Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3229–3236

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis of Betulin Derivatives and Their Protective Effects against the Cytotoxicity of Cadmium

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Received 16 May 2002; accepted 21 May 2002

Abstract—The protecting effect of betulin against cadmium toxicity was investigated using 11 kinds of analogues. It was elucidated by analyzing the analogue activities that both hydroxyl groups on C-3 and C-28 and the isopropenyl group on C-19 played important roles for expressing efficient activities. In addition, the cytotoxicity of betulin was also reduced by being functionalized using the above functional group.

Introduction

In 1999, Naganuma and his colleagues investigated the effects of triterpenes on cadmium toxicity in HepG2 cells and found that three natural products, namely, betulin (**1**), uvaol (**2**), and soyasapogenol B (**3**), have a reducing effect against the toxicity of cadmium chloride among 10 tested triterpenoids (Fig. 1).[1] In particular, betulin (**1**) had the strongest effects, for example in the medium containing 120 μM cadmium chloride, all the untreated cells died, while almost 100% of the cells pretreated with betulin (**1**) survived. In spite of some reports which concerned the reduction of the cadmium toxicity[2–5] and many other effects[6–9] on liver by plant triterpenes, there have been no studies on the detailed mechanisms of these effects. Although the mechanism of the toxicity reducing effect by betulin (**1**) has also not yet been established, the following remarkable features have been suggested by Naganuma et al.: (i) certain protein(s) are induced in cells by treatment with betulin (**1**), (ii) metallothionein, which is known as the representative protein to reduce heavy metal toxicities, was not induced by employed concentrations of betulin (**1**), and (iii) betulin (**1**) promoted the expression of certain genes.[1] To clarify the actual reduction mechanism of cadmium toxicity and to develop the 'probe' for finding the cellular factor(s) which interact with or induce by betulin (**1**) (e.g., betulin-binding protein or betulin-receptor, etc.), we started analyzing the relationship between the expression of the activities and the structure,

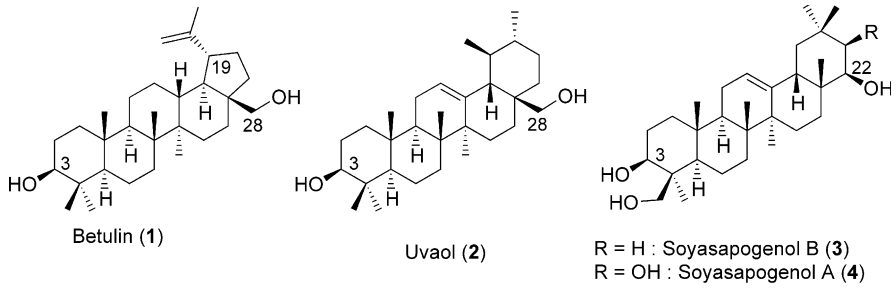

Figure 1.

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0968-0896/02/$ - see front matter
PII: S0968-0896(02)00172-4

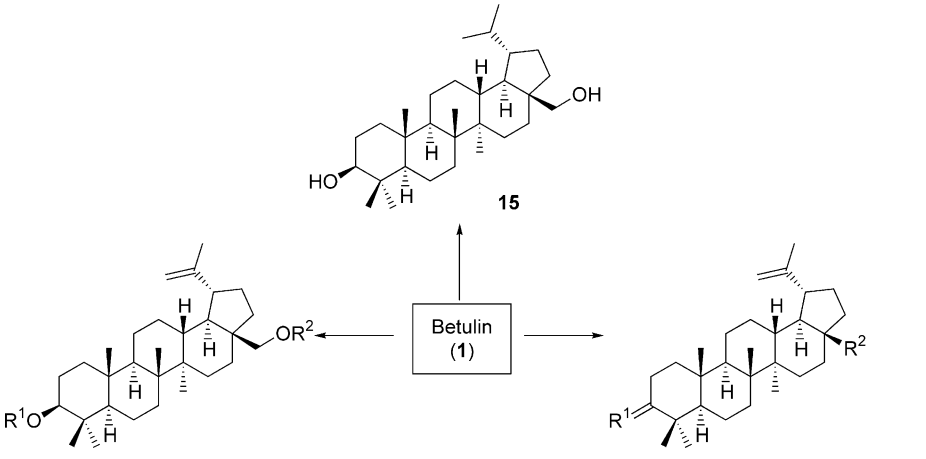
particularly, the functional groups of betulin (**1**) using its analogues.

Chemistry

Before starting the preparation of the betulin analogues, we analyzed the three-dimensional structures of the three effective compounds (**1**–**3**) and one non effective compound (**4**).[7] As the structure of the A-ring moiety of theses compounds closely resembles each other, a comparison of the structure was executed after superposing the A-ring moiety on each compound.[10] By analyzing according to the method described above, it was elucidated that the position of the hydroxyl group on C-28 of both betulin (**1**) and uvaol (**2**) and C-22 of soyasapogenol B (**3**) are very close to each other in a three-dimensional manner. Furthermore, it was also suggested from computational analysis that there will be hydrogen bonding between the two hydroxyl groups on C-21 and C-22 of soyasapogenol A (**4**). The structural difference between soyasapogenol A (**4**, not effective) and B (**3**, effective) is only the presence (soyasapogenol A) or absence (soyasapogenol B) of the hydroxyl group on C-21. Thus, these results suggested that the hydroxyl group on C-28 for **1** and **2** and C-22 for **3** would be crucial for expressing the activity.

Based on these facts, we decided to prepare the analogues and compare the activity basically focused on the following points, (i) C-3 functionalized compound, (ii) C-28 functionalized compound, and (iii) both the C-3 and C-28 substituted compound.

