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Efficient synthesis and biological evaluation of epiceanothic acid and related compounds

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ABSTRACT

Epiceanothic acid (**1**) is a naturally occurring, but very rare pentacyclic triterpene with a unique pentacyclic triterpene (PT) structure. An efficient synthesis of **1** starting from betulin (**3**) has been accomplished in 12-steps with a total yield of 10% in our study. Compound **1** and selected synthetic intermediates were further evaluated as anti-HIV-1 agents, inhibitors of glycogen phosphorylase (GP), and cytotoxic agents. Compound **1** exhibited moderate HIV-1 inhibition. Most importantly, compound **5**, with an opened A-ring, showed significant GP inhibitory activity with an IC_{50} of 0.21 μM, suggesting a potential for development as an anti-diabetic agent. On the other hand, compound **12**, with a closed A-ring, showed potent cytotoxicity against A549 and MCF-7 human tumor cell lines, with IC_{50} values of 0.89 and 0.33 μM, respectively. These results suggest that the A-ring of PTs is an important pharmacophore that could be modified to involve different biological activities.

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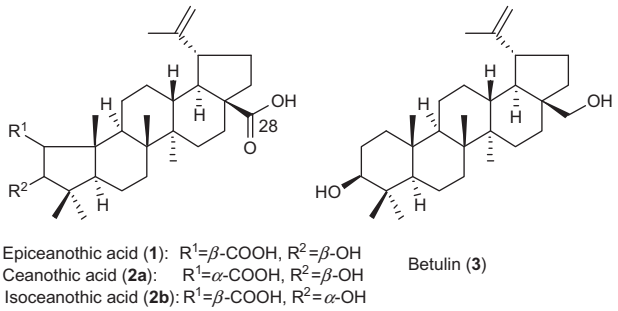
Pentacylic triterpenes (PTs), a group of widespread natural compounds, possess several intriguing biological activities, such as anti-HIV, antitumor, anti-diabetic, anti-inflammatory, antibacterial, antiviral, antiparasitic, hepatoprotective, wound healing, antioxidant, antipruritic, antiangiogenic, antiallergic, and immunomodulatory activities.[1–5] In recent years, PTs have been the focus of much interest due to their significant therapeutic potentials. The anti-HIV and antitumor activities of PTs have received the most attention, as several synthetic PT derivatives have advanced into clinical trials [e.g., PA-457 (DSB, Bevirimat, MPC-4326)[6,7] and PA-1050040 for AIDS therapy, and betulinic acid, CDDO, and CDDO-Me for cancer therapy]. Our previous investigation also showed that PTs represent a new class of glycogen phosphorylase (GP) inhibitors, which may be a key contributing mode of action in their anti-diabetic activity.[8–10]

Epiceanothic acid (EA, **1**) (Fig. 1) is a naturally occurring ceanothane-type PT isolated from the seeds of the traditional Chinese medicine *Ziziphus jujuba* var. *spinosa* (Bunge) Hu and the stings of *Gleditsia sinensis* Lam.[11–13] It is reported to possess strong anti-HIV-1 replication activity in HIV-1_{IIIB} infected C8166 cell lines (EC_{50} <0.064 μg/mL).[12,13] Compound **1** has two natural configurational isomers, ceanothic acid (**2a**)[14–18] and isoceanothic acid (**2b**).[19] Their structures differ from that of **1** only in the orientations of the 2-carboxylic acid (**2a**) and 3-hydroxy group (**2b**) in the A-ring. Compound **2a** was reported to possess anti-microbial and cytotoxic activity,[20–22] and its derivatives were found to be potent cancer chemopreventive agents.[23]



Epiceanothic acid (**1**): R^1=β-COOH, R^2=β-OH
Ceanothic acid (**2a**): R^1=α-COOH, R^2=β-OH
Isoceanothic acid (**2b**): R^1=β-COOH, R^2=α-OH

Betulin (**3**)

Figure 1. Structures of epiceanothic acid (**1**) and related PT compounds (**2a**, **2b**, **3**).

