87 (3H, s, C-3′ OMe), 3.95 (3H, s, C-3″ OMe), 4.80 (1H, t, J = 2.3 Hz, H-21), 5.82 (1H, s, H-4), 6.49 (1H, d, J = 15.8 Hz, H-6), 6.54 (1H, d, J = 15.8 Hz, H-2), 6.93 (1H, d, J = 8.3 Hz, H-5″), 7.06 (1H, d, J = 1.8 Hz, H-2″), 7.10 (1H, d, J = 7.0 Hz, H-5′), 7.11 (1H, s, H-2′), 7.12 (1H, dd, J = 8.3, 1.8 Hz, H-6″), 7.16 (1H, d, J = 7.0 Hz, H-6′), 7.59 (1H, d, J = 15.8 Hz, H-1), 7.61 (1H, d, J = 15.8 Hz, H-7); ^{13}C NMR δ 41.6 (O=C–*CH*$_2$–C=O), 80.4 (C-21), 101.6 (C-4), 109.6 (C-2″), 111.7 (C-2′), 114.8 (C-5″), 120.7 (C-6′), 121.7 (C-6), 123.0 (C-6″), 123.1 (C-5′), 124.4 (C-2), 127.5 (C-1″), 134.4 (C-1′), 139.2 (C-1), 140.7 (C-4′), 141.2 (C-7), 146.8 (C-3″), 148.0 (C-4″), 151.2 (C-3′), 164.5 and 165.5 (O=*C*–CH$_2$–*C*=O), 181.5 (C-3), 184.6 (C-5). HRMS (FAB) calcd for $C_{55}H_{72}O_{10}Na$ [M+Na]$^+$, 915.5023; found, 915.5031. Compound **10**, mp 159–162 °C; $[\alpha]_D$ + 4.1 (c 0.139); ^{1}H NMR δ 3.69 (4H, d, J = 1.1 Hz, O=C–C*H*$_2$–C=O), 3.88 (6H, s, C-3′ and C-3″ OMe), 4.80 (2H, t, J = 2.1 Hz, H-21 and H-21′), 5.84 (1H, s, H-4), 6.56 (2H, d, J = 15.8 Hz, H-2 and H-6), 7.11 (2H, d, J = 8.3 Hz, H-5′ and H-5″), 7.13 (2H, d, J = 1.8 Hz, H-2′ and H-2″), 7.17 (2H, dd, J = 8.3, 1.8 Hz, H-6′ and H-6″), 7.61 (2H, d, J = 15.8 Hz, H-1 and H-7); ^{13}C NMR δ 41.6 (O=C–*C*H$_2$–C=O), 80.4 (C-21, C-21′), 101.9 (C-4), 111.7 (C-2′, C-2″), 120.9 (C-6′, C-6″), 123.0 (C-5′, C-5″), 124.4 (C-2, C-6), 134.2 (C-1′, C-1″), 139.8 (C-1, C-7), 140.9 (C-4′, C-4″), 151.2 (C-3′, C-3″), 164.5 (O=*C*–CH$_2$–*C*=O), 165.5 (O=*C*–CH$_2$–*C*=O), 183.0 (C-3, C-5). HRMS (FAB) calcd for $C_{89}H_{124}O_{14}Na$ [M+Na]$^+$, 1439.8889; found, 1439.8895.