Eleven kinds of betulin analogues (**5**–**15**) were prepared as shown in Scheme 1. As the reactivity of the hydroxyl group on C-28 is much higher than that of C-3, the C-28 monoesters (**5** and **8**) could be directly prepared by using mild esterification conditions (**5**: AcCl, Et_3N, CH_2Cl_2, 69%; **8**: caprylic acid, TMSCl,[11] 84%). The C-3 monoesters (**6** and **9**) were prepared as follows: (i) converted to mono C-28 TBS ether (**16**) (TBSCl, imidazole, DMAP, DMF, 86%), (ii) esterification (**17**: Ac_2O, pyridine, DMAP, 94%; **18**: caprylic acid, DCC, DMAP, CH_2Cl_2, 88%), and (iii) cleavage of TBS ether (**6**: TBAF, THF, 98%; **9**: HF–MeCN, THF, 75%). The diacetate (**7**) was obtained by standard acetylation reaction (Ac_2O, DMAP, pyridine, 94%). 28-Oxobetulin (**10**) and 3,28-dioxobetulin (**12**) were synthesized at the same time by oxidation of betulin (**1**) using a controlled amount of PCC and then separated by column chromatography. 3-Oxobetulin (**11**) was obtained from 28-TBSO-betulin (**16**) by PCC oxidation, followed by deprotection reaction. The C-28 deoxygenated compound (**13**) was synthesized from the C-3 monoacetate



5: R^1 = H, R^2 = Ac
6: R^1 = Ac, R^2 = H
7: R^1 = R^2 = Ac
8: R^1 = H, R^2 = $OC(CH_2)_6CH_3$
9: R^1 = $OC(CH_2)_6CH_3$, R^2 = H
16: R^1 = H, R^2 = TBS
17: R^1 = Ac, R^2 = TBS
18: R^1 = $OC(CH_2)_6CH_3$, R^2 = TBS

10: R^1 = α-H and β-OH, R^2 = CHO
11: R^1 = O, R^2 = CH_2OH
12: R^1 = O, R^2 = CHO
13: R^1 = α-H and β-OH, R^2 = CH_3
14: R^1 = H_2, R^2 = CH_2OH
19: R^1 = O, R^2 = CH_2OTBS
20: R^1 = α-H and β-OAc, R^2 = CHO
21: R^1 = α-H and β-OAc, R^2 = CH_3
22: R^1 = H_2, R^2 = CH_2OTBS

Scheme 1. Reagents and conditions: **1**→**5**: Ac_2O, Et_3N, CH_2Cl_2, 0 °C–rt, 22 h, 69%; **1**→**8**: caprylic acid, TMSCl, rt, 19 h, 84%; **1**→**16**→**17**→**6**: (i) TBSCl, imidazole, DMAP, DMF, 0 °C–rt, 7 h, 86%; (ii) Ac_2O, pyridine, DMAP, 0 °C–rt, 16 h, 94%; (iii) TBAF, THF, reflux, 3 h, 98%; **16**→**18**→**9**: (i) caprylic acid, DCC, DMAP, CH_2Cl_2, rt, 22 h, 88%; (ii) HF–MeCN (1:5), THF, rt, 13 h, 75%; **1**→**7**: Ac_2O, pyridine, DMAP, 0 °C–rt, 5 h, 97%; **1**→**10** and **12**: PCC, CH_2Cl_2, rt, 1.5 h, 17% (**10**), 82% (**12**); **16**→**19**→**11**: (i) PCC, CH_2Cl_2, rt, 1 h, 98%; (ii) TBAF, THF, 70 °C, 3 h, 79%; **6**→**20**→**21**→**13**: (i) PCC, CH_2Cl_2, rt, 0.5 h, 72%; (ii) $TsNHNH_2$, Na_2SO_4, EtOH, 100 °C, 3.5 h; (iii) $NaBH_3CN$, DMF–sulfolane (1:1), *p*-TsOH·H_2O, 100 °C, 1 h, 14% (two steps); (iv) K_2CO_3, MeOH, 50 °C, 13 h, 70%; **19**→**22**→**14**: (i) $TsNHNH_2$, Na_2SO_4, EtOH, 60 °C, 23 h; (ii) $NaBH_3CN$, DMF–sulfolane (1:1), *p*-TsOH·H_2O, 100 °C, 2 h, 40% (two steps); (iii) TBAF, THF, rt, 40 h, 63%; **1**→**15**: H_2, PtO_2, MeOH–$CHCl_3$ (2:1), rt, 8 h, 99%.

(**6**) through the aldehyde (**20**). The deoxydation reaction was carried out by reduction of the corresponding tosylhydrazone using $NaBH_3CN$ in DMF–sulfolane (1:1).[12] Finally, **6** was obtained by solvolysis (K_2CO_3, MeOH, 70%). The C-3 deoxygenated compound (**14**) was obtained from the C-28 TBS ether (**16**) in the same way as the preparation of **13**. Dihydrobetulin (**15**) was synthesized by standard hydrogenation conditions (H_2, PtO_2, MeOH–$CHCl_3$).

Results and Discussion

The procedure for the measurement of the biological activities was carried out using a slightly modified procedure that was previously reported[1] and the results for the individual compounds are summarized in Figure 2.

The protective effect of betulin (**1**) depends on its concentration and showed almost perfect activity beyond