Despite its obvious potential, only limited research has been reported on **1**, because it is very rare in nature. Therefore, it is highly desirable to establish a reliable access to **1**-analogs for biological evaluation. Herein, we report an efficient synthetic route to **1** in 12-steps with a total yield of 10% starting from betulin (**3**), which is easily available at a low price. Compound **1** and the pentacyclic triterpene intermediates[24] were then evaluated for anti-HIV-1, GP inhibitory, and cytotoxic activities.

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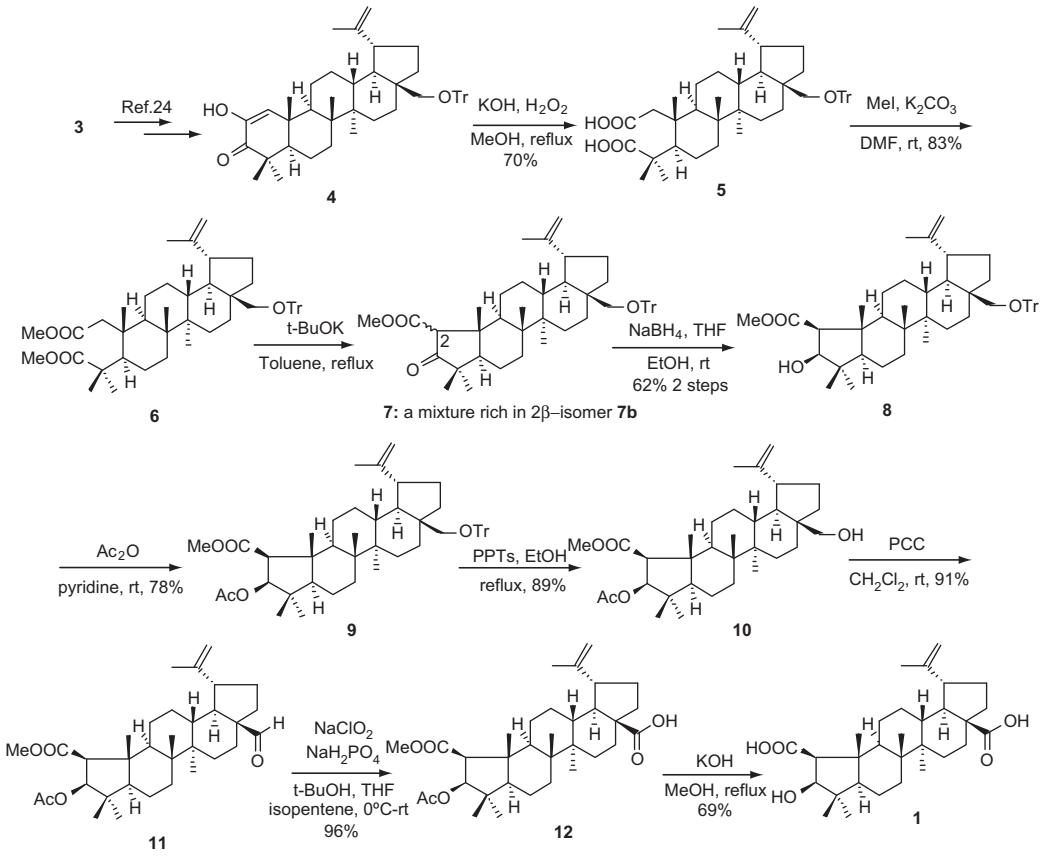

Scheme 1. Synthesis of epiceanothic acid (**1**) from betulin (**3**).