3.2.13. Compounds 11 and 13

To a solution of compound **1** succinic acid (60.6 mg, 0.11 mmol) in dimethylformamide (DMF) (2 mL) were added kojic acid (16.8 mg, 0.12 mmol), 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (WSC·HCl, 24.8 mg, 0.13 mmol), and 1-hydroxybenzotriazole (HOBt) (16.5 mg, 2 mmol) and the mixture was stirred at 60 °C for 1 h under nitrogen atmosphere. To the reaction mixture was added satd-sodium hydrogen carbonate aqueous and the whole was extracted with EtOAc. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 4:1–2:1) to afford compound **11** (12.1 mg, 17%) and compound **13** (27.0 mg, 40%). Compound **11**, mp 168–171 °C; $[\alpha]_D$ + 8.1 (c 0.096); ^{1}H NMR δ 2.79 and 2.95 (each 2H, t, J = 7.1 Hz, –O–CO–*CH*$_2$–*CH*$_2$–CO–O–), 4.50 (2H, s, H-7), 4.71 (1H, t, J = 2.4 Hz, H-21), 6.56 (1H, s, H-3), 7.89 (1H, s, H-6). ^{13}C NMR δ 28.8 and 29.3 (O=C–*CH*$_2$–*CH*$_2$–C=O), 60.7 (C-7), 79.1 (C-21), 113.2 (C-3), 141.0 (C-5), 147.9 (C-6), 167.8 (C-2), 169.7 and 171.5 (O=*C*–CH$_2$–CH$_2$–*C*=O), 172.8 (C-4). HRMS (FAB) calcd for $C_{41}H_{60}O_8Na$ [M+Na]$^+$ m/z 703.4186; found, 703.4184. Compound **13**, mp 130–133 °C; $[\alpha]_D$ +71.1 (c 0.092); ^{1}H NMR δ 2.68 (4H, td, J = 4.4, 1.4 Hz, O–CO–*CH*$_2$–*CH*$_2$–CO–O–), 2.72 (4H, td, J = 4.8, 1.4 Hz, –O–CO–*CH*$_2$–*CH*$_2$–CO–O–), 4.70 and 4.71 (each 1H, t, J = 2.8 Hz, H-21, H-21′), 4.96 (2H, s, H-7), 6.51 (1H, s, H-3), 7.85 (1H, s, H-6). ^{13}C NMR δ 29.3 and 29.4 (O=C–*CH*$_2$–*CH*$_2$–C=O), 61.5 (C-7), 78.9 and 79.1 (C-21 and C-21′), 111.3 (C-3), 138.1 (C-6), 145.8 (C-5), 162.6 (C-2), 171.4, 171.7 and 176.4 (O=*C*–CH$_2$–CH$_2$–*C*=O), 173.9 (C-4). HRMS (FAB) calcd for $C_{76}H_{114}O_{12}Na$ [M+Na]$^+$, 1241.8208; found, 1241.8215.

3.2.14. Compounds 12 and 14

Compounds **12** (30%) and **14** (19%) were synthesized from compound **2** succinic acid similarly to compounds **11** and **13**. Compound **12**, mp 138–140 °C; $[\alpha]_D$ + 4.3 (c 0.10); ^{1}H NMR δ 2.79 and 2.95 (each 2H, t, J = 6.8 Hz, O=C–C*H*$_2$–*CH*$_2$–C=O), 4.50 (2H, s, H-7), 4.71 (1H, t, J = 2.1 Hz, H-21), 6.57 (1H, s, H-3), 7.89 (1H, s, H-6). ^{13}C NMR δ 28.8 and 29.3 (O=C–*CH*$_2$–*CH*$_2$–C=O), 60.8 (C-7), 79.0 (C-21), 113.2 (C-3), 141.0 (C-5), 147.9 (C-6), 167.7 (C-2), 169.7 and 171.5 (O=*C*–CH$_2$–CH$_2$–*C*=O), 172.8 (C-4); HRMS (FAB) calcd for $C_{41}H_{60}O_8Na$ [M+Na]$^+$ m/z 703.4186; found, 703.4192. Compound **14**, mp 124–126 °C; $[\alpha]_D$ −41.2 (c 0.116); ^{1}H NMR δ 2.69 (4H, td, J = 4.1, 1.1 Hz, O=C–*CH*$_2$–*CH*$_2$–C=O), 2.73 (4H, td, J = 4.3, 1.6 Hz, O=C–C*H*$_2$–CH$_2$–C=O), 4.70 and 4.71 (each 1H, t, J = 1.9 Hz, H-21, H-21′), 4.95 (2H, s, H-7), 6.51 (1H, s, H-3), 7.85 (1H, s, H-6). ^{13}C NMR δ 29.2 and 29.4 (O=C–*CH*$_2$–*CH*$_2$–C=O), 61.5 (C-7), 78.9 and 79.1 (C-21 and C-21′), 111.3 (C-3), 138.1 (C-6), 145.9 (C-5), 162.6 (C-2), 171.4, 171.7 and 176.2 (O=*C*–CH$_2$–CH$_2$–*C*=O), 173.9 (C-4). HRMS (FAB) calcd for $C_{76}H_{114}O_{12}Na$ [M+Na]$^+$, 1241.8208; found, 1241.8203.