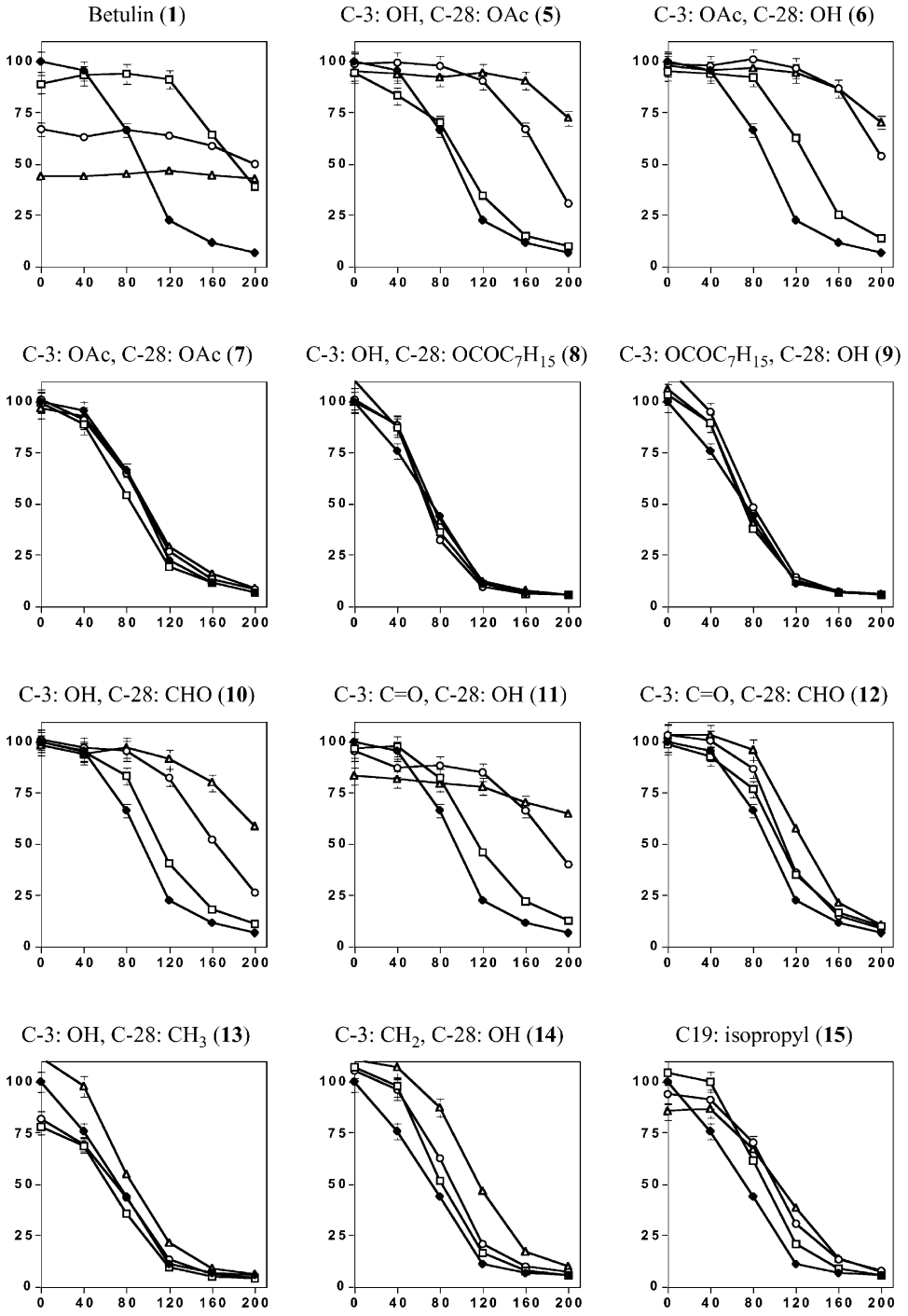


Figure 2. *X*-axis represents concentration of cadmium chloride (μM) and *Y*-axis indicates cell survival (%). Symbols in the graphs display concentration of the each compounds (◆ 0 μM, □ 2 μM, ○ 10 μM, △ 20 μM).

2 μM. However, above a 10 μM concentration of betulin (**1**), almost half of the cells died due to the strong cytotoxicity of betulin itself even if cadmium chloride did not exist in the medium. The monoacetates (**5** and **6**) and the monocarbonyl compounds (**10** and **11**) showed almost identical results. Namely, the protecting effect did not apparently appear at a low concentration of these compounds, but at high concentrations these compounds showed almost the same activities as betulin (**1**). On the other hand, the diacetyl compound (**7**) and the biscarbonyl compound (**12**) did not show any effects even at a high concentration. The monoester derivatives (**8** and **9**), which have a longer alkyl chain at either hydroxyl group (C-3 or C-28) displayed only negligible activities. The monodehydroxylated derivatives (**13** and **14**) also did not show effective activities. These results strongly suggested that the protective effect against cadmium toxicity is closely related to the polarity of the functional groups at C-3 and C-28. In addition to the polarity, it was also noted a large functional group on the either hydroxyl group caused reduced activities, probably due to inhibiting the binding with some receptors.

We anticipated that the isopropenyl group on C-19 did not affect the activity. However, the isopropyl derivative (**15**) did not have any effect and the amount of cell survival was almost identical with the control experiment. By analysis of the three-dimensional structure of betulin (**1**) and the other effective compounds (**2** and **3**), the sp^2 carbon of betulin are farer from that of **2** and **3**. Thus, it is difficult to consider that the reason for the loss in activity is the disappearance of the π–π interaction between the molecule and receptor and the major change by reducing the double bond will be the volume of the functional group. Therefore, it was also concluded that the molecules are strictly recognized by the receptor.

Interestingly, the cytotoxicities of all the derivatives at high concentrations were less than those of betulin (**1**). Especially, for the monoacetate (**5** and **6**) and the monocarbonyl compounds (**10** and **11**), almost 100% of the cells survived at 0 μM cadmium chloride and about 60∼75% of the cells still survived even at 200 μM cadmium chloride in the presence of a 20 μM concentration of the individual compounds. In other words, it was shown that these analogues (**5**, **6**, **10**, and **11**) have the selective activities against cadmium toxicity without any cell damage.

It has already been published that betulin (**1**) and betulinic acid have other interesting bioactivities, for example anti-HIV activity,[13–15] anti-tumor activity,[16,17] spasmogenic activity,[18] and anti-inflammatory activity.[19] The structure–activity relationships for the anti-HIV and the anti-tumor activities have also been well investigated.[13–17] In 1998, Sun et al. reported a spectacular improvement for anti-HIV activities of betulin (**1**) by modification of both hydroxyl groups to the 3′,3″-dimethylglutarate.[14,15] As they mainly focused on carboxylate derivatives, it is difficult to directly compare their work with our results. However, they also reported that the diacetyl compound (**7**) displayed a much weaker activity than betulin (**1**) and the hydrogenated analogues showed a weaker inhibitory activity compared with the corresponding isopropenyl analogues. Furthermore, the importance of the size and electrostatic property of the substituents on C-19 for the cytotoxicity of betulinic acid was also suggested.[17] All of the results described above are consistent with our observations.