As shown in Scheme 1, crude dione compound **4**, which was readily prepared from **3** in 67% yield,[25] was treated with KOH and H_2O_2 in MeOH[26] to give the dicarboxylate compound **5** (70%). Methylation of **5** with iodomethane afforded compound **6** (83%). Dieckmann condensation of **6** (*t*-BuOK/toluene)[27] gave crude five-membered keto ester **7** as a mixture of 2α-ester (**7a**) and 2β-ester (**7b**). This mixture could not be separated by column chromatography, as isomerization could be observed during the purification process. The major component was likely **7b**, based on a comparison with our related work on oleanane-type PTs (unpublished data) and identification of the reduction product **8**. Reduction of the crude **7** in the presence of $NaBH_4$ in THF and EtOH gave 2β-methoxycarbonyl-3β-hydroxy compound **8** as the only product (62% over two-steps). Acetylation of the 3β-hydroxy group was accomplished with acetic anhydride in pyridine in 78% yield. Deprotection of **9** with pyridinium *p*-toluenesulfonate (PPTs) in EtOH gave primary alcohol **10** (89%). Oxidation of **10** with pyridinium chlorochromate (PCC) gave aldehyde **11** in good yield (91%), which was further oxidized with $NaClO_2$ and NaH_2PO_4 in a mixture of *t*-BuOH/THF/2-methyl-2-butene[25] to afford carboxylic acid **12** in high yield (96%). Hydrolysis of **12** afforded epiceanothic acid (**1**) in 69% yield.

Compound **1**, betulin (**3**), four selected pentacylic triterpene intermediates (**9**–**12**) and PA-457 (as a positive control) were tested in acutely HIV-1_{NL4-3} infected MT-4 cells, according to the literature methods.[28–31] However, **3** and **9**–**12** did not exhibit significant antiviral activity in our assay. In addition, despite the previous report, compound **1** demonstrated only moderate anti-HIV activity with an EC_{50} value of 15.6 μM and a therapeutic index (TI) of 2.49 (Table 1). Esterification or acylation of the free carboxylic acid and hydroxy functionalities in A-ring may be a contributing factor to the loss of potency, as seen with **1** versus **9**, **10**, **11**, and **12**. Interestingly, **2a**, which has a 2α-COOH rather than the 2β-COOH in **1**, showed no anti-HIV-1 activity (unpublished data), suggesting that the configuration of the 2-carboxylic acid group has an impact on the anti-HIV-1 activity. Compared with the reported data,[12,13] our results also suggested that different HIV viral strains may have different sensitivity to **1**. The molecular mechanisms underlying this phenomenon remain to be elucidated.

Table 1
Anti-HIV-1 replication activity of PT compounds in HIV-1_{NL4-3} infected MT-4 cell lines

Compound	EC_{50}[a] (μM)	IC_{50}[a] (μM)	TI
PA-457	0.082	15.2	185.4
1	15.6	38.9	2.49

[a] Results are averaged from two experiments. Compounds **3** and **9**–**12** were also tested, but were not active.

Type 2 diabetes mellitus is a severe disease with great economic consequences. Hepatic glucose output is elevated in type 2 diabetic patients, and GP is the enzyme that catalyzes glycogenolysis (release of monomeric glucose from the glycogen polymer storage form, resulting in abnormally high glucose production). GP inhibitors lower glucose level acutely and chronically in diabetic animal models, representing promising new hypoglycemic agents for the treatment of type 2 diabetes mellitus.

In our continuing efforts to find potent GP inhibitors from PT compounds, all of the related compounds (**1**, **3**, **5–6**, and **8–12**) in our study were evaluated for inhibitory activity against rabbit muscle glycogen phosphorylase a (RMGPa).[32] As described previously, the activity of RMGPa was measured by detecting the amount of phosphates released from glucose-1-phosphates in the

Table 2
Inhibition of RMGPa by synthesized PT compounds

Compound	IC_{50}[a] (μM) ± SD	Compound	IC_{50}[a] (μM) ± SD
3	41.5 ± 3.2	10	NI[b]
5	0.21 ± 0.1	11	53.8 ± 4.9
6	2.87 ± 0.1	12	46.4 ± 3.6
8	20.1 ± 1.1	1	194.1 ± 17.5
9	15.2 ± 0.7	Caffeine[c]	75.3 ± 6.6

[a] Values are means of three experiments.
[b] NI = no inhibition.
[c] Caffeine was used as a positive control.

direction of glycogen synthesis.[33] The assay results are summarized in Table 2. Most of the newly synthesized PTs exhibited inhibitory activity against RMGPa.