3.2.15. Compound 15

To a solution of compound **1** succinic acid (40.6 mg, 0.07 mmol) in DMF (2 mL) were added quercetin (23.3 mg, 0.08 mmol), WSC·HCl (19.7 mg, 0.09 mmol), and HOBt (10.7 mg, 0.07 mmol) and the mixture was stirred at 60 °C for 1 h under nitrogen atmosphere. To the reaction mixture was added water and the whole was extracted with EtOAc. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified with silica gel column chromatography (hexane/EtOAc = 4:1–1:1) to give compound **15** (40.5 mg, 86%), mp 187–190 °C; $[\alpha]_D$ + 52.3 (c 0.065); ^{1}H NMR δ 2.92 and 2.96 (each 2H, m, O=C–C*H*$_2$–*CH*$_2$–C=O), 4.80 (1H, t, J = 2.1 Hz, H-21), 6.19 (1H, d, J = 1.9 Hz, H-6), 6.27 (1H, d, J = 1.9 Hz, H-8), 7.12 (1H, d, J = 8.3 Hz, H-5′), 7.92 (1H, d, J = 2.1 Hz, H-2′), 7.94 (1H, dd, J = 8.3, 2.1 Hz, H-6′); ^{13}C NMR δ 29.7 and 30.2 (O=C–*CH*$_2$–*CH*$_2$–C=O), 80.8 (C-21), 94.1 (C-8), 98.9 (C-6), 103.4 (C-4a), 117.8 (C-5′), 122.4 (C-2′), 123.1 (C-1′), 127.0 (C-6′), 135.5 (C-3), 137.4 (C-3′), 144.2 (C-2), 150.1 (C-4′), 156.5 (C-8a), 160.9 (C-5), 162.7 (C-7), 171.7 and 174.0 (O=*C*–CH$_2$–CH$_2$–*C*=O), 174.8 (C-4); HRMS (FAB) calcd for $C_{50}H_{65}O_{11}$ [M+1]$^+$, 841.4527; found, 841.4529.

3.2.16. Compound 16

Compound **16** (50%) was synthesized from compound **2** succinic acid similarly to compound **15**. Mp 207–210 °C; $[\alpha]_D$ −22.2 (c 0.115); ^{1}H NMR δ 2.92 and 2.95 (each 2H, m, O=C–C*H*$_2$–*CH*$_2$–C=O), 4.80 (1H, t, J = 2.3 Hz, H-21), 6.20 (1H, br s, H-6), 6.29 (1H, br s, H-8), 7.13 (1H, d, J = 8.8 Hz, H-5′), 7.92 (1H, d, J = 1.8 Hz,

H-2′), 7.94 (1H, dd, J = 8.8, 1.8 Hz, H-6′); ^{13}C NMR δ 29.7 and 30.2 (O=C–CH_2–CH_2–C=O), 80.7 (C-21), 94.1 (C-8), 98.9 (C-6), 103.4 (C-4a), 117.7 (C-5′), 122.4 (C-2′), 123.1 (C-1′), 127.0 (C-6′), 135.6 (C-3), 137.5 (C-3′), 144.3 (C-2), 150.0 (C-4′), 156.5 (C-8a), 160.9 (C-5), 162.8 (C-7), 171.5 and 174.0 (O=*C*–CH_2–CH_2–*C*=O), 174.9 (C-4); HRMS (FAB) calcd for $C_{50}H_{64}O_{11}Na$ $[M+Na]^+$, 863.4346; found, 863.4350.