In conclusion, we clarified that both the polar functional group on either C-3 or C-28 and the isopropenyl group play an important role in reducing the cadmium toxicity and cytotoxicy of betulin (**1**). Not only in the case of the reduction of cadmium toxicity, but the promotion of the synthesis of certain proteins by betulin (**1**) were also suggested as anti-inflammatory activities.[19] However, it seems difficult to synthesize an efficient probe from betulin itself to clarify the mechanism for any bioactivities by betulin (**1**), because betulin (**1**) does not have any other functional groups except for the hydroxyl and isopropenyl groups which are crucial for its activities. Further studies, which include the asymmetric total synthesis of betulin and new type analogues, are now in progress in our laboratory.

Experimental

General procedure

The IR spectra were measured using a Shimadzu FTIR-8400 spectrophotometer. The ^1H NMR spectra were recorded on a Varian Gemini 2000 (300 MHz), JEOL AL-4000 (400 MHz) and JEOL GX-500 (500 MHz) in $CDCl_3$ as the solvent, unless otherwise stated. The chemical shifts are expressed in δ (ppm) values with tetramethylsilane (TMS) as the internal reference and coupling constants (*J*) are measured in Hz. The ^{13}C NMR spectra were recorded on a JEOL AL-4000 (100 MHz) and JEOL GX-500 (125 MHz). The mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded on JEOL JMS-DX303 and JMS-AX500 instruments.

HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with kanamycin sulfate (60 μg/mL; Life Technologies Inc., Rockville, MD, USA), $NaHCO_3$ (0.1%; Nacalai Tesque), L-glutamine (316 mg/mL; Nacalai Tesque), and FBS (10%; JRH Biosciences, Lenexa, KS, USA) in an atmosphere of 5% CO_2 in air at 37 °C. Alamar Blue™ was obtained from Alamar Biosciences Inc. (Sacramento, CA, USA).

Preparation of the substrates (5–15)

28-Acetoxybetulin (5). To a solution of betulin (15.8 mg, 0.036 mmol) in anhydrous dichloromethane (0.4 mL) was added triethylamine (10.9 mg, 0.11 mmol) and acetic anhydride (5.4 mg, 0.053 mmol) at 0 °C, and the mixture was warmed slowly to room temperature. After being stirred for 22 h, the mixture was diluted with Et_2O, and then the organic solution was washed with saturated NH_4Cl solution. The aqueous phase was extracted again with Et_2O and the combined organic phases were washed with brine. The solution was dried

over $MgSO_4$ and concentrated. The residue was purified by silica gel chromatography eluting with hexane–ethyl acetate (9:1–4:1) to yield **5** (12.1 mg, 69%) as a white solid. IR ν(film) cm^{-1}: 3447, 2943, 2870, 1738, 1240, 1034. ^1H NMR (500 MHz) δ: 0.66 (1H, d, $J=9.2$ Hz), 0.74 (3H, s), 0.80 (3H, s), 0.82–0.91 (1H, m), 0.94 (3H, s), 0.95 (3H, s), 1.01–1.09 (6H, m), 1.15–1.26 (4H, m), 1.34–1.40 (5H, m), 1.49–1.69 (11H, m), 1.74 (1H, dd, $J=12.2$, 7.3 Hz), 1.80–1.84 (1H, m), 1.90–1.98 (1H, m), 2.05 (3H, s), 2.42 (1H, td, $J=11.2$, 5.7 Hz), 3.16 (1H, dd, $J=11.2$, 4.9 Hz), 3.84 (1H, d, $J=11.2$ Hz), 4.23 (1H, dd, $J=11.2$, 1.8 Hz), 4.56 (1H, s), 4.67 (1H, d, $J=1.8$ Hz). MS *m/z* (relative intensity): 484 (37, M^+). HRMS calcd $C_{32}H_{52}O_3$: 484.3916. Found: 484.3904.

28-*tert*-Butyldimethylsiloxybetulin (16). Imidazole (31.4 mg, 0.46 mmol) and DMAP (17.6 mg, 0.16 mmol) were added to a solution of betulin (**1**) (61.5 mg, 0.14 mmol) in anhydrous DMF (2.5 mL) at room temperature. After being stirred for 20 min, the mixture was cooled to 0 °C then TBSCl (41.0 mg, 0.27 mmol) was added. The mixture was warmed to room temperature and stirred for 7 h. Water was added to the mixture and the aqueous phase was extracted with Et_2O. The combined organic solution was washed with brine and dried over $MgSO_4$. The solvent was removed in vacuo and purified by silica gel chromatography (hexane–ethyl acetate, 9:1) to afford **16** (64.3 mg, 86%) as a white solid. IR ν (film) cm^{-1}: 3385, 2943, 2860, 1088. ^1H NMR (500 MHz) δ: 0.02 (6H, s), 0.67 (1H, d, $J=9.2$ Hz), 0.74 (3H, s), 0.81 (3H, s), 0.88 (9H, s), 0.84–1.38 (22H, m), 1.49–1.66 (11H, m), 1.95–1.86 (3H, m), 2.37 (1H, td, $J=10.8$, 5.9 Hz), 3.17 (1H, dd, $J=11.6$, 4.9 Hz), 3.23 (1H, d, $J=9.5$ Hz), 3.65 (1H, d, $J=9.5$ Hz), 4.54 (1H, s), 4.65 (1H, d, $J=1.8$ Hz). MS *m/z* (relative intensity): 556 (7, M^+). HRMS calcd $C_{36}H_{64}O_2Si$: 556.4676. Found: 556.4709.