As shown in Table 2, opening of the A-ring of lupine-type PTs significantly improved the GP inhibitory potency. The A-ring opened compound **6** showed potent GP inhibitory activity with an IC_{50} value of 2.87 μM, and was 14-fold more potent than betulin (**3,** IC_{50} 41.5 μM). Another A-ring opened compound **5** showed the most potent GP inhibitory activity with an IC_{50} value of 0.21 μM. Compound **5** was 14-fold more potent than its methylated parent compound **6** and almost 200-fold more potent than **3**. The trityl ether compounds **8** and **9** were slightly more potent than **3**, and compounds **11** and **12** showed comparable potency to **3**. However, **1** exhibited only weak GP inhibitory activity with an IC_{50} value of 194.1 μM. Overall, the A-ring was proven to be a very important pharmacophore for modifying PTs' GP inhibition activity, and the preliminary SAR analysis showed that opening of the A-ring of lupine-type PTs may enhance GP inhibition. The most potent GP inhibitor, **5** (IC_{50} 0.21 μM), merits further development as a potential anti-diabetic agent.

The cytotoxic activity of **1** and its synthetic intermediates (**5**, **6**, **8–12**) was tested in vitro using the MTT cytotoxicity assay,[34,35] and the results are summarized in Table 3. Five different cancer cell lines were used including PC3 (human prostate cancer), A549 (human lung carcinoma), MCF-7 (human breast cancer), HeLa (human epithelial carcinoma), and BGC-823 (human gastric carcinoma). Adriamycin was used as the reference standard.

Overall, different cancer cell lines showed different sensitivity to the PT compounds. The trityl ether compounds **8** and **9** were inactive against the PC3 cell line, while aldehyde **11** (IC_{50} = 8.51 μM) and carboxylic acid **12** (IC_{50} = 10.8 μM) were more cytotoxic than the corresponding primary alcohol **10** (IC_{50} = 66.3 μM). With a free carboxylic acid and hydroxy group in the A-ring, **1** (IC_{50} = 19.7 μM) showed slightly decreased activity compared with **12**. The A-ring opened compounds **5** and **6** exhibited more potent cytotoxicity against PC3 with IC_{50} values of 6.75 and 5.32 μM, respectively.

Noticeably, natural product **1** showed the greatest cytotoxicity against gastric carcinoma BGC-823 with an IC_{50} value of 2.41 μM. The potency against this cell line was at least 10-fold higher than against all the remaining four tested cancer cell lines, thus, demonstrating selective sensitivity. Compound **12**, with ester-protected carboxylic acid and hydroxy groups in the A-ring relative to **1**, showed three-fold decreased activity with an IC_{50} of 7.64 μM. All of the remaining PTs showed moderate to little cytotoxic activity against BGC-823.

Generally, the A549, MCF-7, and Hela cancer cell lines were insensitive to the tested PT analogs. However, compound **12** showed selective potent cytotoxicity against A549 and MCF-7 with IC_{50} values of 0.89 and 0.33 μM, respectively. Interestingly, the free carboxylic acid and hydroxy groups in the A-ring of **1** decreased its cytotoxic activity against A549 and MCF-7 by more than 20-fold compared with **12**, which is opposite to the activity profile against BGC-823. The activity of **12** against MCF-7 was slightly better than that of adriamycin, suggesting that it merits further SAR and mechanism of action study.

In summary, an efficient access to epiceanothic acid (**1**) starting from betulin (**3**) has been developed in 12-steps with an overall yield of 10%. Because **3** is readily available, this preparation gives practical access to the very rare natural PT epiceanothic acid and enables further pharmacological research and drug development.