3.2.17. Compound 17

To a solution of compound **1** succinic acid (190.1 mg, 0.34 mmol) in DMF (3 mL) were added WSC·HCl (16.5 mg, 0.66 mmol), baicalein (119.0 mg, 0.44 mmol), and DMAP (catalyst), and the mixture was stirred at 60 °C for 4 h under nitrogen atmosphere. The reaction mixture was concentrated to remove solvent, to which was added water and extracted with EtOAc. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 5:1–3:1) to afford compound **17** (100 mg, 36%); mp 187–190 °C; $[\alpha]_D$ −139.2 (*c* 0.116); ^{1}H NMR δ 2.90 and 2.96 (each 2H, m, O=C–CH_2–CH_2–C=O), 4.79 (1H, t, J = 2.5 Hz, H-21), 6.64 (1H, s, H-8), 6.66 (1H, s, H-3), 7.50–7.57 (3H, m, Ar-H (H-3′, 4′and 5′)), 7.88 (2H, dd, J = 8.1, 1.5, H-2′and H-6′), 12.98 (1H, s, O*H*-5). ^{13}C NMR δ 29.5 and 30.4 (each t, O=C–CH_2–CH_2–C=O), 80.7 (C-21), 94.8 (C-8), 105.3 (C-3), 105.4 (C-4a), 121.9 (C-6), 126.3 (C-2′ and C-6′), 129.1 (C-3′ and C-5′), 131.2 (C-1′), 131.9 (C-4′), 153.0 (C-5), 155.0 (C-8a), 155.7 (C-7), 164.3 (C-2), 169.8 and 174.3 (O=*C*–CH_2–CH_2–*C*=O), 182.7 (C-4). HRMS calculated for $C_{50}H_{64}O_9Na$ (M^++Na) 831.4448; found, 831.4442.

3.2.18. Compound 18

Compound **18** (93.5 mg, 32%) was prepared from compound **2** succinic acid (200 mg, 0.36 mmol). The reaction method employed was the same as that of compound **17**. Compound **18**: mp 172–175 °C; $[\alpha]_D$ −62.5 (*c* 0.116); ^{1}H NMR δ 2.92 and 2.98 (each 2H, m, O=C–CH_2–CH_2–C=O), 4.81 (1H, t, J = 2.5 Hz, H-21), 6.65 (1H, s, H-8), 6.68 (1H, s, H-3), 7.52–7.57 (3H, m, Ar-*H* (H-3′, 4′and 5′)), 7.90 (2H, dd, J = 8.2, 1.5, H-2′and H-6′), 13.00 (1H, s, O*H*-5); ^{13}C NMR δ 29.4 and 30.3 (each t, O=C–CH_2–CH_2–C=O), 80.6 (C-21), 94.8 (C-8), 105.3 (C-3), 105.4 (C-4a), 121.9 (C-6), 126.3 (C-2′ and C-6′), 129.1 (C-3′ and C-5′), 131.2 (C-1′), 131.9 (C-4′), 153.0 (C-5), 155.0 (C-8a), 155.7 (C-7), 164.3 (C-2), 169.8 and 174.3 (O=*C*–CH_2–CH_2–C=O), 182.7 (C-4). HRMS calculated for $C_{50}H_{64}O_9Na$ (M^++Na) 831.4448; found, 831.4454.

3.3. Biology

3.3.1. Cells culture

C8166-CCR5 cells, a human $CD4^+$ T-lymphocyte cell line, were grown in RPMI 1640 medium containing 10% (v/v) FBS and antibiotics (50 V/mL of penicillin and 50 μg/mL streptomycin). HeLa cells were grown in DMEM containing 10% (v/v) FBS and antibiotics.

3.3.2. Preparation of the cell-free recombinant virus stocks

HeLa cells (3×10^5 cells) were grown in DMEM with 10% (v/v) FBS in a 25 cm^2 flask for one day and transfected with 3 μg of the HIV-1 NL43 plasmid DNA using FuGENE 6 Transfection Reagent (Roche Diagnostics). The culture medium was replaced with fresh medium after an overnight incubation, and was collected at 48 h after transfection.

3.3.3. Detection of HIV infection by reverse transcriptase (RT) assay

The RT activity of the culture supernatants was measured by the assay method that used a poly (A) template, an oligo (dT) primer, and a [^{32}P]TTP substrate as described previously.[40]

3.3.4. Effects of the compounds on cell viability and HIV infectivity

Assays were carried out in duplicate or triplicate with 96-well plates. C8166-CCR5 cells (3×10^4 cells) were mixed each compound at the appropriate concentrations in 100 μL of culture medium, and were incubated at 37 °C. After 72 h, cell viability was assessed by the cell-associated dehydrogenase activity assay using Cell Counting Kit-8 (DOJINDO). The cell culture containing compounds prepared as described above was infected with HIV-1 by adding NL43 inoculum normalized by RT activity to 1×10^5 cpm. Four days after infection, RT activity of the culture supernatants was measured as described above.

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