3-Acetoxy-28-*tert*-butyldimethylsiloxybetulin (17). Acetic anhydride (13.3 mg, 0.13 mmol) was added to a solution of **16** (49.3 mg, 0.089 mmol) and DMAP (0.7 mg, 6.2 μmol) in pyridine (0.9 mL) at 0 °C and warmed slowly to room temperature. After being stirred for 16 h, Et_2O was added to the mixture and the organic solution was washed with water and the aqueous phase was extracted again with Et_2O. The combined organic solution was washed with brine and dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified by silica gel chromatography (hexane–ethyl acetate, 9:1) to yield **17** (64.3 mg, 94%) as a white solid. IR ν (film) cm^{-1}: 2949, 2858, 1734, 1246, 1088. ^1H NMR (300 MHz) δ: 0.01 (6H, s), 0.81 (3H, s), 0.82 (6H, s), 0.87 (9H, s), 0.94 (3H, s), 0.99 (3H, s), 0.75–1.65 (21H, m), 1.65 (3H, s), 1.81–2.01 (3H, m), 2.01 (3H, s), 2.36 (1H, td, $J=10.7$, 6.1 Hz), 3.23 (1H, d, $J=9.6$ Hz), 3.64 (1H, d, $J=9.6$ Hz), 4.45 (1H, dd, $J=5.8$, 10.4 Hz), 4.54 (1H, dd, $J=2.2$, 1.4 Hz), 4.65 (1H, d, $J=2.5$ Hz). MS *m/z* (relative intensity): 598 (5, M^+). HRMS calcd $C_{38}H_{66}O_3Si$: 598.4781. Found: 598.4743.

3-Acetoxybetulin (6). To a solution of **17** (48.8 mg, 0.081 mmol) in THF (0.3 mL) was added TBAF (1.0 M THF solution, 0.5 mL) at room temperature and the mixture was refluxed for 3 h. The reaction was cooled to room temperature and the solvent was removed in vacuo. The residue was extracted with Et_2O. The organic solution was washed with brine and dried over $MgSO_4$. The solvent was evaporated and the crude product was purified by silica gel chromatography (hexane–ethyl acetate, 4:1) to afford **6** (38.4 mg, 98%) as a white solid. IR ν (film) cm^{-1}: 3421, 2945, 2872, 1732, 1246, 1028. ^1H NMR (300 MHz) δ: 0.78 (1H, d, $J=10.4$ Hz), 0.83 (3H, s), 0.84 (6H, s), 0.97 (3H, s), 1.02 (3H, s), 0.97–2.03 (24H, m), 1.68 (3H, s), 2.03 (3H, s), 2.38 (1H, td, $J=10.6$, 6.0 Hz), 3.33 (1H, d, $J=10.7$ Hz), 3.79 (1H, d, $J=10.7$ Hz), 4.46 (1H, dd, $J=5.5$, 10.7 Hz), 4.58 (1H, s), 4.67 (1H, s). ^{13}C NMR (75 MHz) δ: 14.5, 15.8, 16.0, 16.3, 18.0, 18.9, 20.7, 21.1, 21.2, 23.6, 25.0, 26.9, 27.8, 29.0, 29.6, 33.8, 34.0, 36.9, 37.2, 37.7, 38.2, 40.8, 42.6, 47.7, 48.6, 50.2, 55.3, 60.4, 80.9, 109.7, 150.6, 171.1. MS *m/z* (relative intensity): 484 (30, M^+). HRMS calcd $C_{32}H_{52}O_3$: 484.3916. Found: 484.3913.

3,28-Diacetoxybetulin (7). To a solution of betulin (**1**) (16.5 mg, 0.037 mmol) and catalytic amount of DMAP in pyridine (0.4 mL) was added acetic anhydride (11.3 mg, 0.11 mmol) at 0 °C and warmed slowly to room temperature. After being stirred for 5 h, Et_2O was added to the mixture and the solution was washed with saturated NH_4Cl solution. The aqueous phase was extracted with Et_2O. The combined organic solution was washed with brine and dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel (hexane-ethyl acetate, 9:1) to give **7** (19.0 mg, 97%) as a white solid. IR ν (film) cm^{-1}: 2945, 2872, 1736, 1244, 1023. ^1H NMR (500 MHz) δ: 0.76 (1H, d, $J=9.2$ Hz), 0.81 (3H, s), 0.82 (3H, s), 0.82 (3H, s), 0.94 (3H, s), 1.01–1.08 (6H, m), 1.15–1.28 (4H, m), 1.34–1.39 (5H, m), 1.48 (1H, m), 1.54–1.68 (10H, m), 1.74 (1H, dd, $J=12.5$, 7.6 Hz), 1.80–1.83 (1H, m), 1.98–1.89 (1H, m), 2.01 (3H, s), 2.04 (3H, s), 2.41 (1H, td, $J=11.0$, 6.1 Hz), 3.83 (1H, d, $J=11.0$ Hz), 4.23 (1H, d, $J=11.0$ Hz), 4.44 (1H, dd, $J=11.0$, 5.5 Hz), 4.56 (1H, s), 4.66 (1H, d, $J=1.8$ Hz). MS *m/z* (relative intensity): 526 (7, M^+). HRMS calcd $C_{34}H_{54}O_4$: 526.4022. Found: 526.4020.

28-Capryloxybetulin (8). A few drops of TMSCl was added to a solution of betulin (**1**) (14.0 mg, 0.032 mmol) in caprylic acid (0.32 mL) at room temperature. After being stirred for 19 h, saturated $NaHCO_3$ solution was added and extracted with Et_2O. The combined organic solution was washed with brine and dried over $MgSO_4$. The solution was concentrated and the residue was purified by chromatography on alumina (1.3 g) and silica gel (3.0 g) (hexane–ethyl acetate, 9:1) to afford **8** (15.5 mg, 84%) as a white solid. IR ν (film) cm^{-1}: 3396, 2930, 2870, 1734, 1456. ^1H NMR (300 MHz) δ: 0.68 (1H, d, $J=9.3$ Hz), 0.76 (3H, s), 0.82 (3H, s), 0.88 (3H, t, $J=6.7$ Hz), 0.97 (3H, s), 0.98 (3H, s), 1.03 (3H, s), 0.86–2.04 (34H, m), 1.68 (3H, s), 2.32 (2H, t, $J=7.6$ Hz), 2.45 (1H, td, $J=10.9$, 6.0 Hz), 3.18 (1H, dd, $J=11.0$, 5.2 Hz), 3.84 (1H, d, $J=11.0$ Hz), 4.26 (1H, d, $J=11.0$ Hz), 4.59 (1H, s), 4.69 (1H, d, $J=1.9$ Hz). MS *m/z* (relative intensity): 568 (13, M^+). HRMS calcd $C_{38}H_{64}O_3$: 568.4855. Found: 568.4840.