The synthesized PT derivatives were evaluated biologically as anti-HIV-1 agents, inhibitors of glycogen phosphorylase, and cytotoxic agents. The results showed that **1** has moderate potency against HIV-1_{NL4-3} virus strains. In addition, compound **5** with two free carboxylic acids in an opened A-ring showed potent GP inhibitory activity with an IC_{50} value of 0.21 μM. To our knowledge, it is the most potent PT derived GP inhibitor thus far. On the other hand, compound **12**, with a closed A-ring and protected carboxylic acid and hydroxy groups, exhibited potent cytotoxic activity against A549 and MCF-7 cancer cell lines with IC_{50} values of 0.89 and 0.33 μM, respectively, which were comparable or better than those of adriamycin. These results suggest that the A-ring is an important pharmacophore for both GP inhibitory and cytotoxic activity of PTs. Different modifications on the A-ring could change the bioactivity of epiceanothic acid derivatives. Further mechanistic and pharmacologic studies of these compounds are currently ongoing.

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Table 3
Cytotoxic activity of synthesized PT compounds

Compound	IC_{50}[a] (μM) ± SD for cancer cell lines				
	PC3	A549	MCF-7	HeLa	BGC-823
5	6.75 ± 0.57	136.1 ± 10.1	559.6 ± 45.5	N/A[b]	N/A[b]
6	5.32 ± 0.43	996.5 ± 80.7	373.6 ± 23.7	17.1 ± 0.9	9.54 ± 0.78
8	NA[b]	770.1 ± 65.6	397.5 ± 15.8	NA[b]	19.3 ± 1.66
9	NA[b]	892.5 ± 46.8	565.3 ± 22.4	NA[b]	NA[b]
10	66.3 ± 5.56	62.9 ± 5.69	603.5 ± 44.5	63.1 ± 2.8	25.9 ± 1.49
11	8.51 ± 0.73	194.2 ± 10.6	32.5 ± 2.79	105.4 ± 11.2	12.7 ± 0.85
12	10.8 ± 0.08	0.89 ± 0.07	0.33 ± 0.04	56.7 ± 4.9	7.64 ± 0.19
1	19.7 ± 1.55	16.8 ± 2.13	87.2 ± 8.46	19.1 ± 2.1	2.41 ± 0.22
Adriamycin[c]	0.68 ± 0.07	0.54 ± 0.04	1.65 ± 0.11	1.35 ± 0.08	1.33 ± 0.09

[a] Values are means of three experiments.
[b] NA = no activity.
[c] Adriamycin was used as a positive control.