28-*tert*-Butyldimethylsiloxy-3-capryloxybetulin (18). To a solution of **16** (22.9 mg, 0.041 mmol) in anhydrous CH_2Cl_2 (0.3 mL) was added DCC (13.0 mg, 0.063 mmol) and catalytic amount of DMAP at room temperature. The mixture was stirred until the solution became clear, then caprylic acid (5.9 mg, 0.041 mmol) was added. After being stirred for 6.5 h, another portion of caprylic acid (1.5 mg, 0.011 mmol) was added and the stirring was continued for another 15.5 h. The reaction was diluted with Et_2O, and the mixture was filtered through a pad of Celite™ eluting with Et_2O. The filtrate was concentrated and the residue was purified by chromatography on silica gel (hexane–ethyl acetate, 99:1) to afford **18** (24.4 mg, 88%) as a white solid. IR ν (film) cm^{-1}: 2930, 1732, 1462, 1088. ^1H NMR (300 MHz) δ: 0.04 (6H, s), 0.79 (1H, d, $J=10.4$ Hz), 0.84 (6H, s), 0.85 (3H, s), 0.90 (9H, s), 0.96 (3H, s), 1.02 (3H, s), 0.84–1.68 (33H, m), 1.68 (3H, s), 1.83–1.97 (3H, m), 2.29 (2H, t, $J=7.4$ Hz), 2.34–2.43 (1H, m), 3.25 (1H, d, $J=9.6$ Hz), 3.67 (1H, d, $J=9.6$ Hz), 4.47 (1H, dd, $J=10.4$, 5.5 Hz), 4.57 (1H, d, $J=0.8$ Hz), 4.67 (1H, d, $J=2.2$ Hz). MS *m/z* (relative intensity): 682 (3, M^+). HRMS calcd $C_{44}H_{78}O_3Si$: 682.5720. Found: 682.5692.

3-Capryloxybetulin (9). A solution of HF in MeCN (1:5, 0.1 mL) was added to a solution of **18** (24.4 mg, 0.036 mmol) in THF (0.2 mL) at room temperature. After being stirred for 13 h, saturated $NaHCO_3$ solution was added and extracted with Et_2O. The combined organic solution was washed with brine and dried over $MgSO_4$. The solvent was evaporated and the residue was chromatographed on silica gel (hexane–ethyl acetate, 19:1) to afford **9** (15.2 mg, 75%) as a white solid. IR ν (film) cm^{-1}: 3410, 2941, 1732, 1454, 1375, 1105, 1034. ^1H NMR (500 MHz) δ: 0.75–1.66 (53H, m), 1.86–1.95 (3H, m), 2.26 (2H, t, $J=7.6$ Hz), 2.34–2.37 (1H, m), 3.23 (1H, d, $J=9.5$ Hz), 3.65 (1H, d, $J=9.5$ Hz), 4.45 (1H, dd, $J=11.0$, 5.5 Hz), 4.54 (1H, s), 4.65 (1H, s). MS *m/z* (relative intensity): 568 (18, M^+). HRMS calcd $C_{38}H_{64}O_3$: 568.4855. Found: 568.4829.

28-Oxobetulin (10) and 3,28-dioxobetulin (12). PCC (56.0 mg, 0.26 mmol) was added to a solution of betulin (**1**) (37.8 mg, 0.085 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at room temperature. After being stirred 1.5 h, silica gel and Et_2O were successively added to the mixture and stirred for several min. The mixture was filtered through a pad of Celite™ eluting with Et_2O and the filtrate was concentrated in vacuo. The crude mixture was chromatographed on silica gel (hexane–ethyl acetate, 9:1) to afford **10** (6.1 mg, 17%) and **12** (30.5 mg, 82%) as a white solid, respectively. **10**: IR ν (film) cm^{-1}: 3373, 2941, 2868, 1726, 1454. ^1H NMR (300 MHz) δ: 0.67 (1H, d, $J=8.8$ Hz), 0.75 (3H, s), 0.82 (3H, s), 0.85–2.10 (36H, m), 2.87 (1H, td, $J=11.0$, 5.5 Hz), 3.18 (1H, dd, $J=11.0$, 5.5 Hz), 4.63 (1H, s), 4.76 (1H, s), 9.68 (1H, d, $J=1.4$ Hz). MS *m/z* (relative intensity): 440 (59, M^+). HRMS calcd $C_{30}H_{48}O_2$: 440.3654. Found: 440.3652. **12**: IR ν (film) cm^{-1}: 2943, 2868, 1705, 1458. ^1H NMR (300 MHz) δ: 0.89–0.85 (1H, m), 0.93 (3H, s), 0.95 (3H, s), 0.99 (3H, s), 1.02 (3H, s), 1.07 (3H, s), 1.05–1.12 (1H, m), 1.13–1.52 (10H, m), 1.64–2.35 (13H, m), 2.36–2.55 (2H, m), 2.88 (1H, td, $J=11.1$, 5.7 Hz), 4.64 (1H, s), 4.76 (1H, d, $J=1.8$ Hz), 9.67 (1H, d, $J=1.8$ Hz). MS *m/z* (relative intensity): 438 (74, M^+). HRMS calcd $C_{30}H_{46}O_2$: 438.3498. Found: 438.3524.

28-*tert*-Butyldimethylsiloxy-3-oxobetulin (19). PCC (40.6 mg, 0.19 mmol) was added to a solution of **16** (34.4 mg, 0.062 mmol) in anhydrous CH_2Cl_2 (1.3 mL) at ambient temperature and the mixture was stirred for 1 h. Silica gel and Et_2O were successively added to the mixture and stirred for several min. The mixture was filtered through pad of Celite™ eluting with Et_2O and the filtrate was evaporated. The residue was purified by silica gel chromatography (hexane–ethyl acetate, 9:1) to give **19** (33.8 mg, 98%) as a colorless oil. IR ν (film) cm^{-1}: 2951, 2862, 1707, 1460, 1088. ^1H NMR (500 MHz) δ: 0.00 (6H, s), 0.86 (9H, s), 0.82–1.64 (36H, m), 1.80–1.95 (4H, m), 2.29–2.50 (3H, m), 3.22 (1H, d, $J=9.6$ Hz), 3.63 (1H, d, $J=9.6$ Hz), 4.53 (1H, dd, $J=2.5$, 1.6 Hz), 4.63 (1H, d, $J=1.6$ Hz). MS *m/z* (relative intensity): 554 (6, M^+). HRMS calcd $C_{36}H_{62}O_2Si$: 554.4519. Found: 554.4522.

3-Oxobetulin (11). The mixture of **19** (10.4 mg, 0.019 mmol) and TBAF (1.0 M solution in THF, 0.3 mL) was heated at 70 °C in a sealed tube for 3 h. The solvent was evaporated and the residue was extracted with Et_2O. The organic solution was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (hexane–ethyl acetate, 4:1) to yield **11** (6.7 mg, 79%) as a white solid. IR ν (neat) cm^{-1}: 3396, 2941, 2868, 1705, 1458. ^1H NMR (500 MHz) δ: 0.82–0.88 (2H, m), 0.91 (3H, s), 0.97 (3H, s), 1.01–1.08 (9H, m), 1.17–1.72 (20H, m), 1.82–2.02 (4H, m), 2.35–2.50 (3H, m), 3.33 (1H, d, $J=11.0$ Hz), 3.78 (1H, d, $J=10.4$ Hz), 4.57 (1H, q, $J=1.2$ Hz), 4.67 (1H, d, $J=2.4$ Hz). MS *m/z* (relative intensity): 440 (10, M^+). HRMS calcd $C_{30}H_{48}O_2$: 440.3654. Found: 440.3680.

3-Acetoxy-28-oxobetulin (20). PCC (30.1 mg, 0.14 mmol) was added to a solution of **6** (23.0 mg, 0.047 mmol) in anhydrous CH_2Cl_2 (0.9 mL) at ambient temperature and stirred for 30 min. Silica gel and Et_2O were successively added to the mixture and stirred for several min. The mixture was filtered through pad of Celite™ eluting with Et_2O and the filtrate was evaporated. The residue was purified by silica gel chromatography (hexane–ethyl acetate, 19:1) to afford **20** (16.2 mg, 72%) as a white solid. IR ν (film) cm^{-1}: 2943, 2868, 1730, 1246. ^1H NMR (400 MHz) δ: 0.76 (1H, d, $J=9.0$ Hz), 0.81 (3H, s), 0.82 (6H, s), 0.90 (3H, s), 0.95 (3H, s), 0.81–1.91 (21H, m), 1.68 (3H, s), 1.97–2.08 (2H, m), 2.02 (3H, s), 2.85 (1H, td, $J=11.1$, 5.9 Hz), 4.45 (1H, dd, $J=10.4$, 5.7 Hz), 4.61 (1H, s), 4.74 (1H, s), 9.65 (1H, d, $J=1.5$ Hz). ^{13}C NMR (100 MHz) δ: 14.3, 16.0, 16.3, 16.6, 18.2, 19.1, 20.8, 21.4, 23.8, 25.6, 28.0, 28.8, 29.3, 29.9, 33.3, 34.3, 37.1, 37.8, 38.5, 38.7, 40.9, 42.6, 47.6, 48.1, 50.4, 55.4, 59.4, 80.9, 110.1, 149.6, 170.9, 206.5. MS *m/z* (relative intensity): 482 (28, M^+). HRMS calcd $C_{32}H_{50}O_3$: 482.3760. Found: 482.3763.

3-Acetoxy-28-deoxobetulin (21). To a solution of **20** (16.2 mg, 0.034 mmol), Na_2SO_4 (16.5 mg, 0.12 mmol),

and *p*-toluenesulfonhydrazide (13.0 mg, 0.070 mmol) in EtOH (0.34 mL) was heated at 100 °C for 3.5 h. The mixture was filtered through pad of Celite™ eluting with Et_2O and the filtrate was evaporated. The residue was dissolved in a solution of DMF–sulfolane (1:1, 0.2 mL) containing 1.5 mg of *p*-toluenesulfonic acid monohydrate. $NaBH_3CN$ (9.8 mg, 0.16 mmol) was added to the mixture and heated at 100 °C for 1 h. The mixture was extracted with Et_2O and the organic solution was washed with brine. The organic solution was dried over $MgSO_4$ and the solvent was evaporated. The residue was chromatographed on silica gel (hexane–ethyl acetate, 19:1) to afford **21** (2.2 mg, 14%) as a colorless oil. IR ν (neat) cm^{-1}: 2930, 2856, 1738, 1244. ^1H NMR (400 MHz) δ: 0.79 (3H, s), 0.83 (3H, s), 0.84 (3H, s), 0.85 (3H, s), 0.94 (3H, s), 1.03 (3H, s), 0.75–1.68 (23H, m), 1.68 (3H, s), 1.89–1.94 (1H, m), 2.04 (3H, s), 2.38 (1H, td, $J=10.9$, 5.7 Hz), 4.47 (1H, dd, $J=5.7$, 10.4 Hz), 4.57 (1H, s), 4.69 (1H, d, $J=2.4$ Hz). MS *m*/*z* (relative intensity): 468 (35, M^+). HRMS calcd $C_{32}H_{52}O_2$: 468.3967. Found: 468.3964.

28-Deoxobetulin (13). A solution of **21** (2.2 mg, 0.0047 mmol) and a small amount of K_2CO_3 in MeOH (0.3 mL) was stirred at 50 °C for 13 h. The solvent was evaporated and the residue was purified by silica gel chromatography (hexane–ethyl acetate, 19:1) to yield **12** (1.4 mg, 70%) as a white solid. IR ν (film) cm^{-1}: 3371, 2926, 2855. ^1H NMR (400 MHz) δ: 0.67 (1H, d, $J=9.3$ Hz), 0.75 (3H, s), 0.77 (3H, s), 0.81 (3H, s), 0.93 (3H, s), 0.95 (3H, s), 1.01 (3H, s), 0.75–1.69 (22H, m), 1.66 (3H, s), 1.85–1.96 (2H, m), 2.36 (1H, td, $J=11.0$, 5.9 Hz), 3.17 (1H, dd, $J=11.2$, 4.9 Hz), 4.55 (1H, dd, $J=2.3$, 1.3 Hz), 4.67 (1H, d, $J=2.2$ Hz). MS *m*/*z* (relative intensity): 426 (91, M^+). HRMS calcd $C_{30}H_{50}O$: 426.3862. Found: 426.3874.