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24. All newly synthesized compounds provided satisfactory MS, ^{1}H NMR, and ^{13}C NMR spectra without any discernable impurities. Selected analytical and spectroscopic data are shown as follows. *Compound* **5**: $[\alpha]^D$ +25.0 (*c* 0.18, CH_2Cl_2); IR (film, cm^{-1}): 2950, 2869, 1714, 1693, 1560, 1489, 1449, 1265, 1228, 1063, 883, 743, 706, 632; ^{1}H NMR ($CDCl_3$) δ 0.51 (3H, s), 0.86 (3H, s), 0.89 (3H, s), 1.16 (3H, s), 1.24 (3H, s), 1.64 (3H, s), 0.96–2.63 (23H, m), 2.92 (1H, d, *J* = 8.9 Hz), 3.13 (1H, d, *J* = 8.8 Hz), 4.52 (1H, s), 4.57 (1H, s), 7.21–7.33 (9H, m), 7.48–7.51 (6H, m); ^{13}C NMR ($CDCl_3$) δ 14.8, 15.9, 19.1, 19.3, 20.7, 21.2, 25.0, 27.0, 29.4, 29.9, 30.1, 33.7, 35.2, 37.1, 40.5, 40.9, 41.8, 42.9, 45.7, 47.6, 47.8, 48.4, 48.9, 59.5, 85.9, 109.5, 126.8, 127.7, 128.8, 144.5, 150.8, 178.3, 187.3; ESI-MS *m*/*z*: 753.5 [M+Na]$^+$; HRMS for $C_{49}H_{62}O_5$+Na calcd 753.44895, found 753.4505. *Compound* **6**: $[\alpha]^D$ −5.0 (*c* 0.14, CH_2Cl_2); IR (film, cm^{-1}): 2947, 2868, 1726, 1597, 1449, 1275, 1151, 1064, 764, 706, 632; ^{1}H NMR ($CDCl_3$) δ 0.52 (3H, s), 0.83 (3H, s), 0.92 (3H, s), 1.21 (3H, s), 1.22 (3H, s), 1.62 (3H, s), 0.76–1.74 (16H, m), 2.11–2.40 (7H, m), 2.90 (1H, d, *J* = 8.8 Hz), 3.12 (1H, d, *J* = 8.9 Hz), 3.59 (3H, s), 3.61 (3H, s), 4.50 (1H, s), 4.56 (1H, s), 7.19–7.32 (9H, m), 7.46–7.49 (6H, m); ^{13}C NMR ($CDCl_3$) δ 14.7, 15.9, 19.1, 19.6, 20.8, 21.8, 23.8, 25.3, 27.0, 27.8, 29.9, 30.0, 33.1, 35.2, 37.5, 40.5, 41.6, 41.8, 41.9, 42.9, 46.3, 47.6, 47.7, 48.4, 48.8, 50.7, 51.7, 59.6, 85.9, 109.3, 126.8, 127.7, 128.8, 144.5, 150.8, 171.9, 179.9; ESI-MS *m*/*z*: 781.5 [M+Na]$^+$; HRMS for $C_{51}H_{66}O_5$+Na calcd 781.48025, found 781.48348. *Compound* **8**: $[\alpha]^D$ −12.3 (*c* 0.105, CH_2Cl_2); IR (film, cm^{-1}): 3456, 2945, 2866, 1733, 1632, 1597, 1449, 1375, 1160, 1063, 1030, 775, 741, 706, 632; ^{1}H NMR ($CDCl_3$) δ 0.48 (3H, s), 0.88 (3H, s), 0.91 (3H, s), 1.00 (3H, s), 1.05 (3H, s), 1.63 (3H, s), 0.79–1.68 (18H, m), 2.17–2.32 (4H, m), 2.69 (1H, br s), 2.91 (1H, d, *J* = 8.9 Hz), 3.11 (1H, d, *J* = 8.8 Hz), 3.65 (3H, s), 4.01 (1H, d, *J* = 7.3 Hz), 4.51 (1H, s), 4.57 (1H, s), 7.19–7.32 (9H, m), 7.46–7.49 (6H, m); ^{13}C NMR ($CDCl_3$) δ 13.9, 14.7, 16.5, 18.0, 18.8, 19.2, 23.4, 24.8, 27.1, 30.0, 30.2, 31.7, 34.1, 35.2, 37.0, 41.6, 42.7, 43.0, 47.6, 47.7, 48.9, 49.3, 50.2, 51.4, 59.5, 60.1, 62.0, 82.4, 85.8, 109.4, 126.8, 127.7, 128.8, 144.5, 150.7, 174.2; HRMS for $C_{50}H_{64}O_4$+Na calcd 751.46968, found 751.47246. *Compound* **9**: $[\alpha]^D$ −16.7 (*c* 0.06, CH_2Cl_2); IR (film, cm^{-1}): 3382, 2946, 2866, 1747, 1449, 1375, 1240, 1153, 1063, 764, 705, 632; ^{1}H NMR ($CDCl_3$) δ 0.50 (3H, s), 0.80 (3H, s), 0.88 (3H, s), 1.08 (3H, s), 1.11 (3H, s), 1.62 (3H, s), 1.99 (3H, s), 0.76–1.67 (18H, m), 2.16–2.23 (3H, m), 2.40 (1H, d, *J* = 7.7 Hz), 2.90 (1H, d, *J* = 8.8 Hz), 3.12 (1H, d, *J* = 8.8 Hz), 3.54 (3H, s), 4.50 (1H, s), 4.56 (1H, s), 5.14 (1H, d, *J* = 7.5 Hz), 7.19–7.32 (9H, m), 7.46–7.49 (6H, m); ^{13}C NMR ($CDCl_3$) δ 13.3, 14.7, 16.2, 17.8, 19.0, 19.1, 20.8, 23.7, 24.7, 27.1, 29.9, 30.2, 31.1, 34.0, 35.2, 37.0, 41.4, 42.3, 42.6, 47.5, 47.7, 47.8, 48.9, 50.2, 51.1, 59.5, 59.8, 62.1, 83.6, 85.8, 109.4, 126.8, 127.7, 128.8, 144.5, 150.7, 170.5,171.6; ESI-MS *m*/*z*: 793.7 [M+Na]$^+$; HRMS for $C_{52}H_{66}O_5$+Na calcd 793.48025, found 793.47867. *Compound* **10**: $[\alpha]^D$ −0.67 (*c* 0.06, CH_2Cl_2); IR (film, cm^{-1}): 3355, 2945, 2866, 1749, 1458, 1376, 1242, 1159, 1026, 873, 739; ^{1}H NMR ($CDCl_3$) δ 0.76 (3H, s), 0.97 (3H, s), 1.02 (3H, s), 1.11 (3H, s), 1.18 (3H, s), 1.67 (3H, s), 2.00 (3H, s), 0.76–1.67 (18H, m), 2.32–2.41 (1H, m), 2.44 (1H, d, *J* = 7.7 Hz), 3.33 (1H, d, *J* = 10.8 Hz), 3.57 (3H, s), 3.79 (1H, d, *J* = 10.7 Hz), 4.58 (1H, d, *J* = 1.4 Hz), 4.67 (1H, d, *J* = 1.8 Hz), 5.17 (1H, d, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 14.8, 16.4, 17.8, 19.0, 19.1, 20.8, 23.8, 24.8, 27.3, 29.3, 29.8, 31.1, 34.0, 34.1, 37.1, 41.7, 42.4, 42.9, 47.76, 47.81, 47.85, 48.8, 50.3, 51.2, 59.9, 60.6, 62.2, 83.6, 109.7, 150.4, 170.5, 171.7; HRMS for $C_{33}H_{52}O_5$+Na calcd 551.37070, found 551.37012. *Compound* **11**: $[\alpha]^D$ −4.67 (*c* 0.03, CH_2Cl_2); IR (film, cm^{-1}): 2946, 2867, 1747, 1452, 1377, 1360, 1241, 1061, 1042, 738; ^{1}H NMR ($CDCl_3$) δ 0.81 (3H, s), 0.91 (3H, s), 0.97 (3H, s), 1.10 (3H, s), 1.18 (3H, s), 1.69 (3H, s), 2.00 (3H, s), 0.85–2.11 (20H, m), 2.44 (1H, d, *J* = 7.5 Hz), 2.79–2.89 (1H, m), 3.58 (3H, s), 4.63 (1H, s), 4.75 (1H, s), 5.17 (1H, d, *J* = 7.5 Hz) 9.68 (1H, s); ^{13}C NMR ($CDCl_3$) δ 13.5, 14.3, 16.3, 17.8, 19.00, 19.04, 20.9, 23.7, 25.1, 29.0, 29.3, 30.0, 31.1, 33.4, 34.2, 38.5, 41.7, 42.4, 42.8, 47.6, 47.9, 48.2, 50.4, 51.2, 59.3, 60.0, 62.2, 83.6, 110.3, 149.6, 170.5, 171.6, 206.6; ESI-MS *m*/*z*: 549.0 [M+Na]$^+$; HRMS for $C_{33}H_{50}O_5$+Na calcd 549.35505, found 549.35424. *Compound* **12**: $[\alpha]^D$ −10.67 (*c* 0.09, CH_3OH); IR (film, cm^{-1}): 3346, 2948, 2868, 1748, 1701, 1457, 1377, 1241, 1194, 1158, 1029, 736, 638; ^{1}H NMR (C_5H_5N) δ 0.87 (3H, s), 1.03 (3H, s), 1.06 (3H, s), 1.15 (3H, s), 1.37 (3H, s), 1.76 (3H, s), 2.03 (3H, s), 1.19–1.95 (16H, m), 2.23–2.28 (2H, m), 2.60–2.76 (2H, m), 2.80 (1H, d, *J* = 7.7 Hz), 3.46–3.53 (1H, m), 3.58 (3H, s), 4.73 (1H, s), 4.91 (1H, s), 5.47 (1H, d, *J* = 7.7 Hz); ^{13}C NMR (C_5H_5N) δ 13.8, 14.6, 16.5, 17.8, 19.0, 19.2, 20.4, 24.2, 25.4, 30.2, 30.7, 31.0, 32.7, 34.3, 37.3, 38.1, 41.6, 42.3, 42.8, 47.6, 47.9, 49.5, 50.4, 50.8, 56.3, 59.9, 62.0, 83.8, 109.7, 150.9, 170.1, 171.5, 178.5; ESI-MS *m*/*z*: 565.0 [M+Na]$^+$; HRMS for $C_{33}H_{50}O_6$−H calcd 541.35346, found 541.35249. *Compound* **1** *(Epiceanothic acid)*: $[\alpha]^D$ −14.5 (*c* 0.076, CH_3OH) [lit.