28-*tert*-Butyldimethylsiloxy-3-deoxobetulin (22). A solution of **19** (32.1 mg, 0.058 mmol), a small amount of Na_2SO_4, and *p*-toluenesulfonhydrazide (12.4 mg, 0.067 mmol) in EtOH (0.60 mL) was heated at 60 °C for 23 h. The mixture was filtered through pad of Celite™ eluting with Et_2O and the filtrate was evaporated. The residue was dissolved in a solution of DMF–sulfolane (1:1, 0.3 mL) containing 1.5 mg of *p*-toluenesulfonic acid monohydrate. $NaBH_3CN$ (16.2 mg, 0.26 mmol) was added to the mixture and heated at 100 °C for 2 h. The mixture was extracted with Et_2O and the combined organic solution was washed with brine. The solution was dried over $MgSO_4$ and concentrated. The residue was purified by silica gel chromatography (hexane) to yield **22** (12.7 mg, 40%) as a colorless oil. IR ν (neat) cm^{-1}: 2926, 2862, 1462, 1088. ^1H NMR (400 MHz) δ: 0.04 (6H, s), 0.79 (3H, s), 0.82 (3H, s), 0.84 (3H, s), 0.90 (3H, s), 0.97 (3H, s), 1.02 (3H, s), 0.71–1.68 (23H, m), 1.68 (3H, s), 1.85–1.97 (3H, m), 3.19 (1H, td, $J=10.7$, 6.0 Hz), 3.25 (1H, d, $J=9.4$ Hz), 3.68 (1H, d, $J=9.4$ Hz), 4.56 (1H, s), 4.67 (1H, d, $J=2.0$ Hz). MS *m*/*z* (relative intensity): 540 (7, M^+). HRMS calcd $C_{36}H_{64}OSi$: 540.4726. Found: 540.4768.

3-Deoxobetulin (14). A mixture of **22** (17.5 mg, 0.032 mmol) and TBAF (1.0 M solution in THF, 0.3 mL) was stirred at room temperature for 40 h. The reaction was extracted with Et_2O and combined organic solution was washed with brine. The solution was dried over $MgSO_4$ and the solvent was evaporated. The residue was chromatographed on silica gel (hexane–ethyl acetate, 19:1) to give **14** (8.7 mg, 63%) as a white solid. IR ν (film) cm^{-1}: 3335, 2941, 2868, 1456, 1375, 1026, 760. ^1H NMR (500 MHz) δ: 0.70 (1H, d, $J=12.2$ Hz), 0.77 (3H, s), 0.80 (3H, s), 0.82 (3H, s), 0.97 (3H, s), 1.01 (3H, s), 0.69–1.71 (23H, m), 1.66 (3H, s), 1.84 (1H, dd, $J=12.8$, 7.9 Hz), 1.88–1.98 (2H, m), 2.37 (1H, td, $J=10.8$, 5.7 Hz), 3.31 (1H, d, $J=10.4$ Hz), 3.78 (1H, dd, $J=10.7$, 1.5 Hz), 4.56 (1H, s), 4.66 (1H, d, $J=1.8$ Hz). ^{13}C NMR (125 MHz) δ: 14.8, 16.1, 16.1, 18.6, 18.7, 19.1, 20.7, 21.6, 25.3, 25.6, 27.0, 29.2, 29.8, 33.3, 33.4, 34.0, 34.2, 37.3, 37.5, 40.3, 41.1, 42.1, 42.8, 47.8, 48.8, 50.5, 56.4, 60.6, 109.6, 150.5. MS *m*/*z* (relative intensity): 426 (48, M^+). HRMS calcd $C_{30}H_{50}O$: 426.3862. Found: 426.3860.

20,29-Dihydrobetulin (15). A solution of betulin (**1**) (21.6 mg, 0.049 mmol) and catalytic amount of PtO_2 in MeOH–$CHCl_3$ (2:1, 1.5 mL) was stirred at room temperature under H_2 atmosphere for 8 h. PtO_2 was removed by filtration and the filtrate was concentrated to afford **15** (21.7 mg, 99%) as a white solid. IR ν (film) cm^{-1}: 3298, 2928, 2866. ^1H NMR (500 MHz) δ: 0.67 (1H, d, $J=11.0$ Hz), 0.75 (6H, t, $J=3.4$ Hz), 0.82 (6H, s), 0.94 (3H, s), 0.95 (3H, s), 1.01 (3H, s), 0.77–1.72 (24H, m), 1.79 (1H, dd, $J=12.2$, 7.9 Hz), 1.82–2.15 (2H, m), 3.17 (1H, dd, $J=11.6$, 4.9 Hz), 3.28 (1H, d, $J=11.0$ Hz), 3.75 (1H, d, $J=11.0$ Hz). ^{13}C NMR (125 MHz) δ: 14.7, 14.9, 15.4, 16.0, 16.1, 18.3, 20.8, 21.7, 22.9, 26.9, 26.9, 27.4, 28.0, 29.4, 29.5, 34.0, 34.3, 36.9, 37.2, 38.7, 38.9, 41.0, 42.9, 44.6, 47.9, 48.1, 50.1, 55.3, 60.6, 79.0. MS *m*/*z* (relative intensity): 444 (21, M^+). HRMS calcd $C_{30}H_{52}O_2$: 444.3967. Found: 444.3945.

General procedure for cadmium toxicity assay

HepG2 cells were seeded onto 96-well microplates at a cell density of 1×10^4 cell/well. After being incubated for 24 h, a solution of betulin analogues in DMSO was added. Cadmium chloride was added 24 h later and cultured for further 24 h. To determine of the cell survival, Alamar Blue™ was added to the medium and fluorescence was measured (Ex.: 544 nm, Em.: 590 nm) after 3 h.

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