[4], $[\alpha]^D$ −16.3 (*c* 0.08, CH_3OH)]; IR (film, cm^{-1}): 3476, 2951, 2868, 1696, 1643, 1459, 1377, 1320, 1237, 1187, 1058, 884; ^{1}H NMR (C_5H_5N) δ 1.08 (3H, s), 1.13 (3H, s), 1.15 (3H, s), 1.20 (3H, s), 1.67 (3H, s), 1.74 (3H, s), 1.13–2.22 (18H, m), 2.60–2.64 (1H, m), 2.73–2.80 (1H, m), 2.89 (1H, d, *J* = 7.2 Hz), 3.43–3.49 (1H, m), 4.66 (1H, d, *J* = 7.4 Hz), 4.69 (1H, s), 4.86 (1H, s); ^{13}C NMR (C_5H_5N) δ14.6, 15.0, 16.9, 18.4, 19.5, 19.9, 24.6, 25.9, 30.5, 31.2, 32.1, 33.0, 34.8, 37.6, 38.5, 42.0, 42.9, 43.1, 47.8, 48.1, 49.8, 51.1, 56.5, 62.7, 63.1, 83.1, 110.0, 151.1, 175.7, 178.8; ESI-MS *m*/*z*: 485.0 [M−H]$^-$; HRMS for $C_{30}H_{46}O_5$−H calcd 485.32725, found 485.32683.
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28. *HIV-1$_{NL4-3}$ replication inhibition assay in MT-4 lymphocytes*: A previously described HIV-1 infectivity assay was used.[29–31] A 96-well microtiter plate was used to set up the HIV-1$_{NL4-3}$ replication screening assay. NL_{4-3} variants at a multiplicity of infection (MOI) of 0.01 were used to infect MT-4 cells. Culture supernatants were collected on day four post-infection for the p24 antigen capture using an ELISA kit from ZeptoMetrix Corporation (Buffalo, NY).
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32. *Enzyme assay*: The inhibitory activity of the test compounds against rabbit muscle glycogen phosphorylase a (GPa) was monitored using microplate reader (BIO-RAD) based on the published method.[1] In brief, GPa activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. Each test compound was dissolved in DMSO and diluted at different concentrations for IC_{50} determination. The enzyme was added into 100 L of buffer containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphate, 1 mg/mL glycogen and the test compound in 96-well microplates (Costar). After the addition of 150 L of 1 M HCl containing 10 mg/mL ammonium molybdate and 0.38 mg/mL malachite green, reactions were run at 22 °C for 25 min, and then the phosphate absorbance was measured at 655 nm. The IC_{50} values were estimated by fitting the inhibition data to a dose-dependent curve using a logistic derivative equation.
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34. *MTT assay*: The MTT assay was carried out as described previously.[31] Cells were seeded in 96-well plates and incubated in the CO_2 incubator at 37 °C. When the cells adhered, compounds at different concentrations (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 or 0.1 mmol L^{-1}) were added to every well. After incubation for another 48 h, 20 μL MTT (5%) was added to each well and incubated for an additional 4 h. The viable cells were stained with MTT and scanned with an electrophotometer at 570 nm. Each concentration treatment was done in triplicate wells. The IC_{50} values were estimated by fitting the inhibition data to a dose-dependent curve using a logistic derivative equation.
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